SEED OIL, SEED COAT MUCILAGE AND CUTICULAR WAX DEPOSITION ARE INTERCONNECTED IN ARABIDOPSIS THALIANA

by

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ABSTRACT

Seed oil, seed coat mucilage and cuticular wax are plant-specific metabolites important for plant development and growth. Therefore, understanding biosynthesis, deposition, transport and regulation of these metabolites will benefit our daily life and the environment. The original objectives of my thesis research were to investigate the regulation of seed oil accumulation by a transcription factor GLABRA2 (GL2; chapter 2) and to explore the secretory process involved in the transport of cuticular waxes from the endoplasmic reticulum to the plasma membrane (chapter 3) in *Arabidopsis thaliana*. However, my research revealed two unexpected connections between seed oil and seed coat mucilage deposition and between cuticular wax export and cell wall formation.

At the beginning of chapter 2, I hypothesized that GL2 may regulate seed oil biosynthesis by controlling *PHOSPHOLIPASE D ZETA* (*PLDZ*) genes in the embryo. However, my data demonstrated that GL2, and all of the transcription factors known to be required for GL2 expression, influence seed oil accumulation in the embryo by regulating transcription of a seed coat mucilage biosynthetic gene, *MUCILAGE MODIFIED 4* (*MUM4*) in the seed coat. Based on this evidence, I propose that mucilage biosynthesis in the seed coat competes with oil biosynthesis in the embryo for available photosynthate during seed development. This information suggests a promising way to engineer high oil yields in seeds by blocking seed coat mucilage production.

In chapter 3, I characterized deposition of stem cuticular wax, seed coat mucilage and secondary cell wall columella, and secretion in the *cer11-1* mutant. The pleiotropic *cer11-1* phenotype suggests that CER11 plays a role in secretory trafficking involved in the deposition of apoplastic matrix components, including cuticular wax, seed coat

mucilage and cell wall constituents. Cloning of the *CER11* gene revealed that it encodes C-TERMINAL DOMAIN PHOSPHATASE LIKE 2 (CPL2) that interacts with a vacuolar type H⁺-ATPase (V-ATPase) subunit C (VHA-C) in yeast and plants. I hypothesize that the role of the CER11/CPL2 in secretory trafficking is to determine phosphorylation levels of VHA-C involved in regulation of V-ATPase activity.

PREFACE

A version of chapter 2 has been published as: [Shi, L.], Katavic, V., Yu, Y., Kunst, L., and Haughn, G. (2012) Arabidopsis *glabra2* mutant seeds deficient in mucilage biosynthesis produce more oil. *Plant J* 69, 37-46. © 2011 The Authors. The Plant Journal © 2011 Blackwell Publishing Ltd. The bulk of the text and the figures were reproduced with permission. Prof. Ljerka Kunst and Prof. George Haughn identified the research question. Prof. Ljerka Kunst, Prof. George Haughn, Dr. Vesna Katavic and I designed the experiments. Dr. Yuanyuan Yu generated transgenic lines of *PLDZ1::GUS* and *PLDZ2::GUS*, and she and I examined the expression patterns of *GL2*, *PLDZ1* and *PLDZ2* in seeds by performing GUS activity assays. I conducted all the other experiments, analyzed the data, and wrote the first draft of the manuscript.

For chapter 3, Prof. Ljerka Kunst and Prof. George Haughn identified the research question. Prof. Ljerka Kunst, Prof. George Haughn, Dr. Huanquan (Hugo) Zheng, Dr. Gillian Dean and I designed the experiments. Dr. Huanquan (Hugo) Zheng examined the localization of secGFP in the wild type and *cer11-1* hypocotyl cells (Figure 3.1). Dr. Gillian Dean first identified seed coat mucilage phenotype of *cer11-1*, and she assisted me in the characterization of the *cer11-1* seed coat mucilage phenotypes by conducting ruthenium red staining, monosaccharide analysis with HPAEC and seed coat sectioning. I conducted all the other experiments and analyzed the data with the assistance of Dr. Gillian Dean, Prof. Ljerka Kunst and Prof. George Haughn.

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LIST OF ABBREVIATIONS

| ABA | abscisic acid |
|-------------|---|
| ABC | ATP-binding cassette |
| ABCG | ATP-binding cassette transporter, subfamily G |
| ABI | ABSCISIC ACID INSENSITIVE |
| ABRC | Arabidopsis Biological Resource Center |
| ACCase | Acetyl-CoA Carboxylase |
| ACP | acyl carrier protein |
| AD | GAL4 activation domain |
| AGP | arabinogalactan protein |
| AIR | alcohol insoluble residue |
| Arabidopsis | Arabidopsis thaliana |
| ARF | ADP-ribosylation factor |
| BCCP | BIOTIN CARBOXYL CARRIER PROTEIN |
| BD | GAL4 DNA binding domain |
| bHLH | basic helix-loop-helix |
| bp | base pair |
| BSTFA | N,O-bis(trimethylsilyl)trifluoroacetamide |
| BXL | BETA-XYLOSIDASE |
| C28 | 28 carbons |
| CCD | charge-coupled device |
| cDNA | complementary DNA |
| CER | ECERIFERUM |
| CESA | CELLULOSE SYNTHASE |
| Chr | chromosome |
| cLuc | carboxy-terminus of luciferase protein |
| CMV | Cauliflower Mosaic Virus |
| CoA | Coenzyme A |
| Col | Columbia |
| ConcA | concanamycin A |
| COP | coat protein |
| CPL | CTD PHOSPHATASE LIKE |
| CPT | CDP-choline:diacylglycerol cholinephophotransferase |
| CSC | cellulose synthase complex |
| CTD | carboxy-terminal domain |
| CWLP | CELL WALL-PLASMA EMBRANE LINKER PROTEIN |
| CYP | cytochrome P450 |
| CYTB5 | cytochrome b5 isoforms |
| DAG | diacylglycerol |
| det | de-etiolated |
| DGAT | acyl-CoA:diacylglycerol acyltransferase |
| DMSO | dimethyl sulfoxide |

| DNA | deoxyribonucleic acid |
|----------|---|
| DPA | day post anthesis |
| dsRBM | double stranded RNA binding motif |
| ECR | enoyl-CoA reductase |
| ECH | ECHIDNA |
| EDTA | ethylenediaminetetraacetic acid |
| EE | early endosome |
| EGL | ENHANCER OF GLABRA |
| ELO-LIKE | ELONGATION DEFECTIVE-LIKE |
| ER | endoplasmic reticulum |
| FAE | fatty acid elongase |
| FAE1 | FATTY ACID ELONGATION1 |
| FAR | fatty acyl-CoA reductase |
| FAS | fatty acid synthase |
| FCPH | FCP1 homology domain |
| FID | flame ionization detector |
| FL | full length |
| FLY | FLYING SAUCER |
| FM4-64 | N-(3- triethylammoniumpropyl)-4-(p-diethylaminophenylhexatrienyl) |
| | pyridinium dibromide |
| FRY | FIERY |
| Fuc | fucrose |
| FUS | FUSCA |
| G3P | glycerol-3-phosphate |
| GalA | galacturonic acid |
| GAPC | GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE C |
| | SUBUNIT |
| GATL | GALACTURONOSYLTRANSFERASE-LIKE |
| GC | gas chromatography |
| GEF | GTP exchange factors |
| GK | GABI-Kat |
| GFP | green fluorescent protein |
| GL | GLABRA |
| Glc | Glucose |
| GlcA | glucuronic acid |
| GNL | GNOM-LIKE |
| GPAT | acyl-CoA:glycerol-3-phosphate acyltransferase |
| GPI | Glycosylphosphatidylinositol |
| GT | glycosyltransferase |
| GTP | Guanosine triphosphate |
| GUS | β-glucuronidase |
| HCD | 3-hydroxyacyl-CoA dehydrase |
| HD | homeodomain |
| HD-ZIP | homeodomain-leucine zipper |
| HG | homogalacturonan |

| HPAEC | high-performance anoin exchange chromatography |
|-------|---|
| KAS | 3-ketoacyl-ACP synthase |
| kb | kilo base pair |
| KCR | 3-ketoacyl-CoA reductase |
| KCS | 3-ketoacyl-CoA synthase |
| LEC | LEAFY COTYLEDON |
| Ler | Landsberg erecta |
| LPA | acyl-CoA:lysophosphatidic acid |
| LPAT | LPA acyltransferase |
| LPCAT | acyl-CoA:lysophosphatidylcholine acyltransferase |
| LTPG | Glycosyl-phosphatidylinositol-Anchored Lipid Transfer Protein |
| LUH | LEUNIG_HOMOLOG |
| MAH | MID-CHAIN ALKANE HYDROXYLASE |
| MES | 2-(N-morpholino) ethanesulfonic acid |
| MUM | MUCILAGE MODIFIED |
| MVB | multivesicular body |
| NASC | The European Arabidopsis Stock Centre |
| NDP | nucleoside diphosphate |
| nLuc | amino-terminus of luciferase protein |
| OD | optical density |
| PA | phosphatidic acid |
| PAP | PA phosphatase |
| PAS2 | PASTICCINO2 |
| PC | phosphatidylcholine |
| PCR | polymerase chain reaction |
| PDAT | phospholipid:diacylglycerol acyltransferase |
| PDCT | phoshpatidylcholine:diacylglycerolcholinephosphotransferase |
| PE | phosphatidyl ethanolamine |
| PIN | PIN-FORMED |
| PLDZ | PHOSPHOLIPASE D ZETA |
| PM | plasma membrane |
| PME | PECTIN METHYLESTERASE |
| PMEI | PECTIN METHYL ESTERASE INHIBITOR |
| PUFA | polyunsaturated fatty acid |
| PVC | pre-vacuolar compartment |
| qPCR | quantitative real-time polymerase chain reaction |
| RDR | RNA-DEPENDENT RNA POLYMERASE |
| RFP | red fluorescent protein |
| RG I | rhamnogalacturonan I |
| RG II | rhamnogalacturonan II |
| Rha | rhamnose |
| RHM | RHAMNOSE BIOSYNTHESIS |
| RNA | ribonucleic acid |
| | |

| RNAi | RNA interference |
|----------|---|
| RNAP II | RNA polymerase II complex |
| rRNA | ribosomal RNA |
| RT-PCR | reverse transcription polymerase chain reaction |
| SAPLIP | Saposin B domain-containing protein |
| SAR | Secretion-associated RAS-related protein |
| SD | standard deviation |
| secGFP | secretory GFP |
| SEM | scanning electron microscope |
| SGS | SUPPRESSOR OF GENE SILENCING |
| SNARE | soluble N-ethylmaleimide-sensitive factor attachment protein receptor |
| SOS | saltoverly sensitive |
| SPI | SPIRRIG |
| SSE | SHRUNKEN SEED |
| SYP | SYNTAXIN OF PLANTS |
| TAG | triacyglycerol |
| T-DNA | transfer DNA |
| TGN | trans-Golgi network |
| TT | TRANSPARENT TESTA |
| TTG | TRANSPARENT TESTA GLABRA |
| UTR | untranslated region |
| V-ATPase | Vacuolar-type H ⁺ ATPase |
| VHA-C | V-ATPASE SUBUNIT C |
| VLCFA | very long chain fatty acid |
| WBC | White–Brown Complex |
| WNK | with no lysine (K) |
| WRI | WRINKLED |
| WSD | wax ester synthase/diacylglycerol acytransferase |
| WT | wild type |
| X-gluc | 5-bromo-4-chloro-3-indolyl-β-D-glucuronide |
| YFP | yellow fluorescent protein |

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BACKGROUND AND INTRODUCTION

Plants produce metabolites, such as lipids and carbohydrates, which are essential for plant development and growth, but also have a lot of valuable uses for humans. Therefore, understanding biosynthesis, deposition, transport and regulation of these metabolites will benefit our daily life and the environment. My PhD thesis research focuses on seed oil, cuticular wax and seed coat mucilage in *Arabidopsis thaliana* (Arabidopsis). In the first chapter, I will introduce the biosynthesis and regulation of seed oil, cuticular wax and seed coat mucilage, as well as the secretion of cuticular wax and seed coat mucilage in Arabidopsis to provide the framework for my research questions and research objectives.

1.1 Seed oils

Higher plants produce seeds to propagate and maintain the species. During the process of seed development, plants fill seeds with reserve compounds, which facilitate the germination and seedling growth before photosynthesis is established. In addition to providing nourishment during early stages of plant life, seed oils are important commodities used for human consumption. They can also be used as industrial feed-stocks with a wide range of applications. Furthermore, with the decline of crude oil supplies, plant oils are starting to be exploited as biofuels, replacing the conventional fossil fuels. Given the high value and the overall demand for seed oils, there is considerable interest in increasing the oil production by genetic engineering (Durrett et al., 2008; Dyer et al., 2008; Baud et al., 2008). To accomplish this, a better understanding of the seed oil biosynthetic pathway and its regulation is required.

1.1.1 Seed oil biosynthesis

Seed oils accumulate in embryo cells from the middle until the late stage of Arabidopsis seed development. The main form of seed oil is triacylglycerol (TAG), which is comprised of three fatty acyl chains attached to a glycerol backbone. The fatty acyl chains are generated by *de novo* fatty acid biosynthesis in the plastid. They are then transported to the endoplasmic reticulum (ER), where they can be further elongated into very long chain fatty acids (VLCFAs) and used for TAGs biosynthesis (Baud et al., 2008).

1.1.1.1 De novo fatty acid biosynthesis

Fatty acid biosynthesis has been well studied (Figure 1.1, reviewed by Baud et al., 2008). The key substrate for fatty acid formation is acetyl-coenzyme A (acetyl-CoA). In the plastid, acetyl-CoA is carboxylated to generate malonyl-CoA by an acetyl-CoA carboxylase (ACCase). Before entering fatty acid biosynthetic cycles, the malonyl group is transferred from CoA to an acyl carrier protein (ACP) by malonyl-CoA:ACP malonyltransferase. *De novo* fatty acid biosynthesis begins with acetyl-CoA as an initial starter and malonyl-ACP as a carbon donor, which adds 2 carbons to acyl-ACP in each cycle of a series of condensation reactions. The enzyme complex catalyzing the process is named fatty acid synthase (FAS), and consists of four monofunctional enzymes, including 3-ketoacyl-ACP synthase (KAS), 3-ketoacyl-ACP reductase, 3-hydroxyacyl-ACP dehydratase, and enoyl-ACP reductase. Three KASs function in the different cycles of fatty acid biosynthesis. KAS III catalyzes the first condensing cycle from 2:0-ACP (malonyl-ACP) to 4:0-ACP, KAS I catalyzes reactions from 4:0-ACP to 16:0-ACP, and finally KAS II extends the acyl chain from 16:0-ACP to 18:0-ACP. 18:0-ACP can then

be desaturated to 18:1-ACP. Following their release from ACP by an acyl-ACP thioesterase and transfer to CoA by an acyl-CoA synthase, the major products of fatty acid biosynthesis, 16:0-CoA and 18:1-CoA, are transported from the plastid to the ER for fatty acid elongation and/or incorporation into TAG.



Figure 1.1. Fatty acid synthesis in the plastids in Arabidopsis. Reproduced with permission from Baud et al., 2008; Copyright © 2008, American Society of Plant Biologists.

1.1.1.2 Fatty acid elongation

In the ER, fatty acids can be elongated to very long chain (>18) fatty acids (VLCFAs). Arabidopsis TAGs contain 20-22 carbon-long (C20-C22) VLCFAs (Millar and Kunst, 1997). The fatty acid elongation pathway uses acyl-CoA as a substrate for the acyl chain extension instead of acyl-ACPs used in the *de novo* fatty acid biosynthesis in the plastid. Fatty acid elongation also involves four reactions (Figure 1.2, Kunst and Samuels, 2009; Haslam and Kunst, 2013): condensation of acyl-CoA with malonate, reduction of β -ketoacyl-CoA, dehydration of β -hydroxyacyl-CoA and finally a reduction of enoyl-CoA to form an acyl chain with 2 additional carbons. These four reactions are catalyzed by a complex of four membrane bound enzymes, β -ketoacyl-CoA synthase (KCS, condensing enzyme), β -ketoacyl-CoA reductase (KCR), β -hydroxyacyl-CoA dehydrase (HCD) and enoyl-CoA reductase (ECR), respectively (Kunst and Samuels, 2009).



Figure 1.2. Fatty acid elongation in the ER in Arabidopsis. Reproduced with permission from Kunst and Samuels, 2009; Copyright © 2009, Elsevier.

KCS catalyzes the rate limiting condensing reaction and also determines the chain length of the final products of fatty acid elongation (Millar and Kunst, 1997; Denic and Weissman, 2007). The first identified KCS in Arabidopsis is encoded by FATTY ACID ELONGATION 1 (FAE1). The decreased VLCFA content of storage lipids in the fae1 mutant seeds, fatty acid elongation assays, and the seed-specific expression pattern of the FAE1 gene indicate that FAE1 functions as a condensing enzyme specifically to produce C20-C22 VLCFAs in seed oils (Kunst et al., 1992; James et al., 1995). Based on sequence homology, 21 KCS genes have been found in the Arabidopsis genome (Costaglioli et al., 2005; Haslam and Kunst, 2013). It has been shown that KCS1, ECERIFERUM 6 (CER6)/CUT1 and KCS9 are involved in cuticular lipid biosynthesis (Todd et al., 1999; Millar et al., 1999; Fiebig et al., 2000; Kim et al., 2013), and KCS2/DAISY and KCS9 are involved in suberin production (Franke et al., 2009; Kim et al., 2013). However, KCSs are not the only condensing enzymes in plants. Another protein family called ELONGATION DEFECTIVE-LIKE (ELO-LIKE) proteins, identified based on homology of their sequences to the yeast ELO condensing enzymes was also reported to function in fatty acid elongation. There are four *ELO-LIKE* genes in Arabidopsis, which are not yet well characterized (Haslam and Kunst, 2013).

The other three enzymes, KCR, HCD and ECR, in the fatty acid elongase complex have broad substrate specificities and work with different KCSs to produce VLCFAs for diverse range of lipids in various plant tissues (Kunst and Samuels, 2009; Haslam and Kunst, 2013). In Arabidopsis, β -*KETOACYL-COA REDUCTASE 1(KCR1)* was identified to encode KCR (Beaudoin et al., 2009), *PASTICCINO2 (PAS2)* encodes HCD (Bach et al., 2008), and *CER10* encodes ECR (Gable et al., 2004; Zheng et al., 2005).

1.1.1.3 TAG biosynthesis and assembly

TAGs were traditionally thought to be synthesized only through the Kennedy pathway (Kennedy, 1961). It involves sequential acylation of the glycerol-3-phosphate (G3P) at the sn-1 and the sn-2 positions, catalyzed by acyl-CoA:glycerol-3-phosphate acyltransferase (GPAT) and acyl-CoA:lysophosphatidic acid (LPA) acyltransferase (LPAT), respectively, and followed by removal of the phosphate group of phosphatidic acid (PA) by PA phosphatase (PAP) to allow the acylation of the sn-3 position by the acyl-CoA:diacylglycerol (DAG) acyltransferase (DGAT). More recent work revealed that phosphotidylcholine (PC) also plays an important role in TAG biosynthesis, especially in recruitment of polyunsaturated fatty acids (PUFAs), or unusual fatty acids into TAGs (Bates and Browse, 2012; Bates et al., 2013). Fatty acids can be incorporated into TAGs through PC by three mechanisms. First, by the acyl editing cycle of deacylationreacylation between the acyl-CoA and PC pools catalyzed by the reverse reaction of acyl-CoA:lysophosphatidylcholine acyltransferase (LPCAT; Stymne and Stobart, 1984). Second, a FA can be directly transferred from a PC molecule to *sn*-3 position of DAG to form TAG by the phospholipid:DAG acyltransferase (PDAT; Dahlqvist et al., 2000; Zhang et al., 2009). Third, the interconversion of PC and DAG pools is also possible through the activity of CDP-choline:diacylglycerol cholinephophotransferase (CPT; Li-Beisson et al., 2010) or phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT; Lu et al., 2009), producing PC-derived DAGs, which can serve as the substrates of DGAT and PDAT, as well as the *de novo* DAGs from the Kennedy pathway (Bates and Browse, 2012). Both DGAT and PDAT were demonstrated to be the key enzymes in TAG biosynthesis, as a *PDAT1* RNAi transgenic line in the *dgat1-1* background or a *DGAT1* RNAi transgenic line in the *pdat1-1* background showed a 70% to 80% decrease in oil content and resulted in the disruption of embryo development (Zhang et al., 2009).

After TAGs are synthesized, they are assembled into oil bodies and stored in the cytoplasm of embryonic cells. Plant seed oil bodies are spherical cellular organelles surrounded by a phospholipid monolayer embedded with proteins (Baud et al., 2008; Beaudoin and Napier, 2004; Gutierrez et al., 2007). The most abundant oil body associated proteins identified in Arabidopsis seeds and other oilseeds are oleosins (Baud et al., 2008; Jolivet et al., 2013), which were shown to be important in maintaining the proper size and a high surface-volume ratio of oil bodies, and oil body stability (Siloto et al., 2006; Jolivet et al., 2013).

1.1.2 Regulation of seed oil accumulation

Many plant researchers contributed to the identification of key regulatory proteins involved in seed oil accumulation using forward and reverse genetics. For example, *WRINKLED1* (*WRI1*) encodes a transcription factor reported to regulate the expression of genes involved in glycolysis and fatty acid biosynthesis. The mutant *wri1-1*, in which the *WRI1* gene is disrupted, showed an 80% reduction in seed oil content (Baud et al., 2009; Focks and Benning, 1998). The *WRI1* gene is itself regulated by a transcriptional network comprised of four transcription factors, FUSCA3 (FUS3), ABSCISIC ACID INSENSITIVE 3 (ABI3), LEAFY COTYLEDON 1 (LEC1) and LEC2, which were recognized as the master regulators controlling seed maturation and seed filling (Baud et al., 2007; To et al., 2006). Mutations disrupting any of these four genes result in some common seed phenotypes, including desiccation intolerance, anthocyanin accumulation, and decreased storage compound accumulation (To et al., 2006; Gutierrez et al., 2007).

In addition, a mutation in the *GLABRA 2* (*GL2*) gene, encoding a homeodomain transcription factor, was shown to result in an 8% increase in seed oil content, indicating that GL2 is a negative regulator of seed oil accumulation (Shen et al., 2006). However, the regulatory role of GL2 in seed oil accumulation has not been investigated.

1.2 Cuticular wax

1.2.1 Plant cuticle

Surfaces of the primary aerial tissues of land plants are covered by a highly hydrophobic lipid layer, the cuticle, which protects plants against non-stomatal water loss, serves as a barrier to pathogen invasion and prevents organ fusion. The major components of the plant cuticle are cutin and cuticular wax. Cutin is a polyester composed of glycerol and hydroxy fatty acids with the chain length of 16 or 18 carbons. It is considered the structural backbone of the cuticle. Cuticular wax is embedded in the cutin polymer as intracuticular wax and covers the cutin matrix as a distinct layer called epicuticular wax. Cuticular wax is a complex mixture of aliphatic compounds and secondary metabolites, such as flavonoids and triterpenoids. The major aliphatic components of cuticular wax are derived from VLCFAs, and include fatty acids, primary alcohols, aldehydes, secondary alcohols, ketones and wax esters in Arabidopsis (Samuels et al., 2008; Kunst and Samuels, 2009; Nawrath et al., 2013). My thesis work focuses on the cuticular wax component of the cuticle.

1.2.2 Cuticular wax biosynthesis

1.2.2.1 Fatty acid elongation in cuticular wax biosynthesis

The precursors of cuticular wax biosynthesis are VLCFAs, which are generated by the *de novo* fatty acid biosynthesis in the plastid and fatty acid elongation in the ER of the epidermal cells. The two pathways used for their production are the same as those in the embryo cells used for fatty acid formation during seed oil biosynthesis, introduced in sections 1.1.1.1 and 1.1.1.2 above. However, the wax aliphatic components are derived from VLCFAs with the chain length of 24 to 34 carbons (Samuels et al., 2008), and their biosynthesis requires a unique set of condensing enzymes: KCS1, CER6/CUT1 and KCS9. The severe stem was phenotype of the *cer6/cut1* mutant with accumulation of C24 fatty acids in the stem epidermis, and the expression of the CER6/CUT1 gene exclusively in epidermal cells of the shoot demonstrates its role in cuticular wax biosynthesis and indicates that CER6/CUT1 is the major condensing enzyme responsible for elongation of wax precursors (Millar et al., 1999; Fiebig et al, 2000; Hooker et al., 2002). KCS1 was also reported to be involved in cuticular wax biosynthesis, as evidenced by the reduced amounts C26, C28 and C30 wax components in the kcs1 mutant compared to the wild type (Todd et al., 1999). A recent study showed that KCS9, involved in the elongation of C22 to C24 fatty acids, is also required for wax-related VLCFA production. However, the ubiquitous expression pattern of KCS9 gene and the kcs9 mutant phenotype demonstrated that KCS9 activity is also required for suberin and sphingolipid formation (Kim et al., 2013).

