Metabolic Engineering for Production of Complex Lipids in Tobacco (*Nicotiana benthamiana*) Leaves and Rice (*Oryza sativa*) Endosperm

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Cover: Transmission electron microscopy (TEM) image of chloroplast and leaves of wild type *Nicotiana benthamiana*, simplified schematic carbon allocation in cereal grain, photo of transgenic rice. (TEM by Selcuk Aslan; photo of *N. benthamiana* leaves by Folke Sitbon; a cereal grain by Christer Jansson; photo of transgenic rice plants by Chuanxin Sun).

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Abstract

World chemical and energy supplies for industry are today highly dependent on unsustainable fossil reserves. Vegetable lipids with similar chemical structures to fossil oil could be the ultimate renewable solution to replace fossil oil and provide environmentally friendly feedstocks and energy resources. Wax esters and triacylglycerols (TAG) are two groups of lipid with applications in industry as lubricants, surfactants and biodiesel. However, global production of wax esters and TAG is limited to a few crop species such as jojoba, carnauba, oil palm, soybean and rapeseed. Further, there are restrictions on arable land for oilseed crop growth, and the demand for vegetable lipids is predicted to increase in coming decades. Therefore, there is an urgent need to establish sustainable technologies and develop new alternative oil crop species to meet an expected future vegetable lipid demand.

This thesis examined the potential for production of wax esters and TAG with different qualities in tobacco (*Nicotiana benthamiana*) leaves, and in rice (*Oryza sativa*) endosperm by metabolic engineering. In tobacco leaves, a new metabolic pathway was introduced into chloroplasts by combining bacterial- and plant-derived genes (*AtFAR6*, *AtPES2*, *MaFAR*, *MhWS*, *tpMaFAR::MhWS*, *AtWRI1*) for wax ester biosynthesis. Combinations of the gene functions resulted in production of wax esters with differing composition to a level of 0.9% of leaf dry weight (DW). The newly introduced pathway was further engineered by RNAi inhibition of the *KASII* gene. Additional co-expression of *KASIIRNAi* constructs resulted in increased palmitic/stearic acid ratio, which significantly improved wax ester production in *AtFAR6*-containing combinations. The novel gene fusion *tpMaFAR::MhWS* was further investigated by stable transformation of tobacco plants. Wax ester content in transformants was increased eight-fold compared with wild-type (WT). In rice, overexpression of both full-length and truncated Arabidopsis transcription factor AtWRI1 in endosperm tissues resulted respectively in sevenfold and four-fold accumulation of TAG content compared with WT. Taken together, these results showed that valuable oleochemicals could be increased considerably in plants.

Keywords: Wax ester, triacylglycerol, *Nicotiana benthamiana*, *Oryza sativa,* chloroplast, endosperm.

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Dedication

To my beloved family…

As much as I live I shall not imitate them or hate myself for being different to them…
Orhan Pamuk

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I **Selcuk Aslan**, Chuanxin Sun, Svetlana Leonova, Paresh Dutta, Peter Dörmann, Frédéric Domergue, Sten Stymne, Per Hofvander (2014). Wax esters of different compositions produced via engineering of leaf chloroplast metabolism in *Nicotiana benthamiana*. *Metabolic Engineering* 25, 103-112.

II **Selcuk Aslan**, Per Hofvander, Paresh Dutta, Folke Sitbon, Chuanxin Sun (2014). Transient silencing of the β-ketoacyl-ACP synthase II genes is feasible in *Nicotiana benthamiana* for metabolic engineering of wax ester compositions. *Submitted for publication*.

III **Selcuk Aslan**, Per Hofvander, Paresh Dutta, Chuanxin Sun, Folke Sitbon, (2014). Increased production of wax esters in transgenic tobacco plants by expression of a fatty acid reductase:wax synthase gene fusion. *Submitted for publication*.

IV Yunkai Jin, Jun Su, **Selcuk Aslan,** Lu Jin, Margarita Aili, Chunlin Liu, Paresh Dutta, Per Hofvander, Folke Sitbon, Feng Wang, Chuanxin Sun (2014). Endosperm-specific expression of the *Arabidopsis* gene *WRI1* in transgenic rice plants increases oil contents in the endosperm. *Manuscript*.

The contribution of Selcuk Aslan to the papers included in this thesis was as follows:

I Participated in designing the experiments, carried out the experiments, analysed the data and wrote sections of the paper with co-authors.

II Designed and carried out the experiments, analysed the data and wrote the paper with the guidance of the co-authors.

III Designed and carried out the experiments, analysed the data and wrote the paper with the guidance of the co-authors.

IV Participated in designing the experiments, carried out part of the molecular cloning and biochemical analyses. Joined in writing the paper with the guidance of the co-authors.

Abbreviations

ACCase	acetyl-CoA carboxylase
acyl-ACP	acyl carrier protein
acyl-CoA	acyl-Coenzyme A
AGPase	ADP-glucose pyrophosphorylase
AP2	APETALA2 protein
DAG	diacylglycerol
DGAT	diacylglycerol acyltransferase
DW	dry weight
ER	endoplasmic reticulum
FA	fatty acid
FAE	fatty acid elongase
FALDR	fatty aldehyde reductase
FAR	fatty acid reductase
FAS	fatty acid sythtase
FATA/B	acyl-ACP thioesterase A/B
FW	fresh weight
GC	gas chromatography
GFP	green fluorescent protein
KAS (FAB)	β-ketoacyl-ACP synthase
KCR	β- ketoacyl-CoA reductase
KCS	β-ketoacyl-CoA synthase
MaFAR	marinobacter aquaeolei VT8 fatty acid reductase
MhWS	marinobacter hydrocarbonoclasticus ATCC wax synthase
NADPH	nicotinamide adenine dinucleotide (phosphate)
PES	phytyl ester synthase
PTGS	post-transcriptional gene silencing
p19	growth supressor
q-RTPCR	quantitative real-time PCR
RNAi	RNA interference
SAD	stearoyl-ACP desaturase

TAG	triacylglycerol
TEM	transmission electron microscopy
TLC	thin layer chromatography
tp	transit peptide
VLCFA	very long chain fatty acid
WRI1	WRINKLED1
WS	wax synthase
WS/DGAT	wax synthase/diacylglycerol acyltransferase
WT	wild type
8:0	caprylic acid
10:0	capric acid
12:0	lauric acid
14:0	myristic acid
16:0	palmitic acid
18:0	stearic acid
18:1	oleic acid ($C18:1^{\Delta 9}$)
18:2	linoleic acid ($C18:2^{\Delta 9, 12}$); LA
18:3	alpha-linolenic acid ($C18:3^{\Delta 9, 12, 15}$); ALA
20:0	arachidic acid
20:1	paullinic acid ($C20:1^{\Delta 13}$)
22:0	behenic acid
22:1	erucic acid ($C22:1^{\Delta 13}$)
24:0	lignoceric acid

1 Introduction

Modern society relies directly or indirectly on plants, which are the major constituents of primary food and feed. Plants are also used for non-food materials with applications in creation of renewable and sustainable compounds that are not naturally produced. In addition to plants, humans depend heavily on unsustainable fossil raw materials such as petroleum to meet the energy demand for transportation, generation of heat and electricity, as well as using these as feedstock in the chemical industry for production of solvents, lubricants, plastics and other materials. However, these cheap fossil reserves are finite, unsustainable and not environmentally friendly, *e.g.* combustion of such materials increases the levels of undesired atmospheric carbon dioxide. Therefore, plant-derived oils are being presented as renewable and environmentally friendly alternative energy resources for replacement of fossil oil, due to their similar chemical structure to fossil oil (Vanhercke *et al.,* 2013b; Carlsson *et al*., 2011; Durrett *et al.*, 2008; Dyer *et al.,* 2008; Nikolau *et al*., 2008).

In general, a few crops dominate world plant oil production (Figure 1). These crops are mainly used as food and feed and in industrial applications (Carlsson *et al.,* 2011). However, restrictions on the geographical area available for crop production and different growth requirements place limitations on growing these high-oil content oil crops, which mainly comprise oil palm, soybean, rapeseed and sunflower, to meet global oil demand in the future. Therefore, the possibility of increasing the purity of existing vegetable oil and/or introducing new genes to create alternative oil crops by genetic and metabolic engineering could make great contributions to global oil production.

Vegetable oils are composed principally of energy-rich triacylglycerols (TAGs), which are important for human and animal nutrition and are frequently used in margarines, salad oils and frying oils in the food industry.

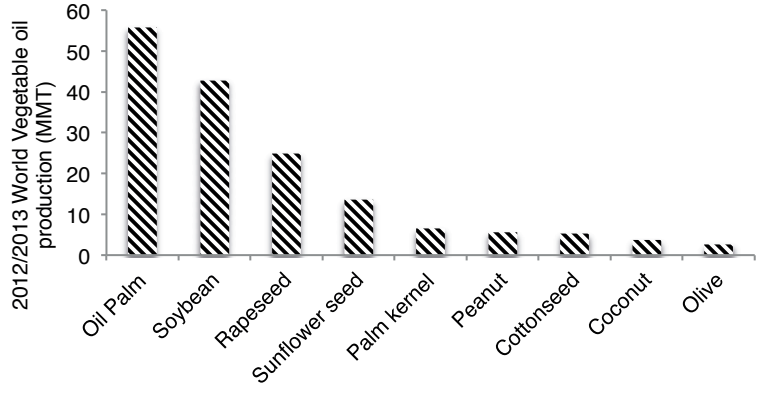

Figure 1. Yearly global oil production from the most common oil crops in the world, 2012-2013 (USDA, 2014).

The energy density of these molecules is crucial not only for biodiesel, which is produced by trans-esterification of their fatty acid components, but also their utilisation in a variety of bio-based industrial formulations such as lubricants and drying oils (Lu *et al.,* 2011). Global vegetable oil production is only equivalent to 3% of the fossil oil amount used globally, and it is thus not realistic to replace total fossil oil consumption with vegetable oils. However, only 10% of fossil oil is used annually in the chemical industry, so alternative ways to increase vegetable oil production could help to meet demand in the chemical industry, replacing fossil oil.

Today's increased knowledge of plant oil biosynthesis, novel gene modification techniques and advances in metabolic engineering have opened the way for reprogramming of carbon allocation in plants or manipulation of biochemical pathways in lipid biosynthesis to increase the quality of existing oil reserves or to generate the new high oil-yielding crops that are needed for replacement of part of fossil oil resources in the near future.

1.1 Plant lipids

Lipids are essential and naturally occurring molecules including fats, fatty acids, waxes, sterols, sterol-containing metabolites, mono/di/triacylglycerols, phospholipids and fat-soluble vitamins (Subramaniam *et al.,* 2011; Fahy *et al.,* 2009). The main role of lipids in organisms is energy storage in the form of fat or oil and structural molecules of biological membranes. In general, TAG is the

main storage lipid group, while sterols and phospholipids are the most crucial examples of lipids participating in the structure of biological membranes. Furthermore, although produced in small quantities, other lipid classes play crucial roles such as electron carriers, cofactors, hormones and intracellular messengers (Nelson *et al.,* 2008). Although thousands of different lipids have been categorised, the focus of this thesis is mainly on wax esters and, to some extent, TAGs.

1.1.1 Production and utilisation in food and non-food applications

Plant lipids derived from oilseed crops constitute one of the most important renewable resources in nature. Globally, the majority of vegetable oils are produced by food oil crops such as oil palm, soybean, rapeseed (canola), sunflower, cottonseed, peanut, maize and olive, together with other minor food oil sources such as safflower, sesame, coconut and linseed. Together, these account for approximately 79% of total production (Vanhercke *et al.,* 2013b; Dyer *et al.,* 2008) (Figure 1). The majority of the oil used in the food industry is composed of five nutritionally important fatty acids (FAs), namely palmitic (C16:0), stearic (C18:0), oleic (C18:1$^{\Delta 9}$), linoleic (C18:2 $^{\Delta 9, 12}$) and α-linolenic acid (C18:3 $^{\Delta 9, 12, 15}$) (Figure 2). These fatty acids differ from each other in terms of acyl chain length and in the number and positions of double bonds or the existence of additional functional groups, resulting in different physical properties (Durrett *et al.,* 2008). Therefore, the properties of oils derived from a mixture of different FAs are affected by the characteristics of these constituent FAs.

Even though plant oil is consumed on a large scale as food and feed, a substantial amount is also used for industrial (non-food) applications (Vanhercke *et al.,* 2013b). Historically, plant oils have been used for generating heat and light. In contrast to other bioenergy resources such as ethanol, vegetable oils are much more favourable in terms of the energy content (Hill *et al.,* 2006). For example, conversion of sugars into ethanol results in 90% of the heat content and lower energy efficiency compared with conversion of plant oils into biodiesel (Dyer *et al.,* 2008).

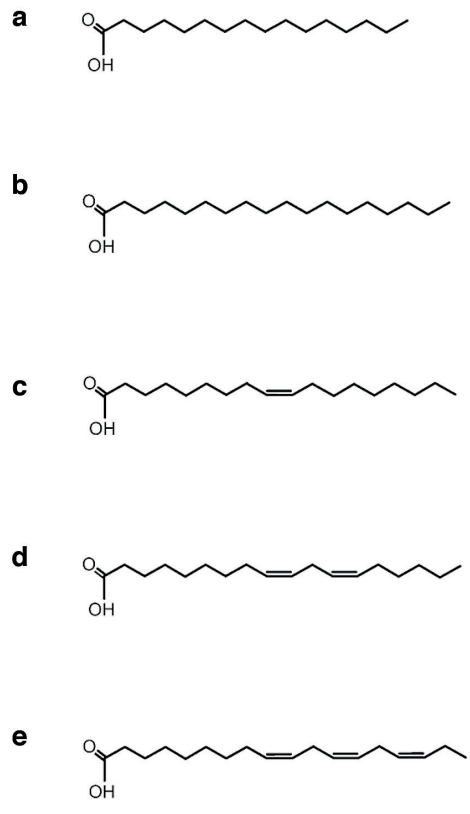

Figure 2. Examples of chemical structure of the fatty acids found in edible oils. (a) palmitic (16:0), (b) stearic (18:0), (c) oleic (18:1), (d) linoleic (18:2), (e) linolenic (18:3) acids. (Reproduced with permission from Vanhercke, 2013).

