Research Article

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Bioproduction of (*Z*,*E*)-9,12-tetradecadienyl acetate (ZETA), the major pheromone component of *Plodia*, *Ephestia*, and *Spodoptera* species in yeast

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Abstract

BACKGROUND: (*Z*,*E*)-9,12-tetradecadienyl acetate (ZETA, Z9,E12-14:OAc) is a major sex pheromone component for many stored-product moth species. This pheromone is used worldwide for mating disruption, detection, monitoring, and mass trapping in raw and processed food storage facilities. In this study, we demonstrate the biological production of ZETA pheromone by engineered yeast *Saccharomyces cerevisiae*.

RESULTS: We mined the pheromone gland transcriptome data of the almond moth, *Ephestia* (*Cadra*) *cautella* (Walker), to trace a novel E12 fatty acyl desaturase and expressed candidates heterologously in yeast and Sf9 systems. Furthermore, we demonstrated a tailor-made ZETA pheromone bioproduction in yeast through metabolic engineering using this E12 desaturase, in combination with three genes from various sources coding for a Z9 desaturase, a fatty acyl reductase, and an acetyltransferase, respectively. Electrophysiological assays (gas chromatography coupled to an electroantennographic detector) proved that the transgenic yeast-produced ZETA pheromone component elicits distinct antennal responses.

CONCLUSION: The reconstructed biosynthetic pathway in yeast efficiently produces ZETA pheromone, leaves an undetectable level of biosynthetic intermediates, and paves the way for the economically competitive high-demand ZETA pheromone's bioproduction technology for high-value storage pest control.

Supporting information may be found in the online version of this article.

Keywords: pest management; pheromone; mating disruption; (*Z*,*E*)-9,12-tetradecadienyl acetate; *Saccharomyces cerevisiae*; bioproduction of pheromone

1 INTRODUCTION

Worldwide, cosmopolitan stored product insect pests inflict uncountable damage and economic losses on stored products such as maize, wheat, and other grains, dates, chocolates, dried cocoa beans, dried fruits, beans, nuts, tobacco, coconut, bananas, and groundnuts in stores.[1] Among these pests, moths belonging to the family Pyralidae (Lepidoptera) contaminate and destroy almost all aforesaid stored food in factories and warehouses. Noticeably, the Indian meal moth, *Plodia interpunctella* (Hübner), Mediterranean flour moth (*Ephestia kuehniella* Zeller), and almond moth (tropical warehouse moth) *Ephestia* (*Cadra*) *cautella* (Walker) are highly destructive and cause a substantial increase in economic costs for pest control, quality loss, and dissatisfaction of consumers.[2,3] Larvae of all species possess glands that secrete silk with which they interlink food products as they move. A considerable amount of damage results from webbing in the grain and on the surface of bags forming large lumps, therefore food is no longer fit for consumption once infested. In the past, the control of these pests depended entirely on pesticides and fumigants, mainly using two universally available fumigants, methyl bromide and phosphine (PH_3). However, there are increasingly strict regulations on using such chemicals; methyl bromide faced a worldwide phase-out by 2015 under the terms of the Montreal Protocol.[4]

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The use of insecticides to control insect populations is increasingly difficult due to governmental regulations, off-target impact on beneficial species, harmful human health and environment, and evolution of insecticide resistance. For example, insecticide residue in fresh and storage date fruit has been a significant concern in the dates industry in Middle Eastern countries in recent years, as importing countries have imposed stringent regulations on permissible pesticide residues.[5–7] As an alternative to conventional pesticides, integrated pest management, including mating disruption (MD) and mass trapping with sex pheromones to prevent insect reproduction, is considered one of the most promising and scalable solutions.[8–10] The major sex pheromone component for the abovementioned pyralid moths is (*Z*,*E*)-9,12-tetradecadienyl acetate (ZETA, Z9,E12-14:OAc). This pheromone is currently commercially available and used worldwide. In addition, there is a high use of ZETA pheromone for MD of other key agricultural pests globally, such as beet armyworm (*Spodoptera exigua* Hübner), Egyptian cotton leafworm (*Spodoptera littoralis* Boisduval), and tobacco cutworm (*Spodoptera litura* Fabricius).[11] Like many commercially available pheromones, large amounts of ZETA are currently produced by chemical synthesis, which requires expensive and often hazardous specialty chemicals as starting materials and may result in toxic waste as byproducts.[9,12] Hence, pest management in warehouse and storage facilities using ZETA pheromone is costly and requires millions of dollars to be spent annually.[13] To account for the increased demand for the ZETA pheromone worldwide, a cost-effective and safe alternative method to synthetic chemical production is needed.[12]

Moth sex pheromones, in general, are fatty acid derivatives produced *de novo* in the pheromone gland with zero to four double bonds in the acyl chain and an oxygenated functional group such as aldehyde, alcohol, and acetate ester.[14,15] Sex pheromones or sex attractants have been identified for thousands of moth species (PheroBase.com). Significant advances have been made during the last 20 years to understand the molecular basis of moth pheromone biosynthesis.[14,16–19] The biological production of several monounsaturated moth sex pheromone components has been achieved in both yeasts and plants recently.[12] High titre of the cotton bollworm and the fall armyworm pheromone precursors in the form of corresponding pheromone alcohols was achieved through fermentation of an oleaginous yeast *Yarrowia lipolytica* on systematic metabolic engineering.[20] A similar approach was used to make the corresponding alcohols of the pheromone of European corn borer in *Y. lipolytica*.[21]

In the present study, we demonstrated the feasibility of producing the ZETA pheromone component in yeast *Saccharomyces cerevisiae* by the concerted expression of a suite of biosynthetic enzymes. First, we functionally characterized a unique Δ12 desaturase essential for the biosynthesis of the ZETA pheromone. We then used biobricks from various sources to successfully assemble the biosynthetic pathway in the yeast, demonstrating the feasibility of producing ZETA by metabolic engineering. Furthermore, we tested the biological activity of the yeast-produced ZETA by gas chromatography coupled to an electroantennographic detector (GC-EAD) using antennae of male *S. exigua* and *P. interpunctella*, of which species the females produce ZETA as their major sex pheromone component.[22,23] Our study demonstrates for the first time that a doubly unsaturated acetate sex pheromone can be produced in yeast and paves the way for green production of an extended portfolio of moth sex pheromones for integrated pest management.

2 METHODS AND MATERIALS

2.1 Identification of pheromone biosynthesis candidate desaturases from an *Ephestia cautella* model

2.1.1 Insect rearing and tissue collection

The *E. cautella* individuals were reared on an artificial diet at 25 ± 2.0 °C under an 16:8 light-dark cycle as previously described.[7] Freshly emerged female adults were collected daily before the scotophase and considered to be 0 days old. The female pupae were collected separately, and the newly emerged adults were maintained in a vial under the same conditions. Laboratory cultures of *S. exigua* and *P. interpunctella* were reared at 23 ± 1 °C under a 17:7 light-dark cycle and 60% relative humidity, and fed on wheatgerm-[24] and beans-based artificial diets,[25] respectively. The male and female pupae were kept separately. Adults were fed with a 10% honey solution, and 2–3 day-old unmated males were used for the electrophysiological experiments.

