Wax ester biosynthetic pathway

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Wax ester biosynthetic pathway

by

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DEDICATION

I dedicate this dissertation to my wife Yanbing Zhang (张燕冰), without whose support I would not have been able to complete this work, and to my children Lola (文澜) and Henry (文治), who are the source of my motivation.
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CHAPTER I: INTRODUCTION

Cuticular wax

In early morphological studies, the plant cuticle wax was described as “cuticular membrane” (Martin and Juniper, 1970; Baker, 1982). The plant cuticle is a hydrophobic layer coating in the epidermis of the aerial plant body. The structure of cuticular wax consists of several layers: epicuticular wax crystalloids or films, the cuticle proper, and a cuticular layer, which interweaves with the epidermal cell wall (from exterior inwards). From chemical perspective, the cuticle has been described as a polyester matrix of hydroxy- and hydroxyl-epoxy-fatty acids, C\textsubscript{16} and C\textsubscript{18} long (cutin) embedded and overlayed with cuticular wax (Kunst and Samuels, 2003). From the view of geneticists who study wax related genes, cuticular wax has been defined as the “lipids which are removed from plant surfaces after brief immersion in an organic [nonpolar] solvent” (Post-Beittenmiller, 1998). In terms of morphology, cuticule wax is considered to be the glaucous or whitish bloom on plant shoots, which corresponds to crystalloid epicuticular wax with light scattering capacity (Koornneef et al., 1989).

Evolution of cuticular wax

Interfacial interactions and physical properties of boundary layers are important for plant life at various scaling levels, and is therefore one of the key adaptations in the evolution of land plants (Raven and Edwards, 2004). Actually, macrofossil records interpreted as cuticles date back to the very earliest terrestrial plant species known (Edwards D et al., 1996). The critical role of this crucial
protective layer is stressed by the fact that it represents one of the largest interfaces between biosphere and atmosphere (Riederer and Schreiber, 1995). In moses, the embryo is surrounded by the maternal gametophyte tissues of the archegonium and subtending gametophyte (Crum, 2001; Shaw, 2003). A recent case study on a moss species *Funaria hygrometrica* suggests that the calyptra and its associated cuticle represent a unique form of maternal care in embryophytes (Budke et al., 2011). This organ can be considered an ancestor of the cuticle.

**Function of cuticular wax**

As the first barrier that a plant presents against to the environment, cuticular wax carries many functions according to its composition or three dimensional wax crystals on the plant surface. Cell forms, sizes and their fine structures have a great influence on several functional impacts on the plant-boundary layer (Koch et al., 2008). The functions of the plant cuticle are summarized in Figure 1. This layer controls non-stomatal water loss (Riederer and Schreiber 2001), protects from UV radiation (Krauss, Markstadter et al. 1997; Solovchenko and Merzlyak 2003; Pfüindel, Agati et al. 2006), keeps surfaces from attaching particles including dust, pollen and pathogen spores, and regulates plant interaction with insects, bacterial and fungal pathogens (Eigenbrode 2004; Carver and Gurr 2008; Leveau 2008; Müller 2008). The cuticle can also prevent organ fusion during plant development (Lolle, Hsu et al. 1998; Sieber, Schorderet et al. 2000).
Figure 1: Schematic summary of the most prominent functions of the cuticle as represented by a hydrophobic microstructured plant surface. (A) Transport barrier: limitation of uncontrolled water loss or leaching from interior and foliar uptake. (B) Water repellency: control of surface water status. (C) Anti-adhesive, self-cleaning properties: reduction of contamination, pathogen attack and control of attachment and locomotion of insects. (D) Signaling: cues for host-pathogens / insect recognition and epidermal cell development. (E) Spectral properties: protection against harmful radiation. (F) Mechanical properties: resistance against mechanical stress and maintenance of physiological integrity (Bargel et al., 2006).

Wax esters

The compounds of cuticular wax consist of very long chain alkanes, fatty acids, alcohols, ketones, aldehydes, terpenoids, and other molecules in addition to wax esters (Jetter et al., 2006). Functions of surface lipid can only be understood on the basis of their characteristic compounds and biosynthetic origin. The biosynthesis of plant surface lipid starts from the elongation of saturated C\textsubscript{16} and C\textsubscript{18} fatty acid CoAs to very long chain fatty acid (VLCFA) wax precursors between
24 and 36 carbons in length. Their subsequent biochemical modification is either via the alkane pathway or primary alcohol pathway (Li et al., 2008). The composition of surface lipid is a result of regulation of these pathway reactions (Figure 2).

**Figure 2**: Simplified pathways for wax biosynthesis in Arabidopsis stems. CER, ECERIFERUM; WSD, wax synthase/diacylglycerol acyltransferase; MAH, mid-chain alkane hydroxylase (Samuels et al., 2008).

**Biological diversity in wax esters among plant species.**

The wax ester content varies among and within species. Arabidopsis leaves only 0.1% to 0.2% of the surface lipids is wax esters, and in stem they account for
between 0.7% to 2.9% of the surface lipid (Jenks et al., 1995). Carnauba palm (Copernicia cerifera) in contrast, accumulates up to 85% wax ester in the surface lipid (Kolattukudy, 1976). In rare cases wax ester also serve as seed storage reserves. Specifically in jojoba (Simmondsia chinensis) 97% of its seed oil is wax ester (Benzioni et al., 2006).

Not only the total level of wax ester affects the characteristic and function of plant surfaces and lipids, but the composition details, such as chain length distribution and isomer composition also contribute to its biological function. So far, only Arabidopsis has been studied to this detail (Lai et al., 2007). The natural biological compositional diversity in wax esters can be used to understand the substrate preference and availability of the pathway that assembles wax esters. Therefore additional studies of wax ester biosynthesis is required.

**Wax ester biosynthesis**

\[
\text{CH}_3(\text{CH}_2)_n\text{-CO-S-CoA} + \text{HO-R} \rightarrow \text{CH}_3(\text{CH}_2)_n\text{-CO-O-R} + \text{HS-CoA}
\]

fatty acyl-CoA  alcohol  wax ester  CoA

**Figure 3:** Wax ester synthesis catalyzed by wax synthase.

The final step of wax ester biosynthetic pathway is catalyzed by wax synthase, which transfers the acyl group from an acyl-CoA to a fatty acyl alcohol (Figure 3). There are three unrelated families of wax synthases found in higher plants, mammals and bacteria (Jetter and Kunst, 2008). The jojoba-like WS family, the first identified plant wax synthase was from jojoba embryos. The jojoba enzyme uses a wide range of saturated and unsaturated acyl-CoAs with a chain length from 14 carbons to 24 carbons, with 20:1 being the preferred substrate, and exhibits
highest activity with C18:1 alcohol (Lardizabal et al., 2000). Later, a second member of this WS family was identified from Euglena. It can utilize fatty acids from 12:0, 14:0, 16:0 and 16:1-9, with 14:0 being the most favored fatty acid substrate; and the preferred primary alcohols substrates are 12:0, 14:0, 16:0 and 16:1-9 with 16:1-9Alc (Teerawanichpan and Qiu, 2010).

The second family type of WS was first identified in *Acinetobacter calcoaceticus*, and is the WS/DGAT (Acyl-Coenzyme A:Diacylglycerol acyltransferase) family. Enzymes in this family exhibit both WS and DGAT activity, thus can utilize both primary alcohols and more complex alcohols (e.g., DAG) as substrates. The *Acinetobacter* WS/DGAT enzyme shows a preference for C14 and C16 acyl-CoA substrates together with C14 to C18 primary alcohols when acting as a WS (Kalscheuer and Steinbuchel, 2003; Stöveken et al., 2005). A plant WS/DGAT type enzyme is the WSD1 gene of Arabidopsis, and it is capable of using C18, C24 and C28 alcohols and C16 fatty acid to produce wax esters (Li et al., 2008).

The third WS family, is the mammalian wax synthase, and it was first identified from mice (Cheng and Russell, 2004). The mammalian WS family of enzymes has the highest activity with acyl-CoAs of between C12 and C16, and primary alcohols shorter than 20 carbons (Cheng and Russell, 2004). There are no obvious plant orthologs of this mammalian WS family (Li et al., 2008).

In general, WSs naturally accept acyl groups with carbon chain length of C16 or C18 and primary alcohols with carbon chain length ranging from C12 to C20 (Shi et al., 2012). Reported activities of WSs with short chain alcohols are low (Stoveken and Steinbuchel, 2008).
Significances

Due to special chemo-physical properties of wax ester, they have many potential applications, such as lubricants, cosmetics, pharmaceutical products, ink, polishes and candles. For example, whale oil, which is primarily composed of wax esters was considered one of the best lubricants and used in specialized lubrication precision instruments. Despite the fact that wax esters are very common in nature, the abundance of wax ester is very low because only a few organisms can accumulate large quantities of wax ester. Most organisms produce just trace amounts of these chemicals. Sperm whale oil contains up to 95% of wax ester consisting of 34 carbons (Crisp, Eaton et al. 1984). Whales were an excellent source of wax ester, until whale hunting was banned internationally in the 1980s. The best alternative to whale oil is now jojoba oil, which occurs in the seed oil from the desert shrub jojoba. This seed oil contains 97% of C38 to C44 wax esters (Jetter and Kunst 2008).

Wax esters are also considered excellent biofuel and biolubricant for the future. However, the high price of wax esters has restricted their applications to high value products, such as cosmetics. Many efforts have been made to improve this situation. Growing jojoba plant in large scale seemed to be an easy solution in the past, but we still have to face the fact that jojoba is far not suitable for our agriculture system. Then genetically modified crop was proposed to massively produce wax ester. By re-constructing the wax ester biosynthetic pathway in genetically modified crop, not only will we be able to harvest sufficient quantities of wax esters, but also be able to manipulate the properties of the produced wax esters by manipulating
the acyl-chain specificity of the wax synthase enzyme. However, the current knowledge about the wax ester biosynthetic pathway is still too rudimentary for such a goal. To date only a few wax synthases have been characterized. Even in the most studied model organism such as Arabidopsis, only one WS/DGAT gene has been identified out of 23 putative WS and WS/DGAT homologs.

The studies reported in this thesis provide new insights to this field. We have set up two platforms for characterizing wax synthase enzymes; one by expressing them in yeast strains which can be fed substrates to characterize the substrate specificity of the wax synthase. The second platform utilizes Arabidopsis seeds in which we also co-expressed 3-ketoacyl CoA synthase and fatty acyl CoA reductase. In total nine genes were tested in this study and three of them were identified as wax synthase and their substrate specificities were characterized.
Dissertation organization

This dissertation includes five chapters. Chapter I provides introduction to wax esters, wax synthases and the significances of wax biosynthetic pathway research. The purpose of this chapter is to do a literature review on wax biosynthesis pathway research.

Chapter II describes wax synthase characterization in a yeast heterologous expression system. In this chapter, I constructed phylogenetic trees for both WS and WS/DGAT family. Nine genes were chosen based on their homology to previously identified WS or WS/DAGT. These characterizations showed that the Arabidopsis At5g55340 encoded WS and a maize WS are able to synthesize wax ester using fatty acids and primary alcohols. These two wax synthase exhibited distinct substrate preference patterns. The maize WS is also capable of producing ethyl esters and benzyl esters when proper substrates were provided.

Chapter III details a manuscript that expressing 9 WS or WS/DGAT genes in Arabidopsis seeds along with jojoba KCS and jojoba FAR. The analysis showed that KCS and FAR successfully increase VLCFA in seed oil and generated noticeable amounts of primary alcohols. Analysis of transgenic line with At5g55380 from Arabidopsis found wax esters in the seed oil. Dr. Ling Li conducted the promoter::GUS experiments in this chapter; this was done under the supervision of Dr. Eve Wurtele.

