**Identification and Characterization of Arabidopsis *ECERIFERUM8* (*CER8*),
a Gene Important for Cuticular Wax Biosynthesis**

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Abstract

As one of the major components of the plant cuticle, cuticular waxes play essential roles in many ecophysiological functions, such as resistance to drought and pathogens. *Arabidopsis thaliana* mutants with abnormal wax load and/or composition are named *eceriferum* (*cer*), which are typically identified by their visibly glossy stems. More than 30 *cer* loci have been identified, but the identification and roles of most of these *CER* genes are unknown.

By using a combination of forward and reverse genetic approaches, I have identified the *CER8* gene as Long-chain Acyl-CoA Synthetase 1 (*LACS1*). The allelism of *CER8* and *LACS1* was confirmed by genetic complementation crosses transcript analysis, and gene sequencing. Scanning electron microscopy (SEM) of *cer8* mutants showed that they have reduced wax crystal density and lack the tube-shaped wax crystals found on wild-type plants. Gas chromatography analysis of *cer8* mutant lines showed that most of the main wax components, including C29 alkanes, C29 ketones, C29 secondary alcohols and C30 primary alcohols are significantly decreased. The total wax load of *cer8* null alleles are decreased to approximately half that of the wild type levels. Conversely, C30 free fatty acids are increased by about 15-fold. This is consistent with a defect in a very long-chain acyl CoA synthetase activity specific for C30 fatty acids. The promoter regions of *CER8/LACS1* and two other epidermal-expressed LACs, *LACS2* and *LACS3* were fused with the GUS reporter gene to analyze the gene expression patterns. *LACS1* was

found to be epidermal specific, while *LACS2* and *LACS3* are expressed in the internal cell layers but accumulate to higher levels in the epidermal cell layers. Finally, the substrate specificities of *LACS1*, -2 and -3 were characterized by using a colourimetric assay with saturated fatty acids of even chain lengths ranging from C16 to C30. *LACS1* shows significantly higher activity for C30 free fatty acids than *LACS2* and *LACS3*, which correlates the increased level of C30 free fatty acids in the *cer8* mutant alleles. These results suggest that *LACS1* and an unknown acyl-CoA thioesterase together provide balanced levels of C30 fatty acyl-CoAs/free fatty acids necessary for the process of cuticular wax biosynthesis .

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List of Abbreviations

AAE: acyl-activating enzyme
ABC transporters: ATP binding cassette transporters
ABRC: Arabidopsis Biological Resource Center
ACP: acetyl-acyl carrier protein
BSTFA: bis-(Trimethylsilyl)trifluoroacetamide
CAPS: cleaved amplified polymorphic sequence
CER: eceriferum
CGSC: *E. coli* Genetic Stock Center
CO: carbon monoxide
DGAT: diacylglycerol acyltransferase
ECR: enoyl-CoA reductase
ER: endoplasmic reticulum
FAE: fatty acid elongase
FAR: fatty acyl-CoA reductase
FAS: fatty acid synthase complex
FAT: fatty acyl-ACP thioesterase
FID: flame ionization detector
GC: gas chromatography
GFP: green fluorescent protein
GPAT: glycerol-3-phosphate acyltransferase
GUS: β -glucuronidase
KCS: β -keto acyl-CoA synthase
KCR: putative β -ketoacyl reductase
LACS: Long-chain acyl-CoA synthetase
LCM: laser capture microtechnique
LTP: lipid transfer proteins
NMD: nonsense mediated mRNA decay
PM: plasma membrane
PTC: premature termination codon
RT-PCR : Reverse-Transcription PCR
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM: scanning electron microscopy
SSLP: simple sequence length polymorphism
VLCFA: very-long chain fatty acids
WS: wax synthase

Chapter 1 Introduction: Plant Cuticle

1.1 Plant cuticle

The plant cuticle is a lipid-based layer that coats the aerial organ surfaces of vascular plants. It is synthesized and secreted by epidermal cells and possesses essential functions including preventing uncontrolled water loss, pathogen and pest resistance, and for plant development. The term 'plant cuticle' can be traced back to the year of 1834, when Brongniart designated "cuticula" to a superficial membrane isolated from cabbage leaf (Brongniart, 1834). Our current concept of the plant cuticle is contributed by von Mohl (1847), who defined the plant cuticle as a two layer-membrane specific to epidermal cells at mature stages. It is now known to consist primarily of a cutin polymer matrix and cuticular waxes.

The structure of a mature plant cuticle can be generally described as a matrix of fatty acids linked together mainly by ester linkages (cutin polymer matrix), which is embedded with intracuticular waxes and overlaid with epicuticular waxes. At mature stages, the arrangement of the cuticular layers, from outer to inner, is the epicuticular layer (either in the form of a wax film or as wax crystals extruding from a wax film), the primary cuticle (also named 'cuticle proper' or 'cuticularized layer'; and containing cutin and intracuticular waxes), and the secondary cuticle (cuticular or cutinized layer, no intracuticular waxes) (Heredia, 2003) (Figure 1).

The main function of the plant cuticle is to prevent non-stomatal water loss

(Kerstiens, 1996). Additionally, the wax crystals that coat the cuticle surface repel water droplets, which serves to shed dust particles and spores (Holloway, 1969a,b, 1970), reflect short wave UV radiation (Krauss et al., 1997), and coordinate interactions between the plant and other organisms including bacteria, fungi and insects (Eigenbrode and Espelie, 1995; Markstädter et al., 2000; Kunst and Samuels, 2003). In addition, the cuticle prevents inappropriate organ fusions during development (Sieber et al., 2000; Nawrath, 2002).

The epicuticular waxes form a variety of ultrastructures as observed by scanning electron microscopy (SEM). The major wax crystalline structures include platelets, tubules, threads, transverse rodlets, dendrites, and longitudinal rodlets (Figure 2) (Jeffree, 2006). For example, wax rich in primary alcohols generally have plate-shaped structures, while wax rich in β -diketones have tubular shapes (Jeffree, 2006). However, the correlation between wax composition and crystalline structure is not strict, because different aliphatic components may result in similar crystalline structures. For instance, *Vitis vinifera* (grape) has oleanolic acid ($C_{30}H_{48}O_3$) as the main wax component, which also gives plate-shaped crystalline structures similar to those generated by primary alcohols (Baker, 1982).

Figure 1 Plant cuticle structure

(A) Schematic image of cuticle structure (adapted from Buchholz, 2006)

EW: epicuticular wax

CP: primary cuticle; also called 'cuticle proper'

CL: secondary cuticle;

PL: pectinaceous layer and middle lamella;

CW: cell wall;

P: plasmalemma.

(B) Cryo-SEM image of cross section of plant epidermal cell including the cuticle plant cuticle (Image kindly provided by A.L. Samuels, University of British Columbia)

Figure 2 Typical wax crystal ultrastructures

Varieties of wax crystal ultrastructures found on the surfaces of different plants as observed by scanning electron microscopy. The chemical names listed next to each panel refer to the major components of the corresponding wax crystal ultrastructure. (adapted from Jetter et al., 2005)

Platelets

Pitcher Plant;
Aldehyde

Tubules

Yew tree;
Secondary
alcohol

Threads

Castor;
Triterpenoid

Transverse Rodlets

Royal fern;
Ketones

Dendrites

Wild cabbage;
Ketone, alkane,
secondary
alcohol

Longitudinal Rodlets

Arabidopsis;
Ketone, alkane,
secondary
alcohol

1.2 Cutin Polymer Matrix

1.2.1 Chemical Composition of Cutin

The cutin polymer matrix is the fundamental framework of the plant cuticle, providing anchoring for intracuticular waxes and interacting with the epidermal cell wall. Cutin makes up 40%~80% of the total cuticle weight and the total load of cutin typically ranges from 10 $\mu\text{g cm}^{-2}$ to 500 $\mu\text{g cm}^{-2}$ (Kolattukudy, 1980; Walton, 1990; Heredia, 2003). Some fruit cutin load can reach as high as 1.5 mg cm^{-2} (Stark and Tian, 2006). Within a given plant species, cutin content varies with organ type and development phase (Holloway, 1982).

Unlike the organic solvent soluble waxes, the ester bonds linking cutin monomers require hydrolysis to release the monomers, which can be further examined by gas chromatography-mass spectrometry to determine the chemical composition (Walton, 1990). The C16 cutin monomers are dominated by 9- or 10, 16- dihydroxyhexadecanoic acid and 16-hydroxyhexadecanoic acid and the most common C18 cutin monomers are 18-hydroxy-9, 10-epoxyoctadecanoic acid and 9, 10, 18-trihydroxyoctadecanoic (Heredia, 2003). In some species, for example *Malus pumila* (apple fruit), unsaturated C18 monomers are also found to be C18 monomers (Stark and Tian, 2006). A common characteristic of cutin monomers is the ω -hydroxylation of C16 and C18 fatty acids with an additional hydroxyl or epoxy group at the middle position of the acyl chain. In the model plant *Arabidopsis thaliana*, however, the major cutin depolymerization products are found to be α , ω -dicarboxylic fatty acids and 2-hydroxy fatty acids, which consist of 40% and 14% of the total cutin weight, respectively (Nawrath, 2006). The more typical ω -hydroxyl

and mid-chain hydroxyl fatty acids only make up 7% and 8% of the total weight, respectively.

It has been observed that in some plant species, a component termed cutan, is identified after all the cuticular wax and cutin materials are removed. It is thought to be formed by cutin monomers with nonester bonds, possibly linked by ether bonds (Jeffree, 1996). It has been reported that cutan is more resistant to the biochemical degradation during the diagenesis process than cutin since cutan is identified in many fossilized plant studies (Tegelaar et al., 1991). However, the exact structure and role of cutan is unknown.

1.2.2 Cutin Monomer Biosynthesis

As described above, the typical chain length of cutin is C16 and C18. Therefore, biosynthesis of cutin monomers begins with the synthesis of long chain fatty acid precursors, followed by oxidative modification to generate the variety of cutin monomers. The biosynthesis of C16 and C18 long chain fatty acids, which occurs in the plastid, is also the start of the biosynthesis of cuticular wax. Therefore, in epidermal cells, part of the C16 or C18 long chain fatty acids synthesized in the plastid are partitioned into cutin monomer biosynthesis, and part will be partitioned into fatty acid elongation leading to cuticular wax biosynthesis, and the rest to other cellular lipid metabolism such as production of membrane lipid molecules.

The chemical pathways of cutin monomer biosynthesis was established in studies using radio-labelled precursors and intermediates (Stark and Tian, 2006). In 2001, Kolattukudy demonstrated that the oxygenated octadecanoates are

derived from oleic acid (C18:1) (or from linoleic acid (C18:2)), which undergoes a series of ordered reactions starting with a ω -hydroxylation, followed by epoxidation of the double bond, and lastly by hydroxylation of the epoxide. A cytochrome P450-dependent enzyme was proposed to catalyze the epoxidation step (Kolattukudy et al., 1981). The details of this transformation process were characterized by Pinot et al. (1992, 1997) using *Vicia sativa*, where it was shown that oleic acid is converted to 9, 10-epoxyoctadecanoic acid followed by hydroxylation to generate diol 9, 10-dihydroxyoctadecanoic acid. The epoxidation reaction is also found to have enantioselectivity; both (9R, 10S) and (9S, 10R) epoxides are detected and the ratio is maintained at 9:1 *in vivo* (Pinot et al., 1999). A cytochrome P450-dependent ω -hydroxylase converts 9, 10-epoxyoctadecanoic acid and diol 9, 10-dihydroxyoctadecanoic acid to 18-hydroxy-9, 10-epoxyhexadecanoic acids and 9, 10, 18-trihydroxyoctadecanoic, respectively (Tijet et al., 1998).

1.2.3 Architecture of Cutin Polymer Matrix

A characteristic difference between cutin and cuticular wax is that cuticular wax is extractable upon brief immersion in organic solvent, while cutin is not since the cutin is cross-linked in three dimensions with ester bonds. Most of the cutin monomers carry a primary hydroxyl group, mid-chain hydroxyl group and a carboxyl group. ω -hydroxy fatty acids are able to be polymerized to form a linear structure. Moreover, the mid-chain hydroxyl group is also able to esterify with carboxyl groups of other fatty acids to form a dendri-form polymerized structure

(Ray et al., 1998a, b). In C16 dominated cutin, half of the mid-chain hydroxyl groups and most of the primary hydroxyl groups are involved in ester bond cross-links (Agullo et al., 1984).

Although some information regarding cutin structure is known, there are still many outstanding questions. One problem is that the cutin monomers generated after ester-bond hydrolysis do not maintain their properties or condition compared to intact cutin. Therefore, some non-destructive analysis approaches have been applied to the study of cutin architecture. Fourier-transform infrared (FT-IR) spectroscopy and X-ray diffraction analysis have demonstrated that cutin has an amorphous structure with two hydrophobic interplanar spaces (Luque et al., 1995; Villena et al., 2000). Moreover, solid-state nuclear magnetic resonance (NMR) further confirmed the finding above and also identified the rigid molecular domain of the cutin polymer matrix, which contains more than 50% methylene groups (Zlotnik-Mazori et al., 1988).

Although likely containing similar aliphatic components, cutan is resistant to degradation by hydrolytic reagents. It is of great importance to understand cutan, as in some plant species, the amount of cutan can be as much as cutin (Schmidt et al., 1982; Villena et al., 1999). In *Clivia miniata* leaves, cutan deposition was found to occur largely after cutin deposition, indicating that cutan deposition does not initiate until cell expansion is complete (Schmidt et al., 1982), which also implies a possible role of cutan in the interaction between cutin and the cell wall. However, the lack of simple and efficient assays for cutan analysis makes the relevant genetic and biochemical research difficult.

1.2.4 Cutin Biosynthetic Enzymes

The number of enzymes involved in cutin biosynthesis is unknown. The currently known genes involved in cutin biosynthesis are summarized in Table 1.

In 2001, Wellesen et al. reported the identification of the *LACERATA* (*LCR*) gene (*At2g45970*). *Lcr* mutants display a series of developmental abnormalities associated with a disrupted cuticle, which is characterized by organ fusions between rosette leaves and between inflorescences. Cloning of the *LCR* gene indicated that it encoded a cytochrome P450 monooxygenase, CYP86A, which is able to catalyze the ω -hydroxylation of fatty acids ranging from C12 to C18:1 with highest activity towards palmitic (C16:1) and oleic acids (C18:1), precursors of cutin monomers (Wellesen et al., 2001).

Another cutin-related cytochrome P450 monooxygenase, CYP86A2 (*ATT1*, *At4g00360*) has been identified (Xiao et al., 2004). The *att1* mutant induces the expression of bacterial type III genes upon inoculation with *Pseudomonas syringae* and shows more severe disease symptoms than wild-type plants (Xiao et al., 2004). Further examination of the *att1* mutant revealed that it has decreased cutin content and a disrupted cuticle membrane ultrastructure, which results in enhanced water permeability. This observation is consistent with the function of the cuticle to control plant water loss. The exact role of *ATT1*, a putative fatty acid hydroxylase in cutin biosynthesis is still unknown. Another interesting observation is that the induction of bacterial type III genes was not observed in another cuticle mutant *wax2* (Chen et al., 2003), indicating either *ATT1* or its product has a specific role in the induction system.

Table 1 Characterized Cutin Biosynthesis Genes

Gene Name	AGI Gene Code	Enzyme encoded	Putative Function	Reference
<i>LCR</i>	At2g45970	cytochrome P450 monooxygenase	ω -hydroxylation of cutin monomers	Wellesen et al., 2001
<i>ATT1</i>	At4g00360	cytochrome P450 monooxygenase	Putative hydroxylation/oxidation	Xiao et al., 2004
<i>GPAT4</i>	At1g01610	glycerol-3-phosphate acyltransferase	Involved in polyester assembly	Li et al., 2007
<i>GPAT8</i>	At4g00400	glycerol-3-phosphate acyltransferase	Involved in polyester assembly	Li et al., 2007
<i>LACS2</i>	At1g49430	Long chain acyl-CoA synthetase	Generate acyl-CoA from free fatty acids for the biosynthesis of cutin monomers	Schnurr et al., 2004; Bessire et al., 2007; Molina et al., 2007; Tang et al., 2007
<i>BDG</i>	At1g64670	α/β -hydrolase fold-containing protein	Proposed to be involved in the polymerization of carboxylic esters in the cuticular layer of cell wall or cuticle proper	Kurdyukov et al., 2006

In 1974, Kolattukudy and Croteau reported that only leaf epidermal and fruit skin were able to generate interesterified polymers and they proposed the presence of an acyltransferase in transferring the hydroxyacyl moieties from CoA ester to the cutin polymer. Two cutin-related acyltransferases were recently reported, glycerol-3-phosphate acyltransferase (GPAT) 4 (*At1g01610*) and GPAT8 (*At4g00400*) (Li et al., 2007). A double mutant of *GPAT4/8* shows significant reduction of cutin monomers, while the single mutant of either gene did not, indicating functional redundancy. Typical cutin-disrupted phenotypes, such as increased water loss, susceptibility to pathogens and altered stomata structure were also observed.

Long-chain acyl-CoA synthetase 2 (*LACS2*) is also involved in cutin biosynthesis. A knockout mutation of *LACS2* results in a thinner abaxial cutin layer on leaves and higher cuticle permeability (Schnurr et al., 2004). The research results regarding *LACS2* are further discussed in section 1.4 on long chain acyl-CoA synthetases.

The *bodyguard* (*bdg*) mutant has irregular cuticle development, while accumulating more cutin and cuticular wax materials (Kurdyukov et al., 2006). The cuticular defects of *bdg* mutant are very similar to transgenic *Arabidopsis* expressing a fungal cutinase (Sieber et al., 2000). The expression of *BDG* gene is epidermal-specific and the protein accumulates in the outermost cell wall of the epidermis. The predicted BDG is an α/β -hydrolase fold-containing protein, but the exact biochemical role is unclear.

