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Review article

The modification of plant oil composition via metabolic engineering—better nutrition by design

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Summary

This article will focus on the modification of plant seed oils to enhance their nutritional composition. Such modifications will include C18 Δ6-desaturated fatty acids such as γ-linolenic and stearidonic acid, omega-6 long-chain polyunsaturated fatty acids such as arachidonic acid, as well as the omega-3 long-chain polyunsaturated fatty acids (often named 'fish oils') such as eicosapentaenoic acid and docosahexaenoic acid. We will consider how new technologies (such as synthetic biology, next-generation sequencing and lipidomics) can help speed up and direct the development of desired traits in transgenic oilseeds. We will also discuss how manipulating triacylglycerol structure can further enhance the nutritional value of 'designer' oils. We will also consider how advances in model systems have translated into crops and the potential end-users for such novel oils (e.g. aquaculture, animal feed, human nutrition).

Introduction

It is now widely accepted that omega-3 long-chain polyunsaturated fatty acids (LC-PUFAs) such as eicosapentaenoic acid (EPA; 20:5Δ5,8,11,14,17) and docosahexaenoic acid (DHA; 22:6 Δ4,7,10,13,16,19) play a crucial role in maintaining optimum human health, in particular reducing our risk of cardiovascular disease (CVD) and related precursor conditions such as metabolic syndrome (which is a clinical indicator for risk of progression towards pathologies such as type-2 diabetes and obesity) (Graham *et al.*, 2004). Given the relentless rise in diseases such as CVD and type 2 diabetes (and the enormous burden these illnesses place on our health systems), regular dietary ingestion of omega-3 LC-PUFAs represent a cheap and simple intervention with which to avert what has been referred to as the 'obesity time bomb' (Logue and Sattar, 2011). This not just based on epidemiological observations but also abundant clinical evidence that increased intake of the long-chain omega-3 LC-PUFAs, EPA and DHA, traditionally from fish oils, has a positive impact on cardiovascular health (Calder, 2004; Saito *et al.*, 2008; Yokoyama *et al.*, 2007). In particular, their effects have been attributed to their modulation of a number of physiological mechanisms, including their antiarrthymic, antithrombotic and anti-inflammatory actions, along with positive effects on the blood lipid profile, on atherosclerotic plaque stability (Saravanan *et al.*, 2010; Tanaka *et al.*, 2008) and on endothelial function and vascular reactivity (Mori *et al.*, 2000; Nestel *et al.*, 2002).

In addition to the importance of omega-3 LC-PUFAs in cardiovascular health, evidence is now also emerging as to the importance of these fatty acids in cognitive function (Beydoun *et al.*, 2007). Cognitive function is a major determinant of quality of life in older age, and decline in cognitive functioning is a primary contributing factor to increasing dependency in the elderly. Omega-3 LC-PUFA has long been known to be important for brain development and normal brain function (Neuringer *et al.*, 1988), with DHA particularly important to brain functioning due to its influence on neural membrane properties, which modulate cell signalling. DHA concentration in the brain decreases with age in humans (Soderberg *et al.*, 1999), leading to the suggestion that this is a causal effect in the age-related deterioration in central nervous system functions (Uauy and Dangour, 2006). This is supported by animal studies; mice fed a low omega-3 LC-PUFA diet displayed cognitive problems (Suzuki *et al.*, 1998) that were ameliorated by DHA supplementation (Chung *et al.*, 2008; Cole and Frautschy, 2006; Moriguchi and Salem, 2003). DHA supplementation also improved memory performance in aged mice (Sugimoto *et al.*, 2002). In humans, it has been consistently reported that a higher intake of fish is related to a reduced cognitive decline and incidence of dementia (reviewed by Kalmijn, 2000). However, it should be noted that only a small number of studies, which examined relationships between cognitive health and dietary intake levels of omega-3 LC-PUFAs, have found significant positive relationships (van Gelder *et al.*, 2007; Morris *et al.*, 2003). Whilst there are examples of studies in cognitively healthy populations which have failed to show efficacy for omega-3 LC-PUFAs in improving cognition (van de Rest *et al.*, 2008), similar omega-3 LC-PUFAs supplementation has been shown to be efficacious in mild cognitively impaired individuals (Chiu *et al.*, 2008; Yurko-Mauro *et al.*, 2010). Thus, there is a growing body of evidence to support an important role for omega-3 LC-PUFAs in aspects of cognition and mental health.

The recognition of potential health benefits of dietary omega-3 LC-PUFA has led to specific recommendations for intake in many countries of the world. For example, in the UK, the Standing Advisory Committee on Nutrition (Kris-Etherton *et al.*, 2009; SACN, 2004) has recommended that adults should be consuming an average of 450 mg/day of omega-3 LC-PUFA. Even against these rather modest levels, adult consumption in the UK is on average half that figure (Givens and Gibbs, 2008) yet there is a growing gulf in what we have available in the oceans, and what we need to consume for a healthier human diet (Collins *et al.*, 2008)—currently, the planet is producing around 1 million tonnes of fish oil/year, yet for every person on the planet to consume

their recommended level of omega-3 LC-PUFA, it would require 2.5 million tonnes/year (FAO, 2010; James and Slaski, 2009). Thus, total global supplies of omega-3 LC-PUFA from all fisheries (both food-grade and feed-grade), marine and freshwater, are finite, limiting and diminishing—the aphorism 'plenty more fish in the sea' is in danger of being rendered obsolete.

Primary production of omega-3 LC-PUFA occurs largely in microbes, particularly microalgae of marine origin, and these fatty acids are passed up (and retained) in the food web. By way of example, Sardine oil contains 16% EPA and 9% DHA (Haraldsson and Hjaltason, 2001). This fact underpins the vital role of fish, and in particular oily fish, as the major source of omega-3 LC-PUFA in the human diet, a fact that is now well appreciated by the consumer. However, over-exploitation of wild fisheries has meant that an increasing proportion of fish and seafood for human consumption is now farmed (around 50% in 2008). Aquaculture is the fastest growing global food production system and is continuing to expand at around 9% per year (Bostock *et al.*, 2010). However, the replacement of capture fisheries by aquaculture in supplying fish for human consumption presents a major paradox as fish-feeds have been traditionally based on fishmeal and fish oil, themselves derived from feed-grade capture fisheries (Pauly *et al.*, 1998; Williams and Burdge, 2006). The resulting demand for marine resources is continually increasing such that requirements for aquaculture feeds now exceed global supplies of fish oil (Tacon and Metian, 2009). This situation has been exacerbated by environmental pollution of the marine environment, which means that for some geographical regions, fish are contaminated with toxic trace elements such as heavy metals, polychlorinated biphenyls (or PCBs) and dioxins, further reducing the available global stock of fish oil. This has also led to significant pressure on lower trophic levels in the marine food webs, most recently with attempts to harvest large volumes of krill for use in fish farming (Bostock *et al.*, 2010). The implications of such uncontrolled exploitation of vital aquatic food webs are extremely serious, both at the level of food security, but also in terms of biodiversity and the protection of ecosystems (Garcia and Rosenberg, 2010). Thus, current aquaculture is significantly dependent on fish oils as an input and represents an unsustainable production system. Attempts to substitute fish oils with vegetable oils have resulted in animals that lack omega-3 LC-PUFAs, reducing their value and appeal to the consumer, and also impacting on the health of the fish (Tocher, 2003). Utilization of marine microalgae as alternative direct supply of EPA and DHA to the aquaculture industry is also possible (Khozin-Goldberg *et al.*, 2011). Indeed, several commercial single-cell sources for LC-PUFA have been developed in the last two decades, for example large-scale arachidonic acid (ARA) production by cultivation of the oleaginous filamentous fungus *Mortierella alpina* to meet the demands of the baby formula industry (Sakuradani *et al.*, 2009). However, economically feasible cultivation of photosynthetic microalgae for large-scale production of LC-PUFA requires substantial advances in photo-bioreactors and breakthrough solutions enhancing growth performance and lipid modification.

