On the way of making plants smell like moths - a synthetic biology approach

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Title and subtitle: On the way of making plants smell like moths — a synthetic biology approach

Abstract: Moth caterpillars are major agricultural pests in many parts of the world. In general female moths attract male mates with their pheromone over long distance. Most of the described moth pheromones belong to the same class of chemical compounds, fatty acid derivatives that are produced de novo in the pheromone gland. The pheromone biosynthesis involves desaturation, chain-shortening by β-oxidation and functional group modification such as reduction, acetylation or oxidation, etc. These enzymes have evolved to function in the production of the complex chemical signals used for sex attraction, thus contributing to the chemical diversity of moth pheromones. In the current thesis, several desaturases were characterized. Firstly, a terminal fatty-acyl-CoA desaturase from winter moth (*Operophtera brumata*) was cloned and expressed heterologously in yeast and proved its ability to convert Z11,Z14,Z17-eicosatrienoic acid to Z11,Z14,Z17,19-eicosatetraenoic acid. This is the first report on methyl terminal desaturase ever. Secondly, desaturation steps in *Cydia pomonella* and *Grapholita molesta*, which use unsaturated dodecenyl alcohol and/or acetate as sex pheromone component(s), were characterized. We found the desaturases in *C. pomonella* work consecutively, account for the production of conjugated double-bond in the fatty acyl chain (E8,E10-12:CoA). But in the case of *G. molesta*, we found Δ10 desaturase on myristic acid. The E/Z10-14:Acyl, which after chain-shortening, reduction and acetylation may produce the *G. molesta* pheromone consisting of Z8-12:OAc, E8-12:OAc and Z8-12:OH. Thirdly, the stereospecificity of two Δ11 desaturases was investigated. A Δ11 desaturase from *Choristoneura rosaceana* takes saturated 14 carbon and produce a mixture of (*E*)-11-tetradecenoate and (*Z*)-11-tetradecenoate with an excess of the Z isomer. A desaturase from *C. parallela* also takes saturated 14 carbon but produce almost pure E11-14C. Reciprocal site-directed mutations on this two desaturases revealed that one amino acid at the C-terminal of the protein is critical for the Z activity of the desaturase (gain or lose of function). This study shed light on cracking the stereospecificity of desaturase. The next study focused on fatty alcohol acetyltransferase that bears great implications in many moth pheromone biosynthesis pathways. It catalyzes the formation of acetate ester by transferring acetate group from the acetyl-CoA to the fatty alcohol. Since no insect-derived pheromone biosynthetic acetyltransferase has been cloned, we heterologously expressed a plant derived acetyltransferase, EaDAcT, in a yeast system, to test the functionality and validity in converting moth pheromone intermediates, fatty alcohols, into final pheromone product, fatty alcohol acetate esters. The results showed EaDAcT could convert various fatty alcohols with chain length range from 10 to 18 carbons, with double bound at varying positions, into their corresponding acetate esters. EaDAcT prefers shorter chain length to the long ones, unsaturated to the saturated ones. The microsome preparations showed an activity pattern similar to the activity observed in the in vivo experiments. Next, through massive sequencing of pheromone producing tissue, we identified genes that might be involved in the pheromone biosynthetic process of the turnip moth (*Agrotis segetum*), such as: fatty acid synthase, β-oxidation enzymes, desaturase, fatty acyl reductase, acetyltransferase, etc. The final study was assembling the parts identified previously in a chassis to make moth pheromones. Using *Nicotiana benthamiana*, as a plant factory, we produced typical moth sex pheromone components by transient expression of up to four genes coding for consecutive biosynthetic steps. We specifically produced biologically active multi-component sex pheromones for two species of small ermine moths. The fatty alcohol fractions from the genetically modified plants were acetylated and mixed to mimic the respective sex pheromones of *Yponomeuta evonymella* and *Y. padella*. Although the composition of the plant-derived mixtures was not optimized, these mixtures were very efficient and specific for trapping of male moths, matching the activity of conventionally produced synthetic pheromones. Our long-term vision is to design tailor-made production of any moth pheromone component in genetically modified plants. Such semi-synthetic preparation of sex pheromones may be a novel and cost-effective way of producing moderate to large quantities of pheromones for integrated pest management, with high purity and a minimum of hazardous waste.

Key words: moth pheromone, transcriptome, fatty acyl desaturase, fatty acyl reductase, acetyltransferase, chain shortening, chain elongation, heterologous expression, synthetic biology, plant expression.

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A doctoral thesis at a university in Sweden is produced either as a monograph or as a collection of paper. In the latter case, the introductory part constitutes the formal thesis, which summarizes the accompanying papers. These have either been published or are in manuscripts in various stages (in press, submitted or manuscript).

It is those who know little, and not those who know much, who so positively assert that this or that problem will never be solved by science.

---Charles Darwin

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Contents

The thesis is based on the following papers

I. Ding BJ, Liénard MA, Wang HL, Zhao C-H, Löfstedt C. (2011) Terminal fatty-acyl-CoA desaturase involved in sex pheromone biosynthesis in the winter moth (Operophtera brumata). Insect Biochem. Mol. Biol. 41: 715–722.

II. Ding BJ, Löfstedt C. (2014) Desaturase orthologues with different specificity, Δ9 and Δ10 respectively, account for differences in sex pheromone biosynthesis between the two tortricid moths *Cydia pomonella* and *Grapholita molesta*. Manuscript.

III. Ding BJ, Löfstedt C. (2014) A Tackle on Sequence Variation Determining the Stereochemistry of a Δ11 Desaturase. Manuscript

IV. Ding BJ, Lager I, Bansal S, Durrett TP, Stymne S, Löfstedt C. (2014) Substrate specificity of a plant-derived acetyl-transferase expressed in yeast: implications for biological production of moth pheromones. Manuscript.

V. Ding BJ, Löfstedt C. (2014) Analysis of the turnip moth *Agrotis segetum* pheromone gland transcriptome in the light of sex pheromone biosynthesis. Manuscript.

VI. Ding BJ, Hofvander P, Wang HL, Durrett TP, Stymne S, Löfstedt C. (2014). A plant factory for moth pheromone production – proof of principle. Accepted. Nat. Commun.

AUTHOR CONTRIBUTIONS TO THE PAPERS

I. CL and HLW conceived the study; BJD, MAL, HLW and CHZ performed the research; BJD, LMA, HLW and CL wrote the paper.

II. CL and BJD conceived the study; BJD performed research; BJD and CL wrote the paper.

III. BJD conceived the study; BJD performed research; BJD and CL wrote the manuscript.

IV. SS, CL and BJD conceived the study; BJD and CL designed the experiments; IL TPD and SB contributed new reagents/analytic tools; BJD and CL wrote the paper.

V. BJD and CL conceived and designed the study; BJD performed research; BJD and CL wrote the manuscript.

VI. BJD, PH, SS, and CL designed research; BJD, HLW, and CL performed research; PH and TPD contributed new reagents/analytic tools; BJD, PH, SS, and CL analyzed data; BJD and CL wrote the paper.

Abbreviations

ACC:	acetyl-CoA carboxylase
ALA:	α-linolenic acid
BAHD:	acetyltransferase super family
Blast:	basic local alignment search tool
cDNA:	complementary DNA
COG:	clusters of orthologous groups
DNA:	deoxyribonucleic acid
DMDS:	dimethyl disulfide
EaDAcT:	*Euonymus alatus* diacylglycerol acetyltransferase
ER:	endoplasmic reticulum
FAD:	fatty acyl desaturase
FAR:	fatty acyl reductase
FAS:	fatty acid synthase
FACES :	fatty acid chain elongation system
GC-MS:	gas chromatography mass spectrometry
KEGG:	Kyoto encyclopedia of genes and genomes
LC-PUFA:	long chain polyunsaturated fatty acid
mRNA:	messenger RNA
MUFA:	monounsaturated fatty acid
NADPH:	nicotinamide adenine dinucleotide phosphate, reduced form
NGS:	next-generation sequencing
Nr:	non-redundant protein sequences database
ORF:	open reading frame
PCR:	polymerase chain reaction
PG:	pheromone gland
PUFA:	polyunsaturated fatty acid
RACE:	rapid amplification of cDNA ends
RNA:	ribonucleic acid
RT-PCR:	reverse transcription PCR
SFA:	saturated fatty acid
SwissProt:	high quality annotated and non-redundant protein sequence database
TAG:	triacylglycerol

Acyl intermediates in the pathway (also throughout the thesis) are depicted as abbreviations, for instance, (Z)-11-tetradecen-1-yl acetate is shortened to Z11-14:OAc where Z denotes the double bond configuration, 11 the double bond position counting from carboxylic end (Δ), 14 the number of carbons in the chain; OAc indicates the functional group as an acetate ester. ACP, acyl carrier protein; CoA, coenzyme A; OH, fatty alcohol; Me: methyl group; One, ketone; H, hydrocarbon.

