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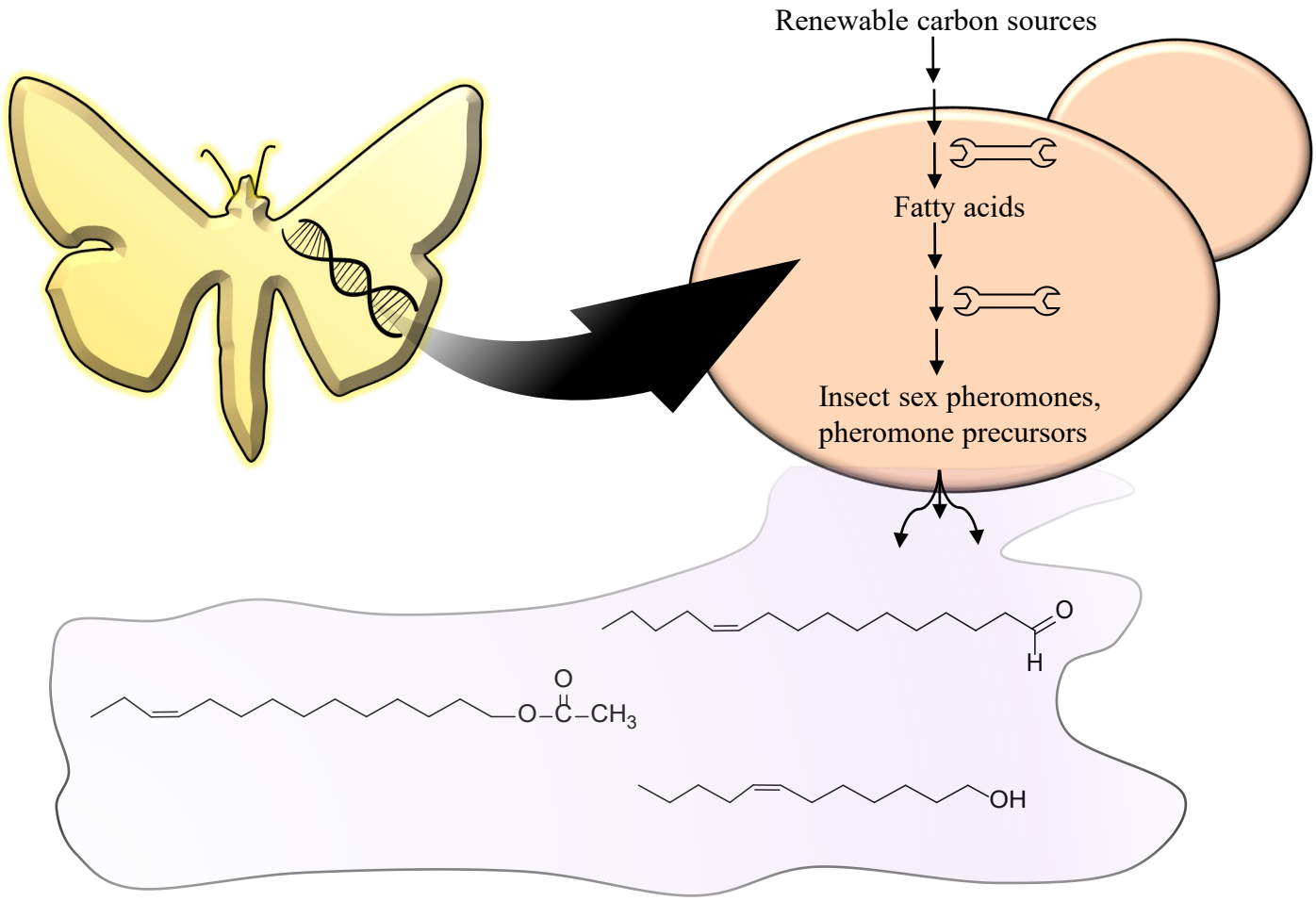
The Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark
BioPhero



Production of Lepidoptera pheromones in yeast cell factories

PhD Thesis by Karolis Petkevicius

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Preface

This PhD thesis serves as a partial fulfilment of the requirements for obtaining a PhD degree at the Technical University of Denmark. The works included in the thesis were carried out at the Novo Nordisk Foundation Center for Biosustainability and company BioPhero in the period of 1st of June 2019 to 31st of August 2022 under the supervision of Prof. Irina Borodina, Dr. Carina Holkenbrink, Dr. Bettina Lorántfy and third-party supervisor Prof. Christer Löfstedt. The PhD project has been funded by the Innovationsfonden under grant agreement number 8053-00179B.

Karolis Petkevicius

Kongens Lyngby, August 2022

Abstract

Crop damage caused by insect pests is a serious problem in agriculture. Conventional control strategies, which rely on the use of chemical insecticides or genetically modified insect-resistant plants, come with drawbacks. Pests develop resistance, making these strategies less efficient with time. Additionally, insecticides lack specificity, giving rise to toxic effects in beneficial insects such as bees as well as bigger animals and humans.

Insect sex pheromones represent a safe and environmentally friendly alternative. These compounds are naturally produced by certain insects to attract a mating partner. This can be utilised to control pests through mating disruption. Pheromone application in crops will confuse the insects, preventing them from finding each other to mate and reproduce. Pheromones are attractive because they are biodegradable, species-specific compounds, which neither harm biodiversity nor induce resistance development.

This thesis describes the development of bio-based production of Lepidoptera pheromones in yeast cell factories. The Lepidoptera order of insects, which comprises moths and butterflies, is interesting from a pest control perspective as it includes major pests such as fall armyworm and cotton bollworm.

To enable bio-based production of insect pheromones, oleaginous yeast *Yarrowia lipolytica* was used as platform. Metabolic engineering redirected the yeast's inherent fatty acid metabolism towards pheromone biosynthesis. This was achieved by screening multiple gene candidates from various insects and other organisms and evaluating their effects by fatty acid and fatty alcohol analyses using gas chromatography-mass spectrometry. Genes of interest included fatty acyl-CoA desaturases, fatty acyl-CoA reductases and peroxisomal oxidases, all known for their essential role in moths' pheromone biosynthesis. Genetic modifications related to decreased formation of triacylglycerides or decreased degradation of fatty acids/alcohols further improved pheromone production.

The work presented in this thesis demonstrates how a bio-based yeast platform can be metabolically engineered for production of insect sex pheromones, offering a viable alternative to conventional chemical synthesis. A wider application of pheromones in agriculture will lead to safer and more sustainable pest management.

Dansk Resumé

Afgrødeskader forårsaget af skadedyr er et alvorligt problem i landbruget. Konventionelle bekæmpelsesstrategier, som er afhængige af brugen af kemiske insekticider eller genetisk modificerede insekt-resistente planter, er forbundet med ulemper. Skadedyr udvikler resistens, hvilket gør disse strategier mindre effektive med tiden. Derudover mangler insekticider specificitet, hvilket giver anledning til toksiske virkninger hos gavnlige insekter såsom bier såvel som større dyr og mennesker.

Insekters sexferomoner repræsenterer et sikkert og miljøvenligt alternativ. Disse forbindelser produceres naturligt af visse insekter for at tiltrække en parringspartner. Dette kan udnyttes til at bekæmpe skadedyr gennem parringsforstyrrelse. Ved anvendelse af feromoner i afgrøder, forvirres insekterne, så de ikke kan finde hinanden og formere sig. Feromoner er attraktive, fordi de er biologisk nedbrydelige, artsspecifikke forbindelser, som hverken skader biodiversitet eller forårsager resistensudvikling.

Dette speciale beskriver udviklingen af biobaseret produktion af Lepidoptera feromoner i gærcellefabrikker. Lepidoptera-ordenen af insekter, som omfatter møl og sommerfugle, er interessant ud fra et skadedyrsbekæmpelsesperspektiv, da den inkluderer væsentlige skadedyr såsom hærlarve og bomuldsugle.

For at muliggøre biobaseret produktion af insektferomoner blev olieholdig gær *Yarrowia lipolytica* brugt som platform. Metabolisk modificering omdirigerede gærens iboende fedtsyremetabolisme mod feromonbiosyntese. Dette blev opnået ved at screene flere genkandidater fra forskellige insekter og andre organismer og evaluere deres virkninger gennem fedtsyre- og fedtalkoholanalyser ved hjælp af gaskromatografi-massespektrometri. Gener af interesse inkluderede fedt-acyl-CoA-desaturaser, fedt-acyl-CoA-reduktaser og peroxisomale oxidaser, alle kendt for deres væsentlige rolle i møls feromonbiosyntese. Genetiske modifikationer relateret til nedsat dannelse af triacylglycerider eller nedsat nedbrydning af fedtsyrer/alkoholer forbedrede feromonproduktionen yderligere.

Det arbejde, der præsenteres i denne afhandling, demonstrerer, hvordan en biobaseret gærplatform kan metabolisk modificeres til produktion af insekters kønsferomoner som et brugbart alternativ til konventionel kemisk syntese. En bredere anvendelse af feromoner i landbruget vil føre til sikrere og mere bæredygtig bekæmpelse af skadedyr.

Acknowledgements

For the last three years and three months I was on a very exciting PhD journey. Now, when this journey had reached an end, I would like to thank to the people who supported me in various ways.

First of all, I would like to say incredibly big thank you to my supervisors Prof. Irina Borodina and Dr. Carina Holkenbrink.

Isaac Newton once said, “If I have seen further [than others], it is by standing on the shoulders of giants.” Metaphorically speaking, you were those giants who provided me with possibilities to see further and develop career-wise and personally.

The nature of Industrial PhD studies implies that the student has to be affiliated with the company. Consequently, I would like to thank CEO of BioPhero Kristian Ebbensgaard for providing the opportunity to do research in this company.

There are plenty of people at BioPhero to whom I am very grateful. I would like to thank Mathias, Leonie, Kanchana and Dan for daily scientific and personal discussions, plus help in the lab.

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Christina and Rune, you are the masters of GC-MS! It was a pleasure to work with you and learn from you.

I am very grateful to Camilla, who helped me to write popular science article in Danish that was published in Dansk Kemi.

I would also like to thank Maria and Dimitris, who were instrumental in testing activities of biologically derived *Z*11-16:Ald and *Z*11-14:OAc.

My appreciation also goes to Prof. Christer Löfstedt. I honestly felt that your input during preparation of manuscripts always significantly improved quality of upcoming papers.

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My biggest thanks go to my family, who unconditionally supported me throughout this journey.

List of publications

Research articles and literature review:

1. Petkevicius K, Löfstedt C, Borodina I: **Insect sex pheromone production in yeasts and plants**. *Curr Opin Biotechnol* 2020 ,**65**:259-267.

2. Holkenbrink C, Ding, BJ, Wang, HL, Dam, MI, Petkevicius K, Kildegaard KR, Wenning L, Sinkwitz C, Lorántfy B, Koutsoumpeli E, França L, Pires M, Bernardi C, Urrutia W, ,Mafra-Neto A, Ferreira BS, Raptopoulos D, Konstantopoulou M, Löfstedt C, Borodina I: **Production of moth sex pheromones for pest control by yeast fermentation**. *Metab Eng* 2020, **62**:312-321.

3. Petkevicius K, Koutsoumpeli E, Betsi PE, Ding BJ 4, Kildegaard KR, Jensen H, Mezo N, Mazziotta A, Gabrielsson A, Sinkwitz C, Lorántfy B, Holkenbrink C, Löfstedt C, Raptopoulos D, Konstantopoulou M, Borodina I: **Biotechnological production of the European corn borer sex pheromone in the yeast *Yarrowia lipolytica***. *Biotechnol J* 2021, **16**:2100004.

4. Petkevicius K, Wenning L, Kildegaard KR, Sinkwitz C, Smedegaard R, Holkenbrink C, Borodina I: **Biosynthesis of insect sex pheromone precursors via engineered β-oxidation in yeast**. *FEMS Yeast Res* 2022, **22**:1-9.

Popular science article:

1. Petkevicius K, **Nu skal der brygges insektferomoner til bæredygtig afgrødebeskyttelse**. *Dansk Kemi*, 103, nr.3, 2022.

Patent applications:

1. WO2020169389 - METHODS AND CELL FACTORIES FOR PRODUCING INSECT PHEROMONES. Inventors: DING, Baojian; LÖFSTEDT, Christer; BORODINA, Irina; WENNING, Leonie; HOLKENBRINK, Carina; KILDEGAARD, Kanchana Rueksomtawin; PETKEVICIUS, Karolis.

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CHAPTER 1

Introduction

1.1. Insect pests – significant problem in agriculture

Plants play significant role in our diet and comprise 80 percent of the food we eat and produce. In order to sufficiently feed increasing population and keep up with rising food demand, efficient farming practises are needed that could result in healthy and high yield harvest. This was highlighted by the Food and Agriculture Organization (FAO) of the United Nations which declared 2020 as the year of plant health [1]. Plant health could be compromised by various pests (bacteria, fungi, viruses, etc.) which might lead to harvest losses up to 50-80% [2]. Among pests, insects are well-recognized contributors to crop damage. Multiple economically relevant plants such as cotton, rice, maize, soybean, and much more are prone to insect infestations leading to reduction in quality and amount of harvest. As example, estimations made by Oliveira et al. indicated that Brazil annually loses approximately 25 million tons of food, fiber, and biofuels due to damage caused by insect pests [3].

During the World War II the need to prevent tropical insect-vectored diseases like malaria motivated to focus on finding effective insecticides. Organic chemicals such as dichloro-diphenyl-tri-chloroethane (DDT), organophosphates and carbamates turned out to be very effective. Even though these chemicals primarily had a purpose of preventing human diseases, it was soon realized that they could serve in agriculture as a tool to protect crops from insect infestations and increase the yield. As noted by Marie Louse Flint and Robert van den Bosch, "*They* [chemical insecticides] *seemed to be truly "miracle" insecticides*." [4] However, as time went by, it became evident that the "miracle" has serious drawbacks. Above-mentioned insecticides are lacking specificity, meaning, that they are deleterious for beneficial insects, for example, bees and can also cause acute and chronic human poisoning [5,6]. Additionally, insects became increasingly resistant towards insecticides. Study by Sparks and Nauen reports that until 2014, 586 species became resistant to at least 1 insecticide [7]. These drawbacks motivated to look for alternatives and develop other, more targeted, pest management techniques. The primary focus of reducing pre-harvest crop losses received very important, additional goal-reduction of pre-harvest losses while minimizing use of chemicals insecticides. One of the strategies which arose was based on implementation of gene coding for *Bacillus thuringiensis* (Bt) toxin to the crops [8]. Once ingested by insects, Bt toxin gets activated in the alkaline gut, attacks epithelial cells, and makes gut leaky [9]. Due to acidic pH in the human gut, Bt toxin is not activated, therefore, does not cause harm to humans. The success of genetically modified (GM) crops could be illustrated by the meta-

analysis, which revealed that switch towards GM crops reduced chemical pesticide use by 37%, improved crop yields by 22% and increased profits of growers by 68% [10]. Despite encouraging numbers, this technology faces certain limitations. Economically important pests such as fall armyworm (*Spodoptera frugiperda*), corn earworm (*Helicoverpa zea*) and European corn borer (*Ostrinia nubilalis*) have been reported to become resistant to Bt crops in particular areas of the world [11–13]. Furthermore, public perception of GM food is often quite negative [14]. Lastly, due to different regional regulations related to commercial release of GM crops, this technology faces limitations in applicability [15].

Even though chemical insecticides and Bt crops are widely used in agriculture nowadays, their above-mentioned down-sides strongly justifies the need for highly specific, environmentally friendly, and broadly applicable tools in insect pest management.

1.2. Insect sex pheromones – tool for efficient crop protection

1.2.1. Brief history of pheromone discovery and their applications

1870s was the time when French entomologist Jean-Henri Fabre observed intriguing phenomena: female great peacock moth placed in a mesh cage inside the house attracted dozens of conspecific males from the outside regardless of where the cage was placed. His conclusion was that female must emit the odour, which cannot be sensed by human nose, however, has very strong attractive power for great peacock moth males [16]. Later, compounds emitted by animals that can modulate behaviour of the same species were named pheromones (Greek φερω pheroo ('I carry') and ὁρμων hormon ('stimulating')). For a long time, odour-causing substances from moths remained elusive until the breakthrough was made by German biochemist Adolf Butenandt. He was the first to identify pheromone from silk moth (*Bombyx mori*) in 1959 which was called Bombykol ((10E,12Z)-Hexadeca-10,12-dien-1-ol) [17]. Structurally, bombykol is sixteen carbon atom length unsaturated straight chain primary fatty alcohol with cis and trans double bonds at positions 10 and 12, respectively. Development of analytical tools such as gas chromatography-mass spectrometry and electroantennography allowed to elucidate sex pheromone components from multiple insects, such as *S. frugiperda*, codling moth (*Cydia pomonella*), cotton bollworm (*Helicoverpa armigera*), diamondback moth (*Plutella xylostella*) and more [18–21]. Up to now, pheromones from more than 600 Lepidoptera species (order of insects which includes moths and butterflies) are elucidated. They can be categorised into 4 classes: from Type 0 to Type III. Type I constitutes 75% of known lepidopteran pheromones and has an interest because of their potential applications in pest management. It turned out that most of Type I

pheromones are relatively similar to Bombykol. They are straight chain unsaturated fatty alcohols/acetates/aldehydes with 10-18 carbon atoms and 1 to 3 double bonds [22].

Ability of pheromones to modulate behaviour of moths is used in agriculture as a tool to monitor insect populations and protect crops [23]. Mating disruption with insect sex pheromones is the technology that has multiple encouraging examples [24]. In order to find mating partner, female moths release specific pheromone (or blend of them) to attract males. Males can sense the released odour plume and navigate themselves towards the female. Application of pheromones with matching composition of the ones released by female masks the original signal, thus, confuses the male. As result, mating is prevented leading to reduced number of larvae in the field of interest. (Figure 1.1). Use of pheromones plays an important role in integrated pest management (IPM) which aims to control pests with the least disruption of the environment. Compared to conventional chemical insecticides and GM crops, pheromones possess several compelling advantages. Unlike insecticides, pheromones are considered as harmless compounds with no adverse effects to humans [25]. Also, they are species-specific which enables targeted pest control. Additionally, so far only single case of resistance to pheromones has been reported compared to hundreds and tens cases of resistance to insecticides and Bt crops, respectively [26]. Over the years pheromone-based mating disruption is gaining more and more popularity. For example, in 11 years (2000-2010) annual production of *C. pomonella* pheromone increased by ~ 3.6-fold [27]. It could be expected that in the future the demand for pheromones will rise. Initiatives such as Farm to Fork driven by European Commission, that ambitiously aims to reduce the use of chemical pesticides by 50% by 2030, or Sustainable Development Goals (SDGs) set up by United Nations (UN) indicate trending shift towards sustainable solutions in agriculture [28,29].

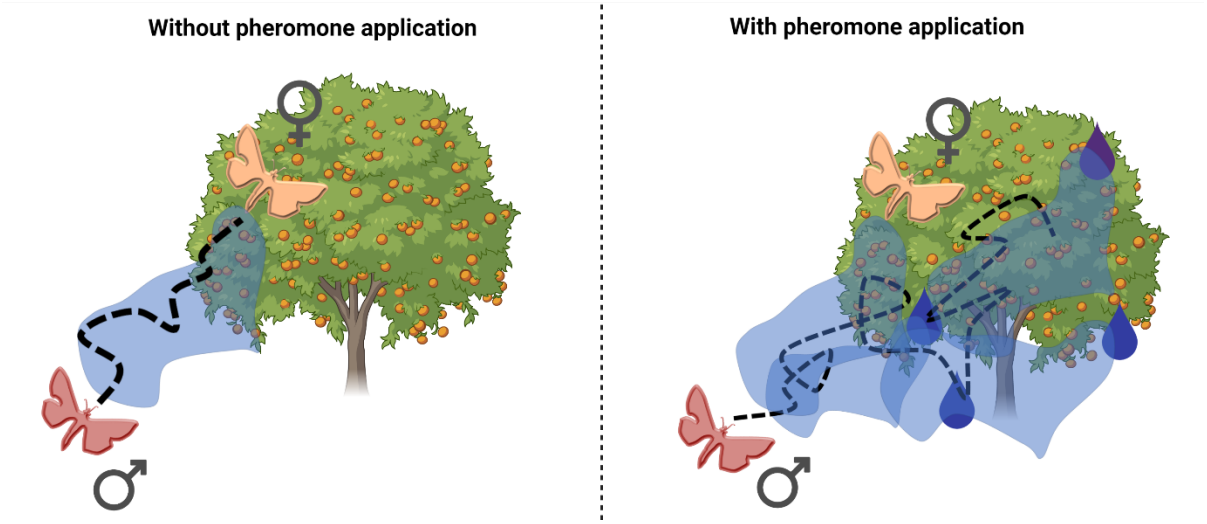

Figure 1.1. Representation of how pheromone application (purple drops) helps to prevent male moth from finding the female for mating.

1.2.2. How do moths produce their sex pheromones?

Biosynthesis of Type I pheromones starts from coenzyme A (CoA) esterified fatty acids such as palmitate or stearate that are produced *de novo* by fatty acid synthase. Aforementioned esterified fatty acids are modified by subset of enzymes in various orders. Enzymatic reactions leading to species-specific pheromone or pheromone blend include fatty acid desaturation, fatty acid chain shortening or elongation, fatty acid reduction to alcohol and fatty alcohol oxidation to aldehyde or acetylation to acetate. Interplay between all of these steps create stunning number of possible chemical structures [30].

Out of above-mentioned reactions, enzymes catalysing desaturation and reduction are the most extensively characterized [31]. Fatty acyl-CoA desaturases (FADs) introduce double bond into the hydrocarbon chain of fatty acyl-CoAs while fatty acyl-CoA reductases (FARs) convert fatty acyl-CoAs into corresponding alcohols. Depending on source organism, FADs can possess different substrate specificities and introduce double bonds in various positions. The most common positions of desaturation appear to be Δ9 and Δ11. However, double bonds could be placed at other positions (Δ5, Δ6, Δ8, Δ10, Δ12, Δ13, Δ14) as well [31]. Characterization of multiple FARs revealed that different FARs tend to have preference for different fatty acids. For example, FAR from *H. armigera* (HarFAR) turned out to be semi-selective enzyme which converts C_8-C_{16} acyl-CoAs into corresponding fatty alcohols and prefers Z9-14:CoA over Z11-16:CoA [32]. Another interesting example described by Lassance et al. revealed differences between two FARs found in *O. nubilalis*. One of them-pgFAR-E, showed high specificity towards (*E*)-11-tetradecenoate, while the second-pgFAR-Z, preferred Z isomer [33].

Compared to FADs and FARs, functional characterization of enzymes involved in fatty acid β-oxidation, elongation, fatty alcohol oxidation and acetylation are lagging behind. Recently, for the first time, Xia and Ding were able to identify and functionally characterize peroxisomal oxidases (POXes) and elongase from Lepidoptera insects [34,35]. In the case of the oxidases, comparative transcriptomics of grapevine moth (*Lobesia botrana*) and heterologous expression of candidate genes in Sf9 insect cells showed that two POXes, Lbo_31670 and Lbo_49602, were required for production of *Z*9-12:acid from *Z*11-14:acid [35]. Regarding elongase, expression of CsupELO4 from rice stem borer (*Chilo suppressalis*) in *Nicotiana benthamiana* significantly improved elongation rate and production of *Z*13-18:acid from *Z*11-16:acid [34]. Fatty alcohol oxidases and acetyltransferases from Lepidoptera remains to be functionally characterized.

In order to dissect sequence of enzymatic steps leading to pheromone biosynthesis, labelling experiments are performed. Deuterium labelled fatty acids are applied to pheromone glands and fate of substrates is analysed. This approach allowed to decipher pathways towards pheromones such as (*E*,*E*)-8,10-dodecadienol, (*Z*,*Z*)-11,13-hexadecadienal, (*Z*)-11-hexadecenal produced by *C. pomonella*, *Amyelois transitella* and *Helicoverpa zea*, respectively and more (Figure 1.2.) .

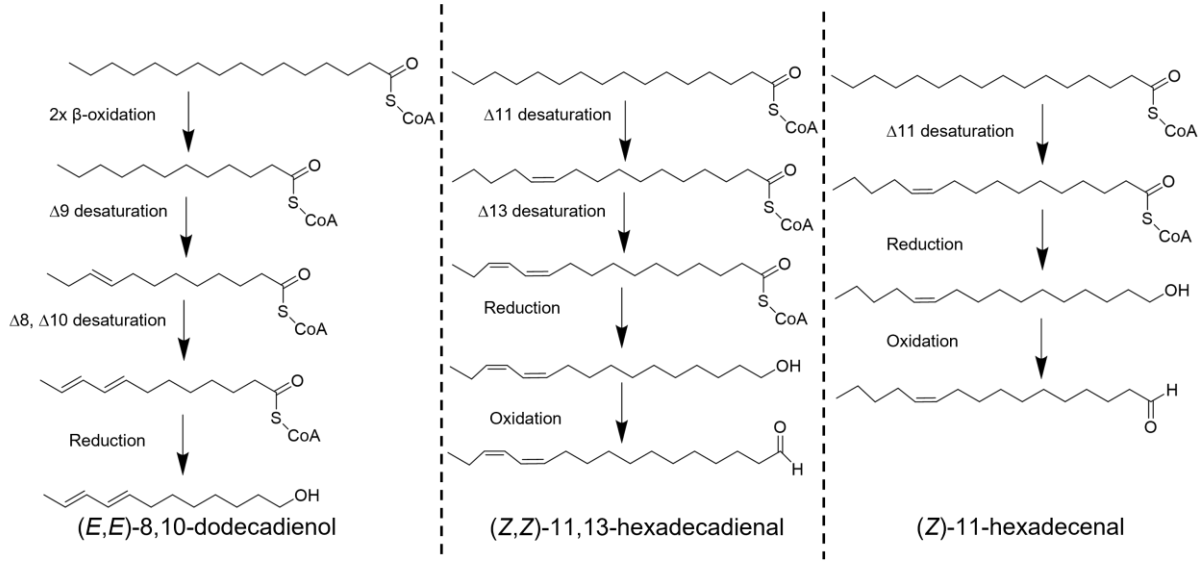

Figure 1.2. Proposed biosynthetic pathways towards three insect sex pheromones based on labelling studies.

1.3. Biotechnological production of insect sex pheromones

1.3.1. Use of plants and yeast for bio-based production of insect sex pheromones

Insect pheromones are derivatives of common fatty acids (palmitate, stearate) which are abundantly found in various living organisms. Discovery of gene candidates and molecular biology tools allow to introduce genes from insects into selected hosts and direct their fatty acid metabolism towards insect pheromone production. So far, yeast species *Saccharomyces cerevisiae* and *Yarrowia lipolytica* and plants *Nicotiana tabacum*, *Nicotiana benthamiana*, *Camelina sativa* have been used for this purpose [30]. "Pheromone brewing", in the case of yeast, and "pheromone farming", in the case of plants, are promising concepts for sustainable production. Conventional chemical synthesis of pheromones relies on fossil fuel derived materials which are finite resources. In order for starting materials to be converted into target compounds, multiple steps are needed which, depending on the synthetic route, might involve toxic chemicals, complex catalysts and high temperatures [36–39]. On the contrary, yeast-based production uses renewable materials such as glucose or glycerol, respectively. These carbon sources could be converted into pheromones or their precursors by fermentation under mild conditions (28-30°C). Fermentation products might need to be derivatized through, typically, one simple chemical reaction [40]. Obtained product is purified, formulated and is ready for application in the field (Figure 1.3.). Number of yeast-derived fatty alcohols, acetates and aldehydes have been proven to be biologically active and modulated behavior of different moths [41].

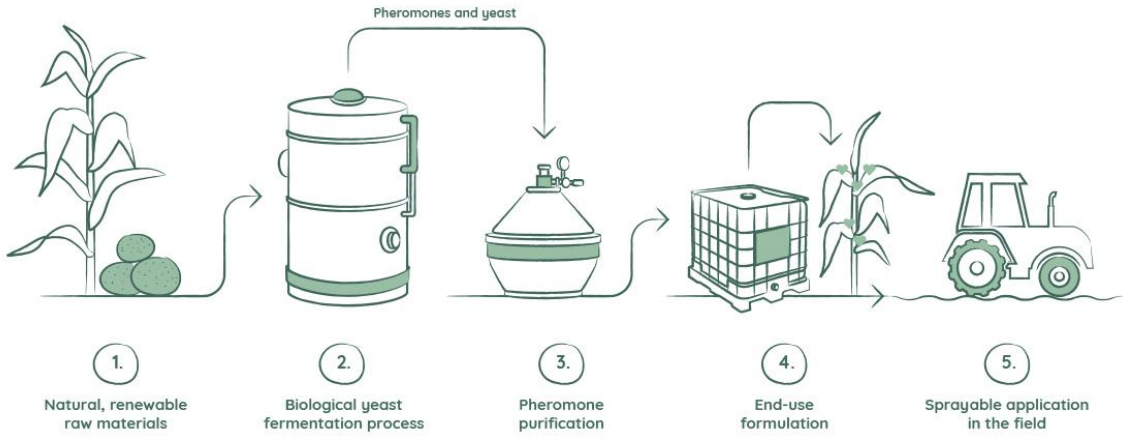

Figure 1.3. Pheromone production from renewable raw materials via yeast fermentation.

1.3.2. *Yarrowia lipolytica*-microorganism of choice for recombinant insect sex pheromone biosynthesis

In this thesis yeast *Y. lipolytica* was used as a host for insect sex pheromone biosynthesis. Genus name "Yarrowia" was given in honour of David Yarrow due to his contribution to yeast systematics, while species name "lipolytica" highlights the ability of this yeast to degrade lipids [42,43]. Indeed, the primary interest in this organism arose in the 1940s from the observations that it was very often isolated from oily/lipid-rich environments, for example cheese, sausages. The natural ability to grow on hydrophobic substrates was later exploited for various purposes such as production of single cell proteins, organic acids or bioremediation [44]. This organism is recognized as non-pathogenic and *Y.lipolytica*-produced citric acid has Generally Recognized as Safe (GRAS) status [45]. Reference genome of *Y. lipolytica* became available in 2004 and contains 6703 genes organized in six chromosomes (A-F) with relatively high G/C content (49%) [46].

Y. lipolytica is suitable for production of fatty acid derived compounds due to its oleaginous properties. Wild type strains can accumulate lipids up to 30-50% of its cell dry weight and metabolic engineering efforts can increase this number up to 90%, as shown by Blazeck et al [47,48]. Portfolio of oleochemicals biosynthesised by this yeast is broad and includes triacylglycerides (TAGs), polyunsaturated fatty acids (PUFAs), fatty alcohols, fatty acid ethyl esters (FAEEs), fatty alkanes, flavour lactones and more [49]. Inherent oleaginous properties of this yeast are provided by set of metabolic pathways. Compared to *S. cerevisiae, Y. lipolytica* has higher fluxes towards acetyl-CoA, and pentose phosphate pathway [50]. Acetyl-CoA serves as the main precursor for fatty acid synthesis while pentose phosphate pathway generates co-factor NADPH which is needed for the intermediate reduction step in the fatty acid synthesis cycle. Another distinguishable feature is the presence of ATP citrate lyase (ACL). This enzyme is considered to be the hallmark of oleaginous yeast [51]. It catalyses the split of citrate into acetyl-CoA and oxaloacetate. Availability of cytosolic citrate is dependent on carbon/nitrogen (C/N) ratio in cultivation media. When this ratio is high, meaning that cells are starving for nitrogen, blockage of tricarboxylic acid (TCA) cycle in mitochondria happens leading to accumulation of citrate. This blockage is caused by deamination of adenosine monophosphate (AMP) which happens in nitrogen-limited conditions. Low levels of AMP inhibit isocitrate dehydrogenase leading to accumulation of

citrate. Excess of citrate is exported from mitochondria to cytosol via citrate-malate shuttle, where it becomes available for ACL [52]. Experimentally, importance of ACL was proven by Dulermo et al., where inactivation of ACL subunit 1 (ACL1) significantly reduced fatty acid content and the size and number of lipid bodies (LBs) [53]. Generated Acetyl-CoA is carboxylated by acetyl-CoA carboxylase (ACC) and malonyl-CoA is produced. These two metabolites serve as substrates for fatty acid synthase (FAS), where C_{16}-C_{18} saturated fatty acids are generated. They can have several fates. Two native desaturases, namely, OLE1 and FAD2, can generate unsaturated fatty acids such as palmitoleate, oleate and linoleate [54]. (Un)saturated fatty acids can be either accumulated in LBs as TAGs, degraded to acetyl-CoA in peroxisomes via β-oxidation or elongated by elongases. Synthesis of TAGs is comprised of four reactions starting from acylation of glycerol 3-phosphate (G3P) by G3P acyltransferase. Generated lysophosphatidic acid (LPA) is further modified by LPA acyltransferase resulting in phosphatidic acid (PA). Dephosphorylation of PA by PA phosphohydrolase (PAP) produces diacylglycerol (DAG) which is finally converted into TAGs by DGA1, DGA2 and LRO1 acyltransferases [54,55]. Fatty acids incorporated into TAGs can be released by intracellular lipases and activated by fatty acyl-CoA synthetases [54]. Degradation of fatty acids occurs in peroxisomes, where β-oxidation cycle comprised of four reactions shortens hydrocarbon chain by two carbons releasing acetyl-CoA [56]. The first, rate liming step, is catalysed by POXes (POX1-6) which are known to have different substrate specificities [57]. POXes produce trans-2-enoyl-CoA which is further converted into 3-ketoacyl-CoA by MFE2 in two sequential reactions. Finally, 3-ketoacyl-CoA thiolase POT1 performs thiolytic cleavage and acetyl-CoA together with two carbons shorter acyl-CoA are synthesised. Deletions of genes coding for proteins involved in β-oxidation and biogenesis of peroxisomes are attractive metabolic engineering strategies for improved production of fatty acid derived compounds [48,50,55]. Contrary to β-oxidation, elongation extends fatty acids by two carbons. In *Y. lipolytica* two elongases, ELO1 and ELO2 have been identified. Experimental data indicates that ELO1 is responsible for elongation of myristic acid into palmitic acid while ELO2 most likely elongates palmitic acid further on [58].

In order to shift native lipid metabolism of *Y. lipolytica* towards insect sex pheromones, EasyCloneYALI genetic engineering toolbox was used. This toolbox is comprised of three main elements: (i) Genome-integrated Cas9 gene from *Streptococcus pyogenes*,(ii) episomal plasmid containing guide RNA (gRNA) which targets specific genomic loci and (iii) repair template for double strand break repair [59]. Previously, this toolbox was successfully

applied to engineer *Y. lipolytica* for production of lactones, fatty alcohols, resveratrol, gibberellins [50,60–62].

Overall, features such as safety, oleaginous properties and accumulated knowledge about fatty acid metabolism together with available genetic engineering tools make *Y. lipolytica* suitable host for insect sex pheromone biosynthesis.

1.4. Thesis structure

The thesis is comprised of six chapters (Figure 1.4.). First of all, Chapter 1 provided the context about the need for sustainable, effective, safe and broadly available tools for insect pest management. Further on, it introduced insect sex pheromones as promising solution, elaborated on general features of their natural biosynthesis and how yeast and plants could be used for production of these fatty-acid derivatives. Finally, oleaginous yeast *Y. lipolytica* was presented as the host which was used for recombinant insect sex pheromone production in this thesis. Chapter 2 presents detailed overview of pheromone pathway discovery and metabolic engineering strategies which have been applied for biosynthesis of insect sex pheromones so far. Chapter 3 focused on engineering of *Y. lipolytica* for production of (*Z*)-11-hexadecenol (*Z*11-16:OH). Yeast-derived *Z*11-16:OH was oxidised into corresponding aldehyde-(*Z*)-11-hexadecenal (Z11-16:Ald) and aldehyde was as effective as commercial synthetic pheromone in *H. armigera* trapping experiments. Study described in Chapter 4 aimed to produce pheromone (*Z*)-11-tetradecenyl acetate (*Z*11-14:OAc) which is used by important maize pest *O. nubilalis*. (*Z*)-11-tetradecenol obtained via fermentation was acetylated and acetate modulated the behaviour of *O. nubilalis* males. Chapter 5 focused on proof-of-concept production of three economically relevant pheromone precursors, namely (*Z*)-7-dodecenol (*Z*7-12:OH), (*Z*)-9-dodecenol (*Z*9-12:OH), and (*Z*)-7-tetradecenol (*Z*7-14:OH) via fatty acid chain shortening. Described study is the first one which employed engineered β-oxidation in yeast for pheromone biosynthesis. Finally, Chapter 6 provides perspectives on how the field of yeast-based pheromone biosynthesis could move further.

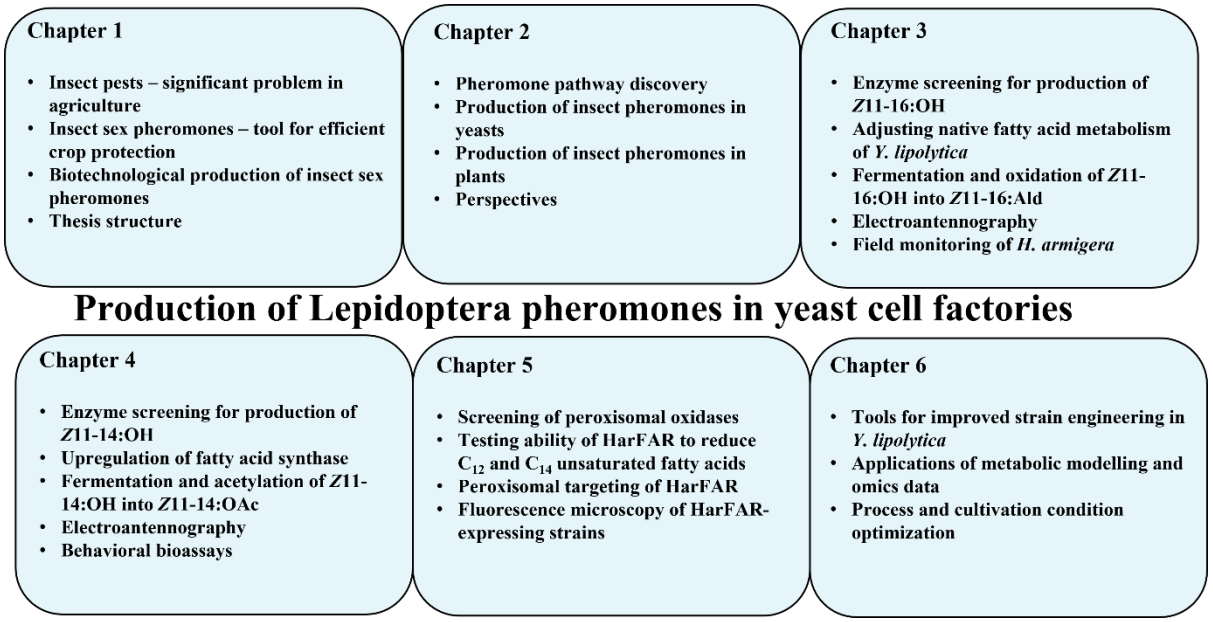

Figure 1.4. Infographics describing thesis structure and key aspects in each chapter.

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CHAPTER 2

Insect sex pheromone production in yeasts and plants

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Current Opinion in Biotechnology

Insect sex pheromone production in yeasts and plants

Karolis Petkevicius[1,2], Christer Löfstedt[3] and Irina Borodina[1,2]

Insect infestation is a major problem in agriculture and forestry addressed primarily with insecticide sprays or genetically modified plant breeds. The problem has aggravated in the last decade due to the emergence of resistance among key insect pests and the removal of multiple insecticides from the market due to their toxicity. Pheromone-based methods for pest management have been in use for over 30 years, though primarily for high-value fruits due to the high cost of the chemical synthesis and pheromone application. As biotechnology solutions for pheromone production are emerging, pheromones will become an economically competitive technology for pest management also in low-value row crops. This review describes the advances in the discovery of pheromone biosynthetic pathways and the recent engineering of yeasts and plants for recombinant production of pheromones.

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Introduction

According to the Food and Agriculture Organization (FAO), agricultural productivity needs to rise by 70% until 2050 to provide sufficient food for the growing population on a limited amount of land and water. At the same time, climate change and the evolution of insect resistance makes pest control increasingly difficult [1,2]. Insect pest control is currently mainly accomplished by spraying chemical insecticides and using genetically modified (GM) crops. However, insects are rapidly developing resistance to both insecticides and GM crops, such as crops containing genes from *Bacillus thuringiensis* (Bt crops), and cause significant losses [2–4]. Moreover, insecticides and GM crops are not allowed in organic farming, while consumer demand for organic products is rising rapidly [5]. Finally, the regulatory demands and financial penalties for the use of insecticides are continuously increasing.

There is a real and urgent need for new effective, economic, sustainable, and safe solutions for pest control among growers, consumers, and agro-chemical companies. The Integrated Pest Management (IPM) concept has been advanced for 50 years, for example, by FAO and the European Commission (Directive 2009/128/EC of the European Parlament and of the Council of 21 October 2009; https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX:02009L0128-20091125). Within IPM, Mating Disruption (MD) with sex pheromones that act by preventing insect reproduction is considered one of the most promising and scalable solutions. The MD was shown effective against many species of moths whose larvae damage a wide variety of crops [6]. This method is highly efficient, environmentally friendly and safe (European Commission, Directive 91/414/EEC). Additionally, compared to large number of resistance cases reported to chemical insteccitides, so far only a single documented case of resistance to MD with pheromones is reported [7]. However, chemically synthesized pheromones with a cost ranging from 500 to several thousand dollars per kg are too expensive for row crop application, and until now, MD has been largely limited to a niche market of pest control in high-value fruits [8,9]. In order to enable wider application of pheromones, it is essential to develop novel more cost effective production methods.

Compared to chemical synthesis, biotechnological microbial and plant-based production has several important advantages. Firstly, cheap renewable feedstocks, such as sugars or glycerol, are used in microbial bio-production instead of the expensive fossil-derived starting chemicals while carbon dioxide serves as carbon source for plants [10••,11••]. Secondly, the biotechnological production typically involves a single-step bio-conversion with living cells as the only catalyst. In some cases, the fermentation/plant-derived products are additionally processed via one or two chemical steps [11••]. In constrast, chemical synthesis of lepidopteran pheromones typically involves multiple steps, each involving chemical catalysts, often expensive as for metathesis [12]. Thirdly, as biotechnological production uses the insect biosynthetic enzymes, the product profile in regard of stereoisomer ratios and major/minor pheromone component ratios will often be similar to the one naturally produced in insect pheromone glands.

This review highlights the recent advances in discovery of the biosynthetic pathways towards sex pheromone

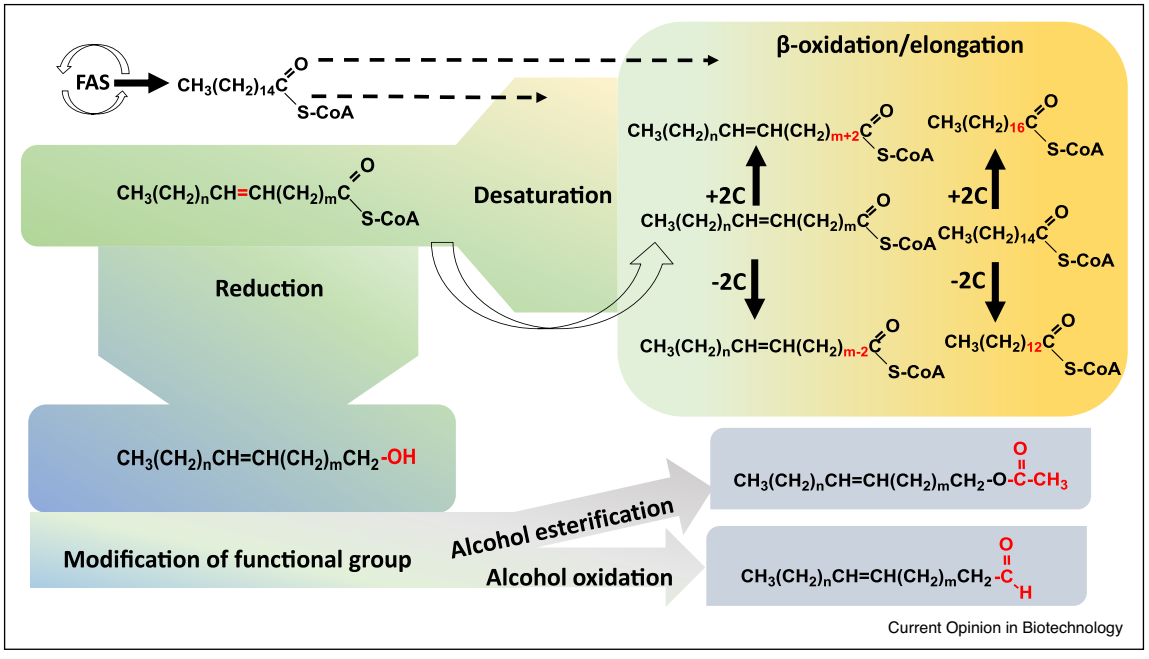
biosynthesis in moths and biotechnological production of pheromones by recombinant yeast and plants.

Pheromone pathway discovery

Because of their economic importance in pest management, sex pheromones of Lepidoptera females are the best studied insect pheromones. Since identification of the first sex pheromone bombykol in 1959, sex pheromones from more than 600 species of Lepidoptera have been identified and attraction of male moths to pheromone compounds have been reported for at least twice as many species [13]. The majority of the lepidopteran sex pheromones are fatty alcohols, aldehydes or fatty alcohol acetates of 10–18 carbon chain length with one to three double bonds [14]. Their biosynthesis starts from the ubiquitous fatty acid metabolite palmitoyl-CoA, which is modified by fatty acid desaturases (FADs), chain shortening via peroxisomal β-oxidation or chain elongation. Subsequently, fatty acid reductases (FARs), acetyltransferases or fatty alcohol oxidases produce the volatile pheromone components from the fatty acyl precursors (Figure 1). The varying specificity of the enzymes and their combinations allow to generate an amazing diversity of these so-called Type I pheromones, which constitute approximately 75% of all known moth pheromones with different chain lengths, double bond and functional group variations [13–15].

The biosynthetic pathways towards many pheromones have been deciphered by studying the fate of labelled pheromone precursors in pheromone glands of female moths and analyzing their incorporation into pheromones. Examples include pheromones from important agricultural pests such as *Cydia pomonella* [16], *Amyelois transitella* [17], *Helicoverpa zea* [18], or *Spodoptera exigua* [19]. In the case of *C. pomonella*, it was concluded that the main sex pheromone component 8*E*,10*E*-dodecadien-1-ol (*E*8, *E*10-12:OH) is produced from palmitic acid, which is shortened by β-oxidation to dodecanoic acid followed by *E*9 desaturation and conversion of the monounsaturated intermediate into the doubly unsaturated precursor, which is finally reduced to the alcohol [16]. Experiments with labelled hexadecanoic and (*Z*)-11-hexadecenoic acids in *A. transitella* demonstrated that the sex pheromone component 11*Z*,13*Z*-hexadecadienal (*Z*11,*Z*13-16:Ald) is produced from palmitic acid, which first undergoes Δ11 desaturation followed by Δ13 desaturation, reduction and oxidation [17]. Regarding *H. zea*, labelling showed that the major sex pheromone component, (*Z*)-11-hexadecenal (*Z*11-16:Ald) is produced by Δ11 desaturation of palmitic acid while the minor components, (*Z*)-9-hexadecenal (*Z*9-16:Ald) and (*Z*)-7-hexadecenal (*Z*7-16:Ald) are derived from stearic acid by Δ11 and Δ9 desaturation respectively, followed by chain shortening [18]. In a recent study, labelling experiments in combination with functional assays confirmed a Δ12 desaturation pathway towards the main sex pheromone of *S. exigua*, 9*Z*,12*E*-

Figure 1



Graphical representation of interplay between biochemical reactions involved in insect pheromone biosynthesis. Red color highlights change made by certain type of enzyme. FAS: fatty acid synthase.

tetradecadienyl acetate (*Z*9,*E*12–14:OAc). Biosynthesis of this compound starts from palmitic acid, which is desaturated at Δ11 position by the desaturase SexiDes5 followed by chain shortening, which results in *Z*9-14: Acid. This intermediate is then used by SexiDes5 and converted into *Z*9,*E*12–14:Acid, which further undergoes reduction and acetylation leading to *Z*9,*E*12–14:OAc [19].

The discovery of the biosynthetic enzymes is now commonly accomplished by comparative analysis of gene expression in the female pheromone glands versus control tissue, which could be the total body or abdomen or labial palps [20–22]. The genes that are overexpressed in the pheromone glands are annotated to identify enzymes that are candidate genes to be involved in pheromone biosynthesis, such as desaturases, reductases, and so on (Figure 1). The enzymes are expressed in heterologous hosts, such as yeast *Saccharomyces cerevisiae* [21], insect cells [23], or plants [11••] and functionally assayed. Transcriptome data from pheromone glands have been reported for multiple lepidopterans, such as the turnip moth (*Agrotis segetum*), the almond moth (*Ephestia cautella*), the pink bollworm (*Pectinophora gossypiella*), the diamondback moth (*Plutella xylostella*), the beet armyworm (*Spodoptera exigua*), and the tobacco cutworm (*Spodoptera litura*) [20,21,24–27]. Transcriptomes from more Lepidoptera and other insects can be found in InsectBase database (http://www.insect-genome.com), which currently contains transcriptomes from 116 insects [28]. Apart from attempts to obtain the data on RNA level, genome sequencing of insects is gaining more and more attention. In 2019, 1,219 insect genome-sequencing projects have been registered with the National Center for Biotechnology Information (NCBI) [29]. The biggest individual project related to insect genomes today is **The i5K Initiative**, which started at 2011 and aims to sequence 5000 high-priority insects genomes. I5K database currently contains genomes from 74 species and is continuously updated (https://i5k.nal.usda.gov/) [30].

Most of the enzymes involved in moth pheromone biosynthesis characterized so far are fatty acyl-CoA desaturases (FADs) and fatty acyl reductases (FARs). According to the review by Tupec *et al.*, more than 50 FADs and 20 FARs are functionally characterized [31]. FADs can have different specificities and introduce double bonds in various positions. The most common desaturation appears to be in positions Δ9 and Δ11, however in some lepidopteran insects less common desaturation reactions occur. Δ5 and Δ6 desaturases acting on myristic acid were found in the genus *Ctenopseustis* [32,33], a Δ8 desaturase obtained from *Dendrolimus punctatus* showed activity towards C12, C14 and C16 saturated fatty acids [34], and a Δ10 desaturase was found in *Planotortrix octo* [35]. In a study by Xia *et al.*, multi-functional Δ11/Δ12 desaturases from *S. exigua* and *S. litura* were characterized [19]. A multi-functional Δ11/Δ13 desaturase acting on palmitic acid was found in *Thaumetopoea pityocampa* [36]. A desaturase from *Ostrinia nubilalis* showed Δ14 activity on palmitic acid when expressed in insect cells [37]. A range of FARs from the genera *Agrotis*, *Bicyclus*, *Bombyx*, *Helicoverpa*, *Heliothis*, *Ostrinia*, *Spodoptera*, and *Yponomeuta* have been functionally characterized [31]. The specificity of the reductases differs a lot. A few reductases have a limited range of substrates, such as *Ostrinia nubilalis* reductases pgFAR-Z and pgFAR-E with a strong preference for *Z*11-14:CoA and *E*11-14:CoA, respectively [38] or reductase pgFAR from *Bombyx mori* with a preference towards *E*10,*Z*12-16:CoA [39]. Reductases from *Helicoverpa* spp. and *Heliothis* spp. can act on a broad range of C8 to C16 fatty acids while having preference for C14 substrates [40]. Four reductases from *Spodoptera* species characterized by Antony *et al.* showed different selectivity for C14 and C16 fatty acids where SexpgFAR I from *S. exigua* and SlitpgFAR I from *S. littoralis* were selective for C16 fatty acids while C14 fatty acids were preferred substrates for SexpgFAR II and SlitpgFAR II [41]. Other reductases, such as AseFAR from *Agrotis segetum* or reductases Yev-pgFAR, Ypa-pgFAR, and Yro-pgFAR from the genus *Yponomeuta* have a broad substrate range [21,42].

A large fraction of moth pheromones are fatty alcohol acetates or fatty aldehydes. Fatty alcohol acetates are postulated to be produced by esterification of fatty alcohols by acetyltransferases. Presently, however, no insect acetyltransferases have been identified that act on fatty alcohols. Ding *et al.* expressed 34 genes potentially coding for acetyltransferases found in *A. segetum*, however, none of the candidates were able to convert fatty alcohols into acetates in *S. cerevisiae* [21]. Interestingly, some background acetylation was detected in *S. cerevisiae* host and was later found to be due to Atf1p, a promiscuous acetyltransferase contributing to production of acetate esters by the yeast [43].

Regarding aldehyde-producing enzymes, gene candidates have been proposed, but none of them are cloned and characterized [26]. Similarly, some β-oxidation enzymes that are supposedly involved in the biosynthesis of multiple pheromones of shorter chain lengths have been identified, but not yet characterized [24,26].

Production of insect pheromones in yeasts

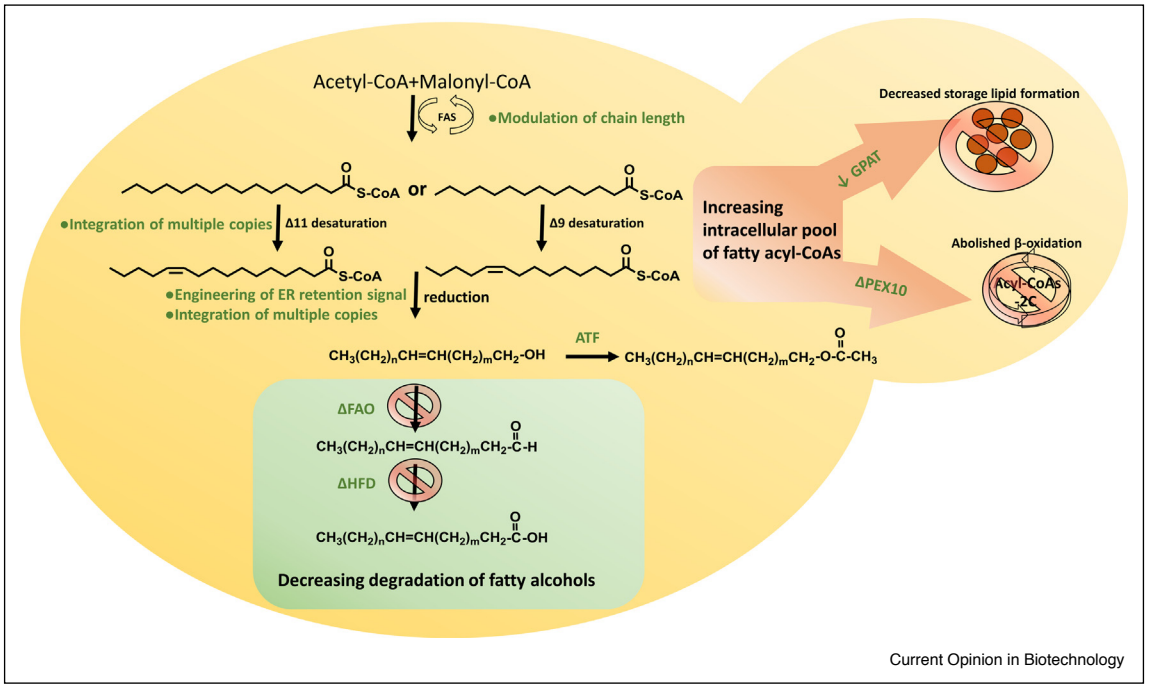
The high cost of chemical synthesis of pheromones encouraged the development of microbial cell factories for the production of pheromones by fermentation of cheap renewable feedstocks.

By expressing a *Z*11-desaturase and a fatty acyl-CoA reductase from *A. segetum* in *S. cerevisiae*, Hagström *et al.* produced 0.195 mg/L of (*Z*)-11-hexadecenol (*Z*11-16:OH) [10••]. The product was extracted and oxidized to the corresponding aldehyde (*Z*11-16:Ald). The aldehyde

elicited specific electrophysiological response from male antennae of the tobacco budworm *Heliothis virescens*. While the titer was several orders of magnitude lower compared to what is required for economical pheromone production at scale, this study was the first proof-of-concept that lepidopteran pheromones can be made from simple carbon feedstock as glucose by recombinant yeasts.

High-level production of lepidopteran pheromones was achieved through metabolic engineering of oleaginous yeast *Yarrowia lipolytica* [44••] (Figure 2). Target compounds were *Z*11-16:Ald, the main sex pheromone component of cotton bollworm *Helicoverpa armigera* and (*Z*)-9-tetradecenyl acetate (Z9-14:OAc), which is the sex pheromone of fall armyworm *Spodoptera frugiperda*. The screening of enzyme combinations was accomplished in *S. cerevisiae*. The combination of a desaturase from *A. transitella* (AtrΔ11) and a reductase from *H. armigera* (HarFAR) was the most efficient for the production of *Z*11-16:OH, which could be chemically oxidized to *Z*11-16:Ald. For the production of *Z*9-14:OAc, a combination of a desaturase from *Drosophila melanogaster* (DmeΔ9), the reductase HarFAR, and the acetyltranferase Atf1p from *S. cerevisiae* gave the best result. The assembled pathways were subsequently expressed in oleaginous yeast *Yarrowia lipolytica*, which was selected as the host for the production of moth pheromones due to its high levels of cytosolic acetyl-CoA and intracellular lipids, which both can serve as precursors for moth pheromone biosynthesis. In *Y. lipolytica*, the degradation of fatty acids and alcohols appeared to be one of the main obstacles for pheromone production in high titres. This problem was solved by deleting genes that are involved in fatty alcohol and fatty acid degradation. Deletion of peroxisomal biogenesis factor 10 encoded by the *PEX10* gene eliminated the formation of peroxisomes, in which β-oxidation of fatty acids takes place. Deletions of fatty aldehyde dehydrogenases encoded by *HFD1-4* genes and fatty alcohol oxidase *FAO1* reduced the oxidation of fatty alcohols into aldehydes and acids. A combination of deletions resulted in a 19-fold increase of the *Z*11-16:OH titer. Another obstacle for pheromone production in *Y. lipolytica* was the chanelling of a large fraction of pheromone precursors fatty acyl-CoAs into storage lipids. This was solved by truncating the promoter of the *GPAT* gene coding for the glycerol-3-phosphate acyltransferase, which resulted in 38% increase of the production. Finally, increasing the copy number of the desaturase and reductase genes led to 9.7-fold titer increase. In a 10 L bioreactor this strain produced 2.6 g/L of *Z*11-16:OH. The versatility of the same platform strain with decreased fatty alcohol degradation and decreased storage lipid formation was demonstrated by expressing the pathway towards *Z*9-14:OH,

Figure 2



Overview of metabolic engineering strategies applied for insect pheromone production in yeast. FAS: fatty acid synthase, ATF: acetyltransferase, HFD: fatty aldehyde dehydrogenase, FAO: fatty alcohol oxidase, GPAT: glycerol-3-phosphate acyltransferase, ER: endoplasmic reticulum, PEX10: peroxisomal biogenesis factor 10.

resulting in 4.9 mg/L titer. *In vivo* produced alcohols can be efficiently converted into acetates chemically by acetylation, as shown by Ding *et al.* [11••] leading to Z9-14:OAc, which is the main sex pheromone component of *S. frugiperda*. In order to improve the supply of tetradecenyl-CoA precursor, the ketoacyl synthase domain of fatty acid synthase *FAS2* was point-mutated as in [44••], resulting in 73.6 mg/L of *Z*9-14:OH or a 15-fold titer increase in *Y. lipolytica* [Holkenbrink et al]. A pheromone mixture containing aldehyde obtained from microbially produced *Z*11-16:OH performed as well as a conventionally produced commercial lure in field trapping experiments targetting *H. armigera*, clearly showing the potential of microbially produced moth pheromones in pest management.

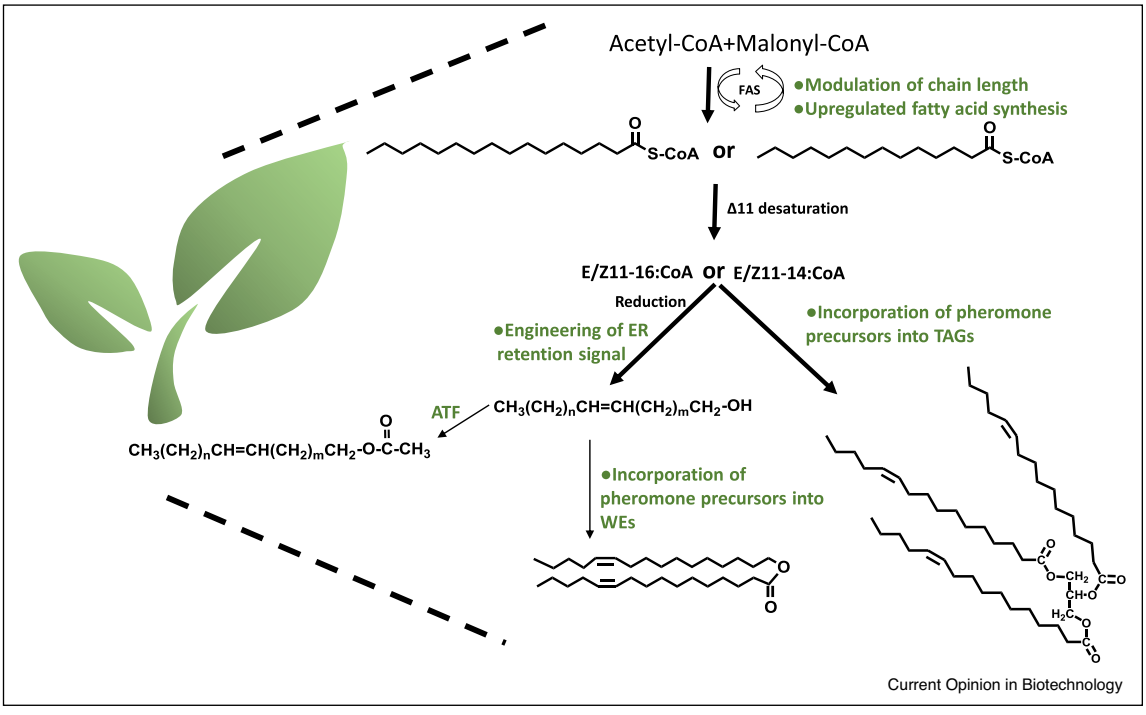
Instead of the direct production of fatty alcohol pheromones in the cell, an alternative approach was described, where the yeast cells are engineered for desaturation and the desaturated fatty acids are accumulated as lipids (Publication Number: WO/2017/214133). The yeast biomass is then dried, lipids extracted, transesterified, distilled, and chemically reduced to the corresponding desaturated fatty alcohols. In addition, exogenous fatty acids can be supplied to the yeast fermentation medium in order to boost the production of unsaturated pheromone precursors.

Production of insect pheromones in plants

Insect pheromones can be produced in plants and it has been proposed to use the plants as natural dispensers of insect pheromones in pest management [45]. Plants can also be used for production of pheromone precursors that can be subsequently extracted and transformed into active pheromone compounds (Figure 3, Table 1). The model plant *Nicotiana benthamiana* [11••] has been used to validate functionality of insect gene products in plants by transient expresion while the oil crop *Camelina sativa* has been engineered for accumulation of pheromone precursors by stable transformation [46•].

The first example of functional expression of lepidopteran enzymes in plants was reported by Nešněrová *et al.*, where a Δ11 insect desaturase was introduced into *Nicotiana tabacum* for semi-synthetic preparation of plant-derived *Z*11-16:OAc. The acetate made from the plant-produced precursor was proven to be effective and attracted the cabbage moth *Mamestra brassicae* in field trials [47••]. Ding *et al.* reconstructed the full pathway towards Z11-16:OAc in *Nicotiana benthamiana* by transient expression [11••]. Additionally, other unsaturated fatty acetates such as (*E*/*Z*)-11-tetradecenyl acetates (*E*/*Z*11-14:OAc) were biosynthesized. Eleven different genes were assayed in different constructs. For production of *Z*11-16:OAc, the most suitable combination consisted of a

Figure 3



Overview of metabolic engineering strategies applied for insect pheromone production in plants. FAS: fatty acid synthase, ER: endoplasmic reticulum, TAGs: triacylglycerides, WEs: wax esters, ATF: acetyltransferase.

Table 1

List of insect pheromones that have been produced in yeast and plants and proven to be biologically active. AveΔ11: *Argyrotaenia velutinana* desaturase, HarFar_KKYR: reductase from *Helicoverpa armigera* with modified ER retention signal, EaDAcT: *Euonymus alatus* acetyltransferase, AtrΔ11: *Amyelois transitella* desaturase, PDesat-TnΔ^{11}Z: *Trichoplusia ni* desaturase, AseΔ11: *Agrotis segetum* desaturase, AseFar: *Agrotis segetum* reductase, pgFAR: pheromone gland-specific reductase from *Bombyx mori*, FatB: acyl carrier protein thioesterase

Insect pheromone	Platform	Expressed genes	Characterization of compound	Reference
*E*11-14:OAc	*Nicotiana benthamiana*	AveΔ11, HarFar_KKYR, EaDAcT	Pheromone blend containing plant-derived *E*11-14:OAc attracted *Y. evonymella* and *Y. padella*	[11••]
*Z*11-14:OAc	*Nicotiana benthamiana*	AveΔ11, HarFar_KKYR, EaDAcT	Pheromone blend containing plant-derived *Z*11-14:OAc attracted *Y. evonymella* and *Y. padella*	[11••]
*Z*11-16:OAc	*Nicotiana benthamiana*	AtrΔ11, HarFar_KKYR, EaDAcT	Pheromone blend containing plant-derived *Z*11-16:OAc attracted *Y. padella*	[11••]
*Z*11-16:OAc	*Nicotiana tabacum*	PDesat-TnΔ^{11}Z	Plant-derived semi-synthetic *Z*11-16:OAc attracted *M. brassicae*	[47••]
*Z*11-16:Ald	*Yarrowia lipolytica*	AtrΔ11, HarFar	Pheromone blend containing yeast-derived *Z*11-16:Ald (semi-synthetically produced from Z11-16:OH) was confirmed to be biologically active against *H. armigera* based on electroantennogram assay and field trapping experiments	[44••]
*Z*11-16:Ald	*Saccharomyces cerevisiae*	AseΔ11, AseFar	Oxidized yeast extracts containing *Z*11-16:Ald elicited electrophysiological signal when tested on *H. virescens*	[10••]
*E*10,*Z*12–16:OH	*Saccharomyces cerevisiae*	pgFAR	Yeast cells producing *E*10,*Z*12–16:OH evoked stereotypical male mating behaviour of *B. mori*	[39]
*Z*11-16:OH *Z*11-16:Ald *Z*11-16:OAc	*Camelina sativa*	FatB, *Z*11 Desaturase	Mixture of plant-derived pheromones (semisynthetically produced from plant-prodced precursor) was as attractive as conventionally produced pheromone in *Plutella xylostella* trapping experiment	[46•]

desaturase from *A. transitella* (AtrΔ11), a reductase from *H. armigera* with modified C-terminus endoplasmic reticulum retention signal (HarFAR_KKYR), and an acetyltransferase from the burning bush *Euonymus elatus* (EaDAcT). Plants expressing these three genes yielded 2 μg of Z11-16:OAc per 1 g of leaf tissue. Best production of *E*/*Z*11-14:OAc was achieved when a thioesterase derived from *Cuphea palustris* (CpFATB2), a desaturase from *Argyrotaenia velutinana* (AveΔ11), HarFAR_KKYR and EaDAcT were used yielding 2.4 μg of *E*/*Z*11-14:OAc mixture per 1 g of leaf tissue. To obtain sufficient amounts of acetates for field tests of biological activity of the plant-derived pheromone compounds, the alcohol fraction from genetically modified plants was acetylated by reaction with acetyl chloride. The plant-derived acetate mixtures were as attractive and specific as chemically produced pheromones for trapping of the small ermine moth species *Yponomeuta padella* and *Y. evonymella* [11••].

Apart from being direct sources of insect pheromones plants hold an opportunity for accumulation of pheromone precursors in the forms of triacylglycols (TAGs) or wax esters (WEs). The oil crop *Camelina sativa* was investigated as a potential platform due to its short life cycle and capability to accumulate up to 49% of oil in seeds [48,49]. Additionally, transformation protocols are well-established [50]. Engineered *C. sativa* expressing palmitoyl-acyl carrier protein-specific thioesterase together with a Δ11 desaturase accumulated Z11-16:Acid at >20% of total fatty acid content in the seed oil, corresponding to around 40 g Z11-16:Acid per kg seeds. The pheromone precursor obtained from the seeds of the engineered plant was chemically converted into the corresponding alcohol, aldehyde and acetate and the mixture was proven to be as effective as their chemically produced equivalent in trapping experiments targeting *Plutella xylostella* [46•].

