

HARNESSING PLANT BIOMASS FOR BIOFUELS AND BIOMATERIALS

Plant triacylglycerols as feedstocks for the production of biofuels

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Received 5 November 2007; revised 25 January 2008; accepted 30 January 2008.

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Summary

Triacylglycerols produced by plants are one of the most energy-rich and abundant forms of reduced carbon available from nature. Given their chemical similarities, plant oils represent a logical substitute for conventional diesel, a non-renewable energy source. However, as plant oils are too viscous for use in modern diesel engines, they are converted to fatty acid esters. The resulting fuel is commonly referred to as biodiesel, and offers many advantages over conventional diesel. Chief among these is that biodiesel is derived from renewable sources. In addition, the production and subsequent consumption of biodiesel results in less greenhouse gas emission compared to conventional diesel. However, the widespread adoption of biodiesel faces a number of challenges. The biggest of these is a limited supply of biodiesel feedstocks. Thus, plant oil production needs to be greatly increased for biodiesel to replace a major proportion of the current and future fuel needs of the world. An increased understanding of how plants synthesize fatty acids and triacylglycerols will ultimately allow the development of novel energy crops. For example, knowledge of the regulation of oil synthesis has suggested ways to produce triacylglycerols in abundant non-seed tissues. Additionally, biodiesel has poor cold-temperature performance and low oxidative stability. Improving the fuel characteristics of biodiesel can be achieved by altering the fatty acid composition. In this regard, the generation of transgenic soybean lines with high oleic acid content represents one way in which plant biotechnology has already contributed to the improvement of biodiesel.

Keywords: biodiesel, triacylglycerol, oilseeds, fatty acid, bioenergy.

Introduction

An increased necessity for energy independence and heightened concern about the effects of increasing carbon dioxide levels have intensified the search for renewable fuels that could reduce our current consumption of fossil fuels. One such fuel is biodiesel, which consists of the methyl esters of fatty acids, usually derived from plant oils, although other sources including animal fat are possible. Plant oils are primarily composed of various triacylglycerols (TAGs), molecules that consist of three fatty acid chains (usually 18 or 16 carbons long) esterified to glycerol (Figure 1a). The fatty acyl chains are chemically similar to the aliphatic hydrocarbons that make up the bulk of the molecules found in petrol (also called gasoline) and diesel (Figure 1c). The hydrocarbons in petrol contain between 5

and 12 carbon atoms per molecule, and this volatile fuel is mixed with air and ignited with a spark in a conventional engine. In contrast, diesel fuel components typically have 10–15 carbon atoms per molecule and are ignited by the very high compression obtained in a diesel engine. Early demonstration versions of the diesel engine were designed to run on peanut oil, reflecting the fact that plant-derived triacylglycerols and petroleum fuels are chemically similar, with structures consisting largely of chains of reduced carbons. However, most plant TAGs have a viscosity range that is much higher than that of conventional diesel: 17.3–32.9 mm² s⁻¹ compared to 1.9–4.1 mm² s⁻¹, respectively (ASTM D975; Knothe and Steidley, 2005). This higher viscosity results in poor fuel atomization in modern diesel

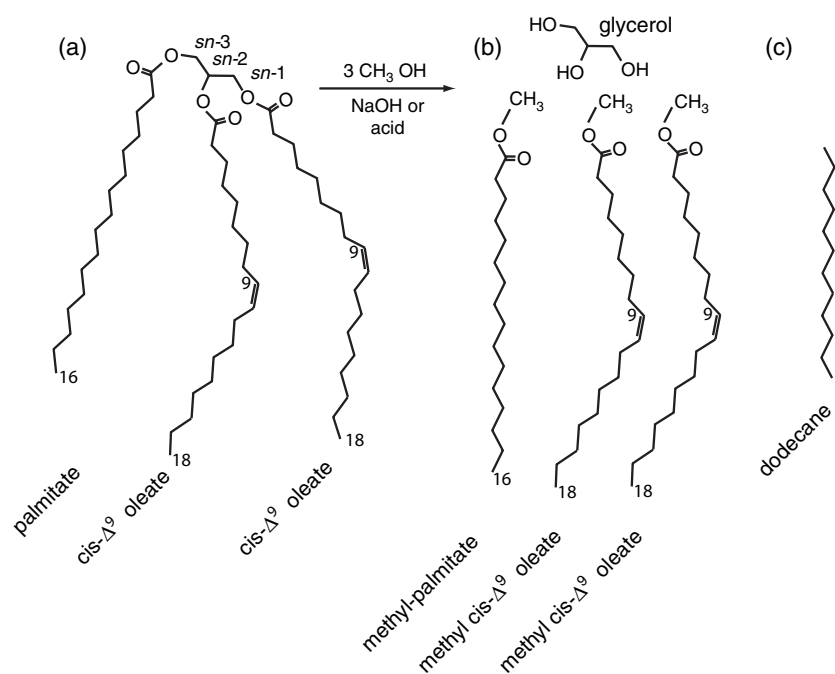


Figure 1. The production of biodiesel from triacylglycerols via esterification with methanol. Triacylglycerol (a) is converted to glycerol and fatty acid methyl esters (b) by reaction with methanol in the presence of an acid or alkali catalyst. Also shown is dodecane (c), a representative hydrocarbon found in conventional diesel, illustrating the similarities in chemical structure.

engines, leading to problems derived from incomplete combustion such as carbon deposition and coking (Ryan *et al.*, 1984). To overcome this problem, TAGs are converted to less viscous fatty acid esters by esterification with a primary alcohol, most commonly methanol (Figure 1b). The resulting fuel is commonly referred to as biodiesel and has a dynamic viscosity range from 1.9 to 6.0 $\text{mm}^2 \text{ s}^{-1}$ (ASTM D6751). The fatty acid methyl esters (FAMES) found in biodiesel have a high energy density as reflected by their high heat of combustion, which is similar, if not greater, than that of conventional diesel (Figure 2; Knothe, 2005). Similarly, the cetane number (a measure of diesel ignition quality) of the FAMES found in biodiesel exceeds that of conventional diesel (Knothe, 2005).

However, some obvious differences exist between the molecules found in conventional diesel and those in biodiesel, which have an impact on the properties of the fuels. For example, biodiesel FAMES contain two oxygen atoms per molecule and often one or more double bonds (depending on the triacylglycerol from which they were derived), whereas the hydrocarbons in conventional diesel tend to be saturated.

Advantages of biodiesel

The higher oxygenated state compared to conventional diesel leads to lower carbon monoxide (CO) production and reduced emission of particulate matter (Graboski and McCormick, 1998). This latter air pollutant is especially problematic in European cities, motivating temporary curfews for diesel-powered vehicles. Biodiesel also contains little or no sulfur or aromatic compounds; in conventional

diesel, the former contributes to the formation of sulfur oxide and sulfuric acid, while the aromatic compounds also increase particulate emissions and are considered carcinogens. In addition to the reduced CO and particulate emissions, the use of biodiesel confers additional advantages, including a higher flashpoint, faster biodegradation and greater lubricity. The higher flashpoint of biodiesel allows safer handling and storage, whereas the biodegradability of biodiesel is particularly advantageous in environmentally sensitive areas where fuel leakage poses large hazards. The lubricity issue has become increasingly important with the widespread mandated adoption of low-sulfur diesel fuels. The elimination of sulfur-containing compounds from conventional diesel also removes the fuel constituents that contribute to the inherent lubricity of the fuel. Biodiesel has greater lubricity than conventional diesel, and blending biodiesel with low-sulfur fuel restores lubricity (Knothe *et al.*, 2005).