Chapter IV contains a genetic study of Arabidopsis T-DNA insertion lines. I characterized two T-DNA mutants in the WS gene At5g55380. This study indicates that At5g55380 may be redundant in the ability of the plant to produce wax esters.
In this chapter, I also discuss the need of combining reverse genetic approaches with heterologous expression studies to best characterize wax synthase genes. Dr. Yuqin Jin discovered morphological phenotypes on the original SALK_060303 T-DNA insertion mutant and it was used for further analysis in this chapter. Dr. Marna Yandeau-Nelson helped design the allelism-test experiment to determine the association between the T-DNA insertion sites, and the mutation causing the morphological phenotypes. The last chapter, Chapter V, contains general conclusions from Chapters II, III, and IV and additionally discusses potential future research.

This dissertation was under the guidance, supervision and support of my major professor, Dr. Basil J. Nikolau.
REFERENCES


CHAPTER II. HETEROLOGOUS EXPRESSION OF WS GENES IN MICROBIAL SYSTEMS

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**ABSTRACT**

Wax esters occur widely among bacteria, plants and mammals. There are three gene families encoding enzymes capable of synthesizing wax esters. Two of them exist in plans; the jojoba-like wax synthase (WS), and the bifunctional wax synthase/diacylglycerol acyl transferase (WS/DGAT). In this paper, we investigated the phylogenetic relationships among and between WS and WS/DGAT, based upon primary sequence homology of the encoded proteins. Nine candidate genes were chosen for experimental characterization using yeast as a heterologous expression host. Three of the expressed gene products were detected immunologically, Arabidopsis WS At5g55340, a maize WS and a moss WS. Moreover, by feeding these transgenic yeast strains with potential fatty acid and fatty alcohol substrates, we demonstrated that At5g55340 and the maize WS were expressed in a functional state, and could direct the synthesis of novel ester lipids. Based upon the ability to form different esters from different precursors, we were able to deduce the substrate preferences of these two enzymes. These characterizations indicate that At5g55340-encoded WS has a rather narrow substrate preference, producing
esters with linear fatty acids and fatty alcohols of about equal chain length. In contrast the maize WS has a wider substrate preference, producing similar linear esters as At5g55340, but also benzyl and ethyl esters.

Introduction

Wax esters are linear molecules consisting of a long chain fatty acid esterified with a long chain alcohol. They are found in a wide variety of organisms including bacteria, plants and animals. Due to their special properties, they have many applications such as lubricants, cosmetics, pharmaceutical products, ink, polishes and candles. For example, whale oil, which is primarily composed of wax esters was considered one of the best lubricants, and used in specialized lubrication precision instruments. Despite the fact that wax esters are very common in nature, the abundance of wax ester is very low because only a few organisms can accumulate wax ester, and most organisms produce just trace amounts of these chemicals. Sperm whale oil contains up to 95% of wax ester, consisting of 34 carbons (Crisp et al., 1984). Whales were an excellent source of wax ester, until whale hunting was banned internationally since the 1980s. The best alternative to whale oil is now jojoba oil, which is the seed oil from the desert shrub jojoba. This seed oil contains 97% of C38 to C44 wax esters (Jetter and Kunst, 2008). Although at much lower amounts, wax esters can also be found in surface cuticular lipids, which form the thin hydrophobic layer that covers outermost surfaces of the aerial tissues of terrestrial plants. Together with other plant surface lipids, they create a barrier between the plant and the environment. This layer controls non-stomatal water loss (Riederer and Schreiber, 2001), protects from UV radiation (Krauss et al.,
Wax esters in plant cuticular lipids vary from species to species, and even within a single species there are variations among different surfaces of a plant. For example, Arabidopsis leaf and stem surfaces contain 0.1% to 0.2% and 0.7% to 2.9% of wax esters, respectively (Jenks et al., 1995), whereas this number could be as high as 85% in leaf wax of the carnauba palm, *Copernicia cerifera* (Kolattukudy, 1976). Wax esters in the same organism are usually a mixture of different carbon chain lengths, consisting of different chain lengths of carboxylic acids and alcohols. The physical and chemical properties of wax esters are determined by the fatty acid and primary alcohol moieties. Thus, understanding the wax ester biosynthetic pathway is essential to generate wax esters with optimal properties for special applications.

The final step of wax ester biosynthetic pathway is catalyzed by wax synthase, which transfers the acyl group from an acyl-CoA to a fatty acyl alcohol. There are three unrelated families of wax synthases found in higher plants, mammals and bacteria (Jetter and Kunst, 2008). The jojoba-like WS family, the first identified plant wax synthase was from jojoba embryo. The jojoba enzyme uses a wide range of saturated and unsaturated acyl-CoAs with a chain length from 14 carbons to 24 carbons, with 20:1 being the preferred substrate, and exhibits highest
activity with C18:1 alcohol (Lardizabal et al., 2000). Later, a second member of this WS family was identified from Euglena. It can utilize fatty acids from 12:0, 14:0, 16:0 and 16:1-9, with 14:0 being the most favored fatty acid substrate; and the preferred primary alcohols substrates are 12:0, 14:0, 16:0 and 16:1-9 with 16:1-9Alc (Teerawanichpan and Qiu, 2010). The second family type of WS was first identified in *Acinetobacter calcoaceticus*, and is the WS/DGAT (Acyl-Coenzyme A:Diacylglycerol) family. Enzymes in this family exhibit both WS and DGAT activity, thus can utilize both primary alcohols and more complex alcohols (DAG) as substrates. The *Acinetobacter* WS/DGAT enzyme shows a preference for C14 and C16 acyl-CoA substrates together with C14 to C18 primary alcohols when acting as WS (Kalscheuer and Steinbuchel, 2003; Stöveken et al., 2005). A second WS/DGAT type enzyme is the WSD1 gene of Arabidopsis, and it is capable of using C18, C24 and C28 alcohols and C16 fatty acid to produce wax esters (Li et al., 2008). The third WS family, is the mammalian wax synthase, and it was first identified from mice (Cheng and Russell, 2004). This third WS family of enzymes, have the highest activity with acyl-CoAs of between C12 and C16 in and primary alcohols shorter than 20 carbons (Cheng and Russell, 2004). There are no obvious plant orthologs in this third WS family (Li et al., 2008). In general, WSs naturally accept acyl groups with carbon chain length of C16 or C18 and primary alcohols with carbon chain length ranging from C12 to C20 (Shi et al., 2012). Reported activities of WSs with short chain alcohols are low (Stoveken and Steinbuchel, 2008).

The most common platforms for studying these WSs are *E. coli* and yeast (*Saccharomyces cerevisiae*). *E. coli* has been successfully used as a heterologous
expression system for some WS/DGAT enzymes (Kalscheuer and Steinbüchel, 2003; Stöveken et al., 2005; Li et al., 2008). Considering that most of these enzymes are from eukaryotic organisms and membrane associated, it's not surprising that successful examples are quite rare. Actually, none of WS from jojoba family has been characterized in E. coli; only a few WS/DGATs have been expressed in E. coli. Another characterized eukaryotic factory, which has been proven to work, better for WSs is yeast. Besides advantages that include the ease of cultivation and genetic manipulation, short generation time and extensive knowledge about its metabolism (Tehlivets et al., 2007; Nielsen, 2009; Beopoulos et al., 2011; Matsuda et al., 2011), for this study a quadruple yeast mutant strain, H1246 was used. This strain carries mutations in four genes that are responsible in the biosynthesis of ester-lipids, specifically triacylglycerol (TAG) and sterol ester biosynthesis. Therefore, in this strain potential substrates will not flow to TAG and sterol ester biosynthesis sinks, which we are not of interest to this study.

In this study, we selected candidate jojoba-like WS and WS/DGAT genes from Arabidopsis, soybean, maize and moss, and we report on their characterization following heterologous expression in yeast. These characterizations identified enzymes with distinct substrate specificities.
Materials and methods

Phylogenetic tree constructions

TBLastn analysis was used to identify protein sequences in the NCBI NR database that are homologous to the jojoba WS-like and WS/DGAT family. The query sequences for this search were the amino acid sequences of experimentally characterized jojoba WS family and WS/DGAT family, and, 12 Arabidopsis WS homologs and 11 Arabidopsis WS/DGAT (Table 1). Homologs were selected based on the score threshold >200, an E-value >0.001. These identified homologs were added to a second query list and the search was repeated. Such iterations of the Tblastn analyses were repeated until no new homologs were identified.

The selected homologs were imported to MEGA 4.0 and amino acid sequences were pairwise aligned by MEGA, and a phylogenetic trees were constructed (Tamura et al., 2007). The phylogenetic tree of the jojoba WS-like homologs was out-grouped with the experimentally characterized bi-functional WS/DGAT sequences, and the phylogenetic tree of the WS/DGAT homologs was out-grouped with the experimentally characterized jojoba WS-like sequences. Each tree was assembled its rigor tested with Neighbor-join method with a bootstrap of N=1000 (Saitou and Nei, 1987).

Codon optimization and chemical gene synthesis

In order to improve protein expression in heterologous systems, all WS sequences were codon-optimized for yeast using the tool provided by GenScript (http://www.genscript.com). Codon optimization significantly reduced codon bias of
the final coding sequences and also eliminated extreme GC content, repetitive sequences, and avoided unfavorable mRNA secondary structure and unnecessary restriction sites. Chemically synthesized, codon-optimized genes were obtained from GenScript, and they were delivered as pUC57 clones.

**Plasmid construction**

All target genes were cloned into expression systems using Gateway Technology (Landy, 1989). Target genes were PCR amplified from pUC57-clones with primers that contained the CACC nucleotide sequence at the beginning of forward primers, at the 5’-end of each gene. PCR products were purified and added to TOPO cloning reaction, cloning the ORF into a Gateway Entry Vector (Invitrogen, Carlsbad, CA). Target genes were then transferred to the Gateway destination vector pYES-DEST52. This vector is capable of performing LR recombination reaction with the entry vector.

**Heterologous Expression in Saccharomyces cerevisiae**

Plasmid pYES-DEST52 carrying different WS cDNA homolog were transformed into yeast (S. cerevisiae) strains H1246 (MATα; are1-Δ::HIS3 are2-Δ::LEU2 dga1-Δ::KanMX4 lro1-Δ::TRP1 ADE2) (Oelkers et al., 2002; Sandager et al., 2002) using electroporation (Neumann et al., 1996). Plasmid pYES2.1 transformed yeast strains were used as negative controls.

Transformed yeast strains were grown at 30°C for 24 hours in 10 ml of the synthetic dropout medium containing 0.17% (w/v) yeast nitrogen base, 0.5% (w/v) ammonium sulfate, 2% (w/v) glucose and 0.06% (w/v) dropout supplement lacking
uracil (DOB + GLU-URA). The yeast cells were collected by centrifugation and resuspended in 25 ml of the same synthetic dropout medium, except glucose was replaced with 2% (w/v) raffinose. After 24 hours growth, the expression of the transgenes was induced by culturing the yeast at 20°C for 24 hours in 25 ml of the same synthetic dropout medium but this time containing 2% (w/v) galactose, 1% (w/v) raffinose as the carbon-source (DOB + GAL + RAF-URA). This induction medium was also supplemented with fatty acids or different alcohols as substrates for the expressed WS. After induction, cells were harvested, and analyzed for protein expression or accumulation of wax esters. For the substrate feeding experiments, 10 mM fatty acids and/or 10 mM alcohols (dissolved in ethanol or DMSO) were used as 200X stock solution, and these substrates were supplemented in the media at a final concentration of 50 uM. In these experiments 10 ml of culture was withdrawn, the cells were collected by centrifugation, and lyophilized for lipid extraction.

