nature biotechnology

Production of very long chain polyunsaturated omega-3 and omega-6 fatty acids in plants

Baoxiu Qi[1,4], Tom Fraser[1], Sam Mugford[2,5], Gary Dobson[3], Olga Sayanova[2,6], Justine Butler[1], Johnathan A Napier[2,6], A Keith Stobart[1] & Colin M Lazarus[1]

We report the production of two very long chain polyunsaturated fatty acids, arachidonic acid (AA) and eicosapentaenoic acid (EPA), in substantial quantities in a higher plant. This was achieved using genes encoding enzymes participating in the ω3/6 Δ^8-desaturation biosynthetic pathways for the formation of C20 polyunsaturated fatty acids. *Arabidopsis thaliana* was transformed sequentially with genes encoding a Δ^9-specific elongating activity from *Isochrysis galbana*, a Δ^8-desaturase from *Euglena gracilis* and a Δ^5-desaturase from *Mortierella alpina*. Instrumental in the successful reconstitution of these C20 polyunsaturated fatty acid biosynthetic pathways was the *I. galbana* C18-Δ^9-elongating activity, which may bypass rate-limiting steps present in the conventional Δ^6-desaturase/elongase pathways. The accumulation of EPA and AA in transgenic plants is a breakthrough in the search for alternative sustainable sources of fish oils.

The very long chain polyunsaturated fatty acids (VLCPUFAs) AA (C20:4 $\Delta^{5,8,11,14}$), EPA (C20:5 $\Delta^{5,8,11,14,17}$) and docosahexaenoic acid (DHA, C22:6 $\Delta^{4,7,10,13,16,19}$) are the subject of much interest, because of their important roles in human health and nutrition. These include neonatal retinal and brain development, as well as cardiovascular health and disease prevention[1–5]. AA and EPA are components of mammalian cell membranes and are also precursors of the eicosanoids, including the prostaglandins, a family of biological effectors involved in inflammatory responses, blood pressure regulation, blood clotting and cell signaling[6]. These fatty acids also inhibit methane production from rumen fermentation, and could help reduce greenhouse gas emissions if used in animal feed[7].

The diet of most modern societies is nowadays relatively low in ω3 PUFAs with a concomitant increased level of ω6 PUFA intake, largely resulting from a preference for plant-seed oils and food products from intensively bred animals. The ω3 PUFAs, EPA and DHA, for example, are usually obtained from oily fish, the consumption of which has decreased significantly in recent years[2]. Fish stocks are also declining, and the oils derived from fish are sometimes contaminated with a range of pollutants. Heavy metals such as methylmercury, for example, have been detected in some fish, and these compounds are known to affect neuropsychological function in adults[8]. A recent global study on farmed salmon revealed high levels of toxins including polychlorinated biphenyls, dioxins and other organochlorine compounds[9]. The production of fish (and fish oils) via aquaculture also requires the supplementation of fish feeds with EPA/DHA, putting additional strain on this diminishing resource[10]. Alternative sources of VLCPUFAs are therefore desirable, and the concept of obtaining them from higher plants in commercial and sustainable quantities is particularly attractive. However, no oil-seed species produces such products naturally, so there is a need to genetically engineer the capacity to synthesize these fatty acids in agronomically viable oil-seed species.

Humans can synthesize VLCPUFAs from the so-called essential fatty acids, linoleic acid (LA, C18:2 $\Delta^{9,12}$) and α-linolenic acid (ALA, C18:3 $\Delta^{9,12,15}$), which must be obtained from the diet. However, biosynthesis of VLCPUFAs is somewhat limited and is regulated by dietary and hormonal changes[11]. VLCPUFA synthesis in mammals proceeds predominantly by a Δ^6-desaturation pathway, in which the first step is the Δ^6-desaturation of LA and ALA to yield γ-linolenic acid (GLA, C18:3 $\Delta^{6,9,12}$) and stearidonic acid (STA, C18:4 $\Delta^{6,9,12,15}$), respectively. Further fatty acid elongation and desaturation steps give rise to AA and EPA (**Fig. 1a**). Accordingly, genes encoding Δ^6-desaturases[12–18], Δ^6-elongase components[19–21] and Δ^5-desaturases[22–27] have been cloned from a variety of organisms including higher plants, algae, mosses, fungi, nematodes and humans.

However, an alternative pathway for the biosynthesis of AA and EPA operates in some organisms. Here, LA and ALA are first elongated specifically to eicosadienoic acid (EDA, C20:2 $\Delta^{11,14}$) and eicosatrienoic acid (EtrA, C20:3 $\Delta^{11,14,17}$), respectively. Subsequent Δ^8 and Δ^5 desaturation of these products yields AA and EPA (**Fig. 1b**). We have recently cloned and functionally identified a specific C18-Δ^9-elongating activity (responsible for the first committed step of this pathway) from the DHA-producing marine microalga, *I. galbana*[28]. A Δ^8-desaturase cDNA has also been characterized from

[1]School of Biological Sciences, University of Bristol, Woodland Road, Bristol, BS8 1UG, England, UK. [2]Long Ashton Research Station, Long Ashton, Bristol, BS41 9AF, England, UK. [3]Scottish Crop Research Institute, Invergowrie, Dundee, DD2 5DA, Scotland, UK. [4]Current address: Department of Biology and Biochemistry, University of Bath, 4 South, Claverton Down, Bath, BA2 7AY, England, UK. [5]Present address: Department of Plant Sciences, University of Oxford, South Parks Road, Oxford, OX1 3RB, England, UK. [6]Current address: Rothamsted Research, Harpenden, Herts AL5 2JQ, England, UK. Correspondence should be addressed to B.Q. (bssbq@bath.ac.uk)

