Characterization of mutants affecting cuticle formation in *Arabidopsis thaliana*

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Abstract

The cuticle is a lipid-based barrier that coats the surfaces of plant aerial organs. It plays numerous essential functions for plant life, such as prevention of uncontrolled water loss and resistance to pathogen invasion. Cuticular waxes, which are largely derived from C20-C34 very-long-chain fatty acids, are a major component of the cuticle. In *Arabidopsis thaliana*, numerous wax-deficient mutants, termed *eceriferum* (*cer*) mutants, have been reported. I characterized a set of novel wax-deficient mutants and identified new alleles of *cer1*, *cer3*, *cer4*, *cer6*, and *cer19*. The novel *cer6-3* allele had flat, irregularly shaped wax crystals on the stem, as opposed to *cer6* null alleles that completely lacked crystals. Sequencing revealed that *cer6-3* has a point mutation that changes alanine to valine at amino acid position 397 of the encoded β -ketoacyl-CoA synthase involved in elongation of C26 acyl chains. The *CER19* gene is uncloned, but wax composition defects in the stems of *cer19* mutants indicated a defect in elongation of C28 acyl chains. The *cer19* mutants were found to have a decrease in leaf cuticular permeability at the seedling stage using a toluidine blue staining assay. I also further characterized the previously identified *cer9-1* mutant and confirmed that it has a major defect in cuticular wax production that affects nearly all wax constituents in leaves and stems. Stem surface imaging by scanning electron microscopy revealed that *cer9-1* had significantly reduced wax crystal density and altered crystal morphology. A major defect in the cuticular permeability of *cer9-1* rosette leaves was revealed using a chlorophyll leaching assay. The gene expression patterns of 10 genes involved in wax and cutin biosynthesis were not significantly affected in *cer9-1*, and thus mis-regulation of these genes likely does not account for the *cer9-1* phenotype. The genomic region containing

the *CER9* gene was narrowed to a 320kbp region on chromosome IV by positional genetic mapping. This is an important step towards the identification and molecular characterization of the *CER9* gene.

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List of Abbreviations

ABC: ATP binding cassette

ABRC: Arabidopsis Biological Resource Center

ACP: acetyl-acyl carrier protein

BSTFA: bis-(trimethylsilyl)trifluoroacetamide

CAPs: cleaved amplified polymorphic sequences

CER: ECERIFERUM

CO: carbon monoxide

dCAPs: dereived cleaved amplified polymorphic sequences

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

FT-IR: fourier-transform infrared

GC: gas chromatography

GC-MS: gas chromatography-mass spectroscopy

GFP: green fluorescent protein

GPAT: glycerol-3-phosphate acyltransferase

HCD: hydroxyacyl-CoA dehydratase n

KAS: ketoacyl-ACP synthase

KCR: β -keto acyl reductase

KCS: β -keto acyl-CoA synthase

LACS: long-chain acyl-CoA synthetase

LCR: LACERATA

LD-RI: Lister and Dean recombinant inbred

LTP: lipid transfer protein

LTPG: glycosylphosphatidylinositol-anchored lipid transfer protein

MAH: mid-chain alkane hydroxylase

NMR: nuclear magnetic resonance

PCR: polymerase chain reaction

PM: plasma membrane

SEM: scanning electron microscopy

SSLP: simple sequence length polymorphism

TB: toluidine blue

VLCFA: very-long-chain fatty acid

WS: wax synthase

WS/DGAT: wax synthase/acyl-CoA diacylglycerol acyl-transferase

Chapter 1 General Introduction

1.1 The plant cuticle

The cuticle is a lipophilic layer that coats the aerial parts of vascular plants. It is synthesized and secreted by epidermal cells. The cuticle structure varies widely among plant species, organ types and their developmental states (Jeffree, 1996, 2006). Despite this variability, all cuticles mainly consist of two types of lipophilic components: 1) cutin, a cross-linked polymer of C16 and C18 chain length fatty acid derivatives, and 2) cuticular wax, a mixture of very-long-chain fatty acids and derivatives that are not polymerized and that can be readily extracted by organic solvents such as chloroform or hexane.

Three distinct layers of the cuticle can be easily identified by transmission electron microscopy (from exterior inwards): epicuticular wax crystalloids, the 'cuticle proper', and the 'cuticular layer' (Kunst and Samuels, 2003). Intracuticular waxes are embedded in the cutin matrix of the cuticle proper, while epicuticular waxes lie above on the outer surface, often in the form of crystals that give the plant a whitish appearance.

The main function of the plant cuticle is to prevent non-stomatal water loss (Kerstiens, 1996; Schreiber *et al.*, 1996). Cutin, owing to the covalent linkages between its monomers, forms the structural backbone of the cuticle (Samuels *et al.*, 2008). The epicuticular wax layer plays important roles in the interaction of the plant with its environment. It repels rainwater, which minimizes the deposition of dust particles, pollen and spores (Holloway, 1969a, b, 1970). The cuticle serves to protect underlying tissues from mechanical damage and from excess levels of ultraviolet radiation (Krauss *et al.*, 1997). It serves as a barrier against bacterial and fungal pathogens (Jenks *et al.*, 1994;

Riederer, 2006) and mediates interactions with insects (Eigenbrode and Espelie, 1995). Furthermore, the cuticle plays pivotal roles in cell-cell interactions, such as preventing fusion of cell walls from adjacent organs during plant development (Sieber *et al.*, 2004).

1.2 Cutin

1.2.1 Chemical composition of cutin

Cutin is the insoluble polymeric component of the cuticle (Kolattukudy, 1981). It is a major constituent (typically 40-80% by weight) of the cuticle. It functions as a densely networked structural support and interacts with the epidermal cell wall.

In the 1970s, the major cutin monomers were identified by using chemical degradation (Kolattukudy, 2001). The monomeric constituents of cutin are mainly C16:0 and C18:0 fatty acids, which are typically hydroxylated (Kolattukudy, 1981) (Figure 1A). The predominant components of the C16 family of monomers are 9- or 10, 16-dihydroxyhexadecanoic acid and 16-hydroxyhexadecanoic acid. Major components of the C18 family of monomers are 18-hydroxy-9, 10-epoxyoctadecanoic acid and 9, 10, 18-trihydroxyoctadecanoic acid (Heredia, 2003). The chemical composition of cutin varies between organ types and location, and even varies the adaxial or abaxial surfaces of the same leaf (Holloway, 1982). For example, cutin of apple fruit contains both C16 and C18 families of monomers, while in flowers the C18 monomers are almost undetectable (Stark and Tian, 2006).

In *Arabidopsis*, the major depolymerization products of cutin are unusual because they consist of high amounts of C16 and C18 dicarboxylic acids, such as α , ω -dicarboxylic fatty acids and 2-hydroxy fatty acids (Bonaventure *et al.*, 2004; Xiao *et al.*, 2004; Franke

(A) Common Cutin Components	(B) Common Cuticular Wax Components
<p>C16 monomers:</p> $\text{CH}_3-(\text{CH}_2)_{14}-\text{COOH}$ $\text{CH}_2\text{OH}-(\text{CH}_2)_{14}-\text{COOH}$ $\text{CH}_2\text{OH}-(\text{CH}_2)_x-\text{CHOH}-(\text{CH}_2)_y-\text{COOH}$ <p>$x, y=6, 7; x+y=13$</p> <p>C18 monomers:</p> $\text{CH}_3-(\text{CH}_2)_7-\text{CH}=\text{CH}-(\text{CH}_2)_7-\text{COOH}$ $\text{CH}_2\text{OH}-(\text{CH}_2)_7-\text{CH}=\text{CH}-(\text{CH}_2)_7-\text{COOH}$ $\text{CH}_2\text{OH}-(\text{CH}_2)_7-\text{CH}-\text{CH}-(\text{CH}_2)_7-\text{COOH}$ $\text{CH}_2\text{OH}-(\text{CH}_2)_x-\text{CHOH}-(\text{CH}_2)_y-\text{COOH}$ <p>$x, y=7, 8; x+y=15$</p>	<p>Very-long-chain free fatty acids:</p> $\text{CH}_3-(\text{CH}_2)_x-\text{COOH}$ <p>Aldehydes:</p> $\text{CH}_3-(\text{CH}_2)_x-\text{CHO}$ <p>Alkanes:</p> $\text{CH}_3-(\text{CH}_2)_x-\text{CH}_3$ <p>Secondary alcohols:</p> $\text{CH}_3-(\text{CH}_2)_x-\text{CHOH}-(\text{CH}_2)_y-\text{CH}_3$ <p>Ketones:</p> $\text{CH}_3-(\text{CH}_2)_x-\text{CO}-(\text{CH}_2)_y-\text{CH}_3$ <p>Primary alcohols:</p> $\text{CH}_3-(\text{CH}_2)_x-\text{CH}_2\text{OH}$ <p>Wax esters:</p> $\text{CH}_3-(\text{CH}_2)_x-\text{O}-\text{CO}-\text{CH}_2-(\text{CH}_2)_y-\text{CH}_3$ <p>$x+y \geq 20$</p>

Figure 1 Chemical structures of cutin and cuticular wax components

(A) Chemical structures of the most common fatty acids that are monomers of plant cutin.

(B) Chemical structures of components typically occurring in plant cuticular wax mixtures.

et al., 2005). Most of the cutin monomers from *Arabidopsis* only carry carboxyl groups, and therefore other hydroxylated fatty acids and glycerol are required to generate the cross-linked matrix (Graca *et al.*, 2002).

In addition to the C16 and C18 major cutin monomers, numerous minor components have been found in cutin of various plant species. For example, hydroxy fatty acids that are longer and shorter than the major components, mid-chain hydroxylated compounds that do not contain a ω -hydroxy group, and mid-chain hydroxylated dicarboxylic acids (Holloway *et al.*, 1972; Hunneman and Eglington, 1972; Deas *et al.*, 1974; Caldicott and Eglington, 1976).

Cutan is the residual material left over when all the cuticular wax and cutin materials have been removed (Heredia, 2003). It is thought to represent cutin monomers that are held together by non-ester bonds and thus are not readily depolymerized. The ratio of cutin and cutan amounts varies between the different stages of cuticle development (Tegelaar *et al.*, 1991). However, the structure, chemical composition, and functions of cutan are not clear.

1.2.2 Molecular architecture of cutin

Cutin is cross-linked in three dimensions mainly by ester bonds (Heredia, 2003). Also, half of the mid-chain hydroxyl groups in the polymer are involved in cross-linking. Most of the primary hydroxyl groups are involved in ester bonds. A very low number of unesterified carboxyl functional groups exists (Heredia, 2003). Other studies indicate that the ω -hydroxy fatty acids can be polymerized to form a linear structure and that the mid-chain hydroxyl group can be esterified with carboxy groups of other fatty acids to form a dendri-form polymerized structure (Ray *et al.*, 1998a, b).

Since cutin soluble products are obtained by chemical depolymerization for subsequent gas chromatography-mass spectroscopy (GC-MS) analysis, they do not represent the properties or condition of the intact cutin matrix. Fourier-transform infrared (FT-IR) spectroscopical analysis and X-ray diffraction analysis have been performed to analyze the intact cuticle, which indicate that cutin has an amorphous structure with two hydrophobic interplanar spaces (Luque *et al.*, 1995; Villena *et al.*, 2000). Additionally, solid-state nuclear magnetic resonance (NMR) confirmed the classical chemical studies that the cutin polyester is held together mostly by primary alcohol ester linkages (Zlotnik-Mazori *et al.*, 1988). Moreover, molecular dynamics calculations have been applied and suggest that cutin is a moderately flexible netting with motional constraints mainly located at the cross-link sites of functional ester groups (Matas *et al.*, 1999).

1.2.3 Biosynthesis of cutin monomers

The initial step of cutin monomer biosynthesis is the synthesis of C16 and C18 long chain fatty acids in the plastid. The C16 and C18 fatty acid precursors are likely shared with wax biosynthesis and other cellular lipid metabolism, such as membrane lipid production. The second step of cutin biosynthesis is oxidative modification, which generates the variety of cutin monomers.

The biosynthetic pathways of both the C16 and C18 family of fatty acids were determined using radio-labeled precursors and identification of intermediates leading to cutin monomers (Kolattukudy, 1981). In *Vicia sativa*, studies indicate that the oxygenated octadecanoates are derived from oleic acid and linoleic acid, which undergo ω -hydroxylation to form diol 9, 10-dihydroxyoctadecanoic acid and then followed by

enantioselective epoxidation of the double bond, and finally hydroxylation of the latter functional group (Kolattukudy *et al.*, 1981; Pinot *et al.*, 1992) (Figure 2).

The identities of the enzymes involved in cutin biosynthesis remain elusive, but recently several key proteins required for the synthesis of cutin have been identified: fatty acyl oxidases (CYP86 family) (Benveniste *et al.*, 2001; Wellesen *et al.*, 2001; Xiao *et al.*, 2004; Rupasinghe *et al.*, 2007; Li *et al.*, 2007, Molina *et al.*, 2008), fatty acyl-activation enzymes (LACS family) (Schnurr *et al.*, 2004; Bessire *et al.*, 2007), and acyltransferases (GPAT family) (Li *et al.*, 2007).

A cytochrome P450-dependent ω -hydroxylase fatty acyl oxidase is proposed to catalyze the epoxidation process (Heredia, 2003). Two genes that encode this class of enzyme have been found to be important for *Arabidopsis* cutin production: *LACERATA* (*LCR*, At2g45970) (Wellesen *et al.*, 2001) and *ATT1* (At4g00360) (Xiao *et al.*, 2004). *lcr* mutants display a series of developmental abnormalities, such as postgenital organ fusions indicative of a cutin defect (Wellesen *et al.*, 2001). The cloned *LCR* gene encodes for cytochrome P450 monooxygenase, CYP86A8, which is able to catalyze ω -hydroxylation of fatty acids ranging from C12:0 to C18:1 *in vitro* (Wellesen *et al.*, 2001). *att1* (aberrant induction of type three genes) mutants show reduced cutin monomer amounts, a loose cuticle membrane ultrastructure and increased permeability to water vapor, indicating that *ATT1* plays an important role in the biosynthesis of cutin monomers (Xiao *et al.*, 2004). Cloning of the *ATT1* gene indicated that it encoded CYP86A2, a cytochrome P450 monooxygenase catalyzing fatty acid oxidation (Xiao *et al.*, 2004).

Figure 2 Biosynthetic pathway of the cutin formation

Proposed biosynthetic pathway of the formation of C16 and C18 cutin monomers begins with biosynthesis of very-long-chain fatty acids. Fatty acid synthesis to C16 and C18 chain lengths occurs in the plastid and then are modified to generate C16 and C18 cutin monomers.

Glycerol-3-phosphate acyltransferase (GPAT) 4 and GPAT8 have been identified as being important for cutin biosynthesis. Double knockout *gpat4 gpat8* mutants display a significantly reduced cutin load and striking defects in stomata structure including a lack of cuticular ledges between guard cells (Li *et al.*, 2007). These evidences indicate that GPAT4 and GPAT8 are not only involved in cutin formation, but also show that cutin integrity is important in stomatal development. Further analysis indicated that GPATs work together with fatty acyl oxidases in lipid polyester synthesis (Li *et al.*, 2007). Additionally, Long-Chain Acyl-CoA Synthetase 2 (LACS2) protein is also involved in cutin biosynthesis. A *lacs2* null mutant displays a reduced thickness of the cutin layer and high cuticle permeability (Schnurr *et al.*, 2004). *Lacs2* mutants (*sma4* and *bre1*) have been shown to have enhanced susceptibility to avirulent *Pseudomonas syringae* and increased resistance to *Botrytis cinerea* (Tang *et al.*, 2007; Bessire *et al.*, 2007). These phenotypes may be due to more rapid leakage of cell fluids in the substomatal region and an increased release of antifungal compounds through a more permeable cuticle. Mutants of *LACS1* (*CER8*) are deficient in C16 cutin monomers, while C18 cutin monomers are increased (Lu *et al.*, 2009). This indicated that LACS1 is also involved in cutin metabolism. Moreover, a double mutant of *lacs1 lacs2* has much lower amounts of both C16 and C18 cutin monomers and a highly disorganized cuticle ultrastructure, revealing that LACS1 and LACS2 have overlapping functions in cutin biosynthesis (Lu *et al.*, 2009). Two other proteins, HOTHEAD (Kurdyukov *et al.*, 2006a) and BODYGUARD (Kurdyukov *et al.*, 2006b), have been identified and are proposed to be involved in di-acid formation and cutin assembly, respectively. SHINE1/WIN1 is the only known regulatory protein of cutin biosynthesis pathway (Kannangara *et al.*, 2007).

SHN1/WIN1 is a transcription factor belonging to a clade of the AP2-domain/ethylene response element binding protein super family of *Arabidopsis* (Aharoni *et al.*, 2004; Broun *et al.*, 2004). LACS2 has been shown to be a direct target of SHN1/WIN1 (Kannangara *et al.*, 2007).

1.3 Cuticular wax

1.3.1 Chemical profiles of plant cuticular waxes

Waxes are the second major chemical component of plant cuticles. Waxes have been defined as the lipids that are removed from plant surfaces after brief immersion in an organic solvent, such as chloroform (Post-Beittenmiller, 1996). Wax is predominantly comprised of very-long-chain fatty acids ($\geq C_{20}$ in length) and derived components. Cuticles also contain triterpenoids and minor secondary metabolites, such as sterols and flavonoids (Kunst and Samuels, 2003). However, the composition of wax varies substantially between organ types and species (Mladenova and Stoyanova-Ivanova, 1977; Baker and Hunt, 1979).

Plant cuticular waxes are typically mixtures of free fatty acids, primary and secondary alcohols, aldehydes, alkanes and ketones with chain lengths ranging from 20 to 34 carbons. Alkyl wax esters with chain lengths ranging from C₃₆-C₇₀ have also been identified in waxes (Figure 1B). In the model plant *Arabidopsis thaliana*, the predominant wax components in stems are C₂₉ alkane, C₂₉ ketone, and C₂₉ secondary alcohols, together with smaller quantities of C₃₀ aldehyde, C₂₆-C₃₀ even chain length primary alcohols, C₂₂-C₃₀ even chain length free fatty acids, and C₄₄-C₅₄ even chain length alkyl wax esters. Alternately, the most abundant cuticular wax

component in leaves of *Arabidopsis* is C31 alkane, and leaves lack ketones and secondary alcohols (Jenks *et al.*, 1995).

1.3.2 Biosynthesis of cuticular wax

Wax biosynthesis consists of two distinct stages. The first stage is the production of very-long-chain fatty acids (VLCFAs) wax precursors, which is accomplished sequentially in two different cellular compartments: fatty acid synthesis in the plastid to produce C16 and C18 fatty acyl precursors and fatty acid elongation in the endoplasmic reticulum to produce C20-C34 VLCFAs. The second stage is modification of monofunctional aliphatics and alkanes. In this latter stage, there are two principal wax biosynthetic pathways: a primary alcohol forming pathway (sometimes called the acyl reduction pathway), which generates primary alcohols and wax esters, and an alkane forming pathway (sometimes called the decarbonylation pathway), which generates aldehydes, alkanes, secondary alcohols and ketones (Figure 3).

