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Review

Commentary: Why don't plant leaves get fat?

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ABSTRACT

Recent pressures to obtain energy from plant biomass have encouraged new metabolic engineering strategies that focus on accumulating lipids in vegetative tissues at the expense of lignin, cellulose and/or carbohydrates. There are at least three important factors that support this rationale. (i) Lipids are more reduced than carbohydrates and so they have more energy per unit of mass. (ii) Lipids are hydrophobic and thus take up less volume than hydrated carbohydrates on a mass basis for storage in tissues. (iii) Lipids are more easily extracted and converted into useable biofuels than cellulosic-derived fuels, which require extensive fractionation, degradation of lignocellulose and fermentation of plant tissues. However, while vegetative organs such as leaves are the majority of harvestable biomass and would be ideal for accumulation of lipids, they have evolved as "source" tissues that are highly specialized for carbohydrate synthesis and export and do not have a propensity to accumulate lipid. Metabolism in leaves is directed mostly toward the synthesis and export of sucrose, and engineering strategies have been devised to divert the flow of photosynthetic carbon from sucrose, starch, lignocellulose, etc. toward the accumulation of triacylglycerols in non-seed, vegetative tissues for bioenergy applications.

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Contents

1. The flow of carbon to (and from) oil in leaves

Leaves capture and reduce carbon from the atmosphere via photosynthesis and provide this reduced carbon to rapidly growing 'sink' tissues and organs, some of which are heterotrophic. The majority of carbon reduced during the day is either exported from the leaves as sucrose or stored in the chloroplasts as starch. Starch is subsequently degraded producing sucrose that supports plant growth during the night [1–3]. A portion of the reduced carbon in leaves is also utilized for the local synthesis of all other organic molecules needed to support cellular activity, including lipids. Although leaf cells typically do not accumulate high amounts of storage lipids, unlike the common case in seeds, they do in fact synthesize small amounts of triacylglycerol, which is thought to serve as a transient storage depot for fatty acids prior to membrane lipid recycling or degradation in peroxisomes [4,5].

A general scheme for the flow of carbon to and from triacylglycerols in a typical mesophyll leaf cell of a C_3 plant is summarized in Fig. 1. Notably, a number of different subcellular organelles are involved in lipid synthesis and degradation. Compartmentation of the respective metabolic pathways helps ensure that carbon flux is directed toward specific lipid metabolites. Initially, fatty acids are

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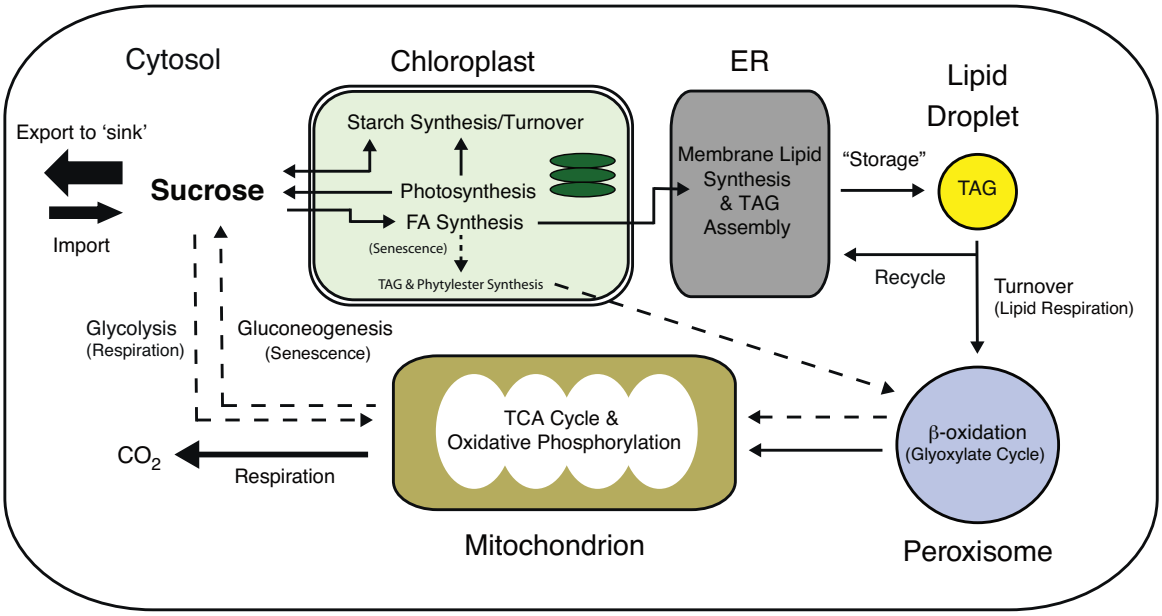

