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The role of desaturases in the biosynthesis of marking pheromones in bumblebee males

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ABSTRACT

Bumblebee males (Hymenoptera) produce species-specific labial gland secretions called marking pheromones (MPs). MPs generally consist of terpenoids and fatty-acid-derived aliphatic compounds with various chain lengths predominantly containing one or no double bonds. The unsaturated fatty-acid-derived MP components were hypothesized to be produced by fatty acid desaturases (FADs) that exhibit diverse substrate specificities. To address this hypothesis, we isolated and functionally characterized FADs from three bumblebee species: *Bombus lucorum*, *Bombus terrestris*, and *Bombus lapidarius*. By employing RNA sequencing of the male labial glands and fat bodies of *B. lucorum* and *B. terrestris*, we identified five paralogous FAD-like sequences but only two FAD lineages were abundant and differentially expressed in the labial glands. We found that abundant FAD lineages were also expressed in the labial gland and fat body of *Bombus lapidarius*. Functional characterization of FADs in a yeast expression system confirmed that Δ4-FADs exhibited a unique Δ4-desaturase activity exclusively on 14-carbon fatty acyls and Δ9-FADs displayed Δ9-desaturase activity on 14- to 18-carbon fatty acyls. These results indicate that Δ9-FADs are involved in the biosynthesis of major unsaturated components of MPs in *B. lucorum* and *B. lapidarius* despite the diverse MP composition of these bumblebee species. The contribution of lipases, acyltransferases, esterases, and fatty acid reductases to production of the species-specific MP composition is also discussed in light of the transcriptomic data obtained in this study.

1. Introduction

In the majority of bumblebee species (Hymenoptera: Apidae: *Bombus*), males exhibit the unique premating behavior of marking prominent objects with pheromones to attract a conspecific female (reviewed by Goulson, 2010). This so-called marking pheromone (MP) is a secretion of the cephalic part of the male labial gland (LG) (Kullenberg et al., 1973; Bergman and Bergstrom, 1997). MPs consist of a mixture of terpenoids or fatty-acid-derived (FA-derived) compounds; their composition is species-specific and can be a valuable taxonomic tool in discriminating bumblebee species and subspecies (e.g., Bertsch et al., 2005; Rasmont et al., 2005; Coppée et al., 2008). Lanne et al. (1987) hypothesized that the biosynthesis of unsaturated FA-derived components of MPs is analogous to the biosynthesis of lepidopteran sex pheromones and that it involves mainly Δ9-fatty acid desaturases (FADs), which introduce a double bond at the Δ9 position of fatty acyl carbon chains. To test this hypothesis, Δ9-FAD from *Bombus lucorum* was cloned and functionally characterized; however, this enzyme was proposed to be involved in primary metabolism rather than MP biosynthesis (Matoušková et al., 2008).

FADs are ubiquitous membrane enzymes localized in the endoplasmic reticulum of all eukaryotic organisms. They play a crucial role in the maintenance of cell membrane structures *via* desaturation of fatty acids in membrane lipids (reviewed by Los and Murata, 1998). The FAD gene family presumably expanded in insects before the divergence of the lepidopteran and dipteran lineages (Roelofs and Rooney, 2003). Many lepidopteran species possess FAD's gene orthologs but extensive functional

Abbreviations: MP, marking pheromone; FAD, fatty acid desaturase; FA, fatty acid; RPKM, reads per kilobase of exon model per million mapped reads; DMDS, dimethyl disulfide; GC/MS, gas chromatography – mass spectrometry; ORF, open reading frame; LG, labial gland; FB, fat body; FAEE, fatty acid ethyl ester; FAR, fatty acid reductase; TM, trans-membrane; qPCR, real-time quantitative PCR; Me, methylester; Et, ethyl ester; MP, marking pheromone.

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characterization of almost 50 lepidopteran FADs indicated that FADs displaying Δ9 function are conserved across this insect order and diversification in sequence and function proceeds preferentially in Δ11 subfamily, which is not present in other insect classes (Knipple et al., 2002; Roelofs and Rooney, 2003; Liénard et al., 2008). Particularly in moths, this subfamily of FADs (further referred to as Δ11-FADs-like subfamily) evolved a broad range of specificities toward: (1) fatty acid chain length, (2) position of the introduced double bond, and (3) double bond configuration. This range enables production of the species-specific FA-derived sex pheromones.

In other insect classes, FADs have received much less attention. To date, very few nonlepidopteran FADs involved in sex pheromone production have been identified; such FADs have been reported in the fruit fly (Diptera) (Dallerac et al., 2000) and the housefly (Diptera) (Eigenheer et al., 2002). Δ9-FADs involved in primary metabolism from the red flour beetle (Coleoptera) (Horne et al., 2010) and the house cricket (Orthoptera) (Riddervold et al., 2002) have been cloned and functionally characterized. We previously cloned and functionally characterized the first hymenopteran Δ9-FAD from the bumblebee *B. lucorum* (Matoušková et al., 2008).

Our current study focuses on three bumblebee species that produce substantially different MPs. The MP of *B. lucorum* (*Bombus s.s.*) predominantly consists of C14-FA-derived ethyl esters (ethyl tetradec-9-enoate) (Bergström et al., 1973; Urbanová et al., 2001). In contrast, the MP of *Bombus terrestris* (*Bombus s.s.*) contains mainly terpenoid compounds (Kullenberg et al., 1970; Šobotník et al., 2008) but no unsaturated FA-derived compounds. In *Bombus lapidarius*, which is representative of the related subgenus *Melanobombus*, the MP is almost exclusively composed of C16-FA-derived alcohols (hexadecanol and hexadec-9-enol) (Kullenberg et al., 1970; Luxová et al., 2003).

In the present work, we describe the isolation, functional characterization, and transcript quantification of two FADs with Δ9- and Δ4-desaturase activity, respectively, from *B. lucorum*, *B. terrestris*, and *B. lapidarius.*

2. Material and methods

2.1. Tissue collection and total RNA isolation

Bombus lucorum, *Bombus terrestris*, and *Bombus lapidarius* males (0–5 days old) were obtained from laboratory colonies established as described by Šobotník et al. (2008). Carefully dissected LGs and fat bodies (FBs) were stored in TRIzol (Invitrogen) at −80 °C prior to RNA isolation. Total RNA was isolated using TRIzol according to the manufacturer's instructions. Genomic DNA contaminants were digested with TURBO DNase (Ambion) at 37 °C for 1 h. The RNA quantity was assessed by NanoDrop ND-100 UV/Vis spectrophotometer (Thermo Scientific) and RNA integrity was confirmed using Agilent 2100 Bioanalyzer (Agilent Technologies).