Stage 1 Synthesis of VLCFA wax precursors

Saturated VLCFAs with chain lengths between C20 and C34 are the precursors for the biosynthesis of aliphatic cuticular wax components. The formation begins with synthesis of C16 and C18 acyl chains in the plastid (Ohlrogge *et al.*, 1993; Ohlrogge and Browse, 1995). In this process, long carbon chains are assembled through a cycle of four reactions: first, condensation of a C2 moiety originating from malonyl-acyl carrier protein (ACP) with an acetyl-CoA. After this condensation step, a series of reactions occurs: reduction of the β -ketoacyl-ACP, dehydration of the β -hydroxyacyl-ACP, and reduction of the *trans*- Δ^2 -enoyl-ACP, resulting in fatty acyl-ACP extended by two carbons longer than the original acetyl molecule. Fatty acid synthesis is catalyzed by

Figure 3 Proposed pathways for wax biosynthesis in *Arabidopsis* stems.

Fatty acid synthesis to generate C16 and C18 chain lengths occurs in the plastid, and further elongation to $C \geq 20$ fatty acyl chain lengths occurs in the endoplasmic reticulum. Also, in the ER, very-long-chain fatty acids precursors are modified to generate wax components through two pathways: a primary alcohol forming pathway and an alkane forming pathway.

ACP: acetyl-acyl carrier protein;

FAS: fatty acid synthase complex;

KASI/ KASII/ KASIII: ketoacyl-ACP synthase I/ II/ III;

FATB/FATA: fatty acyl-ACP thioesterase A/ fatty acyl-ACP thioesterase B;

LACS: long-chain acyl-CoA synthetase;

KCS: β -keto acyl-CoA synthase;

KCR: β -ketoacyl reductase;

ECR: enoyl-CoA reductase;

HCD: hydroxyacyl-CoA dehydratase;

VLCFA: very long chain fatty acid;

FAR: fatty acyl-CoA reductase;

WS/DGAT: wax synthase/acyl-CoA diacylglycerol acyl-transferase;

MAH1: mid-chain alkane hydroxylase;

FAE: fatty acid elongase;

CER: ECERIFERUM;

the well characterized soluble fatty acid synthase complexes (FASs), which are localized in the stroma of the plastid (Shimakata and Stumpf, 1982; Stumpf, 1984). These FASs can be divided into three different types based on which ketoacyl-ACP synthase condensing enzyme is present: ketoacyl-ACP synthase III (KASIII) initiates fatty acid biosynthesis using acetyl-CoA as a substrate (Clough *et al.*, 1992); KASI catalyzes C4-C16 elongation, and KASII extends the chain length to C18 (Shimakata and Stumpf, 1982). Chain length specificity of the FAS is thus determined by the condensing enzyme, and the two reductases and dehydratase lack strict specificity and are shared by all three enzyme complexes (Stumpf, 1984).

Once C16 and C18 fatty acids are synthesized, they are exported from the plastid to the endoplasmic reticulum (ER) for further elongation. To reach this site, C16 and C18 acyl chains are liberated from ACP by fatty acyl-ACP thioesterase and then exported out of the plastid by an unknown mechanism. There are two classes of fatty acyl-ACP thioesterases: fatty acyl-ACP thioesterase A (FATA) and fatty acyl-ACP thioesterase B (FATB). The FATA class specifically cleaves C18:0-ACP, while the FATB thioesterases are able to cleave both C16:0 and C18:0 acyl-chains from acyl-ACP *in vitro* (Voelker *et al.*, 1996). In *Arabidopsis*, the *fatb* mutant has a 20% and 50% reduction in total wax load of leaf and stem cuticular wax, respectively, but without significant changes in the distribution of wax composition (Bonaventure *et al.*, 2003). This indicates that a major portion of C16 and C18 acyl-ACPs are channeled through thioesterase cleavage toward wax biosynthesis (Samuels *et al.*, 2008). Moreover, knockout lines of the FATB gene results in an altered seed morphology, a lower germination rate, reduced growth rate and a reduced level of both palmitate (16:0) and

stearate (18:0) content in flowers, root, leaves, and seeds. All of these phenotypes indicate that FATB plays an important role in production of saturated fatty acids, which are essential for the generation of cellular membrane components, seed oil production as well as cuticular wax biosynthesis. Free C16 and C18 fatty acids become esterified to CoA catalyzed by a long-chain acyl-CoA synthetase (LACS). Nine *LACS* genes have been identified in *Arabidopsis* (Shockey *et al.*, 2002). Among these *LACS* family members, *LACS1*, *LACS2*, *LACS6*, *LACS7*, and *LACS9* 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; Lu *et al.*, 2009). *CER8/LACS1* (At2g47240) plays a direct role in the modification of VLCFAs, with highest activity for C30 acids (Lu *et al.*, 2009). Mutants of *lacs1* have elevated VLCFAs and alkane deficiency of both leaves and stems. The double mutant of *lacs1 lacs2* has much lower total wax load and lower amounts of most wax classes than the single mutants, which revealed that *LACS1* and *LACS2* have overlapping functions associated with the wax biosynthesis in addition to cutin biosynthesis (Lu *et al.*, 2009).

Once the C16:0-CoA and C18:0-CoA are synthesized, they are translocated to the ER for further elongation. This process is catalyzed by fatty acid elongases (FAE) (von Wettstein-Knowles, 1982), multienzyme complexes that are located in the ER (Xu *et al.*, 2002; Zheng *et al.*, 2005; Joubes *et al.*, 2008). Similar to fatty acid synthesis in the plastid, elongation of long-chain acids (C16, C18) to VLCFAs involves a 2-carbon extension cycle. The C2 units for FAE are malonyl-CoA, rather than malonyl-ACP. The four consecutive enzymatic reactions are: condensation of malonyl-CoA with fatty acyl-CoA, reduction of the resulting β -ketoacyl-CoA, dehydration of β -hydroxyacyl-CoA and

reduction of *trans*- Δ^2 -enoyl-CoA (Fehling and Mukherjee, 1991). Several elongation cycles are needed to synthesize C20-C34 acyl chains for further production of aliphatic wax components. The entire elongation process is carried out by several distinct FAE complexes with different chain length specificities (von Wettstein-Knowles, 1993). The substrate specificity of the FAE complex is largely determined by the β -ketoacyl-CoA synthase (KCS), which catalyzes the first step of the cycle (Millar and Kunst, 1997). In the *Arabidopsis* genome, a large family of 21-KCS like sequences have been identified (Lechelt-Kunze *et al.*, 2003; Dunn *et al.*, 2004; Blacklock and Jaworski, 2006; Joubes *et al.*, 2008). Among all the condensing enzymes, CER6 is thus far the only wax-specific KCS and it is involved in elongation of fatty acyl-CoA longer than C22 (Millar *et al.*, 1999; Fiebig *et al.*, 2000; Hooker *et al.*, 2002). Disruption of the *CER6* gene results in only a 19% total wax load compared to wild-type levels and the mutant is completely devoid of epicuticular wax crystals (Millar *et al.*, 1999). In addition, *cer6* mutants are male-sterile indicating that reduced levels of cuticular wax influences stigma-pollen interaction (Millar *et al.*, 1999; Fiebig *et al.*, 2000). Other KCS genes in *Arabidopsis*, *KCS1* and *FIDDLEHEAD*, are upregulated in *Arabidopsis* stem epidermal cells and likely have roles in fatty acid elongation in epidermal cells for wax production (Todd *et al.*, 1999; Yephremov *et al.*, 1999; Suh *et al.*, 2005). Maize GLOSSY8 (*GL8*) is found to be a β -keto acyl reductase (KCR) that catalyzes the reduction of β -ketoacyl-CoAs during VLCFA synthesis (Xu *et al.*, 1997). In the *Arabidopsis* genome, two putative KCRs, AtKCR1 and AtKCR2, were identified and both of them are expressed at high levels in the stem epidermis (Suh *et al.*, 2005; Beaudoin *et al.*, 2009). A knockout of AtKCR1 is embryo lethal, while partial suppression of AtKCR1 activity results in reduced cuticular

wax load in addition to affecting VLCFA composition in sphingolipids, seed triacylglycerols, and root glycerolipids. The role of AtKCR2 is unknown. *Arabidopsis* PASTICCINO2 (PAS2) encodes the fatty acyl-CoA dehydratase, which is limiting for VLCFA synthesis and essential for plant development (Bath *et al.*, 2008). In the *pas2* mutant, lipid fractions of *Arabidopsis* that contain VLCFAs were perturbed, while complete loss of PAS2 function causes embryo lethality. In *Arabidopsis*, CER10 was identified as an enoyl-CoA reductase (ECR) that is required for the synthesis of all the VLCFA-containing lipids (Zheng *et al.*, 2005). *cer10* mutants exhibit reduced organ size, have 40% of the cuticular wax load of wild-type, and a reduction of VLCFAs in seed triacylglycerols and sphingolipids (Zheng *et al.*, 2005). Interestingly, knockout mutants of CER10 are not lethal and still accumulate some VLCFAs and cuticular waxes. This indicates that unknown enzyme(s) function in a partially redundant manner with CER10/ECR (Zheng *et al.*, 2005).

Stage 2 Synthesis of wax compounds

Once the VLCFAs are generated in the ER, two main wax biosynthetic pathways generate the different aliphatic wax components (Samuels *et al.*, 2008).

The primary alcohol pathway is also called the acyl reduction pathway (Kunst *et al.*, 2003). Biochemical studies suggested that generation of primary alcohols can occur via a two-step reaction carried out by two independent enzymes: an NADH-dependent acyl-CoA reductase and an NADPH-dependent aldehyde reductase, such that the aldehyde intermediate can be isolated (Kolattukudy, 1971). Later studies indicated that the reduction of VLCFAs to alcohols can alternatively be carried out by single alcohol-forming FAR enzymes (Kolattukudy, 1970; Vioque and Kolattukudy, 1997; Metz *et al.*,

2000). In the *Arabidopsis* genome, eight FAR-like genes encoding enzymes of this latter type have been identified and one of them is *CER4* (Rowland *et al.*, 2006). *CER4* is the major FAR responsible for primary alcohol formation in *Arabidopsis* cuticular wax (Hannoufa *et al.*, 1993; Jenks *et al.*, 1995; Rowland *et al.*, 2006). Although much of the primary alcohols are secreted to the cuticle in free form, some of them become esterified to acyl groups through the action of a wax synthase (WS) enzyme (Lai *et al.*, 2007). Recently, one gene, *WSD1*, from *Arabidopsis* has been found to be important for cuticular wax ester formation in stems (Li *et al.*, 2008). This gene is a putative bifunctional enzyme, which has high WS activity and substantially lower acyl-CoA:diacylglycerol acyltransferase activity (Li *et al.*, 2008).

The alkane-forming pathway is also called the decarbonylation pathway. A decarbonylation reaction was first proposed by Kolattukudy *et al* (1972) to be part of this pathway. Later biochemical experiments provided support for aldehydes as intermediates leading to formation of alkanes (Kolattukudy *et al.*, 1974). A decarbonylation reaction releases one carbon monoxide (CO) from aldehydes to generate odd chain length alkanes (Cheesbrough and Kolattukudy, 1984; Schneider-Belhaddad and Kolattukudy, 2000). However, the enzymes responsible for aldehyde and alkane formation remain elusive and chemical mechanisms other than decarbonylation are possible. Radioactive labeling studies and genetic evidence indicated that secondary alcohols and ketones were generated by hydroxylation and oxidation, respectively (Kolattududy and Liu, 1970; Khan and Kolattukudy, 1974). In a reverse genetics study, a mid-chain alkane hydroxylase (*MAH1*) responsible for these reactions was identified in *Arabidopsis* (Greer *et al.*, 2007). *MAH1* encodes a

cytochrome P450-dependent enzyme (CYP96A15) and is responsible for secondary alcohol and ketone formation in cuticular wax of *Arabidopsis* stems.

1.3.3 Regulation of wax biosynthesis

Resistance of plants to environmental stresses, microbial pathogens and insects are linked to wax accumulation and wax composition (Post-Beittenmiller, 1996). However, knowledge about the regulatory mechanisms governing wax production is currently limited.

The influence of environmental stresses on the expression of two genes encoding wax biosynthetic enzymes in *Arabidopsis*, *CER2* and *CER6*, has been investigated (Xia *et al.*, 1997; Hooker *et al.*, 2002). *CER6* transcription is induced by light and osmotic stress, while *CER2* expression is rather influenced by cytokinin hormone (Xia *et al.*, 1997; Hooker *et al.*, 2002).

Currently two transcription factors, SHN1/WIN1 and MYB30, have been reported to be involved in wax biosynthesis (Broun *et al.*, 2004; Aharoni *et al.*, 2004; Raffaele *et al.*, 2008). Overexpression of WIN1/SHN1 results in an increased of wax load on both leaves and stems and also the induction of some wax genes, such as *CER1*, *CER2*, and *KCS1* (Aharoni *et al.*, 2004; Broun *et al.*, 2004). Additionally, WIN1/SHN1 can activate genes encoding cutin biosynthetic enzymes, such as *LACS2* and *GPAT4*, (Kannagara *et al.*, 2007). *MYB30* knockout and overexpression lines display alterations in leaf epidermal wax components and DNA microarray analysis of overexpressing *MYB30* lines show that fatty acid elongase (FAE) components are putative target genes of *MYB30* (Raffaele *et al.*, 2008). Additionally, the *MYB41* gene, which encodes an R2R3-MYB transcription factor, has been suggested to be involved in cuticle

biosynthesis based on the fact that over-expression of *AtMYB41* led to higher rates of water loss, faster chlorophyll leaching in ethanol, and a patchy toluidine-blue staining phenotype (Cominelli *et al.*, 2007).

CER7, a subunit of the exosome, is also important for wax biosynthesis. The exosome is involved in RNA processing and degradation (Hooker *et al.*, 2007). An mRNA encoding a *CER3* repressor was postulated to be the target of CER7 ribonuclease in epidermal cells (Hooker *et al.*, 2007). Therefore, specific degradation of the repressor's mRNA by CER7 may allow *CER3* expression and the alkane forming pathway to proceed (Hooker *et al.*, 2007).

1.3.4 Transportation of cuticular wax components

The transport of cuticular waxes can be divided into three stages. First, the wax constituents generated in the endoplasmic reticulum (ER) are delivered to the plasma membrane (PM). Second, all the wax components are released from the PM into the cell wall. Third, the wax components are transported through the cell wall and to the cuticle at the surface.

A number of wax biosynthetic enzymes have been found to be associated with the ER using green fluorescent protein (GFP) as a tag: these are CER6, CER10, CER4, MAH1, and WSD1 (Millar *et al.*, 1999; Fiebig *et al.*, 2000; Zheng *et al.*, 2005; Rowland *et al.*, 2006; Greer *et al.*, 2007; Li *et al.*, 2008). These evidences indicate that both the intermediate metabolites and the final products of the wax biosynthesis pathway are produced in the ER. However, the mechanisms of exportation of wax components from the ER to PM are unknown. Two models have been proposed by analogy with other lipid transport processes (Kunst and Samuels, 2003; Schulz and Frommer, 2004). One

possibility is that wax components are directly transferred to the PM at ER-PM sites of contact. The second model suggests that wax molecules are transported by vesicles moving along the secretory pathway from ER to Golgi and then to the PM.

When wax components reach the PM, they must be exported to the apoplastic environment. Two ATP binding cassette (ABC) transporters, CER5 and WBC11, have been shown to be important for this process (Pighin *et al.*, 2004; Bird *et al.*, 2007; Panikashvili *et al.*, 2007; Ukitsu *et al.*, 2007). Both CER5 and WBC11 ABC transporters are localized to the PM, and *cer5* and *wbc11* mutants accumulate wax molecules inside the epidermal cells (Pighin *et al.*, 2004; Bird *et al.*, 2007; Panikashvili *et al.*, 2007; Ukitsu *et al.*, 2007). Since there is no direct evidence that CER5 and WBC11 can interact with wax molecules as substrates, the details on how these two transporters transport wax molecules across the lipid bilayer remains to be investigated. Moreover, the double mutant *cer5 wbc11* still accumulates wax in stems to a low level (Bird *et al.*, 2007). This suggests that additional transporters are involved in this process.

After reaching the cell wall, wax components are further secreted across the hydrophilic cell wall layer to accumulate and crystallize at the surface. Wax molecules are highly lipophilic and have very low solubility in the cell wall environment. It is possible that this transport process is carried out by special structures that help guide the wax molecules to the cuticle, but evidence for this is lacking. Lipid transfer proteins (LTPs) have also been suggested as being involved in shuttling waxes through the cell wall. LTPs are very small proteins of a large superfamily that can diffuse and move through the pectin matrix of the primary cell wall (Kader, 1996; Arondel *et al.*, 2000; Rogers and Bankaitis, 2000). LTPs are highly expressed in the epidermis and secreted

into the apoplast (Thoma *et al.*, 1993; Pyee *et al.*, 1994). Additionally, the structures of LTPs revealed that they contain a flexible hydrophobic pocket which can bind to linear acyl lipids (Bouliez *et al.*, 2000). A glycosylphosphatidylinositol-anchored lipid transfer protein (LTPG) involved in cuticle development was identified in a reverse genetic screen (Debono *et al.*, 2009). It is highly expressed in the epidermis of *Arabidopsis* stems during cuticle biosynthesis. LTPG is localized in the plasma membrane on the cell wall side, where wax is actively secreted (Lee *et al.*, 2009). Disruption of the LTPG gene resulted in an alteration of cuticular lipid composition, and a more diffuse cuticular layer structure (Debono *et al.*, 2009). All these data indicate that LTPG is involved either directly or indirectly in cuticular lipid deposition and may function as a component of the cuticular lipid export machinery (Debono *et al.*, 2009; Lee *et al.*, 2009).

1.4 Objective of project

The purpose of my thesis project was to identify and characterize novel *cer* mutants and to characterize the uncloned *CER9* gene. The epicuticular wax crystal structures and cuticular wax compositions of a set of novel wax deficient mutants were compared to a set of previously identified *CER* mutants, in order to predict allelism between the sets of mutants. I further phenotypically characterized two potentially novel mutants called *line8* and *line85*. I mapped the two loci to specific chromosome locations and tested for allelism with other *cer* mutant loci mapping to the same region, and found that *LINE8* and *LINE85* were in fact novel alleles of *CER6* and *CER19*, respectively. Finally, I used a map-based cloning approach to localize the position of *CER9* on chromosome IV, and did further phenotypic characterization of the *cer9* mutant to gain insights into the function of *CER9* in cuticular wax biosynthesis.

Chapter 2 Identification of Novel *Eceriferum* Mutants

2.1 Introduction: *eceriferum* (*cer*) mutants in *Arabidopsis thaliana*

Arabidopsis mutants with defects in the production of epicuticular wax are usually called *eceriferum* (*cer*) mutants (Koornneef *et al.*, 1989). These mutants can be easily identified visually because of their brighter green stems and siliques compared to wild-type plants. Studies of wax-deficient mutants in other plant species have been performed, including *Brassica oleracea* (Baker, 1974; Macey, 1974), *Brassica napus* (Holloway *et al.*, 1977), *Zea maize* (Bianchi, 1979), *Hordeum vulgare* (von Wettstein-Knowles, 1979), and *Pisum sativum* (Macey and Barber, 1970).

