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Expanding the docosahexaenoic acid food web for sustainable production: engineering lower plant pathways into higher plants

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Abstract

Background Algae are becoming an increasingly important component of land plant metabolic engineering projects. Land plants and algae have similar enough genetics to allow relatively straightforward gene transfer and they also share enough metabolic similarities that algal enzymes often function in a plant cell environment. Understanding metabolic systems in algae can provide insights into homologous systems in land plants. As examples, algal models are currently being used by several groups to better understand starch and lipid metabolism and catabolism, fields which have relevance in land plants. Importantly, land plants and algae also have enough metabolic divergence that algal genes can often provide new metabolic traits to plants. Furthermore, many algal genomes have now been sequenced, with many more in progress, and this easy access to genome-wide information has revealed that algal genomes are often relatively simple when compared with plants.

Scope One example of the importance of algal, and in particular microalgal, resources to land plant research is the metabolic engineering of long-chain polyunsaturated fatty acids into oilseed crops which typically uses microalgal genes to extend existing natural plant biosynthetic pathways. This review describes both recent progress and remaining challenges in this field.

Long-chain vs. short-chain polyunsaturated fatty acids

Long-chain polyunsaturated fatty acids (LC-PUFA) have a carbon backbone of at least 20 carbons in length and contain multiple double-bond desaturations. Long-chain polyunsaturated fatty acids can be grouped into either an omega-3 (ω3) or ω6 category based on the position of the first double bond from the methyl, or ω, fatty acid terminus. Fatty acid nomenclature can be used to succinctly refer to fatty acids and, as an example, $18{:}4^{\Delta 6,9,12,15}$ refers to the short-chain stearidonic acid which contains only 18 carbons and four double bonds, or desaturations, at the 6, 9, 12 and 15 carbon positions from the carboxyl, or Δ, terminus (Fig. 1). Long-chain polyunsaturated fatty acids have critical roles in human health and development, with studies indicating that deficiencies in these fatty acids can increase the risk or severity of cardiovascular disease (von Schacky 2006), inflammatory diseases and rheumatoid arthritis (Kremer *et al.* 1995; Simopoulos 2002; Nagel *et al.* 2003), hypertension (Ueshima *et al.* 2007) and

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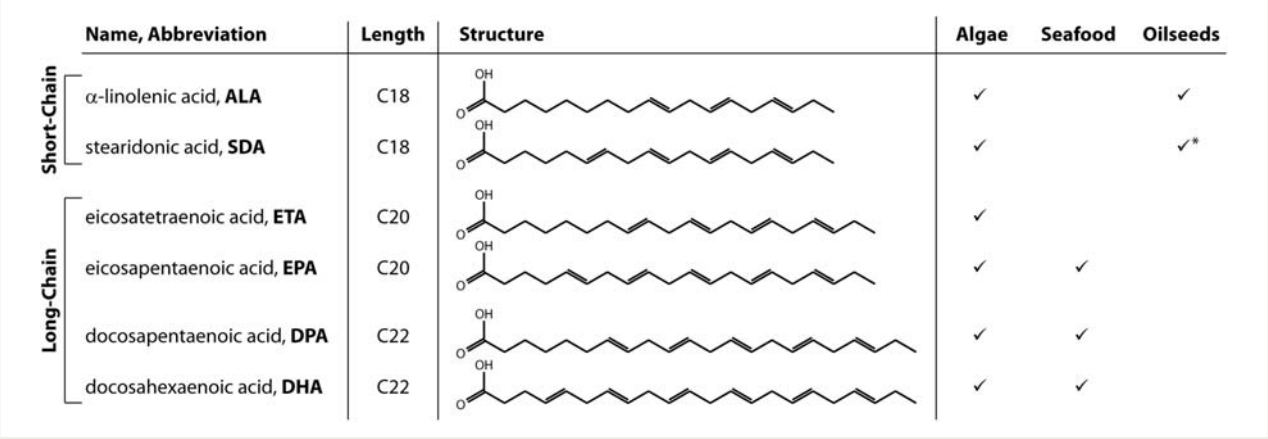

Fig. 1 ω3 long-chain (≥C20) polyunsaturated fatty acids and their predominant natural sources. *Some Boraginaceae such as *Echium plantagineum* contain SDA, although this species is not generally recognized as an oilseed crop species.

neuropsychiatric disorders such as depression or dementia (Freeman *et al.* 2006; Parker *et al.* 2006; Schaefer *et al.* 2006). Recently, the human fatty acid receptor GPR120 was characterized as being an ω3-specific receptor with strong anti-inflammatory and insulin-sensitizing effects (Oh *et al.* 2010). Humans have limited ability to synthesize LC-PUFA, with infants in particular requiring the majority of these fatty acids through the diet. This is particularly the case with the ω3 LC-PUFA eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) and the ω6 LC-PUFA arachidonic acid (ARA), with studies indicating positive effects on infant cognitive development after ARA and DHA supplementation (Birch *et al.* 2000). As a result many infant formulae now contain LC-PUFA, although it is worth noting that ARA supplementation for adults is, in many cases, often considered to be of lesser value since the ω6 LC-PUFA tend to be pro-inflammatory in nature and counteract the strong anti-inflammatory effects of ω3 LC-PUFA.

Just as there are differences in physiological effect between ω3 and ω6 LC-PUFA (i.e. anti- vs. pro-inflammatory, respectively), it is important to note that there are significant differences arising from intake of long-chain vs. short-chain (SC) ω3 PUFA. Alpha-linolenic acid (ALA) is a common ω3 SC-PUFA found in seed oils of flax/linseed, canola/rapeseed, soybean and walnut. Stearidonic acid (SDA), another ω3 SC-PUFA, is less common in plant oils, with the predominant natural plant dietary sources being echium and borage. Human studies along with livestock and aquafeeding trials have demonstrated that SDA in particular can be converted, in part, to the LC-PUFA EPA and, to a lesser extent, DPA (James *et al.* 2003; Tocher *et al.* 2006; Harris *et al.* 2008). Thus, although the direct health benefits of ω3 SC-PUFA are relatively limited when compared with LC-PUFA, they are viewed by some as a surrogate for dietary EPA supplementation (Whelan 2009) due to this *in vivo* conversion and transgenic SDA crops are under development.

It is clear, however, that direct intake of ω3 LC-PUFA results in the greatest health benefits (James *et al.* 2003; Burdge and Calder 2005; Wang *et al.* 2006). Furthermore, the conversion of ω3 SC-PUFA including SDA to DHA has not been observed in either humans or aquaculture species to any significant degree (James *et al.* 2003; Tocher *et al.* 2006; Harris *et al.* 2008). Docosahexaenoic acid fills specific physiological (e.g. Mori *et al.* 1999) and structural roles (such as a neural and retinal component) that cannot be replaced by EPA or other LC-PUFA, and ensuring adequate dietary intake of DHA is therefore required. Consumer awareness of the health benefits of ω3 LC-PUFA is growing (due in part to the efforts of organizations such as the Omega-3 Centre (www.omega-3centre.com) and GOED (www.goedomega3.com)), and there is an increasing demand for these fatty acids which will be difficult for current sources to meet in a sustainable manner.

