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Plants as Factories for Insect Pheromone Production

Deciphering and Reconstructing Sex Pheromone Biosynthetic Pathways of Female Moths

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Plants as Factories for Insect
Pheromone Production

Deciphering and Reconstructing Sex Pheromone
Biosynthetic Pathways of Female Moths

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DEPARTMENT OF BIOLOGY | FACULTY OF SCIENCE | LUND UNIVERSITY



Plants as Factories for Insect Pheromone Production

Deciphering and Reconstructing Sex Pheromone
Biosynthetic Pathways of Female Moths

Yi-Han Xia



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Abstract <p>Compared to other organisms in which the fatty acyl desaturases (FADs) are mostly involved in normal cellular lipid metabolism, moth FADs have evolved extent functions in the biosynthesis of sex pheromones. Female moths release species-specific sex pheromones to attract conspecific males over a long distance for mating. Moth FADs are key enzymes producing the great diversity of moth sex pheromones. They introduce double bonds in specific positions and with specific geometry in the fatty acyl pheromone precursors.</p> <p>In this thesis, I use a variety of experimental approaches including isotope labelling experiments and heterologous expression of gene candidates to characterize several novel FADs involved in pheromone production: The multi-functional <i>SexiDes5</i> from the beet armyworm <i>Spodoptera exigua</i> and <i>SlitDes5</i> from the congeneric <i>Spodoptera litura</i> were found to have $\Delta 12$ desaturase activities. They use palmitic acid to produce (Z)-11-hexadecenoic acid and the subsequently chain-shortened product (Z)-9-tetradecenoic acid to produce (Z,E)-9,12-tetradecadienoic acid. The European grapevine moth, <i>Lobesia botrana</i> was shown to produce its major pheromone precursor (E,Z)-7,9-dodecanoic acid by an $\Delta 7$ FAD. A pheromone gland-specific <i>CsupYPAQ</i> from the rice stem borer <i>Chilo suppressalis</i> was proven to have high activity on palmitic acid to produce (Z)-11-hexadecenoic acid.</p> <p>The highly evolved moth FADs can be used for production of customized pheromone precursors in transformed organisms for a variety of purposes. Compared to the current conventional synthetic approach which producing the hazardous waste during the production process, using semi-synthetic method to produce moth pheromones based on plant-derived pheromone precursors are environmentally friendly I investigated the use of several plant platforms to express a suite of biosynthetic enzymes for moth pheromone precursor production. By employing the <i>Agrobacterium</i>-mediated transformation, I constructed transgenic <i>Nicotiana</i> spp. and <i>Camelina</i> lines for production of C₁₂ to C₁₆ chain length pheromone precursors. The transformed <i>Nicotiana</i> spp. can produce (Z)-11-hexadecenoic acid, (E)-11-tetradecenoic acid, (Z)-11-tetradecenoic acid. The best line from <i>N. benthamiana</i> produced 17.6% (weight%) of (Z)-11-hexadecenoic acid of total fatty acid in vegetative tissue. Also, 7.6% of (E)-9-dodecenoic acid and 6.3% of doubly unsaturated (E,E)-8,10-dodecenoic acid of total fatty acids were produced in seeds of engineered <i>Camelina</i> plants, implying that a significant amount of pheromone precursors might be produced by cultivating these transgenic plants under field conditions.</p> <p>Knowledge of additional pheromone biosynthetic gene functions can improve the possibility and feasibility of synthesizing customized moth pheromones in plant factories. A fatty acyl elongase (ELO) combined with a $\Delta 11$ FAD is thought to provide the fatty acyl pheromone precursors in <i>C. suppressalis</i>. I functionally characterized an ELO gene <i>CsupELO4</i> encoding a protein elongating the major pheromone precursor (Z)-11-hexadecenoic acid into (Z)-13-octadecenoic acid, the precursor of a minor pheromone component. This is the first ELO gene that has been functionally characterized in Lepidoptera. The fatty acyl-CoA pheromone precursors are postulated to be reduced and reoxidized to produce the aldehyde pheromone components. I characterized <i>CsupFAR2</i> from <i>C. suppressalis</i> that encodes a fatty acyl reductase (FAR) reducing the major fatty acyl precursors into corresponding fatty alcohols, which are convert into the fatty aldehyde pheromones by followed-up oxidation.</p> <p>Genetically modified plants actually releasing moth pheromones may be used as part of a push-pull strategy. I made an attempt to engineer <i>Nicotiana</i> spp. plants that would release (Z)-11-hexadecenol and (Z)-11-hexadecenyl acetate. I cloned the promoter CYP71D16, which is a trichome-specific promoter from tobacco <i>Nicotiana tabacum</i>, driving the pheromone biosynthetic genes. I surprisingly found that the production of (Z)-11-hexadecenol increased from 18 to 70 μg per gram fresh leaf when the gene of HarFAR was expressed under CYP71D16 promoter compared to a constitutive promoter CaMV35S. However, no pheromone compounds were found in the plant headspace volatiles.</p>		
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The science of today is the technology of tomorrow

---Edward Teller

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List of papers

The thesis is based on the following paper,

- I. **Yi-Han Xia**, Ya-Nan Zhang, Bao-Jian Ding, Hong-Lei Wang, Christer Löfstedt. (2019) Multi-Functional Desaturases in Two *Spodoptera* Moths with $\Delta 11$ and $\Delta 12$ Desaturation Activities. *Journal of Chemical Ecology* **45**, 378–387. <https://doi.org/10.1007/s10886-019-01067-3>
- II. Bao-Jian Ding*, **Yi-Han Xia***, Hong-Lei Wang, Erik Hedenström, Jürgen Gross, Christer Löfstedt (2020) Biosynthesis of the Sex Pheromone (E,Z)-7,9-dodecadienyl acetate in the European Grapevine Moth *Lobesia botrana* involving $\Delta 11$ Desaturation and an Elusive $\Delta 7$ Desaturase. (*, shared first authorship). Manuscript.
- III. **Yi-Han Xia**, Bao-Jian Ding, Shuang-Lin Dong, Hong-Lei Wang, Christer Löfstedt. (2020) Characterization of genes involved in Sex Pheromone Biosynthesis in the Rice Stem Borer *Chilo suppressalis* (Lepidoptera: Pyraloidea): a Novel Functional Fatty Acyl-CoA Elongase Gene Revealed. Manuscript.
- IV. **Yi-Han Xia**, Bao-Jian Ding, Hong-Lei Wang, Carin Jarl-Sunesson, Per Hofvander, Christer Löfstedt. (2020) Production of Moth Sex Pheromone Precursors in *Nicotiana* spp. by *Agrobacterium*-mediated Stable Transformation. Submitted.
- V. **Yi-Han Xia**, Bao-Jian Ding, Hong-Lei Wang, Carin Jarl-Sunesson, Edgar B. Cahoon, Per Hofvander, Christer Löfstedt. (2020) Metabolic Engineering of Camelina (*Camelina sativa*) for the Production of Mono- and Di-unsaturated C₁₂ Moth Pheromone Precursors. Manuscript.

Authors contributions to the papers

- I. YHX, HLW, YNZ and CL conceived the study. YHX and HLW carried out the labelling experiment and all chemical analysis using GC/MS. YHX and BJD performed molecular work, cell line generation and culturing. YHX, HLW and CL wrote the paper with input from all authors.
- II. BJD, YHX, HLW and CL conceived the study. YHX and HLW carried out the labelling experiment and all chemical analysis using GC/MS. YHX and BJD performed molecular work and bioinformatic analysis. YHX and BJD drafted the manuscript, all authors edited the manuscript and approved the final version.
- III. YHX, BJD and CL conceived the study. YHX performed the research. YHX drafted the manuscript, all authors edited the manuscript and approved the final version.
- IV. YHX, BJD, PH, and CL conceived the study. YHX and BJD carried out vector design, sequencing; YHX performed leaf-disc transformation, plant cultivation and all the sample analysis; YHX drafted the manuscript, all authors edited the manuscript and approved the final version.
- V. YHX, BJD, and CL conceived the study. YHX and BJD carried out vector design, sequencing; YHX performed floral dip transformation, plant cultivation and all the sample analysis; BJD, HLW, PH, EBC, and CL provided technical guidance and suggestions on metabolic engineering strategies, YHX drafted the manuscript, all authors edited the manuscript and approved the final version.

Abbreviations

ACC,	acetyl-CoA carboxylase
ACO,	acyl-CoA oxidase
ACP,	acyl carrier protein
ATF,	acyltransferase
DAG,	diacylglycerol
DGAT,	diacylglycerol acyltransferase
DMDS,	dimethyl disulfide
DNA,	deoxyribonucleic acid
ELO,	long-chain elongase
FAD,	fatty acyl desaturase
FAR,	fatty acyl reductase
FAS,	fatty acyl synthase
FatA,	fatty acyl-acyl carrier protein thioesterase A
FatB,	fatty acyl-acyl carrier protein thioesterase B
FFA,	free fatty acid
GC/MS,	gas chromatography/mass spectrometry
GPAT,	glycerol-3-phosphate acyltransferase 9
LPAAT,	lysophosphatidic acid acyltransferase
MAG,	monoacylglycerol
MCFA,	medium-chain fatty acid
ME,	methyl ester
MUFA,	monounsaturated fatty acid
ORF,	open reading frame
PCR,	polymerase chain reaction
PL,	polar lipid
PUFA,	polyunsaturated fatty acid
RNA,	ribonucleic acid
SE,	sterol ester
SFA,	saturated fatty acid
SIM,	selected ion monitoring
TAG,	triacylglycerol

Abstract

Compared to other organisms in which the fatty acyl desaturases (FADs) are mostly involved in normal cellular lipid metabolism, moth FADs have evolved extensive functions in the biosynthesis of sex pheromones. Female moths release species-specific sex pheromones to attract conspecific males over a long distance for mating. Moth FADs are key enzymes producing the great diversity of moth sex pheromones. They introduce double bonds in specific positions and with specific geometry in the fatty acyl pheromone precursors.

In this thesis, I use a variety of experimental approaches including isotope labelling experiments and heterologous expression of gene candidates to characterize several novel FADs involved in pheromone production: The multi-functional *SexiDes5* from the beet armyworm *Spodoptera exigua* and *SlitDes5* from the congeneric *Spodoptera litura* were found to have $\Delta 12$ desaturase activities. They use palmitic acid to produce (*Z*)-11-hexadecenoic acid and the subsequently chain-shortened product (*Z*)-9-tetradecenoic acid to produce (*Z,E*)-9,12-tetradecadienoic acid. The European grapevine moth, *Lobesia botrana* was shown to produce its major pheromone precursor (*E,Z*)-7,9-dodecanoic acid by an $\Delta 7$ FAD. A pheromone gland-specific *CsupYPAQ* from the rice stem borer *Chilo suppressalis* was proven to have high activity on palmitic acid to produce (*Z*)-11-hexadecenoic acid.

The highly evolved moth FADs can be used for production of customized pheromone precursors in transformed organisms for a variety of purposes. Compared to the current conventional synthetic approach which produces hazardous waste during the production process, using semi-synthetic method to produce moth pheromones based on plant-derived pheromone precursors are environmentally friendly. I investigated the use of several plant platforms to express a suite of biosynthetic enzymes for moth pheromone precursor production. By employing the *Agrobacterium*-mediated transformation, I constructed transgenic *Nicotiana* spp. and *Camelina* lines for production of C_{12} to C_{16} chain length pheromone precursors. The transformed *Nicotiana* spp. can produce (*Z*)-11-hexadecenoic acid, (*E*)-11-tetradecenoic acid, (*Z*)-11-tetradecenoic acid. The best line from *N. benthamiana* produced 17.6% (weight%) of (*Z*)-11-hexadecenoic acid of total fatty acid in vegetative tissue. Also, 7.6% of (*E*)-9-dodecenoic acid and 6.3% of doubly unsaturated (*E,E*)-8,10-dodecenoic acid of total fatty acids were produced in seeds of engineered *Camelina* plants, implying that a significant amount of pheromone precursors might be produced by cultivating these transgenic plants under field conditions.

Knowledge of additional pheromone biosynthetic gene functions can be used to improve the possibility and feasibility of synthesizing customized moth pheromones in plant factories. A fatty acyl elongase (ELO) combined with a $\Delta 11$ FAD is considered to provide the fatty acyl pheromone precursors in *C. suppressalis*. I functionally characterized an ELO gene *CsupELO4* encoding a protein elongating

the major pheromone precursor (*Z*)-11-hexadecenoic acid into (*Z*)-13-octadecenoic acid, the precursor of a minor pheromone component. This is the first ELO gene that has been functionally characterized in Lepidoptera. The fatty acyl-CoA pheromone precursors are postulated to be reduced and reoxidized to produce the aldehyde pheromone components. I characterized *CsupFAR2* from *C. suppressalis* that encodes a fatty acyl reductase (FAR) reducing the major fatty acyl precursors into corresponding fatty alcohols, which are converted into the fatty aldehyde pheromones by followed-up oxidation.

Genetically modified plants actually releasing moth pheromones may be used as part of a push-pull strategy. I attempted to engineer *Nicotiana spp.* plants that would release (*Z*)-11-hexadecenol and (*Z*)-11-hexadecenyl acetate. I cloned the promoter CYP71D16, which is a trichome-specific promoter from tobacco *Nicotiana tabacum*, driving the pheromone biosynthetic genes. I surprisingly found that the production of (*Z*)-11-hexadecenol increased from 18 to 70 µg per gram fresh leaf when the gene of HarFAR was expressed under CYP71D16 promoter compared to a constitutive promoter CaMV35S. However, no pheromone compounds could be found in the plant headspace volatiles.