Plant oils are essential renewable resources for both the chemical and fuel sectors, but their applications in those industries are limited by the high price and lack of availability of similar structures as found in petrochemicals. Oil-producing plants that are important for non-food applications usually produce functionally or structurally different fatty acids than common fatty acids. Utilisation of plant oils in industrial applications is widespread; for instance medium chain fatty acid laurate (C12:0) derived from palm kernel oil and coconut oil has excellent surfactant properties, and is therefore utilised for the production of soaps, detergents and other personal care products (Vanhercke *et al.,* 2013b; Dyer *et al.,* 2008). On the other hand, highly unsaturated oils such as soybean and linseed are potential renewable resources that are used in the production of synthetic drying agents, inks and industrial resins and glues. In

addition to these examples of oils with broad usage, castor oil, tung oil and high-erucic rapeseed oil are specially produced for some specific non-food applications (Vanhercke *et al.,* 2013b).

Plant oils are composed almost entirely of TAGs containing three of the major five fatty acids listed above (Figure 2). However, with some exceptions plants can produce different lipid mixtures such as waxes, which composed of a mixture of different lipid compounds. Wax esters as a predominant component of waxes and primary fatty alcohols are found ubiquitously in many organisms, yet they are produced at low quantities except in a few species. Fatty alcohols with various chemical structures are commonly found either as free forms or in the structure of wax esters or/and phospholipids (Figure 3a). They serve a number of uses such as in cosmetics, agrochemical and pharmaceutical formulations and food products (Domergue *et al.,* 2010). For example, C8-C10 chain length fatty alcohols are used in the plastics industry, C12-C18 are mainly used in the detergent and surfactant industry, C12-C14 are used as components in lubricants and C16-C18 fatty alcohols are used in the cosmetics industry. Moreover, plant-derived waxes with unique physical properties are excellent feedstocks for many industrial applications, especially high temperature and pressure lubricants and hydraulic fluids (Dyer *et al.,* 2008). They are also extensively used in cosmetics and daily care products. However, the high cost and poor agronomic performance of the plants that produce these unusual oils have restricted their usage on a commercial scale (Cuperus *et al.,* 1996).

1.1.2 Triacylglycerol (TAG) occurrences and their biological roles

Triacylglycerol (TAG) is the most common form of lipid classes and is also known as triglyceride, fat or natural fat. It is composed of three fatty acids esterified to a glycerol backbone at the *sn*-1, *sn*-2 and *sn*-3 positions (Figure 3c). The fatty acids in a TAG molecule can differ, but are naturally a mixture of two or three different fatty acids. Most natural fats are composed of a mixture of simple and mixed triacylglycerols.

In most organisms, TAGs function as an energy source and protective molecule. TAG constitutes the major storage lipid in the fruits, nuts, and seeds that serve as energy reserves for seed germination and further seedling development. Beyond high levels in storage tissues, TAG is also present in other tissues of plants such as pollen and vegetative tissues, but at lower levels (Nelson *et al.,* 2008).

TAG is an ultimate source of edible oils for human consumption and a target for replacing petroleum reserves (Lung & Weselake, 2006). It represents a very crucial component in the bio-economy, as it supplies highly reduced

carbon for both food and non-food applications, for instance supplying feedstock materials for the production of petrochemical alternatives.

The physical characteristics of TAG differ between plant species depending on length of fatty acids and degree of saturation. Maize oil and olive oil are two examples that are composed largely of unsaturated fatty acids, which makes them as liquids at the room temperature (Nelson *et al.,* 2008).

1.1.3 Wax esters and their derivatives among organisms

Biological functions and industrial importance

Wax esters are a subclass of naturally occurring lipids with excellent hydrolytic resistance (Figure 3b), in contrast to other groups of lipids. They are esters of aliphatic long-chain fatty alcohols (C16-C30) and long-chain fatty acids (C14-C36) (Iven *et al.,* 2013; Wahlen *et al.,* 2009; Jetter & Kunst, 2008). Naturally occurring wax esters are found in a broad group of organisms with numerous biological functions, and are also highly utilised in commercial and industrial applications (Biermann *et al.,* 2011; Carlsson *et al.,* 2011; Jetter & Kunst, 2008). Wax esters are the predominant components of waxes. In nature, waxes can be categorised as: commercial waxes (beeswax, jojoba, carnauba, wool wax), plant surface waxes and other waxes (such as microbial, marine, bird and insect).

In a broad group of bacteria such as *Acinetobacter*, *Marinobacter*, *Pseudomonas*, *Micrococcus*, *Maroxella*, *Alcanivorax* and *Fundibacter*, wax esters or their derivatives in the form of fatty alcohols serve a number of biological functions (Waltermann *et al.,* 2007; Waltermann & Steinbuchel, 2005; Ishige *et al.,* 2003). Many *Acinetobacter* species accumulate large amounts of wax esters under nitrogen-limited conditions for use as carbon reserves. The same phenomenon has been observed in *Acinetobacter* sp. strain M-1 (Ishige *et al.,* 2003) and in *Rhodococcus opacus* PD630 (Alvarez *et al.,* 2000). *Acinetobacter calcoaceticus* ADP1 is another organism producing intracellular wax esters in the form of insoluble inclusions (Kalscheuer & Steinbuchel, 2003), with a similar chemical structure to wax esters found in sperm whale and jojoba wax (Ervin *et al.,* 1984).

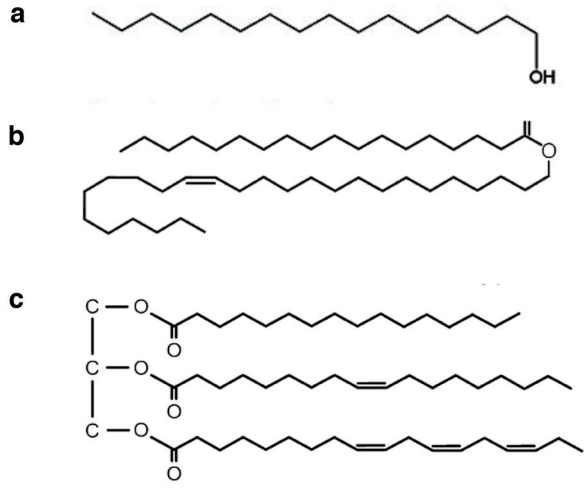

Figure 3. Chemical structure of (a) fatty alcohol, (b) wax ester, and (c) triacylglycerol. (Reproduced with permission from Hofvander, 2011 and Vanhercke, 2013b).

Bacteria with the capacity for accumulating wax esters have been isolated from various geographical locations and from different substrates. This varied evolution has built up large variations in the chemical composition of the wax esters produced (Waltermann *et al.,* 2007). The levels of accumulated wax esters in these species can reach up to 25% of the cellular dry weight (Waltermann & Steinbuchel, 2005), regardless of the carbon source used for wax ester production. The carbon source in *Acinetobacter* species can vary, but the most common compounds are acetate, sugars and sugar acids. The chemical composition of wax esters in bacteria is mostly affected by the chain length of fatty alcohol, rather than fatty acid composition (Kaneshiro *et al.,* 1996). It has been shown that in *Acinetobacter calcoaceticus*, the composition of the wax esters produced by expression of *WS/DGAT* (wax ester synthase/diacylglycerol acyltransferase) is more sensitive to changes in fatty alcohols rather than fatty acid content (Kalscheuer & Steinbuchel, 2003).

In addition to acting as energy reservoirs, wax esters can also function as regulators of buoyancy. In this regard, the location of the wax esters is crucial. In *Acinetobacter* strain MJT/F5/199A it has been shown that wax esters occur in the outer membrane fractions (Thorne *et al.,* 1973), whereas in *Micrococcus cryophilus* they occur within the membrane (Russell & Volkman, 1980). In the latter species, wax esters function as regulators of membrane fluidity.

In terrestrial plants, outer surfaces are often protected with a wax layer that is vital for plant survival in different physical and chemical conditions. The wax layer functions as the first waterproofing component of the cuticle, protecting against UV radiation, pathogen entry and insect attack, and wax can also act as a key controller in plant-insect interactions (Kunst & Samuels, 2003) and in communications between the plant and its environment (Riederer *et al.,* 2006). Because of the protective properties of waxes against non-stomatal water loss, cuticular waxes have played an essential role in adaption of land plants during their evolution (Raven *et al.,* 2004). Wax and the cutin layer is one of the key factors in cell-cell interactions, and play important roles in the plant development and reproductive phases (Borisjuk *et al.,* 2014; Jessen *et al.,* 2011; Wilson *et al.,* 2011; Li-Beisson *et al.,* 2009). Wax-related properties such as wax load and composition are also crucial for plant defence systems against environmental stresses, pathogens or insects (PostBeittenmiller, 1996; Eigenbrode & Espelie, 1995). Thus taken together, understanding the control mechanism of wax accumulation in plants is of interest from many aspects (Hooker *et al.,* 2002).

Plant cuticular waxes are subdivided into intra- and epi-cuticular waxes. They are composed of complex aliphatic molecules of mainly C16 to C34 atoms in length, which occur as free fatty acids, aldehydes, primary and secondary alcohols, alkanes and acid. Wax esters and/or fatty alcohols constitute the majority of the waxes (Samuels *et al.,* 2008; Sturaro *et al.,* 2005). Plant-derived waxes in liquid or solid form have diverse chemical composition. For instance, liquid waxes from jojoba seeds are composed predominantly of wax esters, while solid waxes from carnauba palm are a complex mixture of hydrocarbons (Taylor *et al.,* 2011).

The ratios of major components of waxes can vary between different plant species, tissues/organs and developmental stages (PostBeittenmiller, 1996). For example, the total wax loads on *Arabidopsis* stems are 10-fold greater than that found on leaves (Suh *et al.,* 2005). Even though leaf wax content is generally somewhat low, *e.g.* in Arabidopsis leaves it is 0.1-0.2% (Jenks *et al.,* 1995), several studies have demonstrated that epicuticular wax deposition can be improved by drought treatment in different plant species, including *Arabidopsis thaliana*, cotton, rose, peanut and tree tobacco (Borisjuk *et al.,* 2014). Besides the content of total wax load in different parts of an organism, the composition can also differ. For example, the wax composition of young maize seedlings contains approximately 63% free primary alcohols of C32, whereas that of mature leaves contains 42% wax esters (Bianchi *et al.,* 1985). Short and medium fatty alcohols of C4 to C12 are found in petunia petals, but not in leaf extracts of the same plant (King *et al.,* 2007), although the fatty

alcohol composition of plant cuticular waxes is commonly composed of long chain carbon compounds. In numerous plant species the free and esterified alcohols have a similar chain length pattern, suggesting that the fatty alcohol precursors of these alcohols originated from a common pool (Lai *et al.,* 2007; Rowland *et al.,* 2006; Allebone & Hamilton, 1972; Miwa, 1971).

Carnauba palm (*Copernicia prunifera*) is an unique species which accumulates 85% of leaf dry weight as cuticular waxes on the aerial surfaces of its leaves (Kolattukudy *et al.,* 1976). In general, commercially important waxes are composed of 10-16% free aliphatic fatty alcohols and 80-85% wax esters containing C32-C34 fatty alcohols and C16-C18 fatty acids. Although the main oil storage compound in seeds takes the form of TAG in most major plant species, in jojoba (*Simmondsia chinensis*) approximately 50-60% of the seed by weight consists of wax esters composed of esterified monounsaturated C20-C24 alcohols and unsaturated C18-C24 fatty acids (Metz *et al.,* 2000; Miwa, 1971). Carnauba palm and jojoba are just two examples of species with considerable levels of waxes, which have various uses in the pharmaceutical and cosmetic industries.

Wax esters are not only found in various plant species in the form of cuticle wax, but are also commonly occurring in different organisms, such as ubiquitously in the exoskeleton of insects (Moto *et al.,* 2003), as construction material in honeycombs produced by bees (Aichholz & Lorbeer, 2000) and as waxes in preen gland membranes of avian species (Biester *et al.,* 2012) and of mammals (Cheng & Russell, 2004). Wax esters are found in considerable concentrations in the spermaceti organs of the sperm whale, where they have a regulatory mechanism in buoyancy. These wax esters have excellent chemical and physical properties, making them highly suitable for medical and cosmetic applications and especially as lubricants and food additives (Wahlen *et al.,* 2009; Lardizabal *et al.,* 2000). After the worldwide ban on hunting of sperm whales, plant-derived waxes were proposed as an alternative in industrial applications. However, the high cost and low yield of jojoba seed oil cannot meet the demand for the high-value waxes formerly obtained from sperm whales.

1.1.4 Other lipid groups

There are many classes of lipids in nature with diverse biological functions. Storage (80% of dry cell mass) and structural lipids (5-10% of dry cell mass) play a passive role in the cell, whereas other groups of lipids play an active part in anabolic/catabolic reactions as metabolites and messengers. Despite their diverse roles and physical properties, lipid groups can be categorised into eight classes based on their chemical structures as: fatty acids, glycerolipids,

glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids and polyketides (Nelson *et al.,* 2008).

1.2 Plant lipid biosynthesis

1.2.1 Carbon allocation in cereals

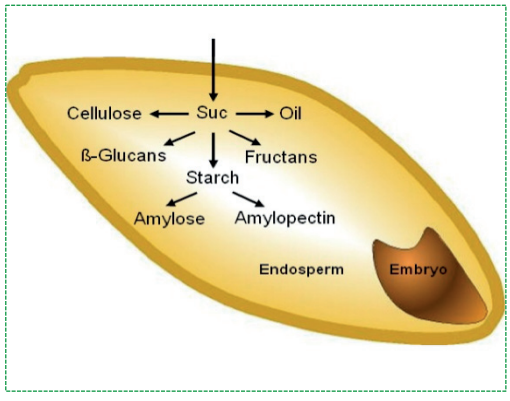

Figure 4. In a cereal seed, sucrose is uploaded and serves as a carbon source for different metabolic pathways (produced by Christer Jansson).

Carbon is a dominant element for many complex pathways that is deposited in cereal seeds in different forms depending on the needs of the organisms (Figure 4). For this reason, understanding carbon allocation in seeds as a shared donor for different pathways is important before proceeding to an analysis of complex lipid biosynthesis.