1. Aims and Purposes

Synthetic biology is about studies on the design and construction of novel genetic circuits and biological devices (system) for useful purposes [2]. It is a combined research area that including and overlapping with a variety of research fields such as metabolic engineering, biomedical engineering, and essentially encompasses anything that concern bioengineering and biotechnology. The tasks for synthetic biologists are finding how the biological system works and how to use it benefit society.

This thesis is dedicated to identify, design, characterize, build and assemble biological parts, devices, and integrating biological systems and develop technologies that enabling the **production of moth pheromones in plant**.

Throughout this thesis, the experiments and studies are designed to serve these steps (Fig. 1):

1) Identify parts for moth pheromone biosynthesis — paper I, II and V.

2) Characterize and optimize parts — paper III and IV.

3) Assemble parts in chassis — paper VI.

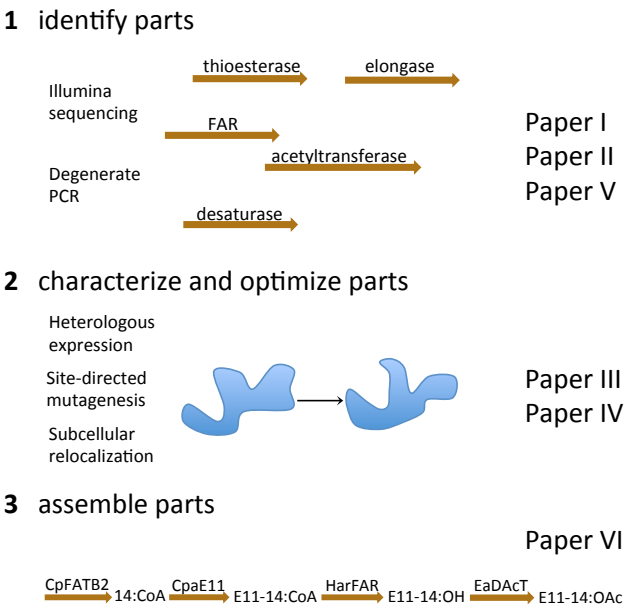


Figure 1. The three steps in synthetic biology. The parts are like "genetic lego blocks", they are gene sequences correspond to traits in organisms. We use various methods and techniques to find the parts and characterize the parts, and then assemble the parts in different "chassis" to achieve a specific biological function or for better understanding the biological phenomenon.

In the following I will first introduce the reader to moth pheromone structures and moth pheromone biosynthetic pathways with a few references to work that is part of my thesis. Secondly, I will briefly introduce the main molecular methods used in my research. I will then review some important enzymatic reaction steps of moth pheromones biosynthesis. The genes that encoding some of these enzymatic reactions have been cloned and characterized intensively, for example the fatty acyl desaturation step and the fatty acyl reduction step. These studies form a potential tool box from which we can pick up elements and build a novel biological system. For some steps, the reactions have been studied by *in vivo* labelling experiment, but the genes have not been cloned yet.

Finally I will provide an example of how we utilize and optimize these biological parts and how they were successfully assembled in a plant chassis for production of moth pheromones and some future perspectives.

In addition to references to the manuscripts included to support my thesis (Papers I-VI) I will also refer to my unpublished work on navel orange worm pheromone biosynthesis and winter moth elongases (summarize: Atr_transcriptome; Obr_elongase) which has not yet been completed and compiled in manuscript form.

2. Overview of moth pheromone

2.1 Type of moth pheromone

Moths generally rely on their highly evolved sex pheromone communication for mate finding. Most of the described moth pheromones belong to the same class of chemical compounds, fatty acid derivatives. Over the last few decades, pheromones from thousands of moth species had been identified, usually 10 to 18 carbons in chain length (0-3 double bonds) with an oxygenated functional group such as alcohol, aldehyde, or acetate ester [3, 34] (the so-called Type I pheromones). For some families (*Geometridae*, *Noctuidae*, and *Lymantriidae*) of moth, their pheromones are composed of polyunsaturated hydrocarbons and their epoxy derivatives with a C17-C25 straight chain. These compounds lacking a terminal functional group are called the Type II pheromones [35]. The variation in chain length, the number, location, and isomeric form of the double bond(s), the type of functional group, and the precise ratios of components in multicomponent pheromones contribute the gigantic diversity of moth pheromones [44, 45, 46].

The starting materials for the type I moth pheromone biosynthesis is palmitate or stearate (the primary fatty acid biosynthesis products), and then modified through a set of tissue specific enzymes working concertedly, making the final pheromone in the pheromone gland. The rate-limiting step of fatty acid biosynthesis [47] is the ATP-dependent carboxylation of acetyl-CoA to malonyl-CoA catalyzed by **acetyl-CoA carboxylase (ACCase)** [48], the first step in saturated long chain fatty acid biosynthesis. ACCase is a large protein with multiple catalytic activities (paper V) working coordinately to provide malonyl-CoA substrate for the biosynthesis of fatty acids [49]. Cytoplasmic **fatty acid synthase (FAS)** plays a major role in the *de novo* synthesis of fatty acids [50]. FAS is also a multifunctional protein (paper V) that produce saturated fatty acids using malonyl-CoA and acetyl-CoA as substrate and require NADPH as reducing agent, in a cyclic process in which an acetyl primer undergoes a series of decarboxylative condensations with several malonyl moieties [51]. The resulting products are palmitic and stearic acid [52, 53, 54] in insect, proved by labeling studies. Whereas for the type II pheromones, the starting material usually is α-linolenic acid which is acquired by the larvae from their feeding on plants [61, 86].

2.2 Moths and their pheromone biosynthesis pathways

In this thesis, the pheromone biosynthetic pathway of several moth species were explored, including those that produce type I pheromones (*Agrotis segetum*, *Cydia pomonella*, *Grapholita molesta*), and type II pheromones (*Operophtera brumata*) and *Amyelois transitella* that produces both type I and type II pheromone compounds.

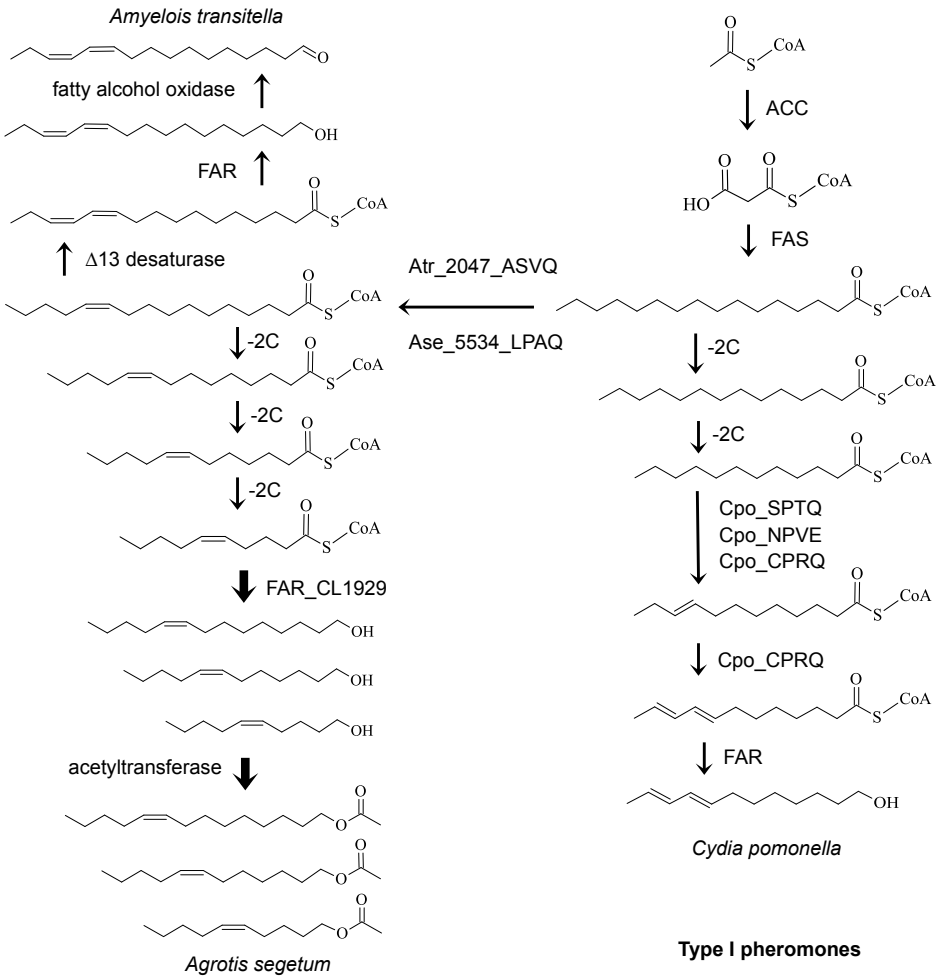


Figure 2. Type I moth pheromone biosynthetic pathways. The starting materials are acetyl-CoA, which are then carboxylated to form malonyl-CoA, and then the malonyl-CoA and acetyl-CoA are entered to a cycle of fatty acid synthesis and end up with common fatty acids stearate and palmitate. The saturated fatty acid will then undergo desaturation and/or limited chain-shortening to form different acyl chains with double bond(s) in various position. Usually the carboxylic group will be reduced by fatty acyl reductase (FAR) to fatty alcohol, and fatty alcohols for many of the moth species are acetylated to form acetate esters.