However, the biggest advantage to using biodiesel, especially given today's environmental and political concerns, is that biodiesel in principle is a sustainable source of liquid transportation fuels and is essentially neutral with respect to the production of carbon dioxide. This is because the energy contained within the reduced hydrocarbon chains of biodiesel is ultimately derived from the sun: plants capture solar energy using photosynthesis, converting carbon dioxide and water to the sugars from which TAGs are derived. Even when taking into account other inputs such as fertilizer and energy for transportation and conversion, biodiesel returns almost double the energy used for its production; its subsequent combustion in place of conventional diesel reduces greenhouse gas emissions by 40% (Hill *et al.*, 2006).

Given the increase in crude oil prices and the introduction of various subsidies, the production of biodiesel in both the EU and the USA has expanded dramatically in recent years. From 2002 to 2006, the production of biodiesel increased 15-fold in the USA, and almost fivefold in the EU (Figure 3a). Despite the relatively large recent increase in production in the USA, European production is still at least six times greater than that in the USA: in 2006, Europe produced approximately 5.6 billion liters of biodiesel compared to 0.86 billion liters produced in the USA. Consequently, the consumption of canola, soybean, palm and other oil crops for biodiesel has grown too. In the USA, it is estimated that approximately 22% of domestic soybean oil production by 2016 will be devoted to biodiesel (US Department of Agriculture, 2007a). The rapid expansion of EU and US plant oil consumption as a feedstock for biodiesel production has played a major role in achieving record high prices for plant oils. For example, soybean oil prices doubled from 2001 to 2007 (Figure 4a).

Of the two major renewable liquid fuels in current use, ethanol and biodiesel, the latter has several advantages over ethanol as a liquid fuel. First, biodiesel has a 25% higher energy content per volume, which translates directly into greater fuel economy (Figure 2). Second, ethanol can lead to corrosion of pipelines and therefore must be stored separately from petrol/gasoline and mixed before use. Third, a

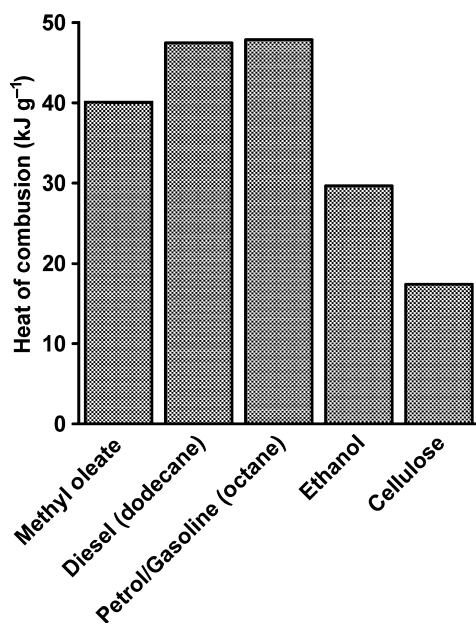


Figure 2. Heat of combustion values for biodiesel and conventional diesel. Values shown are heat of combustion values (in kJ g^{-1}) for representative molecules found in biodiesel (methyl oleate), diesel (dodecane) and petrol/gasoline (octane). For comparison, the values for ethanol and cellulose, other plant-derived sources of energy, are also shown. Values are taken from the CRC Handbook of Chemistry and Physics (2008) and Domalski *et al.* (1987).

fermentation step is required for the conversion of carbohydrate to ethanol, which is then followed by substantial energy inputs to distil ethanol from water. In contrast, plant oils can be extracted and converted to biodiesel by processes that require comparatively low energy. These and other factors result in a 'net energy balance ratio' for biodiesel production that is three to four times more favorable for biodiesel from soybean than for ethanol from maize (Hill *et al.*, 2006). The net energy balance ratio is the energy content of the fuel divided by the agricultural and production energy requirements.

Limitations of biodiesel

Despite the large increase in its use, biodiesel still represents a small percentage of total diesel consumption. For example, in 2005, biodiesel contributed only about 1.6% of the EU diesel fuel consumption (Commission of the European Communities, 2007) and just over 0.21% of that in the USA (US Department of Energy, 2007). For comparison, ethanol provided approximately 5% of US gasoline consumption in 2007. A number of factors, conceptually grouped into two areas, have contributed to the limited adoption of biodiesel. The first group consists of problems with the fuel characteristics of biodiesel, namely poor cold-temperature properties, higher rates of oxidation and increased emission of nitrogen oxides (NO_x) relative to conventional diesel. The second, and more significant, group revolves around the inter-twined factors of cost and supply limitations. The biggest factor affecting the cost of biodiesel production is the price of the input triacylglycerol. For example, in the USA, it has been estimated that the soybean oil input alone contributes 88% of the total production costs of biodiesel (Haas *et al.*, 2006). Glycerol produced during trans-esterification of

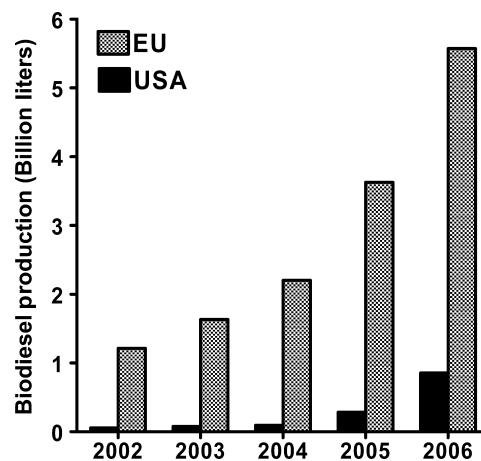


Figure 3. Historical biodiesel production figures. Biodiesel production in the USA and the EU. Data were obtained from the National Biodiesel Board (<http://www.biodiesel.org>) and the European Biodiesel Board (<http://www.ebb-eu.org>) and converted to liters.

plant triacylglycerols has been a valuable side-product that has reduced the overall production cost of biodiesel. Recently, however, the large increases in biodiesel production have caused an excess of glycerol supply over demand, lowering the value of this by-product. Increasing use of glycerol, for example as a fermentation feedstock, will assist the economic viability of biodiesel production.

One of the reasons for the high price of the raw material input is competition from other commercial sectors, such as the food industry, for the use of plant oils. Historically, the low cost of crude oil meant that conventional diesel could be produced at less expense than biodiesel. The recent increases in crude oil prices relative to plant oil prices (Figure 4b), combined with government tax policies or incentives, have greatly increased the economic competitiveness of biodiesel, leading to an increased production of this renewable fuel (Figure 3a). However, even if biodiesel remains economically competitive, the current limited worldwide supply of plant oils prevents biodiesel from replacing conventional diesel to a major extent. Some theoretical examples illustrate the vast amount of plant oil production necessary to replace conventional diesel. Converting the entire 2005 USA soybean crop to biodiesel would replace only 10% of conventional diesel consumed. Even the total world plant oil production of 2005 (approximately 120 million metric tons) would only satisfy approximately 80% of USA diesel demand (US Department of Agriculture, 2007b; US Department of Energy, 2007). As discussed previously, devoting a greater proportion of plant oils for the production of biodiesel has already contributed to higher vegetable oil prices (Figure 4a), not only making biodiesel production more expensive but also having an impact on other sectors of the economy, such as food prices.

