UNIVERSITAT POLITÈCNICA DE VALÈNCIA

PhD in Biotechnology

Bioproduction of insect sex pheromones and related volatile compounds in plants

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El Dr. Diego Orzáez Calatayud, Científico Titular del Consejo Superior de Investigaciones Científicas, perteneciente al Instituto de Biología Molecular y Celular de Plantas (IBMCP, UPV-CSIC) de Valencia, y la Dra. Silvia Gianoglio, investigadora del Instituto de Biología Molecular y Celular de Plantas (IBMCP, UPV-CSIC) de Valencia, CERTIFICAN que Rubén Mateos Fernández, ha realizado bajo su dirección en el Instituto de Biología Molecular y Celular de Plantas, el trabajo titulado "Bioproduction of insect sex pheromones and related volatile compounds in plants", y que autoriza su presentación para optar al grado de Doctor en Biotecnología de la Universitat Politècnica de València.

Y para que así conste, firman el presente certificado en Valencia a 28 de agosto de 2024.

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Abstract

ABSTRACT

Plants are powerful platforms for the biosynthesis of compounds of interest to humans. Through metabolic engineering and synthetic biology, these biofactories can heterologously produce high-value compounds for human use, including industrial, medical, and agricultural applications. Among the latter are insect sex pheromones. Currently, insect pest damage to crops results in significant economic losses every year. As an alternative to pesticides, the use of semiochemicals, particularly insect sex pheromones, is proposed as a useful and more sustainable solution for pest control. However, the production of these compounds is predominantly achieved through chemical synthesis, which generates chemical by-products that are not entirely environmentally friendly. Therefore, alternative synthesis methods are needed to achieve greater sustainability in the process.

The biosynthetic pathways of moth sex pheromones are currently well-described, with many of the genes responsible for their synthesis identified. Recently, in a study published by our group (Mateos-Fernández et al., 2021), stable transgenic *Nicotiana benthamiana* plants, termed 'SexyPlant,' were developed to produce and emit volatile and biologically active compounds such as the pheromones Z11-16OH, Z11-16OAc, and Z11-16Ald. This was accomplished by introducing the biosynthetic pathway of several common pheromone compounds from multiple moth species into *N. benthamiana*, employing genes from both moths and plants, and leveraging part of the plant's endogenous fatty acid metabolism. However, several issues related to toxicity were detected, associated with the accumulation of these compounds and the disruption of the plant's fatty acid metabolic pathway.

In the first chapter, we offer solutions to the unintended toxicity problems observed in the 'SexyPlant.' First, we present a range of alternative acetyltransferases compared to those used in the initial versions of 'SexyPlant,' aimed at improving the conversion efficiency of the pheromone compound Z11-16OH to Z11-16OAc. Additionally, we explore the activation of this biosynthetic pathway, demonstrating the possibility of regulating it not only transiently but also in stable transgenic plants using CRISPR-based transcriptional regulators ('dead' Cas activator, specifically dCasEV2.1)

combined with synthetic promoters. We generated stable lines, termed 'GuidedPathway' and 'NonGuidedPathway', with two different gene configurations that accumulate biomass and do not produce or emit moth sex pheromones until specifically activated by appropriate orthogonal genetic elements. Although the production yield is lower than in constitutive 'SexyPlant' plants, in this chapter, we demonstrate the potential for activating the biosynthetic pathway and explore the bottlenecks in the system.

In the second chapter, we focus on the heterologous production of another family of compounds in plants: irregular monoterpenoids. A significant percentage of these molecules are of great interest as they constitute the sex pheromones of insect species in the Coccoidea superfamily (e.g., scale insects). In this chapter, we explore the potential for heterologous production of irregular monoterpenoids in *N. benthamiana* and *N. tabacum*. However, the biosynthetic pathway leading to these molecules is currently unknown, as the genes involved in this biosynthesis have not been identified. For our work, we expressed plant-derived genes with similar activity. We introduced the genes encoding chrysanthemyl diphosphate synthase (*CPPS*) from *Tanacetum cinerariifolium* and lavandulyl diphosphate synthase (*LPPS*) from *Lavandula x intermedia* into both tobacco and *N. benthamiana* to first demonstrate their transient production. Secondly, we achieved stable expression of these genes, resulting in stable transgenic lines. We characterized the production and emission of these volatile compounds in different tissue types (vegetative and reproductive) and at different leaf development stages. This allowed us to determine that juvenile leaves were the most productive tissue. Additionally, we approached our goal of producing Coccoidea sex pheromones by combining the expression of *Li*LPPS with that of an acetyltransferase from the same species, *Li*AAT4. As a result, we were able to produce and emit relatively high quantities of lavandulyl acetate, a compound that is part of the pheromone blend of the mealybug *Dysmicoccus grassii* and the aggregation pheromone of the thrip *Frankliniella occidentalis,* as well as a proven mosquito larvicide. The emission produced by these 'biodispensers' reached a significant efficiency of up to 0.63 mg of lavandulyl acetate per day, allowing us to

estimate the need for between 200 and 500 of these plants per hectare to match the control achieved by commercial pheromone release devices.

In summary, this thesis provides different strategies for addressing the production of insect sex pheromones and associated volatile compounds in plant biofactories, exploring the limitations of this biosynthesis.

RESUMEN

Las plantas constituyen poderosas plataformas para biosintetizar compuestos de interés para el ser humano. Mediante la ingeniería metabólica y la biología sintética, se puede producir en estas biofactorías y de manera heteróloga compuestos de gran valor para el ser humano, desde moléculas de interés industrial, médico o de uso en agricultura. Entre estos últimos, se encuentran las feromonas sexuales de insectos. En la actualidad, los daños ocasionados por plagas de insectos en los cultivos vegetales suponen enormes pérdidas económicas cada año. Para el control de dichas plagas, como alternativa a los pesticidas, el uso de semioquímicos, entre los cuales destacan las feromonas sexuales de insectos, se plantean como una solución de gran utilidad y mayor sostenibilidad. Sin embargo, la producción de dichos compuestos mayoritariamente tiene lugar mediante síntesis química, la cual genera productos químicos derivados que no son del todo respetuosos con el medio ambiente. Por tanto, se necesitan alternativas de síntesis para lograr una mayor sostenibilidad en el proceso. Las rutas biosintéticas de las feromonas sexuales de polilla se encuentran mejor descritas actualmente, conociéndose gran parte de los genes responsables de dicha síntesis. Recientemente, en un trabajo publicado por nuestro grupo (Mateos-Fernández et al., 2021) se obtuvieron plantas transgénicas estables de *Nicotiana benthamiana*, las denominadas "SexyPlant", productoras y emisoras de los compuestos volátiles y biológicamente activos como feromona Z11-16OH, Z11-16OAc y Z11-16Ald. Esto se consiguió tras introducir la ruta biosintética de varios compuestos feromona comunes a varias especies de polilla, reconstituida en *N. benthamiana* gracias a genes originarios de polilla y de plantas y aprovechando parte de su metabolismo endógeno de ácidos grasos. Sin embargo, se detectaron varios problemas de toxicidad asociados a la acumulación de dichos compuestos y a la alteración de la ruta metabólica de los ácidos grasos de la planta. En el primer capítulo, ofrecemos alternativas a dichos problemas de toxicidad no deseados ocasionados en la "SexyPlant". Para ello, en primer lugar, ofrecemos un abanico de acetiltransferasas alternativas a las planteadas en las primeras versiones de la "SexyPlant", que mejoren la conversión del compuesto feromona Z11-16OH en Z11-16OAc, y con diferentes eficiencias. Por otro lado, exploramos la activación de dicha

ruta biosintética, demostrando la posibilidad de regularla no solo de manera transitoria, sino también en plantas transgénicas estables, gracias al uso de reguladores transcripcionales basados en CRISPR ("dead" Cas9 activadora, concretamente dCasEV2.1) y al empleo combinado de promotores sintéticos. Así, generamos líneas estables, las denominadas "GuidedPathway" y "NonGuidedPathway", con dos configuraciones génicas diferentes que acumulan biomasa y no producen ni emiten feromonas sexuales de polilla hasta que no se activan específicamente con los elementos genéticos ortogonales adecuados. Aunque el rendimiento de producción es menor que en las plantas constitutivas "SexyPlant", en este capítulo demostramos la posibilidad de activar la ruta biosintética y exploramos los cuellos de botella del sistema. En el segundo capítulo, nos centramos en la producción heteróloga en plantas de otra familia de compuestos, los monoterpenoides irregulares. Un gran porcentaje de estas moléculas resultan de enorme interés al constituir las feromonas sexuales de las especies de insecto de la superfamilia Coccoidea (cochinillas, entre otras). En este capítulo exploramos el potencial de producción heteróloga de monoterpenoides irregulares en *N. benthamiana* y *N. tabacum*. Sin embargo, la ruta biosintética que lleva a dichas moléculas resulta en estos momentos desconocida, al no estar identificados los genes implicados en dicha biosíntesis. Para nuestro trabajo, expresamos genes de origen vegetal con actividad parecida. Introdujimos tanto en tabaco como en N. benthamiana los genes de la crisantemil difosfato sintasa (*CPPS*) de *Tanacetum cinerariifolium* y de la lavandulil difosfato sintasa (*LPPS*) de *Lavandula x intermedia*, para demostrar, en primer lugar, su producción transitoria. En segundo lugar, conseguimos la expresión estable de dichos genes, consiguiendo líneas transgénicas estables. Caracterizamos la producción y emisión de dichos compuestos volátiles en distintos tipos de tejido (vegetativo y reproductivo) así como en diferentes estadios de desarrollo de las hojas. Con ello, pudimos determinar que las hojas juveniles eran el tejido más productivo. Por otro lado, conseguimos acercarnos a nuestro objetivo de producir feromonas sexuales de Coccoidea combinando la expresión de *Li*LPPS con la de una acetiltransferasa de la misma especie, *Li*AAT4. Gracias a ello, conseguimos producir y emitir cantidades relativamente altas de lavandulil acetato, el cual

constituye en sí mismo uno de los compuestos de la mezcla feromonal de la cochinilla *Dysmicoccus grassii* y de la feromona de agregación del trip *Frankliniella occidentalis*, así como un demostrado larvicida de mosquitos. La emisión producida por dichas plantas "biodispensadoras" consigue la considerable eficiencia de hasta 0,63 mg de lavandulil acetato al día, permitiéndonos estimar la necesidad de entre 200 y 500 de estas plantas por hectárea para equiparar el control conseguido por dispositivos liberadores de feromonas comerciales. En resumen, esta tesis proporciona diferentes estrategias para abordar la producción de feromonas sexuales de insectos y compuestos volátiles asociados en biofactorías vegetales, explorando los límites de dicha biosíntesis.

RESUM

Les plantes constitueixen poderoses plataformes per a la biosíntesi de compostos d'interés per a l'ésser humà. Mitjançant l'enginyeria metabòlica i la biologia sintètica, es poden produir en aquestes biofactories i de manera heteròloga compostos de gran valor per a l'ésser humà, des de molècules d'interés industrial, mèdic o d'ús en agricultura. Entre aquests últims es troben les feromones sexuals d'insectes. Actualment, els danys ocasionats per plagues d'insectes en els cultius vegetals suposen enormes pèrdues econòmiques cada any. Per al control d'aquestes plagues, com a alternativa als pesticides, l'ús de semioquímics, entre els quals destaquen les feromones sexuals d'insectes, es planteja com una solució de gran utilitat i major sostenibilitat. No obstant això, la producció d'aquests compostos té lloc majoritàriament mitjançant síntesi química, la qual genera productes químics derivats que no són del tot respectuosos amb el medi ambient. Per tant, es necessiten alternatives de síntesi per a aconseguir una major sostenibilitat en el procés.

Les rutes biosintètiques de les feromones sexuals de papallona es troben millor descrites actualment, coneixent-se gran part dels gens responsables d'aquesta síntesi. Recentment, en un treball publicat pel nostre grup (Mateos-Fernández et al., 2021) s'obtingueren plantes transgèniques estables de *Nicotiana benthamiana*, denominades "SexyPlant", productores i emissores dels compostos volàtils i biològicament actius com la feromona Z11-16OH, Z11-16OAc i Z11-16Ald. Això es va aconseguir després d'introduir la ruta biosintètica de diversos compostos feromona comuns a diverses espècies de papallona, reconstituïda en *N. benthamiana* gràcies a gens originaris de papallona i de plantes, aprofitant part del seu metabolisme endogen d'àcids grassos. No obstant això, es detectaren diversos problemes de toxicitat associats a l'acumulació d'aquests compostos i a l'alteració de la ruta metabòlica dels àcids grassos de la planta.

En el primer capítol, oferim alternatives a aquests problemes de toxicitat no desitjada ocasionats en la "SexyPlant". Per a això, en primer lloc, oferim un ventall d'acetiltransferases alternatives a les plantejades en les primeres versions de la 'SexyPlant', que milloren la conversió del compost feromona Z11-16OH en Z11-

16OAc, amb diferents eficiències. D'altra banda, explorem l'activació d'aquesta ruta biosintètica, demostrant la possibilitat de regular-la no sols de manera transitòria, sinó també en plantes transgèniques estables, gràcies a l'ús de reguladors transcripcionals basats en CRISPR ("dead" Cas9 activadora, concretament dCasEV2.1) i a l'ús combinat de promotors sintètics. Així, generem línies estables, les denominades "GuidedPathway" i "NonGuidedPathway", amb dues configuracions gèniques diferents que acumulen biomassa i no produeixen ni emeten feromones sexuals de papallona fins que no s'activen específicament amb els elements genètics ortogonals adequats. Encara que el rendiment de producció és menor que en les plantes constitutives 'SexyPlant', en aquest capítol demostrem la possibilitat d'activar la ruta biosintètica i explorem els colls d'ampolla del sistema.

En el segon capítol, ens centrem en la producció heteròloga en plantes d'una altra família de compostos, els monoterpenoides irregulars. Un gran percentatge d'aquestes molècules resulten de gran interés en constituir les feromones sexuals de les espècies d'insecte de la superfamília Coccoidea (cotxinilles, entre altres). En aquest capítol explorem el potencial de producció heteròloga de monoterpenoides irregulars en *N. benthamiana* i *N. tabacum*. No obstant això, la ruta biosintètica que porta a aquestes molècules resulta en aquests moments desconeguda, en no estar identificats els gens implicats en aquesta biosíntesi. Per al nostre treball, expressem gens d'origen vegetal amb activitat semblant. Introduírem tant en tabac com en *N. benthamiana* els gens de la crisantenil difosfat sintasa (*CPPS*) de *Tanacetum cinerariifolium* i de la lavandulil difosfat sintasa (*LPPS*) de *Lavandula x intermedia*, per a demostrar, en primer lloc, la seua producció transitòria. En segon lloc, aconseguírem l'expressió estable d'aquests gens, aconseguint línies transgèniques estables. Caracteritzàrem la producció i emissió d'aquests compostos volàtils en diferents tipus de teixit (vegetatiu i reproductiu) així com en diferents estadis de desenvolupament de les fulles. Amb això, poguérem determinar que les fulles juvenils eren el teixit més productiu. D'altra banda, aconseguírem acostar-nos al nostre objectiu de produir feromones sexuals de Coccoidea combinant l'expressió de *Li*LPPS amb la d'una acetiltransferasa de la mateixa espècie, LiAAT4. Gràcies a això, aconseguírem produir i emetre quantitats relativament altes de lavandulil acetat, el qual constitueix en si

mateix un dels compostos de la mescla feromonal de la cotxinilla *Dysmicoccus grassii* i de la feromona d'agregació del trips *Frankliniella occidentalis*, així com un demostrat larvicida de mosquits. L'emissió produïda per aquestes plantes "biodispensadores" aconsegueix la considerable eficiència de fins a 0,63 mg de lavandulil acetat al dia, permetent-nos estimar la necessitat d'entre 200 i 500 d'aquestes plantes per hectàrea per a equiparar el control aconseguit per dispositius alliberadors de feromones comercials.

En resum, aquesta tesi proporciona diferents estratègies per a abordar la producció de feromones sexuals d'insectes i compostos volàtils associats en biofactories vegetals, explorant els límits d'aquesta biosíntesi.

Abbreviations

ABBREVIATIONS

ATP: Adenosine triphosphate

bp: base pair

Bt: *Bacillus thuringiensis*

CDS: coding sequence

CoA: Coenzyme A

CPP: chrysanthemyl pyrophosphate

CPPS: chrysanthemyl pyrophosphate synthase

CRISPR: clustered regularly interspaced short palindromic repeats

CRISPRa: CRISPR activation

crRNA: CRISPR RNA

DBS: double strand break

DBTL: design, build, test and learn

dCas9: dead Cas9

dCasEV2.1: dCas9:EDLL-MSV2:VPR/gRNA2.1

DCM: dichloromethane

DDT: Dichlorodiphenyltrichloroethane

DFR: dihydroflavonol 4-reductase

DMAPP: Dimethylallyl pyrophosphate

DNA: Deoxyribonucleic acid

dpi: days post-infiltration

DSB: double-strand break

EDTA: Ethylenediaminetetraacetic acid

FAD: Fatty acid desaturase

FAME: Fatty acid modifying enzymes

FAO: Fatty acid oxidase

FAR: Fatty acid reductase

FBA: flux balance analysis

FW: fresh weight

GABA: γ-aminobutyric acid

GB: Golden Braid

GC: gas chromatography

GC/MS: gas chromatography coupled to mass spectrometry

GFP: Green fluorescent protein

GMO: genetically modified organism

GMP: Good Manufacturing Practices

GP: Guided Pathway

gRNA: guide of RNA

h: hours

HDR: Homology-directed repair

hFAT: hypothetical Fatty Acyl Transferase

HSPME: headspace solid-phase micro-extraction

IDS: Isoprenyl diphosphate synthase

IPM: integrated pest management

IPP: isopentenyl pyrophosphate

IS: internal standard

LB: Luria brooth

LPP: lavandulyl pyrophosphate

LPPS: lavandulyl pyrophosphate synthase

ME: metabolic engineering

MES: 2-(N-morpholino) ethanesulfonic acid

MFA: Metabolic flux analysis

min: minutes

MS: Murashige and Skoog medium

NADH: Nicotinamide adenine dinucleotide

NADPH: Nicotinamide adenine dinucleotide phosphate

NbCPPS: stable transgenic *Tc*CPPS *N. benthamiana*

NBI: Nitrogen Balance Index

NbLPPS: stable transgenic *Li*LPPS *N. benthamiana*

NGP: Non-Guided Pathway

NHEJ: nonhomologous end-joining

Nos: nopaline synthase

Nr: non-redundant

*Nt*CPPS: stable transgenic *Tc*CPPS *N. tabacum*

*Nt*LPPS-AAT4: stable transgenic *Li*LPPS – *Li*AAT4 *N. tabacum*

*Nt*LPPS: stable transgenic *Li*LPPS *N. tabacum.*

OD: optical density

p35S: Cauliflower Mosaic Virus 35 S (CaMV35S) promoter

PAM: protospacer adjacent motif

PCR: polymerase chain reaction

PCSC: plant cell suspension cultures

PEG: Polyethylenglycol

pNOS: nopaline synthase promotor

PNP: plant natural product

PVX: Potato virus X

RNA: Ribonucleic acid

RNAi: RNA interference

rpm: revolutions per minute

RT: Room temperature

s: seconds

SD: standard deviation

sgRNA: small guide of RNA

SxP: Sexy Plant

SynBio: Synthetic Biology

T-DNA: Ti-Plasmid from *Agrobacterium*

TAD: Transcriptional activator domain

TALEs: transcription activator-like effectors

TEV: Tobacco etch virus

TF: transcription factor

TIC: total ion count

tNOS: nopaline synthase terminator

TNT: trinitrotoluene

TPS: Terpene synthase

tracrRNA: trans-activating CRISPR RNA

TU: Transcriptional Unit

UV: Ultraviolet

VIGE: Virus-induced genome editing

VOC: Volatile organic compound

VPR: VP64-p65-Rta

WT: wild type

YFP: yellow fluorescent protein

Z11-16Ald: (*Z*)-11-hexadecenal

Z11-16OAc: (*Z*)-11-hexadecenyl acetate

Z11-16OH: (*Z*)-11-hexadecen-1-ol

ZFPs: Zinc-finger proteins

ZFs: Zinc-fingers

Introduction

INTRODUCTION

1. Synthetic biology: principles and foundations

Synthetic Biology – commonly referred to as SynBio – is a field of study that seeks to harness the astounding richness of metabolic functions found in living organisms to develop novel biological systems implemented with profitable features or applications. Since the first reference to this term in 1980 by Barbara Hobom (Hobom, 1980), the discipline has deeply settled and evolved, becoming a broad field of biotechnological research, in which two primary approaches, referred to as 'bottom-up' and 'top-down', coexist. On the one hand, the long-term objective of SynBio can broadly be conceived – with a bottom-up approach – as the creation of artificial life: a synthetic minimal organism, obtained by a redesign of a biological system starting from its fundamental building blocks, would shed light on the genetic, developmental, and biochemical requirements of living cells. On the other hand, however, SynBio is presently understood mainly with a top-down approach, in which an organism, used as 'chassis', is genetically engineered using appropriate molecular devices to endow it with novel functions (Roberts et *al.,* 2013). These devices, usually genetically encoded, aim to generate user-desired outputs when administering the appropriate preselected inputs, expanding the ability of the chassis to function as a tool or generate products. This can be of great interest for several reasons: *i*) producing high-value molecules with industrial, pharmaceutical, or agricultural applications, among others; *ii*) developing signaling systems linked to biological processes (*i.e.*, sentinel plants or microorganisms); *iii*) gaining a deeper understanding of the requirements and constraints of an organism as a chassis.

Synthetic Biology is fostered by three main principles adopted from engineering, which guide the processes of discovery and tool development: these principles are standardization, decoupling or modularity, and abstraction (Endy, 2005). In this context, modular cloning methods that allow to efficiently build the above-mentioned genetic devices stand out as fundamental elements of SynBio: they all evolved from the initial BioBricks standard, a highly simplified set of rules for assembling DNA

fragments in which all DNA parts are conceived as modular units with standardized sequence edges, resembling lego bricks. In the original Biobricks standard, all DNA parts are idempotent, meaning that composite parts resulting from the assembly of two basic parts can be re-used as input in the following binary assembly, employing exactly the same assembly mechanism as their precursors (Knight, 2003).

SynBio-inspired genetic devices are ideally developed following an iterative engineering framework cycle comprising design, build, test, and learn stages (**DBTL iterative cycle**) (Carbonell et *al.,* 2018, Pouvreau et *al.,* 2018). First, the "design" stage is a planning step where the goal is set, defining the target outcome (e.g., what genes and regulatory elements are necessary to produce a certain compound). Thus, DNA parts (standardized and reusable) are designed as building blocks. Next, in the "build" stage, DNA parts are synthesized, assembled, and then transferred to the host organism. Subsequently, the "test" stage aims to examine the efficiency of the genetic device into the host organism and its effects on it. The last step, "learn", analyzes the outputs and polishes the initial hypothesis, ideally with the interplay serving as feedback for the next DBTL iteration (Figure 1).

For the programming and control of complex biological environments such as cells and organisms, the creation of **synthetic transcriptional genetic circuits** is often required: these are groups of genes and their associated regulatory sequences that efficiently transform a non-transcriptional input signal into a non-transcriptional output response by means of transcriptional messages (Vazquez-Vilar et *al.,* 2023). The ordinary

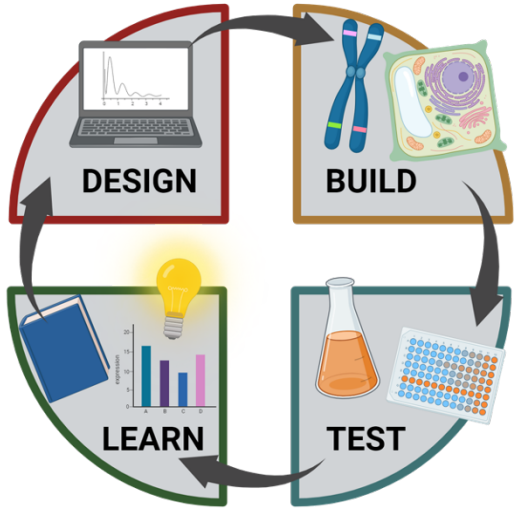


Figure 1. Engineering principles underlying Synthetic Biology. The cyclic process of design-build-test-learn (DBTL).

components of biological genetic circuits are transcriptional units formed by basic genetic parts such as promoters, coding sequences and terminators. Analogously to their electronic counterparts, biological circuits usually comprise defined combinable modules, named: *i*) sensors, involved in the detection of the non-transcriptional input and its conversion into a transcriptional signal, *ii*) processors, in charge of transforming (e.g. operating, amplifying, storing or multiplexing) one or more transcriptional inputs signals into different transcriptional outputs, and *iii*) actuators, which receive operated transcriptional inputs from processors and generate non-transcriptional outputs. Historic milestones place the first examples of implemented synthetic circuits in an *Escherichia coli* chassis and at the beginning of the XXI century, with the seminal works of the genetic toggle switch (Gardner et *al.,* 2000), and the repressilator (Elowitz & Leibler, 2000).

1.2 Orthogonality

A major challenge for SynBio is to obtain **orthogonal** synthetic gene circuits. Orthogonality refers to the absence of problematic interactions with other cellular components (Costello & Badran, 2021) and protects both the host organism, whose physiology should not be overly affected by synthetic genetic circuits, and the performance of the circuit itself, which should be protected from the oscillations in the cellular environment. Moreover, overexpressing endogenous genes could result in cell toxicity or even gene silencing. To avoid these effects, it is common to employ circuit elements using biological parts from evolutionary origin as distant as possible to the chassis organism, thus reducing chances of cross-talk with endogenous elements. Nevertheless, the precise control of any gene circuit is not a trivial task, and finely regulating the spatiotemporal gene expression in a living organism remains a fundamental challenge. Many efforts have been made to provide efficient tools to face these issues.

Synthetic promoters stand out as key orthogonal tools: they are DNA sequences artificially designed with the aim to ameliorate the functions of natural promoters,

taking advantage of existing regulatory sequences, and adapting them to biotechnological interests (Ali & Kim, 2019). To make this possible, the design and construction of synthetic promoters are necessarily based on a strong knowledge of how promoter sequences operate. Basically, synthetic promoters show a core-promoter region or minimal-region at their 3' terminal, and an upstream regulatory region (operator), at the 5' terminal. The minimal promoter region usually contains the TATA box, a consensus sequence recognized by transcription factors forming the preinitiation complex and responsible of RNA polymerase II recruitment. On the other hand, the 5' regulatory region is formed by multiple repeats of *cis* motifs acting as transcription factor binding sites. The number, position, order and orientation of these *cis*-regulatory elements can be designed and finely tuned in a combinatorial way, and they can recruit activators or repressors depending on the specific regulatory interests.

Modular cloning methods can yield a potentially endless repertoire of promoters that can be designed making use of interchangeable parts.

In addition to synthetic promoters, other tools can provide orthogonality, and they can be classified in relation to their contribution to the central dogma of biology, *i.e.*, genetic information storage and replication, transcriptional regulation, and translation. For example, in the first case, synthetic tools providing orthogonality are non-canonical and synthetic nucleotides, as well as synthetic epigenetic changes introduced in DNA bases. In this direction, (Park and collaborators (2019) introduced in eukaryotic cells N6-methyladenine (m6A, originally from prokaryotes) and coupled it with a system of synthetic read-write modules, driving tunable and orthogonal epigenetic modifications.

1.3 Plant Synthetic Biology

The field of SynBio first developed by engineering microbial chassis, a relatively easier task than the engineering of multicellular organisms, because processes can be more easily controlled (Zhang et *al.,* 2022, Clarke & Kitney, 2020, Liu et *al.,* 2020). Soon after

the first developments in microorganisms, plants started to attract the attention of synthetic biologists given their huge potential as chasis for s biomanufacturing. Approximately 80% of the biomass on Earth is from plant origin (Bar-On et *al.,* 2018). Plants have always been paramount sources of bioproducts to be used as food, construction materials, fibers, biofuels and therapeutic compounds. By applying SynBio tools, the plants biofactory potential can be further exploited, and the range of plant-derived products can be expanded.

SynBiologists has especially set its sights on plants for several reasons. First, the metabolic versatility offered by the Plant Kingdom is huge, and some metabolic pathways present in plants are absent in microorganisms. Second, plants are photoautotrophs, which translates industrially into a drastic reduction of their dependence on carbon sources other than CO_2. Plant cultivation can be scaled to reach considerable amounts of profitable biomass, but they can be also grown *in vitro*, which entails controlled conditions and the possibility of propagation of selected tissues. Plant biomass can also typically be exploited for different products at the same time, e.g. for high-value secondary metabolites, fiber, and energy. Finally, their culture and modification for SynBio purposes are mainly free of ethical issues when compared to animal systems, facilitating advances and applications.

Being able to engineer synthetic genetic devices and networks for plants will lead to plant cells displaying predictable and highly performative responses, with repercussions for the design of more sustainable solutions to some of humanity's challenges, such as the active mitigation of climate change, the development of manufacturing supply chains that are more environmentally friendly and enduring, or the ability to rapidly produce antibodies or vaccines in response to a pandemic outbreak (Clarke & Kitney, 2020; Vickers & Freemont, 2022). Still, plants are complex pluricellular organisms, for which genetic engineering can be harder than it is for bacteria or yeast, making it even more important to implement the iterative DBTL pipeline to develop effective genetic circuits.

1.4 Modular cloning standards in Plant SynBio

Most modular cloning systems employed in Plant SynBio make use of type IIS restriction enzymes to facilitate standardized DNA assembly. These are very efficient enzymes that cut at a determined distance from their nonpalindromic recognition site in the DNA sequence and generate a short overhang, whose identity is independent of the recognition site (Figure 2A). This feature can be exploited to design digestion sites so that customized complementary overhangs are created in different DNA parts (vectors or inserts) digested with the same enzyme, and these overhangs can then be enzymatically ligated. In addition, by conveniently designing enzymes recognition sites (scars) in the vector backbone and therefore outside the genetic parts, these sites seamlessly disappear when several genetic parts are assembled. Hence, entry and destination vectors can each be specified by the position of the recognition sites of different type IIS restriction enzymes relative to the insert. From these premises, several cloning strategies emerged, such as the assembly systems Golden Gate (Engler et *al.,* 2009), Golden Gate Modular Cloning (MoClo, Engler et *al.,* 2014), and the here-employed GoldenBraid (Sarrion-Perdigones et *al.,* 2011;Vazquez-Vilar et *al.,* 2017). For many of these initial cloning systems, an effort was made to unify their building blocks and the corresponding syntax into a single assembly standard referred to as "PhytoBricks" (Patron et *al.,* 2015). In practical terms, this means that, with few exceptions, building blocks created for e.g. MoClo can be exchanged with building blocks created by Goldenbraid and vice versa.

The GoldenBraid (GB) DNA assembly system employed in this thesis employs only two type IIS enzymes – *Bsa*I and *Bsm*BI. GB creates transcriptional units (TUs) by joining basic DNA parts, such as promoter + CDS + terminator in multipartite assemblies, and then enables the creation of multigene constructs through bipartite assemblies, in a characteristic double loop (braid) (Figure 2B). To be employed in this system, DNA parts need to be domesticated, which entails eliminating internal recognition sites for type IIS restriction enzymes and adapting the sequence to the PhytoBricks syntax by the addition of the appropriate overhangs. In GB, these DNA parts are assembled in a modular way in hierarchical destination vectors, designated as level 0, level 1 and

level >1. Level 0 comprises vectors containing basic parts such as promoters, CDS, protein domains or terminators, among others. These DNA sequences are cloned into a level 0 entry vector, called pUPD2. Then, basic parts from level 0 vectors are put together in a level 1 entry vector (called an α vector in GB), giving rise to level 1 elements, usually corresponding to complete TUs. From there on, by means of binary assemblies, level 1 elements can be assembled in level 2 vectors (called ω vectors in GB), which can be further combined to generate complex multigene constructs, alternating α- and ω-type destination vectors. The potentially unlimited nature of the system, with endless cycles of double loops and parts being completely exchangeable, offers a great advantage for SynBio. Moreover, to enhance the exchangeability of DNA parts and modules between laboratories and institutions, this and other modular cloning systems also promote the storage and accessibility of DNA parts in public repositories and databases, such as Addgene (https://www.addgene.org/) or the GoldenBraid website itself (https://goldenbraidpro.com/), where parts are described and can be easily shared.

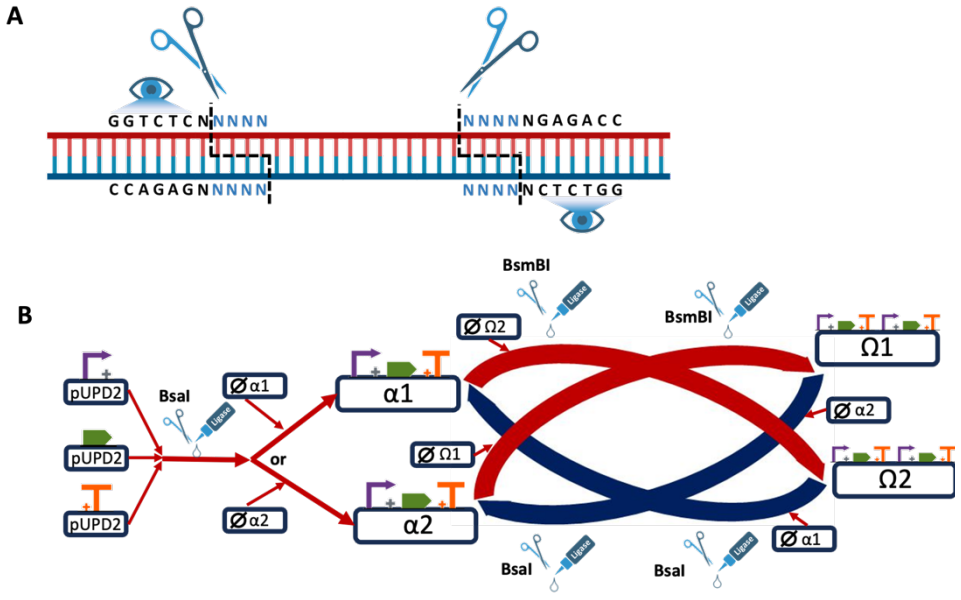


Figure 2 (previous page): Modular cloning tools. (A) Mode of action of type IIS restriction enzymes (REs). Type IIS REs recognize a specific and non-palindromic sequence in the DNA, cutting at a determined distance from the recognition site and generating short overhangs**. (B)** GoldenBraid general process generating infinite loops of constructs. Level 0 standard parts cloned in pUPD2 vectors, such as promoters, coding sequences and terminators, can be assembled through a multipartite assembly by the use of *Bsa*I and ligase into Transcriptional Units (TUs) of level 1 (α1 or α2 destination vectors). Following level > 1 elements can be obtained as a product of binary assemblies into Ω1 or Ω2 vectors by means of BsmBI. Then, alternating α- and ω-type vectors, further combinations can be employed to generate complex multigene constructs.

1.5 Sensors, processors and actuators in Plant Synthetic Biology

Modular cloning systems make it possible to create multi-element DNA constructs in plants, which, combined, generate synthetic gene circuits, aiming at the acquisition of new biological features. One illustrative milestone in plant synthetic circuits was the generation of sentinel *A. thaliana* plants that can detect trinitrotoluene (TNT) in the environment (Antunes et *al.,* 2011). This circuit was developed based on a bacterial sensor that detects TNT by chemotaxis, a processor for the transduction of the transcriptional signal, and an actuator producing a phenotypical response based on the degradation of chlorophyll.

As mentioned above, plant synthetic **sensors** are responsible for the detection of internal or external stimuli, which are non-transcriptional signals, and for their conversion into a transcriptional output. Sensors can be classified as chemical or physical by the nature of the signal they can detect (Vazquez-Vilar et *al.,* 2023). Chemical sensors perceive chemical signals, either external or internal. There is a wide variety of possible chemical signals, such as exogenous small molecules whose use is approved for agriculture (e.g., insecticides, copper or ethanol), and endogenous compounds such as phytohormones. A recent example of a chemical sensor is the obtention of a synthetic oxygen sensor that is based on an existing hypoxia signaling pathway in animals (Iacopino et *al.,* 2019). Conversely, physical sensors react to stimuli such as light and temperature. An interesting example is that of optogenetic

sensors based on phytochromes, which can detect light stimuli of a certain wavelength (Müller et *al.,* 2014).

After detection, **processors** convert a transcriptional input into another transcriptional output of different nature either amplifying, distributing, or storing the signal. Transcription factors (TFs), either natural or synthetic, other programmable transcriptional regulators, such as transcription activator-like effectors (TALEs), artificial zinc-fingers proteins (ZFPs), an inactivated ('dead') version of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) CRISPR/Cas systems and synthetic promoters (see §1.2.4 , Kar et *al.,* 2022) can be used as processor elements in genetic circuits (Li et *al.,* 2012; Osakabe et *al.,* 2010).

Finally, **actuators** transform transcriptional inputs from a connected processor into a non-transcriptional output, including for instance fluorescence reporter signals, recombinant proteins, metabolites, or other type of physiological responses (Osakabe et *al.,* 2010). Several actuators have been engineered, most of them belonging to the reporter gene category. Reporter genes are most useful to test the correct functioning of a complete circuit, ensuring that an output is produced only when the circuit is properly connected from beginning to end. Moreover, they usually return easily measurable outputs, as is the case for fluorescent proteins such as the green fluorescent protein (GFP, Stewart Jr, 2001), the luciferase/renilla reporter (Vazquez-Vilar et *al.,* 2017) or a recently described system based on a fungal autoluminiscence pathway, which can notably be used even to monitor circuit behavior *in vivo* in real time (Calvache et *al.,* 2023).

Once validated with reporters, synthetic gene circuits can be engineered to promote the expression of the enzymes in a metabolic pathway as actuators, placing them at the end of the signal transduction cascade. This can be done with either endogenous metabolic pathways, or with heterologous pathways introduced in the chassis organism as part of the circuit. An example of the first case is the increase in α-tocopherol by targeting an endogenous locus by means of a ZF-based system in *Arabidopsis thaliana* (Van Eenennaam et *al.,* 2004). On the other hand, examples of heterologous metabolic pathways as actuators in the synthetic gene circuit are the

production of heterologous therapeutic proteins in the moss *Physcomitrella patens* thanks to a toggle switch-like system based on red light activation (Müller et *al.,* 2014), or the chemically-induced production of the polyhydroxybutyrate polymer in *Arabidopsis thaliana* (Kourtz et *al.,* 2007). These applications highlight the overlap between the fields of synthetic biology and metabolic engineering, with the first providing tools to enrich and develop the second.

2. Metabolic engineering

Metabolic engineering (ME) is a field that was born with strong links to Synthetic Biology, as well as to genetic engineering, but sets itself apart from the latter due to its distinct emphasis. This approach is thought to have been born in the 1990s (Stephanopoulos & Vallino, 1991; Bailey, 1991). While the strategy followed by genetic engineering is mainly focused on introducing or modifying the activity of an individual gene or a single gene product using recombinant DNA technology, metabolic engineering comprises a more holistic dimension. Metabolic engineering aims to improve the production of a desired product or the development of certain cellular characteristics by studying jointly integrated metabolic pathways and their corresponding genetic regulatory networks (Woolston et *al.,* 2013). By aiming at maximizing yields, it conceives of bioproduction in an industrially oriented manner, aiming to reduce manufacturing costs, or at least make the use of metabolically engineered organisms as viable as other industrial methods.

Being linked and drawing inspiration from SynBio, metabolic engineering shares its basic principles of pathway design, construction, and optimization. Pathway design starts with the elucidation of which enzymes are necessary for the biosynthesis of the target compounds from the precursors already present in the host cells. Also, other factors to be considered in ME are the use of additional genes, which might increase production levels or ensure lower toxicity of the metabolic intermediates, and the capability of the heterologous enzymes to be properly expressed in the host organism (Woolston et *al.,* 2013). Unraveling which genes are responsible for every metabolic

step is crucial: metabolic engineers rely mainly on genomics and transcriptomics data, analyzed by means of bioinformatic tools, to identify candidate sequences, based on their homology with sequences of known function. An important issue concerning ME is the understanding of metabolic fluxes within the organism to be engineered. These fluxes include metabolites, but also the carbon, electron, and energy flows. It is of great importance to understand and quantify metabolic fluxes in order to detect possible bottlenecks in the metabolic pathway, that is to say the key steps that can limit the optimal performance of an entire system.

2.1 Analysis of metabolic fluxes

The methods for the study and quantification of metabolic fluxes *in vivo* revolve around metabolic network models, mathematically represented by a stoichiometry matrix (S) that describes the chemical reactions and transport processes within cells. Advances in genome sequencing and annotation have led to the creation of metabolic network models for numerous organisms. Assuming a pseudo-steady state for intracellular metabolites, one in which their levels remain relatively constant over time, constraints (affecting the flow of carbon, energy and electrons) are set on the metabolism within the network models. The main idea is that the sum of all fluxes producing a metabolite must equal the sum of all fluxes consuming it, mathematically expressed as $S \times v = 0$. There are three main approaches developed in the course of the XXI century to analyze and model metabolic fluxes: these are known as flux balance analysis (FBA), metabolic flux analysis (MFA) and ^{13}C-metabolic flux analysis (^{13}C-MFA). The first approach, the flux balance analysis (**FBA**), is primarily focused on exploring the implications of these stoichiometric constraints on the capabilities of biological systems. The FBA approach is based on the optimal performance of the cell or metabolic system. Therefore, it assumes that only the maximum nutrient uptake rate will limit the maximum growth rate. Conversely, the second approach for the study of metabolic flux, namely metabolic flux analysis (**MFA**), lies on the hypothesis of non-optimal performance of the system; thus, in a more realistic and industry-oriented way, it allows for example the quantification of fluxes in different situations,

such as nutrient deprivation. It also considers the flux of several cofactors, like ATP, NADH and NADPH, adding more information about electron and energy flux to the estimation. However, this approach still implies an estimation of real fluxes taking place in organisms. To get a more precise understanding of flux quantification, the ^{13}C-metabolic flux analysis (**^{13}C-MFA**) can be used. It is based on the labeling of a substrate with the stable isotopes of carbon, Carbon-13 (^{13}C), and on the tracking of their itinerary inside the cell/organism. Isotopes are then quantified in several metabolites by use of mass spectrometry or nuclear magnetic resonance.

2.2 From microbial to plant metabolic engineering

The beginnings of ME historically lie in the efforts to redesign bacterial metabolism. Initially, this meant introducing a few novel enzymes in well-known metabolic pathways, but the complexity of ME soon increased to its current levels, in which any target molecule whose biosynthetic pathway is known can in principle be produced in engineered microorganisms. During the first decades of ME, *Escherichia coli*, as the prokaryotic model, and *Saccharomyces cerevisiae*, as a eukaryotic chassis, have been the object of most of the studies and applications of ME (Pontrelli et *al.,* 2018; Yang et *al.,* 2020; Nielsen et *al.,* 2013; Rahmat & Kang, 2020). This is due mainly to their ease of growth in both laboratory and industrial conditions, to the wealth of information regarding their genetics, metabolism and biology, fostered by the early public availability of genomic sequence data, and to the development of several genetic manipulation and engineering techniques (Duan et *al.,* 2010; Court et *al.,* 2002; Blattner et *al.,* 1997). Soon after developing unicellular prokaryotic and eukaryotic biofactories, metabolic engineers started to turn their attention to pluricellular organisms, which can provide novel metabolic properties, allow for the scale-up of the production of the desired compounds or even offer a more profitable secondary use of the generated biomass after metabolite extraction (e.g., biofuels or fodder). Bacterial or yeast cultures chassis showed some limitations, which more complex organisms could help overcome; these include post-translational

modifications, toxicity concerns arising from metabolite accumulation or low production rates in these microbial biofactories. In this respect, plants possess a set of interesting features: they can successfully be transformed, are metabolically both robust and versatile (possessing a wide variety of specialized secondary metabolic pathways) and, importantly for industrial sustainability, they are autotrophs (Yuan & Grotewold, 2015). Once the possibility of stable genetic transformation of plants via *Agrobacterium tumefaciens* was demonstrated with the stable transfer of the *neo* gene in tobacco (Bevan & Flavell, 1983), the foundations were laid for the further exploration of ME in the Plant Kingdom. After tobacco, more plant species were soon established as chassis for ME, and chosen according to their metabolic features (e.g., production of primary and secondary metabolites, biomass accumulation; Stewart et *al.,* 2018). As for metabolic versatility, most pathways implicated in the biosynthesis of desired products are typically already present in plants, which can provide good amounts of precursors that can be harnessed by increasing the metabolic flux or incorporating new branches to the existing pathways (X. Zhu et *al.,* 2021). It is estimated that the Plant Kingdom contains hundreds of thousands, or even millions of metabolites. However, even with the aid of recent omics technologies (such as genomics, transcriptomics or metabolomics), only a few thousand valuable plant metabolites have been identified, and their metabolic pathways deciphered (Selma et *al.,* 2023). Plant metabolic engineering can thus be defined as the discipline that aims at regulating plant endogenous metabolic pathways, or even introducing new ones, to change the plant metabolic profile (Farré et *al.,* 2014).

2.3 Objectives and applications of plant metabolic engineering

Most plant ME is focused on valuing **plant natural products (PNPs)** or phytochemicals (S. Wu & Chappell, 2008), seeking to produce greater amounts of specific PNPs. This wide range of compounds is of great interest for their value in the pharmaceutical, bioenergetic, plant protection or food industries. PNPs are classified as primary and

secondary metabolites. Primary metabolites are the product of the primary metabolism: they include sugars, fatty acids, amino acids and nucleic acids shared by all living organisms, and also plant-specific molecules such as phytohormones, cell wall components and molecules involved in the photosynthetic process (Maeda, 2019). Conversely, secondary metabolites are low molecular weight compounds comprising a wide range of specialized molecules, not essential for plant growth, development, or reproduction, but which confer an evolutionary benefit by participating in plant defense and in other interactions with the environment (Delgoda & Murray, 2017). Plant secondary metabolites are broadly classified as terpenoids, phenolic compounds, glycosides and alkaloids.

The production of high levels of a certain PNP can be attempted in its natural host or in a closely related organism (e.g., the overproduction of intrinsic tropane alkaloids in belladonna by overexpression of its responsible gene (Zhao et *al.,* 2020)), or in a different chassis with specific technological advantages (e.g., the production of sclareol, a valuable diterpenoid precursor in the fragrance industry, integrated from a *Salvia sclarea* gene into *P. patens* by Pan and collaborators (2015)(X. W. Pan et *al.,* 2015). To optimize the production of PNPs in their natural hosts, a common straightforward ME strategy is the **overexpression of an ortholog gene** encoding an enzyme catalyzing one of the steps in the metabolic pathway. The activity of ortholog genes may differ based on enzymatic efficiency, or the formation of different protein-protein complexes, which can also lead to an alternative product ratio in biosynthesis. Additionally, the use of ortholog genes from other organisms contribute to orthogonality. Sometimes, genes encoding enzymes for a certain metabolic step are found in both plant and microbial sources, so the available repertoire is even more extended. Several successful cases illustrate this approach, e.g. the overaccumulation of β-carotene and lutein by expressing the bacterial *crtB* gene in potato tubers (Ducreux et *al.,* 2005).