Popular science summary

To avoid serious damage to crops, you should pay attention to the moths that are discovered in your field during farming. The female moths lay eggs on the crops and the hatched larvae will feed on the crops, causing serious damage. Moths heavily rely on sex pheromone to communicate between males and females for mating. Pheromones are molecules used for communication between living organisms, across the tree of life from bacteria to humans. Female moths emit species-specific sex pheromone component blends to attract males of the same species over long distances. There are more than 160,000 species described in the order Lepidopteran (moths and butterflies). They are among the most damaging pests of food and fibre crops, capable of adapting fast and evolving resistance to insecticides. Conventional insecticides for pest control do not discriminate between pest and other non-target insects and can in many cases be harmful to other organisms, including humans, and detrimental to plants that are dependent on beneficial insects for pollination. Using pheromones for pest control (such as mass trapping and mating disruption) has become an environmentally friendly alternative because the pheromones are non-toxic, they have no adverse effects on non-target organisms, they do not kill parasitoids or other beneficial insects, and the risks of resistance being developed in the pest is small. Even in terms of profit and reduction in damage, pheromones often compare favourably to the use of insecticides. The global market for pheromone-based control products is currently estimated to more than \$200 millions. However, current standard approaches to pheromone synthesis either require the use of hazardous chemicals or may result in the production of hazardous waste by-products, and it is most difficult to modify the double bonds for production of unsaturated pheromone precursors. The problems inherent to synthetic pheromone production may be overcome by developing an innovative green chemistry alternative, minimizing hazards.

A majority of the identified moth sex pheromone components consists of fatty acid derivatives, which are biosynthesized in species-specific pathways involving successive enzymes activities. Among the enzymes, fatty acyl desaturases (FADs) play an important role to produce the great diversity of sex pheromones between species, introducing double bonds in specific positions of the fatty acyl chain to form the pheromone skeletons. By benefiting from current development of biotechnology and bioengineering, and the functionally characterized pheromone biosynthetic gene toolbox, such as FADs, it has now become possible to synthesize customized pheromone in transformed organisms efficiently. In this thesis, I investigated the use of several plant platforms (tobacco and *Camelina*) to express a suite of biosynthetic enzymes for pheromone precursors production. I established several stable transgenic plant lines for the production of high value pheromones, ranging from the carbon chain length of C_{12} to C_{16} , in either leaves or seeds. The constructed transgenic plant lines produced a significant amount of pheromone

precursors in the greenhouse. This research can be a significant step forward to enable pheromone stable production in plant factories.

In addition to FADs, there are some other important enzymes involved in moth pheromone biosynthesis. For example, fatty acyl elongases (ELOs) and acyl-CoA oxidases (ACO) are expected to determine the pheromone skeletons when combined with FADs. Fatty acyl reductases (FARs) can reduce the pheromone precursors into corresponding alcohol pheromones. To improve the possibility and feasibility of producing customized moth pheromones in plant factories, more functional genes need to be characterized to enlarge the gene toolbox.

In this thesis, first, I elucidated the sex pheromone biosynthetic pathways of the beet armyworm *Spodoptera exigua* and the European grapevine moth *Lobesia botrana*, which use sex pheromone compounds with two double bonds. Furthermore, in order to clarify the molecular mechanism of moth sex pheromone biosynthesis, I functionally characterized several genes from three moth species encoding corresponding pheromone biosynthetic enzymes, including the genes encoding ELO and ACO which are the first time to be reported in Lepidoptera. These findings improve the feasibility of using plant factories for large-scale customized pheromone production.

Moreover, the final goal of this green chemistry alternative for pest control is to be able to grow the pheromone-releasing plant in the field. Thus, it would be important to obtain plants capable of releasing pheromones into the environment. Here, I made some effort to explore this possibility with tobacco. I cloned a gene promoter called CYP71D16 to drive the pheromone biosynthetic genes in tobacco trichomes. It was surprising to find that the pheromone production amount was increased significantly compared to the use of a constitutive promoter, however no pheromones could be collected from the plant headspace volatiles.

Populär sammanfattning på svenska

För att undvika allvarliga skador på grödor bör man vara uppmärksam på de fjärilar som upptäckts i åkrarna. Nattfjärilarnas honor lägger ägg på grödorna och de kläckta larverna kan komma att äta av grödorna och orsaka allvarliga skador. Nattfjärilar förlitar sig starkt på sexualferomoner för att kommunicera mellan hanar och honor i samband med parningen. Feromoner är molekyler som används för kommunikation mellan levande organismer inom hela "tree of life" från bakterier till människor. I allmänhet släpper fjärilshonorna ut artspecifika sexualferomoner för att locka till sig hanar av samma art över långa avstånd. Det finns mer än 160 000 beskrivna arter fjärilar inom insektsordningen Lepidoptera (nattfjärilar och dagfjärilar). De är bland de mest betydande skadedjuren i livsmedels- och fibergrödor och kan anpassa sig snabbt och utveckla motståndskraft mot insekticider. Konventionella insektsbekämpningsmedel skiljer inte på skadedjur och andra insekter utan är i många fall skadliga för andra organismer, inklusive människor, och även för växter som är beroende av nyttoinsekter för pollinering. Att använda feromon för skadedjursbekämpning (genom massfånst och parningsstörning) har blivit ett miljövänligt alternativ eftersom feromonerna är icke-toxiska; de har inga negativa effekter på andra organismer, de dödar inte parasitoider eller andra nyttoinsekter, och riskerna för att resistens utvecklas är små. Även när det gäller skördevinst och minskning av skador utfaller feromoner ofta positivt jämfört med om insekticider används. Den globala marknaden för feromonbaserade bekämpningsmedel beräknas för närvarande till över 200 miljoner dollar. Nuvarande standardmetoder för feromonsyntes kräver ofta användning av farliga kemikalier under syntesen eller kan resultera i farligt avfall som biprodukt och det är svårt att modifiera dubbelbindningarna vid produktion av omättade feromonprekursorer. Problemen som är förknippade med syntetisk feromonproduktion kan övervinnas genom att utveckla ett innovativt alternativ baserat på så kallad grön kemi, samtidigt som riskerna minimeras.

En majoritet av de identifierade feromonkomponenterna hos nattfjärilar består av fettsyra-derivat, som produceras via artspecifika biosyntesvägar. Bland enzymerna spelar fettsyredesaturaser (FAD) en viktig roll för att producera den stora mångfalden av sexualferomoner mellan arter baserat på introduktion av dubbelbindningar i specifika positioner i fettsyrans kolkedja för att bilda feromonskelettet. Genom att dra nytta av den senaste utvecklingen inom bioteknik och bioengineering, och tillgången på gener i verktygslådan, såsom FAD, är det möjligt att effektivt syntetisera anpassade feromoner i transformerade organismer. I min avhandling undersökte jag användningen av flera växtplattformar (tobak och oljedådra) för att uttrycka en serie biosyntetiska enzymer för produktion av feromonprekursorer. Jag etablerade flera stabila transgena växtlinjer för produktion av feromoner med kolkedjelängder från C_{12} till C_{16} , både i blad och frön. De transgena växtlinjerna producerade en betydande mängd feromonprekursorer när

jag odlade dem i växthus. Denna forskning är ett viktigt steg framåt för stabil storskalig produktion av feromoner i växtfabriker.

Förutom FAD:er finns det några andra viktiga enzymer som är involverade i feromonbiosyntes hos fjärilar. Exempelvis kan fettsyrelongaser (ELO) och acyl-CoA-oxidaser (ACO) kombineras med FAD för att modifiera feromonskelettet. Fettsyrereduktaser (FAR) kan reducera feromonprekursorerna till motsvarande alkoholer. För att förbättra möjligheten och genomförbarheten för att producera anpassade fjärilsferomoner i växtfabriker, måste fler gener karakteriseras för att komplettera genverktyslådan.

I min avhandling kartlade jag först biosyntesvägarna för sexualferomonerna hos smalvingat lövfly, *Spodoptera exigua* och vinskottsvecklaren *Lobesia botrana*, som båda använder sexualferomoner med två dubbelbindningar i kolskelettet. För att klargöra den molekylära mekanismen för biosyntes av fjärilsferomoner, karakteriserade jag flera biosyntesgener från tre olika fjärilsarter, inklusive generna som kodar för ELO och ACO, vilka här rapporteras för första gången för Lepidoptera. Dessa resultat ökar möjligheten att utveckla växtfabriker för skräddarsydd feromonproduktion.

Ett annat mål för produktionen av insektsferomoner i växtfabriker skulle kunna vara att utveckla väster som avger feromoner vid odling i fält. Jag försökte producera genmodifierade tobaksplantor som avgav feromoner. Jag klonade en genpromotor som heter CYP71D16 för att reglera produktionen av feromonbiosyntetiska gener i tobakstrikom. Trikom är hår på växtens yta. Till vår förvåning upptäckte vi att feromonproduktionen ökade mycket jämfört med när en konstitutiv promotor användes. Dock kunde vi inte observera att växternas trikomer avgav några feromoner.

中文科学总结

在管理农田作物时，如果发现蛾子在田间飞舞，这预示着你的农田可能要遭殃了。这些雌蛾会在作物上产卵，孵化的幼虫以作物为食，产生大面积危害。信息素是生物体之间用于通讯的化学分子，从低等生物，如细菌，到高等生物，如人、大象都会产生信息素，存在于许多有机生物体中。蛾类昆虫非常依赖性信息素进行种间通讯和交配。一般来说，由雌蛾散发出特定的性信息素组分，来长距离吸引同种雄蛾以完成交配。现如今已经发现了超过十六万种鳞翅目昆虫（飞蛾和蝴蝶），它们其中许多会对粮食类作物和纤维作物产生巨大破坏，并对杀虫剂具有快速而不断发展的抵抗力。传统的杀虫剂不能区分害虫和其他非目标昆虫，并且在许多情况下可以危害包括人类在内的其他生物，有时也会对一些依赖于有益昆虫进行授粉的植物产生负面影响。昆虫信息素无毒，对非目标生物没有不利影响，害虫对信息素产生抗性的可能性也很小，所以利用信息素进行虫害控制（例如大量诱捕和交配破坏）受到越来越多的青睐。目前全球市场的信息素类害虫防控产品供不应求，然而，当前信息素的合成依然依赖于危险化学品作为生产原料，生产过程中产生的一些副产物也会危害环境。因此，克服当前信息素生产的固有问题，开发新的绿色化学生产方式迫在眉睫。

在合成信息素的过程中最费时费力的部分莫过于生产不饱和的信息素前体。大部分已鉴定的蛾类性信息素组分属于脂肪酸衍生物，它们由一系列连续作用的酶来合成。在这些酶中，脱饱和酶对于不同物种之间的性信息素分化起到主要作用，它是生产不饱和信息素前体的关键酶，它主要的功能是在脂肪酸碳链的特定位置引入不饱和的碳碳双键，有时也可以产生碳碳三键。得益于现代生物技术和生物工程学的快速发展，我们可以利用已知功能的信息素合成相关基因，如脱饱和酶，在外源生物体中表达并高效地生产信息素。在这篇论文中，我探究了如何利用烟草和亚麻芥作为植物工厂稳定地生产昆虫性信息素直接前体。我建立了若干个稳定的转基因植物品系，用于在其叶片或种子中生产有较大应用价值的信息素。我所构建的植物品系在温室培养中可以生产大量的信息素前体，这项研究可能是植物工厂信息素稳定生产的重要一步。

除了脱饱和酶，还有一些其它重要的酶也参与蛾类信息素的生物合成。例如，脂肪酸延长酶和酰基辅酶 A 氧化酶通常被认为与脱饱和酶一起作用于生产信息素的脂肪酸结构。脂肪酸还原酶可以将脂肪酸还原为相应的脂肪醇，有些昆虫直接利用脂肪醇作为其主要的信息素组分。因此，为了提高在植物工厂中生产更多特定信息素的可能性和可行性，我们还需要对更多信息素合成相关基因进行功能鉴定，以扩大可使用的功能基因池。

在本论文中，我们首先鉴定了甜菜夜蛾和葡萄花翅小卷蛾的性信息素生物合成途径，它们使用的性信息素组分具有比较特别的碳碳双键结构。为了阐明其性信息素生物合成的分子机制，我们进一步对一些信息素合成相关的候选基因做了功能鉴定。包括二化螟在内，我们总共功能鉴定了数十个编码信息素合成相关酶的基因，其中编码脂肪酸延长酶和酰基辅酶 A 氧化酶的基

因功能在鳞翅目昆虫中是首次得到验证。这些基因功能的鉴定为优化植物信息素工厂提供了更多选择。

另外，利用植物工厂生产信息素用于害虫防治的最终目标是构建出能在田间释放信息素的植物。因此，如何促使植物将信息素释放到外部环境中也是一项重要的研究课题。在本论文中，我初步探索了在烟草中释放信息素的可能性。我克隆了一个名为CYP71D16的基因启动子，以驱动信息素生物合成基因在烟草毛状体中进行表达，与组成型启动子相比，利用CYP71D16启动子的植物产生了更多的信息素，但并未从植物的顶空挥发物中收集到信息素。

Aims and objectives of the thesis

To understand the molecular mechanisms of moth pheromone biosynthesis and extension of the synthetic gene pool will be helpful in order to design tailor-made production of moth pheromones in transgenic organic factories. One significant aim of this thesis is to characterize important enzymes involved in pheromone biosynthesis, especially new FADs that are specialized in performing distinct functions and providing significant molecular evidence for the study about the sequences to functions of FADs.

The second major aim of this thesis work is finding synthetic biology methods to further demonstrate the technical and commercial feasibility of insect pheromone production in plant factories by stable transformation. The proposed strategy has the potential to become an economically sound part of many integrated pest management (IPM) programs. The concept of using transient expression of the necessary and sufficient genes for production of common moth pheromone compounds in *Nicotiana benthamiana* has been proven in a previous study (Ding et al. 2014). This synthetic biology strategy is a “green chemistry”-alternative, which aims for a novel and cost-effective way of producing moderate to large quantities of pheromones with high purity and a minimum of waste. Synthetic biology can be explained as the studies to take the rational design principles of engineering and apply them to the modification and manipulation of living organisms (Gibbs 2004). This has resulted in the construction of increasingly complex genetic circuits and rewired pathways, although the manual construction of these circuits can often be a time-intensive task with complex optimization required (Cloney 2016).

