Review

Biosynthesis and secretion of plant cuticular wax

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Abstract

The cuticle covers the aerial portions of land plants. It consists of amorphous intracuticular wax embedded in cutin polymer, and epicuticular wax crystalloids that coat the outer plant surface and impart a whitish appearance. Cuticular wax is mainly composed of long-chain aliphatic compounds derived from very long chain fatty acids. Wax biosynthesis begins with fatty acid synthesis in the plastid. Here we focus on fatty acid elongation (FAE) to very long chains (C24–C34), and the subsequent processing of these elongated products into alkanes, secondary alcohols, ketones, primary alcohols and wax esters. The identity of the gene products involved in these processes is starting to emerge. Other areas of this field remain enigmatic. For example, it is not known how the hydrophobic wax components are moved intracellularly, how they are exported out of the cell, or translocated through the hydrophilic cell wall. Two hypotheses are presented for intracellular wax transport: direct transfer of lipids from the endoplasmic reticulum to the plasma membrane, and Golgi mediated exocytosis. The potential roles of ABC transporters and non-specific lipid transfer proteins in wax export are also discussed. Biochemical-genetic and genomic approaches in Arabidopsis thaliana promise to be particularly useful in identifying and characterizing gene products involved in wax biosynthesis, secretion and function. The current review will, therefore, focus on Arabidopsis as a model for studying these processes.

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Nomenclature

ACP acyl carrier protein
ACBP acyl-CoA binding proteins
CER eceriferum; gene involved in wax production in Arabidopsis
CoA coenzyme A
ER endoplasmic reticulum
FABP fatty acid binding proteins
FAE fatty acid elongase
FAU fatty acyl-CoA reductase
FAS fatty acid synthase
FAT fatty acyl-ACP thioesterase
KAS plastidial condensing enzyme
KCS β-ketoacyl-CoA synthase
LTP lipid transfer protein
NBD-DAG 1-acyl-2-(N-4-nitrobenzo-2-oxa-1,3-diazole)aminoacyldiacylglycerol
NBD-PC 1-acyl-2-(N-4-nitrobenzo-2-oxa-1,3-diazole)aminoacylphosphatidylcholine
PM plasma membrane
SER smooth endoplasmic reticulum
TAG triacylglycerol
TEM transmission electron microscopy
VLCFA very long chain fatty acids (fatty acids ≥ 20 carbons long)
WS wax synthase
X:Y A fatty acyl group containing X carbon atoms and Y double bonds
1. Introduction

Plant wax is the collective term used to describe lipid components of the cuticle which covers the outer surface of aerial plant tissues. In addition, wax is associated with the suberin matrix in underground and wound tissues [1], as well as lipids of the pollen and seed coat [2–6]. In plant cuticles, wax is predominantly comprised of very long chain aliphatic lipids, but it also includes triterpenoids and minor secondary metabolites, such as sterols and flavonoids. The physical and chemical properties of cuticular wax determine functions vital for plant life. It restricts non-stomatal water loss, protects plants against ultraviolet radiation [7,8] and reduces water retention on the surface of the plant thus minimizing deposition of dust, pollen and air pollutants [9,10]. In addition, surface wax is believed to play important roles in plant defense against bacterial and fungal pathogens [11] and has been shown to participate in a variety of plant-insect interactions [12].

The terminology describing plant surface wax has been first defined by the early morphological work (reviewed by Martin and Juniper [13] and Baker [14]) where the entire waxy coating is called the “cuticular membrane”. It consists of (from exterior inwards) epicuticular wax crystalloids or films, the cuticle proper, and a cuticular layer which interweaves with the epidermal cell wall. More recently, a chemically oriented definition of plant surface lipids has predominated, with the cuticle described as a polyester matrix of hydroxy- and hydroxy epoxy fatty acids C16 and C18 long (cutin) embedded and overlayed with cuticular wax. For molecular genetic analysis of wax-related genes, the wax has been defined as the “lipids which are removed from plant surfaces after brief immersion in an organic [nonpolar] solvent” [15]. Phenotypically wax is considered to be the glaucous or whitish bloom on plant shoots, which corresponds to crystalloid epicuticular wax with light scattering capacity [16].

As our goal in this review is to integrate morphological, biochemical and molecular data, our definition of wax includes aspects of each of the above. A working definition of the total plant cuticle includes the cuticle proper, intracuticular wax and epicuticular wax (Fig. 1). The cuticle proper consists of cutin, the insoluble, covalently cross-linked polymer that forms an electron dense layer over the epidermal cells. The intracuticular wax is defined as the amorphous mixture of lipids embedded in the cutin which links the cuticle with the cell wall matrix, and epicuticular wax refers to the surface lipids forming the crystalloids or smooth film exterior to the cuticle. Distinct chemical compositions of epicuticular vs. intracuticular wax have been demonstrated by selective physical removal and analysis of epicuticular wax, followed by analysis of the remaining intracuticular wax [17,18].

The focus of this review is the biosynthesis and secretion of the aliphatic very long-chain components of plant wax by epidermal cells. They include aldehydes, primary and secondary alcohols, alkanes, ketones and esters, which are derived from saturated very-long-chain fatty acids (VLCFAs) with predominant chain lengths from 20 to 34 carbons (Table 1). Other lipids present in plant cuticular wax, such as terpenoids, flavonoids and sterols will not be considered in this review. For information on these wax components, readers are referred to a review by Walton [19]. The biochemistry of β-diketones, and their derivatives, will not be covered either, as this topic has been extensively reviewed by von Wettstein-Knowles [1,20], and Walton [19]. Instead, recent advances in identification and functional characterization of genes involved in wax synthesis, and new developments in the cell biology of wax secretion will be discussed. Molecular-
genetic approaches in *Arabidopsis thaliana*, coupled with thorough biochemical analysis of mutant phenotypes, have contributed key information to this field of research. Therefore, the current review will emphasize the utility and potential of *Arabidopsis* for studies of wax biosynthesis and function.

Fig. 1. Generic representation of transverse views of wax secreting epidermal cells, showing the components of the cuticle, cell wall domains, and the nonphotosynthetic epidermal cell.

Table 1
Major classes of aliphatic cuticular wax components

<table>
<thead>
<tr>
<th>Wax class</th>
<th>Chain length range in plants&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Predominant chain lengths in <em>Arabidopsis</em> stems&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Content in <em>Arabidopsis</em> stems&lt;sup&gt;c&lt;/sup&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>n</em>-Alkanes</td>
<td>C21–C35</td>
<td>C29, C31, C27</td>
<td>38</td>
</tr>
<tr>
<td>Secondary alcohols</td>
<td>C21–C35</td>
<td>C29, C31, C27</td>
<td>10</td>
</tr>
<tr>
<td>Ketones</td>
<td>C21–C35</td>
<td>C29</td>
<td>30</td>
</tr>
<tr>
<td>Fatty alcohols</td>
<td>C22–C34</td>
<td>C28, C30, C26</td>
<td>12</td>
</tr>
<tr>
<td>Fatty acids</td>
<td>C16–C34</td>
<td>C30, C28</td>
<td>3</td>
</tr>
<tr>
<td>Aldehydes</td>
<td>C21–C35</td>
<td>C30, C28</td>
<td>6</td>
</tr>
<tr>
<td>Wax esters</td>
<td>C32–C64</td>
<td>NA</td>
<td>1</td>
</tr>
</tbody>
</table>

NA, data not available.

<sup>a</sup> Adapted from Kolattukudy [23] and Eigenbrode and Espelie [12].

<sup>b</sup> Data are from Millar et al. [38] for the Columbia ecotype and Rashotte et al. [166] for the Landsberg erecta ecotype, listed in order of abundance.

