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Review article

Metabolic engineering of plant oils and waxes for use as industrial feedstocks

Thomas Vanhercke[1,*], Craig C. Wood[1], Sten Stymne[2], Surinder P. Singh[1] and Allan G. Green[1]

[1]*CSIRO Plant Industry, Canberra, ACT, Australia*

[2]*Swedish University of Agricultural Science, Alnarp, Sweden*

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**Correspondence* (Tel +61 2 6246 4913; fax +61 2 6246 4950; email Thomas.Vanhercke@csiro.au)

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Summary

Society has come to rely heavily on mineral oil for both energy and petrochemical needs. Plant lipids are uniquely suited to serve as a renewable source of high-value fatty acids for use as chemical feedstocks and as a substitute for current petrochemicals. Despite the broad variety of acyl structures encountered in nature and the cloning of many genes involved in their biosynthesis, attempts at engineering economic levels of specialty industrial fatty acids in major oilseed crops have so far met with only limited success. Much of the progress has been hampered by an incomplete knowledge of the fatty acid biosynthesis and accumulation pathways. This review covers new insights based on metabolic flux and reverse engineering studies that have changed our view of plant oil synthesis from a mostly linear process to instead an intricate network with acyl fluxes differing between plant species. These insights are leading to new strategies for high-level production of industrial fatty acids and waxes. Furthermore, progress in increasing the levels of oil and wax structures in storage and vegetative tissues has the potential to yield novel lipid production platforms. The challenge and opportunity for the next decade will be to marry these technologies when engineering current and new crops for the sustainable production of oil and wax feedstocks.

Introduction

Modern society relies heavily and unsustainably on petroleum as a source of many industrial products ranging from fuels and lubricants to specialty chemicals and plastics. Petroleum reserves are finite and nonrenewable, and their widespread use is contributing heavily to undesirable increases in atmospheric CO_2 levels (Le Quéré *et al.*, 2009). As environmental concerns mount and supply constraints emerge, alternative bio-based raw materials to support these industrial needs are gaining increasing strategic importance.

Plant oils represent one of the main opportunities to provide environmentally friendly, renewable and sustainable feedstocks that can potentially substitute for petroleum in many industrial applications. To date, most of the focus has been on using plant oils for biodiesel, providing an alternative source of energy particularly for liquid transport fuel applications. Unfortunately, in spite of their technical suitability for this purpose, and thus their usefulness as a transition technology, in the longer-term plant oils cannot be produced on sufficient scale to make a major contribution to the immense global demand for transport fuels (approx 1.5 billion metric tonnes/year). In the long term, this need must instead be addressed by a mix of other approaches, including greater use of a range of non-carbon-based renewable energy technologies (such as hydro, wind and solar) that are both sustainable and scalable. Petroleum, however, is not only used for energy but is also the primary source of a diverse array of carbon-based molecules (petrochemicals) that are the foundation of the industrial chemical and polymer sectors and that have much greater unit value than hydrocarbon fuels. Diminishing petroleum availability will also impact heavily on the manufacture of these products, and biological carbon is the only alternative raw material. Consequently, a strong resurgence in interest in bio-based alternatives to petrochemicals is now well underway at both the research and the commercial levels.

Crop plants offer substantial potential to provide renewable sources of industrial chemicals through the synthesis and accumulation of specific industrial target molecules in plant vegetative or storage tissues ('crop biofactories'), coupled with subsequent postharvest thermochemical and/or enzymatic conversion ('biorefineries'). Although a wide range of bio-based molecular structures have potential industrial use, lipids—in particular fatty acids, oils and waxes—are especially well suited as replacements for petrochemicals because of the analogous linear carbon chain structures, providing close molecular equivalents or functional equivalents to many existing mainstay industrial feedstock chemicals. Furthermore, global plant oil production, although predominantly for food use, is currently approaching 160 million metric tonnes annually (Oil World Annual, 2012), which equates to around a third of the size of petrochemical production. Importantly, there are now substantial technological opportunities, through metabolic engineering, to greatly increase plant oil production beyond that required to meet future increased food demand and thus to create a significant surplus for deployment into industrial use (Carlsson *et al.*, 2011). Industrial plant oils and waxes could therefore, in the future, feasibly be produced on a scale that could make significant inroads as renewable petrochemical replacements.

In this context, Carlsson *et al.* (2011) have recently framed a technological challenge of trebling global plant oil production over the next two decades to provide sufficient oil to replace 40% of petrochemical usage, without compromising the supply of

plant oils for the food sector. To reach this goal, it will be necessary to develop some of our major oil plants to produce industrial oil compositions that go beyond their natural within-species variation. It will also be necessary to greatly expand plant oil production possibilities and levels, by dramatically increasing oil productivity in current oil crops and by developing and introducing novel oil production platforms. In each of these pursuits, metabolic engineering of plant lipid biosynthesis will clearly play a central role.

Current industrial use of plant oils and waxes

Plant oil production is dominated by the major food oil crops, including oil palm, soybean, rapeseed (canola), sunflower, cottonseed, peanut (groundnut), corn and olive, along with a number of minor sources such as safflower, sesame, coconut and linseed. The majority of these food oils are comprised of only five fatty acids palmitic (C16:0), stearic (C18:0), oleic (C18:$1^{\Delta 9}$), linoleic (C18:$2^{\Delta 9,12}$) and α-linolenic acid (C18:$3^{\Delta 9,12,15}$). However, although global plant oil production is heavily directed towards food use, a significant proportion (around 20%) is already used for industrial (nonfood) applications. One major industrial utilization of plant oils involves medium-chain fatty acids, predominantly laurate (C12:0) from palm kernel oil and coconut oil, in surfactant applications, such as in soaps, detergents and related personal care products. Other food oils that are highly unsaturated, such as linseed and soybean, also find uses as renewable alternatives for synthetic drying agents in surface coatings and inks and are also processed into epoxygenated oils for use in industrial resins and glues.

In addition to these multipurpose oils, there are a few specialty industrial oils obtained from more minor oil plants, such as castor oil, tung oil and high-erucic rapeseed oil. Castor oil is obtained from the castor bean plant (*Ricinus communis*), a difficult crop to manage because of the presence of the highly toxic ricin protein and other allergenic compounds. Castor oil accumulates in the endosperm and contains very high levels (90%) of the hydroxy fatty acid, ricinoleic acid (12-hydroxy C18:$1^{\Delta 9}$). Ricinoleic acid is an increasingly important industrial feedstock as it can undergo pyrolytic cleavage at the reactive Δ12 position to yield undecylenic acid, an alpha olefin used for the production of the polyamide Nylon 11. Castor oil also finds direct use for bio-based polyols in the production of a range of products including polyurethane and as a high-performance lubricant.

Tung oil (or China wood oil) is a highly valued drying oil obtained from the nut of the tung tree (*Vernicia fordii*). Its unique drying properties, highly valued for furniture protection, result from its very high content (82%) of the conjugated fatty acid α-eleostearic acid (C18:$3^{\Delta 9c,11t,13t}$). As for castor, the prospects for expansion of tung production are limited by its restricted agronomic range.

Erucic acid (C22:$1^{\Delta 13}$) is used to produce erucamide, an important slipping agent used in the production of extruded polyethylene and propylene films (Friedt and Luhs, 1998). Erucic acid is now mainly derived from high-erucic acid rapeseed (HEAR) (*Brassica napus*) that has 45%–55% erucic acid. Production of HEAR will become increasing difficult with the continuing expansion of food-grade canola (low-erucic rapeseed), because the two crops are interfertile and HEAR must be grown in the isolation of canola to prevent contamination with erucic acid.

These speciality fatty acids referred to above each have tremendous opportunities for expanded utilization as industry moves increasingly to bio-based products. However, the various agronomic and production limitations that the current crop sources suffer from, and their consequent high price, will present significant constraints to this growth. For this reason, considerable attention has now been directed to exploring whether production of these fatty acids could be engineered in high-performance oilseed crops, rather than using their native sources. Furthermore, the specialty industrial fatty acids currently in production represent only the 'tip of the iceberg' compared with the enormous chemical diversity that exists in nature for fatty acid structure (Badami and Patil, 1980). Several of these novel structures—such as hydroxylated, acetylenated, conjugated, epoxidized, branched chain and cyclic fatty acids (Figure 1)—are now fairly well studied, and their synthesis is generally under relatively simple genetic control, usually involving a single catalytic enzyme (Napier, 2007).

Although fatty acids and triacylglycerols (TAGs) clearly predominate as the plant lipids currently most used for industrial applications, there is also specialized industrial use of a number of plant-derived waxes. Waxes are comprised of a fatty acid esterified to a fatty alcohol and generally range in overall length from C40 to C60 (Figure 1). Most plants produce wax esters (WE) as components of their surface lipid layers, where protection from desiccation and pathogen entry is afforded by their solid state, hydrophobicity and high resistance to hydrolytic degradation. These unique physical properties of waxes are also especially useful for industrial applications. The principal plant wax used industrially is obtained from the leaves of the carnauba palm (*Copernicia prunifera*). Carnauba wax is highly valued as a surface protectant and high-shine polish with a multitude of industrial uses. In contrast to leaf waxes, one plant, the desert shrub jojoba (*Simmondsia chinensis*), is unique in being able to assemble straight-chain WE in its seed where they serve as the predominant storage lipid, accumulating at levels up to 50% of weight of the jojoba bean. The jojoba WE are notably liquid at room temperature and are highly valued ingredients in cosmetics and personal care products.

The specific properties of WE are imparted by the overall chain length and, in some cases, by the presence of functional side groups within the molecule, such as methyl branches or di-esters (Biester *et al.*, 2012; Gamo and Saito, 1971). The ability to modify these characteristics through metabolic engineering opens up the potential to develop improved and specialized waxes designed for particular industrial purposes. In particular, there is now the prospect of engineering plant wax composition to better match the physical and performance properties of modern-day synthetic industrial lubricants, to provide viable renewable and biodegradable alternatives to petrochemically derived products. However, to realize their potential, waxes will need to be produced on much larger scale and at much lower cost than from present sources. The exemplar of WE accumulation in jojoba seed is now providing considerable impetus to metabolic engineering of high-yield oilseed crops to accumulate industrial quality WE. The recent EU-FP7 ICON (Industrial Oil crops producing added value Oils for Novel chemicals) project was established specifically to exploit this potential (http://icon.slu.se).

Plant oil and wax synthesis and accumulation

Fatty acid and triacylglycerol synthesis

Rational and precise engineering of industrial oil and wax production in plants will need to be predicated on a

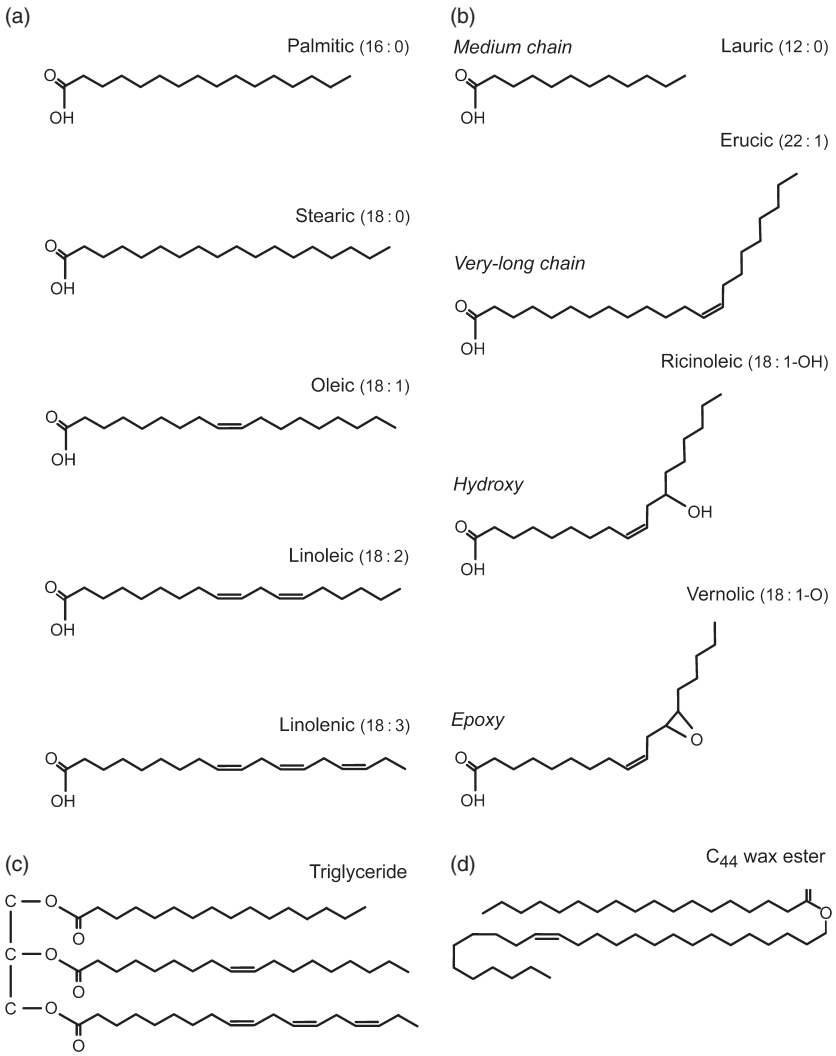

Figure 1 Typical plant lipid structures including (a) fatty acids commonly found in edible plant oils, (b) some unusual fatty acids of industrial interest found in minor crops and wild plants, (c) triacylglycerol and (d) wax ester.

comprehensive understanding of the metabolic pathways for the biosynthesis of fatty acids and their accumulation into TAG and WE storage lipids, and of the genetic control of these pathways. Based on biochemical research over several decades, seed oil biosynthesis had, until relatively recently, come to be viewed as an essentially linear process comprised of sequential fatty acid synthesis, modification and accumulation processes. These pathways and their component enzymes have been described in significant detail in various previous reviews (Baud and Lepiniec, 2010; Li-Beisson *et al.*, 2010; Napier and Graham, 2010; Ohlrogge and Chapman, 2011; Snyder *et al.*, 2009; Wallis and Browse, 2010; Weselake *et al.*, 2009).