Perspectives

Increasing resistance towards *Bt* crops and insecticides with additional negative impact on the environment is a strong motivation to develop alternative approaches in pest management. Microorganisms and plants can provide platforms for sustainable and commercially viable production of insect pheromones but there are challenges to overcome before this technology is ready for the market [10••,11••] (Holkenbrink *et al.* [44••]). So far only relatively simple moth pheromones with a carbon length of 16 or 14 atoms and a double bond in position Δ9 or Δ11 have been produced recombinantly. Among these pheromones are *Z*11-16:Ald, a pheromone of cotton bollworm and rice stem borers, and *Z*9-14:OAc, the main pheromone component of the fall armyworm. These

pheromones are intended for pest control in cotton, rice, soybean, and maize, which are all row crops and where chemically synthesized pheromones are not applicable due to the high cost. There is also a need to establish biotechnological production of more challenging pheromone molecules, the ones that contain multiple double bonds or require chain shortening and other modifications. Examples are pheromones of codling moth, grapevine moth, oriental fruit moth that are already used for fruits protection. If low-cost production of these pheromones via biotechnology is established, it will enable a much wider use of pheromones and reduction of insecticide residues in fruits. In order to establish efficient microbial and plant factories for a wider spectrum of biological pheromone production, including a number of economically important pheromones with 12-carbon backbone (C12) and unusual double bond positions, elucidation of insect biosynthetic pathways and enzymes responsible for them is crucial. This can be largely facilitated by ongoing insect genome projects such as i5k [30]. A bottleneck in recombinant pheromone production is functional characterization. Currently, it is based on heterologous expression in hosts, mostly *S. cerevisiae*, where analysis rely on a low throughput combination of extraction of fatty acids or alcohols and gas chromatography–mass spectrometry. Up to now, many desaturases and reductases are cloned and characterized [31] while functionality of candidate β-oxidation enzymes, acetyltransferases and alcohol oxidases remains to be investigated and confirmed. Combination of genome and transcriptome sequencing efforts together with new high throughput screening technologies would open the door to an immense variety of insect compounds that could be produced in a bio-based manner. For an economically viable process the native metabolism of the production hosts have to be shifted towards desired precursors. This was done for C14 and C16 compounds in yeasts and plants by implementing various metabolic engineering strategies such as elimination of fatty acid/alcohol degradation, redirection of carbon flux from TAGs to acyl-CoAs, or implementation of chain-specific thioesterases [46•,44••]. Further studies towards efficient *in vivo* supply of precursors will be crucial for cost-effective production. Apart from enzyme discovery and metabolic engineering of production hosts, upscaling plays an important role in development of sustainable biological pheromone production for pest management. Regarding microbial production, technical aspects such as determination of optimal fermentation conditions and downstream processing are key points to consider, while in plant-based pheromone production downstream processing as well as regulatory affairs regarding genetically modified crops [51] are very important to address.

Conflict of interest statement

IB and CL are co-inventors on patent applications WO2016207339, WO2018109167, and WO2018109163. CL is a co-inventor on WO/2015/171057. KP and IB have financial interest in BioPhero ApS.

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CHAPTER 3

Production of moth sex pheromones for pest control by yeast fermentation

Carina Holkenbrink, Bao-Jian Ding, Hong-Lei Wang, Marie Inger Dam, Karolis Petkevicius, Kanchana Rueksomtawin Kildegaard, Leonie Wenning, Christina Sinkwitz, Bettina Lorantfy, Eleni Koutsoumpeli, Lucas França, Marina Pires, Carmem Bernardi, William Urrutia, Agenor Mafra-Neto, Bruno Sommer Ferreira, Dimitris Raptopoulos, Maria Konstantopoulou, Christer Lofstedt, Irina Borodina

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ABSTRACT

The use of insect sex pheromones is an alternative technology for pest control in agriculture and forestry, which, in contrast to insecticides, does not have adverse effects on human health or environment and is efficient also against insecticide-resistant insect populations. Due to the high cost of chemically synthesized pheromones, mating disruption applications are currently primarily targeting higher value crops, such as fruits. Here we demonstrate a biotechnological method for the production of (*Z*)-hexadec-11-en-1-ol and (*Z*)-tetradec-9-en-1-ol, using engineered yeast cell factories. These unsaturated fatty alcohols are pheromone components or the immediate precursors of pheromone components of several economically important moth pests. Biosynthetic pathways towards several pheromones or their precursors were reconstructed in the oleaginous yeast *Yarrowia lipolytica,* which was further metabolically engineered for improved pheromone biosynthesis by decreasing fatty alcohol degradation and downregulating storage lipid accumulation. The sex pheromone of the cotton bollworm *Helicoverpa armigera* was produced by oxidation of fermented fatty alcohols into corresponding aldehydes. The resulting yeast-derived pheromone was just as efficient and specific for trapping of *H. armigera* male moths in cotton fields in Greece as a conventionally produced synthetic pheromone mixture. We further demonstrated the production of (*Z*)-tetradec-9-en-1-yl acetate, the main pheromone component of the fall armyworm *Spodoptera frugiperda*. Taken together our work describes a biotech platform for the production of commercially relevant titres of moth pheromones for pest control via yeast fermentation.

1. Introduction

Mating disruption (MD) with sex pheromones is an efficient, safe and environmentally friendly method for pest control instead of using toxic insecticides (Reddy et al., 2010; Benelli et al., 2019). Mating disruption occurs when synthetic pheromones are released into the air of the fields, where they even in modest amounts prevent males from locating females, which disrupts insect reproduction and thereby also the insect infestation. Although the method has been around for over four decades, its usage is still very limited, with about 750,000 ha being treated with MD worldwide, which makes only 0.05% of the total arable and permanent crops area. Most of the MD-treated crops are high-value crops, as apples, grapes, citruses (Ioriatti and Lucchi, 2016). The method is currently too expensive to be applied on the lower-value row crops, such as corn, soybean, cotton, and sorghum.

The majority of identified moth (Lepidoptera) sex pheromone

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components are unsaturated fatty alcohols, alcohol acetates, or aldehydes. These compounds are derived from the insect fatty acid metabolism and have 10–18 carbon-long skeletons. They are called Type I moth pheromone components and constitute approximately 75% of all known moth sex pheromone components (Ando et al., 2004; Löfstedt et al., 2016). The chemical diversity is to a large extent produced by the combined action of specific fatty acyl-CoA desaturases, fatty acyl-CoA reductases, and by chain-shortening (Bjostad and Roelofs, 1983). Lepidopteran desaturases and reductases are embedded in the endoplasmic reticulum. Desaturases act by introducing an oxygen atom into a specific location of a fatty acyl-CoA chain and then removing a water molecule to generate a double bond of *cis*- or *trans*-configuration (Sperling et al., 2003). The remaining oxygen atom from the molecular oxygen is reduced using the electrons obtained from NADH through cytochrome *b*5 reductase and cytochrome *b*5. Lepidopteran fatty acyl-CoA reductases use two NADPH molecules to directly generate fatty alcohol (Matsumoto et al., 1996). Desaturase and reductase activities are typically assayed by expressing them in heterologous systems, e.g., in *S. cerevisiae* devoid of the native elongase Elo1p and desaturase Ole1p activities, in insect cells, or in plant *Nicotiana benthamiana* (Petkevicius et al., 2020).

There is a potential in engineering oil plants to accumulate pheromone precursors in the form of lipids. Proof-of-concept production of several pheromone precursors has been established in *Nicotiana* species,

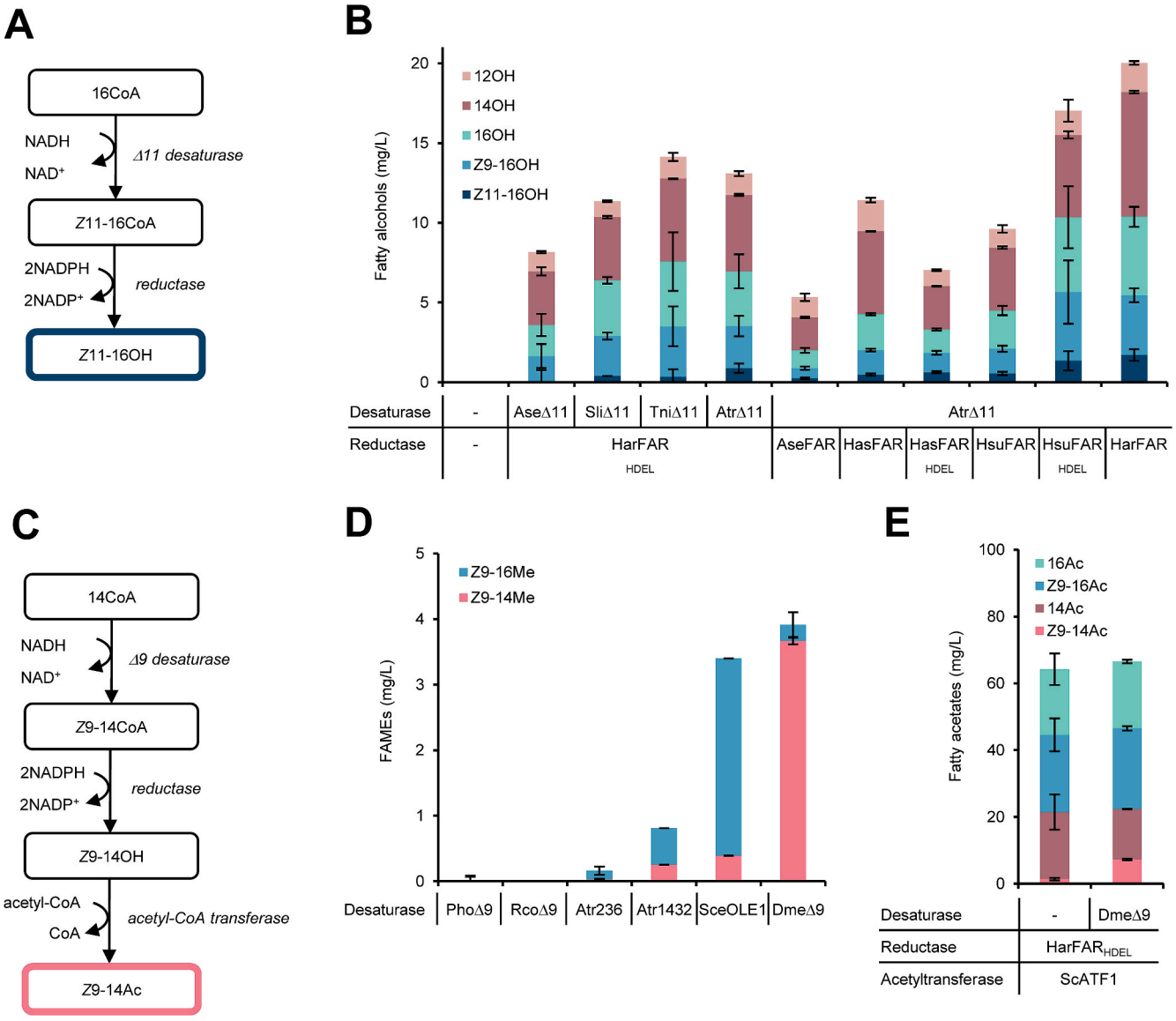

Fig. 1. Biosynthesis of moth sex pheromone compounds in yeast. **A.** Biosynthetic pathway towards (*Z*)-hexadec-11-en-1-ol (*Z*11-16OH). **B.** Concentrations of fatty alcohols in the cultures of *S. cerevisiae* expressing combinations of desaturases and reductases from different lepidopteran species. **C.** Biosynthetic pathway towards (*Z*)-tetradec-9-en-1-yl acetate (*Z*9-14Ac). **D.** Concentrations of fatty acids (analysed as methyl esters) in *S. cerevisiae* cells that express plant and insect desaturases. **E.** Concentrations of fatty alcohol acetates in cultures of *S. cerevisiae* expressing combinations of desaturase, reductase and acetyl-CoA transferase genes. The cultivations were performed at small-scale in tubes or deep-well plates in biological triplicates. The average values and standard deviations are shown.
Abbreviations: AseΔ11 - Δ11 desaturase from Agrotis segetum, SliΔ11 desaturase - Δ11 desaturase from Spodoptera littoralis, TniΔ11 - Δ11 desaturase from Trichoplusia ni, AtrΔ11 - Δ11 desaturase from Amyelois transitella, HarFAR – fatty acyl-CoA reductase from Helicoverpa armigera, AseFAR - fatty acyl-CoA reductase from A. segetum, HasFAR - fatty acyl-CoA reductase from Helicoverpa assulta, HsuFAR - fatty acyl-CoA reductase from Heliothis subflexa, PhoΔ9 - Δ9 desaturase from Pelargonium x hortorum, RcoΔ9 - Δ9 desaturase from Ricinus communis, Atr236 – desaturase 236 from A. transitella, Atr1432 – desaturase 1432 from A. transitella, SceOLE1 - Δ9 desaturase from S. cerevisiae, DmeΔ9 - Δ9 desaturase from Drosophila melanogaster, SceATF1 - alcohol acetyltransferase from S. cerevisiae, FARHDEL – modified desaturase, where the C-terminal insect signal peptide was replaced with 4-amino acid signal peptide from S. cerevisiae.

but the content was too low for commercial exploitation (Ding et al., 2014; Xia et al., 2020). In another study, a model oil plant *Camelina sativa* was genetically modified to express a palmitoyl-acyl carrier protein-specific thioesterase together with a Δ11 desaturase. The content of (*Z*)-hexadec-11-enoic acid (*Z*11-16Acid) in the seed oil was at 20% of the total fatty acids, which corresponds to around 40 g Z11-16Acid per kg seeds (Ortiz et al., 2020). Recombinant production of pheromone precursors in plants is complicated by the long development times of stable plant lines, costly regulatory procedures, and containment measures for growing genetically modified plants. In contrast, microbial cell factories can be readily engineered and cultured at large scale in contained bioreactors. So far, there has been only a single previous study, where insect pheromone pathway was expressed in a microbe with the purpose of establishing a cell factory for pheromone production. Specifically, a Δ11 fatty acyl-CoA desaturase and reductase from the turnip moth *Agrotis segetum* was expressed in *S. cerevisiae* (Hagström et al., 2013). However, as no metabolic engineering had been performed on the host, the titer of the product (*Z*)-hexadec-11-en-1-ol was only at ca. 0.2 mg/L, which is four-five orders of magnitude lower than what is required for economical pheromone manufacturing at scale.

The aim of this study was to optimize the production of unsaturated fatty alcohols in yeast and to validate the biological activity of the yeast-derived moth pheromone components. We have engineered oleaginous yeast *Yarrowia lipolytica* as the cell factory and demonstrated production of two common moth pheromone pheromone components, (*Z*)-hexadec-11-en-1-ol and (*Z*)-tetradec-9-en-1-ol, which are the immediate precursors of the corresponding, economically important, aldehyde and acetate pheromone components.

2. Results and discussion

2.1. Establishing pathways towards moth pheromones in yeast

To establish pathways towards moth pheromone compounds in yeast, we first investigated a range of fatty acyl-CoA desaturases and reductases for the production of (*Z*)-hexadec-11-en-1-ol (*Z*11-16OH) (Fig. 1A) by expressing the enzymes in combinations in baker's yeast *Saccharomyces cerevisiae*. The fermented *Z*11-16OH can be chemically oxidized into (*Z*)-hexadec-11-enal (*Z*11-16Ald), which is the main sex pheromone component of several row crop pests, such as the cotton bollworm *Helicoverpa armigera*, the striped rice stemborer *Chilo suppressalis*, and the yellow rice stemborer *Scirpophaga incertulas* (El-Sayed, 2014). The combination of a desaturase from *Amyelois transitella* and a reductase from *H. armigera* resulted in the highest titre of 1.7 ± 0.4 mg/L *Z*11-16OH (Fig. 1B), which was an order of magnitude enhancement in comparison to the previous study (Hagström et al., 2013). The improvement was likely due to the utilization of a desaturase variant with a higher activity in yeast and due to expression of the genes from constitutive promoters using constructs stably integrated into the yeast genome (Jensen et al., 2014).

Next, we wanted to achieve the biosynthesis of (*Z*)-tetradec-9-en-1-yl acetate (*Z*9-14Ac), which is the main sex pheromone component of the fall armyworm *Spodoptera frugiperda*, a rising pest with a high occurrence of insecticide resistance (Binning et al., 2014; Xia et al., 2020). For this, we searched for a Δ9-desaturase with a higher activity and specificity towards tetradecanoyl-CoA than to hexadecanoyl-CoA (Fig. 1C). The activities of six heterologous desaturase candidates were tested in a *S. cerevisiae ole1*Δ*elo1*Δ strain devoid of the native desaturation and elongation activities. The cells were cultivated with supplementation of methyl tetradecanoate (14Me) and the total lipids were analysed to determine the desaturated fatty acids (Fig. 1D). The strain expressing the desaturase from *Drosophila melanogaster* resulted in the highest concentration of 3.67 ± 0.99 mg/L methyl (*Z*)-tetradec-9-enoate (*Z*9-14Me) and in the highest *Z*9-14Me to methyl (*Z*)-hexadec-9-enoate (*Z*9-16Me) ratio, indicating a higher specificity towards the tetradecanoyl-CoA substrate. To establish the complete pathway towards *Z*9-14Ac in the yeast *S. cerevisiae*, we expressed the *D. melanogaster* Δ9 desaturase together with the *H. armigera* reductase and *S. cerevisiae ATF1* known to catalyse acetylation of fatty alcohols (Ding et al., 2016). The resulting strain produced 7.3 ± 0.2 mg/L of *Z*9-14Ac in comparison to 1.4 ± 0.4 mg/L in an analogous strain lacking the heterologous Δ9 desaturase (Fig. 1E).

2.2. Optimization of the oleaginous yeast Yarrowia lipolytica for moth pheromone production

We rationalized that an oleaginous yeast should be a more suitable cell factory for production of fatty alcohol-based moth pheromones than the baker's yeast that has a low content of the fatty acid precursor acetyl-CoA in the cytosol and can only accumulate small amounts of intracellular lipids. In contrast, the oleaginous yeast *Yarrowia lipolytica* has a naturally high fatty acid metabolism and has been engineered for commercial production of polyunsaturated omega-3 fatty acids (Xue et al., 2013) and for production of lipids (Shaw et al., 2016). Robust genetic tools, including the CRISPR/Cas9 method, have recently been developed for *Y. lipolytica,* and allow the rapid engineering of this yeast species (Holkenbrink et al., 2018; Stovicek et al., 2015).

The first hurdle we encountered when co-opting *Y. lipolytica* for the production of pheromones, was the prevention of endogenous degradation of the target fatty alcohols *Z*11-16OH and (*Z*)-tetradec-9-en-1-ol (*Z*9-14OH). We deleted one by one and in combination the genes encoding the enzymes potentially implicated in fatty alcohol degradation: fatty aldehyde dehydrogenases Hfd1p and Hfd4p (Iwama et al., 2014), as well as fatty alcohol oxidase Fao1p (Iwama et al., 2015) (Fig. 2A). Moreover, we deleted peroxisomal biogenesis factor Pex10p, thus interrupting the correct assembly of peroxisomes and preventing acyl-CoA degradation. Single deletions of *HFD1/HFD4/FAO1/PEX10* genes increased the titre of *Z*11-16OH two-to three-fold, while the combination of four deletions resulted in a 19-fold titre increase (Fig. 2B). The quadruple deletion strain (ST5789) produced 14.9 ± 3.6 mg/L of *Z*11-16OH in comparison to 0.8 ± 0.1 mg/L produced by a reference strain only expressing the biosynthetic pathway towards *Z*11-16OH (ST3844).

When strains ST3844 and ST5789 were incubated with externally supplied 1 g/L *Z*11-16OH and 1 g/L *Z*9-14OH each, strain ST3844 largely degraded the supplied alcohols, with only 6.2 ± 3.8 mg/L *Z*11-16OH left at the end of the cultivation. In contrast to that, strain ST5789 showed a remaining concentration of 630.9 ± 137.1 mg/L *Z*9-14OH and 620.3 ± 73.9 mg/L *Z*11-16OH. Less than 1 g/L of fatty alcohols were recovered probably due to evaporation and some losses during the recovery procedure. A control, which contained only cultivation medium and externally supplied fatty alcohols, showed a remaining concentration of 500.7 ± 135.7 mg/L *Z*9-14OH and 536.9 ± 166.1 mg/L *Z*11-16OH (Fig. S1). The experiment confirmed that the degradation rate of fatty alcohols was much lower in the strain with deletion of *HFD1*, *HFD4*, *PEX10*, and *FAO1* genes.

Another challenge with *Y. lipolytica* as a host was to reduce the channelling of fatty acyl-CoAs, the fatty alcohol precursors, into storage lipids. We hence downregulated the expression of the gene encoding glycerol-3-phosphate acyltransferase (GPAT), which catalyses the first committing step towards glycerolipid- and glycerophospholipid biosynthesis. The downregulation was achieved by truncating the *GPAT* promoter to 100 base pairs and confirmed by qRT-PCR (Fig. S2.A). The downregulation of *GPAT* improved the titre of *Z*11-16OH from 14.9 ± 3.6 mg/L to 20.6 ± 5.4 mg/L (Fig. 2B). At the same time, the total fatty acid content of the cells was reduced by 53% (Fig. S2.B, C). The combination of *Y. lipolytica* genome edits that reduce the fatty alcohol degradation and lipid accumulation thus resulted in a basic platform chassis, where various moth pheromone pathways can be inserted.

The strain, however, predominantly produced fatty alcohols of 16-carbon chain length. In order to enable the production of 14-carbon

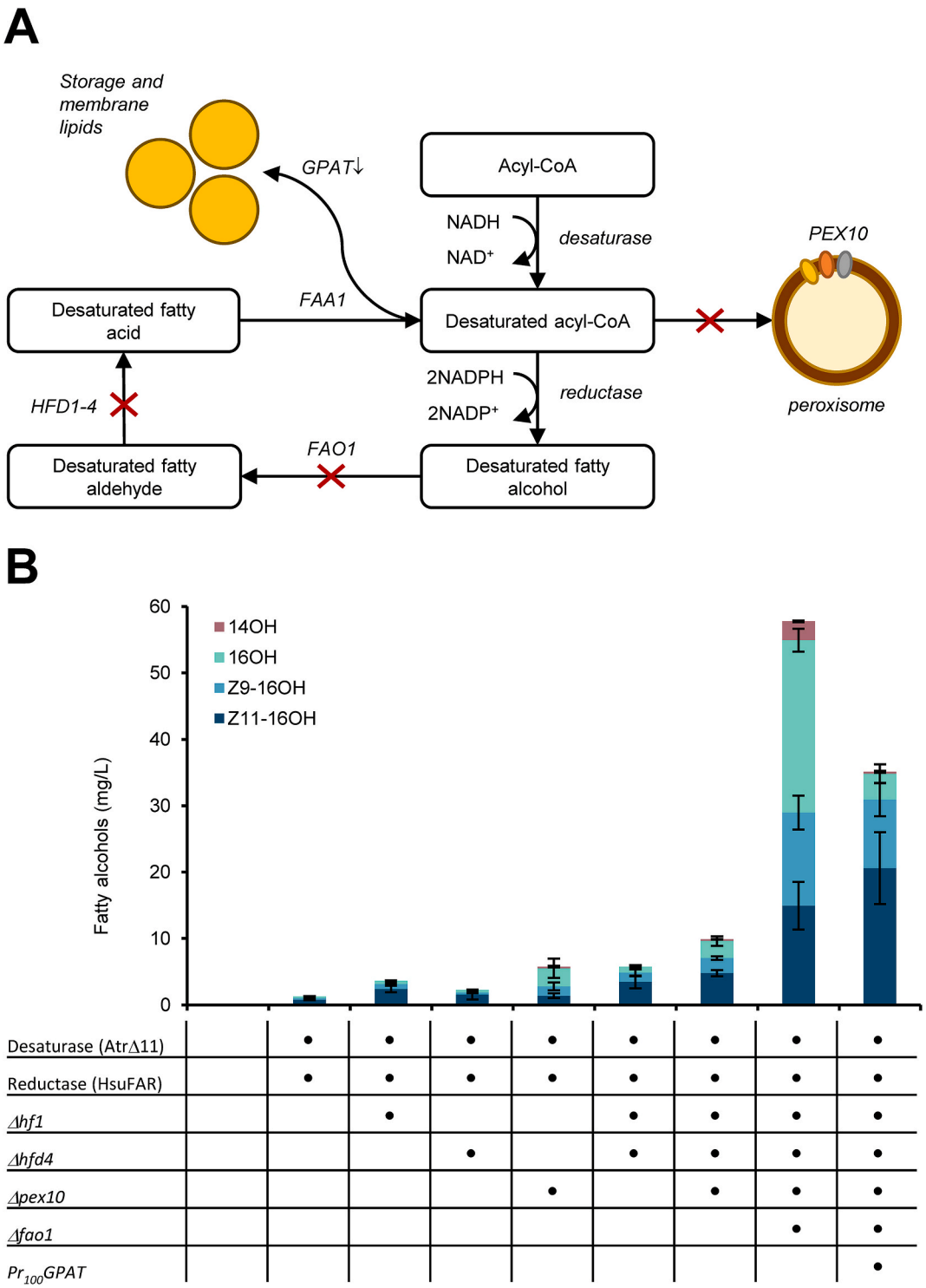

Fig. 2. Metabolic engineering of the oleaginous yeast *Y. lipolytica* towards enhanced production of moth pheromone alcohols. **A.** Overview of metabolic engineering strategies. **B.** Concentrations of fatty alcohols produced by engineered *Y. lipolytica* strains. The cultivations were performed at small-scale in tubes or deep-well plates in biological triplicates. The average values and standard deviations are shown. Abbreviations: *PEX10* – peroxisomal biogenesis factor, *FAO1* – fatty alcohol oxidase, *HFD1-4* – fatty aldehyde dehydrogenases, *FAA1* – fatty acyl-CoA synthetase, *GPAT* - glycerol-3-phosphate acyltransferase. The gene name abbreviations are as in Fig. 1.

pheromones, we introduced a mutation into fatty acid synthase subunit Fas2p^{I1220F}, which was previously reported to benefit the biosynthesis of tetradecanoyl-CoA (Rigouin et al., 2017). We expressed the pathway towards *Z*9-14OH in the engineered *Y. lipolytica* strains and obtained 4.9 ± 1.4 mg/L titre in the basic platform chassis and 73.6 ± 16 mg/L *Z*9-14OH in the platform chassis with additional Fas2p mutation (Fig. 3B). The mutation thus resulted in a 15-fold improvement of a 14-carbon product and should be beneficial for producing also other pheromones derived from tetradecanoyl-CoA.

To further improve the production of *Z*11-16OH, we integrated additional copies of desaturase and reductase genes to pull the flux towards pheromone biosynthesis. Integration of the second copy of the

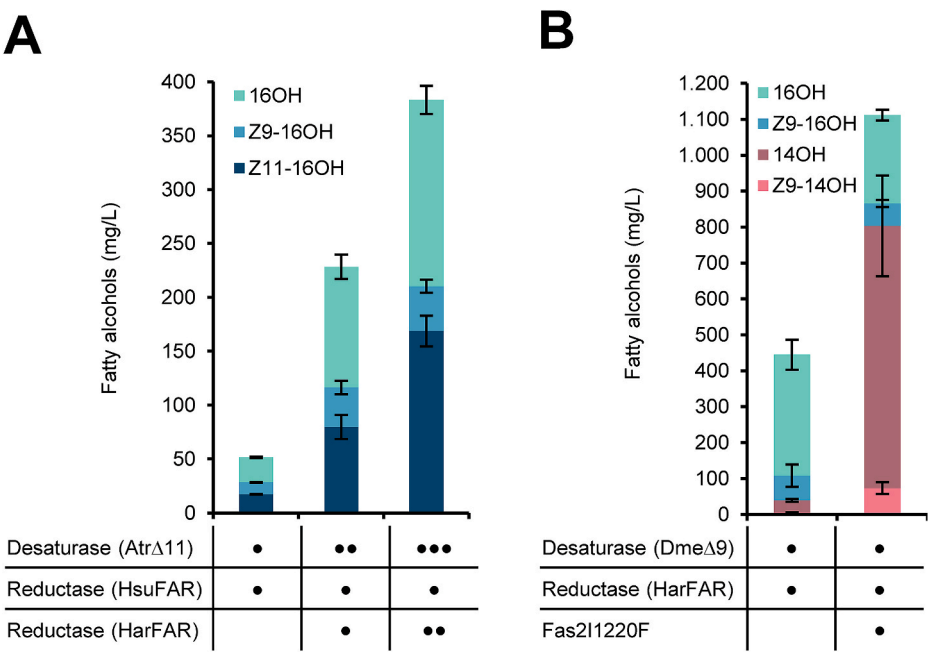

Fig. 3. Production of two lepidopteran pheromone alcohols in engineered *Y. lipolytica* strains. **A.** Production of (*Z*)-hexadec-11-en-1-ol (*Z*11-16OH) in the engineered *Y. lipolytica* host strain (Δ*hfd1*Δ*hfd4*Δ*pex10*Δ*fao1*P_{GPAT}100) harbouring different copy numbers of the pathway. The dots indicate the number of the integrated gene copies. **B.** Production of (*Z*)-tetradec-9-en-1-ol (*Z*9-14OH) in the *Y. lipolytica* host strain additionally engineered by mutating the fatty acid synthase subunit *FAS2*. The cultivations were performed at small-scale in tubes or deep-well plates in biological triplicates. The average values and standard deviations are shown. The gene name abbreviations are as in Fig. 1.

pathway increased the titre 4.6-fold. The strain with three copies of the pathway produced 169 ± 14 mg/L *Z*11-16OH, a 9.7-fold increase in comparison to the single copy strain (Fig. 3A). When the optimized yeast strain was fermented in a 10L-bioreactor, 2.57 g/L of the target product *Z*11-16OH was obtained. The fatty alcohols were extracted from the yeast biomass using organic solvents and purified on a silica column. The eluted fractions with a high content of the product were pooled and oxidized into corresponding aldehyde using tetrakisacetonitrile copper (I) triflate/TEMPO catalyst system (Hoover and Stahl, 2011). The composition of the aldehyde preparation was *Z*11-16Ald, hexadecanal (16Ald), and (*Z*)-hexadec-9-enal (*Z*9-16Ald) in ratio 82:13:5 (Fig. S3, S4). The *Z*11-16Ald is the major and *Z*9-16Ald is the minor pheromone component in *H. armigera* and *C. suppressalis*, where the reported ratios between the two pheromone components in *H. armigera* were from 99:1 to 93:7 (Dunkelblum et al., 1980; Nesbitt et al., 1980; Zhang et al., 2012), in *C. suppressalis* the reported ratio is 90:10 (Tatsuki et al., 1983).16Ald is also present in the pheromone glands of both insect species, but it does not elicit a behavioural response. The composition of the yeast-derived pheromone may thus be close enough and well suited for trapping and mating disruption of these insect species with *Z*11-16Ald as a major and *Z*9-16Ald as a minor pheromone component. The biologically produced pheromone mix was subsequently subjected to activity tests on *H. armigera* in the laboratory and field.

2.3. Electrophysiological responses of male H. armigera

We measured the electroantennographic responses of male *H. armigera* adults to the yeast-derived pheromone blend (Bio-Ald), standard compounds, and mixtures of the standards (Fig. 4). Ald mix #1 contained *Z*11-16Ald, *Z*9-16Ald, tetradecanal (14Ald), and pentadecanal (15Ald) (80:5:5:5, respectively). Ald mix #2 contained equal volumes of each of the same components as Ald mix #1 (25:25:25:25 ratio).

Bio-Ald elicited the same magnitude of response on the male antenna as Ald mix #1 and significantly higher to that of the equivolume Ald mix #2 and to *Z*9-16Ald, the secondary compound of the *H. armigera* pheromone. The major sex pheromone compound, *Z*11-16Ald, yielded a high antennal response, whereas the minor sex pheromone, *Z*9-16Ald, induced a considerably lower response. The significantly lower response to Ald mix #2 is a clear indication that the antennal response is mainly attributed to *Z*11-16Ald and when its quantity in the mixture is lowered,

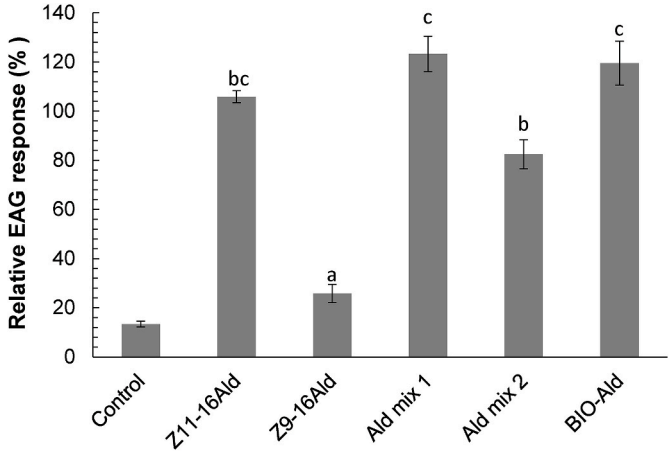

Fig. 4. Electrophysiological responses of male *H. armigera* antennae to yeast-derived pheromone blend (Bio-Ald), standard compounds (*Z*11-16Ald, *Z*9-16Ald) and mixtures of the standard compounds (Ald mix#1, Ald mix#2) (±SEM). Ald mix #1 contained *Z*11-16Ald, *Z*9-16Ald, tetradecanal (14Ald), and pentadecanal (15Ald) (80:5:5:5, respectively). Ald mix #2 contained equal volumes of each of the same components as Ald mix #1 (25:25:25:25 ratio). Means followed by the same letter are not significantly different (P> 0.05, Tukey's studentized range [HSD] test, F=21.491, df=110, P=0.000).

the antennal response also drops. These results indicate that biologically produced *Z*11-16Ald induces the same magnitude of sensory stimulation as the chemically synthesized *Z*11-16Ald, the major compound of the moth's native pheromone.

2.4. Monitoring of H. armigera flight in the field

The pheromone blend was further tested by trap catches of the cotton bollworm *H. armigera* in two cotton fields in Northern Greece. The cotton-growing areas have moderate moth populations with occasional regional and temporal outbursts (Milonas et al., 2016). Mean weekly male catches in traps baited with yeast-derived pheromone (Bio-Ald) and synthetic pheromone (*Z*11-16Ald: *Z*9-16Ald at 97:3 ratio, Control) dispensers are shown in Fig. 5 for two independent trials. Capture data

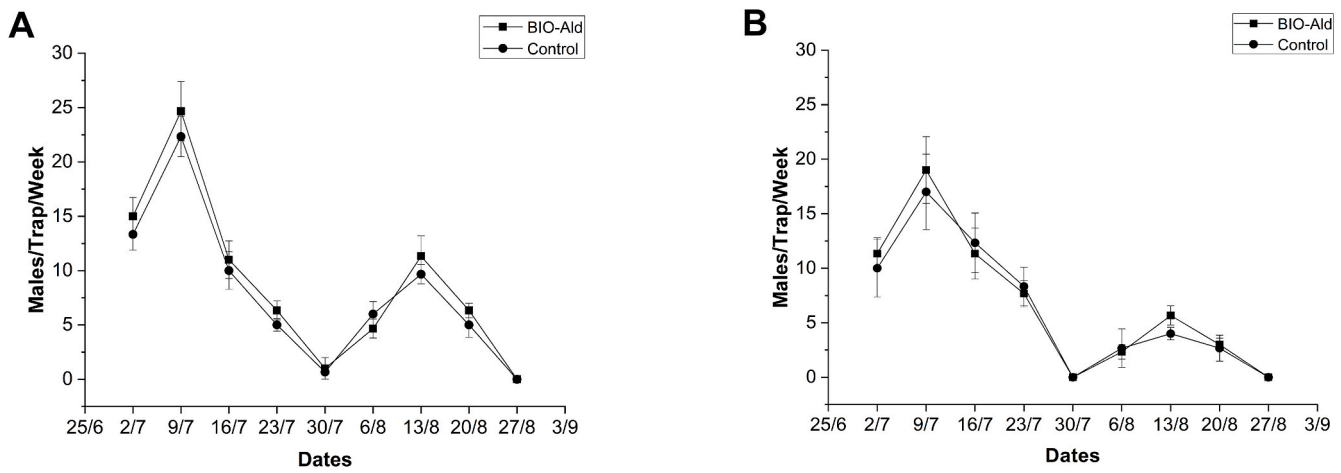

Fig. 5. Monitoring of *H. armigera* flight in Greece (2019). Captures of males in pheromone traps baited with commercial synthetic pheromone (Control and yeast-derived pheromone (Bio-Ald) per week (±SEM): (A) in Thermi, Northern Greece (ANOVA, F = 1.997, df = 5, P = 0.231) and (B) Lamia, Central Greece (ANOVA, F = 0.189, df = 5, P = 0.686).

from the traps indicated that the flight peak occurred on the 2nd week of July (24.7 ± 2.7 males/trap/week for Bio-Ald traps and 22.3 ± 1.8 for the Control traps at Thermi and 19.3 ± 3.0 males/trap/week for Bio-Ald traps and 17.0 ± 3.5 for the Control traps at Lamia). In both regions, the total number of males trapped with the different lures were not significantly different (Bio-Ald: 80.3 ± 4.3 males/trap, Control: 72.0 ± 4.0 males/trap at Thermi and Bio-Ald: 60.3 ± 4.3 males/trap and Control: 57.0 ± 3.6 males/trap at Lamia). It is apparent that the biologically produced pheromone was equally effective under field conditions as the commercially available chemically synthesized one.

3. Conclusions

In summary, we have demonstrated biological production of practically and commercially relevant titers of several lepidopteran sex pheromone components or their precursors in yeast cell factories. A biocatalytic production is particularly advantageous for the production of chemicals for which isomeric composition is critical, such as moth pheromones (Löfstedt et al., 2016). The enzymes can deliver the required stereoisomers, while in chemical synthesis, a mix of isomers is often obtained and may be difficult to separate especially in large quantities. Furthermore, the fermentation is carried out in a cheap medium with glycerol as the sole carbon source, using yeast cells as the only catalyst. This is in contrast to chemical synthesis that will typically require special starting material, expensive catalysts, and several synthesis steps (Yadav and Reddy, 1988; Herbert et al., 2013). Reduced production costs and lower environmental impact of the biotech route in comparison to the chemical synthesis have been established for multiple chemicals, particularly for natural products (Pellis et al., 2018; Jullesson et al., 2015). As an additional advantage, major and minor pheromone components can be produced in a single process in a ratio that is suitable for the target insect. The work creates the foundation for the production of pheromones at a lower cost enabling pheromone-based pest control in row crops, such as rice, cotton, and maize.

4. Materials and methods

4.1. DNA assembly and yeast strain construction

Heterologous genes were codon-optimized for *S. cerevisiae* or *Y. lipolytica* and synthesized (GeneArt, Thermo Scientific). The vectors for gene expression and knock-outs were assembled and transformed into yeast according to the published methodologies (Jensen et al., 2014; Stovicek et al., 2015; Holkenbrink et al., 2018; Jessop-Fabre et al. EasyClone-MarkerFree, 2016). The sequences of the genes and primers, the schemes for gene amplification, cloning and strain assembly are provided in Tables S1-5. The diagrams on Fig. S5 illustrate the workflow for the construction of engineered yeast strains. Yeast strain *Y. lipolytica* GB20 was a kind gift of Volker Zickermann (Goethe-University Frankfurt am Main, Germany). Yeast strain *S. cerevisiae* CEN.PK102–5B was obtained from Peter Kötter (Goethe-University Frankfurt am Main, Germany). Strain *Y. lipolytica* Y-17536 was received from the Agricultural Research Service (NRRL, USA). For removal of selection markers from the genome of *Y. lipolytica*, we used CreA recombinase gene obtained from plasmid pSH66 (EUROSCARF selection).

4.2. Chemicals and media

All chemicals were purchased from Sigma-Aldrich. Pheromone standards were purchased from Pherobank. Nourseothricin was from WERNER BioAgents.

Cultivation of yeast strains.

Fig. 1b: One individual clone of each strain was inoculated into 2 mL medium (100 g/L glucose, 2 g/L yeast extract, 0.33 g/L $(NH_4)_2SO_4$, 1.33 g/L $MgSO_4{\cdot}7H_2O$ 1.33, 0.267 g/L NaCl, 2 mL/L trace metals solution (4.5 g/L $CaCl_2{\cdot}2H_2O$, 4.5 g/L $ZnSO_4{\cdot}7H_2O$, 3 g/L $FeSO_4{\cdot}7H_2O$, 1 g/L H_3BO_3, 1 g/L $MnCl_2{\cdot}2H_2O$, 0.4 g/L $Na_2MoO_4{\cdot}2H_2O$, 0.3 g/L $CoCl_2{\cdot}6H_2O$, 0.1 g/L CuSO4 · $5H_2O$, 0.1 g/L KI, 15 g/L EDTA), 8 mg/L thiamine, 0.67 mg/L biotin, 20 mg/L uracil, 380 mg/L leucine, 76 mg/L histidine, 100 mM potassium hydrogen phthalate buffer) in a 12-mL glass tube (Duran, Wertheim, Germany) with metal labocap lids (Lüdiswiss, Flawil, Switzerland) and incubated for 48 h at 30 °C with shaking at 250 rpm.

Fig. 2b: One individual clone of each strain was inoculated into 5 mL YPD medium with 8% glucose (10 g/L yeast extract, 20 g/L peptone, 80 g/L dextrose) in 12-mL glass tubes (Duran, Wertheim, Germany) with metal labocap lids (Lüdiswiss, Flawil, Switzerland) and incubated overnight at 30 °C with shaking at 250 rpm. The following day the overnight culture was centrifuged, the supernatant was discarded and the pellet was resuspended in 2 mL nitrogen-limited medium (2.9 g/L $(NH_4)_2SO_4$, 1.7 g/L YNB (without amino acids and ammonium sulphate), 380 mg/L leucine, 76 mg/L lysine, 20 mg/L uracil and 60 g/L glucose). The cultures were incubated for 48 h at 30 °C with shaking at 250 rpm.

Fig. 1d: Three individual colonies of strains expressing desaturases were inoculated into 1 mL selective medium (SC-Ura-Leu) and incubated at 30 °C and 300 rpm for 48 h. The cultures were diluted to an OD600 of 0.4 in 5 mL selective medium (SC-Ura-Leu) supplemented with 2 mM

$CuSO_4$ and the 0.5 mM methyl tetradecanoate (14Me) (Larodan Fine Chemicals, Sweden). The methyl tetradecanoate stock solution was prepared to a concentration of 100 mM in 96% ethanol. The yeast cultures were incubated at 30 °C at 300 rpm for 48 h.

Fig. 1e: Strains ST4854 and ST5290 were inoculated into 5 mL synthetic complete medium (SC-His-Leu-Trp supplemented with 20 mg/L uracil and 76 mg/L histidine) and cultivated in 12-mL glass tubes (Duran, Wertheim, Germany) with metal labocap lids (Ludiswiss, Flawil, Switzerland) overnight at 30 °C with shaking at 250 rpm. The following day the overnight culture was centrifuged, the supernatant was discarded and the pellet was resuspended in 2 mL of mineral medium, which had the composition as described elsewhere (Löfstedt et al., 2016). The medium was supplemented with 20 mg/L uracil and 76 mg/L histidine. The cultures were incubated at 30 °C with shaking at 250 rpm for 48 h.

Fig. 2c: One individual clone of each strain was inoculated into 3 mL mineral medium (14.5 g/L KH_2PO_4, 0.5 g/L $MgSO_4$, 2 mL/L trace metals solution (4.5 g/L $CaCl_2{\cdot}2H_2O$, 4.5 g/L $ZnSO_4{\cdot}7H_2O$, 3 g/L $FeSO_4{\cdot}7H_2O$, 1 g/L H_3BO_3, 1 g/L $MnCl_2{\cdot}2H_2O$, 0.4 g/L $Na_2MoO_4{\cdot}2H_2O$, 0.3 g/L $CoCl_2{\cdot}6H_2O$, 0.1 g/L $CuSO_4{\cdot}5H_2O$, 0.1 g/L KI, 15 g/L EDTA), 1 mL/L vitamin solution (50 mg/L biotin, 200 mg/L p-aminobenzoic acid, 1 g/L nicotinic acid, 1 g/L capantotenate, 1 g/L pyridoxine HCl, 1 g/L thiamine HCl, 25 g/L myo-inositol), 6.9 g/L urea, 50 g/L glycerol, 1.9 g/L leucine, 0.38 g/L uracil, 0.38 g/L lysine) in a 24 deep-well plate with air-penetrable lid (EnzyScreen) and cultivated for 24 h at 30 °C with shaking at 250 rpm The following day 3 mL of mineral medium were inoculated with the preculture in a 24 deep-well plate with air-penetrable lid (EnzyScreen) and cultivated for 48 h at 30 °C with shaking at 250 rpm. After 24 h of cultivation, 119 μL of glycerol (corresponding to 50 g/L) were added to each well.

4.3. Metabolite extraction and analysis on GC/MS

Fig. 1b, e; Fig. 2b For extraction, 1 mL of culture was transferred into a 4-mL glass vial and 10 μL of internal standard stock (1 μg/μL methyl (Z)-heptadec-10-enoate in 100% ethanol) was added. The samples were freeze-dried in a freeze dry system (Freezone6 and Stoppering tray dryer, Labconco, Kansas City, USA) at −40 °C and 1 mL chloroform:methanol (2:1, v/v) mixture was added to the cell residues. The mixture was vortexed for 45 s and left at room temperature for 4 h. The organic solvents were slowly evaporated to dryness under a nitrogen stream. One mL of hexane was added to recover the alcohol and acetate content, the samples were vortexed for 10 s, centrifuged and 200 μL of the organic supernatant was transferred to a new glass vial. GC/MS analyses were performed on a Hewlett Packard 6890 GC coupled to HP 5973 mass spectrometer detector. The GC was equipped with an INNOWax column (30 m × 0.25 mm × 0.25 μm), and helium was used as carrier gas (average velocity: 33 cm/s). The MS was operated in electron impact mode (70eV), scanning between m/z 30 and 400, and the injector was configured in splitless mode at 220 °C. The oven temperature was set to 80 °C for 1 min, then increased at a rate of 10 °C/min to 210 °C, followed by a hold at 210 °C for 15 min, and then increased at a rate of 10 °C/min to 230 °C followed by a hold at 230 °C for 20 min. Compounds were identified by comparison of their retention times and mass spectra with those of the corresponding commercially available standards. Data were analysed by the Agilent ChemStation software and iWork Numbers. The concentrations of fatty alcohols were calculated using internal standards.

Fig. 1d One mL of culture was sampled and 3.12 μg of methyl nonadecanoate (19Me) was added as internal standard. Total lipids were extracted using 3.75 mL of methanol/chloroform (2:1, v/v), in a glass vial. One mL of acetic acid (0.15 M) and 1.25 mL of water were added to the tube. Tubes were vortexed vigorously and centrifuged at 2000×g for 2 min. The bottom chloroform phase, about 1 mL, containing the total lipids, was transferred to a new glass vial and the solvent was evaporated to dryness. Fatty acid methyl esters (FAMEs) were made from this total lipid extract by acid methanolysis, as follows. One mL of 2% sulfuric acid in methanol (v/v) was added to the tube, vortexed vigorously, and incubated at 90 °C for 1 h. After incubation, 1 mL of water was added and mixed well, and then 1 mL of hexane was used to extract the FAMEs. The resulting methyl ester samples were subjected to GC/MS analyses on a Hewlett Packard 6890 GC coupled to a HP 5973 mass selective detector as described above. The monounsaturated fatty acid products were identified by comparing their retention times and mass spectra with those of synthetic standards. Data were analysed by the ChemStation software (Agilent, Technologies, USA).

4.4. Fatty alcohol degradation analysis

Cells were cultivated according to the same method as described above for Fig. 2b with the exception that the preculture was incubated for 36 h (instead of overnight) and *Z*9-14OH and *Z*11-16OH were added to a final concentration of 1 g/L (indicated by + Alc). The concentration of fatty alcohols in the whole broth was determined after 48 h of incubation in the nitrogen-limited medium. Extraction of samples was performed as following: 100 μL of broth was extracted with 1 mL of ethyl acetate:ethanol (85:15) and using 10 μL of methyl nonadecanoate (19Me, 2 mg/mL) as internal standard. The samples were vortexed for 20 s and incubated for 1 h at room temperature, followed by 5 min of vortexing. 300 μL of H_2O was added to each sample. The samples were vortexed and centrifuged for 5 min at 21 °C and 3000×g. The upper organic phase was analysed via gas chromatography-mass spectrometry (GC/MS). GC/MS analyses were performed on an Agilent 7820A GC coupled to 5977B mass selective detector. The GC was equipped with a split/spitless injector and a DB-Fatwax UI column (30 m × 0.25 mm × 0.25 μm). The operation parameters were: 1 μL split injection (30:1), injector temperature 220 °C and constant flow 1 mL/min helium. The oven temperature was set to 80 °C for 1 min, then increased at a rate of 15 °C/min to 210 °C, followed by a hold at 210 °C for 7 min, and then increased at a rate of 20 °C/min to 230 °C. Fatty alcohols were analysed in selected ion monitoring (SIM) mode using the following mass-to charge-ratios for quantification: 55.1 and 74.1. Compounds were identified by comparison of retention times with those of the corresponding commercially available standards. Data were analysed by the Mass Hunter software B.08.00.

4.5. Analysis of GPAT expression by qRT-PCR

For qRT-PCR analysis, yeast strains ST5789 (control) and ST5791 (a strain with truncated *GPAT* promoter) were cultivated in triplicates according to method described above for Fig. 2b. The harvesting and pre-treatment before RNA extraction was as described in Dahlin et al. (2019). Total RNA was isolated using RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. First strand cDNA synthesis was performed using SuperScript™ II Reverse Transcriptase (ThermoFisher Scientific). 20 ng of cDNA was used for qRT-PCR analysis which was done using DyNAmo Flash SYBR Green qPCR Kit on a Stratagene Mx3005P (Agilent Technologies). Relative expression level was calculated using double delta method (ΔΔCt), where ΔΔCt = (ΔCtE − ΔCtC).

4.6. Analysis of lipid content in strain with GPAT downregulation

Cells cultivated in triplicates according to method described above for Fig. 2b were subjected to FAMEs analysis. 1 mL of broth was transferred to 4 mL glass vials and centrifuged at 3000 g for 5 min at room temperature, the supernatant discarded and the cell pellet treated with 1 mL of 1M HCl in methanol, vortexed and incubated at 80 °C for 2 h. After methanolysis, the mixture was neutralized with 1 mL of 1M NaOH in methanol, then 0.5 mL of saturated NaCl in water was added and 1 mL of hexane was added together with internal standard (19Me). Mixture was vortexed and centrifuged at 3000 g for 5 min at room temperature and then the upper phase recovered for GC analysis. GC/MS analysis was

done on Agilent 7820A GC system coupled to 5977B mass selective detector. The GC was equipped with DB-Fatwax UI column (30 m × 0.25 mm × 0.25 μm) and helium was used as carrier gas (average velocity 37 cm/s). The MS was scanning between *m/z* 30 and 350 and the injector was configured to split mode (split ratio 10:1) at 220 °C. The oven temperature was set to 80 °C for 1 min, then increased at a rate of 15 °C/min to 210 °C, followed by a hold at 210 °C for 7 min. Then temperature increased at a rate of 20 °C/min to 230 °C. For injection 1 μl of sample was used. Compounds were identified by comparison of retention times with those of reference compounds and quantified based on internal standard. For dry cell weight (DW) measurements 1 mL of culture was taken, centrifuged for 5 min at 16000 g, supernatant discarded, pellet washed with 1 mL sterile water, centrifuged once again and water discarded. Washed pellets were kept in an oven at 65 °C for 48 h and biomass weighted. Effect of GPAT downregulation was evaluated based on ratio between FAMEs and DW normalized to ST-5789. FAMEs which were included in analysis can be seen in Fig. S2.

4.7. Preparation of yeast-derived biological pheromone sample

The fermentations were carried out in a BioFlo 415 bioreactor (Eppendorf/NewBrunswick Scientific), equipped with a *in-situ* sterilized 14 L stainless steel vessel (10 L max working volume). pH was controlled at 5.0 ± 0.1 with automated addition of a 1M solution of H_2SO_4, and a 4M solution of NaOH. Dissolved oxygen was measured using a polarographic electrode and automatically controlled at 20% saturation by changing the stirring speed of three 6-blade Rushton turbines. Strain *Yarrowia lipolytica* ST6379 was inoculated into 6 L of fermentation medium (2 g/L yeast extract, 13.4 g/L yeast nitrogen base, 0.76 g/L lysine, 0.76 g/L uracil, 0.024 mg/L thiamine, 0.002 g/L biotin, and 50 g/L glycerol). After 20 h of fermentation, the culture was supplemented with 750 mL nutrient-rich feed (composed of 16.2 g/L yeast extract, 108.6 g/L yeast nitrogen base, 6.2 g/L lysine, 0.2 mg/L thiamine, 0.02 g/L biotin, and 326 g/L glycerol), followed by a pulse of glycerol to a concentration of 50 g/L in the reactor at 32 h. From 36 h, glycerol was fed continuously keeping a steady glycerol concentration of 20–30 g/L in the bioreactor. The fermentation lasted a total of 48 h. The concentration of *Z*11-16OH was 2.57 g/L.

Liquid-liquid extraction with ethyl acetate was performed on a total of 4.2 L fermentation broth. Fermentation broth was centrifuged at 4000×*g* for 5 min. The supernatant was discarded and the remaining pellet was freeze dried and pulverized. 1 L ethyl acetate was added to the pulverized powder and incubated on a multi-vortexer for 8 h. After filtering off the solvent, the biomass cakes were re-extracted with 0.5 L fresh ethyl acetate. All extracts were combined and the solvent was evaporated to dryness. The extract was resuspended in 25 mL ethyl acetate.

For purification, the crude extract (4.7 g) was passed through a plug of silica gel (approximately 100 g), in a filtration funnel. The silica was washed with hexanes, and then subsequently with a gradient of hexanes: ethyl acetate at the proportions of 95:5 (%) to 80:20 (%), with 5% increments. Fractions were analysed by TLC eluted with a mixture of hexanes:ethyl acetate in the proportion 80:20 and gas chromatography. The purest fractions were combined and the solvent was evaporated, initially by rotary evaporator and then by means of high vacuum pump. The total amount of purified material recovered was 1.8 g. A fraction of the purified material was transformed into the aldehyde, according to the following protocol: TEMPO (2,2,6,6-Tetramethyl-1-piperidinyloxy, 26 mg) and 1-methylimidazole (28 mg) were added to a well-stirred suspension of the alcohol (800 mg), acetonitrile (3 mL), 2,2′-bipyridyl (26 mg) and tetrakisacetonitrile copper(I) triflate (62 mg). The mixture was stirred at room temperature and open atmosphere for 2 h and the completion of the reaction was verified by gas chromatography. The solvent was evaporated and the recovered material was extracted with hexanes, water and $NaHCO_3$ aqueous solution. The organic phase was dried with $MgSO_4$, filtered and the solvent was evaporated under reduced pressure. The resulting material (0.58 g) was dissolved in 10 mL ethyl acetate and analysed as follows. Three replicates 200 μL of the solution were transferred into 5-mL volumetric flasks, and ethyl acetate was added to complete the volume to the meniscus line. The contents of the flasks were mixed by swirling or inverting the flasks up and down several times and aliquots of 1.5 mL were transferred to autosampler vials for GC injection. Analysis was performed in a Agilent 7890 equipped with an FID detector and an HP-5 capillary column. The oven temperature program involved an initial temperature of 115 °C, increased at a rate of 40 °C/min to 162 °C and held for 3 min. The temperature was finally increased to 40 °C/min to 280 °C and held for 3 min. Three concentrations of technical grade *Z*11-16Ald in ethyl acetate were prepared and the solutions were also injected into the GC to create a calibration curve and the equation of the line was used for quantitation of *Z*11-16Ald, according to Table S6. The concentration of Z11-16Ald was determined to be 35.33 mg/mL based on three replicates with the RSD of 0.72% between replicates.

4.8. Electrophysiological responses of male H. armigera

The antennal responses of *Helicoverpa armigera* male adults to the pheromone blend produced from yeast fermentation were evaluated by electroantennography (EAG) using a commercially available electroantennographic system (Syntech, The Netherlands). Antennae of a virgin, two-to-three-days-old male adult were used. The signal was amplified and detected with a data acquisition controller (IDAC-4, Syntech, The Netherlands).

Yeast-derived pheromone blend (Bio-Ald), standard compounds and mixtures of the standards were tested at a total of 39 antennal preparations. As standard compounds, the two pheromone components of *H. armigera* pheromone, *Z*11-16Ald and *Z*9-16Ald, were used. Two mixtures of the two monounsaturated aldehydes with tetradecanal and pentadecanal were also tested on grounds that presence of tetradecanal and pentadecanal has been verified in the pheromone blend produced by yeast fermentation (MS identification, data not shown). The two blends tested were Ald mix 1: *Z*11-16Ald, *Z*9-16Ald, 14Ald, 15Ald at 80:5:5:5 ratio and Ald mix 2: *Z*11-16Ald, *Z*9-16Ald, 14Ald, 15Ald at 1:1:1:1 ratio. Ald mix 1 blend constituents approximate the abundances found in the yeast-derived pheromone blend and notably the ratio of the two monounsaturated aldehydes (94:6) approximate the optimal pheromone blend ratio for *H. armigera*.

Aliquots of 1 μg of each of the compounds (or mixtures) was presented to the male antenna. Stimuli were provided as 0.3 s air puffs into a continuous flow of filtered and humidified air. The air flow, at 25 cm^3/s rate, tube diameter 1 cm, was generated by an air stimulus controller (CS-55, Syntech, The Netherlands). At least 1 min was allowed between successive stimulations in order to allow the antenna to recover. Control stimulus consisted of filter paper and solvent (*n*-pentane). A reference stimulus, consisting of a 1 μg of *Z*11-16Ald (the major sex pheromone of *H. armigera*), was provided at regular intervals during each recording session. The EAG response to each reference stimulus was defined as 100%, and all responses to the test stimuli between adjacent references were normalized in % relative to the references.

4.9. Monitoring of H. armigera flight in the field

Field trials were conducted in Thermi (northern Greece 40°32′11.6″N 23°00′08.0″E) and in Lamia (central Greece 38°87′64.1″N 22°36′81.3″E) on experimental pesticide-free cotton fields (planted area 1.5 ha each). The two discrete geographical regions having similar meteorological conditions (temperature 27-28 °C; rainfall 2–0.5 mm for July and August respectively).

Dispensers used as control were grey rubber septa (bromobutyl elastomers) loaded with 2 mg of *H. armigera* pheromone blend *Z*11-16Ald: *Z*9-16Ald at 97:3 ratio) (provided by Novagrica Hellas SA). Treatment dispensers (Bio-Ald) were similarly bromobutyl elastomers

loaded with 2 mg of the yeast-derived pheromone. BHT and bumetrizole were added as antioxidant and UV absorber respectively at 5% w/w. Six funnel traps (three dispensers loaded with commercially available pheromone, control, and three loaded with the yeast-derived pheromone (Bio-Ald) were installed at 1.2 m height. Baited traps were in operation from early July until early September, the traps were rotated clockwise weekly, and males captured were recorded once per week and removed. Pheromone dispensers were renewed every month (Kikionis et al., 2017).

4.10. Statistical analyses

The electrophysiological and field data were subjected to analysis of variance (ANOVA) (SAS Institute, 2000). The means of electrophysiological data were separated using the Tukey (honestly significant difference, HSD) test at $P = 0.05$. The field data presented as means of male catches per trap per week.

Author contributions

IB and CL conceived the study. CH, BJD, HLW, MID, KP, KRK, LW, CS and BL performed the experiments on pheromone production in yeasts. LF, MP, and BF carried out fermentation in controlled bioreactors and extracted the pheromones. CB, WU, and AM-N purified and oxidized the sample for activity tests. EK, DR, and MK performed laboratory and field tests of pheromones. IB drafted the manuscript and all the authors have contributed to writing.

Declaration of competing interest

IB, CH, CL, BJD, MID, HLW are co-inventors on patent applications WO2016207339, WO2018109167, and WO2018109163. IB, CH, KRK, BL, KP, CS, and LW have financial interest in BioPhero ApS. BSF has financial interest in Biotrend SA. DR has financial interest in Novagrica SA. AM-N has financial interest in ISCA Technologies.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymben.2020.10.001.

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Supplementary Information for

Production of moth sex pheromones for pest control by yeast fermentation

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This PDF file includes:

DNA sequences of synthetic genes and plasmids

>SEQ ID NO: 1. Fatty acyl-CoA reductase from *Agrotis segetum* (WO_2016_207339_A1_3)
ATGCCAGTCTTGACTTCTAGAGAAGACGAAAAATTGTCCGTCCCAGAATTTTACGCTGGTAA
GTCTATTTTTGTTACCGGTGGTACTGGTTTCTTGGGTAAGGTTTTTATCGAAAAGTTGTTGTA
CTGCTGCCCAGATATCGATAAGATCTACATGTTGATCAGAGAAAAAAAGAACTTGTCCATCG
ACGAAAGAATGTCCAAGTTTTTGGATGACCCTTTGTTCTCCAGATTGAAAGAAGAAAGACCA
GGTGACTTGGAAAAGATCGTTTTGATTCCAGGTGATATTACCGCTCCTAATTTGGGTTTGTCT
GCTGAAAACGAAAGAATCTTGTTGGAAAAGGTCAGTGTCATTATTAACTCTGCTGCTACCGTT
AAGTTCAACGAACCATTGCCAATTGCTTGGAAGATTAACGTTGAAGGTACTAGAATGTTGTTG
GCCTTGTCTAGAAGAATGAAGAGAATCGAAGTTTTCATCCATATCTCCACCGCTTACTCTAAT
GCTTCTTCTGATAGAATTGTCGTTGACGAAATCTTGTATCCAGCTCCAGCTGATATGGATCAA
GTTTATCAATTGGTTAAGGACGGTGTCACTGAAGAAGAAACCGAAAGATTATTGAACGGTTT
GCCAAACACTTACACTTTCACTAAGGCTTTGACCGAACATTTGGTTGCTGAACATCAAACTTA
CGTTCCAACCATTATCATCAGACCATCTGTTGTTGCCTCCATTAAGGATGAACCTATTAGAGG
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AGTTTTGTTGGGTAAAGCCTCTAACATCGTTGATGTTATCCCAGTTGATTACGTTGCCAACTT
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CTGCTCCTCTGACTGTAATCCAGTTACTTTGAAGAAGATCATCAAAGAATTCACCGAAGATAC
CATCAAGAACAAGTCCCATATTATGCCATTGCCAGGTTGGTTCGTTTTTACTAAGTACAAATG
GTTGTTGACTTTGTTGACCATCATCTTCCAAATGTTGCCAATGTATTTGGCCGATGTTTACAG
AGTCTTGACCGGTAAAATTCCAAGATATATGAAGTTGCACCACTTGGTCATTCAAACCAGATT
GGGTATTGATTTCTTCACCTCTCATTCTTGGGTTATGAAGACCGATAGAGTCAGAGAATTATT
CGGTTCTTTGTCCTTGGCCGAAAAACACATGTTTCCATGTGATCCATCTTCCATTGATTGGAC
CGATTACTTGCAATCTTACTGCTATGGTGTCAGAAGATTCTTAGAAAAGAAGAAGTAA

>SEQ ID NO: 2. Fatty acyl-CoA reductase from *Heliothis subflexa* (WO_2016_207339_A1_15)
ATGGTTGTCTTGACCTCCAAAGAAACTAAGCCATCTGTTGCTGAATTTTACGCTGGTAAGTCT
GTTTTCATTACTGGTGGTACTGGTTTCTTGGGTAAGGTTTTCATTGAAAAGTTGTTGTACTCC
TGCCCAGATATCGGTAATATCTACATGTTGATCAGAGAAAAGAAGGGTTTGTCCGTTTCCGA
AAGAATCAAGCACTTTTTGGATGATCCTTTGTTCACCAGATTGAAAGAAAAAAGACCAGCCGA
CTTGGAAAAGATCGTTTTGATTCCAGGTGATATTACTGCTCCAGATTTGGGTATTACCTCCGA
AAACGAAAAGATGTTGATCGAAAAGGTCAGTGTCATTATTCATTCTGCTGCTACCGTTAAGTT
CAACGAACCATTGCCAACTGCTTGGAAGATTAACGTTGAAGGTACTAGAATGATGTTGGCCT
TGTCTAGAAGAATGAAGAGAATCGAAGTTTTCATCCATATCTCTACCGCTTACACTAACACCA
ACAGAGAAGTTGTTGACGAAATCTTGTATCCAGCTCCAGCTGATATTGATCAAGTTCACCAAT
ATGTTAAGGACGGTATCTCTGAAGAAGAAACTGAAAAAATCTTGAACGGTAGACCAAACACT
TACACTTTCACTAAGGCTTTGACCGAACATTTGGTTGCTGAAAATCAAGCTTACGTTCCAACC
ATTATCGTTAGACCATCAGTTGTTGCTGCCATTAAGGATGAACCTATTAAGGGTTGGTTGGGT
AATTGGTATGGTGCTACAGGTTTGACTGTTTTTACTGCTAAGGGTTTGAACAGAGTTATCTAC
GGTCACTCTTCTAACATCGTTGATTTGATCCCAGTTGATTACGTTGCCAACTTGGTTATTGCT
GCTGGTGCTAAATCTTCTAAGTCTACTGAATTGAAGGTCTACAACTGCTGTTCTTCTGCTTGT
AACCCAATTACTATCGGTAAGTTGATGTCCATGTTTGCTGAAGATGCTATCAAGCAAAAGTCT
TACGCTATGCCATTGCCAGGTTGGTACATTTTTACTAAGTACAAGTGGTTGGTCTTGTTGTTG
ACCATTTTGTTCCAAGTTATTCCAGCCTACATTACCGACTTGTACAGACATTTGATTGGTAAG
AACCCAAGATATATCAAGTTGCAATCCTTGGTCAATCAAACCAGATCCTCCATTGATTTCTTC
ACCAACCATTCTTGGGTTATGAAGGCTGATAGAGTCAGAGAATTATTCGCTTCTTTGTCTCCA
GCAGATAAGTACTTGTTTCCATGTGATCCAGTCAACATCAATTGGAGACAATATATCCAAGAT
TACTGCTGGGGTGTTAGACATTTCTTGGAAAAAAAGACTTAA

>SEQ ID NO: 3. Fatty acyl-CoA reductase from *Helicoverpa assulta* (WO_2016_207339_A1_11)
ATGGTTGTCTTGACCTCCAAAGAAACTAAGCCATCTGTTGCTGAATTTTACGCTGGTAAGTCT
GTTTTCATTACTGGTGGTACTGGTTTCTTGGGTAAGATCTTCATTGAAAAGTTGTTGTACTCC
TGCCCAGATATCGGTAATATCTACATGTTGATCAGAGAAAAGAAGGGTTTGTCCGTTTCCGA
AAGAATCAAGCAATTTTTGGATGACCCTTTGTTCACCAGATTGAAAGAAAAAAGACCAGCCG
ACTTGGAAAAGATCGTTTTGATTCCAGGTGATATTACTGCTCCAGATTTGGGTATTACCTCCG

AAAACGAAAAGATGTTGATCGAAAAGGTCAGTGTCATTATTCATTCTGCTGCTACCGTTAAGT
TCAACGAACCATTGCCAACTGCTTGGAAGATTAACGTTGAAGGTACTAGAATGATGTTGGCC
TTGTCTAGAAGAATGAAGAGAATCGAAGTTTTCATCCATATCTCTACCGCTTACACTAACACC
AACAGAGAAGTTGTTGACGAAATCTTGTATCCAGCTCCAGCTGATATTGATCAAGTTCACCAA
TATGTTAAGGACGGTATCTCTGAAGAAGAAACTGAAAAAATCTTGAACGGTAGACCAAACAC
TTACACTTTCACTAAGGCTTTGACCGAACATTTGGTTGCTGAAAATCAAGCTTACGTTCCAAC
CATTATCGTTAGACCATCAGTTGTTGCTGCCATTAAGGATGAACCTATTAAGGGTTGGTTGG
GTAATTGGTATGGTGCTACAGGTTTGACTGTTTTTACTGCTAAGGGTTTGAACAGAGTTATCT
ACGGTCATTCCTCTTACATCGTTGATTTGATCCCAGTTGATTACGTTGCCAACTTGGTTATTG
CTGCTGGTGCTAAATCTTCTAAGTCTACTGAATTGAAGGTCTACAACTGCTGTTCTTCTGCTT
GTAACCCAATTACTATCGGTAAGTTGATGTCCATGTTTGCTGAAGATGCTATCAAGCAAAAGT
CTTACGCTATGCCATTGCCAGGTTGGTATGTTTTTACAAAGTACAAGTGGTTGGTCTTGTTGT
TGACCATTTTGTTCCAAGTTATTCCAGCCTACATTACCGACTTGTACAGACATTTGATTGGTA
AGAACCCAAGATATATCAAGTTGCAATCCTTGGTCAATCAAACCAGATCCTCCATTGATTTCT
TCACCTCTCATTCTTGGGTTATGAAGGCTGATAGAGTCAGAGAATTATTCGCTTCTTTGTCTC
CAGCAGATAAGTACTTGTTTCCATGTGATCCAACCGATATTAACTGGACCCATTACATTCAAG
ATTACTGCTGGGGTGTTAGACACTTCTTGGAAAAAAAGACTACCAACAAGTAA

>SEQ ID NO: 4. ∆11-desaturase from *Amyelois transitella* (WO_2016_207339_A1_1)
ATGGTTCCAAACAAGGGTTCCTCTGATGTTTTGTCTGAACATTCTGAACCACAATTCACCAAG
TTGATTGCTCCACAAGCTGGTCCAAGAAAGTACAAAATCGTTTACAGAAACTTGTTGACCTTC
GGTTACTGGCATTTGTCTGCTGTTTATGGTTTGTACTTGTGTTTCACTTGTGCTAAGTGGGCT
ACTATTTTGTTCGCTTTCTTCTTGTACGTTATCGCCGAAATTGGTATTACTGGTGGTGCTCAT
AGATTATGGGCTCATAGAACTTACAAAGCCAAGTTGCCATTGGAAATCTTGTTGTTGATCATG
AACTCCATTGCCTTCCAAGATACTGCTTTTACTTGGGCTAGAGATCATAGATTGCATCACAAG
TACTCTGATACTGATGCTGATCCACATAATGCTACTAGAGGTTTCTTCTACTCTCATGTTGGT
TGGTTGTTGGTTAAGAAACACCCAGAAGTTAAGGCTAGAGGTAAGTACTTGTCTTTGGATGA
CTTGAAGAACAACCCTTTGTTGAAGTTCCAAAAGAAGTACGCCATTTTGGTCATTGGTACTTT
GTGCTTTTTGATGCCAACTTTCGTTCCAGTTTACTTTTGGGGTGAAGGTATTTCTACTGCCTG
GAACATTAACTTGTTAAGATACGTCATGAACTTGAACATGACCTTTTTGGTTAACTCCGCTGC
TCATATTTTTGGTAACAAGCCATACGATAAGTCTATCGCCTCTGTTCAAAACATCTCTGTTTCT
TTGGCTACTTTCGGTGAAGGTTTCCATAACTACCATCATACTTATCCATGGGATTACAGAGCT
GCTGAATTGGGTAACAATAGATTGAATATGACCACCGCCTTCATTGATTTCTTTGCTTGGATT
GGTTGGGCCTACGATTTGAAATCTGTTCCACAAGAAGCTATTGCTAAGAGATGTGCTAAAAC
TGGTGATGGTACTGATATGTGGGGTAGAAAGAGATGA