1.3 Cuticular Wax

1.3.1 Chemical Composition of Cuticular Wax

Waxes have been defined as “lipids that are removed from plant surfaces after brief immersion in an organic solvent”, such as chloroform (Post-Beittenmiller, 1998). Generally speaking, cuticular waxes are complicated mixtures of very-long-chain aliphatic components including fatty acids and derived components. However, the composition varies substantially from species to species and between organ types.

Cuticular waxes are typically fully saturated aliphatic fatty acid and its derivatives generally have one primary functional group, such as a hydroxyl or keto group. The typical ‘waxes’ are free fatty acids, primary and secondary fatty alcohols, alkanes, aldehydes, ketones and alkyl esters (formed by esterification of fatty acids and primary alcohols). These chemical classes are often present as a homologue series of chain lengths (e.g C24, C26, C28 and C30), or one chain length may be predominant. In *Arabidopsis*, n-alkanes, ketones, secondary alcohols and primary alcohols together constitute 90% of the total cuticular wax load on stems (Kunst and Samuels, 2003). The cuticular wax of *Arabidopsis* leaves lacks ketones and secondary alcohols (Jenks et al., 1995).

1.3.2 Biosynthesis of Cuticular Wax

The biosynthetic process can be divided into two stages: (1) the biosynthesis of very-long chain fatty acids (VLCFAs); the products of this process are shared with other plant lipid metabolic pathways such as sphingolipid biosynthesis, and (2)

the modification of very-long chain fatty acids to form the aliphatic wax components described above (Kunst and Samuel, 2008) (Figure 3). This section mainly focuses on the biosynthesis of the major wax components of *Arabidopsis* because most of the recent advances are from research conducted with *Arabidopsis*, due to its ease of molecular genetic analyses and availability of genomic tools including a fully sequenced genome.

Phase 1 Elongation of VLCFA Precursors

Biosynthesis of C16 and C18 fatty acids occurs in the stroma of the plastid (Ohlrogge et al., 1993; Browse and Ohlrogge, 1995). First, acetyl-acyl carrier protein (ACP) receives a C2 moiety from malonyl-ACP by a condensation enzyme. Subsequently, a series of reactions, consisting of a reduction of β -ketoacyl-ACP, dehydration of β -hydroxyacyl-ACP and reduction of *trans*- Δ^2 -enoyl-ACP, generates an acyl-ACP that is elongated by two carbons. This condensation-reduction-dehydration-reduction reaction cycle is repeated, eventually giving acyl-ACPs of chain lengths C16 or C18. The four enzymes catalyzing this 2-carbon elongation are a condensing enzyme, two reductases and a dehydratase, which form a fatty acid synthase complex (FAS) (Kunst and Samuel, 2003). Based on different substrate specificities, the FASs are divided into three types: KASIII, catalyzing C2-C4 elongation (Clough et al., 1992); KASI, catalyzing C4-C16 elongation and KASII, catalyzing C16-C18 elongation (Shimakata and Stumpf, 1982). The substrate specificities of the KASs are determined by the condensing enzyme, and the reductases and dehydratase are shared between the enzyme complexes (Stumpf, 1984).

Figure 3 Chemical pathways of cuticular wax biosynthesis

Biosynthesis of very-long-chain fatty acyl-CoA is first initiated in the plastid, where the chain length reaches to C18, and then further elongated to lengths up to C32 in the endoplasmic reticulum. In epidermal cells, the very-long-chain fatty acyl-CoAs are modified to generate wax components via two proposed pathways: 1) an alcohol forming pathway to produce primary alcohols and wax esters, and 2) an alkane forming pathway to produce aldehydes, alkanes, secondary alcohols and ketones.

FAS: fatty acid synthase;

FAE: fatty acid elongase

After the acyl chain reaches a length of C18, it is cleaved from ACP by an acyl-ACP thioesterase and exported out of the plastid. In order to be further elongated to VLCFAs, the acyl-chain must be activated by being esterified to Coenzyme A (CoA) by a long-chain acyl-CoA synthetase (LACS) to give a fatty acyl-CoA. At this point, the acyl-chains are partitioned into the different lipid metabolic pathways, such as leading to glycerolipids or cuticular wax biosynthesis. The detailed regulatory mechanisms of this partitioning remain unknown. However, it is probably determined by the substrate specificities of the relevant enzymes and potentially their subcellular location. For example, a fatty acyl-ACP thioesterase B (FATB) is able to cleave C16:0 acyl- chain from acyl-ACP, which is further metabolized leading to wax biosynthesis (Bonaventure et al., 2003). Disruption of the *FATB* gene results in a 50% decrease of wax load on *Arabidopsis* stems and a 20% decrease on leaves (Bonaventure et al., 2003). The *LACS2* protein (*At1g49430*) may also be involved in the regulation of partitioning. A loss-of-function mutant of *LACS2* gene shows a disrupted cuticular layer and an increased cuticular wax load on *Arabidopsis* rosette leaves (Schnurr et al., 2004; Bessire et al., 2007). The *LACS2* enzyme was reported to have a substrate preference toward ω -hydroxypalmitic acids (Schnurr et al., 2004), indicating specificity for lipid components destined for cutin biosynthesis.

For fatty acid elongation to chain lengths greater than C18, the cleaved acyl chains are esterified with a CoA group and transported to the endoplasmic reticulum (ER) for further elongation. This process is catalyzed by the fatty acid elongase (FAE) complex (von Wettstein-Knowles, 1982). Similar to elongation in

the plastid, acyl-CoA undergoes a 2-carbon elongation cycle at the ER: condensation of fatty acyl-CoA with malonyl-CoA (rather than malonyl-ACP as in the plastid), which is followed by reduction of β -ketoacyl-CoA, dehydration of β -hydroxyacyl-CoA and reduction of *trans*- Δ^2 -enoyl-CoA (Fehling and Mukherjee, 1991), generating an acyl-CoA that is elongated by two carbons. The four enzymes catalyzing this reaction cycle comprise the FAE complex (von Wettstein-Knowles, 1982). The fatty acyl chain lengths typically reach up to 20-34 carbons after multiple rounds of elongation, which is compatible to the length of the aliphatic wax components. Several distinct FAE complexes with distinct chain length substrate specificities are involved in the entire elongation process (von Wettstein-Knowles, 1993). The condensing enzyme catalyzing the first step determines the substrate specificity of the FAE complex (Millar and Kunst, 1997) and the two reductase and dehydratase are shared between FAE complexes.

Phase 2 Modifications of VLCFAs

In most plants, it has been proposed that there are two pathways to form the different aliphatic wax components using VLCFAs as precursors, the primary alcohol forming pathway and the alkane forming pathway (Samuels et al., 2006).

The primary alcohol pathway, previously called the acyl reduction pathway, yields primary alcohols and wax (alkyl) esters. First, the VLCFAs are reduced to primary alcohols by a fatty acyl-CoA reductase (FAR). This reduction reaction has been proposed to proceed via an aldehyde intermediate, but the aldehyde is not released from the enzyme (Rowland et al., 2006). Although there is a report in the literature proposing that this could occur via a two-step reaction carried out by two

independent enzymes (Kolattukudy, 1971), current research results support the one-step theory. Biochemical evidence for a single enzyme process are from studies using the green alga *Euglena gracilis* (Kolattukudy, 1970), *Simmondsia chinensis* (jojoba) embryo (Pollard et al., 1979) and *Pisum sativum* leaves (Vioque and Kolattukudy, 1997). In addition to biochemical evidence, heterologous expression of alcohol-forming FARs from jojoba (Metz et al., 2000), *Bombyx mori* (Moto et al., 2003), *Mus musculus*, *Homo sapiens* (Cheng and Russell, 2004) and *Arabidopsis* (Rowland et al., 2006) also support that the reduction from acyl-CoA to primary alcohol is carried out by a single FAR enzyme. While some of the primary alcohols are transported out of epidermal cells to form cuticular wax, some of them are esterified with fatty acids to form alkyl esters (wax esters), catalyzed by the action of a fatty acyl-CoA: fatty alcohol acyltransferase (wax synthase, WS) (Li et al., 2008).

The alkane-forming pathway, previously called the decarbonylation pathway, yields aldehydes, alkanes, secondary alcohols and ketones, as shown in Figure 4. This model was first proposed by Kolattukudy (1965, 1967) based on biochemical evidence. In this model, the VLCFAs are reduced to aldehydes by a putative fatty acyl-CoA reductase. A decarbonylase acts to release carbon monoxide (CO) and form odd-chain length alkanes (Cheesbrough and Kolattukudy, 1984; Schneider-Belhaddad et al., 2000). Subsequently, the alkanes are hydroxylated to secondary alcohols and the secondary alcohols are oxidized to ketones.

An up-to-date description of the identified genes encoding the above described enzymes is in section 1.3.5.

1.3.3 Transportation of Cuticular Wax components

The process of wax transportation can be separated into four stages: intracellular transportation from the endoplasmic reticulum (ER) to the plasma membrane (PM), followed by cross-plasma membrane transportation to the cell wall, transportation through the cell wall, and then differential layering in the cuticle.

The intracellular site of wax biosynthesis is proposed to reside in the ER, based on subcellular localization experiments of several wax biosynthetic enzymes, including *Zea mays* β -ketoacyl reductase (Xu et al., 2002), *Arabidopsis* CER6 (β -keto acyl-CoA synthase) (Kunst and Samuels, 2003), *Arabidopsis* CER10 (enoyl CoA-reductase) (Zheng et al., 2005), CER4 (alcohol forming FAR) (Rowland et al., 2006), MAH1 (Greer et al., 2007) and WSD1 (Li et al., 2008). However, the mechanism of intracellular transportation from ER to PM is not understood. Two models have been proposed (Kunst and Samuels, 2003; Schulz and Frommer, 2004). One model suggests that wax molecules are transported to the PM via the Golgi apparatus and mediated by vesicular transport as is typical of the secretory pathway. The second model suggests the wax molecules are transported to the plasma membrane by direct molecular transfer at ER-PM contact sites.

After reaching the PM, wax molecules are further secreted across the lipid bilayer to the apoplastic environment. This process is believed to be primarily carried by ATP binding cassette (ABC) transporters, as shown by the characterization of two wax specific ABC transporters, CER5 and WBC11 (Pighin

et al., 2004; Bird et al., 2007; Panikashvili et al., 2007; Ukitsu et al., 2007). Knockout lines of *CER5* and *WBC11* result in reduced wax loads and accumulation of sheet-like inclusions in the cell cytoplasm (Pighin et al., 2004; Bird et al., 2007; Panikashvili et al., 2007; Ukitsu et al., 2007), which is similar to defects observed in the human disorder, adrenoleukodystrophy (ALD), caused by a defective ABC transporter (Powell et al., 1975). However, the details of the cross membrane transportation still remain unclear. For example, how are the wax molecules delivered to the ABC transporter complex? Are there other macromolecules involved in this cross-membrane transportation? The answer to the latter question is probably yes, because the double mutant of *cer5* and *wbc11* shows a similar wax load as both of the single mutant lines and cuticular wax accumulation is not completely eliminated (Bird et al., 2007). Further research is required to characterize additional members of the transportation system, as well as the physical interactions between the wax molecules and their transporters.

Finally, the hydrophobic wax molecules must travel across the hydrophilic cell wall and finally arrive at their destination, the surface of the epidermal cell, where they will further accumulate and crystallize. Due to the high insolubility of the wax components in the aqueous environment of cell wall, this transportation is likely facilitated by other factors. It was proposed that structures within the cell wall are able to facilitate the wax transport (Kunst and Samuels, 2003). However, such structures had never been physically identified by electron microscopy or other techniques. Another possibility is that lipid transfer proteins (LTPs) are able to “carry” the wax components and move them cross the cell wall (Stern et al., 1991;

Moreau et al., 1998). Plant LTPs belong to a large superfamily and certain members are able to move phospholipids across lipid bilayers *in vitro* (Kader, 1996; Arondel et al., 2000; Rogers and Bankaitis, 2000). LTPs are found to be highly expressed in epidermal cells (Thoma et al., 1993; Pyee et al., 1994) and are as small as 7~9 kDa (Kader, 1996), which enables them to traverse the cell wall. Moreover, three-dimensional structural studies of these LTPs have shown that they have a hydrophobic pocket that can bind long chain fatty acids *in vitro* (Douliez et al., 2000). Although they appear to be good candidates to transport wax molecules across epidermal cell walls, no direct experimental evidence showing that they are involved in this process *in vivo* is currently available. It has recently been shown that the chemical composition of intra- and epicuticular waxes differ (Jetter et al., 2000; Jetter and Schaffer, 2001), indicating differential layering but again the mechanism by which this differential layering occurs is unknown.

1.3.4 Regulation of Cuticular Wax Biosynthesis

As discussed in section 1.2, the main function of the plant cuticle is to serve as a barrier against water loss. It also mediates interactions between plants and microbial pathogens, and between plants and insects. Therefore, it is not surprising that an array of environmental factors, including temperature, solar radiation, humidity and pathogen/insect invasion are able to affect wax biosynthesis (Eignebrode and Espelie, 1995; Post-Beittenmiller, 1996). However, the details of these regulatory mechanisms largely remain unknown.

Another phenomenon is that wax load remains consistent in the rapidly elongating stem (Suh et al., 2005), which gave rise to the idea that wax biosynthesis is correlated tightly with plant development. The *CER2* and *CER6* genes have been confirmed to be transcriptionally regulated through development (Xia et al., 1997; Hooker et al., 2002). Moreover, the transcription of *CER6* can be induced by light and osmotic stresses (Hooker et al., 2002), which are stimuli that are able to increase wax accumulation in *B. oleracea* (Baker, 1974). However, *CER2* does not show a transcriptional response to these stimuli (Xia et al., 1997). Thus far, two transcription factors involved in wax biosynthesis, *WIN1/SHN1* and *MYB30* have been characterized (Broun et al., 2004; Aharoni et al., 2004). Overexpression of *WIN1/SHN1* results in increase wax load on leaves and stems (Aharoni et al., 2004; Broun et al., 2004). Microarray data indicates that some wax biosynthesis genes, including *CER1*, *CER2* and *KCS1* are the direct target genes of *WIN1/SHN1* (Broun et al., 2004). More work is required to fully elucidate the regulatory mechanisms of *WIN1/SHN1* transcription factors and characterize other potential regulators in wax biosynthesis. It appears that the biosynthesis of cutin monomers is also under the control of the WIN1-responsive regulatory network with the finding that five cutin-associated genes, including *LACS2* and *GPAT4*, are the target genes of *WIN1/SHN1* (Kannagara et al., 2007).

MYB30 is another transcription factor that is able to regulate wax biosynthesis (Raffaele et al., 2008). Putative target genes of *MYB30* are the fatty acid elongase (FAE) components, including *KCS1*, *KCS2* and *CER10*. Altered wax chemical composition is observed in both the knockout and overexpression lines of *MYB30*.

In addition to *WIN1/SHN1* transcription regulation, the characterization of *CER7*, a core subunit of the exosome, suggested a unique form of transcriptional regulation of wax biosynthesis (Hooker et al., 2007). The exosome is a multi-protein complex that is able to degrade various types of RNAs. The core unit of the exosome usually has a ring structure consisting of six proteins that all belong to the same class of RNases, the RNase PH-like proteins (Butler, 2002; Raijmakers et al., 2004). The *Arabidopsis cer7* mutant has reduced expression level of *CER3/WAX2/YRE* (Hooker et al., 2007). It was proposed that a target of *CER7* is an mRNA encoding a *CER3*-specific negative regulator. The expression of *CER3* could thereby be increased by specific degradation of this negative regulator by the exosome ribonuclease, allowing the alkane-forming pathway to proceed.

Finally, it is of great interest to consider that cutin and wax biosynthesis are co-regulated due to the fact that the cutin and wax load remain consistent throughout development (Samuels et al., 2008). Moreover, a cutin related gene, *LACS2*, is also found to be the direct target of *WIN1/SHN1* transcriptional factor. However, no further information on the relationship between cutin and wax biosynthesis is currently published.

1.3.5 Wax Biosynthetic enzymes

As described above, the model plant *Arabidopsis thaliana* is a good species to study plant cuticular wax biosynthesis and secretion. It has a fully sequenced genome and a large array of powerful genetic tools. The abundance of wax

crystals on *Arabidopsis* stems confer a whitish, glaucous appearance, while mutants with lower cuticular wax load or altered wax composition, named *eceriferum* (*cer*), have a glossy phenotype on the stem that can be easily distinguished by the naked eye (Koornneef et al., 1989). More than 30 *cer* loci have been identified, but only a small portion of these *CER* genes have been characterised thus far (summarized in Table 2).

In terms of wax biosynthesis, fatty acyl-ACP thioesterase B (*FATB*) was found to be involved in the synthesis of C16:0 and C18:0 free fatty acids in the plastid. Knockout lines of *FATB* gene result in a reduced level of palmitate (16:0) and stearate (18:0) content in flowers, roots, leaves and seeds, as well as a decrease of total wax load on leaves and primary stems without significant changes in the distribution of wax composition (Bonaventure et al., 2003). Moreover, knockout lines showed altered seed morphology, a lower germination rate, and a reduced growth rate. All of these phenotypes indicate that *FATB* serves as a major determinant of saturated fatty acid synthesis, which is essential for the biosynthesis and/or regulation of cellular components critical for plant growth, seed development and cuticular wax biosynthesis.

CER6 and *CER10* were characterized as a β -keto acyl-CoA synthase (KCS) and an enoyl-CoA reductase (ECR) respectively, which are part of the fatty acid elongase catalyzing the first and the last steps of elongation of very-long-chain fatty acids (Fiebig et al., 2000; Millar et al., 1999; Zheng et al., 2005). Knockout mutants of *CER6* have a severe wax-less phenotype; 6~7% of wild-type levels and completely devoid of wax crystals (Millar et al., 1999). In the *cer6* mutant, C24

wax components predominate, indicating it is required for elongation of C24 acyl-CoA to longer chain lengths (Millar et al., 1999). The conditional male sterility implies that reduced levels of cuticular wax influence the stigma-pollen interaction (Millar et al., 1999), which was further confirmed by Fiebig et al. (2000).