In essence, the classical view has been that saturated or monounsaturated acyl chains of various lengths generated via the fatty acid synthesis pathway in the plastid (Figure 2) are exported to the cytosolic compartment from where they are sequentially assembled onto the *sn*-1, *sn*-2 and *sn*-3 positions of the glycerol backbone by the reactions of the Kennedy pathway (Figure 3) to form TAGs that are then sequestered into oil bodies (oleosomes) to serve as energy reserves to support eventual seed germination. Prior to their incorporation into TAG, the acyl chains exported from the plastid are able to undergo a range of enzyme-mediated modifications (Figure 2). The most common of these are the further sequential desaturation at Δ12 and Δ15 positions to produce the polyunsaturated fatty acids (PUFAs) linoleic and α-linolenic. However, in many plants (and other organisms), unusual enzymatic modifications can occur that result in diverse fatty acid structures of potential industrial interest (such as shown in Figure 1). A notable example is the divergent family of *Fad2*-encoded enzymes that have evolved differing catalytic functions from the ancestral Δ12-desaturase (Figure 4). These catalyse the synthesis of a range of hydroxy, epoxy, acetylenic and conjugated fatty acid structures (Broun *et al.*, 1998a; Cahoon *et al.*, 1999; Dyer *et al.*, 2002; Lee *et al.*, 1998; van der Loo *et al.*, 1995; Nam and Kappock, 2007; Qiu *et al.*, 2001; Sperling *et al.*, 2000). Enzymes for the conversion of oleic acid to cyclopropanoic acid fatty acids have also been identified, for example in *Sterculia* (Bao *et al.*, 2002) and cottonseed (Yu *et al.*, 2011). Also, there are fatty acid elongase (FAE) systems that operate in the cytosol for the elongation of oleic acid to eicosenoic acid ($C20:1^{\Delta 11}$) and erucic acid.

In recent years, however, the discovery and characterization of several additional enzymes involved in acyl exchange within the cytosolic compartment has challenged this essentially linear view of TAG assembly. Newly identified acyl exchange enzymes appear to be playing significant roles in the transfer of fatty acids from the site of modification on phosphatidylcholine (PC) to deposition on TAG molecules. They may also be providing specialized

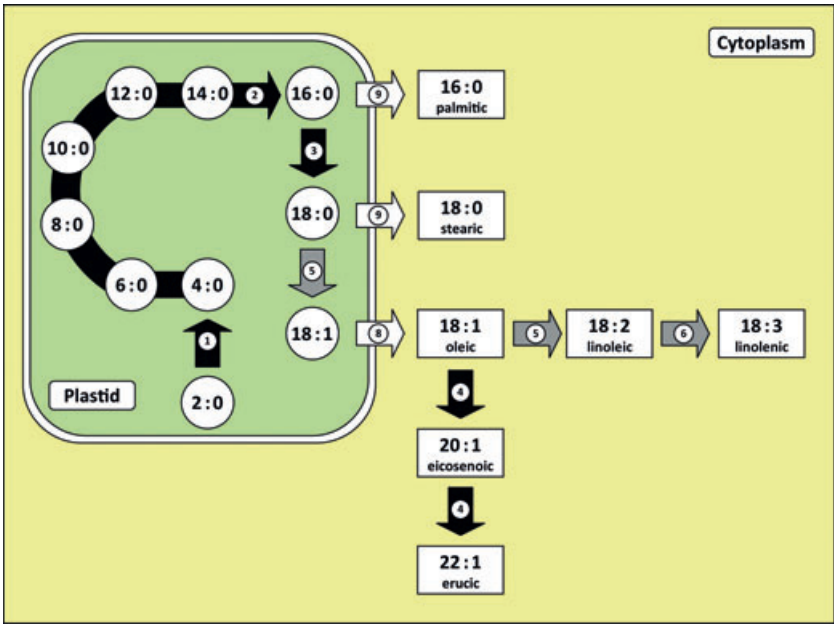

Figure 2 Principal fatty acid biosynthetic pathways in plastidic and cytoplasmic compartments of higher plant cells. Chain elongation enzymes are shown as black arrows: ❶ keto-acyl synthase III (KASIII), ❷ keto-acyl synthase I (KASI), ❸ keto-acyl synthase II (KASII), ❹ Fatty acid elongase (FAE1). Fatty acid desaturases are shown as grey arrows: ❺ Δ9-desaturase (SAD), ❻ Δ12-desaturase (FAD2), ❼ Δ15-desaturase (FAD3). Acyl thioesterases are shown as white arrows: ❽ Fatty acid thioesterase A (FATA), ❾ Fatty acid thioesterase B (FATB).

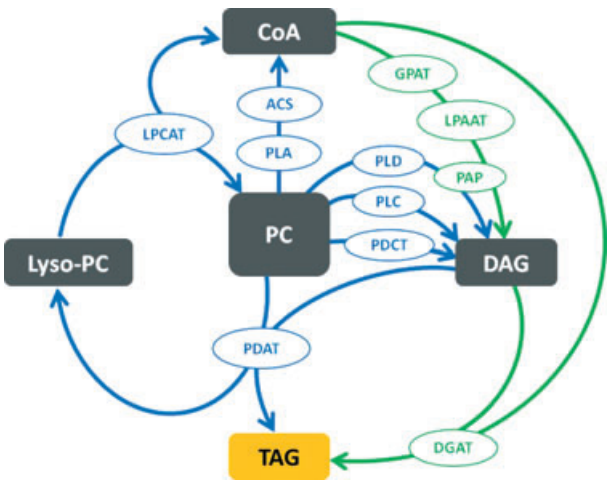

Figure 3 Contemporary diagrammatic representation of the known metabolic routes by which acyl groups from the acyl-PC and acyl-CoA pools can be directly or indirectly channelled to triacylglycerol (TAG). The traditional linear Kennedy pathway is shown in green and more recently defined routes in blue. Acyl pools are shown as solid rectangular boxes, and principal enzymes involved are shown as open oval boxes. ACS, acyl-CoA synthase; DGAT, diacylglycerol acyltransferase; GPAT, glycerol-3-phosphate acyltransferase; LPAAT, lysophosphatidic acid acyltransferase; LPCAT, lysophosphatidylcholine acyltransferase; PAP, 3-*sn*-phosphatidate phosphohydrolase; PDAT, phosphatidylcholine diacylglycerol acyltransferase; PDCT, phosphatidylcholine diacylglycerol cholinephosphotransferase; PLA, phospholipase A; PLC, phospholipase C; PLD, phospholipase D.

pathways for the channelling of unusual, and potentially damaging, acyl groups away from membranes and into storage lipids and thus have potentially significant consequences for attempts to metabolically engineer unusual fatty acid production in plant oils. In particular, a number of enzymes have now been uncovered that mediate the transfer of acyl groups directly from PC to TAG without passage through the acyl-coenzyme A (CoA) pool. One such route involves the enzyme phosphatidylcholine diacylglycerol acyltransferase (PDAT) that transfers an acyl group from the *sn*-2 position of PC directly to the *sn*-3 position of TAG in seed of Arabidopsis (Ståhl *et al.*, 2004). It has recently been shown that PDAT and diacylglycerol acyltransferase (DGAT) are the two main enzymes responsible for TAG synthesis in Arabidopsis seeds (Zhang *et al.*, 2009). Mutation in one gene and down-regulation by RNA interference (RNAi) of the other led to seeds virtually lacking TAG, whereas double mutants were both pollen and embryo lethal (Zhang *et al.*, 2009). Similar to the situation with DGAT, PDAT has been shown to be expressed both in tissues that accumulate TAG (oil palm mesocarp) and in similar tissues that are essentially devoid of TAG (date palm mesocarp) (Bourgis *et al.*, 2011). The relative contribution of DGAT and PDAT in seed TAG synthesis can vary drastically between even evolutionary closely related species, like sunflower and safflower (W. Banas, A. Sanchez, A. Banas and S. Stymne, pers. commun.).

An alternative route, by which acyl groups present on PC can be moved directly to the TAG backbone, is through exchange of the polar head group between PC and diacylglycerol (DAG), the immediate precursor of TAG. The enzyme responsible, phosphatidylcholine diacylglycerol cholinephosphotransferase (PDCT), was recently described in Arabidopsis and shown to act by transferring the complete phosphocholine head group to directly form DAG, thereby effectively providing the entire glycerol backbone from PC as a precursor of TAG (Lu *et al.*, 2009). Mutational inactivation of this gene results in the lowering of oil content and polyunsaturation because of reduced flux of acyl groups from PC to DAG. Furthermore, when the PDCT mutation was combined with mutations in the lysophosphatidylcholine acyltransferase genes (LPCAT1 and LPCAT2) that are responsible for acyl loading and editing of PC, there was a dramatic reduction in PUFA content in the seed TAG, down to one-third the wild-type level. These results indicate that PC acyl editing and phosphocholine head-group exchange between PC and DAG control the majority of acyl fluxes through PC to provide PUFA for TAG synthesis (Bates *et al.*, 2012). Recent work also demonstrates that the acyl-CoA-independent transfer of acyl groups from PC to TAG catalysed by PDAT is dependent on an efficient acylation of lysophosphatidylcholine, the coproduct of the PDAT

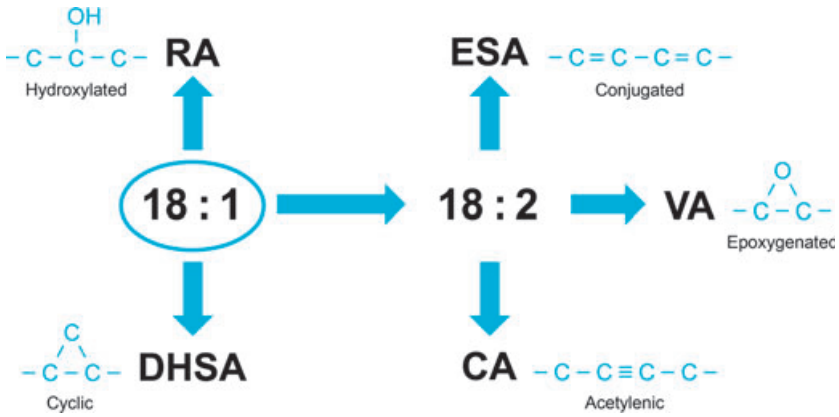

Figure 4 Examples of unusual fatty acid structures originating from modifications to the structure of oleic acid (C18:1) in the acyl-PC pool from the action of divergent *Fad2* enzymes and CPFA synthase. RA, ricinoleic acid; ESA, eleostearic acid; VA, vernolic acid; CA, crepenynic acid; DHSA, dihydrosterculic acid; C18:2, linoleic acid.

reaction. In the absence of a functional DGAT1, the Arabidopsis seed TAG is mainly synthesized by PDAT and disruption of LPCAT2 activity caused drastic reduction in seed TAG content and severely disturbed seed development (Xu *et al.*, 2012).

Another related acyl-CoA-independent route to DAG involves phospholipase D (PLD) that catalyses the removal of just the choline group from PC to form phosphatidic acid (PA), which can then be dephosphorylated by 3-*sn*-phosphatidate phosphohydrolase (PAP) to form DAG. Suppression of PLD in developing soybean seeds has recently been shown to alter both the total content and the level of unsaturation of soybean seed oil, suggesting that it may be playing a role in the conversion of PC to TAG during oil synthesis, either by a direct catalytic action to produce phosphatidic acid (PA), or by exerting a regulatory influence over other acyl exchange enzymes (Lee *et al.*, 2011). In both of these head-group removal reactions (PDCT and PLD), the acyl groups at position *sn*-1 and *sn*-2 of PC retain their positioning on the TAG backbone.

