Pheromone gland-specific fatty-acyl reductase of the silkmoth, *Bombyx mori*

Ken'ichi Moto*, Toyoshi Yoshiga†, Masanobu Yamamoto*, Shunya Takahashi*, Kazuhiro Okano*, Tetsu Ando‡, Tadashi Nakata*, and Shogo Matsumoto*§

*RIKEN, Hirosawa 2-1, Wako, Saitama 351-0198, Japan; †Department of Applied Biological Sciences, Saga University, Saga 840-8502, Japan; and ‡Graduate School of Bio-Applications and Systems Engineering, Tokyo University of Agriculture and Technology, Koganei, Tokyo 184-8588, Japan

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The C_{10}-C_{18} unsaturated, acyclic, aliphatic compounds that contain an oxygenated functional group (alcohol, aldehyde, or acetate ester) are a major class of sex pheromones produced by female moths. In the biosynthesis of these pheromone components, the key enzyme required to produce the oxygenated functional groups is fatty-acyl reductase (FAR). This enzyme converts fatty-acyl pheromone precursors to their corresponding alcohols, which, depending on the moth species, can then be acetylated or oxidized to the corresponding aldehydes. Despite the significant role this enzyme has in generating the species-specific oxygenated constituents of lepidopteran sex pheromones, the enzyme has yet to be fully characterized and identified. In experiments designed to characterize a pheromone-gland-specific FAR in the silkmoth, *Bombyx mori*, we have isolated a cDNA clone encoding a protein homologous to a FAR from the desert shrub, *Simmondsia chinensis*, commonly known as jojoba. The deduced amino acid sequence of this clone predicts a 460-aa protein with a consensus NAD(P)H binding motif within the amino terminus. Northern blot analysis indicated that 2-kb transcripts of this gene were specifically expressed in the pheromone gland at 1 day before adult eclosion. Functional expression of this gene in the yeast *Saccharomyces cerevisiae* not only confirmed the long-chain FAR activity, but also indicated a distinct substrate specificity. Finally, the transformed yeast cells evoked typical mating behavior in male moths when cultured with the pheromone precursor fatty acid, (*E*,*Z*)-10,12-hexadecadienoic acid.

Many species of moths have their own pheromone blends that serve as a medium of communication in mating behavior, with structural/compositional variations of the pheromone blends contributing to species specificity (1). A major class of sex pheromones produced by female moths is the C_{10}-C_{18} unsaturated, acyclic, aliphatic compounds that contain an oxygenated functional group such as aldehyde, alcohol, or acetate ester. These pheromone components are synthesized *de novo* in the pheromone gland from acetyl-CoA through fatty acid synthesis, chain shortening and desaturation (or vice versa), and reductive modification of the carbonyl carbon (2, 3).

In the biosynthesis of this class of sex pheromones, the key enzyme required for production of the oxygenated functional groups is fatty-acyl reductase (FAR), which converts fatty-acyl pheromone precursors to their corresponding alcohols. Depending on the moth species, these alcohols are frequently acetylated by an acetyl-CoA:fatty alcohol transferase or oxidized to the corresponding aldehydes by an alcohol oxidase (3). Despite its significant role in generating the diverse species-specific oxygenated constituents of lepidopteran sex pheromones, the FAR involved has yet to be identified and characterized from this large taxonomic group.

In the silkmoth, *Bombyx mori*, the pheromone gland located between the eighth and ninth abdominal segments initiates production of the sex pheromone bombykol, (*E*,*Z*)-10,12-hexadecadien-1-ol, during photophase starting from the day of adult eclosion (4, 5). Bombykol is synthesized *de novo* from acetyl-CoA through palmitate (16:Acyl), which is stepwise converted to bombykol by Δ11 desaturation, Δ10,12 desaturation, and fatty-acyl reduction (2, 6). In previous experiments using palmitoyl-CoA as a substrate, we detected FAR activity in the homogenate of *B. mori* pheromone glands (7). Further biochemical studies revealed that this enzyme required NADPH, but not NADH, as a reductant and was inhibited by compactin, a specific inhibitor of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase (7). In this article, we describe the isolation of a cDNA from the *B. mori* pheromone gland encoding a protein homologous to the FAR identified in the jojoba plant, *Simmondsia chinensis*, the specific expression of the enzyme's corresponding mRNA in the pheromone gland, and functional expression in *Saccharomyces cerevisiae*. Our results indicate that the cloned gene encodes a pheromone-gland-specific long-chain FAR that preferentially converts the pheromone precursor fatty acid of *B. mori* to the final alcohol product, bombykol.

