Polyunsaturated Fatty Acid Biosynthesis in *Saccharomyces cerevisiae*: Expression of Ethanol Tolerance and the *FAD2* Gene from *Arabidopsis thaliana*

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The *Arabidopsis thaliana* Δ -12 fatty acid desaturase gene (*FAD2*) was overexpressed in *Saccharomyces cerevisiae* by using the *GALI* promoter. *S. cerevisiae* harboring the *FAD2* gene was capable of forming hexadecadienoyl (16:2) and linoleoyl (18:2) residues in the membrane lipid when cultured in medium containing galactose. Gas-liquid chromatography analysis of total lipids indicated that the transformed *S. cerevisiae* accumulated these dienoyl fatty acyl residues and that they accounted for approximately 50% of the total fatty acyl residues. Phospholipid analysis of this strain indicated that the oleoyl (18:1) residue binding phosphatidylcholine (PC) was mostly converted to the 18:2 residue binding PC, whereas 50% of the palmitoleoyl (16:1) residue binding PC was converted to the 16:2 residue binding PC. A marked effect on the unsaturation of 16:1 and 18:1 was observed when *S. cerevisiae* harboring the *FAD2* gene was cultured at 8°C. To assess the ethanol tolerance of *S. cerevisiae* producing polyunsaturated fatty acids, the cell viability of this strain in the presence of ethanol was examined. The results indicated that *S. cerevisiae* cells overexpressing the *FAD2* gene had greater resistance to 15% (vol/vol) ethanol than did the control cells.

Phospholipid synthesis during membrane biogenesis in the yeast *Saccharomyces cerevisiae*, one of the most industrially exploited organisms, has many features characteristic of higher eucaryotic organisms. The fatty acyl residue composition of the phospholipids in the *S. cerevisiae* membrane, like that in other eucaryotic membranes, varies considerably in response to environmental stresses such as changes in temperature (25) and ethanol concentration (7, 12). Low-temperature stress reduces the fluidity of the *S. cerevisiae* plasma membrane. It has been suggested that this organism increases the amount of unsaturated fatty acyl residues in the membrane lipid to maintain membrane fluidity as an adaptation to low temperatures (12). There are many reports related to ethanol stress which suggest a relationship between membrane fluidity and ethanol tolerance (3, 13, 15). *S. cerevisiae* grown in the presence of ethanol appears to increase the amount of unsaturated fatty acyl residues in the membrane lipid, which increases the membrane fluidity (3, 16, 26). This is a supposed adaptation to ethanol stress. However, *S. cerevisiae* also has some differences from other eucaryotes, such as the biosynthesis of unsaturated fatty acids. Under normal growth conditions, many *S. cerevisiae* strains only form the monounsaturated fatty acids palmitoleic (16:1) and oleic (18:1) acids and do not produce the polyunsaturated species linoleic (18:2) and linolenic (18:3) acids, which are present in other fungi and plants (21).

In plants, the biosynthesis of the oleoyl-acyl-carrier protein (ACP) (18:1-ACP) from stearoyl-ACP (18:0-ACP) by stearoyl-ACP desaturase occurs in the chloroplasts, and the conversion of these fatty acyl-ACPs into their coenzyme A (CoA) thioesters by acyl-CoA synthetase then occurs in the outer enve-

lope (5, 22). The resulting acyl-CoAs are eventually utilized in the synthesis of phosphatidic acid, which is a precursor of membrane phospholipids, such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylglycerol, which occur in the endoplasmic reticulum (ER). Direct desaturation of 18:1-PC to 18:2-PC by Δ -12 fatty acid desaturase (*FAD2*) and the subsequent desaturation of 18:2-PC to 18:3-PC by ω -3 fatty acid desaturase (*FAD3*) are also known to occur in the ER membrane of plants (10). Recently, clones of the *Arabidopsis thaliana* genes *FAD2* and *FAD3* were isolated and investigated (1, 19, 27), yet little is known of the biochemical roles of these gene products in vivo. Since *S. cerevisiae* is incapable of polyunsaturated fatty acid formation, it is a suitable host for the genetic study of the role of these enzymes in eucaryotic organisms. A recent observation that linoleic acid formation in the yeast *Candida lipolytica* is similar to the plant desaturation pathway (18:1-PC and not 18:1-CoA is used) suggests that a plant fatty acid desaturase could be active in the yeast *S. cerevisiae* (9). Such studies will contribute to the industrial application of *S. cerevisiae* strains. It has previously been reported that *S. cerevisiae* cultured in medium enriched with linoleic acid showed an increase in cell viability in the presence of ethanol compared with that of cells grown in oleic acid-enriched medium. This suggests that phospholipid enriched with exogenous linoleic acyl residues results in an increase in membrane fluidity and ethanol tolerance (26).