Current and future sources of ω3 LC-PUFA

The main source of ω3 LC-PUFA is wild-harvest marine fish stocks and these, unfortunately, are widely recognized to be in decline. One widely cited paper describes the amount of large predatory fish in the oceans as being at only 10 % of pre-industrial times (Myers and Worm 2003), although this figure has been disputed. One of the authors claimed in a later publication that

the state of the oceans and the effect of this on fish stocks was such that 'all commercial fish and seafood may collapse' by 2048 (Worm *et al.* 2006). This view is hardly unanimous either (see Hölker *et al.* (2007), Wilberg and Miller (2007) and Jaenike (2007) for comment), but efforts are now under way to rebuild fisheries and to improve their sustainability. Regardless, fisheries alone are unlikely to meet the growing demand for ω3 LC-PUFA and alternative sources of these fatty acids must be found. Aquaculture cannot meet the demand since fish themselves do not produce LC-PUFA but, like humans, accumulate LC-PUFA through their diet. In fact, the aquaculture industry itself contributes enormously to demand for LC-PUFA, with ∼90 % of global fish oil production being used in aquafeeds. Logically, the major producers of ω3 LC-PUFA, microalgae, would be a good target for increased production and, indeed, commercial microalgal sources of ω3 LC-PUFA are available. Microbial production tends to supply relatively niche applications such as infant formulae and nutraceuticals due to high production costs and is unlikely to scale up adequately for large-volume applications.

Oilseed crops, with their production capacity and relatively low cost, would be an excellent and sustainable source of ω3 LC-PUFA. For example, several calculations have recently been performed to examine global consumer needs for these oils and using a daily 500 mg requirement, as is recommended by national health bodies, a global population reaching 8 billion by 2025, and assuming fish contain 0.2–3.5 g of ω3 LC-PUFA per 100 g of oil, it was estimated that the current global fish harvest (93 Mt per annum) will fall well short of this requirement. On the other hand, ∼2.5 million hectares of an oilseed crop (about 2 % of total world acreage under cultivation to major oilseed crops) containing 10–15 % DHA and EPA in its oil could replace all the fish oil currently being used globally. Oilseed crop plants do not naturally synthesize these fatty acids, meaning that a metabolic engineering solution would be required before this potential source could become a reality. An oilseed crop with ω3 LC-PUFA production capability would provide an excellent alternative to high-volume, environmentally sensitive marine-based oils. Such an oil could be used in many applications: (i) as an ingredient for aquaculture feeds to provide aquaculture species with ω3 LC-PUFA both for their own developmental requirements and to meet consumer demands for ω3 LC-PUFA content in fish; (ii) as an animal feed to produce ω3 LC-PUFA-enriched meat, eggs and milk; (iii) as a food (e.g. bread or milk) supplement in an oxidation-protected form; (iv) directly, as a nutraceutical supplement similar to the way in which fish oil capsules are currently used. As an additional benefit, an oilseed source would be vegetarian and thus satisfy a requirement for an important sector of the global market.

ω3 LC-PUFA biosynthesis

Biosynthesis of LC-PUFA occurs by either aerobic or anaerobic pathways in nature, with the latter found predominantly in some marine bacteria (e.g. *Vibrio* and *Shewanella*) and protists (e.g. *Schizochytrium* and *Thraustochytrium*). Anaerobic synthesis of LC-PUFA is performed by polyketide synthase (PKS) pathways which are cyclical in nature, with multi-enzyme complexes iteratively performing a series of desaturations and 2-carbon elongations using malonyl-CoA and acetyl-CoA as the source of the carbon units. The complex nature of the PKS pathway and the requirement for associate genes has to date resulted in this being a challenging pathway to introduce into plants. The aerobic pathway, on the other hand, consists of a series of distinct desaturation and elongation steps with the genes coding for each of the enzymes having been isolated and characterized. This review focuses on metabolic engineering of plants by the aerobic pathway, although it is worth noting that considerable progress has been made in understanding and engineering the PKS pathways (Metz *et al.* 2001, 2006, 2009; Lippmeier *et al.* 2009).

Aerobic production of LC-PUFA (Fig. 2) can be thought, in the context of plant metabolic engineering, to commence with the native plant fatty acid oleic acid. This fatty acid is first Δ12-desaturated to produce linolenic acid (LA), which is in turn ω3-desaturated to produce ALA. Linolenic acid and ALA are the respective substrates for the ω6 and ω3 LC-PUFA pathways, with the first committed step in both pathways being a Δ6-desaturation to produce either γ-linoleic acid (GLA) (ω6) or SDA (ω3). All the fatty acids up to this point can be found naturally in angiosperms, although GLA and SDA are relatively rare and efforts have been made to engineer these SC-PUFA into oilseed species (see below). The C18 fatty acids GLA and SDA are then Δ6-elongated to the long-chain (≥C20) fatty acids di-homo-γ-linoleic acid (DGLA) and eicosatetraenoic acid (ETA), respectively. This elongation (and the others described in this review) consists of four consecutive enzymatic steps (condensation, ketoreduction, dehydration and enoyl reduction), although the transgenic introduction of the condensing enzyme or 'elongase' is sufficient to confer specificity to the entire elongation with the other elongation reaction components being supplied by the host organism. The products of the Δ6-elongation, DGLA and ETA, are then Δ5-desaturated to ARA and EPA, respectively. Arachidonic acid marks the end of the traditionally

represented ω6 LC-PUFA pathway, although ARA can be Δ5-elongated to docosatetraenoic acid which can finally be Δ4-desaturated to DPAω6. Similarly, EPA can be Δ5-elongated to DPA (DPAω3) which is then Δ4-desaturated to produce DHA. This 'Δ6' pathway is the most commonly found aerobic pathway, although the alternative 'Δ8' pathway also exists. In this pathway, LA and ALA are Δ9-elongated to eicosadienoic acid (EDA) and eicosatrienoic acid (ETRA), respectively, which are then Δ8-desaturated to DGLA and ETA (Fig. 2). At this point, the Δ6 and Δ8 pathways merge and subsequent desaturations and elongations continue as described above.

