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Review

Carbon flux and fatty acid synthesis in plants

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Abstract

The de novo synthesis of fatty acids in plants occurs in the plastids through the activity of fatty acid synthetase. The synthesis of the malonyl-coenzyme A that is required for acyl-chain elongation requires the import of metabolites from the cytosol and their subsequent metabolism. Early studies had implicated acetate as the carbon source for plastidial fatty acid synthesis but more recent experiments have provided data that argue against this. A range of cytosolic metabolites including glucose 6-phosphate, malate, phosphoenolpyruvate and pyruvate support high rates of fatty acid synthesis by isolated plastids, the relative utilisation of which depends upon the plant species and the organ from which the plastids are isolated. The import of these metabolites occurs via specific transporters on the plastid envelope and recent advances in the understanding of the role of these transporters are discussed. Chloroplasts are able to generate the reducing power and ATP required for fatty acid synthesis by capture of light energy in the reactions of photosynthetic electron transport. Regulation of chloroplast fatty acid synthesis is mediated by the response of acetyl-CoA carboxylase to the redox state of the plastid, which ensures that the carbon metabolism is linked to the energy status. The regulation of fatty acid synthesis in plastids of heterotrophic cells is much less well understood and is of particular interest in the tissues that accumulate large amounts of the storage oil, triacylglycerol. In these heterotrophic cells the plastids import ATP and oxidise imported carbon sources to produce the required reducing power. The sequencing of the genome of *Arabidopsis thaliana* has now enabled a number of aspects of plant fatty acid synthesis to be re-addressed, particularly those areas in which in vitro biochemical analysis had provided equivocal answers. Examples of such aspects and future opportunities for our understanding of plant fatty acid synthesis are presented and discussed.

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Nomenclature

Glc6P	glucose 6-phosphate
PEP	phosphoenol pyruvate
OPP	oxidative pentose phosphate
ACBP	acyl-CoA binding protein
G3P	glycerol 3-phosphate
LPA	lysophosphatidic acid
PA	phosphatidic acid
DAG	diacylglycerol
PC	phosphatidylcholine
TAG	triacylglycerol

1. Introduction

Recent progress in understanding how fatty acid synthesis in plants relates to primary carbon metabolism makes a review of this topic timely. In particular, advances in the understanding of plant genomes, especially that of the oilseed species *Arabidopsis thaliana* [1] present new opportunities and enable new perspectives on old problems, as will be discussed throughout this article. In plants the de novo synthesis of fatty acids occurs primarily in the plastid [2] (Fig. 1). These fatty acids are used for the synthesis of plastidial and other cellular membranes in all cells. In certain plant tissues, most notably in seeds, they are also used for the synthesis of storage oils (TAG: triacylglycerols). This latter aspect of lipid synthesis has attracted much attention because of the potential to produce feedstocks for the chemical industry in planta as components of storage oils. There are two routes to this goal. First, the development of plant species that synthesise novel oils into ''alternative oilseed'' crops. Second, the cloning of genes which determine the synthesis of these novel oils and introduction of these genes into existing crops such as oilseed rape (*Brassica napus* L.) through genetic transformation. Whichever approach is adopted we need

to understand what controls the amount of storage oil accumulated in order to optimise yield. The understanding of the pathways involved in carbon supply to fatty acid synthesis in leaves and oil-storing tissues of plants has improved in recent years. However, we know much less about how the flux of carbon through these pathways is controlled, or indeed how the partitioning of carbon between oil and other storage products that are laid down simultaneously is determined.

The first committed step in fatty acid synthesis is considered to be that catalysed by acetyl-CoA carboxylase (ACCase), which converts acetyl-CoA to malonyl-CoA [2]. Since acetyl-CoA is not imported by plastids [3,4] it must be generated by metabolism within the plastid. In chloroplasts photosynthesis provides an endogenous source of fixed carbon. Whether this fixed carbon can be utilised for the synthesis of acetyl-CoA depends upon the enzyme complement within the chloroplast and is discussed later. In the case of non-photosynthetic cells/organs the plastid is dependent upon import of metabolites from the cytosol in order to synthesise acetyl-CoA. This import process is likely to involve specific transporter proteins on the plastid envelope, as has been reported for non-photosynthetic plastids that carry out starch synthesis (see [5] and references therein). A description of carbon supply to fatty acid synthesis can therefore be broken down into

