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HARNESSING PLANT BIOMASS FOR BIOFUELS AND BIOMATERIALS

High-value oils from plants

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Summary

The seed oils of domesticated oilseed crops are major agricultural commodities that are used primarily for nutritional applications, but in recent years there has been increasing use of these oils for production of biofuels and chemical feedstocks. This is being driven in part by the rapidly rising costs of petroleum, increased concern about the environmental impact of using fossil oil, and the need to develop renewable domestic sources of fuel and industrial raw materials. There is also a need to develop sustainable sources of nutritionally important fatty acids such as those that are typically derived from fish oil. Plant oils can provide renewable sources of high-value fatty acids for both the chemical and health-related industries. The value and application of an oil are determined largely by its fatty acid composition, and while most vegetable oils contain just five basic fatty acid structures, there is a rich diversity of fatty acids present in nature, many of which have potential usage in industry. In this review, we describe several areas where plant oils can have a significant impact on the emerging bioeconomy and the types of fatty acids that are required in these various applications. We also outline the current understanding of the underlying biochemical and molecular mechanisms of seed oil production, and the challenges and potential in translating this knowledge into the rational design and engineering of crop plants to produce high-value oils in plant seeds.

Keywords: biorefining, industrial oils, lipids, seed storage oils, triacylglycerols, unusual fatty acids.

Introduction

Current world production and use of vegetable oil in food and non-food applications

Plant oils represent an important renewable resource from nature. With few exceptions, such as the waxes of jojoba oil, plant oils consist almost entirely of triacylglycerol (TAG) esters containing three fatty acids (FAs) with chain lengths of C8–C24, with C16 and C18 being the most common. Plant oils are used primarily for food and feed purposes, although the oils are increasingly being utilized as renewable sources of industrial feedstocks and fuel. The world production of plant oils amounted to 127 million tonnes in 2006, which represents an increase of about 50 million tonnes compared to only 10 years before (FAOSTAT, 2007). As a point of reference, the annual production of animal fats (tallow, lard and butter) is approximately 22 million tonnes, while fish oils represent about 1 million tonnes (Gunstone and Harwood, 2006). Together with the plant oils, these oils represent the world's natural oil supply. The majority of vegetable oils are produced from just four crops, namely oil palm, soybeans, rapeseed and sunflower, which together account for approximately 79% of the total production. It is estimated that about 14% of the fats and oils are used chemically and 6% as feed material (Patel *et al.*, 2006).

Food and feed

The largest proportion of plant oils is consumed as food and feed, and the oils used in these markets contain various proportions of the five common, nutritionally

Table 1 Fatty acid composition of oils from major and minor oil crops and in wild species

	Fatty acids											
Plants	8:0	10:0	12:0	14:0	16:0	18:0	18:1	18:2	18:3	20:1	Unusual	Note[i]
Major oil crops												
Palm oil[a]				5	36	2	50	8				
Soybean oil[a]					11	4	23	54	8			
Canola oil[a]					4	2	60	21	10	1		High oleic
Sunflower oil[a]					7	5	19	68				High linoleic
Linseed oil					6	2	19	24	47			High α-linolenic
Coconut[a]	7	7	48	18	9	3	6	2				High lauric
Palm kernel[a]	3	4	48	16	8	2	15	2				High lauric
Minor oil crops or wild species												
Borago officinalis[a]					10	4	16	38	<1	4	23	γ-linolenic (isomer of α-linolenic)
Echium plantagineum[a]					6	3	14	13	33		12/17	γ-linolenic/stearidonic
Cuphea hookeriana[d]	50	25	4	1	7		4	5				Caprylic (medium-chain FA)
Cuphea pulcherrima[d]	96	2			1			1				Caprylic (medium-chain FA)
Cuphea lanceolata[d]		83	2	2	3		3	5				Capric (medium-chain FA)
Ricinus communis[b]					1	1	3	4			89	Ricinoleic (hydroxy FA)
Coriandrum sativum[c]					4	3	8	17	1		66	Petroselinic (isomer of oleic)
Crepis alpina				1	4	1	2	18			74	Crepenynic (triple bond)
Vernonia galamensis[e]					3	3	4	21			67	Vernolic (epoxy FA)
Momordica charantia[f]					2	17	15	9			57	Eleostearic (conjugated FA)
Brassica napus[g]					5	1	15	14	9	7	45	Erucic (very-long-chain FA)
Crambe abyssinica[h]					2	1	18	9	6	2	56	Erucic (very-long-chain FA) monounsaturated)

[a]Gunstone *et al.*, 2006; [b]Ogunniyi, 2006; [c]Ramadan and Morsel, 2002; [d]Dehesh, 2001; [e]Baye *et al.*, 2005; [f]Armougom *et al.*, 1998; [g]Ackman, 1990; [h]Lazzeri *et al.*, 1997.
[i]The properties of unusual fatty acids are provided in parentheses (see main text for additional details).

important FAs (Table 1). These five fatty acids are palmitic (16:0), stearic (18:0), oleic (18:1Δ^9), linoleic (18:2$\Delta^{9,12}$) and α-linolenic (18:3$\Delta^{9,12,15}$) acids.[1] The properties of oils depend greatly on their fatty acid composition, and certain compositions are desirable for specific end uses. For example, cooking oils generally contain a higher proportion of mono-unsaturated FAs (such as oleic acid), which are more stable under high temperature, while margarines and spreads are often rich in saturated fatty acids (e.g. palmitic and stearic acids). Other oils, such as salad oils, contain more polyunsaturated FAs (e.g. linoleic and α-linolenic acids). Traditionally, the production of oils for specific applications has been achieved by mixing of various plant oils (Sakurai and Pokorný, 2003) or by partial hydrogenation, whereby double bonds of fatty acids are removed to make the oil more saturated. However, hydrogenation also introduces unwanted *trans* FAs into the oil, which has undesirable effects on human health and nutrition (Ascherio, 2006).

[1]A note regarding nomenclature: the number before the colon designates the total number of carbons in the fatty acid chain, the number after the colon represents the number of double bonds, and the number after Δ indicates the position of the double bond with respect to the carboxyl end of the fatty acid structure. For example, oleic acid (18:1Δ^9) is an 18-carbon long fatty acid with a single double bond at the Δ^9 position. All double bonds are in the *cis* configuration unless otherwise specified.

Fuel/energy

Plant oils have been used to generate heat and light since ancient times. Vegetable oils have a higher energy content than other bioenergy resources such as ethanol, have 90% of the heat content of petroleum-derived diesel, and a favorable energy input/output ratio of about 1:2 to 1:4 for unirrigated crops (i.e. the amount of energy required to produce the crop compared to the amount of energy obtained from the seed oil) (Agarwal, 2007). In light of rising petroleum prices and environmental concerns, the use of plant oils as liquid fuel has seen a strong increase, especially in Europe where biodiesel is already a major fuel derived from oils such as rapeseed, sunflower or palm. For more information on this subject, see the review by Durrett *et al.* (2008).

Industrial feedstocks

As shown in Table 2, there are numerous uses and applications for plant-derived industrial feedstocks.

Table 2 Derivatives produced from plant oils by various processes, together with possible uses and applications

Derivatives	Uses, applications
Fatty acids and derivatives	Metallic soaps, detergents, soaps, cosmetics, alkyd resins, paints, textile, leather and paper industries, rubber, lubricants
Fatty acid methyl esters	Biodiesel, cosmetics, solvents, intermediates in the production of alcohols
Glycerol and derivatives	Cosmetics, toothpaste, pharmaceuticals, food, paints, plastics, synthetic resins, tobacco, explosives, cellulose processing
Fatty alcohols and derivatives	Detergents, cosmetics, textile, leather, and paper industries, duplicator stencils, petroleum additives
Fatty amines and derivatives	Surfactants, fabric softeners, mining, road building, biocides, textile and fiber industries, petroleum additives
Drying oils	Paints, varnish, linoleum
Castor oil, ricinoleic acid	Polyamide 11, alkyd resins

Modified from Patel *et al.* (2006), with permission.