**Western blot analysis**

Yeast cells were extracted using Invitrogen Standard Protocol (Invitrogen, Carlsbad, CA). Protein samples were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and the proteins were transfer to a PVDF (polyvinylidene difluoride) membrane (GE Healthcare Bio-Science KK, Piscataway, NJ). Protein signals were detected using the enhanced chemiluminescence method (ECL, Amersham, Piscataway, NJ). Primary antibody was anti-His tag monoclonal antibody (BioRad, Hercules, CA). Secondary antibody was goat–anti-mouse Ig conjugated to horseradish peroxidase (ECL). Blots were
developed with Pierce Super Signal West Pico Chemiluminescent substrate (Thermo Fisher Scientific, Rockford, IL).

**Yeast lipid extraction**

The yeast lipids were extracted using a modified method previously described (Bligh and Dyer, 1959). Prior to extraction, an aliquot of an internal standard was added to the lyophilized cells. This internal standard was a solution of 5 mg/ml behenyl dodecanoate (C\textsubscript{34}H\textsubscript{68}O\textsubscript{2}), and this was added at a rate of 10 µl per 100 mg dry cell weight. To extract lipids, 1ml of near boiling methanol (60°C) was added to each sample, it was vortexed and immediately incubated at 60°C for 1-min. After cooling to room temperature, 500 µl of chloroform was added to the sample, and mixed by vortexing for 30 seconds. Following the addition of 400 ul of ddH\textsubscript{2}O the sample was further vortexed for 5 minutes. An additional 500 ul of chloroform was added, and again vortexed for 30 seconds. After a final addition of 500 ul of ddH\textsubscript{2}O and vortexing for 30 seconds the mixture was filtered through a 0.45 um PTFE filter. The two phases were separated by centrifugation at 5000g for 4 min, and the lower chloroform layer was recovered and evaporated to dryness using a stream of nitrogen gas.

**Thin-layer chromatography (TLC)**

The dry lipid extract from yeast was dissolved in hexane, and the lipid solution was spotted on silica gel plates (Sigma-Aldrich, St. Louis, MO). The solvent hexane:diethyl ether:acetic acid (90:7.5:1, v/v) was used to develop the plates (Li et al., 2008). TLC reference standards (alkane: C\textsubscript{22}H\textsubscript{46}, sterol ester: Cholesterol
dodecanoate, wax esters: behenyl dodecanoate (C34H68O2), triacylglycerols: canola oil, free fatty acids: palmitic acid, primary alcohols: octadecanol, sterols: ergosterol) were dissolved in hexane at a concentration of 0.5 mg/ml each. The TLC plate was air dried after development. The TLC plate was then sprayed with primuline solution (Taki et al., 1994) (5 mg in 100 ml of acetone/water, 80/20, v/v) with a Sigma glass sprayer. The plate was visualized under UV illumination.

**GC/MS analysis**

Lipid samples were silylated for GC analysis (Martin and Synge, 1941). An aliquot of 1-µL silylated lipid sample was injected into Agilent Technologies Model 6890 Gas Chromatograph equipped with an Agilent 19091S-433 column (30.0m x 250 um x 0.25 um), coupled to a Model 5973 Mass Selective Detector capable of electrical ionization (EI). The temperature programs used in this study were 50 °C for 1 minute, then 25 °C per minute to 200 °C, then 200 °C for 2 minute, then 10 °C per minute to 280 stay for 2 minutes, then 20 °C per minute to 320 stay for 20 minutes.

**Results**

**Phylogenetic classification of WS and WS/DGAT sequences**

Twelve jojoba WS-like genes have been identified in *Arabidopsis* based on sequence homology to the jojoba wax synthase amino acid sequence (Lardizabal et al., 2000; Beisson et al., 2003; Kunst and Samuels, 2003; Klypinaa and Hanson, 2008). These proteins share a high degree of homology, being of similar size, and sharing high degree of sequence similarity and identity (
(Continued on next page)

**Figure**.

Tblastn (Altschul et al., 1990; Altschul et al., 1997) was used to search for additional homologs of the jojoba-like wax synthase. This search conducted in August, 2009 identified 52 homologs of the jojoba-like wax synthase.

The jojoba WS-like family phylogenetic tree was constructed using these 52 putative homologs from many species, using the three known WS/DGAT proteins as the out-group (Figure 2). All but one of the 52 WS family members is sourced from higher plants of both monocots and dicots; the one exception is from a moss (*Physcomitrella patens subsp. patens*). No organisms lower than a moss was included in this tree. The tree can be divided into 3 major clades: moss, monocots and dicots. In the dicots clade, each species has multiple homologs. Homologs from same species did not clustered in the same branch. The monocots clade has only four representative homologs (1 from *S. bicolor* and Z. mays, and 2 from rice).

The WS/DGAT family phylogenetic tree was constructed using 171 putative WS/DGAT homologs identified by Tblastn, using known WS/DGAT amino acid sequences and their homologs in Arabidopsis as the query (Figure 3). Besides the homologs from plants, this phylogenetic tree also contains many homologs from bacteria. Homologs from the same species in the WS/DGAT tree tend to cluster together, indicating that gene duplication events after speciation is probably the evolutionary origins of these homologs. Only one moss WS/DGAT homolog was found, similar to the jojoba-like WS family.
Selecting candidates for functional characterization

Based on the phylogenetic classification of wax ester biosynthesis genes (Figure 2 & Figure 3), six putative jojoba WS-like and three WS/DGAT homologs from several different species were chosen for functional characterization (Table 2). (They will be referred as gene names in this table for the rest of this paper) In Arabidopsis there are 11 WS homologs in the genome, and 8 of them are physically clustered adjoining each other on chromosome 5. We selected 3 of these (At5g55320, At5g55340 and At5g55380) that phylogenetically fall into three different Arabidopsis clades, which would enable us to investigate if these genes that probably arose via gene duplication events have also functionally diverged. At5g55320 was picked because it’s located on another phylogenetic sub-clade and it is specifically expressed in reproductive organs, such as flowers and siliques. At5g55380 was chosen because it has highest expression level among the genes that form its sub-clade (Klypinaa and Hanson, 2008). At5g55340 was also selected based on its high expression among tissues and the fact that it is in a different sub-clade than At5g55320 and At5g55380. We also selected three WS homologs from soybean, maize and moss that are phylogenetically distinct from the Arabidopsis WS clade. WS/DGATs from soybean, maize and moss were also chosen as their WS corresponding genes and there is a good chance that at least one of them is responsible for wax ester synthesis since all of them only have one or two WS and WS/DGAT homologs. It might provide useful information about function redundancy and pathway regulation in soybean, maize and moss.

Protein structure computation
All the homologs are predicted to be integral membrane proteins, and the transmembrane domain topology was calculated with hydropathy analysis using TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) (Emanuelsson et al., 2007). All 6 jojoba-like WS homologs 7 or 8 transmembrane domains, while WS/DGAT homologs usually have 1 or 2 transmembrane domains (Figure 4).

Expression of WS and WS/DGAT homologs in yeast

Earlier published experiences indicate that expression of such integral membrane proteins as WS and WS/DGAT is difficult and risky in microbial hosts (Lardizabal et al., 2000). We therefore utilized a heterologous expression system and strategy that reduced some of the complexity of these experiments, and reduced the risks associated with obtaining functional expression. First, all cDNAs coding for WS and WS/DGAT sequences were codon optimized for yeast expression, and chemically synthesized. Second Gateway technology (Landy, 1989) was used to provide a rapid and highly efficient means of transferring genes from an entry vector to multiple expression vector systems that are capable of expressing the target genes in different hosts. To express the WS and WS/DGAT homologs in yeast, we were only able to clone seven cDNAs into the yeast destination vector pYES-DEST52 (Table 3), and the recombinant vectors were introduced in yeast strain H1246 (Sandager et al., 2002). Due to interruption of four TAG and sterol ester synthesis genes, H1246 is ideal for wax ester study since fatty acyl CoAs in this strain will be directed to wax ester synthesis only.
Heterologous expression of each construct was evaluated by immunological western blot analysis of protein extracts prepared from yeast strains, using anti-his tag serum. This antibody detected the 6xHis-tag that was fused to the C-terminus of the WS and WS/DGAT sequences as they were cloned into pYES-DEST52. Using this assay, the culture condition for expression of each construct was optimized. These analyses established that we obtained detectable expression of At5g55340, At5g55380 and the maize WS (Figure 5). No immunological bands were detected in either yeast strain with empty vector or in strains that were not induced with galactose.

**In vivo functional characterization of WS and WS/DGAT homologs expressed in yeast H1246**

The activity of the putative WS and WS/DGAT was investigated by feeding the yeast H1246 strains carrying each WS or WS/DGAT transgenes with a variety of potential substrates. Fatty acids of different chain lengths and many potential acyl group acceptors were included in the feeding list, including primary alcohols, branched alcohols, sterols, aromatic alcohols, methanol and ethanol. These acyl donors and acceptor were fed into the medium in pairs so that the identification of the ester product would be facilitated.

TLC analysis of the lipids extracted from the recombinant yeast strains that express the WS or WS/DGAT revealed that the lipid profiles remain the same as the empty vector control and non-recombinant strains when grown in induction medium with or without fatty acids supplemented as potential substrates. In
contrast, cultivation of these strains in induction medium containing acyl donor (fatty acid) and acyl acceptors (alcohols), led to the formation of esters (Figure 6).

When all transgenic yeast strains were fed with fatty acid and primary alcohol mixtures including C\textsubscript{13:0}, C\textsubscript{15:0}, C\textsubscript{17:0}, C\textsubscript{19:0} of both kinds, there were spots co-migrated with wax ester standard in strains carrying At5g55340 and maize WS (Figure 6A). In feeding experiment with fatty acids, benzyl alcohol and phenylethyl alcohol mixture provided, there was also a spot co-migrate with wax ester standard in maize WS carrying strain (Figure 6B). Similarly, when maize WS transgenic strain was fed with 0.2% ethanol (v/v) and fatty acid mixture, it also synthesized some lipid that would co-migrate with wax ester standard (Figure 6C).

To further test their substrate specificity with fatty acid and primary alcohols, strains carrying at5g55340 and maize WS, were fed with combinations of one fatty acid with chain length of C\textsubscript{13:0}, C\textsubscript{15:0}, C\textsubscript{17:0}, C\textsubscript{19:0} and one alcohol with chain length of C\textsubscript{13:0}, C\textsubscript{15:0}, C\textsubscript{17:0}, C\textsubscript{19:0} (Figure 7 above; Figure 8 above). To compare enzyme activity with saturated and unsaturated substrates, C\textsubscript{14:0} and C\textsubscript{14:1} fatty acids and C\textsubscript{16:0} and C\textsubscript{16:1} primary alcohol were fed in combinations, too (Figure 7 below; Figure 8 below). GC/MS analysis of yeast lipids revealed that At5g55340 prefers median chain substrates than long chain substrates; maize WS also prefers median chain substrate and it cannot used primary alcohols with chain length longer than 15 carbons. When compare saturated and unsaturated substrates, both of them have high activity with unsaturated primary alcohols.
The maize WS also exhibited activities when fed with ethanol, ethyl ester were found in the lipid profile. Ethyl esters were found ( ). Analyzed by GC/MS, it is most efficient incorporating C17:0 fatty acid into ethyl ester (Figure 9). When synthesizing ethyl ester, it still prefers unsaturated fatty acid with a 4-fold higher activity. It also synthesized aromatic ester when fed with benzyl and fatty acid. GC separated aromatic ester and we were able to identified it was benzyl ester. It is unable to use fatty acids with a chain length longer than 15 carbons (Figure 10). C13:0 fatty acid has much higher activity than C15:0. This enzyme also prefers unsaturated fatty acid when fed with benzyl alcohol and fatty acids.

