



Screening for the genes involved in bombykol biosynthesis: identification and functional characterization of *Bombyx mori* acyl carrier protein

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Species-specific sex pheromones released by female moths to attract conspecific male moths are synthesized *de novo* in the pheromone gland (PG) via fatty acid synthesis (FAS). Biosynthesis of moth sex pheromones is usually regulated by a neurohormone termed pheromone biosynthesis activating neuropeptide (PBAN), a 33-aa peptide that originates in the subesophageal ganglion. In the silkworm, *Bombyx mori*, cytoplasmic lipid droplets (LDs), which store the sex pheromone (bombykol) precursor fatty acid, accumulate in PG cells prior to eclosion. PBAN activation of the PBAN receptor stimulates lipolysis of the stored LD triacylglycerols (TAGs) resulting in release of the bombykol precursor for final modification. While we have previously characterized a number of molecules involved in bombykol biosynthesis, little is known about the mechanisms of PBAN signaling that regulate the TAG lipolysis in PG cells. In the current study, we sought to further identify genes involved in bombykol biosynthesis as well as PBAN signaling, by using a subset of 312 expressed-sequence tag (EST) clones that are in either our *B. mori* PG cDNA library or the public *B. mori* EST databases, SilkBase and CYBERGATE, and which are preferentially expressed in the PG. Using RT-PCR expression analysis and an RNAi screening approach, we have identified another eight EST clones involved in bombykol biosynthesis. Furthermore, we have determined the functional role of a clone designated BmACP that encodes *B. mori* acyl carrier protein (ACP). Our results indicate that BmACP plays an essential role in the biosynthesis of the bombykol precursor fatty acid via the canonical FAS pathway during pheromonogenesis.

Keywords: bombykol, *Bombyx mori*, lipid droplet, triacylglycerol, acyl carrier protein

INTRODUCTION

In most moth species, sex pheromone biosynthesis is regulated by the pheromone biosynthesis activating neuropeptide (PBAN), a neurohormone consisting of 33 amino acid peptide that originates in the subesophageal ganglion and that is characterized by the core C-terminal FSPRLamide sequence. Initially purified and sequenced from the corn earworm, *Helicoverpa zea* (Raina et al., 1989) and the silkworm, *B. mori* (Kitamura et al., 1989), PBAN has since been identified in numerous other lepidopteran species (Rafaeli and Jurenka, 2003). In most species including *B. mori*, PBAN is released into the hemolymph during a species-specific period after adult emergence and acts on the pheromone gland (PG) to trigger the production and release of species-specific sex pheromones (Rafaeli, 2002, 2009). The PG is a functionally differentiated organ in close proximity to the terminal abdominal tip which originates in the intersegmental membrane between the eighth and ninth abdominal segments (Percy and Weatherston, 1974; Fónagy et al., 2000; Matsumoto et al., 2007). In the silkworm, *Bombyx mori*, the sex pheromone, bombykol (*E*, *Z*-10, 12-hexadecadien-1-ol), is synthesized *de novo* within PG cells from acetyl-CoA via conventional long chain fatty acid synthesis (FAS; Bjostad et al., 1987; Jurenka, 2003). The straight

chain fatty acyl intermediate, palmitate, is converted stepwise to bombykol by the actions of a bifunctional Z11-10/12 fatty acyl desaturase, Bmpgdesat1, and a PG-specific fatty acyl reductase, pgFAR (Moto et al., 2003, 2004; Ohnishi et al., 2006). On the day before adult emergence, *B. mori* PG cells rapidly accumulate numerous lipid droplets (LDs) within the cytoplasm (Fónagy et al., 2001). These LDs play an essential role in bombykol biosynthesis by acting as a reservoir for the *de novo* synthesized bombykol precursor, 10,12-hexadecadienoate, which is deposited in the LDs in the form of triacylglycerols (TAGs) with the precursor predominantly sequestered at the *sn*-1 and *sn*-3 positions of the glycerides (Matsumoto et al., 2002). After adult emergence, the stored fatty acid is cleaved and converted to bombykol in response to PBAN stimulation (Matsumoto et al., 2007, 2010).

To identify functional proteins involved in bombykol biosynthesis, we generated a PG expressed-sequence tag (EST) database by constructing a normalized PG cDNA library prepared from newly emerged female *B. mori* moths of the inbred p50 strain (Yoshiga et al., 2000). We next screened *in silico* for PG-specific genes, using our own database as well as the public *B. mori* EST databases (SilkBase and CYBERGATE). Of the approximate 11,000 independent EST clones, we identified a number of clones that are

predominantly or specifically expressed in the *B. mori* PG. Apart from this type of approach, we also established a method of RNAi-mediated *in vivo* gene silencing in *B. mori* (Ohnishi et al., 2006). Using this methodological tool, we have characterized a number of genes that are specifically or predominantly expressed in the *B. mori* PG during pheromonogenesis. These genes, which are up-regulated on the day prior to adult emergence (i.e., *pgFAR*, *Bmpgdesat1*, *pgACBP*, *mgACBP*, *PBANR*, *BmFATP*, and *BmLsd1*), play critical roles in bombykol production triggered by the external PBAN signal (Matsumoto et al., 2001; Moto et al., 2003, 2004; Hull et al., 2004; Ohnishi et al., 2006, 2009, 2011). In the current study, we describe the further identification of eight EST clones involved in bombykol biosynthesis using similar methodologies. Among these clones, we have also determined the functional role of a clone (NRPG0659) that encodes the *B. mori* acyl carrier protein (BmACP) critical for bombykol biosynthesis.

MATERIALS AND METHODS

INSECTS

Larvae of the inbred p50 strain of *B. mori*, originally provided by T. Shimada of the University of Tokyo, were reared on an artificial diet and maintained under a 16L:8D photoperiod at 25°C (Fónagy et al., 1992). Pupal age was determined based on morphological characteristics as described (Matsumoto et al., 2002).

RAPID AMPLIFYING cDNA ENDS (RACE)

Messenger RNA was isolated from about 100 µg of PG total RNA by using a Micro-Fast Track kit (Invitrogen). RACE was performed using a GeneRacer kit (Invitrogen) according to the manufacturer's instructions. Computer-assisted sequence analyses were performed using GENETYX-MAC Ver.15.0.5 (Software Development Co., Tokyo, Japan).