To date, many wax-deficient loci have been identified in *Arabidopsis* including 24 *CER* loci, *FATB*, *KCS1*, *WSD1*, *MAH1*, and *LTPG* (Koornneef *et al.*, 1989; McNevin *et al.*, 1993; Rashotte *et al.*, 2004; Bonaventure *et al.*, 2003; Todd *et al.*, 1999; Li *et al.*, 2008; Greer *et al.*, 2007; Debono *et al.*, 2009) (Table 1). Koornneef *et al.* (1989) identified 89 *cer* mutants in *Arabidopsis* that mapped to 21 different loci (*CER1* to *CER20* and *TRANSPARENT TESTA5*) by screening for plants with glossy stems. A *CER21* locus was reported (McNevin *et al.*, 1993), but later revealed to be allelic to *CER3* (Rowland *et al.*, 2007). Rashotte *et al.* (2004) conducted a non-visual screen using screening by gas chromatography and identified three novel genes that have been designated *CER22*, *CER23*, and *CER24*. In *Arabidopsis*, *CER1-CER8*, *CER10*, *FATB*, *KCS1*, *WSD1*, *MAH1*, and *LTPG* have been cloned so far (Aarts *et al.*, 1995; Negruk *et al.*, 1996; Fiebig *et al.*, 2000; Chen *et al.*, 2003; Bonaventure *et al.*, 2003; Pighin *et al.*, 2004; Zheng *et al.*, 2005; Rowland *et al.*, 2006; Rowland *et al.*, 2007;

Hooker *et al.*, 2007; Greer *et al.*, 2007; Li *et al.*, 2008; Debono *et al.*, 2009; Lu *et al.*, 2009) (Figure 4). The biosynthetic or transport activities of these protein products were described in Chapter 1 and summarized here in Table 1 and Figure 5.

In *Arabidopsis*, the genes disrupted in the *cer* mutant collection are involved in wax biosynthesis, wax intra- or extra-cellular transportation, or regulation. The *cer* loss-of-function mutants generally exhibit changes in the wax crystalline structures and in some cases the wax crystals are absent. These structures are typically observed by scanning electron microscopy (SEM). The major wax crystalline structures observed on plant surfaces are platelets, tubules, threads, transverse rodlets, dendrites, and longitudinal rodlets (Jeffree, 2006). On *Arabidopsis* stems the wax structures are predominated by longitudinal bundles of rodlets. There are no epicuticular wax crystals on wild-type *Arabidopsis* leaves. Studies indicate that the epicuticular wax crystalline structures are largely determined by the composition of wax components (Jeffree, 2006). Epicuticular wax that has a single predominating component usually has a characteristic structural type. For example, the primary alcohol-rich waxes have plate-shaped crystals, while wax rich in β -diketones have a tubular morphology (Jeffree, 2006). However, the correlation between wax composition and crystalline structure is not very strict, especially in complex mixtures of cuticular waxes.

The visual screening method can only identify mutants with a sufficiently low density of crystals that would give a detectable glossy stem phenotype due to a difference in light scattering. Mutants having an altered chemical composition that does not lead to changed visual reflectance are not detected in visual screens. For example, MAH1 is critical for secondary alcohol and ketone formation in *Arabidopsis* stems, but

mah1 mutant still has a glaucous wild-type stem phenotype (Greer *et al.*, 2007). A more rigorous method to screen wax-deficient mutants using gas chromatography (GC) analysis can more sensitively detect alterations in total wax load and/or cuticular wax constituents (Rashotte *et al.*, 2004).

The objective of this chapter was to characterize a set of unpublished wax deficient mutants identified by the Rowland lab. These were temporally termed '*line*' mutants until allelism with existing *cer* mutants could be established. It was anticipated that some of these *line* mutants would represent novel *cer* loci, as the forward screen to identify the original set of *cer* mutants was not done to saturation (Koorneef *et al.*, 1989). The stem wax crystal morphology and stem wax composition of ten *line* mutants and 11 *cer* were characterized by SEM and GC, respectively, to place them into phenotypically similar groups. Genetic complementation crosses were then performed to test for allelism within these groups.

Figure 4 Map positions of wax-deficient mutant loci on the five *Arabidopsis* chromosomes.

Vertical lines and roman numbers represent *Arabidopsis* chromosomes. Mutant loci are placed on the distinct chromosome locations where they mapped relative to SSLP markers on the Lister and Dean recombinant inbred (LD-R) genetic map (Koorneef *et al.*, 1989; Rashotte *et al.*, 2004) and to physical positions (P). These positions are indicated to the left of a mutant designation with P in million base pair (Mbp) and LD-R in centiMorgans (cM). Genes in bold represent genes that have been cloned.

Table 1 Summary of all identified CER loci and cloned genes involve in wax biosynthesis in *Arabidopsis*. An Atgxxxxx below the gene name indicates the gene has been cloned.

Gene	Cuticle phenotype in the stems	Function/prediction	Reference
CER1 At1g02205	Stem wax load 13% of WT. Most stem wax components are reduced. The C30 fatty acid and C30 aldehyde are slightly increased.	May encode for an aldehyde decarbonylase that converts fatty aldehydes to alkanes.	Aarts <i>et al.</i> , 1995
CER2 At4g24510	Stem wax load 25% of WT. All the components in alkane forming pathway are reduced except C28 aldehyde. The C26/C28 acyl groups, primary alcohols and wax esters are increased.	May alter the C26 elongase complex that influences the C26 to C28 fatty acyl elongation step.	McNevin <i>et al.</i> , 1993 Negruk <i>et al.</i> , 1996
CER3/ WAX2/ YRE/ FLP1 At5g57800	Stem wax load 20% of WT. All the components in alkane forming pathway are reduced. The C30 primary alcohol is highly increased.	May alter the aldehyde reductase and aldehyde decarbonylase or affect fatty acid release from fatty acyl-CoA.	Arizumi <i>et al.</i> , 2003; Kurata <i>et al.</i> , 2003; Chen <i>et al.</i> , 2003; Rowland <i>et al.</i> , 2007.
CER4/ FAR3 At4g33790	Stem wax load similar to WT. Primary alcohols and wax esters are almost eliminated. Alkane forming pathway components are slightly increased.	Alcohol-forming fatty acyl-CoA reductase (FAR)	Rowland <i>et al.</i> , 2006
CER5/ WBC12 At1g51500	Stem wax load 41% of WT. All chemical classes are reduced except primary alcohols and esters. The C30 aldehyde and C29 alkane are dramatically reduced.	ABC transporter	Pighin <i>et al.</i> , 2004
CER6/ CUT1/ KCS6 At1g68530	Stem wax load 19% of WT. Reduced levels of all wax components, especially chain lengths longer than C24.	β -keto acyl-CoA synthase (KCS) affecting elongation of C24 fatty acyl-CoA	Millar <i>et al.</i> , 1999; Fiebig <i>et al.</i> , 2000
CER7/ RRP45 At3g60500	Stem wax load 30% of WT. Reduced levels of all stem wax components.	Exosome subunit (RRP45) that affects CER3 transcript stability	Hooker <i>et al.</i> , 2007

CER8/ LACS1 At2g47240	Stem wax load 69% of WT. Alkanes, secondary alcohols, ketones, and wax esters are reduced, while free very-long-chain fatty acids are increased	Long-chain acyl-CoA synthetase that has high activity for C30 free fatty acid	Lu <i>et al.</i> , 2009
CER9	Stem wax load 40% of WT. Alkane forming pathway components are highly reduced, while primary alcohols are increased. C24:0 and C26:0 fatty acids are increased, while C28:0 and C30:0 fatty acids are decreased	May alter activities of the Aldehyde forming fatty acyl reductase and aldehyde decarbonylase or inhibits fatty acid release from fatty acyl-CoA.	Jenks <i>et al.</i> , 1995
CER10/ ECR At3g55360	Stem wax load 36% of WT. Reduced C29 components and increased C30 primary alcohol levels.	Enoyl acyl-CoA reductase (ECR) of fatty acid elongase	Zheng <i>et al.</i> , 2005
CER11	Stem wax load 53% of WT. Reduced C29 components and C30 aldehyde.	May be involved in functions other than wax biosynthesis, such as transport or regulation.	Rashotte <i>et al.</i> , 2001
CER12	Stem wax load 75% of WT. A major reduction in C29 alkane.	May be involved in functions other than wax biosynthesis, such as transport or regulation.	Rashotte <i>et al.</i> , 2001
CER13	Stem wax load 60% of WT. Decrease of C30 aldehyde and subsequent C29 components, along with an increase of both C30 primary alcohol and C31 alkane.	May be involved in reduction of C30 fatty acyl-CoA to C30 fatty Aldehyde.	Rashotte <i>et al.</i> , 2001
CER14	Stem wax load 70% of WT. C29 alkane, secondary alcohol and ketone are reduced.	May encode or modify a decarbonylase, which converts fatty aldehyde to alkanes.	Rashotte <i>et al.</i> , 2001
CER15	Stem wax load 91% of WT. Primary alcohols and esters are moderately reduced, while other components are mostly unaffected.	May regulate the conversion of fatty acids to primary alcohols or be involved in functions other than wax biosynthesis.	Rashotte <i>et al.</i> , 2001
CER16	Stem wax load 10% of WT. Reduced levels of all stem wax components especially alkanes.	May encode or modify a decarbonylase which converts fatty aldehyde to alkanes.	Jenks <i>et al.</i> , 1995
CER17	Stem wax load 95% of WT. C30 aldehyde, C30 primary alcohol and C29 alkane are slightly increased; other components are moderately reduced.	May be involved in reduction of C30 fatty acyl-CoA to C30 fatty Aldehyde, or be involved in functions other than wax biosynthesis.	Rashotte <i>et al.</i> , 2001

CER18	Stem wax load 81% of WT. All the components in alkane forming pathway are slightly decreased; primary alcohols and esters are increased.	May affect C28 fatty acid release from the C28 fatty acyl-CoA elongation complex or be involved in functions other than wax biosynthesis.	Rashotte <i>et al.</i> , 2001
CER19	Stem wax load 30% of WT. All the wax constituents with chain lengths longer than C28 are highly reduced.	May be involved in the C28 fatty acyl-CoA elongation step	Rashotte <i>et al.</i> , 2001
CER20	Stem wax load 70 % of WT. All the components remain unaffected except for reductions in C29 secondary alcohol and C29 ketone levels.	May be involved in the enzymatic oxidation of C29 alkane to C29 secondary alcohol.	Rashotte <i>et al.</i> , 2001
CER21	Allelic to CER3.		McNevin <i>et al.</i> , 1993; Rowland <i>et al.</i> , 2007
CER22	Stem wax load 36% of WT. C30 aldehyde and esters are highly increased; other components are reduced	May encode or regulate the C30 aldehyde decarboxylase that converts C30 aldehyde to C29 alkane.	Rashotte <i>et al.</i> , 2004
CER23	Stem wax load 71% of WT. All the components in primary alcohol forming pathway are increased; C29 alkane is greatly reduced.	May have a defect in C30 fatty acid release from the C30 fatty acyl-CoA elongation complex.	Rashotte <i>et al.</i> , 2004
CER24	Stem wax load 79% of WT. All the components are similar to that of the WT except for the primary alcohols, which show a dramatic reduction of C28 and C30 levels.	May have an effect on the chain-length specificity of a fatty acyl-CoA reductase, affecting reduction of C28 and C30 fatty acids to C28 and C30 primary alcohols.	Rashotte <i>et al.</i> , 2004
FATB At1g08510	Stem wax load 50% of WT. All the wax composition show moderate changes.	Fatty acyl-ACP thioesterase	Bonaventure <i>et al.</i> , 2003
KCS1 At1g01120	C30 fatty acid, aldehyde, and primary alcohol are highly decreased, while other wax components show minor changes	3-ketoacyl-CoA synthase of fatty acid elongase.	Todd <i>et al.</i> , 1999
WSD1 At5g37300	All the components in alkane forming pathway, free fatty acids, and primary alcohols are identical to WT, and there are no accumulation	Wax synthase/DGAT	Li <i>et al.</i> , 2008

	of wax esters		
MAH1 At1g57750	Stem wax load 40% of WT, Alkanes and wax esters are slightly increased, while secondary alcohols and ketones are highly reduced	Midchain alkane hydroxylase (CYP96A15)	Greer <i>et al.</i> , 2007.
PAS2 At5g10480	All aliphatic components are dramatically reduced, such as alkanes and primary alcohols; VLCFAs with chain length shorter than C22 are slightly increased.	3-hydroxy-acyl-CoA dehydratase (HCD).	Bach <i>et al.</i> , 2008.
WBC11 At1g17840	Stem wax load about 50% of WT. The C29 alkane is highly reduced, and C26/C28 primary alcohols and wax esters are moderately reduced.	ATP binding cassette transporter.	Bird <i>et al.</i> , 2007.
LPTG At1g27950	Stem wax load 75% of WT. The C29 alkane is 50% reduced compared with WT, other wax components are not significantly different from WT.	Glycosylphosphatidylinositol-anchored lipid transfer protein.	DeBono <i>et al.</i> , 2009; Lee <i>et al.</i> , 2009.
KCR1 At1g67730	Stem wax load 12% of WT. All compound classes are reduced, especially C29 alkane, C29 secondary alcohol, and C29 ketone.	β -ketoacyl-CoA reductase (KCR).	Beaudoin <i>et al.</i> , 2009.

2.2 Materials and Methods

2.2.1 Plant materials and growth conditions

Two wild-type ecotypes of *Arabidopsis thaliana* were used: Columbia-0 (Col-0) and Landsberg *erecta* (Ler), which were kindly provided by Professor Ljerka Kunst of the Department of Botany, University of British Columbia. *Eceriferum* (*cer*) mutants were obtained from Professor Ljerka Kunst who obtained them from ABRC (Arabidopsis Biological Resource Centre). All the *line* mutants were identified by Professor Owen Rowland.

Seeds were sown onto AT plates. AT medium contents: 50mM Fe(Ethylenediaminetetraacetic Acid), 5mM KNO₃, 2mM Ca(NO₃)₂, 2mM MgSO₄, 2.5mM KH₂PO₄, 1mL/L micronutrients (stock: 70mM H₃BO₃, 15mM MnCl₂x2H₂O, 0.5mM CuSO₄, 1mM ZnSO₄x7H₂O, 0.2mM NaMoO₄x2H₂O, 10mM NaCl, 0.01mM CoCl₂x6H₂O) and 7g/L agar (BioShop, Burlington ON). Plates containing sown seeds were incubated at 4°C for 3-4 days to break dormancy and to give synchronous seed germination. The plates were then moved to a tissue culture cabinet with continuous light and at 22°C temperature for 1-2 weeks. The seedlings were then transferred to soil. Soil was purchased from Berger Peat Moss-Le Group Berger Itee (Saint-Modestem, QC). The soil was soaked with water and autoclaved before using. 45mL of 1 g/L 20-20-20 all purpose fertilizer Plant-Pod[®] (Plant Product Co. Ltd, Brampton ON) was added to each square pot (9cm by 9cm). Plants were grown in an environment-controlled growth chamber (Conviron, Controlled Environment Ltd, Winnipeg, MB) with a long-day photoperiod (18 hours light and 6 hours dark) and at 22°C.

2.2.2 Scanning electron microscopy

Scanning electron microscopy (SEM) was used to examine the densities and shapes of epicuticular wax crystals of each mutant and wild-type plant. One cm stem fragments taken from the middle of the plant inflorescence were cut from 6 week-old plants and placed on an SEM stub, which had been affixed with a double-sided sticky tab. The SEM sample platform was then placed in a sealed plastic container containing a layer of drierite (W.A.Hammond Drierite). Samples were dried for 3-4 days, and then coated with 15nm of gold using a gold sputter coater (Anatech Hummer). The surfaces were subsequently imaged on a JSM-6400 scanning electron microscope (JEOL) located at the Nanoprobe (SEM) facility of Carleton University.

2.2.3 Gas chromatography

Gas chromatography (GC) was performed to determine the total wax load and chemical composition of stems and leaves of each mutant. All stem samples were isolated from six week-old plants and rosette leaf samples were taken from 5-6 week-old plants.

A digital caliper was used to measure the diameter of the stem, taken at 3 positions: the top, the middle, and the bottom of the selected segment and the average was then used to calculate the surface area of the stem. The length of stem used was typically 9cm-12cm. For each repeat, 2-3 stem segments of similar lengths were used in order to ensure a sufficient amount of wax was extracted for GC analysis. Three duplicates of each mutant and wild-type were prepared. For leaves, each repeat contained at least five rosette leaves and three duplicates were prepared.

All the GC extraction tubes were rinsed with HPLC-grade chloroform three times to eliminate contaminants. 10 μ l of 0.1 mg/ml internal standard: methyl heptadecanoate

(17:1), was added to each GC vial by using a 10µl Hamilton syringe. Each tube was then filled up with HPLC-grade chloroform. One extra GC extraction tube containing no wax sample was prepared and used as a blank control.

The stem segments were dipped together in HPLC-grade chloroform for exactly 30 seconds. Leaves were dipped in HPLC-grade chloroform one at a time for exactly 30 seconds, and then the leaves were wrapped in plastic wrap maintaining the shape of each rosette leaf. After dipping, the leaves were imaged on a stereomicroscope and the Zeiss Axio software used to determine the surface area of the leaves.

Following dipping, the chloroform was evaporated under a gentle stream of nitrogen. 100 µl of bis-(Trimethylsilyl) trifluoroacetamide (BSTFA; Pierce) was added to each tube for derivatization, and then incubated at 80°C for 90 min. All the samples were transferred to GC sample vials containing conical glass inserts and then 1µL injected on to 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, increased to 300°C at a rate of 4°C/min, and then maintained at 300°C for 15 min.

2.2.4 Complementation crosses

Complementation crosses were performed to determine allelism between *line* and *cer* mutants. Plants used for crossing were 4-5 weeks old. For the female parent, all the flowers and siliques near the top portion were removed using small scissors. Flower buds, typically 2-3, were identified whose anthers had not begun to shed pollen onto the stigma. The rest of the flower buds were removed. Crossing tweezers were used to open each flower bud. All the sepals, petals and anthers were removed, leaving only the

carpels intact. An open flower from the male parent was removed using crossing tweezers and the convex surface of the anthers was brushed against the stigmatic surface of the exposed carpels on the female parent. Crosses were covered in plastic wrap until the siliques turned yellow. All the siliques were collected separately in sandwich bags (DE LUXE, Mississauga ON), and allowed to dry at room temperature for at least 2 weeks before sowing.