Therefore, there is an urgent need for a completely new source of omega-3 LC-PUFA, which is produced *de novo* by a more sustainable system whose output can increase as required to meet future demands. This review considers research over the last decade to reconstitute the omega-3 LC-PUFA biosynthetic pathway in transgenic plants, highlighting the virtuous circle of knowledge-led iterations to deliver meaningful levels of target fatty acids. Through the fundamental study of plant lipid metabolism, breakthroughs in our capacity to successfully introduce novel traits have been achieved. Equally, by the application of advanced plant metabolic engineering, new insights into the complexity of fatty acid synthesis and modification have been obtained. Together, these advances mean that the ability to generate designer oilseeds via transgenesis is now a reality.

Summary of biosynthetic pathways

All higher plants have the capacity to synthesize the C_{18} polyunsaturated fatty acids (C_{18}-PUFA) linoleic (C18:2Δ9,12, LA) and α-linolenic (C18:3Δ9,12,15, ALA) acids; however, a few plants do have the ability to produce Δ6-desaturated fatty acids, for example stearidonic acid (SDA; 18:4 Δ6,9,12,15) and predominantly γ-linolenic acid (GLA; 18:3 Δ6,9,12). Beyond these fatty acids, higher plants do not possess the enzymatic machinery to undertake the elongation and desaturation steps required to convert C_{18}-PUFA into ω-3 LC-PUFA (Damude and Kinney, 2008; Napier, 2007; Napier and Graham, 2010). The production of PUFA in plants begins with the synthesis of fatty acids (FA) by the multi-subunit fatty acid synthase (FAS) complex in the plastid (Harwood, 1988; Somerville and Browse, 1991). The final products of this enzymatic complex are 16:0- and 18:0-acyl carrier protein (ACP); much of the 18:0-ACP is subsequently desaturated by a soluble stearoyl-ACP desaturase, yielding 18:1 Δ9-ACP (Ohlrogge and Jaworski, 1997). These fatty acids are then hydrolysed from ACP by acyl-ACP thioesterases, exit the plastid and are esterified to coenzyme A (CoA) to form acyl-CoA (Ohlrogge and Jaworski, 1997). Subsequently, some of these acyl moieties will become esterified to phosphatidylcholine (PC) and then undergo desaturation by Δ12- and Δ15-desaturases to form the essential fatty acids LA and ALA (Ohlrogge and Browse, 1995; Ohlrogge and Jaworski, 1997; Sasaki and Nagano, 2004).

To then produce LC-PUFA, a series of alternating desaturation and elongation steps are required, the first of these is carried out by microsomal 'front end' PUFA desaturases which belong to the N-terminal cytochrome b5-fusion superfamily (Sayanova *et al.*, 1997), as opposed to the Δ12 and Δ15-desaturases from plants, algae and some fungi which lack this cytochrome b5 domain. The majority of the microsomal desaturases from lower eukaryotes use glycerolipid-linked substrates, in particular FA esterified to the *sn*-2 position of glycerolipids. The second step in LC-PUFA biosynthesis is microsomal fatty acid elongation, which occurs as a result of four sequential enzymatic reactions: condensation with malonyl-CoA (KCS, β-ketoacyl-CoA synthase), ketoreduction (β-ketoacyl-CoA reductase), dehydration (hydroxyacyl-CoA dehydratase) and enoyl reduction (enoyl-CoA reductase) (Fehling *et al.*, 1992). The initial condensing enzyme is considered to exert strong flux control and is recognized as the regulator of substrate specificity (both chain length and pattern of double bonds). The KCS condensing enzymes themselves are divided into two groups: firstly, the so-called ELO-like sequences, some of which are involved in VLC-PUFA biosynthesis and have been cloned from a number of species including mammals, fungi (e.g. *Mortierella alpine*; Meyer *et al.*, 2004b) and aquatic algae (e.g. *Isochrysis galbana*; Qi *et al.*, 2002). Secondly, there are the FAE1-like enzymes with plant-specific KCS activities involved in the biosynthesis of saturated and monounsaturated fatty acids with C_{18} to C_{22}+ in chain length (James *et al.*, 1995). Both of them exclusively use acyl-CoAs as substrates (Jakobsson *et al.*, 2006).

To produce EPA and DHA, most PUFA-synthesizing eukaryotic organisms operate the so-called Δ6-pathway or the 'conven-

tional' aerobic pathway (schematically represented in Figure 1). The first step in this pathway is the Δ6-desaturation of both 18:2 omega-6 (LA) and 18:3 omega-3 (ALA) resulting in the synthesis of GLA and SDA, respectively (see Figure 1). This step is followed by a Δ6-specific C2 elongation, yielding di-homo γ-linolenic acid (DGLA; 20:3 Δ8,11,14) and eicosatetraenoic acid (ETA; 20:4 Δ8,11,14,17). A final Δ5-desaturation then produces ARA and EPA. Precursors entering the pathway can vary in the number and positions of double bonds. Depending on the primary substrate, sequential Δ6-desaturation, Δ6-elongation and Δ5-desaturation steps produce either omega-6 or omega-3 fatty acids. These two pathways can then be interconnected by ω-3 desaturases, which convert omega-6 fatty acids into their omega-3 counterparts.