Materials and Methods

Insects. Eggs of *B. mori* (Shuko × Ryuhaku) were purchased from Katakura Kogyo (Matsumoto, Japan). Larvae were reared on an artificial diet as described (8).

cDNA Cloning. First-strand cDNA was synthesized by using total RNA from *B. mori* pheromone glands dissected immediately after adult eclosion. PCR was performed by using a sense oligonucleotide primer (5′-GTCTCGAAAGGAGCCGAAAAC-3′) based on the HMG-CoA reductase from the moth *Agrotis ipsilon* (GenBank accession no. AJ009675) and an oligo-dT primer using ExTaq polymerase (Takara Bio, Otsu, Japan). The thermal cycler conditions consisted of 6 cycles at 95°C for 30 sec, 30°C for 30 sec, and 72°C for 1 min, followed by 25 cycles at 95°C for 30 sec, 50°C for 30 sec, and 72°C for 1 min, and then ended at 72°C for 10 min. After separation of the PCR products on a 1% agarose gel, the DNAs corresponding to 1–2 kb were recovered from the gel and reamplified under the same conditions. The PCR products were then cloned by using the pGEM-T Easy Vector System (Promega), and the resultant clones were sequenced.

Sequence analysis indicated the presence of a 1,299-bp clone encoding a protein homologous to the jojoba FAR (JJFAR) (9). To obtain the 5′ end of the clone, 5′ RACE was performed by using the Marathon cDNA amplification kit (CLONTECH) with a gene-specific primer (5′-TCCACGAACCCTCGCACTG-GCTC-3′) and the provided anchor primer according to the manufacturer's instructions. Messenger RNA was isolated from the pheromone gland by using a Micro-FastTrack 2.0 mRNA Isolation Kit (Invitrogen). Conditions for the touchdown PCR

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Abbreviations: FAR, fatty-acyl reductase; JJFAR, jojoba FAR; pgFAR, pheromone-gland FAR; HMG-CoA, 3-hydroxy-3-methylglutaryl CoA; PBAN, pheromone biosynthesis-activating neuropeptide.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AB104896 and AB104897 for *Bombyx mori* pgFAR).

§To whom correspondence should be addressed. E-mail: smatsu@postman.riken.go.jp.

used for 5′ RACE were 94°C for 3 min, followed by 31 cycles at 94°C for 5 sec and 50°C for 3 min. The full-length cDNA of this gene was isolated from a pheromone gland cDNA library prepared from the *B. mori* inbred strain p50 (10). DNA sequencing was done by using a Li-Cor (Lincoln, NE) DNA sequencer (model 4000) and BASE IMAGIR software (Li-Cor).

Northern Blot Analysis. Northern blotting was performed as described (11). Briefly, denatured total RNAs (10 μg of each) prepared from various *B. mori* tissues were separated on a 1.2% gel in 6.6 M formaldehyde/1× Mops buffer and transferred onto a nylon membrane (Hybond N, Amersham Biosciences) by capillary blotting. The 1,299-bp clone was labeled with [α-^{32}P]dCTP by using Ready-To-Go DNA labeling beads (Amersham Biosciences) and used as a hybridization probe.

Plasmid Construction and Yeast Transformation. A plasmid, designated as pESC-LEU-FAR, containing the 460-aa ORF behind the galactose-inducible promoter GAL1 was generated by using the shuttle vector pESC-LEU (Stratagene). For construction of pESC-LEU-FAR, two gene-specific primers were designed: 5′-AAAGGATCCAAGATGTCACACAATGG-3′ (a *Bam*HI site is included) and 5′-AATGTCGACTATAATTTATTTTT-GAACAG-3′ (a *Sal*I site is included). PCR was performed by using KOD -Plus- (Toyobo, Osaka), the conditions of which included 20 cycles at 94°C for 15 sec, 50°C for 30 sec, and 68°C for 90 sec. First-strand cDNA used for PCR was prepared from the p50-strain pheromone gland total RNA. The PCR product was double digested with *Bam*HI and *Sal*I (Takara Bio), separated by agarose gel electrophoresis, and purified from the agarose gel with a QIAquick gel extraction kit (Qiagen, Valencia, CA). The purified DNA was ligated into the linearized pESC-LEU vector, which had been predigested with *Bam*HI and *Sal*I, and then sequenced. Both plasmids, pESC-LEU-FAR and pESC-LEU, were then transformed into the *S. cerevisiae* strain YPH499 (Stratagene) by lithium acetate according to the manufacturer's instructions for pESC yeast epitope tagging vectors. Individual colonies were inoculated in 10 ml of synthetic dextrose (SD) dropout media lacking L-leucine (0.67% yeast nitrogen base without amino acids/2% glucose/0.13% amino acid dropout powder). After overnight incubation at 30°C, the yeast cultures were diluted 1:20 in 100 ml of SD dropout media lacking L-leucine and grown to an OD_{600} of 1.0 at 30°C.

Functional Assays. After centrifugation, the cell pellets were suspended in 100 ml of fresh synthetic galactose (SG) dropout media lacking L-leucine (0.67% yeast nitrogen base without amino acids/2% galactose/0.13% amino acid dropout powder) and incubated at 30°C for 22 h. To examine FAR activity, aliquots of the cultures were diluted 5× to 2 ml in fresh SG dropout media lacking L-leucine and incubated at 30°C for 20 h in the presence of 0.5 mM various fatty acids and 0.2% tergitol (type Nonidet P-40, Sigma). After incubation, 1-ml aliquots of the cultures were dried by using the SPD1010 Integrated SpeedVac System (Thermo Savant) and extracted with 1 ml *n*-hexane. The hexane extracts were concentrated to 30 μl, and 1 μl of each sample was subjected to GC/MS. Electron impact GC/MS was achieved by using a JEOL automass sun mass spectrometer equipped with a HP-5 capillary column (0.25 mm i.d. × 30 m, Agilent, Palo Alto, CA). The column temperature program was 40°C for 2 min, 10°C/min to 140°C, and finally 4°C/min to 220°C. The ionization voltage was 70 eV (1 eV = 1.602×10^{-19} J), and the ion source temperature was 230°C.

