

Fatty Acyl-CoA Reductase and Wax Synthase from *Euglena gracilis* in the Biosynthesis of Medium-Chain Wax Esters

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Abstract *Euglena gracilis*, a unicellular phytoflagellate, can accumulate a large amount of medium-chain wax esters under anaerobic growth conditions. Here we report the identification and characterization of two genes involved in the biosynthesis of wax esters in *E. gracilis*. The first gene encodes a fatty acyl-CoA reductase (*EgFAR*) involved in the conversion of fatty acyl-CoAs to fatty alcohols and the second gene codes for a wax synthase (*EgWS*) catalyzing esterification of fatty acyl-CoAs and fatty alcohols, yielding wax esters. When expressed in yeast (*Saccharomyces cerevisiae*), *EgFAR* converted myristic acid (14:0) and palmitic acid (16:0) to their corresponding alcohols (14:0Alc and 16:0Alc) with myristic acid as the preferred substrate. *EgWS* utilized a broad range of fatty acyl-CoAs and fatty alcohols as substrates with the preference towards myristic acid and palmitoleyl alcohol. The wax biosynthetic pathway was reconstituted by co-expressing *EgFAR* and *EgWS* in yeast. When myristic acid was fed to the yeast, myristyl myristate (14:0–14:0), myristyl palmitoleate (14:0–16:1), myristyl palmitate (14:0–16:0) and palmityl myristate (16:0–14:0) were produced. These results indicate *EgFAR* and *EgWS* are likely the two enzymes involved in the biosynthesis of medium-chain wax esters in *E. gracilis*.

Keywords *Euglena* · Fatty acyl-CoA reductase · Fatty alcohol · Wax ester · Wax synthase

Abbreviations

CoA	Coenzyme A
FAMEs	Fatty acid methyl esters
FAR	Fatty acyl-CoA reductase
RACE	Rapid amplification of cDNA ends
WS	Wax synthase

Introduction

Euglena gracilis is a unicellular phytoflagellate protist that can grow photoautotrophically in a minimal medium as well as heterotrophically in an organic carbon-rich medium. Under light or aerobic conditions, *Euglena* accumulates polysaccharides (mainly β -1,3 glucan, known as paramylon) and wax esters as energy reserves [1, 2]. Once the cell culture is switched from light to dark or from aerobic to anaerobic conditions, the polysaccharide reserve is converted to wax esters in the cytosol and the accumulated wax esters can reach up to 62% of the total lipid content [3]. Wax esters produced in *Euglena* are in a range of the 20- to 36-carbon chains comprised of saturated fatty acids and alcohols of 12–18 carbon chains with myristyl myristate (14:0–14:0) as the major species [4]. Under aerobic growth, *Euglena* mainly accumulates even-numbered wax esters, however an anaerobic growth condition promotes the accumulation of odd-numbered wax esters in some *Euglena* strains [3–6]. In addition to light and oxygen, other environmental factors, such as nutrients and temperature can also affect the production and composition of storage wax esters in *Euglena* cells. The production of wax esters is more efficient when the culture medium contains organic carbon source [7] or when the culture is shifted from low to high temperature (15 °C to 33 °C) [8]. Supplementation of

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the medium with unsaturated fatty acids (oleic acid, linoleic acid, linolenic acid) and unusual fatty acid (ricinoleic acid) leads to the accumulation of these fatty acids in both acyl and alcohol moieties of wax esters [6].

The wax ester biosynthetic pathway consists of two successive steps: conversion of fatty acyl-CoA to fatty alcohol and esterification of fatty acyl-CoA and fatty alcohol [9]. The enzymes responsible for these reactions are fatty acyl-CoA reductase (FAR) and acyl-CoA:fatty alcohol acyltransferase or wax synthase (WS), respectively. The genes encoding FARs have been identified from a variety of living organisms, including mouse [10], human [10], silkworm [11], bean borer moth [12], jojoba [13], *Arabidopsis* [14, 15] and wheat [16]. BmFAR, a fatty acyl-CoA reductase from silkworm (*Bombyx mori*), is responsible for production of sex pheromone bombykol, (*E,Z*)-10,12-hexadecadien-1-ol [11], whereas OsFARXIII, another fatty acyl-CoA reductase from bean borer moth (*Ostrinia scapulalis*), catalyzes the production of (*Z*)-11-tetradecenol, which can be further converted to acetate or aldehyde pheromones [12]. ScFAR, a fatty acyl-CoA reductase from jojoba (*Simmondsia chinensis*), is responsible for producing storage wax esters in developing seeds [13]. *Arabidopsis* fatty acyl-CoA reductase AtCER4 (At4g33790) is involved in the synthesis of cuticular wax lipids [15]. *TaTAA1a*, *TaTAA1b* and *TaTAA1c*, three orthologs of the jojoba ScFAR, were isolated from wheat [16]; they are involved in producing the lipid component in the outer pollen wall.

Wax synthases have also been isolated and characterized from a wide range of living organisms. Some show only wax synthase activity such as mouse WS, human WS [17], jojoba ScWS [18] and petunia PhWS1 [19], while the other exhibit both wax synthase and acyl-CoA:diacylglycerol acyltransferase (DGAT) activities, including *Acinetobacter* WS/DGAT [20] and *Arabidopsis* WSD1 [21]. Mouse and human WSs (*MmWS* and *HsWS*) are highly expressed in sebaceous-rich tissues, such as preputial glands and eyelids [17]. Jojoba ScWS was isolated from developing seeds and is involved in the synthesis of liquid waxes that accumulate in seeds [18]. *Arabidopsis* AtWSD1 (At5g37300) is involved in synthesis of stem epicuticular wax esters as shown by a severe reduction of the wax ester content (44 and 46 carbons) in *Arabidopsis* *wsd1* mutants [21]. Petunia PhWS1 is highly expressed in petals and involved in the production of very long chain fatty acid esters of methyl, isoamyl and short to medium straight chain alcohols (4–12 carbons) [19].

