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Role of very-long-chain fatty acids in plant development, when chain length does matter

*Rôle des acides gras à très longues chaînes dans le développement des plantes, une question de longueur de chaîne*Liên Bach^{a,b}, Jean-Denis Faure^{a,*,b}^a Inra-AgroParisTech, institut Jean-Pierre-Bourgin, UMR1318, route de St. Cyr (RD10), 78026 Versailles cedex France^b Inra, centre de Versailles-Grignon, route de St. Cyr (RD10) 78026 Versailles cedex, France

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ABSTRACT

Very long chain fatty acids (VLCFAs) are essential components for eukaryotes. They are elongated by the elongase complex in the endoplasmic reticulum and are incorporated into four major lipid pools (triacylglycerols, waxes, phospholipids, complex sphingolipids). Functional analysis of several components of the elongase complex demonstrated the essential role of VLCFAs in plants, invertebrates and vertebrates. Although VLCFAs changes in the triacylglycerol pool has no consequence for plant development, modifications of the nature and levels of VLCFAs in waxes, phospholipids and complex sphingolipids have, collectively, profound effects on embryo, leaf, root and flower development. VLCFAs levels in epicuticular waxes are critical for the regulation of epidermal fusions during organogenesis. VLCFAs phospholipids and sphingolipids are involved in membrane structure and dynamics regulating cell size but also division and differentiation. This review summarizes the recent findings in plants but also in other organisms, highlighting the importance of very long acyl chain length during development.

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R É S U M É

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Les acides gras à très longues chaînes (AGTLC) sont des composants essentiels des organismes eucaryotes. Ces AGTLC sont allongés par le complexe elongase au niveau du reticulum endoplasmique et sont ensuite incorporés dans quatre classes majeures de lipides (les triacylglycérols, les cires, les phospholipides et les sphingolipides complexes). Différentes analyses fonctionnelles des enzymes du complexe elongase ont mis en évidence le rôle essentiel des AGTLC dans les plantes, les invertébrés et les vertébrés. Bien qu'une modification de la teneur en AGTLC dans les triacylglycérols n'ait aucune conséquence au niveau du développement de la plante, la nature et les teneurs en AGTLC dans les cires, les phospholipides et les sphingolipides complexes peuvent conduire à de profondes altérations au niveau du développement de l'embryon, de la feuille ainsi que de la fleur. Les teneurs en AGTLC dans les cires épicuticulaires sont critiques pour la régulation des fusions épidermiques au cours de l'organogénèse. De plus, les AGTLC dans les phospholipides et les sphingolipides complexes sont impliqués dans la structure et la

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dynamique membranaire régulant la taille cellulaire mais aussi la division et la différenciation. Dans cette revue, nous résumons les récents résultats mettant en évidence l'importance des AGTLC dans le développement des plantes mais aussi dans le développement d'autres organismes.

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Abbreviations

VLCFAs	very long chain fatty acids
KCS	3-keto-acyl-CoA synthase
KCR	3-keto-acyl-CoA reductase
HCD	3-hydroxy-acyl-CoA dehydratase
ECR	trans 2,3-enoyl-CoA reductase
TAG	triacylglycerol
PCD	programmed cell death
LCB	long chain base
DHS	dihydrosphingosine
3-KDHS	3-ketodihydrosphingosine
PE	phosphatidylethanolamine
PC	phosphatidylcholine
PS	phosphatidylserine
PG	phosphatidylglycerol
MGDG	monogalactosyl-diacylglycerol
DGDG	digalactosyl-diacylglycerol

1. VLCFAs: introduction

Very long chain fatty acids (VLCFAs) are fatty acids with an acyl chain of 18 carbons and longer. The chain length, the degree of unsaturation, the type of polar head and the associated lipids provide the structural and functional diversity of these fatty acids. In plants, VLCFAs are found in triacylglycerols as seed storage lipids. They are involved as membrane constituents and signalling molecules in sphingolipids and phospholipids. Finally, VLCFAs are necessary for the production of cuticular waxes and suberin, two extracellular biopolymers preventing plants from desiccation or external aggressions.

VLCFAs are involved in similar functions in animals, i.e. storage lipids in adipose tissues, the protective lipid barrier of the skin and membrane structural components. Genetic studies on VLCFAs synthesis during the last 10 years are now providing new insights on the cellular functions of VLCFAs. In this review, we will mainly focus on the involvement of VLCFAs in plant development.

2. Biosynthetic pathway and enzymes

VLCFAs are synthesized by the sequential addition of two carbons through four successive enzymatic reactions gathered in the endoplasmic reticulum within a protein complex named the elongase (Fig. 1). The first step of fatty elongation is the condensation of a long chain acyl-CoA with a malonyl-CoA by the 3-keto-acyl-CoA synthase (KCS or condensing enzymes). The resulting 3-keto-acyl-CoA is then reduced by a 3-keto-acyl-CoA reductase (KCR) generating a 3-hydroxy-acyl-CoA. The third step is the dehydration of the 3-hydroxy-acyl-CoA by a 3-hydroxy-acyl-CoA dehydratase (HCD) to a trans-2,3-enoyl-CoA,

which is finally reduced by the trans 2,3-enoyl-CoA reductase (ECR) to yield a two-carbon elongated acyl-CoA. Once acyl-CoA has been elongated from the elongase complex, they can be incorporated into different lipid classes.