The thesis is divided into two major parts, the characterization of novel genes involved in pheromone biosynthesis (**Paper I, II, III**) and the construction of gene cassettes for insect pheromone production in plant (**Paper IV, V**). The objective of the first part is deciphering the molecular mechanism of pheromone biosynthesis in three moth species that are notorious pests, i.e., *Spodoptera exigua* (**Paper I**), *Lobesia botrana* (**Paper II**), and *Chilo suppressalis* (**Paper III**). The second part is dedicated to design, build and assemble an integrated biological system for insect pheromone manufacture in plant factories. In this part, I aim to produce stable lines of transformed plants for the production of C₁₄ and C₁₆ (**Paper IV**) and C₁₂ (**Paper V**) insect pheromone precursors. For the purpose of optimizing the plant factories, storage and release of pheromone compounds from the modified plants is investigated.

Introduction

Lepidoptera fatty acyl desaturases

The membrane-bound fatty acyl desaturases (FADs) belong to a superfamily of oxygen-dependent membrane di-iron-containing enzymes that includes a conserved three-histidine motif, coordinating two iron ions in the protein active center (Behrouzian and Bruist 2002). The enzymes catalyze the removal of hydrogen from a fatty acyl chain at a specific position resulting in the introduction of double bonds into the chain in 'E' or 'Z' configuration by desaturation reaction. Unlike the FADs in mammals, plants and protists that are active in normal cellular lipid synthesis, the Lepidoptera FADs have evolved extensively into different functions involved in producing the great diversity of moth pheromones (Knipple et al. 1998; Knipple et al. 2002; Liu et al. 2002; Roelofs et al. 2002; Jeong et al. 2003; Liénard et al. 2010; Tupec et al. 2017).

Since the 1980s, a variety of enzyme activities of moth FADs has successively been reported (Arsequell et al. 1990; Bjostad and Roelofs 1981; 1983; Foster and Roelofs 1988, 1996; Löfstedt and Bengtsson 1988; Martinez et al. 1990; Zhao et al. 1990). Based on the preference of substrates and product differences, particularly the position of the double bond introduced, the moth FADs can be divided into four subfamilies (Tocher et al. 1998) (Box 1).

BOX 1**Membrane-bound Desaturase subfamily**

Name	Definition	Reference
First desaturase	insert double bond into the saturated fatty acyl chain	Most of the identified moth desaturases belong to this group, $\Delta 10$, $\Delta 11$, $\Delta 13$ desaturases, including a conserved metabolic $\Delta 9$ FAD present in all eukaryotes (Liu et al., 1999; Paper III);
Omega desaturase	introduce double bond into the position between an existing double bond and the methyl end	A terminal FAD from <i>Operophtera brumata</i> (Ding et al., 2011); multi-functional desaturases from two <i>Spodoptera</i> moths (Paper I)
Front-end desaturase	introduce double bond into the position between an existing double bond and the carboxylic end	$\Delta 4$, $\Delta 5$, $\Delta 6$, and bifunctional $\Delta 6$ /sphingolipid $\Delta 8$ desaturases (Hashimoto et al., 2008). $\Delta 7$ desaturases in <i>Lobesia botrana</i> (Paper II)
Sphingolipid desaturase	present solely to the biosynthesis of sphingolipid	sole function is the sphingolipid $\Delta 4$ desaturase (Ternes et al., 2002)

Over the last three decades, genes encoding corresponding FADs have been characterized via heterologous expression systems, e.g., $\Delta 5$ FAD in *Ctenopseustis obliquana* and *C. herana* (Hagström et al. 2014), a $\Delta 6$ FAD in *Antheraea pernyi* (Wang et al. 2010), several $\Delta 9$ FADs from a range of moth species (Liu et al. 2002; Liu et al. 2004; Rodríguez et al. 2004; Rosenfield et al. 2001), a $\Delta 10$ FAD in *Planotortrix octo* (Hao et al. 2002), a $\Delta 11$ FAD in *Trichoplusia ni* which is the first discovered FAD (Knipple et al. 1998), a $\Delta 11/\Delta 13$ multifunctional FAD in *Thaumetopoea pityocampa* (Serra et al. 2007), $\Delta 14$ FAD in *Ostrinia* species (Roelofs et al. 2002), and a terminal FAD in *Operophtera brumata* (Ding et al. 2011), a multifunctional $\Delta 11/\Delta 12$ FADs in two *Spodoptera* moths (**Fig. 1**) (**Paper I**), etc. It is known that moth FADs have evolved multiple functions that introduce conjugated double bonds (Moto et al. 2004; Matoušková et al. 2007; Serra et al., 2006), and produce a triple bond by sequential action (Serra et al. 2007). Still, in many cases, the FADs were reported to use same substrate but the products have strikingly different stereochemistry (Hao et al. 2002; Liu et al. 2004; Buçek et al. 2015).

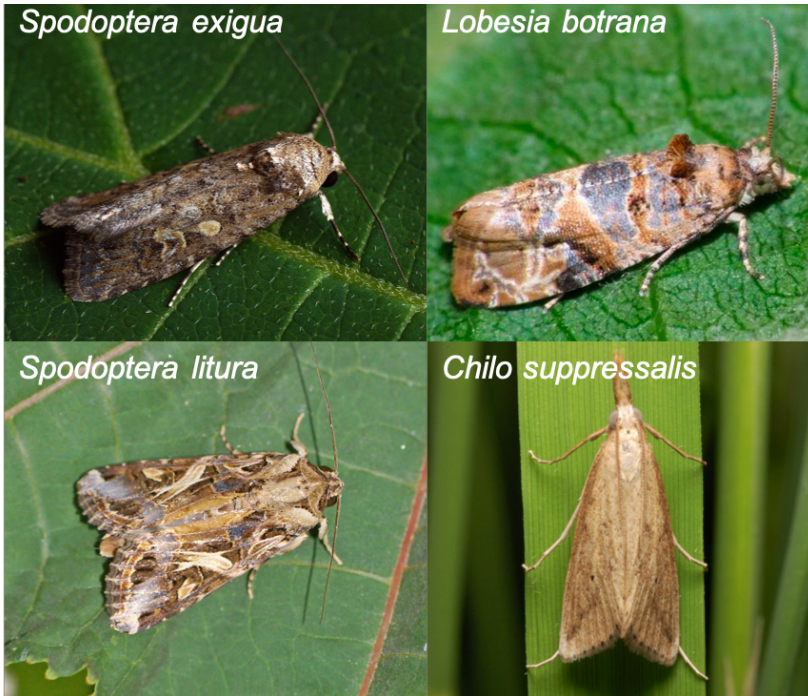


Figure 1. Pictures of the moth species that have been studied of sex pheromone biosynthesis in this thesis.

The Lepidoptera FADs fall into different groups in the phylogenetic tree (**Fig. 2**). The $\Delta 9$ ($C_{16} > C_{18}$) clade with a preference for palmitic acid, and the $\Delta 9$ ($C_{18} > C_{16}$) clade with a preference for stearic acid and contain mostly metabolic FADs for maintaining the fluidity of cell membranes. The $\Delta 11/\Delta 10/\Delta 9$ /bifunctional clade comprises pheromone biosynthetic FADs. The $\Delta 5/\Delta 6$, and $\Delta 14$ clade FADs active in pheromone biosynthesis have a mixture of different signature motifs. In addition, the $\Delta 9$ ($C_{14}-C_{26}$) clade FADs have preferences ranging from myristic acid to long chain (C_{16}) fatty acids and are evolved to produce pheromone compounds as well.

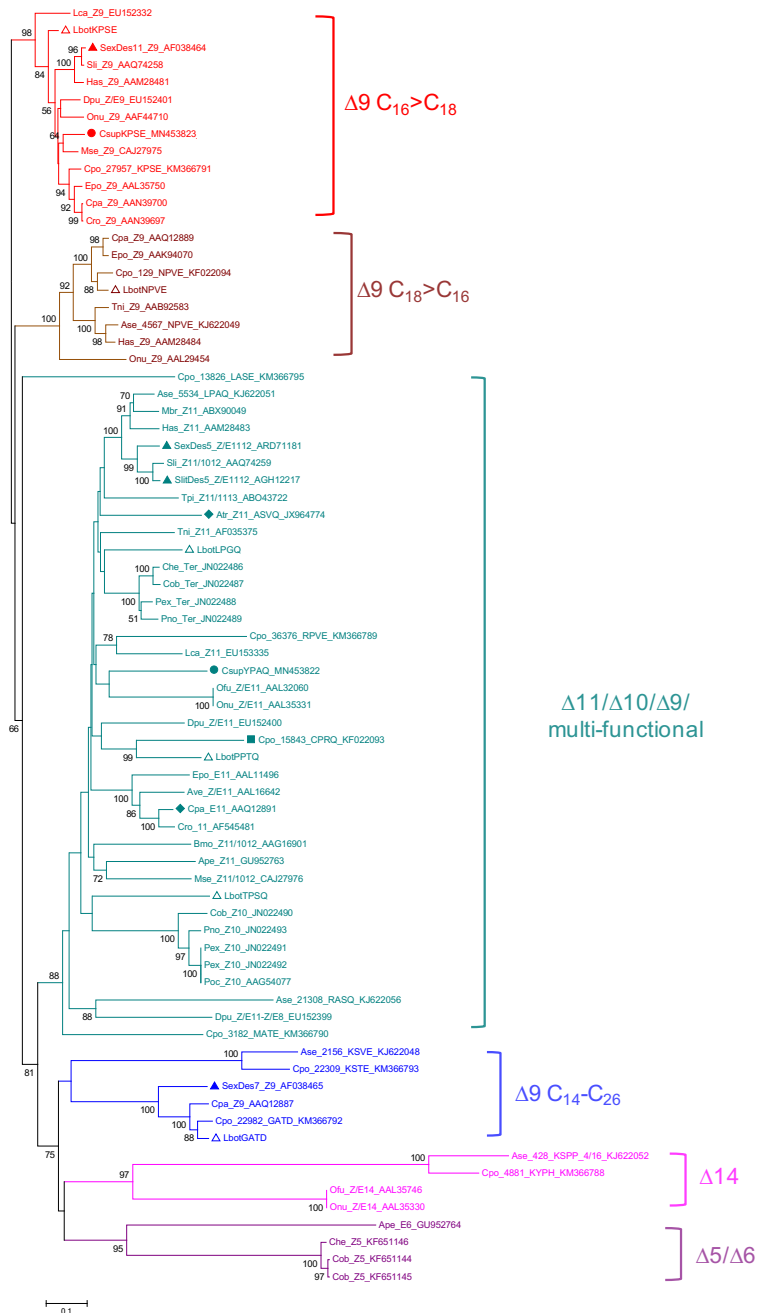


Figure 2. Phylogenetic tree of desaturases. The desaturases tree was constructed by using Lepidoptera desaturases with amino acid sequences. The predicted desaturase genes studied in **Paper I, II, III, IV, V** are marked by triangle, hollow triangle, round, rhombus and square spots, respectively.

Moth pheromones

The name of “Pheromone” comes from “*Perein*” and “*Hormon*” of Greek origin, means “*to carry and to excite*” (Wyatt 2003). In 1959, the first moth (Insecta, Lepidoptera) pheromone bombykol (*E,Z*)-10,12-hexadecadien-1-ol was described from the silk moth *Bombyx mori* (Butenandt et al. 1959). Pheromones are a subclass of semiochemicals that are used by the individuals of the same species to communicate with each other. Pheromone-mediated behaviors are crucial in animals from insects to mammals and contribute significantly to reproductive isolation (Wyatt 2003; Smadja and Butlin 2009). In many branches in the tree of life, from yeast (Michaelis and Herskowitz 1988) to elephants (Rasmussen et al. 1997) pheromones are used. However, insects are definitely the masters of chemical communication, and most of them heavily depend on pheromones for a wide range of different behaviours (Jurenka 2004; Lamprecht et al. 2008).

Female moths emit species-specific pheromone component blends that attract conspecific males over a long-distance, and this kind of pheromone is called sex pheromone. Approximately 75% of known moths use Type I sex pheromone compounds, which are C₁₀-C₁₈ fatty acid (FA) derivatives including mainly acetates, alcohols or aldehydes (Löfstedt et al. 2016). The second most common type, Type II pheromones, are used by ca. 15% of the moth species. Type II pheromone compounds comprise polyunsaturated hydrocarbons and their epoxy derivatives with longer straight chains (C₁₇-C₂₅) (Conner et al. 1980; Löfstedt and Kozlov 1997; Ando et al. 2004). The Type I sex pheromone compounds are generally produced in a specialized tissue named the pheromone gland that is commonly located between the 8th and 9th abdominal segments of the female moths (**Fig. 3**) (Percy 1987; Raina et al. 2000; Ma et al. 2003; Ando et al. 2004). Other identified moth sex pheromone compounds are methyl-branched long chain (C₁₇-C₂₃) saturated or unsaturated hydrocarbons, and functionalized hydrocarbons (Type III) (Löfstedt et al. 2016). Also, short-chain secondary alcohols and ketones (Type 0) have been reported not only in Lepidoptera but also in the sister group Trichoptera, therefore to be considered as the most ancient form of moth pheromones (Visser 1986; Löfstedt and Kozlov 1997; Löfstedt et al. 2016), produced in glands of the 5th abdominal sternite (Löfstedt et al. 1994).

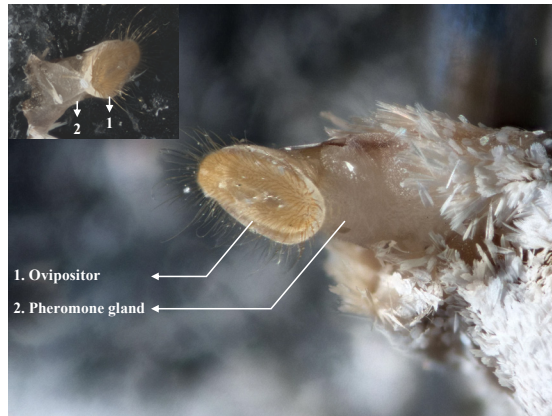


Figure 3. A typical female moth pheromone gland. Reprinted from Xia et al. (2015).