2.2. RNA-seq data generation, assembly, and annotation

RNA-seq was performed with dissected LGs and FBs from both *Bombus lucorum* and *Bombus terrestris*, using 5 μg total RNA isolated from each sample. RNA-seq was outsourced to Fasteris (www.fasteris.com) and was performed using the HiSeqTM 2000 Sequencing System from Illumina (www.illumina.com), multiplexing the four indexed samples in one lane and utilizing single read 100 bp technology. CLC Genomics Workbench (Version 5.0.1) was used for sequence backbone assembly of the resulting 35 Mio sequence reads for both *Bombus lucorum* tissues and 56 Mio sequence reads for both *Bombus terrestris* tissues. First, sequences were trimmed for length and quality with standard settings. Subsequently, they were assembled using the following CLC parameters: nucleotide mismatch cost = 2; insertion/deletion costs = 2; length fraction = 0.3; similarity = 0.9; and any conflict among the individual bases was resolved by voting for the base with highest frequency. Contigs shorter than 250 bp were removed from the final analysis, resulting in a final *de novo* reference assembly (backbone) of 36,162 contigs for *Bombus lucorum* and 38,564 contigs for *Bombus terrestris*. The digital gene expression analysis was performed with QSeq Software (DNAStar Inc.), utilizing the respective mapping tools. Each Illumina sequence was mapped to the obtained reference backbone sequences, which were then used to estimate expression levels and fold-change differences between tissues. The correction for biases in the sequence datasets and different transcript sizes was addressed by using the RPKM (reads per kilobase of exon model per million mapped reads) algorithm (Mortazavi et al., 2008). Due to the lack of a reference genome, exon model information was replaced with contigs derived from the respective transcriptome assemblies (reads per kilobase of contig per million mapped reads). Homology searches (BLASTx and BLASTn) of unique sequences and functional annotation by gene ontology terms (GO; www.geneontology.org), InterPro terms (InterProScan, EBI), enzyme classification codes (EC), and metabolic pathways (KEGG, Kyoto Encyclopedia of Genes and Genomes) were determined using the BLAST2GO software suite v2.4.1 (www.blast2go.de).

2.3. Real-time quantitative PCR

Total RNA (0.25 μg) extracted from LGs and FBs of three-day-old *B. lucorum* and *B. terrestris* males (three biological replicates) served as templates for cDNA synthesis using SuperScript III Reverse Transcriptase (Invitrogen) and random hexamer primers according to the manufacturer's instructions. Real-time quantitative PCR (qPCR) was performed using a LightCycler 480 Real-Time PCR System (Roche). PCR reactions were carried out using Dynamo HS SYBR Green qPCR Master Mix (Finnzymes), 0.625 μM of each primer, and 1 μl of cDNA template. Ten-fold dilutions of cDNA mixture were analyzed to calculate the reaction efficiency for each primer pair. All samples were examined in two technical replicates, and data were exported from the LightCycler 480 SW 1.5 into Microsoft Excel and analyzed using GenEx software (www.multid.se). Relative gene expression was normalized to phospholipase A2 (PLA2) and elongation factor 1α (EEF1A) (Horňáková et al., 2010). qPCR primers for SPVE and NPVE FADs were designed using Primer3 (Rozen and Skaletsky, 2000). Detailed parameters of qPCR analysis and qPCR primer sequences are described in Supplementary Materials and Methods.

2.4. Sequence analysis

The nucleotide sequences of predicted FADs were aligned using the Muscle algorithm (Edgar, 2004) and trimmed manually. Desaturase topology was predicted using the freely available web-based program TMHMM 2.0 (Krogh et al., 2001). The phylogenetic trees were reconstructed with MEGA5 software (Tamura et al., 2011) using the neighbor-joining method, Jones-Taylor-Thorton (JTT) substitution model (Jones et al., 1992) and 1000 bootstrap replicates as a measure of statistical reliability. The publicly available genetic sequence database GenBank (www.ncbi.nlm.nih.gov/genbank) was searched using the BLAST algorithm and was used to retrieve the FADs sequences. Complete and partial coding sequences of FADs reported in this work were deposited in GenBank under accession numbers KC437326–KC437333.

2.5. Cloning of FADs and heterologous expression in S. cerevisiae

The central fragments of Δ9-Blap and Δ9-Bter were isolated using homology-probing PCR according to the procedure described by Matoušková et al. (2008). Briefly, cDNA libraries prepared from total RNA extracted from *B. lapidarius* and *B. terrestris* LGs and FBs were screened using degenerate primers designed against the conserved histidine-rich motifs of membrane FADs. The 3′ and 5′ ends were obtained by rapid amplification of cDNA-ends (RACE) PCR and the composed full-length sequences were verified by sequencing. The SPVE_Blap coding sequence from *B. lapidarius* was obtained using specific primers designed against conserved 3′ and 5′ noncoding regions of Δ4-Bter and Δ4-Bluc obtained from RNA-seq (Table S1).