Taken overall, the relatively recent uncovering of PDAT and PDCT has now provided clear evidence for the existence of metabolic routes for direct (acyl-CoA independent) transfer of acyl groups from PC to each of the three positions on TAG during seed oil biosynthesis. These pathways presumably operate in addition to the classical Kennedy pathway. However, it is tempting to speculate that the full picture is still not elucidated, especially given the significant numbers of uncharacterized acyltransferase genes evident in recent and current genomic and transcriptomic studies of several oil-bearing plants that accumulate unusual fatty acids, such as flax (Wang *et al.*, 2012), *Hiptage benghaliensis* and *Bernardia pulchella* (A.G. Green, S. Singh and X.-R. Zhou, unpublished data). Many of these gene families could harbour additional enzymes for direct acyl exchange either between PC and the various TAG intermediates, or amongst the intermediates themselves. For example, there have been biochemical indications of possible DAG/DAG acyl exchange yielding a monoacylglycerol (MAG) and a TAG (Stobart *et al.*, 1997). However, no gene dedicated to such a function has yet been identified, although it has been reported that a soluble form of yeast PDAT enzyme has some low level of DAG/DAG transacylation activity (Ghosal *et al.*, 2007). Recent work could not detect any DAG/DAG transacylase activity in sunflower or safflower membranes, despite good PDAT activity (W. Banas, A. Sanchez, A. Banas and S. Stymne, pers. commun.) casting some doubts over any significant contribution of such enzyme activity in TAG synthesis in oil seeds. Interestingly, PDAT can also transfer acyl groups from *sn*-1 of PC to TAG, albeit at a lower rate (about 30%) than with acyl groups from the *sn*-2 position (W. Banas, A. Sanchez, A. Banas and S. Stymne, pers. commun.; Ståhl *et al.*, 2004). As acyl groups at *sn*-1 position of PC can undergo desaturation and structural modification at that position (Bao *et al.*, 2003; Stymne *et al.*, 1992), it is possible that PDAT and other enzymes might also be involved in channelling these acyl groups from PC to TAG.

Thus, the picture of TAG synthesis that is now emerging is one of a complex network of acyltransferase reactions mediating the movement of acyl groups between pools of acyl-PC, acyl-CoA and TAG precursors within the cytosolic compartment (Figure 3). Acyl moieties exported from the plastid could take various potential metabolic routes through these networks towards assembly on TAG, the routes taken probably differing between plant species, based on presence, activity and coordinated regulatory control of alternative enzymatic steps. For example, recent studies with soybean (Bates and Browse, 2011) and Arabidopsis (Bates and Browse, 2012) have identified the presence of kinetically distinct DAG pools during oil accumulation. Diacylglycerol (DAG) used in TAG assembly was predominantly derived from PC, whereas DAG produced through *de novo* synthesis (Kennedy pathway) was mainly used to produce PC. As fatty acids esterified to PC are substrates for modifications, such as desaturation, hydroxylation or epoxygenation, the fatty acid composition of the PC-derived DAG pool may be markedly different to that of the *de novo* DAG pool. The extent of this shunting of DAG through PC, compared with direct conversion to TAG, may vary significantly between different species and could be a significant underlying factor in the varying ability of some wild plants to incorporate unusual fatty acids into TAG, as well as the difficulty in engineering some oilseeds to accumulate unusual fatty acids.

Importantly, particular routes through this metabolic network may have become specialized for particular unusual acyl groups in certain plants. This feature could be based on customized substrate specificity of the acyltransferases available, and there is already evidence of divergent substrate specificities within a number of acyltransferase families. For example, it is known that DGAT enzymes in castor bean, *Vernonia galamensis* and tung have strong preferences for DAG and CoA substrates containing ricinoleoyl, vernoleoyl and eleostearoyl moieties, respectively (Kroon *et al.*, 2006; Shockey *et al.*, 2006; Yu *et al.*, 2006).

Wax ester synthesis

In contrast to the complex, and perhaps incompletely defined, metabolic pathways leading to TAG synthesis, the pathways resulting in WE are relatively simple and well understood (Lardizabal *et al.*, 2000). In essence, synthesis of a WE is a two-

step process involving, firstly, the conversion of a fatty acid to a fatty alcohol by the action of a fatty acid reductase (FAR), followed by the esterification of the fatty alcohol to a fatty acid by the action of a wax synthase (WS). This final step in WE synthesis can be viewed as being analogous to the final DGAT-mediated step in the formation of TAG, in that the fatty acid donor becomes esterified either through the primary OH group of the fatty alcohol or the through *sn*-3 OH group of DAG. This commonality has been reinforced by the discovery of DGAT enzymes with dual-function WS/DGAT activity (Kalscheuer and Steinbüchel, 2003; Li *et al.*, 2008) and able to utilize a variety of acyl acceptors as substrates (Yen *et al.*, 2005). Arguably, the best-studied metabolic pathway for WE synthesis is that occurring naturally in jojoba seed wax and its transgenic expression in oil-forming seeds (Lardizabal *et al.*, 2000). Jojoba WE synthesis is located in the extra-plastidial compartments, and both FAR and WS are membrane associated and utilize acyl-CoA (Lardizabal *et al.*, 2000; Metz *et al.*, 2000). The structure of jojoba WE is dominated by C20:1 and C22:1 acyl and alcohol chains, and this is reflected in the substrate specificity of both the FAR and the WS enzymes (Lardizabal *et al.*, 2000; Metz *et al.*, 2000).

Metabolic engineering of fatty acid synthesis and accumulation

For most oleochemical applications of plant oils, it is desirable to have as high a concentration (purity) as possible of the required molecule, to maximize the recovery yield and to minimize potentially difficult or expensive downstream processing and separation costs. However, most of our oilseed species have been chosen for their food use and nutritional value, and they tend to instead have a balanced content of the main five fatty acids, palmitic, stearic, oleic, linoleic and linolenic. The maximum levels of individual fatty acids in these oils are suitable for the food use of the oil, but fall short of very high purity levels preferred for industrial feedstocks. Therefore, a major challenge of metabolic engineering industrial fatty acids into major oil crops is not just to achieve synthesis by introducing genes for the appropriate catalytic enzymes, but also to engineer storage lipid assembly pathways to enable high-level accumulation and purity of the target molecule. We review below recent progress in these endeavours that reveal differing levels of difficulty depending on the type of fatty acid being engineered.

Monounsaturated fatty acids

Monounsaturated fatty acids have significant industrial potential for two main reasons. Firstly, compared with the polyunsaturates that are also present in most plant oils, monounsaturates are relative resistant to oxidative attack and impart better stability for direct use of the oil in industrial products such as biolubricants or biodiesel. Secondly, they can be readily cleaved at their double-bond sites by chemical processing to give rise to products in high demand by the chemical industry, such as monomers for the production of various nylons (polyamides). The predominant monounsaturate present in most plant oils is oleic acid that can be processed to yield azelaic acid (C9) monomers for bionylon production (Höfer, 2003). High-oleic compositions have been developed—using both conventional breeding and gene technology—in several oilseeds (e.g. sunflower, safflower, canola, soybean and peanut) to improve their stability and enable them to be used as replacements for saturated and trans-fatty acid–containing oils. Although the 75%–85% oleic acid levels in these genetically improved oils are ideal for their food use, the significant residual levels of polyunsaturates cause problems in industrial and oleochemical applications.

However, a very recent development of super-high-oleic safflower has now demonstrated the potential to metabolically engineer oilseeds to achieve the sought after oleochemical purity levels. RNAi-mediated gene silencing targeted against the seed-expressed fatty acid thioesterase (FATB) and *FAD2* genes has resulted in the development of safflower oil containing up to 95% oleic acid, with only 2% linoleic acid remaining and with no apparent adverse effects on agronomic performance (C. Wood, Q. Liu, J. Cao, X.-R. Zhou, S. Stephan, S. Singh and A. Green, unpublished data). This achievement using seed-specific gene silencing contrasts noticeably with previous efforts to raise oleic acid using mutagenesis approaches that, although successful in reaching levels around 90% (Skoric *et al.*, 2008), have generally been associated with reduced yield. This yield drag is considered to be due to an undesirable reduction in PUFA content of membranes in vegetative tissues resulting from the mutation of constitutively expressed *FAD2* genes (Clemente and Cahoon, 2009). The development of safflower with seed-specific elevation of oleic acid up to 95% should open the way for greater industrial application of oleic acid as a chemical feedstock and also provide an additional improvement in stability for direct industrial applications, such as in lubricants and in transformer fluids. Importantly, this achievement now serves as a landmark in demonstrating the potential to metabolically engineer highly pure oleochemicals in plant oils. It is interesting that such very high levels of oleic acid and also high levels of gamma-linolenic acid (C18:3$^{\Delta6,9,12}$) (Nykiforuk *et al.*, 2012) could be achieved by relatively straightforward metabolic engineering in safflower, a species that can also naturally accumulate up to 90% linoleic acid. This demonstrates an extreme phenotypic plasticity for this species, at least with respect to tolerance of unsaturated fatty acids.

Metabolic engineering of other monounsaturated fatty acids with different chain lengths is also showing good signs of success in generating quite high levels. Erucic acid is formed through the sequential elongation of oleoyl-CoA by the extraplastidic elongase system, but reaches only 45%–55% of total fatty acids in naturally occurring HEAR, thus limiting the yield of this highly valuable and versatile industrial fatty acid used, for example, in erucamide production. This limitation is due to erucic acid being found only at the outer positions (*sn*-1 and *sn*-3) of TAG in rapeseed, because the rapeseed lysophosphatidic acid acyltransferase (LPAAT) enzyme, responsible for the insertion of fatty acids in the *sn*-2 position, cannot use erucoyl-CoA as the acyl donor (Frentzen, 1993). However, when a *Limnanthes* LPAAT gene that readily utilizes erucoyl-CoA (Cao *et al.*, 1990) was expressed together with a rapeseed FAE in a mutant line having greatly increased oleic acid substrate levels, erucic acid levels increased up to 72% and the total amount of very long-chain fatty acids accounted for up to 77% of all fatty acids (Nath *et al.*, 2009). Importantly, this achievement of high erucic acid content has recently been emulated using a similar strategy in crambe (*Crambe abyssinica*), to create a potential alternative high-erucic crop source that is safely reproductively isolated from edible oil rapeseed (Li *et al.*, 2012b). It is possible that further gains in erucic acid content might be achievable through blocking any acyl-CoA-independent pathways (such as PDAT and PDCT) that may be channelling the remaining oleate directly to TAG, as this could result in this oleate being shunted instead through the acyl-CoA pool and thus being made available for additional elongation to erucic acid (Carlsson *et al.*, 2011). In this context, it might be

noteworthy that the combination of blocks in PDCT and LPCAT in Arabidopsis seeds led to a substantial increase in C20:1 in the seed oil along with greatly decreased level of PUFAs (Bates *et al.*, 2012).

Two other monounsaturated fatty acids, palmitoleic acid (C16:1$^{\Delta 9}$) and its elongation product cis-vaccenic acid (C18:1$^{\Delta 11}$), that are normally present in seed oils at only very low amounts are now of considerable industrial interest following the recent breakthrough development of olefin metathesis, a new organic chemistry process made possible through the development of novel catalysts (Meier, 2009; Rybak *et al.*, 2008). Metathesis reactions utilizing either of these two ω7-unsaturated fatty acids can be used to generate an ω-unsaturated fatty acid and 1-octene that has great value as an industrial chemical feedstock. To capitalize on this opportunity, metabolic engineering strategies for developing very high levels of ω7-unsaturated fatty acids have recently been explored with outstanding success (Nguyen *et al.*, 2010). By simultaneously blocking the ketoacyl synthase II (KASII)-mediated elongation of C16:0 to C18:0 and introducing two Δ9-desaturase genes capable of desaturating C16:0 (one being operative in the plastid and the other in the cytosol), combined levels of the two ω7-fatty acids, palmitoleic and vaccenic acid, of up to 71% of total fatty acids were obtained in Arabidopsis. This matches the levels seen in *Doxantha unguis-cati*, the plant with the highest recorded amount of ω7-fatty acids in its seed oil, with prospects for further increases through engineering additional reduction in C18 unsaturates. A particular feature of this work is that the introduced plastidic C16:0-acyl carrier protein (ACP) Δ9-desaturase had been engineered to convert C16:0 to C16:1$^{\Delta 9}$ with a more than 100-fold higher specificity than that of the naturally occurring enzyme.