After 16:CoA is made by ACC and FAS (Fig. 2), it will undergo various destiny in different moth species. For example, in *C. pomonella*, it is undergone two rounds of chain-shortening, end with 12:CoA. Then three desaturases operate on lauric acid consecutively to produce E8,E10-12:CoA (paper II). The conjugated diene is subjected to reduction by FAR, forming E8,E10-12:OH, the actual pheromone for *C. pomonella*.

In *A. segetum*, the palmitate is first desaturated by a Δ11 desaturase [102,101]. Then three rounds of limited chain-shortening reactions made three fatty acyl intermediates with carbon chain length differ by 2C. Subseqently, they are reduced to three short chain alcohols by one FAR (paper V). Finally, the fatty alcohols are acetylated to the final pheromone components [79,100]. In the case of *A. transitella*, the type I pheromone also start with palmitate, then two desaturases place two double bond in its acyl chain. The Z11,Z13-CoA is reduced to alcohol and oxidized to aldehyde [61].

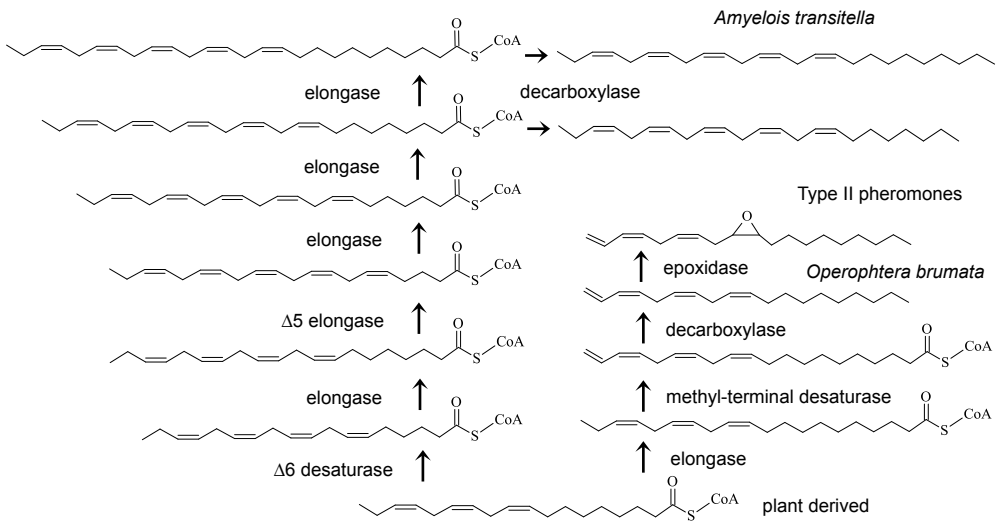


Figure 3. Type II moth pheromone biosynthetic pathway. The starting material is α-Linolenic acid (ALA), which is the most abundant fatty acyl moiety in plant, acquired by insect. ALA under go chain elongations and desaturations become long chain polyunsaturated fatty acid (LC-PUFA), which will then be decarboxylated to form hydrocarbon, and in some species hydrocarbon is epoxidized.

For the type II pheromone (Fig. 3), it starts with α-linolenic acid (ALA), a dietary product obtained from host plant. ALA undergoes several desaturation and elongation steps, become long-chain polyunsaturated fatty acids (LC-PUFA). Hydrocarbon pheromones are produced by decarboxylation [36,103] of the PUFA. In some moths, the hydrocarbons are epoxidized in the pheromone gland before release.

2.3 Site of pheromone biosynthesis

Pheromone gland is usually located as modified epidermal cells between the 8th and 9th abdominal segments [62]. It has been well established that these cells are the site of biosynthesis of type I pheromones. The production of type II pheromones involves another group of cells called oenocytes [63] that are usually associated with epidermal cells throughout the abdomen [75]. After the hydrocarbons are produced they are transported by lipophorin [85] throughout the body and hydrocarbon sex pheromones are picked up by pheromone gland cells for further modification or release directly.

3. Identify, characterize, and optimize the parts of pheromone biosynthesis

3.1 Main Methods

3.1.1 Gene cloning by degenerate PCR approach

Degenerate PCR is a very useful and efficient way to find new genes, because most genes share similar structure across species. One can determine the conserved parts of a gene by aligning the amino acid sequences from a number of related species. Then the amino acid sequence of the conserved motifs can be back-translated to nucleotide motif [89]. In paper I and II, the desaturases were cloned by this approach, using a pair of degenerate primers adapted from the previous studies [90]. One of the drawbacks of degenerate PCR approach is there is no guarantee one can get the full members of a gene family. In both paper I and II, we only got three members of the desaturase gene family, presumably due to their high expression level. If the gene one is looking for is a rare (low expression) transcript, then it depends on your serendipity.

After cloning and sequencing the degenerate PCR products, the "central part" of the targeted genes is obtained. By doing RACE with gene specific primers, the full-length ORF were obtained. Depending on how long and how similar this central part between the gene members, the designing of the gene specific primers for RACE can vary in degree of difficulty. Sometimes, the gene specific primers will pick up other members, even not the ones you found by the degenerate PCR, for example some of the winter moth elongases (section 3.7) were found in this way.

3.1.2 Transcriptomic approach was employed for gene hunting

In order to get all the genes that are possibly involved in the metabolic pathway, the ideal way is using NGS to get snapshot on the transcriptomic level. In paper V, we constructed As_PG and As_AB illumina library, to get a full picture of the turnip moth pheromone biosynthetic pathway. Another two library (At_AB and At_AT)

were constructed to decipher the navel orange worm pheromone biosynthetic pathways (Ding and Löfstedt, unpublished).

To do so, total RNA was extracted from pheromone glands and the abdominal tissues. The mRNA from each samples were enriched and sheared to a certain range of length. cDNA library were constructed and sequenced by illumina Hi-Seq2000. After filtering the raw reads, clean reads are assembled using Trinity [91], and the assembled unigenes were annotated by searching against protein databases: Nr, SwissProt, KEGG, and COG [58] using blastx (paper V).

3.1.3 Construction of expression clones and site-directed mutagenesis

The traditional and classical way of construction expression vectors is using restriction enzyme and ligation reaction [92] and had been successfully used in paper I to construct the winter moth desaturase genes into yeast expression vector. But it is time consuming and low throughput. The more modern way is the gateway™ method [93]. By using this method, hundreds of expression clones were made in this thesis (paper II – VI).

Site-directed mutagenesis were performed in paper III and paper VI on the generation of various version of CroΔ11 gene and HarFAR gene, respectively. We used a phusion PCR [94] combining the gateway cloning approach. Simply put, two fragments were generated by PCR using primers that containing the desired mutations, after hybrid the two fragments by phusion PCR, the full-length ORF were cloned to destination vector by LR reaction.

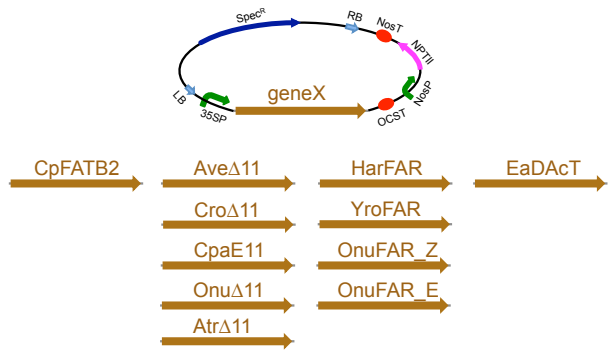
3.1.4 Functional assay by heterologous expression

One of the approaches in reverse genetics is creating of transgene that overexpress the gene of interest. The resulting phenotype may reflect the function of the gene. In this thesis, we used yeast strain that lacking the desaturase (*ole1*) and elongase (*elo1*) through paper I -V. The expression of the foreign insect desaturases were measured by analytical chemistry techniques, GC-MS of fatty acid methylesters and their DMDS derivatives. In paper VI, the expression host is *Nicotiana benthamiana*. We introduced insect genes whose roles are not overlapping with the plant indigenous genes, enable us to measure the resulting effects of the introduced pathways.

3.1.5 Assembly of moth pheromone biosynthetic pathways

We used interchangeable parts to assemble the entire biosynthetic pathway that eliminate the construction of multigene expression vectors and production of

numerous transgenic lines. By doing so, several strains of *Agrobacterium* were mixed together to infect tobacco leaf, each strain carry a biosynthetic gene. This transient expression of a pathway assembled from separate vectors is robust and flexible, enable us to test more than 80 combinations of genes and their modified versions, in a short period of time and a less labor intensive way. The drawback of this approach is only a proportion of cells receive the full set of the pathway transgenes, so there is a reveal of building up pathway intermediates. But we are aiming for proof of principle; this is an ideal way to do so. Once we established a good set of parts that could give good production rate, the generation of stably transformed transgenic lines will be the optimal choice.