Despite its central role in wax biosynthesis, CER6/CUT1 can only elongate VLCFAs to 28 carbons in length. Considering that the most abundant cuticular wax

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components are derived from C30 fatty acids, other protein(s) must be involved. Characterization of the *cer2* mutant deficient in C28 elongation (McNevin et al., 1993; Jenks et al., 1995), and the CER2 protein demonstrated that CER2 works together with CER6/CUT1 in acyl chain extension from C28 to C30 (Haslam et al., 2012). Furthermore, a close CER2 homolog, CER2-LIKE1/CER26, is involved in the elongation of C30 to longer chain lengths and also works redundantly with CER2 in C28 to C30 fatty acid elongation in leaf (Haslam et al., 2012; Pascal et al., 2013).

1.2.2.2 Primary alcohol and alkane pathways

The second stage of wax biosynthesis involves two pathways (Figure 1.3), the primary alcohol pathway, also called the acyl reduction pathway, and the alkane pathway, also named as the decarboxylation pathway (Samuels et al., 2008; Kunst and Samuels, 2009; Bernard and Joubes, 2013). In the primary alcohol pathway, VLCFAs are converted into primary alcohols by two reduction reactions catalyzed by the CER4 fatty acyl-CoA reductase (FAR) (Rowland et al., 2006; Kunst and Samuels, 2009; Rowland and Domergue, 2012). However, the notable amount of C30 fatty alcohols still present in the *cer4* mutant wax suggests that one of the other seven FAR isozymes might also be involved in this process. In addition to being prominent wax components, primary alcohols are also used for the production of wax esters. Wax ester biosynthesis from primary alcohols and fatty acyl-CoAs is catalyzed by a wax ester synthase (Kunst and Samuels, 2009; Rowland and Domergue, 2012). *WSD1*, a member of the bifunctional wax ester synthase/diacylglycerol acyltransferase gene family, was demonstrated to

encode a wax ester synthase involved in wax esters formation in the Arabidopsis stem (Li et al., 2008).

The other branch of wax biosynthesis, the alkane pathway, produces aldehydes, alkanes, secondary alcohols and ketones. Even though several Arabidopsis *cer* mutants show defects specifically in the alkane formation, including *cer1* and *cer3* (Jenks et al., 1995), and the *CER1* and *CER3* genes were cloned several years ago (Aarts et al., 1995; Chen et al., 2003; Rowland et al., 2007), the biochemical functions of CER1 and CER3 in alkane formation have not been established. In addition, reactions involved in alkane formation are not well understood. A recent study, however, showed that CER1 can interact with CER3 to form an alkane synthesis complex, and co-expression of *CER1* and *CER3* in yeast resulted in the production of VLC-alkanes from VLC-acyl-CoAs (Bernard et al., 2012). This work also revealed that ER-localized cytochrome b5 isoforms (CYTB5s) are part of the alkane-forming complex where they likely serve as electron donors of CER1, suggesting that alkane synthesis is a redox-dependent process (Bernard et al., 2012; Bernard and Joubes, 2013).



Figure 1.3. Overview of the cuticular wax biosynthesis. CER, ECERIFERUM; WSD, wax synthase/diacylglycerol acyltransferase; MAH, mid-chain alkane hydroxylase. Reproduced with permission from Samuels et al., 2008; Copyright © 2008, Annual Reviews.

In the final two reactions of the alkane pathway, alkanes are further hydroxylated to yield secondary alcohols, which are subsequently oxidized into ketones. Both reactions have been demonstrated to be catalyzed by a cytochrome P450 enzyme (CYP96A15), encoded by *MID-CHAIN ALKANE HYDROXYLASE 1 (MAH1)* (Greer et al., 2007).

1.2.3 Secretion of cuticular waxes

1.2.3.1 Wax transport from the ER to the PM

The ER localization of wax biosynthesis enzymes, especially the two enzymes catalyzing the last steps of wax biosynthesis, WSD1 (Li et al., 2008) and MAH1 (Greer et al., 2007), suggests that all wax components are likely synthesized in the ER. Thus, the highly hydrophobic wax molecules have to travel through the hydrophilic cytoplasm to the plasma membrane (PM) and, after passing through the PM, traverse the cell wall on their way to the plant surface. Compared to the wax biosynthesis, relatively little is known about the export of the wax components (Figure 1.4; Samuels et al., 2008; Kunst and Samuels, 2009; Bernard and Joubes 2013), especially how they are delivered from the ER to the PM. Two hypotheses have been put forward to explain this process: 1) wax components could be secreted through the Golgi-mediated vesicular trafficking pathway, or 2) wax molecules could be transferred from the ER to the PM directly at the ER-PM contact sites (Figure 1.4; Samuels et al., 2008; Bernard and Joubes, 2013).

Recently, the first piece of evidence has been presented that trafficking of wax components from the ER to the PM is dependent on GNOMLIKE1 (GNL1) and ECHIDNA (ECH) proteins (McFarlane et al. 2014). GNL1 is required for COAT PROTEIN I (COPI) vesicle formation involved in retrograde transport from the Golgi apparatus to the ER (Richter et al., 2007; Teh and Moore, 2007), whereas ECH is a part of a protein complex involved in the trafficking of secretory vesicles from the TGN to the PM (Gendre et al., 2011; Boutte et al., 2013; McFarlane et al., 2013). Mutations in the *ECH* gene result in a dramatic reduction, and those in the *GNL1* result in a moderate reduction, in stem cuticular wax load compared to the wild type, an indication that wax

transport *en route* from the ER to the PM involves the Golgi apparatus and the TGN (McFarlane et al., 2014).

The ER-PM contact sites, also called ER-PM junctions, were observed in several eukaryotes, including mammals, insects, yeast and plants, and they have been shown to play an important role in lipid transfer between the ER and the PM, for example, the transfer of ergosterol in yeast and phosphatidylinositol in Drosophila (reviewed by Carrasco and Meyer, 2011). However, the evidence in support of the function of the ER-PM contact sites in wax export in plants is still lacking.



Figure 1.4. A model of cuticular wax secretion. ABC transporter, ATP binding cassette transporter; CER5, ECERIFERUM 5; FAE, fatty acid elongase; LTP, lipid transfer protein; WBC, WHITE-BROWN COMPLEX. Reproduced with permission from Samuels et al., 2008; Copyright © 2008, Annual Reviews.

1.2.3.2 Wax transport beyond the PM

The first component of the wax export machinery to be identified was CER5, a halfsize ATP-binding cassette (ABC) transporter belonging to ABCG/White-Brown Complex (WBC) subfamily, also named ABCG12 according to the nomenclature of ABC proteins (Pighin and Zheng et al., 2004; Verrier et al., 2008). Wax deficiency of the cer5 mutant associated with an accumulation of lipidic inclusions in the epidermal cells, epidermis-specific expression of the CER5 protein, together with its PM localization led to a proposal that CER5 transporter was involved in the delivery of wax components across the PM to the apoplast (Pighin and Zheng et al., 2004). Another half-size ABC transporter of the same subfamily, ABCG11/WBC11, was reported to be involved in the cuticular lipid export by several research groups (Bird et al., 2007; Luo et al., 2007; Panikashvili et al., 2007; Ukitsu et al., 2007). Characterization of the *abcg11* mutant revealed a deficiency in cuticular wax, but also in cutin deposition. Further work demonstrated that *abcg11cer5* double mutant shows the similar phenotype as the *abcg11* single mutant (Bird et al., 2007) and that these two proteins can interact with each other to form a heterodimer, while ABCG11, but not CER5, can also form a homodimer (McFarlane et al., 2010). Moreover, the PM localization of the CER5 protein is dependent on ABCG11, but not vice versa (McFarlane et al., 2010). Based on all the available evidence it was hypothesized that CER5 and ABCG11 form a heterodimer responsible for cuticular wax export, while ABCG11 homodimer or a heterodimer with another ABC protein is responsible for cutin secretion (McFarlane et al., 2010; Yazaki et al., 2009). The significant amount of the remaining cuticular wax on the cer5 and abcg11 single mutants, or the *cer5abcg11* double mutant indicates that additional ABC proteins,

or possibly another mechanism of wax delivery may be involved in wax transport across the PM (Pighin and Zheng et al., 2004; Bird et al., 2007).

After extrusion across the PM, the hydrophobic wax components have to traverse the hydrophilic cell wall. This function has been proposed to be carried out by cell wall associated lipid transfer proteins (Kunst and Samuels, 2009; Bernard and Joubes, 2013). Two glycosyl-phosphatidylinositol (GPI)-anchored Lipid Transfer Proteins (LTPGs), LTPG1 and LTPG2, were shown to be required for wax export from the PM to the plant surface (DeBono et al., 2009; Kim et al., 2012). Both *ltpg1* and *ltpg2* mutants had reduced cuticular wax load, suggesting that the two LTPGs may work redundantly (Kim et al., 2012). However, the PM localization of two proteins, and the mild wax phenotype of the *ltpg1ltpg2* double mutant (DeBono et al., 2009; Kim et al., 2012) indicate that additional LTPs located in the cell wall are likely involved in wax export as well.

1.2.4 Regulation of cuticular wax deposition

The plant cuticle formation is highly regulated during the development and influenced by environmental stresses, but details of this regulation are not well understood (Nawrath et al., 2013; Bernard and Joubes, 2013). So far, two MYB transcription factors, MYB96 (Seo et al., 2011) and MYB30 (Raffaele et al., 2008), were reported to be involved in the regulation of cuticular wax deposition in the response of drought stress and pathogen infection, respectively. Overexpression of *MYB96* promotes expression of the genes involved in fatty acid elongation (*KCSs*), cuticular wax biosynthesis (*CER1*, *CER2* and *WSD1*) and wax secretion (*ABCG11*), and results in increases accumulation of cuticular wax. In contrast, loss-of-function mutations in the

MYB96 gene was shown to cause wax deficiency (Seo et al., 2011). Similarly, MYB30 was demonstrated to regulate expression of the genes involved in the fatty acid elongation and wax biosynthesis under pathogen attacks. Whether MYB30 is also involved in the regulation of cuticular wax deposition during normal development remains to be determined (Raffaele et al., 2008).

An additional way of regulating wax accumulation in Arabidopsis was discovered through characterization of wax deficient mutant *cer7*. This work demonstrated that the CER7 ribonuclease, a core subunit of the RNA processing and degrading exosome affects wax deposition by controlling the expression of wax biosynthetic gene *CER3* (Hooker et al., 2007). A screen for suppressors of the *cer7* phenotype resulted in the identification of components of small RNA-biosynthetic machinery, RNA-DEPENDENT RNA POLYMERASE1 (RDR1) and SUPPRESSOR OF GENE SILENCING3 (SGS3), indicating that small RNAs are involved in the CER7-mediated regulation of *CER3* expression during cuticular wax biosynthesis (Lam and Zhao et al., 2012).

Furthermore, cloning of the *CER9* gene disrupted in the *cer9* mutant showed that it encodes an E3 ubiquitin ligase. Loss-of-function mutations in the *CER9* cause changes in cutin and wax accumulation (Lu et al., 2012), suggesting that yet another mechanism that involves protein degradation is likely involved in determining the final composition of cuticular lipids.

1.2.5 *Eceriferum* (*cer*) mutants

"Eceriferum" means "not bearing wax". Arabidopsis *cer* mutants were identified because they have brighter green stems and/or siliques due to altered epidermal wax

coating (Dellaert et al., 1979; Koornneef et al., 1982; Koornneef et al., 1989). Using these mutants as a starting point, many *CER* genes have been cloned, including *CER1*, *CER2*, *CER3*, *CER4*, *CER5*, *CER6*, *CER7*, *CER9* and *CER10* mentioned above. Although the functions of some of these *CER* genes still remain to be elucidated, the *cer* mutants have been extremely valuable in the dissection of wax biosynthetic pathways and the characterization of regulatory and transport components involved in wax deposition.

Among the *cer* mutants identified by Koornneef and his coworkers in the initial screen, the *cer11* mutant is quite unique because, in addition to exhibiting wax deficiency, it is dwarf and bushy (Koornneef et al., 1989; see chapter 3). Recent studies in our lab indicated that the *cer11* mutant also has a defect in secretion, which suggests that the CER11 protein may be involved in wax export from the ER to the PM (see chapter 3). However, no extensive characterization of this mutant and the *CER11* gene has been carried out until now.

1.3 Seed coat mucilage

1.3.1 Seed coat epidermal cell development

Seeds include several layers of cells that differentiate from the ovule integuments to form a protective tissue called the seed coat. The seed coat epidermal cells are the outermost layer. The differentiation and development of seed coat epidermal cells begin immediately after fertilization (reviewed by Haughn and Chaudhury, 2005). During the early stages when embryo develops from a zygote to the heart stage, seed coat epidermal cells enlarge by vacuolar expansion. After enlargement of the seed coat epidermal cells stops, the cells start synthesizing pectinaceous seed coat mucilage, which is secreted in a polar fashion to the junction between the radial and the outer tangential primary cell walls, forming the mucilage pocket. As the seed coat mucilage accumulates, the cytoplasm is gradually restricted to the center of the cell forming a volcano-shaped cytoplasmic column surrounded by the donut-shaped mucilage pocket. The seed coat mucilage biosynthesis culminates in the middle stage of seed development (Western et al., 2000), when the embryo is at the bent cotyledon stage and seed oil accumulation in the embryo culminates as well (Baud et al., 2008). During the later stages of seed development, secondary cell wall deposition beneath the mucilage pocket, along the outer tangential and radial sides of the cytoplasmic column, occurs. The secondary cell wall gradually accumulates from the top of the epidermal cell and eventually replaces the cytoplasm to form a volcano-shaped columella, before the cells are dehydrated at the end of maturation.

1.3.2 Seed coat mucilage structure and composition

As mentioned above, seed coat mucilage is synthesized and secreted during seed development and deposited in the mucilage pocket of the seed coat epidermal cells. When seeds imbibe, seed coat mucilage is rehydrated, expands rupturing the primary cell wall and extrudes to encapsulate the seed. The released seed coat mucilage can be easily stained by ruthenium red (Western, 2000). The ruthenium red staining identifies two layers of mucilage in Arabidopsis, an outer loosely attached layer and an inner adherent layer (reviewed by Haughn and Western, 2012).

Pectin represents the major mucilage component of Arabidopsis seed coat mucilage (Western et al., 2001; Haughn and Western, 2012; Kim and Brandizzi, 2014). The three major types of pectin polysaccharides are homogalacturonan (HG), rhamnogalacturonan I
(RG I) and the substituted galacturonan rhamnogalacturonan II (RG II) (Atmodjo et al., 2013). HG is an unbranched homopolymer chain of α -1,4-linked D-galacturonic acids (GalAs), which can be methylesterified or acetylated. Blocks of HG on different molecules, demethylesterified by enzymes, can be crosslinked by Ca²⁺ ions to form a gel. The RG I backbone chain consists of [4)- α -D-GalA-(1,2)- α -L-Rha-(1,]_n, in which the rhamnose (Rha) residues can be substituted with linear or branched oligosaccharides or polysaccharides. The side chains of RG I may include L-arabinan, D-galactan, L-fucrose (Fuc) or D-glucuronic acid (GlcA). RG II consists of an HG backbone with side chains (Haughn and Western, 2012; Atmodjo et al., 2013). In Arabidopsis, the dominant component of seed coat mucilage is relatively unbranched pectin RG I (Western et al., 2001; Haughn and Western, 2012; North et al., 2014). HG has been detected in Arabidopsis seed coat mucilage by JIM5 and JIM7 antibodies that recognize low-medium esterified HG and medium-highly esterified HG, respectively (Willats et al., 2001), and also by chemical analysis (Walker et al., 2011).

Cellulose microfibrils are also present in the seed coat mucilage in Arabidopsis, which can be detected by staining using calcofluor white and pontamine fast scarlet S4B (Willats et al., 2001; Sullivan et al., 2011; Harpaz-Saad et al., 2011; Mendu et al., 2011). A mutation in one of the cellulose biosynthesis gene, *CELLULOSE SYNTHASE 5* (*CESA5*), results in a loosening mucilage phenotype, which suggests the role of cellulose in maintaining mucilage structure and its anchoring to the seed (Sullivan et al., 2011; Harpaz-Saad et al., 2011; Harpaz-Saad et al., 2011; Mendu et al., 2011).

1.3.3 Arabidopsis seed coat mucilage biosynthesis

1.3.3.1 Pectin RG I biosynthesis in seed coat mucilage

As mentioned above, the major component of Arabidopsis seed coat mucilage is RG I, consisting of a backbone of alternating linked L-Rha and D-GalA. The substrates for the biosynthesis of the RG I backbone are the nucleotide sugars, UDP-L-Rha and UDP-D-GalA, which are synthesized from other nucleotide sugar precursors, like UDP-D-Glucose (Glc), by enzymatic reactions (Atmodjo et al., 2013). MUCILAGE MODIFIED 4 (MUM4), also known as RHAMNOSE BIOSYNTHESIS 2 (RHM2), was reported to encode an UDP-L-Rha synthase, which converts UDP-D-Glc to UDP-L-Rha (Western et al., 2004; Usadel et al., 2004; Oka et al., 2007). The mum4/rhm2 mutant seeds release much less mucilage than the wild type after hydration, and the mutant epidermal cells have smaller mucilage pockets (Western et al., 2004; Usadel et al., 2004). In addition, the monosaccharide analysis for mucilage showed that the amount of Rha and GalA decreased to 35% and 45% of the wild type level, respectively (Usadel et al. 2004). The ability of MUM4/RHM2 to convert UDP-D-Glc to UDP-L-Rha has been demonstrated in yeast (Oka et al., 2007). Furthermore, the expression of MUM4 gene is highly regulated during seed development, although it is expressed universally in all tissues (Western et al., 2004). MUM4 transcript level is up regulated in the middle stage of seed development (7 days post anthesis (DPA)), when the mucilage accumulation is at a highest rate (Western et al., 2004). There are another two *RHM* genes homologous to MUM4/RHM2 in Arabidopsis. The remaining amount of Rha in the mum4 mutant mucilage suggests that the three RHMs may work redundantly (Western et al., 2004; Usadel et al., 2004).

Subsequently, UDP-D-GalA and UDP-L-Rha have to be added to the oligo- or polysaccharide acceptor to form RG I backbone chain by the work of glycosyltransferases (GTs) (Atmodjo et al., 2013; North et al., 2014). A GT that may be involved in mucilage RG I backbone synthesis GALACTURONOSYLTRANSFERASE-LIKE5 (GATL5) was identified recently (Kong et al., 2013). The null mutant of *GATL5*, *gatl5-1*, has defects in mucilage production including a decrease in Rha and GalA (Kong et al., 2013). The Golgi localization of GATL5 protein is consistent with the concept that pectin polysaccharide biosynthesis occurs in the Golgi apparatus (Kong et al., 2013; Driouich et al., 2012). However, the GT activity of GATL5 has not yet been demonstrated, and the mild mucilage phenotype of *gatl5* seeds suggests that other GTs involved in seed coat mucilage biosynthesis remain to be identified (Kong et al., 2013).

1.3.3.2 Cellulose biosynthesis in seed coat mucilage

Cellulose microfibrils are synthesized by a PM associated cellulose synthase complex (CSC), which is thought to consist of 6 subunits, each of which contains multiple CESA proteins (reviewed by Somerville, 2006). In Arabidopsis, there are 10 CESAs based on sequence homology (Richmond and Someville, 2000). CESA5 is the first CESA known to be involved in mucilage biosynthesis, in that *cesa5* mutant seeds showed altered mucilage extrusion and reduced and malformed cellulose rays around the hydrated seed (Sullivan et al., 2011; Harpaz-Saad et al., 2011; Mendu et al., 2011). CESA2 and CESA9 do not appear to be involved in mucilage biosynthesis because the *cesa2 and cesa9* single mutants and *cesa2cesa9* double mutant do not have defective seed mucilage and the *cesa5cesa2cesa9* triple mutant showed a similar phenotype to the *cesa5*

single mutant (Mendu et al., 2011). Recently, a study in our lab showed that two mutant alleles of cesa3, ixr1-1 and ixr1-2, produce reduced cellulose in seeds, altered adherent mucilage structure and changed pectin distribution, indicating the involvement of CESA3 in mucilage production (Griffiths, 2013). In addition, another two mutants, *fei2* and *salt* overly sensitive 5 (sos5) also show reductions in the mucilage adhesion and altered cellulose rays similar to cesa5 (Harpaz-Saad et al., 2011). SOS5 encodes a fasciclin-like arabinogalactan protein (AGP) with a glycophosphatidylinositol (GPI) anchor, localized on the outer surface of the PM (Shi et al., 2003). While FEI2 encodes a Leucine-rich repeat receptor-like kinase, which is also localized in the PM (Xu et al., 2008). Both SOS5 and FEI2 are reported to be involved in the cellulose biosynthesis in root and work in the same pathway, because *sos5fei1fei2* triple mutant showed the similar root phenotypes as sos5 or feilfei2 double mutant (Shi et al., 2003; Xu et al., 2008). The similar mucilage phenotype of sos5 and cesa5 suggests that they might function in the same pathway (Harpaz-Saad et al., 2011). However, the more severe mucilage phenotype of sos5cesa5 double mutant compared to the single mutants indicates that SOS5 may be involved in mucilage production through a different pathway from CESA5 (Griffiths, 2013).

1.3.4 Seed coat mucilage modification

The investigations of *MUM2* and *BETA-XYLOSIDASE1* (*BXL1*) genes reveal that seed coat mucilage components are modified after secretion to the apoplast. The *mum2* mutant seeds do not extrude mucilage upon imbibition even when the primary cell wall barrier is manually removed, although the mucilage amount is similar to wild type (Dean

et al., 2007). These data indicate that the mucilage structure is altered by the mutation of *MUM2*. The *MUM2* gene encodes a β -galactosidase, which is believed to remove the side chains of RG I (Dean et al., 2007; Macquet et al., 2007). In support of this hypothesis, the monosaccharide and linkage analyses suggest that the RG I in *mum2* seed coat mucilage is more branched than that of the wild type (Dean et al., 2007; Macquet et al., 2007), consistent with the role of MUM2 in the removal of RG I side chains. The apoplastic localization of MUM2 protein (Dean et al., 2007) and its targeting specifically to the mucilage pocket concomitant with mucilage synthesis in seed coat epidermal cells (Lee, Gilchrist, Haughn, unpublished results, and my results in chapter 3), suggests that the mucilage modification is carried out at the mucilage deposition site after mucilage is secreted. Similarly, BXL1 encodes a bifunctional β -xylosidase/ α -arabinofuranosidase acting as an α -L-arabinofuranosidase in the seed coat (Arsovski et al., 2009). The bxll mutant seeds have patchy and slow mucilage release after hydration in water, but no seed coat epidermal cell development defect or altered mucilage amount was observed (Arsovski et al., 2009). The changed pectic arabinans were also detected in bxll mutant seed mucilage and the epidermal cell wall (Arsovski et al., 2009).

In addition to pectin side chain modification, correct pectin methylesterification has also been demonstrated to be important for proper mucilage release. As mentioned above, pectin HG is believed to be methylesterified by pectin methyltransferase when synthesized in the Golgi and demethylesterified after secretion to the apoplast (reviewed by Atmodjo et al., 2013). It is thought that the correct degree of pectin methylesterification determines the proper cell wall structure and can be achieved by the degree of activity of pectin methylesterases (PMEs) and the inhibition by their proteinaceous inhibitors, Pectin Methyl Esterase Inhibitors (PMEI; Atmodjo et al., 2013; Rautengarten et al., 2008; Saez-Aguayo et al., 2013). Several mucilage mutants that appear to alter the pectin methylesterification of mucilage have been identified (Rautengarten et al., 2008; Saez-Aguayo et al., 2013; Voiniciuc et al., 2013). For example, a mutation in one of the PMEI genes, PMEI6, results in a defect in seed coat mucilage release (Saez-Aguayo et al., 2013). A reverse genetic study demonstrated that loss-offunction mutant seeds of SBT1.7, encoding a subtilisin-like serine protease, failed to release seed coat mucilage when hydrated in water, but released normal mucilage after treated with EDTA (Rautengarten et al., 2008). It was found that the PME activity increases in the *sbt1.7* mutant seeds during the late stages of seed maturation (Rautengarten et al., 2008). Another example is the *flying saucer1* (*fly1*) mutant identified recently (Voiniciuc et al., 2013). The fly1 mutant was identified because the seeds release smaller mucilage halos compared to the wild type and a number of darkly stained small discs were found surrounding the seeds after wetting (Voiniciuc et al., 2013). The cloning of the *FLY1* gene demonstrates that it encodes a transmembrane protein with E3 ubiquitin ligase activity, which appears to be located in the late endosomes (Voiniciuc et al., 2013). The degree of pectin methylesterification was lower in the flyl mutant mucilage relative to the wild type, resulting in the hypothesis that FLY may regulate the pectin methylesterification in the seed coat mucilage by recycling PMEs (Voiniciuc et al., 2013).

