

All fatty acids are not equal: discrimination in plant membrane lipids

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Plant membrane lipids are primarily composed of 16-carbon and 18-carbon fatty acids containing up to three double bonds. By contrast, the seed oils of many plant species contain fatty acids with significantly different structures. These unusual fatty acids sometimes accumulate to >90% of the total fatty acid content in the seed triacylglycerols, but are generally excluded from the membrane lipids of the plant, including those of the seed. The reasons for their exclusion and the mechanisms by which this is achieved are not completely understood. Here we discuss recent research that has given new insights into how plants prevent the accumulation of unusual fatty acids in membrane lipids, and how strict this censorship of membrane composition is. We also describe a transgenic experiment that resulted in an excessive buildup of unusual fatty acids in cellular membranes, and clearly illustrated that the control of membrane lipid composition is essential for normal plant growth and development.

Structural membrane glycerolipids of all plant cells contain almost exclusively 16-carbon and 18-carbon fatty acids, with up to three methylene-interrupted double bonds (16:0, 16:1*, 18:0, 18:1, 18:2, 18:3, and in some species 16:3). These fatty acids are often referred to as common fatty acids.

In contrast with the conservative fatty acid composition of plant membrane lipids, tremendous fatty acid diversity exists in the seed storage lipids. To date, >300 naturally occurring fatty acids have been described in seed oils^{1,2}, and it has been estimated that thousands more could be present throughout the plant kingdom. The structures of these fatty acids can vary in chain length from 8 to 24 carbons, they can have double bonds in unusual positions, or novel functional groups, such as hydroxy, epoxy, cyclic, halogen or an acetylenic group on their acyl chain (Fig. 1). Because their chemical structures deviate significantly from the common fatty acids and they are usually only found in a few plant species, these fatty acids are considered unusual. In many cases, unusual fatty acids are the predominant fatty acid in the seed oil of a plant species.

The reason for such a great diversity of seed oil constituents is unknown, but plants can tolerate high levels of unusual fatty acids in storage lipids because they are sequestered into oil bodies and have no structural function. The special physical and chemical properties of many unusual fatty acids might explain why they are excluded from the membrane lipids of seeds, and are absent from other parts of the plant. It is believed that they would perturb the structural integrity of the membrane bilayer and have deleterious effects on the cell. Consequently, storage and membrane lipids have different fatty acid compositions.

It is puzzling how these different fatty acid compositions become established and are maintained. This question is particularly pertinent as the production of storage and membrane lipids occurs simultaneously and involves common precursors³. Castor bean (*Ricinus communis*), for example, produces seed oil containing nearly 90% ricinoleic acid (18:1-OH), an unusual hydroxy fatty acid (Fig. 1). Analysis of the membrane lipids from developing castor bean seeds indicates that even during its most active period of biosynthesis this fatty acid accounts for only a small proportion of the total fatty acids in the membrane⁴. This observation is

striking because it is a membrane lipid, phosphatidylcholine, which serves as the substrate for 18:1-OH synthesis. Thus, the mechanisms that edit out unusual fatty acids from membrane lipids and then channel them to storage lipids must be extremely selective and efficient. How strict this editing is, probably depends on the structure of the particular unusual fatty acid. For example, the structure of petroselinic acid (18:1Δ6) deviates from common acyl groups only by the position of the double bond. Therefore, 18:1Δ6 can accumulate in membrane lipids to a much greater extent than fatty acids such as 18:1-OH, which contains a polar oxygenated functional group that would be incompatible with the hydrophobic environment of the membrane.

The membrane-editing ability of plants is also important for biotechnology. Many of the unusual fatty acids represent valuable feedstocks for the chemical industry. However, plants that accumulate these fatty acids are often not amenable to agriculture. Therefore, there is an enormous interest in generating transgenic crop plants engineered to accumulate high levels of specific unusual fatty acids. To achieve this goal it might be essential to prevent an accumulation of unusual fatty acids in seed membrane lipids. Thus, engineering projects aimed at generating viable, high-yielding transgenic plants, probably require the introduction of genes involved in the exclusion of unusual fatty acids from membranes⁵. Here we discuss how such exclusion might be accomplished.

Pathways for the synthesis of unusual fatty acids

With the exception of a few commercially valuable unusual fatty acids, relatively little is known about the biosynthesis of these compounds. The proposed pathways leading to the synthesis of the unusual fatty acid discussed in this review are described below.

Medium-chain fatty acids

In plants, *de novo* fatty acid biosynthesis occurs in the plastid where acetate (C2) is elongated by the sequential addition of further C2 units while attached to a soluble acyl-carrier-protein (ACP)³. For common fatty acid formation, the growing acyl chain is terminated when it is 16 or 18 carbons long, by the action of an acyl-ACP thioesterase, which cleaves the acyl group from the ACP to produce a free fatty acid. Plants that synthesize medium-chain fatty acids (MCFA; C8–C14) have an additional acyl-ACP

*In fatty acid nomenclature 16:1 indicates a 16-carbon fatty acid with one double bond.

