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ORIGINAL PAPER

Cloning of Flax Oleic Fatty Acid Desaturase and Its Expression in Yeast

Anna Krasowska · Dorota Dziadkowiec · Anna Polinceusz · Aneta Plonka · Marcin Łukaszewicz

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Abstract Fatty acid desaturases dehydrogenate acyl chains, which results in the formation of a double bond. Using PCR on flax genomic DNA, we cloned a putative Δ12 fatty acid desaturase (Fad2) gene encoding a 378 amino acid protein. Heterologous expression of this protein in yeast as an N-terminal fusion to GFP showed its localization within endoplasmic reticulum. Analysis of membrane lipids revealed the production of dienoic fatty acids, decreased levels of FAD2 substrates and an increased concentration of longer fatty acids. Higher peroxidation of lipids in FAD2-containing strains is not reflected by any visible phenotype in YPD medium. However, FAD2-containing strains with deleted superoxide dismutase genes exhibited significant growth reductions under oxidative stress.

Keywords FAD2 fatty acid desaturase · *Linum usitatissimum* · *Saccharomyces cerevisiae* · Cu–Zn superoxide dismutase · Manganese superoxide dismutase · Omega-6 fatty acids

Abbreviations

GFP	Green fluorescent protein
FAD2	Oleic acid (Δ12) fatty acid desaturase
FA	Fatty acid
PUFA	Polyunsaturated fatty acid
TAG	Triacylglycerols
SOD	Superoxide dismutase
FAMEs	Fatty acid methyl esters

A. Krasowska · D. Dziadkowiec · A. Polinceusz · A. Plonka · M. Łukaszewicz (✉)
Faculty of Biotechnology, Wrocław University,
51-148 Wrocław, Przybyszewskiego 63-77, Poland
e-mail: lukasz@biotrans.uni.wroc.pl

Introduction

Fatty acid desaturation has been studied for over four decades but the reaction mechanism model (recently reviewed in [1]) was adopted only in 1992. In the presence of oxygen, desaturases catalyze stereo-, regio- and chemoselective reactions which are crucial for membrane lipid function and adaptation to various environmental stresses such as temperature [2] or wounding [3], and also for defense signaling pathways [4]. In plants, the first step of FA synthesis is localized in plastids, usually ending with 16:0 (palmitic) or 18:0 (stearic) fatty acid chain. Subsequently, desaturases may catalyze oxygen dependent dehydrogenation which results in the formation of a double (C=C) unsaturated bond. Then the FA may either enter the glycerolipid prokaryotic biosynthesis pathway in plastids or are exported from plastids to enter the eukaryotic pathway [5]. The proportion of lipids entering either pathway is dependent on the plant species. In oil-producing crop plants such as sunflower, cotton, rape or soybean, polyunsaturated fatty acids (PUFA) incorporated in seed storage triacylglycerol (TAG) lipids are probably mostly synthesized via the eukaryotic pathway. The enzyme responsible for the first PUFA (introduction of a second unsaturated bond) in these plants is microsomal FAD2 desaturase, often referred to as Δ12 desaturase as it dehydrogenates the twelfth carbon from the carboxy terminal, or ω6 desaturase (double bond on carbon six from the terminal methyl in 18:2 acyl chain). In plants, linoleic acid (18:2) may be further desaturated, resulting in

the formation of ω3 FA (linolenic acid, 18:3). Linoleic or linolenic FA are essential for human cells which are not able to synthesize them. Appropriate dietary intake of ω6 and ω3 FA therefore has a considerable impact on human health and considerable research effort is focused on their appropriate supplementation, especially in various diseases [6, 7]. It is generally assumed that a modern diet is poor in ω3, and increasing the ω3/ω6 ratio could be beneficial for health.