2.1.2 Desaturase cDNA cloning

Among 22 desaturases transcripts reported in the *E. cautella* PG transcriptome,[7] we chose six highly expressing candidates, *E. cautella* desaturase 2 (abbreviated as Ecau_D2), Ecau_D4, Ecau_D6, Ecau_D9, Ecau_D11, and Ecau_D14, as the potential desaturases for further yeast expression and assays. The full-length open reading frame (ORF) sequences of these desaturases were obtained by amplifying both the 5′ and 3′ cDNA ends using the rapid amplification of cDNA ends (RACE) technique (Table S1). The cDNAs were prepared from the *E. cautella* pheromone gland (PG) total RNA (approximately 1 μg) using a SMARTer RACE Kit (Clontech, Mountain View, CA, USA). The total RNA of *E. cautella* PGs (n = 30; 1–4-day-old female adults dissected at late scotophase) was prepared using a PureLink RNA Mini Kit (Thermo Fisher, Waltham, MA, USA). Touchdown polymerase chain reaction (PCR) [95 °C for 5 min, 35 cycles of 95 °C for 1 min, 65 °C (touchdown to 55 °C) for 30 s and 72 °C for 3 min; and one cycle at 72 °C for 10 min] was carried out using an Advantage 2 PCR kit (Clontech), and the PCR products were gel-purified (Wizard SV Gel purification kit; Promega, Madison, WI, USA) cloned into the pGEM-T vector (Promega) followed by transformation into JM109 competent cells (Promega). The plasmids were isolated and sequenced in both directions (ABI 3500, Thermo Fisher) for sequence verification (see primer details in Table S1).

2.1.3 Quantitative desaturase expression analysis

RT-qPCR assessed the relative expression of six desaturases in the PG. *E. cautella* PG cDNAs were prepared from RNA (~ 1 μg) of different age groups (1, 2, 4, 6, and 10 days old), using a PureLink RNA Mini Kit (Thermo Fisher). According to the manufacturer's instructions, the first-strand cDNA was synthesized using SuperScript IV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). RT-qPCR assays were performed in the Applied Biosystems 7500 Fast Real-Time PCR Systems (SYBR Green PCR Master Mix, Thermo Fisher). Desaturase expression was calculated from three biological replicates obtained from 20 *E. cautella* female PG dissected during late scotophase. Twenty-five microliter technical triplicates were run on the Applied Biosystems 7500 Fast Real-Time PCR System using 100 ng of pheromone gland (PG) cDNA template and 100 mM gene-specific primers (see primer details in Table S1) using the thermal program 50 °C for 20 s (precycling), 95 °C for 5 min (holding), 40 cycles of 95 °C for 15 s and 55 °C for 32 s,

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and finally a continuous melting curve step of 95 °C for 15 s, 60 °C for 1 min, 95 °C for 30 s, and 60 °C for 15 s. Relative expression levels of different desaturase genes were measured with the $2^{-\Delta C}$T method[26] by normalizing them to housekeeping genes, actin, and elongation factor (endogenous control) (Table S1). The qPCR data were analyzed using the software in the 7500 Fast Real-Time PCR System, and significant change in desaturase expression was estimated by one-way analysis of variance (ANOVA), followed by multiple-comparison with the least significant difference (LSD) test ($P < 0.05$) using SPSS v.24 (IBM SPSS Statistics, NY, USA).

2.1.4 *Desaturase phylogenetic analysis*

We aligned amino acid sequences using MAFFT.[27] We used the auto algorithm and BLOSUM30 as the scoring matrix. The final multiple sequence alignment contained 122 sequences with 541 amino acid sites. The phylogenetic tree was constructed in IQ-TREE/2.0-rc2[28] (http://www.iqtree.org). The automatic model search was performed using ModelFInder.[29] The maximum likelihood analysis was performed using default settings and ultrafast bootstrap support[30] with 5000 replicates. We used the Geneious (version 9.1; created by Biomatters, http://www.geneious.com/) for alignment construction, visualizing, and annotating the phylogenetic tree. The terminology for desaturases coined[31] based on the widely used 'signature motif' within a supported grouping of lepidopteran sequences was used when appropriate.

2.2 Heterologous expression

2.2.1 *Functional expression of key PG desaturases: yeast*

For constructing a yeast expression vector containing a candidate desaturase gene, specific primers (Table S1) with attB1 and attB2 sites incorporated were designed to amplify the ORF. The PCR products were subjected to agarose gel electrophoresis and purified using the Wizard SV Gel and PCR Clean-up system (Promega). The ORF was cloned into the pDONR221 vector in the presence of BP clonase (Invitrogen); after confirmation by sequencing, the correct entry clones were selected and subcloned to the pYEX_CHT_DEST vector,[32] and recombinant constructs were analyzed by sequencing again. The final recombinant expression clones harboring the *E. cautella* desaturase genes were introduced into the *ole1/elo1* disruption strain of the yeast *Saccharomyces cerevisiae*[33] using the S.c. easy yeast transformation kit (Invitrogen). For the selection of uracil and leucine prototrophs, the transformed yeast was allowed to grow on an SC-U plate containing 0.7% YNB (without amino acids; Sigma, St. Louis, MO, USA) and a complete drop-out medium lacking uracil and leucine (Formedium, Norfolk, UK), 2% glucose, 1% tergitol (type Nonidet NP-40; Sigma), 0.01% adenine (Sigma) and containing 0.25 mM oleic acid (Sigma) as an additional fatty acid source. After 5 days at 30 °C, individual colonies were picked up to inoculate 1 mL of selective medium (SC-U), which was grown at 30 °C and 300 rpm for 48 h. Yeast cultures were diluted to an OD_{600} (measured by Pharmacia Ultrospec 3000, Cambridge, UK) of 0.4 in 5 mL of fresh selective medium containing 1 mM $CuSO_4$ with supplementation of a biosynthetic precursor. Each fatty acid methyl ester (FAME) precursor [lauric acid methyl ester, 12:Me; myristic acid methyl ester, 14:Me; (*Z*)-9-tetradecenoic acid methyl ester, Z9-14:Me] was prepared in a concentration of 100 mM in 96% ethanol and added to reach a final concentration of 0.5 mM in the culture medium. Compound acronyms refer to geometry across the double bond, position of unsaturation, carbon-chain length, and functionality, e.g. Z9-14:Me = (*Z*)-9-tetradecenoic acid methyl ester (Me = methyl ester, OH = fatty alcohol, OAc = alcohol acetate ester). We used FAMEs as supplemented precursors because they are more soluble in the medium than free fatty acids.[34–36] Yeasts were cultured at 30 °C and 300 rpm. After 48 h, yeast cells were harvested by centrifugation at 4000 *g* and the medium was discarded. Experiments were repeated three times. The pellets were stored at −80 °C until fatty acid analysis.

2.2.2 *Heterologous Expression of Desaturases in Insect Cells*

To further confirm the function of Ecau_D4, we expressed this desaturase in *Spodoptera frugiperda* Sf9 cells using the Baculovirus expression system. The expression construct for Ecau_D4 in the baculovirus expression vector system (BEVS) donor vector pDEST8_Ecau_D4 was made by LR reaction. Recombinant bacmids were made according to instructions for the Bac-to-Bac Baculovirus expression system given by the manufacturer (Invitrogen) using DH10EMBacY (Geneva Biotech, Genève, Switzerland). Baculovirus generation was done using *S. frugiperda* Sf9 cells (Thermo Fisher Scientific), Ex-Cell 420 serum-free medium (Sigma), and baculoFECTIN II (OET, Oxford, UK). The virus was then amplified once to generate a P2 virus stock using Sf9 cells and Ex-Cell 420 medium. Viral titer in the P2 stock was determined using the BaculoQUANT all-in-one qPCR kit (OET) and found to be: 0.8×10^8 pfu mL^{-1} for Ecau_D4_ASVQ. Insect cell lines, Sf9 were diluted to 2×10^6 cells mL^{-1}. Heterologous expression was performed in 20-mL cultures in Ex-Cell 420 medium, and the cells were infected at an MOI of 1. The cultures were incubated in 125-mL Erlenmeyer flasks (100 rpm, 27 °C), with fatty-acid methyl-ester substrates supplemented at a final concentration of 0.25 mM after 1 day. After 3 days, 7.5-mL samples were taken from the culture and centrifuged for 15 min at 4500 *g* at 4° C. The pellets were stored at −80 °C until fatty acid analysis. Sf9 expression experiments were conducted in three replicates.