Published online 16 May 2004; doi:10.1038/nbt972

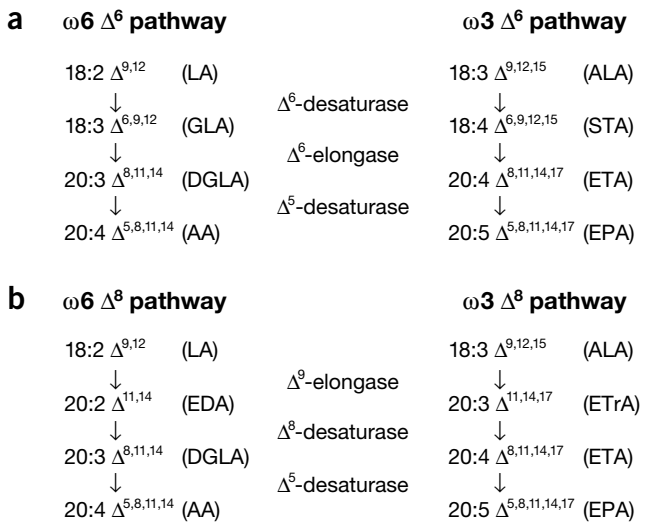

Figure 1 Conventional and alternative desaturation pathways for AA and EPA. (**a**) The conventional Δ^6-desaturation pathways start with the Δ^6-desaturation of LA and ALA, followed by 2-carbon elongation catalyzed by a Δ^6-elongase and further desaturation. (**b**) In the alternative Δ^8-pathways, LA and ALA are first elongated by a C18-Δ^9-specific fatty acid elongase (Δ^9-elongase) to EDA and ETrA, respectively. A Δ^8-desaturase is required to introduce a double bond at the Δ^8 position of the carbon chains to generate DGLA and ETA. Further desaturation by a Δ^5-desaturase results in AA and EPA.

the alga, *E. gracilis*[29]. We now report the reconstitution of the Δ^8-desaturation pathways for VLCPUFA synthesis in *A. thaliana*, and the accumulation of appreciable quantities of AA and EPA in the transgenic plants.

RESULTS

Expression of *IgASE1* in *A. thaliana*

A. thaliana plants were transformed to express *IgASE1*, the gene encoding elongating activity from *I. galbana*. Visual inspection (for a number of different parameters such as growth stature, height, flowering time, number of flowers and seed-set) of all the transgenic plants cultivated revealed no morphological or physiological alteration resulting from transgene expression. Transformed plants were self-fertilized, and lines demonstrating the 3:1 ratio of Liberty-herbicide-resistant to sensitive progeny indicative of a single transformation event were taken through a further round of self-fertilization to establish homozygous single-copy lines. **Figure 2** shows the gas chromatography (GC) profile of total fatty acids extracted from leaves of wild-type and transgenic plants. LA and ALA, the substrates for the *I. galbana* C18-Δ^9-elongating activity IgASE1, are major fatty acids in the leaves of wild-type plants (**Fig. 2a**). Two additional fatty acid species are apparent in the transgenic plants expressing *IgASE1* (**Fig. 2b**). Their retention times are identical to those of the standards EDA and ETrA, the expected C20 elongation products of LA and ALA. These results were similar to those observed previously for *IgASE1* expression in yeast[28]. C20:2 and C20:3 accumulated to 8.4 mol% and 10.4 mol% of total fatty acids, representing conversions of 51% and 22% of their C18 substrates, respectively (**Table 1**).

Coexpression of *EuΔ8* and *IgASE1* in *A. thaliana*

Kanamycin resistance was used as a selectable marker in transformation of the Liberty-herbicide-resistant *IgASE1*-expressing line

