Biosynthesis of the Plant Cuticle

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Contents

Abstract

Cuticular waxes and cutin form the cuticle, a hydrophobic layer covering the aerial surfaces of land plants, mainly preventing non-stomatal water loss and acting as a protective barrier against environmental stresses. Fatty acid-derived

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compounds that compose the building blocks of the cuticle are produced in the endoplasmic reticulum of epidermal cells before being exported to the environmental face of the epidermis. Thirty years of plant genetic studies and the recent development of analytical tools for lipid identification have led to the molecular and biochemical characterization of the enzymes catalyzing the major steps in cuticular compound biosynthesis.

1 Introduction

Four hundred and fifty million years ago, the development of the cuticle, a thin translucent waterproof barrier, represented an important adaptation of plants for colonizing the terrestrial environment. Covering the surface of all aerial organs, the cuticle is strategically located at the plant/air interface, thereby, playing essential functions in plant interactions with the environment mainly by limiting water loss. The protective capacities of the cuticle are based on the physical and biochemical properties of its two highly hydrophobic components, cutin and cuticular waxes, which are assembled ultrastructurally in several layers (Nawrath et al. 2013). The cutin polymer, with embedded intracuticular waxes, constitutes the cuticle proper that is connected to the cell wall by a cuticular layer made of cutin and polysaccharides. Covering the cutin matrix, the outermost layer of the cuticle is composed of epicuticular waxes that can form wax crystal microstructures. Cuticle synthesis starts in early stages of embryo development and is tightly co-regulated with plant growth to provide constant cuticle deposition (Delude et al. 2016). Although the biochemical composition and the thickness of the cuticle vary among different plant species and/or among organs and developmental stages, a set of primary compounds is ubiquitously found in plant cuticles. Cutin, a three-dimensional biopolyester of long-chain polyhydroxy- and epoxyhydroxy-fatty acids cross-esterified to each other or via glycerol backbones, provides the main mechanical strength of the cuticle (Dominguez et al. 2015). Cuticular waxes consist of a complex mixture of homologue series of very-long-chain (VLC) aliphatic compounds, as well as non-acyl lipid cyclic components including terpenoids and flavonoids (Buschhaus and Jetter 2011; Bernard and Joubès 2013). In the past 30 years, the development of sensitive quantitative and qualitative technologies to accurately analyze cuticle composition, coupled with plant reverse genetic analyses, has yielded significant advances toward unraveling the many aspects of plant cuticle metabolism.

Cuticle biosynthetic pathway can be divided into several metabolic blocks (Fig. 1). Initially, the C16:0, C18:0, or C18:1 fatty acids resulting from de novo fatty acid synthesis in the plastids are transferred to the acyl-coenzyme A (CoA) pool, which will be used as precursor for the synthesis of cutin monomers and wax components. The subsequent generation of the cutin polymer can be divided in three successive steps, fatty acyl-chain oxidation, esterification of oxygenated fatty acyl

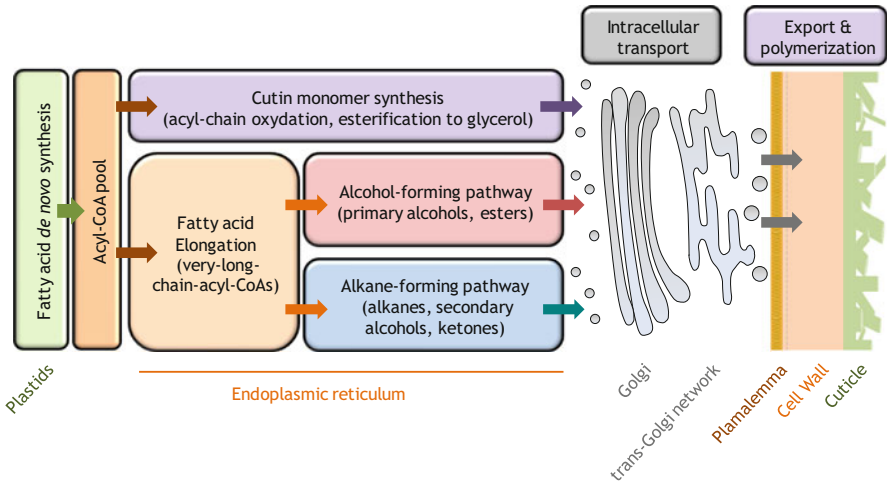

Fig. 1 Cuticle biosynthetic pathway

chains to glycerol, and their export and extracellular polymerization. During wax biosynthesis, VLC acyl-CoAs derived from fatty acid elongation may be converted into primary alcohols and alkyl esters by the alcohol-forming pathway or into aldehydes, alkanes, secondary alcohols, and ketones by the alkane-forming pathway. Cuticle precursors synthesized within the endoplasmic reticulum (ER) are then transported in a directional manner through the Golgi apparatus and trans-Golgi network (TGN) to reach the plasma membrane before being secreted to cover the cell wall of epidermal cells facing the external environment.

