Plant cuticles shine: advances in wax biosynthesis and export
Ljerka Kunst and Lacey Samuels

The plant cuticle is an extracellular lipid structure deposited over the aerial surfaces of land plants, which seals the shoot and protects it from biotic and abiotic stresses. It is composed of cutin polymer matrix and waxes, produced and secreted by epidermal cells. The use of forward and reverse genetic approaches in Arabidopsis has led to the identification of enzymes involved in fatty acid elongation and biosynthesis of wax components, as well as transporters required for lipid delivery to the cuticle. However, major questions concerning wax components, as well as transporters required for lipid delivery to the cuticle. These questions remain to be resolved.

VLCFA biosynthesis
VLCFA precursors for cuticular wax production are synthesized in the epidermal cells by the multienzyme fatty acid elongase (FAE) complex found on the endoplasmic reticulum (ER). Using plastid-generated C_{16} or C_{18} fatty acids and malonyl-coenzyme A (CoA) as substrates, FAE performs a reiterative cycle of four reactions catalyzed by a β-ketoacyl-CoA synthase (KCS), a β-ketoacyl-CoA reductase (KCR), a β-hydroxyacyl-CoA dehydratase (HCD), and an enoyl-CoA reductase (ECR), to produce saturated VLCFAs with 24–36 carbon atoms (Figure 1). The KCS is the rate-limiting enzyme of fatty acid elongation and also determines the length of the final acyl-CoA product [3,4*].

There are two types of KCS enzymes: ELOs, which were discovered in fatty acid elongation defective mutants of Saccharomyces cerevisiae [5,6], and FAE1-type KCSs, which were first characterized in an Arabidopsis mutant deficient in fatty acid elongation of storage lipids [7]. In Arabidopsis, four ELO-type KCS sequences have been annotated [8], but their function has not been experimentally established. In addition, Arabidopsis has a large family of 21 FAE1-type KCS sequences [8,9], consistent with cellular requirements for VLCFAs of different chain lengths. An understanding of how the final acyl chain length is specified by the KCS enzyme is starting to emerge. Work by Denic and Weissman [4**] on the ELO-type KCSs in yeast revealed that the determining factor is the depth of the hydrophobic pocket where the VLCFA sits during elongation. On the basis of this finding, the authors suggest that once the length of the growing acyl chain exceeds the depth of the pocket, further elongation is not possible. However, the confirmation of this hypothesis will require ELO protein structural information. Whether the mechanism proposed for the ELO KCSs also reflects how FAE1-type KCS enzymes discriminate between fatty acid substrates of different chain length remains to be determined. The development of a proteoliposome system for the reconstitution of VLCFA synthesis in vitro [4**] will be a valuable tool in addressing this issue.

Another important unanswered question concerns the number of KCS enzymes involved in the elongation of C_{18}–C_{36} acyl groups required for wax biosynthesis. Each...
KCS usually catalyzes a few successive elongation steps and various KCSs may have partially overlapping acyl chain specificities. At present only the ECERIFERUM6 (CER6) FAE1-type KCS has been shown to be wax-specific and essential for the formation of VLCFAs longer than 22 carbons in Arabidopsis stems [10]. Clearly, other KCSs that participate in the elongation of VLCFA wax precursors need to be identified.

Unlike the KCSs that have strict substrate specificities, the other three enzymes making up the FAE complex, the KCR, HCD, and ECR were hypothesized to have broad substrate specificities, and to be capable of generating the complete repertoire of VLCFAs required by different classes of lipids in various plant tissues [3,11]. This hypothesis could not be experimentally tested until recently because the genes encoding these FAE components were not known. Identification of the ECR, KCR, and HCD in yeast [4**,12–14], led to rapid progress in characterization of the plant enzymes involved in VLCFA formation. A single ECR gene named ECERIFERUM10 (CER10) and a single gene encoding HCD called PASTICCINO2 have been found in Arabidopsis [15,16*]. In contrast, there are two KCR orthologs in the Arabidopsis genome. Surprisingly, only one of these genes, KCR1, encodes a functional β-ketoacyl reductase involved in VLCFA biosynthesis [17]. Thus, Arabidopsis differs from maize, in which two closely related KCRs (GL8A and GL8B) contribute to the activity of the ER-bound elongase [18].

Partial loss of KCR1 or PAS2 function is associated with a general reduction of VLCFA content in seed storage triacylglycerols and complex sphingolipids, as well as reduced levels of cuticular waxes. A complete loss of KCR1 or PAS2 activity is embryo lethal indicating that the capacity to synthesize VLCFAs is crucial at early stages of embryo development [16*,17]. It is also interesting to note that while KCR1 and PAS2 are both essential genes in Arabidopsis, the CER10 gene is not, even though it represents the sole ortholog of the yeast TSC13 ECR [15]. This suggests that a structurally unrelated but functionally equivalent form(s) of the ECR must be present in Arabidopsis.

