Hybrid acyl-CoA desaturases investigated regarding structure in relation to functionality and specificity

Axel Erlansson

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Swedish University of Agricultural Sciences, SLU
Faculty of Landscape Architecture, Horticulture and Crop Production Science
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Axel Erlansson

Supervisor:	**Per Hofvander, SLU, Department of Plant Breeding**
Assistant supervisor:	Ida Lager, SLU, Department of Plant Breeding
Assistant supervisor:	Kamil Demski, SLU, Department of Plant Breeding
Examiner:	Åsa Grimberg, SLU, Department of Plant Breeding

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Swedish University of Agricultural Sciences
Department of Plant Breeding

Abstract

The use of insect sex pheromones in integrated pest management (IPM) systems offers an environmentally friendly alternative to chemical pesticides. However, widespread commercial use of pheromones is limited by high production costs and environmental concerns related to the chemistry of synthetic production. A promising solution is to express insect desaturase enzymes in plants, which could enable biological production of pheromone precursors. Such precursors can be used as raw material for further synthesis to pheromones. This project has investigated if hybrid desaturases, constructed from structural membrane domains of a plant desaturase (ADS1 from *Arabidopsis thaliana*) and the active sites of insect desaturases (AtrΔ11, DmeΔ9, and Cpo_CPRQ) could be transiently expressed in the plant *Nicotiana benthamiana*.

Wild type insect desaturases were functionally expressed in leaf tissue and their expected pheromone precursors were produced, but none of the constructed hybrid desaturases retained functionality. Furthermore, the plant desaturase ADS1, used as a scaffold for the hybrids, also failed to show activity when expressed in *Nicotiana benthamiana*, which suggests compatibility issues or mislocalization in this system. However, a potentially novel finding was made, where leaves infiltrated with the DmeΔ9 wild-type gene produced both the presumed target pheromone precursor 14:1 Z9, as well as a second precursor 16:1 Z11, which likely indicates an elongation of the desaturated products.

The results of the study have demonstrated the complexity of metabolic engineering in plant systems and that successful development of hybrid desaturases is highly dependent on scaffold selection, enzyme localization and host compatibility.

Keywords: Pheromones, insect desaturases; desaturase enzymes; acyl-CoA desaturases, Nicotiana benthamiana, pest management.

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Abbreviations

Abbreviation:	Description:
ACP	Acyl carrier protein
AS	Acetosyringone
CoA	Coenzyme A
ER	Endoplasmic reticulum
FA	Fatty acid
FAD	Fatty acid desaturase
GFP	Green fluorescent protein
(h)	Hybrid
TAG	Triacylglycerol
(WT)	Wild type

1. Introduction

Modern agriculture is facing challenges on multiple fronts. Climate change and growing populations are putting increasing pressure on primary producers to ensure high crop productivity, while there is simultaneously an increasing pressure to reduce the negative environmental impacts of agriculture (Witzgall et al, 2010). One of the concerns about the negative environmental impacts has been that pest management strategies within conventional agriculture have relied heavily on broad-spectrum chemical pesticides. Using this approach has multiple problems. Firstly, these chemicals often have low specificity, meaning a wide range of non-target organisms will be negatively affected, including beneficial insects. Secondly, the application often leave chemical residues in the soil or food, related to environmental toxicity and health issues, and thirdly, increasing resistance of pests to the chemical is also of increasing concern. Despite all the efforts to control pests, herbivorous insects are responsible for more than 20 % of the global agricultural production being lost, which indicates that the industry is in great need of solutions (Wang et al, 2022).

An alternative approach to using broad-spectrum chemical pesticides is a strategy that involves using insect sex pheromones (Petkevicius et al, 2020). Pheromone-based approaches have been adopted to monitor and disrupt mating behaviors of pest insects in integrated pest management (IPM) systems. These are highly species-specific, which allows effective control of pest insects without negatively impacting beneficial insects or ecosystems as a whole. Thereby, they are a lot more environmentally friendly and less concerning regarding both human health and pest resistance. Pheromone-based solutions have been used for more than 30 years, and the global pheromone market for agricultural pest control is projected to exceed 7 billion USD by 2027 (Wang et al, 2022). However, the use of pheromones in pest management is in large part limited by high cost. They are currently used mostly for high value horticultural crops such as fruits and nuts, while being quite inaccessible for low- and middle-income agricultural settings. The production of synthetic pheromones is also a significant challenge. It is a process that involves toxic solvents and generate a large amount of chemical waste which is difficult to recycle or dispose of.

To overcome the challenges of production costs and chemical waste, a potential solution that has been explored is biological production in plants. By inserting insect genes that are involved in pheromone production into plant genomes, these could be used as a sort of biological factories (Ding et al, 2014). Thereby, plants could produce precursors for insect sex pheromones, which has the potential to significantly lower costs and reduce waste.

Insect pheromones mainly consist of small volatile organic compounds like acetate esters, alcohols and aldehydes (Jurenka, 2021). These are typically made up of carbon chains of 10-18 carbon atoms and are derived from the fatty acid synthesis pathways. The position and configuration of the double bonds within fatty acids is one of the most fundamental characteristics of the pheromone precursors. Therefore, the desaturating enzymes, called fatty acid desaturases (FAD), are often the targets in attempts to genetically modify plants to produce pheromone precursors. Nešněrová et al, (2004) was the first study that was able to genetically modify plants to produce precursors for insect pheromones. Despite progress being made since then, there are still significant challenges in being able to efficiently engineer plant pheromone pathways. One hypothesis is that there are issues with compatibility when insect genes are inserted directly into plant cells, which lowers the efficiency of the desaturase enzymes.

This project will explore the possibility of using desaturase enzymes from insects to produce pheromone precursors in plants, and whether combining the active sites of insect genes with structural domains from *Arabidopsis* could lead to a more efficient production. If these issues are to be addressed, it is necessary to have fundamental understanding of the underlying metabolic processes. This would include an understanding of the structure and function of lipids, the synthesis of fatty acids in plants, the mechanisms of desaturase enzymes, the pheromone biosynthesis in insects and how hybrid genes can be engineered.

2. Background

2.1 The role of lipids in plant biology

2.1.1 Overview of lipids

Lipids are a very heterogeneous group of organic compounds that are defined by their hydrophobic characteristics, which means they aren't soluble in water but only soluble in non-polar solvents (Chapman and Ohlrogge, 2012). The biological molecules that are classified as lipids include fatty acids, waxes, phospholipids, glycerolipids, galactolipids, terpenoids and sterols. This diversity of different kinds of lipids also means that there is a wide range of roles that lipids play in living organisms. These roles include, for example, structural integrity of cells, energy storage and signaling.

In plants, lipids are critical for plant development, metabolism and environmental adaptation (Li-Beisson et al, 2013). Lipids are especially important for the formation and function of cellular membranes, including plasma membranes as well as membranes of organelles like chloroplasts, mitochondria and endoplasmic reticulum (ER). These membranes are formed by bilayers of hydrophobic lipids, primarily phospholipids and glycolipids, which create a semi-permeable barrier. This facilitates selective transport through the membrane and localization of membrane associated proteins that are involved in transport, signal reception and enzymatic activities.

Apart from the structural function, lipids also play an important role in energy storage in the form of triacylglycerols (TAG), which is the main component of seed oils (Wasternack and Feussner, 2018). Traditionally, lipids have been classified into either polar lipids or neutral lipids. Polar lipids include the lipids that form membranes, (e.g. phospholipids and glycolipids), while neutral lipids include the storage lipids like triacylglycerols (TAG). In addition to these major categories, lipids can also play a role in plant signaling, like for example phosphatidic acid and jasmonates, which are involved in stress signaling and plant defense responses respectively.

2.1.2 Fatty acids: The building blocks of plant lipids

Fatty acids consist of long hydrocarbon chains with a carboxyl group at the end (Chapman and Ohlrogge, 2012). The chains usually contain an even number of carbon atoms because of how the biosynthesis of fatty acids works. The fatty

acids in plants are mainly between 16 to 22 carbon atoms long and can be divided into groups based on the number of double bonds. Saturated fatty acids contain no double bonds, while monounsaturated and polyunsaturated contain one or multiple double bonds respectively. A fatty acid with 18 carbon and 1 double bond is written 18:1. The position and configuration of the double bond can also be specified with a Z for cis- and E for trans-configuration followed by the number at which carbon the double bond is inserted. As an example, 18:1 Z9 has a cis configuration at the 9th carbon.

The number of double bonds in a fatty acid will have an impact on the physical properties of lipids (Bach and Faure, 2010). Double bonds in a cis-formation within a fatty acid will cause the hydrocarbon chain to bend. This prevents the lipids from being packed tightly together which will improve fluidity and lower the melting point of assemblies of such lipids. Unsaturated lipids thereby serve a crucial function of membrane fluidity and function, particularly under cold stress.

Fatty acids are building blocks that are used to create larger and more complex lipid molecules like phospholipids and TAG (Wasternack and Feussner, 2018). Additionally, fatty acids are precursors for signaling molecules and secondary metabolites, including pheromones. For example, α-linolenic acid (18:3) is precursor to jasmonates, that can influence many different physiological responses, such as plant defenses and reproductive development.

2.1.3 Fatty acid synthesis in plants

The biosynthesis of fatty acids in plants is mainly localized to the plastids, like the chloroplast, and is facilitated by the activity of a multi-enzyme complex called fatty acid synthase (FAS) (Ohlrogge and Browse, 1995). It is a pathway that starts with acetyl-CoA being carboxylated into malonyl-CoA. Subsequently, malonyl-CoA is added to an acyl carrier protein (ACP) to form malonyl-ACP. The fatty acid synthesis will then go through a series of cycles which will use malonyl-ACP as a substrate and extend the chain by two carbon atoms per cycle (Taiz at al, 2015). Each cycle include a condensation, a reduction, a dehydration and a second reduction. Malonyl-ACP reacts with acetyl-CoA for the first cycle, then for the subsequent cycles, malonyl-ACP reacts with the product of the previous cycle. The cycles will typically continue until a 16:0-ACP (palmitoyl-ACP) or a 18:0-ACP (stearoyl-ACP) has been produced (Li-Beisson et al, 2013). Stearoyl-ACP is then often desaturated inside the chloroplast to produce 18:1-ACP (oleoyl-ACP), which is the first unsaturated fatty acid to be synthesized in the pathway.

After the fatty acid chains have gone through the cycles of elongation and reached an appropriate length, they are then cleaved from the ACP protein to either form

free fatty acids or be transferred to another molecule (Li-Beisson et al, 2013). This is an important regulatory point in the pathway of fatty acid synthesis and is mostly facilitated by the enzyme's acyl-ACP thioesterases, like FATA and FATB, but can also be facilitated by acyltransferases. Acyl-ACP thioesterases will initiate a reaction of hydrolysis which will release the free fatty acids into the plastid stroma. FATA primarily hydrolyzes the unsaturated acyl-ACPs like 18:1-ACP, while FATB mostly hydrolyzes saturated acyl-ACPs like 16:0-ACP and 18:0-ACP. Alternatively, to forming free fatty acids, the acyl group of of acyl-ACPs can be transferred from the ACP to an acceptor molecule like glycerol-3-phosphate. The acyl group and the glycerol-3-phosphate will thereby form a glycerolipid which will be incorporated into to plastid membranes.

After synthesis, the free fatty acids that have been cleaved by thioesterase are activated to form acyl-CoA esters by an enzyme called acyl-CoA synthase (ACS) (Schnurr et al, 2002). These enzymes are located at the outer envelope of the plastid. From there, the activated acyl-CoA molecules are sent into the cytosol and onwards to the endoplasmic reticulum (ER). In the ER the acyl-CoAs can be subject to further desaturation (i.e. additional double bonds inserted) by fatty acid desaturases (FAD) and elongation by fatty acid elongases (FAE) (Li-Beisson et al, 2013). It's also in the ER that the assembly of extraplastidial lipids takes place, where the acyl-CoAs serve as the building blocks for larger molecules like phospholipids and triacylglycerols (TAG). This lipid assembly in the ER is essential for expanding cellular membranes, storing energy and facilitating responses to environmental stresses. An overview of the pathways relevant to this study can be seen below in figure 1.

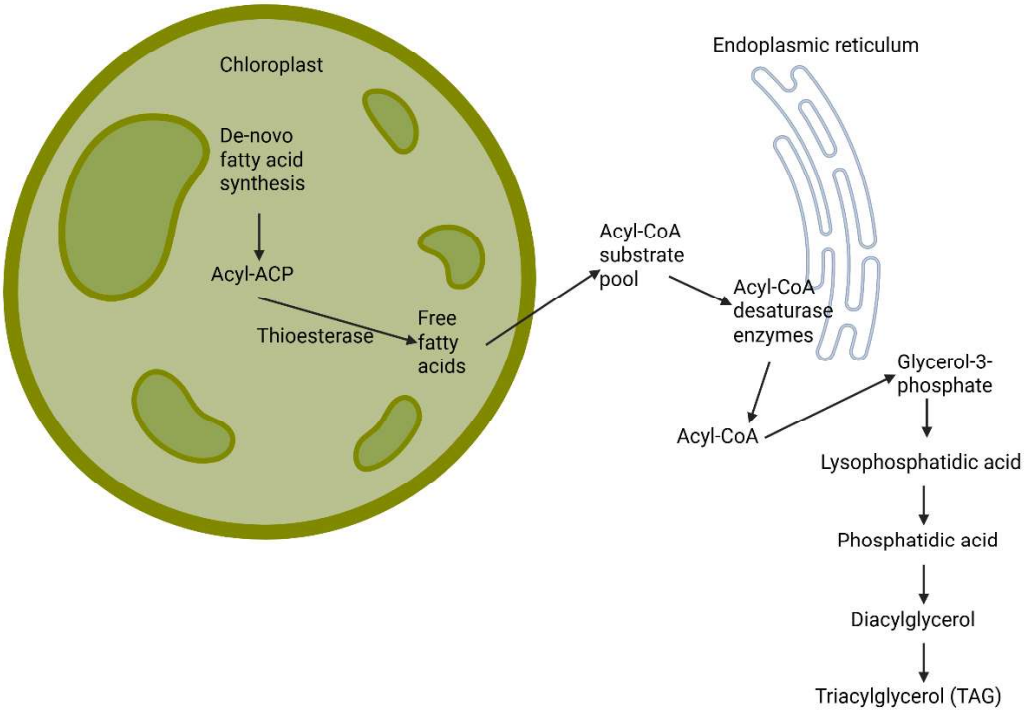

Figure 1. Illustration of the relevant pathways in this study. The acyl-CoA desaturase enzymes at the ER are the main subject of interest for the project. (Created with biorender)

2.1.4 Triacylglycerol (TAG): Structure and function

Triacylglycerols (TAG) are the primary way in which plants store energy, typically in seeds, where TAG accumulates during maturation (Chapman and Ohlrogge, 2012). Structurally, TAG is made up of a backbone of glycerol which is a three-carbon molecule with one hydroxyl group to each carbon. Each of these hydroxyl groups have a fatty acid esterified to it. The three possible positions for fatty acids on the glycerol backbone are called sn-1, sn-2 and sn-3. Any three fatty acids can be attached to the glycerol and form a TAG, which means there is a high chemical diversity among TAG molecules in regard to chain length and the level of unsaturation, which affects their physical characteristics.

The synthesis of TAG in plant cells mainly takes place in the endoplasmic reticulum (ER) (Li-Beisson et al, 2013). This happens through the Kennedy pathway which involves multiple enzymatic steps. First of all, the sn-1 position of glycerol-3-phosphate is acetylated (meaning a fatty acid is added) by glycerol-3-phosphate acyltransferase (GPAT) which forms a lysophosphatidic acid (LPA) molecule. Secondly, another fatty acid is added when LPA is acetylated at the position of sn-2 by lysophosphatidic acid transferase (LPAAT) which produces phosphatidic acid (PA). Thirdly, PA is dephosphorylated by phosphatidic acid phosphatase (PAP), which results in a glycerol molecule with two fatty acids

called diacylglycerol (DAG). Fourthly, the last hydroxyl group of the glycerol molecule at sn-3 is acetylated by diacylglycerol transferase (DGAT), which finally produces a triacylglycerol (TAG).