Biofuels ultimately derive their energy from the sun through photosynthesis; however, one limiting factor to this process is land on which to grow the appropriate crops. Additionally, in some parts of the world, water for irrigation is also scarce. Diverting land and water to higher-yielding energy crops is one, albeit highly simplistic, approach to increase world plant oil production. For example, soybeans are the second biggest source of plant oil in the world, providing 29% of 2005 production total, yet are one of the less efficient crops in terms of oil yield per hectare (Figure 5). Canola (rape) produces at least double the oil per hectare compared with soybean, 1190 l ha⁻¹ compared to 446 l ha⁻¹. Thus, replacing soy with canola could significantly increase plant oil production. In reality, however, such a scenario is unlikely, given not only problems with fungibility but also economic and environmental factors. Historically, soybeans have been grown primarily for their protein yield, and their replacement would greatly affect the supply of inexpensive protein used for animal feed. Additionally, unlike most other oilseed crops, soybean fixes atmospheric nitrogen

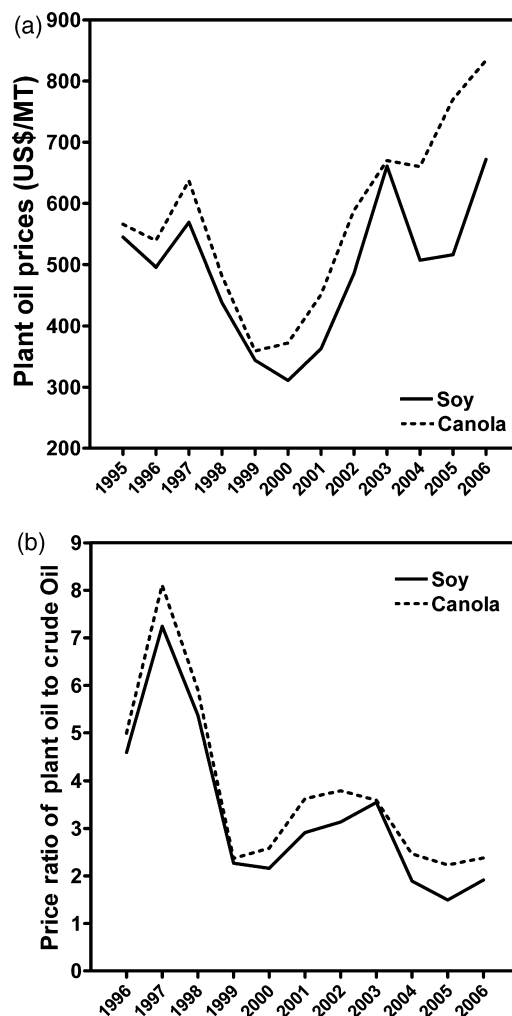


Figure 4. Plant oil prices.

(a) Average oil prices in US\$ per tonne for soybean and canola oils. Dates represent crop years; for example, 1995 represents October 1995 to September 1996. Data are taken from US Department of Agriculture (2007b). (b) Plant oil prices expressed as a ratio of crude oil prices. The cost of purchasing soybean or canola oil containing an equivalent amount of energy to a barrel of crude oil was divided by the average weekly price of a barrel of crude oil. The crude oil prices for crop years representing October to September were averaged. Data were obtained from the Energy Information Administration (http://tonto.eia.doe.gov/dnav/pet/pet_pri_wco_k_w.htm).

(Kinney and Clemente, 2005). Thus biodiesel derived from soybean requires less fertilizer input, reducing its environmental impact. Of all the major oil crops, the oil palm produces the greatest energy per hectare, particularly when net energy is considered (Figure 5). However, oil palm has a limited range of suitable climate conditions, although efforts to expand oil palm growth in Africa and South America and to breed oil palms adapted to drier or cooler climates are underway.

The vast amount of plant oil needed to replace the world's current and future fuel needs creates additional questions. As implied earlier, the conversion of plant oils to biodiesel

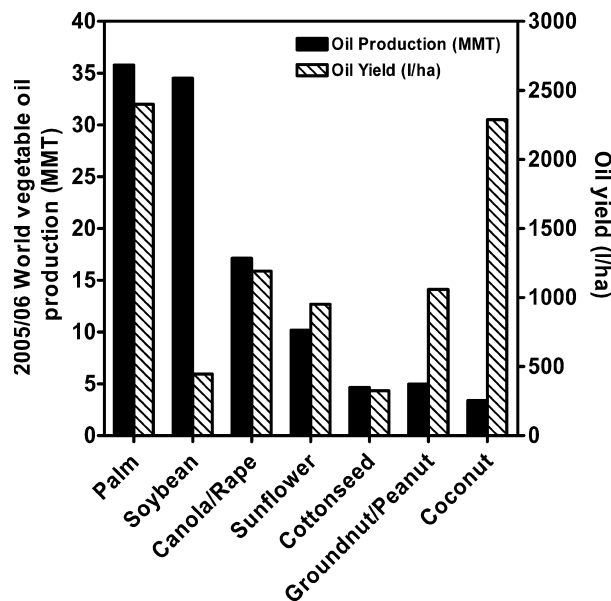


Figure 5. World oil crop production.

The total worldwide oil production (million tonnes) in 2005/2006 for the major oil crops is represented by solid bars. The average oil yield (l ha^{-1}) for each crop is also shown (hatched bars). It is important to note that yield numbers are subject to considerable geographic and temporal variation. Additionally, in the cases of palm and coconut, yield numbers represent production from mature plants and do not reflect periods of lower production during establishment of the oil plantation. Production values are taken from US Department of Energy (2007) and US Department of Agriculture (2007b); yield data are from <http://www.journeytoforever.com> and Fairless (2007).

competes with their use as food. Great care will therefore need to be taken to ensure that a large increase in biodiesel production does not affect the supply of food, especially to the world's poorest citizens. Additionally, the large-scale cultivation of oil crops should ideally occur with minimal environmental impact. The increased use of biodiesel, particularly in Europe, has led to higher amounts of palm oil being imported from south-east Asia. Unfortunately, in order to meet this demand, the expansion of palm plantations has resulted in tropical deforestation. As large-scale biodiesel use grows even further, it will be imperative to expand production without such destruction of natural ecosystems. Equally important will be the implementation of efficient farming practices to maintain soil fertility and minimize the use of increasingly valuable inputs such as fertilizer and water.

One way to increase world plant oil production without the ecosystem destruction involved by expanding crop land is to use marginal or non-arable wasteland. The tropical shrub *Jatropha curcas* is drought-resistant and is capable of growing on land that is unsuitable for other crops (Gubitz *et al.*, 1999). *Jatropha* has been reported to produce large amounts of oil, as much as 1300 l ha^{-1} (Fairless, 2007), but large-scale production may require the development of mechanized harvest and processing technologies.

Tall oil, a by-product from the paper and pulp industry, represents another potential source of TAG for the production of biodiesel. For example, approximately 700 million liters of turpentine and tall oil are produced each year in the USA (Ragauskas *et al.*, 2006). The production of biodiesel from TAGs derived from algae is another possibility, and is described further by Hu *et al.* (2008).

The following sections of this review will discuss ways in which plant biology can address some of the major limitations of biodiesel. Specifically, approaches to increase the production of plant oils and to improve the fuel properties of biodiesel will be described. Additionally, ideas regarding the use of plant oils directly as fuel will be discussed. Before addressing these approaches, a summary of fatty acid and TAG synthesis is presented.

Fatty acid and triacylglycerol production in plants

Most plant oils are derived from triacylglycerols stored in seeds. During seed development, photosynthate from the mother plant is imported in the form of sugars, and the seed converts these into precursors of fatty acid biosynthesis. Glycolysis plays a central role in this conversion (Figure 6). The pyruvate produced by glycolysis is converted to acetyl CoA by the plastidic pyruvate dehydrogenase complex. As there are glycolytic enzyme isoforms in the plastid and the cytosol (Plaxton and Podesta, 2006), it is not entirely clear which pathway is preferred in oil seeds. However, based on EST and microarray analysis in developing *Arabidopsis* seeds (Ruuska *et al.*, 2002; White *et al.*, 2000) and the analysis of mutants inactivated in plastid pyruvate kinase (Andre *et al.*, 2007; Baud *et al.*, 2007b), it seems likely that at least the later reactions of glycolysis relevant to oil biosynthesis proceed in the plastid (Figure 6). In green seeds, a recently discovered Rubisco bypass of the upper part of glycolysis in plastids provides higher carbon-use efficiency and allows re-fixation of CO_2 formed by the plastid pyruvate dehydrogenase complex (Schwender *et al.*, 2004).