This report describes our study in which the *A. thaliana* *FAD2* gene was overexpressed in *S. cerevisiae* and our determination of the biochemical nature of the *FAD2* protein. The contribution of *FAD2* overexpression to ethanol tolerance in *S. cerevisiae* is also discussed.

MATERIALS AND METHODS

Strains and media. *S. cerevisiae* IFO 10150 (*MATa ste-VC9 ura3-52 trp1-289 leu2-3 leu2-112 his3- Δ 1*) and INVSc1 (*MAT α ura3-52 trp1-289 leu2-3 leu2-112*

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FIG. 1. Structure of the plasmid used for the expression of *FAD2* in *S. cerevisiae*. In addition to containing the *FAD2* gene, this plasmid contains the 2 μ m plasmid origin (*ori*), the *URA3* gene marker, the *GAL1* promoter, and the *CYC1* terminator.

his3-Δ1) were used throughout this study. Strain INVSc1 was purchased from Invitrogen Co. YPD medium (1% [wt/vol] Bacto yeast extract, 2% [wt/vol] Bacto Peptone, 2% [wt/vol] glucose), was used in transformation experiments to introduce the *FAD2*-carrying plasmid. Cells harboring the plasmid were cultured in complete minimal (CM) dropout-uracil medium (0.13% dropout powder without uracil [2.5 g of adenine, 1.2 g of L-arginine, 6 g of aspartic acid, 6 g of glutamic acid, 1.2 g of L-histidine, 3.6 g of L-leucine, 1.8 g of L-lysine, 1.2 g of L-methionine, 3 g of L-phenylalanine, 22.5 g of L-serine, 12 g of threonine, 2.4 g of L-tryptophan, 1.8 g of L-tyrosine, 9 g of L-valine], 0.67% Difco yeast nitrogen base without amino acids, 2% [wt/vol] carbon source), which was described previously (2). In the present work, CM dropout-uracil medium containing glucose as a carbon source is referred to as CM(glucose) medium, and those containing raffinose and galactose are referred to as CM(raffinose) and CM(galactose) medium, respectively.

Plasmid construction and transformation. All recombinant DNA techniques used were standard procedures (2, 21). For the isolation of the *FAD2* gene, an *A. thaliana* cDNA library synthesized from *A. thaliana* (Columbia) whole plants was purchased from Stratagene. The *A. thaliana* *FAD2* cDNA was amplified by PCR techniques using *Pfu* DNA polymerase (Stratagene) according to the supplier's instructions. The primers used for PCR were oligonucleotides (5'-CCAGCTT ATGGGTGTCAGGTGGAAGAATGCCGTTCC-3' and 5'-CCGAATTCTCA TAACCTATTGTTGTACCAGTACACAC-3') designed from the *FAD2* cDNA sequence described by Okuley et al. (19). The amplified *FAD2* cDNA was ligated into the *SrfI* site of the vector pCR-Script SK(+) (Stratagene). The sequence of the *FAD2* cDNA was verified by DNA sequencing on an ABI 373A DNA sequencer (Applied Biosystems). Sequencing samples were prepared with a *Taq* Dye Primer Cycle Sequencing Kit (Applied Biosystems). The 1.2-kb *HindIII*-*EcoRI* fragment of *FAD2* was then inserted into the *HindIII*-*EcoRI* site of the expression vector pYES2 (Invitrogen Co.) to produce a new plasmid, pYG-*FAD2*, which was then transformed into *S. cerevisiae* by the lithium acetate method (2).

