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# Terminal fatty-acyl-CoA desaturase involved in sex pheromone biosynthesis in the winter moth (*Operophtera brumata*)

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ABSTRACT

The winter moth (*Operophtera brumata* L., Lepidoptera: Geometridae) utilizes a single hydrocarbon, 1,Z3,Z6,Z9-nonadecatetraene, as its sex pheromone. We tested the hypothesis that a fatty acid precursor, Z11,Z14,Z17,19-nonadecanoic acid, is biosynthesized from α-linolenic acid, through chain elongation by one 2-carbon unit, and subsequent methyl-terminus desaturation. Our results show that labeled α-linolenic acid is indeed incorporated into the pheromone component *in vivo*. A fatty-acyl-CoA desaturase gene that we found to be expressed in the abdominal epidermal tissue, the presumed site of biosynthesis for type II pheromones, was characterized and expressed heterologously in a yeast system. The transgenic yeast expressing this insect derived gene could convert Z11,Z14,Z17-eicosatrienoic acid into Z11,Z14,Z17,19-eicosatetraenoic acid. These results provide evidence that a terminal desaturation step is involved in the winter moth pheromone biosynthesis, prior to the decarboxylation.



## 1. Introduction

The sex pheromone of the winter moth *Operophtera brumata* (Lepidoptera, Geometridae) was identified as 1,Z3,Z6,Z9-nonadecatetraene (1,Z3,Z6,Z9-19:H) (Bestmann et al., 1982; Roelofs et al., 1982). This unusual hydrocarbon belongs to and possesses the common features of so-called type II pheromones of Lepidoptera (Millar, 2000). Type II pheromones are polyene hydrocarbons and related epoxides (and ketones in rare cases) with an odd number of carbons (C17–C25), with double bonds occurring at positions 3,6,9, or 6,9 with Z-configuration (Millar, 2000). They are biosynthetically derived from α-linolenic acid and/or linoleic acid (Millar, 2000) through one or two cycles of 2-carbon unit chain elongation, and the carboxyl group is removed by decarboxylation or decarbonylation (Rule and Roelofs, 1989; Ando et al., 2004; Jurenka, 2004).

Type II pheromone components that consist of hydrocarbons are reported to be produced in oenocyte cells and transported to the gland for release. However, if the pheromone blend comprises epoxides, the final epoxidation occurs in the pheromone gland (Schal et al., 1998a; Wei et al., 2003, 2004; Matsuoka et al., 2006). In the gypsy moth (*Lymantria dispar* L., Lymantridae), biosynthesis of the alkene precursor of its epoxide sex pheromone occurs in oenocyte cells. The alkene is subsequently transported to the pheromone gland and converted into an epoxide (Jurenka et al., 2003). In these species, the role of the pheromone biosynthesis activating neuropeptide (PBAN) is postulated to regulate the precursor uptake into the pheromone gland, rather than regulation of a biosynthetic step or the epoxidation or release of pheromone (Wei et al., 2004; Ando et al., 2008). This is in contrast with the biosynthesis of type I pheromones, which is reported to be under direct PBAN control in many species (Raina et al., 1989; Rafaeli and Jurenka, 2003; Rafaeli, 2009).

Besides chain elongation of the α-linolenic acid and the final decarboxylation reaction to convert the fatty acid to the hydrocarbon, we postulated that the winter moth pheromone biosynthesis includes a methyl-terminus desaturation, which would introduce a terminal double bond at the methyl-end of the fatty acid molecule. Desaturation of fatty acids is a common feature of sex pheromone biosynthetic pathways in the Lepidoptera. The gene family of fatty-acyl-CoA desaturases includes members encoding enzymes displaying different substrate-, stereo- and regio-specificities (Knipple et al., 2002; Roelofs and Rooney, 2003). Two desaturase subfamilies, the so-called "Δ11" and "Δ9 18C > 16C" appear to be Lepidoptera-specific and can account for most of the

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pheromone structure variation among moth species (see for instance Liénard et al., 2008 and references therein). The Δ11 clade contains not only enzymes with Δ11 functionality but also Δ10, Δ11/Δ10,12 and Δ11/Δ11,13 activities (Hao et al., 2002; Moto et al., 2004; Serra et al., 2006, 2007).

In the current study, we investigated pheromone biosynthesis in the winter moth by means of *in vivo* labeling and by heterologous expression of a candidate desaturase gene in yeast. We report on the characterization of a new member of the Lepidoptera-specific Δ11-desaturase gene family, encoding a desaturase that introduces a double bond in the methyl-end of the fatty acid molecule.

## 2. Materials and methods

### 2.1. Insect and tissue collection

Adult wingless winter moth females were obtained from Gabor Szöcs (Department of Zoology, Plant Protection Institute, Hungarian Academy of Science, Budapest, Hungary), or were collected from oak trees (Quercus ssp. L.) (55°42′55″N, 13°12′02″E) in the vicinity of Lund, in November (at night, 4 p.m. to 7 p.m.). The collected wingless female adults were kept under outdoor conditions and the females that displayed the calling behavior were regarded as unmated individuals and used in the experiments. Different body parts were dissected from virgin females and stored separately at −80 °C until RNA extraction.

### 2.2. In vivo labeling

The deuterium labeled α-linolenic acid (9,10,13,13,15,16-$D_6$-Z9,Z12,Z15-octadecatrienoic acid) was diluted in DMSO, at a concentration of 40 μg/μL, and 1μL was injected in the female abdomen, using a 10-μL-Hamilton syringe. After 24-h incubation, the pheromone gland was extracted to monitor label incorporation into the pheromone component. Females injected with a DMSO-only solution were used as controls.