>SEQ ID NO: 5. Fatty acyl-CoA reductase from *Helicoverpa armigera* (WO_2016_207339_A1_7)
ATGGTTGTCTTGACCTCCAAAGAAACTAAGCCATCTGTTGCTGAATTTTACGCTGGTAAGTCT
GTTTTCATTACTGGTGGTACTGGTTTCTTGGGTAAGGTTTTCATTGAAAAGTTGTTGTACTCC
TGCCCAGATATCGGTAATATCTACATGTTGATCAGAGAAAAGAAGGGTTTGTCCGTTTCCGA
AAGAATCAAGCACTTTTTGGATGATCCTTTGTTCACCAGATTGAAAGAAAAAAGACCAGCCGA
CTTGGAAAAGATCGTTTTGATTCCAGGTGATATTACTGCTCCAGATTTGGGTATTACCTCCGA
AAACGAAAAGATGTTGATCGAAAAGGTCAGTGTCATTATTCATTCTGCTGCTACCGTTAAGTT
CAACGAACCATTGCCAACTGCTTGGAAGATTAACGTTGAAGGTACTAGAATGATGTTGGCCT
TGTCTAGAAGAATGAAGAGAATCGAAGTTTTCATCCATATCTCTACCGCTTACACTAACACCA
ACAGAGAAGTTGTTGACGAAATCTTGTATCCAGCTCCAGCTGATATTGATCAAGTTCACAGAT
ATGTTAAGGACGGTATCTCTGAAGAAGAAACTGAAAAAATCTTGAACGGTAGACCAAACACT
TACACTTTCACTAAGGCTTTGACCGAACATTTGGTTGCTGAAAATCAAGCTTACGTTCCAACC
ATTATCGTTAGACCATCAGTTGTTGCTGCCATTAAGGATGAACCTATTAAGGGTTGGTTGGGT
AATTGGTATGGTGCTACAGGTTTGACTGTTTTTACTGCTAAGGGTTTGAACAGAGTTATCTAC
GGTCACTCTTCTAACATCGTTGATTTGATCCCAGTTGATTACGTTGCCAACTTGGTTATTGCT
GCTGGTGCTAAATCTTCTAAGTCTACTGAATTGAAGGTCTACAACTGCTGTTCTTCTGCTTGT
AACCCAATTACTATCGGTAAGTTGATGTCCATGTTTGCTGAAGATGCTATCAAGCAAAAGTCT
TACGCTATGCCATTGCCAGGTTGGTACATTTTTACTAAGTACAAGTGGTTGGTCTTGTTGTTG
ACCATTTTGTTCCAAGTTATTCCAGCCTACATTACCGACTTGTACAGACATTTGATTGGTAAG
AACCCAAGATATATCAAGTTGCAATCCTTGGTCAATCAAACCAGATCCTCCATTGATTTCTTC

ACCTCTCATTCTTGGGTTATGAAGGCTGATAGAGTCAGAGAATTATTCGCTTCTTTGTCTCCA
GCAGATAAGTACTTGTTTCCATGTGATCCAACCGATATTAACTGGACCCATTACATTCAAGAT
TACTGCTGGGGTGTTAGACATTTCTTGGAAAAAAAAAGCTACGAATAA

>SEQ ID NO: 6. Fatty acyl-CoA reductase from *Helicoverpa armigera* with modified C-terminus (WO_2016_207339_A1_9)
ATGGTTGTCTTGACCTCCAAAGAAACTAAGCCATCTGTTGCTGAATTTTACGCTGGTAAGTCT
GTTTTCATTACTGGTGGTACTGGTTTCTTGGGTAAGGTTTTCATTGAAAAGTTGTTGTACTCC
TGCCCAGATATCGGTAATATCTACATGTTGATCAGAGAAAAGAAGGGTTTGTCCGTTTCCGA
AAGAATCAAGCACTTTTTGGATGATCCTTTGTTCACCAGATTGAAAGAAAAAAGACCAGCCGA
CTTGGAAAAGATCGTTTTGATTCCAGGTGATATTACTGCTCCAGATTTGGGTATTACCTCCGA
AAACGAAAAGATGTTGATCGAAAAGGTCAGTGTCATTATTCATTCTGCTGCTACCGTTAAGTT
CAACGAACCATTGCCAACTGCTTGGAAGATTAACGTTGAAGGTACTAGAATGATGTTGGCCT
TGTCTAGAAGAATGAAGAGAATCGAAGTTTTCATCCATATCTCTACCGCTTACACTAACACCA
ACAGAGAAGTTGTTGACGAAATCTTGTATCCAGCTCCAGCTGATATTGATCAAGTTCACAGAT
ATGTTAAGGACGGTATCTCTGAAGAAGAAACTGAAAAAATCTTGAACGGTAGACCAAACACT
TACACTTTCACTAAGGCTTTGACCGAACATTTGGTTGCTGAAAATCAAGCTTACGTTCCAACC
ATTATCGTTAGACCATCAGTTGTTGCTGCCATTAAGGATGAACCTATTAAGGGTTGGTTGGGT
AATTGGTATGGTGCTACAGGTTTGACTGTTTTTACTGCTAAGGGTTTGAACAGAGTTATCTAC
GGTCACTCTTCTAACATCGTTGATTTGATCCCAGTTGATTACGTTGCCAACTTGGTTATTGCT
GCTGGTGCTAAATCTTCTAAGTCTACTGAATTGAAGGTCTACAACTGCTGTTCTTCTGCTTGT
AACCCAATTACTATCGGTAAGTTGATGTCCATGTTTGCTGAAGATGCTATCAAGCAAAAGTCT
TACGCTATGCCATTGCCAGGTTGGTACATTTTTACTAAGTACAAGTGGTTGGTCTTGTTGTTG
ACCATTTTGTTCCAAGTTATTCCAGCCTACATTACCGACTTGTACAGACATTTGATTGGTAAG
AACCCAAGATATATCAAGTTGCAATCCTTGGTCAATCAAACCAGATCCTCCATTGATTTCTTC
ACCTCTCATTCTTGGGTTATGAAGGCTGATAGAGTCAGAGAATTATTCGCTTCTTTGTCTCCA
GCAGATAAGTACTTGTTTCCATGTGATCCAACCGATATTAACTGGACCCATTACATTCAAGAT
TACTGCTGGGGTGTTAGACATTTCTTGGAACATGATGAATTGTAA

>SEQ ID NO: 7. Fatty acyl-CoA reductase from *Heliothis subflexa* with modified C-terminus (WO_2016_207339_A1_17)
ATGGTTGTCTTGACCTCCAAAGAAACTAAGCCATCTGTTGCTGAATTTTACGCTGGTAAGTCT
GTTTTCATTACTGGTGGTACTGGTTTCTTGGGTAAGGTTTTCATTGAAAAGTTGTTGTACTCC
TGCCCAGATATCGGTAATATCTACATGTTGATCAGAGAAAAGAAGGGTTTGTCCGTTTCCGA
AAGAATCAAGCACTTTTTGGATGATCCTTTGTTCACCAGATTGAAAGAAAAAAGACCAGCCGA
CTTGGAAAAGATCGTTTTGATTCCAGGTGATATTACTGCTCCAGATTTGGGTATTACCTCCGA
AAACGAAAAGATGTTGATCGAAAAGGTCAGTGTCATTATTCATTCTGCTGCTACCGTTAAGTT
CAACGAACCATTGCCAACTGCTTGGAAGATTAACGTTGAAGGTACTAGAATGATGTTGGCCT
TGTCTAGAAGAATGAAGAGAATCGAAGTTTTCATCCATATCTCTACCGCTTACACTAACACCA
ACAGAGAAGTTGTTGACGAAATCTTGTATCCAGCTCCAGCTGATATTGATCAAGTTCACCAAT
ATGTTAAGGACGGTATCTCTGAAGAAGAAACTGAAAAAATCTTGAACGGTAGACCAAACACT
TACACTTTCACTAAGGCTTTGACCGAACATTTGGTTGCTGAAAATCAAGCTTACGTTCCAACC
ATTATCGTTAGACCATCAGTTGTTGCTGCCATTAAGGATGAACCTATTAAGGGTTGGTTGGGT
AATTGGTATGGTGCTACAGGTTTGACTGTTTTTACTGCTAAGGGTTTGAACAGAGTTATCTAC
GGTCACTCTTCTAACATCGTTGATTTGATCCCAGTTGATTACGTTGCCAACTTGGTTATTGCT
GCTGGTGCTAAATCTTCTAAGTCTACTGAATTGAAGGTCTACAACTGCTGTTCTTCTGCTTGT
AACCCAATTACTATCGGTAAGTTGATGTCCATGTTTGCTGAAGATGCTATCAAGCAAAAGTCT
TACGCTATGCCATTGCCAGGTTGGTACATTTTTACTAAGTACAAGTGGTTGGTCTTGTTGTTG
ACCATTTTGTTCCAAGTTATTCCAGCCTACATTACCGACTTGTACAGACATTTGATTGGTAAG
AACCCAAGATATATCAAGTTGCAATCCTTGGTCAATCAAACCAGATCCTCCATTGATTTCTTC
ACCAACCATTCTTGGGTTATGAAGGCTGATAGAGTCAGAGAATTATTCGCTTCTTTGTCTCCA
GCAGATAAGTACTTGTTTCCATGTGATCCAGTCAACATCAATTGGAGACAATATATCCAAGAT
TACTGCTGGGGTGTTAGACATTTCTTGCATGATGAATTGTAA

>SEQ ID NO: 8. Fatty acyl-CoA reductase from *Helicoverpa assulta* with modified C-terminus (WO_2016_207339_A1_13)

ATGGTTGTCTTGACCTCCAAAGAAACTAAGCCATCTGTTGCTGAATTTTACGCTGGTAAGTCT
GTTTTCATTACTGGTGGTACTGGTTTCTTGGGTAAGATCTTCATTGAAAAGTTGTTGTACTCC
TGCCCAGATATCGGTAATATCTACATGTTGATCAGAGAAAAGAAGGGTTTGTCCGTTTCCGA
AAGAATCAAGCAATTTTTGGATGACCCTTTGTTCACCAGATTGAAAGAAAAAAGACCAGCCG
ACTTGGAAAAGATCGTTTTGATTCCAGGTGATATTACTGCTCCAGATTTGGGTATTACCTCCG
AAAACGAAAAGATGTTGATCGAAAAGGTCAGTGTCATTATTCATTCTGCTGCTACCGTTAAGT
TCAACGAACCATTGCCAACTGCTTGGAAGATTAACGTTGAAGGTACTAGAATGATGTTGGCC
TTGTCTAGAAGAATGAAGAGAATCGAAGTTTTCATCCATATCTCTACCGCTTACACTAACACC
AACAGAGAAGTTGTTGACGAAATCTTGTATCCAGCTCCAGCTGATATTGATCAAGTTCACCAA
TATGTTAAGGACGGTATCTCTGAAGAAGAAACTGAAAAAATCTTGAACGGTAGACCAAACAC
TTACACTTTCACTAAGGCTTTGACCGAACATTTGGTTGCTGAAAATCAAGCTTACGTTCCAAC
CATTATCGTTAGACCATCAGTTGTTGCTGCCATTAAGGATGAACCTATTAAGGGTTGGTTGG
GTAATTGGTATGGTGCTACAGGTTTGACTGTTTTTACTGCTAAGGGTTTGAACAGAGTTATCT
ACGGTCATTCCTCTTACATCGTTGATTTGATCCCAGTTGATTACGTTGCCAACTTGGTTATTG
CTGCTGGTGCTAAATCTTCTAAGTCTACTGAATTGAAGGTCTACAACTGCTGTTCTTCTGCTT
GTAACCCAATTACTATCGGTAAGTTGATGTCCATGTTTGCTGAAGATGCTATCAAGCAAAAGT
CTTACGCTATGCCATTGCCAGGTTGGTATGTTTTTACAAAGTACAAGTGGTTGGTCTTGTTGT
TGACCATTTTGTTCCAAGTTATTCCAGCCTACATTACCGACTTGTACAGACATTTGATTGGTA
AGAACCCAAGATATATCAAGTTGCAATCCTTGGTCAATCAAACCAGATCCTCCATTGATTTCT
TCACCTCTCATTCTTGGGTTATGAAGGCTGATAGAGTCAGAGAATTATTCGCTTCTTTGTCTC
CAGCAGATAAGTACTTGTTTCCATGTGATCCAACCGATATTAACTGGACCCATTACATTCAAG
ATTACTGCTGGGGTGTTAGACACTTCTTGGAACATGATGAATTGTAA

>SEQ ID NO: 9. Δ11-desaturase from *A. segetum* (WO_2016_207339_A1_42)
ATGGCTCAAGGTGTCCAAACAACTACGATATTGAGGGAGGAAGAGCCGTCATTGACTTTCGT
GGTACCTCAAGAACCGAGAAAGTATCAAATCGTGTACCCAAACCTTATCACATTTGGGTACT
GGCATATAGCTGGTTTATACGGGCTATATTTGTGCTTTACTTCGGCAAAATGGCAAACAATTT
TATTCAGTTTCATGCTCGTTGTGTTAGCAGAGTTGGGAATAACAGCCGGCGCTCACAGGTTA
TGGGCCCACAAAACATATAAAGCGAAGCTTCCCTTACAAATTATCCTGATGATACTGAACTCC
ATTGCCTTCCAAAATTCCGCCATTGATTGGGTGAGGGACCACCGTCTCCATCATAAGTACAG
TGACACTGATGCAGACCCTCACAATGCTACTCGTGGTTTCTTCTATTCTCATGTTGGATGGTT
GCTCGTAAGAAAACATCCAGAAGTCAAGAGACGTGGAAAGGAACTTGACATGTCTGATATTT
ACAACAATCCAGTGCTGAGATTTCAAAAGAAGTATGCTATACCCTTCATCGGGGCAATGTGC
TTCGGATTACCAACTTTTATCCCTGTTTACTTCTGGGGAGAAACCTGGAGTAATGCTTGGCAT
ATCACCATGCTTCGGTACATCCTCAACCTAAACATTACTTTCCTGGTCAACAGTGCTGCTCAT
ATCTGGGGATACAAACCTTATGACATCAAAATATTGCCTGCCCAAAATATAGCAGTTTCCATA
GTAACCGGCGGCGAAGTTTCCATAACTACCACCACGTTTTTTCCTTGGGATTATCGTGCAGC
AGAATTGGGGAACAATTATCTTAATTTGACGACTAAGTTCATAGATTTCTTCGCTTGGATCGG
ATGGGCTTACGATCTTAAGACGGTGTCCAGTGATGTTATAAAAAGTAAGGCGGAAAGAACTG
GTGATGGGACGAATCTTTGGGGTTTAGAAGACAAAGGTGAAGAAGATTTTTTGAAAATCTGG
AAAGACAATTAA

>SEQ ID NO: 10. Δ11-desaturase from *Spodoptera littoralis*
ATGGCTCAATGTGTTCAAACCACCACCATCTTGGAACAAAAAGAAGAAAAGACCGTTACCTT
GTTGGTTCCACAAGCTGGTAAAAGAAAGTTCGAAATCGTCTACTTCAACATCATTACCTTCGC
CTATTGGCATATTGCTGGTTTGTACGGTTTGTATTTGTGTTTCACTTCTACTAAGTGGGCTAC
CGTTTTGTTCTCTTTCTTCTTGTTCGTTGTTGCCGAAGTTGGTGTTACTGCTGGTTCTCATAG
ATTGTGGTCACATAAGACTTACAAGGCTAAGTTGCCATTGCAAATCTTGTTGATGGTCATGAA
TTCCTTGGCTTTCCAAAACACCGTTATCGATTGGGTTAGAGATCACAGATTGCATCACAAGTA
CTCTGATACTGATGCTGATCCACATAATGCTTCTAGAGGTTTCTTCTACTCTCATGTTGGTTG
GTTGTTGGTTAGAAAACACCCAGATGTTAAGAAGAGAGGTAAAGAAATCGACATCTCCGACA
TCTACAACAACCCAGTTTTGAGATTCCAAAAGAAGTACGCCATTCCATTCATTGGTGCTGTTT
GTTTTGTTTTGCCAACCTTGATTCCAGTTTATGGTTGGGGTGAAACTTGGACTAATGCTTGGC
ATGTTGCTATGTTGAGATATATCATGAACTTGAACGTCACCTTCTTGGTTAATTCTGCTGCTC
ATATCTACGGTAAAAGACCATACGATAAGAAGATCTTGCCATCCCAAAACATTGCTGTTTCTA
TTGCTACTTTCGGTGAAGGTTTCCATAACTACCATCATGTTTTTCCATGGGATTACAGAGCTG

CTGAATTGGGTAACAATTCTTTGAACTTCCCTACCAAGTTCATCGATTTTTTCGCTTGGATTG
GTTGGGCCTACGATTTGAAAACTGTCTCCAAAGAAATGATCAAGCAAAGATCTAAGAGAACC
GGTGATGGTACTAATTTGTGGGGTTTGGAAGATGTTGATACCCCAGAAGATTTGAAGAACAC
TAAGGGTGAATGA

>SEQ ID NO: 11. ∆11-desaturase from *Trichoplusia ni* (WO_2016_207339_A1_44)
ATGGCTGTGATGGCTCAAACAGTACAAGAAACGGCTACAGTGTTGGAAGAGGAAGCTCGCA
CAGTGACTCTTGTGGCTCCAAAGACAACGCCAAGGAAATATAAATATATATACACCAACTTTC
TTACATTTTCATATGCGCATTTAGCTGCATTATACGGACTTTATTTGTGCTTCACCTCTGCGAA
ATGGGAAACATTGCTATTCTCTTTCGTACTCTTCCACATGTCAAATATAGGCATCACCGCAGG
GGCTCACCGACTCTGGACTCACAAGACTTTCAAAGCCAAATTGCCTTTGGAAATTGTCCTCA
TGATATTCAACTCTTTAGCCTTTCAAAACACGGCTATTACATGGGCTAGAGAACATCGGCTAC
ATCACAAATACAGCGATACTGATGCTGATCCCCACAATGCGTCAAGAGGGTTCTTCTACTCG
CATGTTGGCTGGCTATTAGTAAAAAAACATCCCGATGTCCTGAAATATGGAAAAACTATAGAC
ATGTCGGATGTATACAATAATCCTGTGTTAAAATTTCAGAAAAAGTACGCAGTACCCTTAATT
GGAACAGTTTGTTTTGCTCTTCCAACTTTGATTCCAGTCTACTGTTGGGGCGAATCGTGGAA
CAACGCTTGGCACATAGCCTTATTTCGATACATATTCAATCTTAACGTGACTTTCCTAGTCAA
CAGTGCTGCGCATATCTGGGGGAATAAGCCTTATGATAAAAGCATCTTGCCCGCTCAAAACC
TGCTGGTTTCCTTCCTAGCAAGTGGAGAAGGCTTCCATAATTACCATCACGTCTTTCCATGG
GATTACCGCACAGCAGAATTAGGGAATAACTTCCTGAATTTGACGACGCTGTTCATTGATTTT
TGTGCCTGGTTTGGATGGGCTTATGACTTGAAGTCTGTATCAGAGGATATTATAAAACAGAG
AGCTAAACGAACAGGTGACGGTTCTTCAGGGGTCATTTGGGGATGGGACGACAAAGACATG
GACCGCGATATAAAATCTAAAGCTAACATTTTTTATGCTAAAAAGGAATGA

>SEQ ID NO: 12. ∆9-desaturase from *Drosophila melanogaster* (WO_2018_109167_A1_9)
ATGGCTCCATACTCTAGAATCTACCACCAAGATAAGTCCTCTAGAGAAACTGGTGTTTTGTTC
GAAGATGATGCTCAAACCGTTGATTCTGATTTGACTACCGATAGATTCCAATTGAAGAGAGC
CGAAAAAAGAAGATTGCCATTGGTTTGGAGAAACATCATCTTGTTCGCTTTGGTTCATTTGGC
TGCCTTGTATGGTTTACATTCCATTTTCACTAGAGCTAAGTTGGCTACTACTTTGTTTGCTGCT
GGTTTGTACATTATCGGTATGTTGGGTGTTACTGCTGGTGCTCATAGATTGTGGGCTCATAG
AACTTACAAAGCTAAATGGCCTTTGAGATTGTTGTTGGTCATCTTCAACACCATTGCTTTCCA
AGATGCTGTTTATCATTGGGCCAGAGATCATAGAGTTCATCACAAATACTCTGAAACCGATG
CTGATCCACATAATGCTACTAGAGGTTTCTTCTTCTCTCATGTTGGTTGGTTGTTGTGCAAGA
AACACCCAGATATCAAAGAAAAGGGTAGAGGTTTGGATTTGTCCGATTTGAGAGCTGATCCA
ATCTTGATGTTTCAAAGAAAGCACTACTACATCTTGATGCCATTGGCTTGTTTTGTTTTGCCAA
CCGTTATTCCAATGGTCTACTGGAACGAAACTTTGGCTTCTTCTTGGTTTGTTGCTACTATGT
TCAGATGGTGCTTCCAATTGAATATGACCTGGTTGGTTAATTCCGCTGCTCATAAGTTTGGTA
ATAGACCATACGATAAGACCATGAACCCAACTCAAAATGCTTTCGTTTCTGCTTTCACTTTTG
GTGAAGGTTGGCATAATTACCATCATGCTTTTCCATGGGATTACAAGACTGCTGAATGGGGT
TGTTACTCTTTGAACATTACTACCGCCTTCATTGATTTGTTCGCTAAAATTGGTTGGGCCTAC
GATTTGAAAACTGTTGCTCCAGATGTTATCCAAAGAAGAGTTTTGAGAACTGGTGATGGTTCT
CATGAATTGTGGGGTTGGGGTGATAAGGATTTGACCGCTGAAGATGCTAGAAACGTTTTGTT
GGTTGACAAGTCCAGATAA

>SEQ ID NO: 13. Hygromycin resistance gene (Addgene_Cat# 106127)
ATGGGTAAAAAGCCTGAACTCACCGCGACGTCTGTCGAGAAGTTTCTGATCGAAAAGTTCGA
CAGCGTCTCCGACCTGATGCAGCTCTCGGAGGGCGAAGAATCTCGTGCTTTCAGCTTCGAT
GTAGGAGGGCGTGGATATGTCCTGCGGGTAAATAGCTGCGCCGATGGTTTCTACAAAGATC
GTTATGTTTATCGGCACTTTGCATCGGCCGCGCTCCCGATTCCGGAAGTGCTTGACATTGGG
GAATTCAGCGAGAGCCTGACCTATTGCATCTCCCGCCGTGCACAGGGTGTCACGTTGCAAG
ACCTGCCTGAAACCGAACTGCCCGCTGTTCTGCAGCCGGTCGCGGAGGCAATGGATGCCAT
TGCTGCGGCCGATCTTAGCCAGACGAGCGGGTTCGGCCCATTCGGACCGCAAGGAATCGG
TCAATACACTACATGGCGTGATTTCATATGCGCGATTGCTGATCCCCATGTGTATCACTGGC
AAACTGTGATGGACGACACCGTCAGTGCGTCCGTCGCGCAGGCTCTCGATGAGCTGATGCT
TTGGGCCGAGGACTGCCCCGAAGTCCGGCACCTCGTGCACGCGGATTTCGGCTCCAACAA
TGTCCTGACGGACAATGGCCGCATAACAGCGGTCATTGACTGGAGCGAGGCGATGTTCGG

GGATTCCCAATACGAGGTCGCCAACATCTTCTTCTGGAGGCCGTGGTTGGCTTGTATGGAG
CAGCAGACGCGCTACTTCGAGCGGAGGCATCCGGAGCTTGCAGGATCGCCGCGGCTCCGG
GCGTATATGCTCCGCATTGGTCTTGACCAACTCTATCAGAGCTTGGTTGACGGCAATTTCGA
TGATGCAGCTTGGGCGCAGGGTCGATGCGACGCAATCGTCCGATCCGGAGCCGGGACTGT
CGGGCGTACACAAATCGCCCGCAGAAGCGCGGCCGTCTGGACCGATGGCTGTGTAGAAGT
ACTCGCCGATAGTGGAAACCGACGCCCCAGCACTCGTCCGAGGGCAAAGGAATAA

>SEQ ID NO: 14. Atrd11 expression cassette and the upstream genomic region of integration site B

Element	**Position (bp)**	
IntB upstream	9-508	Addgene Cat#106124
PEX20 terminator	881-569	Addgene Cat#106124
Atrd11	1862-882	WO_2016_207339_A1_1
GPD promoter	2800-1869	CR382129.1 nt:825834...826766

CGTGCGATCCCACAGTTCTCACTCAGATCATGGAGACTCTAACCTTGAGACATCAATTATCA
GCTCTCGAGGATAATGTTAGTGCAGTTCCAGGACTCATTGTGCAACTGTCACCACGGCATTT
TGGGTCTGTTCTTTTGAAGTACAGAAATATCCTCATTGTTGGTATACTTTGGGACTTTTTCTTG
TTACAGAAGAATAAAAAAACCTCGACTGATGTACTAATTACATGGTTAACATCCCCAAGGTCA
AAGTACAGATATTGTACCGACTTCTGAAATTTGTGGGATCCACACACGGACCTCTGCGATGA
TACAATATCATGGTTCCATGGTCTCTTGAATCACACCACTCAATAATAAACACCAGCTCTTTC
AATCCAACTTAGCTTTCTTCCACTTGAACATAGGCCATCCCCCCTTCTTTCTATTTTACATAAA
TAGCAAGATCCTTACCCCTACATGTCTCATAACACAATCTCAACTTGACTTCCCATAAGAAGT
TCACTCAGTCATGAAAGTCTTAGTACTGGACGTGCAACGCTTCAGATGTGACCATATACTTA
GGCACGCAACTAACATGAATGAATACGATATACATCAAAGACTATGATACGCAGTATTGCAC
ACTGTACGAGTAAGAGCACTAGCCACTGCACTCAAGTGAAACCGTTGCCCGGGTACGAGTA
TGAGTATGTACAGTATGTTTAGTATTGTACTTGGACAGTGCTTGTATCGTACATTCTCAAGTG
TCAAACATAAATATCCGTTGCTATATCCTCGCACCACCACGTAGCTCGCTATATCCCTGTGTT
GAATCCATCCATCTTGGATTGCCAATTGTGCACACAGAACCGGGCACTCACTTCCCCATCCA
CACTTTCATCTCTTTCTACCCCACATATCAGTACCATCACCAGTTTTAGCACATCTCTTAGCAA
TAGCTTCTTGTGGAACAGATTTCAAATCGTAGGCCCAACCAATCCAAGCAAAGAAATCAATG
AAGGCGGTGGTCATATTCAATCTATTGTTACCCAATTCAGCAGCTCTGTAATCCCATGGATAA
GTATGATGGTAGTTATGGAAACCTTCACCGAAAGTAGCCAAAGAAACAGAGATGTTTTGAAC
AGAGGCGATAGACTTATCGTATGGCTTGTTACCAAAAATATGAGCAGCGGAGTTAACCAAAA
AGGTCATGTTCAAGTTCATGACGTATCTTAACAAGTTAATGTTCCAGGCAGTAGAAATACCTT
CACCCCAAAAGTAAACTGGAACGAAAGTTGGCATCAAAAAGCACAAAGTACCAATGACCAAA
ATGGCGTACTTCTTTTGGAACTTCAACAAAGGGTTGTTCTTCAAGTCATCCAAAGACAAGTAC
TTACCTCTAGCCTTAACTTCTGGGTGTTTCTTAACCAACAACCAACCAACATGAGAGTAGAAG
AAACCTCTAGTAGCATTATGTGGATCAGCATCAGTATCAGAGTACTTGTGATGCAATCTATGA
TCTCTAGCCCAAGTAAAAGCAGTATCTTGGAAGGCAATGGAGTTCATGATCAACAACAAGAT
TTCCAATGGCAACTTGGCTTTGTAAGTTCTATGAGCCCATAATCTATGAGCACCACCAGTAAT
ACCAATTTCGGCGATAACGTACAAGAAGAAAGCGAACAAAATAGTAGCCCACTTAGCACAAG
TGAAACACAAGTACAAACCATAAACAGCAGACAAATGCCAGTAACCGAAGGTCAACAAGTTT
CTGTAAACGATTTTGTACTTTCTTGGACCAGCTTGTGGAGCAATCAACTTGGTGAATTGTGGT
TCAGAATGTTCAGACAAAACATCAGAGGAACCCTTGTTTGGAACCATTGTGGCGTTGATGTG
TGTTTAATTCAAGAATGAATATAGAGAAGAGAAGAAGAAAAAAGATTCAATTGAGCCGGCGAT
GCAGACCCTTATATAAATGTTGCCTTGGACAGACGGAGCAAGCCCGCCCAAACCTACGTTC
GGTATAATATGTTAAGCTTTTTAACACAAAGGTTTGGCTTGGGGTAACCTGATGTGGTGCAAA
AGACCGGGCGTTGGCGAGCCATTGCGCGGGCGAATGGGGCCGTGACTCGTCTCAAATTCG
AGGGCGTGCCTCAATTCGTGCCCCCGTGGCTTTTTCCCGCCGTTTCCGCCCCGTTTGCACC
ACTGCAGCCGCTTCTTTGGTTCGGACACCTTGCTGCGAGCTAGGTGCCTTGTGCTACTTAAA
AAGTGGCCTCCCAACACCAACATGACATGAGTGCGTGGGCCAAGACACGTTGGCGGGGTC
GCAGTCGGCTCAATGGCCCGGAAAAAACGCTGCTGGAGCTGGTTCGGACGCAGTCCGCCG
CGGCGTATCGATATCCGCAAGGTTCCATGGCGCCATTGCCCTCCGTCGGCGTCTATCCCGC

AACCTCTAAATAGAGCGGGAATATAACCCAAGCTTCTTTTTTTTTCCTTTAACACGCACACCC
CCAACTATCATGTTGCTGCTGCTGTTTGACTCTACTCTGTGGAGGGGTGCTCCCACCCAACC
CAACCTACAGGTGGATCCGGCGCTGTGATTGGCTGATAAGTCTCCTATCCGGACTAATTCTG
ACCAATGGGACATGCGCGCAGGACCCAAATGCCGCAATTACGTAACCCCAACGAAATGCCT
ACCCCTCTTTGGAGCCCAGCGGCCCCAAATCCCCCCAAGCAGCCCGGTTCTACCGGCTTCC
ATCTCCAAGCACCCCTTTCTCCACACCCCACAAAAAGACCCGTGCAGGACATCCTACTGCGT
CACCTGCACT

>SEQ ID NO: 15. HsuFAR expression cassette

Element	**Position (bp)**	
TEFINTRON promoter	10-540	CR382129.1 nt:1244238.. 1243709
HsuFAR	541-1899	WO_2016_207339_A1_15
LIP2 terminator	1900-2836	Addgene Cat#106124

ACCTGCACTAGAGACCGGGTTGGCGGCGCATTTGTGTCCCAAAAAACAGCCCCAATTGCCC
CAATTGACCCCAAATTGACCCAGTAGCGGGCCCAACCCCGGCGAGAGCCCCCTTCTCCCCA
CATATCAAACCTCCCCCGGTTCCCACACTTGCCGTTAAGGGCGTAGGGTACTGCAGTCTGG
AATCTACGCTTGTTCAGACTTTGTACTAGTTTCTTTGTCTGGCCATCCGGGTAACCCATGCCG
GACGCAAAATAGACTACTGAAAATTTTTTTGCTTTGTGGTTGGGACTTTAGCCAAGGGTATAA
AAGACCACCGTCCCCGAATTACCTTTCCTCTTCTTTTCTCTCTCTCCTTGTCAACTCACACCC
GAAATCGTTAAGCATTTCCTTCTGAGTATAAGAATCATTCAAAATGGTGAGTTTCAGAGGCAG
CAGCAATTGCCACGGGCTTTGAGCACACGGCCGGGTGTGGTCCCATTCCCATCGACACAAG
ACGCCACGTCATCCGACCAGCACTTTTTGCAGTACTAACCGCAGGTTGTCTTGACCTCCAAA
GAAACTAAGCCATCTGTTGCTGAATTTTACGCTGGTAAGTCTGTTTTCATTACTGGTGGTACT
GGTTTCTTGGGTAAGGTTTTCATTGAAAAGTTGTTGTACTCCTGCCCAGATATCGGTAATATC
TACATGTTGATCAGAGAAAAGAAGGGTTTGTCCGTTTCCGAAAGAATCAAGCACTTTTTGGAT
GATCCTTTGTTCACCAGATTGAAAGAAAAAAGACCAGCCGACTTGGAAAAGATCGTTTTGATT
CCAGGTGATATTACTGCTCCAGATTTGGGTATTACCTCCGAAAACGAAAAGATGTTGATCGA
AAAGGTCAGTGTCATTATTCATTCTGCTGCTACCGTTAAGTTCAACGAACCATTGCCAACTGC
TTGGAAGATTAACGTTGAAGGTACTAGAATGATGTTGGCCTTGTCTAGAAGAATGAAGAGAA
TCGAAGTTTTCATCCATATCTCTACCGCTTACACTAACACCAACAGAGAAGTTGTTGACGAAA
TCTTGTATCCAGCTCCAGCTGATATTGATCAAGTTCACCAATATGTTAAGGACGGTATCTCTG
AAGAAGAAACTGAAAAAATCTTGAACGGTAGACCAAACACTTACACTTTCACTAAGGCTTTGA
CCGAACATTTGGTTGCTGAAAATCAAGCTTACGTTCCAACCATTATCGTTAGACCATCAGTTG
TTGCTGCCATTAAGGATGAACCTATTAAGGGTTGGTTGGGTAATTGGTATGGTGCTACAGGT
TTGACTGTTTTTACTGCTAAGGGTTTGAACAGAGTTATCTACGGTCACTCTTCTAACATCGTT
GATTTGATCCCAGTTGATTACGTTGCCAACTTGGTTATTGCTGCTGGTGCTAAATCTTCTAAG
TCTACTGAATTGAAGGTCTACAACTGCTGTTCTTCTGCTTGTAACCCAATTACTATCGGTAAG
TTGATGTCCATGTTTGCTGAAGATGCTATCAAGCAAAAGTCTTACGCTATGCCATTGCCAGGT
TGGTACATTTTTACTAAGTACAAGTGGTTGGTCTTGTTGTTGACCATTTTGTTCCAAGTTATTC
CAGCCTACATTACCGACTTGTACAGACATTTGATTGGTAAGAACCCAAGATATATCAAGTTGC
AATCCTTGGTCAATCAAACCAGATCCTCCATTGATTTCTTCACCAACCATTCTTGGGTTATGA
AGGCTGATAGAGTCAGAGAATTATTCGCTTCTTTGTCTCCAGCAGATAAGTACTTGTTTCCAT
GTGATCCAGTCAACATCAATTGGAGACAATATATCCAAGATTACTGCTGGGGTGTTAGACATT
TCTTGGAAAAAAAGACTTAACTTCTGTTCGGAATCAACCTCAAGGTTAACGGCCACGATCCC
CTCGTTGTTACTCTTGGTCAGCCCATTGTCGGTAACGCTGGCTTTGCTAACTGGGTCGATAA
ACTCTTCTTTGGCCAGGAGAACCCCGATGTCTCCAAGGTGTCCAAAGACCGAAAGCTCTACC
GAATCACCCACCGAGGAGATATCGTCCCTCAAGTGCCCTTCTGGGACGGTTACCAGCACTG
CTCTGGTGAGGTCTTTATTGACTGGCCCCTGATCCACCCTCCTCTCTCCAACGTTGTCATGT
GCCAGGGCCAGAGCAATAAACAGTGCTCTGCCGGTAACACTCTGCTCCAGCAGGTCAATGT
GATTGGAAACCATCTGCAGTACTTCGTCACCGAGGGTGTCTGTGGTATCTAAGCTATTTATC
ACTCTTTACAACTTCTACCTCAACTATCTACTTTAATAAATGAATATCGTTTATTCTCTATGATT
ACTGTATATGCGTTCCTCTAAGACAAATCGAAACCAGCATGCGATCGAATGGCATACAAAAG
TTTCTTCCGAAGTTGATCAATGTCCTGATAGTCAGGCAGCTTGAGAAGATTGACACAGGTGG
AGGCCGTAGGGAACCGATCAACCTGTCTACCAGCGTTACGAATGGCAAATGACGGGTTCAA

AGCCTTGAATCCTTGCAATGGTGCCTTGGATACTGATGTCACAAACTTAAGAAGCAGCCGCT
TGTCCTCTTCCTCGAAACTCTCAAACACAGTCCAGAAGTCCTTTATAGTTTGATCTGTATCCA
GATAGCCTCCGTAATTGGTGTGTGTCTTCAAATCCCAGACGTCCACATTGGCATGTCCTCCA
CTGATAAGCATTTGAAGTTCATCTGCGTTGAACATTGAGACCCACGAAGGGTCAATGAGCTG
GTATAGACCGCCCAAGAATGCATCTGATCTGTCAT

>SEQ ID NO: 16. Ura3 marker cassette fused to 500 bp downstream region for integration into IntB site Addgene Cat#106124

Element	**Position (bp)**
loxP site	1-34
EXP promoter	35-1036
Ura3	1037-1891
Cyc1 terminator	1892-2124
loxP site	2125-2158
IntB downstream	2219-2704

ATAACTTCGTATAATGTATGCTATACGAAGTTATAAGGAGTTTGGCGCCCGTTTTTTCGAGCC
CCACACGTTTCGGTGAGTATGAGCGGCGGCAGATTCGAGCGTTTCCGGTTTCCGCGGCTGG
ACGAGAGCCCATGATGGGGGCTCCCACCACCAGCAATCAGGGCCCTGATTACACACCCACC
TGTAATGTCATGCTGTTCATCGTGGTTAATGCTGCTGTGTGCTGTGTGTGTGTTGTTTGG
CGCTCATTGTTGCGTTATGCAGCGTACACCACAATATTGGAAGCTTATTAGCCTTTCTATTTT
TTCGTTTGCAAGGCTTAACAACATTGCTGTGGAGAGGGATGGGGATATGGAGGCCGCTGGA
GGGAGTCGGAGAGGCGTTTTGGAGCGGCTTGGCCTGGCGCCCAGCTCGCGAAACGCACCT
AGGACCCTTTGGCACGCCGAAATGTGCCACTTTTCAGTCTAGTAACGCCTTACCTACGTCAT
TCCATGCATGCATGTTTGCGCCTTTTTTCCCTTGCCCTTGATCGCCACACAGTACAGTGCAC
TGTACAGTGGAGGTTTTGGGGGGGTCTTAGATGGGAGCTAAAAGCGGCCTAGCGGTACACT
AGTGGGATTGTATGGAGTGGCATGGAGCCTAGGTGGAGCCTGACAGGACGCACGACCGGC
TAGCCCGTGACAGACGATGGGTGGCTCCTGTTGTCCACCGCGTACAAATGTTTGGGCCAAA
GTCTTGTCAGCCTTGCTTGCGAACCTAATTCCCAATTTTGTCACTTCGCACCCCCATTGATCG
AGCCCTAACCCCTGCCCATCAGGCAATCCAATTAAGCTCGCATTGTCTGCCTTGTTTAGTTT
GGCTCCTGCCCGTTTCGGCGTCCACTTGCACAAACACAAACAAGCATTATATATAAGGCTCG
TCTCTCCCTCCCAACCACACTCACTTTTTTGCCCGTCTTCCCTTGCTAACACAAAAGTCAAGA
ACACAAACAACCACCCCAACCCCCTTACACACAAGACATATCTACAGCAATGCCCTCCTACG
AGGCCCGAGCCAACGTCCACAAGTCCGCCTTCGCCGCCCGAGTCCTGAAGCTGGTCGCCG
CCAAGAAGACCAACCTGTGCGCCTCCCTGGACGTCACCACCACCAAGGAGCTGATCGAGCT
GGCCGACAAGGTCGGCCCCTACGTCTGCATGATCAAGACCCACATCGACATCATCGACGAC
TTCACCTACGCCGGCACCGTCCTGCCCCTGAAGGAGCTGGCCCTGAAGCACGGCTTCTTCC
TGTTCGAGGACCGAAAGTTCGCCGACATCGGCAACACCGTCAAGCACCAGTACCGATGCCA
CCGAATCGCCGAGTGGTCCGACATCACCAACGCCCACGGCGTCCCCGGCACCGGCATCAT
CGCCGGCCTGCGAGCCGGCGCCGAGGAGACCGTCTCCGAGCAGAAGAAGGAGGACGTCT
CCGACTACGAGAACTCCCAGTACAAGGAGTTCCTGGTCCCCTCCCCCAACGAGAAGCTGGC
CCGAGGCCTGCTGATGCTGGCCGAGCTGTCCTGCAAGGGCTCCCTGGCCACCGGCGAGTA
CTCCAAGCAGACCATCGAGCTGGCCCGATCCGACCCCGAGTTCGTCGTCGGCTTCATCGCC
CAGAACCGACCCAAGGGCGACTCCGAGGACTGGCTGATCCTGACCCCCGGCGTCGGCCTG
GACGACAAGGGCGACGCCCTGGGCCAGCAGTACCGAACCGTCGAGGACGTCATGTCCACC
GGCACCGACATCATCATCGTCGGCCGAGGCCTGTACGGCCAGAACCGAGACCCCATCGAG
GAGGCCAAGCGATACCAGAAGGCCGGCTGGGAGGCCTACCAGAAGATCAACTGCTAGTCA
TGTAATTAGTTATGTCACGCTTACATTCACGCCCTCCCCCCACATCCGCTCTAACCGAAAAG
GAAGGAGTTAGACAACCTGAAGTCTAGGTCCCTATTTATTTTTTTATAGTTATGTTAGTATTAA
GAACGTTATTTATATTTCAAATTTTTCTTTTTTTTCTGTACAGACGCGTGTACGCATGTAACAT
TATACTGAAAACCTTGCTTGAGAAGGTTTTGGGACGCTCGATAACTTCGTATAATGTATGCTA
TACGAAGTTATCAGCATCGTAATAGCCTCCAAGAGATTGATCATCACTCTGAATGTACAAGCA
ACCCAAGTACCTGCTCCTGCACCTAAGTTCGTCAAAATCGGTTTTACTCAGAGAATCAACAA
CCCTACCAACTGTACATACTGCTAACCCTGATTCTTTGAATAACCCCAATAAGGCTCCTGCTG
ACCCTTCTGCCGTTCTAGGAAACCAGCTTGTTGAGTACGCTGTGAACATCACTCTTGGTATA

CCCGGTCAGCCCTTCTCAGTTCAGATTGACACAGGCTCGTCTGACTTGTGGGTGAAGAGTG
ACGGCTCCTCCGGTGCATTCAACAAGAAGGCTTCTTCTACTTTTCAGGAGGACGTTCCCAAC
GGCTTTGCAATTGCCCACGGAGACAAAACCTCTGCCATTGGAGATTGGGTCAAAGATACCAT
CAATATCGGTGGTGTTAGTATTGACCAGTATAGTATTGACCAGTATGAGTTCGCCATGGCTA
CTCAGACAAATACTGACCCGGTTTTTGGTATCGGCTACCCGAGCAACGAGGCGTCTTATG

>SEQ ID NO: 17. Atrd11 expression cassette

Element	**Position (bp)**	
PEX20 terminator	321-9	Addgene Cat#106124
Atrd11	1302-322	WO_2016_207339_A1_1
GPD promoter	2240-1309	CR382129.1 nt:825834...826766

CGTGCGATACGCAACTAACATGAATGAATACGATATACATCAAAGACTATGATACGCAGTATT
GCACACTGTACGAGTAAGAGCACTAGCCACTGCACTCAAGTGAAACCGTTGCCCGGGTACG
AGTATGAGTATGTACAGTATGTTTAGTATTGTACTTGGACAGTGCTTGTATCGTACATTCTCA
AGTGTCAAACATAAATATCCGTTGCTATATCCTCGCACCACCACGTAGCTCGCTATATCCCTG
TGTTGAATCCATCCATCTTGGATTGCCAATTGTGCACACAGAACCGGGCACTCACTTCCCCA
TCCACACTTTCATCTCTTTCTACCCCACATATCAGTACCATCACCAGTTTTAGCACATCTCTTA
GCAATAGCTTCTTGTGGAACAGATTTCAAATCGTAGGCCCAACCAATCCAAGCAAAGAAATC
AATGAAGGCGGTGGTCATATTCAATCTATTGTTACCCAATTCAGCAGCTCTGTAATCCCATGG
ATAAGTATGATGGTAGTTATGGAAACCTTCACCGAAAGTAGCCAAAGAAACAGAGATGTTTT
GAACAGAGGCGATAGACTTATCGTATGGCTTGTTACCAAAAATATGAGCAGCGGAGTTAACC
AAAAAGGTCATGTTCAAGTTCATGACGTATCTTAACAAGTTAATGTTCCAGGCAGTAGAAATA
CCTTCACCCCAAAAGTAAACTGGAACGAAAGTTGGCATCAAAAAGCACAAAGTACCAATGAC
CAAAATGGCGTACTTCTTTTGGAACTTCAACAAAGGGTTGTTCTTCAAGTCATCCAAAGACAA
GTACTTACCTCTAGCCTTAACTTCTGGGTGTTTCTTAACCAACAACCAACCAACATGAGAGTA
GAAGAAACCTCTAGTAGCATTATGTGGATCAGCATCAGTATCAGAGTACTTGTGATGCAATCT
ATGATCTCTAGCCCAAGTAAAAGCAGTATCTTGGAAGGCAATGGAGTTCATGATCAACAACA
AGATTTCCAATGGCAACTTGGCTTTGTAAGTTCTATGAGCCCATAATCTATGAGCACCACCAG
TAATACCAATTTCGGCGATAACGTACAAGAAGAAAGCGAACAAAATAGTAGCCCACTTAGCA
CAAGTGAAACACAAGTACAAACCATAAACAGCAGACAAATGCCAGTAACCGAAGGTCAACAA
GTTTCTGTAAACGATTTTGTACTTTCTTGGACCAGCTTGTGGAGCAATCAACTTGGTGAATTG
TGGTTCAGAATGTTCAGACAAAACATCAGAGGAACCCTTGTTTGGAACCATTGTGGCGTTGA
TGTGTGTTTAATTCAAGAATGAATATAGAGAAGAGAAGAAGAAAAAAGATTCAATTGAGCCGG
CGATGCAGACCCTTATATAAATGTTGCCTTGGACAGACGGAGCAAGCCCGCCCAAACCTAC
GTTCGGTATAATATGTTAAGCTTTTTAACACAAAGGTTTGGCTTGGGGTAACCTGATGTGGTG
CAAAAGACCGGGCGTTGGCGAGCCATTGCGCGGGCGAATGGGGCCGTGACTCGTCTCAAA
TTCGAGGGCGTGCCTCAATTCGTGCCCCCGTGGCTTTTTCCCGCCGTTTCCGCCCCGTTTG
CACCACTGCAGCCGCTTCTTTGGTTCGGACACCTTGCTGCGAGCTAGGTGCCTTGTGCTAC
TTAAAAAGTGGCCTCCCAACACCAACATGACATGAGTGCGTGGGCCAAGACACGTTGGCGG
GGTCGCAGTCGGCTCAATGGCCCGGAAAAAACGCTGCTGGAGCTGGTTCGGACGCAGTCC
GCCGCGGCGTATCGATATCCGCAAGGTTCCATGGCGCCATTGCCCTCCGTCGGCGTCTATC
CCGCAACCTCTAAATAGAGCGGGAATATAACCCAAGCTTCTTTTTTTTTCCTTTAACACGCAC
ACCCCCAACTATCATGTTGCTGCTGCTGTTTGACTCTACTCTGTGGAGGGGTGCTCCCACCC
AACCCAACCTACAGGTGGATCCGGCGCTGTGATTGGCTGATAAGTCTCCTATCCGGACTAAT
TCTGACCAATGGGACATGCGCGCAGGACCCAAATGCCGCAATTACGTAACCCCAACGAAAT
GCCTACCCCTCTTTGGAGCCCAGCGGCCCCAAATCCCCCCAAGCAGCCCGGTTCTACCGG
CTTCCATCTCCAAGCACCCCTTTCTCCACACCCCACAAAAAGACCCGTGCAGGACATCCTAC
TGCGTCACCTGCACT

>SEQ ID NO: 18. *LEU2* from *Kluyveromyces lactis* (Addgene Cat #67532)
ATGTCTAAGAATATCGTTGTCCTACCGGGTGATCACGTCGGTAAAGAAGTTACTGACGAAGC
TATTAAGGTCTTGAATGCCATTGCTGAAGTCCGTCCAGAAATTAAGTTCAATTTCCAACATCA
CTTGATCGGGGGTGCTGCCATCGATGCCACTGGCACTCCTTTACCAGATGAAGCTCTAGAA
GCCTCTAAGAAAGCCGATGCTGTCTTACTAGGTGCTGTTGGTGGTCCAAAATGGGGTACGG

GCGCAGTTAGACCAGAACAAGGTCTATTGAAGATCAGAAAGGAATTGGGTCTATACGCCAAC
TTAAGACCATGTAACTTTGCTTCTGATTCTTTACTAGATCTTTCTCCTTTGAAGCCTGAATATG
CAAAGGGTACCGATTTCGTCGTCGTTAGAGAATTGGTTGGTGGTATCTACTTTGGTGAAAGA
AAAGAAGATGAAGGTGACGGAGTTGCTTGGGACTCTGAGAAATACAGTGTTCCTGAAGTTCA
AAGAATTACAAGAATGGCTGCTTTCTTGGCATTGCAACAAAACCCACCATTACCAATCTGGTC
ACTTGACAAGGCTAACGTGCTTGCCTCTTCCAGATTGTGGAGAAAGACTGTTGAAGAAACCA
TCAAGACTGAGTTCCCACAATTAACTGTTCAGCACCAATTGATCGACTCTGCTGCTATGATTT
TGGTTAAATCACCAACTAAGCTAAACGGTGTTGTTATTACCAACAACATGTTTGGTGATATTA
TCTCCGATGAAGCCTCTGTTATTCCAGGTTCTTTGGGTTTATTACCTTCTGCATCTCTAGCTT
CCCTACCTGACACTAACAAGGCATTCGGTTTGTACGAACCATGTCATGGTTCTGCCCCAGAT
TTACCAGCAAACAAGGTTAACCCAATTGCTACCATCTTATCTGCAGCTATGATGTTGAAGTTA
TCCTTGGATTTGGTTGAAGAAGGTAGGGCTCTTGAAGAAGCTGTTAGAAATGTCTTGGATGC
AGGTGTCAGAACCGGTGACCTTGGTGGTTCTAACTCTACCACTGAGGTTGGCGATGCTATC
GCCAAGGCTGTCAAGGAAATCTTGGCTTAA

>SEQ ID NO: 19. Fatty acyl-reductase from *Helicoverpa armigera* codon-optimized for *Y. lipolytica* (WO_2018_109167_A1_43)
ATGGTGGTCCTGACCTCTAAGGAGACTAAGCCCTCCGTGGCCGAGTTCTACGCTGGCAAGT
CTGTCTTCATCACCGGCGGAACCGGTTTCCTGGGCAAGGTCTTCATTGAGAAGCTGCTGTA
CTCCTGTCCCGACATCGGCAACATCTACATGCTGATCCGAGAGAAGAAGGGACTGTCTGTG
TCCGAGCGAATTAAGCACTTCCTGGACGACCCCCTGTTCACCCGACTGAAGGAGAAGCGAC
CCGCCGACCTGGAGAAGATCGTGCTGATTCCCGGAGACATCACCGCTCCCGACCTGGGTAT
TACCTCTGAGAACGAGAAGATGCTGATCGAGAAGGTGTCTGTCATCATTCACTCCGCCGCTA
CCGTCAAGTTCAACGAGCCCCTGCCCACCGCCTGGAAGATCAACGTGGAGGGAACCCGAAT
GATGCTGGCTCTGTCTCGACGAATGAAGCGAATTGAGGTCTTCATCCACATTTCCACCGCCT
ACACCAACACCAACCGAGAGGTGGTGGACGAGATCCTGTACCCTGCTCCTGCTGACATTGA
CCAGGTGCACCGATACGTCAAGGACGGTATCTCTGAGGAAGAGACTGAGAAGATTCTGAAC
GGCCGACCCAACACCTACACCTTCACCAAGGCCCTGACCGAGCACCTGGTGGCTGAGAAC
CAGGCTTACGTGCCCACCATCATTGTCCGACCCTCCGTGGTCGCCGCTATCAAGGACGAGC
CCATTAAGGGATGGCTGGGTAACTGGTACGGAGCTACCGGACTGACCGTGTTCACCGCTAA
GGGTCTGAACCGAGTCATCTACGGCCACTCTTCCAACATCGTGGACCTGATTCCCGTGGAC
TACGTCGCCAACCTGGTCATTGCCGCTGGCGCTAAGTCTTCCAAGTCCACCGAGCTGAAGG
TGTACAACTGTTGCTCTTCCGCCTGCAACCCCATCACCATTGGAAAGCTGATGTCTATGTTC
GCCGAGGACGCTATCAAGCAGAAGTCCTACGCTATGCCCCTGCCCGGTTGGTACATCTTCA
CCAAGTACAAGTGGCTGGTCCTGCTGCTGACCATTCTGTTCCAGGTCATCCCCGCCTACATT
ACCGACCTGTACCGACACCTGATCGGCAAGAACCCCCGATACATTAAGCTGCAGTCTCTGG
TCAACCAGACCCGATCTTCCATTGACTTCTTCACCTCTCACTCCTGGGTCATGAAGGCTGAC
CGAGTCCGAGAGCTGTTCGCCTCTCTGTCCCCCGCTGACAAGTACCTGTTCCCCTGTGACC
CCACCGACATCAACTGGACCCACTACATTCAGGACTACTGCTGGGGAGTGCGACACTTCCT
GGAGAAGAAGTCCTACGAGTAG

>SEQ ID NO: 20. Δ9-desaturase from *Drosophila melanogaster* codon.optimized for *Y. lipolytica* (WO_2018_109167_A1_36
ATGGCTCCCTACTCTCGAATCTACCACCAGGACAAGTCGTCCCGAGAGACTGGCGTGCTGT
TCGAGGACGACGCCCAGACCGTGGACTCTGACCTGACCACCGACCGATTCCAGCTGAAGC
GAGCCGAGAAGCGACGACTGCCCCTGGTGTGGCGAAACATCATCCTGTTCGCCCTGGTGC
ACCTGGCCGCTCTGTACGGCCTGCACTCTATCTTCACCCGAGCCAAGCTGGCCACCACTCT
GTTCGCTGCCGGCCTGTACATCATCGGCATGCTGGGCGTGACCGCTGGCGCCCACCGACT
GTGGGCTCACCGAACCTACAAGGCCAAGTGGCCCCTGCGACTGCTGCTGGTGATCTTCAAC
ACCATTGCCTTCCAGGACGCCGTGTACCACTGGGCCCGAGATCACCGAGTGCACCACAAGT
ACTCTGAGACTGACGCTGACCCTCACAACGCTACCCGAGGCTTCTTCTTCTCTCACGTCGGC
TGGCTGCTGTGCAAGAAGCACCCCGACATCAAGGAAAAGGGCCGAGGCCTGGACCTGTCT
GACCTGCGAGCTGACCCCATCCTGATGTTCCAGCGAAAGCACTACTACATTCTGATGCCCCT
GGCCTGCTTCGTGCTGCCCACCGTGATTCCCATGGTGTACTGGAACGAGACTCTGGCCTCT
TCCTGGTTCGTGGCCACCATGTTCCGATGGTGCTTCCAGCTCAACATGACCTGGCTGGTGA
ACTCTGCCGCTCACAAGTTCGGCAACCGACCTTACGACAAGACTATGAACCCCACTCAGAAC

GCCTTCGTGTCTGCCTTCACCTTCGGCGAAGGCTGGCACAACTACCACCACGCATTCCCTT
GGGACTACAAGACCGCCGAGTGGGGCTGCTACTCTCTGAACATCACCACCGCCTTCATCGA
CCTGTTCGCTAAGATCGGCTGGGCCTACGACCTCAAGACCGTGGCTCCCGACGTGATCCAG
CGACGAGTGCTGCGAACCGGCGACGGCTCTCACGAGCTGTGGGGCTGGGGCGACAAGGA
CCTGACCGCTGAGGACGCCCGAAACGTCCTGCTGGTGGACAAGTCTCGATAA

> SEQ ID NO: 21. Δ11-desaturase from from *Amyelois transitella* codon.optimized for *Y. lipolytica* (WO_2018_109167_A1_67)
ATGGTGCCCAACAAGGGTTCTTCCGACGTCCTGTCTGAGCACTCCGAGCCCCAGTTCACCA
AGCTGATTGCTCCCCAGGCTGGCCCCCGAAAGTACAAGATCGTGTACCGAAACCTGCTGAC
CTTCGGATACTGGCACCTGTCTGCCGTCTACGGTCTGTACCTGTGTTTCACCTGCGCCAAGT
GGGCTACCATTCTGTTCGCCTTCTTCCTGTACGTGATCGCTGAGATCGGCATTACCGGCGG
AGCCCACCGACTGTGGGCTCACCGAACCTACAAGGCCAAGCTGCCCCTGGAGATCCTGCT
GCTGATTATGAACTCTATCGCTTTCCAGGACACCGCCTTCACCTGGGCTCGAGATCACCGAC
TGCACCACAAGTACTCTGACACCGACGCTGACCCTCACAACGCTACCCGAGGTTTCTTCTAC
TCCCACGTGGGCTGGCTGCTGGTCAAGAAGCACCCCGAGGTGAAGGCCCGGGGAAAGTAC
CTGTCCCTGGACGACCTGAAGAACAACCCCCTGCTGAAGTTCCAGAAGAAGTACGCTATCC
TGGTCATTGGCACCCTGTGTTTCCTGATGCCCACCTTCGTGCCCGTCTACTTCTGGGGTGAG
GGCATTTCTACCGCCTGGAACATCAACCTGCTGCGATACGTGATGAACCTGAACATGACCTT
CCTGGTCAACTCCGCCGCTCACATTTTCGGCAACAAGCCCTACGACAAGTCTATTGCCTCCG
TGCAGAACATCTCTGTCTCCCTGGCTACCTTCGGAGAGGGTTTCCACAACTACCACCACACC
TACCCTTGGGACTACCGAGCTGCTGAGCTGGGCAACAACCGACTGAACATGACCACCGCCT
TCATTGACTTCTTCGCCTGGATCGGATGGGCTTACGACCTGAAGTCCGTCCCCCAGGAAGC
CATCGCTAAGCGATGCGCTAAGACCGGCGACGGAACCGACATGTGGGGACGAAAGCGATA
G

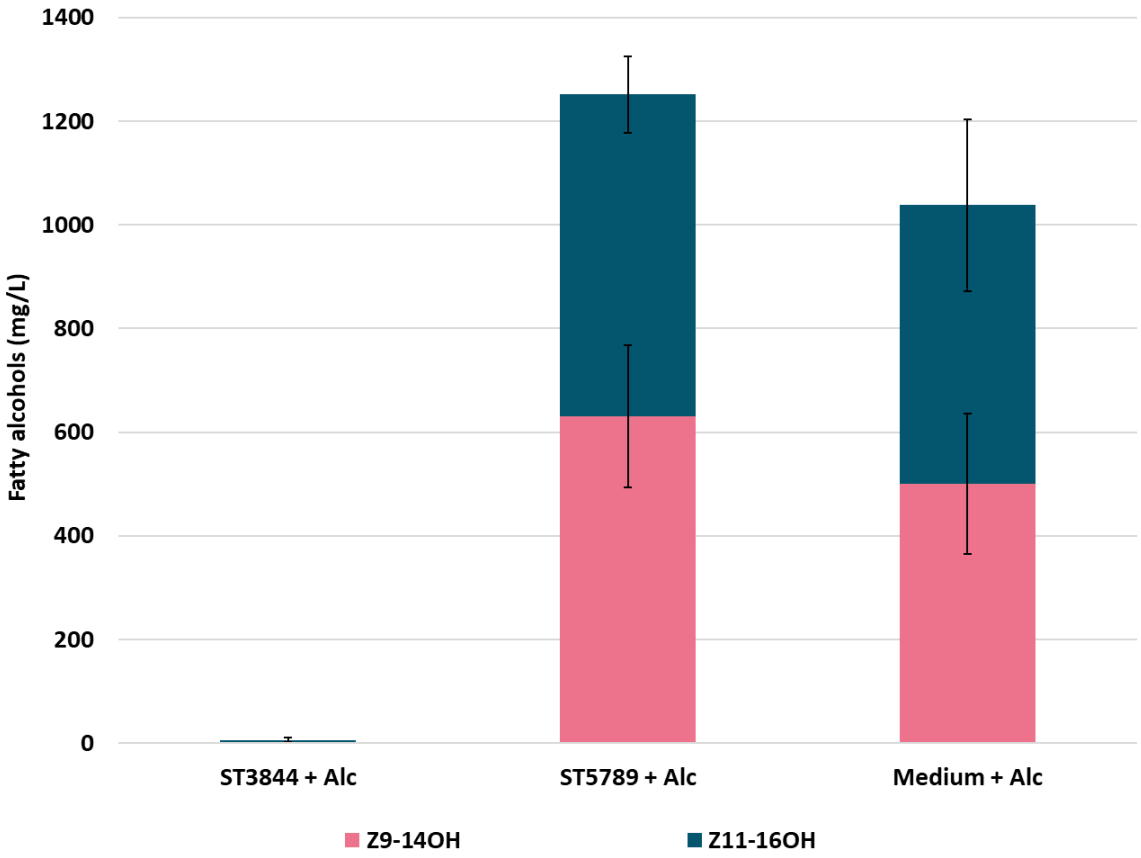

Fig. S1. Analysis of external fatty alcohol degradation in a *Y. lipolytica* strain expressing the heterologous genes HsFAR and Atrd11, and being devoid of the intrinsic genes *HFD1*/*HFD4*/*FAO1*/*PEX10* (ST5789), as well as in a strain only expressing the heterologous genes HsFAR and Atrd11 (ST3844). As a control, external fatty alcohols were added to culture medium (medium + alc) and the alcohol concentration was determined after the same incubation time.

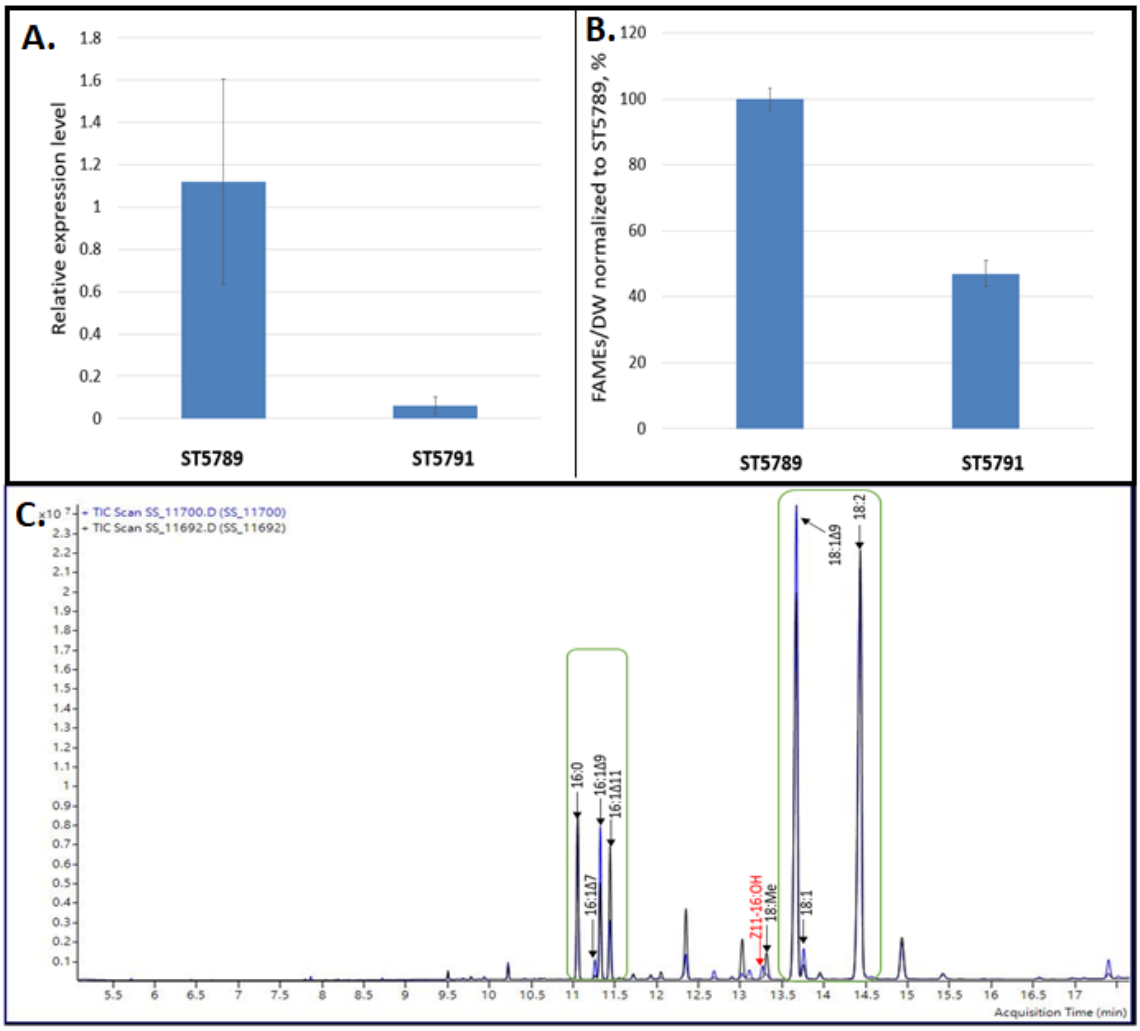

Fig. S2. Expression of glycerol-3-phosphate acyltransferase gene and fatty acid content in the strain with truncated GPAT promoter. **A.** Expression of *GPAT* gene in the control strain (ST5789) and in a strain with truncated *GPAT* promoter (ST5791) measured by qRT-PCR. **B.** Total fatty acid content measured as fatty acid methyl esters. **C.** Overlaid chromatograms of FAMEs extracts from ST5789 (black) and ST5791 (blue) strains. Green circles indicate the methyl esters that were quantified and included in total FAMEs content in panel B. Methyl octadecanoate (18Me) was not included in the FAMEs calculation because it co-eluted with Z11-16OH.

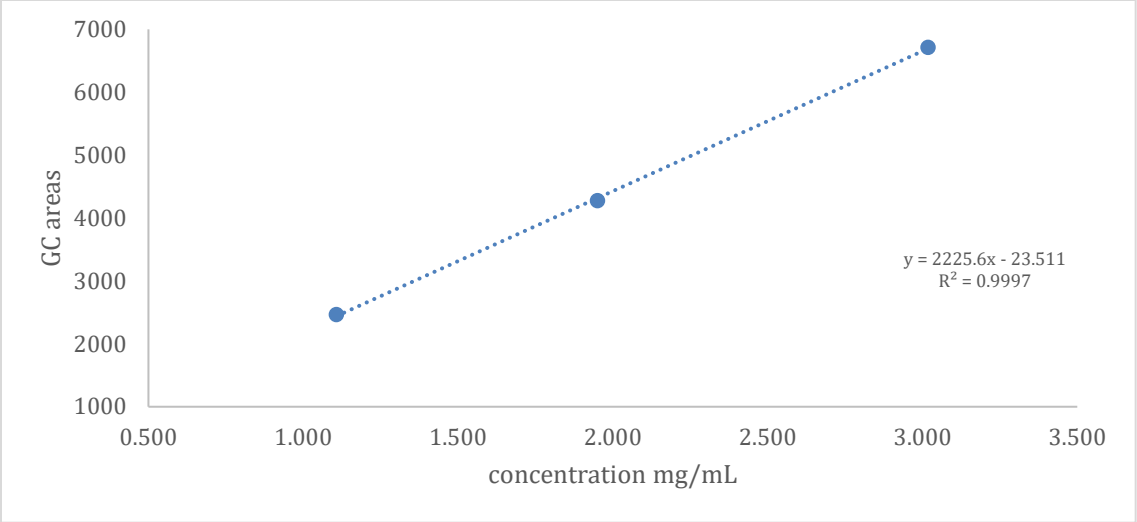

Fig. S3. Calibration curve for *Z*11-16Ald

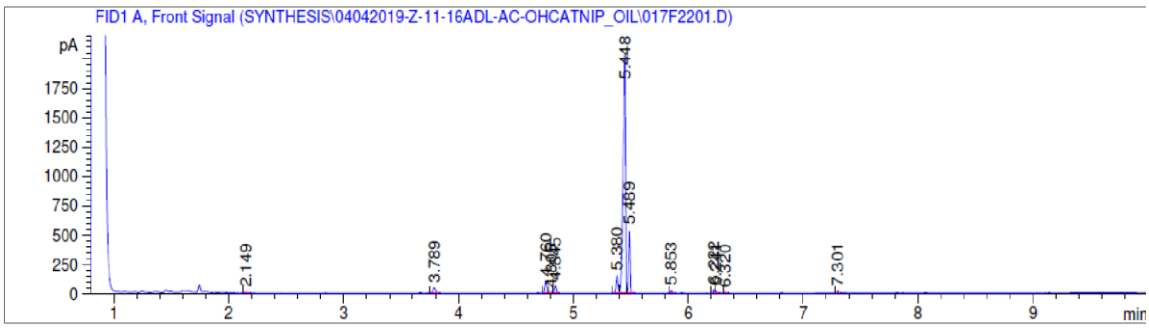

Fig. S4. The GC chromatogram of the biological aldehyde preparation. The peak at 5.448 min corresponds to Z11-16Ald, at 5.489 min 16Ald, and at 5.380 min Z9-16Ald. The ratio of the areas under the curve for the three C16 aldehydes was 82:13:5 (Z11-16Ald : 16Ald : Z9-16Ald).