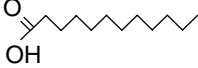
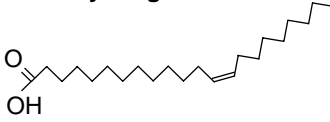
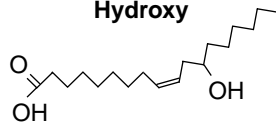
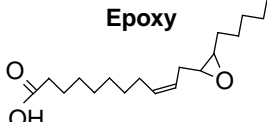
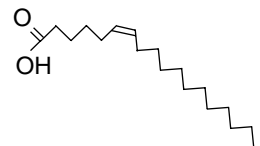
Fatty acid class and example	Name	Principal biosynthetic enzyme	Plant family	% in lipids	
				TAG	Membrane
Medium-chain 	Lauric (12:0)	Thioesterase	Lauraceae Lythraceae <i>Cuphea wrightii</i> Arecaceae Palm oil (<i>Elaeis guineensis</i>)* <i>Cocos nucifera</i> *	54	2.9
Very-long-chain 	Erucic (22:1)	β -Keto-acyl-CoA synthase	Brassicaceae <i>Crambe abyssinica</i> Oilseed rape* <i>Arabidopsis thaliana</i> Limnanthaceae <i>Limnanthes douglasii</i>	60	3.0
Hydroxy 	Ricinoleic (18:1-OH)	Diiron hydroxylase	Euphorbiaceae <i>Ricinus communis</i> * Brassicaceae <i>Lesquerella</i> spp.	85	5.0
Epoxy 	Vernolic (18:1-O)	Epoxidase	Asteraceae <i>Crepis palaestina</i> <i>Vernonia</i> spp. Euphorbiaceae	57	2.0
Double bond position 	Petroselinic (18:1 Δ 6)	Double desaturase	Apiaceae Carrot <i>Coriandrum sativum</i> Araliaceae	70–75	10.0–20.0

Fig. 1. Examples of some of the unusual fatty acids made by plants. Current commercial sources are indicated by an asterisk.

thioesterase, which prematurely cleaves the acyl-chain from ACP, redirecting fatty acid synthesis from long (C16–C18) to medium chains (C8–C14)⁶.

Unusual monounsaturated fatty acids

The synthesis of common monounsaturated fatty acids is catalysed by a soluble plastidial desaturase, which introduces a double bond between carbons 9 and 10 of a C18 acyl-ACP (Δ 9 position, counting from the carboxyl end). Plants that synthesize unusual monounsaturated fatty acids have an additional desaturase, which is closely related to the Δ 9-desaturase, but introduces a double bond at a different location on the acyl-ACP. For example, in coriander (*Coriandrum sativum*), petroselinic acid (Fig. 1) is synthesized by a desaturase that introduces a double bond between carbons 4 and 5 of a C16 acyl-ACP (Δ 4-desaturase). This fatty acid is then extended by two carbons and cleaved from ACP to produce the free fatty acid. These last two steps are thought to require a specialized condensing enzyme and a specialized acyl-ACP thioesterase⁷.