An alternative ME approach to regulate endogenous PNPs consists in overexpressing ortholog **transcription factors (TF)**. Transcription factors are key elements in the complex metabolic network since they orchestrate gene regulation at the

transcriptional level: they are proteins that attach to specific DNA sequences in gene promoters through a DNA-binding domain, and usually recruit the transcriptional machinery that, through an effector domain, activates or represses transcription of that genomic region. Several main plant metabolic pathways have been engineered by this strategy, such as the flavonoid, carotenoid, alkaloid and phenylpropanoid pathways (C. H. Park et *al.,* 2021; Yamada & Sato, 2021; Qiu et *al.,* 2013; (Appelhagen et *al.,* 2018).

When producing a PNP in **a plant which is not the natural host**, it is mandatory to recombinantly impose a diversion in the flux with additional enzymatic steps, by transferring one or multiple transgenes encoding enzymes catalyzing these novel metabolic steps. In this regard, one notable example is the interesting work leading to the *de novo* production of taxadiene, a natural precursor of the anticancer molecule paclitaxel or Taxol®, in *Nicotiana benthamiana*, by introducing the taxadiene synthase gene and thus shunting the terpenoid pathway towards the heterologous metabolite (Hasan et *al.,* 2014). In other cases, plant metabolic engineering is directed at producing a non-endogenous metabolite from a highly phylogenetically distant organism. This is the case, for example, of using plants as biofactories to produce therapeutic molecules of non-plant origin such anti-microbial or anti-cancer drugs, or valuable molecules for pest control (Diego-Martin et *al.,* 2020; Shoji et *al.,* 2012; Orzáez et *al.,* 2009). As for the synthesis of molecules for insect pest management, the heterologous expression of Cry proteins from *Bacillus thuringiensis* (Bt) gave rise to insect-resistant plants, being the first case in a crop the 'NewLeaf' potatoes in 1995, a historic milestone that originated an upcoming agronomic revolution (Sanchis, 2011; Ahmed et *al.,* 2019). Since then, the global area of Bt crops, in increasing number of agronomic varieties and different Cry proteins, has rapidly expanded, counting now with around 100 million cultivated hectares (ISAAA, 2021)

In other cases, a totally '**new-to-nature'** metabolite can be obtained as a product of a combinatorial biosynthetic approach to metabolic engineering (Arendt et *al.,* 2016). These are compounds that have not yet been described in the natural world. They are often structurally similar to other known metabolites and can be obtained by

combining genes involved in close metabolic pathways from different hosts, as well as by employing enzymes showing a relaxed specificity towards their natural substrate combined with a different set of precursors. A brilliant example demonstrating the potential of a combinatorial approach for new-to-nature metabolite biosynthesis is the study developed by Andersen-Ranberg and collaborators (2016). In this combinatorial approach, paired diterpene synthases from different plant organisms were expressed by transient expression in *N. benthamiana*, originating 41 novel metabolites which, in addition to being potentially profitable in themselves, extend the repertoire of scaffolds for further enzymatic modifications for the biosynthesis of molecules of interest.

Finally, another interesting application of ME to PNP metabolism regards the **suppression of the production of a particular compound** or class of compounds, because of their toxicity, or with the aim to redirect the upstream pathway towards alternative, more profitable products. To do so, metabolic engineers' benefit, among other strategies, from RNA interference (RNAi) technology, which acts by silencing or downregulating one or a few target genes at the post-transcriptional level. A representative example of this application is the decrease in the accumulation of gossypol in upland cotton by Rathore and collaborators (2012). This toxic metabolite was not desirable in this crop, and its reduction would make cottonseeds a safer source of proteins for human nutrition or animal feeding. So, levels of gossypol were reduced by RNAi-mediated downregulation of gene coding for the δ-cadinene synthase (*GhCAD1*). Another relevant strategy ME benefits from is the CRISPR/Cas9 technology, used to precisely knockout target sequences (see §2.4). Following this approach, Sánchez-León et *al.,* 2018) obtained low-gluten wheat lines by editing conserved domains throughout α-gliadin genes, and (Li and collaborators (2018) targeted a gene related to ethylene production in tomato, thus generating tomato fruits showing delayed ripening.

2.4 CRISPR-based programmable tools for plant metabolic engineering

Biotechnologists have nowadays at their disposal a few customizable tools that can be easily programmed to target genomic sites in a highly accurate manner, either to mutagenize, edit or induce transcriptional perturbations in the designated target sites. In the case of **genome editing**, the best known is the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated (Cas) system (Doudna & Charpentier, 2014; Belhaj et *al.,* 2015). Briefly, this system, originally derived from a defense system in prokaryotes, is composed of a nuclease (Cas) and two non-coding RNA sequences forming the duplex tracrRNA:crRNA. After processing, while the tracrRNA is recognized by the Cas, the mature crRNA possesses a specific sequence that guides the associated Cas protein to its complementary region in the genome, where the nuclease will act by producing a DNA double-strand break (DSB). This duplex tracrRNA:crRNA can be engineered as a single guide RNA (sgRNA), as well as being customized to match a given target sequence in the genome of the host, guiding the Cas protein complex to act there. Taking advantage of DNA repair systems, such as the error-prone nonhomologous end-joining (NHEJ) or the homology-directed repair (HDR), CRISPR/Cas allows to finely mutagenize and/or edit the target sequence. Small insertions or deletions (indels) leading to a loss-of-function are the most common mutations introduced in target genes, but larger indels or substitutions are also possible (Karlson et *al.,* 2021). This revolutionary tool has enormously expanded the limits of plant metabolic engineering (Sabzehzari et *al.,* 2020). Additionally, the system can be exploited to target and mutagenize more than one gene by multiplexing several gRNAs in the same construct or transformation. An illustrative example of this was the work of R. Li, Li and collaborators (2018), where several tomato genes were edited by a multiplexed CRISPR/Cas9 approach, and hence accumulated 19 times more γ-aminobutyric acid (GABA) than WT plants. Similarly, other programmable tools such as Zinc-fingers (ZFs) and transcription activator-like effectors (TALEs) have been employed for metabolic engineering purposes, precisely editing plant genomes (Clasen et *al.,* 2016; Shukla et *al.,* 2009)

Regarding **transcriptional regulation**, an adaptation of the CRISPR system has equally demonstrated to be very useful. Here, metabolic engineers make use of a 'dead' endonuclease, like dCas9, which retains the ability to target the DNA region specified by the gRNA, but whose endonuclease activity is abolished thanks to two-point mutations in its sequence. The gRNA guides the ribonucleoprotein complex at the promoter sequences of the target gene, whose transcriptional regulation (either activation or repression) is achieved by the dCas protein and the transcriptional regulation domains fused to it (Z. Li et *al.,* 2019). Moreover, the system can be further engineered by protein-fusion strategies or modified gRNA scaffolds to allow the anchoring of different activator domains, achieving an amplified activation or repression response (Kunii et *al.,* 2018; Selma et *al.,* 2019). Making use of this approach, Selma and collaborators (2022) were able to selectively enrich the flavonoid content of *N. benthamiana* leaves, targeting the promoter sequences of genes implicated in their biosynthesis. Also, dCas-based systems can be strategically used in combination with synthetic promoters. Briefly, synthetic promoters can be custom-designed to display sequences recognized and transactivated by the dCas complex, in order to get orthogonal and tailored gene expression. This topic will be further addressed in Chapter 1 (see also Figure 2 in Chapter 1 for further illustration).

2.5 Strategies to improve recombinant protein expression and function in plant hosts

Especially when designing the production of heterologous proteins in evolutionary distant host organisms (for example, human antibodies in plants), **codon optimization** is an important aspect to consider. The genetic code is degenerate, meaning that each amino acid (except for methionine and tryptophan) can be encoded by more than one synonymous codon (Gustafsson et *al.,* 2004). Codon usage is the tendency of each organism to preferentially use a specific synonymous codon during translation. While this bias had long been thought to be of little functional importance, in the last three decades codon usage has been correlated with non-negligible effects in the

expression levels of heterologous proteins, and thus, with the success of metabolic engineering in heterologous hosts (Webster et *al.,* 2017). Adapting the nucleotide sequence of heterologous genes to the codon usage of the chosen biofactory is usually accomplished by different codon optimization algorithms, such as Codon Optimization OnLine (COOL), EuGene, DNA-Tailor (D-Tailor), or the algorithms provided by the DNA synthesis companies. These improvements are reported to increase production 2- to 3-fold (X. Q. Li et *al.,* 2007; Thomas & Walmsley, 2014), but there is no clear consensus and codon optimization is sometimes ineffective. So, the effect of codon optimization should be identified with trial and error and evaluated case by case.

Post-translational modifications represent another level of potential intervention in ME. They are covalent modifications, such as the addition or removal of functional groups, glycosylation or phosphorylation, involving specific surface amino acids in a protein (Kwon et *al.,* 2006; Montero-Morales & Steinkellner, 2018). These modifications lead to important changes in protein structure, sometimes translating to function, interactivity with other proteins, stability, etc. The ability to perform post-translational modifications and the patterns of glycosylation are specific. Even if the patterns of plant glycosylation are not very dissimilar from those of mammals, plants use several different sugar residues, which may potentially be allergenic or antigenic. Engineering post-translational modifications in plant hosts can significantly improve product outcome and provide an advantage in therapeutic applications. Glycosylation in plants has been engineered to reproduce the human glycosylation pattern in therapeutic glucocerebrosidase heterologously produced in carrot cell cultures (Shaaltiel et *al.,* 2007), or to produce humanized N-glycosylated antibodies (Diamos et *al.,* 2020). In this direction, the RNA interference technology was used to generate stable *N. benthamiana* plants showing downregulated xylosyltransferase and fucosyltransferase genes, thus not decorating antibodies with potentially immunogenic plant β 1,2-xylose and core α 1,3-fucose residues (Strasser et *al.,* 2008). These RNAi lines have proved to be an ideal chassis for producing different 'humanized' antibodies, such as monoclonal antibodies against the dengue virus (Dent et *al.,* 2016), the ZMapp antibody cocktail against the Ebola virus (Olinger et *al.,*

2012), and even anti-SARS-CoV-2 antibodies (Diego-Martin et *al.,* 2020). Finally, post-translational modifications can be of crucial importance in metabolic engineering for improving carbon assimilation metabolism, for example, by promoting novel plant growth programs or reinforcing stress tolerance (Balparda et *al.,* 2023).

Subcellular compartmentalization is another key subject to consider in plant ME. Organelles are interesting not only because they can store high amounts of products, but also because the presence of a membrane makes the exchange of intermediates more controlled. Since different subcellular compartments can be involved throughout the biosynthesis of a desired metabolite, several approaches can be adopted to optimize ME in plants (Heinig et *al.,* 2013). Relocating enzymes to different organelles is sometimes a good strategy to improve production, accommodating enzyme activity by taking advantage of naturally available precursor pools, specific pH or redox potential (De La Peña & Sattely, 2021). The most common way to engineer enzyme subcellular localization is by using N-terminal sequences that target the enzyme towards the desired organelles, such as chloroplasts, vacuoles, mitochondria, endoplasmic reticulum, or peroxisomes (Zybailov et *al.,* 2008; Nakamura & Matsuoka, 1993; Heazlewood et *al.,* 2004; Bonifacino et *al.,* 1990; Roggenkamp, 1992). Alternatively, substrate pools could be engineered to increase the availability of precursors in a particular subcellular compartment by overexpression of certain enzymes, which also can lead to enhancing a desired metabolic pathway (Botella-Pavía et *al.,* 2004). In other cases, it is interesting to promote or reduce the transport of metabolic precursors or intermediates occurring between two organelles, to avoid a possible bottleneck (Lynch et *al.,* 2017).

Compartmentalization of metabolites can take place not only at subcellular levels, but also in **specialized plant tissues and organs**. From a ME perspective, structures such as glandular trichomes are of particular interest due to their innate capacity for specific metabolite biosynthesis, accumulation, and secretion (Huchelmann et *al.,* 2017). However, important challenges should be addressed for proper trichome engineering, including trichome-specific promoters controlling the expression of transgenes to avoid pleiotropic effects, and the expression of transporters specialized

in carrying metabolites towards and within these structures (Tissier et *al.,* 2017; Crouzet et *al.,* 2013). Although trichome engineering could offer promising solutions to actual ME challenges, such as tissue toxicity deriving from generalized overexpression of certain metabolites, glandular trichome development needs to be further studied in many plant species.

2.6 Stable transformation of nuclear and organellar genomes

Transforming or editing the **nuclear genome** is the most common strategy in plant ME because of the comparative easiness of the process and the multiple options available to perform it. **Stable transformation** requires the stable and permanent insertion of the transgenes in the plant genome, allowing them to be transferred to the offspring. This represents a very interesting advantage in terms of scalability since, after a proper selection of transformants, a transgenic population producing good levels of the selected metabolite(s) can be obtained (Stephenson et *al.,* 2020). The success of a stable strategy depends on methodological and cellular requirements. At the methodological level, the most important factors are morphogenesis and delivery of the transgenes. Morphogenesis is addressed by choosing the most suitable method for regeneration, including different methods of organogenesis or embryogenesis. As for gene transfer, various methods are available, including *Agrobacterium tumefaciens*-mediated transformation, biolistic methods, and microinjection. At the cellular level, requirements include the competence for regeneration in the tissues of the chosen chassis, as well as competence for transgene integration. Accordingly, their main disadvantages are linked to time-consuming protocols needed for plant regeneration, which often need specific adjustments achieved with trial and error. The availability of efficient methods of DNA assembly for the construction of the transgenic pathway has also been a technical barrier for many years, especially as constructs have begun to consist of increasingly larger fragments (even more so, with the increasing use of CRISPR/Cas and multi-element regulatory systems). Fortunately,

synthetic biology has facilitated the assembly of complex multigene constructs, allowing to largely overcome such barriers (Patron et *al.,* 2015; Vazquez-Vilar et *al.,* 2016). To achieve the permanent integration of the engineered metabolic pathway, several strategies can be followed. The most common and straightforward approach is **multiple gene co-transformation**, in which the genes required for the construction of the desired metabolic pathway are all stacked in the same T-DNA inside a plasmid. This strategy is limited by the number of genes that can be assembled in the same construct. To face this issue, several approaches can be adopted, such as several successive plant transformations (re-transformation), co-transformation with multiple gene constructs, or even sexual crosses of plants carrying different desired genotypes to be combined (Eckert et *al.,* 2006). However, all these strategies (when they imply the transfer of more than one T-DNA), show several non-negligible handicaps. First, there is a need to employ different selection markers, one for each 'plant module'. Moreover, these approaches will demand considerable time, especially in successive plant transformations. In addition, the co-transformation approach with two or more constructs carried in the same *Agrobacterium* strain will require plasmids with different and compatible origins of replication (Pasin et *al.,* 2017). Conversely, **combinatorial transformations** address the construction of the desired metabolic pathway in plants by splitting each gene into a different vector; the various vectors are then transferred to the plant, usually by a biolistic strategy (Bock, 2013). Biolistics normally force the transfer of multiple copies of the DNA constructs in the genome, and hence favor a high frequency of co-integration in the same locus of all the transgenes involved in the transformation, although in different vectors (Altpeter et *al.,* 2005). In this way, a library of plant transformants is generated, each showing a different combination of transgenes (several or all of them) in a wide variety of copy numbers, thus giving rise to different chemotypes and phenotypes. Afterward, a screening is needed to select the best performing transformants. This approach was followed by C. Zhu and collaborators (2008), who thus engineered maize to generate a library of transformants with high carotenoid levels.

Interestingly, metabolic engineering in plants can be performed not only in the nucleus but also in plastid genomes. **Plastid engineering** – giving rise to

transplastomic plants – implies plastid transformation, which is mainly achieved by either biolistic methods or by means of polyethylene glycol (PEG), but other alternative procedures have been described, such as carbon nanotube carriers or by UV-laser microbeams (A. U. Kumar & Ling, 2021). Plastid engineering stands out for several advantages (Hossain & Bakhsh, 2020). First, chloroplasts originated from cyanobacteria, of which they retain several interesting characteristics, such as having genes arranged in operons, whose transcripts are processed in a polycistronic way, avoiding gene silencing. They can also host large amounts of exogenous DNA, which could be potentially regulated by synthetic operons. Second, due to the importance of chloroplasts in primary and secondary metabolism, deriving chiefly from their hosting photosynthesis, there are notable pools of precursor and intermediate metabolites, which can be harnessed for chloroplast engineering to incorporate new transgenic metabolic pathways or, as mentioned before, by N-terminal signals relocating enzymes to these organelles. Finally, since every cell hosts a high number of chloroplasts, transforming the plastidial genome allows to have a greater number of transgenic DNA copies in a single cell. A very recent example illustrating this approach is the work of Tanwar and collaborators (2023) , where tobacco chloroplasts were engineered to enhance the carotenoid pathway by regulating the three heterologous genes under the control of a synthetic operon. Moreover, by choosing plastid engineering, ecological safety issues derived from pollen transmission of the transgenes are overcome, because of the maternal inheritance of these organelles (Bock, 2022).

In order not to saturate one system, and mimicking natural metabolic pathways in which different enzymes are localized in different cellular compartments, the use of the **combined engineering of chloroplast and nuclear genomes** seems a promising strategy. Examples of this approach are the efforts for engineering the biosynthesis of artemisinin - a therapeutic compound for malaria treatment - in a tobacco chassis (Fuentes et *al.,* 2016; Malhotra et *al.,* 2016).

2.7 Transient expression systems

Transient expression shares the same goal of testing and improving a metabolic pathway or incorporating a new one in the chassis organism to produce valuable proteins and metabolites. Transient expression bypasses the time-consuming generation of new plants required for stable transformation. In plants, it is generally obtained by infiltration with recombinant *A. tumefaciens* carrying the desired vector(s) with the genes encoding the custom metabolic pathway. This method was first described by Kapila et *al.,* (1997), and then widely expanded. Here, the T-DNA is delivered to plant cells in multiple copies and leads to high expression levels (Brückner & Tissier, 2013). Besides manual agroinfiltration by syringe, which is only affordable on a small scale (usually in the initial steps of testing a pathway), vacuum infiltration allows higher efficiency and commercial scalability, providing better yields and low-cost production (Simmons et *al.,* 2009). Nowadays, the process has been greatly optimized and scaled up in some companies, such as Leaf Expression Systems (Norwich, UK), where *N. benthamiana* is thus used as a platform to produce a wide variety of recombinant proteins. In basic research contexts, manual agroinfiltration is routinely used to test and screen gene activities and expression conditions. The most used vectors for *Agrobacterium* transient expression are the binary vectors, together with the use of disarmed *Agrobacterium* strains, all of which are an adaptation of the natural *Agrobacterium* Ti plasmids to enhance the transfer of T-DNA to the plant cells. Even though multiple options have emerged over the years, most of them are based on pCambia vectors (www.cambia.org). Alternatively, the vectors carried by *Agrobacterium* and designed to deliver their T-DNA to plant cells and trigger metabolite production can be **virus-based vectors**. Several viruses are used for this purpose, most notably *Potato virus X* (PVX) and *Tobacco mosaic virus* (TMV) (Plchová et *al.,* 2022). These viral vectors are built with wild type viral genomes where additional sequence(s) encoding the product-of-interest is inserted. Once delivered in plant cells, recombinant viral genomes replicate and express their own viral ORFs, together with the ORF encoding the inserted product-of-interest. Because these viral genomes are usually small, they represent an easy platform to engineer. A good

example, among many others, is the engineering of carotenoid production in *N. benthamiana* leaves by Majer and collaborators (2017). Here, a vector based on the Potyvirus *Tobacco etch virus* (TEV) was used to transiently express genes of the heterologous lycopene metabolic pathway, leading to its biosynthesis and accumulation, up to 10% of total carotenoid content. A further step in the use of viral vectors in metabolic engineering strategies is virus-induced genome editing (VIGE), where viral vectors are employed for delivering guide RNAs in stable transgenic plants hosting Cas proteins. This approach has been successfully adapted for boosting endogenous gene activation of the *N. benthamiana* phenylpropanoid pathway, by delivering several gRNAs targeting of different endogenous MYB transcription factors in dCasEV chassis plants (Selma et *al.,* 2022).

2.8 Plant cell cultures

Parallel to the choice of which plant genetic compartment to engineer, it is necessary to choose the most appropriate production system, which can be either a whole plant (with an *in vivo* approach) or a plant cell culture (with an *in vitro* approach) (Sangwan et *al.,* 2018; Selma et *al.,* 2023). The great majority of plant ME efforts have been focused on production in whole plants, relying on a complete network of tissues, specialized organs, and metabolic pathways. In these contexts, only one or a few target step(s) need to be fine-tuned for the biosynthesis of the metabolite or recombinant protein of interest. By contrast, **plant cell suspension cultures (PCSC)** usually consist of undifferentiated plant cells or callus, or even of cambial meristem cells cultured in a liquid medium supplemented with macro- and micro-nutrients under controlled conditions in bioreactors. PCSCs represent an interesting platform for producing both plant metabolites and recombinant proteins (Arya et *al.,* 2020) and show several advantages compared to production in whole plants. They stand out for their biosafety, thus bypassing regulatory issues that affect transgenic plants, and even reaching production under Good Manufacturing Practices (GMP), as well as for their scalability, which is analogous to microbial cell cultures. After a first assessment

at the laboratory scale, the production of the desired metabolite or protein is scaled up to pilot and later industrial-scale bioreactors, which usually allows for lower costs of production. Several therapeutic molecules have been successfully produced by metabolically engineered PCSC, such as resveratrol (known for its potential in fighting cardiovascular diseases and cancer), artemisinin, taliglucerase alfa (a therapeutic enzyme for Gaucher's disease treatment), and Taxol® (for anticancer treatments), among others, in strategies implying either single gene transfer or multigene engineering (Baldi & Dixit, 2008; Chu et *al.,* 2017; Fox, 2012; Lenka et *al.,* 2015). The main disadvantages of PCSCs relate to the need to provide a carbon source and, in some cases, metabolic precursors. Another caveat of PCSCs is the genetic instability derived from mutations accumulated in dividing cells, especially in the case of undifferentiated plant cells.

2.9 Choice and improvement of chassis organisms for plant metabolic engineering

The suitability of a plant species to become a platform for ME depends on several factors. Some important ones are the level of knowledge about its metabolic networks, as well as the availability of protocols for genetic transformation (Hawkins et *al.,* 2021). A deep genetic, developmental, and metabolic knowledge has been built over decades on the model species *Arabidopsis thaliana* (Cao et *al.,* 2011; D'Auria & Gershenzon, 2005); however, this species also has various disadvantages with regards to ME: it is not a crop, it does not accumulate a great biomass, and thus, despite the many available technical resources, it does not represent a suitable biofactory of high-value metabolites. Conversely, other plants, which are edible, industrial or minority crops, would be better suited. For example, tomato, an edible crop cultivated worldwide, has been a target for multiple ME approaches. This was favored by its role as model species for berry development and, thus, by the availability of genetic and metabolic resources, as well as a large body of protocols and methods. Several ME strategies are aimed at improving the nutritional properties of the tomato fruit. This

is the case, for example, of the recent approach followed by Y. Wu and collaborators (2022), in which several endogenous synthetic genes of tomato were introduced in this crop by the control of fruit-specific promoters. On the other hand, ME in tomato can be directed to accumulate therapeutic molecules. For instance, the enrichment of tomato fruit in L-3,4-dihydroxyphenylalanine (L-DOPA), an amino acid used as a drug in the therapy against Parkinson's Disease, was obtained after the expression of a beetroot gene related to the betalain biosynthetic pathway (Breitel et *al.,* 2021). However, the use of crops for ME and as plant biofactories is partially discouraged by the fact that their farming for non-food or -feed purposes would endanger the food chain, considerably affecting their market value (Molina-Hidalgo et *al.,* 2021). The *Nicotiana* genus represents a valuable alternative, being a non-food and non-feed crop with high metabolic versatility and capacity of high-yield cultivation. As in the case of *Arabidopsis*, *Nicotiana* spp. have attracted the attention of plant researchers for nearly a century. The genus counts with more than 75 species, the most notable of which are *Nicotiana benthamiana* Domin (Australian dwarf tobacco) and *Nicotiana tabacum* L. (cultivated tobacco). These two species have been deeply characterized for genetic manipulation, since they have been the model to develop a multitude of techniques and protocols for *in vitro* tissue culture, genetic transformation – both nuclear and chloroplastic –, transient expression, viral infection, and graft-mediated gene and genome transfer, among others. Moreover, high-quality genome assemblies, as well as associated transcriptomic, epigenomic and single nucleotide polymorphism (SNPs) data have been recently published for some *Nicotiana* accessions, which is a hugely profitable resource to develop metabolic engineering approaches (Ranawaka et *al.,* 2023). In terms of metabolism, several recent studies characterizing the metabolic profiles of leaves across plant and leaf development stages have shed light on the metabolomes of *N. benthamiana* and *N. tabacum*, with their implications for metabolic capacity as biofactories of specific classes of compounds (Drapal et *al.,* 2021; L. Zhang et *al.,* 2018). For *Nicotiana benthamiana,* five wild ecotypes distributed across Australia are known, but most *N. benthamiana*-based techniques have been developed in the laboratory (LAB) strain. This is characterized by a mutation in the *Rdr1* gene, which deprives it of major defense

capacities against viral infections, therefore facilitating the transient expression of exogenous DNA, an especially attractive feature for various research applications (Bally et *al.,* 2015). In addition, leaf structure strongly facilitates agroinfiltration. *N. benthamiana* is a vigorous plant of manageable size, easily cultivated indoor (which represents an advantage in terms of biosafety and reduced competitiveness with the food chain) and has a shorter life cycle compared to its relatives in the genus *Nicotiana*. This makes it an appealing plant biofactory, especially from a transient expression perspective (Molina-Hidalgo et *al.,* 2021). On the other hand, tobacco (*Nicotiana tabacum*) has always shone for its high biomass, with adult plants being able to reach a size of more than 3 meters in height, which translates into conspicuous amounts of foliar tissue, for protein or metabolite extraction (Lewis, 2020; Stephenson et *al.,* 2020). Besides its biomass, tobacco has a fast growth cycle and is very easily transformed (actually, tobacco was the first stable transgenic plant obtained in 1983). It shows great robustness and can be grown both in a greenhouse or in the open field with reasonable soil requirements. Moreover, tobacco has a special socioeconomic weight: it is an historically important culture because of its consumption as smoking tobacco, but in EU countries its cultivation has seriously declined, and its traditional use is now being discouraged due to its implications for human health. This decline in tobacco cultivation is unfortunately accompanied by the loss of job opportunities in what used to be dedicated rural areas, and therefore, the search for alternative uses (potentially by means of ME) could revalue this crop, as it was purposed by some European projects such as Newcotiana (https://newcotiana.webs.upv.es/). All these characteristics make it an ideal candidate for metabolic engineering.

While displaying numerous intrinsic positive characteristics, both *Nicotiana* species are still susceptible to **improvements as bioengineering chassis**. Interesting traits to improve are the total plant or foliar biomass and yield, their chemotype (e.g., enrichment in specific precursor compounds), and biosafety aspects. For **biomass improvement**, one possible strategy is delaying or avoiding plant flowering. Several studies have identified certain *Nicotiana* genes as potential targets for gene editing: following the discovery of the crucial importance of *NtFT5* for flowering in *N. tabacum*

(Schmidt et *al.,* 2020), loss-of-function tobacco plants edited at this locus showed an unflowering phenotype and accumulated more biomass. In *N. benthamiana*, *SPL13_1a* has been recently shown to play a crucial role, leading to a significant delay in flowering when knocked out by CRISPR-Cas in stable *N. benthamiana* plants (De Paola et *al.,* 2023). From a perspective of **improving the chemical characteristics** of *Nicotiana* for better downstream processing, transgene-free tobacco plants deprived of nicotine due to CRISPR-Cas editing have been obtained, making it possible to further introduce this character into other tobacco lines by introgression, at the same time bypassing biosafety concerns (Schachtsiek & Stehle, 2019). Also, chassis improvement directed at ameliorating production in glandular **trichomes** can result in higher yields, due to the great potential of these cells in synthesizing and accumulating large amounts of metabolites whose production might otherwise be limited by cellular toxicity in unspecialized tissues (Tošić et *al.,* 2019). In this direction, several works have identified endogenous *Nicotiana* promoter sequences, such as pRbcS-T1 and pMALD1 from *N. tabacum* (Pottier et *al.,* 2020), that could be used in genetic constructs to target the production of a desired metabolite specifically to glandular trichomes.

In summary, plant biofactories have been demonstrated to be very useful platforms to biosynthesize valuable PNPs (already present or not in the plant hosts), new-to-nature metabolites, and even heterologous proteins or metabolites acting as therapeutic molecules as well as profitable compounds in insect pest management (IPM) strategies. Among the latter, this doctoral thesis focuses its interest specifically on the bioproduction of insect pheromones and their precursors.

3. Insect pheromones

Among the natural compounds produced in plant biofactories, this doctoral thesis focuses on insect pheromones and their precursors. Pheromones are semiochemicals, *i.e.*, chemical molecules released by animals that can elicit a behavioral response in other individuals. Depending on their range of action,

semiochemicals can be classified as allelochemicals when transmitting information between individuals from different species (comprising kairomones, allomones, synomones, and apneumones), and pheromones when they act particularly on conspecifics (Bakthavatsalam, 2016; Symonds & Elgar, 2008). Most pheromones are volatile organic compounds (VOCs) and are emitted in small quantities, being active at very low concentrations. The effects that these compounds can exert on other individuals are powerful and impact a wide set of functions, providing signals for sexual attraction of conspecifics, warning against predators, aggregation of members of the same species, marking territories and trails leading to food sources, or demonstrating individual status, among others. Insects particularly exploit pheromones, and especially sex pheromones, for intraspecific communication. The main role of sex pheromones is the identification and location of mating partners, and they are usually, but not exclusively, produced by females. The structures of insect sex pheromones from different major groups of insects have been elucidated, and this thesis will focus on the pheromones of moths (order Lepidoptera) and mealybugs (family: Pseudococcidae). The first insect sex pheromone to be identified, isolated and described was bombykol, produced by the silkworm female moth (*Bombyx mori*) (Butenandt, 1959). From then on, the pheromone identification race started, and many more compounds were identified in numerous insect species, especially moths, thanks to the interest sparked by their potential to control insect behavior in insect pest management strategies. In contrast to the traditional crop pest control methods relying mainly on pesticides, which inflict considerable environmental damage due to their broad-spectrum activity, insect sex pheromones distinguish themselves by their directed mode of action, limited to the targeted insect pest. Furthermore, they exhibit efficacy against pesticide-resistant insects while hardly inducing pheromone resistance or insensitivity (Witzgall et *al.,* 2010).

3.1 Applications of insect pheromones

Pheromones stand out as a sustainable, species-specific alternative to pesticide-based control of insect pests affecting crops and stored goods. The application of

these semiochemicals in the field (Gavara et *al.,* 2020), greenhouses (Cocco et *al.,* 2013) and warehouses (Trematerra, 2012) as part of integrated pest management (IPM) strategies could be addressed by four main approaches, depicted in Figure 3.

Monitoring the presence and abundance of insect pests (Figure 3A) is a preliminary strategy to predict damage to crops, allowing to perform timely treatments. Monitoring involves the use of traps coated with pheromones, enabling the recording of fluctuations in the number of individuals over time, in contrast to manual sampling methods, and has no extermination purpose in itself (Adams et *al.,* 2017). Additionally, it is a very useful technique to detect and track new invasive species, as was demonstrated by Mori & Evenden (2013), where pheromone-baited traps were used to monitor populations of the invasive moth *Coleophora deauratella* in experimental fields.

On the other hand, the **attract-and-kill** strategy (Figure 3B) makes use of the sex pheromone to attract target insect pests to devices for extermination purposes. When insects are not trapped inside the gadget and die after a short period of time due to the effect of killing agents with which they came in contact inside the trap, it is referred to as lure and kill (El-sayed et *al.,* 2009). Conversely, if target pests are retained inside the traps by physical means such as adhesive surfaces, it is considered mass trapping (El-Sayed et *al.,* 2006). Additionally, it is common to use devices that contain both attractant and toxic substances, a strategy which is denominated bait station. In the latter case, the harmful substance will act as a killing, infecting or sterilizing molecule for the pest, and differs from lure and kill for its higher concentrations of killing agent.

Another application of pheromones for pest control is the **push and pull** strategy (Figure 3C). This approach relies on the combined action of both repellent and attractant semiochemical stimuli. The 'push' component is achieved by deterring insects from finding and feeding on the target crop, by means of semiochemicals which directly repel insects, or mask the presence of the crop. Simultaneously, the 'pull' component is achieved by using attractive semiochemicals – such as sex pheromones – to lure and concentrate insects elsewhere, sometimes for further

elimination by traps (Cook et *al.,* 2007). The term 'push and pull' was first used in 1987 by Pyke and collaborators, to describe the efforts for controlling moth species from genus *Helicoverpa*, which affected cotton fields. Additionally, this strategy does not employ only sex pheromones, but it often also uses insect aggregation pheromones or plant volatiles. Plant volatiles can act to attract target pests or, in other cases, their parasitoids and predators. This is one of the reasons why it is fundamental to have a thorough knowledge of pest biology, as well as of their interactions with predators and plant hosts, for an optimal application of push and pull strategies and, in general, of IPM strategies (Reddy & Guerrero, 2004). A recent example of this approach was the study led by Kim et *al.,* (2023), where *Frankliniella occidentalis*, a polyphagous thrip pest, was successfully controlled by 'pushing' insects away from a greenhouse with alarm pheromones, while 'pulling' them to traps by the action of aggregation pheromones.

Finally, **mating disruption (MD)** is a powerful pheromone-based strategy that consists of infusing synthetic sex pheromones into crop areas to block or disturb the chemical communication between male and female insects (Figure 3D), thereby preventing them from mating (Carde & Minks, 1995. Depending on the mechanism on which it relies, we can differentiate between competitive and non-competitive mating disruption. In the first case, the synthetic pheromone 'competes' with natural insect pheromones for the attention and response of individuals; thus, treatment efficacy is dependent on pest density. Conversely, in non-competitive mating disruption, the detection and response to natural pheromones are impaired; this mechanism is sometimes related to the desensitization of males. In this case, treatment becomes independent of pest density (Miller & Gut, 2015). Additionally, there is a wide range of devices that store and release pheromone compounds for mating disruption: the most common are passive dispensers, but there is also flowable microencapsulation for sprayed application, and 'puffers' or aerosol dispensers (Daane et *al.,* 2021). Since the first successful application of MD in 1967, by Gaston and collaborators, where the encounter of male and female individuals of the *Trichloplusia ni* moth was blocked by experimentally flooding the atmosphere with its sex pheromone, many more trials have been performed with an increasing

number of species, mainly moths, where MD actually stands out as the most advanced and promising method employing pheromones to control their populations (Lance et *al.,* 2016; Vacas et *al.,* 2011) and recently, mealybugs (Daane et *al.,* 2021; Lucchi et *al.,* 2019).

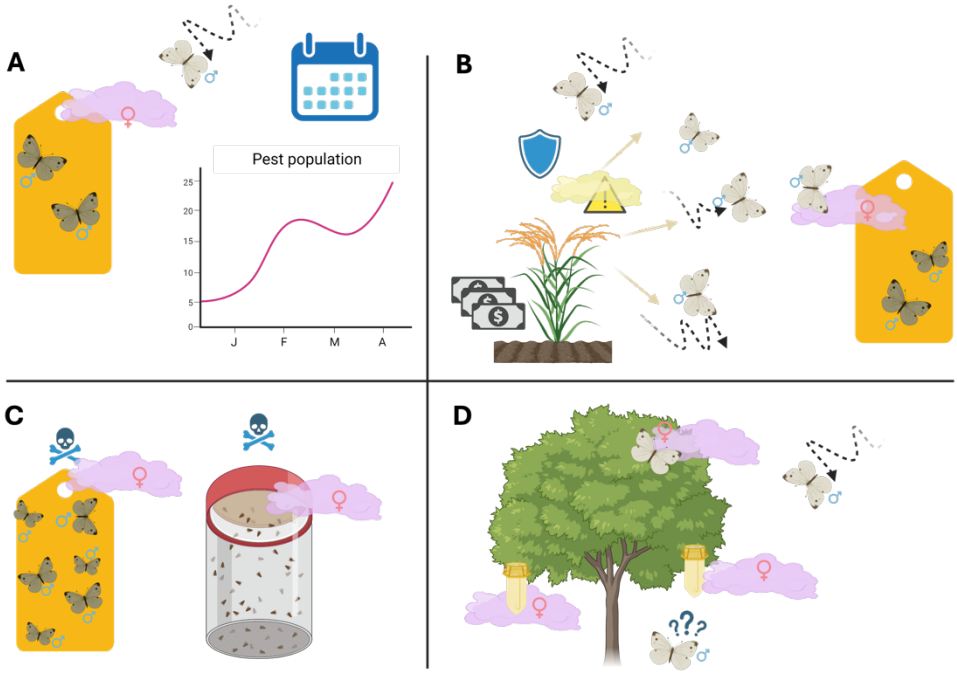


Figure 3: Different strategies for the application of insect sex pheromones in Integrated Pest Management (IPM). (A) Monitoring. **(B)** Push and pull. **(C)** Attract and kill. **(D)** Mating disruption. The figure includes images from Biorender (biorender.com).

3.2 Production of insect pheromones for IPM

Since the first use of pheromone extracts in the 1940s to monitor the activity of a vineyard pest, the moth *Lobesia botrana,* the interest in the applications of insect pheromones started to grow. Momentarily overshadowed by the widespread use of DDT, when this turned out to be highly harmful to the environment, the focus of researchers shifted back to pheromones as more sustainable alternatives for pest

control. Soon, together with the development of analytical chemistry techniques and improvements in equipment, the range of discovered and chemically synthesizable pheromones was broadened. A first mating disruption trial in the 1960s using pheromones highlighted their potential and was followed by multiple applications in different IPM strategies, as mentioned above. Nowadays, given the success of pheromones in crop pest control and the growing concerns about the effects of chemical pesticides on the environment and human health, IPM strategies drive a huge demand in the pheromone production market. Estimates project the global agricultural insect pheromone market to reach more than 8 billion USD by 2028, from a present value of almost 4 billion USD in 2023 (MarketsandMarkets). However, the costs of pheromone production are still the main disadvantage for the large-scale implementation of MD and other pheromone-based strategies in IPM. These depend on several parameters, such as the industrial cost of synthesis of the pheromone molecules themselves, the amount of pheromone required per dispenser considering its release rate, and the number of pheromone dispensers needed per unit area for the success of this pest management strategy (Gavara et *al.,* 2020). Many examples exist of organic synthesis of pheromone compounds, most of them described in detail in Mori's work (K. Mori, 2010), but they still present some limitations. One of the factors affecting pheromone biosynthesis and activity is stereochemistry: insects often respond only to a specific stereoisomer of a pheromone compound, and enantioselective synthesis is often complex and expensive. Thus, commercially available pheromone compounds are often racemic mixtures in which the active ingredient represents only 50% of the total content. Another relevant disadvantage is the generation of hazardous by-products intrinsically created by chemical synthesis. Alternative methods for synthesis and large-scale manufacturing procedures must be explored: biological platforms (biofactories) have recently begun to stand out as sustainable alternatives for the mass production of insect sex pheromones, also relying on the stereoselectivity of enzymes. The rationale behind this thesis is exploring the potential of plant biofactories for the biotechnological production of molecules involved in pest control, focusing on moth and mealybug sex pheromones.

3.3 Moth sex pheromones

3.3.1 Classification of moth pheromone compounds

Lepidoptera represent the second largest insect order, including more than 160.000 identified species of butterflies and moths. Moths are among the most threatening agricultural pests, because of the damage they cause to crops (both directly, by feeding on them as larvae, and indirectly by facilitating mold proliferation and mycotoxin accumulation), and also because of their ability to rapidly develop genetic resistance against insecticides (Denholm & Devine, 2013). Lepidopteran species depend on sex pheromones for long-distance communication and to find mating partners. Each species is characterized by the production of a blend of active pheromone compounds, in which the species-specific identity print is given both by the identity and by the unique relative ratio of the active compounds (Gomez-Diaz & Benton, 2013). To date, more than 700 moth sex pheromones have been described: they usually share a hydrocarbon chain structure of variable length and differ in the number and position of functional groups, methylations and unsaturations. Their names are commonly abbreviated as follows to indicate their structure. First, unsaturations are enumerated: internal double bonds are indicated by "*Z*" or "*E*", corresponding to chirality configurations *Z* or *E*, respectively, and terminal double carbon bonds are indicated by "Δ". On the other hand, triple carbon bonds are indicated by "≡". Then, the position of this unsaturation on the carbon chain is specified, followed by a hyphen and then the length of the carbon chain. For example, the abbreviated form E10,Z12-16:OH is used to describe (10*E*,12*Z*)-10,12-hexadecadien-1-ol (bombykol), counting with two double bonds in position 10 and 12 (*E* and *Z* configuration, respectively) and with a total length of 16 carbons. Then, *cis*-epoxy ring "epo", *trans*-epoxy ring "*t*-epo", or methyl branch "Me" groups should be thus notated. Afterwards, the number of carbons of the straight chain is indicated, preceded by a hyphen. Finally, a colon is used to precede functional groups, which are abbreviated as follows: alcohol (OH), acetate of alcohol (OAc), aldehyde (Ald), ketone (one), ester of carboxylic acid (Ate), and hydrocarbon (H). For instance, Z11-16Ald is the abbreviated name of the pheromone compound used by a large number

of moth species, *Z*-11-hexadecenyl aldehyde, indicating a 16 carbons-length chemical compound, with a double bond in position 11 and *Z* configuration as well as a terminal aldehyde group (Pope et *al.,* 1984; Piccardi et *al.,* 1977). Another example is the Z9,≡11,Δ13–14:Ald, which is used for naming the (*Z*)-9,13-Tetradecadien-11-ynal, a 14 carbon-length fatty chain with a terminal double bound in position 13 (Δ13), a double bound in position 9, a triple bound in position 11 (≡11) and a terminal aldehyde group (Millar et *al.,* 2008). In addition to their chemistry, they are classified according to their proposed biosynthetic pathway into 4 groups, summarized in Figure 4:

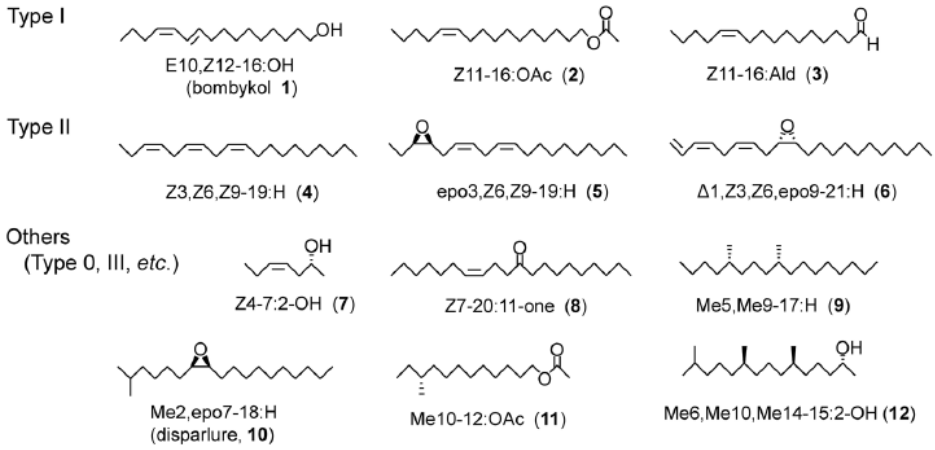


Figure 4: Formulas and abbreviations of representative lepidopteran sex pheromones type I, II, III, 0 and others. Adapted from *Ando & Yamamoto, (2020)*.

- **Type I.** These compounds comprise the majority (around 75%) of the total known moth sex pheromones. Most of them are characterized by hydroxyl, formyl or acetoxyl functional groups in the terminal position of the hydrocarbon chain, measuring C_{10}-C_{18}, as well as showing between 1 and 4 unsaturations.
- **Type II**. Polyunsaturated hydrocarbon semiochemicals, longer than type I (C_{17}-C_{25}), showing 2 to 5 unsaturations, and their corresponding epoxy derivatives, belong to this group, representing around 15% of discovered moth sex pheromones.

- **Type III**. This group includes moth sex pheromone structures carrying at least one methyl branch.
- **Type 0**. This category comprises different short-chain (C_7-C_9) secondary alcohols and related methyl ketones.

3.3.2 Biosynthesis of lepidopteran sex pheromones

As **type I** moth sex pheromones are the most abundant, they have also been the most investigated, especially in terms of biosynthesis. These semiochemicals originate by *de novo* biosynthesis from saturated fatty acids, usually palmitic acid (16C) or stearic acid (18C), using the metabolic precursors acetyl-CoA and malonyl-CoA. Then, the process continues with several steps of desaturation (performed by fatty acid desaturases, FAD), chain shortening or elongation, reduction (by fatty acid reductases, FAR), and acetylation (by fatty acid acetyltransferases, FAT) or oxidation (by fatty acid oxidases, FAO). The general process is depicted in Figure 5. This combination of multiple specific fatty acid modifying enzymes (FAMEs) contributes to the previously mentioned specificity of pheromone compounds in moth species. The role of FADs is especially relevant in generating this wide variety of compounds, thanks to their regioselectivity and stereoselectivity in desaturating fatty acid substrates in the Δ5, Δ6, Δ8, Δ9, Δ10, Δ11, and Δ14 positions. More than 50 FADs from over 20 lepidopteran species have been identified and characterized, which share four transmembrane domains for membrane anchoring, as well as His-rich motifs. On the other hand, FARs are also key enzymes in the biosynthetic pathway and catalyze the reduction of the fatty acids and thus the synthesis of the fatty alcohols: these are already bioactive pheromone components in some cases, while in others they undergo further modifications from other FAMEs. Multiple FAR-like genes have been identified within individual moth species, which exhibit different preferences towards substrate length, determining the ratio of pheromone blend constituents (Rizvi et *al.,* 2021). Conversely, research on FAT genes implied in moth sex pheromone

biosynthesis has not identified any active sequence yet, even if several candidates were suggested after transcriptomic analysis (B. J. Ding & Löfstedt, 2015).