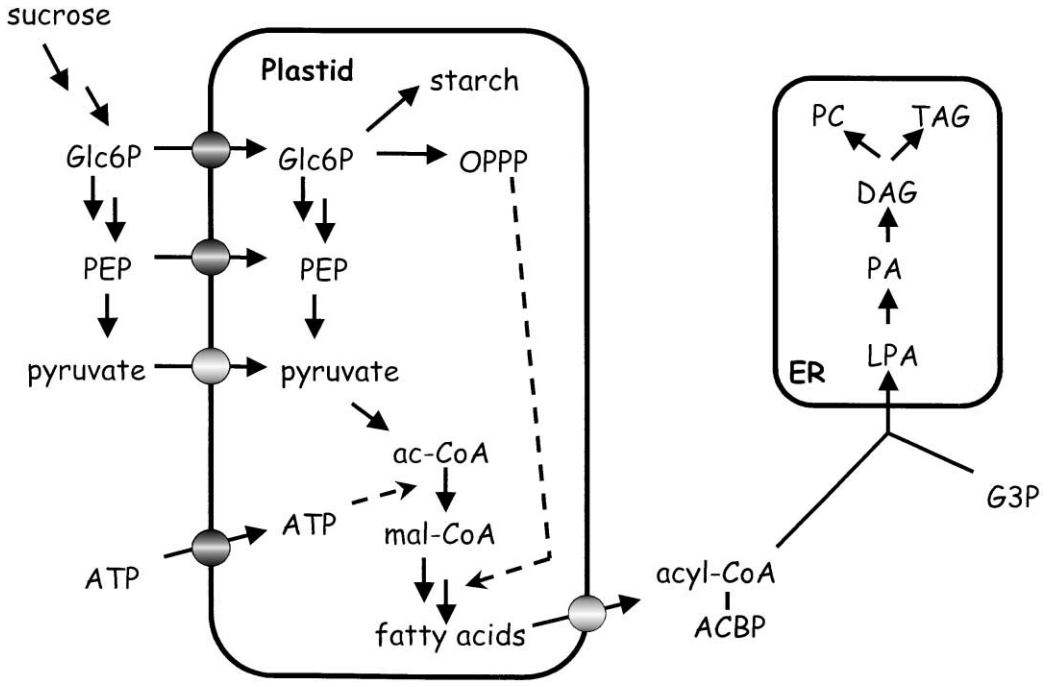

Fig. 1. An overview of the current understanding of metabolism associated with fatty acid synthesis and plastidial carbon partitioning in the developing oilseed rape embryo based on metabolic studies. The scheme presented is similar to that proposed by White et al. [98] for *Arabidopsis* which is based on the detection of ESTs from developing seeds. However, in several cases, ESTs encoding enzymes of metabolic steps that are known to occur were not detected in the screen [98]. The compartmental nature of the metabolism from sucrose to lipids involving the cytosol, plastids and the endoplasmic reticulum is illustrated, as is the interaction between the oxidative pentose phosphate pathway and fatty acid synthesis (dashed arrow). Transporters on the plastid envelope are shown as shaded circles. Transporters for which genes have been cloned and their proteins functionally characterised are indicated by darkened shading. ac–CoA = acetyl–CoA; mal-CoA = malonyl-CoA; OPPP = oxidative pentose phosphate pathway.

two main sections: (1) the enzymes that synthesise or utilise acetyl-CoA in the plastid; and (2) the uptake and conversion of metabolites into the substrate(s) that these enzymes utilise. Both of these aspects will be discussed.

2. Synthesis and utilisation of acetyl-coenzyme A in the plastid

2.1. Synthesis of acetyl-CoA

Four possible routes for the synthesis of acetyl-coenzyme-A (acetyl-CoA) in plastids have been proposed. In the first, free acetate is activated to acetyl-CoA by acetyl-CoA synthetase (ACS) in an ATP-dependent reaction. The activity of ACS has been measured in chloroplasts of several species and the properties of the enzyme are reported to be consistent with a role in de novo fatty acid synthesis [6–8]. More recent studies of expression of the sole ACS gene in *A. thaliana* [9] have revealed a spatial and temporal pattern of expression that is inconsistent with a role for ACS involvement in lipid accumulation, at least in developing seeds. Moreover, preliminary data reported by Nikolau et al. [10] reveal that expression of an antisense construct of ACS using a ubiquitously expressing promoter failed to produce any detrimental morphological phenotype or change in plant fatty acid composition in the transgenic plants. However, they do report that the rate of acetate incorporation into fatty acids is decreased in proportion to the degree of down-regulation of ACS in the antisense lines. These preliminary data provide evidence that ACS does not represent a major or unique route to de novo fatty acid synthesis throughout the plant.

As discussed later pyruvate can be produced by glycolytic activity within the plastid or by cytosolic glycolysis followed by import into the organelle. The pyruvate is converted to acetyl-CoA by the pyruvate dehydrogenase complex (PDC) in a reaction that also generates NADH. In plant cells there are two forms of PDC, one in the mitochondria and another in the plastids. Activity of PDC has been reported in plastids from several species and 30–66% of the total cellular activity is plastidial depending on the tissue [11–17]. Genes encoding subunits of the plastidial PDC have been cloned [18–20] and their expression patterns studied in developing *Arabidopsis* siliques [9]. The spatial and temporal expression patterns were entirely consistent with a role in lipid accumulation in the developing embryos.

