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Fatty Acyl-CoA Reductase of *Ericerus pela* (Hemiptera: Coccoidae): Localization in Insect Cells and Bioinformatic Analysis

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ABSTRACT

Bio-wax components are produced in specialized glands of animals, plants as well as other organisms via well-characterized biosynthetic pathways. Fatty acyl alcohols are the key components for synthesis of wax. A fatty acyl-CoA reductase (FAR) is often essential in this biosynthetic pathway. The subcellular localization of FARs is crucial for understanding the process of synthesis and transport of wax to the surface of body. In this study, we characterized the subcellular localization of EpFAR from the scale insect *Ericerus pela* using an immunofluorescence assay of the insect cells and a bioinformatics analysis. The result of immunofluorescence assay showed EpFAR to be localized to the cytoplasmic region, and the observed fluorescence pattern was characteristic of endoplasmic reticulum (ER) proteins. Comparative analysis of the prediction results showed EpFAR was localized to the ER membrane. These results, combined with the localization of other FARs, suggested the importance of ER as a related subcellular site of white wax biosynthesis.

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Key words
***Ericerus pela*, Fatty acyl-CoA reductase, Subcellular localization**

INTRODUCTION

Ericerus pela Chavannes is one of the well-known economic insects in China, and has been reared for more than a thousand years (Chen and Feng, 2009). The white wax secreted by the male *E. pela* is being used successfully in commercial wax production (Chen and Feng, 2009). Some of the genes associated with wax secretion in *E. pela* have been studied to certain degree (Hu *et al*., 2018; Qi *et al*., 2016). However, the biosynthetic steps (including the detailed cellular pathway) involved in the production of the insect white wax have not been confirmed.

The eukaryotic cells contain various organelles that contain different characteristic proteins and perform specialized function(s). It can be said that subcellular localization information can give an important clue to a protein's function. Although localization signal sequences in mRNA appear to play some role in subcellular localization, the key determinant is the localization residues in the amino acid sequence of a given protein.

Identifying the location of a given protein in the cells plays an important role in understanding their biological function(s). It further helps to explore the proteome in depth, study systems biology, and develop pesticide as well. Even when a number of fatty acyl-CoA reductase genes (*FARs*) from different insects and other organisms have been characterized and the knowledge of their biochemistry pathway are well known, the region of FARs synthesis and the subcellular localization are still sparse. So far, previous studies have only explored the transcription of *FAR* genes by identifying the tissue or organ that was the source of the mRNA corresponding FAR, and not their subcellular localization (Moto *et al*., 2003; Antony *et al*., 2009; Teerawanichpan *et al*., 2010). Only a few of FARs' subcellular localization has been reported hitherto. A previous published study about the subcellular localization of mammalian FAR1 and FAR2 showed that these two enzymes were located in peroxisomes and involved in the synthesis of wax monoesters and ether lipids (Cheng and Russel, 2004). *Arabidopsis thaliana* CER4 with FAR-like biochemical properties, encoding fatty acyl-CoA reductase, was confirmed to be located in the ER membrane and involved in the cuticular wax production (Rowland *et al*., 2006). Among insects, only the subcellular localization of *Heliothis virescens* FAR (HvFAR) and *Drosophila* Waterproof (Wat, encoding fatty acyl-CoA reductase) were studied, ER was the subcellular site for both HvFAR and Wat (Hagström *et al*., 2013; Jaspers *et al*., 2014). It was verified that HvFAR plays a key role in the synthesis of pheromones, and Wat is involved in the wax ester or related hydrophobic substance synthesis in the ER. The

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function of known FARs was in accordance with their localization in cells.

Fatty acyl-CoA reductases of *E. pela* play an important role in the insect white wax biosynthesis (Hu *et al*., 2018), but the synthesizing site of EpFAR has not been explored. In this paper, we used different methods to explore subcellular localization of EpFAR, and further understand the function of EpFAR at cellular level.

