Sealing Plant Surfaces: Cuticular Wax Formation by Epidermal Cells

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Abstract

The vital importance of plant surface wax in protecting tissue from environmental stresses is reflected in the huge commitment of epidermal cells to cuticle formation. During cuticle deposition, a massive flux of lipids occurs from the sites of lipid synthesis in the plastid and the endoplasmic reticulum to the plant surface. Recent genetic studies in *Arabidopsis* have improved our understanding of fatty acid elongation and of the subsequent modification of the elongated products into primary alcohols, wax esters, secondary alcohols, and ketones, shedding light on the enzymes involved in these pathways. In contrast, the biosynthesis of alkanes is still poorly understood, as are the mechanisms of wax transport from the site of biosynthesis to the cuticle. Currently, nothing is known about wax trafficking from the endoplasmic reticulum to the plasma membrane, or about translocation through the cell wall to the cuticle. However, a first breakthrough toward an understanding of wax export recently came with the discovery of ATP binding cassette (ABC) transporters that are involved in releasing wax from the plasma membrane into the apoplast. An overview of our present knowledge of wax biosynthesis and transport and the regulation of these processes during cuticle assembly is presented, including the evidence for coordination of cutin polyester and wax production.

Supplemental Videos

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INTRODUCTION

The plant cuticle is a hydrophobic layer coating the epidermis of the primary plant body. Structurally, the cuticle forms a continuous seal over the outer walls of the epidermal pavement, guard, and trichome cells. Cuticle ultrastructure varies widely among plant species, organ types, and their developmental states, ranging from a procuticle on emerging organs to a mature cuticle that is complete only some time after tissue expansion has ceased (reviewed in References 51 and 52). Despite this variability, all cuticles consist of the same two types of highly lipophilic materials. One of them, cutin, is a polymer consisting mainly of ω- and mid-chain hydroxy and epoxy C_{16} and C_{18} fatty acids, as well as glycerol (44, 95, 124). Owing to covalent linkages between its monomers, cutin resists mechanical damage and forms the structural backbone of the cuticle. In contrast, the second component, cuticular wax, is monomeric and can be extracted by organic solvents. Wax components typically constitute 20%–60% of the cuticle mass (44). Cuticular wax is a complex mixture of straight-chain C_{20} to C_{60} aliphatics and may include secondary metabolites such as triterpenoids, phenylpropanoids, and flavonoids (55). Physical separation and careful constituent analysis have demonstrated that the intracuticular wax, interspersed within the cutin polymer, has a distinct chemical composition from the epicuticular wax lying on the outer surface of the cutin polymer (56). Clearly, this segregation of wax components and the diversity of the wax chemical compositions greatly affect the physical properties of plant surfaces, but the exact implications of this compositional variation for the biological functions of the cuticle are only poorly understood.

Cuticular wax serves the essential function of limiting nonstomatal water loss, and is therefore one of the key adaptations in the evolution of land plants (107). Epicuticular wax, because it is exposed at the outermost surface of plant organs, also plays important roles in the interactions of the plant with its environment. The wax surface influences plant-insect interactions, helps to prevent germination of pathogenic microbes, and causes shedding of water droplets and dust particles as well as spores (110). Together with cutin, wax also plays a pivotal role in cell-cell interactions, e.g., mediating pollen-stigma contact and preventing postgenital organ fusions (128).

The functional importance of the cuticle to the whole plant is evidenced by the significant commitment of epidermal cells to cuticle production. For example, over half of the fatty acids made by epidermal cells of the rapidly expanding *Arabidopsis* stem are estimated to be channeled into the cuticular lipids (cutin and wax), more than intracellular membrane and storage lipids combined (127). The huge investment of epidermal cells to cuticle production is reflected in the epidermis transcriptome during stem expansion (127). Epidermal cells exhibit increased expression of genes encoding proteins involved in lipid

Cutin: a fatty acid–based polyester that forms the structural skeleton of the cuticle

Wax: a mixture of highly lipophilic aliphatics surrounding and covering the cutin and sealing the plant surface

metabolism, as well as membrane-associated and extracellular proteins. The expression of wax biosynthetic genes is also upregulated in epidermal cells, including the *CER* genes that were identified in forward genetic screens for the *Arabidopsis* wax-deficient *eceriferum* mutants (75).

This review focuses on the formation of cuticular wax in *Arabidopsis thaliana*, because most of the recent advances in identification and functional characterization of genes involved in wax biosynthesis and transport have been made in this plant species. Evidence from *Arabidopsis* is complemented with important, mostly biochemical information obtained from other species. For more comprehensive reviews of the biochemical literature and the genetic evidence from other species, readers are referred to previous reviews (14, 67, 68, 139). Because a parallel review summarizes our current knowledge of cutin formation (103), this cuticle component is addressed here only where its formation intersects with wax pathways.

WAX BIOSYNTHESIS

Wax components are synthesized in epidermal cells by joining C_2 building blocks of acetyl-coenzyme A (acetyl-CoA) together into straight-chain aliphatics with 24 to 34 carbon atoms. Wax biosynthesis comprises three distinct stages: First, C_{16} and C_{18} fatty acids are synthesized de novo by the plastid. These ubiquitous fatty acids serve as central intermediates for all lipid classes, and this first stage of wax formation is thus shared with other lipid biosynthetic processes. In contrast, the second stage of the biosynthetic pathway, involving the elongation of C_{16} and C_{18} fatty acids in the endoplasmic reticulum (ER) into very-long-chain fatty acids (VLCFAs) with C_{20}–C_{34} chains, is largely dedicated to generating cuticular waxes (and to a degree to sphingolipid production). In the final stages of wax production in the ER, VLCFAs are modified into the major wax products, including alcohols, esters, aldehydes, alkanes, and ketones (**Figure 1**).

Synthesis of Very-Long-Chain Fatty Acid Wax Precursors

Cuticular lipid formation begins with synthesis of C_{16} and C_{18} fatty acids in leucoplasts, the small nonphotosynthetic plastids found in the epidermis. In this process, long carbon chains are assembled, starting with the condensation of an acetyl-CoA with a C_2 moiety from malonyl-acyl carrier protein (ACP), which originates from acetyl-CoA. After the condensation step, a sequence of reactions including reduction of β-ketoacyl-ACP, dehydration of β-hydroxyacyl-ACP, and reduction of *trans*-Δ^2-enoyl-ACP yields an acyl-ACP product two carbons longer than the original acetyl molecule. Similar elongation cycles, now starting with the condensation of malonyl-ACP with an acyl-ACP and completed by reductive removal of the β-keto group, are repeated six to seven times (43, 97). This process is catalyzed by fatty acid synthases (FASs), complexes of four soluble, dissociable enzymes. Two or three different types of FAS complexes are required for the formation of a C_{16} or C_{18} fatty acid, respectively. FAS complexes differ in their condensing enzymes, which have strict acyl chain length specificities: ketoacyl ACP synthase III (KASIII) (C_2 to C_4) (28), KASI (C_4 to C_{16}), and KASII (C_{16} to C_{18}) (118). In contrast, the two reductases and the dehydratase have no particular acyl chain length specificity and are shared by all three plastidial FAS complexes (126).