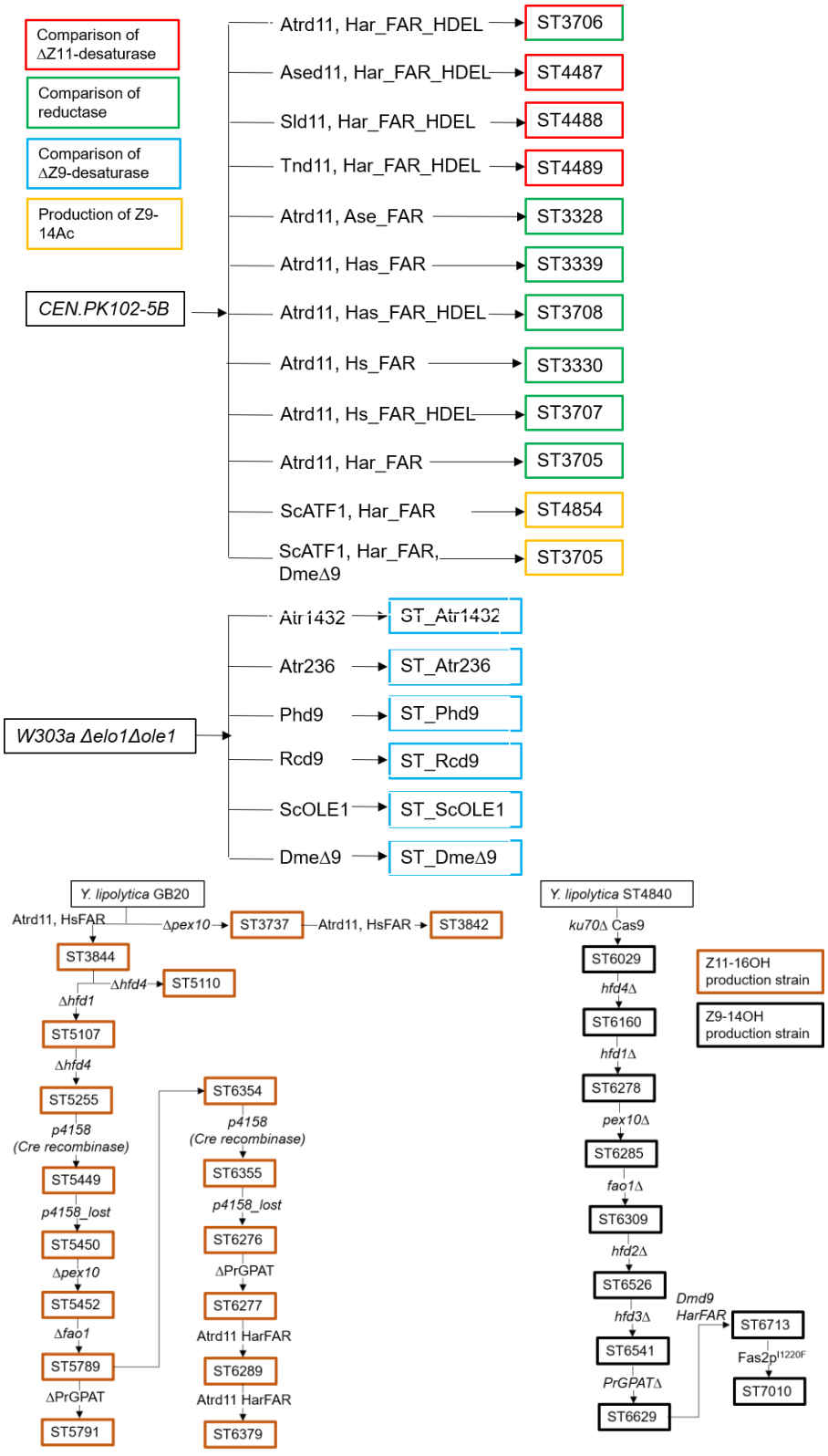

Fig. S5. Graphical diagrams for the construction of the engineered yeast strains. The boxed values are strain names or strain ID numbers. The arrows indicate transformation event resulting in a specific genetic modification.

Table S1. Primers used in this study

Primer name	Primer sequence, 5'->3'
PR-8859 (TPex20_fw)	aagtgtggatggggaagtgag
PR-10350 (atf1_U1_fw)	agtgcagguaaaacaatgaatgaaatcgatgag
PR-10351 (atf1_U1_rev)	cgtgcgauctaagggcctaaaaggagagctttg
PR-10600 (TefYL terminator _rev)	cacgcgaUgaattcggacacgggcatc
PR-10655 (EpiVecYL _fw)	acccattgcUgtagatatgtcttgtgtgtaagg
PR-10656 (EpiVecYL _rev)	atcatgtaaUtagttatgtcacgcttacattc
PR-10702 (Δpex10YL_up _fw)	cattgtaactagtcctggaggg
PR-10703 (Δpex10YL_up _down)	acgaagttaUtttgagccgaggcagatttg
PR-10704 (Δpex10YL_down _up)	acgaagttatUtgacgaggtctggatggaag
PR-10705 (Δpex10YL_down _rev)	cattgctaagaatccaaactggag
PR-10714 (NatMxSynYL-end_fw)	ttgcttgcgaacctaattcc
PR-10718 (TPex20USER _fw)	cgtgcaacgctUcagatgtgaccatatacttagg
PR-10719 (IntBupUSER_rev)	aagcgttgcacgUccagtactaagactttcatgac
PR-10738 (Har_FAR_KKSY E_U1_rev)	cgtgcgaUttattcgtagctttttttttccaagaaatgtctaacac
PR-10739 (Har_FAR_HDEL _U1_rev)	cgtgcgaUttacaattcatcatgttccaagaaatgtctaacac
PR-10740 (Hs_FAR_HDEL_ U1_rev)	cgtgcgaUttacaattcatcatgcaagaaatgtctaacacccc
PR-10741 (Has_FAR_HDEL _U1_rev)	cgtgcgaUttacaattcatcatgttccaagaagtgtctaacac
PR-10766 (NatMxSynYL-start _fw_new)	ataacttcgUataatgtatgctatacgaagttataaggagtttggcgcccg
PR-10767 (NatMxSynYL-start _rev_new)	tcatggacatggcatagac

PR-10851 (Atrd11 expression cassette _fw)	agtgcaggUgacgcagtaggatgtcctgc
PR-10853 (Hs_Far expression cassette _fw)	acctgcacUagagaccgggttgg
PR-11047 (NatMxSynYL-end _rev_new)	aataacttcgUatagcatacattatacgaagttatcgagcgtcccaaaacc
PR-11106 (IntB_up_fw)	cgtgcgaUcccacagttctcactcag
PR-11107 (Hs_Far expression cassette _rev)	atgacagaUcagatgcattcttgggcgg
PR-11108 (URAsynYL _fw)	atctgtcaUataacttcgtataatgtatgc
PR-11109 (URAsynYL _rev)	cacgcgaUcataagacgcctcgttgctc
PR-11110 (E.coli backboneUSER _fw)	atcgcgtgcattcgcggccgcatttaaatcc
PR-11111 (E.coli backboneUSER _rev)	tcgcacgcattcgcggccgcaaatttaaataaaatg
PR-11138 (Hphsyn _fw)	agcaatgggUaaaaagcctgaactcaccgc
PR-11139 (Hphsyn _rev)	attacatgaUtattcctttgccctcggacg
PR-11446 (EpiVecYL (overhang LeuKl)_fw)	agacatUgctgtagatatgtcttgtgtgtaagg
PR-11447 (LeuKL (basic vector)_fw)	aatgtcUaagaatatcgttgtcctacc
PR-11448 (LeuKL (basic vector) _rev)	attacatgaUtaagccaagatttccttgac
PR-11694 (GPAT_up_USER _fw)	CGTGCGAUgcatctaggagctccattcagc
PR-11695 (GPAT_down _USER_rev)	CACGCGAUggacgagcagaccacg
PR-12989 (PrExp _fw)	CGTGCGAUaaggagtttggcgcccg
PR-13336 (pCfB3516 (w/o)_rev)	caacggaatgcgtgcgatcgcgtgcattccttctgttcggaatc
PR-13369 (PrExp _rev)	attggacaUtgctgtagatatgtcttg
PR-13370 (Cre _fw)	atgtccaaUttactgaccgtacacc

PR-13371 (Cre _rev)	aatcgccaUcttccagcaggcgc
PR-13374 (Ttef _fw)	atggcgatUagccccacgttgccggtcttg
PR-13494 (Nat-Tcyc-loxP_fw)	agggtacUactttggatgatactgc
PR-13549 (loxP-PrTefIntron _fw)	ataacttcgUataatgtatgctatacgaagttatagagaccgggttggcggcgc
PR-141 (NB326URA3fwdU)	agaacagcUgaagcttcgtacg
PR-14126 (Ased11_U1_fw)	agtgcaggUaaaacaatggctcaag
PR-14127 (Ased11_U1_rev)	cgtgcgaUttagttgtccttcc
PR-14128 (Sld11_U1_fw)	agtgcaggUaaaacaatggctcaat
PR-14129 (Sld11_U1_rev)	cgtgcgaUtcattcaccctta
PR-14130 (Tnd11_U1_fw)	agtgcaggUaaaacaatggctgttatg
PR-14131 (Tnd11_U1_rev)	cgtgcgaUtcattctttcttagcgtagaaa
PR-142 (NB327URA3Rev2U)	AGGCCACUAGTGGATCTGATATCAC
PR-14269 (UraYL_fw)	atgccctcctacgaggcccg
PR-14270 (UraYL_rev)	ctagcagttgatcttctggtag
PR-14320 (Atf1_U2_fw)	ATCTGTCAUAAAACAATGAATGAAATCGATGAG
PR-14321 (Atf1_U2_rev)	CACGCGAUCTAAGGGCCTAAAAGGAGAGCTTTG
PR-15426 (Δhfd1_up _fw)	CGTGCGAUataagaaaaaaaacag
PR-15427 (Δhfd1_up _rev)	AGCTGTTCUactaaccctacttcctc
PR-15428 (Δhfd1_down _fw)	AGTGGCCUttttattggtggtgtg
PR-15429 (Δhfd1_down _rev)	CACGCGAUgcatagtgcttttcatattc
PR-15438 (Δhfd4_up _fw)	CGTGCGAUagtatcgctactgtactaaaattg
PR-15439 (Δhfd4_up _rev)	AGCTGTTCUagcggacaagtgtcaatgtt
PR-15440 (Δhfd4_down _fw)	AGTGGCCUatgtattttatcagtagtatctc
PR-15441 (Δhfd4_down _rev)	CACGCGAUattggataatacatttccta

PR-1565 (PTEF1)	ATGACAGAUTTGTAATTAAAACTTAG
PR-15974 (Dmd9_U1_fw)	AGTGCAGGUAAAACAatggctccatactctagaatc
PR-15975 (Dmd9_U1_rev)	CGTGCGAUttatctggacttgtcaacc
PR-15976 (attB1_Dmd9_F)	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGCTCCATACTCTAGAATCTAC
PR-15977 (attB2_Dmd9_R)	GGGGACCACTTTGTACAAGAAAGCTGGGTTTATCTGGACTTGTCAACCAACAAAACGTTTCTAG
PR-15978 (attB1_Phd9_F)	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGCCCTGAAGCTGAACCCCTTC
PR-15979 (attB2_Phd9_R)	GGGGACCACTTTGTACAAGAAAGCTGGGTTTACAGCTTCACCTGTCGGTCGAAG
PR-15980 (attB1_Rcd9_F)	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGGCGTCCTGCTGAACATCTG
PR-15981 (attB1_Rcd9_R)	GGGGACCACTTTGTACAAGAAAGCTGGGTTTAGACCTTTCGGTCGAAGATCCA
PR-15982 (attB1_Atr236_F)	GGGGACAAGTTTGTACAAAAAAGCAGGCTACATGCCGCCTCAGGGTCAAGATCGCGAGTC
PR-15983 (att2_Atr236_R)	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTATTATTCATCTTTCGAAGGGTTAAAGATGGTG
PR-15984 (attB1_Atr1432_F)	GGGGACAAGTTTGTACAAAAAAGCAGGCTACATGGCTCCAAATGCCACAGATGCTAATG
PR-15985 (attB2_Atr1432_R)	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTACTAGTCATCTTTCGGGTGTATCCTTATAG
PR-15986 (attB1_OLE1_F)	GGGGACAAGTTTGTACAAAAAAGCAGGCTACATGCCAACTTCTGGAACTACTATTGAATTG
PR-15987 (attB2_OLE1_R)	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAAAAGAACTTACCAGTTTCGTAGATTTCAC
PR-16463 (Δfao1YL_up _fw)	CGTGCGAUTGGGGGAGGATTGCGATGGG
PR-16466 (Δfao1YL_down _rev)	CACGCGAUGTGTTAGTTCCTTGTAGTGTG
PR-16696 (GPAT_up _rev)	agctgttcUTACCGCACTTCCGGAACATC
PR-16698 (GPAT_100bpPr_down _fw)	agtggccUCCGATACTTGTTTGTGTGAC
PR-1852 (PTDH3_fw)	CACGCGAUATAAAAAACACGCTTTTTCAG
PR-1853 (PTDH3_rv)	ACCTGCACUTTTGTTTGTTTATGTGTGTTTATTC
PR-8330 (Ase_FAR_U1_fw)	agtgcagguaaaacaatgccagtcttgacttctagag
PR-8331 (Ase_FAR_U1_rev)	cgtgcgauttacttcttctttcta

PR-8332 (Har_FAR_U1_fw)	agtgcagguaaaacaatggttgtcttgacctccaaag
PR-8336 (Hs_FAR_U1_fw)	agtgcagguaaaacaatggttgtcttgacctc
PR-8337 (Hs_FAR_U1_rev)	cgtgcgauttaagtctttttttcca
PR-8340 (Has_FAR_U1_fw)	agtgcagguaaaacaatggttgtcttgacctc
PR-8341 (Has_FAR_U1_rev)	cgtgcgauttacttgttggtagtct
PR-8350 (Atrd11_U1_fw)	agtgcagguaaaacaatggttccaaacaagggttcc
PR-8351 (Atrd11_U1_rev)	cgtgcgautcatctctttctacccc
PR-8857 (IntB A fragment _fw)	tcaggatgcacaggcacaagtacaatatatcccacagttctcactcagatc
PR-10604 (tracrRNA _rev)	cacgcgaUaccgtacccacacaaaaaaagcaccaccgactc
PR-10607 (PrtRNAGly _fw)	cgtgcgaUagtgaatcattgctaacagatc
PR-11694 (GPAT_up_USER _fw)	CGTGCGAUgcatctaggagctccattcagc
PR-11695 (GPAT_down _USER_rev)	CACGCGAUggacgagcagaccacg
PR-13338 (PrGPD _rev)	ACCTGCACUgttgatgtgtgtttaattc
PR-14148	acgtgcaacgctUacgcaactaacatgaatg
PR-14149	agctgttcUcagatgcattcttgggcggtc
PR-14591 (IntE-4_DW_fw)	agtggccUCACCGAGGGATAGGGAACAC
PR-14592 (IntE-4_DW_rev)	acgcgaUTTAACACTGGACCGTACTGC
PR-15607 (dsOLIGO_pex10 _KO)	aagggagtacgatttatccaccagtacaaggaggagctggagtagcgtccaagtttgcatatctcggtttgtgtacgcttgtgggctcca
PR-15788 (PrtRNA-Gly_rev)	taaccaaccUgcgccgacccggaatcgaac
PR-15789 (crRNA-TRPR_fw)	gttttagagcUagaaatagcaagttaaaataag
PR-15790 (gRNA_cass2_fw)	AGTGCAGGUagtgaatcattgctaacagatc
PR-15791 (gRNA_cass2_rev)	ACCTGCACUaccgtacccacacaaaaaaagcac

PR-15792 (gRNA_cass3_fw)	ATCTGTCAUagtgaatcattgctaacagatc
PR-15793 (gRNA_cass3_re v)	ATGACAGAUaccgtacccacacaaaaaaagcac
PR-15930 (PrGPDPrTefintro n_rev)	acctgcggtUagtactgcaaaaagtgctgg
PR-16463 (Î”fao1YL_up _fw)	CGTGCGAUTGGGGGAGGATTGCGATGGG
PR-16465 (Î”fao1YL_down _fw)	AGTGGCCUGCAAGAGACGAGTTTAGAAATAG
PR-16466 (Î”fao1YL_down _rev)	CACGCGAUGTGTTAGTTCCTTGTAGTGTG
PR-16592 (Atrd11_codonopt YL_U1_fw)	AGTGCAGGUGCCACAATGGTGCCCAACAAGGGTTC
PR-16593 (Atrd11_codonopt YL_U1_rev)	CGTGCGAUCTATCGCTTTCGTCCCCAC
PR-16594 (Har_FAR_codop tYL_U2_fw)	aaccgcaggUGGTCCTGACCTCTAAG
PR-16595 (Har_FAR_codop tYL_U2_rev)	CACGCGAUCTACTCGTAGGACTTCTTCTC
PR-16698 (GPAT_100bpPr_ down _fw)	agtggccUCCGATACTTGTTTGTGTGAC
PR-16724 (Leu2_up _fw)	GGTATTGTGTCACCAACAC
PR-16725 (Leu2_up _down)	ATGTGTGUGGTTGTATGTGT
PR-16728 (YLLeu2-Leu2_down_fw)	ACCCGAAACUAAGAAGACCA
PR-17016 (gRNA1_hfd1_se nse)	CGCAGTCGCTGGATGTCGCCgttttagagct
PR-17017 (gRNA1_hfd1_ant isense)	GGCGACATCCAGCGACTGCGtaaccaacct
PR-17018 (gRNA2_hfd1_se nse)	AAGATTCGGATCAGCACGTTgttttagagct
PR-17019 (gRNA2_hfd1_ant isense)	AACGTGCTGATCCGAATCTTtaaccaacct
PR-17020 (gRNA1_hfd4_se nse)	GGATGCGTACAGGCGCCCTTgttttagagct

PR-17021 (gRNA1_hfd4_antisense)	AAGGGCGCCTGTACGCATCCtaaccaacct
PR-17022 (gRNA2_hfd4_sense)	GACAGAAGCGGTCCCTCCTGgttttagagct
PR-17023 (gRNA2_hfd4_antisense)	CAGGAGGGACCGCTTCTGTCtaaccaacct
PR-17025 (dsOLIGO_hfd1_KO)	ATTGTGTGTATATATATAATCATTCTATGAGGAAGTAGGGTTAGTTTTTATTGGTGGTGTGTTTGAGGAAGGAGGGAGAGCGTTCAAGCT
PR-17026 (dsOLIGO_hfd4_KO)	GGGTTCCTCAGACGACTTCCACGCCTTCTTCCTCACTCGCGAAGATGGAGTTTATGGGTGAGTAATGGATCGTCGCTCATGGGACTAATC
PR-17028 (gRNA_cass4 _fw)	AGCTTGAGUagtgaatcattgctaacagatc
PR-17029 (gRNA_cass4 _rev)	ACTCAAGCUaccgtacccacacaaaaaaagcac
PR-17143 (gRNA3_fao1_sense)	TATTCGATGCCCCGGTAACTgttttagagct
PR-17144 (gRNA3_fao1_antisense)	AGTTACCGGGGCATCGAATAtaaccaacct
PR-17758 (Leu2YL_rev)	AGGCCACUAGTGGATCTGATATCACATAACTTCGTATAGCATACATTATACGAAGTTATAATTGTCATGCCTACAACTC
PR-18042 (gRNA5_hfd1_sense)	GCCATGGTAAGCACCGTAACgttttagagct
PR-18043 (gRNA5_hfd1_antisense)	GTTACGGTGCTTACCATGGCtaaccaacct
PR-18107 (gRNA2_hfd2_sense)	GCCGTGATAAGACCCATAGCgttttagagct
PR-18108 (gRNA2_hfd2_antisense)	GCTATGGGTCTTATCACGGCtaaccaacct
PR-18115 (gRNA2_hfd3_sense)	GCCCCACACGATTCGCCGAGgttttagagct
PR-18116 (gRNA2_hfd3_antisense)	CTCGGCGAATCGTGTGGGGCtaaccaacct
PR-18123 (dsOLIGO_hfd2_KO)	GCCGCTTATAGCTTTGGGCGAACTGTGGTTAGTAAAAATATCATATAATCAGATAATGTGGCGATTGAACTGTATTATTTCACATGATGA
PR-18124 (dsOLIGO_hfd3_KO)	TACATACCCCGCTGTTATCTCTTTATCATTCATAATAAATCATATAGGCTCCAGCTTAAGGGTTAGTGGCGAGACATGTTTTCTTCAGGT
PR-18152 (GPAT_up (direct	aggccacUTACCGCACTTCCGGAACATC

fusion to BB1784)_rev)	
PR-18224 (fao1_up_rev_fusion)	AGGCCACUTGTCAAGTAATCAAGCTAATGC
PR-18913 (gRNA3_PrGPAT_sense)	CTGAGGTCTCATTTATCCAGgttttagagct
PR-18914 (gRNA3_PrGPAT_antisense)	CTGGATAAATGAGACCTCAGtaaccaacct
PR-19018 (Dmd9_U1_rev)	CGTGCGAUTTATCGAGACTTGTCC
PR-19102 (Dmd9_U1_fw)	AGTGCAGGUGCCACAATGGCTCCCTACTCTCG
PR-20733 (Fas2 (I1220F)_sense)	GGTGGTATCACCGCCCTGCGgttttagagct
PR-20734 (Fas2 (I1220F)_antisense)	CGCAGGGCGGTGATACCACCtaaccaacct
PR-20762 (Fas2 (I1220F)_up_CRISPR_repair_fw)	CCAAGTACGAGGACTACCTG
PR-20763 (Fas2 (I1220F)_up_CRISPR_repair_rev)	AGGGCGGUGAAACCACCCATACCGG
PR-20764 (Fas2_down_CRISPR_repair_fw)	ACCGCCCUGCGAGGCATGTTCAAGGACC
PR-20765 (Fas2_down_CRISPR_repair_rev)	GGAGCAGGCACAGATCGG

Table S2. DNA fragments obtained by PCR using the indicated template and primers

DNA fragment ID and name	Description	Fw_primer	Rv_primer	Template DNA
BB0684	Fatty acyl-CoA reductase from *Agrotis segetum*	PR-8330	PR-8331	SEQ ID NO: 1
BB0687	Fatty acyl-CoA reductase from *Heliothis subflexa*	PR-8336	PR-8337	SEQ ID NO: 2
BB0689	Fatty acyl-CoA reductase from *Helicoverpa assulta*	PR-8340	PR-8341	SEQ ID NO: 3
BB0694	Δ11-desaturase from *Amyelois transitella*	PR-8350	PR-8351	SEQ ID NO: 4
BB0914	Fatty acyl-CoA reductase from *Helicoverpa armigera*	PR-8332	PR-10738	SEQ ID NO: 5
BB0915	Fatty acyl-CoA reductase from *Helicoverpa armigera* with modified C-terminus	PR-8332	PR-10739	SEQ ID NO: 6
BB0916	Fatty acyl-CoA reductase from *Heliothis subflexa* with modified C-terminus	PR-8336	PR-10740	SEQ ID NO: 7
BB0917	Fatty acyl-CoA reductase from *Helicoverpa assulta* with modified C-terminus	PR-8340	PR-10741	SEQ ID NO: 8
BB1354	Δ11-desaturase from *A. segetum*	PR-14126	PR-14127	SEQ ID NO: 9
BB1355	Δ11-desaturase from *Spodoptera littoralis*	PR-14128	PR-14129	SEQ ID NO: 10
BB1356	Δ11-desaturase from *Trichoplusia ni*	PR-14130	PR-14131	SEQ ID NO: 11
BB0410	PTDH3 promotor from *S. cerevisiae*	PR-1852	PR-1853	Genomic DNA of *S. cerevisiae* CEN.PK102-5B
BB1143	Alcohol acetyltransferase from *S. cerevisiae*	PR-10350	PR-10351	Genomic DNA of *S. cerevisiae* CEN.PK102-5B
BB1870	Desaturase from *Drosophila melanogaster*	PR-15976	PR-15977	pCfB5316
BB1871	Desaturase from *Pelargonium x hortorum*	PR-15978	PR-15979	pCfB4584
BB1872	Desaturase from *Ricinus communis*	PR-15980	PR-15981	pCfB4585

BB20J	Δ9-desaturase from *Amyelois transitella*	PR-15982	PR-15983	cDNA of *A. transitella* PG
BB19L	Δ9-desaturase from *Amyelois transitella*	PR-15984	PR-15985	cDNA of *A. transitella* PG
BB19J	∆9-desaturase from *S. cerevisiae*	PR-15986	PR-15987	Genomic DNA of *S. cerevisiae* CEN.PK102-5B
BB0464	P_{TDH3} and P_{TEF1} promotors from *S. cerevisiae*	PR-1565	PR-1853	p1977
BB1696	Desaturase from *Drosophila melanogaster*	PR-15974	PR-15975	SED ID NO: 12
BB1422	Alcohol acetyltransferase from *S. cerevisiae*	PR-14320	PR-14321	Genomic DNA of *S. cerevisiae* CEN.PK102-5B
BB1005	Hygromycin resistance gene	PR-11138	PR-11139	SED ID NO: 13
BB1006	pCfB3405 w/o resistance gene	PR-10655	PR-10656	pCfB3405 w/o resistance gene
BB1051	Atrd11 expression cassette and the upstream genomic region of integration site B	PR-10851	PR-11106	SED ID NO: 14
BB1126	Hs_FAR expression cassette	PR-10853	PR-11107	SEQ ID NO: 15
BB1131	Hs_FAR expression cassette	PR-10853	PR-10655	pCfB3465
BB1132	Part of pCfB3465 vector	PR-10656	PR-10851	pCfB3465
BB1135	Vector backbone for propagation in *E.coli*	PR-11110	PR-11111	pCfB2196
BB1137	Ura3 marker cassette fused to 500 bp downstream region for integration into IntB site	PR-11108	PR-11109	SEQ ID NO: 16
BB1338	Hygromycin resistance marker	PR-141	PR-142	pCfB6574
BB1346	Nourseothricin resistance marker	PR-141	PR-142	pCfB4848
BB1349	Genomic region upstream of *pex10* fused to 2/3 Start of nourseothricin resistance cassette	PR-10702	PR-10767	BB1144/BB1347
BB1350	Genomic region downstream of *pex10* fused to 2/3 end of nourseothricin resistance cassette	PR-13494	PR-10705	BB1348/BB1145
BB1427	Ura3 marker cassette	PR-141	PR-142	pCfB4586

BB1543	Genomic region upstream of *hfd1*	PR-15426	PR-15427	Genomic DNA *Yarrowia lipolytica* GB20
BB1544	Genomic region downstream of *hfd1*	PR-15428	PR-15429	Genomic DNA *Yarrowia lipolytica* GB20
BB1549	Genomic region upstream of *hfd4*	PR-15438	PR-15439	Genomic DNA *Yarrowia lipolytica* GB20
BB1550	Genomic region downstream of *hfd4*	PR-15440	PR-15441	Genomic DNA *Yarrowia lipolytica* GB20
BB1757	Genomic region upstream of *fao1* fused to 2/3 start of Ura3 cassette	PR-16463	PR-14270	BB1725/BB1427
BB1758	Genomic region downstream of *fao1* fused to 2/3 end of Ura3 cassette	PR-14269	PR-16466	BB1726/BB1427
BB1782	Genomic region upstream of *gpat*	PR-11694	PR-16696	Genomic DNA *Yarrowia lipolytica* GB20
BB1144	Genomic region upstream of *pex10*	PR-10702	PR-10703	Genomic DNA *Yarrowia lipolytica* GB20
BB1347	2/3 Start of nourseothricin resistance cassette	PR-13549	PR-10767	pCfB4848
BB1348	2/3 End of nourseothricin resistance cassette	PR-13494	PR-11047	pCfB4848
BB1145	Genomic region downstream of *pex10*	PR-10704	PR-10705	Genomic DNA *Yarrowia lipolytica* GB20
BB1048	Integration site B	PR-8857	PR-10719	Genomic DNA *Yarrowia lipolytica* GB20
BB1050	Atrd11 expression cassette	PR-10851	PR-10718	SEQ ID NO: 17
BB1352	Genomic region upstream of *pex10* fused to 2/3 Start of nourseothricin resistance cassette	PR-10702	PR-10767	BB1144/BB1014
BB1353	Genomic region downstream of *pex10* fused to 2/3 end of nourseothricin resistance cassette	PR-10714	PR-10705	BB1351/BB1145
BB1288	EXP promoter	PR-12989	PR-13369	pCfB3405

BB1289	Cre recombinase	PR-13370	PR-13371	pSH66
BB1291	TEF1 terminator	PR-13374	PR-10600	Genomic DNA *Yarrowia lipolytica* GB20
BB1129	LEU2 from Kluyveromyces lactis	PR-11447	PR-11448	SEQ ID NO: 18
BB1130	pCfB3431 w/o resistance gene	PR-11446	PR-10656	pCfB3431
BB1014	2/3 Start of nourseothricin resistance cassette	PR-10766	PR-10767	pCfB3405
BB1351	2/3 End of nourseothricin resistance cassette	PR-10714	PR-11047	pCfB3405
BB1726	Upstream region of *fao1*	PR-16463	PR-18224	Genomic DNA *Yarrowia lipolytica* GB20
BB1784	Genomic DNA downstream of GPAT promoter	PR-16698	PR-11695	Genomic DNA *Yarrowia lipolytica* GB20
BB2081	Genomic DNA upstream of GPAT promoter	PR-11694	PR-18152	Genomic DNA *Yarrowia lipolytica* GB20
BB2102	Downstream region of *fao1*	PR-16465	PR-16466	Genomic DNA *Yarrowia lipolytica* GB20
BB2103	Up- and downstream genomic region around *fao1*	PR-16463	PR-16466	BB2102/BB1726
BB2082	Up- and downstream genomic region GPAT promoter	PR-11694	PR-11695	BB1784/BB2081
BB2311	Upstream genomic region of Fas2p^{I1220}	PR-20762	PR-20763	Genomic DNA *Yarrowia lipolytica* GB20
BB2312	Downstream genomic region of Fas2p^{I1220}	PR-20764	PR-20765	Genomic DNA *Yarrowia lipolytica* GB20
BB2313	Up- and downstream genomic region of Fas2p^{I1220}	PR-20762	PR-20765	BB2311/BB2312
BB1892	gRNA expression cassette targeting *hfd1*	PR-10607	PR-15791	BB1635, BB1636, PR-17016, PR-17017
BB1893	gRNA expression cassette targeting *hfd1*	PR-15790	PR-15793	BB1635, BB1636, PR-17018, PR-17019
BB1894	gRNA expression cassette targeting *hfd4*	PR-15792	PR-17029	BB1635, BB1636, PR-17020, PR-17021
BB1895	gRNA expression cassette targeting *hfd4*	PR-17028	PR-10604	BB1635, BB1636, PR-17022, PR-17023

BB1635	Promoter for gRNA expression	PR-10607	PR-15788	pCfB4589
BB1636	Terminator region for gRNA expression	PR-15789	PR-10604	pCfB4589
BB1687	Fused promoter regions of *gpd and tef1*	PR-13338	PR-15930	pCfB3465
BB1740	HarFAR reductase	PR-16594	PR-16595	SEQ ID NO: 19
BB2206	Dmd9 desaturase	PR-19102	PR-19018	SEQ ID NO: 20
BB1808	Genomic region upstream of *leu2*	PR-16724	PR-16725	Genomic DNA *Yarrowia lipolytica* GB20
BB1947	*leu2*	PR-16728	PR-17758	Genomic DNA *Yarrowia lipolytica* GB20
BB1963	*tef1* promoter region fused to *leu2* of Y. lipolytica	PR-141	PR-142	BB1808, BB1947
BB1739	Atrd11 desaturase	PR-16592	PR-16593	SEQ ID NO: 21
BB1360	Terminator regions of *pex20* and *lip2* fused	PR-14148	PR-14149	pCfB4132
BB1484	Genomic region downstream of integration site IntE_4	PR-14591	PR-14592	Genomic DNA *Yarrowia lipolytica* GB20
BB1243	pCfB3516 (w/o genes or promoters)	PR-8859	PR-13336	pCfB3516

Table S3. Plasmids used in this study

Vector name	Selection marker for yeast	Parent vector	BioBricks	Reference
pCfB2190	KlLEU2	-	-	(1)
pCfB2228	SpHIS5	-	-	(1)
pCfB2501	KlLEU2	pCfB2190	BB0410, BB0684	This study
pCfB2504	KlLEU2	pCfB2190	BB0410, BB0687	This study
pCfB2506	KlLEU2	pCfB2190	BB0410, BB0689	This study
pCfB2537	SpHIS5	pCfB2228	BB0410, BB0694	This study
pCfB3412	KlLEU2	pCfB2190	BB0410, BB0914	This study
pCfB3413	KlLEU2	pCfB2190	BB0410, BB0915	This study
pCfB3414	KlLEU2	pCfB2190	BB0410, BB0916	This study
pCfB3415	KlLEU2	pCfB2190	BB0410, BB0917	This study
pCfB4369	SpHIS5	pCfB2228	BB0410, BB1354	This study
pCfB4370	SpHIS5	pCfB2228	BB0410, BB1355	This study
pCfB4371	SpHIS5	pCfB2228	BB0410, BB1356	This study
pYEX-CHT-DEST	Ura3	-	-	(2)
pYEX-CHT-Atrd1432	Ura3	pYEX-CHT-DEST	BB19L	This study
pYEX-CHT-Atr236	Ura3	pYEX-CHT-DEST	BB20J	This study
pYEX-CHT-Phd9	Ura3	pCfB4584	BB1871	This study
pYEX-CHT-Rcd9	Ura3	pCfB4585	BB1872	This study
pYEX-CHT-OLE1	Ura3	pYEX-CHT-DEST	BB19J	This study
pYEX-CHT-Dmd9	Ura3	pCfB5316	BB1870	This study
pCfB2909	None	-	-	(3)
p1977	None	-	-	(3)
pCfB4580	KlLEU2	pCfB2190	BB0464, BB0915, BB1422	This study

pCfB5316	None	pCfB2909	BB0410, BB1696	This study
pCfB3465	Ura3	-	BB1051, BB1126, BB1135, BB1137	This study
pCfB5110	NatSyn	-	BB1135, BB1346, BB1543, BB1544	This study
pCfB5113	HphSyn	-	BB1135, BB1338, BB1549, BB1550	This study
pCfB3516	HphSyn	-	BB1005, BB1131, BB1132	This study
pCfB5573	HphSyn	-	BB1135, BB1338, BB1757, BB1758	This study
pCfB5750	Ura3	-	BB1135, BB1427, BB1782, BB1784	This study
pCfB2196	BleMX	-	-	(1)
pCfB4158	KlLEU2	pCfB3529	BB1288, BB1289, BB1291	This study
pCfB3529	KlLEU2	-	BB1129, BB1130	This study
pCfB3431	HphSyn	-	BB1006, BB1005	This study
pCfB3405	NatSyn	-	-	(4)
pCfB6574	HphSyn	-	-	(4)
pCfB4848	NatSyn	-	-	(4)
pCfB4586	Ura3	-	-	(4)
pCfB4781	NatSyn	-	-	(4)
pSH66	-	-	-	EuroScarf
pCfB6364	-	-	-	(5)
pCfB3405	-	-	-	(4)
pCfB5878	NatSyn	pCfB3405	BB1892, BB1893, BB1894, BB1895	
pCfB6530	NatSyn	pCfB3405	BB1635, BB1636, PR-18042, PR-18043	
pCfB5790	NatSyn	pCfB3405	BB1833, BB1834	
pCfB6463	NatSyn	pCfB3405	BB1635, BB1636, PR-17143, PR-17144	
pCfB6566	NatSyn	pCfB3405	BB1635, BB1636, PR-18107, PR-18108	
pCfB6570	NatSyn	pCfB3405	BB1635, BB1636, PR-18115, PR-18116	
pCfB6834	NatSyn	pCfB3405	BB1635, BB1636, PR-18913, PR-18914	
pCfB6627	-	-	-	(4)
pCfB6682	-	-	-	(4)

pCfB6969	None	pCfB6682	BB1687, BB1740, BB2206	
pCfB7088	NatSyn	pCfB3405	BB1635, BB1636, PR-20733, PR-20734	
pCfB4589	-	-	-	(4)
pCfB6397	-		BB1135, BB1963, BB1782, BB1784	
pCfB5929	HphSyn	pCfB5219	BB1687, BB17390, BB174	
pCfB5219	HphSyn	-	BB1135, BB1360, BB1338, BB1484	
pCfB4132	-	-	BB1243	
pCfB5390	NatSyn	pCfB4781	BB1687, BB1739, BB1740	

Table S4. Yeast strains

Strain name	Strain description	Parent strain	Plasmids/BioBricks integrated	Reference
Saccharomyces cerevisiae CEN.PK102-5B	MATa *ura3-52 his3□1 leu2-3/112 MAL2-8c SUC2*	-	-	
ST3706	Atrd11, Har_FAR_HDEL	CEN.PK102-5B	pCfB2537, pCfB3413	This study
ST4487	Ased11, Har_FAR_HDEL	CEN.PK102-5B	pCfB4369, pCfB3413	This study
ST4488	Sld11, Har_FAR_HDEL	CEN.PK102-5B	pCfB4370, pCfB3413	This study
ST4489	Tnd11, Har_FAR_HDEL	CEN.PK102-5B	pCfB4371, pCfB3413	This study
ST3328	Atrd11, Ase_FAR	CEN.PK102-5B	pCfB2537, pCfB2501	This study
ST3339	Atrd11, Has_FAR	CEN.PK102-5B	pCfB2537, pCfB2506	This study
ST3708	Atrd11, Has_FAR_HDEL	CEN.PK102-5B	pCfB2537, pCfB3415	This study
ST3330	Atrd11, Hs_FAR	CEN.PK102-5B	pCfB2537, pCfB2504	This study
ST3707	Atrd11, Hs_FAR_HDEL	CEN.PK102-5B	pCfB2537, pCfB3414	This study
ST3705	Atrd11, Har_FAR	CEN.PK102-5B	pCfB2537, pCfB3412	This study
S. cerevisiae W303a *Δelo1Δole1*	MATa *elo1::HIS3 ole1::LEU2 ade2 his3 leu2 ura3*	-	-	(6)
ST_Atr1432	Atr1432	W303a *Δelo1Δole1*	pYEX-CHT-Atrd1432	This study
ST_Atr236	Atr236	W303a *Δelo1Δole1*	pYEX-CHT-Atr236	This study
ST_Phd9	Phd9	W303a *Δelo1Δole1*	pYEX-CHT-Phd9	This study
ST_Rcd9	Rcd9	W303a *Δelo1Δole1*	pYEX-CHT-Rcd9	This study
ST_ScOLE1	ScOLE1	W303a *Δelo1Δole1*	pYEX-CHT-OLE1	This study
ST_DmeΔ9	DmeΔ9	W303a *Δelo1Δole1*	pYEX-CHT-Dmd9	This study
ST4854	ScATF1, Har_FAR	CEN.PK102-5B	pCfB4580	This study
ST5290	ScATF1, Har_FAR, DmeΔ9	CEN.PK102-5B	pCfB4580, pCfB5316	This study
Yarrowia lipolytica GB20	*mus51Δ*, nugm-Htg2, ndh2i, lys11^{-}, leu2^{-}, ura3^{-}, MatB	-	-	(7)
ST3844	IntB::Atrd11 Hs_FAR	*Y. lipolytica* GB20	pCfB3465	This study
ST5107	IntB::Atrd11 Hs_FAR *Δhfd1*	ST3844	pCfB5110	This study

ST5110	IntB::Atrd11 Hs_FAR *Δhfd4*	ST3844	pCfB5113	This study
ST3842	IntB::Atrd11 Hs_FAR *Δpex10*	ST3737	pCfB3516	This study
ST5255	IntB::Atrd11 Hs_FAR *Δhfd1 Δhfd4*	ST5107	pCfB5113	This study
ST5452	IntB::Atrd11 Hs_FAR *Δhfd1 Δhfd4 Δpex10*	ST5450	BB1349/BB1350	This study
ST5789	IntB::Atrd11 Hs_FAR *Δhfd1 Δhfd4 Δpex10 Δfao1*	ST5452	pCfB5573	This study
ST5791	IntB::Atrd11 Hs_FAR *Δhfd1 Δhfd4 Δpex10 Δfao1* ΔPrGPAT	ST5789	pCfB5750	This study
ST6354	IntB::Atrd11 Hs_FAR *Δhfd1 Δhfd4 Δpex10 Δfao1*	ST5789	pCfB4586	This study
ST6355	IntB::Atrd11 Hs_FAR *Δhfd1 Δhfd4 Δpex10 Δfao1*	ST6354	pCfB4158 (replicative)	This study
ST6276	IntB::Atrd11 Hs_FAR *Δhfd1 Δhfd4 Δpex10 Δfao1*	ST6355	pCfB4158 (replicative) lost	This study
ST6277	IntB::Atrd11 Hs_FAR *Δhfd1 Δhfd4 Δpex10 Δfao1* ΔPrGPAT	ST6276	pCfB6397	This study
ST6289	IntB::Atrd11 Hs_FAR *Δhfd1 Δhfd4 Δpex10 Δfao1* ΔPrGPAT IntE4::Atrd11 HarFAR	ST6277	pCfB5929	This study
ST6379	IntB::Atrd11 Hs_FAR *Δhfd1 Δhfd4 Δpex10 Δfao1* ΔPrGPAT IntE4::Atrd11 HarFAR IntC1::Atrd11 HarFAR	ST6289	pCfB5930	This study
ST3737	Δ*pex10*	*Y. lipolytica* GB20	BB1352/BB1353	This study
ST5449	IntB::Atrd11 Hs_FAR *Δhfd1 Δhfd4* pCfB4158	ST5255	pCfB4158 (replicative)	This study
ST5450	IntB::Atrd11 Hs_FAR *Δhfd1 Δhfd4*	ST5449	pCfB4158 (replicative) lost	This study
ST4840 (Y-17536)	*Wild-type Yarrowia lipolytica*			Agricultural Research Service (NRRL, USA)
ST6029	*ku70*Δ Cas9	ST4840	pCfB6364	This study
ST6160	*ku70*Δ Cas9 *hfd4*Δ	ST6029	PCfB5878/PR-17026	This study
ST6278	*ku70*Δ Cas9 *hfd4*Δ *hfd1*Δ	ST6160	pCfB6530/PR-17025	This study
ST6285	*ku70*Δ Cas9 *hfd4*Δ *hfd1*Δ *pex10*Δ	ST6278	pCfB5790/PR-15607	This study

ST6309	*ku70*Δ Cas9 *hfd4*Δ *hfd1*Δ *pex10*Δ *fao1*Δ	ST6285	pCfB6463/BB2103	This study
ST6526	*ku70*Δ Cas9 *hfd4*Δ *hfd1*Δ *pex10*Δ *fao1*Δ *hfd2*Δ	ST6309	PCfB6566/PR-18123	This study
ST6541	*ku70*Δ Cas9 *hfd4*Δ *hfd1*Δ *pex10*Δ *fao1*Δ *hfd2*Δ *hfd3*Δ	ST6526	pCfB6570/PR-18124	This study
ST6629	*ku70*Δ Cas9 *hfd4*Δ *hfd1*Δ *pex10*Δ *fao1*Δ *hfd2*Δ *hfd3*Δ *GPAT_100bpPr*	ST6541	pCfB6834/BB2082	This study
ST6713	*ku70*Δ Cas9 *hfd4*Δ *hfd1*Δ *pex10*Δ *fao1*Δ *hfd2*Δ *hfd3*Δ *GPAT_100bpPr* IntC_2:Dmd9_HarFAR	ST6629	pCfB6627/pCfB6969	This study
ST7010	*ku70*Δ Cas9 *hfd4*Δ *hfd1*Δ *pex10*Δ *fao1*Δ *hfd2*Δ *hfd3*Δ *GPAT_100bpPr* IntC_2:Dmd9_HarFAR Fas2p^{I1220F}	ST6713	pCfB7088/BB2313	This study

Table S5. Strain genotypes on figures

Fig. 1b. The following strains are shown in the order left to right:	
	Saccharomyces cerevisiae CEN.PK102-5B (MATa *ura3-52 his3Δ1 leu2-3/112 MAL2-8c SUC2*)
ST3706	*S. cerevisiae* CEN.PK102-5B Atrd11, Har_FAR_HDEL
ST4487	*S. cerevisiae* CEN.PK102-5B Ased11, Har_FAR_HDEL
ST4488	*S. cerevisiae* CEN.PK102-5B Sld11, Har_FAR_HDEL
ST4489	*S. cerevisiae* CEN.PK102-5B Tnd11, Har_FAR_HDEL
ST3328	*S. cerevisiae* CEN.PK102-5B Atrd11, Ase_FAR
ST3339	*S. cerevisiae* CEN.PK102-5B Atrd11, Has_FAR
ST3708	*S. cerevisiae* CEN.PK102-5B Atrd11, Has_FAR_HDEL
ST3330	*S. cerevisiae* CEN.PK102-5B Atrd11, Hs_FAR
ST3707	*S. cerevisiae* CEN.PK102-5B Atrd11, Hs_FAR_HDEL
ST3705	*S. cerevisiae* CEN.PK102-5B Atrd11, Har_FAR
Fig. 1d. The following strains are shown in the order left to right:	
	S. cerevisiae W303a *Δelo1Δole1* (*MATa elo1::HIS3 ole1::LEU2 ade2 his3 leu2 ura3*)
ST_Atr1432	*S. cerevisiae* W303a *Δelo1Δole1*, Atr1432
ST_Atr236	*S. cerevisiae* W303a *Δelo1Δole1*, Atr236
ST_Phd9	*S. cerevisiae* W303a *Δelo1Δole1*, Phd9
ST_Rcd9	*S. cerevisiae* W303a *Δelo1Δole1*, Rcd9
ST_ScOLE1	*S. cerevisiae* W303a *Δelo1Δole1*, ScOLE1
ST_DmeD9	*S. cerevisiae* W303a *Δelo1Δole1*, DmeD9
Fig. 1e. The following strains are shown in the order left to right:	
ST4854	*S. cerevisiae* CEN.PK102-5B ScATF1, Har_FAR
ST5290	*S. cerevisiae* CEN.PK102-5B ScATF1, Har_FAR, DmeΔ9
Fig. 2a. The following strains are shown in the order left to right:	
	Yarrowia lipolytica GB20 (*mus51Δ*, nugm-Htg2, ndh2i, lys11^{-}, leu2^{-}, ura3^{-}, MatB)
ST3844	*Y. lipolytica* GB20 IntB::Atrd11 Hs_FAR
ST5107	*Y. lipolytica* GB20 IntB::Atrd11 Hs_FAR, *hfd1Δ*
ST5110	*Y. lipolytica* GB20 IntB::Atrd11 Hs_FAR, *hfd4Δ*
ST3842	*Y. lipolytica* GB20 IntB::Atrd11 Hs_FAR, *pex10Δ*
ST5255	*Y. lipolytica* GB20 IntB::Atrd11 Hs_FAR, *hfd1Δ, hfd4Δ*
ST5452	*Y. lipolytica* GB20 IntB::Atrd11 Hs_FAR, *hfd1Δ, hfd4Δ, pex10Δ*
ST5789	*Y. lipolytica* GB20 IntB::Atrd11 Hs_FAR, *hfd1Δ, hfd4Δ, pex10Δ, fao1Δ*
ST5791	*Y. lipolytica* GB20 IntB::Atrd11 Hs_FAR, *hfd1Δ, hfd4Δ, pex10Δ,fao1Δ, P_{GPAT}::100*
Fig. 3a. The following strains are shown in the order left to right:	
ST5791	*Y. lipolytica* GB20 IntB::Atrd11 Hs_FAR, *hfd1Δ, hfd4Δ, pex10Δ,fao1Δ, P_{GPAT}::100*
ST6289	*Y. lipolytica* GB20 IntB::Atrd11 Hs_FAR, *hfd1Δ, hfd4Δ, pex10Δ,fao1Δ, P_{GPAT}::100*, IntE4::Atrd11 HarFAR
ST6379	*Y. lipolytica* GB20 IntB::Atrd11 Hs_FAR, *hfd1Δ, hfd4Δ, pex10Δ,fao1Δ, P_{GPAT}::100*, IntE4::Atrd11 HarFAR, IntC1::Atrd11 HarFAR
Fig. 3b. The following strains are shown in the order left to right:	
ST6713	*ku70*Δ Cas9 *hfd4*Δ *hfd1*Δ *pex10*Δ *fao1*Δ *hfd2*Δ *hfd3*Δ *GPAT_100bpPr* IntC_2:Dmd9_HarFAR
ST7010	*ku70*Δ Cas9 *hfd4*Δ *hfd1*Δ *pex10*Δ *fao1*Δ *hfd2*Δ *hfd3*Δ *GPAT_100bpPr* IntC_2:Dmd9_HarFAR Fas2p^{I1220F}

Table S6. Quantification of Z11-16:Ald

	Sample size (µl)	Volume of flask (ml)	Area GC	Concentration of Z11-16-Ald in diluted sample (mg/ml)	Concentration Z11-16-Ald (mg/ml)
Replicate 1	200	5	3147.6	1.425	35.62
Replicate 2	200	5	3106.6	1.406	35.16
Replicate 3	200	5	3109.9	1.408	35.20

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CHAPTER 4

Biotechnological production of the European corn borer sex pheromone in the yeast *Yarrowia lipolytica*

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RESEARCH ARTICLE

Biotechnological production of the European corn borer sex pheromone in the yeast *Yarrowia lipolytica*

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Abstract

The European corn borer (ECB) *Ostrinia nubilalis* is a widespread pest of cereals, particularly maize. Mating disruption with the sex pheromone is a potentially attractive method for managing this pest; however, chemical synthesis of pheromones requires expensive starting materials and catalysts and generates hazardous waste. The goal of this study was to develop a biotechnological method for the production of ECB sex pheromone. Our approach was to engineer the oleaginous yeast *Yarrowia lipolytica* to produce (*Z*)-11-tetradecenol (*Z*11-14:OH), which can then be chemically acetylated to (*Z*)-11-tetradecenyl acetate (*Z*11-14:OAc), the main pheromone component of the Z-race of *O. nubilalis*. First, a C14 platform strain with increased biosynthesis of myristoyl-CoA was obtained by introducing a point mutation into the α-subunit of fatty acid synthase, replacing isoleucine 1220 with phenylalanine (Fas2p^{I1220F}). The intracellular accumulation of myristic acid increased 8.4-fold. Next, fatty acyl-CoA desaturases (FAD) and fatty acyl-CoA reductases (FAR) from nine different species of Lepidoptera were screened in the C14 platform strain, individually and in combinations. A titer of 29.2 $\pm$ 1.6 mg L^{-1} *Z*11-14:OH was reached in small-scale cultivation with an optimal combination of a FAD (Lbo_PPTQ) from *Lobesia botrana* and FAR (HarFAR) from *Helicoverpa armigera*. When the second copies of FAD and FAR genes were introduced, the titer improved 2.1-fold. The native *FAS1* gene's overexpression led to a further 1.5-fold titer increase, reaching 93.9 $\pm$ 11.7 mg L^{-1} in small-scale cultivation. When the same engineered strain was cultivated in controlled 1 L bioreactors in fed-batch mode, 188.1 $\pm$ 13.4 mg L^{-1} of *Z*11-14:OH was obtained. Fatty alcohols were extracted

Abbreviations: BioPhe, biologically-derived pheromone blend; *E*/*Z*11-14:OAc, (*E*/*Z*)-11-tetradecenyl acetate; *E*/*Z*11-14:OH, (*E*/*Z*)-11-tetradecenol; EAG, electroantennography; ECB, the European corn borer; FAD, fatty acyl-CoA desaturase; FAME, fatty acid methyl ester; FAR, fatty acyl-CoA reductase

from the biomass and chemically acetylated to obtain *Z*11-14:OAc. Electroantennogram experiments showed that males of the Z-race of *O. nubilalis* were responsive to biologically-derived pheromone blend. Behavioral bioassays in a wind tunnel revealed attraction of male *O. nubilalis*, although full precopulatory behavior was observed less often than for the chemically synthesized pheromone blend. The study paves the way for the production of ECB pheromone by fermentation.

KEYWORDS
fatty acyl-CoA desaturases, fatty acyl-CoA reductases, fatty alcohols, insect pheromones, *Yarrowia lipolytica*

1 | INTRODUCTION

Crop damage caused by insects is a severe problem in agriculture. Moths (Lepidoptera) are major insect pests.[1–3] The European corn borer (ECB) *Ostrinia nubilalis* is the main pest of maize *Zea mays* in Europe.[4] It is estimated that in the absence of treatment, up to 20% of the crop may be lost due to the damage by *O. nubilalis* larvae.[5] Like other moth species, females of *O. nubilalis* produce and release a fatty acid-derived sex pheromone, which attracts conspecific males for mating.[6] ECB is polymorphic with respect to its pheromone communication system and two pheromone races are recognized: the Z-race insects use a 97:3 blend of (*Z*)-11-tetradecenyl acetate (*Z*11-14:OAc) to (*E*)-11-tetradecenyl acetate (*E*11-14:OAc), while E-race insects use a 1:99 blend of the same components.[6,7] On maize in Europe, the Z-race is most prevalent.[8]

Mating disruption has been proven an effective and environmentally friendly solution for crop protection against moths. For mating disruption, a more or less species-specific pheromone blend is applied to the fields or orchards to disrupt mating partner detection and, in this way, decrease the propagation of the pest species.[9,10] Currently, pheromones for pest management are produced synthetically from petrol-derived chemicals.[11–13] Chemical synthesis typically comprises multiple steps, uses toxic chemicals and solvents, and expensive catalysts (Figure S1). Biotechnological production of several insect pheromone components has already been established in plants and yeasts.[14–18] Oleaginous yeast *Yarrowia lipolytica* is particularly suitable for production of lepidopteran pheromones due to its naturally high level of fatty acid biosynthesis. Previously, we produced the precursor of the major pheromone component of *Helicoverpa armigera* (*Z*11-16:OH) at 2.5 g L^{-1} in *Y. lipolytica* and proved the effectiveness of yeast-derived insect pheromone in field trapping experiments.[16]

In this study, we aimed to produce *Z*11-14:OAc, the major pheromone component of the ECB Z-race, using engineered *Y. lipolytica* as a host and demonstrate the activity of yeast-derived pheromone on *O. nubilalis* males.

2 | MATERIALS AND METHODS

2.1 | Plasmid construction

Plasmids were constructed according to Holkenbrink et al., 2018.[19] Integration and guide RNA (gRNA) vectors were used to introduce gene expression constructs into characterized genome sites of *Y. lipolytica*.[19] The design of integrative plasmids is illustrated in Figure S2. The plasmids contain two 500-bp-long homology arms. In-between these arms, there is a USER-cloning site flanked by two terminators. The gene of interest was cloned along with *TEF1*intron promoter into the USER-site to generate an expression cassette.[19] Correct cloning was confirmed by Sanger sequencing. The plasmids were linearized with SmiI to generate linear integration fragments. The integration fragments were transformed into *cas9*-expressing *Y. lipolytica* along with a single guide RNA (gRNA) vector targeting the given integration site. The expression of gRNA was controlled by RNA polymerase III promoter and *RPR1* terminator. Additionally, gRNA vectors contained hygromycin or nourseothricin resistance cassettes for selection of yeast transformants. Cas9 protein bound to the gRNA induced double-strand break in the genomic DNA and the integration cassette was introduced into the given site by homologous recombination (Figure S2).

Primers, synthetic DNA, BioBricks, and plasmids used in this study are listed in Tables S1, S2, S3, and S4, respectively. BioBricks were amplified by PCR using Phusion U polymerase (Thermo Fisher Scientific) with the following thermal program: 98°C for 5 min, 30 cycles of (98°C for 20 s, 54°C for 30 s, 72°C for 30 s kb^{-1}), and 72°C for 4 min. After DNA electrophoresis on 1% agarose gel, BioBricks were purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). The integration vectors were digested with FastDigest SfaAI (Thermo Fisher Scientific) and nicked with Nb.BsmI (New England BioLabs). Biobricks with compatible overhangs and nicked integration vectors were assembled and transformed into *Escherichia coli* strain DH5α via Uracil-Specific Excision Reaction (USER) cloning. Clones containing the correct assembly were verified by PCR and Sanger sequencing.

2.2 | Strain construction

All strains constructed in this study are derived from the *Y. lipolytica* strain ST6629, described in Holkenbrink et al.[16] The strain expresses *cas9* gene from *Streptococcus pyogenes*. The gene is codon-optimized for *Y. lipolytica* and is integrated into *KU70* locus, effectively inactivating *KU70* gene involved in DNA repair via the non-homologous end joining pathway. The strain hence has an increased rate of DNA repair via homologous recombination, which facilitates targeted genome editing. Additionally, this strain has modifications related to decreased degradation of fatty acids/alcohols and an increased pool of fatty acyl-CoAs. *Y. lipolytica* strain ST4840, which was obtained from Agricultural Research Service (NRRL, USA), served as a source for genomic DNA (gDNA). gDNA was extracted using Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research). The entire list of the strains is provided in Table S5.

Yeast transformations were performed as described in Holkenbrink et al.[19] with the modifications described below. *Y. lipolytica* strains were streaked on Yeast Peptone Dextrose (YPD) plates (20 g L^{-1} glucose, 10 g L^{-1} peptone, 10 g L^{-1} yeast extract, 15 g L^{-1} agar) and grown for 24 h at 28°C. A small patch of biomass was taken with an inoculation loop and re-streaked on YPD plates containing 0.7 g L^{-1} complete supplement mixture (Formedium). After 24 h incubation at 28°C, the cells were scraped off, resuspended in 1 mL 0.5 M sterile sucrose solution, and centrifuged for 5 min at 3000 × *g* at room temperature. The supernatant was discarded, the cells resuspended in 0.5 M sucrose solution, and a volume corresponding to OD_{600} 2.6 was transferred to a sterile tube for transformation. Tubes were centrifuged for 5 min at 3000 × *g* at room temperature, and the supernatant was removed. 500–1000 ng of gRNA plasmid and integration plasmid, which previously was linearized with SmiI (Thermo Fisher Scientific) and gel-purified prior transformation, were added to the pellet. The mixture was resuspended in 100 μL of transformation mix (Table S6) and incubated at 39°C for 1 h. After the heat shock, the tubes were centrifuged, the supernatant removed, and the pellet was resuspended in 500 μL liquid YPD medium. The cells were incubated for 2 h at 28°C 300 rpm shaking. The pellet was again collected by centrifugation, resuspended in 100 μL of 0.5 M sterile sucrose solution, and plated on YPD plates containing Hygromycin B (200 mg L^{-1}) (Carl Roth) or Nourseothricin (250 mg L^{-1}) (Jena Bioscience) for selection. After 2–3 days of incubation at 28°C, single colonies appeared, which were tested for correct integration by PCR using vector-specific primers and primers complementary to genomic loci close to the integration site (Table S1).

2.3 | Small scale cultivations

Y. lipolytica strains were inoculated from a YPD agar plate to an initial OD_{600} of 0.2 into 2.5 mL YPG medium (10 g L^{-1} yeast extract, 10 g L^{-1} peptone, 40 g L^{-1} glycerol) in 24 well-plate (EnzyScreen). The plate was incubated at 28°C and 300 rpm for 22 h. The plate was centrifuged at 3500 *g* for 5 min at 20°C, the medium was removed and the cells were resuspended in 1.25 mL production medium (50 g L^{-1} glycerol, 5 g L^{-1} yeast extract, 4 g L^{-1} KH_2PO_4, 1.5 g L^{-1} $MgSO_4$, 0.2 g L^{-1} NaCl, 0.265 g L^{-1} $CaCl_2 \cdot 2H_2O$, 2 mL L^{-1} trace elements solution: 4.5 g L^{-1} $CaCl_2 \cdot 2H_2O$, 4.5 g L^{-1} $ZnSO_4 \cdot 7H_2O$, 3 gL^{-1} $FeSO_4 \cdot 7H_2O$, 1 g L^{-1} H_3BO_3, 1 g L^{-1} $MnCl_2 \cdot 4H_2O$, 0.4 g L^{-1} $Na_2MoO_4 \cdot 2H_2O$, 0.3 g L^{-1} $CoCl_2 \cdot 6H_2O$, 0.1 g L^{-1} $CuSO_4 \cdot 5H_2O$, 0.1 g L^{-1} KI, 15 g L^{-1} EDTA). The plate was incubated at 28°C and 300 rpm for 28 h. Each strain was cultivated in triplicate.

2.4 | Bioreactor fed-batch cultivations

Bioreactor fed-batch mode cultivations were carried out in biological triplicates on initial YPG medium (50 g L^{-1} glycerol, 10 g L^{-1} yeast extract (Carl Roth), 10 g L^{-1} peptone) in controlled stirred bioreactor vessels with 1.0 L total aerated end working volume (Infors Minifors 2 systems, Infors AG, Switzerland) at 28°C. All reactors were equipped with pH and optical dissolved oxygen probes (Hamilton AG, Switzerland) and off-gas analyzers (BlueSens GmbH, Germany). pH was controlled at 4.5 ± 0.1 with automated addition of a 4 M solution of NaOH. The dissolved oxygen control was set at a minimum threshold of 20% with a cascade control by gradually increasing the stirrer speed of two six-blade Rushton turbines and the aeration rate using Eve fermentation control software (Infors AG). The initial aeration rate was set to 1 L min^{-1}, with stirring at 400 rpm. The CO_2 (%) and O_2 (%) in the off-gas were monitored continuously during the fed-batch cultivations. Bioreactor cultures of strain *Y. lipolytica* ST9253 were inoculated from shake flask precultures in the exponential growth phase (250 mL baffled shake flasks, 40 mL cultivation volume, YPG 40 g L^{-1} glycerol, 10 g L^{-1} yeast extract, 10 g L^{-1} peptone) to a starter fermentation volume of 620 mL. After the depletion of the initially supplied glycerol carbon source, the cultures were fed with a nutrient-rich feed solution (700 g L^{-1} glycerol, 10 g L^{-1} yeast extract, 10 g L^{-1} $(NH_4)_2SO_4$). The composition of the feed solution was set to facilitate the production of the target compounds after an initial biomass buildup phase. Off-line samples were taken regularly to analyze residual glycerol concentration (Megazyme Inc. assay kit), optical density at 600 nm (Genesys photometer, Thermo Fischer Scientific), cell dry weight, and fatty alcohol concentrations.

2.5 | Extraction and derivatization of lipids

1 mL of cultivation broth was sampled into 4 mL glass vials and centrifuged at 3500 *g* for 5 min at 20°C. The supernatant was removed, and 1 mL of 1 M HCl in anhydrous methanol was added to the pellet. Samples were vortexed for 20 s and incubated at 70°C for 2 h for methanolysis reaction to proceed. Samples were cooled down to room temperature, 1 mL of 1 M NaOH in methanol, 500 μL of saturated NaCl solution in water, 1 mL of hexane, and 10 μL of internal standard (2 g L^{-1} of methyl nonadecanoate (19:Me) in hexane) were added sequentially. Vials were vortexed for 10 s, centrifuged at 3500 *g* for 5 min at 20°C, and the upper organic layer was taken for analysis.

2.6 | Extraction of fatty alcohols from fermentation samples

In small-scale screening experiments, 1 mL of cell culture was sampled into 4 mL glass vials and centrifuged at 3500 *g*, 5 min, 21°C. The supernatant was removed, and 1 mL of ethyl acetate:ethanol mixture (85:15, v/v) was added to the pellet together with 10 μL of internal standard (2 g L^{-1} of methyl nonadecanoate (19:Me) in ethyl acetate). Vials were vortexed for 20 s and incubated for 1 h, followed by 5 min vortexing. 300 μL of water was added, samples vortexed for 10 s, centrifuged at 3500 *g*, 5 min, 21°C, and the upper organic layer was taken for analysis.

Samples from bioreactors were processed as follows. 100 μL of cell culture was taken, and the total broth was processed in the same way as for small-scale samples except that the first centrifugation step was omitted.

2.7 | Extraction and work-up of fatty alcohols from bioreactor cultivation for acetylation reaction

The 2.4 L of broth from the bioreactor cultivation was centrifuged at 3000 *g* for 5 min at room temperature, and the supernatant was separated from the pellet. Fatty alcohols were extracted from the supernatant by adding 2.2 L of ethyl acetate and incubating on a multi-vortexer for 1 h. Organic phase was decanted. To extract fatty alcohols from the pellet, 100 mL of ethyl acetate was added to the pellet, and the mix was shaken on a multi-vortexer for 6 h. The organic phase was decanted, 100 mL of fresh solvent was added to the biomass pellet, and the mixing repeated for 1 h. The organic phase was decanted. All ethyl acetate fractions from the supernatant and pellet extraction were combined and dried with anhydrous sodium sulfate. The solvent was removed in a rotary evaporator.

The extracted crude fatty alcohol mixture was passed through a silica gel column, which was prepared as described in.[20] The silica was washed with hexane and then subsequently with a gradient of hexane/ethyl acetate at the proportion of 95:5 to 40:60 (% v/v). Purest fractions based on thin-layer chromatography were collected, and the solvent was evaporated in a rotary evaporator.

2.8 | Acetylation of fatty alcohols

The purified fatty alcohol mixture was acetylated by using a 1.2 molar equivalent of acetic anhydride and 0.1 equivalent of sodium acetate anhydrous (equivalents were based on 14:OH). The reaction mixture was allowed to stir for 1 h at 80°C in a water bath and then transferred to a separatory funnel where it was diluted with ethyl acetate and washed with a saturated solution of sodium bicarbonate. The organic phase was dried with anhydrous sodium sulfate, and the solvent evaporated in rotary evaporator.

2.9 | Gas chromatography-mass spectrometry (GS-MS) analysis

Analysis was carried out on an Agilent GC 7820A coupled to a MS 5977B, equipped with a split/spitless injector and a DB-Fatwax UI column (30 m x 250 μm x 0.25 μm). The operation parameters were: 1 μL injection, split ratio 20:1 for FAMEs and alcohol samples and 10:1 for acetates, injector temperature 220°C, constant flow 1 mL min^{-1} of helium, oven ramp 80°C for 1 min, 20°C min^{-1} to 150°C, then 1°C min^{-1} to 200°C, then 20°C min^{-1} to 230°C. MS was scanning between *m/z* 30 and 350. For compound identification, reference standards were purchased from Pherobank, and retention times together with mass spectra were compared to the sample of interest. Quantification was made based on 6 points calibration curves. Visual inspection of chromatograms was performed using MassHunter Qualitative Analysis Navigator B.08.00 (Agilent), while quantification was performed using MS Quantitative Analysis (Quant-My-Way) (Agilent).

2.10 | Electrophysiological responses of male *Ostrinia nubilalis*

Antennal responses of male ECB adults to BioPhe were evaluated by electroantennography (EAG) using a commercially available electroantennographic system (Ockenfels Syntech GmbH, Buchenbach, Germany). The antenna of a virgin two-to-three-day old male adult was excised from the head using micro-scissors and attached between two stainless-steel wire electrodes with the assistance of electrically conductive gel (G008, Fiab, Italy). The base of the antenna was connected to the indifferent electrode, whilst the distal end was connected to the recording electrode after cutting off a few segments of the tip. The signal was amplified 10X by a Universal AC/DC pre-amplifier probe connected to the recording electrode, and the analog signal was amplified and detected with a data acquisition controller (IDAC-4, Ockenfels Syntech GmbH).

Test stimulus was the BioPhe containing 7.2% Z11-14:OAc, according to GC-MS analysis. Commercially available Z11-14:OAc, the major sex pheromone of the *O. nubilalis* Z-race, was used as a reference compound (96% purity, Sigma-Aldrich, Germany). The BioPhe blend, as well as commercially available pheromone compounds, were diluted in pentane. For each stimulus, a 10 μL aliquot of the solution was pipetted to a piece of filter paper (7 × 30 mm, Whatman no. (1), and the solvent was allowed to evaporate before being inserted into a Pasteur pipette for odor delivery. Control stimulus consisted of a clean Pasteur pipette with a filter paper impregnated with solvent (pentane). Stimuli were provided as 0.3 s air puffs into a continuous flow of filtered and humidified air. The airflow, at 25 cm^3 s^{-1} rate, tube diameter 1 cm, was generated by an air stimulus controller (CS-55, Syntech, The Netherlands).

The reference stimulus, consisting of a filter paper carrying 100 ng of Z11-14:OAc, was provided at regular intervals during each recording session. The EAG response to each reference stimulus was defined

as 100%, and all responses to test stimuli between adjacent references were normalized in % relative to the references. At least 1 min for weak and moderate stimuli and 5 min for strong stimuli were allowed between successive stimulations to let the antenna recover. The BioPhe blend was tested at a 100 ng dose (containing ~7 ng *Z*11-14:OAc), as well as at a 1 μg dose (i.e., 70 ng *Z*11-14:OAc), in order to reach an amount of major sex pheromone comparable to the 100 ng dose of the *Z*11-14:OAc stimulus.

The minor sex pheromone, *E*11-14:OAc, was also tested, as well as both isomers at a 97:3 Z:E ratio, based on the ratio released from the female moth gland as reported in the literature for ECB Z-race.[7,21] In addition, considering the high amount of tetradecyl acetate (14:OAc) in the BioPhe blend (~50%), the EAG response to 50 ng of this compound was also evaluated (for consistency reasons 50 ng is approx. the amount of 14:OAc contained in the 100 ng BioPhe test dose). All aforementioned stimuli were tested on a total of 20 antennal preparations.