Cereal crops are one of the most important plants for agriculture and human consumption. Of the different tissues of plants, grains are the most valuable part due to their widespread uses in the food and non-food industries, which make them the most economically important structures for biotechnological applications. Seeds are the small embryonic plants where all the genetic materials and nutrients are kept for reproduction of the species (Ekman *et al.,* 2008).

The growth and further development of plants is controlled by the balance of assimilated carbon and stored materials in different organs (Rolland *et al.,* 2006). During daytime, excess carbons from photosynthesis are stored as transitory starch in chloroplasts, and are then utilised to sustain metabolic functions during the night (Zeeman *et al.,* 2010). During photosynthesis, the carbon fixed by the chloroplasts in leaves is either transported to other organs for processing or stored in the form of starch, protein or oil in the seeds (Figure 4). Among plant species, the levels of storage compounds vary based on the carbon allocation regulators in seeds and physiological conditions (Baud & Lepiniec, 2010; Ekman *et al.,* 2008). For example, starch is used at night for respiration by plant organs (Vigeolas *et al.,* 2004).

The form of transported carbon in plants is sucrose (Porter, 1962), which is the main precursor of carbon storage compounds in developing seeds. Carbon allocation to different parts of the plant is a complex metabolic pathway that involves many redundant sets of enzymes. This flux can occur at many different levels such as at the organ level (in seeds), whole plant level (carbon translocation) and tissue level (between different tissues in seeds). There are a number of possible routes for carbon allocation and, in addition, oil biosynthesis in plants can differ among different plant species. The carbon directed to oil biosynthesis in plant cells is shared with other glycolytic pathways, and therefore the amounts of carbon entering oil biosynthesis depend on the competition from these other metabolic pathways. Competing regulation is observed especially between starch and oil biosynthesis in the seeds of oil crops. Several studies have reported increasing oil levels in seeds by redirecting carbon allocation or/and up-regulation of lipid genes or transcription factors (Santos-Mendoza *et al.,* 2008; Vigeolas *et al.,* 2007; Focks & Benning, 1998).

Cereal seed is composed of a large endosperm (70% v/v) surrounded by an aleurone layer, which is approximately one or two cell layers thick. The remaining parts (<30% v/v) of a cereal seed are the embryo and scutellum. Carbon is usually stored in the form of starch and protein in the endosperm, while oil is stored in the embryo and scutellum, thereby making cerelas poor in oil content (Baud & Lepiniec, 2010; Ekman *et al.,* 2008). Attempts have been made to increase the oil content of the seeds, with studies examining *e.g*. the possibility to alter carbon metabolism in the seeds of gymnosperms in order to manufacture desired compounds, such as oil in seeds (Ekman *et al.,* 2008; Oo *et al.,* 1985; Marriott & Northcote, 1975).

1.2.2 Fatty acid biosynthesis

Fatty acids (FAs) are long carbon chains, which constitute the majority of a lipid molecule, providing the hydrophobic characteristics and contribute to extremely diverse lipid structures (*cf*. Figure 2). In plants, unlike in other organisms, fatty acid synthesis occurs in the plastids. The majority of newly synthesised acyl chains are exported to the endoplasmic reticulum (ER) or other sites for further condensation (Browse & Somerville, 1991; Roughan & Slack, 1982).

The *de novo* fatty acid synthesis system consists of two major enzyme systems; acetyl-CoA carboxylase (ACCase) and fatty acid synthase (FAS) (Ohlrogge & Jaworski, 1997) (Figure 5). In plant cells, the first committed step in lipid synthesis begins with irreversible carboxylation of acetyl-CoA to form malonyl-CoA by ACCase activity in the chloroplast or plastid (Harwood,

1988). Because the plastid compartments are not capable of importing acetyl-CoA (Roughan & Slack, 1982; Weaire & Kekwick, 1975), the precursors required for its synthesis must be produced in the plastid or taken up from the cytosol, which is a process that requires specific transporter proteins embedded on the plastid envelope (Martin & Ludewig, 2007; Rolland *et al.,* 2006). More than one carbon source has been proposed for fatty acid synthesis in plastids, *e.g*. Glc-6-P, pyruvate, malate, phosphoenolpyruvate (PEP) and free acetate, which may result in elevated amounts of acetyl-CoA (Rawsthorne, 2002).

There are two types of FAS enzymes in nature, FASI and FASII. FASI is functional in vertebrates and fungi, while FASII is the functional enzyme participating in fatty acid synthesis in plants and bacteria (Nelson *et al.,* 2008). FASI consists of a multifunctional polypeptide, which releases a single product (palmitate) without any release of other intermediates (Nelson *et al.,* 2008). In contrast, in plant cells there is a complex fatty acid synthase (FASII) composed of multi-enzymes, where intermediates including saturated, unsaturated and hydroxy fatty acids are generated. FASII reactions start with utilisation of malonyl-CoA as the central carbon donor for fatty acid synthesis (Ohlrogge & Jaworski, 1997). However, the malonyl group is transferred from CoA to acyl carrier protein (ACP) before entering the pathway by catalysation of malonyl-CoA:ACP transacylase of the FASII enzyme complex. There are at least three condensing enzymes, known as β-ketoacyl-ACP synthases (KASI, II, III), responsible for addition of carbons to the growing acyl chain until a C18:0 fatty acid is obtained. Furthermore, fatty acid synthesis reactions are initiated by the function of KASIII enzyme, resulting in condensation of C2:0-ACP to C4:0-ACP, then KASI catalyses the condensation of C4:0-ACP up to C16:0-ACP, and finally KASII catalyses the extension of C16:0-ACP to form C18:0-ACP (Shimakata & Stumpf, 1982). Before a subsequent cycle of FAS activity, the β-ketoacyl-ACP intermediate is first reduced by β-ketoacyl-ACP reductase, then dehydrated by β-hydroxyacyl-ACP dehydrase, and finally there is an enoyl reduction step that is carried out by enoyl-ACP reductase. Stearoyl-ACP desaturase (SAD), the last modification enzyme of the FASII enzyme complex, adds a double bond at the 9 position of the C18:0-ACP to form C18:1-ACP. The fatty acid synthesis pathway is terminated by the hydrolysis or release of the acyl chain from acyl carrier protein (ACP). There are two types of acyl-ACP thioesterase, known as FATA and FATB, which catalyse the hydrolysis step. Whilst FATA is specific for C18:1-ACP, FATB is particularly used for hydrolysis of saturated acyl-ACPs such as C16:0-ACP and C18:0-ACP. However, other types of thioesterases with specificity to C10 and C12 fatty acyl-ACPs can be found in coconut (*Cocus nucifera*) and Cuphea ssp. (Ohlrogge & Jaworski, 1997). Fatty acids that have been released by

thioesterases are exported from the plastid to enter the eukaryotic pathway for further condensation (Roughan & Slack, 1982). In contrast to thioesterases, acyl transferases trans-esterify the acyl moieties from ACP to glycerolipids or export them to the cytosol for further esterification (Ohlrogge & Jaworski, 1997).

The regulation of this system is balanced with the function of acetyl-CoA carboxylase (ACCase), which is believed to be the rate-limiting enzyme. The majority of the fatty acids synthesised though the fatty acid synthesis pathway meet one of two possible fates; they are either incorporated into the triacylglycerol assembly or join the phospholipid structure of the cell.

1.2.3 Triacylglycerol (TAG) synthesis and putative enzymes

Membrane-bound enzymes can regulate biosynthesis of TAGs that function in the endoplasmic reticulum (ER), in which three primary acyl chains produced via *de novo* fatty acid synthesis are incorporated to a glycerol backbone (Zhang *et al.,* 2009) (Figure 5). This reaction series is known as the Kennedy pathway. The glycerol backbone required for TAG assembly is provided by *sn*-glycerol-3-phosphate (G3P), which is produced by the action of *sn*-glycerol-3-phosphate dehydrogenase, using dihydroxyacetone as a substrate. The reaction series starts with the acyl-CoA-dependent acylation of *sn*-glycerol-3-phosphate (G3P) to obtain lysophosphatidic acid (LPA) by the action of *sn*-glycerol-3-phosphate acyltransferase (GPAT). The second acyl-CoA-dependent step is catalysed by lysophosphatidic acid acyltransferase (LPAAT) activity to generate phosphatidic acid (PA), which is then phosphorylated to form *sn*-1,2-diacylglycerol (DAG) by phosphatidic acid phosphatase (PAP). In the third acyl chain step DAG is then esterified by diacylglycerol acyltransferase (DGAT), which results in the formation of triacylglycerol (TAG). In addition to the Kennedy pathway, TAG can be synthesised via acyl-independent pathways. The synthesis of TAG and further TAG assembly through different routes has been studied in detail within various plant species (Vanhercke *et al.,* 2013b; Taylor *et al.,* 2011). Finally, the TAG produced in ER leads to formation of lipid bodies (0.2-2 microns in diameter) that leave ER for the cytosol. Exported TAGs are usually covered lipid body proteins stored in seeds to form oil bodies (oleosomes) for supplying energy for the further germination of seeds.

Several attempts have been made to understand the metabolic flux network of TAG biosynthesis by both genetic and metabolic engineering of genes/transcription factors participating in TAG assembly to improve energy density. These have included for instance up-regulation of fatty acid

biosynthesis or increasing TAG assembly, and optimising TAG storage or preventing TAG breakdown.

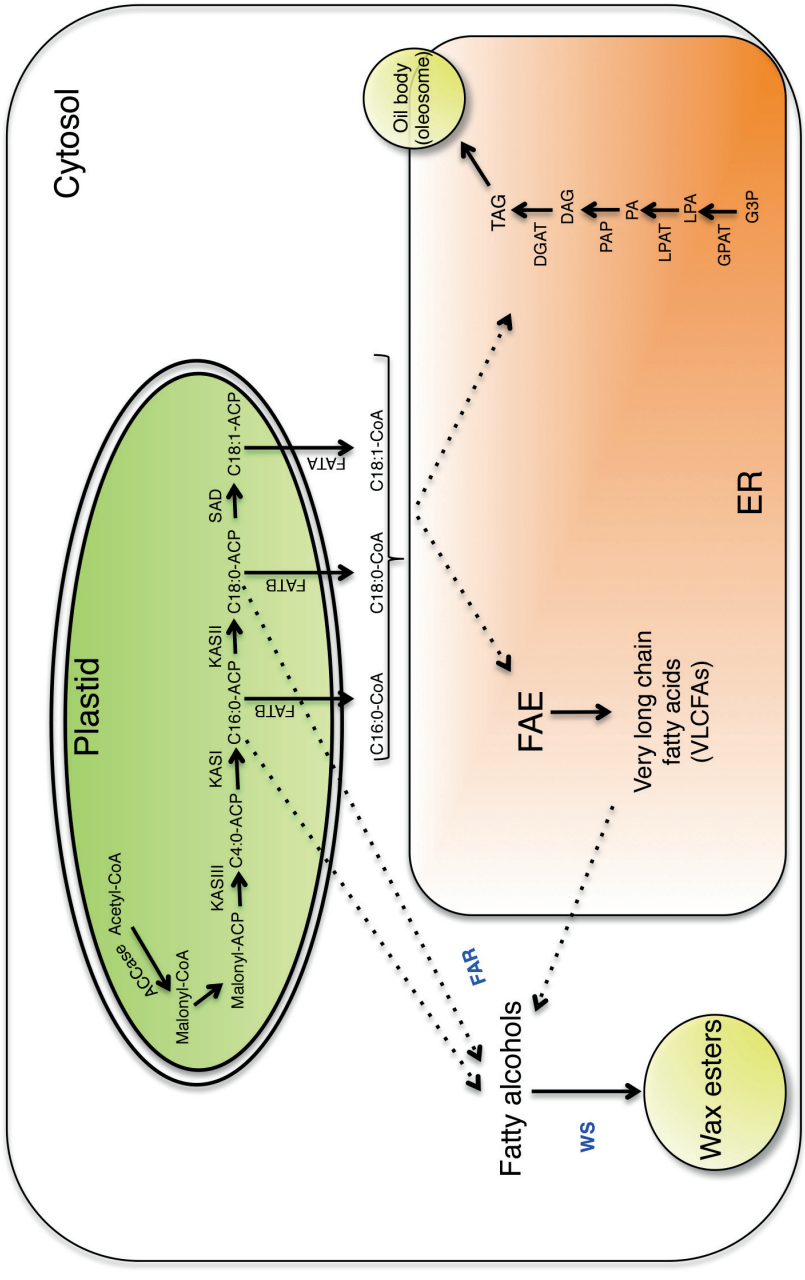

Figure 5. Schematic overview of *de novo* fatty acid synthesis and triacylglycerol (TAG), and wax ester (WE) assembly in a plant cell.

Some insights into the regulatory network have been obtained in studies in different plants and tissues, *e.g.* overexpression of *DGAT1* in leaves (Andrianov *et al.,* 2010; Bouvier-Nave *et al.,* 2000) and of acetyl-CoA carboxylase (ACCase) in tobacco chloroplast and potato tubers (Klaus *et al.,* 2004; Madoka *et al.,* 2002), inhibition of lipid degradation by targeting TAG lipases and β-oxidase (James *et al.,* 2010; Slocombe *et al.,* 2009), mutation of the *TGD1* chloroplast lipid transporter (Xu *et al.,* 2005), and heterologous expression of transcription factors (LEC1, LEC2, WRI1) responsible for seed maturation and development (Liu *et al.,* 2010; Santos Mendoza *et al.,* 2005). In addition, detailed studies on other specific gene/transcription factor network have been reported (Lu *et al.,* 2011; Weselake *et al.,* 2009; Santos-Mendoza *et al.,* 2008).