Knockout mutants of *CER10* have reduced organ size and decreased levels of total cuticular wax load, although not as severe as the *CER6* mutant (Zheng et al., 2005). The reduced levels of very-long-chain fatty acids in seed triacylglycerols and sphingolipids indicated that *CER10*, which encodes an enoyl-CoA reductase, is required for all very-long-chain fatty acid elongation *in planta*. Epidermal- and seed-specific silencing of *CER10* result in reduced levels of total cuticular wax and seed triacylglycerols, respectively, but no abnormal plant morphology, indicating by deduction that the abnormal plant morphology phenotype likely arises from defects in sphingolipid metabolism. Further cellular analysis shows that aberrant endocytic membrane traffic and defective cell expansion are responsible for the morphological abnormalities in *cer10* mutants.

A putative β -ketoacyl reductase (KCR), *At-YBR159* (*At1g67730*), was identified by searching for homologs of the yeast KCR gene *YBR159w* (Beaudoin et al., 2002). The truncated cDNA of *At-YBR159* was reported by Xu et al. (1997) as an ortholog of the maize *GLOSSY8* (*GL8*) gene. Maize *gl8* mutants have reduced levels of wax components longer than C24, suggesting that it encodes a reductase involved in fatty acid elongation (Bianchi et al., 1985; Dietrich et al., 2005). Based on the yeast KCR activity reported by Beaudoin et al. (2002), *At-YBR159* is a good candidate for the wax-associated KCR.

The *CER4* gene was identified as encoding an alcohol-forming fatty acyl-CoA reductase (FAR), generating primary fatty alcohols from very-long-chain fatty acyl-CoA precursors (Rowland et al., 2006). *Cer4* mutants have a complete elimination of C24, C26 and C28 primary alcohols, but overall wild-type level of total wax load due to increases in the alkane-forming pathway components. The lack of wax crystals on *cer4* mutants implies an important role for primary alcohol in the formation of epicuticular wax crystals. The slight amount of C30 primary alcohols in *cer4* indicates that another FAR is also involved in cuticular wax biosynthesis.

WSD1 encodes a wax synthase/acyl-CoA:diacylglycerol acyltransferase 2 (DGAT2) bifunctional enzyme that catalyzes the formation of alkyl esters as its main function (Li et al., 2008). *Wsd1* mutant is the first wax-disrupted mutant with a severely reduced level of only alkyl esters. Although the WSD1 protein is a putative bifunctional enzyme, the expression of WSD1 in yeast results in accumulation of alkyl esters, and not triacylglycerols, demonstrating that its main activity is as a wax synthase. Determination of the subcellular location by fusing *WSD1* to GFP revealed that it is associated with the ER, indicating that the final step of the alcohol forming pathway occurs at ER.

MAH1 encodes a cytochrome P450 enzyme that was demonstrated to catalyze the last two steps of the alkane forming pathway, generating secondary alcohols and ketones from alkanes (Greer et al., 2007). *Mah1* mutants have reduced levels of secondary alcohols and ketones, while the ectopic expression of MAH1 leads to accumulation of secondary alcohols and ketones in leaf wax

where they are not normally present. All of these data indicate that MAH1 is able to catalyze the last two steps of the alkane forming pathway: the hydroxylation leading from alkanes to secondary alcohols and the subsequent hydroxylation leading to ketones. A GFP fusion with MAH1 shows its association with the ER, indicating that the last two steps of the alkane forming pathway occurs at the ER.

CER1, *CER2* and *CER3/WAX2/YRE/FLP1* are also demonstrated to be involved in wax biosynthesis, but their functions remain enigmatic (Aarts et al., 1995; Hannoufa et al., 1993; Jenks et al., 1995; Lai et al., 2007; Negruk et al., 1996; Xia et al., 1996 & 1997; Ariizumi et al., 1995; Chen et al., 2003; Kurata et al., 2003; Ariizumi et al., 2003; Rowland et al., 2007). A common feature of these three mutants is a significantly decreased wax load on primary stems compared with wild-type plant: 19% of wild-type level on *cer1*, 36% on *cer2* and 46% on *cer3/wax2/yre* (Jenks et al., 1995). *Cer1* mutants have a general decrease of all wax components except for a slight increase of C30 aldehyde (Hannoufa et al., 1993; Jenks et al., 1995). Cloning of the *CER1* gene revealed its similarity to a group of integral membrane enzymes, which include monooxygenases and desaturases, but such enzymatic activity for CER1 is unexpected based on the phenotype of *cer1* (Aarts et al., 1995). *CER1* has been proposed to be the aldehyde decarbonylase, but biochemical evidence is lacking and again the phenotype is not consistent with such a role. *Cer2* mutants have a reduced accumulation of alkanes, secondary alcohols and ketones, with an increased accumulation of C26 primary alcohols (Hannoufa et al., 1993; Jenks et al., 1995), implying a block in the elongation of C26/28. Cloning of *CER2* showed no overall

amino acid sequence similarity to any previously known gene products (Xia et al., 1996). However, *CER2* contains a motif that is commonly present in a large family of CoA-dependent acyltransferases (St-Pierre et al., 1998). The exact role of *CER2* thus remains unknown. *Cer3* mutants accumulated C30 primary alcohols, while all other wax components are reduced (Hannoufa et al., 1993; Jenks et al., 1995). *CER3* is identical to *WAX2/YRE/FLP1* (Rowland et al., 2007). Cloning of *CER3/WAX2/YRE/FLP1* revealed that it contains six putative N-terminal localized transmembrane domains, a His-rich di-iron binding region, and a soluble C-terminal domain (Chen et al., 2003). The N-terminal half is similar to members of the sterol desaturase family, as for the case of *CER1*. Indeed, *CER3/WAX2/YRE/FLP1* has 32% identity to *CER1*.

Wax-associated genes from other plant species have also been characterized. Maize wax associated genes *GL1*, *GL2* and *GL8* have been cloned (Sturaro et al., 2005; Tacke et al., 1995; Bianchi et al., 1985; Dietrich et al., 2005). The *GL1* protein contains three histidine-rich domains, characteristic of a family of membrane-bound desaturases/hydroxylases, including fatty acid-modifying enzymes (Sturaro et al., 2005). *GL1* is highly related to Arabidopsis *CER3* (Rowland et al., 2007). *GL2* protein is related to Arabidopsis *CER2*, but like *CER2*, its *in vivo* role in wax biosynthesis is unknown (Tacke et al., 1995; Xia et al., 1996). *GL8* encodes a keto acyl-CoA reductase involved in fatty acid elongation (Bianchi et al., 1985; Dietrich et al., 2005). A tomato (*Lycopersicon esculentum*) β -ketoacyl-CoA synthase *LeCER6* required for fatty acid elongation has been identified; the *lecer6* mutant has decreases in alkanes with chain lengths longer than C28 (Leide et al., 2007).

Table 2 Characterized Wax Biosynthesis Genes

Gene Name	Gene Code	Coded Enzyme	Reference
<i>CER1</i>	<i>At1g02250</i>	Unknown function enzyme;	Aarts et al., 1995; Hannoufa et al., 1993; McNevin et al., 1993
<i>CER2</i>	<i>At4g24510</i>	Unknown function enzyme; putative BAHD CoA-dependent acyltransferase	Jenks et al., 1995; Lai et al., 2007; Negruk et al., 1996; Xia et al., 1996 & 1997
<i>CER3/WAX2/YRE</i>	<i>At5g57800</i>	Unknown function enzyme;	Ariizumi et al., 1995; Chen et al., 2003; Kurata et al., 2003; Rowland et al., 2007
<i>CER4</i>	<i>At4g33790</i>	Fatty acyl-CoA reductase (FAR)	Rowland, et al., 2006
<i>CER5</i>	<i>At1g51500</i>	ABC transporter	Pighin et al., 2004
<i>CER6</i>	<i>At1g68530</i>	β -keto acyl-CoA synthase (KCS), part of fatty acid elongase	Fiebig et al., 2000; Millar et al., 1999
<i>CER7</i>	<i>At3g60500</i>	Exosome subunit; regulator of <i>CER3</i> expression	Hooker et al., 2007
<i>CER10</i>	<i>At3g55360</i>	enoyl-CoA reductase (ECR), part of fatty acid elongase	Zheng et al., 2005
<i>WBC11</i>	<i>At1g17840</i>	ABC transporter	Bird et al., 2007
<i>WIN1/SHN1</i>	<i>At1g15360</i>	AP2-type transcription factor	Broun et al., 2004
<i>MAH1</i>	<i>At1g57750</i>	Midchain alkane hydroxylase (CYP96A15)	Greer et al., 2007
<i>FATB</i>	<i>At1g08510</i>	Acyl-ACP thioesterase	Bonaventure et al., 2003
<i>WSD1</i>	<i>At5g37300</i>	wax synthase/DGAT	Li et al., 2008
<i>KCR</i>	<i>At1g67730</i>	keto-CoA reductase, part of FAE	Xu et al., 1997

1.4 Long chain acyl-CoA synthetase

In order to be incorporated into most lipid metabolism reactions, free fatty acids must be activated to fatty acyl-CoA thioesters (Watkins, 1997). Long chain acyl-CoA synthetases (LACSs, EC 6.2.1.3) are able to catalyze the activation of free fatty acid to fatty acyl-CoA, thus occupying a very important position in lipid metabolism processes. Such an activation process is carried out via a two-step reaction: first, free fatty acids are converted to an acyl-AMP intermediate; then, the activated carbon is coupled to the thiol group of CoA, releasing AMP and the acyl-CoA final product (Figure 17A) (Groot et al., 1976).

LACS enzymes have been identified and characterized in various organisms, including bacteria (*Escherichia coli*; Black et al., 1992), yeast (*Saccharomyces cerevisiae*; for review, see DiRusso and Black, 2007), plants (*Arabidopsis thaliana*; Shockey et al., 2002 & 2003; Fulda et al., 2002 & 2004; Schnurr et al., 2002 & 2004; *Thalassiosira pseudonana*; Tonon et al., 2005) and human (Steinber et al., 2000). They have been demonstrated biochemically and genetically to be involved in a variety of lipid metabolism processes. For example, the sole LACS in *E. coli*, encoded by the *fadD* gene, was found to be associated with the cell plasma membrane and involved in the incorporation of exogenous fatty acids via a vectorial esterification process (Overath et al., 1969). The eukaryotic intracellular fatty acid transportation system also relies on the activation of fatty acids by LACSs, such as for the carnitine shuttle mechanism in mammalian mitochondrial β -oxidation (Eaton et al., 1996) and receptor-mediated importation into yeast peroxisomes (Hettema and Tabak, 2000). Other characterized functions

of LACSs include the initiation of β -oxidation in oilseeds (Beevers 1969; Gerhardt, 1992; Eastmond et al., 2000), and in providing acyl-CoA for the protein N-myristoylation reaction (Gordon et al., 1991; Yalovsky et al., 1999; Ishitani et al., 2000; Martin and Busconi, 2000).

In Arabidopsis, there are nine predicted LACS genes (Shockey et al., 2002), which belong to a larger superfamily of acyl-activating enzymes (AAEs) (Shockey et al., 2003). Among the nine members of the Arabidopsis LACS family members, LACS2, -6, -7 and -9 have been characterized in detail (Fulda et al., 2002; Schnurr et al., 2002; Schnurr et al., 2004; Tang et al., 2007; Bessire et al., 2007).

LACS6 and -7 are associated with the peroxisome (Fulda et al., 2002). Both of these genes are upregulated during germination and in early seedling development (Fulda et al., 2004), indicating that they are involved in β -oxidation in glyoxysomes. Single knockout mutants of these genes did not result in a visible phenotype, whereas a double knockout of LACS6 and -7 has a requirement for exogenous sucrose for normal seedling development (Fulda et al., 2004), indicating its reduced ability to use stored seed lipid. It seems that LACS6 and -7 have an overlapping *in vivo* function regarding activating the seed lipid reserve, but currently the reason why two genes with seemingly identical functions are needed remains unknown.

LACS9 was found to be highly expressed in developing seeds and young rosette leaves and localized to the outer surface of the plastid envelope (Schnurr et al., 2002). Although LACS9 was found to be the major chloroplast LACS, no visible phenotype was observed of a T-DNA knock out line of LACS9. The *in vivo*

function of *LACS9* thus remains unclear.

LACS2 (*At1g49430*) was found to be highly expressed in tissues that undergo rapid cell elongation and expansion, indicating that it may be involved in cuticle biosynthesis. No substantial change of cuticular wax composition was observed in *LACS2* knockout lines. However, a knockout line of *LACS2* has a faster chlorophyll release rate and increased leaf wax load indicating that *LACS2* is required for cutin, rather than cuticular wax biosynthesis. A decreased cuticle thickness and the substrate preference of *LACS2* for ω -hydroxypalmitic acid, a precursor of cutin monomers, support such a proposal (Schnurr et al., 2004). Recently, studies on two *Arabidopsis* mutants, *sma4* and *bre1*, both of which were identified to be allelic to *LACS2*, show an alteration in pathogen resistance, including enhanced susceptibility to avirulent *Pseudomonas syringae* and enhanced resistance to *Botrytis cinerea* (Tang et al., 2007; Bessire et al., 2007). The enhanced resistance was proposed to be due to an increased release of antifungal compounds through a more permeable cuticle due to disrupted cutin formation (Bessire et al., 2007), while the enhanced susceptibility to *Pseudomonas* was more likely to be due to more rapid leakage of cell fluids in the substomatal region, which is a primary point of colonization during *Pseudomonas* infection (Tang et al., 2007). Water stress is believed to be a limiting factor during *Pseudomonas* infection (Wright and Beattie, 2004) and another cutin mutant, *att1*, which has increased rates of water vapor transmission, shows a similar enhanced susceptibility towards *Pseudomonas* (Xiao et al., 2004).

1.5 Overview of Project

The main goal of this Masters thesis was to identify the gene corresponding to the *eceriferum8* (*cer8*) mutant and elucidate the role of the gene product in plant wax biosynthesis. *Eceriferum8* (*cer8*) mutant was first identified by Koornneef et al. (1989) using fast neutron mutagenesis. When grown in winter conditions with a relative humidity (RH) of 60% and 80%, *cer8* shows an almost complete sterility but in summer greenhouse conditions with RH up to 90%, the fertility is close to wild type. Scanning electron microscopy indicated the absence of tube-shaped epicuticular wax crystals that are normally present on wild type stems (Koornneef et al., 1989). The most significant change in the *cer8* mutant wax profile, which distinguished it from other *cer* mutants, was a significant increase of C30 free fatty acids (Jenks et al., 1995). On stems, the amount of C30 free fatty acids is significantly higher than wild type. The leaf wax of a *cer8* mutant also has a significant increase in C30 free fatty acids. Based on the chemical profile, Jenks et al. proposed that *CER8* gene may be involved in acyl chain transfer from the fatty acid elongation site to sites of subsequent modification. However, no further research was published regarding the gene involved and the *in vivo* function of *CER8*.

By using a combination of forward and reverse genetic approaches, I demonstrated that *CER8* gene is *LACS1* (At2g47240). I performed a detailed analysis of the *cer8* mutant phenotype analysis wax crystal formation by SEM and wax composition by gas chromatography. In order to further characterize the role of *LACS1* in cuticular wax biosynthesis, its gene expression profile was analyzed

in comparison with the epidermal-expressed *LACS2* and *LACS3* genes. Finally, the relative substrate specificities of LACS1, -2 and -3 were characterized using saturated fatty acids of even chain lengths ranging from C16 to C30. LACS1 was found to have high activity for C30 free fatty acids consistent with the phenotype of the mutant.

Chapter 2 Materials and Methods

2.1 Plant Materials and Growing Conditions

Two ecotypes of *Arabidopsis thaliana* were used, Columbia-0 (Col-0) and Landsberg *erecta* (Ler). Col and Ler wild type seeds were kindly provided by Prof. Ljerka Kunst of the Department of Botany, University of British Columbia. The *cer8-1* mutant (original Ler-*cer8* mutant; Koorneef et al., 1989) and the SALK T-DNA insertion lines, SALK_127191 (*cer8-2*) and SALK_138782 (*cer8-3*), were obtained from the Arabidopsis Biological Resource Center (ABRC) at Ohio State University.

Plants were grown under various conditions. For the complementation crosses, plants were grown in an controlled-environment growth chamber (Conviron, Controlled Environment Ltd, Winnipeg, MB) with a continuous light photoperiod (24 hours light) and a temperature of 22°C. To analyze wax by gas chromatography and scanning electron microscopy, plants were grown in the same chamber, but with a long-day photoperiod (18 hours light and 6 hours dark) and at 22°C. Except for the two experiments above, plants required for other experiments, such as genomic DNA and total RNA isolation, transformation with *Agrobacterium tumefaciens*, and seed amplification, were grown in a semi-controlled growth room (Nesbitt Biology Building, Room 113A) with a continuous light photoperiod at approximately 22°C which was controlled by a Mitsubishi air conditioner.

The soil used for plant growth was purchased from Berger Peat Moss-Le Groupe Berger Itée (Saint-Modestem QC). The soil was autoclaved prior to use.

The soil was then soaked with water, and fertilized by adding 1 g/L Plant-Pod® 20-20-20 all-purpose fertilizer (Plant Product Co. Ltd, Brampton ON) and packed up in either 72-well pots, square pots (3.5-inch by 3.5-inch) or round pots (5-inch diameter).

After the seeds were sown onto soil, the pots were covered with plastic wrap to maintain high humidity for seed germination. Before germinating, the seeds were incubated at 4°C for 2~4 days to break dormancy (Weigel and Glazebrook, 2002). After moving to a growing environment, the plastic wrap was kept on the pots until the seedlings were established. The plastic wrap was then removed and the plants were watered to keep the top soil wet.

