

## Molecules of Interest

## Eicosapentaenoic acid: biosynthetic routes and the potential for synthesis in transgenic plants

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Accepted 9 October 2003

This review is dedicated to the memory of Dr. David Horrobin (1939–2003).

**Abstract**

Long chain polyunsaturated fatty acids are now known to play important roles in human health. In particular, eicosapentaenoic acid ( $20:5\Delta^{5,8,11,14,17}$ ;  $n-3$ : EPA) is implicated as a protective agent in a range of pathologies such as cardiovascular disease and Metabolic Syndrome (Syndrome X). Eicosapentaenoic acid is currently sourced from fish oils, the presence of this fatty acid being due to the dietary piscine consumption of EPA-synthesising micro-algae. The biosynthetic pathway of EPA has been elucidated, and contains several alternative metabolic routes. Progress in using “reverse engineering” to transgenically mobilize the trait(s) for EPA are considered. In particular, the prospect of producing this important polyunsaturated fatty acid in transgenic oilseeds is highlighted, as is the urgent need for a sustainable replacement for diminishing fish stocks.

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**Keywords:** Polyunsaturated fatty acids; Eicosapentaenoic acid; Metabolic engineering; Metabolic Syndrome; Plant biotechnology

**1. Introduction**

Animal cell membranes primarily consist of a bilayer of phospholipids and cholesterol with imbedded proteins that act as receptors, transporters and enzymes. The phospholipid fatty acid composition determines the physical and functional properties of cell membranes and has important implications for cell integrity and growth, inflammation and immunity. This is defined by the fatty acids esterified to the glycerol backbones, with chain length and unsaturation acting as key determinants (Broun et al., 1999). The role(s) of 20-carbon ( $C_{20}$ ) polyunsaturated fatty acids (PUFAs) with methylene-interrupted double bonds have been the recent focus of intensive research on fatty acid functionality (Napier et al., 1999, 2003; Gill and Valivety, 1997). For the purpose of this review, PUFAs are defined as containing three or more double bonds on a fatty acid chain of 18 or more carbons. PUFAs can be further classified into two families ( $n-6$  or  $n-3$ ), depending on the position of the last double bond proximal to the methyl end of the fatty acid. These  $n-6$  and  $n-3$  fatty acids (also some-

times called omega-3 and omega-6 fatty acids) are derived from the essential fatty acids (EFA) linoleic acid (LA,  $18:2\Delta^{9,12}$ ) and  $\alpha$ -linolenic acid (ALA,  $18:3\Delta^{9,12,15}$ ), respectively. Both of these precursor fatty acids are synthesized by plants, but not mammals; therefore they are essential dietary components of all mammals (Groff et al., 1995) and hence their designation as EFAs. Through a series of acyl desaturation and elongation reactions, LA is metabolized to arachidonic acid (AA,  $20:4\Delta^{5,8,11,14}$ ;  $n-6$ ) and ALA is metabolized to eicosapentaenoic acid (EPA,  $20:5\Delta^{5,8,11,14,17}$ ;  $n-3$ ) and docosahexaenoic acid (DHA,  $22:6\Delta^{4,7,10,13,16,19}$ ). Thus the  $n-6$  (LA) and  $n-3$  (ALA) EFAs yield two different distinct classes of  $C_{20+}$  PUFAs and these two families are not normally interconvertible. This is highlighted by the fact that they are metabolically and functionally distinct, having opposing physiological functions. PUFAs play key roles in cellular and tissue metabolism, including the regulation of membrane fluidity, electron and oxygen transport, as well as thermal adaptation. They are also implicated in prevention and modulation of certain pathological conditions such as obesity and cardiovascular diseases which now appear common in Western society.

Whilst most mammals have a capacity to synthesise  $C_{20+}$  PUFAs from the dietary intake of the precursor

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fatty acids LA and ALA, higher plants in general lack this metabolic pathway. A few taxonomically-unrelated higher plants have the capacity to synthesis  $\Delta^6$ - and  $\Delta^5$ -desaturated fatty acids, though it seems unlikely that this is evolutionarily related to mammalian  $C_{20}$  PUFA biosynthesis. Another dietary source of  $C_{20}+$  PUFAs is delivered by some aquatic micro-organisms which have very active biosynthetic pathways for these lipids. Such marine micro-organisms are consumed by fish and so redistribute the  $C_{20}+$  PUFAs up the food-web, culminating in the accumulation of fish oils rich in these fatty acids.

## 2. PUFAs in human health

### 2.1. PUFA metabolites

From the medical point of view, the most important function of PUFAs is that they are central to the biosynthesis of a class of compounds termed eicosanoids (i.e. metabolites of eicosa [ $C_{20}$ ] PUFAs), serving as precursors for these hormone-like regulatory molecules. The eicosanoids consist of prostaglandins (PGs) and thromboxanes (TXs), which are collectively identified as prostanoids, and the leukotrienes (LTs). These compounds perform a number of essential physiological functions including regulation of the immune system, blood clotting, neurotransmission and cholesterol metabolism (Funk, 2001). The eicosanoids can be formed when physical or chemical insults result in the release of PUFAs from their phospholipid backbone (through the action of phospholipases) and subsequent oxygenation by local oxygenase enzymes. The type of eicosanoids produced (and hence the body's responses) are determined by multiple factors including: cell-type stimulated (platelets, leucocytes and endothelial cells), oxygenase enzymes present (cyclo-oxygenase versus lipoxygenase) and the actual levels of substrate  $C_{20}$  PUFAs in the cell membrane. Eicosanoids derived from  $n-6$  fatty acids have different metabolic properties compared to those derived from  $n-3$  fatty acids. In general, eicosanoids are classified into several different groups, depending on functionality. For example, series-1 and series-3 which are anti-inflammatory whereas series-2 is pro-inflammatory (see Fig. 1). Eicosanoids derived from the 20:4  $n-6$  AA are generally pro-inflammatory, pro-aggregatory and immuno-active (Hwang, 2000). In contrast, eicosanoids derived from 20:5  $n-3$  EPA have little or no inflammatory activity and act to modulate platelet aggregation and immune-reactivity (Funk, 2001). Thus, there is considerable interest in the "positive" (e.g. anti-inflammatory) effects of  $n-3$  (e.g. EPA) derived eicosanoids.