2.11 | Behavioral bioassays

Behavioral studies were performed in a dark room under red light (2.5–3 lux) at 21 ± 2°C and 70 ± 5% relative humidity. Male moths were tested in a wind tunnel as described in.[22] For odor delivery, a piece of triangular-shaped filter paper (2.5 cm base, 4 cm height) was pipetted with appropriate volume of the test solution and, following solvent evaporation, was hung from the suction hook in the tunnel.

Pheromone standards and BioPhe samples were dissolved in pentane. The major and minor sex pheromone components *Z*11-14:OAc and *E*11-14:OAc, respectively, were mixed at a 97:3 ratio and were used as a positive control at a 50 μg dose, which, through preliminary testing, was concluded to be the minimum amount eliciting positive courtship behavior, that is, source-oriented flight pattern, abdominal hair pencils display and abdomen curling upon contact. Additionally, to evaluate potential antagonistic effects of BioPhe blend contaminants on the males' responses, 14:OAc (most prominent component ~50% of BioPhe blend) and *Z*9-16:OAc (~9% of BioPhe blend) have been tested.

A dose of 700 μg BioPhe was used in the bioassays (containing 50 μg *Z*11-14:OAc and 350 μg 14:OAc) and a dose of 350 μg 14:OAc was added to 50 μg pheromone standard (97:3) to emulate abundance and ratio in the BioPhe. Also a dose of 50 μg pheromone standard (97:3) with the addition of 4.5 μg of *Z*9-16:OAc (9% present in the BioPhe blend) was tested, as to our knowledge the role of *Z*9-16:OAc has not been investigated as antagonist to male response. Other components of BioPhe, such as *Z*9-14:OAc have already been reported in the literature as antagonist to male response.[23,24] Behavioral tests were conducted between the 3rd and 5th h in the scotophase.[24,25] 2 h before testing, 2–5 days old male moths were transferred in individual 400 mL clear plastic cups covered with perforated lids and left in the conditions of the dark room to acclimate. For each test, the odor source was attached to the tunnel ceiling, and after 30 s, an individual male was released in the tunnel, and its behavior was recorded for 10 min.

The following sequences of behavior were recorded: close approach (less than 10 cm) and contact with the source (landing). In addition, the approach and landing steps were further analyzed into four grades, and each grade was assigned its corresponding value. For the landing step, grades were discriminated as follows: grade (1) brief contact with the source (no landing), grade (2) landing on source for 1–2 s, grade (3) landing on source for more than 2 s, and grade (4) landing on source accompanied by hairpencils display and/or abdomen curling (copulation attempt). Similarly, for the approach step were discriminated as: grade (1) close approach to source for 1–2 s, grade (2) close approach with zig-zag patterned flight for >2 s, grade (3) close approach with zig-zag patterned flight and hairpencils display for 1–2 s, grade (4) close approach with zig-zag patterned flight and hairpencils display for >2 s.

Between treatments, the flight tunnel inner walls and suction hook were wiped with acetone and left to aerate for 10 min before the next treatment. For each treatment, 30 males were tested. Males were used once and were discarded after testing.

2.12 | Statistical analyses

The electrophysiological and behavioral data were subjected to analysis of variance (ANOVA) (SAS Institute, 2000). The means of electrophysiological data and the response grades were separated using the Tukey (honestly significant difference, HSD) test at $p = 0.05$.

3 | RESULTS

3.1 | Screening of fatty acyl-CoA desaturases

In *O. nubilalis*, a Δ11 desaturase acts on myristoyl-CoA (14:CoA), which in turn is generated by β-oxidation of the abundant fatty acid synthesis product hexadecanoyl-CoA (16:CoA), and generates a mixture of *E*/*Z*11-14:CoA.[6,26] In *Y. lipolytica*, myristic acid is a minor component of the total fatty acid pool, comprising only about 0.25% of the total fatty acids.[27] To increase the supply of 14:CoA in *Y. lipolytica*, we substituted isoleucine 1220 of the fatty acid synthase ketoacyl synthase domain to phenylalanine (Fas2p^{I1220F}). This mutation was proposed to hinder the binding of longer acyl-CoAs in the active site tunnel of the FAS complex and was reported to result in the production of shorter chain acyl-CoAs.[27] The mutation has already been proven to be effective in the production of *Z*9-14:OH, where it increased the titer 15-fold.[16] In this study, we introduced the FAS mutation into *Y. lipolytica* strain ST6629, which was previously developed for decreased degradation of fatty alcohols and decreased storage lipid synthesis.[16] The resulting ST7982 showed 8.4-fold increase in myristic acid production compared to the parental strain (Figure S3).

Next, seven Δ11 desaturases with previously documented *E*/*Z*11-14:CoA activity were tested in strain ST7982 (Figure 1, Figure S4). These were the desaturase Lbo_PPTQ from the grapevine moth *Lobesia botrana*,[28] Onu11 from the European corn borer *O. nubilalis*,[26] EpoE11 from the light brown apple moth *Epiphyas postvittana*,[29]

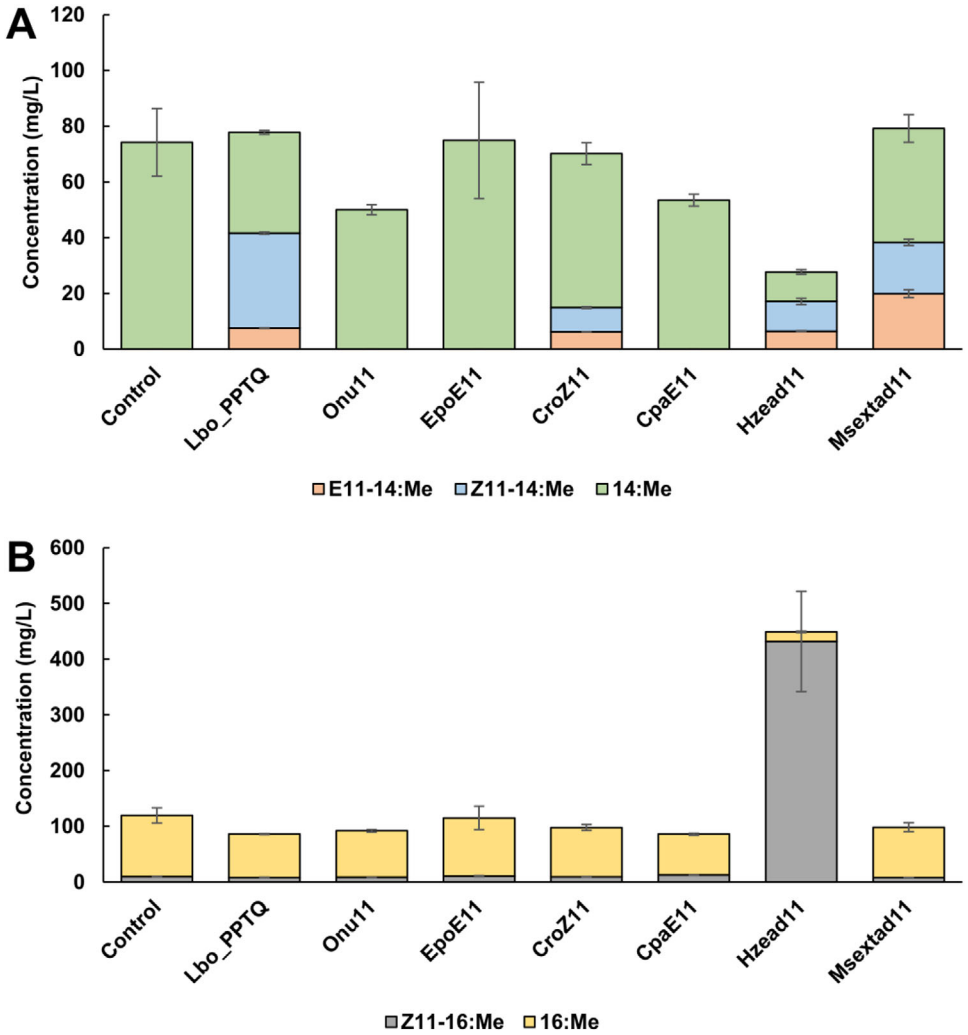

FIGURE 1 Characterization of Δ11 desaturases. (**A**) Production of *E*/*Z*11-14:acid and (**B**) *Z*11-16:acid by *Y. lipolytica* containing different moth fatty acyl-CoA desaturases. Quantification of fatty acids was done after derivatization into methyl esters. Results are obtained from three biological replicates. Error bars indicate standard deviations

CroZ11 from the oblique banded leafroller moth *Choristoneura rosaceana*,[30] CpaE11 from the spotted fireworm moth *Choristoneura parallela*,[31] Hzead11 from the corn earworm *H. zea*,[32] and Msextad11 from the tobacco hornworm *Manduca sexta*.[33] The resulting strains were cultivated, their lipids methanolized into FAMEs, and analyzed on GC-MS. Lbo_PPTQ expression resulted in the highest content of *Z*11-14:acid. The *Z*/*E* isomer ratio was 9:2. For the strain expressing the OnuE11 desaturase, no new compounds could be detected, indicating that this desaturase is most likely not well expressed or active in *Y. lipolytica*. Likewise, no activity was detected for the desaturases EpoE11 and CpaE11, which were previously reported to produce *E*11-14:acid in another yeast, *Saccharomyces cerevisiae*, albeit in minuscule amounts.[29,31] The strain expressing CroZ11 produced a mixture of *E*/*Z*11-14:acid with an excess of the Z isomer; however, the amounts of both isomers were lower compared to the strain expressing Lbo_PPTQ. The desaturase Hzead11 appeared to be a promiscuous Δ11 desaturase with a high preference towards 16:acid (Figure 1). Apart from *Z*11-16:acid, we also detected *E*/*Z*11-14:acid and some other unsaturated fatty acids, which remain to be identified. (Figure S4). The Msextad11 desaturase-expressing strain produced *E*/*Z*11-14:acid in ratio 1:1, where the amount of the E isomer was the highest among the tested desaturases.

In summary, Lbo_PPTQ resulted in the highest content and purity of *Z*11-14:acid and was therefore chosen for establishing the biosynthetic pathway towards ECB pheromone in *Y. lipolytica*.

3.2 | Screening of fatty acyl-CoA reductases

We further screened four FARs to identify the reductase with the highest activity and selectivity towards *Z*11-14:acid. The four reductases were: HarFAR from cotton bollworm *H. armigera*,[34] SlitpgFARII from African cotton leafworm *Spodoptera littoralis*,[35] and two reductases from *O. nubilalis*, OnuFAR_E and OnuFAR_Z.[36] First, we expressed the four reductases individually in the strain ST7982. The resulting strains were cultivated as follows: first, the cells were grown for

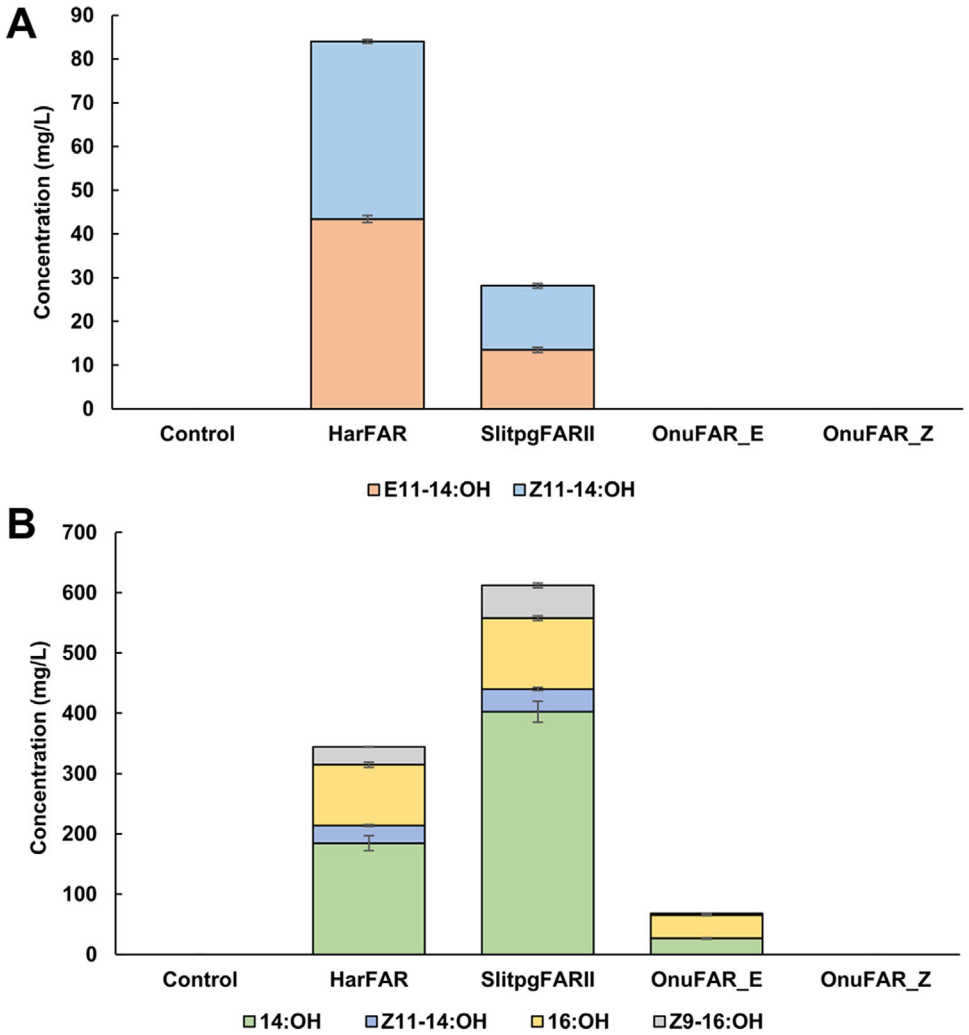

FIGURE 2 Characterization of reductases. (**A**) Profiles of *E*/*Z*11-14:OH produced by *Y. lipolytica* containing different reductases when cultivation media was supplemented with a mixture containing equal amounts (500 mg L^{-1}) of *E*/*Z*11-14:Me. (**B**) Fatty alcohol profiles of *Y. lipolytica* strains expressing Lbo_PPTQ together with different reductases. The control strain has no reductase introduced. Results are obtained from three biological replicates. Error bars indicate standard deviations

biomass propagation for 22 h, followed by a production phase in media supplemented with equal amounts of *E* and *Z*11-14:Me (500 mg L^{-1} of each isomer) lasting for 28 h. Fatty alcohols were extracted from the broth and quantified on GC-MS. HarFAR and SlitpgFARII expressing strains produced around 40 and 14 mg L^{-1} respectively of each isomer, *E*11-14:OH and *Z*11-14:OH, from the corresponding methyl esters, and did thus not exhibit a preference towards any isomer. OnuFAR_E produced very low amounts (<1 mg L^{-1}) of *E*/*Z*11-14:OH, which were below the quantification limit. However, manual inspection of the chromatograms showed that this reductase had a bias towards *E* isomer, as expected. No fatty alcohols were detected in the strain expressing OnuFAR_Z (Figure 2, Figure S5).

Next, for de novo production of *Z*11-14:OH, we expressed the four reductases in the strain ST9992, which contained $Fas2p^{I1220F}$ mutation and expressed desaturase Lbo_PPTQ. Interestingly, while HarFAR resulted in 2.8-fold higher product titer than SlitpgFARII in the feeding assay described above, in this experiment with de novo production of unsaturated fatty acid, a higher *Z*11-14:OH titer was obtained in strain with SlitpgFARII, 37.7 ± 2.6 mg L^{-1} versus 29.2 ± 1.6 mg L^{-1} for HarFAR strain. SlitpgFARII and HarFAR likely have different kinetic parameters, such as substrate affinity, maximum velocity, and potential inhibition by substrate or product. The intracellular concentrations of substrate and product are likely different under conditions when the substrates are taken up from the medium or biosynthesized internally and hence one condition may benefit SlitpgFARII and *vice versa*.

However, the strain expressing SlitpgFARII additionally produced 402.5 ± 17.3 mg L^{-1} of 14:OH and 54.7 ± 4.0 mg L^{-1} of *Z*9-16:OH, which is 2.2- and 1.8-fold higher compared to HarFAR, respectively (Figure 2B). Very high activity towards tetradecanoate of SlitpgFARII was also observed by Antony et al.,[35] who reported that among a wide variety of saturated, mono-, and di-unsaturated fatty acyl substrates, tetradecanoate was converted into alcohol most efficiently.

We chose HarFAR for further strain engineering due to its high activity and better selectivity towards *Z*11-14:CoA than SlitpgFARII.

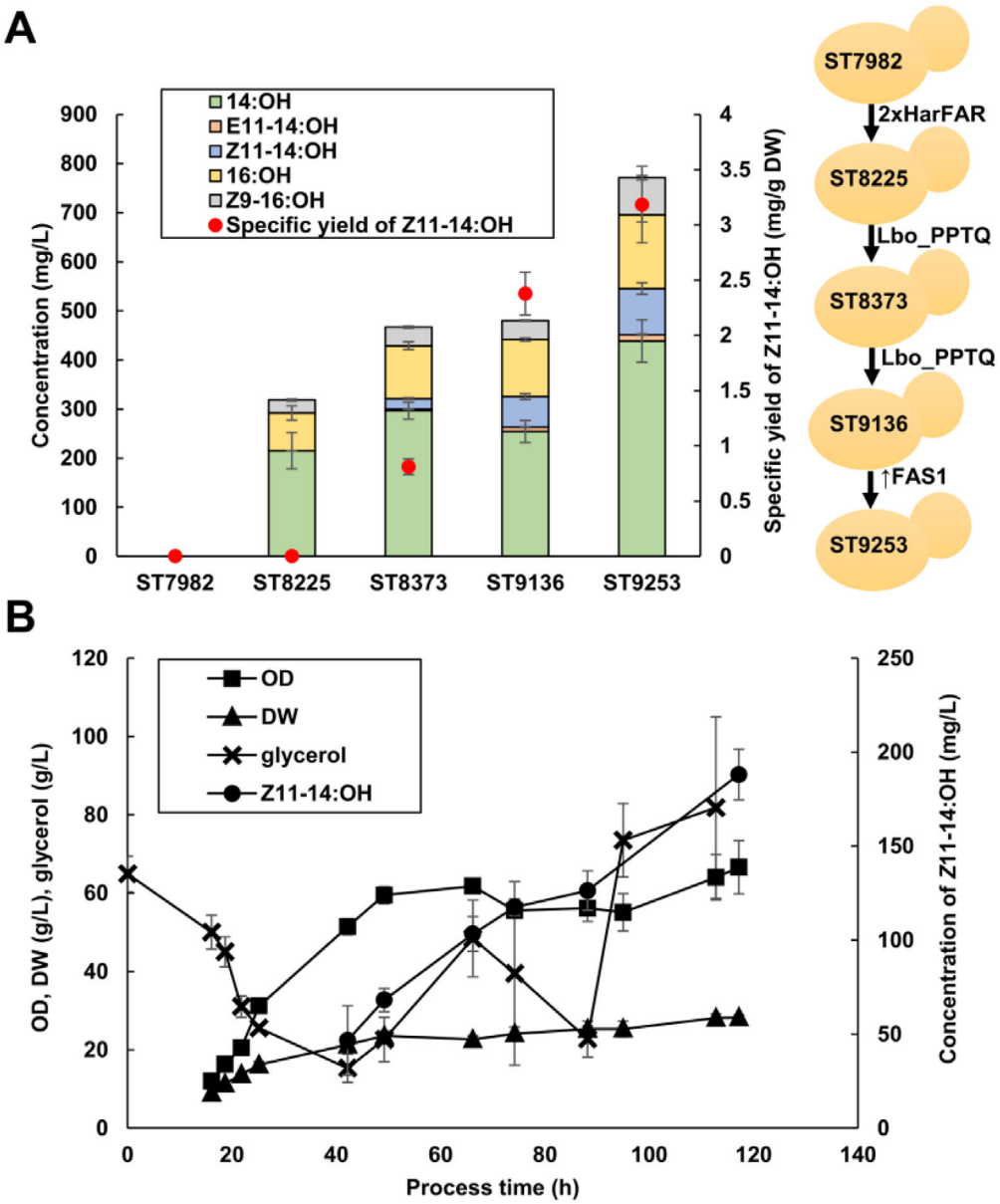

FIGURE 3 Production of Z11-14:OH in small scale cultivations and in 1 L fed-batch fermentation. **(A)** Metabolic engineering of *Y. lipolytica* for improved production of Z11-14:OH. High myristate producing strain ST7982, containing mutation in α-subunit of the fatty acyl synthase complex (Fas2p^{I1220F}) was used as background strain. Results are obtained from three biological replicates. **(B)** Fed-batch fermentation results of ST9253. Data obtained for optical density (OD), cell dry weight (DW), glycerol, and Z11-14:OH concentration are represented as averages from three bioreactors and standard deviations are calculated which are shown as error bars

3.3 | Metabolic engineering for improved production of Z11-14:OH

After selecting Lbo_PPTQ and HarFAR as the biosynthetic enzymes for Z11-14:OH production, we proceeded with further metabolic engineering strategies to increase the product titer and specific yield (Figure 3A). First, we introduced two gene copies of HarFAR into the high myristate producing strain ST7982 to enable biosynthesis of fatty alcohols. The resulting strain ST8225 produced 318.3 ± 52.4 mg L^{-1} total fatty alcohols, among which 14:OH constituted 67.5% and was the most abundant fatty alcohol. In order to produce Z11-14:OH, we expressed Lbo_PPTQ in ST8225 leading to ST8373, which gave 21.5 ± 2.2 mg L^{-1} of the target compound, with a specific yield of 0.8 mg Z11-14:OH per g DW. Compared to the parental strain, this strain produced 46.7% more total fatty alcohols. Then we introduced a second copy of Lbo_PPTQ and this led to a further 3-fold improvement of Z11-14:OH titer and specific yield.

We anticipated that further improvement could be achieved by overexpressing native *FAS1* from *Y. lipolytica*. Previous studies in *S. cerevisiae* showed that the activity of the FAS complex is enhanced when the copy number of *FAS1* gene is increased.[37] Indeed, overexpression of *FAS1* (strain ST9253) resulted in 60.7% improvement in the total

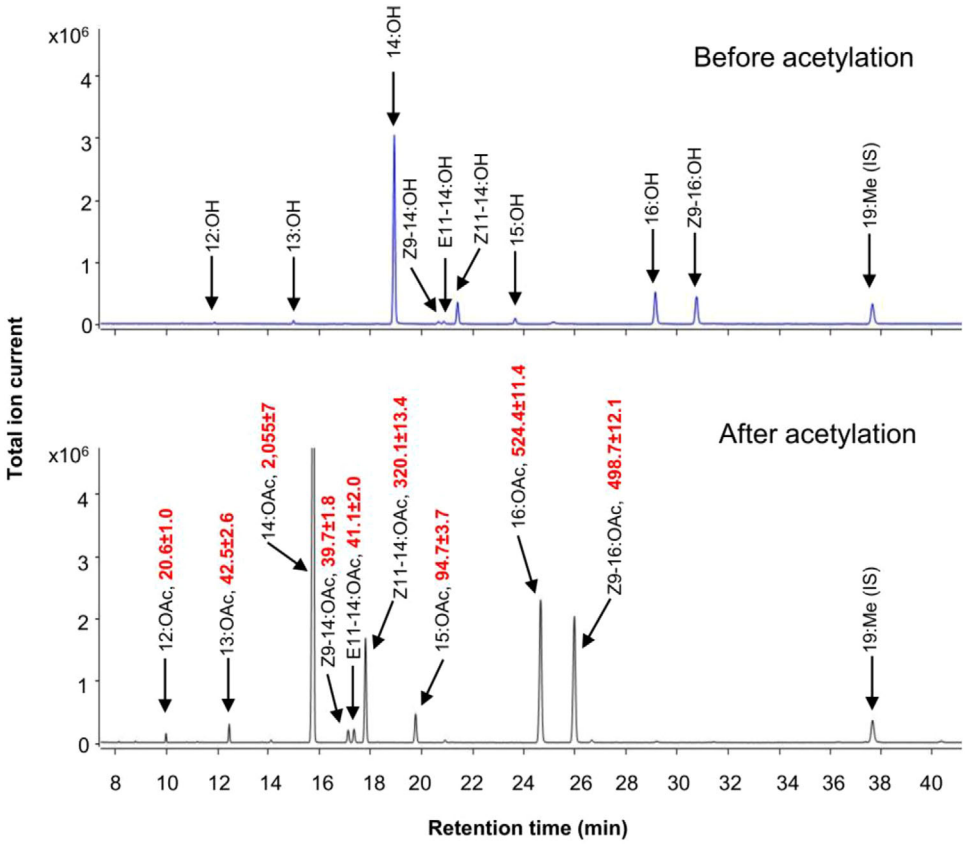

FIGURE 4 GC-MS chromatogram representing fatty alcohol sample before (above) and after (below) acetylation. Numbers in red represent the amount of each fatty acetate (mg ± SD) in the final sample. Measurements were performed in technical triplicates.

fatty alcohol titer and increased production of Z11-14:OH by 54.7%, resulting in 93.9 ± 11.7 mg L^{-1} of Z11-14:OH in small-scale cultivation. The increase of Z11-14:OH specific yield was 34%.

The introduced genome edits did not significantly affect the growth of the yeast strains. The final biomass concentrations were similar, ranging from 26.1 to 29.5 g DW L^{-1}.

3.4 | Production of Z11-14:OAc by fed-batch fermentation and alcohol acetylation

The engineered strain ST9253 (MATa *ku70*Δ *Cas9 hfd4*Δ *hfd1*Δ *pex10*Δ *fao1*Δ *hfd2*Δ *hfd3*Δ GPAT_100bpPr Fas2p^{I1220F} 2x*Lbo_PPTQ* 2x*HarFAR* ↑*FAS1*) was cultivated in controlled bioreactors in fed-batch mode. The initial batch phase served as a biomass propagation phase. After the depletion of the initial carbon source supplied in the batch phase, the composition of the fed-batch feed solution was set to facilitate the production of fatty alcohols. In the first 42 h of fermentation, the accumulation of biomass was targeted, and stationary phase was reached after 49 h (Figure 3B). The start of the fed-batch facilitated a shift towards nitrogen-limited conditions and thereby transition from the biomass build-up phase to production of fatty alcohols.

In the fatty alcohol production phase of the fermentation process, we observed a constant increase in specific yield. The specific production yield of Z11-14:OH peaked at the very end of fermentation and reached 6.7 mg product g^{-1} DW. At this time point, the titer of Z11-14:OH was 188.1 ± 13.4 mg L^{-1}, while the cell dry weight reached 28.2 ± 0.7 g L^{-1} (Figure 3B). The specific yield in bioreactors was 2-fold higher than what was obtained in small-scale cultivation. This could be explained by improved aeration, pH control, and/or glycerol feed control. The titer of 14:OH reached 1,350 ± 188 mg L^{-1}. It was the most abundant fatty alcohol in the fermentation broth.

After 120 h of fed-batch fermentation, fatty alcohols were extracted with organic solvent and purified on a silica column. The resulting fatty alcohol mixture containing approximately 250 mg of Z11-14:OH was acetylated with acetic acid anhydride. It resulted in full conversion, where 320.1 ± 13.4 mg of Z11-14:OAc was obtained, and no traces of alcohols were left at the end of the reaction (Figure 4). The resulting product is referred to as BioPhe, for biologically-derived pheromone.

3.5 | Electrophysiological responses of male *Ostrinia nubilalis*

We studied electrophysiological responses of male *O. nubilalis* insect antennas to pure chemical pheromone components, BioPhe product, and several blends. As expected, EAG responses of ECB Z-race male moths were higher for 100 ng Z11-14:OAc than for E11-14:OAc

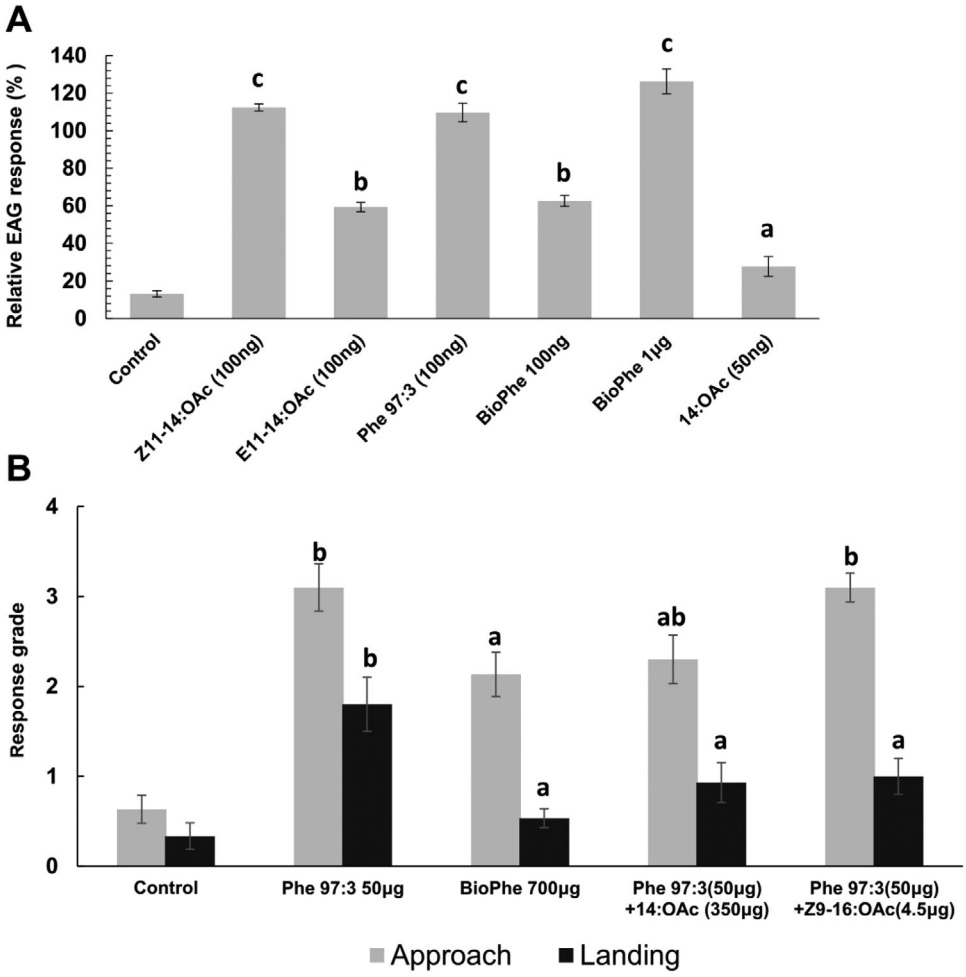

FIGURE 5 Performance of biologically-derived *O. nubilalis* sex pheromone. (**A**) Electrophysiological responses of male *O. nubilalis* antennae to biologically-derived pheromone blend (BioPhe), standard compounds (*Z*11-14:OAc, *E*11-14:OAc and 14:OAc), and mixtures of the standard compounds. Response to 100 ng of *Z*11-14:OAc was defined as 100%, and all responses normalized in % relative to this reference. Means followed by the same letter are not significantly different ($p > 0.5$, Tukey's studentized range [HSD] test, $F = 51.650$, $df = 5$, $p = 0.000$). (**B**) Behavioral responses of *O. nubilalis* males to yeast-derived pheromone blend (BioPhe), standard compounds (*Z*11-14:OAc, *E*11-14:OAc, 14:OAc) and mixtures of the standard compounds. Phe 97:3 represents pheromone composition originally found in *O. nubilalis* Z-race and is generated from standard compounds *Z*11-14:OAc, *E*11-14:OAc in ratio 97:3. Means followed by the same letter are not significantly different ($p > 0.05$, Tukey's studentized range [HSD] test). Approach, $F = 4.579$ $df = 3$, $p = 0.005$, Landing, $F = 5.899$, $df = 3$, $p = 0.001$)

(Figure 5A). Also, the 97:3 (*Z*11-14:OAc/*E*11-14:OAc) mixture at 100 ng dose elicited the same magnitude of response as the major pheromone component (*Z*11-14:OAc). The biologically-derived pheromone BioPhe at 100 ng elicited a significantly lower response, as expected, because Z11-14:OAc is present just at 7.2%, *ergo*, only about 7 ng of *Z*11-14:OAc were directed to the antenna. When the quantity of BioPhe was increased 10-fold, the EAG response was also increased. In this treatment, the amount of *Z*11-14:OAc present in the biologically-derived mixture was of the same order of magnitude as the reference (i.e., ~70 ng). However, it should be noted, that some of the other BioPhe components may also contribute to the augmented response recorded. In this context, we tested the effect of 14:OAc, given the fact that it comprises about 50% of BioPhe. At 50 ng dose, analogous to that contained in 100 ng BioPhe, it was clearly detected by the antenna but to a much lesser degree than the reference compound.

3.6 | Behavioral bioassays

Male ECB moths responded with approach and landing to 50 μg of the pheromone blend corresponding to the *Z*-race (Figure 5B). As BioPhe contains just 7.2% Z11-14:OAc, a 700 μg stimulus of BioPhe was tested in lab bioassays (containing approx. 50 μg Z11-14:OAc). Significantly fewer source-oriented approaches and fewer landings were observed compared to the response elicited by the optimal Z-race pheromone, indicating the presence of compound(s) interfering with the insects' anticipated behavior.

In the BioPhe sample 14:OAc comprises almost 50% of the total amount. To emulate a BioPhe sample, 350 μg 14:OAc were added to the 50 μg pheromone blend (97:3). In this case, males scored significantly lower in both approach and landing. This is an indication that 14:OAc at this dose interferes with the precopulatory behavior of *O. nubilalis*.

Similarly, when 4.5 μg of *Z*9-16:OAc was added to the 50 μg pheromone blend (97:3), significantly fewer landings were recorded, but the frequency of approaches did not differ significantly from what was observed in response to the native pheromone.

4 | DISCUSSION

We have evaluated the performance of seven FADs and four FARs, which are involved in insect sex pheromone biosynthesis, to establish the production of *Z*11-14:OH in *Y. lipolytica*. Among FADs, Lbo_PPTQ showed the highest production of (*Z*)–11–tetradecenoate and was selected for further strain engineering. Several desaturases, namely, Onu11, EpoE11, and CpaE11, were not active. The desaturase Onu11 was previously assayed in other recombinant hosts, such as *S. cerevisiae* and *Nicotiana benthamiana* with divergent results. In *S. cerevisiae*, it generated *E*/*Z*11-14:acid in ratio 4:5 and also produced *Z*11-16:acid,[26] while no activity of this enzyme was observed in *N. benthamiana*.[14] The other two desaturases that were non-functional in our study, EpoE11 and CpaE11, were reported to act on 14:acid and produce *E*11-14:acid in *S. cerevisiae*, but the amounts were very low.[29,31] One possible explanation is that the non-functional desaturases were poorly expressed or misfolded. A recent study by Buček et al. showed that different moth desaturases could have different expression levels.[38] Codon-optimization could also have been suboptimal.[39]

After examining FADs, we screened several FARs for increased specificity towards fatty acids with C14 chain length. Previously, HarFAR was shown to prefer (*Z*)–9–tetradecenoate over (*Z*)–11–hexadecenoate and (*Z*)–9–hexadecenoate.[34] We hypothesized that this reductase should also be able to act on (*E*/*Z*)-11- tetradecenoate, which is structurally similar to (*Z*)-9-tetradecenoate. Indeed, a feeding experiment and co-expression with Lbo_PPTQ confirmed that HarFAR could produce *E*/*Z*11-14:OH from corresponding acids. (*E*/*Z*)-11-tetradecenoate was also converted into alcohol by *Y. lipolytica* expressing SlitpgFARII. This FAR previously produced the highest amounts of *Z*11-14:OH among the four tested reductases from *Spodoptera* spp.[35] Reductases OnuFAR_E and OnuFAR_Z were shown to be selective for E and Z isomers of 11-tetradecenoate, respectively,[36] however, in our study, OnuFAR_E showed little and OnuFAR_Z no activity. The same results were obtained when these enzymes were screened in the plant *N. benthamiana*.[14] One possible explanation for the lack of functionality of these enzymes in yeast and plant expression hosts could be incorrect folding of the reductases in the ER membrane, which has a different lipid composition in yeasts and plants than in insects.[40–43]

In order to improve the production of *Z*11-14:OH, we employed metabolic engineering strategies, such as integration of multiple copies of genes and enhancement of precursor supply by overexpression of *FAS1* subunit of *Y. lipolytica*. In small-scale cultivations of the engineered yeast strain, we achieved 93.9 $\pm$ 11.7 mg L^{-1} of *Z*11-14:OH. On the way of building the production strain (ST9253), we observed that introduction of desaturase Lbo_PPTQ not only contributed to the biosynthesis of *Z*11-14:OH, but it also improved the total fatty alcohol titer. It was previously shown that overexpression of the native *OLE1* desaturase increased fatty acid biosynthesis in general, likely because the fatty acid synthase complex FAS is less inhibited by unsaturated fatty acyl-CoAs than by saturated fatty acyl-CoAs.[44–46]

For collection of pheromone for EAG and behavioral tests, the engineered strain was fermented in controlled 1 L bioreactors in fed-batch mode. We used glycerol as the carbon source with a high carbon-to-nitrogen ratio to favor fatty alcohol production.[47–52] We obtained 188.1 $\pm$ 13.4 mg L^{-1} of *Z*11-14:OH. The strain also produced large amounts (over 1.3 g L^{-1}) of the saturated by-product 14:OH, indicating a significant limitation of the desaturation step. This may be improved by further strain engineering and fermentation optimization in the future. To our knowledge, this is the first study showing the production of *Z*11-14:OH in a microbial host. Previously this pheromone precursor was recombinantly synthesized in plant *N. benthamiana* yielding 14 μg from 1 g leaf tissue.[14] While *Y. lipolytica* has been engineered for the production of fatty alcohols in multiple other studies, the common products are naturally unsaturated or saturated fatty alcohols.[48,49,53–56]

To convert the fermented alcohol into acetate, which is the active sex pheromone component of *O. nubilalis*, a chemical acetylation step was performed, which resulted in full conversion and yielded 320 $\pm$ 13.4 mg of *Z*11-14:OAc. Interestingly, based on the current knowledge about insect pheromone biosynthesis, this reaction should be catalyzed by acyltransferases in insects.[6,57] However, until now, no enzymes from moths catalyzing this reaction have been found even though some gene candidates have been proposed and tested.[58] To achieve in vivo production of fatty alcohol acetates, acetyltransferases from other organisms, such as Atf1p from *S. cerevisiae* or EaDAc from the burning bush *Euonymus alatus*, could be expressed.[14,59] We previously demonstrated that (*Z*)-9-tetradecenyl acetate can be synthesized directly in *S. cerevisiae* strain overexpressing *ATF1* gene (WO/2018/109167).

The yeast-derived pheromone blend caused a response of *O. nubilalis* males in electroantennogram experiments similar to what could be expected based on the responses to synthetic pheromone compounds. Furthermore, the blend was attractive to insects in behavioral bioassays in a wind tunnel, although to a lower level than the chemically synthesized pheromone blend. In addition, full precopulatory behavior was observed less often. In order to induce a complete the ECB precopulatory behavior, a higher purity or more optimal *Z*:*E* isomer ratio may be required. In our study, Z-race of ECB was explored in the EAG and behavioral assays. The strain uses a 97:3 blend of *Z*11-14:OAc to *E*11-14:OAc as sex pheromone.[6] In the yeast-derived pheromone sample, the ratio of these compounds was 89:11, that is, E-isomer was in excess in comparison to the optimal ratio. Potentially, this ratio could be improved by engineering the stereospecificity of the desaturase.[38,60] Notably, reduced precopulatory behavior is not necessarily a hindrance

and may even be a benefit for mating disruption. Therefore, activity studies in the field are warranted.

The effectiveness of managing ECB by mating disruption was previously proven in the USA, where pheromone-dispenser-treated areas had noticeably lower levels of mating compared to untreated control areas.[61] Infestation of many other Lepidoptera pests, including the codling moth *Cydia pomonella* and the grapewine moth *L. botrana*, is also successfully reduced by mating disruption and has been applied commercially for over 20 years.[10] The cost of mating disruption is higher than insecticide treatment,[62] but it is used for high-value fruits due to other advantages, such as absence of toxic residues. For lower-value crops, for example, maize, mating disruption using chemical pheromones is too expensive.[63] Prices of chemically synthesized pheromones range from several hundred to several thousand dollars per kg, therefore reduction of production costs is one of the major driving forces to create efficient microbial or plant factories for biosynthesis of these compounds.[17,63,64] Economic viability of fermentation-based processes for production of specialty oleochemicals has already been shown, where compounds such as omega-3 fatty acids have been produced at commercially relevant titers reaching $\sim$10 g L^{-1}.[65,66]

In summary, we have successfully employed yeast *Y. lipolytica* for production of *O. nubilalis* sex pheromone precursor Z11-14:OH and showed that the resulting yeast-derived pheromone was biologically active in modulating the behavior of *O. nubilalis* males.

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AUTHOR CONTRIBUTIONS

Karolis Petkevicius: Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; validation; visualization; writing-original draft; writing-review & editing. Eleni Koutsoumpeli: Formal analysis; investigation; methodology; writing-review & editing. Petri Christina Betsi: Formal analysis; investigation; methodology; writing-review & editing. Bao-Jian Ding: Resources; validation; writing-review & editing. Kanchana Rueksomtawin Kildegaard: Investigation; methodology; writing-review & editing. Hilbert Jensen: Data curation; investigation; methodology; writing-review & editing. Nora Mezo: Data curation; investigation; methodology; writing-review & editing. Andrea Mazziotta: Data curation; investigation; methodology; writing-review & editing. Anders Gabrielsson: Investigation; methodology; writing-review & editing. Christina Sinkwitz: Data curation; investigation; methodology; validation; writing-review & editing. Bettina Lorantfy: Investigation; methodology; supervision; writing-review & editing. Carina Holkenbrink: Conceptualization; investigation; methodology; resources; supervision; validation; writing-original draft; writing-review & editing. Christer Löfstedt: Conceptualization; funding acquisition; resources; supervision; writing-original draft; writing-review & editing. Dimitris Raptopoulos: Conceptualization; funding acquisition; supervision; writing-review & editing. Maria Konstantopoulou: Conceptualization; funding acquisition; supervision; writing-original draft; writing-review & editing. Irina Borodina: Conceptualization; funding acquisition; resources; supervision; validation; writing-original draft; writing-review & editing.

CONFLICT OF INTEREST

Irina Borodina, Carina Holkenbrink, Christer Löfstedt, Bao-Jian Ding are co-inventors on patent applications WO2016207339, WO2018109167, and WO2018109163. Karolis Petkevicius, Kanchana Rueksomtawin Kildegaard, Hilbert Jensen, Nora Mezo, Andrea Mazziotta, Anders Gabrielsson, Christina Sinkwitz, Bettina Lorantfy, Carina Holkenbrink, Irina Borodina have financial interest in BioPhero ApS. Dimitris Raptopoulos has financial interest in Novagrica SA.

DATA AVAILABILITY STATEMENT

Not applicable.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Supporting information for the research article **Biotechnological production of the European corn borer sex pheromone in the yeast *Yarrowia lipolytica***

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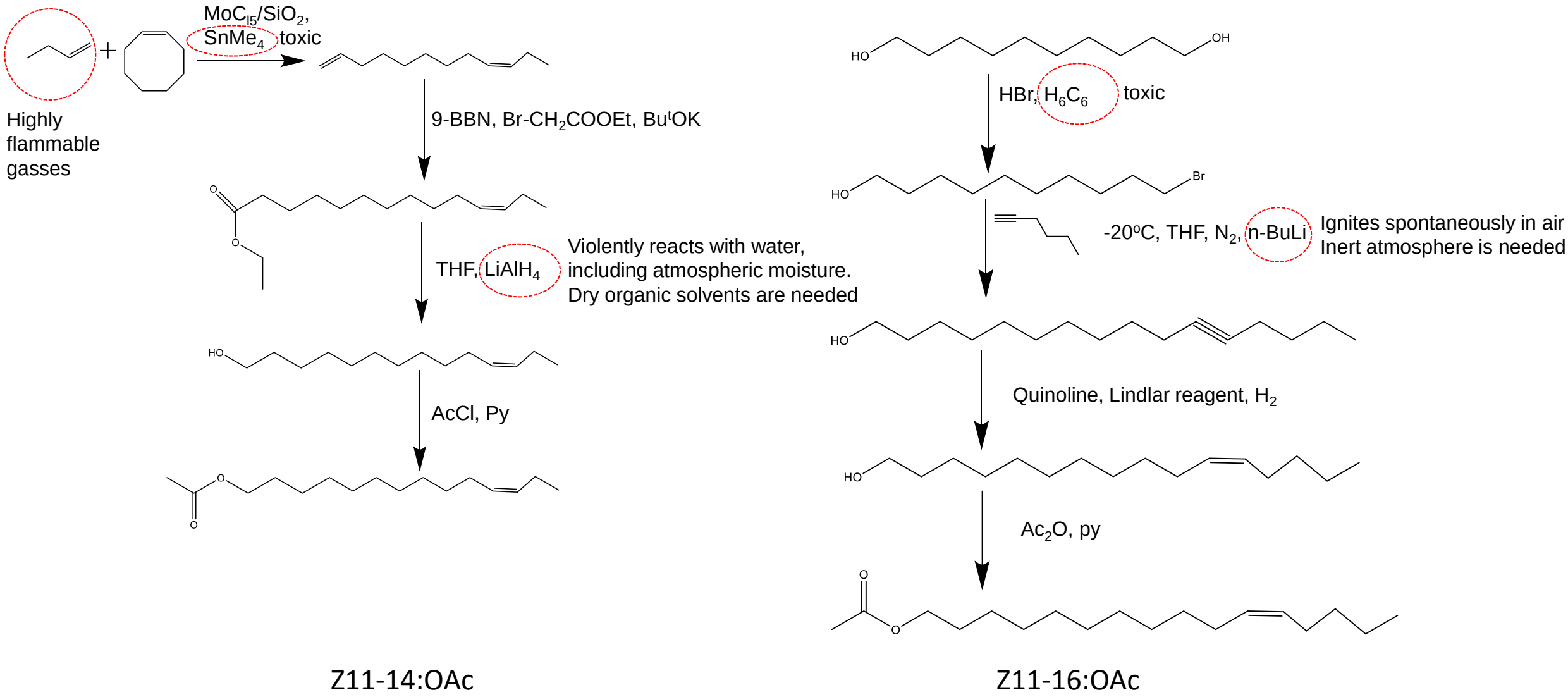

Figure S1. Examples of chemical synthesis of insect sex pheromones Z11-14:OAc and Z11-16:OAc based on supplementary references [1,2].

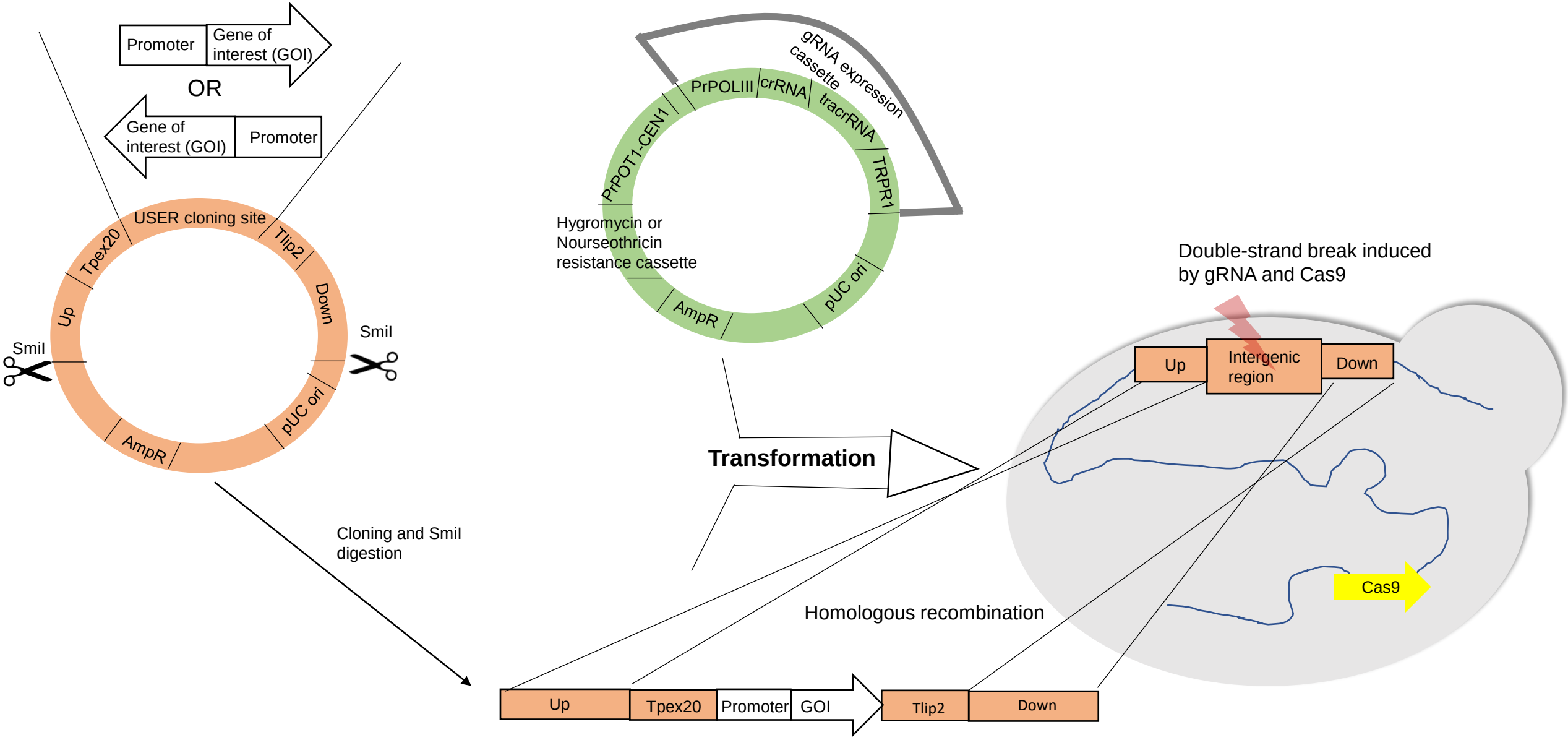

Figure S2. Schematic representation of integration of gene expression constructs into *Y. lipolytica*.

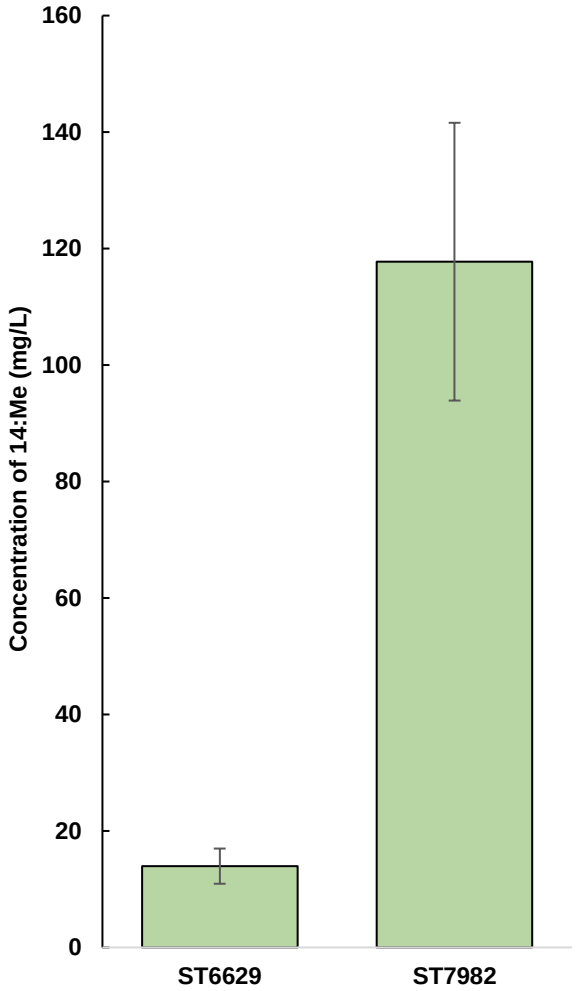

Figure S3. Effect of FAS2 mutation (I1220F) on production of myristic acid. ST6629 has native FAS2 sequence while ST7982 expressed the mutated version.

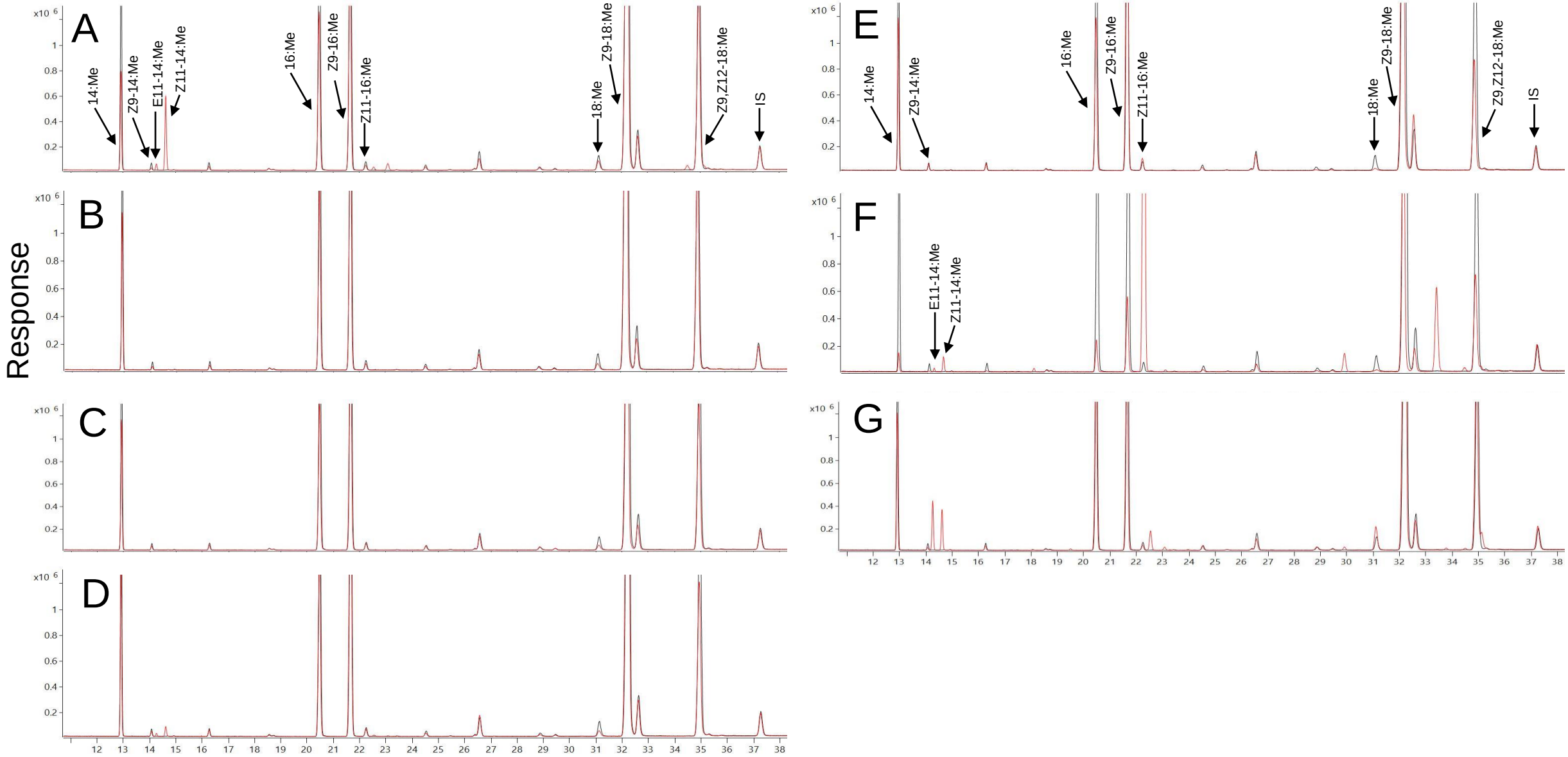

Figure S4. FAMEs profiles of strains containing different desaturases. In each chromatogram control strain (no desaturase) is represented in black color and is overlaid with chromatogram obtained from strain containing: A. Lbo_PPTQ, B. Onu11, C. EpoE11, D. CroZ11, E. CpaE11, F. Hzead11, G. Msextad11, which is represented in red.

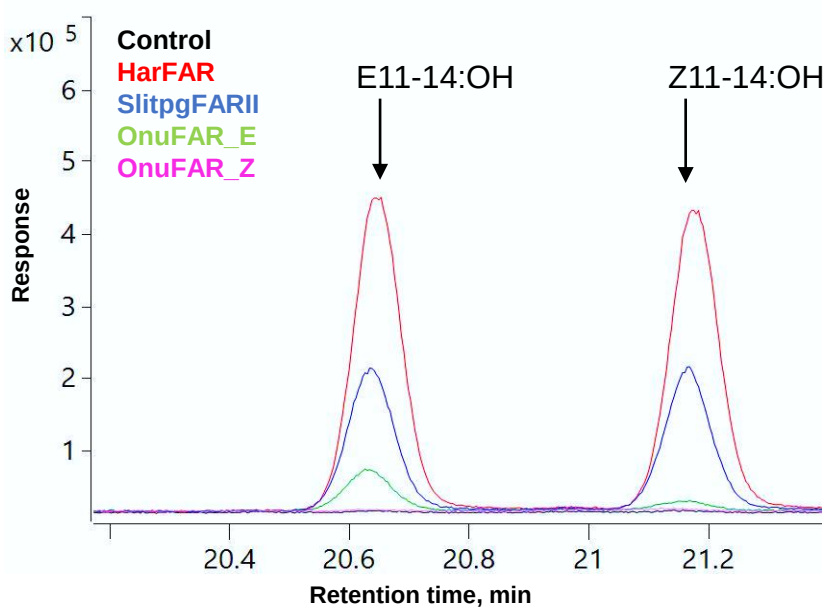

Figure S5. Fragment of overlaid total ion current (TIC) chromatogram from alcohol extracts obtained from control strain (no reductase) and strains containing different reductases during feeding experiment. Strains were fed with mixture containing equal amounts (500 mg/L) of E/Z11-14:Me.

Table S1. Primers used in this study

ID	Description	Sequence 5' to 3'
PR-18241	gRNA2_IntD_1_sense	GCAGACGCAGACGGAGACTGgttttagagct
PR-18242	gRNA2_IntD_1_antisense	CAGTCTCCGTCTGCGTCTGCtaaccaacct
PR-23129	IntF_6_gRNA3_sense	GTCGGAGGAAGGAATCGGTGgttttagagct
PR-23130	IntF_6_gRNA3_antisense	CACCGATTCCTTCCTCCGACtaaccaacct
PR-22536	IntF_6_up_fwd	CGTGCGAUAGAGATACAGAGCGGG
PR-22537	IntF_6_up_rev	AAGCGTTGCACGUCTCTTACCCTGCATTCG
PR-22538	IntF_6_down_fwd	AGTGGCCUTGTTTGTTCATTATTTGTTCTC
PR-22539	IntF_6_down_rev	CACGCGAUGGTCCTAGTGCCAAAC
PR-18928	PrTEF1 <-_U1_fw	CACGCGAUAGAGACCGGGTTGG
PR-18214	PTEFintron_USER_rv	AGTACTGCAAAAAGUGCTG
PR-21723	<-Lbo_PPTQ_U1_fw	ACTTTTTGCAGTACUAACCGCAGGTGCCTCGAGCCGCTTCGG
PR-21724	<-Lbo_PPTQ_U1_rev	CGTGCGAUTTACTCCTTCTTAGCGTG
PR-21727	<-Onu11_U1_fw	ACTTTTTGCAGTACUAACCGCAGGTCCCCTACGCCACTACC
PR-21728	<-Onu11_U1_rev	CGTGCGAUTTACTTCAGCTTGTCGGCCG
PR-21729	<-EpoE11_U1_fw	ACTTTTTGCAGTACUAACCGCAGGCCCCCAACGTGGAGGAGATC
PR-21730	<-EpoE11_U1_rev	CGTGCGAUTTACTCGTCCTGGGTGGAG
PR-21737	<-CroZ11_U1_fw	ACTTTTTGCAGTACUAACCGCAGGCTCCCAACGTCGAGGAC
PR-21738	<-CroZ11_U1_rev	CGTGCGAUTTACTGCAGCACCTCAGAG
PR-21739	<-CpaE11_U1_fw	ACTTTTTGCAGTACUAACCGCAGGCTCCCAACGTCGAGGAC
PR-21740	<-CpaE11_U1_re	CGTGCGAUTTACTGCAGCACCTCAGAG
PR-23717	Hzead11 (forfusionwith Tefintron)_U1_fw	ACTTTTTGCAGTACUAACCGCAGGCCCAGTCTTACCAGTCTACC
PR-23718	(Hzead11)_U1_rev	CGTGCGAUTTAAGAGGACTTGTCCTTCAC
PR-23719	Msextad11 (forfusionwith Tefintron)_U1_fw	ACTTTTTGCAGTACUAACCGCAGGCTCCCAACTTCGGCAACG
PR-23720	Msextad11_U1_rev	CGTGCGAUTTACTCGTGCACGTCGGTAGAC
PR-14279	PrTefintron_fw	cgtgcgaUagagaccggggtt
PR-15930	PrGPD<-->PrTefintron_rev	acctgcggtUagtactgcaaaaagtgctgg
PR-10595	PrTefYL _fw	cgtgcgaUAGAGACCGGGTTGG
PR-23880	Slit/SexpgFARII_forPrTenintron_fw	ACTTTTTGCAGTACUAACCGCAGGTGGTGCTGACCTCTAA
PR-23881	SlitpgFARII_U2_rev	CACGCGAUCTACTTGATCTTCTCGAGGA
PR-24712	OnuFAR_ZEoptmYL_fw_forTefintron	ACTTTTTGCAGTACUAACCGCAGTCTGCCAAC
PR-24714	OnuFAR_EoptmYL_rev_U2	CACGCGAUCTACATAGAGTAGTCGTAG
PR-24713	OnuFAR_ZoptmYL_rev_U2	CACGCGAUCTAGTCGTAGTTGTTGGCG
PR-14149	TPex20-Tlip2_rev	agctgttcUcagatgcattcttgggcggtc
PR-22075	Tefintron_i->_fw	AgaacagcUAGAGACCGGGTTGGCGGCGC
PR-16595	Har_FAR_codoptYL_U2_rev	CACGCGAUCTACTCGTAGGACTTCTTCTC
PR-21923	PR-21923 (YLFas1<- (for fusion Tefintron)_U1_fw)	ACTTTTTGCAGTACUAACCGCAGTACCCTACCACAGGTGTCAAC
PR-18549	Fas1_Ylip _rev	cgtgcgaUttactgcttggactcataagactc
PR-13337	PrGPD _fw	CACGCGAUgacgcagtaggatgtcctg
PR-13338	PrGPD _rev	ACCTGCACUgttgatgtgtgtttaattc
PR-18548	Fas1_Ylip_fw	AGTGCAGGuATGTACCCTACCACAGGTGTC
PR-18549	Fas1_Ylip _rev	cgtgcgaUttactgcttggactcataagactc
PR-8859	TPex20 _fw	aagtgtggatggggaagtgag
PR-14832	intD-1_check_fw	ACTGGTGGCTACAAATGAAG
PR-22291	IntC_3_check_new_fw	CTAGCTCGGTGGTGGTTC
PR-20880	IntE_4_check_new	ATTGCTAAGCGACCATAGAC
PR-22382	IntF_6_seq_fw_2	AACAGATCGGCAGGC

Table S2. Synthetic genes used in this study

ID	Gene	Sequence 5' to 3'	Source
pBP7893	Lbo_PPTQ	ATGGTGCCTCGAGCCGCTTCGGAGGAGACCGACCTTAAGGAGGCTACCCAGCTTGAGCCCC GAAAGTACGAGATCGTGTACACTAACGTGATCTACTTCACCTATTGGCATATCGCCGGACT GTACGGTCTGTACCTGTGTTTTACCTCCGCTAAATGGGAGACCATCGTGTTCGCTTGGGCT TGGTATGTGCTCGGAGAGCTGGGAGTGATTGCCGGCGCTCATAGATTGTGGGCCCACCGAA CCTACAAGGCAAAGATGCCCCTGCAGATCATCCTGATGCTGTTTAATTGTATCGGTTTTCA GAACACCGCTACCGATTGGGTTCGAGATCACCGAGTGCATCACAAGCACTCTGACACCGAC GCCGACCCCCATAACTCTCAGCGAGGCTTCTTTTTCTCTCACGTGGGCTGGCTGCTGACCCG AAAGCATCGGCTGGTGAAGGAGAAGGGAGAAGCTGTTGACATGACTGATATCTACTCTAA CCCTGTTTTAAGATTCCAGAAGAAGTACTCTCTCCCCCTGATCGGCACTCTTTGCTTTGCTC TGCCCACCCTGCTGCCCGCTTACTGCTGGGGAGAGGCCGTCGGCACCGCTTGGAACATTAAC CTGCTGCGATACTGTCTTAACCTGAACGGAACCTTCCTGACTAACTCCGCCGCTCACAAGTT TGGCTCTAAGCCCTATGACAAGACCATTCCTCCCACCCAGAACTTGCTGGTGTCTTTCATG ACTCTGGGAGAAGGATTTCATAATTACCACCACGTCTTCTCGTGGGACTACCGAGCTGCTG AGCTTGGCAACACCTACCTGAACATGACCACTATCTTTATCGACTTCTTCGCTCTTATTGG ATGGGCCTACGACCTGAAGACCGTTCCTGAGGATGTTATTAAAAAGCGAATGGCCCGAACT	GeneArt

		GGAGATGGTACTAACCTGTGGGGTTGGGGAGACAAGGACATGACCAAGGAGGACGTGGTG GACACCGAGATACGATTCCACGCTAAGAAGGAGTAA	
pBP7895	Onu11	ATGGTCCCCTACGCCACTACCGCCGACGGCCACCCCGAAAAGGACGAGTGCTTCGAGGACA ACGAGATCAAGTCTAATTCTCTACCTAAGCTGGAGATTCTGTACTTTAACGTTATGACCTT CACCTTCCTGCACCTGTCTGCTCTGTACGGTCTGTACCTGGGCTTCACCTCTGTGAAGTGGG CTACCATCGGACTGGGCATCATTTTCTACTTCTTCGCCGAGATCGGAATTACCGCCGGAGC CCATCGACTGTGGTCTCACCGATCTTACAAGGCCAAGTTGCCCTTGGAGATCCTGCTGATG GTGTTCAACTCCATGGCCTTCCAGAACACCGCCCTGTCTTGGGCTCGAGACCATCGAGTTCA CCACAAGTGCCCCGACACCAACGGAGACCCTCATAACGCTAATCGAGGATTCTTCTACTCCC ACGTTGGCTGGTTGATGACCAAGAAGTCTGACGAGGTGATTAAGCAGGGAAAGCTGTGTG ACGTGGCTGACCTGTATTCTAACCCCGTTCTGCGATTCCAGAAGAAGTACGCTGTTCCTTT CATTGGAACTCTGTGCTTCGTCCTGCCCACCCTCATCCCCATGTACTTCTGGGGAGAGACCC TGAACAATGCTTGGCATTTCAACATGTTCCGATACGTGATTAACCTGAACGCCACCTTCTG CGTGAACTCTGTCGTGCACAAGTGGGGATACAAGCCCTACGACAAGAACATCTGTCCCACC CAGAACGTGCTGCTGAACTTGGCTGTTCTGGGTGAGGCCTTTCATAACTACCACCATGTGT TCCCTTGGGACTACCGAGCCGCTGAGCTGGGTAATCAGAAGATGAACCCCACTACTCTGTT CATTGATTTCTTTGCCTGGATTGGCTGGGCCTACGACCTTAAGACTGCCTCCAAGGAGATG ATCAAGTCTCGATCTGAACGAACCGGTGACGGAACCGACCTGTGGGGTCACTCGGCCGACA AGCTGAAGTAA	GeneArt
pBP7896	EpoE11	ATGGCCCCCAACGTGGAGGAGATCGAGACTGACCTCACCGAGACTGAGGAGAAATGGGAGA AGCTGGTGGCTCCCCAGGCCGCCCCTAGAAAGCACGAGATCCTGTACACCAACCTGCTGAT TTTCGGCTACGGACACCTGGCCGGACTGTATGGACTGTACCTGTGTTTCACCTCTGCCCGAC TTCAGACCATCATCCTGGCCTTCATTCTGCACGCCATGGCCATCCTGGGAATTACTGCCGGC GCTCACCGACTGTGGACTCACCGTTCTTACAAGGCCACCATGCCCCTGCAGATTATCCTGAT TATCTTCAACTCTCTGTCCTTTCAAAACTCCGCCATCAACTGGGTCCGAGATCACCGATCTC ACCACAAGTACTGTGACACCGACGCAGATCCTCATAACGCCGCTCGAGGCCTGTTCTACAG CCATATCGGATGGCTGCTGGTGAAGAAGCACCCCGAGGTCAAGAAGCGAGGAAAGATGACC GACATGTCTGATGTGTATCGAAACCCCGTGCTGCGATTCCAGAAGAAGTACGCTGTGCCTT TTATCGGAACCATCTGCTTCGTCCTGCCTACCATCATTCCCATGTACTTCTGGGGCGAGTCC CTGAACAACGCCTGGCATATCACCCTCCTGCGATACATTTTCTCCATGCACACTATTTTCCT GGTGAACTCTGTGGCCCATCTGTGGGGCAACCGACCCTACGACAAGAACATTCTTCCCGCT GACAACCGAACCCTGTCTATCGCCACCCTCGGGGAGGCCTCCCACAACTACCACCACACCTT CCCTTGGGATTACCGGTCCACTGAGCTGGGATACCTGCCCACGAACTTTACCACCAACTTT ATCGACTTCTTCGCCTGGATCGGCTGGGCTTACGACCTGAAGACCACCTCTGGAGAGATCA TCAATTCTAGAATCCAGCGAACCGGCGATGGAACCCATTCTCGATCTAAGAAGAACATCTC CACCCAGGACGAGTAA	GeneArt
pBP7907	CroZ11	ATGGCTCCCAACGTCGAGGACATGGAATCTGACCTGCCTGAGTCTGAGGAAAAGCTCGAGA AGCTGGTGGCTCCCCAGGCTGCTCCCCGAAAGTACCAGATCATCTACACCAACCTGCTGACC TTCGGCTACTGGCACATTGCCGGCCTGTACGGACTGTACCTGTGCTTCACCTCTGCCAAGTG GCAGACCATCATTCTGGCCCTGATCCTGAACGAGATGGCCATTCTGGGCATCACCGCTGGC GCCCACCGACTGTGGGCTCACCGATCTTACAAGGCCACCGTGCCTCTGCAGATCATCCTGAT CATCTTCAACTCCCTGTCTTTCCAGAACTCTGCCATCCACTGGATCCGAGATCACCGAATGC ACCACAAGTACTCTGACACCGACGGCGACCCTCACAACGCCTCTCGAGGCTTCTTCTACTCT CACGTCGGCTGGCTGCTGGTGAAGAAGCACCCCGAGGTCAAGAAGCGAGCCAAGACCATCG ACATGTCTGACATCTACTCTAACCCCATCCTGCGATTCCAGAAGAAGTACGCTATCCCCTTC ATCGGCATGATCTGCTTCGTGCTGCCCACTATTATCCCTATGTACTTCTGGGGCGAGACTC TGTCTAACGCCTGGCACATCACCATGCTGCGATACGTGTTCTCTCTGAACTCTATCTTCCTG GTGAACTCCGCCGCTCACCTGTACGGCTACCGACCTTACGACAAGAACATTCTGCCCGCCGA GAACAAGATGACCTTCATTGCCTGCCTGGGCGAGAACTTCCACAACTACCACCACGTGTTC CCTTGGGACTACCGAGCCTCTGAGCTGGGCAACATCGGAATGAACTGGACCGCCAAGTTCA TCGACTTTTTCGCCTGGATCGGCTGGGCCTACGACCTCAAGACCGCCTCTGACGAGAACAT CAAGTCTCGAATGAAGCGAACCGGCGACGGCACCGACGTGTCTGGACAGAAGTACTCTTGC GAGTCCTCTGAGGTGCTGCAGTAA	GeneArt
pBP7908	CpaE11	ATGGCTCCCAACGTCGAGGACATGGAATCTGACATGCCCGAGTCTGAGAAGTGGGAGAAGC TGGTGGCTCCCCAGGCTGCTCCCCGAAAGTACGAGATCATCTACACCAACCTGCTGACCTTC GGCTACGGCCACATTGCCGGCCTGTACGGACTGTACCTGTGCTTCACCTCTGCCAAGTGGCA GACCGTGATCCTGGCCATCATCCTGAACGAGATGGCCATTCTGGGCATCACCGCTGGCGCCC ACCGACTGTGGTCCCACCGATCTTACAAGGCCGCTGTGCCCCTGCAGATCATTCTGATGATC TTCAACTCTCTGGCCTTCCAGAACTCTGCCATCAACTGGGTGCGAGATCACCGAATGCACC ACAAGTACTCTGACACCGACGGCGACCCTCACAACGCCTCTCGAGGCTTCTTCTACTCTCAC GTCGGCTGGCTGCTGGTGAAGAAGCACCCCGAGGTCAAAAAGCGAGGCAAGATGATCGACA TGAGCGACATCTACTCTAACCCCGTGCTGCGATTCCAGAAGAAGTACGCTATCCCCTTCAT CGGCATGATCTGCTTCGTGCTGCCCACTATTATCCCTATGTACTTCTGGGGCGAGACTCTG	GeneArt