GC–MS analyses were performed on a Hewlett–Packard model 5972 MSD in EI mode and interfaced with a Hewlett–Packard 5890 series II gas chromatograph equipped with a HP-INNOWax capillary column (30 m × 0.25 mm × 0.25 μm, Agilent technologies), and helium was used as carrier gas (20 cm/cm). The oven temperature was set to 80 °C for 1 min, then increased at a rate of 10 °C/min up to 210 °C, followed by a hold at 210 °C for 15 min, and then increased at a rate of 10 °C/min up to 230 °C followed by a hold at 230 °C for 20 min.

To detect incorporation of deuterium atoms from the $D_6$-linolenic acid into pheromone components, an acquisition program in selected ion monitoring (SIM) mode was used. The molecular ions at *m/z* 260 and 266 were chosen to monitor unlabeled and $D_6$-labeled 1,Z3,Z6,Z9-19:Hy respectively. The incorporation was evaluated by dividing the relative amount of $D_6$-labeled pheromone to the corresponding native compounds.

### 2.3. Total RNA extraction and cDNA synthesis

Total RNA from various body parts including head, thorax, legs, epidermis (abdominal cuticle), intestines, fat bodies, and pheromone gland were extracted with TRIzol reagent (Invitrogen AB, Lidingö, Sweden) according to the manufacturer's instructions. RNA concentration and purity were checked on a spectrophotometer (Pharmacia, Stockholm, Sweden). First strand cDNAs were synthesized from one microgram of total RNA from each tissue with the Stratagene cDNA synthesis kit and an oligo-dT primer (Stratagene, AH Diagnostics, Skärholmen, Sweden). The cDNAs quality was examined by amplifying a short fragment of both the β-actin gene and the 16S rRNA gene.

### 2.4. Characterization of desaturase-encoding cDNAs

PCR amplification was performed using cDNA from the abdominal epidermal tissue as template with a pair of degenerate primers (Px117: 5′-ACN GCN GGN GCN CAY MGN YTN TGG-3′, Px118: 5′-TGR TGR TAR TTR TGR AAN CCY TCN CC-3′) designed based on the conserved TAGAHR and GEGFH histidine-rich motifs of desaturases (Rosenfield et al., 2001), on a Veriti Thermo Cycler using AmpliTaq Gold polymerase (Applied Biosystems, Stockholm, Sweden). Cycling parameters were as follows: an initial denaturing step at 94 °C for 5 min, 35 cycles at 94 °C for 15 s, 55 °C for 30 s, 72 °C for 2 min followed by a final extension step at 72 °C for 10 min. Specific PCR products were ligated into a TOPO TA pCR 2.1 vector (Invitrogen). The ligation product was transformed into *Escherichia coli* strain Top10 (Invitrogen). Plasmid DNAs were isolated according to standard protocols and recombinant plasmids were subjected to sequencing using universal M13 primers and the BigDye terminator cycle sequencing kit v1.1 (Applied Biosystems). Sequencing products were EDTA/ethanol-precipitated, dissolved in formamide and loaded for analysis on a capillary 3130xl Genetic analyzer (Applied Biosystems). Curated consensus sequences were used for BLAST searches and revealed the presence of 3 distinct cDNA transcripts including two ubiquitous Δ9-desaturase cDNAs. Rapid amplification of 3′- and 5′ cDNA termini was conducted for the last transcript using the BD biosciences RACE kit (Clontech, In vitro Sweden AB, Stockholm) and gene-specific primers (Table 1). In order to verify its integrity and sequence information, the open reading frame (ORF) was amplified using a pair of primers (Table 1) designed spanning before the start codon and after the stop codon. All partial and full-length desaturase cDNA sequences have been deposited in the GenBank database under the accession numbers HQ917684-HQ917686.

### 2.5. Sequence and phylogenetic analyses

Comparison of the newly isolated desaturase sequences to sequence information available publicly was performed using the non-redundant database of Blastx search (Altschul et al., 1997). Transmembrane regions were predicted in the TMHMM server 2.0 (Erik et al., 1998). Prediction of the endoplasmic retention signal was performed using PSORT (http://psort.hgc.jp/form2.html). Desaturase sequences used for phylogenetic reconstructions were retrieved from the GenBank (http://www.ncbi.nlm.nih.gov) and the SilkDB (http://silkworm.genomics.org.cn/) databases. Multiple sequence alignments were run using the Clustal W algorithm (Thompson et al., 1994) and edited in BioEdit (Hall 1999). The Neighbor-Joining tree was constructed using MEGA version 4.0 (Tamura et al., 2007).

**Table 1**
Primers used for desaturase gene cloning, RT-PCR, full-length gene verification and expression.

| Primer name | Sequence[a] |
|---|---|
| 5RACE | 5′-CCCCACATGTGGGCAACGCTATTGATTA-3′ |
| 5RACE_NESTED (RT_S) | 5′-GGGCACTAGCGATGGTATGATGTATGCA-3′ |
| 3RACE | 5′-TACAAGGCCAAGCTGCCTCTGCAGATAT-3′ |
| 3RACE_NESTED (RT_AS) | 5′-GCACCATAAGTATACCGACACTGATGCG-3′ |
| Obr_LPAQ_ORF-F | 5′-CGCATAATGGCGCCCAATACTC-3′ |
| Obr_LPAQ_ORF-R | 5′-TTAAGTGTTCTTCTCGTTTGTCTC-3′ |
| Obr_LPAQ_ORF_BamHI | 5′-gcg**GGATCC**CGCATAATGGCGCCCAATACTC-3′ |
| Obr_LPAQ_ORF_EcoRI | 5′-gcg**GAATTC**TTAAGTGTTCTTCTCGTTTGTCTC-3′ |

[a] Restriction sites are indicated in bold letters, START and STOP codons are underlined.