Transcriptional regulation of the fatty acid synthesis required for TAG biosynthesis is most likely controlled directly by the *WRINKLED1* gene encodes for transcription factor, which has two APETALA2 domains (Baud & Lepiniec, 2010; Ohto *et al.,* 2005; Cernac & Benning, 2004). Focks and Benning (1998) first showed that an Arabidopsis mutant (*wri1*) has a 80% lower oil content, causing a wrinkled appearance of the seed coat, and thus they defined the transcription factor as WRINKLED1 (WRI1). It is considered as a 'master' regulator of the metabolic flux between the sugar import and lipid biosynthesis pathways (Chapman & Ohlrogge, 2012). It most likely regulates lipid biosynthesis by binding to the sequences of promoters of genes encoding enzymes that are involved in glycolysis and FA biosynthesis, thereby activating and enhancing their transcription levels. It was shown that co-expression of *ZmLEC1* and *ZmWRI1* can increase oil content by 48% but with a negative effect on seed germination and leaf growth in maize, while overexpression of *ZmWRI1* does not affect the oil content in endosperm tissues (Shen *et al.,* 2010). In contrast to maize, when Brassica *WRI1* is expressed in Arabidopsis, it results in increased seed weight (about 40%), due to larger embryo size caused by enlarged cells (Shen *et al.,* 2010). Several efforts have been also made to increase the energy density of vegetative tissues by up-regulation of *WRI1*. Such studies, including combined down-regulation of *AGPase* and up-regulation of *WRI1*, did not succeed in increasing oil content by more than 2.1% on a leaf dry weight (DW) basis (Sanjaya *et al.,* 2011). Recently, Vanhercke *et al.* (2014) reported the exciting finding that over-expression of *WRI1*, *DGAT* and *OLEOSIN* genes dramatically increases the TAG content in *Nicotiana tabacum* leaf tissues, by an amount corresponding to 15% of leaf DW, without any detrimental effect on plant development. These

findings open up the possibility of using *WRI1* for increasing seed energy density and also for increasing the energy levels of vegetative biomass.

1.2.4 Biosynthesis of waxes: Enzymes participating in the pathway

Fatty Acid Elongation (FAE) system

In plants, constituents of waxes are exclusively synthesised in the epidermal tissues. Biochemical and genetic insights into wax biosynthesis in plants are generally based on the analysis of plant mutants. Epicuticular wax synthesis can be categorised in three different stages (Figure 5). The acyl-ACPs moieties, produced via *de novo* fatty acid pathway, are used as central intermediates for all type classes of lipids, and thus the first committed step of wax biosynthesis and other lipid biosynthetic pathways share a common pool (Samuels *et al.,* 2008). In the second stage, fatty acid moieties are exported to the cytosol for further elongation in the ER membrane by the membrane-bound fatty acid elongase enzyme (FAE) complex, consisting of β-ketoacyl-CoA synthase (KCS), β- ketoacyl-CoA reductase (KCR), β- hydroxyacyl-CoA dehydratase and enoyl-CoA reductase (ECR), to produce very long chain fatty acids (VLCFAs) with chain length C20-C36 (Borisjuk *et al.,* 2014; Samuels *et al.,* 2008; PostBeittenmiller, 1996). Fatty acid elongation is analogous to fatty acid synthesis. VLCFA synthesis consists of four consecutive reactions, condensation, reduction, dehydration and a second reduction, for each two-carbon elongation. The only difference between the FAS and FAE systems is that the carbon donor for the FAE system is malonyl-CoA rather than malonyl-ACP. Finally, the VLCFAs synthesised are used as substrates for major wax products, *e.g*. alcohols, esters, aldehydes, alkanes and ketones (Samuels *et al.,* 2008).

The VLCFAs produced can either join the alkane-forming pathway or the alcohol-forming pathway, resulting in a range of aliphatic cuticular wax compounds (Rowland & Domergue, 2012; Li *et al.,* 2008; Rowland *et al.,* 2006).

Although the plant cuticular wax synthesis metabolic pathway has been studied intensively, the biochemical reactions of the enzymes involved have not been as well studied. Forward genetic approaches in *Arabidopsis eceriferum* (*cer*), a wax-deficient mutant, and maize (*Zea mays*) *glossy* mutants have assisted in identification of numerous enzymes participating in wax biosynthesis via FAE reactions, including CER6, CER10, GL8A and GL8B (Rowland *et al.,* 2006; Dietrich *et al.,* 2005; Zheng *et al.,* 2005; Hooker *et al.,* 2002; Fiebig *et al.,* 2000; Millar *et al.,* 1999; Xu *et al.,* 1997).

Moreover, plant cuticular wax biosynthesis has been studied under environmental stress conditions, and investigations have been carried out on expression patterns of transcription factors and genes involved in the pathway (Go *et al.,* 2014; Pu *et al.,* 2013; Broun *et al.,* 2004). Since the work presented in this thesis focused on wax ester biosynthesis through the alcohol-forming pathway (Figure 5) and their subsequent esterification, the FAE system and alkane-forming pathway are not described further here, but detailed descriptions can be found elsewhere (Borisjuk *et al.,* 2014; Samuels *et al.,* 2008; Kunst & Samuels, 2003; Hooker *et al.,* 2002; PostBeittenmiller, 1996).

Wax ester biosynthesis: Alcohol-forming pathway

Wax ester biosynthesis, in contrast to that of other lipid classes, is rather simple and well understood (Vanhercke *et al.,* 2013b; Lardizabal *et al.,* 2000). Wax esters are esters of a long-chain fatty alcohol and long-chain fatty acids, which are synthesised by two enzymatic reactions that start with reduction of an activated fatty acid, derived from either acyl-CoA or acyl-ACP, to a fatty alcohol by the function of a fatty acid reductase (FAR), and subsequent esterification of this fatty alcohol to a fatty acid by the function of a wax synthase (WS). Identification of each enzyme group participating in wax biosynthesis has been described in a variety of organisms.

In general, the first committed step in wax ester biosynthesis is the acyl reduction pathway, where an activated acyl group is reduced to a primary alcohol with different chain length (C8-C36) depending on the organism and reaction conditions. Fatty alcohols are commonly found in plants in different forms such as: free fatty alcohols, linked either with an ester bond to a fatty acid to form a wax ester molecule, or with an aromatic compound to form alkyl hydroxycinnamate. These compounds are usually found in plant extracellular lipid barriers such as cuticle, suberin and sporopollenin (Rowland & Domergue, 2012). Reduction reactions can occur either by direct reduction of C16 and/or C18 from *de novo* fatty acid synthesis or reduction of VLCFA. The reduction of VLCFA precursors to primary alcohols was first proposed many years ago (Chibnall *et al.,* 1934), and has been supported in several studies but with two unanswered questions; whether the reduction of fatty acids to corresponding alcohols occurs via an intermediate aldehyde, and whether the reaction is catalysed by two enzymes or whether one fatty acyl-CoA reductase (FAR) can mediate both reaction steps (Kolattukudy, 1996). The reduction pathway of fatty acyl-CoA/ACP is governed differently in prokaryotes and eukaryotes. The biosynthesis of fatty alcohols in bacteria is conducted via two-step reductions by the function of two separate enzymes, with release of an intermediate fatty aldehyde. Firstly, the fatty acyl-CoA/ACP is reduced to an

intermediate fatty aldehyde by fatty acid reductase (FAR), and further reduction of fatty aldehyde to a fatty alcohol catalysed by fatty aldehyde reductase (FALDR) (Wahlen *et al.,* 2009; Reiser & Somerville, 1997; Fixter *et al.,* 1986). However, a recent study characterised Maqu_2220 of *Marinobacter aquaeolei* VT8 protein that is identical to FALDR enzyme and can conduct reductions not only of fatty aldehydes, but also of acyl-CoA and acyl-ACP to fatty alcohols of broad substrate specificity in an NADPH-dependent reaction series in prokaryotes (Hofvander *et al.,* 2011). In contrast to the prokaryotic pathway, fatty acyl reduction in plants is governed by the function of a single enzyme, without releasing intermediate aldehydes (Chen *et al.,* 2011; Shi *et al.,* 2011; Lardizabal *et al.,* 2000). The first studies on plants demonstrated that the green alga *Euglana gracilis* and jojoba FAR produce alcohols with no intermediate aldehydes by the action of one single enzyme using acyl-CoA as substrate (Pollard *et al.,* 1979; Kolattuk.Pe, 1970). This indicates that FAR enzymes function *in vivo* by catalysing both reduction steps without any release of intermediate products (Rowland & Domergue, 2012).

Biochemical investigations on alcohol-forming FARs that catalyse the production of primary alcohols have revealed that these have different substrate specificities with regard to acyl chain length and degree of saturation among different organisms such as garden pea (*Pisum sativum*), jojoba (*Simmondsia chinensis*), Arabidopsis thaliana, wheat (*Triticum aestivum*), mouse (*Mus musculus*), silkmoth (*Bombyx mori*), and human (*Homo sapiens*) cells (Domergue *et al.,* 2010; Doan *et al.,* 2009; Rowland *et al.,* 2006; Cheng & Russell, 2004; Moto *et al.,* 2003; Wang *et al.,* 2002; Metz *et al.,* 2000; Aarts *et al.,* 1997). The identification of FARs in plants and their functions other than conducting reduction reactions have been summarised in detail elsewhere (Domergue *et al.,* 2010). The *in vivo* characterisation of a FAR-like gene, *MS2* (*Male sterile 2*)/*FAR2*, which is localised in the chloroplast and has a function in pollen wall development in Arabidopsis, has shown that it can encode a fatty acid reductase enzyme catalysing primary fatty alcohol synthesis by converting preferred substrate palmitoyl-ACP to C16:0 alcohol in the presence of NADPH (Chen *et al.,* 2011). Moreover, Doan *et al.* (2009) investigated the catalytic activities of Arabidopsis FAR-like genes by expressing five out of eight genes and jojoba FAR gene in *E. coli*. They found that the fatty alcohol profile was noticeably different when *E. coli* endogenous acyl substrates were used, indicating that FARs can also have distinct substrate specificities to degree of saturation and chain length of the substrate. This suggests that the substrate specificities of FAR enzymes are strongly affected by the host expression systems (Rowland & Domergue, 2012). In addition, when *FAR1* and *CER4*/*FAR3* were expressed in a heterologous system, *i.e.* yeast (*S. cerevisiae*),

the fatty alcohol composition is very similar to that found when they are expressed in Arabidopsis, but not to that found when they are expressed in *E. coli*, where much shorter chains are obtained (Domergue *et al.,* 2010). This suggests that the prokaryotic system *E. coli* as a heterologous host is not appropriate for determination of FAR enzymes. Taken together, previous research suggests that the fatty alcohol composition of wax esters is mostly affected by the substrate specificity of the enzyme, the fatty acid composition in the host organism and the availability of acyl-CoA or acyl-ACP substrate ratios (Iven *et al.,* 2013; Samuels *et al.,* 2008; Wang *et al.,* 2002; Lardizabal *et al.,* 2000).

In addition to these, the subcellular localisation of FAR enzymes and the expression pattern of the genes in the cells are crucial factors in determining the substrate specificities of enzymes (Chen *et al.,* 2011; Shi *et al.,* 2011; Domergue *et al.,* 2010; Doan *et al.,* 2009; Rowland *et al.,* 2006). The localisation and function of an enzyme is important and has been shown to allow them to act differently in terms of utilisation of substrates, which results in different ratios of fatty alcohols. Doan *et al.* (2012) demonstrated that when chloroplast-localised Arabidopsis FAR is expressed in *E. coli* and Arabidopsis, different fatty alcohol ratios are obtained.

Esterification of primary alcohols

The last step of wax ester biosynthesis is the esterification of primary fatty alcohols produced and fatty acyl-CoA or acyl-ACP, which is catalysed by wax synthase (WS) enzyme. It has been characterised and identified in a wide range of living organisms such as mice, mammals (Cheng & Russell, 2004), jojoba (Lardizabal *et al.,* 2000) and protozoa (Teerawanichpan & Qiu, 2010). These have only wax synthase activity, while some enzyme forms can have both wax synthase and acyl-CoA:diacylglycerol acyltransferase (DGAT) activity, *e.g*. Acinetobacter (WS/DGAT) (Kalscheuer & Steinbuchel, 2003), petunia (PhWS1) (King *et al.,* 2007) and Arabidopsis (WSD1) (Li *et al.,* 2008), as well as avian wax synthase WS4 (Biester *et al.,* 2012).

The first wax synthase (WS) enzyme to be characterised was jojoba wax synthase (*ScWS*) expressed in Arabidopsis. It has a substrate specificity for unsaturated C18 alcohol and saturated and unsaturated C14-C24 acyl-CoAs (Lardizabal *et al.,* 2000). Twelve other homologues of jojoba wax synthase enzyme in Arabidopsis have since been characterised (Klypina & Hanson, 2008; Costaglioli *et al.,* 2005; Beisson *et al.,* 2003). Another study on *Euglena gracilis* showed that the co-expression of *EgWS* along with *EgFAR* results in production of medium-chain wax esters of saturated and unsaturated C14 to C16:1 (Teerawanichpan & Qiu, 2010).

The second type of wax synthase enzyme to be characterised was WS/DGAT in *Acinetobacter*, which is bifunctional, catalysing both TAG and wax ester synthesis (Kalscheuer & Steinbuchel, 2003). This enzyme is extremely specific for the *Actinomycetes* and can be found within the *Mycobacterium* genus (King *et al.,* 2007), in which higher activity of DGAT rather than WS has been demonstrated (Waltermann *et al.,* 2007; Stoveken *et al.,* 2005). However, this is not the case for *Acinetobacter calcoaceticus* species, which have a broad range of substrates of C2-C30 fatty alcohols. Nevertheless, the best substrates for that enzyme have been shown to be medium chain alcohols of C14-C18 (Waltermann *et al.,* 2007; Waltermann & Steinbuchel, 2005). Another bifunctional enzyme has been characterised from petunia petals, with substrate specificity for C20 and C22 acyl-CoA and medium chain alcohols of C8 to C12, while DGAT activity has not been demonstrated (King *et al.,* 2007). Arabidopsis has 11 homologues of WS/DGAT proteins. One of these, characterised as WSD1, resides in the ER and has 10-fold higher activity of WS than DGAT *in vitro*. The findings for WSD1 are generally consistent with those for the bifunctional enzyme found in ADP1 *A. calcoaceticus*, one difference being that when *Acinetobacter* WS/DGAT enzyme is expressed in yeast accumulation of TAG occurs, while no TAG accumulation occurs when WSD1 is expressed in *E. coli*. This indicates that the substrate specificity of WS enzyme, as is the case for FARs, depends on the organism/tissue and accessibility of substrate for enzyme activity (Li *et al.,* 2008).