RT-PCR ANALYSIS

Pheromone glands and other tissues were dissected into insect Ringer's solution [35 mM NaCl, 36 mM KCl, 12 mM CaCl₂, 16 mM MgCl₂, 274 mM glucose, and 5 mM Tris (pH 7.5)] and mechanically trimmed as described (Ozawa and Matsumoto, 1996). Total RNA was isolated from the trimmed PGs by the method of Chomczynski and Sacchi (1987). First-strand cDNA synthesis was performed with 500 ng total RNA using an RNA PCR kit (Takara Bio, Otsu, Japan) according to the manufacturer's instructions. Fragments of *BmACP* and *Actin* were amplified using a specific oligonucleotide primer set designed from the published sequence [for *BmACP*, NRPG0659-F (sense primer): 5'-GCACAATTAAGCCGCTGTC-3' and NRPG0659-R (antisense primer): 5'-ATGTCTTTTGGTCTGACCAG-3'; for *Actin*, *Actin-F* (sense primer): 5'-AGATGACCCAGATCATGTTT-3' and *Actin-R* (antisense primer): 5'-GAGATCCACATCTGCTGGAAG-3']. PCR was performed using thermocycler conditions consisting of 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s. PCR products (5 µL) were electrophoresed on a 1.5% agarose gel in Tris-acetate-EDTA buffer and stained with ethidium bromide.

SYNTHESIS AND INJECTION OF DOUBLE-STRANDED RNA (dsRNA)

Templates for the synthesis of dsRNAs corresponding to *BmACP* were prepared using gene specific primers containing

T7 polymerase sites [NRPG0659-T7-F (sense primer): 5'-CGGATCCTAATACGACTCACTATAGGGCGGCACAATTA-3', NRPG0659-T7-R (antisense primer): 5'-CCGGATCCTAATACGACTCACTATAGGGCGCCAATACATTGC-3'; nucleotide sequences corresponding to the T7 promoter region are underlined]. PCR was performed using thermocycler conditions consisting of six cycles at 94°C for 30 s, 56.5°C for 30 s, 68°C for 90 s followed by 30 cycles at 94°C for 30 s, 66°C for 30 s, 68°C for 90 s using KOD-Plus – (Toyobo, Osaka, Japan) with the resulting products purified (Wizard SV Gel and PCR Clean-Up kit, Promega) and used as templates to generate dsRNAs using the AmpliScribe™T7 High Yield Transcription kit (Epicentre Technologies, Madison, WI, USA) according to the manufacturer's instructions. After synthesis, the dsRNAs were diluted with diethyl pyrocarbonate (DEPC) – treated H₂O, the RNA concentrations measured (Abs₂₆₀), and the products analyzed by gel electrophoresis to confirm annealing. Samples were diluted to the desired concentration (final volume 2 µL) and injected into 1-day-old pupae (i.e., pupae 1 day removed from the larval-pupal molt) using a 10 µL microsyringe (Hamilton, USA) as described (Ohnishi et al., 2006; Hull et al., 2009, 2010). Control pupae were injected with 2 µL DEPC-treated H₂O alone. After injection, pupae were maintained under normal conditions until adult emergence.

IN VIVO BOMBYKOL ANALYSIS

Adult females were decapitated within 3 h of emergence and maintained at 25°C for 24 h. They were then injected with either 2 µL (2.5 pmol/µL) *B. mori* PBAN in PBS or PBS alone. Abdominal tips were dissected 90 min after injection and bombykol production was measured by HPLC as described (Matsumoto et al., 1990) using a Senshu-Pak NO₂ column [Senshu Scientific Co., Tokyo, Japan; 4.6 mm inner diameter × 150 mm, pore size, 100 Å].

MICROSCOPIC EXAMINATION OF CYTOPLASMIC LDs

PGs were dissected and mechanically trimmed from normal, decapitated, and RNAi-treated females. The excised glands were fixed with a 4% formalin/PBS solution and stained with Nile Red (a fluorescent probe for intracellular neutral lipids; Molecular Probes, Eugene, OR, USA) as described (Fónagy et al., 2001). Fluorescence microscopy was performed with an OLYMPUS BX-60 system equipped with a PM-30 exposure unit and a BH20-RFL-T3 light source (400× magnification). Nile Red imaging was done with a 330–385 nm band pass excitation filter, a 400 nm dichroic mirror, and a 420 nm long pass barrier filter (Olympus cube WU). Images were processed and merged using Photoshop CS (Adobe Systems).

ANALYSIS OF LD TRIACYLGLYCEROLS

Lipid droplets TAGs were analyzed as described previously (Matsumoto et al., 2002). Five trimmed PGs were prepared from the desired stages of female moths and dipped in 100 µL acetone for 10 min at room temperature. The dried acetone extracts were dissolved in *n*-hexane and loaded on either a Senshu-Pak PEGASIL-Silica 120-5 column (Senshu Scientific Co.; 4.6 mm inner diameter × 250 mm, pore size, 120 Å) equilibrated with *n*-hexane/acetic acid (99/1) or a SSC-C₂₂ docosil column (Senshu Scientific Co.; 4.6 mm inner diameter × 250 mm, pore size,

120 Å) equilibrated with acetonitrile/ethanol (6/4). Because various TAG components comprise the cytoplasmic LDs, the entire TAG fraction was separated and detected using an evaporative light scattering detector (ELSD; SEDEX model 75, Sedere, France). Fatty acyl groups in the TAGs were identified by fast atom bombardment mass spectrometry (FAB-MS) and tandem MS (MS/MS) analyses using a JMS HX/HX-110A tandem mass spectrometer (JEOL, Tokyo, Japan) as described (Matsumoto et al., 2002).

RESULTS

EXPRESSION ANALYSIS OF *B. MORI* EST CLONES USING RT-PCR

To identify proteins functionally involved in bombykol biosynthesis, we selected 88 PG-specific genes *in silico* from approximately 11,000 independent clones in our PG EST database and/or the public *B. mori* EST databases. To examine the *in vivo* expression patterns of the 88 gene transcripts, we performed RT-PCR analysis using various tissues including PG, which were prepared from *B. mori* females (p50 strain) before and after eclosion. The results indicated that 26 clones were specifically expressed in the PG and/or up-regulated the day prior to eclosion

(Table 1), suggesting potential functional roles for these genes in pheromonogenesis.