2.2.3 *Fatty acid analysis of yeast and Sf9 cells*

Total lipids were extracted from yeast cells using 1 mL of methanol/chloroform (2:1, v/v, containing 3.12 μg mL^{-1} of 19:Me as internal standard) in a glass tube. One milliliter of HAc (0.075 M) was added to each tube and then vortexed vigorously. Phase separation was achieved by centrifuging at 1500 *g* for 2 min. The bottom chloroform phase, about 0.33 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% (w/w) 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 heptane was used to extract the FAMEs. The methyl ester samples were subjected to gas chromatography–mass spectrometry (GC–MS) analysis on an Agilent 8890 GC equipped with an INNOWax column (30 m × 0.25 mm internal diameter, 0.25 μm film thickness) coupled to a mass detector Agilent 5977B. The GC thermo program was set to be held at 80 °C for 1 min, then ramped up to 230 °C at a rate of 10 °C min^{-1} and held for 15 min followed by a post-run at 240 °C for 3 min. The MS scan range was *m/z* 30 to 350.

Double-bond positions of monounsaturated compounds were confirmed by dimethyl disulfide (DMDS) derivatization followed by GC–MS analysis. DMDS reaction was performed by adding 50 μL of DMDS (Sigma) to 50–100 μL of FAME (biological samples) in the presence of 5 μL of iodine (5% in diethyl ether) as a catalyst. Reactions were incubated at 40 °C overnight. In the case of

identifying diene in the biological samples, incubation was done at 55 °C for 48 h. After that, 50 µL of $Na_2S_2O_3$ solution (5% in water) was used to neutralize the iodine, and the organic phase was transferred to a new tube and condensed to about 40 µL. GC–MS analysis of DMDS product was done in an Agilent 7890A equipped with an HP-5 column (30 m × 0.25 mm internal diameter, 0.25 µm film thickness) coupled to a mass detector Agilent 5975C. The GC thermo program was set at 80 °C for 1 min, raised to 140 °C at a rate of 20 °C min^{-1}, then to 250 °C at a rate of 4 °C min^{-1} and held for 20 min.[37]

2.3 Establishment of the ZETA pathway in a yeast system for bioproduction

2.3.1 Assembly of the biosynthetic pathway in yeast and bioproduction of ZETA

Desaturase Dmel_D9 from *Drosophila melanogaster*[20] Ecau_D4_ASVQ, characterized in this study, fatty acyl reductase SexipgFARII from *Spodoptera exigua*,[38] and acetyltransferase ATF1 from *Saccharomyces cerevisiae*[39] were synthesized [by GeneArt strings (Thermo Fisher) and codon-optimized for *S. cerevisiae*], assembled by fusion PCR[40] and Gateway assembly (Thermo Fisher), cloned to the expression vector pYEX_CHT_DEST (Fig. 7(b)). Yeast strain INVSc1 (Invitrogen) was transformed as described above. Single colonies were inoculated in a shaking flask with 100 mL of SC-U media and cultivated at 30 °C with galactose (2%, final concentration) and Cu^{2+} (1 mM) as an induction agent. Myristic acid methyl ester (final concentration 0.5 mM) was supplemented as the starting material for the sex pheromone biosynthetic pathway. After 4 days of growth, cells were collected by centrifugation (4000 *g*, 10 min). We used heptane to extract 25 mL of the medium and condensed the heptane extract to 1 mL, then 2 µL was injected for GC analysis using the protocols above.

2.3.2 Electrophysiology of ZETA yeast produced pheromone

The antennal electrophysiological activity of the yeast-derived pheromone was tested by GC-EAD. An Agilent 7890 gas chromatograph equipped with a flame ionization detector (FID), an HP-INNOWax column (30 m × 0.25 mm internal diameter and 0.25 µm film thickness; J&W Scientific, Folsom, CA, USA) was used. Antennae of male *S. exigua* and *P. interpunctella* with both tips cut off and associated with the head were mounted on a PRG-2 EAG (10× gain) probe (Syntech, Kirchzarten, Germany) using conductive gel (Blågel, Cefar, Malmö, Sweden). The antennal preparation was put in a flow of charcoal-filtered and humidified air that passed through the column outlet. Hydrogen was used as the carrier gas with a constant flow of 1.8 mL min^{-1}, and the GC effluent was directed to the FID and EAD by a 1:1 division. The GC inlet was set at 250 °C, the transfer line was set at 255 °C, and the detector was set at 280 °C. The GC oven was programmed from 80 °C for 1 min, then increased to 210 °C at a rate of 10 °C min^{-1} and held for 10 min. Data were collected with the software GC-EAD Pro Version 4.1 (Syntech).

3 RESULTS AND DISCUSSION

3.1 Identification of pheromone biosynthesis candidate desaturases from *Ephestia cautella*

3.1.1 Desaturase cDNA cloning

The ORF cDNA transcripts of Ecau_D2, Ecau_D4, Ecau_D6, Ecau_D9, Ecau_D11, and Ecau_D14 cDNA contain 993 bp, 1017 bp, 1059 bp, 1059 bp, 1029 bp, and 1035 bp, respectively, and correspond to proteins of 330 amino acids (Ecau_D2), 338 amino acids (Ecau_D4), 352 amino acids (Ecau_D6), 352 amino acids (Ecau_D9), 342 amino acids (Ecau_D11), and 344 amino acids (Ecau_D14) with predicted molecular weights of 38.63, 39.42, 40.31, 40.70, 39.81, and 39.90 kDa, respectively (GenBank acc nos. MW922324 to MW922329). Ecau_D2 shares 93.27% identity with the *Cydia pomonella* delta-(9)-fatty acyl desaturase (GenBank acc no. AIM40219.1). Ecau_D6 and Ecau_D9 share 89.52% and 94.35% identity, respectively, with the *Amyelois transitella* acyl-CoA delta-(9)-desaturases (EC 1.14.19.2). Ecau_D4, Ecau_D11, and Ecau_D4, Ecau_D11, and Ecau_D14 share 73.61%, 82.35%, and 80.49% identity respectively with the A. transitella acyl-CoA delta-(11)-desaturase (EC 1.14.19.5) (GenBank acc no. NP_001299594.1). The endoplasmic reticulum localization of all these desaturases, a typical characteristic of moth desaturase proteins, was identified using the Euk-mPloc 2.0 server.