However, there are also alternative routes for TAG to be synthesized outside the Kennedy pathway (Bates et al, 2013). It is called the phospholipid:diacylglycerol acyltransferase pathway (PDAT), and in this pathway, an acyl group from a phospholipid is transferred to DAG to form a TAG. This pathway gives plant cells metabolic flexibility, which can be especially important during conditions when the availability of acyl-CoA is scarce.

2.1.5 TAG is stored in oil bodies

In plants, TAG is stored in lipid droplets called oil bodies (Chapman and Ohlrogge, 2012). These are specialized structures in the cytoplasm where a monolayer of phospholipids forms an outer boundary, which is stabilized by structural proteins, primarily oleosins, that prevent oil bodies from joining together. The hydrophilic heads of the phospholipids are directed outward while the hydrophobic tails are directed inwards towards the TAG molecules inside the lipid droplet (Shimada et al, 2018). These oil bodies are formed at the membrane of the ER when phospholipids bud off from the membrane to form the monolayer of phospholipids that encircle the oil body. As these phospholipids bud off from the ER, the TAG that has been produced in the ER is stored inside the oil body.

2.2 Plant desaturases: Enzymes that shape lipid diversity

Desaturases are enzymes that play a critical role in changing the structure and thereby the function of plant lipids (Shanklin and Cahoon, 1998). They are highly specialized in their function to insert double bonds into specific fatty acids at specific positions in the fatty acid chain. This leads to the diversification of the physical and chemical characteristics of membrane and storage lipids, thereby playing a significant role in cellular adaptability, development and signaling. Understanding the structure, function and mechanisms of desaturases enzymes provides important insights into the applications of plant biotechnology and metabolic engineering.

2.2.1 The biochemistry of desaturases

The basic biochemical process of desaturation occurs when a desaturase enzyme removes the hydrogen atoms of two adjacent carbon atoms in a fatty acid (Shanklin and Cohoon, 1998). This leads to the formation of a cis-double bond between these two carbon atoms. The reaction is an oxidative reaction, meaning a

process where atoms or molecules lose electrons, with molecular oxygen playing the role of the final electron acceptor. The process is catalyzed and controlled by the desaturase enzymes and typically happen according to the following stoichiometry: $RH\text{-}CH_2\text{-}CH_2\text{-}R' + O_2 + 2\ e^- + 2\ H^+ \rightarrow RH\text{-}CH{=}CH\text{-}R' + 2\ H_2O$.

The key to the catalytic activity of desaturases is a di-iron center, which is held in place by conserved histidine motifs in the enzyme (Li-Beisson et al, 2013). This di-iron center is the active site that grabs molecular oxygen from the environment and activates it. Once activated, the oxygen molecule is used by the enzyme to remove two hydrogen atoms from the fatty acid chain. However, the desaturase enzyme cannot facilitate this reaction on its own. For the oxygen to be activated and the reaction to be initiated, it requires a donation of electrons. The role of electron donor is typically played by NAD(P)H, which donates electrons to the enzyme cytochrome b5 reductase. From this reductase enzyme, the electrons are then passed to the carrier protein cytochrome b5, which in turn delivers the electrons to the desaturase enzyme.

2.2.2 Types and localization of desaturases

Desaturase enzymes are generally divided into three classes based on their organelle localization and their substrate preferences, namely acyl-ACP desaturases, acyl-lipid desaturases and acyl-CoA desaturases (Cerone and Smith, 2022). Acyl-ACP desaturases are soluble enzymes localized to the plastid stroma. Their substrate are fatty acids bound to ACP and are therefore involved in desaturation of newly synthesized fatty acids in the plastid. Acyl-lipid desaturases are membrane-bound enzymes in the endoplasmic reticulum (ER) and the chloroplast. These desaturases act on fatty acids that are already part of membrane-lipids, like phospholipids and glycerolipids, which is important to maintain membrane fluidity during fluctuations in temperature. Acyl-CoA desaturases are also membrane bound enzymes located mainly in the ER. They act on fatty acids bound to coenzyme A (i.e. outside the plastid) and therefore affect the saturation of a broad range of molecules like phospholipids, TAG and sphingolipids.

2.2.3 Structural features and mechanisms of desaturases

Desaturase enzymes are mainly located inside organell membranes of the ER and chloroplast (acyl-ACP desaturases is an exeption), and they are tied into the lipid bilayers by several hydrophobic transmembrane domains, as indicated below in figure 2 (Ohlrogge and Browse, 1995). These transmembrane domains are spiral-shaped protein stretches, and each desaturase usually has 4 to 6 of them. The active sites of desaturases are also located inside the membrane. The hydrophobic environment within the membrane ensures that the interaction between the

desaturase enzyme and the acyl-lipid or the acyl-CoA molecules are as efficient as possible. The purpose of the transmembrane domains is to position the active sites in such a way to facilitate efficient interaction with the targeted fatty acid tails (Shanklin and Cohoon, 1998).

In the case of acyl-lipid desaturases, the localization of the active sites inside the membrane is crucial since the fatty acid tails on which they act are embedded inside the lipid bilayer (Li-Beisson et al, 2013). However, the case is a bit different for acyl-CoA desaturases. Their structural nature of being membrane bound is the same, but the active sites instead face the cytosolic surface of the ER membrane, shown in figure 2. This is essential because the acyl-CoA molecules (which are the substrates of the acyl-CoA desaturases) are not located inside the membrane, though, they can interact with the surface of the membrane. Thereby the fatty acid tails of acyl-CoA can come into contact with the active sites of the desaturase enzymes.

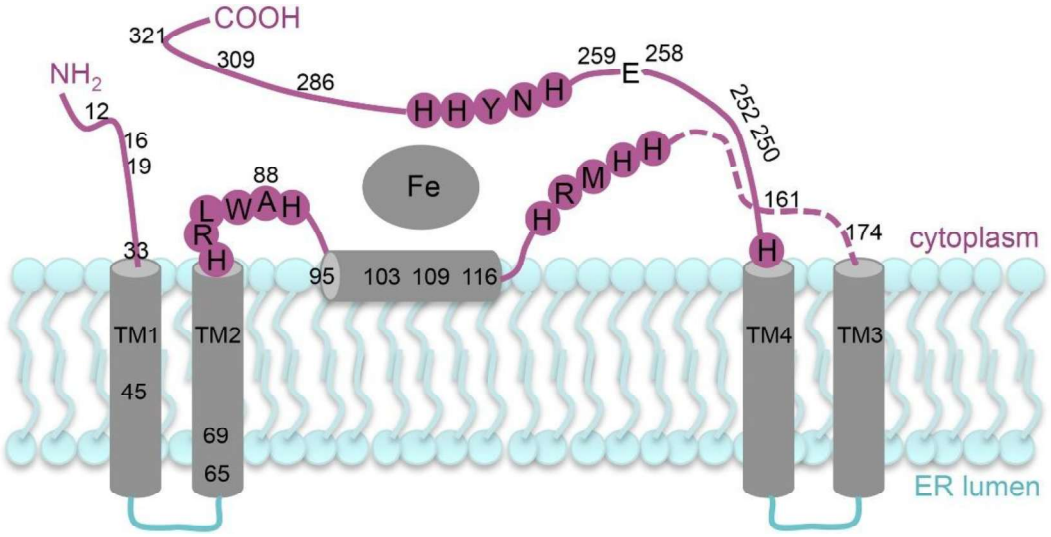

Figure 2. Structural model of desaturase enzyme. The hydrophobic transmembrane domains are labelled TM1, TM2, TM3 and TM4. There are three non-transmembrane domains that shape the active site around the di-iron center. (Image credited to Ding et al, 2016)

2.3 Insect desaturases and pheromone biosynthesis

2.3.1 Chemical communication and the role of pheromones

Communication through the use of chemical signaling between insects is crucial for their survival and to influence behaviors. Pheromone secretion is a highly sophisticated way of signaling and communicating. The term pheromone was originally defined by Karlson and Butenandt (1959) as “substances that are

secreted by an animal to the outside and cause a specific reaction in a receiving individual of the same species, e.g., a release of certain behavior or a determination of physiological development". The word pheromone is of Greek origin, combining the words pherein, which means to carry, with the word hormon, which means to excite/stimulate. Thereby, pheromone is a substance that carries excitement. They are highly potent and volatile molecules (Abd El-Ghany, 2020). Consequently, at very low concentrations, they are able to initiate species-specific changes to behaviors. Through evolution, insects have developed the abilities of both producing and detecting pheromone signaling with precision. Even over long distances and despite the air being filled with pheromones of competing species, the accuracy of communication is extremely high (Yew and Chung, 2015). The behavioral changes that can be affected by pheromones are for example alarm signaling, aggregation, marking trails, social structuring, and most importantly sexual communication.

Insects like Lepidoptera (butterflies and moths) use sex pheromones to find mates. Normally, it is the female moths that produce and release a mix of volatile compounds from pheromone glands (Kaissling, 2014). These glands are a type of secretory organ that is extremely specific in what type of compounds it produces. In turn, the males of the same species receive the pheromone signals through olfactory receptor neurons, which are specialized nerve cells in their antennae and are responsible for detecting smell (McKinney et al, 2015). These typically have a high specificity towards one or a few of the species-specific volatile compounds produced in the pheromone glands of females. When males detect these signals, it can influence the orientation of flight, and trigger landing and courtship.

Insect's sexual pheromone communication is especially complex due to their fine chemical specificity and diversity (Yew and Chung, 2015). Pheromone molecules can be rendered ineffective or even antagonistic because of small differences in chain length, double-bond positioning or stereochemistry. Moths have, therefore, evolved a pheromone production system with extremely high biochemical precision, which facilitates their communication even when their habitats are crowded with insects of other species.

2.3.2 Pheromone biosynthesis in moths

The biosynthesis of moth pheromones usually starts with saturated fatty acids first produced in the cytosol of insect cells (as opposed to the plastids in plants), usually 14:0 or 16:0 (Jurenka, 2004). These will then be modified regarding their level of desaturation, chain length and functional groups by a series of enzymes. As a result, pheromone components like acetates, alcohols and aldehydes with specific patterns of unsaturation are produced. However, the first step of the

enzymatic modifications, desaturation, is typically the most important, since it determines the double-bond's configuration and position in the fatty acid derivatives (Ding et al, 2014). The desaturation of the pheromone precursors usually occurs in the ER of the pheromone producing cells of the pheromone glands, and the occasions when the production of the pheromones are activated is tightly controlled. This means that pheromones will only be produced at the right time at the right place in the insect (Yew and Chung, 2015).

Insect desaturases are enzymes that facilitate the introduction of double-bonds at specific positions of certain fatty acid chains (Ding et al, 2016). Desaturases, therefore, play a key role in the biosynthesis of moth pheromone since the position and configuration of double-bonds determines the biological activity of the final pheromone product. The desaturase enzymes have evolved strong preferences for fatty acid chains of particular lengths, for example, C12, C14 or C16, and a preference for introducing double-bonds at specific positions, for example, Δ9 or Δ11.

2.3.3 Insect desaturase genes of interest: AtrΔ11, DmeΔ9, and Cpo_CPRQ

This project focused on three different insect desaturase enzymes. These are examples of functionally and evolutionarily distinct desaturases that illustrate the diversity of double bond insertion. Each of these enzymes produce different double bonds using different fatty acid substrates, which facilitates the production of pheromone precursors that can enable species-specific communication.

AtrΔ11 comes from *Amyelois transitella*, also known as the navel orangeworm, and inserts a double bond into palmitic acid (16:0-CoA) resulting in 16:1 Z11-acid (Wang et al, 2022). This is a common pheromone precursor for many species of moth, like *Plutella xylostella* (diamondback moth) and *Helicoverpa armigera* (cotton bollworm). AtrΔ11 has a high specificity for this carbon position and configuration which could make it a valuable biotechnological tool.

DmeΔ9 is a desaturase enzyme that come from *Drosophila melanogaster* (fruit fly or banana fly) (Petkevicius et al, 2020). It uses myristic acid (14:0-CoA) to introduce a double bond at the 9th carbon in the chain, which results in 14:1 Z9-acid. DmeΔ9 has previously been expressed in plant tissue in combination with a reductase from *Helicoverpa armigera*, called HarFAR, and an acyltransferase from *Saccharomyces cerevisiae*, called Atf1p, to produce 14:1 Z9-acetate. This is one of primary sex pheromones of *Spodoptera frugiperda*, also called the fall armyworm.

Cpo_CPRQ is a desaturases enzyme from *Cydia pomonella* (codling moth) (Xia et al, 2021). As opposed to AtrΔ11 and DmeΔ9, this desaturase is thought to be bifunctional, meaning it can facilitate desaturation in two steps. First it can convert lauric acid (12:0) into 12:1 E9-acid. Then it can desaturate 12:1 E9-acid in a second step into 12:2 E8,E10-acid (containing two double bonds), which is a precursor of the sex pheromone codlemone which attracts males of the codling moth.

2.4 Engineering hybrid desaturases

Researchers face multiple challenges when attempting to produce pheromone precursors in plants using insect desaturase enzymes. It is logical to think that there could be certain issues with compatibility when introducing insect genes into plants. These issues could include improper folding of protein (which yields a non-functional protein), wrong localization and low enzyme activity, due to differences in membrane composition, redox environments and protein processing systems between insect and plant cells. A potential strategy to overcome these challenges is to engineer hybrid desaturase enzymes, where membrane spanning domains from plant desaturases are combined with active site domains from insect desaturases. This strategy comes from the hypothesis that using structural elements from plants could improve membrane integration and folding efficiency of the enzyme in the endoplasmic reticulum (ER). However, combining the structural elements of plant desaturases with the catalytic domains of the insect enzyme that would retain the original function of the insect desaturases regarding substrate preference, regioselectivity and stereospecificity could present a challenge.

2.4.1 Structural blueprints of desaturases

The plausibility of the idea to construct hybrid desaturases is supported by detailed insights into the three-dimensional structure of desaturase enzymes, which was provided by Bai et al (2015) and Wang et al (2015). These studies describe the secondary structure of SCD1, a closely related desaturase to the desaturases of interest in this project. Their results showed that acyl-CoA desaturases have a well-conserved architecture across species, which is applicable to both animal and a class of plant desaturases. The 3D-structure showed that the desaturase consists of four transmembrane domains, in the shape of α-helices (the most common secondary structure of protein chains), embedded in the ER. These four membrane domains form a sort of hydrophobic tunnel or pocket that guides the fatty acyl chain substrate to the di-iron center at the core of the active site (See Wang et al. 2015 figure 3f for detailed image). Histidine motifs coordinate the di-iron center while the CoA-molecule of the acyl-CoA chain orientate and position the carbon atoms targeted for double bond insertion. The positions of the

transmembrane domains determine the spatial organization of the different components of the enzyme. These insight into the general organization of different domains and active site positioning gives support to the strategy of creating hybrid enzymes.

Another important point from the studies of Bai et al (2015) and Wang et al (2015) is that the detailed mapping of the domains of desaturase enzymes has enabled researchers to determine functional boundaries between different domains. In turn, this has enabled the creation of hybrid genes where domains from different organisms can be swapped but still retain the activity of the active sites. Ideally, this would lead to improved compatibility and function of the enzymes in plant cells, thereby producing higher levels of the pheromone precursors.

2.4.2 ADS1 from *Arabidopsis thaliana* used as a scaffold

In this project, an acyl-CoA desaturase from the plant *Arabidopsis thaliana* was chosen to serve as the backbone of the hybrid constructs. It is a homolog of the selected insect desaturases, and consequently, it shares many similarities in regards to the structure and organization of domains. In its original state, ADS1 desaturates 16:0-CoA into 16:1 Z9-acid (Heilmann et al, 2004). But most importantly, it likely has a high compatibility with the intended ER membrane environment, since its origin is from a plant. ADS1 was therefore combined into different gene constructs with the active sites of AtrΔ11, DmeΔ9, and Cpo_CPRQ. This would hopefully increase the probability of a correct folding and membrane insertion and thereby increase the enzyme efficiency in a plant tissue environment.

3. Research questions

This study has attempted to investigate the structure-function relationship of a specific class of membrane-bound acyl-CoA desaturase enzymes that are involved in the biosynthesis of insect sex pheromones. The specific structure-function relationship of interest is how domain combination affects expression, localization and the catalytic activity of insect desaturases expressed in plant systems.