Discussion

Metabolite profiling experiments indicate that wax esters are widely occurring in many taxonomic classes suggesting that the wax ester biosynthetic pathway is expressed in animals, plants and even microorganisms (Jetter and Kunst, 2008). Yet knowledge concerning the distribution of this pathway is rather limited, with only a few WSs and WS/DGATs enzymes and genes having been functionally identified (Lardizabal et al., 2000; Kalscheuer and Steinbuchel, 2003; Stöveken et al., 2005; Li et al., 2008; Teerawanichpan and Qiu, 2010; Barney et al., 2012). In this study, sequence similarity was used to identify homologs of WSs and WS/DGATs from many organisms, using BLAST (Altschul et al., 1990; Altschul et al., 1997). Amino acid sequence alignment of the selected proteins revealed that these homologs share very high identity and similarity among their own families, even though they may have different types of fatty acyl-CoA and alcohol substrates.
The jojoba-like WS family homologs all have the absolutely conserved histidine and glutamate residues (analogous to H254, E255 in jojoba WS) embedded in a hydrophobic region postulated to be part of the membrane-bound O-acyltransferase superfamily (MBOAT) active site (Hofmann, 2000). In addition, almost all putative plant WS proteins contain a highly conserved asparagine residue (analogous to N210 in the jojoba WS). This residue is conserved in all plant homologs, except At5g51420 where this residue is a threonine. This homolog is predicted to be a larger protein than the other WS, with 435 residues, whereas other plant homologs all have 333-345 residues. Also the expression of the At5g51420 gene has never been detected by RT-PCR or any of the publicly available gene expression database (Costaglioli et al., 2005; Klypinaa and Hanson, 2008). These characteristics suggest that At5g51420 is very likely to be a pseudogene, at least not the best candidate for this research.

The 52 WS homologs that were identified by BLAST analysis were phylogenetically classified based upon primary sequence similarity. The analyses segregated the sequences based upon their taxonomic source, i.e., the monocot, dicot groups and one member from mosses. This finding suggests that these genes emerged before the divergence of moss, monocots and dicots. In the monocots node, there are homologs from maize (Zea mays), rice (Oryza sativa), and milo (Sorghum bicolor). Inside the dicots cluster, the separation among species is not so clear. The homologs from one species usually are separated in to two, even three groups. For example, homologs from Vitis vinifera, some clustered with jojoba WS, which has been characterized (Lardizabal et al., 2000);
some are closer to a branch containing At5g51970, which has been characterized as a sterol acyltransferase (Chen et al., 2007); the rest clustered with soybean and *Ricinus communis*. In *Arabidopsis*, except at5g51970 has been characterized as sterol acyltransferase, the rest homologs from Arabidopsis are located in a different cluster than jojoba WS, together with two WS homologs from *Ricinus communis*. The phylogenetic tree of WS/DGAT family contains much more homologs from bacteria. The plant half of this tree has similar distribution like WS family. Monocots and dicots do not cluster with each other, but inside them, homologs from same specie do not necessarily stay in the same branch. Moss WS/DGAT homolog still stays out of monocots and dicots cluster but there are WS/DGATs from soybean and grape stay close to moss WS/DGAT. After phylogenetic studies, we chose 6 WS and 3 WS/DGAT proteins for this wax biosynthetic pathway study.

The heterologous expression system we set up in this study can be used as a platform for screening wax synthase. This platform covers, codon optimization, optimized protein expression and *in vivo* functional enzyme assays. We have shown that it is capable of expressing proteins that have multiple transmembrane domains to a level that is sufficient for detection by western blot analysis, which has never been achieved in previous studies. Adding the needed substrates to the yeast growth medium and then analyzing the lipid extract for the expected ester products was used to identify the substrate specificities of the individual expressed WS proteins.
The yeast quadruple mutant strain (H1246) was useful in this WS screening platform. Yeast itself does not synthesized wax esters, but the existing triacylglycerol and sterol ester pathways could act as a lipid sink for the fatty acids, and interfered with the \textit{in vivo} assay for WS activity. In the yeast strain H1246, mutations have eliminated TAG and sterol ester synthesis pathway, and this made this yeast strain ideal for this study.

We have characterized two putative proteins in jojoba WS family and have demonstrated that they are indeed WS enzymes. The protein encoded by At5g55340 is capable of using fatty acids and primary fatty alcohols from 13 carbons and 19 carbons into wax ester. Optimum activity was observed when both fatty acid and fatty alcohols have similar chain lengths. When comparing substrates with same chain length but with or without double bounds, this enzyme prefers saturated fatty acids and an unsaturated primary alcohol.

Compared to At5g55340, the maize WS shows broader substrate specificity. Not only can it use primary alcohol as acyl group acceptor, but also ethanol and benzyl alcohols. The ability of utilize long-chain primary alcohols is much higher than utilizing ethanol or benzyl alcohols. This suggests that we shall not only focus on fatty alcohol in the future when studying wax synthases. The ability to synthesize ethyl esters and benzyl esters will be useful to engineering biofuel producing organisms.

Moss WS was also expressed in yeast and could be detected by western blot using anti-His-tag antibody. However, after fed with potential acyl acceptor
groups including primary alcohols, branched alcohols, sterols, aromatic alcohols, methanol and ethanol, we still could not find any types of ester in the transgenic yeast lipids. This could be caused by several reasons. First, we cannot rule out the possibility that moss WS might not be functional in yeast due to protein post-translational regulation differences between moss and yeast. Second, we have no evidence that showed moss WS is wax synthase and based on its location on WS phylogenetic tree, it is distant from all other WS homologs. Third, even moss WS is functional and acts as a wax synthase, we might still need to feed the right substrates in the medium and they might not be included in our feeding list since it is not possible and certainty not practical to provide all possible substrates.

In summary, we demonstrated at5g55340, maize WS and moss WS can be expressed after codon optimization in yeast. By feeding potential substrates into the induction medium, we demonstrated that two of these proteins are capable of assembling wax esters from different fatty acids and alcohols.
Figures and tables:
Table 1: Query amino acid sequences for WS and WS/DGAT family homolog BLAST.

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Figure 1: Multiple sequence alignment of *Arabidopsis* jojoba-like wax synthase homologues and jojoba wax synthase. The multiple alignment of predict amino acid sequences of *Arabidopsis* wax synthases and jojoba wax synthase sequence was made with Clustal W.
Figure 1 continued
### Figure 1 continued

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X non conserved
X similar
X conserved
X all match
Figure 2: Phylogenetic tree of the 12 Arabidopsis and other species proteins related to jojoba WS, rooted with three identified WS/DAGTs. This analysis using MEGA 4.0 was performed using the neighbor-joining tree with 1000 replicates; the handling gap option was pairwise deletion. Sequence alignments were assembled by the ClustalW.
**Figure 3:** Phylogenetic tree of the 233 proteins related to WS/DAGT, rooted with jojoba WS and at5g55380. This analysis using MEGA 4.0 was performed using the neighbor-joining tree with 1000 replicates; the handling gap option was pairwise deletion. Sequence alignments were assembled by the ClustalW. Gene candidates were marked with red frames.
Figure 4: Hydropathy analysis of gene candidates in this study.

Hydropathy analysis was performed on all the 9 putative proteins chosen using algorithm TMHMM (Emanuelsson, Brunak et al. 2007). Regions predicted to be transmembrane domains are shown in bold red.
Table 3: Transgenic yeasts used in this study.

<table>
<thead>
<tr>
<th>Expression Vectors</th>
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<th>Yeast Strains</th>
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<tr>
<td>pYES-DEST52</td>
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<td>H1246</td>
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<tr>
<td>pYES-DEST52</td>
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</table>
**Figure 5**: Western blot; WSs and WS/DGATs expressed in yeast strain H1246. Vector: pYES-DEST52; 6X his-tag antibody. Protein were extracted from 0, 6 and 24 hours after these strains were cultivated in galactose medium.
Figure 6: TLC analysis of neutral lipids synthesized by recombinant yeast H1246 strain carrying WS or WS/DGAT. All the yeast strain were grown in induction medium, with feeding of: A: 50 nM fatty acids and primary alcohol mixtures; B: 50 nM fatty acids and benzyl alcohol and phenylethyl alcohol; C: 50 nM fatty acids and 0.2% ethanol. Fatty acid and primary alcohol mixture contain chain length of 13:0, 15:0, 17:0 and 19:0 carbons.
Figure 7: Wax esters synthesized by At5g55340 in yeast H1246 transgenic strain from feeding experiments. Above: saturated substrates of C_{13:0}, C_{15:0}, C_{17:0} and C_{19:0} fatty acids and primary alcohols were fed in combinations. Below: C_{14:0} and C_{14:1} fatty acids and C_{16:0} and C_{16:1} primary alcohols were fed in combinations.
Figure 8: Wax esters synthesized by maize WS in yeast H1246 transgenic strain from feeding experiments. Above: saturated substrates of C_{13:0}, C_{15:0}, C_{17:0} and C_{19:0} fatty acids and primary alcohols were fed in combinations. Below: C_{14:0} and C_{14:1} fatty acids and C_{16:0} and C_{16:1} primary alcohols were fed in combinations.
Figure 9: Ethyl esters synthesized by maize WS in yeast H1246 transgenic strain from feeding experiments. Above: 0.2% ethanol and fatty acids with C\textsubscript{13:0}, C\textsubscript{15:0}, C\textsubscript{17:0} and C\textsubscript{19:0} chain. Below: 0.2% ethanol and fatty acids with C\textsubscript{14:0}, C\textsubscript{14:1}. 
Figure 10: Wax esters synthesized by maize WS in yeast H1246 transgenic strain from feeding experiments. Fatty acids and benzyl alcohol were fed in combinations.
REFERENCES


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Klypinaa N, Hanson SF (2008) Arabidopsis thaliana wax synthase gene homologues show diverse expression patterns that suggest a specialized role for these genes in reproductive organs. Plant Science 175: 312-320


Prog Lipid Res 42: 51-80


CHAPTER III. ECTOPIC EXPRESSION OF WAX PRODUCING GENES IN TRANSGENIC ARABIDOPSIS SEEDS

In preparation for submission to the Journal of Plant physiology

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ABSTRACT

Two classes of enzyme catalyze the final reaction in the wax ester biosynthetic pathway, the jojoba-like wax synthase (WS) and the bifunctional wax ester synthase/acyl-CoA: diacylglycerol acyltransferase (WS/DGAT). In this study, we chose 6 WSs and 3 WS/DGATs from Arabidopsis, soybean, maize and a moss and expressed them in seeds of Arabidopsis Col-0. These transgenic events were coupled with the co-expression of the jojoba 3-ketoacyl-CoA synthase (KCS) and the jojoba fatty acid reductase (FAR). All three transgenes were under the control of the regulatory sequence of the seed-specific Glycinin promoter. The presence of mRNA of these transformed cDNAs was confirmed by RT-PCR. Gas chromatographic analysis of transgenic seeds indicated that jojoba KCS and FAR generated a substrate pool that contains very long chain fatty acids and long chain primary fatty alcohols. The Arabidopsis transgenic line ectopically expressing the At5g55380 WS produced wax esters in the seed oil.
Introduction

Wax esters are an important industrial commodity and have been considered as having potential as next generation biofuels and biolubricants. Traditionally, wax esters harnessed primarily from marine organisms have been used in the lubricant, food, pharmaceutical and cosmetic industries. For example, a grown whale can easily contain about 2000 liters of the spermaceti oil within its skull that consists mainly of oleyl-oleate ester (Spencer and Tallent, 1973). This oil was widely used as an excellent lubricant in all sorts of machinery, from fine mechanics such as watches, to transmission fluids in modern vehicles. Whaling, as the most efficient means to access large quantities of high quality wax esters directly caused the extinction of many whale species. Whale hunting was banned worldwide in the 1980s in order to conserve whale species. In replacement, jojoba oil was considered a best alternative to whale oil. The seed oil of jojoba contains 97% of C38 to C44 wax esters (Miwa, 1971). However, even after decades of breeding, jojoba is still not suitable for large-scale cultivation. Thus, the quest to search for large scale biological sources for wax esters is still not completed.

