Review

Fatty Acid and Lipid Transport in Plant Cells

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Fatty acids (FAs) and lipids are essential – not only as membrane constituents but also for growth and development. In plants and algae, FAs are synthesized in plastids and to a large extent transported to the endoplasmic reticulum for modification and lipid assembly. Subsequently, lipophilic compounds are distributed within the cell, and thus are transported across most membrane systems. Membrane-intrinsic transporters and proteins for cellular FA/lipid transfer therefore represent key components for delivery and dissemination. In addition to highlighting their role in lipid homeostasis and plant performance, different transport mechanisms for land plants and green algae – in the model systems *Arabidopsis thaliana*, *Chlamydomonas reinhardtii* – are compared, thereby providing a current perspective on protein-mediated FA and lipid trafficking in photosynthetic cells.

FA and Lipid Transport: An Essential Process Throughout the Plant Life Cycle

FAs are building-blocks for the majority of cellular lipids, which are not only essential for membrane function, but also are necessary for growth, development, and plant performance. For example, triacylglycerols (TAGs) in oil seeds represent the major form of carbon storage, and waxes/cutin on the epidermis, as well as suberin in the endodermis/seed coat, regulate water transmission and provide pathogen protection. Moreover, the formation of pollen cell walls is strictly dependent on the delivery of sporopollenin/tryphine material, which is essentially composed of modified FAs [1,2]. The responses of plants to senescence, cold temperatures and phosphate starvation require FA/lipid remodeling and turnover to sequester carbon energies, to protect against freezing and to replace phospholipids by plastid-derived galactolipids, respectively [3,4]. Thus, acyl lipid metabolism is crucial throughout the plant life cycle and in response to abiotic or biotic stresses. In addition, the biotechnological importance of plant-derived lipids is constantly increasing, for example, for the production of biodiesel or the improvement of nutrient quality [5,6].

For these reasons, plant lipid metabolism has been subjected to intensive studies and has been found to require hundreds of enzymatic reactions occurring in several subcellular organelles [7]. The complexity of lipid metabolism is further highlighted by the fact that each membrane within the plant is unique in its acyl and lipid composition. At the same time, all acyl-chains are made in plastids, and are assembled either in plastids or in the endoplasmic reticulum (ER), thus requiring FA/lipid transport. Because lipophilic molecules cannot move freely in an aqueous cellular environment, several modes of transport exist. These include membrane contact sites, diffusion and/or flip transfer within the same membrane system, vesicular trafficking, and protein-mediated transport processes. Considering the presence of a large number of FA and lipid molecular species with different head-group chemistry, and variations in the composition of membranes as well as of the surrounding media, physiochemical properties (e.g., uncharged or charged FAs)

Trends

FAX1, a novel membrane protein in the inner envelope of chloroplasts, mediates FA export.

The discovery and analysis of membrane transporters allows future development of models for transport mechanisms of lipophilic compounds.

The flow of FAs from plastids to their final cellular destination in lipid molecules is controlled by membrane-intrinsic and membrane-attached proteins.

Plant membrane transporters for FAs and lipid derivatives represent essential components in growth, development, and plant performance.

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further impact on the mode of transport. Proteins, however, can be involved in delivery to, flip transfer across, and movement out of membranes [8]. For cellular distribution FA/lipid transport proteins thereby represent an essential prerequisite, controlling lipid homeostasis and affecting plant development and productivity (Figure 1, Key Figure).

In the past decade several **ATP-binding cassette** (ABC) transporter systems (see Glossary) have been described to transport FA/lipid molecules across various membranes [9]. Further, recent identification of FAX1 (fatty acid export 1), a novel protein for FA export across the inner envelope membrane (IE) of chloroplasts [10], represents a breakthrough concerning the question of whether this transport occurs by simple diffusion or if membrane proteins are involved. In vertebrates, FAX1 relatives are mitochondrial membrane proteins of so far unknown function; the protein family thus provides an opportunity to explore novel transport mechanisms. In *Arabidopsis thaliana*, nine **long-chain acyl-CoA synthetases** (LACSs) belong to a large superfamily of acyl-activating enzymes [11], and are involved in FA transport by a process termed **vectorial acylation**. *In vitro*, all LACSs display activity with C₁₈-FA as a substrate, and LACS1–3 show lipid trafficking function in the budding yeast *Saccharomyces cerevisiae* [12]. **Acyl-CoA-binding proteins** (ACBPs) are also implicated in intracellular distribution of acyl-CoA esters and lipids. All six *Arabidopsis* ACBPs bind acyl-CoAs *in vitro* and impact on FA/lipid homeostasis (for non FA/lipid-associated functions and protein partners see [13,14]).

In this review we follow protein-mediated FA/lipid transport pathways in plant cells, focusing on *Arabidopsis* (please note that there are similar data in the monocot model rice, *Oryza sativa*). For lipid transfer proteins (LTPs) [15–18], as well as membrane contact sites, cellular vesicle traffic, or acyl-exchange mechanisms and non membrane-associated processes, we refer to recent advances in the field [19–21]. Furthermore, we compare FA transport mechanisms between prokaryotic and eukaryotic model systems and organelles, and discuss the situation in green microalgae, which have recently emerged as models to engineer oil metabolism [22,23].

From Plastids to the ER

Where It Begins: FA and Lipid Synthesis Pathways

In plants, *de novo* FA synthesis occurs in the plastid stroma, where acyl chains grow attached to the acyl carrier protein (ACP), and become available for lipid assembly mainly in the form of C_{16:0}- and C_{18:1}-ACP [7,24]. A fraction of these **long-chain fatty acids** (LC, C_{16–18}) are integrated into lipids inside plastids (the '**prokaryotic**' pathway), but the majority of the LC-FAs are exported to the ER for further elongation, acyl editing, and lipid assembly (the '**eukaryotic**' pathway). In *Arabidopsis*, about 38% of *de novo* synthesized FAs enter the prokaryotic lipid-synthesis pathway. The remaining 62% of FAs are exported to the eukaryotic pathway, and about the half of these are returned for plastid-intrinsic lipid assembly [25]. In so-called '16:3 plants', such as *Arabidopsis* or *Brassica napus*, these plastid-intrinsic lipids contain a large fraction of C_{16:3} FAs, attached through the prokaryotic pathway by the plastid *sn2*-acyltransferase, which exclusively uses C₁₆-FA substrates [26]. Many vascular plants, such as pea (*Pisum sativum*) and rice, however, mainly use the ER pathway for synthesis of plastid lipids. These are the so-called '18:3 plants' because the ER *sn2*-acyltransferase has a high specificity for C₁₈-FAs. As a consequence, the eukaryotic-type diacylglycerol (DAG) backbone must be re-imported into plastids (Figure 1) [27].

