Biosynthesis of the Sex Pheromone Component (E,Z)
-7,9-Dodecadienyl Acetate in the European Grapevine Moth, Lobesia botrana, Involving Δ11 Desaturation and an Elusive Δ7 Desaturase

Bao-Jian Ding 1 · Yi-Han Xia 1 · Hong-Lei Wang 1 · Fredrik Andersson 2 · Erik Hedenström 2 · Jürgen Gross 3 · Christer Löfstedt 1

Received: 11 November 2020 / Revised: 13 January 2021 / Accepted: 2 February 2021 / Published online: 29 March 2021
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Abstract
The European grapevine moth, Lobesia botrana, uses (E,Z)-7,9-dodecadienyl acetate as its major sex pheromone component. Through in vivo labeling experiments we demonstrated that the doubly unsaturated pheromone component is produced by Δ11 desaturation of tetradecanoic acid, followed by chain shortening of (Z)-11-tetradecenoic acid to (Z)-9-dodecenoic acid, and subsequently introduction of the second double bond by an unknown Δ7 desaturase, before final reduction and acetylation. By sequencing and analyzing the transcriptome of female pheromone glands of L. botrana, we obtained 41 candidate genes that may be involved in sex pheromone production, including the genes encoding 17 fatty acyl desaturases, 13 fatty acyl reductases, 1 fatty acid synthase, 3 acyl-CoA oxidases, 1 acetyl-CoA carboxylase, 4 fatty acid transport proteins and 2 acyl-CoA binding proteins. A functional assay of desaturase and acyl-CoA oxidase gene candidates in yeast and insect cell (Sf9) heterologous expression systems revealed that Lbo_PPTQ encodes a Δ11 desaturase producing (Z)-11-tetradecenoic acid from tetradecanoic acid. Further, Lbo_31670 and Lbo_49602 encode two acyl-CoA oxidases that may produce (Z)-9-dodecenoic acid by chain shortening (Z)-11-tetradecenoic acid. The gene encoding the enzyme introducing the E7 double bond into (Z)-9-dodecenoic acid remains elusive even though we assayed 17 candidate desaturases in the two heterologous systems.

Keywords In vivo labeling experiment · Pheromone gland · Transcriptome · Gene functional characterization · Acyl-CoA oxidase · Chain shortening

Introduction
The European grapevine moth, Lobesia botrana, belongs to the family Tortricidae (Lepidoptera). It feeds on grapes, causing serious yield losses as well as increasing susceptibility to fungal infections (Ioriatti et al. 2011). It is among the most economically serious pests in vineyards in Europe, as well as in Chile, Argentina and California, where L. botrana was accidentally introduced (Gonzales 2010; Varela et al. 2010; Witzgall et al. 2010). The use of sex pheromone-based strategies for pest control is considered an environmentally safe management approach. Pheromone-mediated mating disruption of L. botrana is an effective technique for grape protection and is currently used on about 140,000 ha in the European wine-growing area in the European Union (Ioriatti et al. 2011).

The sex pheromone components of L. botrana were identified in the 1970s and 1980s. The major pheromone component is (E,Z)-7,9-dodecadienyl acetate (E7,Z9–12:OAc) (Buser et al. 1974; Roelofs et al. 1973). Later, (E,Z)-7,9-dodecadienol (E7,Z9–12:OH) and (Z)-9-dodecenoic acid (Z9–12:OAc) were reported as minor pheromone components (Arm et al. 1988). More recently, (E)-7-dodeceny1 acetate, and (E,E)- and (Z,E)-isomers of 7,9,11-dodecatrienyl acetate were identified in the pheromone gland of L. botrana (Witzgall...
et al. 2005). However, to date, the pathway for biosynthesis of sex pheromone has not been investigated, and the enzymes involved in biosynthesis are unknown. Elucidation of the mechanisms of pheromone biosynthesis in *L. botrana* is not only of fundamental interest but could also provide genes necessary for biological production of grapevine moth pheromone in cell and plant factories for the use in pheromone-based pest control (Ding et al. 2014; Hagström et al. 2013; Löfstedt and Xia 2020; Xia et al. 2020).

Compared to other organisms in which fatty acyl desaturases are largely involved in normal cellular lipid metabolism, moth desaturases have evolved to perform specialized functions in the biosynthesis of sex pheromone components. Desaturases introduce double bonds in specific positions of fatty acids, and are responsible for much of pheromone diversity among different moth species. A wide range of desaturases has been characterized in various moths species, including: Δ5 desaturases that introduce double bonds into tetradecanoic acid for production of the fatty acid pheromone precursor (Z)-5-tetradecenoic acid in *C. pernyi* (Wang et al. 2010); several Δ9 desaturases (from a range of species) that introduce double bonds into saturated or unsaturated fatty acids of C12–C18 chain length (Liu et al. 2004; Liu et al. 2002; Rodriguez et al. 2004; Rosenfeld et al. 2001); a Δ10 desaturase that introduces a double bond in hexadecanoic acid to produce the pheromone precursor (Z)-10-hexadecenoic acid in *Planotortrix octo* (Hao et al. 2002); several Δ11 desaturases that produce Δ11-unsaturated fatty acids (Knipple et al. 1998); two Δ11/Δ12 desaturases in *Spodoptera exigua* and *S. litura* that introduce double bonds into both saturated and unsaturated fatty acids to produce (Z)-11-hexadecenoic acid (Z11-16:acid) and (Z,E)-9,12-tetradecadienoic acid (Xia et al. 2019); a Δ11/Δ13 multifunctional desaturase in *Thaumetopoea pityocampa* that produces Z11–16:acid, 11-hexadecenoic acid and (Z)-13-hexadecen-11-yenoic acid (Serra et al. 2007); Δ14 desaturases in *Ostrinia* species that introduce double bonds into palmitic acid to produce (Z)- and (E)-14-hexadecenoic acids (Roelofs et al. 2002); and a terminal desaturase in *Operophtera brumata* that introduces a double bond into the methyl terminus of the carbon chain of Z11,Z14,Z17-eicosatrienoic acid to produce Z11,Z14,Z17,19-eicosatetraenoic acid (Ding et al. 2011).