The synthesis of these two families ( $n-6$  and  $n-3$ ) of  $C_{20}$  PUFAs is mediated by the same enzymes which generally appear to have no particular preference for

substrate. Thus, the balance in intake of  $n-6$  and  $n-3$  fatty acids will therefore determine the types and amounts of eicosanoids in the body and so, influence the strength of the inflammatory response. Consequently, manipulation of the fatty acid composition of cell membranes can theoretically modify the inflammatory, immune and aggregatory responses of tissues, though this understates the complexity of cellular homeostasis. This concept provides the basis for the use of  $n-3$  PUFAs as therapeutic agents in the treatment of chronic inflammatory conditions such as rheumatoid arthritis, asthma, psoriasis and Crohn's disease.  $N-3$  fatty acids may also be involved in the development of non-insulin dependent diabetes as it was shown that a diet low in these fatty acids may favour the development of insulin resistance (Browning, 2003). It is for this reason that dietary intake of  $n-3$  PUFAs is considered protective from Metabolic Syndrome (Clarke, 2001; Groop, 2000). Metabolic Syndrome is a multi-component disorder characterised by reduced insulin sensitivity, alterations in circulatory lipids, hypertension and abdominal obesity, conveying an increased risk to cardiovascular disease. Thus, there is considerable interest in the therapeutic role of  $n-3$  PUFAs as intervention agents in the prevention and treatment of this disorder.

As mentioned above, the PUFA composition of cell membranes is, to great extent, dependent on dietary intake. The typical Western diet is relatively deficient in  $n-3$  fatty acids compared to the diets of our ancestors. Today the ratio of  $n-6$ – $n-3$  EFAs in modern diets is about 25:1, and when compared with a likely ancestral dietary ratio of <2:1, indicative of a current deficiency in  $n-3$  fatty acids (Simopoulos, 1991, 2000). The balance between the intakes of  $n-6$  and  $n-3$  fatty acids has been suggested to be more important than levels of intake of individual fatty acids for homeostasis and has been postulated to lead to decreases in many chronic diseases and improvement in mental health (Horrobin et al., 2002).

### 2.2. Omega-3 PUFAs

There are two key  $n-3$  fatty acids readily used by the body: EPA and DHA; DHA (docosahexaenoic acid, 22:6,  $n-3$ ) is synthesised directly from EPA as discussed below. These PUFAs are highly concentrated in the brain and appear to be particularly important for cognitive and behavioural function; DHA is also found in retinal cells and is likely to play a key role in the acquisition and maintenance of ocular vision (Uauy et al., 2001). Lipid-lowering effects, along with some benefits in reducing platelet aggregation and clotting potential, make  $C_{20}+$   $n-3$  PUFAs very important in the treatment or prevention of cardiovascular disease symptoms (such as high cholesterol and high blood pressure). The decreased blood viscosity and lower fat levels also help reduce the risk of heart attacks. The mild anti-inflammatory effects,

## Metabolic Pathways of Essential Fatty Acids

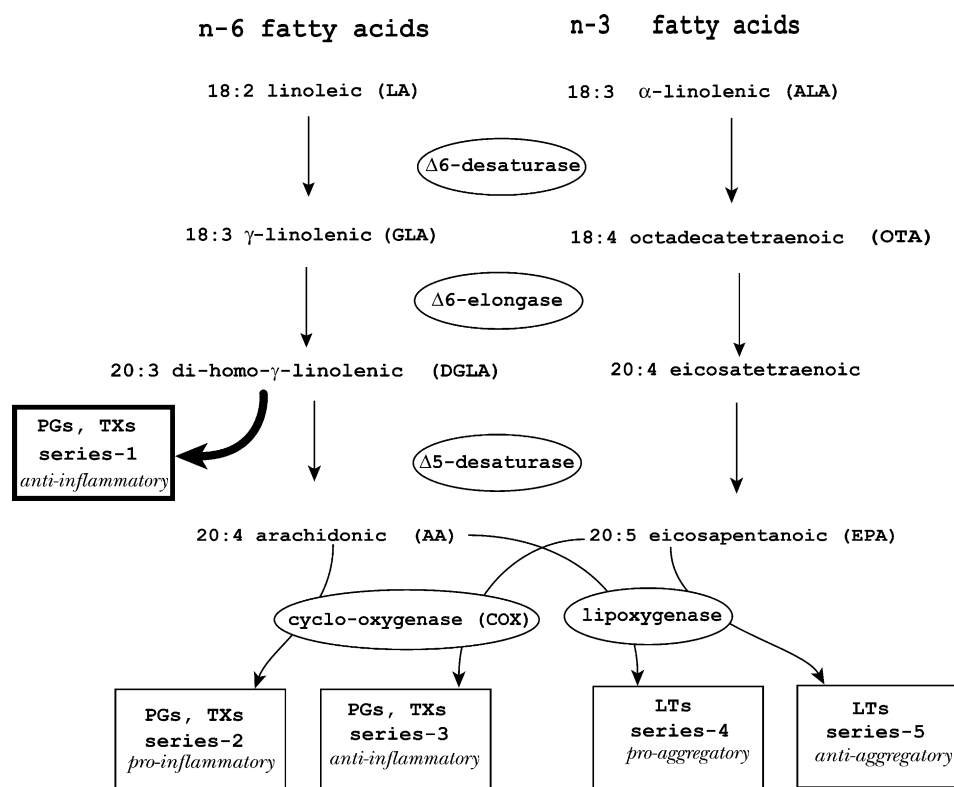


Fig. 1. A simplified scheme of PUFA biosynthesis and eicosanoid metabolism. The two classes (*n*-6, *n*-3) of C<sub>20</sub> PUFAs are synthesised by desaturation and elongation. Oxygenation of these fatty acids by cyclo-oxygenases and lipoxygenases result in the formation of different types of eicosanoids (prostaglandins, PGs; thromboxanes, TXs; leukotrienes, LTs).

possibly a result of increased PG-E1 and PG-E3 prostaglandins, may be helpful in the treatment of arthritis and other inflammatory diseases. In rheumatoid arthritis EPA/DHA supplementation has been shown to reduce joint stiffness and soreness and to improve flexibility. Thrombotic disorders are currently treated and prevented by utilising pharmacological concentrations of EPA (Holman, 1986).