<sup>c</sup> Data are averages from two sources: Hannoufa et al. [28] and Jenks et al. [33] for the Landsberg erecta ecotype.
2. Wax biosynthesis

2.1. Contributions from biochemical and genetic approaches

Wax synthesis requires coordinated activities of a large number of enzymes believed to be organized into multienzyme complexes that carry out the elongation of fatty acid wax precursors and catalyze the formation of a multitude of aliphatic compounds. The reactions of wax production are localized in the epidermal cells [21]. The elucidation of wax biosynthetic pathways in a number of plant species has been possible due to the development of complex techniques, such as gas chromatography/mass spectrometry, that allowed the identification of wax components (reviewed by Walton [19]), and elegant genetic and biochemical studies using radiolabeled tracers [1,22,23] (see Table 2 for reviews). Our knowledge of the enzymes involved in wax biosynthesis, however, remains extremely limited for a number of reasons. Wax biosynthetic enzymes are associated with membranes, and conventional biochemical approaches involving solubilization and purification are not well-suited for membrane-bound enzymes. Furthermore, sufficient amounts of epidermal tissue for enzyme purification are difficult to obtain, and substrates for enzyme activity assays are insoluble in aqueous buffers and not commercially available.

Similarly, genetic approaches alone were initially not successful in revealing the identity of loci involved in wax production. Wax-deficient mutants have been isolated in a number of plant species, including barley (Hordeum vulgare), Arabidopsis, maize (Zea mays), and Brassica napus. The mutant loci in barley and Arabidopsis are termed eceriferum (cer), whereas loci identified in maize and B. napus are referred to as glossy. Barley is the most extensively studied species, with 85 identified cer loci [24]. In Arabidopsis, there are currently 22 known cer mutants displaying varying degrees of waxlessness: cer1-cer10 are very glossy and cer11-cer22 are less so [16,25]. Scanning electron microscopy has shown that the epicuticular wax crystals on many of these mutants are absent or exhibit particular morphological changes (Fig. 2). Chemical analyses demonstrated that mutants have a lower wax load than wild-type plants and/or contain wax with a dramatically altered chemical composition. Most of the mutations, however, do not cause an accumulation of metabolic intermediates due to a specific block in the pathway. Instead, there seems to be an increased flux of precursors into an alternative branch pathway, resulting in more complex changes in wax composition [15,22,26]. Thus, biochemical functions of the mutated gene products are difficult to predict from the alterations in wax compositions in the mutants.

Complex changes in wax composition in the majority of wax-deficient mutants may be a consequence of the visual screens employed to identify lines with reduced wax loads. Only mutants with significant decreases in epicuticular crystalloid accumulation would be isolated in such a screen, most likely those with lesions in regulatory genes, or genes affecting epidermal development. Mutants affecting individual biosynthetic enzymes would be difficult to find due to their more subtle biochemical phenotypes. Nevertheless, attempts to assign tentative functions to genetic loci that affect wax accumulation based on their chemical phenotype have been made for almost every species in which wax-deficient mutants have been identified. Biochemical phenotypes and suggested functions of the gene products of a small subset of Arabidopsis cer mutants are shown in Table 3. Subsequent cloning of genes identified by mutations in these cer lines, and sequence comparisons with sequences in the databases in most cases did not reveal significant
similarity to any gene with a known function. Alternatively, it resulted in different functional assignments altogether of the CER gene products, as described below.

Reduced levels of alkanes, secondary alcohols and ketones and high aldehyde levels on the surface of cer1 mutants were interpreted as being caused by a mutation in the gene encoding an aldehyde decarbonylase [25], a key wax biosynthetic enzyme catalyzing the conversion of aldehydes to alkanes (Fig. 3). This prediction has not been confirmed after cloning of the CER1 gene [27]. Moreover, CER1 has sequence similarity with EPI 23, a Kleinia odora epidermis-specific protein [23] and GLOSSY1 (GL1) protein of maize (Zea mays) [28]. As these proteins contain five to seven predicted transmembrane domains, it was suggested that they may be membrane transporters involved in wax secretion [29].

The cer2 wax composition suggested a block in the elongation of C26 fatty acids [28]. When the CER2 gene was isolated [30,31], the deduced amino acid sequence had no homology to any previously known gene product, providing no clues of its biochemical function. A more recent search of the nucleic acid and protein databases, however, revealed that CER2 may be a member of a large family of coenzyme A-dependent acyltransferases [32], which operate by a catalytic mechanism related to chloramphenicol O-acyltransferase (CAT). This prediction is based on two highly conserved consensus motifs, one of which is found in the catalytic site of CAT. If CER2 indeed encodes an acyltransferase, the specific role for such an enzyme in wax biosynthesis would have to be determined.

Attempts to deduce the biochemical function of CER3 were equally challenging. The surface wax of the cer3 mutant is characterized by a pronounced increase in the chain length of primary alcohols and alkanes, which suggested a defect in the release of fatty acids from fatty acid elongation complexes [28,23]. The isolation of the CER3 gene showed that while its deduced amino acid sequence had no similarities with proteins of known function, it contained a putative nuclear localization sequence (NLS) [34]. In addition, it had two predicted phosphorylation sites, known

<table>
<thead>
<tr>
<th>Authors, reference</th>
<th>Subjects</th>
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<tbody>
<tr>
<td>W. Barthlott et al. (1998) [167]</td>
<td>Classification and terminology of epicuticular wax crystalloid morphology with respect to taxonomy.</td>
</tr>
</tbody>
</table>
to be involved in transport of proteins to the nucleus [35]. The presence of the NLS, together with evidence that CER3 is expressed in all tissues examined, including the roots, led to a proposal that CER3 may encode a novel regulatory protein. Recent database searches, using the predicted amino acid sequence of CER3, identified significant matches with E3-ubiquitin ligases involved in the N-end rule pathway from a wide range of organisms: Schizosaccharomyces pombe, Homo sapiens, Mus musculus, Saccharomyces cerevisiae, Kluyveromyces lactis and Caenorhabditis elegans.

Fig. 2. Scanning electron micrographs of the surface of Arabidopsis thaliana stems. Epicuticular wax crystalloids project from the surface, abundantly in wildtype (Landsberg erecta ecotype), and are either altered or sparse in mutants cer1–cer6 (bars, 10 μm).
<table>
<thead>
<tr>
<th>Eceriferum mutant (additional alleles)(^a)</th>
<th>Sequence/gene similarity</th>
<th>Described chemical phenotype of stem wax(^b)</th>
<th>% Of wildtype stem wax load</th>
<th>Leaf wax</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>–</td>
<td>High C29 alkane, C29 2° alcohol and ketone levels</td>
<td>100</td>
<td>Normal leaf wax</td>
</tr>
<tr>
<td>cer1 (BRL3, BRL11)</td>
<td>72 kDa protein with 3 His-rich motifs; sequence similarity to maize GL1; putative decarbonylase/membrane receptor.</td>
<td>C30 aldehyde accumulation; Reduced levels of both decarbonylation and acyl reduction pathway products</td>
<td>13</td>
<td>Normal leaf wax</td>
</tr>
<tr>
<td>cer2 (BRL5, BRL7, BRL9)</td>
<td>47 kDa protein; sequence similarity to GL2 and acetyl CoA-deacetylvinodline 4–0-acetyl transferase; putative acyl-CoA transferase.</td>
<td>Accumulation of C26/28 acyl groups, 1° OH and wax esters; Reduced levels of decarbonylation pathway products</td>
<td>25</td>
<td>Normal leaf wax</td>
</tr>
<tr>
<td>cer3 (BRL1, BRL6)</td>
<td>90 kDa protein; nuclear localization signal; putative E3-ubiquitin ligase.</td>
<td>Accumulation of C30 1 OH; Reduced levels of C29 alkane, 2° alcohol and ketone</td>
<td>31</td>
<td>Decreased leaf wax</td>
</tr>
<tr>
<td>cer4 (BRL2, BRL10)</td>
<td>Not cloned; putative alcohol-forming acyl-CoA reductase</td>
<td>Reduced levels of 1° OH and wax esters; aldehyde accumulation; No change in decarbonylation product levels</td>
<td>69</td>
<td>Normal leaf wax</td>
</tr>
<tr>
<td>cer6 (BRL4)</td>
<td>56 kDa protein; Putative VLCFA KCS involved in elongation of wax precursors</td>
<td>Accumulation of C24-C26 products; Reduced levels of both decarbonylation and acyl reduction pathway products</td>
<td>19</td>
<td>Decreased leaf wax</td>
</tr>
</tbody>
</table>

\(^a\) Koorneef line in Landsberg erecta ecotype; additional alleles-Lemieux lines with T-DNA insertions in WS ecotype.