To approach this subject, three insect desaturases were selected, AtrΔ11, DmeΔ9, and Cpo_CPRQ. Each of these are involved in the production of important pheromone precursors of moth species, but with different functions regarding regioselectivity and stereospecificity. Genes encoding these enzymes were used, in combination with the homolog desaturase gene ADS1 from *Arabidopsis thaliana*, to create hybrid genes. The hybrid genes consisted of the structural membrane-spanning domains from *Arabidopsis thaliana* combined with the active site regions from the insect genes. DmeΔ9, and Cpo_CPRQ were used to create one hybrid gene each, while AtrΔ11 was used to create three different variants of hybrid genes. The rationale for creating these hybrid genes is based on the hypothesis that structural membrane domains of plant origin may have a better compatibility with plant systems and therefore improve function, leading to increased production of pheromone precursors.

The specific research questions addressed are:

- Can hybrid constructs of desaturase enzymes be successfully expressed and functionally active in plant cells?
- To what degree will the engineered hybrid enzymes maintain functional characteristics, like substrate specificity and positioning and configuration of inserted double bonds, compared to the wild-type insect enzymes expressed in plants?

By answering these questions, the objective of the project is to increase our understanding of how the structural domains of desaturase enzymes contribute to their function, and if these domains can be recombined between different species and organisms. These insights could prove useful for future biotechnological strategies on how to produce insect pheromones in sustainable plant-based systems.

4. Materials & Methods

4.1 Overview of methods

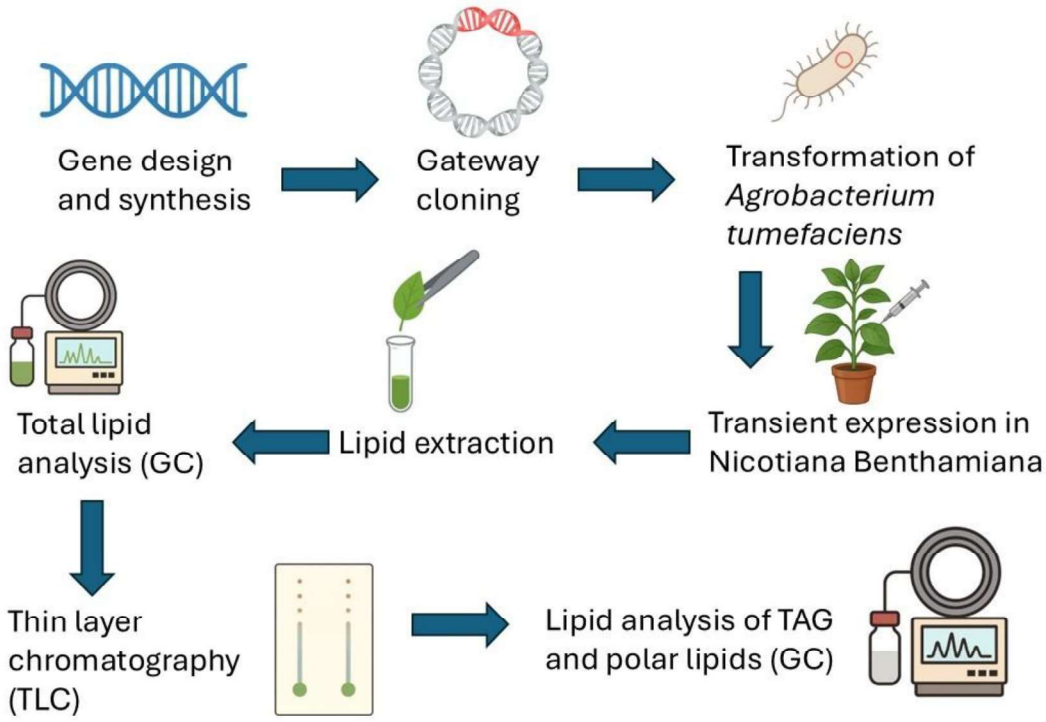

Figure 3. Overview of the methods used. Design and synthesis of hybrid genes had been prepared before the project, so the project technically started with Gateway cloning and finished with lipid analysis of TAG. (Co-created with OpenAI)

4.2 Gene design and synthesis

Before the start of the project, synthetic variants of hybrid desaturase genes had been designed. As previously described, the structural membrane-spanning domains of ADS1, a homolog desaturase from *Arabidopsis thaliana*, were combined with the active sites from AtrΔ11, DmeΔ9, and Cpo_CPRQ. Based on the detailed mapping of the homologous enzyme class SCD in mammalian cells by Bai et al (2015) and Wang et al (2015), it was possible to determine the domain borders of AtrΔ11, DmeΔ9, and Cpo_CPRQ as well as ADS1.

Each desaturase gene consists of two regions coding for the transmembrane domains and two regions coding for the catalytic site, as well as a third non-membrane domain with an unknown function. The Cpo and Dme hybrid genes were both constructed using the two regions coding for transmembrane domains from *Arabidopsis thaliana* in combination with the three non-transmembrane domains from the insect desaturase gene. Atr1 was constructed the same way as

Cpo and Dme, while Atr 2 contained the unknown non-transmembrane domain from *Arabidopsis thaliana*. Atr3 was constructed like Atr2 but with an extra transit peptide added with the purpose to relocate it to the chloroplast. Hybrid gene construction is illustrated in greater detail in the Result-section under "Hybrid gene design".

The hybrid genes were assembled based on a protein level (amino acid sequnce) and after which it was back-translated to DNA sequence and codon-optimized for expression in *Nicotiana benthamiana*. To these assembled synthetic gene sequences, attB sites were added to the ends to make them compatible with Gateway cloning. The final sequences were ordered from ThermoFischer Scientific.

4.3 Gateway cloning

The genes that required to be cloned and transformed into *Agrobacterium tumefaciens* GV3101 were the hybrid constructs for DmeΔ9, Cpo_CPRQ and the three constructs for AtrΔ11, as well as the wild type gene for ADS1 from *Arabidopsis thaliana*. The wild type insect genes for AtrΔ11, DmeΔ9, and Cpo_CPRQ were already available in Agrobacterium and were kept in glycerol stocks.

In summary, Gateway cloning consists of two parts. The first part is the BP reaction where the gene construct is inserted into a donor vector, and the second part is the LR reaction where the gene is transferred from the donor vector to a destination vector.

4.3.1 BP reactions

For the BP reaction, each hybrid gene was combined with the donor vector (pDONR221) and BP clonase II into a reaction mixture and incubated at 25°C for 3 hours. Subsequently, Proteinase K was added to stop the reactions, followed by a shorter incubation at 37°C for 10 minutes. The BP reaction was then followed by a transformation procedure, wherein the BP recombination DNA was added to vials of "Oneshot Top 10" chemically competent *Escherichia coli* cells. Transformation occurred through a standard heat-shock procedure, after which the cells were spread on plates with selective antibiotics and incubated overnight. Successfully grown colonies were selected (3 replicates per gene) and grown in overnight liquid LB cultures. This was followed by plasmid DNA purification with a GENEJET Plasmid Miniprep kit. The DNA concentration and purity was determined with a nanodrop analysis, and the correct recombination of DNA into the donor vector was verified with a restriction digestion with appropriate restriction enzymes. To verify that there had been no mistakes in the gene

synthesis by ThermoFischer Scientific, one replicate for each gene was sent for sequencing with Sanger sequencing.

4.3.2 LR reactions

LR reactions are quite similar to BP reactions. The DNA samples that had been verified through sequencing were then used in the subsequent LR reaction, in which the entry vector DNA was combined into a reaction mixture with the destination vector (pXZP393) and LR clonase II. This was incubated at room temperature for 3 hours and the reaction was stopped by the addition of Proteinase K followed by a second incubation at 37°C for 10 minutes. The LR reaction mixture was subsequently added to vials of chemically competent E. coli cells which were transformed through a standard heat-shock procedure. The E. coli cells were then spread on plates with selective antibiotics and incubated overnight. Successfully grown cultures were selected (3 replicates per gene) and grown in overnight liquid cultures, followed by DNA purification with a GENEJET Plasmid Miniprep kit. The concentration and purity of the DNA was then determined with nanodrop analysis. Verification of a correct recombination of DNA from the entry vector to the expression vector was conducted through a restriction digestion with appropriate restriction enzymes.

4.4 Transformation of *Agrobacterium tumefaciens*

For transformation of *Agrobacterium tumefaciens*, the correctly verified expression vectors that were purified after the LR reaction was used, preferably the replicates with the highest concentration and purity. The expression vector DNA was added to vials of chemically competent Agrobacterium cells GV3101. These were kept on ice for 5 minutes, then in liquid nitrogen for 5 minutes and lastly incubated on a standard shaker at 37°C for 5 minutes. The cell mixture was added to 1 ml of LB media and incubated at 28°C for 2 hours. Subsequently, the transformed Agrobacterium-cells were pelleted and resuspended in LB media, then spread on plates with selective antibiotics and incubated at 28°C for 2 days.

4.5 Transient expression in *Nicotiana benthamiana*

4.5.1 Genes combinations used for infiltration

The means of evaluating the function of genes was through transient expression in *Nicotiana benthamiana*, and the transient expression was achieved by infiltrating Agrobacterium-solutions into leaves. For each gene of interest, a combination of multiple different genes was included in the solutions to improve gene expression, localization of infiltrated tissue, and enhance the biochemical environment for fatty acid modification. These genes included, respectively, P19, GFP, WRINKLED1 and a fatty acid thioesterase chosen from a group of genes

resulting in different specificities (TE12/TE14/TE16). Plants naturally have defense mechanisms that silence foreign RNA expression, and P19 is a viral suppressor of this mechanism of post-transcriptional gene silencing, which prevents RNA degradation and improves transgene expression. GFP is a green fluorescent protein, which is included to confirm successful infiltration and mark out the leaf area where genes are transiently expressed. WRINKLED1 is a transcription factor that upregulates genes in the fatty acid synthesis pathway, which increases the availability of substrates for desaturase activity. The purpose of fatty acid thioesterase is to redirect and enhance the supply of free fatty acid substrates for desaturation. The evaluated desaturase enzymes act on fatty acids of different lengths (12, 14 or 16 C), and the specific thioesterases used facilitates the export of these fatty acid substrates from the chloroplast to the ER, which will optimize substrate flux towards the desaturase pathway. The three fatty acid thioestease genes used were *Uca*FATB1 (exporting 12:0), *Cpa*FATB2 (exporting 14:0) and *Cpu*FATB1 (exporting 16:0).

Table 1. List of genes used in this study.

Gene name	Gene enzymatic action	Origin	Database reference
GFP	Green Fluorescent Protein; Reporter Gene	artificial sequence	ABE28520
P19	RNA Silencing Suppressor	Tomato bushy stunt virus (strain ja6)	P69516.1
WRINKLED 1	FA synthesis transcription factor	*Arabidopsis thaliana*	At3g54320
UcaFATB1	Fatty Acyl-ACP Thioesterase B2	*Umbellularia californica*	Q41635.1
CpaFATB2	Fatty Acyl-ACP Thioesterase B2	*Cuphea palustris*	AAC49180
CpuFATB1	Fatty Acyl-ACP Thioesterase B1	*Cuphea avigera* var. *pulcherrima*	AGG79283
AtrΔ11	ΔZ11 Acyl-CoA Desaturase	*Amyelois transitella*	JX964774
DmeΔ9	ΔZ9 Acyl-CoA Desaturase	*Drosophila melanogaster*	CAB69054
Cpo_CPRQ	ΔE9 Acyl-CoA Desaturase	*Cydia pomonella*	AHW98354
ADS1	ΔZ9 Acyl-CoA Desaturase	*Arabidopsis thaliana*	AT1G06080

4.5.2 Plant material and growth conditions

Plants of *Nicotiana benthamiana* had been grown from seeds and planted in pots. These were grown in greenhouse conditions with 16/8 light hours and naturally fluctuating temperatures (approximately 20-24 °C), and were approximately 5 weeks old at the time of infiltration.

4.5.3 Preparation of *Agrobacterium tumefaciens* for infiltration

Transformed Agrobacterium for P19, GFP, WRINKLED1, *Uca*FATB1, *Cpa*FATB2 and *Cpu*FATB1 as well as the wild type-genes AtrΔ11, DmeΔ9, and Cpo_CPRQ had previously been grown on selective plates from glycerol stocks. In preparation for infiltration, Agrobacterium for all genes, including the ones transformed after gateway cloning, were grown overnight in 20 ml LB media with selective antibiotics at 28°C. In total, the genes included P19, GFP, WRINKLED1, *Uca*FATB1, *Cpa*FATB2, *Cpu*FATB1, AtrΔ11 (WT), Cpo_CPRQ (WT), DmeΔ9 (WT), Atr1 (h), Atr2 (h), Atr3 (h), Cpo (h), Dme (h) and ADS1.

4.5.4 Infiltration of *Nicotiana benthamiana*

To each of the overnight liquid culture, was added 20 µl of Acetosyringone (AS), and then these incubated at 28°C for 3 hours. This is done to activate the viral properties of the bacteria. Meanwhile, the plants were watered and a mixture of 200 ml infiltration media (5 mM $MgCl_2$; 5 mM MES, pH 5.7) and 200µl of AS was prepared (1000:1 V/V of infiltration media+AS). The Agrobacterium in the liquid cultures was then pelleted by centrifuging at 4000rpm for 5 minutes and gently resuspended in 5 ml of the infiltration media+AS-mixture. The optical density of all the agro-solutions was determined with a spectrophotometer as a measure of the concentration of Agrobacterium. The goal is to achieve a concentration of Agrobacterium for each gene in each gene combination corresponding to an optical density of 0,2 in the final infiltration mixture of 20 ml. Therefore, calculations were made to determine the amount of agro-solution needed for each gene in each gene combination. Based on the calculations, infiltration mixtures were combined according to the gene combinations on Table 2, with enough infiltration media+AS mixture to make the final volume of infiltration mixture 20ml.

The infiltration mixtures were then used to make infiltrations in leaves of Nicotiana in replicates of 3 (three individual plants) with a 1 ml needleless syringe. On each plant, two leaves would be infiltrated, and no plant would be infiltrated by the same treatment twice. Younger leaves were selected for infiltration for the highest gene expression. All the leaves younger than the ones infiltrated were removed, and the plants were then placed in greenhouse conditions for 5 days.

4.6 Lipid extraction

After the growth period of 5 days, the infiltrated leaf area was identified with UV-light using a Dual Fluorescent Protein Flashlight which detect the GFP reporter gene. This leaf area was harvested, flash frozen in liquid nitrogen and then freeze dried for 3 days. Leaves were crushed with a spatula and approximately 50 mg of each sample transferred to a Potter Elvehjem homogenizer. 3.75 ml MeOH:$CHCl_3$ (2:1 V/V) and 1 ml HAc (0.15 M) were added and samples were homogenized and transferred to glass tubes. Homogenizers were rinsed with 1.25 ml $CHCl_3$ and 1.25 ml H_2O, which were also transferred to the glass tubes. Samples were vortexed and the phases were separated by centrifugation for 2 minutes at 2000 rpm. The $CHCl_3$ phase (lower phase) was transferred to new tubes, then dried up completely under N_2 and 1 ml $CHCl_3$ was added immediately. These samples were stored in -20°C and used for further analysis.

4.7 Total lipid analysis with gas chromatography (GC)

From the extracted lipid samples, aliquots of one tenth (100 µl) were put into new tubes and 60 µl of 17:0-Me in MeOH (0,84 nmol/µl) was added to each aliquot, which would be used as a reference in the lipid analysis. Samples were dried up under N_2 and 2 ml of methylation mixture (2 % H_2SO_4) was added, and then tubes were methylated on a heating block at 95°C for 45 minutes. 3ml of heptane and 2 ml H_2O was added to each tube, which was vortexed thoroughly and phases were separated by centrifugation for 3 minutes at 3000rpm. The upper heptane phase was pipetted out and into new tubes. These were dried under N_2 until approximately 100 µl remain, which was then transferred to GC-vials and ran on GC. Using the results from the GC, the total amount of fatty acids could be quantified based on the known quantity of 60 µl of 17:0-ME which was added and are not produced naturally in plants.

