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The biochemistry and molecular biology of plant lipid biosynthesis

Antoni R. Slabas and Tony Fawcett
Plant Molecular Biology Group, Department of Biological Sciences, South Road, University of Durham, Durham DH1 3LE, UK

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The importance of lipids

In the areas of cell biology, biophysics and the biochemistry of signal perception and transduction major advances have come about from an appreciation of the importance of biological membranes. Such membranes not only provide a barrier to the outside of the cell but also define the limits of subcellular organelles contained within them. Subcellular organelles have been fractionated and biochemical compartmentalization of enzyme function deduced by classical work of de Duve and colleagues [16]. Initial electron microscopy observations concentrated on the lipid bilayer description of a biological membrane [18]. Subsequent studies by Singer and Nicholson [114] have led to an appreciation of the importance of the proteins in membranes resulting in the fluid mosaic model. The importance of biological membranes has been additionally highlighted with the development of the chemiosmotic hypothesis of energy generation in biological systems. The systems have as a central factor the insertion of an ATPase in a membrane, so allowing vectoral transport [66]. Apart from the lipid bilayer providing the correct environment for ATPase to function, it is now becoming increasingly apparent that many other enzymes require a lipid environment in order to function. An example of this is the bacterial glycerol-3-P acyltransferase (G-3P-AT). The homogeneous protein was found to be inactive, but activity was regained on the addition of sonically disrupted *Escherichia coli* phospholipids, pure phospholipids or mixtures of phospholipids [58]. The enzyme could also be reconstituted in single walled vesicles; chymotrypsin treatment of which caused 95% inactivation of enzymic activity, suggesting that G-3P-AT was inserted assymetrically with the active site facing outwards [24].

The importance of lipids is further exemplified by (1) the cutin coat of plants which forms a barrier against fungal attack [53], (2) sulphated lipo-oligosaccharides of *Rhizobium meliloti* which act as signals to elicit root nodule organogenesis [59] and (3) the effect of desaturation on chilling tolerance, as demonstrated very elegantly by the group of Murata [146]. From an industrial perspective, vegetable oils are a major raw resource for both food and detergent based products and have an increasingly important role in the chemical industry. Major sources are soybean, sunflower, rape and oil palm in which lipids constitute up to 40% of the dry weight of the seed. Different fatty acids may be esterified to the glycerol backbone to generate the enormous variety of natural triglycerides which have been reported [36]. It is thus clear that great potential exsists for genetically engineering plant lipids providing that there is sufficient information on the biochemical pathways involved in addition to the temporal and tissue specific expression of the genes.

It is somewhat surprising, given the central importance of lipids in plants that initially advances in understanding their biosynthesis were restricted to a few pioneering laboratories. In this review we will consider advances made over the last 10 years in the understanding of plant lipid biosynthesis and its regulation. As two excellent reviews have recently appeared we suggest that

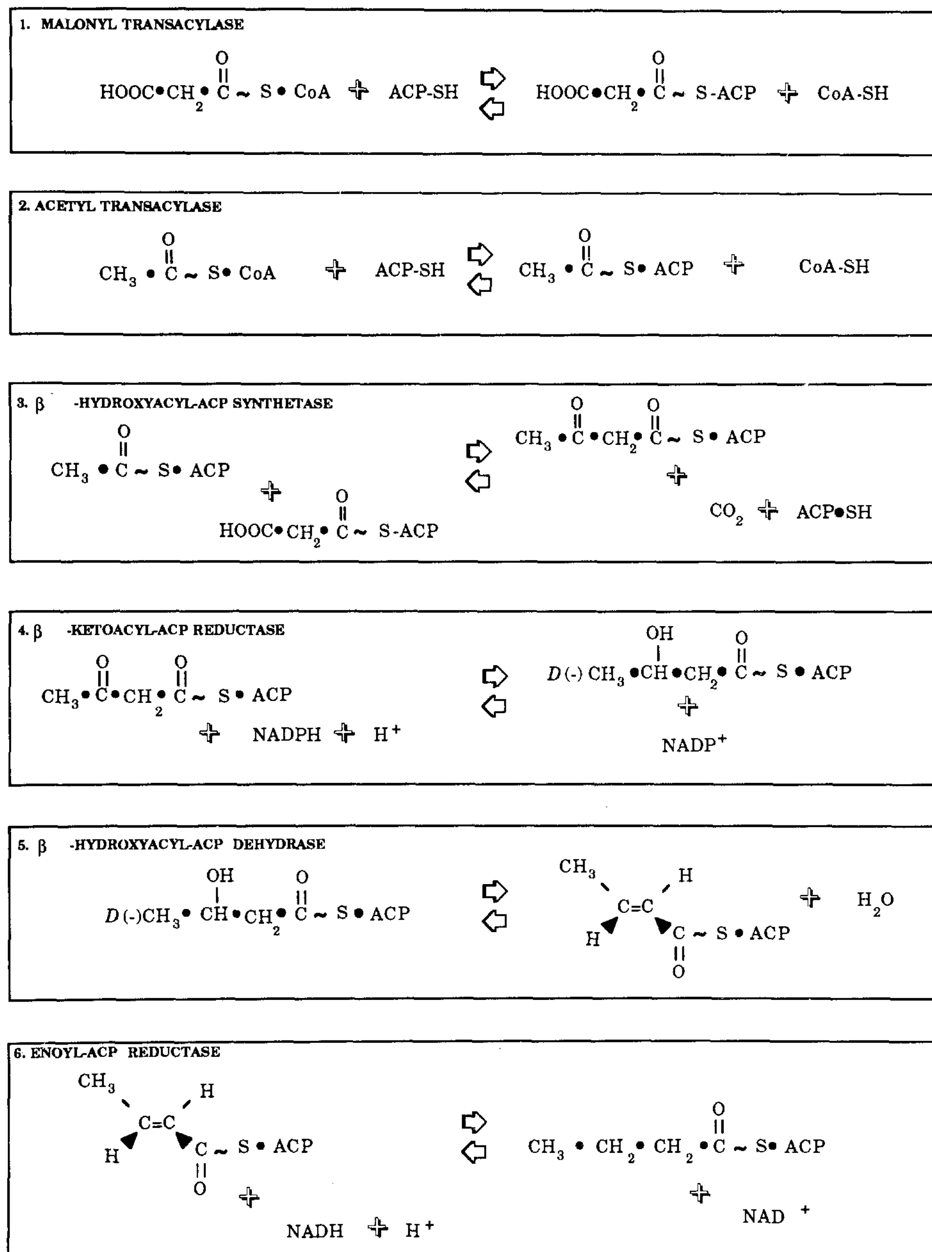

Fig. 1. Enzymic reactions involved in fatty acid synthesis.

additional information can be obtained from these and we have attempted not to duplicate their content. [127, 80].

Historic perspective

Prior to 1982, very little work had been performed on the purification of enzymes involved in plant lipid biosynthesis. It had been known for some time that the biosynthesis of fatty acids up to C_{18} in chain length occurred in chloroplasts [67]. Earlier Stumpf and Barber [134] described the conversion of [^{14}-C] acetate to palmitic and oleic acids by a 10 000 × *g* particulate fraction from avocado mesocarp. In 1975, Canvin [7] reported the biosynthesis of long chain fatty acids in the

developing castor bean and Weaire and Kekwick reported that plastids from avocado mesocarp, prepared by density gradient centrifugation, could incorporate [1-^{14}C]-acetate into palmitate and oleate [148]. In the same period, Yamada and his colleagues documented that the 10 000 × *g* fraction of castor bean endosperm could convert [^{14}C] sucrose to palmitate, stearate and oleate. This indicated that proplastids contained all of the enzymes required to convert sucrose to acetyl-CoA and for the synthesis of fatty acids. It is curious that despite indications that lipid biosynthesis could be studied advantageously in lipid-rich tissues, such as avocado mesocarp and developing oil seeds, this point was overlooked by many laboratories in subsequent years primarily because other systems were more convenient to work with.

Little attention was initially paid to purifying the proteins involved in lipid biosynthesis with one notable exception – that of acyl carrier protein (ACP). The core reactions of fatty acid biosynthesis had been described (see Fig. 1) and it was realized that, like bacterial systems, fatty acid synthesis in crude plant extracts was stimulated by the addition of ACP. ACP is a low-molecular-weight molecule (ca. 10 kDa), first isolated from *E. coli* [86], to which the intermediates of fatty acid biosynthesis are attached via the terminal sulphydryl of the 4′ phosphopantetheine group, which is in turn attached to the protein through a phosphodiester bond to a serine residue; ACP closely resembles coenzyme A in structure (Fig. 2).