2.3 Results

2.3.1 Survey of stem epicuticular wax crystal of *cer* mutants and *line* mutants

Scanning electron microscopy (SEM) was performed to examine the epicuticular crystals of a collection of mutant plants with glossy stems. I analyzed 10 uncharacterized *line* mutants, which had been earlier identified by the Rowland lab (unpublished). For comparison, I also analyzed 11 *cer* mutants, which were previously reported, but in many cases the SEM images of wax crystals were not of high clarity (Koornneef *et al.*, 1989; Aarts *et al.*, 1995; Negruk *et al.*, 1996; Fiebig *et al.*, 2000; Chen *et al.*, 2003; Pighin *et al.*, 2004; Rashotte *et al.*, 2004; Zheng *et al.*, 2005; Rowland *et al.*, 2006; Hooker *et al.*, 2007) (Figure 6 and 7). A variety of alterations were observed in the wax crystal density and/or crystal shapes in the sets of mutants. The cuticle surface features were different for all of the *cer* and *line* mutants compared to wild-type, as described in more detail below when comparing groups of phenotypically related mutants.

2.3.2 Wax load and composition of *cer* mutants and *line* mutants

Gas chromatography was performed to determine the total wax load and wax composition of *cer* mutants, *line* mutants, and corresponding wild type ecotypes (Col-0 or Ler) (Table 2 and 3). As expected, all of the *cer* and *line* mutants had alterations in their total wax load and/or composition. The proportional changes in wax compositions of the *cer* mutants were consistent with that reported in the literature (Jenks *et al.*, 1995; Rashotte *et al.*, 2001). The alterations of wax compositions in the *line* mutants are described in more detail below when comparing groups of phenotypically related mutants.

Figure 6 Scanning electron microscopy images of stem surfaces of *Ler*-WT and 11 *cer* mutants

Epicuticular wax crystals on stems of 11 *Arabidopsis cer* mutants and the corresponding wild type ecotype (*Ler*-WT) were imaged by SEM and images taken at magnifications of 1500X, 5000X, and 10000X, respectively. The scale bars are shown at the bottom of each panel.

Figure 7 Scanning electron microscopy of stem surfaces of 10 *line* mutants and the corresponding wild-type ecotypes

Epicuticular wax crystals on stems of 10 *Arabidopsis line* mutants and the corresponding wild type ecotypes (Col-0 or Ler) were imaged by SEM and images taken at magnifications of 1500X, 5000X, and 10000X, respectively. The scale bars are shown at the bottom of each panel.

Table 2 The total wax load and wax composition from stems of *cer* mutants and wild type ecotypes.

	Total wax load	Total FFA	Total 1°OH	Total ALK	Total ALD	Total KETO	Total 2°OH
<i>Ler</i> -WT	17.78± 1.12	0.83± 0.04	1.21± 0.02	6.87± 0.41	0.74± 0.09	1.73± 0.11	4.25± 0.24
<i>cer1</i>	4.25± 0.98	0.58± 0.11	0.78± 0.21	0.51± 0.15	0.66± 0.02	0.08± 0.02	0.35± 0.08
<i>cer2</i>	27.42± 2.19	0.78± 0.18	3.93± 0.12	11.52± 1.46	0.87± 0.06	2.11± 0.08	6.07± 0.61
<i>cer3</i>	9.36± 1.73	0.61± 0.12	1.03± 0.17	1.61± 0.66	0.08± 0.03	0.18± 0.11	0.83± 0.42
<i>cer4</i>	26.07± 3.11	0.72± 0.02	0.16± 0.03	15.34± 1.25	0.67± 0.19	2.34± 0.21	6.23± 0.44
<i>cer5</i>	12.84± 2.59	0.45± 0.12	1.17± 0.24	1.41± 0.29	0.09± 0.01	0.88± 0.11	2.11± 0.14
<i>cer6</i>	13.01± 2.49	0.58± 0.12	2.19± 0.19	1.21± 0.27	0.78± 0.14	0.11± 0.02	0.36± 0.01
<i>cer7</i>	14.12± 3.17	0.48± 0.17	1.83± 0.19	2.66± 0.18	0.14± 0.01	0.41± 0.03	1.37± 0.15
<i>cer9</i>	11.06± 1.61	1.32± 0.28	2.51± 0.19	2.01± 0.13	0.38± 0.04	0.44± 0.02	1.53± 0.11
<i>cer10</i>	14.61± 2.05	0.41± 0.08	1.01± 0.15	4.62± 0.45	0.25± 0.2	0.57± 0.07	1.63± 0.24
<i>cer19</i>	10.01± 1.98	0.82± 0.21	1.35± 0.11	1.11± 0.04	0.23± 0.03	0.27± 0.01	0.99± 0.05

FFA: Free fatty acid; 1°OH: Primary fatty alcohol; ALK: Alkane; ALD: Aldehyde;

2°OH: Secondary alcohol

All values are the means from three replicates and ± standard deviation is shown. The amounts, presented in µg/cm², of the various chain lengths of the homologue series of each chemical type were combined to give the 'total' amount for each.

Table 3 The total wax load and wax composition from stems of *line* mutants and wild type ecotypes.

	Total wax load	Total FFA	Total 1°OH	Total ALK	Total ALD	Total KETO	Total 2°OH
Col- WT	19.99± 0.84	0.74± 0.05	1.68± 0.14	7.75± 0.27	0.87± 0.09	1.69± 0.06	4.71± 0.24
Ler-WT	17.79± 1.13	0.83± 0.04	1.2± 0.02	6.87± 0.41	0.74± 0.09	1.73± 0.1	4.25± 0.24
<i>line6</i>	21.54± 1.15	0.32± 0.11	0.25± 0.11	8.58± 1.2	1.4± 0.84	1.39± 0.41	3.87± 0.56
<i>line8</i>	10.23± 1.12	0.98± 0.11	2.36± 0.37	1.18± 0.14	0.79± 0.22	0.18± 0.02	0.78± 0.11
<i>line11</i>	11.67± 3.91	0.38± 0.2	1.57± 0.41	2.27± 0.53	0.13± 0.04	0.36± 0.1	1.13± 0.24
<i>line13</i>	9.91± 0.92	0.57± 0.07	1.17± 0.05	2.02± 0.29	0.04± 0.01	0.24± 0.04	1.13± 0.16
<i>line14</i>	10.25± 1.49	0.48± 0.11	1.29± 0.48	2.43± 0.55	0.16± 0.1	0.33± 0.07	1.12± 0.18
<i>line22</i>	17.34± 3.39	0.34± 0.12	0.25± 0.08	6.14± 1.59	0.59± 0.23	1.17± 0.29	3.44 0.84
<i>line23</i>	13.24± 4.16	0.49± 0.21	1.21± 0.56	2.08± 0.67	0.98± 0.27	0.27± 0.01	0.82± 0.3
<i>line28</i>	10.16± 2.21	0.66± 0.18	1.02± 0.21	0.64± 0.29	1.1± 0.12	0.06± 0.02	0.21± 0.04
<i>line85</i>	8.77± 1.3	0.77± 0.11	1.27± 0.12	1.07± 0.08	0.19± 0.03	0.26± 0.02	0.98± 0.09
<i>line98</i>	18.44± 1.63	0.29± 0.16	0.20± 0.11	7.08± 1.01	1.23± 0.26	1.16± 0.15	3.27± 0.71

FFA: Free fatty acid; 1°OH: Primary fatty alcohol; ALK: Alkane; ALD: Aldehyde;

2°OH: Secondary alcohol

All values are the means from three replicates and ± standard deviation is shown. The amounts, presented in $\mu\text{g}/\text{cm}^2$, of the various chain lengths of the homologue series of each chemical type were combined to give the 'total' amount for each.

2.3.3 Comparisons of phenotypically similar *cer* and *line* mutants

After comparing the epicuticular wax crystal density and shape between *cer* and *line* mutants based on SEM images and the cuticular wax compositions of *cer* and *line* mutants based on GC data, I predicted the following: 1) *line6*, *line22*, and *line98* are allelic with *cer4* (Figure 8), 2) *line11*, *line13*, and *line14* are allelic with *cer3* (Figure 9), 3) *line28* is allelic with *cer1* (Figure 10), 4) *line23* is allelic with *cer7* (Figure 11), and 5) *line8* and *line85* are novel loci affecting cuticular wax production (chapter 3).

SEM images of *line6*, *line22*, and *line98* have a similar wax crystal alteration as found for *cer4*. They all contain platelet-shape wax crystals, while the typical tube-shape wax crystals on wild-type were missing (Figure 8A). The total stem wax loads of all four mutants are similar to corresponding wild-type ecotype (Table 2, 3). Primary alcohols with chain lengths C26-C30 of *line6*, *line22*, *line98*, and *cer4* were dramatically reduced, while all the individual components of the alkane forming pathway were slightly increased (Figure 8B). There was considerable variability of the C30 aldehyde, but this peak may be co-eluting with other unknown compounds. The F1 generations of complementation crosses between each *line* mutant and *cer* mutant had a glossy phenotype, confirming that *line6*, *line22*, and *line98* are allelic to *cer4* (Table 4).

The wax crystal density of *line11*, *line13*, *line14*, and *cer3* were significantly reduced and were characterized by dispersed longitudinal rodlets with a cap on top (Figure 9A). The total stem wax loads of *line11*, *line13*, and *line14* were highly reduced. All the components in alkane forming pathway are reduced, while primary alcohols are increased, especially the C30 primary alcohol (Figure 9B). These wax composition defects are similar to that of *cer3*. A complementation cross test confirmed that *line13* is

allelic to *cer3* (Table 4). Because these mutants are partially sterile, the complementation crosses between *line11*, *line14*, and *cer3* were not successful and no definitive conclusion on allelism could be made.

The wax crystals of the stem surfaces of *line28* and *cer1* are completely absent (Figure 10A). The total stem wax load and all wax components of both these two mutants are highly reduced, especially the major wax components, such as C29 alkane, C29 secondary alcohol, and C29 ketone (Table 2, 3; Figure 10B). Complementation crosses confirmed that *line28* is allelic to *cer1* (Table 4).

The SEM images are very similar between *line23* and *cer7*, characterized by similar reductions in wax crystal density and slender longitudinal rodlets capped with dendritic formations (Figure 11A). *line23* and *cer7* have similar reductions in C29 alkanes, secondary alcohols, and dramatic increases in C24 primary alcohols and C33 alkanes (Figure 11B). However, there were some differences. For example, *cer7* had a major increase of C30 primary alcohols while *line23* is decreased in C30 primary alcohols relative to wild-type. Also *cer7* had a reduction in C30 aldehydes, while *line23* was not significantly affected in this chemical class relative to wild-type. The F1 plants derived from complementation crosses between *line23* and *cer7* had glaucous (waxy) stems, indicating that *line23* is in fact not allelic to *cer7* (Table 4).

Figure 8 Comparison of wax crystal shape and cuticular wax composition between *line6*, *line22*, *line98* and *cer4*

(A) Scanning electron microscopy images of *line6*, *line22*, *line98*, *cer4* and the corresponding wild type ecotypes. The SEM images were taken at magnifications of 1500X, 5000X, and 10000X, respectively. The scale bars are shown at the bottom of each panel.

(B) Stem cuticular wax composition of *line6*, *line22*, *line98*, *cer4* and corresponding wild type ecotypes sorted by chemical type.

All values are the means, presented in $\mu\text{g}/\text{cm}^2$, derived from three replicates (n=3). Error bars represent the standard deviation.

Figure 9 Comparison of wax crystal shape and cuticular wax composition between *line11*, *line13*, *line14*, and *cer3*.

(A) Scanning electron microscopy images of *line11*, *line13*, *line14*, *cer3* and the corresponding wild type ecotypes. The SEM images were taken at magnifications of 1500X, 5000X, and 10000X, respectively. The scale bars are shown at the bottom of each panel.

(B) Stem cuticular wax composition of *line11*, *line13*, *line14*, *cer3* and corresponding wild type ecotypes sorted by chemical type.

All values are the means, presented in $\mu\text{g}/\text{cm}^2$, derived from three replicates ($n=3$).

Error bars represent the standard deviation.

Chemical Types and Chain Lengths

FFA: Free fatty acids; OH: Primary alcohols; ALK: Alkanes; ALD: Aldehydes; ZOH: Secondary alcohols; KETO: Ketones

Figure 10 Comparison of wax crystal shape and cuticular wax composition between *line28* and *cer1*.

(A) Scanning electron microscopy images of *line28*, *cer1* and the corresponding wild type ecotype. The SEM images taken at magnifications of 1500X, 5000X, and 10000X, respectively. The scale bars are shown at the bottom of each panel.

(B) Stem cuticular wax composition of *line28*, *cer1* and corresponding wild type ecotype sorted by chemical type.

All values are the means, presented in $\mu\text{g}/\text{cm}^2$, derived from three replicates (n=3).

Error bars represent the standard deviation.

Chemical Types and Chain lengths

FFA: Free fatty acids; OH: Primary alcohols; ALK: Alkanes; 2OH: Secondary alcohols; KETO: Ketones

Figure 11 Comparison of wax crystal shape and cuticular wax composition between *line23* and *cer7*.

(A) Scanning electron microscopy images of *line23*, *cer7* and the corresponding wild type ecotypes. The SEM images taken at magnification of 1500X, 5000X, and 10000X, respectively. The scale bars are shown at the bottom of each panel.

(B) Stem cuticular wax composition of *line23*, *cer7* and corresponding wild type ecotypes sorted by chemical type.

All values are the means, presented in $\mu\text{g}/\text{cm}^2$, derived from three replicates (n=3).

Error bars represent the standard deviation.

Chemical Types and Chain Lengths

FFA: Free fatty acids; OH: Primary alcohols; ALK: Alkanes; ALD: Secondary alcohols; KETO: Ketones

Table 4 Complementation crosses between similar *line* mutants and *cer* mutants

♀ X ♂	No. of successful crosses/total crosses	F1 generation phenotype	Allelism
<i>line6</i> X <i>cer4</i>	2/3	Glossy	Yes
<i>cer4</i> X <i>line6</i>	1/3	Glossy	Yes
<i>line22</i> X <i>cer4</i>	1/4	Glossy	Yes
<i>cer4</i> X <i>line22</i>	1/3	Glossy	Yes
<i>line98</i> X <i>cer4</i>	3/3	Glossy	Yes
<i>cer4</i> X <i>line98</i>	2/3	Glossy	Yes
<i>line13</i> X <i>cer3</i>	6/12	Glossy	Yes
<i>cer3</i> X <i>line13</i>	4/9	Glossy	Yes
<i>line28</i> X <i>cer1</i>	1/11	Glossy	Yes
<i>line23</i> X <i>cer7</i>	2/2	Waxy	No
<i>cer7</i> X <i>line23</i>	1/2	Waxy	No

2.4 Discussion

The cuticular wax phenotypes of a set of new wax deficient mutants, designated *line* mutants were compared to previously characterized *Arabidopsis cer* mutants. Sets of phenotypically similar *line* and *cer* mutants were crossed to test for allelism. From this analysis, I made the following conclusions: 1) *line6*, *line22*, *line98*, and *cer4* are allelic (Figure 8A, B; Table 4), 2) *line13* and *cer3* are allelic (Figure 9A, B; Table 4), 3) *line28* and *cer1* are allelic (Figure 10A, B; Table 4), 4) *line23* and *cer7* are not allelic (Figure 11A, B; Table 4). *Line8* and *line85* were predicted to be novel alleles and are the subject of Chapter 3.

CER4 encodes an alcohol-forming FAR, which is the major FAR responsible for primary alcohol formation in *Arabidopsis* stems (Hannoufa *et al.*, 1993; Jenks *et al.*, 1995; Rowland *et al.*, 2006). To date, several *cer4* mutants lines in different wild-type background have been identified and characterized (*Ler-cer4-1*, *WS-cer4-2*, *Col-cer4-3*, *Col-cer4-4*) (Rowland *et al.*, 2006). However, these are all T-DNA insertion mutants except for *cer4-1*, which is a deletion of most of the gene. Since *line6*, *line22*, and *line98* are all EMS mutagenized mutants, sequencing the *CER4* gene in these mutants may reveal important amino acids for enzyme activity or stability. Characterization of independently derived mutant alleles of *HOTHEAD* revealed important amino acids for activity of the encoded FAD-containing oxidoreductase involved in cutin formation (Krolikowski *et al.*, 2003). A similar approach could be taken with *CER4* as part of a structure function analysis.

CER3, which is also called *WAX2/YRE/FLP1*, has been cloned, but its function remains enigmatic (Chen *et al.*, 2003; Rowland *et al.*, 2007). Also, the predicted protein

is related to the *CER1* gene product, whose function is also currently unknown (Aarts *et al.*, 1995). The sequences of the two predicted proteins contain His-rich motifs characteristic of a class of integral membrane enzymes, and the N-terminal of CER3, which has six putative transmembrane domains, is highly related to that of CER1 (Chen *et al.*, 2003; Rowland *et al.*, 2007). Both CER3 and CER1 protein sequences suggest enzymatic roles in wax biosynthesis and the phenotypes of the mutants suggest roles in the upstream part of the alkane-forming pathway. This is the least understood part of cuticular wax biosynthesis. The identification of *line13* and *line28* as mutant alleles of *CER3* and *CER1*, respectively, may be useful for further molecular and biochemical characterization of *CER3* and *CER1*. For example, the mutations may reside in the His-rich motifs of the predicted proteins.

It was found that *line23* and *cer7* are not allelic. The wax composition profile of *line23* does not precisely match any of the *cer* mutants (this study; Jenks *et al.*, 1995; Rashotte *et al.*, 2001), but there was considerable variability in our wax composition analysis of *line23* and this could be masking phenotypic relationships. Products of both the alcohol-forming and alkane-forming pathways appear to be affected and thus the LINE23 protein may have an upstream role in the biosynthetic pathways, or affect transport or secretion of waxes. It is possible that *line23* is a novel *cer* loci, but more thorough analysis of *line23* wax composition and tests of allelism are required.

In summary, the SEM and GC methods were effective in phenotypically characterizing a collection of new wax-deficient mutants, such that they could be grouped together for subsequent tests of allelism by genetic complementation crosses. The Rowland lab has a much larger collection of uncharacterized mutants that could be

characterized in this manner. SEM analysis of plant surfaces is a relatively quick and easy way to phenotypically group mutants. However, the wax composition analysis is quite time-consuming and subject to distortion when low numbers of repeats are analyzed. A large-scale crossing strategy may be a more prudent approach, after general phenotypic groupings are assigned following imaging of wax crystals by SEM.

Chapter 3 Characterization and Mapping of Two Novel *eceriferum* Mutants

3.1 Introduction

3.1.1 Map-based cloning

Map-based cloning, also known as positional cloning, is widely used to identify the underlying genetic cause of a mutant phenotype by identifying the mutated gene responsible for it (Jander *et al.*, 2002). Map based cloning is based on the fact that genetic recombination and physical distance are directly related. As recombination events between homologous chromosomes decrease, so does the physical distance (Bortiri *et al.*, 2006).

In the past several years, map-based cloning has become a relatively straightforward and time efficient process in *Arabidopsis thaliana* because of three breakthroughs. First, the entire Columbia (Col-0) *Arabidopsis* genome has been sequenced (The *Arabidopsis* Genome Initiative, 2000). Secondly, the partial sequencing of Landsberg *erecta* (Ler) and other ecotypes which revealed a wide selection of randomly distributed genetic markers polymorphisms available for analyses (Alonso and Ecker, 2006). Third, the methods for rapidly detecting DNA polymorphisms have improved (Jander *et al.*, 2002).