Free fatty acids used as substrates were myristic acid (14:Acid; Wako Pure Chemical, Osaka), pentadecanoic acid (15:Acid; Nacalai Tesque, Kyoto), D_3-labeled palmitic acid (hexadecanoic-16,16,16,- D_3 acid, D_3-16:Acid; Isotec), heptadecanoic acid (17:Acid; Sigma), stearic acid (18:Acid; Kanto Chemical, Tokyo), (*E*)-9-palmitoleic acid (*E*9–16:Acid; Funakoshi, Tokyo), and (*Z*)-9-palmitoleic acid (*Z*9–16:Acid; Funakoshi). (*E*)-11-palmitoleic acid (*E*11–16:Acid) and (*Z*)-11-palmitoleic acid (*Z*11–16:Acid) were prepared from their corresponding aldehydes, which were synthesized by using the Wittig reaction with *n*-pentyltriphenylphosphonium bromide and 11-(tetrahydro-2-pyranyloxy)-undecanal. After removal of the protecting groups and separation of the (*E*)- and (*Z*)-isomers with a silvernitrate-silica gel column, both isomers were oxidized to their corresponding free fatty acids by using pyridiniumchlorochromate and the Jones reagent (12). (*E*,*Z*)-10,12-hexadecadienoic acid (*E*,*Z*10,12–16:Acid) was synthesized from the known aldehyde, (*E*)-12-(*tert*-butyldimethylsilyl)oxy-2-dodecen-1-al (13). Briefly, the aldehyde was reacted with the Wittig reagent (14) prepared from *n*-butanyltriphenyl-phosphonium blomide and sodium bis(trimethylsilyl)amide. Subsequent deprotection yielded bombykol (*E*,*Z*10,12–16:OH), 6% of which corresponded to the 12 *E*-isomer and ≈3% to the (10*Z*, 12*Z*)-isomer (^{1}H-NMR analysis). The constituents of this preparation were then subjected to Jones oxidation (12), yielding *E*,*Z*10,12–16:Acid with a >88% geometrical purity upon ^{1}H-NMR analysis. The *E*,*Z*-isomer was purified to homogeneity by HPLC on an octadecyl silica-gel column. The minor *E*,*E*-isomer was also separately purified to afford a 1:1 mixture of *E*,*Z*-isomer and *E*,*E*-isomer.

Results

Isolation of *B. mori* FAR cDNA Clone. We attempted to amplify a portion of the *B. mori* FAR cDNA from pheromone gland RNA of newly emerged *B. mori* female moths by using a sense oligonucleotide primer designed from the HMG-CoA reductase from the moth, *A. ipsilon*, and an oligo-dT primer. Because several PCR products were obtained, only the DNAs corresponding to 1–2 kb were extracted from the gel, reamplified under the same conditions, and cloned. Sequence analysis indicated the presence of a 1,299-bp clone, which exhibited sequence homology with that of a JJFAR (9). To obtain the entire sequence of the ORF, 5′ RACE with a gene-specific primer and isolation of the full-length cDNA from a pheromone gland cDNA library prepared from the inbred p50 strain of *B. mori* (10) were carried out. These results indicated that the ORF encoded a 460-aa protein with a predicted molecular mass of 52,322 Da (Fig. 1). As in other reductases, the deduced amino acid sequence contained the conserved NAD(P)H-binding motif [I, V, F]-X-[I, L, V]-T-G-X-T-G-F-L-[G, A] (15) encompassing residues 23–33. Consequently, we designated this protein as *B. mori* pheromone-gland FAR (pgFAR). A query of the public data banks with the deduced amino acid sequence using BLAST search programs (16) revealed significant, but not high, homology with JJFAR (GenBank accession no. AF149917), with 26% identity over the entire length (Fig. 2). The sequence also showed significant homology with male sterility 2 (MS2) proteins from *Arabidopsis thaliana* (23–24% identity with the C-terminal end of the MS2 proteins) and other MS2-like proteins of unknown function from diverse organisms including *Drosophila melanogaster*, *Anopheles gambiae*, *Torpedo marmorata*, and *Caenorhabditis elegans* (GenBank accession nos. AL009193, EAA03772, AJ272073, and AF098505, respectively).

Northern Blot Analysis. Northern blot analysis of *B. mori* pgFAR demonstrated specific expression of the 2-kb transcripts within the pheromone gland, whereas no detectable signal was observed in the other tissues examined, including whole head, fat body, Malpighian tubule, flight muscle, ovary, and testis (Fig. 3*A*). In the pheromone gland, the transcripts were significantly up-regulated 1 day before adult eclosion; however, the expression level appeared to decrease slightly following eclosion (Fig. 3*B*).