ACP has two other claims to fame – it was one of the first proteins to be synthesized chemically [87] and it is one of the most abundant proteins in *E. coli*, ca. 5×10^4 molecules per cell. It is however rarely seen on two-dimensional gels due to its acidic nature and low binding capacity for Coomassie blue [16, 143].

ACP was purified from avocado mesocarp and spinach leaf by Simoni *et al.* [113]. It was assumed at that time that plant fatty acid synthetase was just like that of *E. coli* i.e. composed of several separate catalytic polypeptides. However despite the fact that the component *E. coli* system enzymes had been resolved from one another and several of them purified to homogeneity, no such attempts had been made with systems. Perhaps

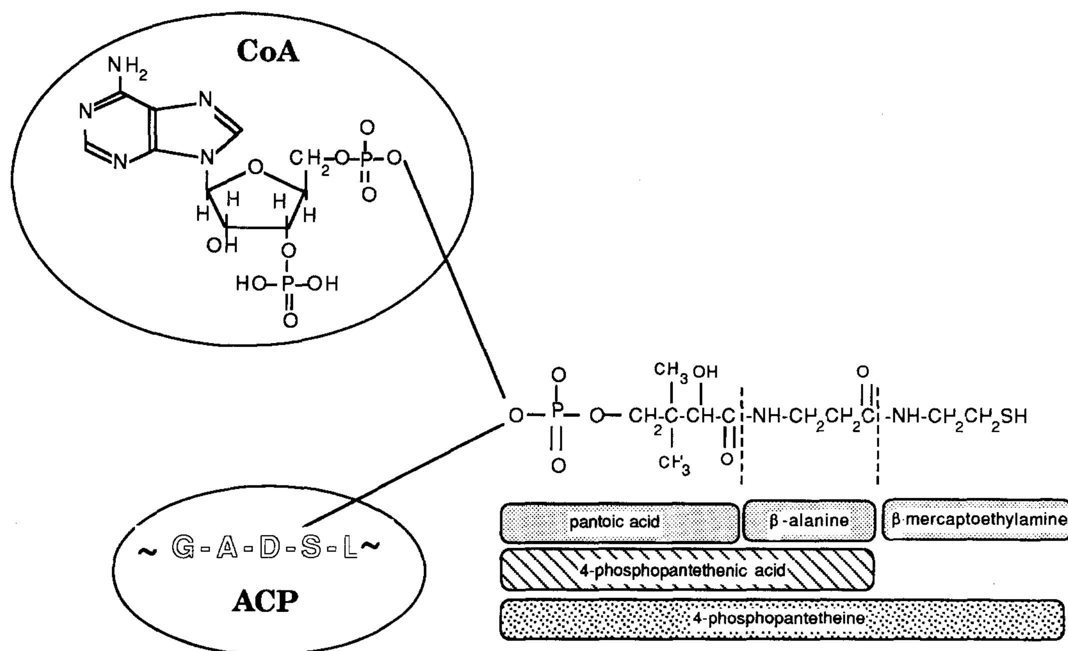

Fig. 2. Structure of coenzyme A and acyl carrier protein. Each has a 4-phosphopantetheine moiety.

because of the attitude 'it would be the same as *E. coli* anyway'. Knowledge of the compartmentalization of lipid biosynthesis had forwarded a model (Fig. 3) in which acetate entered the plastid, was initially converted to acetyl-CoA and then, by acetyl-CoA carboxylase, to malonyl-CoA. Both acetyl- and malonyl-CoA were used to synthesize fatty acids up to C_{18} in chain length as their ACP derivatives. Desaturation then occurred at $C_{18:0}$ by a soluble stearoyl-ACP desaturase. Fatty acid moieties could then either be incorporated into the complex plastid lipids MGDG and DGDG or be exported to the cytoplasm, following hydrolysis to their free fatty acids by acyl-ACP thioesterase. Subsequent desaturation, elongation reactions and synthesis of triglyceride occurs outside of the plastid, predominantly on microsomes. Triacyl glycerols are subsequently stored in discrete oil bodies. Although the broad brush strokes had been painted little was known about the details. This has been the subject of increasingly intense research over the past few years.

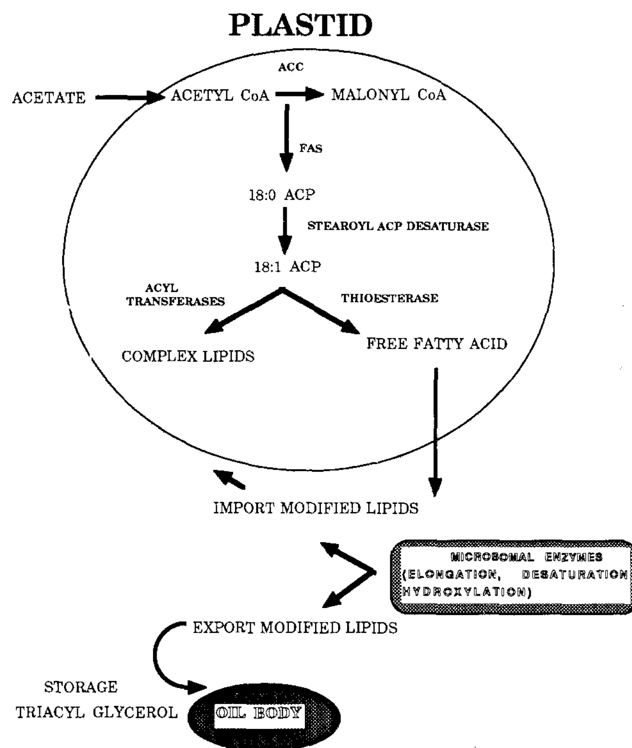

Fig. 3. Compartmentalization of fatty acid synthesizing and modifying enzymes. Acetate enters the plastid, is converted to acetyl-CoA and by acetyl-CoA carboxylase (ACC) to malonyl-CoA. These two substrates are used to synthesize fatty acids up to C_{18} in chain length, as their ACP derivatives, by fatty acid synthetase (FAS). Desaturation can then occur, by the action of soluble stearoyl-ACP desaturase, before the lipids are either incorporated into complex plastid lipids by acyltransferases, or hydrolysed to their free fatty acids by acyl-ACP thioesterase. Following export from the plastid, further elongation, desaturation and hydroxylation may occur (predominantly on microsomes) before the lipid is either returned to the plastid or incorporated in storage triacylglycerols.

The quest for the soluble enzymes and proteins of fatty acid synthetase in plants

There are basically two types of fatty acid synthetase. Type I represented by yeast and mammalian systems have all of their component activities on one or two polypeptide chains. For yeast the functional enzyme is $\alpha_6\beta_6$ and for animals α_2. The second type, type II, is represented by *E. coli* in which the component activities are present on separate polypeptide chains. Whilst the proteins have been isolated and the cDNA cloned from both yeast and animal sources [99, 100], little attention has been paid to the plant enzymes.

In 1982, four independent laboratories reported the separation of component biological activities of plant fatty acid synthetase (FAS) from avocado [8], barley [39], parsley suspension culture [98], safflower [107] and spinach chloroplast [108]. The multi-polypeptide nature of the synthetase was starting to be established but this was only the onset of the quest for these soluble enzymes. To aid readers a schematic representation of plant FAS is given in Fig. 4 showing the relationship of the various components.