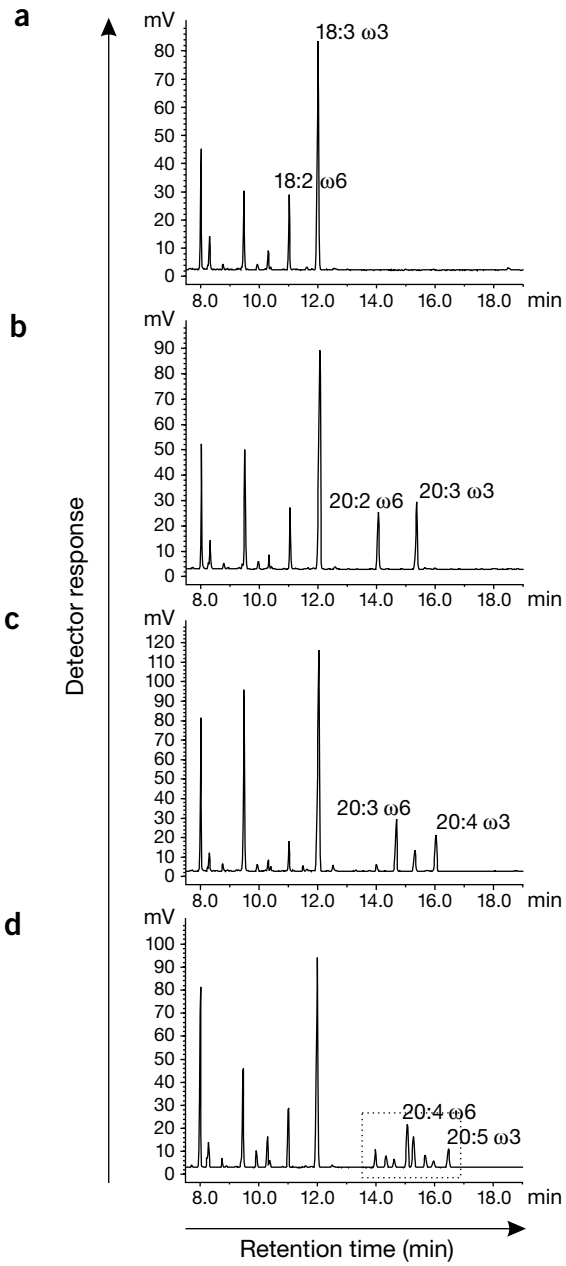

Figure 2 GC profiles of *A. thaliana* leaf fatty acid methyl esters. Fatty acids were extracted from an untransformed plant (**a**); a single-transgenic plant expressing the *I. galbana* Δ^9-elongating activity IgASE1 (**b**); a double transgenic plant expressing IgASE1 and the *E. gracilis* Δ^8-desaturase (EuΔ8) (**c**); a triple transgenic plant expressing IgASE1, EuΔ8 and the *M. alpina* Δ^5-desaturase (MortΔ5) (**d**). The peaks in the boxed region are designated 1 to 8 in the order of increasing retention time; peaks 4 and 8 are 20:4 and 20:5, respectively.

with a construct containing the *E. gracilis* Δ^8-desaturase (*EuΔ8*)[29] coding region under the control of the CaMV 35S promoter. Double-transgenic *A. thaliana* plants, which again exhibited normal morphology (as defined above), were both Liberty- and kanamycin-resistant. Homozygous line D3, an apparent single-copy transformant, had ceased segregating kanamycin-sensitive progeny after two rounds of self-fertilization, and was selected for fatty acid analysis. Two additional peaks are apparent in the GC profile of the leaf fatty acids of the

Table 1 Total fatty acid composition of leaves from wild type, single, double and triple transgenic *A. thaliana* plants

Fatty acid (mol% of total)	Plant source: Wild type	IgASE1 Single transgenic	IgASE1 + EuΔ8 Double transgenic	IgASE1 + EuΔ8 + MortΔ5 Triple transgenic
16:0	17.5 ± 0.3	15.9 ± 0.5	15.5 ± 0.2	16.2 ± 0.4
16:1 Δ^{7}	4.8 ± 0.3	3.9 ± 0.2	2.2 ± 0.1	3.1 ± 0.1
16:3 $\Delta^{7,10,13}$	12.3 ± 0.3	13.5 ± 0.3	19.4 ± 0.3	14.1 ± 0.8
18:0	0.9 ± 0.1	0.8 ± 0.1	0.8 ± 0.04	1.5 ± 0.1
18:1 Δ^{9}	2.9 ± 0.3	2.5 ± 0.3	1.8 ± 0.1	3.5 ± 0.2
18:2 $\Delta^{9,12}$	13.7 ± 0.4	8.2 ±0.5	4.3 ± 0.1	7.2 ± 0.3
18:3 $\Delta^{9,12,15}$	48.0 ± 0.4	36.5 ± 1.0	35.6 ± 0.1	32.0 ± 0.4
20:2 $\Delta^{11,14}$	–	8.4 ± 0.2	1.2 ± 0.1	2.6 ± 0.03
20:3 $\Delta^{11,14,17}$	–	10.4 ± 0.4	3.9 ± 0.2	4.6 ± 0.1
20:3 $\Delta^{8,11,14}$	–	–	8.7 ± 0.3	1.3 ± 0.02
20:4 $\Delta^{8,11,14,17}$	–	–	6.5 ± 0.2	1.2 ± 0.1
20:3 $\Delta^{5,11,14}$	–	–	–	1.4 ± 0.1
20:4 $\Delta^{5,11,14,17}$	–	–	–	1.8 ± 0.1
20:4 $\Delta^{5,8,11,14}$ (AA)	–	–	–	6.6 ± 0.1
20:5 $\Delta^{5,8,11,14,17}$ (EPA)	–	–	–	3.0 ± 0.02
Total C20 PUFAs	–	18.8	20.3	22.5

Single transgenic plants were Liberty-herbicide resistant, expressing the *I. galbana* IgASE1 elongating activity. Double transgenic plants were produced by transforming the single transgenic plants with a T-DNA containing a kanamycin-resistance gene and a Δ^{8}-desaturase gene from *E. gracilis*. Triple transgenic plants were produced by transforming the double transgenic plants with a T-DNA containing a hygromycin-resistance gene and a Δ^{5}-desaturase gene from *M. alpina*. Triple transgenic plants were hemizygous for hygromycin resistance and the linked Δ^{5}-desaturase gene; with this exception, all transgenes in all plants selected for analysis were in the homozygous state. Leaf tissue from rosette stage plants was analyzed, and each value represents the mean ± standard error from two separate measurements of three plants.

double transgenic line (**Fig. 2c**) compared to its single transgenic parent (**Fig. 2b**). These were identified as dihomo-γ-linolenic acid (DGLA, C20:3 $\Delta^{8,11,14}$) and eicosatetraenoic acid (ETA, C20:4 $\Delta^{8,11,14,17}$), respectively, on the basis of the retention times of known standards. DGLA and ETA are the expected products of Δ^{8}-desaturation of the precursors EDA and ETrA, respectively. DGLA and ETA accounted for some 8.7 mol% and 6.5 mol% of the total fatty acids and represented a conversion of 88% and 63% of their respective substrates. The total C20 PUFA content in this double transgenic line was some 20 mol% of the total fatty acids (**Table 1**).