3.2 Desaturases

Desaturases that catalyze the introduction of a double bond at fatty acyl chain, require molecular oxygen and electron donor, mostly, cytochrome b_5 [24]. There are soluble desaturases operating on substrate acyl-acyl carrier protein (ACP-desaturase), usually introducing first double bond, found in plant and localized in plastid [25], forming one group. The other group, including the majority of fatty acid desaturases, are membrane-bound desaturase recognizing acyl chains esterified to CoA or phospholipids, involved in biosynthesis of (poly)unsaturated fatty acids [26], characterized by possessing three histidine boxes lie on the same side of the membrane and 4-6 membrane spanning helices [27]. The two groups are completely unrelated although they use similar cofactor and acting similarly regarding to the outcome product stereochemistry [27, 28]. The second major group, membrane-bond desaturases can be further divided into four categories:

3.2.1 First desaturases

inserting double bond into the saturated acyl chain, for example, the ones in *Cydia pomonella* and *Grapholita molesta* (paper II)

Desaturation of CoA esters of saturated fatty acids is found to be a common feature of sex pheromone biosynthetic pathways in the Lepidoptera. Insect desaturases are

homologous to the ancestral Δ9 desaturases of plants (FAD1), vertebrates and fungi. Several dozens of desaturases, such as Δ6 [55], Δ9 [56, 7], Δ10 [57], Δ11 [4, 5, 6, 59] and Δ14 [60] from many moth species have been characterized.

C. pomonella and *G. molesta* are quite similar regarding to pheromone components. They both use 12 carbon chain length, *C. pomonella* use E8,E10-12:OH, *G. molesta* use E/Z8-12:OH and OAc as sex pheromones. Previously, desaturases involved in such kinds of conjugated diene pheromone biosynthesis had been cloned and functionally characterized in several moth species [59, 68, 69] and they are all Δ11 desaturase and operate on palmitate. The desaturase involved in *C. pomonella* operating on lauric acid, as demonstrated previously *in vivo* labeling studies [104]. By using degenerate PCR approach, three desaturase from *C. pomonella* were found, and subsequent functional assay revealed that these three desaturases work consecutively forming the conjugated double bonds in *C. pomonella* pheromone (paper II). Whereas, in the case of *G. molesta*, it is a multifunctional desaturase with Δ10 activity on palmitate (paper II) produces the E/Z10-14:Acyl which is then undergo chain-shortening to form the E/Z8-12:Acyl.

3.2.2 Omega desaturases

creating double bond between an existing double bond and the methyl end, for example, the ObrTerDes (paper I)

Omega desaturase are commonly found in plants, introducing Δ12 (FAD2) and Δ15 (FAD3) double bonds after the Δ9 double bond (action of the ACP-desaturase) have been put in stearates, forming the α-linolenic acid — the most abundant fatty acid species in plant. In the case of winter moth, α-linolenic acid is obtained from their diet and further processed to make the final pheromone. We found a desaturase that produce double bond between omega-1 and omega-2 carbon of the Z11,Z14,Z17-20:CoA, which is an elongated product of the α-linolenic acid. This is the first desaturase ever found possessing the ability of creating double bond at the methyl terminus of the acyl chain (paper I).

3.2.3 Front-end desaturase

introducing double bond between an existing double bond and the carboxylic end [73], for instance, desaturases in *Amyelois transitella*.

The navel orange worm, *A. transitella* use both the type I and type II pheromones. The type I pheromone is Z11,Z13-16:Ald, and the conjugated double bonds probably produced by Δ11 desaturation of 16:CoA and followed by a Δ13 desaturase on the Z11-16:CoA [61]. The type II pheromones are long chain polyunsaturated

hydrocarbons, and are produced by elongation and desaturation (two times) of α-linolenic acid. From the transcriptome of abdominal tissue, 12 desaturases have been

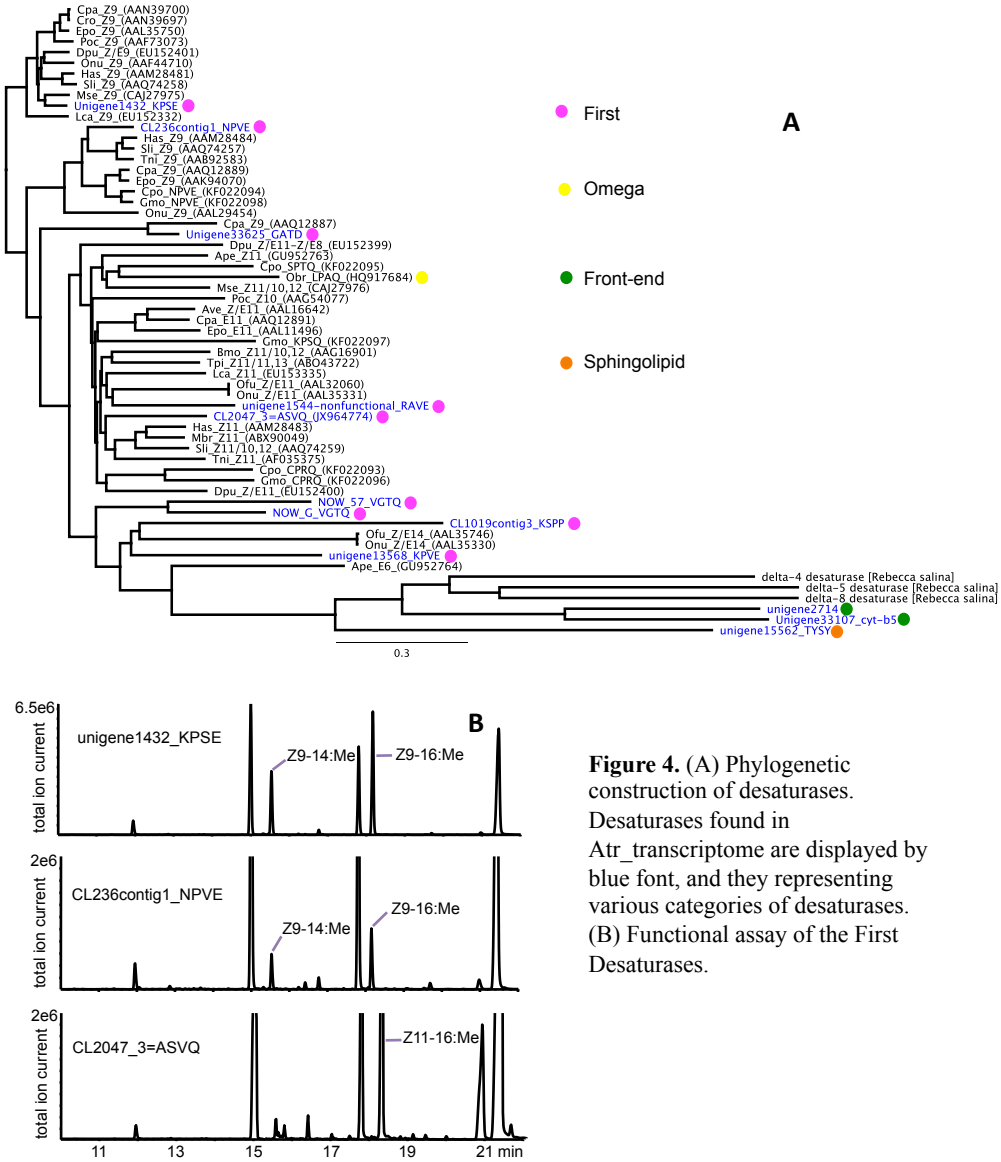


Figure 4. (A) Phylogenetic construction of desaturases. Desaturases found in Atr_transcriptome are displayed by blue font, and they representing various categories of desaturases. (B) Functional assay of the First Desaturases.

identified (Fig. 4A), most of them are clustered to the first desaturase subfamily; two of them are in the front-end desaturase subfamily, where the plant-derived front-end desaturases located, and as usual containing cytochrome b_5 domain. Functional assay (Fig. 4B) revealed that unigene1432 and CL236 are typical Δ9 desaturase of ordinary metabolic pathway. The CL2047 is Δ11 desaturase operating mainly on 16C, suppose

to be the one for type I pheromone biosynthesis. But the two front-end desaturases showed no detectable activity in our yeast expression system (Ding and Löfstedt, unpublished).

Further work will be conducted, such as on optimization of expression conditions, providing agent like electron donors, since previously it was shown overexpression cytochrome b_5 did help improve the expression level of the heterologously expressed genes [64].

3.2.4 Sphingolipid desaturase

The fourth category is sphingolipid desaturases, devoted solely to the biosynthesis of sphingolipid.