In *E. gracilis*, the activities of both FAR and WS were found in the microsomal fraction of cultures grown in the dark [22]. The biochemical assays showed that FAR used 14:0, 16:0 and 18:0 as substrates and required NADH as a cofactor [22, 23]. Although the biosynthesis of wax esters in *Euglena* has been extensively studied, the genes

encoding these enzymes have not been described. Here we report the identification and characterization of two genes, *EgFAR* and *EgWS*, involved in the biosynthesis of wax esters in *E. gracilis*. Heterologous expression of these genes in yeast revealed the unique properties of the enzymes—preferential utilization of medium chain fatty acyl and alcohol as substrates for synthesis of wax esters.

Experimental Procedures

Isolation of Putative *Euglena* Fatty Acyl-CoA Reductase (*EgFAR*) and *Euglena* Wax Synthase (*EgWS*)

Euglena gracilis was grown aerobically in a TSY medium (0.1 g/l sodium acetate, 0.1 g/l beef extract, 0.2 g/l peptone, 0.2 g/l yeast extract, 0.2 µg vitamin B12, 1 µg/l biotin, 100 µg/l thiamine-HCl, 0.1 µg/l niacinamide, pH 7.0 [24]) at 25 °C for 16 h (120 µE m⁻² s⁻¹)/8 h (light/dark) regime. The *Euglena* cells can accumulate up to 28% wax ester under these conditions. Total RNA was extracted from the *E. gracilis* culture using TRIZOL[®] reagent (Invitrogen). The full length cDNAs of *EgFAR* and *EgWS* were obtained using Marathon[™] cDNA Amplification kit under conditions detailed by the supplier (Clontech). The gene-specific primers used for 5'- and 3'-RACE of *EgFAR* are ACR1 (5'-GGCTGGTTGGAGTTGACGTAGCA-3') and ACF1 (5'-GCCATGAACGATTTCTACGCGGG-3') primers, respectively. The gene-specific primers used for 5'- and 3'-RACE of *EgWS* are WSR2 (5'-CTCCGGGTGACCTTTCGGC-3') and WSF2 primers (5'-CCAGCCC TACTTTTCCACATCTCTGAG-3'), respectively.

DNA Sequencing and Analysis

All synthesis and sequencing work was performed by the DNA Technologies Unit at the Plant Biotechnology Institute, National Research Council of Canada. Nucleotide sequence and amino acid sequence comparisons were conducted using Lasergene7 (DNASTAR).

Amino Acid Sequence Alignment and Phylogenetic Analysis

Phylogenetic analysis of functionally characterized FARs and WSs were performed as previously described [25].

Construction of Yeast Expression Vectors Harboring *EgFAR*, *EgWS* and *EgFAR-EgWS*

The *EgWS* open reading frame (ORF) was amplified using primers WSFLF (5'-TTCGCGATGGATTTTTTGGGG-3') and WSFLR (5'-GCCACCCAAGGCACTTGGCCT-3')

and cloned into pYES2.1/V5-His/lacZ vector (Invitrogen), yielding plasmid pPT504. The *EgFAR* ORF was amplified using primers PT0037 (5'-GATCGGATCCATGAACGATTTCTACGCG-3')-PT0041 (5'-ATCAGCTAGCCTATCA CAGCATGGCCCGC-3') and digested with *Bam*HI and *Nhe*I. The amplified fragment was subsequently cloned into the corresponding restriction sites of a yeast expression vector pESC-URA (Stratagene), yielding pPT515. The amplification was performed using 2.5 units of Platinum[®] Pfx DNA polymerase (Invitrogen) in the presence of 5% (v/v) DMSO. PCR conditions for *EgFAR* were 35 cycles of 94 °C for 15 s, 55 °C for 30 s, 68 °C for 1 min with the final extension at 68 °C for 5 min. PCR conditions for *EgWS* were identical to *EgFAR*, except that the extension time was 30 s instead of 1 min. For the co-expression study, the *EgWS* ORF was amplified from pPT504 using primers PT0049 (5'-GATCATCGATATGGATTTCTTAG GTTTTCCTGAC-3')-PT0050 (5'-CTGTAGATCTCTAT CAGACAGACAGACCTAGC-3'). The amplified fragment was digested with *Cla*I and *Bam*HI and subsequently cloned into the corresponding sites of pPT515, yielding pPT516.

Functional Analysis of *EgFAR* and *EgWS* in Yeast

pPT515 (*EgFAR*) and pPT504 (*EgWS*) plasmids were transformed into yeast (*Saccharomyces cerevisiae*) strain H1246 (*MAT α* ; *are1- Δ* ::*HIS3 are2- Δ* ::*LEU2 dgal- Δ* ::*KanMX4 lro1- Δ* ::*TRP1 ADE2*) [26] using S.c. Easy-CompTM transformation kit (Invitrogen). Yeast strains transformed with pPT515 (*EgFAR*), pPT504 (*EgWS*), pPT516 (*EgFAR* + *EgWS*), pESC-URA or pYES2.1 plasmids were grown at 30 °C for 2 days in 10 ml of the synthetic dropout medium containing 0.17% (w/v) yeast nitrogen base, 0.5% (w/v) ammonium sulfate, 2% (w/v) glucose and 0.06% (w/v) dropout supplement lacking uracil (DOB + GLU-URA). After two washes with 10 ml of sterile distilled water, the expression of transgene in yeast were induced by culturing the yeast at 20 °C for 4 days for pPT515 (*EgFAR*) and 30 °C for 2 days for pPT504 (*EgWS*) in 10 ml of the synthetic dropout medium containing 2% (w/v) galactose (DOB + GAL-URA) with or without substrate supplementation in the presence of 0.1% (v/v) tertigol (Nonidet P-40). Two-hundred fifty micromolar of fatty acid was used as a substrate for the pPT515 (*EgFAR*) expression, whereas 250 μ M of fatty acid and 250 μ M of fatty alcohol were used as substrates for the pPT504 (*EgWS*) expression. After induction, the cultures were washed once with 10 ml of 1% (v/v) tertigol and once with 10 ml of distilled water, and then subjected to fatty acid analysis. For the yeast feeding experiment, fatty acid and fatty alcohol substrates were initially prepared as stock solutions in ethanol at the concentration of

500 mM and the appropriate amount of the stock was then diluted in 10% tertigol at the final concentration of 50 mM used for feeding the yeast.