VLCFAs are usually elongated from pre-existing C18 acyl-CoAs produced by the cytosolic fatty acid synthase complex (FAS). However, it has been recently demonstrated that C16-CoA could be also a substrate for the KCS since a mutation in the dehydratase gene led to the accumulation of 3-hydroxyC18-CoA [1].

In yeast and mammals, the KCS proteins are encoded by the multigenic *ELO* gene family. These enzymes were found to have different substrate specificity that depends on their chain length and their degree of unsaturation [2]. For instance in yeast, *Elop2* is responsible for the elongation of VLCFAs up to C24, while *Elop3* is crucial for the conversion of C24 to C26 [3]. In Arabidopsis genome, KCS enzymes are encoded by an *ELO*-like family of four putative condensing enzymes and 21 *FAE1*-like *KCS* genes [4,5] (Table 1). Although *FAE1*-like enzymes are structurally unrelated to the *ELO* enzymes, it has been shown that several *FAE1*-like *KCS* can rescue lethality of the yeast $\Delta elo2/\Delta elo3$ double mutant [2]. Two condensing enzymes were characterized in details: *FATTY ACID ELONGATION 1* (*FAE1*), which encodes a condensing enzyme responsible for the C20 and C22 fatty acid elongation involved in seed triacylglycerol [6] and *CER6*, which catalyzes the elongation of fatty acids longer than C22 specifically in epidermal cells [7,8]. The *KCS CER6* [7], *KCS1* [9] and *FIDDLEHEAD* (*FDH*) [10,11] were found to be involved in the synthesis of VLCFAs precursors of shoot waxes: a complete loss of function of *CER6* and *KCS1* resulted in accumulation of C24 wax derivatives suggesting a potential role of these enzymes in C24 elongation. Similarly, *fdh* mutant presented elevated levels of C20:0 and C22:0 fatty acids in *fdh*-derived cell wall material [12]. Recently, two redundant *KCS*, *KCS20* and *KCS2/DAISY*, have been shown to be necessary for C20 to C22 elongation in the biosynthesis of cuticular waxes and root suberin [13]. In conclusion, the *KCS* multigene family is associated with substrate specificity mainly on the basis of chain length (C20–C22 vs C26+ for instance), but also with the nature of the downstream VLCFAs lipid pool that depends on the tissue-expression pattern (*FAE1* and triacylglycerols vs *DAISY* and suberin).

The 3-ketoacyl-reductase protein is encoded by a single gene in yeast named *YBR159w* and by two genes in maize *GL8A* and *GL8B*. The *gl8agl8b* double mutant was found to be embryo lethal, demonstrating the essential role of VLCFAs in plant development. Analysis of single mutants showed that *KCR* activity is required for cuticular wax production [14]. In Arabidopsis, two *KCR* homologs, *KCR1* and *KCR2*, have also been identified, but only *KCR1* has been demonstrated to be involved in the fatty acyl

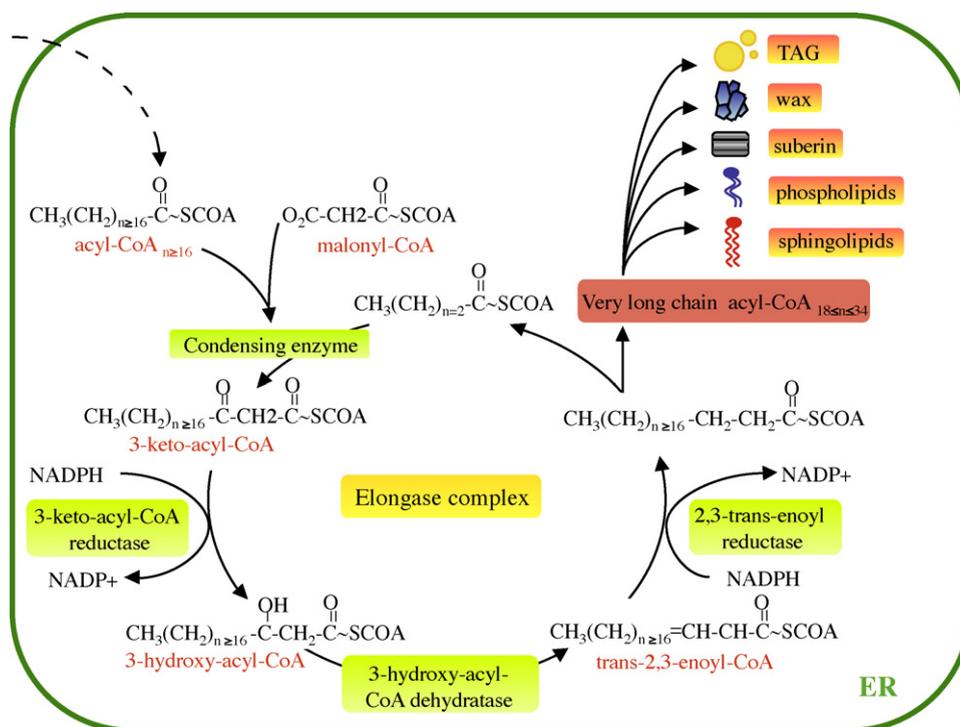


Fig. 1. Very long fatty acid elongation cycle. VLCFAs are elongated from long chain acyl-CoA ($n \geq 16$) by the ER-associated elongase complex. Each two carbon cycle requires four successive enzymatic steps, the condensation of the malonyl-CoA with the acyl-CoA, reduction of the 3-keto-acyl-CoA, dehydration of the 3-hydroxyacyl-CoA and the reduction of the trans-2,3-acyl-CoA. Finally, the enoyl-CoA gives rise to the formation of very long chain acyl-CoA. ER: enoplasmic reticulum.

elongase complex since it complemented the *ybr159w* yeast mutant. *kcr1* null alleles are also embryo lethal in Arabidopsis [15].