Acetyl CoA produced in plastids from pyruvate is activated to malonyl CoA; the malonyl group is subsequently transferred to acyl carrier protein (ACP) giving rise to malonyl ACP, the primary substrate of the fatty acid synthase complex. The formation of malonyl CoA is the committed step in fatty acid synthesis and is catalyzed by the highly regulated plastidic acetyl CoA carboxylase complex (Nikolau *et al.*, 2003).

Plants contain a type II fatty acid synthase complex in plastids that is similar to that found in bacteria (Ohlrogge and Jaworski, 1997; White *et al.*, 2005). *De novo* fatty acid synthesis in the plastids proceeds by a repeated series of condensation, reduction and dehydration reactions that add two carbon units derived from malonyl ACP to the elongating fatty acid chain, which stays conjugated to ACP during this process. In this manner, fatty acid chains containing up

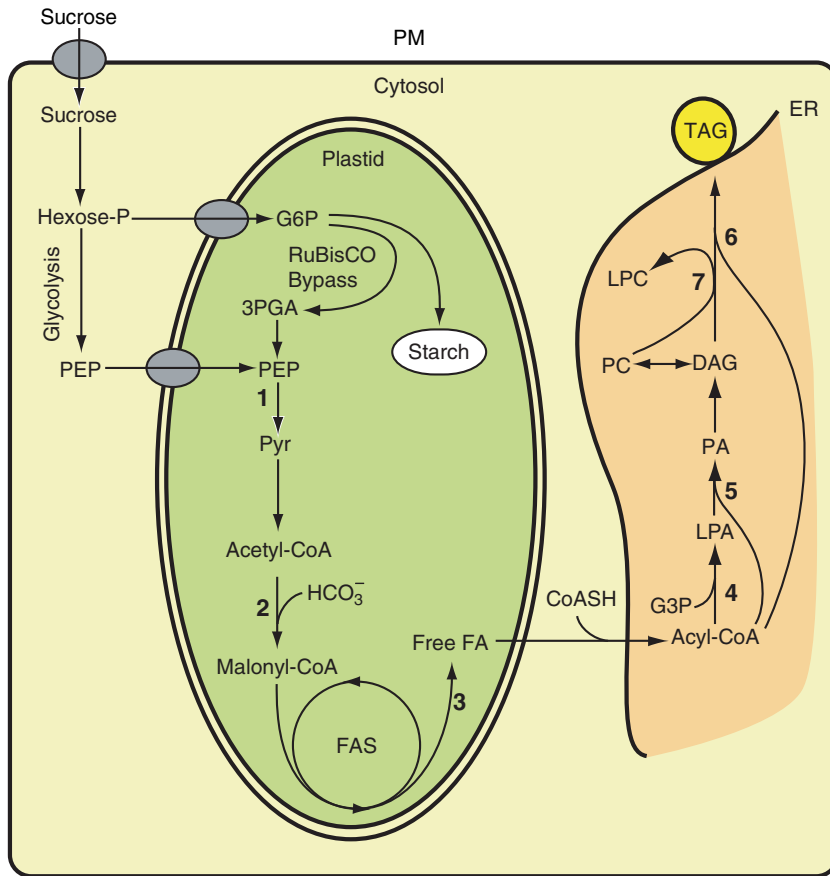


Figure 6. Fatty acid and triacylglycerol production in plants.

For simplicity, not all possible reactions are shown and multiple reactions may be represented by single arrows. Some pathways, for example, the Rubisco bypass pathway are not present in every species. Bold numbers represent key enzymes: 1, plastidic pyruvate kinase; 2, acetyl CoA carboxylase; 3, acyl ACP thioesterases FatA and FatB; 4, glycerol-3-phosphate acyl transferase; 5, lyso-phosphatidylcholine acyl transferase; 6, diacylglycerol acyl transferase; 7, lyso-phosphatidylcholine acyl transferase. 3PGA, 3-phosphoglycerate; DAG, diacylglycerol; ER, endoplasmic reticulum; FAS, fatty acid synthesis; G3P, glycerol-3-phosphate; G6P, glucose-6-phosphate; LPA, lyso-phosphatidic acid; LPC, lyso-phosphatidylcholine; PA, phosphatidic acid; PC, phosphatidylcholine; PEP, phosphoenolpyruvate; PM, plasma membrane; Pyr, pyruvate; TAG, triacylglycerol.

to 18 carbon atoms can be synthesized. The first desaturation step also occurs in the plastid; while the acyl chain is still conjugated to ACP a Δ^9 -desaturase converts stearyl ACP to oleoyl ACP. Termination of fatty acid elongation is catalyzed by acyl ACP thioesterases, of which two main types exist in plants. The FatA class preferentially removes oleate from ACP, whereas FatB thioesterases are active with saturated and unsaturated acyl ACPs, and, in some species, with shorter-chain-length acyl ACPs (Mayer and Shanklin, 2007; Pollard *et al.*, 1991; Salas and Ohlrogge, 2002). After release from ACP, the free fatty acids are exported from the plastid and converted to acyl CoAs. Nascent fatty acids can be incorporated into TAGs in developing seeds in a number of ways. For example, a series of reactions known as the Kennedy pathway results in the esterification of two acyl chains from acyl CoA to glycerol-3-phosphate to form phosphatidic acid (PA) and, following phosphate removal, diacylglycerol (DAG). A diacylglycerol acyltransferase (DGAT), using acyl CoA as an acyl donor, converts DAG to TAG. Two classes of DGAT enzymes have been isolated (Cases *et al.*, 1998, 2001; Lardizabal *et al.*, 2001), and orthologs have been identified in numerous plant species. The two groups of proteins are unrelated, both in terms of sequence and structure. For example, type 1 DGATs are

predicted to contain approximately nine transmembrane domains, whereas the type 2 DGATs probably only contain two such domains.

As an alternative to the simple Kennedy pathway, nascent fatty acids may be first incorporated into membrane lipids at the plastid envelope and/or in the endoplasmic reticulum. DAG and phosphatidylcholine (PC) are interchangeable via the action of cholinephosphotransferase, suggesting one route for the flux of fatty acids into and out of PC. There is evidence to suggest that PC functions as an important intermediate in the formation of TAG, particularly molecular species containing polyunsaturated fatty acids. For example, additional desaturation of oleate (18:1)^a occurs when it is esterified to PC (reviewed by Ohlrogge and Jaworski, 1997). Recent work has also demonstrated that newly synthesized fatty acids can be incorporated directly into PC via an acyl-editing mechanism, rather than through PA and DAG intermediates (Bates *et al.*, 2007). Acyl chains from PC can be incorporated into TAG, either through conversion back to

^aA brief note on lipid nomenclature: fatty acids can be designated as X:Y where X represents the number of carbon atoms and Y represents the number of double bonds. In some cases, the position of the double bond relative to the carboxyl group is designated by delta (Δ). Thus oleate can be abbreviated as 18:1 or 18:1 Δ^9 .

DAG or by the action of a phospholipid diacylglycerol acyltransferase (PDAT) that uses PC as an acyl donor to convert DAG to TAG (Dahlqvist *et al.*, 2000).

