Journal of Experimental Botany, Vol. 63, No. 7, pp. 2397–2410, 2012
doi:10.1093/jxb/err454 Advance Access publication 30 January, 2012

Journal of Experimental Botany
www.jxb.oxfordjournals.org

REVIEW PAPER

Metabolic engineering of the omega-3 long chain polyunsaturated fatty acid biosynthetic pathway into transgenic plants

Noemi Ruiz-López, Olga Sayanova, Johnathan A. Napier and Richard P. Haslam*

Department of Biological Chemistry, Rothamsted Research, Harpenden, Herts AL5 2JQ, UK

* To whom correspondence should be addressed. E-mail: richard.haslam@rothamsted.ac.uk

Received 3 November 2011; Revised 15 December 2011; Accepted 16 December 2011

Abstract

Omega-3 (ω-3) very long chain polyunsaturated fatty acids (VLC-PUFAs) such as eicosapentaenoic acid (EPA; 20:5 Δ5,8,11,14,17) and docosahexaenoic acid (DHA; 22:6 Δ4,7,10,13,16,19) have been shown to have significant roles in human health. Currently the primary dietary source of these fatty acids are marine fish; however, the increasing demand for fish and fish oil (in particular the expansion of the aquaculture industry) is placing enormous pressure on diminishing marine stocks. Such overfishing and concerns related to pollution in the marine environment have directed research towards the development of a viable alternative sustainable source of VLC-PUFAs. As a result, the last decade has seen many genes encoding the primary VLC-PUFA biosynthetic activities identified and characterized. This has allowed the reconstitution of the VLC-PUFA biosynthetic pathway in oilseed crops, producing transgenic plants engineered to accumulate ω-3 VLC-PUFAs at levels approaching those found in native marine organisms. Moreover, as a result of these engineering activities, knowledge of the fundamental processes surrounding acyl exchange and lipid remodelling has progressed. The application of new technologies, for example lipidomics and next-generation sequencing, is providing a better understanding of seed oil biosynthesis and opportunities for increasing the production of unusual fatty acids. Certainly, it is now possible to modify the composition of plant oils successfully, and, in this review, the most recent developments in this field and the challenges of producing VLC-PUFAs in the seed oil of higher plants will be described.

Key words: Lipids, omega-3 fatty acids, polyunsaturated fatty acids, seed oils, transgenic plants.

Introduction

In recent years, considerable focus has been placed on engineering plants for the production of very long chain polyunsaturated fatty acids (VLC-PUFAs) in their seed oils. Typically, VLC-PUFAs are fatty acids (FAs) of ⩾20 carbons in length (C_{20}–C_{22}) with three or more methylene-interrupted double bonds in the *cis* configuration. These FAs are grouped into two main families, omega-6 (ω-6) and omega-3 (ω-3), distinguished by the position of the first double bond counted from the methyl end. The predominant ω-6 and ω-3 C_{18} FAs found in most seed oils are linoleic (LA; 18:2 Δ9,12) and α-linolenic (ALA; 18:3 Δ9,12,15), respectively. Both LA and ALA are required in the diet of higher animals, including humans, as they lack the Δ12- and Δ15-desaturase activities that convert oleic acid (OA; 18:1 Δ9) to LA and ALA, respectively. It is for this reason that LA and ALA are often termed essential

Abbreviations: ω-3, omega-3; ω-6, omega-6; ACP, acyl carrier protein; ALA, α–linolenic acid; ARA, arachidonic acid; CoA, coenzyme A; DAG, diacylglycerol; DGAT, diacylglycerol acyltransferase; DGLA, di-homo γ-linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; ECR, enoyl-CoA reductase; EDA, eicosadienoic acid; EPA, eicosapentaenoic acid; EST, expressed sequenced tag; ETA, eicosatetraenoic acid; ETrA, eicosatrienoic acid; FA, fatty acid; FAS, fatty acid synthase; GLA, γ-linolenic acid; HCD, hydroxyacyl-CoA dehydratase; KCR, β-ketoacyl-CoA reductase; KCS, β-ketoacyl-CoA synthase; LA, linoleic acid; LPAT, lyso-phospholipid acyltransferase; OA, oleic acid; PC, phosphatidylcholine; PDAT, phospholipid:diacylglycerol acyltransferase; PKS, polyketide synthase; PPT, phosphopantetheinyl transferase; PUFA, polyunsaturated fatty acid; ROD1, phosphatidylcholine diacylglycerol cholinephosphotransferase; SDA, stearidonic acid; TAG, triacylglycerol; VLC-PUFA, very long chain polyunsaturated fatty acid.

© The Author [2012]. Published by Oxford University Press [on behalf of the Society for Experimental Biology]. All rights reserved.
For Permissions, please e-mail: journals.permissions@oup.com

Downloaded from https://academic.oup.com/jxb/article/63/7/2397/515583 by U.S. Department of Justice user on 16 August 2022

FAs. The conversion in vertebrates of essential FAs to the ω-6 arachidonic acid (ARA; 20:4 Δ5,8,11,14), ω-3 eicosapentaenoic acid (EPA; 20:5 Δ5,8,11,14,17), and ω-3 docosahexaenoic acid (DHA; 22:6 Δ4,7,10,13,16,19) via iterative rounds of desaturation and elongation is known to be inefficient. Indeed, the majority of dietary essential FAs are β-oxidized to provide energy (Huang *et al.*, 2004), leaving only a small portion available for synthesis of VLC-PUFAs. Although mammals have the necessary enzymes to make VLC-PUFAs from the parent essential FAs, *in vivo* studies in humans show that ~5% of ALA is converted to EPA and <0.5% of ALA is converted to DHA (Williams and Burdge, 2006). These very low conversion efficiencies and lack of sufficient dietary ω-3 VLC-PUFAs could compromise health. There is a substantial literature building in support of the health-beneficial properties of ω-3 VLC-PUFAs (Abeywardena and Patten, 2011, and references therein). In particular the dietary consumption of ω-3 VLC-PUFAs, for example via oily fish, is known to mitigate conditions such as cardiovascular disease, obesity, and metabolic syndrome (Calder, 2004; Nugent, 2004; Williams and Burdge, 2006; Poudyal *et al.*, 2011). This situation is leading to calls for EPA and DHA to be considered as 'conditionally essential' FAs. However, many people have intakes of EPA and DHA that are far below the recommended or suggested intake amounts (Meyer, 2011).

Even if people were to increase their intake of EPA and DHA, providing an adequate sustainable supply is difficult for a number of reasons. Firstly, the primary source of EPA and DHA in the human diet is marine fish, for example tuna and mackerel, yet there is a growing acknowledgement that these sources of ω-3 VLC-PUFAs are under pressure, primarily as a result of overfishing, and cannot meet the ever-increasing global demand (Tocher, 2009). In 2008, of the fish stock groups monitored by the UN FAO (Food and Agriculture Organisation of the United Nations, 2010), 53% were estimated to be fully exploited, 28% overexploited, and 3% depleted or recovering from depletion (1%). Only 15% of the stock groups under observation were categorized as underexploited and able to produce more than their current catches; a situation that gives cause for concern. Secondly, environmental pollution of marine ecosystems has resulted in the accumulation of potential toxins such as heavy metals and dioxins in fish, to the point of questioning the benefits of fish consumption to human health, further limiting availability (Domingo *et al.*, 2007). Thirdly, it might be imagined that the expansion of industrialized aquaculture could solve some of these supply problems; however, marine fish are poor producers of VLC-PUFAs. Indeed the original source of VLC-PUFAs are marine microalgae (e.g. diatoms), which are concentrated through the food chain finally accumulating in fish lipids. Aquaculture, rather than being a solution, requires a source of VLC-PUFAs for the enrichment of fish feed, which represents a further challenge to the dwindling supplies of ω-3 VLC-PUFAs. The statistics are sobering; on average 7.2 Mt of low value or forage fish are removed from the marine food web each year to produce fish meal and fish oil. These fish play an important role in converting plankton into food for higher trophic species, and overexploitation leads to local stress in these species (Tacon and Metian, 2009; Smith *et al.*, 2011). The aquaculture industry continues to expand, with an average annual growth rate of 6.6%. Indeed aquaculture production in 2008 was 52.5 Mt with a value of US$98.4 billion. Such a rapid rate of expansion places a huge demand on the supplies of fish oil. However, catches for the purposes of fish meal and fish oil production have not increased in parallel with demand, a situation that has inevitably led to sharp price rises, for example fish oil prices reached US$590 t^{-1} in March 2010, a price that was 50% higher than a year earlier. In response the ratio of wild fisheries inputs to farmed fish has declined, reflecting the market pressures to reduce fish meal and fish oil inputs in aqua feeds; nonetheless, it still remains as high as 5.0 for Atlantic Salmon (Naylor *et al.* 2009).

A possible alternative might be the substitution of fish oil with vegetable oil in fish feeds, but this detrimentally affects the final fish lipid profile of FAs, altering the ω-6/ω-3 FA ratio and lowering their nutritional value for human consumption. Another option are microalgae, the natural primary producers of VLC-PUFAs. They might offer a direct supply of EPA and DHA to fish oil (Khozin-Goldberg *et al.*, 2011). Indeed, several commercial single-cell sources for VLC-PUFAs have been developed in the last two decades, for example the production of ARA 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 VLC-PUFAs requires substantial advances in photo-bioreactors and breakthrough solutions enhancing growth performance and lipid modification.

