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Article title: Molecular and Functional Analysis of Three Fatty Acyl-CoA Reductases with Distinct Substrate Specificities in Copepod *Calanus finmarchicus*

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Abstract

The marine copepod *Calanus finmarchicus* constitutes the substantial amount of biomass in the Arctic and Northern seas. It is unique in that this small crustacean accumulates a high level of wax esters as carbon storage which is mainly comprised of 20:1n-9 and 22:1n-11 alcohols (Alc) linked with various kinds of fatty acids, including n-3 polyunsaturated fatty acids. The absence of 20:1n-9 Alc and 22:1n-11 Alc in diatoms and dinoflagellates, the primary food sources of copepods, suggests the existence of *de novo* biosynthesis of fatty alcohols in *C. finmarchinus*. Here we report identification of three genes, *CfFAR1*, *CfFAR2* and *CfFAR3*, coding for fatty acyl-CoA reductases involved in the conversion of various fatty acyl-CoAs to their corresponding alcohols. Functional characterization of these genes in yeast indicated that CfFAR1 could use a wide range of saturated fatty acids from C18 to C26 as substrates, CfFAR2 had a narrow range of substrates with only very long chain saturated fatty acid 24:0 and 26:0, while CfFAR3 was active towards both saturated (16:0 and 18:0) and unsaturated (18:1 and 20:1) fatty acids producing corresponding alcohols. This finding suggested that these three fatty acyl-CoA reductase are likely responsible for *de novo* synthesis of a series of fatty alcohol moieties of wax esters in *C. finmarchicus*.

Introduction

Primary fatty acyl alcohols possess important biological functions in living organisms and can be found as free or esterified forms in the cells. The free alcohols and their acetate ester derivatives can function as pheromones in insects (Tillman et al. 1999), while wax esters are found on the surface of plants and animals to serve as a protective barrier against water loss, UV light, pathogen and pest damage (Samuels et al. 2008). Some microorganisms such as *Euglena* and *Acinetobacter* accumulate wax esters as energy reserves (Fixter et al. 1986; Tucci et al. 2010). Honey bees produce comb wax which is used to build hexagonal cells for larvae rearing as well as pollen and honey storage (Cassier and Lensky 1995).

In eukaryotes, the biosynthesis of fatty alcohols is undertaken by a single-enzyme reaction (Kolattukudy 1970; Pollard et al. 1979; Vioque and Kolattukudy 1997). In this reaction, fatty acyl-CoA reductase (FAR) catalyzes reduction of fatty acyl-CoA to fatty alcohol without releasing aldehyde intermediate (Kolattukudy 1970). The genes encoding the fatty acyl-CoA reductases have been isolated from various organisms, including *Euglena* (Teerawanichpan and Qiu 2010), mammals (Cheng and Russell 2004) , insects (Moto et al. 2003; Antony et al. 2009; Lienard et al. 2010; Teerawanichpan et al. 2010), and plants (Metz et al. 2000; Wang et al. 2002; Rowland et al. 2006; Doan et al. 2009; Maes et al. 2010). ScFAR, a fatty acyl-CoA reductase from jojoba (*Simmondsia chinensis*), is responsible for producing storage wax esters in developing seeds (Metz et al. 2000). Arabidopsis fatty acyl-CoA reductase AtCER4 (At4g33790) is involved in the synthesis of cuticular wax lipids (Rowland et al. 2006). Arabidopsis *MALE STERILE 2* (*MS2*) is preferentially expressed in the tapetal cells of male gametophyte and mutation of this gene resulted in an abnormality of pollen development and eventually the male sterility (Aarts et al. 1997). *TaTAA1a, TaTAA1b* and *TaTAA1c*, three orthologs of the jojoba

ScFAR, were isolated from wheat and they are involved in producing the lipid component in the outer pollen wall (Wang et al. 2002). Fatty acyl-CoA reductases have been isolated from silkmoth *Bombyx mori* (Moto et al. 2003), bean borer moth *Ostrinia scapulalis* (Antony et al. 2009) and three different species of ermine moths *Yponomeuta evonymellus*, Y. *padellus* and Y. *rorellus* (Lienard et al. 2010). They are pheromone gland specific and responsible for production of fatty alcohols which serve as sex pheromones directly or are further converted to acetate derivative pheromones.