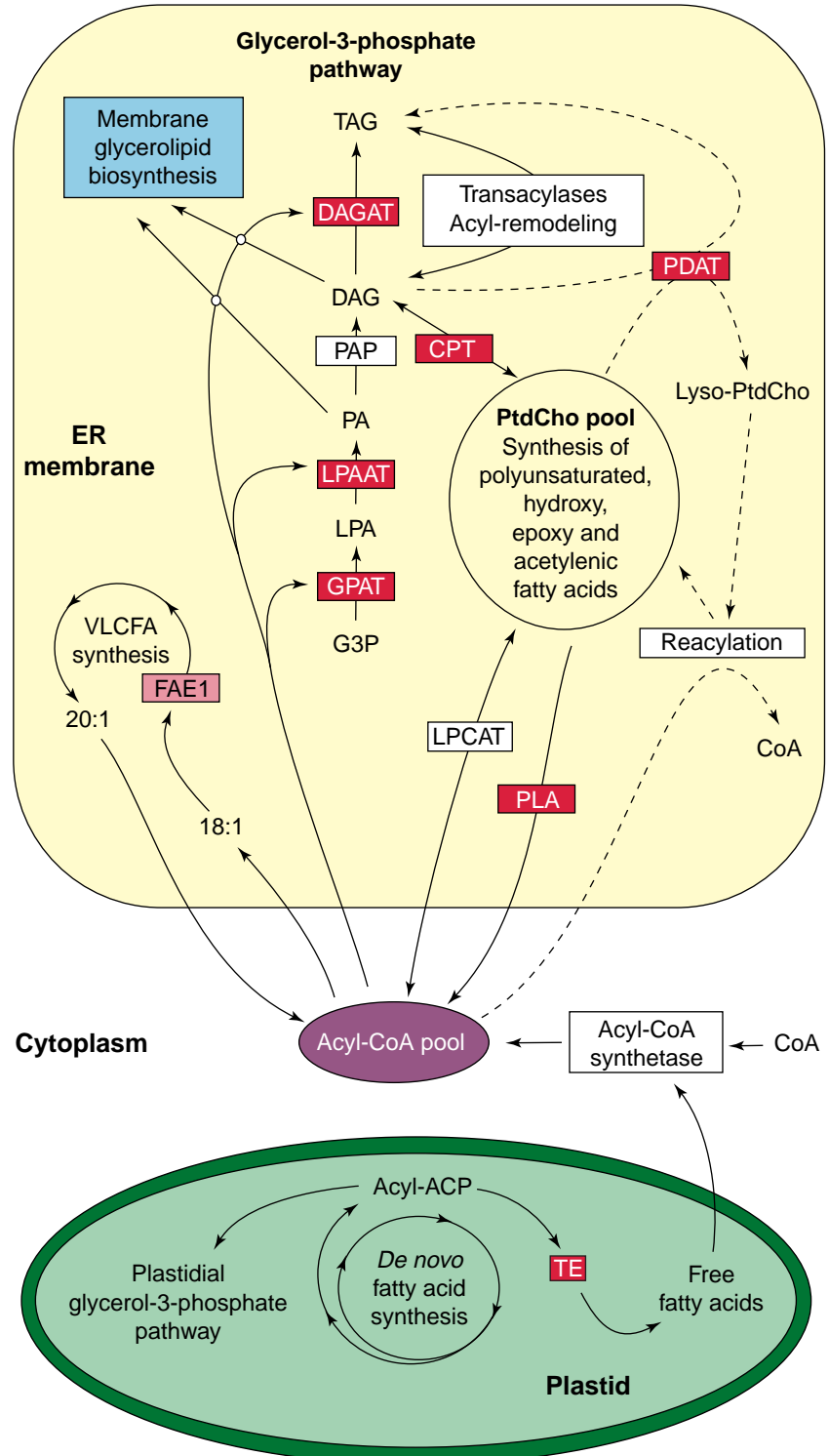
Hydroxy, epoxy and acetylenic fatty acids

A family of related enzymes is responsible for the synthesis of the fatty acids containing hydroxy⁸, epoxy and acetylenic functional groups⁹. These enzymes are structurally similar to extraplastidial membrane-bound Δ 12-desaturases (FAD2), and only four amino acid substitutions are needed to convert an 18:1-desaturase into an 18:1-hydroxylase¹⁰. The synthesis of these fatty acids is thought to take place on the endoplasmic reticulum and use fatty acids esterified to the major membrane lipid phosphatidylcholine (PtdCho) as a substrate (Fig. 2).

Very-long-chain fatty acids

Very-long-chain fatty acids (VLCFAs; >C18) are synthesized extraplastidially by successive rounds of elongation of a C18 fatty acyl precursor by two carbons originating from malonyl-CoA (Fig. 2). Each elongation step requires four enzymatic reactions: condensation between an acyl precursor and malonyl-CoA, followed by a reduction, a dehydration and another reduction. However, in transgenic experiments expression of a single gene encoding

Fig. 2. A simplified diagram of the metabolic pathways of plant lipid biosynthesis. Enzymes discussed in the text are in red boxes. Enzymes whose role in determining the fatty acid composition of membrane lipids is uncertain are in white boxes. In plants, *de novo* fatty acid biosynthesis occurs in the plastid by the addition of C2 units to a growing acyl-chain attached to a soluble acyl-carrier-protein (ACP). To enter the plastidial 'prokaryotic' pathway of glycerolipid synthesis, fatty acids are transferred from ACP to glycerol-3-phosphate (G3P). To enter the 'eukaryotic' pathway, fatty acids are cleaved from the ACP by an acyl-ACP-thioesterase to form free fatty acids. These are exported to the cytoplasm, esterified to CoA and join the acyl-CoA pool. The acyl groups are then used by the acyltransferases of the 'eukaryotic' glycerol-3-phosphate pathway in the endoplasmic reticulum (ER) to produce membrane and storage lipids. The first acylation of glycerol-3-phosphate is catalysed by glycerol-3-phosphate acyltransferase (GPAT), which acylates the *sn*-1 position of the glycerol backbone to produce lysophosphatidic acid (LPA). Subsequently, 1-acyl-glycerol-3-phosphate acyltransferase (LPAAT) acylates the *sn*-2 position to form phosphatidic acid (PA). Dephosphorylation of phosphatidic acid by phosphatidic acid-phosphatase (PAP) yields diacylglycerol (DAG). Diacylglycerol can be acylated at the *sn*-3 position to produce triacylglycerol (TAG) by diacylglycerol acyltransferase (DAGAT) – the only step unique to triacylglycerol biosynthesis. Alternatively, phosphatidic acid and diacylglycerol can both be used as precursors of the major membrane glycerolipids. In addition, diacylglycerol is converted to phosphatidylcholine (PtdCho) by CDP-choline diacylglycerol choline phosphotransferase (CPT). Fatty acids esterified to PtdCho can be modified by membrane-bound desaturases to produce polyunsaturated fatty acids. PtdCho is also the site of synthesis of certain unusual fatty acids (hydroxy, epoxy, acetylenic). Acyl groups can be removed from PtdCho by the action of phospholipases (PLA). Alternatively, fatty acids at the *sn*-2 position of PtdCho can be exchanged for fatty acids from the acyl-CoA pool by the action of acyl-CoA lysophosphatidylcholine acyltransferase (LPCAT). Direct transfer of some fatty acids (ricinoleic acid) from PtdCho to diacylglycerol to produce triacylglycerol, catalysed by phospholipid-diacylglycerol acyltransferase (PDAT), is also thought to occur. The site of synthesis of very-long-chain-fatty acids (VLCFA) is also shown. FAE1 = FATTY ACID ELONGATION 1.