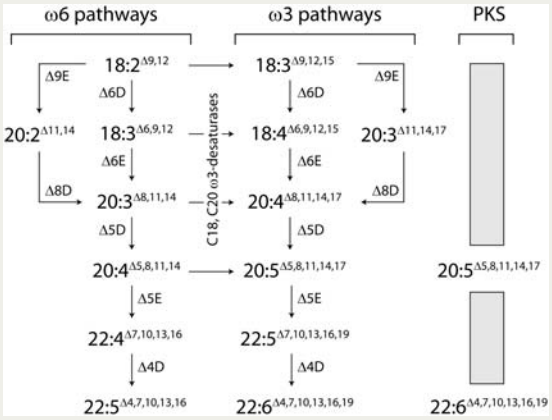

Fig. 2 Long-chain polyunsaturated fatty acid biosynthesis pathways in lower plants, including algae (ω6 and ω3), marine bacteria and protists (PKS). Enzymes are referred to as either 'E' for elongase (e.g. Δ9E is Δ9-elongase) or 'D' for desaturase (e.g. Δ6D is Δ6-desaturase) and belong to the aerobic pathway for LC-PUFA synthesis. A generalized scheme for the processive synthesis of LC-PUFA by the anaerobic PKS pathway is also shown. In this system acetyl-CoA undergoes several rounds of sequential reactions (keto-synthase, keto-reductase, dehydratase and enoyl reductase) that result in repeated elongations by two carbons per cycle of a fatty acyl chain esterified to an acyl carrier protein. Names and abbreviations for the ω3 fatty acids are provided in Fig. 1 and ω6 fatty acids are $18{:}2^{\Delta 9,12}$, LA; $18{:}3^{\Delta 6,9,12}$, GLA; $20{:}3^{\Delta 8,11,14}$, DGLA; $20{:}4^{\Delta 5,8,11,14}$, ARA. The Δ9-elongated fatty acids are $20{:}2^{\Delta 11,14}$, EDA and $20{:}3^{\Delta 11,14,17}$, ETRA.

Long-chain polyunsaturated fatty acid metabolic engineering

It is surprising to some people that fish and other marine species, the predominant source of ω3 LC-PUFA oils in the human diet, are not the source of the genes used in LC-PUFA metabolic engineering applications. Rather, the genes come from microalgae (that fish and other marine species consume), fungi and protists. As already mentioned, genes coding for each step in the LC-PUFA pathways have been isolated and characterized, and several groups have been expressing these in land plants to modify the fatty acid profiles of the plant oil (Fig. 3, Table 1). There are several technical or scientific challenges that must be met before oilseed crops can be engineered to accumulate adequate levels of LC-PUFA. These challenges are mainly focused on increasing the conversion of the native plant fatty acid substrates through to the LC-PUFA of interest with as few intermediate fatty acids as possible. In theory, this simply requires the use of transgenic enzymes which have high conversion efficiencies. In practice,

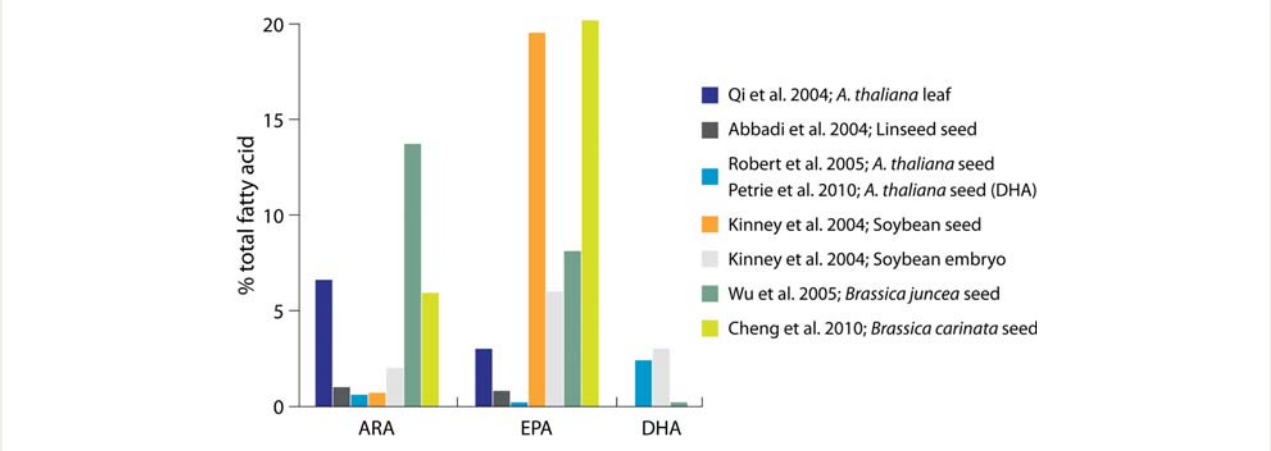

Fig. 3 Some notable LC-PUFA engineering results discussed in this review. See Table 1 for details of genes used in these studies. Format adapted from Venegas-Calerón *et al.* (2010).

Table 1 The genes used in the studies described in Fig. 3. *Denotes an algal gene source.

Publication	Pathway	Host species	Genes
Qi *et al.* (2004)	Δ9	*Arabidopsis thaliana* leaf	*Isochrysis galbana* Δ9-elongase* *Euglena gracilis* Δ8-desaturase* *Mortierella alpina* Δ5-desaturase
Abbadi *et al.* (2004)	Δ6	*Linum usitatissimum* seed	*Phaeodactylum tricornutum* Δ6-desaturase* *Physcomitrella patens* Δ6-elongase *Phaeodactylum tricornutum* Δ5-desaturase*
Robert *et al.* (2005)	Δ6	*Arabidopsis thaliana* seed	*Danio rerio* Δ5/6-desaturase *Caenorhabditis elegans* Δ6-elongase *Pavlova salina* Δ5-elongase* *Pavlova salina* Δ4-desaturase*
Kinney *et al.* (2004)	Δ6	*Glycine max* seed	*Arabidopsis thaliana* Δ15-desaturase *Saprolegnia diclina* Δ17-desaturase *Mortierella alpina* Δ6-desaturase *Mortierella alpina* Δ6-elongase *Mortierella alpina* Δ5-desaturase
Kinney *et al.* (2004)	Δ6	*Glycine max* somatic embryo	As above plus: *Pavlova* sp. Δ5-elongase* *Schizochytrium* Δ4-desaturase*
Wu *et al.* (2005)	Δ6	*Brassica juncea* seed	*Calendula officinalis* Δ12-desatuase *Phytophthora infestans* ω3-desaturase *Thraustochytrium* sp. LPCAT* *Pythium irregulare* Δ6-desaturase *Thraustochytrium* sp. C18 elongase* *Thraustochytrium* sp. Δ5-desaturase* *Oncorhynchus mykiss* C18/C20 elongase *Thraustochytrium* sp. Δ4-desaturase*
Cheng *et al.* (2010)	Δ6	*Brassica carinata* seed	*Pythium irregulare* Δ6-desaturase *Thraustochytrium* sp. C18 elongase* *Thraustochytrium* sp. Δ5-desaturase* *Calendula officinalis* Δ12-desatuase *Phytophthora infestans* ω3-desaturase
Petrie *et al.* (2010a)	Δ6	*Arabidopsis thaliana* seed	*Micromonas pusilla* Δ6-desaturase* *Pyramimonas cordata* Δ6-elongase* *Pavlova salina* Δ5-desaturase* *Pyramimonas cordata* Δ5-elongase* *Pavlova salina* Δ4-desaturase*
Petrie *et al.* (2010b)	Δ9	*Nicotiana benthamiana* leaf	*Pavlova salina:* Δ9-elongase* Δ8-desaturase* Δ5-desaturase* Δ5-elongase* Δ4-desaturase*

the conversion efficiencies of LC-PUFA biosynthesis enzymes are not only affected by actual enzyme activity but also by factors such as substrate dichotomy, the requirement of some enzymes to use substrates from certain metabolic pools.