Calanus finmachicus is a planktonic crustacean (copepod) which represents a significant amount of the biomass in the Arctic and Northern seas (Ringuette et al. 2002). It is a primary food source for juvenile and adult fishes, sea birds and whales (Brown and Gaskin 1988; Michaud and Taggart 2007). This small crustacean accumulates a large amount of wax esters in its oil sac to serve primarily as an energy reserve and aid in buoyancy (Gatten and Sargent 1973; Sargent and Falk-Petersen 1988; Visser and Jónasdóttir 1999). The wax content and composition are influenced by developmental stages and growing seasons (Kattner and Krause 1987; Sargent and Falk-Petersen 1988). The high level of wax esters are generally synthesized in the late stage of copepod development and mainly used as metabolic energy for reproduction over the winter (Sargent and Falk-Petersen 1988). In females, the wax ester is utilised for gonadal development, whereas males consume wax esters for physical activity during reproduction. The alcohol moieties in wax esters are mainly 20:1n-9 and 22:1n-11 alcohols, whereas the acyl moieties in wax esters are varied depending upon the nutritional conditions, mainly the ingested phytoplanktons which are generally rich in ω-3 polyunsaturated fatty acids (PUFAs).

De novo biosynthesis of fatty alcohols in zooplanktons was evident in the feeding experiment using radiolabelled tracers (Morris and Sargent 1973). Activities of fatty acyl-CoA

reductase and wax synthase were detected in the cell-free system of zooplanktons, including *C. finmarchicus* and *Acartia* sp. (Gatten and Sargent 1973) as well as *Euchaeta norvegica* (Sargent et al. 1974). The fatty acyl-CoA reductase activity is NADPH-dependent (Sargent et al. 1974) and is a rate limiting step for the wax ester synthesis in these organisms (Gatten and Sargent 1973; Sargent et al. 1974).

Although the biochemical studies suggest the existence of wax biosynthetic pathways in *C. finmarchicus*, the genes encoding fatty acyl-coA reductase and wax synthase have not been identified. In this study, we started first with analysis of the fatty acid and fatty alcohol composition of *C. finmachicus* sampled from Canadian North Atlantic Ocean, and then went on to clone and functionally characterize three genes encoding fatty acyl-CoA reductases in *Saccharomyces cerevisiae* involved in the biosynthesis of a wide range of fatty alcohols in this copepod.

Materials and Methods

Materials

Calanus finmarchicus samples were harvested from North Atlantic Ocean at Halifax harbour during spring season 2009 (April) using regular conical plankton net towed vertically from 50 meters underneath to the surface. The copepods were either at the female stage (C6F) or at copepodide stage C5 (McLaren et al. 2001). Fatty acids (14:0, 16:0, 18:0, 20:0, 22:0, 24:0, 16:n-9, 18:1n-9, 18:2n-6, 18:3n-3, 20:1n-9, 22:5n-3 and 22:6n-3) and fatty alcohols (14:0Alc, 16:0Alc, 18:0Alc, 20:0Alc, 22:0Alc, 24:0Alc, 16:1n-9Alc, 18:1n-9Alc, 20:1n-11Alc) with 99% purity were purchased from Nuchek-Prep, Inc.

Rapid Amplification of cDNA Ends (RACE)

Total RNA was extracted from *C. finmarchicus* sample using TRIZOL® reagent (Invitrogen) and treated with DNaseI (Invitrogen, Amplification grade) under conditions detailed by the supplier. First-strand cDNA for 3′ RACE was synthesized at 50°C for 1 h, using a 3′ RACE adaptor (Table 1), 1 µg of total RNA of *C. finmarchicus*, and 200 units of SUPERSCRIPT™ III Reverse Transcriptase (Invitrogen). A 2-µl aliquot of reverse transcription reaction was subsequently used in 35 cycles of PCR amplification using the 3′ RACE outer primer and the 3′ RACE gene-specific outer primer under the following cycling conditions: 94 ºC for 30 s, 60 ºC for 30 s, and 72 ºC for 1.5 min. A 2-µl aliquot of outer RACE reaction was further used as a template for the inner 3′ RACE reaction, which was performed using 3′ RACE inner primer and the 3′ RACE gene-specific inner primer. The 3′ RACE products were resolved in 1% agarose gel electrophoresis, purified, cloned into pGEM- T Easy vector (Promega) and sequenced. The primers used for 3′ RACE of *CfFAR1*, *CfFAR2* and *CfFAR3* are listed in Table 1