Sphingolipids are important constituent of plasma membranes of eukaryotic cells and several prokaryotes and viruses. Δ8-sphingolipid desaturase is predominant in higher plants [105], whereas in mammals and yeast desaturation primarily occurs at Δ4 position [106]. Sphingolipid desaturases share a lot of sequence similarity with the other three categories of desaturases and we found representative in both the Ase_PG (CL4584, paper V) and Atr_AB (unigene15562, Fig. 4).

3.2.5 Tackle on the stereospecificity of desaturase

A delta11 desaturase from *Choristoneura rosaceana* takes saturated myristic acid and produces a mixture of (*E*)-11-tetradecenoate and (*Z*)-11-tetradecenoate with an excess of the *Z* isomer. [56]. A desaturase from *C. parallela* also takes saturated 14 carbon but only produces almost pure *E*11-14:CoA [5]. There are only 24 amino acids difference between this two desaturases, so we constructed mutations on all of them, one by one, to narrow down the site where could decide the product stereospecificity. It revealed that one amino acid at the C-terminal of the protein decides the *Z* activity of the desaturase. 258E determined the *Z* activity, lost of 258E in CroΔ11, lost *Z* activity, gain of 258E in CpaE11, gain of *Z* activity (paper III).

Further work would be doing mutations on more sites, and also producing chimeric proteins (hybridize with a pure *Z* desaturase), to find out the sites that determine the *E* activity of the desaturase, in order to control the isomer ratio of the products. That would be very useful for the pheromone production in plants (paper VI), since there is no desaturase with a pure *Z* activity on 14C had been cloned. At the same time, it is also tempting to investigate protein conformational change caused by this mutation from the structural biology point of view.

3.3 Chain shortening

FAS produce fatty acids with chain length of 16C and 18C [54]. Fatty acyl shorter than 16C are products of limited β-oxidation, and the action of specific thioesterase were ruled out since labeling studies showed that longer chain fatty acids were incorporated into shorter chain length pheromone component [95, 96].

β-oxidation is the action of a serial of enzymes, working sequentially, forming a reaction spiral [107], and happening in both the mitochondrion and the peroxisome [97]. Moth pheromones are in general have a specific chain length, suggesting this kind of limited chain shortening may happening in peroxisome [97]. The role of β-oxidation in moth pheromone biosynthesis has been demonstrated by *in vivo* labeling, although it has not been studied in detailed enzymatic level in insects. One example is in the cabbage looper moth (*T. ni*), mutations in the β-oxidation pathway did affect the final pheromone compositions [77, 78]. Another example is that changes in β-oxidation pathway have produced two populations of turnip moth (*A. segetum*) [79], differ in the ratio of their pheromone components, although the influence of FARs cannot be ruled out.

In study V, we found representatives of all the key players and auxiliary enzymes of β-oxidation, and some of them are very high in expression level and differentially expressed among the As_PG and As_AB, forming promising potentials to understand the reason of the altered pheromone composition, either by heterologous expression or by RNAi.

In fact, the combinational actions of desaturases and chain shortening enzymes can produce many of the possible intermediates that can be converted to final pheromone components.

3.4 Fatty acyl Reductase (FAR)

After the double bonds are in place and the acyl chain length is appropriate, the carbonyl carbon is modified to form a functional group. Firstly it requires a step that converts the fatty acyl precursors into fatty alcohols. Great progresses have been made after the first fatty acid reductase gene has been identified in *Bombyx mori* [80]. A bunch *Ostrinia* FARs had been characterized [81, 8], since the gene sequences are conserved to some degree among moth species. The *Ostrinia* FARs are very essential to determine the final pheromone compositions [8], little changes in sequence can cause the pheromone component ratio shift [82]. On the other hand, there are FARs are very versatile in terms of substrate specificity [7, 9], making the pheromone become multicomponent.

In this thesis I investigate a FAR from the turnip moth that converts all the three fatty acyls into fatty alcohols, in a way similar to the FAR from the small ermine moths [7], but towards shorter chain length range (paper V).

In paper VI, we characterized HarFAR [9] by expressing it in plant, with fatty acyl substrates of 14C and 16C available for it because HarFAR prefers the 14C to 16C, unsaturated fatty acyl to saturated acyls, and the production rate were fairly high. By modifying HarFAR with attaching ER retention signal (KKYR) to the C-terminal of the enzyme, it improved the conversion rate for all the fatty acyl species. The plausible reason is this operation made the enzyme associated to the ER [98, 99] better, but we still lacking the knowledge about the original localization of FARs in the native moth pheromone gland cells, although when the insect-derived FAR was expressed in yeast cell, it is demonstrated they are located in ER [98].

3.5 Alcohol oxidation

Fatty alcohols can be the final pheromone components for a substantial number of moth species [3], for example, *C. pomonella*. But more commonly, fatty alcohols will be further processed like oxidized to aldehyde [65, 66, 67] or esterified to form acetate esters [83, 84]. Unlike FARs, these terminal functional group modification enzymes had not been studied extensively. The transcriptome studies on *Heliothis virescens* revealed a number of alcohol oxidase [109]. Efforts had been made on the characterization of alcohol oxidase from *H. virescens* but neither of the tested candidates gene showed valid activity in yeast expression system (Hagström and Löfstedt, personal communication). Gu et al. [108] also found 5 representatives of alcohol oxidase in *Agrotis ipsilon*, although this species did not use aldehyde as sex pheromone. If we search acyl-CoA oxidase in our As_PG library, we can also find many representatives (data not shown).

3.6 Acetyltransferase

This step is the last step in the type I pheromone biosynthesis pathway if the insect uses acetate ester as pheromone components, and supposed not determining the ratio composition of final pheromone compound [10, 11], although in several moth species the Z isomers are produced faster than the E isomers and get more products, when the alcohol substrates were supplemented to the pheromone gland homogenate [12]. The genes involved in this step have not been cloned in any insect species, that it probably belong to a huge family of acyl CoA-utilizing enzymes [13, 14] whose products include a variety of chemicals, such as neurotransmitters [15], plant volatile esters [16,

19], constitutive defense compounds, waxes [17], phytoalexins, lignin, phenolics, alkaloids, anthocyanins [18], make it very difficult to make functional predictions from primary sequence alone. Attempts had been made but end up with getting other member of the family [20]. Then one has to use other alternatives, to transform the fatty alcohols to acetate esters. One example is making biomicroreactor using plant derived wax ester synthase to convert long chain (12C-20C) unsaturated alcohol to its acetate, with a conversion rate about 30% [21, 22].

In this thesis I tested the substrate specificity of a plant-derived acetyltransferase (EaDAcT), that in the original source, catalyzes the formation of 3-acetyl-1,2-diacyl-sn-glycerols (acTAGs) [23]. When expressed in yeast, EaDAcT was able to produce a wide range of acetate esters when supplemented with alcohol substrates (paper IV). EaDAcT can use endogenous yeast acetyl-CoA since no exogenous source was provided. Among these different fatty alcohols with carbon chain length range from 10 to 18, EaDAcT has a preference on 12 carbon level, especially the unsaturated 12C alcohols, about half of the alcohol were esterified to acetate (produced about 15µg per 2mL culture). Similarly, on 14C level, the conversion rates of unsaturated alcohols (15-20%, product quantity about 4µg) were also bigger than the saturated 14:OH (6%). On the 18C level, the conversion efficiency was low (paper IV).

The turnip moth uses a series of medium chain acetate esters as sex pheromones, and there may exist an acetyltransferase that acetylate the fatty alcohols produced by the FAR. By expressing 34 genes annotated to be acetyltransferase in our yeast expression system (which had been used successfully to express the plant-derived acetyltransferase), revealed none of them could acetylate the fatty alcohols (the turnip moth pheromone intermediates, paper V). tBLASTn search with current published BAHDs [13] as query against our As_PG transcriptome got no hit. This maybe suggesting the genes involved in this step had been undergone significant change from the plant-derived genes.

3.7 Chain elongation

Cytoplasmic fatty acid synthase (FAS) plays a major role in the *de novo* biosynthesis of fatty acids. However, the elongation of fatty acids by this enzyme terminates at palmitic acid (C16) and stearic acid (18C).

To gain a longer chain length, the job is handed to fatty acid chain elongation system (FACES), refer to enzymes that are responsible for the addition of two carbon units to the carboxyl end of a fatty acid chain [40]. In both plants and animals, the elongation system is initiated by the condensation of malonyl-CoA with a long chain acyl-CoA, yielding a β-ketoacyl-CoA in which the acyl moiety has been elongated by two carbon atoms. The formed β-ketoacyl-CoA is then reduced by β-ketoacyl CoA reductase [43]

to β-hydroxyacyl-CoA, which is dehydrated by the dehydrase to an enoyl-CoA, and further reduced by the enoyl reductase [43] to yield the elongated acyl-CoA (Fig. 5B).