The biosynthesis of ω3 LC-PUFA in land plants was first reported in 2004 with publications describing the introduction of both the Δ8 and Δ6 pathways. Qi *et al.* (2004) demonstrated the production of 3 % EPA and 6.6 % ARA in *Arabidopsis thaliana* leaf tissue by a Δ8 pathway consisting of the *Isochrysis galbana* Δ9-elongase, *Euglena gracilis* Δ8-desaturase and *Mortierella alpina* Δ5-desaturase with constitutive 35S promoters. This study demonstrated that production of LC-PUFA in land plants was possible, albeit in leaf tissue. Shortly after, Abbadi *et al.* (2004) published the production of LC-PUFA in the seeds of tobacco and linseed by the Δ6 pathway. In this pathway, SDA (11.4 %) and GLA (16.8 %) were produced in linseed, although these fatty acids were not effectively Δ6-elongated with only 0.8 % EPA and 1.0 % ARA being produced. These studies were important proofs of concept that the production of ω3 LC-PUFA in land plants was possible.

ω6 vs. ω3 biosynthesis

These two 2004 publications also showed the complication of parallel ω6 and ω3 pathways in which an individual gene generally functions in both branches (Fig. 2). Using the Δ6 pathway as an example, the Δ6-desaturase is the first committed step of both ω6 and ω3 LC-PUFA production and can desaturate both LA and ALA. The Δ6-desaturation of LA to produce GLA, however, effectively reduces the production of ALA since LA is also the substrate for Δ15-desaturase activity, which produces ALA. One seemingly obvious solution is to increase the production of ALA by introducing more efficient Δ15-desaturase activity, but the success of this approach can be limited by the fact that competition for LA remains between the Δ15- and Δ6-desaturases. This can be overcome by introducing a more broadly functioning ω3-desaturase that not only converts LA to ALA by Δ15-desaturation but can also convert the ω6 fatty acids GLA, DGLA and ARA to their ω3 counterparts. Another approach is to reduce or remove the competition for LA experienced by the Δ15-desaturase by using a Δ6-desaturase that favours ALA as a substrate. A patent describing LC-PUFA synthesis in soybean somatic embryos (Kinney *et al.* 2004) contained an example of the first approach of including an ω3-desaturase gene that could convert ω6 products to their ω3 counterparts. In this work, a Δ6 pathway coupled to a strong Δ17-desaturase resulted in the production of nearly 20 % EPA with very little ARA accumulation due to the ω3-desaturase activity of the Δ17-desaturase. Wu *et al.* (2005) also included a Δ17-desaturase in their Δ6 pathway and this, combined with a lysophosphatidyl acyltransferase (LPAT) which was included as a way of increasing incorporation of novel fatty acids, resulted in the accumulation of up to 15 % EPA in *Brassica juncea* seeds. Similar results were obtained by Cheng *et al.* (2010) who engineered *Brassica carinata* with a Δ6 pathway including an ω3-desaturase and accumulated in excess of 20 % EPA in seed.

The alternative method of reducing ω6 products, namely to use a Δ6-desaturase with preference for the ω3 substrate ALA, has also been demonstrated. Several examples have been reported of genes encoding such Δ6-desaturases (Sayanova *et al.* 2003; García-Maroto *et al.* 2006; Hoffmann *et al.* 2008; Petrie *et al.* 2010a), although the strength of the preference varies. For example, the primula Δ6-desaturase isolated by Sayanova *et al.* (2003) essentially has ω3 specificity with high activity on ALA but very low ω6 activity (Ruiz-López *et al.* 2009). The ω3 preference observed by Hoffmann *et al.* (2008) and Petrie *et al.* (2010a) in their Δ6-desaturases was not as strong as that seen in the primula enzyme, although the Δ6-desaturases were from microalgal sources and seemingly able to access acyl-CoA substrate pools, as will be discussed below. Other microalgal acyl-CoA desaturases such as the *Ostreococcus tauri* Δ6-desaturase (Domergue *et al.* 2005) did not have ω3 preference.

Several Δ9-elongases have now been isolated (Qi *et al.* 2002; Damude *et al.* 2008), although none has a strong ω3 preference. Use of these genes in high-level ω3 LC-PUFA production would therefore likely require the addition of an ω3-desaturase. This is exemplified by the isolation and transgenic plant expression of the entire Δ8 DHA pathway from a single algal species, *Pavlova salina* (Petrie *et al.* 2010b). In this study, a high proportion of the newly produced LC-PUFA were ω6 despite these being almost absent in the native algal fatty acid profile, in which large amounts of ω3 LC-PUFA are present (Zhou *et al.* 2007). Since the *P. salina* Δ9-elongase did not have ω3 preference, it is likely that this alga either has a strong ω3-desaturase or a method of selectively accumulating ω3 LC-PUFA.

Acyl-CoA pathways

Alternative approaches to building ω3 LC-PUFA pathways have been taken with several groups focusing on the construction of acyl-CoA pathways to reduce the 'pool shuffling' that can occur in transgenic LC-PUFA pathways. The LC-PUFA elongation condensing reactions occur using acyl-CoA thioester substrates (the 'acyl-CoA

pool'). In contrast, the majority of desaturases now isolated are lipid-linked desaturases that generally use a phosphatidylcholine substrate (the 'acyl-PC pool'). A desaturated fatty acid must therefore be transferred between the acyl-PC and acyl-CoA pools by acyltransferase enzymes before a subsequent elongation can occur. The synthesis and accumulation of triacylglycerol in seeds are likely more complex than previously thought (Bates *et al.* 2009) and the requirement for acyltransfer between pathway steps presents opportunities for the novel fatty acid to be lost into pools which are, relatively, metabolically inert. This leads to a build-up of intermediate fatty acids along the biosynthetic pathway, with the eventual result being that the terminal product (e.g. DHA) does not accumulate to adequate levels. One solution to this challenge is the addition of acyltransferase enzymes which are capable of rapidly converting the newly elongated or newly desaturated fatty acids to the appropriate pool (e.g. the approach taken by Wu *et al.* (2005) described above in which an LPAT was included in the pathway). Unfortunately, this approach requires the addition of at least one extra gene to an already sizeable transgenic pathway. Fortunately, an alternative solution exists in the form of acyl-CoA desaturases.