There are also a number of postulated roles for EPA which, whilst of great potential interest, are currently less-well defined. For example, the role of EPA in neurological disorders such as schizophrenia and depression; several studies have shown those with schizophrenia often have low levels of the particular EFAs necessary for normal nerve cell membrane metabolism. Similar to diabetes, people with schizophrenia may not be able to convert efficiently ALA to EPA or DHA (Horrobin, 1999). Studies suggested that patients with schizophrenia and depression experience a sustained clinical improvement in symptoms when given EPA supplements (Edwards et al., 1998; Laugharne et al., 1996), though a recent report questioned the efficacy of PUFAs in schizophrenia (Hibbeln et al., 2003). There are also a number of preliminary studies which link

consumption of *n*-3 PUFAs with the development of both infant and adult IQ (Suzuki et al., 2001).

At present the only significant direct human dietary sources of EPA and DHA are cold water fish such as cod, tuna and mackerel. However, EPA and DHA occur widely in many unicellular protist species, especially those of marine origin. EPA comprises up to 25% of the total fatty acids of *Eustigmatophytes* and DHA accounts for up to 11% of the total lipids of *Prymnesiophytes* (Brown, 2002). Some fungi, mosses and bacteria also synthesize significant amounts of EPA and DHA; however, higher plants (the major dietary source of fatty acids) rarely contain PUFAs with more than 18 carbon atoms. According to current opinion, *n*-3 PUFAs are synthesised by the microscopic algae and plankton at the bottom of the marine food chain. They are then passed up the food chain (via consumption by omnivorous and subsequently carnivorous fish species), ultimately to humans. However, it has become evident that EPA/DHA from marine sources no longer represents a sustainable resource. In the case of fish (and their derived oils) produced by aquaculture, it is clear that the use of vegetable oil feed rich in *n*-6 C<sub>18</sub> PUFAs only result in a product rich in *n*-6 fatty acids; this is

clearly not the desired outcome and also generate an EPA-deficiency in the animals. The precipitous decline of European marine fish stocks is well documented, though it is perhaps not appreciated that this not only included primary fish species such as cod, but also so called “trash” species (such as sand eels) which are also utilized (as *n*-3-containing feedstuff) for aquaculture (Sargent and Tacon, 1999). Thus, there is a clear technological push for the development and deployment of a safe, sustainable and cheap alternative source of *n*-3 PUFAs for human health and nutrition. Currently, some microalgal species are cultivated as sources of these fatty acids. However, transgenic oilseed crops engineered to produce EPA and DHA could provide an alternative sustainable source of these important fatty acids (Abbadì et al., 2001). It is for all these reasons that there is great interest in genes encoding enzymes of the PUFA biosynthetic pathway and considerable effort has been expended in their identification. Surprisingly (as outlined below), there is an unexpected diversity in the synthesis of  $C_{20}+$  PUFAs by different organisms.

### 3. Routes for EPA biosynthesis

#### 3.1. Biosynthetic pathways

At present, several alternative pathways for PUFAs biosynthesis resulting in the formation of EPA and DHA have been investigated and genes encoding key enzymatic reaction have been identified (summarised in Fig. 2 and Table 1). The major, or “conventional” aerobic pathway which operates in most (PUFA-synthesising) eukaryotic organisms, starts with  $\Delta^6$  desaturation of both 18:2 *n*-6 and 18:3 *n*-3 resulting in the synthesis of  $\gamma$ -linolenic (GLA, 18:3  $\Delta^{6,9,12}$ ) and octadecatetraenoic (OTA; 18:4  $\Delta^{6,9,12,15}$ ) acids, respectively. This first desaturation step is followed by  $\Delta^6$ -specific  $C_2$  elongation to 20:3  $\Delta^{8,11,14}$  and 20:4  $\Delta^{8,11,14,17}$  and further  $\Delta^5$ -desaturation to produce AA and EPA. From this point, the biosynthesis of DHA may follow two pathways. These are the linear pathway, involving  $C_2$  elongation of EPA to  $C_{22}:5 \Delta^{7,10,13,16,19}$  which is desaturated by  $\Delta^4$ -specific desaturase to yield DHA, and the so-called “Sprecher” pathway which is independent of  $\Delta^4$ -desaturation but involves two consecutive  $C_2$  elongation cycles to yield 24:5  $\Delta^{7,10,13,16,19}$ , followed by  $\Delta^6$ -desaturation and one cycle of  $C_2$ -shortening via  $\beta$ -oxidation in the peroxisome to yield DHA (Sprecher et al., 1995). The existence of the first pathway was confirmed by the isolation of a  $\Delta^4$ -desaturase from the marine protist *Thraustochytrium* spp. and the freshwater species *Euglena* (Meyer et al., 2003; Qiu et al., 2001). The second,  $\Delta^4$ -independent pathway appears more complicated but there is strong experimental evidence that this is the predominant route in mammals (Leonard et al.,

2002). Recently, evidence has been presented by several groups that the  $C_{24} \Delta^6$ -desaturase is the same enzyme as the  $C_{18} \Delta^6$ -desaturase responsible for the synthesis of GLA and OTA (de Antueno et al., 2001; D'andrea et al., 2002). Also, the completion of the human genome sequence reveals the presence of three cytochrome  $b_5$  fusion PUFA desaturases, two of which have been functionally identified as the  $\Delta^5$ - and  $C_{18} \Delta^6$ -desaturases (Cho et al., 1999a,b; Leonard et al., 2000; Marquardt et al., 2000). Whilst it is conceivable that this third cytochrome  $b_5$  fusion sequence encodes a  $C_{22} \Delta^4$ -desaturase, no evidence has been so far provided to confirm this (or a  $C_{24}$ -specific  $\Delta^6$ -desaturase).