Fig. 1. General compartments and cellular pathways of carbon flow to and from stored lipids in a typical C_3 mesophyll cell. See text for details.

synthesized in two carbon increments by a type II, fully dissociable fatty acid synthase complex located in the chloroplast stroma, and they are subsequently delivered as mostly 16- or 18-carbon acyl chains to the endoplasmic reticulum (ER) for membrane lipid synthesis or triacylglycerol assembly [6]. Triacylglycerols are synthesized by enzymes that are believed to reside mostly on the ER membrane [7], after which the nascent triacylglycerols accumulate within the membrane bilayer and form lipid droplets that are deposited in the cytosol [5,8]. Eventually, fatty acyl groups are removed from triacylglycerols via the action of lipases and various lipid droplet-associated proteins, where they are either reused in the ER in membrane lipid synthesis or imported into peroxisomes and degraded by β-oxidation [9]. Citrate appears to be the primary product generated by leaf-type peroxisomes, except in the case of senescing leaves (or young seedlings), where glyoxylate cycle enzymes produce succinate [10] in some species. Regardless, citrate or succinate is either oxidized in the tricarboxylic acid cycle in mitochondria or diverted into the gluconeogenic pathway in the cytosol for eventual synthesis and export of sucrose, especially in senescing tissues [9,11].

2. Competition in the chloroplast between carbohydrate and fatty acid biosynthesis

Chloroplasts convert light energy to usable chemical energy and provide the plant with reduced carbon that supports life. As illustrated in Fig. 2, the fixation of inorganic carbon in the Calvin-Benson cycle within the chloroplast stroma is driven by reductant and ATP generated by photophosphorylation, and the net result is the formation of one molecule of 3-phosphoglycerate for every three molecules of CO_2 fixed. 3-Phosphoglycerate is reduced to triose phosphate, which has at least two major fates in leaf chloroplasts, including export to the cytosol for production of sucrose or utilization for starch synthesis within the stroma. Starch is mobilized at night generating primarily maltose and glucose, which are exported from the stroma to the cytosol for production of sucrose that is subsequently transported to sink tissues.

Fatty acid biosynthesis (and the biosynthesis of other organic molecules) also takes place within the chloroplast stroma, and this competes with carbohydrate synthesis for sources of reduced carbon (Fig. 2). Pyruvate, not acetate, is presumed to be the primary source of carbon for acetyl-CoA formation that feeds into fatty acid biosynthesis [12–14], with the plastidial pyruvate dehydrogenase complex and acetyl-CoA carboxylase considered to be rate-limiting, committed steps for the assembly of fatty acids by the fatty acid synthase complex [6]. Pyruvate appears to be derived primarily from phosphenolpyruvate that is likely imported into the chloroplast directly from the cytosol [15], since chloroplasts seem to lack one glycolytic enzyme to accomplish the conversion of 2-phosphoglycerate to phosphenolpyruvate [16], namely enolase (Fig. 2). It is also possible that pyruvate is transported directly into chloroplasts for fatty acid biosynthesis, but, as discussed below, evaluation of pyruvate transporters in the plastid envelope of plants other than C_4 plants, is still underway [17].

Fatty acids are exported from plastids mostly as 16- or 18-carbon acyl groups and are transported to the ER, by a mechanism that remains unclear, where the synthesis of membrane lipids and storage triacylglycerols occurs (Figs. 1 and 2). There is negative feedback regulation of acetyl-CoA carboxylase by products of fatty acid biosynthesis in the plastid [18] suggesting that the supply of fatty acids for lipid synthesis in leaf cells is tightly regulated. In other words, under normal plant growth and development, whole-plant demand for sucrose appears to command the utmost priority for reduced carbon, and excess carbon is maintained primarily in a transitory storage form of starch supplying soluble carbohydrates at night. Lipid synthesis appears to be controlled at the cellular/tissue level and to depend on the demand for membrane and other acyl lipids. In fact, only small amounts of storage lipids normally accumulate in mesophyll cells, despite the fact that all enzymes for triacylglycerol synthesis are present [7,19], reinforcing the concept that lipid storage is not a favored means of carbon/energy storage in vegetative tissues of plants.