RNAi SCREENING FOR GENES INVOLVED IN *B. MORI* PHEROMONOGENESIS

We previously established a method of RNAi-mediated *in vivo* gene silencing in *B. mori* (Ohnishi et al., 2006). To confirm the *in vivo* function of the 26 genes described above, we performed dsRNA-mediated RNAi knockdown of these target genes and examined their inhibitory effect on bombykol production. Compared to control pupae injected with DEPC-treated H₂O, 1-day-old p50 female pupa injected with 10 µg dsRNA corresponding to the respective genes resulted in a significant reduction in bombykol production for 8 of the 26 genes (NRPG0074, NRPG0604, NRPG0659, NRPG0660, NRPG0873, NRPG0891, NRPG1860, and NRPG1885; Figure 1). Because no decrease in bombykol production was observed in control pupae injected with dsRNAs for unrelated proteins including enhanced green fluorescent protein (EGFP), these findings suggest that disruption of bombykol production results from specific knockdown of the target gene sequences (Figure 1). These eight EST clones include two clones

Table 1 | Expressed-sequence tag clones identified in the *Bombyx mori* pheromone gland.

EST clone name	GenBank homolog description	Accession no. ¹	E-value ²	PG expression ³	Up-regulation ⁴
NRPG0023	Triacylglycerol lipase (<i>Bombyx mori</i>)	DQ311322	5.00E-94	+	+
NRPG0034	Aldehyde dehydrogenase (<i>Bombyx mori</i>)	DQ311433	1.00E-100	+	+
NRPG0074	Chemosensory protein (<i>Bombyx mori</i>)	DQ855507	2.00E-66	+	+
NRPG0098	Scavenger receptor cysteine-rich protein (<i>Culex pungens</i>)	DS232623	1.00E-13	+	+
NRPG0203	B-cell receptor-associated protein 31 (<i>Acromyrmex echinator</i>)	GL888216	2.00E-10	+	
NRPG0204	ABC transporter (<i>Aedes aegypti</i>)	EAT43031	8.00E-43	+	+
NRPG0293	No homolog			+	
NRPG0414	No homolog				+
NRPG0476	No homolog			+	+
NRPG0604	Glycerophosphoryl diester phosphodiesterase (<i>Bombyx mori</i>)	AB115083	3.00E-96	+	+
NRPG0640	Serine protease (<i>Aedes aegypti</i>)	CH477825	3.00E-4	+	+
NRPG0659	Acyl carrier protein (<i>Bombyx mori</i>)	DQ443383	8.00E-76		+
NRPG0660	Cuticle protein (<i>Bombyx mori</i>)	BR000649	3.00E-98	+	+
NRPG0733	Arylsulfatase (<i>Culex quinquefasciatus</i>)	DS232119	2.00E-65	+	+
NRPG0825	No homolog			+	+
NRPG0857	Yellow protein (<i>Bombyx mori</i>)	DQ358081	1.00E-114	+	+
NRPG0873	Glucose dehydrogenase (<i>Aedes aegypti</i>)	EAT44639	4.00E-35	+	+
NRPG0891	Perilipin (<i>Bombyx mori</i>)	DQ311207	1.00E-110	+	+
NRPG1106	Aldehyde oxidase (<i>Bombyx mori</i>)	EU073423	1.00E-127	+	+
NRPG1147	VLC acyl-CoA synthetase (<i>Drosophila melanogaster</i>)	AY118315	6.00E-64	+	+
NRPG1187	Lipase 3 (<i>Drosophila melanogaster</i>)	CAA74737	4.00E-22	+	+
NRPG1431	No homolog			+	
NRPG1860	Fatty acid transport protein (<i>Bombyx mori</i>)	AB451529	1.00E-115	+	+
NRPG1885	Lipase (<i>Aedes aegypti</i>)	EAT48716	2.00E-17	+	+
fpheP04_F_A15	Alcohol dehydrogenase (<i>Bombyx mori</i>)	DQ512730	4.00E-23		+
fpheP07_F_F10	BmorCPR53 cuticle protein (<i>Bombyx mori</i>)	BR000544	3.00E-68	+	

¹Accession number of the GenBank homolog.

²E-value for the comparison of the EST sequence information and the GenBank homolog (BLASTX).

³+ Indicates a gene that is predominantly expressed in the PG.

⁴+ Indicates a gene that is up-regulated from the day prior to eclosion in the PG.

