

Fatty Acid Reductases (FAR): Insights into the Biosynthesis of Fatty Alcohols in Plants and Bacteria

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Abstract

Primary fatty alcohols are present in all phyla, where they serve various unique biological functions, either in structural materials or biochemical compounds. Fatty alcohols and derivatives provided by living organisms are also important feedstock for the oleochemical and lubricant industries. Since they are biodegradable and generally non-toxic, they can also be used in the manufacture of cosmetics and pharmaceuticals.

Fatty alcohols are produced by Fatty Acid Reductases (FAR), which catalyse the reductions of fatty acyl-CoA/ACPs. The reductions are conducted in two consecutive reactions; a fatty acyl-CoA/ACP is first reduced to a fatty aldehyde, which is then further reduced into a fatty alcohol. In prokaryotes, the two-step reduction has until now been considered to be catalysed by two different enzymes, while in eukaryotes it has been found that one enzyme carries out both reduction steps via an intermediate fatty aldehyde, which is thought not to be released during the reductions.

This thesis presents data on the *in vivo* and *in vitro* characterisation of FARs from *Arabidopsis thaliana* (AtFARs) and *Marinobacter aquaeolei* VT8 (Maqu_2220). Contrary to available literature, Maqu_2220 was found to catalyse both steps in the reductions of fatty acyl-CoA/ACPs into fatty alcohols. Thus, at least two biochemical pathways exist among prokaryotes for the reductions of activated fatty acyl chains to fatty alcohols, one of which is the above-identified activity in analogy with eukaryotic FARs. Characterisation of AtFAR6, AtFAR2/MS2 and the bacterial Maqu_2220 revealed that under *in vitro* conditions, intermediate fatty aldehydes were released and free fatty acids were observed, in addition to fatty alcohols produced as end-products.

The *in vitro* characterisation of AtFAR2/MS2 and AtFAR6 showed that the ratio of fatty alcohol/fatty aldehyde produced by these enzymes depends strongly on chain length and saturation state of the substrates and substrate concentration. Both *in vitro* and *in vivo* data consistently showed that the highest activity of AtFAR2/MS2 and AtFAR6 is for the production of C16:0-alcohol from C16:0-CoA/ACP substrates. In a subsequent study, AtFAR6 was found to be a chloroplast-localised FAR enzyme involved in production and accumulation of C16:0-alcohol within this organelle.

Keywords: *Arabidopsis thaliana*, *Marinobacter aquaeolei* VT8, fatty acid reductase, fatty alcohol, fatty aldehyde, wax ester

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Dedication

To my beloved family

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List of Publications

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

- I Doan, T.T.P., Carlsson, A.S., Hamberg, M., Bülow, L., Stymne, S. & Olsson, P. (2009). Functional expression of five Arabidopsis fatty acyl-CoA reductase genes in *Escherichia coli*. *Journal of Plant Physiology* 166(8), 787-796.
- II Doan, T.T.P., Domergue, F., Fournier, A.E., Vishwanath, S.J., Rowland, O., Moreau, P., Wood, C.C., Carlsson, A.S., Hamberg, M. & Hofvander, P. (2011). Biochemical characterization of a chloroplast localized fatty acid reductase from *Arabidopsis thaliana*. *BBA – Molecular and Cell Biology of Lipids*. (Accepted)
- III Doan, T.T.P., Carlsson, A.S. & Hofvander, P. (2011). Functional characterization of AtFAR2, a fatty acid reductase from *Arabidopsis thaliana*. (Manuscript)
- IV Hofvander, P., Doan, T.T.P. & Hamberg, M. (2011). A prokaryotic acyl-CoA reductase performing reduction of fatty acyl-CoA to fatty alcohol. *FEBS Letters* 585, 3538-3543.

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The contribution of Thuy Doan to the papers included in this thesis was as follows:

- Paper I I performed large part of the experiments and data analyses. I discussed the results and wrote the paper together with the co-authors.
- Paper II I planned and performed large part of the experiments and data analyses. I discussed the results and wrote the paper together with the co-authors.
- Paper III I planned and performed all experiments and data analyses. I discussed the results and wrote the paper together with the co-authors.
- Paper IV I was involved in developing the protocol for the *in vitro* assays. I prepared the samples (*in vitro* assay, product derivatization and extraction) for GC-MS analysis.

Abbreviations

aa	Amino acid
AtFAR	<i>Arabidopsis thaliana</i> fatty acid reductase
BSA	Bovine serum albumin
DGAT	Acyl-CoA:diacylglycerol acyltransferase
FAR	Fatty acid reductase
FAS	Fatty acid synthase
FFA	Free fatty acid
GC	Gas chromatography
GC-MS	Gas chromatography – mass spectrometry
GFP	Green fluorescence protein
GUS	β -glucuronidase
IPTG	Isopropyl β -D-1-thiogalactopyranoside
kDa	Kilodalton
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
PCR	Polymerase chain reaction
TLC	Thin layer chromatography
UPGMA	Unweighted Pair Group Method using Arithmetic averages
WS	Wax synthase
YFP	Yellow fluorescence protein

1 Introduction

Primary fatty alcohols are aliphatic hydrocarbons containing a hydroxyl group in the terminal position. In nature, fatty alcohols are found with chain lengths from 8 to 34 carbons, with different levels of branching and/or unsaturation. Primary fatty alcohols are produced by various organisms, such as bacteria, protists, insects, plants, fish, birds, mammals, *etc.* (Domínguez *et al.*, 2011; Svend, 2009; Cheng & Russell, 2004a; Patel *et al.*, 2001; Phleger, 1998; Wang & Kolattukudy, 1995b; Sand *et al.*, 1969). They are present either in free form or in the form of alkyl esters and ether lipids. In many organisms, fatty alcohols are components of epicuticular waxes, which protect the organism from desiccation, ultraviolet light and pathogen attack (Domínguez *et al.*, 2011; Kunst & Samuels, 2009; Svend, 2009; Patel *et al.*, 2001). In addition, fatty alcohols in plants are constituents of suberin and pollen wall and also serve as structural and protective barriers in plant tissues. Besides being structural components, fatty alcohols are the direct substrate for wax ester and ether lipid biosynthesis, and also the precursor of pheromones. These examples illustrate the important roles of fatty alcohols in energy storage and species attraction/recognition (Lassance *et al.*, 2010; Cheng & Russell, 2004b; Kunst & Samuels, 2003; Lardizabal *et al.*, 2000). Since fatty alcohols are synthesised from fatty acids, the central substrates of lipid metabolism, the regulation involved in the biosynthesis of fatty alcohols essentially affects the metabolism of lipids in the cells and thus cell biological functions (Kunst & Samuels, 2009; Kunst *et al.*, 2007; Kunst & Samuels, 2003; Post-Beittenmiller, 1996).

Besides being essential in biological functions, fatty alcohols play important roles in the oleochemistry industry together with fatty acids, glycerine and methyl esters. Here, the major usage for fatty alcohols is in the production of soap, detergents and cosmetics (Rupilius & Ahmad, 2006) and also in the production of inks, surfactants and lubricants. In particular, wax esters,

which possess superior lubrication properties, have great potential as feedstock in the lubricant industry, besides being universally used in cosmetics nowadays (Carlsson *et al.*, 2011).

The conversion of a fatty acid into a fatty alcohol is catalysed by the enzyme fatty acid reductase (FAR) via a fatty aldehyde intermediate. A high diversity of fatty alcohols and their derivatives is found among organisms, species and even tissues, in terms of both quantity and composition. This suggests that there is a high degree of diversity among the enzymes involved in nature.

Although FAR enzymes have been studied in a variety of organisms since the 1970s (Riendeau & Meighen, 1985), it was not until 2000 that the common understanding of fatty alcohol biosynthesis was greatly improved by the first cloning and characterisation of a *FAR* gene from jojoba (*Simmondsia chinensis*), a desert shrub with seeds that accumulate liquid wax esters to up to 60% of seed dry weight (Metz *et al.*, 2000). This jojoba FAR gene and protein sequence has since become the reference for studying fatty acid reductase homologues in other species.

Until now, the biosynthesis of fatty alcohols in eukaryotes and prokaryotes has been believed to be different. In prokaryotes, two enzymes have been reported to be involved in the reduction of fatty acid into fatty alcohol, the first of these being an aldehyde-forming FAR and the second a fatty aldehyde reductase enzyme (Fig. 1)(Wahlen *et al.*, 2009; Reiser & Somerville, 1997). In eukaryotes, one enzyme has been found to catalyse the whole procedure and these enzymes are defined as alcohol-forming FARs (Fig. 1). They have a molecular mass of about 56 to 58 kDa (Metz *et al.*, 2000; Vioque & Kolattukudy, 1997), whereas the aldehyde-forming FARs are smaller, with a molecular mass of around 28 kDa in garden pea (Vioque & Kolattukudy, 1997), 32.5 kDa in *Acinobacter calcoaceticus* and 35 kDa in the green algae *Botryococcus braunii* (Reiser & Somerville, 1997; Wang & Kolattukudy, 1995a).

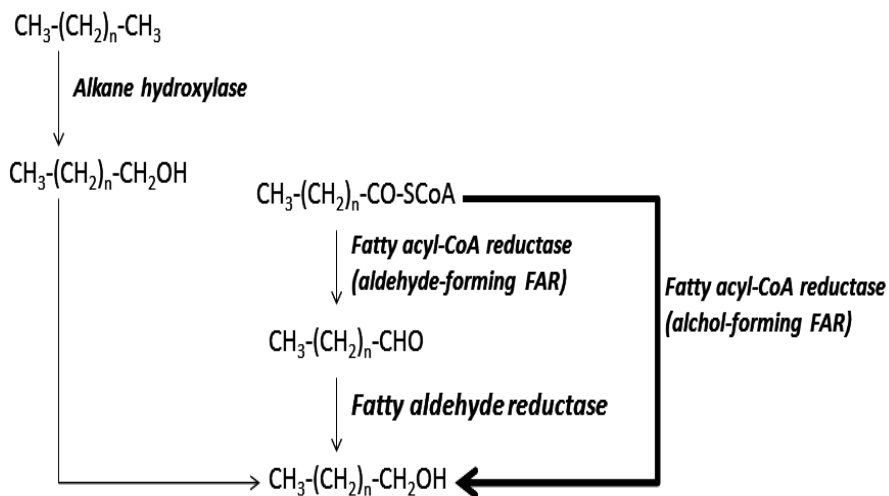


Figure 1: Fatty alcohol biosynthesis in eukaryotes and prokaryotes. Thin arrows: the prokaryotic pathway. Thick arrow: the eukaryotic pathway.

The approach in the present thesis was to study FAR homologues from *Arabidopsis thaliana* (*Arabidopsis*) and *Marinobacter aquaeolei* VT8 with the overall aim of obtaining important knowledge on the biosynthesis of fatty alcohols in both eukaryotic and prokaryotic cells.

Arabidopsis has many advantages as a model system for plants, such as having a short life cycle, a low number of chromosomes ($2n=10$) and a small (114.5 Mb), fully sequenced genome. Intensive research on different aspects of lipid metabolism and biosynthesis has been carried out in *Arabidopsis*, with remarkable progress. It is therefore useful to study fatty alcohol biosynthesis in this plant species. Using jojoba FAR as a reference sequence, eight FAR homologues have been identified from the genome of *Arabidopsis* (Rowland *et al.*, 2006; Costaglioli *et al.*, 2005; Suh *et al.*, 2005). Studying the activity of these FAR homologues from *Arabidopsis* presents an opportunity to obtain insights into the biosynthesis of fatty alcohols in plants in particular and eukaryotes in general.