The richest plant source of essential fatty acids (FA) is flax (*Linum usitatissimum*). Most of its cultivars contain over 60% of linolenic acid, but the high total amount of PUFA is the cause of its rapid peroxidation [8]. PUFA are known to be readily oxidized and the resulting products trigger a chain reaction generating free radicals which could be harmful for the cell. Modification of the PUFA content may thus have a great impact on the response to oxidative stress. Indeed, modification of FA synthesis has been shown to be involved in various physiological stress responses.

Another consequence of the rapid peroxidation of PUFA is the very short shelf life of extracted flax oil; for this reason, only so-called low-PUFA cultivars are used for dietary supplementation. Unfortunately, some of these cultivars, such as Linola, contain only about 3% of linolenic acid, giving rise to a low ω3/ω6 ratio.

To decrease the total amount of PUFA in flax without affecting the ω3/ω6 ratio, the most promising target seems to be the *Fad2* gene responsible for desaturation of oleic acid. Regulation of this gene has been shown to constitute a successful strategy in other crop plants [9, 10]. *Fad2* gene has been cloned and characterized in several plants including most of oil rich ones like sunflower [11], soybean [12] or cotton [9], but it has not yet been cloned from flax. As FAD2 is a membrane bound enzyme very difficult to purify and test in vitro, the method of choice for enzyme characterization is heterologous expression in the yeast *Saccharomyces cerevisiae*. Based on homologies between *Fad2* genes from other plants, we have cloned the *Fad2* gene from flax. This flax FAD2 protein activity and localization in yeast were characterized. The sensitivity to oxidative stress of yeast expressing flax FAD2 has been for the first time evaluated in the *S. cerevisiae* US50-18C strain and isogenic lines lacking SOD1 or SOD2 activity.

Experimental Procedures

Isolation and Cloning of Fad2

PCR amplification was performed for 30 cycles (1 min at 95 °C, 1 min at 52 °C, 1 min at 72 °C) using Taq/Pfu 4/1 mixture on total genomic DNA isolated from a Nike flax cultivar. The following oligonucleotides were used as primers for PCR: AAA ggT ACC AAC ATg ggT gCA ggT ggA AgA ATg C (forward primer AthFad2F) and CCC CTC gAg-TCA TAA CTT ATT gTT gTA CCA g (reverse primer AthFad2R). The amplified DNA fragment was fractionated by agarose gel electrophoresis and inserted into the TA-cloning vector PCR2.1 (Invitrogene, San Diego, USA). Three recombinant plasmids designated pCR2.1A, B and C were amplified in *Escherichia coli* DH5α (Life Technologies, Inc. Gaithersburg, USA) for restriction analysis and DNA sequencing. The amplified fragments present in pCR2.1A, B and C were sequenced completely from both 5′ and 3′ ends. The DNA sequencing was performed using ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, USA). All three plasmids showed an identical sequence of the insert; hence, only pCR2.1A was used for further experiments. The plasmid DNA was isolated using a Qiagen miniplasmid kit according to the manufacturer's protocol. The size of the DNA present in the recombinant plasmid was confirmed by digesting it with *Kpn*I and *Xho*I.

Yeast expression plasmid containing flax desaturase was obtained by digestion of PCR2.1A with *Bam*HI and *Xho*I, gel purification and ligation between the *Bam*H1 and *Xho*I restriction sites of pUG36 (yeast-enhanced green fluorescent protein yEGFP fusion vector, a gift from J.H. Hegemann, Institute of Microbiology, University of Düsseldorf, Germany, Gueldener and Hegemann, manuscript in preparation http://mips.gsf.de/proj/yeast/info/tools/hegemann/pug36_map.html) to give pUG36Fad2. The plasmid was propagated by transformation of *E. coli* DH5α cells. For microscopy, *S. cerevisiae* strains were transformed with pUG36 empty vector and pUG36Fad2 and grown on liquid SD medium supplemented according to auxotrophic requirements without methionine in order to induce the expression of the cloned gene.