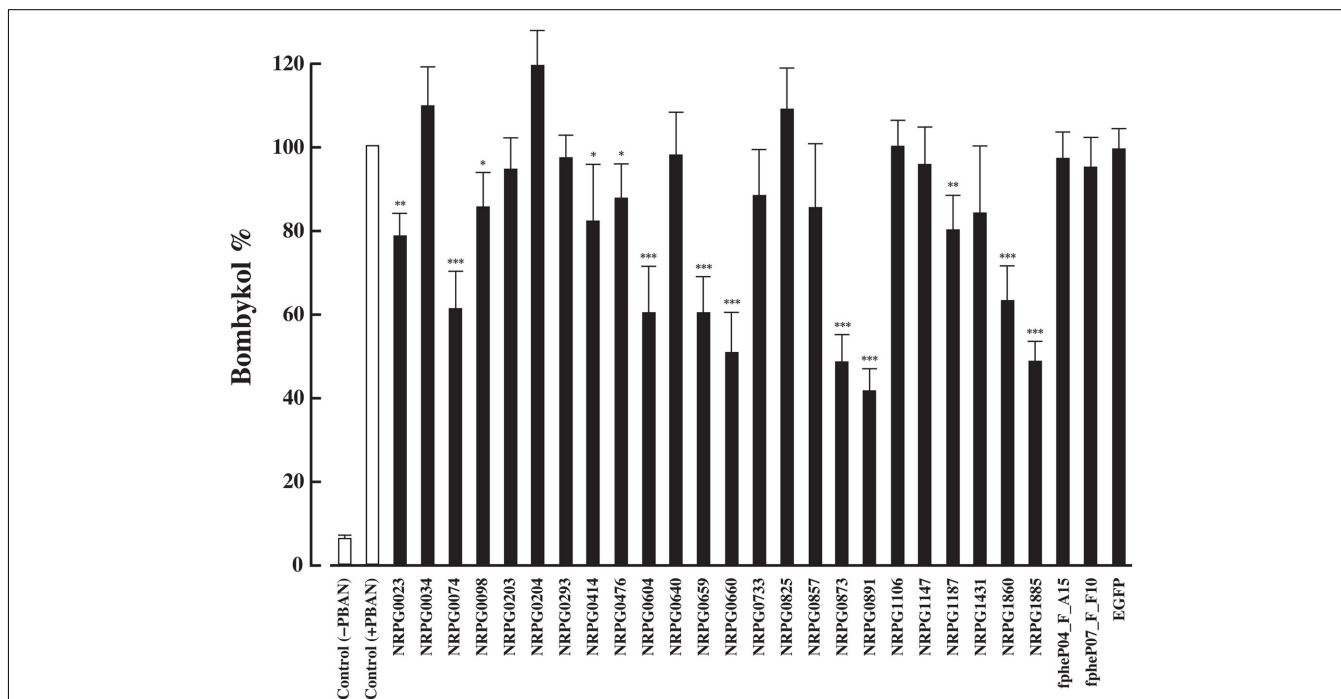


FIGURE 1 | Effect of RNAi treatment on *in vivo* bombykol production. One-day-old pupae were injected with 10 μg dsRNA corresponding to the target genes or *EGFP*. Results are expressed relative to the mean value for

bombykol levels in non-RNAi-treated females following injection with 5 pmol PBAN (**p* < 0.05; ***p* < 0.01; ****p* < 0.001). Bars represent mean values ± SD from independent experimental animals (*n* = > 15).

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1  attaaagggtttggcaaccaggctttcggattc gatgagagagaattgctgaaagaaaaaaagcatttcgtagccttgaagaaatggc  90
1  M A 2
91  tgctgcgaatat tttccgaagtgc tttcggtggtctattacggagatcaacaacgtatagaacgtccgtaacatgctcgtctttcgaccgt 180
3  A A N I F R S A F G G L L R R S T T Y R T S V T C R L S T V 32
181  tcgcttgcaagcaaaagttcagcacaattaaagccgctgtcaaaaatatacaaaagtcaggcccttcagaatgggaacgagaaacatgggat 270
33  A L Q Q K F S T I K A A V K I Y K S Q A L Q N G N E K H G I 62
271  ccgcaagtacagcgggtggccctccctcaccttagaccttataaaaagtagagtactgcttgacttcaactttatgacaaaattaacc 360
63  R K Y S G G P P L T L D L I K S R V L L V L Q L Y D K I N P 92
361  tgaacagttgactgtagatagccacttcatgaatgatctgggtcttgactcactggaccatgttgagggtcataatggcaatggaagatga 450
93  E Q L T V D S H F M N D L G L D S L D H V E V I M A M E D E 122
451  atttgatttgagataccggatggtgatgcagagagactggtcagaccaaagacattgtccaatacattgctgacaaggaagatattta 540
123  F G F E I P D G D A E R L V R P K D I V Q Y I A D K E D I Y 152
541  tgaataataaaaaaaatataaaacttcataat tttggaccagttgtat ttttgaacgataaacaacaaacacaaaattgcattttctta 630
153  E * 153
631  atttacaactggcctaaaagtattg ttttagtgattagacaacttttgttttaattttacacaacgttatcagtgccacttcagaccattg 720
721  tgtaaaaagtgtaaagttttgtttt taattatagcctgttagaatagatcattgtat tttcatttgatctgggtgtacattatctgataa 810
811  taaaatagtaaatagatcaaaaaaaaaaaaaaaaaa 848
    
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FIGURE 2 | cDNA and deduced amino acid sequence of BmACP.

that encode BmLsd1 and BmFATP (NRPG0891 and NRPG1860), which we have recently characterized functionally associated with LD dynamics during *B. mori* pheromonogenesis (Ohnishi et al., 2009, 2011). From this group, five of the genes (NRPG0604, NRPG0659, NRPG0891, NRPG1860, and NRPG1885) are predicted to encode proteins involved in lipid metabolism (Table 1), which is consistent with a role in bombykol biosynthesis. The roles of the other three genes, which encode a chemosensory protein

(NRPG0074), a cuticle protein (NRPG0660), and a glucose dehydrogenase (NRPG0873), in bombykol biosynthesis have yet to be identified.

CHARACTERIZATION OF NRPG0659

Our RNAi-mediated gene silencing screen suggested the involvement of NRPG0659 in bombykol production (Figure 1). NRPG0659 is predicted to encode an acyl carrier protein (Table 1),

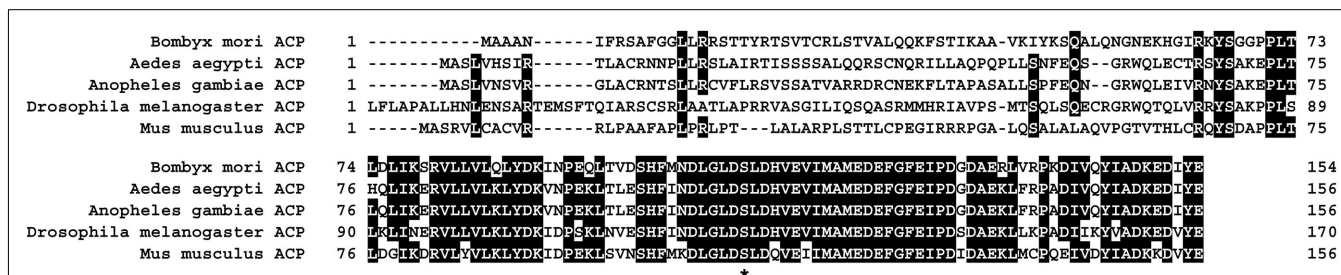


FIGURE 3 | Multiple sequence alignment of BmACP and related proteins. The predicted protein sequence of BmACP is compared with those of the *Aedes aegypti*, *Anopheles gambiae*, *Drosophila melanogaster*, and *Mus musculus* proteins (GenBank accession numbers: DQ443383, EAT36207,

EAA07190, ADB57059, AAH92379). Numbering of each sequence begins at the predicted initiator methionine. Amino acid residues identical to the *B. mori* sequence are highlighted in black. The asterisk (*) indicates the prosthetic group attachment site (Ser).