1.3.5 Regulation of seed coat mucilage production

Seed coat mucilage production is highly regulated during seed development. For example, *MUM4* gene expression in the seed coat begins early (4 DPA), and is up-

regulated during the middle stages (7 DPA), and then is down-regulated again as the seed develops (10 DPA, Western et al., 2004). It has been demonstrated that the regulation of MUM4 expression is controlled by a transcriptional network (Western et al, 2004). The GL2 transcription factor was found to regulate MUM4 during seed development because the MUM4 transcription level is lower in the gl2 mutant seeds at 4 and 7 DPA (Western et al., 2004). GL2 has been previously reported to be involved in the regulation of many pathways, such as trichome development (Koornneef et al., 1982; Rerie et al., 1994; Szymanski et al., 1998), root hair differentiation (Masucci et al., 1996), seed oil accumulation (Shen et al., 2006) and seed coat mucilage production (Koornneef et al., 1982; Western et al., 2001). GL2 expression in several cell types has been reported to be regulated by a transcriptional complex, consisting of three classes of transcription factors, which are a basic helix-loop-helix (bHLH) transcription factor, a MYB transcription factor and a WD40 transcription factor. The WD40 transcription factor is always TRANSPARENT TESTA GLABRA 1 (TTG1) while the bHLH and MYB proteins vary depending on the cell type (Walker et al., 1999; Zhang et al., 2003; Western et al., 2004; Bernhardt et al., 2005; Zhao et al., 2008; Gonzalez et al., 2009; Li et al., 2009). In the seed coat epidermal cells, GL2 is specifically regulated by the complex composed of one of the bHLH proteins GLABRA 3 (EGL3) or TRANSPARENT TESTA8 (TT8), one of the MYB proteins MYB5 or TRANSPARENT TESTA2 (TT2) together with TTG1 (Zhang et al., 2003; Western et al., 2004; Bernhardt et al., 2005; Gonzalez et al., 2009; Li et al., 2009). In addition, a loss-of-function mutation in the AP2 gene results in mucilage defects in ap2 mutant seeds (Western et al., 2001), and a down regulation of GL2 and MUM4 transcript (Western et al., 2004), indicating that AP2 is also involved in the regulation of seed coat mucilage production. Furthermore, AP2 and the TTG1/bHLH/MYB complex also regulate the expression of *TRANSPARENT TESTA GLABRA 2 (TTG2)*, which encodes a WRKY transcription factor (Western et al., 2004; Gonzalez et al., 2009). TTG2 is also involved in seed coat mucilage production, based on the facts that *ttg2* seeds cannot release any mucilage when wetted (Johnson et al., 2002). However, TTG2 controls seed coat mucilage independently of the GL2-MUM4 pathway (Western et al., 2004). Recently, the transcription of a gene encoding another seed mucilage biosynthetic enzyme, GATL5, was demonstrated to be regulated by both TTG2 and GL2 (Kong et al., 2013).

Transcription of the genes encoding enzymes involved in seed coat mucilage modification, such as *MUM2* and *BXL1*, is also regulated. *MUM1* encodes a transcription factor, LEUNIG_HOMOLOG (LUH), and the *mum1* mutant seeds showed a similar mucilage phenotype as *mum2* (Huang et al., 2011). Moreover, the transcription levels of *MUM2* and *BXL1* were down regulated in *mum1* seeds compared to the wild type (Huang et al., 2011), indicating that MUM1 is involved in the regulation of seed coat mucilage modification and maturation by regulating *MUM2* and *BXL1*.

Last but not least, the involvement of FLY1 protein with E3 ubiquitin ligase activity (Voiniciuc et al., 2013) and SBT1.7 protease (Rautengarten et al., 2008) in the seed coat mucilage release indicates that post translational regulatory mechanisms also play an important role in the seed coat mucilage production and modification.

1.3.6 Seed coat mucilage secretion

The major mucilage component, pectin, is synthesized in the Golgi and believed to be secreted to the apoplast by vesicle-mediated secretion (reviewed by Driouich et al., 2012; Kim and Brandizzi, 2014, see section 1.4). Cellulose microfiber, as another component of mucilage and the major component in the primary cell wall and the secondary cell wall columella, is synthesized in the PM by the CESAs, which were recently demonstrated to be present in the vesicle associated complex (Drakakaki et al., 2012). Furthermore, many mucilage modification enzymes active in the apoplast, such as MUM2, PMEs, PMEIs and SBT1.7, are deposited to the apoplast via the secretory pathway (Dean et al., 2007; Saez-Aguayo et al., 2013; Rautengarten et al., 2008). Additionally, the endomembrane system localization of FLY1 protein also suggests that the vesicular trafficking is involved in recycling of mucilage modification enzymes (Voiniciuc et al., 2013). However, our knowledge about the secretion of seed coat mucilage is still limited.

1.4 Secretory pathway

1.4.1 Classical secretory pathway

The classical secretory pathway is an essential pathway conserved in all eukaryotes. It is involved in secretion of proteins, lipids and cell wall components in plants (reviewed by Jurgens and Geldner, 2002; Bassham et al., 2008; Kim and Brandizzi, 2014). Protein secretion starts at the ER, where the secretory proteins are targeted via their N-terminal signaling peptides. At the ER, the proteins are packed into the lumen of ER-derived vesicles if they are soluble, or integrated into the vesicle membrane if they are membrane-bound, and transported to the Golgi cisternae. The secretory proteins will travel through the Golgi apparatus from the *cis* to *trans* cisternae and then move to the *trans*-Golgi network (TGN). Finally, through vesicle fusion to the PM, soluble extracellular proteins will be delivered to the apoplast, whereas membrane-bound ones will be retained in the PM.

The vesicle formation and budding involve coat proteins, which are distinct between different types of coated vesicles (Jurgens and Geldner, 2002; Bassham et al., 2008). Three major types of vesicle coats were identified as COPII, COPI and clathrintype, which are associated with different compartments. For example, the COPII vesicles are derived from the ER and traffic to the Golgi, the COPI vesicles are believed to be involved in retrograde transport from the Golgi to the ER, while the clathrin-coated vesicles are found mainly in the TGN and the PM (Foresti and Denecke, 2008; Bassham et al., 2008).

Other major proteins required for vesicle trafficking in the secretory pathway include coat-GTPases and GTP exchange factors (GEFs), such as Secretion-associated RAS-related protein1 (SAR1) family of small G-proteins and SEC12-family GEFs for COPII, or ADP-ribosylation factor (ARF) family of G-proteins and ARF-GEFs for both COPI and clathrin-type coats (Bassham et al., 2008). In Arabidopsis, GNOM and GNOM-LIKE1 (GNL1) were reported as members of ARF-GEFs, which are involved in the endosomal recycling and endocytosis (Geldner et al., 2003; Teh and Moore, 2007).

After leaving the compartment where they are formed, vesicles are uncoated and directed to the target organelle. Specific vesicle targeting and fusion are mediated by proteins called soluble N-ethylmaleimide-sensitive factor attachment protein receptors

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(SNAREs). The formation of a four-helix *trans*-SNARE complex by three target-SNAREs (t-SNAREs) on the target membrane and one vesicle-associated-SNARE (v-SNARE) determines the destination of the vesicles and initiates membrane fusion (Sollner et al., 1993a; Sollner et al., 1993b; Poirier et al., 1998; McNew et al., 2000; Paumet et al., 2004). There are 54 genes encoding SNAREs in Arabidopsis, among which SYNTAXIN OF PLANTS 61 (SYP61) is localized in the TGN and used as a TGN marker (Uemura et al., 2004). Recently, proteomic analysis of the SYP61 associated compartment provided important information concerning the secretory proteins and cargo of the TGN (Drakakaki et al., 2012). This work identified many proteins, including several CESAs, which further confirmed that trafficking through the TGN plays an important role in cell wall formation (Drakakaki et al., 2012). The presence of the ECHIDNA protein in the SYP61 compartment confirmed the previous data showing that ECHIDNA is co-localized with SYP61 and other TGN markers (Gendre et al., 2011). This is significant, because a recent study on the *echidna* (*ech*) mutant demonstrated that ECHIDNA is required for the secretion of cell wall polysaccharides (Gendre et al., 2013). In addition, the *ech* mutant and the *gnl1* mutant have been reported to have cuticular wax defects (McFarlane et al., 2014), a first indication that wax components could be secreted through the Golgi-mediated vesicular trafficking pathway (See section 1.2.3.1).

1.4.2 The role of vacuolar type H⁺-ATPase (V-ATPase) in vesicular trafficking

V-ATPase is a multi-subunit enzyme complex, which serves as a proton pump by hydrolyzing ATP. V-ATPase structure is highly conserved in eukaryotic cells, and composed of two subcomplexes, the cytoplasmic hydrolysis motor V_1 and the membrane-

associated proton turbine V_0 (Figure 1.5; Reviewed by Forgac, 2007; Saroussi and Nelson, 2009; Marshansky and Futai, 2008; Schumacher and Krebs, 2010). The V_1 sector includes a catalytic hexamer consisting of subunits A and B for ATP hydrolysis, connected by stalks formed by subunits D and F to the proton pathway. Other components of the V_1 sector are subunits C, E, G and H. The V_0 sector is responsible for proton translocation, and in plant cells it is comprised of subunits a, c, c'', d and e (Sze et al., 2002; Schumacher and Krebs, 2010).



Figure 1.5. A schematic model of the V-ATPase. The V-ATPase holoenzyme is composed of the cytoplasmic hydrolysis motor V_1 and the membrane associated proton turbine V_0 . The V_1 sector contains a catalytic hexamer consisting of subunits A and B for ATP hydrolysis, connected by stalks formed by subunits D and F to the proton pathway. V_1 sector also includes subunits C, E, G and H. The V_0 sector is responsible for proton translocation, and is comprised of subunits a, c, c'', d and e. Reproduction with permission from Sze et al., 2002; Copyright © 2002, Elsevier.

The V-ATPase has long been considered to be only a "vacuolar" enzyme responsible for generating the proton gradient and maintaining the pH homeostasis. However, the emerging evidence demonstrates that V-ATPase is also involved in vesicular trafficking (Marshansky and Futai, 2008; Schumacher and Krebs, 2010). In Arabidopsis, three genes encode V-ATPase subunit a (VHA-a), one of which, VHA-a1, is specifically localized in the TGN (Dettmer et al., 2006), and now used as a TGN marker in many studies (for example, Gendre et al., 2013; Shirakawa et al., 2014). In addition, the abnormal Golgi organization was observed in the mutants affecting V-ATPase subunits A (vha-A, Dettmer et al., 2005) and E (vha-El, Strompen et al., 2005), and the RNAi-silenced VHA-a1 (Brux et al., 2008). Defects in cell expansion were also detected in several V-ATPase subunit mutants, including vha-C/det3 (Schumacher et al., 1999; Brux et al., 2008) and transgenic plants in which the expression VHA-c1 and VHA-c3 subunits was down-regulated by RNAi (Padmanaban et al., 2004). The cell expansion defect was suggested to be due to the disrupted secretion of the cell wall components (Brux et al., 2008; Padmanaban et al., 2004). Overall, it was proposed that V-ATPase was involved in the endomembrane trafficking and plays an important role in secretory pathway. However, the exact mechanism of action and the regulation of V-ATPase activity in vesicular trafficking are still not well understood.

1.5 Research questions, objectives and significance of findings

My thesis contains two major parts of the research on plant seed oil, cuticular wax and seed coat mucilage in Arabidopsis. The two main objectives were:

1) To investigate the role of GL2 in seed oil accumulation (Chapter 2)

As introduced above, seed oils not only are essential for plant seedling growth but also have invaluable uses for humans. Understanding biosynthesis and regulation of seed oils will benefit us in many ways and also provide significant knowledge for genetic engineering of high oil seeds. Among the regulators of seed oil accumulation identified so far, GL2 is the first negative regulator identified because gl2 mutant seeds produce more oil (Shen et al., 2006). However, how GL2 is involved in seed oil deposition was still unclear. Therefore, the first objective of my research was to investigate the role of GL2 in seed oil accumulation. Our original hypothesis was that GL2 negatively regulates target genes involved in seed oil biosynthesis in the embryo cells where seed oils are made and deposited. Potential candidates for such genes were PHOSPHOLIPASE D ZETAs (PLDZs), because GL2 negatively regulates PLDZ1 expression in root hair differentiation (Ohashi et al., 2003). Surprisingly, my data do not support the hypothesis, but instead suggest that it is the loss of MUM4 gene expression in the seed coat of the gl2 mutant that is the reason for the high seed oil content. My further investigation of other mutants defective in genes involving seed coat mucilage deposition confirms that the increased seed oil accumulation in the mutants is due to the loss of seed coat mucilage production. The overall results in the chapter 2 lead us to a proposal that the carbon supply normally used for the mucilage production in the seed coat is relocated to the embryo and utilized for seed oil biosynthesis when mucilage is not made. This information suggests a promising way to engineer high oil seeds by blocking seed coat mucilage production.

2) To investigate the function of CER11 in cuticular wax export (Chapter 3)

Relatively little is known about the secretion of apoplastic matrix components including pectin and cuticular wax. Preliminary results concerning the mutant cer11-1 indicated that there were defects in cuticular wax, seed mucilage and the secretion of the secretory green fluorescent protein (secGFP). These data suggest that *CER11* might be required for secretion of apoplastic components. However, the function of CER11 had not been explored in detail. Therefore, the second objective of my research was to clone the CER11 gene and investigate the function of CER11 in cuticular wax deposition, seed coat mucilage release and secretion. I first characterized the phenotypes of cer11-1 mutant in detail. The phenotypes of *cer11-1* suggest that the decreased wax deposition, altered seed coat mucilage release and accumulation, abnormal secondary cell wall formation and delayed MUM2 protein secretion may be all due to a defective secretory pathway in *cer11-1*. I then cloned the *CER11* gene and showed that it encodes C-TERMINAL DOMAIN (CTD) PHOSPHATASE LIKE 2 (CPL2), a phosphatase. Subsequently, I carried out a yeast-2-hybrid screen to identify the substrate of CPL2. The interaction of CPL2 and V-ATPase subunit C (VHA-C) in yeast and tobacco and the colocalization of two proteins in the epidermal cells of the stems and seed coats suggest that VHA-C is a potential substrate of CPL2 in the secretion of cuticular wax and cell wall components.

In the last chapter, chapter 4, I review key results, draw main conclusions of my research in a broader context, and also point out some future perspectives.

CHAPTER 2

ARABIDOPSIS *GLABRA2* MUTANT SEEDS DEFICIENT IN MUCILAGE BIOSYNTHESIS PRODUCE MORE OIL

2.1 Introduction

As discussed in chapter 1, seed oils are not only essential as an energy source for seed germination and seedling growth, but also important for humans as a food constituent, an industrial raw material, and as a feedstock for biodiesels. Thus, many studies have been done to understand how plants can be manipulated to produce seeds with higher oil content. Recently, Shen and his colleagues showed that a mutation in the *GL2* gene resulted in an 8% increase in seed oil content over wild type levels, indicating that GL2 is a negative regulator of seed oil accumulation (Shen et al., 2006).

GL2 encodes a homeodomain (HD) transcription factor which, based on its deduced protein sequence, belongs to the class IV homeodomain-leucine zipper (HD-ZIP) gene family (Rerie et al., 1994; Nakamura et al., 2006). *GL2* has been shown to control characteristics of several epidermal cell types including trichomes (Koornneef et al., 1982; Rerie et al., 1994; Szymanski et al., 1998), atrichoblasts (Masucci et al., 1996), and seed coat epidermal cells (Koornneef et al., 1982; Western et al., 2001). In the seed coat epidermis, GL2 is required for seed coat mucilage biosynthesis at least in part through its positive control over the *MUM4/RHM2* gene (Western et al., 2004). *MUM4/RHM2* encodes a rhamnose synthase needed to produce rhamnose (Western et al., 2004; Usadel et al., 2007), a key substrate for mucilage biosynthesis.

In all cell types, *GL2* expression is activated by a transcription complex that includes polypeptides from three different classes of transcriptional regulators, a basic helix-loop-helix (bHLH) transcription factor, a MYB transcription factor and a WD40 transcription factor TRANSPARENT TESTA GLABRA 1 (TTG1) (Walker et al., 1999; Zhang et al., 2003; Western et al., 2004; Bernhardt et al., 2005; Zhao et al., 2008; Gonzalez et al., 2009; Li et al., 2009). In the seed coat epidermis the components of the *GL2* regulatory complex are one of the bHLH proteins GLABRA 3 (EGL3) or TRANSPARENT TESTA8 (TT8), one of the MYB proteins MYB5 or TRANSPARENT TESTA2 (TT2) together with TTG1 (Zhang et al., 2003; Western et al., 2004; Bernhardt et al., 2005; Gonzalez et al., 2009; Li et al., 2009).

Currently, information concerning downstream targets of the GL2 transcription factor in the seed is limited. In addition to *MUM4*, *in vitro* studies showed that GL2 can bind to the promoter of *PHOSPHOLIPASE D ZETA 1* (*PLDZ1*), and transgenic lines overexpressing *PLDZ1* show an ectopic root hair phenotype similar to the *gl2* mutant, suggesting that GL2 is a negative regulator of *PLDZ1* (Ohashi et al., 2003). The objective of the research described in this chapter was to investigate the potential roles of MUM4 and PLDZ in GL2-mediated regulation of seed oil biosynthesis and deposition.

2.2 Results

2.2.1 gl2 mutant seeds contain more seed oil than wild type seeds.

Previous studies have shown that gl2-1 mutant seeds have higher oil content than wild type seeds (Shen et al., 2006). To verify this result, a T-DNA insertion line, SALK_130213 (gl2-8, Deal and Henikoff, 2010) was obtained. *GL2* transcript cannot be detected in the gl2-8 mutant (Figure 2.1b) in which the T-DNA insertion maps to the second intron of the *GL2* gene (Figure 2.1a). Analysis of seed oil showed a significant increase in oil content in gl2-8 seeds compared with the wild type (Figure 2.1c), similar to that described for the gl2-1 allele (Shen et al., 2006). The gl2-8 mutant also showed abnormal trichomes, an increase in root hair density and no extrusion of seed coat mucilage (Figure 2.2). These results not only indicated that the gl2-8 is a loss-of-function allele, but also confirmed that mutations in the GL2 gene result in increased seed oil accumulation.



Figure 2.1. Plants homozygous for a T-DNA insertion allele of the *GL2* gene show an increased seed oil content. (a) The structure of *GL2* gene (At1g79840) shows exons as black boxes, introns as solid lines and untranslated regions as gray boxes. The positions of the start codon and the stop codon are indicated with vertical arrows. The location of the T-DNA insertion of *gl2-8* (SALK_130213) was mapped and is indicated by the arrow head. The locations of primers used for RT-PCR in (b) are indicated with short horizontal arrows. (b) RT-PCR analysis of steady-state *GL2* transcript levels in wild type (WT (Col)) and mutant (*gl2-8*) leaves. RT-PCR was performed using total leaf RNA, and the expression level of *GAPC* was used as a control. (c) Seed oil analysis of wild type (WT (Col)) and the mutant (*gl2-8*). Data are expressed as mean percentage \pm SD (n=4; replicate analysis performed on seed lots from each line, with 2-2.5 mg seed analyzed per replicate). Student's t-test was applied to the data; the asterisk indicates that, statistically, this value was significantly different from wild type at P < 0.05.



Figure 2.2. Phenotypes of wild type (WT (Col)) (a, c, e) and *gl2-8* mutant plants (b,d,f) in trichome development (a-b) (Scale bar = 2 mm), root hair differentiation (c-d) (Scale bar = 0.2 mm) and mucilage production (e-f) (Scale bar = 0.2 mm).

2.2.2 GL2 control over seed oil levels is exerted through maternal tissues

To investigate the expression pattern of *GL2* in developing seeds, transgenic plants expressing the *uidA* reporter gene encoding β -glucuronidase (GUS) under the control of the *GL2* promoter (*GL2p::GUS*) were kindly provided by Dr. Jin-Gui Chen (see section 2.4). Yuanyuan Yu and I detected GUS activity in the embryo and seed coat indicating that the*GL2* gene is expressed in both locations during early (4 DPA), middle (7 DPA) and late (10 DPA) stages of seed development (Figure 2.3). The seed coat and the embryo hypocotyl expression patterns were consistent with the previous findings using *GFP* expression driven by the *GL2* promoter (Stadler et al., 2005).



Figure 2.3. Seed-specific expression pattern of *GL2* in Arabidopsis seeds. The *uidA* reporter gene encoding β -glucuronidase (GUS) under the control of the *GL2* promoter was expressed in Arabidopsis plants. Developing seeds were incubated in X-gluc assay buffer. GUS activity is indicated by a blue precipitate. (a) Seed coat at 7 DPA. (b-d) Developing embryos at 4 DPA (b), 7 DPA (c) and 10 DPA (d). Scale bars = 0.1 mm.

To determine whether the seed oil phenotype is due to defects in maternal or embryonic tissue I crossed a wild type (WT) plant as a male parent to a *gl2-8* plant as a female parent. The F_1 (*gl2xWT*) progeny were homozygous for *gl2* in the seed coat but heterozygous for *gl2* in the embryo. These results demonstrate that the oil content of the F_1 (*gl2xWT*) seeds is similar to that of *gl2-8* homozygous seeds, and higher than that of wild type seeds (Figure 2.4a) suggesting that it is *GL2* function in the seed coat that influences seed oil levels.



Figure 2.4. (a) Oil content analysis of seeds homozygous for *gl2* in the seed coat and heterozygous in the embryo. *gl2-8* plants were crossed as a female parent (\bigcirc) to wild type (WT (Col)) plants as a male parent (\bigcirc) to generate F1 seeds (F1 (*gl2xWT*)). The genotypes of the seed coat and the embryo of the F1 progeny are shown in the table. The seed oil was analyzed and compared with that of wild type (F1 (WTxWT)) and *gl2-8* plants (F1 (*gl2xgl2*)). Data are expressed as mean percentage \pm SD (n=4; replicate analysis performed on seed lots from each line, with 2-2.5 mg of seed analyzed per replicate). Student's t-test was applied to the data; the asterisk indicates that, statistically, this value was significantly different from wild type at P < 0.05. (b) Fatty acid analysis of mature embryos and seed coats of wild type (WT (Col)) and the mutant (*gl2-8*). Data are expressed as mean performed on seed lots from each line, with 2-0.05. (b) Fatty acid analysis of mature embryos and seed coats of wild type (WT (Col)) and the mutant (*gl2-8*). Data are expressed as mean performed on seed lots from each line, with colored the mutant (*gl2-8*). Data are expressed as mean performed on seed lots from each line, with colored the mutant (*gl2-8*). Data are expressed as mean performed on seed lots from each line).

Seed oils accumulate in both the embryo and the seed coat, although the embryo is the major compartment for seed oil storage (Li et al., 2006). To test whether the loss of GL2 function can affect the oil level in the seed coat or in the embryo, I analyzed the oil content in the embryo and the seed coat separately. The results show that the oil content in the *gl2-8* embryo is higher than that in the wild type embryo, but no difference was observed between wild type and the mutant in the seed coat oil levels (Figure 2.4b). I also compared fatty acid composition in the whole seed, the embryo and the seed coat between wild type and the *gl2-8* mutant. Fatty acid composition in the embryo and the seed coat are different, which is consistent with a previous study (Figure 2.5a, Li et al., 2006). However, no differences in fatty acid composition between wild type and the *gl2-*8 mutant in the whole seed, the embryo or the seed coat were observed (Figure 2.5b-d). Taken together, these results demonstrate that GL2 function in the seed coat influences seed oil accumulation in the embryo.



Figure 2.5. (a) Fatty acid composition in whole seeds, embryos and seed coats of wild type (Col). (b-d) Fatty acid composition in whole seeds (b), embryos (c) and seed coats (d) of wild type (WT (Col)) and the mutant (gl2-8).