Plastid FA Export

Although it is generally agreed that free FAs are shuttled across plastid envelopes, the mode of export was a matter of debate because – until the recent discovery of FAX1 [10] – no membrane-intrinsic transporter could be associated with a direct function in plastid FA export [28]. Facilitated diffusion of free FAs through the lipid membrane was supported by the finding that an acyl-ACP synthetase in the cyanobacterium *Synechocystis* sp. PCC6803 is sufficient for FA

Glossary

Acyl-CoA binding proteins

(ACBPs): small (10 kDa) conserved ACBPs are implicated in the storage and intracellular of acyl-CoA esters or lipids in eukaryotes. In *Arabidopsis*, six ACBP proteins with acyl-CoA binding domains exist, and three of them – ACBP4, 5, and 6 – are localized in the cytosol. Only At-ACBP6 represents a 'classical' 10 kDa ACBP, whereas At-ACBP1–3 are about 38–39 kDa, and At-ACBP4 and 5, with additional kelch motif domains, are about 71–73 kDa in size. Thus, ACBP1–5 represent plant-specific proteins. Whereas ACBP3 is targeted to the extracellular space, ACBP1 and 2 contain ankyrin repeats and have a transmembrane domain that attaches them to the ER and plasma membrane.

ATP-binding cassette (ABC)

transporter: plant ABC transporter ATPases constitute a large membrane-protein family with at least 130 members in *Arabidopsis* and belong to the ABC Superfamily 3.A.1 (Transporter Classification Database, TCDB: www.tcdb.org). The generalized transport reactions for ABC-type uptake and efflux systems are: (i) solute (out) + ATP → solute (in) + ADP + P_i; and (ii) substrate (in) + ATP → substrate (out) + ADP + P_i, respectively. An ABC transporter complex consists of two transmembrane and two nucleotide-binding domains, which can either be assembled by one or two polypeptide chains of the full- and half-size ABC transporters, respectively. Prokaryotic-type ABC transporter complexes are assembled by four separate subunits plus two or more additional substrate-binding proteins (e.g., the TGD1–3 complex in the chloroplast IE). Proteins in the A, D, and G subfamilies in animals and plants are predominantly involved in transport of lipophilic molecules.

Eukaryotic and prokaryotic pathways of lipid synthesis:

starting with LC (C_{16–18}) FAs synthesized in plastids, the eukaryotic pathway of lipid synthesis in plants occurs at the ER where elongation, acyl editing, and lipid assembly take place. Because the ER *sn2*-acyltransferase has high specificity for C₁₈ FAs, eukaryotic DAG backbones in plant glycerolipids mainly contain C₁₈ acyl chains at the *sn2* position. Owing to their endosymbiotic origin,

Key Figure

Fatty Acid (FA) and Lipid Transport in Plant Cells

plastids have retained the so-called prokaryotic lipid synthesis pathway, mainly producing plastid-intrinsic galactolipids and PG. The plastid *sn2*-acyltransferase has an exclusive preference for C_{16} FAs, thus 'prokaryotic' plastid-produced DAG backbones always have C_{16} acyl chains at *sn2*, thereby enabling discrimination of lipid origin. Note that the *sn1*-acyltransferases in both compartments prefer C_{18} FAs.

Long-chain acyl-CoA synthetase

(LACS): LACS enzymes are plant long-chain fatty acid:CoA ligases (AMP-forming) and belong to the IUBMB enzyme class EC 6.2.1.3. LACS or FACS (fatty acid CoA synthetase) are ubiquitously present in pro- and eukaryotic organisms and catalyze the reaction: $ATP + (\text{long-chain}) \text{ fatty acid} + \text{CoA} \rightarrow \text{AMP} + \text{diphosphate} + \text{acyl-CoA}$ (KEGG database: www.genome.jp/kegg/kegg1.html). Owing to their function in FA activation by formation of a thioester with CoA, they play an important role in the so-called process of vectorial acylation.

Long chain (LC) and very long chain (VLC) fatty acids: LC FAs have an alkyl chain length of C_{16-18} , whereas VLC FAs have 20 or more carbon atoms ($C_{\geq 20}$).

Vectorial acylation: the coupling of FA transport across a lipid bilayer membrane – either passive diffusion or transmembrane flip most likely mediated by a membrane-intrinsic protein – with subsequent ATP-dependent FA activation by a LACS/FACS enzyme and delivery for metabolism. Because in this latter step thioester bonds are formed, the process is also referred to as vectorial esterification.

Figure 1. All proteins and pathways depicted are described throughout the text; for details and intermediate steps of FA/lipid synthesis, editing, and assembly (grey lines) we refer to [7,20,24] and <http://aralip.plantbiology.msu.edu/pathways>. Distribution of FAs, lipids, and derivatives within the cell involves transport across membranes of the plastid inner and outer envelopes (IE, OE), endoplasmic reticulum (ER), peroxisome, and plasma membrane (PM). FA/lipid transport pathways in mitochondria and vacuoles – most likely through membrane contact sites and/or vesicle fusion – are not shown because so far no proteins directly involved have been described. Transfer of phospholipids, such as PC, from the ER to the plasma membrane might occur through vesicles and membrane contact sites, but also with the help of ACBP1. Separately encoded subunits of half-size ABC transporters and the TGD1–3 complex are indicated by different shades of color. Open questions (?) include FA transfer from FAX1 across the OE, localization of ER-resident LACS enzymes at the lumen or cytosolic face, interaction of ER LACS with ABC transporters, as well as the export of wax, cutin, suberin, and sporopollenin precursors across the ER membrane. The potential involvement of LACS4 and LACS9 in FA import into plastids most likely