An alternative pathway (or the so-called Δ8-pathway) for the biosynthesis of LC-PUFA has been demonstrated in the protist *Tetrahymena pyroformis*, *Acanthamoeba* spp., *Perkinsus marinus* and *Euglena* (Euglenophyceae) organisms; all of which appear to lack Δ6-desaturase activity (Lees and Korn, 1966; : Ulsamer *et al.*, 1969; Wallis and Browse, 1999). It has also been found in some species of microalgae such as the coccolithophore *Emiliania huxleyi*, *Pavlova* spp. and *Isochrysis* spp. (Qi *et al.*, 2002; Sayanova *et al.*, 2011; Zhou *et al.*, 2007). In this pathway, LA and ALA are first elongated by a specific Δ9-elongase to eicosadienoic (EDA; 20:2 Δ11,14) and eicosatrienoic (ETrA; 20:3 Δ11,14,17) fatty acids. These C_{20} products are then desaturated by a Δ8-desaturase to DGLA and ETA fatty acids. Finally, these PUFA enter the conventional pathway and are desaturated by a Δ5-desaturase yielding ARA and EPA.

From this point, the biosynthesis of DHA may follow two different routes: the linear or 'traditional' pathway and the so-called 'Sprecher' pathway. In DHA-accumulating microbes, the traditional pathway involves C2 elongation of EPA to docosapentaenoic acid (DPA; 22:5 Δ7,10,13,16,19) by a specific Δ5-elongase which is then desaturated by a Δ4-specific desaturase to yield DHA (Figure 1). These activities (Δ5-elongase, Δ4-desaturase) have been isolated and functionally characterized from several organisms and are closely related to the open reading frames required for the synthesis of ARA and EPA (Meyer *et al.*, 2004a; Pereira *et al.*, 2004; Sayanova and Napier, 2004; Wu *et al.*, 2005), whereas in the Sprecher pathway (found in mammals), EPA goes through two consecutive elongation steps producing DPA and subsequently tetracosapentaenoic acid (24:5 Δ9,12,15,18,21), which is then further desaturated by a Δ6-desaturase generating tetracosahexaenoic acid (24:6 Δ6,9,12,15,18,21). This C24 PUFA is then subject to partial peroxisomal β-oxidation to yield DHA. It is thought that the essential translocation from the ER to the peroxisome and then back to the ER may represent a method of regulation of DHA synthesis that is independent from the previous steps of the pathway (Burdge, 2006). From a metabolic engineering point of view, this complex pathway would make the transfer of this system to a heterologous organism difficult (Sprecher *et al.*, 1999).

As mentioned above, most marine organisms utilize the aerobic fatty acid desaturation/elongation pathway to produce DHA. However, some marine microorganisms are able to produce EPA, DPA and DHA in anaerobic conditions. These organisms employ an enzymatic complex similar to bacterial polyketide synthases (PKS) to synthesize LC-PUFA (Metz *et al.*, 2001). Metz *et al.* (2001) revealed that these PKS-like enzymes are capable of *de novo* LC-PUFA synthesis by an iterative extension of the fatty acyl chain, combined with trans-cis isomerization and enoyl reduction in selected cycles. This PKS-like pathway produces EPA or DHA as free fatty acids, which require activation to CoAs prior to incorporation of these products into lipids (Metz *et al.*, 2009). Interestingly these pathways are not exclusive, indeed in some marine eukaryotes the 'traditional' pathway frequently coexists with the PKS pathway.

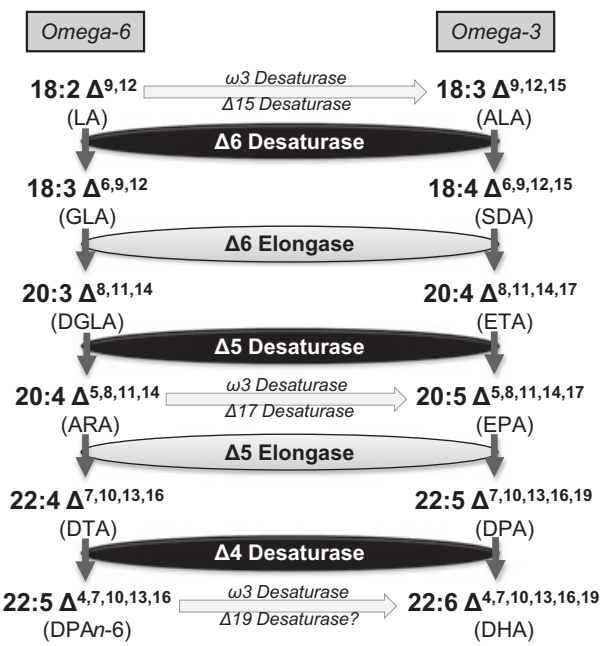

Figure 1 *The biosynthesis of LC-PUFAs*. Schematic representation of the conventional Δ6-desaturation pathway for the synthesis of EPA and subsequent metabolism to DHA via the microbial Δ4-pathway. The alternative pathway (not shown) utilizes a Δ9 elongase and Δ8 desaturase to produce DGLA and ETA, respectively.

Engineering LC-PUFA in higher plants

To date research efforts have demonstrated that positive effects on cardiovascular and other diseases are best established for DHA and EPA; however, these fatty acids are not present in any known higher plants. Yet a small number of plant species do have the faculty to produce Δ6-desaturated fatty acids (e.g. SDA and GLA). Such examples are, however, not appropriate to mass market supply for a number of reasons: for example, these species are not part of modern diets, are difficult to cultivate and have relatively low oil yields. However, the demand for GLA- and SDA-containing oils in the nutritional supplement market (Flider, 2005) has driven interest in transferring the biosynthetic pathway for GLA and SDA directly into commodity crops as a means to produce these high-value fatty acids in a cost-effective manner. The rationale behind this is that the human conversion ratio of dietary SDA to EPA is 3.3–1.0, which is greater than the 14 to 1 ratio of dietary ALA to EPA. Therefore in bypassing the flux control step of Δ6-desaturase activity, oils containing high amounts of SDA are more effective than oils containing ALA in increasing serum EPA levels in humans.

Transgenic production of γ-linolenic and stearidonic acid

Synthesis of GLA and SDA in heterologous systems has been demonstrated. The first published experiments reported an

expression of a Δ6-desaturase isolated from the cyanobacterium *Synechocystis* sp. under the control of a constitutive CaMV 35S promoter in *Nicotiana tabacum* (tobacco) plants, which resulted in the accumulation of low levels of GLA and SDA in transgenic leaves, but not in seeds (Reddy and Thomas, 1996). Much higher levels of GLA and SDA accumulation (a combined FA total of ~20%) were obtained by changing the Δ6-desaturase to one from *Borago officinalis* (Sayanova *et al.*, 1997, 2003). Subsequently, the expression of the *B. officinalis* Δ6-desaturase under the control of a constitutive promoter in *Linium ustatissimum* (linseed) also resulted in the accumulation of GLA (Qi *et al.*, 2002). In both cases, GLA was produced in vegetative tissue and only low levels were detected in seeds. Together these early experiments demonstrated the viability of using transgenic approaches to modify seed oil PUFA content.