The biosynthetic pathways of moth pheromones

The general biosynthetic pathways for Type I pheromones from palmitic acid may include chain elongation or shortening, interspersed with desaturation steps to place double bonds in specific positions. Once the chain is completed, the final steps involve adjustment of the terminal functional group (Löfstedt et al. 2016). Biosynthetic pathways for Type II pheromones usually start from linoleic or linolenic acids. In the oenocytes, different chain lengths may be produced and additional double bonds can be introduced similar to the biosynthesis of the Type I pheromone compounds. The final steps involve decarboxylation to provide odd-numbered chains or oxidation followed by decarboxylation and decarbonylation to produce even-numbered chains. The hydrocarbon products are then transported to the pheromone gland for release directly or after epoxidation (Löfstedt et al. 2016). My thesis is focused on studies of Type I pheromones.

Identification of genes encoding Type I sex pheromone biosynthetic enzymes

Most Lepidopteran sex pheromones share a common progenitor that is *de novo* synthesized from acetyl-CoA via fatty acid synthesis in the PG (Foster 2005). The biosynthesis starts by acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) catalyzing the saturated fatty acid precursor malonyl-CoA from acetyl-CoA in the first committed biosynthesis step (Volpe and Vagelous 1973; Pape et al. 1988). Fatty-acid metabolism enzymes perform desaturation, chain-shortening by β -oxidation, chain-elongation, and functional group modifications by reduction, acetylation or oxidation to finally produce the pheromone components (Strandh et

al. 2008) (**Fig. 4**). Different combinations of these enzymes can produce unique species-specific pheromone blends in different species. The genes encoding two classes of essential enzymes involved in moth pheromone synthesis have been mostly functionally identified. Firstly, the gene encoding FADs that introduce double bonds in selected positions of the carbon chains are most extensively studied (Knipple et al. 2002), and has been described in “Lepidoptera fatty acyl desaturases”.

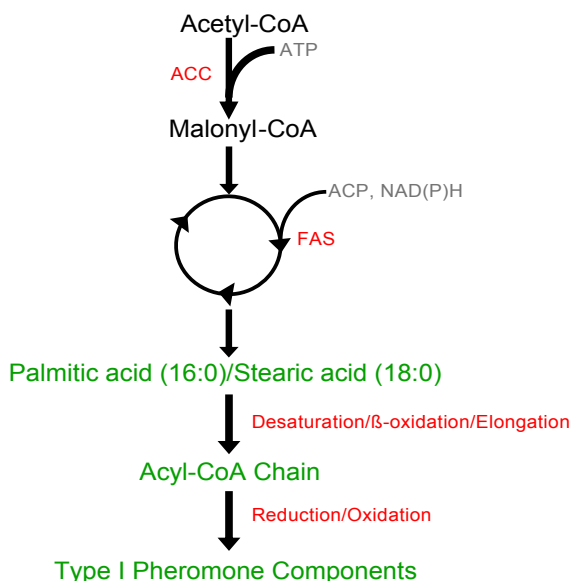


Figure 4. Type I pheromone biosynthetic pathways.

Secondly, the genes encoding fatty-acyl reductases (FAR), responsible for reducing fatty acids to alcohols with different substrate specificities, have been functionally characterized in a few moth species, such as pgFAR-Z/E in *O. nubilalis* (Lassance et al. 2010), pgFAR in *B. mori* (Moto et al. 2003) and pgFAR in *C. suppressalis* (**Fig. 1**) (**Paper III**).

Other important genes postulated to be involved in moth pheromone production remain to be characterized, including

- the genes encoding acetyl-CoA acetyltransferases, which catalyze the conversion of fatty alcohol into acetate ester (Clinkenbeard et al. 1973);
- the genes encoding acyl-CoA oxidases, which are responsible for lipid metabolism by catalyzing the conversion of acyl-CoA into trans-2-enoyl-

CoA during fatty acid β -oxidation, and two novel ACO genes have been reported in *L. botrana* (**Fig. 1**) (**Paper II**);

- the genes encoding alcohol oxidases, which are responsible for converting fatty alcohols into aldehydes;
- the genes encoding elongation of very long chain fatty acid proteins (ELO), which catalyze the reaction of the long-chain fatty acids elongation cycle, and the first lepidoteran ELO gene has been reported in *C. suppressalis* (**Fig. 1**) (**Paper III**);
- the genes encoding fatty acid transport proteins, which are integral membrane-bound proteins found in both the plasma membrane and endoplasmic reticulum, several of which facilitate the uptake and activation of exogenous long chain fatty acids (Stahl 2004; DiRusso et al. 2005; Black and DiRusso 2007; Anderson and Stahl 2013);
- the genes encoding acyl-CoA binding proteins, which bind acyl-CoA esters with high specificity and affinity, and are thought to act as intracellular transporters of acyl-CoA esters between different enzymatic systems (Mogensen et al. 1987; Burton et al. 2005; Færgeman et al. 2007).

Application of moth pheromones for pest control

Lepidoptera is a big order of insects that contains more than 160,000 described moth and butterfly species, and estimated 250,000 species including undescribed species in the whole world (Heppner 1991; Nieukerken et al. 2011). Among the most damaging pests of food and fiber crops, the moths are the super-criminal, and this is also due to the moths' capability of adapting fast and evolving resistance to insecticides (Simmons et al. 2010). It should be noticed that conventional insecticides will not only hurt the pests but are also harmful to other non-target insects, including insects beneficial for pollination or plant protection. Apart from this, in many cases the traditional insecticides are detrimental to humans regarding aspects of food safety and environment injure (Brittain and Potts 2011).

Due to the variety of problems caused by conventional pesticides, synthetic pheromones emerged as an alternative for insect control by monitoring or disruption of pheromone communication in pest insects with many advantages (Wyatt 2003). Moth pheromones are environmentally friendly and non-toxic, they have no adverse effects on non-target organisms and are not harmful to parasitoids or other beneficial insects. In addition, the risks of resistance being developed in the pest are relatively small. Even in terms of profit and reduction in damage, pheromones often compare favourably to the use of insecticides. For example, protecting cabbage from diamondback moth by pheromone was both cheaper, \$62 compared to \$123 per ha,

and more profitable, ca \$800 compared to \$456 per ha than by insecticides (Reddy and Guerrero 2000). Nowadays, there are tons of synthetic pheromones produced for application and it is estimated to be about \$200 millions pheromone-based control products consumed in the global market (Weatherston and Stewart 2002).

Plants as factories for pheromones production

Since the techniques for genetically engineering of plants were developed in the early 1980s, numerous research projects have focused on utilizing transgenic plants to produce high-value recombinant proteins or compounds (Boehm 2007; Karg and Kallio 2009; Lienard et al. 2007; Ma et al. 2005; Mett et al. 2008;). During the past 20 years, producing insect pheromones or their biosynthetic precursors in genetically modified plant factories has been attempted. A moth pheromone precursor was produced in *Nicotiana tabacum* by the introduction of a moth desaturase (Nešněrová et al. 2004), and an aphid alarm pheromone was produced from endogenous plant sesquiterpene by expression of a (*E*)- β -farnesene synthase in *Arabidopsis* (Beale et al. 2006). Moreover, Ding et al. (2014) proved that transient expression of genes coding for consecutive pheromone biosynthetic steps in *N. benthamiana*, resulted in production of biologically active multi-component sex pheromones. The activity of the acetylated sex pheromone mixtures from the fatty alcohol fractions produced by the genetically modified plants have the same activity for trapping of male small ermine moths *Yponomeuta evonymella* and *Y. padella* compared to conventionally produced synthetic pheromones (Ding et al. 2014). These studies have demonstrated that it is feasible to produce highly attractive and species-specific moth pheromones in genetically modified plants.

The potential advantages of using plant-based expression systems include the ability to produce complex proteins that require post-translational modifications, avoiding the possibility of introducing human pathogens during the manufacturing process, and the capability to amplify production efficiently and cost-effectively (Ma et al. 2005; Boehm 2007; Liénard et al. 2007; Mett et al. 2008; Karg and Kallio 2009).

Fatty acids and triacylglycerol biosynthesis in plants

In plants, *de novo* fatty acid biosynthesis takes place in plastids (Ohlrogge et al. 1979), which are double-membrane organelles in plant cells. It starts from the condensation of acetyl-coenzyme A (CoA) and malonyl-acyl carrier protein (ACP) by the β -ketoacyl-ACP synthase (KAS) to produce a four-carbon β -ketoacyl-ACP, which are elongated by sequential condensation of two carbon units from malonyl-ACP by the co-operation of enzymes of fatty acid synthase (FAS) (**Fig. 5**) (Schultz

and Ohlrogge 2001; Voelker and Kinney 2001). Termination of plastid fatty acid chain elongation is catalyzed by fatty acyl-ACP thioesterases (FATs), which hydrolyze acyl chains from ACP to free fatty acids (FFAs). The FFAs are then transported through the plastid and activated to CoA esters, which are assembled into glycerolipids and polar lipids (PL) at the endoplasmic reticulum (ER) (**Fig. 5**), where further modifications such as desaturation, hydroxylation, elongation, etc., occur as well.

In developing seeds, the flux of acyl chains in the ER eventually leads to esterification on all three positions of glycerol to form triacylglycerol (TAG) (**Fig. 5**). The low polarity of TAG is thought to result in the accumulation of this lipid between bilayer leaflets leading to the budding of storage organelles termed oil bodies (Raclot 1997).

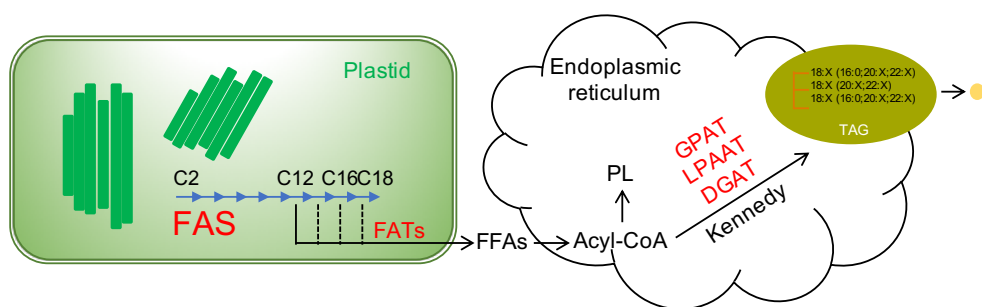


Figure 5. Simplified fatty acid synthesis and TAG assemble pathways in plants.

Fatty acyl-ACP thioesterases (FATs)

According to different substrate preferences, FATs are classified into two families, FatA and FatB (Jones et al. 1995; Salas and Ohlrogge 2002). FatAs generally have activities on C₁₈ saturated or unsaturated fatty acyl-ACP, while FatBs are responsible for releasing C₁₆ acyl chain (Sinchez et al. 2010). FatA orthologues show high activity upon Z9-18-ACP substrate, of which the substrate specificities are similar among different species (Hawkins and Kridl 1998; Knutzon et al. 1992). While FatB enzymes can be further classified into two subclasses, the first is FatB1 that has generally preference for 16:0-ACP, and the second is FatB2 that prefer short- and medium-chain saturated acyl-ACPs (Rodríguez et al. 2014).

FATs are the key enzymes to determine which fatty acids are exported to the cytosol and subsequently incorporated into further glycerolipids biosynthesis (Voelder TA et al. 1996). To date, a variety of specific FAT genes have been functionally demonstrated to effectively modify oil profile in transgenic plants (Salas and Ohlrogge 2002). The engineered fatty acids by FAT genes range from short-chain to long-chain. For instance, e.g., overexpression of FatB2 originally

from California bay laurel (*Umbellularia californica*) in *Brassica napus* and Camelina seeds increased the lauric acid level in the total fatty acids (mol%) from negligible level to 58% (Voelker et al. 1996) and 29% (Kim et al. 2015), respectively; FatB2 from *Cuphea palustris* overexpressed in *N. benthamiana* leaf boosts the production of myristic acid a hundred times (Ding et al. 2014); *B. napus* introduced by FatB1 from *Cuphea* species produced 34% palmitic acid of total fatty acids (mol%) (Jones et al. 1995). Interest in the use of FATs in lipid biotechnology has led to a very active research on their different forms coming from various sources (Mandal et al. 2000; Othman et al. 2000; Serrano et al. 2005; Ghosh et al. 2007).

Metabolic pathway of medium-chain fatty acid synthesis in plant

Medium-chain fatty acids (MCFAs) range from ethanthic acid (C6:0) to myristic acid (C14:0), which are important for a variety of industrial productions, such as cosmetics, detergents, soaps, surfactants, lubricants, etc (Knaut and Richtler 1985; Dyer et al. 2008). The synthesis of MCFAs is a variation on typical *de novo* fatty acid synthesis that takes place in plants that produces primarily C₁₆ and C₁₈ fatty acids. In nature, only a few plants are MCFAs-rich. Therefore, engineered pathways are usually applied to generate MCFAs in non-MCFA-enriched plants.

The MCFA enriched plants are mostly from the tropics, e.g. palm kernel (*Elaeis guineensis* Jacq.) contains ca. 50 (mol) % of lauric acid and 18% of myristic acid of total fatty acids, respectively, as well as coconut (*Cocos nucifera* L.). The seeds from the temperate *Cuphea* genus also produce high amounts of MCFAs (Graham and Kleiman 1992; Graham 1998), of which *C. pulcherrima* can yield more than 90% of C8:0, and *C. viscosissima* accumulates 25% of C8:0 and 64% of capric acid (C10:0). Therefore, *Cuphea* species have been a suitable genetic resource to isolate FAT genes for MCFA production. Establishing oilseed crop lines for MCFA production by introducing *Cuphea* FAT genes have been confirmed to be a useful approach (Dehesh et al. 1996a, b; Leonard et al. 1997; Slabaugh et al. 1998; Filichkin et al. 2006).