An alternative pathway for the biosynthesis of  $C_{20}$  PUFAs has been demonstrated in the protist *Tetrahymena pyroformis*, *Acanthamoeba* spp. and *Euglena gracilis*, organisms which appear to lack  $\Delta^6$ -desaturase activity (Lees and Korn, 1966; Ulsamer et al., 1969; Wallis and Browse, 1999). The first step in this alternative route is elongation of  $C_{18}$  fatty acids, LA and ALA, to eicosadienoic (20:2  $\Delta^{11,14}$ ) *n*-6 and eicosatrienoic (20:3  $\Delta^{11,14,17}$ ) *n*-3 fatty acids, respectively. In turn, these  $C_{20}$  products are desaturated by a  $\Delta^8$ -desaturase to produce 20:3  $\Delta^{8,11,14}$  *n*-6 and 20:4  $\Delta^{8,11,14,17}$  *n*-3 PUFAs which are the intermediates of the conventional pathway. The products of  $\Delta^8$ -desaturation are then subjected to desaturation at the  $\Delta^5$  position to produce AA and EPA and may be elongated with subsequent  $\Delta^4$ -desaturation to DHA. This so called “ $\Delta^8$ -desaturation” pathway has also been found in rat and human testis (Albert and Coniglio, 1977; Albert et al., 1979) and in glioma and breast cancer cell lines (Cook et al., 1991; Bardon et al., 1996) as well as being hypothesised to explain the synthesis of AA in felines, which appears to lack a  $\Delta^6$ -desaturase activity (Sinclair et al., 1981). A  $\Delta^8$ -desaturase has been cloned from *Euglena* and shown to be structurally related to the other cytochrome- $b_5$  fusion desaturases (e.g.  $\Delta^6$ -,  $\Delta^5$ - and  $\Delta^4$ -) involved in PUFA synthesis (Watts and Browse, 1999; Napier et al., 2003).

The role of this alternative route of PUFA metabolism in terms of physiological significance in human health remains unclear but this metabolic route for 20:5 *n*-3 formation could be utilised when the conventional route is impaired (either genetically or pathologically). The desaturation catalysed by the  $\Delta^6$ -desaturase which introduces a double bond at the  $\Delta^6$ -position of LA and ALA is rate limiting and thus is regulated by dietary factors and hormonal changes. Thus, the alternative pathway may fulfil the task of supplying PUFAs in those tissues where these fatty acids are in greater demand, or not subject to similar regulation. Alternatively,  $\Delta^8$ -desaturation may take place in mammalian tissues with reduced or zero  $\Delta^6$ -desaturase activity. Experimental evidence that glioma and breast cancer cells may preferentially elongate 18:3 *n*-3 and that



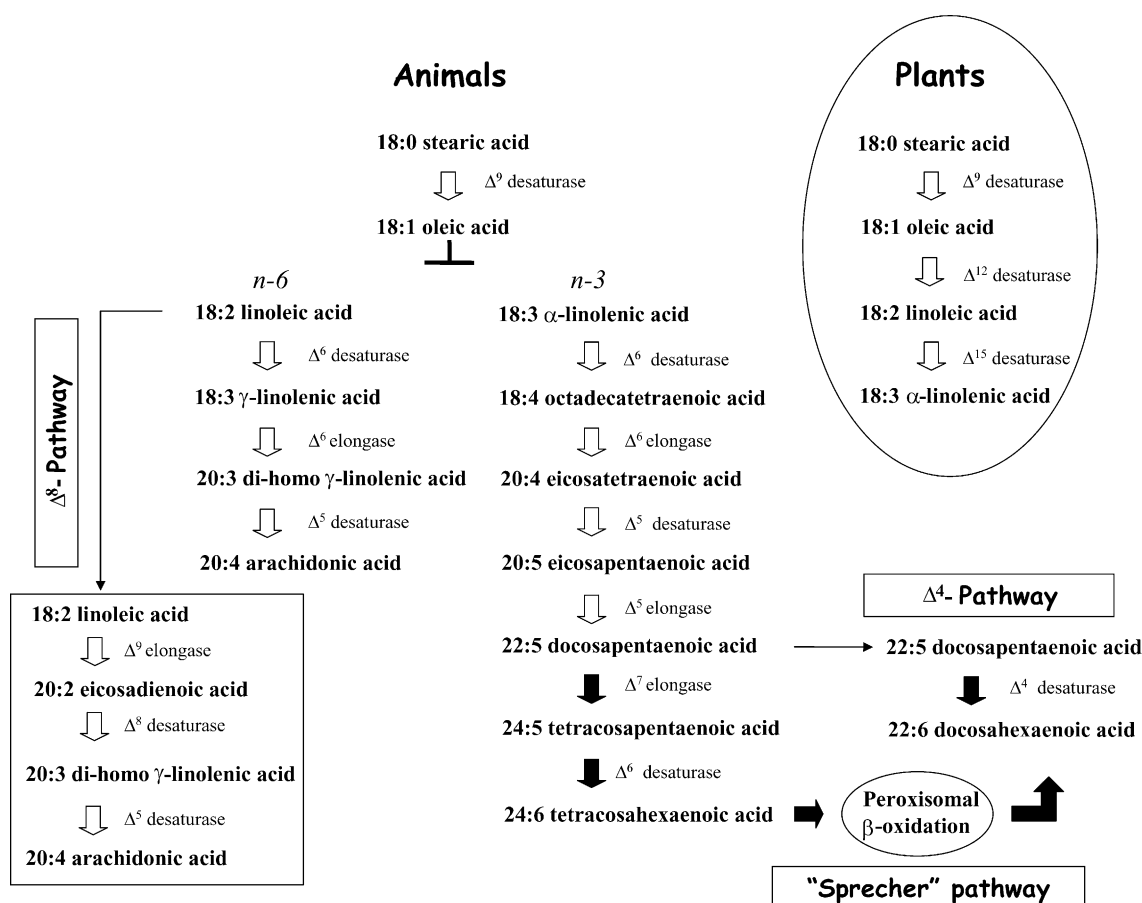


Fig. 2. Biosynthesis of C<sub>20</sub>+ PUFAs. The various different routes for the aerobic biosynthesis are shown. Precursor EFAs linolenic and  $\alpha$ -linolenic acid are the predominant fatty acids synthesised by plants. These then enter the mammalian food web and are subsequently metabolised to C<sub>20</sub>+ PUFAs. The alternative  $\Delta^8$ -desaturase/ $\Delta^9$ -elongase pathway is shown, as are the two alternative routes for DHA synthesis. In the case of EPA/DHA synthesising microalgae, C<sub>20</sub>+ PUFAs are synthesised directly from saturated substrates (since such organisms contain both “plant” and “animal” components of the pathway).