Col-0 crossed to Ler (Col-0 X Ler) is the most commonly used combination for mapping purposes. Thousands of DNA polymorphisms between these two ecotypes have been identified, which allows easy design of new molecular markers (Lukowitz *et al.*, 2000). Simple sequence length polymorphisms (SSLPs) are the most useful markers for first-pass mapping. The SSLP markers exploit the variability of short repetitive sequences in genomes. Additionally, cleaved amplified polymorphic

sequences (CAPs) and derived CAPs (dCAPs) are also effective markers. CAPs markers exploit polymorphic restriction endonuclease sites, while dCAPs marker can exploit almost all single nucleotide changes for mapping purposes. All of these markers are codominant and can be visualized quickly and inexpensively using polymerase chain reaction (PCR), followed by DNA digestion with restriction endonucleases in the case of CAPs and dCAPs markers, and then gel electrophoresis (Peters *et al.*, 2003).

By taking advantage of a sequenced *Arabidopsis* genome and a large collection of genetic markers, map-based cloning becomes a relatively straightforward and time-efficient process. The mapping process can be generally divided into four steps. The first step in map-based cloning is to generate a mapping population. The mutant (e.g. in Col-0 ecotype) is out-crossed to another ecotype (e.g. in Ler ecotype), and then F1 individuals are allowed to self-pollinate. F2 seeds are collected for growth of the mapping population and plants identified that are homozygous mutant (in the case of recessive mutations). Linkage tests to markers polymorphic spread over the five *Arabidopsis* chromosomes are determined during this stage and a three-point cross used to define flanking molecular markers. A dense collection and variety of genetic markers and a larger F2 population are used for fine-resolution mapping in order to narrow down the location of the mutated gene. Once a small genomic region containing the mutation of interest has been determined, typically three procedures are undertaken to identify the gene. One method is sequencing of candidate genes, a second is molecular complementation by transforming wild-type candidate genes into the mutant line(s), and the third is to perform a genetic complementation cross of the mutant with plants that have the candidate genes knocked out by a T-DNA insertion.

3.1.2 Physiological experiments

The cuticle is a lipophylic barrier that protects plants from damage caused by abiotic and biotic stresses. Loss-of-function mutants that affect cuticular wax biosynthesis typically have glossy stems due to altered light reflection from the epicuticular wax crystals. Some of these mutants also display additional phenotypes, such as stunted growth, elevated transpiration rate, increased sensitivity to chemicals, morphological irregularities in cell shape, and strongly perturbed cuticle permeability (Jenks *et al.*, 2002; Yephremov and Schreiber, 2005; Nawrath, 2006). The movement of water between the outer cell wall of the epidermis and the atmosphere adjacent to the plant is largely controlled by the plant cuticle and stomata. Transportation across the cuticle is controlled through simple diffusion along a concentration gradient (Riederer and Schreiber, 2001).

In order to investigate the integrity and permeability properties of the cuticle, specific physiological assays have been developed. These include the fresh water loss assay, chlorophyll leaching assay, and toluidine blue (TB) assay (Lolle *et al.*, 1997; Wellesen *et al.*, 2001; Tanaka *et al.*, 2003). Studies indicate that the epicuticular wax and inner cuticular layers highly influence the rate of water loss (Aharoni *et al.*, 2004). Therefore, water loss can be taken as a measure of cuticular permeability. The mechanism of chlorophyll leaching assay is similar to the water loss assay, except that the non-polar molecule chlorophyll is used. Toluidine blue (TB) is a hydrophilic dye that is generally used for histological staining of plant cell walls. Leaf surfaces without a functional cuticle are more permeable to TB and stain blue (Tanaka *et al.*, 2003).

Therefore applying the TB assay can reveal cuticle defective mutants even as a forward genetic screen.

Line8 and *line85* were described in Chapter 2 and speculated to be novel wax deficient loci based on their surface wax crystals and wax composition. The objective of this chapter was to further characterize *line8* and *line85*. The specific chromosome locations of *line8* and *line85* were determined by linkage analysis to molecular markers. Allelism test with nearby *cer* loci revealed that *line8* and *line85* were unique alleles of *cer6* and *cer19*, rather than novel loci. The permeability defects of these mutants were measured to gain further insights into the nature of the cuticular defects.

3.2 Materials and Methods

3.2.1 Plant materials and growth conditions

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.

3.2.2 Plant genomic DNA extraction

Plant genomic DNA was extracted in two different ways. All the DNA samples used for first-pass mapping were extracted using the 'Whatman FTA protocol'. A young leaf was directly pressed onto a Whatman FTA classic card (Cat. No. WB120205; Whatman International Ltd, England). A piece of parafilm was laid over the leaf. The end of a 50mL falcon tube was used to mash the leaf sample. The material on the FTA card was allowed to dry overnight at room temperature. A 1.2mm Micro-Punch (Harris) was used to punch each sample disc into a tube for subsequent PCR amplification of DNA markers. 50 µL of FTA purification reagent (10mM Tris-HCl, PH 7.5, 2mM EDTA, PH 8.0, 0.1mL/100mL Tween 20) was added to each tube containing the sample disk and incubated for 5 minutes at room temperature. The solution was then removed and this step was repeated. 200µL of 1 X TE buffer (10mM Tris-HCl, 0.1mM EDTA, PH 8.0) was added to each tube and incubated for 5 minutes at room temperature. This solution was removed and this step was repeated. All the reaction tubes containing the prepared disks were dried overnight at room temperature for PCR amplification.

The genomic DNA of samples used for fine-resolution mapping and sequencing was extracted by the 'Quick and Dirty protocol'. One rosette leaf was removed and placed into a 1.5mL microcentrifuge tube. A pestle was used to grind samples by hand

for about 15 seconds. 400µL of extraction buffer (200mM Tris-HCl, pH 7.5, 250mM NaCl, 25mM EDTA, 0.5% SDS) was added and vortexed for 5 seconds. After all the samples were ground, the mixtures were centrifuged at 15000 rpm in a microcentrifuge for 5 minutes. The supernatant was transferred to a new tube and 400µL of isopropanol was added. The solution was vortexed for 2 seconds and incubated for 20-30 min at room temperature. The solution was centrifuged at 15000 rpm for 5 min and the supernatant was discarded. 500 µL of 70% ethanol was added to each tube to remove excess salts and the pellet was allowed to dry overnight. The pellet was dissolved in 50 µL of deionized water (Sigma).

3.2.3 Strategy for designing primers for amplification of polymorphic markers

First, all the files for the *Arabidopsis* polymorphism collection and the Ler sequence were downloaded from the TAIR website: <http://www.arabidopsis.org>. Second, polymorphisms were chosen based on position and Insertion/Deletion (InDel) size. Usually, InDels were chosen that a difference in size of 10-40bp between Col-0 and Ler. Third, the immediate flanking sequences were used to search the Col-0 sequence using the TAIR website BLAST program (<http://www.arabidopsis.org/Blast/index.jsp>), choosing *A. thaliana* GB genomic (DNA) database. The 'hit' from the result was checked for consistency with the information from the original downloaded files, such as BAC name and left-coordinate number. The sequence surrounding the polymorphism was obtained from TAIR Sequence Viewer (<http://www.arabidopsis.org/servlets/sv>). The total sequence copied was around 200bp. This region was pasted into the Primer 3 website: <http://frodo.wi.mit.edu/> to design primers (parameters minimum size: 20; optimum size: 25; maximum size: 27). It was important to verify the polymorphism was

present in the amplified region from Col-0 and *Ler* genomic sequences. For this, a pairwise BLAST using the TAIR BLAST program was used to compare the Col-0 amplified product sequence and the *Ler* amplified product sequence, which was downloaded as described above.

3.2.4 PCR conditions

An Eppendorf AG 22331 Hamburg thermo cycler (No. 5341, Germany) was used to amplify DNA templates. Each 20 μ L volume PCR reaction mixture consisted of 2 μ L of 5ng/ μ L DNA template, 2 μ L of 10mM reverse primer, 2 μ L of 10mM forward primer, 2 μ L of 10 X PCR buffer [100mM KCl, 100mM (NH₄)₂SO₄, 200mM Tris-HCl PH 8.3, 1% Triton X-100], 0.5 μ L of 10mM dNTPs, 2 μ L of 25mM MgCl₂, 0.1 μ L of *Taq* DNA polymerase, and 9.4 μ L of deionized water. PCR reactions were performed with the following reaction conditions: initial denaturation at 94°C for 1 minute, followed by 40 cycles at 94°C for 30 seconds, annealing for 30 seconds (annealing temperature varies depending on the different pairs of primers, typically 55-60°C), and extension at 72°C for 30 seconds. These cycle parameters were followed by incubation at 72°C for 10 minutes, followed by 4°C for an indefinite time.

3.2.5 Agarose gel electrophoresis conditions

Gel electrophoresis was performed to analyze the PCR results. A 4% (w/v) agarose gel comprised of 1 X TAE buffer and 0.05% ethidium bromide was used to electrophoresis all the PCR products. 5 μ L of 5 X blue dye was added to each 20 μ L volume sample. Then, 13 μ L of mixed samples were loaded on to the gel. The agarose gel was run at 80 volts for about an hour. The gel bands were visualized using an Alphamager 2200 gel-imager system (Alpha Innotech, San Leandro, CA). For

amplification of genomic DNA fragments for sequencing, a 0.8% agarose gel was used, and run at 100 volts for about 20 minutes.

3.2.6 Mutation site verification

In order to verify the mutation sites in the *CER6* gene of *line8*, PCR primers were designed to amplify fragments of the *CER6* gene, based on the genomic DNA sequence obtained from TAIR (<http://www.arabidopsis.org>). Genomic annotation obtained from TAIR indicated that the length of the wild type *CER6* coding region was 2220 bps (from 5'-UTR to 3'-UTR). Two pairs of primers were designed using the same strategy for designing primers for amplification of polymorphic markers: LP1 (5'-GCAGCGATGAAAACAAA-3'), LP2 (5'-TGGTGATGTTTGCCTTGA-3'), RP1 (5'-GGCAAACATCACCACAAT-3'), RP2 (5'-TTCCTCTGTTCGCCTTTA-3'). *CER6* can be represented completely by the two PCR products (LP1+RP1 and LP2+RP2), which were each around 1200 bps. DNA fragments generated by PCR were purified using Wizard® Plus SV Minipreps DNA Purification System (Promega). The sequencing reactions were carried out at DNA Landmarks (Montreal, QC). The sequenced DNA fragments were assembled together using Seq Man II Lasergene software (DNASTAR, US).

3.2.7 Toluidine-blue (TB) assay

All the seeds were surface-sterilized with 50% bleach for 10 min and washed with dH₂O 5-6 times. The sterilized seeds were sown on a 0.7% AT plate (Somerville and Ogren, 1982) and incubated at 4°C for 3-4 days. All the plates were then moved to a Conviron chamber for 10 days. Each seedling was taken from the plate and soaked in the toluidine-blue (TB) solution for 2 min. TB solution is an aqueous solution of 0.05%

TB (Fluka) filtered through a 0.2 μm syringe filter (Acrodisc). Stained seedlings were washed gently with dH_2O to remove excess TB from surfaces. The Zeiss Axio program was used to take pictures immediately following staining.

3.2.8 Fresh water loss assay

A fresh water loss assay was performed to analyze epidermal permeability. Stem and leaf samples were used from 4-5 week-old plants. Plants were kept in the dark for at least 4 hours prior to analysis. Detached-rosette leaves and stems with cauline leaves removed were soaked in water for 60 min in the dark. Rosettes and stems were shaken gently to remove excess water and kept in complete darkness to keep stomata closed. The tissues were weighed at regular intervals for 1 hour using a microbalance (TE2145, Sartoris). The rate of water loss per gram of fresh weight tissue was determined by the formula $(\text{initial weight} - \text{measured weight})/\text{initial weight}$.

3.2.9 Chlorophyll-leaching assay

Chlorophyll-leaching was performed using rosette leaves of 3-week old plants. The detached-rosette leaves were weighed and then immersed in 30mL of 80% ethanol at room temperature in the dark. Test tubes with rosette and ethanol mixtures were shaken gently and 200 μL aliquots of supernatant were transferred separately into different 1.5mL tubes at 10, 20, 30, 40, 50, 60, 90, 120 minutes after starting. After all of the samples were taken, 100 μL aliquots were transferred into a 96 microwell plate (Lot No. 05370115, Greiner bio-one) from each 1.5mL tube. The plate was read on a UV microwell spectrophotometer (Molecular Devices Spectra max 340 PC 384) at absorbances of 647nm and 664nm. The total micromoles of chlorophyll leached per

gram of fresh weight tissue were calculated using the equation: total micromoles of chlorophyll = $7.93(A_{664}) + 19.53(A_{647})$ (Lolle *et al.*, 1997).

3.3 Results

3.3.1 Rough mapping: linkage analysis of *line8* and *line85* to molecular markers.

A collection of polymorphic DNA markers were selected that span the five chromosomes (Figure 12). Some of these were taken from Lukowitz *et al* (2000) and some were designed by myself. The detailed information of these markers and primers for amplification by PCR are summarized in Table 5.

Upon first-pass mapping of selected markers from Figure 12, *LINE8* showed linkage to marker nga111 at the bottom of chromosome I, with a recombination frequency of 7.5% based on 50 samples; *LINE85* showed linkage to marker CER449723 at the top of chromosome I, with a recombination frequency of 17.7% based on 50 samples (Figure 12). *LINE8* had a recombination frequency of 40-55% with all of the other markers indicating that they are not linked. *LINE85* had recombination frequencies of 51.8% and 44.7% to markers on the bottom of chromosome IV and V, respectively, indicating that these two markers were unlinked.

Table 5 Polymorphic markers and amplification primers for first-pass mapping

Marker name	Type	Chr.	BAC	Position (Mbp)	Forward Primer	Reverse Primer	Col-0 size (bp)	Ler size (bp)
CER449723	InDel	1	F17F8	11	TTGGATTATCTCTTGAATGGGTTTGG	CCGTCCTTTAGCATTAGGAACTCTGGTG	216	164
NGA280	SSLP	1	F14J16	20.9	CTGATCTCACGGACAATAGTGC	GGCTCCATAAAAAGTGCACC	105	85
NGA111	SSLP	1	F28P22	27.35	TGTTTTTTAGGACAAATGGCG	CTCCAGTTAAGCTAAAGGG	128	162
CER459234	InDel	2	T20F6	0.8	TCCTTTCTTAGCAGCCCTTCACAGATG	TCATGAAGAAGATGTTCTGTAGGAGC	228	179
CER459006	InDel	2	T1B8	14.3	GTCTGGATCTTGTATCGCAAAACCAAA	TGCTTCAGAGCTGATGAACAAAAAGAC	199	154
CER457071	InDel	3	MSJ11	5.3	TAACCACACACATCGTGTTTTGTCC	GGGTCTGCTCATTTCTTCAGTTCTTGT	174	153
CER470856	InDel	3	T25B15	19.4	ATACTTGGGTCCTTCGTCC	GTCGCGAGTTCTCTCCAATC	110	92
CER466221	InDel	4	F18A5	8	TCAAACGCCCAACTGAATCAA	CCTGGTGTGTGTGTGGACT	216	191
CER458597	InDel	4	T16L1	16.1	AAAATAACTTTAGAGAAGGA	GTAGCTAACGATCATGTGAG	191	150
NGA151	SSLP	5	T15N1	4.6	CAGTCTAAAAGCGAGAGTATGATG	GTTTTGGGAAGTTTTGCTGG	150	120
CER462550	InDel	5	MFO20	17.1	AAACATTTGGGCAGGGGTGGATA	GTGGCACTCACTGGGTGTGATGA	237	195

Figure 12 Physical map positions of polymorphic markers for first-pass mapping

Vertical lines and roman numbers represent the five *Arabidopsis* chromosomes. Red values represent recombination frequency of linkage markers to *line8* and purple values represent recombination frequency of linkage markers to *line85*.

3.3.2 Comparisons of crystal morphology and wax composition between *line8* and *cer6*.

First-pass mapping of *line8* showed linkage to the marker NGA111 at position 27.35 Mbp on chromosome I. This position was physically close to the *CER6* locus (Fiebig *et al.*, 2000). Thus, a detailed comparison of the epicuticular wax crystal density and shape were conducted using SEM, and comparison of wax load and composition conducted by GC.

The wax crystals on the stem surfaces of *line8* were reduced in density, but not absent (Figure 13A). The shape of *line8* crystals was very unique, being flattened and irregular in shape reminiscent of fishbones. This, in part, led us to speculate that *line8* was a unique loci affecting cuticular wax metabolism. The stem surfaces of *cer6* mutants are devoid of crystals (Figure 13A).

However, inspection of the cuticular wax composition of *line8* stems revealed much similarity with the reported *cer6-1* phenotype (Jenks *et al.*, 1995). Both *line8* and *cer6* have reductions in C28 and C30 free fatty acids and large increase in C24 and C26 free fatty acids (Figure 13B). Primary alcohols of chain lengths C24 and C26 are also significantly increased in both mutants. Aldehydes, alkanes, secondary alcohols, and ketones (alkane-forming pathway components) with chain lengths beyond C26 are reduced in both mutants, while chain lengths C26 and lower are increased. *CER6* encodes a ketoacyl CoA synthase involved in fatty acid elongation, consistent with this phenotype (Millar *et al.*, 1999; Fiebig *et al.*, 2000).

Figure 13 Comparison of wax crystal shape and cuticular wax composition between *line8* and *cer6*

(A) Scanning electron microscopy images of *line8*, *cer6* and the corresponding wild type ecotype. The SEM images taken at magnifications of 1500X, 5000X and 10000X, respectively. The scale bars are shown at the bottom of each panel.

(B) Stem cuticular wax composition of *line8*, *cer6* and the corresponding wild-type ecotype (Col-0) sorted by chemical type.

All values are means, presented in $\mu\text{g}/\text{cm}^2$, derived from three replicates (n=3). Error bars represent the standard deviation.

Chemical Types and Chain Lengths

FFA: Free fatty acids; OH: Primary alcohols; ALK: Alkanes; ALD: Aldehydes; 2OH: Secondary alcohols; KETO: Ketones

3.3.3 Sequencing of *CER6* gene in the *line8* mutant and alignment of predicted protein sequences

Since the wax composition of *line8* mutant is similar to that of *cer6* and the two loci are linked, it was possible that *line8* was an unusual allele of *CER6*. The *CER6* gene was sequenced in the *line8* mutant to test for the presence of a mutation in order to confirm that *line8* is indeed an allele of *cer6*. The *CER6* gene in *line8* was found to have a one base pair substitution at 1190bp position that changed position 397 alanine, a small hydrophobic amino acid, to valine, a larger hydrophobic amino acid (Figure 14).