Responding to the limited supply and increasing demand for VLC-PUFAs, in particular ω-3 FAs, much research effort has focused on delivering oilseed plants engineered to synthesize EPA and DHA. The potential to expand the use of terrestrial plant-based lipids is great, as such a substitution could alleviate the aquaculture industry's pressures on forage fisheries. It would also reduce human health concerns associated with the presence of dioxins and polychlorinated biphenyls (PCBs) in fish feeds. Such an approach is not without precedence; a few species are able to synthesize the first Δ6-desaturated step in VLC-PUFA synthesis; ω-6 γ-linolenic acid (GLA; 18:3 Δ6,9,12) and ω-3 stearidonic acid (SDA; 18:4 Δ6,9,12,15), in *Borago officinalis* and *Echium plantagineum*, respectively. Yet these sources cannot be utilized for the production of VLC-PUFAs due to their poor agronomic performance. Considerable efforts have been made to improve the composition of vegetable oils, and tremendous progress has been made in improving our (lack of) understanding of the seed oil biosynthetic pathway. However, terrestrial plants do not have the ability to synthesize ω-3 VLC-PUFAs such as EPA and DHA; therefore, any approach must involve the introduction of new desaturase and elongase activities. The introduction of the VLC-PUFA biosynthetic pathways into oilseed crops has been successfully

Downloaded from https://academic.oup.com/jxb/article/63/7/2397/515583 by U.S. Department of Justice user on 16 August 2022

demonstrated, but reaching economically viable levels of EPA and DHA has proved challenging. The pathways and developments being attempted to meet this goal are discussed, as are new insights into characterizing the flux of unusual FAs using emerging technology.

PUFA biosynthetic pathways

The production of VLC-PUFAs in plants begins with the synthesis of FAs by the multisubunit 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). All higher plants have the enzymatic complex to synthesize the C_{18} PUFAs LA and ALA. As discussed, a few plants have the ability to produce Δ6-desaturated FAs, for example SDA and predominantly GLA. However, higher plants do not possess the enzymes to follow the elongation and desaturation steps to convert C_{18} PUFAs into ω-3 VLC-PUFAs (Napier, 2007; Damude and Kinney, 2008; Napier and Graham, 2010). In recent years, several pathways leading to the synthesis of ω-3 VLC-PUFAs have been described from a range of different species, including animals, fungi, plants, and aquatic organisms, demonstrating the significant role of these FAs in various biological systems. Furthermore all the primary genes involved in VLC-PUFA biosynthesis have been identified.

EPA, DHA, and other VLC-PUFAs are traditionally considered to be products of alternating desaturation and elongation steps acting on long-chain polyunsaturated substrates; therefore, two distinct types of primary biosynthetic activities (desaturases and elongases) are required. The first of these steps is carried out by microsomal 'front end' PUFA desaturases which belong to the N-terminal cytochrome *b*5 fusion superfamily, (Sayanova *et al.*, 1997), as opposed to the Δ12- and Δ15-desaturases from plants, algae, and some fungi which lack this cytochrome *b*5 domain. The majority of the microsomal desaturases from lower eukaryotes use glycerolipid-linked substrates, in particular FAs esterified to the *sn*-2 position of glycerolipids. This is in contrast to animals where the substrates for these enzyme activities are thought to be acyl-CoAs (Stymne and Appelqvist, 1978; Griffiths *et al.*, 1988; Jackson *et al.*, 1998). The second step in VLC-PUFA biosynthesis is microsomal FA elongation, which occurs as a result of four sequential enzymatic reactions: condensation of the substrate FA 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). However, the heterologous expression of just the initial condensing enzyme is capable of reconstituting the heterologous elongating activity (Millar and Kunst, 1997; Paul *et al.*, 2006) and for this reason KCS are often referred to as 'elongases'. This condensing enzyme acts in a trans-dominant manner, and is considered to be rate limiting and the regulator of substrate specificity (both chain length and pattern of double bonds). It has been generally assumed that the contribution of the other elongase components to VLC-PUFA synthesis is neutral. The KCS condensing enzymes 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.*, 2004), 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 FAs with C_{18} to C_{22}+ chain length, (James *et al.*, 1995). In contrast to microsomal desaturation, all microsomal elongation described so far, both ELO-type and FAE1-like, exclusively uses acyl-CoAs as substrates (Jakobsson *et al.* 2006).

In order to produce EPA and DHA, most PUFA-synthesizing eukaryotic organisms operate the so-called Δ6-pathway or the 'conventional' aerobic pathway. The first step in this pathway is the Δ6-desaturation of both 18:2 ω-6 (LA) and 18:3 ω-3 (ALA), resulting in the synthesis of GLA and SDA, respectively (see Fig. 1). This step is followed by a Δ6-specific C2 elongation, yielding di-homo γ-linolenic

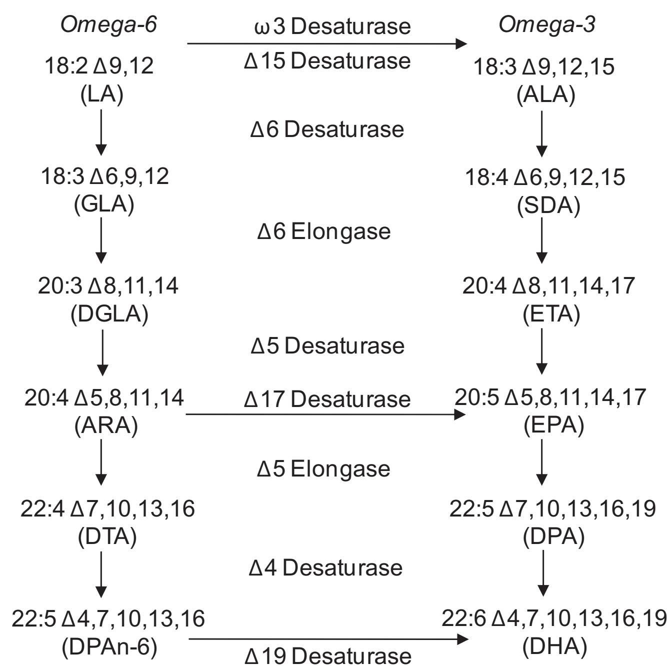

Fig. 1. A schematic representation of the conventional VLC-PUFA biosynthetic pathway from linoleic and α-linolenic acid precursors. The alternative Δ8-pathway (not show in Fig. 1) utilizes a Δ9-elongase and Δ8-desaturase to produce DGLA and ETA, respectively.

Downloaded from https://academic.oup.com/jxb/article/63/7/2397/515583 by U.S. Department of Justice user on 16 August 2022

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 ω-6 or ω-3 fatty acids. The two pathways can be interconnected by ω-3 desaturases, which convert ω-6 FAs into their ω-3 counterparts.

An alternative pathway (or the so-called Δ8-pathway) for the biosynthesis of VLC-PUFAs 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; Zhou *et al.*, 2007; Sayanova *et al.*, 2011*a*). In this pathway, LA and ALA are first elongated by a specific Δ9-elongase to eicosadienoic acid (EDA; 20:2 Δ11,14) and eicosatrienoic acid (ETrA; 20:3 Δ11,14,17). These C_{20} products are then desaturated by a Δ8-desaturase to DGLA and ETA. Finally, these PUFAs 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 (Fig. 1). These activities (Δ5-elongase and 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 (Pereira *et al.*, 2004; Sayanova and Napier, 2004; Wu *et al.*, 2005). In contrast, the Sprecher pathway is found in mammals and is independent of Δ4-desaturation. In this pathway, EPA goes through two consecutive elongation steps initially producing DPA and subsequently tetracosapentaenoic acid (24:5 Δ9,12,15,18,21), that is then further desaturated by a Δ6-desaturase generating tetracosahexaenoic acid (24:6 Δ6,9,12,15,18,21). This C_{24} PUFA is then subject to partial peroxisomal β-oxidation to yield DHA. It is thought that the essential translocation from the endoplasmic reticulum (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 FA 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 synthase (PKS) in order to synthesize VLC-PUFAs (Metz *et al.*, 2001). The first evidence of the existence of such a pathway was demonstrated via the transfer of a 38 kb genomic fragment isolated from the EPA-producing marine bacteria *Shewanella* to *Escherichia coli* and *Synechococcus* sp. which resulted in the production of EPA (Yazawa, 1996; Takeyama *et al.*, 1997, respectively). Later Metz *et al.* (2001) revealed that these PKS-like enzymes are capable of *de novo* PUFA synthesis by an iterative extension of the fatty acyl chain, combined with *trans–cis* isomerization and enoyl reduction in selected cycles. Initially in a manner reminiscent of FAS, these PKS-like complexes synthesize EPA and/or DHA directly from the condensation of malonyl-CoA with a short chain acyl-CoA (Metz *et al.*, 2001; Napier, 2007). Next the acyl chain is subject to successive rounds of reduction, dehydration, further reduction, and condensation, growing by two-carbon units in each round. The introduction of double bonds is via a dehydrase–isomerase mechanism similar to the one found in *E. coli*, which does not require oxygen (Qiu *et al.*, 2001; Metz *et al.*, 2006, 2009). This PKS-like pathway produces EPA or DHA as free FAs, 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 co-exists with the PKS pathway.