A third possible route for intraplastidial synthesis of acetyl-CoA is through a plastidial carnitine acetyltransferase reaction in which acetate is transferred from acetyl-carnitine to CoA. It has been proposed that this activity represents part of a carnitine-dependent acetyl/acyl transfer mechanism in the plant cell [21]. However, as discussed later in considering the uptake and utilisation of metabolites for fatty acid synthesis by isolated plastids, this proposed mechanism is controversial.

The fourth route for acetyl-CoA synthesis is through the ATP-citrate lyase (ACL) reaction. The proposed role of this enzyme in de novo fatty acid synthesis is also somewhat contentious. The activity of ACL in leaves is reported to be predominantly plastidial in leaves of rape (*B. napus* L.) and spinach, while in tobacco and pea it is predominantly cytosolic [22]. However, neither of the two ACL genes in *Arabidopsis* encodes a protein with a predicted organellar targeting sequence, and studies with isoform specific antisera and enzyme assays failed to demonstrate the presence of ACL activity or protein in the plastid [23]. *Arabidopsis* and *B. napus* are close relatives within the

Brassicaceae and would be expected to have broadly similar primary carbon metabolism. The data of the two groups above are therefore inconsistent. Notwithstanding this difference, overexpression of a rat liver ACL in the plastid was reported to increase the fatty acid content of tobacco leaves (mg per g fresh weight of leaf) by 16% compared to control plants [24]. Whether this overexpression actually increases the rate of fatty acid synthesis has not been shown, nor has the source of plastidial citrate been identified.

2.2. Utilisation of acetyl-CoA

The synthesis of malonyl-CoA from acetyl-CoA is catalysed by acetyl-CoA carboxylase (ACCase) in an ATP-dependent step. The knowledge of the type of ACCase involved in this reaction in the plastid has developed considerably over the past 3 years. Two forms of ACCase are known to occur in plants: Type I ACCase, which is a large multifunctional enzyme analogous to that found in yeast and mammals, and type II enzyme which is a multisubunit complex analogous to that found in prokaryotes. It is now known that the plastidial ACCase activity in most higher plants is due to a type II form of enzyme but in the Gramineae the activity is due to a type I enzyme (e.g. [25–27]). All higher plants possess an extraplastidial type I enzyme which is believed to be cytosolic (e.g. [28,29]). This picture has been further complicated by the presence of a type I enzyme in oilseed rape plastids [30,31], although the activity of this isoform in developing embryos is less than 10% of the cellular total of ACCase type I (based on measurement of the propionyl-CoA carboxylase activity of ACCase I: [32]).

A great deal of interest has been focused on ACCase and the role that it might play in regulating the flux of carbon to fatty acids and so to storage oil. There are several experimental lines of evidence that suggest that ACCase does represent a regulatory step. Intermediates of fatty acid synthesis change during the transition to darkness in leaves and chloroplasts in a manner consistent with control at the level of ACCase [33,34]. This observation is entirely consistent with the more recent reports of light-dependent regulation of ACCase II by the redox status of the plastid [35–38] whereby the enzyme is more active under the reducing conditions found in the light. Whether the plastidial type I ACCase found in the Gramineae is also regulated by a similar mechanism is unknown. There is also evidence for regulation of the ACCase II by phosphorylation/dephosphorylation of the β subunit of the carboxyltransferase [39] although a detailed relationship between this observation and the redox regulation remains to be explored.

In leaves, the light-dependent regulation of fatty acid synthesis through ACCase activity is logical if the synthesis of fatty acids from malonyl-CoA is in turn dependent upon the supply of reducing power and ATP from the light reactions of photosynthesis. Whether such fine control of ACCase in organs carrying out storage oil accumulation occurs or is required is more debatable. Other aspects of the control of carbon flux through fatty acid synthesis by ACCase have been addressed in a number of ways. By using specific enzyme inhibitors Page et al. [40] have reported that ACCase exerts strong control over flux into fatty acid synthesis in isolated barley and maize leaf chloroplasts. Roesler et al. [31] have increased total plastidial ACCase activity by up to twofold by targeting a type I ACCase to the plastids in developing oilseed rape embryos. This resulted in an increase in fatty acid content of the seed by up to 5% although whether there was a significant increase depended on the location in which the plants were grown. Indirect evidence for control by ACCase can be drawn from experiments in which fatty acids were supplied to

tobacco suspension cell cultures [41]. This resulted in inhibition of fatty acid synthesis, which was explained by feedback inhibition of ACCase by an undefined mechanism. Much less useful indirect evidence comes from attempts to correlate measurements of maximum catalytic activity of ACCase with the rate of lipid accumulation during seed development (e.g. [42–44]). The occurrence of multiple ACCase isoforms and the relatively low total activities of ACCase reported in this type of experiment shed serious doubt on the reliability of such deductions.