ACP

Initial emphasis on characterization of fatty acid synthesis was placed on ACP. This was partly historic, being based on the work of Simoni *et al.* [113], but also because of a number of well based scientific reasons, namely: (1) it was a small mol-

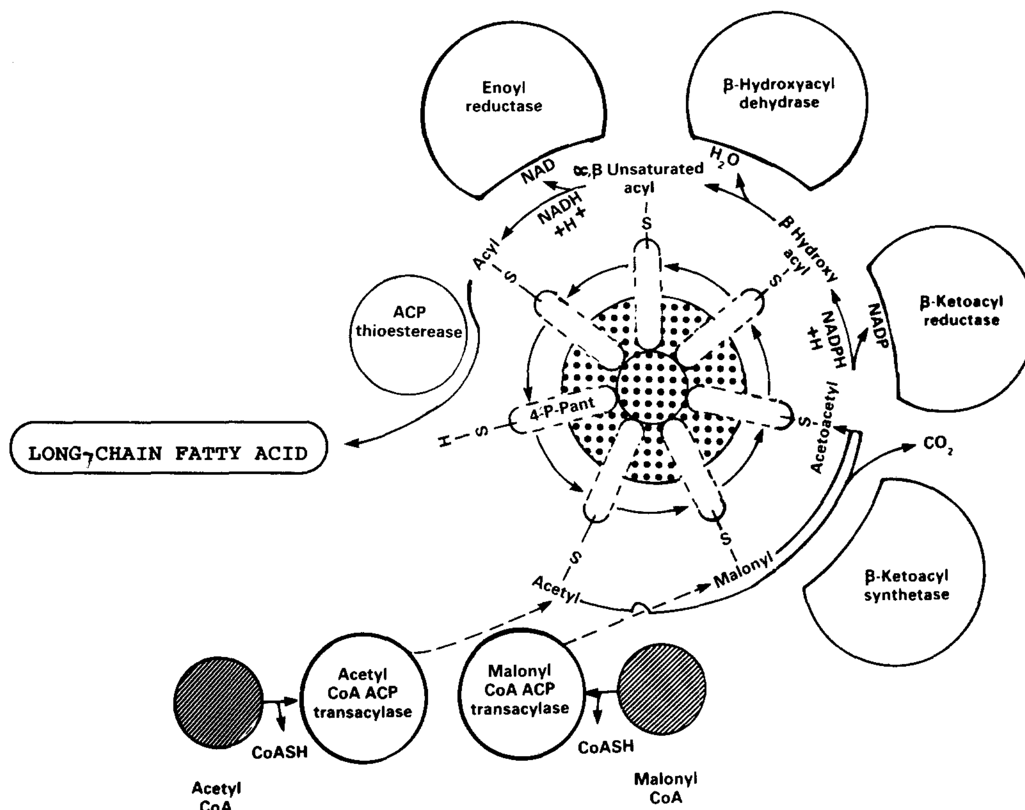

Fig. 4. Schematic representation of plant fatty acid synthetase.

ecule and hence should yield information relatively easily, both at the protein and cDNA level; (2) it appeared to be enzymically relatively stable (the *E. coli* and spinach enzymes were both prepared using acid precipitation and heat denaturation as part of their purification); and (3) it was known to be of central importance, not only as a component of FAS, but also as a substrate for complex lipid biosynthesis. In comparison to other components of plant FAS, ACP would be expected to be present in more than stoichometric quantity due to its many roles. The purification of ACP from spinach leaf [55] and barley leaf [39] was accomplished and the amino acid sequence of both determined. Two independent lines of evidence, from protein purification and western blotting, started to reveal the complexity of ACP types in plants. In barley leaf there are at least three isoenzymes as determined by protein purification. Immunological studies performed by Ohlrogge [78] pointed to two distinct molecular weight forms of ACP in leaf and one in seed. This is somewhat difficult to understand as it would have been predicted that seed would have two forms of ACP, a core FAS component and one involved in storage triglyceride biosynthesis, and that leaf would only have one. The position still remains to be resolved as experiments using ACP as substrates for acyltransferases and acylthioesterases have thrown little light on the matter [27]. The level of ACP activity in developing rape seed [118] was investigated to (1) determine if the activity of ACP was correlated with the deposition of storage lipid (and hence potentially be encoded by a temporally and tissue regulated gene) and (2) select a stage at which to purify the protein from a seed source. ACP activity appeared just prior to the onset of storage lipid biosynthesis and thus the gene was potentially a candidate for both tissue and temporal specific regulation. Studies on ACP levels in developing soybean seed had yielded the same results [78]).

Additional measurements have also been made for several of the enzymes of rape seed FAS for the same reason.

Purification of rape seed ACP provided its own challenges, unlike the leaf counterpart, it was not freely soluble but required detergent solubilization. Unfortunately, it was not as stable as *E. coli* ACP being neither heat- or acid-stable and it rapidly lost biological activity following chromatographic separation. Purification to apparent homogeneity was achieved by using *E. coli* acyl ACP synthetase to specifically radiolabel ACP, in a crude extract, with [^{14}C]palmitic acid. This step introduced a tag with which to follow ACP and convert it from a non-hydrophobic species into a hydrophobic one, and was used as the basis for purification (Fig. 5). Amino acid sequence comparison of ACP from various sources has revealed several areas of high sequence homology (see Fig. 6). Of special note is the region surrounding the phosphopantethylated serine. ACP has not been purified from seed and leaf of the same plant species; the amino acid sequence of leaf forms seem to start AK, only one seed form has been purified to date and this starts AAK which could indicate the existence of a different processing enzyme in seed and leaves.

Ten independent ACP cDNA clones were obtained by screening a size fractionated rape embryo λgt10 cDNA library with redundant oligonucleotides synthesized against the conserved region of ACP. Sequence analysis of these cDNAs demonstrated that the protein (which was nuclear-encoded) had a 51 amino acid N-terminal extension. Amino acid heterogeneity in the mature coding sequences revealed the presence of at least 3 ACP species, which verified heterogeneity seen in sequencing the mature protein [95]. The

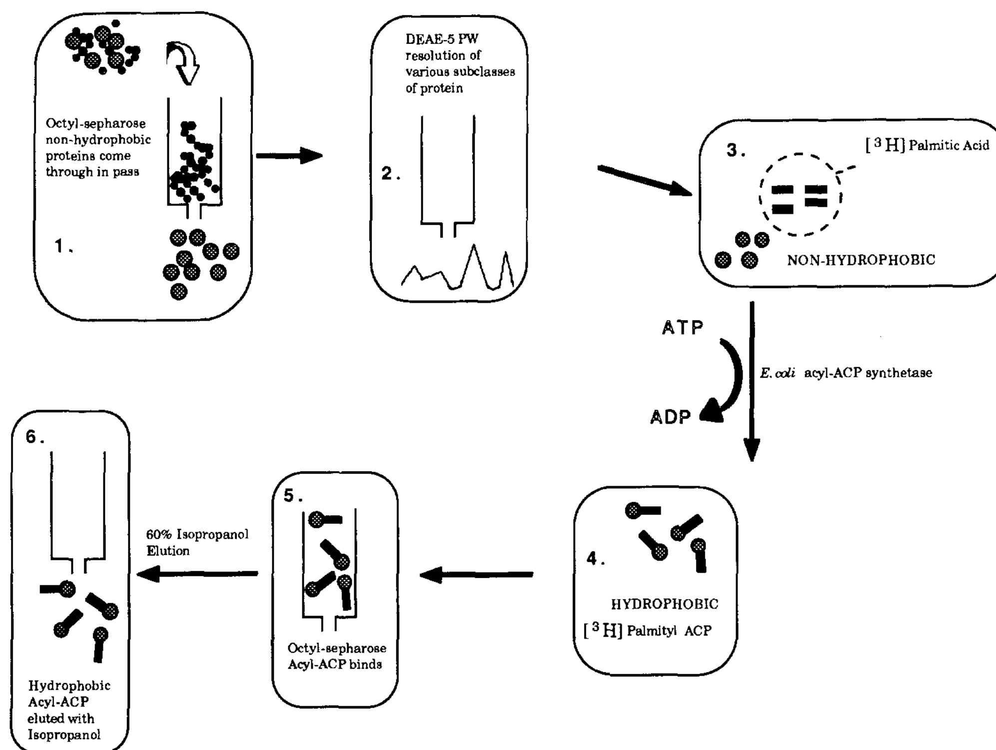

Fig. 5. Purification scheme of rape seed ACP. 1. Hydrophobic proteins (●) are separated from non-hydrophobic proteins by octyl-sepharose chromatography. 2. Ion exchange separation of ACP (followed by biological activity). 4. Quantitative labelling of ACP (a non-hydrophobic species) with radio-labelled palmitic acid using *E. coli* acyl-ACP synthetase. 5. Binding of hydrophobic acyl-ACP to octyl column. 6. Elution by organic solvent.