In canola (*Brassica napus*), the introduction of the fungal (*M. alpina*) Δ6 and Δ12 desaturase genes led to the production of GLA and SDA at 43% and 5%, respectively (Liu *et al.*, 2001), whereas in brown mustard (*Brassica juncea*), expression of a Δ6 desaturase from the fungus *Pythium irregulare* resulted in GLA and SDA levels of up to 40% and 10%, respectively (Hong *et al.*, 2002). Whilst Qi *et al.* (2002) expressed a *B. officinalis* Δ6-desaturase in *B. juncea* producing 3%–9% of GLA from total FA, the expression of the *B. officinalis* Δ6-desaturase was also translated to soybean, where analysis revealed the accumulation of GLA and SDA in seeds as 27.7% and 3.4%, respectively (Sato *et al.*, 2004). One of the transgenic soybean events carrying the *B. officinalis* Δ6 desaturase was subsequently tested under field conditions in Nebraska, USA, and showed no major impact on yield, date to maturity, height, lodging, total oil or total protein content (Clemente and Cahoon, 2009). To modify the FA flux to SDA, Ursin (2003) described the results of iterative engineering, introducing the Δ6 and Δ12 *M. alpina* desaturase genes with a canola Δ15 desaturase transgene in *B. napus*. The coordinated expression of these three transgenes led to the accumulation of GLA and SDA, at 18% and 23%, respectively. Elevation of SDA levels in canola required the three gene combination because 60% of the fatty acid profile of canola oil is represented by oleic acid (Ursin, 2003). Hence, the Δ12 desaturase activity was required to shift the fatty acid pool to LA, thereby permitting the build-up of substrate for GLA production, whilst the expression of the Δ15 desaturase influences the level of ALA to allow for an increased SDA accumulation. In a similar way, Eckert *et al.* (2006) stacked the *B. officinalis* Δ6 desaturase with the Δ15 desaturase from Arabidopsis in soybean. The coordinated expression of the Δ6 and Δ15 genes in a seed-specific fashion produced a fatty acid profile with GLA, ALA and SDA of 5.8%, 33.5% and 21.6%, respectively, under greenhouse conditions (Eckert *et al.*, 2006).

Considerable progress has been made in the transgenic production of these fatty acids, and further studies have shown very high levels (up to 70%) of GLA and/or SDA as a result of seed-specific expression. The production of SDA has attracted attention from biotechnology companies, for example Monsanto and Solae LLC have developed a transgenic soybean (producing 15%–30% SDA and 5%–8% GLA), which is close to commercialization after a successful safety assessment (Hammond *et al.*, 2008), and the award of a Generally Recognized as Safe (GRAS) notice confirming that the omega-3 soybean oil from the bioengineered soybean can be used in foods and beverages (see Monsanto, 2009 for reference). In clinical trials, this SDA-enriched soybean oil has demonstrated efficacy in raising serum EPA levels in humans (Harris *et al.*, 2008; Lemke *et al.*, 2010).

More recently, the seed oil of a high LA cultivated species of safflower (*Carthamus tinctorius*) was modified by transformation with Δ6-desaturase from *Saprolegnia diclina* resulting in levels exceeding 70% (v/v) of GLA (Nykiforuk *et al.*, 2012), with this GM oil now being commercialized in the USA by Arcadia Biosciences. In an attempt to produce SDA alone, linseed lines were generated expressing a Δ6-desaturase from *Primula vialii* (which specifically only utilizes ALA as a substrate). Analysis of individual events demonstrated the accumulation of up to ~13% SDA and the complete absence of GLA in their fatty acid pool. These SDA levels are comparable to those found in the commercial plant source *Echium* spp., but have the advantage of being devoid of the PUFA ω-6 precursor GLA (Ruiz-Lopez *et al.*, 2009). Strikingly, this study also proved that the same construct, which was so successful in transgenic linseed, produced only low levels of SDA in transgenic Arabidopsis, indicating that this model system may not be entirely representative for all oilseed crops.

Metabolic engineering of arachidonic and eicosapentaenoic acid

The effectiveness of GLA and SDA as nutritional fatty acids is largely dependent on the limited ability of humans to convert them to LC-PUFA. Therefore, having established the rationale for the production of specific PUFA in seed oil the next logical step was the metabolic engineering of the entire LC-PUFA biosynthetic route in oilseeds. To be successful, this requires the coordinated expression of multiple genes, as a minimum of three sequential non-native enzymatic reactions (e.g. two desaturations and an acyl-CoA elongation) are involved in the conversion of native plant fatty acids, such as LA and ALA, to LC-PUFA (Figure 1).

Initially, a source of genes encoding the primary LC-PUFA biosynthetic activities was required. Latterly, this challenge was met via the identification of candidate genes from diverse sources (algae, fungi, mosses, plants and mammals). Following the cloning and characterization of these genes in model organisms, the reconstruction, either partially or entirely, of the LC-PUFA biosynthetic pathway in yeast (*Saccharomyces cerevisiae*) demonstrated the viability of such an approach (Beaudoin *et al.*, 2000; Das *et al.*, 2000; Domergue *et al.*, 2002, 2003; Meyer *et al.*, 2004b; Parker-Barnes *et al.*, 2000). However, despite the success of these early engineering attempts in yeast, the translation of the LC-PUFA biosynthetic pathway to seeds was challenging and it was quickly established that the availability of genes was just the first step.

This is best illustrated by the work of Abbadi *et al.* (2004) who engineered a series of plant transformation constructs containing different combinations of Δ6-desaturases from *Physcomitrella patens*, *B. officinalis* and *Phaeodactylum tricornutum*, Δ6-elongases from *Physcomitrella patens* and *Caenorhabditis elegans* and, Δ5-desaturases from *M. alpina* and *Phaeodactylum tricornutum,* where all the genes were under the control of seed-specific promoters. The experiments resulted in a low accumulation of ARA (20:4 Δ5,8,11,14) and EPA in tobacco and linseed transgenic seeds, respectively. Moreover, analysis of the acyl-CoA pools in these seeds showed only trace amounts of SDA, whilst isolated microsomes from these seeds were able to elongate acyl-CoA substrates provided *in vitro*. The application of such detailed biochemical analysis established that the first desaturation in the LC-PUFA biosynthetic pathway was working efficiently, but not the elongation step. This bottleneck was described as a 'substrate dichotomy' (Napier, 2007), and it is the consequence of desaturase

and elongase activities requiring different substrates, that is, phospholipid-linked substrates for desaturases and acyl-CoA for elongases. These different substrate requirements resulted in a rate limiting flux through the alternating desaturation and elongation steps.