In Vitro Assays of *EgFAR* and *EgWS*

To investigate substrate specificity of *EgFAR* and *EgWS* in vitro, the microsomal fractions of yeast expressing *EgFAR* and *EgWS* were prepared as previously described [27, 28]. For the *EgFAR* assay, the enzyme reaction (500 μ l) contained 200 μ g of microsomal proteins, 0.3 M sucrose, 0.1 M MOPS (pH 6.5) or 0.1 M Tris-HCl (pH 7.4), 1 mM EDTA, 2.5 mM DTT, 5 mM MgCl₂, 1 mM PMSF, 100 μ M acyl-CoA, 2.5 mM NADH, 2.5 mM NADPH. The reaction was carried out at 30 °C with gentle shaking for 30 min to 2 h. For the *EgWS* assay, the same amount of protein and the reaction buffer (pH 7.4) were used. One-hundred micromolar of acyl-CoA and 100 μ M fatty alcohol were added as substrates. The reaction was carried out at 30 °C with gentle shaking for 2 h. The enzyme reactions were terminated by addition of 100 μ l of 6 M HCl. After adding 1 ml of phosphate buffered saline and 1 ml of 0.9% (w/v) NaCl solution, lipids were extracted twice with 2 ml hexane. The known amount of methyl eicosanoate (20:0-ME) was added as the internal standard. The solvent was subsequently removed under a nitrogen stream and the lipid samples were resuspended in 20 μ l of hexane, which was derivatized with bis(trimethylsilyl)-acetamide (TMS) and analyzed by GC for the *EgFAR* assay or was directly analyzed by GC for the *EgWS* assay (see below). Acyl-CoA stock solutions were prepared in 10 mM sodium acetate buffer (pH5.2)/ethanol (1:1, v/v) at a concentration of 50 mM.

Co-Expression of *EgFAR* and *EgWS* in Yeast

To reconstitute the *Euglena* wax biosynthesis pathway in yeast, the plasmid pPT516 harboring the two-gene cassette (*EgFAR* and *EgWS*) was transformed into the yeast strain H1246. The transformants were grown in the DOB + GLU-URA medium at 30 °C for 2 days and the expression of *EgFAR* and *EgWS* was induced in the DOB + GAL-URA medium supplemented with 500 μ M 14:0 in the presence of 0.1% (v/v) tertigol at 20 °C for 4 days. The yeast cells were harvested and analyzed for fatty alcohol and wax monoester production as described above.

Analysis of Fatty Acids, Fatty Alcohols and Wax Esters of Yeast Transformants

Fatty acids of yeast cells were transmethylated with 2 ml of methanol/HCl (3 M) at 80 °C for 2 h and the reaction was terminated by adding 1 ml of 0.9% NaCl solution. Total fatty acid methyl esters (FAMES) and fatty alcohols were

then extracted twice with 2 ml of hexane and the hexane phase was transferred to a new tube, evaporated under a nitrogen stream and resuspended in 200 μ l of hexane. Fifty microliters of samples were derivatized with 50 μ l of TMS/pyridine (1:1, v/v) at 80 °C for 30 min and the derivative was analyzed by gas chromatography (GC). For wax ester analysis, the total lipid was extracted by homogenizing yeast cells in the presence of 6 ml of chloroform/methanol (2:1, v/v). The organic phase was dried under a stream of nitrogen gas, resuspended in 50 μ l of hexane and analyzed by GC, GC–mass spectrophotometry (MS) and thin layer chromatography (TLC).

GC, GC–MS and TLC Analysis

Fatty acids (10:0, 12:0, 14:0, 16:0, 16:1n-9, 18:0, and 18:1n-9), fatty alcohols (10:0Alc, 12:0Alc, 14:0Alc, 16:0Alc, 16:1n-9Alc, 18:0Alc and 18:1n-9Alc), wax esters (14:0–12:0, 14:0–14:0, 14:0–16:0, 14:0–16:1n-9, 14:0–18:0, 14:0–18:1n-9, 12:0–14:0, 16:0–14:0, 16:1n-9–14:0, 18:0–14:0, 18:1n-9–14:0) and TLC reference standard (cholesterol, cholesteryl oleate, triolein, oleic acid, methyl oleate) with 99% purity were purchased from Nuchek-Prep, Inc. For GC analysis, one-microliter samples were analyzed on an Agilent 6890 N GC equipped with a DB-5 column (10 m \times 0.25 mm) (J&W Scientific). The following temperature programs were employed: 70 °C for 1 min, then 10 °C/min to 300 °C, and 300 °C for 10 min with H₂ as the carrier gas. For MS analysis, the mass selective detector was run under standard electron impact conditions (70 eV), scanning an effective *m/z* range of 40–700 at 2.26 scans/s. Identities of FAMES, fatty alcohols-TMS derivatives and wax esters were identified by comparing their retention times with those of the standards and confirmed by GC–MS on the basis of their fragmentation patterns. For TLC analysis, total lipid samples and standards were spotted on 60- \AA silica gel SIL G-25 plates (Macherey–Nagel) and resolved in hexane/diethylether/acetic acid (90:7.5:1, v/v/v) [21]. The plate was air-dried and the lipid metabolites were detected by primuline spraying (0.05% w/v; Sigma) and the pictures were taken under UV light at 254 nm.

