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Functional Expression of the Extraplastidial *Arabidopsis thaliana* Oleate Desaturase Gene (*FAD2*) in *Saccharomyces cerevisiae*[1]

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The functional expression in yeast of the *Arabidopsis thaliana FAD2* gene, encoding the extraplastidial oleate desaturase (1-acyl-2-oleoyl-*sn*-glycero-3-phosphocholine Δ12-desaturase) is reported. Dienoic fatty acids constituted up to 11% (w/w) of the total fatty acids in transformed *Saccharomyces cerevisiae* cells and were confirmed to be linoleic acid and Δ^9,Δ^{12}-hexadecadienoic acid by gas chromatography-mass spectrometry.

Plants possess fatty acyl desaturases both in the plastid and the ER with a range of substrate and positional specificities (for recent reviews, see Heinz, 1993; Murphy, 1994; Ohlrogge and Browse, 1995). Unlike most other higher eukaryotes, they produce polyunsaturated fatty acids, particularly linoleic (18:2) and α-linolenic (18:3), which are thought to be essential for membrane function. One of the most important enzymes for the production of polyunsaturates in plants is the oleate desaturase (1-acyl-2-oleoyl-*sn*-glycero-3-phosphocholine Δ12-desaturase) of the ER. This enzyme is thought to be an integral membrane protein that accepts 1-acyl-2-oleoyl-*sn*-glycero-3-phosphocholine as a substrate and requires NADH, NADH:Cyt b_5 oxidoreductase, Cyt b_5, and oxygen for activity (Smith et al., 1990; Kearns et al., 1991). The gene for the oleate desaturase has been recently cloned from *Arabidopsis thaliana* by Okuley et al. (1994). Sequence analysis of this and other desaturase genes has identified a number of conserved His-containing motifs that are thought to be involved in binding active-site iron atoms (Shanklin et al., 1994). An enzyme from castor (*Ricinus communis*) with a high degree of sequence similarity to plant fatty acid desaturases, including the His motifs, catalyzes the hydroxylation of 1-acyl-2-oleoyl-*sn*-glycero-3-phosphocholine at the 12 position in a reaction that is thought to be closely related to desaturation (see van de Loo et al., 1995, and refs. therein).

The biochemical mechanism(s) and structure/function relationships of the plant fatty acid desaturases are still not well understood. The determinants of positional specificity are not known, and the structural relationships between desaturases and other fatty acid modifiers such as the castor hydroxylase are not known. Some progress in this area has been made in the study of other plant and mammalian fatty acyl desaturases (Fox et al., 1993, 1994; Hitz et al., 1994; Shanklin et al., 1994) and a cyanobacterial Δ-12 desaturase (Avelange-Macherel et al., 1995) expressed in heterologous hosts. Heterologous expression has allowed for structure/function studies through the use of site-directed mutagenesis. However, no extraplastidial fatty acyl desaturase from plants has previously been expressed in a microbial host. Functional expression requires the presence of a suitable membrane environment (ER) and electron donor (Cyt b_5) for the enzyme. Consequently, a eukaryote is likely to be the most suitable host. In this paper, we report the expression the *A. thaliana FAD2* gene (encoding oleate desaturase) in *Saccharomyces cerevisiae* and confirm the regioselectivity of the enzyme.

MATERIALS AND METHODS

Expression Vector Construction

The open reading frame of the *FAD2* gene, originally cloned by Okuley et al. (1994), was amplified by PCR in a 100-μL volume containing 250 ng of *Arabidopsis thaliana* (var Landsberg) DNA, 50 pmol each of the oligonucleotide primers GCCGAATTCATGGGTGCAGGTGGAAGA and GCCGAATTCCACCATCATGCTCATAACT, 20 mM Tris-HCl, 1.5 mM $MgCl_2$, 25 mM KCl, 0.05% Tween 20, 50 μM each dATP, dCTP, dGTP, and TTP, and 2.5 units of *Taq* DNA polymerase. Thirty cycles of denaturation (96°C, 15 s), annealing (55°C, 30 s), and extension (72°C, 90 s) were performed. The resulting 1.2-kb PCR product was digested with *Eco*RI and ligated with *Eco*RI-digested pSE936 (kindly provided by Ronald W. Davis [Stanford University, Stanford, CA]; Elledge et al., 1991) to give the plasmid pDR1. The pSE936 plasmid is a *Saccharomyces cerevisiae* centromeric expression vector containing the *GAL1* promoter. The sequence of the insert of pDR1 was confirmed to be identical to that previously reported (Okuley et al., 1994) and to be in the sense orientation relative to the *GAL1* promoter using the PRISM DyeDeoxy Terminator Cycle

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Abbreviations: CM -ura gal, minimal medium lacking uracil with Gal replacing Glc; X:Y, a fatty acid with X carbon atoms and Y *cis* double bonds (if necessary, the position of the double bonds relative to the carboxyl is indicated as a superscript).