To construct expression vectors, the open reading frames (ORFs) were amplified from LG cDNA libraries using specific expression primers (Table S1) and ligated in-frame with an N-terminal hexahistidine tag into the pYEXTHS-BN vector under control of the CUP1 promoter (Holz et al., 2002). The resulting expression vectors were verified by sequencing. Desaturase- and elongase-deficient *Saccharomyces cerevisiae* cells (MAT**a** *elo1*:*HIS3 ole1*:*LEU2 ade2 his3 leu2 ura3*) (Schneiter et al., 2000) were transformed with expression vectors or an empty pYEXTHS-BN vector (control) using the *S.c.* EasyComp transformation kit (Invitrogen) according to the manufacturer's instructions. Transformed colonies were selected on YNB agar plates lacking uracil (YNB-U: 0.67% yeast nitrogen base without amino acids, 2% glucose, 2% agarose, supplemented with Brent supplement mix without uracil (ForMedium) according to the manufacturer's instructions) and inoculated into 20 ml of YNB-U liquid medium supplemented with 0.5 mM $CuSO_4$ for induction of FAD expression. When indicated, methyl myristate, methyl laurate, palmitoleic acid, or oleic acid were added to a final concentration of 0.3 mM, together with 1% tergitol as a solubilizer. The yeast cells were cultivated at 30 °C for 3 days until the late stationary growth phase was reached. To evaluate heterologous protein expression, yeast cells were harvested by centrifugation (3 min, 3000 g, room temperature), resuspended in lysis buffer (50 mM Tris, pH 6.8, containing 1.5% β-mercaptoethanol, 2% SDS, 10% glycerol, and bromophenol blue) and sonicated. Cellular extracts corresponding to 100 μl of yeast culture were separated by SDS–PAGE (12% gel). His-tagged FADs were detected by Western blot using anti-poly-histidine peroxidase-conjugated antibodies (1:2000) (Sigma–Aldrich) and West Femto chemiluminescent substrate (Thermo Scientific) according to the manufacturer's instructions.

2.6. FAME preparation, DMDS derivatization, and GC/MS analysis

S. cerevisiae cells were harvested by centrifugation (3 min, 3000 g, room temperature) and washed with 0.1% tergitol solution and water. The cell pellet was lyophilized and extracted by shaking with 1 ml of a dichloromethane:methanol solution (2:1) and 0.3 g of glass beads (1 h, room temperature). Fatty acid methyl esters (FAMEs) were prepared from the extract of total cellular lipids using the transesterification procedure described by Matoušková et al. (2008). The resulting FAMEs were extracted with 600 μl of hexane and analyzed by gas chromatography coupled with mass spectrometric detection (GC/MS) under the conditions described below.

The double bond position in all detected unsaturated FAs was identified using dimethyl disulfide (DMDS) derivatization of FAME extracts according to the procedure described by Murata et al. (1978). The retention behavior and mass spectra were compared with those of synthetic standards of methyl myristoleate (Sigma–Aldrich), methyl palmitoleate (Sigma–Aldrich), methyl oleate (Sigma–Aldrich), a 92:8 mixture of methyl *Z*4-tetradecenoate and methyl *E*4-tetradecenoate (prepared as described below). The FAME extracts and corresponding DMDS adducts were analyzed with a 7890A gas chromatograph coupled to a 5975C mass spectrometer equipped with electron ionization (EI) and a quadruple analyzer (Agilent Technologies) using DB-5MS or DB-WAX capillary columns (both J&W Scientific; 30 m × 0.25 mm, film thickness 0.25 mm). Conditions for analysis were as follows: carrier gas: He at a flow rate of 1 ml/min; split ratio: 1:10; injection volume: 2 μl; injector temperature: 220 °C; and thermal gradient: 140 °C–245 °C at 3 °C/min, then 8 °C/min to 280 °C, and final temperature held for 5 min. The temperature program was terminated at 245 °C and held for 10 min when the DB-WAX column was used.

2.7. Synthesis of a (4Z)- and (4E)-methyl tetradec-4-enoate mixture (Z4-14:1Me/E4-14:1Me)

A 92:8 mixture of *Z*4-14:1Me and *E*4-14:1Me was synthesized by a Wittig reaction of methyl 4-oxobutanoate with decyltriphenylphosphorane, prepared from the reaction of decyltriphenylphosphonium bromide with butyllithium in tetrahydrofuran (THF). The configuration of the major double bond isomer *Z*4-14:1Me was determined from the ^{1}H NMR spectrum (doublet of triplets at 5.42 ppm, with a coupling constant of 10.8 Hz).

n-Butyllithium (1.6 M in hexane, 0.25 ml, 0.400 mmol) was added dropwise at −78 °C to a suspension of decyltriphenylphosphonium bromide (200 mg, 0.414 mmol) in dry THF (2 ml) under a nitrogen atmosphere, and the resulting deep orange mixture was stirred at −78 °C for 30 min. The cooling bath was removed, the reaction mixture was warmed to room temperature and stirred for 10 min, whereupon almost all salt dissolved. The reaction mixture was again cooled to −78 °C, and a concentrated solution of methyl 4-oxobutanoate (46 mg, 0.4 mmol) in THF (0.2 ml) was added slowly. During the addition of the aldehyde, the color of the reaction mixture discharged. The reaction mixture was stirred for 5 min, then warmed to room temperature and stirred for an additional 3 h. The reaction was quenched by addition of a half-saturated ammonium chloride solution (2 ml). Diethyl ether (5 ml) was added, and the organic layer was separated. The aqueous phase was extracted with diethyl ether (2 × 2 ml). The combined organic layers were washed with brine (5 ml) and dried over magnesium sulfate. The solvent was removed *in vacuo*. Flash column chromatography (20:1 pentane:diethyl ether) afforded an inseparable 92:8 mixture of (*4Z*)- and (*4E*)-methyl tetradec-4-enoate (41 mg, 43%) as a colorless oil. Spectroscopic data are shown in the Supplementary Materials and Methods.

3. Results

3.1. Description of predicted FAD sequences

We utilized system based on four-amino-acid (aa) signature motif (Knipple et al., 2002), i.e., GPTE, KPTE, LPQD, NPVE, and SPVE (Fig. 1) for initial analysis of FAD lineages from transcriptomic data, since little was known about FAD specificities from hymenopteran *species*. We predicted five paralogous FAD lineages in *B. lucorum* and *B. terrestris* LG and FB. The FAD orthologs from *B. terrestris* and *B. lucorum* were highly conserved, sharing over 98% aa sequence similarity. The abundances of predicted FAD transcripts were assessed according to the calculated RPKM values.

