A desaturase-like protein from white spruce is a Δ^9 desaturase

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Abstract ***Gymnospermae*** **seed lipids are characterized by a high degree of desaturation, most having a Δ^9 double bond. By degenerate polymerase chain reaction (PCR) we have isolated a white spruce (*Picea glauca*) cDNA clone that encodes an amino acid sequence sharing a high degree of homology with other putative plant acyl-coenzyme A (CoA) Des9 desaturases. Both in vivo and in vitro expression studies in a Δ^9 desaturase-deficient yeast strain demonstrated the desaturation functionality of the white spruce clone, and gas chromatography-mass spectrometry (GC-MS) analyses confirmed the regioselectivity of the encoded enzyme. This is the first report of the functional characterization of a plant membrane-bound acyl-CoA-like protein Δ^9 desaturase by heterologous expression in yeast.** © **2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Gymnosperm; Conifer; Δ^9 Desaturase; Fatty acid; Gene expression

1. Introduction

Plastidic fatty acids are important components of membrane lipids synthesized by the fatty acid synthase complex with most acyl intermediates being esterified to acyl carrier protein (ACP). The further desaturation of C_{16} and C_{18} fatty acyl moieties to those highly unsaturated fatty acids characteristic of plant cell membrane components are carried out by two types of desaturases, those in the chloroplast (plastid) and those in the endoplastic reticulum (ER). Desaturases differ not only by their regioselectivity, but also by their substrate specificity [1], a characteristic used in their classification. Both acyl-lipid and acyl-coenzyme A (CoA) desaturases are membrane-bound enzymes. In yeast and mammals, acyl-CoA desaturases react with CoA thioesters [2,3], while in plants and cyanobacteria, acyl-lipid desaturases utilize fatty acids esterified to glycerolipids [4,5].

Several plant Δ^9 desaturases have been identified and characterized in species as diverse as *Brassica napus* [6], safflower [7], castor and cucumber [8], spinach [9] and cat's claw [10]. All of these plant *Des9* desaturase genes encode soluble plastidial acyl-ACP desaturases. However, clones from rose [11], *Arabidopsis* [12] and the red alga *Cyanidioschyzon merolae* [13] whose amino acid sequences share some homology with yeast or mammalian Δ^9 acyl-CoA desaturases, have been reported, but none have been functionally characterized. Recently, the first functional characterization of a plant acyl-CoA desaturase gene and its expression in soybean somatic embryos were reported for a *Limnanthes Des5* [14].

Gymnospermae seeds are rich in highly polyunsaturated lipids, the most abundant fatty acids in conifers having double bonds on the Δ^5 and Δ^9 carbons [15]. Although several biochemical and nutritional studies have shown the beneficial effects of conifer seed oil, such as lowering cholesterol in blood serum [16], very little has been done so far at the molecular level to exploit this genetic source for seed oil modification.

In this study, we report the cloning of a desaturase gene isolated from white spruce and its functional characterization as an acyl-CoA-like protein *Des9* gene by heterologous expression in yeast. This is, to our knowledge, the first report on the isolation of a desaturase gene in *Gymnospermae*, and most importantly, the first functional identification of a membrane-bound acyl-CoA-like Des9 protein isolated from plants.

2. Materials and methods

2.1. Substrates

Non-radiolabeled lipid chemicals were purchased from NuChek Prep Inc. Other biochemicals or high performance liquid chromatography (HPLC)-grade solvents were obtained from Sigma Aldrich Canada and EM Science, respectively. Radiolabeled ^{14}C-18:0-CoA was synthesized from the corresponding ^{14}C-18:0 (Amersham) as described previously [17].

2.2. Gene cloning and yeast transformation

Using total RNA from white spruce mature somatic embryos (*Picea glauca* (Moench) Voss, courtesy of Drs D. Dunstan and J.-Z. Dong, PBI/NRC) and degenerate primers designed from conserved histidine motifs of putative *Des9* desaturase genes (*Arabidopsis thaliana*, rose, *Anabaena*, yeast and human), a 500 bp fragment was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) and sequenced. A BLAST search revealed some sequence similarity to other *Des9* desaturases and acyl-CoA desaturase-like genes. The missing termini were produced by 3′ and 5′ rapid amplification of cDNA ends (RACE) (Gibco BRL) and the full-length clone amplified by PCR with a pair of gene-specific primers (forward primer: GCCATGGCAGCATTAGTATTATCA and reverse primer: CTACAACCCTGGCTTGGAAGACA) adding a Kozak consensus sequence to the 5′ end of the gene for enhanced gene expression in yeast [18]. This putative gene for the spruce Δ^9 desaturase was cloned into pYES2.1/TOPO 10F′ (Invitrogen) behind the galactose-inducible *GAL1* promoter, and the AMY-2α yeast strain ([19] courtesy of Dr. C. Martin) was transformed using the LiCl method [20]. This yeast