Yeast Strains

Mutant strains with deleted *SOD1* and *SOD2* [13] were constructed in the genetic background of parental *S. cerevisiae* strain US50-18C (MATα, PDR1-3, ura3, his1) [14]. Deletions of respective *SOD1* and *SOD2* genes were performed by the short flanking homology method [15] with replacement of the marker by the loxP-kanMX-loxP cassette [16].

Exposure to Oxidants

Twenty-four hours yeast cultures on YPD were diluted to OD 0.01 with YPD medium, 2 mM *tert*-butyl hydroperoxide (TBHP) or 70 μM methyl viologen (paraquat) or 2% ethanol was added and the mixtures were incubated at 30 °C without agitation for 24 h before OD_{600} evaluation.

Isolation of Plasma Membrane Lipids

Yeast strains transformed with empty vector pUG36 or containing flax desaturase, pUG36FAD2, were cultured in YPD medium to the optical density 0.5 was spun down for 5 min at 1,000*g*, resuspended in Tris buffer pH 9.3 and after β-mercaptoethanol addition (50 µl/10 ml cells) the suspension was shaken for 30 min. at 37 °C, spun down for 5 min at 1,000*g* and washed twice with 1.2 M sorbitol. The pellet was suspended in RT medium (1.2 M sorbitol, 10 mM EDTA, 10 mM Tris–HCl pH 7.5), Zymolase (50 mg/ml) was added and the sample was incubated for 1 h at 37 °C. The resulting spheroplasts were washed twice in 1.2 M sorbitol. Lipids were isolated by a method adapted from [17]. Spheroplasts were diluted (1:4) with distilled water, sonicated for 3 min and spun down for 7 min at 1,000*g* at 4 °C. The collected supernatant was centrifuged for 1 h at 4 °C at 28,000*g*. The plasma membranes collected in the pellet were suspended in chloroform–methanol (1:2, v/v) and stirred vigorously for 1 min. An equal volume of 0.9% NaCl was added and the mixture was again vigorously shaken for 5 min and left to settle for 24 h at RT. After separation, lipids in the chloroform lower phase were collected and chloroform was added to the upper phase. The sample was shaken vigorously, left to settle down and the lower phase was again collected after 24 h at RT. After evaporation of chloroform the lipids were weighed and resuspended in chloroform:methanol (1:2) mixture.

Determination of Yeast Fatty Acid Composition

The fatty acid composition of the total yeast fat was examined by gas chromatography. Methyl esters of the fatty acids (FAMEs) were obtained by esterification of fat samples by a modified method from [18]. This method is suitable for small lipid quantities with large amounts of unsaponifiables. Fifty milligram of lipids was saponified at 70 °C for 1 h with 1 ml of 2 M KOH in 75% aqueous methanol. The unsaponifiable material was extracted two times with petroleum ether and then with 1 ml 1.25 M HCl for 30 min (both solutions in anhydrous methanol). The FAMEs were extracted with hexane. The methyl ester mixture was separated on a CP-Sil 88 Chrompack capillary column (50 m × 0.25 mm). Helium was used as carrier gas and the separation was carried out at a temperature programmed from 150 °C (for 6 min) to 235 °C; the temperature increased at a rate of 6 °C/min. Particular fatty acids were identified by comparison with external standards.