In spite of the significant recent success with engineering very high levels of the aforementioned monounsaturates, not all monounsaturate fatty acids have proved so amenable to metabolic engineering. For example, C16:1$^{\Delta 6}$ and C18:1$^{\Delta 6}$ (petroselenic acid) that constitute about 80% of all fatty acids in the seed oils of *Thunbergia alata* and *Coriander sativum*, respectively, can be split by ozonolysis to yield adipic acid (C6:0) and either capric (C10:0) or lauric acid. Adipic acid is one of the building blocks in 6,6-nylon, which has an annual production of about 2.5 million metric tonnes and is today made from petroleum. The acyl-ACP desaturase enzymes responsible for the production of these monounsaturated fatty acids have been identified some time ago (Cahoon *et al.*, 1992, 1994); however, when expressed in transgenic plants, the synthesis of these fatty acids was very low. It was concluded that the poor synthesis was probably due to either the lack of, or the incorrect assembly of, a necessary multicomponent enzyme association (Suh *et al.*, 2002).

Medium-chain saturates

Medium-chain saturated fatty acids—in particular caprylic (C8:0), capric and lauric acid—have a range of industrial applications. Although palm kernel oil and coconut oil already provide significant plant sources of lauric acid, there are no commercially viable plant sources rich in caprylic acid and capric acid. These fatty acids are currently fractionated out as minor components of palm kernel and coconut fats, or synthesized from petroleum via oxidation of the corresponding aldehydes. An oil crop producing high amounts of caprylic or capric acid in its oil could be an economically viable alternative to the organic chemical synthesis.

Medium-chain saturates are produced abundantly in seed oils of some wild plants, notably in the genus *Cuphea* where they can reach extremely high levels (Badami and Patil, 1980). They are synthesized by termination of chain elongation during plastidial fatty acid synthesis through the intervention of chain-length-specific acyl-ACP thioesterases (Dehesh, 2001; Voelker *et al.*, 1992, 1997) followed by export to the cytosol for incorporation into TAG. One of the earliest examples of metabolic engineering of oil quality was the production of over 60% of lauric acid in rapeseed (Voelker *et al.*, 1992; Wiberg *et al.*, 2000), achieved by transgenically expressing a single gene encoding a C12:0-ACP-specific thioesterase obtained from Californian bay tree. Similar strategies were subsequently attempted in rapeseed using *Cuphea* thioesterase genes with specificities towards C8 and C10 acyl-ACPs, but resulted in much lower synthesis of the medium-chain fatty acids (Wiberg *et al.*, 2000). A range of biochemical investigations (Bafor and Stymne, 1992; Bafor *et al.*, 1990; Eccleston and Ohlrogge, 1998; Larson *et al.*, 2002; Poirier *et al.*, 1999; Wiberg *et al.*, 1994) all suggested that the transgenic seeds lacked the enzymes required to efficiently acylate the medium-chain fatty acids onto TAG backbones, leading to a build-up of these fatty acids in the acyl-CoA pool and their resultant shunting to beta-oxidation. This contrasts with the situation in wild *Cuphea* plants where the concerted action of specialized glycerol-3-phosphate acyltransferase (GPAT) and LPAAT allows the production of exclusively di-medium-chain DAGs, which are preferentially used by DGAT to form TAG (Bafor and Stymne, 1992; Bafor *et al.*, 1990). It is interesting to contrast the disappointing results obtained for C8 and C10 with the high-level accumulation obtained for C12. It would appear that the TAG assembly enzymes present in *Brassicaceae* plants, such as Arabidopsis and rapeseed, may be competent on saturated fatty acids in the range C12 to C18 but much less so with shorter chain lengths such as C8 and C10. However, as for C16 and C18 saturated fatty acids, the C12 lauric acid is more or less excluded from the *sn*-2 position of the TAG.

Unusual fatty acids

The aforementioned saturates and monounsaturates are relatively innocuous fatty acids, and it is perhaps unsurprising that high levels of purity are being achieved or approached. However, engineering accumulation of many of the more unusual modified fatty acids found in wild plants and other organisms is proving to be a much more challenging endeavour. Fatty acids containing reactive functional groups, such as hydroxy groups, epoxy bridges or carbocyclic structures, or unusual bond structures, such as acetylenic or conjugated bonds, represent valuable starting materials for industrial chemistry. It is notable that in nature, there are many instances where plants accumulate storage lipids with very high concentrations of these unusual fatty acid structures, for example 90% of ricinoleic acid in *R. communis* (Badami and Patil, 1980) and 92% of vernolic acid (an epoxy fatty acid) in *Bernardia pulchella* (Spitzer *et al.*, 1996). Initial attempts to engineer production of these, and other unusual fatty acids, in oilseeds through transgenic expression of key biosynthetic (catalytic) enzymes were relatively promising in achieving synthesis of the target fatty acids. However, in all cases, the product levels fell well short of achieving the ideal purity levels and generally failed to even emulate the levels found in the organisms from which the genes for the biosynthetic enzymes were obtained (Cahoon *et al.*, 2006; Dyer *et al.*, 2008; Thomaeus *et al.*, 2001).

Many of these modified fatty acids are known to be synthesized from oleic acid or linoleic acid substrates esterified to PC on the membranes (Figure 4), where their unusual or reactive

structure could be damaging to membrane integrity. Wild plants that produce such fatty acids must therefore have developed highly effective pathways for their removal from PC and sequestration into inert storage lipids, presumably via the concerted action and specialization of enzymes involved in PC editing, PC to DAG conversion and TAG assembly that have co-evolved with the catalytic enzyme (Napier and Graham, 2010). It is therefore not surprising that the lipid metabolic machinery of recipient oil crops that do not normally produce these unusual fatty acids is not optimized for their handling.

Given the increased complexity of acyl exchange networks responsible for this channelling now becoming apparent, it is clear that alternative specialization strategies are possible, and different organisms may have undergone different evolutionary adaptations to handle these fatty acids. The metabolic engineering challenge therefore for achieving high-level synthesis and accumulation of these unusual fatty acids in oil crops is to uncover these specialized metabolic pathways and engineer them into the crop platform alongside the required catalytic enzymes. Recent progress in this regard has focussed primarily on the acyltransferases DGAT and PDAT (Figure 2) involved in the acylation at the *sn*-3 position on DAG as they are considered to be two key enzymes for TAG formation in seeds. It is therefore important that these two enzymes are competent to handle engineered unusual fatty acids in the target oilseed. Indeed, both a DGAT (*RcDGAT2*) and a PDAT (*RcPDAT1A*) from castor have been shown to have a preference for ricinoleic acid containing substrates and have been implicated in the incorporation of high amounts (>90%) of this fatty acid into TAG in castor. Not surprisingly, co-expression of either of these enzymes alongside the fatty acid Δ12-hydroxylase (FAH12) from castor led to significant increases in the accumulation of ricinoleic acid in the seed oil of Arabidopsis, increasing from 17% to around 28% in both cases (Burgal *et al.*, 2008; van Erp *et al.*, 2011; Kim *et al.*, 2011), but with reductions in total oil content. However, co-expressing both *RcPDAT1A* and *RcDGAT2* restored the oil content to nearly wild-type level and gave rise to a major increase in the mass of hydroxy fatty acids accumulating in the seed. Likewise, co-expression of DGAT1 and DGAT2 from *Vernonia galamensis*, a plant that accumulates high levels of the Δ12-epoxygenated vernolic acid in its oil, along with the Δ12-epoxygenase resulted in a significant (fourfold) increase in the accumulation of vernolic acid in soybean embryos and reversed the depression in oil and protein content previously seen when the Δ12-epoxygenase was expressed alone (Li *et al.*, 2010b,c, 2012a).

Further upstream, the lack of efficient removal of the unusual fatty acids from the site of synthesis on PC has also been identified as a bottleneck in a number of instances. For example, transgenic soybean and Arabidopsis seed expressing fatty acid conjugases accumulated nearly 25% conjugated fatty acids in their PC pool (where they are synthesized) compared with the native tung tree that typically retains <1% of the conjugated fatty acids in its PC pool (Cahoon *et al.*, 2006). Similar results were also reported in plants transformed with castor FAH12—the transgenics had more than twice the amount of hydroxylated fatty acids in the PC pool than that found in castor (Bates and Browse, 2011; van Erp *et al.*, 2011). Enzymes such as phospholipases C and D, LPCAT and PDCT, sourced from native species that accumulate high levels of unusual FAs, may be useful for their removal from PC via acyl editing and exchange mechanisms (Stålberg *et al.*, 2009; Zheng *et al.*, 2012). For example, when a castor PDCT was co-expressed with FAH12 in Arabidopsis, ricinoleic acid in PC was significantly reduced and accumulation of hydroxylated fatty acids in the oil was doubled (Hu *et al.*, 2012). In addition, it has been observed that the overall fatty acid synthesis rate of Arabidopsis seed expressing castor FAH12 is reduced by >30% and it is possible that this could be due to the feedback inhibition mechanism, linked with the plastidial acyl-CoA carboxylase (ACCase), identified recently (Andre *et al.*, 2012) but also by the catabolism of DAG with ricinoleoyl groups (Bates and Browse, 2011). Facilitating efficient removal and channelling of unusual fatty acids to TAG might alleviate this phenomenon and overcome reduced oil content in these lines.

To date, co-expression of specialized TAG assembly genes seems to have been performed mainly as the addition of a pathway rather than as a substitution for an endogenous equivalent. As such the introduced specialized enzymes will be competing against the endogenous network and thus may not have the full opportunity to express their potential. It would be interesting to see whether, for instance, silencing endogenous DGAT in combination with introducing specialized DGATs will further enhance the accumulation of unusual fatty acids. Furthermore, such silencing might not just be restricted to the counterpart endogenous gene(s), but could be used to eliminate entire networks of activity operating on normal fatty acids, to provide a 'blank page' for the expression of TAG assembly pathways favouring accumulation of unusual fatty acids (Chapman and Ohlrogge, 2012). Such an approach has been successfully employed for the characterization of genes involved in TAG synthesis in a yeast strain that is totally deficient in TAG assembly genes (Sandager *et al.*, 2002).

Another possible bottleneck to accumulation that is yet to be fully explored is the level of expression of the fatty acid-modifying enzyme in the transgenic plants. In some species that naturally accumulate high levels of some unusual fatty acids, it has been shown that mRNA levels for the fatty acid-modifying enzymes are at similar high levels to those for storage proteins (Li *et al.*, 2010a; Nam and Kappock, 2007). It is unknown whether the levels of expression of these genes in transgenic plants so far developed are limiting to the accumulation of the unusual fatty acid products. As the transgenes are often driven by seed storage gene promoters, it is likely that expression levels are adequate, at least for low-level synthesis, but might need to be further increased to provide high levels of synthesis should impediments to accumulation in storage lipids be successfully alleviated. Another aspect that has to be taken into account is that these enzymes need cytochrome b5 and b5 reductase for their activity. It has been shown that down-regulating cytochrome b5 reductase lowers the hydroxylation of oleic acid to ricinoleic acid in Arabidopsis seeds, although it has little effect on the desaturation of oleic acid to linoleic, a reaction that also requires this reductase (Kumar *et al.*, 2006).

Engineering wax ester synthesis

Triacylglycerols have attractive physical and biodegradability features for use as lubricants but have limited ability to replace petroleum-derived lubricants because of their generally poorer hydrolytic and oxidative stability. In contrast, WE have excellent resistance to hydrolysis (Li *et al.*, 2010a). In fact, WE harvested from the spermaceti whale were widely used in high-pressure and high-temperature lubricants before a global ban on hunting the whale was introduced in 1972. Following this, the desert shrub jojoba (*Simmondsia chinensis*) was investigated as an alternative biological source of WE, because its seed oil is unique in being

composed almost entirely of WE. However, the jojoba plant is low yielding and labour intensive, and therefore, its WE are very expensive and unable to compete with cheaper petroleum-based products in the lubricant market. Developments in gene technology stimulated early interest to engineer oilseeds to provide a lower-cost source of jojoba-type WE. In principle, a minimum of only two additional enzymatic activities need to be introduced to produce WE in seeds—a FAR to produce the fatty alcohol and a WS to esterify the fatty alcohol to a fatty acid. This was confirmed when expression in Arabidopsis of the jojoba FAR and jojoba WS (in combination with an FAE to produce C20:1 to C24:1 fatty acids) resulted in a major proportion of the seed TAG being replaced by jojoba-type WE (Lardizabal *et al.*, 2000). It is notable that in these experiments, the introduced WE pathway was operating in competition with the endogenous TAG assembly pathway. It will be interesting to see the outcome of more sophisticated approaches involving the simultaneous suppression of TAG assembly, such as by silencing DGAT and PDAT activity.