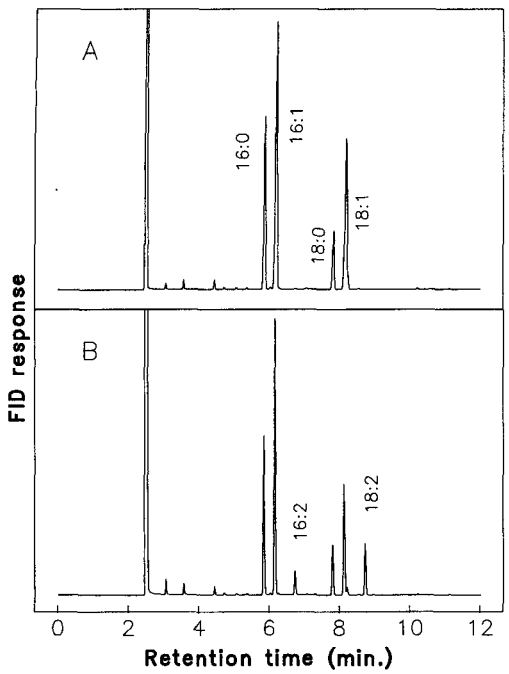

Figure 1. Gas chromatographic analysis of fatty acid methyl esters from yeast transformed with pSE936 (A) and pDR1 containing the *A. thaliana FAD2* gene (B) and grown in CM -ura gal at 15°C. FID, Flame ionization detector.

Sequencing System (Perkin-Elmer/Applied Biosystems) and a model 373 DNA sequencer (Applied Biosystems). The *S. cerevisiae* strain MKP-o (*MATα can1–100 ade2–1 lys2–1 ura3–52 leu2–3, 112 his3-Δ200 trp1-Δ901*; kindly provided by Wei Xiao [University of Saskatchewan, Saskatoon, Canada]) was transformed with pSE936 and pDR1 by the method of Gietz et al. (1992) and selected on minimal agar plates lacking uracil (Ausubel et al., 1995).

Growth and Biochemical Analysis of Transformed Yeast

Minimal medium lacking uracil and containing Glc and CM -ura gal were inoculated with yeast and incubated at a range of temperatures until the stationary phase was reached. For fatty acid analysis, the cells of 10-mL aliquots of saturated cultures were collected by centrifugation and washed once with 10 mL of distilled water. The pellets were dried under vacuum, and fatty acids were transmethylated, extracted, and analyzed by GC as described by Taylor et al. (1992). For double-bond position analysis, fatty acid methyl esters were saponified and the resulting fatty acids were derivatized with diethylamine according to Nilsson and Liljenberg (1991). GC-MS analyses of the diethylamide derivatives were performed as described by Carrier et al. (1995).

RESULTS AND DISCUSSION

Production of Dienoic Acids in Yeast Transformants

S. cerevisiae cells transformed with pDR1 containing the *A. thaliana FAD2* gene showed accumulation of 16- and 18-carbon dienoic fatty acids that are not normally present in wild-type yeast (see Fig. 1). 16:2 and 18:2 were not detected in control cultures of yeast transformed with pSE936 grown in the presence of Gal (Fig. 1) and of the pDR1 transformant grown under Glc repression of the *GAL1* promoter (data not shown). This indicates that the plant oleate desaturase gene was functionally expressed in the yeast cells and acted on the endogenous monounsaturated substrates to give 16:2 and 18:2. To confirm the regioselectivity of the *FAD2* gene product in yeast, diethylamides of fatty acids from the DR1 strain were analyzed by GC-MS (Nilsson and Liljenberg, 1991). Figure 2 shows a mass spectrum of the compound identified as 18:2, which was present in the DR1 strain and absent from the SE936