### 2.6. Tissue specificity of candidate desaturase gene

A two-step reverse transcriptase-PCR (RT-PCR) was performed using total RNA isolated from various parts of the female body including head, thorax, legs, abdominal epidermal tissue, intestines, fat bodies, and pheromone gland. One microgram of total RNA was first reverse-transcribed into cDNA for 1 h at 42 °C using the Stratagene cDNA synthesis kit with an oligo-dT primer (Invitrogen) in a 10-μL reaction. Subsequently, 1/10 of each prepared cDNA were subjected to PCR with desaturase gene-specific primers (Table 1, RT_S, RT_AS). The following temperatures were used: 94 °C for 2 min, 35 cycles at 94 °C for 15 s, 66 °C for 30 s, and 68 °C for 30 s, followed by a final extension at 68 °C for 2 min. Amplification products were analyzed by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide, and visualized by ultraviolet illuminance.

### 2.7. Functional assay of the epidermal-specific candidate desaturase by complementation in mutant yeast using the expression vector pYEX-CHT

For the construction of a yeast expression vector containing the candidate desaturase gene (named *Obr-TerDes*), specific primers with either *Eco*RI and *Bam*HI restriction sites incorporated (Table 1) were designed for amplifying the ORF. The PCR product was double digested along with the empty pYEX-CHT yeast expression vector (Patel et al., 2003). The digested products were subjected to agarose gel electrophoresis and purified using the Wizard® SV Gel and PCR Clean up system (Promega Biotech AB, Nacka, Sweden). The ORF was subcloned into the linearized pYEX-CHT expression vector in presence of T4 DNA ligase (Promega). The ligation product was transformed into Top10 *E. coli* competent cells (Invitrogen) and recombinant constructs were analyzed by sequencing. The pYEX-CHT recombinant expression vector harboring the winter moth desaturase gene was introduced into the double deficient *ole1 elo1* strain (*MATa elo1::HIS3 ole1::LEU2 ade2 his3 leu2 ura3*) of the yeast *Saccharomyces cerevisiae*, defective in both desaturase and elongase functions (Schneiter et al., 2000) using the S.c. easy yeast transformation kit (Invitrogen). For selection of uracil and leucine prototrophs, the transformed yeast was allowed to grow on SC plate containing 0.7% YNB (w/o aa, with ammonium sulfate) and a complete drop-out medium lacking uracil and leucine (Formedium™ LTD, Norwich, England), 2% glucose, 1% tergitol (type Nonidet NP-40, Sigma–Aldrich Sweden AB, Stockholm, Sweden), 0.01% adenine (Sigma) and containing 0.5 mM oleic acid (Sigma) as extra fatty acid source. After 4 days at 30 °C, individual colonies were picked up to inoculate 10 mL selective medium at 30 °C and grown at 300 rpm for 48 h. Yeast cultures were diluted to an $OD_{600}$ of 0.4 in 10 mL fresh selective medium containing 2–4.5 mM $CuSO_4$ with supplementation of a biosynthetic precursor, i.e. the Z9,Z12,Z15-octadecatrienoic methylester (Z9,Z12,Z15-18:Me) or the Z11,Z14,Z17-eicosatrienoic methylester (Z11,Z14,Z17-20:Me) (Larodan Fine Chemicals, Malmö, Sweden). Each FAME precursor was prepared at a concentration of 100 mM in 96% ethanol and added to reach a final concentration of 0.5 mM in the culture medium (Liénard et al., 2010). Yeasts were cultured in a range of temperatures (15-30 °C) with $Cu^{2+}$-induction. After 48 h yeast cells were harvested by centrifugation at 3000 rpm and washed 3 times with sterile water. Cells were dried (at 40 °C for 30 min) and lysed by addition of 500 μL of chloroform-methanol (2:1, v/v), vortexed vigorously for a few seconds and incubated at room temperature for 1 h. After a brief centrifugation step, the organic phase was transferred into a new glass tube and the solvent was evaporated under a gentle nitrogen stream. Potassium hydroxide (0.5 M in methanol) was added and the tubes were shaken vigorously, and incubated at 40 °C for 1 h to allow fatty acid esterification with KOH present as catalyst. The reaction was stopped by addition of 500 μL HCl (0.5 M in methanol). The methylesters were extracted with hexane three times and washed with deionized water two times to remove the inorganic salt. The hexane layer was dried by adding anhydrous sodium sulfate ($Na_2SO_4$) and concentrated under a gentle nitrogen stream. The methylester samples were subjected to GC–MS analyses on a Hewlett–Packard 6890 GC coupled to a mass detector HP 5973. The GC was equipped with an INNOWax column (30 m × 0.25 × 0.25), and helium was used as carrier gas (velocity: 20 cm/s). The oven temperature was set to 80 °C for 1 min, then increased at a rate of 10 °C/min up to 210 °C, followed by a hold at 210 °C for 15 min, and then increased at a rate of 10 °C/min up to 230 °C followed by a hold at 230 °C for 20 min. Statistical analyses were carried out in Excel and MiniTab 9.0 (one-way ANOVA).

## 3. Results

### 3.1. Labeling experiments

The abdominal injection of $D_6$-linolenic acid resulted in incorporation of the label into the pheromone component, 1,Z3,Z6,Z9-19:H with an incorporation rate of 82.20% ± 18.90% ($N = 3$).