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the condensing enzyme is sufficient for the synthesis of VLCFAs. It appears that the other three enzymes of the pathway are present in all cells and at sufficiently high levels, thus VLCFA biosynthesis is regulated primarily by the expression of the condensing enzyme¹¹.

Common themes of unusual fatty acid biosynthesis

The pathways described above share several common features. Firstly, the synthesis of these unusual fatty acids involves just one additional or alternative enzymatic step from primary lipid metabolism^{6,8,9,11,12}. However, although only one enzyme is

needed for their synthesis, other specialized fatty acid biosynthetic enzymes or components are required for their high accumulation¹³. Secondly, all the enzymes identified to date that are involved in unusual fatty acid biosynthesis are structurally related to enzymes of primary lipid metabolism. Thus, it appears that 'housekeeping genes' have been recruited and specialized for the development of enzymes, which either have altered substrate specificities, or can catalyse closely related but modified reactions¹⁴. Many of the unusual fatty acids are found in taxonomically dispersed families (Fig. 1), implying that the recruitment of enzymes for the

synthesis of these unusual fatty acids might have occurred a number of independent times during angiosperm evolution. What selective advantages are associated with high levels of accumulation of any of the given unusual fatty acids is unknown.

Mechanisms for the exclusion of unusual fatty acids from membrane glycerolipids

Higher plants have two distinct pathways for the synthesis of membrane lipids: the prokaryotic pathway in the plastid envelope and the eukaryotic pathway in the endoplasmic reticulum. In both pathways, the first and second steps in membrane lipid synthesis are the sequential acylation of glycerol-3-phosphate (G3P) at the *sn-1* and *sn-2* positions to produce phosphatidic acid. Phosphatidic acid is then either activated to cytidine diphospho-diacylglycerol (CDP-DAG) or converted to diacylglycerol by a phosphatase for the synthesis of the major membrane phospholipids and glycolipids. In developing seeds, diacylglycerol is also further acylated to produce triacylglycerol. The production of membrane and storage lipids therefore shares a common pathway referred to as the glycerol-3-phosphate pathway or the Kennedy pathway¹⁵ (Fig. 2).

Two hypotheses have been proposed to explain the targeting of unusual fatty acids to storage lipids and their exclusion from membrane lipids: enzyme specificity and compartmentation. However, it is highly likely that there is no single sorting mechanism in operation in any given plant species, and that multiple mechanisms are working in concert. Because plants accumulating unusual fatty acids have evolved independently, it is likely that these mechanisms, or their relative importance, differ significantly in different species.

Enzyme specificity

One possibility is that phosphatidic acid and diacylglycerol molecular species containing fatty acids that are incompatible with membrane structure are either not accepted by phospholipid or glycolipid biosynthetic enzymes or that they are rapidly edited out of the membranes. We will discuss several studies that have examined the *in vitro* specificities of enzymes involved in lipid metabolism to assess their potential role in fatty acid distribution between membrane and storage lipids.

Thioesterases

Thioesterases remove newly formed acyl chains from ACP. Their activity thus prevents the entry of these acyl groups into the prokaryotic pathway of glycerolipid synthesis, because the glycerol-3-phosphate acylating enzymes require an acyl-ACP substrate. This is the case in plants such as *Cuphea* spp.: medium-chain fatty acids are released in the plastid in a form that cannot be used by the prokaryotic pathway. By contrast, unusual monounsaturated fatty acids, such as 18:1Δ6, are synthesized while esterified to ACP. As a result 18:1Δ6-ACP is generated, which readily enters plastidial membrane lipids¹⁶. The 18:1Δ6-producing plants possess an additional acyl-ACP thioesterase, which is highly specific for 18:1Δ6, and might be involved in enhancing the flux of 18:1Δ6 out of the plastid⁷.