For purposes of increasing MCFA content, divergent FatB enzymes were characterized and transgenically investigated, predominantly focusing on the engineering of lauric acid (C12:0) (Eccleston and Ohlrogge 1998; Knutzon et al. 1999; Voelker et al. 1992; Reynolds et al. 2017). In plants, the biosynthesis of MCFA is a variation on typical *de novo* fatty acid synthesis that generates primarily C₁₆ and C₁₈ fatty acids. Chain-lengths of fatty acids are primarily determined by acyl-ACP thioesterases, in including FatB thioesterases that typically release C₁₆ acyl chains from *de novo* fatty acid biosynthesis (Li-Beisson et al. 2013). Variant forms of FatB, found in selected plant species, are able to release fatty acids of chain lengths shorter than C₁₆, as demonstrated by transgenic expression in seeds (Pollard

et al. 1991; Jones et al. 1995; Voelker 1996; Tjellström et al. 2013; Kim et al. 2015). In previous study, a FatB gene *UcTE* from California bay laurel (*Umbellularia californica*) was found to have high activity for production of 12:0 in rapeseed (*Brassica napus*) (Voelker et al. 1992). When the MCFAs are exported into the cytoplasm from the plastid in oilseeds, they become available for incorporation into TAG, which is formed most directly by the Kennedy pathway enzymes of glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidic acid acyltransferase (LPAAT) and diacylglycerol acyltransferase (DGAT) continuously (Thelen and Ohlrogge 2002; Cahoon et al. 2007; Dyer et al. 2008; Kim et al. 2015a). A recent report showed that the co-expression of a variant FatB thioesterase with LPAT in *Camelina* seeds, the MCFA accumulation was improved (Kim et al. 2015a).

Plant platforms for pheromone production

In this thesis, I worked on three different plant platforms: *N. tabacum*, *N. benthamiana* and *Camelina sativa* for pheromone production.

N. tabacum is also called cultivated tobacco which is an herbaceous plant and it is only found in cultivation. *N. tabacum* is the most commonly grown plant in the *Nicotiana* genus. It is commercially grown in many countries and the leaves are used to produce tobacco. The height of matured tobacco plants is between 1 and 2 meters. The leaves vary in size and the lower leaves are the largest with a length of up to 60 cm. *N. benthamiana* is a close relative of *N. tabacum*, and the mature plants show a big variation in height, ranging from as tall as 1.5 meters to shorter than 200 mm. In our greenhouse, the height of *N. benthamiana* was about 300 mm. The two *Nicotiana* species are both favourable to work with in metabolic engineering aiming at production of pheromone compounds as they have relatively short production times, large area of leaves to output volatiles and are relatively easy to grow in controlled growth conditions. In addition, there is less concern about contaminating food supplies as they are not food crops.

Camelina was chosen as the oilseed production platform for our studies because it is limited use as a food crop and is considered an ideal system for rapid introduction and evaluation of fatty acid and other oil-related traits (Iskandarov et al. 2014). Foremost, transgenes can easily be introduced into *Camelina* using a simple *Agrobacterium*-based method (Lu and Kang 2008), and it has a relatively short life cycle that allows up to three generations in a year for evaluation of engineered traits (Bansal and Durrett 2016). *Camelina* is also closely related to *Arabidopsis thaliana*, with a wealth of transgenic and genomic data for optimizing endogenous biosynthetic pathways for production of desired oil traits in seeds that typically are 30% to 40% oil by weight (Nguyen et al. 2013).

In many plants, trichomes are tiny specialized hair structures for secondary metabolite production and release. For instance, biosynthesis of the diterpenes takes place in trichome heads, where secretory vesicles and cells are located (**Fig. 6**) (Kandra and Wagner 1988; Duke 1994; Guo et al. 1995). CYP71D16 was confirmed as a trichome-specific promoter leading the downstream gene to be specifically expressed in plant trichome (Wang et al. 2002). To explore the possibility of releasing moth pheromones from *Nicotiana* leave's trichome cells, a *N. tabacum* trichome specific promoter pCYP71D16 was used for driving pheromone biosynthetic gene expression.

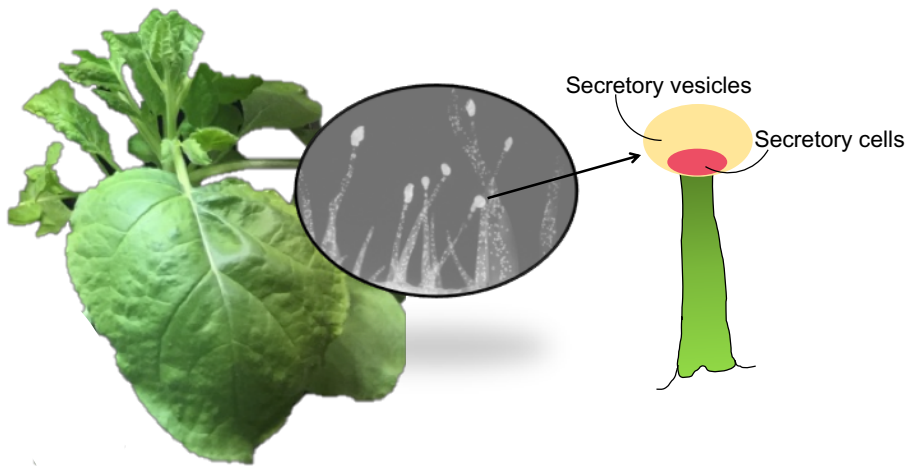


Figure 6. Graphs of plant trichomes.

General Methodology

Labelling experiment

Isotopic labelling was the technique used to identify moth pheromone biosynthetic pathways by tracking the passage of an isotope through the metabolic pathways in this thesis. The deuterium-labelled precursors were separately dissolved in dimethylsulphoxide (DMSO) (Bjostad and Roelofs 1983; Yamaoka et al. 1984) and topically applied to the female abdominal tip where the pheromone gland is located. After incubation of a half to several hours, pheromone glands were excised and extracted (Bjostad and Roelofs 1984), and the samples could be analyzed by gas chromatography/mass spectrometry (GC/MS) (Christie 1998) (**Fig. 7**). The double bond positions in the fatty acid chain were confirmed by dimethyl disulfide (DMDS) derivatization (Dunkelblum et al. 1985).

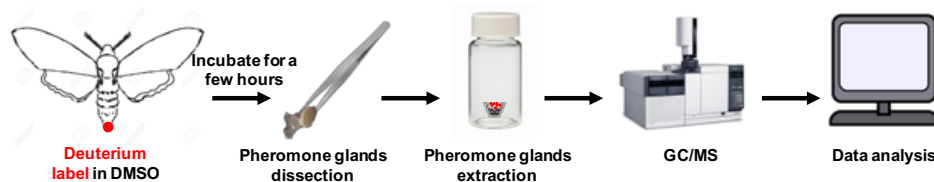


Figure 7. Experimental workflow of *in vivo* isotopic labelling experiment performed in this thesis.

Cloning and plasmid constructs for assays

Amplification of genes was performed by PCR either from cDNA templates synthesized from total RNA (**Paper I**) or genome DNA extracted directly from plant materials (for trichome specific promoter pCYP71D16, of which the results are not yet complete and compiled in manuscript form). For some genes of the unavailable biological source in our lab, I ordered the custom DNA synthesized by Invitrogen. (**Paper III-V**). All the genes contain the Gateway[®] cloning site attB (Gateway cloning system, Invitrogen) and were subsequently cloned to Gateway[®] entry vector in first step by BP reaction. For constructing co-expression clones, the Phusion PCR (Atanassov et al. 2009) was performed by putting two fragments that containing several dozens of homologue sequence bases and the DNA polymerase together.

After hybridizing of the two fragments, the recombined sequence was cloned to Gateway® vector. The expression clones in this thesis were also constructed by using Gateway® method (Katzen 2007). After the entry clones were confirmed by sequencing (Schuster 2007), all of them were sub-cloned to a destination vector by LR reaction with different multigene combinations for a variety of purposes.

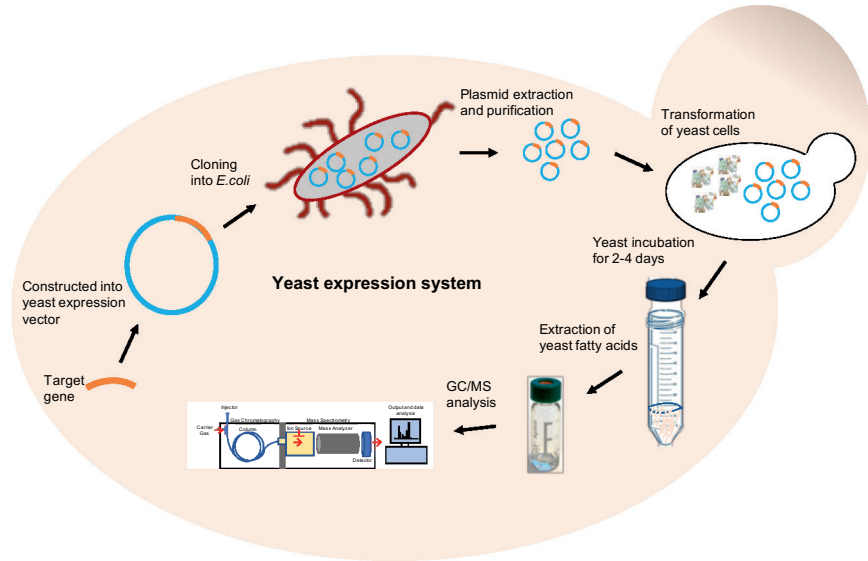
Functional assays

In this thesis, we have used three platforms for gene functional characterization. One was the yeast expression system (**Fig. 8a**). I used yeast expression vectors of pYEX-CHT and pYES52 for the functional assays. The expression clones contained FADs were introduced into the double deficient *ole1/elo1* strain (MATa *elo1::HIS3 ole1::LEU2 ade2 his3 leu2 ura3*) of the yeast *Saccharomyces cerevisiae*, while the expression clones contained FARs were introduced into the *INVSc* strain of yeast *S.c.* (MATa *HIS3 LEU2 trp1-289 ura3-52*). The transformation of yeasts was carried out using the *S.c.* easy yeast transformation kit (Life technologies). The detailed protocol was described in **Paper I** and **III**.

The second platform for gene functional assay was the plant transient expression system. I used *N. benthamiana* as the plant platform for gene expression (**Fig. 8b**). The plant expression clones in pXZY393 vector containing the target genes were first introduced into *Agrobacterium tumefaciens* GV3101 strain (MP90RK) by electroporation (1700 V mm⁻¹, 5 ms, Eppendorf 2510). Meanwhile, a viral silencing suppressor protein P19 was introduced into the same *A. tumefaciens* strain as well in order to inhibit the transgene silencing of the host cells and extend transgene expression over a longer period of time with a higher degree of expression (Voinnet et al. 2003). Subsequently the transformed *A. tumefaciens* was incubated for several days until the culture concentration was high enough for infiltration of *N. benthamiana*. The infiltration experiment was carried out by using a 1 mL syringe without needle, containing the *A. tumefaciens* cells, to inject the lower side of a suitable four-week-old *N. benthamiana* leaf, with a gentle squeeze on the plunger and modest pressure on the leaf using a finger. The detailed protocol was written in **Paper III**.

The last platform we used for gene functional assay was the insect cell expression system. The expression construct for candidate gene in the BEVS donor vector pDEST8 was made by LR reaction. Recombinant bacmids were made according to instructions for the Bac-to-Bac™ system given by the manufacturer Invitrogen using DH10MEmbacY (Geneva Biotech). Baculovirus generation was done using Sf9 cells (Invitrogen), Ex-Cell 420 medium (Sigma) and baculoFECTIN II (OET). The detailed protocol was written in **Paper II**.

a



b

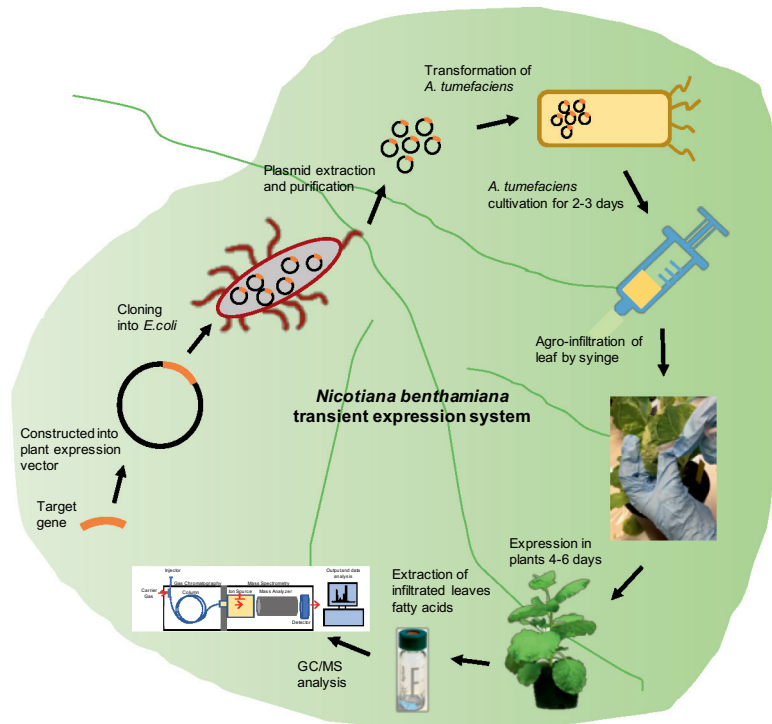


Figure 8. Yeast (a) and *Nicotiana benthamiana* transient (b) expression system applied in the thesis studies.