$\Delta^8$ -desaturation follows this elongation gives support to the hypothesis that  $\Delta^8$ -desaturation may play an important role in cancerogenesis (Cook et al., 1991; Bardon et al., 1996). However, as mentioned above, the human genome contains only three candidate “front-end” PUFA desaturases, two of which have been functionally characterised. Thus, if the third cytochrome b<sub>5</sub> fusion desaturase is not the C<sub>22</sub>  $\Delta^4$ -desaturase, it is conceivable that it encodes a C<sub>20</sub>  $\Delta^8$ -desaturase activity. Alternatively, the presence of  $\Delta^8$ -desaturase activity in cancerous cells could possibly represent a loss of specificity (either directly or indirectly) by the  $\Delta^5$ - or  $\Delta^6$ -desaturases. Recently Qi et al. (2002) isolated  $\Delta^9$ -elongase from C<sub>22</sub>-PUFA synthesising microalga *Isochrysis galbana* which had the ability to elongate the C<sub>18</sub> fatty acids, LA and ALA to 20:2 *n*-6 and 20:3 *n*-3 (i.e. substrates for  $\Delta^8$ -desaturation). This suggests that the PUFA biosynthetic pathway in *I. galbana* may be similar to that of *Euglena* and utilise the alternative route to provide substrates for  $\Delta^5$ -desaturation.

In contrast to plants and mammals, some insects and invertebrates such as the nematode *C. elegans* have

retained the ability to synthesize C<sub>20</sub> PUFAs from C<sub>16–18</sub> saturated and monounsaturated fatty acid, with this latter organism containing all of the enzymes required for the de novo synthesis of *n*-6/*n*-3 C<sub>20</sub> PUFAs (Hutzell and Krusberg, 1982). The nematode contains  $\Delta^5$ - and  $\Delta^6$ -desaturases as well as PUFA elongase activities found in animals and genetic analysis has revealed the importance of PUFA biosynthesis in normal worm development (Napier and Michaelson, 2001; Watts and Browse, 2002; Watts et al., 2003). Moreover, *C. elegans* can perform the crucial step in de novo PUFA biosynthesis by converting 18:1  $\Delta^9$  to LA, via a  $\Delta^{12}$  acyl lipid desaturase (Hutzel and Krasberg, 1982; Tanaka et al. 1996); the *C. elegans* gene for this desaturase was recently identified (Peyou-Ndi et al., 2002). This desaturase activity is found in plants but absent in mammals, and its presence (or absence) is likely to represent a key step in the acquisition of dietary requirement for EFAs. *C. elegans* can also convert a range of C<sub>18</sub> and C<sub>20</sub> *n*-6 PUFAs into *n*-3 PUFAs through the action of a C<sub>18–20</sub> *n*-3 desaturase which provides an additional mechanism in the regulation of

Table 1

Enzyme	Type of organism	Species	Refs.
<i>Aerobic desaturases</i>			
$\Delta^4$ -desaturase	Algae	<i>Thraustochytrium</i> sp. <i>Euglena gracilis</i>	Qui et al., 2001 Meyer et al., 2003
$\Delta^5$ -desaturase	Mammals	<i>Homo sapiens</i>	Cho et al., 1999b
	Nematode	<i>Caenorhabditis elegans</i>	Michaelson et al., 1998b
	Fungi	<i>Mortierella alpina</i> <i>Pythium irregulare</i>	Michaelson et al., 1998a Hong et al., 2002a
$\Delta^6$ -desaturase	Algae	<i>Thraustochytrium</i> sp.	Qui et al., 2001
	Mammals	<i>Homo sapiens</i> <i>Mus musculus</i>	Cho et al., 1999a Cho et al., 1999a
	Nematode	<i>Caenorhabditis elegans</i>	Napier et al., 1998
	Plants	<i>Borago officinales</i> <i>Echium</i> <i>Primula</i> <i>Anemone</i>	Sayanova et al., 1997 Garcia-Maroto et al., 2002 Sayanova et al., 2003 Whitney et al., 2003
	Mosses	<i>Ceratodon purpureus</i> <i>Physcomitrella patens</i>	Sperling et al., 2000 Girke et al., 1998
	Fungi	<i>Mortierella alpina</i> <i>Pythium irregulare</i>	Chaudhary et al., 1999 Hong et al., 2002b
Bifunctional $\Delta^5/\Delta^6$ desaturase	Fish	<i>Danio rerio</i>	Hastings et al., 2001
$C_{20}$ $\Delta^8$ -desaturase	Protist (green algae)	<i>Euglena gracilis</i>	Wallis and Browse, 1999
$C_{18-20}$ $n-3$ desaturase	Nematode	<i>Caenorhabditis elegans</i>	Spychalla et al., 1997
<i>Aerobic elongases</i>			
$\Delta^6$ -elongases	Nematode	<i>Caenorhabditis elegans</i>	Beaudoin et al., 2000
	Mosses	<i>Physcomitrella patens</i>	Zank et al., 2000
	Fungi	<i>Mortierella alpina</i>	Parker-Barnes et al., 2000
PUFA-elongase	Mammals	<i>Homo sapiens</i>	Leonard et al., 2002
$\Delta^9$ -elongase	Algae	<i>Isochrysis galbana</i>	Qi et al., 2002
<i>Anaerobic enzymes</i>			
EPA-polyketide synthase	Bacteria	<i>Shewanella putrefaciens</i>	Takeyama et al., 1997
DHA-polyketide synthase	Algae	<i>Schizochytrium</i> sp.	Metz et al., 2001
	Bacteria	<i>Moritella marinus</i>	Metz et al., 2001

$n-3$  PUFAs supply. Thus, the ratio of  $n-3/n-6$  PUFAs is higher in *C. elegans* than in mammals because the presence of the (additional)  $n-3$  desaturase activity makes the two parallel  $n-6/n-3$  PUFA biosynthetic pathways more independent from the dietary intake of  $n-6$  and  $n-3$  fatty acids.