4.8 Thin layer chromatography (TLC)

Based on the results of the total lipid analysis, an aliquot corresponds to approximately 1000 nmol of fatty acids was taken from the extracted lipids in $CHCl_3$. These were dried up completely under N_2 and dissolved in 100 µl of $CHCl_3$ then loaded onto a silica gel plate. A standard for neutral lipids was also loaded onto the plate as a reference. The fatty acids were separated through thin layer chromatography (TLC) by placing the silica plates in neutral solvent composed of heptane:diethylether:Hac (70:30:1 V/V/V). The plates were sprayed with primulin (5 %) and viewed under UV-light to mark out the area of TAG. For each sample, the area of TAG, the area of polar lipids at the bottom and the rest of the lipids were scraped off separately into three different tubes.

4.9 Fatty acid analysis of TAG and polar lipids (GC)

To the tubes with silica gel scrapes from the TLC, 50 nmols of 17:0Me in MeOH and 2 ml of methylation mixture (2 % H_2SO_4 in water-free MeOH) was added, and then methylated at 95°C for 45 minutes. 2 ml of both heptane and H_2O was added to all tubes, which were vortexed thoroughly and phases were separated by centrifugation for 2 minutes at 2000 rpm. The upper heptane phase was moved to new tubes and evaporated under N_2 until approximately 100 µl remain, which was transferred to glass vials and analysed with gas chromatography (GC). The GC analysis was preformed with a Agilent 8860 system using a CP-Wax 58 FFAP CB column, with an injection temperature of 240 °C and a detector temperature of 260 °C. Each sample's injection volume was 1 µl and the intital temperature for each run was 150 °C which is held for 0.2 minutes. Then follows Ramp 1 where the temperature increases 4 °C per minute until 210 °C is reached, and ramp 2 where the temperature is increased 10 °C until 250 °C is reached and held for 10 minutes. A software from Agilent called OpenLab Data Analysis was then used to analyze the results. The amount of each fatty acid in the samples were calculated based on the peak area which was multiplied with a response factor and then compared to the peak area of 17:0 which was a known amount.

5. Results

5.1 Hybrid gene design

The design and synthesis of the hybrid genes had been conducted before the start of the project, but alignments between the hybrid proteins, the insect desaturase genes and the *Arabidopsis* scaffold were performed as part of the project to illustrate the construction process. Alignments were conducted on protein sequences and illustrate the regions of the transmembrane domains, originating from *Arabidopsis thaliana*, and the catalytic domains originating from the insect desaturases, as well as other functional elements.

The structure of the Dme hybrid and the Cpo hybrid were very similar, as seen in figures 4 and 5. There are two regions which determine the transmembrane domains and each of these regions code for two consecutive α-helices (four α-helices in total). The active site of the enzyme consists of two catalytic regions that folds into one united structure, which facilitates the function of the desaturases. At the start of the sequence (N-terminus), however, there is a third non-transmembrane region which function is unknown.

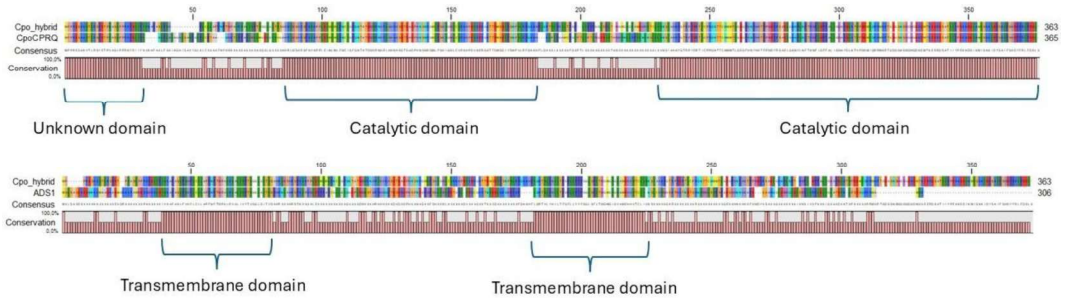

Figure 4. Amino acid sequence alignment between the Cpo hybrid and the Cpo insect gene on top, and between the Cpo hybrid and the ADS1 scaffold (from Arabidopsis thaliana) on the bottom. The level of conservation illustrates the origin of the domains. Cpo hybrid consist of 362 amino acids.

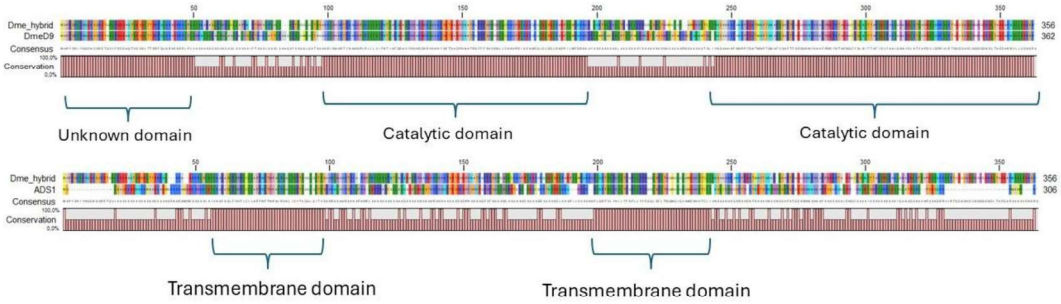

Figure 5. Amino acid sequence alignment between the Dme hybrid and the Dme insect gene on top, and between the Dme hybrid and the ADS1 scaffold (from Arabidopsis thaliana) on the bottom. The level of conservation illustrates the origin of the domains. Dme hybrid consist of 355 amino acids.

The Atr-desaturase enzyme was, as previously mentioned, constructed into three different variations of hybrid genes. In figure 6, the two alignments on top illustrate that Atr1 was constructed similarly to the Cpo- and Dme-hybrids, with the two trans-membrane regions originating from *Arabidopsis thaliana* and the two catalytic regions, plus the unknown non-transmembrane domain, originated from the Atr-insect desaturase. The third alignment illustrate that Atr2 is similar to Atr1, except that the unknown (N-terminus) domain was taken from *Arabidopsis thaliana* instead of the insect gene. The fourth alignment illustrate that Atr3 is the same as Atr2 but with a transit peptide sequence, which should cause it to locate to the chloroplast instead of the ER.

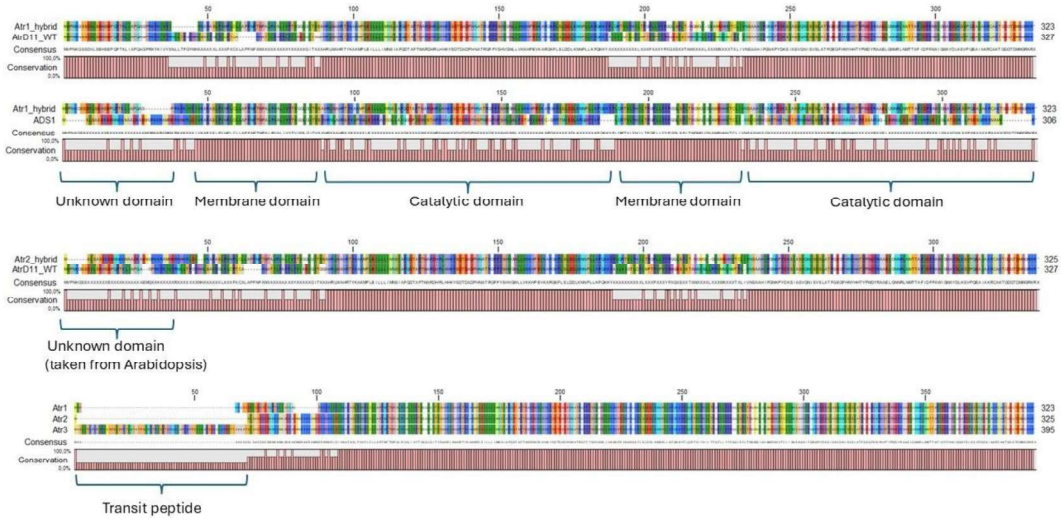

Figure 6. Amino acid sequence alignment illustrating differences between hybrids Atr1, Atr2 and Atr3. Based on the level of conservation, in comparisons between the hybrid genes, the Atr-wild-type gene and ADS1, the different components have been identified and highlighted. Atr 1, 2 and 3 consist of 322, 324 and 394 amino acids respectively.

5.2 Gateway cloning

As mentioned in the method, the genes that required cloning were Atr1 (h), Atr2 (h), Atr3 (h), Dme (h), Cpo (h) and ADS1 (WT). The procedure of gateway cloning included a BP reaction and an LR reaction.

5.2.1 Restriction digestions of BP reactions

After the BP reactions, the recombinant plastids were transformed into E.coli. The plasmid DNA was subsequently purified and correct recombination was verified through a restriction digestion, as seen in figures 7, 8 and 9. The restriction enzymes used were HindIII and EcoRV for Atr1; only EcoRV for Atr2, Atr3 and ADS1; EcoRV and BgIII for Cpo and EcoRV and StuI for Dme.

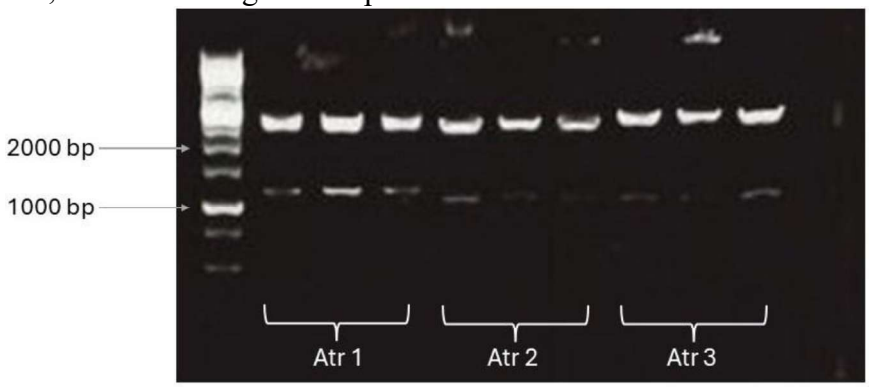

Figure 7. Agarose gel electrophoresis image of the restriction digestion of BP reactions of hybrid genes Atr1, Atr2 and Atr3. Digestion was conducted in replicates of three. The order of samples on the gel is indicated in the image. Correct recombination was verified. Expected DNA fragment lengths were approximately 1000 and 2500 bp.

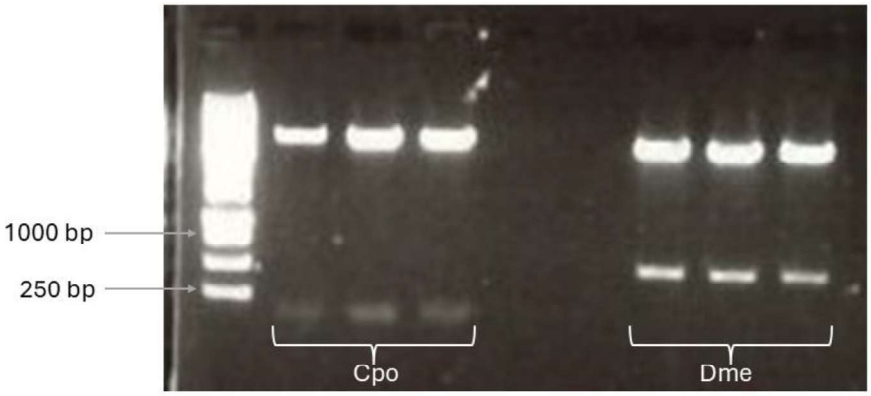

Figure 8. Agarose gel electrophoresis image of the restriction digestion of BP reactions of hybrid genes Cpo and Dme. Digestion was conducted in replicates of three. The order of samples on the gel is indicated in the image. Digestion verified correct recombination. Expected DNA fragment lengths were approximately 3500 and 160 bp for Cpo and 3200 and 440 for Dme.

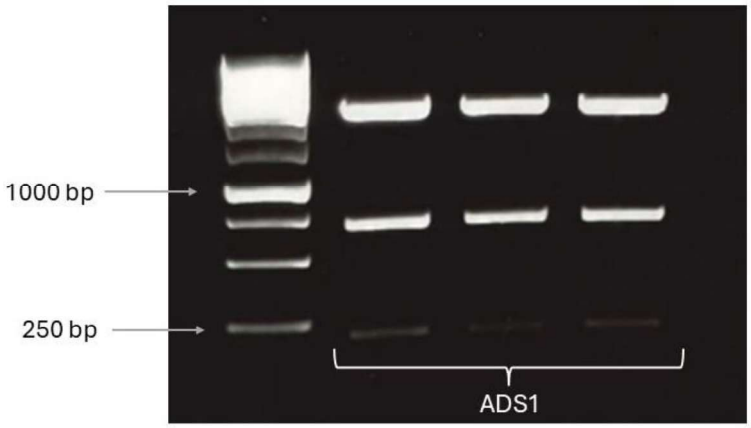

Figure 9. Agarose gel electrophoresis image of the restriction digestion of BP reactions of the scaffold gene from Arabidopsis thaliana ADS1. Digestion was conducted in three replicates and verified a correct recombination in the BP reaction. Expected DNA fragment lengths were approximately 2500, 700 and 220 bp.

The purified DNA from the BP reactions was also sequenced with Sanger sequencing to verify both the correct synthesis of the original hybrid genes, and the correct recombination into the donor vector.

5.2.2 Restriction digestions of LR digestion

The LR reactions recombined the hybrid gene from the donor vector to the destination vector. This reaction was also verified with a restriction digestion. The restriction enzymes used were EcoRV and HindIII for Atr1, EcoRV for Atr2 and Atr3, BgIII for Cpo, StuI for Dme and BamHI for ADS1.

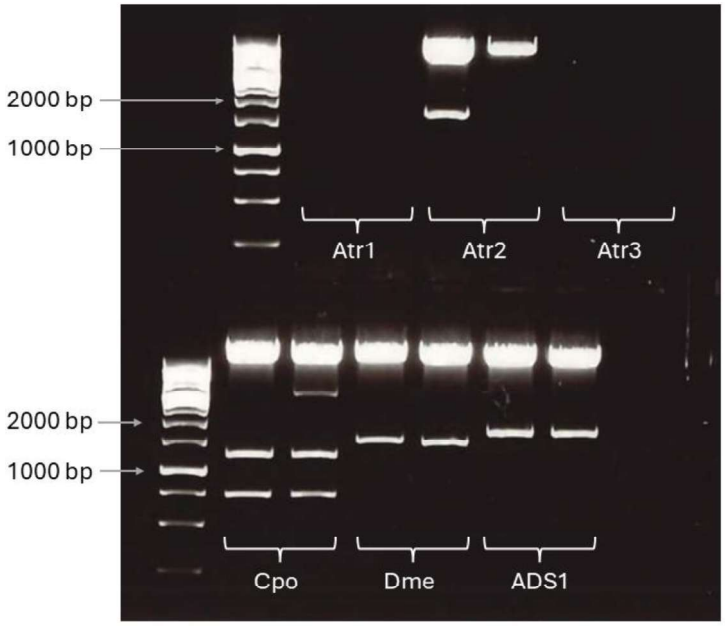

Figure 10. Agarose gel electrophoresis image of the restriction digestion of LR reactions of Atr1, Atr2 Atr3, Cpo, Dme and ADS1. Digestion was conducted in two replicates and the order of samples on the gel is indicated in the figure.

Of the restriction digestion of LR reactions, both replicates of Cpo, Dme and ADS1, as well as the first replicate of Atr2 verified correct recombination in the LR reactions, as seen in figure 10. However, Atr1 and Atr3 did not show any evidence of the expected DNA, which indicates the LR reactions were unsuccessful. Expected DNA fragment lengths were approximately 7500, 5600 and 1700 for Atr1, Atr2 and Atr3; 13000, 1300 and 750 for Cpo; 13000 and 1600 for Dme; and 13000 and 1800 for ADS1.

