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A fatty acyl-CoA reductase highly expressed in the head of honey bee (*Apis mellifera*) involves biosynthesis of a wide range of aliphatic fatty alcohols

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ABSTRACT

Honey bees (*Apis mellifera*) are social insects which have remarkable complexity in communication pheromones. These chemical signals comprise a mixture of hydrocarbons, wax esters, fatty acids, aldehydes and alcohols. In this study, we detected several long chain aliphatic alcohols ranging from C18-C32 in honey bees and the level of these alcohols varied in each body segment. C18:0Alc and C20:0Alc are more pronounced in the head, whereas C22:0Alc to C32Alc are abundant in the abdomen. One of the cDNAs coding for a fatty acyl-CoA reductase (*AmFAR1*) involved in the synthesis of fatty alcohols was isolated and characterized. *AmFAR1* was ubiquitously expressed in all body segments with the predominance in the head of honey bees. Heterologous expression of *AmFAR1* in yeast revealed that AmFAR1 could convert a wide range of fatty acids (14:0–22:0) to their corresponding alcohols, with stearic acid 18:0 as the most preferred substrate. The substrate preference and the expression pattern of *AmFAR1* were correlated with the level of total fatty alcohols in bees. Reconstitution of the wax biosynthetic pathway by heterologous expression of *AmFAR1*, together with *Euglena* wax synthase led to the high level production of medium to long chain wax monoesters in yeast.

1. Introduction

Aliphatic fatty alcohols are naturally found in many organisms and can be present in the free form or incorporated into ether lipids and wax esters. The enzyme that catalyzes the reduction of fatty acyl-CoAs to alcohols is fatty acyl-CoA reductase (FAR) and genes coding for this enzyme have been isolated from various organisms, including protozoa (Teerawanichpan and Qiu, 2010), insects (Antony et al., 2009; Moto et al., 2003), animals (Cheng and Russell, 2004a) and plants (Metz et al., 2000; Rowland et al., 2006). Some of them are engaged in epicuticular wax synthesis, such as *Arabidopsis* fatty acyl-CoA reductase AtCER4 (Rowland et al., 2006) and others are involved in production of storage wax esters, such as *Euglena* fatty acyl-CoA reductase EgFAR (Teerawanichpan and Qiu, 2010) and jojoba fatty acyl-CoA reductase ScFAR (Metz et al., 2000). Mammalian FARs are likely to engage the biosynthesis of both ether lipids and wax esters (Cheng and Russell, 2004a; Honsho et al., 2010). So far, only two *FAR* genes were isolated from insects and both of them are pheromone gland-specific and involved in the pheromone biosynthesis. BmFAR, fatty acyl-CoA reductase from silkmoth (*Bombyx mori*), is responsible for the production of sex pheromone bombykol, (*E*,*Z*)-10,12–hexadecadien-1-ol (Moto et al., 2003), while OsFARXIII, fatty acyl-CoA reductase from bean borer moth (*Ostrinia scapulalis*), catalyzes the production of (*Z*)-11-tetradecenol, which is further converted to its acetate or aldehyde derivative pheromones (Antony et al., 2009).

In the wax biosynthetic pathway, the fatty alcohol is esterified with fatty acyl-CoA, yielding wax ester, which is catalyzed by acyl-CoA:fatty alcohol acyltransferase or wax synthase (WS) (Fig. 1). The genes encoding WSs have been isolated from several organisms, including bacteria (Kalscheuer and Steinbuchel, 2003), protozoa (Teerawanichpan and Qiu, 2010), animals (Cheng and Russell, 2004b) and plants (King et al., 2007; Lardizabal et al., 2000; Li et al., 2008). However, only three of them have been successfully expressed in yeast, including *Arabidopsis* AtWSD1 (Li et al., 2008), petunia PhWS1 (King et al., 2007) and *Euglena* EgWS (Teerawanichpan and Qiu, 2010). AtWSD1 mainly synthesizes wax esters with 16:0 fatty acid and 18:0Alc, 24:0Alc and 28:0Alc alcohols, PhWS1 produces wax esters with very long chain fatty acids and methyl, isoamyl short to medium chain alcohols (4–12 carbons), whereas EgWS prefers medium to long chain substrates (12–18 carbons).

Abbreviations: CoA, coenzyme A; FAMEs, fatty acid methyl esters; FAR, fatty acyl-CoA reductase; MUFA, monounsaturated fatty acids; SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids; WS, wax synthase.

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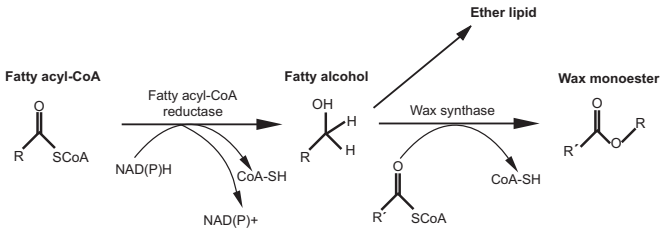

Fig. 1. Biosynthesis of fatty alcohols and the derivatives (wax esters and ether lipids).