membrane transfer [29]. However, several ABC transporters for lipids or FAs/acyl-CoAs as well as eukaryotic FA transport systems (Figure 2) provide evidence for the direct participation of membrane proteins in plastid FA transport. Nevertheless, before transport acyl-ACP thioesterases on the stroma-side of the plastid IE catalyze the hydrolysis of acyl-ACP to free FAs (Figures 1, 2D). According to acyl-ACP synthesis rates and thioesterase specificity in *Arabidopsis* chloroplasts, oleic acid ($C_{18:1}$) is the major FA exported, followed by palmitic acid ($C_{16:0}$) and only very small amounts of stearic acid ($C_{18:0}$) [25]. In general, toxic free FAs have a short half-life [28,30], and their pool in chloroplasts is marginal, requiring either quick flow into metabolism or export from the organelle.

Insight into the potential mechanism of plastid FA export was provided by the characterization of FAX1, which inserts into the chloroplast IE via α -helical membrane-spanning domains [10]. Detailed phenotypic and ultrastructural analyses of FAX1 mutants in *Arabidopsis* showed that FAX1 is crucial for biomass production, male fertility, and the distribution of FA-derived compounds such as lipids, ketone waxes, and pollen cell-wall material. Quantification of lipid, FA, and wax contents has revealed that levels of ER-derived lipids decrease when FAX1 is absent, but levels of plastid-produced lipid species increase. FAX1 overexpressing lines showed the opposite, including a pronounced rise of TAGs in flowers and leaves. Furthermore, the cuticular layer of stems from *fax1* knockouts was specifically reduced in C29-ketone waxes. Transcripts of *Arabidopsis* FAX1 are present at all developmental stages, and peak in leaf tissues as well as in early pollen development [10]. Consequentially, the strongest phenotype of *fax1* knockouts is observed during growth (e.g., these plants have reduced biomass) and in particular in pollen grains/tapetum cells of anthers, leading to absence of outer pollen cell-wall structures and impaired pollen release by anthers. Because sporopollenin of the exine pollen cell wall and tryphine material of the pollen coat are derived from modified FAs/lipids (Figure 1), this *fax1* phenotype is again linked to FA/lipid metabolism. Differential gene expression in FAX1 mutants confirms phenotypes and metabolic imbalances and might lead to the identification of plastid-to-nucleus signaling pathways. Because FAX1 in *Arabidopsis* belongs to a family of seven proteins, the plastid-predicted FAX2, FAX3, and FAX4 most likely can bypass the loss of FAX1 function. Based on their relative expression profiles, in particular in seeds, FAX2–4 should play a more prominent role than FAX1. Future research to increase seed oil content must therefore consider differences and similarities in developing oilseeds and green vegetative organs [31,32], concerning expression patterns and metabolic, physiological as well as functional specificities of proteins and tissue types.

When expressed in yeast, FAX1 complements for FA transport with a specificity range of $C_{16:0} > C_{18:1} \sim C_{18:0}$ [10], and thus *in vivo* most likely mainly exports free palmitic acid. Nevertheless, FAX1 can also transport oleic acid, which in the plastid stroma is provided at highest substrate concentrations. In IE membranes, FAX1 is thereby most likely involved in vectorial acylation, and presumably functions in a passive, carrier-like mode, driven by availability of free FAs and LACS enzyme activity at the outer envelope (OE; Box 1, Figure 2).

occurs through ER–plastid contact sites, and thus is not depicted. Please note that although TGD4 and TGD2 bind PA, the actual nature of the eukaryotic lipid backbone (eLB) re-imported into plastids is still uncertain ([21] for discussion). In plastids, however, the eukaryotic DAG moiety is derived from the imported eLB, and synthesis steps for PA, DAG, and MGDG in general occur at the IE, whereas DGDG is made at the OE. Note that transfer of PG, MGDG and DGDG to thylakoid membranes is not depicted. Abbreviations: ABC, ATP-binding cassette; ACB, acyl-CoA-binding domain-containing protein; ACP, acyl carrier protein; β -ox: β -oxidation; CoA, coenzyme A; DAG, diacylglycerol; DGDG, digalactosyl-diacylglycerol; ER, endoplasmic reticulum; Ex, exine layer; FAX1, fatty acid export 1; FFA, free fatty acid; G3P, glycerol-3-phosphate; Int, intine layer; LACS, Long-chain acyl-CoA synthetase; LC, long chain (C_{16-18}) acyl residue; lip-c, lipophilic compound; LPA, lysophosphatidic acid; MGDG, monogalactosyl-diacylglycerol; Nex, nexine layer; PA, phosphatidic acid; PC, phosphatidylcholine; Pc, pollen coat; PE, phosphatidyl-ethanolamine; PG, phosphatidyl-glycerol; PLs, phospholipids; Prec., precursors; TAG, triacyl-glycerol; TGD, trigalactosyl-diacylglycerol; VLC, very long chain ($C_{\geq 20}$) acyl group. Prefixes: e, derived from eukaryotic pathway; p, derived from prokaryotic pathway.