The majority of desaturases are lipid linked, although there are published examples of enzymes which are able to function on acyl-CoA thioesters (Kajikawa *et al.* 2004; Domergue *et al.* 2005; Hoffmann *et al.* 2008; Petrie *et al.* 2010*a*) and several of these have been used in DHA pathways. Using such acyl-CoA desaturases means that elongation and desaturation can occur in the same metabolic pool, and thus avoid the efficiency and accumulation losses associated with the 'pool shuffling' described above. Acyl-CoA LC-PUFA pathways can often be characterized by high elongation efficiency. Petrie *et al.* (2010*a*), for instance, described a large increase in Δ6-elongation efficiency when the Δ6-elongase substrate, SDA, was produced by an acyl-CoA Δ6-desaturase when compared with SDA produced by an acyl-PC Δ6-desaturase. Similarly, Hoffmann *et al.* (2008) were able to construct an acyl-CoA pathway with efficient elongation, although the amounts of LC-PUFA produced were very low.

Improving Δ5-elongation

A final area of recent focus has been the Δ5-elongation step. Although Wu *et al.* (2005) and Cheng *et al.* (2010) demonstrated significant levels of EPA production (15 and 25 %, respectively), there have, to date, been no reports of high levels of DHA production in transgenic plants. This is mostly due to low Δ5-elongation efficiency and it has been suggested that the inclusion of enzymes with suitable acyl-transfer function (such as the acyltransferases described above) may be required to achieve adequate efficiency. Indeed, the authors of this review have previously had similar difficulty in obtaining adequate Δ5-elongation efficiency (Robert *et al.* 2005), although the recent isolation of a highly efficient enzyme from the microalga *Pyramimonas cordata* is encouraging (Petrie *et al.* 2009). This enzyme functions extremely well in plants (Fig. 3) and may be an important component of a transgenic DHA biosynthesis pathway for oilseed crops. Figure 3 demonstrates the importance of careful enzyme selection when constructing transgenic pathways, with a clear increase in EPA elongation observed with the *P. cordata* Δ5-elongase. Furthermore, Fig. 3 also demonstrates the effectiveness of using a Δ6-desaturase with ω3 preference (i.e. extremely low ω6 fatty acid levels without using an ω3-desaturase) and an acyl-CoA-like nature (i.e. the subsequent Δ6-elongation is extremely efficient). The high conversion efficiency of the *P. salina* Δ5-desaturase used in this pathway, both in terms of desaturation and provision of the Δ5-desaturated products to the Δ5-elongase, indicates that this enzyme may also be able to access acyl-CoA substrates although this is not yet biochemically proven. If so, this demonstrates the importance of using a pathway in which most of the desaturases are acyl-CoA-dependent for DHA synthesis. It is also worth noting that all transgenes in this pathway are from algal sources.

Long-chain polyunsaturated fatty acid production and accumulation in oilseed crops

Figure 4 describes results from a rapid transient expression system in a model plant, *Nicotiana benthamiana* (Wood *et al.* 2009; Petrie *et al.* 2010*c*). This transient expression of a DHA pathway alongside the *A. thaliana* DGAT1 yielded an impressive level of EPA and DHA accumulation in the leaf triglycerides. Such an oil composition, if replicated in oilseeds, would yield oils whose fatty acid profile will compare very favourably to that of a fish. Other groups rely extensively on *A. thaliana*, cultured soybean embryos or similar, relatively rapid, systems to test hypotheses and constructs before transforming crop species. The transition from an interesting result in a model system to a good result in an oilseed crop species is not necessarily trivial. Whilst this transition is broadly applicable to most crop metabolic engineering projects, the introduction of LC-PUFA to crops presents some specific problems. LC-PUFA, including DHA, are unusual fatty acids in a land plant context and may

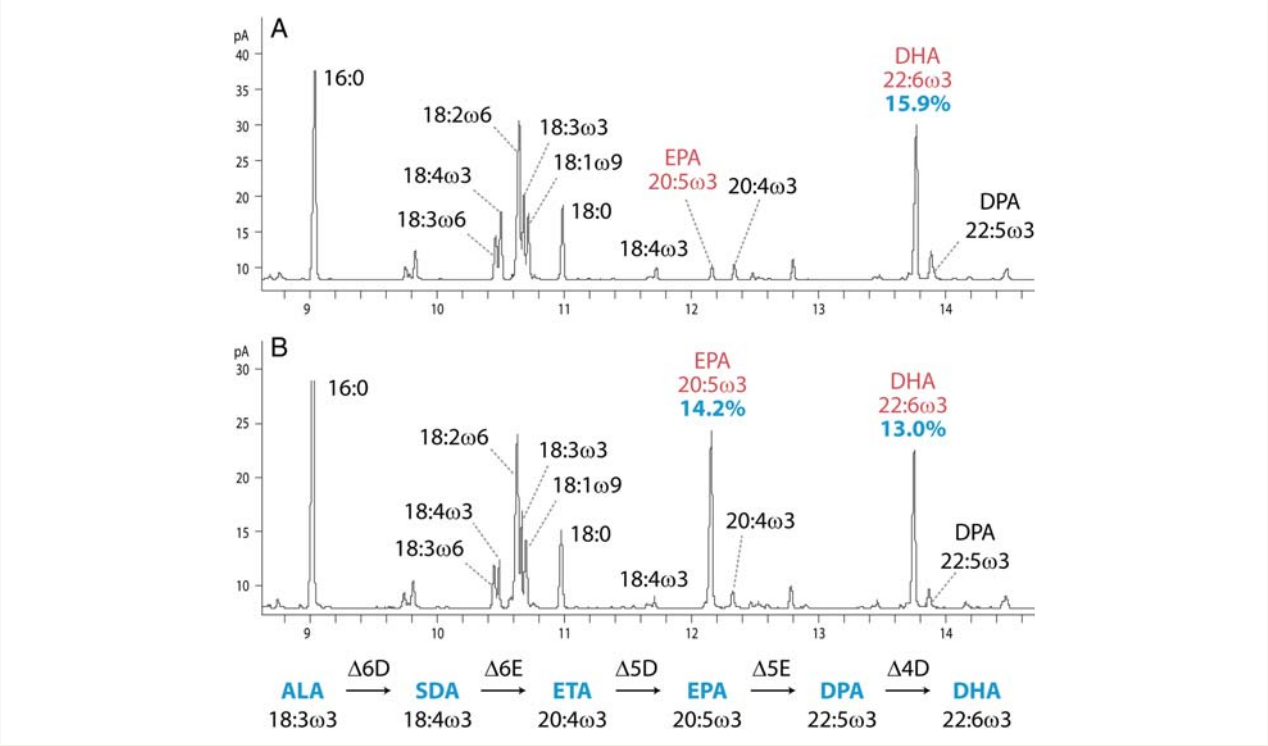

Fig. 4 Docosahexaenoic acid production in plant leaf. Gas chromatography (GC) traces of fatty acid methyl esters (FAME) produced from triacylglycerol in *N. benthamiana* leaf tissue transiently expressing single-gene Cauliflower mosaic virus 35S promoter-driven binary constructs containing the P19 gene silencing suppressor, *Micromonas pusilla* Δ6-desaturase, *P. cordata* Δ6-elongase, *P. salina* Δ5-desaturase, *P. cordata* Δ5-elongase (A) or *P. salina* Δ5-elongase (B), and the *P. salina* Δ4-desaturase. The accumulation of EPA in the sample using the *P. salina* Δ5-elongase demonstrates the manner in which metabolic pathways can be tailored by careful selection of a single gene in the pathway.