Engineering VLC-PUFAs in higher plants

Transgenic production of stearidonic and γ-linolenic acid

As described above, positive effects on cardiovascular and other diseases are best established for DHA and EPA; however, these FAs are not present in any known higher plants. Yet a small number of plant species do have the faculty to produce Δ6-desaturated FAs (e.g. SDA and GLA). Such examples are, however, not appropriate to mass market supply as these species are not part of the modern diet, they are difficult to cultivate, and they have relatively low oil yields. Moreover, the composition of their oils can also be problematic, for instance borage (*B. officinalis*) contains a small amount of the undesirable ω-9 erucic acid (22:1 Δ13) (Guil-Guerrero *et al.*, 2006). However, there is a requirement for GLA- and SDA-containing oils for the nutritional supplement market (Flider, 2005), and it is for that reason that there has been a large research interest in using high-yielding crops to produce oils with a significant GLA or SDA content.

Thus the discovery and cloning of Δ6-desaturases inevitably led to the expression in plants which started as soon as the genes became available. The first published experiments reported an expression of a Δ6-desaturase isolated from the cyanobacterium *Synechocystis* sp. under the control of a constitutive *Cauliflower mosaic virus* (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

Downloaded from https://academic.oup.com/jxb/article/63/7/2397/515583 by U.S. Department of Justice user on 16 August 2022

higher levels of GLA and SDA accumulation (a combined FA total of ~20%) were obtained by changing the Δ6-desaturase to one from *B. officinalis* (Sayanova *et al.*, 1997, 2003). Later on, 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 (Qiu *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.

Considerable progress has been made and further studies have shown very high levels (up to 70%) of GLA and/or SDA as a result of seed-specific expression. Qiu *et al.* (2002) expressed a *B. officinalis* Δ6-desaturase in *Brassica juncea*, producing 3–9% of GLA from total FA. Liu *et al.* (2001) expressed a fungal Δ6-desaturase from *M. alpina* along with a Δ12-desaturase from the same fungus, producing up to 43% GLA in seeds of *Brassica napus*. Similar levels of GLA were produced in *B. juncea* seeds expressing a Δ6-desaturase from the fungus *Pythium irregulare* (Hong *et al.*, 2002). The production of SDA has attracted attention from biotechnology companies, for example Monsanto and Solae LLC have a transgenic soybean (producing 15–30% SDA and 5–8% GLA) close to commercialization after successful safety assessment (Hammond *et al.*, 2008) and a generally recognized as safe (GRAS) notice confirming that the ω-3 soybean oil from the bioengineered soybean can be used in foods and beverages. 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.*, 2011). In an attempt to produce SDA alone, linseed lines expressing a ω6-desaturase from *Primula vialii* (which specifically only utilizes ALA as a substrate), accumulated up to ~13% SDA and a complete absence of GLA in their lipids. These SDA levels are comparable with those found in the commercial plant source *Echium* spp., but have the advantage of being devoid of the PUFA ω-6 precursor GLA (Table 1; 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.

Reconstitution of VLC-PUFA synthesis in transgenic plants

Having established the rationale for the production of specific PUFAs in seed oil, the logical next step was the metabolic engineering of the entire VLC-PUFA biosynthetic route in oilseeds. To be successful this required 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; see Fig. 1) are involved in the conversion of native plant FAs such as LA and ALA to VLC-PUFAs such as ARA and EPA. Of course before any such engineering attempt could be made, the genes encoding the primary VLC-PUFA biosynthetic activities had to be identified and characterized. Indeed over recent years this challenge was met with extensive progress in 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, researchers firstly endeavoured to reconstruct either partially or entirely the VLC-PUFA biosynthetic pathways in the yeast *Saccharomyces cerevisiae*, demonstrating the

Table 1. Comparison of published transgenic lines producing VLC-PUFAs and biosynthetic intermediates

NP, data not provided.

Reference	Plant species	Tissue	Fatty acid GLA	SDA	DGLA	ARA	ETA	EPA	DPA	DHA
Conventional pathway										
Abbadi *et al.* (2004)	*N. tabacum*	Seed	29.3	–	1.8	1.5	–	–	–	–
	L. usitatissimum	Seed	16.8	11.4	1.2	1.0	0.9	0.8	–	–
Kinney *et al.* (2004)	*G. max*	Embryo	22.7	3.1	4.0	0.4	3.3	13.3	0.9	–
	G. max	Embryo	2.7	3.6	3.1	2.5	2.1	5.2	1.0	3.3
	G.max	Seed	11.7	1.1	10.1	2.2	2.4	19.6	0.8	–
Wu *et al.* (2005)	*B. juncea*	Seed	27.3	2.2	1.9	4.0	1.1	8.1	0.1	0.2
Ruiz-Lopez *et al.* (2009)	*L. usitatissimum*	Seed	>0.5	11.8	–	–	–	–	–	–
Cheng *et al.* (2010)	*B. carinata*	Seed	26.9	5.4	2.2	5.7	2.5	20.4	4.0	–
Alternative pathway										
Qi *et al.* (2004)	*A. thaliana*	Leaves	–	–	1.3	6.6	1.2	3.0	–	–
Using acyl-CoA desaturases										
Robert *et al.* (2005)	*A. thaliana*	Seed	0.6	1.8	1.9	1.6	0.4	3.2	0.1	–
	A. thaliana	Seed	0.4	1.5	1.5	1.0	0.8	2.4	0.1	0.5
Hoffmann *et al.* (2008)	*A. thaliana*	Seed	>0.5	>0.1	0.8	0.1	0.9	0.05	–	–
Petrie *et al.* (2010)	*N. benthamiana*	Leaves	2.1	1.5	–	0.6	0.6	10.7	0.3	–
PKS system										
Metz *et al.* (2006)	*A.thaliana*	Seed	NP	NP	NP	NP	NP	NP	1.8	2.4

Downloaded from https://academic.oup.com/jxb/article/63/7/2397/515583 by U.S. Department of Justice user on 16 August 2022

feasibility of such an approach (Beaudoin *et al.*, 2000; Das *et al.*, 2000; Parker-Barnes *et al.*, 2000; Domergue *et al.*, 2002, 2003; Meyer *et al.*, 2004). However, despite the success of these early engineering attempts, it was quickly established that the availability of genes was just the first step in a successful approach.

Abbadi *et al.* (2004) engineered a series of plant transformation constructs containing different combinations of Δ6-desaturases from *Physcomitrella patens*, *B. officinalis*, and *Phaeodactylum tricornutum*, Δ6-elongases from *P. patens* and *Caenorhabditis elegans*, and Δ5-desaturases from *M. alpina* and *P. tricornutum*; where all the genes were under the control of seed-specific promoters. The experiments resulted in a low accumulation of ARA and EPA in tobacco and linseed transgenic seeds, respectively (Table 1). Moreover, analysis of the acyl-CoA pools in these seeds showed only trace amounts of SDA, but the 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 VLC-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, namely 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.

The efficiency with which endogenous acyltransferases accept non-native substrates could play an important role in avoiding this bottleneck, and it is also likely that different activities [lyso-phospholipid acyltransferases (LPATs); phospholipid:diacylglycerol acyltransferase (PDAT), etc.] have different affinities for these novel FAs (Dyer *et al.*, 2008). In addition, many of these acyl exchange acyltransferases can work in both forward and reverse directions (Fig. 2), and it is for that reason that the pool sizes of the different metabolites are expected to prove decisive in determining the leading enzyme activity. Interestingly, the severity of this bottleneck appears to vary from one host plant to another. This was demonstrated when higher levels of EPA and ARA were obtained in transgenic soybean (*Glycine max*) and Indian mustard (*B. juncea*) than in linseed or tobacco. It seems that the endogenous acyltransferases from transgenic soybean and *Brassica* spp. have a broader substrate specificity than linseed or tobacco. In an attempt to alleviate this bottleneck researchers sought candidate genes encoding the enzyme LPCAT (lysophosphatidylcholine acyltransferase), which catalyses a bidirectional exchange between the PC and the acyl-CoA pool. Examples of LPCAT have been functionally characterized from yeast and animals (reviewed in Ståhl *et al.*, 2008). However, in all cases it was only the forward reaction that was demonstrated (i.e. acyl-CoA-dependent acylation of lyso-PC, e.g. X. Chen *et al.*, 2006; Nakanishi *et al.*, 2006). The identification of a plant or algal form of LPCAT remains elusive, and clear evidence for the role of the reverse reaction (release of a FA 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-PUFAs in plants is required. Other opportunities for manipulating acyl exchange are being identified as our knowledge of fundamental lipid metabolism improves. An example of this is the recent 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.