MATERIALS AND METHODS

Antibody and insect cells

The recombinant nuclear polyhedrosis baculovirus strain containing full length *EpFAR* sequence and the virus strain generated with empty pFastBac HT B vector were prepared as described previously (Hu *et al*., 2018) and conserved at -80°C in our laboratory. The preparation of polyclonal antibody against *EpFAR* in the rabbits has been described previously (Hu *et al*., 2014) and conserved at -80°C in our laboratory. The secondary antibody (tetramethyl rhodamine isothiocyanate labeled affinity purified antibody to rabbit IgG (H+L)) was raised in goat (KPL, USA).

The *Spodoptera frugiperda* Sf9 cells used in this study were obtained from the Department of Biotechnology, Research Institute of Resource Insects of the Chinese Academy of Forestry. Sf9 insect cells were cultured with Sf-900 II SFM (Invitrogen), a serum-free media optimized for the growth and maintenance of Sf9 cells, in T25 sterile culture bottles at 28 °C in CO_2 incubator. An inverted microscope with a 10 or 20X phase contrast objective was used to inspect the cells every two days. Normally, the medium was relatively clear and cells were somewhat refractive under the microscope. The cells were subcultured with a 1:2 split ratio at 5-day intervals when the cells grew and reached confluence. Once the cells reached confluence in the flasks, these mature cells were gently blown by pipet into cell suspension, half the suspension was dispensed into a new sterile flask, and an equivalent amount of fresh Sf-900 II SFM was added into this old flask, as well as the new flask. Then, these flasks were placed into the CO_2 incubator at 28 °C.

Immunofluorescence staining

Infection of Sf9 insect cells was performed as follows. On day 0, 1 mL Sf9 insect cells from the culture bottle (approximately 1.5×10^6 cells/mL, >96% viability) was plated into 6-well dishes on a piece of sterile glass cover slip (a diameter of 20 mm) in each well and grown in Sf900 II SFM medium supplemented with 5% fetal calf serum (v/v), 50 units/mL penicillin, and 50 µg/mL streptomycin sulfate, at 28°C. After 24 h, the medium was removed and infection was performed with infectious recombinant nuclear polyhedrosis baculoviral stocks containing *EpFAR* or virus generated with empty pFastBac HT B vector or nothing added. Incubated at 28 °C for an additional 48 h, cells were washed thrice with ice-cold PBS (pH 7.4) gently, followed by methanol fixation at -20°C for 15 min. Cells were washed three times with ice-cold PBS and then incubated in a blocking solution (PBS containing 2% BSA) for 30 min at room temperature. Post blocking, the cells were incubated overnight with the purified primary antibody at a 1:500 dilution or with diluted pre immune serum. After incubation, cells were given three PBS washes, treated for 1 h with the secondary antibody diluted with PBS (contain 0.1% BSA) at 1:300. After rinsing these cells for three times with PBS, we stained them with DAPI (10 µg/mL) for 5 min at room temperature. Then, cells were washed thrice with PBS and deionized distilled H_2O subsequently. The cover slip was mounted on a glass slide using a ProLong® Gold Antifade Reagent (thermofish scientific) and examined using laser scanning confocal microscope (LSCM, Fluoview FV10i, OLYMPUS).

Bioinformatic analysis

Five amino acid sequences coding for fatty acyl-CoA reductase were aligned using Bioedit 7.0 (ClustalW multiple alignment), and the N terminal and C terminal of their conserved regions were analyzed. The sequence of *EpFAR* has been obtained beforehand by PCR and sequencing (Hu *et al*., 2018). Other FARs including *Heliothis virescens* FAR (GenBank: ACX53790.1), *Drosophila* Wat (GenBank: NP_651652.2), *Mus musculus* FAR1 (GenBank: AAH07178.1) and *Mus musculus* FAR2 (GenBank: AAH55759.1), were downloaded from GenBank (www.ncbi.nlm.nih.gov/protein). Subcellular localization analysis of the proteins was performed using the following tools: PSORT II based on various sequence features of known protein sorting signals (Horton and Nakai, 1997). TargetP 1.1 based on the predicted presence of any of the N-terminal presequences: chloroplast transit peptide (cTP), mitochondrial targeting peptide (mTP) or secretory pathway signal peptide (SP) (Emanuelsson *et al*., 2000, 2007). PTS1 addressed the peroxisomal targeting signal 1 (Neuberger *et al*., 2003). Euk-mPLoc 2.0 covers cases where proteins may have one or more subcellular locations (Chou and Shen, 2010). ProtComp 9.0 was used to predict the subcellular localization for the animal proteins by combining several methods of protein localization prediction (http://www.softberry.com/). TMpred (Hofmann, 1993), TOPCONS (Bernsel *et al*., 2009) and TMHMM Server v. 2.0 (Krogh *et al*., 2001) were used to explore the topology of EpFAR using hydropathy analysis.