Further extension of C_{16} and C_{18} fatty acids to VLCFA chains requires their liberation from ACP by an acyl-ACP thioesterase, activation to CoA thioesters by a long-chain acyl-CoA synthetase (LACS), and transfer to the ER. These processes are important for partitioning of C_{16} and C_{18} acyl chains between pathways leading to cuticlar lipid formation and those leading to membrane

***eceriferum* (*cer*):** *Arabidopsis* and barley mutants with altered cuticular wax amounts and/or composition (literally, "not carrying wax")

CoA: coenzyme A

VLCFAs: very-long-chain fatty acids (C_{20}–C_{34})

ACP: acyl carrier protein

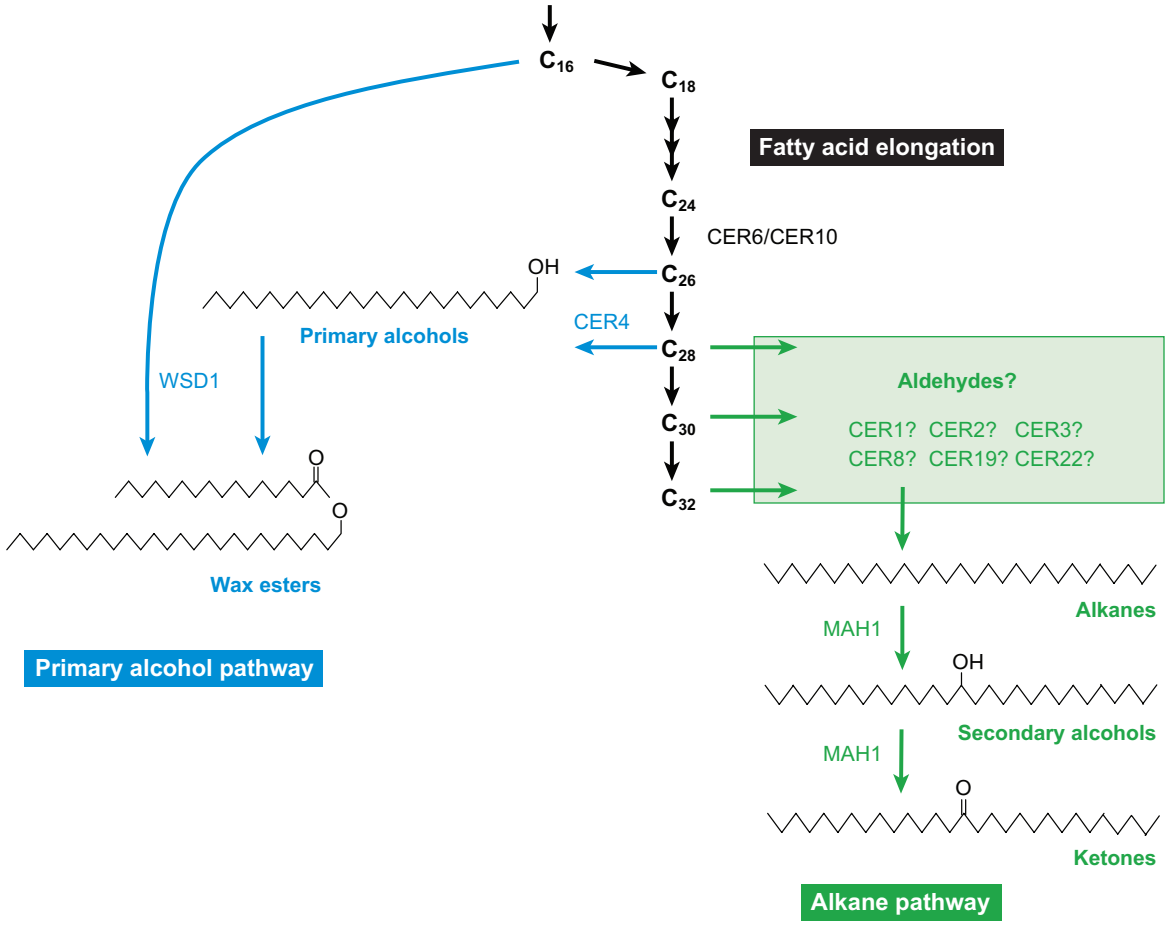

Figure 1

Simplified pathways for wax biosynthesis in *Arabidopsis* stems. CER, ECERIFERUM; WSD, wax synthase/diacylglycerol acyltransferase; MAH, mid-chain alkane hydroxylase.

glycerolipid synthesis within and outside of the plastid of epidermal cells.

Details of fatty acid partitioning into different biosynthetic pathways at this control point are still vague, but substrate flux seems to be determined by activities and specificities of the enzymes involved, including the KASII enzyme (101), acyl-ACP acyltransferases, thioesterases, and the $C_{18:0}$ acid desaturase in the plastid, as well as extraplastidial acyltransferases and fatty acid elongase enzymes in the ER. For example, for C_{16} acyl-ACP, the possible fates are further elongation to C_{18} acyl-ACP by KASII, transfer onto a glycerol backbone in the plastid to form a prokaryotic-type membrane lipid, or cleavage by a fatty acyl–ACP thioesterase B (FATB) with subsequent export of the free fatty acid out of the plastid (43, 136). In *Arabidopsis*, the *fatb* loss-of-function mutant shows a 50% reduction in total wax load in stems and a 20% reduction in leaves (**Table 1**) (20). Based on this mutant wax phenotype it can be speculated that, in an elongating epidermal cell actively synthesizing cuticle, a major portion of C_{16} (and an unknown proportion of C_{18}) acyl-ACPs are channeled through thioesterase cleavage toward wax biosynthesis.

FATA and FATB: class A and class B fatty acyl-ACP thioesterases

Table 1 Summary of genes reported to encode proteins involved in wax biosynthesis and secretion in *Arabidopsis*