In a similar approach, Kinney *et al.* (2004) combined the expression of a Δ6-desaturase, a Δ5-desaturase and a Δ6-elongase from the fungus *M. alpina* in transgenic soybean seeds and embryos. To maximize accumulation of omega-3 LC-PUFA, they in turn co-expressed both a ω-3 Δ17-desaturase from *Saprolegnia diclina* and an ω-3 Δ15-desaturase from Arabidopsis, converting the ω-6 PUFA metabolites into their ω-3 counterparts. In these experiments, they achieved EPA levels of 9.3% in somatic soybean embryos. Replacing the *M. alpina* Δ6-desaturase with a *S. diclina* Δ6-desaturase appeared to increase EPA levels in embryos slightly (EPA levels up to 13.3%) and plants derived from some of these embryos produced seeds with total FA EPA levels reaching up to almost 20%. It is still unclear why this event yielded higher production of EPA in soybean compared with linseed, and it should be noted that other attempts to produce EPA in transgenic soybeans have not been as successful (Chen *et al.*, 2006).

Other studies have also demonstrated the effective production of ARA and EPA in oilseeds. For example, Wu *et al.* (2005) transformed *B. juncea* with a construct that contained only the minimal set of genes required to produce ARA and EPA: a Δ6-desaturase from the fungus *Pythium irregulare*, a Δ6-elongase from a moss (*Physcomitrella patens*) and a Δ5-desaturase from *Thraustochytrium*. The transgenic seeds contained high levels of GLA (27%), but relatively low levels of C_{20} PUFA, highlighting the poor performance of the reactions required for Δ6-elongation. However, these seeds also contained 7% ARA and 0.8% EPA because of the high conversion efficiency of the Δ5-desaturase. *B. juncea* oil is rich in LA, a ω-6 substrate in the PUFA synthesis pathway, and this could be the reason why seeds of transgenic plants accumulated much higher amounts of the ω-6 fatty acid ARA than the ω-3 EPA. In the second construct, a *Calendula officinalis* Δ12-desaturase was added to ensure an increase in LA. This experiment resulted in notably higher levels of ARA (up to 17.7% of total FA) as well as EPA (averaged 1.3%). The next step was the addition of a second Δ6-elongase from *Thraustochytrium* spp. to this construct, and it only produced a low increase in elongation efficiency (small increases in both EPA and ARA). These results suggested that overall elongation is not limited strictly by the level of substrate present in an acyl-CoA form. The final addition of a *Phytophtora infestans* ω-3/Δ17-desaturase produced oil that contained higher levels of EPA (8.1%) with a concomitant reduction in ARA.

A further iteration of the production LC-PUFA in oil seeds was the reconstitution of the alternative Δ8-desaturase pathway (Qi *et al.*, 2004). Arabidopsis plants were sequentially transformed with a Δ9-elongase from the algae *I. galbana*, a Δ8-desaturase from the protist *Euglena gracilis* and a fungal Δ5-desaturase from *M. alpina*; all of which were under the control of the constitutive 35S promoter. The subsequent analysis of leaf tissue showed an accumulation of 6.6% ARA and 3.0% EPA in total lipids, representing the first 'proof-of-concept' demonstration of the alternative pathway in plants. However, the choice of the constitutive 35S promoter limited the expression of the transgenes to vegetative tissues and detailed analyses of leaf lipids indicated an inefficient transfer of these non-native fatty acids from the acyl-CoA pool into extra-plastidial phospholipids. (Fraser *et al.*, 2004; Sayanova *et al.*, 2006). Further progress has been reported since these early experiments; for example, the identification and functional characterization of the five *E. huxleyi* genes direct the synthesis of DHA. Surprisingly, *E. huxleyi* is using the alternative Δ8-desaturation route which has previously only been observed in a few unrelated microorganisms. Given that *E. huxleyi* accumulates significant levels of the Δ6-desaturated fatty acid SDA, it was speculated that the biosynthesis of DHA is likely to be metabolically compartmentalized from the synthesis of stearidonic acid in this algae (Sayanova *et al.*, 2011). Moreover, the seed-specific expression of the alternative pathway has now been demonstrated in both *B. napus* and *Arabidopsis* seed resulting in the production of ARA and low levels of intermediate C20 fatty acids (Petrie *et al.*, 2012a), emphatically illustrating the efficacy of this route for LC-PUFA synthesis.

Production of a marine-like oil in seeds

The production of DHA in plants could only be attempted after appreciable levels of EPA in seeds were demonstrated. Kinney *et al.* (2004) was the first author reporting DHA synthesis in oilseeds (subsequently Kinney *et al.*, 2011). They observed that the transgenic soybean embryos producing EPA were unexpectedly also accumulating up to 4% DPA, as a result of the *M. alpina* elongase. Subsequently, six different cDNAs were co-expressed in somatic soybean embryos to produce DHA: three genes required for the synthesis of EPA, a Δ4-desaturase from *Schizochytrium aggregatum* and a specific Δ5-elongase from *Pavlova* sp. Disappointedly, low DHA accumulation was achieved (2.0–3.3% of DHA in total FA), likely a result of the poor acyl-exchange between the Δ5-elongase and the Δ4-desaturase.

Further attempts by Wu *et al.*, 2005 added three extra genes to the transformation vector: a Δ6/Δ5-elongase (from the fish species *Oncorhynchus mykiss*), plus both a Δ4-desaturase and a putative lysophosphatidic acid acyltransferase from *Thraustochytrium* sp and transformed *B. juncea* seeds. The transgenic seeds showed an accumulation of 8.1% (highest value was 15% EPA); however, there was a reduced conversion of EPA into DPA, which was rate limiting for the Δ4-desaturase producing DHA (the highest recorded value was 1.5% DHA of total FA). Moreover, these experiments could not prove if the acyltransferase activity contributed to the enlarged synthesis of VLC-PUFA. To obtain a higher and more uniform expression of all genes, the same strong seed-specific promoter was then used for each gene. Counter intuitively, the levels of FA then went down in following generations indicating that the presence of many copies of the same promoter sequence could be causing some silencing of the genes (Chen *et al.*, 2006).

Bottlenecks and solutions

As the possibility of producing VLC-PUFA in plants has developed, the influence of the host crop has become more apparent—the concept of 'cutting and pasting' algal genes into an oilseed needs to better consider the metabolic context of the host organism. With hindsight, this seems obvious, given that a developing seed is likely to be expressing (at least) several hundred genes involved lipid metabolism, yet the transgenes represent a tiny (<5%) proportion of these. Thus, there is a need for greater understanding as to how best integrate endogenous and transgene-derived metabolism. To that end, initial studies by Cheng *et al.* (2010) investigating the effects of different host species, genes and promoters on EPA biosynthesis using the conventional

pathway have proved informative. Zero-erucic acid *Brassica carinata* appeared to be a better host species for EPA production than *B. juncea,* yielding an average EPA level of 20.4% in transgenic seeds (25.0% in an individual seed). However, in both species, the inefficiency of the Δ6-elongation step was evidenced by the high levels of GLA and SDA remaining in transgenic seeds. The levels of these intermediates in EPA accumulating seeds were still very high, with the averaged levels of GLA and SDA 26.9% and 5.4% of total FA, respectively.