Phylogenetic analysis of functionally characterised FARs from various organisms, including *Arabidopsis* FAR homologues, has revealed that there are two groups of FARs. Group I contains copepod, insect, animal and *Euglena* FARs (Fig. 2), while Group II contains plant FARs (Fig. 2). In each group, sub-groups with similarity either in functionality or species have been found. For example in Group I, sub-groups of the pheromone gland-specific FARs and animal FARs have been identified. The copepod FARs

and honeybee FAR, which have a wide range of substrate specificity, are closely related, thus gathering in another sub-group. As for Group I, several sub-groups are also present in Group II (Fig. 2), thus suggesting that these enzymes potentially have different characteristics.

A BLAST search among microbe genomes revealed that *Marinobacter aquaeolei* VT8 also contains a gene (Maqu_2220) with relatively high amino acid sequence similarity to jojoba FAR and the homology level of this gene to jojoba FAR is higher than that of mouse (Cheng & Russell, 2004a), copepod (Teerawanichpan & Qiu, 2011), insect (Lassance *et al.*, 2010; Liénard *et al.*, 2010; Teerawanichpan *et al.*, 2010; Antony *et al.*, 2009; Moto *et al.*, 2004) and even Euglena FARs (Teerawanichpan & Qiu, 2010) (Fig. 2). It is therefore expected to have the activity of an alcohol-forming FAR.

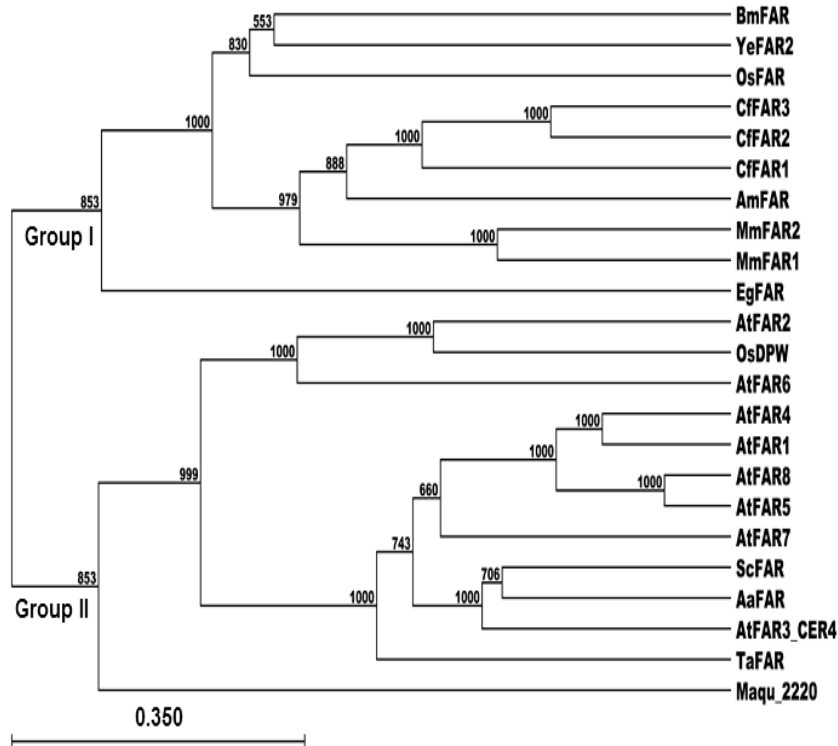


Figure 2: Phylogenetic analysis of functional characterized FARs from different organisms and FAR homologues from *Arabidopsis thaliana* and *Marinobacter aquaeolei* VT8. The analysis was conducted using UPGMA algorithm with 1000 bootstrap replicates (CLC DNA Workbench 6.1). The details of the analyzed protein sequences are listed in Appendix 1.

Specific objectives of the work in this thesis were to:

- Investigate whether the FAR homologues from *Arabidopsis thaliana* show fatty acyl-CoA reductase activity and characterise any differences in the activity of these FAR homologues (**Paper I**).
- Characterise biochemical aspects of AtFAR enzymes that contain putative chloroplast transit peptides (**Papers II, III**).
- Investigate the activity of Maqu_2220, a FAR homologue from *Marinobacter aquaeolei* VT8 (**Paper IV**).

The FAR enzymes were characterised here using different approaches. Enzymes were characterised *in vivo* using different expression systems such as *Escherichia coli*, *Saccharomyces cerevisiae* and *Nicotiana benthamiana*, in addition to fusion expressions with reporter genes such as β -glucuronidase (GUS) and fluorescence protein genes (YFP and GFP). The *in vitro* characterisations were carried out using purified enzymes.

2 Occurrences of primary fatty alcohols and derivatives in plants and bacteria

There are three outputs of fatty alcohols in plants and bacteria: the free form of fatty alcohols; wax esters that are the monoester of a fatty acid and a fatty alcohol; and ether lipids that are created from the dehydration of a fatty alcohol and another alkyl moiety such as triacylglycerol or phospholipids. While free fatty alcohols and wax esters are present in abundance in plants and bacteria, ether lipids are not present in appreciable amount in plants, if they occur at all (Weber & Mangold, 1987; Weber & Benning, 1985; Mangold & Paltauf, 1983). In bacteria, ether lipids are found in anaerobic bacteria as membrane constituents (Fritz, 1994; Goldfine & Langworthy, 1988).

In this thesis, the focus was on free form primary fatty alcohols and to some extent on wax esters (Fig. 3).

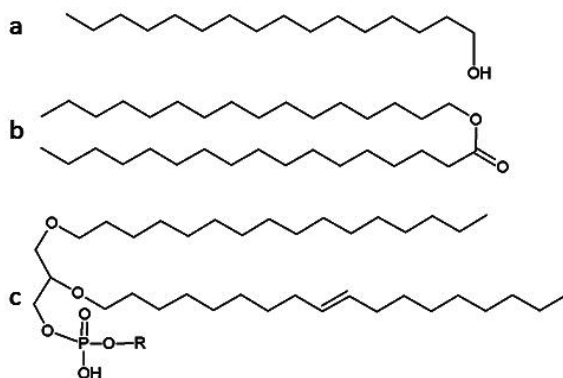


Figure 3: Example structures of fatty alcohol (a), wax ester (b), ether phospholipid (c)

2.1 Occurrences of primary fatty alcohols and derivatives in plants

Primary fatty alcohols and their derivatives with diverse chemical structures and biological functions are present in all plant species. They are commonly found in cuticular waxes, suberin, sporopollenin or seeds, with carbon chain length ranging from 14 to 34 carbons depending on the compound of which they form part (Chen *et al.*, 2011; Shi *et al.*, 2011; Domergue *et al.*, 2010; Molina *et al.*, 2006; Lardizabal *et al.*, 2000).

2.1.1 Primary fatty alcohols and derivatives for protective functions

Cuticular waxes are a complex mixture of fatty acids, fatty aldehydes, alkanes, ketones, wax esters and primary and secondary fatty alcohols (Buschhaus & Jetter; Jetter *et al.*, 2006). Cuticular waxes are components of plant cuticle, which covers the aerial parts of the plant, protecting it from adverse conditions such as UV light, drought and attack by pathogens and herbivores (Buschhaus & Jetter, 2011; Suh *et al.*, 2005; Riederer & Schreiber, 2001). In cuticular waxes, the fatty alcohols are found with carbon chain length from 18 to 34 carbons, either in a free form or in the form of wax esters (Jetter & Kunst, 2008; Allebone & Hamilton, 1972b; Miwa, 1971). However, in petunia petals, moieties of shorter chain fatty alcohols \leq C12:0 and even C4:0, C6:0 alcohols are present in the wax esters (King *et al.*, 2007). Instead of being accumulated at low quantities as commonly seen in plants, the carnauba palm (*Copernicia prunifera*) accumulates a massive amount of cuticular waxes on the leaf surfaces. The composition of these waxes includes free aliphatic fatty alcohols to 10-16%, wax esters of C32 and C34 alcohols and fatty acids of C16 to C20 carbon chain to 80-85%. In several plant species, the chain length patterns of the free and esterified alcohols are similar, indicating that they are derived from a common pool of fatty alcohol precursors (Lai *et al.*, 2007; Rowland *et al.*, 2006; Allebone & Hamilton, 1972b; Miwa, 1971; Miwa & Wolff, 1962).

Suberin is a complex mixture of fatty acids, ω -hydroxyl fatty acids, glycerol, α,ω -dicarboxylic acids, hydroxycinnamic acids and fatty alcohols. Fatty alcohols of suberin have been found to contain 14 to 22 carbons in their chain length (Pollard *et al.*, 2008; Molina *et al.*, 2006). Suberin is deposited at specific cell wall locations during plant development of both external and internal tissues such as bark tissues (the periderm of secondary growth stems and roots), the endoderm of roots and the bundle sheath cells

of monocots (Pollard *et al.*, 2008; Li *et al.*, 2007; Kolattukudy *et al.*, 2001). Suberin synthesis is also induced in sealing wounds and in stressed tissues (Domergue *et al.*, 2010; Pollard *et al.*, 2008). Besides serving as a protection barrier for plants against abiotic and biotic stresses, root suberin is also important in controlling water and ion uptake (Schreiber, 2010; Pollard *et al.*, 2008; Allebone & Hamilton, 1972a).

Sporopollenin is the major constituent of the outer cell wall of pollen grains and is made up of a mixture of fatty acid derivatives and phenolic compounds. Besides serving as a protective layer, sporopollenin influences pollination through controlling the release of proteins involved in pollen-pistil interaction (Hesse, 2000). Recently, C16:0-alcohol was found to be an important component of sporopollenin of the exine layer (Chen *et al.*, 2011; Shi *et al.*, 2011). A lack of C16:0-alcohol results in abnormality of the pollen exine layer, thus affecting pollination (Chen *et al.*, 2011; Shi *et al.*, 2011; Dobritsa *et al.*, 2009; Aarts *et al.*, 1997).

Plant fatty alcohols and wax esters evidently not only play an important role in plant self-protection, but also contribute to regulating the anatomy, morphology and thus the integrity of plant tissues.

2.1.2 Primary fatty alcohols and derivatives for storage functions

Seeds in most cases accumulate TAG as the storage lipid. In contrast, the species jojoba (*Simonsia chinensis*) accumulates about 60% wax esters of seed dry weight. These wax esters are the esterified forms of monounsaturated C20 to C24 alcohols and unsaturated C18 to C24 fatty acids, with the majority of the wax ester consisting of a C22:1-alcohol and a C20:1 fatty acid (Metz *et al.*, 2000; Miwa, 1971).

2.2 Occurrences of fatty alcohols and derivatives in bacteria

Fatty alcohols, commonly in the form of wax esters, are found in a number of bacterial species, for example *Mycobacterium tuberculosis*, *Micrococcus cryophilus*, *Norcadia* and *Corynebacterium*, as well as bacteria in the genus *Acinetobacter* and some bacterial strains belonging to the genera *Marinobacter*, *Pseudomonas*, *Neiseria*, *Micrococcus* and *Fundibacter* (Wältermann & Steinbüchel, 2006; Ishige *et al.*, 2003). Wax esters can be produced under nitrogen-limited conditions to serve as energy storage in cells and are also involved in the regulation of membrane fluidity, as seen in *Micrococcus cryophilus* (Wahlen *et al.*, 2009; Reiser & Somerville, 1997; Lloyd & Russell, 1983).