GPTE_Bluc and GPTE_Bter FAD transcripts had low abundance and were present at comparable levels in both LG and FB cDNA libraries. A partial transcript termed KPTE_Bter was sequenced in low abundance only in *B. terrestris* LG and FB libraries, but we were able to amplify a KPTE_Bluc ortholog from the *B. lucorum* cDNA

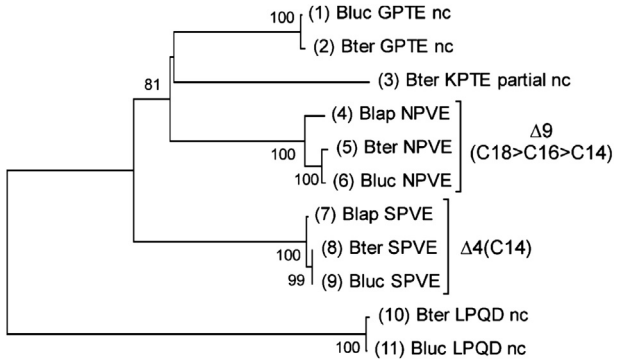

Fig. 1. Phylogenetic relationship between FAD amino acid sequences predicted from RNA-seq of LG and FB cDNA libraries from *B. lucorum* and *B. terrestris*. NPVE_Blap and SPVE_Blap sequences were obtained by PCR using *B. lapidarius* FB and LG cDNA libraries. Accession numbers are as follows: (1) KC437331, (2) KC437330, (3) KC437329, (4) CAT01313, (5) CAW34805, (6) CAM96720, (7) KC437327, (8) KC437328, (9) KC437326, (10) KC437332, and (11) KC437333. Numbers along branches indicate bootstrap percentage support from 1000 replicates. Bootstrap support values <50% are not shown. nc, not characterized.

library using PCR primers designed against KPTE_Bter. The KPTE_Bluc sequence was not obtained in RNA-seq, probably due to its low abundance in the *B. lucorum* LG. The presence of a KPTE_Bluc amplicon indicates that the KPTE lineage is not specific for *B. terrestris* (data not shown). Additional predicted partially sequenced FAD transcripts were designated LPQD_Bluc and LPQD_Bter and were significantly more abundant in the fat body than in the labial gland (data not shown). The full-length sequences of LPQD_Bter and LPQD_Bluc were obtained using PCR with gene-specific primers (data not shown). Their sequences did not contain the catalytically essential third histidine-rich motif of membrane FADs (Shanklin et al., 1994). This finding suggests that LPQD transcript might not encode functional FAD or might code for an enzyme with other than desaturase activity.

The two remaining FAD lineages, designated NPVE and SPVE, were significantly more abundant in LGs than FBs, according to RNA-seq data. The NPVE_Bluc and NPVE_Bter transcripts were 43-fold and 14-fold, respectively, more abundant in LGs than FBs of the corresponding bumblebee species. SPVE_Bluc and SPVE_Bter were 12-fold and 29-fold, respectively, more abundant in LGs than FBs. The differentially high abundance in LGs was confirmed by qPCR analysis (Fig. 2). We therefore selected NPVE and SPVE FADs, along

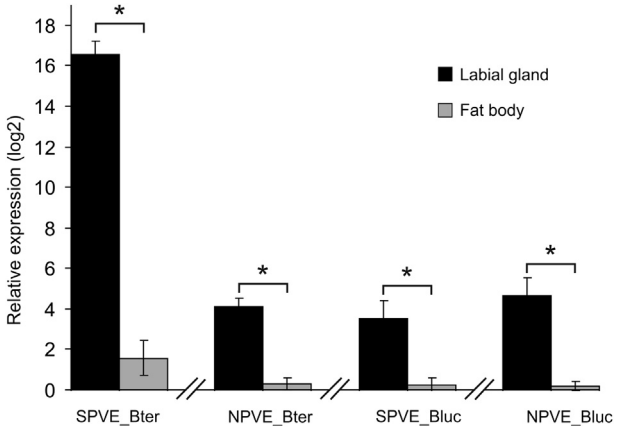

Fig. 2. Relative abundance of Bluc_SPVE, Bter_SPVE, Bluc_NPVE, and Bter_NPVE transcripts in labial glands (LG) and fat bodies (FB) quantified by qPCR. Data are shown as mean ± SD of three biological replicates. $^{*}p < 0.05$.

with the NPVE and SPVE orthologs from *B. lapidarius* obtained by PCR amplification of the respective LG cDNA library, for further characterization.

3.2. Sequence analysis of NPVE and SPVE FADs

The NPVE_Bluc sequence obtained in this study was identical to that of the previously characterized Δ9-FAD from *B. lucorum* (Matoušková et al., 2008). Both the NPVE_Blap and NPVE_Bter ORFs encode proteins with 351 aa residues. These proteins are almost identical to the sequence of Δ9-FAD from *B. lucorum*, sharing 97% and 98% aa sequence similarity, respectively. The most similar functionally characterized non-bumblebee sequence found in GenBank was a *Z*9-FAD from the house cricket *Acheta domesticus* that prefers an 18-carbon fatty acyl substrate (80% and 81% aa sequence similarity, respectively, NCBI GenBank: AAK25796, Riddervold et al., 2002).

The SPVE_Bluc and SPVE_Bter ORFs encode an identical protein with 368 aa residues, further referred to as SPVE_Bluc/Bter, which is almost identical to the 368-aa SPVE_Blap protein (99% aa similarity). The SPVE-Bluc/Bter and SPVE_Blap proteins share high sequence similarity with a *Z*9-desaturase from the housefly *Musca domestica* that produces a 1:1 ratio of *Z*9-16:1 and *Z*9-18:1 (64% and 66% aa sequence similarity, respectively, NCBI GenBank: AAN31393, Eigenheer et al., 2002) and *Z*9-FADs from *A. domesticus* (66% and 64% aa sequence similarity, respectively). Topology predictions indicate the presence of four transmembrane helices in NPVE FADs, whereas six transmembrane helices are predicted in SPVE FADs (Fig. S1).

NPVE and SPVE FAD orthologs from *B. lucorum*, *B. terrestris*, and *B. lapidarius* share high aa sequence similarities with uncharacterized hymenopteran FADs, e.g., those from the bumblebee *Bombus impatiens* (over 97% aa sequence similarity; NCBI RefSeq: XP_003492439 and XP_003492440), the leafcutter bee *Megachile rotundata* (over 85% aa sequence similarity; XP_003703958 and XP_003703934), the European honeybee *Apis mellifera* (over 86% aa sequence similarity; XP_624557 and XP_395629), and several ant species, such as the leafcutter ant *Acromyrmex echinatior* (over 80% aa sequence similarity; EGI70555 and EGI70557).