The manipulation of competition between carbohydrate and lipid synthesis within the chloroplast probably depends on regulating multiple, coordinated processes, including the regulation of starch accumulation/turnover, the export of carbohydrate from the chloroplast stroma, the production of reducing equivalents, the synthesis and export of sucrose from the cytosol, and the biosynthesis of fatty acids within the stroma. For example, the combination of

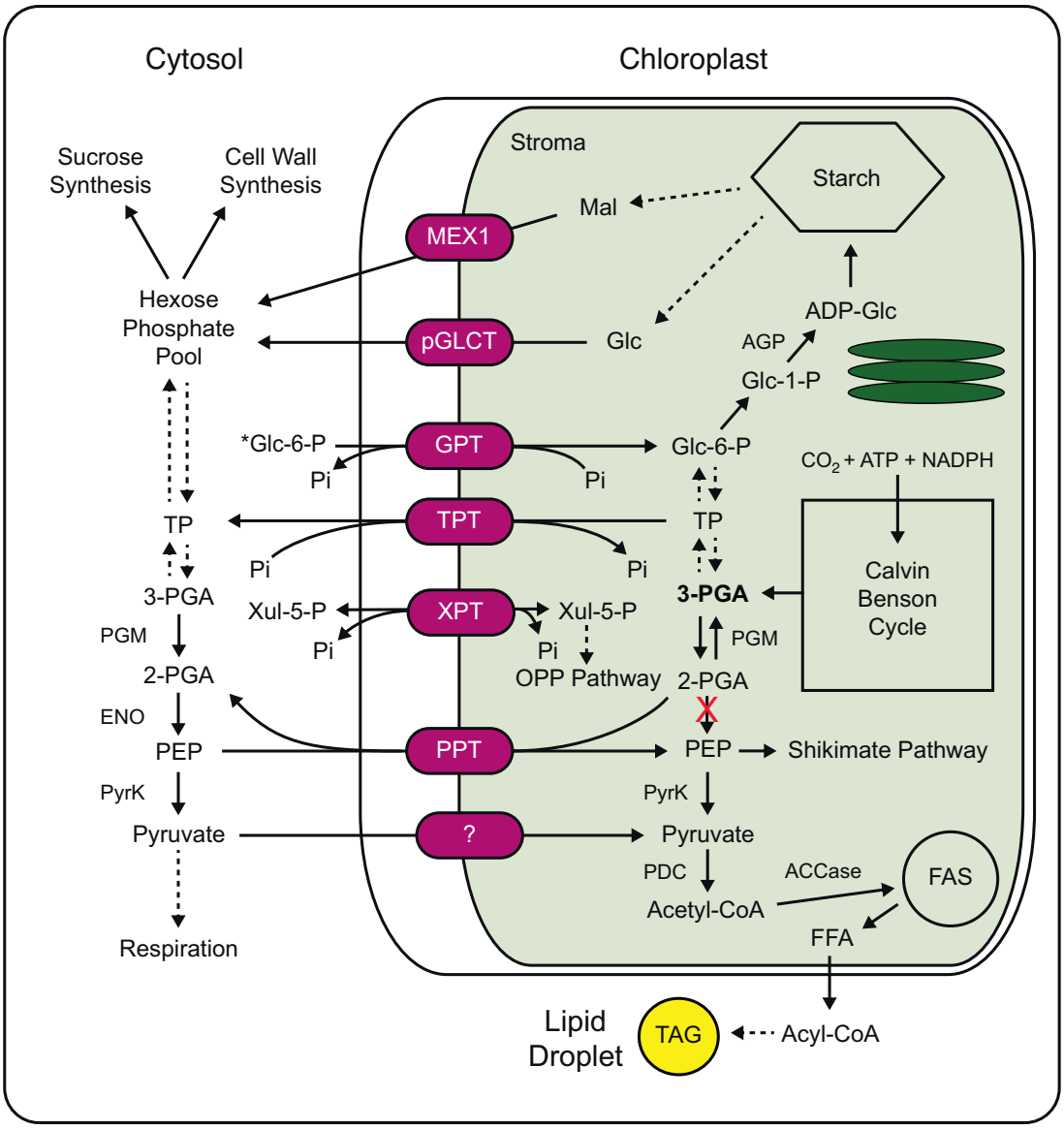

Fig. 2. Major paths for the transport of glycolytic intermediates into and out of chloroplasts during sucrose, starch and/or lipid synthesis and turnover in a leaf cell. See text for details. Asterisk denotes that the import (via GPT) of Glc-6-P from the cytosol into the stroma occurs mainly in heterotrophic tissues.

the up-regulation of the fatty acid biosynthesis pathway (through ectopic expression of the transcription factor *WRINKLED1*) and an inhibition of starch accumulation (through RNAi suppression of a subunit of ADP glucose pyrophosphorylase) resulted in a marked increase in triacylglycerol accumulation in cytosolic lipid droplets in leaves [20]. The tradeoff between starch accumulation and the amounts of stored triacylglycerol has been particularly well examined in developing seeds [21,22]. Developing seeds are a carbon sink where sucrose is imported and utilized. Sucrose made in leaves is mostly exported. Hence, the applications of metabolic concepts and engineering strategies in seeds versus leaves must consider these very different source-sink relationships.