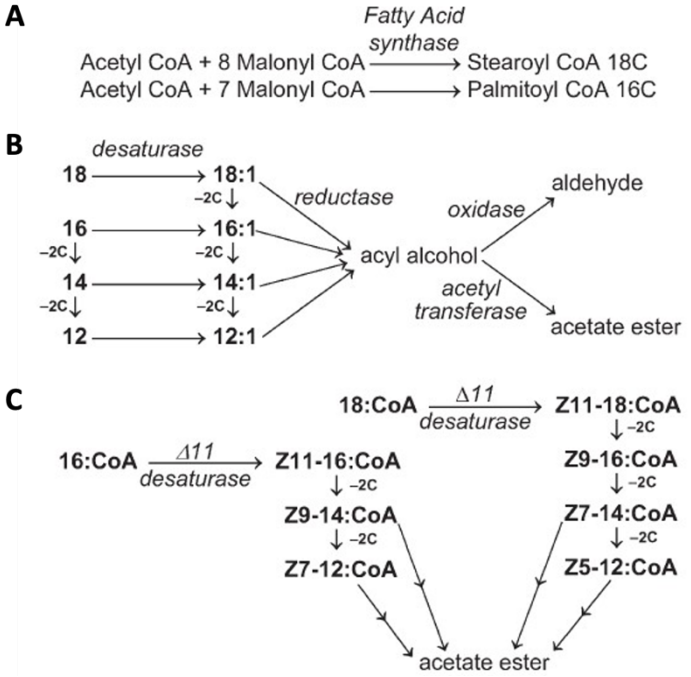


Figure 5: **General biosynthetic pathways for the production of alcohol, aldehyde, and acetate ester pheromone components in female moths**. **(A)** Production of saturated fatty acids. **(B)** Production of monounsaturated fatty acids and limited chain shortening produces intermediate compounds that can be reduced to an alcohol. Aldehyde and acetate ester pheromones are produced by an oxidase and acetyl-transferase, respectively. **(C)** Biosynthetic pathway for the production of the acetate ester pheromone components in the cabbage looper moth, *Trichoplusia ni*. The CoA derivatives are reduced and acetylated to form the acetate esters. Additional pheromone components include 12:OAc and 11–12:OAc. (Adapted from Mori (2004).

Regarding **type II** pheromones, double bonds are supposed to come directly from dietary unsaturated fatty acids, which then suffer subsequent modifications such as chain elongations, decarboxylations and epoxidations. Even if much less is known about this class of moth sex pheromones, to date epoxidation has been suggested by several cytochrome P450 enzymes (Rong et *al.,* 2014; Rong, Fujii, Naka, et *al.,* 2019; Rong, Fujii, & Ishikawa, 2019).

3.4 Mealybug pheromones

3.4.1 Classification of mealybug sex pheromones

Mealybugs (Hemiptera: Pseudococcidae) form an insect family that comprises more than 1900 species, now broadly distributed across the world, becoming damaging agricultural pests, affecting fruit orchards, rice, sugarcane, potato and other value crops (C. Franco et *al.,* 2004; J. F. Franco et *al.,* 2009). This situation is especially pronounced in the Mediterranean basin, where the Valencian Community registers a high impact of these pests in citrus and persimmon orchards and vineyards, among others (Tena et *al.,* 2015; Pérez-Rodríguez et *al.,* 2017). Mealybugs display a marked sexual dimorphism: adult males are winged, have no mouthparts, and have a short lifespan, while females are wingless, sedentary with retrogressed legs, and produce a protective surface of powdery wax, hence their common name "mealybugs" (J. F. Franco et *al.,* 2009; Ross & Shuker, 2009; Tabata, 2020). Most of these sap-feeding insects reproduce sexually, and adult virgin females emit sex pheromones in the order of a few nanograms per day to guide winged males to find them. Once mated, their pheromone production decreases or even completely stops. Considering the significant role that pheromones play in guiding males over long distances, these semiochemicals have probably come under substantial selective pressure, enhancing the process of mating and reproduction, thereby driving mealybug evolution and speciation events.

In moths, the differences between the sex pheromone blends of different species rely on the ratio of each sex pheromone compound, as well as on their identity, meaning that species-specific pheromone blends may be composed of different ratios of the same compounds. On the other hand, in mealybugs, sex pheromones often constitute exclusive species-specific molecules, and are often formed by a single component, even if there are some exceptions (Tabata, 2020). This structural variety is one of the reasons contributing to the challenges encountered in describing these pheromone compounds, together with the minimal amounts biosynthesized by females. However, in the last decades, more than twenty sex pheromone compounds have been characterized and identified from around as many mealybug species Zou &

Millar, 2015). Some features are shared among mealybug pheromones. In chemical terms, they are based on terpene structures, mostly irregular monoterpenes, a product of the coupling of two isoprene units in an unusual non-head-to-tail manner. These are called irregular because most monoterpenes found across both Prokaryotes and Eukaryotes are biosynthesized by the regular head-to-tail condensation of IPP and DMAPP units. Interestingly, some irregular monoterpenes are also found in aromatic plant species. Exceptions have been described in mealybugs, such as the ester of a hemiterpene alcohol in the pheromone of *Crisicoccus matsumotoi*, or the aldehyde cyclopentane in *Dysmicoccus brevipes*. Based on their monoterpene skeleton, four main groups of mealybug pheromones have been identified.

1. Lavandulol-related pheromone structures. Mealybug pheromones with an acyclic structure are all based on this 2-isopropenyl-5-methyl-4-hexenol structure, commonly named lavandulol. This monoterpene alcohol, present in lavender essential oil, is naturally found in plants as the result of a "head-to-middle" condensation of two molecules of DMAPP, as is the case for the reaction catalyzed by the lavandulyl diphosphate synthase of *Lavandula intermedia* (*Li*LPPS). In many active pheromone compounds, lavandulol is esterified (Figure 6A). This is the case of pheromone compounds such as (*S*)-lavandulyl acetate and (*S*)-lavandulyl senecioate in *Planococcus ficus* (Hinkens et *al.,* 2001), (*R*)-Lavandulyl (*S*)-2-methylbutyrate as a minor pheromone component in *Maconellicoccus hirsutus* (A. Zhang et *al.,* 2004), its isomer (*R*)-lavandulyl (*R*)-2-methylbutyrate as a minor pheromone component in *Phenococcus madeirensis* (Ho et *al.,* 2009), and (*R*)-lavandulyl propionate and (*R*)-lavandulyl acetate in *Dysmicoccus grassii* (De Alfonso et *al.,* 2012). In other cases, mealybug pheromones are based on the same lavandulol skeleton, with double bonds in different positions (Figure 6B). To this category belong the esters β, δ and γ-lavandulyl acetate, corresponding to the pheromone compounds of *Planococcus minor* (Ho et *al.,* 2007), *Dysmicoccus neobrevipes* (Tabata & Ichiki, 2015; Tabata & Ohno, 2015) and *Planococcus kraunhiae* (Tabata, 2013), respectively. Other variations have been described: in *Pseudococcus comstocki* the alcohol moiety of the pheromone is 3-acetoxy-2,6-dimethyl-1,5-heptadiene, an analog of lavandulol (Bierl-

Leonhardt et *al.,* 1982). All these structures, showing a common lavandulol skeleton, suggest the probable implication of this monoterpenoid in pheromone biosynthesis, as well as a possible evolutionary common origin in mealybugs.

2. Cyclopropane-related pheromone structures. All cyclic structures with three-carbon rings found in mealybug pheromones are esters of chrysanthemol (2,2-dimethyl-3-(2-methylprop-1-enyl)cyclopropyl)methanol). Like lavandulol, chrysanthemol is the product of a non-head-to-tail condensation of two units of DMAPP, which involves a c1'-2-3 cyclopropanation. To date, three esters of chrysanthemol have been identified as mealybug pheromones: (1R,3R)-chrysanthemyl (R)-2-methylbutanoate from *Phenococcus madeirensis* (Ho et *al.,* 2009), (1R,3R)-chrysanthemyl (R)-2-acetoxy-3-methylbutanoate in the case of *Pseudococcus calceolariea* (El-Sayed et *al.,* 2010), and (1S,3R)-chrysanthemyl tiglate from *Ferrisia virgata* (Tabata & Ichiki, 2017) (Figure 6C). As is the case for lavandulol, a biosynthetic pathway for chrysanthemol is also found in plants, specifically in the Antemidae tribe of the Asteraceae family. Notably, in *Tanacetum cinerariifolium* chrysanthemol is the precursor for the biosynthesis of *trans*-chrysanthemic acid, which is implicated in the biosynthesis of pyrethrins, which are important insecticide compounds.

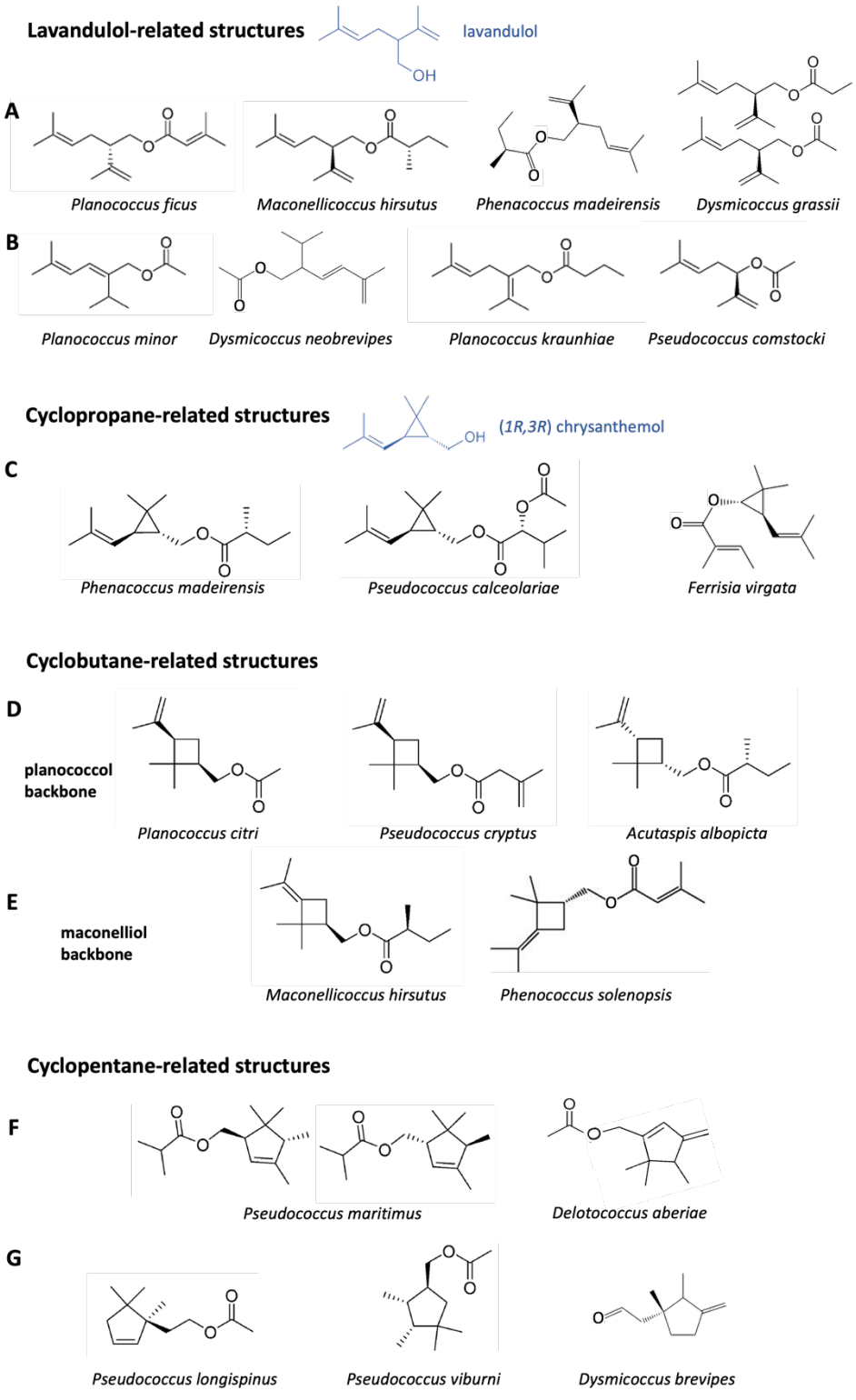
3. Cyclobutane-related pheromone structures. Other monoterpenoid cyclic structures contributing alcohol skeletons for the synthesis of mealybug pheromones are based on four-carbon (cyclobutane) rings, also the putative product of irregular non-head-to-tail condensation and cyclization of isoprene blocks. Depending on the position of a double bond between carbons in this cyclobutane ring, pheromones are classified as carrying planococcol (Figure 6D) or maconelliol groups (Figure 6E). The first class includes planococcyl acetate from *Planococcus citri* (Bierl-Leonhardt et *al.,* 1981), planococcyl 3-methyl-3-butenoate from *Pseudococcus cryptus* (Arai et *al.,* 2003), and (*1S,3S*)-3-isopropenyl-2,2-dimethylcyclobutylmethyl (*R*)-2-methylbutanoate, from *Acutaspis albopicta* (Millar et *al.,* 2012). In the latter, the alcohol moiety is constituted by an enantiomer of planococcol. On the other hand, the second class of cyclobutane-containing pheromone structures comprises the

pheromone compounds (*R*)-maconelliyl (*S*)-2-methylbutanoate from *M. hirsutus* (A. Zhang et *al.,* 2004) and (*R*)-maconellyl senecioate from *Phenacoccus solenopsis* (Tabata & Ichiki, 2016).

The four-membered carbocycle motif is also found in many other natural products, from the first discovered α- or β-pinene, to β-caryophyllene and more complex structures such as dimeric compounds showing cyclopropanes in their structures or the cyclobutaned-sesquiterpenes produced by some fungi (Antonsen et *al.,* 2018). However, none of these structures, especially those from plants, apparently derive from irregular couplings, so the biosynthetic pathways to achieve such a specific condensation remain unknown.

4. Cyclopentane-related pheromone structures. Five-carbon rings are also present in the skeleton structures building mealybug sex pheromones. These cyclopentyl or cyclopentenyl rings exhibit a wide variety of linkage types, opposite to previous cycled structures, which showed similar connections inside every group. Among the molecules described to date, pheromones displaying this structure are in some cases based on a necrodol alcohol moiety (Figure 6F): α-necrodol for *Pseudococcus maritimus*, where the active pheromone blend is composed by the two enantiomers (*R,R*)- and (*S,S*)- *trans*-(3,4,5,5-tetramethyl-2-cyclopentenyl) methyl isobutyrate (Figadère et *al.,* 2007); β-necrodol for *Delottococcus aberiae*, whose main pheromone compound is 4,5,5-trimethyl-3-methylenecyclopent-1-en-1-yl)methyl acetate (Vacas et *al.,* 2019). Other cyclopentane-related structures are 2-(1,5,5-trimethytl-2-cyclopentenyl) ethyl acetate from *Pseudococcus longispinus* (Millar et *al.,* 2009), (1S,2S,3R)-(2,3,4,4-tetramethylcyclopentyl)-methyl acetate, typical of *Pseudococcus viburni* (Hashimoto et *al.,* 2008), and (*1S,2S*)-(1,2-dimethyl-3-methylenecyclopentyl)acetaldehyde, from *D. brevipes* (K. Mori, 2016) (Figure 6G).

Figure 6 (next page): Classification of mealybug sex pheromone structures according to backbone. Schematical representation of several molecular structures from mealybug sex pheromones according to backbone structure. Structures with esterifications in lavandulol skeleton **(A)** and based on lavandulol with double bonds in different positions **(B)**, chrysanthemol-related structures **(C)**, planococcol-related structures **(D)**, maconelliol-related structures **(E)**, necrodol-related structures **(F)** and other cyclopentane-related structures **(G)** (Adapted from Zou et *al.,* 2014).


Lavandulol-related structures
lavandulol
OH
A
Planococcus ficus
Maconellicoccus hirsutus
Phenacoccus madeirensis
Dysmicoccus grassii
B
Planococcus minor
Dysmicoccus neobrevipes
Planococcus kraunhiae
Pseudococcus comstocki
Cyclopropane-related structures
(1R,3R) chrysanthemol
OH
C
Phenacoccus madeirensis
Pseudococcus calceolariae
Ferrisia virgata
Cyclobutane-related structures
D
planococcol backbone
Planococcus citri
Pseudococcus cryptus
Acutaspis albopicta
E
maconelliol backbone
Maconellicoccus hirsutus
Phenococcus solenopsis
Cyclopentane-related structures
F
Pseudococcus maritimus
Delotococcus aberiae
G
Pseudococcus longispinus
Pseudococcus viburni
Dysmicoccus brevipes

Scale insects, a related group of insects belonging to the same Coccoidea superfamily, show similar chemical structures for their sex pheromones. In some cases, they are based on polyketides (Lanier et *al.,* 1989), on esters of regular monoterpenoids such as geraniol and nerol (Gieselmann et *al.,* 1979a), esters of sesquiterpenols and solanone (Ho et *al.,* 2014; Gieselmann et *al.,* 1979b) and in yet other cases, similarly to mealybugs, on esters of cyclic monoterpenes (Einhorn et *al.,* 1998).

3.4.2 Biosynthesis of mealybug sex pheromones

The biosynthetic pathways of monoterpenoid pheromones remain unclear, due to the many uncommon aspects of their chemistry. First, these are the only irregular monoterpenoids known in the Animal Kingdom, and act as pheromones primarily in mealybugs and scale insects but are also found in some thrips as aggregation pheromones (X. Li et *al.,* 2019). In the Plant Kingdom, monoterpenes are usually synthesized as the product of regular head-to-tail coupling condensation reactions. However, it is also in plants where several irregular exceptions have been described (e.g., LPPS, CPPS), but visibly the evolutionary distance between Plants and Insects is such that homology-based identification of candidate genes is not straightforward. In monoterpene biosynthesis, a first coupling step is needed, followed by one or several further steps of modification (substitutions, cyclizations, desaturations) and thus, synthesis of the monoterpene (F. Zhou & Pichersky, 2020). Some of the monoterpene biosynthetic pathways well described in plants count with separate enzymes catalyzing each step, *i.e.*, one enzyme responsible for the prenyltransferase/isoprenyl diphosphate synthase (IDS) activity and one or more responsible for the terpene synthase (TPS) activity. However, in other cases such as that of CPPS, the same enzyme is responsible for both steps (T. Yang et *al.,* 2014). While a great number of enzymes have been described as TPSs in plants (Christianson, 2017), only a few examples of insect enzymes acting as TPSs have been identified so far. Among these, the complete biosynthetic pathway responsible for the production of the iridoid monoterpene sex pheromone in pea aphids was recently discovered (Köllner et *al.,* 2022), as well as the terpene synthases from members of the Hemiptera (Lancaster

et *al.,* 2018 and 2019) and Coleoptera (Beran et *al.,* 2016) orders. These examples support a *de novo* hypothesis for terpene biosynthesis in insects. Insect TPSs described to date show more sequence similarity to plant and microbe prenyltransferases than to plant or microbe TPSs, which contributed to the difficulties in their identification (Lancaster et *al.,* 2019). Therefore, it was suggested that irregular monoterpenoids such as lavandulyl-PP or chrysanthemyl-PP may be synthesized *ex novo* by mealybug irregular IDSs similarly to what happens in plants but, to date, no functional sequences have been identified in mealybugs, despite the identification of many candidates (Juteršek et *al.,* 2024). In addition, Tabata, (2022) determined that the synthesis of *P. citri* sex pheromone is fenced in a single trait locus.

Conversely to the *de novo* hypothesis for the biosynthesis of irregular monoterpenes in mealybugs (Millar et *al.,* 2005), alternative origins were also hypothesized for such molecules, considering their presence in both plants and bacteria. One of these hypotheses supports the sequestering of these irregular monoterpenes from host plants during feeding, as it was suggested for several animals, such as some mollusks, which sequester characteristic terpenes from their algal diet (Tholl et *al.,* 2023). Alternatively, others have proposed the biosynthesis of irregular monoterpene pheromones by means of the endosymbiotic bacteria hosted in mealybugs (J. F. Franco et *al.,* 2009). These endosymbionts are believed to be crucial for nutrition as well as in various physiological processes in mealybugs, so pheromone biosynthesis or at least precursor contribution has been hypothesized. However, no evidence has been found supporting either dietary or endosymbiotic participation for irregular monoterpene biosynthesis so far in mealybugs. With all this, given the mentioned difficulties found in searching for the original mealybug genes implied in monoterpene-based sex pheromones, alternative strategies are being developed to reach their biosynthesis or, at least, to understand requirements underlying enzyme sequence and structure. Accordingly, some strategies for the elucidation of biosynthetic mechanisms have been based on the directed mutagenesis of regular coupling enzymes from other sources, like the tomato neryl diphosphate synthase (NDPS), with the aim to shift coupling mechanisms from regular to irregular activity,

resembling *Li*LPPS (Gerasymenko et *al.,* 2022). Other strategies have been focused on creating chimeric prenyltransferases showing different combinations of residues from regular and irregular coupling enzymes, aiming to determine which residues are critical to shift activity towards irregular coupling. This was the case of a chimeric chrysanthemyl diphosphate synthase/farnesyl diphosphate synthase (CPPS/FPPS), built using plant enzyme sequences and based on the hypothesis of a common ancestor and recent new functionalization of CPPS from FPPS (Thulasiram et *al.,* 2007). This chimeric protein surprisingly performed a series of irregular couplings and, between other products, monoterpenoid structures unique to insects and absent in plants, such as planococcyl or maconellyl diphosphates (characteristic of the backbone of several mealybug sex pheromones) were detected. Although models are being developed, to date a clear *in silico* identification of regular and irregular coupling enzymes has not been achieved. For the following esterification steps in the biosynthesis of mealybug pheromones, acyltransferases are thought to be involved but, as is the case for Lepidoptera, no sequences have been validated so far in insects.

4. Biomanufacturing of natural products for pest control

4.1 Biomanufacturing of insecticidal and insect repellent compounds

Given the minimal amounts of pheromone compounds produced by individual insects when isolated, synthesis of these semiochemicals is required for both elucidation of their chemical structure and producing employable quantities for further application in insect pest management strategies (K. Mori, 2010b). Specific and laborious organic chemistry reactions have demonstrated the feasibility of the synthesis of many insect sex pheromones, as described in K. Mori (2010b). However, the number of successful cases where procedures can be applied on an industrial scale is not high, mainly

because of the production of hazardous by-products. One key step in pheromone biosynthesis is the generation of C=C double bonds, which represents a costly, polluting, and time-consuming methodology due to the need to employ both extreme temperatures and noble metals as catalysts (Gayon et *al.,* 2023). Presently, alternative methods are being assessed, including the substitution of these metals by other non-noble metals.

Another critical point is the enantioselectivity of the synthesized chemical compounds. Even if some sex pheromones don't show chirality, such as bombykol, many others show stereoisomery. When employing synthetic racemic mixtures, in around 60% of cases the opposite enantiomer acts as a non-inhibitory molecule for the attractivity of the blend (K. Mori, 2007). Unfortunately, it is also possible that its opposite enantiomer or its diastereomer inhibits the response to the pheromone (K. Mori, 2010b). That is why efforts have been directed at developing enantioselective synthesis methods, which can be performed with three strategies: *i*) starting from natural products acting as chiral pools of enantiopure molecules and derivating them; *ii*) performing enantiomer separation; and *iii*) synthesis by asymmetric reactions. In this context, the use of enzymes, which ensure stereoselectivity, would constitute a notable advantage.

A considerable number of specialized plant natural products constitute interesting molecules that can be harnessed for pest control strategies. Since direct extraction from the producer plant is often unaffordable, given the minute quantities in which these compounds are usually naturally biosynthesized by host plants, synthetic biology and metabolic engineering approaches can be developed in order to reach industrial-scale production. Different biological chassis have been identified for these aims. As mentioned before, microorganisms such as bacteria – i.e. mainly *E.coli* – and yeasts have been used as biofactories for the biosynthesis of these IPM products, by using metabolic engineering strategies. Several terpenoids display insecticidal effects, and efforts in heterologous metabolic engineering of terpenoids are now starting to yield results. Among them, cembratriene-ol and cembratriene-diol are two diterpene compounds with insecticidal as well as anti-fungal properties, naturally

produced by tobacco, whose heterologous production was successfully obtained in the yeast *Saccharomyces cerevisiae* (X. Zhang et *al.,* 2021). However, metabolic engineers have used not only traditional model organisms, but also other fungi, such as the oleaginous yeast *Yarrowia lipolytica*, which has been engineered to heterologously overproduce nootkatone, a sesquiterpenoid with several properties, including insecticidal (Guo et *al.,* 2018). Moving to an example of plant biofactories, engineered tomato plants demonstrated to be able to produce *trans*-chrysanthemic acid in type IV glandular trichomes (Y. Wang et *al.,* 2022). This constitutes the acid moiety of pyrethrins, a powerful and sustainable insecticide which is, to date, extracted from natural sources with limited yields which do not satisfy market needs.

4.2 Biomanufacturing of sex pheromones and other volatiles

As suggested above, alternatives to chemical synthesis need to be found for insect pheromones to support a more sustainable industrial production. Even if insect sex pheromone biosynthetic pathways are normally absent from the commonly used heterologous hosts, it is true that certain common routes in the metabolism of the biological chassis could be leveraged to shunt the metabolic flux towards the production of the desired pheromone compound once the required steps are engineered.

Regarding Lepidopteran insect sex pheromones, which, as described before, are mainly constituted by hydrocarbon chains with hydroxyl, formyl or acetoxyl functional groups, parallel metabolic engineering efforts have been made both in yeast and in plants. Sometimes, the bioengineered product is a pheromone precursor; in other cases, the biosynthesis of bioactive sex pheromone compounds has been achieved (Xia et *al.,* 2020; Mateos-Fernández et *al.,* 2021).

As for the **fungal** bioproduction platforms, *S. cerevisiae* represents again a first test line. After the successful testing of a candidate FAR which was expressed in *S.*

cerevisiae supplemented with appropriate fatty acid precursors (Moto et *al.,* 2003), many other assays followed. The biosynthesis of the alcohol component from a moth sex pheromone was obtained by Hagström and collaborators (2013), after co-expressing both FAD and FAR moth enzymes in *S. cerevisiae*. Recently, double unsaturations in sex pheromones were achieved for the first time in a biological production platform through the co-expression of two FADs with different specificities, a FAR and a FAT enzyme (Ding et *al.,* 2022). On the other hand, a monoterpenoid acting as the sex pheromone of a beetle, 8-hydroxygeraniol, has been accumulated in high titers after metabolic engineering in *S. cerevisiae,* which demonstrates the potential of this platform to produce sex pheromones of different chemical origins (H. Wang et *al.,* 2023).

Alternatively, the already mentioned *Y. lipolytica* has emerged as an attractive chassis species due to its propensity to accept a wide variety of substrates as a carbon source, as well as metabolic precursors, and is used to produce a great range of bioproducts such as biodiesels, fatty acids or secreted proteins (Darvishi et *al.,* 2018). For all these reasons, numerous engineering attempts have been made, especially to promote lipid production in bioreactors of, among others, moth sex pheromones and their precursors (Holkenbrink et *al.,* 2020; Zeng et *al.,* 2018; Hambalko et *al.,* 2023). This was the case of the heterologous production of several alcohol and acetate forms of moth sex pheromones and precursors by Holkenbrink and collaborators (2020) in *Y. lipolytica*. In this study, this fungal chassis was specifically engineered to enhance biosynthesis and direct the metabolic flux towards the desired compounds, by knocking out several genes related to target fatty alcohol degradation while downregulating a gene related to precursor storage in undesired structures. Thus, the authors of this study obtained high titers of moth pheromones, which were successfully tested on pests in the field. A similar strategy was followed by BioPhero, a spin-out of the Technical University of Denmark, in a recent work where *Y. lipolytica* was metabolically engineered to promote β-oxidation (Petkevicius et *al.,* 2022). This chassis improvement, together with the co-expression of several FAMEs, led to the production of various alcohol moth sex pheromone components. All these fungal chassis are depicted in Figure 7.

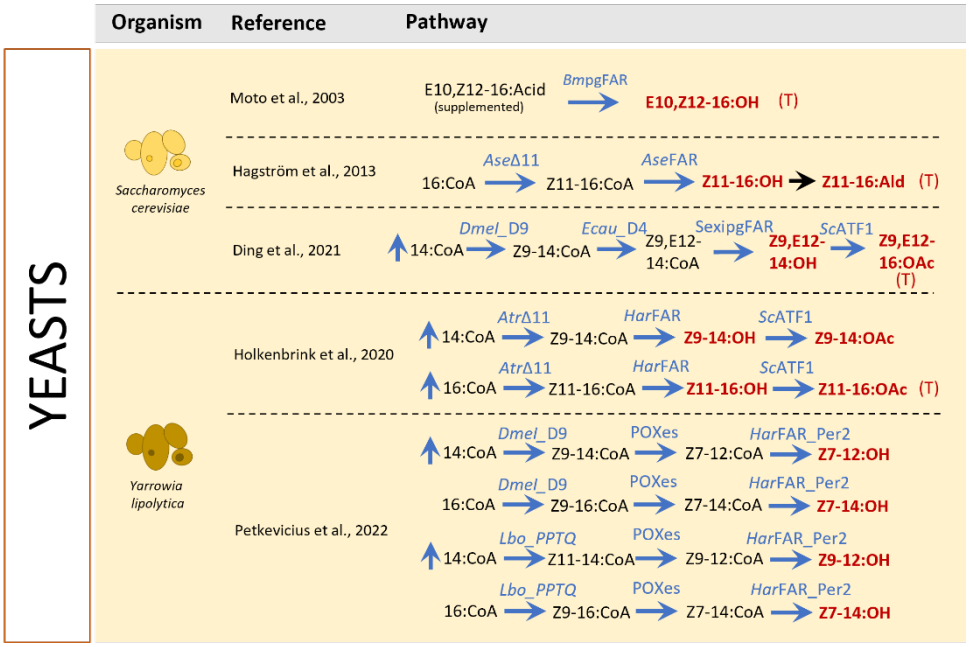


Figure 7: Strategies for the biosynthesis of lepidopteran sex pheromones in yeast organisms. Blue arrows represent synthesis by a heterologous enzyme; black arrows represent chemical synthesis; brown arrows represent synthesis by an endogenous enzyme. Metabolites in red text are known to act as active sex pheromones. (T) indicates experimental assays have shown that the molecule affected the behaviour of moths. *Bmpg*FAR = *Bombyx mori* pheromone gland fatty-acyl reductase; *Ase*Δ11 = *Agrotis segetum* Δ11 fatty-acyl desaturase; *Ase*FAR = *Agrotis segetum* fatty-acyl reductase; *Dmel*_D9 = *Drosophila melanogaster* desaturase 9; *Ecau*_D4 = *Ephestia cautella* desaturase 4; *Sexipg*FAR = *Spodoptera exigua* pheromone gland fatty acyl reductase; *Sc*ATF1 = S*accharomyces cerevisiae* alcohol acetyltransferase; *Atr*Δ11 = *Amyelois transitella* Δ11 fatty-acyl desaturase; *Har*FAR = *Helicoverpa armigera* fatty-acyl reductase; POXes = peroxisomal oxidases; *Har*FAR_Per2 = *Helicoverpa armigera* fatty-acyl reductase with peroxisomal targeting sequence peptide; Lbo_PPTQ = *Lobesia botrana* desaturase. Adapted from Mateos-Fernández et al. (2022).

Yeast-based biofactories are meant for subsequent extraction of the sex pheromone, its optional chemical modification, and its application or formulation in appropriate formats (traps, dispensers, etc.) in the field. Conversely, **plant biofactories** could potentially allow not only a similar approach based on extraction and formulation, but also direct emission from the producer plant, which would act as a living biodispenser for IPM strategies. This was proven by engineering plants to release (*E*)-β-farnesene, which is an aphid alarm pheromone. (*E*)-β-farnesene release was

implemented first in *Arabidopsis* as a model species (Beale et *al.,* 2006), and later in wheat (Bruce et *al.,* 2015). The possibility of synthesizing and emitting this VOC to reduce aphid attacks was also demonstrated in indoor conditions (Sun et *al.,* 2017).

Regarding sex pheromones, the first historical milestone in the field of plants metabolically engineered to synthesize insect sex pheromones was the work of Nešněrová et *al.,* (2004), where tobacco was engineered to produce a moth sex pheromone precursor, methyl (Z11)-hexadec-11-enoate. Following this, several other works reached similar objectives, such as the biosynthesis of pheromone precursors in *Nicotiana* spp. by Xia and collaborators (2020). Another chassis, the oil crop *Camelina sativa*, has lately attracted the attention of metabolic engineers because of its high capacity for the accumulation of fatty acids in seeds, reaching up to 40% of seed dry weight (Iskandarov et *al.,* 2013). Moreover, *Camelina* has several other qualities, such as its short growing season and seeding, stress tolerance and low agronomic requirements, as well as the easiness of genetic transformation (Murphy, 2016). Thus, moth sex pheromone precursors were accumulated in *Camelina* seeds and, after chemical processing, were tested to monitor and disrupt mating for several Lepidopteran species (H. L. Wang et *al.,* 2022). Attempts to biosynthesize final, active sex pheromone products started with the seminal work of Ding and collaborators (2014), who achieved not only the production of the bioactive alcohol by transient expression in *N. benthamiana* leaves, but also, and for the first time, the final acetylated pheromone. To reach this hallmark, the authors identified and transiently expressed an appropriate plant acetyltransferase, the *Ea*DAcT from *Euonymus alatus*. A few years later, *Nicotiana* species were used again to stably express different combinations of FADs and FARs to obtain fatty acid precursors of moth sex pheromones (Xia et *al.,* 2020). Recently, our group generated the so-called “Sexy Plant”, the first stable transgenic *N. benthamiana* plant producing and emitting the three bioactive forms of a pheromone cluster, Z11-16OH, Z11-16OAc and Z11-16Ald (Mateos-Fernández et *al.,* 2021). This was the first example of a living stable biodispenser of moth sex pheromones in a plant chassis. Shortly after, this idea was taken up by Xia and collaborators (2022), who also assayed the release of moth sex pheromones by *N. benthamiana* plants transiently transformed with a different

combination of FAD and FAR genes than in the previous stable approach, demonstrating again the feasibility of these biosynthetic strategies. The different plant chassis examples are shown in Figure 8.

Pheromones are not the only kind of volatiles that plants can emit to control pests; actually, some endogenous plant volatiles could act synergistically with insect pheromones to induce a behavioral response (Gregg et *al.,* 2018). Herbivore-induced plant volatiles (HIPVs) are another very useful kind of VOCs, as they play an important role in tritrophic relationships between plants, herbivores, and their natural predators, thus acting as a signal for predators and parasitoids to prey on herbivore insects and defend the damaged crop (Turlings & Erb, 2018). Some metabolic engineering attempts have been made in this direction, such as the engineering of *Lotus japonicus* synthesizing (*E,E*)-geranyllinalool and other HIPV volatiles (Brillada et *al.,* 2013). However, these types of interconnections are probably too complex to serve as pest control systems through metabolic engineering interventions at this stage.

In sum, all these pieces of evidence are indicative of the utility of biological biofactories for the synthesis of sex pheromones and other interesting semiochemicals and highlight the potential of plant biofactories as promising biodispensers of volatile semiochemicals in Integrated Pest Management approaches.

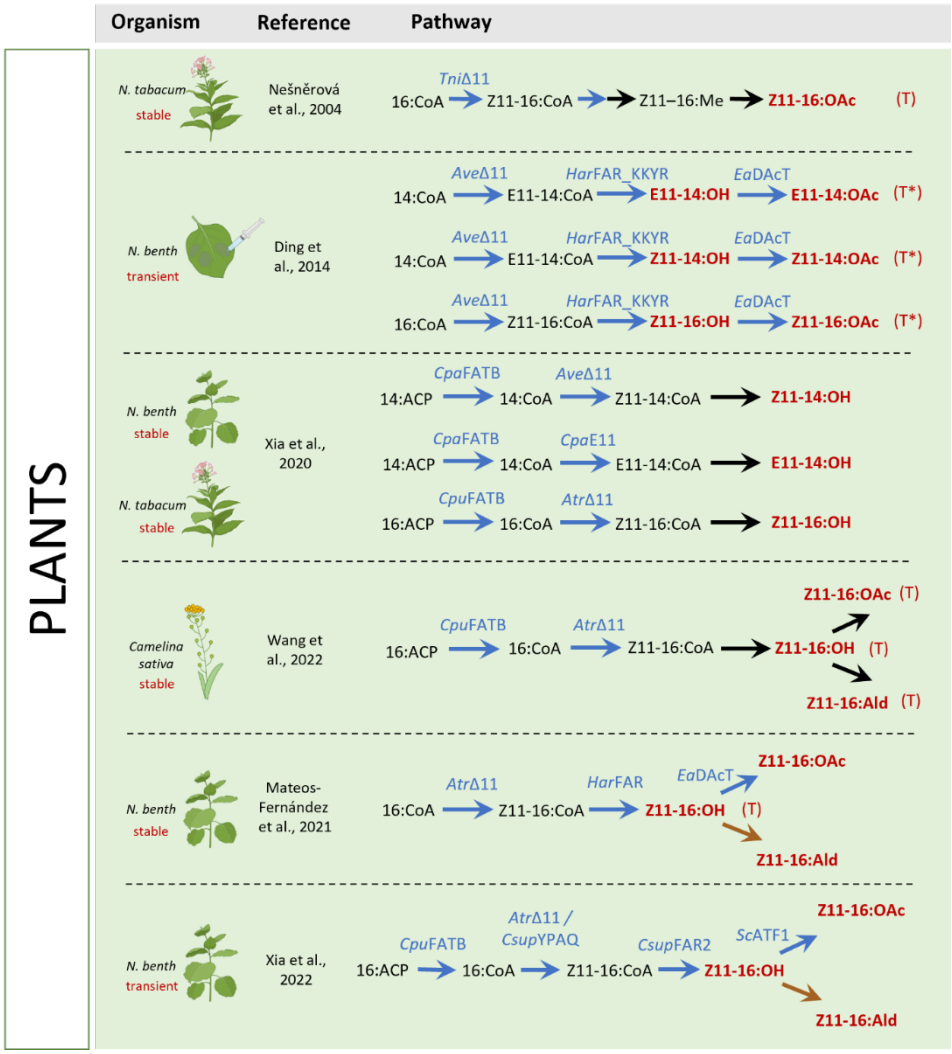


Figure 8: Strategies for the biosynthesis of lepidopteran sex pheromones. Blue arrows represent synthesis by a heterologous enzyme; black arrows represent chemical synthesis; brown arrows represent synthesis by an endogenous enzyme. Metabolites in red text are known to act as active sex pheromones. (T) indicates experimental assays have shown that the molecule affected the behaviour of moths and (T*) indicates that the behaviour assay was conducted with a chemically synthesized molecule, even if also produced via biosynthesis. *Tni*Δ11 = *Trichoplusia ni* Δ11 fatty-acyl desaturase; *Ave*Δ11 = *Argyrotaenia velutinana* Δ11 fatty-acyl desaturase; *Har*FAR_KKYR = *Helicoverpa armigera* fatty-acyl reductase with C-terminal endoplasmic reticulum retention signal; *Ea*DAcT = *Euonymus alatus* acetyltransferase; *Cpa*FATB *Cuphea palustris* 14:ACP fatty acid thioesterase; *Cpa*E11 = *Choristoneura parallela* E11 desaturase; *Cpu*FATB = *Cuphea pulcherrima* fatty acid thioesterase; *Atr*Δ11 = *Amyelois transitella* Δ11 fatty-acyl desaturase; *Har*FAR = *Helicoverpa armigera* fatty-acyl reductase; *Csup*YPAQ = *Chilo suppressalis* Δ11 fatty acyl desaturase; *Csup*FAR2 = *Chilo suppressalis* fatty-acyl reductase; *Sc*ATF1 = *Saccharomyces cerevisiae* alcohol acetyltransferase. Adapted from Mateos-Fernández et al. (2022).

Objectives

OBJECTIVES

The **main objective** of this thesis is to investigate the suitability of plants of the genus *Nicotiana* as biofactories of insect sex pheromones and other related volatile compounds using synthetic biology and metabolic engineering strategies.

To achieve this goal, the following **specific objectives** are outlined:

1. To improve and regulate the production of moth sex pheromones in *Nicotiana benthamiana* plants, especially with the aid of the CRISPRa technology, with the aim to overcome the constraints of the previously developed 'SexyPlant' *N. benthamiana* biofactories constitutively expressing a moth sex pheromone pathway.

2. To assess the ability of stable transgenic *N. benthamiana* and *N. tabacum* biofactories to produce irregular monoterpenes related to mealybug sex pheromone compounds.

Chapter 1

Optimization and regulation of moth pheromone production in 'SexyPlant' *Nicotiana benthamiana* biofactories

Part of this work was published in the following article, for which my work was essential:

Kalyani Kallam, Elena Moreno-Giménez, **Rubén Mateos-Fernández**, Connor Tansley, Silvia Gianoglio, Diego Orzáez, and Nicola Patron (2023): *Tunable control of insect pheromone biosynthesis in* Nicotiana benthamiana. Plant Biotechnology Journal, 21 (7), 1440-1453, DOI: 10.1111/pbi.14048

This chapter is an extract of the above-mentioned publication and describes those parts of the published work for which I contributed as the main experimental author. My contribution includes performing plant stable transformations, genotyping, and conducting the experiments related to volatile measurements, as well as the search, design and assembling of acetyltransferases genes.

ABSTRACT

Due to the substantial damages inflicted by Lepidoptera on crops and the abundance of Lepidopteran species, moth sex pheromones presently occupy the greatest share in pheromone market and research. Attempts at producing moth sex pheromones in biofactories had generally relied on constitutive expression of the related biosynthetic pathways. However, it has been demonstrated that such strategies usually lead to unintended toxicity in the biofactories, especially in plant-based strategies, which can compromise their viability and efficiency. Building on our previous work, in which we engineered two versions of a stable transgenic *Nicotiana benthamiana* producing and emitting moth sex pheromone compounds, this study explores alternative activatable strategies, in order to overcome the developmental impact of constitutive pheromone production. The CRISPR-based transcriptional regulators, combined with synthetic promoters, emerge as promising orthogonal tools for controlled heterologous expression.

Here, we contribute new metabolic engineering strategies for improving bioproduction of moth sex pheromones in plants. First, we explore the efficiency of new acetyltransferases in acetylating fatty alcohols, improving the conversion of the Lepidopteran pheromone compound Z11-16OH to Z11-16OAc. Second, we achieve the production of pheromone compounds via different approaches based on the dCasEV2.1 system in transient expression in *N. benthamiana*. Finally, we demonstrate the possibility to stably express an activatable insect pheromone pathway in this species and explore the regulatory bottlenecks of the system. These approaches advance the implementation of a biotechnologically based Integrated Pest Management (IPM) strategy for crop protection.

Keywords: transcriptional activation, dead-Cas, synthetic promoters, moth sex pheromones, metabolic engineering, *Nicotiana benthamiana*.

INTRODUCTION

Every year, insect pests cause huge damage to crops, heavily affecting food production yields. Strategies for the protection of plants and stored goods are required to face the problem, especially in the present scenario of climate change and rise of global population. Projections expect crop losses to become even more pronounced, with an estimated increase of 10-25% for every one-degree increase in the global mean surface temperature (Deutsch et *al.,* 2018). Agriculture has traditionally made use of pesticides (at first inorganic, and then synthetic organic compounds) to keep crop pests at bay. However, the use and abuse of these compounds have raised concerns about human health, sustainability, and loss of biodiversity, as well as about the insurgence of genetic resistance to pesticides among target pests. Alternative integrated pest management (IPM) strategies, such as the use of pheromones, have thus become crucial to guarantee environmental health and high productivity. Insect pheromones stand out for their species-specific activity, their effectiveness against insecticide-resistant insect populations, and their lower environmental impact compared to pesticides. Per contra, synthetic pheromone production is often unfortunately costly, with final products ranging in price from 500 to several thousands of USD kg^{-1}, and it can generate polluting residues (Petkevicius et *al.,* 2020).

The bulk of pheromone production is destined for the control of Lepidoptera, which stand out as one of the biggest insect orders damaging food and fiber crops, due to their great adaptability and to the rapid insurgence of genetic resistance to insecticides (Löfstedt & Xia, 2021). Lepidoptera is the second largest insect order, and it includes more than 160,000 species (Ando et *al.,* 2004). Given the great number of species of this order acting as crop pests, extensive research has been dedicated to studying lepidopteran sex pheromones: following the key discovery of bombykol in the silkworm female moth (*Bombyx mori*) in the late 1950s (Butenandt, 1959), pheromones were identified for over 720 lepidopteran species worldwide (Ando, 2023). Presently, research on lepidopteran sex pheromones still overshadows the advances in the discovery of pheromones of other insect orders. Most lepidopteran sex pheromones can be characterized as mono- or di- unsaturated long straight-chain

(C10-C18) fatty acids, derived from palmitic and stearic acids, featuring functional groups such as primary alcohols, aldehydes and acetates (Löfstedt et *al.,* 2016). Their biosynthesis is based on three essential and sequential enzymatic steps, following fatty acid biosynthesis: *i*) the introduction of double bonds at specific positions in the carbon chains (most commonly Δ9 and Δ11) by fatty acid desaturases (FADs); *ii*) the generation of fatty alcohols, by fatty acyl reductases (FARs) exhibiting different substrate specificities; *iii*) an alternative step of oxidation or esterification of these fatty alcohols, by fatty alcohol oxidases (FAOs) or fatty acyl transferases (FATs), respectively. Furthermore, additional modifications, such as chain elongation or shortening, alongside desaturation steps, contribute to determining the carbon backbone structure prior to the specification of terminal functional groups (Löfstedt et *al.,* 2016). The engineering of these metabolic pathways in heterologous biological production platforms would bypass the previously mentioned disadvantages associated with pheromone chemical synthesis. For this aim, it is necessary to identify the genes responsible for the desired enzymatic activities, either from the original organisms producing the pheromone compounds, or from other sources. Subsequently, these genes must be meticulously engineered within the designated biofactory to ensure proper functionality and expression, following strategies such as codon optimization, addition of signal peptides for subcellular compartmentalization of enzymes, or the use of promoters with the appropriate strength and expression pattern.