Regulation of oil synthesis occurs at multiple levels (Hills, 2004). The conversion of sugars into TAG in developing seeds is a classic example for the developmental and tissue-specific regulation of metabolism. Global analysis of mRNAs in developing seeds of *Arabidopsis* provided a snapshot of specific expression profiles of genes encoding enzymes that are predicted or known to be involved in the underlying pathways (Ruuska *et al.*, 2002). For example, a transcription factor, WRINKLED 1 (WRI1), proposed to be involved in controlling oil biosynthesis was identified in *Arabidopsis* (Baud *et al.*, 2007a; Cernac and Benning, 2004; Masaki *et al.*, 2005). The corresponding *wri1* mutant is deficient in oil biosynthesis and shows a reduction in the activity of glycolytic enzymes (Focks and Benning, 1998). Other transcription factors are involved in the complex regulation of metabolism in developing oil seeds; a more detailed overview of the transcriptional network in developing seeds is provided by Santos-Mendoza *et al.* (2008). Additional levels of control certainly involve allosteric enzyme regulation, for example at the level of acetyl CoA carboxylase (Hunter and Ohlrogge, 1998; Nikolau *et al.*, 2003) or plastid pyruvate kinase (Andre *et al.*, 2007; Baud *et al.*, 2007b). Metabolite (sugar) sensing mechanisms might provide input for integration of primary metabolism in seeds, but are not yet understood at the mechanistic level (Rolland *et al.*, 2006). Identifying the factors and understanding the mechanisms that control oil biosynthesis in plants will ultimately be the basis for successful engineering of oil content in vegetative plant tissues.

Improving the fuel properties of biodiesel

Plant oils are mostly composed of five common fatty acids, namely palmitate (16:0), stearate (18:0), oleate (18:1), linoleate (18:2) and linolenate (18:3), although, depending on the particular species, longer or shorter fatty acids may also be major constituents. These fatty acids differ from each other in terms of acyl chain length and number of double bonds, leading to different physical properties. Consequently, the fuel properties of biodiesel derived from a mixture of fatty acids are dependent on that composition. Altering the fatty acid profile can therefore improve fuel properties of biodiesel such as cold-temperature flow characteristics, oxidative stability and NO_x emissions.

Cold-temperature flow characteristics

When diesel fuels are cooled, fine crystals will form at the temperature defined as the cloud point (CP). These solid crystals can plug fuel filters and block fuel lines, leading to problems with engine operation. If the fuel is cooled further,

it will eventually become gel-like and can no longer be poured; the temperature at which this occurs is defined as the pour point (PP). No. 2 diesel fuel (the type most used in automobiles) has CP and PP values of around -16 and -27°C, respectively, whereas biodiesel derived from soybeans has much higher CP and PP values, around 0 and -2°C, respectively (Dunn and Bagby, 1995; Lee *et al.*, 1995). These high CP and PP values make current forms of pure biodiesel impractical for use in colder climates. The presence of saturated methyl esters longer than C12 significantly increases both the CP and PP, even when blended with conventional diesel fuel (Serdari *et al.*, 1999; Stournas *et al.*, 1995).

Many solutions have been proposed to improve cold-temperature flow characteristics of biodiesel: winterization, the use of additives, esterification with branched alcohols, and altering the fatty acid composition of the input oil. Given the ineffectiveness and/or greater cost associated with these approaches (Dunn *et al.*, 1996; Lee *et al.*, 1995, 1996), probably the best way to improve the cold-temperature flow properties of biodiesel is to alter the fatty acid composition of the raw material. As long-chain saturated fatty esters significantly increase the CP and PP, whereas unsaturated fatty acids have little effect when blended with diesel (Serdari *et al.*, 1999; Stournas *et al.*, 1995), reducing the saturated fatty acid content of plant oil can improve the cold-temperature flow properties of the biodiesel derived from it. Indeed, the CP and PP of FAMES derived from low-palmitate soybean oil are -7 and -9°C respectively, at least 5°C lower than for FAMES from normal soybean oil (Lee *et al.*, 1995).

Oxidation

Biodiesel, particularly from highly unsaturated sources, oxidizes more rapidly than conventional diesel. The oxidation of long-chain methyl esters typical of those found in biodiesel results in the initial accumulation of hydroperoxides, which eventually polymerize forming insoluble sediments that are capable of plugging filters, fouling injectors and interfering with engine performance. The influence of molecular structure on the rate of oxidation of biodiesel is greater than the influence of environmental conditions such as air, light and the presence of metal (Knothe and Dunn, 2003). In particular, the number and position of double bonds in a fatty acid ester affect the rate of this auto-oxidation. For example, esters of linoleate are 40 times more reactive than oleate esters because they contain an easily oxidized *bis*-allylic methylene group between the two double bonds (Frankel, 1998; Holman and Elmer, 1947). Even low concentrations of polyunsaturated fatty esters have a disproportionately large effect on the oxidative stability of biodiesel (Knothe and Dunn, 2003).

Therefore, one approach to improving oxidative stability will involve reducing the amounts of unsaturated (particu-

larly polyunsaturated) fatty acids present in the input oil. Another approach is adding anti-oxidants to the fuel. Tertiary butylhydroquinone (TBHQ) can improve the oxidative stability of compounds containing fatty acyl chains (Canakci *et al.*, 1999; Duplessis *et al.*, 1985). Even more importantly, recent studies have shown that tocopherols (vitamin E) present naturally in soybean oil can reduce the rate of biodiesel oxidation by more than a factor of 10 (Canakci *et al.*, 1999; Knothe *et al.*, 2005). An increased understanding of the pathways involved in the synthesis of tocopherols and tocotrienols (another major form of vitamin E in plants) has provided transgenic strategies to manipulate the levels of these anti-oxidants in soybeans and other crops (Cahoon *et al.*, 2003; Karunanandaa *et al.*, 2005); such alterations could therefore be incorporated when developing oil crops for biodiesel.

NO_x emissions

As mentioned previously, diesel engines emit less carbon monoxide and particulate matter when operated with biodiesel compared to regular conventional diesel. However, NO_x emissions are increased (Graboski and McCormick, 1998), probably as a result of higher combustion temperatures. Emissions studies of the constituent FAMEs typically present in biodiesel revealed that methyl palmitate, methyl laurate and methyl stearate all produce less NO_x than standard diesel, as do the methyl and ethyl esters of hydrogenated soybean oil. On the other hand, progressively increasing the number of double bonds in FAMEs resulted in a corresponding increase in NO_x production. Further, methyl esters with longer fatty acid chains had lower NO_x emissions (McCormick *et al.*, 2001). Therefore, reducing the unsaturated (particularly the polyunsaturated) fatty acid content of the input oil should result in lower NO_x emissions from the resulting biodiesel.

Engineering fatty acid profiles to optimize biodiesel fuel characteristics

Improving cold-temperature flow characteristics requires a fuel with low saturated fatty acid levels, whereas increasing oxidative stability and reducing NO_x emissions requires decreasing the amounts of unsaturated and polyunsaturated fatty acids. Additionally, the ignition quality (as measured by cetane number) is also adversely affected by increased unsaturation (Klopfenstein, 1985; Serdari *et al.*, 1999; Stournas *et al.*, 1995). Given the antagonistic requirements between cold-temperature flow characteristics on the one hand and oxidative stability, NO_x emissions and cetane number on the other, there is no fatty acid profile that will provide a fuel for which all these parameters are optimal (Table 1). However, a very good compromise can be reached by considering a fuel high in the mono-unsaturated fatty

Table 1 Comparison of the fuel properties of FAMEs commonly found in biodiesel with the fuel properties of conventional diesel

	Palmitate	Stearate	Oleate	Linoleate	Linolenate
Carbons	16	18	18	18	18
Double bonds	0	0	1	2	3
Cold flow ^a	Worse	Worse	Similar	ND	ND
Fuel stability ^b	Good	Good	Satisfactory	Poor	Poor
NO _x emissions ^c	Lower	Lower	Similar	Higher	Higher
Ignition quality ^d	Higher	Higher	Higher	Similar	Lower ^e

ND, no data.

^aEvaluation of cold-flow properties based on the CP and PP of FAMEs blended with conventional diesel (Serdari *et al.*, 1999).