### 3.2. Screening and characterization of desaturase cDNAs

Three distinct desaturase cDNAs were cloned by using *O. brumata* epidermal cDNA in PCRs with degenerate oligonucleotide primers (Rosenfield et al., 2001) targeting the characteristic histidine-rich domains of fatty acid desaturases (Shanklin et al., 1994; Shanklin and Cahoon, 1998). Sequence analysis revealed two transcripts, named *Obr-Δ9-KPSE* and *Obr-Δ9-NPVE* to be highly homologous to sequences encoding Δ9-desaturases in other moths. Their deduced protein sequences shared about 90% identity with Δ9-desaturases that typically catalyze the formation of palmitoleic acid and oleic acid, as notably shown in the corn earworm, *Helicoverpa assulta* and the cabbage looper moth, *Trichoplusia ni* (Liu et al., 1999; Knipple et al., 2002). A full-length sequence was obtained for the third desaturase transcript only, because *in vivo* labeling did not predict pheromone biosynthesis to proceed through Δ9-desaturation, similarly to many other moths where the ubiquitous Δ9-desaturases are not required for pheromone biosynthesis (Roelofs and Rooney, 2003). The full-length cDNA for the third transcript (*Obr-LPAQ*) spanned 1196 bp and encompassed an ORF of 963 bp (320 aa residues). The *Obr-LPAQ* encoded-protein had a predicted molecular weight of 36.7 kDa and a theoretical isoelectrical point at 9.01. Its amino-acid sequence shared approximately 50% identity with other insect Δ11-desaturases and related desaturases of the same gene subfamily such as the *Ofu*-Δ11-desaturase (44%), the *Poc*-Δ10-desaturase (49%) (Hao et al., 2002), the bifunctional *Bmo*-Δ11-Δ10/12 desaturase/conjugase (46%) (Moto et al., 2004) and the multifunctional *Tpi*-Δ11-Δ11/13 desaturase (49%) (Serra et al., 2007) (Fig. 1). Phylogenetic analysis of lepidopteran desaturases further indicated that *Obr-LPAQ* clustered into the Δ11-desaturase subfamily (Fig. 2).

### 3.3. Desaturase tissue distribution

RT-PCR experiments were performed from total RNA from various tissues of female winter moths and showed the *Obr-LPAQ* transcript to be highly expressed in the abdominal epidermal tissue, as well as in the intestine tissue, whereas the transcript was barely detectable in the other tissues investigated (Fig. 3).

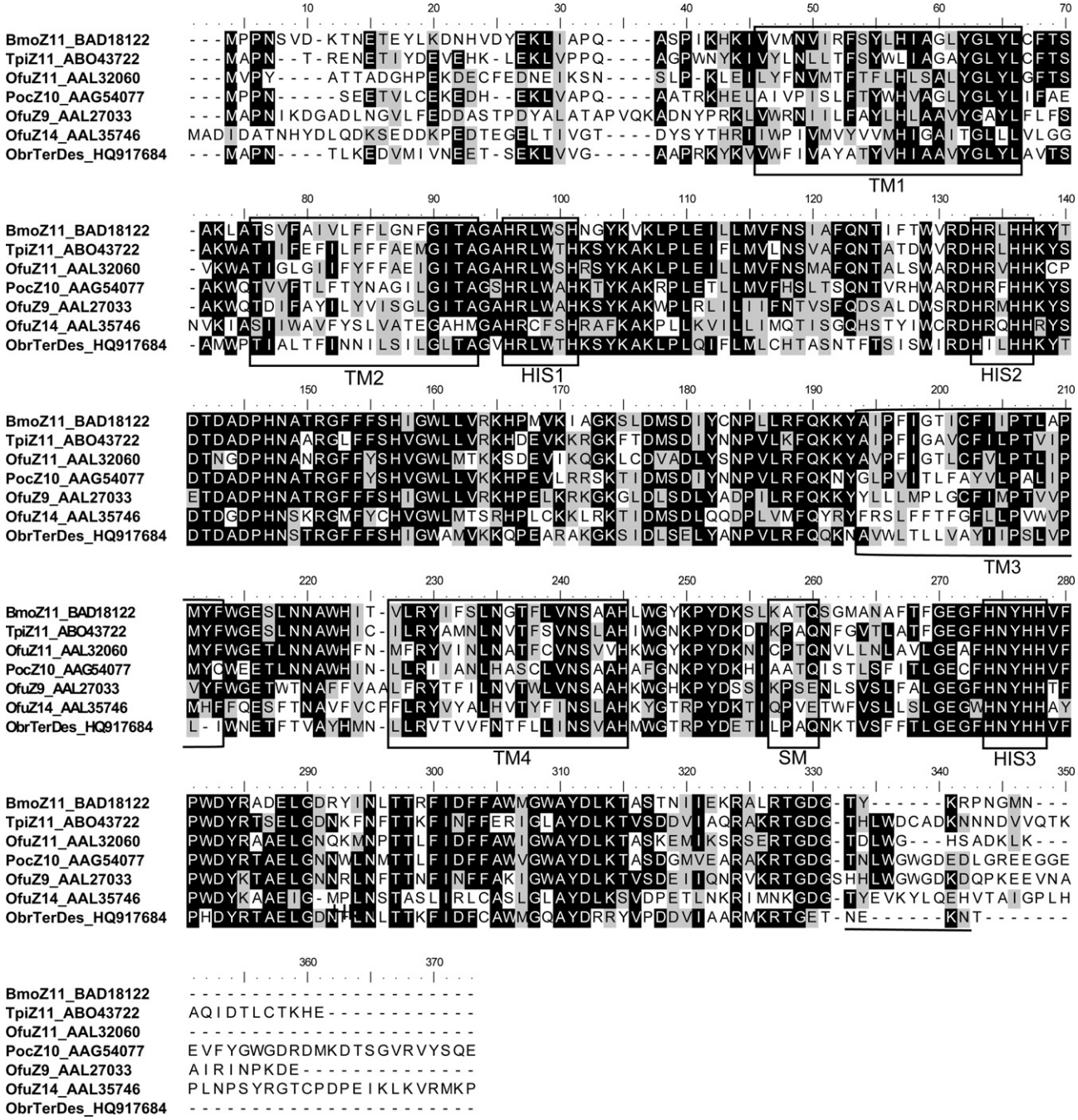


**Fig. 1.** Amino-acid sequence comparison of acyl-CoA desaturases from various lepidopteran insects. The abbreviated sequence names correspond to Bmo, *Bombyx mori*; Tpi, *Thaumetopoea pityocampa*; Ofu, *Ostrinia furnacalis*; Poc, *Planotortrix octo* and Obr, *Operophtera brumata*. Following the abbreviated species name is indicated the desaturase biochemical activity as well as the GenBank accession number. Black background indicates aa identities and gray background indicates aa similarities. Boxed regions indicate the three conserved histidine domains (HIS) of desaturases, the four predicted protein transmembrane domains (TM1 to TM4) and the desaturase signature motif (SM), respectively. The proposed ER retention signal is underlined (aa positions 316–319).