During the past two decades, many efforts have been directed at assaying combinations of newly discovered candidate genes for pheromone biosynthesis, identified in moth transcriptomes and genomes, expressing them in yeast and plant biofactories (Mateos-Fernández et *al.,* 2022; Petkevicius et *al.,* 2020). In fact, several moth FADs, such as *Ase*Δ11 from *Agrotis segetum* (Hagström et *al.,* 2013) and *Csup*YPAQ or *Csup*KPSE from *Chilo suppressalis* (Xia et *al.,* 2022), yielded high heterologous activities when expressed, respectively, in yeast and in both yeast and *N. benthamiana* leaves. On the other hand, the enzymatic activity of numerous lepidopteran FARs, such as *Ase*FAR from *A. segetum* (Hagström et *al.,* 2013) and *Csup*FAR2 from *C. suppressalis* (Xia et *al.,* 2022), resulted in good conversion rates for

several substrates. However, the parallel search of active FATs did not lead to any active candidate gene, as showed by B. J. Ding & Löfstedt, (2015), who assayed 34 candidate acetyltransferases, none of which yielded the desired activity when expressed in yeast with proper fatty alcohol supplementation. Fortunately, some active FAT enzymes have been identified from other sources. One successful example of the recreation of a functional moth sex pheromone pathway is that of the genetically engineered 'SexyPlant' or 'SxP', a *Nicotiana benthamiana* biofactory developed in our group (Mateos-Fernández et *al.,* 2021). SxP served as proof of concept for the creation of stable biodispensers emitting volatile pheromone compounds, and constitutes the premise to further explore the requirements and constraints of heterologously producing moth sex pheromones in plant biofactories, which will be addressed in this chapter.

The pheromone blend of SxPs is composed of (*Z*)-11-hexadecen-1-ol (Z11-16OH), (*Z*)-11-hexadecenyl acetate (Z11-16OAc), and (*Z*)-11-hexadecenal (Z11-16Ald), and it was obtained expressing a desaturase from *Amyelois transitella* (*Atr*Δ11), a reductase from *Helicoverpa armigera* (*Har*FAR), and the plant diacylglycerol acetyltransferase *Ea*DAcT from *Euonymus alatus*. These genes were expressed under the control of the constitutive CaMV35S promoter, and the corresponding transcriptional units assembled in one single GoldenBraid construct. The choice of biosynthetic genes was, in turn, based on the work of Ding et *al.,* (2014), where their transient expression resulted in the successful production of these compounds in *N. benthamiana* leaves. The first version of this pheromone-producing plant (SxPv1.0) was shown to produce Z11-16OH at appreciable levels, while Z11-16OAc was present only in very modest amounts; unexpectedly, the production of Z11-16Ald was also detected, which correlated with Z11-16OH levels. Since no specific gene was introduced for the biosynthesis of this compound, it was assumed that it was produced by an endogenous alcohol oxidase. It was also very clear from the onset of this work that plant size and biomass were severely affected by the levels of production of pheromone compounds. Two populations were clearly identified in the transgenic progeny, called high- and low-producers, whose phenotypes were clearly distinguishable: chlorotic and stunted for the high producers, and wild type-like for

the low producers. Through transcriptomic analyses, several truncations were discovered in the *EaDAcT* gene, explaining the low levels of Z11-16OAc detected in SxPv1.0 plants. So, an improved version of the biofactory, called SxPv1.2, was produced, ensuring it harbored a complete and intact pheromone biosynthetic pathway. When analyzed by metabolite solvent extraction, SxPv1.2 indeed produced moderate levels of Z11-16OAc (on average, 11.8 µg g^{-1} FW), while still biosynthesizing high levels of Z11-16OH (on average, 111.4 µg g^{-1} FW). Also remarkable was the capability of SxPv1.2 plants to act as living biodispensers, being able to release the alcohol and acetate forms of the pheromone at a considerable rate (estimated on average at 8.48 ng g^{-1} FW per day for Z11-16OH and 9.44 ng g^{-1} FW per day for Z11-16OAc), and highlighting the – predictable – higher volatility of the acetates, which have a reduced affinity for the aqueous environment of the mesophyll compared with the alcohols. Moreover, this new SxPv1.2 genotype also showed a slight recovery from the severe dwarfism affecting SxPv1.0 high pheromone producers. In general, despite success in producing the desired compounds, we noticed several bottlenecks in this constitutive overexpression strategy. As suggested in another recent work stemming from this project (Juteršek et *al.,* 2022), these bottlenecks are intrinsically related to several harmful transcriptional and metabolic disturbances, deriving from the constitutive overexpression of this metabolic pathway as well as from a signaling pathway disorder. One of the severest negative effects was the reduction of plant growth and of biomass accumulation by up to 90%, leading to premature death. Other phenotypes, as can be seen in Figure 1, include yellowing and leaf petioles growing at a steeper angle than in wild type individuals, in a 'cabbage-like' fashion uncharacteristic of *N. benthamiana*.

Toxicity in SxP was possibly related to the accumulation of Z11-16OH in the leaves, as earlier pointed out by Xia et *al.,* (2020). Therefore, as a first strategy for the improvement of the biofactory, we aimed at decreasing the Z11-16OH levels in the plant. To do so, we explored the activity of alternative acetyltransferases that might lead to an improvement in the alcohol:acetate conversion rate and possibly have a detoxification effect, based on the higher volatility of the acetates and the correspondingly lower accumulation in plant tissues. This higher acetate:alcohol ratio

is desirable not only for better fitness, but also for achieving compound ratios closer to the active blends of known moths. For example, the most active blend for *Sesamia nonagrioides* (Lefèbvre) is a 90:10:5 blend of Z11-16OAc:Z11-16OH:Z11-16Ald.

Figure 1: Comparison of the phenotypes of SxPv1.0 T_3 SxPv1.2 T_1 and WT plants. Physiological development between SxPv1.0 5_1_7_X (T_3), SxPv1.2 4_X (T_1) and WT *Nicotiana benthamiana* plants at the young and adult stage. Pictures were taken from representative individuals at young (4 weeks after transplant) and adult (7 weeks after transplant) stages. (Adapted from Mateos-Fernández *et al.,* 2021)

A second, more radical approach to remedy the fitness loss is to avoid constitutive overexpression and explore conditional activation instead, turning the pheromone biosynthetic pathway into an activable pathway, whose expression is induced only when sufficient biomass has been accumulated. This requires a synchronized induction of the different pheromone biosynthetic genes.

The coordinated activation of several genes in a heterologous pathway poses a recurrent engineering problem in plant SynBio. Whereas conditional activation of a single gene can be easily achieved using a (e.g. chemically) inducible promoter, the limited availability of such promoters often leaves as only engineering option the use of the same repeated promoter controlling all transcriptional units. This is a far from ideal solution since repeated sequences often lead to gene silencing in subsequent generations.

Recently it was shown that conditional transcriptional activation can be engineered with the interplay of CRISPR-based activation (CRISPRa) tools, a sort of orthogonal and programable synthetic transcriptional regulators that allow simultaneous activation of several downstream genes (X. Ding et *al.,* 2022; Selma et *al.,* 2022; Vazquez-Vilar et *al.,* 2021).This strategy is based on the use of inactivated ('dead') endonucleases like dCas9, in which the nuclease activity is removed through point mutations. The inactivation of the endonuclease domains does not impact the ability to bind DNA in a programmable fashion. Thus, by attaching transcriptional regulatory domains (activators or repressors) to the dCas9, gene promoters can be targeted via a custom-designed 20-nucleotide guide RNA (gRNA) and consequently activated or repressed. This approach also allows the configuration of several gRNAs in a multiplexed fashion, expressing them as a polycistronic transcript. Multiplexed gRNAs can either target the same gene to achieve higher activation levels or, alternatively, and especially relevant in the context of this work, be directed at different promoters to regulate several genes at the same time. CRISPRa systems are based on fusions of dCas proteins with transcriptional activator domains, which are carried by the inactivated endonuclease to the target promoter (Figure 2). Moreover, the transcriptional response can be further amplified by using modified gRNA scaffolds to allow the anchoring of further activator domains. Among other CRISPRa tools, the development of the dCasEV2.1 system by Selma et *al.,* (2019) is remarkable for our purposes (Figure 2A). This programmable transcriptional activator comprises the following elements: (i) a dCas9 fused to the EDLL transcriptional activation domain (Tiwari et *al.,* 2012), (ii) a gRNA with two aptamers in its scaffold that bind the viral coat protein MS2 (Kunii et *al.,* 2018), and (ii) the MS2 protein itself fused to the so-

called VPR activation domain (a tandem combination of three activation domains, namely VP64, p65 and Rta) (Chavez et *al.,* 2015). The dCasEV2.1 system has been shown to achieve strong and specific activation of endogenous *N. benthamiana* genes, as described by Selma et *al.,* (2019) and (Selma, Sanmartín, et *al.,* 2022).

The dCasEV2.1 system has also been successfully combined with GoldenBraid-based minimal synthetic promoters (GB_SynP) (Moreno-Gimenez et *al.,* 2022). The GB_SynP promoter collection of custom-designed promoters based on the model sequence of the tomato *SlDFR* promoter (p*SlDFR*) (Figure 2B), which conserve TATA and 5'UTR regions in the named A3 minimal promoter part, fused to one or multiple target sequence(s) of the gRNA (A2 proximal part) and a longer random sequence at their 5' end (A1 distal part). The co-expression of the selected gRNAs next the dCasEV2.1 specifically activate the synthetic promoters containing the gRNA target. This system has been shown to possess negligible basal expression levels in the absence of the corresponding gRNAs/dCasEV2.1 pair (Moreno-Giménez et *al.,* 2022).

In this work, we aimed at advancing towards the improvement of the SxP moth pheromone bioproduction concept in plants by (i) improving the acetylation of fatty-acid derivatives suing alternative acetyltransferases an (ii) approaching cascade regulation of the moth sex pheromone metabolic pathway using the dCasEV2.1 system in combination with GB_SynP promoters.

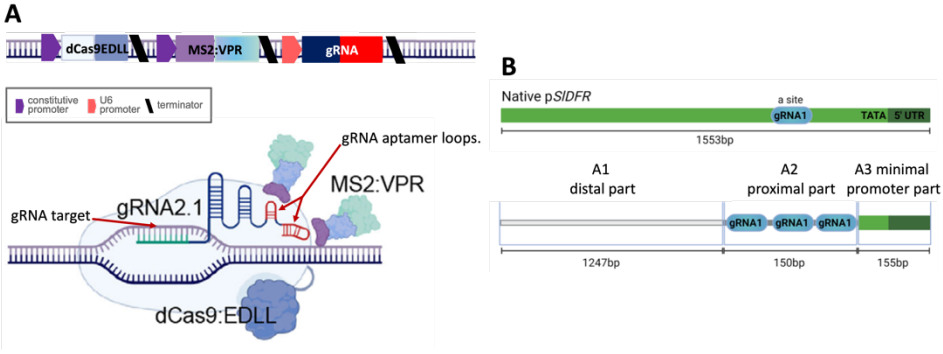


Figure 2 (previous page): Schematic representation of CRISPRa tools exemplified by dCasEV2.1-mediated transcriptional activation system and synthetic promoters. (A) dCasEV2.1 genomic sequence (upper part) codes for a "dead" version of Cas9 (dCas protein, lower part), fused to the EDLL transcriptional activation domain. The programable transcriptional activator also counts with the viral coat protein MS2, fused to the three activation domains (abbreviated, VPR), and that binds the gRNA2.1 by the two aptamer loops in gRNA scaffold. By means of all these elements, dCasEV2.1 system can achieve strong activation of a target genomic region, determined by the specificity provided by gRNA target design. **(B)** In the upper part, schematic representation of the native *SlDFR* promoter (p*SlDFR*) employed as a model sequence for the design of the GB_SynP collection. In the lower part, schematic representation of promoter parts in GB_SynP collection: A1 distal part includes a long genomic random sequence, followed by a A2 proximal part including one or multiple gRNA target sequences, interspersed by short random sequences, and a A3 minimal promoter part (mDFR), including TATA box and 5'UTR from *SlDFR*. Adapted from Selma et al. (2022) and Moreno-Giménez et al. (2022).

MATERIALS AND METHODS

DNA assembly and cloning

All constructs used for plant transformation were assembled using the GoldenBraid (GB) cloning system (Sarrion-Perdigones et *al.,* 2011; Vazquez-Vilar et *al.,* 2017). Standardized DNA parts (promoters, coding sequences and terminators) were designed, synthesized, and cloned as Level 0 parts using the GB domestication strategy described by Sarrion-Perdigones et *al.,* (2013). Once cloned into a pUPD2 vector, these new DNA elements were verified by enzymatic digestion and by sequencing. Transcriptional units (level 1 parts) were then assembled via multipartite *Bsa*I restriction-ligation reactions from level 0 parts, while level > 1 modules were produced via binary *Bsa*I or *Bsm*BI restriction–ligation. All level ≥ 1 parts were confirmed by restriction enzyme analysis. All GB constructs created and/or employed in this study are provided in Table S1 at the end of this chapter and have been deposited at Addgene. Details about their sequences are publicly available at https://goldenbraidpro.com/.

The *Escherichia coli* TOP 10 strain was used for cloning of all constructs, and transformation was performed by employing the Mix & Go kit (Zymo Research) following the manufacturer's instructions. The final expression vectors were transformed into electrocompetent *Agrobacterium tumefaciens* strains GV3101 or

EHA105 for transient expression, or *A. tumefaciens* strain LBA4404 for stable transformations.

Sequence search and filtering

BLASTn or BLASTx searches (https://blast.ncbi.nlm.nih.gov/Blast.cgi) were conducted to identify additional moth candidate sequences. Searches were conducted against non-redundant (nr) NCBI databases, filtering for butterfly and moth sequences (taxid 7088). The remaining parameters were set as default (*e*-value cut-off 1.0E5). The Softberry prediction software (http://www.softberry.com/berry.phtml) was used for trimming the identified sequences and predict start and stop codons. Finally, candidate sequences were adapted to *N. benthamiana* codon usage with the IDT Codon Optimization Tool (https://eu.idtdna.com), and domesticated using GB (https://goldenbraidpro.com/) cloning tools. Domains were annotated using blastp and the Prosite MyDomains Image Creator tool. The phylogenetic tree of the candidate sequences was created with Phylogeny.fr (www.phylogeny.fr/index.cgi), using the "OneClick" option (Dereeper et *al.,* 2008).

Transient expression assays and activation of moth sex pheromone pathways in *Nicotiana benthamiana*

Transient expression assays to validate gene activity and to activate incomplete metabolic pathways were carried out through infiltration of *N. benthamiana* leaves mediated by *A. tumefaciens*, strain GV3101. Precultures of *A. tumefaciens* carrying the constructs of interest were grown from glycerol stocks for 2 days at 28 °C 250 rpm with the appropriate antibiotics until saturation, then refreshed by diluting them 1:1000 in LB liquid medium supplemented with appropriate antibiotics and grown overnight in the same conditions. Cells were pelleted by centrifugation at 3400 x *g* for 15 min at RT and resuspended in agroinfiltration buffer containing 10 mM 2-(N-morpholino) ethanesulfonic acid (MES), pH 5.7, 10 mM $MgCl_2$, and 200 µM

acetosyringone, then incubated for 2 h in dark and RT conditions under slow shaking. The OD_{600} of each culture was adjusted to reach a value of 0.05-0.06 in the final culture mixtures. Each mixture had a final OD_{600} value of 0.2. Equal volumes of each culture were mixed, including the silencing suppressor P19 for co-infiltration to reduce post-transcriptional gene silencing (Garabagi et *al.,* 2012). The relative abundance of each *A. tumefaciens* culture was kept constant in all infiltration mixtures by adding an *A. tumefaciens* culture carrying an empty vector when needed. Agroinfiltration was carried out with a 1 mL needless syringe through the abaxial surface of the three fully expanded leaves of 4–5-week-old *N. benthamiana* plants. In activation assays, half of the leaf was agroinfiltrated with *A. tumefaciens* cultures harboring activator constructs, while the other half was agroinfiltrated with cultures carrying control constructs. Plants were then grown at 24 °C (light)/20 °C (darkness) with a 16:8 h light:darkness photoperiod. Samples were collected 5 days post-infiltration (dpi) using a 1.5-2 cm corkborer and snap frozen in liquid nitrogen.

Stable plant transformation

Stable transgenic plants were generated following the transformation protocol reported by Clemente, (2006). Leaf disks were isolated from 4–5-week-old WT, T_3 dCasEV2.1 or T_1 NGP *N. benthamiana* plants grown at 24 °C (light)/20 °C (darkness) with a 16 : 8 h light : darkness photoperiod, disinfected by washing in a 2.5% sodium hypochlorite solution for 15 min, then rinsed in 70% ethanol for 10 s and washed 3 times for 15 min in sterile distilled water. Around 200 disks per transformation were cut employing a Ø 0.8-1.2 cm corkborer, then transferred to co-culture plates (Murashige and Skoog medium supplemented with vitamins, 8 g/L phytoagar and enriched with 1 mg/L 6-benzylaminopurine and 0.1 mg/L naphthalene acetic acid). After a 24 h incubation on this medium, a LBA4404 *A. tumefaciens* culture carrying the desired construct and grown overnight to an OD_{600} of 0.2-0.5 in TY medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl, pH = 5.6) supplemented with 2 mM $MgSO_4 \cdot 7H_2O$, 200 µM acetosyringone and the appropriate antibiotics was used to infect disks during 15 min. After infection, disks were returned to co-culture plates

and incubated for 48 h in the dark. Shoot development was induced by incubation of disks on MS medium plates supplemented with vitamins, 8 g/L phytoagar, 1 mg/L 6-benzylaminopurine and 0.1 mg/L naphthalene acetic acid, carbenicillin for *A. tumefaciens* eradication and the appropriate antibiotic for selection of the transformants. After 3-4 weeks of growth in shooting-induction medium with weekly transfers to fresh medium, shoots were transferred to a root-inducing medium (MS supplemented with vitamins, 8 g/L phytoagar and the selection agent when appropriate). Plant tissue culture was carried out in a growth chamber (16:8 h light:darkness photoperiod, 24 °C, 60 %–70 % humidity, 250 µmol /(m^2/s). Rooted plantlets were transferred to soil and grown in a greenhouse at 24 : 20 °C (light : darkness) with a 16 : 8 h light : darkness photoperiod.

Plant growth and selection

Transgenic seeds were disinfected by incubating 50 mg of seeds under suspension in 10% trisodium phosphate dodecahydrate for 20 min, followed by incubation under suspension in 3% sodium hypochlorite for 20 min. Then, seeds were washed in sterile distilled water and sown on germination medium (4.9 g/L MS with vitamins, 30 g/L sucrose, 9 g/L Phytoagar, pH = 5.7) supplemented with 100 mg/L kanamycin for positive transgene selection. Control plants were obtained similarly, by placing seeds on a non-selective germination medium. WT and antibiotic-resistant seedlings were transferred to the greenhouse 15 days after germination, where they were grown at 24 : 20 °C (light : darkness) with a 16 : 8 h light : darkness photoperiod.

The best T_0 plants selected for further characterization in the T_1 generation were chosen based on their production rates after induction by agroinfiltration with dCasEV2.1, as well as for the early formation of capsules and seeds.

The segregation of transgenes and the estimation of transgene copy number were determined by germinating seeds on selective media and by calculating survival percentages. T_0 lines were assumed to be multiple copy lines for the transgene when

segregation of the transgene was not possible in the T_1 progeny, and no segregation was detected in the following transgenic generations.

dCasEV2.1:YFP *N. benthamiana* seeds were kindly provided by Dr Sara Selma, being the progeny of the élite line presented in Selma et *al.,* (2022)**.**

Crosses between transgenic lines

Flowers of *N. benthamiana* transgenic lines used as female parentals were emasculated 1-2 days before anthesis, then manually pollinated with pollen of plants used as male parentals. Pollinated pistils were quickly covered with a transparent film to maintain humidity, protect fecundation, and prevent undesired pollen from fertilizing the female organ.

Seeds from successfully fertilized flowers were disinfected as detailed above. Then, seeds were sown on germination medium (4.9 g/L MS with vitamins, 30 g/L sucrose, 9 g/L Phytoagar, pH = 5.7) supplemented with both 100 mg/L kanamycin and 50 mg/L hygromycin for double positive transgene selection. Controls for the crossing assay were taken by fertilizing female flowers with pollen of the same plant and sowing in a medium with the same double antibiotic selection as done in real crosses between lines.

Analysis of volatile organic compounds (VOCs)

Standards, extraction methods and analysis of pheromone compounds were as previously described (Mateos-Fernández et *al.,* 2021). Briefly, synthetic samples of Z11-16OH were obtained as described by Zarbin et *al.,* (2007) and purified by column chromatography using silica gel and a mixture of hexane: Et_2O (9 : 1 to 8 : 2) as an eluent. Acetylation of Z11-16OH was carried out using acetic anhydride (1.2 eq) and trimethylamine (1.3 eq) as a base in dichloromethane (DCM), generating the corresponding acetate in 95% yield, whose spectroscopical data was fully coincident

with that described in the literature (Zarbin et *al.,* 2007). Oxidation with pyridinium chlorocromate of a 100 mg sample of Z11-16OH was carried out following the method described by Zakrzewski et *al.,* (2007), generating 62 mg (60%) of Z11-16Ald, whose spectroscopical data was fully coincident with that described in the literature (Zakrzewski et *al.,* 2007).

For biological samples, 50 mg of frozen, ground leaf samples were weighed in a 10 mL headspace screw-cap vial and stabilized by adding 1 mL of 5M $CaCl_2$ and 150 µL of 0.5M EDTA (pH = 7.5). Tridecane was here added to a final concentration of 10 ppb for use as an internal standard, after which they were immediately bath-sonicated for 5 min. Volatile compounds were captured by means of headspace solid phase microextraction (HS-SPME) with a 65 µm polydimethylsiloxane/divinylbenzene (PDMS/DVB) SPME fiber (Supelco, Bellefonte, PA, USA). Volatile extraction was performed automatically by means of a CombiPAL autosampler (CTC Analytics). Vials were first incubated at 80 °C for 3 min with 500 rpm agitation. The fiber was then exposed to the headspace of the vial for 20 min under the same conditions of temperature and agitation. Desorption was performed at 250 °C for 1 min (splitless mode) in the injection port of a 6890N gas chromatograph (Agilent Technologies) coupled to a 5975B mass spectrometer (Agilent Technologies). Chromatography was performed on a DB5ms (60 m, 0.25 mm, 1 µm) capillary column (J&W) with helium as the carrier gas at a constant flow of 1.2 mL/min. Data were recorded in 5975B mass spectrometer in the 35–300 m/z range at 20 scans/s, with electronic impact ionization at 70 eV. For initial identification of the pheromone peaks, oven programming conditions were 40 °C for 2 min, 5 °C/min ramp until reaching 280 °C, and a final hold at 280 °C for 5 min. Once the target peaks were identified, the oven conditions were changed to an initial temperature of 160 °C for 2 min, 7 °C/min ramp until 280 °C, and a final hold at 280 °C for 6 min, to reduce the overall running time without losing resolution of the desired compounds. Identification of compounds was performed by the comparison of both retention time and mass spectrum with pure standards (for pheromones) or by comparison between the mass spectrum for each compound with those of the NIST 2017 Mass Spectral library. Relative quantifications were assessed by integrating peak areas for each compound, for a selected Quantifier Ion (QI). The

chosen QIs were the following: Z11:16OH QI = 95; Z11:16OAc QI = 95; Z11:16Ald = 82. All pheromone values were divided by the tridecane value of each sample for normalization. Alternatively, in Figure 3F, pheromone values were normalized using the total ion count (TIC) of the corresponding sample (Y. Wu & Li, 2016). Chromatograms were processed by means of the Agilent MassHunter software (Agilent Technologies). For estimation of the yields, a calibration curve was constructed for each pheromone from a set of 7 peak areas ranging from 0.005 to 20 ppm, normalized with the tridecane values. Yields were estimated by applying this curve to the mean value of the three biological replicates.

Statistical analysis

Statistical analyses were performed using the Statgraphics v. 19 and GraphPad Prism v8.0.2 (GraphPad Software, San Diego, CA, USA).

RESULTS

1. Improving the acetylation activity of the original SexyPlant with alternative acetyltransferases

Heterologous production of moth sex pheromone compounds from endogenous C16 fatty acyl CoA has previously been achieved by constitutive expression of a Δ11 desaturase, a fatty acid reductase and a diacylglycerol acetyltransferase (Figure 3A). The genes encoding moth acetyltransferases involved in pheromone biosynthesis have not yet been identified, because all putative candidates did not prove to be active when tested in different biological hosts (Fujii et *al.,* 2010; B. J. Ding & Löfstedt, 2015). In previous examples of heterologous moth pheromone biosynthesis, the enzymes employed were derived from plants (*Euonymus alatus EaDAcT*; EC 2.3.1.20) (B. J. Ding et *al.,* 2014), or from yeast (*Saccharomyces cerevisiae ScATF1*; EC 2.3.1.84)

(B. J. Ding et *al.,* 2016). We wanted to explore new alternative enzymes putatively able to yield this enzymatic activity.

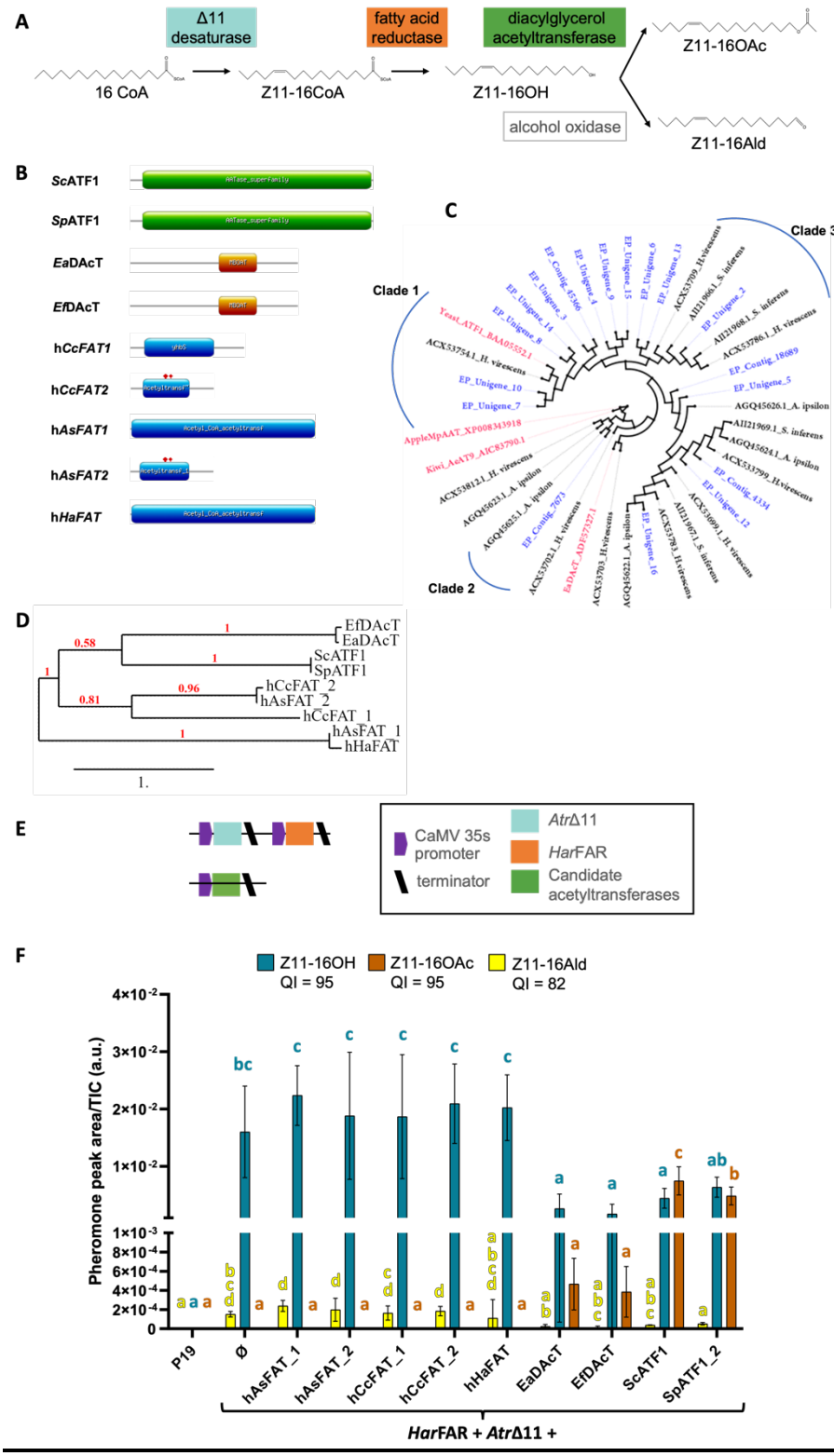
Candidate sequences were selected from plant, yeast and insect sources (Figure 3B). The plant diacylglycerol acetyltransferase from *Euonymus fortunei* (*EfDAcT*) (Tran et *al.,* 2017) appeared to fit as a good candidate, given the similarities with the here-functional *EaDAcT.* As the *Euonymus alatus* acetyltransferase, *Ef*DAcT shows a promising sequence domain from the same MBOAT (membrane-bound O-acyltransferase, pfam13813) family, as well as the asparagine and histidine residues presumably related to this catalytic function. Similarly, the alcohol acetyltransferase *SpATF1-2*, from *Saccharomyces pastorianus*, could also be efficient in enzymatic conversion (Yoshimoto et *al.,* 1999), given its high similarity with *ScATF1*. In addition, we looked for candidate genes with the desired acetyltransferase activity in several moth transcriptomes. The search for potential fatty acyl transferases (FAT) was based on the work of Antony et *al.,* (2015), where several sequences were identified as candidates for different steps of pheromone biosynthesis in the transcriptome of the pheromone gland of *Cadra cautella.* We selected representative *C. cautella* sequences and other related sequences either to test *in planta* or as queries to conduct further homology-based searches. A phylogenetic tree grouping *C. cautella*-derived candidate acetyltransferase sequences with sequence of insect, yeast and plant origin is shown in Figure 3C.

A first clade observed in the tree is that of sequences grouping with *S. cerevisiae* ATF1, which is known to be a functional FAT for moth pheromone production (B. J. Ding et *al.,* 2016). The EP_Unigene_10_FAT gen belongs to this clade and was chosen to be functionally tested *in planta* and named *hCcFAT_1* (hypothetical *C. cautella* FAT_1).

A second clade is that of transcripts grouping with FAT-like sequences from the moth *Agrotis ipsilon*, which produces pheromone blends containing C12, C14 and C16 acetates, and should therefore possess versatile acetyltransferases accepting a variety of substrates of different lengths. In this group, EP_contig_7673 was chosen for its high shared identity (95%) with a FAT-like *A. ipsilon* sequence and named *hCcFAT_2*. Using *hCcFAT_2* as query in a BLASTn search against sequences from other moths, one *Agrotis segetum* sequence (*hAs*FAT_1) was identified as a putative fatty alcohol

acetyltransferase, sharing an 81.25% identity with *hCcFAT_2*. Like *A. ipsilon*, *A. segetum* also biosynthesizes several acetate pheromone compounds, making *hAs*FAT_1 an interesting acetyltransferase candidate to act on substrates of different lengths.

Figure 3 (next page): Testing of potential acetyltransferases for the heterologous production of Lepidopteran sex pheromones. (A) Pathway for the production of the two main volatile components of many Lepidopteran sex pheromones (*Z*)-11-hexadecen-1-ol (Z11-16OH) and (*Z*)-11-hexadecenyl acetate (Z11-16OAc) in plants from endogenous 16C fatty acyl CoA (Z11-16CoA). Successful examples included a Δ11 desaturase, a fatty acid reductase and a diacylglycerol acetyltransferase. The accumulation of (Z)-11-hexadecenal (Z11-16:Ald) was also observed, presumably catalyzed by an endogenous alcohol oxidase (Mateos-Fernández et *al.*, 2021). **(B)** Selected candidates for plant, yeast and insect acetyltransferases. Yeast candidates belong to the alcohol acetyltransferase superfamily, while plant candidates show distinctive MBOAT (membrane-bound O-acyltransferase) domains. Insect sequences show either the acetyltransferase_1 domain (pfam00583, h*Cc*FAT2 and h*As*FAT2), the acetylCoA acetyltransferase domain (COG0183, h*As*FAT1 and h*Ha*FAT) or the yhbS domain (COG3153, h*Cc*FAT1). Red dots in h*Cc*FAT2 and h*As*FAT2 indicate residues constituting a coenzyme-A binding pocket. **(C)** Phylogenetic tree grouping C. cautella candidate acetyltransferase sequences with sequence of insect, yeast and plant origin, adapted from Antony et *al*. (2015). **(D)** Phylogenetic tree of the candidate genes tested in this assay. Bootstrap value n = 1000 iterations. **(E)** Schematics of the design of related GB constructs used for screening of acetyltransferase activity by transient expression in *N. benthamiana*. **(F)** Differences in the relative quantities and ratios of Z11-16OAc, Z11-16OH and Z11-16Ald obtained by co-expression of diacylglycerol transferases from *Euonymus alatus* (*Ea*DAcT), E. fortunii (*Ef*DAcT), *Saccharomyces cerevisiae* (*Sc*ATF1) and *S. pastorianus* (*Sp*ATF1-2) with a fatty acid reductase from *Helicoverpa armigera* (*Har*FAR) and a Δ11 desaturase from *Amyelois transitella* (*Atr*Δ11) providing the Z11-16OH precursor. Compounds were measured by HS-SPME GCMS in agroinfiltrated *N. benthamiana* ground leaves, at 5 dpi. Values shown are the mean and standard deviation of n=3 biological replicates (independent infiltrations), normalized with the total ion count (TIC) area. QI = Quantifier Ions for relative quantification. Samples annotated with a common letter are not significantly different by a one-way ANOVA with post-hoc Fisher LSD at the 5% level of significance. The same color is used to denote bars and letters representing classes of significance for each compound.


A
Δ11 desaturase
fatty acid reductase
diacylglycerol acetyltransferase
16 CoA
Z11-16CoA
Z11-16OH
Z11-16OAc
alcohol oxidase
Z11-16Ald
B
ScATF1
SpATF1
EaDAcT
EfDAcT
hCcFAT1
hCcFAT2
hAsFAT1
hAsFAT2
hHaFAT
C
Clade 1
Clade 2
Clade 3
D
EfDAcT
EaDAcT
ScATF1
SpATF1
hCcFAT_2
hAsFAT_2
hCcFAT_1
hAsFAT_1
hHaFAT
0.58
0.81
0.96
1.
E
CaMV 35s promoter
terminator
AtrΔ11
HarFAR
Candidate acetyltransferases
F
Z11-16OH QI = 95
Z11-16OAc QI = 95
Z11-16Ald QI = 82
Pheromone peak area/TIC (a.u.)
P19
Ø
hAsFAT_1
hAsFAT_2
hCcFAT_1
hCcFAT_2
hHaFAT
EaDAcT
EfDAcT
ScATF1
SpATF1_2
HarFAR + AtrΔ11 +

Finally, a third clade was identified as containing transcripts clustering with FAT-like sequences from the moth *Sesamia inferens*, which also produces acetate pheromone compounds. The *C. cautella* EP_Unigene_2_FAT sequence was chosen as a reference for this group, given its low *e*-value and high % identity with a putative acetyltransferase from *Danaus plexippus*, and used as query for a BLASTX search against moth sequences, obtaining the putative *hAs*FAT_2 from *A. segetum* and *hHa*FAT from *Helicoverpa assulta*. These were also selected for further testing because both moth species are known to produce C16 acetates among their pheromone compounds. The similarities and relationships between the selected candidates are summarized in the phylogenetic tree in Figure 3D. The details of all selected sequences are reported in the Supplementary Data at the end of this chapter.

To assay their potential as acetyltransferases accepting C16 fatty alcohols as substrates each candidate gene was assembled in a separate GoldenBraid vector, regulated by the strong Cauliflower mosaic virus 35S promoter (pCaMV35S) and the nopaline synthase terminator (tNOS). Then, each candidate was co-expressed with a construct containing the coding sequences of the Δ11 desaturase from *Amyelois transitella* (*Atr*Δ11) and the fatty acid reductase from *Helicoverpa armigera* (*Har*FAR) (Figure 3E).

The total volatile organic compound (VOC) profile of samples was analyzed at 5 dpi. We found that none of the sequences from moth transcriptomes showed any activity, so the fatty alcohol Z11-16OH generated by the other two enzymes of the pathway (*Atr*Δ11 and *Har*FAR) remained intact (Figure 3F). Nevertheless, the acetylation of Z11-16OH into Z11-16OAc was achieved by both yeast enzymes (*Sc*ATF1 and *Sp*ATF1-2), at similar rates, and by both plant enzymes (*Ea*DAcT and *Ef*DAcT), whose activities were also comparable between them. The production of Z11-16OAc using both yeast enzymes was much higher than that with the plant enzymes. The fluctuation in aldehyde levels is also remarkable: these are notable when the acetyltransferase is not present or is not functional, and they decrease to trace levels when a functional acetyltransferase comes into play, probably because it pulls from the Z11-16OH pool.

Given these results, *Sc*ATF1 was chosen as the preferred acetyltransferase to act upon a C16 fatty alcohol substrate in further experiments.

2. Building a pheromone biosynthesis pathway controlled by CRISPRa

The design of an improved pheromone biofactory continued with the incorporation of CRISPR-mediated control, now including *ScATF1* acetyltransferase. To this end, each of the three genes responsible for pheromone biosynthesis (*AtrΔ11*, *HarFAR* and *ScATF1*) was set under the control of an orthogonal synthetic promoter from the GB_SynP collection. The three synthetic promoter were different in most of their sequence, and all three of them comprised three parts, from 3´to 5´: (i) a constant minimal promoter sequence based on the model sequence of the tomato *SlDFR* gene promoter (pSlDFR); (ii) a proximal region containing multiple copies of the target sequence for a single gRNA, acting as cis boxes; and (iii) a distal region with a randomized sequence, accounting for the variability between promoters. Combining these target sequences with the dead Cas9 activator system (dCasEV2.1, Selma et *al.,* 2019) carrying the appropriate gRNA (g1DFR), will allow dCasEV to precisely recognize gRNA targets within the synthetic promoters, positively regulating the transcription of the three genes of the pheromone pathway. So, with these premises, two alternative strategies were designed to regulate the moth pheromone biosynthetic pathway (Figure 4A). In the first strategy, the three biosynthetic genes were co-assembled in a single T-DNA together with the specific g1DFR, and the CRISPRa function was provided in a different construct. This strategy was designated as Guided-Pathway (GP, GB3897) due to the presence of the gRNA in the design. In the second strategy, a construct was assembled only with the three transcriptional units containing the biosynthetic genes, while g1DFR was provided in a separated construct together with the rest of CRISPRa elements. This strategy was named Non-Guided-Pathway (NGP, GB3898) because of the absence of the guide RNA in the pheromone pathway construct. Both strategies were conceived to depend on the exogenous

addition of, at least, the dCasEV2.1 element to activate pheromone biosynthesis, and to include the kanamycin resistance gene for further employment in stable plant expression.

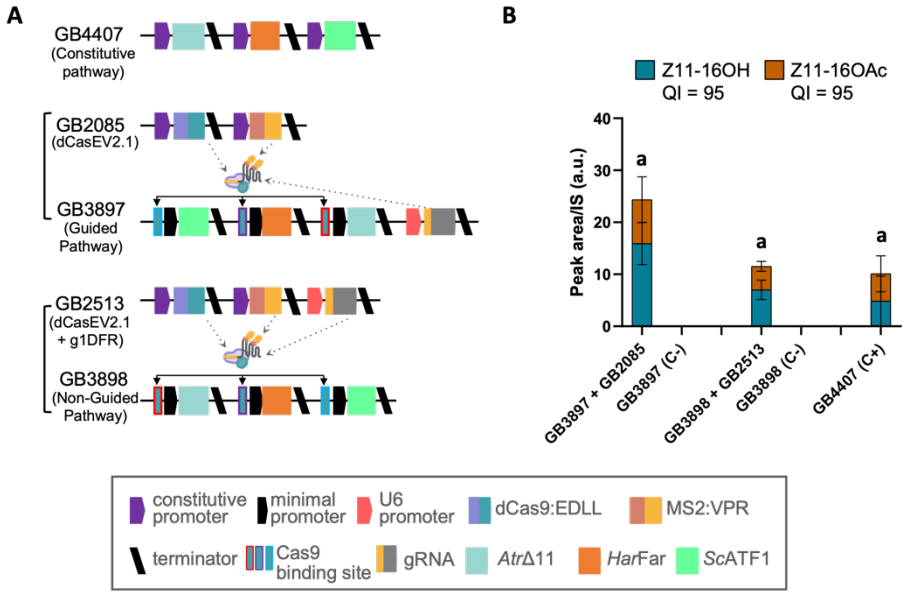


Figure 4: CRISPR/Cas9-mediated control of pheromone biosynthesis. (A) Schematic representation of the constructs for constitutive (GB4407) or dCasEV2.1-activated expression of AtrΔ11, HarFAR and ScATF1 (GB2085+GB3897 and GB2513+GB3898). For dCasEV2.1-mediated regulation, synthetic promoters consisting of a minimal DFR core promoter fused to one of three unique sequences containing the conserved gRNA target sites are used. **(B)** Yields of pheromone components (Z11-16OH and Z11-16OAc) obtained following transient agroinfiltration. Compounds were measured by HS-SPME GCMS in agroinfiltrated *N. benthamiana* ground leaves, at 5 dpi. Production values represent the mean and standard deviation of n = 3 biological replicates (independent agroinfiltrated leaves) peak areas, normalized with the corresponding internal standard (IS) peak area. QI = Quantifier Ions for relative quantification. The sum of the production values of both compounds is represented in stacked bars. The means of stacked bars that are annotated with a common letter are not significantly different by a one-way ANOVA with post-hoc Fisher LSD at the 5% level of significance.

We first compared these two construct configurations in transient expression. The pheromone pathway without the guide RNA (NGP, GB3898) was co-infiltrated with a construct containing both dCasEV2.1 and the g1DFR (GB2513). On the other hand, when the gRNA was co-assembled with the pheromone biosynthetic genes (GP, GB3897), this module was co-infiltrated with a construct containing the dCasEV2.1

system alone (GB2085) (Figure 4A). Both configurations proved to be functional, and yields were comparable to those obtained from the CaMV35S promoter (construct GB4407) (Figure 4B). The best yields were obtained from the GP (constructs GB3897 + GB2513, estimated yields 384.4 µg Z11-16OH/g FW and 175.8 µg Z11-16OAc/g FW), even surpassing the performance of the constitutive pathway. As a control of activation, the GP construct was co-infiltrated with only P19; on the other hand, the NGP construct was co-infiltrated with a mix of *A. tumefaciens* cultures carrying an unspecific gRNA for a sequence which was not present in the construct, dCasEV2.1 and P19. No pheromone compounds were detected in any of these control samples, which demonstrates the specificity of the system being activated only by the correct gRNA.

3. CRISPR-mediated control of moth sex pheromone biosynthesis in stable transgenic *Nicotiana benthamiana* lines

Given the positive results obtained in transient expression, the constructs containing the genes for the moth sex pheromone pathway controlled by synthetic promoters were used to stably transform *N. benthamiana* plants (Figure 5A and 5B). In the design of an activable pathway, we pursued a modular approach, this creating stable transgenic *N. benthamiana* lines encoding either the Guided or the Non-Guided dCasEV2.1-activable Pathway (GP and NGP, respectively), which could be later trans-activated with the conditional addition of the remaining elements of the synthetic gene circuit (dCasEV2.1 for GP and dCasEV2.1 plus gRNA for NGP respectively.

We first started with the stable transformation of GP and NGP modules. Twenty-five PCR-positive regenerated T_0 plants (7 GP and 18 NGP) were obtained. To test their functionality, T_0 plants were agroinfiltrated with the dCasEV2.1 regulatory elements (dCasEV2.1 alone for GP, or with also g1DFR in NGP; Figure 5A and 5B). As a control of activation, NGP *N. benthamiana* plants were also agroinfiltrated with a mix of *A. tumefaciens* cultures containing an unspecific gRNA, dCasEV2.1 and P19, and GP

plants with only the P19 culture. No pheromone activity was detected in control samples, corroborating the specificity of the system. In contrast, when agroinfiltrated with the appropriate dCasEV2.1 regulatory elements, pheromone biosynthesis was switched on, and products were detected (Figure 5C). In general, NGP plants reached the highest pheromone yields for both Z11-16OH and Z11-16OAc. Regarding biosynthesis of the acetate form, NGP plants were clearly outstanding, reaching in the best individual T_0 plant around 1/3 of its total pheromone content, while in GP plants Z11-16OAc production was not detected except for one plant, where its accumulation was still quite low. The maximum yield was obtained in plant NGP38, estimated at 3.01 µg Z11-16OH g^{-1} FW and 0.48 µg Z11-16OAc g^{-1} FW. Even if these numbers remain still far from the highest values obtained expressing GP and NGP transiently, they demonstrate the possibility to establish, in stable transgenic plants, a pheromone biosynthetic pathway controlled by synthetic promoters. It was remarkable that all generated plants showed a "WT-like" growth, and no detrimental phenotype was detected. This holds particular significance, as one of our main objectives was to overcome the growth penalties observed in our SxPv1.0 and v1.2.

Considering the positive results observed in T_0 GP and NGP *N. benthamiana* generation, we pursued their validation in the next transgenic generation, hoping that pheromone biosynthesis could be improved in the T_1. Four pheromone-accumulating lines were selected, two GP with different T-DNA copy number, and two multiple-copy NGP lines showing very different production levels. Pheromone production following agroinfiltration of dCasEV2.1 regulatory elements was analyzed by HS-SPME GC/MS in the T_1 progeny, as it had been done in T_0 plants. The measured relative levels of moth sex pheromone compounds were higher in this second transgenic generation, for both the alcohol and acetate forms (Figure 5D). In the selected GP lines, T_1 Z11-16OAc levels now became detectable but were still low, while their respective parentals were not producing the acetate at measurable levels. The best T_1 GP plant produced almost ten times more Z11-16OH than its corresponding T_0 parental, while the best T_1 NGP plant reached 4-fold higher production levels for the same molecule than reported in the previous transgenic generation.