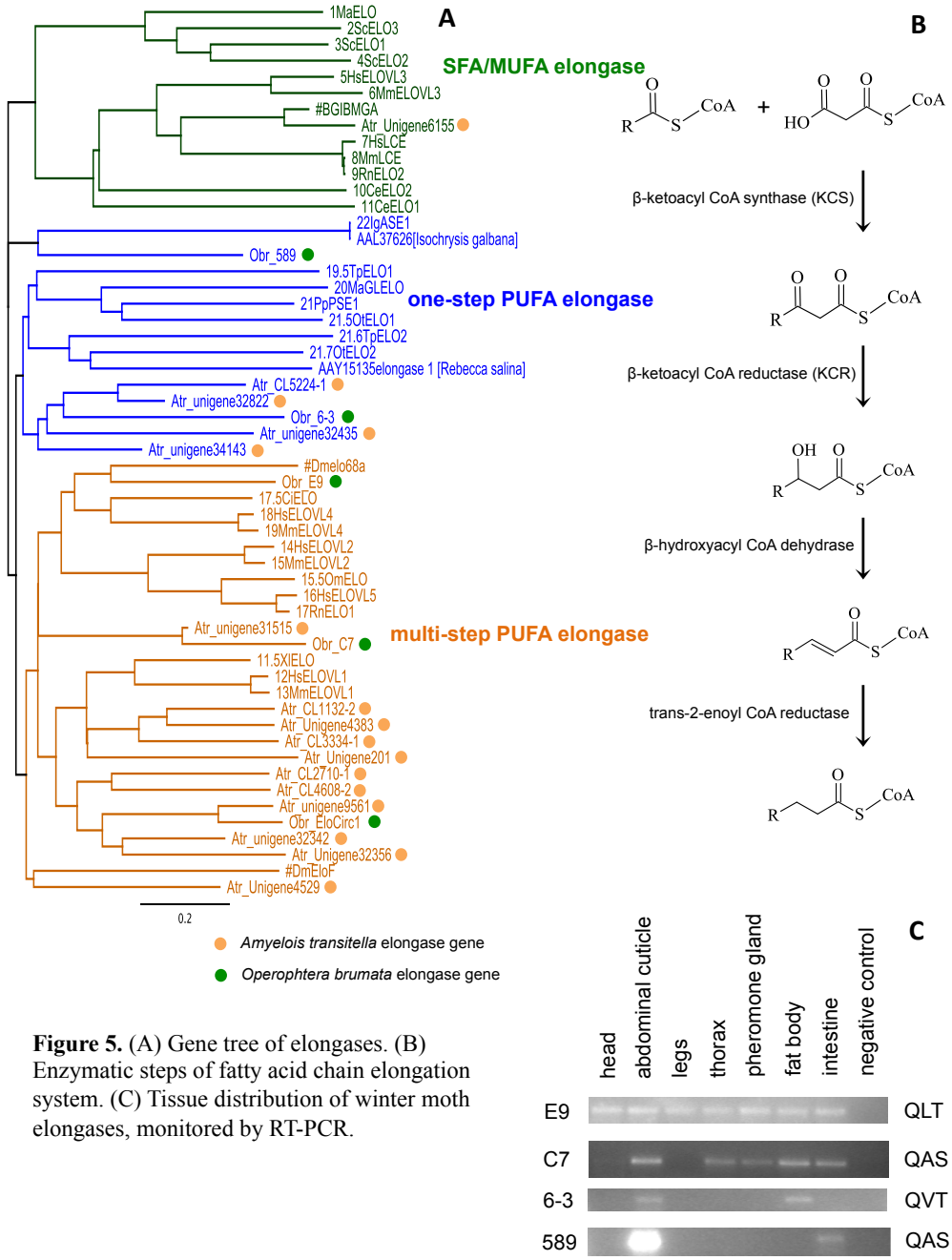


Figure 5. (A) Gene tree of elongases. (B) Enzymatic steps of fatty acid chain elongation system. (C) Tissue distribution of winter moth elongases, monitored by RT-PCR.

Biochemical studies have indicated that the condensing enzyme of the elongation system is the rate-limiting enzyme, and this enzyme also regulates the specificity of the substrate fatty acids in term of chain length and degree of unsaturation [41, 42], so "elongase" is frequently used to refer to the condensing enzyme [70, 71]. The remaining three enyzmes are more or less the same across kingdoms, forms the basis for heterologous expression.

Based on their specificity to substrate fatty acids, elongases can be classified largely into three groups: one is specific to saturated and monounsaturated fatty acids, another to PUFA of fixed chain-length ("single-step"), and the other is to PUFA with variable chain-lengths ("multi-step") [72]. In *Drosophila*, the elongases involved in long-chain hydrocarbon biosynthesis [114] cluster in this multi-step PUFA clade (Fig. 5A).

By designing degenerate primers (eloF: TTY YTN CAY KKN TWY CAY CA and eloR: NAR NSC RTA RTA NSH RTA CAT) based on the conserved motif [33, 74] QxxFLHxYHH and HxxMYxYY of elongase, 140bp fragment was amplified from cDNA of winter moth abdominal tissue. After cloning this fragment and sequencing it, five distinct transcripts were identified. By employing rapid amplification of cDNA ends, obtained the full-length open reading frame of all the five elongases in winter moth abdomen.

From the transcriptome data of navel orange worm abdominal tissue, 16 elongases gene objects were hunted out, 9 of them were full-length ORF of elongase. By looking at the flanking amino acids adjacent to conserved histidine box region [33], only the unigene6155 (PLI) is supposed to be the S/MUFA elongase among all these newly found elongases.

Phylogenetic tree were constructed based on data retrieved from [76] by combining elongase sequences from both winter moth and navel orange worm. The three groups of elongases stand out clearly, shown by different colors of branches.

Tissue expression distribution of the winter moth elongases were examined by RT-PCR (Fig. 5C), using gene specific primers. Obr_E9 is ubiquitously expressed, suppose to be the normal metabolic enzyme, whereas the other three genes showed various degree of differential expression pattern among the examined tissues, with Obr_589 and Obr_6-3 being the most promising ones involved in the pheromone biosynthetic pathway (Ding and Löfstedt, unpublished).

3.8 Decarboxylation

This is a cytochrome P450 dependent reaction. Hydrocarbon formation involves two steps conversion: 1) reduction of the long chain fatty acid to an aldehyde intermediate; 2) cytochrome P450 catalyze oxidation of the aldehyde into hydrocarbon, and release

of CO_2 [36, 37, 38, 39]. In the winter moth (*O. brumata*), *in vivo* labeling experiment showed that the LC-PUFA is incorporated into the hydrocarbon in the abdominal cuticles [103]. Alpha oxidation has been proven for the production of an even numbered hydrocarbon in the winter moth (*Erannis bajaria*) [110]. In the navel orange worm, we found two candidates of P4504G, unigene33263 and unigene3241, with high expression level in the abdominal cuticle tissue and antenna respectively. Further RNAi analysis is needed to prove their involvement in the formation of hydrocarbon in *Amyelois transitella* abdominal tissue.

3.9 Epoxidation

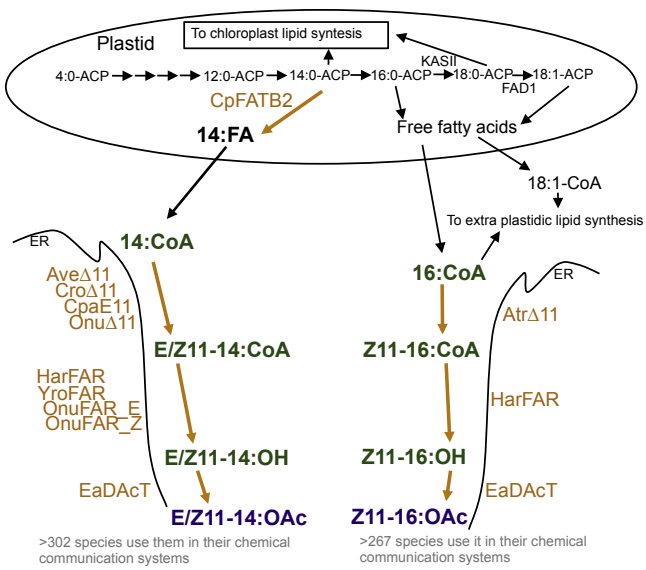
After the hydrocarbon pheromone compound has been produced in the oenocytes, it is transported to pheromone gland [88], and in some moths, it is epoxidized before release. *In vivo* study proved that monooxygenase that responsible for making the epoxide in the Japanese giant looper is regiospecific (always attack the Z3-double bond) regardless the hydrocarbon chain length and degree of unsaturation [87]. In this thesis, I did not address this step since none of the insect I studied uses epoxide as sex pheromone.