### 3.4. Functional assay of Obr-LPAQ desaturase in yeast

The *Obr-LPAQ* ORF was subcloned into the $Cu^{2+}$-inducible vector pYEX-CHT and functional expression was conducted in ole1 elo1 yeast cells. After two days of incubation under copper induction and in the presence of pheromone precursors, the Z9,Z12,Z15-18:Me or Z11,Z14,Z17-20:Me, yeast cells were harvested and subjected to fatty acid analysis. GC–MS analysis of fatty acid methylesters from yeast harboring the gene of interest and supplemented with Z9,Z12,Z15-18:Me did not result in the production of any new unsaturated biosynthetic intermediate such as Z9,Z12,Z15,17-18:Me (Fig. 4A, B). In contrast, GC–MS results indicated that yeast harboring the same genetic construct and supplemented with the Z11,Z14,Z17-20:Me converted it into the Z11,Z14,Z17,Z19-20:Me (Fig. 4C). The same substrate was not converted in control yeast carrying an empty pYEX-CHT vector, thereby demonstrating the substrate conversion to result from the action of the exogenous winter moth desaturase gene (Fig. 4C, D). The *Obr-LPAQ* encoded enzyme thus displayed a terminal desaturase activity, and we hereafter designate it as *Obr-TerDes*. Mass