RESULTS

Localization of EpFAR in Sf9 insect cells

Concerning the result of immunofluorescence study, distinctive red fluorescence exhibiting cloud-like formations could be seen in the cytoplasm between the cell membrane and nuclear membrane of Sf9 insect cells infected with recombinant nuclear polyhedrosis baculovirus under the laser scanning confocal microscope. The observed fluorescence pattern was characteristic for ER proteins, while nucleus showed only the blue and no red fluorescence (Fig. 1). However, the images did not reveal the organellar localization of EpFAR in the cytoplasm. As expected, control cells infected with the virus generated from empty pFastBac HT B vector, as well as in cells without any treatment using identical parameters, were devoid of any red signal. The control sets stained positive for nuclear marker only.

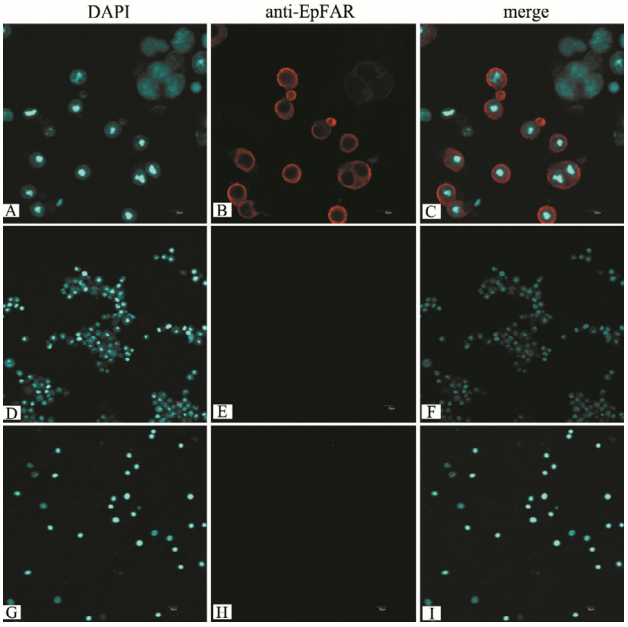

Fig. 1. Localization of EpFAR in Sf9 cells. Sf9 cells were infected with the recombinant baculovirus and fluorescence imaged by LSCM. A, B, and C, cells were infected with the recombinant nuclear polyhedrosis baculovirus including EpFAR. D, E, and F, cells treated with the virus generated with an empty pFastBac HT B vector were used as a control. G, H, and I, cells cultured in the normal culture medium were used as another control.

Subcellular localization based on bioinformatics

The in silico prediction tools were used to forecast the subcellular localization of EpFAR and other protein sequences coding fatty acyl-CoA reductase, Wat, HvFAR and other FARs from *Mus musculus* were included in the predictive study. The results from the tools showed different subcellular localization of these proteins. The only exception was PSORT II, which predicted EpFAR, as well as the other four proteins, to be localized to the cytoplasm (Table I). Meantime, the results of PSORT II suggested that EpFAR had an ER membrane retention signal (KKXX-like motif) in the C-terminus, LKYF. The ER membrane retention signals were also found in HvFAR, Wat and MmFAR2 (Fig. 2). Euk-mPLoc 2.0 and ProtComp proposed EpFAR to be localized to ER or plasma membrane while the others to be localized to ER, peroxisome, plasma membrane and cytoplasm (Table I). There was no peroxisomal targeting signal in EpFAR, HvFAR and Wat. Aligning the amino acid sequences revealed that, EpFAR, as well as Wat, MmFAR1 and MmFAR2 contain a segment of 41-66 amino acids in the C-terminal region, and this region does not occur in the HvFAR. In addition, the EpFAR contained less residues at its C-terminus, and Wat is the same too, compared with MmFAR1 and MmFAR2 (Fig. 2).