3. Transporters at the chloroplast membrane

The chloroplast stroma and the cytosol have parallel glycolysis and gluconeogenesis pathways and there is considerable flux of metabolites through the outer and inner envelope that meets cellular, tissue and whole plant demands (Fig. 2). For instance, at least two types of "phosphate" translocators (antiporters) are relevant when one considers the integration between carbohydrate metabolism and fatty acid synthesis in leaves. The triose phosphate translocator (TPT), which transports triose phosphate to the cytosol, as well as Pi (and 3-phosphoglycerate) into the chloroplast, appears to operate mostly in support of sucrose synthesis. An interesting feature of chloroplasts is the lack of a complete glycolytic pathway, and as such, phosphenolpyruvate must be imported to satisfy several biosynthetic pathways inside the organelle [16]. The phosphenolpyruvate/phosphate translocator (PPT), which transports phosphenolpyruvate into chloroplasts and 2-phosphoglycerate (and Pi) to the cytosol, operates to support the synthesis of fatty acid (and isoprenoids and branched chain amino acids) inside the stroma [23].

In addition to the triose phosphate and phosphenolpyruvate/phosphate translocators, there is evidence for one or more pyruvate transporters in the chloroplast inner envelope, and these have mostly been associated with C_4 plants [15]. Molecular evidence suggests this pyruvate transporter family may be widespread among land plants [17], and while it remains to be determined what role, if any, pyruvate transporters might play in chloroplast fatty acid synthesis, this is one area that could provide some new strategies for channeling carbon into lipids in leaves.

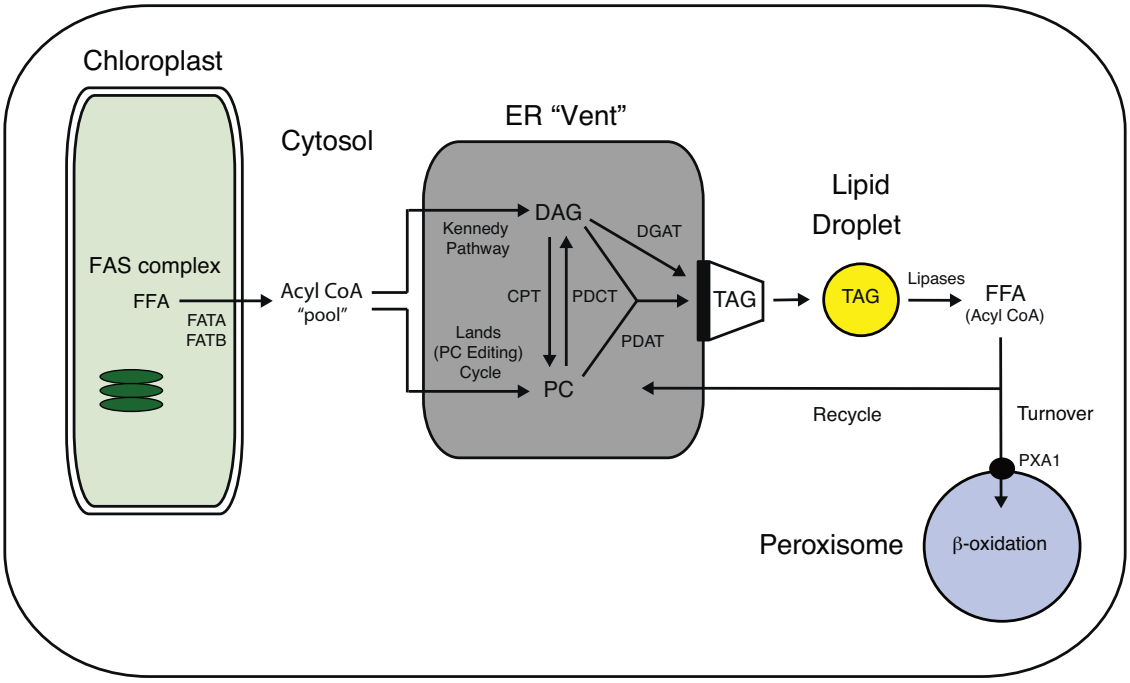

Fig. 3. General, simplified model for the cellular assembly and turnover of TAGs in a leaf cell. See text for details.