2.2 Genetic complementation cross

Genetic complementation crosses were performed to check the allelism between *LACS1* (*At2g47240*) and *CER8*. The two SALK T-DNA insertion lines each carrying a T-DNA insertion in the *LACS1* gene were identified using the SALK T-DNA Express website (<http://signal.salk.edu/cgi-bin/tdnaexpress>). Homozygous insertion lines of SALK_127191 and SALK_138782 were crossed with the *cer8-1* mutant; *cer8-1* as female and *cer8-2/-3* as males. Seeds were collected from successfully developed siliques and sown out to grow the F1 generation. Genomic DNA was isolated from young leaf tissue using the 'Dellaporta' plant genomic DNA extraction protocol as describe below (Dellaporta et al., 1983), to verify the offspring of successful crosses by polymerase chain reaction (PCR) using the T-DNA verification primers SALK_127191_LP

(5'-AAACCCTAAAAAGGATTGTTTGC-3'), SALK_127191_RP (5'-ATCCATGAGG GGATTCAAAAG-3'), SALK_138782_LP (5'-CATTTTTCAAAAGTATTCATCAAGT G-3'), SALK_138782_RP (5'-CGTAACACGTTCAAATTCTACATAAATC-3') and LBb1 (5'-GCGTGGACCGCTTGCTGCAACT-3'), which were designed by the online tools of the Salk Institute Genomic Analysis Laboratory (<http://signal.salk.edu/tdnaprimers.2.html>). The stem cuticular wax phenotype of the F1 plants from successful crosses were photographed together with the parent plants as well as the corresponding wild type plants, all of which were grown under the same condition.

2.3 Plant Genomic DNA Extraction

The plant genomic DNA extraction protocol was modified from Dellaporta et al. (1983). Approximately 100 mg of plant leaves were collected and immediately frozen in liquid nitrogen in a mortar followed by grinding to a fine powder using a pestle. The fine frozen powder were transferred to a new 1.5-ml microcentrifuge tube and 0.5 ml extraction buffer (100 mM Tris, pH 8.0; 50 mM EDTA, pH 8.0; 500 mM NaCl; 10 mM β -mercaptoethanol which was added right before use) was added and the mixture left on ice while the other samples were processed. After all the samples had been ground, 35 μ l of 20% sodium dodecyl sulfate (SDS) was added, vortexed to mix well and incubated for 10 min at 65 - 70 °C. Then, 130 μ l of 5 M potassium acetate were added to each sample after which the samples were left on ice for 5 min and centrifuged at 13000 rpm for 5 min. The supernatant was transferred to a fresh tube containing 60 μ l of 3M sodium acetate and 640 μ l

of isopropanol. The solution was vortexed and centrifuged at 13000 rpm for 10 min. The supernatant was discarded and the pellet was resuspended in 200 µl of BTE (50 mM Tris, pH 8.0; 1 mM EDTA pH 8.0). The solution was centrifuged at 13000 rpm for 5 min. The supernatant was transferred to a new 1.5-ml microcentrifuge tube containing 20 µl of 3M sodium acetate and 450 µl of 100% ethanol, mixed well by vortexing and then centrifuged for 10 min. The pellet was rinsed with ice-cold 70 % ethanol. The pellet was dissolved in 100 µl of TE (10 mM Tris-Cl, pH 8.0; 1 mM EDTA) containing 10 mg/ml RNase A (Bioshop Canada, Burlington, ON). The solution was incubated for 1 hr at 37 °C, followed by addition of 10 µl of 3M sodium acetate and 220 µl of 100% ethanol and then centrifuged at 13000 rpm for 10 min. The pellet was rinsed with ice-cold 70% ethanol and allowed to air dry. The pellet was dissolved in 50 µl of TE and 0.1 µl was used for PCR.

2.4 Mutation Site Verification

In order to verify the mutation sites of each *cer8* alleles, PCR primers were designed to amplify fragments of *LACS1* gene, based on the genomic DNA sequence obtained from TAIR (www.arabidopsis.com). Genomic annotation obtained from TAIR indicated that the length of the wild type *LACS1* coding region was 3782 bps (from ATG start codon to TGA stop codon). Three pairs of primers were designed to amplify three ~1500 bps fragments which were assembled together to represent a complete *LACS1* gene: *At2g47240_Seq_F1* (5'-TAACTCCGTCGGTTGGATTC-3'), *At2g47240_Seq_R1* (5'-ACCTAATGCCG

TCAAGTTGG-3'), *At2g47240*_Seq_F2 (5'-TAACGTGAGCGACGAGCTTA-3'), *At2g47240*_Seq_R2 (5'-TAACGTGAGCGACGAGCTTA-3'), *At2g47240*_Seq_F3 (5'-GGTCATCACATAAACCTGAC-3') and *At2g47240*_Seq_F3 (5'-GGTAGTTACGTGGATTTTGG-3'). Three additional sequencing primers were also designed for each fragment to complete sequencing of the amplified DNA fragments: *At2g47240*_Seq_F4 (5'-CAGTGGATCATAGCAATGGAG-3'), *At2g47240*_Seq_F5 (5'-CACACAAAGCGACGTTGATA-3') and *At2g47240*_Seq_F1 (5'-TTGTTGTCGACTATCCTTAATGCTT-3'). The sequencing reactions were carried out at DNA Landmarks (Montreal, QC). After sequencing, the DNA sequences were assembled together using Seq Man II Lasergene software (DNASTAR, Inc. Madison, US). The mutant *CER8* genomic sequence was compared with sequenced *Ler* wild-type *At2g47240* gene sequence to identify the location of the lesion in *cer8-1*.

2.5 Semi-Quantitative Reverse-Transcription PCR (RT-PCR)

Semi-quantitative reverse-transcription PCR (RT-PCR) was performed to determine the relative gene expression levels of *LACS1* in the *cer8* alleles compared to respective wild-type ecotypes. Total RNA from stem tissue was harvested from the three mutants and wild-type ecotypes using TRIzol reagent (mixture of phenol, guanidine isothiocyanate, red dye and other proprietary components; Invitrogen, Burlington ON). Approximately 100 mg of plant stem tissue was harvested, placed in aluminum foil and immediately frozen in liquid nitrogen. Frozen tissues were ground to a fine powder in pre-chilled 1.5-ml

microcentrifuge tubes with plastic micropestles. 600 µl of TRIzol reagent was added and tissues were further ground to form a fine homogenate. Another 400 µl of TRIzol was added to the homogenate, the solution mixed by vortexing and incubated at room temperature for 5 min. Then, 200 µl of chloroform was added and mixed vigorously by hand for 15 sec. The mixture was incubated at room temperature for 3 min and centrifuged at 12,000 g for 15 min at 4 °C. The aqueous layer was transferred to a new 1.5 ml microcentrifuge tube and 500 µl of isopropyl alcohol added. The solution was mixed by vortexing and incubated at room temperature for 10 min. Then, the solution was centrifuged at 12,000 g for 10 min. The supernatant was discarded and the RNA pellets were rinsed in 70% ethanol (v/v) (prepared with diethylpyrocarbonate-treated water). The RNA was dissolved in 50 µl of RNase-free water (Sigma, Oakville, ON) and stored in a -80°C freezer. The RNA concentration was quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Nepean, ON).

One µg of total RNA was used as template to synthesize *LACS1* cDNA using Superscript III reverse transcriptase (Invitrogen Burlington Ontario). The cDNA was further used as template to amplify the *LACS1* transcript using PCR. The primers used were *At2g47240_RT_F1* (5'- GATTTTCTCCATATGGGACGTG-3'), and *At2g47240_RT_R1* (5'- CGCGACAAGCATAGATTTGA-3'); *At2g47240_RT_F2* (5'- GACCTACCGGATTCAAACCA-3') and *At2g47240_RT_R2* (5'- CAATCTGGCTCA AGAAGTCCTTT-3'). The primers were designed to anneal to flanking exons in order to eliminate any amplification from genomic DNA. The initiation factor gene *eIF4A-1* (*At3g13920*) was used as a constitutive control

using primers *At3g13920_RT_F1* (5'- CCAGAAGGCACACAGTTTGATGCA-3') and *At3g13920_RT_R1* (5'- TCATCATCACGGGTCACGAAATTG-3') (Beisson et al., 2007). All the PCR products were run on a 0.8% (w/v) agarose electrophoresis with ethidium bromide as dye. The gel image was photographed using an Alphamager 2200 gel-imager system (Alpha Innotech, San Leandro, CA).

2.6 Scanning Electron Microscopy

In order to examine the epicuticular wax crystals of *cer8* mutant lines versus wild-type, scanning electron microscopy (SEM) was performed. Approximately one cm fragments of plant stem were cut from 4 week-old plants and placed on an SEM stub that had a double-sided sticky tab affixed to it. A layer of drierite (W.A.Hammond Drierite) one cm thick was placed in a sealed container and the SEM sample platform with plant stem samples were placed in the sealed container and allowed to dry for at least four days. The samples were coated with 15 nm of gold and subsequently photographed on a JSM-6400 scanning electron microscope (JEOL) located at the Nanoprobe (SEM) facility of Carleton University.

2.7 Gas Chromatography

All the cuticular wax samples were isolated from six week-old plants, which had developed reproductive organs. The diameter of the stem was measured using a digital caliber and by taking 5 values along the length of the selected segment and the average was used to calculate the surface area of the stem ($s = \pi \cdot d \cdot h$; s: surface area, d: diameter, h: length). The selected region of the stem

was cut and dipped in HPLC-grade chloroform for exactly 30 sec. After dipping, the stem length was measured immediately. The chloroform, which contains intra- and epicuticular waxes, was dried under a gentle stream of nitrogen. At least 5 duplicates of each mutant line and wild type were prepared. The dried wax samples were derivatised by adding 100 μ l of bis-(Trimethylsilyl)trifluoroacetamide (BSTFA; Pierce) and incubated at 80°C for 90 min. The samples were then run directly on a Varian GC-3900 gas chromatograph, equipped with a flame ionization detector (FID). The oven was programmed to maintain an initial temperature of 150°C for 1 min and increased to 300°C at a rate of 4°C /min and finally maintained at 300°C for 15 min. In order to quantify the amount of each wax components, 1 μ g of internal standard, methyl heptadecanoate (17:1), was added to each sample vial containing chloroform prior to wax isolation.

2.8 GUS Reporter Gene Analysis

In order to determine the gene expression patterns of *LACS1*, -2 and -3 gene, the promoter region of each gene was translationally fused with the β -glucuronidase (GUS) reporter gene to indicate the gene expression location following incubation with X-Glucuronide (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid, cyclohexylammonium salt), which is transformed into a blue precipitate by the action of GUS. The promoter regions of each gene were amplified from BAC genomic DNA T8I13 (*LACS1*), F13F21 (*LACS2*) and F15H21 (*LACS3*) using proof reading DNA polymerase Pwo (Roche, Mississauga, ON). The primers

used for amplification were (restriction sites are underlined in the primer sequences):

*At2g47240_GUS_F1**Sall* (5'-GACGTCGACGCTCCTAGCTGGCTATTTT-3'),

*At2g47240_GUS_R1**Xbal* (5'-GACTCTAGACTCTTCTACCTTAGCCGC-3'),

*At1g49430_GUS_F1**HindIII* (5'-GACAAAGCTTTGAGAAACCGTGTCTGACCAA-3'),

*At1g49430_GUS_R1**BamHI* (5'-CTAGGATCCCAACAACACATTATCCGC-3'),

*At1g64400_GUS_F1**HindIII* (5'-GACAAAGCTTTCGTACGGACTTTCGTTCTTG-3'),

*At1g64400_GUS_F1**Sall* (5'-CTAGGATCCAAACCTCAACGATGTATCGAC-3'). Two

different flanking restriction enzyme sites were introduced into each of the amplified products: *Sall/XbaI* for the *LACS1* promoter and *HindIII/BamHI* for the *LACS2* and *-3* promoters. Both the promoter and pBI101 vector (Stratagene, La Jolla, CA) were digested using the corresponding restriction enzymes to generate 'sticky' ends, ligated using T4 DNA ligase (Bioshop Canada, Burlington, ON), and then the ligation mixtures were transformed into competent DH5α *E. coli* to generate recombinant plasmids pBI101_*LACS1*pro::GUS, pBI101_*LACS2*pro::GUS and pBI101_*LACS3*pro::GUS. The vector maps of these recombinant plasmids are shown in Appendix A. Recombinant plasmids containing inserts were identified by colony PCR screening. After purification using a Wizard® Plus SV Minipreps DNA Purification System (Promega, Madison, WI), the promoter regions were PCR amplified from recombinant plasmids and sequenced to verify that there were no mutations introduced during PCR amplification with additional sequencing primers: *At2g47240_GUS_F2*seq (5'-GACCGGCGGTTACTTGGAATAA-3'), *At2g47240_GUS_F3*seq (5'-CACGTGACGGTAACTGGCTA-3'), *At2g47240_GUS_F4*Seq (5'-CCGACCTACCGGATTCAAACC-3'), *At1g494300_GUS_F2*seq (5'-ATAGGGAG

CAGGTCGGAGACTCA-3') and *At1g64400_GUS_F2seq* (5'-CGATTCTAGGTGAC TCACTTGTG-3'). The recombinant plasmids were subsequently transformed into Col-0 wild-type plants via *Agrobacterium*-mediated transformation using the floral dipping method (Clough and Bent, 1998). One single *agrobacterium* colony from LB+Kan/Get/Rif (50/40/100 µg/ml) plate was inoculated into 5 ml of liquid LB medium containing the same antibiotic and grown overnight. The 5 ml overnight culture was subsequently inoculated into 25 ml of LB medium containing the same antibiotics and grown overnight. 12.5 ml of the overnight culture was finally inoculated into 500 ml of LB liquid medium containing the same antibiotics and grown to stationary phase ($OD_{600}=2.0$). The cells were then harvested by centrifugation at 7000 rpm for 10 min and resuspended in 500 ml of 5% sucrose and 0.05% Silwet L-77. Col-0 wild-type plants were dipped directly into the *agrobacterium* solution for 2~3 sec and placed on their side to drain excess liquid. A plastic dome was placed over dipped plants to retain humidity for 12-24 hrs and kept in low light until the following day. The dipped plants then were allowed to self-fertilized and seeds were collected. Successful transformants were selected on AT medium plates containing 40 µg/ml Kanamycin and transformants containing a single T-DNA insertion loci were screened based on a segregation ratio of 3:1 (resistant versus non-resistant) of the T2 generation. Non-segregating homozygous T-DNA lines were identified in the T3 generation by identifying lines that gave 100% Kanamycin resistant progeny.

'GUS staining' was performed on different tissues at different development phases, including rosette leaves, cauline leaves, flower buds, matured flowers

(open but with no silique developed), matured stem, young siliques (3 days after sepals, petals and stamens had shed and the carpel had begun to elongate), matured siliques (no elongation observed for 3 days and seeds were observed in the silique) and roots (3-day, 7-day and 11-day old). The harvested tissue was immersed in heptane and incubated for 5 min. The heptane was then removed and collected for re-use. The tissues were allowed to air-dry for 30 sec to 1 min and washed in pre-chilled staining buffer (50 mM NaH_2PO_4 , 100 mM $\text{KFe}(\text{CN})_6$, 0.1% Triton X-100) and kept on ice for 2 min. The solution was replaced with 1-2 ml of staining buffer, which contained X-Glucuronide (1 mM) covering the tissues completely and incubated in a 37°C oven for 8 -10 hrs (for roots, incubation time was shortened to 2 hrs). After incubation, the staining solution was replaced with 70% ethanol and the samples allowed to sit at room temperature overnight. This ethanol was removed and fresh 70% ethanol was added. The stained tissues were photographed on a Carl Zeiss Discovery V12 SteREO microscope equipped with an AxioCam MRC camera.

The stained stems were embedded in wax and cross sectioned to determine the cell-layer specific gene expression patterns. The stained stems were first soaked in fixative solution (50% of ethanol, 5% of acetic acid and 3.7% of formaldehyde) for 4 hrs. The stems were then washed in 70% of ethanol for 30 min, 85% for 30 min and 95% ethanol with 0.1% of eison stain overnight. On day 2, the stems were cleared in 100% ethanol for 1 hr with two changes of fresh 100% ethanol for 30min each. Then, the stems were permeated with xylenes by incubating in ascending concentrations of xylene:ethanol solutions for 30 min

each, starting from 25% xylene:75% ethanol, 50% xylene:50% ethanol, 75% xylene:25% ethanol and finally to 100% xylene. Following the final 30 min incubation in 100% xylene, the xylene was exchanged for fresh xylene, filled to half of the vial and incubated for another 30 min. Twenty paraplast (paraffin wax) embedding medium chips were added and incubated at room temperature overnight. On day 3, the vial was placed in a 42°C water bath until the chips had melted. The vial was then moved to a 60°C oven and incubated for at least 4 hrs (usually until the end of the day). The xylene/wax solution was changed with 100% pre-melted paraplast and incubated in a 60°C oven overnight. From day 4 to day 6, six wax changes spaced at least 6 hrs were performed. On day 7, the wax solution containing the sample was poured into a petri-plate, which was pre-warmed to 60-65°C in a water bath. It was topped up with molten wax. A dissecting needle was used to separate the tissues within the wax and the petri-plate was moved to a cold platform until the wax was completely solidified. Cross sections were obtained on a Rotary Microtome HM325 sectioning machine (MICROM International GmbH). The 10 µm-thick slim wax slices containing sectioned sample were placed on a FisherBrand® Superfrost®/Plus microscope slide and incubated on a slide warmer (FisherScientific, Nepean, ON) overnight. The slide was further dewaxed by dipping up and down in the following solutions for about 1 min each: 100% xylene (2×1 min); 100% ethanol; 95% ethanol; 90% ethanol; 80% ethanol+0.85% NaCl; 60% ethanol+0.85% NaCl; 30% ethanol+0.85% NaCl; 0.85% NaCl. Finally, the samples were mounted with 50% glycerol, covered with a piece of cover glass and sealed with nail polish. When the nail

polish had dried, the slide was cleaned and photographed using a Carl Zeiss Discovery V12 SteREO microscope equipped with an AxioCam MRC camera.