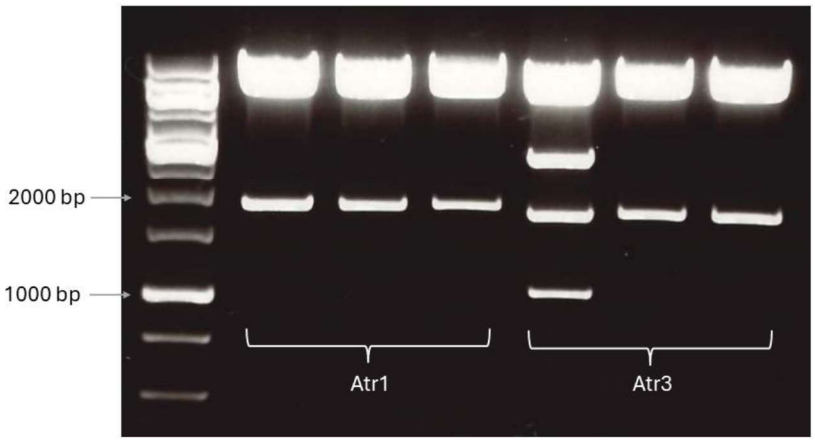

Figure 11. Agarose gel electrophoresis image of the restriction digestion of the repeated LR reactions of Atr1 and Atr3. Digestion was conducted in replicates of three and the order of samples on the gel is indicated in the figure.

LR reactions were repeated for Atr1 and Atr3, in which the restriction digestion showed that all replicates except the first replicate of Atr3 was verified as correct recombination into the destination vector, based on the expected lengths of the DNA fragments, as seen in figure 11.

5.3 Transient expression in *Nicotiana benthamiana*

Below in table 2 and 3 is a list of the gene combinations for all treatments, as well as gene of interest length, presumed substrate and the presumed target pheromone precursors. Figure 12 includes a few images to illustrate the infiltration process.

Table 2. List of the gene combinations included in each treatment. Genes or treatments listed as (WT) are from the wild-type insect genes and genes or treatments listed as (h) are hybrids.

Treatment name	RNA silencing suppressor	Reporter gene	Fatty acid synthesis transcription factor	Fatty acid thioesterase	Gene of interest
Control	P19	GFP	WRINKLED1		
Atr (WT)	P19	GFP	WRINKLED1	*Cpu*FATB1	AtrΔ11 (WT)
Cpo (WT)	P19	GFP	WRINKLED1	*Uca*FATB1	Cpo_CPRQ(WT)
Dme (WT)	P19	GFP	WRINKLED1	*Cpa*FATB2	DmeΔ9 (WT)
Atr1 (h)	P19	GFP	WRINKLED1	*Cpu*FATB1	Atr1 (h)
Atr2 (h)	P19	GFP	WRINKLED1	*Cpu*FATB1	Atr2 (h)
Atr3 (h)	P19	GFP	WRINKLED1	*Cpu*FATB1	Atr3 (h)
Cpo (h)	P19	GFP	WRINKLED1	*Uca*FATB1	Cpo (h)
Dme (h)	P19	GFP	WRINKLED1	*Cpa*FATB2	Dme (h)
ADS1	P19	GFP	WRINKLED1		ADS1

Table 3. Gene of interest length, presumed substrate and presumed target pheromone precursors for each treatment.

Treatment name	Gene of interest length (in bp)	Presumed substrate	Presumed target pheromone precursor
Atr (WT)	978	16:0-CoA	16:1 Z11
Cpo (WT)	1095	12:0-CoA	12:1 E9
Dme (WT)	1083	14:0-CoA	14:1 Z9
Atr1 (h)	966	16:0-CoA	16:1 Z11
Atr2 (h)	972	16:0-CoA	16:1 Z11
Atr3 (h)	1182	16:0-CoA	16:1 Z11
Cpo (h)	1086	12:0-CoA	12:1 E9
Dme (h)	1065	14:0-CoA	14:1 Z9
ADS1	915	16:0-CoA	16:1 Z9

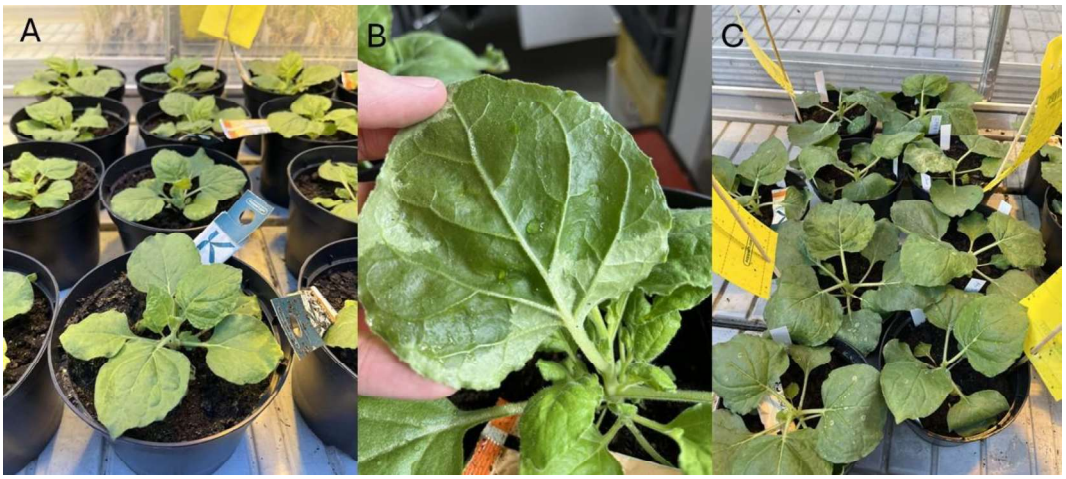

Figure 12. Image A shows the developmental stage of the plants at the time of infiltration. Image B illustrates the infiltrated leaf area, which was approximately 70-90 % of each leaf. Image C shows the state of the plants at the time of harvest.

The first observation that could be made about the effects of the treatments was that treatment Dme (h) appeared to cause chlorotic spots on the leaves, as shown in figure 13. All three replicates were affected to varying degrees, but all other treatments had no visual symptoms.

Figure 13. Treatment Dme (h) induced chlorotic spots to varying degrees to all three replicates.

5.4 Lipid separation with TLC

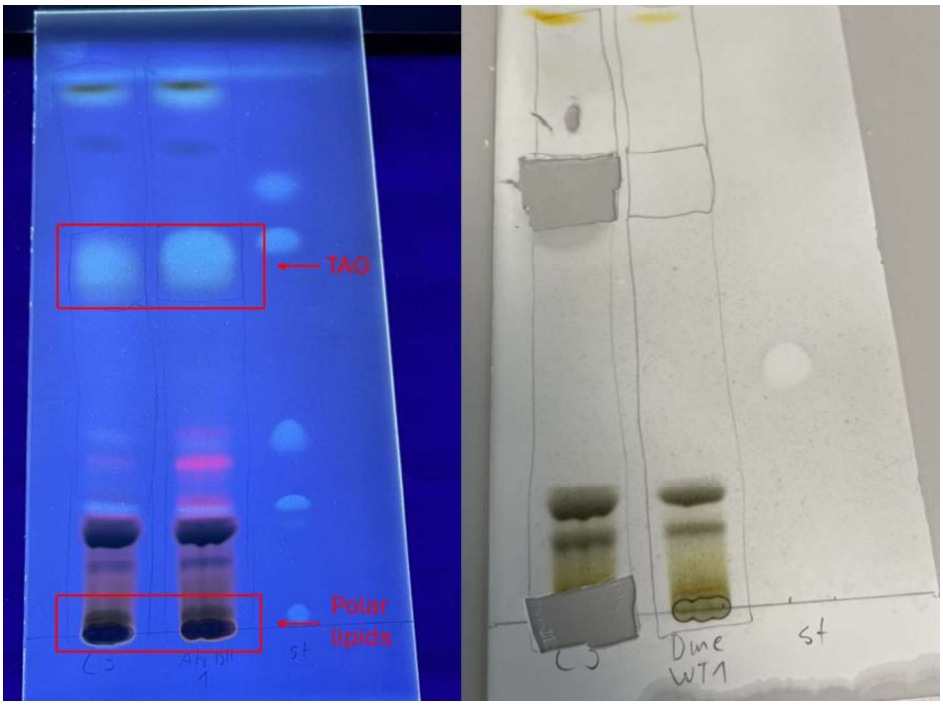

Figure 14. Lipid separation with thin layer chromatography (TLC). On the left is a TLC plate view under UV-light with areas with TAG and polar lipids are marked in red. The right image illustrate areas of TAG and polar lipids scraped off and included in the lipid analysis.

After separation on TLC plates, the silica containing the TAG and the polar lipids were scraped off into separate tubes, illustrated in figure 14.

5.5 Lipid analysis of TAG and polar lipids (GC)

The lipid analysis of TAG and polar lipids using GC generated results regarding multiple different aspects of lipid synthesis.

5.5.1 Hybrid desaturase enzymes did not retain function

The hybrid genes were evaluated by analyzing the fatty acid composition (%) of TAG and polar lipids in infiltrated Nicothiana bethamiana leaves. Comparisons were conducted between the hybrid gene treatments and the treatments containing their respective original insect desaturase genes. Hybrid gene treatment Atr2 (h) was compared with Atr (WT), Cpo (h) was compared with Cpo (WT) and Dme (h) was compared with Dme (WT). However, due to time constraints, hybrid gene treatments Atr1 (h) and Atr3 (h) were not analyzed.

Figures 15 and 16 show the results of the fatty acid compositions of TAG and polar lipids of Atr2 (h) and Atr (WT). As a first observation, it can be noted that

the wild-type insect desaturase of Atr proved to be functional in Nicotiana, which is indicated by the TAG composition containing 4,2 % of 16:1 Z11. However, the target pheromone precursor 16:1 Z11 was not produced in the leaves infiltrated with the hybrid gene. The TAG the Atr-hybrid did contain approximately 50 % of the fatty acid substrate 16:0, but it is unclear to what degree this was available as a substrate for the desaturase enzyme. Figure 16 of the polar lipids showed that the 16:1 Z11 was also incorporated into membrane lipids to a certain degree.

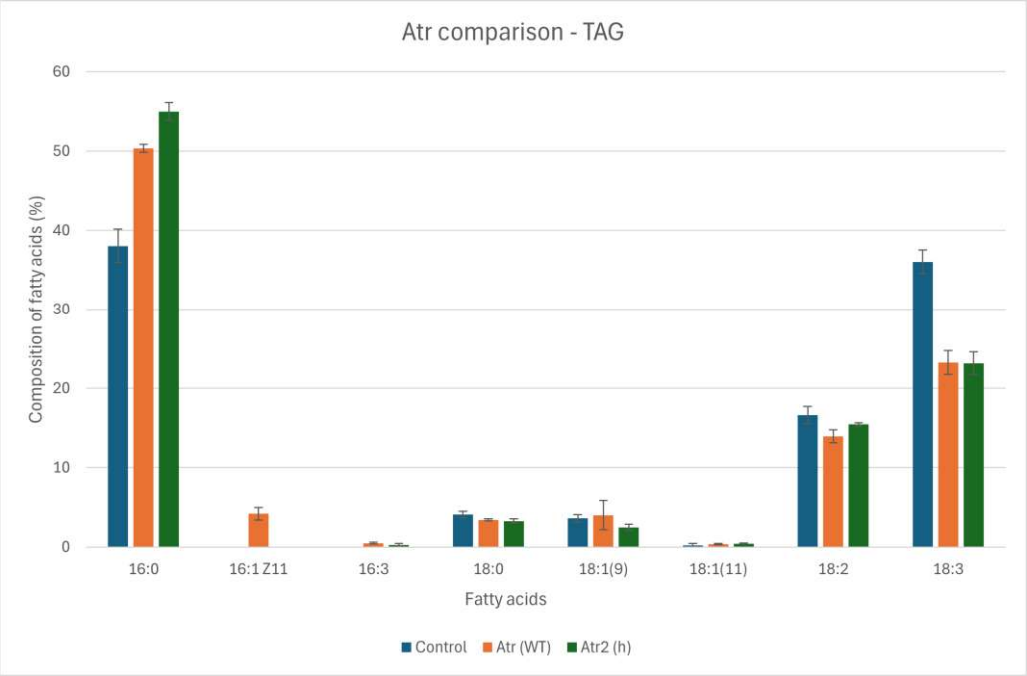

Figure 15. Comparison of fatty acid composition (%) in TAG in Nicotiana benthamiana leaves infiltrated with treatments control, Atr (WT) and Atr2 (h). n = 3 biological replicates. Error bars represent standard deviation.

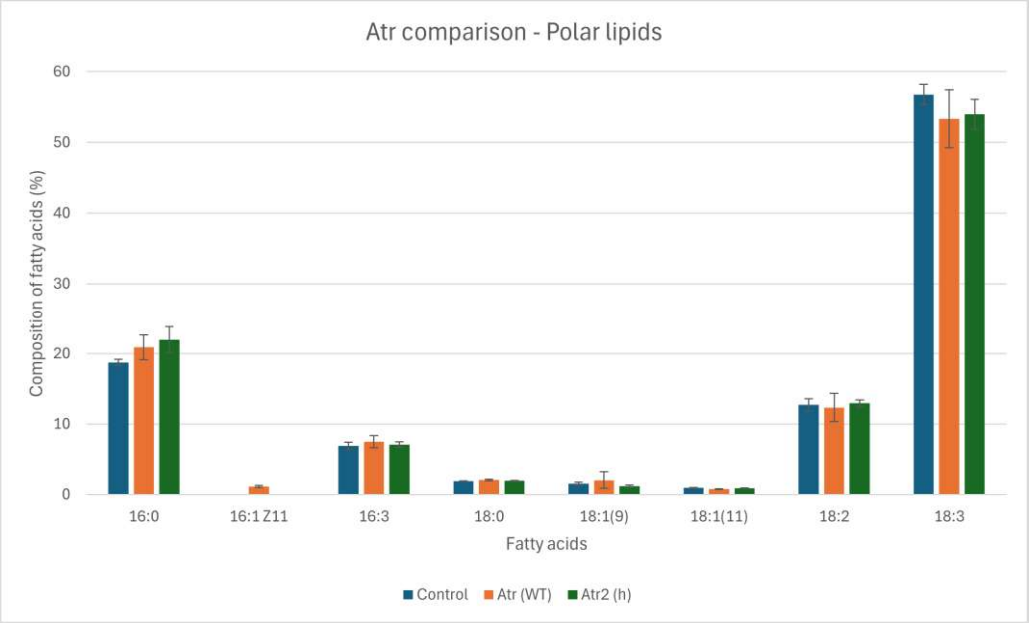

Figure 16. Comparison of fatty acid composition (%) of polar lipids in Nicotiana benthamiana leaves infiltrated with treatments control, Atr (WT) and Atr2 (h). n = 3 biological replicates. Error bars represent standard deviation.

Figures 17 and 18 show the results of the fatty acid compositions of TAG and polar lipids of Cpo (h) and Cpo (WT). Similarly to Atr, the Cpo hybrid gene did not appear to retain the function of the insect desaturase enzyme, or at least to a significantly lower degree. The TAG extracted from the leaves infiltrated with the treatment containing the Cpo (WT) gene was composed of approximately 2.8 % of the target pheromone precursor 12:1 E9. In the TAG from the leaves infiltrated with the Cpo (h) treatment, the target precursor was detectable but averaged approximately 0.2 %. Another observation is that the TAG of both the treatment Cpo (WT) and Cpo (h) contained less of the substrate (12:0) compared to the substrate (16:0) in the Atr-treatments.