In terms of starch mobilization, nighttime needs for carbon are met largely through the turnover of transitory starch in the stroma and the subsequent export of maltose and glucose via the maltose excess protein 1-like/maltose exporter (MEX1) and the plastidic glucose translocator (pGLCT), respectively (Fig. 2). Other transporters in the plastid envelope include the glucose-6-phosphate/phosphate and xylulose-5-phosphate/phosphate translocators (GPT and XPT). GPT supplies glucose-6-phosphate for the oxidative pentose phosphate pathway and starch synthesis in heterotrophic tissues, while the role of the XPT is less clear [15]. The oxidative pentose phosphate pathway can also have an indirect impact on fatty acid biosynthesis in some non-green plastids by providing reducing equivalents to drive fatty acid synthesis [21,24]. In chloroplasts, it is likely that most of the required reducing power and ATP needed to drive fatty acid synthesis is provided by photosynthetic electron transport.

4. Triacylglycerol assembly in the ER: an oil "vent"?

Fatty acyl groups synthesized in the plastid stroma that are exported to the ER are released as free fatty acids by chain terminating fatty acyl ACP thioesterases (FATA and FATB) in the stroma (Fig. 3). These free fatty acids are subsequently converted to acyl-CoAs on the plastid outer envelope and exported to the cytosolic acyl-CoA pool where they are incorporated into membrane phospholipids or triacylglycerols. The synthesis of triacylglycerol was believed to proceed by the relatively straightforward Kennedy pathway, whereby fatty acyl groups are transferred to the *sn*-1 and *sn*-2 positions of glycerol-3-phosphate to eventually form diacylglycerol [25]. Diacylglycerol (DAG) can subsequently be interconverted to membrane phospholipids such as phosphatidylcholine (PC) by enzymes such as cholinephosphotransferase (CPT) and phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT), or esterified on the *sn*-3 position producing triacylglycerol by the enzyme diacylglycerol acyltransferase (DGAT) (Fig. 3). The pathways for membrane and storage lipid synthesis are far more complex, involving an acyl-editing pathway (also known as the Lands cycle), as well as an acyl-CoA independent pathway for triacylglycerol synthesis [26,27]. The latter reaction is catalyzed by the enzyme phospholipid:diacylglycerol acyltransferase (PDAT) (Fig. 3). Kinetic evidence suggests that much of the newly synthesized fatty acids are shuttled through phosphatidylcholine [28,29]. The fatty acyl groups may be modified while esterified to phosphatidylcholine (e.g., desaturation) or while they are in the acyl-CoA pool (e.g., elongation), but ultimately they are utilized in membrane or storage lipid synthesis. In developing seeds, it appears that phospholipid:diacylglycerol acyltransferase and diacylglycerol acyltransferase cooperate in a redundant fashion to synthesize triacylglycerols [30].

There is little information on the precise pathways that are involved in triacylglycerol synthesis in leaves. There may be a combination of pathways that are similar to or different from those in seeds [27,28,31], and these may be compartmentalized between chloroplasts and the cytosol. There is evidence that diacylglycerol acyltransferase is up-regulated during leaf senescence [32], presumably to convert membrane acyl lipids to triacylglycerol prior to mobilization and conversion to sucrose for export, but this was reported to be associated with chloroplast membranes. Indeed, triacylglycerols and phytylesters both accumulate dramatically inside chloroplasts during the normal progression of leaf senescence (Fig. 1), and this process appears to be mediated in large part by two plastidial phytyl ester synthases that scavenge fatty acids released from plastidial galactolipids [33]. It remains unclear how the diacylglycerol acyltransferase participates in the process of stress- or senescence-induced nonpolar lipid accumulation inside chloroplasts during senescence [32], and there is almost no information about the interaction between plastoglobule formation inside chloroplasts and triacylglycerol pools that accumulate in the cytosol of leaf cells during stress or senescence (Fig. 1).