Processing of plant oils is achieved through conversion reactions, which result in the production of derivatives of the original TAG molecules (Metzger and Bornscheuer, 2006). The composition of fatty acids in the oil, with regard to fatty acid composition, chain length or the presence of additional functional groups, influences the characteristics and end uses of the derived oleochemicals. For example, medium-chain length fatty acids such as lauric acid (12:0), derived primarily from palm kernel and coconut oils, are excellent surfactants that are used extensively for the production of soaps and detergents. The market for lauric acid alone is estimated to be worth more than $1.4 billion annually. Another example of an industrially important fatty acid derived from plants is erucic acid ($22{:}1\Delta^{13}$), which is a very-long-chain fatty acid derived from oilseed rape. Erucic acid is used to produce erucamide, which is used as a slipping agent for production of extruded polyethylene and propylene films such as shopping or refuse bags (Friedt and Luhs, 1998; Wang *et al.*, 2003). Global demand for erucic acid and the related behenic acid (22:0) is expected to continue to increase, rising from 18 and 15 million tonnes in 1990 to 35 and 46 million tonnes, respectively, by 2010 (Jadhav *et al.*, 2005).

The double bonds present in fatty acids also represent excellent starting points for modification of the hydrocarbon chain for the production of new types of feedstocks. For example, plant oils can be treated with a variety of chemicals to convert the double bonds of fatty acids into hydroxyl groups, and the resulting 'polyols' can be mixed with compounds such as isocyanate to form polyurethanes. These environmentally friendly, renewable alternatives to petroleum-derived polyurethane have excellent physical characteristics and perform well in a variety of applications, such as spray insulating foams, rigid foams, flexible foams such as interior car parts, coatings, sealants, adhesives and elastomers (Mielewski *et al.*, 2005). These and other types of chemical modifications to plant oils can create new and novel compounds with interesting properties, including medium- and long-chain diacids, ω-hydroxy fatty acids and ω-unsaturated fatty acids (Biermann *et al.*, 2000; Metzger and Bornscheuer, 2006; Wagner *et al.*, 2001). The treatment of plant oils with chemicals, however, adds to the overall cost and environmental footprint of utilizing these oils for industrial purposes.

A wide variety of structurally diverse fatty acids occurs in the seed oils of wild plant species (Aitzetmüller *et al.*, 2003; Badami and Patil, 1980; Smith, 1971), and many of these 'unusual' fatty acids represent outstanding (potential) feedstocks for industry (Table 1). They include unusual monounsaturated fatty acids, medium, short, or very-long-chain fatty acids, fatty acids with additional functional groups such as epoxy and hydroxy groups, or fatty acids with conjugated or acetylenic bonds, and significant research has been conducted into their biosynthesis in plant seeds (Figure 1). Other unusual plant oils are those composed of wax esters instead of TAGs. Importantly, a single unusual fatty acid may account for up to 90% of the seed oil composition, which greatly simplifies downstream processing and purification. The commercial production of these seed oils, however, is hampered by the poor agronomic traits of the plants (e.g. small seeds, limited geographical growing areas), which significantly increase the costs associated with their production (Cuperus *et al.*, 1996).

Consequently, if the production of industrially important fatty acids in high-yielding oil crops can be developed successfully through genetic engineering approaches, there is a huge market volume available. Assuming that these designer oils can compete efficiently with petroleum-based alternatives with regard to both improved functional qualities and price, a major growth potential for plant-derived industrial feedstocks will be created.

Lubricants

Lubricants represent a large non-food product area in which plant oils can be increasingly utilized. In 2003, the worldwide consumption of lubricants totaled 36 million

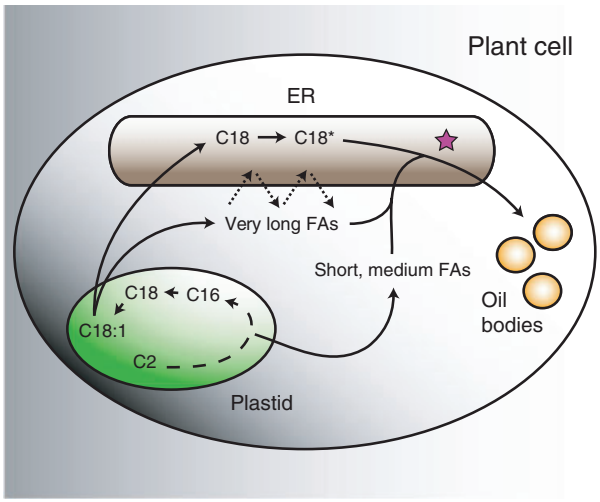

Figure 1. General overview of the organelles and metabolic pathways involved in production of industrially important oils in plant seeds.
Fatty acid biosynthesis occurs in the plastids of plant cells, with consecutive attachment of two carbon units to a growing fatty acid chain resulting in the production of C16, C18, and C18:1. The seeds of some plants contain enzymes that terminate the chain-elongation process early, resulting in the production of short- or medium-chain fatty acids that can be incorporated into storage oil (oil bodies). In other plants, C18:1 exported to the cytosol may be further elongated to produce very-long-chain fatty acids, or can be incorporated into the phospholipids of the endoplasmic reticulum (ER) to produce unusual fatty acids (C18*) containing hydroxy or epoxy groups, or acetylenic or conjugated bonds. Novel biochemical pathways may also be engineered in plants, for example through transfer of genes from microalgae to produce nutritionally important, very-long-chain polyunsaturated fatty acids (VLC-PUFAs). These complex biochemical pathways require transfer of fatty acids between the CoA and phospholipid pools for repeated rounds of fatty acid elongation and desaturation, respectively (dashed arrows). Once produced, the various fatty acids are incorporated into TAGs by a variety of acyltransferase reactions (star), and TAGs eventually accumulate in cytosolic oil bodies.

tonnes (not including marine oils), valued at about $28 billion. More than 70% of the total lubricant volume is used in motor oils for automotive engines, and roughly 10% is used as hydraulic oils. Plant oils (and their derivatives) are relatively inert and have a high viscosity index, high flash point and low volatility, making them especially attractive for use as lubricants for total loss applications such as chainsaw bar lubricants, drilling muds and oils, open gear oils, hydraulic fluids, outboard engine lubricants, mould release oils and other situations where environmental concerns are an issue (Erhan and Asadauskas, 2000). The chief disadvantages with using the main vegetable oils as lubricants, however, are their poor low-temperature fluidity and their sensitivity to oxidation at high temperatures (Wagner *et al.*, 2001). They therefore currently capture only a narrow segment of the total lubricants market (Whitby, 2004). The low oxidative stability of vegetable oils can be improved through chemical modification of plant oils or by using specialty oils, such as castor bean oil (*Ricinus communis*), for high-temperature applications (Schneider, 2006). Despite a widespread demand for castor oil, however, cultivation of this crop is restricted due to the presence of a toxin (ricin) and allergenic proteins, and thus the cost of castor oil is relatively high.

Wax esters also constitute another target molecule for lubricant applications. The high linearity of wax esters enhances the viscosity index of the oil and imparts specific desirable characteristics such as anti-rust, anti-foam, anti-wear and friction reduction properties to the lubricant (Bisht *et al.*, 1993; El Kinawy, 2004). These properties make wax esters excellent feedstocks for production of high-temperature and pressure lubricants as well as hydraulic fluids. Wax esters are produced in the seeds of the desert shrub jojoba (*Simmondsia chinensis*), and, despite the presence of market niches for this oil, the low yields and high costs of jojoba production preclude use of the wax in these applications.