	Gene	MIPS Accession number	Cuticle phenotype	Reference
Biosynthesis				
Fatty acyl-ACP thioesterase, B class	*FATB*	At1g08510	Stem wax load 50% of WT. Leaf wax load 80% of WT	(20)
β-keto acyl-CoA synthases (KCS)	*CER6*	At1g68530	Stem wax load 10% of WT. Reduced levels of all wax components; accumulation of C_{24}/C_{26} acyl groups	(35, 92)
Enoyl-CoA reductase (ECR)	*CER10*	At3g55360	Stem wax load 40% of WT. Reduced levels of all wax components	(150)
Fatty acyl-CoA reductase (FAR)	*CER4*	At4g33790	Stem wax load similar to WT. Reduced primary alcohols and wax esters in stem wax. When expressed in yeast, makes C24 and C26 primary alcohols	(113)
Wax synthase/ DGAT (WSD)	*WSD1*	At5g37300	Stem wax load similar to WT. Reduced wax esters in stem wax	F. Li, X. Wu, L. Samuels, R. Jetter & L. Kunst, unpublished.
Midchain alkane hydroxylase (CYP96A15)	*MAH1*	At1g57750	Stem wax load 60–85% of WT. Reduced levels of secondary alcohols and ketones in stem wax	(38)
Secretion				
ABC transporter	*CER5*	At1g51500	Stem wax load 45% of WT. Reduced levels of alkanes in stem wax	(102)
ABC transporter	*WBC11*	At1g17840	Stem wax load 44–60% of WT. Reduced levels of alkanes in stem wax	(17, 87, 98, 133)
Regulation				
Exosome subunit (RRP45)	*CER7*	At3g60500	Stem wax load 30% of WT. Reduced levels of all stem wax components	(49)
Unknown function				
	CER1	At1g02205	Stem wax load 13% of WT. Reduced levels of all stem wax components; C_{30} aldehyde accumulation	(1, 42, 90)
	CER2	At4g24510	Stem wax load 25% of WT. Reduced levels of alkanes, secondary alcohols and ketones; accumulation of C_{26}/C_{28} acyl groups, primary alcohols and wax esters	(54, 79, 96, 142, 143)
	CER3/WAX2/ YRE/FLP1	At5g57800	Stem wax load 20% of WT. Reduced levels of aldehydes, alkanes, secondary alcohols and ketones; higher levels of C_{30} primary alcohols	(5, 25, 54, 77, 112)

The process of fatty acid export from the plastid is not well understood. Fatty acids released from ACP by a thioesterase in the plastid must undergo conversion to acyl-CoAs by LACSs in the outer envelope membrane (4, 57, 116). Based on the kinetics of labeled fatty acid export from the plastid, the fatty acyl movement from the thioesterase to LACS was

PLAMs: plastid-ER associated membranes

FAEs: fatty acid elongases

KCS: β-ketoacyl-CoA synthase

proposed to occur by some type of facilitated diffusion (74), but the mechanism of transfer between the two enzymes has not been established. There are nine *LACS* genes annotated in the *Arabidopsis* genome (119), and one of them, *LACS2*, has been implicated in cuticle biosynthesis (115). A recent analysis of the *lacs2* phenotype demonstrated that the cuticular defect is in the cutin, rather than the wax, of rosette leaves (12). Indeed, the wax levels on the surface of *lacs2* mutants are increased rather than decreased, a common phenomenon in plants with disrupted cutin (12, 78, 115, 121). Thus, the LACS isozyme(s) that would be primarily responsible for CoA esterification of fatty acids en route to wax biosynthesis has yet to be identified.

Once the fatty acids are esterified to CoA, they are translocated to the ER, where additional acyl chain elongation and modification of VLCFAs to diverse aliphatic wax components occur. Recent work has experimentally verified the intimate connection between the plastid and the ER of *Arabidopsis* leaf protoplasts. Physical manipulation of green fluorescent protein (GFP)-labeled ER strands, using laser scalpels and optical tweezers, demonstrated that ER membranes are firmly attached to isolated plastids (3). Sensitivity to proteases and the strength of the attachment is consistent with protein-protein interactions maintaining these plastid-ER associated membranes (PLAMs), which may be major routes for lipid transfer between the two organelles.

The extension of fatty acids from long (C_{16}, C_{18}) to very long chains ($\geq C_{20}$) is carried out by fatty acid elongases (FAEs), multienzyme complexes residing on the ER membrane (76, 145, 150). Analogous to plastidial fatty acid synthesis, VLCFA formation involves four consecutive enzymatic reactions, and results in a two-carbon extension of the acyl chain in each elongation cycle. However, unlike the FAS, which uses malonyl-ACP as a C_2 donor, FAE utilizes C_2 units from malonyl-CoA. Extraplastidial malonyl-CoA is generated by the multifunctional acetyl-CoA carboxylase (ACCase). In *Arabidopsis*, two genes, *ACC1* and *ACC2*, encode the multifunctional ACCases. Even though both genes appear to be ubiquitously expressed, studies of mutants disrupted in the *ACC* genes unequivocally established that ACC1 is involved in supplying malonyl-CoA for VLCFA elongation (8, 9).

Multiple elongation cycles are needed to generate C_{24} to C_{34} acyl chains for the production of aliphatic wax components (**Figure 1**). These elongation cycles are carried out by several distinct elongases with unique substrate chain length specificities (137, 138). The specificity of each elongation reaction lies in the condensing enzyme (β-ketoacyl-CoA synthase, KCS) of the elongase complex (18, 81, 93, 99, 132). Because single condensing enzymes can catalyze a few consecutive elongation steps, and various FAEs may have overlapping ranges of substrate chain lengths, it is not clear how many condensing enzymes participate in the elongation of a C_{18} to C_{34} fatty acid. Consistent with the requirement for multiple FAEs to handle fatty acyl precursors of various chain lengths, a large family of 21 *KCS*-like sequences has been identified in *Arabidopsis* (18, 34, 82). A second gene family of putative condensing enzymes, related to the *Saccharomyces cerevisiae* condensing enzymes *ELONGASE 1* (*ELO1*), *ELO2*, and *ELO3*, has also been annotated (34).

The only wax-specific KCS characterized to date is CER6, involved in elongation of fatty acyl-CoAs longer than C_{22} (35, 50, 92) (**Table 1**). In addition to *CER6*, seven other *KCS* genes were found to be upregulated in *Arabidopsis* stem epidermal cells in a microarray experiment (127), including *FIDDLEHEAD* and *KCS1*, although the functions of these *KCS* genes remain enigmatic (131, 146). Because a major reduction of CER6 activity in *cer6* mutants or in transgenic sense-suppressed plants nearly abolishes stem wax accumulation (35, 92), there is clearly no significant functional overlap of CER6 with other condensing enzymes in *Arabidopsis* stems. Thus, KCSs may have cell-type-specific functions and may not be expressed in

the epidermal pavement cells, but instead may be expressed only in trichomes or guard cells, as shown for the condensing enzyme named HIC (for high carbon dioxide) (37).

The identification of the additional enzymes of the plant FAE has recently become possible due to progress in research on yeast sphingolipid biosynthesis, beginning with the isolation of the β-keto acyl reductase (KCR) (10, 41) and the enoyl-CoA reductase (ECR) (62) genes from *Saccharomyces cerevisiae*.

The wax phenotype of the maize *glossy8* mutant led to the proposal that GLOSSY8 functions as a KCR, catalyzing the reduction of β-keto acyl-CoA during VLCFA synthesis for wax production (144). The sequence homology between maize GLOSSY8 and the yeast KCR, whose biochemical function was demonstrated (10), further supported this proposal. More recently, a closely related gene designated *GL8b* was identified in maize, which shows overlapping expression patterns and function with the original *GL8a* (33). Loss of both GL8a and GL8b activities is embryo lethal, a phenotype attributed to the essential role of VLCFAs in sphingolipid assembly. A BLAST search using the yeast KCR sequence resulted in the identification of two putative homologs in the *Arabidopsis* genome, both of which show high levels of expression in the stem epidermis (127). One of these sequences, At1g67730, rescues the growth phenotype of the yeast *ybr159w* mutant defective in KCR (41). Taken together, this information makes it very likely that the two *Arabidopsis* genes encode KCRs. However, additional molecular and biochemical characterization is needed to establish their biochemical and biological role in cuticular wax formation.