In conclusion, ACCase has properties that enable it to regulate carbon flux to fatty acids in leaves but there is little conclusive evidence to suggest that it can regulate flux in storage organs.

3. Carbon flow to acetyl-CoA in the plastid

If plastids are to utilise carbon from the level of hexose for the synthesis of pyruvate and so acetyl-CoA, they require a complete glycolytic pathway. A complete glycolytic pathway has been reported to be present in non-photosynthetic plastids from pea and rapeseed embryos, cauliflower buds, and wheat and castor (*Ricinus communis*) seed endosperm [15,17,45–49]. In several of these studies the activities of some of the glycolytic enzymes are reported to be low. In fact, phosphoglycerate mutase is reported to be absent from the plastids of pea roots and sycamore cells and from pea leaf chloroplasts [50–52]. It is also uncertain if hexokinase and NAD-glyceraldehyde 3-phosphate dehydrogenase activities are present in the pea root plastids since conflicting evidence is presented by Trimming and Emes [51] and Borchert et al. [53]. Clearly, the capacity of plastids to convert carbon sources at the level of hexose, triose or PEP to pyruvate in order to supply fatty acid synthesis differs between plant tissues. An additional route for the synthesis of pyruvate is through decarboxylation of imported malate by the plastidial NADP-dependent malic enzyme (NADP-ME). Activity of NADP-ME has been reported in plastids from oilseed rape embryos [17] and castor endosperm [54].

4. Utilization of metabolites for fatty acid synthesis by isolated plastids

Almost all of the research that has so far addressed the carbon source for plastidial fatty acid synthesis has relied on the isolation of intact plastids and the study of their ability to incorporate exogenously supplied metabolites into fatty acid products. This approach has led to a great deal of potentially valuable information (e.g. Fig. 1). However, this information requires careful interpretation. First, it is possible that the properties of the plastids may be altered during isolation. Second, for reasonable conclusions to be drawn the plastids should be capable of rates of fatty acid synthesis that are comparable to the rates required for in vivo fatty acid synthesis. Third, in vitro studies of isolated organelles are in general simplistic: single substrates are supplied at saturating concentrations for fatty acid synthesis so that interpretation of the data is straightforward. This is a very different situation from that in vivo where multiple substrates are available for which the in vivo concentrations are not known, and where there is potential competition for the carriers and enzymes. Despite such reservations the studies to date have revealed that a broad range of metabolites are taken up and utilised by plastids for fatty acid synthesis, depending on the plant species, organ and stage of development.

In all studies reported so far acetate is taken up by isolated plastids and utilised for fatty acid synthesis. The relative rates of utilisation of acetate and of other potential substrates for fatty acid synthesis in vitro depends upon the tissue from which the plastids were extracted. Moreover the rates of acetate utilisation tend to be low when compared to the other substrates. Nevertheless, acetate has been widely claimed to be an important carbon source for fatty acid synthesis in chloroplasts (e.g. [3,55,56]). Roughan and Ohlrogge [57] have proposed that there is a "metabolon" in chloroplasts of spinach which channels acetate through to long chain fatty acids and involves acetyl-CoA synthetase, ACCase, and FAS. Their studies are based upon experiments with plastids that are partially lysed and have lost some enzymes, and yet show high rates of fatty acid synthesis, implying retention of a macromolecular complex within the "leaky" plastids that can convert acetate to fatty acids. They also proposed that this complex would preferentially utilise acetyl-CoA that was derived from ACS rather than pyruvate dehydrogenase. Whether such a "metabolon" exists in other plastids remains to be determined. Recent studies by Bao et al. [58] have re-addressed the dogma that acetate is the immediate precursor for fatty acid synthesis. When whole leaves were fed $^{14}CO_2$ and the appearance of label measured in the fatty acid and acetate fractions, the data were wholly inconsistent with a significant pool of free acetate, or with the involvement of an acetate pool in the movement of label from CO_2 to acetyl-CoA [58]. This result was true for leaves of a range of plant species and provides good evidence that the flow of photosynthetically fixed carbon into fatty acids is not via a free acetate pool. This still leads the debate back to whether the carbon remains within the chloroplast and pyruvate is formed using the reactions of glycolysis from triose phosphates to PDC, or whether triose phosphate is exported to the cytosol before conversion to another metabolite (e.g. pyruvate) prior to import and utilisation in fatty acid synthesis.