2.2.3 *PLDZ* genes are expressed in the cotyledons and are up-regulated in *gl2-8* mutant seeds

A previous study suggested that *PLDZ1* is negatively regulated by GL2 during root cell differentiation (Ohashi et al., 2003). Since PLDZ activity could contribute to seed oil synthesis, I tested the hypothesis that *PLDZ* is a target of GL2 in seeds by examining the seed expression of *PLDZ* genes in wild type and the *gl2* mutant. In Arabidopsis there are two *PLDZ* genes, *PLDZ1* (At3g16785) and *PLDZ2* (At3g05630). Constructs expressing *GUS* under the control of *PLDZ1* (*PLDZ1p::GUS*) and *PLDZ2* (*PLDZ2p::GUS*) promoters, made by Yuanyuan Yu, were introduced into wild type plants. The detection of GUS activity in these transgenic plants indicated that both *PLDZ* genes were expressed in the developing embryos. *PLDZ1* was mainly expressed in the cotyledons in the early, middle and late stages of seed development (Figure 2.6b-d), whereas the *PLDZ2* promoter was active in the cotyledons only in the middle and late developmental stages (Figure 2.6f-h). In addition, GUS activity was detected in the funiculus (Figure 2.6a, e), but was never observed in the seed coats of transgenic plants expressing either construct.



Figure 2.6. Seed-specific expression pattern of *PLDZ1* (a-d) and *PLDZ2* (e-h) in Arabidopsis seeds. The *uidA* reporter gene encoding β -glucuronidase (GUS) under the control of the *PLDZ1* or *PLDZ2* promoter was expressed in Arabidopsis plants. Developing seeds were incubated in X-gluc assay buffer. GUS activity is indicated by a blue precipitate. (a, e), Seed coat at 7 DPA; (b-d, f-h), Developing embryos at 4 DPA (b, f), 7 DPA (c, g) and 10 DPA (d, h). Scale bars = 0.1 mm.

To test whether *PLDZ* genes are the targets of GL2 in seeds, I determined the expression levels of *PLDZ* genes in gl2-8 mutant developing seeds using quantitative real-time PCR (qPCR). Results showed that *PLDZ1* and *PLDZ2* expression levels in the gl2-8 mutant seeds during the early (4 DPA) and middle (7 DPA) stages were the same as those in the wild type, but in the late stage (10 DPA) seeds, both *PLDZ* genes were upregulated in the gl2-8 mutant compared with wild type (Figure 2.7). These results suggest that the *PLDZ* genes are GL2 targets in seeds in the late stage of development.



Figure 2.7. qPCR analysis of *PLDZ1* (a) and *PLDZ2* (b) expression in developing seeds. Total RNAs from 4 DPA, 7 DPA or 10 DPA developing seeds of wild type (WT (Col)) and the mutant (*gl2-8*) were analyzed for expression of *PLDZ1* (a) and *PLDZ2* (b) genes by qPCR using gene-specific primer pairs. *PLDZ1* and *PLDZ2* gene expression values were normalized to the *GAPC* expression. Error bars represent SD (n=4).

2.2.4 PLDZ genes are not required for GL2-mediated seed oil accumulation

To determine if PLDZ is involved in oil biosynthesis, the seed oil content of *pldz* mutants was analyzed. T-DNA insertion lines were obtained for *pldz1* (SALK 083090) and *pldz2* (SALK 094369) from the Arabidopsis SALK collection (Alonso et al., 2003), and the sites of T-DNA insertions were determined. The insertion in PLDZ1 gene was mapped to the second exon, while the insertion in PLDZ2 gene was located in the eleventh exon (Figure 2.8a). Wild type transcript of PLDZ1 and PLDZ2 genes could not be detected in plants homozygous for *pldz1* and *pldz2* (Figure 2.8b). Considering the possibility that PLDZ1 and PLDZ2 might work redundantly, I generated the *pldz1pldz2* double mutant plants. The seed oil content of either single mutant plant was not significantly different from wild type seeds (Figure 2.8c). The *pldz1pldz2* seeds also had wild type oil content (Figure 2.8d), suggesting that PLDZ does not play a role in seed oil biosynthesis. Furthermore, I constructed the gl2-8pldz1pldz2 triple mutant and compared its seed oil levels to gl_2 and the $pld_2 lpld_2 2$ double mutant to determine whether the high seed oil phenotype of the gl2 mutant was dependent on PLDZ function. The oil content of the gl2-8pldz1pldz2 seeds was similar to that of the gl2-8 mutant (Figure 2.8d), demonstrating that *PLDZ* genes are not required for GL2-mediated seed oil accumulation.





Figure 2.8. T-DNA insertion alleles of *pldz1* and *pldz2* mutants have a wild type seed oil phenotype. (a) The structures of *PLDZ1* gene (At3g16785) and *PLDZ2* gene (At3g05630) show exons as black boxes, introns as solid lines and untranslated regions as gray boxes. The start codon and the stop codon are indicated with vertical arrows. The locations of T-DNA insertion of *pldz1* (SALK_083090) and *pldz2* (SALK_094369) were mapped and are indicated by arrow heads. The locations of primers used for RT-PCR in (b) are indicated with short horizontal arrows. (b) RT-PCR analysis of steady-state *PLDZ1 and PLDZ2* transcript levels in mRNA from wild type (WT (Col)) and the double mutant (*pldz1pldz2*) leaves. RT-PCR was performed using total leaf RNA, and the expression level of *GAPC* was used as a control. (c) Oil analysis of wild type (WT (Col)) and the mutants (*gl2-8, pldz1 and pldz2*). (d) Oil analysis data are expressed as mean percentage \pm SD (n=4; replicate analysis performed on seed lots from each lines, with 2-2.5 mg seeds analyzed per replicate). The Student's t-test was applied to the data; the asterisk indicates that, statistically, this value was significantly different from wild type at P < 0.05.

2.2.5 MUM4 influences seed oil deposition

MUM4 encodes a rhamnose synthase, a mucilage biosynthetic enzyme, and was reported to be a target of GL2 in seed coat mucilage production (Western et al., 2004). To verify whether this is the case, I tested the expression level of MUM4 in the developing seeds by qPCR. My results show that MUM4 is down regulated in gl2-8 mutant seeds during the middle stages (7 DPA) of seed development (Figure 2.9a), which is consistent with previous data obtained using RT-PCR (Western et al., 2004). To directly assess whether MUM4 is involved in seed oil accumulation, I analyzed the oil content of mum4-*I* seeds. As shown in Figure 2.9b, *mum4-1* seeds contain more oil than the wild type seeds, implicating the involvement of MUM4 in seed oil accumulation. I also investigated the genetic relationship between GL2 and MUM4 by generating and analyzing gl2-8mum4-1 double mutant plants. The gl2-8mum4-1 seed oil levels were similar to those in *mum4-1* and *gl2-8* mutants (Figure 2.9b), and the double mutant also had reduced mucilage extrusion as observed in the *mum4-1* and *gl2-8* mutants (Figure 2.10). These results are consistent with the hypothesis that the high seed oil phenotype of the gl2 mutant is due to the loss of expression of MUM4.



Figure 2.9. Expression level of the *MUM4* gene in developing seeds and levels of seed oil in the *mum4-1* mutant. (a) Total RNAs from 7 DPA developing seeds of wild type (WT (Col)) and the *gl2-8* mutant were analyzed for expression of the *MUM4* gene and the *GAPC* gene as a standard, by qPCR. *MUM4* gene expression was normalized to *GAPC* expression values. Error bars represent SD (n=4). (b) Oil analysis of wild type (WT (Col)), the single mutants (*gl2-8* and *mum4-1*) and the double mutant (*gl2-8mum4-1*). Data are expressed as mean percentage \pm SD (n=4; replicate analysis performed on seed lots from each lines, with 2-2.5 mg seeds analyzed per replicate). The Student's t-test was applied to the data; the asterisk indicates that, statistically, this value was significantly different from wild type at P < 0.05.



Figure 2.10. Seed coat mucilage phenotypes of wild type (WT (Col)) (a), gl2-8 (b), mum4-1 (c) and gl2-8mum4-1 (d). The mature wild type and mutant seeds were stained with ruthenium red. Note the red staining of the mucilage capsule surrounding seeds. Scale bar = 0.2 mm.

2.2.6 Mucilage deficient mutants do not always have higher oil content than the wild type

To determine whether the high oil phenotype is due to the loss of mucilage or more specifically the loss of MUM4 function I investigated the seed oil content of mutants disrupted in the components of the regulatory pathway for mucilage production including TTG1, MYB5, TT2, EGL3, TT8, GL2, MUM4 and TRANSPARENT TESTA GLABRA2 (TTG2) (Figure 2.11) (Western et al., 2004; Gonzalez et al., 2009). My results show that *ttg1-1*, *myb5-1tt2* and *egl3tt8* have higher seed oil contents than the wild type (Figure 2.12a-c). The oil content of *myb5-1tt2* double mutant seeds is higher than that of the single mutants *myb5-1* or *tt2* (Figure 2.12b), which indicated that MYB5 and TT2 function redundantly in controlling seed oil levels. Similarly, the *egl3tt8* double mutant had higher oil than either single mutant *egl3* or *tt8* (Figure 2.12c). In contrast, the

oil content of ttg2 seeds was indistinguishable from the wild type (Figure 2.12d) suggesting that it is not the loss of mucilage per se, but more specifically the loss of MUM4 function that results in the overproduction of seed oil.



Figure 2.11. (a) The proposed pathway for the regulation of seed coat mucilage (Western et al., 2004; Gonzalez et al., 2009). (b) Mucilage phenotypes of wild type (WT) and the mutants of *myb5- 1tt2*, *egl3tt8*, *ttg1-1* and *ttg2-1*. Scale bar = 0.2 mm.



Figure 2.12. Oil phenotypes of the mutants, disrupted in genes that decrease seed mucilage. (a) Oil phenotypes of wild type (WT (Ler)) and the mutant (*ttg1-1*). (b) Oil phenotypes of wild type (WT (Col)), the single mutants (*gl2-8, myb5-1* and *tt2*) and the double mutant (*myb5-1tt2*). (c) Oil phenotypes of wild-type (WT (Col)), the single mutants (*gl2-8, egl3* and *tt8*) and the double mutant (*egl3tt8*). (d) Oil phenotypes of wild-type (WT (Ler)) and the mutants (*gl2-1* and *ttg2-1*). Oil analysis data are expressed as the mean percentage \pm SD (n=4; replicate analysis performed on seed lots from each lines, with 2-2.5 mg seeds analyzed per replicate). Student's t-test was applied to the data; the asterisk indicates that, statistically, this value was significantly different from wild type at P < 0.05.

2.3 DISCUSSION

GL2, a homeodomain transcription factor, negatively regulates the level of storage oil in the seed (Shen et al., 2006), a result I have confirmed and extended by showing that the positive regulators of GL2 act in a similar manner, and that the influence occurs primarily through maternal tissue. To investigate the mechanism by which GL2 controls oil levels, I examined known targets of GL2 that could influence seed oil, the *PLDZ* genes and *MUM4*. My data reveal that while the *PLDZ* genes are not involved in seed oil biosynthesis or deposition, *MUM4* may be the primary target through which GL2 acts to control seed oil content.

2.3.1 GL2 negatively regulates *PLDZ* genes during the late stages of seed development

PLDZ catalyzes the hydrolysis of membrane phospholipids to generate a free hydrophilic head group and phosphatidic acid that can serve as a second messenger and/or as a substrate for triacylglycerol synthesis (Wang, 2000). Since the *PLDZ1* gene is negatively regulated by GL2 in the root (Ohashi et al., 2003), I hypothesized that *PLDZ* genes may be involved in GL2-mediated control of seed oil levels. My results demonstrated that *PLDZ1* and *PLDZ2* are expressed in the embryo and funiculus and are up-regulated in *gl2* mutant seeds during the late stages (10 DPA) of seed development (Figures 2.6 and 2.7). Thus, in addition to controlling *PLDZ1* expression in the root, GL2 is a negative regulator of *PLDZ1* and *PLDZ2* expression in seeds. However, genetic analysis revealed that PLDZs are not involved in the seed oil biosynthesis, because the seed oil contents of *pldz* single mutants or *pldz1pldz2* double mutant did not exhibit any

change compared with the wild type seeds (Figure 2.8). Consequently, the roles of *PLDZs* in the seed remain to be elucidated.

2.3.2 *MUM4* is a downstream target of GL2

MUM4 encodes an NDP-L-rhamnose synthase, which converts NDP-D-glucose to NDP-L-rhamnose, a key substrate for RG I, the major constituent of Arabidopsis seed mucilage (Western et al., 2004; Usadel et al., 2004; Oka et al., 2007). My qPCR results showed that the MUM4 gene is down-regulated in gl2 seeds (Figure 2.9a), consistent with previous RT-PCR results (Western et al., 2004). Recent analysis of the MUM4 promoter sequences suggests that within the seed, MUM4 expression is found primarily in the seed coat (Esfandiari and Haughn, unpublished data). These data suggest that GL2 is a positive regulator of MUM4 in the seed coat. I have shown that, like GL2, MUM4 activity in the seed coat is required for normal seed oil accumulation, since loss of MUM4 function results in higher seed oil. In addition, the fact that the phenotype of the gl2mum4 double mutant is similar to that of both parents suggests that GL2 and MUM4 function in the same pathway to influence seed oil biosynthesis (Figure 2.9b). Taken together, my data are consistent with the idea that GL2 negatively regulates seed oil by positively regulating MUM4. Further work is required to determine whether MUM4 is a direct target of GL2 and whether other genes downstream of GL2 are also involved in controlling seed oil levels.

2.3.3 Carbon partitioning between seed oil biosynthesis and seed coat mucilage production

In oilseeds like Arabidopsis the seed storage oil is synthesized and deposited in the embryo. Surprisingly, my data suggest that both GL2 and its downstream target MUM4 negatively regulate seed oil biosynthesis, but act in the seed coat. Thus, this regulation must be indirect. Sucrose, as a major carbon source, is transported from photosynthetic tissues into the developing seeds to support seed storage compound accumulation. It has been suggested that sucrose is unloaded first into the seed coat through the funiculus, and then imported into the endosperm before reaching the embryo (Stadler et al., 2005). In the embryonic cells, sucrose is converted through glycolysis into acetyl-CoA, the key substrate of fatty acid biosynthesis required for oil production (Baud et al., 2008). Sucrose is also the carbon source for mucilage biosynthesis in the seed coat. Sucrose is first catabolized to glucose and fructose, and the monosaccharides used for cell wall polymer biosynthesis. For example, the glucose can be converted into rhamnose by MUM4 for use in the production of RG I, the key component of mucilage (Western et al., 2004; Usadel et al., 2004; Oka et al., 2007). Therefore, seed oil and seed coat mucilage production share the same carbon source. An attractive hypothesis to explain the role of GL2 and MUM4 in negatively regulating oil biosynthesis is that seed mucilage and oil are competing sinks for photosynthate, and that photosynthate in the seed is limiting. In the absence of mucilage formation (gl2 and mum4 mutants) the sucrose normally used for the production of mucilage is instead used for oil biosynthesis.
2.3.4 *ttg2* mutant seeds have a wild type oil content

TTG2 gene encodes a WRKY transcription factor, which is reported to be involved in regulation of several development processes in Arabidopsis (Johnson et al., 2002; Ishida et al., 2007; Gonzalez et al., 2009; Western et al., 2004). It has been previously established that, like GL2, TTG2 is positively regulated by the TTG1-bHLH-MYB complex and is required for seed coat mucilage biosynthesis (Gonzalez et al., 2009; Western et al., 2004, Figure 2.11). However, unlike GL2, TTG2 does not regulate MUM4 and therefore must influence mucilage biosynthesis through its control of other genes needed for mucilage biosynthesis (Figure 2.11a). A very recent study demonstrated that the GATL5 gene, TTG2 positively regulates which encodes а putative galacturonosyltransferase, an enzyme adding GalA into mono- or polysaccharide receptor to form the RG I backbone (Kong et al., 2013). Thus, TTG2 may regulate mucilage biosynthesis at least partially through GATL5, and not MUM4. Interestingly, I have shown that ttg2 mutant seeds have wild type oil content indicating that the loss of seed mucilage is not sufficient to increase oil biosynthesis. These data raise the question as to why the effect of gl2/mum4 on seed oil is different from ttg2. One possible explanation is that since *mum4* disrupts the conversion from glucose to rhamnose, the unused glucose can easily be transported to and used by the embryo for oil production. If *ttg2* is required for a later step, such as adding GalA into RG I backbone during mucilage biosynthesis, the unused carbohydrate (rhamnose, for example) may not be as useful a substrate as glucose for oil biosynthesis in the embryo.

2.3.5 Blocking mucilage biosynthesis might be a way to increase seed oil production

Seed oils are of great value in many food and non-food applications, including biodiesel production, but their supply is limited. Therefore, numerous attempts have been made to increase the seed oil content in plants. Some of these attempts focused on increasing the carbon flow to triacylglycerol biosynthesis (Weselake et al., 2009; Ekman et al., 2008; Iyer et al., 2008) at the expense of other major seed storage compounds, starch and protein. The biosynthetic pathways involved in the production of seed storage compounds compete with each other for the carbon resources during embryo development (Weselake et al., 2009). For example, *shrunken seed 1 (sse1)* mutant seeds with a disrupted triacylglycerol biosynthetic pathway produced more starch than the wild type (Lin et al., 2004), whereas embryo-specific overexpression of the *BIOTIN* CARBOXYL CARRIER PROTEIN 2 (BCCP2) resulted in a decreased seed oil production and an increased carbon flow into seed storage protein synthesis (Chen et al., 2009). My results reveal a regulatory mechanism of carbon partitioning that occurs between the maternal seed coat and the filial embryo. Mutations in GL2 and MUM4 do not appear to affect other cell types producing mucilage (root cap and transmitting tract cells) nor have obvious deleterious effects on the growth and functioning of Arabidopsis. While there are reports that germination of some mucilage mutants can be delayed relative to wild type under conditions of limited water supply (Penfield et al., 2001; Arsovski et al., 2009), most species of plants including some species of Brassicaceae lack seed mucilage, and some cultivars of *Brassica napus* extrude little mucilage when imbibed suggesting that seed mucilage is not critical for agronomic performance. Thus it seems possible that the

manipulation of MUM4 function can be exploited to increase the seed oil content in oil crops with myxospermous seed by blocking conversion of glucose to rhamnose in the seed coat.

2.4 Materials and methods

2.4.1 Plant material and growth conditions

Arabidopsis ecotypes Columbia (Col-0) and Landsberg erecta (Ler) were used in this study. SALK T-DNA insertional lines, SALK 130213 (gl2-8, Deal and Henikoff, 2010), SALK 083090 (pldz1), SALK 094369 (pldz2), SALK 030942 (myb5-1, Li et al., 2009) and SALK_030966 (tt8, Gonzalez et al., 2009) were obtained from the Arabidopsis Biological Resource Center (ABRC, www.arabidopsis.org). mum4-1, ttg1-1 and ttg2-1 mutants have been previously described (Western et al., 2004; Walker et al., 1999; Johnson et al., 2002). SALK_005260 (tt2), SALK_019114 (egl3), myb5-1tt2 and egl3tt8 mutants were kindly provided by Dr. Antonio Gonzalez (Gonzalez et al., 2009). Homozygous lines for each gene were identified by genotyping using primers listed in Table 2.1. Double and triple mutants were obtained by crossing homozygous lines and identifying the appropriate lines from the F_2 generation. The *GL2p::GUS* line was kindly provided by Dr. Jin-Gui Chen (University of British Columbia, Vancouver, BC, Canada) and has been previously described (Szymanski et al., 1998). The PLDZ1::GUS and PLDZ2::GUS lines were generated by Yuanyuan Yu in our lab and have been described (Shi et al., 2012). Arabidopsis plants were transformed using the floral spray method (Chung et al., 2000). Seeds were germinated on AT medium (Haughn and Somerville 1986) supplemented with agar (7 g/L) and appropriate antibiotics. The 10-day-old

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seedlings were transferred to soil and grown at 20 $^{\circ}$ C under continuous light with a light intensity of 100 µmol m⁻²s⁻¹. The plants for oil content analysis in each biological replicate were grown in the same chamber and the positions of the plants were rotated every other day.

2.4.2 RNA isolation, RT-PCR and qPCR

Rosette leaves and developing seeds of the wild type (Col-0) and the *gl2-8* mutant or the *pldz1pldz2* double mutant were collected and immediately frozen in liquid nitrogen. RNA isolation and reverse transcription were performed by using the protocols as previously described (Zhao et al., 2010). Gene specific primers used in RT-PCR and qPCR are listed in Table 2.1. iQ SYBR Green Supermix (Bio-Rad) was used to perform qPCR in an iQ5 Multicolor Real-Time PCR Detection System as specified by the manufacturer (Bio-Rad, <u>www.bio-rad.com</u>).

2.4.3 GUS histochemical assays

The seeds at different developmental stages were collected from transgenic plants containing *GL2p::GUS*, *PLDZ1::GUS* or *PLDZ2::GUS* constructs. The seed coats and embryos were carefully separated with needles under the dissecting microscope. Tissues were immersed in the GUS staining buffer (100mM Na-phosphate, PH 7.0, 0.5mM K3[Fe(CN)6], 0.5mM K4[Fe(CN)6], 0.2% Triton X-100 and 1mM 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-gluc)) and incubated for 1-4 hours or overnight. Chlorophyll was removed by incubating with 70% ethanol. Stained samples were examined under an Olympus SZX16[®] stereomicroscope (Olympus).

2.4.4 Seed oil content analysis

The seed oil content analyses were carried out as described by Li et al. (2006) with minor modifications. Briefly, for each of four replicates 2-2.5 mg of dry seeds from each line were weighed and transferred into 1x10 cm glass tubes (pre-rinsed thoroughly with chloroform and dried) with Teflon screw caps. 1 ml of freshly prepared 5% (v/v) sulfuric acid in methanol and 300 μ l of 0.1 μ g/ μ l triheptadecanoin in toluene as internal standard were added to each tube. The samples were mixed and incubated at 90 °C for 1.5 hr. After cooling on ice, 0.5 ml of 0.9% NaCl was added to each tube and fatty acid methyl esters were extracted with 0.2 ml of hexane. 50 μ l of hexane was transferred into glass vials. Fatty acid methyl esters were analyzed by an Agilent 6890 gas chromatography-flame ionization detection (GC-FID) with a DB-23 capillary column (Agilent Technologies) as described previously (Kunst et al., 1992). The program ran with a split mode (10:1), began from 180 °C for 1 min, increased temperature by 4 °C/min to 240 °C and held for 7 min. Each experiment was repeated at least twice.

For oil analysis in the embryo and the seed coat, mature seeds were imbibed in water at $4 \,^{\circ}$ overnight. The imbibed seeds were broken by gently pressing between two slides. The embryos and the seed coats were carefully separated under the dissection microscope. 10 embryos or 100 seed coats per replicate were collected for fatty acid methyl ester analysis.