Wax esters are also found in surface lipids of aerial tissues of terrestrial plants. Together with other plant surface lipids, they create a barrier between the plant and the environment. This layer controls non-stomatal water loss (Riederer and Schreiber, 2001), protects from UV radiation (Krauss et al., 1997; Solovchenko and Merzlyak, 2003; Pfüindel et al., 2006), keeps surfaces from attaching particles including dust, pollen and pathogen spores, and regulates plant interaction with insects, bacterial and fungal pathogens (Eigenbrode, 2004; Carver and Gurr, 2008;
Leveau, 2008; Müller, 2008). The cuticle can also prevent organ fusion during plant development (Lolle et al., 1998; Sieber et al., 2000). Although the concentration of wax esters in plant surface lipids are very low, understanding their synthetic pathway can provide valuable knowledge including the substrate preference, and efficiency of the enzymes that assemble these lipids. This information can play a key role in generating a genetically modified crop for wax ester production.

The final step of wax ester biosynthetic pathway is catalyzed by an enzyme, which transfers the acyl group from an acyl-CoA to a fatty acyl alcohol. There are three unrelated families of wax synthases found in higher plants, mammals and bacteria (Jetter and Kunst, 2008). The jojoba-like wax synthase (WS) family, the first identified plant wax synthase was from jojoba embryo. The jojoba enzyme uses a wide range of saturated and unsaturated acyl-CoAs with a chain length from 14 carbons to 24 carbons, with 20:1 being the preferred substrate, and exhibits highest activity with C18:1 alcohol (Lardizabal et al., 2000). Later, a second member of this WS family was identified from Euglena. It can utilize fatty acids from C_{12:0}, C_{14:0}, C_{16:0} and C_{16:1-9}, with C_{14:0} being the most favored fatty acid substrate; and the preferred primary alcohols substrates are C_{12:0}, C_{14:0}, C_{16:0} and C_{16:1-9} with C_{16:1-9} alcohol being the best substrate (Teerawanichpan and Qiu, 2010).

The second family type of WS was first identified in *Acinetobacter calcoaceticus*, and is the bifunctional WS/DGAT (wax ester synthase/acyl-CoA : diacylglycerol acyltransferase) family. Enzymes in this family exhibit both WS and DGAT activity, thus can utilize both primary alcohols and more complex alcohols (DAG) as substrates. The *Acinetobacter* WS/DGAT enzyme shows a preference for
C14 and C16 acyl-CoA substrates together with C14 to C18 primary alcohols when acting as WS (Kalscheuer and Steinbuchel, 2003; Stöveken et al., 2005). A second identified WS/DGAT type enzyme is the WSD1 gene of Arabidopsis, and it is capable of using C18, C24 and C28 alcohols and C16 fatty acid to produce wax esters (Li et al., 2008). The third wax ester producing enzyme family, is the mammalian wax synthase, and it was first identified from mice (Cheng and Russell, 2004). This mammalian WS family of enzymes has the highest activity with acyl-CoAs of between C12 and C16 and primary alcohols shorter than 20 carbons (Cheng and Russell, 2004). There are no obvious plant orthologs in this mammalian WS family (Li et al., 2008).

Besides our interest of exploring the basic knowledge about wax ester biosynthetic pathway, this study also provides precious experiences of engineering a genetically modified plant to produce wax esters. This is also the critical step for generating genetically modified crops. An agronomically suitable oil crop, grown in large scale, can significantly lower the high price of wax esters in the market right now. The price is the major factor that limits the use of wax esters to high profit applications, such as cosmetic and pharmaceutical industries. With wax esters at an affordable cost, large scale industrial applications such as biofuels and biodegradable lubricants will become possible. We anticipate that some of these transgenes will be useful in the commercial level wax ester production in the future.

**Materials and methods**

**Plant materials**
*Arabidopsis thaliana* ecotype Columbia (Col-0) was used for transgenic expressions. Seeds were planted on MS medium then stratified for 24 hours. After two weeks, seedlings were transferred to LC1 soil mix (Sungro Horticulture, Agawam, MA). Plants were grown under continuous white fluorescent light at 22°C.

**Vector construction and Arabidopsis transformation**

A binary vector carrying 3 genes: 1) jojoba 3-ketoacyl CoA synthase, which is involved in fatty acid elongation of monounsaturated fatty acids; 2) jojoba fatty acyl-CoA reductase, which is involved in the formation of fatty alcohols; and 3) jojoba wax synthase, was obtained from the Dr. Ed Cahoon (University of Nebraska–Lincoln). The plant marker gene – DsRed that was in this vector was replaced with the hygromycin resistant gene. The jojoba wax synthase gene was removed by restriction enzyme digestion. The resulting vector was named pKF, which was used as a control vector. Six WS and 3 WS/DGAT genes (Table ) were cloned into this vector. These gene sequences were chemically synthesized by GenScript (Piscataway, New Jersey). They resulting vectors were named pKFW1 to pKFW9 respectively. All genes in this vector (3-ketoacyl CoA synthase, fatty acyl-CoA reductase and the wax synthesis gene) were under the control of seed specific, *Glycinin* promoter (Ding et al., 2006).

Ten plant binary vectors were introduced into *Agrobacterium tumefaciens* strain c58C1 via electroporation. These Agrobacterium strains were used to transform Arabidopsis using a floral dip method (Clough and Bent, 1998). Siliques, rosette leaves and seeds from confirmed individual transgenic Arabidopsis lines
were collected for genetic and biochemical analysis.

**Reverse transcript PCR**

Siliques at the mid-green stage (Mansfield and Briarty, 1991; Siloto et al., 2006) were collected, and RNA was extracted using RNeasy RNA extraction kit (Qiagen, Valencia, CA). The integrity of the isolated RNA was evaluated by agarose gel electrophoresis. The RNA preparation was treated with DNase I (Invitrogen, Carsbad, CA) to digest genomic DNA contamination. RNA samples were then used for first strand cDNA synthesis with oligo (dT)$_{20}$ primers and SuperScript III First Strand Synthesis System for RT-PCR (Invitrogen, Carsbad, CA), while same samples were treated identically only the reverse transcriptase was replaced with distilled water. The products from the first strand synthesis were then amplified by gene-specific primers (Table 2) designed for each of the wax synthase transgene sequences. Genomic DNA extract from rosette leaves from the same individual Arabidopsis plant was also amplified by same primers as a positive control.

**Seed lipid extraction**

For seed oil extraction (Heilmann et al., 2012), 5 mg of desiccated Arabidopsis seeds were homogenized using a glass homogenizer in 1 ml chloroform and 1 ml methanol. Docosyl dodecanoate (C$_{34}$H$_{68}$O$_2$) was added as internal standard when the samples were going to be analyzed by GC/MS. The homogenates were then transferred to a glass tube with Teflon lined screw cap, and shaken for 20 minutes. Then 1.3 ml of hexane and 700 ul diethyl ether (2 ml in total) was added, and the mixture was shaken for 20 minutes. The mixture was
centrifuged for 5 minutes at 450g, and the insoluble pellet on the bottom of the tube was discarded. The solvent was collected and evaporated under nitrogen gas and the oil was dissolved in 1 ml chloroform.

**Thin layer chromatography**

Lipid extracts from Arabidopsis seeds were dissolved in hexane, and the lipid solution was spotted on silica gel TLC plate (Sigma-Aldrich, St. Louis, MO). The plate was developed with the solvent hexane:diethyl ether:acetic acid (90:7.5:1, v/v) (Li et al., 2008). TLC reference standards (alkane: C\textsubscript{22}H\textsubscript{46}, sterol ester: cholesterol dodecanoate, wax esters: Docosyl dodecanoate (C\textsubscript{34}H\textsubscript{68}O\textsubscript{2}), triacylglycerol: canola oil, free fatty acids: palmitic acid, primary alcohols: octadecanol, and sterols: ergosterol) were dissolved in hexane (0.5 mg/ml each). The TLC plate was air dried after development, and then sprayed with primuline solution (5 mg in 100 ml of acetone/water, 80/20, v/v) from a Sigma glass sprayer. The plate can be visualized under UV light.

**GC/MS analysis**

For GC analysis, 1-µl lipid samples were injected into Agilent Technologies Model 6890 Gas Chromatograph equipped with a Agilent 19091S-433 column (30.0m x 250 um x 0.25 um), coupled to a Model 5973 Mass Selective Detector capable of electrical ionization (EI). The temperature programs used in this study were 50 °C for 1 minute, then 25 °C per minute to 200 °C, then 200 °C for 2 minute, then 10 °C per minute to 280 stay for 2 minutes, then 20 °C per minute to 320 stay for 20 minutes.
GUS activity analysis

The organ and tissue specific expression pattern of the At5g55380 gene was assessed using transgenic plants that carried a reporter genes (GFP-GUS) fused to different promoter elements from At5g55380. Three different constructs were generated (Figure 5) in which different promoter fragments were fused to a chimeric GFP-GUS gene in the expression transformation vector, pBGWFS7 (Karimi et al., 2002). Two of these constructs fused 1.55-kb or 0.28-kb of genomic DNA immediately upstream of the “ATG” translational start codon of the At5g55380 gene to the GFP-GUS chimeric gene, to generate WaxSp1, and WasSp2 constructs. In the third construct (WaxSp1tar), the GFP-GUS chimeric gene was fused to the 3’-end of the At5g55380 gene.

Results:

Expression of WS and WS/DGAT in transgenic Arabidopsis.

We chose to investigate 9 genes originally isolated from Arabidopsis, soybean, maize and moss. 6 are from the WS family and 3 are from the bifunctional WS/DGAT family. To ensure expression would be optimized, the codon usage of the moss WS and WS/DGAT genes were modified for Arabidopsis expression. Arabidopsis WS homologs were also redesigned to a different DNA sequences, so we can design RT-PCR primers unique to each transgene (Table 2).

The 10 plasmids constructed for plant transformation all carried the jojoba 3-ketoacyl-CoA synthase (KCS) and the jojoba fatty acid reductase (FAR) under the control of Glycinin regulatory sequences. Jojoba KCS was chosen because it can
elongate fatty acyl CoA up to 24 carbons (Lassner et al., 1996), and these acyl-CoAs can be reduced by jojoba FAR to generate primary long chain alcohols (Metz et al., 2000). One plasmid (pKF) that did not carry any wax biosynthesis gene was used as a negative control. The other 9 plasmids, named pKFW1 to pKFW9, all contain one WS or WS/DGAT gene sequence also under the control of Glycinin regulatory sequences. Glycinin is a soybean (G. max) promoter which specifically regulates the expression of transgenes in embryos of transgenic plants (Sims and Goldberg, 1989; Ding et al., 2006).