3.3. NPVE FADs display Δ9-desaturase specificity

To test the enzymatic properties of NPVE_Blap, NPVE_Bter, and NPVE_Bluc, their ORFs were cloned with an N-terminal hexahistidine tag into the pYEXTHS-BN plasmid under control of the Cu^{2+}-inducible CUP1 promoter. The desaturase- and elongase-deficient *elo1*Δ *ole1*Δ yeast strain was transformed with the resulting NPVE vectors or an empty pYEXTHS-BN vector (control). Only the strains transformed with vectors bearing NPVE ORFs grew well without addition of unsaturated fatty acids to the cultivation medium, indicating the complementation of unsaturated FA auxotrophy by the NPVE ORFs. Western blot analysis demonstrated that His-tagged NPVE FADs with a molecular weight of ~40 kDa were expressed in the NPVE transformants (Fig. 3). GC/MS analysis of transesterified lipidic extracts showed almost identical FAME profiles for all NPVE strains with dominant peaks of methyl palmitoleate (*Z*9-16:1Me) and methyl oleate (*Z*9-18:1Me), along with a minor peak of methyl myristoleate (*Z*9-14:1Me). Accordingly, the NPVE FADs were termed Δ9-FADs. As the relative amount of myristic acid (14:0) in the yeast strains accounted for less than 1%, its concentration was increased by supplementing the cultivation medium with 0.3 mM methyl myristate (Fig. 4A). The conversion rates of myristic acid, palmitic acid, and stearic acid to their corresponding Z9-unsaturated products were calculated as the ratio of relative abundance of unsaturated FAs to the total relative amount

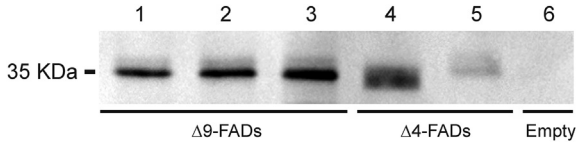

Fig. 3. Expression of Δ9-FADs and Δ4-FADs in yeast strains monitored by Western blot. Cell lysates prepared from 100 μl of yeast culture were separated by SDS–PAGE, and the histidine-tagged FADs were detected by anti-poly-histidine antibodies. Lane 1, Δ9-Bter; lane 2, Δ9-Bluc; lane 3, Δ9-Blap; lane 4, Δ4-Bter/Bluc; lane 5, Δ4-Blap; lane 6, yeast strain bearing empty plasmid (control). The molecular weight marker is shown on the left.

of saturated and unsaturated FAs. All Δ9-FADs desaturated 18:0 with the highest conversion rate (98%), followed by 16:0 (85%–88%) and 14:0 (62%–63% in Δ9-Bter and Δ9-Bluc, respectively; 47% in Δ9-Blap) (Fig. 4B).

3.4. SPVE FADs display Δ4-desaturase specificity

Because the protein sequences encoded by SPVE_Bter and SPVE_Bluc ORFs were identical, only the SPVE_Bter ORF was amplified and ligated into the pYEXTHS-BN vector, and the resulting expression vector is further referred to as the SPVE_Bter/Bluc vector. The enzymatic properties of SPVE_Bter/Bluc and SPVE_Blap FADs were tested analogously as the NPVE FAD activities and specificities. However, SPVE transformants did not grow in the YNB-U + $CuSO_4$ cultivation medium. To investigate whether the SPVE FADs possess any desaturation activity, the transformants were cultivated in the presence of a mixture of *Z*9-16:1 and *Z*9-18:1 (1:1) to maintain their growth. Western blot analysis revealed that both SPVE FADs were expressed in the yeast system, although SPVE_Blap was expressed in lower amounts than SPVE_Bter/Bluc (Fig. 3). GC/MS analysis of FAME extracts revealed trace amounts of unsaturated 14:1Me (0.01%) as a sole novel product of SPVE FADs. In order to increase its amount, the yeast cultivation medium was supplemented with methyl myristate, the presumed precursor of 14:1Me. Under these conditions, the amount of 14:1Me increased to 0.19% and 0.22% in SPVE_Bter/Bluc and SPVE_Blap transformants, respectively.

The double bond position was determined to be Δ4 using DMDS derivatization, based on the presence of characteristic fragments at *m/z* 147 and 187. Δ4-14:1Me was partially separated into two isomers with identical mass spectra using a non-polar DB5 column. The comparison with the retention behavior of synthetic *Z*4-14:1Me containing minor amounts of *E*4-14:1Me indicated that both the *Z*4- and *E*4-isomers are present in the extract from SPVE transformed strains in an approximately 5:1 ratio (Fig. 5).

To further test the specificity of Δ4-FADs toward a short chain fatty acyl substrate, the cultivation medium was supplemented with methyl laurate (12:0). However, no novel desaturation products of 12:0 were detected (data not shown), indicating that Δ4-FADs exclusively desaturate myristate.

3.5. Phylogenetic analysis

To infer the evolutionary relationship between insect FADs, we constructed a phylogenetic tree based on sequences comprising functionally characterized insect Δ9-FADs; representatives of the moth Δ11-FADs-like lineage; the bumblebee Δ9 (NPVE) and Δ4 (SPVE) FADs reported here; and their homologs from *B. impatiens*, *A. mellifera*, *M. rotundata*, and *A. echinatior* (Fig.S2). The hymenopteran FADs formed a Δ9-lineage clustering with insect Δ9-FADs, and a novel Δ4-lineage. The lepidopteran lineage of Δ9(C18 > C16)-FADs with preference for stearate and Δ9(C16 > C18)-FADs with preference for palmitate were well separated (Knipple et al., 2002; Rooney, 2009). The bumblebee Δ9- lineage was not statistically supported to cluster with the lepidopteran Δ9(C18 > C16) or Δ9(C16 > C18) lineages (Fig. S2).