In addition to desaturases, moth pheromone biosynthesis involves other enzymes that contribute to structural diversity. β-Oxidases and elongases are considered to combine with desaturases to determine the basic structures of pheromone fatty acyl precursors (Löfstedt et al. 2016) but, so far, no enzymes involved in chain-shortening have been identified and characterized. Fatty-acyl reductases (FARs) with different substrate specificities are responsible for reducing fatty acyl moieties to alcohols, and have been functionally characterized in several moth species (Lassance et al. 2010; Löfstedt et al. 2016; Moto et al. 2003).

In the present study, we performed *in vivo* labeling experiments to investigate the pheromone biosynthetic pathway in *L. botrana*. We did high-throughput sequencing of the *L. botrana* pheromone gland transcriptome and identified candidate genes that might be involved in pheromone biosynthesis. Finally, we functionally characterized several of these candidate genes in yeast and Sf9 heterologous systems.

### Methods and Materials

#### Insects

Pupae of *L. botrana* were obtained from a rearing facility at the Julius Kühn Institut (JKI), Federal Research Centre for Cultivated Plants, Institute for Plant Protection in Fruit Crops and Viticulture, Siebeldingen, Germany. Larvae were reared in 500 ml polypropylene (PP) cups (Huthamaki, Alf, Germany) on a semi-synthetic diet, according to the protocol described by Markheiser et al. (2018). Male and female pupae were kept separately in a climate chamber at 23 ± 1 °C under a 17 h:7 h light: Dark photoperiod and 70% RH. After emergence, adults were fed with 10% honey solution, with two- to three-day-old virgin females being used for experiments throughout this study.

#### Chemicals

\begin{align*}
[12,12,12-\text{D}_3\text{H}_3] \text{ Dodecanoic acid (D}_3\text{-12:acid),} \\
[14,14,14-\text{D}_3\text{H}_3] \text{ Tetradecanoic acid (D}_3\text{-14:acid),} \\
[16,16,16-\text{D}_3\text{H}_3] \text{ Hexadecanoic acid (D}_3\text{-16:acid) were purchased from Larodan AB (Malmö, Sweden).} \\
(\text{Z})\text{-11-[13,13,14,14,14-\text{D}_5\text{H}_5]} \text{ Tetradecenoic acid (D}_5\text{-Z11-14:acid) was synthesized as described in Zhu et al. (1996).} \\
(\text{Z})\text{-9-[12,12,12-\text{D}_9\text{H}_9]} \text{ Dodecanoic acid (D}_9\text{-Z9-12:acid) was synthesized as described in Supplementary File 1.} 
\end{align*}

Other fatty acid and pheromone standards were available in our laboratory and were of various origin.

#### Labeling Experiments and Sample Preparation

The deuterium-labeled potential precursor acids D3-16:acid, D3-14:acid, D3-12:acid, D5-Z11-14:acid, and D9-Z9-12:acid were dissolved separately in dimethylsulphoxide (DMSO) at 40 μg/μl. About 1 h into scotophase, 0.4 μl of a solution of a potential precursor was applied topically to the pheromone gland of females in a group. The same volume of DMSO was applied as a control to females in another group. After 1 h incubation, pheromone glands were excised and five glands pooled in a 250 μl insert (in a 1.5 ml glass vial) to
which 20 µl n-heptane was added. After extracting glands for 30 min at room temperature, the solvent, which contained pheromone components, was transferred into a new vial. The remaining lipids in the residue were subsequently extracted with 100 µl chloroform/methanol (2:1 v:v) at room temperature overnight. After extraction, base methanolysis was conducted as described in Bjostad and Roelofs (1984).

Gas Chromatography/Mass Spectrometry (GC/MS)

Pheromone gland extracts and methylated fatty acyl compounds were analyzed using a Hewlett-Packard (HP) 5975 mass selective detector coupled to an HP 6890 series gas chromatograph, equipped with a polar column (HP-INNOWax, 30 m × 0.25 mm, 0.25 µm film thickness) or an Agilent 5975C mass selective detector coupled to an Agilent 7890A series gas chromatograph equipped with a non-polar column (HP-5MS, 30 m × 0.25 mm, 0.25 µm film thickness). Helium was used as carrier gas (average velocity: 33 cm sec⁻¹). The injector was configured in splitless mode at 250 °C.

For analysis of pheromone gland extracts, the column oven temperature was set at 80 °C for 1 min, then increased to 190 °C at 10 °C min⁻¹, held for 10 min, and finally increased to 230 °C at 4 °C min⁻¹, then held for 10 min. Incorporation of deuterium label into the pheromone components was detected by selected ion monitoring (SIM) mode (Table 1).

For fatty acid methyl esters (FAMEs), the oven temperature was set at 80 °C for 1 min, then increased to 230 °C at 10 °C min⁻¹ and held for 10 min. Incorporation of deuterium label into fatty acid methyl esters was detected in the SIM mode, as described in Table 2. Full scans, from m/z 30–400, were for mass spectra. Compounds were identified by comparison of retention times and mass spectra with corresponding standards.