Trends in Plant Science

Figure 2. Fatty Acid (FA)-Transport Systems in Model Organisms and Plant Organelles. Note the different transport directions related to the cytosol (cyt, light yellow) of the respective cells. (A) In *E. coli*, uptake of free FFAs (FFA) is mediated by the β -barrel protein FadL in the outer membrane (OM) and FadD, a fatty-acyl CoA synthetase (FACS) at the cytosolic face of the plasma membrane (PM), which couples FFAs as thioesters to coenzyme A (CoA). Most likely, FFAs cross the periplasmic space (PPM), partition into the PM and are sensed by ATP-activated FadD in their protonated form (cf. [8]). In Gram-positive bacteria such as *B. subtilis*, which lack the OM, the protein YnfL is described as FACS enzyme [107]. (B) In the eukaryotic model *S. cerevisiae*, FFAs are transported into the cell by the concerted action of the membrane protein Fat1p and an interacting FACS enzyme partner – Faa1 or Faa4. Fat1p integrates into the PM with four predicted hydrophobic α -helices (green cylinders), and contains ATP/AMP-binding (grey rectangles) and FACS motifs at the cytosolic face (cf. [108]), which suggest enzyme activity and formation of acyl-adenylate intermediates before handover to Faa1/Faa4. Whereas the contribution of the membrane-intrinsic Fat1p domains during flip transfer of FFAs across the membrane is unclear (red box [8]), the cytosolic domains, together with Faa1 or Faa4, mediate acyl-CoA synthesis. (C) The *Arabidopsis* full-size ABC transporter ABCD1 cleaves cytosolic acyl-CoA, transports FFA, and interacts with the FACS enzymes LACS6 and/or LACS7 that activate FFAs to acyl-CoA [64]. Whether ABCD1 releases CoA to the cytosol or peroxisome lumen (lum, pink) is still unknown. (D) In plastids of higher plants (in the model *Arabidopsis*), FFAs are released from the acyl carrier protein (ACP) by FATA/FATB thioesterases in the stroma (str, green). In a proposed model, the FAX1 protein (fatty acid export 1), which is anchored in the inner envelope membrane (IE) by three hydrophobic α -helices (purple cylinders), might bind FFAs by an amphiphilic α -helix at the lipid bilayer/stroma interface (light blue cylinder) and flip transfer across the membrane. Further, FAX1 contains an N-terminal stretch that might fold into an additional, non-membrane associated α -helix (grey cylinder) and could mimic the structure of the ‘helical up-and-down bundle’ of the human apolipoprotein APOE3 ([10] for discussion). APOE3 is involved in lipid transport and binding during formation of lipoprotein particles by a conformational change of its N-terminal helix bundle that allows detergent-like solubilization of lipids and the formation of lipoprotein particles [109]. Thus, the N-terminal stretches of plastid FAX1 proteins might act in a similar way during FA transport. At the cytosolic face of the outer envelope membrane (OE) the FACS enzyme LACS9 could drive vectorial acylation and plastid FA export by feeding FA-CoA thioesters into lipid metabolism (cf. Figure 1). However, only recently LACS9 was described to be involved in FA import into plastids ([37], see below). Thus, the direct impact of LACS9 on plastid FA export, whether other proteins are involved in this process, and how FFAs cross the intermembrane space (IMS) and are finally transferred over the OE remain questions to be answered by future research. In general, FA transport by FAX1 should be possible in both directions, depending on substrate concentrations present at each side of the membrane and the action of a vectorial component. In plastids this would be constant delivery of FFAs at the stromal side of the IE, in combination with ATP-dependent LACS activity and feeding into lipid metabolism at the cytosolic side of the plastid. In yeast complementation assays, however, high external FFA concentrations, together with a cytosolic Faa1/Faa4 FACS activity (B), would lead to FAX1-mediated FA uptake (cf. [10]).

On the Way to the ER

LACS9 at the OE is unique to plastids [33–35] and might drive vectorial acylation for plastid FA export (Figure 2D). A test with isolated *lacs9* knockout chloroplasts using $^{14}\text{C}_{16:0}$, $^{14}\text{C}_{18:1}$ substrates revealed that LACS9 catalyzes about 90% of the chloroplast activity for acyl-CoA synthesis [34]. Because *lacs9* knockouts show a wild-type phenotype, other enzymes may compensate for this activity, although so far no additional plastid LACS have been found.

Box 1. FA Transport and the Concept of Vectorial Acylation

Protein-mediated FA transport was first discovered in *E. coli* [110,111]. In this organism two components are involved in uptake of exogenous FAs (Figure 2A): FadL, an outer membrane β -barrel protein, and FadD, a protein with fatty acyl CoA synthetase (FACS) activity attached to the inner leaflet of the plasma membrane [8,112]. Because neither FadL nor FadD *E. coli* mutants can import FAs [110,111], both proteins are indispensable for FA traffic by the so-called process of vectorial acylation [8]. Whereas FadD represents an ATP-dependent driving force that forms FA-CoA thioesters and releases acyl-CoA into the cytoplasm, FadL is a true membrane transporter, functioning by FA binding/diffusion and conformational changes that allow FAs to traverse the β -barrel [113]. Because in cyanobacteria an acyl-ACP synthetase can also drive FA uptake [29,114], FA activation by thioesters and subsequent integration into metabolism – in other words vectorial acylation – seems to be sufficient for transport across the plasma membrane of prokaryotes. *S. cerevisiae* is the eukaryotic model organism, where again exogenous FAs must be activated to their CoA thioesters [8,112,115] by the cytosolic FACS enzymes Faa1 or Faa4. In contrast to prokaryotes, however, the membrane-intrinsic Fat1p (FATP/SLC27 proteins in mammals [116]) is involved in vectorial acylation across the plasma membrane and builds a complex with Faa1/Faa4 subunits (Figure 2B) [108,117,118]. Interestingly, in the oleaginous yeast *Yarrowia lipolytica*, Fat1p may also be involved in FA export from lipid bodies [119]. FA uptake into peroxisomes is mediated by ABC transporters of subfamily D and energized by cytosolic ATP [56]. Functional complementation of yeast $\Delta pxa1/pxa2/faa2$ – lacking peroxisomal FA transport and FACS activity – and isolation of native membrane complexes [64] have shown that the *Arabidopsis* ABC transporter ABCD1 interacts with the LACS enzymes LACS6 and/or LACS7 that activate FFAs to acyl-CoA for subsequent β -oxidation (Figure 2C). Whether, besides ATP hydrolysis by ABCD1, LACS6/7 are necessary to drive transport via vectorial acylation still remains unclear. In the plastid IE membrane, FAX1 is the membrane-intrinsic protein for FA transport (Figure 2D). Functional complementation of yeast $\Delta fat1$, but not of $\Delta faa1/faa4$ mutants [10], indicates that FAX1 is acting only in membrane transfer of FAs and not – as yeast Fat1p (Figure 2B) – in FA activation. Vectorial acylation across the two envelope membranes could finally be driven by feeding FAs into cellular metabolism (Figure 1) and a LACS enzyme at the cytosolic face of the plastid, although the OE-associated LACS9 [33–35] recently was described to be involved in FA import into plastids ([37]; for discussion see below and Figure 2D).