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

First stranded cDNA synthesis was performed at 55°C for 1 h, using 0.5 µg of oligo(dT)$_{12\text{-}18}$ primers, 1-µg total RNA isolated from *C. finmachicus*, and 200 units of SUPERSCRIPT™ III Reverse Transcriptase (Invitrogen). Two microlitres of the first-strand reaction was subsequently used as a template for 50-µl PCR reaction in the presence of 2.5 units of Platinum® *Pfx* DNA polymerase (Invitrogen). The full-length CfFAR1, CfFAR2 and CfFAR3 were amplified using primers PT122-PT123, PT124-PT125, PT126-PT127, respectively (Table 1). The PCR conditions were 35 cycles of 94 °C for 15 sec, 55 °C for 30 sec, 68°C for 1 min. The amplified products were gel-purified, 3′A-overhang added, using *Taq* DNA polymerase (Invitrogen) and cloned into pYES2.1/V5-His-TOPO® (Invitrogen).

DNA Sequencing and Analysis

All DNA 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).

The previously characterized FAR amino acid sequences were aligned with ClustalW as hosted at the European Bioinformatics Institute (Chenna et al. 2003) using default parameters, including the Gonnet scoring matrix, a gap penalty of 10, and a gap extension penalty of 0.2. The resulting alignment was used to generate a distance-based phylogram using the neighbour-joining method performed using PROTDIST and NEIGHBOR in the PHYLIP software suite, version 3.6 (Felsenstein 1989) as hosted by the Institute Pasteur, Paris, France. Parameters for PROTDIST included the use of the Dayhoff PAM matrix and George/Hunt/Barker amino acid categories. The tree was visualized using TREEVIEW (Page 1996). The analysis was repeated with bootstrap analysis using 100 iterations and an extended majority rule tree was constructed using CONSENSE. Hydropathy analysis of CfFARs was performed using TopPredII (Claros and von Heijne 1994) and the Goldman Engleman Steitz (GES) scale (Engelman et al. 1986) with default parameters. The potential membrane-associated domains with a score greater than 0.5 GES were shown in Fig. 1.

Functional Analysis of Putative Fatty Acyl-CoA Reductases from *C. finmarchicus* in Yeast

The pYES2.1 plasmids harbouring CfFAR1, CfFAR2 or CfFAR3 were transformed into yeast (*Saccharomyces cerevisiae*) strain INVSc1 (*MATa his3Δ1 leu2 trp1-289 ura3-52 MATα his3Δ1 leu2 trp1-289 ura3-52*; Invitrogen) using S.c. EasyComp™ transformation kit (Invitrogen). For functionally analysis, the yeast transformants were grown at 30 °C for 2 days in 10 ml of

synthetic dropout medium containing 0.17% (w/v) yeast nitrogen base, 0.5% 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 was induced by culturing the yeast at 20 °C for 4 days in 10 ml of synthetic dropout medium containing 2% (w/v) galactose and 0.06% (w/v) dropout supplement lacking uracil (DOB+GAL-URA) with 250 μM substrate fatty acid supplementation in the presence of either 0.1% tergitol (Nonidet P-40; for 14:0,16:0,18:0, 20:0,16:1n-9,18:1 n-9 and 20:1 n-9) or 2.5% ethanol (for 22:0 and 24:0). The fatty acid and fatty alcohol substrates were individually dissolved as stock solutions in ethanol at the final concentration of 500 mM. For 14:0, 16:0, 18:0, 20:0, 16:1n-9, 18:1n-9, 18:2n-6, 18:3n-3 and 20:1n-9 substrates, the appropriate amount of fatty acids were further dissolved in 10% tergitol at the final concentration of 50 mM. For 22:0 and 24:0, the appropriate amount of fatty acids was further dissolved in ethanol at the final concentration of 10 mM and heat until completely dissolved. The fatty acid solutions were heated till completely dissolved before adding into the yeast culture. After four days of induction, the cultures were washed once with 10 ml of 1% tergitol and once with 10 ml of distilled water and subjected to fatty acid analysis.