3.1.2 Relative expression level of E. cautella *desaturses*

Identifying the PG-enriched expression of the desaturase gene can provide valuable insights into its role in pheromonogenesis.[41] We quantitatively measured the expression of all the six

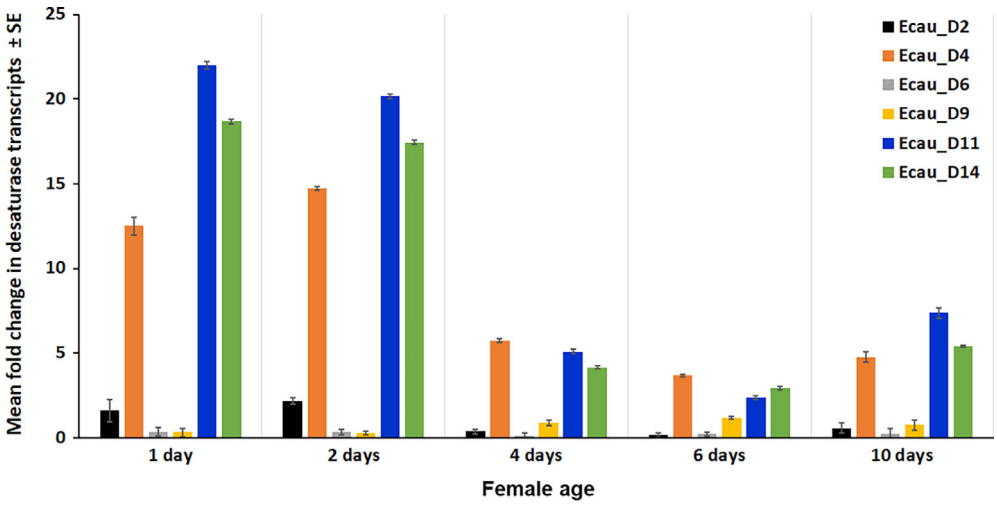

Figure 1. The expression levels of desaturases in the pheromone gland of *E. cautella*. The mean relative fold-change expression of different desaturase genes was measured from raw cycle threshold (Ct) values relative to the expression of the endogenous control genes, i.e. actin and elongation factor (Table S1).

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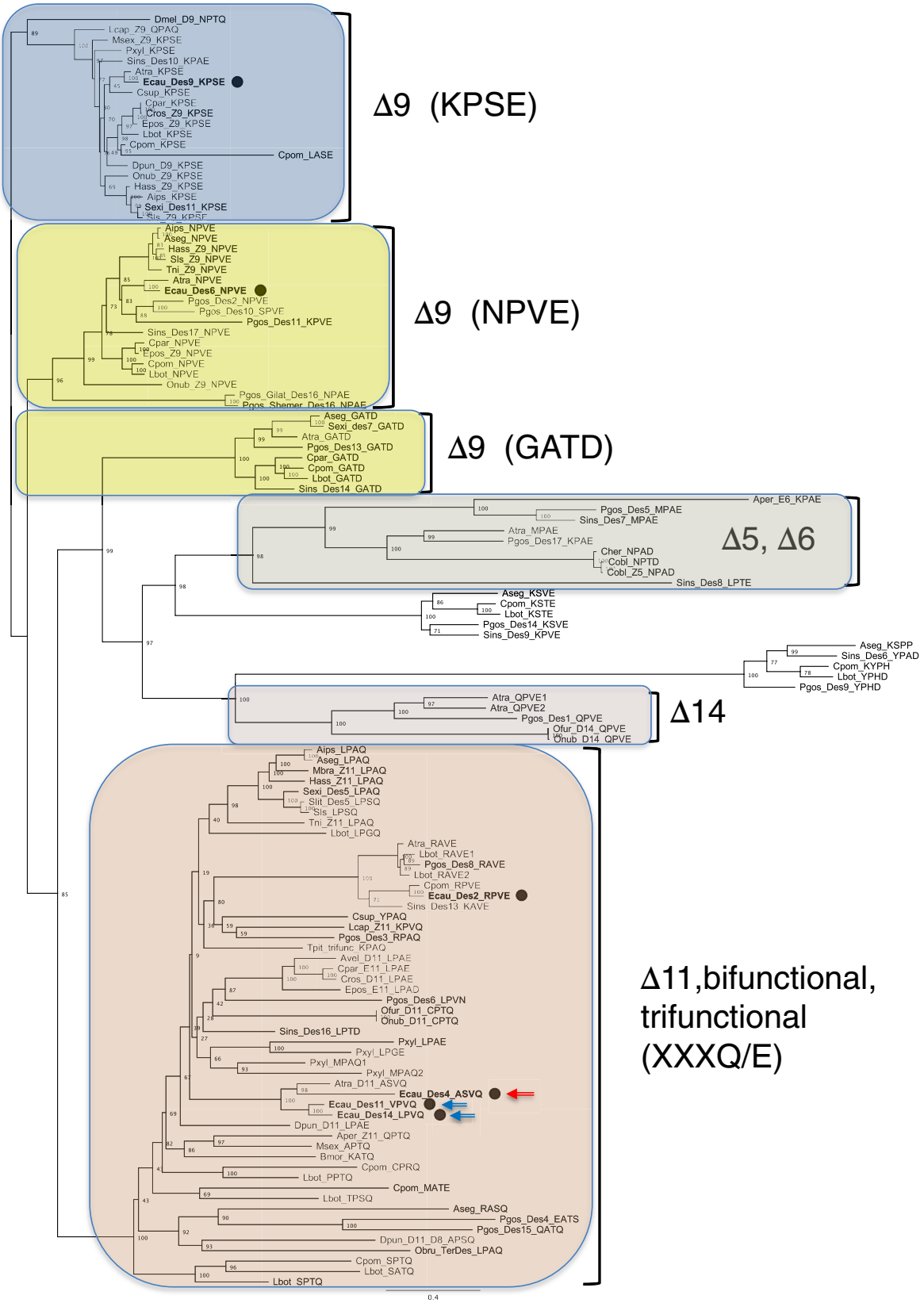

Figure 2. Phylogeny of desaturase genes. The maximum likelihood tree was constructed using amino acid sequences of 122 fatty acyl desaturase genes of 30 insect species. The *E. cautella* desaturases are indicated by a solid dot. Desaturase groups with similar activities are indicated in parenthesis (represented by branches of the same color) and bootstrap values are shown at the node of each branch. The arrows indicate the Δ11 desaturases involved in *E. cautella* pheromone biosynthesis, with red and blue arrows indicating E12 and Z11 desaturase, respectively.