which is known to be a universal and highly conserved carrier of acyl intermediates during FAS (Byers and Gong, 2007). Consequently, we sought to characterize the functional role of the BmACP expressed in the PG. Structural analysis using both 3'- and 5'-RACE confirmed that *BmACP* is comprised of a 848-nt ORF encoding a 153-aa protein flanked by a 85-nt 5'-UTR and a 304-nt 3'-UTR (Figure 2). Comparison of the BmACP sequence with sequences from other organisms revealed high similarities with the *Aedes aegypti* ACP, *Anopheles gambiae* ACP, *Drosophila melanogaster* ACP, and *Mus musculus* ACP (59, 59, 54, and 54% similarity, respectively; Figure 3). These ACPs including BmACP are characterized by a highly conserved C-terminal domain sequence that contains a conserved serine residue necessary for interacting with acyl proteins (Figure 3; Byers and Gong, 2007). While RT-PCR analysis revealed that *BmACP* is ubiquitously expressed in adult tissues (Figure 4A), it undergoes up-regulation 1 day prior to adult emergence in the PG (Figure 4B). This up-regulation profile is reminiscent of PG proteins crucial to bombykol biosynthesis (Matsumoto et al., 2001; Moto et al., 2003, 2004; Hull et al., 2004; Ohnishi et al., 2006, 2009, 2011).

IN VIVO EFFECT OF RNAi-MEDIATED BmACP KNOCKDOWN ON BOMBYKOL PRODUCTION

To confirm the *in vivo* function of BmACP, we returned to dsRNA-mediated RNAi knockdown and examined the effect on transcript levels. When we injected 10 μg *BmACP* dsRNA into newly emerged adult p50 females, *BmACP* mRNA levels 2 days after injection in the PG were selectively reduced compared to those in other tissues as well as control females injected with DEPC-treated H₂O alone (Figure 5A). Although the reason for the PG-selective knockdown of BmACP is presently unknown, a similar PG-selective decrease in transcript levels was observed following knockdown of BmFATP and BmLsd1 (Ohnishi et al., 2009, 2011). Because *BmACP* dsRNA injections had no effect on pupal development or adult emergence, we next injected varying concentrations (1, 5, and 10 μg) of *BmACP* dsRNA into newly emerged female moths. A dose-dependent reduction in bombykol production was observed with 1, 5, and 10 μg dsRNA injections resulting in bombykol levels 73, 63, and 59% of the control level, respectively (Figure 5B). To confirm the knockdown effect by *BmACP*, we also examined other dsRNAs corresponding to non-overlapping 5' and 3' regions

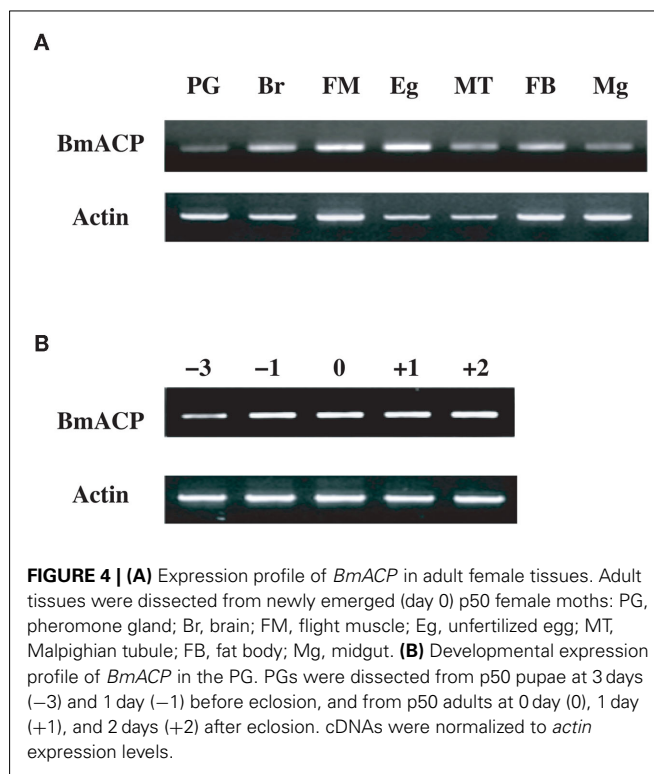
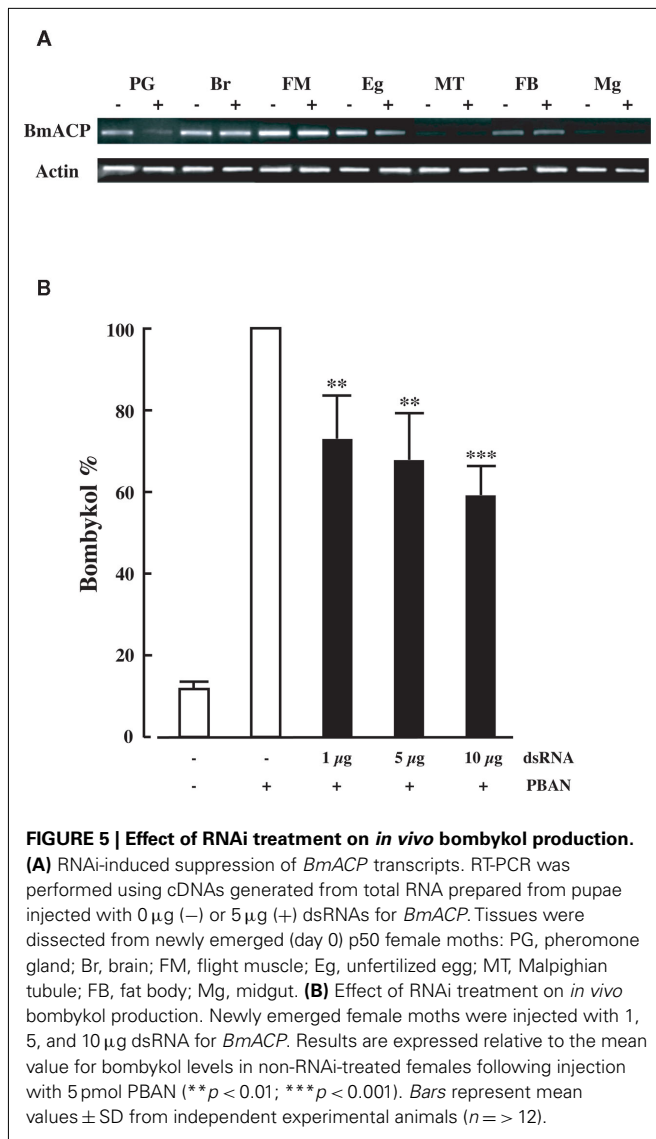


FIGURE 4 | (A) Expression profile of *BmACP* in adult female tissues. Adult tissues were dissected from newly emerged (day 0) p50 female moths: PG, pheromone gland; Br, brain; FM, flight muscle; Eg, unfertilized egg; MT, Malpighian tubule; FB, fat body; Mg, midgut. **(B)** Developmental expression profile of *BmACP* in the PG. PGs were dissected from p50 pupae at 3 days (-3) and 1 day (-1) before eclosion, and from p50 adults at 0 day (0), 1 day (+1), and 2 days (+2) after eclosion. cDNAs were normalized to *actin* expression levels.