Inks

A recent success story regarding industrial usage of plant oils is 'soy ink', which is produced from soybean oil that is blended with pigments, resins and waxes to make environmentally friendly printing inks (Erhan *et al.*, 1992). This product has now in great part replaced petroleum-based ink, such that almost one-third of America's 10 000 newspaper printers use it, and >90% of daily newspapers are using soy inks for color printing.

Nutritionally important, very-long-chain polyunsaturated fatty acids

Examples of high-value fatty acids for the food and feed industry include the very-long-chain polyunsaturated fatty acids (VLC-PUFAs) such as arachidonic acid (AA, $20{:}4\Delta^{5,8,11,14}$), eicosapentaenoic acid (EPA, $20{:}5\Delta^{5,8,11,14,17}$) and docosahexaenoic acid (DHA, $22{:}6\Delta^{4,7,10,13,16,19}$). VLC-PUFAs confer flexibility, fluidity and selective permeability to cellular membranes, and may also be metabolized to produce lipid signalling molecules such as eicosanoids, and, as such, have been shown to be vital for brain development and beneficial for reducing cardiovascular disease risk and improving a range of other human and animal health conditions (Colquhoun, 2001; Das, 2006; de Urquiza *et al.*, 2000; Funk, 2001; Kroes *et al.*, 2003). VLC-PUFAs (i.e. FAs with 20 or more carbons) are produced from linoleic (LA) and α-linolenic (ALA) acid precursors (Figure 2; Sayanova and Napier, 2004), and as LA and ALA cannot be synthesized in mammals, they are by definition essential dietary fatty acids. LA and ALA (and their respective VLC-PUFA derivatives) are also commonly referred to as omega-6 (ω6) and omega-3 (ω3) fatty acids, as they contain double bonds located six or three carbons from the methyl (omega) end of the fatty acids, respec-

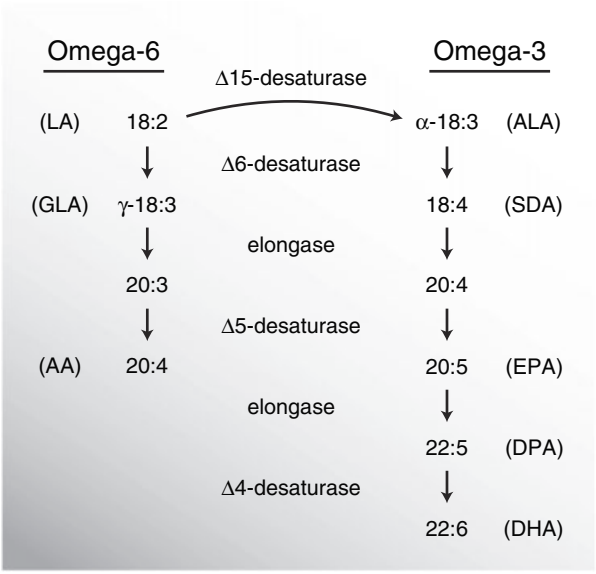

Figure 2. Production of nutritionally important, very-long-chain polyunsaturated fatty acids from linoleic and α-linolenic acid precursors.
Linoleic acid (LA, 18:2; an omega-6-type fatty acid) is converted into α-linolenic acid (ALA, α-18:3; an omega-3-type fatty acid) by a Δ^{15} fatty acid desaturase (curved arrow). As neither LA or ALA can be produced in mammals, they are considered essential dietary fatty acids. LA and ALA can be further desaturated and elongated (enzymes listed vertically between the relevant arrows) to produce a variety of omega-6- or omega-3-type VLC-PUFAs, including arachidonic acid (AA, 20:4), eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6). These fatty acids serve as important structural components of biological membranes (particularly in the brain and retina), or can be further metabolized to produce a variety of lipid signalling molecules that affect growth, development and physiological well-being. Production of VLC-PUFAs in mammals is inefficient, and these beneficial fatty acids are often obtained from other sources, such as fish oils. GLA, gamma-linolenic acid (γ-18:3); SDA, stearidonic acid (18:4); DPA, docosapentaenoic acid (22:5).

tively. As shown in Figure 2, LA is metabolized to produce the ω6 FAs γ-linolenic acid (GLA, 18:3$\Delta^{6,9,12}$) and AA, while ALA is converted to the ω3 FAs stearidonic acid (SDA, 18:4$\Delta^{6,9,12,15}$), EPA, docosapentaenoic acid (DPA, 22:5$\Delta^{7,10,13,16,19}$) and DHA. EPA and AA can be further metabolized in mammals to produce a group of hormone-like compounds called eicosanoids, and the eicosanoids derived from EPA are generally found to have more beneficial effects on cardiovascular health in comparison with those derived from AA (Lee and Lip, 2003). As the production of eicosanoids from EPA and AA is determined in part on availability of ALA and LA precursors, the ratio of ω6 to ω3 in the diet is an important consideration for human health and nutrition. The recommended ratio of ω6/ω3 fatty acids in the human diet is approximately 2:1 to 6:1 (Simopoulos, 2000; Wijendran and Hayes, 2004), and the much higher ratio of ω6 fatty acids in the typical Western diet (approximately 20:1) is thought to be a major contributor to cardiovascular disease (Simopoulos, 2000).

VLC-PUFAs are found in many food applications, including infant formulas, adult dietary supplements, animal feed and food additives, and are used as precursors for the production of pharmaceuticals. These applications represent an extensive market that is predicted to be in the range of US $1 billion (Domergue *et al.*, 2005). As an example of the growth potential, the world wholesale market for infant formula alone is estimated to be valued at $10 billion per year (Ward and Singh, 2005). There is only a very limited capability to synthesize VLC-PUFAs from LA or ALA in mammals (Ursin, 2003), and it is therefore important that they are obtained through the diet (especially AA, EPA and DHA). However, none of the VLC-PUFAs are normally produced in higher plants. Some mosses have been reported to contain AA and EPA (Girke *et al.*, 1998; Kaewsuwan *et al.*, 2006), but the main organisms responsible for producing the EPA and DHA present in the human diet are marine microalgae (Carlsson *et al.*, 2007). Fatty acids produced in these organisms make their way up the food chain to accumulate in fish oils, which are the primary source of these fatty acids for human nutrition. An increased intake of this food has been recommended as a way towards a more balanced ratio of ω6 to ω3 fatty acids. However, from the long-term perspective, this solution is hampered by the steady decrease in global fish populations (Sargent and Tacon, 1999) in combination with various problems associated with commercial fish farming (Naylor *et al.*, 2000). Generation of plants that produce high amounts of 'fish oil'-type fatty acids, therefore, would be highly desirable for sustainable production of these important fatty acids (Abbadi *et al.*, 2001; Ursin, 2003).

The potential for plant oils in the emerging bioeconomy

Both common and unusual fatty acids present in seed oils are capable of satisfying demand from a variety of existing markets currently served by petrochemical and specialty nutritional sources, and their use can also lead to the development of new market applications. However, the successful development of oleochemical-based products and nutritionally important fatty acids for global markets is critically dependent on the effectiveness and cost competitiveness of the strategies employed for the production of these industrial oils, as well as the development of knowledge-based approaches for their production in high-yielding oil crop species. Provided below is a description of our current understanding of seed oil production in several wild plant species that naturally produce industrially important seed oils, the challenges and potential in transferring these metabolic pathways to higher-yielding oil crop species, and remaining areas of research and emerging technologies that are required to establish the knowledge base and

molecular tools for producing high-value industrial oils in plant seeds.