Similarly, the isolation of the *TSC13* gene encoding the yeast ECR (62) allowed the identification of a single-copy *ECR* gene from *Arabidopsis*, responsible for the reduction of enoyl-CoA during FAE. Functional complementation of the yeast *tsc13-1elo2*Δ mutant with the *Arabidopsis* sequence demonstrated that it is indeed a true ECR, and that this protein can physically interact with the yeast Elo2p and Elo3p condensing enzymes (36). The *Arabidopsis ECR* gene is identical to the *CER10* gene (150) (**Table 1**), which is defective in the *cer10* mutant originally identified by Koornneef and colleagues (75). *CER10* is ubiquitously expressed in *Arabidopsis*; analysis of the *cer10* mutants demonstrated that *CER10/ECR* is required for the synthesis of all the VLCFA-containing lipids, including cuticular waxes, seed triacylglycerols (TAGs), and sphingolipids (150). Surprisingly, the ECR-deficient *cer10* mutants still accumulate 40% of wild-type cuticular wax as well as VLCFAs in sphingolipids and seed TAGs, suggesting that another ECR exists in *Arabidopsis*. Alternatively, unknown enzymes functionally similar to the ECR may complement the *cer10* deficiency to maintain critical levels of VLCFA synthesis (150).

The last of the enzymes in the FAE complex, the β-hydroxyacyl-CoA dehydratase, was for many years elusive in both yeast and plants (129). Recent studies using proteoliposome reconstitution of the *Saccharomyces cerevisiae* FAE have demonstrated that PHS1p, an ER-localized transmembrane protein, has dehydratase activity (30). This work is expected to prompt rapid progress in the identification of the dehydratase enzyme in plants.

KCR: β-keto acyl reductase

ECR: enoyl CoA reductase

Synthesis of Primary Alcohols and Wax Esters

One branch of the wax biosynthetic reactions, generally called the acyl reduction pathway, is responsible for the formation of constituents with predominantly even numbers of carbons (**Figure 1**). In diverse plant species and organs, the most important of these compounds are primary alcohols, synthesized with a chain length preference of C_{26} or C_{28} and in some systems C_{30} or C_{32} (7). The alcohols are frequently found in free form and esterified to various acyl groups, including aromatic and short (C_2), long (C_{16}, C_{18}), or very-long-chain aliphatic acids (55). Biosynthesis has long been investigated, and both *Brassica oleracea* and *Arabidopsis* have proven to be suitable model

FAR: fatty acyl-CoA reductase

systems for these investigations, because in both species the cuticles of all above-ground organs accumulate high concentrations of primary alcohols and their esters (6, 42, 54).

Closely matching chain length distributions between alcohols and acids in the waxes of diverse plant species suggest that these compounds are biosynthetically related (14). Consequently, Chibnall & Piper (27) proposed that alcohols are generated by reduction of VLCFA precursors and there is now direct evidence that supports this hypothesis. The reduction of fatty acids to the corresponding alcohols must occur via intermediate aldehydes, and much research has focused on whether both reactions are catalyzed by two independent enzymes, or whether one fatty acyl-CoA reductase (FAR) can catalyze both steps (68).

Biochemical support for the two-step process leading to alcohol formation comes from experiments in *B. oleracea*, where an aldehyde intermediate could be isolated (66). The chemical phenotype of the *gl5* mutant of *Zea mays* was initially thought to provide further support for the two-step reduction hypothesis (15), but was later found to be caused by mutations in two genes, *GL5* and *GL20* (139). Because neither gene has been cloned, it is not clear whether one or both of them code for relevant reductases, and a case cannot be made for or against the two-step hypothesis for wax alcohol biosynthesis in *Z. mays*.

There is also substantial evidence for the direct reduction of acyl CoAs to primary alcohols. Initially, the green alga *Euglena gracilis* was found to generate alcohols from acyl CoA precursors without releasing aldehyde intermediates (65). Subsequent biochemical studies showed that single FARs were also responsible for direct reduction to wax alcohols in jojoba embryos (*Simmondsia chinensis*) (104) and pea leaves (*Pisum sativum*) (135). Functional expression of genes specifying alcohol-forming FARs from jojoba (91), silkmoth (*Bombyx mori*) (94), mouse (*Mus musculus*), and human (*Homo sapiens*) cells (26) in heterologous systems confirmed that the reduction of VLCFAs to alcohols in these species is also carried out by single alcohol-forming FARs.

Based on the biochemical results from experiments performed in various species, it seemed likely that a similar FAR enzyme was also involved in primary alcohol biosynthesis in *Arabidopsis*. Eight FAR-like genes were identified in the *Arabidopsis* genome, one of which is *CER4* (113) (**Table 1**). *cer4* mutants exhibit major decreases in primary alcohols and wax esters, suggesting that *CER4* encodes an alcohol-forming FAR (42, 54). Molecular characterization of *CER4* alleles, genomic complementation, and heterologous expression in yeast confirmed that CER4 is indeed the major FAR responsible for primary alcohol formation in *Arabidopsis* stems (113). The enzyme is capable of carrying out both reduction steps from acyl precursors to primary alcohols, thus showing that the direct reduction process is operating in this species.

The alcohols generated by CER4 likely serve as precursors for ester biosynthesis (**Figure 1**). This hypothesis implies that a pool of primary alcohols is generated in the epidermal cells, which are available either for direct export to the cuticle or for ester formation. Detailed analyses of esterified and free alcohols of various mutants of *Arabidopsis* show a clear correlation of alcohol chain lengths in both types of compounds. This study demonstrated that the alcohols formed by CER4 are indeed incorporated into the wax esters, and that the levels of alcohols limit ester production (79).

Ester biosynthesis in higher plants, mammals, and bacteria is catalyzed by wax synthase (WS) enzymes, which fall into three groups based on sequence information. The first class is exemplified by jojoba WS, which is capable of using a wide array of saturated and unsaturated acyl CoAs, ranging between C_{14} and C_{24}; unsaturated C_{18} alcohol is the prefered second substrate (80). In *Arabidopsis* twelve homologs of the jojoba type wax synthase exist, but none have yet been characterized. In contrast, the second class, which includes the mammalian WS enzymes, has

no known homologs in plants. They have the highest activity with acyl-CoAs between C_{12} and C_{16} in length and efficiently use alcohols shorter than C_{20} (26). Finally, in *Acinetobacter calcoaceticus*, a third class of WS has been identified as a bifunctional WS/DGAT enzyme with a preference for C_{14} and C_{16} acyl-CoA together with C_{14} to C_{18} alcohols (125). Nearly one hundred WS/DGAT homologs from over twenty different microorganisms have been identified so far (140), as well as ten sequences in the *Arabidopsis* genome.