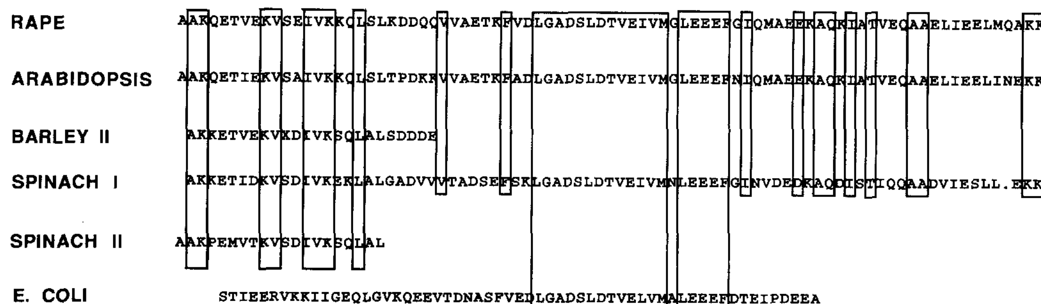

Fig. 6. Sequence homologies of various ACPs. The rape, *Arabidopsis*, spinach I and *E. coli* sequences are translated from their nucleotides, whilst the spinach II and barley II data were obtained by direct amino acid sequencing of the proteins.

ACPs could be broadly classified into two classes on the basis of heterogeneity in the transit peptide [112].

Whilst investigating potential similarity of *Brassica napus* seed and leaf ACP a rape seed cDNA clone was used as a probe on a northern blot containing both embryo and leaf poly(A)$^+$ RNA [95]. The probe hybridized to the embryo mRNA but, unexpectedly, did not hybridize to the leaf mRNA. The use of totally degenerate oligonucleotide probe to the conserved amino acid motif 'LEEEF' resulted in strong hybridization to both seed and leaf derived poly(A)$^+$ RNA. Although there is an apparent strong conservation of amino acid sequence between the leaf and seed forms of ACP from different species, this is not reflected at the nucleotide level. A spinach leaf ACP cDNA has been isolated [96]; when used as a probe for a northern blot, low hybridization to the embryo mRNA resulted, further supporting a concept of different codon usage between leaf and seed isoforms.

NMR studies have been performed on *E. coli* ACP to determine the three dimensional structure [40, 49]. Such investigations have become possible with plant ACP obtained from *E. coli* expression systems [28, 50]. Genomic clones for ACP have been reported from *Arabidopsis* [57, 84] and *B. napus* [112]. The genes cloned from *B. napus* are known to be expressed, because of absolute sequence identity to two cDNAs isolated from a rape embryo library. They contain three introns which are in the same positions in the two genes. Intron 1 occurs in the transit peptide, intron 2 occurs between amino acids 1 and 2 of the mature protein and intron 3 is situated in the middle of the conserved phosphopantetheine binding region. The *Arabidopsis* gene appears to have the same pattern of introns based on protein sequence alignments [84]. Oil seed rape contains approximately 35 copies of seed expressed ACP gene per haploid genome [112].

We will not go into further complexities of the compartmentalization/organellar location of ACP at this stage but will mention three significant observations in the literature: (1) following a demonstration of ACP in *Neurospora* mitochondria [5], ACP has also been immunologically localized to plant mitochondria [11], suggesting a wider metabolic role than that reported to date. Recent experiments from Walkers' lab indicate it is a component of the NADH-ubiquinone oxidoreductase from bovine heart mitochondria [93]; (2) ACP has been found in multiple copies in the erythromycin synthesizing gene of *Saccharopolyspora erythraea* [13] and hence has an important role in the synthesis of macrocylic compounds; (3) sequence similarity between the NodF gene product of *Rhizobium leguminosarum* and ACP has been reported [102] indicating a role of fatty acids in host recognition in the nodulation process. Recent evidence [105] shows that the β-keto-acyl [ACP] reductase from *B. napus* has sequence similarity to the NodG gene product, supporting the contention that some Nod genes encode components of fatty acid synthase.

Condensation reactions and the nature of the first step in plant fatty acid synthetase: the role of acetyl CoA:ACP transacylase

The initial condensation reaction in both bacterial and plant fatty acid synthesis has been held to be the condensation between acetyl-ACP and malonyl-ACP with the formation of acetoacetyl-ACP. Acetyl-CoA:ACP transacylase is considered to be the rate-limiting step in fatty acid synthetase [133]. Following this initial condensation, the same enzyme, β-ketoacyl-ACP synthase (KAS), elongates the acyl-ACP by further addition of C2 units from malonyl-ACP until $C_{18:0}$ ACP is synthesized. Shimakata and Stumpf [109] clearly resolved two KAS isoforms in spinach leaf extracts and carried out reconstitution experiments with purified spinach leaf FAS components. Using hexanoyl-ACP or palmitoyl-ACP as the primers and [^{14}C]-malonyl-CoA, KAS I was shown to be involved in synthesizing fatty acids up to C_{16}, and KAS II to be responsible for the C_{16} to C_{18} conversion. Further, KAS I was shown to have little activity when palmitoyl-ACP was the primer and KAS II had a substrate specificity for chain lengths C_{12} or greater. The reconstituted system, with either KAS I or KAS II, could not extend stearoyl-ACP, indicating that other condensation enzymes must be involved in this process. Condensation reactions are of central importance in plant metabolism, they are involved in elongation to long-chain fatty acids [149], and represent the key reaction of chalcone synthase [29] and resveratrol synthase [97] amongst others. β-ketoacyl synthase I has been shown to have high amino acid sequence homology to the *nodE* gene product [41].

KAS I and KAS II, from spinach leaf are sensitive to covalent modification and inactivation by the antibiotic cerulenin; KAS I being much more sensitive than KAS II. Purification of KAS I to homogeneity was first achieved from rape seed [63], the key to the procedure being the development of a rapid *in vitro* complementation assay in which fractions were assayed for their ability to restore fatty acid synthesis to a cerulenin-inhibited *E. coli* extract. A similar assay had been used to purify bacterial KAS I [91]. Cerulenin labelling has also been used to purify the condensing enzyme from barley chloroplasts; here it was essential to radiolabel the chloroplasts whilst they were intact, with [^{3}H]-cerulenin [111]. Barley KAS I is composed of α and β subunits; using oligonucleotides derived from amino acid sequence data for the pure β subunit, cDNA was amplified using the PCR reaction. This approach yielded a 311 bp cDNA sequence which was used as a probe to isolate the full-length cDNA clone. The deduced primary structure of the β subunit and the *fabB*-encoded β-ketoacyl-ACP synthase from *E. coli* share 49% similarity, including 35% identity [114], but the barley protein has less similarity to other plant condensing type enzymes such as chalcone synthetase.

The use of an antibiotic proved vital in the isolation of KAS I and in the separation of KAS I from KAS II activity. Antibiotics have also played a key role in the identification of a new condensing enzyme, KAS III, involved in the initial reaction of fatty acid synthesis in both bacteria and plants.

Bacterial fatty acid synthesis is strongly inhibited by the action of cerulenin. However, Jackowski and Rock [44] demonstrated, in *E. coli*, that acyl-ACP formation *in vivo* was not blocked by this antibiotic and short-chain (4–8-carbon) acyl-ACPs accumulated in cerulenin-treated cells, indicating the presence of a short-chain condensing enzyme. Following this, a cerulenin-insensitive short-chain condensing enzyme was demonstrated in spinach leaves [45] and in other plant species [147].

Earlier experiments [74, 75] showed that the antibiotic thiolactomycin selectively inhibits type II, dissociable, fatty acid synthases and that in the *E. coli* system, acetyl-CoA:ACP transacylase and KAS were the thiolactomycin-sensitive enzymes. Lowe and Rhodes [61] have purified acetyl CoA:ACP transacylase from *E. coli* and found it not to be thiolactomycin-sensitive; this difference may be explained by an observation of Jaworski *et al.* [46], which showed that inhibition of KAS III is actually caused by an oxidative breakdown product of thiolactomycin.