Results

Isolation of the Flax Fad2

Genes coding for FAD2 desaturase have been identified in several higher plants including nearly all oil producing crop plants but not in flax. FAD2 enzymes are closely related in terms of their overall amino acid sequence. In addition, genes encoding microsomal oleic acid desaturase in species such as *Arabidopsis*, *Gossypium* or *Brassica* do not have introns within the coding sequence. Thus it was possible to design PCR primers within the conserved N and C terminals of *Fad2* based on the *Arabidopsis thaliana* gene (GenBank acc. no L26296) and dicot AUG context [19] with the aim of amplifying complete *Fad2* open reading frame on the genomic DNA of another species. Using AthFad2F and AthFad2R primers and Taq/Pfu polymerases mixture we have amplified on the genomic DNA from flax a 1137 nucleotide fragment and cloned it into pCR2.1 vector. The cloned fragment was sequenced on both strands. The isolated genomic DNA fragment (GenBank acc. No DQ222824) contains a complete open reading frame encoding a putative FAD2 flax protein. Encoded protein of 378 amino acids (Fig. 1) has the highest identity (79%) to *Ricinus communis*, gbABK59093.1, (78%) to *Sesamum indicum* gbAAF80560.1, *Vernicia fordii* gbAAN87573.1, *Cucurbita pepo* gbAAS19533.1, *Jatropha curcas* gbABA41034.1, *Hevea brasiliensis* gbAAY87459.1 and (74%) to *A. thaliana* (AAA32782.1). *S. indicum* FAD2 enzyme has been shown to be seed specific and responsible for desaturation of oleic to linoleic acid [20]. Flax FAD2 enzyme contains domains highly conserved among the desaturase family (His-rich boxes, membrane-spanning domains) but is 5-amino acid shorter than the majority of FAD2. The shorter length could result from a 5-amino acid insertion/deletion (indel) at position 12 from the N-terminus. To characterize this putative FAD2 protein prior to the introduction into flax, the sequence was introduced into yeast to confirm its role as FA desaturase.

Localization of GFP–FAD2 Fusion in Yeast

To check the subcellular localization of the flax FAD2 desaturase, the coding sequence has been introduced within the *Bam*H1 and *Xho*I restriction sites of pUG36 vector as N-terminal fusion with yEGFP, yielding pUG36Fad2 plasmid. Characteristic endoplasmic reticulum staining has been observed upon induction of the expression of FAD2 in yeast cells (Fig. 2). Superimposing the localization of vacuole using Nomarski contrast and FM4-64-staining clearly showed that the GFP staining extends to