Very recently, one of the WS/DGATs from *Arabidopsis* was characterized and shown to be responsible for the formation of cuticular wax esters in stems of this species (F. Li, X. Wu, L. Samuels, R. Jetter & L. Kunst, unpublished observations) (**Table 1**). This enzyme utilizes mostly C_{16} acyl-CoA precursors, indicating that these very early intermediates of wax biosynthesis must come together with the alcohols formed as much later products of the pathway by acyl elongation and reduction.

Synthesis of Alkanes, Secondary Alcohols, and Ketones

A second branch of the wax biosynthetic pathway is responsible for the formation of compounds with predominantly odd numbers of carbons. Among these, the alkanes are the most prominent because they are ubiquitous in the wax mixtures from various plants and organs, where they frequently accumulate to high concentrations. Secondary alcohols and ketones with similar chain length distributions often accompany alkanes, pointing to a direct biosynthetic relationship between all three classes of compounds (55). Biochemical experiments in the 1960s and 70s addressed the biosynthesis of these compounds and led to a model describing the pathway as a series of reactions leading first from VLCFA precursors to alkanes and then from alkanes to ketones (**Figure 1**). Subsequent experiments confirmed that this pathway indeed occurs in two sets of reactions, with the alkanes as central intermediates or as end products (if the second set is not carried out). Although the second part of the pathway is relatively well characterized, the first part remains poorly understood.

WS/DGAT: dual function wax synthase and diacylglycerol acyl transferase

Compelling evidence exists that the VLCFA precursors described above are utilized for alkane biosynthesis. This conclusion was first drawn from feeding experiments in diverse plant species that showed incorporation of elongation products into various classes of wax compounds, including alkanes, secondary alcohols, and ketones (63, 64, 69, 72). These biochemical results were confirmed by molecular genetic studies in *Arabidopsis*, where two mutants known to be defective in VLCFA elongation, *cer6* and *cer10*, showed drastic reductions of the longer acyl chains together with depleted levels of all the odd-numbered wax constituents (alkanes, secondary alcohols, and ketones) (54, 105). Based on combined biochemical and molecular genetic evidence, it is now well-established that elongation precedes decarbonylation and that, therefore, both the primary alcohol and the alkane pathways compete for acyl-CoA precursors of various chain lengths. However, a comparison between the wax phenotypes of *Arabidopsis* wild-type and *cer6* stems shows that the primary alcohol pathway can utilize C_{26} precursors quite well, whereas the alkane pathway cannot (92).

Although the conversion of VLCFA precursors into alkanes as the first odd-numbered products could formally proceed in one step, both experimental data and theoretical considerations make it very unlikely that a direct decarboxylation reaction occurs. Instead, it is generally accepted that the net decarboxylation of the acyl substrates is brought about by a sequence of transformations. Accordingly, a number of different *Arabidopsis* mutants showing a stem wax phenotype with reduced alkane levels have been described, at least some of them likely due to defects in genes coding for enzymes of the first stage of this pathway (42, 54, 105, 106). Unfortunately, several of these genes have not been cloned (*CER8*, *CER19*, *CER22*), and the biochemical function of the

corresponding proteins cannot be deduced on the basis of changes in wax composition alone. Three other genes have been isolated (*CER1*, *CER2*, *CER3/WAX2/YRE/FLP*) (**Table 1**), but all attempts to characterize their products have failed so far (1, 5, 25, 77, 96, 112, 142, 143). Nevertheless, both deduced protein sequence similarity of selected domains to known enzymes, and double mutant phenotypes, make it very plausible that these genes encode pathway enzymes.

The central reaction of the alkane-forming pathway (the step that makes the transition from even- to odd-numbered carbon chains) is thought to involve the loss of one carbon atom from the acyl precursors, rather than C_1 addition. Early indications for C_1 loss came from wax analyses of diverse species, in which the compound classes with odd-numbered carbon chains have homolog patterns shifted one carbon down from the accompanying even-numbered classes, including fatty acids (27). A similar correlation was found in *Arabidopsis* mutants affected in stem wax biosynthesis, in which a reduction in alkane-derived C_{29} compounds is sometimes accompanied by an accumulation of C_{30} compounds (42, 54, 105). The C_1 loss was further confirmed by biochemical experiments showing that, in vitro, labeled carboxyl carbons are lost in the reaction, whereas in-chain labels are retained. For example, C_{30} and C_{32} acids are converted into C_{29} alkane and C_{31} alkane by *B. oleracea* leaf discs (69) and *P. sativum* epidermal peels (61), respectively, whereas *Allium porrum* leaf microsomes form C_{23} alkane from C_{24} acid (23).

Although the overall C_1 loss is thus well established, the reaction details are not understood. Various hypotheses have been proposed, mostly differing in the central reactions in which the C_1 unit is cleaved off (14, 19, 27), but only one of the hypotheses has been tested to a certain degree. In this model, the central C-C cleavage step is decarbonylation (i.e., the loss of a CO molecule) by an aldehyde intermediate, and therefore alkane formation is frequently described as the decarbonylation pathway (19). Several biochemical experiments have been carried out with the explicit goal of confirming decarbonylation, and evidence supporting this hypothesis has been acquired (22, 24, 31, 32, 114, 135, 141). However, final biochemical proof for decarbonylation is currently missing, because the two enzymes predicted to be involved in the process (an aldehyde-forming acyl-CoA reductase and an aldehyde decarbonylase) have only been partially purified and some of their most important characteristics remain to be determined.

A multistep pathway hypothesis also seems to be supported by a number of *Arabidopsis* mutants that show correlations between alkane and aldehyde levels. Most notably, stem waxes of *cer1* and *cer22* have decreased levels of wax alkanes, partially compensated by increased levels of aldehydes (42, 54, 106). *CER1* and *CER22* were therefore postulated to encode enzymes catalyzing aldehyde decarbonylation. However, the observed correlation between aldehyde and alkane levels could also be explained by aldehydes being a side product of the pathway, with disrupted alkane formation leading to a backup into this side branch. Consequently, on the basis of the phenotype alone it cannot be decided whether CER1 or CER22 is indeed a decarbonylase involved in alkane formation. Even the cloning of the *CER1* gene did not reveal the function of the protein that it encodes (1). In summary, experimental evidence has not fully validated the decarbonylation hypothesis, and alkane formation remains by far the least understood part of wax biosynthesis.

In *Arabidopsis* leaves, alkanes are the final product of this pathway, whereas in the stem wax of this species, as well as in leaf wax of *B. oleracea*, the alkanes are accompanied by corresponding secondary alcohols and ketones (6, 54). In these instances additional reactions are carried out, in which the alkanes are modified by midchain hydroxylation to secondary alcohols and a second oxidation into ketones. Direct evidence for the oxidation reactions was

provided by the incorporation of labeled alkanes and secondary alcohols into ketones, and by inhibitor studies pointing to mixed function oxidases (70, 71, 73).