not necessarily be metabolized by germinating seed of oilseed crop species. The physiological impact of this would likely vary, depending on both the amount of oil present and available in the seed during germination and the proportion of LC-PUFA accumulated in the seed oil. High-oil species such as rapeseed or flaxseed naturally have a greater energy store than lower oil crops which can then be accessed during germination since these species have been selectively bred to produce oil in excess of germination requirements. The effective removal of a fraction of these 'surplus' triacylglycerols would likely have a relatively small effect on germination and seedling vigour when compared with lower oil crop species such as soybean. Similarly, it could reasonably be expected that the production of high levels of LC-PUFA in lower oil crop species could dramatically affect germination rates and seedling vigour.

It will also be interesting to observe the positional distribution of LC-PUFA in the triacylglycerols constituting high LC-PUFA crop oil, especially since studies have indicated that LC-PUFA located on the *sn*-2 position are more stable under storage and also more bioavailable to human infants, and possibly adults, than those on the *sn*-1,3 positions (Berry 2009).

Acceptance of crop-based ω3 LC-PUFA

As with many projects in the broader field of land plant metabolic engineering, the scientific and technical hurdles around the production of LC-PUFA are only part of the challenge. The real-world delivery of a crop-based source of ω3 LC-PUFA will inevitably require acceptance of the technology by the broader public. A genetically modified ω3 LC-PUFA crop will have tremendous environmental sustainability credentials due to the role it will play in relieving pressure on global fish stocks. Furthermore, such a crop will have direct health benefits for the consumers and, given the increasing awareness of the importance of ω3 LC-PUFA in the general public, these factors are likely to play an important part in increasing consumer acceptance.

Some regions have a high level of aversion to transgenic technologies due to perceived risks

(Hansen *et al.* 2003; Food and Agriculture Organization 2004; Cox *et al.* 2008*a*, *b*), although this does not appear to be the case in other countries. One study, for example, has found that 99 % of consumers in the USA were more concerned with hygiene, sanitation and food-borne illnesses than with biotechnology (IFIC 2005). Regardless, it is useful to review consumers' attitudes to transgenic ω3 LC-PUFA in particular: Cox *et al.* (2008*a*, *b*, 2011) have investigated both US and Australian consumer attitudes toward ω3 LC-PUFA and also ω3 LC-PUFA produced from transgenic oilseeds. They found that whilst a minority had concerns about the use of transgenic technology, the majority of study participants, generally, were either ambivalent or positive about the use of transgenic ω3 LC-PUFA oilseeds. This agrees with earlier data which also suggested that consumers are likely to accept transgenic technology where there is a direct benefit to the consumer (Hossain *et al.* 2003; Hossain and Onyango 2004). Interestingly, consumers tended to prefer a transgenic ω3 LC-PUFA oilseed as the preferred source of oil to be incorporated into bread, milk and supplements above oils sourced from fish and algae (Cox *et al.* 2011).

Conclusions and forward look

Algal genes have contributed significantly to the LC-PUFA metabolic engineering efforts described in this short review. Similarly, research towards the *in planta* production of carotenoids, and in particular astaxanthin, continues to require the use of genes from algal species that naturally synthesize these compounds (see Misawa (2009) for a review). In a broader context, algal model species such as *Chlamydomonas reinhardtii* are now being used to better understand metabolism and catabolism in plants and plant-like species (Wijffels and Barbosa 2010). Recent focus on finding or engineering sustainable sources of biofuels has seen increased use of algal models to investigate mechanisms of lipid accumulation and starch conversion. Wang *et al.* (2009) and Moellering and Benning (2010) have described the formation of lipid droplets in *C. reinhardtii* after nitrogen starvation, with Miller *et al.* (2010) then describing changes in transcript abundance in these systems. It is likely that findings such as these will have relevance in land plants due to the high degree of similarity between the organisms (Hicks *et al.* 2001). There has been considerable effort under way by the US Department of Energy-funded Joint Genome Institute to sequence the genomes of a number of algal species. This data set is publically available at http://genome.jgi-psf.org/. Detailed analyses of algal genomes not only provide important information for research into functional genomics, but also increase our understanding of the physiology, environmental adaptation mechanisms, morphogenesis and evolution of organisms (especially the origin of photosynthetic organisms) and their evolution into land plants.

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Conflicts of interest statement

None declared.

References

Abbadi A, Domergue F, Bauer J, Napier J, Welti R, Zähringer U, Cirpus P, Heinz E. 2004. Biosynthesis of very-long-chain polyunsaturated fatty acids in transgenic oilseeds: constraints on their accumulation. *The Plant Cell* **16**: 2734–2748.

Bates P, Durrett T, Ohlrogge J, Pollard M. 2009. Analysis of acyl fluxes through multiple pathways of triacylglycerol synthesis in developing soybean embryos. *Plant Physiology* **150**: 55–72.

Berry S. 2009. Triacylglycerol structure and interesterification of palmitic and stearic acid-rich fats: an overview and implications for cardiovascular disease. *Nutrition Research Reviews* **22**: 3–17.

Birch E, Garfield S, Hoffman D, Uauy R, Birch D. 2000. A randomized controlled trial of early dietary supply of long-chain polyunsaturated fatty acids and mental development in term infants. *Developmental Medicine and Child Neurology* **42**: 174–181.

Burdge G, Calder P. 2005. Conversion of α-linolenic acid to longer-chain polyunsaturated fatty acids in human adults. *Reproduction Nutrition Development* **45**: 581–597.

Cheng B, Wu G, Vrinten P, Falk K, Bauer J, Qiu X. 2010. Towards the production of high levels of eicosapentaenoic acid in transgenic plants: the effects of different host species, genes and promoters. *Transgenic Research* **19**: 221–229.

Cox D, Evans G, Lease H. 2008*a*. Australian consumers' preferences for conventional and novel sources of long chain omega-3 fatty acids: a conjoint study. *Food Quality and Preference* **19**: 306–314.

Cox D, Evans G, Lease H. 2008*b*. Predictors of Australian consumers' intentions to consume conventional and novel sources of long-chain omega-3 fatty acids. *Public Health Nutrition* **11**: 8–16.

Cox D, Evans G, Lease H. 2011. The influence of product attributes, consumer attitudes and characteristics on the acceptance of: (1) novel bread and milk, and dietary supplements and (2) fish and novel meats as dietary vehicles of long chain omega 3 fatty acids. *Food Quality and Preference* **22**: 205–212.