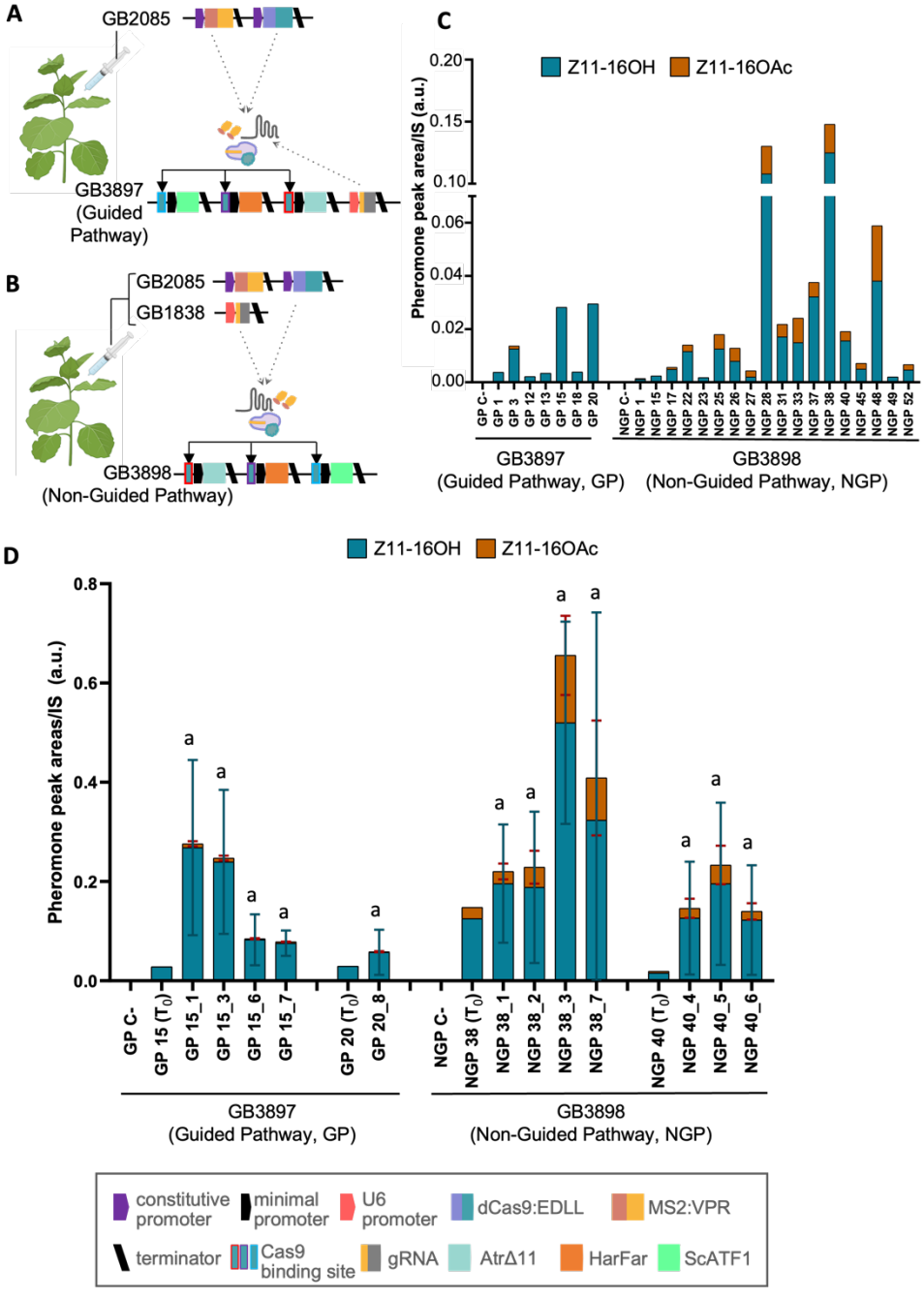

A
GB2085
GB3897
(Guided
Pathway)
B
GB2085
GB1838
GB3898
(Non-Guided Pathway)
C
Z11-16OH
Z11-16OAc
Pheromone peak area/IS (a.u.)
0.20
0.15
0.10
0.06
0.04
0.02
0.00
GP C-
GP 1
GP 3
GP 12
GP 13
GP 15
GP 18
GP 20
NGP C-
NGP 1
NGP 15
NGP 17
NGP 22
NGP 23
NGP 25
NGP 26
NGP 27
NGP 28
NGP 31
NGP 33
NGP 37
NGP 38
NGP 40
NGP 45
NGP 48
NGP 49
NGP 52
GB3897
(Guided Pathway, GP)
GB3898
(Non-Guided Pathway, NGP)
D
Z11-16OH
Z11-16OAc
Pheromone peak areas/IS (a.u.)
0.8
0.6
0.4
0.2
0.0
a
GP C-
GP 15 (T0)
GP 15_1
GP 15_3
GP 15_6
GP 15_7
GP 20 (T0)
GP 20_8
NGP C-
NGP 38 (T0)
NGP 38_1
NGP 38_2
NGP 38_3
NGP 38_7
NGP 40 (T0)
NGP 40_4
NGP 40_5
NGP 40_6
GB3897
(Guided Pathway, GP)
GB3898
(Non-Guided Pathway, NGP)
constitutive promoter
minimal promoter
U6 promoter
dCas9:EDLL
MS2:VPR
terminator
Cas9 binding site
gRNA
AtrΔ11
HarFar
ScATF1

Figure 5 (previous page): Pheromone biosynthesis in T_0 *Nicotiana benthamiana* transgenics. Schematics of the constructs used for expression of moth pheromone compounds in transgenic lines containing **(A)** the Guided Pathway (GP GB3897, sgRNA integrated) or **(B)** the Non-Guided Pathway (NGP GB 3898, sgRNA infiltrated). **(C)** Pheromone relative levels obtained from T_0 transgenics agroinfiltrated with constructs expressing dCasEV2.1 regulatory elements. **(D)** Pheromone relative levels obtained from the T_1 progeny of four independent T_0 lines encoding either the Guided or Non-Guided Pathway agroinfiltrated with constructs expressing regulatory elements: only dCasEV2.1 in the first case (GB2085), and dCasEV2.1 and g1DFR (GB1838) in the second case, respectively. For GP negative controls (GP C-), GP plants were agroinfiltrated only with the P19 silencing suppressor. For NGP negative controls (NGP C-), NGP plants were agroinfiltrated with an unspecific sgRNA, dCasEV2.1 and P19. Compounds were measured by HS-SPME GCMS in agroinfiltrated *N. benthamiana* ground leaves, at 5 dpi. Relative quantifications were normalized using tridecane as an internal standard (IS). In panel **(D)**, values represent the mean and standard deviation of n = 3 biological replicates (independent infiltrations). The sum of the production values of both compounds is represented in stacked bars. The means of stacked bars that are annotated with a common letter are not significantly different by a one-way ANOVA with post-hoc Fisher LSD at the 5% level of significance. The figure includes images from Biorender (biorender.com).

Contrary to the transient assay, where the GP construct reached the highest pheromone biosynthesis yields, the stable NGP strategy seems to work better in stable plants, since it reached the highest Z11-16OAc levels, while maintaining also high Z11-16OH levels. Acetate production turned out to be more than notable in T_1 NGP plants, correlating with the highest total pheromone yields detected in activatable stable plants. The maximum yields were obtained from line NGP_38_3, estimated at 12.5 µg Z11-16OH g^{-1} FW and 2.8 µg Z11-16OAc g^{-1} FW. This represents 13-fold less alcohol and 3-fold less acetate than the highest-yield stable constitutive plant presented in Mateos-Fernández et *al.,* (2021), although without associated growth penalty. These values also demonstrate a higher acetate:alcohol ratio in *N. benthamiana* stable transformants with the regulated pathway (around 1:5) than in those with the constitutive pathway (around 1:20).

4. Combining the complete set of an activable metabolic pathway in a single plant

The generation of these Guided Pathway (GP) and Non-Guided Pathway (NGP) *N. benthamiana* lines allowed us to successfully regulate the activation of the moth sex pheromone metabolic pathway in stable plants through CRISPR-mediated control, bypassing the growth penalties observed in our previous pheromone-producing 'SexyPlant' (Mateos-Fernández et *al.,* 2021). This achievement encouraged us to undertake the next challenge, which is the introduction of both modules encoding the metabolic pathway and the dCasEV2.1 system in a single plant, activable via the delivery of the gRNA. The rationale behind this strategy was to ultimately control the pheromone production with the addition of the gRNA, a small molecule that can be easily delivered non-transgenically e.g. using viral-based delivery systems (Selma, Gianoglio, et *al.,* 2022).

To combine the two transgene modules together, we planned three strategies, summarized in Figure 6: (i) super-transformation of our best NGP lines with a construct carrying the dCasEV2.1 elements and a compatible antibiotic resistance; (ii) super-transformation of a homozygous dCasEV2.1 elite line previously generated in our group (Selma et *al.,* 2022) with a construct carrying the NGP and a compatible antibiotic resistance; (iii) sexual crossing between transgenic stable lines NGP and dCasEV2.1. The GP genotype was not selected for this approach because, together with the dCasEV2.1 in a stable plant, it would result in a plant continuously producing pheromone compounds, incurring in the same growth penalties already observed in 'SexyPlant' v1.0 or v1.2.

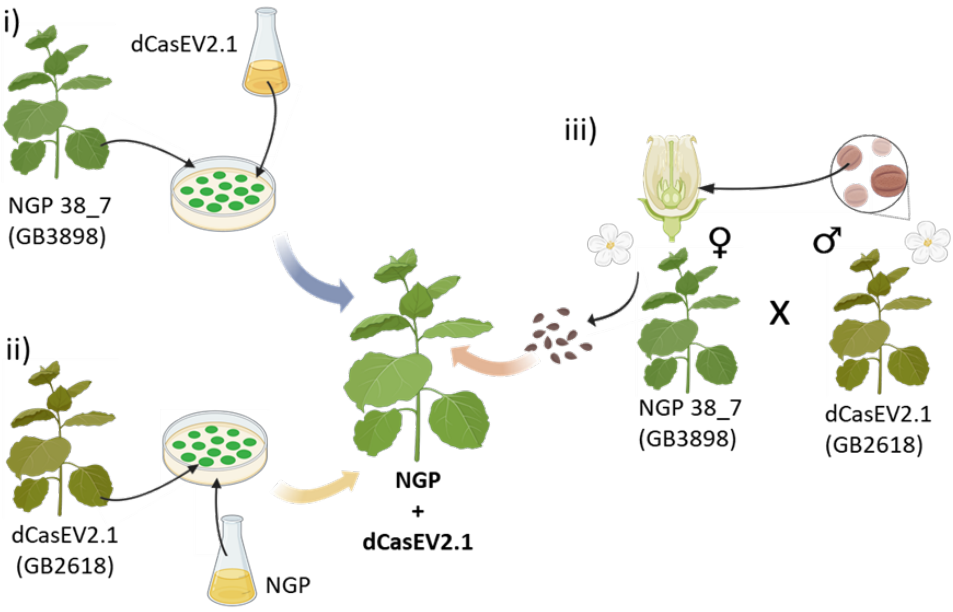


Figure 6: Strategies followed to obtain *N. benthamiana* stable plants carrying the Non-Guided Pathway and dCasEV2.1 modules. **i)** Leaf tissue disks proceeding from the good pheromone producer NGP *N. benthamiana* are regenerated and transformed via *A. tumefaciens* with dCasEV2.1 construct. **ii)** dCasEV2.1 *N. benthamiana* plant tissue is regenerated and transformed via *A. tumefaciens* with NGP construct. **iii)** Pollination cross between emasculated female flowers of NGP 38_7 *N. benthamiana* plant and pollen from a dCasEV2.1 *N. benthamiana*, generating transgenic F_1 seeds and finally a NGP-dCasEV2.1 plant. The figure includes images from Biorender (biorender.com).

For the first strategy, the NGP 38 transgenic line was selected, based on it having the highest pheromone bioproduction levels (Figure 5D). Leaf discs from plant NGP 38_7 were used for transformation with a construct containing the dCasEV2.1 elements, the YFP fluorescence marker and a hygromycin antibiotic marker for positive selection. Eleven T_0 NGP-dCasEV2.1-YFP regenerated plants were obtained. Unfortunately, none of them produced any detectable levels of Z11-16OH or Z11-16OAc when supplemented with g1DFR.

For the second strategy, leaf discs from a T_4 homozygous dCasEV2.1 *N. benthamiana* was used for re-transformation with a construct carrying the NGP module and a hygromycin antibiotic marker for positive selection. Two transformation and regeneration experiments were carried out, generating nineteen transformants. Only

one of these, dCasEV2.1-NGP_9, produced detectable, albeit low, levels of pheromone compounds upon activation with a g1DFR sgRNA (Supplementary Figure S1). However, pheromone levels obtained when activated with the g1DFR sgRNA decreased dramatically in the T_1 population obtained from this plant, and the compounds were hardly detected as traces (Supplementary Figure S1).

In the third strategy, we tried pollination crosses between the two plant modules, using the NGP 38_7 individual as female parental and T_4 dCasEV2.1 as male parental. In the F_1 progeny, the presence of both modules was confirmed by PCR genotyping and visually by detecting YFP fluorescence. A trial was made, trying to activate pheromone production in the F_1 population by agroinfiltration of g1DFR, but no pheromone compounds were detected (data not shown).

5. Understanding the limiting factors of the stable strategy using trans-supplementation analysis

The lack of pheromone production in the F_1 generation of *N. benthamiana* plants stably carrying the Non-Guided Pathway and the dCasEV2.1 system (NGP x dCasEV2.1 F_1) when agroinfiltrated with g1DFR was not expected, given the positive results previously obtained by combining stably integrated and transiently expressed modules in the same plants. One of the hypotheses was that, because of hemizygosity, the transgene dosage in the F_1 population was too low, as compared to that obtained in transient expression. If this was true, transiently supplementing any of the modules may increase expression levels, and the production of pheromone compounds should be rescued. Potentially, this supplementation might also allow us to describe which is the limiting factor in this approach.

To this end, three NGP x dCasEV2.1 F_1 plants (P1 to P3) were agroinfiltrated using one of the following culture mixes:

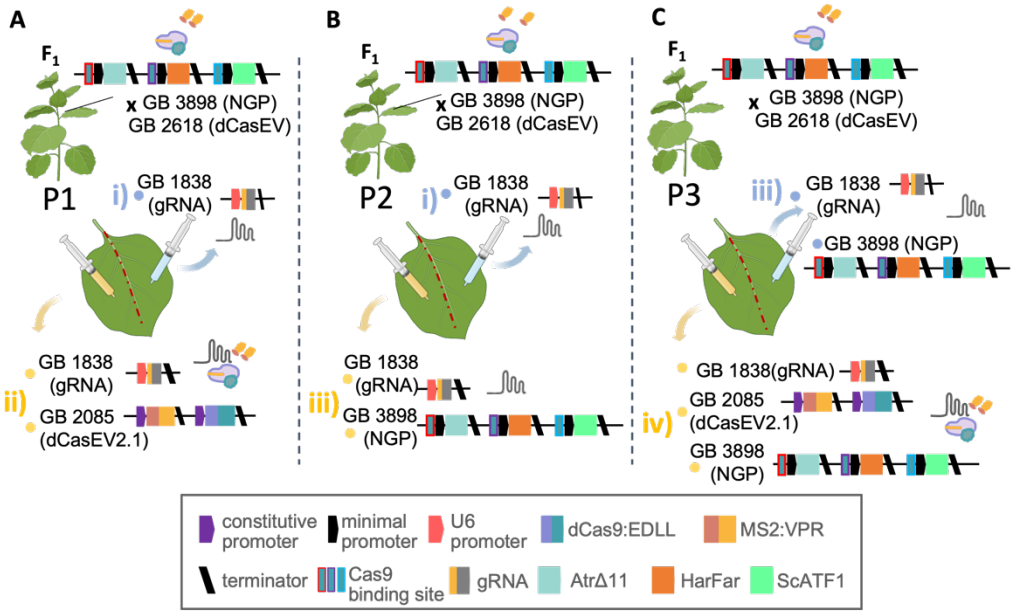
i) g1DFR alone (1 element)
ii) g1DFR + dCasEV2.1 (2 elements)

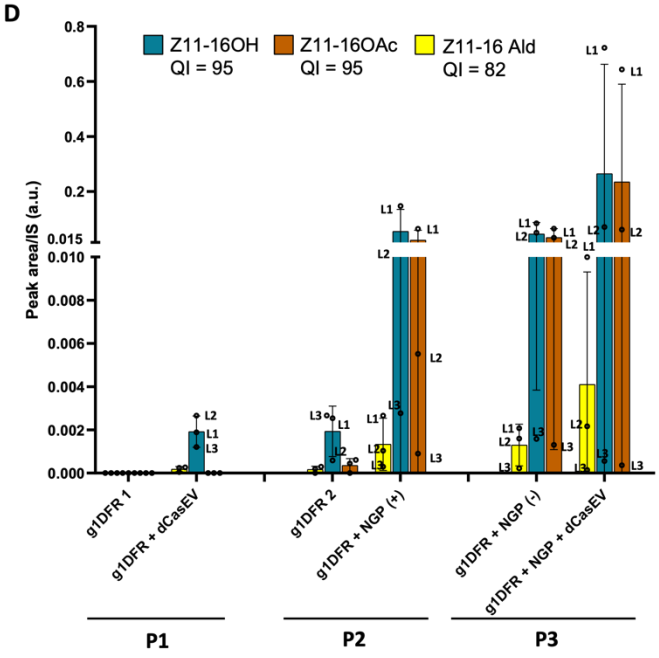
iii) g1DFR + NGP (2 elements)

iv) g1DFR + dCasEV2.1 + NGP (3 elements)

The agroinfiltration approaches for each plant (P1 to P3) are summarized in Figure 7A-C, and the results of the analysis are shown in Figure 7D. For each plant, three leaves of different age were agroinfiltrated (L1-oldest to L3-youngest), each leaf agroinfiltrated in two different areas with different construct combinations. In those leaf areas infiltrated only with g1DFR (strategy *i*) or g1DFR plus dCasEV (strategy *ii*), the levels of pheromone compounds were only detectable in traces and did not follow any evident trend. For strategies *iii* and *iv*, the production of pheromone compounds was clearly detected, and a general trend of decreasing yield could be observed as the infiltrated leaf position progressed from the oldest leaf (leaf 1) to the youngest (leaf 3). Although the differences among the different culture combinations were clearly observable in leaves 1 and 2, the low pheromone production achieved in younger leaves for all three approaches resulted in average values with low statistical significance. Despite this, the results clearly and unexpectedly showed that high pheromone production levels were recovered only when the NGP construct was supplemented to the NGP x dCasEV2.1 plants via agroinfiltration, indicating that the limiting factor in the transgenic approach was the genes encoding the biosynthetic pathway. Indeed, the supplementation of the genome-integrated pathway with gRNA alone or with gRNA in combination with dCasEV2.1 was unable to produce significant pheromone levels in those plants.

Figure 7 (next page): Complementation assay in F1 generation NGP x dCasEV2.1: (A), **(B)** and **(C)**: Schematics of strategies i), ii), iii) and iv) followed in 3 F1 NGP x dCasEV *N. benthamiana* plants (P1, P2 and P3) in the complementation assay. **(A)** P1 leaves were agroinfiltrated on one half of the leaves with a g1DFR culture (strategy i)), and on the other half with g1DFR + dCasEV (strategy ii)). **(B)** P2 leaves were agroinfiltrated on one half of the leaves with a g1DFR culture (strategy i)), and on the other half with g1DFR + NGP (strategy iii)). **(C)** P3 leaves were agroinfiltrated on one half of the leaves with g1DFR + NGP (strategy iii)) and on the other half with g1DFR + NGP + dCasEV (strategy iv)). **(D)** Pheromone relative levels obtained from each of the 3 agroinfiltrated F1 NGP x dCasEV plants. Compounds were measured by HS-SPME GCMS in agroinfiltrated *N. benthamiana* ground leaves, at 5 dpi. The values shown are the mean and standard deviation of n=3 biological replicates (independent agroinfiltrated leaves) of peaks, normalized with the internal standard (IS) peak area. Each point represents the value of every individually agroinfiltrated leaf, labeled as L1, L2 or L3, meaning leaf #1, leaf #2 or leaf #3, bottom-up, respectively. QI = Quantifier Ions for relative quantification. The figure includes images from Biorender (ender.com).


A
B
C
F1
x GB 3898 (NGP)
GB 2618 (dCasEV)
P1
P2
P3
i) GB 1838 (gRNA)
ii) GB 1838 (gRNA)
GB 2085 (dCasEV2.1)
iii) GB 1838 (gRNA)
GB 3898 (NGP)
iv) GB 1838(gRNA)
GB 2085 (dCasEV2.1)
GB 3898 (NGP)
constitutive promoter
minimal promoter
U6 promoter
dCas9:EDLL
MS2:VPR
terminator
Cas9 binding site
gRNA
AtrΔ11
HarFar
ScATF1


D
Z11-16OH QI = 95
Z11-16OAc QI = 95
Z11-16 Ald QI = 82
Peak area/IS (a.u.)
0.8
0.6
0.4
0.2
0.015
0.010
0.008
0.006
0.004
0.002
0.000
g1DFR 1
g1DFR + dCasEV
g1DFR 2
g1DFR + NGP (+)
g1DFR + NGP (-)
g1DFR + NGP + dCasEV
P1
P2
P3

DISCUSSION

Plants have been demonstrated to be appropriate platforms for the heterologous production of a wide variety of high-value products by expressing sequences from other organisms, through either transient or stable transgene expression. To date, plant biofactories have proven to efficiently biosynthesize biopharmaceuticals such as vaccines, antibodies, and therapeutic enzymes (Lomonossoff & D'aoust, 2016), molecules of interest for industry and nutritional feedstocks (Schwarzhans et *al.,* 2017, Cahoon et *al.,* 2007), and products for agriculture such as pheromones or their precursors (Petkevicius et *al.,* 2020, Mateos-Fernández et *al.,* 2022). In fact, the production of moth sex pheromones in plant biofactories shows several appealing advantages, such as the possibility to leverage the precursors already available in plant cells redirecting the metabolic flux towards the desired heterologous pathway, as well as the use of plants as biodispensers, directly releasing volatile pheromone compounds to the surrounding atmosphere.

In this study we first explored different FA acetyltransferases, initially aiming at identifying a moth acetyltransferase sequence, which should naturally transfer the acetyl group to the desired pheromone metabolites, in our case Z11-16OH, therefore generating Z11-16OAc. Undoubtedly, many efforts had been previously directed at searching acetyltransferases directly implied in moth sex pheromone biosynthesis across moth transcriptome and genome databases. Among them B. J. Ding & Löfstedt, (2015) found 34 candidate sequences in the *Agrotis segetum* pheromone gland transcriptome, but none of them satisfactorily esterified fatty alcohols into the desired acetates when tested in a yeast expression system. In fact, functional prediction from primary sequences remains unclear, given the wide variety of products and metabolic pathways in which acetyltransferases can be implied (St-Pierre & De Luca, 2000; D'Auria & Gershenzon, 2005; Günther et *al.,* 2011). Therefore, we broadened that search and decided to assay five yet untested sequences retrieved from the transcriptomes of *Agrotis segetum, Cadra cautella* and *Helicoverpa assulta.* In alternative to moth FATs, previous studies used plant acetyltransferases. One such enzymes was *Ea*DAcT (B. J. Ding et *al.,* 2014), which was used in stable transformants

in our previous work (Mateos-Fernández et *al.,* 2021). *Ef*DAcT, from a related plant species, had never been tested with this aim (Tran et *al.,* 2017). Moreover, the activity of the yeast *Sc*ATF1 had already been confirmed for pheromone production in yeast and in plant transient expression (B. J. Ding et *al.,* 2016; Xia et *al.,* 2022), but had never been assayed in stable transgenic plant biofactories. After assaying all putative acetyltransferase genes, *Sc*ATF1 was selected as the best sequence to build stable constructs for further assays. With this work, we have expanded the range of FA-acetyltransferases compatible with moth sex pheromone production, showing the potential of the *Sp*ATF1-2 and *Ef*DAcT enzymes for this use. The here-assayed set of acetyltransferases enriches the general pool of known FA-modifying enzymes, and allowed us to make an informed choice, considering the different conversion efficiencies, of the most appropriate enzyme for the pheromone blend we wanted to produce.

As a second optimization approach, we pursued an improvement of the live biodispenser approach. As discussed in Mateos-Fernández et al. (2021), the biomass accumulated by constitutive SxPs (around 9 g FW per plant) was clearly insufficient for a realistic application as a bioproduction system. Although the growth penalty of SxPv1.0 was rescued to some degree with the introduction of an intact intact *EaDAcT* gene, a deleterious metabolic perturbation was still evident in SxPv1.2 plants, especially in the individuals producing the highest pheromones levels. Transcriptomic analysis showed a severe transcriptional reprogramming, consisting especially of a strong stress-like response, evidenced by unusual expression patterns in genes related to hormonal signaling, secondary metabolism and photosynthesis (Juteršek et *al.,* 2022).

All these pieces of evidence pointed to the need to control the expression of the biosynthetic genes, enabling biomass to accumulate before energy is redirected to the biosynthesis of the desired products. Using orthogonal synthetic elements, it becomes possible to control both the timing and the expression levels, limiting undesired impacts on normal development and growth (Ali & Kim, 2019). Furthermore, while several approaches have made use of constitutive

overexpression, to date no activatable heterologous pheromone pathway reconstruction has been achieved in stable plants. The dCasEV2.1 system has already been validated for the regulation of endogenous genes (Selma et *al.,* 2019), and later satisfactorily applied for the fine enrichment of *N. benthamiana* leaves in selected endogenous metabolites (Selma, Sanmartín, et *al.,* 2022). In a recent study, Moreno-Giménez and collaborators (2022) showed the first application of dCasEV2.1 in regulating an exogenous metabolic pathway, the LUZ autoluminescence pathway, used as a reporter system. In the present work, this CRISPRa strategy has equally been exploited for the regulation of an exogenous metabolic pathway: both here-explored configurations of constructs harboring the moth sex pheromone pathway controlled by synthetic promoters, with or without the gRNA included in the construct design, induced the efficient transient production of pheromone compounds (Figure 4). The high activation levels, which in the case of GP even surpassed the constitutive transient expression, evidenced the functionality of the dCasEV2.1 system in this application. This augmentation probably lies in the capability of amplifying gene expression, provided by autonomous transcriptional activation domains (TADs) intrinsic to the dCasEV system (Kunii et *al.,* 2018), surpassing the limits previously described for constitutive expression. This led to the highest observed yields, estimated at 384.4 µg Z11-16OH/g FW and 175.8 µg Z11-16OAc/g FW during transient expression. These estimations fall within the same range as previously reported transient expression experiments, which reached 381 µg Z11-16OH /g FW (B. J. Ding et *al.,* 2014) and 335 µg Z11-16OH /g FW (Xia et *al.,* 2020). It is worth noting that those studies determined their yields through absolute quantification based on solvent extractions, while our measurements are estimations derived from HS-SPME relative quantifications. Notably, the obtained yields are understandably higher than those reported for stable transgenics, which yielded up to 164.9 µg Z11-16OH/g FW and 9.6 µg Z11-16OAc/g FW through absolute quantifications (Mateos-Fernández et *al.,* 2021). The tight control exerted by synthetic promoters and the specificity of the dCasEV2.1 activation system is also evident, given the absence of pheromone production in plants agroinfiltrated with unspecific gRNAs.

Once we had identified an efficient acetyltransferase and a reliable activatable expression system, the following step was to produce the NGP and GP transgenic stable plants, which carry the synthetic pathway alone or in combination with the g1DFR, respectively. As in transient expression, both strategies proved functional, and several T_0 plants of each genotype produced the desired pheromone compounds when activated with the appropriate regulatory element(s) via agroinfiltration. As in transient assays, we confirmed that one of the main advantages of the system is the absence of background production (leakiness) observed in both genotypes until supplemented with the remaining elements of the system. This tight regulation is to be attributed to the accurate design of the synthetic promoters employed to control pheromone gene expression. This prevented any growth penalties, and plants showed normal development until agroinfiltrated for activation, allowing the plant to accumulate biomass, as desired. The results obtained in stable trans-activated plants are still far from the production levels per biomass unit achieved in full transient expression (between 30 and 60 times lower) and to those of SxP1.2. However, when the growth penalty is put in the equation, the regulated strategy is still advantageous as compared to SxPv1.2. We estimated 3.5 times less NGP than SxPv1.2 plants are needed for the extraction of one gram of the acetate compound. Although this advantage cannot be exploited in practical terms

The differences between stably and transiently expressed regulated pathways could potentially be explained by taking into consideration the reduced copy number of the pathway genes implied in the stable approach. Hence, the feasibility of heterologous metabolite production hinges on the capability of the production system to support inducible, high-level expression of all the elements in the dCasEV2.1/activatable pathway system. Finally, the specificity of the system and its tight dependence on the identity of the sgRNA was evidenced when agroinfiltrated with an unspecific sgRNA in the case of NGP (a sgRNA targeting the NOS promoter), which resulted in a total absence of pheromone biosynthesis (Figure 5). This is important because once pathway regulation is optimized, it could be exploited to generate transgenic plants carrying different insect pheromone biosynthetic pathways, each being activated by a different gRNA. In this scenario, it would be possible to specifically activate the

production of different pheromones or the other, depending on which gRNA is delivered. To make this strategy manageable, the system should depend only on the addition of the sgRNA signal, which could be easily delivered by different means, including plant viruses. In fact, this approach has already been used to edit plant genomes in a precise way using gRNAs delivered by viral vectors (Uranga et *al.,* 2021). What is more interesting, viral vectors have been used to deliver gRNAs to activate gene transcription (Selma, Sanmartín, et *al.,* 2022): here, a Potato Virus X (PVX)-derived vector was employed as a delivery system for gRNAs, triggering the transcriptional activation of endogenous genes and leading to a transient metabolic reprogramming. Similarly, a PVX-vector could be used to deliver a sgRNA activating the heterologous moth sex pheromone pathway on dCasEV2.1-NGP plants.

With this strategy in mind, our last objective was that of creating and evaluating stable transgenic plants harboring both dCasEV2.1 elements and the activatable pathway. Unexpectedly, all the strategies that we followed (over-transformation in both senses and crosses between genotypes) failed in yielding dCasEV2.1-NGP plants producing pheromone compounds. The size of the T-DNA for each of the plant modules (around 12.5 kb in NGP and 13 kb in dCasEV2.1 constructs) is considerable, and this makes more difficult to assess the presence of an intact transgenic construct. A more detailed inspection of the NPG in the super-transformation approach would be required in the future to ensure that no rearrangements or small deletions have occurred during integration the transgenic constructs leading to misfunction. For constructs of similar size, we have previously encountered problems in recovering a complete and intact T-DNA insert in regenerated plants, as was the case in the first version of the 'Sexy Plant' (Mateos-Fernández et *al.,* 2021), probably the result of an inefficient T-DNA transfer from bacteria to infected plant cells during transformation. The generation of a greater number of transgenic events could have ensured that they harbored both intact modules.

In the case of sexual crossing approach, even though the F_1 was a homogenous population fully genotyped as positive for both the NGP and dCasEV2.1, and showed YFP fluorescence, the resulting pheromone response was indetectable by GC/MS

when activated by agroinfiltrating *A. tumefaciens* cultures carrying the active gRNA. We hypothesized that a first weakness might be the low transgene dose in the F_1: during the cross, only half of the copies of the transgene are transferred to the gametes and therefore, for each transgene, F_1 individuals carry only half the dose of their respective parentals. This hypothesis was discussed above, and it is partially supported by results shown in Figure 7, where supplementation assays evidenced a gene dose effect, especially for NGP, which was then pinpointed as a limiting factor of the system. This means that, with a low NGP:dCasEV2.1 ratio (strategies *i* and *ii*), activation is low because not enough targets are available for the dCasEV2.1 system to act upon, while, with a more balanced NGP:dCasEV2.1 ratio (strategy *iii*), dCasEV2.1 could potentially cover more targets and trigger a higher activation response. Apparently, this configuration is still not optimal from a stoichiometric point of view, and that is why with a transiently supplied extra dCasEV2.1 dose (strategy *iv*) activation and pheromone production reach a maximum. dCasEV2.1 availability could be expectedly improved in homozygous plants in the following generation. As for NGP availability, this cannot be enhanced by focusing on transcriptional levels but, at the DNA level, increasing the copy number of the activatable NGP pathway would probably increase the efficiency of the system Alternatively, to achieve a better induction than the one observed in our dCasEV-NGP stable plant system, further activation strategies could be explored, such as the recently described CRISPR-Act3.0 strategy (C. Pan et *al.,* 2021). This third generation of the CRISPRa allows a wider multiplexing capacity and has been demonstrated to simultaneously activate multiple genes. In addition, it has been probed to act by maximizing the recruitment of activation domains. CRISPR-Act3.0 has also been adapted to a sibling version of the CRISPR-Cas9, the CRISPR-Cas12b, as well as to a variant that does not require the recognition of the protospacer adjacent motif.

In sum, this study provides some valuable insights into the factors regulating the efficiency and sustainability of pheromone production in plants. The first is the exploration of alternative acetyltransferases to employ in moth sex pheromone production in plants, which show different alcohol:acetate conversion efficiencies. The second is the possibility of efficiently controlling the production of sex

pheromones by combining synthetic promoters regulating the biosynthetic genes and the dCasEV2.1 CRISPRa system, conforming an activatable synthetic pathway. This can be done in plants which stably integrate the activatable pathway and in which the regulatory elements are delivered transiently. Notably, this strategy could be transferred to other heterologous pathways, to produce insect pheromones or other high-value compounds in plants, to avoid the frequent and serious detrimental effects associated with constitutive heterologous overexpression. Finally, this work also advances our understanding of the constraints affecting pheromone production in our pathway, demonstrating that, in our case, the foremost limiting factor is the availability of enough copies of the target activatable pathway, and only secondarily of the dCasEV2.1 regulatory elements. Altogether, our findings represent a significant step forward toward our goal of generating plant biofactories which are viable living biodispensers of insect pheromones.

SUPPLEMENTARY MATERIAL

Table S1: GoldenBraid constructs employed in this study. In blue are constructs already present in the GB collection, while in black are constructs assembled specifically for this work.

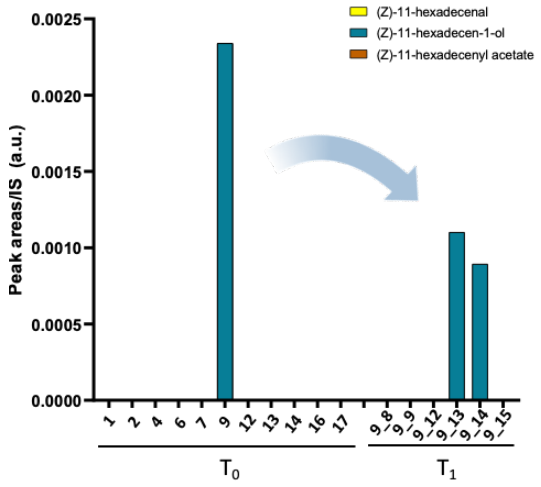
GB ID	Name	Description
GB0030	pUPD:p35S	CaMV 35S promoter
GB0037	pUPD:tNos	*Agrobacterium tumefaciens terminator* NOS
GB0107	pEGB SF	Twister plasmid to swap inserts from an alpha1 or alpha1R vector to any omega level vector
GB0108	pEGB p35s:P19:tNos	TU for the expression of the silencing suppressor P19
GB0235	pEGB 1Alpha1R tNos:HygroR:pNos	Hygromycin TU for stable transformation
GB0466	pEGB SF (TU)	Twister plasmid to swap inserts from an omega1 or omega1R vector to any alpha level vector
GB1022	pDGB2_Alpha2_35s:AtrΔ11:35s + 35s:HarFAR:35s	Transcriptional unit for expression of diacylglycerol acetyltransferase from *Euonymus alatus*.
GB1024	pDGB2_Omega1_35s:AtrΔ11:35s + 35s:HarFAR:35s	Module for the constitutive expression of the Δ11 desaturase from *Amyelois transitella* and a fatty acid reductase from *Helicoverpa armigera*.
GB1181	pEGB 3Ω1 tNos:nptII:pNos-SF	TU for kanamycin resistance gene (*nptII*) plant expression under the regulation of the Nos promoter
GB1203	pEGB p35s:P19:tNos	TU for the constitutive expression of the silencing suppressor P19 driven by the 35S promoter
GB1724	pDGB3_alpha1_U6-26:gRNA 4 (pNos):MS2 F6x2 aptamer	GB-cassette for the expression of a guide RNA targeting the Nopaline Synthetase Promoter with the Ms2 recognition loop in position 3' in the scaffold
GB1838	pDGB3alpha1_U6-26-1gRNA-DFR F6x2 MS2scf	GB-cassette for the expression of a guide RNA targeting the DFR promoter with two Ms2 aptamer copies in the 3' of the scaffold
GB2085	pDGB3_Omega1_35s-Ms2:VPR-Tnos-35s-dCas9:EDLL-Tnos	Module for the expression of Ms2 protein fused to VPR and dCas9 fused to EDLL
GB2414	pDGB3_alpha2_dCas9:EDLL -Ms2 VPR -SF	Module for the expression of Ms2 protein fused to VPR and dCas9 fused to EDLL
GB2513	pDGB2_Omega2_35s:dCas9:EDLL:nos +35s:MS2:VPR:nos + U626:gRNA1 (pDFR)	Module for constitutive expression of dCas9:EDLL, Ms2:VPR and a gRNA targeting the DFR promoter.
GB2528	pDGB3-alpha1 Tnos:NptII:Pnos-Tnos:YFP:P35S -SF	Module for constitutive expression of Yellow Fluorescent Protein (YFP) and kanamycin resistance (NptII)
GB2618	pDGB3_Omega1 NPTII-YFP-SF dCas9EV	Module for the expression of Ms2 protein fused to VPR and dCas9 fused to EDLL and the KanR with YFP protein
GB3676	pUPD2_ATF1	pUPD2 containing CDS of alcohol O-acetyltransferase from *Saccharomyces cerevisiae* S288C, codon optimized Nicotiana.
GB3679	pUPD2_ATF1-2	pUPD2 containg CDS of alcohol O-acetyltransferase from *Saccharomyces pastorianus* strain CBS 1483 chromosome SeVIII-SeXV, codon optimized Nicotiana.
GB3680	pUPD2_EfDAcT	pUPD2 containing CDS of 1,2-diacyl-sn-glycerol:acetyl-CoA acetyltransferase from *Euonymus fortunei*, codon optimized for Nicotiana

GB ID	Name	Description
GB3681	pDGB3_Alpha2_p35s:ScATF1:t35s	Transcriptional unit for expression of alcohol O-acetyltransferase from *Saccharomyces cerevisiae* S288C, codon optimized for Nicotiana.
GB3682	pDGB3_Alpha2_ p35s:ScATF1-2:t35s	Transcriptional unit for expression of alcohol O-acetyltransferase from *Saccharomyces pastorianus* strain CBS 1483 chromosome SeVIII-SeXV, codon optimized for Nicotiana.
GB3683	pDGB3_Alpha2_p35s:EfDAcT:t35s	Transcriptional unit for expression of 1,2-diacyl-sn-glycerol:acetyl-CoA acetyltransferase from *Euonymus fortunei*, codon optimized for Nicotiana.
GB3897	pDGB3_Alpha2_nptII+ SF+INS+ATF1+HarFar+AtrD11 +gRNA1 DFR	Module for the inducible expression of AtrD11, HarFar and ATF1 using dCasEV2.1 system, the nptii gene for selection and the gRNA-1DFR (SexyPlant's Guided-pathway)
GB3898	pDGB3_Omega1_nptII +AtrD11+HarFar+INS+ATF1	Module for the inducible expression of AtrD11, HarFar and EaDAct using dCasEV2.1 system, and the nptii gene for selection (SexyPlant's Non-guided-pathway)
GB3899	pDGB3_Omega1_HygR+AtrD11 +HarFar+INS+ATF1	Module for the inducible expression of AtrD11, HarFar and ATF1 using dCasEV2.1 system, and the hygromycin resistance gene for selection (SexyPlant's Non-Guided pathway with hygromycin resistance gene)
GB4601	pDGB3_Omega1_HygR-dCasEV-SF	Module containing dCasEV with hygromycin resistance
GB5397	pUPD2_hAsFAT_1	pUPD2 containing CDS of hypotethical Fatty Acyl Transferase from *Agrotis segetum* sequence 1, codon optimized Nicotiana.
GB5398	pUPD2_hAsFAT_2	pUPD2 containing CDS of hypotethical Fatty Acyl Transferase from *Agrotis segetum* sequence 2, codon optimized Nicotiana.
GB5399	pUPD2_hCcFAT_1	pUPD2 containing CDS of hypotethical Fatty Acyl Transferase from *Cadra cautella* sequence 1, codon optimized Nicotiana.
GB5400	pUPD2_hCcFAT_2	pUPD2 containing CDS of hypotethical Fatty Acyl Transferase from *Cadra cautella* sequence 2, codon optimized Nicotiana.
GB5401	pUPD2_hHaFAT	pUPD2 containing CDS of hypotethical Fatty Acyl Transferase from *Helicoverpa assulta*, codon optimized Nicotiana.
GB5402	pDGB3_Alpha2_hAsFAT_1	Transcriptional Unit for expression of hypotethical Fatty Acyl Transferase from *Agrotis segetum* sequence 1, codon optimized Nicotiana.
GB5403	pDGB3_Alpha2_hAsFAT_2	Transcriptional Unit for expression of hypotethical Fatty Acyl Transferase from *Agrotis segetum* sequence 2, codon optimized Nicotiana.
GB5404	pDGB3_Alpha2_hCcFAT_1	Transcriptional Unit for expression of hypotethical Fatty Acyl Transferase from *Cadra cautella* sequence 1, codon optimized Nicotiana.
GB5405	pDGB3_Alpha2_hCcFAT_2	Transcriptional Unit for expression of hypotethical Fatty Acyl Transferase from *Cadra cautella* sequence 2, codon optimized Nicotiana.
GB5406	pDGB3_Alpha2_hHaFAT	Transcriptional Unit for expression of hypotethical Fatty Acyl Transferase from *Helicoverpa assulta*, codon optimized Nicotiana.

Table S2. Candidate FAT genes tested in this chapter.

Candidate name	Sequence functional annotation	Organism	Sequence source
h*Cc*FAT_1	EP_Unigene_10_FAT	*Cadra cautella*	*C. cautella* transcriptome (Antony *et al.,* 2015)
h*Cc*FAT_2	EP_contig_7673	*Cadra cautella*	*C. cautella* transcriptome (Antony *et al.,* 2015)
h*As*FAT_1	Fatty alcohol acetyltransferase	*Agrotis segetum*	BLASTX with EP_Unigene_2_FAT GBXH01082849 (*C. cautella*)
h*As*FAT_2	*Agrotis segetum* clone CL7064 fatty alcohol acetyltransferase (ATF) mRNA, complete cds	*Agrotis segetum*	BLASTN with EP_contig_7673 (*C. cautella*)
h*Ha*FAT	Acetyltransferase 19 (*Helicoverpa assulta*) Ac. No. ATJ44587.1	*Helicoverpa assulta*	BLASTX with EP_Unigene_2_FAT GBXH01082849 (*C. cautella*)
*Sc*ATF1	Alcohol acetyltransferase	*S. cerevisiae*	Ding *et al.* (2016)
*Sp*ATF1_2	Alcohol acetyltransferase	*S. pastorianus*	Yoshimoto *et al.* (1999)
*Ef*DAcT	Diacylglycerol acetyltransferase	*E. fortunei*	Tran *et al.* (2017)

Supplementary Figure S1. Pheromone biosynthetic response in T_0 and T_1 dCasEV + NGP *Nicotiana benthamiana* transgenics after activation by agroinfiltration with g1DFR *A. tumefaciens* culture.



Chapter 2

Assessment of Tobacco (*Nicotiana tabacum*) and *N. benthamiana* as Biofactories of Irregular Monoterpenes for Sustainable Crop Protection

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My contribution to this work was essential for its publication. I performed agroinfiltration, plant transformations, genotyping, and most of the experiments related to phenotypic assessment of the transgenic biofactories. I also contributed significantly to manuscript writing. The manuscript is reported in its entirety.

ABSTRACT

Irregular monoterpenes are important precursors for a set different compounds employed in pest control, such as insecticides and insect sex pheromones. In nature, irregular monoterpenes are synthesized mostly by mealybugs as sex pheromones, and by some aromatic plants, as part of their secondary metabolism. Since, despite ongoing efforts, no mealybug sex pheromone biosynthetic pathway has been elucidated so far, we decided to employ metabolically engineered plants as biofactories for these compounds. Plants are generally appealing hosts for the heterologous production of a plethora of high-value chemicals with medical, industrial and agricultural applications. In the case of pheromones, the appeal of achieving bioproduction in plants is enhanced by the possibility of using them as live biodispensers of bioactive volatiles, in a strategy similar to that of traditional pheromone emitters. Here, we assessed the feasibility of using cultivated tobacco (*Nicotiana tabacum*) and its relative *Nicotiana benthamiana* as biofactories for the irregular monoterpene alcohols chrysanthemol and lavandulol, expressing the *CPPS* gene from *Tanacetum cinerariifolium* and the *LPPS* gene from *Lavandula intermedia*, respectively. Acknowledging the toll that terpene overexpression takes on plant metabolism, we evaluated the impact of high levels of constitutive metabolite production on the physiology and biomass accumulation in both chassis. We also traced the biosynthetic dynamics of the target compounds in different plant tissues and developmental stages, from juvenile leaves to flowers. Metabolite production was assessed both by solvent extraction and by collection of the emitted fraction under dynamic conditions. Lavandulol and chrysanthemol are important precursors for many valuable compounds, yet are not biologically active as pheromones in themselves. As an example of an active pheromone compound, we super-transformed the best lavandulol-producing tobacco line with a lavender acetyltransferase gene, *LiAAT4*, obtaining a tobacco lavandulyl acetate biodispensers. (*R*)-lavandulyl acetate is one of the components of the sex pheromone blend of the banana mealybug *Dysmicoccus grassii*, as well as of the aggregation pheromone of the thrips *Frankliniella occidentalis*. In addition, it is also an effective

mosquito larvicide, and can be safely used to treat waters. According to our analyses, our tobacco lavandulyl acetate biodispensers can emit up to 0.63 mg of lavandulyl acetate per plant every day. We estimate that with these levels of volatile emission, between 200 and 500 plants per hectare would be sufficient to ensure a daily emission of pheromones comparable to commercial lures throughout a season, a density which might be feasible in some cropping systems. This is an important step towards plant-based sustainable solutions for pest control. Although some improvements can be envisaged for increasing metabolite production, enhancing volatilization and/or reduce pleiotropic effects, we demonstrate that this straightforward overexpression strategy, in which the bulk of metabolite production is carried out by mesophyll cells, is a valuable entry approach to the concept of plant pheromone biodispensers. Our results lay the ground for future applications and represent a framework for further development of biofactories for other irregular monoterpenoid pheromones, whose biosynthetic genes are yet unknown.

Keywords: Biofactories, irregular monoterpenes, mealybugs, *Nicotiana benthamiana*, pheromones and tobacco.