Jojoba WE are mainly composed of monounsaturated C20 and C22 fatty acid and alcohol carbon units and have too high a melting point (about 9 °C) for widespread lubricant use, especially in cold climates. On the other hand, WE of very diverse composition and melting points are found throughout nature, and a vast pool of FAR and WS genes is therefore available to enable the transgenic synthesis of other types of WE suited to a range of applications and conditions (Rowland and Domergue, 2012). Metabolic engineering to achieve synthesis of WE with the required properties is also likely to need engineering of the fatty acid composition of the oil crop, to ensure that the required chain lengths and functional modifications are inherent in the fatty acid and fatty alcohol components of the WE. An important recent development has been the demonstration that transgenic expression of engineered mouse FAR1 and WS genes in yeast and Arabidopsis enabled synthesis of WE containing >65% oleoyl-oleate, provided the genes were cotargeted to the oil body membrane. This involved the removal of normal peroxisomal targeting signal from the FAR1 gene and expressing it as an oleosin fusion protein (Heilmann *et al.*, 2012).

In addition to providing valuable biolubricants, WE can also be hydrolysed to yield their component fatty acids and fatty alcohols, of which the latter have substantial use in chemical industry and an economic value about twice that of the fatty acid. As WE biosynthesis, in contrast to TAG assembly, is a relatively simple process and does not share any glycerolipid intermediate with membrane lipid synthesis, it may ultimately prove to be a more amenable system for engineering accumulation of unusual fatty acids such as those that include hydroxy, epoxy or other functionalities. This would still, however, require the unusual fatty acid to be efficiently removed from PC and transferred to the acyl-CoA pool to be available for processing by FAR and WS enzymes.

Other alternative pathways for engineered waxes may now be possible. One such approach is suggested by the recent finding that fatty alcohols are also produced by FAR enzymes located in the chloroplast and operating on fatty acids bound to ACP (Doan *et al.*, 2012). This finding in combination with the identification of chloroplast-localized WS genes (Lippold *et al.*, 2012) opens up the prospect for potential synthesis and deposition of WE within the plastids, rather than the cytosol.

Protein engineering

Metabolic engineering is not limited to just the transgenic expression of genes encoding naturally occurring biosynthetic enzymes; there is also the potential to engineer the protein structure of these enzymes to alter aspects such as substrate specificity, double-bond positioning (regioselectivity) and reaction outcome. This capability provides potential to improve effectiveness in existing reaction mechanisms and even to engineer enzymes that undertake reactions at novel positions in the carbon chain. It also opens up the possibility of synthesizing novel fatty acids with multiple functional groups, thereby paving the way for designer lipids that currently lie beyond the reach of most metabolic engineering strategies.

Fatty acid desaturases are prime targets for protein engineering because of their central and well-defined role in the modification of acyl chains within the plant cell. The resolution of the crystal structures of the soluble plastidic C16:0-ACP Δ9-desaturase from castor and C16:0-ACP Δ4-desaturase from *Hedera helix* (poison ivy) resulted in a leap forward in the understanding of substrate-binding pocket geometry as well as key residues that determine chain length specificity of soluble desaturases (Guy *et al.*, 2007; Lindqvist *et al.*, 1996). It also provided valuable insights into structural determinants that govern the regioselectivity within the acyl chain of the fatty acid substrate (Guy *et al.*, 2011). Structure–function informed strategies as well as directed *in vitro* evolution have yielded new soluble desaturase variants displaying altered catalytic reaction, regioselectivity and changes in substrate specificity (Cahoon *et al.*, 1997; Whittle and Shanklin, 2001; Whittle *et al.*, 2008). Acyl-ACP desaturases with engineered substrate specificity are already finding their way into metabolic engineering projects, such as in enabling synthesis of high levels of palmitoleic acid in Arabidopsis as described earlier herein (Nguyen *et al.*, 2010).

In contrast to plastidial acyl-ACP desaturases, the current structure–function understanding of membrane-bound fatty acid desaturases remains extremely limited. Owing to the absence of a crystal structure, protein engineering studies focussing on this large and diverse class of fatty acid-modifying enzymes have relied on domain swapping, the exchange of differentially conserved amino acid residues between homologous members, or more random approaches such as directed molecular evolution. This has provided some insights into residues and regions that influence the reaction outcome (desaturation, hydroxylation and acetylenation), regioselectivity, stereochemistry and chain length specificity (Broadwater *et al.*, 2002; Broun *et al.*, 1998b; Gagne *et al.*, 2009; Hoffmann *et al.*, 2007; Hongsthong *et al.*, 2004; Meesapyodsuk *et al.*, 2007; Rawat *et al.*, 2012; Vanhercke *et al.*, 2011). Similar to acyl-ACP desaturases, these studies have also proven that in most cases, a limited number of substitutions are sufficient to confer significant changes in the specificity of membrane-bound desaturases. However, the lack of a detailed structural model as well as the limited number of high-throughput screening assays available mean that future progress in engineering membrane-bound desaturases is likely to remain limited for the foreseeable future.

Recently, acyltransferases have attracted significant attention as another target for protein engineering. This interest has been fuelled in part by the development of a high-throughput Nile Red assay in yeast and an elegant yeast complementation selection system that allows for the isolation of functional DGAT variants within large random libraries (Siloto *et al.*, 2009b). Both systems have been used to improve the activity of the *Brassica napus* DGAT1 acyltransferase in yeast (Siloto *et al.*, 2009a) although functional characterization and validation in model and target plants is still needed. Other target enzymes and applications

envisaged include engineering PDAT acyltransferases, screening cDNA libraries for novel TAG synthesizing enzymes and the engineering of acyl donor specificity of DGAT acyltransferases revealed by supplying different unsaturated and unusual fatty acids during selection. A glimpse of the potential of engineered acyltransferases can be found in the work of Xu *et al.* (2008) where targeted mutagenesis of a putative serine/threonine kinase site in the *Tropaeolum majus* DGAT1 acyltransferase increased the activity *in vitro* and resulted in increased seed oil content when expressed in Arabidopsis.

Engineering expanded oil production

The ability to engineer industrial fatty acid production in plants will, on its own, be insufficient to achieve substantial replacement of petrochemicals with bio-based alternatives. It will also be necessary to greatly expand the global supply of plant oils to well beyond the future food needs of an increasing population to create the additional supply volume needed (Carlsson *et al.*, 2011). This will involve increasing the oil productivity of existing oil crops, introducing additional oilseed crops and creating novel oil production platforms. Metabolic engineering has a central role to play in each of these pursuits and is already making significant contributions to addressing these goals, as has been well reviewed in recent years (Carlsson *et al.*, 2011; Lu *et al.*, 2011; Ohlrogge and Chapman, 2011; Taylor *et al.*, 2011; Weselake *et al.*, 2009). A wide array of metabolic engineering approaches have been employed to increase the oil content in a variety of plant tissues, including diverting carbon flow from starch to TAG, up-regulating fatty acid synthesis, modifying expression of individual TAG biosynthetic enzymes and transcription factors, and slowing the turnover of TAG. These approaches have met with various degrees of success, but it is fair to conclude that they show a lot of promise for expanding the production of plant oils.

A promising new approach to engineer leaf oil has involved the expression of a mammalian monoacylglycerol acyltransferase (MGAT) in *Nicotiana benthamiana* leaves (Petrie *et al.*, 2012a). Based on *in vitro* yeast assays and expression results in *N. benthamiana*, the authors propose that co-expression of a MAG-synthesizing enzyme such as *Arabidopsis* GPAT4 (Yang *et al.*, 2010b) and an MGAT or bifunctional MGAT/DGAT can result in DAG and TAG synthesis from glycerol-3-phosphate via a route that is independent and complementary to the endogenous Kennedy pathway and other TAG synthesis routes. This possibility of recruiting MAG as an alternative/additional substrate for TAG biosynthesis presents a novel approach for engineering and increasing oil in a range of plant tissues.

Overexpression of the AtLEC1 transcription factor, or its rapeseed orthologs BnLEC1 and BnL1L, is known to raise fatty acid levels in transgenic Arabidopsis plants, but with severe developmental abnormalities. However, the use of truncated napin A promoters, which retain the seed-specific expression pattern but with a reduced expression level, to drive the expression of BnLEC1 and BnL1L in rapeseed, resulted in increases in the seed oil content by 2%–20% without any detrimental effects on major agronomic traits (Tan *et al.*, 2011). This highlights the possibility of a similar approach to drive the ectopic expression of other transcription factors like LEC2 whose expression has previously resulted in lipid increase but with associated phenotypic abnormalities (Santos Mendoza *et al.*, 2005; Stone *et al.*, 2001). Another lipid regulatory approach to raise oil content is suggested by the recent identification of 18:1-ACP as the signal molecule and plastidic ACCase as the target for product feedback inhibition on fatty acid biosynthesis in rapeseed (Andre *et al.*, 2012). Recent studies have also pointed to other novel approaches for directing more carbon flux to lipid synthesis (Hua *et al.*, 2012; Myer *et al.*, 2012; Sanjaya *et al.*, 2011; Shi *et al.*, 2012), including through the manipulation of genes not normally associated with oil synthesis such as haemoglobin (Vigeolas *et al.*, 2011), opening up even further biotechnological approaches for engineering oil increase in plants. Perhaps most importantly, the ongoing additions of transcriptomes from a growing list of oil-producing tissues from higher plants and algae promise to continue to generate new insights into oil accumulation (Brown *et al.*, 2012; Guarnieri *et al.*, 2011; Hajduch *et al.*, 2011; Jiang *et al.*, 2012; Merchant *et al.*, 2012; Miller *et al.*, 2010; Troncoso-Ponce *et al.*, 2011; Yang *et al.*, 2010a).

Future prospects

Recent progress in metabolic engineering of plant lipids has been strong and sustained. In fact, the engineering of plants to produce long-chain PUFA, involving the introduction of a completely new pathway comprised of five additional enzymatic steps, plus enhancement of endogenous precursor pathways (Petrie *et al.*, 2010, 2012b; Ruiz-López *et al.*, 2012), is arguably already the most complex metabolic engineering goal so far achieved in plants. Importantly, metabolic engineering of industrial oils and waxes, as well as other plant lipids, is now on the verge of a significant acceleration as a result of several major recent advances in underlying technologies.

Firstly, as evidenced herein, our understanding of biochemical pathways governing fatty acid, oil and wax pathways is now proceeding at a much more rapid rate, resulting from the integration of genomic, transcriptomic, transgenic and biochemical investigative approaches. This capability is built on recent major increases in the efficiency of gene sequencing that has made it feasible and affordable to generate full-sequence information from any individual species in a relatively short period of time. Whole genome sequencing can now be conducted at a genotype level rather than a species level, and comparative transcriptomics between contrasting tissues, genotypes or species is being routinely used to uncover genetic associations with lipid biosynthetic phenotypes.

Secondly, a number of highly effective transient assay systems are now available that enable *in planta* metabolic engineering to proceed on a rapid time cycle and high-throughput basis. In addition to somatic embryo assay systems that have been applied in soybean and rapeseed for some time, a significant new capability has recently emerged through the development of the *Nicotiana benthamiana* transient leaf assay system into a sophisticated system for high-throughput experimental metabolic engineering (Wood *et al.*, 2009). This system was instrumental in evaluating alternative multistep transgenic pathways for DHA synthesis (Petrie *et al.*, 2010) and is now in widespread use within the plant lipid research community.

Thirdly, lipidomic analysis has been continually improved in both sensitivity and reliability and now enables simultaneous tracking of the multitude of intermediates involved in oil and wax biosynthetic pathways (Hummel *et al.*, 2011). Lipidomic technologies are currently undergoing some further fascinating developments including the use of mass spectroscopic (MS) imaging to provide detailed lipid species composition across tissue sections, or even individual cellular organelles or oil drops isolated from oil-bearing tissues (Horn and Chapman, 2012; Horn *et al.*, 2011,

2012). These developments will provide increased precision for unravelling the interplay of pathways with developmental biology.

Coupled together, these significant advances now enable, for the first time, the application of combinatorial metabolic engineering strategies in oil plants, to maximize efficiencies and specificities of component biosynthetic enzyme pathways. This technological step change will enable a move from the slow and laborious step-by-step testing and assembly of relatively simple transgenic pathways that has characterized the gene discovery period, to a rigorous, high-throughput and multistep combinatorial strategy. This may herald a move towards synthetic biology approaches (French, 2009) with the promise of further significant advances in engineering of plant-based oils and waxes for future industrial use.

References

Andre, C., Haslam, R.P. and Shanklin, J. (2012) Feedback regulation of plastidic acetyl-CoA carboxylase by 18 : 1-acyl carrier protein in *Brassica napus*. *Proc. Natl Acad. Sci. USA*, **109**, 10107–10112.

Badami, R.C. and Patil, K.B. (1980) Structure and occurrence of unusual fatty acids in minor seed oils. *Prog. Lipid Res.* **19**, 119–153.

Bafor, M. and Stymne, S. (1992) Substrate specificities of glycerol acylating enzymes from developing embryos of two *Cuphea* species. *Phytochemistry*, **31**, 2973–2976.

Bafor, M., Jansson, L., Stobart, A.K. and Stymne, S. (1990) Regulation of triacylglycerol biosynthesis in embryos and microsomal preparations from the developing seeds of *Cuphea lanceolata*. *Biochem. J.* **272**, 31–38.