In *Acinetobacter calcoaceticus*, the wax esters can account for 25% of cellular dry weight (Wältermann *et al.*, 2005). These wax esters are combinations of fatty acids (C14:0, C16:0, C18:0) and fatty alcohols (C14:0, C16:0, C16:1, C18:0, C18:1, C20:0) (Fixter *et al.*, 1986). It has been shown that the chemical structure of wax esters depends strongly on the assimilated carbon sources as well as the growing temperature (Wältermann & Steinbüchel, 2006). Feeding experiments with *Acinetobacter* show that the carbon chain length of the wax esters is significantly affected by the chain length of the exogenous fatty alcohols, whereas the changes in the chain length of exogenous fatty acids show a minor influence on the wax esters (Kaneshiro *et al.*, 1996). It has also been found that the wax ester synthase activity of *Acinetobacter calcoaceticus* WS/DGAT (wax ester synthase/acyl-CoA:diacylglycerol acyl transferase) is more sensitive to changes in fatty alcohols than to changes in fatty acids (Kalscheuer *et al.*, 2003). This suggests that the chain length of the fatty alcohols determines the chain length of the wax esters in this species (Rontani & Timmis, 2010; Kalscheuer & Steinbüchel, 2003; Kaneshiro *et al.*, 1996).

3 Enzymes of fatty acid, fatty alcohol and wax ester biosynthesis in plants and bacteria

3.1 Enzymes in *de novo* biosynthesis of fatty acids in plants and bacteria

It is well known that activated fatty acids, fatty acyl-CoA and fatty acyl-ACP, are substrates in the biosynthesis of fatty alcohols. In plants, there is high homology in chain length distribution of fatty acids and fatty alcohols in waxes across diverse plant species, suggesting a tight correlation between the biosynthesis of fatty alcohols and fatty acids of plant waxes (Samuels *et al.*, 2008). In plant cells, *de novo* lipid synthesis is initiated in chloroplasts or other types of plastids using acetyl-CoA as the precursor. Acetyl-CoA is first carboxylated to form malonyl-CoA by the enzyme acetyl-CoA carboxylase (ACCase). The resulting malonyl-CoA then becomes the 'carbon donor' for elongation reactions catalysed by the complex of multiple enzymes fatty acid synthase (FAS II). By the catalysation of malonyl-CoA: ACP transacylase of the FAS complex, malonyl-CoA is transformed into malonyl-ACP. The addition of two carbons from malonyl-ACP into the backbone of the acyl-ACPs is then catalysed by one of three 3-ketoacyl-ACP synthases (KAS I, II, III). KAS III is strictly specific for the condensation of C2:0-ACP to produce C4:0-ACP, KAS I is responsible for the extension of C4:0-ACP to form up to C16:0-ACP and KAS II catalyses the condensation of C16:0-ACP to form C18:0-ACP. Each of the condensation steps is consequently followed by β -keto reduction, dehydration and an enoyl reduction reaction carried out by enzyme units of the FAS complex (3-ketoacyl-ACP reductase, hydroxyacyl-ACP dehydrase and enoyl-ACP reductase, respectively) to finally produce saturated fatty acyl-ACPs with up to 16 and 18 carbons (Fig. 4). The fatty acyl-ACPs produced are to a high degree

desaturated by acyl-ACP desaturase (commonly stearyl-ACP desaturase) to form 18:1-ACP. The acyl chains are finally hydrolysed from ACP by acyl-ACP thioesterase. There are two types of acyl-ACP thioesterase, FATA specific for 18:1-ACP; FATB has high specificity to fatty acyl-ACPs with saturated acyl groups such as C16:0-ACP and 18:0-ACP. In some cases such as California bay tree (*Umbellularia California*), *Cuphea* spp. and coconut (*Cocos nucifera*), other thioesterases with specificity to C10 and C12 fatty acyl-ACPs are also found (Ohlrogge & Jaworski, 1997). The released fatty acids by thioesterases are then transferred to glycerolipid or exported to the cytosol for further elongation (Ohlrogge & Browse, 1995) (Fig. 4).

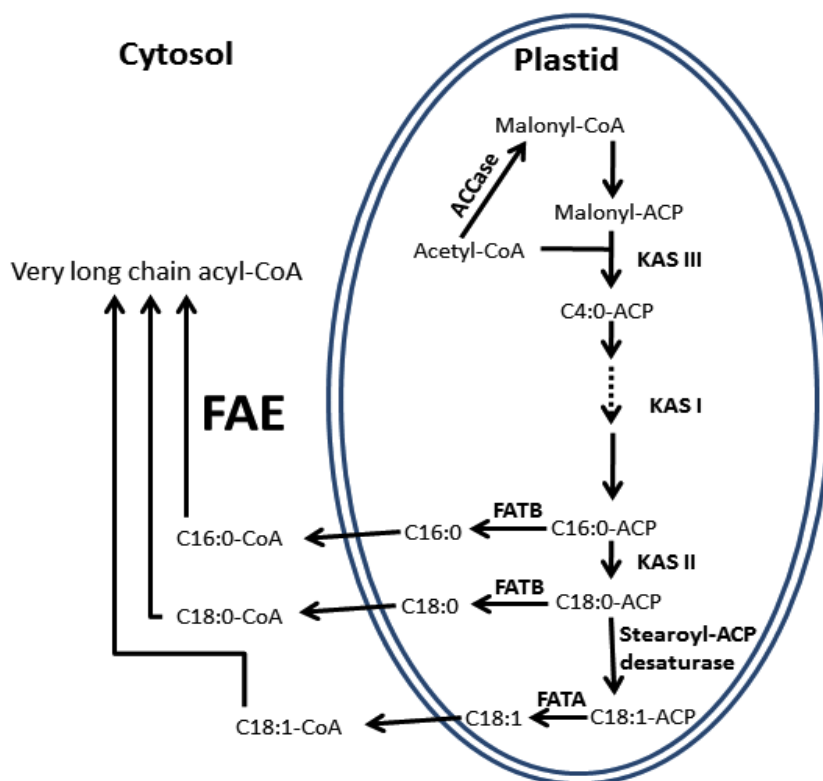


Figure 4: *De novo* fatty acid biosynthesis in plant cells.

It has been found that lipid biosynthesis conducted in the chloroplast is similar to that of prokaryotic cells, *i.e.* bacteria. Although it is still not clear whether there is the presence of bacterial thioesterase with high specificity

to ACP linked fatty acyls (Gong *et al.*, 2011; Klinke *et al.*, 1999; Magnuson *et al.*, 1993). Lipid biosynthesis in the chloroplast is often called the prokaryotic pathway to differentiate it from the eukaryotic pathway of lipid biosynthesis that occurs in the endoplasmic reticulum (ER) (Ohlrogge & Browse, 1995; Magnuson *et al.*, 1993; Heemskerk & Wintermans, 1987; Warwick *et al.*, 1986).

In plants, fatty acids exported from the chloroplast can be further elongated in the ER by membrane-bound fatty acyl-CoA elongase enzymes (FAE) (Kunst & Samuels, 2003). In this process, successive condensations of malonyl-CoA with acyl-CoAs are carried out; these reactions are similar to that catalysed by FAS II in the chloroplast, with the exception that the growing fatty acid chain is anchored to CoA instead of ACP (Kunst & Samuels, 2003; Cassagne *et al.*, 1994; Fehling & Mukherjee, 1991; Pollard & Stumpf, 1980) (Fig. 4). The very long-chain fatty acyl-CoA (VLCFA) synthesised can then act as the substrate for a decarbonylation pathway to form fatty aldehydes, odd-chain alkanes, secondary alcohols and ketones (Kunst & Samuels, 2009; Schneider-Belhaddad & Kolattukudy, 2000; Cheesbrough & Kolattukudy, 1984). Alternatively, the VLCFA can join an acyl reduction pathway to be converted into fatty alcohols and wax esters (Domergue *et al.*, 2010; Kunst & Samuels, 2009; Jetter & Kunst, 2008; Rowland *et al.*, 2006; Post-Beittenmiller, 1996) (see following sections for details).

3.2 Enzymes in biosynthesis of fatty alcohols in plants and bacteria

The biosynthesis of fatty alcohols is conducted via two-step reductions of fatty acyl-CoA/ACP. In bacteria, these two reactions are catalysed by two separate reduction enzymes (Wahlen *et al.*, 2009; Reiser & Somerville, 1997). The substrate is first reduced into a fatty aldehyde by a fatty acyl CoA/ACP reductase (aldehyde-forming FAR) and the fatty aldehyde is then reduced further into a fatty alcohol by a fatty aldehyde reductase (Wahlen *et al.*, 2009; Kalscheuer & Steinbuchel, 2003; Reiser & Somerville, 1997) (Fig. 1). In plants, the two-step process is carried out by a single fatty acyl CoA/ACP reductase (alcohol-forming FAR) and the free fatty aldehyde intermediate is not released (Chen *et al.*, 2011; Shi *et al.*, 2011; Lardizabal *et al.*, 2000; Vioque & Kolattukudy, 1997) (Fig. 1). Besides alcohol-forming FAR, aldehyde-forming reductase is also found in plants, although it is supposed to be involved in a different catabolism pathway, *e.g.* a decarbonylation pathway (Vioque & Kolattukudy, 1997). The fatty alcohols

produced by FARs are determined by the substrate specificity of the enzyme and influenced by the fatty acid profiles of the organisms (Samuels *et al.*, 2008; Wang *et al.*, 2002; Lardizabal *et al.*, 2000). In eukaryotic and multicellular organisms, the fatty alcohol profiles are also influenced by the subcellular location of FAR in the cells, as well as the expression pattern of *FAR* genes (Chen *et al.*, 2011; Shi *et al.*, 2011; Domergue *et al.*, 2010; Rowland *et al.*, 2006).

In addition to the fatty alcohol biosynthesis by FAR enzymes mentioned above, some bacteria can synthesise primary fatty alcohols from n-alkane by the hydroxylation reaction catalysed by alkane hydroxylase enzymes (Kalscheuer & Timmis, 2010; Rojo & Timmis, 2010; Rontani & Timmis, 2010; Ishige *et al.*, 2003; Ishige *et al.*, 2002) (Fig. 1). In plants, there are also enzymes that catalyse the hydroxylation of n-alkane, but this leads to the formation of only secondary fatty alcohols (Domínguez *et al.*, 2011; Kunst & Samuels, 2009; Samuels *et al.*, 2008; Kunst & Samuels, 2003; Post-Beittenmiller, 1996).

3.2.1 Enzymes involved in the biosynthesis of fatty alcohols in plants

Activity of plant FAR enzymes

Although plant FARs have been studied since the 1970s, this had been conducted on heterologous enzymes. In 1997, the first purification and characterisation of FAR from garden pea leaves revealed that garden pea possess a 58 kDa alcohol-forming FAR and that this enzyme utilises NADPH as reductant for the production of fatty alcohol from fatty acyl-CoA (Vioque & Kolattukudy, 1997). Later, the cloning and characterisation of a jojoba FAR revealed that this is also a NADPH-dependent FAR, with a molecular mass of 56 kDa (Metz *et al.*, 2000). More recently, an alcohol-forming enzyme from rice, which was named defective pollen wall (DPW), was also identified as being a FAR enzyme utilising NADPH. The molecular and biochemical data on FAR from garden pea, wheat, jojoba and rice suggest that alcohol-forming FAR enzymes are NADPH-dependent enzymes. The characterisation of wheat, jojoba and rice FARs has also revealed that while the reduction of fatty acyl-CoA/ACP into fatty alcohols by these FARs is conducted via the formation of a fatty aldehyde intermediate, the free fatty aldehyde intermediate has not ever been observed.