Galactose induction. Cultures of transformed *S. cerevisiae* cells were grown at 30°C for 16 h in CM(raffinose) medium. Test cultures were then incubated aerobically for 4 generations in CM(raffinose) medium which was supplemented with appropriate concentrations of galactose.

Lipid analysis. The cells were harvested, and the total lipids were extracted with chloroform-methanol (1:2, vol/vol) and then methylated with 5% hydrochloric acid in methanol at 85°C for 3 h. The phospholipids were separated by two-dimensional thin-layer chromatography on silica gel plates (0.25 mm thick). The plates were developed in the first dimension with a chloroform-methanol-28% ammonia (65:35:8, vol/vol/vol) solvent system and in the second dimension with a chloroform-acetone-methanol-acetate-water (10:4:2:3:1, vol/vol/vol/vol/vol) solvent system. Phospholipids were detected by spraying the plates with 0.1% primulin in acetone vapor. Each phospholipid spot was scraped from the thin-layer chromatography plates with a sharp razor blade and methylated as described above. The fatty acid methyl esters were analyzed by gas-liquid chromatography (model GC-8A chromatograph; Shimadzu, Tokyo, Japan) on a capillary column (model TC-70; GL Sciences, Tokyo, Japan). Arachidic acid methyl ester was used as an internal standard.

Cell viability test. Plate counts were performed to determine the ethanol tolerance of *S. cerevisiae* (26). A cell suspension was first diluted to a concen-

tration of 10^7 cells per ml in an aqueous solution containing 67 mM KH_2PO_4 (pH 4.5) and an appropriate concentration of ethanol. The *S. cerevisiae* cells in these solutions were incubated anaerobically at 30°C for 0, 2, 4, or 6 h, and the viability of the population was determined. The number of viable cells was determined by plating dilutions on three YPD agar plates and incubating them aerobically at 30°C for 48 h.

RESULTS

Expression of *FAD2* gene in *S. cerevisiae*. The plasmid pYG-*FAD2* carrying the *A. thaliana* *FAD2* gene was constructed from the vector pYES2 as described in Materials and Methods (Fig. 1). Since the *FAD2* cDNA had been inserted into the *HindIII*-*EcoRI* site located at the 3' end of the *GAL1* promoter, *FAD2* expression could be induced by the addition of galactose. The plasmid pYG-*FAD2* was introduced into *S. cerevisiae* IFO 10150 and INVSc1. In preliminary experiments, it was observed that the IFO 10150 strain could not grow in medium containing 2% raffinose and 2% galactose, but growth

FIG. 2. Capillary gas-liquid chromatography analysis of the total lipid fatty acids from *S. cerevisiae* IFO 10150 transformants. The IFO 10150 strain harboring pYG-*FAD2* (a) or pYES2 (b) was cultured in CM(raffinose) medium supplemented with 0.2% galactose at 30°C for 4 generations.

TABLE 1. Major fatty acyl residue composition of total lipids in *S. cerevisiae* transformants cultured at 30°C for 4 generations

Strain	Plasmid ^a	Galactose (%) ^b	Fatty acyl residue content (%) ^c						
			Saturated			Unsaturated			
			Total	C _{16:0}	C _{18:0}	Total	C _{16:1}	C _{16:2}	C _{18:1}
IFO 10150	pYG-FAD2	0.02	20.5	15.3	5.2	79.0	41.8	10.0	9.6
	pYES2	0.02	19.6	15.2	4.4	79.7	56.8		22.9
	pYG-FAD2	0.2	22.9	14.4	8.5	76.5	20.7	19.8	7.0
	pYES2	0.2	21.4	16.5	4.9	78.0	53.5		24.5
INVSc1	pYG-FAD2	2	35.8	25.8	10.0	60.2	30.1	6.1	9.6
	pYES2	2	32.0	22.2	9.8	67.9	38.1		29.8

^a Plasmids were introduced into *S. cerevisiae* IFO 10150 and INVSc1.^b Galactose concentrations of the CM(raffinose) medium are given.^c Results were determined from the peak areas of methyl esters. Values are means of two replicate analyses.

was restored when the galactose concentration was reduced to 0.2%. Therefore, the INVSc1 transformant was cultured in CM(raffinose) medium plus 2% galactose and the IFO 10150 transformant was cultured in CM(raffinose) medium plus 0.2% or 0.02% galactose.