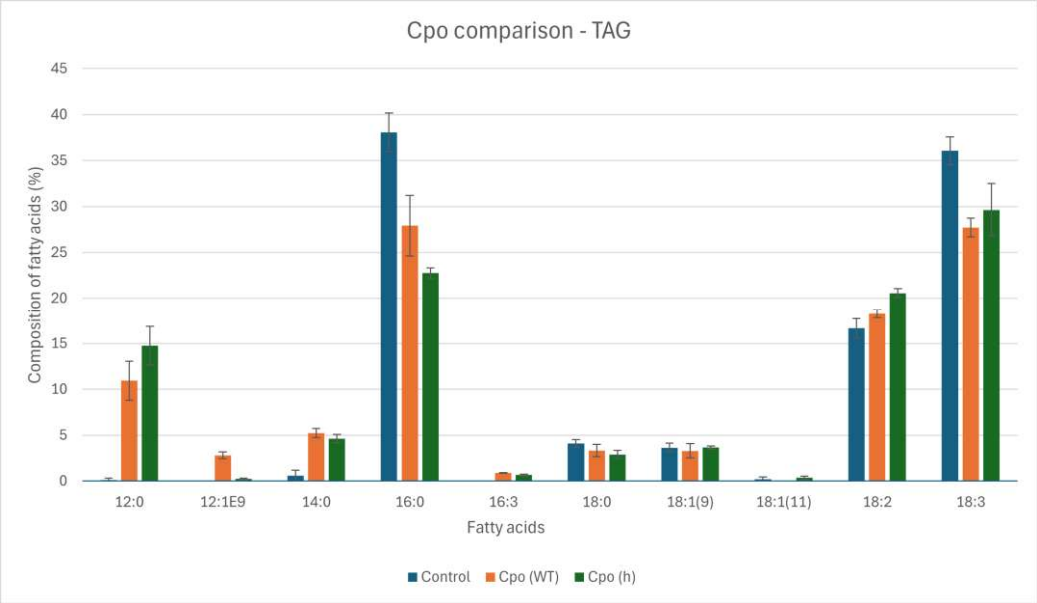

Figure 17. Comparison of fatty acid composition (%) in TAG in Nicotiana benthamiana leaves infiltrated with treatments control, Cpo (WT) and Cpo (h). n = 3 biological replicates. Error bars represent standard deviation.

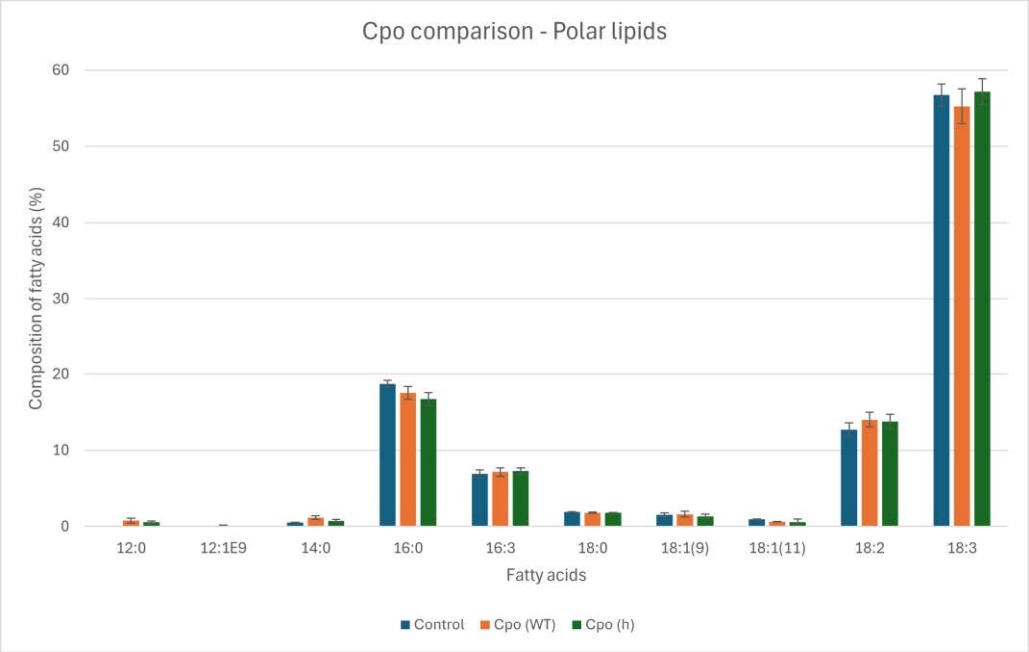

Figure 18. Comparison of fatty acid composition (%) of polar lipids in Nicotiana benthamiana leaves infiltrated with treatments control, Cpo (WT) and Cpo (h). n = 3 biological replicates. Error bars represent standard deviation.

Figures 19 and 20 show the results of the fatty acid compositions of TAG and polar lipids of Dme (h) and Dme (WT). The Dme (h) gene did not retain the function of the Dme (WT) gene, since the target pheromone precursor 14:1 Z9 was absent from both the TAG and the polar lipids of the leaves infiltrated with the Dme (h) gene. However, two observations can be made about the Dme (WT)

treatment. Firstly, the amount of target pheromone precursor 14:1 Z9 produced by Dme (WT) was higher than then target pheromone precursor produced by both the Atr (WT) and the Cpo (WT). The TAG produced by treatment Dme (WT), was composed of 10.2 % of the target pheromone precursor 14:1 Z9. Secondly, the leaves infiltrated with the Dme (WT) treatment also contained the target pheromone precursor of Atr (WT) 16:1 Z11, which was not expected.

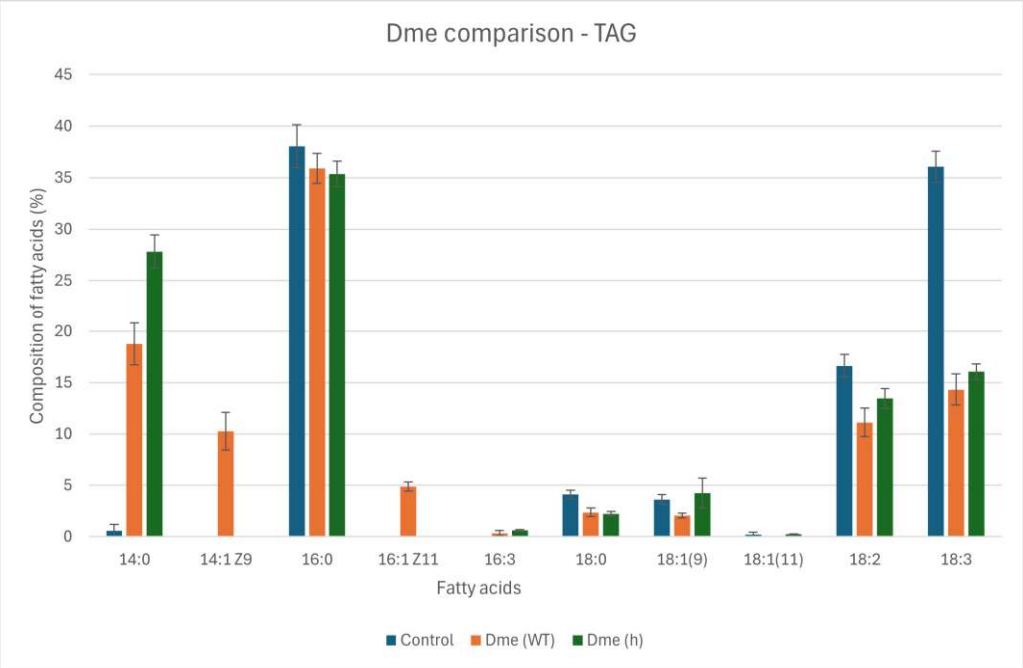

Figure 19. Comparison of fatty acid composition (%) in TAG in Nicotiana benthamiana leaves infiltrated with treatments control, Dme (WT) and Dme (h). n = 3 biological replicates. Error bars represent standard deviation.

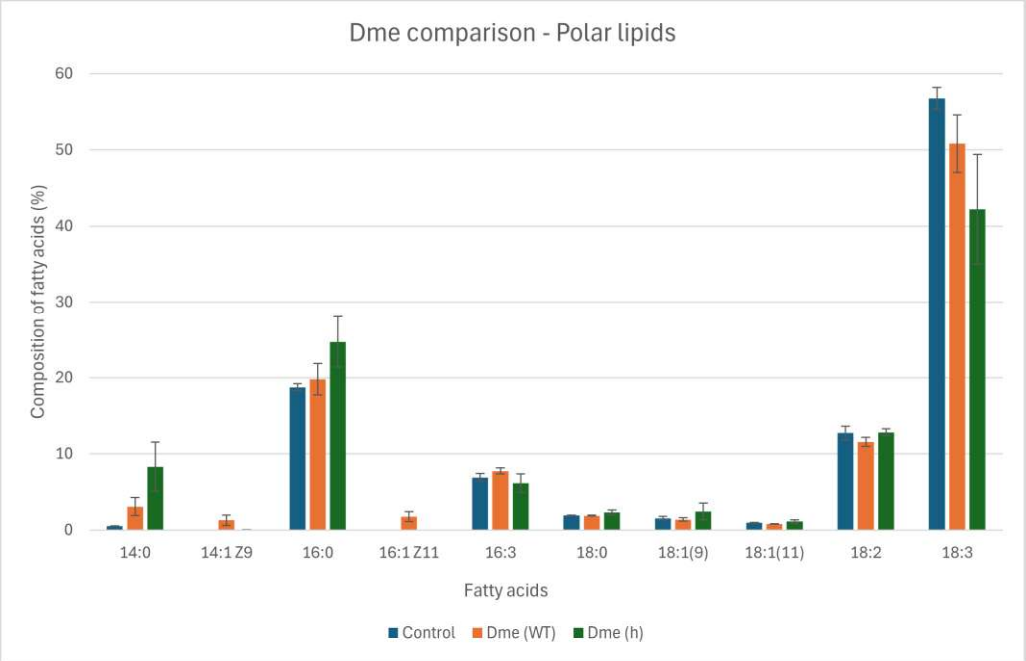

Figure 20. Comparison of fatty acid composition (%) of polar lipids in Nicotiana benthamiana leaves infiltrated with treatments control, Dme (WT) and Dme (h). n = 3 biological replicates. Error bars represent standard deviation.

5.5.2 ADS1 scaffold gene is non-functional

The gene ADS1 from *Arabidopsis thaliana* was used as a scaffold in the construction of the hybrid genes. This was included as a treatment and control for functionality for transient expression as a second control group. Unfortunately, when expressed in *Nicotiana benthamiana* leaves, ADS1 did not retain the original function of ADS1 in *Arabidopsis thaliana*. In its native state, ADS1 acts on 16:0 as a substrate and produces 16:1 Z9, which was absent in both the TAG and the polar lipids of the leaves infiltrated with ADS1, as seen in figures 21 and 22. This has large implications for the possible conclusions to draw from the results.

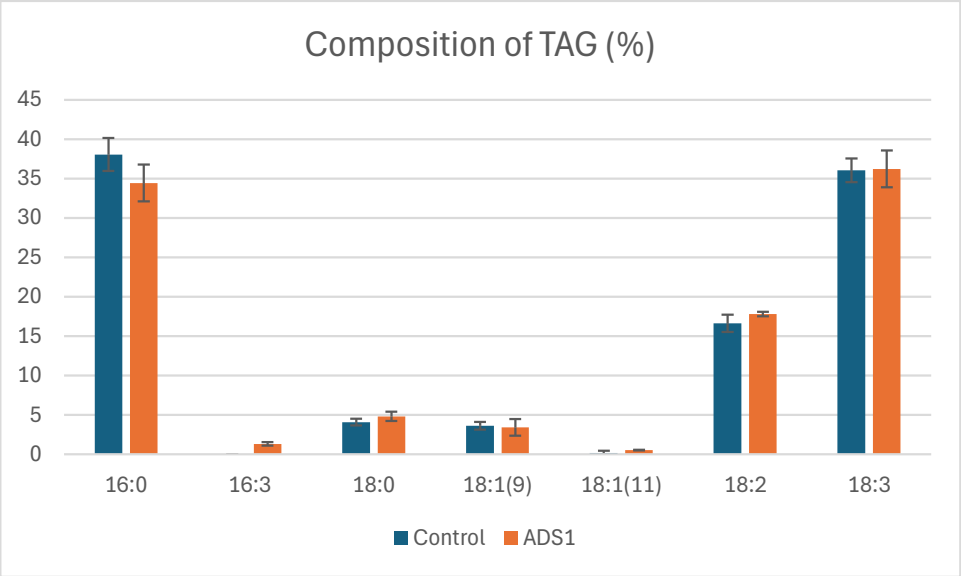

Figure 21. Fatty acid composition in TAG in Nicotiana benthamiana leaves infiltrated with the treatment ADS1 compared to the control. n = 3 biological replicates. Error bars represent standard deviation.

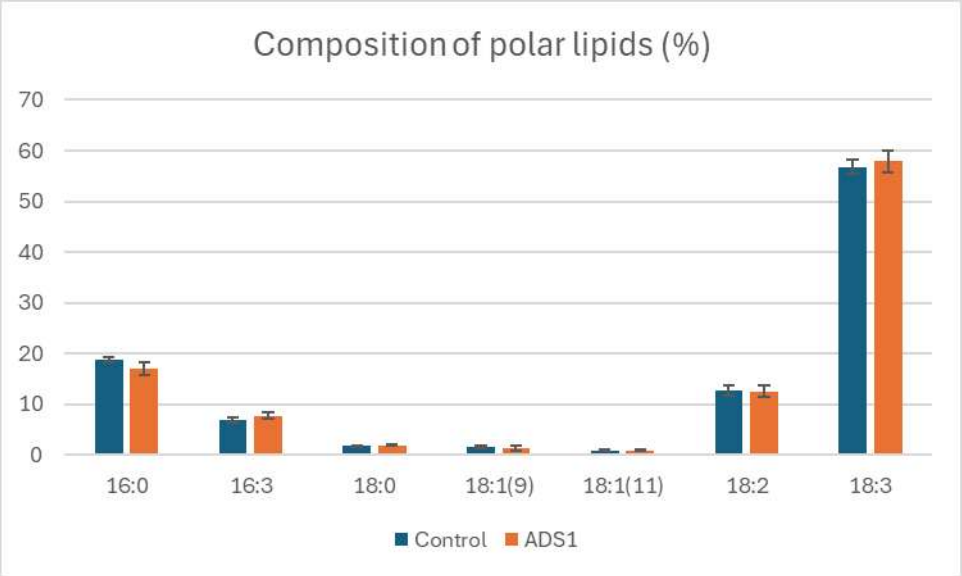

Figure 22. Fatty acid composition of polar lipids in Nicotiana benthamiana leaves infiltrated with the treatment ADS1 compared to the control. n = 3 biological replicates. Error bars represent standard deviation.

5.5.3 Total lipid content in TAG

The results of lipid analysis showed that the amount of TAG in nmol per mg of leaf dry weight differed to a certain extent both between the treatments and between the replicates within treatments. However, treatment Dme (h) was the only treatment that differed significantly from the control group, as indicated by the letters above the bars in figure 23. Dme (h) contained on average 87 nmol of

TAG per mg leaf dry weight, while the control group contained on average 16 nmol per mg leaf dry weight.

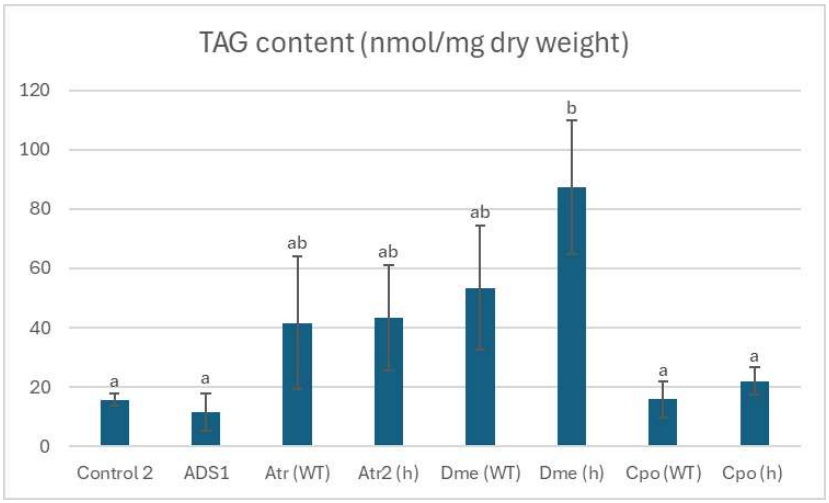

Figure 23. Comparison of TAG content in leaves infiltrated with each treatment. n = 3 biological replicates. Error bars represent standard deviation. Significance is illustrated by the letters above each bar, based on an analysis of variance (ANOVA). Bars not sharing a letter are significantly different.