Results

Isolation of Two cDNAs Encoding Putative Fatty acyl-CoA Reductase and Wax Synthase from *E. gracilis*

The partial cDNA sequences of *Euglena* putative fatty acyl-CoA reductase *EgFAR* and wax synthase *EgWS* were

obtained by a homology search of the *Euglena* EST database using jojoba ScFAR and ScWS as query sequences and the full-length putative *EgFAR* and *EgWS* cDNAs were obtained by 5'- and 3'-RACE using *Euglena* cDNAs as the template. Sequence analysis indicated putative *EgFAR* encodes a polypeptide of 514 amino acids in length with the predicted molecular mass of 56.5 kDa. The deduced protein *EgFAR* contains a Rossmann-fold NAD/NADP binding domain (NABD; 289 amino acids) [29] linked with a Male Sterile 2 domain (MS2, 97 amino acids) [30] at the carboxyl end (Fig. 1a). A conserved motif (I/V/F)-X-(I/L/V)-T-G-X-T-G-F-L-(G/A), found in other fatty acyl-CoA reductases, was also observed in the NABD domain of *EgFAR* (Fig. 1b). The hydropathy analysis indicated that the putative *EgFAR* contains five high hydrophobic regions; one located at the N-terminus and the rest present in the central portion of the protein. Phylogenetic analysis of the putative *EgFAR* and related sequences reveals that *EgFAR* is more closely related to plant fatty acyl-CoA reductases, including *Arabidopsis* AtCER4 [15], wheat TaTAA1 [16] and jojoba ScFAR [31], whereas the insect and mammalian fatty acyl-CoA reductases [10] form a distant group (Fig. 2a). Sequence analysis of the putative *EgWS* indicated that it encodes a polypeptide of 368 amino acids with the predicted molecular mass of 41.2 kDa. The deduced protein *EgWS* contains six distinct hydrophobic regions. Phylogenetic analysis of *EgWS* and related sequences reveals that *Euglena* *EgWS* clusters with jojoba ScWS [18], which is distantly related to the rest members of wax synthases, including the mammalian WSs [17], *Acinetobacter* AcWS/DGAT [20], *Arabidopsis* AtWSD1 [21] and petunia PhWS1 [19] (Fig. 2b).

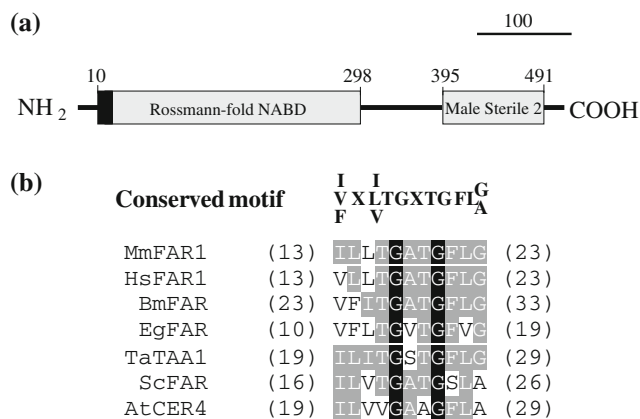


Fig. 1 A schematic diagram illustrating the functional domains (a) and a conserved motif (b) of *EgFAR* and other related sequences. The numbers indicate amino acid positions. The accession numbers of these sequences are indicated in Fig. 2

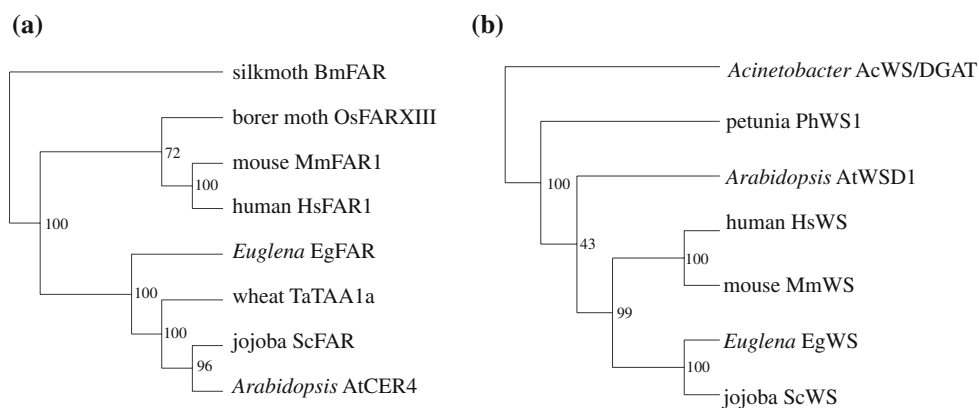


Fig. 2 Phylogenetic analysis of EgFAR (a) and EgWS (b) and their related sequences. The GenBank accession numbers of the sequences are as follows: mouse MmFAR1, BC007178; mouse MmFAR2, BC055759; human HsFAR1, AY600449; human HsFAR2, BC022267; silkmoth BmFAR, AB104896; borer moth OsFARXIII, EU817405; wheat FAR

(TaTAA1a), AJ459249; jojoba ScFAR, AF149917; *Arabidopsis* AtCER4, AY070065-At4g33790; mouse MmWS, AY611032; human HsWS, AY605053; *Arabidopsis* AtWSD1, NM_123089-AT5G37300; *Acinetobacter* AcWS/DGAT, AF529086; jojoba ScWS, AF149919; petunia PhWS1, DQ093641

Functional Analysis of *EgFAR* and *EgWS* in Yeast

To determine the function of putative *EgFAR* and *EgWS*, the full-length cDNAs were cloned into the yeast expression vectors separately and the recombinant plasmids were transformed into the *Saccharomyces cerevisiae* quadruple mutant strain H1246 [26], in which four acyltransferase genes involved in the triacylglycerol (TAG) and sterol ester biosynthesis were disrupted. This strain appeared to have diminished capacity for wax ester biosynthesis (Fig. 3b), thus can serve as a good host system to examine the function of genes involved in the biosynthesis of wax esters.

The activity of the putative *Euglena* fatty acyl-CoA reductase was investigated by feeding the yeast strain carrying *EgFAR* with a variety of probable fatty acid substrates. Analysis of total fatty alcohols in the cells indicated that the transformant expressing *EgFAR* produced new peaks only when myristic acid (14:0) and palmitic acid (16:0) were used as substrates, compared with the vector control (Fig. 3a). The chemical structures of these new peaks were determined as myristyl alcohol (14:0Alc) and palmityl alcohol (16:0Alc) by GC-MS. The conversion efficiencies of the two alcohols were 34 ± 4 and $24 \pm 3\%$, respectively. No other alcohol products were detected when the yeast strain was fed with other saturated fatty acids (10:0, 12:0 and 18:0) or unsaturated fatty acids (16:1n-9, 18:1n-9 and 18:2n-6). A similar result was obtained when *EgFAR* was expressed in yeast strain INVSc.