Although the physiological significance of triacylglycerol accumulation in the cytosol of plant leaves is presently unclear, one possibility is that the ER acts as a cellular "vent" for excess acyl groups [34], and these acyl groups can be most easily and stably sequestered as triacylglycerols, partitioned first between the two bilayer leaflets, and then as nascent lipid droplets in the cytosol [7,8]. Depending upon cellular needs, the acyl groups stored

in triacylglycerols may be recycled back into membrane lipids or metabolized (via β-oxidation) in peroxisomes (Fig. 3). While this is an intriguing hypothesis, there is circumstantial evidence to support that triacylglycerol formation in leaves does indeed serve as "buffer" for the overflow of cellular acyl lipids. It is clear that up-regulating triacylglycerol formation can lead to increases in cytosolic lipid droplets in leaves [35,36]. Similarly, interfering with the normal breakdown of fatty acids in leaves, which results in an elevation of fatty acid content, also results in an increase in cytosolic lipid droplets and triacylglycerol content [37–39]. A soluble acyltransferase (named DGAT3) was recently identified in Arabidopsis mutants defective in acyl turnover [40], suggesting that a recycling pathway exists in plant cells that "scavenges" excess acyl-CoAs into triacylglycerol. This DGAT3 is homologous to another soluble DGAT identified previously in peanut [41], and indicates that much more remains to be understood about the lipid metabolic pathways that synthesize, recycle and/or turnover triacylglycerols in plant cells.

A model for the ER as an oil "vent" is not too difficult to envision and this general scheme for triacylglycerol synthesis in the ER might have been exaggerated during plant evolution and specialized (with specific subcellular machinery) allowing oil storage in certain tissue types such as those in oilseeds or oleaginous fruits. In fact, recent transcriptome profiling of fruit tissues from two related palm species [42], oil palm that accumulates large amounts of triacylglycerols, and date palm that accumulates large amounts of soluble sugars, revealed that there was little-to-no difference in the abundance of transcripts encoding the ER acyltranferase machinery (i.e., PDATs or DGATs). Oil palm had markedly elevated transcript abundances compared to date palm of plastidial transporters and all enzymes directing carbon to and through fatty acid biosynthesis [42]. In other words, the ER machinery in oil palm appears to have adequate acyltransferase capacity for triacylglycerol assembly to accommodate up-regulated fatty acid synthesis of 100-fold more than date palm. Hence, the acyltransferase capacity helps to facilitate the "venting" of large amounts of triacylglycerol into lipid droplets into the cytosol of mesocarp cells (triacylglycerol accumulates to 88% dry weight in oil palm versus less than 1% in date palm). Certainly there are proteins yet to be identified that contribute to lipid droplet packaging (since oleosins are not expressed in most non-seed tissues), and these mechanisms will need to be considered when attempting to engineer large amounts of triacylglycerol in leaf tissues. In addition, redirecting carbon flux from carbohydrate to fatty acid synthesis in leaves must consider that leaves are source tissues that are specialized in carbon production and export, unlike seeds or oleaginous fruits that are sink tissues specialized in efficient carbon import and utilization.

5. Triacylglycerol turnover in leaves

Most of the models for the turnover of triacylglycerols in higher plants come from studies of triacylglycerol mobilization in germinated oilseeds [8], but this may be inadequate to explain the normal turnover of triacylglycerols in cells of vegetative tissues such as leaves [19]. It is generally believed that the hydrolysis of triacylglycerols in cytosolic lipid droplets produces free fatty acids, and these free fatty acids are transported into peroxisomes (either as acyl-CoAs or as free fatty acids) by the peroxisomal ABC transporter 1 (PXA1), where they are ultimately metabolized to acetyl-CoA by the β-oxidation pathway [9] (Fig. 3). In germinated seeds and in senescing leaf tissues, the glyoxylate cycle enzymes, malate synthase and isocitrate lyase, participate in the conversion of acetyl-CoA into organic acids that are exported from the peroxisome and metabolized in the cytosol and mitochondria and support gluconeogenesis and sucrose synthesis (Fig. 1). Conversely, mesophyll leaf cells that are not senescing appear to utilize acetyl-CoA derived from β-oxidation for citrate synthesis. The cells then use this as a source of respiratory carbon in the mitochondrial tricarboxylic acid cycle, especially in lipid respiration at night [23]. The extent of this respiratory pathway in plant leaves is not well understood, nor are the dynamics of acyl recycling from triacylglycerol stores into membrane lipid synthesis at the ER, which was only revealed recently in mutants with defects in β-oxidation of fatty acids [40]. Consequently, the efficient and stable engineering of elevated triacylglycerol content in leaf tissues requires a better understanding of the function and regulation of triacylglycerol turnover.