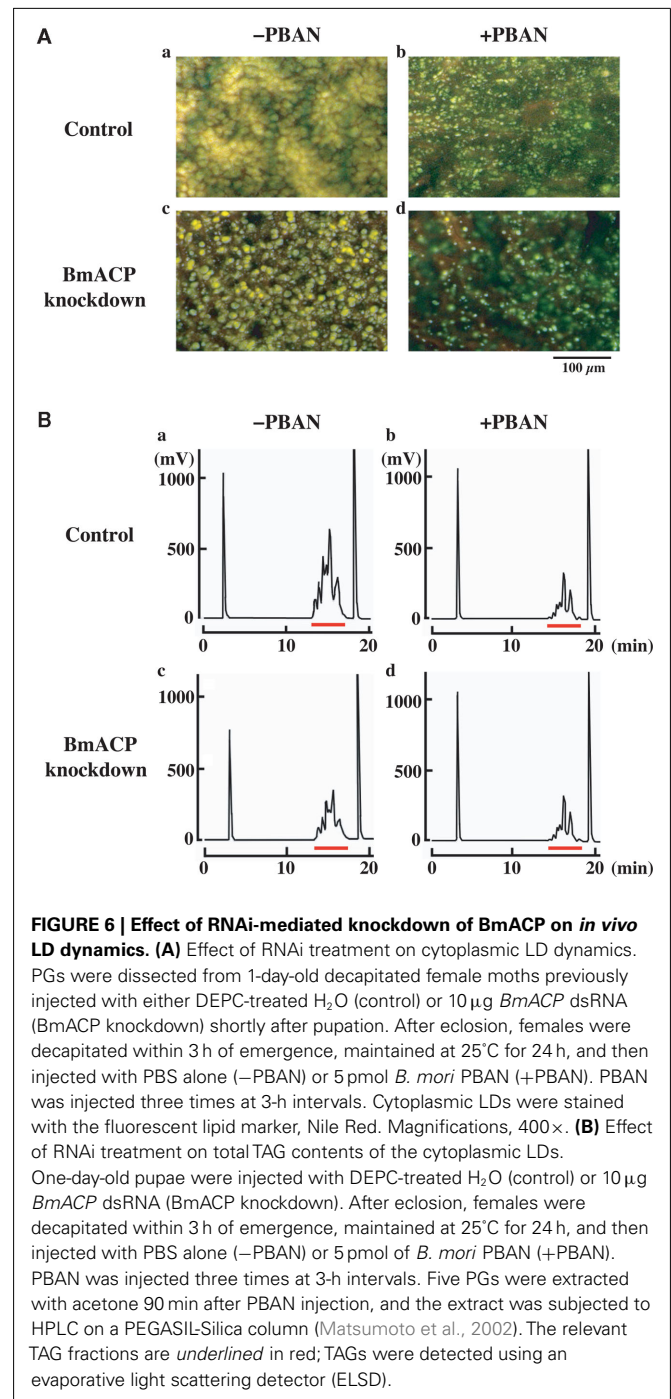
of *BmACP*; these dsRNAs had a similar knockdown effect on bombykol production (data not shown).

IN VIVO EFFECT OF RNAi-MEDIATED BmACP KNOCKDOWN ON CYTOPLASMIC LD DYNAMICS

We further examined the knockdown effect of BmACP on LD dynamics during pheromonogenesis (Figure 6). The most obvious characteristic feature of PG cells is the abundance of LDs within the cytoplasm (Fónagy et al., 2000, 2001). The role of the LDs in bombykol biosynthesis is to store the bombykol precursor fatty acid in the form of TAGs and release it in response to the external signal of PBAN (Matsumoto et al., 2002). To monitor the LD dynamics during pheromonogenesis, we have previously stained the LDs with the fluorescent lipid marker, Nile Red, and found that they appear 1–2 days before eclosion, rapidly



accumulate on the day of eclosion, and decrease in size and number after eclosion in response to PBAN (Fónagy et al., 2001). In RNAi-untreated controls, female moths accumulated numerous large LDs in the PG cell, which underwent a striking reduction following PBAN-induced LD lipolysis (Figure 6A,a,b, also see Ohnishi et al., 2009, 2011). In contrast, PGs of female moths treated with *BmACP* dsRNA accumulated noticeably smaller LDs (Figure 6A,a,c). Because these LDs store the bombykol precursor in the form of TAGs (14), we further examined the effects of BmACP knockdown on the fluctuation of total TAGs. Following extraction with acetone from dissected PGs, total TAG levels were measured by normal-phase HPLC on a PEGASIL-Silica column (Matsumoto et al., 1990). In BmACP knocked-down PGs, PBAN-induced TAG lipolysis was comparable to that of the non-RNAi-treated control (Figure 6B,b,d). In contrast, accumulation of TAG content was significantly reduced (38%) in BmACP-knockdown PGs compared to the RNAi-untreated control (Figure 6B,a,c). Taken together, these results indicate that BmACP is integral to



the bombykol biosynthetic pathway in that it plays an essential role in accumulating the bombykol precursor for TAG storage in the cytoplasmic LDs before eclosion.