The activity of the putative *EgWS* was investigated by feeding the yeast strain expressing *EgWS* with 14:0 and 14:0Alc substrates. The total neutral lipids including triacylglycerols and wax esters in the transformant were first

analyzed by TLC. In contrast to the vector control, the yeast expressing *EgWS* produced wax esters in presence of 14:0 and 14:0Alc that were detected on the TLC plate (Fig. 3b). Like the control, the transformant expressing *EgWS* did not produce any triacylglycerols, indicating *EgWS* possess wax synthase activity, but not diacylglycerol acyltransferase activity as seen in bacterial WS/DGAT [32]. Wax composition analysis by GC and GC-MS indicated that the major components of wax esters produced in the transformant were myristyl myristate (14:0–14:0), myristyl palmitate (14:0–16:0) and myristyl pamitoleate (14:0–16:1) (Fig. 3c). It was also noted that the vector control could produce a trace amount of myristyl myristate (14:0–14:0) when the yeast was fed with substrates (Fig. 3b, c).

To examine the fatty acid substrate specificity of *EgWS*, the yeast strain was fed with 14:0Alc in combination with a range of fatty acids including 10:0, 12:0, 14:0, 16:0, 16:1n-9, 18:0, and 18:1n-9. The wax analysis showed that *EgWS* could incorporate 12:0, 14:0, 16:0 and 16:1–9 fatty acids into wax esters, but it could not use 10:0, 18:0 or 18:1n-9 as the substrate. Quantitative analysis of the ratio of products versus substrates indicated that the highest conversion efficiency of fatty acids was observed on 14:0, which was followed by 12:0, 16:0 and 16:1n-9 (Table 1). In order to examine fatty alcohol substrate specificity, the yeast strain was fed with 14:0, the most preferred acyl-CoA substrate for *EgWS* in combination with a range of fatty alcohols including 10:0Alc, 12:0Alc, 14:0Alc, 16:0Alc, 16:1n-9Alc, 18:0Alc and 18:1n-9Alc. Wax analysis indicated that *EgWS* could utilize 12:0Alc, 14:0Alc, 16:0Alc and 16:1n-9Alc fatty alcohols, but not 10:0Alc, 18:0Alc and 18:1n-9Alc as the substrates. The preferred fatty alcohol was 16:1n-9Alc, which was followed by 14:0Alc, 12:0Alc and 16:0Alc (Table 1).

Fig. 3 Activity of *EgFAR* (a) and *EgWS* (b, c) in yeast. **a** GC analysis of fatty acid methyl esters and fatty alcohol TMS ethers prepared from the yeast transformed with the pESC-URA vector control or *EgFAR*. **b** TLC analysis of the total lipid of the yeast transformed with the pYES2.1 vector control (lanes 5 and 6) or *EgWS* (lanes 7 and 8) in the absence (lanes 5 and 7) and presence (lane 6 and 8) of substrates (14:0 and 14:0Alc). Lane 1–4, 9 are standards: DAG diacylglycerol, TAG triacylglycerol (c) GC analysis of the total lipid prepared from yeast transformed with *EgWS* or the pYES2.1 vector control and fed with 14:0 and 14:0Alc substrates