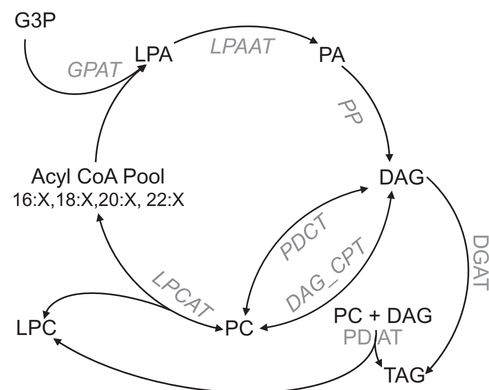

Fig. 2. A schematic representation of the main lipid classes and biochemical pathways involved in the production of triacylglycerols. The acyl-CoA pool is shown as the central route for TAG synthesis; however, it is clear that fatty acids are routed through PC/DAG and directly from PC; the influence of each route varying with species. The primary activities (see www. arabidopsisacyllipids.plantbiology.msu.edu/pathways for a full description of *Arabidopsis* acyl lipid metabolism) are: glycerol-3-phosphate acyltransferase (GPAT); 1-acylglycerol-3-phosphate acyltransferase (LPAAT); phosphatidate phosphatase (PP); phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT); diacylglycerol cholinephosphotransferase (DAG-CPT); acyl-CoA:diacylglycerol acyltransferase (DGAT); phospholipid:diacylglycerol acyltransferase (PDAT); and 1-acylglycerol-3-phosphocholine acyltransferase (LPCAT).

A further iteration of the production VLC-PUFAs in oil seeds was the reconstitution of the alternative Δ8-desaturase pathway (Qi *et al.*, 2004). *Arabidopsis* plants were sequentially transformed with genes expressing a Δ9-elongase from the alga *Isochrysis galbana*, a Δ8-desaturase from the protist *Euglena gracilis*, and a fungal Δ5-desaturase from *M. alpina*; all under the control of the constitutive 35S promoter. The accumulation of 6.6% ARA and 3.0% EPA in total lipids of leaf tissues represented a 'proof-of-concept' demonstration for the plant synthesis of VLC-PUFAs and for the functionality 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 FAs from the acyl-CoA pool into extraplastidial phospholipids (Fraser *et al.*, 2004; Sayanova *et al.*, 2006*a*).

Downloaded from https://academic.oup.com/jxb/article/63/7/2397/515583 by U.S. Department of Justice user on 16 August 2022

The seed-specific expression of the alternative pathway would allow proper evaluation of the efficacy of this route to VLC-PUFAs.

As the possibility of producing VLC-PUFAs in plants has developed, the influence of the host crop has become more apparent. Cheng *et al.* (2010) investigated the effects of different host species, genes, and promoters on EPA biosynthesis using the conventional pathway. 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; GLA and SDA averaged 26.9% and 5.4% of total FA, respectively.

Only once an appreciable level of EPA production in seeds was possible could the production of DHA be realistically attempted. The first report of DHA synthesis in oilseeds was carried out by Kinney *et al.* (2004, 2011). They demonstrated in a patent application filed by Dupont the expression of a Δ6-desaturase, a Δ5-desaturase, and a Δ6-elongase from the fungus *M. alpina* in soybean. In this case individual genes were under the control of different seed-specific promoters, and both a ω-3 Δ17-desaturase from *S. diclina* and a ω-3 Δ15-desaturase from *Arabidopsis* were included in constructs to maximize the accumulation of ω-3 VLC-PUFAs by turning 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 an *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 (R. Chen *et al.*, 2006).

Unexpectedly, these embryos also produced up to 4% DPA, the elongation product of EPA, demonstrating that the *M. alpina* elongase had some activity on C_{20} fatty acids when expressed in plants. Subsequently, and in order to produce DHA in somatic embryos, five different cDNAs were co-expressed: the three genes required for the synthesis of EPA, a Δ4-desaturase from *Schizochytrium aggregatum* and a specific Δ5-elongase from *Pavlova* sp. Disappointedly, only 2.0–3.3% of DHA in total FA was achieved; these low yields were presumably a result of a poor acyl exchange between the Δ5-elongase and the Δ4-desaturase.

Reconstitution of the entire DHA synthesis in *B. juncea* using a series of binary vectors containing three, four, five, six, and nine genes—all individually controlled by the same strong seed-specific promoter (Wu *et al.*, 2005)—allowed individual steps of the pathway to be observed. The first construct contained only the minimal set of genes required to produce ARA and EPA: a Δ6-desaturase from the fungus *P. irregulare*, a Δ6-elongase from a moss (*P. 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. *Brassica 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 (average 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.

Latterly more ω-3-desaturases have been identified from a number of fungal species (Damude *et al.*, 2008). In particular, fungi have been a source of bifunctional Δ12- and Δ15-desaturases, for example *Fusarium moniliforme* (Damude *et al.*, 2006), *Acanthamoeba castellanii* (Sayanova *et al.*, 2006*b*), *Claviceps purpurea* (Meesapyodsuk *et al.*, 2007), and *Coprinus cinereus* (Zhang *et al.*, 2007). The co-expression of a Δ12/Δ15-desaturase from *F. moniliforme* with the primary VLC-PUFA biosynthetic enzymes has shown an appreciable increase in the levels of EPA in yeast (*Yersinia lipolytica*) and plants (soybean) (Damude *et al.*, 2006), opening the door to higher yields of ω-3 LC-PUFAs. An alternative option would be the inclusion of a VLC-PUFA desaturase with strong preferences for ω-3 substrates, as seen in *Mantoniella squamata*, *Primula*, and *Echium* (Sayanova *et al.*, 2003; Garcia-Maroto *et al.*, 2006; Hoffmann *et al.*, 2008). The expression of a *Primula* Δ6-desaturase in linseed seeds has already shown a high accumulation of SDA (13.4% of FA) without the concomitant accumulation of GLA in the triacylglycerol (TAG) of seeds (Ruiz-Lopez *et al.*, 2009).

In attempting the synthesis of DHA in *B. juncea* seeds, 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. The roles of acyltransferases on FA composition through substrate preferences have been discussed (e.g. Snyder *et al.*, 2009) and it is conceivable that the acyltransferase from a DHA-rich organism might improve the exchange between different pools of intermediate substrates. However, these experiments could not prove if the acyltransferase activity contributed to the increased synthesis of VLC-PUFAs. The transgenic seeds showed an accumulation of 8.1% (the highest value was 15% EPA); however, there was a poor conversion of EPA into DPA

Downloaded from https://academic.oup.com/jxb/article/63/7/2397/515583 by U.S. Department of Justice user on 16 August 2022

which was rate limiting for the Δ4-desaturase producing DHA (the highest recorded value was 1.5% DHA of total FAs). In order to have a high and uniform expression of all genes, the same strong seed-specific promoter was used for each gene. However, the levels of FA went down in the following generations (X. Qiu *et al.*, unpublished data), indicating that the presence of many copies of the same promoter sequence could be causing some silencing of the genes (also reported by R. Chen *et al.*, 2006). Most recently, an increase in the oil content in *Arabidopsis* seeds has been demonstrated by the individual expression of two LPAT sequences from *B. napus* (Maisonneuve *et al.*, 2010). It was predicted that such increases in TAGs resulted from the increased flux of acyl-chains. The potential of specific acyltransferases to improve the production of VLC-PUFAs in plants remains unclear because acyl-channelling is the sum of multiple and different acyl exchange activities and it is highly likely that each plant species has a different combination of such activities.

Other available options might include the use of an acyl-CoA-independent enzyme such as a phospholipid:diacylglycerol acyltransferase or PDAT (Dahlqvist *et al.*, 2000), which catalyses the transfer of acyl groups from the *sn*-2 position of the major phospholipids to diacylglycerols (DAGs), and ultimately forming TAGs and lyso-phospholipids. Several genes for PDAT activity have been identified in plants; *Arabidopsis* contains several PDAT-like genes, including one (PDAT1) that is expressed only during seed development. Although PDAT1 has been shown to have a role (in combination with a diacylglycerol acyltransferase; DGAT1) in *Arabidopsis* seed oil biosynthesis (Zhang *et al.*, 2009), it is still unknown if this enzyme does play a major quantitative or qualitative role in VLC-PUFA seed accumulation. A DAGAT2 from castor (*Ricinus communis*) may offer potential for manipulation of the TAG pool (Burgal *et al.*, 2008), but perhaps the search for TAG biosynthetic enzymes with affinity for VLC-PUFAs should focus on lower eukaryotes with the desired activities. Finally, the discovery of a new enzyme, phosphatidylcholine diacylglycerol cholinephosphotransferase (ROD1) that catalyses a previously unknown reaction, the interconversion exchange between DAG and PC by phosphocholine head group exchange, might provide a new mechanism by which neutral lipids are enriched in VLC-PUFAs (Lu *et al.*, 2009).

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} PUFAs). 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 T_1 plants. The authors suggested that the use of an acyl-CoA-dependent 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} PUFAs. 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 *M. squamata* Seed-specific promoters were used to co-express these enzymes with the Δ6-elongase PSE1 from 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 TAGs (10.7% in total FAs) using a putative acyl-CoA-dependent Δ6-desaturase with strong ω-3 preference from the marine microalga *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-PUFAs such as EPA in leaf tissue. This new approach could certainly enhance our capacity to identify some optimal combinations of FA biosynthetic activities for the production of VLC-PUFAs 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 ω-6 GLA, linked to PC), but the seed levels of target VLC-PUFAs such as EPA and DHA have been disappointingly low (Robert *et al.*, 2005; Hoffmann *et al.*, 2008; Sayanova *et al.*, 2011*b*). 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 if the co-expression of this activity would enhance the activity of the algal acyl-CoA Δ6-desaturases in transgenic plants.