INTRODUCTION

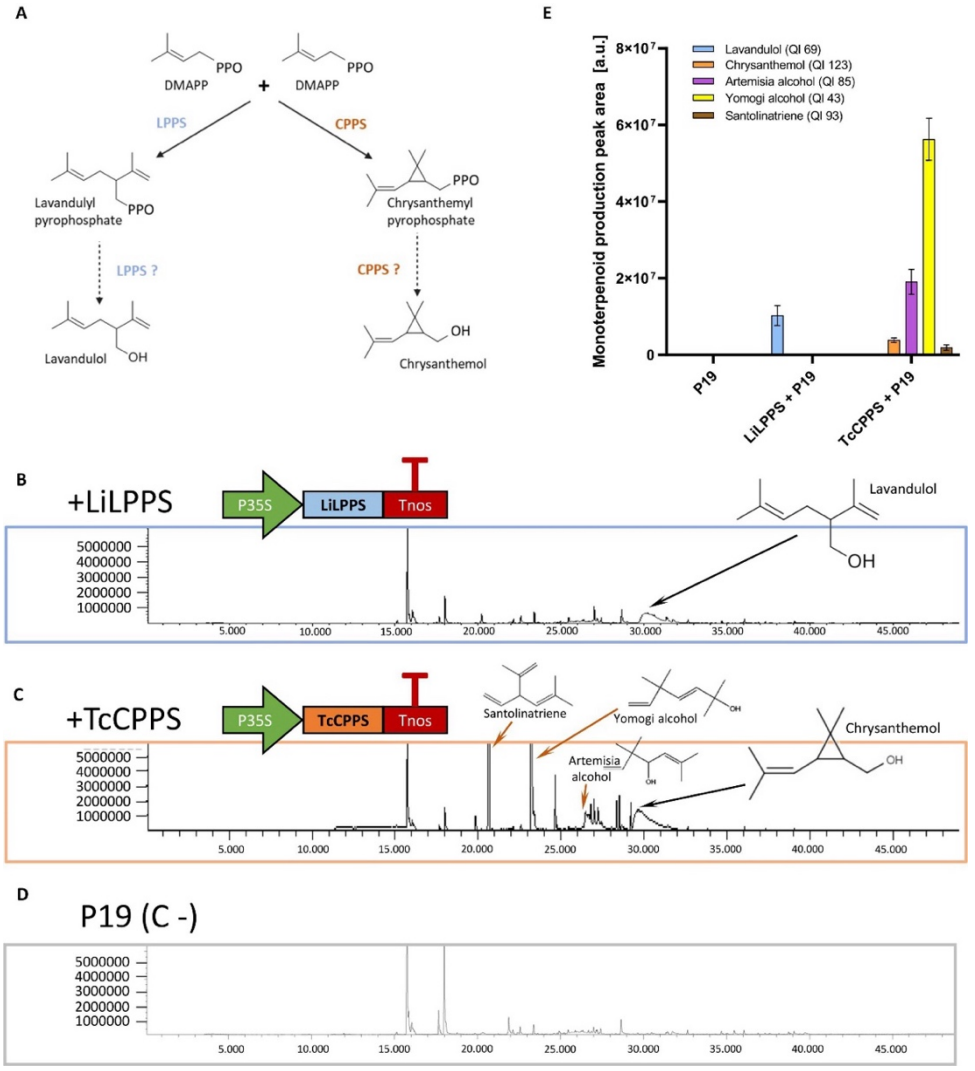
Sustainability in agri-food systems, as in any other sector of the economy, is achieved by balancing the long-term – and sometimes competing – interests of environmental protection, economic profitability and social equity. The protection of crops and stored goods from damage induced by insect pests is an indispensable aspect of increasing agricultural yields and reducing food waste, and the way in which pest control is achieved is fundamental for sustainability. Insect pheromones are sustainable alternatives to traditional pesticides because they are effective, safe, and pose very limited risks of insurgence of genetic resistance, with virtually inexistent effects on non-target populations (Rizvi et *al.,* 2021). Pheromones are volatile organic compounds (VOCs) produced by insects that function at very low concentrations as semiochemicals modulating the behavior of conspecifics. The most interesting for pest control are sex pheromones, usually produced by females, and aggregation pheromones, mainly produced by males to attract both males and females. Both types of pheromones can be used as lures to construct traps to attract and affect individuals, or to monitor population levels. Sex pheromones are also employed in mating disruption strategies, where the release of the pheromone to the environment masks the signal produced by females, preventing or delaying mating (Miller & Gut, 2015). Despite their advantages, the chemical synthesis of pheromone compounds and the formulation of traps can be complex and costly, making them affordable for expensive end products (like high-value orchard productions), but far less accessible for row crops (Bento et *al.,* 2016; Ioriatti & Lucchi, 2016; Petkevicius et *al.,* 2020). Thus, research has focused on developing pheromone biofactories through the engineering of biological hosts like yeasts and plants (Mateos-Fernández et *al.,* 2022). Bioproduction of pheromones has, in principle, several advantages over chemical synthesis: renewable feedstocks benefit the production pipeline and generate fewer polluting by-products; production costs are reduced; finally, chemical synthesis produces racemic mixtures, while enzymes ensure stereoselectivity, which is crucial for pheromone activity (Mateos-Fernández et *al.,* 2022). Biofactories can be used either to synthesize active pheromone compounds (B. J. Ding et *al.,* 2014; Holkenbrink

et *al.,* 2020; Mateos-Fernández et *al.,* 2021), or to produce precursors to be extracted and modified chemically, resulting in hemisynthetic preparations that still enhance sustainability (Nešněrová et *al.,* 2004; Xia et *al.,* 2020; H. Wang et *al.,* 2023). While yeasts can provide greater yields and ease of extraction, plants may be used as pheromone biofactories following two different strategies: one is to synthesize molecules (active compounds or precursors) to be extracted; the second is to engineer plants to be live pheromone emitters (Bruce et *al.,* 2015). The appropriate plant host for each strategy depends on plant biomass, on specialized metabolisms supporting the production of target compounds and, for bioemitters, on the ability to volatilize them.

The bulk of pheromone bioproduction has focused on Lepidopteran sex pheromones, because of the enormous economic relevance of these pests and because these molecules have relatively simple structures and their biosynthetic pathways are known (Löfstedt et *al.,* 2016). Still, an immense potential exists to produce a wide variety of pheromones for different targets. Mealybugs (Pseudococcidae) are a family of insects which constitute a relevant threat to crops in sub-tropical and Mediterranean climates. Their mating behavior strongly depends on sex pheromones: these typically contain various monoterpene-derived esters and many species synthesize irregular monoterpenes, which are unusual in nature, resulting from the non-head-to-tail coupling of two DMAPP units instead of the regular (head-to-tail) condensation of an IPP and a DMAPP unit (Kobayashi & Kuzuyama, 2019). (Zou & Millar, 2015) provide an extensive review of mealybug sex pheromones and their chemistry. Unfortunately, their biosynthesis remains unclear and insect genes responsible for their production are yet to be identified (Tabata, 2022; Juteršek et *al.,* 2024). In the absence of known mealybug biosynthetic genes, alternative approaches to the bioproduction of their sex pheromones rely on other organisms producing analogous compounds. This is the case of plants producing lavandulyl pyrophosphate (LPP) and chrysanthemyl pyrophosphate (CPP), irregular branched and cyclic monoterpenoids, respectively (Figure 1A). LPP and its derivatives lavandulol and lavandulyl acetate are produced by various lavender species (Lamiaceae) and by some Apiaceae and are important fragrances, while CPP and the alcohol chrysanthemol are

produced by members of the Anthemidae tribe within the Asteraceae family (Minteguiaga et *al.,* 2023). LPP and CPP are the precursors of a variety of bioactive compounds important for pest management. Both LPP and CPP are valuable as the monoterpene moieties of the sex pheromone compounds of various mealybug species (such as *Planococcus ficus* Signoret, *Dysmicoccus grassii* Leonardi and *Phenacoccus madeirensis* Green, among others) and can be easily esterified to give an active product (Zou & Millar, 2015). In particular, lavandulyl acetate is an active pheromone compound for the mealybug *D. grassii* (De Alfonso et *al.,* 2012), and a component of the aggregation pheromone of the Western flower thrips *Frankliniella occidentalis* Pergrande (Hamilton et *al.,* 2005). Finally, lavandulyl acetate has also been identified as a mosquito larvicide with low toxicity towards non-target organisms (Govindarajan & Benelli, 2016), and CPP is a precursor for the biosynthesis of pyrethrins, a class of important natural insecticides (Xu, Moghe, et *al.,* 2018).

Monoterpenoids are highly accumulated by common aromatic plants, which store essential oil compounds in glandular trichomes. However, these plants are not ideal bioproduction platforms for heterologous compounds, since they are not easy to transform genetically, and their biomass and growth rate are lower than other wide-leaf species, such as tobacco (*Nicotiana tabacum* L.) and *Nicotiana benthamiana* Domin. Tobacco represents a versatile chassis for genetic manipulation with high biomass production, and *N. benthamiana* allows efficient testing of multiple gene combinations through agroinfiltration (Molina-Hidalgo et *al.,* 2021). Thus, we used the LPP synthase gene from *Lavandula* x *intermedia* Emeric ex Loisel. (*LiLPPS*; Demissie et *al.,* 2013) and the CPP synthase gene from *Tanacetum cinerariifolium* (Trevir.) Sch.Bip. (*TcCPPS*; T. Yang et *al.,* 2014) to transform tobacco and *N. benthamiana*, aiming at assessing the potential of these species as producers and emitters of irregular monoterpenoids. We also transformed *Li*LPPS-expressing tobacco plants with the AAT4 acetyltransferase from *L. intermedia* (L. S. Sarker & Mahmoud, 2015), successfully esterifying lavandulol to lavandulyl acetate.


A
PPO
DMAPP
+
PPO
DMAPP
LPPS
CPPS
Lavandulyl pyrophosphate
PPO
PPO
Chrysanthemyl pyrophosphate
LPPS ?
CPPS ?
Lavandulol
OH
OH
Chrysanthemol
E
Monoterpenoid production peak area [a.u.]
8×10^7
6×10^7
4×10^7
2×10^7
0
Lavandulol (QI 69)
Chrysanthemol (QI 123)
Artemisia alcohol (QI 85)
Yomogi alcohol (QI 43)
Santolinatriene (QI 93)
P19
LiLPPS + P19
TcCPPS + P19
B
+LiLPPS
P35S
LiLPPS
Tnos
Lavandulol
OH
5000000
4000000
3000000
2000000
1000000
5.000
10.000
15.000
20.000
25.000
30.000
35.000
40.000
45.000
C
+TcCPPS
P35S
TcCPPS
Tnos
Santolinatriene
Yomogi alcohol
OH
Chrysanthemol
Artemisia alcohol
OH
OH
5000000
4000000
3000000
2000000
1000000
5.000
10.000
15.000
20.000
25.000
30.000
35.000
40.000
45.000
D
P19 (C -)
5000000
4000000
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45.000

Figure 1 (previous page): Production of the volatile monoterpenoids lavandulol and chrysanthemol in transient expression in *N. benthamiana*: (A) Biosynthetic metabolic pathway of the irregular monoterpenoids lavandulyl pyrophosphate and chrysanthemyl pyrophosphate via the non-head-to-tail condensation of two DMAPP by the LPPS or CPPS enzyme. Production of the alcohols might be due to host endogenous phosphatases or to a bifunctional activity of these irregular IDSs. **(B)** The T-DNA construct used for transient expression of *Li*LPPS controlled by the CaMV35S promoter and Nos terminator, and the HS-SPME GC-MS profile of *N. benthamiana* ground leaf tissue 5 dpi. **(C)** The T-DNA construct used for transient expression of *Tc*CPPS controlled by the CaMV35S promoter and Nos terminator, and the HS-SPME GC-MS profile of *N. benthamiana* ground leaf tissue 5 dpi. **(D)** The HS-SPME GC-MS profile of *N. benthamiana* ground leaf tissue 5 dpi in negative control plants infiltrated only with the P19 silencing suppressor. **(E)** Lavandulol, chrysanthemol, and chrysanthemol-derived compounds measured by HS-SPME GC-MS in agroinfiltrated *N. benthamiana* ground leaves. Values represent the mean and SD of n =3 biological replicates (independent agroinfiltrated leaves). QI =Quantifier Ions for relative quantification of peaks.

MATERIALS AND METHODS

DNA assembly and cloning

All DNA parts used for plant transformation were domesticated and assembled using the GoldenBraid (GB) standard as described by Sarrion-Perdigones et al. (2011). All constructs were verified by Sanger sequencing and/or restriction analysis. All GB constructs designed and employed in this study are available at https://goldenbraidpro.com/ under their corresponding IDs, which are listed in Supplementary Table S1. All constructs were cloned using the *Escherichia coli* TOP 10 strain. The final expression vectors were transformed into electrocompetent *Agrobacterium tumefaciens* GV3101 or LBA4404 for transient or stable transformations, respectively.

Transient expression assays in *Nicotiana benthamiana*

Transient expression assays to validate gene activity were carried out through infiltration of *N. benthamiana* leaves mediated by *Agrobacterium tumefaciens*. Pre-

cultures were grown from glycerol stocks for two days at 28°C at 250 rpm with the appropriate antibiotics until saturation, then refreshed and grown overnight in the same conditions. Cells were pelleted and resuspended in an agroinfiltration buffer containing 10 mM 2-(N-morpholino) ethanesulfonic acid (MES), pH 5.7, 10 mM $MgCl_2$, and 200 µM acetosyringone, then incubated for 2h at RT under slow shaking. The OD_{600} of each culture was adjusted to reach a value of 0.05-0.06 in the final culture mixtures. Each mixture had a final OD_{600} value of 0.2. Equal volumes of each culture were mixed, including the silencing suppressor P19 for co-infiltration to reduce post-transcriptional gene silencing (Garabagi *et al.,* 2012). The relative abundance of each *A. tumefaciens* culture was kept constant in all infiltration mixtures by adding an *A. tumefaciens* culture carrying an empty vector when needed. Agroinfiltration was carried out with a 1 mL needle-free syringe, through the abaxial surface of the three youngest fully expanded leaves of 4-5 weeks old *N. benthamiana* plants, grown at 24°C (light)/20°C (darkness) with a 16:8 h light:darkness photoperiod. Samples were collected 5 days post-infiltration (dpi) using a Ø 1.5-2 cm corkborer and snap frozen in liquid nitrogen.

Generation and selection of stable transformants

Stable transgenic plants were generated following the transformation protocol described by Kallam et al. (2023). The same procedure was used for both *Nicotiana* spp. For the selection of transgenic progenies, seeds were disinfected by incubation under suspension in 10% trisodium phosphate dodecahydrate for 20 min and then in 3% sodium hypochlorite for 20 min. Then, seeds were washed in sterile distilled water and sown on germination medium (5 g/L MS with vitamins, 30 g/L sucrose, 9 g/L Phytoagar, pH = 5.7) supplemented with 100 mg/L kanamycin for positive transgene selection. *NtLPPS-AAT4* tobacco seeds were supplemented with both 100 mg/L kanamycin and 20 mg/L hygromycin for simultaneous transgenic selection. Control plants were obtained similarly, by placing seeds on a non-selective germination medium. Untransformed wild type (WT) and antibiotic-resistant seedlings were

transferred to the greenhouse 15 days after germination, where they were grown at 24 : 20°C (light : darkness) with a 16 : 8 h light:darkness photoperiod. The segregation of transgenes and the estimation of transgene copy number were initially determined by calculating survival percentages of seedlings germinated on selective media. Transgene copy number was estimated using the Chi squared test. T_0 lines were assumed to be multiple copy lines for the transgene when segregation of the transgene was not possible in the T_1 progeny, and no segregation was detected in the following transgenic generations. Finally, transgene copy numbers were determined using quantitative PCR in plants used for phenotypic characterization.

Transgenic plants within a population (e.g., *NbLPPS N. benthamiana* plants transformed with the *LiLPPS* transgene) were identified using successive numbers referring to their lineage: e.g., in *NbLPPS*_11_2_4, '11' indicates the identity of the T_0 plant self-fertilized to obtain the T_1 plant '11_2', from which the T_2 plant '11_2_4' was generated.

Plant sampling

Samples for VOCs analysis in the T_0, T_1 and T_2 generations were collected from the youngest fully expanded leaves of 35-40 days-old *N. benthamiana* or *N. tabacum* plants using a Ø 1.5-2 cm corkborer and snap frozen in liquid nitrogen.

For the analysis of T_3 *N. benthamiana* plants, the first collection of leaf tissue was performed just before the first flower reached anthesis (-1 day), choosing the youngest leaves ranging in length from 3 to 5 cm (henceforth named young leaves), and middle-stem fully expanded leaves (henceforth named adult leaves). The second collection of leaf tissue (post-flowering stage) was carried out after 90 days in soil, following the same criteria adopted in the first collection for the selection of young and adult leaves. For senescent leaves, leaves in the lower part of the stem were sampled when turning slightly yellow. Three leaves per leaf type were sampled as biological replicates.

For the analysis of T_3 *NtLPPS* and *NtCPPS* tobacco plants, and of T_1 *NtLPPS-AAT4* tobacco plants, leaf tissue collection was performed just before the first flower reached anthesis (-1 day), sampling upper-stem leaves ranging in length from 15 to 25 cm (henceforth named young leaves), middle-stem fully expanded and deep green leaves (henceforth named adult leaves), and lower-stem leaves, turning slightly yellow, for senescent leaves. Three leaves per leaf type were sampled as biological replicates.

For the biomass calculation and estimation of total plant production, four-month-old tobacco plants were harvested at the end of the assay and their leaves classified according to leaf age; a correction factor based on production levels measured at different leaf ages was used to estimate total yields.

N. benthamiana leaf samples for intact tissue HSPME VOCs analysis were collected from young leaves ranging in weight from 150 to 350 mg and rolled inside the vials. Emission values were later normalized using leaf weight. Tobacco leaf samples for volatile release in dynamic condition assays were represented by young leaves from the upper part of the plant, 30-35 cm long.

Flower sampling was carried out at pre-anthesis (-1 day for *N. benthamiana*) and with completely open flowers, for flowers of both *Nicotiana* spp.

Analysis of volatile organic compounds (VOCs)

For powdered samples, 50 mg of frozen, ground leaf or flower tissue were weighed in a 10 mL or 20 mL headspace screw-cap vial and stabilized by adding 1 mL of 5M $CaCl_2$ and 150 µL of 0.5 M EDTA, pH=7.5, after which they were immediately bath-sonicated for 5 min. Volatile compounds were captured by means of headspace solid phase microextraction (HS-SPME) with a 65 µm polydimethylsiloxane/divinylbenzene (PDMS/DVB) SPME fiber (Supelco, Bellefonte, PA, USA). Volatile extraction was performed automatically by means of a CombiPAL autosampler (CTC Analytics, Zwingen, Switzerland).

For the T_0, T_1 and T_2 generations in *N. benthamiana*, and for the T_0 and T_1 generations in *N. tabacum*, analyses were made using a PEGASUS 4D mass spectrometer (LECO Corporation, St. Joseph, MI, USA). Vials were first incubated at 50°C for 10 min under 500 rpm agitation. The fiber was then exposed to the headspace of the vial for 20 min under the same conditions of temperature and agitation. Desorption was performed at 250°C for 1 min (splitless mode) in the injection port of a 6890 N gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled to PEGASUS 4D mass spectrometer (LECO Corporation). After desorption, the fiber was cleaned in a SPME fiber conditioning station (CTC Analytics) at 250°C for 5 min under a helium flow. Chromatography was performed on a BPX-35 (30 m, 0.32 mm, 0.25 µm) capillary column (SGE) with helium as the carrier gas at a constant flow of 2 mL/min. The oven conditions started with an initial temperature of 40°C for 2 min, 5°C/min ramp until 250°C, and a final hold at 250°C for 5 min. Data were recorded in a PEGASUS 4D mass spectrometer (LECO Corporation) in the 35-300 m/z range at 20 scans/s, with electronic impact ionization at 70 eV. Chromatograms were processed by means of the ChromaTOF software (LECO Corporation).

For the T_3 generation *N. benthamiana LPPS* and *CPPS* transformants, the T_2 and T_3 generation tobacco *LPPS* and *CPPS* transformants, and the T_0 and T_1 *LiLPPS LiAAT4* tobacco transformants, desorption was performed at 250°C for 1 min (splitless mode) in the injection port of a 6890 N gas chromatograph (Agilent Technologies) coupled to a 5975B mass spectrometer (Agilent Technologies). Chromatography was performed on a DB5ms (60 m, 0.25 mm, 1 µm) capillary column (J&W) with helium as the carrier gas at a constant flow of 1.2 mL/min. Oven conditions were the same indicated above. Data were recorded in 5975B mass spectrometer in the 35-300 m/z range at 20 scans/s, with electronic impact ionization at 70 eV. Chromatograms were processed by means of the Agilent MassHunter software (Agilent Technologies).

For intact tissue assays, leaf or flower tissue samples were weighed and placed in a 20 mL headspace screw-cap vial. Volatile compounds were captured by means of headspace solid phase microextraction (HS-SPME) with a 65 µm polydimethylsiloxane/divinylbenzene (PDMS/DVB) SPME fiber (Supelco). Volatile

extraction was performed automatically by means of a CombiPAL autosampler (CTC Analytics). Vials were first incubated at 30°C for 10 min under 500 rpm agitation.

Chromatograms were processed by means of the Agilent MassHunter software. Identification of compounds was made by comparison of both retention time and mass spectrum with pure standards (lavandulol, chrysanthemol, linalool and lavandulyl acetate) and by comparison between mass spectrum for each compound with those of the NIST 2017 spectral library. Relative quantifications were assessed by integrating peak areas for each compound, for a selected Quantifier Ion (QI). The chosen QIs were the following: lavandulol QI = 69; chrysanthemol QI = 123; artemisia alcohol QI = 85; Yomogi alcohol QI = 43; santolinatriene QI = 93; lavandulyl acetate QI = 69. Every compound quantification was corrected with the value of the daily deviation of a master mix, processed and analyzed every day. A linalool internal standard (IS) was always added as a control of hour drift.

The quantification of monoterpenoid compounds emitted either by whole plants, or by detached leaves or flowers was carried out by volatile collection in dynamic conditions. Individual plants for *N. benthamiana* or 50 g of tobacco leaves were placed inside 5 L glass reactors (25 cm high × 17:5 cm diameter flask) with a 10 cm open mouth and a ground glass flange to fit the cover with a clamp. The cover had a 29/32 neck on top to fit the head of a gas washing bottle and to connect a glass Pasteur pipette downstream to trap effluents in 400mg of Porapak-Q 80-100 (Waters Corporation, Milford, MA, USA) adsorbent. For tobacco flowers, around 3 g of flowers were placed inside 1.3 L glass chambers (50 cm length × 6 cm diameter cylinder). Plant and leaf samples were collected continuously for 72 h, and flower samples for 48 h, by using an ultrapurified-air stream, provided by an air compressor (Jun-air Intl. A/S, Norresundby, Denmark) coupled with an AZ 2020 air purifier system (Claind Srl, Lenno, Italy) to provide ultrapure air (amount of total hydrocarbons < 0:1 ppm). In front of each glass reactor, an ELL-FLOW digital flowmeter (Bronkhorst High-Tech BV, Ruurlo, The Netherlands) was fitted to provide an air push flow of 100 mL/min during sampling. Volatiles trapped in the Porapak Q cartridges were eluted with 3 mL pentane. Solvent extracts were concentrated under a gentle nitrogen stream up to

500 µL and 25 µL of an internal standard solution (100 µg/mL in dichloromethane) were added to the sample prior to the chromatographic analysis for quantification of the target molecules.

Solvent extraction from plant tissues

The total quantity of pheromone compounds accumulated in each plant or leaf bunch was extracted with toluene (TLN). Plant samples (ca. 3 g), mixed with fine washed sand (1 : 1, plant : sand, w/w), were manually ground with a mortar to aid in tissue breakdown and facilitate the extraction. The resulting material was then transferred to 50 mL centrifuge tubes with 10 mL TLN. The extraction process was assisted by magnetic agitation for 12 h and finally by ultrasound in a Sonorex ultrasonic bath (Bandelin electronic, Berlin, Germany) for 30 min. A 1 mL sample of the resulting extract was filtered through a PTFE syringe filter (0.25 µm). Twenty-five µL of an internal standard solution (100 µg/mL in dichloromethane) were added to the sample prior to the chromatographic analysis for quantification of the target molecules.

Quantification of target compounds

The quantification was performed by gas chromatography coupled to mass spectrometry (GC-MS), using an internal standard. A straight chain fluorinated hydrocarbon ester (heptyl 4,4,5,5,6,6,7,7,8,8,9,9,9-tridecafluorononanoate; TFN) was selected as the internal standard to improve both sensitivity and selectivity for MS detection (Gavara et *al.,* 2020).

One µL of each extract was injected in a Clarus 690 gas chromatograph (Perkin Elmer Inc., Wellesley, MA) coupled to a Clarus SQ8T MS instrument operating in full scan mode and using EI (70 eV). The GC was equipped with a ZB-5MS fused silica capillary column (30m × 0.25mm i.d. × 0.25 µm; Phenomenex Inc., Torrance, CA). The oven was held at 60°C for 1 min then was raised by 10°C/min up to 120°C, maintained for 4 min, raised by 10°C/min up to 130°C, and finally raised by 20°C/min up to 280°C held for 2 min. The carrier gas was helium at 1 mL/min. The GC injection port and transfer line

were programmed at 250°C, whereas the temperature of the ionization source was set at 200°C. Chromatograms and spectra were recorded with GC-MS Turbomass software version 6.1 (PerkinElmer Inc.).

The amount of each compound and the corresponding chromatographic areas were connected by fitting a linear regression model, $y = a + bx$, where y is the ratio between compound and TFN areas and x is the amount of compound.

Chlorophyll Index measurements

For T_3 generation *LPPS* and *CPPS* transformants of both *Nicotiana* spp., and T_1 *NtLPPS-AAT4* tobacco transformants, chlorophyll index (C.I.) data were collected with a Dualex-A optical sensor (Dualex Scientific® (Force-A, Orsay, France). Three leaves per plant were sampled for each leaf stage: young and adult leaves in pre-flowering plants, and young, adult and early senescent leaves in post-flowering plants, using the same criteria specified for plant sampling.

Synthesis of pure standards for GC-MS

Linalool. Standard sample of linalool was commercially acquired from Sigma-Aldrich.

Racemic Lavandulol. A solution of methyl 3,3-dimethylacrylate (1.5 g, 0.013 mol) in dry tetrahydrofuran (5 ml) was slowly added to a cooled solution of lithium diisopropylamide at -40°C (2.0 M in THF, 7.9 ml, 1.2 eq) under nitrogen. After 60 min of continuous stirring, prenyl bromide (2.22 g 0.014 mol, 1.15 eq) were added, and the solution was warmed up to room temperature and stirred for an additional 5 h. The reaction was quenched by the addition of saturated ammonium chloride solution (5 ml) and extracted with Et_2O (3 X 15 ml). The combined organic phases were successively washed with HCl 1M (1 X 5 ml), $NaHCO_3$ 10 % (1 X 5 ml) and brine (1 X 10 ml), dried with anhydrous magnesium sulphate, and the solvent evaporated under vacuum. The crude material was dissolved in anhydrous tetrahydrofuran (4 ml) and

slowly added to a suspension of $LiAlH_4$ (0.67 g, 0.017 mol, 1.35 eq.) in dry THF (5 ml) at 0°C under argon atmosphere. After 3 h of continuous stirring, sodium sulphate decahydrate (Glauber's salt) was carefully added to the suspension until a clear solid was formed (hydrogen formed during the quenching was removed with a continuous stream of nitrogen). The solid was filtered off through a celite pad, and the solvent evaporated under vacuum. The crude material was purified by column chromatography using a mixture of hexane and ethyl acetate in a 8 to 2 ratio (hexane:acetate 8:2) as eluent. Evaporation of the solvent of the corresponding fractions afforded pure lavandulol (1.4 g, 96 % purity by GC-FID, 70 % overall yield for two steps). The spectroscopical properties of lavandulol were fully coincident with those described in the literature (Pepper et *al.,* 2014).

Lavandulyl acetate. Triethyl amine (1.05 ml, 7.6 mmol, 1.8 eq.) and acetic anhydride (0.46 ml, 4.8 mmol, 1.15 eq.) were subsequently added to a solution of lavandulol (0.65 g, 4.2 mmol) in dry dichloromethane (8 ml) at room temperature. After 5 h of continuous stirring, the reaction was poured in dichloromethane (15 ml) and subsequently washed with HCl 1M (1 X 10 ml), $NaHCO_3$ 10 % (1X 10 ml) and brine (1 X 10 ml), dried with anhydrous magnesium sulphate, and the solvent evaporated under vacuum. The crude material was purified by column chromatography using a mixture of hexane:acetate (9:1) as eluent. Evaporation of the solvent of the corresponding fractions afforded pure lavandulol (0.78 g, 98 % purity by GC-FID, 95 % yield). The spectroscopical properties of lavandulyl acetate were fully coincident with previously reported data (Cross et *al.,* 2004).

Chrysanthemol. A solution of chrysanthemic acid (mixture of isomers, 3 g, 18 mmol) in anhydrous tetrahydrofurane (6 ml) was slowly added to a suspension of $LiAlH_4$ (1.4 g, 38 mmol, 2 eq.) in dry THF (20 ml) at 0°C under argon atmosphere. The reaction mixture was warmed up to room temperature and, after 5 h of continuous stirring, sodium sulphate heptahydrated (Glauber's salt) was carefully added to the suspension until a clear solid was formed (hydrogen formed during the quenching was removed with a continuous stream of nitrogen). The solid was filtered off through a celite pad, and the solvent evaporated under vacuum. The crude material was purified

by column chromatography using a mixture of hexane:acetate (7:3) as eluent. Evaporation of the solvent of the corresponding fractions afforded pure (+)-*trans*-chrysanthemol (2.3 g, 96 % purity by GC-FID, 85 % yield). The spectroscopical properties of (+)-*trans*-chrysanthemol were fully coincident with those described in the literature (Dufour et *al.,* 2012).

Assessment of transgene copy number and of the transcriptional levels of biosynthetic genes

Transgene copy number was determined by quantitative PCR (qPCR) by amplifying the *nptII* gene in single transformants, and the *nptII* and *AAT4* genes in double transformants, as well as a species-specific calibrator endogenous gene (*N. benthamiana* actin and *N. tabacum* actin). Genomic DNA was extracted using a CTAB protocol (Murray & Thompson, 1980) and 10 ng of DNA were used as template in each reaction.

The transcriptional expression levels of each biosynthetic gene were determined by qRT-PCR. Total RNA was isolated from 100 mg of leaf or flower tissue using the Gene Jet Plant Purification Mini Kit (Thermo Fisher Scientific, Massachusetts, USA) according to the manufacturer's instructions. Before cDNA synthesis, total RNA was treated with the rDNAse-I Invitrogen Kit according to the manufacturer's instructions. An aliquot of 1 µg of DNAse-treated RNA was used for cDNA synthesis using the PrimeScript™ RT-PCR Kit (Takara, Kusatsu, Japan) in a final volume of 20 µL according to the manufacturer's indications. In *N. benthamiana*, the F-box gene was used as an internal reference, while the actin gene was used for tobacco.

For both experiments (qPCR and qRT-PCR) reactions were carried out in technical triplicates, in the presence of a fluorescent dye (TB Green® Premix Ex Taq) using the Applied Biosystems 7500 Fast Real-Time PCR system. Both mRNA fold change calculations and transgene copy numbers were determined using the comparative $\Delta\Delta C_T$ method (Livak & Schmittgen, 2001). For transgene copy number, a plant with

one copy of the transgene was used as calibrator. The sequences of all the primers used in quantitative PCRs are reported in Supplementary Table 2.

Statistical analysis

Statistical analyses were performed using the Past4 software (Hammer et *al.,* 2001) and GraphPad Prism v8.0.2 (GraphPad Software, San Diego, CA, USA).

RESULTS

1. Volatile irregular monoterpenoid alcohols lavandulol and chrysanthemol are efficiently produced in tobacco and *N. benthamiana*.

The production of irregular monoterpenoids from their DMAPP precursor (Figure 1A) in non-specialized leaf cells was first assayed by transient agroinfiltration in *N. benthamiana* of the genes encoding the corresponding isoprenyl transferases. The coding sequences of *Li*LPPS and *Tc*CPPS were assembled in separate GoldenBraid vectors, each of them regulated by the Cauliflower Mosaic Virus 35S promoter (pCaMV35S) and the nopaline synthase terminator (tNOS) (Figure 1B,C). The volatile organic compound (VOC) composition of infiltrated leaves was analyzed 5 days post-infiltration (dpi) by headspace solid-phase micro-extraction (HSPME) gas chromatography/mass spectrometry (GC/MS). Production of the volatile monoterpenoid alcohols lavandulol and chrysanthemol was successfully detected in *LiLPPS*- and *TcCPPS*-agroinfiltrated samples, respectively (Figure 1B,C). Relative quantifications are shown in Figure 1E. None of these products was detectable in negative controls (Figure 1D,E). In *TcCPPS*-infiltrated leaves other related volatile monoterpenoids were detected, namely Artemisia and Yomogi alcohols and santolinatriene, as identified by the NIST mass spectral library (2017). Both Artemisia and Yomogi alcohol are known to derive from the chrysanthemyl cation in aqueous environments, through the rupture of its C(1')-C(3') cyclopropane bond (Poulter et *al.,* 1977; Rivera et *al.,* 2001).

Given the success obtained in transient expression, the generation of stable lines producing lavandulol and chrysanthemol was attempted both for both *Nicotiana* spp. Twelve T_0 *LiLPPS N. benthamiana* (*NbLPPS*) plants and four T_0 *LiLPPS* tobacco (*NtLPPS*) plants were recovered on selective media. Similarly, seven *TcCPPS N. benthamiana* (*NbCPPS*) and three *TcCPPS* tobacco (*NtCPPS*) T_0 plants were obtained. The production of targeted monterpenoids in all four transformation experiments was followed for individual plants in T_0, T_1 and T_2 generations, and the results are shown in Figure S1.

Selection for best-performing lines through generations was made based on production/growth balance, and general trends remained stable up to the T_2 generation. *NtLPPS* plants (especially line *NtLPPS*_3_1) were more productive than their *N. benthamiana* counterparts, with up to 7-fold the lavandulol levels detected in *NbLPPS* plants, except for line *NbLPPS*_11_2_4, whose levels reached 50% those of the best tobacco producers. *NtCPPS* plants produced over 4 times more chrysanthemol than their *NbCPPS* counterparts. In both species, plants producing lavandulol showed yellowing and slower growth, in contrast to non-producer transgenic plants, which were comparable to WTs, and a similar trend was observed for *NbCPPS* and *NtCPPS* plants. Especially for *NbCPPS* and *NtCPPS*, production was associated with premature blossom drop and frequent failure to reach fruit set (not shown). In all chrysanthemol-producing plants, the derived products Artemisia and Yomogi alcohols and santolinatriene were detected (Figure S2), most likely being breakdown products generated during sample processing, as reported in various instances (Xu, Lybrand, et *al.,* 2018). This points to a likely under-estimation of chrysanthemol in these samples. However, while taking this factor into consideration, at this point we used these assays only as relative quantification methods to compare different plants within the *NbCPPS* and *NtCPPS* populations.

2. Lavandulol and chrysanthemol production is higher in the earlier developmental stages and in young leaves of *N. benthamiana* and tobacco transgenic lines

For a thorough characterization of transgenic *N. benthamiana* and tobacco plants, we analyzed the levels of the target compounds in leaves at different developmental stages of the plant, as well as at different stages of leaf development, in a T_3 generation. Two tobacco (*NtLPPS*_1_3_2 and *NtLPPS*_3_1_3) and two *N. benthamiana* (*NbLPPS*_11_2_4 and *NbLPPS*_5_2_2) lines were selected based on their lavandulol production and general plant fitness levels. Determination of their transgene copy number by quantitative PCR (qPCR) revealed that all *NtLPPS* plants carry multiple copies of the transgene (Figure S3). In *N. benthamiana*, plants of the

*NbLPPS*_11_2_4 line seem to carry three co-segregating copies of the transgene (always resulting in a 2:1 resistant:susceptible ratio when germinating on selective medium), while plants of the *NbLPPS*_5_2_2 line only possess one copy of the transgene (Figure S3). All selected *NbCPPS* and *NtCPPS* carried one copy of the transgene, as repeatedly determined by segregation on selective media and confirmed by qPCR (Figure S3). One vigorous line with a single T-DNA insertion, characterized by high production rates, was selected for each *Nicotiana* species. Overaccumulation of monoterpenes is known to affect plant growth and fertility, a trend we had observed in the T_1 and T_2 generations of plants producing lavandulol and chrysanthemol. As expected, all T_3 transgenic lines showed a delay in flowering time compared to WTs (Figure 2A,E). This delay consists of a 20-30% increase in the number of days in soil before flowering in *N. benthamiana*, while in tobacco the delay can be greater, reaching 93% in *NtLPPS*_3_1_3. No significant differences between genotypes are found regarding plant height except for *NtLPPS*_3_1_3 (Figure 2B,F), but a reduction in biomass is observed. In *N. benthamiana*, both *NbLPPS* lines show a significant reduction in biomass estimated as fresh weight (FW) after 100 days in soil, which can range between 60 and 80% compared to both WT and *NbCPPS* (Figure 2C,D). In tobacco, differences in size and plant biomass are not significant between WT and *NtCPPS*, but they are for *NtLPPS*, whose biomass is reduced between 35 and 75% (Figure 2G,H). The chlorophyll index (C.I.) is another parameter for measuring loss of fitness due to overexpression of monoterpenoids, since these compounds often induce chlorosis. In *N. benthamiana*, no significant differences are found in the C.I. of *NbCPPS* compared to WTs, while for *NbLPPS* there is a reduction at both the pre- and post-flowering stages (Figure S4). In tobacco, both *NtLPPS* and *NtCPPS* show a reduction in C.I. at the pre-flowering stage, which disappears post-flowering (Figure S4). In all cases, differences correlate with higher monoterpenoid production (see also Figure 3).

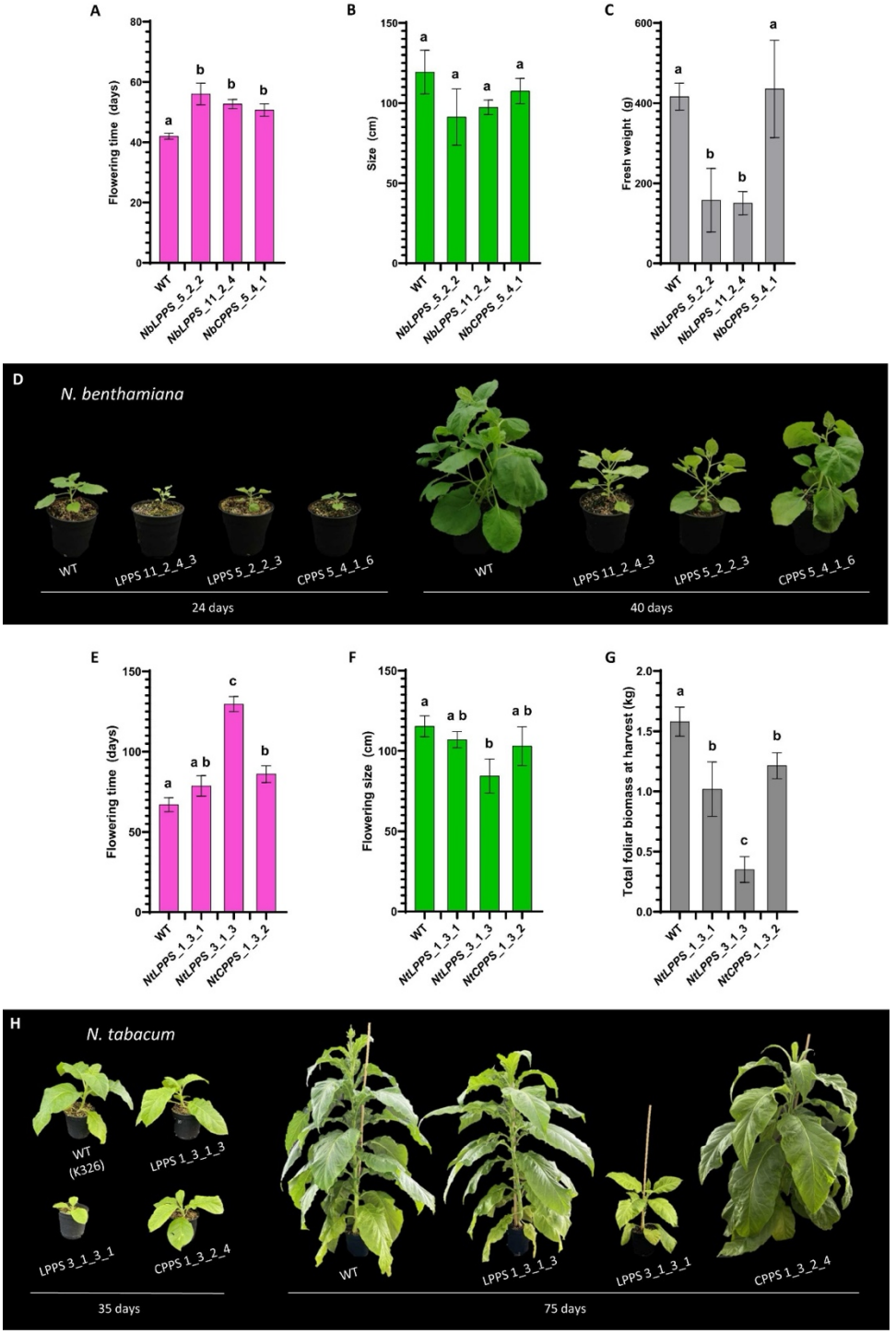

A
Flowering time (days)
a
b
b
b
WT
NbLPPS_5_2_2
NbLPPS_11_2_4
NbCPPS_5_4_1
B
Size (cm)
a
a
a
a
C
Fresh weight (g)
a
b
b
a
D
N. benthamiana
WT
LPPS 11_2_4_3
LPPS 5_2_2_3
CPPS 5_4_1_6
24 days
40 days
E
Flowering time (days)
a
a b
c
b
NtLPPS_1_3_1
NtLPPS_3_1_3
NtCPPS_1_3_2
F
Flowering size (cm)
a
a b
b
a b
G
Total foliar biomass at harvest (kg)
a
b
c
b
H
N. tabacum
WT (K326)
LPPS 1_3_1_3
LPPS 3_1_3_1
CPPS 1_3_2_4
35 days
75 days

Figure 2 (previous page). Physiological effect of irregular monoterpene production in T_3 *N. benthamiana* and tobacco plants. Comparison of flowering time **(A)**, plant size at 100 days **(B)** and total biomass reached at 100 days **(C)** in *NbLPPS*, *NbCPPS* and WT plants. In panel (A), time measured as days from transfer to soil to anthesis of the first flower. **(D)** Phenotype of *NbLPPS* and *NbCPPS* plants compared to WT at 24 and 48 days in soil. Comparison of flowering time **(E)**, plant size at flowering **(F)** and total foliar biomass accumulated at harvest time (140 days) **(G)** in *NtLPPS*, *NtCPPS* and WT plants. In panel **(E)**, time measured as days from transfer to soil to formation of the floral meristem. **(H)** Phenotype of *NtLPPS* and *NtCPPS* plants compared to WT at 35 and 75 days in soil. Values are the mean and standard deviation of at least 3 independent plants of each line. Error bars with the same letter are not significantly different (one-way ANOVA with post-hoc Tukey HSD at the 5% level of significance).

The monoterpenoid content of leaves was assessed pre- and post-anthesis in *N. benthamiana* plants. Pre-anthesis, two types of leaves were analyzed (Y=young and A=adult), while post-anthesis a third type of leaf (S=senescent) was included. The analysis of the monoterpenoid content in different individuals and tissues (Figure 3A,B and Data File S1) shows that the factor affecting productivity the most is the type of tissue. Plants within each line show homogeneous phenotypes in terms of production levels and fitness, and no significant differences are found between plants descending from the same T_2 parental. Overall, young leaves of pre-anthesis plants are the most productive vegetative tissues in both *NbLPPS* and *NbCPPS* lines, followed by the adult leaves of these pre-anthesis plants (Figure 3A,B). We observed a decrease in the levels of the target monoterpenoids in fully flowering plants, diminishing with the increase in leaf age (Figure 3A,B). In *NbLPPS* and *NbCPPS*, adult leaves of pre-flowering plants and young leaves of post-anthesis plants usually show similar production levels. Senescent leaves are still productive, even if a clear decline was observed for all analyzed compounds. In tobacco, only one sampling was performed just prior to anthesis, and three leaf types were collected (Y=young, A=adult and S=senescent). The same decrease in monoterpenoid production with increasing leaf age was identified in *NtLPPS* and *NtCPPS* plants: young leaves stand out as the most productive vegetative tissue in tobacco (Figure 3C,D and Data File S1). *NbLPPS*_11_2_4 and *NtLPPS*_3_1_3 have comparable levels of lavandulol, especially in young leaves. By contrast, tobacco produced more chrysanthemol than *N. benthamiana*, with young leaves of *NtCPPS*_1_3_2 producing more than twice the

levels detected in the best performing *NbCPPS*_5_4_1 young pre-flowering leaves. Estimation of mRNA levels through qRT-PCR in young leaves of pre-flowering plants generally highlighted a correlation between transgene copy number, transcriptional regulation of the biosynthetic genes and monoterpenoid production (Figure S5). The only exception is represented by *NtLPPS* plants, for which greater variability is detected: these plants all carry multiple copies of the transgene and differences in gene expression levels are possibly due to the silencing of some of these copies, also depending on positional effects. In *NbCPPS*, all assayed plants carry one copy of the transgene, and the modest differences in gene expression levels parallel their variation in chrysanthemol production.

For a more accurate measure of monoterpenoid production, solvent extraction was used to obtain absolute quantifications. From *NbLPPS*_11_2_4 whole young T_3 plants, almost 35 µg/g FW of lavandulol were obtained, in contrast to 0.6 µg/g FW of chrysanthemol in *NbCPPS*_5_4_1 plants (Table 1). These quantifications correlate with the accumulation of lavandulol and chrysanthemol observed in relative quantifications (Figure 3A,B). A similar trend was observed in tobacco: an average of 22.55 µg/g FW of lavandulol were retrieved from young leaves of *NtLPPS*_1_3_1 plants, while extraction from young leaves of *NtCPPS*_1_3_2 plants yielded an average of 0.6 µg/g FW of chrysanthemol (Figure 3C,D and Table 1).

