Plant Science 249 (2016) 84–92

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Biochemical characterization and substrate specificity of jojoba fatty acyl-CoA reductase and jojoba wax synthase

Magdalena Miklaszewska [a,*], Antoni Banaś [b]

[a] Department of Plant Physiology and Biotechnology, University of Gdańsk, ul. Wita Stwosza 59, 80-308, Gdańsk, Poland
[b] Intercollegiate Faculty of Biotechnology of University of Gdansk and Medical University of Gdańsk, ul. Abrahama 58, 80-307 Gdańsk, Poland

ARTICLE INFO

Article history:
Received 28 March 2016
Received in revised form 2 May 2016
Accepted 13 May 2016
Available online 13 May 2016

Keywords:
Wax synthase
Fatty acyl-CoA reductase
Wax esters
Fatty alcohols
Simmondsia chinensis

ABSTRACT

Wax esters are used in industry for production of lubricants, pharmaceuticals and cosmetics. The only natural source of wax esters is jojoba oil. A much wider variety of industrial wax esters-containing oils can be generated through genetic engineering. Biotechnological production of tailor-made wax esters requires, however, a detailed substrate specificity of fatty acyl-CoA reductases (FAR) and wax synthases (WS), the two enzymes involved in wax esters synthesis. In this study we have successfully characterized the substrate specificity of jojoba FAR and jojoba WS. The genes encoding both enzymes were expressed heterologously in *Saccharomyces cerevisiae* and the activity of tested enzymes was confirmed by *in vivo* studies and *in vitro* assays using microsomal preparations from transgenic yeast. Jojoba FAR exhibited the highest *in vitro* activity toward 18:0-CoA followed by 20:1-CoA and 22:1-CoA. The activity toward other 11 tested acyl-CoAs was low or undetectable as with 18:2-CoA and 18:3-CoA. In assays characterizing jojoba WS combinations of 17 fatty alcohols with 14 acyl-CoAs were tested. The enzyme displayed the highest activity toward 14:0-CoA and 16:0-CoA in combination with C16-C20 alcohols as well as toward C18 acyl-CoAs in combination with C12-C16 alcohols. 20:1-CoA was efficiently utilized in combination with most of the tested alcohols.

1. Introduction

Wax esters are esters of long chain fatty alcohols and fatty acids synthesized in a wide range of organisms. They fulfill a number of functions, including energy storage, protection from desiccation, UV light, and pathogens as well as buoyancy regulation. In industry wax esters are used to produce high pressure lubricants, pharmaceuticals, cosmetics and inks [1]. *Simmondsia chinensis* (jojoba) is a perennial desert shrub native to North America, which accumulates wax esters as seed storage lipids, instead of triacylglycerols. Wax esters account for up to 60% of the dry weight of their cotyledons and are composed of very long-chain (C20, C22, and C24) monounsaturated fatty acids and alcohols [2,3]. In plants the biosynthesis of wax esters occurs in cytosol and uses activated fatty acids as substrates. Fatty acids, exported from plastids, are converted to acyl-CoAs and can be further elongated by fatty acyl-CoA elongase (FAE) system. Alcohol-forming fatty acyl-CoA reductase (FAR) reduces acyl-CoAs to corresponding alcohols and wax synthase (WS) catalyzes the esterification reaction of a fatty acyl-CoA with a long-chain fatty alcohol [4].

Alcohol-forming fatty acyl-CoA reductases are membrane-associated enzymes, which contain a Rossmann-fold NAD(P)H binding domain and a fatty acyl-CoA reductase (FAR_C) domain [5]. Wax synthases are membrane-bound enzymes with several transmembrane domains. Bifunctional wax synthases, exhibiting additionally a DGAT activity, were identified in TAG/WE synthesizing bacteria as well as in plants, such as Arabidopsis [6,7].

In *S. chinensis* wax esters are formed in the cytosol of developing embryo cells. FAR from *S. chinensis* contains 493 amino acid residues and has predicted molecular mass of 56,2 kDa [8]. Jojoba wax synthase is a 352-amino-acids protein and contains seven to nine transmembrane domains. In *in vitro* assays with microsomal membrane fractions isolated from jojoba embryos, wax synthase showed significant activity toward C14-C24 acyl-CoAs and C8-C24 fatty alcohols in combination with 16:0-OH and 16:0-CoA, respectively. In the studied combinations of substrates the 20:1-CoA from tested acyl-CoA and 18:1-OH and 18:2-OH from the tested alcohols were preferentially utilized by the tested enzyme [4].