\(^b\) Based on Hannoufa et al. [28], Jenks et al. [33], Rashotte et al. [166].
E3-ubiquitin ligases mediate the critical step of substrate recognition that results in substrate ubiquitination and its targeting for degradation by proteasomes [37].

The only case in which the alteration of wax composition gave a relatively accurate prediction of biochemical function is the cer6 mutant, where accumulation of C24 fatty acid suggested a mutation affecting microsomal fatty acid elongation [33]. Similarly, in the CER6-suppressed plants, the levels of C24 acyl groups increased by more than seven fold over wild type levels, suggesting that the product of the CER6 gene (originally called CUT1) is involved in the elongation of acyl chains longer than 24 carbons [38]. Cloning of the CER6 gene [38,39] demonstrated that CER6 encodes an elongase condensing enzyme required for the synthesis of very long chain fatty acid precursors for stem and pollen wax production (see Section 2.2.2).

Fig. 3. Proposed metabolic pathways for wax biosynthesis in Arabidopsis stems. VLCFAs in the epidermal cells are converted through two biosynthetic pathways to all of the aliphatic wax components. The decarbonylation pathway produces aldehydes, alkanes, secondary (2°) alcohols, and ketones, whereas the acyl reduction pathway yields primary (1°) alcohols and esters. Widths of the arrows indicate relative metabolic fluxes through different reactions. Reproduced, with permission, from Ref. [38].
The functions of *Arabidopsis* CER genes, which were first identified on the basis of their waxless phenotypes by M. Koornneef and colleagues over a decade ago [16], can now be reassessed using the large amounts of sequence information deposited in expressed sequence tag (EST) and genomic sequence databases. Access to this wealth of information, together with greatly expanded range of methods to isolate and identify new plant genes, has created new opportunities to examine genetic mechanisms that control wax production. The following section will summarize progress in characterization of genes and gene products involved in fatty acid elongation and wax biosynthesis.

2.2. Biosynthesis of very long chain fatty acids

2.2.1. Pathways for elongation of the fatty acyl chains

Aliphatic components of cuticular wax are synthesized in the epidermal cells from saturated very long chain fatty acids (VLCFA) (Fig. 3). Formation of VLCFA wax precursors is a complex process that is accomplished in two stages in different cellular compartments. The first stage, the de novo fatty acid synthesis of C16 and C18 acyl chains, occurs in the stroma of plastids by soluble enzymes forming the fatty acid synthase complex (FAS) [40,41]. During synthesis, the growing acyl chain is attached to acyl carrier protein (ACP), an essential protein cofactor usually considered a component of FAS. Fatty acid synthesis proceeds through a series of four reactions: condensation of a C2 moiety originating from malonyl-ACP to acetyl-CoA, followed by the reduction of \( \beta \)-ketoacyl-ACP, the dehydration of \( \beta \)-hydroxyacyl-ACP, and the reduction of trans- \( \Delta^2 \)-enoyl-ACP. These reactions result in a fully reduced acyl chain extended by two carbons. Three different types of FAS complexes are required for the synthesis of 18-carbon fatty acids in the plastid. They differ in their condensing enzymes which have strict acyl chain length specificities: KASIII (C2–C4) [42], KASI (C4–C16) and KASII (C16–C18) [43]. In contrast, the two reductases and the dehydratase apparently have no particular acyl chain length specificity and are shared by all three plastidial elongation complexes [44].

The second stage of fatty acid elongation, the extension of the ubiquitous C16 and C18 fatty acids to VLCFA chains that are used for the production of aliphatic wax components, is catalyzed by extra-plastidial membrane-associated multienzyme complexes, known as fatty acid elongases (FAE) [45], (see Sections 2.2.2 and 3.2.2). Similar to the de novo fatty acid synthesis in the plastid, a series of four consecutive enzymatic reactions results in a two carbon extension of the chain: condensation between a CoA-esterified fatty acyl substrate and malonyl-CoA, followed by a \( \beta \)-keto reduction, dehydration and a final enoyl reduction [46]. Since chain lengths of aliphatic wax components are typically in the range of 20–34 carbons, multiple rounds of elongation are needed to extend the acyl chain to the desired length. The differential effects of inhibitors on incorporation of radiolabeled precursors into wax components of various chain lengths, and analyses of mutants with defects in fatty acid elongation, demonstrated that sequential acyl chain extensions are carried out by several distinct elongases with unique substrate chain length specificities [20]. Specificity of each elongation reaction has been attributed to the selectivity of the elongase condensing enzyme [47] (see Section 2.2.3). Consistent with the requirement for fatty acyl precursors of various chain lengths for the synthesis of cuticular wax lipids, a large family of 21 elongase condensing enzyme-like sequences has been identified in the *Arabidopsis* genome. It is not known how many of these putative condensing enzymes participate in wax production and
how many different condensing enzymes are needed for the elongation of a C18 to C34 fatty acid. Since elongase condensing enzymes can catalyze multiple elongation steps [48], and because wax biosynthesis takes place in several different plant tissues at different stages of plant development, a detailed analysis of the substrate specificity and expression patterns of individual condensing enzymes will be necessary to determine their exact metabolic roles.

2.2.2. Enzymes of the extraplastidial FAE and their functional organization

The membrane-bound nature of the fatty acid elongation enzymes has hindered both their biochemical characterization, and studies into their functional organization and subcellular localization by cellular fractionation. Partial purification of elongases from several plants [49–51], resulted in each case in an enrichment of several protein bands. This observation suggested that elongases do not consist of a single high molecular weight multifunctional polypeptide as proposed by Agrawal and Stumpf [52], but rather of several individual protein components.

Extensive mutant screens for changes in very long chain fatty acid composition in Arabidopsis seeds resulted in isolation of a single class of mutants with a lesion in the gene designated FATTY ACID ELONGATION1 (FAE1) [48,53,54]. A mutation in FAE1 affected only seed VLCFA content, suggesting that FAE1 was specifically involved in VLCFA elongation reactions in the seed [48]. Cloning [55] and subsequent characterization of the FAE1 gene [47] indicated that it, indeed, encoded only a condensing enzyme and not all four activities of the elongase. Ectopic expression of FAE1 in Arabidopsis and tobacco, and expression in a heterologous yeast system, demonstrated that the condensation reaction is both the substrate-specific step and the rate-limiting step of FAE [47]. In addition, these experiments revealed that the introduction of FAE1 alone in these systems is sufficient for the production of VLCFAs, suggesting that the other three components of the elongase (two reductases and a dehydrase) are found ubiquitously in all cells [47].

The isolation of this first gene encoding an elongase condensing enzyme (β-keto acyl-CoA synthase, abbreviated KCS) led to a rapid identification of additional sequences with a high degree of sequence similarity to FAE1 in the Arabidopsis EST database. To date, three condensing enzymes: KCS1 [56], FIDDLEHEAD (FDH [57,58]) and CER6 [38,39] have been implicated in the synthesis of VLCFA precursors for wax production in shoots. Interestingly, mutations at the KCS1 and FDH loci did not result in visible surface glossiness on any organ, indicating that surface wax load was not severely reduced. In contrast a major reduction of CER6 activity in cer6 mutants and sense-suppressed CER6 plants nearly abolished stem wax accumulation, and resulted in conditional male sterility [38]. The fact that KCS1 and FDH cannot “rescue” the severe waxless phenotype and male sterility of cer6 mutants and CER6-suppressed plants indicates that these condensing enzymes do not functionally overlap with CER6 in the stem and anther of Arabidopsis. Furthermore, a recently identified VLCFA condensing enzyme CER60, with high amino acid sequence identity to CER6 does not appear to significantly contribute to the synthesis of stem and pollen surface lipids [39]. This may be due to a very low level of CER60 expression in mature Arabidopsis tissues [59]. Taken together, these data suggest that CER6 is the major condensing enzyme for stem wax and pollen coat lipid biosynthesis. Accordingly, CER6 is highly transcribed throughout development, exclusively in the epidermal cells of the Arabidopsis shoot [59]. The only exception to the epidermal expression was observed in the anthers nearing maturity, in which CER6 transcript was localized in the tapetum.
Until very recently virtually nothing was known about the two reductases and the dehydratase activities of the fatty acid elongase. Mutant screens in *Arabidopsis* were not successful in finding mutants defective in these three enzyme activities, implying that these enzymes are either essential or functionally redundant [47]. Although the dehydratase remains uncharacterized, our understanding of the reductases has benefited from recent work on the VLCFA biosynthetic pathway in yeast and animal cells. In *Saccharomyces cerevisiae*, the β-keto acyl reductase and enoyl reductase genes involved in sphingolipid fatty acid elongation have been identified and characterized, providing sequences for searching the *Arabidopsis* genome, and a heterologous mutant system for testing candidate *Arabidopsis* genes.