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Abbreviations: ACP, acyl carrier protein; DEA, diethylamide; *Des9*, the white spruce gene encoding a Δ9 desaturase; FAME, fatty acid methyl ester; FFA, free fatty acid; GC-MS, gas chromatography-mass spectrometry; HPLC, high performance liquid chromatography; MGDG, monogalactosyldiacylglycerol; PC, phosphatidylcholine

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strain carries a mutation in the stearoyl-CoA desaturase gene (*ole1*) leading to the production of a defective Des9 desaturase.

2.3. Yeast growth and biochemical characterization

Transformed yeast cells were grown at 28°C on minimal medium lacking uracil but containing glucose (SD-ura, glucose), and supplemented with ricinoleic acid (200 μg/ml culture) in 0.1% tergitol. The AMY2α yeast strain containing the empty plasmid vector pYES2.1 was used as a negative control. At growth saturation, the cultures were transferred into 50 ml of SD-ura, with glucose/galactose (50/50 v/v) and grown at 20°C for 3 days, then at 15°C for another 3 days. These growth conditions have been shown to favor the accumulation of unsaturated fatty acids in yeast [21]. Fatty acid methyl esters (FAMEs) and diethylamide (DEA) derivatives were produced as described [22,23] and analyzed by gas chromatography (GC) and/or HPLC followed by GC-mass spectrometry (MS).

2.4. Analysis of lipid fractions

Total lipid extracts (TLE) were prepared and saponified as described [24]. An aliquot (10%) of the free fatty acid (FFA) fraction was transmethylated [22] to produce FAMEs for GC analysis. DEA derivatives were prepared on the remaining FFAs according to Nilsson and Lijenberg [23]. HPLC fractions containing the $16{:}1^{\Delta 9}$ and $18{:}1^{\Delta 9}$ DEAs were collected and the expected retention times verified by GC prior to establishing the position of the double bonds in the acyl derivatives by GC-MS.

2.5. Stearoyl-CoA desaturase in vitro assay

Yeast transformants and plasmid-only negative control cultures were grown as described above, at either 15 or 20°C. Cells were centrifugated, washed and lysed in 1 ml grinding buffer (80 mM HEPES (pH 7.4), 1 mM EDTA, 1 mM DTT, 0.32 M sucrose) with glass beads [25]. Cell wall breakage was verified with a microscope before filtering the lysate. The protein concentration was adjusted to 5 μg/μl lysate and the stearoyl-CoA desaturase assay performed, in duplicate, for 1 h at 25°C by adding [1-^{14}C]-18:0-CoA (180 μM, 10 nCi/nmol) and 0.5 mM NADH and shaking at 100 rpm, in open tubes. The reaction was stopped by adding 3 ml of 10% KOH in methanol (tube capped) and heated at 80°C for 1 h. FAMEs were produced as above for reverse phase radio-HPLC analysis [22].

3. Results and discussion

3.1. Structural characterization of the white spruce Des9 gene

Based on DNA sequence information from other known *Des9* genes, a full-length clone was generated by PCR, sequenced in both directions and the nucleotide sequence submitted to the GenBank database (accession number AF438199). The nucleotide sequence had an open reading frame of 1149 bp encoding a 383 amino acid polypeptide with a calculated molecular weight of 43.7 kDa. A BLAST search showed that this clone was most similar to other putative plant acyl-lipid or acyl-CoA *Des9* desaturases (*A. thaliana* and *Rosa hybrida*) and to the *Limnanthes* acyl-CoA *Des5* gene, but not to plant acyl-ACP desaturases. At the amino acid level, the white spruce clone shared 57.1% with an *Arabidopsis* clone (AB017071) hypothesized to be a *FAD5* gene by its chromosomal location [26], but also 47.8% similarity to the rose putative acyl-CoA *Des9* (AAB50679), 48.2% with the putative *Arabidopsis* acyl-CoA *Des9* (BAA25181) and 43.8% with the *Limnanthes* acyl-CoA *Des5* gene (AF247133), the only clone so far to be functionally characterized. This similarity was particularly strong in three histidine-rich motifs (**HXXXXH**, **HXXHH** and **HXXHH**) that are highly conserved among membrane-bound acyl-CoA and acyl-lipid desaturases and are believed to correspond to a diiron active site of the enzyme [27]. The predicted conifer protein was largely hydrophilic, except for the central domain which contained four hydrophobic sections, probably representing the membrane-spanning regions also typical of other membrane-bound desaturases (Kyte-Doolittle plots, data not shown). There was no evidence of a cytochrome b_5 domain, the electron donor required for desaturation by microsomal enzymes. Finally, computer-based analyses using various software led to contradictory results and therefore no definite conclusion could be drawn as to the exact localization of the enzyme encoded by the white spruce clone.