There has been debate in the literature as to whether acetyl-carnitine represents a true in vivo substrate for plastidial fatty acid synthesis. The work of Masterson et al. [59,60] has suggested that this might be the case for chloroplasts isolated from pea leaves. In these experiments the rate of fatty acid synthesis from acetyl-carnitine was more than four-fold greater than that from acetate. However, Roughan et al. [61] were unable to demonstrate incorporation of acetate from acetyl-carnitine into fatty acids by chloroplasts from spinach and pea leaves. The publication of the *Arabidopis* genome sequence [1] has enabled a search for plant homologues of the enzymes and transporters that form the carnitine shuttle as outlined by Wood et al. [21]. To date only two candidate genes have been identified, both encoding putative acetyl/acyl-carnitine transporters. None of the genes encoding the other components of the pathway have been identified. Until the actual transport properties of the proteins have been determined and their cellular locations defined the debate on the role of carnitine metabolism remains open.

Glucose 6-phosphate (Glc6P), glucose, dihydroxyacetone phosphate, malate and pyruvate can support high rates of fatty acid synthesis by isolated plastids, their relative utilisation depending on the plant species and tissue studied [17,49,54,62,63]. Based upon such studies it is tempting to propose that the substrate that is utilised best in vitro is the most likely substrate in vivo. However, for the reasons given at the introduction to this section, caution should be applied when making such deductions from experiments carried out at a single time point in the ontogeny of the tissue. While still imperfect, greater certainty can be attached to deductions of which metabolites might be used in vivo by looking for correlations between their utilisation by isolated plastids in vitro and the flux of carbon through fatty acid synthesis as it changes in vivo. For

example, Eastmond and Rawsthorne [49] have shown that the relative utilisation of metabolites by plastids from developing rapeseed embryos is correlated with the patterns of starch and fatty acid synthesis. During the early stages of development the embryos accumulate both starch and lipid and during this period the isolated plastids display relatively high rates of utilisation of Glc6P for fatty acid and starch synthesis. Later in embryo development when lipid synthesis predominates and the starch is degraded, the plastids use pyruvate at high rates for fatty acid synthesis and Glc6P is used preferentially for the OPP pathway (see later).

5. Import of metabolites for fatty acid synthesis by plastids

Despite many years of investigation of plastidial fatty acid synthesis little attention has been given to the import of exogenous substrates into the plastids for this pathway. The lack of information on the role of transporters in fatty acid synthesis is surprising given the interest in fatty acid synthesis, and in the background knowledge of the plastid transporters associated with starch synthesis and carbon supply for the OPP pathway in non-photosynthetic plastids. The movement of acetate into plastids is believed to occur via diffusion across the membranes of the plastidial envelope. A role for the carnitine/acetyl-carnitine exchange transporter in moving acetate equivalents into the plastids has been proposed by Wood et al. [21], but this route for metabolic flux remains controversial as discussed above. Recent studies by Rawsthorne, Hills and colleagues [49,64–69] have significantly advanced the knowledge of transporters involved in fatty acid synthesis.

Consistent with the utilisation of Glc6P by plastids of oilseed rape embryos [17,63] the activity of a Glc6P transporter (GPT) was identified in the envelope of the plastids from this tissue [64]. The activity of the GPT decreases slightly during embryo development but is in excess of the combined rates of Glc6P metabolism via fatty acid and starch synthesis and through the OPP pathway [49]. The metabolite transport properties of the rapeseed GPT are very similar to those reported for that from pea embryos and roots and cauliflower bud plastids [70–72]. Interestingly, the rapeseed embryo GPT is sensitive to inhibition by long chain acyl-CoAs [68], which leads to feedback inhibition of fatty acid synthesis during in vitro incubations where these acyl-CoAs are allowed to accumulate [67,68]. The feedback is mediated by inhibition of Glc6P uptake that decreases both carbon supply to fatty acid synthesis and the provision of reducing power to fatty acid synthesis through the OPP pathway [67,68]. The concentration at which the acyl-CoA inhibition occurs is in the nM range (IC_{50} of 250 nM) and presents a physiologically meaningful basis for a model for in vivo feedback of fatty acid synthesis by the plastid [68].

Pyruvate can support fatty acid synthesis by plastids isolated from both oilseed rape embryos and castor seed endosperm [17,54,63,73]. In oilseed rape embryos the uptake of pyruvate is reported to involve a carrier with a K_m for pyruvate of 0.2 mM that increases in activity during embryo development in parallel with the increase in ability of isolated plastids to utilise pyruvate for fatty acid synthesis [49]. Although preliminary studies have suggested that the characteristics of the rapeseed pyruvate transporter are like those of the monocarboxylate transporter (MCT) family of proteins [74], there is no obvious homologue of a gene encoding an MCT-like protein in the genome of *Arabidopsis* ([1], I. Paulsen, personal communication). The precise mechanism of pyruvate uptake by plastids therefore remains elusive.