The amino acid sequence of CER6(A397V) was compared with wild-type CER6 (At1g68530), KCS17 (DAISY, At1g04220), and FAE1 (KCS18, At4g34520), three genes from *Arabidopsis* that are highly expressed in epidermis, roots, and seeds, respectively (Millar *et al.*, 1999; Rossak *et al.*, 2001; Franke *et al.*, 2009). Two KCSs from other species, StKCS6 from *Solanum tuberosum* (potato) (Serra *et al.*, 2008) and LfKCS45 from *Lesquerella fendleri* (Moon *et al.*, 2005), were also included. Comparison of sequence alignment revealed the presence of six conserved cysteine residues and four histidines (Figure 14), which are important for enzyme activity (Ghanevati and Jaworski, 2001). A397 was highly conserved in all the aligned putative KCSs. Moreover, the A397V mutation site is in close proximity to the conserved cysteine residue at position 390.

3.3.4 Complementation crosses between *line8* and *cer6*

To test for allelism between *line8* and *cer6*, reciprocal complementation crosses between *line8* and *cer6* were performed. The glossy phenotype in the F1 generation from complementation crosses confirmed that *line8* is allelic to *cer6* (Figure 15). We

therefore re-name *line8* as *cer6-3*, continuing from the allelic numbering described in Fiebig *et al* (2000) for *cer6* mutants.

Figure 14 Amino acid sequence comparison of CER6(A397V), CER6(WT), KCS17, FAE1, LfKCS45, and StKCS6

GenBank accession numbers of the sequences used are: *Arabidopsis* CER6(WT), NP_177020; *Arabidopsis* KCS17, NP_171918; *Arabidopsis* FAE1, Q38860; *Lesquerella fendleri* LfKCS45, AAU05611; potato StKCS6, ACF17125. Identical residues are highlighted on a black background; similar amino acids are shown on a gray background. Conserved cysteine and histidine residues are labeled with red triangles and green diamonds, respectively. The mutation site of CER6(A397V) is labeled on a yellow background.

CER6 (WT) 1 M-----PCAPMPEFSSSVKLYVKLGYOYLVNHFLLPIMAVAVE
 CER6 (A397V) 1 M-----PCAPMPEFSSSVKLYVKLGYOYLVNHFLLPIMAVAVE
 StKCS6 1 M-----E-EPVPMFSSSVKLYVKLGYOYLVNHFLLPIMAVAVE
 RCS17 1 MNENH-QSDHMMNTIHVTKKLPNELLSVALKYVKLGYYLSSNAYYLPPVGLPAITS
 FAE1 1 M-----TSVNVKLLRYVLTNFFNLCLEPDTAFAGK
 LfKCS45 1 M-----ANVKLLRYVLTNFFNLCLEPDTAFAGK

▽

CER6 (WT) 45 LLRMGPEEILNWNLSQFDLVQVLCSSFFIFISTVYFMSKPRRIYLVDSYCKPPVTCR
 CER6 (A397V) 45 LLRMGPEEILNWNLSQFDLVQVLCSSFFIFISTVYFMSKPRRIYLVDSYCKPPVTCR
 StKCS6 44 LRLGPEEILNWNLSQFDLVQVLCSSFFIFISTVYFMSKPRRIYLVDSYCKPPVTCR
 RCS17 61 SSFSLTLLILLYNHLKHFHLSSTLFAALLIFLLTLYFTTRPRRIYLVDSYCKPPVTCR
 FAE1 33 ASRLTINQLENFLSYLCHNLTITVLLFAFTTGLVLYLTPRPVYLVDSYCYLPPPHL
 LfKCS45 31 ASSLDCEELQNLWFLCHNLTITVLLFAVTTTSAIYFTTRPRVYLVDSYCHLPPPHQ

CER6 (WT) 105 VPFAIFME-----HSRLILKDKPKSEFQMRILERSGLGEETCLPPAIHYPPPT
 CER6 (A397V) 105 VPFAIFME-----HSRLILKDKPKSEFQMRILERSGLGEETCLPPAIHYPPPT
 StKCS6 104 VPFAIFME-----HSRLILKDKPKSEFQMRILERSGLGEETCLPPAIHYPPPT
 RCS17 121 CTRETFME-----RSQVNGIFTEDNRFQCIILERSGLGCKTYPEALLRPMPNP
 FAE1 93 VSVSKVMTIFYQIRKADTSSRNACDDPSSEFLRLKICERSGLGDETYSPEGLIHPPRK
 LfKCS45 91 ITVKKIENNVNKNRELDASHNSKIP--SSLDVRLRLERSGLGEETVPTSLNPPITQ

CER6 (WT) 155 TMDAARSEACVIFERADDLEKTKGKPKKDILIVNCSLSEPTPSLSAMVVKYKLRSN
 CER6 (A397V) 155 TMDAARSEACVIFERADDLEKTKGKPKKDILIVNCSLSEPTPSLSAMVVKYKLRSN
 StKCS6 154 TMDAARSEACVIFERADDLEKTKGKPKKDILIVNCSLSEPTPSLSAMVVKYKLRSN
 RCS17 171 CMSEARKEAETVIFCAIDALEKTKGKPKKDILIVNCSLSEPTPSLSAMVVKYKLRSN
 FAE1 153 TFAASREETEKVIFCAIDALEKTKGKPKKDILIVNCSLSEPTPSLSAMVVKYKLRSN
 LfKCS45 149 TMDAARSEETEKVIFCAIDALEKTKGKPKKDILIVNCSLSEPTPSLSAMVVKYKLRST

▽

CER6 (WT) 215 IKSFNLSGMGCSAGLISDLARDLLQVHPNSNAITVSTEITPNYYQGNERRAMLLPNCLE
 CER6 (A397V) 215 IKSFNLSGMGCSAGLISDLARDLLQVHPNSNAITVSTEITPNYYQGNERRAMLLPNCLE
 StKCS6 214 IKSFNLSGMGCSAGLISDLARDLLQVHPNSCALVVSTEITPNYYQGNERRAMLLPNCLE
 RCS17 231 WLSNLSGMGCSAGLISDLARDLLQVCPNSYALVVSTENITLNYHLGNDRSMLLSNCLF
 FAE1 213 IKSFNLSGMGCSAGLISDLARDLLQVHPNSYALVVSTENITCGIYAGNRRSMYNSCLF
 LfKCS45 209 IKSFNLSGMGCSAGLISDLARDLLQVHPNSYALVVSTENITRNMYVGNRRSMLVNCLE

▽

CER6 (WT) 275 RMGGAALFISNRRSDRRRAKYKLSHLVRTHGADDKSEFCVYEQEDPKCH--VGINLSKD
 CER6 (A397V) 275 RMGGAALFISNRRSDRRRAKYKLSHLVRTHGADDKSEFCVYEQEDPKCH--VGINLSKD
 StKCS6 274 RMGGAAILLSNRRSDRRRAKYKLSHLVRTHGADDKSEFCVYEQEDPKCH--VGINLSKD
 RCS17 292 RMGGAAILLSNRRSDRRRAKYKLSHLVRTHGADDKSEFCVYEQEDPKCH--VGINLSKD
 FAE1 273 RMGGAAILLSNRRSDRRRAKYKLSHLVRTHGADDKSEFCVYEQEDPKCH--VGINLSKD
 LfKCS45 269 RMGGAAILLSNRRSDRRRAKYKLSHLVRTHGADDKSEFCVYEQEDPKCH--VGINLSKD

▽

CER6 (WT) 333 LMAIAGEALKANITTGPLVLPASEQLLEFLSLIGRKIFNP-KWKPY-IPDFKLAFAEHFC
 CER6 (A397V) 333 LMAIAGEALKANITTGPLVLPASEQLLEFLSLIGRKIFNP-KWKPY-IPDFKLAFAEHFC
 StKCS6 332 LMAIAGEALKANITTGPLVLPASEQLLEFLSLIGRKIFNP-KWKPY-IPDFKLAFAEHFC
 RCS17 351 LMAIAGEALKANITTGPLVLPASEQLLEFLSLIGRKIFNP-KWKPY-IPDFKLAFAEHFC
 FAE1 331 LMAIAGEALKANITTGPLVLPASEQLLEFLSLIGRKIFNP-KWKPY-IPDFKLAFAEHFC
 LfKCS45 327 LMAIAGEALKANITTGPLVLPASEQLLEFLSLIGRKIFNP-KWKPY-IPDFKLAFAEHFC

CER6 (WT) 391 IHAGGRAVIDELCKNLQLSGEHVEASRMTLHRFGNTSSSSLWYELSYIESKGRMRAGDR
 CER6 (A397V) 391 IHAGGRAVIDELCKNLQLSGEHVEASRMTLHRFGNTSSSSLWYELSYIESKGRMRAGDR
 StKCS6 390 IHAGGRAVIDELCKNLQLSGEHVEASRMTLHRFGNTSSSSLWYELSYIESKGRMRAGDR
 RCS17 410 IHAGGRAVIDELCKNLQLSGEHVEASRMTLHRFGNTSSSSLWYELSYIESKGRMRAGDR
 FAE1 390 IHAGGRAVIDELCKNLQLSGEHVEASRMTLHRFGNTSSSSLWYELSYIESKGRMRAGDR
 LfKCS45 385 IHAGGRAVIDELCKNLQLSGEHVEASRMTLHRFGNTSSSSLWYELSYIESKGRMRAGDR

▽

CER6 (WT) 451 WQIAFGSGFKCNSAVWKCNRITIKTPKD-----GPWSDCIDRYPVFIPEVVKL-----
 CER6 (A397V) 451 WQIAFGSGFKCNSAVWKCNRITIKTPKD-----GPWSDCIDRYPVFIPEVVKL-----
 StKCS6 450 WQIAFGSGFKCNSAVWKCNRITIKTPD-----GPWSDCIDRYPVFIPEVVKL-----
 RCS17 470 WQIAFGSGFKCNSAVWVALRTIDPSKPKKKKTNPWIDSLIEHPVPVPTSPITSSSES-
 FAE1 450 WQIAFGSGFKCNSAVWVALRTIDPSKPKKKKTNPWIDSLIEHPVPVPTSPITSSSES-
 LfKCS45 445 WQIAFGSGFKCNSAVWVALRTIDPSKPKKKKTNPWIDSLIEHPVPVPTSPITSSSES-

Figure 15 Stem phenotypes of wild-type, *cer6* and F1 plants from complementation crosses between *line8* and *cer6*.

The stems from left to right are Col-WT, Ler-WT, *cer6*, *line8*, and F1 of *cer6* X *line8*.

3.3.5 Comparisons of crystal morphology and wax composition between *line85* and *cer19*.

First-pass mapping of *line85* showed linkage to the marker CER449723 at position 11.0 Mbp on chromosome I. This position was physically close to the reported *CER19* locus (Rashotte *et al.*, 2004), but the gene has not been cloned yet. Thus, a detailed comparison of the epicuticular wax crystal density and shape was conducted using SEM, and comparison of wax load and composition conducted by GC.

The wax crystals on the stem surfaces of *line85* were similar in shape to crystals on the stem surface of *cer19* (Figure 16A). However, the density of crystals was significantly lower on *line85* compared to *cer19* causing us to initially think that they are phenotypically distinct and representing different loci.

However, perturbations of cuticular wax composition in *line85* and *cer19* were nearly identical (Figure 16B). Both mutants have dramatically reduced levels of wax components that are C29 or higher, which is anything ultimately derived from C30 or longer fatty acyl-CoA precursors. Both the primary alcohol and alkane forming pathways were affected. C24 and C26 primary alcohols and C27 alkanes are both increased in *line85* and *cer19* compared to wild-type.

3.3.6 Complementation crosses between *line85* and *cer19*

The close proximity of the *line85* and *cer19* loci and similar wax composition defects suggested that they are in fact allelic. To test for allelism between the two mutants, reciprocal complementation crosses between *line85* and *cer19* were performed. The glossy phenotype in the F1 generation from complementation crosses confirmed that *line85* is allelic to *cer19* (Figure 17). Both mutants are recessive alleles

(data not shown; Koornneef *et al.*, 1989). We therefore re-name *line85* as *cer19-2*, with the original *cer19* mutant identified by Koornneef *et al* as *cer19-1*.

Figure 16 Comparison of wax crystal shape and cuticular wax composition between *line85* and *cer19*

(A) Scanning electron microscopy images of *line85*, *cer19* and the corresponding wild type ecotype (*Ler*-WT). The SEM images were taken at magnifications of 1500X, 5000X, and 10000X, respectively. The scale bars are shown at the bottom of each panel.

(B) Stem cuticular wax composition of *line85*, *cer19* and corresponding wild-type ecotype (*Ler*-WT) sorted by chemical type.

All values are means, presented in $\mu\text{g}/\text{cm}^2$, derived from three replicates ($n=3$). Error bars represent the standard deviation.

Chemical Types and Chain Lengths

FFA: Free fatty acids; OH: Primary alcohols; ALK: Alkanes; 2OH: Secondary alcohols; KETO: Ketones

Figure 17 Stem phenotypes of wild-type, *line85*, *cer19* and F1 plants from complementation crosses between *line85* and *cer19*.

The stems from left to right are Col-WT, Ler-WT, *line85*, *cer19*, and F1 of *line85* X *cer19*.

3.3.7 Cuticular permeability analysis

We extended the phenotypic analysis of *cer19-1* and *cer19-2* (*line85*) by measuring effects on cuticular permeability. Three measurements of cuticular permeability were performed: 1) toluidine blue (TB) assay (Figure 18), 2) rate of water loss from fresh tissue (Figure 19), and 3) rate of chlorophyll-leaching from fresh tissue (Figure 20).

The staining by TB was significantly increased in *cer19-1* and *cer19-2* in 10 day old seedlings compared to wild-type (Figure 18). This is indicative of a continuous defect in the developing cuticle of these seedlings. This is a class I staining pattern according to Tanaka *et al* (2003), whereas, mutants of other classes (II to V) have patchy, proximal-only, distal-only, or trichome-only staining patterns. There were no differences in the TB staining of adult plants between the *cer19* mutants and wild-type (data not shown).

The water loss assay measures the diffusion of non-stomatal water loss from plant tissues to the environment. The rosette leaves of *line85* and *cer19* both showed a slightly increased rate of water loss over time compared to wild-type (Figure 19A). But the error bars are huge, so the results may not be statistically significant. While stems showed similar rates of water loss between mutant and wild-type (Figure 19B).

A chlorophyll-leaching test of *cer19-1*, *cer19-2*, and Ler-WT plants was also performed as an additional measurement of cuticular permeability in adult plants. The detached rosette leaves of both *cer19-1* and *cer19-2* exhibited increased chlorophyll leaching in 80% ethanol compared to wild-type ecotype, especially apparent at 60 minutes (Figure 20). At two hours following immersion of tissue in 80% ethanol, the concentration of chlorophyll leached from *cer19-1* and *cer19-2* rosette leaves was 2-3

times greater than that of rosette leaves from *Ler*-WT plants. According to T-test statistical analysis, with $df=8$, t must be at least 2.5 to reach $p\text{-value}<0.05$. Our result was $t_{line85}=1.9225$; $t_{cer19}=1.8932$, so by conventional criteria, this difference is considered not to be statistically significant.

Figure 18 Toluidine blue staining of Ler-WT, *line85*, and *cer19* seedlings

TB stain on leaves of 10 day old seedlings of (A) Ler-WT, (B) *cer19-1*, and (C) *cer19-2*

Figure 19 Rate of water loss in fresh tissue of *cer19-1*, *cer19-2*, and *Ler-WT*

(A) Fresh weight assay with leaves of 5-week old *cer19-1*, *cer19-2*, and *Ler-WT* plants, where the rate of water loss per gram of fresh weight tissue from the rosette leaves was measured at the indicated time points (n=5; $\pm$ standard deviation). The plants were kept in the dark for at least four hours prior to measurement to make sure stomata were closed.

(B) Fresh weight assay with stems of 5-week old *cer19-1*, *cer19-2*, and *Ler-WT* plants, where the rate of water loss per gram of fresh weight tissue from stems was measured at the indicated time points (n=5; $\pm$ standard deviation). The plants were kept in the dark for at least four hours prior to measurement to make sure stomata were closed.

Figure 20 Chlorophyll-leaching assay of *cer19-1*, *cer19-2*, and *Ler*-WT leaves.

The concentration of chlorophyll leached over 2 hours into a solution of 80% ethanol from rosette leaves taken from 4 week old plants of *cer19-1*, *cer19-2*, and *Ler*-WT (n=10; $\pm$ standard deviation).

3.4 Discussion

I originally speculated that the mutants *line8* and *line 85* represented novel loci based on their unique defects in wax crystal formation and cuticular wax composition (Chapter 2). To test this, I first roughly mapped their respective genetic positions by linkage analysis to molecular markers spread out over the five Arabidopsis chromosomes. *LINE8* mapped near to the *CER6* locus and *LINE85* mapped near to the *CER19* locus. In addition, closer inspection of the cuticular wax defects revealed phenotypic relationships between the respective mutants. Complementation tests confirmed that *line8* is allelic to *cer6* and this was confirmed by the presence of a non-silent point mutation in the *CER6* gene present in the *line8* background. We therefore renamed *line8* as *cer6-3*. In addition, complementation tests confirmed that *line85* is allelic to *cer19*, and *line85* was renamed *cer19-2*.

CER6 encodes a β -ketoacyl-CoA synthase (KCS) or condensing enzyme involved in fatty acid elongation of fatty acyl-CoAs longer than C26 in epidermal cells (Millar *et al.*, 1999; Fiebig *et al.*, 2000; Hooker *et al.*, 2002). There was a low density of wax crystals that accumulated on the stem surfaces of *cer6-3*, while wax crystals were not detectable on the stem surfaces of *cer6-1*. It is likely that *cer6-3* is a weaker allele than *cer6-1*, accounting for this difference in phenotype. The mutation in the *CER6* gene of *cer6-3* is a one-base pair substitution at nucleotide 1190bp, relative to the start codon, changing amino acid residue 397 from alanine to valine. This amino acid is near to one of the active-site residues necessary for enzymatic activity, but is not thought to be directly involved in catalysis (Ghanevati and Jaworski, 2000; Joubes *et al.*, 2008). Also, the change from a small hydrophobic residue (alanine) to a larger one (valine) is relatively

conservative and thus activity of the enzyme may not be completely knocked out in *cer6-3*. Consistent with this hypothesis, the C29 alkanes, C29 secondary alcohols, and C29 ketones, which make up the majority of stem cuticular wax were more reduced in *cer6-1* than *cer6-3*. The lesion in *cer6-1* is a three-base deletion eliminating codon 319 of the *CER6* coding region and is thought to be a null mutation (Fiebig et al., 2000). Fiebig et al., 2000 also characterized the *cer6-2* allele and found that it contained an A-to-C transversion in the *CER6* gene that changes the histidine at amino acid position 148 to proline. In addition, this study reported an intragenic suppressor of *cer6-2* called *cer6-2R* that has a C-to-T transition at this same codon converting it to a serine in the encoded protein. This is a partial suppressor and there is only slight restoration of wax crystals relative to *cer6-2* stems. The density and shape of the wax crystals on *cer6-2R* are very similar to the density and shape of crystals on *cer6-3*, again consistent with *cer6-3* being a relatively weaker allele than the null mutation.