2 Cutin Biosynthesis

Although the cutin monomer acyl composition is now well characterized, its macromolecular structure, as well as its association with the outermost cell wall layer of epidermal cells, remains poorly described (Yeats and Rose 2013). Through the use of particular plants and organs for which the physical isolation of cuticle by chemical and/or hydrolytic enzyme treatments had been achieved, the first analyses of cutin composition revealed that long-chain polyhydroxy- and epoxyhydroxy-fatty acids represent the major components of the polymer (Kolattukudy 1980). In the early twenty-first century, the adaption of isolation procedures to the plant model *Arabidopsis thaliana* (Franke et al. 2005), as well as the establishment of protocols based on extensive delipidation of plant tissues to eliminate all soluble lipids (Bonaventure et al. 2004), resulted in rapid progress toward elucidation of the major biochemical pathways generating cutin.

2.1 Cutin Monomer Synthesis

The most recent discoveries concerning cutin synthesis suggest that the basic building block of the cutin polymer is *sn*-2 mono(oxygenated)acyl-glycerol (2MAG; Yeats et al. 2014). In most plants, the major oxidized acyl chains are long-chain ω-hydroxy- and polyhydroxy-fatty acids, of which the latter are hydroxylated at positions 9 and/or 10 as well as at the ω-position (Fig. 2). In that respect, the cutin of *Arabidopsis* leaves and stems, which is richer in dicarboxylic acids (DCA) than in hydroxy-fatty acids, may represent a unique composition. If it is highly likely that fatty acids produced in the plastids are first activated, then oxidized, and finally transferred to glycerol in the ER, the sequential order of the different enzymatic reactions yielding 2MAGs has not been unambiguously determined.

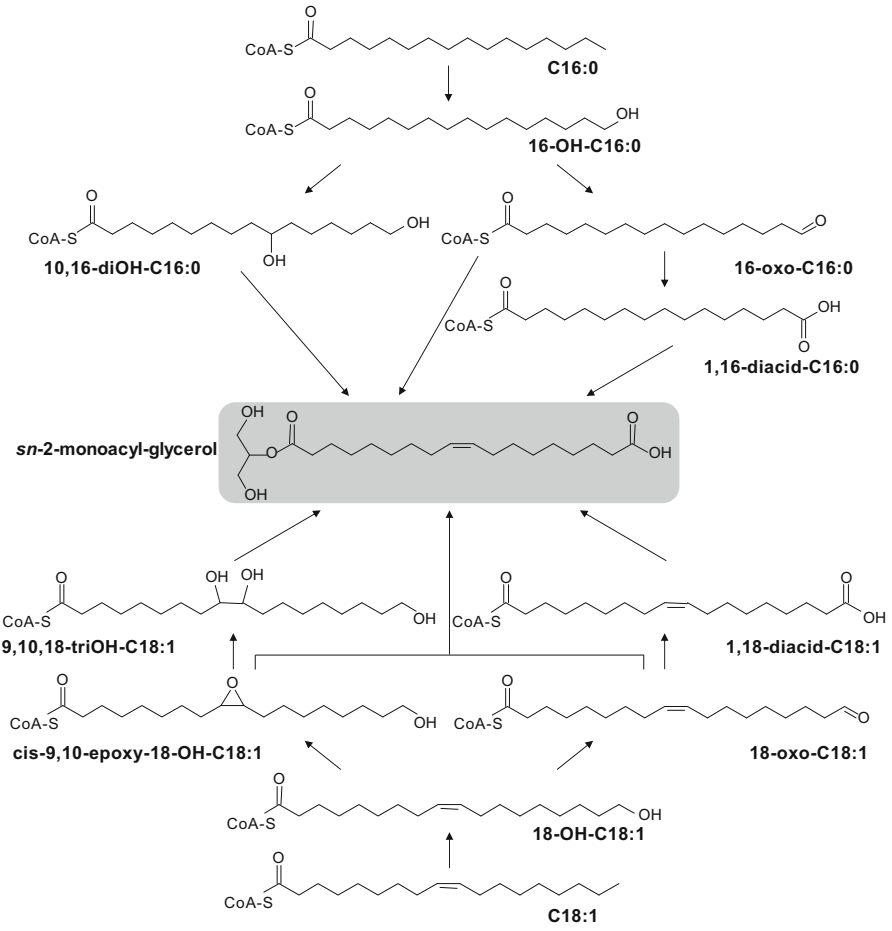

Fig. 2 Cutin monomer biosynthetic pathways

In *Arabidopsis*, the C16:0, C18:0, and C18:1 free fatty acids derived from the de novo synthesis within plastids are activated in the form of acyl-CoAs by two ER-resident, long-chain acyl-CoA acyltransferases, LACS1 and LACS2 (Weng et al. 2010). Mutations in *LACS2* globally impact cuticle development and especially wax content (Schnurr et al. 2004), whereas mutations in *LACS1* affect both cutin and wax loads (Lü et al. 2009). The double *lacs1lacs2* mutant is more afflicted than either single mutant, and the many pleiotropic effects, including the fusion of organs, suggest that LACS1 and LACS2 have overlapping activities that are obligatory for normal cuticle establishment (Weng et al. 2010; Lü et al. 2009). However, the exact role of these enzymes remains elusive as the type of acyl chains activated *in planta* remains unknown. In addition, the observation that LACS2 displays more activity toward 16-hydroxy-palmitic acid than to palmitic acid in vitro (Schnurr et al. 2004) raises further questions about the exact position of these enzymes within the metabolic pathway.