6. Discussion

6.1 Non-functionality of ADS1 in *Nicotiana benthamiana*

The most critical result from this project was that the infiltration of *Nicotiana benthamiana* leaves with the ADS1 treatment did not result in detectable levels of the presumed product 16:1 Z9, neither in the TAG nor the polar lipids. There appeared to be a high amount of the presumed substrate of ADS1, 16:0, in both the TAG and the polar lipids but no apparent conversion into 16:1 Z9. This was quite unexpected since the hypothesis, related to the concept of hybrid gene construction, was founded on the expectation that a plant desaturase would have a higher degree of compatibility with another plant system compared to an insect desaturase and had even proven functional in yeast expression (Heilman et al 2004).

The lack of functionality of ADS1 have a number of possible explanations. First of all, the lipid analysis indicated a high level of the presumed substrate 16:0, but the availability and compartmentalization of the substrate is unclear. The infiltrated gene combination with ADS1 did not include a thioesterase exporting the substrate 16:0, but 16:0 appears present in *Nicotiana benthamiana*. However, it is possible that the naturally occurring 16:0 might not be available as free 16:0-CoA in the endoplasmic reticulum (ER), but might be incorporated into complex lipids or localized to parts of the cell which are inaccessible to the ADS1 desaturase.

A second possible explanation is that the ADS1 protein might have been misfolded in *Nicotiana benthamiana*. In its native state, ADS1 is a membrane-bound enzyme in the ER of *Arabidopsis thaliana* but when transiently expressed in a different system, like Nicotiana, it could cause problems. For example, there might be variations between species in the composition of membranes or protein processing which could cause misfolding of the enzyme or incorrect insertion into the ER membrane. This could lead to a reduction in activity or render the enzyme completely non-functional. This seems unlikely as it has been shown active in other expression systems, including yeast.

Thirdly, another possible explanation is that there might be native acyl-CoA desaturases or other enzymes in *Nicotiana benthamiana* which compete for the same substrate as ADS1. Additionally, there might be differences between the metabolic flux through fatty acid biosynthesis pathways in *Arabidopsis thaliana*

and *Nicotiana benthamiana* which reduces the formation or recycling of 16:0-CoA in desaturation pathways.

It is also possible, however unlikely, that there were some more basic problems concerning post-transcriptional or translational regulation of the transient expression. Even though the genes were expressed with a 35S promotor, transcriptional silencing, suboptimal codon usage, or issues with mRNA stability might have contributed to low levels of expression. The inclusion of P19 is supposed to mitigate this, but the effects of post-transcriptional regulation cannot be entirely ruled out as a contributing factor.

6.2 Litterature context: ADS1 activity and transferability

The unexpected non-functionality of ADS1 in *Nicotiana benthamiana*, indicated in this study, is contradictory to previous studies that have described the enzyme's activity. It is therefore reasonable to question the level of compatibility, regarding structure and function, when ADS1 is expressed in non-native plant systems. In the literature, there appears to be a certain ambiguity regarding the localization and enzymatic function of ADS1 depending on the context of expression. It is important to understand these discrepancies when interpreting the results of this project.

Heilmann et al. (2004) was early to report ADS1 as a Δ9-palmitoyl-CoA desaturase in *Arabidopsis thaliana*, converting 16:0-CoA to 16:1 Z9. The study was conducted on both yeast and in *Arabidopsis thaliana* and reported ADS1 to be cytosolic, meaning bound to the ER membrane, and with strong substrate specificity and regioselectivity. However, when expressed with a transit peptide relocating the enzyme to the chloroplast, the regiospecificity changed from Δ9 to Δ7, which indicates a level of complexity regarding the context of where it is expressed. Though, in this project, ADS1 was expected to behave as reported without the transit peptide, and thereby display a high level of compatibility with the plant system.

Smith et al. (2013) evaluated ADS1 in yeast and concluded that it can act on both 16:0 and 18:0 to catalyze either Δ9 or Δ7 desaturation. Smith et al. (2013) also evaluated ADS1 localization through transient expression in *Nicotiana benthamiana* and, similarly to Heilmann et al. (2004), reported that ADS1 was located to the endoplasmic reticulum (ER), as would be expected of a acyl-CoA desaturases. In certain contexts, the study also showed that ADS1 can be involved in desaturation of longer fatty acids, like for example 26:1. The findings by Smith et al. (2013) proves that transient expression of ADS1 in Nicotiana bethamiana is

possible, and also indicate that ADS1 is functionally versatile which potentially could be adapted to different plant systems.

In contrast with earlier reports, Chen and Thelen (2016) has provided evidence that when ADS1 is expressed in its native plant *Arabidopsis thaliana*, it localizes to the chloroplast rather than the ER as reported earlier. By tagging the expression of ADS1 with fluorescent protein in leaf protoplast of *Arabidopsis thaliana*, there were strong indications that ADS1 co-localizes with chlorophyll autofluorescence. Chen and Thelen (2016) further speculate that the discrepancy in results compared to Smith et al. (2013) could be due to the fact that epidermal cells of *Nicotiana benthamiana* lack functional chloroplast. Thereby their results indicate that ADS1 naturally occurs in the chloroplast of *Arabidopsis thaliana* and functions as an acyl-lipid desaturase acting on membrane lipids. Though, in the absence of functioning chloroplast, ADS1 will be located to other subcellular compartments like the ER.

The ambiguity around the subcellular localization and functional versatility of ADS1 appear to be a central point of discussion for this project. The purpose of the study was to transiently express genes encoding desaturases in *Nicotiana benthamiana*, and then to target these enzymes to the ER in order to access cytosolic acyl-CoA substrates. However, if ADS1 instead locates to the plastids, or if membrane targeting signals are unclear, this could cause mislocalization and loss of function.

The varying localizations and functions of ADS1 reported by Heimann et al. (2004), Smith et al. (2013) and Chen and Thelen (2016) illustrate the challenges in genetically engineering plant systems. The ambiguity of what mechanisms determine function makes it complicated to make assumptions about compatibility between heterologous systems. Plant systems like *Nicotiana benthamiana* and *Arabidopsis thaliana* may differ in multiple critical ways which are crucial to the function of ADS1, like membrane structure and protein processing and trafficking. While ADS1 was selected as a backbone for the construction of hybrid enzymes due to presumptions of compatibility between plant systems, the conflicting evidence in the literature indicate that its subcellular behavior may be more complex than previously thought. The discrepancies in the literature may help to explain why ADS1 did not appear to be functional as expected in this project. It also emphasizes the importance of validating both enzyme activity and localization in the selected plant system.

6.3 Hybrid enzyme evaluation

The absence of the expected product of ADS1, 16:1 Z9, is, as discussed, a strong indicator of the non-functionality of ADS1 in infiltrated *Nicotiana benthamiana* leaves. This makes it challenging to accurately evaluate the hybrid enzymes and instead creates questionmarks around ADS1's ability to act as scaffold for hybrid construction. Since the hybrid genes are constructed with the transmembrane domains of ADS1, its apparent inactivity undermines the idea that it could provide a more compatible membrane environment for the catalytic domains of insect desaturases. If ADS1 itself seemed to be non-functional in this experiment, then the inactivity of the hybrid enzyme may not stem from domain incompatibility, but from a flawed scaffold. Thereby, conclusions around the concept of hybrid gene construction and the impact of domain swapping must be drawn cautiously. Without verification of ADS1 being functional in *Nicotiana benthamiana*, the true cause of the inactivity of the hybrid enzymes remains uncertain.

6.4 Observation on fatty acid dynamics and pheromone precursor formation

There are several observations that can be made from the results that are not directly related to the original research questions but still provide valuable insight.

6.4.1 Treatment Dme (WT) produced two pheromone precursors

The first, and perhaps most noteworthy of these observations, is that the fatty acid profile of treatment Dme (WT) contained both its presumed target pheromone precursor 14:1 Z9 as well as a second pheromone precursor 16:1 Z11. Interestingly, the amount of 16:1 Z11 in the Dme (WT) treatment is even higher than in the Atr (WT) treatment, despite 16:1 Z11 being the presumed target of AtrΔ11. This is potentially a novel finding that has previously not been reported in the literature. These insights could prove relevant for future studies related to the enzymatic and metabolic activities of DmeΔ9.

However, the mechanisms of this occurrence are unclear. Based on the configuration and position of the double bond, the more likely explanation is that 14:1 Z9 is being elongated by an endogenous elongase enzyme into 16:1 Z11. It is not uncommon that monounsaturated fatty acids in plants can be extended by two-carbon units via fatty acid elongases, which makes this a plausible explanation. Though, it is not beyond the realm of possibility that DmeΔ9 can act on 16:0 as a substrate to produce 16:1 Z11, but this explanation is likely less plausible.

Further research is needed to verify the mechanisms involved in the production of both 14:1 Z9 and 16:1 Z11 in the Dme (WT) treatment. However, the presence of 16:1 Z11 highlights the possibility of unintended pathway interactions in heterologous expression systems. Regardless of the mechanisms involved, this gives plausibility to a future scenario where multiple pheromone precursors could be produced and extracted from the same plant.

6.4.2 Substrate levels suggest fixed export independent of desaturase activity

Another observation was made when comparing fatty acid profiles of TAG between hybrid and wild-type desaturase treatments. It can be noted that the wild-type desaturase treatments have a lower amount of substrate available compared to their respective hybrid treatments. As previously discussed, the hybrid treatments were non-functional and the wild-type treatments were functional, thereby substrate conversion only took place in the wild-type treatments. When comparing the fatty acid profiles, the difference in the level of substrate between wild-type and hybrid treatments is roughly offset by the amount of target pheromone precursors produced by the functional wild-type desaturases. For example, the TAG profile of the Dme (WT) treatment contained approximately 19 % 14:0 and 10 % 14:1 Z9, while the Dme (h) treatment contained approximately 28 % 14:0 and no detectable 14:1 Z9. The relationships between the Atr- and Cpo-treatments are similar. This indicates that thioesterase-mediated export of saturated substrate into the ER is relatively fixed and unaffected by whether desaturation occurs. This implies that desaturation acts downstream of substrate export and that there is no feedback mechanism which increase the flux of substrate in response to conversion by the desaturases.

6.4.3 Visual symptoms correlate with elevated TAG accumulation

There seemed to be an interesting connection regarding the visual phenotype that the plants displayed and the total lipid content of the TAG analysis. Treatment Dme (h) was the only treatment that had a significantly higher amount of TAG per mg of leaf dry weight compared to the control. At the same time, Dme (h) was the only treatment that displayed any visual phenotypic effects the plants. Infiltration of Dme (h) seemed to induce chlorotic spots to varying degree on the leaves, which developed pale yellow-grey areas. It would appear plausible that the unusually high production of TAG and the development of chlorotic leaf areas might be linked.

A potential explanation for this is that the high accumulation of TAG was perhaps excessive to the point where it disrupted membrane integrity or signaling

pathways. Another possibility might be that non-functional or misfolded desaturase protein negatively affected the lipid metabolism which caused a stress response. These are merely speculations regarding the underlying mechanisms, however, the correlation seem plausible.

6.5 Future direction and broader perspectives

Multiple aspects of this study have shown areas of research that would be appropriate for future studies. Based on the results of this study, there are also a few aspects of the method that could be changed, if repeated. One limitation in the evaluation of ADS1 was the absence of a thioesterase in the gene combination. The lack of a thioesterase aimed at exporting the presumed substrate of ADS1 likely limited the pool of available substrate in the ER. This was potentially a contributing factor to the non-functionality of ADS1. If the evaluation of ADS1 were to be repeated through transient expression in *Nicotiana benthamiana*, including a thioesterase for 16:0 could provide sufficient substrate availability, and thereby lead to a more accurate assessment of desaturase activity.

The study also suggests that scaffold selection in hybrid desaturase construction is critical. Since ADS1 didn't display any activity in *Nicotiana benthamiana*, it might be appropriate to reconsider the gene selection for which scaffold to use for domain swapping. When researching the literature there was an apparent ambiguity around both the localization, the preferred substrate and regioselectivity based on the context of expression of ADS1. Therefore, it would be beneficial for future studies to verify the function and localization of candidate scaffold genes in the chosen host system before construction of hybrid genes.

The occurrence of two different pheromone precursors in the Dme (WT) treatment and the seemingly fatal effects of the Dme (h) treatment, causing chlorotic leaf development, indicate that heterologous expression can interact with endogenous systems in unforeseen ways. In this context of creating functional hybrid genes, it is therefore essential to create balanced pathways, which will manage metabolic fluxes appropriately and avoid stress responses.

In a broader perspective, the concept of hybrid gene construction is an exciting idea. This study, however, highlights the complexity of metabolic engineering in plants. Future studies could try to broaden the evaluation and combine metabolic profiling, localization studies and protein activity assays. This could provide the deeper knowledge needed for creating hybrid enzymes and, subsequently, developing plant-based pheromone production to reach its full potential.

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Popular science summary

In order to make our food production system more sustainable, scientists are exploring ways to reduce the reliance on harmful chemical pesticides. A promising alternative is to use insect sex pheromones, which are natural scent signals that insects use to find each other and mate. These pheromones can be used to confuse pest insects and prevent them from reproducing and would be an eco-friendly way to protect crops.

The problem is that producing these pheromones synthetically is both expensive and has a negative environmental impact. A promising alternative is to use plants as “mini-factories” to produce the building blocks of pheromones using genes from insects. These insect genes code for enzymes, called desaturases, which shape the chemical structure of pheromones. However, insect desaturases are not adapted to function in plants, so when inserted into plants, they often have low efficiency and therefore the intended building blocks of pheromones are not produced in the plant tissue. Therefore, this project has explored the idea of creating hybrid genes between plant and insect genes. The hypothesis is that when such genes are inserted into plants, the corresponding hybrid enzymes could show increased compatibility and efficiency in plants.

The chosen method to evaluate this idea was to insert both the hybrid-, insect-, and plant enzymes into tobacco plants and see if they were able to produce the pheromone building blocks. The results showed that the original insect enzymes were functional, but none of the hybrid genes functioned as hoped. The original plant enzyme did not work either, which was surprising, since the whole idea was built on the assumption that including plant enzyme components would improve efficiency. This led to the conclusion that enzyme function is very sensitive to the biological conditions which might differ between plant species.

However, the study made a surprising and potentially new discovery when one of the insect enzymes turned out to produce not just one, but two different pheromone building blocks. This opens up the possibility of producing multiple pheromone components in the same plant.

Overall, this study has demonstrated the complexities of genetic engineering. There are still challenges to overcome in trying to produce insect pheromones in plants, but hopefully the lessons of this study will bring our food production system one step closer to a sustainable future.

Appendix 1

Sequences of the constructed desaturase hybrid enzyme genes (including att-sites for gateway cloning).