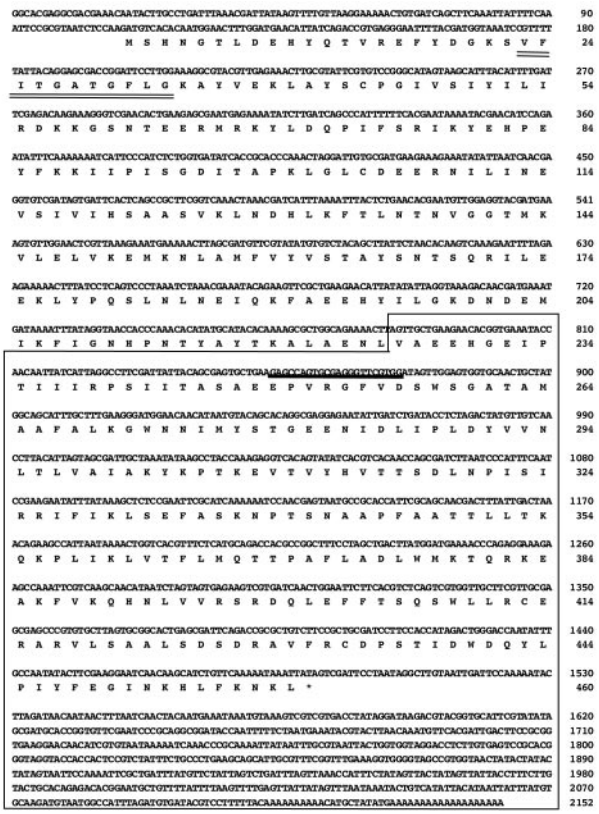

Fig. 1. cDNA and deduced amino acid sequence of *B. mori* pgFAR. The region corresponding to the primer sequence used for 5′ RACE is underlined. The C-terminal part obtained by the first PCR is boxed. The proposed NAD(P)H binding motif is double underlined.

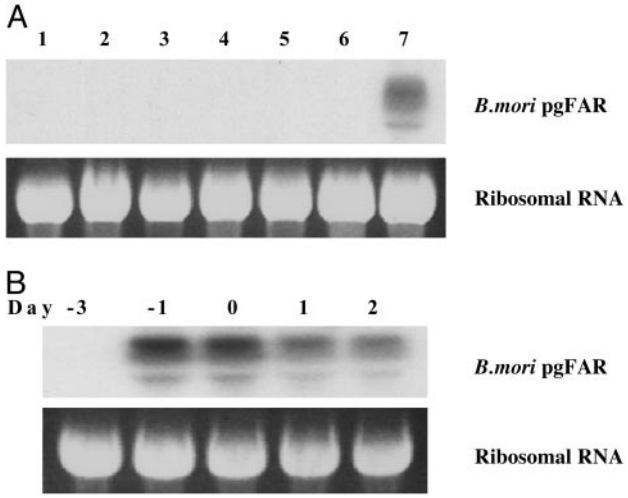

Fig. 3. Northern blot analysis of *B. mori* pgFAR. (*A*) Expression in adult tissues. Lanes: 1, head; 2, fat body; 3, Malpighian tubule; 4, flight muscle; 5, ovary; 6, testis; 7, pheromone gland. Each tissue was dissected from a newly emerged female moth (day 0). Testis was dissected from a newly emerged male moth. (*B*) Expression in the pheromone gland. Pheromone glands dissected from females before and after adult eclosion were used for total RNA extraction. Day 0 corresponds to the day of eclosion. Ribosomal RNA band stained with ethidium bromide is shown as a loading control. The heterogeneity of the transcripts is caused by the differences in their 3′-untranslated regions (data not shown).

Functional Expression of *B. mori* pgFAR in *S. cerevisiae*. To investigate the reductase activity and substrate specificity of *B. mori* pgFAR, we constructed a plasmid, referred to as pESC-LEU-FAR, containing the 460-aa ORF, and expressed the *B. mori* pgFAR protein under the control of the inducible GAL1 promoter in the *S. cerevisiae* strain YPH499. After induction with galactose and incubation at 30°C for 22 h, we observed that hexadecanol (16:OH) was produced by those yeast cells transformed with the pESC-LEU-FAR, but not by those cells transformed with the empty vector pESC-LEU (Fig. 4 *A* and *B*). Because we observed that incubation with media containing D_3-labeled palmitic acid also resulted in incorporation and reduction to the corresponding alcohol D_3-hexadecanol (D_3-16:OH) (Fig. 4*C*), we further investigated the substrate specificity of the expressed *B. mori* pgFAR by adding various fatty acids to the incubation media. In these experiments, we estimated the substrate specificity by comparing the production of fatty alcohols derived from the added fatty acids with the internal 16:OH, which was produced from endogenous substrates by the action of the introduced *B. mori* pgFAR as indicated in Fig. 4 *A* and *B*. In a series of experiments using saturated long-chain fatty acids (Fig. 4 *C*–*G*), 15:Acid was preferentially reduced to the corresponding alcohol (15:OH). Although reduction of the 17:Acid and 18:Acid to their corresponding alcohols, 17:OH and 18:OH, did occur, the reduction was not as efficient as that of the 15:Acid or 16:Acid. Furthermore, we found that reduction of the 14:Acid to its