Analysis of Fatty Acid Methyl Esters and Alcohols of Copepod and Yeast Transformants

C. finmarchicus (30 individuals) samples were crushed in a mortar and directly transmethylated in 1% H_2SO_4 in methanol at 60°C for 2 h. Fatty acid methyl esters (FAMEs) and fatty alcohols were extracted with 2 ml of hexane and the hexane was subsequently removed by nitrogen gas. Total FAMEs and fatty alcohols were resuspended in 50 μl of hexane and derivatized with 50 μl of *N*,*O*-bis(trimethylsilyl)-acetamide (TMS)/pyridine (1:1, v/v) at 80 °C for 30 min and the derivative was analyzed by gas chromatography (GC). 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 % (w/v) NaCl solution. Total 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 µl of hexane. Fifty microlitres of samples were derivatized with TMS as described above.

GC and GC-Mass Spectrometry (MS) Analysis

FAMEs and TMS derivatives of alcohols of *C. finmarchicus* were analyzed on an Agilent 6890N GC-MS equipped with a DB-23 column (30 m×0.25 mm) (J&W Scientific). The following temperature programmes were employed: 120 °C for 1 min, then 4°C/min to 300 °C, and 240 °C for 15 min with He as carrier gas. For GC and GC-MS analysis of yeast transformants, samples were analyzed on an Agilent 6890N GC equipped with a DB-5 column (30 m×0.25 mm) (J&W Scientific). The following temperature programmes were employed: 125 °C for 1 min, then 5°C/min to 300 °C, and 300 °C for 10 min with He as 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 to 700 at 2.26 scans/s. Identities of FAMEs were identified by comparing their retention times and their fragmentation patterns with those of the standards.
The conversion efficiencies (%) of substrates in yeast were calculated as [(product)/(substrate+ product)] × 100 using the values corresponding to the weight percentage of fatty acid substrates and the fatty alcohol products inside the yeast cells.

Results

Analysis of the lipid composition in *C. finmarchicus*

Wax esters of *C. finmarchicus* samples harvested at female stage (C6F) or copepodide stage C5 constitute more than 70% of the total lipids (Kattner and Graeve 1991; Michaud and Taggart 2007). The composition analysis of total lipids indicated that *C. finmarchicus* contains a wide range of fatty acids and fatty alcohols. It contains approximately 24% saturated fatty acids, 42% monounsaturated fatty acids and 34% polyunsaturated fatty acids (PUFAs). Eicosapentaenoic acid (20:5n-3; 18.4% of total fatty acids), docosahexaenoic acid (22:6n-3; 12.7% of total fatty acids) and stearidonic acid (18:4n-3; 2.5% of total fatty acids) are three major ω-3 PUFAs detected in the *C. finmarchicus* sample. These ω-3 fatty acids in *C. finmarchicus* could be derived directly from the diet (Morris and Sargent 1973; Sargent and Falk-Petersen 1988). Unlike the fatty acid profile, the fatty alcohols in *C. finmarchicus* consist of only saturated and monounsaturated hydrocarbons. It produces 20:1n-9Alc (52.4% of total fatty alcohols) and 22:1n-11Alc (31.9% of total fatty alcohols) as major fatty alcohols with 16:0Alc, 16:1n-9Alc, 18:1n-9Alc and 18:1n-6Alc as minor components together accounting for 12.8% of total fatty alcohols (Table 2).