Oxidation of the acyl chains is catalyzed by various cytochrome P450 enzymes (Fig. 2). First, members of the *Arabidopsis* CYP86 subfamily produce ω-hydroxy-fatty acids. In *Arabidopsis* leaves and stems, CYP86A8 (LCR) and CYP86A2 (ATT1) were shown to be the major cytochrome P450s involved in the acyl oxidation of cutin precursors (Wellesen et al. 2001; Xiao et al. 2004). Although the cutin composition of these tissues contains substantial amounts of 16:0, 18:1, and 18:2 ω-hydroxy-fatty acids, it is strongly dominated by the corresponding DCA (Li-Beisson et al. 2013). Nevertheless, the enzymes responsible for the conversion of ω-hydroxy-fatty acids to DCA remain uncharacterized. Since cytochrome P450 enzymes that are able to oxidize the terminal methyl group of a fatty acid to a carboxyl group have been previously characterized, at least in vitro (Le Bouquin et al. 2001), it is possible that CYP86A8 (LCR), CYP86A2 (ATT1), or another cytochrome P450 enzyme produces DCA from ω-hydroxy-fatty acids and/or generates oxo-fatty acids. Oxidoreductases, such as HOTHEAD (HTH), might also be involved in this process, since the corresponding mutants have reduced levels of DCAs (Krolikowski et al. 2003; Kurdyukov et al. 2006). In *Arabidopsis* flowers, CYP86A4 converts palmitic acid to 16-hydroxy-palmitic acid. Another cytochrome P450 enzyme, CYP77A6, catalyzes the in-chain hydroxylation to produce 10,16-dihydroxypalmitate, which represents 80% of acyl monomers in floral cutin (Li-Beisson et al. 2009). The cytochrome P450 enzymes from this subfamily might also produce epoxy-fatty acids, since microsomes from yeast expressing CYP77A4 are able to catalyze the epoxidation of monounsaturated fatty acids (Sauveplane et al. 2009).

The generation of 2MAGs relies on a specific family of glycerol-3-phosphate: acyl-CoA acyltransferases (GPAT) that are only present in land plants and that are specifically involved in lipid polyester synthesis (Yang et al. 2012). GPATs involved in cutin biosynthesis differ from the classical acyltransferases involved in membrane and storage lipid biosynthesis by specifically acylating the *sn*-2 position of glycerol-3-phosphate instead of the *sn*-1 position for classical GPATs. Additionally, they carry a phosphatase activity allowing subsequent conversion of 2-acyl-lysophosphatidic acids into 2MAGs. In addition, in vitro assays using yeast microsomes from cells

expressing these GPATs showed a clear preference for ω-hydroxy-fatty acids and DCAs. These special features most probably allow the epidermis to separate the glycerolipid precursors of cutin from those of membrane glycerolipids (Yang et al. 2010, 2012). In *Arabidopsis*, the *gpat4 gpat8* double mutant has strongly reduced cutin loads in both leaves and stems (Li et al. 2007), whereas the *gpat6* mutant is affected in the cutin load of flowers (Li-Beisson et al. 2009), thus indicating that this small clade of GPATs is specifically devoted to cutin biosynthesis. It should be noted that these acyltranferases are not responsible for the incorporation of phenylpropanoids into the cutin polyester; instead, this reaction is carried out by the BAHD acyltransferases. In particular, DCF (for Deficient in Cutin Ferulate) was shown in *Arabidopsis* to control cutin ferulate content and, in vitro, to transfer ferulic acid to ω-hydroxyacids (Rautengarten et al. 2012). Another BAHD, DCR (for Deficient in Cuticular Ridges), was shown to strongly affect flower cutin content (Panikashvili et al. 2009), although its principal activity remains unknown (Molina and Kosma 2015).

2.2 Cutin Polymerization

Whereas all the reactions yielding 2MAGs have been shown to occur in the endoplasmic reticulum, the enzymes responsible for the polymerization process of cutin have been localized within the cell wall (Girard et al. 2012; Yeats et al. 2012). Therefore, cutin precursors must be transported within the epidermal cell and, then, through both the plasmalemma and the cell wall to reach their final location. Some components of these steps have been characterized, but the entire transport of cutin monomers remains poorly described as discussed in Sect. 4.