A putative β-ketoacyl reductase of the FAE in *S. cerevisiae* was identified by searching the genome for oxidoreductases or sequences containing a diagnostic NADH binding motif, predicted transmembrane topology and a dilysine ER-retention motif. Elongation activity assays of mutants with deletions in identified genes demonstrated that the yeast gene YBR159w is required for the activity of the microsomal elongase, and that it encodes a β-ketoacyl reductase [60]. Despite a pronounced reduction in elongation activity, Ybr159 loss-of-function mutants are viable. This is surprising, because there are no functionally redundant sequences found in the yeast genome. By contrast, two sequences homologous to YBR159w are present in *Arabidopsis*. One of these sequences designated *At-YBR159* (MIPS Accession number Table 4) functionally complements the *ybr159/C1* mutant [60]. A second related sequence (MIPS Accession number Table 4) which is 44% identical, 68% similar to the first polypeptide, detected in a BLAST query of the *Arabidopsis* genome database, has not yet been characterized. A truncated *Arabidopsis* cDNA corresponding to the *At-YBR159* gene has been previously identified by Xu et al. [61] as an ortholog of the maize *GLOSSY8 (GL8)* gene. In maize, a mutation at the *GL8* locus results in decreased levels of wax ester components longer than C24 [24,62,63,64]. The *GL8* gene was isolated by transposon tagging and suggested to encode a reductase involved in fatty acid elongation [61]. Based on evidence presented by Beaudoin et al. [60], it is clear that the maize GL8 functions as a β-ketoacyl-reductase of the FAE involved in wax production.

The availability of these genes should allow us to determine the structures of the β-ketoacyl reductase enzymes, the specific elongases they are associated with, and their site of action. Furthermore, experimental data of Beaudoin et al. [60] suggest that overexpression of YBR159w and *At-YBR159* results in moderate increases in the overall elongation activity. It will be interesting to determine if this is also the case in *Arabidopsis*, or whether the condensing enzyme is the only rate-limiting step of fatty acid elongation.

The *TSC13* gene encoding the yeast enoyl reductase was isolated from a temperature sensitive mutant defective in sphingolipid biosynthesis [65]. *TSC13* is essential for viability, and as there are no *TSC13* homologous sequences in the *S. cerevisiae* genome, it is likely that Tsc13p catalyzes a step in the fatty acid elongation cycle for acyl-CoA substrates of all chain lengths. Co-immunoprecipitation of the *TSC13* gene product with the elongase condensing enzymes Elo2p and Elo3p demonstrated that Tsc13p physically interacts with the other components of the elongation machinery, supporting the hypothesis that FAE enzymes function as a large multimeric protein complex in yeast. Tsc13p was localized to the endoplasmic reticulum, specifically at the nuclear-vacuolar junction [65]. A similarity search using the deduced amino acid sequence encoded by *TSC13* identified a single sequence in the *Arabidopsis* genome with significant homology over its entire length (30% identity, 46% similarity). Functional complementation of the yeast mutant
### Table 4
Summary of database accession numbers for genes encoding wax biosynthetic enzymes

<table>
<thead>
<tr>
<th>Known genes</th>
<th>GenBank accession number</th>
<th>MIPS accession number</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fatty acid elongation</strong></td>
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<tr>
<td><strong>β-keto acyl-CoA synthases (KCS)</strong></td>
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<td>KCS1</td>
<td>AF053345</td>
<td>At1g01120</td>
<td>[56]</td>
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<tr>
<td>FIDDLEHEAD</td>
<td>AF337910</td>
<td>At2g26250</td>
<td>[57,58]</td>
</tr>
<tr>
<td>CER6 = CUT1</td>
<td>AF129511</td>
<td>At1g68530</td>
<td>[38,39,59]</td>
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with the *Arabidopsis* sequence and reverse genetic approaches in *Arabidopsis* should establish its role in fatty acid elongation.

2.3. Pathways for cuticular wax biosynthesis

VLCFA products of the elongases generated in the epidermis are used for the synthesis of other wax components. In most plants, including *Arabidopsis*, there are two principal wax biosynthetic pathways: an acyl reduction pathway, which gives rise to primary alcohols and wax esters, and a decarbonylation pathway, leading to the formation of aldehydes, alkanes, secondary alcohols and ketones (Fig. 3). Comprehensive reviews describing the pathways of wax biosynthesis, including the less widespread β-diketone pathway, are available [1,66] (see Table 2—summary of wax reviews). Therefore, we will limit our discussion to the recent results which shed new light on our understanding of the wax biosynthetic process, and point out where additional research is needed.

2.3.1. The acyl reduction pathway

The fatty acyl reduction of VLCFacyl-CoA esters to primary alcohols has been examined in a variety of plants [67–70]. Partial purification of reducing activities from *B. oleracea* initially led to a proposal that primary alcohol production is a two-step process carried out by two separate enzymes, an NADH-dependent acyl-CoA reductase required for a reduction of VLCFAs to aldehydes, and an NADPH-dependent aldehyde reductase required for a further reduction of aldehydes to primary alcohols [68]. Subsequent work of Vioque and Kolattukudy [69] demonstrated that, at least in pea (*Pisum sativum*), this is not the case, and that alcohol formation from VLCFAs is carried out by a single fatty acyl-CoA reductase (FAR). Furthermore, even though alcohol biosynthesis likely proceeds via an aldehyde intermediate, the free aldehyde is not released. Solubilization and biochemical characterization of a fatty acyl-CoA reductase involved in wax ester formation in developing embryos of jojoba (*Simmondsia chinensis*), further substantiated this conclusion [70]. Cloning of the cDNA encoding the jojoba enzyme and expression in *E. coli* and *B. napus* verified its biochemical role and resulted in the accumulation of fatty alcohols. The pea and the jojoba alcohol-forming fatty acyl reductases are both integral membrane proteins, which appear to be associated with the ER [70] and have similar molecular masses in the range of 56–58 kDa. FAR-related sequences from corn (*Z. mays*), rice (*Oryza sativa*), cotton (*Gossypium hirsutum*) and *B. napus* found in the public databases, and a family of eight FAR-like proteins in *Arabidopsis* (MIPS accession numbers Table 4) suggest that alcohol-generating reductases are ubiquitous in plants. One of these FAR-related *Arabidopsis* genes, *MS2* (MIPS accession number Table 4), encodes a tapetum-specific protein essential for pollen fertility [71,72].

A unique type of plastids found in tapetal cells called elaioplasts is known to accumulate neutral lipids, such as TAGs, wax and sterol esters [73,74]. After lysis of the tapetal cells these wax and sterol esters get deposited onto the surface of mature pollen. It is, therefore, attractive to propose that MS2 protein is involved in the formation of fatty alcohols required for wax ester production in the tapetum of the anther.