RNA Isolation, cDNA Library Construction and Illumina Sequencing

Approximately 50 pheromone glands of two- to three-day-old virgin females of L. botrana were collected for transcriptome sequencing. Total RNA of the glands was extracted using the TRIzol® reagent (Invitrogen). As control tissue, 25 abdominal tips from males were collected and treated the same way. We used Agilent 2100 Bioanalyzer system to check the RNA integrity and quantitation. Total RNA was sent to Novogene (Hong Kong) for Illumina sequencing.

De Novo Assembly and Bioinformatics Analysis

Transcriptome assembly was accomplished using Trinity (Grabherr et al. 2011) to assemble the clean reads de novo. Gene function was annotated based on the databases of Nr (NCBI non-redundant protein sequences), Nt (NCBI non-redundant nucleotide sequences), Pfam (Protein family), KOG/COG (Clusters of Orthologous Groups of proteins), Swiss-Prot (a manually annotated and reviewed protein sequence database), KO (KEGG Ortholog database) and GO (Gene Ontology).

Quantification of Gene Expression Level

Gene expression levels were estimated by RSEM (Li and Dewey 2011) for each sample: clean data were mapped back onto the assembled transcriptome and read count for each gene was obtained from the mapping results. Gene Ontology (GO) enrichment analysis of the differentially expressed genes (DEGs) was implemented by the GOSeq R packages based on Wallenius non-central hyper-geometric distribution (Young et al. 2010), which can adjust for gene length bias in DEGs. They were converted into values of FPKM (expected number of Fragments Per Kilobase of transcript sequence per Millions base pairs-sequenced in RNA-seq). FPKM is the most common method of estimating gene expression levels, which considers the effects of both sequencing depth and gene length on counting of fragments (Van Verk et al. 2013).

Phylogenetic Analysis

Local BLAST was performed using Geneious software. Desaturase sequences used for phylogenetic reconstructions were retrieved from the GenBank (http://www.ncbi.nlm.nih.gov) database. MAFFT alignment (Katoh and Standley 2013; Katoh et al. 2002) with scoring matrix Blosum62 was performed in Geneious. Phylogenetic analysis was performed in IQtree 2.0-rc2 (http://www.iqtree.org; last accessed: Nov-11,

| Table 1 Selected ion monitoring (SIM) mode used for detecting incorporation of deuterium label into the pheromone components |
|---------------------------------------------------------------|
| Compound | SIM (m/z) | Compound | SIM (m/z) | Compound | SIM (m/z) |
| Z9−12:OAc1 | 166, 61 | E7,Z9−12:OAc | 164, 224, 61 | E7,Z9−12:OH | 164, 182, 31 |
| D2,Z9−12:OAc | 169, 61 | D2−E7,Z9−12:OAc | 167, 227, 61 | D2−E7,Z9−12:OH | 167, 185, 31 |
| D3,Z9−12:OAc | 171, 61 | D3−E7,Z9−12:OAc | 169, 229, 61 | D3−E7,Z9−12:OH | 169, 187, 31 |

1 Compound acronyms refer to geometry across double bond, position of unsaturation, carbon chain length, functionality and total number of D labels; e.g., D2−Z9−12:OAc = (Z)-9-dodecenyl acetate with five D atoms.
Table 2  Selected ion monitoring (SIM) mode used for detecting incorporation of deuterium label into the fatty acyl methyl ester (FAME) pheromone precursors

| Saturated FAME  | SIM (m/z) | Mono-unsaturated FAME  | SIM (m/z) | Di-unsaturated FAME  | SIM (m/z) |
|----------------|-----------|------------------------|-----------|----------------------|-----------|
| 16:Me         | 270       | Z11-14:Me              | 166, 240  | E7,Z9–12:Me          | 136, 210  |
| D1–16:Me      | 273       | D9-Z11–14:Me           | 169, 243  | D9,E7,Z9–12:Me       | 139, 213  |
| 14:Me         | 242       | D7-Z11–14:Me           | 171, 245  | D7,E7,Z9–12:Me       | 141, 215  |
| D1–14:Me      | 245       | Z9–12:Me               | 138, 212  | –                    | –         |
| 12:Me         | 214       | D5-Z9–12:Me            | 141, 215  | –                    | –         |
| D1–12:Me      | 217       | D5-Z9–12:Me            | 143, 217  | –                    | –         |

FAME acronyms refer to geometry across double bonds, position of unsaturation, carbon chain length, esterification and total number of D labels; e.g., D9,E7,Z9–12:Me refers to methyl (E,Z)-7,9-dodecadienoate with five D atoms.

Functional Assay in Sf9 Cells

The expression construct for Lbo_ACOs in the BEVS donor vector pDEST8 was made by LR reaction. Recombinant bacmids were made according to instructions for the Bac-to-Bac™ system given by the manufacturer Invitrogen using DH10EMEmBacY (Geneva Biotech). Baculovirus generation was done using Sf9 cells (Invitrogen), Ex-Cell 420 medium (Sigma) and baculoFECTIN II (OET). Virus was then amplified once to generate a P2 virus stock using Sf9 cells and Ex-Cell 420 medium. The virus titer in the P2 stock was determined using the BaculoQUANT all-in-one qPCR kit (OET). Insect cells lines Sf9 were diluted to 2 × 10^6 cells/ml. Expression was done in 20 ml cultures in Ex-Cell 420 media, at virus additions (MOI 1). The cultures were incubated in 125 ml Erlenmeyer flasks (100 rpm, 27 °C), with Z11–14:Me supplemented at a concentration of 0.25 mM on the second day. On the fourth day of expression, 7.5 ml samples were taken from the culture and centrifuged for 15 min at 4500 x g at 4 °C. The pellets were stored at –80 °C until fatty acid analysis. Aliquots were also taken for visualization in the fluorescence microscope of YFP expression from the virus backbone.
Fatty Acid Analysis of Yeast and Sf9 Cells