Functional characterization of cDNA clones corresponding to *C. elegans*  $\Delta^5$ -,  $\Delta^6$ -,  $\Delta^{12}$ -(designated FAT-3, FAT-4 and FAT-2, respectively) and the  $C_{18-20}$   $n-3$  (designated FAT-1) desaturase genes have confirmed their enzymatic activities (Napier et al., 1998; Michaelson et al., 1998a,b; Watts and Browse, 1999; Sychalla et al., 1997; Peyou-Ndi et al., 2002). One intriguing aspect of *C. elegans* PUFA biosynthetic pathway is the obvious evidence of gene duplication; this is true for both the related FAT-1/FAT-2 genes and the  $\Delta^5$ - and  $\Delta^6$ -desaturases. This may serve as a paradigm for the evolution of distinct enzyme activities from an ancestral prototypic (bifunctional) enzyme (Napier and Michaelson, 2001). In that respect, the identification of a bi-functional  $\Delta^6/\Delta^5$ -desaturase from zebrafish may represent such an archetype (Hastings et al., 2001).

Whilst most flowering plants have no capacity to synthesis  $C_{20}$  PUFAs, some other (lower) organisms can

synthesize PUFAs from saturated fatty acids. The moss *Physcomitrella patens* synthesises EPA via the conventional  $n-3$  PUFAs pathway involving  $\Delta^6/\Delta^5$ -desaturation and elongation steps, as witnessed by the isolation of genes encoding these activities (Girke et al., 1998; Zank et al., 2000). The fungus *Mortierella alpina* and many species of algae possess all the enzyme activities to synthesize EPA from saturated substrates (Parker-Barnes et al., 2000; Chaudhary et al., 1999). Studies of PUFAs biosynthesis with radiolabeled precursors have shown that *M. alpina*, *Porphyridium cruentum*, *Cryptocodinium cohnii* and *Nannochloropsis* use a similar pathway leading to EPA biosynthesis (Khozin et al., 1997; Henderson and Mackinlay, 1991; Schneider and Roessler, 1994).

Recently a very different and much simpler anaerobic biosynthetic pathway has been identified for both prokaryotic and eukaryotic marine organisms (Metz et al., 2001; Napier, 2002). It is catalysed by a multifunctional complex that is analogous to polyketide synthases (PKS) and does not require PUFA-specific desaturases and elongases. Several PKS gene clusters from  $C_{20}$ -PUFA accumulating marine bacteria *Shewanella* and *Vibrio marinus* were expressed in *E. coli* and *Synechococcus* result-

ing in the accumulation of EPA and DHA (Takeyama et al., 1997; Tanake et al., 1996). The identification of a PKS-like pathway in a marine protist *Schizochytrium*, a member of the *Thraustochytriaceae*, indicates that it can be widespread in marine ecosystem (Metz et al., 2001). It also raises a question as to the evolutionary relevance of the aerobic desaturase/elongase pathway present in *Thraustochytrium* (another member of the *Thraustochytriaceae*) (Qiu et al., 2001; Napier, 2002).

### 3.2. Identification of genes involved in *n*-3 PUFA biosynthesis

During last few years most of the genes responsible for the biosynthesis of the *n*-3 PUFAs have been cloned from various organisms including algae, fungi, mosses, plants and mammals (summarised in Table 1). The desaturation enzymes required for PUFA biosynthesis are membrane-bound proteins which have three strongly conserved histidine-rich sequences (His boxes) comprising the general motifs H–X<sub>[3–4]</sub>H, H–X<sub>[2–3]</sub>H–H and H/Q–X<sub>[2–3]</sub>H–H (Shanklin et al., 1994; Shanklin and Cahoon, 1998; Sperling et al., 2003). Based on regioselectivity these desaturases can be separated into two different groups: the “methyl-end” desaturase introducing the next double bond between the existing one and the methyl end of the fatty acid chain, and the “front-end” desaturases, inserting a new double bond between an existing one and the carboxyl end of the acyl group in a methylene-interrupted pattern (Somerville and Browse, 1996; Napier et al., 1997). All current examples of “front end” desaturases (introducing  $\Delta^6$ -,  $\Delta^5$ -,  $\Delta^4$ - and  $\Delta^8$ -double bonds) contain a N-terminal cytochrome b<sub>5</sub> domain which serves as the electron donor for desaturation (Napier et al., 1997, 1999, 2003; Sperling et al., 2003). Another characteristic of the “front end” desaturases is the substitution of histidine by glutamine in the third histidine box. Site-directed mutagenesis of this glutamine to (a consensual) histidine resulted in loss of the desaturase activity; thus, the consensus sequence for the third His box of front-end desaturases is more correctly given as Q–X<sub>[2–3]</sub>H–H (Sayanova et al., 2001).

The first example of the gene encoding the key reaction in both *n*-6 and *n*-3 metabolic pathways, the microsomal  $\Delta^6$ -fatty acid desaturase, was cloned from borage (*Borago officinalis*) and expressed in tobacco plants and in yeast (Sayanova et al., 1997, 1999). These studies demonstrated the formation of GLA and OTA in transgenic plants and also in yeast (in the presence of exogenous substrates, LA and ALA). Orthologs of the  $\Delta^6$ -desaturase have been identified from many different species including *C. elegans* (Napier et al., 1998; Watts and Browse, 1999), mammals (Cho et al., 1999a,b, fungi (Huang et al., 1999; Hong et al., 2002a,b), mosses (Girke et al., 1998; Sperling et al., 2000) and plants

(Garcia-Maroto et al., 2002; Whitney et al., 2003). All these  $\Delta^6$ -desaturases showed no major preference for *n*-6 or *n*-3 substrates (Browse et al., 1999; Huang et al., 1999; Girke et al., 1998; Sperling et al., 2000), though recently we have cloned and characterized two members of  $\Delta^6$ -fatty acid desaturases from *Primula* with strong specificity towards *n*-3 substrates (Sayanova et al., 2003). As mentioned above, the presence of 24:6 *n*-3 metabolites in mammalian cells supported the hypothesis of the existence of two distinct  $\Delta^6$ -desaturase activities specific to chain length (C<sub>18</sub> v. C<sub>24</sub>). Until recently, there was little conclusive evidence that the  $\Delta^6$ -desaturase which acts on the C<sub>18</sub> unsaturated fatty acids is also capable of desaturating C<sub>24</sub> substrates. Two recent studies have demonstrated that both human and rat single  $\Delta^6$ -desaturases act on C<sub>18</sub> and C<sub>24</sub> PUFAs (de Antueno et al., 2001; d'Andrea et al., 2002). Interestingly, the rat  $\Delta^6$ -desaturase is more active on C<sub>18</sub> *n*-3 than C<sub>24</sub> *n*-3 (d'Andrea et al., 2002). However, these results do not exclude the possibility of the existence of another (substrate-specific)  $\Delta^6$ -desaturase activities (or even a  $\Delta^4$ -desaturase) in other mammals.