Biosynthesis of high-value oils in native plant species and transgenic plants

General pathway of oil biosynthesis and its manipulation in engineered oilseed crops

The biosynthesis of seed storage oils containing the five major fatty acids occurs primarily in two subcellular compartments (Figure 3). The *de novo* synthesis of palmitic (16:0) and stearic (18:0) acids and the desaturation of stearic to oleic acid (18:1Δ^9) occur in plastids, with nascent fatty acids esterified to a small soluble protein called acyl carrier protein (ACP). These three fatty acids are then exported to the cytosol and into the acyl CoA and acyl-lipid pools. Oleic acid incorporated into phosphatidylcholine (PC) in the endoplasmic reticulum (ER; reaction A in Figure 3) is available for conversion to linoleic (18:2$\Delta^{9,12}$) and then α-linolenic (18:3$\Delta^{9,12,15}$) acid by the sequential action of substrate-specific desaturases (Somerville and Browse, 1991; Stymne and Stobart, 1987). A proportion of the resulting fatty acid products is retained as structural components of cellular membranes (e.g. phospholipids of the ER and galactolipids of plastids), and the rest are transferred to TAG by either (i) re-entry into the acyl CoA pool and attachment to all three positions of TAG by the consecutive actions of glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidic acid acyltransferase (LPAAT) and diacylglycerol acyltransferase (DGAT; also known as the 'Kennedy pathway'; reaction B in Figure 3; Griffiths *et al.*, 1985), (ii) direct transfer from phospholipids to diacylglycerol by an enzyme called phospholipid:diacylglycerol acyltransferase (PDAT; reaction C in Figure 3; Dahlqvist *et al.*, 2000; Ståhl *et al.*, 2004), or (iii) removal of the phospholipid headgroup to form DAG (reaction D in Figure 3), which can subsequently be utilized by DGAT or PDAT to form TAG (Stobart and Stymne, 1985).

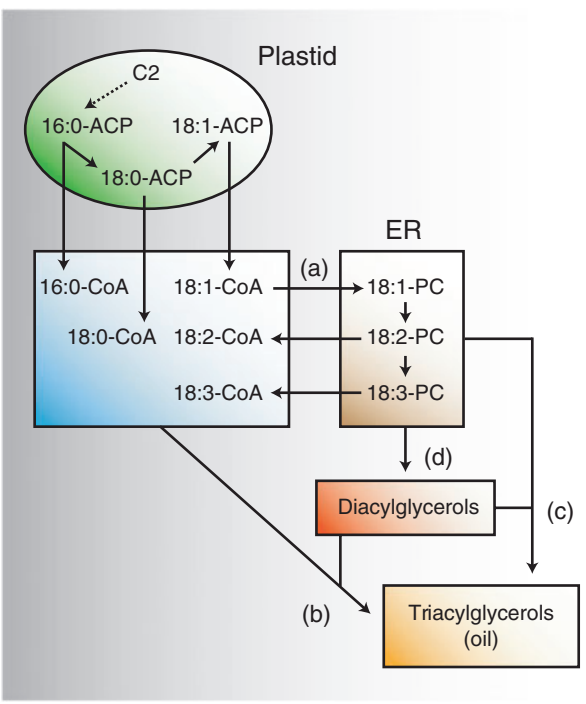

Figure 3. Main lipid classes and biochemical pathways involved in the production of storage oils containing the five common, nutritionally important fatty acids.
The first three fatty acids (16:0, 18:0 and 18:1) are produced during *de novo* synthesis of fatty acids in plastids, where fatty acid elongation and introduction of the first double bond occur while fatty acids are attached to acyl-carrier protein (ACP). Fatty acids are then exported to the acyl CoA pool, and oleic acid is transferred to the phosphatidylcholine (PC) fraction of the ER (reaction A) for production of 18:2 and 18:3 by fatty acid desaturases. The resulting five fatty acids may be incorporated into TAG by at least three potential routes, including (i) the traditional Kennedy pathway (reaction B), where fatty acids are transferred from the acyl CoA pool to all three positions of triacylglycerol, (ii) direct transfer of fatty acids from PC to diacylglycerol (reaction C) to produce TAG, or (iii) removal of the headgroup of PC to produce diacylglycerol (reaction D), which is subsequently consumed in reaction B or C to produce TAG.

All the genes involved in biosynthesis of the five common fatty acids are known, and a number of acyltransferase genes that are putative candidates for the reactions leading to the synthesis of TAG with these five fatty acids have been cloned. However, it should be noted that only one of these genes (DGAT1) has been shown unambiguously to be involved in TAG synthesis *in vivo* (Zhang *et al.*, 2005; Zou *et al.*, 1999). The uncertainty of the actual genes/enzymes involved in TAG synthesis and their relative contribution, even in the model plant Arabidopsis, are of course a major obstacle with regard to interpretation of the outcome of attempts to create transgenic plants with more exotic fatty acids, as described in the next section. Most domesticated oilseed crops, however, have been successfully modified through either breeding or genetic engineering approaches to optimize the ratio of endogenous fatty acids in the storage oil for specific end uses (reviewed by Drexler *et al.*, 2003). For example, suppression of the oleate Δ^{12}-desaturase gene (which normally converts 18:1 to 18:2) in soybean, sunflower, cotton and canola has resulted in the production of oils with a high oleic acid content, which have greater oxidative stability and improved performance in high-temperature cooking applications. Oils with a high oleic acid content are also desired by the chemical industry, as oleic acid can be used in a variety of applications including detergents, soaps, lubricants, cosmetics and emulsifying agents, and as a source of C9 monomers for plastics (Metzger and Bornscheuer, 2006).

Superimposed on the general pathway of TAG biosynthesis with common fatty acids (Figure 3) are other enzymatic steps leading to the accumulation of TAGs containing uncommon fatty acids in a number of plant species. We will

deal with only a few of these oil traits, where the biosynthesis and genetics have been studied in some detail and which are of particular interest for industrial or nutritional use.

Oils containing medium-chain fatty acids

Some plant species accumulate oil containing high amounts of medium-chain fatty acids, i.e. fatty acids with fewer than 16 carbons. Of these, the most commercially important oils are palm kernel and coconut oil, which contain predominantly lauric acid (12:0). Various species of the genus *Cuphea* also accumulate high amounts of medium-chain fatty acids in their seed oil, ranging from C8 to C14, usually with only one chain length dominating in any one species (Graham, 1989). Fatty acid chain length is determined during the *de novo* synthesis of fatty acids in plastids by the operation of chain-length-specific thioesterases (Dehesh *et al.*, 1998; Voelker *et al.*, 1992, 1997) that cleave the fatty acids from the growing acyl-ACP, thus terminating their elongation (reaction A in Figure 4) (Dörmann *et al.*, 1995; Jones *et al.*, 1995).