The *CER19* gene has not yet been cloned and thus molecular characterization of the two *cer19* alleles is not yet possible. The *cer19-2* appears to be a stronger allele than *cer19-1*, due to the more dramatic reduction of wax crystals in *cer19-2*. However, the wax load and composition was not significantly different between the two alleles. It is possible that there was subtle difference in wax production that could affect the amount of wax crystals formed. Alternatively, a background mutation in either of these alleles could be modifying the phenotype. Neither mutant has been backcrossed to eliminate backgrounds, which is important for eliminating background mutations that are not tightly linked.

We performed three different physiological assays to test whether the *cer19* mutants were significantly altered in cuticular permeability. Both alleles showed high permeability to TB stain compared to wild-type, but only in young seedlings. It is possible that the seedlings, which have a developing cuticle, are more sensitive to perturbations in cuticle composition than adult plants. The other two physiological assays, water loss and chlorophyll-leaching assays, revealed slightly increased cuticular permeabilities in tissues of adult *cer19* mutants compared to wild type. However, there was considerable variation and it is uncertain if these differences are significant. Further replicates are needed to more conclusively investigate the cuticle permeability in *cer19* mutants. Investigation of cuticle structure by transmission electron microscopy (TEM) may also reveal differences between *cer19* and wild-type (Lu et al., 2009). However, studies have pointed out that cuticle permeability is largely determined by the nanomolecular structure and packing arrangement of cutin and wax molecules within the cuticle membrane itself (Reynhardt, 1997; Schreiber *et al.*, 1997), and thus TEM analysis may not reveal microscale perturbations.

The cloning of *CER19* is key to further understand its role in wax biosynthesis. The rough map position reported by Rashotte et al. (2001), which was further refined here, is an important starting point for this endeavor. The identification of *cer19-2* as a second allele of *cer19* will facilitate the identification of the *CER19*. For example, the two *cer19* alleles are expected to have distinct mutations in the *CER19* gene and this is good evidence of identification. The wax composition defect of *cer19* suggests that the *CER19* protein is involved in fatty acid elongation from C28 to C30 (Rashotte et al., 2001). There were significant decreases in all wax components derived from fatty acyl

chains of C30 or higher. Wax components of chain lengths C28 or lower were either not affected or were increased. A block in fatty acid elongation at the C28 to C30 step would be expected to yield increases in the smaller intermediates. This type of accumulation is observed in *cer6* mutants that are apparently blocked in fatty acid elongation at the C26 to C28 step. Therefore, it is possible that *CER19* encodes for a KCS and there are candidate KCSs on the top of chromosome I. However, none of these have thus far been implicated in cuticular wax metabolism, at least as single mutations. A knockout of *KCS2/DAISY* (*At1g04220*), which is located at the top of chromosome I, does not affect wax accumulation, but a double mutation of *kcs2* with *kcs20* does moderately reduce wax load (Lee et al., 2009b). The transcripts of *KCS1* (*At1g01120*) and *KCS3* (*At1g07720*) are enriched in the epidermis (Suh et al., 2005), indicative of roles in cuticular wax metabolism, but they are relatively far away from the mapped *CER19* locus. *KCS5* (*At1g25450*) is much closer to the *CER19* locus and its mRNA is also enriched in the epidermis. Thus, *KCS5* is a good candidate for *CER19*. It is also possible that *CER19* is a regulator that influences the C28 to C30 elongation step by modifying the expression, stability or activity of a KCS involved in this elongation step. It is more difficult to identify candidate genes for such a regulator.

Chapter 4 Characterization of *cer9* Phenotype and Mapping of the *CER9* Loci

4.1 Introduction

The *cer9-1* mutant was identified by Koornneef *et al.* (1989) and mapped to the lower portion of chromosome IV between 59.8 cM and 76.0 cM. *cer9-1* was derived from ethyl methanesulfonate (EMS) mutagenized plants in the *Ler* ecotype and found to be a recessive mutation (Koornneef *et al.*, 1989).

Scanning electron microscopy analysis revealed that the wax density of *cer9-1* is significantly reduced and morphologically altered compared to wild-type (Koornneef *et al.*, 1989). The mutant is characterized by decreased tubule-like wax structures and altered conformation of the platelets (Koornneef *et al.*, 1989). Jenks *et al.* (1995) analyzed the leaf wax composition and found that C24:0 and C26:0 free fatty acids are significantly increased (23-fold and 27-fold, respectively) compared to *Ler*-WT, while free fatty acids with chain lengths longer than C26 were not detectable. There are also large reductions in the alkane and primary alcohol components of all chain lengths. Conversely, C26 and C28 primary alcohol amounts in cuticular wax of *cer9-1* stems are highly increased (more than two times), while C30 primary alcohols are decreased. All components in alkane forming pathway of *cer9-1* stems are significantly reduced including all chain lengths. Thus, there are tissue-specific differences in cuticular wax composition tissues in *cer9-1*. In stems, it is possible that the alkane forming pathway in *cer9-1* stems is blocked, which causes C26 and C28 fatty acyl-CoAs to be more available for acyl reduction, generating more primary alcohols. The production or availability of C30 acyl-CoAs may be affected in *cer9*, affecting both pathways. *Cer9-1* seems to be affected in a key upstream part of wax metabolism, between elongation

and enzymatic modification of elongated chains. Therefore, cloning and characterization of *CER9* may be essential to elucidate this part of wax biosynthesis.

The objective of this chapter was to further characterize the defects of *cer9-1* to gain further insights into CER9 function, and to clone the *CER9* gene using a map-based cloning strategy.

4.2 Materials and Methods

4.2.1 Plant materials and growth conditions

Cer9-1 and wild type (*Ler* ecotype) were grown in a semi-controlled growth room (Nesbitt Biology Building, Room 113A) with a continuous light photoperiod and at a temperature of approximately 22°C, which was controlled by a Mitsubishi air conditioner.

4.2.2 Mapping population generation

Cer9-1 (Koorneef *et al.*, 1989), which is in *Ler* ecotype, was crossed with Col-0 wild-type ecotype using *cer9-1* as the female and Col-0 as the male. All seeds were collected separately from each successfully developed silique derived from a cross and sown out to grow the F1 generation. The F2 generation seeds were collected separately from individual selfed F1 plants. Homozygous *cer9-1* in the F2 mapping population were identified by their visible glossy stem phenotype. These plants were genotyped for genetic mapping of the *CER9* locus. Genomic DNA extractions and PCR analysis were performed as described in Chapter 3.

4.2.3 Phenotypic characterization of *cer9-1*

Scanning electron microscopy and gas chromatography were performed as described in Chapter 2. Measurements of cuticular permeability, including fresh water loss assay, chlorophyll-leaching assay, and toluidine-blue staining, were performed as described in Chapter 3.

4.2.4 Reverse-transcription PCR (RT-PCR)

Reverse-transcription PCR (RT-PCR) analysis was used to compare the expression levels of genes known to be involved in wax and cutin biosynthesis in the *Ler*-WT and *cer9-1* mutant. Total RNA from rosette leaves and stems were isolated

from *Ler*-WT and *cer9-1* using the TRIzol protocol (Invitrogen), according to the manufacturer's instructions. The RNA concentrations were quantified using a Nanodrop-1000 spectrophotometer (Thermo Fisher Scientific, Nepean, ON). One μg of RNA was reverse transcribed into cDNA using ImProm-II™ Reverse Transcription System (Promega, Madison, USA), according to the manufacturer's protocol. First, the RNA was denatured at 68°C for 5 min to remove secondary structure. Then the RNA was snap cooled on ice. Each 15 μl reaction mixture, containing 4 μl of ImProm-II™ 5X reaction buffer (Promega), 1.6 μl of 25mM MgCl_2 , 1 μl of 10mM dNTP, 1 μl of 30 μM oligo(dT), 0.25 μl of ImProm-II™ reverse transcriptase (Promega) and 13 μl RNase-free water, was added directly to the RNA. The mixture was incubated at 50°C for 60min, and then the cDNA stock was diluted (1:1) for PCR amplification. The PCR reaction in a 20 μl final volume contained 2 μl of diluted cDNA, 1 μl of 10mM reverse primer, 1 μl of 10mM forward primer, 2 μl of 10X PCR buffer, 1.6 μl of 25mM MgCl_2 , 0.1 μl of *Taq* DNA polymerase, and 11.7 μl of deionized water. The sequences of the gene specific primers used are listed in Table 6. The PCR reaction was started with an initial step at 94°C for 1 min, followed by 28 cycles at 94° for 30 seconds, annealing at 57°C for 30 seconds, and extension at 72°C for 30 seconds. These cycle parameters were followed by incubation at 72°C for 10 min, followed by 4°C for an indefinite time. All the PCR products were separated on a 0.8% (w/v) agarose gel by electrophoresis with ethidium bromide as DNA intercalating agent for fluorescent detection. The gel image was photographed using an Alphamager 2200 gel-imager system (Alpha Innotech, San Leandro, CA).

Table 6 Gene-specific primers used in RT-PCR

Primer	Sequence
CER1-LP	5'-AGATAAGAAAGGAGTGAAGGTGCTT-3'
CER1-RP	5'-AGAGATTTTGGAACTATCAATGCTG-3'
CER2-LP	5'-CTACTCCAATCTTATCACCACGTCT-3'
CER2-RP	5'-CTATCAATGTGGTTGAGGCTAAAAT-3'
CER3-LP	5'-TCCAATACTTCTTACCGTTTGCTAC-3'
CER3-RP	5'-CGTAAGTGCAGTTCCTTCTAAACTT-3'
CER4-LP	5'-GAAGTATGGTCCCAATCTAAATCAA-3'
CER4-RP	5'-ATTTTCTCTTTTGTCCCTACCATC-3'
CER5-LP	5'-AAGAGAGATTGAGTGGTTACTACGG-3'
CER5-RP	5'-TGATAAGTCCCACCACTTAGAATGT-3'
CER6-LP	5'-CCTAATTACTATCAAGGCAACGAGA-3'
CER6-RP	5'-ATGTAGCTAAGCTCGTACCATAACG -3'
CER8-LP	5'-GATTTTCTCCATATGGGACGTG -3'
CER8-RP	5'-CGCGACAAGCATAGATTTGA-3'
LACS2-LP	5'-GTAATCTTAAACAACGCAGCAATTT-3'
LACS2-RP	5'-AACTCTCAGTCAATCCATAACCTTG-3'
ATT1-LP	5'-ACAACCTACCCTAAAGGTCCTACGTG-3'
ATT1-RP	5'-CAGCAATTCTTGCTTACGTGTATTA-3'
KCS1-LP	5'-CGAAGTCGGAATCTTGATAGTAAAC-3'
KCS1-RP	5'-GTCCTAAAGTCGTGATGTTTGTGTTT-3'
GAPC-F	5'-TCAGACTCGAGAAAGCTGCTAC-3'
GAPC-R	5'-GATCAAGTCGACCACACGG-3'

4.3 Results

4.3.1 Analysis of epicuticular wax crystals and wax composition of *cer9-1* mutants

4.3.1.1 Altered epicuticular wax crystal formation of *cer9-1*

Scanning electron microscopy was performed to analyze the epicuticular crystals on *cer9-1* mutants. The surface imaging of *cer9-1* performed by Koornneef *et al.* (1989) was of relatively low resolution. Compared with the corresponding wild-type *Ler*-WT ecotype, the total wax crystal density of *cer9-1* was significantly decreased (Figure 21A, D). The typical tube-shape wax crystals found on *Ler*-WT stems were missing on *cer9-1* stems (Figure 21C, F). Instead, the major wax crystal shapes on *cer9-1* stem were flat irregular shaped protrusions. These wax crystal phenotypes were consistent with and expand upon the previous analysis by SEM of *cer9-1* stem surfaces (Koornneef *et al.*, 1989).

Figure 21 Altered epicuticular wax crystals on stems of *cer9-1*.

Wax crystals on stems of six week-old *Ler*-WT and *cer9-1* plants were imaged by scanning electron microscopy.

(A) *Ler*-WT at magnification of 1500X; (B) *Ler*-WT at magnification of 5000X; (C) *Ler*-WT at magnification of 10000X;

(D) *cer9-1* at magnification of 1500X; (E) *cer9-1* at magnification of 5000X; (F) *cer9-1* at magnification of 10000X

The scale bars are shown at the bottom of each panel

4.3.1.2 Cuticular Wax Composition on stems and leaves of *cer9-1*

Gas chromatography was performed to analyze the total cuticular wax load and composition of *cer9-1* stems and leaves, compared to the corresponding wild-type ecotype (*Ler*-WT). The major wax components on *Ler*-WT leaves and stems are different (Jenks *et al.*, 1995). C29 alkane, C29 secondary alcohol, and C29 ketone are the major wax components of *Arabidopsis* wild-type stems, while leaves have a much higher proportion of C31 alkane than C29 alkane and there are no detectable secondary alcohols and ketones (Table 7; Jenks *et al.*, 1995). C24-C26 free fatty acids and primary alcohols are present on both stems and leaves (Figure 22; Jenks *et al.*, 1995). The total wax load on *cer9-1* stems was decreased to 62% of wild type (*Ler*) levels, while the total wax load on *cer9-1* leaves was similar to the load on *Ler*-WT leaves (Table 7). On *cer9-1* stems, C24:0 and C26:0 free fatty acids were significantly increased, while C28:0 and C30:0 free fatty acids were highly decreased compared to wild type (Figure 22A). Primary alcohols of chain lengths C24, C26, and C28 were increased on *cer9-1* stems, whereas the C30 primary alcohol was decreased to 42% of wild-type levels (Figure 22A). With regards to alkane-forming pathway products, all components were decreased by about 75%, except for C26/C28 aldehydes, whereas C27 alkanes were not significantly affected (Figure 22C). On *cer9-1* leaves, C24:0 and C26:0 free fatty acids were increased 57- and 31- fold, respectively, relative to that on wild-type leaves, while C28:0 and C30:0 free fatty acids were decreased to nearly undetectable levels. Primary alcohols of chain length C24, C26, and C28 were decreased on *cer9-1* leaf wax, while C30 primary alcohol was unaffected (Figure 22B). On leaves, all wax components

of all chain lengths produced in the alkane-forming pathway were dramatically decreased (Figure 22D).

Table 7 Chemical composition of stem and leaf cuticular wax of *Ler*-WT and *cer9-1*

	Ler-WT Stems	<i>cer9-1</i> Stems	Ler-WT Leaves	<i>cer9-1</i> Leaves
Total Wax Load	17.79±1.12	11.06±1.61	2.28±0.15	2.29±0.51
C24 Free Fatty Acid	0.04±0.003	0.29±0.05	0.006±0.002	0.35±0.03
C26 Free Fatty Acid	0.02±0.001	0.28±0.03	0.01±0.006	0.31±0.03
C28 Free Fatty Acid	0.07±0.002	0.04±0.005	0.007±0.003	0±0
C30 Free Fatty Acid	0.34±0.02	0.12±0.01	0.039±0.01	0.004±0.001
C24 Primary Alcohol	0.05±0.003	0.06±0.006	0.01±0.005	0.001±0.002
C26 Primary Alcohol	0.28±0.01	0.99±0.07	0.014±0.005	0.001±0.0008
C28 Primary Alcohol	0.55±0.01	1.31±0.01	0.017±0.004	0.004±0.0008
C30 Primary Alcohol	0.31±0.003	0.13±0.01	0.029±0.009	0.03±0.003
C26 Aldehyde	0.02±0.002	0.05±0.01	0.001±0.0002	0.001±0.0001
C28 Aldehyde	0.11±0.01	0.12±0.04	0.009±0.004	0.001±0.0008
C30 Aldehyde	0.61±0.07	0.21±0.03	0.018±0.007	0.01±0.003
C27 Alkane	0.06±0.004	0.08±0.007	0.006±0.002	0.002±0.001
C29 Alkane	6.63±0.41	1.89±0.12	0.057±0.01	0.01±0.006
C31 Alkane	0.18±0.007	0.04±0.009	0.05±0.01	0.006±0.002
C33 Alkane	0.0075±0.003	0.03±0.002	0.13±0.001	0.03±0.007
C29 Secondary Alcohol	4.17±0.23	1.51±0.11	0.005±0.001	0.001±0.0009
C31 Secondary Alcohol	0.08±0.009	0.03±0.004	0.01±0.004	0.007±0.004
C29 Ketone	1.73±0.11	0.45±0.02	0±0	0±0

Unit: $\mu\text{g}/\text{cm}^2$. Each value is the average of five replicates (n=5), and $\pm$ standard deviation is shown.

Figure 22 Stem and leaf cuticular wax compositions of *cer9-1* and the corresponding wild type ecotype (*Ler-WT*).

(A) Primary alcohols and free fatty acids components of *cer9-1* stem wax sorted by chemical class and chain length.

(B) Primary alcohols and free fatty acids components of *cer9-1* leaf wax sorted by chemical class and chain length.

(C) Alkane forming pathway components of *cer9-1* stem wax sorted by chemical class and chain length.

(D) Alkane forming pathway components of *cer9-1* leaf wax sorted by chemical class and chain length.

All values are mean, presented in $\mu\text{g}/\text{cm}^2$, derived from five replicates ($n=5$). Error bars represent the standard deviation.

FFA: Free fatty acids; OH: Primary alcohols

ALK: Alkanes; ALD: Aldehydes; 2OH: Secondary alcohols; KETO: Ketones

4.3.2 Cuticular permeability of *cer9-1*

A TB assay was performed in young seedlings to test for defects in *cer9-1* cuticle permeability. The leaves of wild-type seedlings did not show TB staining, as expected for plants with a complete cuticle (Figure 23A). The leaves of *cer9-1* seedlings showed staining in the distal portions of the leaves (Figure 23B), indicative of a cuticle permeability defect of the class II type according to the classification nomenclature of Tanaka *et al* (2003).

Measurements of non-stomatal water loss from plant tissues to the environment were performed. Rosette leaves and stems showed no difference in the rate of water loss between *cer9-1* and *Ler-WT* (Figure 24A, B). According to T-test statistical analysis, with $df=6$, t must be at least 4.3 to reach $p\text{-value} < 0.05$. Our result was $t=0.3503$, so by conventional criteria, this difference is considered to be not statistically significant.

As showed in Figure 25, chlorophyll leached much faster from the detached rosette leaves of *cer9-1* plants in 80% ethanol compared to leaves of wild-type plants. After 40 minutes of being immersed in 80% ethanol, the concentration of chlorophyll leached from *cer9-1* rosette leaves was almost 12 times greater than that from *Ler-WT* rosette leaves. At two hours following immersion in 80% ethanol, $8.3 \pm 0.3 \mu\text{mol/g}$ of chlorophyll had leached from *cer9-1* rosette leaves, while only $1.3 \pm 0.2 \mu\text{mol/g}$ of chlorophyll had leached from *Ler-WT* rosette leaves.

Figure 23 Toluidine blue staining on leaves of *Ler*-WT and *cer9-1* seedlings

The TB staining assay was conducted on two week old seedlings of (A) *Ler*-WT, and (B) *cer9-1*.