The final step of the acyl reduction pathway is the synthesis of wax esters, a reaction catalyzed by a fatty acyl-CoA: fatty alcohol acyltransferase (wax synthase, WS). WS activity from several different sources has been characterized biochemically [75–77], and described to be membrane-bound. However, very little was known about the proteins associated with the WS activity and
the genes encoding them. A major breakthrough was a recent report of a partial purification of a WS polypeptide from jojoba embryos [78], which also resulted in identification of a cDNA encoding the WS enzyme. The identity of the cloned cDNA was demonstrated by expression in Arabidopsis, which resulted in high levels of WS activity in seeds of transgenic plants. Hydrophathy analysis of the deduced protein sequence revealed seven to nine transmembrane domains indicating that jojoba WS is an integral membrane protein. Twelve Arabidopsis sequences (MIPS accession numbers Table 4) have considerable sequence similarity to the jojoba WS protein. Several of these genes are differentially expressed in inflorescences and seeds of Arabidopsis [78]. One can anticipate that WS enzymes in these tissues would be involved in the formation of wax esters for pollen coats, and synthesis of seed storage lipids, respectively. In addition, several of the WS genes are probably required for cuticular wax production in Arabidopsis shoots. However, the exact biochemical roles of these enzymes are yet to be determined.

Of the 22 wax-deficient cer mutants in Arabidopsis identified to date, only a mutation in the CER4 gene has been suggested to affect the synthesis of products via the acyl reduction pathway. A major reduction in primary alcohol and wax ester levels in the cer4 mutant, partially compensated for by an increase in aldehydes, has been attributed to a lesion in an aldehyde reductase of the acyl-reduction pathway [28,33]. If, however, primary alcohols are generated from fatty acids without release of an aldehyde intermediate, as suggested by Vioque and Kolattukudy [69], there would be no aldehyde reductase enzyme in the acyl reduction pathway. Therefore, the cer4 phenotype can only be explained by a mutation in the alcohol-forming reductase of the acyl reduction pathway. The observed increase in aldehyde levels in the cer4 mutant may then be a result of an increased flux of fatty acyl precursors into the decarbonylation pathway.

2.3.2. The decarbonylation pathway

The decarbonylation pathway is initiated by the production of aldehydes from VLCFA precursors by a membrane-bound fatty acyl-CoA reductase. This aldehyde-forming reductase has been resolved and purified to homogeneity from pea leaves, and shown to be an enzyme with distinct molecular weight (28 kDa) and biochemical properties from the reductase involved in the production of primary alcohols [69]. However, a gene encoding this enzyme has not yet been isolated. The generated aldehydes are then decarbonylated to odd-chain alkanes with a release of carbon monoxide. This reaction is catalyzed by an aldehyde decarbonylase [79]. Two plant aldehyde decarbonylases have been studied in some detail, one from pea, and one from a green colonial alga Botryococcus braunii [79–81]. Both enzymes need metal ions for their function and are integral membrane proteins, with the pea decarbonylase proposed to be associated with the cuticle (however, see Section 3.2.2) and the Botryococcus decarbonylase activity located in the microsomes. However, technical difficulties encountered during solubilization of decarbonylases have delayed molecular characterization of these enzymes and elucidation of the catalytic mechanism of decarbonylation.

A molecular genetic approach to identifying and characterizing a plant decarbonylase is an attractive alternative to conventional biochemical approaches. In Arabidopsis stems, products of the decarbonylation pathway account for ~90% of the total wax [38]. Thus, a lesion in a key biosynthetic step of this pathway, such as decarbonylation, is likely to result in a significantly reduced wax load, and such a mutant could be easily identified on the basis of its increased glossiness. Surprisingly, of the 22 different genetic loci identified by mutation to date, only CER1 was reported
to specifically affect the decarbonylation pathway. Stem wax of cer1 mutants shows a dramatic reduction in alkane, secondary alcohol and ketone levels, accompanied by an increase in aldehydes. Based on this phenotype, CER1 was suggested to encode an aldehyde decarbonylase [25,26,28]. Aarts et al. [27] cloned the CER1 gene, but because aldehyde decarbonylase genes have not been cloned previously, the CER1 sequence was not useful in assigning a biochemical function to its gene product. Moreover, as described in Section 2.1, CER1 shares considerable sequence similarity with the products of the maize GL1 gene and the epi23 gene from Kleinia odora, that have been proposed to belong to a family of membrane-bound receptors [29]. Sequence similarity, however, between GL1 and CER1 is restricted to the NH2-terminal region, and the CER1 protein is much larger than the GL1 polypeptide. Furthermore, mutations in these two genes confer different phenotypic changes on the cuticular wax compositions of Arabidopsis and maize. Taken together, these data suggest that, despite their similar structures, CER1 and GL1 proteins may perform different roles in cuticular wax biosynthesis. Further work is clearly needed to resolve the functional identities of these proteins.

Subsequent steps of the decarbonylation pathway, the hydroxylation of alkanes to secondary alcohols and oxidation of secondary alcohols to ketones, have been demonstrated in broccoli (Brassica oleracea) using radiolabeled alkanes [82,83]. An absence of secondary alcohols and ketones in Arabidopsis cer1 mutant blocked in alkane synthesis supports the conclusion that these wax components are derived from alkanes [25]. Our knowledge of the enzymes catalyzing these reactions, however, is extremely limited. In broccoli, the introduction of the hydroxyl group required oxygen and was inhibited by phenanthroline. Because this inhibition was reversed by Fe2+, it was suggested that a mixed function oxidase was involved in this hydroxylation [83]. However, whether the alkane is indeed the substrate for hydroxylation is not known with certainty. No mutants affecting this part of the decarbonylation pathway have been identified in Arabidopsis to date, and only a single gene in barley, cer-soh is known that affects the synthesis of secondary alcohols [22,84]. Progress in this area awaits identification and characterization of genes encoding the enzymes of this pathway.

3. Wax secretion

3.1. Considerations of wax precursors as secretory products

Wax components are unusual secretory products because of their hydrophobicity that sets them apart from the “conventional” products of Golgi-mediated secretion, such as polysaccharides and proteins. Hamilton [85] has reviewed the physical processes governing fatty acid transport across lipid bilayers in each step of the transport including adsorption, transmembrane “flip-flop”, and desorption. Measurements of pure fatty acids crossing small unilamellar vesicles (SUV) show that long chain fatty acids spontaneously partition into bilayers and flip-flop across the bilayer on a time scale of seconds [86]. The tendency of fatty acids to partition into a nonpolar environment has been quantified as a large negative drop in free energy, which increases logarithmically with chain length [87]. Thus, the longer chain fatty acids found during wax synthesis will strongly favour a nonpolar environment. Conversely, measurements of the rate of fatty acid removal or desorption from a model phospholipid bilayer showed a logarithmic decline in deso-
rption rate for each additional \((-\text{CH}_2\text{)}_2\) unit on the aliphatic chain [86]. As wax precursors mature from long chains produced in the plastid to very long chain fatty acids and their derivatives, they become increasingly hydrophobic and more difficult to remove from a nonpolar environment. It is clear that very long chain, saturated hydrocarbons will not be freely soluble in an aqueous environment, yet a typical epidermal wax-secreting cell consists of a collection of aqueous spaces separated by membrane bilayers.

The nonpolar environments available to the very long chain fatty acid wax precursors are the interior of organelle membrane bilayers or the interior of carrier protein hydrophobic pockets. The cell must control the partitioning of fatty acids into membranes to avoid deleterious effects on bilayer structure and to maintain the organelle’s unique lipid milieu. In mammalian cells, abundant fatty acid-binding proteins (FABP) in the cytoplasm control the intracellular pool of free long chain fatty acids, acting as a buffer to constrain fatty acid interactions to those required by the cell [88–90]. FABP can be “membrane active”, removing fatty acids from membranes during transient collisions and delivering fatty acids to specific membranes and target enzymes [91]. The mammalian cytosolic 14 kD FABP gene family is made up of a suite of tissue-specific isoforms, but homologs of FABP have not been found in the \(S.\text{cerevisiae}\) or the \(Arabidopsis\) genomes.