		TCTAACGCCTGGCACATCACCATGCTGCGATACGTGTTCTCTCTGAACTCTATCTTCCTGGT GAACTCCGCCGCTCACCTGTACGGCTACCGACCTTACGACAAGAACATTCTGCCCGCCGAGA ACAAGATCGCCCTGATCGCCTGCCTGGGCGACTCTTTCCACAACTACCACCACGTGTTCCCT TGGGACTACCGAGCCTCTGAGCTGGGCAACATCGGAATGAACTGGACCGCTCAGTTCATCG ACTTTTTCGCCTGGATCGGCTGGGCCTACGACCTCAAGACCGCCTCTGACGAGAACATCAA CTCTCGAATGAAGCGAACCGGCGACGGCACCGACATCTCTGGACAGAAGTACTCTTGCGAG TCCTCTGAGGTGCTGCAGTAA	
pBP8835	Hzead11	ATGGCCCAGTCTTACCAGTCTACCACCGTGCTGTCTGAGGAAAAGGAACTGACCCTGCAGC ACCTGGTGCCTCAGGCTTCGCCCCGAAAGTACCAGATCGTGTACCCCAACCTGATCACCTTC GGCTACTGGCACATTGCCGGCCTGTACGGACTGTACCTGTGCTTCACCTCTGCCAAGTGGG CCACCATCCTGTTCTCTTACATTCTGTTCGTGCTGGCCGAGATCGGCATCACCGCTGGCGCC CACCGACTGTGGGCTCACAAGACCTACAAGGCCAAGCTGCCCCTCGAGATCCTGCTGATGG TGTTCAACTCTATCGCCTTCCAGAACTCTGCCATCGACTGGGTGCGAGATCACCGACTGCA CCACAAGTACTCTGACACCGACGCTGACCCTCACAACGCCTCTCGAGGCTTCTTCTACTCTC ACGTCGGCTGGCTGCTGGTGCGAAAGCACCCCGAGGTGAAGAAGCGAGGCAAGGAACTCAA CATGTCTGACATCTACAACAACCCCGTGCTGCGATTCCAGAAGAAGTACGCTATCCCCTTC ATCGGCGCCGTGTGCTTCGCTCTGCCCACTATGATCCCCGTGTACTTCTGGGGCGAGACTTG GTCTAACGCCTGGCACATCACCATGCTGCGGTACATCATGAACCTGAACGTGACCTTCCTG GTGAACTCTGCTGCCCACATCTGGGGCAACAAGCCCTACGACGCCAAGATTCTGCCCGCTCA GAACGTGGCCGTGTCTGTGGCCACCGGCGGAGAGGGCTTCCACAACTACCACCACGTGTTC CCTTGGGACTACCGAGCCGCCGAGCTGGGCAACAACTCTCTGAACCTGACCACCAAGTTCA TCGACCTGTTCGCCGCCATCGGCTGGGCCTACGACCTCAAGACCGTGTCCGAGGACATGATC AAGCAGCGAATCAAGCGAACCGGCGACGGCACCGACCTCTGGGGCCACGAGCAGAACTGCG ACGAGGTGTGGGACGTGAAGGACAAGTCCTCTTAA	GeneArt
pBP8836	Msextad11	ATGGCTCCCAACTTCGGCAACGAGGTGTCGTCGCCCATCGTGGCCGAGGAATCTTACGAGA AGCTGATCCCTCCTCAGGCTGCTCCCCGAAAGTACAAGTACCTGTACGCCAACATGATCTA CTTCACCTACTGGCACATTGCCGGCCTGTACGGCATCTACCTGGCCATCACCACCGCCAAGT GGGCCACCATCATCCTGGCCTACCTGCTGTTCGTGGCCGGCGAGATCGGCATCACCGCTGGC GCCCACCGACTGTGGGCTCACAAGTCTTACAAGGCCAAGCTGCCCCTGCAGATCCTGCTGA TGCTGTTCAACTCTACCGCCTTCCAGAACTCTGTGATCACCTGGGTGAAGGACCACCGAAT GCACCACAAGTACTCTGACACCGACGCTGACCCTCACAACGCTACCCGAGGCTTCTTCTACT CTCACGTCGGCTGGCTGATGGTGAAGCGACACCCCGAGGCCATCAAGCGAGGCAAGTCTCT GGACATGTCTGACATCTACAACAACCCCGTGCTGAAGTTCCAGAAGAAGTACGCTATTCCC CTGATTACCACCGTGGCCTTCGTGCTGCCCACTATTATCCCTATGTACTTCTGGGACGAGTC TTTCAACGTGGCCTGGCACATGACCATGCTGCGGTACATCATCAACCTGAACACCATCTTT CTGGTGAACTCTGTCGCTCACATGTGGGGCTACAAGCCCTACGACAAGAACATTGCTCCCA CTCAGAACTACATTGCCACCTTCGCTACCCTCGGCGAGGGCTTCCACAACTACCACCACGCA TTCCCTTGGGACTACCGAGCCTCTGAGCTGGGCAACAACTACCTGAACCTGACTACCAAGT TCATCGACTTTTTCGCCTGGATCGGCTGGGCCTACGACCTCAAGACCGTGCCTGAGGACCT GCTGCAAAAGCGAATGGAACGAACCGGCGACGGCACCAACCTGTGGGGCCGAGGCGATAAG AACATGAAGAAGGACTACGTCAAGTCTACCGACGTGCACGAGTAA	GeneArt
pCfB5547	HarFAR	ATGGTGGTCCTGACCTCTAAGGAGACTAAGCCCTCCGTGGCCGAGTTCTACGCTGGCAAGT CTGTCTTCATCACCGGCGGAACCGGTTTCCTGGGCAAGGTCTTCATTGAGAAGCTGCTGTA CTCCTGTCCCGACATCGGCAACATCTACATGCTGATCCGAGAGAAGAAGGGACTGTCTGTG TCCGAGCGAATTAAGCACTTCCTGGACGACCCCCTGTTCACCCGACTGAAGGAGAAGCGAC CCGCCGACCTGGAGAAGATCGTGCTGATTCCCGGAGACATCACCGCTCCCGACCTGGGTAT TACCTCTGAGAACGAGAAGATGCTGATCGAGAAGGTGTCTGTCATCATTCACTCCGCCGCT ACCGTCAAGTTCAACGAGCCCCTGCCCACCGCCTGGAAGATCAACGTGGAGGGAACCCGAA TGATGCTGGCTCTGTCTCGACGAATGAAGCGAATTGAGGTCTTCATCCACATTTCCACCGC CTACACCAACACCAACCGAGAGGTGGTGGACGAGATCCTGTACCCTGCTCCTGCTGACATT GACCAGGTGCACCGATACGTCAAGGACGGTATCTCTGAGGAAGAGACTGAGAAGATTCTGA ACGGCCGACCCAACACCTACACCTTCACCAAGGCCCTGACCGAGCACCTGGTGGCTGAGAAC CAGGCTTACGTGCCCACCATCATTGTCCGACCCTCCGTGGTCGCCGCTATCAAGGACGAGCC CATTAAGGGATGGCTGGGTAACTGGTACGGAGCTACCGGACTGACCGTGTTCACCGCTAAG GGTCTGAACCGAGTCATCTACGGCCACTCTTCCAACATCGTGGACCTGATTCCCGTGGACT ACGTCGCCAACCTGGTCATTGCCGCTGGCGCTAAGTCTTCCAAGTCCACCGAGCTGAAGGT GTACAACTGTTGCTCTTCCGCCTGCAACCCCATCACCATTGGAAAGCTGATGTCTATGTTC GCCGAGGACGCTATCAAGCAGAAGTCCTACGCTATGCCCCTGCCCGGTTGGTACATCTTCA CCAAGTACAAGTGGCTGGTCCTGCTGCTGACCATTCTGTTCCAGGTCATCCCCGCCTACATT ACCGACCTGTACCGACACCTGATCGGCAAGAACCCCCGATACATTAAGCTGCAGTCTCTGG TCAACCAGACCCGATCTTCCATTGACTTCTTCACCTCTCACTCCTGGGTCATGAAGGCTGAC CGAGTCCGAGAGCTGTTCGCCTCTCTGTCCCCCGCTGACAAGTACCTGTTCCCCTGTGACCC CACCGACATCAACTGGACCCACTACATTCAGGACTACTGCTGGGGAGTGCGACACTTCCTG GAGAAGAAGTCCTACGAGTAG	GenScript ,[16]
pBP8892	SlitpgFARI I	ATGGTGGTGCTGACCTCTAAGGAAAAGTCTAACATGTCTGTGGCCGACTTCTACGCCGGCA AGTCTGTGTTCATTACCGGCGGCACCGGATTCCTCGGCAAGGTGTTCATCGAGAAGCTGCT GTACTCTTGCCCCGACATCGACAAGATCTACATGCTGATCCGAGAGAAGAAGGGCCAGTCT ATCCGAGAGCGACTGACCAAGATCGTGGACGACCCTCTGTTCAACCGACTGAAGGACAAGC GACCCGGCGACCTGGGAAAGATCGTGCTGATCCCCGGCGACATCACCGTGCCTGGCCTGGG CATCTCTGAGGAAAACGAGACTATCCTGACCGAGAAGGTGTCTGTGGTGATCCACTCTGCC GCCACCGTGAAGTTCAACGAGCCCCTGGCCACCGCCTGGAACGTGAACGTCGAGGGCACCC GAATGATCATGGCCCTGTCTCGACGAATGAAGCGAATCGAGGTGTTTATCCACATCTCTAC CGCCTACACCAACACCAACCGAGCCGTGATCGACGAGGTGCTGTACCCTCCTCCTGCCGACA TCAACGACGTGCACCAGCACGTGAAGAACGGCGTGACCGAGGAAGAGACTGAGAAGATCCT	GeneArt

		GAACGGACGACCCAACACCTACACCTTCACCAAGGCTCTGACCGAGCACCTGGTGGCCGAG AACCAGTCTTACATGCCCACCATCATCGTGCGACCCTCTATCGTGGGCGCCATCAAGGACG ACCCCATCCGAGGCTGGCTGGCCAACTGGTACGGCGCCACCGGCCTGTCCGTGTTCACCGCC AAGGGCCTGAACCGAGTGATCTACGGCCACTCTAACCACGTCGTGGATCTGATTCCCGTGG ACTACGTGGCCAACCTGGTGATCGTGGCTGGCGCCAAGACCTACCACTCCAACGAGGTGAC CATCTACAACTCTTGTTCTTCTTCTTGCAACCCCATCACCATGAAGCGACTGGTGGGCCTGT TCATTGACTACACCGTCAAGCACAAGTCTTACGTGATGCCCCTGCCTGGCTGGTACGTGTA CTCTAACTACAAGTGGCTGGTGTTCCTGGTGACCGTGATCTTCCAGGTGATCCCCGCCTACC TGGGCGACATCGGCCGACGACTGCTGGGCAAGAACCCTCGGTACTACAAGCTGCAGAACCT GGTCGCTCAGACCCAAGAGGCCGTGCACTTCTTCACCTCTCACACCTGGGAGATTAAGTCT AAGCGAACCTCTGAGCTGTTCTCTTCTCTGTCCCTGACCGACCAGCGAATGTTCCCCTGCGA CGCCAACCGAATCGACTGGACCGACTACATCACCGACTACTGCTCTGGCGTGCGACAGTTCC TCGAGAAGATCAAGTAG	
pBP9205	OnuFAR_E	ATGTCTGCCAACACCATGGAAACCGACGAGCAGTTCACCTACAACTCTCCCATCGTGAACT TCTACTCTGGCAAGTCTGTGTTCGTGACCGGCGCTACCGGCTTCCTGGGCACCGTGCTGGTC GAGAAGCTGCTGTTCTCTTGCAAGGGCATCAACAACATCTACATCCTGATCAAGCAGACCG AGGACCTGACCATCGAGGCCCGAATCCTGAACTACCTGAACTCTAAGGCCTTCCACCGAGT GAAGAACACTAACCCCGAGCTGATGAAGAAGATCATCCCCATCTGCGGCAACCTCGAGGAC AAGAACCTGGGCATCTCTGACTCTGACATGAAGACCCTGCTGGAAGAGGTGTCTATCGTGT TCCACGTGGCCGCCAAGCTGCTCTTTAAGATGTCTCTGACCGCCGCTGTGAACATCAACACC AAGCCTACCGAGCAGCTGATCGCCATCTGCAAGAAGATGCGACGAAACCCCATCTTCATCT ACGTGTCCTCTGCCTACTCTAACGTGAACGAGCAGATCATCGACGAGAAGGTGTACAACAC CGGCGTGCCCCTCGAGACTATCTACGACACCCTGGACACCGAGAACACCCGAATTACCGAC ATCTTCCTGGACAAGCGACCCAACACCTACACCTACTCCAAGGCTCTGGCCGAGGTGGTCG TCGAGAAGGAATTCGACGAGTCTGCCGCCATCGTGCGACCCTCTATCATCGTGTCCTCCAT TCGAGAGCCCATTCCAGGCTGGCTGTCTGGCTCTCACGGCTTCCCTCGAGTGGTGGGAGCCG CCTGCAAGGGCCTGCTGCTCCGATGGCACGGCGACGGCACCGTGGTGTGCGACCTGATTCCT GTGGACCACGTCGCCAACCTGATCATTGCCGCCGCTTGGGAGTCTAACGAGCGACGACTGA TGGGCAACAAGGGCGTGAAGGTCTACAACTGTTGTTCTTCTCTGCGAAACCCTATCGACGT GATCACCGTGGTCAAGACCTGCATCAAGTACCGAAAGTACTTCGGCACCCGAACCATGTCT ATCTTCACCCCTCGATTCATCATGAAGAAGAACTACTTCATCTACAAGCTGCTGTACTTCA CCTACCACACTATCCCCGCTGCTATTATCGACGGCTTCTTCTGGCTGACCGGACGAACCCCT ATCATGCTCAAGACCCTCGACAAGCTGGGCAAGATCTCTTCTGTGCTCGAGTACTTTACCC ACCACCAGTTCATCTTTCTGGACTCCAACGTGCGAGGACTGCTGCGACGAATGGAAGGCAC CGACCGACAGACCTTCAACTTCGACGTGACCGAGATCGAGTGGGAGCCCTACCTGCAGAAC TTCGTGCGAGGCATTGCCAACAACTACGACTACTCTATGTAG	GeneArt
pBP9206	OnuFAR_Z	ATGTCTGCCAACACCATGGAAACCGACGAGCAGTTCACCTACAACTCTCCCATCGTGAACT TCTACTCTGGCAAGTCTGTGTTCGTGACCGGCGCTACCGGCTTCCTGGGCACCGTGCTGGTC GAGAAGCTGCTGTTCTCTTGCAAGGGCATCAACAACATCTACATCCTGATCAAGCAGACCG AGGACCTGACCATCGAGGCCCGAATCCTGAACTACCTGAACTCTAAGGCCTTCCACCGAGT GAAGAACACTAACCCCGAGCTGATGAAGAAGATCATCCCCATCTGCGGCAACCTCGAGGAC AAGAACCTGGGCATCTCTGACTCTGACATGAAGACCCTGCTGGAAGAGGTGTCTATCGTGT TCCACCTGGCCGCCAAGCTGCTCTTTAAGATGTCTCTGGCCGCTGCCGTGAACATCAACACC AAGTCTACCGAGCAGCTGATCGCCATCTGCAAGAAGATGCGACGAAACCCCATCTTCATCT ACGTGTCCTCTGCCTACTCTAACGTGAACAAGCAGATCATCGACGAGAAGGTGTACTCTAC CGGCGTGCCCCTCGAGACTATCTACGACACCCTGGACGCCAAGAACACCCGACTGATGGAC ATCTTCCTGGACAAGCGACCCAACACCTACACCTACTCCAAGGCTCTGGCCGAGGTCCTGGT TGAGAACGAGTTCGACGAGTCTGCCGCCATCGTGCGACCCTCTATCATTGCCTCTTCTATTC GAGAGCCCATTCCTGGCTGGCTGTCTGGCTCTCACGGCTTCCCTCGAGTGGTCGAGGCCGCC TGCAAGGGCCTGCTGCTCCGATGGCACGGCGACGGCACCGTGGCCTTCGGCATCATCCCCGT GGACCACGTGGCCAACCTGATCATTGCCGCCGCTTGGGAGTCTAACGAGCGACGACTGATC GGCAACAAGGGCGTGAAGGTCTACAACTGCTGCTCTGGCCTGCGAAACCCTATCGACGTGT CTACCGTGATGAACACCTGTCTGAAGTACCGAAAGTACTTCGGCACCCGAACCATGTCTAT CATCACCCCTCGATTCATTATGAAGAAGAACTACTTCCTGTACAAGCTGCTGTACTTCACC TACCACACTATCCCCGCTGCTATTATCGACGGCTTCTTCTGGCTGACCGGACGAACCCCTAT GATGCTGAACACCCTGCACAAGCTCCGAAAGCTGTCCTCTGTGCTCGAGTACTTTACCCTG CGACAGTTCCTGTTCCTGGACTCCAACGTGCGAGGACTGCTGCGACGAATGGAAGGCACCG ACCGACAGACCTTCAACTTCGACGTGACCGAGATCGAGTGGGAGCCCTTCCTGCAGAACTG TGTGCGAGGAATCGCCAACAACTACGACTAG	GeneArt

Table S3. Biobricks used in this study

ID	Description	Fw_primer	Rv_primer	Template DNA	Reference
BB1135	Easy Clone vector backbone	See ref.	See ref.	See ref.	[19]
BB1631	TPex20-TLip2	See ref.	See ref.	See ref.	[19]
BB1635	PrtRNA-Gly	See ref.	See ref.	See ref.	[19]
BB1636	crRNA-TRPR	See ref.	See ref.	See ref.	[19]
BB8388	IntF_6_Up	PR-22536	PR-22537	Genomic DNA of ST4840	This study
BB8389	IntF_6_Down	PR-22538	PR-22539	Genomic DNA of ST4840	This study
BB2719	{-PrTefintron_USER	PR-18928	PR-18214	Genomic DNA of ST4840	This study
BB2693	{-Lbo_PPTQ	PR-21723	PR-21724	pBP7893	This study
BB2695	{-Onu11_forPrTefin	PR-21727	PR-21728	pBP7895	This study

BB2696	<-EpoE11	PR21729	PR-21730	pBP7896	This study
BB2700	<-CroZ11	PR-21737	PR-21738	pBP7907	This study
BB2701	<-CpaE11	PR-21739	PR-21740	pBP7908	This study
BB8834	Hzead11{-for fusionwithPrTefintron	PR-23717	PR-23718	pBP8835	This study
BB8835	Msextad11{-for fusionwithPrTefintron	PR-23719	PR-23720	pBP8836	This study
BB1688	->PrTefintron	PR-14279	PR-15930	pCfB3465	This study
BB1740	Har_FAR_codoptYL	See ref.	See ref.	See ref.	[16]
BB2093	PrTEFintron_USER-}	PR-10595	PR-18214	pCfB3516	This study
BB8906	SlitpgFARII_forPrTefintron	PR-23880	PR-23881	pBP8892	This study
BB9247	OnuFAR_EoptmYL	PR-24712	PR-24714	pBP9205	This study
BB9248	OnuFAR_ZoptmYL	PR-24712	PR-24713	pBP9206	This study
BB8212	PrTefintron->HarFAR-Tlip	PR-15095	PR-14149	pBP7980	This study
BB8213	i_PrTefintron->HarFAR	PR-22075	PR-16595	pBP7980	This study
BB7970	YLFas1<- (for fusion Tefintron)	PR-21923	PR-18549	pCfB6809	This study
BB1244	{-PrGPD	PR-13337	PR-13338	Genomic DNA of ST4840	This study
BB2134	Fas1_Ylip	PR-18548	PR-18549	Complementary DNA of ST3683	This study

Table S4. Plasmids used in this study

ID	Description	Parent plasmid	Biobricks/Primers	Reference
pCfB3405	pORI1001-Nat-CEN1-USER	See ref.	See ref.	[19]
pCfB3431	pORI1001-Hphsyn-CEN1-USER	See ref.	See ref.	[19]
pCfB4783	pIntE_3-Nat-TPex20-TLip	See ref.	See ref.	[19]
pCfB6630	pNat-YLgRNA3_IntC_3	See ref.	See ref.	[19]
pCfB6631	pNat-YLgRNA2_IntD_1	See ref.	See ref.	[19]
pCfB6638	pNat-YLgRNA2_IntE_4	See ref.	See ref.	[19]
pCfB7088	pNat-YLgRNA1_Fas2 (AA1220)	See ref.	See ref.	[19]
pBP8033	pHph-YLgRNA2_IntD_1	pCfB3431	BB1635, BB1636, PR-18241, PR-18242	This study
pBP8646	pHph_YLgRNA3_IntF_6	pCfB3431	BB1635, BB1636, PR-23129, PR-23130	This study
pCfB6684	pIntD_1-TPex20-TLip2	See ref.	See ref.	[19]
pCfB6371	pIntC_3-TPex20-TLip2	See ref.	See ref.	[19]
pCfB6679	pIntE_4-TPex20-TLip2	See ref.	See ref.	[19]
pBP8264	IntF_6_Up_TPex20-USER-TLip2_IntF_6_Down	-	BB1135, BB8388, BB1631, BB8389	This study
pBP7912	pIntD_1-TPex20-Lbo_PPTQ	pCfB6684	BB2719, BB2693	This study
pBP7914	pIntD_1-TPex20-Onu11	pCfB6684	BB2719, BB2695	This study
pBP7915	pIntD_1-TPex20-EpoE11	pCfB6684	BB2719, BB2696	This study
pBP7919	pIntD_1-TPex20-CroZ11	pCfB6684	BB2719, BB2700	This study
pBP7920	pIntD_1-TPex20-CpaE11	pCfB6684	BB2719, BB2701	This study
pBP8839	pIntD_1-TPex20-Hzead11{-PrTefintron-TLip2	pCfB6684	BB2719, BB8834	This study
pBP8840	pIntD_1-TPex20-Msextad11{-PrTefintron-TLip2	pCfB6684	BB2719, BB8835	This study
pBP7980	pIntC_3-Tefintron-> HarFAR	pCfB6371	BB1688, BB1740	This study
pBP8898	pIntC_3-PrTefintron-}SlitpgFARII	pCfB6371	BB2093, BB8906	This study
pBP9336	pIntC_3-Tefintron_OnuFAR_EoptmYL	pCfB6371	BB2093, BB9247	This study
pBP9337	pIntC_3-Tefintron_OnuFAR_ZoptmYL	pCfB6371	BB2093, BB9248	This study
pBP8071	pIntE_4-TPex20-Tefintron-HarFAR-Tlip-PrTefintron->HarFAR-Tlip	pCfB6679	BB8212, BB8213	This study
pBP8680	pIntC_3-TPex20-Lbo_PPTQ_PrTefIntron_TLip2	pCfB6371	BB2719, BB2693	This study
pBP8655	pIntF_6-YlFAS1{-PrTefintron	pBP8264	BB7970, BB2719	This study
pCfB6809	pIntE_3-Nat-TPex20-Pr-Fas1_Ylip-TLip2	pCfB4783	BB1244, BB2134	
pCfB3465	See ref.	See ref.	See ref.	[16]
pCfB 3516	See ref.	See ref.	See ref.	[16]

Table S5. Strains used in this study

ID	Genotype	Parent strain	Added elements	Reference/source

ST4840 (Y-17536)	Wild-type *Yarrowia lipolytica*	N/A	N/A	Agricultural Research Service (NRRL, USA),
ST6629	MATa ku70Δ Cas9 hfd4Δ hfd1Δ pex10Δ fao1Δ hfd2Δ hfd3Δ GPAT_100bpPr	See ref.	See ref.	[16]
ST7982	MATa ku70Δ Cas9 hfd4Δ hfd1Δ pex10Δ fao1Δ hfd2Δ hfd3Δ GPAT_100bpPr Fas2p^{I1220F}	ST6629	pCfB7088, BB2313	This study
ST9992	MATa ku70Δ Cas9 hfd4Δ hfd1Δ pex10Δ fao1Δ hfd2Δ hfd3Δ GPAT_100bpPr Fas2p^{I1220F} IntD_1::TPex20-Lbo_PPTQ<-PrTefintron-TLip2	ST7982	pB8033, pBP7912	This study
ST9993	MATa ku70Δ Cas9 hfd4Δ hfd1Δ pex10Δ fao1Δ hfd2Δ hfd3Δ GPAT_100bpPr Fas2p^{I1220F} IntD_1::TPex20-Onu11<-PrTefintron-TLip2	ST7982	pB8033, pBP7914	This study
ST9994	MATa ku70Δ Cas9 hfd4Δ hfd1Δ pex10Δ fao1Δ hfd2Δ hfd3Δ GPAT_100bpPr Fas2p^{I1220F} IntD_1::TPex20-EpoE11<-PrTefintron-TLip2	ST7982	pB8033, pBP7915	This study
ST9995	MATa ku70Δ Cas9 hfd4Δ hfd1Δ pex10Δ fao1Δ hfd2Δ hfd3Δ GPAT_100bpPr Fas2p^{I1220F} IntD_1::TPex20-CroZ11<-PrTefintron-TLip2	ST7982	pB8033, pBP7919	This study
ST9996	MATa ku70Δ Cas9 hfd4Δ hfd1Δ pex10Δ fao1Δ hfd2Δ hfd3Δ GPAT_100bpPr Fas2p^{I1220F} IntD_1::TPex20-CpaE11<-PrTefintron-TLip2	ST7982	pB8033, pBP7920	This study
ST10142	MATa ku70Δ Cas9 hfd4Δ hfd1Δ pex10Δ fao1Δ hfd2Δ hfd3Δ GPAT_100bpPr Fas2p^{I1220F} IntD_1::TPex20-Hzead11<-PrTefintron-TLip2	ST7982	pB8033, pBP8839	This study
ST10143	MATa ku70Δ Cas9 hfd4Δ hfd1Δ pex10Δ fao1Δ hfd2Δ hfd3Δ GPAT_100bpPr Fas2p^{I1220F} IntD_1::TPex20-Msextad11<-PrTefintron-TLip2	ST7982	pB8033, pBP8840	This study
ST8524	MATa ku70Δ Cas9 hfd4Δ hfd1Δ pex10Δ fao1Δ hfd2Δ hfd3Δ GPAT_100bpPr IntC_3::TPex20-PrTefintron->HarFAR-TLip2	ST6629	pCfB6630, pBP7980	This study
ST10152	MATa ku70Δ Cas9 hfd4Δ hfd1Δ pex10Δ fao1Δ hfd2Δ hfd3Δ GPAT_100bpPr IntC_3::TPex20-PrTefintron->SlitpgFARII-TLip2	ST6629	pCfB6630, pBP8898	This study
ST10153	MATa ku70Δ Cas9 hfd4Δ hfd1Δ pex10Δ fao1Δ hfd2Δ hfd3Δ GPAT_100bpPr IntC_3::TPex20-PrTefintron->OnuFARE-TLip2	ST6629	pCfB6630, pBP9336	This study
ST10154	MATa ku70Δ Cas9 hfd4Δ hfd1Δ pex10Δ fao1Δ hfd2Δ hfd3Δ GPAT_100bpPr IntC_3::TPex20-PrTefintron->OnuFARZ-TLip2	ST6629	pCfB6630, pB9337	This study
ST10155	MATa ku70Δ Cas9 hfd4Δ hfd1Δ pex10Δ fao1Δ hfd2Δ hfd3Δ GPAT_100bpPr Fas2p^{I1220F} IntD_1::TPex20-Lbo_PPTQ<-PrTefintron-TLip2 IntC_3::TPex20-PrTefintron->HarFAR-TLip2	ST9992	pCfB6630, pBP7980	This study
ST10156	MATa ku70Δ Cas9 hfd4Δ hfd1Δ pex10Δ fao1Δ hfd2Δ hfd3Δ GPAT_100bpPr Fas2p^{I1220F} IntD_1::TPex20-Lbo_PPTQ<-PrTefintron-TLip2 IntC_3::TPex20-PrTefintron->SlitpgFARII-TLip2	ST9992	pCfB6630, pBP8898	This study
ST10157	MATa ku70Δ Cas9 hfd4Δ hfd1Δ pex10Δ fao1Δ hfd2Δ hfd3Δ GPAT_100bpPr Fas2p^{I1220F} IntD_1::TPex20-Lbo_PPTQ<-PrTefintron-TLip2 IntC_3::TPex20-PrTefintron->OnuFARE-TLip2	ST9992	pCfB6630, pBP9336	This study
ST10158	MATa ku70Δ Cas9 hfd4Δ hfd1Δ pex10Δ fao1Δ hfd2Δ hfd3Δ GPAT_100bpPr Fas2p^{I1220F} IntD_1::TPex20-Lbo_PPTQ<-PrTefintron-TLip2 IntC_3::TPex20-PrTefintron->OnuFARZ-TLip2	ST9992	pCfB6630, pB9337	This study
ST8225	MATa ku70Δ Cas9 hfd4Δ hfd1Δ pex10Δ fao1Δ hfd2Δ hfd3Δ GPAT_100bpPr Fas2p^{I1220F} IntE_4::Tpex20-PrTefintron->HarFAR-TLip2-PrTefintron->HarFAR-TLip2	ST7982	PCfB6638, pBP8071	This study
ST8373	MATa ku70Δ Cas9 hfd4Δ hfd1Δ pex10Δ fao1Δ hfd2Δ hfd3Δ GPAT_100bpPr Fas2p^{I1220F} IntE_4::Tpex20-PrTefintron->HarFAR-TLip2-PrTefintron->HarFAR-TLip2 IntD_1::TPex20- Lbo_PPTQ<-PrTefintron-TLip2	ST8225	pCfB6631, pBP7912	This study
ST9136	MATa ku70Δ Cas9 hfd4Δ hfd1Δ pex10Δ fao1Δ hfd2Δ hfd3Δ GPAT_100bpPr Fas2p^{I1220F} IntE_4::Tpex20-PrTefintron->HarFAR-TLip2-PrTefintron->HarFAR-TLip2 IntD_1::TPex20- Lbo_PPTQ<-PrTefintron-TLip2 IntC_3::TPex20- Lbo_PPTQ<-PrTefintron-TLip2	ST8373	pCfB6630, pBP8680	This study
ST9253	MATa ku70Δ Cas9 hfd4Δ hfd1Δ pex10Δ fao1Δ hfd2Δ hfd3Δ GPAT_100bpPr Fas2p^{I1220F} IntE_4::Tpex20-PrTefintron->HarFAR-TLip2-PrTefintron->HarFAR-TLip2 IntD_1::TPex20- Lbo_PPTQ<-PrTefintron-TLip2	ST9136	pBP8655, pBP8646	This study

	IntC_3::TPex20- Lbo_PPTQ<-PrTefintron-TLip2 IntF_6::TPex20- YlFAS1<-PrTefintron-TLip2			

Table S6. Composition of transformation mix

Component	mix for 1x transformation
PEG (Stock 50%; sterile-filtrated);	83.5 µl
LiAc (Stock 2M; sterile-filtrated; end 0.1 M)	5.0 µl
ssDNA (Stock 10 mg/ml; end 0.25 g/l)	2.5 µl
DTT (stock 2M; sterile-filtrated; end 100 mM)	5.0 µl
EDTA 0.05 M	2 µl
Tris-HCl 0.5 M pH 8	2 µl

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CHAPTER 5

Biosynthesis of insect sex pheromone precursors via engineered β-oxidation in yeast

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Research Article

Biosynthesis of insect sex pheromone precursors via engineered β-oxidation in yeast

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One sentence summary: The authors engineered the yeast *Yarrowia lipolytica* for production of insect sex pheromones precursors.
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Abstract

Mating disruption with insect sex pheromones is an attractive and environmentally friendly technique for pest management. Several Lepidoptera sex pheromones have been produced in yeast, where biosynthesis could be accomplished by the expression of fatty acyl-CoA desaturases and fatty acyl-CoA reductases. In this study, we aimed to develop yeast *Yarrowia lipolytica* cell factories for producing Lepidoptera pheromones which biosynthesis additionally requires β-oxidation, such as (Z)-7-dodecenol (Z7-12:OH), (Z)-9-dodecenol (Z9-12:OH), and (Z)-7-tetradecenol (Z7-14:OH). We expressed fatty acyl-CoA desaturases from *Drosophila melanogaster* (Dmd9) or *Lobesia botrana* (Lbo_PPTQ) and fatty acyl-CoA reductase from *Helicoverpa armigera* (HarFAR) in combinations with 11 peroxisomal oxidases of different origins. Yeast cultivations were performed with supplementation of methyl myristate (14:Me). The oxidase Lbo_31670 from *L. botrana* provided the highest titers of (Z)-7-dodecenoate, (Z)-9-dodecenoate, and (Z)-7-tetradecenoate. However, no chain-shortened fatty alcohols were produced. The mutation of fatty acid synthase ($Fas2p^{I1220F}$) to increase myristate production did not lead to targeted fatty alcohol production. The problem was solved by directing the reductase into peroxisomes, where the strain with Dmd9 produced 0.10 ± 0.02 mg/l of Z7-12:OH and 0.48 ± 0.03 mg/l of Z7-14:OH, while the strain with Lbo_PPTQ produced 0.21 ± 0.03 mg/l of Z9-12:OH and 0.40 ± 0.07 mg/l of Z7-14:OH. In summary, the engineering of β-oxidation in *Y. lipolytica* allowed expanding the portfolio of microbially produced insect sex pheromones.

Keywords: β-oxidation, insect pheromones, *Yarrowia lipolytica*, fatty alcohols, fatty acids, peroxisomal oxidases

Introduction

Lepidoptera is the second largest order in the Insecta class and includes moths and butterflies (Wahlberg et al. 2013). Lepidopteran sex pheromones are fatty acid-derived metabolites that are biosynthesized and released by females to attract conspecific males for reproduction. Most of these pheromones are C_{10}–C_{18} straight chain hydrocarbons with one to three double bonds and an oxygen-containing terminal group (alcohol, aldehyde, and alcohol acetate; Ando et al. 2004). The use of these oleochemicals is recognized as an environmentally friendly and effective method for pest control in agriculture. Their potential has been shown in several mating disruption studies, where the application of specific pheromone helped to reduce insect infestation (Alfaro et al. 2009, Hummel et al. 2015, Ioriatti and Lucchi 2016). Currently, chemical synthesis is the primary source of pheromones. However, in recent years, microbial and plant-based production has also been developed (Ding et al. 2014, Hagström et al. 2013a, Holkenbrink et al. 2020, Jiang et al. 2021, Petkevicius et al. 2021, Xia et al. 2020).

The fatty acid metabolism of yeast and plants can be engineered to enable the biosynthesis of pheromones and their precursors. Modifications needed to redirect common fatty acids, such as palmitate and stearate, toward pheromone biosynthesis include fatty acid desaturation, reduction, chain-shortening, alcohol acetylation, or oxidation (Petkevicius et al. 2020). A continuously growing list of sequenced insect genomes and transcriptomes facilitates enzyme discovery and characterization (Ding et al. 2014, 2021, Ding and Löfstedt 2015, Lassance et al. 2021). Fatty acyl-CoA desaturases (FADs) and fatty acyl-CoA reductases (FARs) are the most studied groups of enzymes related to pheromone biosynthesis. More than 50 FADs and 20 FARs from various insects have been characterized (Tupec et al. 2017). FADs introduce a double bond into a hydrocarbon chain while FARs are converting fatty acyl-CoAs into corresponding alcohols. Metabolic engineering efforts in yeast previously allowed to obtain *Saccharomyces cerevisiae* and *Yarrowia lipolytica* strains capable of producing insect pheromones and their precursors such as (Z)-9-tetradecenyl acetate (Z9-14:OAc), (E/Z)-11-tetradecenol (E/Z11-14:OH), (Z)-11-hexadecenol (Z11-16:OH), and (Z)-11-hexadecenal (Z11-16:Ald). Hagström et al. 2013b, Holkenbrink et al. 2020, Jiang et al. 2021, Petkevicius et al. 2021). The plants *Nicotiana benthamiana*, *N. tabacum*, and *Camelina sativa* have been used for production of Δ11 C_{14} and C_{16} fatty acid derivatives as well (Ding et al. 2014, Mateos-Fernández et al. 2021, Nešněrová et al. 2004, Ortiz et al. 2020). Additionally, a recent study in *C. sativa* demonstrated the production of a more challenging pheromone precur-

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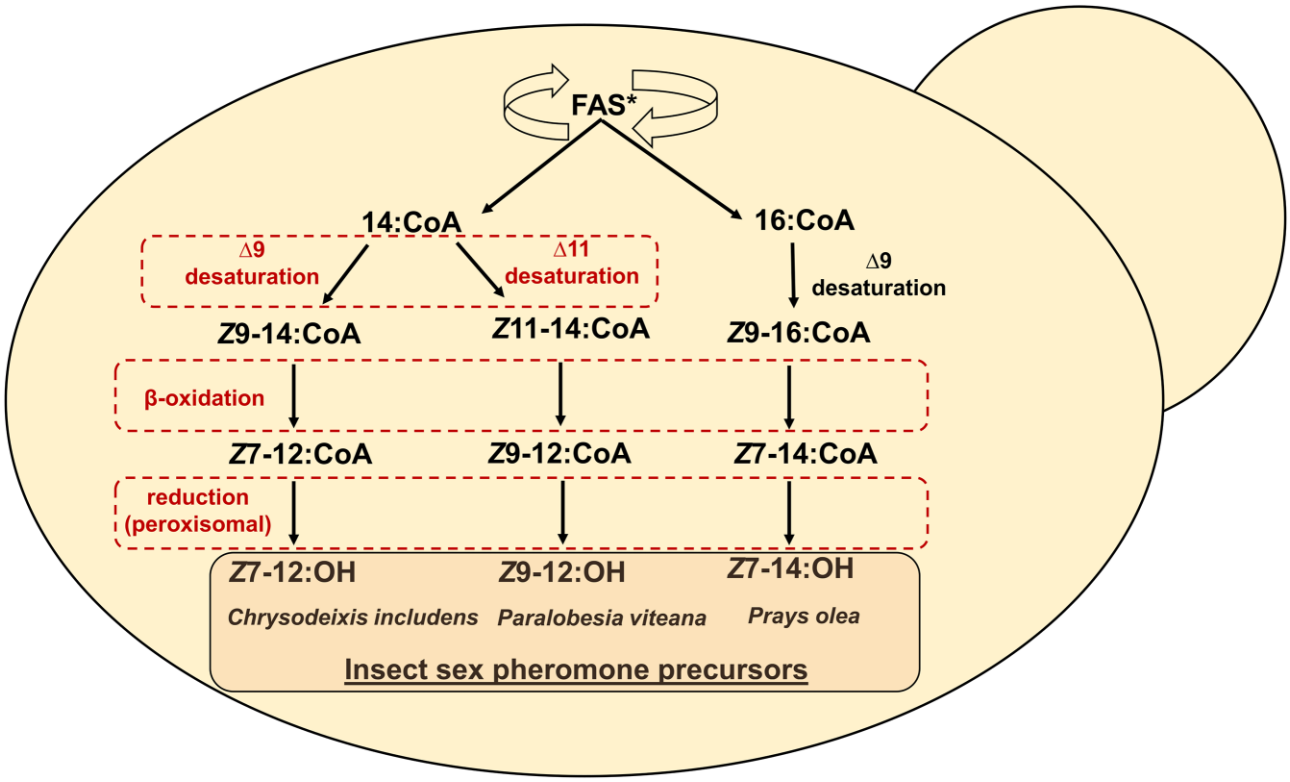

Figure 1. Schematic representation of metabolic pathways leading to three target insect sex pheromone precursors. Red color indicates steps, which were engineered in this study. FAS*: fatty acid synthase, where isoleucine was replaced to phenylalanine in the α chain ($Fas2p^{I1220F}$) for increased production of 14:CoA.

sor of the codling moth *Cydia pomonella*-(*E*,*E*)-8,10-dodecadienoic acid (Xia et al. 2021). Our previous studies showed that strategies related to decreased degradation of fatty acids/alcohols and improved acyl-CoA supply are beneficial for the biosynthesis of C_{14} and C_{16} pheromones (Holkenbrink et al. 2020, Petkevicius et al. 2021).

Compared to fatty acid desaturation and reduction, chain-shortening, alcohol acetylation, and oxidation are underexplored, and information about enzymes performing these reactions in moths is scarce (Petkevicius et al. 2020). For the insects to produce pheromones of a specific carbon length, the oxidation of fatty acids needs to terminate after one to three β-oxidation cycles generating acyl-CoA of 10–14 carbons. This contrasts with the metabolic oxidation that proceeds to completion and generates acetyl-CoA. One β-oxidation cycle is composed of four reactions that take place in peroxisomes. Each cycle shortens the fatty acyl chain by two carbons and releases acetyl-CoA (Hiltunen et al. 2003). Peroxisomal oxidases (POXes) perform the first reaction converting acyl-CoA into trans-2-enoyl-CoA and hydrogen peroxide. The second and third steps are performed by a multifunctional enzyme (MFE2), which oxidizes trans-2-enoyl-CoA into 3-ketoacyl-CoA. In the final step, thiolytic cleavage catalyzed by 3-ketoacyl-CoA thiolase generates two-carbon shorter acyl-CoA and acetyl-CoA. POXes may vary in their chain length substrate specificities and, in this way, control the number of β-oxidation cycles (Luo et al. 2002). Multiple Lepidoptera POXes have been identified from genome and transcriptome sequencing data, however, until now, only several of them have been characterized (Antony et al. 2015, Chen et al. 2017, Ding et al. 2021, Dou et al. 2019).

While there are so far no reports in the literature on the production of pheromones by engineering peroxisomal β-oxidation, this engineering strategy has been applied for making some other fatty acid-derived chemicals. Oleaginous yeast *Y. lipolytica* and *Candida tropicalis* have been successfully engineered to produce fatty acid derivatives such as flavor lactones and adipic acid, respectively (Ju et al. 2020, Marella et al. 2020). In the case of *Y. lipolytica*, Marella et al. (2020) demonstrated that by replacing native POXes with a heterologous oxidase from *Rhinolophus sinicus* (RsAcox2), production of γ-dodecalactone from oleic acid could be increased 6-fold. RsAcox2 preferentially acted on acyl-CoAs of 14 carbons and above, and this resulted in the degradation of hydroxylated oleic acid to C_{12} precursor of γ-dodecalactone. Ju et al. (2020) showed that adipic acid production from methyl laurate could be increased by 5.4-fold when *C. tropicalis* broad-spectrum oxidase AOX4 was substituted by native oxidase AOX5, which was shown to have narrow substrate specificity (C_{12}–C_{10}). In some instances, it is beneficial to abolish β-oxidation and completely prevent fatty acid degradation. This was demonstrated by the biotechnology company Verdezyne, where *C. tropicalis* lacking POX4 and POX5 produced sebacic (C_{10}) and dodecanedioic (C_{12}) acids from the corresponding monocarboxylic acids without any significant degradation products (patent application number 201615272104). Such examples illustrate that β-oxidation can be engineered to enable biosynthesis of fatty acid derivatives with desired chain length.

In this study, we have selected three insect pheromone precursors as targets, namely, Z7-12:OH, Z9-12:OH, and Z7-14:OH, and constructed metabolic pathways towards them (Fig. 1). Acetate and aldehyde derivatives of these fatty alcohols are the main sex pheromone components of important pests, such as the soybean looper *Chrysodeixis includens*, the grape berry moth *Paralobesia viteana*, and the olive moth *Prays olea*, respectively (Campion et al. 1979, Roelofs et al. 1971, Tumlinson et al. 1972).

We aimed to expand the portfolio of microbially produced insect sex pheromone precursors by engineered β-oxidation. Until now, this step was not implemented in the recombinant biosynthesis of insect pheromones, limiting the spectrum of possible products. To alleviate this bottleneck, we have chosen to work with the yeast *Y. lipolytica* due to its oleaginous properties and the availability of genetic engineering tools (Darvishi et al. 2018, Holkenbrink et al. 2018).

Materials and methods

Plasmid construction

Plasmid construction was performed based on the EasyCloneYALI toolbox described by Holkenbrink et al. (2018). Integrative and gRNA vectors were constructed and used to obtain chromosomal integration of expression cassettes in defined genomic loci of *Y. lipolytica*. Lists of primers, synthetic genes, biobricks, and plasmids used in this study are provided in Tables S1–S4 (Supporting Information), respectively. Biobricks were amplified by using Phusion U polymerase under the following conditions: 98°C for 5 min, 30 cycles of (98°C for 20 s, 54°C for 30 s, and 72°C for 30 s/kb), 72°C for 7 min. PCR products were purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). Assembly of plasmids was performed by USER® cloning. Parent vectors were treated with FastDigest SfaAI (Thermo Fisher Scientific) restriction enzyme and Nb.BsmI (New England BioLabs) nicking endonuclease. Opened vector and compatible biobricks/primers were transformed into *Escherichia coli* strain DH5α. The correct assembly of plasmids was confirmed by colony PCR and Sanger sequencing.

Yeast strain construction

Strains generated in this study were obtained using a lithium acetate-based transformation protocol described previously (Petkevicius et al. 2021). The list of strains is provided in Table S5 (Supporting Information). We used *Y. lipolytica* ST9138 as the background strain. The strain is derived from ST4840 (Y-17536), obtained from Agricultural Research Service (NRRL, USA). ST9138 has all six native POXes deleted (Δ*pox1-6*), which provides a suitable background for testing different POX variants.

Cultivation conditions

Yeast strains were inoculated from a YPD plate to an initial OD_{600} of 0.2 into 2.5 ml YPG medium (10 g/l yeast extract, 10 g/l peptone, and 40 g/l glycerol) in 24 well-plates with an air-penetrable lid (EnzyScreen; three replicates per strain). The plates were incubated at 28°C, shaken at 300 rpm at 5 cm orbit cast. After 22 h, the plates were centrifuged for 5 min at room temperature at 3000 × *g*. The supernatant was discarded, and the cells were resuspended in 1.25 ml production medium per well (50 g/l glycerol, 5 g/l yeast extract, 4 g/l KH_2PO_4, 1.5 g/l $MgSO_4$, 0.2 g/l NaCl, 0.265 g/l $CaCl_22H_2O$, and 2 ml/l trace elements solution: 4.5 g/l $CaCl_22H2O$, 4.5 g/l $ZnSO_47H_2O$, 3 g/l $FeSO_47H_2O$, 1 g/l H_3BO_3, 1g/l $MnCl_24H_2O$, 0.4 g/l Na_2MoO_4, 0.3 g/l $CoCl_26H_2O$, 0.1 g/l $CuSO_45H_2O$, 0.1 g/l KI, and 15 g/l EDTA). Production medium was supplemented with methyl myristate (Sigma-Aldrich). Specific amounts are provided in the section of "Results and discussion." The plates were incubated for 28 h at 28°C, shaken at 300 rpm.

Sample preparation for fatty acid and alcohol analysis

For analysis of fatty acids, 500 µl of cultivation broth was transferred to 4 ml glass vials and centrifuged for 5 min at room temperature at 3000 × *g*. The supernatant was decanted and the cell pellet exposed to transmethylation by 1 ml 1 M HCl in methanol (anhydrous). The samples were incubated at 70°C for 2 h. Every 30 min, the samples were vortexed for 10 s. After cooling down the samples to room temperature, 1 ml of 1 M NaOH in methanol (anhydrous), 500 µl of saturated NaCl solution in water, 500 µl of hexane, and 5 µl of methyl nonadecanoate (19:Me; 2 g/l) as internal standard were added. The samples were vortexed and centrifuged for 5 min at room temperature at 3000 × *g*. The upper organic phase was analyzed via gas chromatography–mass spectrometry (GC–MS).

For analysis of fatty alcohols, 500 µl of broth was transferred to 4 ml glass vials and centrifuged for 5 min at room temperature at 3000 × *g*. The pellet was treated with 500 µl of a mixture of ethyl acetate:ethanol (85:15, v/v) and 5 µl of 19:Me (2 g/l) was added as an internal standard. The samples were vortexed for 20 s and incubated for 1 h at room temperature followed by 5 min of vortexing. A volume of 300 µl of water were added, the samples were vortexed and centrifuged for 5 min at room temperature at 3000 × *g*. The upper organic phase was analyzed via GC–MS or gas chromatography–flame ionization detector (GC–FID).

Analysis of fatty acid methyl esters and fatty alcohols by GC–MS and GC–FID

In this study, two gas chromatography systems were used-Agilent 7820A and Agilent 7890B. For fatty acid methyl esters (FAMEs) analysis, the Agilent 7820A system was coupled to a 5977B mass detector and equipped with a DB-Fatwax UI column (30 m × 250 μm × 0.25 μm). The operation parameters were: 1 μl injection, split ratio 20:1, injector temperature 220°C, constant flow 1 ml/min of helium, oven ramp 80°C for 1 min, 15°C/min to 210°C, 7 min hold time, and 20°C/min to 230°C. MS was scanning between m/z 30 and 350. The samples obtained from the supplementation assay (Fig. 3) were analyzed using previously described settings and temperature program (Petkevicius et al. 2021). The fatty alcohol profile shown in Fig. 4(A) was obtained as follows: all fatty alcohols except Z7-12:OH have been analyzed under the same conditions as FAMEs samples. Z7-12:OH has been identified and quantified using a HP-5 column (30 m × 320 μm × 0.25 µm) under the following conditions: 1 μl injection, split ratio 20:1, injector temperature 220°C, constant flow 1 ml/min of helium, oven ramp 80°C for 1 min, 15°C/min to 150°C, 7 min hold time, 10°C/min to 210°C, then 20°C/min to 300°C, and hold time for 5 min. MS was scanning between m/z 30 and 350. The chromatograms in Figures S1 and S2 (Supporting Information) were obtained by analyzing samples on a Agilent 7890B system equipped with a HP-5 column under the following conditions: 1 μl injection, split ratio 40:1, injector temperature 220°C, constant flow 30 ml/min of hydrogen, oven ramp 150°C for 3 min, 10°C/min to 210°C, and 20°C/min to 300°C. The quantification of compounds was performed based on the internal standard (19:Me) and the identity of compounds was confirmed based on comparison of retention times and mass spectra with reference standards. Reference standards were purchased from Pherobank.

Fluorescence microscopy

Selected strains were cultivated as described in section "Cultivation conditions." The cultivation broth was diluted 5-fold and 5 µl of the sample were loaded onto a microscope slide. Microscopy was performed using 100x magnification and immersion oil in a Leica DFC300 FX microscope equipped with a Leica EL600 external light source. Green fluorescence images were obtained using a

GFP filter cube, while the red signal was obtained using a Y3 filter cube.

Results and discussion

Engineering β-oxidation in *Y. lipolytica* by replacement of native POXes with heterologous ones

We envisioned that screening multiple POX variants from different sources in the Δ*pox1*-6 *Y. lipolytica* background strain would reveal the most suitable candidates to obtain fatty acids with the desired chain length and position of desaturation. More specifically, our goal was to find an oxidase that could efficiently convert the unsaturated C_{14} and C_{16} fatty acids [(*Z*)-9-tetradecenoic acid (Z9-14:acid), (*Z*)-11-tetradecenoic acid (Z11-14:acid), and (*Z*)-9-hexadecenoic acid (Z9-16:acid)] into two carbons shorter respective products: [(*Z*)-7-dodecenoic acid (Z7-12:acid), (*Z*)-9-dodecenoic acid (Z9-12:acid), and (*Z*)-7-tetradecenoic acid (Z7-14:acid)]. A total of 11 POX candidates from various sources were screened to select the most suitable POX. The list of POXes included three native *Y. lipolytica* oxidases, YliPOX2 (YALI0F10857g), YliPOX3 (YALI0D24750g), and YliPOX5 (YALI0C23859g), which are the major contributors to the overall peroxisomal acyl-CoA oxidase activity in this yeast (Wang et al. 1999). Additionally, POXes from *Aspergillus nidulans* (AniPOX), *Cucurbita maxima* (CmaPOX), *Homo sapiens* (HsaPOX), *Paenarthrobacter ureafaciens* (PurPOX), and *Rattus norvegicus* (RnoPOX) were selected due to their reported activities towards C_{14} and C_{16} acyl-CoAs (Bakke et al. 2007, Hayashi et al. 1998, Miyazawa et al. 1987, Oaxaca-Castillo et al. 2007, Reiser et al. 2010). Lastly, three oxidases from the insects *Agrotis segetum* (AsePOX) and *Lobesia botrana* (Lbo_31670, Lbo_49554) were included. In these moths, β-oxidation is postulated to be essential for producing the pheromone precursors, Z7-12:acid in the case of *A. segetum* and Z9-12:acid in the case of *L. botrana* (Ding and Löfstedt 2015, Ding et al. 2021).

POXes were screened in yeast strains either expressing Dmd9 FAD from *Drosophila melanogaster* or Lbo_PPTQ FAD from *L. botrana*, which provide the unsaturated precursors, Z9-14:CoA and Z11-14:CoA, respectively, while native *Y. lipolytica* desaturase YliOLE1 (YALI0C05951g) is responsible for the biosynthesis of Z9-16:CoA from 16:CoA.

In order to produce the target fatty alcohols, the FAR from *Helicoverpa armigera* (HarFAR) was considered a potentially suitable candidate. Previously, it was shown to act on a wide variety of fatty acyl-CoAs with a chain length ranging from C_8 to C_{16} (Hagström et al. 2012). We rationalized that activity of the FADs and FAR in combination with different POXes could result in biosynthesis of Z7-12:OH, Z9-12:OH, and Z7-14:OH (Fig. 1).

Strains containing different POXes combined with HarFAR and Dmd9 or Lbo_PPTQ did not produce any of the three target alcohols (Figures S1/S2, Supporting Information). Hence, we decided to evaluate the fatty acid profiles instead and determine if Z7-12:acid, Z9-12:acid, and Z7-14:acid are produced by yeast strains expressing different POXes (Fig. 2). The introduction of Dmd9 into parental strain ST9138 (Δ*pox1*-6) resulted in a 6-fold increase in Z9-14:acid titer, reaching 12.4 ± 0.7 mg/l. Some background levels of this fatty acid in the parental strain could be explained by the endogenous activity of *Y. lipolytica* YliOLE1 desaturase. In the strain containing YliPOX2 no Z9-14:acid was detected. This oxidase was reported to have the highest activity towards 14:CoA among the set of tested fatty acyl-CoAs (8:CoA, 10:CoA, 12:CoA, 14:CoA, and 16:CoA), which is in agreement with the obtained results (Luo et al. 2002). The absence of Z7-12:acid suggests that YliPOX2 has a promiscuous activity, and β-oxidation does not stop after one cycle. The highest titer of Z7-12:acid was achieved with the oxidase Lbo_31670, which reached 1.72 ± 0.53 mg/l. The yeast strains expressing Dmd9 in combination with YliPOX3, YliPOX5, or AniPOX did not produce any Z7-12:acid, while the rest of the oxidases, CmaPOX, HsaPOX, PurPOX, RnoPOX, AsePOX, and Lbo_49554 generated between 0.09 and 0.91 mg/l of Z7-12:acid.

Introduction of Lbo_PPTQ (ST9314) yielded 6.0 ± 1.2 mg/l of Z11-14:acid and 0.6 ± 0.1 mg/l of (*E*)-11-tetradecenoic acid (E11-14:acid). Expression of Lbo_PTTQ was combined with expression of the individual POXes listed above and screened for production of Z9-12:acid. Production of Z9-12:acid was detected in strains expressing one of the POXes CmaPOX, HsaPOX, PurPOX, RnoPOX, AsePOX, Lbo_31670, or Lbo_49554, but not in strains expressing YliPOX2, YliPOX3, YliPOX5, or AniPOX. The strain with the highest production of Z9-12:acid, 0.93 ± 0.3 mg/l, combined the expression of the desaturase Lbo_PPTQ with the *L. botrana* oxidase Lbo_31670. Transcriptomics studies have shown that this POX is specifically expressed in the pheromone glands of *L. botrana* and contributes to the production of Z9-12:acid in this insect (Ding et al. 2021). A second POX from *L. botrana*, Lbo_49554, did not show strong expression bias towards pheromone glands, and produced approximately 10-fold lower amounts of Z9-12:acid than Lbo_31670 in this study. The differences between the two mentioned oxidases have been observed not only in the production levels of Z7-12:acid and Z9-12:acid, but also in the titers of (*Z*)-7-hexadecenoic acid (Z7-16:acid), which is the β-oxidation product of oleic acid (Z9-18:acid). In the set of strains expressing Dmd9, the level of Z7-16:acid was 3.7-fold higher in the Lbo_49554 expressing strain compared to the strain expressing Lbo_31670 (Figure S3, Supporting Information). A similar result was observed for the strains expressing Lbo_PPTQ, the difference was 3.1-fold (Figure S4, Supporting Information). This result supports the data obtained from transcriptomics studies and implies that Lbo_49554 is a metabolic oxidase while Lbo_31670 contributes to sex pheromone biosynthesis in *L. botrana*.

The highest titer of Z7-14:acid was achieved in the strain ST9347 (expresses Lbo_31670 POX) and reached 0.17 ± 0.03 mg/l. The ratio between the β-oxidation product (Z7-14:acid) and the precursor (Z9-16:acid) was 0.005, while in the best Z7-12:acid and Z9-12:acid producing strains (ST9347 and ST9350, respectively) the product/precursor ratios were approximately 36-fold higher. This shows that even the oxidase which provided the highest titer of Z7-14:acid among the tested variants is suboptimal, and in the future, more candidates could be screened for more efficient conversion of Z9-16:acid into Z7-14:acid.

In summary, fatty acid profiles revealed that strains containing Lbo_31670 POX provided the highest titers of Z7-12:acid, Z9-12:acid, and Z7-14:acid and the absence of corresponding alcohols most likely has other reasons than the lack of substrates.

Validating activity of HarFAR on chain-shortened desaturated fatty acyl-CoAs

Since no target alcohols have been observed in the first round of strain screening, we have decided to test the ability of HarFAR to convert Z7-12:acid, Z9-12:acid, and Z7-14:acid into corresponding alcohols by supplying methyl esters of the acids mentioned above to the cultivation media as substrates. Previously, this reductase proved its versatility and has been used to produce various unsaturated fatty alcohols such as Z9-14:OH, *E*/Z11-14:OH, and Z11-

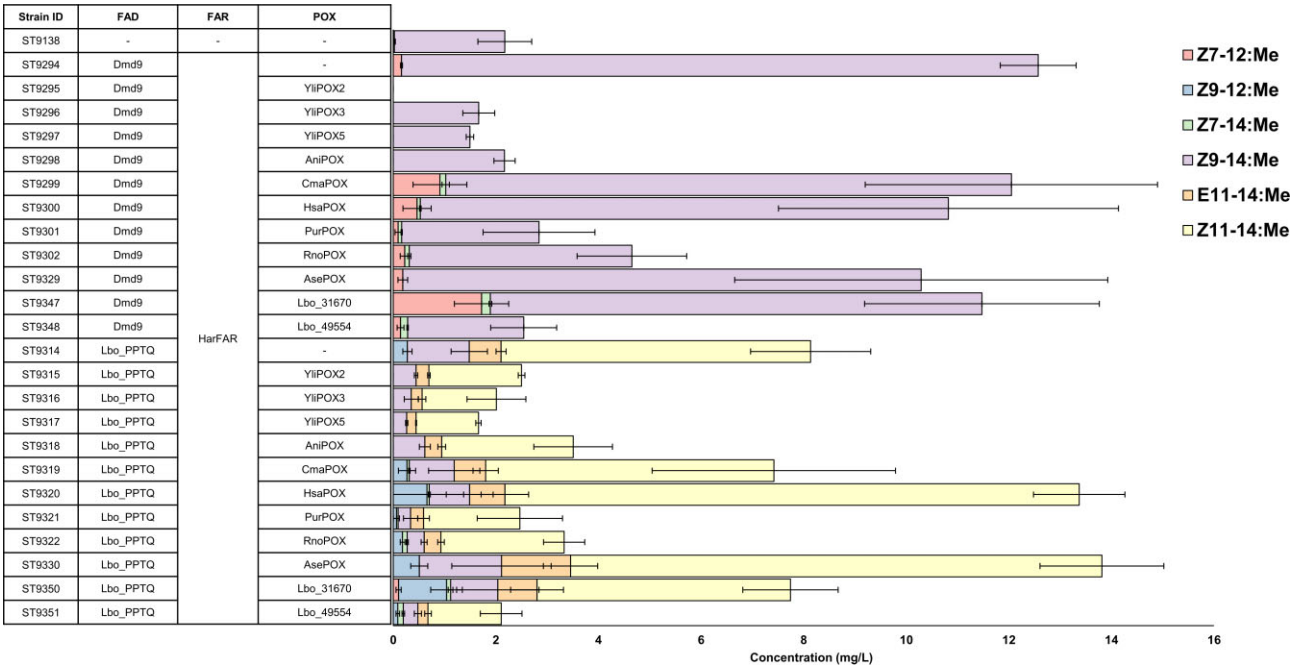

Figure 2. Profiles of C_{12} and C_{14} unsaturated fatty acids in the form of methyl esters obtained from the *Y. lipolytica* strains containing different FADs and POXes. Cultivation media was supplemented with 0.24% (v/v) of 14:Me. Error bars represent standard deviations from three technical replicates.

16:OH. However, there was a lack of direct evidence if it can accept Z7-12:acid, Z9-12:acid, and Z7-14:acid as substrates (Holkenbrink et al. 2020, Petkevicius et al. 2021). Certain insect FARs are known to have very strict substrate specificities, such as reductases from *Ostrinia nubilalis*, while others have a broad substrate range (Tupec et al. 2017).

A *Y. lipolytica* strain expressing HarFAR under the strong constitutive *TEF1*intron promoter was used, and equal amounts of methyl esters of Z7-12:acid, Z9-12:acid, and Z7-14:acid were added to the culture medium (500 mg/l of each compound). Fatty alcohols were extracted from the cell pellet and subjected to GC–MS analysis. The strain expressing HarFAR converted supplied methyl esters into fatty alcohols while no production of these compounds was observed in the negative control strain without FAR. At the end of the cultivation, Z7-12:OH, Z9-12:OH, and Z7-14:OH reached titers of 12.1 ± 0.8 mg/l, 17.9 ± 1.2 mg/l, and 7.0 ± 0.7 mg/l, respectively, confirming that HarFAR is suitable for the biosynthesis of the three target compounds (Fig. 3).

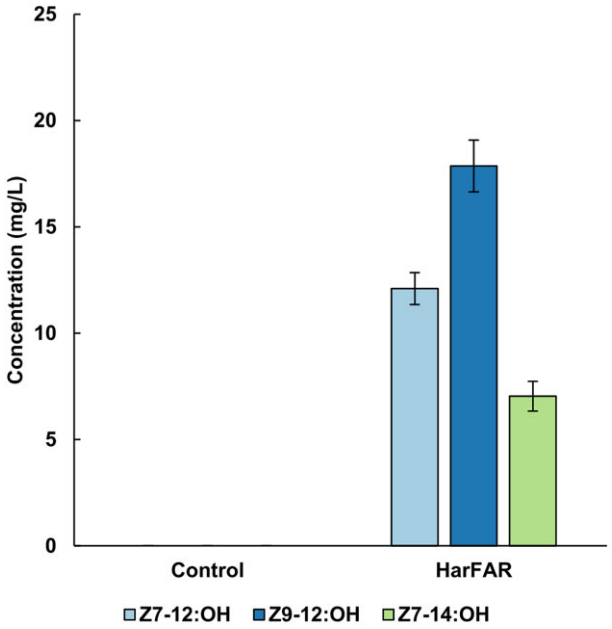

Figure 3. Production of Z7-12:OH, Z9-12:OH, and Z7-14:OH in the *Y. lipolytica* strain expressing HarFAR. Control is the strain lacking HarFAR. Cultivation media was supplemented with equal amounts (500 mg/l) of Z7-12:Me, Z9-12:Me, and Z7-14:Me. Error bars represent standard deviations from three technical replicates.