It is likely that the efficiency with which endogenous acyltransferases accept non-native substrates is playing an important role in avoiding this bottleneck, and it is also likely that different activities [e.g. lyso-phospholipid acyltransferases, LPAT; phospholipid:diacylglycerol (DAG) acyltransferase, PDAT, etc.] have different affinities for these novel fatty acids (Dyer *et al.*, 2008). Furthermore, many of these acyltransferases work in both forward and reverse directions, and the pool sizes of the different metabolites are likely responsible in defining the leading enzyme activity. Moreover, the severity of this bottleneck appears to vary from one host plant to another. It was revealed that higher levels of EPA and ARA were achieved in transgenic soybean and Indian mustard than in linseed or tobacco, indicating that the endogenous acyltransferase system in soybean and *Brassica* spp. have a broader substrate specificity than in linseed or tobacco.

Several attempts to alleviate this bottleneck and to manipulate acyl-exchange have been carried out as our knowledge of fundamental lipid metabolism has increased. The enzyme LPCAT (lysophosphatidylcholine acyltransferase), which catalyses a bidirectional exchange between the PC and the acyl-CoA pool, has been functionally characterized from yeast and animals (reviewed in Ståhl *et al.*, 2008). However, in all cases, only the forward reaction has thus far been demonstrated (i.e. acyl-CoA-dependent acylation of lyso-PC for example Chen *et al.*, 2006; Nakanishi *et al.*, 2006). Latterly, LPCAT have been identified in *B. napus* (Zheng *et al.*, 2012). Specifically three *B. napus* LPCAT homologs were identified, of which BnLPCAT1-1 and BnLPCAT1-2 are orthologous to Arabidopsis AtLPCAT1 (At1g12640), whilst BnLPCAT2 is an ortholog of AtLPCAT2 (At1g63050). The proteins encoded by BnLPCAT1-1 and BnLPCAT2 were chosen for further study. Enzymatic assays demonstrated that both proteins exhibited a substrate preference for LPCs and unsaturated fatty acyl-CoAs. In addition to the enzymatic properties of plant lysophosphatidylcholine acyltransferases uncovered in this study, this report describes a useful technique that facilitates subsequent analyses into the role of LPCATs in PC turnover and seed oil biosynthesis. However, evidence for the role of the reverse reaction (release of a fatty acid from the *sn*-2 position of PC and activation to acyl-CoA) and its effectiveness in alleviating the bottleneck for improving the transgenic production of VLC-PUFA in plants is required.

Another example of the manipulation of the acyl-CoA pool was the characterization of a 10 kDa acyl-CoA-binding protein from *B. napus,* which demonstrated the role these proteins might have in the acyl-exchange between PC and the acyl-CoA pool (Yurchenko *et al.*, 2009). Thus, it is likely that this acyl-CoA-binding protein facilitates (or even modifies) the activity of plant acyltransferases such as LPCAT. All these efforts were not only practical demonstrations of applied biotechnology, but also provided insights into how biochemical pathways respond to perturbation.

The most obvious way to bypass the acyl-exchange bottleneck is to target both desaturase and elongase activities to one pool. This was made possible by the identification of an acyl-CoA-dependent Δ6-desaturase from the microalgae *Ostreococcus tauri* (Domergue *et al.*, 2005), which when co-expressed in yeast with a Δ6-elongase resulted in high levels of C_{20} PUFA). Whilst Robert *et al.* (2005) used a bi-functional Δ5/Δ6-desaturase from the fish *Danio rerio* that was also thought to act on acyl-CoA substrates. Arabidopsis plants then transformed with this desaturase combined with PEA-1 (a Δ6-elongase from *C. elegans*; Beaudoin *et al.*, 2000) accumulated levels of ARA and EPA up to 1.6% and 3.2%, respectively. Further co-transformation with a Δ4-desaturase and a Δ5-elongase from the alga *Pavlova salina* produced 0.5% DHA in seeds of T1 plants. The authors suggested that the use of an acyl-CoA-dependant desaturase may have reduced the need for exchange of intermediates between the acyl-CoA and phospholipid pools, leading to a more efficient synthesis of C_{20} PUFA. Yet although 67% of SDA was elongated, only 17% of EPA was converted to DPA, suggesting that the availability of an acyl-CoA-linked substrate alone did not overcome problems with elongation efficiencies.

Hoffmann *et al.* (2008) also tried to avoid the acyl-exchange bottleneck by only using acyl-CoA-dependent desaturases: a Δ6-and a Δ5-desaturase from *Mantoniella squamata*. Seed-specific promoters were used to co-express these enzymes with the Δ6-elongase PSE1 of the moss *P. patens* (Zank *et al.*, 2002) in Arabidopsis plants. Transgenic seeds accumulated low levels (<0.5%) of EPA, but lacked the accumulation of Δ6-desaturation products previously observed. Most recently, Petrie *et al.* (2010) have produced up to 26% EPA in *N. benthamiana* leaf triacylglycerol (TAGs) (10.7% in total FA) using a putative acyl-CoA-dependent Δ6 desaturase with strong omega-3 preference from the marine micoralga *Micromonas pusilla*. These experiments make use of a very useful and exciting tool reported by Wood *et al.* (2009), where a transient expression system is used to reconstitute the synthesis of VLC-PUFA such as EPA in leaf tissue. This new approach could certainly enhance our capacity to identify some optimal combinations of fatty acid biosynthetic activities for the production of VLC-PUFA in plants, provided that any differences between leaf cells and developing cotyledon cells in seeds are accounted for.

Presently, all the published examples using an acyl-CoA-dependent route have resulted in a significant reduction in the accumulation of biosynthetic intermediates (most notably omega-6 GLA, linked to PC), but the seed levels of target VLC-PUFA such as EPA and DHA have been disappointingly low (Hoffmann *et al.*, 2008; Robert *et al.*, 2005; Sayanova *et al.*, 2012). Substrate availability, the use of non-optimized sequences or the presence of some unidentified metabolic bottlenecks could be some of the causes; in addition, many approaches lack an unambiguous acyl-CoA-dependent Δ5-desaturase from lower eukaryotes. Zhou *et al.* (2008) identified two acyl-CoA-dependent Δ12-desaturases from insects (house cricket (*Acheta domesticus*) and red flour beetle (*Tribolium castaneum*); it will be interesting to see whether the co-expression of this activity would enhance the activity of the algal acyl-CoA Δ6-desaturases in transgenic plants.