Bao, X.M., Katz, S., Pollard, M. and Ohlrogge, J. (2002) Carbocyclic fatty acids in plants: Biochemical and molecular genetic characterization of cyclopropane fatty acid synthesis of *Sterculia foetida*. *Proc. Natl Acad. Sci. USA*, **99**, 7172–7177.

Bao, X.M., Thelen, J.J., Bonaventure, G. and Ohlrogge, J.B. (2003) Characterization of cyclopropane fatty-acid synthase from *Sterculia foetida*. *J. Biol. Chem.* **278**, 12846–12853.

Bates, P.D. and Browse, J. (2011) The pathway of triacylglycerol synthesis through phosphatidylcholine in *Arabidopsis* produces a bottleneck for the accumulation of unusual fatty acids in transgenic seeds. *Plant J.* **68**, 387–399.

Bates, P.D. and Browse, J. (2012) The significance of different diacylglycerol synthesis pathways on plant oil composition and bioengineering. *Front. Plant Sci.* **3**, 147.

Bates, P.D., Fatihi, A., Snapp, A.R., Carlsson, A.S., Browse, J. and Lu, C. (2012) Acyl editing and headgroup exchange are the major mechanisms that direct polyunsaturated fatty acid flux into triacylglycerols. *Plant Physiol.* **160**, 1530–1539.

Baud, S. and Lepiniec, L. (2010) Physiological and developmental regulation of seed oil production. *Prog. Lipid Res.* **49**, 235–249.

Biester, E.M., Hellenbrand, J., Gruber, J., Hamberg, M. and Frentzen, M. (2012) Identification of avian wax synthases. *BMC Biochem.* **13**, 4.

Bourgis, F., Kilaru, A., Cao, X., Ngando-Ebongue, G.F., Drira, N., Ohlrogge, J.B. and Arondel, V. (2011) Comparative transcriptome and metabolite analysis of oil palm and date palm mesocarp that differ dramatically in carbon partitioning. *Proc. Natl Acad. Sci. USA*, **108**, 12527–12532.

Broadwater, J.A., Whittle, E. and Shanklin, J. (2002) Desaturation and hydroxylation. Residues 148 and 324 of Arabidopsis Fad2, in addition to substrate chain length, exert a major influence in partitioning of catalytic specificity. *J. Biol. Chem.* **277**, 15613–15620.

Broun, P., Boddupalli, S. and Somerville, C. (1998a) A bifunctional oleate 12-hydroxylase: desaturase from *Lesquerella fendleri*. *Plant J.* **13**, 201–210.

Broun, P., Shanklin, J., Whittle, E. and Somerville, C. (1998b) Catalytic plasticity of fatty acid modification enzymes underlying chemical diversity of plant lipids. *Science*, **282**, 1315–1317.

Brown, A.P., Kroon, J.T.M., Swarbreck, D., Febrer, M., Larson, T.R., Graham, I. A., Caccamo, M. and Slabas, A.R. (2012) Tissue-specific whole transcriptome sequencing in castor, directed at understanding triacylglycerol lipid biosynthetic pathways. *PLoS One*, **7**, e30100.

Burgal, J., Shockey, J., Lu, C., Dyer, J., Larson, T., Graham, I. and Browse, J. (2008) Metabolic engineering of hydroxy fatty acid production in plants. RcDGAT2 drives dramatic increases in ricinoleate levels in seed oil. *Plant Biotechnol. J.* **6**, 819–831.

Cahoon, E.B., Shanklin, J. and Ohlrogge, J.B. (1992) Expression of a coriander desaturase results in petroselinic acid production in transgenic tobacco. *Proc. Natl Acad. Sci. USA*, **89**, 11184–11188.

Cahoon, E.B., Cranmer, A.M., Shanklin, J. and Ohlrogge, J.B. (1994) Δ6 hexadecenoic acid is synthesized by the activity of a soluble Δ 6 palmitoyl-acyl carrier protein desaturase in *Thunbergia alata* endosperm. *J. Biol. Chem.* **269**, 27519–27526.

Cahoon, E.B., Lindqvist, Y., Schneider, G. and Shanklin, J. (1997) Redesign of soluble fatty acid desaturases from plants for altered substrate specificity and double bond position. *Proc. Natl Acad. Sci. USA*, **94**, 4872–4877.

Cahoon, E.B., Carlson, T.J., Ripp, K.G., Schweiger, B.J., Cook, G.A., Hall, S.E. and Kinney, A.J. (1999) Biosynthetic origin of conjugated double bonds: production of fatty acid components of high-value drying oils in transgenic soybean embryos. *Proc. Natl Acad. Sci. USA*, **96**, 12935–12940.

Cahoon, E.B., Dietrich, C.R., Meyer, K., Damude, H.G., Dyer, J.M. and Kinney, A.J. (2006) Conjugated fatty acids accumulate to high levels in phospholipids of metabolically engineered soybean and *Arabidopsis* seeds. *Phytochemistry*, **67**, 1166–1176.

Cao, Y.Z., Oo, K.C. and Huang, A.H.C. (1990) Lysophosphatidate acyltransferase in the microsomes from maturing seeds of meadowfoam (*Limnanthes alba*). *Plant Physiol.* **94**, 1199–1206.

Carlsson, A.S., Lindberg Yilmaz, J., Green, A.G., Stymne, S. and Hofvander, P. (2011) Replacing fossil oil with fresh oil—with what and for what? *Eur. J. Lipid Sci. Technol.* **113**, 812–831.

Chapman, K.D. and Ohlrogge, J.B. (2012) Compartmentation of triacylglycerol accumulation in plants. *J. Biol. Chem.* **287**, 2288–2294.

Clemente, T.E. and Cahoon, E.B. (2009) Soybean oil: genetic approaches for modification of functionality and total content. *Plant Physiol.* **151**, 1030–1040.

Dehesh, K. (2001) How can we genetically engineer oilseed crops to produce high levels of medium-chain fatty acids? *Eur. J. Lipid Sci. Technol.* **103**, 688–697.

Doan, T.T., Domergue, F., Fournier, A.E., Vishwanath, S.J., Rowland, O., Moreau, P., Wood, C.C., Carlsson, A.S., Hamberg, M. and Hofvander, P. (2012) Biochemical characterisation of a chloroplast localized fatty acid reductase from *Arabidopsis thaliana*. *Biochim. Biophys. Acta*, **1821**, 1244–1255.

Dyer, J.M., Chapital, D.C., Kuan, J.C., Mullen, R.T., Turner, C., McKeon, T.A. and Pepperman, A.B. (2002) Molecular analysis of a bifunctional fatty acid conjugase/desaturase from tung: implications for the evolution of plant fatty acid diversity. *Plant Physiol.* **130**, 2027–2038.

Dyer, J.M., Stymne, S., Green, A.G. and Carlsson, A.S. (2008) High-value oils from plants. *Plant J.* **54**, 640–655.

Eccleston, V.S. and Ohlrogge, J.B. (1998) Expression of lauroyl-acyl carrier protein thioesterase in *Brassica napus* seeds induces pathways for both fatty acid oxidation and biosynthesis and implies a set point for triacylglycerol accumulation. *Plant Cell*, **10**, 613–621.

van Erp, H., Bates, P.D., Burgal, J., Shockey, J. and Browse, J. (2011) Castor phospholipid:diacylglycerol acyltransferase facilitates efficient metabolism of hydroxy fatty acids in transgenic *Arabidopsis*. *Plant Physiol.* **155**, 683–693.

French, C.E. (2009) Synthetic biology and biomass conversion: a match made in heaven? *J. R. Soc. Interface*, **6**, S547–S558.

Frentzen, M. (1993) Acyltransferases and triacylglycerols. In *Lipid Metabolism in Plants* (Moore, T.S., ed), pp. 195–230. Boca Raton, FL, London: CRC Press.

Friedt, W. and Luhs, W. (1998) Recent developments and perspectives of industrial rapeseed breeding. *Fett/Lipid*, **100**, 219–226.

Gagne, S.J., Reed, D.W., Gray, G.R. and Covello, P.S. (2009) Structural control of chemoselectivity, stereoselectivity, and substrate specificity in membrane-bound fatty acid acetylenases and desaturases. *Biochemistry*, **48**, 12298–12304.

Gamo, M. and Saito, K. (1971) The distribution of diol waxes in preen glands of some birds. *Comp. Biochem. Physiol.* **39B**, 151–157.

Ghosal, A., Banas, A., Ståhl, U., Dahlqvist, A., Lindqvist, Y. and Stymne, S. (2007) *Saccharomyces cerevisiae* phospholipid:diacylglycerol acyl transferase (PDAT) devoid of its membrane anchor region is a soluble and active enzyme retaining its substrate specificities. *Biochim. Biophys. Acta*, **1771**, 1457–1463.

Guarnieri, M.T., Nag, A., Smolinski, S.L., Darzins, A., Seibert, M. and Pienkos, P. T. (2011) Examination of triacylglycerol biosynthetic pathways via *de novo* transcriptomic and proteomic analyses in an unsequenced microalga. *PLoS One*, **6**, e25581.

Guy, J.E., Whittle, E., Kumaran, D., Lindqvist, Y. and Shanklin, J. (2007) The crystal structure of the ivy Δ4-16:0-ACP desaturase reveals structural details of the oxidized active site and potential determinants of regioselectivity. *J. Biol. Chem.* **282**, 19863–19871.

Guy, J.E., Whittle, E., Moche, M., Lengqvist, J., Lindqvist, Y. and Shanklin, J. (2011) Remote control of regioselectivity in acyl-acyl carrier protein-desaturases. *Proc. Natl Acad. Sci. USA*, **108**, 16594–16599.

Hajduch, M., Matusova, R., Houston, N.L. and Thelen, J.J. (2011) Comparative proteomics of seed maturation in oilseeds reveals difference in intermediary metabolism. *Proteomics*, **11**, 1619–1629.

Heilmann, M., Iven, T., Ahmann, K., Hornung, E., Stymne, S. and Feussner, I. (2012) Production of wax esters in plant seed oils by oleosomal co-targeting of biosynthetic enzymes. *J. Lipid Res.* **53**, 2153–2161.

Höfer, R. (2003) Oleochemical polyols: new raw materials for polyurethane applications. *Coatings*. Hannover, Germany: de, Vincentz Network.

Hoffmann, M., Hornung, E., Busch, S., Kassner, N., Ternes, P., Braus, G.H. and Feussner, I. (2007) A small membrane-peripheral region close to the active center determines regioselectivity of membrane-bound fatty acid desaturases from *Aspergillus nidulans*. *J. Biol. Chem.* **282**, 26666–26674.

Hongsthong, A., Subudhi, S., Sirijuntarat, M. and Cheevadhanarak, S. (2004) Mutation study of conserved amino acid residues of *Spirulina* Δ^6-acyl-lipid desaturase showing involvement of Histidine 313 in the regioselectivity of the enzyme. *Appl. Microbiol. Biotechnol.* **66**, 74–84.

Horn, P.J. and Chapman, K.D. (2012) Lipidomics in tissues, cells and subcellular compartments. *Plant J.* **70**, 69–80.

Horn, P.J., Ledbetter, N.R., James, C.N., Hoffman, W.D., Case, C.R., Verbeck, G.F. and Chapman, K.D. (2011) Visualization of lipid droplet composition by direct organelle mass spectroscopy. *J. Biol. Chem.* **286**, 3298–3306.

Horn, P.J., Korte, A.R., Neogi, P.B., Love, E., Fuchs, J., Strupat, K., Borisjuk, L., Shulaev, V., Lee, Y.J. and Chapman, K.D. (2012) Spatial mapping of lipids at cellular resolution in embryos of cotton. *Plant Cell*, **24**, 622–636.

Hu, Z., Ren, Z. and Lu, C. (2012) The phosphatidylcholine diacylglycerol cholinephosphotransferase is required for efficient hydroxy fatty acid accumulation in transgenic Arabidopsis. *Plant Physiol.* **158**, 1944–1954.

Hua, W., Li, R.-J., Zhan, G.-M., Liu, J., Li, J., Wang, X.-F.Liu, G.-H. and Wang, H.-Z. (2012) Maternal control of seed oil content in *Brassica napus*: the role of silique wall photosynthesis. *Plant J.* **69**, 432–444.

Hummel, J., Segu, S., Li, Y., Irgang, S., Jueppner, J. and Giavalisco, P. (2011) Ultra performance liquid chromatography and high resolution mass spectrometry for the analysis of plant lipids. *Front. Plant Sci.* **2**, 54.

Jiang, H., Wu, P., Zhang, S., Song, C., Chen, Y., Li, M., Jia, Y., Fang, X., Chen, F. and Wu, G. (2012) Global analysis of gene expression profiles in developing physic nut (*Jatropha curcus* L.) seeds. *PLoS One*, **7**, e36522.