2.9 Protein Expression in *E. coli* and LACS Activity Determination

A K27 strain of *E. coli* that carries a knock out mutation of the *E. coli* acyl-CoA synthetase *FadD* gene (Overath et al., 1969) was obtained from the *E. Coli* Genetic Stock Center (CGSC) at Yale University (<http://cgsc2.biology.yale.edu/index.php>). The K27 (*fadD*) *E. coli* was modified to express T7 RNA polymerase using a λ DE3 lysogenization kit (Novagene, Mississauga, ON). The K27 (*fadD*) strain was grown in LB medium (Bioshop Canada, Burlington, ON) that had been supplemented with 0.2% maltose, 10 mM MgSO_4 to an OD_{600} of 0.5. A mixture of 10^8 plaque forming units (pfu) λ DE3, 10^8 pfu Helper Phage, and 10^8 pfu Selection Phage was incubated with 1–10 μl host cells. 5 μl and 10 μl of host cell culture were used here to make two plates with different amounts of candidate lysogens colonies. The chosen 10 colonies were grown in LB medium (Bioshop Canada, Burlington, ON), supplemented with 0.2% maltose, 10 mM MgSO_4 , to an OD_{600} of 0.5. In duplicate tubes (one to be induced by IPTG and the other for non-induction negative control; see below), 100 μl of the host cells were mixed with 100 μl of T7 Tester Phage, which was pre-diluted in 1 $\times$ Phage Dilution Buffer (20 mM Tris-HCl, pH 7.4; 100 mM NaCl; 10 mM MgSO_4) to $1\text{--}2 \times 10^3$ pfu/ml. The mixtures were incubated at room temperature for 10 min with 3 ml of molten top agarose (1% tryptone; 0.5% NaCl; 0.6% agarose) that was added to each tube and the contents of one duplicate was poured onto an LB

plate and the other duplicate onto an LB plate with 0.4 mM isopropyl β -D-1-thiogalactopyranoside (IPTG, Bioshop Canada, Burlington, ON). The top agarose was allowed to solidify and incubated at room temperature overnight. Successful lysogen should produce large plaques with diffuse edges on LB+IPTG. Glycerol stocks of successful lysogen cultures were made by mixing 700 μ l of overnight culture with 300 μ l of 50% sterile glycerol.

The lysogens were further verified by Western blotting to detect the expression of T7 RNA polymerase upon induction with IPTG. The successful λ DE3 lysogen candidates were grown on LB plate and a single colony was inoculated in 5 ml of liquid LB medium and grown overnight. Three ml of the overnight culture was inoculated into 50 ml of LB medium, grow at 37°C for 2 hrs and IPTG added to a final concentration of 1 mM to induce the expression of T7 RNA polymerase for 16 hrs. Cells were harvested by centrifugation at 5000 rpm for 15 min and resuspended in 5 ml of lysis buffer (50 mM of Tris-Cl, pH 8.0; 50 μ l of Bioshop protease inhibitor cocktail), followed by sonicating for 1 min at 50% duty cycle, level 4.5 using a microtip. The cell lysate was centrifuged at 5000 rpm for 15 min. The supernatant was collected and 40 μ l was loaded onto a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a Bio-Rad Mini-Protean Tetra Cell system (Bio-Rad, Hercules, CA) at 150 volts for 1 hr. The proteins were transferred to GE Pure Nitrocellulose transfer membrane (0.45 micron; GE Osmonics Labstore, Minnetonka, MN) in a Bio-Rad Mini Trans-Blot[®] Electrophoretic Transfer Cell system (Bio-Rad, Hercules, CA) in a cold room overnight.

The protein (Western) blot for detection of T7 RNA polymerase was performed using an Amersham ECL Advance Western Blotting Detection Kit (GE Healthcare, Baie d'Urfé, QC). Blocking solution was prepared by dissolving ECL Advance Blocking Agent (supplied by kit) in Tris buffered saline-T (TBS-T) solution (0.8% of sodium chloride; 20 mM of Tris-HCl, pH 7.6; 0.1% of Tween 20) to make a final concentration of 2% (w/v). The membrane containing separated proteins was immersed in 20 ml of blocking solution and incubated on an orbital shaker for 1 hr at room temperature. It was rinsed with two fresh change of TBS-T buffer, and then the membrane was incubated with diluted T7 RNA polymerase monoclonal antibody (1:10000) in blocking solution (Novagen, Mississauga, ON) on an orbital shaker for 1 hr. The membrane was then rinsed with two fresh changes of TBS-T buffer and washed in $>4 \text{ ml/cm}^2$ of TBS-T buffer for 15 min and subsequently washed 3×5 min with fresh changes of TBS-T buffer at room temperature. After this washing step, the membrane was incubated with diluted Amersham ECL anti-mouse IgG, horseradish peroxidase-linked species-specific whole antibody (1:10000 in blocking solution; GE Healthcare, Baie d'Urfé, QC) on an orbital shaker for 1 hr at room temperature. After incubation, the membrane was rinsed with two fresh changes of TBS-T buffer and washed in $>4 \text{ ml/cm}^2$ of TBS-T buffer for 15 min and subsequently 3×5 min with fresh changes of TBS-T buffer at room temperature. The ECL Advance Solution A (Tris buffer in 3.2% ethanol) and ECL Advance Solution B (proprietary substrate in Tris buffer; supplied by kit; pre-equilibrate to room temperature) were mixed in a ratio of 1:1. The excess TBS-T buffer was drained by gently touching the edge of the

membrane on a piece of Kimwipe and then the membrane placed protein side up on a clean surface. The mixed ECL solution was gently pipetted onto the membrane and incubated for 5 min at room temperature. The excess detection solution was drained by gently holding the membrane with forceps and touching the edge against a Kimwipe tissue. The membrane protein side was placed down onto a piece of clean plastic wrap. The membrane was wrapped up and any air bubbles were removed. The wrapped membrane was placed with the protein side up into an X-ray film cassette. One piece of Kodak® Scientific Imaging film (Burnaby, BC) was placed on top of the membrane and the film was exposed for 5 sec, and then put into the ILFORD Film Developer solution (1:10 dilution; Concord On) for 5 min, rinsed with running water for 1 min, followed by putting the film into ILFORD Rapid Fixer solution (1:5 dilution; Concord, ON) for 5 mins, then washed with running water for another 1 min.

To heterologously express LACS1, -2 and -3 in *E. coli*, cDNAs of each gene were amplified from clone U14264 (*LACS1*), U18235 (*LACS2*) and U09658 (*LACS3*) which were obtained from ABRC. The primer used here were:

LACS1_FORBamHI (5'-AGAGGATCCATGAAGTCTTTGCGGCTAAG-3'),
LACS1_REVSaII (5'-AGAGTCGACTCAGATTTTCTTTGAGGCCAAT-3'),
LACS2_FORBamHI (5'-AGAGGATCCATGTCTTTAGCCGCGGATAAT-3'),
LACS2_REVSaII (5'-AGAGTCGACCTATGCCATGGACCTCTTTG-3'),
LACS3_FORBamHI (5'-AGAGGATCCATGGCGACTGGTCGATACAT-3') and
LACS3_REVSaII (5'-AGAGTCGACTTACACTCGTAGCTGCACTTC-3'). After digestion with *BamHI* and *SaII* restriction enzymes, the cDNAs of *LACS1*, -2 and

-3 were cloned between the corresponding restriction sites of pET28a vectors (Novagen, Mississauga, ON), to generate pET28a_LACS1, pET28a_LACS2 and pET28a_LACS3 recombinant plasmids. I acknowledge Nigel Tan for building these three constructs. The plasmids were transformed into chemical competent K27(DE3) strain of *E. coli* generated as described above. An empty pET28a vector was also transformed into the same *E. coli* strain to serve as a negative control. To express the LACS enzymes, 5 ml starter cultures for each construct were grown overnight with shaking at 225 rpm at 37°C in LB medium, containing 50 µg/ml Kanamycin. Three ml of the overnight cultures were inoculated into 50 ml of LB medium containing 50 µg/ml Kanamycin and the cells grown overnight under the same conditions as for the starter culture. 30 ml of the 50 ml overnight culture for each construct was inoculated into 500 ml of LB medium containing 50 µg/ml Kanamycin and grown at 30°C with shaking at 225 rpm until mid-log phase ($OD_{600}=0.8$) had been reached. When mid-log phase was reached, IPTG was added into each culture to a final concentration of 1 mM to induce the expression of the LACS enzymes. The expression was then allowed to proceed for exactly 16 hours.

Cells were harvested by centrifugation at 5,000 rpm for 15 min at 4°C. The cell pellets were rinsed in 100 ml of 0.85% sodium chloride and then the cells were harvested again as above. Thirty ml of buffer A (50mM of Na_2HPO_4 , 50 mM of NaH_2PO_4 , 10 mM of Imidazole) with 30 µl of Protease Inhibitor Cocktail lacking EDTA (Bioshop Canada, Burlington, ON) and 1 mg/ml of lysozyme (Sigma, Oakville, ON) were added to the cell pellet. The cell pellets were thoroughly

resuspended in Buffer A and incubated in the cold for 30 min, and then subsequently sonicated for 90 sec (sonicating for 15 sec with rest intervals of 15 for a sec total of six repeats). The cell debris was removed by centrifugation at 5,000 rpm at 4°C for 15 min. The supernatants were transferred to Beckman Quick-Seal ultracentrifuge tubes (Beckman Coulter, Inc.) and membrane fractions were isolated by ultracentrifugation at 100,000g at 4°C for 1 hr. The brown pellets were gently re-dissolved in phosphate buffer (20mM, pH7.4, 0.5M NaCl, containing 0.1% Triton X-100 (Sigma, Oakville, ON) by sonication in an ice-bath at 50% duty cycle, level 4.5 using a microtip and further purified by passing over a nickel-containing column (Bio-Rad, Hercules, CA, Hercules, CA) column charged with 0.1 M of nickel sulfate (Sigma, Oakville, ON). The protein elutions were quantified using Bio-Rad Protein Assay Kit II (Bio-Rad, Hercules, CA) and the presence of LACS enzyme was verified by SDS-PAGE electrophoresis with Coomassie Brilliant Blue staining and Western blotting as described above, By using a T7 Tag Antibody (Novagen, Mississauga, ON) instead of T7 RNA polymerase monoclonal antibody as primary antibody.

The LACS enzyme activities towards saturated fatty acid substrates of various chain lengths were determined by a colourimetric assay (Ichihara et al., 1991). The substrates tested were hexadecanoic acid (C16:0), octadecanoic Acid (C18:0), eicosanoic acid (C20:0), docosanoic acid (C22:0), tetracosanoic acid (C24:0), hexacosanoic acid (C26:0), octacosanoic acid (C28:0) and triacontanoic acid (C30:0) (all obtained from Sigma, Oakville, ON). The potassium salts of each fatty acid were prepared by mixing each fatty acid in HPLC-grade hexane (Sigma,

Oakville, ON) with 0.01 M of methanolic KOH to neutralize, and then followed by evaporation of the solvent under reduced pressure. The vials containing fatty acids salt powders need to be filled with nitrogen gas to minimize oxidation. The assay medium contained 0.15 M MOPS/NaOH (pH 7.6), 0.5 mM fatty acid potassium salt, 0.5 mM CoA, 4.5 mM ATP, 12 mM MgCl₂, 1 mM dithiothreitol (DTT), 0.55 mM Triton X-100, 1% methanol, 0.5 U acyl-CoA oxidase (from *Candida* sp.; Sigma, Oakville, ON), 1,000 U catalase (from bovine liver; 1340 U/mg solid; Sigma, Oakville, ON), and 10 µl of enzyme extract in a total volume of 50 µl. After a 15 min incubation at 30°C, the reaction was stopped by the addition of 50 µl of 2 M KOH. To this solution was added 0.5 µl of 0.6% 4-amino-3-hydrazino-5-mercapto-1,2,4-tzole (AHMT; Sigma, Oakville, ON) and then 0.5 M HCl was added to the mixture. The mixture was incubated at 37°C for 10 min, and then 200 µl of 0.75% KIO₄ was added. The total volume was 350 µl. After dilution with 700 µl of water, the absorbance at 550 nm was measured at room temperature using a Varion Cary 300 spectrometer (Varian). The molar absorption coefficient, $\epsilon = 29,200 \text{ M}^{-1}\text{cm}^{-1}$ of the resulting purple dye was used to calculate the amount of acyl-CoA synthesized. The activity of each enzyme towards each substrate was determined as the average of 3 duplicates.

Chapter 3 Results

3.1 Identification of *CER8*

The *cer8* mutant locus was previously mapped to the bottom of chromosome 2 relative to simple sequence length polymorphism (SSLP) markers on the Lister and Dean Recombinant Inbred map (Rashotte et al., 2004). We selected candidate genes for *CER8* based on the approximate position of the *cer8* locus relative to nearby physical positions of known candidate genes (Figure 4). The candidate genes were narrowed down to those that have epidermal-specific gene expression determined by DNA microarray analysis (Suh et al., 2005). Two distinct SALK T-DNA insertion lines, preferably with an insertion in an exon, for each candidate gene were obtained from ABRC and grown on soil to check if they had a 'glossy' phenotype.

Among the various SALK lines tested, SALK_127191 and SALK_138782, both of which carry a T-DNA insertion in the gene *At2g47240* (*LACS1*; Shockey et al., 2002), were observed to have a typical 'glossy' phenotype on their stems (Figure 5A). To test for allelism between *LACS1* and *CER8*, each SALK T-DNA insertion line was crossed with the original *Ler-cer8* mutant (Koornneef et al., 1989). The *cer8-1* was reported to be a recessive mutant by Koornneef and co-workers (1989) and I verified a 3:1 Mendelian segregation ratio typical of a single recessive loci in a *cer8* F2 population derived from a cross of *cer8-1* with a wild type parent (148 glossy plants out of 606 total plants). The homozygotes of SALK T-DNA insertion lines were verified by the T-DNA verification primer designed by the online tools of the Salk Institute Genomic Analysis Laboratory.

Figure 4 Selection of *CER8* candidate genes

The chromosome on the left shows genetic mapping positions of *cer8* and other loci on *Arabidopsis thaliana* chromosome 2, based on the Lister and Dean recombinant inbred map (Rashotte et al., 2004). The chromosome on the right indicates the physical map positions of genes known or suspected to be involved in acyl lipid metabolism that are up-regulated in the epidermis of stems, relative to internal cell layers (Suh et al., 2005). *At2g47240*, which was further demonstrated to be allelic to *CER8*, is circled in a red rectangle square. The red round spots represent the position of the centromere on chromosome representations. *rga*, *RNS1*, *PhyB* and *COP1* are cleaved amplified polymorphic sequence (CAPS) markers with the positions indicated on both maps.

I acknowledge Adel Al-Shammari for accomplishing the selection of *CER8* candidate genes, as part of his undergraduate thesis project.

Table 3 Candidates for *CER8* Gene (adapted from Al-Shammari, 2006 and Suh et al., 2005)

Gene Name/Physical position	Description of putative encoded function	SALK lines and T-DNA insertion sites
<i>At2g38470</i> 16.11 Mb	WRKY family transcription factor	SALK_006602.47.80.x (Exon) SALK_075255.54.25.x (300-UTR5)
<i>At2g39400</i> 16.46 Mb	Monoacylglycerol lipase	SALK_135501.18.35.x (Intron) SAIL_75_F12 GS803577 (300-UTR5)
<i>At2g38530</i> 16.13 Mb	Lipid transfer protein type I	SALK_026257.47.35.x (1000-promotor)
<i>At2g39350</i> 16.43 Mb	ABC transporter	SALK_146494.27.10.x (Exon) SALK_017391.43.25.x (Exon)
<i>At2g41540</i> 17.33 Mb	Dihydroxyacetone phosphate reductase	SALK_020444.44.10.x (Exon) SALK_020437.20.60.x (Exon)
<i>At2g45970</i> 18.92 Mb	Fatty acid ω -hydroxylase	SALK_020444.44.10.x (Exon) SALK_020437.20.60.x (Exon)
<i>At2g47240</i> 19.40 Mb	Long-Chain acyl-CoA synthetase	SALK_127191.49.80.x (Exon) SALK_138782.39.85.n (Intron)
<i>At2g47630</i> 19.54 Mb	Monoacylglycerol lipase	SALK_008237.56.00.x (300-UTR5) SALK_123054.15.75.x (Intron)

Figure 5 Identification of *CER8* as *LACS1* (*At2g47240*)

(A) Wild-type, mutant and F1 plants from complementation crosses.

The stems from left to right are Ler-WT, *cer8-1*, Col-WT, *cer8-2* (SALK_127191), F1 of *cer8-1*×*cer8-2*, *cer8-3* (SALK_138782) and F1 of *cer8-1*×*cer8-3*.

(B) Schematic map of *At2g47240* gene with mutation sites indicated.

Blue boxes represent 5' and 3' untranslated regions (UTRs); red boxes represent exons; lines between red boxes represent introns; ATG is the start codon, TGA is the stop codon and the mutation site of each mutant line is marked by an arrow. *Cer8-1* is a 2bp deletion in exon 8. *Cer8-2* (SALK_127191) and *cer-3* (SALK_138782) are T-DNA insertion in exons 12 and 8, respectively.

(C) Semi-quantitative RT-PCR of *CER8/LACS1* in wild-type and mutant lines.

The upper lane shows the *LACS1/CER8* transcript in all three mutant lines as well as corresponding wild-type ecotype to the left. The lower lane shows the expression of the constitutive control gene, *elf4A-1* (*At3g13920*). The primers used for amplification were *At2g47240_RT_F2* (5'- GACCTACCGGATTCAAACCA -3') and *At2g47240_RT_R2* (5'- CAATCTGGCTCA AGAAGTCCTTT-3') and their binding sites are shown in Figure 5B.

The SALK lines were used as male (pollen donor) and *Ler-cer8* as female. If allelic, the F1 generation of the cross should have a glossy mutant phenotype; otherwise, the F1 should have a glaucous wild-type phenotype. As shown in Figure 5A, the F1 plants have a 'glossy' phenotype compared with wild-type plants. Genomic DNA from 10 F1 plants of each cross were extracted and amplified using T-DNA verification primers to verify the existence of a hemizygous T-DNA insertion in the F1 plant, indicating that the glossy plants obtained in the F1 generation were indeed successful crosses and not self progeny. Based on these results, I concluded that *At2g47240* (*LACS1*) is allelic to *CER8*. The three *cer8* mutant lines, *Ler-cer8*, SALK_127191 and SALK_138782, were named as *cer8-1*, *cer8-2* and *cer8-3*, respectively.