Whilst measuring the activity of a number of lipid synthesis enzymes in plant material, Stumpf and Shimakata [135] concluded that (1) acetyl-CoA:ACP transacylase was probably rate limiting and (2) elevation of its level in reconstitution experiments gave rise to the ability to synthesize medium-chain fatty acids. If there is indeed an alternative KAS, KAS III, which utilizes acetyl-CoA and not acetyl ACP, what then is the function of acetyl CoA:ACP transacylase and does such an activity really exist? The results concerning a potential role for acetyl-CoA:ACP transacylase in the synthesis of medium-chain fatty acids should be treated with some caution as rape seed, which does not make medium-chain fatty acids, has a high *in vitro* level of this activity [135]. Lowe and Rhodes [61] recently reinvestigated acetyl-CoA:ACP transacylase activity from *E. coli* and found that it only represented a minor component in the original purification. Some insight into what could be occurring comes from consideration of another bacterial enzyme, acyl-ACP synthetase, which we have previously mentioned in the isolation of seed ACP. The requirement for this biological activity in *E. coli* was questioned, as acyl-ACP are the synthetic product of fatty acid synthetase, it is now clear that it is a partial reaction of another enzyme involved in phosphatidyl ethanolamine metabolism [12]. There are strong indications that acetyl-CoA:ACP transacylase could be a partial activity of KAS III, but only purification of KAS III to homogeneity will resolve this question. What then is the function of acetyl-CoA:ACP transacylase activity? Is it really required for lipid synthesis in plants? It may well be that plants are plastic in their metabolism having alternative metabolic routes [89]. In *E. coli* KAS III (*fabH*) has recently been cloned (C.O. Rock, unpublished) and experiments are underway to construct a strain in which this gene is totally inactive to test if its activity is essential (C.O. Rock, pers. comm.). Figure 7 shows the relationship between the three different KAS activities in the synthesis of fatty acids.

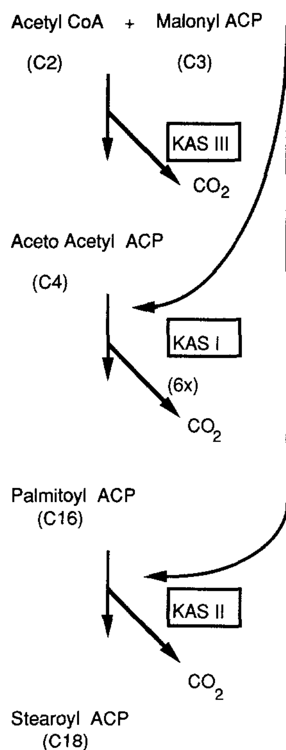

Fig. 7. Role of β-ketoacyl [ACP] synthase isoforms in the synthesis of saturated fatty acids.

Malonyl-CoA:ACP transacylase and β-hydroxyacyl dehydratase

Malonyl-CoA:ACP transacylase has been purified to homogeneity from avocado (Hilt, 1984, cited [133]) and a number of other plant sources have been used to produce partially purified preparations: barley [38]; leek [60], spinach [129] and soybean [26]. In the case of soybean and leek, evidence for the presence of isoforms was found, but these have no ascribed functional significance. A gene for malonyl-CoA:ACP transacylase has recently been cloned from *E. coli* by complementation of the *fabD* mutant [30; A.R. Stuitje, pers. comm.), so it may be possible to clone the plant gene by complementation of the *E. coli* mutant (cf. the complementation of an *E. coli* enolase-deficient mutant with a maize cDNA [56]). The dehydratase has been purified to homogeneity from spinach leaves [110] and partially purified from developing safflower seeds

[107], but since this work there has been little activity in this area.

The reductive steps of fatty acid synthesis

Two reductive steps are required for fatty acid biosynthesis. The enzymes for both steps have been purified, using acyl-CoA substrate analogues, but it has subsequently been shown that ACP is by far the preferred substrate. The first reductive step is catalysed by β-ketoacyl-ACP reductase. This enzyme has been purified to homogeneity from spinach [110], avocado [105] and rape [103]. Amino acid sequence data has been obtained for the avocado enzyme which is NADPH-specific. The N-terminus shows homology to Cyt f of *Marchantia* and there is extensive internal sequence homology to the NodG gene product [105]. The rape enzyme, like the avocado enzyme, is both cold-labile and dilution-sensitive [106].

Recently, a cDNA clone has been obtained for the rape seed enzyme and this was used to clone a full-length cDNA from *Arabidopsis*. Extensive nucleotide sequence homology has been found between the rape and *Arabidopsis* cDNAs. The amino acid sequence of the β-ketoacyl-ACP reductase is highly homologous to that of the NodG gene product (Fig. 8).

The NodG gene product was previously of unknown biochemical function and it can now be concluded that it is probably a β-ketoacyl-ACP reductase. The structure of the sulphated lipo-oligosaccharide involved in *R. meliloti* host specificity for nodulation has recently been described [140]. From the structure (Fig. 9) it can be seen that both ACP and β-ketoreductase could have an important role in the synthesis of the lipid moiety. Significant homology is also observed between NodE and condensing enzyme [41], adding evidence to the hypothesis that some Nod genes encode for components of fatty acid synthase (A. Downie, pers. comm.). Amino acid

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nod G              M F E L I G R K A L V • • • • • • • • • • • • • • •   11
Ar-R   M A A A V A A P R L I S L K A V G K L G F R E I S Q I R Q L A P   32

nod G  • • • • • • • • • • • • • • • • • • • • • • • • • • • • • • • •
Ar-R   I H S A I P H F G M L A C R S R Q P F S T S V V K A Q A T A T E   64

nod G  • • • • • • • • • • • • • • • • T G A S G A I G G A I A R V L   26
Ar-R   Q S P G E V V Q K V E S P V V V I T G A S R G I G K A I A L A L   96

nod G  • • H A Q G A I V G L H G T Q I E K L E T L • • A T E L G D R V   54
Ar-R   G K A G C K V I V N W A R S A K E A E E V A K Q I E E W G G Q A  128

nod G  K L F P A N L A N R D E V K A L G Q R A E A D L E G V D I L V N   86
Ar-R   I T F G G D V S K A T D V D A M M K T A L D K W G T I D V V V N  160

nod G  N A G I T K D G L F L H M A D P D W D I V L E V N L T A M F R L  118
Ar-R   N A G I T R D T L L I R M K N S Q W D E V I A L N L T G V F L C  192
Br-R                                                         G V F L C    5

nod G  T R E I T Q Q M I R R R N G R I I N V T S V A G A I G N P G Q T  150
Ar-R   T Q S A A V K M M K K K R G R I I N I S S V V G L I G N I G Q A  224
Br-R   S Q A A T K I M M K K R K G R I I N I A S V V G L I G N I G Q A   37

nod G  N Y C A S K A G M I G F S K S L A Q E I A T R N I T V N C V A P  182
Ar-R   N Y A A A K G G V I F F S K T A A R E G A S R N I N V N V V C P  256
Br-R   N Y A A A K A G V I G F S K T A A R E G A S R N I N V N V V C P   69

nod G  G F I E S A M T D K L N H K Q K E K I M V A I P I H R M G T G T  214
Ar-R   G F I A S N M T A V L G E D M E K K I L G T I P L G R Y G E A G  288
Br-R   G F I A S E M T A K L G E D M E K K I L G T I P L G R Y G Q P E  101

nod G  E V A S A V A Y L A S D H A A • Y V T G Q T I H V N G G M A M I  245
Ar-R   G V A G L V E F L A L S P A A S Y I T G Q A F T I D G G I A I    319
Br-R   D V A G L V E F L A L S P A A S Y I T G Q T F T I D G G I A I    132
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Fig. 8. Comparison of NodG and β-ketoreductase protein sequences.

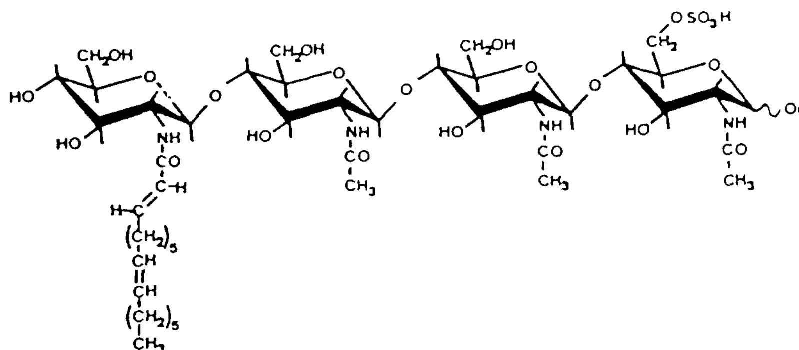

Fig. 9. Sulphated lipo-oligosaccharide involved in *Rhizobium meliloti* host specificity for nodulation.

sequence homology derived from genes involved in lipid synthesis have thus revealed the probable function of certain nodulation genes. What is somewhat unclear is why the endogenous bacterial fatty acid synthesis components are unable to perform these reactions. This is probably due to absolute substrate specificity of the individual reactions involved in the synthesis of the signalling molecule.