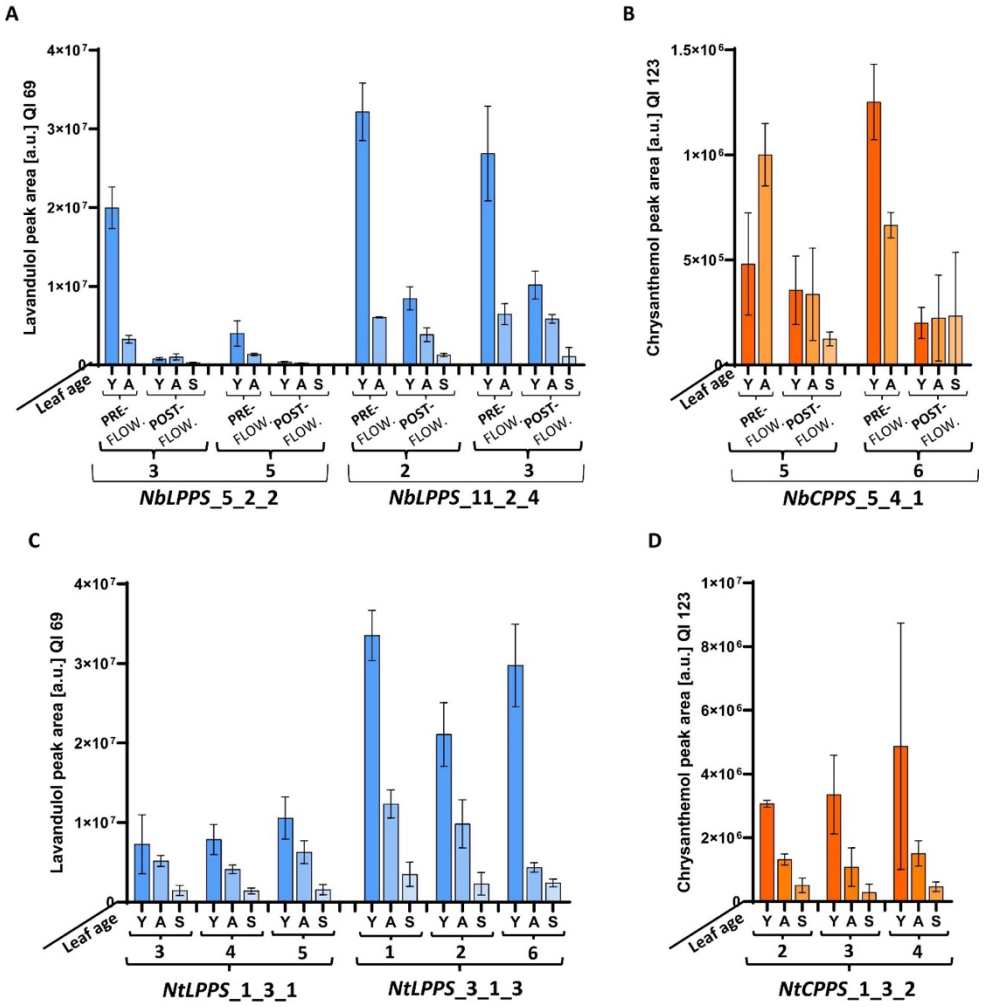


Figure 3: Comparison of lavandulol and chrysanthemol production in ground leaves analyzed by HS-SPME GC-MS at different developmental stages of T_3 *N. benthamiana* and tobacco plants. (A) Lavandulol production in *NbLPPS*_11_2_4 and *NbLPPS*_5_2_2 lines. **(B)** Chrysanthemol production in *NbCPPS*_5_4_1 line. **(C)** Lavandulol production in *NtLPPS*_1_3_1 and *NtLPPS*_3_1_3 lines. **(D)** Chrysanthemol production in *NtCPPS*_1_3_2 line. In panels (A) and (B) values are reported for two plant growth stages (pre-flowering and post-flowering) and for three leaf developmental stages (Y=young, A=adult, S=senescent). In panels (C) and (D) values are reported for three types of leaves (Y=young, A=adult, S=senescent). Production values represent the mean and standard deviation of n = 3 biological replicates (independent leaves). All data for this figure, together with statistical analyses (two-way ANOVA) are reported in Supplementary Data File S1.

Table 1: Quantity (µg) of lavandulol and chrysanthemol obtained from young whole T_3 *NbLPPS* and *NbCPPS* plants and from ground leaves of young T_3 *NtLPPS* and *NtCPPS* plants by solvent extraction and GC/MS/MS quantification.

Plant	µg lavandulol g^{-1} FW	µg chrysanthemol g^{-1} FW
Nb LPPS 11_2_4_7	30.1	-
Nb LPPS 11_2_4_8	29.7	-
Nb LPPS 11_2_4_9	44.6	-
Mean ± sd	34.8 ± 8.5	-
Nb CPPS 5_4_1_7	-	0.61
Nb CPPS 5_4_1_8	-	0.49
Nb CPPS 5_4_1_9	-	0.61
Mean ± sd	-	0.60 ± 0.07
Nt LPPS_1_3_1_3	29.68	-
Nt LPPS_1_3_1_4	21.96	-
Nt LPPS_1_3_1_5	16.02	-
Mean ± sd	22.55 ± 6.85	-
Nt CPPS_1_3_2_2	-	0.43
Nt CPPS_1_3_2_3	-	0.68
Nt CPPS_1_3_2_4	-	0.76
Mean ± sd		0.6 ± 0.2

3. The potential for volatilization of lavandulol and chrysanthemol in vegetative and reproductive tissues of transgenic lines

Volatilization from stabilized ground tissue in HSPME vials was adopted as a high-throughput screening method to estimate the content of VOCs, requiring minimal amounts of manipulation and reagents, as described in the previous section. This allowed us to compare a great number of samples in parallel, while also getting a general picture of the tissue volatilome beyond extraction of single classes of compounds. However, other approaches were deemed necessary to characterize the production and emission of monoterpenoid alcohols more accurately, keeping in mind the prospective use of plants as live bio-dispensers. Two strategies were

followed: *i*) incubating intact samples in HSPME vials and analyzing the emitted compounds and *ii*) analyzing volatiles emitted by intact plants or leaves under dynamic conditions. Flowers emit great quantities of volatiles in many plant species (Loughrin et *al.,* 1991; Dudareva et *al.,* 2013; Adebesin et *al.,* 2017): we wondered if *N. benthamiana* or tobacco flowers could be responsible for a considerable percentage of the total volatile monoterpenoid production of our transgenic plants. In addition to leaves, both intact and ground flowers from transgenic plants were analyzed by HSPME GC/MS.

For *NbLPPS*, the amount of lavandulol detected in ground samples was always higher than the amount emitted by intact tissues (Figure 4A). Leaves were always more productive than flowers, but flowers emitted a proportionally greater amount of lavandulol. In ground samples, leaves contained four times the lavandulol detected in flowers per biomass unit. However, the emitted fraction is higher in flowers than in leaves: intact flowers emit around 30% of the lavandulol detected in ground samples, while this fraction is only around 12% in leaves (Figure 4A). An analogous trend was observed for *NbCPPS*: chrysanthemol emission was almost undetectable in intact leaves, while being considerably higher in ground tissue (Figure 4B), and it was also detected in intact flowers, which emit almost ten times as much chrysanthemol as leaves per biomass unit. The production of lavandulol and chrysanthemol did not depend on the developmental stage of *N. benthamiana* flowers, since no differences were found between flowers before and after anthesis for either compound (Figure S6). When quantifying emission from young intact *N. benthamiana* plants under dynamic conditions, 0.16 µg/g FW/day lavandulol were detected in *NbLPPS* plants, four times the amount of chrysanthemol emitted by *NbCPPS* plants (0.04 µg/g FW/day, Table 2).

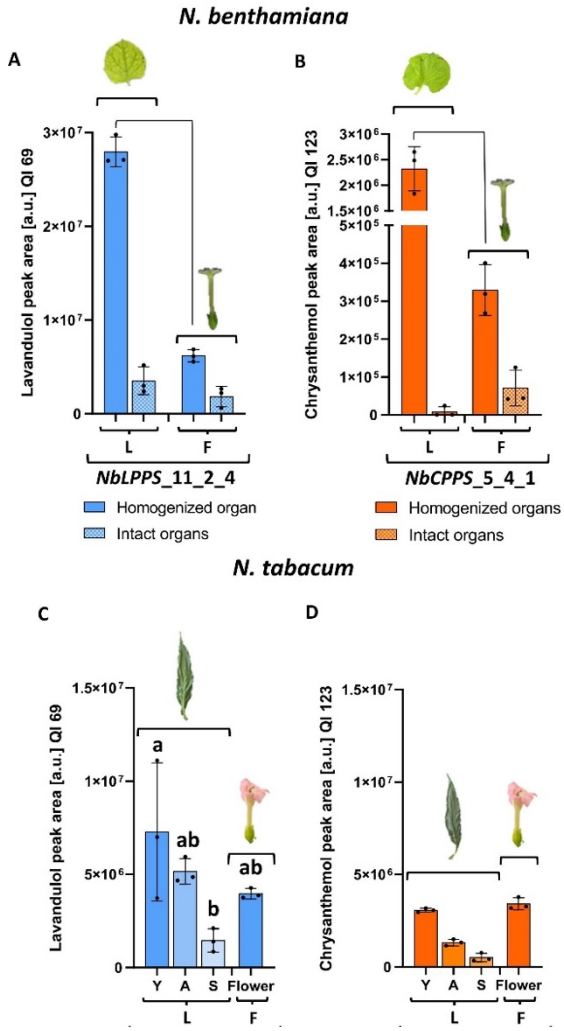


Figure 4. Production of lavandulol and chrysanthemol in T_3 *N. benthamiana* and tobacco plants in different tissue types analyzed by HS-SPME GCMS. (A) Lavandulol production in vegetative and reproductive tissue in line *NbLPPS*_11_2_4, measured in homogenized and intact organs. **(B)** Chrysanthemol production in reproductive and vegetative tissue in line *NbCPPS*_5_4_1, measured in homogenized and intact organs. **(C)** Lavandulol production in vegetative and reproductive tissues in line *NtLPPS*_1_3_1, measured in homogenized leaves and flowers. **(D)** Chrysanthemol production in vegetative and reproductive tissue in line *NtCPPS*_1_3_2, measured in homogenized leaves and flowers. In all panels, leaf tissues are denoted as "L" and flower tissues as "F". In panels (C) and (D) values are reported for three types of leaves (Y=young, A=adult, S=senescent). Values represent the mean and SD of n=3 biological replicates (independent organs). Comparisons (Student's *t* and Mann-Whitney's test for *N. benthamiana* and one-way ANOVA with HSD Tukey's post hoc test, $p<0.05$) were carried out between samples undergoing the same treatment (homogenized or intact). Letters identify significance groups. No significant differences are found in *N. benthamiana* samples.

Table 2: Quantity (µg) of lavandulol and chrysanthemol released by whole T_3 *NbLPPS* and *NbCPPS* young individuals, and by T_3 *NtLPPS* and *NtCPPS* intact young leaves and flowers, measured by volatile collection under dynamic conditions onto Porapak and GC/MS/MS quantification.

Plant	Material	µg lavandulol g^{-1} FW day^{-}	µg chrysanthemol g^{-1} FW
Nb LPPS	Full plant	0,22	-
Nb LPPS	Full plant	0,96	-
Nb LPPS	Full plant	0,16	-
Mean ± sd	Full plant	0,16 ± 0,06	-
Nb CPPS 5_4_1_7	Full plant	-	0,06
Nb CPPS 5_4_1_8	Full plant	-	0,02
Nb CPPS 5_4_1_9	Full plant	-	0,05
Mean ± sd	Full plant	-	0,04 ± 0,02
Nt LPPS_1_3_1_3	Young leaves	0,05	-
Nt LPPS_1_3_1_4	Young leaves	0,06	-
Nt LPPS_1_3_1_5	Young leaves	0,06	-
Mean ± sd	Young leaves	0,05 ± 0,005	-
Nt LPPS_1_3_1_3	Flowers	0,17	-
Nt LPPS_1_3_1_4	Flowers	0,35	-
Nt LPPS_1_3_1_5	Flowers	0,39	-
Mean ± sd	Flowers	0,30 ± 0,12	-
Nt CPPS_1_3_2_2	Young leaves	-	0,02
Nt CPPS_1_3_2_3	Young leaves	-	0,02
Nt CPPS_1_3_2_4	Young leaves	-	0,01
Mean ± sd	Young leaves	-	0,02 ± 0,004
Nt CPPS_1_3_2_2	Flowers	-	0,05
Nt CPPS_1_3_2_3	Flowers	-	0,05
Nt CPPS_1_3_2_4	Flowers	-	0,01
Mean ± sd	Flowers	-	0,03 ± 0,02

In tobacco transgenic lines, the content of both monoterpenes is comparable in ground samples of both flowers and leaves (Figure 4C,D) but, in terms of volatilization,

flowers are more efficient emitters. *NtLPPS* flowers emit around six times more lavandulol than leaves per biomass unit, while *NtCPPS* flowers emit up to twice more chrysanthemol than leaves per biomass unit (Table 2). Yet, at the transcriptional level, the expression of *LiLPPS* and *TcCPPS* in tobacco flowers is lower than in leaves (Figure S5), paralleling the contents detected in ground samples. This further shows that flowers have a higher propensity than leaves for volatilization. Comparing emission of lavandulol and chrysanthemol, under dynamic conditions the lavandulol released by *NtLPPS* young leaves (0.05 µg/g FW/day) is almost three times more abundant than the chrysanthemol released by *NtCPPS* leaves (0.02 µg/g FW/day). Interestingly, the chrysanthemol-derived compounds Artemisia and Yomogi alcohols found in ground samples are barely detectable, if at all, in the emitted volatilome of intact samples, supporting the hypothesis that they were technical artifacts produced by manipulation of ground HSPME samples (Data File S2).

Overall, comparing the two biofactories, *N. benthamiana* plants often show higher emission rates per biomass unit than tobacco leaves (three times more lavandulol and twice more chrysanthemol, Table 2). The two sets of values are not fully comparable (whole *N. benthamiana* plants *vs*. detached tobacco leaves), yet we can hypothesize that the greater biomass of adult tobacco plants would allow a considerably greater total emission. Specifically, the biomass of the analyzed *NtLPPS* tobacco leaves is 5.6 times higher than that of *NbLPPS* plants, and 3 times higher for *NtCPPS* leaves than for *NbCPPS* plants (Figure 2).

4. Lavandulol can be esterified to lavandulyl acetate by the LiAAT-4 acetyltransferase in tobacco

We wanted to test the ability of our plants to produce lavandulyl acetate as an example of an active pheromone compound derived from one of the assayed precursors. In addition to its value as a fragrance, (R)-lavandulyl acetate is a semiochemical found in the aggregation pheromone of the thrips *F. occidentalis* (Hamilton et *al.,* 2005) and in the sex pheromone of the mealybug *D. grassii* (De

Alfonso et *al.,* 2012). Notably, Govindarajan & Benelli, (2016) also highlighted its effectiveness as a mosquito larvicide with a LC50 of around 4 µg/ml in aqueous solution. The AAT4 acetyltransferase from *L. intermedia* (*Li*AAT4) acetylates monoterpenoid alcohols, including lavandulol (L. S. Sarker & Mahmoud, 2015). We first tested *LiAAT4* in *N. benthamiana* leaves: a construct containing the P35S::*LiAAT4*::tNOS transcriptional unit was agroinfiltrated on T_3 *NbLPPS* plants (Figure 5A) and high levels of acetylation of the lavandulol substrate were obtained (Figure 5B), with an average 70% of the substrate detected in the absence of *Li*AAT4 converted to lavandulyl acetate.

Given the greater biomass and general robustness of tobacco compared to *N. benthamiana*, we used the T_1 tobacco line producing the highest levels of lavandulol, *NtLPPS*_3_1, for stable transformation with *LiAAT4*. In the *NtLPPS_AAT4* T_0 population, different efficiencies were observed for the conversion of lavandulol to lavandulyl acetate, measured by HSPME GC-MS (Figure S7). Some individuals showed especially good acetylation rates, producing levels of lavandulyl acetate comparable to those of lavandulol in their parental line, with accordingly lower levels of lavandulol. As for production of monoterpenoid alcohols, the production of metabolites is directly correlated with the abundance of transcripts (Figure S7). However, a strong negative correlation was observed between production levels and fitness, and the plants with the highest lavandulyl acetate production were not able to produce viable seeds. For phenotypic characterization, the progeny of the plant with the highest production levels which allowed to collect viable seeds (*NtLPPS_AAT4*_8) was chosen. As for *NtLPPS* plants, we evaluated accumulation in different plant tissues. Like in plant biofactories of monoterpenoid alcohols, young leaves of T_1 *NtLPPS-AAT4* plants were the most productive tissues (between 2- and 7-fold more than adult leaves), while the lowest levels of lavandulyl acetate were observed in senescent leaves, paralleling lavandulol availability (Figure 5C). Again, the greatest source of variation was the type of tissue. The estimation of transgene copy number in these plants confirmed the presence of multiple copies of the *LiLPPS* transgene in the *NtLPPS* background, while one or two copies of the *LiAAT4* transgene were detected, coherently with the 1:3 susceptible:resistant ratio observed when

germinating plantlets on selective medium. No significant differences were found between the levels of lavandulyl acetate in the three tested T_1 individuals, while modest differences were found at the transcriptional level (Figure S5). Again, production and emission were determined by solvent extraction and under dynamic conditions, respectively. Toluene extractions from leaves showed a markedly greater accumulation of lavandulol than of lavandulyl acetate (Table 3), with the alcohol being tens of times more abundant than the acetate. These data contrast sharply with the results of the analyses of homogenized tissue, in which the acetate:alcohol ratio is always higher and favors the acetate (Figure 5C). On the other hand, lavandulyl acetate is emitted at a much higher rate than lavandulol (Table 4). This different distribution can be attributed to the greater polarity of lavandulol compared to lavandulyl acetate, determining for the latter a greater volatilization due to a lower affinity for the aqueous environment of the cell. The same can be observed in the analysis of ground samples by HSPME, in which lavandulyl acetate moves at higher rates from the aqueous $CaCl_2$ EDTA buffer to the headspace.

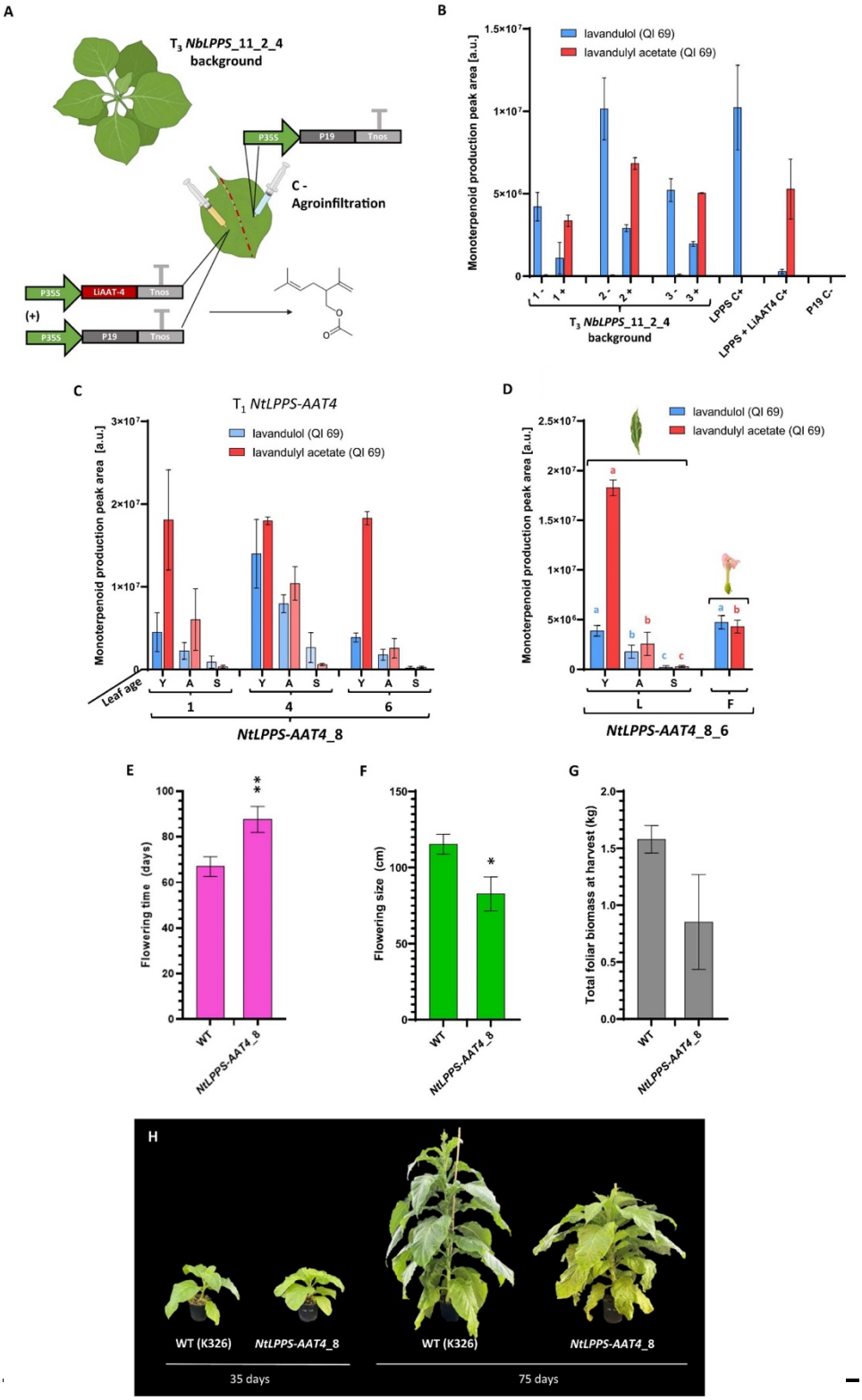

A
T3 NbLPPS_11_2_4 background
P35S
P19
Tnos
C - Agroinfiltration
P35S
LiAAT-4
Tnos
(+)
P35S
P19
Tnos
B
lavandulol (OI 69)
lavandulyl acetate (OI 69)
Monoterpenoid production peak area [a.u.]
1.5×10^7
1×10^7
5×10^6
0
1 -
1 +
2 -
2 +
3 -
3 +
T3 NbLPPS_11_2_4 background
LPPS C+
LPPS + LiAAT4 C+
P19 C-
C
T1 NtLPPS-AAT4
lavandulol (OI 69)
lavandulyl acetate (OI 69)
Monoterpenoid production peak area [a.u.]
3×10^7
2×10^7
1×10^7
0
Leaf age
Y
A
S
1
4
6
NtLPPS-AAT4_8
D
lavandulol (OI 69)
lavandulyl acetate (OI 69)
Monoterpenoid production peak area [a.u.]
2.5×10^7
2×10^7
1.5×10^7
1×10^7
5×10^6
0
Y
A
S
L
F
NtLPPS-AAT4_8_6
E
Flowering time (days)
100
80
60
40
20
0
**
WT
NtLPPS-AAT4_8
F
Flowering size (cm)
150
100
50
0
*
WT
NtLPPS-AAT4_8
G
Total foliar biomass at harvest (kg)
2.0
1.5
1.0
0.5
0.0
WT
NtLPPS-AAT4_8
H
WT (K326)
NtLPPS-AAT4_8
WT (K326)
NtLPPS-AAT4_8
35 days
75 days

Figure 5 (previous page). Esterification of lavandulol to lavandulyl acetate. (A) Constructs carrying the *LiAAT4* transgene controlled by the CAMV35s promoter and NOS terminator and the silencing suppressor P19, and the design of the agroinfiltration assay. **(B)** Levels of lavandulyl acetate obtained by infiltrating *Li*AAT4 in *NbLPPS* T_3 plants (*NbLPPS*_11_2_4 background) and analyzed by HS-SPME GC-MS; for each individual, '-' indicates infiltration with P19 alone, while '+' indicates infiltration with *Li*AAT4 and P19. Positive controls are represented by WT leaves infiltrated with *Li*LPPS alone (LPPS C+) and with *Li*LPPS and *Li*AAT4 (LPPS AAT4 C+), always combined with P19. The negative control is represented by WT plants infiltrated only with P19 (C-). **(C)** Production of lavandulol and lavandulyl acetate in the T_1 progeny of *NtLPPS_AAT4*_8, analyzed in ground leaves by HS-SPME GC-MS; values are reported for three types of leaves (Y=young, A=adult, S=senescent). **(D)** Production of lavandulol and lavandulyl acetate in reproductive (indicated as F) and vegetative (indicated as L) tissues (ground samples) of T_1 *NtLPPS_AAT4*_8 plants, analyzed by HS-SPME GC-MS. Three types of leaves are analyzed (Y=young, A=adult, S=senescent). Comparison of flowering time **(E)**, plant size at flowering **(F)** and total foliar biomass accumulated at harvest time (140 days) **(G)** in *NtLPPS_AAT4* and WT plants. In panel (E), time measured as days from transfer to soil to formation of the floral meristem in T_1 *NtLPPS_AAT4* and WT plants. **(H)** Phenotype of T_1 *NtLPPS_AAT4*_8 plants compared to the WT at 35 and 75 days in soil. In panels (B), (C) and (D) values are the mean and standard deviation of 3 independent samples. In panels (E), (F) and (G), values are the mean and standard deviation of at least 3 independent plants of each line. *P*-values were calculated using Student's *t*-test; $*P \leq 0.05$, $**P \leq 0.01$. The figure includes images from Biorender (biorender.com).

Table 3. Quantity (µg) of lavandulol and lavandulyl acetate obtained from ground young leaves of T_1 *NtLPPS-AAT4* plants by solvent extraction and GC/MS/MS quantification.

Plant	**µg lavandulol g^{-1} FW**	**µg lavandulyl acetate g^{-1} FW**
Nt LPPS-AAT4_4_4	16.37	0.49
Nt LPPS-AAT4_4_5	17.85	0.70
Nt LPPS-AAT4_4_6	16.85	0.56
Mean ± sd	17.02 ± 0.75	0.58 ± 0.11
Nt LPPS-AAT4_8_1	10.55	0.85
Nt LPPS-AAT4_8_4	15.88	0.64
Nt LPPS-AAT4_8_6	6.49	0.46
Mean ± sd	10.97 ± 4.71	0.65 ± 0.20

Regarding flowers, the levels of lavandulol and lavandulyl acetate in homogenized samples are very similar (Figure 5D), also being comparable to those of lavandulol in *NtLPPS* flowers (Figure 4C). While in *NtLPPS* flowers the transcriptional levels of *LiLPPS* were lower than those reported in the leaves, in *NtLPPS_AAT4* flowers both *LiLPPS* and *LiAAT4* are expressed as much as in leaves (Figure S5). Volatilization from intact flowers again favors lavandulyl acetate over lavandulol. In absolute terms, the emission rate from flowers is on average 2.5-fold less abundant than it is from young leaves (0.25 *vs*. 0.63 µg/g FW/day, see Table 4).

Table 4. Quantity (µg) of lavandulol and lavandulyl acetate released from intact organs (leaves and flowers) of T_1 *NtLPPS-AAT4* plants, obtained by volatile collection under dynamic conditions onto Porapak, and GC/MS/MS quantification.

Plant	Material	µg lavandulol g^{-1} FW day^{-1}	µg lavandulyl acetate g^{-1} FW day^{-1}
Nt LPPS-AAT4_4_4	Leaves	0,06	0,11
Nt LPPS-AAT4_4_5	Leaves	0,12	0,39
Nt LPPS-AAT4_4_6	Leaves	0,11	0,34
Mean ± sd	Leaves	0,1 ± 0,03	0,28 ± 0,15
Nt LPPS-AAT4_8_1	Leaves	0,09	0,86
Nt LPPS-AAT4_8_4	Leaves	0,09	0,56
Nt LPPS-AAT4_8_6	Leaves	0,04	0,47
Mean ± sd	Leaves	0,07 ± 0,03	0,63 ± 0,20
Nt LPPS-AAT4_8_1	Flowers	0,17	0,17
Nt LPPS-AAT4_8_3	Flowers	0,19	0,41
Nt LPPS-AAT4_8_6	Flowers	0,12	0,18
Mean ± sd	Flowers	0,16 ± 0,04	0,25 ± 0,14

The heterologous production of lavandulyl acetate, too, was accompanied by pleiotropic effects such as yellowing leaves, a delay in flowering and a lower biomass at flowering time (Figure 5E,F,G,H and Figure S4). Interestingly, when compared to the T_3 tobacco plants derived from the same parental used for stacking the *LiAAT4* transgene, the aberrant phenotype of the plants producing only lavandulol (Figure

2H) was overall more severe than that of those producing high levels of lavandulyl acetate: *NtLPPS*_3_1_3 plants accumulate 22% of the leaf biomass of WT tobacco, while *NtLPPS_AAT4*_8 T_1 plants reach 53%. However, the greatest reduction in chlorophyll index was found in *NtLPPS-AAT4* plants (Figure S4).

DISCUSSION

In this study, we demonstrated the potential of tobacco and *N. benthamiana* plants as biofactories of irregular monoterpenes and their derivatives. Several reports show the heterologous production of mono- and sesquiterpenes in bacteria, yeasts and plants (Q. Wang et *al.,* 2019; Xie et *al.,* 2019; Dusséaux et *al.,* 2020; Fuentes et *al.,* 2016). However, very few examples exist of the production of irregular monoterpenoids, and many of their biosynthetic pathways remain unknown (Minteguiaga et *al.,* 2023). As case studies, we chose (*R*)-lavandulyl pyrophosphate and (1*R*,3*R*)-chrysanthemyl pyrophosphate and their alcohols, which are among the few irregular monoterpenoids whose biosynthetic pathways are known. The chirality of the products of *Li*LPPS and *Tc*CPPS, which is important for their biological activity, had been previously determined by heterologous expression and purification (Demissie et *al.,* 2013; Rivera et *al.,* 2001). The most widely studied among irregular monoterpene derivatives is chrysanthemic acid, for its importance as the monoterpene moiety of type I pyrethrins. Its full biosynthetic pathway was elucidated (Xu, Moghe, et *al.,* 2018) and heterologous expression was achieved in fruits and glandular trichomes of tomato (Xu, Lybrand, et *al.,* 2018; Y. Wang et *al.,* 2022). T. Yang et al. (2014) also expressed *Tc*CPPS in tobacco to demonstrate its ability to produce chrysanthemol *in planta*, but this was done mainly as a proof of concept rather than to estimate the yield of tobacco biofactories. Concomitantly, heterologous production of insect pheromones has focused almost exclusively on moth sex pheromones, with a few notable exceptions, like the production of 8-hydroxygeraniol in engineered yeast (H. Wang et *al.,* 2023), and that of the sesquiterpenoid aphid alarm pheromone (*E*)-β-farnesene in wheat, the first crop engineered to release an insect pheromone

(Bruce et *al.,* 2015). In this respect, our work moves towards filling this niche and establishing plant-based biofactories for irregular monoterpenoids with prospective uses for extraction and formulation of a variety of products, as well as for live emission in greenhouses or the open field. Thus, we focused on understanding the phenotypic effects associated with bioproduction in unspecialized cells following a constitutive expression strategy, as well as in estimating the biosynthetic potential.

Bioproduction of monoterpenes in *Nicotiana* using a constitutive overexpression strategy is not physiologically innocuous. For instance, (Yin et *al.,* 2017) found early flowering and increased branching when overexpressing the peppermint geranyl diphosphate synthase small subunit in tobacco. Other deleterious effects were observed for different terpenoids, including chlorosis, dwarfism, and a reduction in fertility (Huchelmann et *al.,* 2017). Because of the similarity of the phenotypes observed in different reports, cytotoxicity of the new metabolites is considered not to be the only cause, and perhaps plant depletion of its essential terpenoid precursors (IPP/DMAPP) is also playing a role. Many of the observed deleterious effects in our transgenic plants were dose dependent. We observed a reduction in size and leaf biomass of transgenic plants producing lavandulol and lavandulyl acetate, correlating with the greatest reductions in chlorophyll index. In *N. benthamiana*, the reduction in leaf biomass for *NbLPPS* was due mostly to the observed reduction in number of lateral shoots (data not shown). Despite the observed effects, in general plants showing moderate production levels (e.g., chrysanthemol producers in this study) also showed moderate phenotypic effects, suggesting that a balance between volatile productivity and biomass production can be reached. Whether such balance is favorable in technoeconomic terms will depend on the absolute production levels and the concentrations required for achieving a biological effect.

The analysis of productivity across tissues and developmental stages was useful to understand the dynamics of biosynthesis, as well as the design of the best conditions for biofactory use. Metabolite accumulation depends, at least in part, on precursor availability at a specific growth stage and on the activation of competing metabolic pathways (Drapal et *al.,* 2021). Developmental information is also crucial to determine

at what stage plant tissues should be harvested or used as biodispensers to maximize product yield. We found that young leaves at the pre-flowering stage are most productive tissue in most instances. We also observed that, at least in the constitutive overexpression strategy followed here, flowers do not provide a special advantage in terms of accumulation or volatile emission. While emission per biomass unit is often higher in flowers than it is in vegetative tissues, the total biomass of tobacco flowers represents on average less than 5% of the leaf biomass of an adult plant: an adult tobacco plant can carry around 100 flowers, each weighing around 500 mg, for a total flower biomass of approximately 50 g, while leaf biomass in an adult tobacco plant is usually around 1 kg. Also, flowers are relatively short-lived organs, while tobacco has the ability to produce new leaves and shoots when older leaves are harvested, extending the production cycle. In the light of these results, it could be advisable to grow plants in short cycles, harvesting before flowering to maximize productivity. Also, non-flowering tobacco varieties (Schmidt *et al.,* 2020) could be suitable candidates for plant biofactories, since genetically impeded flowering would increase their biosafety profile when grown in the open field or in association with other crops. Other suggested strategies to increase product yields and to reduce pleiotropic effects in heterologous hosts include accumulation in trichomes using specific promoters and transporters (Huchelmann et *al.,* 2017). This might not represent an ideal solution for the accumulation of volatile compounds in tobacco, since it does not possess peltate trichomes such as those of aromatic plants, but rather, its capitate trichomes are specialized for the secretion on the leaf surface of non-volatile diterpenes and phytoalexins (Tissier et *al.,* 2017). While it might be a worthwhile approach to increase volatilization for bioemitters, it is possible that the greater mesophyll biomass still guarantees greater yields using ubiquitous promoters, given that fitness loss is kept within acceptable levels.

As expected, absolute product yields were found to be metabolite dependent. Lavandulol yields were consistently higher than those of chrysanthemol (38- and 58-fold more lavandulol than chrysanthemol was extracted from tobacco and *N. benthamiana* tissues, respectively). Previous reports of heterologous expression of *Tc*CPPS found that chrysanthemol may be glycosylated (Xu, Lybrand, et *al.,* 2018) and

this could account for these consistently lower levels, as well as, possibly, for the lower fitness loss observed in *NbCPPS* and *NtCPPS* plants. Based on the biomass data and the quantification of monoterpenoids, we could estimate the average production per plant for each transgenic line as 0.3 mg chrysanthemol in *NtCPPS* plants, around 16 mg lavandulol for *NtLPPS* plants, and around 3.4 mg lavandulol from a *NtLPPS-AAT4* plant. For lavandulyl acetate, only 0.20 mg of compound per plant could be extracted. Based on toluene extraction levels, approximately 6 kg of young tobacco leaves (6 plants) would be necessary to produce enough lavandulyl acetate to treat 1 L of water against mosquito larvae at its LC50 (Govindarajan & Benelli, 2016). Altogether, it seems clear that at the extractable yields obtained using constitutive overexpression are currently too low to provide a competitive advantage to alternative production systems.

In contrast with the modest technoeconomic perspectives of the constitutive expression strategy in terms of extraction yields, our data suggest a high potential of the tobacco platform as volatile live biodispensers. For this, it is important to put the data obtained here in the context of pest control strategies conducted in the field with related pheromones. Comprehensive assessments of mating disruption strategies to control the mealybug *Planococcus ficus* using lavandulyl senecioate were recently reported by Daane et al. (2020) and Lucchi et al. (2019) using field deployed dispensers containing chemically synthetized racemic compounds. Here it was found that significant results in pest control were obtained with dispensers loaded with a total of 4.15 g/ha over a season. Likely, the conclusions drawn in these studies may apply to other mealybugs and other pheromones comprising irregular monoterpene esters. According to our daily emission estimations of lavandulyl acetate in tobacco plants (correcting for leaf age and number of leaves at each stage in an adult plant), a few hundred (200-500) plants per hectare would be sufficient to ensure similar release levels as those reported by (Lucchi et *al.,* 2019). Depending on the crop to which they would be coupled, this might represent a feasible density. The factors relevant for the effectiveness of pheromone dispensers include a steady emission rate (which can be more important than absolute pheromone concentration) and constant coverage during the season, to ensure pheromone emission during all periods of peak

flight activity. Plant bioemitters, in this respect, represent interesting solutions because their emission depends on renewable metabolic resources, and it is not restricted to the initial load of the dispenser. The life cycle of a tobacco plant, for example, is compatible with that of other crops to which it may be coupled as producer of pheromones. Also, all available dispensers use racemic mixtures, only half of which is the active ingredient, while biosynthesis ensures stereospecificity.

In conclusion, we show here that tobacco plants producing irregular monoterpenoids, and particularly lavandulyl acetate, are a valuable model to understand the feasibility of using live pheromone emitter plants as tools for mating disruption. Further improvements might be envisioned to increase productivity and volatilization. However, compared to previous works on plant-based production of lepidopteran pheromones (Mateos-Fernández et *al.,* 2021), tobacco plants producing monoterpene esters appear to be a more viable tool for plant-based pest control. Finally, in addition to being biofactories and live emitters, these plants represent a versatile metabolic and genetic tool for the combinatorial assessment of a variety of enzymatic activities (e.g., acyltransferases from different sources) acting upon the constitutively expressed monoterpenoid precursors to yield an array of pheromone compounds and other bioactive molecules.

Supplementary Data

Supplementary Table S1. List of the GB constructs used or generated in this study.
Supplementary Table S2. List of the DNA primers used in quantitative PCRs.
Supplementary Figure S1. Stable production of lavandulol and chrysanthemol in transgenic *N. benthamiana* and tobacco T_0 – T_2 plants.
Supplementary Figure S2. Levels of Artemisia alcohol, Yomogi alcohol and santolinatriene detected in transgenic *TcCPPS N. benthamiana* and tobacco T_0 – T_2 plants.
Supplementary Figure S3. Transgene copy number determined by quantitative PCR.
Supplementary Figure S4. Chlorophyll Index (C.I.) of transgenic *N. benthamiana* and tobacco plants producing irregular monoterpenoids.
Supplementary Figure S5. Transcriptional levels of the monoterpene biosynthetic genes in young leaves and flowers.
Supplementary Figure S6. Production of volatile monoterpenoids in transgenic *N. benthamiana* T_3 lines in flowers at different development stages.
Supplementary Figure S7. Esterification of lavandulol to lavandulyl acetate in T_0 stable tobacco plants.
Supplementary Data File S1. All data and statistical analysis relative to figures in the main text.
Supplementary Data File S2. All data and statistical analysis relative to supplementary figures.
Supplementary Data File S3. Data of qPCRs and qRT-PCRs.

Data availability

All data reported in this study, as well as statistical analyses, are available in Supplementary Files S1, S2 and S3, deposited at Zenodo: https://doi.org/10.5281/zenodo.8427571. The sequences of the plasmids used for transformation can be consulted at https://goldenbraidpro.com/.

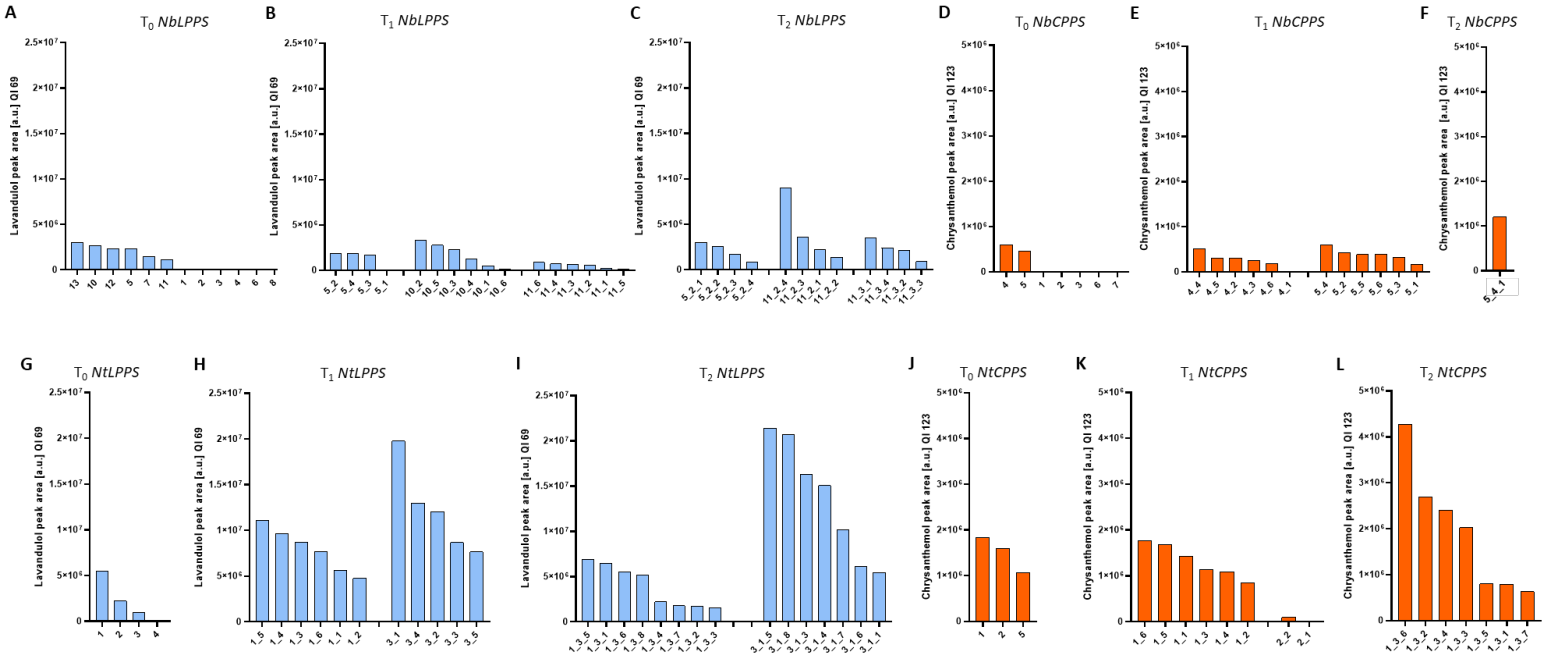
SUPPLEMENTARY INFORMATION

Table S1. Existing GoldenBraid constructs used in this study (in blue) and novel GoldenBraid constructs assembled in this study (in black).

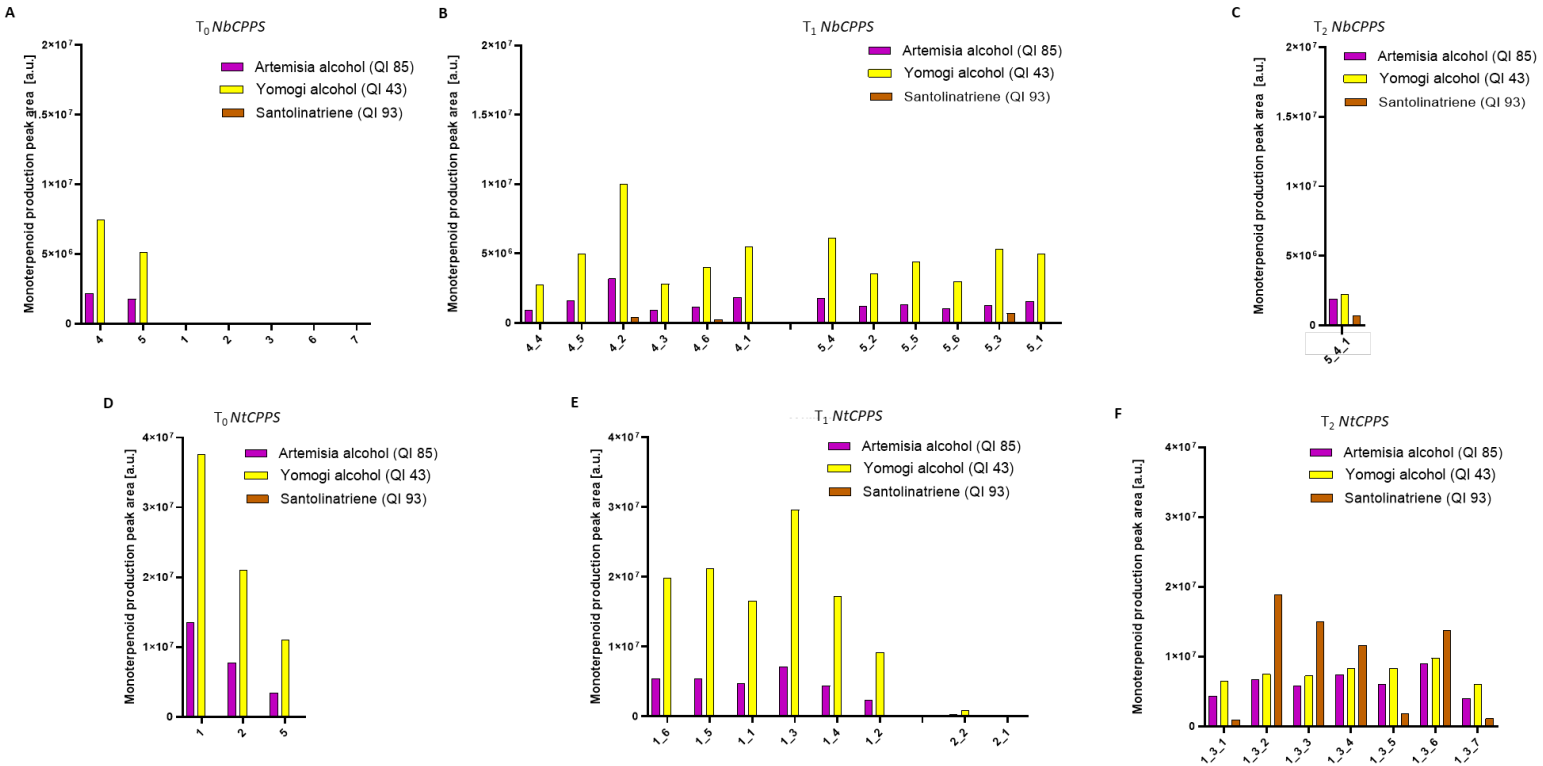
GB ID	Name	Description
GB0030	pUPD:p35S	CaMV 35S promoter
GB0037	pUPD:tNos	*Agrobacterium tumefaciens terminator* NOS
GB0107	pEGB SF	Twister plasmid to swap inserts from an alpha1 or alpha1R vector to any omega level vector
GB0108	pEGB p35s:P19:tNos	TU for the expression of the silencing supressor P19
GB0235	pEGB 1Alpha1R tNos:HygroR:pNos	Hygromycin TU for stable transformation
GB1181	pEGB 3Ω1 tNos:nptII:pNos-SF	TU for kanamycin resistance gene (*nptII*) plant expression under the regulation of the Nos promoter
GB3059	pUPD_CPPS (B4)	pUPD containg CDS of chrysantemyl pyrophosphate synthase from *Tanacetum cinerariifolium*
GB3060	pUPD_LPPS (B4)	pUPD containing CDS of lavandulyl pyrophosphate synthase *from Lavandula x intermedia*
GB3061	3α1 p35s:CPPS-His:tNos	TU for the expression of chrysanthemyl pyrophosphate synthase (CPPS) with 8xHis
GB3062	3α1 p35s:LPPS-His:tNos	TU for the expression of lavandulyl pyrophosphate synthase (LPPS) with 8xHis
GB3418	3Ω2 p35s:LPPS-His:tNos-SF	TU for expression of lavandulyl pyrophosphate synthase (LPPS) with a Stuffer Fragment (SF)
GB3419	3Ω2 p35s:CPPS-His:tNos-SF	TU for expression of chrysanthemyl pyrophosphate synthase (CPPS) with a Stuffer Fragment (SF)
GB3420	3α1 p35s:LPPS-His:tNos-SF-tNos:nptII:pNos-SF	TU for expression of lavandulyl pyrophosphate synthase (LPPS) with a Stuffer Fragment (SF) with kanamycin resistance gene (*nptII*)
GB3421	3α1 p35s:CPPS-His:tNos-SF-tNos:nptII:pNos-SF	TU for expression chrysanthemyl pyrophosphate synthase (CPPS) with a Stuffer Fragment (SF) with kanamycin resistance gene (*nptII*)
GB4092	pUPD2 LiAAT4	CDS of monoterpene acetyltransferase of *Lavandula x intermedia* LiAAT4
GB4093	3α2 p35s:LiAAT4:tNos	TU containing LiAAT4 monoterpenoid acetyltransferase from *Lavandula x intermedia*
GB4157	3Ω1 tNos:HygroR:pNos p35s:LiAAT4:tNos	Module for expression of LiAAT4 monoterpenoid acetyltransferase from *Lavandula x intermedia* with hygromycin resistance gene

Table S2. Primers used in this study for quantitative PCRs.