Figure 1

Fatty acid elongase (FAE) complex synthesizes very long chain fatty acids (C20 and longer) by repeated cycles of two-carbon addition. In one cycle, the four enzymes of the FAE complex work sequentially to carry out the condensation of two carbons from malonyl-CoA (carbon donor) to the growing acyl-CoA chain (carbon acceptor), followed by reduction, dehydration, and another reduction reaction, resulting in a fully saturated acyl-CoA.
**Biosynthesis of aliphatic wax constituents**

Despite the great diversity of chemical compositions among plant cuticles from different species, various plant organs and across developmental states, the aliphatic VLCFA derivatives are ubiquitously present wax components. They are generated from VLCFAs by two biosynthetic pathways, the alcohol-forming (acyl reduction) pathway involved in the production of primary alcohols and wax esters, and the alkane-forming (decarbonylation) pathway, which gives rise to aldehydes, alkanes, secondary alcohols, and ketones (Figure 2). Even though most of the major biosynthetic steps leading to aliphatic wax products have been established both genetically and biochemically, the enzymes that catalyze biosynthetic reactions have not been identified until very recently. Using the *Arabidopsis* wax-deficient cer4 mutant, the CER4 gene was cloned and shown to encode a fatty acyl-CoA reductase, which catalyzes the two-step

Figure 2

Wax biosynthesis and export of wax component to the plant cuticle. Long chain fatty acyl groups (C16–C18) are generated by a fatty acid synthase (FAS) in the plastid. They are released by an acyl-ACP thioesterase (FATB), activated to a CoA thioester by a long chain acyl-CoA synthetase (LACS) and exported from the plastid to the ER. In the ER, fatty acids are extended to very long chain fatty acids (VLCFAs) by a fatty acid elongase (FAE) comprised of four enzymes as depicted in Figure 1, a β-ketoacyl-CoA synthase (CER6), a β-ketoacyl-CoA reductase (KCR1), a β-hydroxyacyl-CoA dehydratase (PAS2) and an enoyl-CoA reductase (CER10), and modified to diverse aliphatic wax components. Enzymes of the primary alcohol pathway, fatty acyl-CoA reductase (CER4) and wax synthase (WSD1) generate primary alcohols and wax esters, respectively. Enzymes and steps involved in the conversion of VLCFAs to alkanes have not been identified (indicated by question marks). Formation of secondary alcohols and ketones is catalyzed by a midchain alkane hydroxylase (MAH1). Wax components are mobilized from the ER to the plasma membrane (PM) by an unknown mechanism and exported from the cell by a process that requires ABC transporters. A recently discovered glycosylphosphatidylinositol-anchored lipid transfer protein (LTPG) is also involved in wax export to the cuticle, but its precise function has not been determined.
reduction of VLCFAs to primary alcohols in Arabidopsis shoots [19]. Primary alcohols together with acyl groups are then used as substrates for the biosynthesis of alkyl esters [20], a reaction catalyzed by a wax synthase. A member of the WAX SYNTHASE/DIACYLGlycerol ACYL-TRANSFERASE (WS/DGAT) family, homologous to the WS/DGAT bifunctional protein from Acinetobacter calcoaceticus, was shown to carry out wax ester formation in the Arabidopsis stem [21*].

In contrast to the enzymes of the alcohol-forming pathway, which have now been identified, the enzymes involved in the formation of alkanes and the biosynthetic steps that they catalyze remain enigmatic (Figure 2). A number of different Arabidopsis mutants with reduced alkane levels in their stem wax have been described, for example, cer1, cer2, cer3, cer8, cer19, and cer22 [22*,23*], but the genes identified in these mutants have either not been cloned (CER19, CER22), or if cloned, have not provided clues about the biochemical role of the proteins that they encode (CER1, CER2, CER3). An intriguing new report identifies CER8 as LACS1, a member of the long chain acyl-CoA synthetase (LACS) family that joins free fatty acids to CoA [23*]. The cer8/lacs1 mutation results in reduced accumulation of products of the alkane pathway and a hyperaccumulation of C_{26}–C_{30} free fatty acids in the cuticle. Enzyme activity assays using recombinant LACS1 expressed in E. coli demonstrated a corresponding substrate preference for C_{30} fatty acids. However, why the CoA activation of VLCFA would be required for alkane production remains a mystery. The only currently known enzyme of the alkane-forming pathway is the recently discovered MAH1, a cytochrome P450-dependent midchain hydroxylase, which catalyzes two successive reaction steps, a conversion of alkanes to secondary alcohols and secondary alcohols to ketones [24*].