Two types of enoyl-ACP reductases have been reported for plant material. The NADH-specific enzyme has been purified from avocado [8], spinach [110] and rape [117]. In rape seed, this enzyme is induced prior to the deposition of lipid and the biological activity and protein appearance shows the same type of kinetics as ACP. It is therefore also a potential target for a seed-specific and temporally regulated gene. Initial experimentation demonstrated that the enzyme is tetrameric. Based on the presence of two separate bands on SDS-PAGE it was concluded that the enzyme was $\alpha_2\beta_2$ [117]. Subsequent separation of the α and β components by reversed-phase chromatography and N-terminal amino acid sequencing showed that the two polypeptides differed by a six amino acid amino terminal extension [14]. Analysis by western blotting on freshly homogenized rape seed indicated that the enzyme actually has an α_4 structure.

The lower β band was an isolational artefact due to an endogenous protease which cleaved between a serine and lysine residue [120]. Recent experimentation in our laboratory with higher-resolution gels has demonstrated that the situation in rape is not so simple. In crude extracts of both rape leaf and rape seed two bands are present. These have an approximately equal intensity. The newly identified polypeptide runs above α and we have termed it α'. The nature of this new species still remains to be elucidated and is the subject of current investigations. It could be either a membrane-bound form or, alternatively, a long acyl chain-specific NADPH form which has been reported from two separate laboratories. The latter enzyme requires long-chain substrates for detection and could have been lost in the original purifications which used the short-chain crotonyl (C_4) substrate.

Extensive amino acid sequence data is available on the rape seed enzyme [121]. This enzyme has a strong affinity for ACP [120], and covalent modification of an arginine residue in enoyl reductase using phenylglyoxal destroyed this interaction [15]. The cDNA has been cloned from a rape embryo library and its complete sequence determined showing a transit peptide of 73 amino acids [48]. This has enabled the peptides obtained by amino acid sequencing to be ordered [121].

Two independent genomic clones from *Arabidopsis* have been isolated, by the group of Stuitje, and fully sequenced (A.R. Stuitje, pers. comm.). *Arabidopsis* cDNA clones have also been isolated by the group at Durham using a rape enoyl reductase cDNA probe provided by Stuitje (in preparation). Northern analysis on leaf, seed and flower RNA indicates that the rape gene is constitutively expressed, whereas protein extracts from leaf and flower reveal relatively little immunological cross-reaction on western blots [48]. This is somewhat different from the situation with ACP where the embryo cDNA isolated failed to hybridize to leaf mRNA. If a seed specific enoyl reductase gene does exist then it may require a more sophisticated probe for its detection, possibly derived from the transit peptide sequence or the 5′ untranslated region.

Import and export of lipids in the plastid

Early experimentation demonstrated that acetate could enter plastids and be incorporated into lipids [132]. However, is acetate the true substrate which is imported into plastids? Recent results from the laboratory of Thomas have indicated that acetyl groups could enter chloroplasts as acetylcarnitine. This is similar to the mitochondrial systems [64], where a specific carnitine translocator exists. In feeding experiments on isolated chloroplasts L-[1-^{14}C acetyl] carnitine gave a five-fold greater incorporation of radioactivity into fatty acids than [1-^{14}C] acetate [64].

Following the synthesis of long-chain acyl-ACPs they are either hydrolysed to free fatty acids, by acyl-ACP thioesterase, and are exported from the plastid – possibly by a carnitine route. Alternatively, they are incorporated into the plastid lipids by acyl transferases [92]. Independently the acyl-ACP thioesterase has been purified to homogeneity from rape seed [34] and to near homogeneity from squash [42]. In rape, enoyl-ACP reductase copurified with acyl-ACP thioesterase through several successive steps indicating a close affinity between the two enzymes (A. Hellyer and A.R. Slabas, unpublished observation). Interest in the thioesterase has arisen for two reasons: (1) the balance of thioesterase and acyltransferase has been hypothesized to be a regulatory point for the prokaryotic versus eukaryotic pathway [92] and (2) as a possible mechanism, by analogy with animal systems, for the synthesis of medium-chain fatty acid biosynthesis in plants.

Nature of the chain termination mechanism and the synthesis of medium-chain fatty acids.

Several mechanisms have been proposed for the synthesis of medium-chain fatty acids in plants. They include: specific thioesterases; specific acyltransferases; high levels of acetyl-CoA:ACP transacylase; separate compartmental synthetases; controlled β-oxidation and specific β-keto acyl synthetase [133]. There is no definitive evidence for the mechanism in plants, although extensive studies have been performed in crude systems. In rats and mallard duck the synthesis of medium-chain fatty acids is brought about by the premature chain termination of fatty acid synthesis by interaction with a specific medium-chain thioesterase-thioesterase II, alternatively called medium-chain hydrolase [126, 86]. The protein has been purified from both duck and rat and the cDNA cloned [94]. Transformation of rat fibroblast 3T3 cells with the gene resulted in the synthesis of medium-chain fatty acids. It should be borne in mind, however, that this is not a universal mechanism of medium-chain fatty acid synthesis in animals. Goat fatty acid synthetase is capable of synthesizing medium-chain fatty acids merely by supplementation with microsomes and therefore has no apparent requirement for thioesterase II [52]. Recently a medium-chain specific acyl-ACP thioesterase from California bay laurel has been isolated [83]. Introduction of this gene into the *fadD* mutant of *E. coli* has resulted in the production of high quantities of medium-chain fatty acids (T. Volker, pers. comm.). It will be interesting to see in the future if the gene encoding this enzyme will be capable of altering the lipid profile of plants.

Regulation of the rate and extent of lipid synthesis

In animal systems it is well documented that the rate of fatty acid biosynthesis is limited by acetyl-CoA carboxylase (ACC). This enzyme has a subunit M_r of 250 000 and has recently been cloned both from chicken and rat [20]. ACC is highly regulated, in response to hormonal balance, by phosphorylation. The sequence of the phosphorylation sites has been determined [81]. Many of the interactions of animal FAS with other components, when investigated *in vitro*, have required rate limitation on the availability of malonyl-CoA [51]. What then is the situation in plants both with respect to the subunit size and regulation of ACC? There are reports in the literature of varying subunit M_r of the basic polypeptide of ACC (summarized in [33]) and this has led to the belief that there is possibly more than one form of ACC. Perhaps note should have been taken on the first reports of the purification of ACC as a high-molecular-weight polypeptide subunit from parsley, wheat germ [21] and rape seed [115]. In these instances there was clearly one high-molecular-weight species. We have used antibodies raised against wheat germ ACC to probe leaf material and found similar high M_r (220 kDa plus) cross-reacting proteins in leaf material [122]. Also direct purification of rape leaf ACC has demonstrated that it is a high M_r species of 220 kDa [116]. This draws us to the conclusion that in rape and wheat a single high-M_r form is present in both leaf and seed. Other biotin-containing peptides, which are carboxylases, have been found in animals [9] and plants [150], but these probably have other functions. The possibility cannot, however, be ruled out that a different cytoplasmic form of ACC exists which has a lower molecular weight. Indeed, Nikolau has purified a 50 kDa protein from embryogenic carrot cells and analysis of the cDNA clone isolated has indicated that this is possibly a cytoplasmic ACC [73]. Proof of this will come from immunogold localization studies. Attempts have been made to look for phosphorylation and the regulation of plant ACC by citrate, but there is no evidence that such regulation exists. Is such regulation however required in plants? ATP, generated by photophosphorylation, is one of the key substrates of ACC which is apparently located within plastids [133]. Any reduction in the pool size of ATP would rapidly reduce the rate of the carboxylase and hence slow down lipid synthesis. Perhaps no other control at this point is required. If a separate cytoplasmic form of ACC exists it will probably be subject to a different regulatory mechanism.

Measurement of the major acyl-ACP intermediates of fatty acid biosynthesis in light- and dark-grown material has shown an increase of acetyl-ACP in the dark. This is consistent with the light/dark control of the rate of fatty acid biosynthesis being by ACC [85].

Two further points are worthy of mention here: (1) a biotin-binding sequence from tomato has been identified [37] and it contains the amino acid sequence A-M-K-M, a highly conserved motif in carboxylase sequences; and (2) acetyl-CoA carboxylase seems to be the site of action of differential herbicide tolerance between monocots and dicots [82]. The latter point and its potential regulatory role has led to a number of groups concentrating on the cloning of ACC. In rape seed the level of ACC activity has been measured during seed maturation [141] like other enzymes of lipid synthesis the activity rises prior to the onset of lipid deposition and rises continually through lipid synthesis. However, by contrast, whilst the level of other enzymes of lipid metabolism remains high [119] that of ACC is rapidly reduced once maximal lipid accumulation has been achieved [141]. ACC could be acting as a central regulator, effectively shutting down lipid synthesis even though an active FAS is present, by removing the substrate malonyl-CoA.