Enabling production of chain-shortened desaturated fatty alcohols by targeting the reductase into peroxisomes

The absence of the target fatty alcohols motivated us to improve the strains towards *in vivo* precursor supply and increased expression of HarFAR. Before increasing the expression of HarFAR, the best performing strains selected from the screening of POXes (ST9347 and ST9350) were mutated in the fatty acid synthase (FAS) α chain ketoacyl synthase domain. This resulted in ST10313 and ST10314, respectively. Replacement of isoleucine 1220 in Fas2p to phenylalanine ($Fas2p^{I1220F}$) has been shown to increase myristic acid levels up to 8.4-fold (Petkevicius et al. 2021). Even though strains were engineered for increased myristic acid production, cultivation media was also supplemented with inexpensive 14:Me to ensure efficient precursor supply. Myristoyl-CoA is the precursor for production of Z7-12:OH and Z9-12:OH (Fig. 1). $Fas2p^{I1220F}$ mutation did not increase C_{14} fatty alcohols titer, indicating that the reduction reaction and not precursor supply is limiting the flux towards the product (Fig. 4A). Next, an additional copy of HarFAR under the strong *TEF1*intron promoter was integrated into the genome of ST10313 and ST10314, resulting in strains ST10383 and ST10387, respectively. Compared to the GPD promoter, which was used for expression of HarFAR in ST10313 and ST10314, *TEF1*intron was reported to provide a 7-fold higher fluorescence signal when humanized Renilla Green Fluorescent

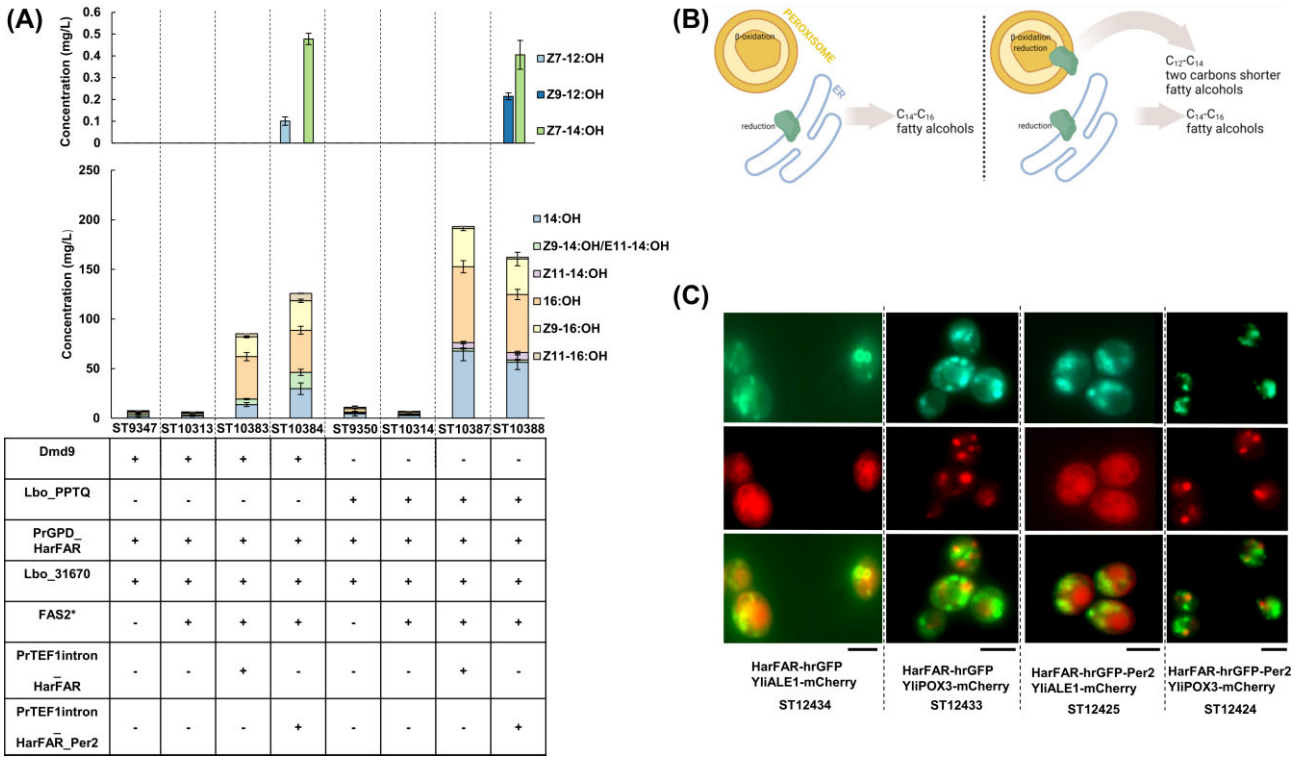

Figure 4. Production of Z7-12:OH, Z9-12:OH, and Z7-14:OH via peroxisomal targeting of HarFAR. (A) Fatty alcohol profiles of engineered *Y. lipolytica* strains. "–" and "+" indicate absence or presence of corresponding genetic change. In the strains expressing Dmd9, Z9-14:OH/E11-14:OH consists only of Z9-14:OH. Cultivation media was supplemented with 0.4% (v/v) of 14:Me. Error bars represent standard deviations from three technical replicates. (B) Schematic representation indicating coupling of β-oxidation and reduction by redirection of HarFAR (green) to peroxisomes. (C) Fluorescence microscopy images of strains coexpressing HarFAR fusion proteins (top row, green) and ER and peroxisomal markers (middle row, red). Bottom row shows overlay between the two images. Scale bars below the images correspond to 5 μm. FAS2*: modified α chain of fatty acid synthase ($Fas2p^{I1220F}$).

Protein (hrGFP) was used as a reporter gene (Holkenbrink et al. 2018). For the expression of HarFAR under the *TEF*1intron promoter we have selected IntE_4 integration site located on chromosome E. Previously, a comparison of 12 integration sites showed that the highest expression of hrGFP is obtained from the IntE_4 site (Holkenbrink et al. 2018). This genome edit allowed to increase total fatty alcohol levels by 16.2-fold in the strain containing Dmd9 (ST10313 versus ST10383), while a 27.8-fold increase was observed in the case of Lbo_PPTQ expressing strain (ST10314 versus ST10387; Fig. 4A). Despite a significant increase in the total fatty alcohol titers, no production of Z7-12:OH, Z9-12:OH, or Z7-14:OH was detected.

This motivated us to explore the possibility of directing HarFAR into peroxisomes, which would allow β-oxidation and reduction reactions to occur in the same cellular compartment. In order to be shortened by two carbons, unsaturated acyl-CoAs have to access the lumen of peroxisomes and, after a β-oxidation cycle, travel back to the proximity of ER for reduction by FAR. We anticipated that the presence of HarFAR in peroxisomes would eliminate the need to export fatty acids out of peroxisomes and enable the production of the target fatty alcohols. To redirect HarFAR to the peroxisome, a 16-amino-acid-long peroxisomal targeting sequence peptide GGGSAAVKLSQAKSKL was C-terminally fused to HarFAR (HarFAR_Per2). This signal was used previously to target cytosolic FAR from *Marinobacter aquaeolei* (FaCoAR) into peroxisomes and helped to increase fatty alcohol production by 2.7-fold in *S. cerevisiae* compared to cytosolic FaCoAR (Zhou et al. 2016). Comparison between ST10383 and ST10384, both containing Dmd9, shows that HarFAR_Per2 improved total fatty alcohol titer by 19% compared to nonmodified HarFAR and enabled the production of Z7-12:OH and Z7-14:OH, which reached titers of 0.10 ± 0.02 mg/l and 0.48 ± 0.03 mg/l, respectively (Fig. 4A; Figure S5, Supporting Information). Among 14:OH, Z9-14:OH, 16:OH, Z9-16:OH, and Z11-16:OH, the biggest fold change was observed in Z9-14:OH, which increased by 3.2-fold. In general, the degree of unsaturation (calculated as the ratio between the sum of unsaturated fatty alcohols and total fatty alcohols) was higher in ST10384 (0.43 ± 0.01) compared to ST10383 (0.34 ± 0.01). The same trend was observed previously when this parameter was compared between the *S. cerevisiae*-produced fatty alcohols by the action of cytosolic and peroxisomal FaCoAR (Zhou et al. 2016). Expression of HarFAR_Per2 in the strain containing Lbo_PPTQ enabled the production of Z9-12:OH, and Z7-14:OH, and titers reached 0.21 ± 0.03 mg/l and 0.40 ± 0.07 mg/l, respectively (Fig. 4A; Figure S6, Supporting Information). These compounds were not produced in the strain containing nonmodified HarFAR (ST10387).

Interestingly, the strains containing Lbo_PPTQ had higher total fatty alcohol levels than respective Dmd9 strains (ST10383 versus ST10387 and ST10384 versus ST10388). Additionally, HarFAR_Per2 did not provide higher total fatty alcohol levels, while the degree of unsaturation was still higher (0.25 ± 0.01 in ST10387 and 0.30 ± 0.05 in ST10388). The differences in total fatty alcohol levels between the respective Dmd9 and Lbo_PPTQ expressing strains could be related to the fatty acid levels in the parent strains [Figure S3 (Supporting Information) ST9347, Figure S4 (Supporting Information) ST9350]. ST9350 had 37.2% higher total FAMEs content compared to ST9347. It was previously reported that the expression of heterologous desaturases increases lipid production in *Y.*

lipolytica, possibly through alleviating feedback inhibition caused by saturated fatty acyl-CoAs (Yan et al. 2020). It could be hypothesized that different unsaturated fatty acids have distinct effects on the FAS complex to modulate the fatty acid profile differently. In addition to deep-well cultivations, shake flask cultivations were performed (Figure S7, Supporting Information), and production of the target fatty alcohols reconfirmed. The titers and specific yields can be found in Table S6 (Supporting Information).

Previously, insect sex pheromone alcohols such as Z11-16:OH, Z9-14:OH, and Z11-14:OH have been microbially produced in *S. cerevisiae* and *Y. lipolytica*. In most of these studies, production of unsaturated fatty alcohols was achieved by expression of FADs and FARs. Expression of FAD and FAR from turnip moth (*A. segetum*) in *S. cerevisiae* enabled the production of Z11-16:OH at 0.195 mg/l (Hagström et al. 2013a). The same compound was later produced in the yeast *Y. lipolytica* by expressing multiple copies of Atr∆11 FAD from *Amyelois transitella* together with FARs from *Helicoverpa* spp. Additionally, modifications preventing fatty alcohol degradation (∆*FAO1*), fatty acid degradation (∆*PEX10*), and triacylglycerol formation (Pr_{100}GPAT) were combined resulting in the strain capable of producing 2.57 g/l of Z11-16:OH. In the same study, combining FAD from *D. melanogaster* with HarFAR and *FAS2* mutation ($Fas2p^{I1220F}$) yielded 73.6 mg/l Z9-14OH (Holkenbrink et al. 2020). Z11-14:OH reached the titer of 188.1 mg/l in *Y. lipolytica*, where Lbo_PPTQ desaturase and HarFAR reductase served as enzymes converting 14:CoA into Z11-14:OH (Petkevicius et al. 2021). Recently, a different approach was demonstrated by Jiang et al. (2021). Instead of FARs, which use acyl-CoAs as substrates for fatty alcohol formation, the authors employed carboxylic acid reductase (CAR) that acts on free fatty acids and results in aldehyde production. Use of FAD from *H. armigera* in combination with CAR from *Mycobacterium marinum* enabled biosynthesis of (Z)-11-hexadecenal (Z11-16:Ald) at the level of 22.7 mg/l (Jiang et al. 2021). The present study employed yet another element-peroxisomal β-oxidation to expand the list of microbially produced insect sex pheromone alcohols.

Chemically, the synthesis of target fatty alcohols (Z7-12:OH, Z9-12:OH, and Z7-14:OH) could be accomplished by cross-metathesis where α,ω-diols and α-olefins serve as starting materials (patent application number 20200039900). In order to obtain the desired isomer, complex metal catalysts ensuring correct double bond configuration have to be used (Quigley and Grubbs 2014). In contrast, bio-based production employs renewable feedstocks, such as glycerol or glucose, and the specificity of desaturases ensures the desired stereochemistry.

In order to visually inspect the cellular localization of HarFAR and HarFAR_Per2, HarFAR was C-terminally tagged with hrGFP or hrGFP_Per2. The yeast strains were additionally transformed with either mCherry-tagged YliALE1 (YALI0F19514g) or YliPOX3, serving as ER membrane and peroxisomal marker proteins, respectively (Fig. 4C). In the strains expressing HarFAR-hrGFP (ST12434 and ST12433), a distinct circular green signal was obtained, i.e. typical for ER membrane proteins. This signal colocalized with YliALE1-mCherry signal (ST12434) but not with YliPOX3-mCherry (ST12433), indicating that HarFAR is present in the ER membrane, but not in peroxisomes. This result is in agreement with previous studies that have shown that FARs from mouse or noctuidae family of insects are ER membrane resident proteins (d'Espaux et al. 2017, Hagström et al. 2013b). Strains expressing HarFAR-hrGFP-Per2 showed a pattern of small green granules distinct from ER signal (ST12425), and in certain places overlaid with the punctate signal from YliPOX3-mCherry (ST12424), demonstrating peroxisomal localization of reductase tagged with hrGFP_Per2. However, redirection from ER to peroxisomes appeared to be suboptimal, since a green signal was also observed in the locations where YliPOX3-mCherry signal was absent. SKL tripeptide at the C-terminus of Per2 sequence is known as canonical peroxisomal targeting sequence 1 (PTS1). This sequence is recognized by Pex5p cytosolic receptor, which guides SKL-containing proteins into peroxisomes (Walter 2019). Apart from PTS1, peroxisomal targeting sequence 2 (PTS2) can also direct proteins to peroxisomes. PTS2 is located at N-terminus and has the following sequence: (R,K)-(L,V,I)-X_5-(H,Q)-(L,A,F). This signal is recognized by Pex7p, which carries proteins to peroxisomes (Schafer et al. 2004). Interestingly, the study by Rosenthal et al. (2020) showed that Pex5p cargo proteins have different targeting priorities. Proteins such as Cat2p and Lys2p have been shown to have high targeting priority. Targeting priority turned out to be related to the high binding affinity of those proteins' last 10 amino acids to Pex5p (Rosenthal et al. 2020). Alternative designs could be explored in the future for more efficient HarFAR targeting. For example, instead of Per2, C-terminal sequences from Cat2p or Lys2p could be used or/and effects of PTS2 signal could be explored. Furthermore, overexpression of PEX5 and PEX7 might improve targeting since those two receptors are involved in protein trafficking to peroxisomes.

Both fusion proteins (HarFAR-hrGFP and HarFAR-hrGFP-Per2) were enzymatically active and generated around 350 mg/l of fatty alcohols (Figure S8, Supporting Information). Interestingly, apart from the typical ER pattern, HarFAR-hrGFP also provided bright, dotted signals that were distinct from ER and peroxisomal signals. Previously, it was shown that proteins with predicted transmembrane domains and ER localization can also be found in lipid droplets. This was experimentally proven for *Y. lipolytica* lipid-modifying enzymes such as DGA1 (YALI0E32769g) and SLC1 (YALI0E18964g; Bredeweg et al. 2017). Lipid droplets are generally believed to be derivatives of the ER, where synthesized neutral lipids aggregate with ER membrane and form separate cellular compartments surrounded by a membrane monolayer (Jacquier et al. 2011). Studies in *S. cerevisiae* show that lipid droplet formation is initiated at specific ER regions which contain Fld1 and Nem1 proteins. Fld1-Nem1 site recruits TAG-producing enzymes such as Lro1 or Dga1, leading to neutral lipid synthesis and lipid body growth and maturation (Choudhary et al. 2020).

Conclusions

In this study, we have established bioproduction of insect sex pheromone alcohols, namely, Z7-12:OH, Z9-12:OH, and Z7-14:OH. This was achieved by engineering β-oxidation, where the native *Y. lipolytica* POXes were replaced by the *L. botrana* oxidase Lbo_31670. Unsaturated fatty acid precursors were generated by the action of FADs, Dmd9, Lbo_PPTQ, and YliOLE1, while peroxisomally expressed FAR was used for fatty alcohol formation. The work paves the way toward a broader spectrum of biologically produced insect sex pheromone components.

Authors' contributions

K.P., I.B., and C.H. conceived and designed the study. L.W. and K.R.K. participated in molecular biology work (plasmid design and construction, sequencing analysis, and so on), C.S. and R.S. participated in the GC–MS analysis. K.P. performed the experiments, analyzed the data, and drafted the manuscript. I.B. and C.H. edited and proof-read the manuscript. All authors read and approved the final manuscript.

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Supplementary data

Supplementary data are available at FEMSYR online.

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Supplementary material for the research article

Biosynthesis of insect sex pheromone precursors via engineered β-oxidation in yeast

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Supplementary table S1. Primers used in this study

ID	Description	Sequence 5' to 3'
PR-18928	PrTEF1intron <-_U1_fw	CACGCGAU AGAGACCGGGTTGG
PR-18975	<-PrTEF1_fw	ACCTGCACUTTTGAATGATTCTTATAC
PR-18066	Har_FAR_U2_fw	ATCTGTCAUGCCACAATGGTGGTCCTGACCTCTAAG
PR-16595	Har_FAR_codoptYL_U2_rev	CACGCGAUCTACTCGTAGGACTTCTTCTC
PR-18930	PrTEF1intron <-_forfusion_U1_fw	ATCAGTAGCU AGAGACCGGGTTGG
PR-18214	PrTEF1intron_USER_rv	AGTACTGCAAAAAGUGCTG
PR-23004	PrGPD_forfusion_U1_fw	ATCAGTAGCUGACGCAGTAGGATGTCCTGC
PR-22213	PrGAPHD->_U2_rev	ATGACAGAU TGTTGATGTGTGTTTAATTCAAGAATG
PR-19018	Dmd9_U1_rev	CGTGCGAUTTATCGAGACTTGTCC
PR-21723	<-Lbo_PPTQ_U1_fw	ACTTTTTGCAGTACUAACCGCAG GTGCCTCGAGCCGCTTCGG
PR-21724	<-Lbo_PPTQ_U1_rev	CGTGCGAU TTACTCCTTCTTAGCGTG
PR-23435	Ase_POX_U1_fw	AGTGCAGGUGCCACA ATGCCCATCTTCATCTG
PR-23436	Ase_POX_U1_rv	CGTGCGAUTTACAGCTTAGACTGCA
PR-21755	Lbo31670->_U2_fw	ATCTGTCAU GCCACA ATGACTGAGGTGACCAAGG
PR-21756	Lbo31670->_U2_rev	CACGCGAU TTACAGCTTACCCTGCATG
PR-15521	PrExp_fw	CGTGCGAUAAGGAGTTTGGCGCCCGTT
PR-15522	PrExp_rev	ATGACAGAUTGCTGTAGATATGTCTTGT
PR-21757	Lbo49554->_U2_fw	ATCTGTCAU GCCACA ATGGAGTCTAAGGACTTTG
PR-21758	Lbo49554->_U2_rev	CACGCGAUTTACAGCTTGGCGGGCACG
PR-22827	Yli_POX2_fw	AGTGCAGGUGCCACAATGAACCCC
PR-22828	Yli_POX2_rv	CGTGCGAUCTATTCCTCATCAAG
PR-22829	Yli_POX3_fw	AGTGCAGGUGCCACAATGATCTCCC
PR-22830	Yli_POX3_rv	CGTGCGAUCTATTCCTCGTCCAG

PR-22833	Yli_POX5_fw	AGTGCAGGUGCCACAATGAACAACAAC
PR-22834	Yli_POX5_rv	CGTGCGAUCTACTCGTCCAGGTC
PR-22841	Ani_POX_fw	AGTGCAGGUGCCACAATGCCCAACC
PR-22842	Ani_POX_rv	CGTGCGAUCTACAGCTTAGACTTG
PR-22843	Cma_POX_fw	AGTGCAGGUGCCACAATGGCCGCTG
PR-22844	Cma_POX_rv	CGTGCGAUCTACAGCTTAGACGAAG
PR-22845	Hsa_POX1-2_fw	AGTGCAGGUGCCACAATGAACCCCG
PR-22846	Hsa_POX1-2_rv	CGTGCGAUCTACAGCTTCGAAGAAG
PR-22847	Pur_POX_fw	AGTGCAGGUGCCACAATGACCGAGG
PR-22848	Pur_POX_rv	CGTGCGAUCTACAGCTTCGAAGAAG
PR-22849	Rno_POX-2_fw	AGTGCAGGUGCCACAATGAACCCCG
PR-22850	Rno_POX-2_rv	CGTGCGAUCTACAGCTTCGAAGAAG
PR-10595	PrTEF1intron _fw	CGTGCGAUAGAGACCGGGTTGG
PR-24919	HarFAR_Per2_U2_rev	CACGCGAUCTAAAGCTTAGACTTGGCCTGAGAAAGCTT AACGGCGGCGGAGCCTCCGCCCTCGTAGGACTTCTTC
PR-23172	IntD2_dwn_fwd	AGTGGCCUGACCAACCTTGTTTGG
PR-23173	IntD2_dwn_rev	CACGCGAUGCATATACGATTTGACTG
PR-23171	IntD2_up_fwd	CGTGCGAUCGTTTCGGAATGTGTC
PR-23170	IntD2_up_rev	AAGCGTTGCACGUTAAGTTGAGAGAGAACGC
PR-23167	IntE_7_up_fwd	CGTGCGAUGAATCTTGGTGCTCAAC
PR-23166	IntE_7_up_rev	AAGCGTTGCACGUGCAATCCGAAGAAGC
PR-23168	IntE_7_dwn_fwd	AGTGGCCUTACTCACATCAGATGGTC
PR-23169	IntE_7_dwn_rev	CACGCGAUGACACATGTGTCTACG
PR-22532	IntF_5_up_fwd	CGTGCGAUGAAGGCTACAACAAGGG
PR-22533	IntF_5_up_rev	AAGCGTTGCACGUTTTTTACATCACGTGCC
PR-22534	IntF_5_down_fwd	AGTGGCCUTGCGCTCACTCGTGATG
PR-22535	IntF_5_down_rev	CACGCGAUCTTGTTTCCCATAGTTTAATG
PR-26919	HarFARforhrGFP_rv	ACCACCCTCGUAGGACTTCTTCTC
PR-26920	hrGFPforHarFAR_fw	ACGAGGGTGGUGGTTCTGTGAGCAAGCAGATC
PR-15506	hrGFP_U2_rev	CACGCGAUTTACACCCACTCGTGCA
PR-26921	hrGFPPer2_rv	CACGCGAUTTAAAGCTTAGACTTGGCCTGAGAAAGCTTAACGG CGGCGGAGCCTCCGCCCACCCACTCGTGCAG
PR-24936	POX3 Y.l. for TEF1intron fw	ACTTTTTGCAGTACUAACCGCAGATCTCCCCCAACCTC
PR-26932	YliPOX3formCherry_rv	ACCACCTUCCTCGTCCAGCTC
PR-26926	YliALE1forTEF1intron_fw	ACTTTTTGCAGTACUAACCGCAGGCCTTTCCATGG
PR-26933	YliALE1formCherry_rv	ACCACCCTUGGTCTTGATGG
PR-26934	mCherryforYliPOX3_fw	AAGGTGGUGGTTCTGTGTCTAAGGGCGAAG
PR-26936	mCherry_U2_rv	CACGCGAUCTACTTGTACAGCTC
PR-26935	mCherryforYliALE1_fw	AAGGGTGGUGGTTCTGTGTCTAAGGGCGAAG

Supplementary table S2. Synthetic genes used in this study

ID	Gene	Sequence 5' to 3'
Sequence No. 1	Fatty acyl reductase from *Helicoverpa armigera*	ATGGTGGTCCTGACCTCTAAGGAGACTAAGCCCTCCGTGGCCGAGTTCTACGCTGGCAAGTCTGT CTTCATCACCGGCGGAACCGGTTTCCTGGGCAAGGTCTTCATTGAGAAGCTGCTGTACTCCTGTC CCGACATCGGCAACATCTACATGCTGATCCGAGAGAAGAAGGGACTGTCTGTGTCCGAGCGAATT AAGCACTTCCTGGACGACCCCCTGTTCACCCGACTGAAGGAGAAGCGACCCGCCGACCTGGAGAA GATCGTGCTGATTCCCGGAGACATCACCGCTCCCGACCTGGGTATTACCTCTGAGAACGAGAAGA TGCTGATCGAGAAGGTGTCTGTCATCATTCACTCCGCCGCTACCGTCAAGTTCAACGAGCCCCTG

		CCCACCGCCTGGAAGATCAACGTGGAGGGAACCCGAATGATGCTGGCTCTGTCTCGACGAATGAA GCGAATTGAGGTCTTCATCCACATTTCCACCGCCTACACCAACACCAACCGAGAGGTGGTGGACG AGATCCTGTACCCTGCTCCTGCTGACATTGACCAGGTGCACCGATACGTCAAGGACGGTATCTCT GAGGAAGAGACTGAGAAGATTCTGAACGGCCGACCCAACACCTACACCTTCACCAAGGCCCTGAC CGAGCACCTGGTGGCTGAGAACCAGGCTTACGTGCCCACCATCATTGTCCGACCCTCCGTGGTCGC CGCTATCAAGGACGAGCCCATTAAGGGATGGCTGGGTAACTGGTACGGAGCTACCGGACTGACCG TGTTCACCGCTAAGGGTCTGAACCGAGTCATCTACGGCCACTCTTCCAACATCGTGGACCTGATT CCCGTGGACTACGTCGCCAACCTGGTCATTGCCGCTGGCGCTAAGTCTTCCAAGTCCACCGAGCTG AAGGTGTACAACTGTTGCTCTTCCGCCTGCAACCCCATCACCATTGGAAAGCTGATGTCTATGTT CGCCGAGGACGCTATCAAGCAGAAGTCCTACGCTATGCCCCTGCCCGGTTGGTACATCTTCACCA AGTACAAGTGGCTGGTCCTGCTGCTGACCATTCTGTTCCAGGTCATCCCCGCCTACATTACCGACC TGTACCGACACCTGATCGGCAAGAACCCCCGATACATTAAGCTGCAGTCTCTGGTCAACCAGACC CGATCTTCCATTGACTTCTTCACCTCTCACTCCTGGGTCATGAAGGCTGACCGAGTCCGAGAGCT GTTCGCCTCTCTGTCCCCCGCTGACAAGTACCTGTTCCCCTGTGACCCCACCGACATCAACTGGAC CCACTACATTCAGGACTACTGCTGGGGAGTGCGACACTTCCTGGAGAAGAAGTCCTACGAGTAG
Sequence No. 2	Fatty acyl desaturase from *Drosophila melanogaster*	ATGGCTCCCTACTCTCGAATCTACCACCAGGACAAGTCGTCCCGAGAGACTGGCGTGCTGTTCGA GGACGACGCCCAGACCGTGGACTCTGACCTGACCACCGACCGATTCCAGCTGAAGCGAGCCGAGA AGCGACGACTGCCCCTGGTGTGGCGAAACATCATCCTGTTCGCCCTGGTGCACCTGGCCGCTCTGT ACGGCCTGCACTCTATCTTCACCCGAGCCAAGCTGGCCACCACTCTGTTCGCTGCCGGCCTGTACA TCATCGGCATGCTGGGCGTGACCGCTGGCGCCCACCGACTGTGGGCTCACCGAACCTACAAGGCC AAGTGGCCCCTGCGACTGCTGCTGGTGATCTTCAACACCATTGCCTTCCAGGACGCCGTGTACCAC TGGGCCCGAGATCACCGAGTGCACCACAAGTACTCTGAGACTGACGCTGACCCTCACAACGCTAC CCGAGGCTTCTTCTTCTCTCACGTCGGCTGGCTGCTGTGCAAGAAGCACCCCGACATCAAGGAAA AGGGCCGAGGCCTGGACCTGTCTGACCTGCGAGCTGACCCCATCCTGATGTTCCAGCGAAAGCAC TACTACATTCTGATGCCCCTGGCCTGCTTCGTGCTGCCCACCGTGATTCCCATGGTGTACTGGAAC GAGACTCTGGCCTCTTCCTGGTTCGTGGCCACCATGTTCCGATGGTGCTTCCAGCTCAACATGACC TGGCTGGTGAACTCTGCCGCTCACAAGTTCGGCAACCGACCTTACGACAAGACTATGAACCCCAC TCAGAACGCCTTCGTGTCTGCCTTCACCTTCGGCGAAGGCTGGCACAACTACCACCACGCATTCCC TTGGGACTACAAGACCGCCGAGTGGGGCTGCTACTCTCTGAACATCACCACCGCCTTCATCGACC TGTTCGCTAAGATCGGCTGGGCCTACGACCTCAAGACCGTGGCTCCCGACGTGATCCAGCGACGA GTGCTGCGAACCGGCGACGGCTCTCACGAGCTGTGGGGCTGGGGCGACAAGGACCTGACCGCTGA GGACGCCCGAAACGTCCTGCTGGTGGACAAGTCTCGATAA
Sequence No. 3	Fatty acyl desaturase from *Lobesia botrana*	ATGGTGCCTCGAGCCGCTTCGGAGGAGACCGACCTTAAGGAGGCTACCCAGCTTGAGCCCCGAAA GTACGAGATCGTGTACACTAACGTGATCTACTTCACCTATTGGCATATCGCCGGACTGTACGGTC TGTACCTGTGTTTTACCTCCGCTAAATGGGAGACCATCGTGTTCGCTTGGGCTTGGTATGTGCTC GGAGAGCTGGGAGTGATTGCCGGCGCTCATAGATTGTGGGCCCACCGAACCTACAAGGCAAAGAT GCCCCTGCAGATCATCCTGATGCTGTTTAATTGTATCGGTTTTCAGAACACCGCTACCGATTGGG TTCGAGATCACCGAGTGCATCACAAGCACTCTGACACCGACGCCGACCCCCATAACTCTCAGCGA GGCTTCTTTTTCTCTCACGTGGGCTGGCTGCTGACCCGAAAGCATCGGCTGGTGAAGGAGAAGGG AGAAGCTGTTGACATGACTGATATCTACTCTAACCCTGTTTTAAGATTCCAGAAGAAGTACTCTC TCCCCCTGATCGGCACTCTTTGCTTTGCTCTGCCCACCCTGCTGCCCGCTTACTGCTGGGGAGAGG CCGTCGGCACCGCTTGGAACATTAACCTGCTGCGATACTGTCTTAACCTGAACGGAACCTTCCTG ACTAACTCCGCCGCTCACAAGTTTGGCTCTAAGCCCTATGACAAGACCATTCCTCCCACCCAGAAC TTGCTGGTGTCTTTCATGACTCTGGGAGAAGGATTTCATAATTACCACCACGTCTTCTCGTGGGA CTACCGAGCTGCTGAGCTTGGCAACACCTACCTGAACATGACCACTATCTTTATCGACTTCTTCG CTCTTATTGGATGGGCCTACGACCTGAAGACCGTTCCTGAGGATGTTATTAAAAAGCGAATGGCC CGAACTGGAGATGGTACTAACCTGTGGGGTTGGGGAGACAAGGACATGACCAAGGAGGACGTGG TGGACACCGAGATACGATTCCACGCTAAGAAGGAGTAA
Sequence No. 4	Peroxisomal oxidase from *Agrotis segetum*	ATGCCCATCTTCATCTGCATCATCACCTCTCAGGCCATCATCCGATCTAACGTCGAGCGAGTGGCC GTGATCCTGAACATCAACATGGGCAAGGTGAACGAGGACCTGGTGCGAGAGCGAGCCAAGTGCAC CTTCAACATCGAGGAACTGACCTACTTCCTGGACGGCGGCAAGGACAAGACCCTCGAGCGAAAGG AAACCGAGCGAGCTATGCTGACCAAGCGAGAGGAACTGTTCGGCGGCGTGCCCGACGAGTACCTG TCTCACAAGGAAAAGTACGAGAACTCTATGCGAAAGGCCGTGATTCTGTTCGGCATCCTGCGAAA GATCCAGAAGGACAACAACACCGACCTGACCAACTACCGAAACCTGCTGTCTGGCGTGCTGTCTG TGTCTATCTCTCAGGACGGCTCTCCCTTCGGCCTGCACTACATCATGTTCATGCCCGTGCTGCTGT CTCAGGCCGACGAGAAGCAGCAAGAGAAGTGGCTGAAGCGAGCCATGAACTGCGAGATCATCGGC TCTTACGCCCAGACCGAGCTTGGCCACGGCACCTTCATCCGAGGACTCGAGACTACCGCCACTTAC GACCCCGCCACTCAAGAGTTCGTGCTGCACTCTCCCGCTCTGTCCTCTTACAAGTGGTGGCCCGGT GGCCTGGGCAACACCGTGAACTACTGCATCGTGATCGCCCAGCTGTACTCTAAGGGCGTGTGCCA CGGCATCCACTCGTTCATCGTGCAGGTCCGAGATGAGGACACCCACATGCCTCTGCCTGGCATCA AGGTGGGCGAGATCGGCGTCAAGATGGGCCTGAACTCTGTGAACAACGGCTTCCTGGGCTTCGAG AACGTGCGAATTCCCCGAGTGAACATGCTGATGAAGCACGCCAAGATTCTCGAGGACGGCACCTA CGTGAAGTCTAAGAACAACAAGCTGATCTACGGCGCCATGGTGTTCGTGCGAGTGGTGATCGTGT TCGACTCTGTCAACTACCTGGCCAAGGCCATCACTATCGGAGCCCGATACTCTCTGGTGCGACGA CAGTCTCAGCTGAAGGCCGGCGAGCCCGAGCGACAGATCCTGGACTACGTGACCCAGCAGCACAA GATTCTGCCCGCCATTGCCGGCTGCTACGCCATGAAGATGAACGCCTGGCGACTGTGGGACACCT TTAACCTGATCAACGGCCAGCTGCACCAGGGCAACATGGAACGACTGGGCGAGCTGCACGCCCTG GCCTGCTGCCTGAAGGCCATCTCTACCACCGACGCCGCTATGTTCACCTCTCTGTGCCGACTCGGC TGTGGCGGCCACGGCTACATGACCTCTTCTAACCTGCCTCCTACCTACGCTCTGACCTCTGCCTCT TGCACCTACGAGGGCGACAACACCGTTCTGCTGCTGCAGACCGCTCGATTCCTGCTCAAGACCTG GCGACAGATTGACACCCATCCTCTGACTCGAACCGTGGCCTACCTCAAGACCGTGTCTGCTCCCGG CTTCTCTGACCGATGGGAGTCCTCTGTCGAGGGCATCATTCGAGGCTTCCAGACCGTGGCTATGA AGAAGATCTCTTCTTGCCTGGACATCATGACCTCCAAGGTGATGTCTGGCATGTCTCAAGAGGAC GCCTGGAACGCCATCTCCATCCAGCTGGTGTCTGCCGCCGAGTCTCACTCTCGAGGCACCGTGATC

		TCTACCTTCTACGAGGACATGTCTAAGGCCATGCGATCTATGACCGCTCCTCTGGCTAAGGTGAT GGGCCAGCTGGTCGAGCTGTACGCTGTGTACTGGACTCTCGAGCGACTGGGAGACATGCTGCAGT ACACCTCTATCTCCCACACCGACGTGGTGGACCTGCGATCTTGGTACGAGGAACTCCTCCGAAAG ATTCGACCCAACACCATCGGCCTGGTGGACGCCTTCGACATCATCGACGAGCTGCTCCAGTCTACC CTGGGCGCCTACGACGGCCGAGTGTACGAGCGACTCATGGAAGAGGCCCTGAAGTCTCCCCTGAA CGCTGAGCCCGTGAACCAGTCTTTCCACAAGTACCTGAAGCCTTTCATGCAGTCTAAGCTGTAA
Sequence No. 5	Peroxisomal oxidase 31760 from *Lobesia botrana*	ATGACTGAGGTGACCAAGGTGAACCCCGACCTCCAGCGAGAACGAGACAACTGTACATTCAACGT TACCGAGCTGACTAACCTGATCGACGGCGGTGTGCAGAAGACCGAGGAGCGACGGAAGCGAGAGG AGATGGTGCTGAAAGAAGGTATCCACCTGGACGAGGTTCCCTCAGAGTACCTGTCCCACAAGGAG AAGTACGAGCTCGCTGTGAAGAAGGCATGCTACCTGTTCAAGATGATCCGACGACTGCAGGAGGA AGAGAACACTGGAATGGAGAATTACCAGGAGGTGCTGGGCGGATCCCTGGGATCCGCTATCCTGC GAGACGGCTCCCCTCTCACCCTGCATTACGTGATGTTCATCCCTACCATCATGGGCCAGGCTACGG TTGAGCAGCAAGTGCTTTGGATCGGCCGAGCCTTCAATTGCGATATTATCGGAACTTACGCCCAG ACTGAGCTTGGTCACGGAACCTTCATTCGAGGACTGGAAACCACCGCCACTTACGATCCCTCTAC AAAGGAGTTCGTCCTGCACTCCCCCACTTTGACTTCCTACAAGTGGTGGCCCGGAGGCCTTGCTC ACACGGCCAATTACTGTATCGTGGTGGCTCAACTTTACACACAAGGCAAGTGCCATGGCATTCAC CCCTTTATCGTCCAGCTACGAGACGAAGAGACCCACATGCCCCTGCCCGGCATCAAGGTGGGCGA AATCGGCGCTAAACTGGGAATGAACGGCACCAACAACGGCTTCCTTGGATTCGATAAGGTGCGAA TCCCCCGAGAGCACATGCTGATGAAGAACGCCAAGGTTCTCGAGGACGGTACCTATGTGCGAGCC CCCAGCAGTAAGCTGACATACGGTACTATGATGTTTGTGCGAGTTGTGCTGGTGAACGACGTGTG CTCGTATATGGCAAAGGCCGTTACCATCGCTACTAGATACTCTGCTGTGCGACACCAGTCTCAGC CTAAGCCCGACGAGCCCGAGCCCCAAATCCTGGAGTACGTGACTCAACAGCACAAGCTTGCCATC GGCATTGCAACCGTGCACGCCTTTCGACTATCCGCCTCTTGGCTGTGGAACATGTACAACAACGT GACAGCCGAGCTGGACGCCGGCGACTTAGAGCGACTTCCAGAACTGCACGCTCTGTCTTGCTGTC TGAAGGCCGTTTCGACTGCTGATGCCTCTGAATGCGTCGAGCGATGCCGACTTGCTTGTGGAGGC CACGGATACATGTTGTCCTCTAACTTCCCTCTGATGTACGGCATGGTGACCGCCGCTTGTACTTA CGAAGGCGAGAACACCGTGATGCACCTGCAGACCGCCCGGTATCTGGTCAAGGCCTGGCAGCAGG CCCAGGGTGGCAACCCCCTGACGCCTACTGTTGCCTACATCGGATCTATTGGCGCCGGACGACGA AGCCCACCTTGGGATAACACCGTGGAGGGCATTATCCTGGGCTTCCAGCGCGTGGCTGCCGGTAA GATTGCCCAGTGTGTTGCTAATATCGAAAAACGACAGCGAACCGGAATGTCTTACGAGGATGCTT GGAACATGACCTCAGTGCAGCTTGTGTCTGCTAGCGAGGCCCACTGTCGAGCTTTCATCCTCCGG ACCTACTTCGAGGAGACCGAAAACCAAATCGGTTCCGTCTCTCCCGCTCTGCGAGCAGTGCTGCT GCAACTGGTCGATTTGTACGTTGTGTTTTGGGCTCTGCAGCGTGTCGGAGACCTTCTGAGATTCA CCTCTATCTCTGAGCGAGACATCGAGCAGCTTCAGTCTTGGTACGAGGATCTTCTGATCAAGCTG CGAGTCAACGCTGTCGGACTGGTCGACGCTTTCGACATCCGAGACGAGATCTTGAACTCTGCTCT CGGAGCCTACGACGGGCGAGCTTACGAGCGGCTTATGGATGAAGCCCTTAAGTCTCCCCTGAACG CTGAACCTGTGAACCAGTCTTTCCATAAGTATCTGAAGCCTTTCATGCAGGGTAAGCTGTAA
Sequence No. 6	Peroxisomal oxidase 49554 from *Lobesia botrana*	ATGGAGTCTAAGGACTTTGACCCTGTGTCCGATGCTGAGTTGAAAGACTACTTCCCCGACCTCCC TTCTGGTCCTCTGGATAAGTTCCGAAAGAAGGCCACCTTCGACTGGAGACGAATGAAGCTAGTGT ACGACTCTAAGCAGTCTATTGAGACCAAGGATAAGGTGTGGAAGTTCATGCTTGCTCACCCTCTG TTCAAGCACTCTGTCGCCACCCCCACCCTGGATGAGCAGCGACAGATTGCTACCAAACGTATGTA TCTGCTGCATAACGCTGACCTGGTCCCTCTGGAGGAAATCGCTATGCATCCCCGACTCTTTCAGTC GTTGACTGAGGCCATTTTCATGTTCGACTCTTCGGTGGCCGTTAAGCTGTCTCTTACCTTCCGAA TGTTCACCAACACAATTCGCGGATCGGGCAGACAGCATCACTACCACTTGATTGAGGACTGCGAC AACGGCAAGATCGGCGGATGCTTCGCCCTGACTGAGATCGCCCACGGATCTAATGCTAAGGGCAT GCGAACCACCGCAACCTACGACGTGGAGGGCCGATGTTTCGTCATGCACACCCCCGACTTTGAAG CTGCCAAGTGTTGGGTTGGCTCCCTGGGTAAGTGTGCTACCCACGCCATTGTCTACGCCATGCTG ATCTCTAAGGGCAAAAACCATGGTCTTCATTCCTTCGTGGTGCCCATCCGAGATCCTAAGACACT CCGACCTTTTGCAGGCGTGACTGTCGGAGACATCGGCGAGAAGATCGGACTGAACGGAGTGGACA ACGGTTTCCTGATGTTCAACAAGTACCGACTGCCCAAGGAGGCCGCTCTCGACAAGCTGGGTGGA GTTGACGAAAACGGCGACTACAAGACTCCTTTCAGAGATCCTTCCAAGCGGTTCGGTGCTTCCCT GGGCATTCTGTCTGGCGGCCGAGTGCACATTACCTCTATCTCTACCAACTACCTCCAGAAGGCTG TGGTGATCGCTGTGCGATACTCTGCCGTGCGCCGACAGTTTGGCCCCGAGAACGCCGTCGAGGAG ACTCCTGTCCTTGAGTACCAGCAACAGCAGATTCGACTTCTGCCCTACCTGGCCGCTACTTTCGCC ATGCGAGTGTTCTGTAACTGGTTCGGCGAAGTGCACGTGCAGATGACCATCGATAACCTGGTTGG TGCCGACGAGCAGGCTGCGGCCCGAGGTATCGAGATGCATGCCCTGTCTTCCGCCGTGAAGCCCG TGTGCGGTTGGACCGCCCGAGACGGACTGCAGAACTGTAGAGAATGTTGCGGTGGTCATGGATAC CTCCGAGCCTCCGCCATTGGCGACCTGCGAAACGACAACGACGCAAACTGTACCTACGAGGGCGA GAACTCTATGCTCCTCCAGCAGACTTCTAACTGGCTGCTGGGCGTCTGGGCTAGACGACGACAGC CCGGTTTCGCCGACACACCTTTCGGATCTATCCAATTCCTGGATGGCGCTCAGGCCCTGCTGGACG AGTCTTGTCAGTGGAACTCCGCTGAAGAGATCGTGCAGCCTTCCAACGTCGTCCACATGTACAAG TGGCTAACCACCTACATGCTCAAGCTCACCGCTGATAAGGTGTCCCAGCTTCGATCTCGAGGTTC GTCTAACTACGAGGTCCGAAACGATTCGCAGTCCTACAACGCTGTGACTCTGTCTGTGGTGTATG GAGAGCACTTTATTGTGAATCACTTCTACAAGACTGCACTGCAGTTCCAGGACGCCGCCTGTAGA GCTGTCCTGCTGAAGCTGGTGGCCCTGTACGGAGCCTTCCTTCTCGAGAAGCACATGGCTACCCT TTATATCGGAGGATTCTTTACCAAGCAGCAGGGACTGCTCCTGCGCGAAGGAGTTCTCTCTCTCT GCAGCGCCCTGTCACCCCAGGCCGTGTCGCTGGCCGACACCCTCGCTCCCCCTGATTGGTGTCTGA ACTCTGTGCTGGGATGCGCTGACGGCGAGGCGTACAAACATATCCAGGATTCTATTATGACTTAC CCTGGCTCGATGACCCGGCCTGAGTGGTGGCGAGATGTTGCCCACTGGGAGACTTACGTGCCCGC CAAGCTGTAA
Sequence No. 7	Peroxisomal oxidase from *Aspergillus nidulans*	ATGCCCAACCCTCCGCCTGCCTGGGTGCAAGCTCTGAAGCCCGCTTCGCCCCAGGGCACCGAGCTG CTGACCCAAGAGCGAGCCCAGTCTAACATCGACGTGGACACCCTGGGCGACCTGCTGCACACCAA GGAAGCCCTGAAGAAGCAGGACGAGATCCTGTCTGTGCTGAAGTCTGAGAAGGTGTTCGACAAG TCTCGAAACCACGTGCTGGGCCGAACCGAGAAGATCCAGCTGGCCCTGGCTCGAGGCAAGCGACT

		GCAGCAGCTGAAGAAGGCCCACAACTGGTCTGACGAGGACGTCCACGTGGCCAACGACCTGGTGT CTGAGCCCACTCCTTACGGCCTGCACGCCTCTATGTTTCTGGTGACCCTGCGAGAGCAGGGCACCC CTGAGCAGCACAAGCTGTTCTACGAACGAGCCCGAAACTACGAGATCATCGGCTGCTACGCCCAG ACCGAGCTTGGCCACGGCTCTAACGTGCGAGGACTCGAGACTACCGCCACTTGGGACCCCTCTGA CCAGACCTTCATCATTCACTCTCCCACTCTGACCGCCTCTAAGTGGTGGATCGGCTCTCTGGGACG AACCGCCAACCACGCCGTGGTGATGGCCCAGCTGTACATCGGCGGCAAGAACTACGGACCCCATC CTTTCGTGGTGCAGATCCGAGACATGGAAACCCACCAGCCTCTCGAGAACGTGTACGTGGGCGAC ATCGGCCCCAAGTTCGGCTACAACACCATGGACAACGGCTTTCTGCTGTTCAACAAGCTGAAGAT TCCCCACGTGAACATGCTGGCCCGATTCGCCCAGGTGGACAAGGCCACCAACAAGTACATTCGAC CCGCTTCTCCCTCTCTGATGTACGGCACCATGACCTGGGTGCGATCCAACATCGTGCTGCAGGCTG GCGGCGTGCTCGCCCGAGGCGTGACCATTGCCGTGCGATACTGCGCCGTGCGACGACAGTTCCAG GACCGAGATGCCAAGGCCAACGCCGAAGAGAACCAGGTGCTGAACTACAAGATGGTCCAGATTCG ACTGCTGCCCCTGCTGGCCGCCATGTACGCCCTGCACTTCACCGGCCGAGGCATGATGCGACTGTA CGAAGAAAACCAAGAACGAATGAAGGCTGCCGCTCAGGCCGACCAAGAGAAGCGAGGCGCTGGCC CCGAGCAGCTGCGAGCCGGATCTGACCTGCTGGCTGACCTGCACGCTACCTCTTGCGGCCTGAAG GCCCTGGCCTCTACCACCGCTGGCGAGGGCCTCGAGGTGTGCCGACGAGCCTGTGGCGGCCACGG ATACTCTAACTACTCTGGCATCGGACCCTGGTACGCCGACTACCTGCCTACTCTGACCTGGGAGG GCGACAACTACATGCTGACTCAGCAGGTTGCCCGATACCTGCTCAAGTCTGCCCGAGCCGTGCTG GCCGGCAAGGGCACCGCCAACGACACCTCTCGAATCCTGCAGGCCTACCTCGCTCGACGAGACAA GGGCGCCTCTTTCGACATCCTGGGCAACGACGCCGACATTGTGGCCGCCTTCGCCTGGCGAACCGC TCACCTGACCTTCGAGACTCTGAAGTACCGAGATGTCGAGAAGCGATCTTGGAACTCTCTGCTGA TCAACTTCTGGCGACTGTCTACCGCTCTGTCTCAGTACCTGGTGGTGAAGAACTTCTACGAGGCC GTGAACTCTCCCGAGATCCGATCTTCTCTGGACAAGGACACTGCTTCTACCCTGCGATCTCTGTTC CGACTGCACGCTCTGCACACCCTGGACCGAGAGGCCTCCGAGTTCTTCTCTTCTGCCGCCGTGACC GTGCGACAGATCGGACTGACCCAGACCTCTGAGGTGCCCAAGCTGCTGGACGAGATTCGACCCCA CGCCGTCCGACTGGTGGACTCTTGGAAGATCCCCGACTGGCAGCTGGACTCTGCCCTGGGCCGATC TGACGGCGACGTGTACCCCGACCTGTTCAAGCGAGCCTCTATGCAGAACCCCGTGAACGACCTCG TGTTCGACCCCTATCCTTGGAACGAGAACGTCCTGAAGAACGCCGGTGAGATCAAGTCTAAGCTG TAG
Sequence No. 8	Peroxisomal oxidase from *Cucurbita maxima*	ATGGCCGCTGGCAAGGCCAAGGCTAAGATCGAGGTGGACATGGGATCTCTGTCTCTGTACATGCG AGGCAAGCACCGAGAGATCCAAGAGCGAGTGTTCGAGTACTTCAACTCTCGACCCGAGCTGCAGA CCCCTGTGGGCATCTCTATGGCCGACCACCGAGAGCTGTGCATGAAGCAGCTGGTCGGCCTGGTG CGAGAGGCCGGCATTCGACCCTTCCGATTCGTGAACGAGGACCCCGCCAAGTACTTCGCCATCAT GGAAGCCGTGGGCTCTGTGGACGTGTCTCTGGCCATCAAGATGGGCGTGCAGTTCTCTCTGTGGG GCGGCTCTGTGATCAACCTGGGCACCAAGAAGCACCGGGACCGATTCTTCGACGGCATCGACAAC GTGGACTACCCCGGCTGCTTCGCCATGACTGAGCTGCACCACGGCTCTAACGTGCAGGGCCTGCA GACCACCGCCACTTTCGACCCCATCACCGACGAGTTCATCATCAACACCCCTAACGACGGCGCCAT CAAGTGGTGGATCGGCAACGCCGCCGTCCACGGCAAGTTCGCCACCGTGTTCGCCAAGCTGGTGC TGCCCACTCACGACTCTCGAAAGACCGCCGACATGGGAGTGCACGCCTTCATCGTGCCCATCCGAG ATCTGAAGTCTCACAAGACCCTGCCTGGCATCGAGATCCACGACTGCGGCCACAAGGTGGGCCTG AACGGCGTGGACAACGGCGCCCTGCGATTCCGATCTGTGCGAATTCCCCGAGACAACCTGCTGAA CCGATTCGGCGAGGTGTCTCGAGATGGCAAGTACAAGTCCTCTCTGCCCTCTATCAACAAGCGAT TCGCCGCCACTCTGGGCGAGCTGGTTGGCGGCCGAGTCGGACTGGCCTACTCTTCTGCCTCTGTGC TGAAGATCGCCTCTACTATCGCCATCCGATACTCCCTGCTGCGACAGCAGTTCGGCCCTCCTAAGC AGCCCGAGGTGTCCATCCTGGACTACCAGTCTCAGCAGCACAAGCTGATGCCCATGCTGGCCTCT ACCTACGCCTTCCACTTCTCTACCATGCAGCTCGTCGAGAAGTACGCCCAGATGAAGAAGACCCA CGACGAGGAACTGGTGGGCGACGTGCACGCCCTGTCTGCCGGCCTGAAGGCCTACGTGACCTCTT ACACCGCCAAGTCTCTGTCTACCTGCCGAGAGGCCTGTGGCGGCCACGGATACGCCGTGGTCAAC CGATTTGGCACCCTGCGAAACGACCACGACATCTTCCAGACCTTCGAGGGCGACAACACCGTGCT GCTCCAGCAGGTCGCCGCCTACCTGCTCAAGCAGTACCAAGAGAAGTTCCAAGGCGGCACCCTGG CCGTGACCTGGAACTACCTGCGAGAATCTATGAACACCTACCTCTCGCAGCCCAACCCTGTGACC GCTCGATGGGAGTCTGCCGACCATCTGCGAGATCCCAAGTTTCAGCTGGACGCTTTCCAGTACCG AACCTCTCGACTGCTGCAGTCTGTGGCCGTGCGACTGCGAAAGCACACCAAGAACCTGGGATCTT TCGGCGCCTGGAACCGATGCCTGAACCATCTGCTGACCCTGGCTGAGTCTCACATCGAGTCTGTG ATTCTGGCCCAGTTCATCGAGTCCGTGCAGAGATGTCCCAACGCTAACACCCAGGCTACCCTGAA GCTGGTGTGCGACCTGTACGCTCTGGACCGAATCTGGAACGACATCGGCACCTACCGAAACGTCG ACTACGTGGCTCCCAACAAGGCAAAGGCCATCCACAAGCTCACCGAGTACCTGTGCTTCCAGGTG CGAAACATTGCCCAAGAGCTGGTGGACGCCTTCGACCTGCCTGACCACGTGACTCGAGCCCCTAT TGCCATGAAGTCTAACGCCTACTCTCAGTACACCCAGTACATCGGCTTCGACCAGATTACCTCTG TGGGATCTTCGTCTAAGCTGTAG
Sequence No. 9	Peroxisomal oxidase from *Homo sapiens*	ATGAACCCCGACCTGCGACGAGAGCGAGACTCTGCCTCTTTCAACCCCGAGCTGCTGACCCACATC CTGGACGGCTCTCCCGAAAAGACCCGACGACGACGAGAAATCGAGAACATGATCCTGAACGACCC CGACTTCCAGCACGAGGACCTGAACTTTCTGACCCGATCTCAGCGATACGAGGTGGCCGTGCGAA AGTCTGCCATCATGGTGAAGAAGATGCGAGAGTTCGGAATCGCTGACCCCGACGAGATCATGTGG TTCAAGAACTTCGTGCACCGAGGACGACCCGAGCCTCTGGACCTGCACCTGGGCATGTTTCTGCC CACTCTGCTGCACCAGGCCACCGCCGAGCAGCAAGAGCGATTCTTCATGCCCGCCTGGAACCTCGA GATCATCGGCACCTACGCTCAGACCGAGATGGGCCACGGCACCCACCTCCGAGGACTCGAGACTA CCGCCACCTACGATCCCGAGACTCAAGAGTTCATCCTGAACTCTCCCACCGTGACCTCTATCAAGT GGTGGCCCGGTGGACTGGGCAAGACCTCTAACCACGCCATCGTGCTGGCCCAGCTGATCACCAAG GGCAAGTGCTACGGCCTGCACGCCTTCATCGTGCCCATCCGAGAGATCGGAACCCACAAGCCTCT GCCTGGCATCACCGTGGGCGACATCGGCCCCAAGTTCGGCTACGACGAGATTGACAACGGCTACC TGAAGATGGACAACCACCGAATTCCTCGAGAGAACATGCTGATGAAGTACGCCCAGGTGAAGCCC GACGGAACCTACGTGAAGCCCCTGTCTAACAAGCTGACCTACGGAACCATGGTGTTCGTGCGATC TTTCCTGGTCGGCGAGGCCGCTCGAGCCCTGTCCAAGGCCTGCACCATTGCCATCCGATACTCTGC

		CGTGCGACACCAGTCTGAGATCAAGCCCGGCGAGCCTGAGCCTCAGATCCTGGACTTTCAGACCC AGCAGTACAAGCTGTTCCCTCTGCTGGCCACCGCTTACGCCTTCCAGTTCGTGGGCGCCTACATGA AGGAAACCTACCATCGAATCAACGAAGGCATCGGCCAGGGCGACCTGTCTGAGCTGCCCGAACTG CACGCCCTGACCGCCGGACTGAAGGCTTTCACCTCTTGGACCGCCAACACCGGCATCGAGGCCTGC CGAATGGCCTGTGGCGGCCACGGCTACTCTCACTGCTCTGGACTGCCCAACATCTACGTGAACTTC ACCCCTTCGTGTACCTTCGAGGGCGAGAACACCGTGATGATGCTGCAGACCGCTCGATTCCTCAT GAAGTCTTACGACCAGGTGCACTCTGGCAAGCTGGTGTGCGGCATGGTGTCTTACCTGAACGATC TGCCCTCTCAGCGAATTCAGCCTCAGCAGGTTGCCGTGTGGCCCACCATGGTCGACATCAACTCTC CCGAGTCTCTGACCGAGGCCTACAAGCTGCGAGCTGCTCGACTGGTCGAGATCGCCGCCAAGAAC CTGCAGAAGGAAGTCATCCACCGAAAGTCTAAGGAAGTGGCTTGGAACCTGACCTCTGTGGACCT GGTGCGAGCTTCTGAGGCCCACTGCCACTACGTGGTGGTGAAGCTGTTCTCTGAGAAGCTGCTGA AGATCCAGGACAAGGCCATCCAGGCCGTGCTGCGATCTCTGTGCCTGCTGTACTCTCTGTACGGC ATCTCTCAGAACGCCGGCGACTTCCTGCAGGGCTCTATCATGACTGAGCCCCAGATTACCCAGGT CAACCAGCGAGTGAAGGAACTGCTCACCCTGATCCGATCTGACGCCGTGGCTCTGGTGGACGCCT TCGACTTTCAGGACGTGACCCTGGGCTCTGTGCTGGGCCGATACGACGGCAACGTGTACGAGAAC CTGTTCGAGTGGGCCAAGAACTCGCCCCTGAACAAGGCCGAGGTGCACGAGTCTTACAAGCACCT GAAGTCTCTGCAGTCTAAGCTGGACCAGATTACTTCTGTGGGATCTTCTTCGAAGCTGTAG
Sequence No. 10	Peroxisomal oxidase from *Paenarthrobacter ureafaciens*	ATGACCGAGGTGGTGGACCGAGCCTCTTCTCCCGCCTCTCCTGGCTCTACCACCGCCGCTGCCGAC GGCGCCAAGGTGGCCGTCGAGCCTCGAGTGGACGTGGCCGCTCTGGGCGAGCAGCTCCTCGGCCG ATGGGCCGACATCCGACTGCACGCCCGAGATCTGGCCGGACGAGAGGTGGTGCAGAAGGTCGAGG GACTGACCCACACCGAGCACCGATCTCGAGTGTTCGGCCAGCTGAAGTACCTGGTGGACAACAAC GCCGTGCACCGAGCTTTCCCTTCTCGACTCGGCGGATCTGACGACCACGGCGGCAACATTGCCGGC TTCGAGGAACTGGTGACTGCTGACCCCTCGCTGCAGATCAAGGCCGGCGTCCAGTGGGGCCTGTT CGGCTCTGCCGTGATGCACCTGGGCACCCGAGAGCACCACGACAAGTGGCTGCCCGGCATCATGT CTCTCGAGATCCCCGGCTGCTTCGCCATGACCGAGACTGGCCACGGCTCTGACGTGGCCTCTATCG CCACCACCGCCACCTACGACGAAGAGACTCAAGAGTTCGTGATCGACACACCCTTCCGAGCCGCCT GGAAGGACTACATCGGCAACGCCGCCAACGACGGCCTGGCCGCCGTCGTGTTCGCTCAGCTGATC ACCCGAAAGGTGAACCACGGCGTCCACGCCTTCTACGTGGACCTGCGAGATCCCGCCACCGGCGA CTTTCTGCCCGGAATCGGCGGCGAGGACGACGGCATCAAGGGCGGCCTGAACGGCATCGACAACG GACGACTGCACTTCACCAACGTGCGAATTCCCCGAACTAACCTGCTGAACCGATACGGCGACGTG GCTGTGGACGGCACCTACTCTTCTACCATCGAGTCTCCCGGCCGACGATTCTTCACCATGCTGGGA ACCCTGGTGCAGGGCCGAGTGTCTCTGGACGGCGCTGCCGTGGCCGCCTCTAAGGTGGCCCTGCA GTCTGCCATCCACTACGCCGCCGAGCGACGACAGTTCAACGCCACCTCTCCTACCGAGGAAGAGG TGCTGCTGGACTACCAGCGACATCAGCGACGACTGTTTACCCGACTGGCTACTACCTACGCTGCC TCTTTCGCCCACGAACAGCTGCTGCAAAAGTTCGACGACGTGTTCTCTGGCGCCCACGACACCGA CGCCGACCGACAGGACCTCGAGACTCTGGCTGCCGCTCTGAAGCCCCTGTCTACCTGGCACGCCCT GGACACCCTGCAAGAGTGCCGAGAGGCCTGCGGCGGAGCCGGCTTCCTGATCGAGAACCGATTCG CCTCTCTGCGAGCTGACCTGGACGTGTACGTGACCTTCGAGGGCGACAACACCGTGCTGCTGCAG CTGGTGGCCAAGCGACTGCTGGCCGACTACGCCAAGGAATTCCGAGGCGCCAACTTCGGCGTGCT GGCCCGATACGTCGTGGACCAGGCCGCTGGCGTGGCTCTGCACCGAACCGGCCTGCGACAGGTGG CCCAGTTCGTGGCCGACTCCGGCTCTGTGCAGAAGTCTGCCCTGGCTCTGCGAGATGAGGAAGGC CAGCGAACCCTGCTGACCGACCGAGTGCAGTCTATGGTGGCCGAGGTGGGCGCTGCCCTGAAGGG CGCTGGCAAGCTGCCCCAGCACCAGGCTGCTGCCCTGTTCAACCAGCATCAGAACGAGCTGATCG AGGCCGCTCAGGCCCACGCCGAGCTGCTCCAGTGGGAAGCCTTCACCGAGGCTCTGGCCAAGGTC GACGACGCCGGCACCAAGGAAGTGCTGACCCGACTGCGGGACCTGTTCGGACTGTCTCTGATTGA GAAGCACCTGTCTTGGTATCTGATGAACGGCCGACTGTCTATGCAGCGGGGACGAACCGTGGGCA CCTACATCAACCGACTGCTCGTGAAGATTCGACCCCACGCTCTGGACCTGGTCGACGCCTTCGGCT ACGGCGCTGAGCATCTGCGAGCCGCCATTGCCACCGGTGCCGAGGCCACTCGACAGGACGAGGCC CGAACCTACTTCCGACAGCAGCGAGCCTCTGGATCTGCCCCTGCCGACGAAAAGACCCTGCTGGCC ATTAAGGCCGGCAAGTCCCGAGATCAGATTACCTCTGTGGGATCTTCTTCGAAGCTGTAG
Sequence No. 11	Peroxisomal oxidase from *Rattus norvegicus*	ATGAACCCCGACCTGCGAAAGGAACGAGCCTCTGCCACTTTCAACCCCGAGCTGATCACCCACAT CCTGGACGGCTCTCCCGAGAACACCCGACGACGACGAGAAATCGAGAACCTGATCCTGAACGACC CCGACTTCCAGCACGAGGACTACAACTTTCTGACCCGATCTCAGCGATACGAGGTGGCCGTGAAG AAGTCTGCCACCATGGTCAAGAAGATGCGAGAGTACGGCATCTCTGACCCCGAAGAGATCATGTG GTTCAAGAACTCTGTGCACCGAGGACACCCTGAGCCTCTGGACCTGCACCTGGGCATGTTTCTGC CCACTCTGCTGCACCAGGCTACCGCCGAGCAGCAAGAGCGATTCTTCATGCCCGCCTGGAACCTCG AGATCACCGGCACCTACGCTCAGACCGAGATGGGCCACGGCACCCACCTCCGAGGACTCGAGACT ACCGCCACTTACGACCCCAAGACTCAAGAGTTCATCCTGAACTCTCCCACCGTGACCTCTATCAAG TGGTGGCCCGGTGGCCTGGGCAAGACCTCTAACCACGCCATCGTGCTGGCCCAGCTGATTACCCA GGGCGAGTGCTACGGCCTGCACGCCTTCGTGGTGCCCATCCGAGAGATCGGAACCCACAAGCCAC TGCCTGGCATCACCGTGGGCGACATCGGCCCCAAGTTCGGCTACGAGGAAATGGACAACGGCTAC CTGAAGATGGACAACTACCGAATTCCTCGAGAGAACATGCTGATGAAGTACGCCCAGGTGAAGCC CGACGGAACCTACGTGAAGCCCCTGTCTAACAAGCTGACCTACGGAACCATGGTGTTCGTGCGAT CTTTCCTGGTGGGCAACGCCGCTCAGTCTCTGTCTAAGGCCTGCACCATTGCCATCCGATACTCTG CCGTGCGACGACAGTCTGAGATCAAGCAGTCTGAGCCCGAGCCTCAGATCCTGGACTTTCAGACC CAGCAGTACAAGCTGTTCCCTCTGCTGGCCACCGCCTACGCCTTCCACTTCGTGGGCCGATATATG AAGGAAACCTACCTGCGAATCAACGAGTCTATCGGCCAGGGCGACCTGTCTGAGCTGCCCGAGCT GCACGCCCTGACCGCCGGACTGAAGGCTTTCACCACCTGGACCGCCAACGCCGGCATCGAGGAAT GCCGAATGGCCTGTGGCGGCCACGGCTACTCTCACTCCTCTGGCATCCCCAACATCTACGTGACCT TCACTCCCGCCTGCACCTTCGAGGGTGAGAACACCGTGATGATGCTGCAGACCGCTCGATTCCTG ATGAAGATCTACGACCAGGTGCGATCTGGCAAGCTGGTCGGCGGCATGGTGTCTTACCTGAACGA TCTGCCCTCTCAGCGAATTCAGCCTCAGCAGGTTGCCGTGTGGCCCACTATGGTGGACATCAACT CGCTCGAGGGCCTGACCGAGGCCTACAAGCTGCGAGCCGCTCGACTGGTCGAGATCGCCGCCAAG AACCTGCAGACCCACGTGTCTCACCGAAAGTCTAAGGAAGTGGCTTGGAACCTGACCTCTGTGGA

		CCTGGTGCGAGCTTCTGAGGCCCACTGCCACTACGTGGTGGTGAAGGTGTTCTCTGACAAGCTGC CCAAGATCCAGGACAAGGCTGTCCAGGCCGTGCTGCGAAACCTGTGCCTGCTGTACTCTCTGTAC GGAATCTCTCAGAAGGGCGGCGACTTCCTCGAGGGCTCTATCATCACCGGCGCTCAGCTGTCTCA GGTCAACGCTCGAATCCTCGAGCTGCTGACCCTGATTCGACCCAACGCCGTGGCTCTGGTGGACG CTTTCGACTTCAAGGACATGACCCTGGGCTCTGTGCTGGGACGATACGACGGCAACGTGTACGAG AACCTCTTCGAGTGGGCCAAGAAGTCTCCCCTGAACAAGACCGAGGTGCACGAGTCTTACCACAA GCACCTGAAGCCTCTGCAGTCTAAGCTGGACCAGATTACCTCCGTGGGATCTTCTTCGAAGCTGT AG
Sequence No. 12	Humanized renilla green fluorescent protein	ATGGTGAGCAAGCAGATCCTGAAGAACACCTGCCTGCAGGAGGTGATGAGCTACAAGGTGAACCT GGAGGGCATCGTGAACAACCACGTGTTCACCATGGAGGGCTGCGGCAAGGGCAACATCCTGTTCG GCAACCAGCTGGTGCAGATCCGCGTGACCAAGGGCGCCCCCCTGCCCTTCGCCTTCGACATCGTG AGCCCCGCCTTCCAGTACGGCAACCGCACCTTCACCAAGTACCCCAACGACATCAGCGACTACTT CATCCAGAGCTTCCCCGCCGGCTTCATGTACGAGCGCACCCTGCGCTACGAGGACGGCGGCCTGG TGGAGATCCGCAGCGACATCAACCTGATCGAGGACAAGTTCGTGTACCGCGTGGAGTACAAGGGC AGCAACTTCCCCGACGACGGCCCCGTGATGCAGAAGACCATCCTGGGCATCGAGCCCAGCTTCGA GGCCATGTACATGAACAACGGCGTGCTGGTGGGCGAGGTGATCCTGGTGTACAAGCTGAACAGCG GCAAGTACTACAGCTGCCACATGAAGACCCTGATGAAGAGCAAGGGCGTGGTGAAGGAGTTCCCC TCCTACCACTTCATCCAGCACCGCCTGGAGAAGACCTACGTGGAGGACGGCGGCTTCGTGGAGCA GCACGAGACCGCCATCGCCCAGATGACCAGCATCGGCAAGCCCCTGGGCAGCCTGCACGAGTGGG TGTAA
Sequence No. 13	mCherry	ATGGTGTCTAAGGGCGAAGAGGACAACATGGCCATCATCAAGGAATTCATGCGATTCAAGGTGC ACATGGAAGGCTCTGTGAACGGCCACGAGTTCGAGATCGAAGGCGAAGGCGAGGGACGACCCTAC GAGGGCACCCAGACCGCCAAGCTGAAGGTGACCAAGGGCGGACCCCTGCCTTTCGCCTGGGACAT TCTGTCTCCCCAGTTCATGTACGGCTCTAAGGCCTACGTGAAGCACCCCGCCGACATTCCCGACTA CCTGAAGCTGTCGTTCCCCGAGGGCTTCAAGTGGGAGCGAGTGATGAACTTCGAGGACGGCGGCG TGGTGACCGTGACTCAGGACTCTTCGCTGCAGGACGGCGAGTTCATCTACAAGGTGAAGCTGCGA GGCACCAACTTTCCCTCTGACGGCCCCGTGATGCAAAAGAAGACCATGGGCTGGGAAGCCTCTTC TGAGCGAATGTACCCCGAGGACGGTGCCCTGAAGGGCGAGATCAAGCAGCGACTGAAGCTCAAGG ACGGTGGCCACTACGACGCCGAGGTCAAGACCACCTACAAGGCCAAGAAGCCCGTCCAGCTGCCT GGCGCCTACAACGTGAACATCAAGCTGGACATCACCTCTCACAACGAGGACTACACCATCGTCGA GCAGTACGAGCGAGCCGAGGGCCGACACTCTACCGGCGGCATGGACGAGCTGTACAAGTAG

Supplementary table S3. Biobricks used in this study

ID	Description	Forward primer	Reversed primer	Template DNA	Reference
BB8302	{-PrTEF1intron	PR-18928	PR-18975	Genomic DNA of ST4840	This study
BB2068	Har_FAR_Ylop->	PR-18066	PR-16595	Sequence No. 1	This study
BB2720	{-PrTEF1intron_USER_forfusion	PR-18930	PR-18214	Genomic DNA of ST4840	This study
BB8644	PrGPD_forfusion-}	PR-23004	PR-22213	Genomic DNA of ST4840	This study
BB8640	Dmd9{-PrTEF1intron_forfusion	PR-19018	PR-18930	Sequence No. 2	This study
BB2693	{-Lbo_PPTQ	PR-21723	PR-21724	Sequence No. 3	This study
BB1135	Vector backbone	See ref.	See ref.	See ref.	Holkenbrink 2018
BB8769	{-Ase_POX	PR-23435	PR-23436	Sequence No. 4	This study
BB2709	Lbo31670->	PR-21755	PR-21756	Sequence No. 5	This study
BB1558	PrExp-}	PR-15521	PR-15522	Genomic DNA of ST4840	This study
BB2710	Lbo49554->	PR-21757	PR-21758	Sequence No. 6	This study
BB1635	PrtRNA-Gly	See ref.	See ref.	See ref.	Holkenbrink 2018
BB1636	crRNA-TRPR	See ref.	See ref.	See ref.	Holkenbrink 2018
BB8516	{-Yli_POX2	PR-22827	PR-22828	Genomic DNA of ST4840	This study
BB8517	{-Yli_POX3	PR-22829	PR-22830	Genomic DNA of ST4840	This study
BB8519	{-Yli_POX5	PR-22833	PR-22834	Genomic DNA of ST4840	This study
BB8523	{-Ani_POX	PR-22841	PR-22842	Sequence No. 7	This study
BB8524	{-Cma_POX	PR-22843	PR-22844	Sequence No. 8	This study
BB8525	{-Hsa_POX	PR-22845	PR-22846	Sequence No. 9	This study
BB8526	{-Pur_POX	PR-22847	PR-22848	Sequence No. 10	This study
BB8527	{-Rno_POX	PR-22849	PR-22850	Sequence No. 11	This study
BB1688	->PrTEF1intron	See ref.	See ref.	See ref.	Petkevicius 2021