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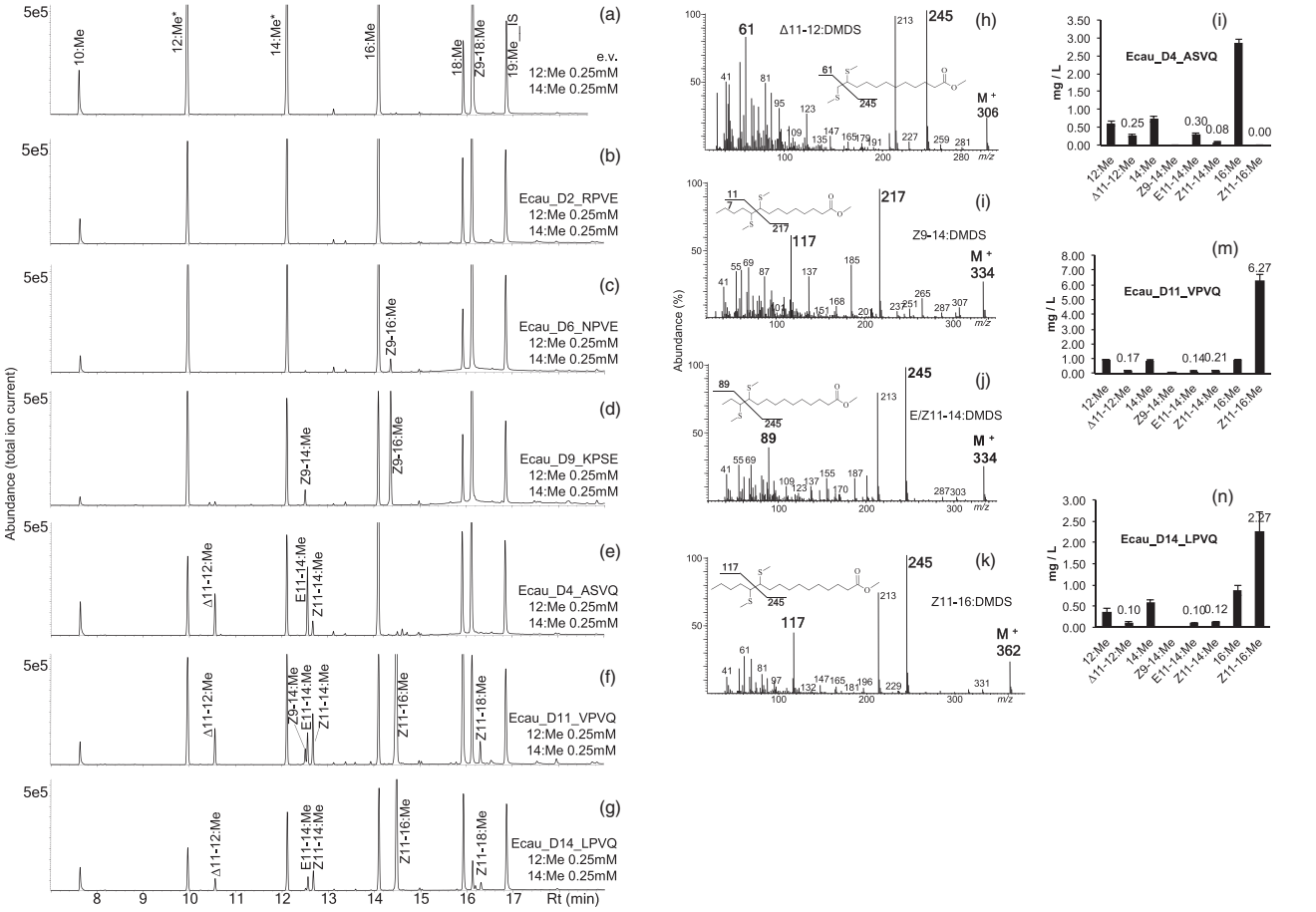

Figure 3. GC–MS analysis of FAMEs from yeast expressing *Ephestia cautella* desaturase supplemented with 12:Me* and 14:Me*. FAMEs of (a) control yeast transformed by empty vector, (b) Ecau_D2_RPVE, (c) yeast expressing Ecau_D6_NPVE, (d) Ecau_D9_KPSE, (e) Ecau_D4_ASVQ, (f) Ecau_D11_VPVQ, and (g) Ecau_D14_LPVQ. DMDS derivatives of FAME from yeast expressing Ecau_D11_VPVQ: (h) mass spectrum of DMDS adduct of Δ11-12:Me, (i) mass spectrum of DMDS adduct of Z9-14:Me, (j) mass spectrum of DMDS adduct of E/Z11-14:Me, (k) mass spectrum of DMDS adduct of Z11-16:Me. (l–n) Quantification of saturated and unsaturated products made by the desaturases Ecau_ D4_ASVQ, Ecau_ D11_VPVQ, and Ecau_D14_LPVQ. The experiments were replicated three times and the error bars represent standard error.

candidates using qRT-PCR, and the rate of the desaturase expression between different ages (1–10 days) was compared. We observed a significantly higher expression level ($P < 0.001$) of Ecau_D11 and a slightly lower expression of Ecau_D14 in the 1–2-day-old female *E. cautella*. When compared to Ecau_D2 expression in 1-day-old *E. cautella*, we found 14 times higher expression in Ecau_D11 ($F = 2106$; df = 1, 10; $P < 0.0001$), followed by Ecau_D14 ($F = 2041$; df = 1, 10; $P < 0.0001$) with 12 times higher expression (Fig. 1). Our data also showed a higher expression of Ecau_D4 in 1-day-old females, which is approximately eight times higher than Ecau_D2 ($F = 547$; df = 1, 10; $P < 0.0001$) (Fig. 1). Ecau_D11 was slightly down-regulated in the 2-day-old females compared to 1-day-old insects. In contrast, up-regulation of Ecau_D4 was observed in the same stage. However, the mRNA expression level of all these three genes was much lower (in 4–10-day-old females (Fig. 1). In contrast, the other three desaturases, Ecau_D2, Ecau_D6, and Ecau_D9, were found to be poorly expressed in the *E. cautella* PG.

3.1.3 *Desaturase phylogenetic analysis*

Desaturase sequences obtained from transcriptome and genome projects of various moth species were selected for building the phylogenetic tree (Fig. 2). Our sampling of desaturase sequences included the functional classes previously identified in moths (i.e. Δ5, Δ6, Δ8, Δ9, Δ10, Δ11, Δ12, Δ14). The results revealed that Ecau_Des6_NPVE and Ecau_Des9_KPSE are categorized as metabolic desaturases essential for lipid metabolism, cell signaling, and maintaining membrane fluidity in response to temperature fluctuation.[42] Most likely, these desaturases are not involved in pheromone biosynthesis. Ecau_Des4_ASVQ, Ecau_Des11_VPVQ, and Ecau_Des14_LPVQ fall into the Δ11 desaturase clade with signature motif XXXQ/E, the most relevant clade of desaturases to moth sex pheromone biosynthesis. This group contains many genes involved in the biosynthesis of diene pheromone, e.g. Atra_ASVQ in *Amyelois transitella*,[43] Bmor_KATQ in *Bombyx mori*,[44] Msex_APTQ in *Manduca sexta*,[45] Lcap_KPVQ in *Lampronia capitella*,[46] Dpun_LPAE in *Dendrolimus punctatus*,[47] Sexi_LPAQ, Slit_LPAQ,[48] and Sls_LPSQ in *Spodoptera* spp,[49] and Cpo_CPRQ in *Cydia pomonella*.[50] Ecau_Des2_RPVE falls into the same desaturase clade and is close to Lcap_KPVQ, which has been proven to desaturate Z9-14:Acyl to Z9,Z11-14:Acyl, the immediate fatty acyl precursor of the *Lampronia capitella* pheromone.[46]

3.2 Heterologous expression

3.2.1 *Functional expression of candidate PG desaturases in yeast and Sf9 cells*

We expressed all six desaturases in our yeast expression system. In the first round of experiments, the yeast was fed with 12:Me and

