Isolation and characterization of a cDNA from *Cuphea lanceolata* encoding a β-ketoacyl-ACP reductase

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Received October 29, 1991

Summary. A cDNA encoding β-ketoacyl-ACP reductase (EC 1.1.1.100), an integral part of the fatty acid synthase type II, was cloned from *Cuphea lanceolata*. This cDNA of 1276 bp codes for a polypeptide of 320 amino acids with 63 N-terminal residues presumably representing a transit peptide and 257 residues corresponding to the mature protein of 27 kDa. The encoded protein shows strong homology with the amino-terminal sequence and two tryptic peptides from avocado mesocarp β-ketoacyl-ACP reductase, and its total amino acid composition is highly similar to those of the β-ketoacyl-ACP reductases of avocado and spinach. Amino acid sequence homologies to polyketide synthase, β-ketoreductases and short-chain alcohol dehydrogenases are discussed. An engineered fusion protein lacking most of the transit peptide, which was produced in *Escherichia coli*, was isolated and proved to possess β-ketoacyl-ACP reductase activity. Hybridization studies revealed that in *C. lanceolata* β-ketoacyl-ACP reductase is encoded by a small family of at least two genes and that members of this family are expressed in roots, leaves, flowers and seeds.

Key words: *Cuphea* – Fatty acid synthase – β-Ketoacyl-ACP reductase – Polyketide synthase – Short-chain alcohol dehydrogenase family

Introduction

Fatty acid synthase (FAS) is a key enzyme in cellular lipid metabolism, comprising a multifunctional enzyme complex that catalyses consecutive condensations and reductions of acetyl residues to form fatty acids. Two differently organized types of FAS, types I and II, have been described (Brindley et al. 1969; Bloch and Vance 1977). In type I FAS, which is found in animals, yeast, mycobacteria and *Euglena gracilis* (cytoplasmatic; Hendren and Bloch 1980), the various enzyme functions are performed by different domains of a multifunctional polypeptide. In contrast, type II FAS consists of a complex of dissociable enzymes and is found in prokaryotes such as *Escherichia coli*, cyanobacteria, and also in *Euglena gracilis* (plastidic; Hendren and Bloch 1980) and plants. Because of the importance of the FAS enzyme, both types have attracted much research interest. Biochemical approaches have been used to describe the enzymatic functions and to study the regulation of enzyme activity (for review see Wakil et al. 1983; Harwood 1988).

The organization of FAS from rat and yeast was deduced from cDNA (Schweizer et al. 1989; Amy et al. 1989) or gene (Schweizer et al. 1986, 1987) sequences, respectively. However, little is known about the genetics and molecular structure of FAS-encoding genes in plants. The first component of plant FAS to be cloned and investigated in detail was its structural component, acyl carrier protein (ACP). ACP has been cloned from various plant species: *Arabidopsis thaliana* (Post-Beittemiller et al. 1989; Lamppa and Jacks 1991), *Brassica campestris* (Rose et al. 1987), *B. napus* (Safford et al. 1988; de Silva et al. 1990), *Hordeum vulgare* (Hansen 1987; Hansen and von Wettstein-Knowles 1989), and *Spinacea oleracea* (Scherer and Knauf 1987). In addition, two enzymatic functions of plant FAS have been cloned recently: the β-ketoacyl-ACP synthase I (EC 2.3.1.41) of *H. vulgare* (Sigaarda-Andersen et al. 1991) and the enoyl-ACP reductase (EC 1.3.1.9) of *B. napus* (Kater et al. 1991). The β-ketoacyl-ACP synthase I catalyses the condensation of an acetyl residue and the nascent ACP-bound acyl chain after decarboxylation of malonyl-CoA and release of coenzyme A resulting in β-ketoacyl-ACPs. These intermediates are reduced in the subsequent steps of the FAS reaction. The first reduction is catalysed by a β-ketoacyl-ACP-reductase or 3-oxoacyl-[acyl-carrier protein] reductase (EC 1.1.1.100).

Biochemical studies with a plant enzyme have revealed an NADH- and an NADPH-dependent form in avocado (Caughley and Kekwick 1982) and spinach (Shimakata and Stumpf 1982). The only structural informa-
tion available for this type of enzyme concerns amino acid sequences of the amino-terminal region and two tryptic fragments of the avocado enzyme (Sheldon et al. 1990). In this paper we describe the isolation and characterization of a cDNA encoding the β-ketoacyl-ACP reductase (NADPH dependent) from Cuphea lanceolata (Lythraceae), a plant that is noteworthy for the high content (83%) of capric acid in its seed storage lipids (Graham 1989).

Materials and methods

Plant material. Immature embryos were dissected from seeds of C. lanceolata K− (non-sticky trichoms) (Hirsinger and Röbbelen 1980), which had been collected from field-grown plants at the Institut für Pflanzenbau und Pflanzenzüchtung, Universität Göttingen. Seeds were selected by their colour, being dark green or showing spots that were already coloured on the seed coat. Plantlets for DNA isolation were grown under greenhouse conditions at 20°C with a 15 h photoperiod.