The dehydratase gene was only discovered recently in yeast as the *YJL097w/PHS1* gene [16]. This gene is essential for cell viability mainly during cell division since weak *phs1* allele showed G2/M transition defects. Like for *KCR*, Arabidopsis genome contains two *PHS1* homologs, *PAS-TICCINO2 (PAS2)* and At5g59770. *PHS1* expression complements *pas2-1* defects, however, only *PAS2* was able to rescue *phs1* lethality [1] (Fig. 2A). A complete loss of *PAS2* activity led also to embryo lethality stressing the essential role of VLCFAs (Fig. 2B). Partial loss of *PAS2* function led to the general reduction of VLCFAs in triacylglycerols, complex sphingolipids and cuticular waxes while it accumulates long chain bases and precursors of sphingolipids. Biochemical phenotype was associated with strong developmental defects. Surprisingly, whereas *KCS* enzyme was thought to be the only limiting step for VLCFAs elongation, the overexpression of dehydratase activity in Arabidopsis led to enhanced VLCFAs mainly in epicuticular waxes suggesting that several limiting enzymatic steps were present among the elongase complex [1].

The last reduction step in the elongase complex is carried out by the trans-2,3-enoyl-reductase *TSC13* gene in yeast and was so far associated with only one gene in Arabidopsis named *CER10* [17]. Like *PAS2*, the absence of *ECR* activity resulted in general reduction of VLCFAs. Even though *CER10* is the only ortholog of the yeast *TCR13 ECR*, this gene is not essential unlike *KCR1* or *PAS2*, indicating

the potential existence of functionally equivalent isoforms of *ECR* in Arabidopsis [15].

Once elongated, the very long chain acyl-CoA is incorporated into various lipid pools comprising triacylglycerols, epicuticular waxes, phospholipids and sphingolipids. The relative roles of these different pools for plant growth and development are variable, but recent contributions highlight the importance of VLCFAs and the potential role of fatty acyl chain length in plant development (Table 1).

3. TAG are not involved in plant development

The triacylglycerols (TAG) are formed by the Kennedy pathway from a single molecule of glycerol, in which each hydroxy group is acylated with up to three acyl chains ranging from eight to 26 carbons long. They are stored in oleosomes during the embryo or/and endosperm maturation. These lipids act as a reserve of energy for germinating seeds and they can represent about half of the total seed dry weight. In the majority of seeds, acyl groups from TAG are similar to those found in membrane lipids. For instance, the most abundant fatty acids in seeds are palmitic acid (16:0), stearic acid (18:1), oleic acid (18:0), linoleic acid (18:2) and linolenic acid (18:3). However, VLCFAs can be also present in TAG in significant amount. Members of Brassicaceae family for instance preferentially incorporate fatty acids from mono-insaturated VLCFAs. Millar et al. have determined the seed oil fatty acyl composition for 100 different Arabidopsis accessions [18]. They showed that all

Table 1
Arabidopsis elongase genes.