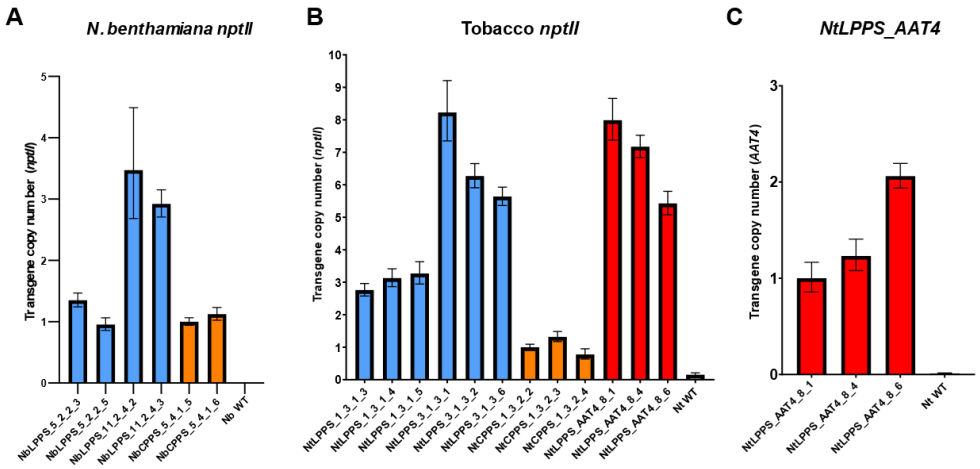
Primer	**Sequence**
N. benthamiana actin forward	TATGGAAACATTGTGCTCAGTGG
N. benthamiana actin reverse	CCAGATTCGTCATACTCTGCC
N. benthamiana Fbox forward	GGCACTCACAAACGTCTATTTC
N. benthamiana Fbox reverse	ACCTGGGAGGCATCCTGCTTAT
Tobacco actin forward	CCTCACAGAAGCTCCTCTTAATC
Tobacco actin reverse	ACAGCCTGAATGGCGATATAC
nptII forward	TTGCCGAATATCATGGTGGA
nptII reverse	TCAGCAATATCACGGGTAGC
LiLPPS forward	CACAAAATTCCCAAGCCCGG
LiLPPS reverse	CCTCGCCAAAATCCGGAAAC
TcCPPS forward	CCGAGGTTGGAGTAGTTGCT
TcCPPS reverse	GAGGTCCAGAAGATGCACGT
LiAAT4 forward	AGTGTCCATCGCCAGTCATG
LiAAT4 forward	CGGCCCTCAATCTCGAATGA



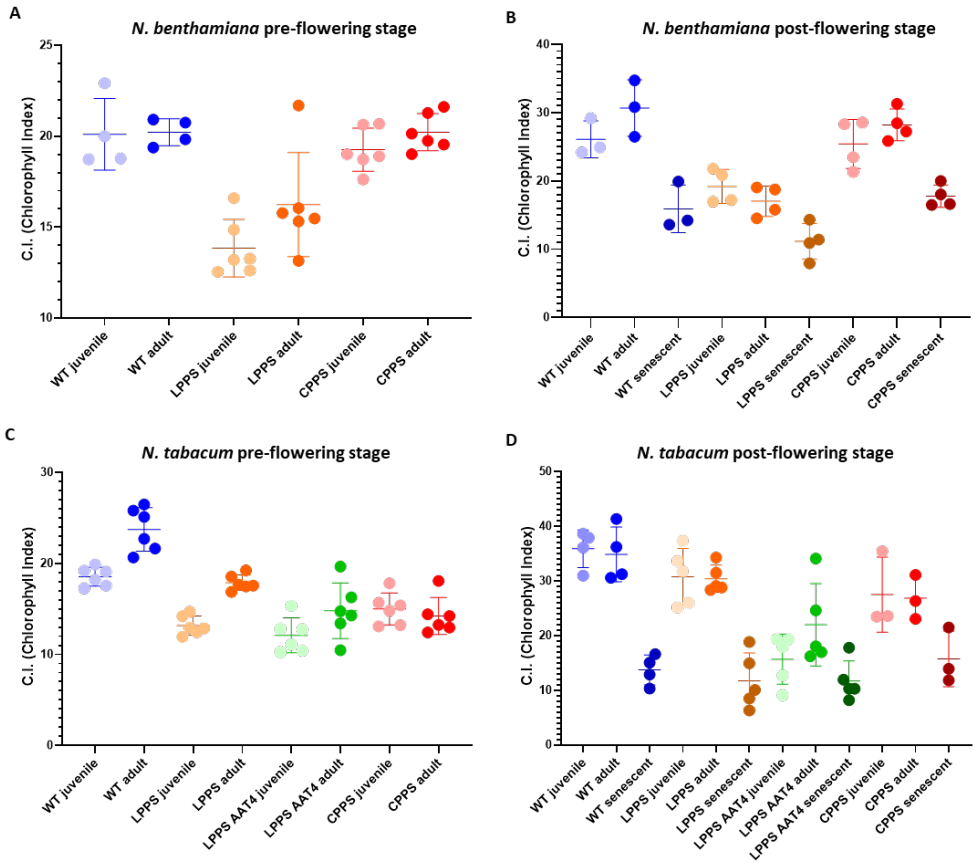
Supplementary Figure S1. Stable production of lavandulol and chrysanthemol in transgenic *N. benthamiana* and *N. tabacum* $T_0 - T_2$ plants. Lavandulol production levels in individual *NbLPPS* T_0 plants (**A**), T_1 plants (**B**), and T_2 plants (**C**). Chrysanthemol production levels in individual *NbCPPS* T_0 plants (**D**), T_1 plants (**E**), and T_2 plants (**F**). Lavandulol production levels in individual *NtLPPS* T_0 plants (**G**), T_1 plants (**H**) and T_2 plants (**I**). Chrysanthemol production levels in individual *NtCPPS* T_0 plants (**J**), T_1 plants (**K**), and T_2 plants (**L**). Each value represents the production of the mix of the three youngest fully expanded leaves of 35-40 days old plants (ground samples analyzed by HS-SPME GC-MS).



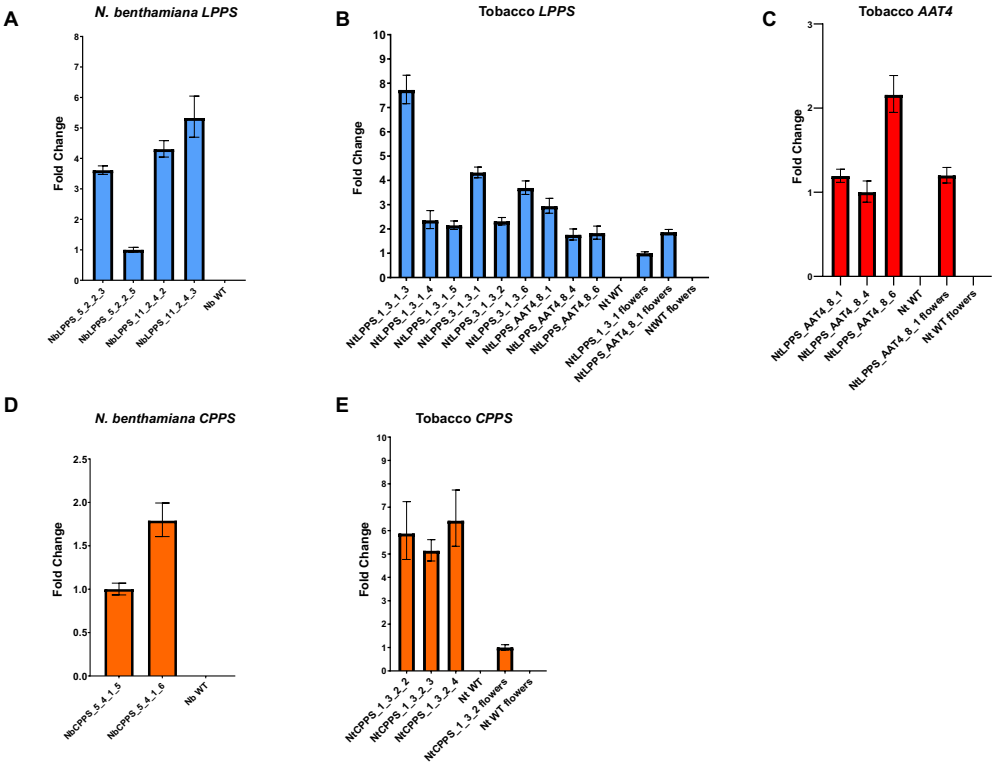
Supplementary Figure S2. Levels of Artemisia alcohol, Yomogi alcohol and santolinatriene detected in transgenic *Tc*CPPS *N. benthamiana* and *N. tabacum* $T_0 - T_2$ plants. Artemisia alcohol, Yomogi alcohol and santolinatriene levels in individual *NbCPPS* T_0 plants (**A**), T_1 plants (**B**), and T_2 plants (**C**). Artemisia alcohol, Yomogi alcohol and santolinatriene production levels in individual *NtCPPS* T_0 plants (**D**), T_1 plants (**E**) and T_2 plants (**F**). Quantification Ions (QI) selected for peak area integration are specific for each molecule, making areas are not fully comparable between compounds. Each value represents the production of the mix of the three youngest fully expanded leaves of 35-40 days old plants (ground samples analyzed by HS-SPME GC-MS).



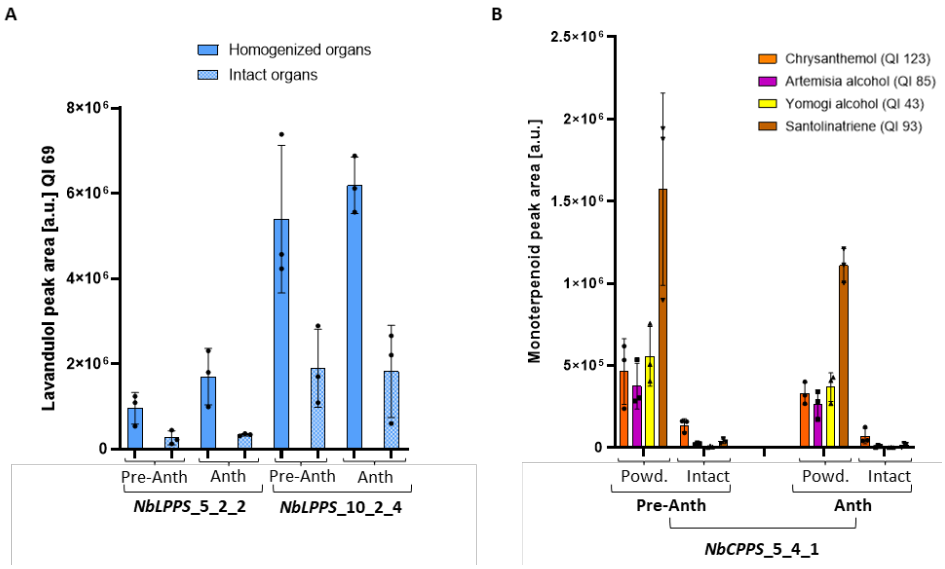
Supplementary Figure S3. Transgene copy number in *N. benthamiana* and *N. tabacum* biofactories of irregular monoterpenoids. (**A**) Determination of the copy number of the *nptII* resistance marker in *NbLPPS* and *NbCPPS* T_3 plants used for phenotypic assessments. (**B**) Determination of the copy number of the *nptII* resistance marker in *NtLPPS, NtCPPS* T_3 plants and NtLPPS_AAT4 T_1 plants used for phenotypic assessments. (**C**) Determination of the copy number of the *AAT4* transgene in *NtLPPS_AAT4* T_1 plants used for phenotypic assessments. Values are determined using the *N. benthamiana* and *N. tabacum* actin gene as reference for each species, respectively, and using the single-copy *NbCPPS* and *NtCPPS* plants (as repeatedly determined by segregation on selective media) as calibrators in the $\Delta\Delta C_T$ method.



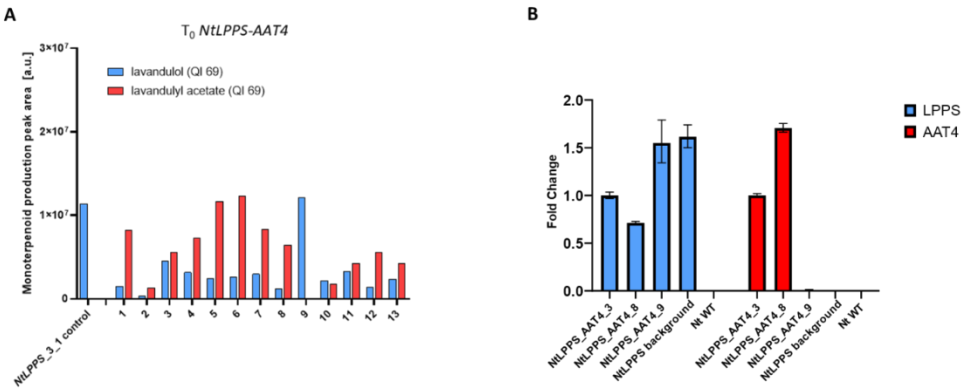
Supplementary Figure S4. Chlorophyll Index (C.I.) in transgenic *N. benthamiana* and *N. tabacum* plants producing irregular monoterpenoids. (**A**, **B**) Chlorophyll Index measured in juvenile, adult and senescent leaves of T_3 *NbLPPS* and *NbCPPS,* and WT *N. benthamiana* in the pre-flowering (A) and post-flowering (B) stages. (**C**, **D**) Chlorophyll Index measured in juvenile, adult and senescent leaves of T_3 *NtLPPS* and *NtCPPS,* T_1 *NtLPPS-AAT4* and WT *N. tabacum* in the pre-flowering (C) and post-flowering (D) stages. Values represent the mean and standard deviation of n = 3-6 biological replicates.



Supplementary Figure S5. Transcriptional levels of the monoterpene biosynthetic genes in young leaves and flowers. (**A**) Expression levels of the *LiLPPS* transgene in *NbLPPS* T_3 plants. (**B**) Expression levels of the *LiLPPS* transgene in *NtLPPS* T_3 plants and in *NtLPPS_AAT4* T_1 plants. (**C**) Expression levels of the *LiAAT4* transgene in *NtLPPS_AAT4* T_1 plants. (**D**) Expression levels of the *TcCPPS* transgene in *NbCPPS* T_3 plants. (**E**) Expression levels of the *TcCPPS* transgene in *NtCPPS* T_3 plants. For each data set, the sample with the lowest expression was used as calibrator in the $\Delta\Delta C_T$ method.



Supplementary Figure S6. Production of volatile monoterpenoids in transgenic *N. benthamiana* T_3 lines in flowers at different development stages. (**A**) Lavandulol detected by HS-SPME GC-MS in ground samples or emitted from intact flowers of two T_3 *NbLPPS* lines, at the pre-anthesis and anthesis stages. (**B**) Chrysanthemol and derived compounds detected by HS-SPME GC-MS in ground samples or emitted from intact flowers of the T_3 *NbCPPS*_5_4_1 line, at the pre-anthesis and anthesis stages. Values represent the mean and standard deviation of n = 3 biological replicates (independent flowers). Quantification Ions (QI) selected for peak area integration are specific for each molecule, thus making areas are not directly comparable between compounds.



Supplementary Figure S7. Esterification of lavandulol to lavandulyl acetate in T_0 stable tobacco plants. (**A**) Production of lavandulol and lavandulyl acetate in T_0 *NtLPPS-AAT4* tobacco plants, as determined by HS-SPME GC-MS in ground samples. Each value represents mixed leaf tissue from the three youngest fully expandend leaves of 35-40 days old plants. (**B**) The transcriptional levels of the *LiLPPS* and *LiAAT4* transgenes in a pool of T_0 *NtLPPS-AAT4* tobacco plants and in their *NtLPPS* parental (background). All transcriptional levels are referred to those of plant *NtLPPS_AAT4_*3 in the $\Delta\Delta C_T$ method.

General discussion

GENERAL DISCUSSION

• Metabolic engineering towards pheromone products

The pheromone market, valued at nearly 5 billion USD in 2024, is poised for significant growth, with projections suggesting an increase to almost USD 18 billion by 2032, according to Fortune Business Insights (www.fortunebusinessinsights.com). This expansion is driven largely by the reduced toxicity of pheromones compared to synthetic pesticides, aligning with a global shift towards more eco-friendly agricultural practices. Moreover, economies like India and China are tightening regulations on chemical pesticides, positioning pheromones as strategic alternatives. However, pheromone production faces substantial cost barriers, primarily due to the expensive synthesis processes and additional expenses related to packaging in dispensers or traps, which complicates their adoption, particularly among small-scale farmers and for non-specialty crops. In the marketplace, sex pheromones hold a dominant share, significantly ahead of aggregation pheromones. The market is segmented by application into dispensers, traps, and spray methods, with dispensers currently leading. Orchards are the primary cropping system employing pheromones, driven by the significant losses inflicted by Lepidopterans, and relying on the high value of the end products to amortize the costs of treatments.

Regarding production methods, while chemical synthesis remains prevalent, there is a growing shift towards alternative bioproduction strategies. A standout in this field is BioPhero, a company founded in 2016 by Irina Borodina as a spinout from the Technical University of Denmark, and now part of FMC since 2022. The company uses yeasts, such as *Yarrowia lipolytica*, to produce a range of pheromones. This approach is currently among the most viable, both at the regulatory level and industrially, since it allows for greater scalability (Petkevicius et *al.,* 2022). BioPhero exemplifies what is feasible at the present moment, yet, like many companies in the biotech sector, it faces the ongoing challenge of scaling and market integration. In 2022, the company contracted their first agreement with a customer to produce a moth pheromone compound active on a major rice pest. As more customers request their services, bio-

based companies like BioPhero will be able to consolidate their presence in the market. Scalability is one of the major challenges faced by biotech companies: the efficiency achieved on a laboratory scale does not always translate to industrial-scale efficiency, and genetically modified microorganisms or plants may exhibit high productivity in controlled lab or greenhouse environments but struggle with consistency and stability on a larger scale. On the other hand, achieving economic feasibility can be difficult, because the costs of methods, as well as the development and maintenance of the biofactories, can be very high. Also, competing with the established chemical synthesis methods which are often more cost-effective in the short term, and have a longer track record of commercial success, adds to the difficulty. Thus, marketing and education efforts are necessary to build trust and demonstrate the benefits of bio-synthesized pheromones.

When comparing the biosynthetic yields of plants with those of yeast or cell cultures, microorganisms usually perform better. Petkevicious and collaborators (2022) achieved notable yields of 0.21 mg/L of the alcohol Z7-12:OH and 0.48 mg/L of Z7-14:OH using metabolically engineered *Y. lipolytica*. In contrast, plant biofactories normally have to rely on accumulation in specific organs to be competitive: for instance, metabolically engineered *Camelina sativa* has been shown to accumulate fatty acid pheromone precursors for up to 40% of seed dry weight, making extraction presumably easier (Ortiz et *al.,* 2020). One complication of production in plant tissues is represented by the presence of a wide variety of other metabolites (e.g., phenolics, chlorophylls, sugars) that can interfere with the extraction process (Jones & Kinghorn, 2012). Therefore, plants can be valuable biofactories if the expression levels are high and the extraction process is straightforward. Additionally, in line with the principles of circular and bioeconomy, plant biofactories may serve further purposes, such as producing other metabolites, generating biomass, or aiding in soil remediation. The practical limitations of pheromone production in plants are akin to the reasons synthetic pyrethroids are favored over pyrethrins: although there is a known source of pyrethrins that produces them in specialized organs at relatively high concentrations by plant metabolism standards, the challenges of cultivation, extraction and purification often make synthetic alternatives more viable.

However, plant biofactories have a potential application never achievable in a fermentation tank: they can be used as live biodispensers, as was firstly conceptualized and demonstrated by Beale and collaborators in 2006 with *Arabidopsis thaliana* (and later wheat) plants emitting (*E*)-β-farnesene. Despite the advantages of this strategy, including application in the field, their use in Europe is restricted by GMO regulations. Even with the latest prospects of acceptance of the New Genomics Techniques by the EU, the plants that we have presented in this thesis, by including a heterologous metabolic pathway, are excluded from this liberalization. A different scenario may be in place for their confined use in greenhouses, where transgenic biodispensers may work as companion crops to non-transgenic and non-sexually compatible productions.

- **The "biodispenser" strategy in pheromone-engineered plants**

In our vision, the most attractive feature of generating transgenic plants biosynthesizing insect sex pheromones is their power to release semiochemicals to the surrounding atmosphere. It is a unique and remarkable characteristic of this kind of biofactory, intrinsically not achievable in bacteria or fungi. In fact, producing pheromones in these engineered plants avoids the needs, expenses, and residues related to producing a trap or a dispenser (Mateos-Fernández et *al.,* 2022). These living biodispensers are devised to be employed following an intercropping strategy, where the emitter plant works as a companion of the crop to be protected (Lopes et *al.,* 2016). Moreover, bioemitters can be used following two strategies: attraction and mating disruption. In the first strategy, biodispensers protect crops from pests by attracting them to other areas. Attraction requires lesser quantities of pheromone compared to mating disruption, but high specificity is needed in turn, which means avoiding the presence of other compounds competing with the active pheromone. Attraction strategies may fit with mealybug pheromone-producing plants, such as *NtLPPS-AAT4*: Pseudococcidae require tiny amounts of pheromones, on which they

rely heavily, and their pheromone blends contain fewer components than those of Lepidoptera, often with one major component which is sufficient for attraction (Tabata, 2020). On the other hand, the mating disruption strategy, which requires overall higher quantities of the semiochemicals to be effective, could potentially fit with both Lepidopteran and mealybug pheromone-producing plants. The SexyPlant prototypes, producing moth pheromone compounds, would probably only be useful within a mating disruption strategy but, to ensure effective doses, emission rates should be increased.

There are evident differences in the performance of the moth and mealybug plant pheromone biodispensers presented in this thesis. The most promising SxPv1.2 released as far as 79.3 ng Z11-16OH/plant/day and 88.3 ng Z11-16OAc/plant/day. Monoterpenoid emitter plants are much more efficient: for *N. benthamiana* plants, which were measured in similar dynamic conditions as SxPv1.2, T_3 *NbLPPS* released an average of 1.7 µg lavandulol/plant/day, and T_3 *NbCPPS* 0.3 µg chrysanthemol/plant/day. In tobacco biodispensers, estimations of approximate total emissions point to an average of 16.84 µg lavandulol/plant/day in *NtLPPS* plants, and to an average of 4.7 µg chrysanthemol/plant/day in *NtCPPS* plants. Importantly, these are not active pheromone compounds: *NtLPPS-AAT4* plants are estimated to emit up to 92.46 µg lavandulyl acetate/plant/day. Not surprisingly, active mealybug pheromone compounds are highly volatile, and are emitted more efficiently than their precursors. In Chapter 2 we estimated the number of plants per hectare necessary for setting up a proper mating disruption strategy: using between 200 and 500 lavandulyl acetate tobacco plants per hectare would ensure an emission comparable to that of typical pheromone dispensers, loading 4.15 g pheromone/ha over a season. Moreover, when using an approach of constitutive expression, plants provide a continuous emission, which is sometimes preferred to higher concentrations of pheromones over a limited period of time.

- **Future approaches to pheromone biosynthesis in plants**

The most evident disadvantage encountered in plant pheromone biofactories is the toxicity associated with the accumulation of pheromone-related metabolites, with a possible consequent deregulation of host metabolism, which we first investigated in plants producing moth sex pheromones (Mateos-Fernández et *al.,* 2021; Juteršek et *al.,* 2022). The mentioned growth penalty was also registered to a lower extent in the *N. benthamiana* and tobacco plants overexpressing monoterpenoid metabolites presented in Chapter 2: for example, *NtLPPS3* accumulated as little as 20% the biomass of wild-type plants. Solutions to this may be found with spatiotemporal regulation of product biosynthesis. Spatially, this would require restricting biosynthesis to specific tissues and cell types, avoiding generalized toxicity. In tobacco and *N. benthamiana*, however, this does not appear practical, since these species lack peltate glandular trichomes for storage of volatile compounds, and also do not produce oily seeds where fatty-acid derivatives may be accumulated. Temporal regulation, inducing biosynthesis at appropriate times, would be more viable, although technologically more challenging.

In Chapter 1, we explored the possibility of activating a full metabolic pathway using CRISPRa, causing the 'detonation' of pheromone production at a desired time. Other collaborators and members of our team have explored activation of metabolic pathways through copper-mediated induction (Garcia-Perez et *al.,* 2022; Kallam et *al.,* 2023). This induction system is particularly appealing because sprayable copper sulfate is a user-friendly and widely accepted treatment even in field conditions (V. Kumar et *al.,* 2021). Thus, as a complementation of the results presented in Chapter 1, Kallam et al. (2023) activated moth sex pheromone biosynthesis in plants employing two different copper-based systems. In the first one, we assembled multigene constructs in which the genes for the biosynthesis of moth sex pheromone compounds are controlled by minimal synthetic promoters containing CUP2 binding sites (CBS); the transcriptional activator system CUP2:GAL4, formed by the yeast copper responsive factor CUP2 fused to the non-viral yeast activator domain Gal4, is also added. When copper is administered, CUP2 suffers a conformational change,

which enables it to specifically bind to the CBS in the promoters, activating transcription of the target genes. In the absence of copper, the system is characterized by a low background expression. This approach was tested transiently by agroinfiltrating WT *N. benthamiana* leaves with the construct carrying the synthetic metabolic pathway and CUP2:GAL4, and spraying three days after agroinfiltration with copper sulfate. The second successful strategy was equally based on the CUP2-GAL4 system, this time coupling it to the dCasEV2.1 system: by regulating the expression of the dCasEV2.1 with CBS-containing synthetic promoters, the CRISPRa system is expressed in the presence of copper, and this precisely activates the moth sex pheromone pathway genes (which were, in turn, under the control of synthetic promoters with dCasEV2.1 binding sites). This double regulation strategy, when tested transiently, was also successful, yielding considerable levels of pheromones after activation and a tight control of expression in the absence of the copper inductor.

Another interesting strategy for pathway regulation could make use of molecular memory switches, such as the genetic switch based on the phage ϕC31 integration system (Bernabé-Orts et *al.,* 2020). This orthogonal tool allows to alternatively activate, in a mutually exclusive fashion, the expression of two genes of interest (GOI). The genetic toggle switch is composed of a central invertible element composed of a promoter (CaMV35S) and a terminator, which serves as an insulator sequence. The invertible element is flanked by *att* recombination sites and a gene of interest is set on each side of the device (Figure 1A). Thus, the promoter initially points at one of the genes, driving its expression, while the other is turned off. When recombination of the central element is induced administering the ϕC31 integrase, the promoter comes to face the opposite coding sequence, switching the first gene off. Switch recombination can also be reversed when adding the ϕC31 integrase with its recombination directionality factor (RDF). In contrast to transcriptional activation requiring constant supply of inductors (such as gRNAs or copper sulfate), toggle switches are memory devices whose changes are conserved until intentionally reversed, and whose state is also inherited by the progeny (Smith et *al.,* 2010). In the master thesis of Corbalán-Acedo (2022), conducted in our laboratory, a toggle switch was constructed to regulate the expression of the dCasEV2.1 system, and assayed for

the regulation of the same moth sex pheromone Guided Pathway (GP, GB3897) described in Chapter 1 (Figure 1B). The activation of the downstream pathway was successful, but the great disadvantage we encountered with this strategy was the very high basal expression levels of the target metabolites even when the dCasEV2.1 activator system was supposedly switched off by the toggle switch, pointing to very loose regulation. To improve the system, which holds an enormous potential in controlling the expression of potentially toxic metabolic pathways, several synthetic designs can be proposed. These include using longer insulation sequences and/or weaker promoters in order to minimize basal expression. If this were achieved, it would be possible to activate the synthesis of pheromone compounds only when the target insect is detected in the field, or even to have alternative insect pheromone pathways activated at different times according to the pests present in the environment at a given time (Figure 1C).

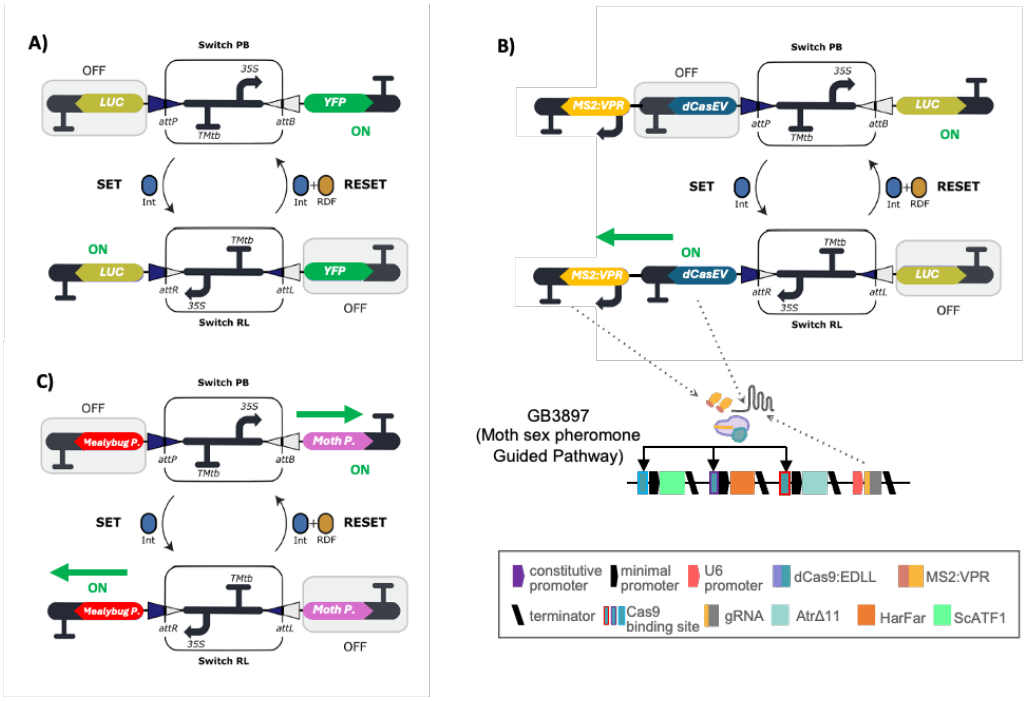


Figure 1 (next page). Alternative strategies for the use of the memory switch based on the phage ϕC31 integration system for activatable pheromone production in plant biofactories. (A) Basic functioning of the genetic memory switch design with a simple reporter1:reporter2 configuration. In the upper part, the switch displays a PB configuration and allows the transcription of Yellow fluorescent protein (YFP), while Luciferase (LUC) transcription remains inactivated. When the ϕC31 integrase (Int) is administrated (SET operation, at the bottom), recombination of the central element is induced and YFP transcription turns OFF, while luciferase transcription switches ON. To come back to the original configuration, administration of both Int and recombinant directionality factor (RDF) is needed. **(B)** Memory switch design in which the promoter initially points at the transcription of a reporter (LUC). When integrase is administrated, the switch inverts itself allowing the transcription (ON) of the transcriptional activator dCasEV2.1, which translated product binds a preexistent in genome activatable insect sex pheromone biosynthetic pathway such as the already presented SxP GuidedPathway, allowing the biosynthesis of the related pheromone compounds and turning OFF the reporter/growth factor. **(C)** Memory switch design in which the promoter initially points at the transcription of a certain insect sex pheromone biosynthetic pathway (ON, e.g. moth sex pheromones, Moth P.). When integrase is administrated, the switch inverts itself allowing the transcription (ON) of another insect sex pheromone biosynthetic pathway (e.g. mealybug sex pheromones, Mealybug P.), while the moth sex pheromone pathway is turned OFF. This strategy could be profitable in crop pest changing situations. Adapted from Bernabé-Orts et al. (2020).

- **Strategies and challenges in pathway reconstruction and in the identification of candidate genes**

The two classes of pheromone compounds considered in this thesis derive from relatively simple metabolic pathways which exploit ubiquitous intermediates shared by all plant species, namely fatty acids and hemiterpenes, so a limited number of heterologous activities need to be introduced to yield the final products. Lepidopteran sex pheromones are typically composed of a blend of different fatty acid derivatives (e.g., alcohols, aldehydes and acetates), where fatty alcohol esters represent a big share of the bioactive compounds (Leal-Soares et *al.,* 2020; Tamaki et *al.,* 1977). Mealybug pheromone blends are often composed of just two monoterpenoid esters (Zou & Millar, 2015), mostly derived from irregular monoterpenes. Such blends can be composed of either different esters of the same monoterpenoid alcohol (like (*R*)-(-)-lavandulyl propionate and acetate in *D. grassii*), or of molecules with a different monoterpenoid moiety and the same substituent group (like (*R*)-lavandulyl (*S*)-2-methylbutanoate and (*R*)-maconellyl (*S*)-2-methylbutanoate in *Maconellicoccus hirsutus*). Alcohol moieties, as detailed in the introduction, include lavandulol, chrysanthemyl alcohol, planococcol, maconelliol, necrodol, and others (A. Zhang et *al.,* 2004; Ho et *al.,* 2009; Vacas et *al.,* 2019; Bierl-Leonhardt et *al.,* 1981). Substituent groups, on the other hand, include acetates, propionates, senecioates, methylbutanoates, isovalerates, and others (De Alfonso et *al.,* 2012; Tabata & Ichiki, 2016; Zada et *al.,* 2003; Ho et *al.,* 2009). For moth sex pheromones, various insect genes have been successfully characterized for the biosynthesis of unsaturated fatty acids and their reduction to alcohols, while, as we have seen, no effective insect acetyltransferase has been tested so far. We originally expected the biosynthetic pathways of monoterpenoid-derived mealybug pheromones to be relatively simpler, involving in principle down to only two steps added to the plant 'wild-type' metabolic context: one for the coupling of isoprene units for the biosynthesis of an irregular monoterpenoid, and another for its esterification. Unfortunately, their elucidation has proven extremely difficult and, to date, the only means to produce compounds with a pheromone-like activity for mealybugs in a heterologous host rest on the use of

plant genetic resources, thanks to convergent evolution between a few plant and insect enzymatic activities.

When setting out to identify pheromone biosynthetic enzymes, approaches are normally directed at the analysis of genomic and/or transcriptomic data from the target insect species. The 'standard' procedure involves the isolation of RNA from pheromone glands and the comparison of transcript abundance between individuals which produce the pheromones (typically, unmated females) and others which do not (mated females and/or males), as well as between pheromone glands and control tissues. This approach was successful in identifying several Lepidopteran and Coleopteran sex pheromone biosynthetic genes, including desaturases and reductases (Y. N. Zhang et *al.,* 2017; Buček et *al.,* 2015; Lancaster et *al.,* 2019). Suitable candidate genes are subsequently tested in heterologous organisms such as *E. coli*, *S. cerevisiae* or, in our case, plants. Matters are rather more complicated in the case of mealybugs, not least because the site of pheromone biosynthesis in their bodies has not been identified and their very small size makes the isolation of organs extremely laborious, requiring collecting thousands of individuals to isolate enough biological material for analysis.

In the SUSPHIRE project, in whose framework this thesis was developed, our group and collaborators aimed to unravel the biosynthetic pathway of the pheromone of the *Planococcus citri* mealybug. Even with the generation of high-quality transcriptomic data, and relying on an available genomic sequence, no conclusive results were reached (Juteršek et *al.,* 2024). Eighteen candidate sequences corresponding to putative *cis* and *trans* IDS enzymes were identified after contrasting the transcriptomes of virgin females, which produce the pheromone, and mated females, where pheromone biosynthesis is completely absent. Such candidates were tested, alone or in combination, with *in vitro* assays providing various suitable substrates, as well as in biological hosts (*E. coli* and *N. benthamiana*), but none yielded pheromone-related compounds. Our failures fit into the context of a general lack of knowledge regarding mealybug pheromone biosynthetic pathways. Mealybugs synthesize highly unusual irregular, and often cyclic, monoterpene derivatives, which

for the most part have not been described in other organisms. Animals normally possess a farnesyl diphosphate synthase which also produces geranyl diphosphate, but lack further enzymes coupling two C5 isoprene units. Conversely, IDS and terpene synthases are widespread in the Plant Kingdom, but the great phylogenetic distance between the two taxa implies that, beyond a general prediction of function, they cannot be used to search for distinctive domains or motives within insect data, nor can common biosynthetic mechanisms be assumed a priori. The identification of a *P. citri* irregular IDS enzyme would have meant a major step forward in mealybugs biology and biochemistry and would have shed light on insect monoterpene biosynthesis, facilitating the identification of new activities in related species, as well as opening the doors to metabolic engineering for pest control. As it is, our options for bioproduction are currently limited to the exploitation of irregular plant IDSs, like LPPS and CPPS. Relying on plant enzymes, another approach to gaining insight into mealybug pheromone biosynthesis was based on introducing mutations in known IDS enzymes, aiming at shifting their activity and specificity. Yet again in the framework of SUSPHIRE, our collaborators engineered the *Solanum lycopersicum* trichome-specific neryl diphosphate synthase (*Sl*NPPS), a regular *cis* IDS (Gerasymenko et *al.,* 2022). The N88H *SlNPPS* mutant did in fact produce up to 13.1% of irregular monoterpenoids when fed exclusively with DMAPP. Interestingly, one of these compounds was (1*R*, 3*S*)-planococcyl diphosphate, the *trans* isomer of planococcyl-PP. This achievement, far from allowing us to produce irregular compounds such as mealybug pheromones in large quantities, allows us to get closer to identifying the key amino acid residues responsible for non-head-to-tail coupling and cyclization.

• Acyltransferases: evasive enzymes

We have seen how important esterifications are for both mealybug and moth pheromones. From the point of view of chemical synthesis, the most limiting, time-consuming, and expensive reactions are those leading up to the fatty or monoterpene alcohols to esterify. In this sense, setting up biofactories to produce these alcohols

would already be an improvement in pheromone production pipelines, and esterifications could easily be performed chemically. However, this is of course inviable when seeking to develop biodispensers. Unfortunately, the identification of such activities has proved particularly arduous. Similarly to IDS enzymes and monoterpene synthases, plants possess well-described enzyme families, such as BAHD- and SCPL-type acyltransferases, which lack an equivalent in animal genomes (Bontpart et *al.,* 2015; Ciarkowska et *al.,* 2019; W. Zhang et *al.,* 2024). From a biocatalytic perspective, there is an abundance of enzymes, such as the *Candida antarctica* lipase B (CALB), which can perform a multitude of esterifications when provided with specific substrates, but they are hardly suited for expression in complex biological environments with many competing substrates (L. Li et *al.,* 2015; Cha et *al.,* 2019). More specific enzymes are thus required.

In Lepidoptera, a fatty acyl transferase is presumably responsible for the transfer of the acetyl groups to fatty alcohol substrates. Despite the many efforts made to uncover a functional sequence, no acetyltransferase naturally implied in moth pheromone biosynthesis has ever been found (B. J. Ding & Löfstedt, 2015). Insect acetyltransferases other than those involved in acetylating fatty alcohols are the arylalkylamine N-acetyltransferases (AANAT), involved in neurotransmitter kinetics or cuticle formation (C. Y. Wu et *al.,* 2020; Tsugehara et *al.,* 2007), and protein acetyltransferases, a wide enzyme category mainly involved in histone acetylation for controlling chromatin dynamics and epigenetic regulation (Lee & Workman, 2007). The plant diacylglycerol *O*-acyltransferases like *Ea*DAcT (B. J. Ding et *al.,* 2014) or *Ef*DAcT (Tran et *al.,* 2017) employed to bypass this issue are categorized with the Enzyme Commission number EC 2.3.1.20, while yeast alcohol *O*-acetyltransferases such as *Sc*ATF1 (B. J. Ding et *al.,* 2016) or *Sp*ATF1-2 (Salazar et *al.,* 2019; Yoshimoto et *al.,* 1999) are coded as EC 2.3.1.84. Their success is probably due a certain tolerance for different substrates.

On the other hand, the esterification of monoterpenoid alcohols is regarded as the final step in the biosynthesis of active mealybug pheromones, which is thought to be performed by specific acyltransferases. It is likely that, based on available knowledge,

insect genes encoding these enzymes would not be easily identifiable *in silico* from their catalytic domains. It is also possible that this step is performed by enzymes with other catalytic activities, which also accept pheromone precursors as substrates. In this thesis, we show that the plant BAHD-type acetyltransferase *Li*AAT4 can successfully acetylate the product of *Li*LPPS in a heterologous biofactory (*N. tabacum*) in order to obtain (*R*)-lavandulyl acetate, a molecule with different applications as pheromone compound (for the mealybug *D. grassii* and for the thrip *F. occidentalis*) and as larvicidal agent against mosquitoes. L. S. Sarker & Mahmoud, (2015) show that BAHD alcohol acetyltransferases can accept various monoterpenoid substrates, since both *Li*AAT3 and *Li*AAT4 are able to produce acetates of geraniol, lavandulol and nerol. This enzyme is therefore expected to act also on other monoterpenoid alcohols involved in mealybug pheromone biosynthesis. While *Li*AAT4 is a very valuable addition to our toolkit, the variety of mealybug pheromones also depends on the diversity of the substituent groups. Many known plant acyltransferases act on short chain fatty acids, sugars and phenylpropanoids (Yauk et *al.,* 2017). To date, no other plant acyltransferase active on monoterpenoid alcohols has been characterized, but many monoterpenoid esters (e.g., isovalerates, propionates, methylbutanoates) have been described in a variety of plant species, especially aromatic and medicinal plants. The identification of these enzymatic activities in plants is, from our perspective, an effort well-worth undertaking for both plant biology and biotechnology. Genomes and transcriptomes are available for some of these species, but the great chemical diversity between cultivars and landraces of these plants, as well as the seasonal variability in secondary metabolite concentration in their tissues, make it extremely important to select the correct database for candidate search. Also, these specific esters sometimes represent a small percentage of the total metabolite composition of plant extracts, complicating the identification of associated transcripts.

Finally, one additional point remains unclear in the biosynthesis of monoterpenoid esters. As discussed in Chapter 2, after expression in *N. benthamiana* and in tobacco of LPPS and CPPS we detected the monoterpenoid alcohols lavandulol and chrysanthemol. However, *in vitro* assays indicate that IDS enzymes catalyze the biosynthesis of a non-volatile isoprenyl diphosphate, which needs to be

dephosphorylated to yield the corresponding volatile alcohol. This is how the activities of GPPS, NDPS and LPPS are understood (J. Zhou et *al.,* 2015; Schilmiller et *al.,* 2009; Demissie et *al.,* 2013). Only for CPPS, and only under very specific conditions, a bifunctional activity has been reported (T. Yang et *al.,* 2014), describing CPPS as capable of dephosphorylating chrysanthemyl diphosphate to chrysanthemol. We do not know whether dephosphorylation of lavandulyl diphosphate to lavandulol is catalyzed by LPPS or whether it is the product of an endogenous phosphatase in the hosts. *In vitro* tests conducted so far (by (Demissie et *al.,* 2013) and by us, not shown) seem to indicate that LPPS produces only lavandulyl-PP, yet it remains unclear why no other similar monoterpenoid alcohol (such as geraniol) is present in the volatilomes of *N. benthamiana* and tobacco if such an activity is present in the hosts. Investigating this specific step in the pathway may lead to greater efficiency in the production of downstream compounds.

Conclusions

CONCLUSIONS

1. Several optimized versions of the moth sex pheromone-producing plant "SexyPlant" were developed and characterized, with the aim to bypass the toxicity handicaps encountered in the previous SxPv1.0 and v1.2 biofactories. Alternative acetyltransferase genes were characterized for pheromone heterologous expression in plants, with *ScATF1* having the best conversion rate of the lepidopteran pheromone compound Z11-16OH to Z11-16OAc. Alternatively, other acetyltransferases were assayed, which reported a range of conversion efficiencies, eligible under convenience.

2. A strategy for conditional moth pheromone production comprising the dCasEV2.1 programable activation system targeting specifically and simultaneously and all three synthetic promoters driving the biosynthetic genes was shown to function efficiently in transiently transformed *N. benthamiana* leaves. The pheromone biosynthesis pathway was activated only in the presence of a specific gRNA targeting the synthetic promoters.

3. Stable *N. benthamiana* transgenic lines carrying the conditional pheromone biosynthesis pathway either with its activating gRNA (referred to as Guided Pathway, GP) or without the gRNA (referred to as Non-Guided Pathway, NGP) were generated. These transgenic lines yielded high pheromone production levels when activated with orthogonal elements: dCasEV2.1 in the case of GP, or dCasEV2.1 plus RNA guide in the case of NGP.

4. Heterologous overproduction of irregular monoterpenoids (i.e. lavandulol, lavandulyl acetate, chrysanthemol and compounds related to chrysanthemol), with a role as precursors in mealybug sex pheromones, was established in *N. benthamiana* and *N. tabacum* transgenic lines. The trends of accumulation of irregular monoterpenes were described in different tissue

types and developmental stages, highlighting greater accumulation in juvenile leaves.

5. Tobacco plants producing lavandulyl acetate represent a valuable model to understand the feasibility of using live pheromone biodispenser plants as tool for pest management. At the current production levels, the volatilization of lavandulyl acetate by *NtLPPS-AAT4* plants points to an estimated average of 200-500 plants per hectare necessary to achieve pheromone concentrations in the environment similar to those used for traditional mating disruption approaches.

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