A possible alternative to FABPs for binding hydrophobic VLCFA wax precursors is acyl CoA-binding proteins (ACBP). ACBPs have been characterized in a variety of organisms and demonstrated to bind cytoplasmic acyl-CoAs [92–94]. In \(Arabidopsis\), both cytoplasmic acyl CoA-binding proteins [95,96], and membrane-associated forms of acyl CoA-binding proteins [97] have been described. The latter were detected in developing siliques associated with the plasma membrane and vesicles of epidermal cells of the embryos, which was interpreted as indicating that this acyl-CoA binding protein plays a role in embryo cuticle development. The role of acyl CoA-binding proteins in binding CoA esters of very long chain fatty acids during wax synthesis has not been experimentally tested.

3.2. Wax biosynthesis in the context of the epidermal cell

3.2.1. FAS and thioesterase activities in the plastid produce long chain fatty acid wax precursors

The typical epidermal cell that is active in wax secretion contains a large central vacuole surrounded by a very thin layer of cortical cytoplasm. The cytoplasm contains small leukoplasts, smooth and rough endoplasmic reticulum (ER), Golgi, mitochondria, and cytoskeletal elements. What cellular structures support the biosynthesis and transport of hydrophobic wax precursors?

The first cellular structure to support wax biosynthesis is the plastid, where fatty acid synthesis occurs. Here, elongating acyl chains are esterified to acyl carrier proteins (ACP) in the stroma [98]. Once they reach C16 or C18 in length, the partitioning of fatty acids for membrane glycerolipid synthesis from the fatty acids destined for wax biosynthesis must occur. This is an important control point in epidermal cells resulting in a massive flow of saturated fatty acids into wax production [66]. For use in membrane lipids, acyl chains can either be transferred directly from the ACP to glycerol-3-phosphate in the plastid (“prokaryotic pathway”) or they can be released from the ACP by a thioesterase and exported from the plastid to the ER (“eukaryotic pathway”) [41]. For wax production, the 16:0 or 18:0 acyl groups are released from ACP by an acyl-ACP thioesterase and move to the ER for further elongation. The thioesterase activity involved in this
process has not been identified. This is partly due to the fact that it is not known with certainty whether 16:0 or 18:0 are the precursor fatty acids exported from the plastid to be used for wax biosynthesis. One possibility is that 16:0 is released by the thioesterase in the plastid, with subsequent elongation steps occurring in the cytosol by elongases anchored in the ER membranes. Alternatively, 16:0 could be elongated to 18:0 by 3-ketoacyl-ACP synthase II (KASII) activity in the plastid [43], in which case 18:0 would be exported for extraplastidial fatty acid elongation, as postulated by Post-Beittenmiller [66]. A single acyl-ACP thioesterase that preferred 18:0 was purified from the wax producing epidermis of leek (*Allium porrum*) [99].

Acyl-ACP thioesterases have been classified into FatA and FatB groups based on gene sequence analysis and substrate preferences [100,101]. FatA thioesterases are ubiquitously expressed housekeeping genes with oleoyl-ACP (18:1-ACP) as their preferred substrate and with minor activity towards 16:0 and 18:0. FatB thioesterases prefer saturated acyl-ACP substrates of various chain lengths, and are further subdivided into a seed specific subgroup with specificity towards medium chain (C10-C14) fatty acyl-ACPs and a widely expressed subgroup with specificity towards saturated C14–18 acyl-ACPs [101]. It is this latter subgroup, FatB1, which would logically be expected to be involved in cleaving C16:0-ACP or C18:0-ACP for wax biosynthesis. When the *Arabidopsis* FatB1 gene was expressed in *E. coli*, the preferred substrate determined *in vitro* was 16:0 > 18:1 > 18:0 > 14:0-ACP [102]. *Arabidopsis* FatB1 is highly expressed in flowers, with lesser expression in leaves, roots, and stems. FatB1 is responsible for generating the membrane pool of 16:0 found in flower membrane lipids, and overexpression of the FatB1 gene in *Arabidopsis* led to accumulation of 16:0 in seeds [103].

It is important to note that the acyl-ACP thioesterases in the plastid are competing with the acyltransferases and an 18:0 desaturase for substrates by mechanisms that are not well understood and, therefore, the in vitro activity of the thioesterase alone may not accurately predict what its in vivo substrate will be. If the wax producing cell has relatively low desaturase activity, high KASII activity and a typical FatB1 thioesterase, then 18:0 could still be the primary export product of its plastids.

Following liberation from ACP, the long chain fatty acids (C16 or C18) are thiol-esterified to CoA by acyl-CoA synthetases in the chloroplast envelope [104]. Trapping of fatty acids in an intracellular pool by addition of CoA has been termed “vectorial acylation” since the presence of the CoA esterified to the carboxyl group of the fatty acid prevents “flip-flop” of fatty acids back across the bilayer [85].

3.2.2. *FAE* to very long chain fatty acids is associated with the endoplasmic reticulum

Long chain fatty acid wax precursors are further elongated by the microsomal elongase complexes to very long chain fatty acids (Section 2.2.1). The close physical association between plastids and endoplasmic reticulum may facilitate fatty acyl transfer from the plastid envelope to the ER [105]. Because some domains of the ER are sensitive to fragmentation by aldehyde fixatives used to prepare cells for electron microscopy, the most reliable reports of the plastid-ER relationship come from studies using cryofixation [106]. For example, cryofixed epidermal cells from *Arabidopsis* stems contain plastids that are often wrapped in a domain of ER (Fig. 4). The mode of transfer of fatty acids between the plastid and the ER has not been established. Fatty acids can spontaneously partition into lipid bilayers, so acyl-CoA esters destined for further elongation in the ER could partition into the endomembrane system. Alternatively, a protein carrier, such as acyl-CoA binding
protein, may be involved. In *S. cerevisiae*, deletion of the gene encoding ACBP led to accumulation of C18:0, and dramatically reduced levels of C26:0, suggesting that ACBP is involved in transport of acyl-CoAs to the fatty acid elongation system associated with sphingolipid biosynthesis [107].

There are several lines of evidence that FAE occurs in the endoplasmic reticulum (Fig. 5). When the *Arabidopsis* condensing enzyme CER6, tagged with GFP, was transiently expressed in the epidermis of *Nicotiana benthamiana*, the fluorescence appeared in a pattern which is characteristic of the endoplasmic reticulum (Fig. 6). In the *cer6* mutant, a proliferation of the SER in epidermal cells suggests a buildup of blocked substrate at this site (Samuels and Kunst, unpublished observations). Elongation activity has also been demonstrated in the microsomal fractions carrying ER marker enzymes from epidermal cells in a variety of plants [49,50]. Jenks et al. [108] quantitatively demonstrated the proliferation of ER membranes when wax synthesis was

Fig. 4. (A) The close physical relationship between the endoplasmic reticulum (ER) and plastid (arrow) is seen in this transmission electron micrograph of cryofixed *Arabidopsis* epidermis. In the epidermal cells, the non-green plastids, leukoplasts, are small and not photosynthetically active. CW, cell wall; v, vacuole; PM, plasma membrane. (B) Freeze fracture replica of a tobacco (var. BY-2) suspension cultured cell showing the close association (arrow) between the ER and the plasma membrane (PM). At these sites, the ER presses against the cytoplasmic face of the PM but does not fuse with it. (bars, 0.25 μm). (Samuels and Staehelin, unpublished data).
Fig. 5. Wax biosynthetic pathways placed in the context of the epidermal cell. Fatty acid synthesis to C18 occurs in the plastid, and fatty acid elongation to VLCFA takes place in association with the membranes of the endoplasmic reticulum (ER). Four enzymes working sequentially carry out each 2C unit addition in the plastid fatty acid synthase and the ER fatty acid elongase. Acyl reduction and decarbonylation reactions may occur on domains of the ER closely associated with the plasma membrane and linked to the cell wall (CW).
Fig. 6. Transient expression of green fluorescent protein (GFP) tagged proteins in epidermis of tobacco (*Nicotiana benthamiana*) leaves. (a) HDEL-GFP, ER resident protein retention signal fused to green fluorescent protein gives control characteristic pattern for ER. (b) Subcellular localization of CER6-GFP, showing the distribution of β-ketoacyl-CoA synthase (condensing enzyme of fatty acid elongation producing very long chain fatty acids for aliphatic wax components) in the ER (Zhang and Kunst, unpublished data).
switched on by light in sorghum cork cells. Further support for the ER localization of elongase enzymes comes from reports in 	extit{S. cerevisiae}. The VLCFA enoyl reductase in this organism has been shown to reside in the ER membrane, in a novel ER subdomain between the nucleus and the vacuole [65]. There is also good evidence that the β-ketoacyl reductase required for VLCFA production is an ER membrane protein [60].