Gene names	Others names	Arabidopsis accessions	Yeast homologs	Biochemical function ^a	Activity ^b	Phenotype ^c
<i>KCS1</i>	<i>KCS1</i>	At1g01120	–	3-keto-acyl-CoA synthase	[50,51]	Growth retardation [9]
<i>KCS2</i>	<i>DAISY</i>	At1g04220	–	Wax/suberin biosynthesis (c26) [9] 3-keto-acyl-CoA synthase Wax/suberin biosynthesis (c22) [13]	[8,51]	Glossy appearance [13] Root growth retardation/Suberin layer alteration
<i>KCS3</i>	–	At1g07720	–	Putative 3-keto-acyl-CoA synthase	–	–
<i>KCS4</i>	–	At1g19440	–	Putative 3-keto-acyl-CoA synthase	–	–
<i>KCS5</i>	<i>CER60</i>	At1g25450	–	Putative 3-keto-acyl-CoA synthase	[51]	–
<i>KCS6</i>	<i>CER6, CUT1</i>	At1g68530	–	3-keto-acyl-CoA synthase Wax/suberin biosynthesis [7,52,53]	–	Glossy appearance/ Reduced fertility [7,51,53]
<i>KCS7</i>	–	At1g71160	–	Putative 3-keto-acyl-CoA synthase	–	–
<i>KCS8</i>	–	At2g15090	–	Putative 3-keto-acyl-CoA synthase	–	–
<i>KCS9</i>	–	At2g16280	–	Putative 3-keto-acyl-CoA synthase	[8]	–
<i>KCS10</i>	<i>FDH</i>	At2g26250	–	Putative 3-keto-acyl-CoA synthase	–	Postgenital organ fusions/Reduced fertility Trichome defects [10,12]
<i>KCS11</i>	–	At2g26640	–	Putative 3-keto-acyl-CoA synthase	–	–
<i>KCS12</i>	–	At2g28630	–	Putative 3-keto-acyl-CoA synthase	–	–
<i>KCS13</i>	<i>HIC</i>	At2g46720	–	Putative 3-keto-acyl-CoA synthase	–	Stomatal density augmentation in response to elevated CO ₂ [54]
<i>KCS14</i>	–	At3g10280	–	Putative 3-keto-acyl-CoA synthase	–	–
<i>KCS15</i>	–	At3g52160	–	Putative 3-keto-acyl-CoA synthase	–	–
<i>KCS16</i>	–	At4g34250	–	Putative 3-keto-acyl-CoA synthase	–	–
<i>KCS17</i>	<i>KCS2</i>	At4g34510	–	Putative 3-keto-acyl-CoA synthase	[50,51]	–
<i>KCS18</i>	<i>FAE1</i>	At4g34520	–	3-keto-acyl-CoA synthase Seed storage lipids (c20/c22) [6]	[2,8,50,55]	–
<i>KCS19</i>	–	At5g04530	–	Putative 3-keto-acyl-CoA synthase	–	–
<i>KCS20</i>	–	At5g43760	–	3-keto-acyl-CoA synthase Wax/suberin biosynthesis (c22) [13]	[8,51]	Glossy appearance [13] Root growth retardation/Suberin layer alteration
<i>KCS21</i>	–	At5g49070	–	Putative 3-keto-acyl-CoA synthase	–	–
<i>Elop-like</i>	<i>FEN1</i>	At3g06460	ELO2	Unknown	–	–
<i>Elop-like</i>	<i>FEN1</i>	At3g06470	ELO2	Unknown	–	–
<i>Elop-like</i>	<i>GNS1/SUR4</i>	At1g75000	ELO3	Unknown	–	–
<i>Elop-like</i>	<i>GNS1/SUR4</i>	At4g36830	ELO3	Unknown	–	ABA hypersensitivity (reduced root growth inhibition reduced germination) reduced water loss from leaves [56]
<i>KCR1</i>	<i>KCR1</i>	At1g67730	YBR159w	3-keto-acyl-CoA reductase General reduction VLCFA content [15]	–	Embryo lethality (null allele) Glossy appearance/postgenital organ fusions Trichome defects Lack of lateral roots/reduced hair roots [15]
<i>KCR2</i>	<i>KCR2</i>	At1g24470	YBR159w	Unknown	–	–
<i>HCD</i>	<i>PAS2</i>	At5g10480	PHS1	3-hydroxy-acyl-CoA dehydratase General reduction VLCFA content 3-hydroxy-acyl-CoA increase [1]	–	Embryo lethality (null allele) Glossy appearance/Postgenital organ fusions Cell proliferation defects [1]
<i>HCD2</i>	<i>PAS2-like</i>	AT5G59770	PHS1	Unknown	–	–
<i>ECR</i>	<i>ECR10</i>	At3g55360	TCS13	Trans-2,3-enoyl-CoA reductase General reduction VLCFA content [17]	–	Glossy appearance/Postgenital organ fusions/Reduced fertility Trichome defects Cell expansion defects [17]

^a Biochemical function is indicated (with acyl-chain specificity).

^b Activity: when enzymatically active KCS is demonstrated a reference is provided.

^c Phenotype of mutant or RNAi lines.

Arabidopsis accessions contained a significant proportion of VLCFAs averaging 31% w/w of total seed fatty acid. The most abundant and specific VLCFAs in TAG are the eicosenoic acid C20:1 (20% of the total seed fatty acid) and the erucic acid C22:1 (2% of the total seed fatty acid).

To date, no real physiological or biochemical role of VLCFAs in TAG composition has been demonstrated. The *fae1* mutant, which contains less than 1% w/w of VLCFAs [6], is still able to germinate. Interestingly, even if *FAE1* gene expression is restricted to the seed, *fae1* mutations modified vegetative development with early flowering transition and slower leaf growth [19]. Similarly, *Canola* rapeseed varieties

are completely devoid in VLCFAs in their seed oil composition and do not show significant modification in terms of growth. Thus, these results suggest that the VLCFAs in TAG are not essential for plant development.

In animals, VLCFAs are also used to generate energy since they can be oxidized by beta-oxidation in peroxisomes and by the cytochrome P450 CYP4A. Several studies have demonstrated that an accumulation of VLCFAs in the liver resulting from a disrupted VLCFAs oxidation can be an important cause of disease [20]. Deficient mice in peroxisomal fatty acyl-CoA oxidase (AOX) present hepatic steatosis and steatohepatitis.