Leaf disc transformation via *Agrobacterium*

The method of *Agrobacterium*-mediated leaf disc transformation (Clemente et al. 2006) was used for *Nicotiana* spp. stable transformation (**Fig. 9**). First, the *A. tumefaciens* culture containing the construct was incubated at 30 °C in LB medium supplemented with suitable antibiotics, until the optical density (A600nm) can be adjusted to 0.9-1. Plant material was obtained from 4-5 weeks old *Nicotiana* plants grown under sterile conditions on MS medium (Murashige and Skoog 1962) in a climate chamber. Subsequently, the transgenic lines were obtained by *Agrobacterium*-mediated leaf-disc transformation. Leaf discs (20 mm x 20 mm) were cut out and incubated 5 min in an *A. tumefaciens* solution, dried with sterile napkin paper and transferred to Petri dishes with MS medium (Horsch et al. 1985). After 2-3 days incubation in darkness, leaf discs were transferred to selection medium. Then after 2-3 weeks of incubation, the callus produced on the leaf edges were transferred to shoot-inducing medium. After 2-3 weeks of incubation, the shoots were transferred to root-inducing medium. The shoots were finally transferred into soil and grown in greenhouse until maturity. The detailed protocol is written in **Paper IV**.

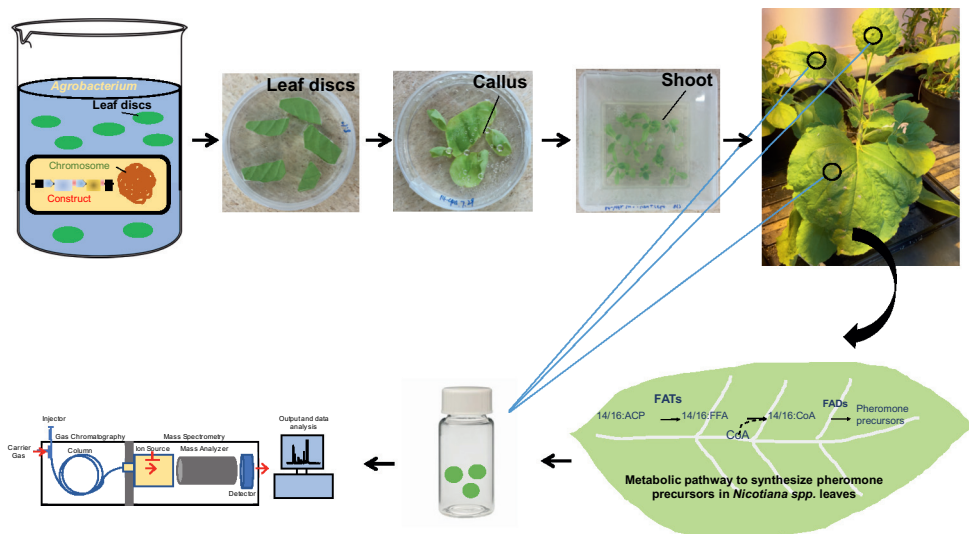


Figure 9. Experimental Workflow of *Agrobacterium*-mediate leaf-disc stable transformation.

Floral dip transformation via *Agrobacterium*

The method of *Agrobacterium*-mediated floral dip transformation was used for *Camelina* stable transformation (**Fig. 10**). The constructed expression vectors were introduced into *Agrobacterium tumefaciens* strain GV3101 (MP90RK) by electroporation (1700Vmm^{-1} , 5 ms, Eppendorf 2510). The transformed *Agrobacterium* cells were grown on solid LB medium supplemented with antibiotics (50 mg/L rifampicin, 50 mg/L gentamicin and 50 mg/L spectinomycin) after incubating at 30°C for 36 h. Afterwards, a single clone from each expression clone was incubated in 2 mL liquid LB medium with suitable antibiotics as described above at 30°C for 36 h. Then the *Agrobacterium* solution was transferred to 30 mL medium for a 36 h incubation, and after that, the solution was transferred to 1 L medium for a 24 h incubation. Subsequently, the five weeks old *Camelina* plants were then transformed (Lu and Kang 2008) by the floral dip/vacuum infiltration method as described in Liu et al. (2012). After eight to ten weeks, the seeds were collected and sown in soil, and herbicide was used to select the transformants. The detailed protocol was written in **Paper V**.

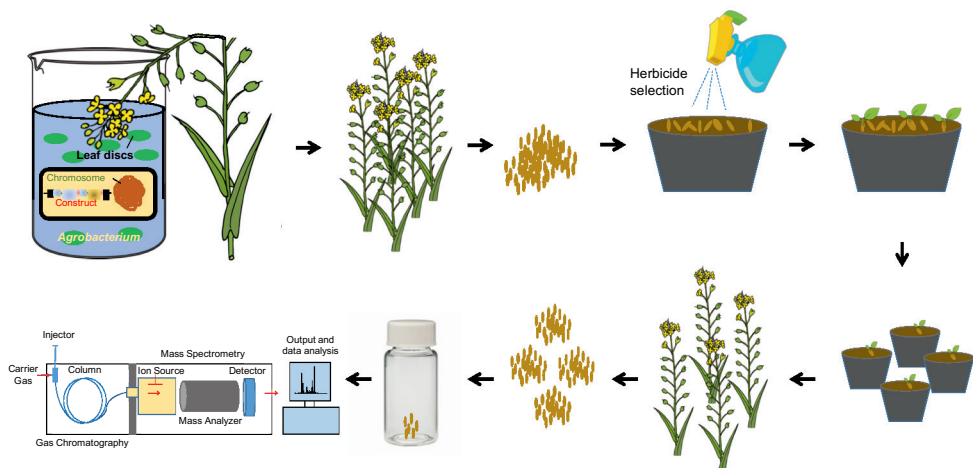


Figure 10. Experimental Workflow of *Agrobacterium*-mediate floral dip stable transformation.



Figure 11. Experimental Workflow of *Agrobacterium*-mediated floral dip stable transformation.

Lipid analysis

In order to analyze the gene functions in transformed yeast, insect cells and plants, as well as to detect the pheromone precursor production in *Nicotiana* spp. leaves and *Camelina* seeds, the lipid analysis was performed. For the lipid analysis, first the pheromone gland tissues (**Paper I**), yeast cells (**Paper I** and **III**), insect cells (**Paper II**) or plant leaves (**Paper III-V**) were extracted by chloroform:methanol (2:1 v:v) at room temperature overnight. For fatty acids (pheromone precursors) analysis, this was followed by base-methanolysis and transesterification as previously described (Dunkelblum and Kehat 1987) to convert all fatty acids to their corresponding methyl esters. The products were then recovered in n-heptane prior to GC/MS analysis. While the samples for pheromone compounds such as alcohol, aldehyde and acetate analysis were followed by adding n-heptane to re-dissolve the compounds after the total lipid dryness by nitrogen, which were then transferred to new glass vials for GC/MS analysis. The seed fatty acids extraction and methanolysis was performed directly by adding 2% sulphuric acid in methanol and incubated at 90°C for 1 h, and then water and n-heptane were added to extract the fatty acid methyl ester products prior to GC/MS analysis (**Paper V**).

Headspace volatile collection

To perform the experiment of plant headspace volatile collection, the equipment was set up as shown in below. For the use of air flow to collect dynamic headspace volatile, the experiment was carried out as described in Raguso and Pellmyr (1998). For the use of solid phase micro extraction (SPME) to collect volatile, the experiment was performed as described in Centini et al. (1996).

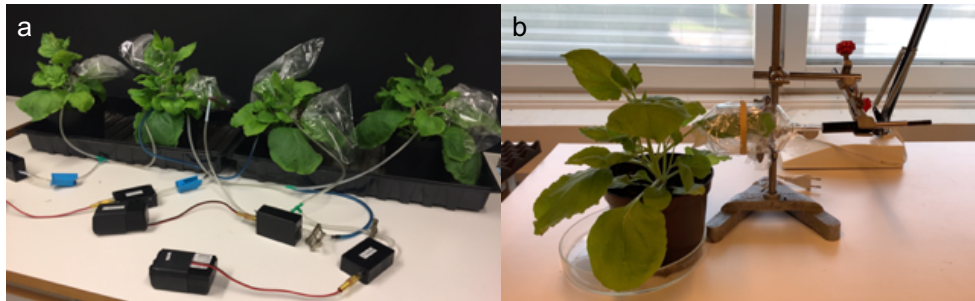


Figure 12. Equipment for leaf volatile collection. a) a dynamic headspace technique using air flow. b) solid phase micro extraction (SPME) technique.

Results and discussion

Labelling experiments reveal new FADs activities (**Paper I and II**)

Tracking the pheromone biosynthetic pathways of *S. exigua* and *L. botrana* by isotope-labelling experiment, we confirmed an unusual $\Delta 12$ (**Paper I**) and a novel $\Delta 7$ (**Paper II**) desaturation activity to be involved, respectively. In the *S. exigua*, the *in vivo* labelling experiment showed that deuterium atoms from [16,16,16- $^2\text{H}_3$] hexadecanoic acid (D_3 -16:acid) were incorporated into all the detected acetates and alcohols, including (*Z*)-11-hexadecenyl acetate (Z11-16:OAc), (*Z*)-11-hexadecenol (Z11-16:OH), (*Z*)-9-tetradecenyl acetate (Z9-14:OAc), (*Z*)-9-tetradecenol (Z9-14:OH), (*Z,E*)-9,12-tetradecadienoic acetate (Z9,E12-14:OAc) and (*Z,E*)-9,12-tetradecadienol (Z9,E12-14:OH) (**Fig. 13**), as well as into the corresponding FAMES, i.e., methyl (*Z*)-11-hexadecanoate (Z11-16:Me), methyl (*Z*)-9-tetradecenoate (Z9-14:Me), methyl (*Z,E*)-9,12-tetradecadienoate (Z9,E12-14:Me) and methyl myristate (14:Me) (**Fig. 12**). Notably, when non-labeled (*Z*)-9-tetradecenoic acid (Z9-14:acid) was applied to the pheromone gland, the amount of Z9,E12-14:OAc increased significantly. In contrast, when [14,14,14- $^2\text{H}_3$] tetradecanoic acid (D_3 -14:acid) and (*E*)-12-[14,14,14- $^2\text{H}_3$] tetradecenoic acid (D_3 -E12-14:acid) were applied, label incorporation was not detected in any of the above components. These results confirm that the production of major pheromone component Z9,E12-14:OAc in *S. exigua* starts from palmitic acid and a $\Delta 11$ FADs acts on palmitic acid to produce Z11-16:acid, which is then chain-shortened to Z9-14:acid, followed by the second desaturation at the $\Delta 12$ position to form Z9,E12-14:OAc (**Fig. 14**). This pathway is in line with previous study of *S. littoralis* (Jurenka, 1997).

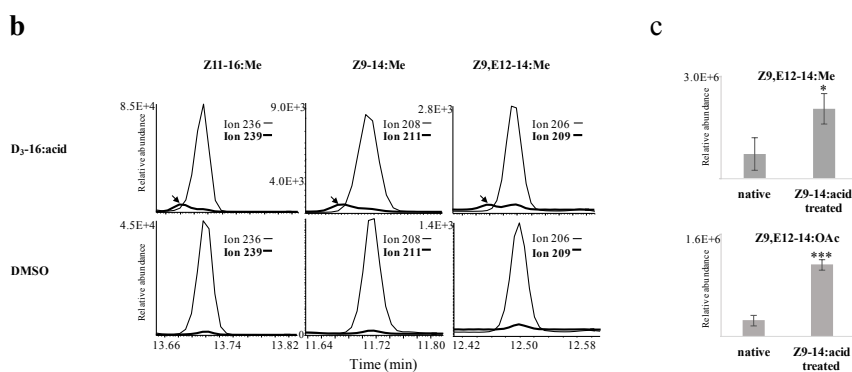
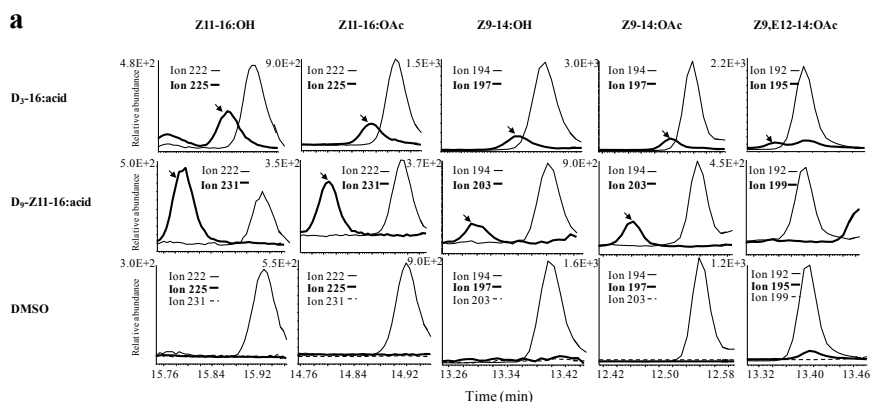


Figure 13. Incorporation of deuterium labels into (a, c) pheromone gland components and (b, c) fatty acyl precursors in *Spodoptera exigua*. Reprinted from Paper I.

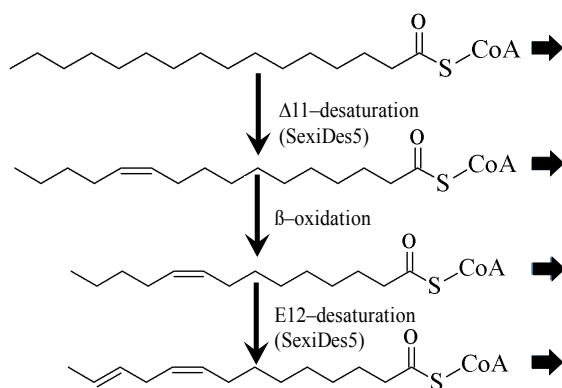


Figure 14. Biosynthetic pathway for sex pheromone of *Spodoptera exigua*. Reprinted from Paper I.