Coexpression of *MortΔ5*) + *IgASE1* + *EuΔ8* in *A. thaliana*

The relatively high content of C20 fatty acids in the double-transgenic plants indicated that it might be possible to obtain AA and EPA by further transformation. In order to assess this, the coding region of the *M. alpina* Δ^{5}-desaturase gene (*Mort*Δ5)[26] was placed under the control of the CaMV 35S promoter in the binary vector pCAMBIA 1300, and the Liberty- and kanamycin-resistant homozygous line D3 was transformed to hygromycin resistance. The triple-transgenic plants obtained remained phenotypically indistinguishable from the wild type. Leaf fatty acids were determined in 24 plants, and the results for the highest C20 PUFA producers are presented in **Figure 2d** and **Table 1**. Compared to the double transgenic plants, a further four fatty acid peaks are apparent. Two of these, peaks 4 and 8, correspond to AA and EPA, the expected Δ^{5}-desaturation products of DGLA and ETA, respectively. The yields of AA and EPA were 6.6 mol% and 3.0 mol% of the total fatty acids, or 29% and 13% of the total C20 PUFA products, respectively, representing 84% conversion of substrate DGLA and 71% of ETA. Small amounts (3.2 mol% of total fatty acids) of two other C20 fatty acids were also present and these were identified as sciadonic acid, C20:3 $\Delta^{5,11,14}$, and juniperonic acid, C20:4 $\Delta^{5,11,14,17}$ (see below). The total C20 PUFA content was some 22 mol% of total fatty acids (**Table 1**).

Confirmation of C20 PUFA identities in triple transgenic *A. thaliana* plants

Nitrogen-containing derivatives of fatty acids give informative mass spectra because the nitrogen atom carries the charge during ionization, resulting in radical-induced cleavage at every carbon-carbon bond along the alkyl chain. In the mass spectra obtained by gas chromatography-mass spectrometry (GC-MS) of 4,4-dimethyloxazoline (DMOX) derivatives of unsaturated fatty acids, if a double bond occurs between carbons *n* and *n* + 1 then a gap of 12 atomic mass units (amu) between ions corresponding to fragments containing *n* – 1 and *n* carbons is observed[30]. In saturated parts of the chain there are gaps of 14 amu because of cleavage of adjacent methylene groups.

Figure 3 shows the mass spectra of six of the eight peaks indicated in **Figure 2d** that were putatively identified as the intermediates and final products of the reconstituted ω6/3 Δ^{8} pathways. In the mass spectrum of the DMOX derivative of peak 1 the molecular ion at *m/z* 361 indicates a diunsaturated 20-carbon acid, whereas gaps of 12 amu between *m/z* 264 and 276, and between *m/z* 224 and 236 indicate double bonds at Δ^{14} and Δ^{11}, respectively. This confirms peak 1 as EDA. In the mass spectrum of the DMOX derivative of peak 3, the molecular ion at *m/z* 359 indicates a triunsaturated 20-carbon acid, whereas the gaps of 12 amu between *m/z* 182 and 194, 262 and 274, and 222 and 234 indicate a double bond at the Δ^{8} position, in addition to the double bonds at Δ^{14} and Δ^{11}. Peak 3 is thus confirmed as DGLA. The molecular ion at *m/z* 357 in the mass spectrum of the DMOX derivative of peak 4 indicates a tetraunsaturated 20-carbon acid; an odd-mass peak at *m/z* 153 is characteristic of a Δ^{5} double bond, whereas additional double bonds at Δ^{8}, Δ^{11} and Δ^{14} confirm peak 4 as AA. Similarly, the mass spectra of the DMOX derivatives of peaks 5, 7 and 8 confirm them to be the products of the ω3 Δ^{8} desaturation pathway, namely ETrA, ETA and EPA, respectively.

Figure 4 shows the mass spectra of the remaining two peaks indicated in **Figure 2d**, which do not appear to be components of the ω3/6-Δ^{8} desaturation pathways. In the mass spectrum of the DMOX derivative of peak 2, the molecular ion at *m/z* 359 indicates a triunsaturated 20-carbon acid, whereas gaps of 12 amu between *m/z* 262 and 274, and between *m/z* 222 and 234 indicate double bonds at Δ^{14} and Δ^{11}, respectively. The odd-mass peak at *m/z* 153 is characteristic of a Δ^{5} double bond, so this confirms peak 2 as sciadonic acid. The molecular ion at *m/z* 357 in the mass spectrum of the DMOX derivative of peak 6 indicates a tetraunsaturated 20-carbon acid. The gaps of 12 amu between *m/z* 302 and 314, *m/z* 262 and 274, and *m/z* 222 and 234 indicate double bonds at Δ^{17}, Δ^{14} and Δ^{11}, whereas the odd-mass peak at *m/z* 153 is characteristic of a Δ^{5} double bond. This confirms peak 6 as juniperonic acid.

All the mass spectra shown in **Figures 3** and **4** have been described previously (C20:3 $\Delta^{5,11,14}$ in ref. 30 and C20:4 $\Delta^{5,11,14,17}$ in ref. 31). All eight are similar to mass spectra, as described (http://www.lipid.co.uk/infores/masspec.html).