In glycolysis, pyruvate is formed by the action of pyruvate kinase from phosphoenolpyruvate (PEP). The importance of import of PEP for plastidial metabolism is revealed by the *A. thaliana cue1* mutation [75], alleles of which are in the PEP transporter (PPT) gene [76]. The PPT was proposed to support the plastidial shikimate pathway in plants by supplying PEP as an essential precursor [77]. Consistent with this proposal, *cue1* mutants are disrupted in the production of aromatic amino acids but can be rescued by feeding these compounds to mutant plants in tissue culture [76]. Streatfield et al. [76] have reported that there is no effect on the overall lipid levels in leaves or on seed lipid content of the *cue1* mutant plants and conclude that there is not a major flux of PEP into fatty acids. The lack of effect on seed lipid content could be explained if the activity of the PPT was relatively low and therefore import of PEP made little contribution to overall carbon flux into the glycolytic pathway in the oilseed embryo. Recently Kubis and Rawsthorne [66] have reported that the activity of the PPT is comparable to that of the GPT and pyruvate transporters in the oilseed rape embryo. Incorporation of carbon from PEP into fatty acids by isolated rapeseed embryo plastids also occurs at rates that are comparable to Glc6P and pyruvate (Everett, Kubis and Rawsthorne, unpublished data). Thus the two types of data support opposite conclusions. It is possible that the growth of *cue1* mutants and control plants, and so seed yield, under tissue culture conditions is less than that occurring under optimal conditions in the soil. If so the effect of reducing PPT in the embryo may not be manifested if import of other substrates for fatty acid synthesis were sufficient. Further work is clearly required to resolve this question.

The highest rates of fatty acid synthesis by plastids from castor endosperm are supported by exogenously supplied malate [54]. Consistent with this observation a novel malate transport activity was identified on the envelope of castor plastids [78]. This transporter activity appears to be via a phosphate counter-exchange mechanism, similar to that reported for mitochondria [79], and not the well characterized dicarboxylate exchange transporter of chloroplasts [80].

From the above studies it can be argued that the activities and properties of transporters may be important (1) in determining the metabolic routes by which carbon is imported into the plastid and utilised for fatty acid synthesis and their relative importance, and (2) in providing a means for feedback regulation of plastidial fatty acid synthesis. Further studies of the transporters will continue to identify what appear to be metabolic steps that are of potential importance in vivo. Ultimately it is the manipulation of the activity of these transporters in vivo that will reveal their relative importance in determining how plastidial and cytosolic metabolism interact during fatty acid synthesis.

6. Sources of ATP and reducing power for fatty acid synthesis

The synthesis of fatty acids requires stoichiometric amounts of ATP and acetyl-CoA, and NADPH and NADH for each C2 addition to a growing acyl chain in the reactions catalysed by acetyl-CoA carboxylase and fatty acid synthetase [81]. In chloroplasts, light energy can be used to provide the NADPH and ATP required for fatty acid synthesis. In heterotrophic tissues, the plastids need to import these cofactors or to generate them intraplastidially through carbohydrate oxidation or metabolite shuttles.

Indirect evidence for ATP import comes from studies of fatty acid and starch synthesis in isolated plastids. For example, starch or fatty acid synthesis by plastids isolated from heterotrophic organs is widely reported to be dependent on the provision of exogenous ATP (e.g.

[54,63,71,82,83]). Direct evidence for import of ATP into plastids has been reported for plastids isolated from pea roots and sycamore cells, and for spinach chloroplasts [69,84,85]. This uptake of ATP is carried out by ATP/ADP exchange translocators that are located on the plastid envelope [86–88]. In *Arabidopsis* there are two genes encoding plastidial adenylate exchange transporters, and the proteins encoded by them have similar biochemical properties when recombinantly expressed [86]. The roles of these multiple isoforms and their relative expression patterns are to date unknown.

Developing embryos of a number of species have photosynthetic characteristics and therefore the potential to produce ATP within the plastid through photophosphorylation. Acetate incorporation into fatty acids by plastids from linseed and rapeseed is stimulated by light [65,89]. However, in rapeseed exogenous ATP supports the greatest rates of fatty acid synthesis and Glc6P-dependent fatty acid synthesis shows no light dependency [65]. Moreover, Eastmond et al. [90] calculated that in rapeseed transmission of light to the developing embryo through both the silique wall and the testa would be low. In contrast Willms et al. [91] have argued that in soybean light energy does stimulate fatty acid synthesis based on their studies of developing soybean fruits using isotope discrimination studies and gas exchange measurements.