4. Production of moth pheromones in plants — proof of principle

4.1 Assembly of moth pheromone biosynthetic pathway in plant

In plant leaf tissue, the fatty acid biosynthesis is happening in the chloroplast, and the carbon source for synthesizing fatty acid is from the C3 products of photosynthetic carbon fixation [1]. Palmitic and oleic acid are released into the cytosol as precursor for extra-plastid lipid synthesis. We took advantage of the pool of 16:CoA in the cytosol and introduced a Δ11 desaturase that specifically recognizes 16:CoA (AtrΔ11 desaturase) to produce Z11-16:CoA. A FAR from another moth (HarFAR) was introduced to reduce the Z11-16:CoA into Z11-16:OH. Finally the plant derived acetyl transferase gene EaDAcT was used to transfer an acetate group to form the Z11-16:OAc.

By introducing a thioesterase, specifically releasing the 14:ACP from the chloroplast to the cytosol, we produced Z11-14:OAc and E11-14:OAc. 14:CoA was desaturated by an insect Δ11 desaturase (AveΔ11) to form a mixture of E- and Z11-14:CoA. The fatty acid reductase HarFAR was used to produce the alcohols and finally EaDAcT was used to form the acetates E- and Z11-14:OAc.



This work use *Nicotiana benthamiana* as a plant factory, through extensively modification of plant lipid metabolism, we made complicated, hard-to-synthesize, multiple component moth sex pheromones, proved the principle that plant can be used to make moth pheromones. The long-term

vision is to design tailor-made production of any moth pheromone component in stably transformed plants.

4.2 Test of attractiveness of the plant-derived pheromone

Fatty alcohols produced by the engineered tobacco was purified by thin layer chromatogram, and then acetylated chemically to get acetate esters. The esters from two different treatments (16C and 14C) were mixed to form a blend to mimic the pheromone composition of female moth, *Yponomeuta padella*. The blend, about 20µg was loaded to rubber septa, hanging out in the field in a trap, to test their attractiveness to male moths. For trapping *Y. evonymella*, only the acetates from the 14C plant were used. Field trap experiments reveled great success; the plant-derived pheromones are as effective as the organically synthetic ones (Fig. 6).

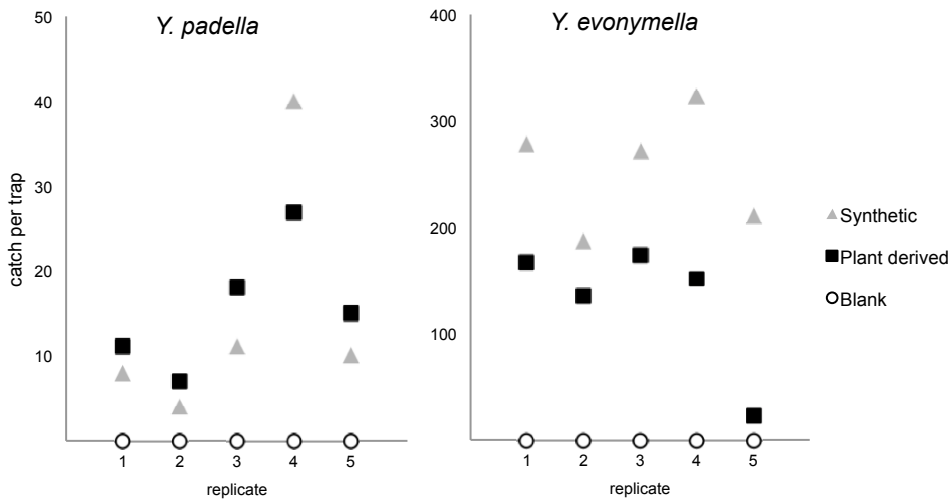


Figure 6. Trap catches of males of two small ermine moth species obtained with synthetic pheromone (positive control), plant-derived pheromone, and blank traps (negative control) (N=5).

5. Conclusions and Perspectives

Pheromones are environmentally friendly alternatives to the use of traditional pesticides for control of insect pests and indeed synthetic pheromones are annually produced in huge amounts for this purpose [113]. The study of moth pheromones has been a hot spot since the first identification of the sex pheromone in *Bombyx mori* [115]. Thousands of sex pheromones have been chemically identified [3], and the biosynthetic pathways of hundreds of moth pheromones have been elucidated [34,44,45]. Moreover, the enzymes and even the genes encoding these enzymes have been studied intensively [46], especially the desaturation step [111] and reduction step [112].

In this thesis, I contributed to the identification, characterization and optimization of the genes that can be used for the biosynthesis of moth pheromones. Desaturases from *Operophtera brumata* (paper I) and *Cydia pomonella* (paper II) were identified which are specialized on long chain fatty acyl (20C) and short chain fatty acyl (12C) respectively. By mutagenesis we identified an amino acid that is important for Z activity in a desaturase operating on myristic acid (paper III).

Although the moth pheromone biosynthetic acetyltransferase have not been cloned yet, we instead expressed a plant-derived acetyltransferase (EaDAcT) in yeast expression system. EaDAcT was characterized *in vivo* and *in vitro* and revealed its ability of converting various fatty alcohol intermediates into acetate esters (paper IV). At the same time, we noticed that the yeast endogenous acetyltransferase can also acetylate long chain fatty alcohols (10C-18C) but previous studies only focused on the short chain range (4C-10C). Similarly, in the plant species, like kiwi, apple, banana, acetyltransferases have also been characterized but also focused on short chain alcohols. It is tempting to test the ability of these acetyltransferase on esterifying moth pheromone related fatty alcohols.

Moreover, by using the massive sequencing technology on *Agrotis segetum* pheromone gland, I generated lists of gene candidate that may be useful for the characterizing the acetyl-CoA carboxylase, fatty acid synthetase, β-oxidation enzymes, and the acetylation enzymes involved in pheromone biosynthesis. I found that the pgFAR in *A. segetum* is a versatile FAR that can reduce all the three fatty acyl intermediates into fatty alcohols (paper V).

The genes and techniques available to us constitute a potent toolbox from which we can pick up elements to be used in the construction of genetically modified plants with an ability to produce insect pheromones. I used this synthetic biology approach, to combine these elements and produced multi-component moth pheromones in tobacco plant by transiently expressing 3-4 biosynthetic genes (paper VI). We produced microgram amounts of sex pheromones specific for two species of moths. Importantly, these *in planta* synthesized compounds matched conventionally produced pheromones with regard to attractiveness and specificity in field trapping experiments.

This study paves the way for future production of insect pheromones in plant factories. Such a synthetic biology approach represents a novel and environmentally friendly way of producing moderate to large quantities of moth sex pheromones (high-value product) with high purity and a minimum of hazardous waste. This method therefore has the potential to become an economically sound part of many integrated pest management (IPM) programs.

With the continuous advancing and efforts of large-scale genome and transcriptome sequencing, more and more useful information will be provided on the knowledge of pheromone production of various moth species. With this information, synthetic biologist will construct and test more parts and devices that will enable the production of wide range of moth pheromone components.

Systematic modifications of yeast and plant lipid metabolism are needed in order to achieve a better production of moth pheromones in yeast cell factory [101] and plant cell factory (paper VI). In yeast, desaturation occurs on the acyl moiety of phospholipid (sn-2 position [31]), elongation occurs on the acyl moiety of the acyl-CoAs, and the acyl-CoA pool is limited, leading to lower conversion efficiency by elongases [29]. The source of acyl-CoAs, the key intermediates in TAG biosynthesis, is both newly synthesized fatty acids and fatty acids released from TAGs by lipases. Acyl-CoAs can then be incorporated into either TAGs or phospholipids by various acyltransferases or degraded by β-oxidation. Increased expression of diacylglycerol acyltransferase (DGAT) and cholinephosphotransferase (CPT) markedly increased the lipid production in *Yarrowia lipolytica* yeast [30]. Lipid content is also determined by the relative strength of the synthesis and degradation of acyl-CoAs. In a pex10Δ strain, β-oxidation was impaired, fatty acids might be released from TGAs, but acyl-CoAs would be used as substrates for fatty acid elongases and acyltransferases only, as there is no β-oxidation, resulting in higher and stable lipid content [32]. These are fields worthwhile to explore with the aim of improving semisynthetic production of insect pheromones in different chassis.

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Svensk sammanfattning

Nattfjärilslarver är viktiga jordbruksskadedjur i många delar av världen. Men det finns nattfjärilarar som odlas för deras silkeskokong, till exempel den anmärkningsvärda silkesfjärilen, *Bombyx mori*. Denna art är den första och bäst studerade insektsarten som använder flyktiga organiska molekyler att kommunicera med, och med oerhört hög sensitivitet och specificitet. I allmänhet är det nattfjärilshonorna som lockar hannarna med sitt könsferomon över långa avstånd. De flesta beskrivna nattfjärilskönsferomonerna hör till samma klass av kemiska föreningar, nämligen fettsyrederivat som produceras *de novo* i feromonkörteln. Deras feromonbiosyntes innebär desaturation, kedjeförkortning med β-oxidation och slutligen reduktion, acetylering eller oxidation. Dessa enzymer har utvecklats för att fungera i produktionen av de komplexa kemiska signaler som används för sexuell attraktion, vilket bidrar till den kemiska mångfalden av nattfjärilsferomoner.