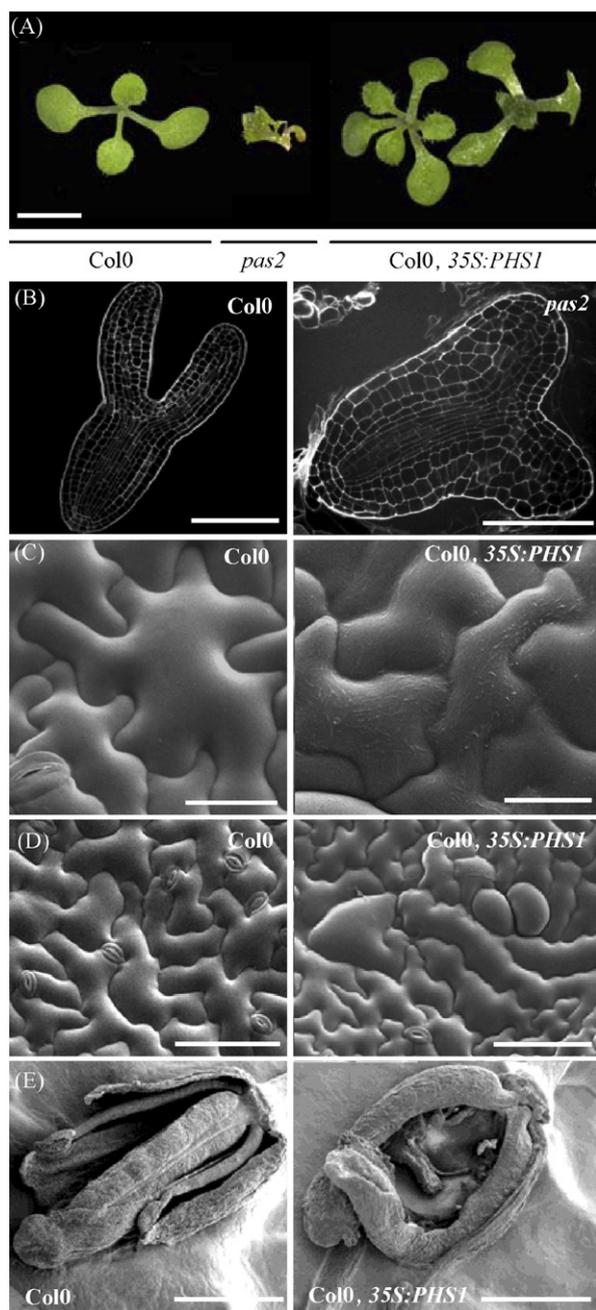


Fig. 2. PAS2 is essential for plant development. A. *pas2* mutation and expression of 35S:PHS1 lead to abnormal leaf morphology (fusions, left) and phyllotaxy (triple cotyledons, right) (scale bar, 1 mm). B. The *pas2* embryo (right) at the torpedo stage showed defective cotyledon development compared to wild-type (left) (scale bar, 40 μ m). Embryos were fixed and stained with propidium iodide and imaged with a scanning confocal microscope. C. Scanning electron microscopy (SEM) showed increase of epicuticular waxes in *PHS1* expressing epidermal cells (right) compared to wild-type (left) (scale bar, 100 μ m). D. *PHS1*-expressing plants have an increased wax load on epidermal cell surface (right) compared to wild type (left) (scale bar 50 μ m). E. *PHS1*-expressing plants showed unfused carpels (right) compared to wild type (left) (scale bar, 1 mm).

4. VLCFAs in extracellular biopolymers and development

The cuticle forms a hydrophobic barrier, which covers the aerial parts of land plants. The cuticle consists of a polyester matrix of C16 or C18 hydroxy and epoxy-hydroxy-fatty acids named cutin embedded and overlaid by cuticular waxes. These waxes are made by a mixture of very long chain lipids of 26 to 34 carbons long such as primary and secondary alcohols, aldehydes, alkanes, ketones and esters.

Elongated acyl-CoA are substrates of two distinct biosynthetic pathways leading to wax synthesis: the decarbonylation pathway and the reduction pathway. The decarbonylation pathway begins with the production of aldehydes from acyl-CoA by the acyl-CoA reductase. Then, aldehydes are decarbonylated by an aldehyde decarbonylase to produce alkanes with odd-chains. These alkanes are then hydroxylated to form secondary alcohols, which are finally oxidized in ketones [21]. The reduction pathway is used for primary alcohols and waxes ester synthesis. Several reports showed that primary alcohols are directly synthesized from acyl-CoA by an acyl-CoA reductase. These primary alcohols can then be combined with free fatty acids to form wax esters by an acyl-CoA alcohol transferase.

Specific acyl chain length predominates in each lipid class conferring different physical/chemical properties to waxes, in particular modifying their form and appearance. For instance, *Arabidopsis* stems show a crystalline wax structure whereas leaves have a thin wax layer. There is also a report suggesting that similar crystalline wax structure may originate from different lipid compositions [22].

The proportion of the major classes differs among plant species, and also among tissues and organs. For instance, alkanes and ketones are mainly found in leek leaves and in *Arabidopsis* stems, whereas they are undetectable in maize leaves. Specific differences can even be found between the adaxial and abaxial leaf surface of *Pisum sativa* that show 10-fold difference in the levels of alkanes [23]. Finally, wax composition depends on development stages. Young maize leaves have 63% of primary alcohols whereas old leaves have 42% of predominantly wax esters [24].

In many plants, cuticular waxes confer a glaucous or grayish appearance on the plant surface. This phenotype led to a powerful genetic screen that allowed the isolation of the *eciferum* mutants (*cer*) characterized by reduced epicuticular waxes. Several mutants turned out to be mutations in elongase genes. Indeed, *cer6* mutant abolished wax production on *Arabidopsis* stems [7] and *cer10* mutant showed 60% reduction of wax load [17]. Although the rest of the elongase mutants have not been identified as *cer* mutants, they also exhibit a waxless phenotype correlated with reduced wax loads. The *kcs1* mutant presents 80% less aldehydes and alcohols of C26 and C30 length on leaves, stem and siliques [9]. In *pas2* plants, all the wax aliphatic components are strongly reduced [1]. Finally, the *AtKCR1*-RNAi lines, which are defective in the keto-acyl-CoA reductase activity, present a glossy aspect and a reduced cuticular wax load [15].