Lately, the reconstitution of ARA and EPA biosynthesis in transgenic Arabidopsis expressing an acyl-CoA-dependent Δ6-desaturase from *O. tauri* has been demonstrated (Ruiz-Lopez *et al.*, 2012). These studies re-affirmed previous studies, all of which showed the benefits of using the *O. tauri* acyl-CoA-dependent Δ 6-desaturase to avoid the accumulation of unwanted C18 intermediates (such as GLA and SDA) without significantly reducing the accumulation of EPA. However, it was also shown the

unexpected channelling of heterologous C20 polyunsaturated fatty acids into minor phospholipid species, and also the apparent negative metabolic regulation of phospholipid-dependent Δ6-desaturases. These data indicated that the accumulation in TAG of non-native omega-3 LC-PUFAs is primarily via the phosphatidylcholine (PC) to DAG, route (Bates *et al.*, 2009), analogous to that recently shown for hydroxylated fatty acids (Bates and Browse, 2011). This has consequences for the efficient accumulation of EPA synthesized by the acyl-CoA pathway (Figure 2).

Finally, it has been established that phosphatidylcholine:DAG cholinephosphotransferase (PDCT) plays an important role in directing fatty acyl fluxes during TAG biosynthesis (Lu *et al.*, 2009; Zhaohui *et al.*, 2012). PDCT mediates the inter-conversion between PC and DAG, thus enriching PC-modified fatty acids in the DAG pool prior to forming TAG (Figure 2). PDCT is known to be required for the efficient metabolism of engineered hydroxy fatty acids in Arabidopsis seeds. These results add a new gene in the genetic toolbox for efficiently engineering unusual fatty acids in transgenic oilseeds, but it still needs to be proved if it will efficiently re-direct the accumulation of LC-PUFA in to TAG when expressing acyl-CoA desaturases.

New technologies—solutions to old problems?

The emerging science of synthetic biology provides a new paradigm for plant lipid engineering, allowing one to not just modify existing seed oil metabolism, but rather seeks to build a bespoke system by re-designing metabolic pathways from scratch to create entirely new biosynthetic pathways *de novo* within cells, thereby enabling production of valuable molecules. This approach may lead to crops with novel traits, including seed oils with greater nutritional potential. As an example, Petrie *et al.* (2012b) have recently taken the first steps in building a pathway for TAG assembly in plants that uses MAG as an intermediate. The 'MAG pathway' for TAG synthesis is a well-established facet of mammalian lipid metabolism, where the digestion of dietary fat by pancreatic lipase generates *2*-MAG and FFAs, which are absorbed in the intestine and reassembled into TAG by MAG and DAG acyltransferases, several of which have duel activity (Cheng *et al.*, 2008; Yen *et al.*, 2002). Indeed, Tumaney *et al.* (2001) proposed that this pathway might also exist in plants, given their discovery of a MGAT activity in developing peanut cotyledons. For such a pathway to function in plants, a *de novo* source of MAG is required. Interestingly, a family of *sn-2*-specific glycerol-3-phosphate acyltransferases (*2*-GPATs) have recently been characterized in plants that are involved in the synthesis of aliphatic monomers, which are key components of polyesters such as cutin and suberin (Yang *et al.*, 2010b, 2012). Several of these proteins harbour an additional phosphatase activity and can therefore generate *2*-MAG (Yang *et al.*, 2010b, 2012). It follows that TAG could be assembled in plants using a *2*-GPAT/phosphatase and a bifunctional MGAT/DGAT, as an alternative to the 'conventional' Kennedy pathway (Petrie *et al.*, 2012b). Although this pathway was proposed to provide a means of increasing TAG production in plant tissues (Petrie *et al.*, 2012b), it also has the potential to bypass several of the bottlenecks described in the preceding section that concerns the efficient incorporation of 'unusual' fatty acids into TAG (cf. Figure 2). Indeed, there is significant variation in substrate specificity within the *2*- GPAT (Yang *et al.*, 2010b, 2012) and MAG and DAG acyltransferase families (Carlsson *et al.*, 2011; Cheng *et al.*, 2008). Use of the appropriate enzymes may allow the synthesis of TAG with a specific fatty acid composition and perhaps even stereoisomeric structure. These properties can be particularly important for a number of high-value 'speciality lipids' that have applications in the food industry, including those containing VLC-PUFA (Xu and Akoh, 2002).

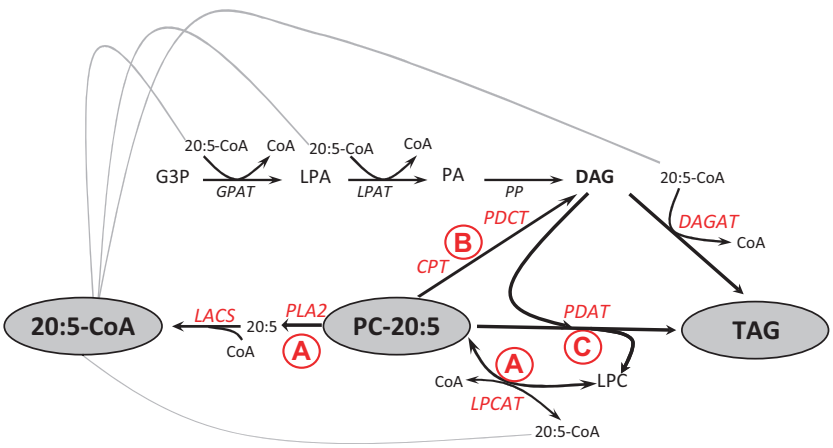

Figure 2 Schematic diagram of the main lipid classes and biochemical pathways involved in the production of TAG in developing seeds. There are three mechanisms for the removal of LC-PUFA from PC to make it then available for incorporation into TAG (mechanisms A, B and C). For mechanism A, FAs esterified to PC are under a constant dynamic exchange with the acyl-CoA pool in a process described as acyl editing. Removal of FAs from PC can proceed by the reverse action of acyl-CoA:lysophosphatidylcholine acyltransferase (LPCAT) or the combined action of phospholipase A2 PLA_2 and long-chain acyl-CoA synthetase, LACS. Once in the acyl-CoA pool, acyl-CoAs and glycerol-3-phosphate (G3P) can be converted into TAG by the consecutive action of acyl-CoA:glycerol 3-phosphate acyltransferase (GPAT), acyl-CoA:lysophosphatidic acid acyltransferase (LPAT), phosphatidic acid phosphatase (PAP) and acyl-CoA:diacylglycerol acyltransferase (DGAT). For mechanism B, the PC head group can be removed, producing a DAG molecule containing the same FAs. This reaction can proceed by four enzymatic mechanisms: phospholipase C, phospholipase D along with PAP, the reverse action of CDP- choline: diacylglycerol cholinephosphotransferase (CPT), or the recently identified phosphatidylcholine:diacyglycerol cholinephosphotransferase (PDCT). The DAG produced by these mechanisms can then be utilized to produce TAG. For mechanism C, direct transfer of the sn-2 FA of PC to the sn-3 of DAG produces TAG via a phospholipid:diacylglycerol acyltransferase (PDAT). DAG, diacylglycerol; TAG, triacylglycerol.