Biochemical investigations of TAG formation *in vitro* using microsomal membrane fractions from developing *Cuphea lanceolata* seeds, which are rich in caproate (10:0), indicate that *Cuphea* has two sets of acylation enzymes in the Kennedy pathway (Bafor and Stymne, 1992; Bafor *et al.*, 1990). One set of GPAT and LPAAT enzymes has specificity for medium-chain fatty acids in such a way that it promotes the formation of diacylglycerols with two medium-chain fatty acids, but very little mixed medium-chain/long-chain diacylglycerols. The *Cuphea* DGAT, the last step in TAG biosynthesis, has a very high selectivity towards di-medium-chain diacylglycerols (Wiberg *et al.*, 1994). The other GPAT and LPAAT enzymes are specific for long-chain acyl groups and produce diacylglycerols that contain only long-chain fatty acids. As the *Cuphea* DGAT has such high selectivity for medium-chain diacylglycerols and removes these substrates from the DAG pool, the remaining DAGs are enriched in long-chain acyl groups that are utilized for membrane phospholipid synthesis. Further, the *Cuphea* seed membranes have phospholipases that selectively hydrolyse medium-chain fatty acids out of membranes (Ståhl *et al.*, 1995). Thus, combination of the selective removal of medium-chain fatty acids from the diacylglycerol pool and the activity of the specific phospholipases efficiently prevents these fatty acids from accumulating in organelle membranes. In this context, it is important to mention that medium-chain fatty acids, like most unusual fatty acids found in seed oils, occur in very low abundance in the membrane lipids of the seeds, whereas they can be the dominating fatty acid in TAG. It is anticipated that the presence of these unusual fatty acids in the membrane lipids of transgenic plants would severely perturb membrane function of the cell due to the disruptive physical properties induced by these fatty acids compared to the normal fatty acid composition. There are several exceptions to this general observation, however, such as accumulation of relatively high amounts of γ-linolenic (18:3$\Delta^{6,9,12}$) and stearidonic (18:4$\Delta^{6,9,12,15}$) acids in leaf lipids of certain *Primula* species (Sayanova *et al.*, 1999) and in the phospholipids of certain engineered plants (Abbadi *et al.*, 2004; Napier, 2007), with no apparent negative effects on membrane function and integrity. Moreover, not all unusual fatty acids that are edited out from the membrane lipids are bilayer-disturbing fatty acids. For example, crepenynic acid, an acetylenic fatty acid, has a very similar three-dimensional structure as oleic acid but is not present at all in the seed membrane lipids of the plant species that accumulate them to very high levels in the seed oil (Thomaeus *et al.*, 2001).

One of the earliest successes in engineering unusual fatty acid composition in oilseeds involved the production of oils with a high lauric acid (12:0) content in Arabidopsis and rapeseed. Transgenic expression of a laurate-specific acyl-ACP thioesterase gene from the California bay tree (*Umbellularia californica*), firstly in Arabidopsis (Voelker *et al.*, 1992) and subsequently in rapeseed (Wiberg *et al.*, 2000), led to the accumulation of over 50% laurate in the seed TAG. Analysis of triglycerides in the transgenic rapeseed oils, however, revealed that laurate was present in high amounts at the *sn*-1 and *sn*-3 positions, but was very poorly incorporated at *sn*-2. To overcome this limitation, the gene for a laurate-specific LPAAT obtained from coconut (a high-

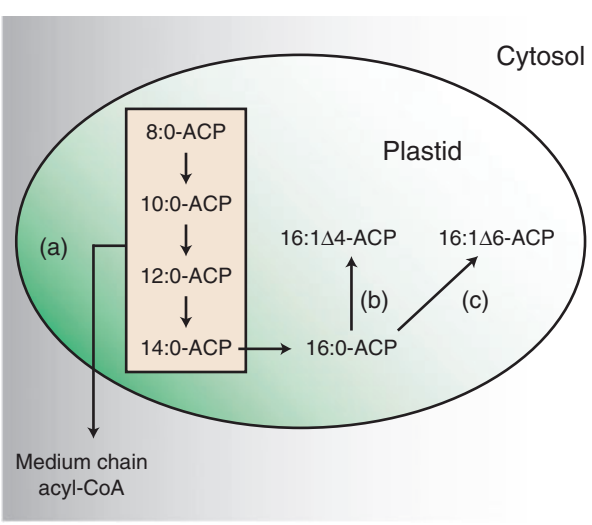

Figure 4. Model for the production of short- and medium-chain fatty acids. Short- and medium-chain fatty acids (8:0–14:0; boxed area) are released from the growing acyl-ACP chain during fatty acid synthesis by specific thioesterase enzymes (reaction A), thereby terminating the elongation process and making these fatty acids available for incorporation into TAG. In some plants, 16:0-ACP is further modified by a soluble enzyme (representing a diverged form of the soluble desaturase that is typically involved in conversion of 18:0-ACP to 18:1Δ^9-ACP) that introduces a double bond at the Δ^4 or Δ^6 position (reactions B and C, respectively). These fatty acids may be further elongated or directly incorporated into TAG.

laurate oil source) was also introduced and resulted in the synthesis of oils with significant incorporation of laurate at the *sn*-2 position, which further increased the total laurate levels up to 67% (Knutzon *et al.*, 1999). Similar attempts to produce rapeseed oils that were rich in other more valuable medium-chain fatty acids such as caprylic (8:0) and capric (10:0) acids yielded up to 8% and 30% of these fatty acids in seed oil, respectively (Wiberg *et al.*, 2000). These levels of accumulation are significantly lower than that observed for production of lauric acid in transgenic plants, and also much lower than the amounts observed in the native plant species. The bottleneck in the accumulation of capric and caprylic acids in transgenic plants seems to be an inability to acylate them efficiently to the glycerol backbone. Evidence for this bottleneck is that these fatty acids dominate the fatty acyl CoA pool in the developing seeds of transgenic plants, whereas they are minor components of this pool in the native plants that accumulate them (Larson *et al.*, 2002). The build-up of medium-chain fatty acids in the acyl CoA pool induces β-oxidation of these fatty acids (Poirier *et al.*, 1999), and thus creates a futile cycle in which much more medium-chain fatty acid is produced than accumulates in TAG.

Unusual monoenoic fatty acids

Among species that accumulate unusual monoenoic C16 or C18 fatty acids in their seed oil and that have been the subject of both biochemical and genetic analysis are coriander (*Coriandrum sativum*), with 80% petroselinic acid (18:1Δ^6) in its seed oil, and *Thunbergia alata*, with about 80% 16:1Δ^6. Petroselinic acid and 16:1Δ^6 can be cleaved at the double bond to yield adipic acid, a dicarboxylic acid that is used as a component in many plastics that is currently synthesized from fossil oil. Both petroselinic acid and 16:1Δ^6 fatty acids are synthesized by acyl-ACP desaturases related to the common stearoyl ACP Δ^9-desaturase that produces oleic acid (Cahoon *et al.*, 1992, 1994). The genes for these divergent desaturases have been cloned from both species and shown to be palmitoyl-ACP (16:0-ACP) desaturases. In the case of the coriander desaturase, the double bond is inserted at the Δ^4 position of 16:0-ACP, and the fatty acid is then elongated to 18:1Δ^6, whereas, in *T. alata*, the enzyme is a Δ^6-desaturase (Cahoon *et al.*, 1992, 1994; reactions B and C in Figure 4). The channelling of petroselinic acid into TAG, once exported from the plastid, appears to occur via a small phospholipid pool, and involves poorly understood biochemical reactions (Cahoon and Ohlrogge, 1994).

Attempts to genetically engineer high levels of medium to long-chain (C14–C18) monoenoic fatty acids have so far met with little success, despite considerable effort. Separate seed-specific expression in Arabidopsis of genes encoding the 16:0-ACP Δ^4-desaturase from coriander, the 16:0-ACP Δ^6-desaturase from *Thunbergia alata*, and the 14:0-ACP Δ^9-desaturase from geranium [*Pelargonium* × *hortorum*] each resulted in accumulation of monoene products at relatively low levels, ranging from 1 to 15%, compared to the > 80% levels present in the species from which the genes were sourced (Suh *et al.*, 2002). It was concluded that actual synthesis of the monoenes may be the limiting factor, and it was postulated that this was caused by failure of the introduced acyl-ACP desaturase to correctly interact with other components of the plastidic enzyme machinery. It is interesting to note that in both coriander and *T. alata* seeds, the unusual monoenes appear to transit through PC prior to incorporation into TAG (Cahoon and Ohlrogge, 1994; Schultz and Ohlrogge, 2000), similar to what has been shown recently for the common fatty acids (Bates *et al.*, 2007).