Deuterium labelled D_3 -16:acid, D_3 -14:acid, (Z)-11-[13,13,14,14,14,- 2H_3] tetradecenoic acid (D_5 -Z11-14:acid), (Z)-9-[12,12,12- 2H_3] dodecenoic acid (D_3 -Z9-12:acid) and [12,12,12- 2H_3] dodecanoic acid (D_3 -12:acid) were topically applied to the pheromone glands of *L. botrana* to track the biosynthetic pathway. The results showed that deuterium atoms from D_3 -16:acid were incorporated into all the pheromone components, including (Z)-9-dodecenyl acetate (Z9-12:OAc), (E,Z)-7,9-dodecadienol (E7,Z9-12:OH) and (E,Z)-7,9-dodecadienyl acetate (E7,Z9-12:OAc) (**Fig. 15**). In the fatty acyl compounds analysis, deuterium atoms from D_3 -16:acid were incorporated into methyl palmitate (16:Me), 14:Me, methyl (Z)-11-tetradecanoate (Z11-14:Me), methyl lauritate (12:Me) and methyl (E,Z)-7,9-dodecadienoate (E7,Z9-12:Me). Application of D_3 -14:acid showed the same incorporation results as observed for D_3 -16:acid, whereas no label incorporation was found from D_3 -14:acid into 16:acid. Labels from D_5 -Z11-14:acid were incorporated into Z9-12:OAc, E7,Z9-12:OH and E7,Z9-12:OAc (**Fig. 15b**), as well as into Z9-12:Me and E7,Z9-12:Me intermediates (**Fig. 15a**). Additionally, labels from D_3 -Z9-12:acid were extremely highly incorporated into E7,Z9-12:OAc and incorporated into Z9-12:OAc and E7,Z9-12:OH as well (**Fig. 15-16**). In contrast, when D_3 -12:acid was applied, label incorporation was not detected in any of the above mentioned compounds (**Fig. 15**). The incorporation of deuterium labels from D_3 -Z9-12:acid into E7,Z9-12:OAc, E7,Z9-12:OH, and E7,Z9-12:Me (**Fig. 16**) strongly suggests an elusive $\Delta 7$ desaturation on Z9-12:acid to form the major pheromone precursor E7,Z9-12:acid in *L. botrana*.

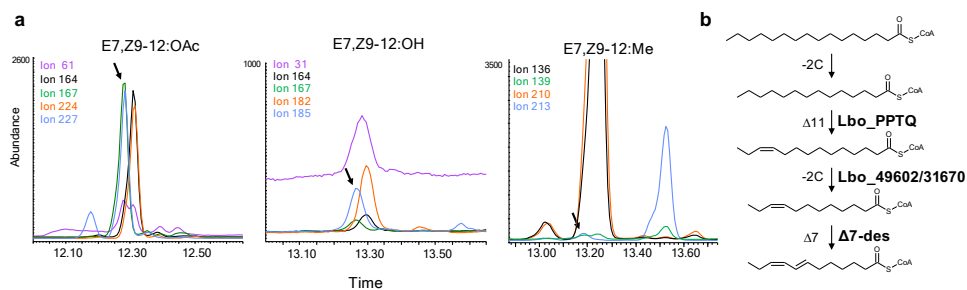


Figure 16. (a) Incorporation of deuterium labels from (Z)-9-[12,12,12- 2H_3] dodecenoic acid (D_3 -Z9-12:acid) into pheromone gland components in *Lobesia botrana*. (b) Biosynthesis pathway for fatty acyl intermediate of *L. botrana*.

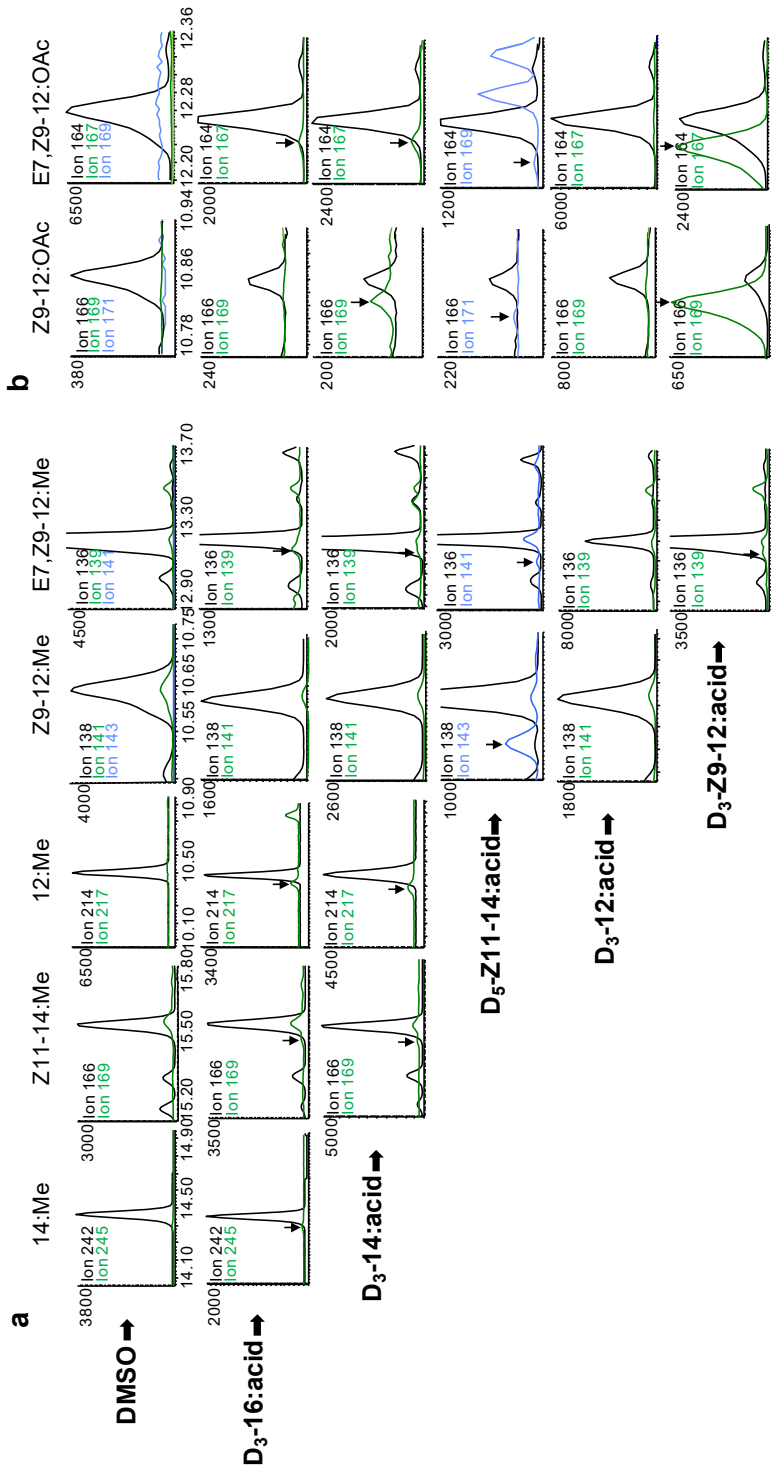


Figure 15. Incorporation of deuterium labels into (a) fatty acyl precursors and (b) pheromone gland components in *Lobesia botrana*.

Functional assays of novel FAD genes (**Paper I, II and III**)

Here, I report several novel FAD genes with a variety of functions. First, involving the $\Delta 12$ desaturation activity, two unusual $\Delta 11/\Delta 12$ FAD genes *SexiDes5* and *SlitDes5* were characterized in *S. exigua* and the congeneric *S. litura*, with the same functions using palmitic acid and the subsequently chain-shortened product (*Z*)-9-tetradecenoic acid (*Z9*-14:acid) as substrates to produce (*Z,E*)-9,12-tetradecadienoic acid (*Z9,E12*-14:acid), respectively (**Paper I**). A $\Delta 11$ FAD gene *Lbo_PPTQ* from *L. botrana* can produce important pheromone intermediate *Z11*-14:acid from myristic acid (**Paper II**). *C. suppressalis* utilizes (*Z*)-11-hexadecenal (*Z11*-16:Ald) as its major pheromone component. By heterologous expression in yeast and plants, a $\Delta 11$ FAD gene *CsupYPAQ* presents very high substrate specificity to palmitic acid for production of (*Z*)-11-hexadecenoic acid (*Z11*-16:acid) with great activity. Another FAD gene *CsupKPSE* from *C. suppressalis* has preference for C_{16} . It is interesting, however, that *CsupKPSE* switches the preference for C_{16} to C_{18} to form oleic acid when the culture nutrition was limited (**Paper III**).

The predicted ER retention signature motif in $\Delta 11/\Delta 12$ FADs *SexiDes5*, *SlitDes5*, and $\Delta 11$ FAD *CsupYPAQ* is “LPAQ”, “LPSQ”, and “YPAQ” respectively. It is noticed that the motif difference in FADs is to some extent related to the functions, e.g., FADs within the “KPSE” group are $\Delta 9$ desaturases having preference for C_{16} , while the “NPVE” group are mainly modifying C_{18} (Rosenfield et al. 2001; Liu et al. 2002; Liu et al. 2004; Rodríguez et al. 2004). The “xxxQ” are most likely to be the $\Delta 11$, $\Delta 10$ and multi-functional FADs and a few exceptions are $\Delta 9$ FADs, which are exclusively involved in pheromone biosynthesis (Knipple et al. 1998; Hao et al. 2002; Serra et al. 2007; Xia et al. 2015). The $\Delta 6$ and $\Delta 14$ groups contain a mixture of different signature motifs from the $\Delta 9$ and $\Delta 11$ groups, and their biological functions are diverged from $\Delta 9$, and $\Delta 11$ FADs (Roelofs et al. 2002; Wang et al. 2010). On the other hand, there were some exceptions to the “motif” signal hypothesis. For example, Ding et al. (2016) reported that one amino acid at the cytosolic carboxyl terminus of the protein (258E), which is outside of the motif region, is critical for the ‘Z’ activity of the *Choristoneura rosaceana* FAD. In this thesis study, *SexiDes5* and *SlitDes5* were showing same functions in the yeast although with different ER motifs. The motif of *CsupYPAQ* has only one amino acid different from *SexiDes5*. However, *CsupYPAQ* is highly specific to palmitic acid (**Paper III**), whereas *SexiDes5* can form a variety of products with wide preferences for both saturated and unsaturated fatty acids (**Paper I**). Thus, making mutations at different sites for these unique FADs could be a possible approach to explore the relationship between sequences and functions of FADs.

Production of pheromone precursors in plants (**Paper IV and V**)

Many functionally diversified FADs were characterized by previous studies, making it possible to design tailor-made pheromone precursor production in engineered plants. In this thesis, *N. tabacum*, *N. benthamiana* and *C. sativa* were successfully transformed to obtain stable transgenic lines producing pheromone precursors.

The successful production of several insect sex pheromone precursors in *N. tabacum* and *N. benthamiana* by stable transformation was demonstrated in this study. Levels of up to 17.6% Z11-16:acid in the total fatty acids was achieved. All of the transformed plastid FatBs and FADs were functionally active in producing pheromone precursor in both *Nicotiana* species. This is the first report of Z/E11-14:acid production in a plant by stable transformation. The performance of Z11-16:acid production in *N. benthamiana* was better than in *N. tabacum*. The average value of Z11-16:acid production in T0 *N. tabacum* was 0.2% whereas in T0 *N. benthamiana* it was 1.8%. The best *N. benthamiana* transgenic line #025 produced as high as 13.6% of Z11-16:acid of the total fatty acids in T2 plants (**Fig. 17**), which is much higher than the production amount reported from Nešnerová et al. 2004, claiming that 6% of Z11-16:acid of total fatty acids was produced in their transgenic *N. tabacum* NtD15B line. The quantity of Z11-16:acid in the form of methyl ester in *N. benthamiana* determined to be 335 μg per gram fresh leaf is also higher, compared to 32 μg per gram of *N. tabacum* fresh leaf in Nešnerová et al. 2004. The results suggest that *N. benthamiana* has potential to be more efficient than *N. tabacum* as a plant factory for Z11-16:acid production. In the report of Ding et al. 2014, 381 μg per gram fresh leaf of Z11-16:acid was produced in *N. benthamiana* by transient expression, which is a massive overexpression of exogenous genes over a few days that ignores the health of the plant. Here, the production of 335 μg per gram leaf of Z11-16:acid by stable transformation shows the ability of vegetative material to function with the expression of *CpuFatB1* and *Atr Δ 11* and yield compounds over development, providing the potential for further commercial production.

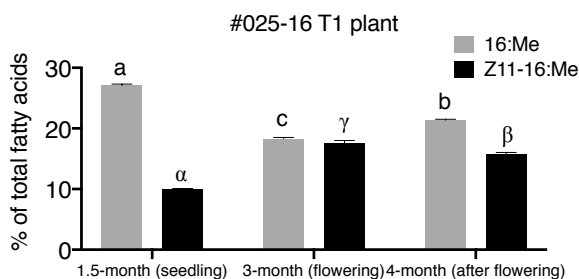


Figure 17. Percentage of palmitic acid (16:0) and (Z)-11-hexadecenoic acid (Z11-16:acid) of total fatty acids in T1 plant #025-16 leaves during different development stages of plant.

To investigate the feasibility of a “plant factory production” in *Camelina*, we established four different types of transformant lines by using different exogenous gene cassettes (**Paper V**). In the regenerated *Camelina* seeds, the mono-unsaturated E9-12:acid with small amount of (*Z*)-9-dodecenoic acid (Z9-12:acid) and diunsaturated E8,E10-12:acid were produced in all the four types of transformant lines (**Fig. 18**). *Camelina* was genetically modified for production of E8,E10-12:acid via stable integration of gene cassettes using *Agrobacterium*-mediated floral dip transformation. This is the first report on production of di-unsaturated pheromone precursors in plants. Furthermore, the production amount of 6.3% of E8,E10-12:acid of total fatty acids is quite high. Because the oil content of the *Camelina* seeds, on a dry weight basis, is typically between 35 to 45% and the yields of *Camelina* are in a range of 336 to 2240 kg of seeds per hectare (Moser 2010). This means that 7.4 to 63.5 kg (minimum to maximum) of E8,E10-12:acid might be produced by cultivating our best *Camelina* line. Moreover, in this study we also investigated four strategies for optimization of the plant factory for production of E8,E10-12:acid. We demonstrated that co-expression of the desaturase with *P19* and multiple gene copies can increase the production of C₁₂ pheromone precursors significantly. Also, it was confirmed that stably expressing *P19* regulated by the seed-specific *napin* promoter would not cause observable harm of plant development.