Regardless of attempts to build new pathways, a detailed understanding of underlying biochemistry and gene regulation networks is essential for pathway construction. Therefore, research effort has focused on building on the strong traditions of plant lipid biochemistry to deliver advances in technology, both instrumentation and techniques that, when applied, have greatly improved our knowledge of the metabolism of oilseed lipids. For example, advances in genomic applications have shown that acyl-lipid metabolism in Arabidopsis requires at least 120 enzymatic reactions and more than 600 genes to encode the proteins and regulatory factors involved (Li-Beisson *et al.*, 2010). Outside *Arabidopsis*, a number of oil crops have been sequenced, for example flax (Wang *et al.*, 2012), soybean (Schmutz *et al.*, 2010) and castor (Chan *et al.*, 2010), providing a reference genomes for mapping reads from next-generation sequencing. Analysis of high-quality RNA-Seq data from different tissues of castor has enabled a better understanding of the compartmentalization of tri-ricinolein TAG synthesis (Brown *et al.*, 2012) and has supplemented information on expression of lipid metabolic genes in soybean (Severin *et al.*, 2010), *Jatropha curcus* (Costa *et al.*, 2010), and bitter melon (Yang *et al.*, 2010a). Another approach has been analysis of expressed sequenced tag (EST) data from seeds sequestering oil, which allowed the identification of genes actively involved in TAG production (Cahoon and Kinney, 2005). Specifically, the application of the latest pyrosequencing technology generates huge numbers of ESTs offering opportunities for gene discovery in fatty acid (TAG) biosynthesis. For example, the analysis of ESTs from the developing seeds of four species differing in their TAG structure, content and storage tissue revealed both conserved and distinct species-specific expression patterns for genes involved in the synthesis of glycerolipids and their precursors (Troncoso-Ponce *et al.*, 2011). Notably, the EST levels of several genes, for example GPAT, LPAT and PDAT, potentially involved in the accumulation of unusual TAG structures that might be associated with VLC-PUFA accumulation, were distinct. This might suggest that the acyltransferases required for TAG accumulation have a separate expression pattern not coordinated with fatty acid synthesis or DGAT. All of these studies have clearly demonstrated how seed lipid metabolism is the result of the coordinated cooperation of multiple genes and any attempt at metabolic engineering must adapt to the existing biochemical landscape.

The exponential growth in the number of genes now known to be associated with lipid metabolism has driven researchers towards the systematic analysis of the lipidome to connect these genes to a specific function. Lipidomics can be described as the detailed analysis and global characterization, both spatial and temporal, of the lipids within a living system. The data obtained from such analyses can therefore be utilized to define lipid pathways, roles of specific genes and identify those lipid species that serve as substrates and products, underpinning a systems biology approach to oil seed engineering. The development of comprehensive analytical lipidomic strategies can be categorized into two approaches. The first is based on the separation of different lipid categories using extraction and chromatographic separation prior to class-specific mass analysis, whereas the second directly infuses a total lipid extract into the mass spectrometer and employs different monitoring methods to provide lipid class-specific analysis. An example of the former is the application of multi reaction monitoring (MRM) using hybrid triple mass spectrometers for the characterization of acyl-CoA species. Utilizing a specific CoA extraction method (Larson and Graham, 2001) combined with chromatography (Haynes *et al.*, 2008) and mass spectrometry, it is now possible to identify CoA species with acyl chain lengths of four to thirty-two carbons and specifically species that previously co-eluted using conventional chromatographic approaches. Understanding the composition of the acyl-CoA pool is crucial to any engineering attempt as they are key substrates for lipid biosynthesis and acyl-exchange, as evidenced by the identification of the 'substrate bottleneck'. Examples of the second approach, the analysis of lipids directly from extracts by direct-infusion MS, include methods for phosopholipids, galactolipids (Devaiah *et al.*, 2006; Xiao *et al.*, 2010) and TAG (Clauß *et al.*, 2011; Krank *et al.*, 2007). This methodology has been particularly effective and now plays a central role in experimental analysis of oilseeds engineered for altered lipid metabolism. Whereas the typical analysis of fatty acids from seed oil is limited to approximately eight or 10 individual fatty acids, the number of individual species of phospholipids, TAG and galactolipids rises into the hundreds reflecting the various editing activities of numerous acyltransferases and ensuing metabolic exchange. This type of detailed analysis allows insight into how plants are incorporating the expression of transgenes for VLC-PUFA biosynthesis into routine TAG synthesis. It is now possible to describe the substrate and product lipid pools to an unprecedented level of detail using MS/MS targeted lipid analysis, allowing precise quantification of each iterative engineering attempt (Sayanova *et al.*, 2011). Such global analysis can reveal the novel consequences of gene expression on lipid composition; for example, the straightforward suppression of phospholipase D in soybean seeds showed increased levels of polyunsaturated di18:2 PC and PE (Lee *et al.*, 2011). Latterly, direct-infusion mass spectrometry has been performed on a miniature scale suitable for the analysis of individual subcellular compartments (discussed in Horn and Chapman, 2012). The application of this approach has identified the heterogeneity of lipid droplets in oilseeds, which could explain compartmentalization limitations in metabolic engineering approaches. This type of characterization allows rational decision-making based on how LC-PUFA is channelled into TAG and where the likely bottlenecks and diversions are limiting yield. It is imperative to any successful engineering approach that losses of LC-PUFA substrates and intermediates are characterized and mitigated. The adoption of high-throughput approaches to lipidomic analysis also facilities the establishment of an iterative approach to trait engineering, in which each successive round of metabolic engineering is informed by the detailed biochemical insights provided by interrogation of the seed lipidome.

Conclusions and summary

The targeted manipulation of plant seed oil composition represents some of the most complex metabolic engineering in transgenic plants to date—up to 10 transgenes have been coordinately expressed in developing seeds, resulting in the accumulation of high-value fatty acids. However, it is equally clear that we are still some way off from being able to alter seed oil composition in an entirely predictable manner. In that respect, whilst a number of studies have 'failed' to achieve headline targets of accumulation of non-native fatty acids, such efforts have equally indicated that plant lipid metabolism is significantly more complicated than previously imagined. It is also now apparent that some aspects of seed lipid synthesis and deposition are under strong homoeostatic regulation and, as a whole, these

metabolic pathways form an integrated network. The challenge for the future will be to take the new tools and approaches described above and apply these further to the significant advances we have already witnessed in the last decade. Given that the benchmark for transgenic accumulation of EPA currently stands at 20% (in *B. carinata*; Cheng *et al.*, 2010) yet only 6 years earlier the highest level was 0.8% (in linseed; Abbadi *et al.*, 2004), it is obvious the immense progress the field has made. It does therefore not seem unreasonable to predict further significant advances (such as the high level accumulation of both EPA and DHA in seed oil) in the coming years, leading to the ultimate goal of a viable terrestrial alternative to fish oils.

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