6. What factors support the diversion of carbon to oil?

Systems-style approaches will likely accelerate the pace at which regulatory clues for triacylglycerol synthesis may be gleaned. For example, recent comparisons of the transcriptomes between oil-rich and oil-poor palm species (e.g., oil date palms) have helped to identify candidate transcription factors related to *WRINKLED1* that may globally up-regulate triacylglycerol synthesis machinery in this non-seed tissue [42]. Others made comparisons of proteomes of isolated "embryoplasts" with isolated chloroplasts in *Brassica napus* and identified qualitative and quantitative differences in fatty acid biosynthetic and glycolytic enzymes in these two different types of plastids [43]. Elsewhere, comparative proteomics of chloroplasts from C_4 leaves revealed a relative spatial separation of lipid synthesis and starch synthesis between mesophyll and bundle sheath cell plastids [44]. Transcriptomics have been used to identify new candidate transporters that may be relevant in pyruvate metabolism and fatty acid biosynthesis in maize leaves [45]. Metabolic flux analysis of developing seeds indicates that changes in glycolytic flux, as well as plastidial pyruvate kinase and pyruvate dehydrogenase, are most important for regulating triacylglycerol accumulation [46,47]. These latter findings are consistent with other data from the literature pointing to these steps as key to influencing triacylglycerol accumulation in oil-rich tissues, but it remains to be seen how this process actually operates to influence fatty acid synthesis in leaf chloroplasts.

It is unlikely that a simple "transplantation" of processes that operate in sink tissues such as seeds and fruits will be entirely satisfactory to divert carbon to oil in leaves. Plant leaves do not normally accumulate triacylglycerols. Leaves are genetically programmed to synthesize and export carbohydrates that support overall plant growth and development. Instead, the synthesis of lipids in leaves appears to be used in cellular maintenance of source tissues rather than in general energy storage. Nonetheless, the accumulation of 10% oil (by weight) in leaves of an energy crop such as switchgrass is estimated to result in a 30% increase in usable liquid fuel energy [7] Hence, there has been considerable enthusiasm to elevate lipid content in plant leaves, and while results are often short of 10% triacylglycerol on a dry weight basis, increases in triacylglycerol in leaves have been possible (see below), implying that strategies for altering carbon partitioning in favor of triacylglycerol accumulation in leaves should be pursued. Still, success is likely to depend upon a more broad and careful consideration of prevailing carbohydrate metabolism and transport mechanisms in leaf cells.

7. Strategies for the accumulation of triacylglycerol in leaves

Lipid content in plant vegetative tissues has been elevated by several groups. For instance, a general up-regulation of lipid synthesis by ectopic expression of *WRINKLED1* showed an additive effect in triacylglycerol and cytosolic lipid droplet accumulation when *WRINKLED1* expression was combined with suppression of starch synthesis [20]. Further increases in lipid accumulation might be

achieved by a reduction in sucrose export from cells, perhaps by reducing the activity of sucrose transporters or by suppressing the demand for carbon by a specific 'sink', such as developing flowers.

Blocking the breakdown of fatty acids can also increase triacylglycerol content in leaves. Mutant plants defective in the peroxisomal transporter PXA1, which is responsible for the uptake of fatty acids into peroxisomes for β-oxidation (Fig. 3), accumulated significant amounts of triacylglycerols in leaves and this phenotype was exacerbated with extended dark treatment [38]. Extended dark treatment also ultimately led to necrosis, perhaps from the accumulation of free linolenic acid. The accumulation of more triacylglycerol in extended darkness is explained by increased demand for respiratory carbon being met by mobilization of esterified fatty acids; however, since fatty acids cannot be taken up by peroxisomes for further oxidation, these acyl groups accumulate in triacylglycerol in leaves, especially in extended darkness where transitory starch has been depleted. Likewise, an elevation of triacylglycerol in leaves of plants that were defective in peroxisomal β-oxidation also was demonstrated [37] indicating a block in the utilization of fatty acids for respiratory carbon results in accumulation of triacylglycerol. The ectopic expression of the seed transcription factor, *LEAFY COTYLEDON 2* (*LEC2*) induced triacylglycerol formation in leaves, including triacylglycerols containing fatty acids that are typically found in seed oil [37]. Combining the β-oxidation mutant with ectopic expression of *LEC2* resulted in an accumulation of triacylglycerol content that was similar to the total amount of triacylglycerol observed in either parental line alone, suggesting that the amount of triacylglycerol produced was limited at least in part by carbon partitioning into the fatty acid biosynthetic pathway.