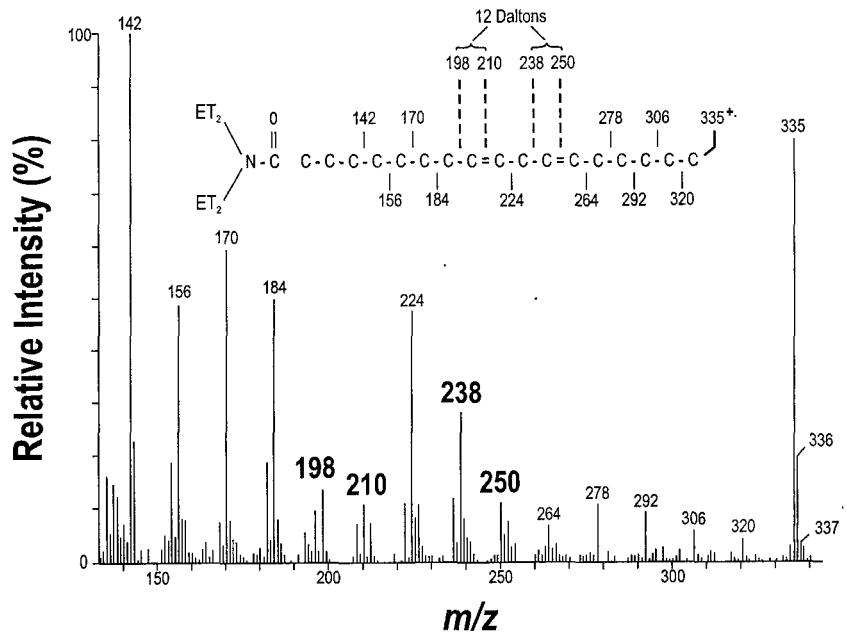

Figure 2. Mass spectrum of 18:2 peak from yeast transformed with pDR1 (see Fig. 1B). The structure of the diethylamide of 18:2 is shown with *m/z* values for ions that include the amide moiety. Note the two pairs of ions at 198 to 210 and 238 to 250, which are diagnostic for double bonds at the Δ9 and Δ12 positions, respectively.

strain. Major fragments of n and $n+1$ carbons differing by 12 D are diagnostic of a double bond between carbons $n+1$ and $n+2$. Thus, the fragments of 198, 210, 238, and 250 D indicate double bonds at the Δ9 and Δ12 positions. Similar data (not shown) identify the 16:2 peak as $^{\Delta 9,\Delta 12}$16:2 and confirm the identity of the other fatty acids shown in Figure 3. Together the MS data are consistent with the *FAD2* gene product being a Δ12-desaturase with regioselectivity determined by the double bond (Δ9 position) and carboxyl group of the substrate (Howling et al., 1972; Miquel and Browse, 1992; Heinz, 1993).

Initially, dienoic acid accumulation was limited to less than 1% (w/w) of total fatty acids when pDR1 transformants were grown at the customary 28 to 30°C. However, it was subsequently found that in cultures grown at lower temperatures there was a greater accumulation of these fatty acids (Fig. 3). At 15, 22, and 28°C, linoleic acid accumulates to 9.2, 6.4, and 0.6% (w/w), respectively, of total fatty acids in the DR1 strain. There could be a number of explanations for this temperature effect. For example, at the higher temperatures there could be a detrimental effect of polyunsaturates or decreased enzyme stability.

A few aspects of the behavior of the DR1 strain are notable (data not shown). At given temperatures, growth rates and total fatty acid levels were not noticeably different between SE936 and DR1 strains. In Gal-grown DR1 cultures, dienoic acid levels as a fraction of total fatty acids were maximal at the stationary phase. Apparently, the *FAD2* gene product was not highly expressed, since polypeptide profiles of the MKP-o and DR1 strains determined by polyacrylamide electrophoresis were not qualitatively different.

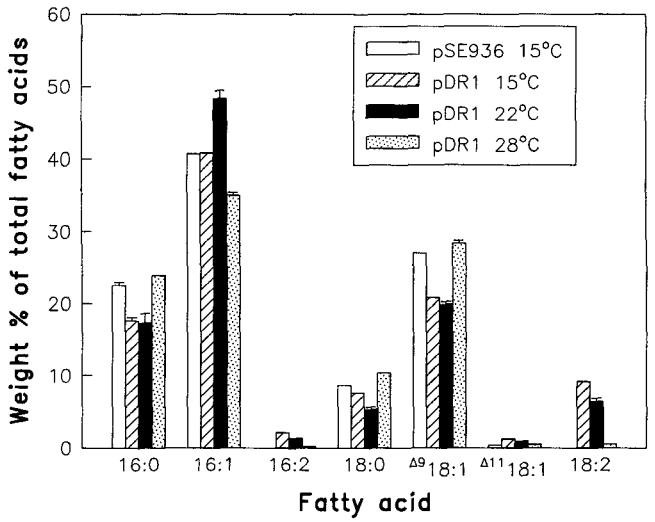

Figure 3. Effect of temperature on the accumulation of dienoic acids in the DR1 yeast strain. Fatty acid levels as a fraction of total fatty acids are indicated for pSE936 and DR1 transformants grown in CM -ura gal at the temperatures shown. The additional fatty acids 20:0, 20:1, 22:0, and 24:0 were detected in the 0.01 to 1.0% (w/w) range in both SE936 and DR1 cultures. Where greater than 0.3% of total fatty acids, SE values of triplicate samples are shown.

The fact that we have obtained functional expression of a plant membrane-bound fatty acyl desaturase in yeast suggests that this host provides a suitable membrane and redox environment for the enzyme. It is likely that at least some of the heterologous gene product finds its way to the yeast ER, where it interacts with Cyt b_5. The effective expression of *FAD2* and similar genes in yeast, combined with site-directed mutagenesis, should provide a powerful system for the study of structure-function relationships of plant membrane-bound desaturases and related enzymes such as the fatty acyl hydroxylases.

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