However, overlapping function of LACS9 was demonstrated with the ER-targeted LACS1 and LACS4 (Figure 1) [36,37]. First, *lacs1/lacs9* double mutants showed an 11% decrease in seed FAs, implying that during seed TAG synthesis, LACS1 can partly take over the function of LACS9 [36]. Second, LACS9 was described to cooperate with LACS4, resulting in even higher FA reduction (by 27%) of seed TAGs in *lacs4/lacs9* [37]. Most importantly, mutation of the ER enzyme LACS8 in the *lacs4/lacs9* mutant background was lethal. Double mutants in *lacs4/lacs9* showed a specific and strong increase in $C_{18:2}$ FA content, and *in vivo* pulse-chase labeling experiments in leaves revealed that less ER-derived galactolipids were present in plastids. Thus, a model was proposed in which LACS9 together with LACS4 is involved in FA transfer from the ER to chloroplasts, most likely in the form of $C_{18:2}$ in eukaryotic phosphatidic acid (PA) at plastid–ER contact sites ([21,37] for discussion). Detailed future research on mutant plants as well as *in vitro/in vivo* enzyme activity and transport assays will be necessary to clarify whether LACS9 is acting in plastid FA export and/or import, and if interaction with FAX1 takes place.

Mutations in LACS1 at the ER reduce the amount of almost all chemical classes of waxes, as well as of C_{16} cutin monomers, on the epidermis of stems and leaves (Figure 1). *lacs1/2* double mutants revealed lower amounts of wax and cutin than either parent, demonstrating an overlapping function of both enzymes in wax and cutin biosynthesis [38]. Investigation of *lacs1/lacs4* double knockouts further demonstrated that a LACS1/LACS4 combination is required for formation of tryphine pollen coat lipids [39].

After being esterified to CoA through the activities of LACSs, members of acyl-CoA binding proteins (ACBPs) have also been found to be involved in shuffling FAs to the ER. Cytosolic ACBP4–6 prefer $C_{18:1}$ - over $C_{16:0}$ -CoA; however, *in vitro* affinities are different for ACBP4 and 5 compared to ACBP6 [40–42]. All three proteins can bind phosphatidyl choline (PC), indicating that, besides transfer of LC-CoA, they might be implicated in intracellular lipid distribution (Figure 1). Insight into their *in planta* role was provided by analysis of ACBP4–6 triple mutants with reduced seed weight, germination sensitivity to abscisic acid, and accumulation of C_{18} -CoAs in *acbp6* embryos and seedlings [42]. Mutant *acbp4/5/6* pollen grains display reduced *in vitro* germination and defects in oil bodies and the outer pollen cell wall [43], the

latter being reminiscent of *fax1* knockouts (see above). ACBP6 is further associated with lipid remodeling during freezing tolerance [44,45].

ABCA9 – one of 12 half-size *Arabidopsis* ABC transporters of subfamily A – was described to be involved in FA/acyl-CoA import into the ER (Figure 1) [46]. ABCA9 is localized to ER membranes and is expressed in developing seeds during the stages when storage lipids accumulate. The amount of TAG (as well as the weight and size of seeds) is reduced in *abca9* knockout seeds but increased in ABCA9-overexpressing plants. Feeding experiments with ¹⁴C-acetate, ¹⁴C-oleoyl-CoA, and ¹⁴C-oleic acid demonstrated reduced FA incorporation in TAGs of *abca9* seeds, revealing a key role for ABCA9 during FA/acyl-CoA import into the ER.

From the ER Back to Plastids

For the assembly of plastid-intrinsic galactolipids, DAG-precursors of both prokaryotic and eukaryotic pathways are used (in *Arabidopsis* about 50% of DAG-backbones are made in the plastid, and the other half are transported into plastids from the ER). In consequence, lipid re-import across the plastid OE, inter-membrane space, and IE is required [25,47]. For this transport, perhaps in the form of ER-derived PA, an ABC transporter system [TGD1–3 (TRIGALACTOSYLDIACYLGLYCEROL1–3) of prokaryote-type subfamily I] at the IE and the PA-binding β -barrel lipid transfer protein TGD4 in the OE are required [20,27]. Mutation of TGD1 (ABCI14) impairs ER-to-plastid lipid trafficking and leads to abnormal accumulation of trigalactosyldiacylglycerol (TGDG) [48,49]. Subsequently, it was shown that the TGD1–3 complex (ABCI14–16) mediates lipid transfer from the intermembrane space to the stroma [50,51]. TGD1 represents the IE membrane-intrinsic permease, TGD2 binds the substrate PA in the intermembrane space, and the stroma-localized ATPase TGD3 provides the energy for transport (Figure 1). Because TGD4 mutations also accumulate TGDG [52], the protein can bind PA [53] and forms a β -barrel in the plastid OE [54], and TGD4 most likely has a similar function as the *E. coli* FadL β -barrel (Figure 2A) in PA transport across the OE. Only very recently the fifth TGD protein, TGD5, was identified in a forward genetic screen for lipid trafficking mutants in *Arabidopsis*. Based on extensive biochemical and genetic analyses, TGD5 is hypothesized to facilitate lipid transfer from the OE to the IE by bridging TGD4 with the TGD1–3 complex via protein–protein interactions (Figure 1) [55].