In **Papers II** and **III**, the *in vitro* characterisation of AtFAR2/MS2 and AtFAR6 from Arabidopsis was conducted using purified enzymes. This

revealed that similarly to the alcohol-forming FARs from jojoba and pea, the AtFARs characterised (AtFAR2/MS2 and AtFAR6) are also NADPH-dependent FAR enzymes. Interestingly, the *in vitro* data showed that besides fatty alcohol being produced as a final product, fatty aldehyde was also released through the reduction reaction (**Papers II, III**) (Fig. 5). Using fatty aldehyde (C18:0-aldehyde) as the substrate for the AtFAR6 enzyme revealed its ability to convert this substrate into C18:0-alcohol (**Paper II**). In addition to the release of fatty aldehyde, free fatty acid was also observed.

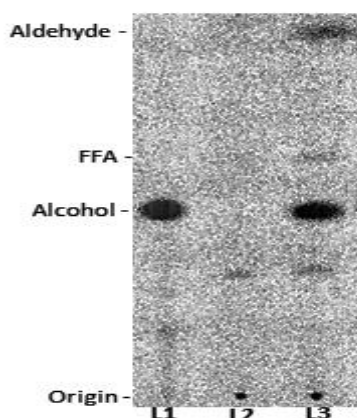


Figure 5: Electronic radiochromatogram of TLC showing the alcohol, aldehyde and free fatty acid produced by mAtFAR6. Assays were conducted in 100mM potassium phosphate buffer (pH 7.0), 50 μ M [14 C] 16:0-CoA, 10mM NADPH and 3 mg/ml BSA at 30°C. L1: [14 C] 18:1-OH standard, L2: assay without adding enzyme, L3: assay using 50 μ g/ml mAtFAR6.

However, neither AtFAR6 nor AtFAR2/MS2 could utilise free fatty acid (C16:0) as a substrate. The results therefore suggested that although the conversion of fatty acyl-CoA/ACP is conducted via the formation of free fatty acid and fatty aldehyde, the CoA and ACP groups in the substrates are necessary for the substrate:enzyme interaction/or recognition of these FARs. It should also be noted that in cells fatty acids are almost never present in the form of free fatty acids (Ohlrogge & Browse, 1995). It was observed that the alcohol/aldehyde ratio of the reduction reaction by AtFAR2 and AtFAR6 depends significantly on the substrate chain length, as well as the concentration of the substrates. The highest reduction products as well as the highest ratio of alcohol/aldehyde were achieved only in optimal conditions (**Papers II, III**). When using C14:0-CoA as a substrate, only fatty aldehydes and free fatty acids were produced (**Papers II, III**). This would suggest that C14:0-CoA is accepted by the enzymes as a

substrate but a substantial portion of the acyl groups are not bound to the protein after cleavage from CoA and thus released as free fatty acids. The proportion of the 14:0 acyl chains that is bound to the enzymes only undergo the first reduction step, with release of the fatty aldehyde. It was also found that the micelle formation of substrates severely affects the activity of AtFAR6 and AtFAR2/MS2, as shown by the significant decrease in enzyme activity at high substrate concentrations. This adverse effect of substrate concentration was removed by the addition of BSA in the assays (**Papers II, III**). At high substrate concentration, the stimulation effect of BSA on enzyme activity was increased up to an optimal BSA concentration, after which it decreased. The results demonstrated that these enzymes can only utilise the free form of substrates and not the micelle form and BSA-bound form of substrates (**Papers II, III**).

Substrate specificity of plant FAR enzymes

Substrate specificity of a FAR enzyme is one of the most important factors in controlling the fatty alcohol profile of the cells and also in determining the biological function of the enzyme. The substrate specificity of plant FAR enzymes was mainly studied at *in vivo* level. In this method, the conclusions regarding the substrate specificity of a certain enzyme are either based on the fatty alcohol profiles of the corresponding mutants or the expression of *FAR* in a heterologous expression system.

The expression of jojoba *FAR* in *E. coli* predominantly results in the production of C14:0, C16:0 and C18:1 alcohols (**Paper I**). The expression of this jojoba *FAR* in *Brassica napus* results in the predominant production of C22:1-OH and the analysis of jojoba wax ester composition of the seeds suggests that jojoba *FAR* is responsible for the production of monounsaturated C20, C22 and C24 alcohols from corresponding fatty acyl-CoAs (Metz *et al.*, 2000). Those results therefore indicate that although jojoba *FAR* has the ability to use various forms of substrates (medium-chain and very long-chain substrates, monounsaturated and saturated substrate), the highest specificity is for the monounsaturated form of very long-chain fatty acyl-CoAs. The fatty alcohols produced by *FAR* then serve as substrates for wax ester biosynthesis in seeds. Similarly to jojoba *FAR*, the heterologous expression of TAA1 from wheat shows that this enzyme has the capacity to use different forms of substrates for producing alcohols through the accumulation of monounsaturated C18 to C22 alcohols, as well as C24:0, C26:0 alcohols, when expressed in tobacco; and the production of C14:0, C16:0 and C18:1 alcohols when expressed in *E. coli* (Wang *et al.*, 2002).

It should be noted that since the jojoba genome sequence is still unknown, it is not known whether the jojoba FAR reported by Metz *et al.* (2000) is the only enzyme responsible for the production of fatty alcohols in seeds. The same holds true for the alcohol-forming FAR from garden pea, which is suggested to be responsible for the production of C26:0 and C28:0 alcohols in pea leaves based on the composition of cuticular leaf waxes (Vioque & Kolattukudy, 1997; Kolattukudy, 1970).

Using the jojoba FAR sequence as the reference, eight FAR homologues have been identified from the genome of Arabidopsis (Rowland *et al.*, 2006; Costaglioli *et al.*, 2005; Suh *et al.*, 2005). **Paper I** presented data on the preliminary characterisations of five Arabidopsis genes in *E. coli*. The data showed that with the exception of AtFAR7, which is suspected to be a pseudogene (due to the presence of stop codon after the 95th amino acid), the expression of AtFAR3/CER4, AtFAR1, AtFAR2/MS2 and AtFAR8 yielded significant amounts of fatty alcohols. However, the quantity and profiles of fatty alcohols produced by these enzymes were different to each other and to jojoba FAR (**Paper I**). It is therefore suggested that these enzymes could have different substrate specificity *in planta*.

The expression of AtFAR3/CER4 in *E. coli* resulted in the production of predominantly C18:1-OH, as well as C16:0-OH and C14:0-OH and this was the enzyme which produced the lowest quantity of fatty alcohols in *E. coli* in comparison to the enzymes characterised in **Paper I**. This could be explained by the results for the expression of AtFAR3/CER4 gene in yeast and analysis of the corresponding Arabidopsis mutant (*cer4*) which shows that AtFAR3/CER4 is responsible for the production of fatty alcohols with chain length from C24 to C28 (Rowland *et al.*, 2006). Accordingly, AtFAR3/CER4 has high specificity to the very long-chain fatty acyl-CoAs which are not available in *E. coli*.

Using *E. coli* as the expression system is fast and efficient, although it apparently still has limitations judging from the discrepancy in the pool of fatty acyl chains present in *E. coli* compared with plants. For example, *E. coli* only contains endogenous fatty acyl chains up to 18 carbons in length and *E. coli* C18:1 has a different type of desaturation than that in plants (11c-C18:1 in bacteria compared with 9c-18:1 in plants) (Shaw & Ingraham, 1965). In addition, the fatty acyl-ACP pool is dominant to the fatty acyl-CoA pool (Ohlroge & Browse, 1995; Magnuson *et al.*, 1993). Moreover, in an *E. coli* system, the results obtained from heterologous expression of FARs could be influenced by the presence of endogenous fatty aldehyde reductase activity that reduces fatty aldehyde into fatty alcohol (Schirmer *et*

al., 2010; Reiser & Somerville, 1997). As a consequence, the fatty alcohol profiles of genes expressed in *E. coli* might deviate from the substrate specificity of the FAR enzymes *in planta* (Domergue *et al.*, 2010). In yeast, the eukaryotic expression system, the differences in the fatty acyl-CoAs pools for FARs are still observed. For example, while high production of C18:0-alcohol followed by C20:0-alcohol is observed in yeast expressing AtFAR4, a study on the *atfar4* mutant showed that only the contents of C20:0 and C22:0 alcohols are reduced in this mutant (Domergue *et al.*, 2010). Despite the above discrepancy, characterisations of AtFAR4, AtFAR5 and AtFAR1 in yeast and the corresponding Arabidopsis mutants still show that these enzymes have the highest specificity to C20:0, C18:0 and C22:0, respectively (Domergue *et al.*, 2010).

The characterisation of the aforementioned enzymes, *i.e.* jojoba FAR, TAA1, AtFAR3/CER4 and the group of AtFAR1, AtFAR4 and AtFAR5, revealed that the fatty alcohol profiles produced by the FARs varied among the expression systems. However, the findings also indicate that these enzymes have the capacity to utilise a wide range of substrates.

In **Paper I**, AtFAR6 and AtFAR2/MS2 were shown to be the most efficient enzymes in producing fatty alcohols in *E. coli*. To further investigate the activity of these enzymes, the heterologous expression of *AtFAR6* in yeast and also the transient expression of AtFAR6 and *AtFAR2/MS2* in *Nicotiana benthamiana* were studied. Contrary to the enzymes discussed above, *in vivo* characterisation of *AtFAR6* and *AtFAR2/MS2* consistently showed that in all expression systems, *i.e.* *E. coli*, yeast and *N. benthamiana*, C16:0-alcohol was the predominant product of these enzymes (**Papers II, III**; (Chen *et al.*, 2011).

Although the substrate specificity of the AtFARs mentioned above had been demonstrated previously, the relative importance of enzyme specificity *per se* in relation to the pool of acyl substrates available to the enzyme for determination of the fatty alcohol composition produced in a given cell type was not known. In **Papers II and III**, the activity of AtFAR6 and AtFAR2/MS2 was further characterised *in vitro*. In these characterisations, *AtFAR6* and *AtFAR2/MS2* were expressed in *E. coli*, after which the enzymes were purified and used for *in vitro* assays. Consistent with the results from *in vivo* characterisation, AtFAR6 revealed high specificity to the substrate with 16 carbons. Interestingly, this enzyme was able to utilise both C16:0-CoA and C16:0-ACP as substrate at approximately similar K_m values. This AtFAR6 was later identified to be an enzyme resident in

chloroplasts, organelles with high availability of C16:0-ACP substrate (see next section) (**Paper II**).

Similarly to AtFAR6, AtFAR2 also showed the specificity to both C16:0-CoA and C16:0-ACP substrates. The K_m value of enzyme to C16:0-CoA was found to be $5.31 \pm 1.15 \mu\text{M}$ (**Paper III**). Chen *et al.* (2011) also reported on characterisation of AtFAR2/MS2 but in contrast they showed that this enzyme had very strict specificity to C16:0-ACP, with a K_m value for this substrate of $23.3 \pm 1.15 \mu\text{M}$ and with no activity to C16:0-CoA substrate observed.