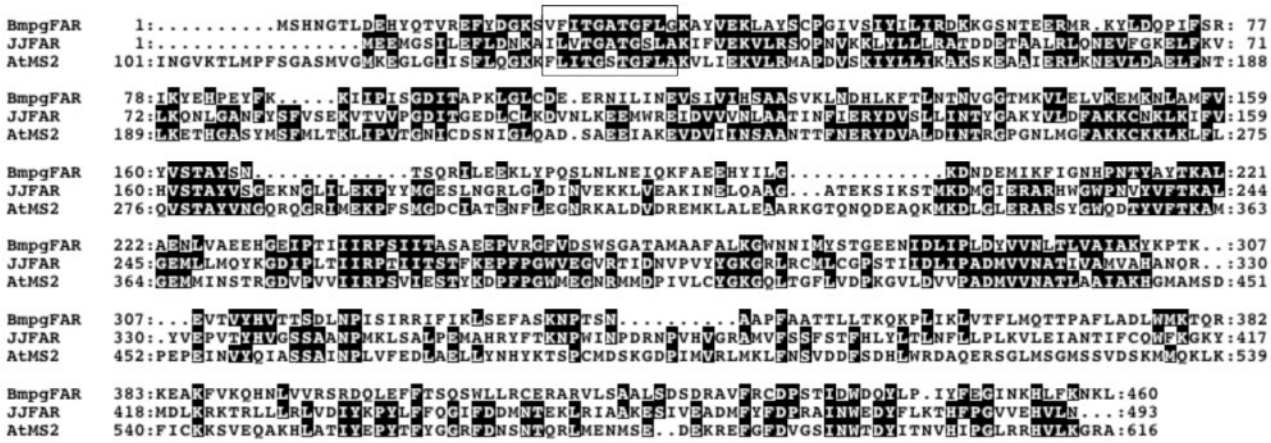

Fig. 2. Multiple alignment of *B. mori* pgFAR and its related proteins. The identical residues are shaded black. The proposed NAD(P)H binding motif is boxed. BmpgFAR, *B. mori* pgFAR; AtMS2, *A. thaliana* male sterility 2 protein (GenBank accession no. X73652). JJFAR is from GenBank accession no. AF149917.