Alongside all these multistep engineering efforts there is the anaerobic PKS-like pathway, which has also been used for DHA production in plants (Metz *et al.*, 2006). *Arabidopsis* seeds accumulated up to 0.8% DHA and 1.7% DPA when four genes were co-expressed in these seeds: the three subunits (ORFA, B, and C) of the PKS pathway from *Schizochytrium* with a phosphopantetheinyl transferase (PPT) from *Nostoc*. This PPT activity is essential to activate the acyl carrier protein domains of the PKS. The possibility of producing high levels of EPA or DHA in plants with low levels of intermediates and/or by-products using this pathway would promote their commercialization. Hence, further optimization of this pathway in commercial oilseeds is ongoing.

Downloaded from https://academic.oup.com/jxb/article/63/7/2397/515583 by U.S. Department of Justice user on 16 August 2022

However, the control of gene expression and high levels of enzyme activity in plants may prove tough due to the significant differences between this pathway and the endogenous plant pathway which involve ER-localized fatty acyl chain elongation or desaturation. It also remains to be determined whether there will be problems associated with the export of those novel free FAs from plastids (Metz *et al.*, 2009).

The biosynthesis of EPA and DHA in transgenic plants has been clearly demonstrated by a number of groups; however, the levels of VLC-PUFAs are not as high as those seen in fish oil. The goal now is to generate a vegetable oil substitute for fish oils optimizing the accumulation of VLC-PUFAs and avoiding the accumulation of intermediates and by-products. In order to do this, our understanding of the regulation, organization, and assembly of the elongase complex must improve. The performance of the FAE1-like alternative pathway Δ9-elongase from *P. marinus* (Venegas-Caleron *et al.*, 2007) and finding new FAE-1-like KCS activities might achieve higher elongation of target FAs. Beyond KCS activity, it is possible that the isolation and co-expression of the additional core components of the elongases from suitable EPA- or DHA-accumulating organisms will improve the production of these FAs. It is also important to note that the observed variation in VLC-PUFA levels could be partly due to the different promoters used (R. Chen *et al.*, 2006; Damude *et al.*, 2008), and that the use of promoters whose activity coincides with maximal oil synthesis and accumulation could enhance the overall levels of target VLC-PUFAs. Finally, it is also possible that the lack of a subdomain in the endomembrane system or the lack of co-location of critical activities in the pathway could be another cause of limited production of VLC-PUFAs.

Increasing VLC-PUFA yield in seeds: emerging technology

Much of the discussion above has revolved around approaches to produce effectively high levels of unusual FAs in plants that differ in chain length and desaturation. However, it is becoming apparent that a better understanding of the enzymes and genes involved in seed oil biosynthesis might help circumvent the limitations on the production of unusual FAs. For example, it is now known that acyl-lipid metabolism in *Arabidopsis* requires at least 120 enzymatic reactions and >600 genes to encode the proteins and regulatory factors involved (Li-Beisson *et al.*, 2010). Beyond *Arabidopsis*, host oil crops are poorly characterized at the genomic level; only the analysis of expressed sequenced tag (EST) data from seeds sequestering oil allows the identification of genes actively involved in TAG production (Cahoon and Kinney, 2005). The application of the latest pyrosequencing technology generates huge numbers of ESTs, offering opportunities for gene discovery in FA (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 FA synthesis or DGAT.

As already discussed, the choice of the host crop and, therefore, the metabolic backdrop influences the relative yield from any engineering attempt. Understanding the inter-relationships between lipid metabolites and their pathways is essential to improve VLC-PUFA yield. Yet our understanding of the accumulation of target VLC-PUFAs in storage TAGs is partial; only recently has the application of detailed kinetic analysis to the channelling of novel fatty acids into soybean embryo TAGs revealed the central acyl exchange between PC and the acyl-CoA pool (Bates *et al.*, 2009). Further work showed how the production of unusual FAs, for example hydroxy FAs, in *Arabidopsis* and soybean was limited by the flux of DAGs through PC (Bates and Browse, 2011), but not in castor (utilizing *de novo* DAGs; Bafor *et al.*, 1991). These approaches are set to illuminate the interaction and exchange between lipid pools; a greater appreciation of the different mechanisms of TAG biosynthesis will allow the application of specific engineering strategies to individual host crops.

Labelling studies such as those described above are re-defining the understanding of the inter-relationships between lipid metabolites. Complementary to these focused approaches, the application of mass spectrometry-based techniques and the ability to survey all the major lipid classes involved in oil production comprehensively is proving invaluable in attempts to engineer VLC-PUFA production. Specific methods have been developed for phosopholipids, galactolipids (Devaiah *et al.*, 2006; Xiao *et al.*, 2010), acyl-CoAs (Larson and Graham, 2001), and TAGs (Krank *et al.*, 2007; Clauß *et al.*, 2011). Indeed the application of multireaction monitoring (MRM) using hybrid triple mass spectrometers has enabled the identification of acyl-CoA species that previously co-eluted using conventional chromatographic approaches (Haynes *et al.*, 2008; see Fig. 3A, B). Whereas the typical analysis of FAs from seed oil is limited to ~8 or 10 individual FAs, the number of individual species of phospholipids, TAGs, 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 (Sayonova *et al.*, 2011*b*). This type of characterization allows rational decision making based on how VLC-PUFAs are channelled into TAGs and where the likely bottlenecks and diversions are limiting yield. It is imperative

Downloaded from https://academic.oup.com/jxb/article/63/7/2397/515583 by U.S. Department of Justice user on 16 August 2022

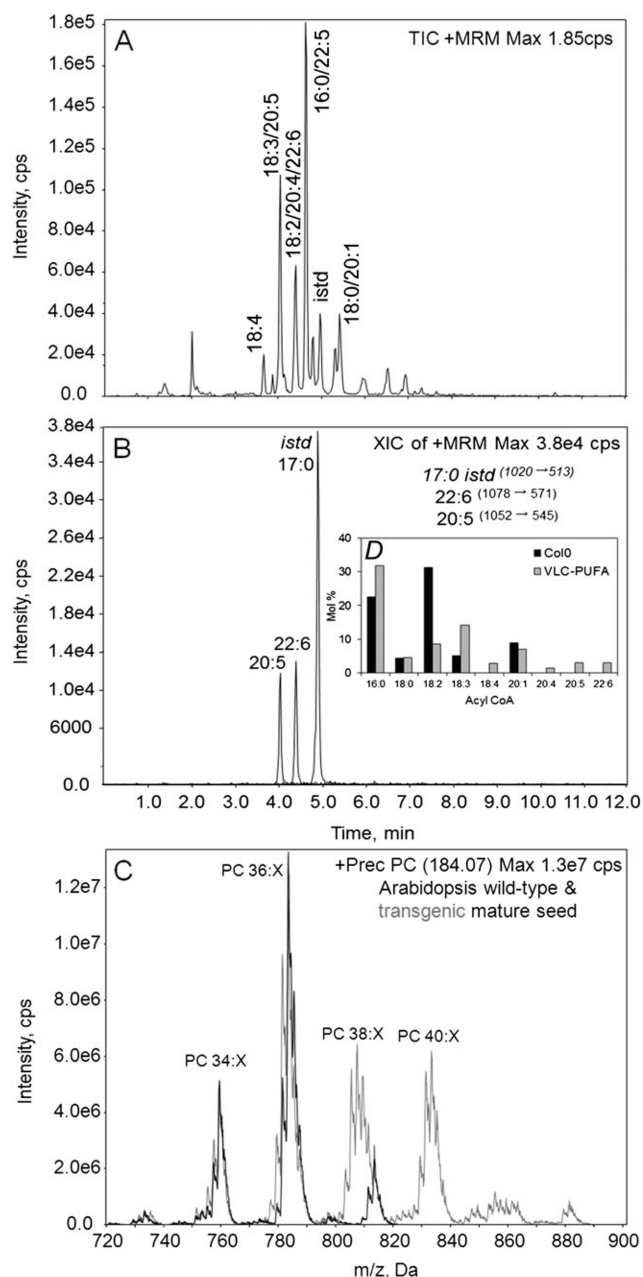

Fig. 3. The application of emerging technology to the analysis of lipids in *Arabidopsis* seed expressing the VLC-PUFA biosynthetic pathway. Firstly the separation and identification of acyl-CoA species by LC-MS/MS. (A) Total ion count for multireaction monitoring (MRM); (B) extracted MRM traces for EPA and DHA allowing the quantification of co-eluting acyl-CoA species (D). The analysis of the acyl-CoA pool allows unambiguous identification of individual acyl-CoA species and therefore the efficacy of both desaturase and elongase genes *in planta* (transgenic Arabidopsis plants are described in Sayanova *et al*., 2011*b*). Secondly the use of ESI-MS/MS systematically to characterize discrete lipid pools enumerates each iterative engineering attempt (C; PC head group positive precursor ion scan for engineered and wild-type mature *Arabidopsis* seed).

to any successful engineering approach that losses of VLC-PUFA substrates and intermediates to multifarious lipid pools are characterized and mitigated.