A reverse genetic approach led to the recent discovery of a cytochrome P450-dependent enzyme (CYP96A15) involved in secondary alcohol and ketone formation in *Arabidopsis* stems (38). The protein is a mid-chain alkane hydroxylase (MAH1) that can catalyze both reaction steps by first hydroxylating alkanes on the central -CH_2- group, and then likely rebinding the resulting secondary alcohol for the second oxidation reaction (**Table 1**). Based on all the available biochemical and molecular genetic information, these two steps of the wax biosynthetic pathway are now well characterized, and confirm the original hypothesis that the pathway proceeds via alkanes as the central intermediates that may or may not be further oxidized depending on plant species and organ type.

TRANSPORT OF CUTICULAR WAX

Intracellular Sites of Wax Synthesis

Enzymes that catalyze the initial steps of wax synthesis, the formation of VLCFA wax precursors, are associated with the ER in all plant species investigated to date. For example, fatty acid elongation activities found in the microsomal fraction of leek (*Allium porrum*) colocalize with ER markers (13, 84). Furthermore, subcellular fractionation in *Zea mays* reveals that the β-ketoacyl reductase resides in the ER (145). Similarly, *Arabidopsis* CER6 condensing enzyme and enoyl-CoA reductase GFP fusion proteins localize to the ER (76, 150).

The ER was recently reported to also be the subcellular compartment in which VLCFAs are further metabolized via the primary alcohol and the alkane pathways. The site of primary alcohol formation was determined by localization of the *Arabidopsis* CER4 FAR, the enzyme that catalyzes the conversion of acyl-CoA to primary alcohols, after expression in yeast (113). Similarly, the mid-chain alkane hydroxylase MAH1, which catalyzes the oxidation of alkanes to secondary alcohols and ketones in *Arabidopsis* stems, was localized to the ER of stem epidermal pavement cells (38). MAH1 is the last enzyme on this wax biosynthetic pathway, and thus its intracellular location implies that both the intermediate metabolites and the final products of the pathway are located in the ER, and must be delivered from this compartment to the plasma membrane for export toward the cuticle.

MAH1: mid-chain alkane hydroxylase, a cytochrome P450-dependent enzyme that forms secondary alcohols and ketones

Delivery of Wax Constituents to the Plasma Membrane

Because conventional sample preparation techniques for electron microscopy extract lipophilic compounds (109), it is difficult to visualize cell structures that may be involved in intracellular wax transport. For this reason, the mechanisms for transport of wax molecules within epidermal cells are currently unknown. On the basis of circumstantial evidence and by analogy with other intracellular lipid transport processes, two hypothetical routes for wax molecules from the ER to the PM have been suggested (76, 117): 1) Golgi-mediated vesicular traffic through the secretory pathway, or 2) direct molecular transfer at ER-PM contact sites (**Figure 2**).

Consistent with the first hypothesis, epidermal cells of sorghum (*Sorghum bicolor*) and rice (*Oryza sativa*) contain dark-staining vesicles (46, 53). However, further cytochemical investigations in deep-water rice showed that these osmiophilic particles tested negative for cutin and candidate proteins for cuticle formation (e.g., lipid transfer proteins) although they did contain other unidentified proteins (47). Thus, the role of these vesicles in the transport of cuticle precursors from the ER to the PM remains unresolved.

In agreement with the second hypothesis that wax components may be transferred from the ER to the PM by a nonvesicular route, membrane contact sites have been

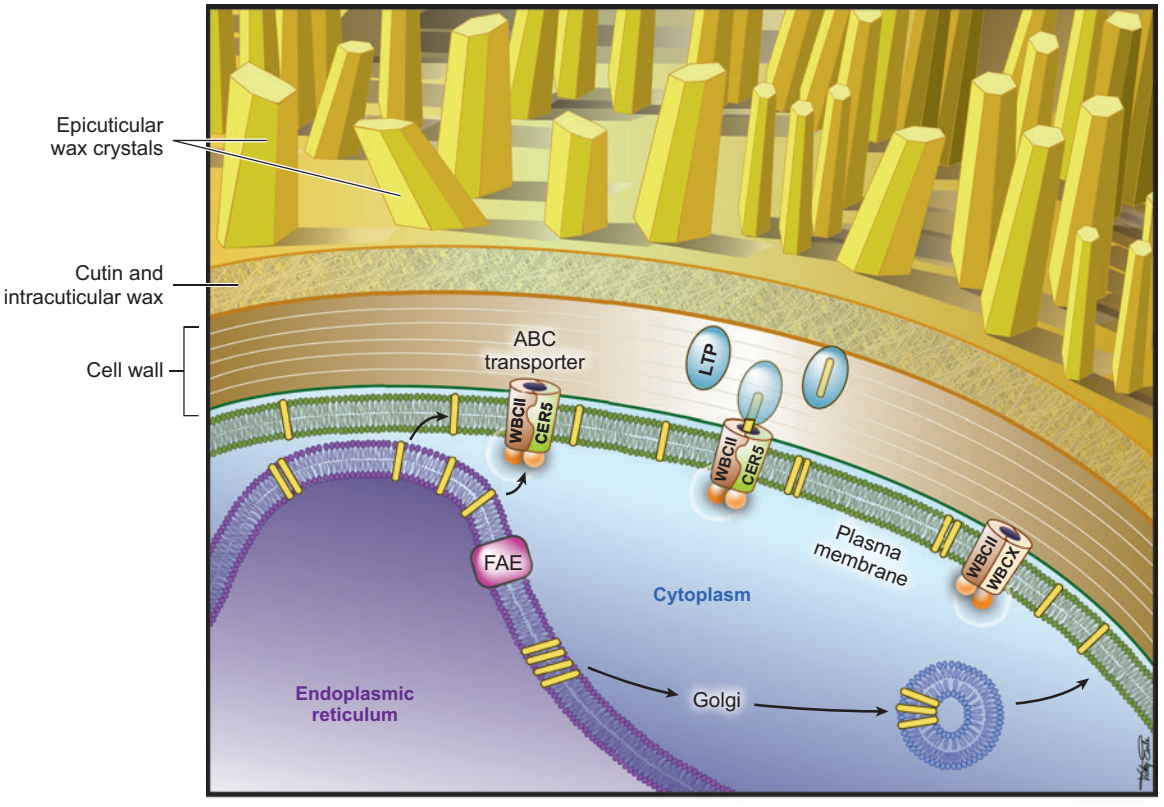

Figure 2

A current model of wax export from an epidermal cell to the cuticle. ABC transporter, ATP binding cassette transporter; CER5, ECERIFERUM 5; FAE, fatty acid elongase; LTP, lipid transfer protein; WBC, WHITE-BROWN COMPLEX.

described in plant cells where cortical ER is in close proximity (within 10 nm) to the PM (122, 123). Similar contact sites in *S. cerevisiae* have been designated as PM-associated membranes (PAMs) (48, 100). PAMs have been defined morphologically by transmission electron microscopy and biochemically by fractionation, which demonstrates the enrichment of some lipid biosynthetic proteins in these domains (100). Cargo selection proteins, such as intracellular lipid transfer proteins, are postulated to be enriched at these sites to facilitate nonvesicular lipid traffic (85). In plants, the monomolecular exchange of lipids at such sites is a suggested mechanism for membrane recycling (123) and/or wax trafficking (76).