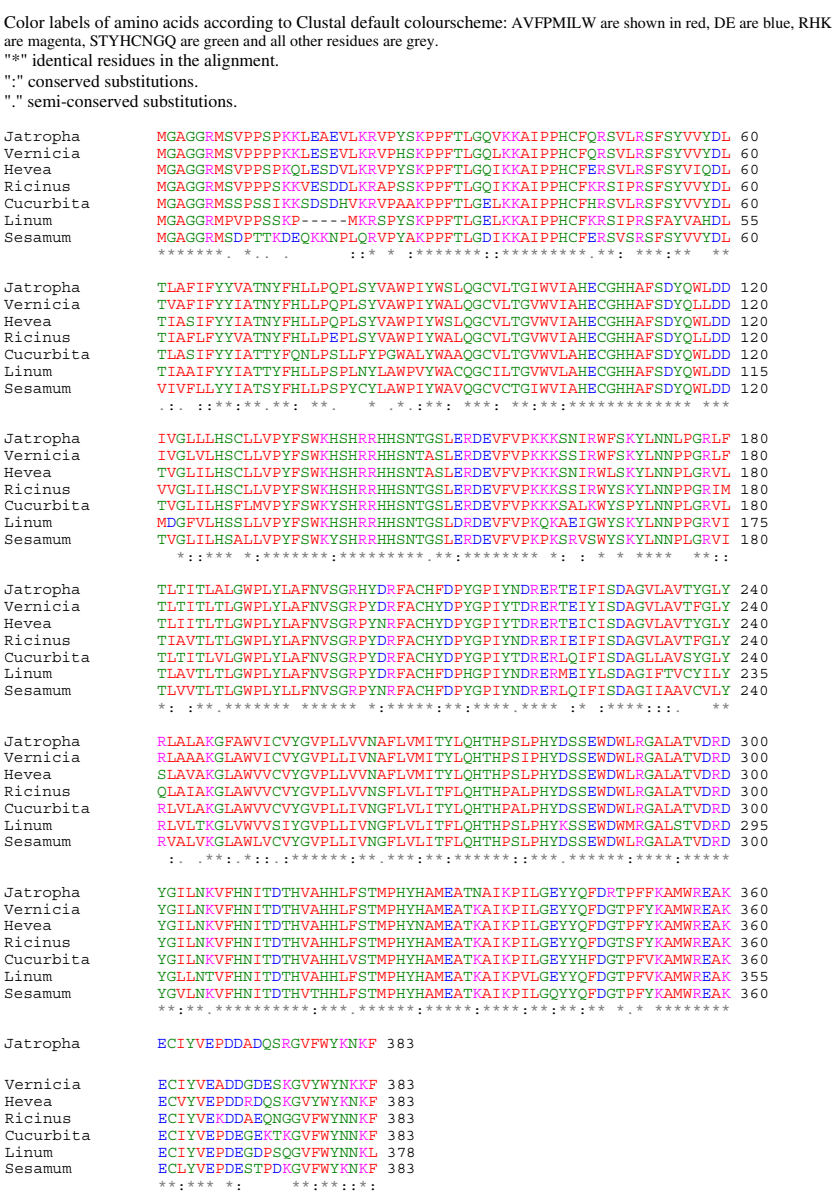

Fig. 1 CLUSTAL alignment [32] of FAD2 proteins from *Jatropha*, *Vernicia*, *Hevea*, *Ricinus*, *Cucurbita*, *Linum* and *Sesamum*

membranes other than vacuolar. Longer incubation resulted in relocalization of the GFP-tagged desaturase similar to that previously described for non-permissive conditions by Tatzer [21]. After longer incubation, the cytoplasm was becoming slightly fluorescent and high intensity fluorescent bodies appeared in some yeast cells. Yeast transformed with the control plasmid (pUG36) containing only GFP when cultured under inducing conditions showed the fluorescence to be uniformly distributed in the cytoplasm.

Enzyme Activity in Yeast Cells

Heterologous expression of flax FAD2 did not affect the growth of yeast culture under standard conditions. To evaluate the activity of the flax desaturase the yeast was cultured in the YPD medium without methionine repression of the met25 promoter. *S. cerevisiae* US50-18C cells transformed with pUG36Fad2 containing the *L. usitatissimum Fad2* gene showed increased accumulation of 16:2 and 18:2 fatty acids while the precursor level (16:1 and 18:1) decreased (Table 1). In the control culture of yeast transformed with pUG36 grown in the same conditions, 16:2 FA were below detection limits and the level of 18:2 FA was three times lower. This indicates that the cloned gene is functional and has activity similar to other plant oleate desaturases. Interestingly, the most marked difference in yeast with expression of FAD2 was increased amount of saturated fatty acids (16:0, 18:0). Besides the described above changes FAD2 expression also resulted in the increased accumulation of some longer fatty acids (22:0 and 22:1).

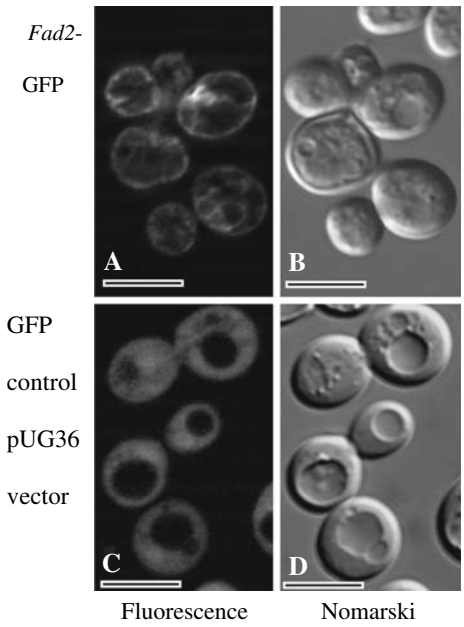

Fig. 2 Subcellular localization in *S. cerevisiae* cells of the flax GFP–FAD2 desaturase and GFP alone. The strains expressing pUG36Fad2 or pUG36 was cultivated at 30 °C under inducing conditions to the early logarithmic phase. The characteristic endoplasmic reticulum staining was observed for pUG36Fad2 (**a**), while the entire cytoplasm was stained in pUG36 (**c**). Nomarski views of the corresponding visual fields are shown on the *right* (**b**, **d**). *Bars* 10 μm