Total lipids were extracted using 3.75 ml of methanol/chloroform (2:1, v/v) in a glass tube. One ml of HAc (0.15 M) and 1.25 ml of water were added to the tube to wash the chloroform phase. Tubes were vortexed vigorously and centrifuged at 2000 rpm for 2 min. The bottom chloroform phase (ca. 1 ml), containing the total lipids, was transferred to a new glass tube. FAMEs were made from this total lipid extract. The solvent was evaporated to dryness under gentle nitrogen flow. One milliliter of sulfuric acid, 2% in methanol, was added to the tube, vortexed vigorously, and incubated at 90 °C for 1 h. After incubation, 1 ml of water was added, mixed well, and then 1 ml of n-hexane used to extract FAMEs. FAME samples were subjected to GC/MS analysis on an Agilent 8890 GC/Agilent 7693MS.

Results

Biosynthetic Pathway

Fatty acyl moieties identified from gland extracts are shown in Fig. 1. Relatively high amounts of monounsaturated Z11–14:acid, Z9–12:acid and doubly unsaturated E7,Z9–12:acid were found, together with small amounts of several other unsaturated fatty acids, including (E)-9-dodecenoic acid (E9–12:acid), (Z)-5-tetradecenoic acid (Z5–14:acid), (Z)-7-tetradecenoic acid (Z7–14:acid), (Z)-9-tetradecenoic acid (Z9–14:acid), (E)-11-tetradecenoic acid (E11–14:acid), (Z)-7-hexadecenoic acid (Z7–16:acid) and (Z,E)-7,9-dodecadienoic acid (Z7,E9–12:acid). No doubly unsaturated C14 or C16 fatty acids were detected (Fig. 1).

Label from D3–16:acid was incorporated into 14:acid, 12:acid, Z11–14:acid, and E7,Z9–12:acid (Fig. 2a), as well as the major pheromone compound E7,Z9–12:OAc (Fig. 2b). Similarly, label from D3–14:acid was incorporated into 12:acid, Z11–14:acid, E7,Z9–12:acid (Fig. 2a), and the pheromone compounds E7,Z9–12:OAc and Z9–12:OAc (Fig. 2b). Label from D3–Z11–14:acid was incorporated into Z9–12:acid and E7,Z9–12:acid (Fig. 2a), and the corresponding acetates (Fig. 2b). Label from D3–Z9–12:acid was incorporated into E7,Z9–12:acid (Fig. 2a), E7,Z9–12:OAc and Z9–12:OAc (Fig. 2b), as well as the corresponding alcohol E7,Z9–12:OH (Fig. 3). Label incorporation into acyl precursors was generally low in this study, except from D3–Z11–14:acid into (chain-shortened) D5–Z9–12:acid (Fig. 2a). Compared to other labeling results, the incorporation rates from D3–Z9–12:acid into the doubly unsaturated acetate and alcohol were remarkably high (Fig. 3). However, when D3–12:acid was applied, no incorporation of label was detected in any of the abovementioned compounds (Fig. 2).

Transcriptome Assembly

A total of more than 78 million raw reads were generated by Illumina HiSeq™ 2500 from the pheromone glands of L. botrana, resulting in about 75 million clean reads after clustering and redundancy filtering of the raw reads. Data were deposited in NCBI database under accession code PRJNA663283. The clean reads were assembled into 75,207 unigenes with a mean length of 1247 bp and the N50 length of 2061 bp (Table 3). BUSCO completeness for the assembled transcripts was 96% (Simao et al. 2015).

Gene Ontology (GO) Annotation

The 75,207 unigenes were classified into different functional groups using BLAST2GO for Gene Ontology (GO) annotation. Based on sequence homology, 29,065 unigenes (38.64%) could be annotated. After GO annotation, the successfully annotated genes were grouped into three main GO domains: Biological Process (BP), Cellular Component (CC), Molecular Function (MF). One unigene could be annotated...
tetradecanoic acid (D3-14:acid), [14,14,14-2H3] tetradecanoic acid (D3-16:acid), (Z)–11-[13,13,14,14,14-2H5] tetradeconoic acid (D3-12:acid) and (Z)–9-[12,12,12-2H3] dodecenoic acid (D3-12:acid) were indicated by arrows in comparison with that from a DMSO solvent control. a. Ions at m/z 242 and 214 were used to monitor the native methyl tetradecanoate (14:Me) and dodecanoate (12:Me), respectively. Ions at m/z 245 and 217 monitored three deuterium atoms incorporated into 14:Me and 12:Me, respectively. Ions at m/z 166, 138, 136 monitored native methyl (Z)-11-tetradeconoate (Z11–14:Me), methyl (Z)-9-dodecenoate (Z9–12:Me), methyl (E,Z)-7,9-dodecadienoate (E7,Z9–12:Me), respectively. Ions at m/z 169, 141 and 139 monitored three deuterium atoms incorporated into Z11–14:Me, Z9–12:Me and E7,Z9–12:Me, respectively. Ions at m/z 143 and 141 monitored five deuterium atoms incorporated into Z9–12:Me and E7,Z9–12:Me, respectively. b. Ions at m/z 166 and 164 monitored native (Z)-9-dodecenyacetate (Z9–12:OAc) and (E,Z)-7,9-dodecadienyl acetate (E7,Z9–12:OAc), respectively. Ions at m/z 169 and 167 monitored three deuterium atoms incorporated into Z9–12:OAc and E7,Z9–12:OAc. Ions at m/z 171 and 169 monitored five deuterium atoms incorporated into Z9–12:OAc and E7,Z9–12:OAc. The deuterium-labeled compounds elute earlier than unlabeled compounds because of isotope effects (Matucha et al. 1991).