Enzymes catalyzing terminal steps of both wax biosynthetic pathways, WSD1 and MAH1, have been localized in the ER of the epidermis [21*]. Since all the VLCFA-forming and all the known wax biosynthetic enzymes reside in the ER, it is likely that this is the subcellular compartment where all of the cuticular wax components are generated.

**Cuticular wax export**

In epidermal cells, cuticular lipids must be transported from sites of synthesis on the ER to the plasma membrane (PM), moved through the PM and, finally, translocated across the outer periclinal cell wall to the cuticle where they apparently self-assemble into the cuticle proper.

The intracellular traffic of the VLCFAs and their derivatives is still a matter of speculation. For example, do the cuticular lipids move in vesicles from the ER to the Golgi and on to the PM, or is there direct nonvesicular lipid traffic between the ER and PM? Studies in *S. cerevisiae* demonstrated the nonvesicular movement of lipids between the PM and the ER [25,26] and this could occur at membrane contact sites where the membranes are adjacent but not mixing or fusing (reviewed by [27]).

The subsequent transport step from the PM to the cell exterior appears to be carried out by PM bound transporters (Figure 2), although experimental evidence that the cuticular lipids are the actual transport substrates is still lacking. Forward genetic studies, using the *cer5* mutant [28], identified a PM-localized ATP binding cassette (ABC) transporter, CER5, required for wax export from the stem epidermal cells of Arabidopsis [29]. This ABC transporter of the ABCG subfamily is related to human ABCG transporters that export a variety of lipid substrates (reviewed by [30]) and *Drosophila* eye pigment transporters such as WHITE, BROWN, and SCARLET complex (WBC) ABC transporters [31]. The plant ABC transporter nomenclature has recently been revised and, due to the conserved nature of these transporters, the Human Genome Organization subfamily designations could be adopted [32*]. For example, because the *Arabidopsis* CER5 ABC transporter is a member of the ABCG subfamily, its name was changed to ABCG12.

Functional ABC transporters are made up of two ABC domains and two transmembrane domains (TMD), which may be present on one polypeptide or on two ‘half-transporter’ polypeptides that contain one ABC and one TMD domain each. To function, half-transporters can form homodimers, as is the case for the well-characterized human xenobiotic exporter ABCG2 [30], or heterodimers, such as the *Drosophila* eye pigment transporters WHITE/BROWN and WHITE/SCARLET [33]. ABCG12/CER5 could form a homodimer or a heterodimer with one of the other 27 ABCG half-transporters encoded in the *Arabidopsis* genome. Two approaches have been used to look for other ABCG transporters that could act in cuticular lipid export: ABCG transporters highly expressed in the epidermis during cuticular lipid secretion were characterized using reverse genetics approaches [34*,35], or the expression of all *Arabidopsis* ABCG half-transporters was systematically disrupted using RNA interference (RNAi) [36]. In each case, *abcg11* mutants were identified with reduced wax loads on the stem surface, similar to the *abcg12/cer5* mutant lines. In addition, and in contrast to the *abcg12/cer5* mutants, *abcg11* mutants displayed organ fusions, a phenotype correlated with disorganized cutin components [37], and the most severe *abcg11* alleles were shown to have reduced cutin levels in their cuticles [34*,35,36]. Surprisingly, the *abcg11* mutants were also dwarf and
sterile, suggesting that the ABCG11 transporter has more extensive roles than just cuticular lipid export.

Double abcg11abcg12 mutants had similar wax defects as the abcg1 single mutant lines [34*], indicating that the phenotypes were not additive, suggesting that these gene products may act in the same process or pathway. On the basis of this genetic evidence, and by analogy with the Drosophila eye pigment ABC transporters, a working model has been proposed where heterodimers of ABCG11 and ABCG12 act in wax transport. In addition, ABCG11 could dimerize with other partners to translocate diverse substrates, such as cutin precursors [38].

Once highly hydrophobic wax molecules are extruded from the PM of epidermal cells, they must somehow cross the hydrophilic cell wall. It has long been postulated that cell wall specific carriers, such as lipid transfer proteins (LTPs), may be involved in the delivery of wax components to the cuticle as LTPs are highly expressed in Arabidopsis during cuticle formation [39]. Only recently, the isolation and characterization of an Arabidopsis mutant disrupted in a glycosylphosphatidylinositol-anchored LTP, named LTPG, revealed reduced alkane accumulation at the plant surface [40*,41], confirming that this PM-bound LTP is required for lipid export. While LTPG has lipid binding capacity in vitro [40*], the question remains as to whether this LTP shuttles across the cell wall to deliver lipids to the cuticle.