In addition to the 16:0-ACP Δ^4-desaturase, coriander has a whole set of specialized proteins in the plastid in order to efficiently synthesize petroselinic acid, such as specialized ACP, ferredoxin, 3-ketoacyl-ACP synthase and thioesterase (Suh *et al.*, 2002). The accumulation of petroselinic acid in coriander seeds demonstrates that some plants accumulating unusual oils have recruited a large number of specialized genes in order to achieve this. This raises interesting questions with regard to how these genes have co-evolved, and also what advantage this oil has conferred in order to drive plant selection in this direction. Plant seeds are known to contain a variety of anti-physiological compounds that discourage animal predation, including protease inhibitors, poorly digestible seed storage proteins (often lacking essential amino acids), unusual sugars that disrupt the gastrointestinal tract, toxins etc, and perhaps seed oils containing high amounts of unusual fatty acids represent another facet of this protective process. Certain fatty acids (and their secondary metabolites) are known to have protective effects against fungi, spider mites and aphids (Cahoon *et al.*, 2003; Schultz *et al.*, 1996), and the ability of castor oil to induce colitis in both animals and humans is well recognized. Voelker and Kinney (2001) have further suggested that certain structural features of fatty acids, such as medium chain length or storage as wax esters, might allow more rapid mobilization or enhanced stability at elevated temperatures, respectively, which could provide a competitive edge under certain environmental conditions. It is also possible that the accumulation of unusual fatty acids does not confer a selective advantage or disadvantage, but rather is something that just occurred. For example, the main evolutionary pressure on seed oils is to serve as a reservoir of reduced carbon and energy for germinating seedlings, and the structural features of fatty acids are less important with regard to this function. This is in contrast to biological membranes, where the structural properties of fatty acids are essential for determining the integrity and fluidity of the lipid bilayer, and thus the fatty acid composition of membranes is strictly conserved. Expression of genes for unusual fatty acid synthesis in transgenic plants may result in

accumulation of the unusual fatty acids in biological membranes, which can impair seed development and subsequent germination rates (Cahoon *et al.*, 2006). This observation may provide a rationale for the co-evolution of genes that promote exclusion of unusual fatty acids from biological membranes and the channelling of these fatty acids into TAG. Regardless of the exact mechanism(s) that drives this evolutionary process forward, the ability of certain plants to accumulate high amounts of unusual fatty acids in their seed oil provides an important precedent for attempts to rationally engineer these traits into high-yielding oilcrop species.

Unusual fatty acids produced in the cytosol

Most of the unusual fatty acids found in the seed oils of various plants are synthesized in the cytosolic compartment using common fatty acid precursors exported from the plastid (Figures 1 and 5). These reactions include elongation of fatty acids to very long chain lengths (i.e. longer than C18), and one such fatty acid, erucic acid (22:1Δ^{13}), is dominant in the seed oil of many Brassicaceae species. These elongation steps are performed while the acyl group is attached to CoA in a reaction sequence analogous to those of *de novo* fatty acid synthesis in plastids, except that all of the extra-plastidial enzyme activities are membrane-bound, whereas in the plastid they are soluble and utilize acyl-ACP as substrates (Domergue *et al.*, 2000).

For industrial use, erucic acid must be obtained in high concentration in order to reduce purification costs. Thus, increasing the erucic acid content in *Brassica napus* (rapeseed) by genetic engineering has been a goal for several groups. Rapeseed oil contains erucic acid predominantly in the outer (*sn*-1 and *sn*-3) positions of the triglyceride, which limits the accumulation of erucic acid to 66%. It was therefore assumed that the rapeseed LPAAT enzyme responsible for the acylation at the *sn*-2 position lacks specificity for erucic acid. An LPAAT gene from *Limnanthes alba*, encoding an enzyme that is known to acylate erucic acid at the *sn*-2 position, was transgenically expressed in a rapeseed with high erucic acid content, but only a small increase in the total percentage of erucic acid was obtained, despite redistribution of erucic acid from the outer to the middle position of TAGs (Lassner *et al.*, 1995; Weier *et al.*, 1997). However, when transgenic plants expressing the *Limnanthes* LPAAT gene and an extra copy of the rapeseed *FAE1* elongase gene were crossed with a rape variety with high oleic acid content, individual seeds with over 70% erucic acid were obtained (Nath *et al.*, 2006). Thus, it can be concluded that, provided there is sufficient erucic acid synthesis, the inactivity of the rapeseed LPAAT with respect to erucic acid was the only endogenous metabolic impediment to high-level accumulation in TAG.

Fatty acids with additional functional groups

Fatty acids with additional functional groups in the fatty acid chain represent excellent feedstocks for industry, and some plant species are known to accumulate high amounts of hydroxylated, epoxidated, conjugated or acetylenic fatty acids in their seed oils (Table 1). Of these plants, only the tung tree (*Vernicia fordii*), yielding conjugated fatty acids, and castor bean, yielding hydroxylated fatty acids, are cultivated on a commercial scale. In most cases, these fatty acids are formed by enzymes related to, and probably evolved from, the FAD2 oleoyl-PC Δ^{12}-desaturase, which is responsible for production of the common fatty acid linoleic acid (Cahoon *et al.*, 1999; Dyer *et al.*, 2002; Lee *et al.*, 1998; van de Loo *et al.*, 1995). Although all of these fatty acids are synthesized while bound to PC (Figure 5), they are rapidly and specifically removed from this phospholipid and channelled to TAGs, a process that has been suggested to be performed by acyl-specific phospholipases (Ståhl *et al.*, 1995), PDAT (Dahlqvist *et al.*, 2000) and/or an enzyme (possibly phospholipase C or the reverse activity of choline phosphotransferase) that converts phosphatidylcholine into diacylglycerol (a substrate for subsequent production of TAG). As the common linoleic and linolenic acids are also synthesized while bound to PC and subsequently transferred to TAGs, the question arises as to whether the unusual fatty acids synthesized on PC utilize variants of these same

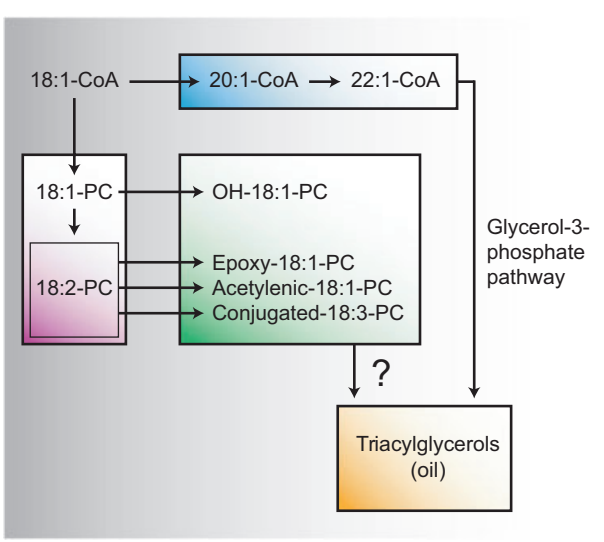

Figure 5. Production of industrially important fatty acids from oleic or linoleic acid.
Oleoyl CoA (18:1-CoA) may be elongated in the cytosol to produce very-long-chain fatty acids (20:1- or 22:1-CoA), which can be transferred into TAG by the acyl CoA-dependent acyltransferases of the glycerol-3-phosphate pathway (Kennedy pathway). Oleic acid may also be transferred into the PC fraction of the ER for conversion to linoleic acid by a membrane-bound oleate Δ^{12}-desaturase. Many plants contain a divergent form of the Δ^{12}-desaturase that is capable of producing unusual fatty acids such as hydroxy, epoxy, acetylenic or conjugated fatty acids. The transfer of unusual fatty acids from PC to TAG is significantly more complex than for the common fatty acids, and, as such, details of the relevant enzyme activities are only beginning to emerge.

enzymes or whether specialized enzyme reactions have evolved. It has been suggested that lysophosphatidylcholine acyltransferases (LPCAT) working in both forward and reversible modes are responsible for the channelling of oleic acid into PC for subsequent desaturation, and the transfer of linoleic and linolenic acid products out of PC into the acyl CoA pool (Stymne and Stobart, 1984). The recent identification of Arabidopsis LPCAT genes might allow this hypothesis to be tested experimentally (Ståhl *et al.*, 2007).