Once elongation is complete, VLCFAs are reduced to fatty alcohols by an acyl-CoA reductase of the acyl-reduction pathway, which appears to be associated with the ER [70]. The primary alcohols can then be linked to fatty acids to give rise to wax-esters. Wax synthase, catalyzing this reaction, is an integral membrane protein [78], but its site of action is not known.

Alternatively, VLCFA precursors are reduced to aldehydes by a membrane-bound acyl-CoA reductase before they are used by the enzymes of the decarbonylation pathway. The decarbonylation reaction has been localized by subcellular fractionation to a heavy “cell wall-cuticle region”. This is surprising, because the pH optimum of the enzymatic activity showed a sharp peak near 7, suggesting a cytoplasmic location [79]. It is possible that the decarbonylase exists on the cytoplasmic face of the ER or plasma membrane (PM) in a domain that is physically associated with the cell wall. Strong physical links between the ER, PM, and cell wall have been demonstrated [109,110]. This physical interaction can be resolved after plasmolysis of cells. In one type of association, shrinkage of the protoplast from the wall reveals thin strands of cytoplasm that remain attached to the cell wall. These strands, first described by Hecht in 1912 [111], are long thin extensions of the cytoplasm that attach to the wall in discrete spots and include endoplasmic reticulum, ribosomes, and cytoskeletal elements [112,113]. Localization of the decarbonylase using a decarbonylase-GFP/confocal microscopy and immuno-gold/TEM will be informative, especially in cells that have been plasmolyzed.

Alkanes can be further processed into secondary alcohols and ketones by enzymes that are entirely uncharacterized either biochemically or molecularly.

3.2.3. Transport from the ER to the plasma membrane and export from the cell

It is currently not known how molecules such as very long chain fatty acids, alcohols, aldehydes or alkanes are moved from the ER to the plasma membrane. It is even more difficult to envision how they are exported from their “comfortable” nonpolar environment to the aqueous apoplast. In the absence of fatty acid binding proteins in plants, it appears that molecules this hydrophobic would partition into membrane bilayers. Based on our current knowledge, there are at least two nonexclusive mechanisms which could move wax components from the ER to the PM. It must be stressed that there is no experimental evidence supporting any of these two potential mechanisms, and they are presented as hypotheses to be tested, rather than well-established fact.

**Hypothesis 1.** Wax components could be transferred directly to the PM via sites of close apposition of ER domains with the protoplasmic face of the plasma membrane. In rapidly frozen, freeze fractured preparations of plant cells, plasma membrane domains in tight association with the cortical ER are observed [114,115] (Fig. 4, Samuels and Staehelin, unpublished observations). No membrane fusion occurs in these sites but the ER membrane is in close (10 nm) proximity to the PM. These sites have been postulated to represent regions where molecular recycling of lipids could occur following addition of lipids to the plasma membrane during exocytosis [114]. During conventional chemical fixation for electron microscopy, the osmotic disruptions created by
inactivation of plasma membrane pumps would disrupt these ER-PM associations, so cryofixation is necessary to preserve them. Likewise, thin sectioning for TEM makes it difficult to visualize these associations as the 70 nm thick sections typically only contain disjointed strands of ER. If direct transfer of lipid molecules from the ER to the PM exists, it must be carefully controlled to maintain the unique lipid composition of each compartment. The membrane lipids of the PM contain more saturated fatty acids and there are more sterols in the PM than the ER [116]. Evidence that lipids can move in the opposite direction, from the PM to the ER without a vesicular intermediate comes from observations in suspension cultured soy cells treated with fluorescent membrane lipids. Application of fluorescent phosphatidylcholine (NBD-PC) led to rapid conversion of NBD-PC to NBD-diacylglycerol (NBD-DAG), and translocation directly from the PM to the ER [117].

During wax biosynthesis, very long chain fatty acyl-CoA molecules could be converted to alkanes, secondary alcohols, ketones or primary alcohols at ER sites adjacent to the plasma membrane followed by direct translocation into the plasma membrane.

**Hypothesis 2.** Wax components could be carried by vesicular traffic from the ER to the Golgi and finally PM. Following elongation in the ER, acyl reduction or decarbonylation reactions could also occur in the ER, or anywhere along the secretory pathway, and the wax products could be exported by exocytosis of Golgi derived vesicles. The “cell wall/cuticle” fraction containing the decarbonylase could just as easily be a plasma membrane fraction tightly associated with the cell wall, as suggested above, except in this scenario decarbonylation could be occurring in the plasma membrane [22]. Sphingolipids, which carry very long chain fatty acyl tails, have been shown to follow the classical ER-Golgi-PM secretory route to the plasma membrane in mammals, *S. cerevisiae*, and plants (reviewed by Moreau et al. [118]). The acyl chains found in sphingolipids are typically C24:0 and may associate with sterols to form “lipid rafts” [119], transient iceberg-like microdomains in the PM. In yeast, there is evidence that lipid rafts assemble in the ER, and that they are involved in ER-Golgi-PM traffic [120]. VLCFAs in membrane bilayer can cause strong perturbations in the acyl chain environment within the bilayer [121,122]. Association of the very long chain acyl groups into lipid rafts with sterols could be a mechanism for preventing disruption of the lipid bilayer structure by VLCFA. If wax precursors follow the ER-Golgi-PM route, a physical sorting via lipid rafts can be a mechanism to separate these potentially disruptive molecules from the housekeeping domains of the ER.

As stated above, these are hypothetical routes of very long chain acyl groups leaving the endomembrane system. There is little evidence to support either a direct ER-PM or the Golgi-mediated route. Jenks et al. [108] looked for ultrastructural changes in sorghum cork cells when wax production was stimulated by light. They showed that, while there was a proliferation of the ER, the Golgi and the associated vesicles did not change. It should be noted, however, that the Golgi associated vesicles are difficult to quantify using the conventional chemical fixation electron microscopy techniques employed in this study. In the analysis of the Golgi and the ER of waxless *Arabidopsis* mutants cer1–cer10, there were no mutants with accumulations of Golgi vesicles or altered Golgi morphology [123].

Once the wax components are in the plasma membrane, they must be removed from the bilayer and moved into the aqueous apoplast. Moving VLCFacyl derivatives from the hydrophobic core of the bilayer to the aqueous apoplast would require an input of energy. Members of the “ABC transporter” protein family pump a variety of lipophilic molecules out of yeast and mammalian cells [124,125]. The
characteristics of ABC transporters that make them potential candidates for wax exporters are their homology to known lipid transporters, location of one subset of ABC transporters in the plasma membrane, and their ability to hydrolyze ATP to provide energy for energetically unfavourable processes. In the Arabidopsis genome, 129 sequences have been identified as putative ABC transporters [126,127]. Many of these have strong sequence similarity to drug resistance proteins and pigment transporters of mammalian and yeast systems, and they can be classed into six ‘families’ of related sequences (summarized at www.arabidopsisABC.net). A role for ABC transporters in the export of secondary metabolites has been postulated, based on ATP dependent export of a diterpene [128]. If ABC transporters are involved in diterpene export, it is logical to extrapolate a role for ABC transporters in export of triterpenoids in cuticles rich in these compounds, such as Prunus laurocerasus [18]. With T-DNA insertion lines of Arabidopsis available, it should be possible to examine the lines with disruptions to their ABC transporters for waxless phenotypes.