IN VIVO EFFECT OF RNAi-MEDIATED BmACP KNOCKDOWN ON DE NOVO SYNTHESIS OF THE BOMBYKOL PRECURSOR

The TAGs that compose cytoplasmic LDs in the PGs comprise various TAG species in which the bombykol precursor is predominantly sequestered at the *sn*-1 and/or *sn*-3 position as a major

fatty acyl component (Matsumoto et al., 2002). Consequently, we sought to assess the effects of BmACP silencing on *de novo* synthesis of the bombykol precursor via the conventional FAS pathway. In agreement with our previous results (Matsumoto et al., 2002) and **Figure 7**, overall TAG contents in PGs of both control and RNAi-treated moths were dramatically reduced in response to PBAN (**Figures 6B** and **7**). In addition, the total amount of TAGs in RNAi-treated PGs was again reduced $\sim 34\%$ compared to that of

the non-RNAi-treated PGs (**Figures 7A,C**). Furthermore, there was a significant difference in the TAG elution profiles between non-RNAi-treated and RNAi-treated PGs before PBAN injection; silencing BmACP brought about a striking reduction in specific TAG peaks (see peaks 1, 1a, 2, 3 in **Figure 7**). MS analysis indicated that the TAG species eluted in these peaks are mainly comprised of the bombykol precursor fatty acid (**Table 2**; Matsumoto et al., 2002).

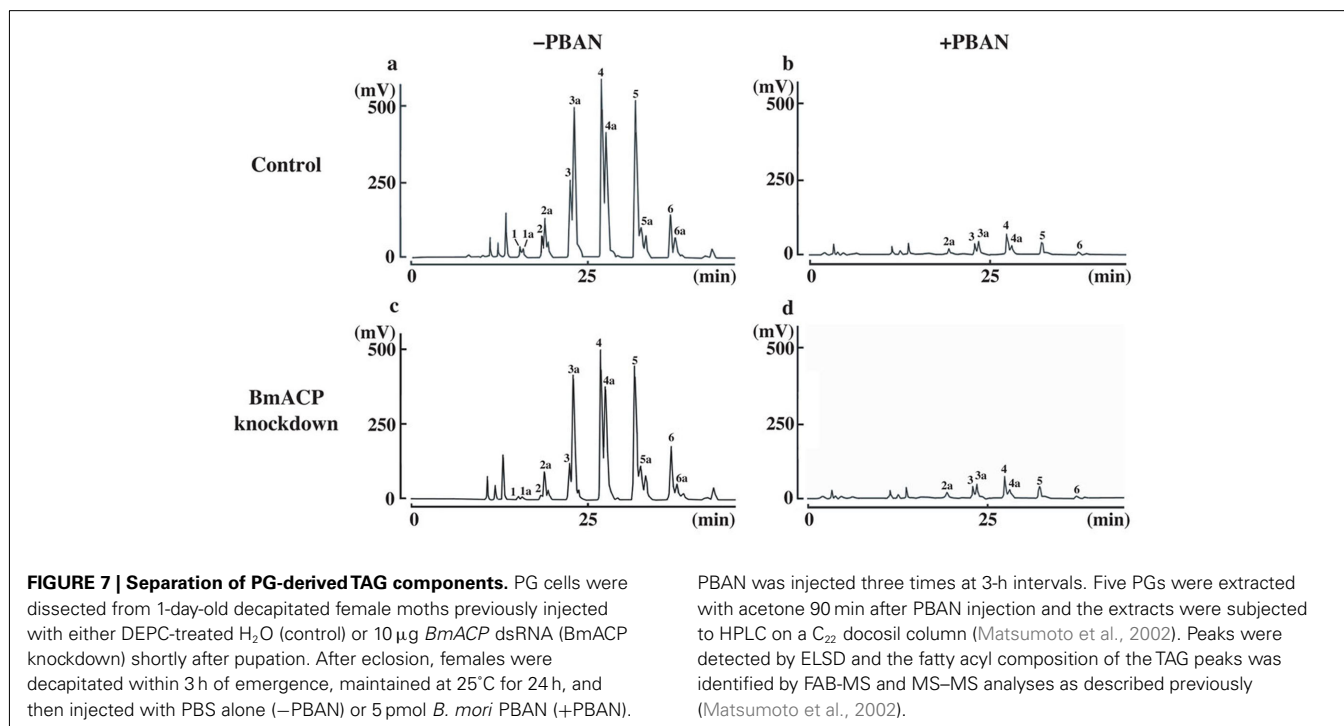


Table 2 | Comparison of peak areas in Figures 7A,C.