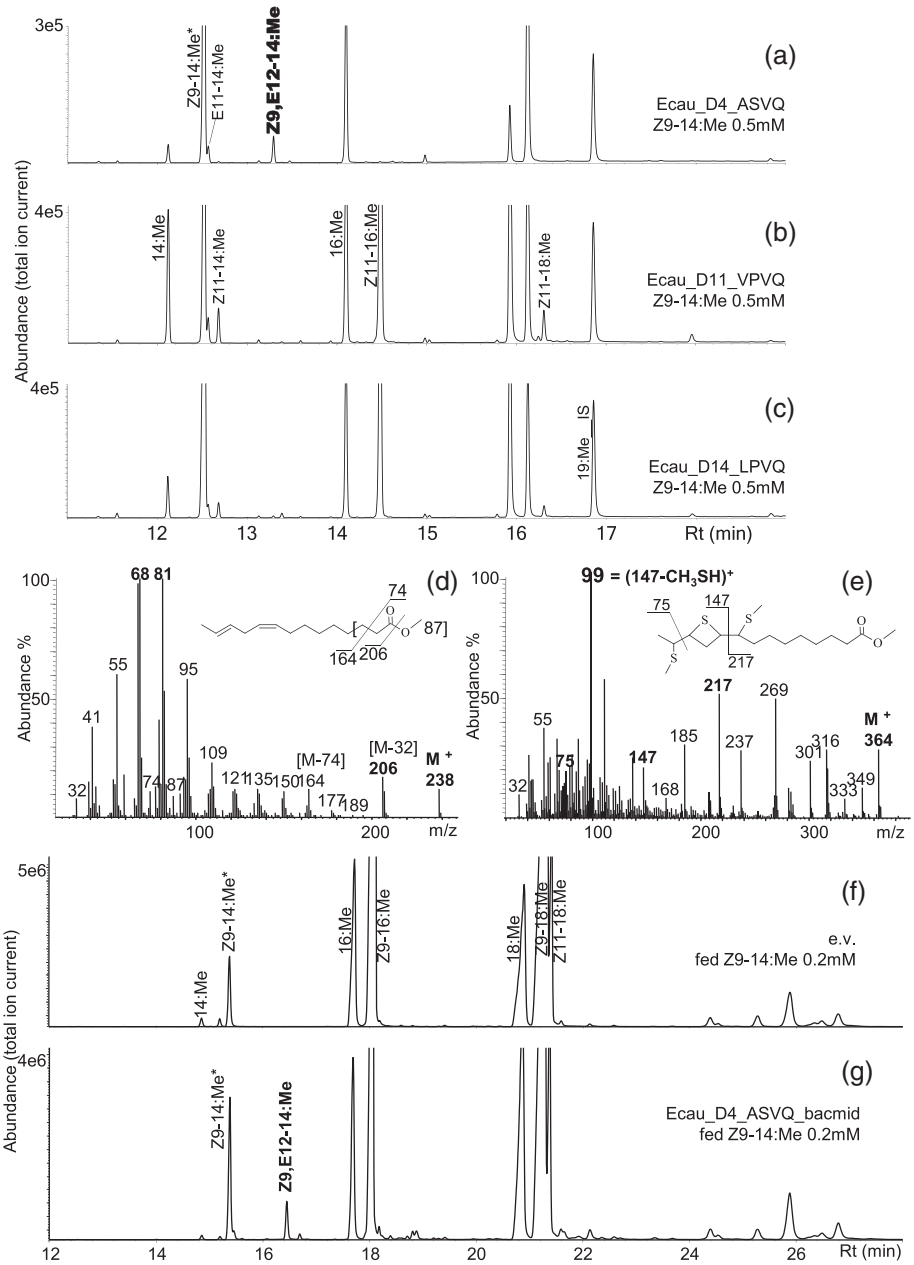

Figure 4. GC-MS analysis of FAMEs from yeast cells expressing Ecau_D4, Ecau_D11, and Ecau_D14 desaturases supplemented with Z9-14:Me*. GC chromatogram of FAME derived from (a) yeast cells transformed by Ecau_D4_ASVQ, (b) yeast cells expressing Ecau_D11_VPVQ, (c) yeast cells expressing Ecau_D14_LPVQ. Yeast cells are supplemented with Z9-14:Me*. (d) Mass spectrum of Z9,E12-14:Me from Ecau_D4_ASVQ yeast sample. (e) Mass spectrum of DMDS derivative of Z9,E12-14:Me from Ecau_D4_ASVQ yeast sample. (f) the empty virus infected Sf9 cell fed with Z9-14:Me. (g) Ecau_D4_ASVQ_bacmid infected Sf9 cell fed with Z9-14:Me.

14:Me [yeast cells have enough C16 (palmitic acid) and C18 (stearic acid) for desaturase to use as substrate] to get an overview of the activity of each desaturase. As shown in Fig. 3a–g, Ecau_D6_NPVE and Ecau_D9_KPSE are metabolic desaturases producing Z9-14:Acyl, (*Z*)-9-hexadecenoic acid (Z9-16:Acyl) and (*Z*)-9-octadecenoic acid (Z9-18:Acyl). Ecau_D6_NPVE and Ecau_D9_KPSE are categorized as metabolic desaturases.[42] Most likely, these desaturases are not involved in pheromone biosynthesis, with a few exceptions reported in previous studies. For example, the Dpu_KPSE from *Dendrolimus punctatus* (Lepidoptera: Lasiocampidae) produces a range of Δ9 monounsaturated products. When supplemented with Z7- and E7-14:Acyl [(*Z*)-7-tetradecenoic acid and (*E*)-7-tetradecenoic acid], Dpu_KPSE can introduce a second desaturation to produce Δ7Δ9-14:Acyl, illustrating the formation of conjugated double bonds by this type of fatty acyl desaturase.[47] In our yeast assay, Ecau_D9_KPSE produced a small amount of Z9-14:Acyl, which can be used as a substrate for the Ecau_D4_ASVQ to produce the doubly unsaturated product Z9,E12-14:Me. Maybe Ecau_D9_KPSE can also contribute to the pheromone biosynthesis in the live insect. When Ecau_D4_ASVQ is expressed in yeast, it produces more of the *E* isomer than the *Z* isomer of the monounsaturated Δ11–14C products.

Moreover, this desaturase showed no activity on C16 in yeast (Fig. 3e). On the contrary, the other two closely related

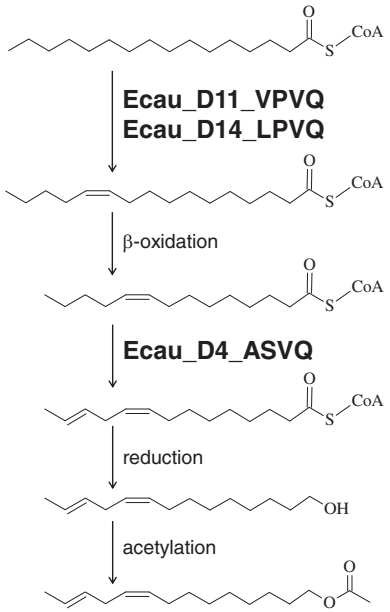

Figure 5. ZETA pheromone biosynthetic pathway in *Ephestia cautella*. Palmitate is (first) desaturated to Z11-16:Acyl, which is then chain shortened (*β*-oxidation) to Z9-14:Acyl. Z9-14:Acyl is (omega) desaturated to Z9,E12-14:Acyl, which is reduced and acetylated to the final ZETA pheromone. The genes characterized in this study are denoted in bold type.

desaturases show high activity on C16 (Fig. 3f,g). Ecau_D11_VPVQ and Ecau_D14_LPVQ mainly produce (*Z*)-11-hexadecenoic acid Me, Z11-16:Me, which is a precursor for pheromone production. These desaturases were also found to act on C14:Acyl, making small amounts of (*E*)-11-tetradecenoic acid (E11-14:Acyl) and (*Z*)-11-tetradecenoic acid (Z11-14:Acyl). The Z9-14:Acyl in the Ecau_D11_VPVQ yeast sample could be the chain-shortening product from Z11-16:Acyl since this desaturase produces a large amount of Z11-16:Acyl. The DMDS reaction confirmed the double-bond positions of all the unsaturated products (Fig. 3h–k). The relative quantity of desaturated product was calculated to understand the enzymatic activities on different carbon chain length substrates (Fig. 3l–n). Ecau_D2_RPVE did not show any activity in our yeast expression system, which is expected since all its immediate neighbors are also nonfunctional desaturases (Fig. 2).