The long-running mystery of cutin polymerization was recently solved by two groups, both of which simultaneously reported the identification of an extracellular enzyme that strongly influences cutin cross-linking in tomato, and which is capable of polymerizing 2MAGs in vitro (Girard et al. 2012; Yeats et al. 2012). This enzyme, now referred to as cutin synthase, belongs to the Gly-Asp-Ser-Leu family of esterases/acylhydrolases, commonly called GDSL-lipases. Fruits from *GDSL1*-silenced tomato lines have a much thinner cuticle with strongly reduced amounts of cutin monomers and display increased brightness, permeability, and post-harvested water loss, as well as significant decreases in cutin polymerization according to FTIR (Fourier transform infrared spectroscopy) analyses (Girard et al. 2012). At the same time, the *cd1* mutant (for *cutin deficient 1*), with a point mutation resulting in an early stop codon in the same gene, shows a similar fruit phenotype with cutin load being reduced by 90 to 95% (Yeats et al. 2012). Most importantly, these authors also reported that the CD1 protein, upon heterologous expression in *Nicotiana benthamiana* and purification, was able to polymerize 2-mono(10,16-dihydroxyhexadecanoyl)glycerol in vitro, confirming its acyltransferase activity and fundamental role in cutin polymerization (Yeats et al. 2012). A putative *Arabidopsis* orthologue, AtCUS1/LTL1, was shown to have similarly strong polyester synthase activity but displayed negligible hydrolytic activity (Yeats et al. 2014).

In addition, plants expressing an artificial microRNA that silences *LTL1* show flowers with fused petals devoid of adaxial nanoridges (Shi et al. 2011). The fact that the oligomers synthesized in vitro by cutin synthases are linear may, nevertheless, suggest that other enzymes are responsible for the branching and cross-linking of the polymer.

3 Pathways for Cuticular Wax Biosynthesis

Cuticular wax aliphatic compounds consist of a mixture of VLC molecules ranging from 22 to 38 carbon atoms. Produced by the elongase complexes, VLC acyl-CoAs can be transformed into free VLC fatty acids (VLCFAs) and/or processed through two distinct pathways. The alcohol-forming pathway produces even-numbered primary alcohols and alkyl esters, and the alkane-forming pathway yields aldehydes as well as odd-chain-numbered alkanes, secondary alcohols, and ketones (Fig. 1).

In the last 30 years, visual screens of ethyl methanesulfonate (EMS) mutant libraries identified wax-deficient mutants such as *eceriferum* (*cer*, waxless), *glossy* (*gl*), *bloomless* (*bm*), or *wax crystal-sparse* leaf (*wsl*) in *Arabidopsis*, barley, maize, sorghum, or rice. In *Arabidopsis thaliana*, 89 *cer* mutants were isolated and shown to be affected on 21 independent loci, defining a set of 21 genes with potential function in wax biosynthesis, transport, or regulation (Koornneef et al. 1989). Moreover, new candidate genes with a role in the wax pathways were identified based on gene co-regulation data and gene expression enrichment in the epidermis of young developing organs as compared to the entire organs, a typical expression pattern of wax associated genes, allowing the characterization of the major steps in wax biosynthetic pathways (Lee and Suh 2015).

3.1 Fatty Acid Elongases Produce Very-Long-Chain Fatty Acids

Fatty acid elongation initially uses C16:0 and C18:0 fatty acids resulting from the plastidial de novo synthesis that are esterified to coenzyme A before entering the ER-bound, multienzyme fatty acid elongase (FAE) complexes (Fig. 3). Each FAE cycle catalyzes four successive reactions generating an acyl chain extended by two carbon atoms: formation of β-ketoacyl-CoA by condensation of malonyl-CoA with an acyl-CoA catalyzed by a β-ketoacyl-CoA synthase (KCS), reduction of β-ketoacyl-CoA to β-hydroxyacyl-CoA by a β-ketoacyl-CoA reductase (KCR), dehydration of β-hydroxyacyl-CoA to enoyl-CoA by a β-hydroxyacyl-CoA dehydratase (HCD), and reduction of enoyl-CoA by an enoyl-CoA reductase (ECR).

Studies, including photoperiod and chemical inhibition of elongase activities, initially raised the idea for existence of multiple elongase complexes, each with a distinct chain-length specificity that perform sequential and/or parallel reactions to produce the broad chain-length range of VLCFAs found in plants (von Weisstein-Knowles 1982). The KCR, HCD, and ECR enzymes are thought to have broad substrate specificity and may be shared by all FAE complexes (Kunst and Samuels 2009).