The second key enzyme in the *n*-3 PUFAs pathway is the  $\Delta^5$ -desaturase that catalyses the last desaturation step of EPA biosynthesis from 20:4 *n*-3 and sequences encoding this activity have been identified from various organisms (Table 1). The cloned  $\Delta^5$ -desaturases were expressed in yeast in the presence of exogenous substrates and demonstrated their ability to produce  $\Delta^5$ -unsaturated fatty acids. The  $\Delta^5$ -desaturases cloned from *M. alpina* and *C. elegans*, when expressed in heterologous systems, acted on a range of substrates, inserting double bonds in a non-methylene-interrupted pattern. Expression of the *M. alpina*  $\Delta^5$ -desaturase in transgenic canola resulted in the production of 18:2  $\Delta^{5,9}$  (taxoleic acid) and C18:3  $\Delta^{5,9,12}$  (pinolenic acid) (Knutzon et al., 1998), whereas the nematode enzyme (when expressed in yeast in the presence of exogenous substrates) produced 20:3  $\Delta^{5,11,14}$  and 20:4  $\Delta^{5,11,14,17}$  in addition to endogenously produced 18:2  $\Delta^{5,9}$  (Watts and Browse, 1999). The presence of two  $\Delta^5$ -desaturases from the slime mould *Dictyostelium discoideum* have also been reported to produce taxoleic acid and 18:2  $\Delta^{5,11}$  with one of these enzymes able to act on the saturated substrate, 16:0 (Saito and Ochiai, 1999). At present there is no experimental evidence for the use of C<sub>20</sub> PUFAs as the substrates for *Dictyostelium discoideum*  $\Delta^5$ -desaturases. However, small amounts of 20:3  $\Delta^{5,11,14}$  and 20:4  $\Delta^{5,11,14,17}$  have been identified in several species of slime molds (Rezanka, 1993) which leaves the possibility that these two  $\Delta^5$ -desaturases can act on the C<sub>20</sub> conventional  $\Delta^5$  substrates.

As already described, the  $\Delta^5$ -desaturation of eicosatetraenoic acid (20:4 *n*-3) is the last step on the biosynthetic pathway of EPA. The previous step, in which 20:4 *n*-3 is synthesized from OTA, is catalyzed by a C<sub>2</sub>

elongase complex. Biochemical evidence suggests that the fatty acid elongation consists of four steps: condensation, reduction, dehydration and a second reduction (Cinti et al., 1992). At present, identified condensing enzymes (3-keto-acyl synthases) can be divided into two groups. A first group includes the FAE-like plant enzymes involved in the biosynthesis of saturated and monounsaturated fatty acids with C<sub>18–22</sub> chain length, with these enzymes showing homology to other condensing enzymes such as chalcone synthase (Millar and Kunst, 1997); so far, there is no evidence that this FAE1-like class of enzymes is involved in the synthesis of PUFAs. However, a second class of (presumptive) condensing enzymes has been defined by the ELO gene family of yeast, which are required for the synthesis of VLCFA components of sphingolipids (Oh et al., 1997). Apparent paralogs of the ELO-type class of (sphingolipid) VLCFA elongases have recently been demonstrated to be involved in PUFA biosynthesis. For example, several C<sub>18</sub>  $\Delta^6$ -specific elongases from fungus, moss and nematode have been cloned and characterized by heterologous expression in yeast (Beaudoin et al., 2000; Parker-Barnes et al., 2000; Zank et al., 2000), whilst a related C<sub>18</sub>  $\Delta^9$ -elongating activity of the alternative  $\Delta^8$ -desaturation pathway has also recently been identified from *Isochrysis* (Qi et al., 2002). The molecular basis for this substrate recognition ( $\Delta^6$ - versus  $\Delta^9$ -C<sub>18</sub> PUFAs) is currently undefined, as is the precise enzymatic function of these ELO-like ORFs, though as mentioned above, they are assumed to be condensing enzymes.

#### 4. Biotechnology of PUFA production

The existence of different pathways for PUFAs biosynthesis offers a wide range of alternatives to budding plant biotechnologists in their quest to produce desired fatty acids in transgenic oilseed crops. At present, the most obvious approach is based on the use of aerobic “front-end” desaturases and elongases. The C<sub>20</sub> PUFA biosynthetic pathway has been successfully reconstituted in yeast by the co-expression of the  $\Delta^6$ -elongase with  $\Delta^6$ - and  $\Delta^5$ -fatty acid desaturases resulted in small but significant accumulation of AA and EPA from exogenously supplied LA and AL (Beaudoin et al., 2000; Zank et al., 2000).

The first step towards engineering oilseeds to produce PUFAs such as EPA was expression of the borage  $\Delta^6$ -fatty acid desaturase in transgenic tobacco and *Arabidopsis* plants (Sayanova et al., 1997, 1999). Data obtained from these experiments indicated that the (constitutive) expression of borage  $\Delta^6$  desaturase in transgenic plants resulted in the production of 18:3 *n*-6 and 18:4 *n*-3 which clearly indicated that the unusual fatty acids required for PUFAs biosynthesis can be

incorporated into glycerolipids of the host plants (unlike other non-native fatty acids in transgenic plants; Suh et al., 2002). A step forward in the assembling of the PUFAs pathway in oilseeds was the co-expression of the *M. alpina*  $\Delta^6$ - and  $\Delta^{12}$ -desaturases in canola plant resulted in the accumulation of up to ~50% of GLA (Huang et al., 2001). More recently, we have obtained transgenic soybeans expressing the borage  $\Delta^6$ -desaturase under the control of a seed-specific promoter, resulting in the accumulation of GLA to almost 50% of seed fatty acids (A.J. Kinney, personal communication). These data indicate that it is very likely that GLA is not restricted to a single position (such as sn-2) on the glycerolipids backbone. Since accumulation of high levels of GLA is a prerequisite for the successful reconstitution of the C<sub>20</sub> PUFA biosynthetic pathway (Fig. 2), these results bode well for future experiments. Currently, considerable effort is focussing on the combined expression in plants of the  $\Delta^6$ -desaturase with the  $\Delta^6$ -elongase and the  $\Delta^5$ -desaturase; publication of these data is eagerly awaited. However, since the “reverse engineering” of any PUFA biosynthetic pathway requires the transgenic mobilisation of multiple different enzyme activities, this will require the heterologous expression of a minimum of three transgenes. The different reverse engineering strategies for the synthesis of C<sub>20</sub>+ PUFAs in transgenic plants are summarized below.