Recently, another type of wax synthase enzyme, Phytyl Ester Synthase1 and 2 (PES1 and 2), was identified in Arabidopsis. Both isomers of the enzymes have been shown to participate in phytyl ester synthesis in chloroplasts and have a capacity for diacylglycerol acyltransferase activity for TAG biosynthesis. As a result, fatty acid phytyl esters and intracellular wax esters were accumulated in the plastoglobules of chloroplasts in plant cells. Moreover, during senescence and nitrogen-limited conditions, both enzymes, which have a wide range of substrate specificity to acyl-CoAs, acyl carrier proteins and galactolipids, have been shown to be highly expressed and a considerable amount of medium fatty acids is present in the phytyl ester fraction (Lippold *et al.,* 2012).

1.3 Metabolic engineering of vegetable oil as a renewable alternative

Increasing environmental concerns and the declining fossil raw resources are two major issues to be overcome in future (Steen *et al.,* 2010). Plant lipids have the potential to provide environmentally friendly, sustainable and renewable

energy feedstocks that can replace petroleum resources in many industrial applications (Carlsson *et al.,* 2011; Nikolau *et al.,* 2008). Crop plants can be adapted to provide substantial renewable amounts of industrially important molecules in plant storage or vegetative tissues via metabolic engineering approaches (Vanhercke *et al.,* 2013b).

Consumption of plant oils with a high value for food and non-food applications has increased by 50% during the past decade due to the greater energy demand of a rapidly growing global population and high consumption of biodiesel for transportation (Lu *et al.,* 2011). In order to reduce the dependency on fossil-based resources in the chemical industry, 40% of these resources must be replaced with alternative energy resources in two decades, without affecting the food oil sector. To achieve this goal, it has to be borne in mind that alternative plant-derived oils have to be cheaper and as secure as fossil oil (Carlsson *et al.,* 2011). Increasing the levels of plant oils through biotechnological and metabolic engineering approaches can be achieved in different ways, such as increasing the oil content of existing oil crops, domestication of new vegetable oil crops with the desired composition, redirection of carbon sources into oil instead of starch, or oil synthesis/accumulation in vegetative tissues (Carlsson *et al.,* 2011). In this regard, industrial plant oils, especially waxes, TAGs and fatty alcohols, have potential for replacement of petrochemical materials (Vanhercke *et al.,* 2013b), thereby reducing the dependency on fossil oil in society.

Metabolic engineering of waxes, TAG and other plant lipids has already been achieved at rapid speed with the advent of new technologies. A combination of genomic, transcriptomic, transgenic and biochemical investigations has helped in the understanding of complex biochemical lipid pathways. Furthermore, the availability of sophisticated transient expression systems such as agro-infiltration for high-throughput experiments has been instrumental for monitoring more than one gene function in a short period (Wood *et al.,* 2009). By taking advantage of these elegant systems, simultaneous engineering of multiple pathways can be achieved.

Approaches have been applied to increase the oil content in different plant tissues by enhancing the fatty acid supply or substrate availability of glycerol-3-phosphate via up-regulation of genes participating in fatty acid biosynthesis (Durrett *et al.,* 2008). However, very few studies have reached the goal of increasing energy density by over 50% (Carlsson *et al.,* 2011; Shen *et al.,* 2010; Weselake *et al.,* 2009). An accumulated level of starch in leaves of up to 10% of dry weight can be achieved (Ekman *et al.,* 2007), and if starch mobilisation is inhibited in fully expanded leaves, 50% of the dry weight of leaves can be starch. By channelling starch to oil production, the theoretical

yield of oil from tobacco leaves can be similar to that of high oil yielding oil palm (Carlsson *et al.,* 2011; Andrianov *et al.,* 2010).

TAGs are biodegradable products and their physical features allow them to be used as lubricants in the chemical industry, although their poor hydrolytic and oxidative properties are limiting factors. On the other hand, wax esters (WEs) are excellent compounds with high hydrolytic resistance (Li *et al.,* 2010), making it possible to engineer inexpensive wax esters in oilseed crops comparable with jojoba-type WE in terms of their suitability as lubricants. The linear structure of wax esters improves their viscosity and results in specific properties such as anti-rust, anti-foam, anti-wear and friction reduction characteristics of the lubricants (El Kinawy, 2004; Bisht *et al.,* 1993). Because of the excellent stability and lubrication properties of wax esters, the engineering of WE production in commercial oilseed crops and the production in vegetative tissues has been intensively studied (Taylor *et al.,* 2011). The first approach, in Arabidopsis seeds, showed that co-expression of alcohol-forming *Arabidopsis FAR* and jojoba *WS* resulted in replacement of TAGs with wax esters in seeds (Lardizabal *et al.,* 2000). Moreover, expression of a combination of jojoba-type wax synthase, a fatty acyl-CoA reductase and cDNA encoding for KCS in *Brassica napus* resulted in accumulation of 49% wax ester in seed oil by diversion of FAs from TAG assembly to wax ester biosynthesis, yet commercially interesting levels were not reported (Taylor *et al.,* 2011). More recently, simultaneous expression of *FAR* and *WS* increased wax ester levels in Arabidopsis seeds (Heilmann *et al.,* 2012). These findings demonstrate the feasibility of engineering wax esters in seeds and other organs of plants on a larger scale in order to contribute valuable bio-lubricants. Thus, meeting the demand for fatty alcohols, wax esters and TAGs for industrial applications by producing these materials with desired properties in large amounts would be highly advantageous (Jenkins *et al.,* 2011; Rude & Schirmer, 2009; Jetter & Kunst, 2008; Kalscheuer *et al.,* 2006).

Overall, in order to make plant lipids attractive for utilisation in many industrial aspects, some basic demands must be met. For example, the tailored new molecules should be as pure as possible, the recovery yield should be maximised and the cost of separation and downstream processing should be minimised (Carlsson *et al.,* 2011). For food supply, existing oil crops are composed of one of the major fatty acids and the content of these oils is sufficient for using these crops as a food supply, while it is not pure enough for utilisation in industrial feedstocks. Therefore, it has been suggested that engineering of storage lipid assembly is required, as well as introducing new genes/transcription factors, in order to be able to engineer industrially important fatty acids into major oil crops (Vanhercke *et al.,* 2013b). However,

the replacement of natural petrochemicals with bio-based alternatives at a substantial level will be inadequate with only metabolic engineering of industrial fatty acids into oil crops. The growing world population and its food and energy demands will require global oil supplies in larger volumes in future, which could be achieved by improving the oil productivity of existing oil crops, creating new oil crops and creating new oil production platforms by metabolic engineering approaches (Vanhercke *et al.,* 2013b; Carlsson *et al.,* 2011; Lu *et al.,* 2011; Weselake *et al.,* 2009).

1.4 Two model crops for increasing the energy density

Tobacco

Plants have not only been providing humans with food, but also with other natural products such as medicines, dyes, poisons and materials like paper, rubber and cotton, for thousands of years. With the birth of modern biotechnology around the 1980s, plants were used for the first time for production of specific products such as industrial enzymes, specific proteins and molecular biology reagents (Hood, 2002; Fischer & Emans, 2000). *Nicotiana benthamiana* (a tobacco species) is one of the most widely used model plant species for understanding fundamental issues in molecular and plant biology. It is an herbaceous plant that originates from Australia and its accessions are very similar or even originated from a single source (Goodin *et al.,* 2008). Being a member of the Solanaceae family, *N. benthamiana* is a close relative of tomato (*Solanum lycopersicum*), and potato (*Solanum tuberosum*), two economically important species in global vegetable and food consumption whose genomes have been sequenced in recent years (Tomato Genome Consortium 2012; Potato Genome Sequencing Consortium 2011). The whole genome of *N. benthamiana* comprises an estimated 3 Gb (gigabases) consisting of 19 chromosomes (Bombarely *et al.,* 2012; Narayan, 1987) and is now available on the SolGenomics Network (http://solgenomics.net). The genus *Nicotiana* (*Solanaceae*: Linnaeus 1753) consists of 76 species from four continents, with the majority occurring in South America and Australia, mainly distributed in tropical and subtropical areas (Knapp *et al.,* 2004). *Nicotiana benthamiana* is an allotetraploid from the *Suaveolentes* section (Clarkson *et al.,* 2004; Knapp *et al.,* 2004; Chase *et al.,* 2003). Commercial tobacco (*N. tabacum*) is also an allotetraploid, and one of its progenitors is closely related to *N. sylvestris* from the *Sylvestris* section.

Nicotiana benthamiana was not a widely used model crop before the advent of major technical advances such as transformation and agro-infiltration. The large leaves of the plants and its susceptibility to pathogens make it attractive

to biologists aiming to express proteins through the intermediate organism *Agrobacterium* (Ma *et al.,* 2012; Wagner *et al.,* 2004; Van der Hoorn *et al.,* 2000; Tang *et al.,* 1996; Chapman *et al.,* 1992). Prior to large-scale production in a stable transformation system, the function of the gene/s can be tested in transient expression systems in *N. benthamiana* plants (Torres *et al.,* 1999).

Commercial tobacco, which is closely related to *N. benthamiana*, produces large amounts of biomass, and by genetic transformation it could produce seeds rich in desired products for long-term storage. Tobacco is one of the unique plant classes, which can produce up to 170 metric tonnes of biomass per hectare (Cramer *et al.,* 1996; Sheen, 1983). Globally, the tobacco plant is grown in 119 countries, and the leaves of the plant are used for producing cigarettes in the tobacco-processing industries (US Department of Agriculture). In the search for conventional crops to replace petroleum resources for sustainable bioenergy resources, tobacco represents a promising alternative crop due to generation of large amounts of inexpensive biomass that could be used for energy production instead of smoking. It is believed that transgenic tobacco plants producing high levels of lipids useful in industry can be cultivated on a commercial scale. Production of biofuel is usually correlated with seeds, where oil accumulation occurs, usually in the form of TAGs, rather than with green biomass. The applicability of tobacco seed oil, which comprises 40% of seed dry weight, as fuel for diesel engines has been demonstrated (Usta, 2005), but uptake is rather low compared with traditional oil producers such as soybean or rapeseed (Andrianov *et al.,* 2010). Oil biosynthesis machinery generally begins in green tissues and tobacco leaves contain 1.7%-4% of oil by dry weight (Koiwai *et al.,* 1983) that can be extractable as fatty acids (Vicente *et al.,* 2007). Despite the low levels of oil in biomass compared with seeds, tobacco is a promising alternative energy crop for biofuel production and could be used as a model crop for other high-biomass plants in metabolic engineering approaches.

Rice

Rice (*Oryza sativa*) is the main staple food for a large proportion of the total population in the world, ranked as second with regard to global production after maize (FAOSTAT, 2010). Rice is an annual (can survive as a perennial in tropic areas), diploid (2n=24), short-day plant (Izawa & Shimamoto, 1996). It has been cultivated for more than 7000 years, and was first domesticated in China (Izawa & Shimamoto, 1996). There are many varieties of rice, but cultivated rice comprises two main species, *Oryza sativa* (Asian rice*)* and *O. glaberrima* (African rice). The genus *Oryza* consists of many wild species, and it is believed that cultivated rice through a domestication process has evolved

from one of these wild species (*Oryza rufipogon*) (Chang, 1976). Subspecies of *O. japonica* and *O. indica* are used in cultivation and have also been used in constructing molecular maps.

Even though dicotyledons provide most insights in system biology, having a model crop from the monocotyledons is also crucial due to noteworthy differences during developmental phases. Moreover, it is important to understand metabolic fluxes in crops that provide food for billions of people every day. Maize (*Zea mays*) has been valuable for many studies in biology as a model crop, yet transformation of the plant is not optimal. Rice has been demonstrated to be an efficient model monocotyledon plant in molecular biology due to the efficient and time-wise *Agrobacterium*-mediated transformation (Hiei *et al.,* 1994), and its fairly small genome size. Furthermore, the endosperm part of the rice grain, where mainly starch and proteins are accumulated, is relatively bigger than embryo. These properties of rice make it suitable for studying carbon partitioning in the endosperm for accumulation of lipids instead of starch, in order to increase the energy density of the grains by molecular biology and genetic approaches. Therefore rice has a bright future as an alternative energy-rich monocotyledon model plant in future research.

2 Aims of the study

The general aims of the thesis were to genetically manipulate the regulatory lipid pathway with emphasis on producing wax esters and triacylglycerol in monocot and dicot plants for creating alternative oil crops. In order to understand the metabolic pathway of lipid metabolism, genes and/or transcription factors involved in wax ester and triacylglycerols biosynthesis in plant cells, the following specific objectives were established:

- To increase the energy levels of *Nicotiana benthamiana* leaf tissues by transient over-expression of wax ester biosynthetic genes directed to chloroplast compartments.

- To manipulate *de novo* fatty acid synthesis by down-regulating the *β-ketoacyl-ACP synthase II* (*KASII*) gene in order to increase palmitic acid levels and their further incorporation to wax ester biosynthesis.

- To create transgenic *Nicotiana benthamiana* plants with emphasis on wax ester production by using a tobacco transformation approach.

- To increase the oil in rice endosperm, with the ultimate aim of using cereal seeds as oil-producing tissues.

3 Results and Discussion

3.1 Wax esters of different compositions produced via engineering of leaf chloroplast metabolism in *Nicotiana benthamiana* (I)

In order to study wax ester production in chloroplast compartments of *Nicotiana benthamiana* leaves, we tested a number of combinations of putative genes that take part in wax ester biosynthesis by co-expressing them via an agro-infiltration transient system in Paper I. We used plant- and/or bacterial-derived *FAR* (*fatty acid reductase*) and *WS* (*wax synthase*) genes, as well as WRINKLED1 (WRI1) transcription factor. To prevent plant defence system interference, a short protein, p19, was co-expressed together in all combinations and GFP (green fluorescent protein) was used as an indicator for the infiltrated area.

A number of physical and chemical factors have great effects on the agro-infiltration method in order to obtain a stable application. Agro-infiltration is a rapid and straightforward way of screening a large number of transgene constructs in response to different stimuli (Schöb *et al.,* 1997). Many genes and their end products can be tested using this method without the need for generation transgenic plants, which can be challenging for many plant species (Horn *et al.,* 2004; Fischer *et al.,* 1999). Expression of transgenes is highly used by agro-infiltration systems but the effect is limited because of RNA silencing of the plant defence system, which is inevitably triggered when a foreign gene is introduced. In order to minimise the limitation of the plant defence system, we used the p19 protein (Voinnet *et al.,* 2003). Moreover, we observed that the reproducibility and quality of the experiments was greatly affected by plant age and leaf positioning. Based on the results of different leaf positions and ages tested, we conducted the experiments with 5- to 6-week-old

plants, and observed the best expression levels when using the terminal leaves of the first seedlings.