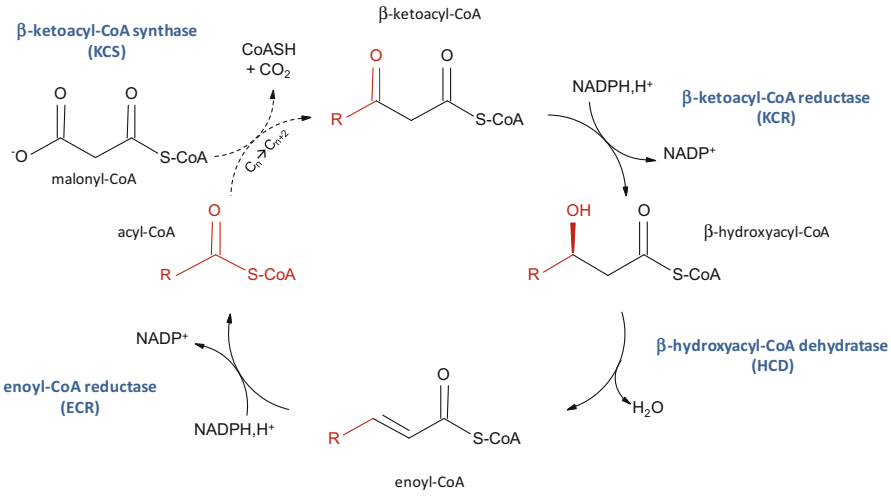

Fig. 3 Fatty acid elongation

In contrast, KCS enzymes determine the chain-length substrate specificity of each elongation reaction.

Consistent with the coexistence of multiple FAE complexes, approximately 20 KCS members have been annotated in several angiosperm genomes (Guo et al. 2016). Expression pattern analyses and heterologous expression in yeast of a subset of the 21 *Arabidopsis* KCS indicated that some of them have overlapping functions, or they are specialized to particular environmental conditions (Joubès et al. 2008; Haslam and Kunst 2013). To date, several KCS have been shown to be involved in cuticle precursor synthesis (KCS1, KCS2, KCS5/CER60, KCS6/CER6, KCS9, KCS10/FDH, KCS13/HIC, KCS16, and KCS20) (Haslam and Kunst 2013; Hegebarth et al. 2017). Nevertheless, based on expression patterns and mutant phenotypes, KCS5/CER60 and KCS6/CER6 appear as major actors involved in the elongation of fatty acids longer than C_{26} for the production of cuticular waxes (Haslam et al. 2015). However, KCS are not the only factors that dictate elongation of VLCFAs, as the functional characterization of the *Arabidopsis* and rice CER2-LIKE proteins has suggested a role of these proteins in the regulation of FAE activities for the elongation of VLCFAs (Haslam et al. 2012, 2015; Pascal et al. 2013; Wang et al. 2017). As such, they could facilitate the formation of VLC acyl-CoAs by stabilizing the FAE complexes, by enhancing their activity, or by allowing the newly elongated acyl-CoA to be presented back to the KCS enzyme after an elongation cycle. However, further work is needed to determine the mode of action of these proteins. The *Arabidopsis* PAS1 protein is another example of a FAE complex regulator (Roudier et al. 2010). PAS1 is a member of the immunophilin family of chaperones that are known to target protein complexes and regulate their

assembly or activity. Similar to a loss of one of the elongase core components, a defect in PAS1 was shown to reduce the amounts of all VLCFA-containing lipids.

In the last decade, major findings on VLCFA synthesis in yeast allowed the identification of the corresponding KCR, HCD, and ECR enzymes in *Arabidopsis*. Complementation assays of yeast mutants revealed that *KCR1* and *PASTICCINO2* (*PAS2*) encode a functional KCR and HCD, respectively (Bach et al. 2008; Beaudoin et al. 2009). Complementation of the *Arabidopsis cer10* mutant and the yeast *tsc13-1elo2*Δ mutant by expression of *AtECR* demonstrated that *CER10* encodes a functional ECR (Zheng et al. 2005). Consistent with the assumption that the three enzymes are common to all FAE complexes, the total loss of KCR1 or PAS2 is embryo lethal, whereas the loss of CER10 and partial loss of KCR1 or PAS2 activity result in a severe reduction of all major classes of VLCFA-containing lipids (waxes, triacylglycerols, and sphingolipids) causing major developmental impairment (Zheng et al. 2005; Bach et al. 2008; Beaudoin et al. 2009).

3.2 Alcohol-Forming Pathway

The alcohol-forming pathway, also called reduction pathway, produces even-numbered primary alcohols and alkyl esters (Fig. 4).

The first biochemical studies of primary alcohol formation suggested a two-step reaction in which fatty acyl-CoA reductase (FAR) reduces VLC acyl-CoAs to aldehydes, which are further reduced to primary alcohols by an aldehyde reductase (Kolattukudy 1971). However, biochemical studies on jojoba seeds and pea leaves, as well as expression of genes encoding alcohol-forming activities in heterologous systems, revealed that a single enzyme produced fatty alcohols, with the intermediate aldehyde remaining bound to the enzyme (Rowland and Domergue 2012). In *Arabidopsis*, the *cer4* mutant shows a severe reduction of primary alcohols and wax esters, suggesting that CER4 (also called FAR3) could play a role in this biosynthetic pathway (Jenks et al. 1995). Expression of CER4 in yeast results in the production of VLC primary alcohols confirming the FAR activity of CER4 (Rowland et al. 2006).