Kalscheuer, R. and Steinbüchel, A. (2003) A novel bifunctional wax ester synthase/acyl-CoA:diacylglycerol acyltransferase mediates wax ester and triacylglycerol biosynthesis in *Acinetobacter calcoaceticus* ADP1. *J. Biol. Chem.* **278**, 8075–8082.

Kim, H.U., Lee, K.R., Go, Y.S., Jung, J.H., Suh, M.C. and Kim, J.B. (2011) Endoplasmic reticulum-located PDAT1-2 from castor bean enhances hydroxy fatty acid accumulation in transgenic plants. *Plant Cell Physiol.* **52**, 983–993.

Kroon, J.T., Wei, W., Simon, W.J. and Slabas, A.R. (2006) Identification and functional expression of a type 2 acyl-CoA:diacylglycerol acyltransferase (DGAT2) in developing castor bean seeds which has high homology to the major triglyceride biosynthetic enzyme of fungi and animals. *Phytochemistry*, **67**, 2541–2549.

Kumar, R., Wallis, J.G., Skidmore, C. and Browse, J. (2006) A mutation in Arabidopsis cytochrome b5 reductase identified by high-throughput screening differentially affects hydroxylation and desaturation. *Plant J.* **48**, 920–932.

Lardizabal, K.D., Metz, J.G., Sakamoto, T., Hutton, W.C., Pollard, M.R. and Lassner, M.W. (2000) Purification of a jojoba embryo wax synthase, cloning of its cDNA, and production of high levels of wax in seeds of transgenic *Arabidopsis*. *Plant Physiol.* **122**, 645–655.

Larson, T.R., Edgell, T., Byrne, J., Dehesh, K. and Graham, I.A. (2002) Acyl CoA profiles of transgenic plants that accumulate medium-chain fatty acids indicate inefficient storage lipid synthesis in developing oilseeds. *Plant J.* **32**, 519–527.

Le Quéré, C., Raupach, M.R., Canadell, J.G., Marland, G., Bopp, L., Ciais, P., Conway, T.J., Doney, S.C., Feely, R.A., Foster, P., Friedlingstein, P., Gurney, K., Houghton, R.A., House, J.I., Huntingford, C., Levy, P.E., Lomas, M.R., Majkut, J., Metzl, N., Ometto, J.P., Peters, G.P., Prentice, I.C., Randerson, J.T., Running, S.W., Sarmiento, J.L., Schuster, U., Sitch, S., Takahashi, T., Viovy, N., van der Werf, G.R. and Woodward, F.I. (2009) Trends in the sources and sinks of carbon dioxide. *Nat. Geosci.* **2**, 831–836.

Lee, M., Lenman, M., Banas, A., Bafor, M., Singh, S., Schweizer, M., Nilsson, R., Liljenberg, C., Dahlqvist, A., Gummeson, P.-O., Sjodahl, S., Green, A. and Stymne, S. (1998) Identification of non-heme diiron proteins that catalyze triple bond and epoxy group formation. *Science*, **280**, 915–918.

Lee, J., Welti, R., Schapaugh, W.T. and Trick, H.N. (2011) Phospholipid and triacylglycerol profiles modified by PLD suppression in soybean seed. *Plant Biotechnol. J.* **9**, 359–372.

Li, F., Wu, X., Lam, P., Bird, D., Zheng, H., Samuels, L., Reinhard Jetter, R. and Kunst, L. (2008) Identification of the wax ester synthase/acyl-coenzyme A: diacylglycerol acyltransferase WSD1 required for stem wax ester biosynthesis in Arabidopsis. *Plant Physiol.* **148**, 97–107.

Li, W., Kong, X.H., Ruan, M., Ma, F.M., Jiang, Y.F., Liu, M.Z., Chen, Y. and Zuo, X.H. (2010a) Green waxes, adhesives and lubricants. *Philos. Transact. A Math. Phys. Eng. Sci.* **368**, 4869–4890.

Li, R., Yu, K. and Hildebrand, D.F. (2010b) DGAT1, DGAT2 and PDAT expression in seeds and other tissues of epoxy and hydroxy fatty acid accumulating plants. *Lipids*, **45**, 145–157.

Li, R., Yu, K., Hatanaka, T. and Hildebrand, D.F. (2010c) Vernonia DGATs increase accumulation of epoxy fatty acids in oil. *Plant Biotechnol. J.* **8**, 184–195.

Li, R., Yu, K., Wu, Y., Tateno, M., Hatanaka, T. and Hildebrand, D.F. (2012a) *Vernonia* DGATs can complement the disrupted oil and protein metabolism in epoxygenase-expressing soybean seeds. *Metab. Eng.* **14**, 29–38.

Li, X., van Loo, E.N., Gruber, J., Fan, J., Guan, R., Frentzen, M., Stymne, S. and Zhu, L.-H. (2012b) Development of high-erucic acid oil in the industrial oil crop *Crambe abyssinica*. *Plant Biotechnol. J.* **20**, 862–870.

Li-Beisson, Y., Shorrosh, B., Beisson, F., Andersson, M., Arondel, V., Bates, P., Baud, S., Bird, D., DeBono, A., Durrett, T., Franke, R., Graham, I., Katayama, K., Kelly, A., Larson, T., Markham, J., Miquel, M., Molina, I., Nishida, I., Rowland, O., Samuels, L., Schmid, K., Wada, H., Welti, R., Xu, C., Zallot, R. and Ohlrogge, J. (2010) Acyl-lipid metabolism. *Arabidopsis Book*, **8**, e0133.

Lindqvist, Y., Huang, W., Schneider, G. and Shanklin, J. (1996) Crystal structure of Δ^9 stearoyl-acyl carrier protein desaturase from castor seed and its relationship to other di-iron proteins. *EMBO J.* **15**, 4081–4092.

Lippold, F., vom Dorp, K., Abraham, M., Hölzl, G., Wewer, V., Yilmaz, J.L., Lager, I., Montandon, C., Besagni, C., Kessler, F., Stymne, S. and Dörmann, P. (2012) Fatty acid phytyl ester synthesis in chloroplasts of Arabidopsis. *Plant Cell*, **24**, 2001–2014.

van der Loo, F.J., Broun, P., Turner, S. and Somerville, C. (1995) An oleate 12-hydroxylase from *Ricinus communis* L. is a fatty acyl desaturase homolog. *Proc. Natl Acad. Sci. USA*, **92**, 6743–6747.

Lu, C.F., Xin, Z.G., Ren, Z.H., Miquel, M. and Browse, J. (2009) An enzyme regulating triacylglycerol composition is encoded by the ROD1 gene of Arabidopsis. *Proc. Natl Acad. Sci. USA*, **106**, 18837–18842.

Lu, C., Napier, J.A., Clemente, T.E. and Cahoon, E.B. (2011) New frontiers in oilseed biotechnology: meeting the global demand for vegetable oils for food, feed, biofuel, and industrial applications. *Curr. Opin. Biotechnol.* **22**, 252–259.

Meesapyodsuk, D., Reed, D.W., Covello, P.S. and Qiu, X. (2007) Primary structure, regioselectivity, and evolution of the membrane-bound fatty acid desaturase of *Claviceps purpurea*. *J. Biol. Chem.* **282**, 20191–20199.

Meier, M.A.R. (2009) Metathesis with oleochemicals: new approaches for the utilization of plant oils as renewable resources in polymer science. *Macromol. Chem. Phys.* **210**, 1073–1079.

Merchant, S.S., Kropat, J., Liu, B., Shaw, J. and Warakanont, J. (2012) TAG, you're it! *Chlamydomonas* as a reference organism for understanding algal triacylglycerol accumulation. *Curr. Opin. Biotechnol.* **23**, 352–363.

Metz, J.G., Pollard, M.R., Anderson, L., Hayes, T.R. and Lassner, M.W. (2000) Purification of a jojoba embryo fatty acyl-coenzyme A reductase and expression of its cDNA in high erucic acid rapeseed. *Plant Physiol.* **122**, 635–644.

Miller, R., Wu, G., Deshpande, R.R., Vieler, A., Gartner, K., Li, X., Moellering, E. R., Zauner, S., Cornish, A.J., Liu, B., Bullard, B., Sears, B.B., Kuo, M.-H., Hegg, E.L., Shachar-Hill, Y., Shui, S.-H. and Benning, C. (2010) Changes in transcript abundance in *Chlamydomonas reinhardtii* following nitrogen deprivation predict diversion of metabolism. *Plant Physiol.* **154**, 1737–1752.

Myer, K., Stecca, K.L., Ewell-Hicks, K., Allen, S.A. and Everard, J.D. (2012) Oil and protein accumulation in developing seeds is influenced by the expression of a cytosolic pyrophosphatase in Arabidopsis. *Plant Physiol.* **159**, 1221–1234.

Nam, J.W. and Kappock, T.J. (2007) Cloning and transcriptional analysis of *Crepis alpina* fatty acid desaturases affecting the biosynthesis of crepenynic acid. *J. Exp. Bot.* **58**, 1421–1432.

Napier, J.A. (2007) The production of unusual fatty acids in transgenic plants. *Annu. Rev. Plant Biol.* **58**, 295–319.

Napier, J.A. and Graham, I.A. (2010) Tailoring plant lipid composition: designer oilseeds come of age. *Curr. Opin. Plant Biol.* **13**, 330–337.

Nath, N.K., Wilmer, J.A., Wallington, E.J., Becker, H.C. and Möllers, C. (2009) Increasing erucic acid content through combination of endogenous low polyunsaturated fatty acids alleles with Ld-LPAAT + Bn-Fae1 transgenes in rapeseed (*Brassica napus* L.). *Theor. Appl. Genet.* **118**, 765–773.

Nguyen, H.T., Mishra, G., Whittle, E., Pidkowich, M.S., Bevan, S.A., Merlo, A. O., Walsh, T.A. and Shanklin, J. (2010) Metabolic engineering of seeds can achieve levels of ω-7 fatty acids comparable with the highest levels found in natural plant sources. *Plant Physiol.* **154**, 1897–1904.

Nykiforuk, C.L., Shewmaker, C., Harry, I., Yurchenko, O.P., Zhang, M., Reed, C., Oinam, G.S., Zaplachinski, S., Fidantsef, A., Boothe, J.G. and Moloney, M. M. (2012) High level accumulation of gamma linolenic acid (C18:3Δ6, 9,12 cis) in transgenic safflower (*Carthamus tinctorius*) seeds. *Transgenic Res.* **21**, 367–381.

Ohlrogge, J. and Chapman, K. (2011) The seeds of green energy—expanding the contribution of plant oils as biofuels. *The Biochemist*, **33**, 34–38.

Oil World Annual (2012). *ISTA Mielke GmbH*. Hamburg, Germany: Oil World Annual.

Petrie, J.R., Shrestha, P., Liu, Q., Mansour, M.P., Wood, C.C., Zhou, X.R., Nichols, P.D., Green, A.G. and Singh, S.P. (2010) Rapid expression of transgenes driven by seed-specific constructs in leaf tissue: DHA production. *Plant Methods*, **6**, 8–13.

Petrie, J.R., Vanhercke, T., Shrestha, P., El-Tahchy, A., White, A., Zhou, X.R., Liu, Q., Mansour, M.P., Nichols, P.D. and Singh, S.P. (2012a) Recruiting a new substrate for triacylglycerol synthesis in plants: the monoacylglycerol acyltransferase pathway. *PLoS One*, **7**, e35214.

Petrie, J.R., Shrestha, P., Zhou, X-R., Mansour, M.P., Liu, Q., Belide, S., Nichols, P.D. and Singh, S. (2012b) Metabolic engineering plant seeds with fish oil-like levels of DHA. *PLoS One*, **7**, e49165.

Poirier, Y., Ventre, G. and Caldelari, D. (1999) Increased flow of fatty acids toward beta-oxidation in developing seeds of *Arabidopsis* deficient in diacylglycerol acyltransferase activity or synthesizing medium-chain-length fatty acids. *Plant Physiol.* **121**, 1359–1366.

Qiu, X., Reed, D.W., Hong, H., Mackenzie, S.L. and Covello, P.S. (2001) Identification and analysis of a gene from *Calendula officinalis* encoding a fatty acid conjugase. *Plant Physiol.* **125**, 847–855.

Rawat, R., Yu, X.-H., Sweet, M. and Shanklin, J. (2012) Conjugated fatty acid synthesis. Residues 111 and 115 influence product partitioning of *Momordica charantia* conjugase. *J. Biol. Chem.* **287**, 16230–16237.

Rowland, O. and Domergue, F. (2012) Plant fatty acyl reductases: enzymes generating fatty alcohols for protective layers with potential for industrial applications. *Plant Sci.* **193–194**, 28–38.