Atr1 (h)
GGGGACAAGTTTGTACAAAAAAGCAGGCTTCAACATGGTGCCGAACA
AGGGCTCATCTGATGTGCTTTCTGAGCACTCTGAGCCGCAGTTCACCA
AGCTTATTGCTCCTCAAGCTGGCCCGAGGAAGTACAAGATCGTTTACA
TCGTGAAGGCTTTCGCCAGCCTGTTCGTGCATTTCCTTTGTCTGCTTGC
CCCGTTCAATTTCACCTGGCCTGCTCTTAGAGTGGCCCTTATTGTGTAC
ACCGTTGGTGGTCTTGGTATCACCGTTTCTGCTCATAGGCTTTGGGCTC
ACAGGACCTACAAGGCTAAGATGCCTCTTGAGATCCTGCTGCTGATCA
TGAACTCTATCGCTTTCCAGGACACCGCATTCACCTGGGCTAGAGATC
ATAGGTTGCACCACAAGTACAGCGATACCGATGCTGATCCTCATAACG
CTACTCGGGGCTTCTTCTACTCTCACGTTGGTTGGCTGCTTGTGAAGAA
GCACCCTGAAGTTAAGGCTAGGGGCAAGTTCCTGTCTCTGGATGACCT
TAAGAACAACCCGCTGCTGAAGTTCCAGAAGAAGTACTTTCTGCAGCG
GACCGTGCTTTACCACATTCTGACTTTCGGCTTTCTGCTGTACTACTTC
GGTGGCCTTTCTTTCCTTACCTGGGGTATGGGTATTGGCGTGGCAATGG
AACATCATGTGACCTGCCTTATCGTGAACTCCGCTGCTCACATCTTCGG
CAACAAGCCTTACGACAAGTCTATCGCCTCTGTGCAGAACATCTCTGT
GTCTCTTGCTACCTTCGGTGAGGGCTTCCATAATTACCACCACACTTAC
CCTTGGGACTACAGGGCTGCTGAGCTTGGTAACAACAGGTTGAATATG
ACCACCGCCTTCATCGATTTCTTCGCTTGGATTGGCTGGGCCTACGATC
TTAAGTCTGTTCCGCAAGAGGCTATCGCTAAGAGGTGTGCTAAGACCG
GTGATGGAACTGATATGTGGGGCAGAAAGAGGTAGGACCCAGCTTTC
TTGTACAAAGTGGTCCCC

Atr2 (h)
GGGGACAAGTTTGTACAAAAAAGCAGGCTTCAACATGAGCCTGAGCG
CCTCTGAAAAAGAAGAGAACAACAAGAAGATGGCCGCCGACAAGGCT
GAGATGGGTAGAAAGAAAAGGGCTATGTGGGAGCGTAAGTGGAAGA
GGCTTGATATCGTGAAGGCTTTCGCCTCTCTGTTCGTGCACTTCCTTTG
TCTGCTTGCCCCTTTCAATTTCACCTGGCCTGCTCTTAGAGTGGCCCTT
ATTGTGTACACCGTTGGTGGTCTTGGTATCACCGTTTCTGCTCATAGGC
TTTGGGCTCACAGGACCTACAAGGCAAAGATGCCTCTTGAGATCCTGC
TGCTGATCATGAACTCTATCGCTTTCCAGGACACCGCATTCACCTGGG
CTAGAGATCATAGGTTGCACCACAAGTACAGCGATACCGATGCTGATC
CTCATAACGCTACTCGGGGCTTCTTCTACTCTCACGTTGGTTGGCTGCT
TGTGAAGAAGCACCCTGAAGTTAAGGCTAGGGGCAAGTTCCTGTCTCT

GGATGACCTTAAGAACAACCCGCTGCTGAAGTTCCAGAAGAAGTACTT
TCTGCAGCGGACCGTGCTTTACCACATTCTGACTTTCGGCTTTCTGCTG
TACTACTTCGGTGGCCTTTCTTTCCTTACCTGGGGTATGGGTATTGGCG
TGGCAATGGAACATCATGTGACCTGCCTTATCGTGAACTCCGCTGCTC
ACATCTTCGGCAACAAGCCTTACGACAAGTCTATCGCCTCTGTGCAGA
ACATCTCTGTGTCTCTTGCTACCTTCGGTGAGGGCTTCCATAATTACCA
CCACACTTACCCTTGGGACTACAGGGCTGCTGAGCTTGGTAACAACAG
GTTGAATATGACCACCGCCTTCATCGATTTCTTCGCTTGGATTGGCTGG
GCCTACGATCTTAAGTCTGTTCCGCAAGAGGCTATCGCTAAGAGGTGT
GCTAAGACCGGTGATGGAACTGATATGTGGGGCAGAAAGAGGTAGGA
CCCAGCTTTCTTGTACAAAGTGGTCCCC

Atr3 (h)
GGGGACAAGTTTGTACAAAAAAGCAGGCTTCAACATGGCCAGCCTGC
TGACTAAGCCTAAGCCTGTTTTCCTGTGCAGCCCTTCACTTTCTCCTCG
GACTCTTAATACCGCCACTCCGTCTTTGAACTTCACCCGGATTTCTTTC
ACCCACCACCAAAAGCTTGCTCCGTTCAAGCCTCCTTCTCTTGTGGTTG
CTTTCAGCGAGAAGGGTCTGAAGAGGGATGTTACTACAGCTGCTGCTG
CTACCTCTCTGAGCGCTTCTGAAAAAGAAGAGAACAACAAGAAGATG
GCCGCCGACAAGGCTGAGATGGGAAGAAAGAAAAGGGCTATGTGGGA
GCGTAAGTGGAAGAGGCTTGATATCGTGAAGGCTTTCGCCTCTCTGTT
CGTGCACTTCCTTTGTCTGTTGGCCCCATTCAATTTCACCTGGCCTGCT
CTTAGAGTGGCCCTTATTGTGTACACCGTTGGTGGTCTTGGTATCACCG
TTTCTGCTCATAGGCTTTGGGCTCACAGGACCTACAAGGCAAAGATGC
CTCTTGAGATCCTGCTGCTGATCATGAACTCTATCGCTTTCCAGGACAC
CGCATTCACCTGGGCTAGAGATCATAGGTTGCACCACAAGTACAGCGA
TACCGATGCTGATCCTCATAACGCTACTCGGGGCTTCTTCTACTCTCAC
GTTGGTTGGCTGCTTGTGAAGAAGCACCCTGAAGTTAAGGCTAGGGGC
AAGTTCCTGTCTCTGGATGACCTTAAGAACAACCCGCTGCTGAAGTTC
CAGAAGAAGTACTTTCTGCAGCGGACCGTGCTTTACCACATTCTGACT
TTCGGCTTTCTGCTGTACTACTTCGGTGGGCTTTCTTTCCTTACCTGGG
GTATGGGTATTGGCGTGGCAATGGAACATCATGTGACCTGCCTTATCG
TGAACTCCGCTGCTCACATCTTCGGCAACAAGCCTTACGACAAGTCTA
TCGCCTCTGTGCAGAACATCTCTGTGTCTCTTGCTACCTTCGGTGAGGG
CTTCCATAATTACCACCACACTTACCCTTGGGACTACAGGGCTGCTGA
GCTTGGTAACAACAGGTTGAATATGACCACCGCCTTCATCGATTTCTTC
GCTTGGATTGGCTGGGCCTACGATCTTAAGTCTGTTCCGCAAGAGGCT
ATCGCTAAGAGGTGTGCTAAGACCGGTGATGGAACTGATATGTGGGG
CAGAAAGAGGTAGGACCCAGCTTTCTTGTACAAAGTGGTCCCC

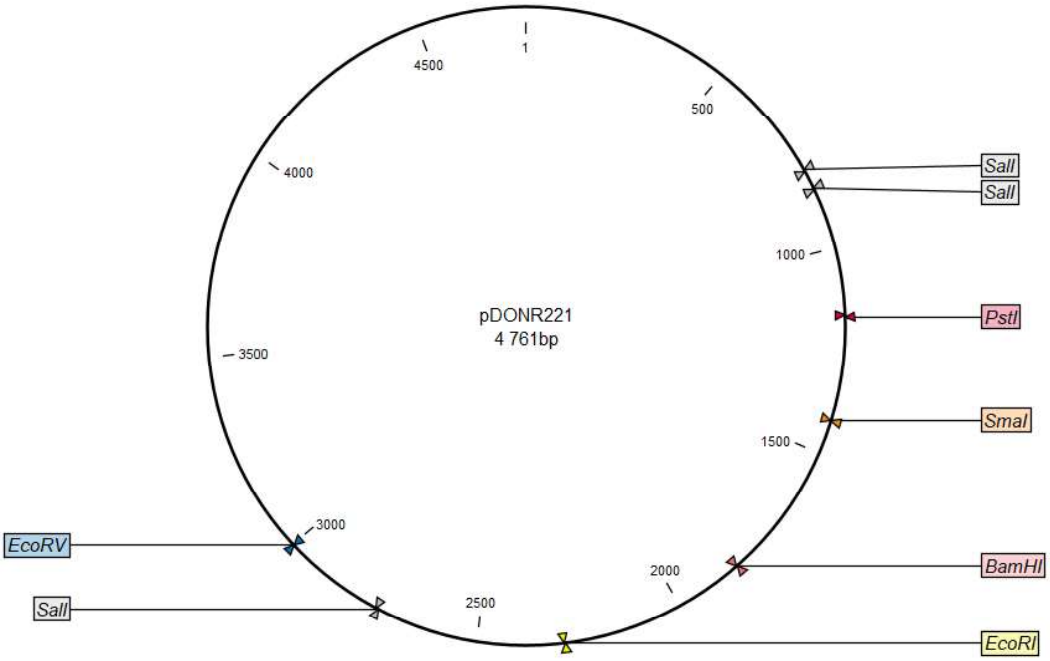
Cpo (h)
GGGGACAAGTTTGTACAAAAAAGCAGGCTTCAACATGCCTCCGCGTG
AGTCTAAGAAGGTGACCCTTAGGTCTTACGAGACTCCTGTGGCTTCTTT
GCCTCCTAGGAAGTACGAGATCATCTACATCGTGAAGGCTTTCGCCTC
TCTGTTCGTGCACTTCCTTTGTCTGCTTGCCCCTTTCAATTTCACCTGGC
CTGCTCTTAGAGTGGCCCTTATTGTGTACACCGTTGGTGGTCTTGGTAT
CACCGTTTCTGCTCACAGGCTTTGGAGCCACAGATCCTTCAAGGTTAA
GCCTCCGCTTGAGATCATGCTGATGCTGTTCAACTGCATCGGCTTCCAG
AATACCGCTACCGATTGGGTGAGAAACCATCGGTTGCATCACAAGCAC
AGCGATACCGATGCTGACCCTCATAACTCTAACAGGGGCATGCTGTTC
TCTCACATTGGTTGGCTGTGCGTGAGAAAGCACCCTGATGTGAAAGAG
AGGGGTAAGACCACCGACATGAGCGACATCTACTCTAACCCTGTGCTG
CGGTTTCAGAAGAAGCACTTCTTGCAGAGGACCGTGCTTTACCACATT
CTGACCTTCGGCTTTCTGCTGTACTACTTCGGTGGCCTTTCTTTCCTTAC
CTGGGGTATGGGTATTGGCGTGGCAATGGAACATCATGTGACCTGCCT
TATCGTGAACTCTATCGCTCACAAGTACGGCACCAGGCCTTACGATAG
AACTATTTGCCCTAGGCAGAACACTACCTGCAACATGATGACTCTTGG
TGAGGGCTTCCACAACTACCACCATACCTTTCCATGGGACTACAGGTC
TGCTGAGCTGGGTAAGAACTACCTGAACTTCACCAAGTGGTTCATCGA
TTTCTTCGCCCTGATCGGCTGGGCTTACGATCTTAAGACTGTGCCTGAT
GACATGATCCAGCGGAGAATGAAGAGGACCGGTGATGGTTCTAATAG
CTGGGGTTGGGGCGATAAGGACATGACCAAAGAAGAGAGGGACAGCG
CCACCATTATCTACCCTGAGAAGAAAGACGACATCAAGATGATCAGC
AAGAAGATCGACTACAGCAAGATCTTCAGCCACGACTACTTCAGGCTG
TTCGATGAGCTTTAGGACCCAGCTTTCTTGTACAAAGTGGTCCCC

Dme (h)
GGGGACAAGTTTGTACAAAAAAGCAGGCTTCAACATGGCCCCGTACA
GCAGGATCTATCACCAGGATAAGTCCAGCCGTGAGACTGGTGTTCTGT
TCGAAGATGATGCTCAGACCGTGGATTCTGATCTGACCACTGATAGGT
TCCAGCTGAAGAGAGCTGAGAAGAGAAGGCTTCCTCTGGTGATCGTTA
AGGCTTTCGCTTCTCTGTTCGTGCACTTCCTTTGCCTGCTTGCTCCTTTC
AATTTCACCTGGCCTGCTCTTAGAGTGGCCCTTATTGTGTACACCGTTG
GTGGTCTTGGTATCACCGTTTCTGCTCATAGGCTTTGGGCTCACAGGAC
CTATAAGGCTAAGTGGCCTCTTAGGCTGCTGCTGGTGATCTTCAACAC
CATTGCTTTCCAGGACGCTGTGTACCACTGGGCTAGAGATCATAGGGT
GCACCACAAGTACAGCGAGACTGATGCTGATCCTCATAACGCTACTCG
GGGCTTCTTCTTCTCTCACGTTGGTTGGTTGCTGTGCAAGAAGCACCCT
GACATCAAAGAGAAAGGCAGGGGCCTTGATCTTAGCGATCTTAGGGC
TGATCCGATCCTGATGTTCCAGAGAAAGCACTTCTTGCAGAGGACCGT
GCTTTACCACATTCTGACCTTCGGCTTTCTGCTGTACTACTTCGGTGGT

CTGTCTTTCCTTACCTGGGGTATGGGTATTGGTGTGGCCATGGAACACC
ATGTGACCTGCTTGATTGTGAACTCCGCTGCTCACAAGTTCGGCAACA
GGCCTTACGACAAGACTATGAACCCTACTCAGAACGCCTTCGTGAGCG
CTTTCACTTTTGGTGAAGGTTGGCACAACTACCACCACGCATTCCCTTG
GGATTACAAGACTGCTGAGTGGGGATGCTACAGCCTGAATATTACCAC
CGCTTTCATCGACCTGTTCGCCAAGATTGGTTGGGCCTACGATCTTAAG
ACTGTGGCTCCTGATGTGATCCAGCGTAGGGTTTTGAGAACCGGTGAT
GGTTCTCATGAGCTTTGGGGTTGGGGTGATAAGGATCTTACTGCTGAG
GACGCTAGGAACGTGCTGCTTGTTGATAAGAGCCGTTAGGACCCAGCT
TTCTTGTACAAAGTGGTCCCC

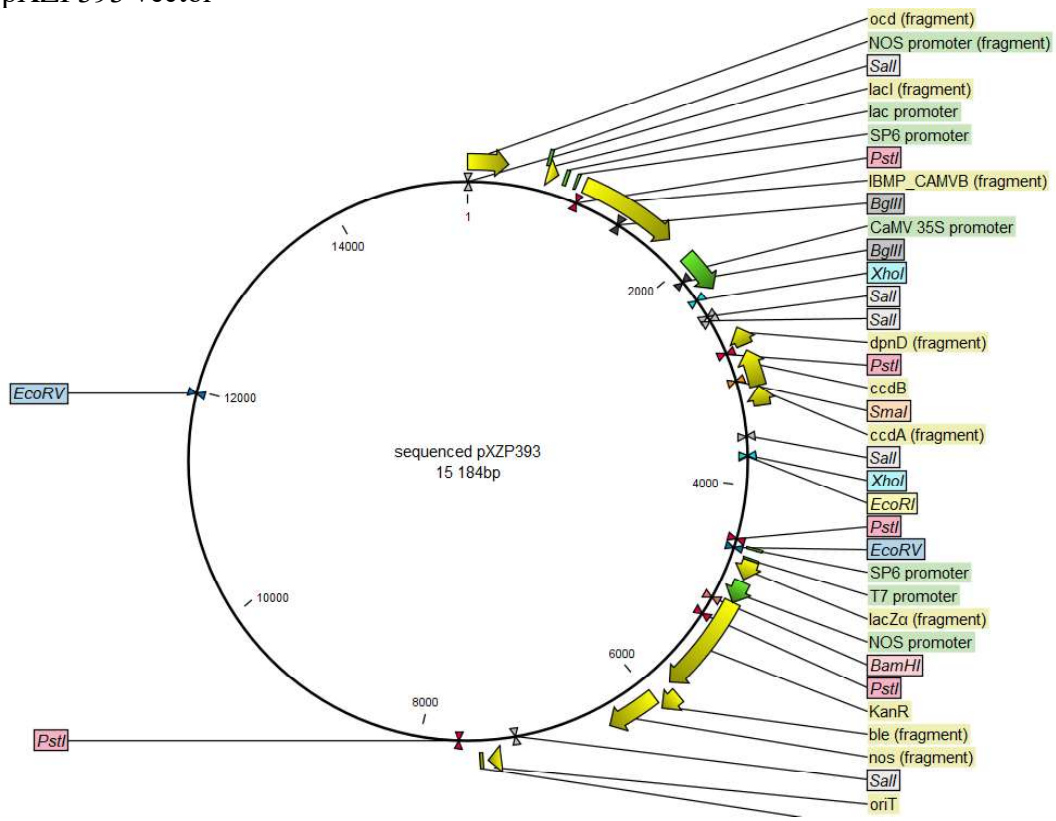
Appendix 2

pDONR221 vector



Appendix 3

pXZP393 vector



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