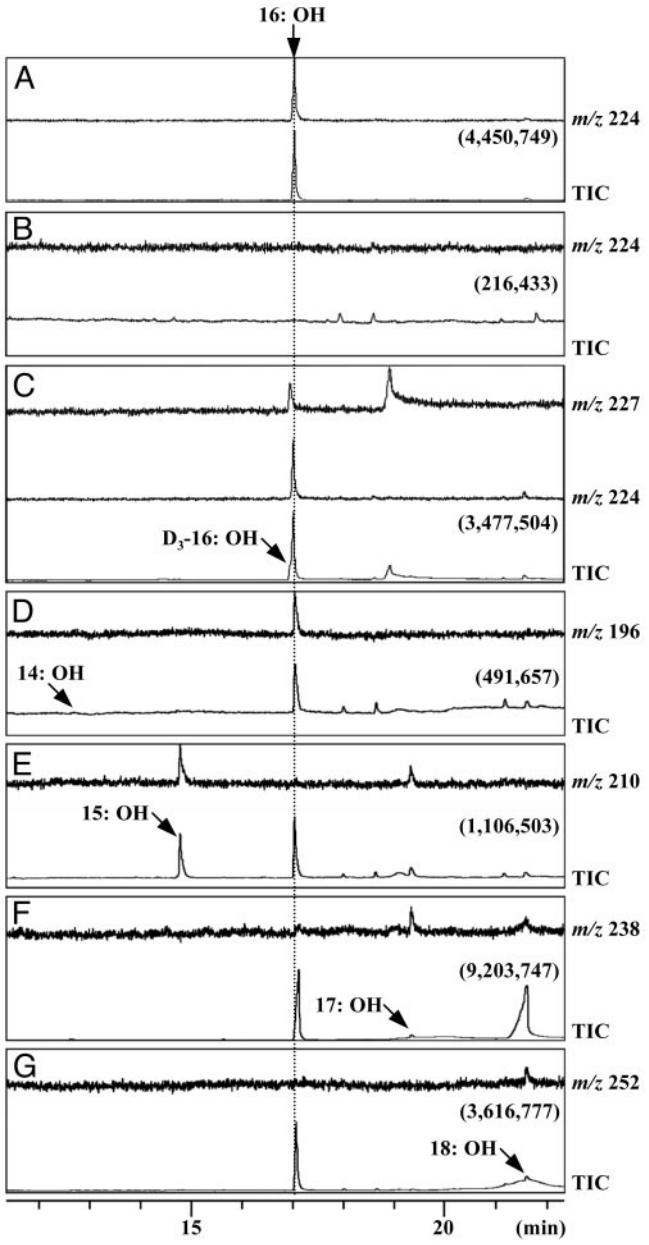

Fig. 4. GC/MS analysis of fatty alcohols in the yeast-cell extracts by using total ion chromatograms (TICs) and mass chromatograms. (*A*) Yeast cells transformed with pESC-LEU-FAR. (*B*) Control yeast cells transformed with pESC-LEU. The fragment ion of m/z 224 corresponds to $[M\text{-}18]^+$ of 16:OH. (*C–G*) Yeast cells transformed with pESC-LEU-FAR were incubated in the presence of 0.5 mM saturated fatty acids. (*C*) D_3-16:Acid. The m/z 227 corresponds to $[M\text{-}18]^+$ of D_3-16:OH. (*D*) 14:Acid. The m/z 196 corresponds to $[M\text{-}18]^+$ of 14:OH. (*E*) 15:Acid. The m/z 210 corresponds to $[M\text{-}18]^+$ of 15:OH. (*F*) 17:Acid. The m/z 238 corresponds to $[M\text{-}18]^+$ of 17:OH. (*G*) 18:Acid. The m/z 252 corresponds to $[M\text{-}18]^+$ of 18:OH. Fatty alcohols were extracted with *n*-hexane from lyophilized yeast cells. The value in parentheses indicates total peak abundance in each TIC.

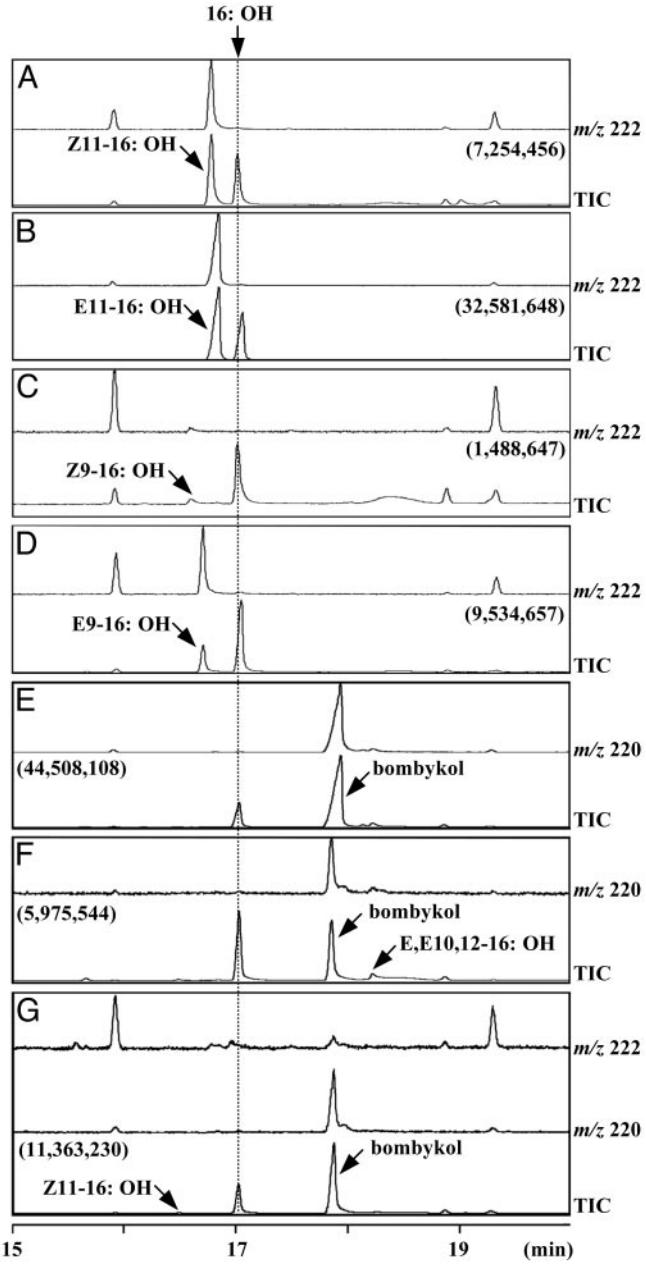

Fig. 5. GC/MS analysis of fatty alcohols in the yeast-cell extracts by using TICs and mass chromatograms. Yeast cells transformed with pESC-LEU-FAR were incubated in the presence of 0.5 mM monoene C_{16} fatty acids. (*A*) *Z*11–16:Acid. (*B*) *E*11–16:Acid. (*C*) *Z*9–16:Acid. (*D*) *E*9–16:Acid. The m/z 222 corresponds to $[M\text{-}18]^+$ of monoene 16:OH. (*E–G*) Conversion of *E*,*Z*10,12–16:Acid to *E*,*Z*10,12–16:OH (= bombykol). Yeast cells transformed with pESC-LEU-FAR were incubated in the presence of 0.5 mM *E*,*Z*10,12–16:Acid (*E*), 1:1 mixture of 0.25 mM *E*,*Z*10,12–16:Acid and *E*,*E*10,12–16:Acid (*F*), or 1:1 mixture of 0.5 mM *E*,*Z*10,12–16:Acid and *Z*11–16:Acid (*G*). The value in parentheses indicates total peak abundance in each TIC.

corresponding alcohol was barely detectable (Fig. 4*D*), whereas the 19:Acid and the 20:Acid failed to be reduced (data not shown). These results indicate that *B. mori* pgFAR indeed exhibits long-chain FAR activity with a distinct substrate specificity.