In comparison, the affinity of AtFAR2/MS2 to the most preferred substrate (C16:0-ACP) reported by Chen *et al.* (2011) is four-fold lower than the affinity of this enzyme to C16:0-CoA substrate as reported in **Paper III**. This difference indicates that the activity of the enzyme described by Chen *et al.* (2011) is much lower than the activity of the enzyme used in **Paper III**. On the other hand, in **Paper III** no enzyme activity was observed when free palmitic acid was used in the assay, indicating that this enzyme can only utilise activated fatty acids (ACP or CoA) as substrates and not the free fatty acids. Accordingly, the observation on C16:0-CoA utilisation reported by Chen *et al.* (2011) can be suspected to have been influenced by the purification conformation differences of the purified enzyme or by the use of improperly prepared C16:0-CoA substrate, which is degraded into free palmitic acid in the assays.

Moreover, the results presented in **Paper III** showed that AtFAR2/MS2 could only utilise the free form of the substrate and not the micelle form. In the test conditions used, there was almost no enzyme activity at $10 \mu\text{M}$ of C16:0-CoA substrate and activity of enzyme at high C16:0-CoA concentration was only observed with the presence of BSA in the assay. Assuming that the K_m value of AtFAR2/MS2 to C16:0-ACP ($23.3 \pm 1.15 \mu\text{M}$) is similar to that of C16:0-CoA substrate which according to the results in **Paper III** would then be trapped in micelle form at this concentration and therefore be inaccessible to AtFAR2/MS2. Whereas, the activity of the enzyme to C16:0-ACP at this concentration might not be affected since it is known from the literatures that the critical micelle concentration of C16:0-ACP is much higher than that of C16:0-CoA (Lueking & Goldfine, 1975).

Apparently, the results presented demonstrated that although they are chloroplast localised enzymes, AtFAR2/MS2 and AtFAR6 had a high capacity to use both types of substrates, ACP and CoA (**Papers II, III** and the next section) (Chen *et al.*, 2011). Similarly, another chloroplast localised

alcohol-forming FAR enzyme from rice is shown to have the capacity to use both CoA and ACP (Shi *et al.*, 2011). According to the results from the characterisation of AtFAR2/MS2, AtFAR6 and DPW, these enzymes apparently have the ability to use both activated fatty acid forms, ACP and CoA, as the substrates. In order to investigate whether this is the common character of Arabidopsis FARs, the *in vitro* assays using purified AtFAR5 were conducted in the presence of either C18:0-CoA or C18:0-ACP as the substrate. The results showed that AtFAR5 could efficiently utilise C18:0-CoA but not C18:0-ACP (Fig. 6). This indicates that the utilisation of ACP substrate is not a universal property among the Arabidopsis FARs.

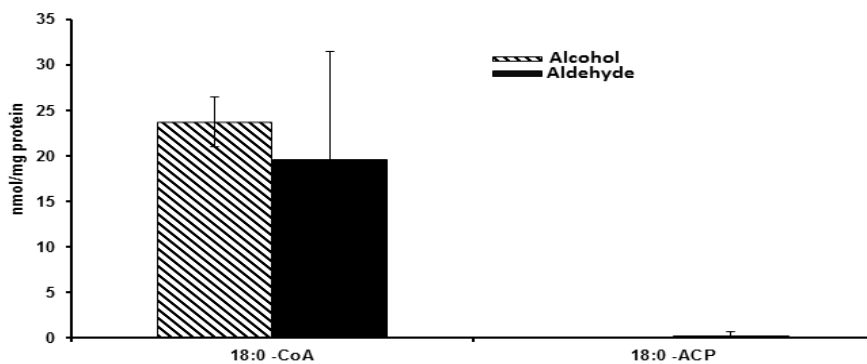


Figure 6: Activity of AtFAR5 to C18:0-ACP and C18:0-CoA substrates. Assays were conducted in 100mM potassium phosphate buffer (pH 7.0), 10mM NADPH and 1.25 μ M of either [14 C] 18:0-CoA or [14 C] 18:0-ACP as substrate. Assays were incubated at 30°C. Error bars indicate 95% confidence limits.

Subcellular localisation of plant FAR enzymes

The activity and substrate specificity decide the biochemical identity of an enzyme, while the regulation of enzyme activity in the cell is determined by its subcellular localisation, which influences parameters such as enzyme stability, substrate availability and the flux of products produced by the enzyme. Although the subcellular location of jojoba FAR has not been studied, analysing the alcohol moiety of jojoba wax esters demonstrates that jojoba FAR has high specificity toward very long-chain fatty acyl-CoAs (C20:1, C22:1 and C24:1-CoA), the products of the FAE enzyme complex in the endoplasmic reticulum (ER). This suggests that jojoba FAR is an enzyme with a subcellular localisation in the ER of the cell (Metz *et al.*, 2000). Studies on the subcellular location of AtFAR3/CER4 suggest that this enzyme is also an ER enzyme, but since there is little evidence of a membrane-bound domain in the protein sequence, the membrane

association of AtFAR3/CER4 is assumed to be through intermediate molecules, *e.g.* post-translational attached lipids (Rowland *et al.*, 2006). This explains why in the *E. coli* system, an expression system with similar acyl-linked substrates (fatty acyl-ACP) to that of the chloroplast, the jojoba FAR and AtFAR3/CER4 produced lower amounts of fatty alcohols than were produced by AtFAR6 and AtFAR2/MS2 (which are discussed later in this section as chloroplast enzymes) (**Paper I**).

For AtFAR5, AtFAR4 and AtFAR1, there are no available data about their subcellular locations, but it has been shown that they have a preference for saturated substrates with 18, 20 and 22 carbons, respectively (Domergue *et al.*, 2010). In plants, there are two forms of activated C18:0 available, the cytosolic form C18:0-CoA and the plastidic form C18:0-ACP. As discussed in the previous section, AtFAR5 could only utilise 18:0-CoA as the substrate and not C18:0-ACP (Fig. 6). This finding excluded the possibility of AtFAR5 using a chloroplast form of C18:0 substrate. Considering AtFAR4 and AtFAR1, C20:0 and C22:0 are known to be the abundant types of fatty acids outside the chloroplast and it is therefore likely that this group of AtFARs (AtFAR1, AtFAR4 and AtFAR5) resides in another compartment in the cell than the chloroplast. However, since no membrane-bound domain was found in AtFAR1, AtFAR4 or AtFAR5 using *in silico* prediction (**Paper I** and unpublished data), it is not clear in which specific compartment these enzymes are localised.

Among the AtFAR enzymes identified in **Paper I**, AtFAR6 and AtFAR2/MS2 are unique in that they contain N-terminal extensions of 72 aa and 118 aa respectively. This has been shown by the protein alignments of these enzymes to other alcohol-forming FAR homologues (**Paper I**). Our *in silico* prediction using ChloroP and PCLR (Schein *et al.*, 2001; Emanuelsson *et al.*, 1999) revealed that the N-terminal extensions of AtFAR6 and AtFAR2/MS2 are predicted to comprise chloroplast transit peptide sequences (**Paper I**). The chloroplast transit peptide is essential for the recognition and localisation of the attached protein into the chloroplast. Once the protein is imported and located inside the chloroplast, the transit peptide sequence is cleaved off so that the protein becomes a functional enzyme (Bruce, 2000; Archer & Keegstra, 1990). Recently, Chen *et al.* (2011) reported AtFAR2/MS2 to be a FAR enzyme and localised in chloroplast.

To investigate whether the AtFAR6 enzyme is also chloroplast localised, the characterisation of AtFAR6 was carried out using two different forms of protein sequences: (1) the full-length protein sequence, AtFAR6; and (2)

the truncated protein sequence without the chloroplast transit peptide, *mAtFAR6* (**Paper II**). In this characterisation, the transient expression experiments in *Nicotiana tabacum* leaves were conducted using constructs carrying various parts of *AtFAR6* fused to the coding region of yellow fluorescent protein (YFP). When the full-length *AtFAR6* sequence was fused at the N-terminus of the YFP, fluorescence appeared in a distinct pattern that is typical of chloroplast localisation (**Paper II**) (Fig. 7). In contrast, when a sequence lacking the first 71 amino acids of the *AtFAR6* protein was used, the YFP fluorescence was more diffuse and resembled that of cytosolic proteins (**Paper II**) (Fig. 7). To confirm that the N-terminal sequence of *AtFAR6* could target a protein to the chloroplast, a fusion of the first 71 amino acids of the *AtFAR6* protein to YFP was produced. Expression of this fusion protein resulted in a similar punctuated pattern to that observed with the full-length *AtFAR6* sequence (**Paper II**) (Fig. 7).

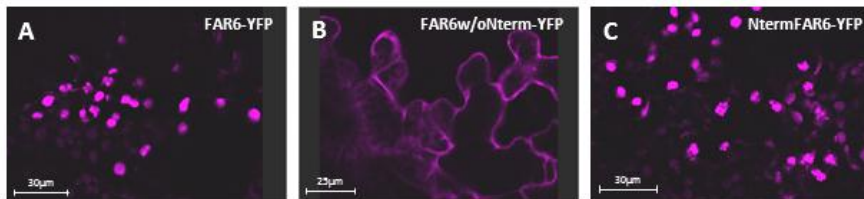


Figure 7: Subcellular localization of full-length *AtFAR6* and truncated variants. Yellow fluorescent protein (YFP) fusions of full-length *AtFAR6* (**A**), *AtFAR6* lacking the N-terminal 71 amino acids (**B**), and the N-terminal 71 amino acids of *AtFAR6* (**C**), were transiently expressed in *Nicotiana tabacum* leaves and fluorescence imaged by laser scanning confocal microscopy.

To further confirm these results, *mAtFAR6* and *AtFAR6* were expressed transiently in *N. benthamiana*, followed by analysis of the fatty alcohols produced in the leaves and in chloroplast from the leaves. The results showed significant accumulation of C16:0 and C18:0 alcohols in the chloroplasts of leaves expressing *AtFAR6*, while a negligible quantity of fatty alcohols was detected in the control and the chloroplast of leaves expressing *mAtFAR6* (**Paper II**) (Fig. 8). It was observed that the leaves expressing *AtFAR6* showed chlorotic lesions after three days of infiltration (**Paper II**). The transient expression of *AtFAR2/MS2* in *N. benthamiana* also resulted in the production of C16:0-alcohol in the leaves (**Paper III**). However, a low amount of C16:0-alcohol was detected in the chloroplast of leaves expressing *AtFAR2/MS2* and there was no difference in appearance between leaves expressing *AtFAR2/MS2* and those of the control plants (**Paper III**).