4. Discussion

MP biosynthesis in *B. lucorum*, *B. terrestris*, and *B. lapidarius* was previously studied *in vivo* and *in vitro* by tracking the incorporation of labeled precursors into MP components. Enzymatic activities such as FA reduction, FA esterification, *de novo* terpenoid synthesis, triacylglycerol hydrolysis, or FA desaturation were detected in the investigated species (Luxová et al., 2003; Matoušková et al., 2008; Žáček et al., 2013). The most detailed study on the biosynthesis of FA-derived MPs was performed in *B. lucorum*, but no desaturase activity was detected during the *in vitro* experiments, thus preventing the determination of LG FAD specificities (Luxová et al., 2003; Matoušková et al., 2008; Žáček et al., 2013). Although Δ9-desaturation was observed during *in vivo* experiments after applying palmitic acid to the abdomen or head capsule of *B. lucorum* males, transport of fatty acyls occurred between the FB and LG; therefore, it could not be established whether LG or FB is the site of Δ9-desaturation (Luxová et al., 2003).

The desaturase multigene family evolution is well described in Lepidoptera. The desaturase family originated before split among Lepidoptera, Diptera, and Orthoptera (Roelofs and Rooney, 2003).

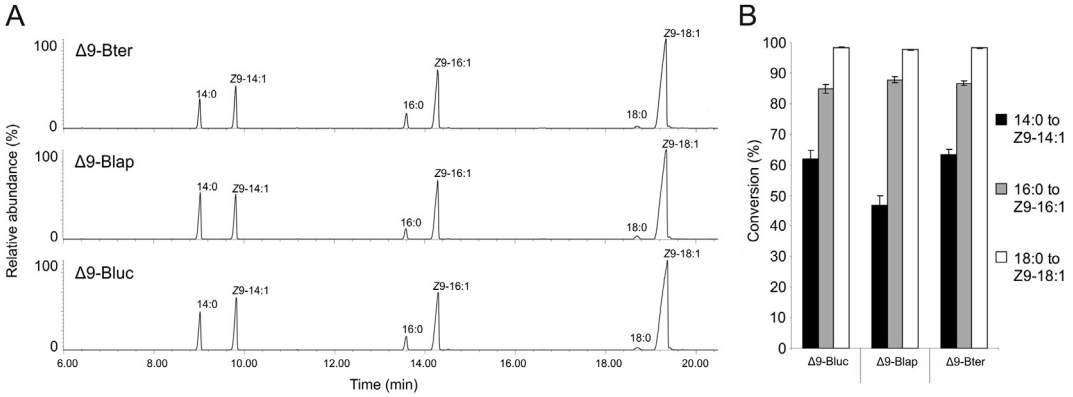

Fig. 4. GC/MS analysis of the FAME extracts of yeast strains expressing Δ9-FADs. (A) Chromatograms represent FAMEs prepared from total lipidic extracts of strains expressing Δ9-Bter, Δ9-Blap, and Δ9-Bluc using a DB-WAX column. (B) Conversion rates of myristic (14:0), palmitic (16:0), and stearic (18:0) acids to their corresponding monounsaturated fatty acids (*Z*9-14:1, *Z*9-16:1, and *Z*9-18:1, respectively) calculated from relative abundances of individual FAMEs produced in the yeast strains. Bars represent mean ± S.D. of three cultivation replicates. The cultivation medium was supplemented with 0.3 mM methyl myristate.

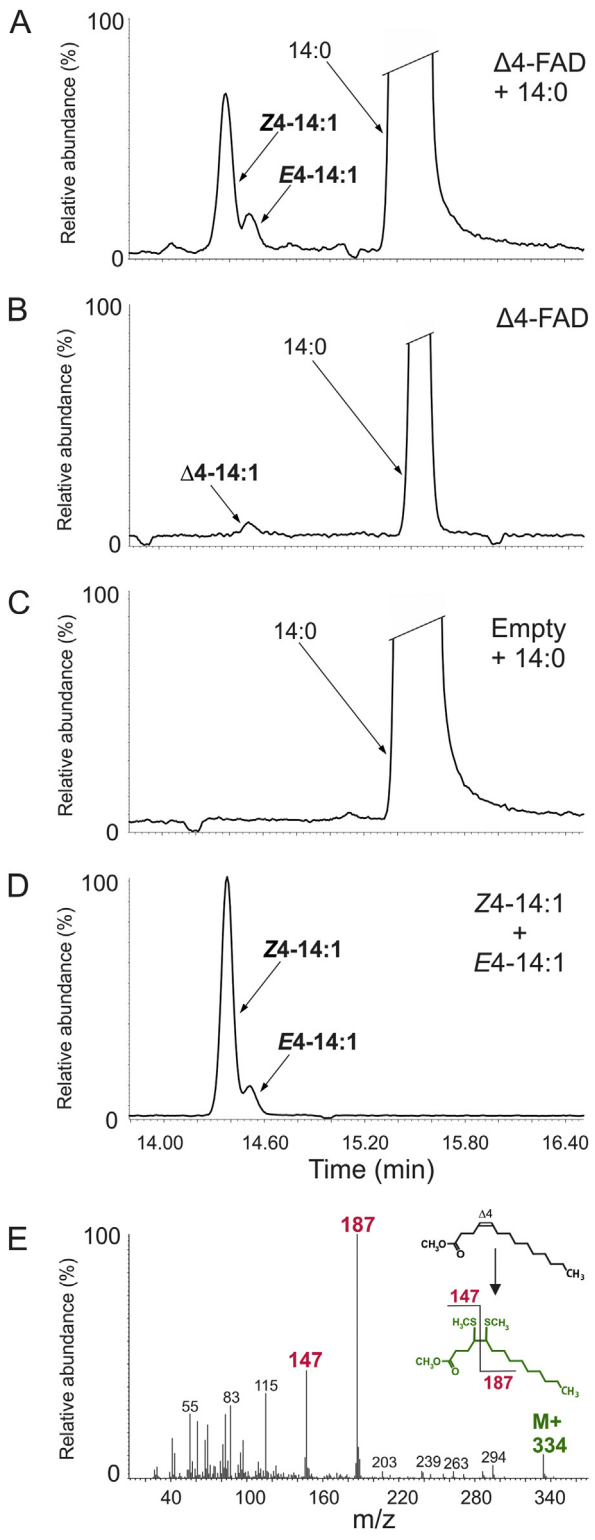

Fig. 5. GC/MS analysis of FAME extracts from yeast strains expressing Δ4-FADs. Sections of chromatograms represent analysis of FAMEs prepared from strains expressing Δ4-FADs (A and B) and a control strain transformed with an empty plasmid (C), which were cultivated in the presence (indicated as "+14:0") or absence of supplemental methyl myristate. The profile and quantity of FAMEs isolated from strains expressing Δ4-Bluc/Bter and Δ4-Blap were virtually identical, and for clarity, only the chromatogram obtained from GC/MS analysis of the Δ4-Blap strain is shown. (D) Chromatogram of a mixture of *Z*4-14:1Me and *E*4-14:1Me synthetic standards. (E) The mass spectrum of a DMDS adduct of 14:1Me present in the extract of SPVE yeast strains. Characteristic fragments and molecular ions are highlighted. A DB-5 column was used in all analyses.