Abbreviation: ACAT, acyl-CoA:cholesterol acyltransferase; DGAT, acyl-CoA:diacylglycerol acyltransferase; DW, dry weight; FAR, fatty acyl-CoA reductase; MGAT, acyl-CoA:monoacylglycerol acyltransferase; Ric, ricinoleic acid; TAG, triacylglycerols; WE, wax esters; WS, wax synthase; fatty acyl-CoA, fatty alcohol acyltransferase.

* Corresponding author.
E-mail address: magdalena.miklaszewska@biol.ug.edu.pl (M. Miklaszewska).

http://dx.doi.org/10.1016/j.plantsci.2016.05.009
0168-9452/

In this study we successfully expressed genes encoding jojoba fatty acyl-CoA reductase and wax synthase in *Saccharomyces cerevisiae*. Both *in vivo* and *in vitro* substrate specificity of tested enzymes was determined. The activity of FAR and WS enzymes toward substrates with different carbon chain lengths, different saturation degrees and different side chains was characterized using *in vitro* assays with microsomal fractions isolated from transgenic yeast.

2. Materials and methods

2.1. Chemicals and materials

All chemicals and solvents were purchased from Merck (Darmstadt, Germany) or Sigma-Aldrich (St. Louis, MO, USA), unless stated otherwise. Restriction enzymes and molecular biology reagents were purchased from Thermo Scientific (Waltham, MA, USA), unless otherwise stated. Unlabelled acyl-CoAs were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Lipid standards, fatty alcohols were purchased from Larodan Fine Chemicals (Malmö, Sweden) and phytol was purchased from Sigma-Aldrich (St. Louis, MO, USA). The [^{14}C]acyl-CoAs were synthesized using [^{14}C]fatty acids (Biotrend, Cologne, Germany) according to the modified method described by Sánchez et al. [9]. 1,2-dihexanoyl-*sn* glycerol and cholesterol were purchased from Sigma-Aldrich (St. Louis, MO, USA); 1,2-[^{14}C]-dioleoyl-*sn* glycerol and 1-[^{14}C]oleoyl-*sn* glycerol were obtained by partial treatment of [^{14}C]-triolein (Perkin Elmer, Waltham, MA, USA) with a lipase from *Rhizopus arrhizus* (Sigma-Aldrich, St. Louis, MO, USA).

2.2. Yeast strains

The *Saccharomyces cerevisiae* BY4742 strain and the quadruple mutant strain H1246 (*MATα are1-Δ:HIS3 are2-Δ:LEU2 dga1-Δ:KanMX4 lro1-Δ:TRP1 CEE2*) [10] were used as heterologous hosts to study the substrate specificity of tested enzymes.

2.3. Plasmid construction and yeast transformation

DNA manipulations were carried out in *Escherichia coli* DH5α as described by Green et al. [11]. The gene encoding jojoba wax synthase was amplified from the pBinGlyRed-FWS3 plasmid (provided by Prof. Edgar B. Cahoon, University of Nebraska-Lincoln) using Phusion High-Fidelity DNA Polymerase (Finnzymes, Helsinki, Finland) and primers 5′-TATATAAGCTTATGGAGGTGGAGAAGGAG-3′ and 5′-TATATCTAGATCACCACCCCAACAAACC-3′. The PCR fragment was digested with Hind*III*/Xba*I* and ligated into yeast expression vector pYES2 (Invitrogen, Carlsbad, CA, USA) containing a galactose inducible promoter (GAL1) yielding plasmid pYES2-ScWS. The pYES2-ScFAR plasmid carrying jojoba FAR gene was donated by Dr. Peter Denolf from Bayer Crop Science NV (Ghent, Belgium). The plasmids pYES2-ScFAR and pYES2-ScWS, carrying a jojoba FAR and WS gene respectively, as well as the empty pYES2 plasmid (control) were introduced into BY4742 and H1246 yeast strains according to the modified LiAc/SS carrier DNA/PEG method [12].

2.4. Growth conditions

Yeast transformed with pYES-ScFAR were cultured on a rotating platform (220 rpm) in synthetic dropout (SD) medium lacking uracil containing 2% (w/v) galactose for 48 h at 30 °C. Yeast harboring pYES2-ScWS plasmid were cultured in SD medium lacking uracil containing 2% (w/v) galactose supplemented with 12.5 mg oleic alcohol in the presence of 1% (v/v) Tween 20.