3.3. Transport of wax precursors across the cell wall to the cuticle

3.3.1. Lipid transfer proteins

Hydrophobic wax components must move through the hydrophilic cell wall matrix to reach the cuticle. Lipid transfer proteins (LTPs) have been suggested to mediate this movement during wax biosynthesis [66,129,130]. They may be specific for one lipid class, such as phosphoinositol, or, more typically for plants, have broad substrate binding specificity (nonspecific lipid transfer proteins; nsLTP). The nsLTPs are abundant in plant wax. For example, the broccoli LTP WAX9 (73% identical to Arabidopsis LTP1) represented more than 90% of the wax-associated protein on broccoli (B. oleracea) leaves [131]. LTPs were originally defined by their ability to transfer phospholipids between membranes in vitro, leading to a hypothesized role in lipid transfer within the cell (reviewed by Kader [132]). However, this hypothesis is not supported in plants where nsLTP have hydrophobic N-terminal signal sequences, suggesting that LTPs are targeted to the secretory pathway [133] and ultimately to extracellular locations in the cell wall and cuticle [131,134].

LTPs are small (9 kd), basic proteins with eight conserved cysteines which are joined by disulfide bonds to form a hydrophobic tunnel [135–138]. High resolution x-ray crystallography of maize nsLTP has revealed that the hydrophobic tunnel accommodates a variety of ligands from C10-C18 by allowing some flexibility in the ligand binding cavity [139]. This would be consistent with nsLTP carrying C16 and C18 cutin precursors to the plant surface. However, the modeling to date suggests that the very long chain fatty acid wax precursors would not fit in the hydrophobic cavity of the LTP [140]. Furthermore, in direct competition experiments, maize LTP showed substrate preference for C18 over C20, C22 or hydroxylated C18 acyl chains [141].

Although there has been no direct demonstration of a role for the LTPs in transferring wax components or cutin across the apoplast, there is circumstantial evidence for their involvement in cuticular lipid synthesis. Data from RNA blotting experiments, in situ hybridizations, promoter-GUS fusions and EST database projects demonstrate that LTPs are expressed in an epidermis-specific manner, especially in organs active in cuticle production such as developing flowers, leaves and stems [131,132,134,142,143]. In addition, positive correlations have been reported between conditions that stimulate wax synthesis, such as drought [144], light [145], or heavy metal treatment [146], and lipid transfer protein expression.
If LTPs do, indeed, carry cuticular lipid components to the plant surface, they could be loaded with their “cargo” in the lumen of the ER and follow the secretory pathway out into the apoplast. Immuno-gold localization and cell fractionation studies, however, suggest that the lipid transfer proteins are primarily present in the apoplast, and not the endomembrane system [131,134]. It is possible that these wax associated proteins represent “spent” carriers, although if each LTP is capable of carrying one or two fatty acids on a one-way trip, then massive amounts of protein would be required. Alternatively, lipid transfer proteins could interact with the plasma membrane lipid bilayer and extract lipids directly into their hydrophobic cavities. An intriguing preliminary study has shown that LTP from wheat (T. aestivum) binds to plasma membrane vesicles with saturation kinetics, suggesting the presence of a plasma membrane receptor for the LTP [147].

3.3.2. The physical path of wax components through the epidermal cell wall

The unique cell wall structure of the typical epidermal cell may give clues as to the path that wax must take through the cell wall (reviewed by Martin and Juniper [13]). Between the symplasm and the epicuticular surface, there are distinct subdomains of cell wall around the wax-secreting cell (Fig. 1). The innermost zone is a typical primary cell wall of about 0.25–0.5 μm, which encases the epidermal cell. The current models depict the primary cell wall as a hydrophilic, reinforced gel: 2–3 layers of cellulose microfibril layers, complexed with hemicelluloses, enclosed in a matrix of pectins and proteins [148–150]. On the periclinal surface facing the plant surface is a cap of secondary cell wall layer over the cell, which may play a mechanical role in preventing deformation of the epidermis. Overlying this, and interacting directly with the cuticular lipids, is a pectin rich layer with cellulose microfibrils branching through it, which is in continuity with the pectin-rich middle lamella of the anticlinal walls between the adjacent cells. The porosity of the wall seems to be determined by the properties of the pectin matrix [151], a mixture of homogalacturonans and rhamnogalacturonans variably methylsterified or crosslinked by calcium through their carboxyl groups.

So how the hydrophobic wax components make their way through the layers of the epidermal cell wall to the plant surface? Epidermal cells do not show strong structural polarity in their organelle distribution, i.e. accumulation of Golgi and secretory vesicles on the periclinal surface of the cell. If Golgi vesicles are carrying wax components, there must be targeting information which is specific to the apical surface of the cell to facilitate vesicle fusion in this area. Following release, the wax components could move through the primary cell wall and into the middle lamella which is continuous with the apical pectic layer. It is conceivable that they ‘travel’ within the hydrophobic cavity of LTPs, which are highly abundant in the apoplast and below the size exclusion limit of the cell wall. Once at the cuticle/pectin interface, hydrophobic wax components could partition into the nonpolar phase of the cuticular layer. When the concentration of a certain wax component reaches a critical threshold, epicuticular crystalloids of that component could form on the aerial surface from the amorphous wax mixture embedded in the crosslinked cutin [152–154].

An alternative possibility is that the precursors move through the cell wall in direct contact with the cell wall matrix. Cell wall proteins or polysaccharides may create relatively hydrophobic subdomains within the cell wall where passage of free hydrophobic components could be facilitated and wax components could self-associate into a narrow ’river’ of hydrophobic compounds.
moving to the cell surface. Although the cell wall is predominantly hydrophilic, hydrophobic domains could be formed by cell wall proteins such as glycine rich proteins (GRP), whose secondary structure has been predicted to form β-pleated sheets with a large hydrophobic surface [155,156]. GRPs have been localized in vascular tissues but also in the epidermis of maize [157] and Arabidopsis [158]. Potential polysaccharide components that could contribute to hydrophobic domain formation are highly methyl-esterified pectins. There are cases where polysaccharides bind hydrophobic compounds. The cyclic oligosaccharide from a bacterium, cyclodextrin, is used as a tool in animal cell biology to selectively remove and bind cholesterol from the plasma membrane [159]. The α-(1-4) glycosidic ring of cyclodextrin has the hydroxyl groups projecting outward, creating a hydrophobic pocket for lipid binding [160]. A homologous cell wall polysaccharide held under tension to project the hydrophobic domain along a passage could contribute to an area of the cell wall where wax constituents could pass. The movement through the aqueous apoplast has been difficult to examine experimentally. The process cannot be observed with conventional electron microscopy because sample preparation procedures such as dehydration and resin embedding extract lipidic components. As well, saturated long chain fatty acid derivatives such as alkanes lack chemical groups that would react with heavy metal stains.

Historically, debate has centred on whether pores exist in the cuticle that could be responsible for extrusion of wax crystals [14,161]. More recent descriptions of the phase behaviour and crystallinity characteristics of the cuticle studied by Fourier transform infrared spectroscopy [162] confirm earlier nuclear magnetic resonance results [163] showing that plant cuticular wax components organize themselves into crystalline and amorphous zones. Amorphous regions might allow freer movement of epicuticular wax through the cuticle, resulting in regions which act as functional “pores” without a physical opening occurring. Once in the hydrophobic realm of the cuticle, wax behaves as a self-organizing system [164]. Based on movement of wax through isolated cuticles, a mechanism for movement of epicuticular wax components in the cuticular water vapour stream has been proposed [165].

4. Conclusions

The attention devoted to the plant cuticular wax is a reflection of its importance to plant biology. The wax on the outside of a plant is the fossil record of the metabolic activity of that epidermis. Its examination has contributed valuable information about the chemical diversity of wax composition within a single species and amongst taxa. The pioneering contributions of Kolattukudy, von Wettstein-Knowles, and others have shown us the basic wax biochemical pathways. The isolation of mutants in Arabidopsis and other plant species, combined with biochemical analyses of mutant phenotypes, resulted in identification gene products involved in wax production, and cloning of a number of wax biosynthetic genes. However, not all genes involved in the biochemistry, regulation and transport of wax to the plant surface have been identified by genetic approaches. The challenge now is to take the genomic tools available to find additional genes whose functions are essential, redundant, or more subtle, and investigate their specific contributions to cuticular wax production during the life cycle of the plant. The path to greater understanding of how wax is synthesized and secreted will come from delving into the active plant cell.
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