The IFO 10150 and INVSc1 *FAD2* transformants were grown at 30°C for 4 generations, and then the total lipids were extracted and subjected to gas-liquid chromatography analysis (Fig. 2 and Table 1). Total lipids from IFO 10150 and INVSc1 carrying the vector pYES2 were also subjected to GLC analysis as a control. The trace obtained with the controls showed the general distribution of fatty acyl residues in the wild-type *S. cerevisiae* strain, and these were unaffected by the addition of galactose. The proportion of unsaturated fatty acyl residues in IFO 10150 was 1.16 times greater than that in INVSc1. On the other hand, the appearance of 18:2 and 16:2 in the *FAD2* transformants of both strains demonstrated that the *A. thaliana* *FAD2* protein functioned normally in *S. cerevisiae*. IFO 10150 *FAD2* transformants grown in CM(raffinose) medium containing 0.2% galactose produced almost 50% of the total fatty acyl residues as 16:2 and 18:2. When induced by 0.02% galactose, these dienoic fatty acyl residues accounted for about 25% of the total fatty acyl residues. These results confirm that the galactose-regulated expression system for pYG-FAD2 operated normally. INVSc1 *FAD2* transformants induced with 2% galactose only produced approximately 20% of the total fatty acyl residues as dienoic fatty acyl residues. Of particular interest is that the *A. thaliana* *FAD2* protein expressed in *S. cerevisiae* was active with not only oleoyl residues (18:1) but also palmitoleoyl residues (16:1). IFO 10150 transformants grown in 0.2% galactose synthesized about 20% of the total fatty acyl residues as 16:2, and the proportion of 16:1 in these cells was reduced to about 40% of that in the control cells, although the proportion of 16:0 in the wild type was similar to that in the transformant. The ratio of 16:2 to 16:1 was 1:1, and that of 18:2 to 18:1 was 4:1.

As the IFO 10150 *FAD2* transformant had a greater level of *A. thaliana* *FAD2* expression in the presence of 0.2% galactose, this transformant was used in subsequent experiments.

Appearance of fatty acids in major membrane lipids. The fatty acyl residues in the major membrane phospholipids PC and PE were chromatographed and analyzed (Table 2). In the *FAD2* producing cells, 18:2 accounted for about 30% of the total fatty acyl residues, while 18:1 accounted for 7% of the PC and PE classes. The proportion of 18:1 in both the PC and PE classes was reduced 7.5-fold relative to the control. The proportion of 16:2 in PC contrasted sharply with that in PE.

The ratio of 16:2 to 16:1 was approximately 1:1 in PC and

0.4:1 in PE. In contrast to the unsaturated fatty acyl residues, no significant differences in the saturated fatty acyl residue 16:0 were observed between the *FAD2*-producing cells and the control, apart from an increase in 18:0 in the *FAD2*-producing cells. The increase in 18-carbon fatty acyl residues (i.e., 18:0, 18:1, and 18:2) is also shown in Table 2.

Temperature-dependent synthesis of 16:2 and 18:2. Temperature has long been known to alter the fatty acyl residue composition of cell lipids. The accumulation of unsaturated fatty acyl residues in the IFO 10150 *FAD2* transformants and in the control was investigated at different temperatures (Fig. 3). The relative amount of 16:1 was found to increase in the control with decreasing temperature, while that of 16:0 decreased and that of 18:0 remained unchanged. These temperature-dependent characteristics of the *S. cerevisiae* IFO 10150 strain were retained in the *FAD2*-producing cells. However, although little difference was observed in the relative amounts of 18:2 and 16:2 at 30 and 15°C, the *FAD2* transformants grown at 8°C had less 18:2 and 16:2 than cells grown at 30 or 15°C. A peculiar feature of the *FAD2* transformants was the decrease in the relative amount of unsaturated fatty acids at 8°C, in contrast with the widely accepted phenomenon that lipid unsaturation is associated with low temperatures.