3.2 Identification of mutation sites in *cer8* alleles

The mutation type and location of each mutant was determined by isolating genomic DNA samples from *Ler*-WT, *Col*-WT, *cer8-1*, *cer8-2* and *cer8-3*, followed by amplification by PCR of the *At2g47240* gene and sequencing. The sequencing results indicated that *cer8-1* carries a two 2 nucleotide deletion (1580-T and 1581-A, relative to start codon at position +1) in the 8th exon, which results in a premature stop codon at position 1615 in the middle of 8th exon. *Cer8-2* carries a T-DNA insertion in the 12th exon between nucleotides 2650-G and 2651-C and *cer8-3* carries a T-DNA insertion in the 8th exon between nucleotides 1609-C and 1610-A (Figure 5B).

3.3 Transcript Profile of *CER8/LACS1* in *cer8* alleles

The transcript profile of *CER8/LACS1* (*At2g47240*) in each of the mutant alleles was analysed by semi-quantitative reverse transcription PCR (RT-PCR) using total mRNA isolated from stem tissues as template. While the control gene *eIF4A1* (*At3g13920*) was equally expressed in the *cer8* alleles and wild-type plants, the transcript of *CER8/LACS1* was absent in all three *cer8* mutant lines (Figure 5C). The experiment was repeated twice using two independent sets of plants for mRNA extraction and two pairs of RT-PCR primers with different binding sites. The *At2g47240_RT_F1* and *At2g47240_RT_R1* pair was flanking the mutation region; and the *At2g47240_RT_F2* and *At2g47240_RT_R2* pair binds to a region upstream of all three mutation sites. The absence of transcript in *cer8-1* is likely the result of nonsense-mediated mRNA decay (El-Bchiri et al., 2008). These results indicate each of the alleles are null alleles.

3.4 Altered epicuticular wax crystal formation of *cer8*

Scanning electron microscopy was performed to examine the epicuticular crystals on the *cer8* alleles. Koornneef et al. (1989) performed SEM analysis of *cer8-1* previously, but the quality was poor. The images shown here represent at least three duplicate stems for each mutant line and corresponding ecotype wild-type, all of which showed consistent results. The overall epicuticular wax crystals density was significantly decreased on *cer8-1* (Figure 6A), compared with *Ler*-WT at 1500× magnification (Figure 6B). The typical 'tube-shape' wax crystals on *Ler*-WT stems (Figure 6C), were found to be largely missing on *cer8-1* stems

(Figure 6D). Instead, the most abundant wax crystal type was a small 'trunk' shape.

The *cer8-2* and *cer8-3* mutant lines in Col-0 ecotype background have a similar wax crystal alteration as found for *cer8-1* (Figure 7). These wax crystal phenotypes were consistent with those reported by Koornneef et al. (1989).

Figure 6 Scanning electron microscopy images of *Ler*-WT and *cer8-1*

(A) *Ler*-WT at magnification of 1500×

(B) *Cer8-1* at magnification of 1500×

(C) *Ler*-WT at magnification of 5000×

(D) *Cer8-1* at magnification of 5000×

Plants were grown in a controlled-environment growth chamber with a long-day photoperiod (18 hours light and 6 hours dark) and at 22°C. The scale bars are shown at the bottom of each panel.

Figure 7 Scanning electron microscopy images of Col-WT, *cer8-2* and *cer8-3*

(A) Col-WT at magnification of 1500×

(B) *cer8-2* at magnification of 1500×

(C) *cer8-3* at magnification of 1500×

(D) Col-WT at magnification of 5000×

(E) *cer8-2* at magnification of 5000×

(F) *cer8-3* at magnification of 5000×

Plants were grown in a controlled-environment growth chamber with a long-day photoperiod (18 hours light and 6 hours dark) and at 22°C. The scale bars are shown at the bottom of each panel.

3.5 Cuticular wax load and chemical composition of *cer8*

The total stem wax load and composition of each *cer8* allele was analyzed by gas chromatography with detection by flame ionization detector (FID). The identity of peaks was achieved by running standards of the major components and comparing retention times. The quantification of each component was done by comparison with an internal standard (methyl heptadecanoate, C17:1) added prior to wax extraction.

The total wax load on *cer8-1* decreased to 73.02%±4.94% of wild-type (*Ler*) level and the loads of *cer8-2* and *cer8-3* were 58.83%±6.04% and 56.57%±3.29% of wild type (*Col*) level, respectively (Table 4 and Figure 8). The three major wax components, alkanes, secondary alcohols and ketones, were decreased to 50.93%, 43.67% and 51.54% of wild-type levels on *cer8-1* respectively (Figure 9). Similar decreases were observed for *cer8-2* and *cer8-3*. Nearly all the individual components of the alkane forming pathway decreased on *cer8* mutant lines except for C27 alkane, which showed a slight increase (Figure 10A). C28:0 and C30:0 aldehydes both were slightly increased on *cer8* mutant lines (Figure 10C). The C30 free fatty acids showed a significant increase on *cer8* alleles (10.5-fold on *cer8-1*, 16-fold on *cer8-2* and 15-fold on *cer8-3*; Figure 10B and Table 4). The C28 free fatty acids were decreased relative to wild-type. Primary alcohols of chain lengths C24, C26 and C28 were slightly increased, except for the C30 primary alcohol which was decreased to 44.03% of wild-type levels (Figure 10B).

In summary, C30 free fatty acids are significantly increased on *cer8* alleles and all the components derived from C30 acyl-CoA are reduced, except for C30 aldehyde.

Table 4 Chemical composition of cuticular wax of *cer8* alleles

	<i>Ler</i> -WT	<i>cer8-1</i>	<i>Col</i> -WT	<i>cer8-2</i>	<i>cer8-3</i>
Total Wax Load	159.23±16.41	116.28±7.88	259.90±12.55	152.90±15.70	147.04±8.57
C27 Alkane	0.77±0.09	1.03±0.12	1.39±0.06	1.31±0.22	1.35±0.09
C29 Alkane	77.05±5.21	39.04±2.33	121.35±5.15	48.49±5.65	47.74±2.11
C31 Alkane	1.61±0.15	0.38±0.04	2.30±0.21	0.46±0.06	0.48±0.08
C29 Ketone	37.00±4.90	19.07±2.46	65.31±3.39	29.90±3.61	28.29±2.32
C29 Secondary Alcohol	15.89±2.15	6.94±0.79	26.00±1.41	9.62±1.03	9.11±0.70
C31 Secondary Alcohol	0.52±0.04	0.31±0.02	1.11±0.15	0.54±0.06	0.51±0.05
C24 Primary Alcohol	1.01±0.39	1.70±0.27	0.71±0.06	1.10±0.07	1.19±0.18
C26 Primary Alcohol	3.93±0.38	6.26±0.32	5.80±0.69	7.61±0.93	7.61±0.39
C28 Primary Alcohol	5.95±0.67	7.38±0.36	10.05±1.31	9.86±1.05	9.30±0.60
C30 Primary Alcohol	2.18±0.31	0.96±0.11	3.39±0.43	1.23±0.13	1.11±0.10
C24 Free Fatty Acid	0.49±0.06	0.68±0.11	0.99±0.11	1.13±0.28	1.28±0.12
C28 Free Fatty Acid	0.64±0.16	0.29±0.07	1.21±0.13	0.37±0.09	0.32±0.07
C30 Free Fatty Acid	1.63±0.48	17.14±0.97	1.14±0.17	18.74±2.27	17.25±1.41
C28 Aldehyde	2.22±0.42	3.05±0.32	4.46±0.49	4.85±1.15	5.28±0.71
C30 Aldehyde	8.36±1.95	12.04±2.60	14.69±1.30	17.76±4.41	16.23±4.6

Unit: ng/mm²; Each value is the average of five repetitions (n=5) and ± standard deviation is shown.

Figure 8 Total cuticular wax loads of *cer8* alleles

Total cuticular wax load of *Ler*-WT, *cer8-1*, *Col*-WT, *cer8-2* and *cer8-3*. All *cer8* alleles showed a decreased total wax load. The plants examined were 6-weeks old and stems were selected from the middle of the bolts. Values are averages of 5 duplicates (n=5). Error bars represent the standard deviation. The red bars correspond to plants of the *Ler* ecotype background, while the blue bars correspond to plants of the *Col* ecotype background.

Figure 9 Cuticular wax composition of *cer8* alleles

Wax components of each *cer8* allele and the corresponding wild-type ecotypes were sorted by chemical type. All *cer8* alleles show an overall decrease of alkanes, secondary alcohols and ketones, slight increases of primary alcohols and aldehydes and a significant increase of free fatty acids.

The plants examined here were 6-weeks old and stems were selected from the middle of the bolts. Values are averages of 5 duplicates (n=5). Error bars represent the standard deviation.

Figure 10 Cuticular wax composition of *cer8* alleles sorted into chemical class and chain length distributions

(A) Alkane forming pathway components

All the products of this pathway decreased in *cer8* alleles except for C27 alkanes which had a slight increase. The C27 alkanes, C29 alkanes and C31 secondary alcohols are shown in the inset in order to expand the scale.

(B) Primary alcohols and free fatty acids

Primary alcohols with chain lengths of C24, C26 and C28 slightly increased while C30 primary alcohol decreased; C30 free fatty acid were significantly increased amongst all *cer8* alleles, while C28 free fatty acids decreased. C24 free fatty acid did not show a significant change. The C26 free fatty acid was not positively identified in our chromatograms.

(C) Aldehydes

Both the C28 and C30 aldehydes slightly increased relative to wild-type.

The plants examined here were 6-weeks old and stems were selected from the middle of the bolts. Values are averages of 5 duplicates (n=5). Error bars represent the standard deviation.

This suggests that C30 aldehyde are derived from C30 free fatty acid, rather than C30 acyl-CoA. Other wax components were not changed, except for C30.

3.6 Analysis of *LACS1*, -2 and -3 gene expression patterns

To analyze the gene expression patterns of *LACS1*, -2 and -3, the promoter regions of each gene were fused with the GUS reporter gene in recombinant plasmids, which were transformed into Col-WT Arabidopsis. For *LACS2* and *LACS3*, 1833 bps and 2221 bps, respectively, upstream of the start codon were fused in frame with the GUS reporter gene. For *LACS1*, 3458 bps upstream of the start codon was fused in frame as there is a 1068 bp intron in the 5' untranslated region. Three generations of transformants were screened to obtain homozygous T-DNA insertion lines and multiple independent transgenic lines (>10) were analyzed to obtain the representative stained images of a T3 generation line shown here (Figures 11, 12 and 13).

LACS1 was expressed along the entire stem (Figure 11A) and specifically in the epidermal layer (Figure 11B). In cauline leaves, *LACS1* (Figure 11D) was generally expressed over the leaf surface but most strongly in trichomes, which are specialized epidermal cells. In siliques, *LACS1* was not expressed in the peel (Figure 11E), nor seeds (Figure 11F). In flowers, the expression was detected in the stigma and filaments of the stamens (Figure 11C). In the stigma, GUS staining appeared as “dark spots” along the stigma, which are likely to be ovules (Figure 11C). In roots, *LACS1* was expressed in the primary (Figure 11I) and lateral roots (Figure 11J), but not in the root tips (Figure 11H). Along the primary root, *LACS1*

was strongly detected in the elongation zone, above the root tips (Figure 11I), but not in the maturation zone (Figure 11G). In the elongation zone, the expression was detected in all the cell layers, and was not epidermal-specific as in the stem (Figure 11I).

LACS2 showed gene expression along the length of the stem as for *LACS1* (Figure 12A), but it was not epidermal specific. *LACS2* promoter-driven GUS activity was also detected in internal cell layers of stem, but accumulated to higher levels in the epidermal layer (Figure 12B), indicating additional functions for *LACS2* in internal tissues. In other aerial organs, *LACS2* was expressed in leaves (Figure 12D), flowers (sepals, petals, stamens, filaments and carpel; Figure 12C), and silique peels (Figure 12E). *LACS2* expression was not detected in seeds (Figure 12F). In roots, expression of *LACS2* was detected in an internal cell layer, likely the endodermal layer (Figure 12G). It was not detected in the root epidermis (Figure 12G). *LACS2* was detected in the root cap and elongation zone (Figure 11G, H), but not in the meristematic and maturation zones (Figure 12I, J).

Similar to *LACS2*, *LACS3* was also expressed along the entire length of stem (Figure 13A), with expression in the internal layers, but with higher expression in the epidermal layer (Figure 13B). *LACS3* was also expressed in leaves (Figure 13D). The expression of *LACS3* in the flower was similar to *LACS1*; the staining appeared in filaments of the stamen and as dark spots, which are likely to be the ovules, in the carpel (Figure 13C). *LACS3* has a distinct expression pattern in silique compared to *LACS1* and -2. *LACS3* expression was detected in silique peel (Figure 13E) as for *LACS2*. However, unlike *LACS1* and *LACS2*, *LACS3*

expression was detected in matured seed (Figure 13F). The expression in roots was detected in the root cap, meristematic and maturation zones of root in an internal layer, likely the endodermal layer (Figure 13G, H). It was not expressed in the epidermal layer (Figure 13G, I). Lateral roots showed a similar expression profile as the primary root (Figure 13J).

Figure 11 Expression of GUS reporter gene driven by *LACS1* promoter

- (A) Stem: expression is along the entire stem
- (B) Cross section of stem: epidermal-specific expression
- (C) Flower: expressed in ovules within carpel
- (D) Leaf: generally low expression level in pavement cells and high level in trichomes (inset)
- (E) Silique: no expression detected
- (F) Seed: no expression detected
- (G) Root: 11-day old matured section; no expression detected
- (H) Root tip: expression is detected in the meristematic and elongation zone
- (I) Root: 11-day old elongation section, expression is detected in both the internal tissue and epidermal layer
- (J) Lateral root: expression is similar to central root

Plants were grown in a semi-controlled growth room (Nesbitt Biology Building, Room 113A) with a continuous light photoperiod at approximately 22°C which was controlled by a Mitsubishi air conditioner.

Figure 12 Expression of GUS reporter gene driven by *LACS2* promoter

- (A) Stem: expression is along the entire stem
- (B) Cross section of stem: expression in all cell layers but highest in epidermal layer
- (C) Flower: expression is detected in all parts with lower expression in petals
- (D) Leaf: high expression level all over the pavement cells and trichomes (inset)
- (E) Silique: expression is detected on the peel
- (F) Seed: no expression detected
- (G) Root: 11-day old matured section; expression is detected only in an internal cell layer
- (H) Root tip: expression is detected in the root cap and meristem
- (I) Root: 11-day old matured section with a developing lateral root; high level of expression is detected in the young lateral root, but not detected in the matured central roots flanking the young lateral root
- (J) Matured lateral root and primary roots: high level of expression in the young lateral roots is reduced at this phase; no expression is detected in the mature primary root.

Plants were grown in a semi-controlled growth room (Nesbitt Biology Building, Room 113A) with a continuous light photoperiod at approximately 22°C which was controlled by a Mitsubishi air conditioner.

Figure 13 Expression of GUS reporter gene driven by *LACS3* promoter

- (A) Stem: expression is along the entire stem
- (B) Cross section of stem: expression in all cell layers but highest in epidermal layer
- (C) Flower: expressed in filaments and ovules within the carpel, with 'dark spot' along the carpel
- (D) Leaf: expression level all over the leaf tissue but lower than observed for *LACS2*, and extremely low expression in trichome (inset)
- (E) Silique: expression is detected on the peel
- (F) Seed: expression is detected on the surface
- (G) Root: 11-day old matured section, expression is detected only in an internal cell layer
- (H) Root tip: expression is detected in the root cap and meristem
- (I) Root: 11-day old elongation section of central root; no expression is detected
- (J) Matured lateral root 11-day old: expression profile is similar to primary root; expression is detected in matured section, root tip and meristematic zone, but not in elongation region

Plants were grown in a semi-controlled growth room (Nesbitt Biology Building, Room 113A) with a continuous light photoperiod at approximately 22°C which was controlled by a Mitsubishi air conditioner.

3.7 Substrate specific of LACS1, -2 and -3 enzymes

To determine the substrate specificity of LACS1, -2 and -3 enzymes. cDNAs of each gene were cloned into the pET28a vector for expression in *E. coli*. The open reading frames were fused with a histidine tag for protein purification and T7 epitope for protein detection by Western blotting. Initial experiments indicated that the background levels of acyl-CoA synthetase activity in wild-type *E. coli* (BL21(DE3)) were too high (data not shown). Therefore, we obtained the K27 strain of *E. coli*, which has a mutation in the *fadD* gene encoding the prokaryotic acyl-CoA synthetase. For heterologous expression of Arabidopsis LACS proteins, it was necessary to genetically modify the K27 strain to contain T7 RNA polymerase under the control of the *lacUV5* promoter (DE3 lysogen). This was done using a commercial kit available from Novagen. The expression of T7 RNA polymerase was detected by Western blot using antibodies against T7 RNA polymerase (Figure 14). A high expressing lysogen was chosen as the expression host for analysis of LACS activity. After purification by nickel chromatography from *E. coli* lysates, the enzyme activities were analyzed by a colourimetric assay (Ichihara et al., 1991) using saturated free fatty acids with even chain lengths from C16 to C30.

The expression of LACS1, -2 and -3 proteins were detected by Coomassie Brilliant Blue staining of the SDS-PAGE gel and Western blot using antibody against the T7-tag (Figure 15). As shown in Figure 16, LACS1 showed a significant higher activity towards C30 free fatty acid, the precursor of major wax components C29 alkane, C29 secondary alcohol and C29 ketone, than did

LACS2 and LACS3. LACS1 also shows high activity towards C16 and C20 free fatty acids while relatively lower activity on C18, C22, C24, C26, C28 free fatty acids. LACS2 shows a similar activity towards all the substrates. LACS3 has the highest activity towards C16 free fatty acids. As the substrate chain lengths increased, LACS3 showed a similar, relatively lower activities towards the C18, C20, C22 and C24 free fatty acids and even lower when the chain length reach to C26 and C30. The lowest activity of LACS3 appears on the C28 free fatty acids.