Stumpf and Shimakata [135] have measured the rate of the component enzymes in plant FAS in various plant extracts and have come to the conclusion that probably acetyl-CoA:ACP transacylase was rate-limiting. With the current confusion over the role of acetyl-CoA:ACP transacylase in plants this whole question requires re-evalution. It is important to remember that these are *in vitro* results and may not reflect the

in vivo situation which will be dependent on subcellular location of substrate and enzyme. It is also not clear what controls the extent of lipid synthesis (it could be ACC as alluded to above) and little information is available concerning the partitioning between lipid synthesis and the synthesis of other major storage products: starch and protein. Alterations to the total quantity of lipid deposited and the ratio of the storage products in seeds is a potential agronomic target for the future.

Desaturases in higher plants

Desaturation has a marked effect on the physical properties of lipids. Indeed, several plants specifically change their degree of unsaturation in response to cold acclimatization so that the membranes can retain their degree of fluidity [62, 69]. Alterations in the chilling sensitivity of plants are correlated with the degree of unsaturation of phosphatidylglycerol (PG). Plants, such as squash which contain a low level of Δ12-*cis* fatty acids in PG are chilling-sensitive, whilst those typified by *Arabidopsis*, which have a higher level of Δ12-*cis* in PG, are more chilling-tolerant. The composition of these lipids will be regulated by both the specific fatty acid composition of the acyl pool and the selectivity of the acyltransferases.

Plants have two types of desaturases, a soluble chloroplast Δ9 desaturase and membrane-bound desaturases which are involved in Δ12 and Δ15 product synthesis. From a biochemical point of view the latter present a much greater difficulty in isolation, due to their membrane requirement.

The Δ9 (stearoyl) desaturase has recently been cloned from cucumber and castor seeds [104] using antibodies made against the purified avocado protein. The cDNA from safflower embryos has been cloned and includes a 33 amino acid transit peptide [139]. Modulation of the stearoyl-ACP desaturase level, using antisense technology, in transgenic rapeseed has resulted in a marked decrease in the level of oleic acid with an increase in the level of stearic acid [54]. This is a soluble ferrodoxin requiring enzyme and should not be confused with other desaturases which are membrane-bound, such as the Δ12 and Δ15 desaturases which respectively form linoleate (18:2) and linolenate (18:3). It is well documented that such desaturation occurs on phosphatidylcholine and monogalactosyldiacylglycerol backbones respectively [123, 136] and components of the electron transport chain have been identified [125]. Purification of these enzymes would present a formidable challenge even to the most experienced biochemists; Somerville and Browse, with some foresight, have created a series of mutants of lipid metabolism in *Arabidopsis* (Fig. 10) and experimentation is currently underway to isolate the genes by gene walking. This area is exceptionally important, but outside the scope of this review (for details, see [127]). Other approaches, including T-DNA tagging, are also expected to provide a significant way forward [22].

Cyanobacterial desaturases and temperature acclimatization

Desaturases are important in temperature adaptation, yet how does one obtain the enzymes and subsequently the genes involved in the production of unsaturated lipids if the proteins are membrane bound and rapidly lose activity on purification? Murata and colleagues of NIBB, such as Okasaki, have taken a lateral approach. They selected for mutants in the cyanobacterium *Synechocystis* PCC 6803 that were defective in the desaturation of fatty acids and were chilling-sensitive, having a lower growth rate than the wild type at 22 °C [145]. A mutant was shown, by lipid analysis, to be deficient in Δ12 desaturation. By complementation they were able to clone a gene which allowed growth at low temperatures and restored Δ12 desaturation. This gene could be either a structural or regulatory gene, as is the case for the desaturase mutants in *Arabidopsis*. Insertion of the gene into *Anacystis nidulans* R2-SPc, a species of *Cyanobacterium* completely lacking in Δ12 desaturation, resulted in the synthesis of Δ9,12 unsaturated fatty acids. This was at the expense Δ9 monounsaturated fatty acids and concomitant in-

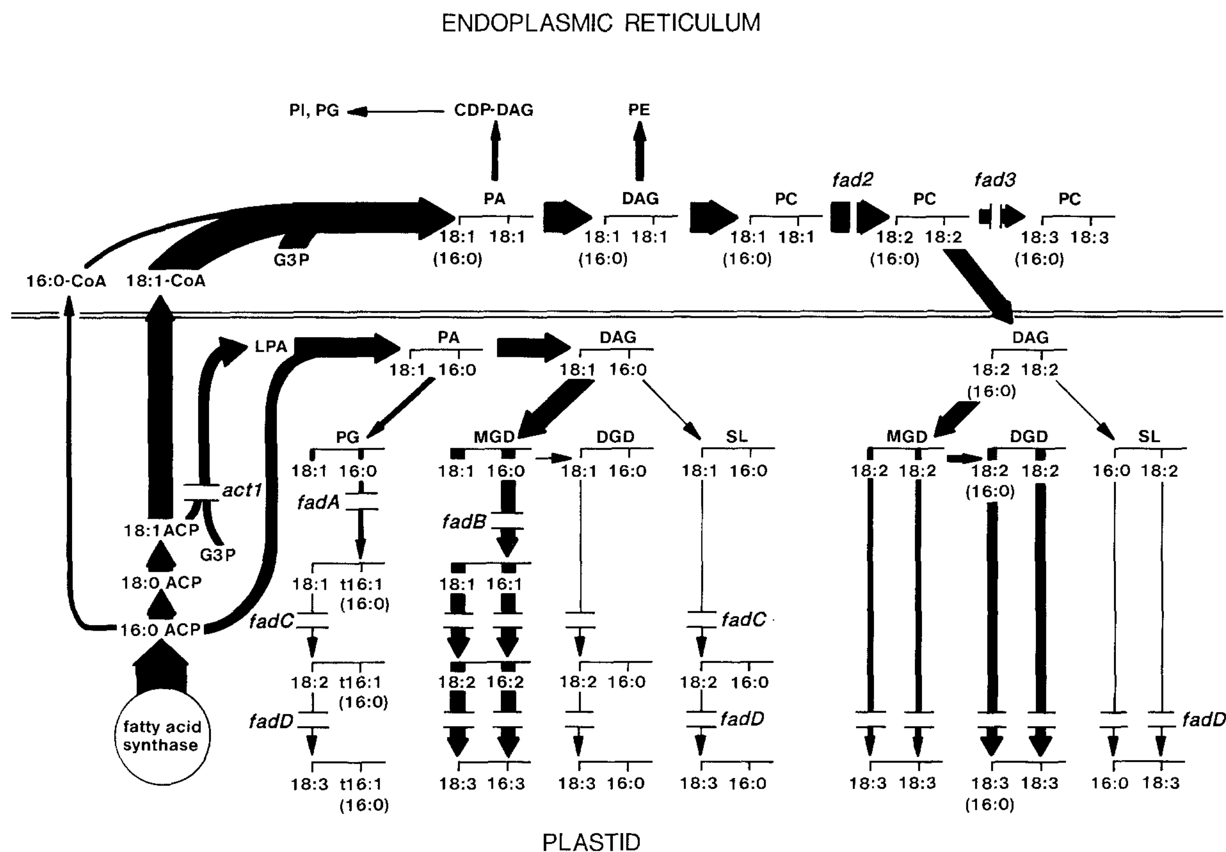

Fig. 10. Mutants in lipid biosynthesis of *Arabidopsis*, reprinted from Somerville and Browse, [Science 252: 80–87 (1991)] with permission (copyright 1991 by the AAAS). Seven classes of mutant in glycerolipid metabolism are shown. These were identified by measuring the fatty acid composition of approximately 10 000 individuals, by gas chromatography, from randomly chosen plants in a mutagenized population.

crease in the chilling tolerance of the transformant [146]. This represents the first example of the alteration of lipid composition by genetic engineering.

Experimentation on rye protoplasts, utilizing a 'membrane engineering approach' have demonstrated the importance of unsaturated lipids, specifically in phosphatidylcholine with respect to increasing the stability of plasma membrane during freezing-induced dehydration [130].