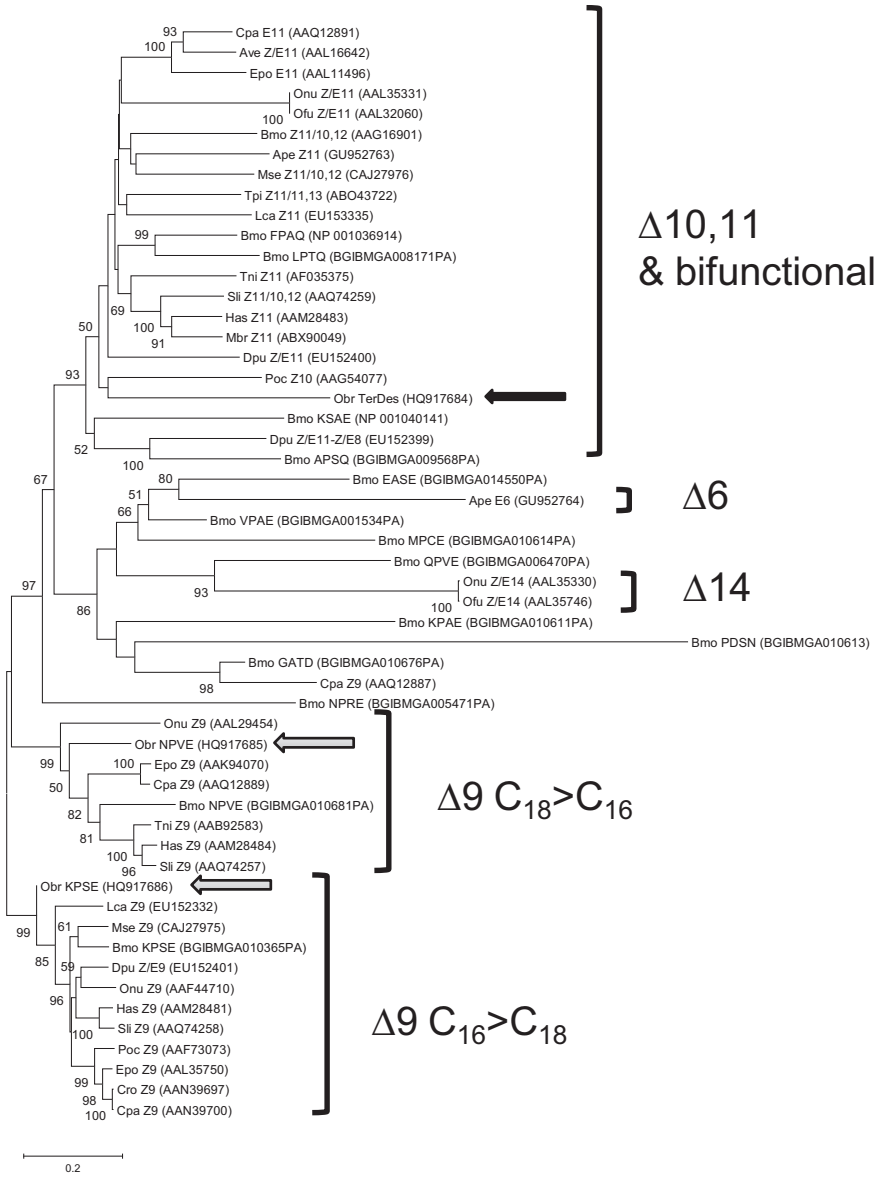


**Fig. 2.** Evolutionary tree of lepidopteran desaturase genes. The Neighbor-Joining tree was reconstructed using amino-acid sequences and the JTT algorithm (MEGA 4.0). Numbers along branches indicate bootstrap support from 1500 replicates. Only desaturases from species for which complete sequences (except the two partial Δ9-desaturases from this study (indicated by unfilled arrows) and biochemical activity (except sequences retrieved from SilkDB) have been reported were used in the tree construction. The desaturases found in *O. brumata* are indicated with black arrows (Obr_LPAQ = *Obr-TerDes*). Biochemical activities are indicated in connection to the species name, followed by accession numbers in parentheses. The tree was rooted on the Δ9-desaturase $C_{16} > C_{18}$ functional class. The abbreviated species names correspond to: Ape, *Antheraea pernyi*; Ave, *Argyrotaenia velutinana*; Bmo, *Bombyx mori*; Cpa, *Choristoneura parallela*; Cro, *Choristoneura rosaceana*; Dpu, *Dendrolimus punctatus*; Epo, *Epiphyas postvittana*; Has, *Helicoverpa assulta*; Lca, *Lampronia capitella*; Mbr, *Mamestra brassicae*; Mse, *Manduca sexta*; Obr, *Operophtera brumata*; Ofu, *Ostrinia furnacalis*; Onu, *Ostrinia nubilalis*; Poc, *Planotortrix octo*; Sli, *Spodoptera littoralis*; Tni, *Trichoplusia ni*; Tpi, *Thaumetopoea pityocampa.*

spectrometry analysis of the putative Z11,Z14,Z17,Z19-20:Me revealed this compound to possess all characteristic fragment ions demonstrating the positions of double bonds of 1,Z3,Z6,Z9-tetraenes, i.e., at *m/z* 79 (hydrocarbon ion of general formula $[C_nH_{2n-5}]^+$), a tropylium rearrangement ion at *m/z* 91 (Meyer and Harrison, 1964), an ω ion that defines an $n-3$ terminal group (Brauner et al., 1982; Fellenberg et al., 1987) at *m/z* 106 (Yamamoto et al., 2008), an α ion at *m/z* 264 that contains the carboxyl group and the first two double bonds, an ion at *m/z* 287 $[M-31/32]^+$ that corresponds to the loss of a methoxyl group, and the molecular ion at *m/z* 318 (Fig. 4E). When the *Obr-TerDes* desaturase gene was expressed under growth conditions optimal for *S. cerevisiae* (30 °C), there was a relatively low production of tetra-unsaturated fatty acid, i.e., the mean conversion rate between precursor/product was 0.7% and therefore we ran an experiment where yeast cells were cultured at different temperatures (15, 18, 24 and 30 °C) and with different $Cu^{2+}$ concentrations (2.5–4.5 mM) to see if we could optimize the production of the

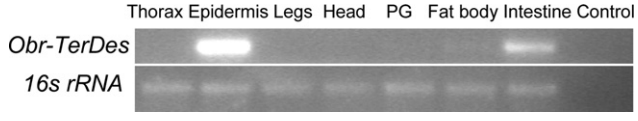


**Fig. 3.** Tissue distribution of the winter moth terminal desaturase, *Obr-TerDes* monitored by RT-PCR. *Obr-TerDes* is expressed in epidermal and intestine tissues. Amplification without template was used as negative control. Amplicon sizes: *Obr-TerDes*, 226 bp; *16s RNA*, 397 bp.