Sensitivity of Yeast to Oxidative Stress

Synthesis of polyunsaturated fatty acids (PUFA) in *S. cerevisiae* could influence their membrane fluidity and susceptibility to oxidation and might render the cells more susceptible to oxidative stress. Thus, in addition to the US50-18C strain, pUG36Fad2 plasmid has also been introduced into isogenic strains lacking cytoplasmic (SOD1) or mitochondrial (SOD2) superoxide dismutase. The growth of these three strains after pUG36Fad2 transformation has been compared on YPD medium (Fig. 3). The growth of original US50-18C strain and a *sod2* mutant was not significantly affected by FAD2 expression under the experimental conditions tested. In contrast, the growth of strain with deletion of *SOD1* was significantly poorer when transformed with pUG36Fad2 plasmid (Fig. 4). This inhibition was much stronger when the medium was supplemented with the oxidants TBHP, paraquat or ethanol and was visible also for the strain lacking SOD2. The Sod1

Table 1 Fatty acid composition (expressed as mass fraction of identified FA) in *S. cerevisiae* transformed with empty vector (control) and containing flax *Fad2* desaturase

	Fatty acids (% total)	
	Empty vector	FAD2 desaturase
14:0	3.2 ± 0.038	4.4 ± 0.005
16:0	18.9 ± 0.017	27.0 ± 0.006
16:1	26.9 ± 0.061	10.8 ± 0.013
16:2	0.0 ± 0.002	0.5 ± 0.012
18:0	9.0 ± 0.017	22.2 ± 0.021
18:1	38.8 ± 0.019	22.2 ± 0.010
18:2	0.9 ± 0.053	2.8 ± 0.023
18:3	0.0 ± 0.013	0.7 ± 0.005
20:0	0.4 ± 0.007	0.5 ± 0.001
20:1	0.0 ± 0.001	0.6 ± 0.008
22:0	1.0 ± 0.008	1.9 ± 0.006
22:1	0.7 ± 0.003	5.0 ± 0.001
24:0	0.2 ± 0.008	1.0 ± 0.041

The data represent mean values of three analyses ± SE

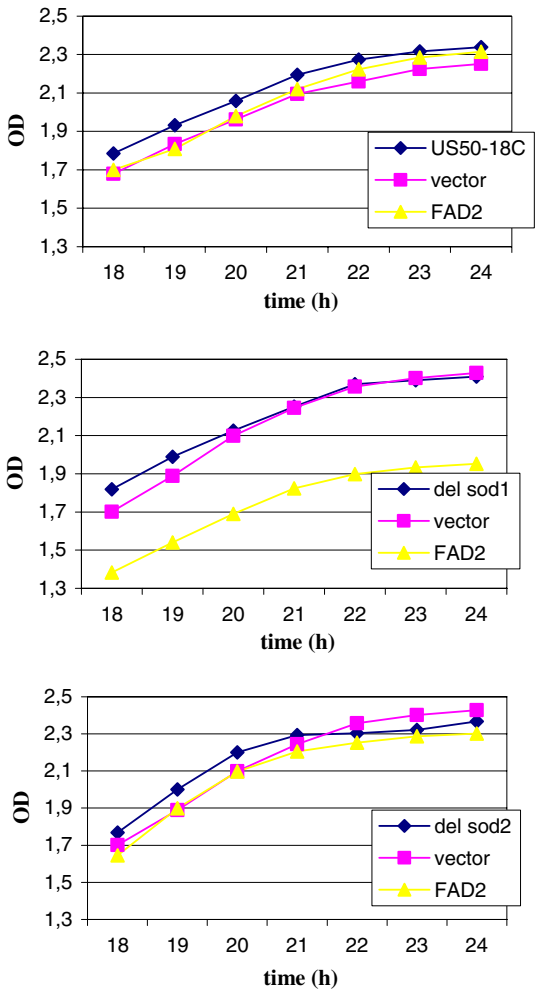

Fig. 3 Growth curves of analyzed strains with or without flax desaturase. Yeast strains (US50-18C and isogenic strains without *SOD*1 or *SOD*2 genes) were transformed with the pUG36 (vector) and pUG36Fad2 (FAD2) plasmids. Strains were diluted to OD_{600} 0.01 and optical density was measured. Data represent average from three independent experiments