**Phylogenetic Analysis**

By searching the transcriptome data using desaturase His 1, 3 family motifs and the cytochrome b5 domain (Marquardt et al. 2000; Napier et al. 1999), we found 17 full-length desaturase-like genes. We next performed phylogenetic reconstructions with the 17 desaturase-like genes identified in *L. botrana*. Five genes fall into the front-end/cytochrome-b5-related clade (Fig. S2). These were subsequently treated separately due to their low similarity with first desaturases. Our analyses indicate that three of the *L. botrana* first desaturase candidates cluster into

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**Fig. 2** Incorporation of deuterium labels into fatty acyl precursors and pheromone components in *Lobesia botrana*. Representative chromatograms obtained on INNOWax column. Fatty acyl precursors were analyzed as corresponding methyl esters. Label incorporation from [16,16,16-2H3] hexadecanoic acid (D3–16:acid), [14,14,14-2H3] tetradecanoic acid (D3–14:acid), (Z)–11-[13,13,14,14,14-2H5] tetradeconoic acid (D3–12:acid) and (Z)–9-[12,12,12-2H3] dodecenoic acid (D3–12:acid) were indicated by arrows in comparison with that from a DMSO solvent control. a. Ions at m/z 242 and 214 were used to monitor the native methyl tetradecanoate (14:Me) and dodecanoate (12:Me), respectively. Ions at m/z 245 and 217 monitored three deuterium atoms incorporated into 14:Me and 12:Me, respectively. Ions at m/z 166, 138, 136 monitored native methyl (Z)-11-tetradeconoate (Z11–14:Me), methyl (Z)-9-dodecenoate (Z9–12:Me), methyl (E,Z)-7,9-dodecadienoate (E7,Z9–12:Me), respectively. Ions at m/z 169, 141 and 139 monitored three deuterium atoms incorporated into Z11–14:Me, Z9–12:Me and E7,Z9–12:Me, respectively. Ions at m/z 143 and 141 monitored five deuterium atoms incorporated into Z9–12:Me and E7,Z9–12:Me, respectively. Ions at m/z 166 and 164 monitored native (Z)-9-dodecenyacetate (Z9–12:OAc) and (E,Z)-7,9-dodecadienyl acetate (E7,Z9–12:OAc), respectively. Ions at m/z 169 and 167 monitored three deuterium atoms incorporated into Z9–12:OAc and E7,Z9–12:OAc. Ions at m/z 171 and 169 monitored five deuterium atoms incorporated into Z9–12:OAc and E7,Z9–12:OAc. The deuterium-labeled compounds elute earlier than unlabeled compounds because of isotope effects (Matucha et al. 1991).

\[ \text{Sex Pheromone Biosynthesis Gene Candidates} \]

By homology search, 41 genes putatively related to sex pheromone biosynthesis were obtained, including candidate genes encoding 17 desaturases, 13 FARs, 1 fatty acid synthase (FAS), 3 acyl-CoA oxidases (ACOs), 1 acetyl-CoA carboxylase (ACC), 4 fatty acid transport proteins (FATPs) and 2 acetyl-CoA binding proteins (ACBPs) (Table 4).

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**Phylogenetic Analysis**

By searching the transcriptome data using desaturase His 1, 3 family motifs and the cytochrome b5 domain (Marquardt et al. 2000; Napier et al. 1999), we found 17 full-length desaturase-like genes. We next performed phylogenetic reconstructions with the 17 desaturase-like genes identified in *L. botrana*. Five genes fall into the front-end/cytochrome-b5-related clade (Fig. S2). These were subsequently treated separately due to their low similarity with first desaturases. Our analyses indicate that three of the *L. botrana* first desaturase candidates cluster into
the Δ9 desaturases clade, seven fall into the clade of Δ11, Δ10 and bifunctional desaturases, and the last two did not cluster into any functionally-characterized desaturase clade (Fig. 5).

**Functional Assay of Desaturase Candidates**

We heterologously expressed all the desaturase candidates in our Δole1/elo1 yeast system. In the first round of experiments, we fed the yeast with 14:Me as substrate; the yeast naturally produces a high amount of saturated C16 fatty acid precursor. Lbo_KPSE, Lbo_NPVE and Lbo_GATD produced Z9–14:acid and Z9–16:acid. Lbo_PPTQ produced Z11–14:acid, Lbo_LPGQ produced Z11–16:acid and (Z)-11-octadecenoic acid (Z11–18:acid). Lbo_TPSQ showed Δ12 desaturation activity, producing (Z)-12-tetradecenoic acid (Z12–14:acid), (Z)-12-hexadecenoic acid (Z12–16:acid), and (Z)-12-octadecenoic acid (Z12–18:acid). We assigned the name “group A” for these 6 desaturases (Fig. 6a). For those not showing any activity in this round, the name “group B” was assigned. In the second round of experiments, we fed the yeast expressing each desaturase candidate with Z9–12:Me, but none of the desaturases showed any evidence of Δ7 desaturation (Fig. 6b). The chromatogram is from Lbo_PPTQ, but is representative of all the desaturases from Group A and Group B. We further supplemented group B desaturases with E9–14:Me and Z11–14:Me, but did not see any doubly unsaturated product (E9,Z11–14:Me). We also expressed all the group B desaturases in the Sf9 system (Fig. 7). Thus, in the third round of experiments, all the “group B” desaturase were fed with 14:Me to test if there were any activity at all in the Sf9 cells, since they are not active in the yeast system. Figure 7c is a chromatogram from Lbo_SPTQ fed with 14:Me, which is representative of all the candidates from “group B”, with all resulting in similar chromatograms.