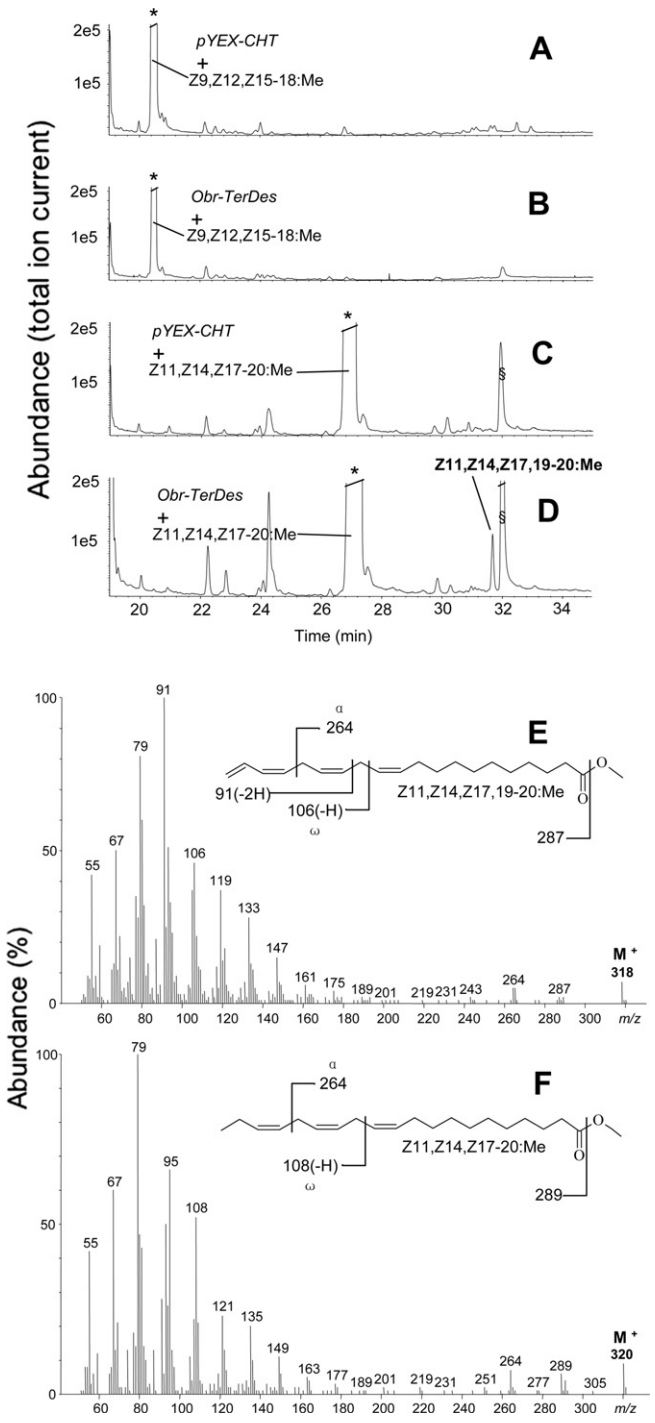


**Fig. 4.** GC–MS analyses of methanolyzed lipid extracts from $Cu^{2+}$-induced yeast transformed with the *Obr-TerDes* desaturase gene from *O. brumata* grown at 24 °C. The asterisks (*) indicate the supplemented polyunsaturated fatty acyls. The panels represent the total ion current chromatograms (TICs) from control pYEX-CHT (A and C) and pYEX-CHT-*Obr-TerDes* (B and D). In (A) and (B), yeast are supplemented with 0.5 mM Z9,Z12,Z15-18:COOH whereas in (C) and (D) they are supplemented with 0.5 mM Z11,Z14,Z17-20:Me. (E, F) Mass spectra of methyl-terminus desaturated product, Z11,Z14,Z17,19-20:Me, and the substrate of the terminal desaturase, Z11,Z14,Z17-20:Me, respectively. The § shows the free palmitic acid, the remnant of the base methanolysis reaction. This figure shows that the substrate of the *Obr-TerDes* is Z11,Z14,Z17-20:CoA, rather than Z9,Z12,Z15-18:CoA.

tetra-unsaturated acyl. When the culture temperature cooled down to 15 °C, the overall yeast growth was too slow to allow sampling. In contrast, the best substrate conversion (1.6%) was obtained at 24 °C with 2.5 mM $Cu^{2+}$ (Fig. 5).

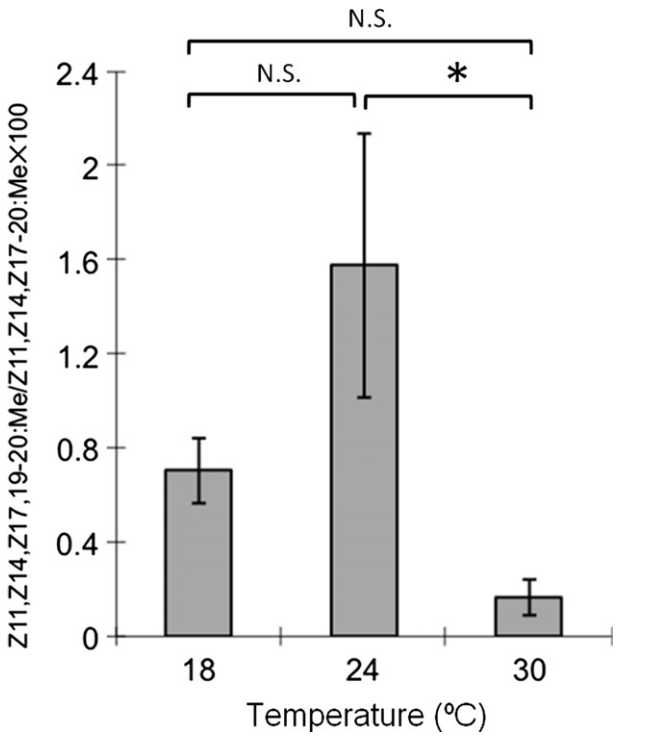


**Fig. 5.** Production of Z11,Z14,Z17,19-20:Me according to variable growth temperatures. Yeast cells expressing the pYEX-CHT-*Obr-TerDes* were incubated with 2.5 mM $Cu^{2+}$. The temperature affects the relative production of the terminal desaturated pheromone intermediate. The *Y*-axis represents the mean conversion rate precursor/product. The number of replicates for each treatment is 3. The asterisk (*) represents a significant difference and N.S. means non-significant (Fisher's test $p < 0.05$). The error bars represent the standard errors of the mean (SEM).

## 4. Discussion

Long-chain hydrocarbon compounds (C17-C22) with two to four unsaturations are widely used as sex pheromones or sex attractants in different families of moths including more than a hundred Geometrid species (Millar, 2000; Ando et al., 2004). While Z6,Z9-19:H and Z3,Z6,Z9-19:H are widely used among geometrids, 1,Z3,Z6,Z9-19:H serves as sex pheromone component only in *O. brumata* and *Operophtera bruceata* (Arn et al., 1992). Using *in vivo* labeling, we first demonstrated that the biosynthesis of 1,Z3,Z6,Z9-19:H is initiated from the diet-derived linolenic acid. Linolenic acid is also reported to serve as initial precursor for the biosynthesis of hydrocarbon pheromones in the pyralid wax moth *Galleria mellonella* (L.) (Stanley-Samuelson et al., 1987) and the arctiid moths *Estigmene acrea* (Drury) and *Phragmatobia fuliginosa* (L.) (Rule and Roelofs, 1989), as well as *Utetheisa ornatrix* (L.) (Choi et al., 2007). This supports that most polyunsaturated hydrocarbon pheromones in various moth families are likely to occur via chain elongation, desaturation and decarboxylation of linolenic acid (Ando et al., 2008; Wang et al., 2010a,b).