^bFuel stability comparisons are between the various FAMEs rather than with conventional diesel, and are derived from Frankel (1998) and Knothe and Dunn (2003).

^cDerived from McCormick *et al.* (2001).

^dEvaluation based on cetane numbers from Knothe (2005).

^eBased on data for ethyl linolenate.

acids, such as oleate or palmitoleate (16:1 Δ^9), and low in both saturated and polyunsaturated fatty acids. The presence of a single double bond greatly enhances the cold-temperature flow properties of methyl oleate compared to methyl stearate (Serdari *et al.*, 1999; Stournas *et al.*, 1995). Various studies suggest that biodiesel with high levels of methyl oleate will have excellent, if not optimal, characteristics with regard to ignition quality, NO_x emissions and fuel stability. For example, while unsaturation tends to reduce the cetane number of biodiesel, that of methyl oleate is higher than the minimal biodiesel standard (ASTM D6751; Knothe, 2005). Additionally, it has been estimated that biodiesel fuels with an average of 1.5 double bonds per molecule will produce an equivalent amount of NO_x to conventional diesel (McCormick *et al.*, 2001), thus a fuel high in oleates (one double bond per molecule) should not result in higher NO_x emissions. Finally, given that polyunsaturated fatty acids have a disproportionately large effect on the auto-oxidation of biodiesel (Knothe and Dunn, 2003), reducing the polyunsaturated fatty acid content will improve the stability of the fuel.

Soybean lines with high levels of oleic acid and low levels of saturated and polyunsaturated fatty acids have been developed using a transgenic strategy that results in down-regulation of two genes involved in fatty acid synthesis (Buhr *et al.*, 2002). Down-regulation of the *FAD2-1* gene, encoding a Δ^{12} fatty acid desaturase, prevented the conversion of oleic acid to polyunsaturated fatty acids, resulting in increased levels of oleic acid. Additionally, preventing the release of saturated fatty acids from ACP by down-regulating *FatB*, which encodes a palmitoyl ACP thioesterase, lowered the levels of saturated fatty acids. In this manner, five soybean lines were generated that had oleic acid levels

greater than 85% and saturated fatty acid levels less than 6%, compared to wild-type levels of 17.9% and 13.1%, respectively (Buhr *et al.*, 2002). Consistent with predictions, biodiesel synthesized from these high-oleic soybeans demonstrated improved fuel characteristics with regard to cold-temperature flow properties and NO_x emissions (Tat *et al.*, 2007). For example, compared to biodiesel derived from conventional soybean oil, the CP was lowered from 1 to -5°C, and the PP from 0 to -9°C. Also, NO_x emissions from the high oleic acid biodiesel were only 7.5% higher than those from No. 2 diesel fuel (compared to 13.5% higher for biodiesel derived from conventional soybeans). Further improvement in CP and PP might be obtained by engineering mono-unsaturated oils with shorter chain lengths. Some plant species, for example *Doxantha unguis*, accumulate high levels of 16:1 Δ^9 (Chisholm and Hopkins, 1965), and the identification and subsequent transformation of a variant acyl ACP desaturase from this species into *Arabidopsis* and canola resulted in transgenic lines with increased levels of 16:1 Δ^9 (Bondaruk *et al.*, 2007; Cahoon *et al.*, 1998). Similar results were obtained in *Arabidopsis* plants expressing a castor ACP desaturase with enhanced activity towards 16:0 ACP (Cahoon and Shanklin, 2000). However, with both strategies, the total accumulation of 16:1 Δ^9 was low; higher levels of accumulation of 18:1 Δ^{11} and 20:1 Δ^{13} suggest that elongation of the desired 16:1 Δ^9 product resulted in accumulation of these other unusual fatty acids.

Direct use of plant oils as fuel

Altering the fatty acid composition of TAGs could also lead to the development of low-viscosity plant oils. As mentioned previously, the high viscosity of plant oils leads to poor fuel atomization, preventing their direct use as fuel in most modern diesel engines. The development of low-viscosity plant oils that could be used in engines directly would eliminate the need for chemical modification, thus improving the cost-effectiveness of biofuels. Viscosity increases with the number of acyl carbons, and is decreased by the presence of double bonds (Allen *et al.*, 1999). Thus, low-molecular-weight TAGs and acetyl TAGs have lower viscosities than those typically found in conventional plant oils.

Low-molecular-weight TAGs, such as tributyrin (4:0) up to tricaprין (10:0), are predicted to have better fuel atomization characteristics than conventional TAGs (Goodrum and Eiteman, 1996). Indeed, fuel studies specifically examining the coking index (a measure of engine carbon deposition) revealed that Captex 355, an artificial blend of tricaprין and tricaprilyn (8:0), has a lower coking index than No. 2 diesel (Geller *et al.*, 1999a). Seeds from the genus *Cuphea* are enriched for low-molecular-weight TAGs, containing saturated medium-chain fatty acids with lengths of 8–14 carbon atoms (Graham, 1989), and could therefore be a source of such low-viscosity TAGs. Seed oil with elevated levels of

tricaprין (6:0) and tricaprilyn from a mutant of *Cuphea viscosissima* had a coking index comparable to that of No. 2 diesel (Geller *et al.*, 1999a,b). However, one problem with the direct use of low-molecular-weight triacylglycerols is their poor cold-temperature flow properties. Indeed, as saturated TAGs with acyl chains longer than eight carbons are solid at room temperature, it is not surprising that simulated *Cuphea* oil tends to solidify (Geller *et al.*, 1999a,b). Blending is one possible approach to overcoming this problem: a 20% mixture of *Cuphea* oil with conventional diesel had a coking index similar to that of No. 2 diesel (Geller *et al.*, 1999a). An alternative, but admittedly more challenging, solution would be to develop plants that produce high levels of TAGs with a molecular weight less than that of tricaprilyn.

Work is underway to develop *Cuphea* as a crop; for example, breeding lines with reduced seed shattering and increased seed oil have been developed (Knapp and Crane, 2000a,b). An alternative strategy is transferring the genes responsible for the accumulation of medium-chain fatty acids in *Cuphea* to an existing oil crop. One class of enzymes important in regulating the length of fatty acid chains in plants is the FatB acyl ACP thioesterases, which remove elongating fatty acid chains from ACP (Pollard *et al.*, 1991). Such an acyl ACP thioesterase, *ChFatB2*, has been isolated from *Cuphea hookeriana*, which accumulates up to 75% 8:0 and 10:0 in its seed oil. Subsequent expression of this gene in canola resulted in the accumulation of up to 11% 8:0 and 27% 10:0 in the transgenic seed (Dehesh *et al.*, 1996). However, these proportions are much lower than in *Cuphea*, and mean that additional purification of the low-molecular-weight TAGs would have to occur prior to use as fuel. The low yield of novel fatty acids in transgenic plants is unfortunately a common problem and is one of the main reasons limiting the widespread production of unusual fatty acids using such approaches (Thelen and Ohlrogge, 2002b). A greater understanding of how plants direct the flux of fatty acids into TAGs is therefore needed before these approaches become commercially practical. In this regard, co-expression with additional components of the fatty acid synthesis pathway appears to have some effect: for example, expressing a 3-ketoacyl ACP synthase (KAS) from *C. hookeriana* with *ChFatB2* enhanced the accumulation of caprylate and caprate by an additional 30–40% (Dehesh *et al.*, 1998).