When genes encoding divergent FAD2 enzymes have been expressed transgenically in Arabidopsis and oilseed plants, they generally result in relatively low levels of accumulation of the unusual fatty acid, usually <20% compared to the 60–90% typically found in the source species (Broun and Somerville, 1997; Cahoon *et al.*, 1999; Lee *et al.*, 1998). Interestingly, oleic acid content is considerably increased in all these transgenic plants (Cahoon *et al.*, 2006; Singh *et al.*, 2000; Thomaeus *et al.*, 2001), suggesting that production of the unusual fatty acids in some way inhibits the desaturation of oleic to linoleic acid. This is problematic for the production of many unusual fatty acids in transgenic plants, as linoleic acid is the direct precursor used for their synthesis. In Arabidopsis plants expressing the *Crepis palaestina* Δ^{12}-epoxygenase gene (which produces an epoxy fatty acid called vernolic acid), this depression in Δ^{12}-desaturation could be alleviated by down-regulating the activity of enzymes competing for the substrate and also by expressing additional Δ^{12}-desaturase activity, and this resulted in an increase in vernolic acid accumulation from 6 to 21% (Zhou *et al.*, 2006). Nonetheless, even with high levels of substrate availability, accumulation remains at relatively low levels.

It is notable that the levels of unusual fatty acids present on PC in the transgenic plants is substantially higher than that observed in the source plants (Cahoon *et al.*, 2006; Thomaeus *et al.*, 2001), supporting the general contention that accumulation of these fatty acids in transgenic plants is primarily limited by their inefficient removal from PC as well as their probable inefficient passage through the Kennedy pathway. The eventual route to high-level accumulation of these unusual fatty acids in transgenic oilseeds might therefore involve the discovery and introduction of genes encoding specialized forms of the key enzymes such as phospholipases (Bafor *et al.*, 1991; Ståhl *et al.*, 1995), LPCAT (Ståhl *et al.*, 2007; Stymne and Stobart, 1984), PDAT (Dahlqvist *et al.*, 2000) and DGAT (Shockey *et al.*, 2006). It has already been shown that the DGAT enzymes in *Vernonia galamensis*, castor bean and tung seed have preferences for transacylating vernolic acid onto di-vernoleoyl DAG (Yu *et al.*, 2006), ricinoleic acid onto di-ricinoleoyl DAG (Kroon *et al.*, 2006), and eleostearic acid onto di-eleostearoyl DAG (Shockey *et al.*, 2006), respectively. It is therefore likely that transgenic expression of appropriately specialized DGATs will enhance the synthesis of TAGs that are rich in unusual fatty acids. It is entirely feasible that similar specialization exists in many, if not all, of the key enzymes involved in accumulation of unusual fatty acids in TAG, and thus the multigenic transfer of a specialized 'metabolome' of enzymes may ultimately be necessary to achieve very high level accumulation in transgenic oilseeds.

Wax esters

The seeds of the desert shrub jojoba (*Simmondsia chinensis*) accumulate oil in the form of liquid wax esters instead of the usual TAGs. Wax esters are esters between a fatty acid and a fatty alcohol, and their biosynthesis includes reduction of a fatty acid to a primary alcohol, catalysed by a fatty acid reductase, and then esterification of a fatty acid from acyl CoA to this alcohol, which is performed by a wax synthase. Jojoba seeds convert mainly C20 and C22 mono-unsaturated fatty acids to fatty alcohols and esterify them to C20 and C22 carbon fatty acids to form the wax esters (Pollard *et al.*, 1979). Both wax synthase and fatty acid reductase have been purified from developing jojoba seeds and their amino acid sequences utilized to clone the corresponding genes (Lardizabal *et al.*, 2000). Introducing these genes into Arabidopsis led to the production of a high amount of wax esters in the transgenic seeds (Lardizabal *et al.*, 2000). Given the initial successful report of production of wax esters in transgenic plants and the high value of the product, it is surprising that no further attempts to produce these compounds in transgenic seeds have been reported. Wax esters also provide an as yet unexplored potential for accumulation of unusual fatty acids in combination with industrially useful alcohols rather than as triglycerides.

Very-long-chain, nutritionally important polyunsaturated fatty acids

Considerable efforts have been devoted to produce AA, EPA and DHA in transgenic plants and have met with highly encouraging success (Abbadi *et al.*, 2004; Kinney *et al.*, 2004; Qi *et al.*, 2004; Robert *et al.*, 2005; Wu *et al.*, 2005). Contrary to the situation in which the altered lipid metabolism of the transgenic plants is intended to mimic the metabolism of lipids produced in other plant seeds, most attempts to produce VLC-PUFA mimic the lipid metabolism in marine algae or animals. The production of VLC-PUFA consists of cycles of alternating desaturation and chain elongation, in which the desaturation reactions occur on acyl-PC substrates and the elongation reactions occur on acyl CoA substrates (Figure 1). This necessitates an acyltransferase-mediated switch of the acyl group between substrate pools at each step, and while the precise mechanisms for this shuttling are unknown, LPCAT enzymes are considered likely mediators as is the case with the common linoleic and linolenic acids (Domergue *et al.*, 2005).

Alternatively, it may be possible to utilize enzymes that desaturate acyl CoAs, rather than acyl-PC substrates, which would circumvent the need to move the fatty acid between the PC and CoA pools (Sayanova *et al.*, 2007). An additional challenge to production of VLC-PUFAs in plants is that these fatty acids are known to naturally accumulate in both the membrane lipid and TAG of microalgae, and whether a similar accumulation of these fatty acids in seed oils of plants will be detrimental to agronomic performance remains to be determined. Nevertheless, the transfer of up to nine genes to plants to catalyze the production of VLC-PUFAs represents a significant step forward in the field of plant lipid metabolic engineering. For more information, readers are directed to two recent review articles (Graham *et al.*, 2007; Napier, 2007).

An alternative approach to the production of VLC-PUFA in plants that may bypass the accumulation of these fatty acids in membrane lipids is to introduce an entirely different biosynthetic process that is observed in some bacteria. These bacteria synthesize VLC-PUFA not via normal fatty acid synthesis but rather through a polyketide pathway, producing only the end product and with no intermediates that could enter the membrane lipid system (Metz *et al.*, 2001). It remains to be seen whether this pathway for synthesis can be successfully transferred into plants by genetic engineering.

Strategy for obtaining added-value oils in transgenic plants

Developing a better understanding of the cellular and regulatory aspects of oil biosynthesis

We have described the biosynthesis of various unusual fatty acids in plants and have pinpointed the enzyme reactions involved and described whether these reactions occur in the plastid or outside. However, this is a simplistic and possibly misleading depiction, as it envisages that all enzymes in a certain compartment will have access to all substrates present in this compartment. In reality, there are likely to be very specific metabolic channelling activities, for example involving sub-domains of the ER. It has recently been shown that nearly all palmitic and oleic acid exported out from the plastid is first acylated to PC, presumably by transfer to lysophosphatidylcholine, and thus these acids do not enter the glycerol-3-phosphate pathway directly (Bates *et al.*, 2007). Consequently, these fatty acids have to be released into the acyl CoA pool for net synthesis of lipids via the Kennedy pathway, probably at a site different from the acylation of PC. This suggests that there are several acyl CoA pools outside the plastid, and certain pools are available only for certain enzymes. The demonstration that DGAT1 and DGAT2 from tung tree, enzymes that both catalyze the acylation of diacylglycerols to TAGs, are localized at distinct sites in ER membranes is another indication that there is a spatial separation of lipid-metabolizing enzymes that is probably essential for correct channelling of the substrates into the final oil reserve (Shockey *et al.*, 2006). This compartmentalization has important implications with regard to the possibilities for creating transgenic plants with desired oil qualities that have largely been neglected in the past and require significantly more research.