In the second round of the experiments, the three desaturases Ecau_D4_ASVQ, Ecau_D11_VPVQ, and Ecau_D14_LPVQ were fed with Z9-14:Me to investigate if they could produce the diene pheromone precursor. As shown in Fig. 4a, Ecau_D4_ASVQ can convert the Z9-14:Acyl into the (*Z*,*E*)-9,12-tetradecadienoic acid (Z9,E12-14:Acyl), a direct precursor for the subsequent reduction and acetylation steps. The mass spectrum of the Z9,E12-14:Me has *m/z* 68 and 81 as base peaks, supporting diene hydrocarbon moiety. Molecular ion *m/z* 238 has a relatively low abundance, typical for a diene with isolated double bonds (Fig. 4d). The DMDS results confirmed the positions of the double bonds to be Δ9 and Δ12 (Fig. 4e). The other two desaturases, Ecau_D11_VPVQ and Ecau_D14 _LPVQ, did not produce the Z9,E12-14:Acyl (Fig. 4b,c), hence they are not likely to be involved in the second desaturation step in the pheromone biosynthesis.

We further confirmed the activity of Ecau_D4_ASVQ by expressing it in the insect cell line Sf9. Even though the catalytic activity of Ecau_D4_ASVQ is reasonably good in the yeast, we were curious to see if the insect cells could provide a better cellular environment. When the Ecau_D4_ASVQ was expressed in the Sf9 cell system and supplemented the culture medium with Z9-14:Me we observed a consistent production of Z9,E12-14:Acyl, demonstrating that Ecau_D4_ASVQ indeed catalyzes the biosynthesis of Z9,E12-14:Acyl (Fig. 4f,g). We did not detect any activity of this desaturase on longer chain length (C16, C18) substrates in Sf9 cells, which is in line with the previous results in yeast. The mass spectrum of the Z9,E12-14:Me peak was identical to the spectrum obtained from the yeast product.

3.3 Establishment of the ZETA pathway in the yeast system for bioproduction

The proposed biosynthesis of Z9,E12-14:OAc in *E. cautella* (Fig. 5) is based on earlier *in vivo* labeling experiments.[51] We conclude that Ecau_D11_VPVQ and Ecau_D14_LPVQ may contribute to the substrate pool of Z11-16: Acyl, which is then chain-shortened to Z9-14:Acyl, subsequently serving as a substrate for Ecau_-D4_ASVQ to produce Z9,E12-14:Acyl (Fig. 5). This biosynthetic pathway involves at least two distinct desaturases, which is different from the previous study of a single bifunctional desaturase being responsible for producing monoene and diene products (with chain shortening in between) in *Spodoptera* spp.[48]

To construct the biosynthetic pathway for ZETA bioproduction, we used Dmel_D9 from *D. melanogaster*, which mainly produces Z9-14:Acyl when expressed in yeast.[20] We do not have access to the PG-specific fatty acyl reductase (pgFAR) from *E. cautella*, and there has not been an insect-derived acetyltransferase characterized so far. Therefore, we used SexipgFARII[38] and yeast ATF1[39] to assemble the biosynthetic pathway. SexipgFARII is specialized for reducing the Z9,E12-14:Acyl, as previously demonstrated.[38] ATF1 is very active in acetylating (*Z*, *E*)-9,12-tetradecadien-1-ol (Z9,E12-14:OH).[39] When the entire biosynthetic pathway (Fig. 6a,b) was expressed in yeast and supplemented with 14:Me in the yeast medium, the final pheromone components Z9,E12-14:OAc and (*Z*)-9-tetradecenyl acetate (Z9-14:OAc) were detected in the medium (Fig. 6c,d) after 4 days of cultivation.

Interestingly, the acetylation step is very efficient, leaving no detectable level of intermediate Z9,E12-14:OH in the medium or yeast cells. Unfortunately, the activity of the Ecau_D4_ASVQ is low in the yeast cellular environment, and thus this becomes a rate-limiting step in the biosynthetic pathway. Besides producing the ZETA pheromone, (*Z*,*E*)-9,11-tetradecadienyl acetate (Z9,E11-14:OAc, identity confirmed by comparing retention time with synthetic standard) was also produced in substantial amounts in the yeast strain (INVSc1), expressing the biosynthetic pathway. This may be caused by the different order of actions of the two desaturases (Fig. 6a). In route 1, Z9-14:Acyl is produced by Dmel_D9 first, then used as a substrate for the Ecau_D4_ASVQ, followed by reduction and acetylation to make ZETA pheromone. In route 2, Ecau_D4_ASVQ uses 14:Acyl first to produce E11-14:Acyl, then Dmel_D9 uses the E11-14:Acyl as a substrate to produce Z9,E11-14:Acyl; this will lead to the production of Z9,E11-14:OAc. One way of suppressing route 2 would be to use a more potent promoter for the Dmel_D9. Hence, the Z9-14:Acyl would accumulate in the cells to provide substrate for the Ecau_-D4_ASVQ, which under an inducible promoter could be activated after Z9-14:Acyl has reached a certain level. Along the same lines, the reductase and the acetyltransferase expression could be turned on at a later stage through the onset of the Gal1 promoter to avoid competing with the second desaturation step for the use of the Z9-14:Acyl pool. We calculated that the production titer in

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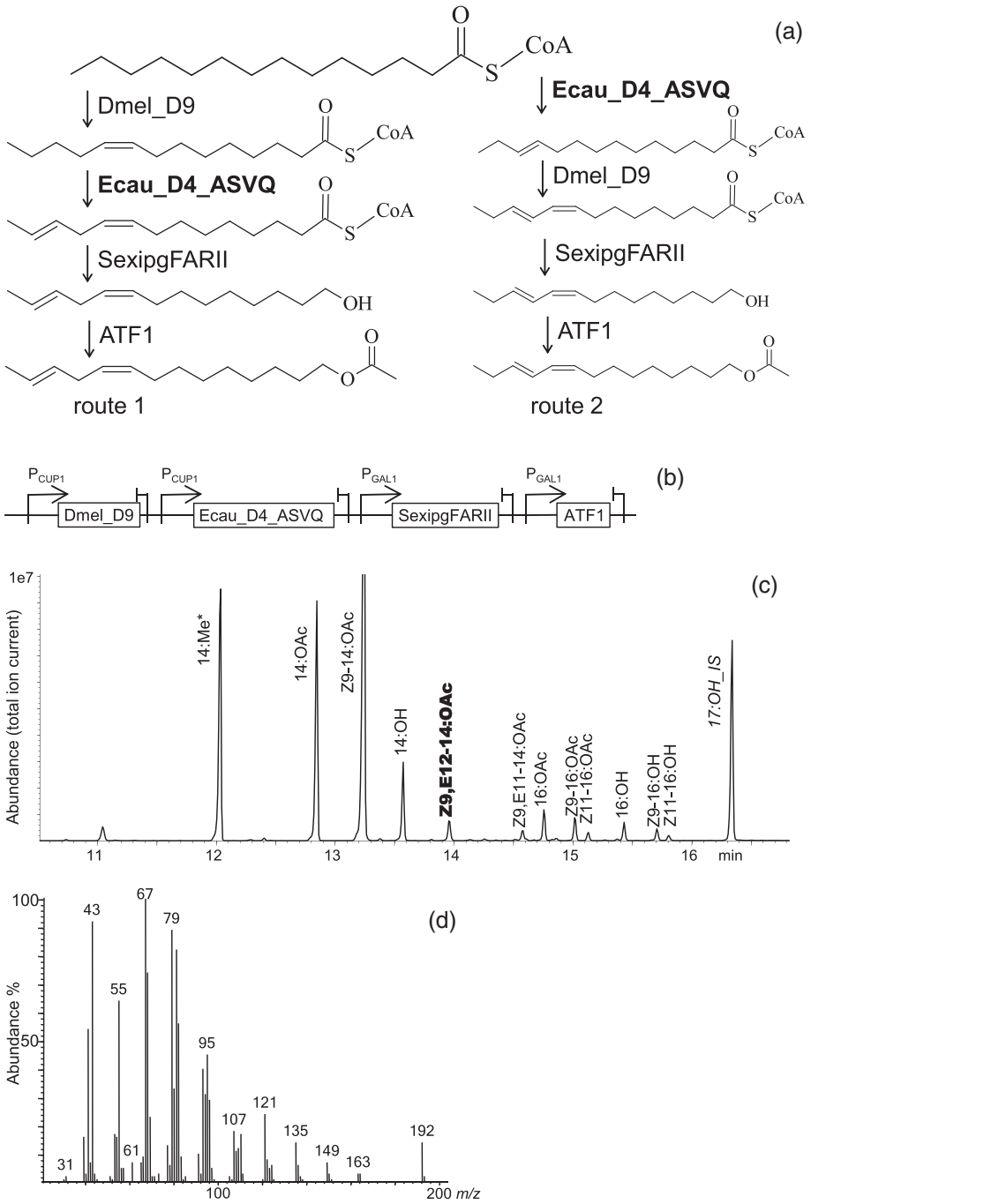