Another potential source of ATP in the plastid is that generated by substrate level phosphorylation in the reactions of glycolysis. Kleppinger-Sparace and colleagues [83,92] have investigated whether the ATP required for fatty acid synthesis can be generated within the plastid in this manner. Their results have shown that at least for glycolysis between dihydroxyacetone phosphate (DHAP) and 2-phosphoglycerate this is possible. By setting up a triose phosphate shuttle (provision of 2 mM DHAP, 2 mM oxaloacetic acid and 4 mM inorganic phosphate as defined in Kleppinger-Sparace et al. [83]) in their incubations they demonstrated that rates of fatty acid synthesis from acetate were up to 44% of those obtained in the presence of exogenous ATP alone. Exogenous PEP also stimulates acetate-dependent fatty acid synthesis by plastids, probably through the generation of ATP in the plastidial pyruvate kinase step, the stimulation relative to that supported by exogenous ATP being dependent upon the particular plastid type [92–94]. The recent data on uptake and incorporation of PEP into fatty acids ([66]; Rawsthorne et al., unpublished) would suggest that carbon skeletons, ATP and some of the reducing power required for fatty acid synthesis (see later) could all be generated in the plastid by metabolism of PEP through pyruvate kinase and pyruvate dehydrogenase.

To illustrate the difficulty of interpreting these in vitro experiments in an in vivo context, other studies suggest that plastidial glycolytic metabolism is not capable of supporting fatty acid synthesis. Qi et al. [62] and Kang and Rawsthorne [63] have demonstrated that carbon from Glc6P can be incorporated into fatty acids by plastids isolated from pea roots and developing rapeseed embryos. In both cases this incorporation was strongly dependent upon exogenous ATP with rates reduced by at least 90% when ATP was excluded. It is reasonable to assume that the Glc6P was being metabolised by glycolysis within the plastid during these incubations. This should yield the ATP required to drive the incorporation of acetyl-CoA into fatty acids and yet ATP had to be provided in order to see this. The problem with interpreting these experiments is that other metabolism with an ATP demand might be occurring simultaneously in the same plastids and so compete for ATP. This is certainly the case for plastids from developing rapeseed embryos, which can synthesise starch and fatty acids simultaneously [63]. Indeed, the pathways for starch or fatty acid synthesis can compete for exogenously supplied ATP [95]. Given the

evidence to date it is very likely that fatty acid synthesis by plastids from heterotrophic organs is to a large extent dependent upon provision of ATP from the cytosol.

The source of reductant for fatty acid synthesis in heterotrophic tissues is not clear. Inclusion of exogenous reduced pyridine nucleotides in incubations made with isolated plastids gives variable results. For example several studies have shown little effect of manipulating exogenous pyridine nucleotides (e.g. [54,62,63]). These results are usually associated with Glc6P-, malate-, or pyruvate-dependent fatty acid synthesis. In contrast, provision of reduced NAD(P) in incubations where the carbon source is acetate can lead to significant stimulation in the rate of fatty acid synthesis [54,96]. These data can be interpreted to suggest that certain substrates that are imported by the plastids can be metabolised to produce not only the acetyl-CoA for fatty acid synthesis but also the reducing equivalents. Smith et al. [54] have argued that the intraplastidial conversion of malate to acetyl-CoA is extremely efficient for fatty acid synthesis because the reactions catalysed by NADP-ME and PDC form acetyl-CoA, NADPH and NADH, which are all required for this pathway.

Sources of reducing power within the plastid are certainly capable of sustaining fatty acid synthesis. For example ATP-dependent incorporation of acetate in the absence of light can occur in rapeseed embryo plastids at measurable rates over incubations of 1 h, suggesting that an intraplastidial source of reductant is available [65]. Apart from malate and pyruvate metabolism another potential source of this reductant is the plastidial OPP pathway. The OPP pathway has been shown to provide reductant for nitrogen assimilation in pea root plastids [97]. For lipid storing seed tissues the measurement of activity of enzymes of the plastidial OPP pathway and of $^{14}CO_2$ release from [1-^{14}C]Glc6P supplied to isolated plastids (e.g. [17,48,63]), and the detection and expression analysis of Glc6P dehydrogenase cDNAs in *Arabidopsis* [98,99] all provide indirect evidence that the OPP pathway may be linked to fatty acid synthesis. More direct evidence can be drawn from the observations of increased utilisation of carbon sources by plastids when Glc6P is included in incubations [63,100], and from increased liberation of $^{14}CO_2$ from [1-^{14}C]Glc6P when substrates for fatty acid synthesis in addition to Glc6P are provided [49].

On balance, it is most likely that plastids in heterotrophic organs import metabolites and then oxidise them in order to generate the reductant that is required for fatty acid synthesis.