Acetyl TAGs are a modified TAG with a short two-carbon acetyl group rather than a longer acyl chain at the *sn*-3 position. This structure results in an oil of lower viscosity that has the potential to directly replace No. 4 diesel (a heavier grade). These unusual triacylglycerols are abundant in the seeds of some plant species, such as members of the genus *Euonymus* where they can form up to 98% of the seed oil. As with *Cuphea*, these species are not currently suitable for development as oilseed crops. Therefore, the transfer of enzyme(s) necessary for acetyl TAG production has been

explored. To this end, a type 1 diacylglycerol transferase (DGAT1) involved in the production of acetyl glycerides has been isolated from developing seeds of *Euonymus alatus* (Milcamps *et al.*, 2005). However, as with the previous examples, the heterologous expression of *EaDGAT1* failed to yield high levels of acetyl TAGs.

Design of new energy crops for biodiesel production

While improving the fuel characteristics of biodiesel will make its use more acceptable to consumers, the major obstacle to the large-scale adoption of biodiesel is producing sufficient amounts of plant triacylglycerols for fuel without greatly affecting the supply and cost of food. This situation has led to numerous efforts and proposals to develop alternative sources of biofuels. Although a large proportion of these efforts are focused on conversion of lignocellulosic feedstocks to ethanol, we outline below some speculative strategies for the design of new crops to produce biodiesel.

Historically, humans have bred plants for use as food, so agriculture has traditionally focused on increasing the yield and nutritional quality of the edible portion. New crop plants intended for production of biofuels will require different properties from those developed for food or fiber. One major focus of future research will be to produce plants with elevated levels of oils or other hydrocarbons in vegetative tissues that can be easily harvested and converted to biofuels. Thus, we can imagine that ideal biofuel crops will have increased levels of high-energy-content oils that will provide more usable energy per unit of land than crops that were designed for food production.

Increasing plant oil production

Increasing seed and/or oil yield per hectare is the obvious approach to providing sufficient oil for biodiesel needs without diverting land from other uses. An increased understanding about how plants control the flux of carbohydrates to synthesize fatty acids and TAGs, combined with genetic engineering, offers new strategies to increase oil yield. These efforts have typically focused on increasing the supply of fatty acid or glycerol-3-phosphate substrates or up-regulating the activity of one of the enzymes involved in attaching the fatty acids to the glycerol backbone.

For example, evidence suggests that oil synthesis may be limited by the production of fatty acids (Bao and Ohlrogge, 1999), the production of which is regulated by acetyl CoA carboxylase (ACCase). Reduction of ACCase activity lowered the fatty acid content in transgenic seeds (Thelen and Ohlrogge, 2002a). However, substantially increasing ACCase activity may be quite complex. Not only is the enzyme feedback-inhibited and post-translationally regulated, but it is encoded by multiple genes (Nikolau *et al.*, 2003). Some

success has been achieved by expressing a cytosolic version of the enzyme that is encoded by a single gene. Addition of a plastid transit sequence targeted the introduced protein to chloroplasts, ultimately resulting in a 5% increase in seed oil (Roesler *et al.*, 1997).

More recent work has focused on the supply of glycerol-3-phosphate, which appears to limit the synthesis of triacylglycerols in rape seed (Vigeolas and Geigenberger, 2004). Subsequently, increasing the glycerol-3-phosphate levels in developing seeds by over-expression of a yeast gene encoding a cytosolic glycerol-3-phosphate dehydrogenase (*GPD1*) resulted in a substantial increase in seed oil levels in transgenic rape grown in greenhouses (Vigeolas *et al.*, 2007).

Success in increasing plant oil levels has also been achieved by altering steps that acylate the glycerol backbone. For example, lysophosphatidate acyltransferase (LPAT) catalyzes the addition of the second acyl chain to the *sn*-2 position of lysophosphatidic acid (LPA), forming phosphatidic acid (PA, Figure 6). Over-expression of an LPAT from yeast in *Arabidopsis* and *Brassica* resulted in an increase in seed oil content, as much as 48% in some lines (Zou *et al.*, 1997). Additional work has confirmed that oil increases, although more modest, can also be achieved in the field (Taylor *et al.*, 2002), thus demonstrating the potential agricultural utility of such genetic modifications.

Altering the final step in TAG synthesis also affects the amount of seed oil produced. This reaction, which adds the third acyl chain, is catalyzed by a diacylglycerol acyltransferase (DGAT, Figure 6). Mutations in the *Arabidopsis* DGAT1 gene result in a reduction in oil content (Katavic *et al.*, 1995; Routaboul *et al.*, 1999; Zou *et al.*, 1999). Conversely, over-expression of the *Arabidopsis* DGAT1 gene in wild-type plants led to increased seed oil levels (Jako *et al.*, 2001). Similarly, transformation of the same gene into tobacco resulted in increases in TAG levels in the leaves of various transgenic lines (Bouvier-Nave *et al.*, 2000). Similarly, expression of a DGAT2 gene from the oleaginous fungus *Mortierella rammanniana* in soy reportedly resulted in small but significant increases in seed oil content (Lardizabal *et al.*, 2004).

While these advances are exciting, much of this research is at an early stage. In some cases, the increased oil trait needs to be transferred from a model species to an appropriate oil crop. Additionally, rigorous field testing at various locations and over multiple growth seasons is necessary not only to determine whether percentage oil content increases are also reflected in increased oil yield per hectare but also whether increases are stable and sustainable in a genetic background of elite, high-yielding commercial varieties.

Strategies to produce oil in vegetative tissues

Photosynthesis in leaves is the ultimate source of almost all biofuels. Once a leaf is developed, it undergoes a develop-

mental change from sink (absorbing nutrients) to source (providing sugars). In food crops, most sugars are translocated out of source leaves to support growth of new leaves, roots and fruits. Because translocation of carbohydrate is an active process, there is a loss of carbon and energy during translocation. Furthermore, after the developing seed takes up carbon from the mother plant, there are additional carbon and energy losses associated with the conversion of carbohydrate into the oil, protein or other major components of the seed (Goffman *et al.*, 2005). For example, sunflower seeds are estimated to release as CO₂ approximately 40% of the carbon taken up by the developing embryo (Alonso *et al.*, 2007). Suppression of flowering can significantly reduce energy usage for flower and fruit development, and is known to increase biomass yields in a number of crops including sugar cane, maize, root crops and tobacco (Berding and Hurney, 2005; Salehi *et al.*, 2005).

Thus, one objective for the design of new energy crops is to maximize the energy content of leaves and/or stems such that the whole above-ground plant is harvested and less carbon and energy is lost through translocation, flowering and seed production. A number of genetic modifications are known that block carbon export from leaves, resulting in up to 50% starch and/or soluble carbohydrate by dry weight in leaves (Lu and Sharkey, 2004; Maeda *et al.*, 2006; Russin *et al.*, 1996). For example, reduction of the sucrose transporter blocks carbon export from potato leaves, resulting in 50% soluble carbohydrate and starch by dry weight (Riesmeier *et al.*, 1994). One potential problem with these systems is that photosynthesis can be inhibited by starch, sucrose or other end-product accumulation (Paul and Foyer, 2001). Nevertheless, after exporting sucrose from the leaves, sugar cane accumulates very high levels of sucrose in vacuoles of the stalk (up to 50% of stalk dry weight). In general, increasing the capacity for sucrose sequestration or other sink synthesis or accumulation results in increased photosynthesis (Laporte *et al.*, 1997). For example, initial results indicate that sugar content can be doubled by conversion of sucrose to isomaltulose in sugar cane transformed with a bacterial sucrose isomerase (Wu and Birch, 2007). Thus, it may be possible to engineer leaves or stems to block carbon export or to sequester carbon in ways that do not lead to inhibition of photosynthesis. If biofuel accumulation in leaves or stems is inhibitory to photosynthesis or growth, it will be desirable to engineer plants in which carbon accumulation begins only after the leaves have reached their maximum size. Developmentally regulated promoters and senescence-induced promoters can counteract senescence while polymers are accumulating in leaves. For example, a senescence-related promoter can extend the period of carbon fixation of leaves (Gan and Amasino, 1995).