2.4.5 Ruthenium red staining for seed coat mucilage

The dry seeds from each line were shaken in water for an hour before they were stained in a 0.01% (w/v) aqueous solution of ruthenium red (Sigma-Aldrich). The stained seeds were inspected with a Zeiss AxioScop 2 light microscope (Carl Zeiss).

| Genotyping primers for mutants | | | | | |
|--|--------------------------------|----------------|-----------|--|--|
| Primer name | Primer sequence | Mutant line | | | |
| LBb1 | 5'-GCGTGGACCGCTTGCTGCAACT-3' | All Salk lines | | | |
| gl2-8LP | 5'-ACCACCGATCAGATCAGACAC-3' | SALK_130213 | | | |
| gl2-8RP | 5'-GGAGTTTTCGAGGTGGAGATC-3' | | | | |
| pldz1-083090LP | 5'-TGAAAAGCATGGAAATTTTCG-3' | SALK_083090 | | | |
| pldz1-083090RP | 5'-GTGATCGTCTCTGTCTCTCGC-3' | | | | |
| pldz2-094369LP | 5'-CTTCATGAGCCTTCAGAATGC-3' | SALK_094369 | | | |
| pldz2-094369RP | 5'-CGGCATTTACCTCTGGTACAG-3' | | | | |
| myb5-1LP | 5'-AACGTTAGCACTTAGCTAGCCTG-3' | SALK_030942 | | | |
| myb5-1RP | 5'-GGTTCTGATTTTAGCTTGATCCC-3' | | | | |
| tt8_030966LP | 5'-GGCCCATAAAACGACAAGAAG-3' | SALK_030966 | | | |
| tt8_030966RP | 5'-TACCACGTTTTCGTATCTCCG-3' | | | | |
| tt2Salk_LP | 5'-GCGGGTCAGAATCTAGTTTCC-3' | SALK_005260 | | | |
| tt2Salk_RP | 5'-AAGCAGATGGTCGTTGATAGC-3' | | | | |
| egl3SALK-LP | 5'-TCGTTAAATTCAAGCGATTGC-3' | SALK_019114 | | | |
| egl3SALK-RP | 5'-TATCCATGCAACCCTTTGAAG-3' | | | | |
| mum4-1LP | 5'-CAGATCTGAAGAATCTTGATCC-3' | mum4-1 | | | |
| mum4-1RP | 5'-TAGGCCCATAAACATTGTTCC-3' | | | | |
| ttg1-1LP | 5'-GAAAGACATCAGGCTAGTGTG-3' | ttg1-1 | | | |
| ttg1-1RP | 5'-TAGTGTCAAATCTCTAGTGGC-3' | | | | |
| ttg2-1LP | 5'-ACTTGCCACGAGATTGTAGAC-3' | ttg2-1 | | | |
| ttg2-1RP | 5'-CCAAGTTTGTTGACGATTCC-3' | | | | |
| Tag1F | 5'-TGGGTTTAGTGCTAAAACCC-3' | | | | |
| Tag1R | 5'-AAAGAGGCCACGAGAGTATAG-3' | | | | |
| Primers for RT-PCR or quantitative Real-time PCR | | | | | |
| Primer name | Primer sequence | Gene Name | ATID | | |
| GL2RT-F | 5'-AGACACATGGAAGCGCTATTC-3' | GL2 | AT1G79840 | | |
| GL2RT-R | 5'-TGTTCTTGATCGTCGGAGCATG-3' | | | | |
| PLDZ1RT-F | 5'-CGGCGATATTAGCCCTGTACTCTT-3' | PLDZ1 | AT3G16785 | | |
| PLDZ1RT-R | 5'-GCATTGCATGCTTTCCTCTA-3' | | | | |
| PLDZ1QRT-F | 5'-GATAATTGGCAAAAGGTGTGG-3' | | | | |
| PLDZQRT-R | 5'-CGGTTTCCAGATGTTACCTTG-3' | | | | |
| PLDZ2RT-F | 5'-AGACAGGAGAAAGTACCCGCGAAT-3' | PLDZ2 | AT3G05630 | | |
| PLDZ2RT-R | 5'-TCACTCTGCTTCCCAATCTG-3' | | | | |
| PLDZ2QRT-F | 5'-GATTGCCGGTGTCAAATAATCAG-3' | | | | |
| PLDZ2QRT-R | 5'-GGAGATGAAGAATTGGTTCTC-3' | | | | |
| MUM4QRT-F | 5'-AAGTTACAGGACAGATCAGG-3' | MUM4 | AT1G53500 | | |
| MUM4QRT-R | 5'-TAGGCCCATAAACATTGTTCC-3' | | | | |

Table 2.1. PCR primers used in chapter 2.

CHAPTER 3

CER11/CTD PHOSPHATASE LIKE 2 (CPL2) IS REQUIRED FOR SECRETORY TRAFFICKING OF CUTICULAR WAX AND SEED COAT MUCILAGE IN ARABIDOPSIS

3.1 Introduction

The primary aerial surfaces of land plants are covered by a lipidic cuticle, which protects them against non-stomatal water loss, serves as a barrier to pathogen invasion and prevents organ fusion. The cuticle consists of cutin and cuticular waxes made in the epidermal cells. The biosynthesis of cuticular waxes has been studied for many years and the major biosynthetic enzymes have been identified, and localized to the ER (reviewed by Samuels et al., 2008; Bernard and Joubes, 2013; Haslam et al., 2012). By comparison, relatively little is known about the export of wax molecules from their site of synthesis in the ER to the PM, and from the PM through the cell wall to the plant surface. Currently, the only known components of the wax export machinery are two ABC transporters, CER5/ABCG12 and ABCG11, that function as heterodimers in the PM (McFarlane et al., 2010), and two GPI-anchored lipid transport proteins which also reside in the PM (DeBono et al., 2009; Kim et al., 2012). However, how waxes reach the PM has not been resolved.

One of the attractive hypotheses is that wax components are transported from the ER to the PM by Golgi-mediated vesicular traffic through the secretory pathway (reviewed by Kunst and Samuels, 2003). The secretory pathway, as well as endomembrane system, is conserved in eukaryotes. However, the plant endomembrane system is distinct from the mammalian system in several important ways. For example, although the ER, the Golgi apparatus, and the trans-Golgi network (TGN) are organelles of the plant endomembrane system, there is no distinct endosome as in animal cells. Instead, the TGN, an independent and highly mobile organelle in plant cells serves as the main secretory and endocytic hub equivalent to early endosomes (Dettmer et al., 2006;

Viotti 2010). addition. plant cell et al.. In the has а prevacuolar compartment/multivesicular body (PVC/MVB), which acts as a late endosome, and vacuole, not found in animal cells (Nebenfuhr, 2002; Carter et al., 2004; Viotti et al., 2010). Although secretion of proteins and cell wall polysaccharides via secretory trafficking in plants has been studied to some extent (Viotti et al., 2010; Driouich et al., 2012; reviewed by Drakakaki and Dandekar, 2013), the question of whether cuticular waxes are transported to the plasma membrane along the secretory pathway is still a matter of debate. Recently, however, wax secretion has been shown to be dependent on the function of GNOM-LIKE1 (GNL1) and ECHIDNA (ECH), providing evidence that the vesicle trafficking is involved (McFarlane et al., 2014).

The availability of wax-deficient *eceriferum* (*cer*) mutants (Koornneef et al., 1989) led to the identification of a number of genetic loci required for the biosynthesis and deposition of wax (reviewed by Samuels et al., 2008). One of these mutants, *cer11-1*, stood out from the rest because its wax deficient phenotype co-segregated with dwarf bushy stature similar to other mutants with secretory defects (e.g. *echidna*, Gendre et al., 2011). Another aspect of the *cer11-1* phenotype that co-segregated with the wax deficiency was a failure of the seed coat epidermal cells to properly extrude mucilage (preliminary test done by Dr. Gillian Dean, research associate in the Haughn lab). During differentiation, these cells synthesize and secrete to the apoplast, a large volume of pectinaceous mucilage, forming a donut-shaped pocket between the cell membrane and the outer-tangential cell wall. Upon exposure of mature seeds to an aqueous solution, the mucilage expands, ruptures the cell wall and forms a gel-like capsule surrounding the seed (reviewed by Haughn and Western, 2012).

A possible explanation for the complex *cerl1-1* phenotype is that the mutant is defective in some aspect of secretion. To test this hypothesis, Dr. Huanquan Zheng, a former postdoctoral fellow in the Kunst lab, generated a transgenic cer11-1 plant expressing secGFP, a secreted form of *Aequorea victoria* green fluorescent protein that serves as a tool for genetic analysis of secretory trafficking in plant cells (Batoko et al., 2000; Zheng et al., 2004). Many studies of secretory trafficking have taken advantage of secGFP, a visual marker transported from the ER to the apoplast where it generates a very weak fluorescent signal in the acidic apoplastic environment (Batoko et al., 2000; Zheng et al., 2004). If the secretion from the ER to the PM is inhibited, increased secGFP accumulation will result in a strong fluorescence signal at the site of the block. To determine if the *cerl1* mutation affects secGFP movement along the secretory pathway to the PM, Dr. Zheng compared the *secGFP* transgene expression and GFP signal in the wild type cells and the *cerl1-1* cells. The *secGFP* transgene was transcribed in both wild type and *cer11-1* mutant backgrounds (Figure 3.1b). However, as shown in Figure 3.1a, cer11-1 mutant exhibited an enhanced intracellular GFP signal compared to the wild type, indicative of compromised ER to PM trafficking.

To further investigate the hypothesis that CER11 protein plays a role in secretory trafficking of cuticular wax and seed coat mucilage, I characterized the *cer11-1* mutant phenotypes in detail, and identified the protein encoded by *CER11*. My results reveal that *CER11* encodes a phosphatase with a role in secretion, and suggest that it acts, at least in part, by dephosphorylating subunit C of V-ATPase, a proton pump involved in secretory trafficking.



Figure 3.1. Comparison of GFP fluorescence and *secGFP* transcript levels between the wild type (WT) and mutant (*cer11-1*) plants expressing *secGFP*. (a) Confocal laser scanning microscopy images of WT and *cer11-1* hypocotyl cells expressing *secGFP* show different levels of GFP fluorescence in the ER network and the fusiform bodies. Scale bar = 20 μ m. (b) RT-PCR analysis of *secGFP* transcript levels in the WT and *cer11-1* seedlings expressing *secGFP*. RT-PCR was performed using total RNA from seedlings, with ribosomal RNA (rRNA) used as a control.

3.2 Results

3.2.1 The *cerl1* mutant is defective in wax deposition

The *cer11-1* mutant was originally identified as a wax deficient mutant by a visual screen for stem surface glaucousness (Koornneef et al., 1989). Detailed analysis of stem and leaf wax by gas chromatography with flame ionization detection (GC-FID) revealed that the mutant accumulated only 53% of the wild type stem wax, and 75 % of the wild type leaf wax. This reduction in the wax load was reported to be primarily due to lower levels of C29 molecular species of alkanes, secondary alcohols and ketones, as well as C30 aldehyde, whereas leaf cuticular wax mixtures contained lower levels of alkanes and primary alcohols (Rashotte et al., 2001). The *cerl1-1* mutant also exhibited severe wax deficiency under our growth conditions, as evidenced by its glossy, bright green stems (Figure 3.2b). Reduced stem was accumulation was verified by scanning electron microscopy (SEM), which demonstrated that fewer epicuticular wax crystals were present on the stem surface (Figure 3.2c,d), and GC-FID analysis which confirmed that the cer11-1 stem was load was reduced by ~50% in comparison to wild type (Figure 3.2e). However, compositional analyses of the stem wax did not reveal any changes in the *cer11-1* stem wax profile (Figure 3.2f).



Figure 3.2. Phenotypes of the *cer11-1* mutant. (a) Images of 5-week-old WT (Ler) and *cer11-1* plants show that the *cer11-1* plant is dwarf and bushy. Scale bar = 5 cm. (b) Enlarged images of WT and *cer11-1* stems demonstrate that the *cer11-1* stem is glossy. Scale bar = 0.1 cm. (c-d) SEM images showing wax crystals deposited on the surface of WT (c) and *cer11-1* (d) stems. Scale bar = 5 μ m. (e-f) Cuticular wax load and composition determined by GC-FID. Wax analysis data are expressed as the mean \pm SD (n=4). Student's t-test was applied to the data; the asterisk indicates that, statistically, this value was significantly different from WT at P < 0.05.

3.2.2 Mucilage release and cell wall formation are defective in the seed coat epidermal cells of *cer11*

During the preliminary analysis of the *cer11-1* mutant, we noticed that upon imbibition the adherent seed coat mucilage halo was much smaller than that of wild-type seeds. I further investigated the *cer11-1* seed coat phenotypes in detail. As shown in Figure 3.3a, less mucilage was detected around the *cer11-1* seeds after shaking in water.



Figure 3.3. Seed coat mucilage phenotypes of the *cer11-1* seeds. (a) WT (Ler) and *cer11-1* seeds after hydration with shaking in water or EDTA, and subsequent staining with ruthenium red. Lower magnification images show phenotypes of multiple seeds and higher magnification images show single representative seeds. Scale bar = 0.5mm. (b) Monosaccharide analysis of mucilage by HPAEC. The monosaccharide composition of mucilage extracted from the seed by gentle shaking in water (water extracted mucilage) and extraction by shaking in Na₂CO₃ (Na₂CO₃ extracted mucilage) or of whole seeds was determined. Differences in rhamnose (rha) and galacturonic acid (galA) content between WT and *cer11-1* are indicated by black arrows. Values are the mean \pm SD (n=4; replicate analysis performed on seed lots from each line, with 5 or 20 mg of seeds analyzed per replicate) and are expressed as nmoles of sugar normalized to milligrams of seed used for mucilage extraction or milligrams of Alcohol Insoluble Residue derived from whole seed (whole seed). Student's t-test was applied to the data; the asterisk indicates that, statistically, this value was significantly different from WT at P < 0.05.

In EDTA, a chelator that loosens mucilage pectin, a larger halo was observed, although the halo did not appear to be as big as wild type. Previous analyses of mutants with a similar phenotype have shown that the absence of a mucilage halo can result from either loosening of the mucilage, so that the mucilage is no longer adherent (such as *cesa5-1*; Sullivan et al., 2011), a compositional change in pectin that prevents mucilage extrusion (such as *mum2*; Dean et al., 2007), or a decrease in the amount of pectin made (such as mum4; Western et al., 2004). To distinguish between these possibilities I quantified monosaccharide composition by high-performance anion exchange chromatography (HPAEC; Figure 3.3b) with the assistance of Dr. Gillian Dean. The HPAEC data revealed that there was little qualitative difference between the spectrum of sugars found in *cerl1*-I and wild type mucilage extracted from seeds using either water, or Na_2CO_3 a mild base used to extract cell wall carbohydrates. However, the amount of rhamnose and galacturonic acid, the backbone sugars of the major component of the seed coat mucilage RG I, was significantly decreased (Figure 3.3b), indicating that there is less extractable mucilage in *cerl1-1* seeds. To determine if this decrease in extractable sugar amount was due to a defect in mucilage extrusion or synthesis, monosaccharide analysis was done on samples prepared from whole seeds. As shown in Figure 3.3b, the mucilage extracted from the whole *cer11-1* seeds also contains significantly less rhamnose and galacturonic acid than wild type, suggesting that less mucilage is present in *cerl1-1* seeds.

I further investigated the mucilage-producing seed epidermal cells of *cer11-1* by SEM. The SEM images revealed that the columella, a volcano-shaped secondary cell wall, is not properly formed in the *cer11-1* seed epidermis (Figure 3.4a). Sectioning of the *cer11-1* and wild type developing seeds showed that there were no major anatomical

differences between the *cer11-1* and the wild type during the early (4 DPA), middle (7 DPA) and late (10 DPA) stages of epidermal cell development. However, at maturity (15 DPA), the *cer11-1* columella was hollow (Figure 3.4b), suggesting that the columella secondary cell wall deposition of *cer11-1* seed is slower than in the wild type and fails to complete development by seed maturity.

In summary, seed coat mucilage deposition and release, as well as secondary cell wall formation in the *cerl1-1* seeds are impaired.



Figure 3.4. Phenotypes of the *cer11-1* seed coat epidermal cells. (a) SEM images of WT and *cer11-1* whole seeds (upper panel) and individual seed coat epidermal cells (lower panel). Columellas are indicated by white arrows. (b) Light micrographs of cross-sections through WT (upper panel) and *cer11-1* (lower panel) seed coats stained with toluidine blue showing epidermal cells at 4 DPA, 7 DPA, 10 DPA and 15 DPA. Development of the *cer11-1* seed coat epidermal cells at 4 DPA, 7 DPA and 10 DPA is indistinguishable from the wild type. However, at 15 DPA, the *cer11-1* columella formation is not complete and the seed coat does not rupture upon hydration. Mucilage pockets are indicated by black arrowheads; columellas are indicated by black arrows. Scale bar = 20 μ m.

3.2.3 Secretion of MUM2, a cell wall modifying enzyme, but not CESA5, or waxrelated proteins, is delayed in the *cer11* mutant

Stunted growth of the *cer11-1* mutant, reduced stem wax accumulation on the stem surface and mucilage deposition in the seed epidermis, delayed columella secondary cell wall deposition, together with the deficient secretion of secGFP protein suggest that CER11 may be required for secretory trafficking of wax molecules, or of the wax transporters, or both, to the PM, as well as the transport of cell wall components or proteins required for seed coat development. I first examined whether the mutation in the *CER11* changed the localization of proteins involved in wax export, CER5/ABCG12, ABCG11 and LTPG (Pighin and Zheng et al., 2004; Bird et al., 2007; DeBono, et al., 2009) and seed coat mucilage deposition, MUM2 and CESA5 (Dean, et al., 2007; Harpaz-Saad et al., 2011; Mendu et al., 2011; Sullivan et al., 2011). I performed this experiment by introducing CER5/ABCG12-GFP, YFP-ABCG11, YFP-LTPG, MUM2-YFP or GFP-CESA5 into the cer11-1 background, and determining the sub-cellular localization of these proteins compared to the wild type by confocal microscopy. No obvious differences in the localization of the wax-related proteins and GFP-CESA5 were apparent in the *cer11-1* background in comparison to the wild type (Figure 3.5).



Figure 3.5. Localization by confocal microscopy of CER5-GFP, YFP-ABCG11 and YFP-LTPG in wild type (WT) and the mutant (*cer11-1*) stem epidermal cells, and CESA5-GFP localization in wild type (WT) and the mutant (*cer11-1*) seed coat epidermal cells at 7 DPA. Scale bar = $20 \mu m$.

However, the localization of MUM2-YFP in the seed coat epidermal cells during seed development was altered in *cer11-1* with respect to the wild type (Lee, Gilchrist, Haughn, unpublished results, Figure 3.6). In the seeds with embryos at the heart stage, the YFP fluorescence in the wild type seed coat epidermal cells was present in the primary cell wall, while no YFP signal was detected in the *cer11-1* cells (Figure 3.6a, e).



Figure 3.6. MUM2-YFP localization by confocal microscopy in WT (a-d) and the *cerl1-1* mutant (e-h) seed coat epidermal cells at the heart stage (a, e), the bent cotyledon stage of embryo development (b, f) and at maturity (d, h) in transverse section. The YFP-MUM2 localization in wild type (c) and the *cerl1-1* (g) seed coat epidermal cells at the bent cotyledon stage is also shown in longitudinal section. The FM4-64 PM staining (i) and the overlay with the YFP-MUM2 signal in the *cerl1-1* seed coat epidermal cells (j) are shown as well. The developmental stages of the embryo are indicated in the inserts. The white arrows in the images (c, g, i, j) indicate mucilage pockets. Scale bar = 20 μ m.

During the embryo bent cotyledon stage, MUM2-YFP fluorescence in the wild type seeds was evenly distributed throughout the mucilage pocket and in the primary cell wall (Figure 3.6b), while the YFP signal in the *cer11-1* seeds was concentrated at the inner edge of the mucilage pocket close to the PM (Figure 3.6f). Longitudinal sections through the epidermal cells combined with staining of the PM marker FM4-64, confirmed the

observation that the MUM2 protein in *cer11-1* seeds was concentrated on the inner side of the mucilage pocket (Figure 3.6c, g, i, j). Finally, at embryo maturity, the MUM2-YFP signal was detected in the columella secondary cell wall of wild type and *cer11-1* seed coats, but the signal was much weaker in the *cer11-1* seeds than in the wild type (Figure 3.6d, h). Overall, the time course of MUM2-YFP sub-cellular localization in the developing seed coat epidermal cells is consistent with the hypothesis that secretion of the MUM2 protein in the *cer11-1* mutant is delayed compared to the wild type, resulting in uneven distribution of MUM2 signal in the primary cell wall and mucilage pocket.

3.2.4 Positional cloning of the *CER11* gene

To determine the molecular identity of CER11 and obtain clues about its biochemical function, a positional cloning approach was used to isolate the *CER11* gene. The *cer11-1* mutant in the Landsberg *erecta* background was crossed to the Columbia-0 wild type to generate a mapping population. Segregation analysis of the *cer11* phenotype relative to known InDel markers using the F_2 progeny revealed that the *cer11* wax deficiency was linked to a ~300kb region between 21,140bp and 325,700bp on chromosome 5 (Figure 3.7), which contains 87 genes.



Figure 3.7. Map position of *cer11-1* mutation in a ~300 kb region between 21,140 bp and 325,700 bp on chromosome 5 (Chr 5). The InDel marker positions and the corresponding recombination rates are shown.

The cer11-1 mutant was generated by fast neutron mutagenesis (Koornneef et al., 1982), and therefore the mutation could be a deletion or a chromosomal rearrangement. If that was the case, I reasoned that the mutation might be detectable by changes in PCR amplification relative to wild type. I therefore designed a series of primers, and PCRamplified different chromosomal fragments in the 300 kb region of interest. Using this strategy, I identified the gene At5g01800 as having a break point on chromosome 5 at position 308,246 bp in the *cer11-1* line. Furthermore, using inverse PCR I identified two additional break points in the *cerl1-1* mutant, one on chromosome 1 at position 714,924 bp and the other on chromosome 5 at position 110,325 bp (Figure 3.8a). The identified chromosomal rearrangements include an inversion of 197,912 bps on chromosome 5 between positions 110,335 bp and 308,246 bp and a reciprocal translocation between chromosomes 5 and 1 joining chromosome 5 110,325 bp to chromosome 1 714,927 bp and chromosome 1 714,924 bp to chromosome 5 308,246 bp (Figure 3.8a). Furthermore, 2 nucleotides (AA at chromosome 1 714,925 to 714,926 bp), 9 nucleotides (TTACTTGAA at chromosome 5 110,326 to 110,334 bp) and 2 nucleotides (AG at chromosome 5 308,247 to 308,249 bp) are missing at the three break points, respectively (Figure 3.8a). The rearrangements were confirmed by PCR-amplification using three pairs of primers (Figure 3.8b).

(a) WT Chr 1 714,924bp ----ditte PI +P2 At1g03070 At1g03055 At1g03060 110,325bp Chr 5 308,246bp HHI/4-8-8 P3 P4 P6 P5 At5g01280 At5g01810 At5g01260 At5g01790 At5g01800 At5g01270 cer11-1 /AG TTACTIGAA // +++++ 14 At5g01790 At5g01810 At1g03055 → ← P1 P5 At5g01280 P4 P6 At5g01270//At5g01800 At1g03060//At5g01800 /AA ---------+P2 P3 At5g01260 At1g03070 At5g01270//At1g03060 (b) WT cer11-1 WT cer11-1 P1+P2 P1+P5 P3+P4 P3+P2 P5+P6 P4+P6



3.2.5 *CER11* encodes the CTD PHOSPHATASE LIKE 2 (CPL2)

Three genes in the *cer11-1* mutant were affected by the described chromosomal rearrangements: At1g03060 (*SPIRRIG*, *SPI*), At5g01270 (*C-terminal domain* (*CTD*) *PHOSPHATASE LIKE 2, CPL2*) and At5g01800 (*SAPOSIN B DOMAIN-CONTAINING PROTEIN, SAPLIP*), according to the TAIR database (http://www.arabidopsis.org). To determine which of these three genes is *CER11*, I examined the phenotypes of lines homozygous for T-DNA insertional mutations in *SPI* or *CPL2* (no T-DNA mutant of *SAPLIP* is available) and carried out transgene complementation tests for *CPL2* and *SAPLIP*.

The *SPI* gene was eliminated as a *CER11* candidate because two T-DNA mutants in *SPI* (*spi-1* and *spi-2*) with insertions in the tenth exon and the seventeenth exon, respectively (Figure 3.9a), did not show any of the *cer11-1* phenotypes (Figure 3.9b-d).

To determine whether *CPL2* or *SAPLIP* is *CER11*, two constructs, *CPL2p::CPL2* and *SAPLIPp::SAPLIP*, were generated in which the genomic coding sequence of each of these genes is driven by their native promoters. These constructs were transformed into the *cer11-1* mutant. The *CPL2p::CPL2* transgene rescued all the *cer11* phenotypes, including the dwarfism, the stem wax deficiency, the seed coat mucilage phenotype and the altered columella morphology (Figure 3.10a-e), whereas the *SAPLIPp::SAPLIP* did not. In addition, the high GFP fluorescence of the *cer11-1* mutant carrying the *secGFP* transgene was also suppressed by *CPL2p::CPL2*, indicating that *CPL2* is able to complement the *cer11* secretory defect (Figure 3.10f, g). These data strongly suggested that *CER11* and *CPL2* are the same gene.



Figure 3.9. Structure of the *SPI* gene and phenotypes of *spi-1* and *spi-2* mutants. (a) Exon-intron structure of the *SPI* gene (At1g03060) with exons shown as black boxes, introns as solid lines and untranslated regions as black triangles. The locations of T-DNA insertions in *spi-1* (SALK_131421) and *spi-2* (SALK_116367) were mapped and are indicated by arrowheads. The location of the break point in the *SPI* gene in the *cer11-1* mutant is indicated by a black arrow. (b) Images of 5-week-old WT (Col-0) and *spi-1* and *spi-2* mutant plants show that the *spi-1* and *spi-2* plants are similar to wild type in stature. Scale bar = 5 cm. (c) Enlarged images of WT, *spi-1* and *spi-2* stems exhibit similar glaucous phenotypes. Scale bar = 0.1 cm. (d) Images *spi-1* and *spi-2* seeds stained with ruthenium red after immersion in water show no mucilage phenotypes. Scale bar = 0.5 mm.