**Acyl-CoA Oxidase (ACO)**

We found three full-length acyl-CoA oxidases (Table 4) in the *L. botrana* transcriptome and functionally expressed the two

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**Table 3** Distribution of Unigenes size in *Lobesia botrana* female glands transcriptome assembly

| Transcript length interval | 200–500 bp | 500–1kbp | 1 k–2kbp | >2kbp | Total |
|---------------------------|------------|----------|----------|-------|-------|
| Number of transcripts    | 25,735     | 19,473   | 16,075   | 13,940| 75,223|
most highly expressed ones, i.e., ACO_31670 and ACO_49602 (Table 5) in the Sf9 expression system (Fig. 8). We found a significant peak of Z9–12:Me in the chromatograms of cells expressing ACO_31670 and ACO_49602 when Z11–14:Me was added, but only a tiny peak in cells harboring the empty virus (two replicates). Hypothesizing that Lbo_PPTQ introduces the first double bond in tetradeconoic acid and that the second double bond is introduced by another desaturase immediately after chain shortening of the Z11–14:acid intermediate, we also co-expressed Lbo_PPTQ, ACO_31670, ACO_49602 and all “group B” desaturases in Sf9 cells in a separate experiment. We did not find any trace of methyl E7,Z9–12:Me (data not shown).

**Discussion**

In the present study, we investigated the sex pheromone bio-synthetic pathways in *L. botrana*. As shown in Fig. 9, our *in vivo* labeling experiment proved that the sex pheromone is biosynthesized from chain-shortening of 16:acid to 14:acid, followed by Δ11 desaturation to produce Z11–14:acid, which is further chain-shortened to Z9–12:acid. Subsequently, an unusual Δ7 desaturation occurs on Z9–12:acid to produce the precursor, E7,Z9–12:acid, which undergoes further reduction and acetylation to the corresponding alcohol and acetate.

Three geometrid moths, *Idaea aversata*, *I. straminata* and *I. biselata*, use different 7,9-dodecadienyl acetates as pheromone components, with (Z,Z)-9,11-tetradecadienyl acetate acting synergistically in field trapping of *I. aversata* (Ando et al. 1987; Biwer et al. 1975; Szöcs et al. 1987; Zhu et al. 1996). When D3–16:acid was applied to pheromone glands of *I. aversata*, label was incorporated into both Z9,Z11–14:OAc and Z7,Z9–12:OAc suggesting that, in this case, the dodecadienyl precursor is a chain-shortening product of the longer C14 homolog (Zhu et al. 1996). However, there is no evidence for a similar pathway in *L. botrana* because no (E,Z)-9,11-tetradecadienoic acid, neither native compound nor deuterium-labeled, was found in the pheromone gland after application of D3–Z11–14:acid. The incorporation of deuterium label from D3–Z11–14:acid into Z9–12:acid indicated chain-shortening of the monounsaturated tetradeccenyl precursor to produce the dodecenyl intermediate (Fig. 2a). Trace-level label incorporation was found from D3–16:acid and D3–14:acid into E7,Z9–12:acid but not into Z9–12:acid, possibly because the amount of labeled monounsaturated intermediate was below the detection limit, or the Δ7 desaturase was highly active converting all the Z9–12:acid.

The subsequent functional assays of candidate genes in *Aole1/elo1* yeast and Sf9 expression systems demonstrated that Lbo_PPTQ is a Δ11 desaturase working on 14:acid to produce Z11–14:acid, which is consistent with the results of the labeling experiments. In addition, our functional assays of all the other desaturase gene candidates confirmed that Lbo_KPSE, Lbo_NPVE, and Lbo_GATD are Δ9 desaturases, as suggested by phylogenetic analysis, and Lbo_LPGQ is a Δ11 desaturase forming predominantly Z11–18:acid and Z11–16:acid from 18:acid and 16:acid, respectively. Lbo_TPSQ is a Δ12 desaturase working on 14:acid, 16:acid and 18:acid. Although these showed desaturase activity in the functional assays, there was no evidence that these five Δ9, Δ11 and Δ12 desaturases are...
Table 4  Transcripts identified as putative pheromone biosynthesis genes in the pheromone gland of *Lobesia botrana*