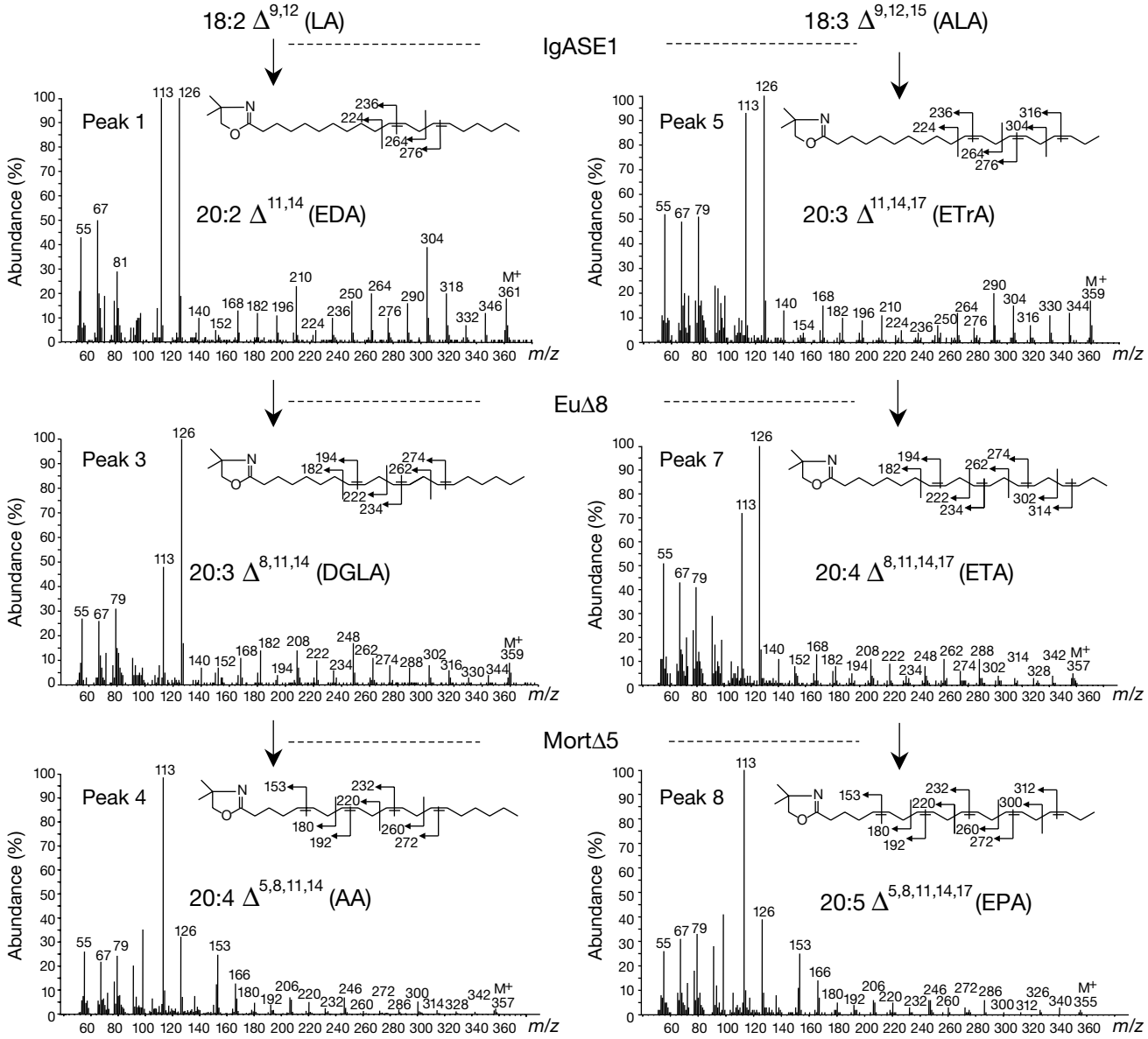

Figure 3 Positional analysis (by GC-MS) of the carbon double bonds of the C20 PUFAs (as their DMOX derivatives) in triple transgenic *A. thaliana* leaf tissues. Peaks 1, 3, 4, 5, 7 and 8 correspond to the peaks indicated in **Figure 2d**. The fatty acids are presented in their order of formation in the ω6/3 Δ^8-desaturation pathways.

DISCUSSION

The biosynthesis of appreciable quantities of the important VLCPUFAs, AA and EPA, has been achieved in the model higher plant *A. thaliana* by transformation and constitutive expression of three genes that encode components of the alternative Δ^8-desaturation pathways (**Fig. 1b**). The genes used encode a specific C18-Δ^9-fatty acyl elongating activity from the microalga *I. galbana*[28], a Δ^8 desaturase from *E. gracilis*[29] and a Δ^5-desaturase from the filamentous fungus *M. alpina*[26]. Although the first two of these genes originated in organisms in which the Δ^8-desaturation pathways may operate, *M. alpina* produces AA perhaps exclusively via the Δ^6-desaturation pathway. Δ^5-desaturation is however the point of convergence of both the conventional Δ^6- and alternative Δ^8-pathways (**Fig. 1**). *Agrobacterium tumefaciens*–mediated gene transfer to *A. thaliana* was sequential, with selectable resistance markers used in the order of Liberty herbicide, kanamycin and hygromycin. Plants expressing only *IgASE1* accumulated appreciable amounts of EDA and ETrA (**Fig. 2b** and **Table 1**), and these C20 PUFAs were converted to DGLA and ETA in the double transgenic plants also expressing *EuΔ8* (**Fig. 2c**). Triple-transgenic *A. thaliana* plants, additionally expressing *MortΔ5*, accumulated appreciable quantities of AA and EPA in their leaf tissues, and the sum of these two fatty acids accounted for 43% of the total C20 PUFA products (**Fig. 2d** and **Table 1**).