All 10 plasmids were introduced into Agrobacteria tumefaciens, and subsequently used to transform Arabidopsis. The seed progenies from the transformation event were identified as the T1 generation. For each transgenic line each batch of these T1 seeds were germinated on Hygromycin selective MS plates, and several individuals were identified, and confirmed by PCR genotyping with gene specific primers for KCS, FAR, WS and WS/DGAT. The T1 plants were grown to maturity, and T2 seeds were harvested. RNA was extracted from siliques of T2 plants, and T3 seeds were collected and used for oil content analysis.

RT-PCR was performed on RNA isolated from developing T3 seeds to determine the expression of transgenes using primers specific to each transgenes. Gene specific primers (Table 2) were designed to recognize unique sequences for each the transgene, and the specificity of these primer pairs was tested using wild type Arabidopsis to make sure that they would not show false positive results due to unspecific primer binding (data not shown).
RNA was extracted from Arabidopsis siliques at early and middle maturation stages, reverse transcribed using the poly-A tail as the primer site, and the resulting cDNA was used as the template for PCR. Duplicate reactions without reverse transcribed RNA samples were used as controls for genomic DNA contamination. The Arabidopsis Actin-2 gene (At3g18780), which is constitutively expressed in every organ, served as an ideal positive control for RT-PCR (Czechowski et al., 2005). Individual transgenic Arabidopsis plants were tested from each transgenic line.

We tested at least 4 individual plants for each transgene resulted in same band intensity, and one set of results are given in Figure 1. No PCR products were observed in any of the non-reverse transcribed treatments indicating that none of the RNA preparations contained genomic DNA contamination. Actin-2 mRNA was detected in all reverse transcribed samples showing that all the reverse transcription reactions were successful. Genomic DNA samples all showed the existence of transgenes in all samples analyzed. RT-PCR analysis detected the mRNA for every transgene assayed, with the exception of the moss WS/DGAT showed no positive results indicating undetectable gene expression. Although, the RT-PCR analysis we conducted is not quantitative, the variation of the band intensity differences suggest different expression levels even though the transcription of all transgene were under the regulation of the same promoter.

**Seed oil analysis**

Mature seeds collected from T2 were extracted with an organic solvent, and TLC was used separated the lipid classes. The TLC plates were sprayed with
premulin, and visualized under UV light. Seed oils from transgenic lines expressing the Arabidopsis At5g55380 (pKFW3) contained a significant amount of lipid class that co-migrated with the wax ester standard (Figure 2).

The lipids from all the 10 transgenic lines were also analyzed by GC/MS. Samples from transgenic lines expressing only KCS and FAR have novel GC peaks that were identified as elongated fatty acids C_{20:0} and C_{24:0} and primary alcohols (Figure 3), which are not present in wild type Col-0 Arabidopsis. These primary alcohols are monounsaturated and of 20, 22, and 24 carbon atoms in lengths. In the transgenic lines expressing At5g55380, besides these novel elongated fatty acids and primary alcohols, wax esters were also been identified (Figure 4). These wax esters consist of 16 and 18 carbon fatty acids esterified with long chain unsaturated primary alcohol.

**Organ and tissue specific expression of At5g55380**

The organ and tissue specific expression of At5g55380 was investigated in transgenic Arabidopsis plants carrying a GUS transgene whose expression was driven by a 1-kb promoter fragment from At5g55380. The expression patterns obtained from the resulting three sets of transgenic plants were near identical, thus only the observations from the waxSp2 construct are shown (Figure 6). In young seedlings, GUS expression, driven by the promoter of At5g55380 gene was expressed in cotyledons and leaves, and although expression is detectable in mesophyll cells of these organs, expression appears to be concentrated in the vasculature (Figure 6AD). Just before bolting, expression was stronger in the older leaves than in young expanding leaves, but it was not expressed in the shoot
meristem (Figure 6B). Strong expression was seen in the opened flower (Figure 6F), and within these flowers it was concentrated in the sepals and pedicles (F&G). Strong expression was also seen in the roots of young seedlings (10-day old plants); particularly in the primary roots (Figure 6H). Within the root system expression was particularly enhanced at the initiation of the lateral roots, and in the root vasculature, but not in root tip (Figure 6I).

Discussions

Wax esters are assembled by the reaction between an acyl-CoA and a fatty alcohol, a pathway that exists in bacteria, plants and animals (Lardizabal et al., 2000; Ishige et al., 2003; Cheng and Russell, 2004; Stöveken et al., 2005). The final reaction in this pathway is catalyzed by wax synthase, which belongs to the membrane-bound O-acyl transferase (MBOAT) super family (Matsuda et al., 2008; Shindou et al., 2009). As an important constituent of the surface lipids of terrestrial plants, there is limited knowledge about wax synthases from plants. The first wax synthase was identified and characterized from jojoba, which stores wax ester as major lipid in its seed oil (Lardizabal et al., 2000). By sequence similarity to the jojoba WS sequence, 12 jojoba-like WS homologs have been annotated in the Arabidopsis genome. To date however, none of them has been identified as a WS, and indeed, one of them, At3g51970 has been characterized as a sterol O-acyltransferase (Chen et al., 2007). More recently, another member of the MBOAT super family, a WS/DGAT has been reported to be responsible for wax ester synthesis in Arabidopsis stem (Li et al., 2008), which appears to be the only enzyme characterized as responsible for wax ester biosynthesis in terrestrial plants.
In this study we have successfully expressed both WS and WS/DGAT genes in Arabidopsis ecotype Col-0 seeds. Using the soybean glycinin promoter sequence (Sims and Goldberg, 1989; Ding et al., 2006) we have specifically ectopically co-expressed a 3-gene pathway in transgenic Arabidopsis seeds. In addition to the wax ester producing genes, the three reaction pathway included a gene for elongating fatty acids (KCS), and a gene for reducing fatty acyl-CoAs to fatty alcohols (FAR) (Lassner et al., 1996; Metz et al., 2000).

Platforms for characterizing wax synthase can be divided into microorganisms and oilseed plants. Microorganisms can be easily manipulated and usually with a simple and well-known background, such as yeast. And substrates can be fed into the medium to get rid of the influence from endogenous lipids. However, microorganisms like yeast also have their own shortcomings. Because wax synthases are all plant proteins with multiple transmembrane domains, the protein expression can be difficult. Within a few successful attempts, previous study found the substrate specificity was not the same as the same enzyme expressed in oil seed. On the other hand, oil seed plants have no concerns about the gene expression. The lipids accumulate during in oil seed development can provide a large potential substrate pool for wax synthase. Arabidopsis seed has been frequently used for study lipid metabolic processes. Arabidopsis seeds usually stores lipids mostly as triacylglycerol. During the synthesis of triacylglycerol, an enormous pool of fatty acids is present in the embryo. Expressing wax synthase in developing oil seed would take advantage of the substrate pool in the seed. However, in order to synthesize wax esters in the seed, fatty acyl CoAs produced
by Arabidopsis may not be of sufficient chain length. Not only may Arabidopsis lack
diversity of fatty acyl CoAs, but these seeds also lack the primary alcohols that
would be needed for wax ester biosynthesis. To make fatty acyl CoA and primary
alcohols available in Arabidopsis seeds, jojoba KCS and FAR were transformed
under the control of seed specific promoter. GC analysis showed new fatty acid and
primary alcohol peaks, which were specifically identified from their mass spectra.
The lipid profile showed 20 carbon and 24 carbon fatty acids; 20:1, 22:1 and 24:1
primary alcohols, which is consistent with previous studies of these two enzymes
(Lassner et al., 1996; Metz et al., 2000).

The transgenic lines of Arabidopsis Col-0 were confirmed by PCR using
gene specific primers on genomic DNA prepared form rosette leaves. To further
confirm that these genes have been transcribe in Arabidopsis seeds, siliques from
early and middle maturation were collected for RNA extraction. The RT-PCR results
revealed jojoba KCS, FAR and 8 out of 9 WS or WS/DGAT genes have been
successfully transcribed; the only one without detectable mRNA present was moss
WS/DGAT. Individual plants from independent transformation events showed
consistency, indicating the glycinin regulatory sequence has met our expectation
and minimum variation among different gene insert locations.

In this study, Arabidopsis transgenic line carrying At5g55380 was able to
accumulate significant amounts of wax ester in its seed oil. Further GC analysis
revealed the occurrence of C_{36}, C_{38}, and C_{40} esters. These wax esters consist of
saturated and unsaturated 16 or 18 carbon fatty acyl moieties, esterified to
unsaturated primary alcohols with chain length from 20 to 24 carbons. These
findings indicate that the WS encoded by At5g55380 prefers to esterify C\textsubscript{16:0}, C\textsubscript{18:0},
C\textsubscript{18:1} and C\textsubscript{18:2} fatty acyl CoA, with C\textsubscript{20:1}, C\textsubscript{22:1} and C\textsubscript{24:1} primary alcohols.

We have also studied the expression of At5g55380 in Arabidopsis, using promoter::GUS gene fusion method. We found this gene is expressed among all tissue types, even in the root vasculature. Previously similar conclusions were drawn from RT-PCR studies (Klypinaa and Hanson, 2008), with the exception that they did not check expression in roots.

Wax esters have been widely used in foods, cosmetic, perfume and pharmaceutical industries. More recently more attention has been focused on their potential as biorenewable biofuel and bio-lubricant. However, because of limited sources of natural wax esters, these molecules have a high price, which severely restricts applications to high value products. This study demonstrates an approach of modifying oil seeds to generate genetically modified plants to produce wax ester in the seed oil. This approach can be transplanted to an oil seed crop, and wax esters can be produced in large quantities to provide an economically viable source of these molecules for applications.
Figures and tables:

Table 1: List of genes chosen for ectopic expression in transgenic Arabidopsis seeds.

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<td>Maize WS/DGAT</td>
<td>TGAACATAGCACCACATCTAGGA</td>
<td>TGAACATAGCACCACATCTAGGA</td>
</tr>
<tr>
<td>A. thaliana Actin-2</td>
<td>GGCTCTCTTTAACCCAAAGG</td>
<td>GGGCATCTGAATCTCTCAGC</td>
</tr>
</tbody>
</table>
Figure 1: Expression of transformed genes in A. thaliana seeds. Gene names are indicated at right, control is Col-0 line with KCS and FAR but no WS or WS/DGAT. Genes examined are labeled at the bottom, GSP: gene specific primers. RT-PCR (lane a), minus RT control (lane b), and genomic DNA as positive control (lane c), products of these three PCR reactions are shown. The actin-2 gene was used as a positive control for RT-PCR efficiency.
Figure 2: TLC analysis of seed oil from transgenic Arabidopsis lines.