This family is composed from at least five gene clusters that are correlated with desaturase functions (Roelofs and Rooney, 2003; Liénard et al., 2008; Rooney, 2009). In this study, we identified five distinct transcripts encoding putative FADs in the LG transcriptomes of *B. lucorum* and *B. terrestris*. Two FAD genes with the signature motif NPVE and SPVE were significantly more highly expressed in LGs than in FBs. We therefore selected these as candidate genes possibly involved in the biosynthesis of FA precursors of MPs.

The heterologous expression of NPVE_Bter, NPVE_Blap, and the previously characterized NPVE_Bluc (Matoušková et al., 2008) provided evidence that all NPVE FADs are Δ9-FADs. The substrate preference of Δ9-FADs was highly conserved, with 18:0 being the preferred substrate, followed by 16:0 and 14:0. Previously, we functionally characterized Δ9-Bluc in an *ole1*Δ yeast strain using an expression vector with a galactose-inducible promoter. In that expression system, 14:0 was desaturated to *Z*9-14:1 with a low conversion rate. This observation led to the hypothesis that Δ9 NPVE_Bluc participates mainly in the primary metabolism of C16 and C18 fatty acids (Matoušková et al., 2008). However, the experiments presented in this work indicated a higher conversion of 14:0, along with increased conversion rates of 16:0 and 18:0, compared with rates in the previously employed expression system. We attribute these observed differences to the use of the Cu^{2+}-induced expression of Δ9-Bluc and the use of the *elo1*Δ *ole1*Δ yeast strain, which presumably resulted in higher desaturase production and activity.

We propose that Δ9-FADs are involved in the production of MPs in *B. lucorum* and *B. lapidarius* because: (1) Δ9-FAD transcripts are highly abundant in the LG of *B. lucorum* and also present in the *B. lapidarius* LG (not quantified), and (2) in the yeast expression system, Δ9-FADs produced Δ9-14:1 and Δ9-16:1, which are presumed precursors of major MP components in *B. lucorum* and *B. lapidarius*, respectively. However, the species-specific composition of MP is apparently not determined by the specificity of FADs but rather by the selectivity of other enzymes involved in preferential transport of 14:0 in LGs or in *de novo* biosynthesis of 14:0 in LGs, as ethyl tetradec-9-enoate is the major component (53%) of *B. lucorum* MP. Additional MPs are present here in lower concentrations: ethyl dodecanoate (6%), ethyl hexadec-9-enoate (4%), ethyl tetradecanoate (2%), and ethyl octadec-9-enoate (2%) (Bergström et al., 1973; Urbanová et al., 2001). The composition of FA ethyl esters found in the LG extract correlates well with the content of free FAs, with *Z*9-14:1 being the major free FA in LGs (Matoušková et al., 2008). Given the low abundance of *Z*9-14:1 in *B. lucorum* FBs (Cvacka et al., 2006), we initially presumed that a Δ9-FAD with preference for 14:0 was expressed exclusively in the *B. lucorum* LG and was responsible for the major content of *Z*9-14:1Et in *B. lucorum* MP. Indeed, pheromone-specific FADs exhibiting a substrate preference toward 14:0 were identified in the fruit fly (Dallerac et al., 2000) and numerous moth species (e.g., Liu et al., 2002; Fujii et al., 2011). Nevertheless, our results provide evidence that the Δ9-Bluc enzyme, whose transcript is highly abundant in the LG, does not prefer 14:0 as a substrate but rather exhibits broad substrate specificity toward 18:0, 16:0, and 14:0.

Myristic acid (14:0) can be transported to LGs from FBs in the form of diacylglycerols and then cleaved by a selective enzyme (Žáček et al., 2013). Simultaneously, 14:0 can be desaturated in the LG to *Z*9-14:1 *via* abundant NPVE_Bluc, resulting in accumulation of *Z*9-14:1 and depletion of 14:0. Low content of 14:0 in the LG was indeed observed by Matoušková et al. (2008). Importantly, the proposed biosynthetic pathway would lead to the accumulation of *Z*9-14:1 without the necessity of a 14:0-specific FAD.

Our preliminary analysis of *B. lucorum* transcriptomic data supports both the "*de novo*" and "selective transport" hypotheses of

supplying the LG with a sufficient amount of 14:0 substrate for MP biosynthesis. In the transcriptomic data, we found that lipase and acyltransferase transcripts were highly abundant and differentially expressed in *B. lucorum* LGs, which could indicate an association with FA transport and storage (reviewed by Arrese et al., 2001; Yen et al., 2008; respectively). Lipase and acyltransferase are promising candidates for enzymes involved in the selective release and accumulation, respectively, of 14:0 in *B. lucorum* LGs (Table S2 and Supplementary List S1).

In *B. lucorum*, the terminal biosynthetic reaction is fatty acid ethyl esterification. The specificity of this reaction step toward a 14-carbon-long FA substrate may further contribute to the prevalence of ethyl myristoleate in *B. lucorum* MP (Matoušková et al., 2008). Recently, α/β-3 and EsLi enzymes from the honeybee (*A. mellifera*) were shown to exhibit FA ethyl ester synthase activity and to produce ethyl oleate, a honeybee prime pheromone (Castillo et al., 2012). The preliminary transcriptomic data analysis indicates the presence of homologs of α/β-3 and EsLi in *B. lucorum* LGs and FBs. However, these enzymes are not differentially expressed in the LG, and therefore they are likely not the principal enzymes of LG-localized biosynthesis of ethyl esters (data not shown).