BB1740	Har_FAR_codoptYL	See ref.	See ref.	See ref.	Holkenbrink 2020
BB9309	PrTEF1intron_HarFAR_Per2	PR-10595	PR-24919	pBP8236	This study
BB8682	IntD_2_dwn	PR-23172	PR-23173	Genomic DNA of ST4840	This study
BB8681	IntD_2_up	PR-23171	PR-23170	Genomic DNA of ST4840	This study
BB2313	Fas2 (I1220F)	See ref.	See ref.	See ref.	Petkevicius 2021
BB8679	IntE_7_up	PR-23167	PR-23166	Genomic DNA of ST4840	This study
BB8680	IntE_7_dwn	PR-23168	PR-23169	Genomic DNA of ST4840	This study
BB1631	TPex20-TLip2	See ref.	See ref.	See ref.	Holkenbrink 2018
BB8386	IntF_5_Up	PR-22532	PR-22533	Genomic DNA of ST4840	This study
BB8387	IntF_5_Down	PR-22534	PR-22535	Genomic DNA of ST4840	This study
BB10144	PrTEF1intronHarFARforhrGFP	PR-10595	PR-26919	pBP8236	This study
BB10145	hrGFPforHarFAR	PR-26920	PR-15506	Sequence No. 12	This study
BB10146	hrGFPforHarFAR_Per2	PR-26920	PR-26921	Sequence No. 12	This study
BB2093	PrTEF1intron_USER-}	See ref.	See ref.	See ref.	Petkevicius 2021
BB10154	YliPOX3formCherry	PR-24936	PR-26932	Genomic DNA of ST4840	This study
BB10155	YliALE1formCherry	PR-26926	PR-26933	Genomic DNA of ST4840	This study
BB10156	mCherryforYliPOX3	PR-26934	PR-26936	Sequence No. 13	This study
BB10157	mCherryforYliALE1	PR-26935	PR-26936	Sequence No. 13	This study

Supplementary table S4. Plasmids used in this study

ID	Description	Parent plasmid	Biobricks/primers	Reference
pCfB6630	pNat-YLgRNA3_IntC_3	See ref.	See ref.	Holkenbrink 2018
pBP8754	pIntF_3-Ase_POX_GeneArt{-PrEXP	pBP8009	BB2721, BB8769	This study
pBP8627	pIntD_2-Dmd9{-PrTEF1intron-PrGPD-}HarFAR	pBP8620	BB8640, BB2720, BB8644, BB2068	This study
pBP8400	pIntC_3-TPex20-PrEXP-}Lbo31670-TLip2	pCfB6371	BB2709, BB1558	This study
pBP8401	pIntC_3-TPex20-PrEXP-}Lbo49554-TLip2	pCfB6371	BB2710, BB1558	This study
pBP8802	pIntD_2-Lbo_PPTQ{-PrTEF1intron-PrGPD-}HarFAR	pBP8620	BB2693, BB2720, BB8644, BB2068	This study
pCfB7088	pNat-YLgRNA1_Fas2 (AA1220)	See ref.	See ref.	Holkenbrink 2020
pBP8900	pHph_YLgRNA5_IntE_4	pCfB3431	BB1635, BB1636, PR-23285, PR-23286	This study
pBP8340	pIntC_3-TPex20-Yli_POX2{-PrTEF1-TLip2	pCfB6371	BB8516, BB8302	This study
pBP8341	pIntC_3-TPex20-Yli_POX3{-PrTEF1-TLip2	pCfB6371	BB8517, BB8302	This study
pBP8343	pIntC_3-TPex20-Yli_POX5{-PrTEF1-TLip2	pCfB6371	BB8519, BB8302	This study
pBP8347	pIntC_3-TPex20-Ani_POX{-PrTEF1-TLip2	pCfB6371	BB8523, BB8302	This study
pBP8348	pIntC_3-TPex20-Cma_POX{-PrTEF1-TLip2	pCfB6371	BB8524, BB8302	This study
pBP8349	pIntC_3-TPex20-Hsa_POX{-PrTEF1-TLip2	pCfB6371	BB8525, BB8302	This study
pBP8350	pIntC_3-TPex20-Pur_POX{-PrTEF1-TLip2	pCfB6371	BB8526, BB8302	This study
pBP8351	pIntC_3-TPex20-Rno_POX{-PrTEF1-TLip2	pCfB6371	BB8527, BB8302	This study
pBP8003	pNat-YLgRNA4_IntF_3	pCfB3405	BB1635, BB1636, PR-22039, PR-22040	This study
pBP8623	pNat_YLgRNA1_IntD_2	pCfB3405	BB8736	This study
pBP8576	pHph_YLgRNA1_IntD_2	pCfB3431	BB1635, BB1636, PR-23192, PR-23193	This study

pBP8032	pHph-YLgRNA3_IntC_3	pCfB3431	BB1635, BB1636, PR-18239, PR-18240	This study
pBP8236	pIntE_4-PrTEF1intron->HarFAR	pCf6679	BB1688, BB1740	This study
pBP9438	pIntE_4_PrTEF1intron_HarFAR_Per2	pCf6679	BB9309	This study
pBP8009	pIntF_3-TPex20-TLip2		BB1135, BB1631, BB8031, BB1480	This study
pBP6371	pIntC_3-TPex20-TLip2	See ref.	See ref.	Holkenbrink 2018
pBP3405	pORI1001-Nat-CEN1-USER	See ref.	See ref.	Holkenbrink 2018
pBP3431	pORI1001-Hphsyn-CEN1-USER	See ref.	See ref.	Holkenbrink 2020
pBP6679	pIntE_4-TPex20-TLip2	See ref.	See ref.	Holkenbrink 2018
pBP8620	pIntD_2-TPex20-TLip2		BB1135, BB8682, BB8681	This study
pBP8575	pNat_YLgRNA1_IntE_7		BB1635, BB1636, PR-23190, PR-23191	This study
pBP8645	pHph_YLgRNA4_IntF_5		BB1635, BB1636, PR-23127, PR-23128	This study
pBP8662	pIntE_7-TPex20-TLip2		BB1135 ,BB8679, BB8680, BB1631	This study
pBP8263	IntF_5_Up_TPex20-USER-TLip2_IntF_5_Down		BB1135 ,BB8386, BB1631, BB8387	This study
pBP10672	pIntE_7-TPex20-PrTEF1intron_HarFAR_hrGFP-TLip2	pBP8662	BB10144, BB10145	This study
pBP10669	pIntE_7-TPex20- PrTEF1intron _HarFAR_hrGFP_Per2-TLip2	pBP8662	BB10144, BB10146	This study
pBP10676	IntF_5_Up_TPex20- PrTEF1intron _YliPOX3_mCherry-TLip2_IntF_5_Down	pBP8263	BB2093, BB10154, BB10156	This study
pBP10677	IntF_5_Up_TPex20- PrTEF1intron _YliALE1_mCherry-TLip2_IntF_5_Down	pBP8263	BB2093, BB10155, BB10157	This study

Supplementary table S5. Strains used in this study

ID	Relevant features	Parent strain	Added elements	Reference/source
ST4840	Wild-type *Yarrowia lipolytica*			Agricultural Research Service (NRRL, USA)
ST6629	See ref.	See ref.	See ref.	Holkenbrink 2020
ST8524	See ref.	See ref.	See ref.	Petkevicius 2021
ST9138	ΔPOX1-6	See ref.	See ref.	Patent application WO/2020/169389
ST9199	ΔPOX1-6, IntC_3-Yli_POX2{-TEF1	ST9138	pCfB6630, pBP8340	This study
ST9200	ΔPOX1-6, IntC_3-Yli_POX3{- TEF1	ST9138	pCfB6630, pBP8341	This study
ST9202	ΔPOX1-6, IntC_3-Yli_POX5{- TEF1	ST9138	pCfB6630, pBP8343	This study
ST9206	ΔPOX1-6, IntC_3-Ani_POX{- TEF1	ST9138	pCfB6630, pBP8347	This study
ST9207	ΔPOX1-6, IntC_3-Cma_POX{- TEF1	ST9138	pCfB6630, pBP8348	This study
ST9208	ΔPOX1-6, IntC_3-Hsa_POX{- TEF1	ST9138	pCfB6630, pBP8349	This study
ST9209	ΔPOX1-6, IntC_3-Pur_POX{- TEF1	ST9138	pCfB6630, pBP8350	This study
ST9210	ΔPOX1-6, IntC_3-Rno_POX{- TEF1	ST9138	pCfB6630, pBP8351	This study
ST9284	ΔPOX1-6, IntF_3-Ase_POX{-PrEXP	ST9138	pBP8754, pBP8003	This study
ST9294	ΔPOX1-6, IntD_2-Dmd9{-PrTEF1intron-PrGPD-}HarFAR	ST9138	pBP8627, pBP8623	This study
ST9295	ΔPOX1-6, IntC_3-Yli_POX2{-PrEXP, IntD_2-Dmd9{-PrTEF1intron-PrGPD-}HarFAR	ST9199	pBP8627, pBP8576	This study
ST9296	ΔPOX1-6, IntC_3-Yli_POX3{-PrEXP, IntD_2-Dmd9{-PrTEF1intron - PrGPD-}HarFAR	ST9200	pBP8627, pBP8576	This study
ST9297	ΔPOX1-6, , IntC_3-Yli_POX5{-PrEXP IntD_2-Dmd9{- PrTEF1intron-PrGPD-}HarFAR	ST9202	pBP8627, pBP8576	This study
ST9298	ΔPOX1-6, IntC_3-Ani_POX{-PrEXP, IntD_2-Dmd9{-PrTEF1intron-PrGPD-}HarFAR	ST9206	pBP8627, pBP8576	This study
ST9299	ΔPOX1-6, IntC_3-Cma_POX{-PrEXP, IntD_2-Dmd9{- PrTEF1intron-PrGPD-}HarFAR	ST9207	pBP8627, pBP8576	This study
ST9300	ΔPOX1-6, IntC_3-Hsa_POX{-PrEXP, IntD_2-Dmd9{- PrTEF1intron-PrGPD-}HarFAR	ST9208	pBP8627, pBP8576	This study

ST9301	ΔPOX1-6, IntC_3-Pur_POX{-PrEXP, IntD_2-Dmd9{- PrTEF1intron-PrGPD-}HarFAR	ST9209	pBP8627, pBP8576	This study
ST9302	ΔPOX1-6, IntC_3-Rno_POX{-PrEXP, IntD_2-Dmd9{- PrTEF1intron-PrGPD-}HarFAR	ST9210	pBP8627, pBP8576	This study
ST9329	ΔPOX1-6, IntF_3-Ase_POX{-PrEXP, IntD_2-Dmd9{- PrTEF1intron-PrGPD-}HarFAR	ST9284	pBP8627, pBP8576	This study
ST9347	ΔPOX1-6, IntD_2-Dmd9{-PrTEF1intron-PrGPD-}HarFAR, IntC_3-PrEXP-}Lbo_31670	ST9294	pBP8400, pBP8032	This study
ST9348	ΔPOX1-6, IntD_2-Dmd9{-PrTEF1intron-PrGPD-}HarFAR, IntC_3-PrEXP-}Lbo_49554	ST9294	pBP8401, pBP8032	This study
ST9314	ΔPOX1-6, ΔPOX1-6, IntD_2-Lbo_PPTQ{- PrTEF1intron -PrGPD-}HarFAR	ST9138	pBP8802, pBP8623	This study
ST9315	ΔPOX1-6, IntC_3-Yli_POX2{-PrEXP, IntD_2-Lbo_PPTQ{- PrTEF1intron-PrGPD-}HarFAR	ST9199	pBP8802, pBP8576	This study
ST9316	ΔPOX1-6, IntC_3-Yli_POX3{-PrEXP, IntD_2-Lbo_PPTQ{- PrTEF1intron-PrGPD-}HarFAR	ST9200	pBP8802, pBP8576	This study
ST9317	ΔPOX1-6, IntC_3-Yli_POX5{-PrEXP, IntD_2-Lbo_PPTQ{- PrTEF1intron-PrGPD-}HarFAR	ST9202	pBP8802, pBP8576	This study
ST9318	ΔPOX1-6, IntC_3-Ani_POX{-PrEXP, IntD_2-Lbo_PPTQ{- PrTEF1intron-PrGPD-}HarFAR	ST9206	pBP8802, pBP8576	This study
ST9319	ΔPOX1-6, IntC_3-Cma_POX{-PrEXP, IntD_2-Lbo_PPTQ{-PrTEF1intron-PrGPD-}HarFAR	ST9207	pBP8802, pBP8576	This study
ST9320	ΔPOX1-6, IntC_3-Hsa_POX{-PrEXP, IntD_2-Lbo_PPTQ{- PrTEF1intron-PrGPD-}HarFAR	ST9208	pBP8802, pBP8576	This study
ST9321	ΔPOX1-6, IntC_3-Pur_POX{-PrEXP, IntD_2-Lbo_PPTQ{- PrTEF1intron-PrGPD-}HarFAR	ST9209	pBP8802, pBP8576	This study
ST9322	ΔPOX1-6, IntC_3-Rno_POX{-PrEXP, IntD_2-Lbo_PPTQ{- PrTEF1intron-PrGPD-}HarFAR	ST9210	pBP8802, pBP8576	This study
ST9330	ΔPOX1-6, IntF_3-Ase_POX{-PrEXP, IntD_2-Lbo_PPTQ{- PrTEF1intron-PrGPD-}HarFAR	ST9284	pBP8802, pBP8576	This study
ST9350	ΔPOX1-6, IntD_2-Lbo_PPTQ{-PrTEF1intron -PrGPD-}HarFAR, IntC_3-PrEXP-}Lbo_31670	ST9314	pBP8400, pBP8032	This study
ST9351	ΔPOX1-6, IntD_2-Lbo_PPTQ{-PrTEF1intron -PrGPD-}HarFAR, IntC_3-PrEXP-}Lbo_49554	ST9314	pBP8401, pBP8032	This study
ST10313	ΔPOX1-6, IntD_2-Dmd9{-PrTEF1intron -PrGPD-}HarFAR, IntC_3-PrEXP-}Lbo_31670, FAS2 (I1220F)	ST9347	pCfB7088, BB8908	This study
ST10383	ΔPOX1-6, IntD_2-Dmd9{-PrTEF1intron -PrGPD-}HarFAR, IntC_3-PrEXP-}Lbo_31670, FAS2 (I1220F), IntE_4- PrTEF1intron ->HarFAR	STST10313	pBP8900, pBP8236	This study
ST10384	ΔPOX1-6, IntD_2-Dmd9{-PrTEF1intron -PrGPD-}HarFAR, IntC_3-PrEXP-}Lbo_31670, FAS2 (I1220F), IntE_4_ PrTEF1intron _HarFAR_Per2	STST10313	pBP8900, pBP9438	This study
ST10314	ΔPOX1-6, IntD_2-Lbo_PPTQ{-PrTEF1intron -PrGPD-}HarFAR, IntC_3-PrEXP-}Lbo_31670, FAS2 (I1220F)	ST9350	pCfB7088, BB8908	This study
ST10387	ΔPOX1-6, IntD_2-Lbo_PPTQ{-PrTEF1intron -PrGPD-}HarFAR, IntC_3-PrEXP-}Lbo_31670, FAS2	ST10314	pBP8900, pBP8236	This study

	(I1220F), IntE_4- PrTEF1intron ->HarFAR			
ST10388	ΔPOX1-6, IntD_2-Lbo_PPTQ{-PrTEF1intron -PrGPD-}HarFAR, IntC_3-PrEXP-}Lbo_31670, FAS2 (I1220F), IntE_4- PrTEF1intron ->HarFAR_Per2	ST10314	pBP8900, pBP9438	This study
ST12413	ΔPOX1-6, IntE_7- PrTEF1intron _HarFAR_hrGFP	ST9138	pBP8575, pBP10672	This study
ST12410	ΔPOX1-6, IntE_7- PrTEF1intron _HarFAR_hrGFP_Per2	ST9138	pBP8575, pBP10669	This study
ST12433	ΔPOX1-6, IntE_7- PrTEF1intron _HarFAR_hrGFP, IntF_5-PrTEF1intron _YliPOX3_mCherry-	ST12413	pBP8645, pBP10676	This study
ST12434	ΔPOX1-6, IntE_7- PrTEF1intron _HarFAR_hrGFP, IntF_5-PrTEF1intron _YliALE1_mCherry-	ST12413	pBP8645, pBP10677	This study
ST12424	ΔPOX1-6, IntE_7- PrTEF1intron _HarFAR_hrGFP_Per2, IntF_5-PrTEF1intron _YliPOX3_mCherry-TLip2_IntF_5_Down	ST12410	pBP8645, pBP10676	This study
ST12425	ΔPOX1-6, IntE_7- PrTEF1intron _HarFAR_hrGFP_Per2, IntF_5-PrTEF1intron _YliALE1_mCherry-TLip2_IntF_5_Down	ST12410	pBP8645, pBP10677	This study

Supplementary materials and methods relevant for generation of data presented in supplementary table S6 and supplementary figure S7.

Y. lipolytica strains were inoculated into 37.5 mL of YPG media in 250 mL shake flasks at OD_{600}=0.2 and cultivated for 22 h at 28°C, shaken at 300 rpm at 5 cm orbit cast. Cell growth over time was monitored by measuring optical density using GENESYS™ 10S UV-Vis Spectrophotometer (Thermo Scientific). After 22 h of growth, cultivation broth was transferred into 50 mL falcon tube, centrifuged for 5 min at room temperature at 3000x*g*. The supernatant was discarded, and the cells resuspended in 20 mL production medium. Resuspended cells were transferred back to shake flasks and 0.4% (v/v) of 14:Me supplied. For cell dry weight and glycerol measurements, 1 mL cultivation broth was taken and centrifuged for 5 min at room temperature at 12500x*g*. Supernatant was used for glycerol measurements. Cell pellet was resuspended in 1 mL 70% ethanol and centrifuged for 5 min at room temperature at 12500x*g*. Liquid was removed and pellet was kept for 72 h in the 70°C degrees oven before weight measurements. Glycerol was measured using Randox GY105 glycerol assay. Quantification of 14:Me and Z7-12:OH was performed using GC-FID under the same settings as for data presented in Figures S1 and S2. Quantification of Z9-12:OH was performed under the same settings as for data presented in Figure 2.

Supplementary table S6. Titers and specific yields (g of product/g of dry weight) of Z7-12:OH, Z9-12:OH and Z7-14:OH at the end of shake flask cultivations (52h after media exchange).

Product / Strain	Z7-12:OH	Z9-12:OH	Z7-14:OH
ST10384	1.13±0.05 mg/L Y_{xp}=4.8x10^{-5}±4.4x10^{-6}	0 mg/L Y_{xp}=0	0.15±0.04 mg/L Y_{xp}=6.4x10^{-6}±2.1x10^{-6}
ST10388	0 mg/L Y_{xp}=0	0.33±0.07 mg/L $Y_{xp=}$1.1x10^{-5}±1.9x10^{-6}	0.13±0.06 mg/L $Y_{xp=}$4.3x10^{-6}±2.2x10^{-6}

Supplementary references:

Holkenbrink C, Dam MI, Kildegaard KR *et al*. EasyCloneYALI: CRISPR/Cas9-Based Synthetic Toolbox for Engineering of the Yeast *Yarrowia lipolytica*. *Biotechnol J* 2018; 13:1–8.

Holkenbrink C, Ding BJ, Wang HL, *et al*. Production of moth sex pheromones for pest control by yeast fermentation. *Metab Eng* 2020, DOI:10.1101/2020.07.15.205047.

Petkevicius K, Koutsoumpeli E, Betsi PC *et al*. Biotechnological production of the European corn borer sex pheromone in the yeast *Yarrowia lipolytica*. *Biotechnol J* 2021, DOI:10.1002/biot.202100004

Chromatogram number	Strain ID	FAD	FAR	Peroxisomal oxidase
1	ST9294	Dmd9	HarFAR	-
2	ST9295			YliPOX2
3	ST9296			YliPOX3
4	ST9297			YliPOX5
5	ST9298			AniPOX
6	ST9299			CmaPOX
7	ST9300			HsaPOX
8	ST9301			PurPOX
9	ST9302			RnoPOX
10	ST9329			AsePOX
11	ST9347			Lbo_31670
12	ST9348			Lbo_49554

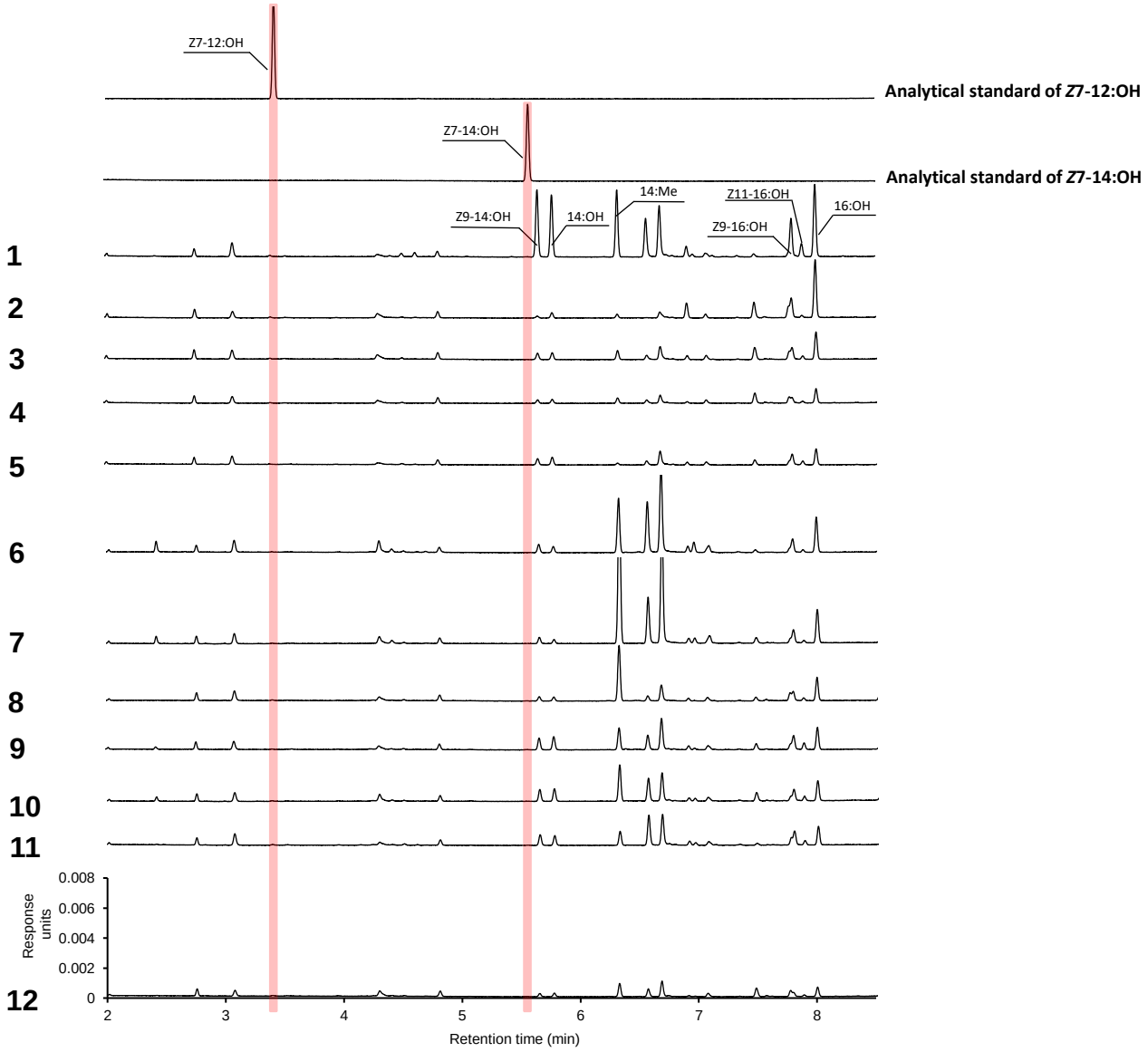

Figure S1. Fatty alcohol profiles obtained from the *Y. lipolytica* strains containing Dmd9, HarFAR and different POXes. Cultivation media was supplemented with 0.24% (v/v) of 14:Me.

Chromatogram number	Strain ID	FAD	FAR	Peroxisomal oxidase
1	ST9314	Lbo_PPTQ	HarFAR	-
2	ST9315			YliPOX2
3	ST9316			YliPOX3
4	ST9317			YliPOX5
5	ST9318			AniPOX
6	ST9319			CmaPOX
7	ST9320			HsaPOX
8	ST9321			PurPOX
9	ST9322			RnoPOX
10	ST9330			AsePOX
11	ST9350			Lbo_31670
12	ST9351			Lbo_49554

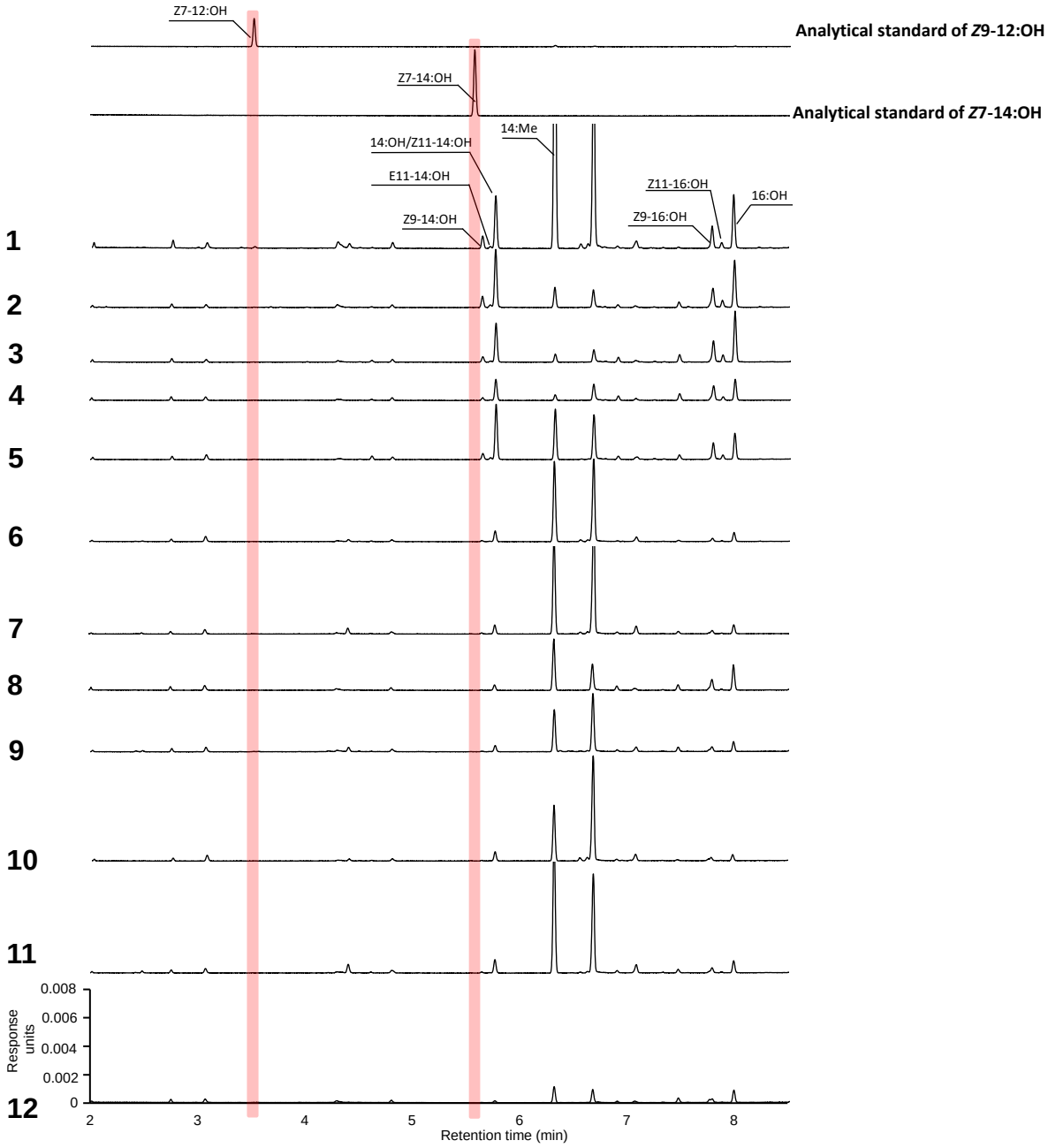

Figure S2. Fatty alcohol profiles obtained from the *Y. lipolytica* strains containing Lbo_PPTQ, HarFAR and different POXes. Cultivation media was supplemented with 0.24% (v/v) of 14:Me.

% of Z7-12:Me in total FAMEs	0.004	0.032	0	0	0	0	0.185	0.093	0.195	0.046	0.036	0.370	0.0.03
% of Z9-12:Me in total FAMEs	0	0	0	0	0	0	0	0	0	0	0	0	0
% of Z7-14:Me in total FAMEs	0	0	0	0	0	0	0.023	0.012	0.014	0.021	0	0.037	0.032

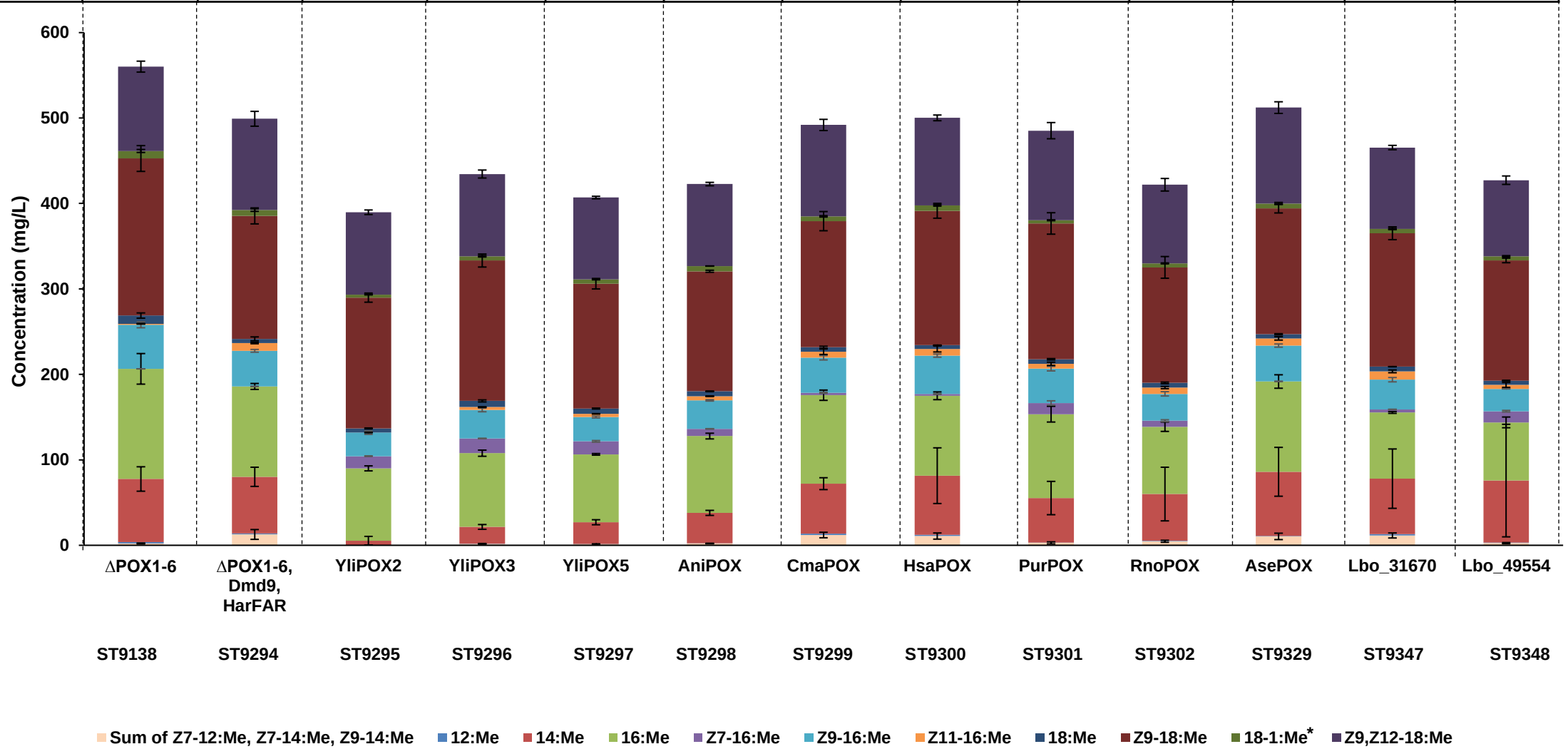

Figure S3. Fatty acid profiles in the form of methyl esters obtained from the *Y. lipolytica* strains containing Dmd9, HarFAR and different POXes. Cultivation media was supplemented with 0.24% (v/v) of 14:Me. *: Position of double bond remains to be identified. Error bars represent standard deviations from three technical replicates

% of Z7-12:Me in total FAMEs	0	0	0	0	0	0	0	0	0	0	0.014	0
% of Z9-12:Me in total FAMEs	0.037	0	0	0	0	0.037	0.090	0.010	0.029	0.081	0.126	0.011
% of Z7-14:Me in total FAMEs	0	0	0	0	0	0.006	0.006	0.005	0.015	0	0.010	0.014

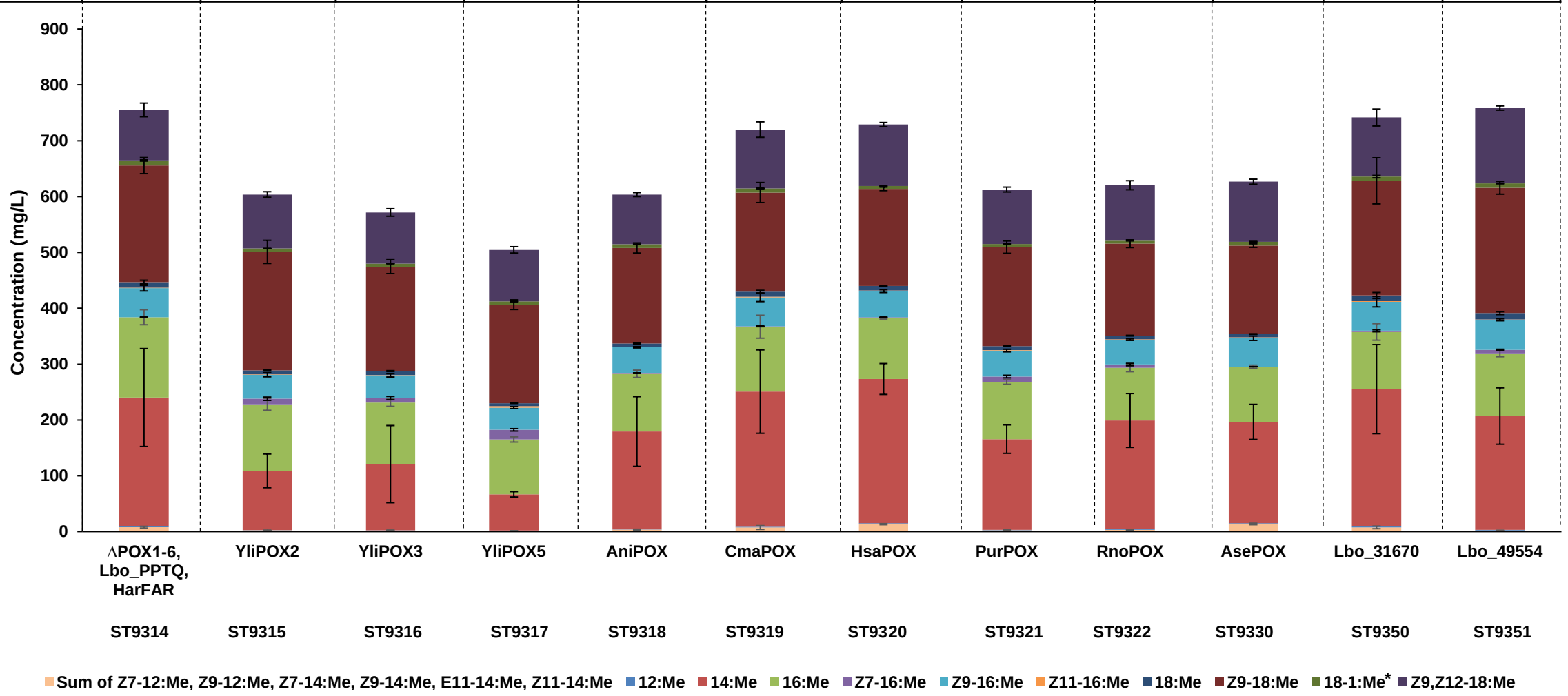

Figure S4. Fatty acid profiles in the form of methyl esters obtained from the *Y. lipolytica* strains containing Lbo_PPTQ, HarFAR and different POXes. Cultivation media was supplemented with 0.24% (v/v) of 14:Me. *: Position of double bond remains to be identified. Error bars represent standard deviations from three technical replicates

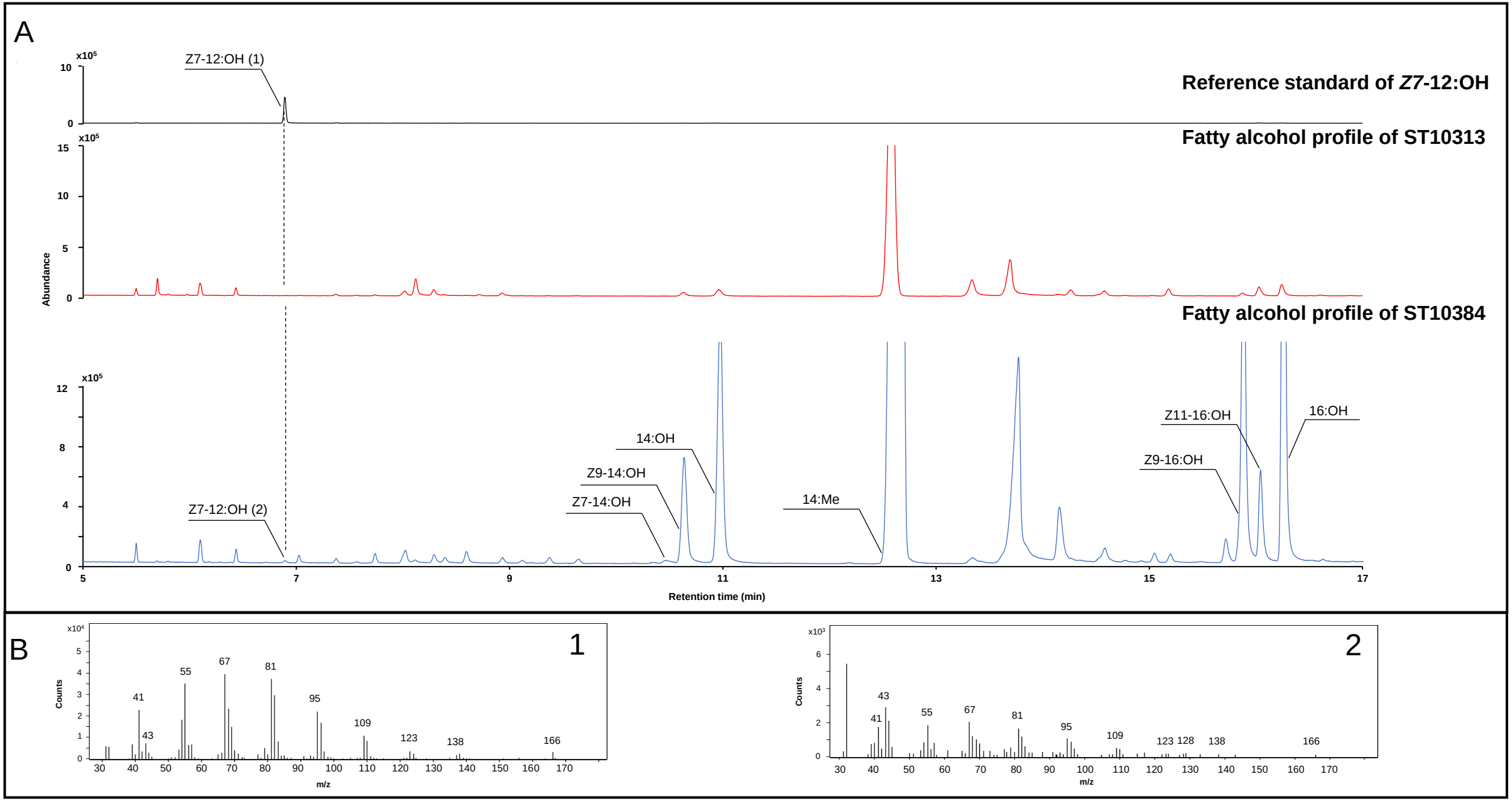

Figure S5.A. GC-MS chromatograms of Z7-12:OH reference standard and fatty alcohol profiles of ST10313 (red) and ST10384 (blue). B. Mass spectra of Z7-12:OH reference standard (1) and biologically produced Z7-12:OH (2). Cultivation media was supplemented with 0.4% (v/v) of 14:Me.

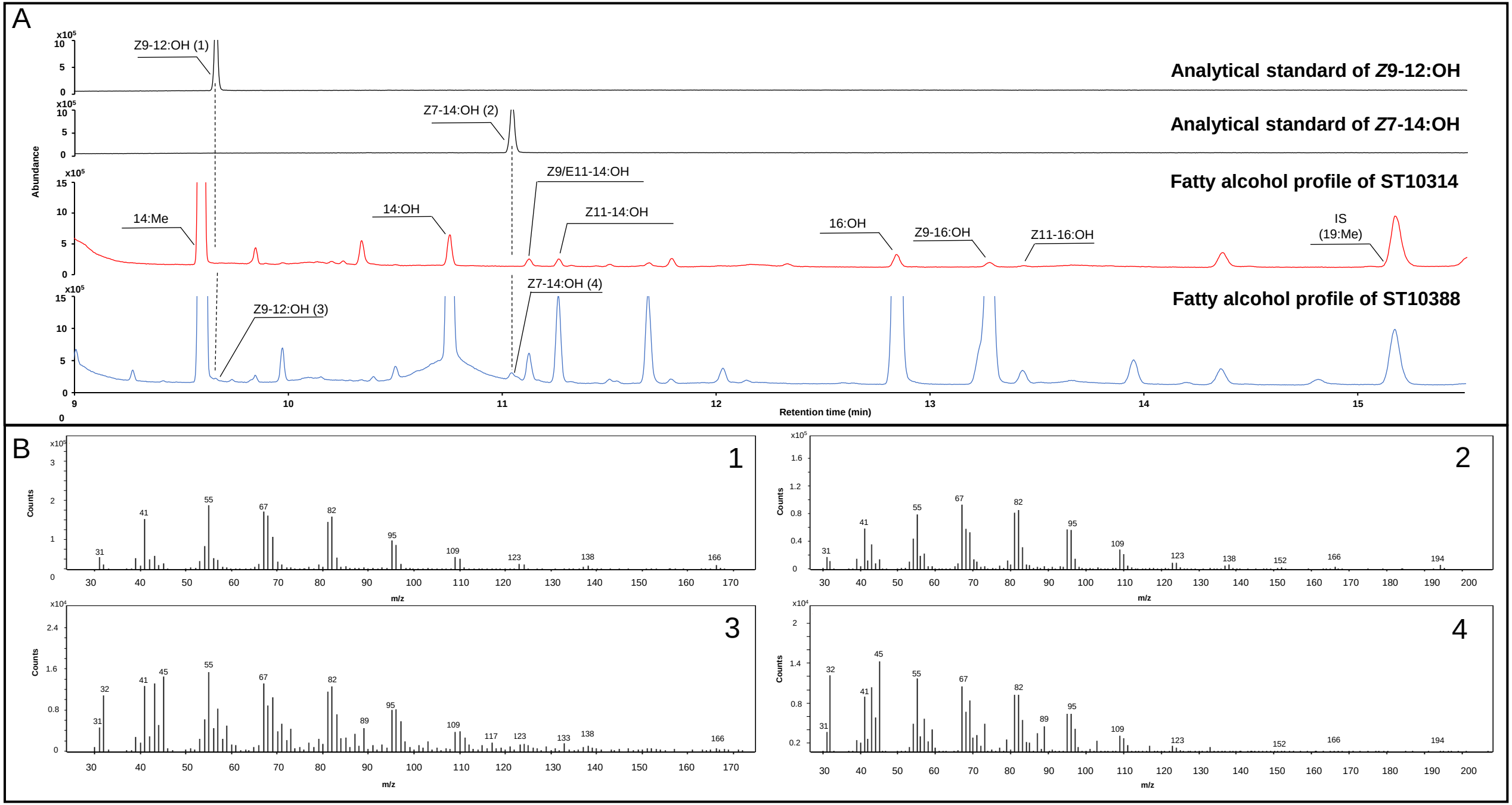

Figure S6.A. GC-MS chromatograms of Z9-12:OH and Z7-14:OH reference standards together with fatty alcohol profiles of ST10314 (red) and ST10388 (blue). B. Mass spectra of Z9-12:OH reference standard (1) and Z7-14:OH reference standard together with mass spactra of biologically produced Z9-12:OH (3) and Z7-14:OH (4). Cultivation media was supplemented with 0.4% (v/v) of 14:Me.

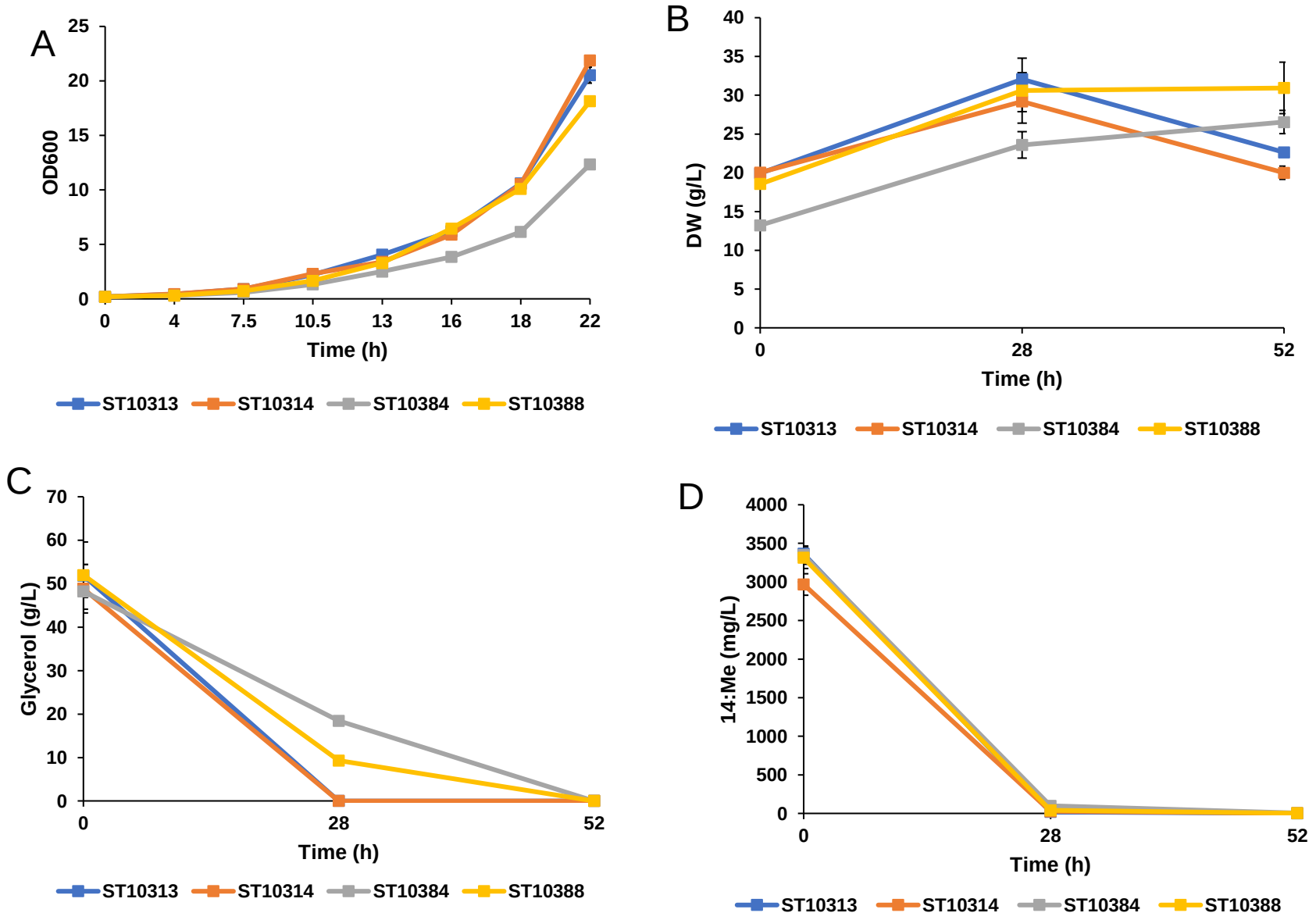

Figure S7. Growth characteristics and utilization of glycerol and 14:Me by *Y. Lipolytica* strains ST10313, ST10314, ST10384, ST10388 in shake flasks. A. Growth curves during the first 22h in YPG media. B. Dry weight (DW) measurements at 0, 28 and 52h after media exchange. C. Glycerol measurements at 0, 28 and 52h after media exchange. D. 14:Me measurements at 0, 28 and 52h after media exchange. Error bars represent standard deviations. OD measurements were performed in technical dublicates. DW, glycerol and 14:Me measurements were performed in technical triplicates.

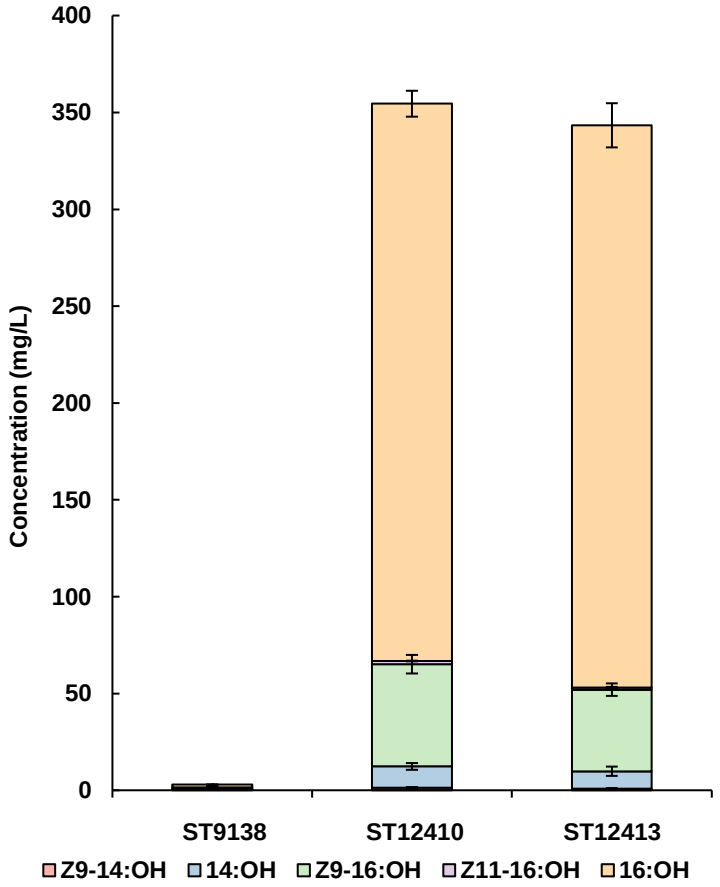

Figure S8. Fatty alcohol profiles of the *Y. lipolytica* strains expressing HarFAR-hrGFP-Per2 (ST12410) and HarFAR-hrGFP (ST12413) fusion proteins. ST9138 is the strain that does not have FAR. Error bars represent standard deviations from three technical replicates

CHAPTER 6

Perspectives

6.1. A briefing on perspectives

Perspectives chapter will cover aspects associated with (i) tools for improved strain engineering in *Y. lipolytica*, (ii) applications of metabolic modelling and omics data and (iii) process and cultivation condition optimization. More specifically, part (i) will discuss implementation of genetically encoded biosensors, additionally, possible improvements in transformation efficiencies and targeted genome editing will be suggested. If/when implemented, these tools might boost discovery of novel genetic engineering targets for improved production of various metabolites in *Y. lipolytica*. Part (ii) will cover genome scale models and use of multi omics data as another type of tools which can guide development of cell factories. Finally, Part (iii) will propose some alternative cultivation condition options which might improve process economy and pheromone production in yeast.

6.2. Tools for improved strain engineering in *Y. lipolytica*

6.2.1. Implementation of biosensors

In this thesis several native genes were targeted to improve insect sex pheromone biosynthesis in yeast. In Chapter 3, deletions of two fatty aldehyde dehydrogenases (*HFD1* and *HFD4*), peroxisomal biogenesis factor 10 (*PEX10*) and fatty alcohol oxidase (*FAO1*) have been performed together with truncation of promoter controlling expression of glycerol-3-phosphate acyltransferase ($P_{GPAT}100$). In addition, mutation exchanging isoleucine to phenylalanine in fatty acid synthase α-chain ($Fas2p^{I1220F}$) was done in order to enable production of C_{14} pheromone precursor *Z*9-14:OH. In chapter 4, overexpression of fatty acid synthase β chain (*FAS1*) helped to improve the titer of *Z*11-14:OH. In chapter 5, background strain devoid of six peroxisomal oxidases (Δ*pox1-6*) have been used as a background strain for screening POXes from different organisms. Apart from above-mentioned selections, number of other genes could be revealed as potential contributors to higher pheromone production levels.

HFD1, *HFD4*, *PEX10*, *FAO1*, $P_{GPAT}100$, $Fas2p^{I1220F}$, *FAS1*, *POX1-6* have been selected as targets due to their direct involvement in fatty acid metabolism. Alternatively, more engineering targets, which at first glimpse would not be obvious, could be revealed by implementing biosensors. Typically, biosensor system is composed of three elements: (i) plasmid containing the gene coding for bacterial-derived transcription factor (TF) (ii) plasmid containing reporter gene whose transcription is regulated by the promoter containing TF binding site and (iii) library of potential candidate genes [1–3]. The most TFs in biosensor circuits are repressors [1]. Metabolite of choice can influence the binding of TF to the

promoter and in this way control the signal which is obtained from the reporter. In repressor-based biosensors, higher concentration of a certain metabolite decreases the binding of TF to the promoter driving expression of a reporter gene, so, as a result, higher concentrations of the metabolite become directly proportional to the measurable signal obtained from the reporter. In *S. cerevisiae*, several fatty acid-related biosensor systems had been developed. Dabirian and colleagues had constructed an acyl-CoA-responsive system [1]. In this system, FadR from *Escherichia coli* was used as repressor. Natively, FadR controls expression of genes which are involved in fatty acid metabolism [4]. Expression of the reporter gene (green fluorescent protein) was controlled by P_{TEF1} promoter which had FadR binding sites integrated. Potential candidate genes for improved production of acyl-CoA were provided in the form of Molecular Barcoded Yeast Open Reading Frame library (MoBY-ORF), which contained 4956 genes. Fluorescence-activated cell sorting (FACS)-based screening combined with the overexpression of a selected sub-set of candidates in fatty alcohol-producing strain showed that overexpression of three genes (*RTC3, LPP1* and *GGA2*) significantly increased fatty alcohol levels [1]. Before this study, none of these genes had been reported to be directly involved in fatty acyl-CoA metabolism, which highlights the potential of biosensors-guided metabolic engineering for finding new targets. Apart from the acyl-CoA sensing system, *S. cerevisiae* had been also employed for finding the targets which can be beneficial for production of malonyl-CoA-direct substrate for FAS system. Study by Li et al. employed malonyl-CoA responsive TF FapR in combination with tdTomato reporter gene driven by *GPM1* promoter containing FapR binding site [5]. A strain expressing the above-described sensing system was transformed with a *S. cerevisiae* cDNA library and screened by FACS. Screening revealed two novel candidates (*TPI1* and *PMP1*) whose overexpression in the strain containing heterologous enzyme converting malonyl-CoA into 3-hydroxypropionic acid (3-HP) helped to improve 3-HP levels by 100%. Compared to *S. cerevisiae*, there are significantly fewer studies related to biosensors in *Y. lipolytica*. For this yeast, systems sensing naringenin and erythritol have been employed [6,7]. In the study by Lv and co-authors, a naringenin sensing system was used to implement metabolite addiction, where presence of naringenin was coupled to expression of auxotrophic marker [6]. In another study, Qiu et al. developed a screening system for erythritol production, where supernatants from ultraviolet light (UV) and an atmospheric room temperature plasma (ARTP)-mutant library were screened in erythritol sensing *E. coli* fermentation broth [7].

Implementation of acyl-CoA/malonyl-CoA sensing systems in *Y. lipolytica* potentially could help reveal novel targets for improved production of insect sex pheromones.

6.2.2. Improvements in transformation efficiency and targeted genome editing

Compared to *S. cerevisiae*, transformation efficiencies in *Y. lipolytica* are noticeably lower [8,9]. This factor could be a significant limitation in biosensor-based library screening, where in order to cover as much variants as possible, a high number of colonies is desired. In this thesis chemical, a lithium acetate (LiAc)-based transformation protocol, was used [10]. This method can provide up to $6x10^5$ colonies per µg replicative plasmid DNA [8,11]. In comparison, in *S. cerevisiae* this number could reach up to $1.2x10^7$ [9]. Alternatively, electroporation is used to introduce exogenous DNA into the yeast [12]. However, based on comparison of different transformation protocols used for *Y. lipolytica*, LiAc-based procedure turns out to be the most effective [8]. In order to improve transformation efficiencies further, several relatively simple adjustments of the used protocol could be considered. Supplementation of fatty acids prior transformation could be performed , or the composition of the transformation mix modified [13,14].

Y. lipolytica uses the non-homologous end joining (NHEJ) pathway over homologous recombination (HR) for DNA repair [15,16]. This inherent feature is not desirable when precise genetic modifications are needed. During NHEJ, an exogenous linear DNA fragment is prone for random integration while HR relies on recognition of homologous regions, thus, facilitating targeted integration. In order to reduce the frequency of NHEJ and improve precision of genetic modifications, *Y. lipolytica* background strains devoid of Ku70p (Δ*ku70*) are constructed [17–19]. In this thesis background strains where *KU70* was disrupted by replacing it with *CAS9* gene from *Streptococcus pyogenes* were used [17]. Ku70p is a part of Ku70p/Ku80p heterodimer which is the principal component of NHEJ DNA repair pathway [20]. There is a clear consensus in the literature that *KU70* deletion improves homologous recombination in *Y. lipolytica* while mixed results can be found on the effect of *KU80* deletion [15,16,18,19]. Deletion or disruption of genes coding for proteins involved in NHEJ can compromise cells viability, thus, methods that would not require *KU70* deletion, but still improve HR are desirable. Expression of gene coding for Rad52p from *S. cerevisiae*, which is a crucial element in HR in this yeast, turned out to significantly improve HR efficiency in *Y. lipolytica*. Additionally, the use of longer homology arms resulted in higher HR frequency [19]. Apart from genetic methods, HR can be favoured by treating the cells with metabolites arresting cell cycle at specific stage. Arresting cells in S-phase (cell cycle phase where HR dominates over NHEJ) by using hydroxyurea turned out to be promising approach [15].

Another factor which plays significant role in CRISPR-Cas9-based genome editing is the design of guide RNAs (gRNAs) [21]. gRNA consists of two main parts: (i) crispr RNA

(crRNA), alternatively called protospacer, and trans-activating crRNA (tracrRNA). crRNA guides Cas9p endonuclease to specific site in the genome while tracrRNA serves as scaffold linking the crRNA to Cas9p [22]. Expression of gRNAs can be based on polymerase II (pol II) or polymerase III (pol III) promoters. In the pol II-based design, gRNA sequence is flanked by hammerhead (HH) at 5' and Hepatitis delta virus (HDV) ribozyme at 3' to release desired gRNA. In the pol III-based design, pol III promoter fused to tRNA coding sequence is used to drive transcription of gRNA. After transcription, mature gRNA is obtained by releasing tRNA by the action of RNase P and RNase Z ribonucleases [21,23]. In the study performed by Schwartz and colleagues, comparison of two designs showed that pol III-based design provided better results when *PEX10* was selected as target for disruption [23]. The toolbox used in this thesis has pol III-based gRNA expression design, where native $tRNA^{gly}$ promoter together with $tRNA^{gly}$ coding sequence is used to drive expression of gRNAs. $tRNA^{gly}$ coding sequence is connected with protospacer by the linker consisting of 9 nucleotides, 5'GGTTGGTTA3' [17]. Even though EasyCloneYALI toolbox is recognized as one of the most efficient for *Y. lipolytica* genome editing, several approaches could be exploited to improve it even further [21]. Several reports indicate that removal of the linker provides improved genome editing efficiencies, thus motivating to change current gRNA expression design [21,24]. Another potentially promising research area could be exploration of different pol III promoters to provide efficient transcription of gRNAs.

Overall, adjustments in transformation protocol, gene modifications related to improved HR or exploration of different gRNA designs could boost transformation efficiencies and targeted genome editing.

6.3. Applications of metabolic modelling and omics data

Use of *in silico* computational tools in combination with various omics data can be a powerful combination for optimization of cell factories [25]. Available annotated genomes and accumulated knowledge about enzyme functions allow to reconstruct genome-scale metabolic models (GEMs) and use them for various purposes, such as metabolic flux prediction, cell factory design, culture condition optimization or comparative analysis of several yeast species [26]. In general, GEMs are a mathematical representation of chemical reactions occurring in the given organism. Stoichiometric matrix (S) together with vector defining fluxes (v) through all reactions are the core of GEM. In the stoichiometric matrix rows represent unique metabolites and each column represents reaction. In the steady state, where metabolite concentrations are not changing over time, multiplication Sxv=0. Given the

objective function and using linear programming, flux balance analysis (FBA) can provide flux distribution values which maximizes objective function [27]. In the most cases, objective function is biomass growth, however, in some instances it could be production of metabolite of interest [28,29]. Up to now, there are at least six *Y. lipolytica* GEMs [30]. The first one (iNL895) was published in 2012 and includes 2002 metabolic reactions, 895 genes and 1847 metabolites [31]. The reconstruction of this model was based on *S. cerevisiae* GEM, where adjustments had been made based on available knowledge about differences between *S. cerevisiae* and *Y. lipolytica* metabolism. As example, *Y. lipolytica* is alkane- assimilating yeast, so the pathway, which leads to alkane degradation to free fatty acids, had to be added [32]. The same approach was used by Kavšček and colleagues, where based on *S. cerevisiae* GEM as a scaffold, *Y. lipolytica* GEM was reconstructed (iYLI647) and used for FBA with a purpose to optimize lipid production [29]. Simulation suggested that reduced glucose uptake rate would prevent citrate secretion, whose overproduction is not desirable when the objective is lipid synthesis. Model prediction was validated experimentally, where no citrate was detected when glucose feeding was done according to prediction. Additionally, the model suggested that reduced O_2 consumption is positively correlated with lipid synthesis. Indeed, when dissolved oxygen was reduced from 50% to 1% during fermentation, a 67% higher lipid content was obtained. Furthermore, GEMs can also be used for predicting gene targets whose deletions or overexpressions could increase the production of a target metabolite. By using iYL_2.0 GEM in combination with a gene knockout and overexpression algorithms, Wei and colleagues identified six deletion and six overexpression targets for improved TAG production [33]. Among deletion targets, deletion of carbonate hydrolyase (YALI0F21406g) was predicted to improve the TAG production rate by 6.4-fold, while overexpression of six genes involved in fatty acid (YALI0C11407g, YALI0D17864g) and amino acid (YALI0F08415g, YALI0C01859g, YALI0E18238g, YALI0D07986g) metabolism provided 34.1% improvement in production rate. Another example of GEM-guided strain design for improved metabolite production is provided by Mishra and colleagues [28]. In this case, dodecanedioic acid was selected as target molecule. GEM iYLI647 was used to identify metabolic engineering targets. Overexpression of malate dehydrogenase and malic enzyme were predicted to provide 47.8% yield improvement, while overexpression of glutamate dehydrogenase showed 22.2% improvement. These examples illustrate that *in silico* predictions could provide targets for growth condition optimization together with metabolic engineering targets which further on might be implemented in a fermentation set-up and strain construction, respectively.

In order to improve accuracy of GEMs, proteomics data together with kinetic parameters of enzymes can be integrated. This approach is abbreviated as GECKO (GEM with Enzymatic Constraints using Kinetic and Omics data) and was first applied to *S. cerevisiae* GEM Yeast7 [34]. Determination of protein concentrations together with their k_{cat} values allows to obtain more strict flux values which results in improved simulations. As example, enzyme-constrained Yeast7 (ecYeast7) could capture experimentally validated effect of NADH dehydrogenase (NDI1) deletion, while Yeast7 failed to do so [34]. Recently, GECKO model was developed for *Y. lipolytica* (eciYali) as well [35].

In addition to GEMs, strain design could be guided by multi-omics data such as proteomics, transcriptomics, metabolomics and fluxomics. One example of that is the study by Dahlin and colleagues, where combination of ^{13}C-flux analysis, metabolomics and transcriptomics were employed in order to obtain insights into differences between *S. cerevisiae* and *Y. lipolytica* fatty alcohol producing and non-producing strains under different cultivation conditions [36]. Comparative transcriptomics between fatty alcohol producing and non-producing strains of *Y. lipolytica* revealed that expression of several formate dehydrogenases was upregulated in fatty alcohol producing strains. These enzymes oxidize formate to carbon dioxide in $NAD(P)^+$-dependent manner. Comparative metabolomics between growth and nitrogen-depletion phases in non-producing *Y. lipolytica* strain highlighted significant organic acid secretion in the latter condition. Based on this finding the authors proposed several metabolic engineering targets which potentially could lead to retention of organic acids inside the cell and their conversion into fatty alcohol precursors. Finally, ^{13}C-flux analysis indicated significant differences in terms of flow of metabolites in central carbon metabolism of *S. cerevisiae* and *Y. lipolytica*. *Y. lipolytica* had significantly higher flux towards pentose phosphate pathway and acetyl-CoA generation. Considering NADPH and acetyl-CoA demands for fatty acid/fatty alcohol synthesis, this result provides strong argument why *Y. lipolytica* is chosen over *S. cerevisiae* for production of fatty acid derivatives. In another case, where proteomics, phosphoproteomics and metabolomics were employed, Pomraning with co-authors shed a light on regulation of acetyl-CoA and malonyl-CoA forming enzymes, ATP-citrate lyase (ACL) and acetyl-CoA carboxylase (ACC), respectively, during nitrogen starvation [37]. During nitrogen starvation authors observed significant increase in abundance of protein kinases while this trend was not seen for ACL and ACC proteins. This implies that during lipid accumulation phase, regulation of ACL and ACC happens post-translationally rather than transcriptionally. A study performed in *S. cerevisiae* supports this notion, where removal of two phosphorylation sites from ACC1 increased enzymatic activity by 3-fold

[38]. These findings provide meaningful directions for optimization strategies and provide experimentally validated hints where efforts have to be focused (in the case of ACC, enzyme engineering with the aim to create constitutively active and phosphorylation-insensitive version, most likely, would be more fruitful in comparison to attempt to increase transcript levels of native version).

Overall, described systems-level approaches could be considered in the future to obtain deeper insights into regulation of metabolic networks of the strains producing different fatty acids/alcohols constructed in this thesis.

6.4. Process and cultivation condition optimization

Apart from strain engineering, which involves gene discovery and metabolic pathway implementation/optimization, cultivation condition optimization also plays crucial role in bio-based manufacturing. In order to establish an economically viable fermentation-based process, the price difference between substrate supplied and product produced has to be, preferably, as high as possible. In this thesis pure glycerol was used as carbon source based, on findings that *Y. lipolytica* has higher growth rate on glycerol than on glucose [39]. In order to reduce substrate costs, crude glycerol, obtained as a side-product from oil transesterification during biodiesel production, could be considered as cheaper alternative. Crude glycerol has already been used for production of lipids, erythritol and citric acid in *Y. lipolytica* [40,41]. Even though crude glycerol contains impurities such as methanol or heavy metals which potentially could decrease the performance of cell factory, comparable results in terms of growth and citric acid production yield were observed, when compared to pure glycerol [40,42]. This indicates that use of crude glycerol as a carbon source for pheromone production could be promising strategy to improve process economy.

In order to obtain high-level production of fatty acid derivatives, a common cultivation strategy is to use two-step process, where at first, cells are cultivated without nutrient limitation. This results in biomass accumulation. In the second stage, nutrient limitation, most frequently, in the form of nitrogen starvation, is implemented in order to induce lipophilic phenotype [10,43–45]. A successful example of that is demonstrated by DuPont, where a two-step fermentation strategy allowed to obtain economically relevant levels of ω-3 cis-5,8,11,14,17-eicosapentaenoic acid (EPA) which culminated in commercialization under the product name New Harvest™ [45]. Generally, in the second stage carbon source remains the same, as could be seen in multiple studies [10,43–45]. However, it might be beneficial to provide different carbon source. In the case of insect pheromone production, which starts

from C_{16}-C_{14} fatty acids, alternative strategy could be to feed oils rich in those fatty acids instead of glucose or glycerol. This strategy provides several benefits. Fatty acids released from *ex novo* provided lipids by *Y. lipolytica*-secreted lipases could be used as direct precursors for fatty alcohol biosynthesis while glucose or glycerol has to go through multiple enzymatic reactions in order to be converted into fatty acids and finally corresponding alcohols [36,46]. *Y. lipolytica* is very well-known for its lipase secretion which is induced under exposure to lipids [47,48]. Oil feeding strategy has been explored for production of limonene, lactones, lipids/biodiesel and citric acid in *Y. lipolytica* [49–52].

In summary, exploration of utilization of carbon sources which are cheaper or structurally closer to fatty alcohols could be performed in the future.

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