In *B. mori*, the sex pheromone bombykol is biosynthesized through *Z*11–16:Acyl, which is further desaturated to *E*,*Z*10,12–16:Acyl, and finally reduced to bombykol, *E*,*Z*10,12–16:OH (6). Consequently, we further examined the substrate specificity by using unsaturated C_{16} fatty acids. Among the monounsaturated C_{16} fatty acids tested, both *Z*11- and *E*11–16:Acids were reduced to their corresponding alcohols (Fig. 5 *A* and *B*). In contrast, we observed a diminished reduction to the corresponding alcohol when either *Z*9- or *E*9–16:Acid was used as a substrate, although the *E*9–16:Acid exhibited a greater reduction to its correspond-

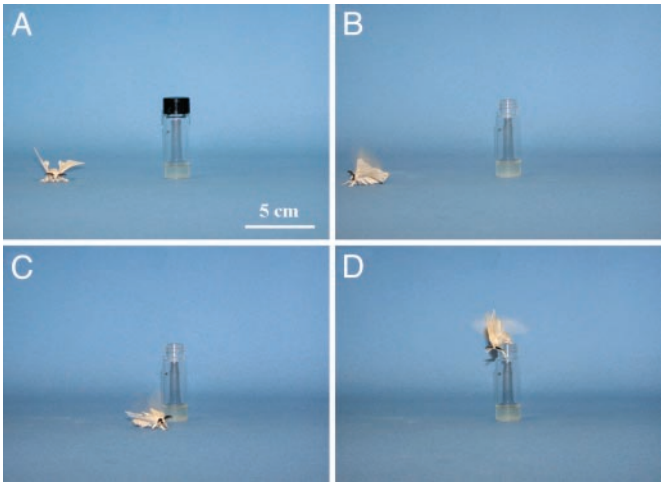

Fig. 6. Mating behavior of the male *B. mori* moth evoked by yeast cells expressing *B. mori* pgFAR. Yeast cells were incubated in the presence of 0.5 mM *E*,*Z*10,12–16:Acid. (*A*) Before exposure to volatile contents of the vial containing transformed yeast cells. (*B–D*) Sequential behavior of the male moth for the first 3 min after removal of the cap. This behavior was consistent with stereotypical male mating behavior, which is characterized by fluttering of the male's wings as he approaches the female and ultimately culminates copulation.

ing alcohol (Fig. 5 *C* and *D*). Examination of the diunsaturated fatty acid *E*,*Z*10,12–16:Acid (i.e., the bombykol precursor fatty acid) indicated that this substrate was preferentially reduced and converted to the final alcohol product, bombykol (Fig. 5*E*). If we added a 1:1 mixture of *E*,*Z*10,12–16:Acid and *E*,*E*10,12–16:Acid to the incubation medium, *E*,*Z*10,12–16:Acid was selectively reduced and bombykol was the predominant product (Fig. 5*F*). Likewise, when a 1:1 mixture of *E*,*Z*10,12–16:Acid and *Z*11–16:Acid was added to the incubation medium, *E*,*Z*10,12–16:Acid was selectively reduced and converted to bombykol (Fig. 5*G*). These results indicate that *B. mori* pgFAR exhibits a strong substrate preference for the bombykol precursor fatty acid. Throughout these expression analyses, there were no detectable amounts of any intermediate aldehydes. Finally, typical mating behavior of the male *B. mori* moth was evoked by yeast cells expressing *B. mori* pgFAR (Fig. 6).

Discussion

Long-chain primary alcohols occur both as free alcohols and, more commonly, in a combined state, as is the case with wax in a variety of organisms (17). These alcohols can also serve as precursors of ether lipids, which are especially abundant in animal nervous system tissues (18). In insects, although long-chain fatty alcohols are widely used as wax to protect the outer surface of the cuticle (19), they also have a significant role as the precursors of species-specific pheromone components in many moth species (3).

The biochemistry of fatty alcohol synthesis has demonstrated that long-chain fatty alcohols are primarily biosynthesized through reduction of the corresponding fatty acyl-CoAs. Differing from aldehyde-generating FARs, alcohol-generating FARs are commonly integral membrane proteins that catalyze a four-electron reduction of fatty acyl-CoAs to yield free primary alcohols without the release of intermediate aldehydes (9, 20). With the aim to understand the molecular mechanisms underlying sex pheromone production in moth species, we had demonstrated long-chain FAR activity in the homogenate of *B. mori* pheromone glands. By using palmitoyl-CoA as a substrate, we observed reduction to the corresponding hexadecanol without the release of the intermediate aldehyde (7). We also observed that this enzyme could be solubilized from the microsomal fraction of the pheromone gland by using 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate as a detergent (21). These findings suggest that the FAR involved in sex-pheromone production of *B. mori* is an alcohol-generating one. With the exception of our current findings, the most widely published alcohol-generating FAR gene in the literature is that of JJFAR, which converts seed wax fatty acids to their corresponding fatty alcohols (9).