Conclusion

The successful introduction into plants of the primary biosynthetic enzymes for the production of nutritionally important VLC-PUFAs such as ARA, EPA, and DHA has been amply demonstrated. Equally the numerous engineering iterations that have arisen during this process have highlighted our incomplete appreciation of the pathways of FA synthesis and lipid remodelling. The identification of enzymes that mediate the flux of unusual FAs, for example DGAT2, could unlock the potential of seeds to produce large amounts of high-value FAs. The desire to produce VLC-PUFAs has focused attention on fundamental metabolic processes and the application of new technology to characterize their interaction; further advances in our knowledge of plant lipid biochemistry will undoubtedly occur. Although the target VLC-PUFAs have been produced in crop plants, a plant-based equivalent of marine oil remains a challenge. This is because in seeds producing VLC-PUFAs, the levels of EPA and DHA need to be closer to that found in marine oils, and the accumulation of ω-6 and ω-3 intermediates must decline. Refining the composition of VLC-PUFAs in seeds to obtain a fish oil substitute is the most pressing objective; once achieved, then a viable alternative source to the diminishing natural reserves of fish oils would be available. The benefits of producing VLC-PUFAs in plants are clear: a sustainable and non-contaminated source of important FAs essential to human nutrition. Once a route for a VLC-PUFA trait through performance field trials and regulatory approval is secured, the entry of transgenic VLC-PUFAs into the human food chain is possible. This might occur directly through products formulated with transgene-derived VLC-PUFAs (e.g. yoghurt or margarine) or indirectly, in animal feeds containing transgene-derived VLC-PUFAs (e.g. most obviously in farmed fish fed on a diet of modified terrestrial oils). Whatever route is eventually accepted, transgene-derived VLC-PUFAs have an important role in the sustainability of fish stocks, food security, and human nutrition.

Acknowledgements

This research is funded by the Biotechnology and Biological Sciences Research Council (BBSRC).

References

Abbadi A, Domergue F, Bauer J, Napier JA, 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.

Abeywardena MY, Patten GS. 2011. Role of n-3 long chain polyunsaturated fatty acids in reducing cardio-metabolic risk factors.

Downloaded from https://academic.oup.com/jxb/article/63/7/2397/515583 by U.S. Department of Justice user on 16 August 2022

Endocrine, Metabolic and Immune Disorders Drug Targets **11,** 232–246.

Bafor M, Smith MA, Jonsson L, Stobart K, Stymne S. 1991. Ricinoleic acid biosynthesis and triacylglycerol assembly in microsomal preparations from developing castor bean (*Ricinus communis*) endosperm. *Biochemical Journal* **280,** 507–514.

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

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

Beaudoin F, Michaelson LV, Hey SJ, Lewis MJ, Shewry PR, Sayanova O, Napier JA. 2000. Heterologous reconstitution in yeast of the polyunsaturated fatty acid biosynthetic pathway. *Proceedings of the National Academy of Sciences, USA* **97,** 6421–6426.

Burdge GC. 2006. Metabolism of alpha-linolenic acid in humans. *Prostaglandins, Leukotrienes and Essential Fatty Acids* **75,** 161–168.

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

Cahoon EB, Kinney AJ. 2005. The production of vegetable oils with novel properties: using genomic tools to probe and manipulate fatty acid metabolism. *European Journal of Lipid Science and Technology* **107,** 293–243.

Calder PC. 2004. Fatty acids and cardiovascular disease: evidence explained and mechanisms explored. *Clinical Science* **107,** 1–11.

Chen R, Matsui K, Ogawa M, Oe M, Ochiai M, Kawashima H. 2006. Expression of Δ6, Δ5-desaturase and GLELO elongase genes from *Mortierella alpina* for production of arachidonic acid in soybean [*Glycine max* (L.) Merrill] seeds. *Plant Science* **170,** 399–406.

Chen X, Hyatt BA, Mucenski ML, Mason RJ, Shannon JM. 2006. Identification and characterization of a lysophosphatidylcholine acyltransferase in alveolar type II cells. *Proceedings of the National Academy of Sciences, USA* **103,** 11724–11729.

Cheng BF, Wu GH, 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.

Clauß K, Roepenack-Lahaye E, Böttcher C, Roth MR, Welti R, Erban A, Kopka J, Scheel D, Milkowski C, Strack D. 2011. Over-expression of sinapine esterase BnSCE3 in oilseed rape seeds triggers global changes in seed metabolism. *Plant Physiology* **155,** 1127–1145.

Dahlqvist A, Stahl U, Lenman M, Banas A, Lee M, Sandager L, Ronne H, Stymne S. 2000. Phospholipid:diacylglycerol acyltransferase: an enzyme that catalyzes the acyl-CoA-independent formation of triacylglycerol in yeast and plants. *Proceedings of the National Academy of Sciences, USA* **97,** 6487–6492.

Damude HG, Kinney AJ. 2008. Enhancing plant seed oils for human nutrition. *Plant Physiology* **147,** 962–968.

Damude HG, Zhang H, Farrall L, Ripp KG, Tomb JF, Hollerbach D, Yadav NS. 2006. Identification of bi-functional Δ12/omega3 fatty acid desaturases for improving the ratio of omega-3 to omega-6 fatty acids in microbes and plants. *Proceedings of the National Academy of Sciences, USA* **103,** 9446–9451.

Das T, Thurmond JM, Bobik E, Leonard AE, Parker-Barnes JM, Huang YS, Mukerji P. 2000. Polyunsaturated fatty acid-specific elongation enzymes. *Biochemical Society Transations* **28,** 658–660.

Devaiah SP, Roth MR, Baughman E, Li M, Tamura P, Jeannotte R, Welti R, Wang X. 2006. Quantitative profiling of polar glycerolipid species from organs of wild-type Arabidopsis and a PHOSPHOLIPASE Da1 knockout mutant. *Phytochemistry* **67,** 1907–1924.

Domergue F, Abbadi A, Ott C, Zank TK, Zähringer U, Heinz E. 2003. Acyl carriers used as substrates by the desaturases and elongases involved in very long-chain polyunsaturated fatty acids biosynthesis reconstituted in yeast. *Journal of Biological Chemistry* **278,** 35115–35126.

Domergue F, Abbadi A, Zahringer 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,** 483–490.

Domergue F, Lerchl J, Zahringer U, Heinz E. 2002. Cloning and functional characterization of *Phaeodactylum tricornutum* front-end desaturases involved in eicosapentaenoic acid biosynthesis. *European Journal of Biochemistry* **269,** 4105–4113.

Domingo JL, Bocio A, Falcó G, Llobet JM. 2007. Benefits and risks of fish consumption Part I. A quantitative analysis of the intake of ω-3 fatty acids and chemical contaminants. *Toxicology* **230,** 219–226.

Dyer JM, Stymne S, Green AG, Carlsson AS. 2008. High-value oils from plants. *The Plant Journal* **54,** 640–655.

Eckert H, La Vallee B, Schweiger BJ, Kinney AJ, Cahoon EB, Clemente T. 2006. Co-expression of the Borage Δ6-desaturase and the Arabidopsis Δ15-desaturase results in high accumulation of stearidonic acid in the seeds of transgenic soybean. *Planta* **224,** 1050–1057.

Fehling E, Lessire R, Cassagne C, Mukherjee KD. 1992. Solubilization and partial purification of constituents of acyl-CoA elongase from *Lunaria annua*. *Biochimica et Biophysica Acta* **1126,** 88–94.

Food and Agriculture Organisation of the United Nations. 2010. *The state of world fisheries and aquaculture*. Rome: Food and Agriculture Organisation.

Flider JF. 2005. GLA: uses and new sources. *Inform* **16,** 279–283.

Fraser TCM, Qi B, Elhussein S, Chatrattanakunchai S, Stobart AK, Lazarus CM. 2004. Expression of the isochrysis C_{18}-Δ9 polyunsaturated fatty acid specific elongase component alters arabidopsis glycerolipid profiles. *Plant Physiology* **135,** 859–866.

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

Griffiths G, Stobart AK, Stymne S. 1988. Delta 6- and delta 12-desaturase activities and phosphatidic acid formation in microsomal

Downloaded from https://academic.oup.com/jxb/article/63/7/2397/515583 by U.S. Department of Justice user on 16 August 2022

preparations from the developing cotyledons of common Borage (*Borago officinalis*). *Biochemical Journal* **252,** 641–647.

Guil-Guerrero JL, López-Martínez JC, Navarro-Juárez R, Gómez-Mercado F. 2006. Gamma-linolenic acid from Cape Verdian Boraginaceae. *Natural Products Reearch* **20,** 9–12.

Hammond BG, Lemen JK, Ahmed G, Miller KD, Kirkpatrick J, Fleeman T. 2008. Safety assessment of SDA soybean oil: results of a 28-day gavage study and a 90-day/one generation reproduction feeding study in rats. *Regulatory Toxicology and Pharmacology* **52,** 311–323.