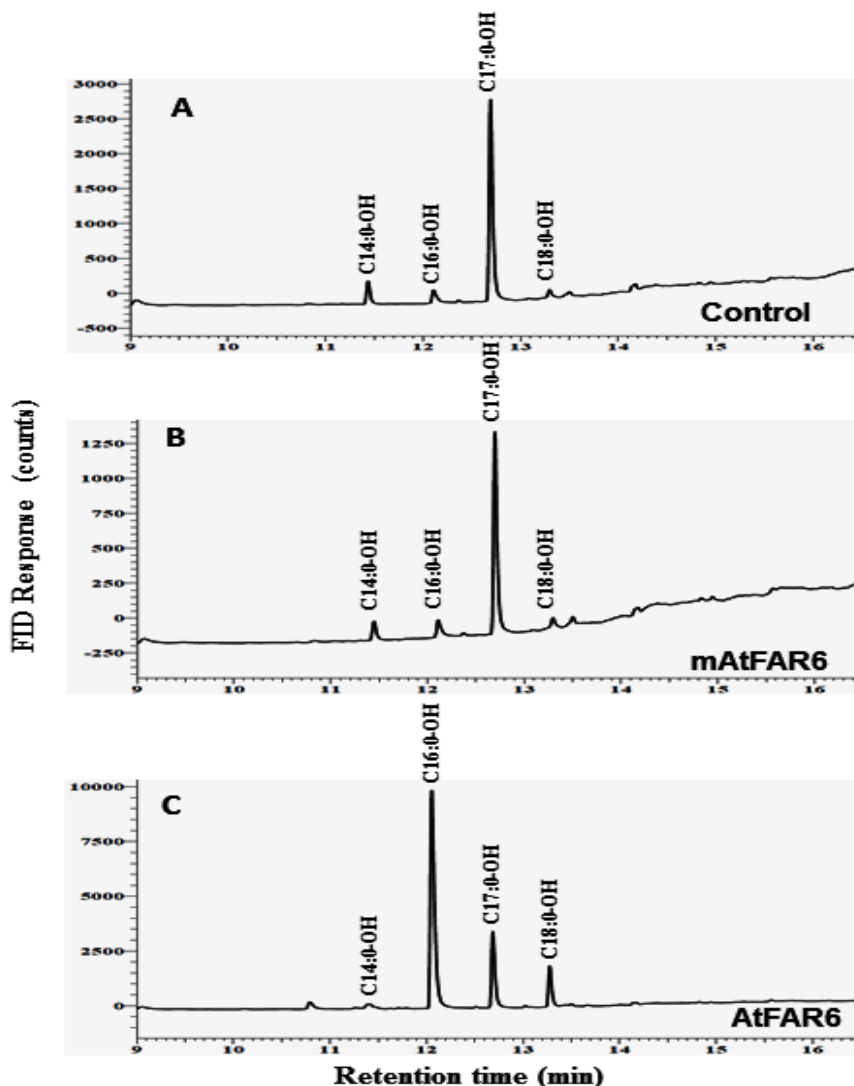


Figure 8: GC analysis of fatty alcohol produced in chloroplasts of *Nicotiana benthamiana* leaves expressing *mAtFAR6* and *AtFAR6*. C17:0-OH (3 nmol) was added as internal standard. A. Control: chloroplasts of leaves expressed *GFP* and *P19*. B. *mAtFAR6*: chloroplasts of leaves expressed *mAtFAR6*, *GFP* and *P19*. C. *AtFAR6*: chloroplasts of leaves expressed *AtFAR6*, *GFP* and *P19*.

The above results are contrary to the results from heterologous expression of *AtFAR6* and *mAtFAR6* in yeast, in which expression of *mAtFAR6* yielded a much higher level of fatty alcohols than *AtFAR6* (**Paper II**). Previous studies shows that since yeast cells do not possess chloroplasts or any other

type of plastids, they have difficulties in discriminating between mitochondrial and chloroplast targeting signals, which can lead to plant proteins with a chloroplast targeting peptide being introduced into the mitochondria (Fischer, 2011; Loddenkötter *et al.*, 1993; Hurt *et al.*, 1986). It is therefore likely that the yeast protein sorting machinery sent the full-length AtFAR6 protein to the mitochondria, where it became spatially separated from the C16:0-CoA and C18:0-CoA substrates in the cytosol. Conversely, in *N. benthamiana*, the expression of AtFAR6, but not of *mAtFAR6*, showed significant accumulation of C16:0-OH and C18:0-OH (**Paper II**). This indicates that in contrast to the yeast system, the full-length AtFAR6 protein was most probably correctly targeted and imported into the chloroplast in the leaves, where its targeting signal is cleaved. The presence of significant amounts of fatty alcohols within isolated chloroplasts of *N. benthamiana* leaves expressing AtFAR6 strongly suggests that AtFAR6 is a chloroplast localised enzyme and is much more active in this compartment than in the cytosol, and that a large proportion of the fatty alcohols produced are contained in this organelle (**Paper II**).

In plant cells, *de novo* lipid synthesis takes place in the chloroplast or other types of plastids and results in the production of saturated fatty acyl-ACPs with 16 to 18 carbons. These fatty acyl-ACPs are then desaturated (in the case of 18:0-ACP) and/or acylated to glycerolipid in the chloroplast or hydrolysed from ACP by acyl-ACP thioesterases, exported to the cytosol and activated to fatty acyl-CoAs for further utilisation in the cytosol (Ohlrogge & Browse, 1995). To obtain insights into the subcellular localisation of AtFAR6 via identification of substrate specificity, *in vitro* assays using purified mAFAR6, AtFAR6 and AtFAR2/MS2 were conducted. The results showed that AtFAR6 and mAtFAR6 had the same specific activity, but that the activity of mAtFAR6 was higher than that of AtFAR6. Therefore the mAtFAR6 was used for further characterisation. These assays showed that mAtFAR6 and also AtFAR2/MS2 have the capacity to utilise acyl-ACP as substrate (for details see **Papers II, III** and (Chen *et al.*, 2011)). These findings are consistent with previous experiments showing that AtFAR2/MS2 and AtFAR6 were the most efficient enzymes in producing alcohols in *E. coli* (**Paper I**), where acyl-ACP substrates are more likely to be available (Ohlrogge & Browse, 1995). This further confirmed that AtFAR6 and AtFAR2/MS2 are enzymes that reside in chloroplast. Similarly to AtFAR6 and AtFAR2/MS2, DPW (defective pollen wall), a FAR enzyme from rice, has also been identified as a chloroplast enzyme and the chloroplast transit peptide is essential in enzyme targeting (Shi *et al.*, 2011).

Biological functions of plant FAR enzymes

The overproduction of fatty acids could be toxic to the cell and therefore using these fatty acids as a source for production of fatty alcohols, which then serve as substrates for wax ester biosynthesis, is one way to overcome this problem (Turkish & Sturley, 2009). Besides being an intermediate substance in preventing the toxicity effect of fatty acids, fatty alcohols produced by FARs are also known to be involved in a variety of biological functions.

It is well known that jojoba FAR is important for producing fatty alcohols for wax ester biosynthesis in jojoba seeds. These wax esters then serve as energy storage and are used during seed germination via β -oxidation (Huang *et al.*, 1978; Moreau & Huang, 1977). This is the only case among plants where wax esters are predominantly accumulated in seeds as a storage compound. Since the fatty acyl-CoAs are the common substrates of both TAG and wax ester biosynthesis, the alteration of TAG and wax ester biosynthesis could significantly affect the composition of storage lipids in cells (Voelker & Kinney, 2001). One example of this is the expression of jojoba FAR, WS and KCS in Arabidopsis leading to the production of up to 70% of wax esters in transgenic Arabidopsis seeds (Lardizabal *et al.*, 2000), indicating that there is a redirection from TAG formation as usual into wax ester formation in this case.

In Arabidopsis, although eight FAR homologues are present, each of them possesses distinct characteristics; they act either individually or interactively during plant development. AtFAR3/CER4 is involved in the production of C24:0 to C28:0 alcohols in Arabidopsis, and these fatty alcohols participate to build the structure of cuticular waxes both in free form and the form of wax esters (Rowland *et al.*, 2006). The *cer4* mutant of Arabidopsis shows glossy stems with absence of wax crystals and, instead, a thick, smooth film of waxes covering the stem surface (Rowland *et al.*, 2006). Also involved in building the protective layers of the plant, the three enzymes AtFAR1, AtFAR4 and AtFAR5 produce C22:0, C20:0 and C18:0 alcohols, respectively, in suberin (Domergue *et al.*, 2010). Besides the expression during suberin deposition in plant tissues, expressions of *AtFAR1*, *AtFAR4* and *AtFAR5* are also induced by wounding and salt stress (Domergue *et al.*, 2010).

While AtFAR3/CER4, AtFAR1, AtFAR4 and AtFAR5 mostly produce fatty alcohols in vegetative tissues, the fatty alcohols produced by Arabidopsis AtFAR2/MS2 are similar to wheat and rice FARs, which are

essential for pollen wall development (Chen *et al.*, 2011; Shi *et al.*, 2011; Wang *et al.*, 2002). The *ms2* mutant displays the male sterile phenotype, while the knockout T-DNA insertion mutant displays the abnormal pollen wall phenotype (Dobritsa *et al.*, 2009; Aarts *et al.*, 1997). Interestingly, the Arabidopsis *ms2* mutant can be rescued by the expression of rice DPW, which indicates that this DPW is most likely an orthologous enzyme of Arabidopsis AtFAR2/MS2 (Shi *et al.*, 2011).

It should be noted that plants that are homozygous for a T-DNA insertion mutation in a FAR gene, *AtFAR2/MS2* (SAIL_92_C07), still produce seeds (Dobritsa *et al.*, 2009) and occasionally a fertile phenotype is also found in the *ms2* mutant (Aarts *et al.*, 1997). It is therefore postulated that at least one homologous *AtFAR* gene can complement *AtFAR2/MS2* to some extent in these cases, as seen in the sterility recovery of *ms2* mutant by heterologous expression of *DPW* (Shi *et al.*, 2011). According to the results in **Paper III** and Chen *et al.* (2011), AtFAR2/MS2 is the enzyme responsible for the production of C16:0-alcohol in chloroplast of Arabidopsis. The biological functions of most AtFARs among the eight AtFAR homologues in the Arabidopsis genome have been discussed earlier. Among the Arabidopsis FAR homologues, AtFAR6 appeared to be the only one which showed similar substrate specificity and biochemical properties to those of AtFAR2/MS2 (**Paper II**). Moreover, similarity to AtFAR6 was also determined in terms of being a chloroplast localised enzyme (for details see 3.2.1-Subcellular localisation of plant FAR enzymes). The similarity in enzyme activity of AtFAR2/MS2 and AtFAR6, as well as the subcellular location of these enzymes, *i.e.* enzymes of chloroplasts, indicates that there could be a functional redundancy among AtFAR2/MS2 and AtFAR6 in the development of Arabidopsis and that AtFAR6 could be an enzyme that is functionally complementary to AtFAR2/MS2 or *vice versa*. The analysis of *AtFAR6* promoter:GUS fusions showed that besides stem epidermis and root cap, expression was also found in anthers (for details see **Paper II**). In addition, data from the gene expression database (AtGenExpress Development) show that expressions of both *AtFAR2/MS2* and *AtFAR6* are found in mature pollen and stem (Schmid *et al.*, 2005). It therefore appears that with similar subcellular localisation, tissue expression and enzyme activity, AtFAR6 is the only candidate among AtFAR homologues which could act in a complementary way to AtFAR2/MS2.

Phylogenetic analysis of functionally characterised FARs (Fig. 1) revealed that DPW (Shi *et al.*, 2011), AtFAR6 (**Paper II**) and AtFAR2/MS2 (Chen *et al.*, 2011) **Paper III**), the chloroplast localised FAR enzymes, were

closely related and thus formed a group. The group of AtFAR1, AtFAR4 and AtFAR5 (Domergue *et al.*, 2010) and AtFAR8 (**Paper I**) are grouped in another clade. In this group, *AtFAR8* is possibly the result of duplication of *AtFAR5* and enzymes of this group comprise FARs for suberin fatty alcohols. It was also apparent that jojoba FAR (Metz *et al.*, 2000), AtFAR3/CER4 (Rowland *et al.*, 2006) and AaFAR (Maes *et al.*, 2010), the specific FAR enzymes for very long-chain fatty acyl-CoAs, were in the same group. The wheat FAR (TaFAR) (Wang *et al.*, 2002), with medium- and very long-chain substrate specificity, was closer to the suberin group and jojoba group. The studies on plant FARs revealed that although these enzymes are ubiquitous, each enzyme apparently possesses distinct biochemical characteristics and they act either interactively or independently during the development of plants. From an evolutionary point of view, the presence of multiple homologous genes encoding for enzymes with similar catalytic activity and also overlapping biological functions might be a possible way to improve the adaptation capacity of plants to changes in the environment (Zhang, 2003; Lynch, 2002; Long & Thornton, 2001; Lynch & Conery, 2000). The occurrence of different FAR homologues in one genome has also been reported in other eukaryotic organisms, such as insects and copepods (Teerawanichpan & Qiu, 2011; Lassance *et al.*, 2010; Liénard *et al.*, 2010).