Isolation of putative *CfFARs* from *C. finmarchicus*

Using the sequence similarity search of expressed sequence tag (EST) databases prepared from *C. finmarchicus* with the fatty acyl-CoA reductase previously identified from honey bee (AmFAR1) as a query sequence (Teerawanichpan et al. 2010), we identified six EST clones (EL774137, EL965618, EL586057, FK867560, FG632629, FK041312) representing three different putative *CfFAR* genes, named *CfFAR1* (EL774137 and EL965618), *CfFAR2* (EL774137 and EL965618) and *CfFAR3* (FG632629 and FK041312). To retrieve the missing ends of these ESTs, rapid amplification of cDNA ends (RACE) approaches were then adopted to obtain the full-length cDNAs, *CfFAR1, CfFAR2* and *CfFAR3* that encode putative fatty acyl-coA

reductases in *C. finmarchicus*. *CfFAR1* encodes a polypeptide of 467 amino acids with a molecular mass of 52.1 kDa, while *CfFAR2* and *CfFAR3* encode slightly larger polypeptides with 498 amino acids with a molecular mass of 56.2 kDa and 495 amino acids with a molecular mass of 55.8 kDa, respectively.

Sequence comparison of these three CfFARs revealed that CfFAR2 and CfFAR3 share 65% identity at the amino acid level, whereas CfFAR1 shares 51.9% and 42.6% amino acid identity to CfFAR2 and CfFAR3, respectively. All of them contain Rossmann-fold NAD/NADP binding domains (NABD) (Rossmann et al. 1974) linked with Male Sterile 2 domain (MS2) (Aarts et al. 1997) at the carboxyl end (Fig. 1). A conserved motif (I/V)-(F/L)-(I/L/V)-T-G-X-T-G-F-(M/L/V)-(G/A), found in other fatty acyl-CoA reductases, was also observed in the NABD domain of CfFARs (Fig. 1). Hydropathy analysis showed that CfFAR1 contains one potential hydrophobic region in the middle of the polypeptide, whereas CfFAR2 and CfFAR3 bear two potential hydrophobic regions; one at about two thirds of the sequences and the other at the carboxyl termini (Fig. 1).

Sequence comparison between copepod FARs and other functionally characterized reductases showed that the copepod FARs have the highest sequence similarity to honeybee AmFAR1 (40-42% amino acid identity), followed by mammalian FARs (35-38% identity), insect pheromone gland-specific FARs (28-35% identity) and finally plant FARs (21-28% identity). Phylogenetic analysis of these reductases revealed that the *C. finmarchicus* FARs form a group with bee AmFAR1, which is closely related to the mammalian reductases (human FARs and mouse FARs), and distantly related to the pheromone gland-specific insect reductases, *Euglena* EgFAR1 and plant reductases (Fig. 2).

Functional characterization of putative *CfFARs* in yeast

Activities of putative *CfFAR*s were examined by expressing these genes under the *GAL1* inducible promoter in yeast *Saccharomyces cerevisiae*. To test whether any of these genes is involved in producing eicosanol (20:1n-9 Alc), the major alcohol species in *C. finmarchicus*, the yeast strains expressing *CfFAR1*, *CfFAR2* or *CfFAR3* were respectively fed with eicosenoic acid (20:1n-9). The fatty acid and fatty alcohol analysis of transformants showed that, the yeast expressing *CfFAR1* produced five novel peaks, compared to the vector control (Fig. 3). GC and GC-MS analysis confirmed that these peaks have identical retention times and mass spectra to those of TMS derivatives of stearyl alcohol (18:0Alc), eicosanoyl alcohol (20:0Alc), behenyl alcohol (22:0Alc), lignoceryl alcohol (24:0Alc) and ceryl alcohol (26:0Alc). The yeast expressing *CfFAR2* produced two new peaks, compared to the vector control, and these peaks showed identical retention times and mass spectra to those of TMS derivertives of lignoceryl alcohol (24:0Alc) and ceryl alcohol (26:0Alc). The yeast expressing *CfFAR3* produced four novel peaks, compared to the vector control, and those peaks are palmitoyl alcohol (16:0Alc), stearyl alcohol (18:0Alc), oleyl alcohol (18:1n-9 Alc) and eicosanol (20:1n-9 Alc) confirmed by GC-MS (Fig. 3).