Interestingly, the core elongase mutants (*pas2*, *kcr1*, *cer10*) display another obvious phenotype, which consist in postgenital organ fusions. Usually, such fusions are observed in angiosperm during the formation of the carpel. Studies in *Catharanthus roseus* suggested that organ fusion is caused by specific epidermal cell interactions involving exchange of small water-soluble morphogenetic factors [25]. Several Arabidopsis mutants showing organ fusion were characterized by a permeability change of the outer epidermal cell wall and cuticle [12]. As the epidermal layer is considered as an active site of lipid synthesis necessary for deposition of cuticle and waxes, it has been postulated that alteration of the cell wall or the cuticle could enhance the diffusion of fusion promoting signals. Conversely, the overexpression of *PHS1* led to an increased wax load on the epidermal surface of leaves, stems and carpels (Fig. 2C). Leaves were smaller, more digitated and showed abnormal cell shape [1]. Carpels were unfused demonstrating that epicuticular waxes at the epidermal surface are essential to prevent organ fusion (Fig. 2D and E).

Mutants with reduced waxes do not always present organ fusion. For instance, the most dramatical reduction in wax load was observed in *CER6*-silenced plants (7% of that of the WT) without any consequence on growth or on plant development [7]. Mutation in *FDH*, which has also been implicated in the synthesis of VLCFAs precursors for wax production [12], leads to ectopic fusion phenotype but without glossy leaves or stems that are typical of *cer* phenotype [10].

The fact that *cer6* mutant is missing specifically acyl-CoA C26 or longer suggests that at least a specific acyl chain length might be necessary for preventing postgenital organ fusion. Thus, examining the involvement of each component of the cuticle and epicuticular waxes will be necessary to understand how epidermal fusion is initiated.

VLCFAs are also major components of another extracellular biopolymer, the suberin that can be found in diverse tissues but mainly in the root endodermal cells as characteristic lamella structure that contains aliphatic chains and phenolic components. The main aliphatic monomers are glycerol associated with ω -hydroxyacids (ω -OH-acids), and α,ω -dicarboxylic acids (α,ω -diacids), ranging from C16 to C30 in chain length. Unsubstituted fatty acids, primary alcohols and an aromatic domain rich in ferulic acid are also commonly found in suberin.

Suberin biosynthesis begins in the symplast where its building blocks are found. Depending on the species, the fatty acids can undergo mid-chain modification leading to unsaturated and to epoxy-fatty acids, whereas saturated fatty acids are elongated up to C30 by the elongase complex. The α,ω -diacids and ω -hydroxyacids are produced by ω -oxidation catalized by a NADPH dependent cytochrome P450 mono-oxygenase. After export to the apoplast, the monomers are extensively polymerized in suberin macromolecule.

In Arabidopsis roots, free fatty acids are mainly C22:0 while primary alcohols includes 18:0, 20:0 and 22:0. However, VLCFAs are less abundant in ω -OH-acids (mainly 18:1 but 22:0 and 24:0 species can be detected) and absent in α,ω -diacids (only 18:1 and 16:0) [26].

Little is known on the role of the suberin during plant development. Recent studies have demonstrated that a modification of suberin structure or composition could alter root development. The double mutant *kcs20/daisy-1* that showed specific reduction of C20+ acyl chains in suberin exhibited reduced root growth indicating that specific VLCFAs in suberin are required for root development [12]. The *kcr1* RNAi lines present also an abnormal root phenotype, with an inhibition of lateral root elongation and a strong reduction of root hair length that are associated with reduced levels of C20+ in root fatty acids. Three novel peaks in suberin composition corresponding to C16 and C18 α,ω -diacids and C18 ω -hydroxy fatty acids were also observed [15].

Like plant epidermal waxes, animal skin is coated with a protective hydrophobic barrier against external aggression. Interestingly, the mammalian ELOVL3 condensing enzyme has been suggested to be involved in the formation of fatty acyl chains containing up to 24 carbons required in the epidermis for hair and skin formation. Indeed, *Elov3* ablated mice exhibited a sparse hair coat and a general hyperplasia of the sebaceous glands that led to loss of the protective function of the skin. These phenotypes correlated with significant modifications of the lipid content of the epidermis, which is a mixture of triglycerides, wax and sterol esters. More precisely, the *elov3* mice displayed high levels of eicosenoic acid (20:1), which was more accentuated in triglycerides fractions. Thus, VLCFAs, by being components of protective extracellular biopolymers, are essential during development in both plants and vertebrates.

5. VLCFAs in membrane lipids: signal molecules and membrane structural components

Biological membranes consist in a bilayer of polar lipids, predominantly glycerolipids associated with sterols and sphingolipids. In plant membranes, two distinct glycerolipid groups are found: the phospholipids, which contain a phosphate as polar head group, and the glycolipids, harbouring a carbohydrate moieties on their head group. Among plant phospholipids, phosphatidylethanolamine (PE) and phosphatidylcholine (PC) are the major components of the plasma membrane, mitochondrial membrane and endoplasmic reticulum, whereas phosphatidylserine (PS) is a minor lipid accounting for less than 1% of the total glycerolipids. On the other hand, glycolipids such as monogalactosyl-diacylglycerol MGDG and digalactosyl-diacylglycerol DGDG predominate in the chloroplast envelope and the thylakoid membrane. Phosphatidylglycerol (PG) is specific to the thylakoid membrane. The nature of lipid classes as well as the acyl chain length varied greatly among different plant organs. For instance, Arabidopsis leaves and flowers have the highest levels of phospholipids while roots have the least [26]. PC, PE and PS are the phospholipids that contain significant amounts of VLCFAs. It has been demonstrated that these three phospholipids are composed mostly of 18-carbon species combined with a very long acyl chain ranging from 20 to 26 carbons.