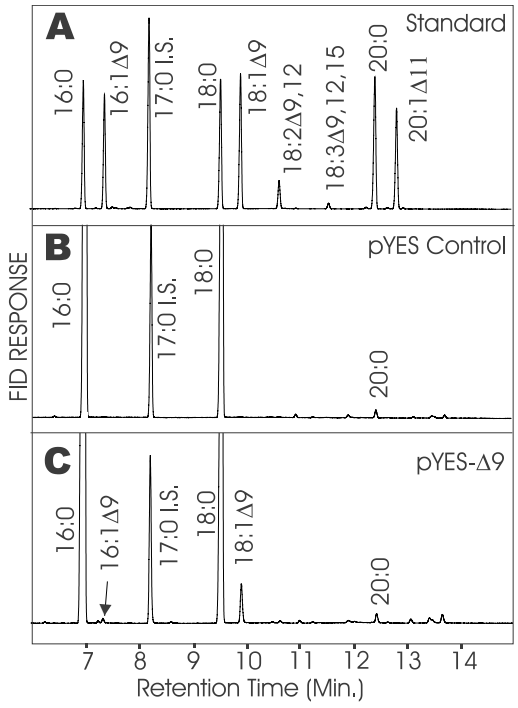

Fig. 1. Gas chromatogram of FAMEs. A: Authentic standards. B: FAMEs from the plasmid-only control yeast lysate. C: FAMEs from the lysate of yeast transformed with the putative white spruce *Des9* gene. The relevant section of the chromatogram corresponding to the C_{16}-C_{18} FAMEs is shown, but not the one for the ricinoleic acid FAME (elution time 19.31 min). IS, internal standard.

3.2. In vivo analyses

Yeast has been successfully used as an expression system to characterize several enzymes involved in fatty acid biosynthesis, especially plant membrane-bound fatty acid desaturases [21,28,29]. In this study, yeast cells transformed with the putative *Des9* gene were able to produce a significant proportion of $18{:}1^{\Delta 9}$ (1.30%) and, to a lesser extent, $16{:}1^{\Delta 9}$ (0.15%) from 18:0 (26.4%) and 16:0 (69.6%) respectively. These values are calculated as relative proportions among the relevant C_{16}-C_{18} saturated and monounsaturated FAMEs. This is illustrated on the GC chromatogram (Fig. 1C) which shows the presence of two new peaks with retention times corresponding to $16{:}1^{\Delta 9}$ and $18{:}1^{\Delta 9}$. Both monounsaturated fatty acids were absent in the plasmid-only control (Fig. 1B). Mass spectrometry analyses conducted on DEA derivatives confirmed that the double bonds in the new compounds detected in the transformed yeast cell extracts were positioned at the Δ^9 carbon (Fig. 2). The ricinoleic acid FAME (data not shown in Fig. 1) eluted at a retention time of 19.31 min and, because it was added to the culture medium at a relatively high concentration (670 μM), it typically constituted about 62% of the full FAME spectrum.