FA Transport into Peroxisomes

FA β -oxidation, which is exclusively located in plant peroxisomes, represents the major pathway for sequential breakdown of FAs to acetyl-CoA and is essential for providing germinating seeds/growing seedlings of oil seed plants with carbon energy. Thus, FA import across the peroxisomal membrane is necessary, and this is mediated by ABCD transporters [56]. Because, in *Arabidopsis*, independent forward genetic screens [57–59] identified the full-size ABCD1 to be responsible for this task, the protein is also known as PXA1 (PEROXISOMAL ABC TRANSPORTER 1), CTS (Comatose), or PED3 (PEROXISOMAL DEFECTIVE 3). In addition to FAs, ABCD1 can transport other metabolites such as lipophilic hormone precursors and is also involved in acetate metabolism [60]. Knockout of ABCD1 thus evokes pleiotropic phenotypes, such as fewer lateral roots, impaired seed dormancy and germination, reduced fertilization, or severe leaf necrosis after extended darkness [30,57–59,61,62], the latter resulting from accumulation of toxic free FAs. In the yeast $\Delta pxa1 pxa2$ mutant, which is deficient in import of exogenous FAs into peroxisomes, AtABCD1 complements for oleic acid-dependent growth and restores FA β -oxidation [63]. However, its cytosolic ATPase function could be stimulated only by acyl-CoA, and not by free FAs, leaving open the question whether the substrates are FAs or acyl-CoAs. Experiments in the yeast triple mutant $\Delta pxa1/pxa2/faa2$ [lacking peroxisomal FA transport and fatty acyl CoA synthetase (FACS) activity] and isolation of native membrane complexes [64] nicely demonstrated interaction of the membrane-intrinsic ABCD1 and LACS6/7 on the matrix side of the peroxisome membrane (Figure 1). LACS6/7 are the peroxisome LACS

enzymes and, upon double mutation, reveal defects in seed lipid mobilization as well as seedling establishment [65,66], indicating involvement in FA β -oxidation. Furthermore, ABCD1 possesses an unusual intrinsic acyl-CoA thioesterase activity [64], introducing a model in which ABCD1 cleaves cytosolic acyl-CoA and transports free FAs that in turn are activated by LACS6/7 (Figure 2C). In addition, ABCD1 interacts with CGI-58, a regulator of lipid metabolism and signaling in mammals and plants [67]. In leaves (but not seeds), ABCD1 and CGI-58 thereby regulate TAG content and probably lipid signaling. Fueling of FAs into β -oxidation in leaf tissue most likely occurs via TAG intermediates stored in lipid droplets (Figure 1) to protect against the toxic effects of free FAs. TAG synthesis occurs via cytosolic lipins and PDAT1 (phospholipid: diacylglycerol acyltransferase 1), whereas TAGs accumulated in lipid droplets are broken down by the sequential activity of lipases, beginning with SDP1 (SUGAR-DEPENDENT 1) [68,69]. Subsequently liberated FAs enter peroxisomes through the activity of ABCD1, or via an alternative bypass route for free FAs, discussed in [64].

Transport to and across the Plasma Membrane

In plants, three complex hydrophobic biopolymers also called 'surface lipids' are deposited within the cell wall to restrict water flow and protect against biotic/abiotic stresses: (i) waxes/cutin in the cuticular layer of epidermis cells, (ii) suberin, in the periderm, root endodermis and seed coat, and (iii) sporopollenin/tryphine of the pollen cell wall (Figure 1). Because synthesis of their constituents occurs in the ER, transport of precursors towards/across the plasma membrane and distribution within the cell wall are required. Further, membrane constituents themselves have to be shuttled from ER to the plasma membrane. Most likely this occurs through vesicle traffic and membrane contact sites, however, evidence for involvement of proteins is also accumulating.

ACBP1 (acyl-CoA-binding domain-containing protein 1) and ACBP2 have a transmembrane domain that attaches them to the ER and plasma membrane [13]. Thus, most likely they are involved in intermembrane FA/lipid traffic (Figure 1). Recombinant ACBP1 binds C₁₈-LC and **very long chain** (VLC) acyl-CoAs as well as PC and PA [70,71]. Although highly similar in sequence, ACBP2 prefers C₁₆ acyl-CoA and binds lysoPC [72,73]. *ACBP1* and 2 single mutants are affected in seed development and germination [74,75], and *acbp1/2* double mutants are embryo lethal [76]. For ACBP1, physiological functions in wax/cutin formation (Figure 1) by possible VLC acyl-CoA trafficking [71], as well as lipid remodeling upon cold stress – probably by maintenance of membrane-associated PC/PA-pools – are described [70,77]. ACBP2 is implicated in signaling during drought response and phospholipid repair [75] as well as in lysoPC detoxification upon oxidative stress [73,78]. Interestingly, ACBP3, which binds saturated C_{20:0–24:0}-CoA as well as PC and phosphatidylethanolamine (PE), is targeted to the apoplast and thus could shuttle them to their destination within the cell wall [79]. Mutation of ACBP3 suggests a role in senescence-induced FA/lipid remodeling [80] and plant defense [81,82]. LTP lipid-transfer proteins, which specifically evolved in land plants and bind lipophilic ligands in a hydrophobic cavity, are also excreted into the extracellular space. Evidence for involvement of LTPs in assembly of cuticular wax, suberin, and sporopollenin (Figure 1) is constantly accumulating in recent literature, and concerns glycosylphosphatidylinositol (GPI)-anchored LTPs attached to the external side of the plasma membrane [15,17,18] as well as completely secreted proteins (e.g., from anther tapetum cells to the locule and pollen surface) [16].

Active plasma membrane export of precursors for surface lipids is mediated by ABC transporters of subfamily G, as described in the following section. Because their function is mainly deduced from analysis of loss-of-function phenotypes, the identity of the exact substrates remains an open question. Answering remains challenging because it is extremely difficult to perform direct *in vitro* transport assays owing to the technical difficulty of producing and purifying these large, membrane-intrinsic, hydrophobic proteins in heterologous systems, as well as the scarce commercial availability and low solubility of lipophilic substrates.