Production of oil, rather than carbohydrate, in leaves or stems has several potential advantages. First, oils have

double the energy content per carbon atom compared with carbohydrates (Figure 2) and can be extracted with low energy inputs and low costs. Because no microbial fermentation is required, net energy yields from oil crops are inherently high. Second, the higher energy content stored in oil potentially provides a larger sink for photosynthesis, thereby reducing the likelihood of feedback suppression of photosynthesis. Third, when carbohydrates are fermented to ethanol, one third of the carbon is lost as CO₂. However, when plants synthesize oil, CO₂ is released inside the cell in a concentrated form where it can be re-fixed by photosynthesis. This is important because uptake of CO₂ normally requires plants to open their stomata and there is a loss of 100–1000 water molecules for every molecule of CO₂ taken up. As a consequence, water often limits photosynthesis and crop yields. The recapture of CO₂ released during oil synthesis (or other metabolism) does not 'cost' the plant water. Fourth, the conversion of carbohydrate to oil can be extremely efficient if reducing power is available. The Rubisco bypass pathway (Schwender *et al.*, 2004) allows greater carbon-use efficiency, increasing the carbon recovered in oil from 66% obtainable via glycolysis to 80% via the bypass. Under agricultural cropping systems, light availability and thus reducing power are usually not limiting to yield, and therefore adding energy to biomass by converting carbohydrate to oil can in principal occur without added agricultural inputs.

Because of their very high biomass yields and low fertilizer or other inputs, perennial grasses are projected to be a major future source of biofuels (Heaton *et al.*, 2004). As the following calculation indicates, if 20–25% of the dry matter of such a harvested crop is oil instead of lignocellulose, the energy value of the crop is nearly doubled. Taking 15 tonnes per hectare as an average dry matter yield for a perennial grass (Heaton *et al.*, 2004), an oil content of 20–25% by weight will produce about 3400 l of biodiesel. This is roughly three and six times the yield of canola and soybean per hectare, respectively. The energy content of 3400 l of plant oil is 113 GJ. To recover the energy content of the cell wall/lignin as ethanol requires hydrolysis, fermentation and distillation; a rough estimate is that the gross energy recovered would be approximately 117 GJ. Thus, with 20–25% oil in the harvested material, the recoverable energy content of the crop would be substantially higher. Because the extraction of oil and conversion to biodiesel requires less energy than lignocellulose hydrolysis, fermentation to ethanol and distillation, the net energy balance and greenhouse gas benefits for biodiesel are even more favorable (Hill *et al.*, 2006).

Is it feasible to produce oil in leaves?

Although seeds (and surrounding mesocarp; e.g. palm, olive, avocado) are by far the greatest current commercial

sources of plant oils, many other tissues are capable of abundant oil synthesis. Some wild species of tomato secrete up to 20% of leaf dry weight as acylated sugars (Fobes *et al.*, 1985), tubers of *Cyperus esculentus* accumulate 26% oil (Zhang *et al.*, 1996), and phloem tissue of *Tetraena mongolica* stems contains approximately 10% oil (Wang *et al.*, 2007). TAG also accumulates during senescence of leaves (Kaup *et al.*, 2002) and under stress (Sakaki *et al.*, 1990a,b), and in Arabidopsis mutants disrupted in endoplasmic reticulum-to-chloroplast lipid trafficking (Xu *et al.*, 2005). As mentioned previously, the ectopic over-expression of Arabidopsis DGAT1 in tobacco led to increases in leaf TAG levels (Bouvier-Nave *et al.*, 2000). Another noteworthy mutant of Arabidopsis is *pickle*, which is deficient in a chromatin remodeling factor and in which roots become embryo-like and produce seed storage compounds including oil (Ogas *et al.*, 1997, 1999). Thus, accumulation of oils in non-seed tissues clearly occurs in a number of plants.

The discovery of transcription factors and other regulatory systems that control plant oil biosynthesis (Cernac and Benning, 2004; Ohlrogge and Jaworski, 1997; Ruuska *et al.*, 2002) has suggested ways of producing oil in tissues other than seeds. Particularly promising is the WRI1 transcription factor of Arabidopsis, which controls primary metabolism in the seeds and is required for seed oil biosynthesis (Baud *et al.*, 2007a; Cernac and Benning, 2004; Cernac *et al.*, 2006; Focks and Benning, 1998; Masaki *et al.*, 2005; Ruuska *et al.*, 2002). Ectopic expression of the WRI1 transcription factor can lead to accumulation of oil in developing seedlings of Arabidopsis (Cernac and Benning, 2004). Similar results have been reported for over-expression of the *LEC2* gene (Santos Mendoza *et al.*, 2005) which acts upstream of WRI1 (Baud *et al.*, 2007a). In addition, double mutants in *HSI2* and *HSL1*, two B3 domain transcriptional repressors, accumulate oil in seedlings. Furthermore, callus derived from these seedlings continues to produce oil and storage proteins for at least 14 days (Tsukagoshi *et al.*, 2007). In all these examples, oil accumulation required a supply of carbohydrate, a situation that mimics developing seeds.

The availability of transcription factors controlling genes involved in seed maturation and storage accumulation provides interesting targets to engineer the production of oil in vegetative tissues or with which to develop a process to convert sugar into oil by seedling fermentation. As the ectopic expression of either *WRI1* or *LEC2*, or the repression of *HSI2* and *HSL1*, leads to embryos or embryo-like tissues producing oil when fed with sugars, one could envisage a process in which, for example, seeds over-expressing WRI1 are germinated in a reactor that can be supplied with sugar-containing liquid substrate. Under such conditions, embryo-like tissues or seedlings should be able to accumulate oil as already shown for Arabidopsis seedlings expressing a *WRI1* cDNA (Cernac and Benning, 2004). Thus, combining agricultural and industrial processes based on plant seedlings

might provide an alternative to the production of high-yielding oil crops in the field.

Conclusion

Given the increasingly large quantities of fossil fuels consumed, there is concern about the long-term availability of these non-renewable energy sources. Additionally, for much of the industrialized world, this energy has to be imported, resulting in negative trade balances. Biodiesel, produced from renewable and often domestic sources, represents a more sustainable source of energy and will therefore play an increasingly significant role in providing the energy requirements for transportation. Equally important, the production and use of biodiesel results in reduced CO₂ production. Thus, both the EU and the USA have set goals of deriving 10% and 30%, respectively, of their transportation energy needs from biofuels. However, with respect to biodiesel, a limited supply of plant oil feedstocks represents the biggest challenge to reaching these goals. Therefore, the production of plant oils needs to be expanded, preferably without major increases in food prices or damage to natural environments. Plant biology therefore has an important and perhaps critical part to play in the large-scale development of biodiesel. Already, an increased knowledge of how plants synthesize fatty acids has enabled successful engineering of the composition of plant oils, thereby providing a biodiesel with better fuel properties. Viable strategies for increasing oil production in seeds have also been demonstrated, although additional work is necessary to translate these to yield increases in the field. In addition, research at an early stage has also suggested ways of producing oil in vegetative tissue rather than in seeds. Combining these approaches to develop high-yielding energy crops will increase the production of plant oils suitable for biodiesel.

Acknowledgements

We thank Tom Sharkey and Mike Pollard for helpful discussions. Work on plant and algal oil metabolism in the authors' laboratories was supported by a United States Department of Energy grant DE-FG02-87ER13729 (J.O.), a United States National Science Foundation grant MCB 0453858 (C.B.), a United States Air Force Office of Scientific Research grant FA9550-07-1-0212 (C.B.), by BASF Plant Sciences (C.B.) and by the Michigan Agricultural Experiment Station (C.B. and J.O.). This work was supported by the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service, grant number 2005-35504-16195.

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