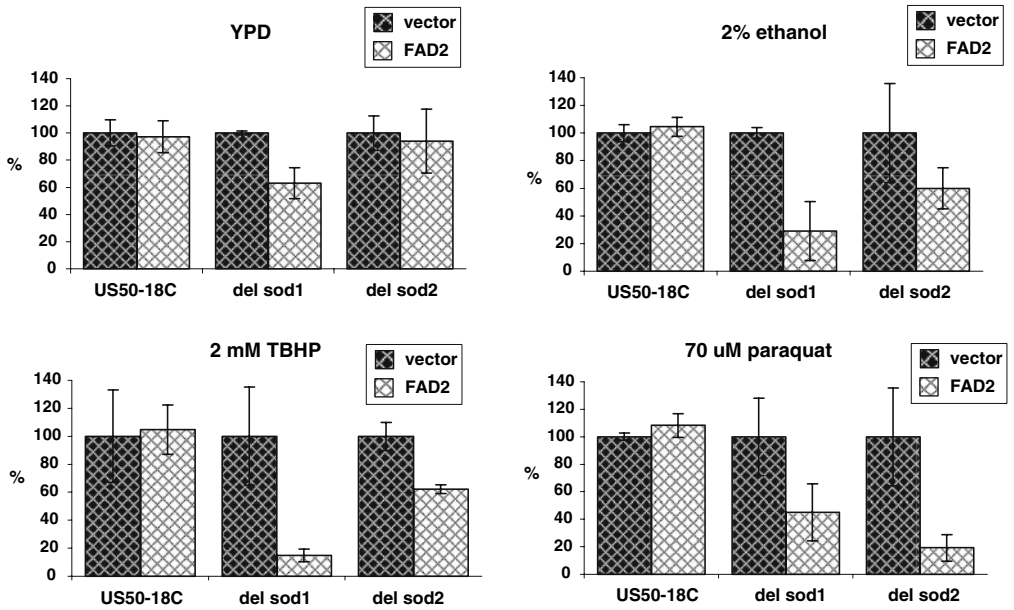

Fig. 4 Yeast growth inhibition by FAD2 activity under oxidative stress. Yeast strains (US50-18C and isogenic strains without *SOD*1 or *SOD*2 genes) were transformed with the pUG36 (vector) and pUG36Fad2 (FAD2) plasmids. Strains were diluted to OD_{600} 0.01 and optical density was measured after 24 h of incubation. Results are in percent relative to vector. Data represent three independent experiments ± SD

deletion mutant usually shows a stronger phenotype under oxidative stress. This was the case on YPD alone or supplemented with TBHP or ethanol. However, when the YPD medium was supplemented with paraquat, stronger growth inhibition was found for the strain without SOD2 dismutase. Taken together, our results suggest that under conditions of oxidative stress, PUFA production is deleterious to yeast growth.