Figure 14 Western-blot detection of T7 RNA polymerase in K27(DE3) *E. coli*

HMS174 is the control strain provided by the kit, which is able to express T7 RNA polymerase upon induction with IPTG. Numbers of 11, 20, 23 and 27 refer to independent isolates of the candidate lysogen lines; '+' means the induction of expression of T7 RNA polymerase by IPTG and '-' means no IPTG induction; arrow shows the 99kDa T7 RNA polymerase. T7 RNA polymerase was detected in all of the induction lanes and absent from no induction lanes. Lower molecular weight bands were observed, which may be degradation products. No.27 was chosen for the subsequent expression host of LACS enzymes.

Ladder kDa	HMS 174	No. 11	No. 20	No. 23	No. 27
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Figure 15 Detection of LACS1, -2 and -3 in K27(DE3) *E. coli*

(A) Coomassie Brilliant Blue staining of protein SDS-PAGE showing the induced expression of LACS1, -2 and -3. Arrows shows the expected bands representing LACS1, -2 and -3 enzymes while no band was present at the same location of the pET18a empty vector control.

(B) Western-blotting showing the expression of the enzymes was detected in LACS1, -2 and -3 lanes using T7-tag antibody, while no enzyme was detected in the empty vector control pET28a.

pET28a refers to the empty vector control which is transformed into competent K27(DE3) and induced by IPTG, which was also done for cells containing pET28a with LACS1, -2 and -3.

A

B

Figure 16 Comparison of substrate specificities of LACS1, -2 and -3

Substrates of saturated free fatty acids with even chain length from C16 to C30 are shown on X-axis and enzyme activity determined by colourimetric assay (Ichihara et al., 1991) shown on Y-axis (unit: nmol/min/mg protein). Values are averages of 3 duplicate assays (n=3); error bars represent standard deviation.

Chapter 4 Discussion and Future Research

Arabidopsis mutants with altered wax load and composition have a 'glossy' appearance compared to wild-type. Such mutants are named *eceriferum* (*cer*). More than 30 *cer* loci have been indentified (Koornneef et al. 1989; Rashotte et al., 2004), but only a fraction of these have been cloned (Table 2). In this study, I have indentified the *CER8* gene as *LACS1* (*At2g47240*) and based on my characterization propose models for the role of *CER8/LACS1* in cuticular wax biosynthesis.

The identification of *CER8* gene was facilitated by screening mutant plants that contain T-DNA insertions in genes that were physically close to the genetic location of *cer8*. The evidence supporting the conclusion that *CER8* is allelic to *LACS1* are: (1) plants carrying T-DNA insertions in *LACS1* (*At2g47240*) have a typical 'glossy' phenotype as the original *cer8-1* mutant and complementation crosses of *cer8-1* and *cer8-2/-3* failed to rescue the mutant phenotype indicating allelism; (2) a 2bp deletion was identified in the *LACS1* gene of *cer8-1* mutant determined by sequencing and (3) RT-PCR showed that the transcript of *LACS1* is absent from all three *cer8* mutant alleles. Although molecular complementation was not done in this study, the evidence shown here is adequate to support the conclusion that *CER8* is *LACS1*.

Sequencing of *At2g47240* in *cer8-1* revealed a 2bp deletion in the 8th exon that gives rise to a premature in-frame stop codon (PTC) in the same exon. PTCs were also found in the T-DNA segments inserted in the *At2g47240* gene of *cer8-2* and *cer8-3* (sequence of T-DNA available at website of SALK T-DNA Express:

<http://signal.salk.edu/cgi-bin/tdnaexpress>). The presence of PTCs indicated that absence of transcript could be the result of nonsense-mediated mRNA decay (NMD). NMD is an evolutionarily conserved mechanism of mRNA surveillance to recognize and eliminate aberrant mRNAs containing PTC, thereby preventing the accumulation of potentially deleterious truncated proteins (Isken and Maquat, 2007). This mechanism is well-characterized in mammals and yeast (Nagy and Maquat, 1998; Gatfield et al, 2003; Amrani et al, 2004; Longman et al, 2007), but poorly understood in plants (Kerényi et al., 2008). Although not extensively characterized in plants, it was shown that PTCs localized >50 bps upstream of an exon-exon junction are able to elicit NMD (Nagy and Maquat, 1998), which is just the case of *cer8-1* (PTC is localized at 85 bps upstream the 8th-9th exons junction). Therefore, the absence of the *LACS1* transcript in the three *cer8* alleles was probably due to the NMD in plants. The absence of transcript also indicates that these are null alleles.

In order to begin characterization of the *in vivo* function of *CER8/LACS1*, the *cer8* mutant phenotype was analyzed in detail by SEM and gas chromatography. The wax chemical profile, both of leaves and stems, of the *cer8-1* allele was previously investigated by Jenks et al. (1995). In terms of total wax load, *cer8* mutants have 80.46% of wild-type levels on rosette leaves and 53.57% of wild-type levels on stems (Jenks et al., 1995). The decrease of total stem wax load is due to decreases of the major wax components, C29 alkanes, C29 ketones and C29 secondary alcohols. Conversely, a minor wax component, C30 free fatty acid, is increased by 60 times on stems. In the cases of these major wax

components, *cer8* stem wax has decreases of 66.53% for C29 alkanes, 69.11% for C29 ketones and 59.71% for C29 secondary alcohols respectively (calculated based on data of Jenks et al., 1995). Alkanes, secondary alcohols and ketones with chain lengths longer than C30 also show decrease on *cer8* mutant, but due to their small proportion in the wild type plant wax, their contribution to the total wax load decrease is not that significant. The *cer8* leaf wax profile also showed a similar decreased trend as stem wax. However, the predominant chain length of alkane on leaves is C31 and this component is significantly decreased. These decreases indicate that *LACS1* is important for generating acyl-CoA pools of 30 carbons and longer. The GC data presented here is consistent with that reported by Jenks and co-workers (1995), in terms of distribution while the absolute amounts were lower. This may be due to different growing conditions of the plants and different experimental conditions. For example, the solvent to dissolve cuticular wax used by Jenks et al was hexane while chloroform was used in this study. Growing conditions could have a major influence since it was reported that wax biosynthesis is regulated by environmental factors such as temperature, humidity and illumination intensity (Eignebröde and Espelie, 1995; Post-Beittenmiller, 1996; Hooker et al., 2002). The changes of cuticular wax composition of *cer8-2* and *cer8-3* alleles were consistent with *cer8-1*, which further verify the conclusion that *LACS1* is allelic to *CER8*. The dramatic increase of C30 free fatty acid is consistent with the biochemical assay results showing that *LACS1* enzyme has higher activity towards C30 free fatty acid than free fatty acids with shorter chain lengths. Most of the wax components with chain lengths

shorter than C30 are not significantly influenced in *cer8* alleles, indicating that other *LACS*s, such as *LACS3*, may be important for generating the C24-C28 acyl-CoAs. The wax composition of *lacs3* mutant should be carefully characterized in future research, and characterization of *lacs1/lacs3* double mutant may be particularly revealing. The wax load and composition is not significantly affected in the *lacs2* mutant (Schnurr et al., 2004).

Another interesting observation of the *cer8* wax profile is the increase of C28 and C30 aldehydes. Based on the current proposed biosynthetic pathway, aldehydes are derived from acyl-CoA and they serve as precursors of alkane production (Figure 4; Kolattukudy et al., 1966, 1967). Therefore, aldehydes would be expected to be decreased in *cer8* mutants, since increased accumulation of C30 free fatty acids would be accompanied by decreased levels of C30 acyl-CoAs. This suggests that the biosynthesis of aldehydes may be from free fatty acids and in a pathway distinct from the production of alkanes, which require acyl-CoA as substrate. *Cer1* and *cer22* mutants also exhibit reductions in alkanes, but increased levels of aldehydes (Jenks et al., 1995; Rashotte et al., 2004). A common feature between *cer1*, *cer8* and *cer22* is that the increased accumulation of C30 aldehyde is accompanied by a decreased level of C30 primary alcohols (Jenks et al., 1995; Rashotte et al., 2004; this study). Another *cer* mutant, *cer3*, carries reciprocal wax changes with decreased levels of C30 aldehyde and increased levels of C30 primary alcohols (Jenks et al., 1995). All four of these *cer* mutants have decreased levels of other alkane forming pathway products (Jenks et al., 1995; Rashotte et al., 2004; this study). These data imply that

biosynthesis of aldehydes is possibly via a pathway distinct from alkane formation. This would be different than the current paradigm of acyl-CoAs being reduced to aldehyde followed by decarbonylation to alkanes. The unknown aldehyde-forming reductase would use free fatty acid, rather than acyl-CoA as substrate to generate aldehyde. Moreover, null mutants of the Arabidopsis alcohol forming reductase, *CER4*, do not show significant alterations in the levels of C30 aldehydes and C30 free fatty acids (Rowland et al., 2006), indicating that biosynthesis of aldehyde is distinct from the alcohol forming pathway.

The gene expression profile of *LACS1* was examined by fusing the promoter with the GUS reporter gene, and also compared with two other *LACS* genes, *LACS2* and *LACS3*. The gene expression profiles of *LACS2* and *LACS3* were also examined because they were determined to be highly up-regulated in the epidermal layer as determined by DNA microarray data (Suh et al., 2005). Consistent with the microarray data of Suh et al. (2005), the expression of *LACS1* on stem was exclusive to the epidermal layer; however, *LACS2* and *LACS3* were also expressed in internal tissues, although epidermal expression was higher than internal tissues. Together with its higher enzyme activity toward C30 free fatty acids than that of *LACS2* and *LACS3*, it is likely that *LACS1* serves as the major wax-associated *LACS* on the stem. However, *LACS2* and/or *LACS3* could also contribute to activation of C30 fatty acids in epidermal cells since the C29 alkanes, C29 secondary alcohols and C29 ketones are not completely eliminated on the *cer8/lacs1* mutant. Biochemical analysis showed that *LACS2* and -3 have activity towards C30 free fatty acids, although much lower than that of *LACS1*. To verify

the possible redundant roles between *LACS1*, *LACS2* and *LACS3*, double mutants of *lacs1/lacs2*, *lacs1/lacs3* and a triple mutant *lacs1/lacs2/lacs3* should be generated by crossing single mutant lines and the chemical composition of wax of these double/triple mutant plants determined. If the double/triple mutants show a more severe decrease of C29 alkanes, C29 secondary alcohols and C29 ketones and higher increase of C30 free fatty acids than *lacs1/cer8* single mutant, the redundant roles of *LACS1*, -2 and/or -3 could be demonstrated. Otherwise, *LACS2* and *LACS3* may have other functions. Indeed, *LACS2* has been characterized to be important for activation of fatty acids for cutin biosynthesis (Schnurr et al., 2004)

All three *LACS* were also expressed in leaf, but *LACS1* were highly restricted to trichomes while *LACS2* and *LACS3* were expressed more ubiquitously, which implies different roles of *LACS1*, -2 and -3 in leaf cuticular wax biosynthesis. As reported by Jenks et al., (1995), leaf cuticular wax composition in *cer8-1* is different from stem cuticular wax. The dominant component of leaf cuticular wax is C31 alkane, with lesser amount of C29 alkanes; the C29 secondary alcohols and C29 ketones are present in trace amounts in leaf cuticular wax (Jenks et al., 1995). Moreover, the decrease of leaf cuticular wax composition on *cer8-1* is not as dramatic as the decrease of the stem cuticular wax (Jenks et al., 1995). Together with the limited gene expression levels of *LACS1* on leaf, *LACS1* appears not to play as important a role in leaf cuticular wax biosynthesis as it does for stem wax biosynthesis. Other *LACS* genes, for example *LACS3*, may be more important for leaf cuticular wax biosynthesis. Therefore, in future research, leaf cuticular wax

composition should be examined in single mutants of *lacs1*, -2 and -3, as well as in the double and triple mutants. The other possibility that needs to be considered is the potential role of LACS1 in cutin biosynthesis. LACS2 has been shown previously to be important for cutin biosynthesis (Schnurr et al., 2004). The biochemical assay shown here indicate that LACS1 and LACS3 have high activity towards the C16 and C18 free fatty acid, the characteristic chain length of cutin monomers. Therefore, it is reasonable to investigate if they are also involved in the cutin biosynthesis. However, cutin monomers are modified with ω -carboxylic groups and in chain hydroxyl groups that could affect specificity. LACS2 has been shown to have higher activity for 16-hydroxy palmitate than palmitate, consistent with its specific role in cutin biosynthesis (Schnurr et al., 2004). However, TEM images indicated that the cutin layer is not completely disrupted in *lacs2*, which further highlights the possibility that other LACS, including LACS1, may contribute to cutin biosynthesis. In future research, cutin structure and composition of *lacs1* and *lacs3* single mutants should be determined to check if there are any cutin defects. Considering the possible functional redundancy, it is also necessary to check the integrity of the cutin biopolyester structure and chemical composition of double and triple mutants. Other experiments to check the cuticle structure, such as chlorophyll leaching (Schnurr et al., 2004) should be also performed on the single, double and triple mutants. Together with the cuticular wax data of leaf and stem from all these mutants, we will then have clearer understanding of the functions of LACS1, -2 and -3, as well as the different contributions of cutin and cuticular wax to the physiological functions of the plant cuticle.

All three *LACS*s were also expressed in root, although with different expression patterns. *LACS1* was expressed in the elongation region and appeared to be in all cell layers. I did not perform a cross section of roots, but analysis of digital *in situ* data indicated that *LACS1* is expressed in all cell layers of roots, from inner stele to the outer lateral root cap (Birnbaum et al, 2003). This expression pattern is very similar to the previous report of *CER4* (Rowland et al., 2006). Several other genes associated with cuticular wax biosynthesis are also be detected in roots (Birnbaum et al., 2003 online supplemental data), including *CER1* (Aarts et al., 1995; Hannoufa et al., 1993; McNevin et al, 1993); *CER2* (Negruk et al., 1996; Xia et al., 1996 & 1997; Lai et al., 2007), *CER3* (Kurata et al., 2003; Rowland et al., 2007), *CER5* (Pighin et al., 2004), *CER10* (Zheng et al., 2005), *WBC11* (Bird et al., 2007), *WIN1/SHN1* (Bround et al., 2004), *MAH1* (Greer et al., 2007), *FATB* (Bonaventure et al., 2003) and *WSD1* (Li et al., 2008.). Moreover, *LACS2* and *LACS3* also showed expression in roots, although restricted to the endodermal layer. This suggests that *LACS1*, -2 and -3 may also involved in the biosynthesis of other lipid materials in roots. One possibility is suberin. Suberin is deposited in various inner and outer tissues at specific locations during plant growth (Kolattukudy, 2001; Bernards, 2002; Nawrath, 2002; Kunst et al., 2005; Stark and Tian, 2006); for example, in the seed coat (Espelie et al., 1980; Ryser and Holloway, 1985; Moire et al., 1999) and root and stem endodermis (Espelie and Kolattukudy, 1979a; Zeier et al., 1999; Enstone et al., 2003). Moreover, the waxes associated with suberin also contain components commonly found in cuticular wax, such as very-long chain primary alcohols

(Espelie et al., 1980), and participate in the important role of suberin in constituting the major diffusion barrier formed during wound healing (Soliday et al., 1979). The overlap in chemical composition of suberin-associated waxes may explain why cuticular wax genes are also expressed in the roots. *LACS2* had been previously identified to have a substrate preference for a common suberin monomer precursor, ω -hydroxyacids (Schnurr et al., 2004). Together with its expression in the endodermal layer in roots, this indicates that *LACS2* may play a role in suberin biosynthesis. The expression of *LACS3*, which is also restricted to the endodermis where suberin is deposited, also implies a possible role in suberin biosynthesis, while the more broad expression of *LACS1* in roots indicates that it may be involved in root lipid metabolism other than for suberin associated lipids. Current data cannot predict the roles of *LACS1*, *LACS2* and *LACS3* in suberin biosynthesis or other lipid metabolism in roots. Future research will require the determination of suberin composition of *lacs1*, *lacs2* and *lacs3* mutants to check if any suberin associated phenotype is observed.

LACS1 and *LACS3* exhibited a very similar expression pattern in flowers (Figure 11C and 13C), suggesting that they may have redundant functions in generating fatty acyl-CoA pools of floral tissues. *LACS1* expressed in ovules but not in seeds, while *LACS3* is expressed in mature seeds, which may suggest that *LACS1* is only involved in the initial phase of seed development while *LACS3* is involved throughout the seed development process.

The gene expression patterns observed by the GUS assay were generally consistent with previously reported DNA microarray data examining gene

expression patterns in developing tissues of *Arabidopsis thaliana* (Schmid et al., 2005). There were some exceptions, however, such as in floral organs where the DNA microarray data indicated that *LACS1* was expressed at comparable levels in petals and stamens; *LACS1* was also detected in seeds and expression in roots were very low (Schmid et al., 2005). However, no GUS activity was observed in petals and seeds in the *LACS1* promoter:GUS lines, while the staining in roots was very strong (Figure 11). *LACS2* was also found to be expressed in seeds according to the DNA microarray data (Schmid et al., 2005), but GUS staining was not observed in seeds (Figure 12). *LACS3* showed a higher expression levels on petals than carpels in the DNA microarray experiments (Schmid et al., 2005), but GUS staining was not observed in petals (Figure 13). The developmental phases of the organs analyzed in the experiments may be important for the timing of gene expression and these differ slightly between these experiments, as do the growth conditions. It is important to verify that the promoter region used in this assay is functional by molecular complementation. In this experiment, the promoter regions would be fused to the LACS cDNAs and the constructs transformed into the corresponding mutant backgrounds to test for complementation. Other experiments, such as *in situ* hybridization should also be conducted to verify the gene expression profile determined by analysis of promoter:GUS lines.