Ethanol tolerance. The viability of *FAD2* transformants in the presence of ethanol was measured to determine ethanol tolerance. *FAD2* transformants cultured for 4 generations were suspended in aqueous solutions of 67 mM KH₂PO₄ to which ethanol, at concentrations of 0, 5, 10, 15, and 20%, had been added. The viability of the transformants was then compared with that of the control cells (Fig. 4). In 15% ethanol, the *FAD2* transformants remained more viable than the control

TABLE 2. Major fatty acyl residue composition of PC and PE in *S. cerevisiae* IFO 10150 harboring pYG-FAD2 or pYES2

Plasmid ^a	Phospholipid	Fatty acyl residue content (%) ^b							
		Saturated			Unsaturated				
		Total	C _{16:0}	C _{18:0}	Total	C _{16:1}	C _{16:2}	C _{18:1}	C _{18:2}
pYG-FAD2	PC	21.3	11.6	9.7	77.5	22.9	24.5	2.2	27.9
	PE	9.9	8.4	1.5	89.3	34.7	13.9	5.3	35.4
pYES2	PC	16.8	9.5	7.3	81.8	63.3		18.5	
	PE	7.2	6.6	0.6	92.0	55.4		36.6	

^a Plasmids were introduced into *S. cerevisiae* IFO 10150.^b Results were determined from the peak areas of methyl esters. Values are means of two replicate analyses. Transformed cells were cultured in CM(raffinose) medium supplemented with 0.2% galactose at 15°C for 4 generations.

FIG. 3. Relationship between temperature and the fatty acyl residue composition of total lipids in *S. cerevisiae* IFO 10150 harboring pYG-FAD2 (a) or pYES2 (b). These cells were cultured in CM(raffinose) medium supplemented with 0.2% galactose for 4 generations. Values are means of two replicate analyses. Bars indicate the percent content of fatty acyl residues. Black boxes indicate the percentages of other fatty acyl residues.

cells. After 6 h of incubation, the viability of the *FAD2* cells was four times greater than that of the control cells. At lower ethanol concentrations, both types of cell retained most of their viability. However, when cell suspensions were exposed to 20% ethanol, both types of cell died within 1 h of incubation (data not shown).

DISCUSSION

By the introduction of an inducible *A. thaliana* *FAD2* gene into *S. cerevisiae*, we succeeded in producing an *S. cerevisiae* strain capable of endogenously synthesizing polyunsaturated fatty acids. Our results show that the *A. thaliana* *FAD2* gene functions normally in *S. cerevisiae*.

Unlike the *S. cerevisiae* Δ -9 desaturase (*OLE1*), which has a cytochrome *b*₅-like domain on its carboxyl terminus (17), the *FAD2* protein is thought to interact with cytochrome *b*₅ to produce its desaturase activity (14). It is possible that the *FAD2* protein is inserted into the ER membrane posttranslationally, as this protein lacks the retention signal domain found in integral ER membrane proteins and has no recognizable sequence for targeting to the ER (19). Thus, it would appear that the *A. thaliana* *FAD2* protein is inserted into the ER membrane and interacts with yeast cytochrome *b*₅ in *S. cerevisiae*. This view is supported by the observation that a large amount of dienoic fatty acyl residues appeared in the PC. To understand the exact mechanism of formation of the active *FAD2* protein in *S. cerevisiae*, further study is required.

It was previously reported that exogenous 18:2 in *S. cerevisiae* considerably repressed transcription of the *OLE1* gene (4). The expression of the *FAD2* gene in *S. cerevisiae* showed that a large amount of dienoic fatty acids was synthesized and that the proportion of unsaturated fatty acyl residues was slightly reduced. Hence, this could result from a reduction in *OLE1* mRNA levels.