Transport for Cutin, Wax, and Suberin Assembly

All epidermal cells of plant tissues are coated by the hydrophobic cuticular layer composed of a cutin matrix in the outer face of the epidermal cell wall, and waxes, divided into intracuticular (within the cutin matrix) and superimposed epicuticular waxes (Figure 1). Whereas cutin forms a rigid network of acylglycerols and polyesters of mainly LC hydroxy or epoxy FAs, wax is usually made of a mixture of straight-chain alkanes, alcohols, aldehydes, ketones, esters, and free FAs [83–85]. Cutin/wax precursors are produced and secreted by epidermal cells; for example, more than 50% of FAs synthesized in *Arabidopsis* stem epidermal cells are exported across the plasma membrane [86]. The first ABC transporter described to be involved, CER5/ABCG12, was discovered in *eceriferum* mutants, which means ‘not bearing wax’ [87]. The half-size ABCG12 is exclusively expressed in epidermal cells, and *abcg12* knockouts show more than 50% decline of stem cuticular waxes. Epidermal cells of *abcg12* plants accumulate abnormal, sheet-like wax deposits in the cytoplasm, similarly to knockouts of the closely related ABCG11 [88–90]. In addition to missing epicuticular waxes, *abcg11* mutants exhibited more pleiotropic phenotypes, implicating involvement in cutin, wax, and suberin metabolism in reproductive organs and roots, respectively [91]. In stem epidermal cells, homo- and heterodimers of ABCG11 and 12 are present, and are important for wax export [92]. Because ABCG13 also seems to be required for secretion of cutin building-blocks in flowers [93], tissue-dependent combinations of different half-size ABCG proteins for transport of wax, cutin, and/or suberin components are possible (Figure 1). Further, ABCG11 can form dimers with ABCG9 and ABCG14, which links it to a function in lipid/sterol homeostasis regulation in vascular organs [94]. ABCG32/PEC1 (PERMEABLE CUTICLE 1) belongs to the full-size ABCG subfamily [9], but by contrast is only involved in transport of cutin precursors because knockout mutants display a permeable cuticle phenotype, and reduction of cutin monomers is accompanied by lipidic inclusions in flower epidermis cells [95].

Similar to cutin, suberin, which is mainly deposited in the inner face of cell walls, is a mixture of acylglycerols, hydroxy/epoxy fatty acids joined by ester linkages, fatty alcohols and VLC aliphatics. In addition, suberin contains phenolic compounds, mostly hydroxycinnamic acids [84]. Again, ABCG transporters play an important role in the construction of suberin layers [96]. Recently, it was reported that half-size ABCG2, ABCG6 and ABCG20 are involved in the formation of suberin layers in root endodermis and seed coat tissues (Figure 1) [97]. Seed coats and roots of *abcg2/6/20* triple mutants had increased permeability, distorted suberin structure, and reduced aliphatic components.

Transport for Sporopollenin/Tryphine Building-Blocks of the Outer Pollen Cell Wall

Pollen cells are surrounded by a cell wall, which functions not only in protection from UV radiation and pathogen attack, but also mediates interaction with the stigma during pollination. Thus, interference with pollen cell wall assembly often affects plant male fertility. Pollen cell walls consist of three layers: (i) an intine layer, composed of cellulose; (ii) an exine layer, primarily composed of sporopollenin; and (iii) a tryphine pollen coat, covering exine cavities and containing carotenoid/flavonoid pigments (Figure 1) [1,2]. Sporopollenin and tryphine are complex, hydrophobic biopolymers mainly composed of FA/lipid derivatives, although their exact composition is still unclear [98,99]. During pollen development, most precursors are exported from tapetum cells in anthers, and thus are deposited from the exterior onto the pollen grain. Whereas FA (via FAX1 proteins, see above) and fatty alcohol delivery from tapetum cell plastids is crucial for exine and pollen coat assembly [10,100], transport across the plasma membrane is also accomplished by half-size ABCG transporters (Figure 1). ABCG26/WBC27, that is expression-regulated in tapetal cells at early pollen stages [101], was identified to mediate sporopollenin precursor transport from tapetum to anther locules. *abcg26* knockouts show severely reduced fertility and defects in pollen exine formation [102–104], and recently the protein was linked to the transport of polyketides derived from plastid FAs [105]. Further, *abcg1/abcg16* double mutants are defective

Box 2. The Situation in Green Microalgae: Differences in FA/Lipid Transport between *Chlamydomonas* and *Arabidopsis*

In contrast to land plants, until recently FA and lipid biosynthesis in green microalgae has been studied only rarely [23,120–122]. The general framework for the metabolic networks involved in FA synthesis, modification, glycerolipid assembly, and FA turnover in microalgae can be deduced based on the presence of orthologs of known proteins of lipid metabolism [23]. Members of the major protein families (ABC transporter, LTP, ACBP, LACS, as well as FAX proteins) known to be involved in FA/lipid transport in plants are also encoded in the genome of *Chlamydomonas*; however, for none of them has activity been demonstrated experimentally. Considering the large evolutionary diversity in microalgae, we limit our discussion to the best-studied model microalga *Chlamydomonas reinhardtii*, with focus on three potential differences between lipid trafficking in *Chlamydomonas* and *Arabidopsis*.