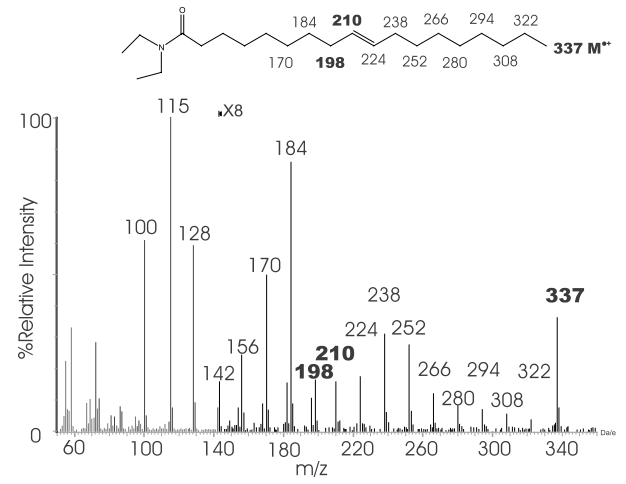

Fig. 2. Mass spectral analysis of fatty acyl-DEA derivative of the 18:1$^{\Delta 9}$ peak from yeast transformed with the putative white spruce Δ^9 desaturase. Fragments of *n* and *n*+1 carbons differing by 12 Da indicate a double bond between carbons *n*+1 and *n*+2. In the spectrum of fatty acyl-DEA derivatives from the transgenic yeast culture, the fragments at *m/z* = 198 (*n*+1) and 210 (*n*+2) are diagnostic for a double bond at the Δ^9 position in an 18-carbon monounsaturated fatty acid DEA derivative ($M^+ = 337$).

3.3. In vitro analyses

Results of the stearoyl-CoA desaturase assay showed Des9 desaturase activity in the yeast cells transformed with the white spruce *Des9* clone (237.5 ± 20.22 pmol/h/mg protein), a 20-fold increase compared to the relatively insignificant activity observed in the plasmid-only control (11.18 ± 0.80 pmol/h/mg protein). These results demonstrated that the white spruce clone functions as a Des9 desaturase capable of using stearoyl moieties. However, the degree of activity obtained with the cells grown at 15°C was not higher than of that grown at 20°C (data not shown), suggesting that, most likely, the temperature switch is more important for the accumulation of polyunsaturated acids than the absolute growth temperature, an observation previously reported in transgenic plants [12].

While a BLAST search revealed some amino acid sequence homology between our conifer desaturase cDNA and an *Arabidopsis* Δ^9 desaturase-like protein clone that was subsequently hypothesized to be a putative *FAD5* gene [26], our experimental data do not support such an assessment at the biochemical level: *FAD5* is localized in the plastid, and requires 16:0-ACP as the acyl donor to be placed in the *sn*-2 position of monogalactosyldiacylglycerol (MGDG) to be desaturated by *FAD5* to give 16:1$^{\Delta 7}$ [29,30]; it does not exhibit desaturase activity with stearoyl moieties. Furthermore, we transformed *ole1* mutant yeast with the conifer *Des9*, and found that 'new' fatty acids, 16:1$^{\Delta 9}$ and 18:1$^{\Delta 9}$, were produced which were not present in the control. The position of the double bonds in 16:1 and 18:1 were confirmed by DEA derivatization, followed by GC-MS. When lysates of control and transformed (conifer *Des9*) yeast cells were supplied with 1-^{14}C-18:0-CoA in vitro, we observed conversion to radiolabeled 18:1$^{\Delta 9}$ only in the conifer *Des9* transformant. Finally, it is important to note that our expression system, yeast, has neither acyl-ACP nor MGDG, the necessary substrates for *FAD5*. Collectively, our data strongly support our assessment that the conifer clone encodes an acyl-CoA-like protein Δ^9 desaturase, not a *FAD5* homolog.

There is a possibility that a complex glycerolipid such as phosphatidylcholine (PC) is involved as an intermediate substrate, with the stearoyl group of the stearoyl-CoA being first incorporated into the glycerolipid by a yeast acyltransferase and then the stearoyl moiety being desaturated. However, the *lyso*-PC acyltransferase mediating the migration of acyl moieties into the *sn*-2 position of PC to be desaturated shows a very strong preference for 18:1 or 18:2 thioesters, with virtually no affinity for 18:0-CoA [31]. Moreover, plant PCs are typically devoid of 18:0 at the *sn*-2 position, thus PC is not likely to be a primary substrate.

In conclusion, our data show that the clone isolated in *P. glauca* is a stearoyl-CoA-like protein Δ^9 desaturase, probably extra-plastidial, and the first from plants, and *Gymnospermae* in particular, to be functionally characterized. Also, it is reasonable to infer from our studies that the heretofore putative plant acyl-lipid or acyl-CoA *Des9* desaturases from *A. thaliana* and *R. hybrida* are also likely to be extra-plastidial stearoyl-CoA Δ^9 desaturases.

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