Damude H, Kinney A, Ripp K, Zhu Q. 2008. Multizymes and their use in making polyunsaturated fatty acids. Patent WO2008124048.

Domergue F, Abbadi A, Zähringer U, Moreau H, Heinz E. 2005. *In vivo* characterization of the first acyl-CoA Δ6-desaturase from a member of the plant kingdom, the microalga *Ostreococcus tauri*. *Biochemical Journal* **389**: 483.

Food and Agriculture Organization (FAO). 2004. State of Food and Agriculture: Agricultural biotechnology: Meeting the needs of the poor. **http://www.fao.org/docrep/006/Y5160E/y5160e11.htm#P15_5987**.

Freeman M, Hibbeln J, Wisner K, Davis J, Mischoulon D, Peet M, Keck P, Marangell L, Richardson A, Lake J, Stoll A. 2006. Omega-3 fatty acids: evidence basis for treatment and future research in psychiatry. *The Journal of Clinical Psychiatry* **67**: 1954–1967.

García-Maroto F, Mañas-Fernández A, Garrido-Cárdenas J, Alonso D. 2006. Substrate specificity of acyl-Δ6-desaturases from Continental versus Macaronesian *Echium* species. *Phytochemistry* **67**: 540–544.

Hansen J, Holm L, Frewer L, Robinson P, Sandøe P. 2003. Beyond the knowledge deficit: recent research into lay and expert attitudes to food risks. *Appetite* **41**: 111–121.

Harris W, Lemke S, Hansen S, Goldstein D, DiRienzo M, Su H, Nemeth M, Taylor M, Ahmed G, George C. 2008. Stearidonic acid-enriched soybean oil increased the omega-3 index, an emerging cardiovascular risk marker. *Lipids* **43**: 805–811.

Hicks G, Hironaka C, Dauvillee D, Funke R, D'Hulst C, Waffenschmidt S, Ball S. 2001. When simpler is better. Unicellular green algae for discovering new genes and functions in carbohydrate metabolism. *Plant Physiology* **127**: 1334.

Hoffmann M, Wagner M, Abbadi A, Fulda M, Feussner I. 2008. Metabolic engineering of ω3-very long chain polyunsaturated fatty acid production by an exclusively acyl-CoA-dependent pathway. *The Journal of Biological Chemistry* **283**: 22352–22362.

Hossain F, Onyango B. 2004. Product attributes and consumer acceptance of nutritionally enhanced genetically modified foods. *International Journal of Consumer Studies* **28**: 255–267.

Hossain F, Onyango B, Schilling B, Hallman W, Adelaja A. 2003. Product attributes, consumer benefits and public approval of genetically modified foods. *International Journal of Consumer Studies* **27**: 353–365.

Hölker F, Beare D, Dörner H, di Natale A, Rätz H-J, Temming A, Casey J. 2007. Comment on 'Impacts of biodiversity loss on ocean ecosystem services'. *Science* **316**: 1285; author reply 1285.

IFIC (International Food Information Council Foundation). 2005. Food biotechnology survey questionnaire. **http://www.ific.org/research/qualhealthclaimsres.cfm**.

Jaenike J. 2007. Comment on 'Impacts of biodiversity loss on ocean ecosystem services'. *Science* **316**: 1285; author reply 1285.

James M, Ursin V, Cleland L. 2003. Metabolism of stearidonic acid in human subjects: comparison with the metabolism of other n-3 fatty acids. *American Journal of Clinical Nutrition* **77**: 1140–1145.

Kajikawa M, Yamato KT, Kohzu Y, Nojiri M, Shimizu S, Sakai Y, Fukuzawa H. 2004. Isolation and characterization of Δ6-desaturase, an ELO-like enzyme and Δ5-desaturase from the liverwort *Marchantia polymorpha* and production of arachidonic and eicosapentaenoic acids in the methylotrophic yeast *Pichia pastoris*. *Plant Molecular Biology* **54**: 335–352.

Kinney A, Cahoon E, Damude H, Hitz W, Kolar CLZ. 2004. Production of very long chain polyunsaturated fatty acids in oilseed plants. Patent US20040057.

Kremer J, Lawrence D, Petrillo G, Litts L, Mullaly P, Rynes R, Stocker R, Parhami N, Greenstein N, Fuchs B. 1995. Effects of high-dose fish oil on rheumatoid arthritis after stopping nonsteroidal antiinflammatory drugs. Clinical and immune correlates. *Arthritis and Rheumatism* **38**: 1107–1114.

Lippmeier J, Crawford K, Owen C, Rivas A, Metz J, Apt K. 2009. Characterization of both polyunsaturated fatty acid biosynthetic pathways in *Schizochytrium* sp. *Lipids* **44**: 621–630.

Metz J, Roessler P, Facciotti D, Levering C, Dittrich F, Lassner M, Valentine R, Lardizabal K, Domergue F, Yamada A. 2001. Production of polyunsaturated fatty acids by polyketide synthases in both prokaryotes and eukaryotes. *Science* **293**: 290.

Metz J, Flatt J, Kuner J. 2006. PUFA polyketide synthases and uses thereof. Patent WO2006135866.

Metz J, Kuner J, Rosenzweig B, Lippmeier JC, Roessler P, Zirkle R. 2009. Biochemical characterization of polyunsaturated fatty acid synthesis in *Schizochytrium*: release of the products as free fatty acids. *Plant Physiology and Biochemistry* **47**: 472–478.

Miller R, Wu G, Deshpande R, Vieler A, Gaertner K, Li X, Moellering E, Zauner S, Cornish A, Liu B, Bullard B, Sears BB, Kuo M-H, Hegg E, Shachar-Hill Y, Shiu S-H, Benning C. 2010. Changes in transcript abundance in *Chlamydomonas reinhardtii* following nitrogen-deprivation predict diversion of metabolism. *Plant Physiology* **154**: 1737–1752.

Misawa N. 2009. Pathway engineering of plants toward astaxanthin production. *Plant Biotechnology* **26**: 93–99.

Moellering E, Benning C. 2010. RNA interference silencing of a major lipid droplet protein affects lipid droplet size in *Chlamydomonas reinhardtii*. *Eukaryotic Cell* **9**: 97–106.

Mori T, Bao D, Burke V, Puddey I, Beilin L. 1999. Docosahexaenoic acid but not eicosapentaenoic acid lowers ambulatory blood pressure and heart rate in humans. *Hypertension* **34**: 253–260.

Myers R, Worm B. 2003. Rapid worldwide depletion of predatory fish communities. *Nature* **423**: 280–283.

Nagel G, Nieters A, Becker N, Linseisen J. 2003. The influence of the dietary intake of fatty acids and antioxidants on hay fever in adults. *Allergy* **58**: 1277–1284.