Intermediates and products of wax biosynthesis are expected to partition into membrane bilayers within the epidermal cells due to their high hydrophobicity (29, 148). However, nothing is known about the local concentrations and the physical behavior of waxes in the complex lipid mixtures found in biological membranes. High wax concentrations in the epidermal ER could locally disturb the membrane structure (39, 45, 134) and have detrimental effects on housekeeping ER membrane domains. To maintain membrane homeostasis, specialized domains of ER might be dedicated to wax production in the epidermis, similar to oil domains dedicated to storage triglyceride biosynthesis in seeds (120).

Export of Wax from the Plasma Membrane

After wax molecules have been delivered to the PM, they must be released from the lipid bilayer into the apoplastic environment. First progress was recently made toward a more detailed understanding of wax export when two transporters, CER5 and WBC11, required for this process were discovered in *Arabidopsis* stems (17, 102). These ATP binding cassette (ABC) transporters from the ABCG/WHITE-BROWN COMPLEX (WBC) subfamily were shown to be involved in cuticle formation, because both *cer5* and *wbc11* mutants have reduced surface wax loads combined with intracellular wax accumulation (17, 87, 98, 102, 133). Both transporters were localized to the PM of stem epidermal cells using functional transporter–fluorescent protein fusions and confocal microscopy (17, 98, 102, 133).

Although the phenotypes of *cer5* and *wbc11* mutants show that the two ABC transporters are required for wax export from the PM, there is no direct evidence that these proteins actually move wax molecules as substrates. However, given the sequence similarity between CER5 and WBC11 with known lipid transporters of the ABCG subfamily in humans, it seems probable that CER5 and WBC11 are handling wax molecules. Additional questions remain, such as how is substrate presented to the transporter? Can the wax molecules be extracted directly from the ER? The presence of intracellular inclusions in the *cer5* or *wbc11* mutants, rather than PM defects, is consistent with substrate pooling in intracellular membranes such as the ER.

If the CER5 and WBC11 transporters function independently to export wax, then knocking both out should produce a more severe surface wax phenotype than the loss of each protein alone. When *cer5wbc11* double mutants were generated and analyzed, their stems were found to have wax loads and compositions similar to the single mutants, suggesting that the two transporters act in the same pathway or complex (17). ABC transporters of the WBC subfamily are half-transporter proteins requiring dimerization to function (16, 108). An attractive hypothesis is that CER5 and WBC11 form a heterodimer which transports wax across the plasma membrane of the epidermal cells (**Figure 2**). *cer5wbc11* double mutants still accumulate some stem wax; thus, further research is needed to characterize additional protein(s) that are involved in the export of wax from the PM of *Arabidopsis* epidermal cells.

wbc11 mutant lines, but not the *cer5* mutants, also exhibit organ fusions and decreased cutin levels (17, 87, 98), suggesting that WBC11 additionally functions in cutin export. This function might be accomplished by WBC11 homodimers, or by heterodimers formed by WBC11 and members of the WBC subfamily other than CER5. The latter model, in which CER5/WBC11 complexes transport wax components and WBC11/WBCX complexes transport cutin (**Figure 2**), does have a precedent in the eye pigment transporter system of *Drosophila melanogaster*. There, WHITE and BROWN proteins, members of the WBC subfamily, form a heterodimer to transport the red drosopterin pigment precursors, whereas the WHITE/SCARLET heterodimer transports the brown ommochrome pigment precursors (88). Future investigations will show whether another ABC transporter is indeed involved in cutin export in *Arabidopsis*, and how it interacts with WBC11.

Transport of Wax Through the Cell Wall to the Cuticle

Once wax components have been exported from the epidermal cell, they must cross the hydrophilic cell wall to reach the cuticle. Even though lipid transfer proteins (LTPs) have long been attractive candidates for wax transport across the cell wall (58), because they are abundantly expressed in the epidermis, are secreted into the apoplast (130), are small

ABC transporters: ATP binding cassette transporters

WBC: WHITE-BROWN COMPLEX subfamily of ABC transporters, equivalent to the ABCG subfamily

LTPs: lipid transfer proteins

enough to traverse the pores of the cell wall, and contain a hydrophobic pocket that binds long-chain fatty acids in vitro (147), no experimental evidence exists for this hypothesis. This lack of information is partially due to difficulties in verifying roles for LTPs in planta, and partially due to the large number of candidate genes. Of the 72 LTP genes annotated in the *Arabidopsis* genome, only *DIR1* has been characterized, and mutant plants with defects in this type 3 LTP (as defined by Reference 11) are defective in long distance signaling for systemic acquired resistance upon pathogen attack (89). From the remaining 71 LTPs, candidates involved in wax transport can be selected on the basis of their epidermis-specific expression. Recently published microarray data indicate that a number of type 1 and type 5 LTPs are highly expressed in the epidermis (127). Reverse genetic approaches may soon reveal whether these proteins indeed function as cuticular lipid carriers within the epidermal cell wall.

REGULATION OF WAX BIOSYNTHESIS

Cuticular wax formation is known to be tightly regulated in response to both developmental and environmental cues. For example, wax loads on rapidly elongating *Arabidopsis* stems remain remarkably constant, indicating that wax production is closely matched to surface area expansion (127). Furthermore, expression analyses carried out for two *Arabidopsis* genes encoding wax biosynthetic enzymes, *CER2* and *CER6* (50, 143), confirmed that these genes are transcriptionally regulated during development. *CER6* transcription is also induced by light and osmotic stress, environmental factors known to stimulate wax accumulation (50). Somewhat unexpectedly, *CER2* expression is not light, heat, cold, or wound inducible, and is unaffected by osmotic stress (143). Despite solid evidence that wax production is under transcriptional control, transcription factors regulating this process have not yet been identified.

Currently, the only transcription factors known to affect wax biosynthesis are the WAX INDUCER (WIN)/SHINE family in *Arabidopsis* and WAX PRODUCTION 1 (WXP1) in *Medicago truncatula*. Overexpression of these APETALA2 (AP2)/ethylene-responsive element binding protein (EREBP)-type transcription factors dramatically enhances wax accumulation in transgenic plants and results in a strikingly glossy leaf phenotype (2, 21, 149). A detailed examination of *35S:WIN1/SHINE 1* (*SHN1*) transgenic plants demonstrated that WIN1/SHN1 overexpression results in the induction of several wax-related genes, including *CER1*, *CER2*, and *KCS1*, and consequently results in a dramatic increase in leaf alkane levels (2, 21). Extreme leaf glossiness and increased wax deposition on leaves and stems are also detected in transgenic plants overexpressing the *WIN1/SHN1* paralogs *SHN2* and *SHN3* (2). In addition, RNA-blot and microarray analyses showed that other genes predicted to encode lipid biosynthetic enzymes and proteins involved in cellular trafficking, including one unidentified ABC transporter, are upregulated in the *35S:WIN1/SHN1* overexpressors (21).