Figure 24 Cuticular transpiration of Ler-WT and *cer9-1*

(A) Fresh weight assay with leaves of 5-week old *cer9-1* and Ler-WT plants, where the rate of water loss per gram of fresh weight tissue from rosette leaves was measured at the indicated time points (n=5; $\pm$ standard deviation).

(B) Fresh weight assay with stems of 5-week old *cer9-1* and Ler-WT plants, where the rate of water loss per gram of fresh weight tissue from stems was measured at the indicated time points (n=5; $\pm$ standard deviation).

Figure 25 Rate of chlorophyll leaching in *cer9-1* and Ler-WT leaves

The concentration of chlorophyll leached over 2 hours into solution from 3 week-old rosette leaves of *cer9-1* and Ler-WT immersed in 80% ethanol (n=10; $\pm$ standard deviation).

4.3.3 Positional mapping of *cer9-1*

As described above, the alterations in cuticular wax on stems and leaves of *cer9-1* are very dramatic. Identification of the *CER9* gene may give important insights into biosynthesis, regulation and/or transport of cuticular waxes. We therefore initiated a map-based cloning approach to identify *CER9*.

Genetic analysis confirmed that the glossy phenotype of *cer9-1* is recessive by observing F1 plants derived from a cross between *Ler*-WT and *cer9-1* mutants (data not shown). The F1 plants were glaucous. Furthermore, 200 segregating F2 plants showed 152 wild-type and 48 glossy plants. A chi-squared statistical analysis was used to determine if the segregation data fits the hypothesis that *cer9-1* is a recessive mutation. χ^2 was 0.11 and therefore lies confidently within the range that is to be expected for 95% of sample sets occurring from segregation of a single recessive gene. So we can accept that *cer9-1* segregates at a 3:1 ratio. Therefore, it is a recessive mutation.

To determine the position of the *CER9* loci, an F2 mapping population was constructed containing 1046 glossy individuals derived from a cross of *cer9-1* with Col-0. By analyzing 40 glossy plants from this population, the mutation causing the glossy phenotype was found to be linked to the simple sequence length polymorphism (SSLP) marker nga1107, which is at position 18.09 Mbp on the bottom of chromosome IV (Figure 26A, B). This result was consistent with previous genetic linkage analysis that indicated *CER9* was located at the bottom of chromosome IV (Koornneef *et al.*, 1989). For fine mapping, new insertion-deletion (InDel) markers were developed near to this mapping region. *CER9* gene was mapped to a region flanked by markers CER452834 and CER460758, which are located at positions 12.85 Mbp and 18.51 Mbp, respectively

(Figure 26C). This gave a recombination frequency of 7% and 14%, respectively, based on 100 samples each. To further narrow down the locus, more InDel markers were designed between the flanking markers (Table 8) and the entire mapping population of 1046 individuals was used to identify recombinants. The *CER9* locus mapped to a region between marker CER458597 and marker CER460644, which are located between 16.21 Mbp and 16.53 Mbp, respectively (Figure 26D). Five bacterial artificial chromosomes (BACs) covered that region: T16L1, F17I5, F28A23, F10M10, and T4L20 (Figure 26E).

During the course of this mapping procedure, it came to our attention that a collaborator, the lab of Professor Matthew Jenks (Purdue University, West Lafayette, Indiana, USA), had also established a mapping population for *CER9*. We needed additional recombinants to further narrow down the region containing *CER9*. Therefore, we shared out mapping information with the Jenks laboratory and they were able to further narrow down the region containing *CER9* to 61 Kbp using their larger mapping population. They sequenced the entire 61 Kbp region in the *cer9-1* mutant and found a single non-silent missense mutation (personal communication). However, further experimentation is needed to confirm the identification of *CER9* and this is beyond the timeframe and scope of this M.Sc. thesis.

Figure 26 Genetic mapping of *CER9* the locus

(A and B) First-pass mapping of *CER9*. *CER9* was found to be linked to SSLP marker nga1107. The recombination frequency (RF) was based on 40 samples. The physical position of the marker is indicated.

(C) Flanking markers of *CER9* loci. *CER9* was mapped to a genetic interval between markers CER452824 and CER460758. The recombination frequency (RF) of each marker was based on 100 samples and is indicated below the marker names.

(D) Fine genetic mapping of *CER9*. Analyzing 1046 F₂ mapping population plants, *CER9* was further narrowed down to a region between markers CER458597 and CER460644. Markers and the numbers of recombination events identified are indicated.

(E) Five bacterial artificial chromosomes (BACs) covered the region between markers CER458597 and CER460644. Yellow lines represent five different BACs. Red lines in the BAC represent the position of each marker in the BAC.

(A) Chromosome IV

18.1 Mbp RF=15%

nga1107

CER460758

RF=14%

CER452834

RF=7%

CER454977

CER452101

CER451776

CER448549

CER454915

CER452246

CER451505

CER452316

CER458597

CER449794

CER451699

CER460644

Marker names

Recombinants 37

Marker names

BACs/Recombinants T16L1 (2/1046)

CER458597

CER449794

F17I5 (0/1046)

CER451699

F10M10

F28A23 (0/1046)

T4L20 (7/1046)

CER460644

Table 7 Polymorphic markers for fine-pass mapping of cer9

Marker name	BAC	Position (Mbp)	Forward Primer	Reverse Primer
CER452834	F6I7	12.85	GCTCCTTGTTTGGTAATAT	TACAGTATAAATACCGTAGT
CER454977	M4I22	13.66	TGACTTGAGTTTGTTTATTGATTGA	CCCATCTCCAAAAATCGAAA
CER542101	F3L17	15.29	TGGTGGTCTTGGGAAGATGAA	ACGTACGTTAAATGGCAAAGA
CER451776	F28M20	15.38	GTGATTCGGATCAATAAAAAATGCT	CTCATCAAAGTGGTGATGTGTTGT
CER448549	F10N7	15.47	TGAAAGGTATAATCTGAATGAATCTCT	CACATTCTGTGGGTTACTAATTG
CER453188	F8B4	15.68	CCACCAAAATGTTGAATTGTAATAA	TTGTTTCCAAAAATACCATAAGCTGT
CER454915	L23H3	15.69	ATACGGCGAGATCCTTACCAG	CTGATAATTTCTCTTCTCCGACAGA
CER452246	F4D11	15.75	TGTTTAAATGCATACCAAATAGACG	CATCCATCCATGCTTTATATGTTG
CER451505	F26P21	15.91	GAGGACTTGATGAGGTGTTTTTCTCT	TATCTTTTGGTCATGACATGGTTGT
CER452316	F4I10	15.96	AAGAAGTGTAATTGCTAGCGTCAAA	TACTATAAGAAAGCAAACCCCAAAAC
CER458597	T16L1	16.21	AAAATAACTTTAGAGAAAGGA	GTAGCTAACGATCATGTGAG
CER449794	F17I5	16.29	AGGATCTGCTGACTGCTCAAACT	ACAGTGTGGAAAAATGCTATAGTGG
CER451699	F28A23	16.39	CAGGATCTGCTGACTGCTCA	CAGTGTGGAAAAATGCTATAGTGG
CER460644	T4L20	16.53	ATATCACAACTTGTTTTTCTGATGAA	AAAGAAAAAGGAAGAAACAATCA
NGA1107	T9A14	18.09	GCGAAAAAACAAAAAATCCA	CGACGAATCGACAGAATTAGG
CER460758	T5I17	18.51	CCAGAAAGGCACAGTTTGATGC	TCATCATCAGGGTCACGAAATT

4.3.4 Gene expression patterns of wax and cutin-associated genes in *cer9-1*

The candidate *CER9* gene described above is not predicted to be a metabolic enzyme, based on its complex wax-altered phenotype. It may be a regulator and therefore we decided to monitor the gene expression levels of cuticle-associated genes in *cer9-1* to test if they were up or down regulated. RT-PCR was performed on the set of cuticle-associated genes and a constitutive control (*GAPC*) in both leaves and stems, comparing transcript levels in wild-type versus *cer9-1*. We analyzed the expression of *KCS1* (Todd *et al.*, 1999) and *CER6* (Fiebig *et al.*, 2000), which are involved in elongation of fatty acyl chains; *ATT1* (Xiao *et al.*, 2004) is a cytochrome P450 enzyme involved in biosynthesis of cutin; *LACS2* (Schnurr *et al.*, 2004) and *CER8/LACS1* (Lu *et al.*, 2009) are acyl-activating enzymes involved in both wax and cutin biosynthesis; *CER2* (Negruk *et al.*, 1996; Xia *et al.*, 1996; St-Pierre *et al.*, 1998) encodes a putative CoA-dependent acyltransferase involved in cuticle biosynthesis; *CER4* (Rowland *et al.*, 2006) is an alcohol-forming fatty CoA reductase involved in production of primary alcohols in the cuticle; *CER5* (Pighin *et al.*, 2004) encodes an ABC transporter required for wax transport to the cuticle; *CER1* (Aarts *et al.*, 1995) and *CER3* (Chen *et al.*, 2003; Rowland *et al.*, 2007) are postulated to be integral membrane enzymes that contain a di-iron active site involved in biosynthesis of wax, and *GAPC* is the constitutively expressed control. However, none of these genes showed detectable changes in *cer9-1* compared to *Ler*-WT (Figure 27).

Figure 27 RT-PCR analysis of transcripts encoding proteins involved in wax and cutin biosynthesis in *Ler*-WT and *cer9-1*

Each panel is the amplification product generated by RT-PCR and is representative of the transcript levels of the genes indicated on the left. RNA for RT-PCR was extracted from 27 day-old rosette leaves of *Ler*-WT and *cer9* (column 1), and 40 days old rosette leaves and stems of *Ler*-WT and *cer9* (column 2 and 3, respectively). The last row is the RT-PCR product of *GAPC*, which is constitutively expressed, and thus is a loading control for the corresponding lanes in the panels above.

4.4 Discussion

The *cer9-1* mutant has a dramatic and very unique alteration in its cuticular wax composition (Jenks et al., 1995; and results shown here). In wild-type *Arabidopsis* plants, the major leaf wax constituents are C29-C33 alkanes, whereas in *cer9-1* these constituents are nearly absent, and instead C24 and C26 free fatty acids predominate almost to the exclusion of all other waxes. In stem wax, the C24 and C26 free fatty acids are also elevated in *cer9-1*. However, in contrast to the leaf phenotype, stem wax also has higher levels of C26 and C28 primary alcohols but lower levels of C30 primary alcohols relative to wild-type. Wax esters of *cer9-1* stems are increased relative to wild-type, but the chain length distribution has not been reported. Alkanes, secondary alcohols and ketones of chain lengths C29 and higher are substantially reduced. Thus, both chemical class and chain lengths of wax constituents are altered in *cer9-1*, and there are substantial differences in the effects between the alterations exhibited in stem and leaf wax. Jenks et al. (1995) speculated that the *CER9* gene product is involved in chain-length specific transfer of fatty acyl-chains through the wax biosynthetic pathway, but that the chain length affected is different depending on the tissue type. Proteins involved in such a transfer process have not been identified, nor is there evidence that such a transfer process needs to be facilitated. It is also possible that *CER9* is a regulator of wax biosynthesis and that the complex alterations in wax accumulation observed in *cer9-1* leaves and stems may be due to misregulation of multiple genes and/or proteins involved in wax biosynthesis.

We also investigated whether *cer9-1* has a defect in the permeability of its cuticle using three different assessments of cuticular permeability. Although there was no major effect on cuticular transpiration (water loss assay) and a relatively minor effect on

staining by toluidine blue, *cer9-1* did exhibit a large difference from wild-type in the rate of chlorophyll leaching. Differences in cuticle permeability at different developmental stages may account in part for these conflicting results. The rosette leaves used in the chlorophyll-leaching assay were taken from 3-week old plants, which still have a developing cuticle, whereas the leaves used in the fresh water loss assay were taken from 5 week-old plants. The water loss assay should be repeated on younger leaves to determine if the cuticle is more dramatically affected, in terms of permeability, in younger plants. The general integrity of the cuticle structure can also be investigated at various developmental stages using transmission electron microscopy (TEM), but this is a relatively crude measurement of cuticle structure. Also, it will be important to determine if there are alterations in cutin composition in *cer9-1*, which can also affect the ultrastructure of the cuticle and thus the passage of chlorophyll through the cuticular membrane. For example, *lacs1/cer8* and *lacs2* mutants affect both cutin and wax composition (Lu et al., 2009) and the same may be true in the *cer9-1* mutant.

Important progress has been made towards the cloning of *CER9* by my localization of the interval containing *CER9* to an approximately 320 Kbp region on chromosome IV. As mentioned above in the Results section, the interval containing *CER9* was further localized to 61 Kbp in collaboration with the laboratory of Matthew Jenks and a candidate gene for *CER9* has been identified through sequencing of this region. Future work is needed to verify the identity of the *CER9* gene, which is work in progress between the two labs. Ultimately, detailed characterization of the *CER9* gene and gene product will hopefully reveal the precise role of *CER9* in cuticular wax metabolism.

Towards this end, I tested the possibility that the *cer9-1* mutant was affecting the expression of other genes involved in the biosynthesis and/or secretion of wax and cutin components. This analysis did not require knowing the identity of the *CER9* gene. As described above, the complicated phenotype of *cer9-1* suggests that *CER9* may be a regulator of cuticle development rather than a biosynthetic enzyme. Also, the candidate gene for *CER9* and genes in the vicinity of the *CER9* loci are not predicted to encode enzymes involved in cutin or wax metabolism. We tested 10 genes involved in wax or cutin production, but the transcript levels of these genes were not altered in the *cer9-1* mutant. However, we did not exhaustively evaluate all genes involved in cuticle development. These other genes could be tested in the future or a DNA microarray may reveal alterations of gene expression on a genome-wide scale. Alternatively, the effects of *cer9-1* could be exerted at the protein level, with changes in the levels or activities of proteins involved in wax and/or cutin metabolism. This is more complicated to evaluate than changes at the transcriptional level. There are also a myriad of indirect effects on cuticle development that could be taking place in the *cer9-1* mutant, which would cause the dramatic alterations in wax composition in leaves and stems. Again, identification of *CER9* is critical for evaluating this possibility further.

Chapter 5 General Conclusion and Discussion

Mutants of *Arabidopsis* that have wax deficiencies are often easily identified by their glossy stems. Detailed analyses of the wax composition defects in these mutants, which are typically called *eceriferum* (*cer*), have provided enormous insights into the nature of wax biosynthetic pathways. Furthermore, the cloning of *CER* genes has allowed the characterization of key wax biosynthetic enzymes and transporters (Samuels et al., 2008). However, not all of the known *CER* loci have been cloned and screens for *cer* mutants have never been done to saturation. Therefore, much remains to be elucidated in understanding cuticular wax biosynthesis and its regulation. To this end, I examined a set of previously uncharacterized *Arabidopsis* mutants affected in cuticular wax production.

In Chapter 2, the goal was to phenotypically group the new wax deficient mutants, termed *line* mutants, which were previously identified by the Rowland lab, and then use this information to guide complementation crosses to determine which *line* mutants were alleles of existing *cer* mutants and which were potentially mutants of novel *cer* loci. The phenotypes analyzed were wax crystal density and shape by scanning electron microscopy and wax composition by gas chromatography. This approach was successful, although in hindsight complementation crosses, without detailed phenotypic characterization of these *line* mutants, may have been just as quick. The Rowland lab possesses over 50 additional *line* mutants that have not yet been characterized, but complementation crosses between all these mutants and the nearly 30 known *cer* mutants would be cumbersome. Therefore, my approach may be useful in analyzing

this larger set. The key is to generate robust data on the wax compositions by gas chromatography and have an effective means to compare the profiles.

In Chapter 3, I began further characterization of what were thought to be mutants of two novel *CER* loci. More detailed phenotypic characterization, positional mapping, and complementation crosses revealed, however, that they were instead new alleles of *cer6* and *cer19*, which we termed *cer6-3* and *cer19-2*, respectively. *Cer6-3* is a weak allele, which is what caused me to initially think it was not a mutation in *CER6*. Sequencing of the *CER6* gene in this mutant background revealed that amino acid 317 of the encoded β -ketoacyl-CoA synthase is important for enzymatic activity, and that changing the residue from a small hydrophobic residue (alanine) to a larger hydrophobic residue (valine) does not completely abolish activity. The importance of this amino acid for activity of this class of enzyme has not been tested before, although it is a conserved residue. Identification of the new *cer19-2* allele will be important for the subsequent cloning and characterization of *CER19*. This follow-up work is being carried out in collaboration between the Rowland lab and the laboratory of Professor Ljerka Kunst at the University of British Columbia. The presence of independent mutations in *cer19-1* and *cer19-2* that reside in the same candidate gene will be good evidence for *CER19* identification. Also, phenotypic characterization is typically carried out in two independent allelic lines to eliminate the possibility of linked background mutations modifying the phenotype. Careful examination of the wax composition defect in *cer19-1* and *cer19-2* indicated that *CER19* is likely involved in elongation of C28 acyl chains to C30 acyl chains. This is a key step in wax biosynthesis leading to production of C30 and

longer acyl-CoAs, which are the major substrates for modification in the alkane-forming pathway.

In Chapter 4, I initiated a map-based cloning approach to identify the *CER9* gene. The cuticular wax phenotype of *cer9-1* is intriguing, with alterations in every major chemical class and chain length. In *cer9-1* leaves, the major wax components are C24 and C26 free fatty acids rather than alkanes and primary alcohols. This caused an alteration in the permeability of the leaf cuticle, which had not been reported before. Future work needs to be done to determine if leaf cutin is also affected in *cer9-1*. Interestingly, there are tissue-specific wax alterations in *cer9-1*. In *cer9-1* stems, C26 and C28 primary alcohols are not reduced as in leaves, but are instead significantly increased. It is possible that regulation of wax metabolism is different in leaves and stems of Arabidopsis. Indeed, *CER9* itself may encode a regulator. If so, it does not seem to act at the transcriptional level, at least for the 10 genes tested here, which are known to be important for wax or cutin metabolism. Using linkage to molecular markers on chromosome IV, I narrowed down the region of genomic DNA containing *CER9* to a 320kbp interval. I could not narrow down the region further because there were no more useful recombinants in my mapping population. A larger mapping population was needed. The laboratory of Professor Matthew Jenks at Purdue University also had a mapping population for *cer9*, and so we shared our mapping information and they were able to further narrow the segment containing *CER9* using their larger mapping population. A candidate gene has been identified, but further molecular characterization is required to determine if this gene is indeed *CER9*. Molecular complementation of the *cer9-1* mutant by transformation of the wild-type gene is being carried out. Additional

alleles have not been identified in this gene (e.g. T-DNA inserts) and silencing of the gene using RNA interference is also being carried out. The subsequent characterization of *CER9* will continue in collaboration between the Rowland and Jenks laboratories.

In closing, this thesis has made important progress in the phenotypic characterization of *Arabidopsis cer9* and *cer19* mutants affected in cuticular wax metabolism. The future cloning and characterization of *CER9* and *CER19* is anticipated to provide valuable insights into the molecular mechanisms governing cuticular wax production.

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