Fig. 2. FAR amino acid sequence alignment. Alignment of EpFAR and other known amino acid sequences corresponding to the sequences specified in the Table I. The ER membrane retention signals are marked with a box. On top of the alignment, the regions corresponding to the predicted transmembrane domains (TMH) of EpFAR are marked as follows: TMpred TMH are marked with a solid line (top: 23-39aa, 256-276aa, 354-372aa, 377-395aa, 500-520aa), predictions by TOPCONS are marked with a dotted lines (second from the top: 22-42aa, 379-399aa, and 498-518aa), while the TMHMM predictions are marked with dashed line (bottom: 494-516aa).

Table I. An overview of the results from various subcellular prediction tools.

Protein	TargetP1.1/RC	PTS I	PSORT II	Euk-mPLoc 2.0	ProtComp-Version 9
EpFAR	-/1	Not targeted	cytoplasm	Endoplasmic reticulum	Plasma membrane
HvFAR	-/2	Not targeted	cytoplasm	Endoplasmic reticulum	Plasma membrane
Wat	-/1	Not targeted	cytoplasm	Cytoplasm Endoplasmic reticulum	Plasma membrane
MmFAR1	-/2	Not targeted	cytoplasm	Endoplasmic reticulum Peroxisome	Peroxisomal
MmFAR2	-/3	Not targeted	cytoplasm	Endoplasmic reticulum Peroxisome	Peroxisomal

Note: Genbank accession numbers: HvFAR, ACX53790.1; Wat, NP_651652.2; MmFAR1, AAH07178.1; MmFAR2, AAH55759.1. TargetP 1.1, "-" means neither mitochondria/Chloroplast nor secretory pathway, "RC" means realiability class.

TMpred and TOPCONS were used to study the membrane spanning segments of EpFAR. TMpred strongly suggested a model (total score: 4627) that the N-terminus of EpFAR was outside, and there were 5 strong transmembrane helices (23-39aa, 256-276aa, 354-372aa, 377-395aa, 500-520aa), with the region around amino acids 500-520 being especially hydrophobic (Fig. 2). TOPCONS predicted three TMH: residues 22-42 as a putative TMH, and residues 379-399, and 498-518 as certain TMHs, and these TMHs overlapped with the three among the five TMHs strongly predicted by TMpred. We further used TMHMM to predict transmembrane topology and the result indicated that the C-terminus of EpFAR at residues 517-520 was located on the inner of membrane and the N-terminus outside, with one TMH in-between, and this hydrophobic region was at residues 494-516.

DISCUSSION

The different characteristic proteins were localized to different organelles in the eukaryotic cells via one of the alternative pathways including co-translational (Johnson and van Waes, 1999; Hedge and Keenan, 2011), post-translational (Hedge and Keenan, 2011), and other targeting pathways (Iwashita *et al*., 2010). However, these pathways have not been extensively investigated. Consequently, it is very difficult to research their involvement in subcellular targeting of proteins. In this study, the results of the immunofluorescence assay showed that EpFAR was localized in the cytoplasmic region, where different organelles are located, and not to the nuclear region. Our insilico predictions suggested that EpFAR was localized to the region of cytoplasm, which especially pointed to ER. Sequence alignment of EpFAR and other *FAR* proteins with known subcellular localization implied that EpFAR was localized to the ER.