PS is the lipid class that contains the highest proportion of VLCFAs. It can represent 69% of total PS found in roots whereas VLCFA-PC average only 1%. The amount of VLCFA-PE is also abundant in roots, while VLCFA-PS presents a slight increase in roots and a significant increase of PS in seeds. In the report of Millar et al., the authors demonstrated that the overexpression of seed specific KCS FAE1 increases the levels of VLCFAs in leaf glycerolipids where VLCFA-PC and PE can reach respectively 18% and 27% of phospholipids compared to 2 and 5% in WT [28].

Sphingolipids are also important structural components of membrane, accounting for more than 40% of plasma membrane lipids in some plants, but also found in the Golgi network and in endosomes [29]. They are composed of a long chain base (amino alcohol) and an amide linked fatty acyl chain. More complex sphingolipids are formed with the addition of a glycosylated polar head. Plant sphingolipids include four main lipid classes: the simple sphingolipid-ceramide and hydroxy-ceramide and the more complex forms, glucosylceramide and glucosylinositolphosphorylceramide. Plant sphingolipid diversity reflects their distribution among plant tissues. For instance, seed glucosylceramides are enriched with dihydroxy long chain bases and C16 to C20 saturated hydroxy-fatty acids while leaves are enriched with trihydroxy long chain bases and saturated very long acyl chains (C20 to C26) [30]. Sphingolipids show also structural specificity among organisms. Sphingomyelin, which has phosphocholine as the polar head group and is the major phosphosphingolipid in animals, has not been detected in plants. On the other hand, yeast contains exclusively inositolphosphorylceramides. Our knowledge of sphingolipid and glycerolipid functions are mainly learned from studies in mammals and yeast where both classes of lipids serve as structural membrane components and precursors of signaling molecules. Elevated levels of complex sphingolipids were associated with cell apoptosis, terminal differentiation, or cell cycle arrest [31]. The reduction of complex sphingolipid levels by contrast can lead to an increase of sphingoid long chain base leading to cell proliferation [32].

One of the first uncovered roles of sphingolipids in general concerned programmed cell death (PCD) as a mechanism of defence against pathogens. *ASC-1* gene has been demonstrated to be homologous to *LAG1* (longevity assurance life) and *LAC1*, which both encode the yeast ceramide synthase. *Alternaria alternat f.sp. lycopersici* is a host-selective toxin-producing fungus able to induce programmed cell death in *asc/asc* tomato plants. This process has been shown to correlate with a strong reduction of sphingolipids and increased levels of LCB (dihydrosphingosine [DHS] and 3-ketodihydrosphingosine [3-KDHS]), indicating that programmed cell death might be linked to sphingolipid synthesis [33].

Recently, Raffaele et al. showed the involvement of VLCFAs in the activation of the hypersensitive cell death response in Arabidopsis when they explored the role of MYB30 transcription factor during this process [34]. Two hypotheses were emphasized to explain the role of VLCFAs in the regulation of cell death: since the overexpression of MYB30 slightly modifies the wax composition, VLCFAs

could compromise plant response to pathogens by modifying the cuticle [34]. Several studies suggested that cuticular waxes could directly inhibit pathogen growth or just provide a physical barrier to the pathogen entrance. However, as the overexpression of MYB30 results also in a larger change in VLCFAs containing sphingolipids, another hypothesis would be that VLCFAs sphingolipids could be involved in PCD in response to pathogen infection. VLCFAs sphingolipids could control PCD by structuring the plasma membrane allowing recognition of the pathogen and the initiation of the PCD signaling pathway. Alternatively, VLCFAs sphingolipids could act directly as signalling molecules as observed in animal apoptosis. Interestingly, the work of Reina-Pinto et al. showed that a misexpression of *FAE1* in epidermal cells under the control of *FDH* promoter leads to the death of a specific cell type, which are the trichomes. However, this *FAE1*-dependent cell death was found to be sphingolipid-independent. An increase of long chain acyl-CoAs esters was observed in *FDH:FAE1* plants and a chemical genetic screen indicated a role for phospholipase A2 and phospholipids, analogous to so-called “lipoapoptosis” observed in vertebrates [35]. Recently, the role of VLCFAs in plant cell proliferation has been highlighted by the further characterisation of the elongase dehydratase encoded by *PASTICCINO2* gene (Fig. 2A). Indeed, *pas2* exhibits severe developmental defects correlated with ectopic cell division, cell dedifferentiation and defective hormonal response, reminding one of the cell cycle defective phenotype observed in *phs1* yeast mutant. Conversely, the overexpression of *PAS2* in plants led to leaf serration and abnormal leaf development reminiscent of cell cycle inhibition and premature differentiation [1,36].

The role of sphingolipids in the deregulation of cell cycle and differentiation was also observed in animals and yeast. The *lag1* gene was discovered in a screen for yeast longevity genes [37]. Furthermore, the *Lass* gene family, which are homologous to *lag1* in animals, has been involved in breast cancer [38]. *In vitro* studies have correlated breast cancer progression with increased ceramide levels due to an upregulation of specific *LASS* genes. In particular, increased levels of 16:0-Cer, 24:1-Cer and 24:0-Cer were found in malignant tumors compared with benign tumors and normal tissue. Similarly, a study demonstrated that exogenous application of short chain ceramide led to the dephosphorylation of *Retinoblastoma* (Rb) gene, an event that blocks the cell cycle [39]. However, *ELOVL4* deficient mice exhibited depletion in the epidermis of ceramides with very long chain fatty acids (C28+) and accumulation of ceramides with C26 fatty acids. This accumulation in VLCFAs-ceramides is correlated with defective skin permeability, leading to early postnatal death [40]. Altogether, these data indicate that LCFA/VLCFA-ceramides act as a rheostat controlling cell division and differentiation [41,42].