Ruiz-López, N., Sayanova, O., Napier, J.A. and Haslam, R.P. (2012) Metabolic engineering of the omega-3 long chain polyunsaturated fatty acid biosynthetic pathway into transgenic plants. *J. Exp. Bot.* **63**, 2397–2410.

Rybak, A., Fokou, P.A. and Meier, M.A.R. (2008) Metathesis as a versatile tool in oleochemistry. *Eur. J. Lipid Sci. Technol.* **110**, 797–804.

Sandager, L., Gustavsson, M.H., Ståhl, U., Dahlqvist, A., Wiberg, E., Banas, A., Lenman, M., Ronne, H. and Stymne, S. (2002) Storage lipid synthesis is non-essential in yeast. *J. Biol. Chem.* **277**, 6478–6482.

Sanjaya, Durrett, T.P., Weise, S.E. and Benning, C. (2011) Increasing the energy density of vegetative tissues by diverting carbon from starch to oil biosynthesis in transgenic Arabidopsis. *Plant Biotechnol. J.* **9**, 874–883.

Santos Mendoza, M., Dubreucq, B., Miquel, M., Caboche, M. and Lepiniec, L. (2005) *LEAFY COTYLEDON 2* activation is sufficient to trigger the accumulation of oil and seed specific mRNAs in Arabidopsis leaves. *FEBS Lett.* **576**, 4666–4670.

Shi, L., Katavic, V., Yu, Y., Kunst, L. and Haughn, G. (2012) Arabidopsis *glabra2* mutant seeds deficient in mucilage biosynthesis produce more oil. *Plant J.* **69**, 37–46.

Shockey, J.M., Gidda, S.K., Chapital, D.C., Kuan, J.C., Dhanoa, P.K., Bland, J. M., Rothstein, S.J., Mullen, R.T. and Dyer, J.M. (2006) Tung tree DGAT1 and DGAT2 have nonredundant functions in triacylglycerol biosynthesis and are localized to different subdomains of the endoplasmic reticulum. *Plant Cell*, **18**, 2294–2313.

Siloto, R.M.P., Truksa, M., Brownfield, D., Good, A.G. and Weselake, R.J. (2009a) Directed evolution of acyl-CoA:diacylglycerol acyltransferase: development and characterization of *Brassica napus* DGAT1 mutagenized libraries. *Plant Physiol. Biochem.* **47**, 456–461.

Siloto, R.M.P., Truksa, M., He, X., McKeon, T. and Weselake, R.J. (2009b) Simple methods to detect triacylglycerol biosynthesis in a yeast-based recombinant system. *Lipids*, **44**, 963–973.

Skoric, D., Jocic, S., Sakac, Z. and Lecic, N. (2008) Genetic possibilities for altering sunflower oil quality to obtain novel oils. *Can. J. Physiol. Pharmacol.*, **86**, 215–221.

Snyder, C., Yurchenko, O.P., Siloto, R.M.P., Chen, X., Liu, Q., Mietkiewska, E. and Weselake, R.J. (2009) Acyltransferase action in the modification of seed oil biosynthesis. *N. Biotechnol.* **26**, 11–16.

Sperling, P., Lee, M., Girke, T., Zahringer, U., Stymne, S. and Heinz, E. (2000) A bifunctional delta-fatty acyl acetylenase/desaturase from the moss *Ceratodon purpureus*. A new member of the cytochrome b5 superfamily. *Eur. J. Biochem.* **267**, 3801–3811.

Spitzer, V., Aitzetmüller, K. and Vosmann, K. (1996) The seed oil of *Bernardia pulchella* (Euphorbiaceae)—a rich source of vernolic acid. *J. Am. Oil Chem. Soc.* **73**, 1733–1735.

Ståhl, U., Carlsson, A.S., Lenman, M., Dahlqvist, A., Huang, B.Q., Banas, W., Banas, A. and Stymne, S. (2004) Cloning and functional characterization of a Phospholipid: Diacylglycerol acyltransferase from Arabidopsis. *Plant Physiol.* **135**, 1324–1335.

Stålberg, K., Ståhl, U., Stymne, S. and Ohlrogge, J. (2009) Characterization of two *Arabidopsis thaliana* acyltransferases with preference for lysophosphatidylethanolamine. *BMC Plant Biol.* **9**, 60.

Stobart, K., Mancha, M., Lenman, M., Dahlqvist, A. and Stymne, S. (1997) Triacylglycerols are synthesised and utilized by transacylation reactions in microsomal preparations of developing safflower (*Carthamus tinctorius* L.) seeds. *Planta*, **203**, 58–66.

Stone, S.L., Kwong, L.W., Yee, K.M., Pelletier, J., Lepiniec, L., Fischer, R.L., Goldberg, R.B. and Harada, J.J. (2001) *LEAFY COTYLEDON2* encodes a B3 domain transcription factor that induces embryo development. *Proc. Natl Acad. Sci. USA*, **98**, 11806–11811.

Stymne, S., Tonnet, M.L. and Green, A.G. (1992) Biosynthesis of linolenate in developing embryos and cell-free preparations of high-linolenate linseed (*Linum usitatissimum*) and low-linolenate mutants. *Arch. Biochem. Biophys.* **294**, 557–563.

Suh, M.C., Schultz, D.J. and Ohlrogge, J.B. (2002) What limits production of unusual monoenoic fatty acids in transgenic plants? *Planta*, **215**, 584–595.

Tan, H., Yang, X., Zhang, F., Zheng, X., Qu, C., Mu, J., Fu, F., Li, J., Guan, R., Zhang, H., Wang, G. and Zuo, J. (2011) Enhanced seed oil production in canola by conditional expression of *Brassica napus LEAFY COTYLEDON1* and *LEC1-LIKE* in developing seeds. *Plant Physiol.* **156**, 1577–1588.

Taylor, D.C., Smith, M.A., Fobert, P., Mietkiewaska, E. and Weselake, R.J. (2011) Metabolic engineering of higher plants to produce bio-industrial oils. In *Comprehensive Biotechnology*, 2nd edn, Vol. 4 (Moo-Young, M., ed), pp. 67–85. The Netherlands: Elsevier B.V.

Thomaeus, S., Carlsson, A.S. and Stymne, S. (2001) Distribution of fatty acids in polar and neutral lipids during seed development in *Arabidopsis thaliana* genetically engineered to produce acetylenic, epoxy and hydroxy fatty acids. *Plant Sci.* **161**, 997–1003.

Troncoso-Ponce, M.A., Kilaru, A., Cao, X., Durrett, T.P., Fan, J., Jensen, J.K., Thrower, N.A., Pauly, M., Wilkerson, C. and Ohlrogge, J.B. (2011) Comparative deep transcriptional profiling of four developing oilseeds. *Plant J.* **68**, 1014–1027.

Vanhercke, T., Shrestha, P., Green, A.G. and Singh, S.P. (2011) Mechanistic and structural insights into the regioselectivity of an acyl-CoA fatty acid desaturase via directed molecular evolution. *J. Biol. Chem.* **286**, 12860–12869.

Vigeolas, H., Huhn, D. and Geigenberger, P. (2011) Nonsymbiotic hemoglobin-2 leads to an elevated energy state and to a combined increase in polyunsaturated fatty acids and total oil content when overexpressed in developing seeds of transgenic Arabidopsis plants. *Plant Physiol.* **155**, 1435–1444.

Voelker, T.A., Worrell, A.C., Anderson, L., Bleibaum, J., Fan, C., Hawkins, D.J., Radke, S.E. and Davies, H.M. (1992) Fatty acid biosynthesis redirected to medium chains in transgenic oilseed plants. *Science*, **257**, 72–74.

Voelker, T.A., Jones, A., Cranmer, A.M., Davies, H.M. and Knutzon, D.S. (1997) Broad-range and binary-range acyl-acyl-carrier-protein thioesterases suggest an alternative mechanism for medium-chain production in seeds. *Plant Physiol.* **114**, 669–677.

Wallis, J.W. and Browse, J. (2010) Lipid biochemists salute the genome. *Plant J.* **61**, 1092–1106.

Wang, Z., Hobson, N., Galindo, L., Zhu, S., Shi, D., McDill, J., Yang, L., Hawkins, S., Neutelings, G., Datla, R., Lambert, G., Galbraith, D.W., Grassa, C.J., Geraldes, A., Cronk, Q.C., Cullis, C., Dash, P.K., Kumar, P.A., Cloutier, S., Sharpe, A.G., Wong, G.K., Wang, J. and Deyholos, M.K. (2012) The genome of flax (*Linum usitatissimum*) assembled de novo from short shotgun sequence reads. *Plant J.* **72**, 461–473.

Weselake, R.J., Taylor, D.C., Rahman, M.H., Shah, S., Laroche, A., McVetty, P.B. E. and Harwood, J.L. (2009) Increasing the flow of carbon into seed oil. *Biotechnol. Adv.* **27**, 866–878.

Whittle, E. and Shanklin, J. (2001) Engineering Δ^9-16:0-acyl carrier protein (ACP) desaturase specificity based on combinatorial saturation mutagenesis and logical redesign of the castor Δ^9-18:0-ACP desaturase. *J. Biol. Chem.* **276**, 21500–21505.

Whittle, E.J., Tremblay, A.E., Buist, P.H. and Shanklin, J. (2008) Revealing the catalytic potential of an acyl-ACP desaturase: tandem selective oxidation of saturated fatty acids. *Proc. Natl Acad. Sci. USA*, **105**, 14738–14743.

Wiberg, E., Tillberg, E. and Stymne, S. (1994) Substrates of diacylglycerol acyltransferase in microsomes from developing oil seeds. *Phytochemistry*, **36**, 573–577.

Wiberg, E., Edwards, P., Byrne, J., Stymne, S. and Dehesh, K. (2000) The distribution of caprylate, caprate and laurate in lipids from developing and mature seeds of transgenic *Brassica napus* L. *Planta*, **212**, 33–40.

Wood, C.C., Petrie, J.R., Shrestha, P., Mansour, M.P., Nichols, P.D., Green, A.G. and Singh, S.P. (2009) A leaf-based assay using interchangeable design principles to rapidly assemble multistep recombinant pathways. *Plant Biotechnol. J.* **7**, 1–11.

Xu, J., Francis, T., Mietkiewska, E., Giblin, E.M., Barton, D.L., Zhang, Y., Zhang, M. and Taylor, D.C. (2008) Cloning and characterization of an acyl-CoA-dependent *diacylglycerol acyltransferase 1* (*DGAT1*) gene from *Tropaeolum majus*, and a study of the functional motifs of the DGAT protein using site-directed mutagenesis to modify enzyme activity and oil content. *Plant Biotechnol. J.* **6**, 799–818.

Xu, J., Carlsson, A.S., Francis, T., Zhang, M., Hoffman, T., Giblin, M.E. and Taylor, D.C. (2012) Triacylglycerol synthesis by PDAT1 in the absence of DGAT1 activity is dependent on re-acylation of LPC by LPCAT2. *BMC Plant Biol.* **12**, 4.

Yang, P., Li, X., Shipp, M.J., Shockey, J.M. and Cahoon, E.B. (2010a) Mining the bitter melon (*Momordica charantia* L.) seed transcriptome by 454 analysis of non-normalized and normalized cDNA populations for conjugated fatty acid metabolism-related genes. *BMC Plant Biol.* **10**, 250–269.

Yang, W., Pollard, M., Li-Beisson, Y., Beisson, F., Feig, M. and Ohlrogge, J. (2010b) A distinct type of glycerol-3-phosphate acyltransferase with sn-2 preference and phosphatase activity producing 2-monoacylglycerol. *Proc. Natl Acad. Sci. USA*, **107**, 12040–12045.

Yen, C.L., Monetti, M., Burri, B.J. and Farese, R.V. (2005) The triacylglycerol synthesis enzyme DGAT1 also catalyzes the synthesis of diacylglycerols, waxes, and retinyl esters. *J. Lipid Res.* **46**, 1502–1511.

Yu, K., McCracken, C.T., Li, R. and Hildebrand, D.F. (2006) Diacylglycerol acyltransferases from *Vernonia* and *Stokesia* prefer substrates with vernolic acid. *Lipids*, **41**, 557–566.

Yu, X.-H., Rawat, R. and Shanklin, J. (2011) Characterization and analysis of the cotton cyclopropane fatty acid synthase family and their contribution to cyclopropane fatty acid synthesis. *BMC Plant Biol.* **11**, 97.

Zhang, M., Fan, J., Taylor, D.C. and Ohlrogge, J.B. (2009) DGAT1 and PDAT1 acyltransferases have overlapping functions in *Arabidopsis* triacylglycerol biosynthesis and are essential for normal pollen and seed development. *Plant Cell*, **21**, 3885–3901.

Zheng, Q., Li, J.Q., Kazachkov, M., Liu, K. and Zou, J. (2012) Identification of *Brassica napus* lysophosphatidylcholine acyltransferase genes through yeast functional screening. *Phytochemistry*, **75**, 21–31.