It was surprising that the amounts of 18:2 and 16:2 in the INVSc1 *FAD2* transformants, grown and induced in 2% galactose, were smaller than those found in IFO 10150 transformants grown in CM(raffinose) medium and induced by 0.2 or 0.02% galactose. To investigate the time course of *FAD2* ex-

pression, we carried out Northern (RNA) blot analysis of total RNA obtained from the IFO 10150 *FAD2* transformants grown for different time periods. Although galactose induction is known to occur within a few minutes of exposure to galactose (23), it was not until after 21 h (3 generations) of cultivation that the *FAD2* transcript could be detected as a high-level signal in our experiment (data not shown). These results suggest that IFO 10150, which is incapable of metabolizing galactose, transported minimal amounts of galactose and only accumulated large amounts of galactose after a long incubation period.

The *FAD2* desaturase protein found in many plants catalyzes only the conversion of 18:1-PC to 18:2-PC, since 16:1 is not found in the plant ER membrane (18). However, the *S. cerevisiae* *FAD2* transformants synthesized 16:2-PC in addition to 18:2-PC, as 16:1-PC is present in the ER. Thus, the *FAD2*

FIG. 4. Decrease in cell viability of populations of *S. cerevisiae* IFO 10150 harboring pYG-FAD2 (circles) or pYES2 (triangles) when incubated at 30°C in either 67 mM KH_2PO_4 (open symbols) or 67 mM KH_2PO_4 containing 15% ethanol (closed symbols). Vertical bars represent the standard errors for three replicate experiments.

protein expressed in *S. cerevisiae* is capable of recognizing both 18:1-PC and 16:1-PC as substrates. On the other hand, the enzyme appears to react preferentially with a specific substrate structure, because the ratio in PC of 18:2 to 18:1 was much greater than that of 16:2 to 16:1 (Table 2). The presence of the dienoic fatty acyl residues in PE was also shown by our experiments. As PC is the end product of the *S. cerevisiae* phospholipid biosynthetic pathway, acyl-PE is not synthesized from acyl-PC (6). The PE dienoic fatty acyl residues probably originated from the dienoic fatty acyl group, a derivative of acyl exchange between acyl-CoA and acyl-PC (24).

FAD2 transformants grown at 8°C contained a relatively larger amount of unsaturated fatty acyl residues than did transformants grown at 30°C, which is in general agreement with the phenomenon of cellular adaptation to cold (25). However, the proportion of polyunsaturated fatty acyl residues decreased. The reduction in the 18:2 and 16:2 fatty acyl residue amounts is probably due to a reduction in *FAD2* expression from the *GAL1* promoter and/or in the activity of the *FAD2* protein in *S. cerevisiae*.

In general, ethanol tolerance in yeasts has been examined by using three parameters, yeast growth rate, fermentation rate, and cell viability (7). We tested the effect of ethanol on the cell viability of the IFO 10150 *FAD2* transformants. This strain showed a lower rate of viability loss than did the control strain when suspended in 15% ethanol. This result supported previous reports that cells enriched in linoleoyl residues remained more viable than cells enriched in oleoyl residues (26). This suggested that not only exogenous but also endogenous polyunsaturated fatty acyl residues lead to an increase in cell viability. The two other parameters could not be used to determine ethanol tolerance, because the *FAD2* transformants had a growth rate lower than that of the control strain in the absence of exogenous ethanol. Overexpression of the *FAD2* gene appears to be a burden on the cell. To determine ethanol tolerance on the basis of the two parameters, further study is required to produce *S. cerevisiae* cells with an appropriate level of *FAD2* gene expression.

As described above, the concentration of polyunsaturated fatty acids accounted for 50% of the total fatty acids in *S. cerevisiae* IFO 10150 carrying the *FAD2* gene. It is therefore possible that other foreign desaturase genes, such as *FAD3*, could be expressed in *S. cerevisiae*. Biotechnology may provide new possibilities of changing the fatty acyl residue composition in the membrane lipids of *S. cerevisiae* and may provide a means of determining the response mechanism of the fatty acyl residue composition of eucaryotic membranes to environmental stress.

At the completion of this study, another group reported an expression system similar to the one described in this work (8).

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