Acyltransferases

Acyltransferases are central to the synthesis of both membrane lipids and triacylglycerol. A wide variety of *in vitro* studies have indicated that these enzymes show considerable selectivity for acyl groups both on the activated acyl substrate (e.g. acyl-CoA) and on the molecule to which they transfer the acyl group. The selectivity of the acyltransferases might therefore be a key factor in determining the partitioning of common and unusual fatty acids into membrane and storage lipids, respectively^{17,18}.

The biosynthesis of triacylglycerol in *Cuphea* is a good example of how partitioning might be achieved. All three acyltransferases

involved in triacylglycerol synthesis can use medium-chain and common fatty acids. Consequently, the fatty acyl group at the *sn-1* position generally depends on the composition of the acyl-CoA pool^{19,20}. However, the acyl group incorporated at the *sn-1* position affects the acyl-CoA specificity and the selectivity of the *sn-2* acylating enzyme, 1-acyl-glycerol-3-phosphate acyltransferase (LPAAT). When a C18 fatty acid is at the *sn-1* position, LPAAT preferentially uses a C18 fatty acid for the *sn-2* position, whereas if a MCFA is at the *sn-1* position, LPAAT exclusively uses a MCFA. The mechanism for this specificity is thought to be the co-existence of different isoforms of LPAAT (Refs 18,21). As a result, the formation of mixed-chain phosphatidic acid species, esterified with both medium and common fatty acid chains, is largely prevented. The *sn-3* acylating enzyme, diacylglycerol acyltransferase (DAGAT), then displays a specificity and selectivity for 10:0/10:0-diacylglycerol species and almost exclusively directs a medium-chain to the *sn-3* position^{20,22}. Thus, the seeds of *Cuphea* have acyltransferases with optimal properties for the efficient incorporation of MCFA into triacylglycerols.

In vitro enzyme studies and genetic engineering have demonstrated that acyltransferases from other plant species containing unusual fatty acids have evolved similar properties directed at efficient targeting of their own unusual fatty acids into triacylglycerols²²⁻²⁴. DAGAT is especially important in this respect. Because rapid equilibration between the diacylglycerol and PtdCho pools is thought to occur, the high selectivity of DAGAT for diacylglycerols with unusual fatty acids effectively reduces the levels of unusual fatty acids in PtdCho. By contrast, acyltransferases from plant species that accumulate triacylglycerols with predominantly common fatty acids, such as sunflower (*Helianthus*) or maize, are not good at using unusual fatty acids²⁵.

Cytidine diphosphocholine diacylglycerol choline phosphotransferase

Cytidine diphosphocholine diacylglycerol choline phosphotransferase (CDP-CPT) catalyses the reversible conversion of diacylglycerol to PtdCho. Therefore, it could potentially prevent diacylglycerol species containing unusual fatty acids from moving into the PtdCho pool, as well as channeling PtdCho-containing unusual fatty acids into the diacylglycerol pool (Fig. 2). It has been hypothesized that CPT might be particularly important in plants where the unusual fatty acid is actually made on PtdCho. To investigate whether this is true, the specificity of CPT was examined *in vitro* using extracts from plants that accumulate medium-chain, hydroxy and very-long-chain fatty acids. None of the enzymes tested was able to discriminate between diacylglycerol molecular species containing either common or unusual fatty acids. Thus, the specificity of CPT is clearly not responsible for the observed bias between triacylglycerol and membrane fatty acid composition that exists in those plant species where the unusual fatty acid is actually made on PtdCho (Ref. 26).

In fact, it has now been demonstrated that in developing coriander seeds, petroselinic acid, an unusual monoenoic fatty acid synthesized in the plastid, is metabolized through PtdCho during periods of rapid triacylglycerol biosynthesis¹⁶. Based on our current understanding of lipid metabolism this would appear unnecessary and raises many puzzling questions. It suggests that PtdCho plays a role in triacylglycerol metabolism other than its involvement in modification reactions^{16,22}. If that is the case, CPT would not need to exclude this class of unusual fatty acids from the PtdCho pool.

Phospholipases

There is considerable evidence that certain unusual fatty acids can be removed from membrane lipids by the action of acyl-specific phospholipases. Moreover, it appears that plants that contain

unusual fatty acids have phospholipase activities that are specific for the same unusual fatty acids that they produce. For instance castor bean, *Cuphea* species and *Euphorbia lagascae* have acyl-specific phospholipase A₂ (PLA₂) activities that selectively hydrolyse hydroxy, medium-chain and epoxy fatty acids, respectively, from their membrane lipids²⁷. Surprisingly, studies using 18:1-OH showed that microsomes from plants that do not produce this fatty acid also had phospholipase activity specific for 18:1-OH (Ref. 4). These observations suggest that microsomal PLA₂s might have a general role in releasing membrane-incompatible fatty acids from PtdCho to protect the integrity of the membrane. PLA₂s have been postulated to have a similar function in animal cells by removing oxygenated fatty acids from membrane lipids²⁸.