In the *B. lapidarius* LG, the content of major free FAs correlated reasonably well with the content of volatile alcohol components of MP. The dominant free FA in LG is *Z*9-16:1 (90%), followed by minor amounts of *Z*11-18:1 (7%) and 16:0 (2%) (Fig.S3 and Supplementary Materials and Methods). Although we demonstrated that Δ9-Blap exhibits a significantly decreased preference toward 14:0 compared with Δ9-Bluc and Δ9-Bter, this shift in desaturase specificity toward longer FA substrates alone does not explain the dominant content of free *Z*9-16:1 in LGs. More likely, the composition of free FAs in the *B. lapidarius* LG suggests a specific mechanism responsible for the accumulation of *Z*9-16:1 and 16:0 in LGs, analogous to the accumulation of C14 fatty acids in the *B. lucorum* LG discussed above. The observation that C16 fatty acids are the major constituents of triacylglycerols in the FB of *B. lapidarius* and the major fatty acyl chains of MP volatiles led to the hypothesis that *Z*9-16:1 and 16:0 could be transported from the FB to the LG in the form of diacylglycerol and serve there as MP precursors (Cvacka et al., 2006). Alternatively, 16:0 could be the major transported FA, which undergoes rapid desaturation in the LG by Δ9-Blap to give rise to the specific MP composition of *B. lapidarius*.

The proposed terminal step of MP biosynthesis in *B. lapidarius* is FA reduction catalyzed by a fatty acid reductase (FAR). The strict specificity of the *B. lapidarius* FAR toward C16 fatty acids could explain the fact that *Z*11-18:1 is present as a free FA in the LG but that the corresponding alcohol is absent in the MP. An analogous mechanism of determination of pheromone blend composition was proposed for several moth FARs, which exhibit preferences for the chain length and double bond position of the sex pheromone precursor FAs (Moto et al., 2003; Lassance et al., 2013).

In *B. terrestris*, GC analysis of the LG extract coupled with electroantennographic detection indicated that Δ9-unsaturated fatty acid derivatives were not present as active components of the extract (Šobotník et al., 2008). In contrast to this previous observation, the NPVE_Bter transcript encoding Δ9-FAD is highly abundant in the *B. terrestris* LG. One possible explanation is post-transcriptional downregulation of Δ9-Bter. This mechanism was proposed to explain the presence of highly abundant yet enzymatically inactive desaturase transcripts in moth pheromone glands (Roelofs and Rooney, 2003; Park et al., 2008). Post-transcriptional downregulation might result in the low abundance of the Δ9-Bter enzyme and explain the absence of Δ9-unsaturated compounds in the *B. terrestris* LG. However, Δ9-Bter mRNA is one of the most abundant transcripts in the *B. terrestris* LG (data not shown), and further experiments will be required to explain the apparent discrepancy between high abundance of Δ9-Bter transcript and absence of Δ9-FA-derived compounds in MP of *B. terrestris*.

Identification of desaturase with Δ4-specificity was surprising because occurrence of the Δ4-monounsaturated FAs (Δ4-16:1) is limited in nature. It is present in seeds of *Umbelliferae*, *Araliaceae*, and *Garryaceae* plant species (Cahoon et al., 1992) and in the sexually deceptive orchid *Ophrys sphegodes*, in which Δ4-16:1 is a proposed intermediate of alkene biosynthesis (Schlüter et al., 2011). The unusual Δ4-specificity might be underlined by the presence of the 5th and 6th predicted transmembrane (TM) helices, which deviates from the expected membrane topology of acyl-CoA desaturases (Stukey et al., 1990; Man et al., 2006). The additional predicted helices likely represent membrane-associated regions, which might play a role in FA substrate recognition, as previously suggested by Diaz et al. (2002). Concerning the highly unusual Δ4-FAD activity, it cannot be excluded that the enzymatic activity of Δ4-FADs in bumblebee tissue differs from the Δ4-desaturase activity observed in the yeast expression system. Based on the conserved histidine-rich motifs $HX_{3-4}H$, $HX_{2-3}HH$, and $(H/Q)X_{2-3}HH$ (reviewed by Shanklin and Cahoon, 1998), the Δ4-FADs belong to the large family of non-heme iron enzymes. This gene family comprises not only enzymes that introduce double bonds into fatty acyl chains but also FA hydroxylases and sphingolipid-modifying enzymes (Shanklin and Cahoon, 1998). However, none of the previously described non-heme iron-containing enzymes was found to be homologous to Δ4-FADs (data not shown). The absence of Δ4-14:1 in the bumblebee LG might be explained by the mechanism of post-transcriptional downregulation of Δ4-FADs discussed above. To address this question, future development of FAD-specific antibodies may unequivocally establish the abundance of Δ9-FAD and Δ4-FAD enzymes across bumblebee tissues.

5. Conclusion

Next-generation sequencing of the *B. terrestris* and *B. lucorum* LG and FB transcriptomes enabled the identification of five paralogous FAD-like sequences and selection of two candidate FADs paralogs, which are differentially and abundantly expressed in the LGs and therefore presumably involved in MP biosynthesis. Functional characterization of Δ4-FADs and Δ9-FADs from *B. terrestris* and *B. lucorum*, along with their homologs from *B. lapidarius*, revealed that FAD substrate specificities and amino acid sequences are highly conserved across bumblebee species, even though the FA compositions of bumblebee MPs are diverse. Although *B. terrestris* contains Δ9-FADs and Δ4-FADs, which display *in vitro* activities, only trace amounts of desaturated FA-derived compounds are present in the pheromone blend of this bumblebee. These desaturases are likely post-transcriptionally downregulated in this species. Our data further indicate that Δ9-FADs are involved in the biosynthesis of MPs in *B. lapidarius* and *B. lucorum*; however, their substrate specificities do not regulate the species-specific composition of the MPs. Specific MP composition may instead be determined by enzymes that participate in accumulation of FAs with a particular chain length in the bumblebee LGs, e.g., by fatty acyl lipases and acyltransferases. Additionally, enzymes involved in terminal step of pheromone biosynthesis, i.e. fatty-acyl reductases in *B. lapidarius* and fatty acid esterases in *B. lucorum* might further contribute to the species specific MP composition.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2013.05.003.

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