Conclusions
Cuticular wax deposition on the aerial plant surfaces requires the coordinated regulation of a complex metabolic network of enzymes and transporters. Molecular genetic approaches in Arabidopsis have been successfully employed in perturbing this network at various points to determine the role of individual proteins in wax biosynthesis, establish the ER as the subcellular compartment where wax components are generated and identify proteins involved in wax translocation across the PM to the apoplast. Gaps remain in our understanding of alkane formation, the delivery of wax molecules from the ER to the PM, the mechanism of ABCG-mediated wax export and the identity of the proteins involved in shuttling wax between the PM and the cuticle. Finally, the regulation of cuticular wax deposition in response to developmental and environmental cues remains obscure. There is good evidence that wax biosynthesis is under transcriptional control [22*], yet the transcription factors regulating this process in the epidermis have not been identified. Currently the only known wax regulatory component is the Arabidopsis CER7 ribonuclease [42**], a core subunit of the exosome, which controls stem wax production via the alkane-forming pathway. Identification of the mRNA target of the CER7 ribonuclease, and information on additional components involved, are essential prerequisites for defining the steps involved in this regulatory process.

Conflict of interest statement
The authors are not aware of any bias or conflicts of interest that might be perceived as affecting the objectivity of this review.

Acknowledgements
The authors acknowledge the artwork of Vicki Earle, UBC Media Group (Figures 1 and 2) and thank David Bird and Patricia Lam for their helpful comments on the manuscript. The research in Ljerka Kunst’s and Lacey Samuels’ laboratories is supported by grants from the Natural Sciences and Engineering Research Council of Canada.

References and recommended reading
Papers of particular interest published within the period of review have been highlighted as:
• of special interest
•• of outstanding interest


This report presents the discovery of the β-hydroxyacetyl dehydratase, the final and so far elusive enzyme of the FAE complex involved in VLCFA biosynthesis in yeast. Furthermore, this study describes the reconstitution of the FAE complex in liposomes and reveals how the final length of the fatty acyl-CoA chain is determined by the FAE condensing enzyme.

12. Kohlwein SD, Eder S, Oh C, Martin CE, Gabke K, Bacikova D, Dunn T: Tsc13p is required for fatty acid elongation and


The authors demonstrate that the plant \( \beta \)-hydroxacyl dehydratase of the fatty acid elongase is encoded by the PASTICCINO2 (PAS2) gene. PAS2 resides in the ER and physically interacts with the CER10 enoyl reductase. It is not only an essential enzyme for plant growth and development, but also a limiting activity for VLCFA synthesis because PAS2 over-expression results in increased VLCFA levels.


The authors identify a member of the Arabidopsis WS/DGAT enzyme family as the wax synthase that catalyzes the biosynthesis of wax esters, dimeric cuticular wax components found on the surface of the plant shoot. This enzyme is localized on the ER and uses long chain and very long chain alcohols with C16 fatty acid for wax ester production.


This is a critical and comprehensive look at our present understanding of cuticular wax biosynthesis and transport, and the regulation of these processes during cuticle assembly in Arabidopsis.


This study is noteworthy, not only because it characterizes one of classic *eceriferum* mutants of Arabidopsis (*cer8*), identifying the mutant gene, LACS1, as a member of the long chain acyl-CoA synthetase family, but also because the authors further explore the relationship between LACS1 and LACS2, a cutin-related LACS. This work integrates the mutant phenotypes with biochemical activities and points to the intimate relationship between these cuticular components.


Using a reverse genetic approach, the authors identify ER-associated cytochrome P450 enzyme CYP96A15 as the midchain alkane hydroxylase, which carries out the biosynthesis of secondary alcohol and ketone components of cuticular wax in Arabidopsis inflorescence stems.


Analysis of the *abcg11* mutants deficient in the plasma membrane localized ABCG11 transporter showed that this protein is required for the export of both cutin and wax to the cuticle. This work reveals yet another close link between the two lipidic components of the cuticle.


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This study demonstrates that a GPI-anchored LTP has a role in the export of wax components to the cuticle in *Arabidopsis* stems. Although this evidence ends a long-standing controversy over whether LTPs act as carriers of wax components to the plant surface, the plasma membrane localization of the LTPG leads to many other questions, such as what is the role of the GPI anchor in LTPG function.


The positional cloning of the *CER7* gene identified a ribonuclease, a core subunit of the exosome, as a regulatory protein which controls wax biosynthesis. The proposed target of the *CER7* ribonuclease is an mRNA specifying a transcriptional repressor of the key wax biosynthetic gene *CER3*. In wax producing epidermal cells, the *CER7* ribonuclease is suggested to degrade the target repressor mRNA, thereby boosting *CER3* expression and wax production by the alkane-forming pathway of wax biosynthesis.