Figure 3.10. CPL2p::CPL2, but not SAPLIPp::SAPLIP, can complement the phenotypes of the *cer11-1* mutant. (a) Shoot morphology, Scale bar = 5 cm, (b) Stem appearance, Scale bar = 0.1 cm, and (c) Stem wax content of 5-week-old WT (Ler), cer11-1 and the transgenic cer11-1 plants expressing CPL2p::CPL2 or SAPLIPp::SAPLIP. Wax analysis data are expressed as the mean \pm SD (n=4). Student's t-test was applied to the data; the asterisk indicates that, statistically, this value was significantly different from wild type at P < 0.05. (d) Images of seeds from WT (Ler), cer11-1 and transgenic cer11-1 plants expressing CPL2p::CPL2 or SAPLIPp::SAPLIP stained with ruthenium red after hydration in water. Scale bar = 0.5 mm. (e) SEM images of seed coat epidermis of seeds from WT, *cer11-1* and the transgenic *cer11-1* plants expressing *CPL2p::CPL2* show that the *cerl1-1* columella phenotype was complemented. Scale bar = 20 μ m. (f) Comparison of secGFP fluorescence in the hypocotyl cells of WT (Ler), the *cer11-1* mutant and the transgenic *cerl1-1* plants expressing *CPL2p::CPL2*. Confocal microscopy shows that the GFP signal is enhanced in the mutant (cer11-1) cell, but not in the WT (Ler) or the cer11-*I* expressing *CPL2p::CPL2*, indicating that *CPL2p::CPL2* can complement the secretion phenotype of the *cerl1-1* mutant. Scale bar = 20 μ m. (g) RT-PCR analysis of *secGFP* transcript levels in the WT (Ler), the *cer11-1* mutant and transgenic *cer11-1* seedlings complemented with CPL2::CPL2 expressing secGFP. RT-PCR was performed using total RNAs from seedlings, and the expression level of GAPC was used as a control.

Additional evidence that *CPL2* is the *CER11* gene was obtained through phenotypic analyses of plants homozygous for two SALK T-DNA alleles, *cpl2-1* (SALK_149234) and *cpl2-2* (SALK_059753) (Ueda et al., 2008), and a GABI-KAT T-DNA allele (GK-433F07) that I designate *cpl2-3* (Figure 3.11a). Normal *CPL2* transcripts were not detected in any of the three mutants (Figure 3.11b). All three mutants were bushy and dwarf in stature compared to the wild type, similar to the *cer11-1* mutant (Figure 3.11c), have reduced total wax loads on the stem surface (Figure 3.11d), and exhibit a *cer11-*like defective mucilage phenotype (Figure 3.11e). Defects in *cpl2-1* and *cpl2-2* seed mucilage were not as apparent as in *cpl2-3*, possibly due to the fact that *cpl2-1* and *cpl2-2* are partial loss-of-function alleles (Ueda, et al., 2008). Moreover, the seeds of all three *cpl2* alleles have collapsed columellas, like the *cer11-1* mutant (Figure 3.11f). In summary, the *cpl2* alleles phenocopy the *cer11-1* mutant. Therefore, I conclude that *CPL2* and *CER11* are the same gene. I refer to the gene as *CER11/CPL2* and rename *cer11-1* allele as *cpl2-4*.



Figure 3.11. Phenotypes of the *cpl2* mutants. (a) The structure of *CPL2* gene (At5g01270) shows exons as black boxes, introns as solid lines, 5'-UTR as a white box and 3'UTR as a black triangle. Locations of the T-DNA insertions in the cpl2 mutantscpl2-1 (SALK 149234), cpl2-2 (SALK 059753) and cpl2-3 (GK-433F07) were mapped and are indicated by arrowheads. The location of the break point in the *cerl1-1* mutant is indicated as a vertical black arrow. Locations of primers used for RT-PCR in (b) are indicated with short horizontal arrows. (b) RT-PCR analysis of steady-state CER11/CPL2 transcript levels in WT (Col-0) and the mutant (cpl2-1, cpl2-2 and cpl2-3) leaves. RT-PCR was performed using total leaf RNA, and the expression level of GAPC was used as a control. (c) Images of 5-week-old WT (Col-0) and the mutant (cpl2-1, cpl2-2 and cpl2-3) plants showing that all the *cpl2* mutant plants are dwarf and bushy compared to the wild type. Scale bar = 5 cm. (d) Cuticular wax analysis for WT and the mutant stems by GC-FID. Wax analysis data are expressed as the mean \pm SD (n=4). Student's t-test was applied to the data; the asterisk indicates that, statistically, this value was significantly different from wild-type at P < 0.05. (e) Mucilage extrusion from WT (Col-0) and the cpl2-1, cpl2-2 and cpl2-3 seeds after hydration in water and staining with ruthenium red. Scale bar = 0.5 mm. (f) SEM images of seed coat epidermis of seeds from WT (Col-0) and the mutants. Scale bar = $20 \mu m$.

3.2.6 *CER11/CPL2* is expressed throughout the plant and CER11/CPL2 protein is localized in both the nucleus and the cytoplasm

A previous study revealed that *CER11/CPL2* encodes a CTD phosphatase, which can dephosphorylate RNA polymerase II complex (RNAP II) *in vitro* (Ueda, et al., 2008). However, the CER11/CPL2 role in secretion, wax export and cell wall formation has not been previously described. As a first step towards investigating the CER11/CPL2 function in this context, I examined the transcription profile of the *CER11/CPL2* gene in Arabidopsis by qPCR. The qPCR results demonstrate that the *CER11/CPL2* gene is universally expressed in all the plant organs tested, including leaf, stem, flower, root, seed, as well as in developing seedlings (Figure 3.12a). The highest levels of expression were detected in leaves, seeds and 10–day-old seedlings.

To determine the sub-cellular localization of the CER11/CPL2 protein, I generated a translational fusion between the *CER11/CPL2* genomic sequence and GFP (*CER11/CPL2p::CER11/CPL2-GFP*) and transformed this construct into the *cer11-1/cpl2-4* plants. I then examined the CER11/CPL2-GFP localization in the stem (Figure 3.12b) and the seed coat (Figure 3.12c-e) epidermal cells by confocal microscopy. In both organs, the CER11/CPL2-GFP-related fluorescence was present in both the nucleus and the cytoplasm, which is consistent with results obtained in a previous study using a *CER11/CPL2-GFP* transgene driven by the CaMV 35S promoter (Koiwa, et al., 2004).



Figure 3.12. Expression levels of the *CER11/CPL2* gene in different organs and subcellular localization of CER11/CPL2-GFP. (a) Total RNAs from WT (Col-0) leaf, stem, flower, root, 10-day-old seedlings and developing seeds at 4 DPA, 7 DPA and 10 DPA of WT were analyzed for expression of the *CER11/CPL2* gene and the *GAPC* gene, by qPCR. *CER11/CPL2* gene expression was normalized to *GAPC* expression values. Error bars represent SD (n=4). (b-e) Localization of the CER11/CPL2-GFP in the stem epidermal cells (b) and the seed coat epidermal cells at the early (c), the middle (d) and the late (e) stages of seed development by confocal microscopy. Representative embryos for each of the developmental stages are shown in the small panels. Scale bar = 20 µm.

3.2.7 VHA-C binds to CER11/CPL2 and co-localizes with it in the epidermal cells

To identify the potential substrate(s) of CER11/CPL2, I carried out a yeast-2-hybrid screen with Arabidopsis cDNA library using GAL4 binding domain fused CER11/CPL2 (BD-CER11/CPL2) as bait, and identified 23 genes as potential interactors (Table 3.1). Two of these genes, the *CELL WALL-PLASMA MEMBRANE LINKER PROTEIN* (*CWLP*, At3g22120) and *V-ATPASE SUBUNIT C* (*VHA-C/DET3*, At1g12840) were further investigated because *CWLP* was identified multiple times in the screen, and V-ATPase was reported to be involved in secretory trafficking (Dettmer, et al., 2006; Marshansky and Futai, 2008).

| ATID | Description | Number of times each gene was |
|-----------|--|-------------------------------------|
| | | identified |
| At3g22120 | Cell wall-plasma membrane linker protein (CWLP) | 6 |
| At1g12840 | V-type proton ATPase subunit C (VHA-C/DET3) | 1 |
| At1g02500 | S-adenosylmethionine synthetase 1 (SAM1) | 2 |
| At3g17390 | S-adenosylmethionine synthase 4 (MTO3) | 1 |
| At1g60950 | Ferredoxin-2 (FED A) | 3 |
| At5g66570 | 33 kDa oxygen evolving polypeptide 1 (PSBO1) | 2 |
| At1g72150 | Patellin-1 (PATL1) | 1 |
| At1g22530 | Patellin-4 (PATL2) | 1 |
| At3g47370 | 40S ribosomal protein S20-2 | 3 |
| At3g45030 | 40S ribosomal protein S20-1 | 1 |
| At5g62360 | Plant invertase/pectin methylesterase inhibitor domain- containing protein | 1 |
| At1g53645 | Hydroxyproline-rich glycoprotein-like protein | 1 |
| At2g18170 | Mitogen-activated protein kinase 7 (MAPK7) | 1 |
| At2g20890 | Photosystem II reaction center PSB29 protein | 1 |
| At1g74470 | Encodes a multifunctional protein with geranylgeranylreductase activity shown to catalyze the reduction of prenylatedgeranylgeranyl-chlorophyll a to phytyl-chlorophyll a (chlorophyll a) and free geranylgeranyl pyrophosphate to phytyl pyrophosphate. | 1 |
| At1g47128 | Putative cysteine proteinase RD21A | 1 |
| At3g23570 | Dienelactone hydrolase family protein | 1 |
| At2g47710 | Adenine nucleotide alpha hydrolases-like protein | 1 |
| At1g66200 | Glutamine synthetase cytosolic isozyme 1-2 (GSR2) | 1 |
| At3g11400 | Eukaryotic translation initiation factor 3G1 (EIF3G1) | 1 |
| At3g48590 | Nuclear factory Y subunit C1 (NF-YC1) | 1 |
| At5g14610 | DEAD-box ATP-dependent RNA helicase | 1 |
| At4g35060 | Heavy metal transport/detoxification domain-containing protein | 1 |

Table 3.1. Genes identified by yeast-2-hybrid screening. Gene description is from TAIR database (<u>http://www.arabidopsis.org</u>).

Each of the prey clones recovered for *CWLP* contains only the part of the gene that encodes the C-terminus (CWLP¹⁶⁶⁻³³⁴). To verify that the CWLP is a true CER11/CPL2 interactor in the yeast 2-hybrid system, I generated a construct containing a full-length CWLP (FL-CWLP) conjugated with a GAL4 activation domain (AD). As shown in Figure 3.13a, the C-terminal CWLP (CWLP¹⁶⁶⁻³³⁴), but not the FL-CWLP, can bind to CER11/CPL2 in yeast.



Figure 3.13. CER11/CPL2 and CWLP do not interact in the epidermis. (a) Yeast-2hybrid assay shows that C-terminal CWLP (AD-CWLP¹⁶⁶⁻³³⁴), but not full length CWLP (AD-CWLP) can bind to CER11/CPL2 (BD-CER11/CPL2). AD, GAL4 activation domain fusions; BD, GAL4 DNA binding domain fusions; -L-T-H, medium without leucine, tryptophan and histidine. Serial 1:10 dilutions are shown. (b-g) Localization of CER11/CPL2-RFP and YFP-CWLP in the wild type stem epidermal cells (b-d) and seed coat epidermal cells (e-g) by confocal microscopy. YFP-CWLP was not detected in the stem or seed coat epidermal cells and, therefore, CER11/CPL2 and CWLP are not colocalized in these tissues. Scale bar = 20 μ m.

I also tested whether CWLP and CER11/CPL2 are co-localized *in vivo*. For this experiment, a full length CWLP with YFP inserted immediately following the signal peptide and driven by the CWLP native promoter (*CWLPp::YFP-CWLP*), as well as the CER11/CPL2-RFP driven by the CER11 native promoter (*CER11/CPL2p::CER11/CPL2-RFP*) were co-expressed in wild type (Col-0) plants. Visualization of the YFP-CWLP and CER11/CPL2-RFP fluorescence patterns in the stem and the seed coat epidermal cells by confocal microscopy demonstrated that YFP-CWLP is not expressed in the stem or seed coat epidermis (Figure 3.13b-g), although the YFP signal was observed in the stem cortical cells.

In addition, two T-DNA alleles of *CWLP*, *cwlp-1* (GK-382D01) and *cwlp-2* (Sk_31605), were obtained for examination of their wax and seed coat mucilage phenotypes. Insertions in both *cwlp-1* and *cwlp-2* were mapped to exons, and wild type *CWLP* transcripts could not be detected in either *cwlp-1* or *cwlp-2* (Figure 3.14a-b). The GC-FID wax analysis data show that stem wax loads of the *cwlp* mutants are similar to the wild type (Figure 3.14c), while ruthenium red staining of *cwlp* mutant seeds immersed in water demonstrate that they release normal amount of mucilage (Figure 3.14d). Taken together, these data suggest that CWLP is not a substrate of CPL2 in cuticular wax export or seed coat mucilage release.



Figure 3.14. Phenotypes of the *cwlp* mutants. (a) The structure of *CWLP* gene (At3g22120) shows the exon as a black box, the 5'-UTR as a white box and 3'UTR as a black triangle. The locations of the T-DNA insertion of *cwlp-1* (GK-382D01) and *cwlp-2* (SK_31605) were mapped and are indicated by arrowheads. Locations of primers used for RT-PCR in (b) are indicated with short horizontal arrows. (b) RT-PCR analysis of steady-state *CWLP* transcript levels in WT (Col-0) and the *cwlp-1* and *cwlp-2* mutant leaves. RT-PCR was performed using total leaf RNA, and the expression level of *GAPC* was used as a control. (c) Cuticular wax analysis for WT and the *cwlp-1* and *cwlp-2* stems by GC-FID. Wax analysis data are expressed as the mean \pm SD (n=4). (d) Mucilage extrusion from WT (Col-0) and the *cwlp-1* and *cwlp-2* seeds after hydration in water and staining with ruthenium red. Scale bar = 0.5 mm.

To confirm binding of VHA-C to CER11/CPL2 in yeast, the N-terminal fragment (VHA-C¹⁻¹⁷⁵), C-terminal fragment (VHA-C¹⁷⁶⁻³⁷⁵), or full length VHA-C protein conjugated with AD domains were co-expressed with BD-CER11/CPL2 in yeast cells. Results of this experiment revealed that only the C-terminal VHA-C fragment could bind to CER11/CPL2 in the yeast-2-hybrid system (Figure 3.15a). To demonstrate that these two proteins also interact in plants, I carried out a split-luciferase assay. For this purpose

VHA-C was fused with the C-terminal Luciferase (*cLuc-VHA-C*) and CER11/CPL2 was fused with the N-terminal Luciferase (*CER11/CPL2-nLuc*) driven by the CMV35S promoters and transiently co-expressed in tobacco leaves. Luciferase activity was detected only in the cells co-expressing *cLuc-VHA-C* and *CER11/CPL2-nLuc*, but not in the cells carrying *cLuc-VHA-C* and *nLuc* or *cLuc* and *CER11/CPL2-nLuc* (Figure 3.15b), an indication that VHA-C can bind to CER11/CPL2 *in planta*. To further delineate the binding domain of CER11/CPL2, I generated a series of truncations of CER11/CPL2 and examined binding activity towards VHA-C by yeast-2-hybrid assay. As shown in Figure 3.15c, the N-terminal portion of CER11/CPL2 (CER11/CPL2¹⁻⁵⁹⁹) is sufficient for binding to VHA-C.

To determine whether VHA-C and CPL2 co-localize in Arabidopsis epidermal cells, VHA-C genomic sequence was fused to the *YFP* reporter gene (*VHACp::VHAC-YFP*) and co-expressed with *CER11/CPL2p::CER11/CPL2-RFP* in wild type (Col-0) plants. Detection of fluorescence by confocal microscopy established that VHA-C-YFP and CER11/CPL2-RFP co-localize in the stem and seed coat epidermis (Figure 3.16).



Figure 3.15. Interaction between CER11/CPL2 and VHA-C. (a) Yeast-2-hybrid assay shows that C-terminal domain of VHA-C (AD-VHA-C¹⁷⁶⁻³⁷⁵) can bind to CER11/CPL2 (BD-CPL2). AD, GAL4 activation domain fusions; BD, GAL4 DNA binding domain fusions; -L-T-H, medium without leucine, tryptophan and histidine. Serial 1:10 dilutions are shown. (b) Split-luciferase assay. Luciferase activity in tobacco leaves co-infiltrated with the Agrobacterial strains containing CPL2-nLuc and cLuc-VHA-C or empty vectors (nLuc and cLuc). The areas of infiltration are indicated by arrows. nLuc, N-terminal Luciferase; cLuc, C-terminal Luciferase. (c) Yeast-2-hybrid assay shows that the N-terminal domain of CPL2 (CPL2¹⁻⁵⁹⁹) is responsible for VHA-C binding. The CPL2 domain structure and two major functional domains are shown. FCPH, catalytic FCP1 homology domain; dSRBM, double-stranded RNA binding motif.


Figure 3.16. Co-localization of CPL2-RFP and VHA-C-YFP in stem epidermal cells (a-c) and seed coat epidermal cells (d-f). Scale bar = $20 \mu m$.

3.2.8 VHA-C protein localization is not altered in the *cer11/cpl2* mutant

Even though VHA-C and CER11/CPL2 can interact, the effect of their interaction on the function of VHA-C is not known. One possibility is that CER11/CPL2 binding to VHA-C might change the VHA-C localization in the cell. To test this hypothesis, *VHACp::VHAC-YFP* construct was introduced into the *cpl2-3* mutant background, and the VHA-C-YFP sub-cellular localization in both wild type and *cpl2-3* genetic backgrounds was evaluated by confocal microscopy. The microscopy results show that the VHA-C-YFP localization in the *cpl2-3* mutant stem and seed coat epidermal cells is the same as in the wild type cells (Figure 3.17).



Figure 3.17. VHA-C-YFP localization in WT (Col-0) (a-d) and *cpl2-3* mutant (e-h) stem epidermal cells (a, e) and seed coat epidermal cells during the early (b, f), the middle (c, g) and the late (d, h) stages of seed development. Representative embryos for each of the developmental stages are shown in the small panels. Scale bar = $20 \mu m$.

3.2.9 *det3-1* mutation does not affect stem wax and seed coat mucilage deposition

V-ATPase has been shown to be essential for secretory trafficking, and null alleles of the Arabidopsis V-ATPase genes cause gametophytic or embryo lethality (Dettmer et al., 2005, Strompen et al., 2005). However, a weak allele of V-ATPase subunit C, *det3-1*, is viable (Schumacher et al., 1999). The *det3-1* carries a T/A transversion in the first intron of the *VHA-C* gene (Figure 3.18a), resulting in a reduced *VHA-C* transcript level, and an additional longer transcript (Figure 3.18b, Schumacher et al., 1999), indicating that the mutation in *det3-1* altered the *VHA-C* transcript splicing. As a result, *det3-1* exhibits a 2-fold lower V-ATPase activity than the wild type, and this defect in the trans-Golgi network/early endosome (TGN/EE) causes severe growth retardation (Figure 3.18c, Schumacher, et al., 1999, Brux et al., 2008). To investigate the function of VHA-C in wax and seed coat mucilage secretion, I determined the surface wax load and seed coat mucilage accumulation and extrusion in the *det3-1* mutant (Figure 3.18d-f). These analyses showed that the total wax load on the *det3-1* stem surface was comparable to wild type levels (Figure 3.18d), and that *det3-1* mutant seeds released similar amounts of seed coat mucilage as the wild type after immersion in water or EDTA with shaking (Figure 3.18e). Furthermore, the columella structure in the mutant seed coat epidermal cells was indistinguishable from that in the wild type (Figure 3.18f).

3.2.10 *cpl2-3det3-1* double mutant is as dwarf as the *det3-1*, but shows *cpl2* wax and seed coat mucilage phenotypes.

To investigate the genetic relationship between *CPL2* and *VHA-C*, I generated the *cpl2-3det3-1* double mutant. The surface wax load and seed coat mucilage extrusion in water were determined (Figure 3.19). The results demonstrated that the growth of the *cpl2-3det3-1* plant is inhibited as well, and the plant is more similar as the *det3-1* and smaller than the *cpl2-3* (Figure 3.19a). However, the amount of stem surface wax and the amount of seed coat mucilage released in water in *cpl2-3det3-1* are reduced as much as those in the *cpl2-3* compared to the wild type (Figure 3.19b, c).



Figure 3.18. Wax and seed coat phenotypes of the *det3-1* mutant. (a) The structure of the *VHA-C* gene (At1g12840) shows exons as black boxes, introns as black lines, 5'-UTR as a white box and 3'UTR as a black triangle. The location of the point mutation in the *det3-1* is indicated by a vertical arrow. Locations of primers used for RT-PCR in (b) are indicated with short horizontal arrows. (b) RT-PCR analysis of steady-state *VHA-C* transcript levels in WT (Col-0) and the *det3-1* leaves. RT-PCR was performed using total leaf RNA, and the expression level of *GAPC* was used as a control. Two different sizes of *VHA-C* transcripts were detected in the *det3-1*, which are indicated by black arrows. (c) A comparison of the 5-week-old WT (Col-0) and *det3-1* plants shows that the *det3-1* mutation causes a severe growth defect. Scale bar = 5 cm. (d) Cuticular wax analysis of the 5-week old WT and *det3-1* stems by GC-FID. Wax analysis data are expressed as the mean \pm SD (n=4). (e) Mucilage extrusion from the WT and *det3-1* seeds after hydration in water or EDTA, and staining with ruthenium red. Scale bar = 0.5 mm. (f) SEM images of seed coat epidermis of seeds from the wild type (WT) and the mutant (*det3-1*). Scale bar = 20 µm.



Figure 3.19. Phenotypes of the double mutant (*cpl2-3det3-1*) (a) A comparison of the 5-week-old WT (Col-0), the single mutants (*cpl2-3* and *det3-1*), and the double mutant (*cpl2-3det3-1*) plants shows that the *cpl2-3det3-1* double mutant has a severe growth defect, similar to the *det3-1* single mutant. Scale bar = 5 cm. (b) Cuticular wax analysis of the 5-week old WT (Col-0), the single mutants (*cpl2-3* and *det3-1*), and the double mutant (*cpl2-3det3-1*) stems by GC-FID. Wax analysis data are expressed as the mean \pm SD (n=4). Student's t-test was applied to the data; the asterisk indicates that, statistically, this value was significantly different from wild-type at P < 0.05. (c) Mucilage extrusion from WT (Col-0), the single mutants (*cpl2-3* and *det3-1*), and the double mutant (*cpl2-3det3-1*) seeds after hydration in water, and staining with ruthenium red. Scale bar = 0.5 mm.

3.3 Discussion

3.3.1 The primary role of CER11/CPL2 is in secretion

The *cer11* mutant is a dwarf with defects in cuticular wax deposition in the stem epidermis, mucilage and cellulosic secondary cell wall deposition and MUM2 secretion in seed coat epidermal cells, and in secretion of secGFP, a visual protein marker of intracellular trafficking in plant cells. Although cause and effect are difficult to establish with certainty, all aspects of the phenotype could be explained if the primary role of CER11/CPL2 is in secretion.

Cuticular wax formation has been extensively studied (reviewed by Samuels et al., 2008; Kunst and Samuels, 2009; Bernard and Joubes, 2013) and the biosynthesis of very long chain fatty acid wax precursors and wax molecules in plant cells is now fairly well understood. However, transport of cuticular wax to the plant surface, especially delivery of wax constituents from their biosynthetic origin in the ER to the PM, is still poorly defined. One of the attractive hypotheses previously proposed is that wax molecules reach the plasma membrane by vesicular trafficking through the Golgi apparatus (Kunst and Samuels, 2003). My data are consistent with this hypothesis since a mutation in the *CER11/CPL2* gene that disrupts normal secretory function (Figure 3.1 and Figure 3.10) also results in markedly reduced amount of cuticular wax deposited on the stem surface (Figure 3.2 and Figure 3.10). Similarly, cell wall components, such as hemicelluloses and pectins, are believed to be trafficked from the Golgi apparatus to the apoplast by secretory vesicles (reviewed by Driouich et al., 2012). My work on the cer11/cpl2 revealed that there is a reduction in the amount of pectin delivered to the mucilage pocket consistent with the hypothesis that its secretion is impaired (Figure 3.3).