However, it is unlikely that phospholipase activity is the primary mechanism by which plants with unusual fatty acids censor their membrane lipids. In the metabolism of some unusual fatty acids, such as 18:1Δ6, reliance on phospholipases would set up a futile cycle²⁶. These unusual fatty acids are esterified onto lipids from the acyl-CoA pool, and their release from PtdCho by phospholipases (followed by re-esterification to CoA) would return them to the same acyl-CoA pool (Fig. 2). Thus, phospholipases might well have a role in removing unusual fatty acids from cellular membranes, but are probably of lesser importance in directing them to triacylglycerols.

Transacylation reactions

Observations made over several years have suggested that, in addition to the glycerol-3-phosphate pathway, plants might use alternative transacylation pathways for the synthesis and acyl remodeling of triacylglycerol^{5,29}. Although the true role of transacylation enzymes in storage lipid biosynthesis remains uncertain, recent experiments indicate that transacylation might be an important mechanism for efficiently channeling unusual fatty acids out of membrane lipids into triacylglycerols. In castor bean, for example, an enzyme designated phospholipid diacylglycerol acyltransferase (PDAT) was recently described. This activity appears to be responsible for the transfer of 18:1-OH from the *sn*-2 position of PtdCho to diacylglycerol to produce lyso-PtdCho and triacylglycerol (Ref. 30; A. Dahlqvist, pers. commun.; Fig. 2). Transfer of 18:1 by this mechanism was considerably less efficient suggesting that PDAT had specificity for the hydroxy fatty acid. Because 18:1-OH is synthesized on the *sn*-2 position of PtdCho, such PDAT-mediated transacylation reaction would not only reduce 18:1-OH accumulation in diacylglycerol (via CTP activity), but also prevent it from entering the acyl-CoA pool for incorporation into membrane lipids.

Compartmentation of triacylglycerol biosynthesis

It has been argued that the specificities of the enzymes of lipid metabolism are not discriminating enough to account for the strong bias between membrane and storage lipids²⁶. As an alternative, it has been proposed that membrane lipid and triacylglycerol assembly might be carried out by distinct subsets of enzymes located in separate domains of the endoplasmic reticulum (ER)^{16,26,31}. Subcellular fractionation of developing oilseed rape (*Brassica napus*) embryos has provided evidence that the localization of enzymes involved in triacylglycerol biosynthesis does not exactly coincide with enzymes involved in membrane lipid biosynthesis in the ER (Ref. 31). In addition, analysis of petroselinic acid accumulation in coriander using [1-¹⁴C]acetate-labelling of developing seeds has suggested that there might be a distinct pool of PtdCho dedicated to triacylglycerol assembly in this plant¹⁶. However, the evidence to support the compartmentation hypothesis is limited and further experiments are needed. Comparing immunolocalization of enzymes involved in storage lipid biosynthesis (e.g. DAGAT) with enzymes involved in membrane lipid biosynthesis might help to resolve this question.

Production of unusual fatty acids in transgenic crop plants

Although plants have evolved mechanisms to correctly target the unusual fatty acid that they naturally accumulate, will the same be true when foreign fatty acids are introduced into transgenic plants? The best-studied example to date is the laurate-producing rape (Calgene™), in which a California bay tree (*Umbellularia californica*) medium-chain fatty acid thioesterase (*FatB1*) was expressed using the seed-specific Napin promoter. In some of these *Napin-FatB1* lines, 12:0 accumulated to >55 mol% of the seed triacylglycerols. By contrast, only 6 mol% 12:0 was found in PtdCho of the membrane lipids of mature seeds, suggesting that 12:0 was correctly targeted in these transgenic plants. However, in developing oilseed rape during rapid triacylglycerol deposition, 12:0 levels could sometimes reach up to 46 mol% of the fatty acids in PtdCho (Ref. 5). By contrast, in species naturally accumulating 12:0, the levels were only between 1 and 4 mol% (Ref. 32). Thus, 12:0-targeting appears less effective in oilseed rape than in plants naturally evolved to synthesize 12:0-rich oils. This implies that if transgenic rape plants are to accumulate >90% of 12:0 in their seed oil, it might also be necessary to engineer the pathways to exclude unusual fatty acids from the membranes. Otherwise, the seed membrane levels of 12:0/12:0-PtdCho could increase dramatically, resulting in a compressed membrane bilayer, and in all likelihood non-viable seeds⁵.