Arabidopsis lines transformed with which wax ester producing gene were marked at the bottom of the TLC plate. Control: Col-0 line with KCS and FAR but no WS or WS/DGAT.
**Figure 3**: Fatty acids and primary alcohols produced in Arabidopsis Col-0 transgenic line carrying jojoba KCS and jojoba FAR.
Figure 4: Wax esters synthesized in Arabidopsis Col-0 transgenic line carrying At5g55380, jojoba KCS and jojoba FAR. Red line represents Col-0 line with KCS and FAR transformed.
Figure 5: Constructs of At5g55380::GUS transgene. Three sets of transgenic lines were generated (WaxSp1, WaxSp2, and WaxSp1tar), which fused a GFP-GUS chimeric gene to different portions of the At5g55380 gene. The lengths and position of the fragments cloned into the constructs were: WaxSp1, 1551-bp upstream from position -1; WaxSp2, 280-bp upstream from position -1. WaxSp1tar, the promoter region plus the At5g55380 ORF. GUS activity assays for all three were very similar, and only data from the WaxSp2 transgenic lines are shown here.
**Figure 6:** At5g55380 GUS staining results. The expression of the At5g55380 gene visualized by GUS activity stains in young seedlings (A), in plants just before bolting (B), isolated cotyledon (C), isolated young leaf (D), flowers and buds (E and F), and roots (G, H, I). pd = pedicle, sp = sepal, sty = style, rv = root vasculature. Scale: red bar: 1mm blue bar: 0.1mm
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CHAPTER IV. MUTATIONAL ANALYSIS OF JOJOBA-LIKE WAX ESTER SYNTHASE IN ARABIDOPSIS

In preparation for submission to the Journal of BMC Plant Biology

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ABSTRACT

The Arabidopsis genome carries twelve homologs of genes encoding jojoba-like wax ester synthase. Reverse genetic approaches facilitate the characterization of the physiological functions of such multi-gene families, a process that is greatly enabled in Arabidopsis by the availability sequence-indexed, T-DNA mutagenized lines. In this paper, two T-DNA insertion lines (SALK_060303 and RATM54-0095-1_H) that carried mutations in the jojoba-like wax synthase gene, At5g55380 were characterized. We showed that the short root-length phenotype observed in SALK_060303 allele was due to mutation approximately 18 cM away from the At5g55380 locus. The genetic segregation studies enabled the separation of the short root-length-phenotype allele from the SALK_060303 allele in At5g55380. Biochemical characterization of the wax esters in the segregated SALK_060303 line, and of the RATM54-0095-1_H line established that these mutations did not affect the wax ester chemotype, suggesting that At5g55380 is redundant in the ability of Arabidopsis to produce wax esters.
Introduction

As will all terrestrial plants, aerial organs of Arabidopsis are covered by the cuticle, a hydrophobic layer coating the epidermis of the plant body. The commonality of all cuticles is that they consist of two types of highly lipophilic materials—cutin and surface lipids. Cutin is a polymer consisting mainly of $\omega$- and mid-chain hydroxyl and epoxy C$_{16}$ and C$_{18}$ fatty acids, and glycerol (Heredia, 2003; Nawrath, 2006; Stark and Tian, 2008). The surface lipids are outside and embedded in the cutin layer and can be extracted by organic solvents. The surface lipid is a complex of straight-chain C$_{20}$ to C$_{60}$ aliphatic molecules and may include secondary metabolites such as triterpenoids, phenylpropanoids and flavonoids (Jetter et al., 2006).

Surface lipids are thought to prevent nonstomatal water loss, and is therefore one of the key adaptation in the evolution of terrestrial plants (Raven and Edwards, 2004). Surface lipids are exposed to the environment as the outermost frontier of plant organs, therefore it is critical in how a plant interacts with its environment including plant-insect interactions, preventing germination of pathogenic microbes, and causing shedding of water droplets and dust particles as well as spores (Riederer and Muller, 2008). Surface lipids and cutin are also involved in cell-cell interactions, such as mediating pollen stigma contact and preventing post-genital organ fusion (Tanaka and Machida, 2008).

Wax esters are a component of the plant’s cuticular lipids, and their exact composition varies from species to species, and even within a single species there
are variations among different surfaces of a plant. For example, Arabidopsis leaf and stem surfaces contain 0.1% to 0.2% and 0.7% to 2.9% of wax esters, respectively (Jenks et al., 1995), whereas this number could be as high as 85% in leaf wax of the carnauba palm, Copernicia cerifera (Kolattukudy, 1976). Wax esters in the same organism are usually a mixture of different carbon chain lengths, consisting of different chain lengths of a carboxylic acid and alcohol. The physical and chemical properties of wax esters are determined by the component fatty acid and primary alcohol moieties.

The final step of wax ester biosynthetic pathway is catalyzed by wax synthase, which transfers the acyl group from an acyl-CoA to a fatty acyl alcohol. There are three unrelated families of wax synthases found in higher plants, mammals and bacteria (Jetter and Kunst, 2008). The jojoba-like WS family, the first identified plant wax synthase was isolated from jojoba embryos. The jojoba enzyme uses a wide range of saturated and unsaturated acyl-CoAs with a chain length from 14 carbons to 24 carbons, with 20:1 being the preferred substrate, and exhibits highest activity with C18:1 alcohol (Lardizabal et al., 2000). Later, a second member of this WS family was identified from Euglena. It can utilize fatty acids from 12:0, 14:0, 16:0 and 16:1-9, with 14:0 being the most favored fatty acid substrate; and the preferred primary alcohols substrates are 12:0, 14:0, 16:0 and 16:1-9 with 16:1-9Alc (Teerawanichpan and Qiu, 2010). The second family type of WS was first identified in Acinetobacter calcoaceticus, and is the WS/DGAT (Acyl-Coenzyme A:Diacylglycerol) family. Enzymes in this family exhibit both WS and DGAT activity, thus can utilize both primary alcohols and more complex alcohols (DAG) as
substrates. The *Acinetobacter* WS/DGAT enzyme shows a preference for C14 and C16 acyl-CoA substrates together with C14 to C18 primary alcohols when acting as WS (Kalscheuer and Steinbuchel, 2003; Stöveken et al., 2005). A second WS/DGAT type enzyme is the WSD1 gene of Arabidopsis, and it is capable of using C18, C24 and C28 alcohols and C16 fatty acid to produce wax esters (Li et al., 2008). The third WS family, is the mammalian wax synthase, and it was first identified in mice (Cheng and Russell, 2004). This third WS family of enzymes, have the highest activity with acyl-CoAs of between C12 and C16 in and primary alcohols shorter than 20 carbons (Cheng and Russell, 2004). There are no obvious plant orthologs in this third WS family (Li et al., 2008). In general, WSs naturally accept acyl groups with carbon chain length of C16 or C18 and primary alcohols with carbon chain length ranging from C12 to C20 (Shi et al., 2012). Reported activities of WSs with short chain alcohols are low (Stoveken and Steinbuchel, 2008).

Even through *Arabidopsis* has as many as 12 jojoba-like WS homologs and 11 WS/DGAT homologs, only one gene from WS/DGAT family has been identified as wax synthase. For the jojoba-like WS family, the only one homolog identified was a sterol O-acyltransferase (Chen et al., 2007). In this paper, we will discuss the mutant study we have done to understand wax ester biosynthetic pathway in *Arabidopsis*, specifically the role of At5g55380 in forming the wax esters of the cuticular lipids.
Materials and methods

Plant material and growth conditions

T-DNA insertion mutant lines SALK_060303 and RATM54-0095-1_H were obtained from the ABRC (www.arabidopsis.org) (Alonso et al., 2003). Seeds were planted on MS plate then stratified for 24 hours. After two weeks, seedlings were transferred to LC1 soil mix (Sungro Horticulture, Agawam, MA). Plants were grown under continuous white fluorescent light at 22°C.

Root phenotype analysis

Seeds were aligned on MS plates and germinated, while the plates were maintained vertically in a growth room with continuous fluorescent light at 22°C. Root length of Arabidopsis seedlings were measured from images taken regularly from MS plates. Software ImageJ (http://rsbweb.nih.gov) was used to measure each root length in image pixles, which was then converted into mm using a reference object length.

DNA isolation and PCR genotyping

Genomic DNA was prepared from rosette leaves. 1-2 leaves stored frozen in a 1.5 ml centrifuge tube were homogenized in the same tube in the presence of 500 ul shorty buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA), and the extract was clarified by centrifugation at 15,300 g for 5 minutes. One volume of chilled isopropanol was added to the supernatant and DNA was recovered by centrifugation at ca. 15,300 g for 10 minutes. The pellet was washed with 0.5 ml 70% (v/v) ethanol
at room temperature, followed by a one minute centrifugation. The pellet was then dried and dissolved in 50 ul distilled water.

Specific primers were designed and synthesized for genotyping. For allele SALK_060303:

left boarder PCR primers:
- 380ATG: 5’-ATGGAAGAAAAAGTTTAGAAACTTAATCG-3’,
- LBa1: 5’-TGGTTTCACGTAGTGGGCCATCG-3’;
right board primers:
- Out80Re: 5’-TTTGGCAGAGTTTTTTATTTT-3’,
- RB2-SALK: 5’-GACAGGTCGGTCTTGACAAAA-3’.

For allele RATM54-0095-1_H:

left board primers:
- LP-801H: 5’-GACAAATCCAGACAAACGACG-3’,
- Ds5-2a: 5’-TCCGTTCCGTTCGTTTTTAC-3’;
right board primers:
- RP-801H: 5’-CATGGCTTGCAAACTTTAAGC-3’,
- Ds3-2a: 5’-CCGGATCGTATCGGTTTTCG-3’.

**Lipid metabolite analysis**

The Arabidopsis lipids were extracted using a modified method previously described (Bligh and Dyer, 1959). Leaves, flowers, siliques and stem were collected then lyophilized for lipid extraction. To extract lipids, 2 ml of near boiling methanol (60°C) was added to each sample, it was vortexed and immediately incubated at
60°C for 1 min. After cooling to room temperature, 1 ml of chloroform was added to the sample, and mixed by vortexing for 30 seconds. Following the addition of 800 ul of ddH2O the sample was further vortexed for 5 minutes. An additional 1 ml of chloroform was added, and again vortexed for 30 seconds. After a final addition of 1 ml of ddH2O and vortexing for 30 seconds the mixture was filtered through a 0.45 um PTFE filter. The two phases were separated by centrifugation at 5000g for 4 min, and the lower chloroform layer was recovered and evaporated to dryness using a stream of nitrogen gas.

**GC/MS analysis**

For GC analysis, one microliter lipid samples were injected into Agilent Technologies Model 6890 Gas Chromatograph equipped with a Agilent 19091S-433 column (30.0m x 250 um x 0.25 um), coupled to a Model 5973 Mass Selective Detector capable of electrical ionization (EI). The temperature programs used in this study were 50 °C for 1 minute, then 25 °C per minute to 200 °C, then 200 °C for 2 minute, then 10 °C per minute to 280 stay for 2 minutes, then 20 °C per minute to 320 stay for 20 minutes.

**Results and discussion**

**Insertion location of SALK_060303 and RATM54-0095-1_H alleles**

The insertion sites of the T-DNA of in both alleles were identified by sequencing the PCR product generated from genomic DNA using specific primers for At5g55380 and both boarders of the T-DNA insert (Figure 1). At5g55380 has only one exon and no introns. In the SALK_060303 allele the T-DNA element is
inserted at between nucleotide position 1000 of the At5g55380 ORF, and this insertion event deleted 42 nucleotides. Seventeen of the deleted nucleotides are located within the computer-predicted 3` non-coding region of the gene and the remaining 25 are located immediately upstream of the stop codon. In RATM54-0095-1_H, the T-DNA element is inserted between nucleotide position 565 and 592 of the ORF, and thus deletes 27 nucleotides of the ORF.

**Growth phenotypes associated with SALK_060303 and RATM54-0095-1_H**

To ascertain the effect of the SALK_060303 allele on the growth morphology of Arabidopsis, sterile seeds homozygous for SALK_060303 and their wild type siblings were plated together on Murashige and Skoog solid media, and the growth of the two genotypes were compared (Figure 2). The mutant has narrower leaves and is less tolerant of drought situation. Mutant seedlings also have shorter main roots, with less secondary roots as compared to their wild type siblings and wild type columbia-0 seedlings. RATM54-0095-1_H allele was also compared with its wild type Nossen siblings. In contrast to the SALK_060303, no noticeable difference in growth and development was observed in RATM54-0095-1_H homozygous plants.