Besides the predicted fatty acids of the ω3/6 Δ^8-pathways (**Fig. 1b**) two other products were observed in GC analysis (**Fig. 2d**, peaks 2 and 6). GC-MS of the DMOX derivatives identified them as 20:3 $\Delta^{5,11,14}$ and 20:4 $\Delta^{5,11,14,17}$, respectively (**Fig. 4**). It is most probable that these arose by Δ^5-desaturation of EDA and ETrA, since the Δ^5-desaturase

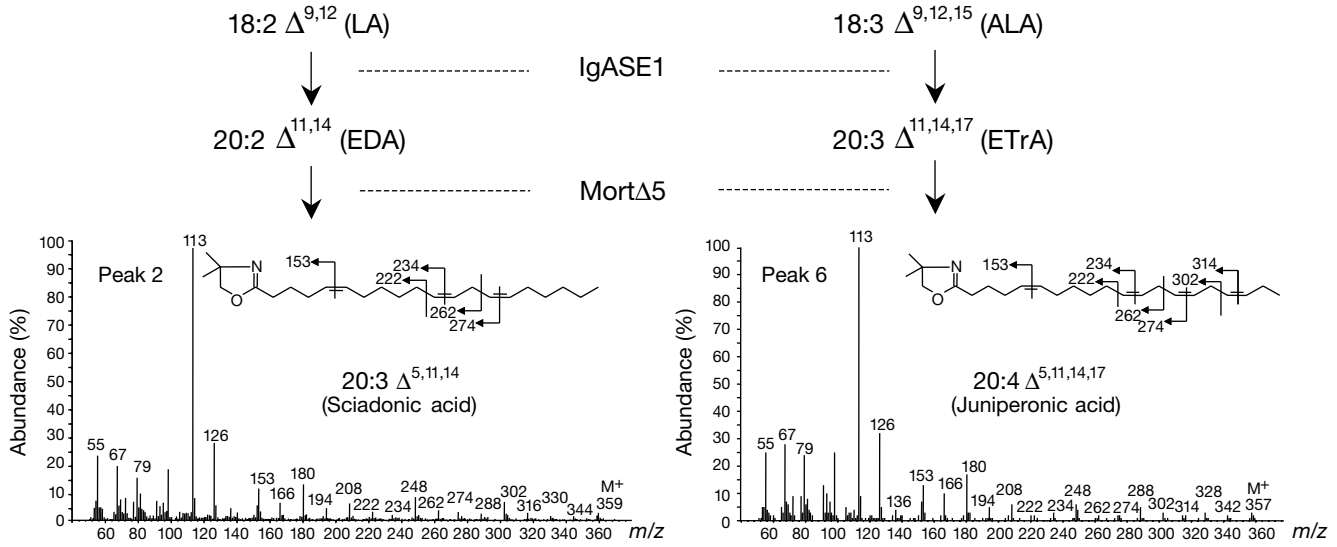

Figure 4 Positional analysis (by GC-MS) of the carbon double bonds of the two nonmethylene-interrupted C20 PUFAs (as their DMOX derivatives) in triple transgenic *A. thaliana* leaf tissues. Peaks 2 and 6 correspond to the peaks indicated in **Figure 2d**.

has previously been observed to display promiscuity in heterologous systems[24,27]. This may account, therefore, for these unusual products in the transgenic *A. thaliana* plants. However no significant Δ^5-desaturation of C18 fatty acids was apparent in transgenic *A. thaliana* leaves, which is in contrast to the production of taxoleic acid (C18:2 $\Delta^{5,9}$) and pinolenic acid (C18:3 $\Delta^{5,9,12}$) in canola transformed with the same gene[27]. It is likely that the two unusual C20 fatty acids are end products and not used in further fatty acid biosynthesis. It is also interesting that they are not observed in *I. galbana*, the source of the Δ^9-specific elongase component[28]. *I. galbana* is an EPA/DHA-producing microalga and we have suggested that these products are synthesized via the so-called ω3 Δ^8-desaturation pathway[28,32] (**Fig. 1b**).

Our results demonstrate that both the ω3 Δ^8- and ω6 Δ^8-desaturation biosynthetic pathways for VLCPUFA production can operate in higher plants, yielding the potentially valuable products AA and EPA. The key step would appear to be the C18-Δ^9-PUFA-specific elongating activity IgASE1 from *I. galbana*, which specifically catalyzes the elongation of the essential C18-Δ^9-PUFAs, LA and ALA. These fatty acids are in abundance in green vegetative tissues of higher plants, and in *A. thaliana* their elongation resulted in appreciable levels of EDA and ETrA. The efficiency of this alternative pathway may hinge on the presence of available cytoplasmic pools of LA-CoA and ALA-CoA, which have previously been shown to be present in *A. thaliana* leaf tissue[33]. Since microsomal fatty acid elongation commences with the condensation of malonyl-CoA with an acyl-CoA primer, these LA- and ALA-CoAs are suitable substrates for elongation to EDA and ETrA by the *I. galbana* Δ^9 elongase. In contrast, recent attempts to reconstitute the conventional ω6 and ω3 Δ^6-desaturation pathways in yeast, using genes encoding Δ^6-desaturase, Δ^6-elongating activity and Δ^5-desaturase met with limited success[19,34]. Elongation uses 'cytosolic' acyl-CoA, whereas most plant PUFA desaturases use acyl substrates esterified to glycerolipids, especially microsomal phosphatidylcholine[35], where acyl substrate is channeled into the *sn-2* position for subsequent desaturation and the products returned to the acyl-CoA pool via an acyl exchange process[36]. To explain their relatively low yield of AA in yeast, it has been argued[34] that insufficient GLA substrate was made available from the site of Δ^6-desaturation to the acyl-CoA pool for elongation, and that this explained a similar poor activity of the reconstituted conventional pathway in transgenic plants. This does not appear to present a problem in the reconstituted alternative pathway, but whether it is the different sequence of elongation and desaturation events found in the Δ^8-pathways that circumvent these perceived defects remains to be determined.