| Name          | Gene       | Length (aa) | Best Blastx Match                                                                 |
|---------------|------------|-------------|-----------------------------------------------------------------------------------|
| **Desaturase (DES)** |            |             |                                                                                   |
| Lbo_NPVE     | Cluster-6761.35600 | 350         | delta 9 desaturase                                                                |
| (A)           |            |             | AIM40222.1 [Cydia pomonella] 0.0 87                                               |
| Lbo_GATD     | Cluster-6761.9745 | 384         | desaturase                                                                        |
| (A)           |            |             | AIM40222.1 [Cydia pomonella] 0.0 87                                               |
| Lbo_PPTQ     | Cluster-6761.26702 | 337         | acyl-CoA delta (11) desaturase like                                              |
| (A)           |            |             | AER29851.1 [Ctenopseustis herana] 1.00E-169 69                                    |
| Lbo_TPSQ     | Cluster-6761.53556 | 346         | acyl-CoA delta (11) desaturase like                                              |
| (A)           |            |             | XP_01319513-2.1 [Amyelois transitella] 0.0 95                                       |
| Lbo_LPGQ     | Cluster-6761.38020 | 350         | desaturase                                                                        |
| (A)           |            |             | AIM40218.1 [Cydia pomonella] 2.00E-179 84                                         |
| Lbo_KPSE     | Cluster-6761.53650 | 374         | desaturase                                                                        |
| (A)           | g1_i1      |             | AIM40223.1 [Cydia pomonella] 0.0 86                                               |
| Lbo_KSTE     | Cluster-6761.50373 | 352         | delta 9 desaturase                                                                |
| (B)           |            |             | AIM40221.1 [Cydia pomonella] 0.0 92                                               |
| Lbo_SATQ     | Cluster-6761.3834 | 345         | terminal desaturase                                                               |
| (B)           |            |             | AER29851.1 [Ctenopseustis herana] 0.0 70                                           |
| Lbo_SPTQ     | Cluster-6761.40238 | 360         | acyl-CoA delta (11) desaturase like                                              |
| (B)           |            |             | XP_01319513-2.1 [Amyelois transitella] 0.0 96                                       |
| Lbo_RAWE1    | Cluster-6761.25528 | 330         | fatty acyl desaturase                                                             |
| (B)           |            |             | AHW98359.1 [Grapholitha molesta] 0.0 92                                           |
| Lbo_RAWE2    | Cluster-6761.28035 | 305         | fatty acyl desaturase                                                             |
| (B)           |            |             | AHW98358.1 [Grapholitha molesta] 1.00E-180 72                                       |
| Lbo_44,979   | Cluster-6761.44979 | 337         | desaturase                                                                        |
| (B)           |            |             | AIM40218.1 [Cydia pomonella] 0.0 84                                               |
| Lbo_21731    | Cluster-6761.21731 | 448         | desaturase                                                                        |
| (B)           |            |             | AIM40226.1 [Cydia pomonella] 0.0 73                                               |
| Lbo_44136    | Cluster-6761.44136 | 451         | desaturase                                                                        |
| (B)           |            |             | AIM40226.1 [Cydia pomonella] 0.0 77                                               |
| Lbo_36936    | Cluster-6761.36936 | 447         | desaturase                                                                        |
| (B)           |            |             | AIM40226.1 [Cydia pomonella] 0.0 66                                               |
| Lbo_28799    | Cluster-6761.28799 | 462         | cytochrome b5-related protein                                                     |
| (B)           |            |             | XP_00492400-8.1 [Bombyx mori] 0.0 65                                              |
| Lbo_29833    | Cluster-6761.29833 | 459         | cytochrome b5-related protein-like                                               |
| (B)           |            |             | XP_02815852-8.1 [Ostrinia furnacalis] 0.0 65                                          |
| **Fatty-acyl reductase (FAR)** |            |             |                                                                                   |
| Lbo_FAR_11462| Cluster-11,426.0 | 508         | putative fatty acyl-CoA reductase CG5065                                         |
|               |            |             | KPI96398.1 [Papilio xuthus] 0.0 69                                               |
| Lbo_FAR_11487| Cluster-6761.11487 | 519         | putative fatty-acyl CoA reductase CG5065                                        |
|               |            |             | XP_01436632-2.1 [Papilio machaon] 0.0 68                                           |
| Lbo_FAR_17149| Cluster-6761.17149 | 600         | fatty-acyl reductase 5                                                           |
|               |            |             | ATJ44463.1 [Helicoverpa armigera] 0.0 74                                           |
| Lbo_FAR_22678| Cluster-6761.22678 | 530         | putative fatty-acyl CoA reductase 5                                              |
|               |            |             | ALJ30239.1 [Spodoptera litura] 2.00E-171 47                                        |
| Lbo_FAR_22999| Cluster-6761.22999 | 546         | fatty-acyl CoA reductase 2                                                      |
|               |            |             | ADI82775.1 [Ostrinia nubilalis] 0.0 76                                            |
| Lbo_FAR_30064| Cluster-6761.30064 | 510         | putative fatty acyl-CoA reductase CG8306                                         |
|               |            |             | XP_02282419-4.1 [Spodoptera litura] 0.0 73                                          |
| Lbo_FAR_33934| Cluster-6761.33934 | 523         | fatty-acyl CoA reductase 4                                                    |
|               |            |             | AKD01782.1 [Helicoverpa assalata] 0.0 64                                           |
| Lbo_FAR_34479| Cluster-6761.34479 | 515         | putative fatty-acyl CoA reductase CG5065 iso-form X1                             |
|               |            |             | XP_01155696-3.1 [Plutella xylostella] 0.0 73                                         |
|               |            |             | Cluster-6761.37174 | putative fatty acyl-CoA reductase CG5065                                |
actually involved in pheromone biosynthesis in *L. botrana* based on the labeling experiments. Furthermore, no desaturase showed Δ7 desaturation activity on Z9–12:Me in our functional assays; E7,Z9–12:acid was not detected in any desaturase-transformed yeast when Z9–12:Me was added. Conjugated double bonds are formed in different ways in different moth species. For instance, many cloned and functionally characterized desaturases in moths are bi- or multi-functional Δ11 desaturases, preferably using 16:acid as substrate (Matoušková et al. 2007; Moto et al. 2004; Serra et al. 2006; Xia et al. 2019). In the codling moth, *Cydia pomonella*, (E,E)-8,10-dodecadienol (E8,E10–12:OH) is biosynthesized by a bifunctional E9 desaturase working on 12:acid (Löfstedt and Bengtsson 1988).