3.2.2 Fatty acyl-CoA reductase in bacteria (Paper IV)

In prokaryotes, the medium- and long-chain fatty alcohols for the biosynthesis of wax esters are believed to be produced by two-step reduction of either acyl-CoA or acyl-ACP via an intermediate fatty aldehyde. A study of mutants of *Acinetobacter calcoaceticus* deficient in wax ester biosynthesis revealed a mutant impaired in the first reduction step leading to fatty aldehyde (Reiser & Somerville, 1997). In a recent paper, a putative gene sequence (Maqu_2220) of fatty aldehyde reductase enzyme was identified in *Marinobacter aquaeolei* VT8, a Gram-negative bacteria strain isolated from a sample acquired at the head of an offshore oil well in southern Vietnam (Huu *et al.*, 1999). The corresponding 513 aa protein product of Maqu_2220 was shown to reduce added aldehyde substrates to fatty alcohol. Thus in combination with an acyl-CoA reductase, the identification of this fatty aldehyde reductase enzyme (FALDR) could be seen as closing the gap in the reduction from fatty acyl-CoA to fatty alcohol (Wahlen *et al.*, 2009). Accordingly, the biosynthesis of fatty alcohols of prokaryotes is different to that of eukaryotes, where a single enzyme can

perform both reduction steps (Lardizabal *et al.*, 2000; Vioque & Kolattukudy, 1997).

Maqu_2220 produces fatty alcohols from fatty acyl-CoA/ACP substrates

Maqu_2220 is reported to be an aldehyde-reducing protein (FALDR) which catalyses the reduction of fatty aldehyde into fatty alcohol (Wahlen *et al.*, 2009). However, the protein sequence of Maqu_2220 FALDR showed high homology to other identified alcohol-forming FARs such as jojoba FAR and Arabidopsis AtFAR3/CER4 with similar conserved domains in the protein sequence, *i.e.* the predicted Rossmann-fold which is suggested to be the NAD(P)H-binding site and also the C-terminal region with the so-called male sterility domain. From the similarity in protein sequence, the Maqu_2220 FALDR was suspected to be an alcohol-forming FAR, therefore eliminating the need for producing fatty aldehyde by an acyl-CoA reductase (ACR) in *Marinobacter aquaeolei* VT8.

In **Paper IV**, Maqu_2220 was expressed in *E. coli* and the corresponding enzyme was purified. The *in vitro* assays using purified enzyme were conducted using saturated [¹⁴C]-fatty acyl-CoA substrates of C10 to C22 carbons in the chain length for the assays and reaction products were separated by TLC. Of the substrates tested, Maqu_2220 had the highest activity in production of fatty alcohols with C20:0-CoA, followed by C18:0-CoA and then C16:0-CoA. Fatty alcohol production with saturated substrates C10:0 to C14:0-CoA and C22:0-CoA, although detectable, was low (Fig. 9). These results showed that in conditions with non-preferred substrates, considerable amounts of free fatty acids and aldehydes were produced. This would suggest that although accepted as substrates, the full reaction does not take place efficiently, which led to the formation of intermediate products: free fatty acids after an initial thioesterase-like activity and fatty aldehyde after the first reduction step.

In addition to the ability for using saturated substrates, Maqu_2220 could also efficiently reduce unsaturated substrates C18:1- Δ 9-CoA to produce fatty alcohol and C22:1- Δ 9-CoA. In fact, C18:1- Δ 9-CoA was found to be the substrate most efficiently reduced into fatty alcohol. The enzyme was also able to utilise ricinoleoyl-CoA (the substrate with the addition of a Δ 12-hydroxyl group to C18:1-*cis*- Δ 9) for alcohol production (Fig. 9). Maqu_2220 also reduced branched substrate, 2-methyl-C18:0-CoA, into fatty alcohol at a low but detectable level, but no activity was detected with 2-methyl-C16:0-CoA.

In prokaryotes, the CoA-activated acyl chains are mainly derived from oxidative reactions with medium- and long-chain hydrocarbons, while the ACP-linked acyl chains are produced from the *de novo* biosynthesis of fatty acids. C16:0-ACP was therefore tested as a substrate. The results showed that Maqu_2220 was able to produce C16:0-alcohol from C16:0-ACP. The difference in activity of Maqu_2220 with C16:0 activated by CoA or ACP was in fact much less than the difference in activity of C16:0-CoA to 14:0 and 18:0-CoA substrates (Fig. 9). The minor difference in activity between 16:0-ACP and 16:0-CoA might indicate that *Marinobacter aquaeolei* VT8 is prepared to produce fatty alcohols leading to wax esters from both *de novo* fatty biosynthesis, as well as utilising activated substrates from degradation of available hydrocarbons in the environment. The above results showed that Maqu_2220 is a protein capable of performing both reduction steps from fatty acyl-CoA/ACP to fatty alcohol (Fig. 9). Therefore it is the first prokaryotic FAR enzyme with similar catalytic activity to the alcohol-forming FAR found in eukaryotes. The findings also demonstrated that there are at least two biochemical pathways exist among prokaryotes for the reduction of activated fatty acyl chains to fatty alcohols, one of which is analogous activity to the eukaryotic FARs identified above.

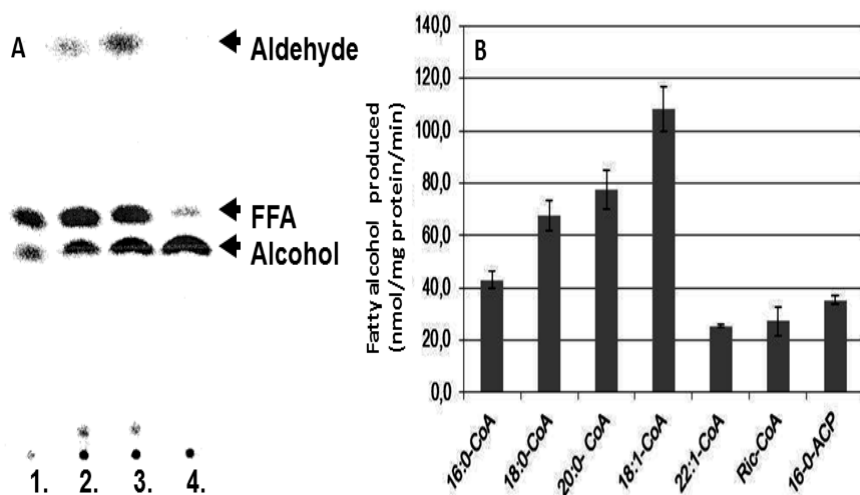


Figure 9: A. Distribution of radioactive products on TLC-plate after incubation of the Maqu_2220 protein with [¹⁴C]-fatty acyl-CoA substrates and subsequent separation. The identity of reaction products were established by GC-MS (Appendix C). Lane 1: 10:0, Lane 2: 12:0, Lane 3: 14:0, Lane 4: 16:0. B. Activity of the Maqu_2220 protein with different activated [¹⁴C]-fatty acyl substrates at 30°C. Bars represent rate of alcohol production in nmol/mg protein/min and are means of two independent experiments. Ric-CoA (ricinoleoyl-CoA)

Maqu_2220 FAR utilises a wide range of substrates

The results on the characterisation of *Marinobacter* FAR showed that this enzyme has considerable activity, with a highly diverse range of substrates with different carbon chain lengths, activated forms (ACP and CoA linked acyls) and levels of modification (hydroxylated, unsaturated, branched substrates). This is in contrast to most other FAR homologues, which have a narrow range of substrate utilisation. For example, *Euglena gracilis* FAR mainly produces saturated 14 and 16 carbon alcohols (Teerawanichpan *et al.*, 2010). Insect FARs relating to pheromone synthesis are also quite specific, with a preference for 14 or 16 carbon substrates (Lassance *et al.*, 2010). The honeybee FAR (AmFAR), copepod FARs (CfFAR) and mouse FAR1 (MmFAR1) are the exceptions, with a wider range of substrate utilisation (Teerawanichpan & Qiu, 2011; Teerawanichpan *et al.*, 2010; Cheng & Russell, 2004a). For plant FARs, substrate utilisation depends strongly on subcellular location and on the biological function of the enzymes (discussed above). In these organisms, the observed specificities are most likely a function of the requirements placed on the end-product *e.g.* protective surface waxes or signalling pheromones, but also depend on the substrates that are spatially and temporally available in the system studied.

In plants and animals, the fatty alcohols and wax esters commonly serve very specialised functions where physical properties are of importance. Therefore a high stringency FAR enables the organism to fine-tune the contribution to the specific properties of the compound, or mixture of compounds, in which the fatty alcohol is utilised. In marine bacterial species, energy storage is the primary function of wax esters, which could mean that it is an advantage if a wider range of substrates can be utilised either from internally synthesised resources or from scavenging of hydrocarbons available in the environment.

The *Marinobacter* FAR described here showed high homology to the reported alcohol-forming FARs from eukaryotes. In addition, a multitude of other sequences with a similar primary structure were found but are so far uncharacterised. On a wider search through the eukaryotes, homologous sequences were found in the moss *Physcomitrella patens* (Acc. XP_001771307 and XP_001758118) and among protists such as *Euglena gracilis*, *Trypanosoma cruzi* (Acc. XP_809421) and *Phytophthora infestans* (based on the incomplete sequences). Surprisingly, sequences with domains typical for FARs could not be found among algae and fungi.

Using BLAST search for proteins in the fully sequenced microbe section of NCBI an additional five putative bacterial FARs which are of a similar size (480-550 aa) and contain the same conserved domains as proven eukaryotic FARs can be found. These bacterial species are closely related, which could mean that this type of gene and enzyme is very rare and confined to a small section of bacterial species. Interestingly among all over 1800 microbial genomes which can be subjected to BLASTP, all five genomes containing an ORF with significant homology to Maqu_2220 FAR belong to marine bacteria. Other amino acid sequences found with homology to FAR fall into two classes of 750-820 aa and 1470-1600 aa. They are essentially homologous to other FAR, but with an extension in the C-terminal. It was also found that the C-extensions of these two classes are not conserved, thus indicating diverging functions of these two groups.

Fatty alcohols are the substrate for biosynthesis of wax esters, which are suggested to be important compounds for survival during dehydration in the water-air boundary, facilitating terrestrial life (Finkelstein *et al.*, 2010). Maqu_2220 of *Marinobacter aquaeolei* VT8 has now been proven to have the same type of activity as eukaryotic FARs and may together with homologous sequences from marine bacteria be the prototypical version of this enzyme, where specialisation of enzyme function may have occurred upon migration to land and evolution of more complex organisms.

3.3 Enzymes in biosynthesis of wax esters

One of the most important biochemical roles of fatty alcohols is as precursors for the biosynthesis of wax esters. The esterification of fatty alcohol and fatty acyl-CoA/ACP to form wax ester is catalysed by wax ester synthase (WS) enzyme (fatty acyl-CoA: fatty alcohol acyltransferase). To date, three types of WS enzymes have been identified in plants and bacteria.