There Is Probably No Lipid Import back into the *Chlamydomonas* Chloroplast

Chloroplast membranes of *Chlamydomonas*, similar to those of cyanobacteria and land plants, are essentially composed of MGDG (monogalactosyl-diacylglycerol) and DGDG (digalactosyl-diacylglycerol). Interestingly, these two galactolipids are entirely produced by so-called 'prokaryotic' species, in other words with a C₁₆-FA present at the *sn*-2 position [123]. This sets *Chlamydomonas* apart from most land plants, where either a mixture of C₁₆-, C₁₈-FAs are present on the *sn*-2 position of a galactolipid (the so-called 16:3 plants, such as *Arabidopsis*), or where only *sn*-2 C₁₈-FAs are present (the so-called 18:3 plants, such as *Pisum sativum* [47]). The fact that both chloroplast- and ER-derived lipids can be used as precursors for chloroplast-specific galactolipids demonstrates the need for lipid trafficking through the two plastid envelopes and integration into thylakoid membranes. This traffic is fairly active: for example, around 30–50% of acyl-chains exported out of the plastid are re-imported, involving the molecular players TGD4 at the OE and TGD1–3 at the IE membrane (see above and Figure 1). The lack of 'eukaryotic' galactolipid species in the chloroplast of *Chlamydomonas* indicates that the import of lipids back to chloroplasts is probably not necessary, and this is consistent with the absence of TGD4 in the *Chlamydomonas* proteome. Genes encoding proteins orthologous to the *Arabidopsis* TGD1–3 ABC transporter complex are present in *Chlamydomonas*, and the corresponding proteins have further been detected in a lipid-droplet proteome of *Chlamydomonas* [124]. *Chlamydomonas* TGD1–3 could be involved in trafficking galactolipids assembled in the plastid envelope to thylakoid membranes where they are the major components. Experimental evidence for this or other roles, however, needs to be demonstrated. The lack of lipid re-import into plastids in an evolutionarily primitive alga supports the hypothesis that, with evolution of the eukaryotic lipid synthesis pathway, the plastid pathway has been diminished in some higher plants [47]. A note of caution to this interpretation is that the two-pathway theory is largely based on the different substrate specificities of the *sn*2 acyltransferases (i.e., lysophosphatidic acid acyltransferase LPAT). The acyl specificities of *Chlamydomonas* LPAT proteins, however, have not been demonstrated.

The Metabolic Consequence of the Absence of PC in *Chlamydomonas*

In addition to the flow of acyl-chains from their site of *de novo* synthesis (i.e., the plastid) to their distribution and deposition into different subcellular membrane systems, acyl-chains are also constantly exchanged between different lipid classes from one membrane to another. The latter can be even more prominent under stress conditions. PC has been found to play a central role in acyl-chain redistribution in higher plants [32]. PC is also the major substrate where acyl modifications occur and also likely a carrier for shuffling acyl-chains between subcellular compartments and membrane subdomains. However, one of the defining features of the lipidome of *Chlamydomonas* is the apparent lack of PC and the occurrence of the betaine lipid DGTS (diacylglycerol-trimethylhomoserine [123]). This raises the issue of the metabolic organization of acyl-exchange mechanisms in *Chlamydomonas*. Whether DGTS can take up such a role in lipid trafficking as PC is not clear because, except for DGTS synthase, enzymes involved in DGTS metabolism have not been identified.

Under Stress, Galactolipids Can Be Used as a Source To Supply Acyl-Chains for TAG Synthesis in Both Organisms, but Likely via Different Routes?

Cold-stressed *Arabidopsis* leaves accumulate TAGs, which are derived from a DAG backbone produced from MGDG through the action of a galactolipid:galactolipid galactosyltransferase (also named SENSITIVE TO FREEZING 2, SFR2 [125]). *Chlamydomonas* also evolved a way to supply acyl-chains present in chloroplast MGDG to TAG synthesis. This is achieved via the action of the galactolipid lipase PGD1 (phosphoglycerate dehydrogenase 1) [126]. Under nitrogen starvation, the null mutant *pgd1* accumulated only 50% of wild-type TAG levels. Genes encoding proteins showing some sequence similarity to PGD1 can be identified in the genome of *Arabidopsis*, but functional demonstration for its role in lipid remodeling remains to be tested. Studies on lipid-droplet assembly in *Chlamydomonas* further show the presence of chloroplast envelope membrane proteins, and thus indicate a partial origin of lipid droplets from chloroplast membranes [122,127,128].

in the formation of inner exine and carbohydrate intine layers [97], and ABCG9 and ABCG31 contribute to sterol ester accumulation in tryphine pollen coat [106]. Because half-size ABC transporters are flexible for dimerizing, different combinations of subfamily G proteins might define the substrate and tissue specificity of transport.

Concluding Remarks

In summary, FA/lipid transport proteins play a crucial role in the distribution of lipophilic compounds within plant cells, and thus strongly affect plant growth, development, and performance. Considering that everything starts in plastids, it is fascinating that acyl residues can travel via FAX, LACS, ACBP, TGD, ABCA, ABCG proteins to their final destinations. Future research therefore offers exciting prospects for understanding transport mechanisms and their physiological impact (see Outstanding Questions). The combination of transport with steps in biosynthesis further provides an opportunity to improve lipid production in the green lineage, including green microalgae (Box 2), which have recently emerged as models for engineering oil metabolism.

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Outstanding Questions

Plastid FA export: FAX1 function affects TAG content in flower and leaf tissue, and future research must therefore clarify the role of FAX proteins in seed oil accumulation, allowing biotechnological applications in oilseed crops. Besides detailed functional tests in heterologous systems, *in planta* assays to determine the true substrate specificity of FAX1 are needed. Further open questions are the involvement of other proteins besides FAX1, the function of FAX2–FAX7 proteins, and how FAs cross the intermembrane space and are finally transferred over the OE. Detailed studies should clarify whether LACS9 acts in plastid FA export and/or import, and whether interaction with FAX1 takes place.

Plastid re-import of eukaryotic lipids: although TGD4 and TGD2 bind specifically to PA, the chemical nature of eukaryotic DAG backbones for plastid re-import is still unclear. It should also be tested whether TGD4 functions similarly to the *E. coli* FadL in PA transport, and whether other proteins are needed for lipid import. Membrane contact sites, in particular between ER and chloroplasts, complicate the picture of FA and lipid traffic between membranes. Future studies should therefore clarify the exact subcellular localization of proteins not only by error-prone GFP-targeting but also by different independent approaches (e.g., immunostaining) and by *in vivo* discrimination and quantification of traffic mediated by membrane transporters.

Transport of lipophilic compounds by ABC transporters: similarly to ABCD1 in peroxisomes, more direct functional assays will be necessary to determine substrate specificity and interaction partners for other ABC transporters. Future research should address whether ER-resident LACs interact with ABC transporters and how ER export of lipophilic compounds is accomplished.

FA/lipid transport in green microalgae: future studies should determine the FA/lipid transport processes operating in model systems based on analyses of lipid molecular species, metabolic data, and protein sequence similarities. Proteins showing sequence similarity to known transporters of plant surface lipids are also present in the algal genome, but their function remains

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