The incorporation of acyl groups into complex lipids and the genetic engineering of altered acyl lipid composition in plants

There is an enormous variety of complex lipids within cells such as sphingolipids, MGDG, DGDG, phosphatidylcholine, phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine and triacylglycerols. The incorporation of fatty acids onto the backbone of these lipids requires the action of acyltransferases. Acyltransferases, responsible for the synthesis of plastid lipids, use ACP derivatives preferentially [23] whilst those involved in triacylglycerol biosynthesis use CoA substrates [25].

The soluble plastid glycerol-3-P acyltransferase (G-3-P-AT) has been purified from a number of plant sources: squash [76], pea [4, 19] and spinach [4]. Recently the cDNA for the squash [43], *Arabidopsis* [76] and pea G-3-P-AT have been cloned and sequenced.

In *E. coli*, the G-3-P-AT is membrane-bound, the enzyme was very labile during purification and was inactive until inserted into a biological

membrane [24] – a case of a lipid synthesizing enzyme requiring a lipid environment! The protein has several membrane-spanning domains and insertion into the membrane was topicologically correct. This gives an optimistic view to reactivation of the plant membrane-bound enzymes upon insertion into the appropriate biological membrane. The use of chaperonins to refold denatured protein may also prove advantageous if applied during the isolation of membrane proteins.

The acyltransferases for position *sn*-2 and *sn*-3 in plants, like their *E. coli* equivalents, are membrane-bound; the 1-acylglycerol-3-phosphate acyltransferase of pea, which catalyses the acylation of the *sn*-2 position, has a marked selectivity for palmitate [23]. 1-acylglycerol-3-phosphate acyltransferases of seed tissue have a similar high selectivity for certain acyl chains and as such are seen as a potential target for genetic engineering. Since acyltransferases have different substrate specificities it should be possible to selectively alter the composition of plant lipids by manipulating the type and level of acyltransferases in plants. Murata introduced the squash and *Arabidopsis* G-3-P-AT gene into tobacco plants. This resulted in marked alteration in the lipid composition of the phosphatidylglycerol, verifying that *in vitro* selectivities are also seen *in vivo* under conditions where the acyl pool is the same [70].

Work is currently underway on the acyltransferases from plant mitochondria and other systems; they appear to have different substrate properties. It will be of interest to see if the mitochondrial enzyme also uses ACP as this has been reported in plant mitochondria [11]. A microsomal acyltransferase has recently been partially purified from etiolated shoots of pea and this will not use ACP substrates [31].

Fatty acid-binding proteins/membrane formation and targeting

Free fatty acids can be highly toxic within the cell, hence there are particular species of proteins which bind to them. Initially, in animals, it was believed that acyl-CoA- and fatty acid-binding proteins were one and the same. However, more recent studies have shown these functions reside in separate proteins [89]. In plants non-specific lipid-binding proteins have been well characterized [144, 151], appearing during germination. cDNAs have been isolated from maize [137], barley [2, 68] and spinach [3] for non-specific lipid transfer proteins. Amino acid sequences deduced from cDNA data show that the polypeptides are produced by cleavage of a preprotein and evidence suggests that processing is by the secretory pathway [3]. Originally it was believed that lipid-binding proteins were possibly involved in lipid transfer between organelles. Three recent observations would strongly question this conclusion: (1) maize lipid transfer protein has been immunolocalized and found predominantly in the external cellular layers and around the leaf veins [128]; (2) the EP2 lipid transfer protein in embryogenic carrot suspensions is secreted into the medium [131]; (3) the *Arabidopsis* lipid transfer protein has an extracellular location [138]. It is possible that lipid transfer proteins are involved in wax or cutin synthesis. Apart from the lipid transfer proteins referred to above, phospholipid-transfer proteins have been the subject of much recent research. These can exchange the lipid molecules between membranes without altering their own lipid content. There seem to be many different transfer proteins in eukaryotic cells which are capable *in vitro* of directing net lipid movement between organelles. Recent experiments in yeast systems [1] have demonstrated that the *SEC14* gene, which is essential for transport of proteins from the golgi complex, encodes a phosphoinositol/phosphatidylcholine transfer protein. These results establish an *in vivo* function for phospholipid transfer proteins, namely compartment-specific stimulation of protein secretion.

It is of interest to know if either fatty acid-binding proteins or lipid-binding proteins are present in seeds during lipid accumulation to act as carriers between cellular compartments.

Formation of oil bodies and oil body proteins

The mechanism of oil body formation has been described in detail elsewhere (see [71] for review). Early studies by Slack *et al.* [123] found that two major proteins co-purified with safflower and linseed oil bodies; these hydrophobic proteins are now known as oleosins. cDNA, gene and protein sequence data are available for two maize proteins [142, 88], carrot [32], rape and radish [72]. All of these proteins have similar amino acid sequences, sharing a central hydrophobic domain. It has been suggested that oleosins prevent coalescence of oil droplets during desiccation of the seed and may contain a lipase-binding site [72]. Clearly, there are other proteins associated with oil bodies. Herman *et al.* [35] have reported a hydrophilic oil body-associated protein from soybean, but as yet no function has been ascribed to this protein.

Differential gene expression and lipid synthesis

Perhaps the most advanced study in this area concerns the regulation of rape embryo ACP expression. The rape embryo clones were broadly divided into two classes based on differences in the transit sequence (group I and group II). It has been demonstrated that group II shows both temporal and tissue-specific expression [112]. Recent experiments from the Unilever laboratory have shown that a 1.4 kb 5′ flanking sequence of a rape seed ACP gene will confer temporal and seed specific expression on reporter genes which have this promoter (R. Safford, pers. comm.). Since napin, cruciferin and the oleosins [71] are deposited later during seed development than the onset of lipid deposition it is highly likely that promoter elements from these genes would not have the same temporal expression as the lipid synthesis genes. Other ACP genes exist which are apparently constitutively expressed [80]. In future it will be interesting to find the mechanism involved in such temporal and tissue-specific expression. Analysis of the 5′ sequences of several genes specifically expressed in seeds at the same time should provide information about common *cis* elements and hence lead to the identification of *trans*-acting factors which might regulate their expression.

Current directions and key questions

Despite an increased activity in the area of plant lipids, little is still known about the structure of many of the component enzymes of complex lipid biosynthesis (see review by Joyard *et al.* [47]). In part this is due to the problems of purifying membrane-bound enzymes. However this could be possible if a concerted scientific effort is made. Generating mutants of lipid metabolism in *Arabidopsis* and the ability to 'gene-walk' is one approach that aims to overcome this. Such mutants will arise from lesions in both structural and regulatory genes; mutations in *de novo* fatty acid synthesis up to $C_{18:0}$ the FAS genes are likely to be lethal. Since the fundamental reactions of fatty acid synthesis are conserved it is possible that antibody and gene probes derived from soluble proteins could be used to clone membrane-bound counterparts of central fatty acid synthesis that are involved in chain elongation.

The essential requirement of particular fatty acids in certain structural relationships in the cell can be tested by mutation and/or transformation studies. This has already been achieved for *trans* Δ3-hexadecenoic acid, by characterization of a mutation at a single nuclear locus (*fadA*), the carriers of which completely lack *trans*-16:1 [6]. This acyl chain was thought to play an important role in some aspect of photosynthesis, but its loss did not have a significant effect on chloroplast function. In the longer term mutational studies can also be extended to the importance of certain lipid classes, as is being undertaken in studies with *E. coli* [10].

With the availability of a number of cDNA and genomic clones for plant FAS components it will be possible to study the transcriptional regulation of these genes.

At the biochemical level, a number of questions remain unresolved. Are components of the FAS

reaction associated *in vivo*? What is the exact nature of the number and stoichiometry of components of plant FAS? What are the physiological triggers stimulating the expression of genes for lipid synthesis in plants? What is the exact nature of the substrate which enters the plastid and the nature of the product which is exported?

The fruits of this in time will not only be a greater understanding of lipid biochemistry, which is scientifically satisfying, but will eventually lead to genetically engineered lipid compositions, both of membranes and storage lipids. The feasibility of this has already been demonstrated by Murata [70] and Kridl *et al.* [54]. It may well be an important consideration in the genetic engineering of the lipid content of seeds to alter the specificities of the lipases involved in the germination process. If newly produced lipids are not readily mobilized by the endogenous lipase, problems may occur on germination.

Alteration of the lipid content of plants will eventually supply key intermediates for the chemicals industry which will allow for sustainable agriculture, renewable raw resources and an environmentally cleaner route to chemical production.

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