Harwood JL. 1988. Fatty acid metabolism. *Annual Review of Plant Physiology and Plant Molecular Biology* **39,** 101–138.

Haynes CA, Allegood JC, Sims K, Wang EW, Sullards CM, Merrill AH. 2008. Quantitation of fatty acyl-coenzyme A in mammalian cells by liquid chromatography-electrospray ionization tandem mass spectrometry. *Journal of Lipid Research* **49,** 1113–1125.

Hoffmann M, Wagner M, Abbadi A, Fulda M, Feussner I. 2008. Metabolic engineering of ω-3 very long chain polyunsaturated fatty acid production by an exclusively acyl-CoA-dependent pathway. *Journal of Biological Chemistry* **283,** 22352–22362.

Hong H, Datla N, Reed DW, Covello PS, MacKenzie SL, Qiu X. 2002. High-level production of γ-linolenic acid in *Brassica juncea* using a Δ6 desaturase from *Pythium irregulare*. *Plant Physiology* **129,** 354–362.

Huang YS, Pereira SL, Leonard AE. 2004. Enzymes for transgenic biosynthesis of long-chain polyunsaturated fatty acids. *Biochimie* **86,** 793–798.

Jackson FM, Fraser TC, Smith MA, Lazarus C, Stobart AK, Griffiths G. 1998. Biosynthesis of C_{18} polyunsaturated fatty acids in microsomal membrane preparations from the filamentous *fungus Mucor circinelloides*. *European Journal of Biochemistry* **252,** 513–519.

Jakobsson A, Westerberg R, Jacobsson A. 2006. Fatty acid elongases in mammals: their regulation and roles in metabolism. *Progress in Lipid Research* **45,** 237–249.

James DW, Lim E Jr, Keller J, Plooy I, Ralston E, Dooner HK. 1995. Directed tagging of the Arabidopsis FATTY ACID ELONGATION1 (FAE1) gene with the maize transposon activator. *The Plant Cell* **7,** 309–319.

Khozin-Goldberg I, Iskandarov U, Cohen Z. 2011. LC-PUFA from photosynthetic microalgae: occurrence, biosynthesis, and prospects in biotechnology. *Applied Microbiology and Biotechnology* **94,** 910–915.

Kinney AJ, Cahoon EB, Damude HG, Hitz WD, Kolar CW, Liu ZB. 2004. *Production of very long chain polyunsaturated fatty acids in oilseed plants* International Patent Application WO 2004/071467 A2.

Kinney AJ, Cahoon EB, Damude HG, Liu Z. 2011. *Production of very long chain polyunsaturated fatty acids in oilseed plants* United States Patent Application 20110269983.

Krank J, Murphy R C, Barkley R M, Duchoslav E, McAnoy A. 2007. Qualitative analysis and quantitative assessment of changes in neutral glycerol lipid molecular species within cells. *Methods in Enyzmology* **432,** 1–20.

Larson TR, Graham IA. 2001. A novel technique for the sensitive quantification of acyl CoA esters from plant tissues. *The Plant Journal* **25,** 115–125.

Lees AM, Korn ED. 1966. Metabolism of unsaturated fatty acids in protozoa. *Biochemistry* **5,** 1475–1481.

Li-Beisson Y, Shorrosh B, Beisson F, *et al*., 2010. Acyl-lipid metabolism. *The Arabidopsis book* **8,** e0133. doi:10.1199/tab.0133.

Liu JW, Huang Y-S, DeMichele S, Bergana M, Bobik E, Hastilow C, Chuang LT, Mukerji P, Knutzon D. 2001. Evaluation of the seed oils from a canola plant genetically transformed to produce high levels of γ-linolenic acid. In: Huang YS, Ziboh VA, eds. *γ-Linolenic acid: recent advances in biotechnology and clinical applications*. Champaign, IL: AOCS Press, 61–71.

Lu CF, Xin ZG, Ren ZH, Miquel M, Browse J. 2009. An enzyme regulating triacylglycerol composition is encoded by the ROD1 gene of Arabidopsis. *Proceedings of the National Academy of Sciences, USA* **106,** 18837–18842.

Maisonneuve S, Bessoule JJ, Lessire R, Delseny M, Roscoe TJ. 2010. Expression of rapeseed microsomal lysophosphatidic acid acyltransferase isozymes enhances seed oil content in Arabidopsis. *Plant Physiology* **152,** 670–684.

Meesapyodsuk D, Reed DW, Covello PS, Qiu X. 2007. Primary structure, regioselectivity, and evolution of the membrane-bound fatty acid desaturases of *Claviceps purpurea*. *Journal of Biological Chemistry* **282,** 20191–20199.

Metz JG, Flatt JH, Kuner JM. 2006. *The genes for the enzymes of the polyunsaturated fatty acid polyketide synthase of Schizochytrium and their use in the manufacture of polyunsaturated fatty acids* International Patent Publication WO 2006135866; 2006.

Metz JG, 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.

Metz JG, Roessler P, Facciotti D, *et al*., 2001. Production of polyunsaturated fatty acids by polyketide synthases in both prokaryotes and eukaryotes. *Science* **293,** 290–293.

Meyer A, Kirsch H, Domergue F, *et al*., 2004. Novel fatty acid elongases and their use for the reconstitution of docosahexaenoic acid biosynthesis. *Journal of Lipid Research* **45,** 1899–909.

Meyer BJ. 2011. Are we consuming enough long chain ω-3 polyunsaturated fatty acids for optimal health? *Prostaglandins, Leukotrines and Essential Fatty Acids* **85,** 275–280.

Millar AA, Kunst L. 1997. Very-long-chain fatty acid biosynthesis is controlled through the expression and specificity of the condensing enzyme. *The Plant Journal* **12,** 121–131.

Nakanishi H, Shindou H, Hishikawa D, Harayama T, Ogasawara R, Suwabe A, Taguchi R, Shimizu T. 2006. Cloning and characterization of mouse lung-type acyl-CoA:lysophosphatidylcholine acyltransferase 1 (LPCAT1). Expression in alveolar type II cells and possible involvement in surfactant production. *Journal of Biological Chemistry* **281,** 20140–20147.

Napier JA. 2007. The production of unusual fatty acids in transgenic plants. *Annual Review of Plant Biology* **58,** 295–319.

Napier JA, Graham IA. 2010. Tailoring plant lipid composition: designer oilseeds come of age. *Current Opinion in Plant Biology* **13,** 330–337.

Downloaded from https://academic.oup.com/jxb/article/63/7/2397/515583 by U.S. Department of Justice user on 16 August 2022

Naylor RL, Hardy RW, Bureau DP, *et al*., 2009. Feeding aquaculture in an era of finite resources. *Proceedings of the National Academy of Sciences, USA* **106,** 15103–15110.

Nugent AP. 2004. The metabolic syndrome. *Nutrition Bulletin* **29,** 36–43.

Nykiforuk CL, Shewmaker C, Harry I, *et al*., 2011. High level accumulation of gamma linolenic acid (C18:3Δ6.9,12 cis) in transgenic safflower (*Carthamus tinctorius*) seeds. *Transgenic Research* (in press).

Ohlrogge J, Browse J. 1995. Lipid biosynthesis. *The Plant Cell* **7,** 957–970.

Ohlrogge JB, Jaworski JG. 1997. Regulation of fatty acid synthesis. *Annual Review of Plant Physiology and Plant Molecular Biology* **48,** 109–136.

Parker-Barnes JM, Das T, Bobik E, Leonard AE, Thurmond JM, Chaung LT, Huang YS, Mukerji P. 2000. Identification and characterization of an enzyme involved in the elongation of n-6 and n-3 polyunsaturated fatty acid. *Proceedings of the National Academy of Sciences, USA* **97,** 8284–8289.

Paul S, Gable K, Beaudoin F, Cahoon E, Jaworski J, Napier JA, Dunn TM. 2006. Members of the Arabidopsis FAE1-like 3-ketoacyl-CoA synthase gene family substitute for the Elop proteins of *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **281,** 9018–9029.

Pereira SL, Leonard AE, Huang YS, Chuang LT, Mukerji P. 2004. Identification of two novel microalgal enzymes involved in the conversion of the omega-3-fatty acid, eicosapentaenoic acid, into docosahexaenoic acid. *Biochemical Journal* **384,** 357–366.

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

Plourde M, Cunnane SC. 2007. Extremely limited synthesis of long chain polyunsaturates in adults: implications for their dietary essentiality and use as supplements. *Applied Physiology, Nutrition, and Metabolism* **32,** 619–634.

Poudyal H, Panchal SK, Diwan V, Brown L. 2011. Omega-3 fatty acids and metabolic syndrome: effects and emerging mechanisms of action. *Progress in Lipid Research* **50,** 372–387.

Qi B, Beaudoin F, Fraser T, Stobart AK, Napier JA, Lazarus CM. 2002. Identification of a cDNA encoding a novel C_{18} Δ-9 polyunsaturated fatty acid-specific elongating activity from the docosahexaenoic acid (DHA)-producing microalga, *Isocarysis galbana*. *FEBS Letters* **510,** 159–165.