Detailed analysis of wax ester chain lengths from the stems of *Arabidopsis cer4* mutants indicated that primary alcohols formed by CER4 are substrates for subsequent alkyl ester formation (Lai et al. 2007). Wax synthase (WS) enzymes catalyze the esterification of primary alcohols to acyl-CoAs in higher plants, mammals, and bacteria (Lardizabal et al. 2000; Cheng and Russell 2004; Stoveken et al. 2005). In *Arabidopsis*, the search for sequences similar to the jojoba WS and bifunctional WS/diacylglycerol acyltransferases (DGATs) from *Acinetobacter calcoaceticus* revealed 12 and 11 sequences, respectively. Analysis of a WS/DGAT-encoding gene (*WSD1*), which is highly expressed in the epidermis, subsequently confirmed its involvement as the major WS in cuticular wax synthesis (Li et al. 2008).

3.3 Alkane-Forming Pathway

The alkane-forming pathway, also called the decarbonylation pathway, produces aldehydes and odd-numbered alkanes, secondary alcohols, and ketones (Fig. 4).

Analyses of *Arabidopsis cer* mutants and complementary biochemical experiments proposed an alkane-forming pathway in which VLC acyl-CoAs are used as precursors to form alkanes through aldehydes (Bernard and Joubès 2013). However, how even-numbered VLCFAs are transformed into odd-numbered alkanes remains an intriguing question, although significant advances have been made recently in the characterization of the alkane-forming complex (Bernard et al. 2012). Initially, biochemical analyses led to the proposal that alkanes could be produced from VLCFAs through the formation of an intermediate aldehyde (Cheesbrough and Kolattukudy 1984; Dennis and Kolattukudy 1991; Vioque and Kolattukudy 1997; Schneider-Belhaddad and Kolattukudy 2000). Although this hypothesis has never been demonstrated for plants, it is the only one that has been experimentally tested in microsomal fractions from pea leaves (Vioque and Kolattukudy 1997) and from the green alga *Botryococcus braunii* (Cheesbrough and Kolattukudy 1984; Dennis and Kolattukudy 1991; Schneider-Belhaddad and Kolattukudy 2000). Contrary to the NADPH-cytochrome P450 reductase mechanism of hydrocarbon synthesis in insects, in which aldehydes are converted to alkanes by a decarboxylation mechanism that releases a CO_2 molecule (Reed et al. 1994; Qiu et al. 2012), the coproduct of the aldehyde conversion in pea and *Botryococcus braunii* was a CO molecule

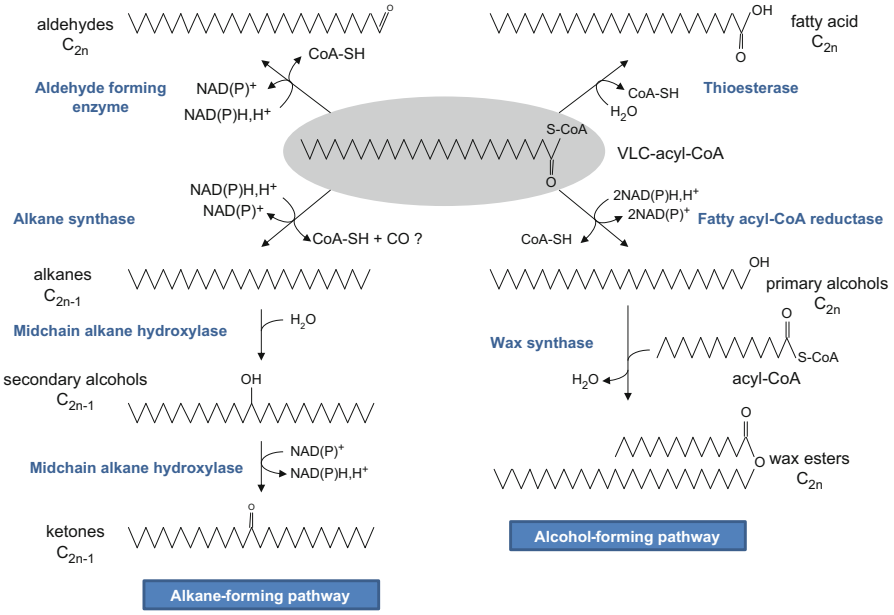

Fig. 4 Cuticular wax compounds biosynthetic pathways

suggesting a decarbonylation mechanism. Therefore, it was proposed for plants that VLC acyl-CoAs are reduced by a fatty acyl-CoA reductase into corresponding even-numbered aldehydes, which are converted by an aldehyde decarbonylase into alkanes with the loss of one carbon atom.