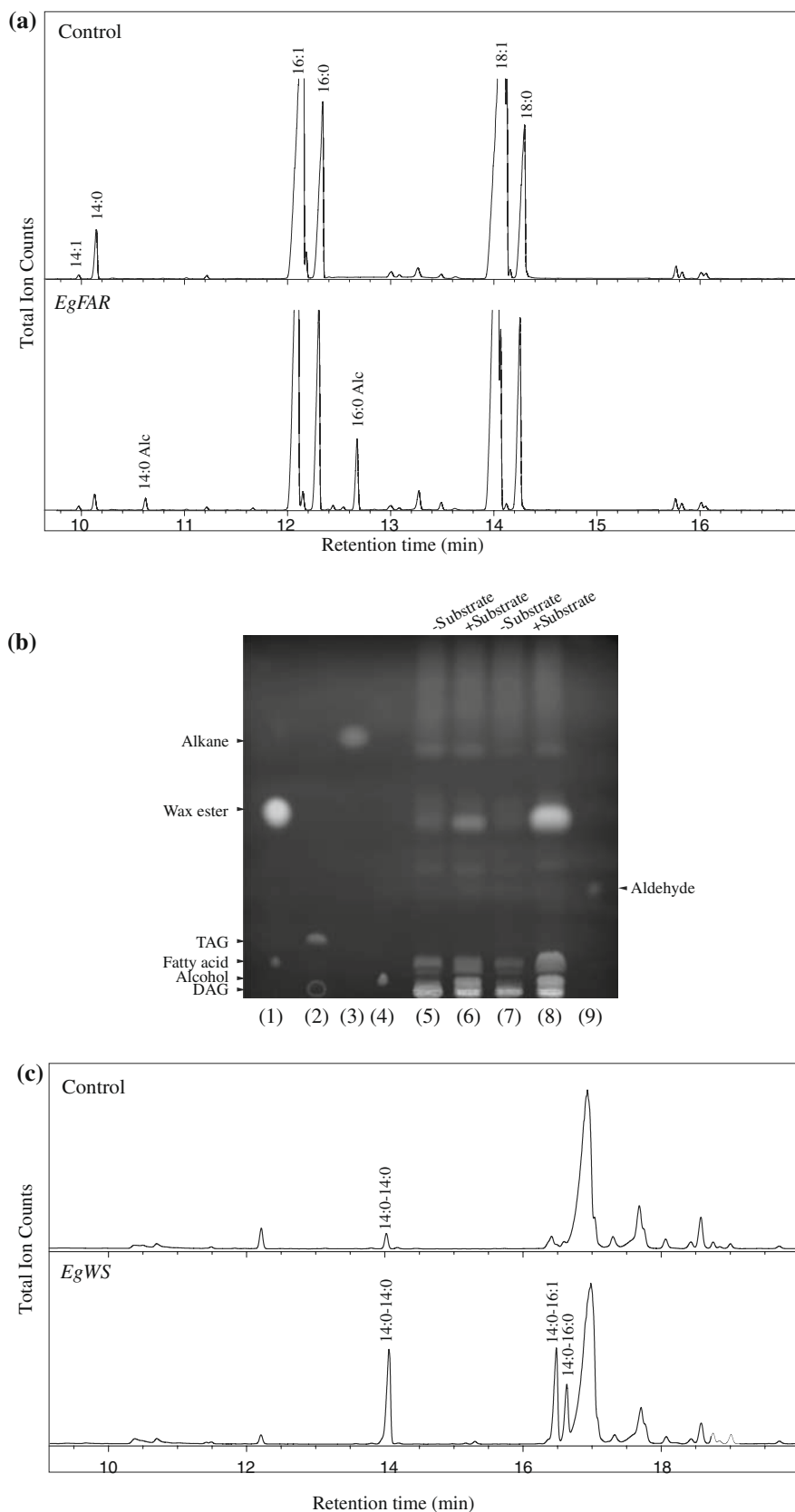


Table 1 Substrate specificity of EgWS for fatty acids and fatty alcohols

Substrate	Conversion efficiency (%)	
	Fatty acid	Fatty alcohol
12:0	37 ± 8	21 ± 2
14:0	47 ± 4	47 ± 6
16:0	31 ± 1	25 ± 2
16:1n-9	33 ± 3	68 ± 5
18:0	n.d.	n.d.
18:1n-9	n.d.	n.d.

The transformant yeast was fed with 14:0Alc in combination with different fatty acids to examine the fatty acid specificity and fed with 14:0 in combination with different fatty alcohols to examine the fatty alcohol specificity. The conversion efficiencies (%) of wax esters were calculated as $[(\text{product})/(\text{substrate} + \text{product})] \times 100$ using the values corresponding to the weight percent of fatty alcohol substrates and the wax ester products inside the yeast cells. The values are the average derived from triplicates ± standard deviation (SD)

n.d. not detected

To study the substrate specificity of EgFAR and EgWS in vitro, the microsomal fractions of yeast expressing *EgFAR* or *EgWS* were incubated with a series of fatty acids or fatty acid and alcohol combinations. However, only small activity of wax ester synthesis was detected in the in vitro assay of EgWS, which was basically in agreement with the result from the in vivo experiment. Furthermore, we could not detect any fatty acyl-CoA reductase activity in the in vitro assay of EgFAR with all possible substrates. The reason why the in vitro EgWS and EgFAR activities in

yeast were low is unknown. It is noteworthy that so far only three reports have described the in vitro fatty acyl-CoA reductase and wax synthase activities in heterologous systems, including mammalian FARs and WSs in the HEK293 human cell line [10, 17] and *Acinetobacter* WS/DGAT in *E. coli* [20]. The difficulty in the yeast in vitro assays of EgFARs and EgWSs might be related to the nature of this type of enzymes or simply the low activities of the two enzymes in yeast.

Reconstitution of the *Euglena* Wax Biosynthetic Pathway in Yeast

To reconstitute the *Euglena* wax biosynthetic pathway in yeast, *EgFAR* and *EgWS* were cloned into a yeast expression vector, pESC-URA, in which the expression of *EgFAR* and *EgWS* were driven by *GALI* and *GAL10* inducible promoters, respectively. The recombinant plasmid was then transformed into the yeast strain H1246 and the transformants were grown in the selective medium supplemented with 14:0, a common preferred substrate for both *EgFAR* and *EgWS*. Wax ester analysis indicated that the co-expressing yeast, like the *EgFAR*-expressing yeast, could convert 14:0 and 16:0 to 14:0Alc and 16:0Alc, respectively (Fig. 4). In addition, the co-expressing yeast produced three new wax ester peaks which were not detected in either *EgFAR*-expressing yeast or vector control yeast. Identities of these peaks were determined by comparing their retention times and mass spectra to those of standards. Peak 1 had the identical retention time and mass spectrum to

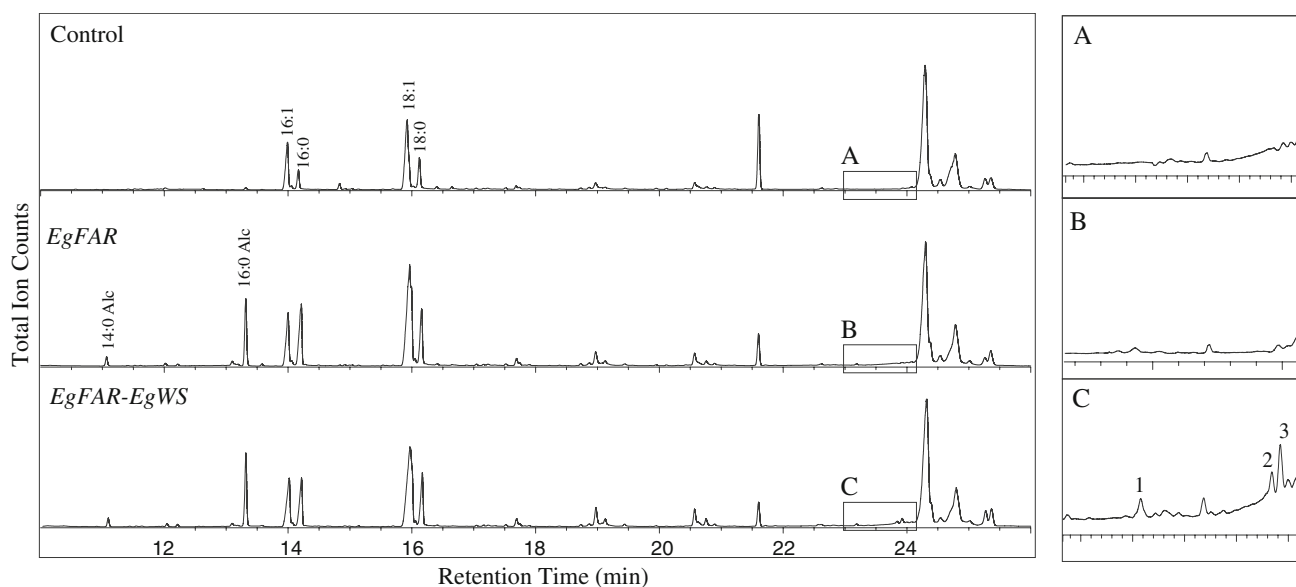


Fig. 4 Co-expression of *EgFAR* and *EgWS* in yeast. GC analysis of the total lipid showing wax ester production prepared from yeast transformed with pESC-URA (empty vector), *EgFAR* and *EgFAR-EgWS* and fed with 14:0 substrate. *Peak 1* myristyl-myristate (14:0–