Five days after the agro-infiltration of gene combinations in *N. benthamiana* leaves, GFP-indicating areas were excised for further experiments. The expression of individual gene was confirmed by qRT-PCR (quantitive real time PCR). Wax ester or alcohol fractions were separated by thin layer chromatography (TLC) and analysed by gas chromatography (GC). In general, total wax ester levels were high when *MaFAR* (*Marinobacter aquaeolei* VT8) was combined with *AtPES2* (*Phytyl ester synthase2*), resulting in a top level of 1.62 nmol/mg fresh weight (FW), corresponding to approximately 0.9% of leaf dry weight (DW). When *MaFAR* was co-expressed with *MhWS* (*Marinobacter hydrocarbonoclasticus* ATCC49840), wax esters were found at a level of 0.66 nmol/mg FW, corresponding to approximately 0.4% of leaf DW. Expression of *AtFAR6* (Arabidopsis thaliana fatty acyl reductase) with *AtPES2* gave the highest level of wax esters, with an amount of 0.9 nmol/mg FW, whereas *AtFAR6* in combination with *MhWS* resulted in 0.42 nmol/mg FW. We concluded that MaFAR is more efficient in production of fatty alcohols and AtPES2 is more efficient in esterification. AtPES2 was efficiently used to pool up medium chain fatty acyl (mainly C12:0-C14:0) groups compared with MhWS, and therefore gave high levels of wax ester production.

The substrate specificity of FAR enzymes has an important role in determining the fatty alcohol composition of cells, which differs between organisms (Wang *et al.,* 2002; Metz *et al.,* 2000). Expression of two *FAR* (*AtFAR6* and *MaFAR*) genes produced different levels of fatty alcohols with different composition (Figure 6). AtFAR6 produced mainly 16:0-OH alcohols, in agreement with the previous results from *in vitro* studies (Doan *et al.,* 2012), while MaFAR produced both 16:0-OH and 18:0-OH in almost equal amounts. We concluded that a high level of the two types of alcohols from expression of *MaFAR* was the reason for high wax ester levels. It has been shown that MaFAR produces medium chain alcohols *in vitro* (Hofvander *et al.,* 2011), but this was not the case *in vivo*.

In addition to the substrate-specific activity of the alcohol-forming enzymes, wax synthase plays a crucial role in determining the characteristics of the wax esters produced. To examine this, we tested two wax synthase enzymes, MhWS and AtPES2, to reach high levels of wax esters with different composition. In general, co-expression of *AtPES2* with *MaFAR* resulted in higher wax ester amounts. We showed that MhWS has narrow preferences, to mainly 16:0 and 18:0 acyl groups, while PES2 has a broad substrate range, mainly 12:0 and 14:0 as well as 16:0 and 10:0 at a low but considerable level.

Surprisingly, MhWS was not used for shorter fatty acids than 16:0 as has been suggested previously (Barney *et al.,* 2012).

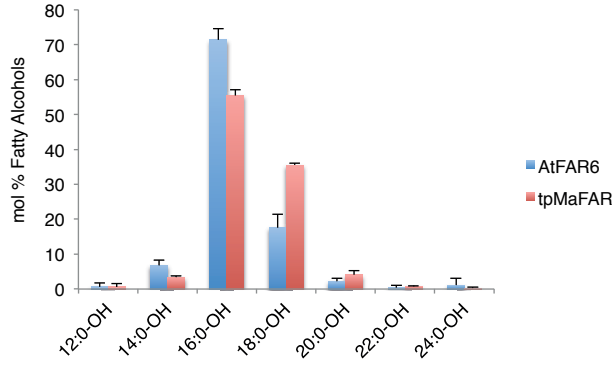

Figure 6. Fatty alcohol composition in leaves when FAR genes were expressed individually in *Nicotiana benthamiana* leaves.

We also tested a fusion construct (*tpMaFAR::MhWS*) harbouring the catalytic parts of *MaFAR* and *MhWS* with an *AtFAR6* chloroplast transit peptide. The expression of this construct resulted in production of wax esters, confirming the functional sites of individual enzymes. The wax esters produced via the fusion function were similar in quality and quantity to the product produced when the *MaFAR* and *MhWS* were expressed individually.

Moreover, it is known that *Arabidopsis thaliana* WRINKLED1 (AtWRI1) is an essential transcription factor that has a direct or indirect effect on the regulation of fatty acid biosynthesis (Focks & Benning, 1998). Therefore, we also investigated whether AtWRI1 would enhance wax ester biosynthesis via activating gene expression in lipid biosynthesis. In the *AtWRI1* combinations, the only combination with *AtFAR6* + *AtPES2* gave a 36% increase in wax ester production. In contrast, there were significant decreases in other combinations. For all *AtWRI1*-containing combinations, TAG accumulation was increased by about five-fold over control leaves. The *de novo* fatty acid biosynthesis in the plastid compartment of plant cells is a shared pathway among all other lipid biosynthetic pathways. Based on this fact, with the decreased levels of wax esters and increased levels of TAG accumulation it could be assumed that additional co-expression of the *WRI1* might be more efficient for activating genes which take part in TAG assembly.

Upon agro-infiltration of *N. benthamiana* leaves, a phenotypic change was observed (Figure 7). In general, control leaves appeared paler in regions upon infiltration, but otherwise healthy. Infiltration of leaves with some

combinations resulted in severe damage, with areas entering a state of cell death resembling senescence.

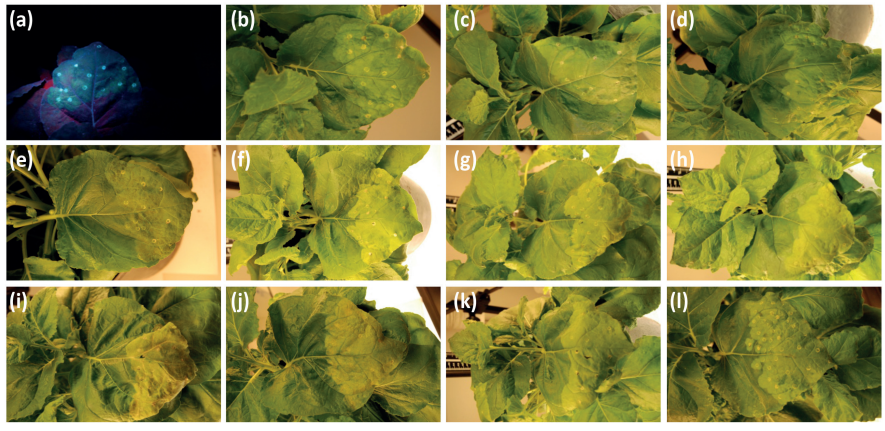

Figure 7. Photos of leaf tissues of *N. benthamiana* five days post infiltration; (a) P19+GFP viewed under blue LED light, (b) P19+GFP, (c) AtFAR6+AtPES2, (d) AtFAR6+AtPES2+AtWRI1, (e) AtFAR6+tpMhWS, (f) AtFAR6+tpMhWS +AtWRI1, (g) tpMaFAR+PES2, (h) tpMaFAR+PES2+AtWRI1, (i) tpMaFAR+MhWS, (j) tpMaFAR+MhWS+AtWRI1, (k) tpMaFAR::MhWS and (l) tpMaFAR::MhWS+AtWRI1.

This observation led us to investigate whether the cell death occurred due to accumulation of free alcohols produced by FARs. The free alcohols in all combinations and in the control leaves with only FARs were subjected to GC analysis. This showed that MaFAR produced greater levels of free alcohols than AtFAR6. The leaves infiltrated with *MaFAR* in combination with *MhWS* were observed to be very necrotic on the infiltrated areas (Figure 7i), whereas this effect was not as obvious when *AtPES2* was co-infiltrated with *MaFAR* (Figure 7g). The amounts of free alcohols in the *MhWS*-infiltrated leaves were twice those found in *AtPES2*-infiltrated leaves. However, the levels of free alcohols when *AtFAR6* was expressed with either *AtPES2* (Figure 7c) or *MhWS* (Figure 7e) were similar. The results confirmed that MaFAR was superior to AtFAR and that MhWS has a narrower substrate specific activity and low capacity for pooling fatty alcohols for further esterification than AtPES2. Therefore, unincorporated free alcohols in wax ester assembly may cause cell death and result in high levels of necrotic lesions on the leaves.

The enzymes responsible for lipid biosynthesis are usually membrane integral or peripheral proteins. This implies that functions of these enzymes for lipid biosynthesis may not occur in the chloroplast matrix. However, one of the alcohol-forming *FAR* in Arabidopsis (*AtFAR6*) and a wax synthase (*AtPES2*) have been shown to be localised in the chloroplast compartment of Arabidopsis

plants (Doan *et al.,* 2012; Lippold *et al.,* 2012). The bacterial enzymes used were directed to the chloroplast by using Arabidopsis plastid transit peptide to introduce a new wax ester production pathway in the chloroplast. With transmission electron microscopy (TEM), we were able to locate the wax esters produced in the chloroplast matrix (Figure 8). The results indicated that metabolic engineering of chloroplast lipid metabolism for production of different energy-rich oleochemicals is possible.

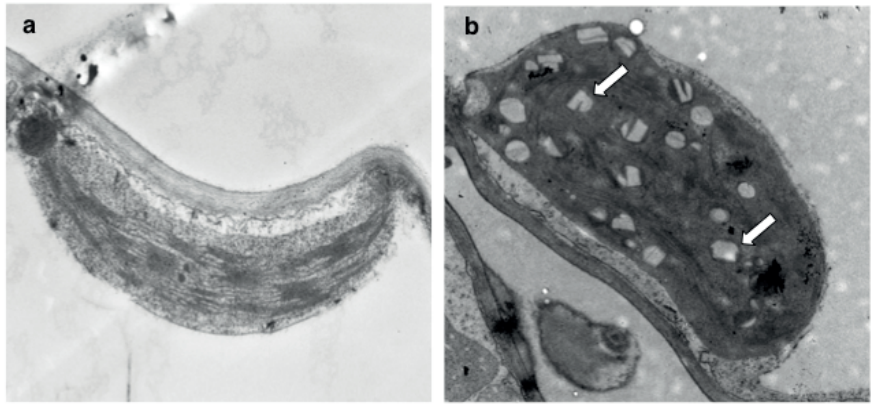

Figure 8. Transmission electron microscopy (TEM) images of (a) Control (*P19+GFP*) and (b) combinations of *tpMaFAR+MhWS.* White arrow shows wax esters. Bar = 500 nm.

All in all, we showed the feasibility of a novel concept: via application of *Agrobacterium*-mediated transient expression system, wax esters can be produced to a high level with different origin FAR and WS enzymes with different substrate specificities. We also showed production of wax esters to a maximum level of 0.9% of leaf DW, which can in future be improved by a number of strategies in order to increase the energy density of biomass. In order to get attractive levels of wax esters and fatty alcohols, identification of a FAR enzyme that can use medium chain substrates is of interest. It is clear that AtPES2 has a substrate preference for shorter chains, and it would be of great interest to engineer an alternative wax synthase that can utilise longer acyl groups such as the 18:0-OH produced by MaFAR. Our results demonstrated that the transient expression system in *N. benthamiana* can ultimately be used for testing multiple candidate genes responsible for production of oleochemicals that are important for industrial applications.

3.2 Transient silencing of β-ketoacyl-ACP synthases II genes is feasible in *Nicotiana benthamiana* for metabolic engineering of wax ester compositions (II)

The investigation of wax ester production indicated that wax esters were produced in the chloroplast matrix (Paper I). The results raised the possibility for further increasing wax ester levels by metabolic engineering of chloroplast enzymes. In Paper II, we focused on β-ketoacyl-ACP synthases II (KASII). As a member of the small β-ketoacyl-ACP synthase (KAS) family, KASII is responsible for adding C2-units to growing acyl chain, which elongates 16:0-ACP to 18:0-ACP in *de novo* fatty acid synthesis (Carlsson *et al.,* 2002). We hypothesised that down-regulation of *KASII* would provide more 16:0-ACP substrates by inhibiting production of 18:0-ACP, which could simultaneously be used by the activity of wax ester-forming enzymes for wax ester production with desired compositions.

Blast results using the DNA sequences of *Arabidopsis thaliana KASII* (AY081285) revealed two *KASII* genes in the *Nicotiana benthamiana* genome at SolGenomics (http://www.solgenomics.net). In order to test whether both isomers can be silenced, the individual gene sequences and a common region of the two genes were cloned and three independent *KASIIRNAi* constructs were made using intron-spliced hairpin technology (Helliwell & Waterhouse, 2005). The constructs are termed as *KASIIRNAi-1*, *2*, *3*, respectively. Based on the results presented in Paper I, we conducted a set of gene combinations via agro-infiltration. For the alcohol-forming gene function, we selected both AtFAR (Arabidopsis thaliana fatty acid reductase) and MaFAR (*Marinobacter aquaeolei* VT8). For esterification enzymes, we selected only AtPES2 (Arabidopsis thaliana phytyl ester synthase2). Six combinations of genes (including *AtFAR6* + *AtPES2* or *MaFAR* + *AtPES2* with and without *KASIIRNAi*) were mixed, with p19+GFP included in every mixture, and used as controls. The combinations were then infiltrated on *N. benthamiana* leaves to assess the transcript levels and their effect on chloroplast lipid metabolism. Five days after infiltration, expression of genes was assessed by quantitative real-time PCR (qRT-PCR). We observed high expression of wax ester-producing genes upon infiltration, and severe inhibition of *KASII* genes. The expression of *KASII* was silenced almost completely when the RNAi constructs were co-expressed together with wax ester biosynthesis genes. Among three constructs, the best inhibition was recorded with *KASIIRNAi-3* in all combinations. It has been reported that the complete inhibition of *KASII* in Arabidopsis *FAB* (*KASII*) transformants with strong seed-specific hairpin-RNAi reductions can cause death and that less severe inhibition results in normal embryo development (Pidkowich *et al.,* 2007). Here, we showed that

infiltration of *N. benthamiana* leaves after 5 days post infiltration did not cause any significant differences from the controls.