The *cer11/cpl2* phenotypes may be due not only to impaired secretion of the extracellular matrix materials, wax and cell wall carbohydrates themselves, but the proteins required for their processing, movement across the membrane or synthesis of non secreted matrix material such as cellulose as well. My results demonstrate that the *cer11/cpl2* mutant exhibits delayed MUM2 protein secretion (Figure 3.6). MUM2 is a β-galactosidase that is secreted to the apoplast during mucilage synthesis in seed coat epidermal cells (this study; Dean et al., 2007; Lee, Gilchrist and Haughn, unpublished data). It catalyzes the removal of galactose groups from mucilage polysaccharides to loosen the mucilage and permit extrusion following maturity (Dean et al., 2007). The observed defects in extrusion of seed mucilage (Figure 3.3) could be explained, in part, by the lack of delivery of sufficient MUM2 to properly process the pectin.

The defect in seed coat epidermal columella synthesis in *cer11/cpl2* (Figure 3.4) could be due to a disruption in cellulose synthase complexes that must be secreted to the plasma membrane. CESA5 is a cellulose synthase subunit associated with the production of cellulose in the secondary walls of seed coat epidermal cells (Harpaz-Saad et al., 2011; Mendu et al., 2011; Sullivan et al., 2011), so impaired secretion of CESA5 could explain the columella defects. A decrease in wax deposition could occur not only through a defects in delivery of wax to the PM through vesicular traffic, but also by a reduction in the delivery of the ABC transporters CER5/ABC12, ABCG11, and the lipid binding protein LTPG to the plasma membrane since all of these are needed to transport wax to the cuticle (Pighin and Zheng et al., 2004; Bird et al., 2007; DeBono, et al., 2009). Although my qualitative analysis of CESA5, CER5/ABC12, ABCG11, and LTPG localization did not show any differences between wild type and the *cer11/cpl2* mutant,

subtle perturbations in the timing or amount of these proteins could easily have been missed in my analysis.

In addition to the mucilage and wax deficiencies, the *cer11/cpl2* plants have a dwarf stature. Defects in secretion are expected to have a variety of effects on cell biology that could lead to stunted growth. Indeed, defects in the endomembrane system typically result in a dwarf phenotype (e.g. *de-etiolated3-1 (det3-1)*, Schumacher et al., 1999; *echidna*, Gendre et al., 2011). The exact nature of any additional defects in the *cer11/cpl2* plant remains to be determined.

It is clear from the *cer11/cpl2* phenotype (Figure 3.2, 3.3, 3.4, 3.6, 3.10, 3.11) that secretion is only partially blocked even though the mutations appear to be null alleles. I propose that the disruption of *CER11/CPL2* function in the *cer11* mutant does not completely block secretory trafficking, but slows down the secretion process resulting in the delayed secretion of MUM2 protein, the cell wall components in the seed coat, cuticular waxes on Arabidopsis stems, as well as other products transported by the general secretory pathway, as evidenced by the pleiotropic effect of the *cer11* mutation and its profound influence on the growth and development of this mutant.

3.3.2 The function of CER11/CPL2 in secretion

Cloning of the *CER11* gene revealed that it encodes CPL2, predicted to be a CTD phosphatase according to its deduced protein sequence containing a FCPH catalytic domain homologous to the yeast FCP1 CTD phosphatase (Koiwa et al., 2002; Koiwa et al., 2004; Figure 3.15c). In Arabidopsis, more than 20 genes are predicted to encode plant

CTD phosphatases (Bang et al., 2006). Among them, CPL1/FIERY2 (FRY2) and CER11/CPL2 are unique to plants and belong to the CPL1-like class, because in addition to the FCPH domain, these proteins also contain two or one double stranded RNA binding motifs (dsRBMs), respectively (Koiwa et al., 2004; Figure 3.15c). CPL1/FRY2 and CER11/CPL2 have *in vitro* dephosphorylation activity (Koiwa et al., 2004), and were demonstrated to be involved in plant growth and response to abiotic stresses, such as cold, salt and abscisic acid (ABA) (Koiwa et al., 2002; Ueda et al., 2008). In addition, the male gamete lethality of *cpl1cpl2* double mutant indicates that they function redundantly (Koiwa et al., 2004).

However, the phenotypes of *cer11/cpl2* mutant plants are distinct from *cpl1/fry2*, and include additional leaf morphology, early flowering (Ueda et al., 2008), dwarfism and stem wax-deficiency (this study) that were not observed in the *cpl1/fry2* mutants. Furthermore, CPL1 protein is localized only in the nucleus, while CPL2 is localized in both the nucleus and cytoplasm (Koiwa et al., 2004; Figure 3.12b). Taken together, these results suggest that CPL1/FRY2 and CER11/CPL2 are only partially redundant, and that CER11/CPL2 likely has functions that are distinct from CPL1/FRY2. My research data demonstrate that this is indeed the case, and provide evidence that CER11/ CPL2 plays an important role in general secretory trafficking required for plant growth and development, stem cuticular wax export, seed coat mucilage accumulation and extrusion, and columella secondary cell wall formation.

To elucidate the CER11/CPL2 function in secretory trafficking, I carried out a yeast-2-hybrid screen in an attempt to identify CER11/CPL2 substrate(s). Using this approach, I recovered a number of potential binding partners of CER11/CPL2. Among

them, VHA-C subunit of the vacuolar H⁺-ATPase (V-ATPase) with the demonstrated role in the secretory trafficking was shown to be co-expressed with the CER11/CPL2 in the stem and seed coat epidermis, and interact with CER11/CPL2 in yeast and in planta (Figure 3.15a-b). Results from the binding study with truncated fragments further indicated (Figure 3.15c) that CER11/CPL2 via its N-terminal domain can interact with VHA-C. Thus, even though it remains to be determined if CER11/CPL2 can dephosphorylate VHA-C by a direct assay, VHA-C is an attractive potential target of CER11/CPL2.

V-ATPase is a multi-subunit enzyme comprised of the cytosolic ATP hydrolysis "motor" V_1 and the membrane-integral proton "turbine" V_0 . In addition to the tonoplast, this enzyme is found in all compartments of the endomembrane system (Gaxiola et al., 2007). V-ATPase not only functions in acidification of the cellular compartments and maintains the proton gradient across membranes, but also is directly involved in endocytic and secretory transport from the TGN (Schumacher and Krebs, 2010; Dettmer et al., 2006). My data show that CER11/CPL2 can bind to the subunit C of V-ATPase (VHA-C) and that CER11/CPL2 is required for the normal secretion function in plant cells, which suggests that CER11/CPL2 may influence secretion by affecting the phosphorylation state of VHA-C. Interestingly, the VHA-C was shown to be phosphorylated by a WNK (With No lysine (K)) kinase, WNK8 (Hong-Hermesdorf et al., 2006), however, the effect of the phosphorylation on the function of VHA-C remains obscure. In yeast and insect cells, phosphorylation and dephosphorylation control reversible assembly of the V-ATPase, suggested to be an important mechanism controlling V-ATPase activity (Toei et al, 2010).

V-ATPase is an essential enzyme and null alleles of Arabidopsis single copy V-ATPase genes cause gametophytic or embryo lethality (Dettmer et al., 2005, Strompen et al., 2005). The only viable mutant of VHA-C is a knockdown line *det3-1*, originally identified based on its altered photomorphogenesis (Cabrera y Poch et al., 1993). The *det3-1* plants have a dwarf phenotype (Cabrera y Poch et al., 1993; Figure 3.18), and the *det3-1* plants have a dwarf phenotype (Cabrera y Poch et al., 1993; Figure 3.18), and the *det3-1* hypocotyls are conditionally shorter than the wild type. The retarded growth of *det3-1* was demonstrated to result from defective cell expansion due to reduced V-ATPase activity in the TGN (Schumacher et al., 1999; Brux et al., 2008). In addition, *det3-1* also exhibits cellulose synthesis defects (Cano-Delgado et al., 2003), and the mutant is hypersensitive to the cellulose synthesis inhibitor isoxaben (Brux et al., 2008). Interestingly, the isoxaben treatment led to a rapid loss of CESA6 from the PM, suggesting that isoxaben may affect the trafficking of CESAs to the PM (Paredez et al., 2006) leading to a reduced cellulose synthesis and decreased cell expansion.

In an attempt to establish a functional link between the CER11/CPL2 and V-ATPase in secretion, I examined the phenotypes of the *det3-1* mutant. However, even though *det3-1* mutants show pronounced dwarfism, as well as defects in secretion and cell wall formation (Figure 3.19a), they do not exhibit wax and seed coat phenotypes observed in the *cer11/cpl2* line. This may be due to the fact that the *det3-1* still has 40% of V-ATPase activity (Schumacher et al., 1999), which may be sufficient for normal cuticular wax export and seed coat development. Furthermore, *det3-1* is a conditional mutant sensitive to the growth conditions, such as temperature and nutrient levels (Brux et al., 2008), which makes the interpretation of *det3-1* phenotypes more challenging. It also needs to be taken into consideration that VHA-C may not be the only target of CER11/CPL2.

3.4 Materials and Methods

3.4.1 Plant material and growth conditions

Arabidopsis ecotypes Columbia (Col-0) and Landsberg *erecta* (Ler) and the *cer11-1* mutant (Koornneef et al., 1989) were used in this study. SALK T-DNA insertional lines, SALK 149234 (cpl2-1, Ueda et al., 2008), SALK 059753 (cpl2-2, Ueda et al., 2008), SALK_131421 (spi-1), and SALK_116367 (spi-2) were obtained from the ABRC (www.arabidopsis.org). The T-DNA lines, GK-433F07 (cpl2-3), GK-382D01 (cwlp-1) and det3-1 (Cabrera y Poch et al., 1993) were obtained from The Nottingham Arabidopsis Stock Centre (NASC, www.arabidopsis.info). The SK T-DNA line SK_31605 (cwlp-2) was obtained from Agriculture and Agri-Food Canada (http://aafcaac.usask.ca/FST/, Robinson and Parkin, 2009). Homozygous lines for each gene were identified by genotyping using primers listed in Table 3.2. The secGFP, CER5-GFP and GFP-CESA5 lines have been previous described (Batoko et al., 2000; Pighin and Zheng et al., 2004; Bischoff et al., 2011). The YFP-ABCG11 and YFP-LTPG lines were kindly provided by Dr. Lacey Samuels (University of British Columbia, BC, Canada; Bird et al., 2007; DeBono et al., 2009). The MUM2-YFP lines were generated in our lab (Gilchrist and Haughn, University of British Columbia, BC, Canada). Seeds were germinated on AT medium (Haughn and Somerville 1986) supplemented with agar (7 g/L) and appropriate antibiotics. The 7- to 10-day-old seedlings were transferred to soil and grown at 20 °C under continuous light with a light intensity of 100 μ mol m⁻²s⁻¹.

3.4.2 Plasmid construction and plant transformation

To generate the *CER11/CPL2p::CER11/CPL2* construct, a 6770 bp long genomic fragment of CER11/CPL2 genomic sequence, including the 2117 bp upstream sequence before the start codon of the CER11/CPL2 gene, the 3981 bp long coding region and the 672 bp long 3'UTR sequence, was amplified from the Columbia (Col-0) genomic DNA using the forward primer (5'- ACGCGTCGAC AAGCTCGGAATCAAGTGTCAAC -3') and the reverse primer (5'- TTT GGCGCGCCTCAAGTTTGGACCTTTGGTGC-3'). The fragment was recombined into the binary vector pCAMBIA1380 between the AscI and SalI cutting sites. To generate CER11/CPL2p::CER11/CPL2-GFP construct, a 6095 bp long fragment of *CER11/CPL2* genomic sequence, including the 2117 bp upstream sequence before the start codon of the CER11/CPL2 gene and the 3978 bp long coding region without the amplified plasmid stop codon. was from the CER11/CPL2p::CER11/CPL2 using the forward primer (5'- GGGG ACA AGT TTG TAC AAA AAA GCA GGC TTT AAGCTCGGAATCAAGTGTCAAC -3') and the reverse primer (5'- GGGG AC CAC TTT GTA CAA GAA AGC TGG GTG CAACTCTGAAATGTTTTCTTTAGGCGC -3'). The fragment was recombined into the vector pDONR221 (Invitrogen Life Technologies) and then into the binary vector pGWB4 with a GFP tag (Nakagawa et al., 2007) using GATEWAY cloning strategy (http://www.lifetechnologies.com). To generate CER11/CPL2p::CER11/CPL2-RFP construct, the 6095 bp long genomic fragment of CPL2 was amplified from the plasmid CER11/CPL2p::CER11/CPL2 using the forward primer (5'- ACGCGTCGAC AAGCTCGGAATCAAGTGTCAAC -3') and the reverse primer (5'- ACGCGTCGAC CAACTCTGAAATGTTTTCTTTAGGCGC -3') and the *mRFP* coding sequence was

amplified from the plasmid pCAMBIA2300-pUBQ1-mRFPL (kindly provided by Dr. Geoffrey Wasteneys, University of British Columbia, BC, Canada) using the forward primer (5'- ACGC<u>GTCGAC</u> ATGGCCTCCTCCGAGGACGTCATC -3') and the reverse primer (5'- TTT <u>AAGCTT</u> TCA GGCGCCGGTGGAGTGGCG -3'). The *mRFP* and *CER11/CPL2* fragments were sequentially ligated into the SalI and HindIII sites and the AscI and SalI sites of pCAMBIA1380, respectively.

To generate the *SAPLIPp::SAPLIP* construct, a 3234 bp long fragment of *SAPLIP* genomic sequence, including the 1747 bp upstream sequence before the start codon of the *SAPLIP* gene, the 1225 bp long coding region and the 262 bp long 3'UTR sequence, was amplified from the Columbia (Col-0) genomic DNA using the forward primer (5'- GGG <u>GAATTC</u> TGTGTGCATGAGACATATGGAG -3') and the reverse primer (5'- ACGC<u>GTCGAC</u> GAGTACACGCATCAACGTAAC -3'), and ligated into the EcoRI and SalI sites of binary vector pCAMBIA1380.

To generate the *CWLPp::YFP-CWLP* construct, a 2804 bp long fragment of SAPLIP genomic sequence, including the 2723 bp upstream sequence before the start codon of the *SAPLIP* gene and the 81 bp long sequence encoding the signal peptide of SAPLIP, was first amplified from the Columbia (Col-0) genomic DNA using the forward primer (5'- TTT <u>AAGCTTGATTAAGCTGCTGCATCAGAGG</u> -3') and the reverse primer (5'- TTT <u>TCTAGA</u> AAA <u>GCGGCCGC</u> C ACAGTCGCAAGCATAGGAGAC - 3'), and ligated into the HindIII and XbaI sites of binary vector pBI101 to generate the *pBI101-CWLPp::CWLPsig* construct. At the same time, a NotI cutting site in the reverse primer was introduced into the plasmid before the XbaI site. The YFP coding sequence was amplified from the pEarleyGate104 plasmid (Earley et al., 2006) using the forward

primer (5'- TTT <u>GCGGCCGC</u> ATG GGC AAG GGC GAG GAG CTG TT -3') and the reverse primer (5'- TTT TCTAGA CTT GTA CAG CTC GTC CAT GCC G -3'), and ligated into the NotI and XbaI sites of the construct *pBI101-CWLPp::CWLPsig* generate the *pBI101-CWLPp::CWLPsig-YFP*. Finally, a 1289 bp long fragment of *CWLP* genomic sequence, including the 924 bp long remaining coding sequence and the 365 bp long 3'UTR sequence, was amplified from Columbia (Col-0) genomic DNA using the forward primer (5'- TTT <u>TCTAGA</u> ACCCCTCCTAAACCATCACCAG -3') and the reverse primer (5'- GGG <u>GGATCC</u>TATGCATCAGTGCAATGCGCATG -3'). The PCR fragment was ligated into the XbaI and BamHI sites of the construct *pBI101-CWLPp::CWLPsig-YFP* to generate the construct for expressing the *YFP-CWLP* under the control of the *CWLP* native promoter.

To generate the *VHACp::VHAC-YFP* construct, a 3164 bp long fragment of VHA-C genomic sequence, including the 530 bp long upstream sequence before the start codon of the *VHA-C* gene and the 2634 long coding sequence before the stop codon, was amplified from the Columbia (Col-0) genomic DNA using the forward primer (5'- GGGG <u>ACA AGT TTG TAC AAA AAA GCA GGC TTT</u> AGATGAATCAATCTTCTTACTTCA GAG-3') and the reverse primer (5'- GGGG <u>AC CAC TTT GTA CAA GAA AGC TGG GTG</u> AGCAAGGTTGATAGTGAAGGAG -3'). The PCR fragment was recombined into pDONR221 and then into pGWB40 with an YFP tag (Nakagawa et al., 2007) using GATEWAY cloning strategy (http://www.lifetechnologies.com).

To generate constructs for the yeast-2-hybrid assay, the full length and the truncated fragments of the *CPL2* sequence were amplified from cDNAs prepared from RNA isolated from 4-week-old Columbia (Col-0) wild type leaves, and recombined into the

BD vector pGBK-T7 (kindly provided by Dr. Xin Li, University of British Columbia, BC, Canada). The full length fragment of CWLP, and the full length and the truncated fragments of VHA-C were amplified from the Columbia (Col-0) cDNAs made from RNA also derived from 4-week-old leaves, and recombined into the AD vector pGAD-T7 (kindly provided by Dr. Xin Li, University of British Columbia, BC, Canada). The PCR primers are listed in Table 3.2.

To generate the *CPL2-nLuc* construct for the split luciferase assay, a 2310 bp long fragment of *CPL2* cDNA coding sequence without the stop codon was amplified from the Columbia (Col-0) cDNAs derived from 4-week-old leaf RNA, using the forward primer (5'- CGG <u>GGTACC</u> ATGAATCGTTTGGGTCATAAATCCG -3') and the reverse primer (5'- ACGC<u>GTCGAC</u> CAACTCTGAAATGTTTTCTTTAGGCGC -3'). The PCR fragment was ligated into the KpnI and SalI sites of vector 35S::NLuc (Chen et al., 2008). To generate the *cLuc-VHA-C* construct, a 1128 bp long fragment of *VHA-C* cDNA coding sequence was also amplified from the Columbia (Col-0) cDNAs from 4-week-old leaves using the forward primer (5'-CGG <u>GGTACC</u> ATGACTTCGAGATATTGGG TGG -3') and the reverse primer (5'-ACGC<u>GTCGAC</u> TTAAGCAAGGTTGATAGTG AAGGAG -3'). The PCR fragment was ligated into the KpnI and SalI sites of the vector 35S::CLuc (Chen et al., 2008).

For the complementation test or intracellular localization, the construct *CER11/CPL2p::CER11/CPL2, SAPLIPp::SAPLIP, CER11/CPL2p::CER11/CPL2-GFP, CER11/CPL2p::RFP, CWLPp::YFP-CWLP* or *VHACp::VHAC-YFP* was electro-transformed into *Agrobacterium tumefaciens* GV3101 cells. The transformed Agrobacteria were grown to the log phase, after which the cells were spun down, washed

and resuspended in 5% sucrose containing 0.025% silwet 1-77. 4-week-old wild type (Col-0) plants or *cer11-1* mutant plants were sprayed with the Agrobacterial suspension. The T_1 transgenic seeds were screened on AT medium (Haughn and Somerville 1986) supplemented with agar (7 g/L) and appropriate antibiotics.

3.4.3 Wax analysis by GC-FID

The top 10 cm, or full length if not longer than 10 cm, of 4- to 5-week-old primary inflorescence stems were used for wax analysis. The tissues were cut and submerged in chloroform containing 10µg of tetracosane as an internal control for 30 sec. Chloroform was then evaporated under a stream of nitrogen and the waxes were silylated in pyridine and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (Sigma-Aldrich) by incubating at 80 °C for 1 h. The solvent was blown off under nitrogen gas, and the wax pellet was re-dissolved in 30µl of chloroform. 1 µL of each sample was injected and analyzed on an Agilent 7890A gas chromatograph with a flame ionization detector (GC-FID) using an HP1 methylsiloxane column (Agilent Technologies). Samples were analyzed in a split mode (2.7:1) using a program running at 50 °C for 2 min, followed by an increase in temperature by 4 °C/min to 200 °C and held for 1 min, and then a final increase by 3 °C/min to 320 °C and held for 15 min. The total wax load per stem surface area was calculated based on the two-dimensional area measured in Adobe Photoshop and multiplied by π . Four biological replicates were analyzed for each line.

3.4.4 Monosaccharide analysis by HPAEC

To prepare alcohol insoluble residue (AIR), ~5 mg of whole seeds from wild type (Ler) and *cer11-1* plants were ground into fine powder in liquid nitrogen. The samples were suspended in 1 ml of 70% ethanol and incubated at 65 $\$ for 10 min to deactivate enzymes. After washing three times with fresh 70% ethanol with shaking at room temperature for 30 min, the AIR was dried under nitrogen gas at 60 $\$ and weighed before 40 μ l of 5 mg/ml erythritol was added as an internal control. The AIR was then hydrolyzed with 72% (w/v) sulfuric acid on ice for 2 h. After adding 1.93 ml of water to give a final concentration of 4% sulfuric acid, the samples were autoclaved at 121 $\$ for 1 h and filtered using a 0.45 μ m syringe filter before being used for HPAEC.

To extract seed coat mucilage by water, ~20 mg of wild type (Ler) and *cer11-1* seeds (exact weight recorded) were hydrated in 1.4 ml of water with 10 μ l of 5 mg/ml erythritol as an internal control. The samples were gently shaken for 2 h. The mucilage in the supernatant (1 ml) was transferred to a glass tube and dried under nitrogen gas at 60 °C. The mucilage was hydrolyzed with sulfuric acid as described above.

To extract seed coat mucilage by Na₂CO₃, ~20 mg of wild type (Ler) and *cer11-1* seeds (exact weight recorded) were mixed with 500 μ l of 20 mM of Na₂CO₃ and gently shaken for 25 min before 900 μ l of water and 10 μ l of 5 mg/ml erythritol were added. The samples were gently shaken for another 2 h. The mucilage supernatant (1 ml) was dried and hydrolyzed as described above.

Neutral sugar standards (fucose, arabinose, rhamnose, galactose, glucose, mannose, and xylose) and the acid sugar standard (galacturonic acid), prepared with and without Na_2CO_3 as required, were also processed in the same way as the samples.

The composition and concentration of neutral monosaccharide sugars were analyzed using a high-performance liquid chromatograph (DX-600; Dionex) equipped with an ion-exchange PA1 (Dionex) column, a pulsed amperometric detector (ED 40) with a gold electrode, and a Spectra AS 3500 autoinjector (Spectra-Physics). The column was eluted with deionized water at a flow rate of 1 ml/min. 20 µl of each sample and standards were injected. Optimization of baseline stability and detector sensitivity was achieved by post-column addition of 0.2 M NaOH. For acid sugars, the column was eluted at a flow rate of 1 ml/min with 0.1 M NaOH with a linear gradient of 0 to 400 mM sodium acetate from 5 to 40 min, followed by a 10 min 0.3 M NaOH wash. The column was then equilibrated to 0.1 M NaOH prior to the next injection.

3.4.5 Positional cloning

3.4.5.1 Map-based cloning

To clone the *CER11* gene, the *cer11-1* mutant in the Landsberg *erecta* background was crossed to the Columbia-0 wild type to generate a mapping population. 21 F_2 generation plants with *cer11* wax phenotype were used for rough mapping, and 1100 F_2 generation plants with *cer11* wax phenotype were used for fine mapping. The four InDel markers used for fine mapping on chromosome 5 are shown in Figure 3.7. They are M21140 at position 21,140 bp, M82750 at position 82,750 bp, F7A7-1 at position 211,000 bp and T20L15 at position 325,700bp. The primer sequences are shown in Table 3.2.

3.4.5.2 Inverse PCR

1 µg of genomic DNAs from the wild type (Landsberg *erecta*) or *cer11-1* were digested by Taq I restriction enzyme at 65 $^{\circ}$ C for 2 h, followed by the inactivation of the enzyme at 80 $^{\circ}$ C for 20 min. After purification, the digested DNAs were re-ligated with T4 DNA ligase at 18 $^{\circ}$ C overnight. The self-ligated DNAs were used as templates for inverse PCRs with primers annealing to the known sequences close to the chromosome break point.

3.4.6 qPCR

Rosette leaves, stems, flowers, roots, seedlings and developing seeds of the wildtype (Col-0) were collected and immediately frozen in liquid nitrogen. Total RNAs were isolated by using TRIZOL[®] Reagent (Invitrogen Life Technologies), and reverse transcription were performed by using iScript[™] cDNA Synthesis Kit (Bio-Rad). Gene specific primers used in qPCR are listed in Table 3.2. iQ SYBR Green Supermix (Bio-Rad) was used to perform qPCR in an iQ5 Multicolor Real-Time PCR Detection System as specified by the manufacturer (Bio-Rad, <u>www.bio-rad.com</u>).