14:0), *Peak 2* myristyl-palmitoleate (14:0–16:1), *Peak 3* myristyl palmitate (14:0–16:0) and palmityl myristate (16:0–14:0). Selected regions of the chromatograms of control, *EgFAR* and *EgFAR-EgWS* expressing yeast are magnified in boxes A, B and C, respectively

myristyl myristate (14:0–14:0) which accounted for ~23% of total wax esters (Fig. 5a) while peak 2 had the identical retention time and mass spectrum to myristyl palmitoleate (14:0–16:1) (Fig. 5b) which accounted for ~22% of total wax esters. Peak 3 appeared to contain two wax ester products, myristyl palmitate (14:0–16:0) and palmityl myristate (16:0–14:0) together they accounted for ~55% of total wax esters. GC and GC–MS analysis showed that it had the same retention time as myristyl palmitate (14:0–16:0) and palmityl myristate (16:0–14:0) and possessed the mass spectrum (Fig. 5c) with diagnostic ions representing the mass spectra of both myristyl palmitate (14:0–16:0) (Fig. 5d) and palmityl myristate (16:0–14:0) (Fig. 5e). Based on the abundance of the diagnostic fragments and the relative response factors of 14:0–16:0 (m/z 257) and 16:0–

14:0 (m/z 229), peak 3 contained ~58% of 14:0–16:0 and ~41% of 16:0–14:0. Collectively, these results indicated that EgWS in the co-expressing yeast could esterify 14:0Alc and 16:0Alc produced by EgFAR with 14:0, 16:0 and 16:1n-9 fatty acids, producing medium chain wax esters, myristyl myristate (14:0–14:0), myristyl palmitate (14:0–16:0), myristyl palmitoleate (14:0–16:1) and palmityl myristate (16:0–14:0).

Discussion

The biosynthesis of wax esters comprises two consecutive catalytic steps, reduction of fatty acyl-CoA to fatty alcohol and subsequent esterification of fatty acyl-CoA and fatty

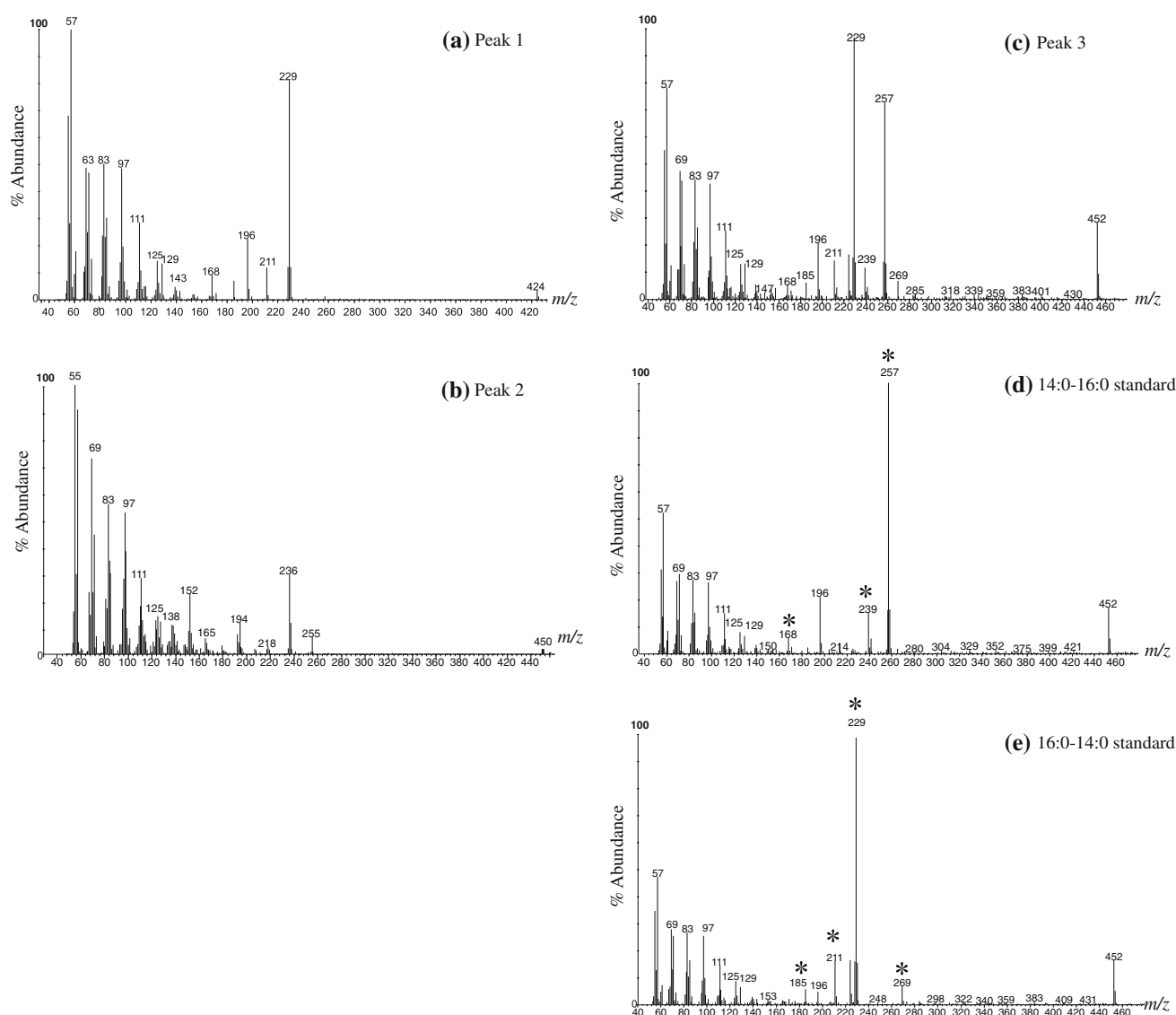


Fig. 5 GC/MS analysis of wax ester peak 1 **a**, peak 2 **b** and peak 3 **c** in Fig. 4, myristyl palmitate (14:0–16:0) standard **d** and palmityl myristate (16:0–14:0) standard **e**. Asterisks indicate diagnostic ions of the wax esters