Several *Arabidopsis cer* mutants with a decreased alkane load have been biochemically characterized. The *cer3* mutant shows a dramatic reduction in aldehydes, alkanes, secondary alcohols, and ketones. The *cer1* mutant exhibits a dramatic decrease in alkanes and a near abolition of secondary alcohol and ketone production, accompanied by a slight increase in aldehyde content (Aarts et al. 1995; Chen et al. 2003; Kurata et al. 2003; Bourdenx et al. 2011). It has been proposed from these phenotypes that CER3 may encode the VLC-acyl-CoA reductase that produces aldehydes, whereas CER1 may encode the alkane-forming enzyme that catalyzes the presumed decarbonylation of aldehydes to alkanes. Wax analyses of CER1 over-expressors have revealed a specific increase in alkanes with chain lengths of between 27 and 33 carbon atoms, which is consistent with CER1 encoding an alkane-forming activity with a strict substrate specificity for compounds containing more than 27 carbon atoms (Bourdenx et al. 2011). Recently, the proof that CER1 and CER3 act synergistically as a heterodimer complex that catalyzes the conversion of VLC-acyls-CoAs to alkanes, with the intermediate aldehyde remaining bound to the complex, was provided by co-expression of the two proteins in yeast (Bernard et al. 2012).

Early characterization of the aldehyde decarbonylase partially purified from pea leaves indicated that this activity requires metal ions and is inhibited by 0_2 and reducing powers, suggesting that the reaction would be redox independent (Schneider-Belhaddad and Kolattukudy 2000). Nevertheless, CER1 was shown to interact with the ER-localized cytochrome b_5 (CYTB5), and co-expression of CYTB5-B with the CER1/CER3 complex in yeast was shown to increase VLC-alkane production. This suggests that CYTB5 is a redox cofactor of the CER1/CER3 alkane-forming activity (Bernard et al. 2012). The observation that CER1 contains catalytic histidine-clusters typical of a di-iron-binding site and that CYTB5 physically interacts with CER1 highlights the analogy between CER1 and the cyanobacterial aldehyde decarbonylase. This latter is structurally related to nonheme di-iron enzymes, uses iron as the only active metal for its activity, and appears to catalyze a redox-neutral aldehyde decarbonylase reaction even though a reducing system is strictly required (Warui et al. 2011; Das et al. 2011). Experimental evidence and functional analogy to cyanobacterial aldehyde decarbonylase strongly suggest that CER1 serves as the alkane-forming enzyme even though the enzymatic activity of the protein is still unknown. On the other hand, as neither aldehydes nor other potential intermediates were detected in yeast expressing CER3 alone, or co-expressed with CER1, the role of CER3 in VLC-alkane synthesis remains unknown.

As mentioned above, alkanes can be further modified, for instance, in *Arabidopsis* stems, by consecutive oxidation to produce secondary alcohols and, subsequently, ketones (Fig. 4). Evidence for alkanes as precursors of these compounds has been provided by the following experiments. Feeding experiments have

demonstrated that alkanes and secondary alcohols can be transformed into ketones in *Brassica oleracea* (Kolattukudy et al. 1973). There exists an apparent correlation between secondary alcohols, ketones, and alkane chain lengths in *Arabidopsis*, as well as mutant phenotypes showing a deficit in alkanes accompanied by a similar deficit in these compounds (Aarts et al. 1995; Chen et al. 2003). By looking for genes upregulated in the *Arabidopsis* stem epidermis, and which encode proteins potentially involved in lipid oxidation, a cytochrome P450 encoding gene, *CYP96A15*, was identified as a candidate for a catalytic role in secondary alcohol and ketone formation (Greer et al. 2007). Ectopic expression of *CYP96A15* in *Arabidopsis* leaves yielded the production of secondary alcohols and ketones demonstrating that CYP96A15 functions as a mid-chain alkane hydroxylase (MAH1) that can produce secondary alcohols and catalyze their subsequent oxidation into ketones (Greer et al. 2007).

3.4 Other Compounds

Wax mixtures typically contain homologous series of VLC saturated alkanes or alcohols derived from the alkane- and alcohol-forming pathways, but VLC acyl-CoAs that are produced by the FAE complexes can be also converted to free VLCFAs or aldehydes (Fig. 4). Indeed, detection of significant amounts of homologous series of these compounds in extracellular lipids in almost all plant wax mixtures indicated that at least a proportion of VLC acyl-CoAs can be converted to VLCFAs by an unknown, ER-localized VLC-acyl-CoA thioesterase. The other possibility is that they are reduced to aldehydes, potentially independently, from the alcohol- or alkane-forming pathway, by an unknown aldehyde-forming enzyme (Fig. 4).

Furthermore, wax compounds with modified carbon chains have been reported in many species. Iso- and anteiso-alkanes or alcohols have been found in several *Brassicaceae* or *Solanaceae* species (Busta and Jetter 2017), alkanes with within-chain methyl branches or cyclopropyl rings have been identified in barley (von Wettstein-Knowles 2007), and β-diketones have been found in waxes of many *Gramineae* species (von Wettstein-Knowles 2012). However, the biochemical reactions yielding these unusual wax components and the sequence of the corresponding metabolic steps are not fully understood, and the enzymes involved in these biosynthetic pathways remain uncharacterized.