In this study, the results of Euk-mPLoc 2.0 suggested that EpFAR was localized to the ER, and PSORT II predicted cytoplasmic localization. At the same time, PSORT II indicated the ER membrane retention signal (Teasdale and Jackson, 1996), the LKYF was KKXX-like motif in the C-terminus of EpFAR. For the sequences of HvFAR and Wat, the KKXX-like motif in their C-terminus (LEKK and LKLF, respectively) was also found. Consistently, it was verified that HvFAR and Wat were localized to ER by the recombinant expression assay in cells (Hagström *et al*., 2013; Jaspers *et al*., 2014). Hydrophobicity analysis showed there was a hydropathy profile for EpFAR which was similar to the hydropathy profile of *Drosophila* Wat, suggesting functional conservation or similarity between the two proteins to certain degree. MmFAR1 contained one high hydrophobic region at its carboxyl end, as well as MmFAR2, the hydrophobic regions in mouse FARs were thought to be a requirement for targeting the enzymes to peroxisomes, although MmFAR2 was not just distributed in the peroxisomes (Cheng and Russell, 2004; Honsho *et al*., 2010). In contrast to MmFARs, this high hydrophobic region at carboxyl end is not found in known insect pgFARs, including HvFAR. Hagström *et al*. (2013) studied the subcellular localization of HvFAR, a fatty acyl-CoA reductase, involved in pheromone biosynthesis in the tobacco budworm, which suggested localization to the ER. In the comparison and analyses of protein sequences, including EpFAR, HvFAR, Wat, and MmFARs, we hypothesized that EpFAR was located in the ER, which was consistent with our finding that EpFAR localized to the cytoplasmic region. Our immunolocalization image is also consistent with previous reports for Wat and HvFAR, since the observed fluorescence pattern is characteristic for ER proteins in *S. cerevisiae* (Huh *et al*., 2003; Lau *et al*., 1999).

Protein subcellular localization is considered to predict the action site of a given protein in the cell. The different types of organelles contain diverse characteristic proteins and perform specialized functions in the eukaryotic cells. Based on the function of Lepidoptera pheromone-gland-specific FARs known to catalyze the reduction of a fatty acid to an intermediate or final product of fatty acyl alcohol in insect pheromone, fatty alcohols are main components of pheromone production (Liénard

et al., 2008, 2010; Lassance *et al*., 2010), together with the reported data on the localization of desaturases (Shanklin and Cahoon, 1998; Stuckey *et al*.,1990; Serra *et al*., 2007), it was revealed that the ER acts as a subcellular site of pheromone production. Thus, ER has proved to be very important for the insect chemical communication and its control of behavior (Shin *et al*., 1991; Moto *et al*., 2003; Anthony *et al*., 2009; Hagström *et al*., 2013). Some genes in the FARs family play a key role in forming of protective layer by providing very long chain fatty alcohols that serve as potential substrates for wax ester synthesis or related hydrophobic substances (Cheng and Russell, 2004; Rowland *et al*., 2006; Jaspers *et al*., 2014). This conclusion is also consistent with ER as the site of lipid biosynthesis and the secretory and endocytic pathway (Kaiser *et al*., 2005; Vitale and Denecke, 1999; Hedge and Keenan, 2011). Our previous data have shown that the EpFAR could catalyze the very long chain fatty acyl-CoA to its corresponding fatty alcohol (Hu *et al*., 2018). Yang *et al*. (2012a, 2012b) deduced that EpFAR was a key gene for the biosynthesis of insect white wax on the tegumental surface. These results highlighting role of EpFAR and immunolocalization study of EpFAR were helpful for considering the importance of the ER as the subcellular site of white wax production. Further research is needed to confirm ER as the definitive subcellular location of EpFAR.

CONCLUSION

In conclusion, the present study provided new information about the expression location of EpFAR at cellular level. EpFAR was localized to the cytoplasmic region, and the observed fluorescence pattern was the characteristic of ER proteins. Based on the bioinformatics analysis, EpFAR was localized to the ER membrane. The expression site of EpFAR could elucidate the mechanism underlying insect white wax synthesis.

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Statement of conflict of interest

The authors have declared no conflict of interests.

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