To study the substrate specificity of these fatty acyl-CoA reductases, the yeast strains expressing these genes were separately fed with various probable fatty acids. The results showed that none of these CfFARs could utilize polyunsaturated fatty acids such as 18:2n-6, 18:3n-3 or 20:5n-3 and middle chain fatty acids such as14:0 as substrates. CfFAR1 had very high conversion efficiencies towards saturated fatty acids from 20:0 to 24:0, which was then followed by 26:0. Stearic acid (18:0) is the least preferred substrate. CfFAR2 utilized 24:0 and 26:0 as effectively as CfFAR1. Whereas, CfFAR3 showed the high conversion efficiency towards 18:0,

followed by a very long chain monounsaturated fatty acid 20:1n-9. 16:0, 18:1 n-9 and 16:1 n-9, are minor substrates for CfFAR3 (Table 3).

Discussion

Copepod is a small crustacean that lives upon phytoplankton in water system. It represents the largest animal biomass in ocean and major food source for small fish and whales (Ringuette et al. 2002). *C. finmarchicus* is one of the most occurring copepods in the Arctic and Northern seas and plays an important role in the ocean ecosystem by linking between the primary producer of organic compounds and higher trophic levels of animals. Like other calanoid copepods, *C. finmarchicus* can transform low-energy carbohydrates to high-energy wax esters. The stored wax esters in the copepod can reach up to 70% of the total lipids (Kattner and Krause 1987) where very long chain ω-3 polyunsaturated fatty acids are major components (Table 2).

Although the copepod has been known to produce wax esters and their biosynthetic pathways have been proposed (Sargent and Falk-Petersen 1988), genes that are directly involved in the biosynthesis have yet to be cloned. In this report, we identified three genes encoding fatty acyl-CoA reductases from *C. finmarchicus* which are involved in converting fatty acids to corresponding fatty alcohols, which can then be used for the synthesis of wax esters. When expressed in yeast, these FARs showed distinct difference in their substrate preponderances. CfFAR1 could use a wide range of saturated long or very long chain fatty acids (18C to 26C) as substrates, but not any unsaturated fatty acids as substrates. CfFAR2 could only effectively utilize two very long chain saturated fatty acids 24:0 and 26:0 as substrates, not any shorter chain saturated fatty acids or unsaturated fatty acids as substrates. CfFAR3 that could use unsaturated

fatty acids such as 20:1n-9 and 18:1n-9 in addition to saturated 18:0 and 16:0 as substrates. The substrate specificity of CfFAR3 towards 20:1n-9, 16:0, 18:1n-9 and 16:1n-9, is consistent with the fatty alcohol composition of *C. finmarchicus*, suggesting the possible involvement of CfFAR3 in producing these fatty alcohols for the wax biosynthesis of this organism. However, based on the conversion efficiency of CfFAR3 on 20:1n-9 which is lower than that of C18:0, it is conceivable that there might be other unidentified FARs in *C. finmarchicus* to preferably reduce this fatty acid for the wax synthesis. Alternatively, we could not also exclude the possibility that the yeast feeding assay is not so reflective of *in vivo* substrate specificity of the FAR in the copepod, as it has been previously shown that the cellular micro-environment difference in host systems could have a strong effect on the substrate specificity of the wax-synthetic enzymes (Wang et al. 2002; Rowland et al. 2006; Doan et al. 2009). It is noteworthy that *C. finmarchicus* contains a high level of 22:1n-11 alcohol in the wax esters (Table 2), however, due to the commercially unavailability of this fatty acid, we were unable to test this important substrate for CfFARs.

Hydropathy analysis revealed a hydrophobic region at the carboxyl terminus of CfFAR2 and CfFAR3. The hydrophobic region was also found in mammalian FARs and a honey bee FAR (Cheng and Russell 2004; Teerawanichpan et al. 2010) and was previously assumed to play a role in targeting the FARs to peroxisome where the ether lipids are synthesized (Cheng and Russell 2004; Honsho et al. 2010). If so, CfFAR2 and CfFAR3 may likely destine to cellular compartments different from that of CfFAR1 as it does not have this hydrophobic region.