Discussion

In crop plants, at least two FAD2 isoforms have been found which contribute in various degrees to the synthesis of seed storage TAGs. While several results have been published on *Fad2* genes of crop plants such as sesamus [20], cotton [9], soybean [12], rape [22] or sunflower [11], this is to our knowledge the first report on flax *Fad2* desaturase. In this study, based on *Fad2* sequence homologies between species, we designed primers and amplified on genomic DNA a full-length ORF of putative flax *Fad2* gene. Sequence comparison of a 378-amino acid putative protein showed the highest homology to *S. indicum* and *V. fordii* desaturases which have been shown to be specifically involved in linoleic acid synthesis in seeds. As these species are not closely related, the high homology could indicate that the cloned flax putative desaturase is also seed specific. Heterologous expression of this protein in yeast has shown characteristic localization within endoplasmic reticulum. Analysis of fatty acids extracted from yeast membranes showed an increased level of dienoic FA. This supports the assumption that the cloned gene encodes an active desaturase. Increased levels of both 16:2 and 18:2 fatty acids suggests that the enzyme is able to dehydrogenate palmitoleic and oleic acid. This feature has been observed in a various degree in other *Fad2* genes [23–25]. In contrast to other *Fad2* genes expressed in yeast the extent of accumulation of dienoic acids was lower. This could be due to isolation of lipids from YPD medium where expression of the introduced *Fad2* gene induction was weak.

It has been shown that FA desaturase is a very plastic enzyme and substrate specificity may be affected by the environment [26]. Also only minor changes in primary structure may result in a modification of the catalyzed reaction, promoting, e.g. hydroxylation instead of desaturation [27]. Thus, it is very interesting that flax FAD2 GFP-tagged protein conserved its activity.

Expression of the flax *Fad2* gene and accumulation of dienoic fatty acids triggers a severe response in yeast. Higher desaturation of FA affects membrane fluidity; to compensate for this, the synthesis of precursors (monoenoic FA) could be blocked and elongation of the fatty acid chain stimulated. In fact, FA analysis showed lower levels of monoenoic FA and higher levels of saturated and longer FA. In some experiments, expression of *Fad2* genes also results in the increase of saturated fatty [21] but changes are not so dramatic as in our experiments. The differences could be due to different media or strains

used and weak overexpression which allows cells to adapt to the appearance of dienoic fatty acids. It could also result from inhibition of the first unsaturation event. It has been found that *OLE1* performing the first desaturation step is inhibited by unsaturated fatty acids including 18:2 [28]. It is well established that the composition of cell membranes is dynamically adapted to environmental conditions. It has been shown that FA desaturation level and length play important roles in maintaining appropriate yeast membrane fluidity at various temperatures [29]. Recently, growing attention has been paid to the role of very long fatty acids (VLFAs) in *S. cerevisiae* (more than 18 carbons) and new functions of VLFAs have been discovered [30]. Although genes responsible for the synthesis of VLFAs are essential, VLFAs have often not been included in the published composition of FA isolated from *S. cerevisiae* because of their low amounts. Here we show that a higher amount of dienoic FA might result in a higher accumulation of VLFAs. This could be the response to modified membrane fluidity and/or oxidative stress. In fact, under oxidative stress we have observed modified composition of lipids and fatty acids (increase of longer and unsaturated FA) isolated from yeast membranes.

Lipid membranes are also the target of a free radical attack during oxidative stress. PUFA easily undergo peroxidation [8]. Among the first products of FA peroxidation are transient conjugated dienes. As FA peroxides are potent signaling molecules of oxidative stress [31], it was interesting to find out how expression of flax *Fad2* desaturase affects cell growth. It was very surprising that the growth of our control strain US50-18C in YPD medium alone or supplemented with oxidants was not significantly affected by the expression of desaturase. However, when the effect of FAD2 expression was tested in strains devoid of *Sod1* and *Sod2* and thus compromised for antioxidant defenses, these strains showed growth inhibition which increased after the addition of oxidants. Accumulation of oxidative stress factors (inside and outside the cell) strongly inhibits the growth of yeast cells with higher amount of unsaturated fatty acids. Our results suggest that both superoxide dismutases play an active role in protecting membranes against oxidative stress resulting in increased yeast mortality; this was also observed on spot-tests analyses (data not shown). One of the possible explanations of the severe *sod* phenotype is that dienoid FAs are incorporated into mitochondrial membranes where, in the absence of superoxide dismutases PUFA are not efficiently protected and impair energy production.

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