In order to observe the effects of decreased transcript levels of *KASII* on the fatty acid profile in *N. benthamiana* leaves, we ran the total leaf extracts on TLC (Thin Layer Chromatography) and GC (Gas Chromatography). We found significant increases in C16 levels upon *KASII* inhibition. The highest 16:0 levels were recorded with a 58% increase in *KASIIRNAi-3* when infiltrated on its own as control. These results are in agreement with findings in Arabidopsis that when the *KASII* gene is knocked out, there is a 53% increase in 16:0 levels (Pidkowich *et al.,* 2007).

Moreover, we quantified the total wax esters in six different combinations. The GC results revealed that the highest wax ester amounts were obtained in the *MaFAR*-containing combinations. Total wax esters in *AtFAR6*-containing samples where *KASII* was inhibited were improved significantly (Figure 9a). Wax esters in the combination of *AtFAR6+PES2+RNAi-3* were improved by as much as 73% compared with the control (*AtFAR6+PES2*). Regarding the changes in wax ester composition, the fatty acid shift of wax esters was altered by an increase in C16:0 of up to 72% in this combination. A significant increase in C16/C18 ratio in the *AtFAR6*-containing combinations was recorded, further indicating the direct role of *KASII* inhibition for providing palmitoyl substrates in *de novo* fatty acid synthesis in *N. benthamiana* for wax ester production. However, the ratio of C16/C18 levels was not significantly altered in the *MaFAR*-containing combinations. These results demonstrate that AtFAR6 is more efficient than MaFAR in the presence of more available C16 substrate. We concluded that the changes observed in the total wax ester amounts were due to changes occurring in the palmitoyl compounds (Figure 9).

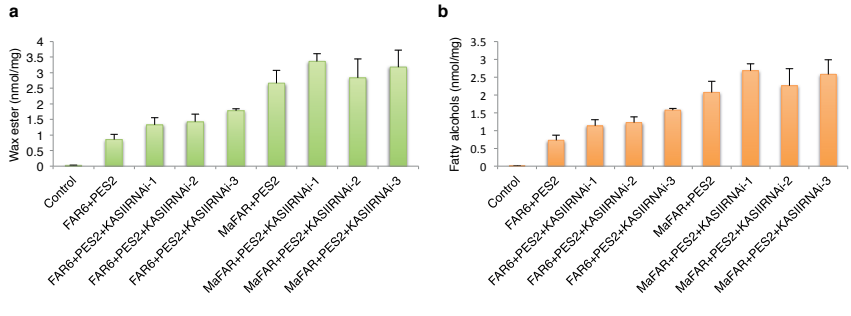

Figure 9. Total wax ester amounts in relation to increased palmitoyl substrates in *N. benthamiana* leaf tissues after *KASII* inhibition. (a) Total wax ester levels and (b) changes in 16.0-OH composition.

In order to understand the limiting factor in wax ester formation, we also checked the free alcohol content in the total leaf extracts. We found that the total free alcohol amounts were not significantly changed by *KASII* inhibition, whereas upon individual *KASIIRNAi* inhibition the C16/C18 ratio was significantly altered. In general, total free alcohols were present at higher levels in *MaFAR*-containing samples than *AtFAR6*-containing samples. The composition of the free alcohols was similar to that found in the wax esters. The 16:0-OH species were the dominant compounds in the *AtFAR6*-infiltrated leaves, while a similar amount of C16:0-OH and C18:0-OH was observed in the *MaFAR*-infiltrated leaves.

Apart from being involved in wax ester production, *AtPES2* gene has also been shown to take part in TAG assembly in the ER (Lippold *et al.,* 2012). We suggest that any release of fatty acids from the chloroplast, which in theory should be elevated, can be captured by AtPES2 for TAG production. The total amounts of TAGs were not altered significantly by additional *KASII* silencing in any of the combinations, while the C16/C18 ratio was significantly changed only in *KASIIRNAi-3* combinations. This demonstrates that wax ester assembly was efficient within the plastid before high levels of fatty acids were exported to the cytosol for TAG assembly.

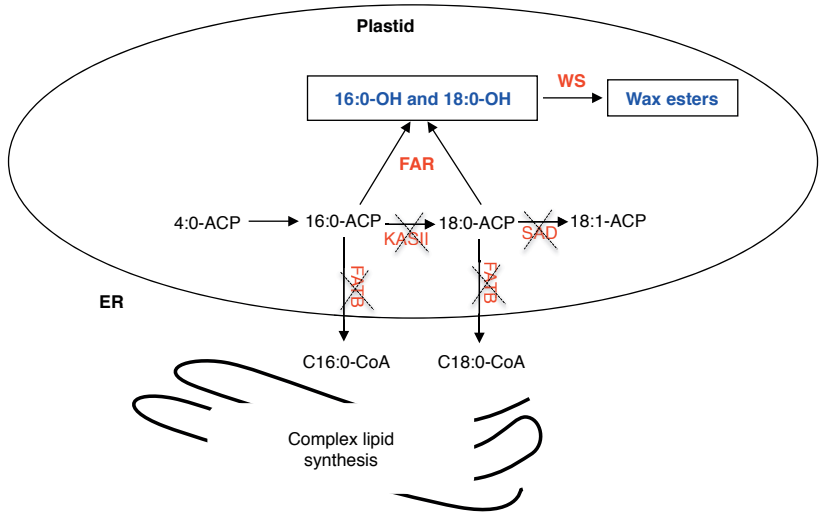

Figure 10. Schematic illustration of the complex lipid metabolic pathway in the plastid compartment, indicating possible directions to increase wax ester content by inhibition studies.

Taken together, the results in Paper II showed the impact of KASII enzyme in *de novo* fatty acids synthesis and further on the wax ester biosynthesis

pathway. By down-regulating the different KASII isomers, the elevated 16.0-ACP levels were incorporated to wax esters mainly by AtFAR6 rather than MaFAR in the chloroplast of *N. benthamiana* leaves. The results showed the feasibility of using an *Agrobacterium*-mediated transient expression system for metabolic engineering of chloroplast wax ester synthesis. Through continuous utilisation of this elegant system, wax ester levels can be elevated to an even greater extent by metabolic engineering measures such as down-regulation of *FatB* (*Acyl-ACP thioesterase B*) or/and *SAD* (*stearoyl-ACP desaturase*), which play an active role in *de novo* fatty acid biosynthesis (Figure 10).

3.3 Increased production of wax esters in transgenic tobacco plants by expression of a fatty acid reductase:wax synthase gene fusion (III)

In Papers I and II, we tested the transient production of wax esters in the chloroplast compartment of *Nicotiana benthamiana* plants by metabolic engineering of genes that take part in the process. These novel approaches led us to create transgenic *N. benthamiana* plants producing wax esters for longer-term energy purposes. The possibility of creating new crops in terms of wax ester biosynthesis in Arabidopsis seeds was first shown in the beginning of this millennium (Lardizabal *et al.,* 2000) and again quite recently (Heilmann *et al.,* 2012). In Paper III, we examined whether lipid engineering in transgenic tobacco plants for bioenergy purposes is realistic and feasible in the near future. Wax ester production in green biomass of plants such as tobacco with potential biomass production of approximately 170 t/ha can contribute to global energy supply.

Tobacco transformation was first achieved more than 20 years ago. The transformation process involves introducing the gene of interest into *Agrobacterium tumefaciens*, which is then used for infecting discs of tobacco leaves. Callus proliferation and the creation of subsequent explants take approximately 6-8 weeks. To generate stable transformation for wax ester production, we selected the fusion gene (*tpMaFAR::MhWS*) (Paper I). We were able to generate 14 tobacco transformants from the T1 lines, 12 of which were viable and yielded seeds, while two were lost. The seeds of second-generation transformants were applied to kanamycin resistance screening, resulting in nine resistant lines. Three of the transgenic lines from the second generation (transformants 1, 2 and 6) were found to have a high *tpMaFAR::MhWS* expression level in leaves (unpublished data). The seeds of the third-generation transgenic *N. benthamiana* plants were selected for

kanamycin resistance again, resulting in two homozygous transformants of lines 2.10 and 6.1 being used for further analyses.

The expression of *tpMaFAR::MhWS* transgene in selected transgenic lines was confirmed by qRT-PCR (quantitative real time PCR) analysis at three positions (top, middle and basal). The results showed that the relative expression levels appeared differently between both transformants, with a variable expression pattern, confirming the integration of the fusion gene into the *N. benthamiana* genome. The expression levels were generally higher in line 6.1 than in line 2.10, and no expression was recorded from wild-type plants (Figure 11a). We observed a basipetal increase of the relative expressions at three positions in both transformants.

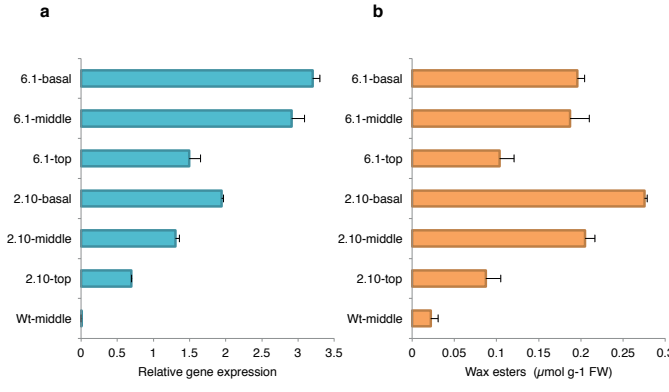

Figure 11. Correlation between gene expression and wax ester content. (a) Relative gene expression and (b) total wax esters at different positions of transgenic *Nicotiana benthamiana* plants.

To confirm the function of the transgene expression for wax ester production, we quantified the wax esters in transgenic lines by GC. It has been shown previously that the degree of production of lipids in plants can vary among tissues or organs (Li *et al.,* 2008). This can be due either to enzyme accessibility to substrates in different parts of tissues/organs or different expression levels at different levels of tissues. Therefore, we conducted analyses at three positions of both leaf and stem tissues, and found that total wax esters were produced at the same composition of fatty alcohols and methyl esters, with different extents in leaves and stem tissues in the same transformants. In general, the high wax esters were produced in transgenic line 2.10, and relatively low amounts in line 6.1. Overall, the degree of wax ester production was basipetal, as was shown in gene expression analyses (Figure 11b). The highest wax ester amount in basal leaves of the line 2.10

transformant was 0.28 of μmol g^{-1} of fresh weight (FW), while it was 0.2 μmol g^{-1} of FW in basal leaves of line 6.1. The levels of wax esters at three positions of stems in the two lines were also different from each other, and even differed within the same transformant. In contrast to leaf wax ester content, the highest wax esters in stem tissues were produced in line 6.1 in the middle, top and basal levels, respectively. Production from the middle level of transformant 6.1 was 0.26 μmol g^{-1} of FW, while it was 0.14 μmol g^{-1} of FW from the middle level of transformant 2.10. According to calculations for total leaf and stem FW, total wax ester level was approximately 8 mg per plant (85 g FW), corresponding to 0.15% dry weight (DW) per plant, or an eight-fold increase compared with wild-type (WT). The positive correlation between gene expression and biochemical analyses (Figure 11) indicates that there is sufficient substrate for wax ester biosynthesis in tobacco and that the total amounts can be further improved, for instance by using stronger promoters or minimising transgene post-transcriptional silencing for optimisation of tobacco transformation.

In addition to total wax ester levels, we analysed whether the composition of the wax ester profile was altered when *tpMaFAR::MhWS* was expressed in transgenic plants compared with transient expression. The results demonstrated that in both systems, MaFAR consistently produced mainly 16:0-OH (palmitoyl alcohol) and 18:0-OH (stearoyl alcohol) alcohols, while fatty acid species of wax esters consisted mainly of 16:0 and 18:0, as well as 20:0 and 22:O. Interestingly, the fatty acid compounds of 20:0 and 22:0 were not significant when *tpMaFAR::MhWS* was expressed transiently. This suggests that part of the wax ester biosynthesis in the current transgenic lines might have occurred outside the plastid for VLCFA (very long chain fatty acid) synthesis. The mechanism behind this unexpected result should be further investigated for optimisation of tobacco transformation in the future.

In Paper I, we showed that MaFAR was sufficient to provide fatty alcohols for wax ester production and that high level of free alcohols that were unbound to wax esters caused necrotic lesions on the leaf surface. The transgenic plants were grown and developed slightly differently than WT plants, and both transformants showed reduced chlorophyll levels in leaves. We investigated the free alcohol levels, which might have been the cause of obtaining transgenic plants with abnormal phenotype. The free alcohol level at the middle position of transformant 2.10 was 0.44 μmol g^{-1} of FW, while it was 0.39 μmol g^{-1} of FW in middle leaves of line 6.1. The highest level of free alcohols was found in the top-level stem tissue of transformant 6.1, where it comprised 0.29 μmol g^{-1} of FW, while it was 0.2 μmol g^{-1} of FW in the top-

level tissue of transformant 2.10. The composition of the residual alcohols consisted of 16:0-OH and 18:0-OH.

In order to investigate the negative effects of free fatty alcohols, we created single transformants expressing *MaFAR* gene construct driven by CaMV35S promoter. We generated 10 *MaFAR*-expressing transgenic tobacco plants, which showed reduced fertility and pollen production and could be rescued by hand-pollination. During the second regeneration, they were mainly killed (approximately 80%) by over-expression of *MaFAR*. The lethal phenotype was observed even within non-selective media without kanamycin, where the majority of the seedlings died. Total free fatty alcohol analyses of the dead and live seedlings showed that total free alcohols were six-fold higher in dead seedlings than in their live counterparts. These results strongly suggest that the free alcohols unbound to wax esters affected plant survival. Therefore cell death and obvious chlorophyll degradation might also be the reason for the phenotypically abnormal *tpMaFAR::MhWS* transgenic plants observed (Figure 12).