In addition to knowing about the presence and importance of such sub-domains, it is also important to understand the regulation of associated enzyme activities. While gene expression is an essential first step for conferring a desired oil trait, targeting and accumulation of the encoded protein in the correct location is also essential for enzyme activity. Studies of both fatty acid desaturases (Horiguchi *et al.*, 2000; Tang *et al.*, 2005) and DGAT1 enzymes (He *et al.*, 2004) have indicated that these proteins are regulated at the post-transcriptional level, and developing a better understanding of the regulatory mechanisms of oil biosynthetic enzymes should increase the effectiveness of their utilization in engineered, transgenic systems (Dyer and Mullen, 2008).

Emerging technologies for sensitive metabolic flux analysis

The field of plant lipid biochemistry is deeply rooted by radiolabeling tracer studies that provide powerful methods for understanding the inter-relationships of lipid metabolites and metabolic pathways in plant cells. There is a need to return to these types of studies to help understand the complex, nonlinear pathways of storage oil formation and fatty acid channelling in both native and engineered plant species. The development of advanced metabolic flux analyses using various precursors labelled with radioactivity or heavy stable isotopes, and measurement of label, including isotopomers, in various metabolite pools has provided new insights into central carbon metabolism in plants (Allen *et al.*, 2007; Alonso *et al.*, 2007). Importantly, these studies can be carried out in unperturbed whole plants, providing an unprecedented view of metabolic flux under *in vivo* conditions (Romisch-Margl *et al.*, 2007). The value of these types of experiments is clearly demonstrated by a recent study of fatty acid flux from plastids to PC, which demonstrates that fatty acids are transferred directly to the PC fraction rather than first being incorporated into glycerol-3-phosphate to produce phosphatidic acid (Bates *et al.*, 2007). These data create a shift in our understanding of glycerolipid metabolism, and the identification of enzymes involved in this 'acyl-editing' mechanism is awaited with great anticipation.

A second technological advancement that provides an unprecedented level of detail with regard to plant lipid metabolism is the usage of mass spectrometry-based techniques for identification of individual lipid molecular species. 'Lipidomic' profiling has now been developed for each of the major classes of lipids involved in storage oil production, including phospholipids (Devaiah *et al.*, 2006),

fatty acyl CoAs (Larson and Graham, 2001) and TAGs (Leskinen *et al.*, 2007), although determining the positional specificity of fatty acids on the glycerol backbone remains a challenge. Unlike the fatty acid composition of common vegetable oils, which may be limited to five or six individual fatty acids (Table 1), the individual glycerolipid species comprising phospholipids, TAGs and galactolipids in seeds are far more complex, with hundreds of lipids (containing various combinations and positional distributions of fatty acids) representing the collective activities of many acyltransferase enzymes. Knowledge of these individual molecular species will provide insight into how plants respond to transgene expression for oil synthesis, and also allow more sensitive analysis of enzyme function, for example through knockout of putative acyltransferase enzymes. Furthermore, utilization of heavy isotopes for pulse–chase analysis should provide an unprecedented view of fatty acid trafficking during storage oil synthesis, which will guide subsequent attempts to identify key enzymes that are important for channelling of fatty acids into storage oil.

The power of pyrosequencing technology and the integration of genomics and proteomics strategies for gene discovery

While many of the plants that produce industrially important fatty acids are poorly characterized at the genomic level, generation of expressed sequence tag (EST) libraries from seeds that are actively engaged in oil synthesis has proven to be a successful strategy for identification of genes involved in industrial oil production (Cahoon and Kinney, 2005). The impact of this approach is likely to be greatly expanded with the development of massively parallel pyrosequencing technology, which provides much greater coverage of DNA sequences. Pyrosequencing has recently been evaluated as a strategy for sequencing the genomes of barley (Wicker *et al.*, 2006), soybean (Swaminathan *et al.*, 2007) and *Medicago truncatula* (Cheung *et al.*, 2006), and also for generating EST libraries from maize (Emrich *et al.*, 2007) and Arabidopsis (Weber *et al.*, 2007). The latest pyrosequencing technology generates reads of up to 500 bp, which helps to alleviate the problem of building contigs that was encountered with earlier machines (Mashayekhi and Ronaghi, 2007). The sequences obtained from pyrosequencing technology provide significant insight into gene expression in a given tissue, and the relatively low cost and short time associated with pyrosequencing analysis suggest that this technology will be widely utilized for gene discovery programs in many plant species.

Availability of transcriptome information will not only assist in the identification of divergent gene family members, but will also provide a robust database for aiding proteomic analysis through peptide mass fingerprinting. As a result, we will soon observe a convergence of genomic and proteomic approaches for elucidating previously unknown aspects of storage oil biosynthesis. No industrial crop is currently better suited for this type of analysis than castor bean. With the recent completion of a fourfold coverage of the castor genome, which is publicly available at the TIGR website (http://castorbean.tigr.org/), and an in-depth analysis of seed-specific ESTs derived from full-length cDNAs (Lu *et al.*, 2007), there is now an unprecedented level of detail available regarding the genes involved in production of one of the most highly sought after, naturally produced industrial oils in the marketplace (castor oil). Furthermore, proteomic strategies have already been developed for analysis of proteins derived from the ER of castor bean (Maltman *et al.*, 2002), and these studies could be greatly aided by studying specific protein complexes that might be associated with key proteins known to be involved in castor oil biosynthesis (e.g. oleoyl-PC Δ^{12}-hydroxylase, DGAT2). Although castor bean has not yet been stably transformed (which would permit the introduction of affinity-tagged 'bait' proteins), new methods have been developed to assist in membrane protein complex analysis, including rate-zonal centrifugation of large protein complexes (Hartman *et al.*, 2007) and 'top-down' mass spectrometry of integral membrane proteins (Whitelegge, 2004; Whitelegge *et al.*, 2006). Collectively, these approaches will greatly assist in the identification of proteins involved in castor oil biosynthesis, and should provide significant insight into the production of industrially important oils in the seeds of plants.

Conclusions

Plant oil (TAG) is the bioproduct that is chemically most similar to fossil oil, and therefore has the greatest potential to replace it in the chemical industry. In fact, fossil oil is believed to be derived from ancient, lipid-rich organic material, such as spores and planktonic algae, sedimented and transformed under high pressure and temperature over millions of years (Hunt *et al.*, 2002). There is increasing appreciation that the production of TAG in plant cells is far more complex than the simple linear pathway represented by the traditional Kennedy pathway (Napier, 2007). Elucidation of enzyme activities responsible for controlling the flux of fatty acids between phospholipid and acyl CoA pools, in particular, will improve not only the transfer of unusual fatty acids into storage oils, but will also assist in the rational improvement of pathways for VLC-PUFA production, where enzymes act upon fatty acid substrates attached either to phospholipids or CoA (Abbadi *et al.*, 2004). A major challenge that has only just started to receive attention is the spatial organization of the lipid biosynthetic machinery within the various

subcellular compartments. Until a better picture of the metabolic channelling occurring within the cell is obtained, the full potential of genetic engineering of plant oil quality cannot be realized.

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