2.5. Lipid extraction and gas chromatography

Cells of 25 ml culture were harvested, washed twice with distilled water and disrupted in a glass-bead shaker. Lipids were extracted into chloroform according to Blight & Dyer [13]. Extracts obtained from yeast harboring pYES2-ScFAR were concentrated and transmethylated with 2% (v/v) sulphuric acid in dry methanol for 40 min at 90 °C. Extracts obtained from yeast harboring pYES2-ScWS were separated by thin-layer chromatography (TLC) on silica gel 60 plates (Merck, New York, USA) using hexane/diethyl ether/acetic acid (70/30/1, v/v/v) as the solvent system. The lipid areas were visualized by brief exposure to I_2 vapors and identified by means of appropriate standards. Bands co-chromatographing with the wax ester standard were transmethylated as described above. The methyl esters were extracted with hexane, concentrated and analyzed on Shimadzu GC-2010 equipped with a flame ionization detector and a 60 m × 0.25 mm CP-WAX 58 CB fused-silica column (Agilent Technologies, Santa Clara, CA, USA). Methyl ester of heptadecanoic acid (Sigma-Aldrich, St. Louis, MO, USA) was used as an internal standard. Total fatty acids of the transgenic yeast acyl-lipids were determined by gas chromatography after prior incubation of freeze-dried aliquots of yeast with a methylation mixture for 90 min at 90 °C.

2.6. Microsomal membrane preparation

Membrane preparation were performed as described previously [14]. Briefly, transgenic yeast were harvested, washed with distilled water and disrupted by shaking with glass beads. Cell debris was sedimented (1,500 g, 10 min, 4 °C) and the resulting supernatant was centrifuged (100,000 *g*, 1.5 h, 4 °C). The pellet was suspended in 0.1 M potassium buffer (pH 7) and stored at −80 °C until use for further analyses.

2.7. In vitro *FAR and WS assay*

In order to establish the optimal *in vitro* assay conditions the effect of various factors, such as reaction time, temperature, buffer pH and BSA concentration on the activity of tested enzymes was investigated. The activity of jojoba FAR was assayed by measuring the formation of [^{14}C]16:0-OH from [^{14}C]16:0-CoA. For jojoba WS [^{14}C]16:0-CoA and 16:0-OH were used as substrates.

After the optimization step, the detailed study of the substrate specificity of jojoba FAR was performed with fourteen [^{14}C]acyl-CoAs: decanoyl-CoA (10:0), lauroyl-CoA (12:0), myristoyl-CoA (14:0), palmitoyl-CoA (16:0), stearoyl-CoA (18:0), oleoyl-CoA(18:1), linoleoyl-CoA (18:2), linolenoyl-CoA (18:3), arachidoyl-CoA (20:0), eicosenoyl-CoA (20:1), erucyl-CoA (22:1), ricinoleyl-CoA, 2-methylhexadecanoyl-CoA (2-Me-16:0) and 2-methyloctadecanoyl-CoA (2-Me-18:0). Reaction mixture contained, in a total volume of 100 μl, 0.1 M phosphate buffer (pH 6.5), 44 μg membrane protein, 2 μmol NADPH, 1 mg BSA and 5 nmol [^{14}C]acyl-CoA. Incubation was carried out for 30 min at 40 °C in Eppendorf Thermomixer Compact with shaking (1250 rpm). Reactions were terminated by addition of 375 μl chloroform/methanol (1:2, v/v), 5 μl acetic acid, 125 μl chloroform, and 125 μl water, and the lipids were extracted into chloroform.

The characterization of substrate specificity of jojoba WS was performed with fourteen above-mentioned [^{14}C]acyl-CoAs in combination with seventeen unlabelled fatty alcohols: decanol (10:0), dodecanol (12:0), tetradecanol (14:0), hexadecanol (16:0), hexadecenol (16:1), octadecanol (18:0), octadecenol (18:1), linoleyl alcohol (18:2), linolenyl alcohol (18:3), eicosanol (20:0), eicosenol (20:1), docosanol (22:0), docosenol (22:1), tetracosanol (24:0), tetracosenol (24:1) and phytol. The fatty alcohols (100 nmol/assay) were added to the freeze-dried microsomal membranes (22 μg of