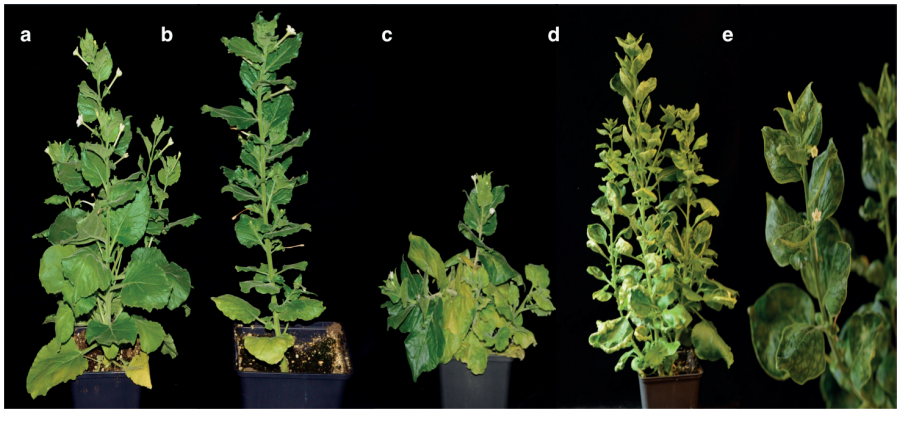

Figure 12. General appearance of transgenic *Nicotiana benthamiana* plants. (a) Wild type, (b) a representative 35S:*tpMaFAR::MhWS* line 2.10 transformant, (c) a representative 35S:*tpMaFAR::MhWS* line 6.1 transformant and (d-e) a representative 35S:*tpMaFAR* line. Plants depicted at eight weeks from sowing.

Earlier reports and Papers I-III indicate a bright future for creating new oil crops with designer products such as wax esters in order to preserve natural resources by providing alternative energy crops for the chemical industry. While still far from being possible on a large scale for commercial use, we demonstrated the possibility of producing wax esters in green tissues of *N. benthamiana* plants and of developing this elegant system for large-scale production of tailor-made products for a changing environment.

3.4 Endosperm-specific expression of the *Arabidopsis* gene *WRI1* in transgenic rice plants increases oil contents in the endosperm (IV)

The most common form of vegetable oil is stored as triacylglycerol (TAG) in the storage tissues of plant organs such as seeds and fruits. While seed TAG content is rather high, in order to provide energy for seedlings after germination, there is not a considerable amount of oil stored in the leaves. TAG is usually stored in the embryo and scutellum compartments of the cereal grain, while the endosperm is rich in starch granules. In order to increase the energy density of cereal grains, carbon channelling from starch to TAG in endosperm can be achieved by metabolic engineering of seed lipid metabolism. Earlier studies on maize showed that a transcription factor, WRINKLED1 (WRI1), plays a pivotal role in the flow of carbon to oil by directly activating genes involved in fatty acid synthesis and controlling genes for assembly and storage of TAG. The overexpression of *WRI1* shows clear increases in the embryo but no significant changes in the endosperm (Shen *et al.,* 2010). In Paper IV, the aim was to over-express *Arabidopsis WRI1* in endosperm of rice seeds and also transiently express it in tobacco leaf tissues.

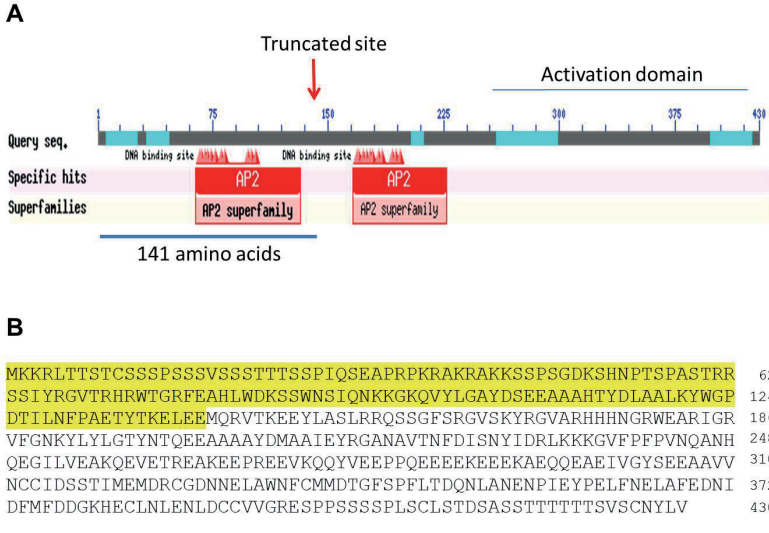

Figure 13. (A) The primary schematic structure of WRI1. Truncated site is shown with red arrow. (B) Amino acid (aa) sequence of AtWRI1. The missing 141aa is indicated in yellow.

The *Arabidopsis WRINKLED1* (GenBank ACC No: NM_202701.2 for the corresponding gene) encodes for a transcription factor that consists of two

APETALA2 (AP2) domains at the protein N-terminus, including the N-terminal AP2 domain for its functionality (Figure 13). We constructed a truncated WRI1 without 141 N-terminal amino acids, and also a full-length *WRI1*. In order to test these two constructs in *N. benthamiana* and rice, they were fused to *35S* and *HvSBEIIb* promoters, respectively. Four constructs were made: *p35S:Full-WRI1, p35S:Tr-WRI1, pHvSBEIIb:Full-WRI1* and *pHvSBEIIb:Tr-WRI1,* respectively.

In order to test the functionality of *WRI1* in *N. benthamiana* leaves, we infiltrated Tr-*WRI1* and Full-*WRI1* together with p19 protein as performed earlier (Papers I and II). Five days after inoculation, the infiltrated areas were excised and used for detection of transcripts and further lipid extraction. The semi-PCR data demonstrated the presence of both types of *WRI1* in *N. benthamiana* leaves. Total lipid extracts were then separated on TLC (Thin Layer Chromatography) plates, which clearly showed accumulation of TAG upon expression of both versions of *WRI1*. The TAG accumulation areas were scraped and quantified with GC (Gas Chromatography). The results showed that both individual expressions Tr-WRI1 and Full-WRI1 were capable of accumulating TAG in leaves of *N. benthamiana*. The total TAG amounts were recorded as 0.42% of leaf dry weight (DW) upon infiltration of Tr-*WRI1* and 1.4% of leaf DW upon infiltration of Full-*WRI1*. In other words, there was a five-fold increase with expression of Tr-*WRI1* and a 17-fold increase with Full-*WRI1* expression compared with the corresponding controls. The composition of TAG on leaf tissues was dominated by 16:0, 18:0, 18:1, 18:2 and 18:3, as reported previously (Vanhercke *et al.,* 2013a). However, the change range in 18:1 between truncated and full-length *WRI1* was noticeably higher in Paper IV.

By using the same sequences of Tr-*WRI1* and Full-*WRI1* driven *HvsbeIIb* p, we generated transgenic rice plants using the Minghui 86 (MH86) cultivar by *Agrobacterium*-mediated transformation. We generated 10 lines for Full-*WRI1* and seven lines for Tr-*WRI1*. Screening of homozygotes is ongoing. Southern blot analysis showed that almost every line carries a single insertion. One line from each transformation (Full-*WRI1*-18 and Tr-*WRI1*-35) was selected for gene expression analysis in endosperm tissues during rice seed development. Gene expression analysis showed that expression of *AtWRI1* was significantly higher than wild-type (WT), with an expression peak around 11 days after flowering (daf) in both full-length and truncated transformants. Carefully dissected endosperm tissues from rice seeds were used for TLC and GC analysis to quantify TAG. The results showed that both of the *AtWRI1* versions were capable of enhancing TAG production in rice endosperm. Tr-*WRI1* was active for enhancing TAG synthesis. By overexpression of Tr-*WRI1* four-fold

increase of TAG was found and seven-fold with overexpression of Full-Wri1 compared to the control. The composition of TAG in rice endosperm was slightly different than that in tobacco leaves. The dominant compounds consisted of 16:0, 18.1 and 18.2, and the level of difference with truncated and full length *WRI1* with regard to 18.1 was not as considerable as observed in tobacco leaves. The production of TAG molecules was also seen under TEM (Transmission Electron Microscopy) in the Tr-*WRI1*-15 transformants, but not in the control.

In conclusion, we showed in Paper IV that both truncated and full length *Arabidopsis WRI1* were able to activate the genes taking part in TAG assembly in tobacco leaf and rice endosperm. Further molecular and biochemical analyses will be needed in order to identify the mechanism of how Tr-*WRI1* functions without 141 aa from the N-terminal. It would also be interesting to analyse the individual expression of genes that are regulated by *WRI1*, in order to understand why 18:1 composition was higher in tobacco. One reason might be that *WRI1* is still functional for a number of genes in TAG assembly, and some were not activated because of the missing aa sequence. Therefore, this might cause for the many-fold changes in total TAG accumulation between the two constructs. All in all, the data demonstrate that both constructs are capable of enhancing TAG biosynthesis in the leaf tissue of tobacco and rice endosperm. Paper IV made progress in understanding the AtWRI1 structural functionality and in increasing TAG content in cereal endosperm tissues.

4 Conclusions

- Wax ester production at a maximum rate of 0.9% DW was achieved by introducing a fatty acyl-reductase (FAR) and a wax ester synthase (WS) into chloroplast lipid metabolism in *N. benthamiana* leaves via an agro-infiltration system. It was possible to express both bacterial- and plant-derived genes for wax ester biosynthesis *in vivo*, resulting in different fatty acid profiling.
- *Marinobacter* FAR (MaFAR) proved superior to *Arabidopsis* FAR (AtFAR6) for production of primary fatty alcohols, mainly consisting of C16:0-OH and C18:0-OH. AtPES2 was more efficient in esterifying short- and medium-chain primary fatty acids than bacterial WS (MhWS).
- A fusion polypeptide (tpMaFAR::MhWS) consisting of catalytic domains of MaFAR and MhWS was able to produce wax esters in the chloroplast. AtWRI1 generally did not enhance wax ester biosynthesis.
- The RNAi hairpin construct of two isomers of *KASII* was able to significantly reduce the two gene expression levels in *N. benthamiana*. The RNAi construct with a common region of the isomers had the best effect for inhibiting total transcript levels of the genes.
- AtFAR6 proved more efficient than MAFAR in utilising C16 fatty acid pools after inhibition of *KASII*, which in consequence increased wax esters more efficiently. There was a good correlation between C16/C18 ratio and total wax ester content in *AtFAR6*-containing combinations. The total level of wax esters reached 0.9% of DW in *AtFAR6* combinations and 2% of DW in *MaFAR*-containing combinations.
- The fusion polypeptide was successfully used to generate stable transformed *N. benthamiana* plants by a tobacco transformation

system. Wax esters of similar quantity and quality were produced in leaf and stem tissues. The phenotypes of the transgenic plants were somewhat different due to remaining fatty alcohols. The free fatty alcohols caused a lethal phenotype in *MaFAR* transformants.

- TAG accumulation can be improved by over-expression of Tr-*WRI1* and Full-*WRI1* in rice endosperm and tobacco leaf tissues. The relationship between the structure and function of *AtWRI1* is under investigation.

5 Future Perspectives

The aim of this thesis was to increase the energy density of selected plant tissues by reprogramming the carbon flux to TAG biosynthesis in the endosperm part of the monocotyledonous model crop rice (*Oryza sativa*); and to achieve wax ester biosynthesis both transiently and with stable transformation in tobacco plants (*Nicotiana benthamiana*). We introduced a new metabolic pathway where manipulation of gene expression was possible with the final target of wax ester biosynthesis in chloroplast of tobacco. I suggest the following perspectives for future research:

- Transient expression of RNA interference with *KASII* genes for instance showed the possibility of increasing palmitoyl substrates. In this context, tests on suppression of saturation (SAD) or thioesterase enzymes such as FatA or FatB would be interesting in order to achieve greater yields of palmitoyl and stearoyl substrates in the chloroplast for extending wax ester metabolic engineering.
- We demonstrated that both plant- and bacterial-derived FARs resulted in production of medium chain fatty alcohols. To increase the production of primary fatty alcohols with different chain length and their usefulness in wax ester biosynthesis, it would be interesting to identify another FAR enzyme with high activity that can conduct reduction reactions of shorter or longer fatty acids when expressed transiently. Transient expression in *N. benthamiana* would then be an elegant system to test new variants of FARs, which could produce fatty alcohols with desired substrate specificities.
- Wax esters consisting of medium chain fatty alcohols and fatty acids are of interest for industrial applications. We found that AtPES2 has clear substrate specificity for shorter chain fatty acids than C16, while MhWS has preferences for medium chain fatty acids consisting of C16 and C18. However, the high levels of free alcohols, even in the

presence of MhWS, indirectly indicated that MhWS is not sufficient for esterification purposes in our system. Therefore, it would be of great interest to identify another wax synthase with higher activity to C16 and C18 fatty acids in order to produce industrially important medium-chain wax esters at greater extents.

- The accumulation of free fatty alcohols in the chloroplast of *N. benthamiana* leaves expressing *AtFAR6* and especially *MaFAR* resulted in necrotic lesions on the leaf surface of both transient and transgenic approaches. Moreover, individual expression of *MaFAR* in tobacco resulted in lethality in transgenic lines. These indicate that fatty alcohols are toxic compounds for cell survival. To overcome this complexity, introduction of a new wax synthase enzyme to prevent detrimental effects of primary alcohols would be great of interest for future work on stabilisation of the wax ester producing plants. Moreover, new variants of promoters, such as inducible promoters to decrease the negative effect of fatty alcohols for plant survival, would be interesting for commercial applications of wax esters at larger scales. It would also be interesting to study how the seed viability of the transgenic lines expressing an alcohol-forming gene is affected.
- The accumulation of TAGs in the endosperm of rice was successful, although the levels obtained were too low for commercial interests. It would be interesting to test other transcription factors/genes participating in TAG biosynthesis by a modularity of new promoters to reach commercially interesting levels. It will be necessary to investigate the functionality of truncated WRI1 and its effect on TAG assembly at enzymatic level. Inhibition/reduction of starch biosynthesis and redirection of carbon flux from starch to TAG biosynthesis is another issue to investigate in rice endosperm.

The results presented in this thesis, such as the newly introduced wax ester pathway in vegetative tissues and TAG accumulation in seeds, will hopefully help in the identification of new possible paths for increasing energy levels in other plants, for creation of a bio-based environment. The new possible routes for understanding lipid metabolism can encourage scientists to efficiently manipulate new classes of lipids with desired chemical structures and also to increase the energy density in tissues that do not naturally contain oils.

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