Exclusion of unusual fatty acids from leaf membranes

We have mainly focused on how unusual fatty acids are excluded from the membrane lipids of the seed, because this is where they occur in nature. However, the use of transgenic plants allows us to create unique situations in plant cells. Recently, seed-specific enzymes responsible for the synthesis of medium-chain, hydroxy and very-long-chain fatty acids have all been expressed in the vegetative tissues of plants. The results of these experiments have been somewhat surprising and have given new insights into the regulation of lipid metabolism.

When the medium-chain fatty acid thioesterase gene, *FatB1*, is expressed in oilseed rape under the control of the 35S promoter³³, some of the 35S-*FatB1* transgenic lines accumulate high levels of MCFAs in the seed triacylglycerols. However, the MCFAs are absent completely from all other parts of these plants, even though higher levels of *FatB1* activity are present in the vegetative tissues than in the seeds³⁴. *FatB1* was correctly targeted to the chloroplasts of the 35S-*FatB1* plants, and the isolated chloroplasts can efficiently produce MCFAs *in vitro*. Thus, it is likely that MCFAs are being synthesized in the leaves of 35S-*FatB1* plants, but are then probably rapidly broken down. In support of this hypothesis, enzymes from both the β-oxidation pathway (breakdown of fatty acids) and the glyoxylate cycle pathways (metabolism of fatty acids) are induced in the leaves of transgenic 35S-*FatB1* plants^{33,34}. Furthermore, this degradation is specific for MCFAs because the 12:0-CoA oxidase activity, which is responsible for the breakdown of 12:0, increases up to sixfold, whereas 16:0-CoA oxidase activity remains constant between transgenic and control plants. This idea is supported by studies in *E. coli*. Whereas wild-type *E. coli* cells expressing *FatB1* do not accumulate MCFAs, expression of *FatB1* in *E. coli* mutants blocked in β-oxidation results in high levels of MCFAs, which are secreted from the cells in the form of free fatty acids³⁵. Therefore, MCFAs are probably excluded from membrane lipids in the leaf, and the presence of these excess fatty acids then induces pathways in the cells for their disposal.

Similar results have been obtained with transgenic tobacco and *Arabidopsis* plants expressing the castor bean fatty acid hydroxylase (*FAH12*) gene under the control of the 35S promoter^{8,36}. In both transgenic systems 18:1-OH accumulates in their seed