Figure 6. Reconstructed ZETA biosynthetic pathway in *Saccharomyces cerevisiae*. (a) Biosynthetic pathway leading to the bioproduction of ZETA pheromone. (b) Genetic circuit for the yeast transformation. (c) GC trace of the heptane extract of the yeast media. (d) Mass spectrum of the yeast-produced ZETA pheromone.

this study is 0.32 mg L^{-1}. Further engineering of the host strain similar to previous studies[20,21] and an optimized design of the biosynthetic pathway is necessary to bring the ZETA pheromone production to a commercially viable level.

3.3.1 *Electrophysiology activity of yeast-produced ZETA pheromone*

The acetate products found in extracts of both yeast cell pellet and medium elicited clear responses from antennae of male *P. interpunctella* and *S. exigua* (Fig. 7). Both species responded strongly to ZETA and the monounsaturated Z9-14:OAc. The latter was produced in a much higher amount in the yeast compared to ZETA production. A minor response to the saturated tetradecanyl acetate was also observed from male *P. interpunctella*, whereas both species showed no response to other yeast-derived components.

To summarize, we successfully demonstrated the biological production of (*Z*,*E*)-9,12-tetradecadienyl acetate, the fatty acid-derived diunsaturated sex pheromone in yeast cell factories. Noticeably, the yeast produced ZETA pheromone in its correct

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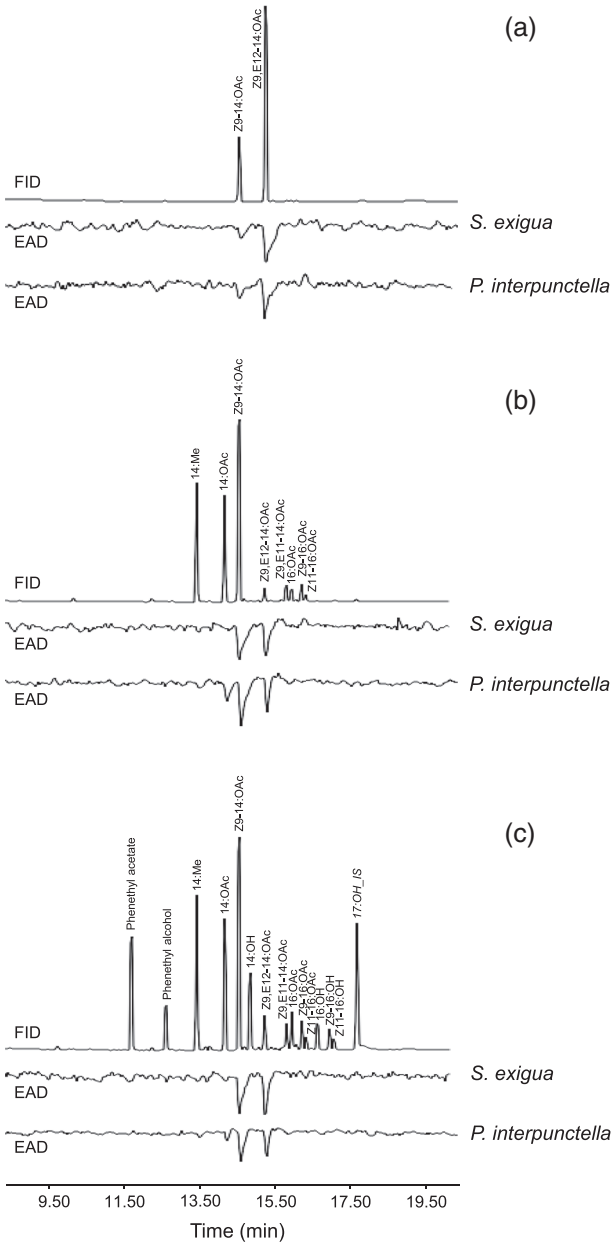

Figure 7. GC-EAD bioassay of the yeast-produced pheromone Z9,E12-14:OAc. Antennal responses (EAD) from male *Plodia interpunctella* and *Spodoptera exigua* elicited by GC elutes (FID) of synthetic compounds Z9-14:OAc and Z9,E12-14:OAc (a), ester fraction of yeast cell pellet extract (b), and crude extract of medium (c).

isomeric form. Following confirmation of the chemical structure of the ZETA pheromone, electrophysiological studies proved ZETA pheromone induced typical antennal responses in male moths. The behavioral activity of the yeast-produced pheromone and the need for downstream processing of the raw product remains to be investigated. The practical application of bioproduction of ZETA pheromone is that the three enzymes used in the current study can effectively deliver the pheromone component. It is important to note that the yeast fermentation leftovers and byproducts in the present study are primarily biodegradable, and we used a standard medium in the experiment. In contrast, the chemical synthesis of ZETA will typically require unique starting material, expensive catalysts, and several synthesis steps, and above all it produces several environmental pollutants.[12,52,53] Our studies lay the foundation for the bioproduction of ZETA pheromones to be used in pheromone-based pest control of key agricultural and stored product pests such as *Spodoptera* spp., *P. interpuctella*, *E. cautella*, and *E. kuehniella*. Still, further metabolic engineering, an optimized fermentation protocol generating commercially relevant titres (>5 g L^{-1} ZETA pheromone), and further tests of the biological activity of the product are required for large-scale commercial bioproduction of ZETA pheromone.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

BA, BJD, and CL conceived the study. BA and MAL carried out cloning of the desaturase genes and RT-qPCR. BJD and BA did functional assay of the desaturases in the yeast and Sf9 cell, chemical analyses, and assembled the biosynthetic pathway in the yeast. HLW did the GC-EAD analyses. BJD and BA wrote the paper with input from CL and HLW. All the authors approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

The Ecau_D2, Ecau_D4, Ecau_D6, Ecau_D9, Ecau_D11, and Ecau_D14 sequences reported in this paper have been deposited in the GenBank database (accession no. MW922324 to MW922329).

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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