**Confirm correlation between T-DNA insertion and morphological phenotype**

A genetic allelism test was conducted to evaluate whether the apparent phenotype associated with the SALK_060303 allele, was due to the insertion of the T-DNA in At5g55380. Mutant homozygous plants from both T-DNA insertion lines
were intercrossed, and in parallel a control cross was conducted between Col-0 and Nossen wild types generated from sibling lines of the mutants. The morphological phenotype that was scored in the progeny was the short-root length of Arabidopsis seedling, and the segregation of the T-DNA insertion alleles was genotyped by PCR using specific designed primers described above. The root length of the mutants and their wild type siblings are shown in Figure 3. The roots of RATM54-0095-1_H allele in the Nossen background showed no significant difference as compared to the wild-type siblings, while the roots of SALK_060303 mutants are significantly shorter than the Col-0 wild-type. Also, plants carrying both alleles (obtained by intercrossing SALK_060303 and RATM54-0095-1_H) were compared to seedlings from the Nossen and Col-0 wild type cross, and no significant differences in root length were found.

Locating mutation causing the short-root length phenotype

To further identify the mutation that caused the morphological short-root length (r) phenotype associated with the SALK_060303 allele, progeny seeds from a heterozygotes for T-DNA insertion (Ww) and morphological phenotype (Rr) (identified by offspring segregation) were planted, and a total of 104 such plants were evaluated. The T-DNA insertion was genotyped by PCR and short-root length phenotype was also recorded. These 104 plants can be categorized into 6 groups: WWR_ : WwR_ : wwR_ : WWrr : Wwrr : wwrr. When only the T-DNA insertion or morphological phenotype is considered separately, they should both segregate at a ratio of 1:2:1 or 3:1. Statistical analysis (chi-square test) of the ratio of the T-DNA insertion (Table 1) and short-root length (r) phenotype (Table 2) among the
evaluated 104 progeny, showed a p-value > 0.05; thus both failed to reject the null hypothesis that it agrees with the Mendelian segregation for a recessive alleles.

If T-DNA insertion and the mutation causing the short-root length phenotype are not linked, they should also obey Mendel’s Law of Independent Assortment; namely the expected ratio of WWR_ : WwR_ : wwR_ : WWrr : Wwrr : wwrr should equal to 3:6:3:1:2:1 (Polynomial expansion of (1:2:1)(3:1)). The p-value of the chi-square test is <<0.005, suggesting the mutation causing phenotype is linked with the T-DNA insertion (Table 3).

The linkage between the SALK_060303 T-DNA insertion and the mutation can be calculated using many approaches. In this case, using offspring of selfed heterozygous plants, the linkage can be calculated by sum of complementary classes method, weighted mean method, product method, method of maximum likelihood and method of maximum $\chi^2$ (Fisher and Balmukand, 1928). The distance between SALK_060303 T-DNA insertion in At5g55380 and the mutation causing the short-root length phenotype is listed in Table 4; the best estimate of the linkage is around 18 centiMorgans.

Chemotype of At5g55380 mutants of Arabidopsis

From the segregation genetic crosses we were able to recover via recombination a line that carried the SALK_060303 T-DNA insertion but did not carry the short-root length phenotype trait. This new segregated SALK_060303 line (DAT-18) was analyzed for wax esters. The total lipid was extracted and analyzed by GC/MS (Figure 4). Lipid profiles from the DAT-18 line (homozygous for the
SALK_060303 allele, the sibling DAT-9 line (heterozygous for the SALK_060303 allele), and DAT-4 wild type sibling are shown in Figure 4; there are no new or missing peaks among these profiles.

**Summary of reverse genetics study**

This reverse genetic study failed to demonstrate that At5g55380 is required for *in vivo* wax ester biosynthesis in Arabidopsis. There are several potential explanations for this observation. One possibility is that At5g55380 does not encode for a wax synthase. But the findings presented in Chapter 3 of this thesis, the ectopic expression of this gene in Arabidopsis seeds, clearly establish that At5g55380 encodes a wax synthase.

Clearly, the explanation lies elsewhere. The most likely explanation is that At5g55380 is redundant in the production of wax esters. This is apparent considering that At5g55380 is one of 12 such genes in the Arabidopsis genome. Although genetic studies in Arabidopsis have identified over 30 genes (*cer* mutants) that are needed for surface lipid deposition, none of them to date have been in the 12 jojoba-like wax synthase genes. The fact that wax esters are only a small portion of surface lipid constituent is the probable explanation as changes in wax ester accumulation can be easily missed in the visual screen used to identify the *cer* mutants.

Therefore as demonstrated in this thesis, characterization of wax synthase paralogs in Arabidopsis will require a combination of a reverse genetic approach, coupled with a heterologous expression system, either in yeast or Arabidopsis seed.
Figures and tables:

Figure 1: T-DNA insertion of two At5g55380 knock-out mutants.
Figure 2: Effect of mutation in At5g55308 on growth morphology. The growth morphology of sibling plants that were homozygous for the wild type or SALK_060303 allele was compared at different stages of development. A: Wild type and mutant seedlings transferred from plates; B: Plants at about 7 days after flowering; C: Seedlings 12 DAP on MS plate; D: 6 DAP seedlings.
Figure 3: Root length of SALK(SALK_060303) and RIKEN(RATM54-0095-1_H) compared to their wildtype siblings. SALK/RIKEN was generated by crossing SALK_060303 and RATM54-0095-1_H homozygotes; Col-0 and Nossen were also crossed then used as control.
**Table 1**: Chi-square test of T-DNA insertion segregation of SALK_060303.

<table>
<thead>
<tr>
<th></th>
<th>WW</th>
<th>Ww</th>
<th>ww</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed</td>
<td>33</td>
<td>50</td>
<td>21</td>
<td>104</td>
</tr>
<tr>
<td>Expected</td>
<td>26</td>
<td>52</td>
<td>26</td>
<td>104</td>
</tr>
</tbody>
</table>

Chi-square: 2.923  
Degree of freedom: 2  

p-value: 0.2318792
Table 2: Chi-square test of morphological phenotype segregation in SALK_060303 line.

<table>
<thead>
<tr>
<th></th>
<th>R_</th>
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</tr>
</thead>
<tbody>
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<td>83</td>
<td>21</td>
<td>104</td>
</tr>
<tr>
<td>Expected</td>
<td>78</td>
<td>26</td>
<td>104</td>
</tr>
</tbody>
</table>

Chi-square: 1.282

degree of freedom: 1

p-value: 0.2575179
Table 3: Chi-square test of independent assortment of T-DNA insertion and phenotypic mutation.

<table>
<thead>
<tr>
<th></th>
<th>WWR_</th>
<th>WwR_</th>
<th>wwR_</th>
<th>WWrr</th>
<th>Wwrr</th>
<th>wwrr</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed</td>
<td>31</td>
<td>44</td>
<td>8</td>
<td>2</td>
<td>6</td>
<td>13</td>
<td>104</td>
</tr>
<tr>
<td>Expected</td>
<td>19.5</td>
<td>39</td>
<td>19.5</td>
<td>6.5</td>
<td>13</td>
<td>6.5</td>
<td>104</td>
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</tbody>
</table>

Chi-square: 27.59
Degree of freedom: 5
p-value: 0.0000437
Table 4: Linkage between T-DNA insertion and morphological mutation calculated by different methods.

<table>
<thead>
<tr>
<th>METHOD</th>
<th>RECOMBINATION VALUE (CM)</th>
</tr>
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<tr>
<td>Sum of complementary classes</td>
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</tr>
<tr>
<td>Weight mean</td>
<td>23</td>
</tr>
<tr>
<td>Product</td>
<td>19</td>
</tr>
<tr>
<td>Maximum likelihood</td>
<td>18</td>
</tr>
<tr>
<td>Minimum $X^2$</td>
<td>18</td>
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</table>
**Figure 4:** GC analysis of lipids from SALK_060303 homozygote (DAT-18), heterozygote (DAT-9) and wild type sibling (DAT-4).
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CHAPTER V: GENERAL CONCLUSIONS

The goals of the work presented in this thesis are to expand the limited knowledge on wax ester biosynthetic pathway, and to generate the technology base for the future production of wax esters in microorganisms or seed crops as biorenewable chemicals. To achieve these goals, we studied 9 wax ester biosynthetic genes. Based on sequence homology, 6 of these genes were classified as jojoba-like WS, and 3 were classified as bi-functional WS/DGAT enzymes. These nine genes were tested by transgenic expression in yeast and Arabidopsis platforms.

Expression in yeast resulted in the functional characterization of the Arabidopsis At5g55340 WS gene and of a maize WS gene. The substrate specificity of these two enzymes was characterized by supplying potential substrates in the medium supporting the growth of the transgenic yeast. These experiments revealed that the At5g55340-encoding WS utilizes fatty acid and primary alcohols substrates with chain length of between 13 and 19 carbons. The enzyme exhibits a substrate preference pattern in which the fatty acid and the primary alcohol have similar chain length, and a preference for saturated rather than unsaturated fatty acids and alcohols.

The maize WS that was also active in yeast displayed a different substrate preference. This enzyme can use fatty acid substrates from 13 to 19 carbons, but the primary alcohol substrate has to be shorter than 17 carbons in chain length. This enzyme also favors unsaturated substrates. The maize WS is also capable of
synthesizing two biologically unusual esters, namely ethyl esters and benzyl ester. The significance of this finding is that fatty acid ethyl esters have applications in biofuels, as a biodiesel. The utility of benzyl esters would need additional applications research as its chemo-physical properties are not well understood.

All the 9 candidate genes were also expressed in Arabidopsis seeds, along with jojoba KCS and FAR. All three components were under the regulation of the seed-specific Glycinin promoter. Analysis of the resulting seed oil revealed that At5g55380 is capable of assembling wax esters using 16 and 18 carbon fatty acid and C20:1, C22:1 and C24:1 primary alcohols.

The two expression platforms (yeast and seeds) were complementary in functionally demonstrating the wax synthase activity of three gene products. At5g55380, which was active in seeds, requires substrates that were not available for the yeast feeding experiments, namely very long chain unsaturated primary alcohols. In contrast, in the transgenic Arabidopsis lines carrying At5g55340 and the maize WS (which were active in yeast) did not synthesize any wax ester in the seed oil. This is probably because the KCS and FAR we chose were unable to provide the appropriate chain length of the alcohol substrate. The At5g55340 WS has low activity when the chain length of substrates is increased, and the shortest primary alcohol found in the Arabidopsis lines carrying KCS and FAR was C20:1. Similarly, the maize WS showed no activity in Arabidopsis seed for the same reason since it can only utilize C13 and C15 alcohols in yeast.

These results indicate that yeast and plant expression systems cannot replace each other, and are therefore complementary. One advantage of the yeast
expression platform is that we can manipulate the substrate pool as long as we are able to obtain the substrates. The substrate pool can be expanded to short alcohol, branched chain alcohol, aromatic alcohols, sterol and much more; this is impossible for the plant system. However, plant expression system can also provide substrates that are difficult to purchase. Moreover, by using different KCS and FAR it may be possible to expand the substrate pools that can be provided in seeds.

A reverse genetic study, using T-DNA insertion mutants was also conducted, with the Arabidopsis gene At5g55380. Biochemical analysis of plant lipids showed that the At5g55380 mutants did not differ from the wild type, indicating that this gene is redundant in producing wax esters.