In bacteria such as *Acinetobacter calcoaceticus* ADP1 and *Mycobacteria tuberculosis*, there is a bifunctional WS/DGAT which is involved in the production of both wax ester and triacylglycerol (TAG) (Wältermann *et al.*, 2007; Stoveken *et al.*, 2004; Kalscheuer & Steinbuchel, 2003). The WS/DGAT from *Mycobacteria tuberculosis* has much higher DGAT activity than WS activity (Wältermann *et al.*, 2007; Stoveken *et al.*, 2004). Whereas, the bifunctional enzyme from *Acinetobacter calcoaceticus* has higher WS activity than DGAT activity and regarding the WS activity, this enzyme can utilise a broad range of fatty alcohols (C2-C30). However, the highest specificity is for C14:0 to C18:0 alcohols, which are esterified to C14:0, C16:0 acyl-

CoAs (Wältermann *et al.*, 2007; Wältermann *et al.*, 2005). Proteins with high homology to bacterial WS/DGAT are also found in plants. In petunia (petals), the enzyme has high homology to bacterial WS/DGAT but only the WS activity is detected with high specificity to C22-C28 acyl-CoAs and C10:0-C12:0 alcohols (King *et al.*, 2007). In the Arabidopsis genome, there are 11 homologues of WS/DGAT. The identification and characterisation of a putative Arabidopsis WS/DGAT (WSD1) revealed that this enzyme is located in the endoplasmic reticulum with 10-fold higher WS activity than DGAT activity *in vitro*. However no DGAT activity has been detected *in vivo* (Li *et al.*, 2008). In Arabidopsis, WSD1 is responsible for the production of wax esters of stem epidermis (Li *et al.*, 2008). The presence of these bi-functional WS/DGAT enzymes in plants therefore indicates that there is an evolutionary relationship in the regulation of wax ester biosynthesis and TAG biosynthesis in plants.

The second type of WS is specialised for only synthesising wax esters and is found in jojoba. This enzyme has specificity to unsaturated C18 alcohol and both saturated and unsaturated C14 to C24 acyl-CoAs (Lardizabal *et al.*, 2000). There are also 12 genes in Arabidopsis with high homology to jojoba WS (Klypina & Hanson, 2008; Costaglioli *et al.*, 2005; Beisson *et al.*, 2003), but the activity of enzymes encoded by these genes has not been determined.

Another type of WS found in Arabidopsis is phytyl ester synthase (PES). This enzyme catalyses the esterification of phytol and fatty acids to form fatty acid phytyl esters in chloroplasts during senescence (Ischebeck *et al.*, 2006). However, the corresponding gene of PES has not been identified so far.

4 Fatty alcohols and derivatives: Industrial applications, problems and prospects

Together with fatty acids, fatty alcohols are important basic oleochemicals. Fatty alcohols are the starting material for producing other oleochemical products by being further processed by esterification, ethoxylation, sulphation, amidation and other modifications. Fatty alcohols with 8 to 10 carbons in the chain are material for the plastics industry and are also used as solvents for printing inks and lacquers, while C12-C18 alcohols are mostly used in the detergent and surfactant industry. The C12-C14 alcohols are also used as components in lubrication, bearing and hydraulic oils. For the cosmetic industry, C16-C18 alcohols are mostly used. Wax esters, a derivative of fatty alcohols, appear to be the best option for using as high value lubricants due to their high resistance to hydrolysis (Carlsson *et al.*, 2011; Carlsson, 2006; Rupilius & Ahmad, 2006; Gervajio, 2005).

Fatty alcohols and wax esters are ubiquitous among organisms, but are generally produced in low quantities except in some cases such as sperm whales, carnauba palm and jojoba. In order to meet the demand for fatty alcohols and wax esters for industrial applications, the development of living systems which produce these compounds with desired properties in large quantities would be highly advantageous (Jenkins *et al.*, 2011; Rude & Schirmer, 2009; Jetter & Kunst, 2008; Kalscheuer *et al.*, 2006). Progress in biotechnology has enabled us to efficiently modify the biochemical pathway of cells for target oil products. However, although precise and efficient, genetic engineering requires a detailed understanding of the metabolic pathways involved so that target enzymes with the right specificity and subcellular location are manipulated for efficient synthesis of a certain desired product (Carlsson *et al.*, 2011).

Since fossil oil (petroleum) is also the feedstock for fatty alcohols and wax esters, producing fatty alcohols and derivatives from living systems can help

establish the balance in using oleochemical materials over petrochemical materials and therefore reduce the dependency of our society on fossil oil. More importantly, the development of living systems that produce fatty alcohols and derivatives will help to produce sustainable, renewable, non-toxic and biodegradable materials for industrial applications, thus reducing the environmental consequences of fossil oil consumption.

5 Conclusions and future considerations

5.1 Conclusions

AtFAR6 and AtFAR2/MS2 are alcohol-forming enzymes localised in the chloroplast. These enzymes utilise C16:0-CoA/ACP as substrates for C16:0-alcohol production.

The present *in vitro* characterisation of AtFAR6 and AtFAR2/MS2 confirmed these enzymes as FAR enzymes that produce fatty alcohols as end-products via intermediate fatty aldehydes, which were also released. In addition, free fatty acids were observed. The ratio of fatty alcohol:fatty aldehyde produced by this enzyme under *in vitro* conditions depends strongly on the availability of substrates regarding chain length, as well as free form substrates. The highest ratio of fatty alcohol:fatty aldehyde was achieved when 16:0-ACP/CoA was used as the substrate.

AtFAR6 and AtFAR2/MS2 possess similar activity, subcellular localisation and specific tissue expression, and are therefore likely to complement each other in functionality, for example in stratification of the exine layer during pollen development.

Characterisation of plant FARs demonstrated that although ubiquitous in plants, different FARs have distinct biochemical characteristics. They act either interactively or independently during the development of plants. The biosynthesis of fatty alcohols and wax esters in plant cells is summarised in Figure 10.

Maqu_2220 from *Marinobacter aquaeolei* VT8 encodes an alcohol-forming FAR with the capacity to use a broad range of substrates, *i.e.* saturated, unsaturated substrates, CoA- and ACP-activated substrates, branched acyl substrates and hydroxyl acyl substrate (ricinoleoyl-CoA). The finding also demonstrated that there are at least two biochemical pathways exist among

prokaryotes for the reduction of activated fatty acyl chains to fatty alcohols, one of which is analogous activity to eukaryotic FARs identified here.

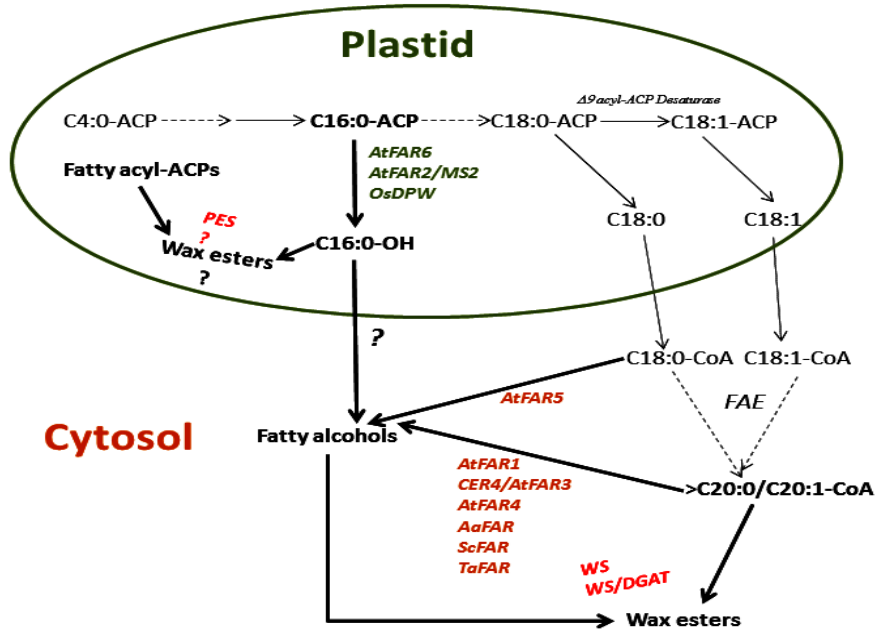


Figure 10: The biosynthesis and metabolism of fatty alcohols in plant cells. Thin arrows: fatty acid biosynthesis. Thick arrows: fatty alcohol metabolism.

5.2 Future considerations

The reduction power for reactions catalysed by FARs is supplied by NADPH, which is generated from plant photosynthesis and the pentose phosphate pathway. In addition, NADPH is also the reductant of the reactions in the Calvin cycle, lipid biosynthesis and oxidation-reduction in the cell. It would therefore be interesting to investigate whether regulation of the NADPH flux affects fatty alcohol production and the related metabolism pathways.

The study on *AtFAR6* and *AtFAR2/MS2* revealed the possibility that these enzymes are complementary to each other in biological function. Therefore the overexpression of *AtFAR6* in the *ms2* mutant as well as the analysis of the double mutant *atfar6/ms2* should clarify any relationship. We would expect that the *atfar6/ms2* plants would be sterile and the *AtFAR6* overexpressing/*ms2* plants would have normal pollen development thus

being fertile. If not then the lack of C16:0-alcohol gives a serious influence on pollen formation but plants can still stay fertile.

A considerable quantity of C16:0-alcohol was accumulated in the chloroplasts of *N. benthamiana* leaves expressing AtFAR6. In addition, chlorotic lesions were observed on these leaves compared with the control leaves. This indicates that the accumulation of fatty alcohols in chloroplasts is toxic to the cells. Within cells, one way to overcome this toxicity is by producing the ester form of alcohols, as seen in the formation of wax esters in bacteria during overproduction of fatty alcohols and fatty acids. Another possible way is to export the fatty alcohol to the cytosol for further modification. A small quantity of C16:0-alcohol moieties was detected in the wax ester fraction of *N. benthamiana* leaves expressing AtFAR6, but it is not known whether this wax ester is synthesised inside or outside the chloroplast. In contrast to AtFAR6 expression, the leaves expressing AtFAR2/MS2 were similar to the control. Therefore it would be interesting to investigate how the cells cope with the high amount of C16:0-alcohol produced by AtFAR2/MS2 and AtFAR6 in *Arabidopsis* cells.

Phylogenetic analysis of characterised FARs showed that there is a relationship in protein sequence of the FARs with species specificity, subcellular specificity and enzyme-specific activity. This suggests that there are corresponding conserved domains in the FAR amino acid sequences and therefore further investigations to define these domains could provide a better understanding for the engineering of FAR proteins.

Considering the evolution perspective, FAR homologues are ubiquitous in plants but limited to a few bacterial species and protists and have so far not been found in algae. Therefore, further research on additional FARs from various organisms could lead to interesting evolutionary perspectives. For example, if this type of enzyme is not found among algae how the marine bacteria or genes contained within these prokaryotes fit in with the accepted evolutionary tree of eukaryotes such as plants.

In combination with other reported research, the results from the studies in this thesis demonstrate that there is a vast resource of FAR enzymes with different substrate specificities as well as subcellular localisation in different organisms. The understanding of the availability and the biochemical properties of FARs will enable us to efficiently modify lipid metabolism for the production of fatty alcohols and derivatives with desired chemical structures in the cells.

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