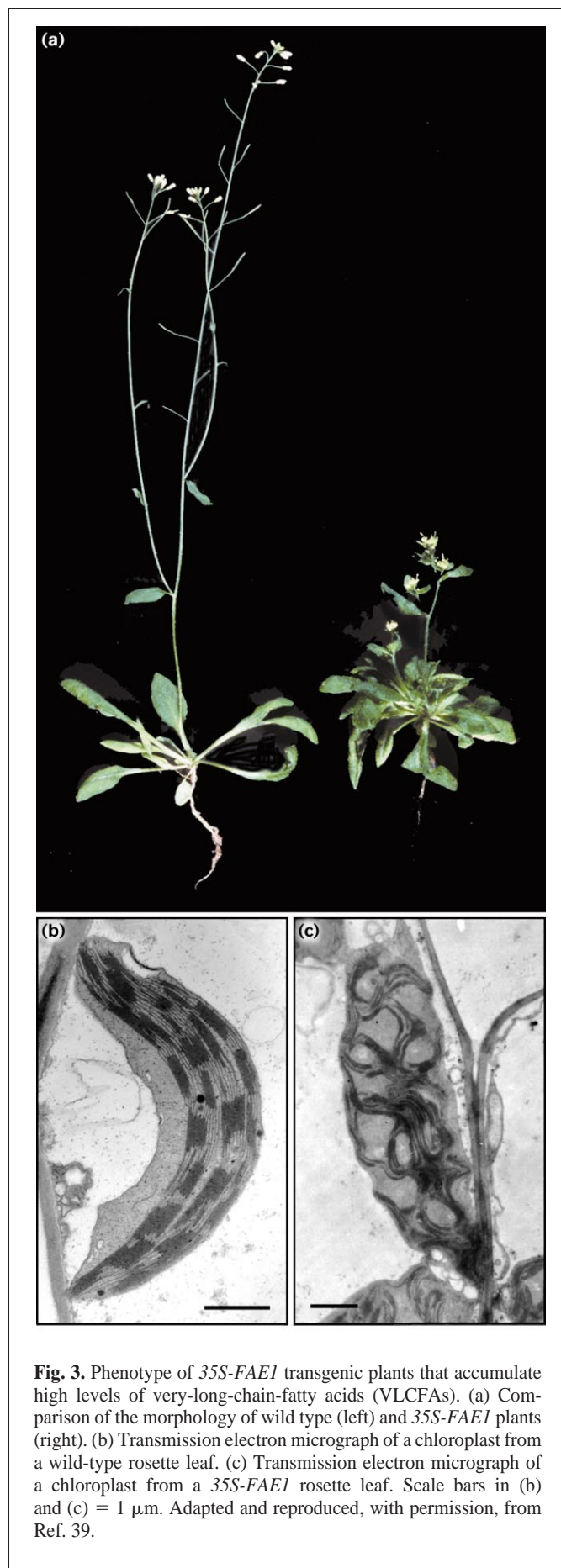


Fig. 3. Phenotype of *35S-FAEI* transgenic plants that accumulate high levels of very-long-chain-fatty acids (VLCFAs). (a) Comparison of the morphology of wild type (left) and *35S-FAEI* plants (right). (b) Transmission electron micrograph of a chloroplast from a wild-type rosette leaf. (c) Transmission electron micrograph of a chloroplast from a *35S-FAEI* rosette leaf. Scale bars in (b) and (c) = 1 μm . Adapted and reproduced, with permission, from Ref. 39.

triacylglycerols, but is not present anywhere else in the plants. RNA blot analysis has revealed that the *FAH12* transcript is present at high levels in non-seed tissues, and *in vitro* assays have demonstrated hydroxylase activity in roots and leaves³⁷. The failure of MCFAs and hydroxy fatty acids to accumulate in non-seed tissues is further evidence that mechanisms exist in plant cells that can deal with fatty acids, which are incompatible with membrane structure and function.

The accumulation of VLCFAs in membrane lipids

By contrast with *35S-FatB1* and *35S-FAH12* transgenic plants, *35S* promoter-directed ectopic expression of *FATTY ACID ELONGATION 1 (FAEI)*¹¹, a gene encoding a VLCFA-condensing enzyme required for the synthesis of C20 and C22 seed oil constituents in *Arabidopsis*³⁸, resulted in plants accumulating high levels of unusual fatty acids in membrane lipids³⁹. VLCFAs were incorporated into all major membrane lipid classes, and in some *35S-FAEI* transgenic lines accumulated to >30% of the total leaf fatty acid, thus completely changing the basic fatty acid makeup of the plant³⁹. This result suggests that the plant does not perceive VLCFAs as unusual and that mechanisms that exclude medium-chain and hydroxy fatty acids from leaf membrane lipids do not operate on VLCFAs. However, such mechanisms must be present in the seed because VLCFAs are not found in membranes of developing embryos.

Those *35S-FAEI* plants with low levels of VLCFAs appear wild type in all respects. By contrast, plants with high levels of VLCFA content in their membrane glycerolipids undergo a dramatic morphological transformation (Fig. 3), with the transgene causing abnormalities both at the whole plant and subcellular levels. The severity of the observed morphological changes is correlated tightly with the levels of VLCFAs, with extreme levels of VLCFAs resulting in lethality.

It is puzzling why VLCFAs can accumulate in leaf lipids when other unusual fatty acids cannot. The simplest explanation is that VLCFAs have a role to play in the membrane bilayer. Indeed, C24–C26 VLCFAs are found in sphingolipids. These lipids are structurally distinct from glycerolipids and are thought to be present in the plasma membrane of most, if not all eukaryotic cells. There is evidence suggesting that VLCFAs are required for the formation of highly curved membrane structures⁴⁰. The fact that the *35S-FAEI* plants have curved thylakoid membranes supports this notion, and suggests that thylakoid membrane lipids containing VLCFAs might be mimicking the structural role of sphingolipids. This is possible in view of a report⁴¹ that yeast cells can survive without sphingolipids by producing new structurally similar glycerolipids containing C26 fatty acids. In conclusion, hyperaccumulation of VLCFAs in membrane lipids in the *35S-FAEI* plants appears to perturb the structure of cellular membranes, which results in pleiotropic effects on plant growth and development³⁹. The striking outcome of the *35S-FAEI* transgenic experiment described here indicates just how inadequate our understanding is of the exact relationship between fatty acid composition of membrane lipids and membrane structure and function.

Future prospects

Because of their unique chemical properties, many of the unusual fatty acids described here have important industrial applications. For these fatty acids to be economically attractive, conventional oilseed crops will have to be genetically engineered to produce oils with a single predominant unusual fatty acid. The existence of wild species, such as castor, which contains oil with 80–90% 18:1-OH, suggests that this is a feasible goal. However, the inability to specifically target unusual fatty acids to seed triacylglycerols, and their excessive accumulation in membrane lipids, might disrupt seed membrane integrity and impair seed development

or germination. Initial results indicate that this might not be a problem in crop species that produce only moderate quantities of unusual fatty acids in their seeds. However, as transgenic plants are generated that can produce higher levels of unusual fatty acids, it might be necessary to introduce additional enzymes, such as acyltransferases and phospholipases, into plants to achieve correct targeting or exclusion of unusual fatty acids from cell membranes and to obtain viable transgenic seeds. We believe that this topic is of great importance and will be fundamental for our ability to create new oil-crop species with the desired oil composition.

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