3.4.7 Microscopy

3.4.7.1 Confocal Laser Scanning Microscopy

Sub-cellular localization was carried out on a Perkin Elmer UltraviewVoX spinning disk confocal microscope using glycerol immersion lens for live imaging. Dissected segments from the apical ~3 cm of stem or developing seeds were mounted in distilled water and immediately imaged. *cer11-1* developing seeds expressing *MUM2-YFP* were stained with 5 µg/ml of FM4-64 (Molecular Probes) for 10 min. GFP was detected using a 488 nm laser with a 507 to 543 nm filter, YFP was detected using a 514 nm laser with a 525 to 555 nm filter, and RFP and FM4-64 were detected using a 561 nm laser with a 570 to 620 nm filter. The confocal images were taken using the Volocity software (Perkin Elmer) and edited using the Fiji software.

3.4.7.2 SEM

Dry seeds or segments from the apical 1 cm of dry stem were mounted onto stubs and sputter coated with gold particles for 10 min in a SEM Prep 2 sputter coater (Nanotech). The coated samples were viewed under a Hitachi S4700 field emission SEM (Hitachi) at an accelerating voltage of 5 kV and a working distance of 12 mm.

3.4.7.3 Mucilage staining

Dry seeds were hydrated in water or 50 mM EDTA with shaking at room temperature for 1 h, and washed with water twice before staining with 0.01% (w/v) aqueous solution of ruthenium red (Sigma-Aldrich) for 1 h. The low magnification

images were taken with an Olympus SZX16[®] stereomicroscope (Olympus) and the high magnification images were taken with a Zeiss AxioScop 2 light microscope (Carl Zeiss).

3.4.7.4 Resin embedding and sectioning

Developing seeds from wild type (Ler) and *cer11-1* plants were dissected and fixed with 3% glutaraldehyde (Canemco) in 0.1 M phosphate buffer overnight at 4 °C. The samples were then post-fixed in 1% (v/v) osmium tetroxide in 0.05 M phosphate buffer for 2 hr. After dehydration in a series of increasing concentrations of ethanol, the seeds were embedded in Spurr's resin. The embedded samples were sectioned (50 μ m) using a Reichert Ultracut E microtome (Reichert) and stained with 1 % (w/v) toluidine blue. Images were taken under a Zeiss AxioSkop 2 light microscope (Carl Zeiss).

3.4.8 Yeast-2-hybrid screen

Yeast-2-hybrid screening was carried out using the GAL4-based yeast-2-hybrid system. The CER11/CPL2 was fused to the C-terminal end of the *GAL4* promoter binding domain (BD-CER11/CPL2). The BD-CER11/CPL2 as bait was introduced into the yeast strain YPH1347 (kindly provided by Dr. Xin Li, University of British Columbia, BC, Canada), in which *GAL4* promoter drives the histine synthetic gene, and used to screen an Arabidopsis cDNA expression library (kindly provided by Dr. Yuelin Zhang, University of British Columbia, BC, Canada) encoding Arabidopsis proteins as C-terminal fusions to *GAL4* transcription activation domain (AD). Putative CER11/CPL2 interacting proteins were selected based on the histine prototrophy.

3.4.9 Split-luciferase assay

The split-luciferase assay system has been previously described (Chen et al., 2008; Gehl et al., 2011). Briefly, *CER11/CPL2-nLuc, cLuc-VHA-C* and empty vectors were transformed into *Agrobacterium tumefaciens* GV3101 cells. The Agrobacterial cells were grown into log phase. The cells were collected and resuspended in an infiltration medium (1x Murashige and Skoog, 10 mM MES, pH 5.6, 150 μ M acetosyringone) to a final concentration of OD₆₀₀ = 0.6. The 3- to 4-week-old tobacco (*Nicotiana benthamiana*) leaves were infiltrated using 1 ml syringes with different combinations of bacterial suspensions. The plants were incubated at room temperature in the dark for 48 h. 1 mM of D-Luciferin (Sigma-Aldrich) dissolved in DMSO, 10 mM MgCl2 and 10 mM MES, pH 5.6, were infiltrated into the piece of the Agrobacteria-infiltrated leaves before cutting the leaves for exposure. Luminescence was visualized after a 20-30 min exposure using a CCD camera with a low-light imaging system (ChemiDocTM XRS+; Bio-Rad, http://www.bio-rad.com/) using a 3x3 binning settings for all images.

| Genotyping primers for mutants | | | | |
|--|----------------------------------|----------------|-----------|--|
| Primer name | Primer sequence | Mutant line | | |
| LBb1.3 | 5'-ATTTTGCCGATTTCGGAAC-3' | All Salk lines | | |
| cpl2-1LP | 5'-AAAGCCTAATTGAGGTGGAGC-3' | SALK_149234 | | |
| cpl2-1RP | 5'-CTCCGTGAAGCTTGTTTTCAG-3' | | | |
| cpl2-2LP | 5'-AAGGCTGCAGCTGATCATTCTAC-3' | SALK_059753 | | |
| cpl2-2RP | 5'-CCTAAACTCCACCTGCAAAATCG-3' | | | |
| spi-1LP | 5'-TTCACATAGTTAGCATGGGTGGG-3' | SALK_131421 | | |
| spi-1RP | 5'-AACGCTTCTCTGTGTTTTGAGG-3' | | | |
| spi-2LP | 5'-CCTGGTTCTATCTTCCTTGCC-3' | SALK_116367 | | |
| spi-2RP | 5'-CGATGGGCATCAGATATCAAG-3' | | | |
| GABI-inF2 | 5'-ATATTGACCATCATACTCATTGC-3' | All GK lines | | |
| cpl2-3LP | 5'-ATTTACGTTTGGGGGGAACTTG-3' | GK-433F07 | | |
| cpl2-3RP | 5'-GAGCATAATCCCTTTCAGCCATG-3' | | | |
| cwlp-1LP | 5'-ACCCCTCCTAAACCATCACCAG-3' | GK-382D01 | | |
| cwlp-1RP | 5'-AAGCGGGACATTTGAAGTCAG-3' | | | |
| SK-L3 | 5'-ATACGACGGATCGTAATTTGTCG-3' | All SK lines | | |
| cwlp-2LP | 5'-ATAACTCCACCAACACCAACC-3' | SK 31605 | | |
| cwlp-2RP | 5'- TATGCATCAGTGCAATGCGCATG-3' | | | |
| det3-1LP-WT | 5'-CGTGTCTACGATTCTAATTACGAGCT-3' | det3-1 | | |
| det3-1LP-Mut | 5'-CGTGTCTACGATTCTAATTACGAGCA-3' | | | |
| det3-1RP | 5'-TAATGGCATTGAGTTGACCACG-3' | | | |
| P1 | 5'-GGTTCTCACTATTCAAGTGCTGG-3' | cer11 | | |
| P2 | 5'-TTCACATAGTTAGCATGGGTGGG-3' | | | |
| P3 | 5'-TAGTTGCTCACATCCGGTGATGG-3' | | | |
| P4 | 5'-TTTATCAGACAGCCCTTGTCG-3' | | | |
| P5 | 5'-TTAACCTCTGTCAAGCTGTGAC-3' | | | |
| P6 | 5'-GGCAAAAGTCAATCTTCAGCAGC-3' | | | |
| Primers for RT-PCR or quantitative Real-time PCR | | | | |
| Primer name | Primer sequence | Gene Name | ATID | |
| mGFP-F | 5'-GTGAAGGTGATGCAACATACG-3' | secGFP | | |
| mGFP-R | 5'-TGTGTGGACAGGTAATGGTTG-3' | | | |
| CPL2RT-P1F | 5'-AATTCCTATGCAGCCACCTTC-3' | CER11/CPL2 | AT5G01270 | |
| CPL2RT-P1R | 5'-TCTTCTCACCCGTGAACAACAC-3' | | | |
| CPL2RT-P2F | 5'-ATCTACACGCAGTCTGTTTCC-3' | | | |
| CPL2RT-P2R | 5'-GAGCATAATCCCTTTCAGCCATG-3' | | | |
| CPL2QRT-F | 5'-TGTGCTTGTAAAACTGAGACCAGC-3' | | | |
| CPL2QRT-R | 5'-TTCTTGCTACGCAGAGATGTG-3' | | | |
| CWLPRT-F | 5'-ATAACTCCACCAACACCAACC-3' | CWLP | AT3G22120 | |
| CWLPRT-R | 5'-TTAAGCGGGACATTTGAAGTCAG-3' | | | |
| VHA-CRT-F | 5'-CCTTAGATTCTCTACTCGCTCTC-3' | VHA-C | AT1G12840 | |
| VHA-CRT-R | 5'-TAATGGCATTGAGTTGACCACG-3' | | | |

Table 3.2. PCR primers used in chapter 3

| Primers for generating constructs for Y-2-H | | |
|---|--|--|
| Primer name | Primer sequence | |
| BD-CPL2FP | 5'-GGG GAATTC ATGAATCGTTTGGGTCATAAATCCG-3' | |
| BD-CPL2RP | 5'-ACGC GTCGAC TCACAACTCTGAAATGTTTTCTTTAGGCGC-3' | |
| BD-CPL2 ¹⁻³⁹² RP | 5'-ACGC GTCGAC TCA GTAACCTCTGACATTGCATGC-3' | |
| BD-CPL2 ¹⁻⁵⁹⁹ RP | 5'-ACGC GTCGAC TCA AAATGGACCAACAGGAGCAGAAC-3' | |
| BD-CPL2 ³⁶⁶⁻⁷⁷⁰ FP | 5'-GGG <u>GAATTC</u> TATGCTCCACAAGCAGAGACAG-3' | |
| BD-CPL2 ⁶⁵⁶⁻⁷⁷⁰ FP | 5'-GGG <u>GAATTC</u> TTATTTGTGAGTGCACTGCAAG-3' | |
| AD-CWLPFP | 5'-GGG <u>GAATTC</u> ATGGGGTCTCGCTCTCAAAAC-3' | |
| AD-CWLPRP | 5'-GGG <u>GGATCC</u> TTAAGCGGGACATTTGAAGTCAG-3' | |
| AD-VHA-CFP | 5'-GGG <u>GAATTC</u> ATGACTTCGAGATATTGGGTGG-3' | |
| AD-VHA-CRP | 5'-GGG <u>GGATCC</u> AGCAAGGTTGATAGTGAAGGAG-3' | |
| AD-VHA-C ¹⁻¹⁷⁵ RP | 5'-GGG <u>GGATCC</u> TTA AATATCTTCTGGCTTAACCAAGTTTG-3' | |
| InDel Marker primers | | |
| InDel Marker name | Primer sequence | |
| M21140 | 5'-GTCGTTAACCTCTACAACACCTC-3' | |
| | 5'-TGACTTGGCCCAGACATAGTATC-3' | |
| M82750 | 5'-AATGACCAACTAGAATCAATGCG-3' | |
| | 5'-ATGTCTTATCAGCTGAGAATCATG-3' | |
| F7A7-1 | 5'-GGCTGGTTCAACGATCAAA-3' | |
| | 5'-GAATGTGGCGGGGGGGGGGGAGGTA-3' | |
| T20L15 | 5'-TTTTGCGTCATCAATCATAC-3' | |
| | 5'-TGAGTTGTCGGCATCGTA-3' | |

 Table 3.2. PCR Primers used in chapter 3 (continued)

CHAPTER 4

CONCLUDING REMARKS AND FUTURE DIRECTIONS

4.1 Concluding remarks

The original goals of this research were to elucidate the regulation mechanism of seed oil deposition by the transcription factor GL2 and investigate the secretory process involved in the deposition of cuticular waxes in Arabidopsis. It is an interesting twist of fate that both GL2 and CER11 not only influence lipids, but plant cell walls as well. However, the interconnections between seed oil and seed coat mucilage deposition and between cuticular wax export and cell wall formation are distinct. The effect of GL2 on seed oil appears to be indirect through its regulation of *MUM4* transcription. CER11, on the other hand, appears to impact wax export and cell wall formation as a consequence of its more general effect on secretion.

4.1.1 The production of seed coat mucilage competes with the embryo for photosynthate reserves

In chapter 2, I describe my studies of the mechanism involved in GL2-mediated increase of seed oil content. My data demonstrate that GL2, and all of the transcription factors known to be required for *GL2* transcription, influence seed oil accumulation in the embryo through the regulation of *MUM4* transcription in the seed coat. The exact manner by which MUM4 exerts its influence on seed oil is unclear. MUM4 is a biosynthetic enzyme, NDP-L-rhamnose synthase, which catalyzes the production of RG I, the major component of seed coat mucilage. The substrate of MUM4, NDP-D-Glucose, comes from sucrose, the major carbon source produced by photosynthesis (Western et al., 2004; Usadel et al., 2004; Oka et al., 2007; Bar-Peled and O'Neill, 2011). On the other hand, sucrose is also the carbon source for seed oil biosynthesis in the embryo. The substrate of

de novo fatty acid biosynthesis, acetyl-CoA, is generated from sucrose through glycolysis (Baud et al., 2008). It has been suggested that sucrose is unloaded first into the seed coat through the funiculus, and then imported into the endosperm before reaching the embryo (Stadler et al., 2005). Therefore, I propose that the unused sucrose from the mutant (*gl2 and mum4*) seed coat mucilage production is imported into the embryo and utilized for seed oil biosynthesis. My results are consistent with the idea that mucilage biosynthesis in the seed coat competes with the embryo for available photosynthate reserves during seed development.

4.1.2 CER11/CPL2 functions in secretory pathway involved in both cuticular wax export and seed coat cell wall formation

In chapter 3, I show that the CER11/CPL2 protein is required for normal secretion. CER11/CPL2 impacts multiple processes including the deposition of wax and seed coat mucilage, and formation of seed coat columella, all of which involve polarized secretory events. It is likely that other processes are also affected, as evidenced by the fact that the *cer11-1* mutant is a dwarf. Therefore CER11/CPL2 may function in polarized secretory events in general.

CER11/CPL2 is a putative CTD phosphatase (Ueda et al., 2008). However, the exact role and the substrate of CER11/CPL2 in secretory trafficking are not yet known. In chapter 3, I demonstrate that a subunit of V-ATPase, VHA-C, can interact with CER11/CPL2, and two proteins are co-localized *in vivo*. It has been demonstrated that V-ATPase plays an important role in the endomembrane trafficking (Marshansky and Futai,

2008; Schumacher and Krebs, 2010). In addition, the dwarfism and the cell expansion defects of *det3-1*, a mutant of *VHA-C* (Schumacher et al., 1999; Brux et al., 2008), suggest that VHA-C is involved in secretory pathway. Therefore, the overall evidence leads to the logical hypothesis that CER11/CPL2 may function in secretion by affecting VHA-C activity. Certainly, further studies, such as investigation of the ability of CER11/CPL2 to dephosphorylate VHA-C, are still required.

4.2 General discussion and future directions

4.2.1 Engineering oilseed crops with high oil content may be achieved by suppressing seed coat mucilage production

The rapid increasing energy consumption versus the decline of crude oil supplies, and the concern about global climate change by greenhouse gas emission from burning fossil fuels have been driving a lot of interest in research and production of renewable energy, such as biofuels. Plant seed oils are one of the major feedstocks for biofuel production (Karp and Shield, 2008; Youngs and Somerville, 2012), as well as for diverse industrial applications. My research in chapter 2 demonstrates that the deficiency in seed coat mucilage production caused by mutation in *GL2* or *MUM4* genes results in a high seed oil content. There are no obvious negative pleiotropic effects resulting from mutation in these genes apart from the loss of seed coat mucilage. Many species of plants, including some species of Brassicaceae lack seed mucilage, and some cultivars of *Brassica napus* extrude little mucilage when imbibed, an indication that seed mucilage is not critical for agronomic performance. Therefore, my data suggest a promising way to

engineer high oil content in oilseed crops by down-regulating seed coat mucilage production.

Camelina sativa, a close relative of Arabidopsis, has been cultivated as an oilseed crop for decades, mainly in Europe (Zubr, 1997). The genome of *C.sativa* was recently sequenced by scientists from Agriculture and Agri-Food Canada and the National Research Council of Canada through a collaborative effort funded by Genome Prairie. The genome sequence is available online (http://www.camelinadb.ca). In addition, it was shown that *C. sativa* seeds produce a considerable amount of mucilage when hydrated in water, and that the mucilage is mainly composed of pectin RG I as in Arabidopsis (Western, 2012; North et al., 2014). Therefore, it may be possible to increase the oil content of *C. sativa* seeds by manipulating seed coat mucilage biosynthesis. This could be achieved by silencing Camelina *GL2* or *MUM4* homologous genes specifically in the seed coat.

4.2.2 Polar secretion of cuticular wax, seed coat mucilage and columella cell wall

Polar secretion plays an essential role for plant growth and development. In the stem epidermal cells, cuticular wax components are secreted, in a polar manner, and deposited on the outer surface of the stem (Kunst and Samuels, 2009). In addition, in the seed coat epidermal cells, mucilage is secreted in a polar manner to the mucilage pocket, localized at the junction between the radial and the outer tangential primary cell walls (Haughn and Western, 2012; North et al., 2014). Furthermore, the secondary cell wall in the seed coat epidermal cell is deposited progressively beginning at the top of the

cytoplasmic column and ending at the bottom, eventually replacing the cytoplasm with the columella, suggesting that the polar secretion is involved (Haughn and Western, 2012; North et al., 2014). Moreover, the mucilage pocket localization of MUM2 suggests that the secretion of MUM2 protein may be polar as well, although the mechanism of MUM2 secretion is still under investigation (Lee, Gilchrist, Haughn, unpublished results, Figure 3.5 in chapter 3). My data described in chapter 3 demonstrate that CER11/CPL2 is coincidentally involved in all these secretory events, suggesting that CER11/CPL2 may play a general role in polar secretory trafficking. However, it is still not clear what determines polarity in these secretion events. It is possible that the receptors or transporters on the PM, such as ABC transporters for wax export, are localized in a polar manner. However, the distribution of CER5, ABCG11 or LTPG protein does not support this hypothesis (Pighin and Zheng et al., 2004; Bird et al., 2007; DeBono et al., 2009; Figure 3.6 in chapter 3). A recent study suggested that apoplast localization of LTPG, one of the proposed wax carriers, is dynamic and responsive to changes in cell shape and wall curvature during cell growth and differentiation (Ambrose et al., 2013). Whether the dynamic localization of LTPG plays a role in the polar secretion of wax remains to be determined.

When discussing polar secretion, one must recall the typical polar localization of PM associated auxin efflux transporters, PIN-FORMED (PIN) proteins. Auxin plays a fundamental role in embryonic and post-embryonic axis formation. In addition to auxin biosynthesis, polar auxin transport is critical for auxin-regulated plant development (reviewed by Grunewald and Friml, 2010). It has been demonstrated that the polar auxin transport is achieved mainly by the asymmetric localization of PIN proteins in the PM.

However, PIN proteins are initially secreted in a non-polar manner, while the PIN polarity is established by lateral endocytic recycling (Dhonukshe et al., 2008) that involved ARF-GEFs, GNOM and GNL1 proteins (see chapter 1) (Geldner et al., 2003; Teh and Moore, 2007). A recent study demonstrated that a mutation in *GNL1* results in a reduced wax secretion (McFarlane et al., 2014). Therefore, the polar transport of auxin and wax secretion may share a similar mechanism. The transcriptional profiling on the *cer11/cpl2* mutant demonstrated that many auxin responsive genes were down-regulated in this mutant compared with wild type (Ueda et al., 2008). Furthermore, overexpression of ARF5, one of the auxin responsive genes, partially complements the *cer11/cpl2* leaf phenotype (Ueda et al., 2008), suggesting that CER11/CPL2 is involved in the auxin signaling pathway. Thus, I speculate that CER11/CPL2 is also involved in polar secretion of PINs.

4.2.3 Regulation of cuticular wax deposition in response to environmental conditions

Cuticular wax on the surface of plants plays an important role in plant/environment interactions. The wax deposition is highly regulated in response to the different environmental conditions. For example, *CER6* expression is up-regulated under several stresses, such as drought, salt, ABA and also requires light (Hooker et al., 2002). Furthermore, the expression of other *KCS*s as well as *KCR* and *CER10* is induced under drought, salt, dehydration, dark or low-temperature conditions (Joubes et al., 2008).

In chapter 3, I demonstrate that CER11/CPL2 is involved in secretion, and that mutations in CER11/CPL2 lead to reduced wax deposition. Several lines of evidence show a connection between CER11/CPL2 and a response to salt stress. First, a previous study (Ueda et al., 2008) showed that the expression of stress responsive reporter gene *RD29a-LUC* in the *cpl2-2* mutant is hypersensitive to cold, ABA and salt treatments, and sensitivity to salt is most prominent. A germination test for cpl2-2 seeds also indicated that *cpl2-2* is hypersensitive to high concentration of NaCl compared to wild type (Ueda et al., 2008). Therefore, CER11/CPL2 may be specifically involved in the response to high salinity. Whether wax secretion is regulated in response to osmotic stress, such as salinity, via CER11/CPL2 remains to be investigated. Second, CER11/CPL2 can bind to VHA-C, a subunit of V-ATPase. An increase in V-ATPase activity or transcript levels occurs in response to salt tolerance (Janicka-Russak and Klobus, 2007; Batelli et al., 2007; Zhang et al., 2012). V-ATPase has been shown to be directly involved in the salt overly sensitive (SOS) pathway by interacting with one of the components in the pathway, SOS2 (Batelli et al., 2007). Another study on V-ATPase subunit a (VHA-a) indicated that a mutant of TGN-localized VHA-a1, but not the double mutant of vacuole-localized VHAa2 and VHA-a3, is hypersensitive to salt treatment, suggesting that V-ATPase activity in TGN leads to salt tolerance (Krebs et al., 2010). Moreover, two of the previously mentioned studies (Batelli et al., 2007; Krebs et al., 2010) tested the salt sensitivity of det3-1 mutant and demonstrated that det3-1 mutant is hypersensitive to the high concentration of NaCl. Therefore, it is possible that CER11/CPL2 is used to regulate wax secretion in response to salt stress through its regulation of VHA-C.

4.2.4 The role of V-ATPase in endomembrane trafficking

V-ATPase V₁ sector hydrolyzes ATP and promotes rotation of V₂ sector to open a proton channel, driving protons across the membrane. Thus, it plays an important role in generating the proton gradient and maintaining pH homeostasis. (Reviewed by Forgac, 2007; Saroussi and Nelson, 2009; Marshansky and Futai, 2008; Schumacher and Krebs, 2010). Over the past decade, it became apparent that V-ATPase also plays a role in endomembrane trafficking (Marshansky and Futai, 2008; Schumacher and Krebs, 2010). In Arabidopsis, the "a" subunit of V-ATPase in the membrane associated V₂ sector, VHA-a, is encoded by three genes, VHA-a1, VHA-a2 and VHA-a3 (Sze et al., 2002). One of them, VHA-a1, is localized in the TGN, demonstrating that V-ATPase is not only associated with the tonoplast, but is also present elsewhere in the endomembrane system (Dettmer et al., 2006). In support of this hypothesis, use of V-ATPase inhibitor, concanamycin A (ConcA), revealed a crucial function of V-ATPase in secretory and endocytic trafficking (Dettmer et al., 2006; Viotti et al., 2010). Treatment with ConcA leads to deformation of Golgi structure, accumulation of large vesicles, aberrant secretion of secGFP and PM-localized BRI protein, and accumulation of xyloglucans in the ConcA induced aggregates (Dettmer et al., 2006; Viotti et al., 2010). In chapter 3 of my thesis, I describe the function of CER11/CPL2 in the secretory pathway and demonstrate that CER11/CPL2 can bind to VHA-C subunit of V-ATPase. Therefore, it is plausible that the role of CER11/CPL2 in vesicular trafficking is in the regulation of V-ATPase activity. However, to determine how CER11/CPL2 regulates V-ATPase activity via VHA-C requires further investigation.

VHA-C was demonstrated to play a role in the assembly of V-ATPase complex (Schumacher et al., 1999). Furthermore, it has been shown that VHA-C isolated from the tobacco hornworm (*Manduca sexta*) can bind to cytoskeletal actin filaments (Vitavska et al., 2005). In addition, one study on WNK kinases indicates that Arabidopsis WNK8 can phosphorylate VHA-C, as well as some other subunits in the V-ATPase V₁ sector, although the role of the phosphorylation for VHA-C function or V-ATPase activity is still not clear (Hong-Hermesdorf et al., 2006). In my thesis, the binding of VHA-C to the phosphatase CER11/CPL2, and the role of CER11/CPL2 in secretory trafficking suggest that VHA-C may be the substrate of CER11/CPL2. If this is true, it can be proposed that the V-ATPase activity and its role in endomembrane trafficking can be regulated by dynamic changes of the phosphorylation state of VHA-C through the combined influence of WNK8 and CER11/CPL2.

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