The enzyme activities of LACS1, -2 and -3 were previously studied using saturated and unsaturated fatty acid substrates with chain lengths up to C20 (Shockey et al., 2002). In the cases of the commonly used substrates C16:0 and C18:0 free fatty acids, the data shown in this study is consistent with Shockey's

report. As shown in Figure 16, both LACS1 and LACS3 show higher activity towards C16 and C18 free fatty acids than LACS2. Therefore, LACS2 is likely to be specific for cutin biosynthesis while LACS1 and/or LACS3 may be involved in partitioning C16:0 and C18:0 free fatty acids into fatty acid elongation and wax biosynthesis at the plastid.

LACS1 has a significantly higher activity towards C30 free fatty acids than for lower chain lengths. Conversely, LACS2 and LACS3 have relatively low activity for C30 free fatty acid. The high activity for C30 is consistent with the *lacs1* phenotype, with significant increases in C30 free fatty acid and decreases in products that depend on a high C30 fatty acyl-CoA pool for synthesis. However, there are no big differences in substrate specificity between the three enzymes below C30. This implies that factors other than substrate specificity may determine the different functions of LACS enzymes. For example, the subcellular location of these enzymes may be different. A plastid-localized LACS, LACS9, was proposed to be the major chloroplast LACS (Schnurr et al., 2002), although it did not show particular substrate preferences (Shockey et al., 2002). Therefore, the subcellular location of LACS1, -2 and -3 should be examined by, for example, fusing the proteins to green fluorescent protein (GFP) and examining protein location by confocal microscopy. For example, if LACS3 is localized in the plastid and LACS1 accumulates in ER, it is possible that LACS3 is involved in the CoA ligation and exportation of fatty acids out of plastid while LACS1 is specific to maintain the elongated acyl-CoA pools in the ER important for subsequent modification to wax components. It has also recently been shown that different

DGAT enzymes important for triacylglycerol production reside in different subdomains of the ER (Shockey et al., 2006), and this may be the case for LACS enzymes related to cuticle development.

Another key experiment is to examine the *in planta* acyl-CoA levels by removing the plant epidermal layer using either physical peeling (Suh, et al., 2005) or laser capture microdissection (LCM). It is reasonable to expect a reduced level of acyl-CoA in the *cer8* mutants compared with wild-type plant.

Here, I have demonstrated that *LACS1* is involved in wax biosynthesis and important for synthesis of the C30 fatty acyl-CoA pool. However, this is not expected to be required as during elongation the CoA moiety of the fatty acyl chains is not expected to be lost. As shown in Figure 3, when the C18 free fatty acids are synthesized in the plastid as acyl-ACP, the ACP (acyl carrier protein) groups are cleaved by acyl-ACP thioesterase (FATB). The C18 free fatty acids are transported out of the plastid and then a plastid-associated LACS, possibly *LACS9*, catalyzes the formation of C16:0 and C18:0-CoA. The fatty acyl-CoA are then transported to the endoplasmic reticulum and further elongated to various chain lengths, ranging from C24 to C34, by fatty acid elongases (FAE) (von Wettstein-Knowles, 1982; Kunst & Samuel, 2003; Zheng et al., 2005). The results are fatty acyl-CoAs ranging in chain length from C24 to C34. The elongation, as described in section 1.3.2, is processed by condensation of fatty acyl-CoA with malonyl-CoA, which is followed by reduction of β -ketoacyl-CoA, dehydration of β -hydroxyacyl-CoA and reduction of trans- Δ^2 -enoyl-CoA (Fehling and Mukherjee, 1991), generating an acyl-CoA that is elongated by two carbons. There is no loss

of CoA group in the final products. Therefore, it would seem that wax biosynthesis does not require a LACS enzyme, since the CoA group is not cleaved from the acyl-CoA during elongation. Therefore, in this context, what is the exact role of *LACS1* in the plant cuticular wax biosynthesis?

The observation that a knockout of *LACS1* results in cuticular wax disruption leads to my proposal that *LACS1* is a regulator of wax biosynthesis (Figure 17). In wild-type cuticular wax mixtures, C30 free fatty acids exist as a minor component (1%, Table 4). Since very-long-chain fatty acids are elongated as acyl-CoA, an acyl-CoA thioesterase is likely involved in wax biosynthesis to cleave the CoA group from acyl-CoA to generate free fatty acids. The thioesterase and *LACS1* may work together to maintain balanced levels of free fatty acid and relevant acyl-CoAs, which could ensure the normal progress of wax biosynthesis. As shown in Figure 17, when the chain length of acyl-CoA reaches C30:0, an acyl-CoA thioesterase may remove the CoA group and release the free fatty acids. However, since the FAE enzymes and wax modification enzymes prefer acyl-CoA as their substrates, this acyl-CoA thioesterase actually terminates the elongation of the carbon chain length and the production of other wax components. In this way, *LACS1* is required to add the CoA groups back to the free fatty acids to re-generate acyl-CoAs, which promotes the process of the subsequent chain length elongation and very-long-chain acyl-CoA modification.

This model could also explain the *lacs1/cer8* mutant phenotype. In the *lacs1/cer8* mutant, the CoA group cannot be added back to the free fatty acid. In this way, the thioesterase removes the CoA group from the C30 acyl-CoA

elongation intermediate, generating C30 free fatty acid. As a result, the free fatty acids would increase, while other wax components that depend on C30 acyl-CoAs would decrease. Due to the redundant function with other *LACS* genes, such as potentially *LACS3*, the level of acyl-CoA is not completely eliminated. A similar mechanism exists in plastid, where the initial elongation of C2 to C18 occurs. When the chain length of acyl-ACP reaches C18, FATB removes the ACP group thus terminating the elongation process. The substrate specificity of FATB is the major determinant of chain length and level of saturated fatty acids in the plastid (Salas and Ohlrogge, 2002). The C18 free fatty acids are then transported out of the plastid and CoA is added by a plastid-associated *LACS* enzyme for further lipid metabolism, including elongation at endoplasmic reticulum. It is reasonable to deduce that a similar regulation mechanism also exists in the downstream phase of the wax biosynthetic pathways, since fatty acid elongation in the ER share a similar 4-step reaction as the chain length elongation in the plastid.

However, another question arises regarding this proposed model. Since C30 free fatty occupies such a small proportion in the wild-type wax mixture, why does the plant develop such an acyl-CoA thioesterase/*LACS* mechanism to generate C30 free fatty acids? This repeating dethioesterification/thioesterification seems to be energy-wasting. Therefore, C30 free fatty acids may have other important functions rather than just as a minor wax mixture component. One may be for production of cuticular aldehydes as proposed above. Another possibility is that C30 free fatty acid may be able to regulate the expression of lipid metabolism-related genes (Figure 18).

Figure 17 LACS enzyme function in wax biosynthesis and proposed regulation model

(A) Two-step reaction catalyzed by formation of acyl-CoA by LACS enzyme (Groot et al., 1976).

(B) Proposed model of interplay between *LACS1/CER8* activity and thioesterase activity in cuticular wax biosynthesis.

When the chain lengths of acyl-CoA reach C30, the CoA group is removed by an unknown acyl-CoA thioesterase to form C30 free fatty acids, part of which will be secreted out as a minor wax component and possibly used in other reactions, such as production of aldehydes. *LACS1/CER8* is able to add the CoA group back to re-generate acyl-CoA, which will be further elongated to longer chain lengths or modified via either the alcohol-forming pathway or the alkane forming pathway.

A knockout mutation of *LACS1/CER8* will reduce the formation of acyl-CoA, which will results in the accumulation of C30 free fatty acid and a shortage of acyl-CoA substrate for further modification and elongation. This is consistent with the phenotype observed in the *cer8* mutant, which is reported in this thesis. A knockout mutation of the putative thioesterase would be predicted to reduce free fatty acids levels in the cuticle and possible increase products of alkane and alcohol-forming pathways.

A

B

As has been demonstrated in animals, in the cytosol, HNF-4 α and PPARs are both nuclear receptors (transcription factors) that bind to the direct repeat 1 (DR1) motif of gene promoter sequences to regulate gene expression (see Figure 18A) (Desvergne et al., 1999; Hertz et al., 1998; Elholm et al., 2001; Murakami et al., 2001). Most of the target genes are involved in lipid metabolism, adipocyte differentiation, apolipoproteins, coagulation factors, HNF-1 and other processes (Schoonjans et al., 1996; Desvergne et al., 1999; Michalik et al., 1999;). It has been demonstrated that acyl-CoA acts as a positive ligand for HNF-4 α (Hertz et al., 1998) while antagonistic for PPARs (Elholm et al., 2001; Murakami et al., 2001). In contrast, free fatty acid is a positive ligand for PPARs. The different effects of acyl-CoA and free fatty acid on HNF-4 α and PPARs indicates that the level of acyl-CoA/free fatty acid is able to regulate gene expression via these two pathways. It is possible that a cytosolic acyl-CoA thioesterase in conjunction with an acyl-CoA synthetase is involved in the regulation of wax biosynthetic gene expression via maintaining balanced levels of C30 fatty acyl-CoA/free fatty acid (Figure 18B).

In summary, I propose that an unknown acyl-CoA thioesterase and LACS1 are able to maintain the level of acyl-CoAs/free fatty acids, which are able to indirectly regulate the expression of lipid metabolism genes via coordinate transcription factors, thus initiating differing lipid metabolism in different cell types by generating C30 acyl-CoAs. To test this functional mode, a number of experiments could be done. In one aspect, characterization of the unknown acyl-CoA thioesterase could directly test this model. Candidate genes have been

identified by searching the Arabidopsis Information Resource (TAIR, <http://www.arabidopsis.org/index.jsp>) database using the NCBI Basic Local Alignment Search Tool (BLAST) 2.2.8 program. First, the *E. coli* acyl-CoA thioesterase-II TesB protein sequence (Naggert et al., 1991) was used to perform such database searching and there are two putative acyl-thioesterase proteins: At1g01710 and At4g00520, showing 44% and 41% identities to TesB protein sequence. Another acyl-CoA thioesterase protein of type I, *E. coli* TesA, was used to search but no candidate Arabidopsis thioesterases were found. Another mouse acyl-CoA thioesterase ACT12 (Suematsu et al., 2002), which belongs to a newly identified type of acyl-CoA thioesterase was used for searching the Arabidopsis genome for homologs. Two more putative Arabidopsis acyl-CoA thioesterases were obtained: *At2g30720* and *At5g48370*.

Tilton et al. (2000) previously reported four putative Arabidopsis acyl-CoA thioesterases, ACH1, ACH2, ACH4 and ACH5, which were separated into two families. Thus far, only the identity of ACH2 was reported and the protein function studied (Tilton et al. 2004). This protein sequence is identical to At2g01710. Biochemical characterization of ACH2 showed that it is not involved in β -oxidation, as it is for its yeast and human homologs (Tilton et al., 2004; Hunt et al., 2002; Jones et al., 1999). Taken together, these four putative Arabidopsis thioesterases serve as candidates for the proposed model.

Experiments can thus be designed based on the functional model. First, it can be deduced that a knockout of the unknown acyl-CoA thioesterase will result in the reduced level of C30 free fatty acids. Knockout alleles can be obtained by

Figure 18 Model: Acyl-CoA/free fatty acids regulate gene expression

(A) Acyl-CoA/free fatty acid regulates lipid metabolism by regulating transcription factors HNF-4 α and PPARs in animal cells.

(B) Proposed acyl-CoA/free fatty acid regulation model in plant cells

In plant cells, an acyl-CoA/free fatty acid pool plays an important role in regulating wax metabolism. The chain length distribution of such a pool is regulated by fatty acid elongase (FAE) and the composition (ratio of acyl-CoA and free fatty acids) is under the control of acyl-CoA thioesterase/long chain acyl-CoA synthetase. Acyl-CoA and free fatty acid are able to modulate the activities of transcriptional factors (HNF-4 α -like and PPARs-like) or distinct homologs, that can further regulate wax metabolism.

A

B

screening SALK T-DNA insertion lines. If a knockout of any these four putative acyl-CoA thioesterases have this phenotype, a double knockout of *LACS1* and the thioesterase may give an intermediate phenotype, but regulatory control would be lost and the final phenotype is difficult to predict.

In another aspect, identifying wax-associated transcription factors, the equivalents of HNF-4 α and PPARs, could also support the proposed model. Thus far, two wax-associated transcriptional factors have been reported, *WIN1/SHN1* targeting *CER1*, -2 and -3 and *MYB30* targeting FAE genes (Bround et al., 2004; Raffaele et al., 2008). It is not known how the activities of these two transcriptional factors are regulated. Varieties of methods could be performed to test if their activities are associated with acyl-CoA/free fatty acids, such as sequence comparison with HNF-4 α or PPARs or direct biochemical analysis. Identifying functional homologs of HNF-4 α and PPARs in *Arabidopsis* could also help to verify the model.

Moreover, if the acyl-CoA/free fatty acids are indeed able to regulate the gene expression levels, transcript levels are expected to be altered in the *lacs1/cer8* mutant, which can be easily detected by candidate genes or whole genome DNA microarray analysis.

Besides the model described above, an alternative *in vivo* role of *LACS1* is that it is involved in the activation and mobilization of free fatty acids between ER compartments. In the yeast *Saccharomyces cerevisiae*, the uptake of exogenous fatty acids into cells requires both very-long-chain acyl-CoA synthetase Fat1p and an acyl-CoA synthetase (ACS), either Faa1p or Faa4p (Faergeman et al., 1997;

2001) via a vectorial acylation pathway (Overath et al., 1969). Fat1p is specific for fatty acids longer than C20 (Watkins et al., 1998; Choi et al., 1999; Dirusso et al., 2000), while Faa1p or Faa4p are specific for C8 and C20 fatty acids (Faergeman et al., 2001). This scenario is reminiscent of LACS1 and LACS3. First, LACS1 shows higher activity towards C30 fatty acids and LACS3 shows highest activity towards C16 free fatty acids, representing Fat1p and ACS, respectively. Second, the C16 and C18 fatty acids need to be transported to the ER, although it is not known whether it is transported as acyl-CoA or free fatty acid. The acceptance of C16 and C18 carbon chain into the ER is likely to be a transmembrane transportation into the ER lumen since the final product will be transported to the plasma membrane, possibly via the Golgi apparatus. Therefore, another possibility is that LACS1 and/or LACS3 may be involved in the transportation and activation of fatty acids leading to waxes through the ER. In yeast, Fat1p and ACS form a physical complex to perform the transportation function (Zou et al., 2003). Therefore, the same experiments, split ubiquitin yeast two-hybrid system and co-immunoprecipitation can be applied on LACS1 and LACS3 to examine if they also have a similar physical interaction. GFP-fusions to identify the precise subcellular localization of LACS1 and LACS3 may also help to verify this functional model.

Another important point in future research derived from my findings in this study, is that the biosynthesis of aldehyde may be derived from free fatty acids in a separate pathway from the alkane-forming pathway, in contrast to previous models. Therefore, identification of the putative aldehyde-forming reductase

would generate precious information regarding wax biosynthesis. The results of such a study would make a key contribution to understanding that aspect of wax biosynthesis, which is currently the least understood.

Appendix A Constructs for overexpression of *LACS1*

Construction of transgenic overexpression lines is a commonly used method to study gene function. Here, I made plant binary constructs that contain *LACS1* cDNA under the control of the cauliflower mosaic virus 35S promoter or the strong epidermal-specific *CER6* promoter to generate overexpression lines. Due to time limitations, I generated the constructs but was not able to characterize the transgenic lines. The experimental procedure for plasmid construction and plant transformation is reported here.

Construction of recombinant plasmid

LACS1 cDNA was amplified from clone U14264 (ordered from ABRC) using primers At2g47240_gene_FoxbaI (5'-GACTCTAGAGCTCCTAGCTGGCTATTTT-3') and At2g47240_gene_RevSacl (5'-GACGAGCTCAGTGGTAGTTACGTGGATTTTGG-3') and proof reading DNA polymerase PRIME Taq DNA Polymerase (Bioshop Canada, Burlington, ON). The *LACS1* fragment and the vectors pBAR_CER6pro::SHN1 (kindly generated by undergraduate student Catherine Chung of the Rowland lab), pVKH18 (Zheng et al., 2005) (contains 35S promoter with transcription terminator sequence) were digested using restriction enzymes *Xba*I and *Sac*I. The *LACS1* fragment was ligated together with each binary vector using T4 DNA ligase (Bioshop Canada, Burlington, ON) to generate recombinant plasmids: pBAR_CER6pro::*LACS1* and pVKH18_35Spro::*LACS1*. The ligation mixtures were transformed into competent DH5 α *E. coli* and colonies containing recombinant plasmid were screened by PCR. After purification from *E. coli*, *LACS1* cDNA insert was amplified using the primers described above and

sequenced to verify that no mutations were introduced during the PCR reaction. The recombinant plasmids were subsequently transformed into Col-WT, *cer8-1* and *cer8-2* plants via *Agrobacterium*-mediated transformation using the floral dipping method (Clough and Bent, 1998).

Screening of successful transformants

Transformants of pVKH18_35Spro::LACS1 were screened on normal AT agarose plates supplemented with 20 µg/ml hygromycin B (Bioshop Canada, Burlington, ON). Successful transformants will be transplanted to soil. Transformants of pBAR_CER6pro::LACS1 were screened on soil by spraying with BASTA herbicide (Farnam Companies, Inc, Phoenix AZ).

Phenotype analysis and predicted results

Some results may be predicted based on current knowledge, largely generated in this study. First, the glossy phenotypes of *cer8-1* and *cer8-2* should be rescued with both of the overexpression constructs. GC results of *cer8-1* and *cer8-2* overexpression lines should have wild-type levels of cuticula wax, or at least significantly higher levels than in *cer8-1* and *cer8-2* mutants. The GC results of Col-WT overexpression lines may shows a reduced level of C30 free fatty acids and increased levels of C29 alkane, C29 secondary alcohols, C29 ketones and C30 primary alcohols due to an increased level of C30 acyl-CoA pool.

Appendix B Recombinant Plasmid Maps

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