Honey bees (*Apis mellifera*) are well known for the production of honey and comb wax. Comb wax comprises 45–55% of wax esters and 13–17% of hydrocarbons (Blomquist et al., 1980). Wax esters in comb wax are mainly 16:0 or 15-hydroxy-16:0 (15-OH-16:0) esterified with very long chain fatty alcohol (24Alc-34Alc) (Blomquist et al., 1980; Garnier et al., 2002). In addition, honey bees also produce shorter chain alcohols (<24:0Alc) for other biological functions. For example, a blend of fatty alcohols (17:0Alc to 22:0Alc) and aldehydes (19:0Ald-22:0Ald) were found in the cuticular layer of drone cocoon which induce a strong arrestment response in varroa mites (Donzé et al., 1998). The highly volatile short chain alcohols (4–9 carbon chains), their acetate derivatives and a very long chain fatty alcohol [Z]-11-eicosenol are found in alarm pheromones (Boch et al., 1962; Collins and Blum, 1983; Pickett et al., 1982). The release of these chemicals alerts bees to act and sting the target.

Plasmalogen (1-O-1′-alkenyl-2-acylglycerophospholipids) are commonly found in a trace amount in the cell membrane of animals (Nagan and Zoeller, 2001). In humans, the level of plasmalogens varies with tissues and a higher amount of plasmalogens are found in immunological cells, cardiac tissues, skeletal muscles, intestine mucosa and nervous tissues (Nagan and Zoeller, 2001) where the fatty acyl-CoA reductases are also highly expressed (Burdett et al., 1991; Cheng and Russell, 2004a). The biosynthesis of this ether lipid in mammals consists of multiple catalytic steps including reduction of fatty acids to fatty alcohols and formation of the ether linkage of fatty alcohols to the glycerol backbone, which are believed to take place in peroxisomes (Hajra, 1995).

In this paper, we report the identification and characterization of a honey bee fatty acyl-CoA reductase (AmFAR1) that can convert a wide range of fatty acids to their corresponding alcohols, which are the essential components of pheromones, wax esters or ether lipids. Reconstitution of the wax biosynthetic pathway using *AmFAR1* along with *Euglena* wax synthase resulted in a high level production of wax monoesters in yeast.

2. Materials and methods

2.1. Organisms

The worker bees used in this study were obtained during the fall of 2008. A selected colony (Saskatraz-28) of domestic honey bees, *Apis mellifera* L. (Hymenoptera, Apidae) was used to stimulate the wax synthesis by feeding with 50% (w/v) sucrose solution. Worker bees showing and not showing (controls) wax flakes extruding from abdominal segments were collected and immediately frozen in liquid nitrogen.

2.2. Isolation of putative fatty acyl-CoA reductase cDNAs

Total RNA was extracted from 100 mg honey bee using RNeasy kit (Qiagen) and treated with DNaseI (Amplification grade, Invitrogen) under conditions detailed by the supplier. First-strand cDNA was synthesized at 50 °C for 1 h, using 0.5 μg of oligo(dT)$_{12-18}$ primers, 1 μg total RNA isolated from bees, and 200 units of SUPERSCRIPT™ III Reverse Transcriptase (Invitrogen). Two μl of the first-strand reaction was subsequently used as a template for a 100 μl PCR reaction in the presence of 2.5 units of Platinum® *Pfx* DNA polymerase (Invitrogen). The primers used to amplify six putative reductases are listed in Supplementary Table S1. The PCR conditions for all these amplicons were 35 cycles of 94 °C for 15 s, 55 °C for 30 s, 68 °C for 1 min. Amplified products were gel purified, 3′A-overhang added, using *Taq* DNA polymerase (Invitrogen), cloned into pYES2.1 TOPO® TA expression kit (Invitrogen) and sequenced.

2.3. 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 Inc., Madison, WI, USA).

2.4. Amino acid sequence alignment and phylogenetic analysis

The previously characterized FARs 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.

2.5. Functional analysis of putative bee reductases in yeast

The pYES2.1 plasmids harbouring putative bee reductases 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 functional 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 30 °C for 2 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). For substrate specificity test, the yeast transformants were grown at 30 °C for 2 days in DOB + GLU-URA and 30 °C for 2 days in DOB + GAL-URA supplemented with 250 μM fatty acid substrate in presence of either 0.1% tergitol (Nonidet P-40; for 14:0, 16:0, 18:0, 20:0, 16:1n-9, 18:1n-9, 18:2n-6, 18:3n-3, 18:1 12-OH and 20:1n-9) or 2.5% ethanol for 22:0 and 24:0. After two 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.

2.6. Expression analysis of AmFAR1

Approximately 100 mg of head, thorax and abdomen segments of worker bees were homogenized in 1 ml TRIzol® reagent