I denna avhandling har flera desaturaser undersökts. Först är det en terminalfett-acyl-CoA-desaturas från frostfjärilen *Operophtera brumata* som klonats och uttryckts heterologt i jäst och visat sin förmåga att omvandla Z11,Z14,Z17-eikosatriensyra till Z11,Z14,Z17,19-eikosatetraensyra. Detta är den första rapporten om metyl-terminal-desaturas någonsin.

I det andra manuskriptet har jag visat de desaturationssteg i *Cydia pomonella* och *Grapholita molesta* som använder omättad dodekenylalkohol och/eller -acetat som sexualferomonkomponent(er). Jag fann desaturaser i *C. pomonella* som arbetar i följd och bidrar till att producera en konjugerad dubbelbindning i fettsyrekedjan (E8,E10-12:CoA). Denna studie är ett exempel på hur en konjugerad dien kan produceras i en nattfjärilferomonkörtel. Men för *G. molesta* upptäckte jag att Δ10 desaturaser använder mättat 14-kol. Den E/Z10-14:Acyl genomgå kedjeförkortning, slutligen reduktion och acetylering kan producera *G. molesta* feromon som består av Z8-12:OAc, E8-12:OAc, och Z8-12:OH.

Det tredje manuskriptet handlar om stereospecificiteten hos Δ11-desaturaser. En Δ11-desaturas från *Choristoneura rosaceana* använder mättat 14-kol och producerar en blandning av Z/E11(65:35) C14-syra. En desaturas från *C. parallela* tar också mättad 14-kol, men producerar enbart ren E11-14C. Ömsesidiga riktade mutationer på dessa desaturaser avslöjade att en aminosyra vid C-terminalen av proteinet är kritisk för Z-aktiviteten av desaturaset (vinst eller förlust av funktion). Denna studie är fundamental för förståelsen av stereospecificiteten av desaturas.

Den fjärde studien fokuserade på fettsyrealkohol-acetyltransferas som har stor påverkan på många nattfjärilsferomoners biosyntesvägar. Den katalyserar bildningen av acetat-ester genom att överföra acetatgruppen från acetyl-CoA till fettsyrealkohol. Eftersom inget feromonbiosyntetiskt acetyltransferas som härstammar från insekter klonats, uttryckte jag en heterologiskt acetyltransferas, *EaDAcT*, utvecklat från växter istället, i ett jästsystem för att testa funktionaliteten och giltigheten i att omvandla fjärilsferomonintermediärer, fettsyrealkoholer, till den slutliga feromonprodukten, nämligen fettsyrealkohol-acetatestrar. Resultaten visade att *EaDAcT* kunde konvertera olika fettsyrealkoholer med kedjelängd från 10 till 18 kolatomer, med dubbelbindningar vid olika positioner i motsvarande acetatestrar. *EaDAcT* föredrar kortare kedjelängd över de långa, dvs. omättade över mättade.

I den femte studien använde jag ”next-generation sequenzing” av feromonproducerande vävnad för att kunna identifiera gener som kan vara involverade i den feromonbiosyntetiska processen för sädesbroddflyet, *Agrotis segetum*, till exempel fettsyresyntas, β-oxidationsenzymer, desaturaser, fettacylreduktaser , acetyltransferaser, etc.

Den sista studien var ”montering” av delar som tidigare identifierats i ett ”chassi” för att göra nattfjärilsferomoner. Genom att använda tobaksplantan, *Nicotiana benthamiana*, som en ”växtfabrik” producerade vi typiska nattfjärilsferomon-komponenter genom ”transient expression” av upp till fyra gener som kodar för konsekutiva biosyntetiska steg. Vi producerade specifikt biologiskt aktiva flerkomponentsferomoner för två arter spinnmalar, *Yponomeuta*. Fettsyrealkohol-fraktioner från de genetiskt modifierade växterna acetylerades och blandades för att efterlikna respektive könsferomon från *Y. evonymella* och *Y. padella*. Även om sammansättningen av de från växter härstammande blandningarna inte var optimerad, var dessa blandningar mycket effektiva och specifika för fångst av nattfjärilshanar, och matchar mycket väl aktiviteten av konventionellt framställda syntetiska feromoner. Vår långsiktiga vision är att utforma skräddarsydd produktion av valfria nattfjärilsferomonkomponenter i genetiskt modifierade växter. Sådan semisyntetisk beredning av könsferomoner kan vara ett nytt och kostnadseffektivt sätt att producera måttliga till stora mängder feromoner för integrerat växtskydd, med hög renhet och ett minimum av farligt avfall.

中文摘要

在世界許多地方蛾類幼蟲是主要的農業害蟲，但也有蛾類養殖業為獲取其絲綢蠶繭，例如家蠶。其實它也是第一個也是最好的研究揮發性有機分子用於傳遞信息的昆蟲研究模型。一般來說是雌蛾釋放信息素通過很長的距離來吸引雄性同類，而且具有出色的靈敏度和特異性。至今有記載的蛾類信息素都屬於同一類化合物(脂肪酸衍生物), 在信息素腺體內合成。信息素的生物合成涉及到脂肪酸的脫飽合，碳鏈縮短，並最終還原，乙酰化或氧化成醛等。這些反應過程中的酶已經進化到具有專一性，專門用於生產複雜的化學信號，使得蛾類的信息素存在豐富的化學多樣性。在本論文中，首先對幾個脫飽和酶進行研究。第一篇文章對冬尺蠖的脫飽合酶進行克隆並在酵母中通過異源表達證明了它具有把順十一,順十四,順十七-二十碳三烯酸轉化成順十一,順十四,順十七,十九-二十碳四烯酸的能力。這是有史以來第一個對甲基端脫飽合酶的報導。第二篇文章報導了來自蘋果蠹蛾和梨小食心蟲的脫飽合酶。它們都使用不飽和的十二碳烯醇和乙酸酯作為性信息素成分。在蘋果蠹蛾中發現兩個脫飽和酶連續工作產生共軛雙鍵。但在梨小食心蟲中，發現是由同一個脫飽和酶工作兩次產生的共軛雙鍵。這項研究提供了在蛾信息系素腺體內可以生產共軛二烯的又一個例子。第三篇文章對十一位脫飽和酶的立體特異性進行了調查。薔薇斜條捲葉蛾的十一位脫飽和酶作用於飽和十四酸，產生的順十一十四酸比反十一十四酸等於一百比四十七的混合物; 而來自於斑點捲葉蛾的脫飽和酶也作用於十四個碳飽和酸，但只生產純的反十一十四酸。通過對這兩個脫飽和酶的定點突變研究證明，一個在蛋白的羧基末端的氨基酸對脫飽和酶的順式活性至關重要。第四篇文章的研究集中在脂肪醇乙酰轉移酶，其在許多蛾類性信息素的生物合成途徑中承擔很大的作用。它催化的反應是轉移乙酰基給脂肪醇形成乙酸酯。由於此酶沒有在昆蟲中被克隆到，本研究異源表達了植物來源的乙酰基轉移酶，在酵母系統中測試其轉化蛾類性信息素脂肪醇的功能。結果表明其可以轉化碳鍊長度範圍從十個碳到十八個碳，不同雙鍵位置的各種脂肪醇，成為其相應的乙酸酯。第五篇文章利用下一代測序技術對黃地老虎信息素腺體進行分析，鑑定出許多與信息素合成相關的基因及過程，例如脂肪酸合成酶、脫飽合酶、貝塔氧化、還原酶、乙酰基轉移酶等等。在第六篇文章中利用煙草作為生物反應器來生產蛾類信息素來證明這個理論是可行的。通過瞬時表達四個蛾類信息素合成基因本研究成功生產出典型的蛾類信息素並證明了其具有能吸引雄蛾的生物活性。雖然利用植物生產的信息素的成份和比例沒有經過優化，但還是誘捕到大量的稠李巢蛾和蘋果巢蛾，其效果與有機合成的信息素相匹配。我們的長期目標是利用轉基因植物來生產任何類型的蛾類信息素。這種新穎的利用合成生物學的技術來製備信息素的方法將來也許會成為害蟲綜合防治的首選。

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PLANT-DERIVED MOTH PHEROMONES ATTRACTED MALE MOTHS, *YPONOMEUTA EVONYMELLA.*

We demonstrated that different moth pheromone components could be produced in the *Nicotiana benthamiana* by transient expression of the necessary biosynthetic genes. We produced microgram amounts of sex pheromones specific for two species of moths in *N. benthamiana*. Importantly, these *in planta* synthesized compounds matched conventionally produced pheromones with regard to attractiveness and specificity in field trapping experiments. Such a synthetic biology approach represents a novel and environmentally friendly way of producing moderate to large quantities of moth sex pheromones (high-value product) with high purity and a minimum of hazardous waste. Our method therefore has the potential to become an economically sound part of many integrated pest management (IPM) programs.

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