##### 4.1. Routes for C<sub>20</sub>+ PUFA synthesis

###### Aerobic

###### Synthesis of EPA

- Conventional aerobic desaturation  
 $\Delta^6$ -desaturase,  $\Delta^6$ -elongase, Yields AA, EPA  
 $\Delta^5$ -desaturase

- Alternative aerobic desaturation  
 $\Delta^9$ -elongase,  $\Delta^8$ -desaturase, Yields AA, EPA  
 $\Delta^5$ -desaturase

###### Additional pathway “skews”

- *N*-3-specific  $\Delta^6$ -desaturase Yields OTA
- C<sub>18–20</sub> w-3 desaturase Yields OTA, EPA

###### Conversion of EPA to DHA

- Conventional aerobic desaturation  
 $\Delta^5$ -elongase,  $\Delta^4$ -desaturase Yields DPA, DHA
- “Sprecher” peroxisomal shunt  
 $\Delta^5$ -elongase,  $\Delta^7$ -elongase, Yields THA, DHA  
 $\Delta^6$ -desaturase  
(requires 1 cycle of  $\beta$ -oxidation to convert THA to DHA)

###### Anaerobic

- Polyketide synthase-like Yields EPA/DHA  
processive synthesis



At present, the availability of PUFA elongases and desaturases makes real the possibility of producing a wide range of PUFAs in oilseed crops. However, this is likely to be sub-optimal in efficiency when compared with the original gene sources, as has been observed in other attempts to engineer (more simple) fatty acid traits in transgenic plants (Drexler et al., 2003; Suh et al., 2002). This could be due to multiple factors, such as those resulting from the synthesis of non-native fatty acids, variations in substrates used for desaturation (glycerolipid versus acyl-CoA) (Domergue et al., 2003), as well as the random nature of transgenes integration into plant genomes. Another consideration is the promiscuous behaviour of enzymes in heterologous systems, resulting in activities towards either new or unexpected substrates. For example, the expression of the *M. alpina*  $\Delta^5$ -desaturase in transgenic canola resulted in the accumulation of  $C_{18}$   $\Delta^5$ -desaturated fatty acids, even though these products are not detected in the endogenous lipids of the fungus (Knutzon et al., 1998). Therefore, the selection of genes encoding enzymes with high chain-length selectivity becomes a particularly important issue. Whilst  $\Delta^6$ - or  $\Delta^5$ -unsaturated metabolites as 16:1 $\Delta^6$  or taxoleic, pinolenic acids and  $C_{20:2}$   $\Delta^{5,11}$  are not considered to be normal intermediates in  $C_{20}$  PUFA biosynthesis, their physiological effects (as minor compounds in a transgenic plant) would still certainly need to be considered from human nutritional and biochemical perspectives. Therefore, an additional target for biotechnological applications would be the identification of high-fidelity enzymes for production of PUFAs without the formation of unintended by-products resulting from substrate promiscuity.

## 5. Future prospects

Whilst the synthesis of EPA requires only three enzyme activities (Table 1), the longer term objective might be the synthesis of DHA. Since this  $C_{22}$  PUFA is synthesised by additional elongation and desaturation of EPA, synthesis in a heterologous host will require additional enzyme activities (and hence, transgenes). Initial attempts to heterologously reconstitute DHA synthesis in yeast appear to have demonstrated low but significant levels of this PUFA, marking an important “proof-of-concept” A. Abbadi and E. Heinz, oral presentation. It remains to be seen if this (or other) aerobic desaturase/elongase pathways are the best system for the transgenic synthesis of  $C_{20+}$  PUFAs, or if the anaerobic PKS-like system is a viable alternative.

Whatever routes are used for the transgenic synthesis of  $C_{20+}$  PUFAs such as EPA, it is clear that there is an urgent need for alternative and sustainable sources of these fatty acids (Sargent and Tacon, 1999). However, it is equally clear that continued consumer antipathy

(exacerbated by co-ordinated lobbying from unelected pressure groups) currently limits the possibility for deploying transgenic plants nutritionally-enhanced with  $C_{20}$  PUFAs into the human food chain. One alternative for the provision of the health-beneficial PUFAs such as EPA sourced from transgenic plants would be to use them as feedstuffs in commercial aquaculture. As described above, fish accumulate  $C_{20+}$  PUFAs primarily as a result of dietary intake of EPA and DHA-rich micro-organisms, and not as a result of endogenous biosynthetic capacity. This translates into an absolute requirement for EPA/DHA in the supplements used to feed fish under aquaculture. Thus, it is possible to envisage a situation in which EPA-enriched oils, derived from transgenic plants, are used to replace current (less sustainable) “trash” fish sources of these PUFAs in aquaculture. In this way, the significant health benefits of these fatty acids could be delivered into the human diet, without the requirement for the direct ingestion of GM food.

## 6. Conclusions

Virtually all the genes encoding the enzyme activities required for primary  $C_{20+}$  PUFA biosynthesis have now been cloned and functionally characterised, and the possibility of heterologous reconstitution of this pathway has been demonstrated in yeast. The first steps towards the “reverse engineering” of EPA synthesis in transgenic plants look extremely promising and it is to be anticipated that further advances (via the introduction of additional PUFA synthesising activities) will be made. The use of transgenic plants to synthesise fish oils may not only provide a sustainable source of these important fatty acids, but may also help demonstrate the utility of GM technology to enhance human health and nutrition.

## Acknowledgements

Rothamsted Research receives grant-aided support from BBSRC (UK).

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