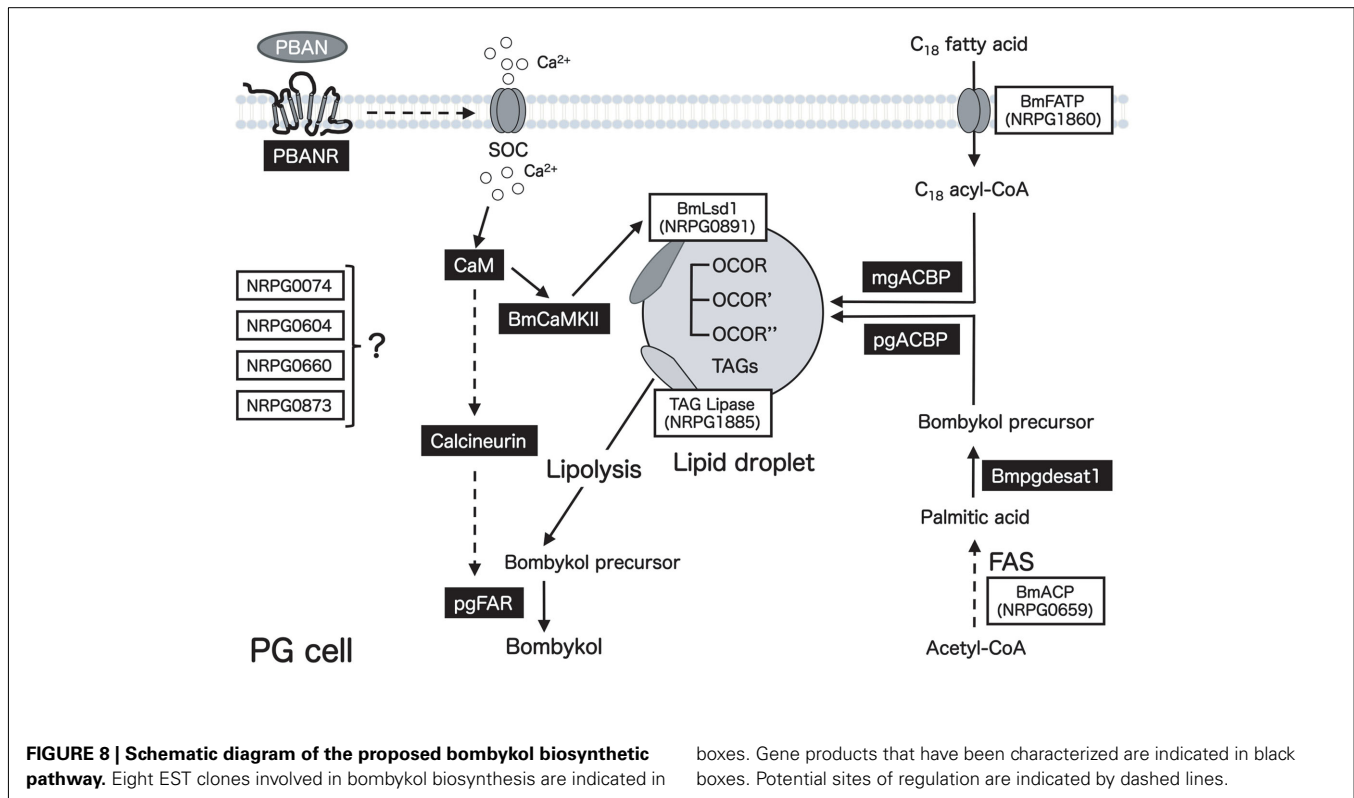
Peak#	Control (a) Avg. peak area (AU) SD	BmACP knockdown (c) Avg. peak area (AU) SD	Ratio (%) B/A \times 100	Student's <i>t</i> -test ^a	Fatty acyl compositions ^b
1	143 \pm 8	50 \pm 6	35.0	***	(C16:2, C16:2, C16:2)
1a	134 \pm 3	57 \pm 11	42.2	***	(C16:2, C16:2, C18:3)
2	211 \pm 12	118 \pm 21	55.6	***	(C16:2, C18:2, C16:2)
2a	941 \pm 166	678 \pm 155	72.0	*	(C16:1, C18:3, C18:3) and (C16:2, C18:2, C18:3)
3	1,622 \pm 204	492 \pm 62	30.3	***	(C16:2, C18:1, C16:2)
3a	2,022 \pm 318	1,691 \pm 270	80.1	*	(C16:2, C18:1, C18:3)
4	2,169 \pm 296	1,550 \pm 329	71.5	**	(C16:2, C18:1, C16:1) and/or (C16:1, C18:1, C16:2)
4a	1,831 \pm 318	1,333 \pm 270	72.8	*	(C16:1, C18:1, C18:3), (C16:1, C18:2, C18:2), and (C16:2, C18:1, C18:2)
5	2,140 \pm 248	1,638 \pm 399	76.5	*	(C16:2, C18:1, C18:1) and/or (C18:1, C18:1, C16:2)
5a	689 \pm 67	595 \pm 116	86.4	NS	(C18:1, C18:1, C18:3) and (C18:1, C18:2, C18:2)
6	624 \pm 72	552 \pm 42	88.5	NS	(C16:1, C18:1, C18:1) and/or (C18:1, C18:1, C16:1)
6a	301 \pm 49	221 \pm 45	73.3	NS	(C18:1, C18:1, C18:2)

C16:1, Δ 11-hexadecenoate; C16:2, Δ 10,12-hexadecadienoate; C18:1, Δ 9-octadecenoate; C18:2, Δ 9,12-octadecadienoate; C18:3, Δ 9,12,15-octadecatrienoate. NS, not significant.

^aStatistical analysis of avg. peak area control samples vs. BmACP knockdown samples performed using Student's *t*-test ($n = 5$).

^bFatty acyl compositions of the TG(s) in each peak were identified with Fast Atom Bombardment MS and MS–MS analyses (Matsumoto et al., 2002).

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.



DISCUSSION

We have developed a method to facilitate identification of the genes involved in bombykol biosynthesis, using RT-PCR expression analysis and RNAi-mediated *in vivo* gene silencing in conjunction with EST databases. In the current study, we identified eight EST clones that likely encode various proteins that play a role in bombykol production (Table 1; Figure 1). These eight EST clones include two clones that encode BmFATP and BmLsd1 (NRPG1860 and NRPG0891), two proteins that we have recently identified and shown to be functionally associated with LD dynamics during *B. mori* pheromogenesis (Ohnishi et al., 2009, 2011). We have reported that BmFATP plays a role in stimulating the TAG synthesis required for LD accumulation via a process similar to the so-called vectorial acylation that couples the uptake of extracellular fatty acids with activation to CoA thioesters (Ohnishi et al., 2009). In contrast, BmLsd1 plays an essential role in the TAG lipolysis associated with bombykol biosynthesis via phosphorylation with activation of BmCaMKII by influx of extracellular Ca^{2+} (Ohnishi et al., 2011).

Of the eight clones identified in the current study, we further characterized the clone that encodes BmACP. ACP is a highly conserved 70–100-aa carrier of acyl intermediates during FAS (Byers and Gong, 2007). In yeast and mammals, ACP exists as a separate domain within a large multifunctional fatty acid synthase polyprotein (type I FAS), whereas it is a small monomeric protein in bacteria and plastids (type II FAS). Because many insect sex pheromones are synthesized *de novo* from acetyl-CoA via the conventional long chain FAS pathway within PG cells (Bjostad et al., 1987; Jurenka, 2003), identification of BmACP in the PG suggests that it plays a significant role in the early

steps of bombykol biosynthesis. Accordingly, RNAi-mediated gene silencing of BmACP *in vivo* resulted in a significant reduction in bombykol production, suggesting the functional relevance of BmACP in bombykol biosynthesis (Figure 5). In addition, RNAi treatment resulted in accumulation of noticeably smaller LDs in the PG (Figure 6), indicating that it plays an essential role in LD accumulation prior to eclosion. While similar results was observed when BmFATP gene expression was suppressed (Ohnishi et al., 2009), the analysis of TAG contents revealed a significant difference. BmACP suppression mainly reduced the TAGs containing the bombykol precursor fatty acid, whereas the BmFATP suppression mainly reduced the TAGs containing the essential C_{18} -fatty acids derived from diet (Figure 7). These results are consistent with the general properties of ACPs and strongly suggest that BmACP plays an essential role in the biosynthesis of the bombykol precursor palmitic acid via the canonical FAS pathway in PG cells during pheromogenesis. These results also indicate that BmACP and BmFATP function cooperatively to populate the TAGs comprising the cytoplasmic LDs (Figure 8). In addition, our present results validate the methodological approach we have taken to mine EST databases for unidentified functional molecules that play a critical role in moth sex pheromone production.

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