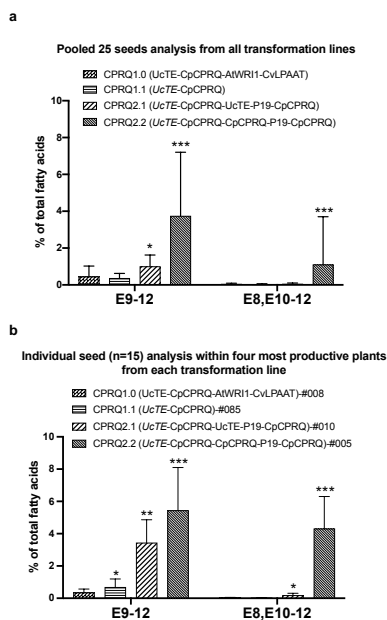


Figure 18. Comparison of mean percentage of E9-12:acid and E8,E10-12:acid of total methylated fatty acid between four strategies. a) analyzed from pooled 25 seeds of each transformant; b) analyzed from 15 individual seeds from most productive plant of each strategy.

New platform for functional assay of pheromone biosynthetic genes (**Paper III**)

In this thesis, a new concept for molecular mechanism exploration of pheromone biosynthesis is provided, which is using *N. benthamiana* transient expression platform. The idea emerged from the principle of plant factories. Transient expression of various insect genes in plant leaves as factories for pheromone production has been demonstrated for a couple of years (Ding et al. 2014), but utilizing this platform for gene function studies has never been explored. In this study, we confirm that the plant transient expression system is efficient and useful.

In **paper III**, we use plants as a platform for functional characterization of the *C. suppressalis* biosynthetic gene candidates in order to compare the results to the yeast expression system. The observed functions of these genes in yeast and plant are similar and complementary. For instance, it is interesting that *CsupKPSE* showed C₁₈>C₁₆ substrate preference when oleic acid was absent in *ole1/elo1* yeast. However, in the plant, *CsupKPSE* shows its preference on C₁₆>C₁₈ because the leaf does not lack oleic acid. The phylogenetic analysis indicated that *CsupKPSE* belongs to the C₁₆>C₁₈ clade. This implies that *CsupKPSE* can adjust its function to produce oleic acid similar to the function of ancestral metabolic desaturase when the nutrition is limited. Therefore, expression of desaturase genes in plant leaves for identification of different functions has the advantage of avoiding the problem of supplemented nutrition inference, especially for Δ^9 desaturase identification. A FAR gene *CsupFAR2* expressed low activity in yeast, while in plants it shows very high activity. Because the plant lipids are different from yeast, it offers an alternative way for gene function studies. The pgFAR *CsupFAR2* showed additional minor activities on polyunsaturated fatty acids of linoleic acid and α -linolenic acid in *N. benthamiana*, which has never been reported in previous FAR function studies. Moreover, the algae ELO *IgalASE1* elongated linoleic acid (18:2) and α -linolenic acid (18:3) to eicosadienoic acid (20:2) and eicosatrienoic acid (20:3) in *N. benthamiana*, show the same function as reported in yeast *S. cerevisiae* expression system (33), indicating that heterologous expression of ELO in plant leaves for functional characterization is reliable and feasible.

New ACO, ELO and FAR findings (**Paper II and III**)

The specific skeleton of an unsaturated fatty acyl chain is generally produced by combination of FAD and ELO or ACO. Among the functionally characterized pheromone biosynthetic genes in *L. botrana* and *C. suppressalis*, there were two novel ACO genes *Lbo_31670*, *Lbo_49602* (**Paper II**) and a first reported ELO gene *CsupELO4* (**Paper III**) showing functions. The former two genes encoding two acyl-CoA oxidases that may produce Z9-12:acid by chain shortening of Z11-14:acid.

The later gene encoding an elongase may produce Z13-18:acid, an immediate precursor of a pheromone component in *C. suppressalis*.

We also functionally characterized a new FAR gene (**Paper III**) from *C. suppressalis*, *CsupFAR2* that shows very high activity to Z11-16:acid, producing corresponding Z11-16:OH. It also has other minor activity with C₁₆-C₁₈ fatty acids, similar to *HarFAR* from *Helicoverpa armigera* (Hagström et al. 2012; 2013), but having more specific activities on Z11-16:acid. The high activity and substrate specificity of *CsupFAR2* suggests it to be a valuable candidate for further pheromone production in both yeast and plant factories.

Increase the production amount of pheromone compounds- trichome specific promoter

A long-term vision is to produce genetically modified plants that eventually can be used in intercropping as natural dispensers of pheromones and as part of a push-pull (Cook et al. 2007) strategy, providing an innovative and environmentally friendly approach for pest management. Production of a high yield of pheromones in plants by stable transformation is still challenging. With the purpose of producing fatty alcohols or acetates especially for releasing these compounds from the leaf, a trichome specific promoter derived from *N. tabacum* was cloned and used for FAR and ATF gene expression.

In moths, after the immediate acyl-CoA precursors are produced, FAR can catalyze the reduction of acyl-CoA into fatty alcohols, which either are used as pheromones for some moth species, or in some other moths, converted to corresponding acetates after trans-acetylation by ATFs. Therefore, in order to explore the possibility of releasing pheromone alcohol and acetate in transformed plants, the FAR gene *HarFAR* and the ATF gene *ATF1* was constructed with a trichome specific promoter CYP71D16, respectively, producing the pheromones Z11-16:OH and Z11-16:OAc (**Fig. 19**).

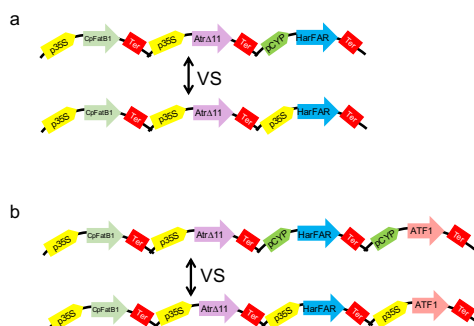


Figure 19. Scheme of constructed vectors for a) Z11-16:OH and b) Z11-16:OAc production in *Nicotiana* spp.

CYP71D16 was confirmed as a trichome-specific promoter leading the downstream gene to be specifically expressed in plant trichomes (Wang et al. 2002). After 3-5 days, the infiltrated *N. benthamina* plant expressing *HarFAR* controlled by CYP71D16 promoter produced 70 μg Z11-16:OH in per gram fresh leaf, while the plant expressing *HarFAR* driven by CaMv35S promoter only produced a tiny amount of Z11-16:OH (**Fig 20**). The same combination of *CpFatB1*, *Atr Δ 11* and *HarFAR* driven by 35S promoter were also tested in the study of Ding et al. 2014, which reported 18 μg Z11-16:OH in per gram fresh leaf was produced. Also, *ATF1* controlled by CYP71D16 expressing in the plant produced much more Z11-16:OAc than 35S (**Fig. 20**). This result suggested that CYP71D16 promoter can increase the pheromone production amount significantly compared to 35S promoter. However, until now, no pheromone compounds from the plant headspace volatiles was collected.

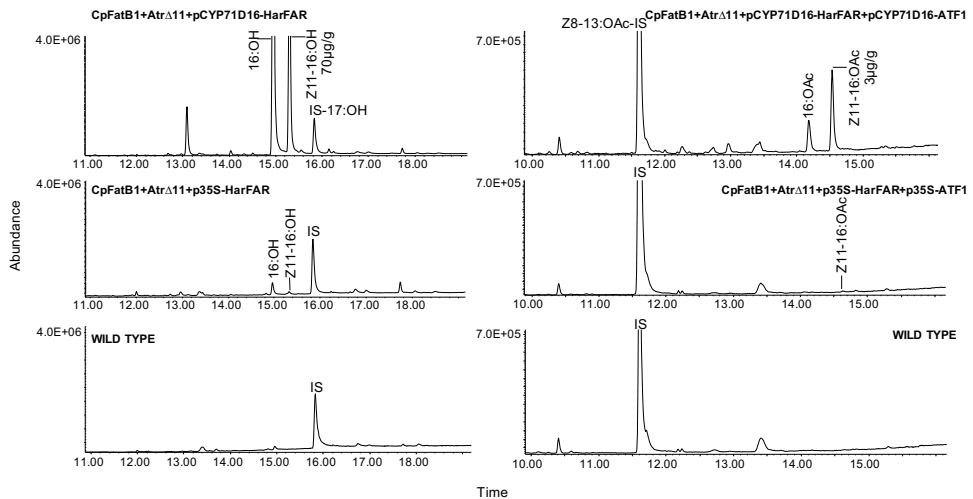


Figure 20. Expression of *HarFAR* and *ATF1* with pCYP71D16 promoter or 35S promoter.

Conclusions and perspectives

In this Ph.D. project, the first contribution is that we characterized several key pheromone biosynthetic genes in the serious pest species, including two beet armyworm *Spodoptera* species (**Paper I**), European grapevine moth *L. botrana* (**Paper II**) and the rice stem borer *C. suppressalis* (**Paper III**). We enriched the functional pheromone biosynthetic gene database. These findings will not only help understanding the mechanisms of pheromone biosynthesis, but will also provide many putative candidates for pheromone production in bio-factories.

In the perspective of utilizing results from basic science for application, the second contribution of this thesis is that we established several stable transgenic plant lines for pheromone precursors production, ranging from the carbon chain length of 12 (**Paper V**) to 16 (**Paper IV**), either in the leaves or seeds. This is the first report on an extended production of pheromone precursors (C₁₂ and C₁₄) over generations in plants. We confirm that *N. benthamiana* is a suitable platform for stable production of C₁₆ pheromone precursors. We also established productive *Camelina* line for di-unsaturated C₁₂ pheromone precursor production. Up to 6.3% of E8,E10-12:acid of total fatty acids was achieved. Because the oil content of the *Camelina* seeds, on a dry weight basis, is typically between 35 to 45% and the *Camelina* yields anywhere are from 336 to 2240 kg of seeds per hectare, which means 7.4 to 63.5 kg (minimum to maximum) of E8,E10-12:acid might be produced by cultivating our best *Camelina* line.

A further aim is to enable stable production of final pheromone components in bio-factories, which remains to be explored. First, increasing the pheromone precursor production to provide more substrate for pheromone biosynthesis is one of the possible approaches. How to increase the precursor production, for instance by elevating the TAG accumulation in plant cells, needs further efforts. Our long-term goal is to design the “tailor-made” production of any moth pheromone in stably transformed plants. However, much remains unknown about the molecular mechanisms underlying the pheromone biosynthesis. The identification of genes encoding pheromone biosynthesis enzymes is a crucial step in ensuring the compatibility of bio-factories. Prior to this thesis, only FAD and FAR genes have been functionally characterized, but we now have found several functional genes encoding ACOs (**Paper II**) and ELO that were involved in pheromone biosynthesis (**Paper III**). We also developed the plant platform for insect gene functional

characterization, contributing an efficient way for further fatty acid metabolic and pheromone biosynthesis studies.

Ultimately, plant factory for pheromone production could be used in a “push-pull” strategy for pest control, which is using pheromones that act to make the protected resource unattractive to the pests (push) while luring them toward an attractive source (pull) from where the pests are subsequently removed. This requires that the plants can release pheromones into the atmosphere which is still challenging. We intended to accomplish this by using a trichome-specific promoter CYP71D16 to drive pheromone biosynthetic genes. It was surprising to find that this strategy increased the pheromone production amount quite substantially. However, no detectable pheromone compounds were collected from the plant headspace volatiles. To understand the mechanism of volatile release could be the next step to go forward.

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List of papers

- I. Yi-Han Xia, Ya-Nan Zhang, Bao-Jian Ding, Hong-Lei Wang, Christer Löfstedt. (2019) Multi-Functional Desaturases in Two *Spodoptera* Moths with $\Delta 11$ and $\Delta 12$ Desaturation Activities. *Journal of Chemical Ecology* 45, 378–387. <https://doi.org/10.1007/s10886-019-01067-3>
- II. Bao-Jian Ding*, Yi-Han Xia*, Hong-Lei Wang, Erik Hedenström, Jürgen Gross, Christer Löfstedt (2020) Biosynthesis of the Sex Pheromone (E,Z)-7,9-dodecadienyl acetate in the European Grapevine Moth *Lobesia botrana* Involving $\Delta 11$ Desaturation and an Elusive $\Delta 7$ Desaturase. (*, shared first authorship). Manuscript.
- III. Yi-Han Xia, Bao-Jian Ding, Shuang-Lin Dong, Hong-Lei Wang, Christer Löfstedt. (2020) Characterization of genes involved in Sex Pheromone Biosynthesis in the Rice Stem Borer *Chilo suppressalis* (Lepidoptera: Pyraloidea): a Novel Functional Fatty Acyl-CoA Elongase Gene Revealed. Manuscript.
- IV. Yi-Han Xia, Bao-Jian Ding, Hong-Lei Wang, Carin Jarl-Sunesson, Per Hofvander, Christer Löfstedt. (2020) Production of Moth Sex Pheromone Precursors in *Nicotiana* spp. by *Agrobacterium*-mediated Stable Transformation. Submitted.
- V. Yi-Han Xia, Bao-Jian Ding, Hong-Lei Wang, Carin Jarl-Sunesson, Edgar B. Cahoon, Per Hofvander, Christer Löfstedt. (2020) Metabolic Engineering of Camelina (*Camelina sativa*) for the Production of Mono- and Di-unsaturated C12 Moth Pheromone Precursors. Manuscript.