RNA isolation. Total RNA was isolated from plant tissue based on the procedure of Schröder et al. (1988), except for the following modification to the homogenization step: prior to addition of 1 ml extraction buffer (0.4 M TRIS-HCl, pH 8.0, 0.1 M NaCl, 0.04 M EDTA, 5% SDS) per gram of tissue, plant material was homogenized in liquid nitrogen. Poly(A)⁺ RNA for library construction and polymerase chain reaction (PCR) was isolated from total RNA using oligo-dT cellulose (Boehringer, Mannheim) according to the recommendations of the manufacturer. Total RNA for Northern blots was isolated using QIAGEN-tip 500 (Diagen, Düsseldorf) according to the manufacturer’s protocol.

DNA isolation. DNA was extracted from leaves of non-flowering plantlets essentially according to the method of Dellaporta et al. (1983). However, after isopropanol precipitation, phenolic compounds were extracted with 0.5 g of Polyclar AT (Serva, Heidelberg) per gram of tissue.

Molecular techniques. Molecular cloning, polymerase chain reaction including first strand cDNA synthesis, and Southern and Northern blots were performed according to standard methods as described by Sambrook et al. (1989). Dideoxynucleotide-DNA sequencing was based on the method of Sanger et al. (1977) using the T7 sequencing kit (Pharmacia LKB Biotechnology, Freiburg). Deletion clones for sequencing were created using the ExoIII/mung bean deletion kit of Stratagene (Heidelberg).

cDNA library. For poly(A)⁺ mRNA isolation, embryos, approximately 1.5 to 2 mm in size, were dissected from immature seeds of C. lanceolata, collected in liquid nitrogen and stored at −70°C until use. A directional cDNA library was constructed in the expression vector lambda ZAP II (Stratagene) according to the manufacturer’s protocol. The library was screened following standard protocols (Sambrook et al. 1989). Prior to characterization of the cDNA inserts, the pBluescript vector was released from lambda ZAP II by in vivo excision with the helper phage R408 as described by Stratagene.

Glutathione-S-transferase fusion. A region of 33 bp encoding a part of the presumed transit peptide together with the coding region for β-ketoacyl-ACP reductase of cDNA C1KR27 was cloned in-frame with the glutathione-S-transferase (GST) of the E. coli expression vector pGEX-KG (kindly provided by Dr. J. Dixon; Guan and Dixon 1991). Mutagenic oligonucleotides (5'-CCATG-GCTACTCTCCGTATAAG-3' and 3'-CCGTACTGGTACATTCCTCGAG-5', corresponding to positions 186–207 and 983–1003, respectively, in Fig. 1) were synthesized in order to amplify by PCR the sequence encoding the mature protein, thus creating restriction endonuclease cleavage sites for NcoI and SstI at the termini. The 818 bp fragment obtained was sequenced from both ends and, to ensure that no mutations had occurred during PCR, its core region was replaced by the authentic Smal-EcoRV fragment from the isolated cDNA clone C1KR27. The fusion protein was purified by single-step affinity chromatography using glutathione-S Sepharose, according to Smith and Johnson (1988), resulting in an electrophoretically pure protein of 53 kDa. The protein was assayed for β-ketoacyl-ACP reductase activity immediately after elution from the affinity column.

β-ketoacyl-ACP reductase enzyme assay. β-ketoacyl-ACP reductase activity was monitored by measuring the decrease of A₃₄₀ according to the protocol of Sheldon et al. (1990). Prior to addition of the substrate, the reaction mixture (1 ml final volume), consisting of 100 mM potassium phosphate (pH 7.0), 100 μM NADPH, and 1–10 μl purified fusion protein, was preincubated for 5–10 min at 30°C. The reaction was started by the addition of acetoacetyl-CoA to a final concentration of 2.5 mM.

Sequence evaluation. Analysis of sequence data was performed using the software package of the University of Wisconsin Genetics Computer Group (Devereux et al. 1984).

Results

Isolation of cDNA C1KR27

In order to screen a cDNA library, a gene-specific probe for β-ketoacyl-ACP reductase of C. lanceolata was synthesized by PCR. Degenerate oligonucleotides, 5'-ACIGCIGTIGA[CT][GCTGGGGG-3' and 3'-TT[AG]-TA[AG]TT[AG]CITA[AG]CGTA[AG]CGTG-5' (see Fig. 1; priming regions are shaded) to be used as primers for PCR were deduced from parts of the amino acid sequence of two tryptic fragments of the β-ketoacyl-ACP reductase of avocado mesocarp (Sheldon et al. 1990). Using poly(A)⁺ RNA as template, the PCR resulted in an amplified DNA fragment of 340 bp, which when cloned and sequenced, was found to code for a