The fact that the total C20 PUFA content was almost the same in all the transgenic lines generated suggests that elongation was the rate-limiting step in AA and EPA production. However this conclusion assumes that the level of *IgASE1* expression was constant in single-, double- and triple-transgenic lines. Although some 20 mol% of the total C20 PUFAs accumulated in the transgenic plants, it appears to have no effect on the growth and morphology. This is in contrast to the dramatic morphological abnormalities observed[37] in ectopic expression of the seed-specific Fatty Acid Elongation1 (*FAE1*) gene using the CaMV 35S promoter in transgenic *A. thaliana*. Such abnormalities were ascribed to the incorporation of very long chain fatty acids (VLCFAs), including saturated and monounsaturated fatty acids, into glycerolipids, although these phenotypic effects were only observed in a few highly expressing lines. Analysis of our transgenic plants has also shown substantial incorporation of C20 fatty acids into chloroplast and extraplastidic glycerolipids[38]; however, here they are all polyunsaturated. Thus, although a very similar constitutive promoter was used in both studies on the accumulation of VLCFAs in plants, it is clear that the incorporation of C20 PUFAs into membrane glycerolipids is benign, as distinct from the incorporation of C20 saturated and monounsaturated fatty acids.

The results reported here demonstrate clearly that 'pathway engineering' for the viable production of VLCPUFAs in plants is possible. In order to exploit these observations more fully it will be necessary to express the genes in question in a seed-specific manner and in a lipid background rich in the fatty acid substrates that are specific for the elongase component, IgASE1. To this end, oilseed species such as soy and linseed should provide appropriate experimental material for further study.

We have successfully reconstituted the Δ^8-desaturation pathways for both ω6 and ω3 VLCPUFA biosynthesis in a higher plant. This has

been achieved by sequential transfer and expression of three genes in the model plant *A. thaliana*. Our data indicate that the use of these alternative Δ^8-desaturation pathways is likely to be more appropriate for genetic modification of oilseeds than the previously described conventional Δ^6-desaturase/elongase route. The triple-transgenic plants accumulate both AA and EPA in amounts that suggest that, if their incorporation into seed storage oils can be achieved, the production of these fatty acids in oil seed crops could become an economically viable proposition.

METHODS

Plasmid constructs for expressing fatty acid metabolism genes in transgenic plants. All coding regions used were placed in CaMV 35S promoter–nos terminator expression cassettes. The coding region of *I. galbana* elongating activity, *IgASE1*[28], was inserted as a *Kpn*I fragment into pBlueBac 4.5 (Invitrogen) and excised with *Pst*I plus *Eco*RI for insertion into pCB302-1 (ref. 39). The *M. alpina* Δ^5-desaturase coding region[26] was inserted as a *Kpn*I-*Sac*I fragment into the expression site of pCAMBIA-1300EC, a plasmid that had been constructed by replacing the pUC18 polylinker of pCAMBIA-1300 (CAMBIA) with an expression cassette (*Hin*dIII-*Eco*RI fragment) from pBI220. The *E. gracilis* Δ^8-desaturase coding region[29] was amplified from total RNA extracted from cultures of *E. gracilis* strain Z by RT PCR using the following primers (restriction site extensions underlined, initiating and stop codons in bold): forward primer (*Bam*HI) 5′-ATGGATCCACC**ATG**AAGTCAAAGCGCCAA-3′ and reverse primer (*Xho*I) 5′-ATCTCGAG**TTA**TAGAGCCTTCCCCGC-3′. The PCR product was cloned and sequenced, revealing the presence of an open reading frame of 1,266 base pairs, encoding a protein of 421 amino acid residues and a stop codon. This differs slightly from the sequence presented elsewhere[29], which describes a polypeptide of 422 residues. The difference results from a small frame shift at the 5′ end of the open reading frame, but it is unclear whether this represents an earlier sequencing error or a bona fide polymorphism between different accessions of *E. gracilis* strain Z. However, functional characterization of this new (421 residue) form of the *E. gracilis* Δ^8-desaturase expressed in yeast confirmed an identical function to that described earlier[29]. The cDNA was excised from vector pESC-Trp (Stratagene) with *Bam*HI plus *Xho*I, passaged through pBlueBac 4.5 (Invitrogen) and excised as a *Bgl*II-*Bam*HI fragment for insertion into the *Bam*HI site of $pBECKS_{19}$.6 (ref. 40).