VLCFAs are also involved in cellular homeostasis via their involvement structural membrane lipids influencing membrane dynamics (membrane bending and fusion, membrane microdomains). For instance, C26 fatty acid, which is the major VLCFAs in yeast, has been demonstrated to be critical for membrane bending [43]. The isolation of a

conditional acyl-CoA carboxylase mutant showed that reduced amounts of 26:0 lipids are linked to defects of the nuclear pore, mostly consisting in the separation of the inner and the outer membranes of the envelop. More precisely, these defects were the result of the absence of C26-phosphatidylinositol, which biophysical properties were associated with the stabilization of highly curved membrane domains [44]. The importance of the structural role of VLCFAs at the plasma membrane is also illustrated during cytokinesis. Cytokinesis in animals is carried out by the constriction of an actomyosin ring coupled to plasma membrane ingression. Szafer-Glusman et al. reported that the loss of function of the *BOND* gene, a member of the VLCFAs condensing enzyme Elov1 blocks or strongly delays the cleavage furrow during early telophase in dividing *Drosophila* spermatocytes [45]. Furthermore, they showed that the *bond* phenotype differs from those of membrane trafficking mutants such as *arf6* or *rab11*. Thus, the authors concluded that the *BOND* function might contribute to the cytokinesis by controlling the ability of membrane formation or bending during furrow ingression rather than being involved in membrane trafficking. In plants, a similar observation was made with *FAE1* overexpressing plants that display an accumulation of VLCFAs in membrane glycerolipids associated with severe defects in plant morphology [27]. In particular, the authors noticed a highly curved thylakoid membrane. Millar et al. speculated that the accumulation of high levels of VLCFAs in the major plasma membrane lipids (PC and PE) might be responsible for the alteration of membrane curvature [27].

Sphingolipids are also contributing to the formation of membrane microdomains or lipid rafts. In mammals and yeast, these lipids rafts are involved in endocytosis, and protein sorting. In yeast, C26-ceramide-bound is critical for the routing of newly synthesized protein to the plasma membrane [46,47]. However, it has been recently demonstrated that yeast are still viable in the complete absence of sphingolipids [48]. In plants, reduction of CER10 activity led to strong VLCFAs reduction in all lipid classes (waxes, triacylglycerols, sphingolipids) causing cell expansion defects in epidermal cells and trichomes. The lack of cell expansion was correlated with reduced endocytosis. To discriminate between the different VLCFAs lipid classes, RNAi lines targeting CER10 in epidermal cells or in the embryo, led to specific reduction of 40% of wax load and 30% of seed triacylglycerols without any consequence on plant morphogenesis suggesting that sphingolipids were most probably involved in endocytic membrane dynamic associated with aberrant cell expansion [17].

6. Conclusion

Considerable progress in our understanding of VLCFAs has been made in the past 5–10 years mainly thanks to the isolation of the elongase mutants. Nevertheless, further investigations of the biophysical properties of VLCFAs will be needed to better understand their roles in cell physiology. It is clear now that, by being involved in such diverse lipid pools, VLCFAs are associated with very different cellular functions, cell proliferation, differentia-

tion or death. The identification of the molecules involved in epidermal cell proliferation during postgenital organ fusion or cell death will be some of the challenges of the years to come. Convergent evolution is remarkable in a way that complete absence of VLCFAs in wax esters led to abnormal epidermal cell response, both in plants with cell proliferation during postgenital fusions and in vertebrates with cell death during skin development. In animals, increased levels of VLCFAs lead to several neurodegenerative diseases. For instance, studies demonstrated that autism was correlated with increased levels of plasma VLCFAs potentially due to impaired mitochondrial fatty acid oxidation [41]. X-adrenoleukodystrophy (ALD), which is a severe neurodegenerative disorder, is characterized by progressive demyelination within the central and peripheral nervous system. This disease is caused by a deficiency in *Abcd1* peroxisomal transporter resulting in an impairment of peroxisomal beta-oxidation and the accumulation of saturated very long chain fatty acids in plasma, fibroblasts and tissues [42].

In plants, even if most of the elongase mutants present similar developmental defects (postgenital fusions, glaucous appearance, trichome defects), specific phenotypes are also observed such as cell proliferation for *pas2* mutant, cell expansion defects for *cer10* and absence of lateral roots for silenced-*KCR1* plants. Although, these pleiotropic phenotypes could be due to different allele strength, we cannot exclude crosstalk with other pathways. Indeed, the presence of a protein tyrosine phosphatase (PTP) motif in PAS2 protein led originally to the definition of PAS2 being a PTP-like protein involved in cell cycle regulation by interacting with cyclin dependent kinase A [49]. The conservation of this motif across eukaryotes suggests that phosphorylation dependent protein interaction and regulation might be involved in fatty acid elongation. The fact that mutation in PAS2 mammalian homolog PTPLA led to centronuclear myopathy that is reminiscent to phosphoinositide phosphatase myotubularin MTM1 phenotype supports this hypothesis [1].

Functional genetics has opened the way to a better characterization of the enzymes involved in VLCFAs synthesis but many exciting challenges remain to understand the role of each lipid molecules, its cellular compartmentalization and its dynamics during growth and development.

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