Subsequently, we showed that linolenic acid is elongated to eicosatrienoic acid, Z11,Z14,Z17-20:Acyl, followed by a terminal desaturation to form the direct tetraenoic fatty-acyl intermediate, Z11,Z14,Z17,Z19-20:Acyl (Fig. 6). Evidence from the terminal desaturation taking place on Z11,Z14,Z17-20:Acyl comes from our *in vitro* expression experiments, which demonstrated that the Z11,Z14,Z17-20:Acyl substrate can be used by a methyl-terminus desaturase, *Obr-TerDes* to form the immediate pheromone precursor, the Z11,Z14,Z17,19-20:Acyl (Fig. 4). Since the linolenic acid could not be used as a substrate by *Obr-TerDes*, our *in vitro*

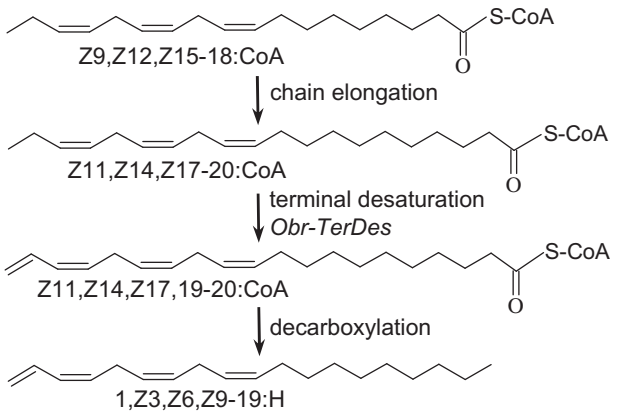


**Fig. 6.** Predicted biosynthetic pathway leading to the hydrocarbon sex pheromone of *O. brumata*. *In vivo* labeling experiments support the initial substrate to be linolenic acid whereas the desaturase gene identified in this study accounts for the methyl-terminal desaturation step (Obr-TerDes), taking place after elongation and before decarboxylation.

expression data clearly support that chain elongation takes place prior to the desaturation event, i.e., linolenic acid has to be elongated by one 2-carbon unit into Z11,Z14,Z17-20:Acyl to form the *Obr-TerDes* desaturase substrate. The Z11,Z14,Z17,19-20:Acyl immediate precursor is then most likely converted to 1,Z3,Z6,Z9-19:H by reductive decarboxylation to produce the active hydrocarbon pheromone (Fig. 6).

While moth fatty acid (type I) sex pheromones are biosynthesized in the pheromone gland through the successive action of specific pheromone gland enzymes (Jurenka et al., 2003; Tillman et al., 1999; Ando et al., 2004), the biosynthesis of long-chain hydrocarbons typically seem to occur in specialized epidermal cells called oenocytes in moths (e.g., Millar, 2000), as well as in locusts (Diehl, 1975) or flies (e.g., Ferveur et al., 1997). Evidence for oenocytes as the site of biosynthesis of long-chain monounsaturated or di-unsaturated hydrocarbons was demonstrated in *Drosophila melanogaster*, where transgenic flies lacking oenocyte cells were shown to be devoided of hydrocarbons, which supported these cells to process hydrocarbon production (Ferveur et al., 1997; Billeter et al., 2009). Gene silencing experiments of two pheromone biosynthetic desaturase genes (desat1 and desatF) recently provided further evidence that hydrocarbon biosynthesis in flies takes place directly in oenocyte cells and not in fat bodies (Wicker-Thomas et al., 2009). Evidence from the winter moth shows that the pheromone biosynthetic terminal desaturase *Obr-TerDes* is highly expressed in the epidermal tissue (Fig. 3), which supports that the site of pheromone biosynthesis may also be associated with cuticular oenocyte cells in this species. Whereas transport is likely limited in dipteran species since hydrocarbons are distributed directly onto the cuticle (Wicker-Thomas et al., 2009), ready-to-emit hydrocarbons in moths need to be transported to the pheromone gland for release or epoxidation prior to release (Schal et al., 1998a; Jurenka and Subchev, 2000; Jurenka et al., 2003; Wei et al., 2003, 2004; Matsuoka et al., 2006). Hydrocarbon transportation from oenocytes to the gland presumably occurs across the hemolymph through the action of lipophorins (Ando et al., 2008) and was notably demonstrated in *E. acrea* and *P. fuliginosa* (Rule and Roelofs, 1989), in the Holomelina tiger moth (Schal et al., 1998b) as well as in the gypsy moth (Jurenka et al., 2003).

Species-specific moth pheromone biosynthetic Δ11-desaturases have likely evolved from the metabolic acyl-CoA Δ9-desaturases (Roelofs and Rooney, 2003; Liénard et al., 2008). They catalyze the formation of uncommon unsaturated fatty-acyl-CoA esters with variable chain lengths, different locations of unsaturations, and either the ordinary Z or the unusual E double bond geometry. Altogether the latter clade contributes to the formation of a majority of unsaturated intermediates of species-specific blends of pheromones in combination with carbon-chain shortening or elongation enzymes dedicated to pheromone biosynthesis (Bjostad and Roelofs, 1984; Knipple et al., 1998, 2002; Liu et al., 1999, 2004, 2002a,b; Tillman et al., 1999; Rosenfield et al., 2001; Hao et al., 2002; Jeong et al., 2003; Moto et al., 2004; Serra et al., 2006, 2007; Matoušková et al., 2007; Liénard et al., 2008, 2010; Wang et al., 2010a,b). Our study reveals an atypical enzyme of this family of genes (Fig. 2) displaying a methyl-terminus desaturase activity (Fig. 4). Although a similar desaturation activity has been demonstrated by *in vivo* labeling studies in several other moth species (Stanley-Samuelson et al., 1988; Gehlsen et al., 2009), *Obr-TerDes* is the first methyl-terminus desaturase gene to be functionally characterized in insect pheromone biosynthesis. The pheromone biosynthesis in *O. brumata* thus involves an atypical methyl-terminus desaturation step accounted for by the *Obr-TerDes*. Interestingly, this gene not only has evolved a specific cuticular expression pattern, but it encodes a particular pheromone biosynthetic Δ11-like desaturase gene duplicate. Besides, it has acquired a novel methyl-terminus activity, but has lost the usual Δ11-function, thereby conferring *O. brumata* with a unique pheromone signal among moths.

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