The aliphatic carbon chain length in moth pheromone compounds is adjusted by limited β-oxidation (Jurenka et al. 1994), which is considered to be performed by four enzymes, an acyl-CoA oxidase, an enoyl-CoA hydratase, a L-3-hydroxyacyl-CoA dehydrogenase, and a thiolase, as discussed.
Fig. 5 Phylogenetic tree of desaturases identified from Lobesia botrana and other Lepidoptera species. The maximum likelihood tree of selected desaturase genes constructed using amino-acid sequences.

The L. botrana desaturases are indicated by solid dot, with expression level indicated as FPKM_PG (pheromone gland)_FPKM_MAT (male abdominal tip)
in Ding and Löfstedt (2015), with several candidate genes suggested in *Agrotis segetum* in the same study. The first step of this β-oxidation is catalyzed by an acyl-CoA oxidase with different specificities (Osumi and Hashimoto 1978). As mentioned above, our labeling experiments demonstrated that, in the *L. botrana* pheromone gland, a β-oxidation enzyme was involved in producing the biosynthetic intermediate Z9–12:acid through chain shortening of Z11–14:acid. We found three full-length acyl-CoA oxidase (ACO) gene candidates from the transcriptome data, and heterologously expressed the two ACOs with the highest expression levels in the Sf9 system. The results showed that both Lbo_31670 and Lbo_49602 could chain-shorten Z11–14:acid to Z9–12:acid, but no shorter chain-length acids were found (Fig. 8).
Studies of pheromone biosynthetic pathways have demonstrated that chain-shortening is a crucial step in pheromone biosynthesis in many moth species (Bjostad and Roelofs 1983; Bjostad et al. 1987; Jurenka et al. 1994; Jurenka 1997; Xia et al. 2019; Wolf and Roelofs 1983; Wu et al. 1998). Differences in chain-shortening result in the production of different sex pheromone component ratios in two strains of the cabbage looper, *Trichoplusia ni*, i.e., the normal *T. ni* preferentially chain-shortened Z11–16:acid through two cycles of β-oxidation to Z7–12:acid, whereas mutant strain females had a reduced ability to chain-shorten (Jurenka et al. 1994). In the turnip moth, *Agrotis segetum*, successive β-oxidations starting from Z11–16:acid produced Z9–14:acid, Z7–12:acid, and Z5–10:acid. These three acids were then reduced and acetylated into the pheromone components Z9–14:OAc, Z7–12:OAc and Z5–10:OAc (Löfstedt et al. 1986). Differences in chain-shortening activity account for different ratios of these pheromone components in Swedish (12:59:29) and Zimbabwean (78:20:2) populations of *A. segetum* (Wu et al. 1998).

Identification of the genes encoding limited β-oxidation enzymes should help us understand the molecular control of chain shortening. The most important step here is the first, with the acyl-CoA oxidase catalyzing the formation of a double bond between the second and third carbon. However, this functionality had not previously been characterized in any moth species in the context of pheromone biosynthesis. This is the first study to report functional ACO genes involved in pheromone biosynthesis. We suggest that the subsequent three reactions are performed by the Sf9 cell machinery and most likely by a protein with three functions and less specific to substrate chain length (Hashimoto 1996). To date, no acetyltransferase gene has been characterized in the context of moth sex pheromone biosynthesis. By homology, searching in *L. botrana*, we failed to find any novel candidate genes to test for this activity other than the ones from *A. segetum* that had previously been tested with negative results (Ding and Löfstedt 2015).

In conclusion, we reveal the biosynthetic pathway for the pheromone of the European grapevine moth, *L. botrana*, including evidence that an unusual Δ7 desaturation activity is involved. We found six functional desaturase genes, of which Lbo_PPTQ exhibits high Δ11 desaturase activity on
tetradecanoic acid in sex pheromone biosynthesis in *L. botrana*. We also confirmed that Lbo_31670 and Lbo_49602 are key genes involved in chain shortening of Z11–14:acid for production of the biosynthetic intermediate Z9–12:acid, which sheds light on the enzymes involved in β-oxidation in pheromone biosynthesis in Lepidoptera. The molecular mechanism for introduction of the second double bond remains enigmatic. Possible explanations for our failure to identify and characterize the enzyme responsible for this reaction include the A7 desaturase not having the ‘normal’ motifs of other desaturases preventing its identification from the transcriptome or that our heterologous expression systems do not possess the necessary components to allow the successful functional expression of this desaturase.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s10886-021-01252-3.

**Acknowledgments** Thanks to LP3 in Department of Biology, Lund University for the technical support for S9 expression. The phylogenetics in this paper was enabled by resources provided by the Swedish National Infrastructure for Computing (SNIC) at Raackham hosted at UPPMAX partially funded by the Swedish Research Council through grant agreement no. 2018-05973. Thanks to Sonja Anslinger and Christoph Hoffmann (JKE, Germany) for rearing *Lobesia botrana* and to Jean-Marc Lassance for advice. Thanks to the Chinese Scholarship Council for supporting Yihan Xia’s PhD scholarship. This project has received funding from the European Union’s Horizon 2020 research and innovation program under grant agreement No. 760798 (OLEFINE), and the Swedish Foundation for Strategic Research (grant No. RBP 14–1909-2) for supporting Yihan Xia’s PhD scholarship. This project has received funding from the European Union’s Horizon 2020 research and innovation program under grant agreement No. 760798 (OLEFINE), and the Swedish Foundation for Strategic Research (grant No. RBP 14–1909-2).

**Funding** Open access funding provided by Lund University.

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