One important concern with elevating triacylglycerol accumulation in leaves is the potential for negative impacts on plant growth and development. For example, seedling establishment of *pxa1* mutants is sucrose dependent [48], and ectopic expression of seed-specific transcription factors such as LEC2 resulted in abnormal growth [49]. The negative effects of *LEC2* expression in leaves were successfully circumvented by placing it under control of an inducible promoter [36]. This facilitated an increase of triacylglycerol in tobacco leaves by more than 20-fold following induction [36]. Another approach that led to increased accumulation of triacylglycerol-containing lipid droplets in leaves, but did not appear to compromise growth and development, was the disruption in a homologue of the mammalian Comparative Gene Identifier-58 (CGI-58) in Arabidopsis plants [39]. CGI-58 activates lipolysis in animal tissues, and although its function in plants is not entirely clear, it is postulated to have a role in regulating lipid turnover in vegetative tissues [39].

Other strategies to increase triacylglycerol levels in leaves have focused on enhancing acyltransferase activity and boost overall triacylglycerol synthesis in leaf cells. For instance, increasing the expression of DGAT in tobacco leaves resulted in a marked increase in leaf triacylglycerol content [36]. Similarly, the heterologous expression of an algal diacylglycerol acyltransferase resulted in altered carbon partitioning in leaves toward the synthesis of a broad range of triacylglycerols with different chain lengths [50]. A new approach to introduce an acyltransferase-mediated pathway into tobacco leaves that utilizes monoacylglycerol for channeling into the synthesis of triacylglycerols has been reported [35]. This pathway modification resulted in up to a 7-fold increase in leaf triacylglycerol content in stably-transformed lines and may have promise in combination with other strategies that channel carbon to lipid.

Perhaps one of the more obvious ways to alter leaf metabolism in favor of oil accumulation would be to restore a complete functional glycolytic pathway in the chloroplast such that the 3-phosphoglycerate produced from photosynthesis could be converted to phosphenolpyruvate in the stroma. This would directly connect carbon fixation to fatty acid biosynthesis within the same organelle. It is conceivable that the loss of the plastidial glycolytic enolase enzyme in green tissues during evolution conferred a strong growth advantage to early photosynthetic organisms, since more of the photosynthate captured by the chloroplast would be available for export and utilization in supporting growth, rather than being incorporated directly into fatty acid and/or other molecules within the chloroplast.

Restoration of the glycolytic pathway in chloroplasts might also come with some significant challenges. For instance, the diversion of photosynthate to fatty acid would likely reduce overall carbon availability supporting plant growth, as is observed in plant mutants that are engineered to accumulate higher amounts of starch in the stroma [1]. Using inducible or senescence-specific promoters that support the temporal regulation of starch accumulation resulted in a 7- to 20-fold increase in starch content without any apparent effects on overall plant biomass [1]. Thus, similar approaches might be used for obtaining oil accumulation in leaves at later stages of the plant life cycle by using inducible or senescence-specific promoters to drive expression of the missing glycolytic gene.

Finally, relatively little is known about the assembly, regulation and turnover of the organelles that store triacylglycerol in vegetative cells. It is generally believed that lipid droplets in green tissues are dynamic organelles that are involved in transient triacylglycerol storage, lipid signaling and/or lipid turnover [5,11]. As such, any attempts to increase triacylglycerol content might need to be coupled with complementary approaches to "stabilize" the triacylglycerol within lipid droplets, removing them from access to turnover machinery. One possibility is to express oleosin proteins or other structural proteins to coat the surface of the lipid droplet [51], thereby reducing availability of the triacylglycerol to catabolic enzymes such as lipases. While some might suspect that oleosins could act as recognition sites for lipases for triacylglycerol hydrolysis [8], this remains to be tested in leaf tissues. Alternatively, biogenetic mechanisms might be upregulated to cause proliferation of lipid droplets in leaves, which could provide greater "sink capacity" within cells allowing triacylglycerol deposition. As more is learned about lipid droplet biogenesis and regulation in leaves, there will undoubtedly be new opportunities to couple organelle biogenesis with enhanced triacylglycerol synthesis, and accomplish the end-goal of producing high amounts of energy-dense oils that can be recovered for a multitude of purposes including biofuels.

8. Conclusions and perspectives

There appear to be a number of possibilities for enhancing the lipid content of vegetative tissues. Success will require informed strategies that not only consider the metabolic pathways leading to the synthesis and turnover of triacylglycerol and their transcription or biochemical control, but also an appreciation of overall plant physiology. Tissue source-sink relationships and the specific integration of carbohydrate synthesis with lipid metabolism in leaf (source) tissues will be important to address. Hence, insights from single cell systems (like microalgae) or oil-rich sink tissues like seeds, while informative, may need to be extrapolated to leaf-based systems with caution.

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