Surprisingly, an investigation into the mode of action of the WIN1/SHN1 transcription factor revealed that it rapidly activates genes encoding cutin biosynthetic enzymes and that this activation, at least for *LACS2*, is a result of direct binding of WIN1/SHN1 to the target gene promoter. This study also confirmed that WIN1/SHN1 is capable of activating wax biosynthetic genes, but that their induction lags behind genes involved in cutin formation (59). This delayed induction of wax-related genes suggests that the control of wax formation by WIN1/SHN1 may be indirect and may require additional transcription factors acting downstream of WIN1/SHN1. Alternatively, wax biosynthetic genes may be regulated in a similar way as the genes encoding enzymes of the cutin biosynthetic pathway, but are induced more slowly than the cutin genes.

Finally, WIN1/SHN1 may impact wax production indirectly by affecting the composition or amount of cutin produced, thus altering the physical properties of the cuticle. This in turn may, possibly through facilitated transport of signaling molecules, activate wax biosynthesis (59). *Arabidopsis* mutants with altered cutin, including *bodyguard* (78) and *lacs2* (12, 115), as well as transgenic plants expressing a fungal cutinase (121), all accumulate more cuticular wax than wild-type plants.

While the search for the elusive components involved in transcriptional activation of wax biosynthesis continues, an intriguing new regulatory pathway controlling cuticular wax accumulation was recently discovered in *Arabidopsis* (49). The key component of this pathway is the CER7 ribonuclease, a core subunit of the exosome, the evolutionarily conserved complex of 3′-5′ exoribonucleases involved in RNA processing and degradation (**Table 1**). In addition to performing general exosomal functions, as described for other eukaryotes, the CER7 ribonuclease has a unique role in epidermis-specific control of wax biosynthesis. The proposed target of this ribonuclease in epidermal cells is a mRNA encoding a repressor of transcription of the key wax biosynthetic gene *CER3*. In wild-type cells, at the onset of wax biosynthesis, the CER7 ribonuclease degrades the mRNA specifying the repressor, thereby allowing CER3 expression and wax production via the alkane-forming pathway to proceed (49). Further work in this area is needed to identify the mRNA target of the CER7 ribonuclease and define the individual steps involved in this process.

Finally, it is interesting to consider how cuticle biosynthetic pathways are regulated in the epidermis during organ development so that wax deposition is synchronized with cutin formation. The constant amounts and compositions of both wax and cutin along all areas of the elongating inflorescence stem of *Arabidopsis* (127) indicate that synthesis and transport of these two components of the cuticle are closely coordinated. Further proof of cross talk between both pathways is that reduced cutin amounts in *Arabidopsis* mutants and transgenics cause increased wax accumulation. Although this result may be explained by the fact that the two pathways share common fatty acid precursors, it may also point to regulatory interactions between the pathways, to common biosynthetic steps, or to (partially) shared mechanisms for transport of cuticle components. A first example for common steps between wax biosynthesis and a cutin-like pathway was provided by the characterization of glycerol 3-phosphate acyltransferase 5 (GPAT5), an acyltransferase involved in the synthesis of aliphatic suberin and waxes in *Arabidopsis* roots (86). Ectopic expression of *GPAT5* results in accumulation of free VLCFAs and corresponding monoacylglycerols in *Arabidopsis* stem cuticular wax at the expense of standard stem wax components (86), implying that GPAT5 is present in the same subcellular compartment and competes with wax biosynthetic enzymes for the same pool of very-long-chain acyl-CoA substrates.

Exosome: multiprotein complex with RNase activity implicated in control of mRNA levels in eukaryotes

SUMMARY POINTS

1. Primary plant surfaces are covered with a cuticle consisting of cutin and wax, and developing epidermal cells make a strong commitment to cuticle biosynthesis during cell expansion.
2. A central stage in wax biosynthesis is elongation of C_{16} and C_{18} acids to very-long-chain fatty acids ($C_{>20}$), mediated by elongase complexes. There has been exciting progress in characterization of three out of the four components of the complex, namely the β-keto acyl-CoA synthase condensing enzyme (KCS), the β-keto acyl-CoA reductase (KCR), and the enoyl-CoA reductase (ECR).

3. On one branch of the wax biosynthetic pathway, even-numbered primary alcohols are formed by reduction of very-long-chain fatty acyl-CoAs. The fatty acyl-CoA reductase (FAR) responsible for this reaction in *Arabidopsis* has been recently identified, showing that both reduction steps from very-long-chain fatty acids to aldehydes and on to the primary alcohols can be catalyzed by one enzyme.
4. On a second branch of the wax biosynthetic pathway, alkanes are formed and further converted into secondary alcohols and ketones by two consecutive oxidation steps. A reverse genetic approach recently led to the discovery of a cytochrome P450–dependent mid-chain alkane hydroxylase (MAH1) that catalyzes these final steps of the pathway.
5. All the wax biosynthetic enzymes investigated to date are localized to the endoplasmic reticulum. Because some of these enzymes catalyze early steps, and some catalyze final steps in the pathway, the entire process of wax biosynthesis likely occurs in this single intracellular compartment.
6. Export of cuticular lipids from the plasma membrane of epidermal cells has been demonstrated to be carried out by CER5 and WBC11 proteins in *Arabidopsis*, members of the ABCG/WHITE-BROWN COMPLEX (WBC) subfamily of ATP binding cassette (ABC) transporters.
7. Wax accumulation in *Arabidopsis* is, at least in part, controlled by the mRNA stability of a proposed repressor of transcription of the key wax biosynthetic gene *ECERIFERUM 3* (*CER3*). At the onset of wax biosynthesis, the mRNA specifying the repressor is recognized and degraded by the CER7 ribonuclease, a core subunit of the exosome, which results in *CER3* expression and wax production via the alkane-forming pathway.

FUTURE ISSUES

1. Wax biosynthesis requires unique lipid biochemical machinery and unique regulation mechanisms in epidermal cells. What are the proportions and how is the flux of C_{16} and C_{18} saturates leaving the plastid controlled?
2. How many KCS homologs are involved in the various elongation rounds during fatty acid elongation? How do chain length preferences of KCSs overlap? Even more importantly, the fourth enzyme of the plant elongase complex, the β-hydroxyacyl-CoA dehydratase, must be identified.
3. Whether one or two FARs are operating in the alcohol pathway of various plant species remains unclear. Intriguing *Arabidopsis* mutant phenotypes and biochemical data from other systems suggest multiple mechanisms could be at work. Is there more than one reductase involved in wax alcohol formation in *Arabidopsis*?
4. An examination of earlier biochemical data on the early steps in the alkane pathway leaves many unanswered questions. This remains the least understood part of wax biosynthesis, despite an array of *Arabidopsis* mutants impaired in this pathway. These mutants provide excellent tools to test the various possible mechanisms for alkane biosynthesis, including decarbonylation.

5. Many aspects of wax export remain enigmatic. How are the highly hydrophobic aliphatic wax components accommodated in cellular membranes? Is wax transport from the endoplasmic reticulum to the plasma membrane vesicular or nonvesicular? How are cuticular lipids transferred across the cell wall?
6. Identification of the mRNA target of the CER7 ribonuclease is essential for defining the mechanism of CER7-based regulation of wax biosynthesis.

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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