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

Qiu X, Hong H, Datla N, MacKenzie SL, Taylor DC, Thomas TL. 2002. Expression of Borage Δ6-desaturase in *Saccharomyces cerevisiae* and oilseed crops. *Canadian Journal of Botany* **80,** 42–49.

Qiu X, Reed DW, Hong H, MacKenzie SL, Covello PS. 2001. Identification and analysis of a gene from *Calendula officinalis* encoding a fatty acid conjugase. *Plant Physiology* **125,** 847–855.

Reddy AS, Thomas TL. 1996. Expression of a cyanobacterial Δ6-desaturase gene results in gamma-linolenic acid production in transgenic plants. *Nature Biotechnology* **14,** 639–642.

Robert S, Singh SP, Zhou XR, Petrie JR, Blackburn SI, Mansour PM, Nichols PD, Liu Q, Green A. 2005. Metabolic engineering of Arabidopsis to produce nutritionally important DHA in seed oil. *Functional Plant Biology* **32,** 473–479.

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' ω-3 fatty acids. *Plant Biotechnology Journal* **7,** 704–716.

Sakuradani E, Ando A, Ogawa J, Shimizu S. 2009. Improved production of various polyunsaturated fatty acids through filamentous fungus *Mortierella alpina* breeding. *Applied Microbiology and Biotechnology* **84,** 1–10.

Sasaki Y, Nagano Y. 2004. Plant acetyl-CoA carboxylase: structure, biosynthesis, regulation, and gene manipulation for plant breeding. *Bioscience, Biotechnology and Biochemistry* **68,** 1175–1184.

Sayanova OV, Beaudoin F, Michaelson LV, Shewry PR, Napier JA. 2003. Identification of Primula fatty acid Δ6-desaturases with n-3 substrate preferences. *FEBS Letters* **542,** 100–104.

Sayanova O, Haslam RP, Calerón MV, López NR, Worthy C, Rooks P, Allen MJ, Napier JA. 2011*a*. Identification and functional characterisation of genes encoding the omega-3 polyunsaturated fatty acid biosynthetic pathway from the coccolithophore *Emiliania huxleyi*. *Phytochemistry* **72,** 594–600.

Sayanova O, Haslam RP, Guschina I, Lloyd D, Christie WW, Harwood JL, Napier JA. 2006*b*. A bi-functional Δ12, 15-desaturase from *Acanthamoeba castellanii* directs the synthesis of highly unusual n-1 series unsaturated fatty acids. *Journal of Biological Chemistry* **281,** 36533–36541.

Sayanova O, Haslam RP, Qi B, Lazarus CM, Napier JA. 2006*a*. The alternative pathway C_{20} Δ8-desaturase from the non-photosynthetic organism *Acanthamoeba castellanii* is an atypical cytochrome b5-fusion desaturase. *FEBS Letters* **580,** 1946–1952.

Sayanova OV, Napier JA. 2004. Eicosapentaenoic acid: biosynthetic routes and the potential for synthesis in transgenic plants. *Phytochemistry* **65,** 147–158.

Sayanova O, Ruiz-Lopez N, Haslam RP, Napier JA. 2011*b*. The role of Δ6-desaturase acyl-carrier specificity in the efficient synthesis of long-chain polyunsaturated fatty acids in plants. *Plant Biotechnology Journal* (in press).

Sayanova O, Smith MA, Lapinskas P, Stobart AK, Dobson G, Christie WW, Shewry PR, Napier JA. 1997. Expression of a *B. officinalis* desaturase cDNA containing an N-terminal cytochrome b5 domain results in the accumulation of high levels of Δ6-desaturated fatty acids in transgenic tobacco. *Proceedings of the National Academy of Sciences, USA* **94,** 4211–4216.

Smith ADM, Brown CJ, Bulman CM, *et al*., 2011. Impacts of fishing low-trophic level species on marine ecosystems. *Science* **333,** 1147–1150.

Snyder CL, Yurchenko OP, Siloto RMP, Chen X, Liu Q, Mietkiewsk E, Weselake RJ. 2009. Acyltransferase action in the modification of seed oil biosynthesis. *New Biotechnology* **26,** 11–16.

Downloaded from https://academic.oup.com/jxb/article/63/7/2397/515583 by U.S. Department of Justice user on 16 August 2022

Somerville C, Browse J. 1991. Plant lipids: metabolism, mutants and membranes. *Science* **252,** 80–87.

Sprecher H, Chen Q, Yin FQ. 1999. Regulation of the biosynthesis of 22:5 n-6 and 22:6 n-3: a complex intracellular process. *Lipids* **34,** 153–156.

Ståhl U, Stålberg K, Stymne S, Ronne H. 2008. A family of eukaryotic lysophospholipid acyltransferases with broad specificity. *FEBS Letters* **582,** 305–309.

Stymne S, Appelqvist LA. 1978. The biosynthesis of linoleate from oleoyl-CoA via oleoyl-phosphatidylcholine in microsomes of developing safflower seeds. *European Journal of Biochemistry* **90,** 223–229.

Tacon AGI, Metian M. 2009. Fishing for aquaculture: nonfood use of small pelagic forage fish, a global perspective. *Review of Fish Science* **17,** 305–317.

Takeyama H, Takeda D, Yazawa K, Yamada A, Matsunaga T. 1997. Expression of the eicosapentaenoic acid synthesis gene cluster from *Shewanella* sp. in a transgenic marine cyanobacterium, *Synechococcus* sp. *Microbiology* **143,** 2725–2731.

Tocher D. 2009. Issues surrounding fish as a source of ω-3 long chain polyunsaturated fatty acids. *Lipid Technology* **21,** 13–16.

Troncoso-Ponce MA, Kilaru A, Cao X, Durrett TP, Fan J, Jensen JK, Thrower NA, Pauly M, Wilkerson C, Ohlrogge JB. 2011. Comparative deep transcriptional profiling of four developing oilseeds. *The Plant Journal* **68,** 1014–1027.

Ulsamer AG, Smith FR, Korn ED. 1969. Lipids of *Acanthamoeba castellanii*. Composition and effects of phagocytosis on incorporation of radioactive precursors. *Journal of Cell Biology* **43,** 105–114.

Venegas-Calerón M, Beaudoin F, Sayanova O, Napier JA. 2007. Cotranscribed genes for long chain polyunsaturated fatty acid biosynthesis in the protozoon *Perkinsus marinus* contain a plant-like FAE1 3-ketoacyl-CoA synthase. *Journal of Biological Chemistry* **282,** 2996–3000.

Wallis JG, Browse J. 1999. The Δ8-desaturase of *Euglena gracilis*: an alternate pathway for synthesis of 20-carbon polyunsaturated fatty acids. *Archives of Biochemistry and Biophysics* **365,** 307–316.

Williams CM, Burdge G. 2006. Long-chain n-3 PUFA: plant v. marine sources. *Proceedings Nutritional Society* **65,** 42–50.

Wood CC, Petrie JR, Shrestha P, Mansour MP, Nichols PD, Green AG, Singh SP. 2009. A leaf-based assay using interchangeable design principles to rapidly assemble multistep recombinant pathways. *Plant Biotechnolgy Journal* **7,** 914–924.

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.

Xiao S, Gao W, Chen Q-F, Chan S-W, Zheng S-X, Ma J, Wang M, Welti R, Chye M-L. 2010. Overexpression of Arabidopsis acyl-CoA binding protein ACBP3 promotes starvation-induced and age-dependent leaf senescence. *The Plant Cell* **22,** 1463–1482.

Yazawa K. 1996. Production of eicosapentaenoic acid from marine bacteria. *Lipids* **31,** Suppl, S297–300.

Yurchenko OP, Nykiforuk CL, Moloney MM, Stahl U, Banas A, Stymne S, Weselake RJ. 2009. A 10-kDa acyl-CoA-binding protein (ACBP) from *Brassica napus* enhances acyl exchange between acyl-CoA and phosphatidylcholine. *Plant Biotechnology Journal* **7,** 602–610.

Zank TK, Zähringer U, Beckmann C, Pohnert G, Boland W, Holtorf H, Reski R, Lerchl J, Heinz E. 2002. Cloning and functional characterisation of an enzyme involved in the elongation of Δ6-polyunsaturated fatty acids from the moss *Physcomitrella patens*. *The Plant Journal* **31,** 255–268.

Zhang M, Fan JL, Taylor DC, Ohlrogge JB. 2009. DGAT1 and PDAT1 acyltransferases have overlapping functions in Arabidopsis triacylglycerol biosynthesis and are essential for normal pollen and seed development. *The Plant Cell* **21,** 3885–3901.

Zhang S, Sakuradani E, Ito K, Shimizu S. 2007. Identification of a novel bifunctional delta12/delta15 fatty acid desaturase from a basidiomycete, *Coprinus cinereus* TD#822-2. *FEBS Letters* **581,** 315–319.

Zhou XR, Horne I, Damcevski K, Haritos V, Green A, Singh S. 2008. Isolation and functional characterization of two independently-evolved fatty acid Δ12-desaturase genes from insects. *Insect Molecular Biology* **17,** 667–676.

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

Downloaded from https://academic.oup.com/jxb/article/63/7/2397/515583 by U.S. Department of Justice user on 16 August 2022