4 Export of Cutin Monomers and Wax Compounds

As indicated by the subcellular localization of the cuticular biosynthetic pathway enzymes, it is now well established that the biosynthesis of the different cuticular compounds occurs in the endoplasmic reticulum (Fig. 1). The recent use of *Arabidopsis* mutants defective in vesicle trafficking and protein secretion suggests that the transfer of these hydrophobic molecules through the hydrophilic cytoplasm

involves vesicles that transit through the ER-Golgi interface and the trans-Golgi network to deliver cargo to the plasma membrane (Fig. 1) (McFarlane et al. 2014).

Once the cuticle compounds have reached the plasmalemma, their export is carried out by ABC transporters (ATP-binding cassette transporters). The gene encoding the first ABC transporter identified in *Arabidopsis* facilitating wax transport was ABCG12/CER5 (Pighin et al. 2004). A search for ABC protein-encoding genes with an expression pattern similar to ABCG12 revealed ABCG11 as a candidate for wax export (Bird et al. 2007; Panikashvili et al. 2007). The *abcg11* mutant and the double mutant *abcg11 abcg12* show similar wax composition, suggesting that both transporters act in the same pathway or ABC transporter unit. Furthermore, *abcg11* showed organ fusions, defects in cuticle permeability, and a reduced cutin load, indicating that ABCG11 is also involved in cutin monomer export (Panikashvili et al. 2007, 2010). ABCG11 and ABCG12 are half-transporters, and, whereas ABCG11/ABCG12 heterodimers have a function in wax export, ABCG11 may also homodimerize or heterodimerize with unknown component (s) to transport cutin monomers (McFarlane et al. 2010). However, residual export of waxes and cutin monomers onto the plant surface in the absence of ABCG11 and ABCG12 indicates that other ABC transporters might also export these compounds. Recently, several other ABCG transporters have been characterized in *Arabidopsis*. ABCG13, which is closely related to ABCG11 and ABCG12, contributes to cutin formation in flowers (Panikashvili et al. 2011). ABCG9 and ABCG14 can also form dimers with ABCG11, and these, as well as ABCG31, affect sterol ester levels in vegetative tissues or pollen grains (Le Hir et al. 2013; Choi et al. 2014). In contrast, ABCG32/PEC1, which is a full-length transporter, is required for hydroxy-fatty acid transport in leaves and flowers (Bessire et al. 2011).

Based on transcriptome analysis of *Arabidopsis* stems and stem epidermal cells, seven candidate LTPs (lipid transfer proteins), which could play a role in cuticular precursor transport, were isolated (Suh et al. 2005). The cuticle phenotype of *ltpg1* and *ltpg2* mutants suggests that both of these glycosylphosphatidylinositol-anchored LTPs (LTPGs) could be involved in cuticle formation (DeBono et al. 2009; Lee et al. 2009; Kim et al. 2012). However, the subtle changes in wax composition observed in *ltpg1* or *ltpg2* mutants have given rise to the proposition that multiple, specialized LTPs, but with overlapping functions, are required to deliver the entire diversity of cuticle compounds to the epidermal surface.

5 Research Needs

Over the last years, significant progress has been made in the understanding of the molecular and biochemical mechanisms underlying cuticle biosynthesis. These advances have greatly benefitted from the development of molecular reverse and forward genetic tools in different species and the simultaneous development of analytical methods for detailed lipid characterization. However, several processes still remain to be clarified, especially those following:

1. The exact order of the reactions involved in cutin monomer synthesis needs to be unambiguously established. For this purpose, it will be important to establish when LACS activity is exactly required, to determine the substrates of the different cytochrome P450 enzymes identified thus far, and to discover the enzymes responsible for the other reactions of the proposed pathways.
2. The processes whereby VLC acyl-CoAs are generated and subsequently converted to the VLC aliphatic wax compounds found on the surfaces of many plant species are now well understood. However, the catalytic activities of several enzymes involved in these metabolic pathways have to be characterized in more detail. The activities of the KCS subunits of the different FAE complexes and the regulation of these activities, for example, by the CER2-LIKE proteins, have to be puzzled out. Similarly, the exact enzymatic activity of the alkane-forming complex remains to be solved. Furthermore, the enzymes involved in the biosynthesis of free VLCFAs and aldehydes must be identified and characterized.
3. As many unusual compounds are found in the plant wax mixtures, the biosynthetic pathways and the related genes or enzymes involved in their generation have to be discovered. The development of molecular and biochemical tools to characterize the synthesis of these components in species that accumulate them will be a major challenge for the next few years.
4. If some information concerning the transport of cuticular compounds across the plasma membrane are now available, future reporting must take into account the intra- and extracellular transport of the cuticular components to provide a cell-wide understanding of these processes.

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