7. Interaction between pathways within plastids

Apart from the examples described above that have related the activity of the OPP pathway to other metabolisms, the interaction of pathways within plastids has received relatively little attention. This is perhaps not surprising since the experiments required to address these issues are inevitably more complex than those in which incorporation of carbon from a single metabolite into a single product are studied. Common approaches with respect to fatty acid synthesis are to provide a radiolabelled substrate in the presence or absence of an unlabelled one and to look for interaction. This approach often reveals that one metabolite competes with another and they are therefore entering a common pathway.

An investigation of the partitioning of imported Glc6P to starch and fatty acids, and to CO_2 via the OPP pathway in plastids isolated from developing rapeseed embryos has revealed that this changes during development [49,63]. Up to the early-mid cotyledon stage of development, when

the embryo is accumulating starch and oil, about 50% of the carbon from Glc6P is utilised for starch synthesis with the remainder divided equally between fatty acid synthesis and the OPP pathway. Later, as the embryo becomes more active in oil accumulation the fluxes of imported Glc6P to starch and fatty acids decrease while oxidation through the OPP pathway increases. This observation emphasises the need to address developmental effects on plastidial metabolism (see earlier for carbon sources for fatty acid synthesis). These studies also showed that total flux to fatty acids could be increased by provision of pyruvate and Glc6P simultaneously and that this did not affect flux of Glc6P to starch. Notably, the in vitro rate of fatty acid synthesis can only account for the calculated in vivo rate of fatty acid synthesis when multiple substrates are provided to the plastids. Such an observation leads to the suggestion that in vivo more than one carbon source is imported from the cytosol for use in fatty acid synthesis by the plastid.

Embryos of oilseeds can accumulate starch and evidence for metabolic links between the synthesis of both compounds is emerging. In oilseed rape embryos the synthesis of starch is transient and mature seeds contain negligible amounts of starch [64]. The precise role of this starch accumulation has not been defined but evidence of starch synthesis in developing embryos during the period of net starch degradation [49] suggests that the starch is turning over. To begin to address the roles of starch synthesis in the oilseed embryo a known *Arabidopsis* mutant that lacks starch synthesis has been studied. The *pgm-1* mutant lacks plastidial phosphoglucomutase [101] and embryos of the mutant line accumulate 40% less oil than the wild type [102]. While this result provides a clear indication that starch synthesis is linked to oil synthesis, the nature of the interaction is completely unknown.

A study of metabolism in oilseed rape plants expressing a medium chain thioesterase (MCTE) has revealed that the interaction between the biosynthesis and degradation of fatty acids pathways can induce compensatory changes in metabolism to maintain carbon flux to oil. The expression of MCTE in oilseed rape leads to the synthesis of laurate in the seeds [103]. This accumulation of laurate is linked to the induction of fatty acid β-oxidation [104]. Surprisingly, even though β-oxidation is induced the final oil content of the transgenic lines is unaltered compared to the wild type [105]. An explanation for this is that the flux through fatty acid synthesis increases to compensate for the degradation and increased expression of enzymes of fatty acid synthesis was reported [105]. To what extent the rate of carbon supply to fatty acid synthesis would have changed in response to such perturbation was not studied.

8. Conclusion

Our understanding of the pathways involved in carbon flux to fatty acids in plants has increased greatly in recent years. Cytosolic carbon sources such as Glc6P, PEP, pyruvate and malate are now seen as important substrates for plastidial fatty acid synthesis rather than acetate. Although not all species will use Glc6P for fatty acid synthesis, its utilisation for generation of reducing power to drive fatty acid synthesis is seen as important for heterotrophic plastids. Set against this progress a number of questions remain to be resolved, particularly those relating to how flux to fatty acid synthesis is controlled, and to interactions that occur (1) at the level of relative metabolite contribution to a given pathway and (2) between pathways themselves. Isolated plastids continue to be a valuable tool in providing basic information that enables the

creation of hypotheses that can be tested in vivo. One route to testing these hypotheses will be the selection from tagged populations of knock-out mutants that contain insertions in genes of interest. Such populations of *Arabidopsis* are already accessible and they will provide a valuable resource. However, in some cases, and particularly for studies of seed metabolism, knock-out lines (e.g. the *cue1* mutant described above) with wide pleiotropies that impose maternal effects on seed development may not be the best tools for studying seed metabolism. In such cases the route to manipulation will be through transgenic lines with embryo-specific up or down regulation of the expression of the target gene. These targeted investigations can be supplemented by approaches such as that of Girke et al. [99] by seeking genes that show different patterns of expression between seeds and other plant organs. The same approach could equally be applied to studying differential expression of genes between different development stages or to lines that have altered metabolism with as yet not fully understood phenotypes, e.g. the *pgm-1* line or the MCTE transgenic lines described above. As discussed by White et al. [98], an additional value of investigating the expression of genes within the developing seed is that regulatory genes as well as those encoding the enzymes of biosynthetic pathways are also detected and can then be studied. Undoubtedly there is still much for us to learn.

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