Long and very long chain saturated fatty alcohols are mostly present as esterified forms in epicuticular waxes of plants and animals (Hargrove et al. 2004). They are also found as components of glyceryl ether lipids in the liver of ratfish (C18-C24) (Hayashi et al. 1983). The

alcohol moieties in epicuticular waxes of plants and honey bees tend to be very long chain (C24-C34) (Hargrove et al. 2004). Epicuticular lipids in cereals, such as wheat and oat, however, contain relatively high level of free alcohols which are usually either C26 or C28 in length (Hargrove et al. 2004). Free forms of long and very long chain alcohols are also found in epicuticular layer of some arthropods, such as Arizona vejovid scorpions (C18-C32) (Toolson and Hadley 1977) and grasshoppers (C24-C30) (Soliday et al. 1974). For *C. finmarchicus*, the structure of the cuticular layer has been examined (Raymont et al. 1974), but the lipid composition of the layer remains unknown. In this study we did not detect any very long chain saturated alcohol in copepod samples used. However, it will be interesting to see if CfFAR1 and CfFAR2 that have substrate preference towards very long chain saturated fatty acids are actually involved in the biosynthesis of cuticular lipids and glyceryl ether lipids in *C. finmarchicus*.

Short to long chain fatty alcohols are found in pheromone blends in social insects (Alaux et al. 2010). The genes encoding pheromone gland-specific reductases have been isolated from three types of moths (Moto et al. 2003; Antony et al. 2009; Lienard et al. 2010). BmFAR, a fatty acyl-CoA reductase from silkmoth (*Bombyx mori*), is responsible for production of sex pheromone bombykol, (*E*,*Z*)-10,12–hexadecadien-1-ol (Moto et al. 2003). 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 (Antony et al. 2009). Fatty acyl-CoA reductase (FARII) from ermine moths (*Yponomeuta* sp.) converts C14 or C16 fatty acids to their corresponding alcohol derivatives which are components of sex pheromone blends of yponomeutids (Lienard et al. 2010). The communication between potential mates in copepods is restricted due to its small size, limited metabolic capability and high diffusion rate of the surrounding water (Snell and Morris 1993). The existence of sex

pheromones in copepods is evident based on mating behaviour studies (Snell and Morris 1993). However, the diffusible signals for mate recognition in copepods have yet to be identified. Therefore, the involvement of fatty alcohols synthesized by the acyl-CoA reductases identified here in this process remains to be elucidated.

Biological wax, an ester of a fatty acid and a fatty alcohol, has been widely used in pharmaceuticals, cosmetics, lubricants and coatings. Current capacity of natural wax production 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 (Jetter and Kunst 2008). This study identified three functional fatty acyl-CoA reductases which can convert distinct sets of fatty acids to their corresponding alcohols, especially, CfFAR3 that has high activity towards monounsaturated fatty acids such as 20:1n-9 and 18:1n-9 which widely occur in plant oilseeds. Therefore, it would be a good candidate for transgenic production of wax esters in plants for industrial use.

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Legends

Fig. 1. Amino acid sequence alignment of three putative fatty acyl-CoA reductases. The identical residues are shaded in black. An open box with dotted line indicates the conserved motif, (I/V)-(F/L)-(I/L/V)-T-G-X-T-G-F-(M/L/V)-(G/A) which might be directly involved in the catalytic reaction. The open box with solid line indicates the hydrophobic domain. A dotted line indicates the Rossmann-fold NABD domain while a solid line denotes the Male Sterility 2 domain.

Fig. 2 Phylogenetic analysis of CfFARs and other related sequences. The GenBank accession numbers of the sequences are as follows: copepod CfFAR1, JN243755; copepod CfFAR2, JN243756; copepod CfFAR3, JN243757; mouse MmFAR1, BC007178; mouse MmFAR2, BC055759; human HsFAR1, AY600449; human HsFAR2, BC022267; silkmoth BmFAR, AB104896; bean borer moth OsFARXIII EU817405; ermine moth YevFARII, GQ907232; *Euglena* EgFAR GU733919; honey bee AmFAR, NM_001193290; Arabidopsis Male Sterile 2 (AtMS2), X73652; wheat FAR (TaTAA1a), AJ459249; jojoba ScFAR, AF149917; Arabidopsis AtCER4, AY070065-At4g33790.

Fig. 3 Functional expression of CfFARs in yeast. Gas chromatography analysis of trimethylsilyl derivatives of fatty acid methyl esters prepared from the yeast transformed with pYES2.1 vector control, CfFAR1, CfFAR2 and CfFAR3, respectively.