alcohol. The reduction of fatty acid to fatty alcohol occurs through aldehyde intermediate and is catalyzed by either one or two enzyme reactions. In the two-enzyme reaction, acyl-CoA reductase first converts the fatty acyl-CoA to aldehyde and then the aldehyde reductase catalyzes the reduction of the aldehyde to alcohol. Evidence supporting the two-step reaction came from the early identification of aldehyde reductase from *Brassica oleracea* [33] and the recent isolation of the *acyl-CoA reductase* gene from *Acinetobacter*, catalyzing the production of fatty aldehyde [34]. However, support for the existence of the two-enzyme reduction is scarce in eukaryotes. In fact, a single enzyme reaction for the fatty acid reduction appears to occur widely in nature where the FAR catalyzes reduction of fatty acyl-CoA to fatty alcohol directly, although aldehyde intermediates could be detected by indirect trapping assays [23]. This type of fatty acyl-CoA reductases has been identified from jojoba cotyledon, pea leaves [23, 35, 36] and many others [37–39], including the one we describe here from *Euglena*.

Although *E. gracilis* has long been known to accumulate a large amount of medium-chain wax esters under dark and anaerobic growth conditions, the genes involved in the biosynthesis of these wax esters have yet to be identified. In this study, we report the isolation and characterization of two cDNAs, *EgFAR* and *EgWS*, from *E. gracilis* encoding two enzymes involved in the biosynthesis of medium chain wax esters. The pairwise sequence comparison of *EgFAR* and previously identified FARs revealed that *EgFAR* shares low amino acid identity with other FARs (23–27.4%). Functional analysis of *EgFAR* in yeast indicated that it could effectively convert 14:0 and 16:0 fatty acids to their corresponding alcohols. Compared with other biochemically characterized FARs, *EgFAR* possesses a narrower substrate range, only using saturated fatty acids with 14 and 16 carbon chains as substrates with the preferred fatty acid being 14:0 when expressed in yeast.

The pairwise sequence comparison of *EgWS* and related wax synthases revealed that *EgWS* also shares low amino acid identity to its related enzymes (in the range of 13–27%). It was noted that *EgWS* does not contain an N-terminal domain with the proposed active site (HHXXXD) found in *Acinetobacter* AcWS/DGAT [20], *Arabidopsis* AtWSD1 [21] and petunia PhWS1 [19]. This domain was believed to be essential for their acyl-CoA acyltransferase activities for the synthesis of wax esters and TAGs [20]. It will be interesting to know how *EgWS* functions without this domain. To date, three wax synthases including *EgWS* we report here have been functionally characterized in yeast. AtWSD1 mainly synthesizes wax esters with 16:0 fatty acid and 18:0Alc, 24:0Alc and 28:0Alc alcohols, and PhWS1 produces wax esters with very long chain fatty acids and methyl, isoamyl short to

medium chain alcohols (4–12 carbons), whereas *EgWS* prefers 14 carbon chain fatty acid as the substrate.

Wax esters consisting of medium chain saturated fatty alcohols are sporadically found in animals and microorganisms [40–43]. *E. gracilis* can accumulate up to 28 or 62% of the total lipid as wax esters when grown in aerobic or anaerobic conditions [3] and the major molecular species of the wax esters accumulated is myristyl myristate [4, 6]. Functional expression of *EgFAR* and *EgWS* separately in yeast revealed that both enzymes could use 14 and 16 fatty acid substrates and the co-expression of *EgFAR* and *EgWS* in yeast resulted in production of medium-chain wax esters. These results are consistent with the composition of wax esters naturally present in *Euglena*, suggesting an important role of *EgFAR* and *EgWS* in synthesizing the wax esters in *Euglena*. *EgFAR* and *EgWS* were identified by the homology search of an EST database using jojoba *ScFAR* and *ScWS* as query sequences and only one EST of each *EgFAR* and *EgWS* was found in the database. However, it could not be excluded that there are additional genes involved in the biosynthesis of medium-chain wax esters in *Euglena* that were not present in the EST database. It should also be noted that *Euglena* can accumulate unsaturated fatty acid (e.g. 18:1n-9) wax esters when the culture medium is supplemented with such fatty acid [6]. Although we have not observed any activity of *EgFAR* and *EgWS* towards 18:1n-9 when they were expressed in yeast, this should not exclude the possibility that the difference of the cellular micro-environment between the native host and yeast could have effect on the substrate specificity of the enzymes. Alternatively, there might be additional FARs and WSs present in *Euglena* that can utilize unsaturated fatty acids as substrates.

Medium- and long-chain wax esters have been widely used in food, pharmaceuticals, textiles, perfumes and flavoring. They can also be used in the production of fatty sulfate salts and alcohol ethoxylates in the detergent industry. The current supply of wax from natural sources is limited due to the high production cost and cannot meet the growing demand for its widespread uses. Metabolic engineering of oilseed plants to produce wax esters has been viewed as an attractive alternative to provide cost-effective sources for biological wax. The first attempt of wax metabolic engineering in plants was undertaken by expressing jojoba *ScFAR* and *ScWS*, along with *Lunaria annua* β -ketoacyl-CoA synthase (a component of fatty acid elongase) resulting in the production of very long chain wax esters in *Brassica napus* seeds [18]. Here, we describe two genes *EgFAR* and *EgWS* from *Euglena* encoding fatty acyl-CoA reductase and wax synthase that have a substrate preference towards medium chain substrates. It will be interesting to see how these genes perform in the production of industrially important medium-chain wax esters in oil seed crops.

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