In the present experiments, which were designed to characterize the pgFAR of *B. mori*, we reasoned that the pgFAR might exhibit sequence homology with other HMG-CoA reductases, because previous observations indicated that compactin, a specific inhibitor of HMG-CoA reductases, inhibited pgFAR activity in an *in vitro* assay (7). Consequently, we designed a pair of degenerate primers based on two conserved sequences (GDR/AMGMNM and GQDPAQNV) found in insect HMG-CoA reductases from *A. ipsilon*, *D. melanogaster*, and *Blattella germanica*, and we performed PCR accordingly. Although this attempt succeeded in obtaining the expected 360-bp PCR product with some sequence homology to that of the *A. ipsilon* HMG-CoA reductase, we were unable to obtain cDNA homologs corresponding to HMG-CoA reductase, despite repeated screenings of a pheromone gland cDNA library in which this PCR product was used as a probe (data not shown). Finally, we chose a sense oligonucleotide primer that was designed based on the consensus sequence between the *A. ipsilon* HMG-CoA reductase and the 360-bp PCR product, and performed 3′ RACE using oligo-dT. Likewise, this attempt was not successful in isolating a 3′-end clone exhibiting sequence homology to the HMG-CoA reductase; however, we unexpectedly were able to obtain a 1,299-bp clone that exhibited sequence homology with JJFAR. Isolation of the full-length cDNA revealed that the encoded protein, designated as *B. mori* pgFAR, had 26% identity to JJFAR over the entire length and, like many other reductase proteins, contained one consensus NAD(P)H binding motif at the N-terminal region (Figs. 1 and 2). Northern blot analysis of *B. mori* pgFAR revealed that its expression occurs specifically in the pheromone gland starting shortly before adult eclosion (Fig. 3), suggesting that *B. mori* pgFAR is likely involved in the pheromone biosynthetic pathway of this species. Furthermore, functional expression in *S. cerevisiae* confirmed that *B. mori* pgFAR is an alcohol-generating long-chain FAR and that it exhibits a distinct substrate specificity (Figs. 4 and 5). Among the saturated long-chain fatty acids assayed with 14–20 carbons in chain length, 15:Acid and 16:Acid were found to be preferentially reduced to their corresponding alcohols. Further elucidation of the substrate specificity by using unsaturated C_{16} fatty acids confirmed that *B. mori* pgFAR strictly recognized the location and isomeric nature of the double bonds and showed a strong preference for the bombykol precursor *E*,*Z*10,12–16:Acid (Fig. 5). Finally, yeast cells expressing *B. mori* pgFAR evoked typical mating behavior in the male moth of *B. mori* (Fig. 6). These findings altogether indicate that *B. mori* pgFAR is indeed the pheromone-gland-specific enzyme involved in the fatty-acyl reduction step of bombykol biosynthesis that preferentially reduces the precursor fatty acid to the final alcohol product, bombykol.

Biosynthesis of pheromone components in many moth species is normally stimulated by a specific 33-aa peptide hormone termed pheromone biosynthesis-activating neuropeptide (PBAN), which originates from the subesophageal ganglion (22, 23). In *B. mori*, PBAN directly acts on the pheromone gland to stimulate bombykol production (8, 24). Because bombykol production occurs shortly after adult eclosion in response to PBAN (5, 24), the pheromone-producing cells must be primed for pheromone production upon adult eclosion. Consequently, there is a rapid accumulation of a large number of lipid droplets in the

cytoplasm 1 day before adult eclosion (5, 25), the function of which is to store the bombykol precursor fatty acid in the form of triacylglycerols (TG) (26). Lipolysis of the TG takes place upon adult eclosion, resulting in liberation of the bombykol precursor fatty acid, which is further reduced to bombykol via the fatty acyl-CoA intermediate (27). In this pathway, PBAN stimulates the steps of TG lipolysis and fatty-acyl reduction (25, 28). In the latter step, *B. mori* pgFAR is the key enzyme under PBAN control (7). Although the precise regulatory mechanisms exerted by PBAN on *B. mori* pgFAR remain to be determined, it is obvious that the peptide regulates this enzymatic activity at the translational or posttranslational level, as up-regulation of the *B. mori* pgFAR gene occurs before the release of PBAN during the pupal stage (Fig. 3*B*). We have reported that up-regulation of the pheromone-gland-specific genes encoding acyl-CoA desaturase homologues, and two acyl-CoA binding proteins, pgACBP and mgACBP, occurs 1 day before adult eclosion (10, 11). Because up-regulation of the *B. mori* pgFAR gene also occurs shortly before adult eclosion, coordinated and organized mechanisms must have evolved in the pheromone glands of *B. mori* to provide the molecular basis required for pheromone production.

Reduction of pheromone precursor fatty acids to their corresponding alcohols is the common step in the biosynthesis of oxygenated sex-pheromone components, regardless of the moth species (3). Contrary to our expectations, the present results revealed that *B. mori* pgFAR exhibited a strong substrate specificity for the bombykol precursor fatty acid (Fig. 5). This finding could imply that long-chain pgFAR from different moth species constitute a family of pgFAR with varying substrate specificity. Many moth species have their own pheromone blends, and the precise ratios of components in multicomponent pheromones collectively allow their distinct species specificities (29). Accordingly, the substrate specificity of individual pgFAR may eventually define the precise ratios of components in the pheromone blends of individual moth species.

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