Oh D, Talukdar S, Bae E, Imamura T, Morinaga H, Fan W, Li P, Lu W, Watkins S, Olefsky J. 2010. GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. *Cell* **142**: 687–698.

Parker G, Gibson N, Brotchie H, Heruc G, Rees A-M, Hadzi-Pavlovic D. 2006. Omega-3 fatty acids and mood disorders. *The American Journal of Psychiatry* **163**: 969–978.

Petrie J, Liu Q, Mackenzie A, Shrestha P, Mansour P, Robert S, Frampton D, Blackburn S, Nichols P, Singh S. 2009. Isolation and characterisation of a high-efficiency desaturase and elongases from microalgae for transgenic LC-PUFA production. *Marine Biotechnology*. doi: 10.1007/s10126-009-9230-1.

Petrie J, Shrestha P, Mansour M, Nichols P, Liu Q, Singh S. 2010*a*. Metabolic engineering of omega-3 long-chain polyunsaturated fatty acids in plants using an acyl-CoA Δ6-desaturase with ω3-preference from the marine microalga *Micromonas pusilla*. *Metabolic Engineering* **12**: 233–240.

Petrie J, Mackenzie A, Shrestha P, Liu Q, Frampton D, Robert S, Singh S. 2010*b*. Isolation of three novel long-chain polyunsaturated fatty acid Δ9-elongases and the transgenic assembly of the entire *Pavlova salina* docosahexaenoic acid pathway in *Nicotiana benthamiana*. *Journal of Phycology* **46**: 917–925.

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

Qi B, Beaudoin F, Fraser T, Stobart A, Napier J, Lazarus C. 2002. Identification of a cDNA encoding a novel C18-Δ9 polyunsaturated fatty acid-specific elongating activity from the docosahexaenoic acid (DHA)-producing microalga, *Isochrysis galbana*. *FEBS Letters* **510**: 159–165.

Qi B, Fraser T, Mugford S, Dobson G, Sayanova O, Butler J, Napier J, Stobart K, Lazarus C. 2004. Production of very long chain polyunsaturated omega-3 and omega-6 fatty acids in plants. *Nature Biotechnology* **22**: 739–745.

Robert S, Singh S, Zhou X, Petrie J, Blackburn S, Mansour P, Nichols P, Liu Q, Green A. 2005. Metabolic engineering of *Arabidopsis* to produce nutritionally important DHA in seed oil. *Functional Plant Biology* **32**: 473.

Ruiz-López N, Haslam RP, Venegas-Calerón M, Larson TR, Graham IA, Napier JA, Sayanova O. 2009. The synthesis and accumulation of stearidonic acid in transgenic plants: a novel source of "heart-healthy"omega-3 fatty acids. *Plant Biotechnology Journal* **7**: 704–716.

Sayanova OV, Beaudoin F, Michaelson LV, Shewry PR, Napier J. 2003. Identification of Primula fatty acid delta 6-desaturases with n-3 substrate preferences. *FEBS Letters* **542**: 100–104.

von Schacky C. 2006. A review of omega-3 ethyl esters for cardiovascular prevention and treatment of increased blood triacylglycerol levels. *Vascular Health and Risk Management* **2**: 251–262.

Schaefer E, Bongard V, Beiser A, Lamon-Fava S, Robins S, Au R, Tucker K, Kyle D, Wilson P, Wolf P. 2006. Plasma phosphatidylcholine docosahexaenoic acid content and risk of dementia and Alzheimer disease: the Framingham Heart Study. *Archives of Neurology* **63**: 1545–1550.

Simopoulos A. 2002. Omega-3 fatty acids in inflammation and autoimmune diseases. *Journal of the American College of Nutrition* **21**: 495–505.

Tocher D, Dick J, MacGlaughlin P, Bell J. 2006. Effect of diets enriched in Δ6 desaturated fatty acids (18:3n-6 and 18:4n-3), on growth, fatty acid composition and highly unsaturated fatty acid synthesis in two populations of Arctic charr (*Salvelinus alpinus* L.). *Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology* **144**: 245–253.

Ueshima H, Stamler J, Elliott P, Chan Q, Brown I, Carnethon M, Daviglus M, He K, Moag-Stahlberg A, Rodriguez B, Steffen L, Van Horn L, Yarnell J, Zhou B. 2007. Food omega-3 fatty acid intake of individuals (total, linolenic acid, long-chain) and their blood pressure: INTERMAP study. *Hypertension* **50**: 313–319.

Venegas-Calerón M, Sayanova O, Napier JA. 2010. An alternative to fish oils: metabolic engineering of oil-seed crops to produce omega-3 long chain polyunsaturated fatty acids. *Progress in Lipid Research* **49**: 108–119.

Wang C, Harris W, Chung M, Lichtenstein A, Balk E, Kupelnick B, Jordan H, Lau J. 2006. n-3 Fatty acids from fish or fish-oil supplements, but not alpha-linolenic acid, benefit cardiovascular disease outcomes in primary- and secondary-prevention studies: a systematic review. *The American Journal of Clinical Nutrition* **84**: 5–17.

Wang Z, Ullrich N, Joo S, Waffenschmidt S, Goodenough U. 2009. Algal lipid bodies: stress induction, purification, and biochemical characterization in wild-type and starchless *Chlamydomonas reinhardtii*. *Eukaryotic Cell* **8**: 1856–1868.

Whelan J. 2009. Dietary stearidonic acid is a long chain (n-3) polyunsaturated fatty acid with potential health benefits. *Journal of Nutrition* **139**: 5–10.

Wijffels R, Barbosa M. 2010. An outlook on microalgal biofuels. *Science* **329**: 796–799.

Wilberg M, Miller T. 2007. Comment on 'Impacts of biodiversity loss on ocean ecosystem services'. *Science* **316**: 1285.

Wood C, Petrie J, Shrestha P, Mansour M, Nichols P, Green A, Singh S. 2009. A leaf-based assay using interchangeable design principles to rapidly assemble multistep recombinant pathways. *Plant Biotechnology Journal* **7**: 914–924.

Worm B, Barbier E, Beaumont N, Duffy J, Folke C, Halpern B, Jackson J, Lotze H, Micheli F, Palumbi S, Sala E, Selkoe K, Stachowicz J, Watson R. 2006. Impacts of biodiversity loss on ocean ecosystem services. *Science* **314**: 787–790.

Wu G, Truksa M, Datla N, Vrinten P, Bauer J, Zank T, Cirpus P, Heinz E, Qiu X. 2005. Stepwise engineering to produce high yields of very long-chain polyunsaturated fatty acids in plants. *Nature Biotechnology* **23**: 1013–1017.

Zhou XR, Robert SS, Petrie JR, Frampton DMF, Mansour MP, Blackburn SI, Nichols PD, Green AG, Singh SP. 2007. Isolation and characterization of genes from the marine microalga *Pavlova salina* encoding three front-end desaturases involved in docosahexaenoic acid biosynthesis. *Phytochemistry* **68**: 785–796.