Plant transformation. Binary plasmids were transferred to *A. tumefaciens* strain GV3101 by electroporation and kanamycin-resistant colonies were selected in all cases. *A. thaliana* ecotype Columbia 4 plants were transformed by the floral dipping method of Clough and Bent[41], with two rounds of dipping at 5-d intervals. To select for transformants harboring the elongase component approximately 10,000 seeds from dipped wild-type plants were germinated on compost. Seedlings at the 2–4 true-leaf stage were sprayed with 0.5% Liberty herbicide (0.5 g glufosinate-ammonium l^{-1}; Aventis), and spraying was repeated 1 week later. Twelve herbicide-resistant plants (T1) were selected and potted on and after two rounds of self-fertilization T3 seed was germinated on Liberty plates (half-strength Murashige and Skoog (MS) medium containing 5 mg glufosinate-ammonium l^{-1}) to identify homozygous lines that had ceased segregating herbicide-sensitive progeny. The line with the greatest C20 fatty acid content was selected for transformation with the Δ^8-desaturase gene. *A. tumefaciens*–mediated transformation was performed as described above, and ~2,000 seeds from dipped plants were spread on half-strength MS medium containing 50 μg ml^{-1} kanamycin. Twelve kanamycin-resistant plants were selected and line D3, which had the greatest content of Δ^8-desaturated products, was self-fertilized to T3 to obtain homozygous transformants for further transformation with the Δ^5-desaturase gene. Hygromycin (10 μg ml^{-1})-resistant plants were selected, transplanted to soil and grown for fatty acid analysis.

Fatty acid analysis. Fatty acids were extracted and methylated as described[42]. The fatty acid methyl esters were analyzed by GC on a 30 m × 0.25 mm fused silica DB-23 column (J and W Scientific) using heptadecanoic acid (C17:0) as internal standard and quantified by flame ionization detection. The chromatograph was programmed for an initial temperature of 140 °C for 5 min followed by a 20 °C min^{-1} temperature ramp to 185 °C and a secondary ramp of 1.5 °C min^{-1} to 220 °C. The final temperature was maintained for 2 min. Injector and detector temperatures were maintained at 230 °C and 250 °C, respectively. The initial head pressure of the carrier gas (He) was 90 kPa; a split injection was used.

For positional analysis of carbon double bonds, fatty acid methyl esters were hydrolyzed with 0.1 M potassium hydroxide in 90% aqueous ethanol at 22 °C for 16 h. After acidification and addition of water, the free fatty acids were extracted with diethyl ether-isohexane (1:1 by vol). The free fatty acids were converted to DMOX derivatives by heating with 2-amino-2-methyl-1-propanol at 190 °C for 16 h. On cooling, water was added and the DMOX derivatives were extracted with diethyl ether-isohexane (1:1 by vol). The solvent extract was washed with water and dried over anhydrous sodium sulphate. The fatty acid DMOX derivatives were subjected to GC-MS on a Hewlett Packard 5890 Series II Plus gas chromatograph fitted with an on-column injector and a Supelcowax 10 (0.25 mm × 30 m, 0.25 μm) capillary column, connected to a Hewlett Packard 5989B MS Engine quadrupole mass spectrometer. The column temperature was held at 80 °C for 3 min, temperature-ramped to 170 °C at 20 °C min^{-1}, then to 240 °C at 2 °C min^{-1} and finally to 280 °C at 5 °C min^{-1}. Helium was the carrier gas, at a flow rate of 1.4 ml min^{-1}, and pressure programming was used in constant flow mode. The mass spectrometer was operated in electron impact mode at an ionization energy of 70 eV.

ACKNOWLEDGMENTS

The authors gratefully acknowledge financial support from BASF Plant Sciences GmbH, Germany, and the Scottish Executive Environment and Rural Affairs Department. Long Ashton Research Station (1903–2003) and Rothamsted Research receive grant in aid from the Biotechnology and Biological Sciences Research Council (BBSRC) UK.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Biotechnology* website for details).

Received 16 December 2003; accepted 19 February 2004
Published online at http://www.nature.com/naturebiotechnology/

1. Carlson, S.E., Werkman, S.H., Peeples, J.M., Cooke, R.J. & Tolley, E.A. Arachidonic acid status correlates with the first year growth in preterm infants. *Proc. Natl. Acad. Sci. USA* **90**, 1073–1077 (1993).
2. Gill, I. & Valivety, R. Polyunsaturated fatty acids, part 1: occurrence, biological activities and applications. *Trends Biotechnol.* **15**, 401–409 (1997).
3. Crawford, M. Placental delivery of arachidonic acid and docosahexaenoic acids: implication for the lipid nutrition of preterm infants. *Am. J. Clin. Nutr.* **71**, 275S–284S (2000).
4. Lauritzen, L., Hansen, H.S., Jurgensen, M.H. & Michaelsen, K.F. The essentiality of long chain n-3 fatty acids in relation to development and function of brain and retina. *Prog. Lipid Res.* **40**, 1–94 (2001).
5. Thies, F. *et al.* Association of n-3 polyunsaturated fatty acids with stability of atherosclerotic plaques: a randomized controlled trial. *Lancet* **361**, 477–485 (2003).
6. Kinsella, J.E., Lokesh, B., Broughton, S. & Whelan, J. Dietary polyunsaturated fatty acids and eicosanoids: potential effects on the modulaton of inflammatory and immune cells: an overview. *Nutrition* **6**, 24–44 (1990).
7. Fievez, V., Dohme, F., Danneels, M., Raes, K. and Demeyer, D. Fish oils as potent rumen inhibitors and associated effects on rumen fermentation *in vitro* and *in vivo*. *Anim. Feed Sci. Technol.* **104**, 41–58 (2003).
8. Yokoo, E.M. *et al.* (2003) Low level methylmercury exposure affects neuropsychological function in adults. *Environmental Health: A Global Access Science Source* **2**, 1–11 (http://www.ehjournal.net/content/2/1/8) 2003.
9. Drexler, H. *et al.* Metabolic engineering of fatty acids for breeding of new oilseed crops: strategies, problems and first results. *J. Plant Physiol.* **160**, 779–802 (2003).
10. Hites, R.A. *et al.* Global assessment of organic contamination in farmed salmon. *Science* **303**, 226–229 (2004).
11. Yamazaki, K., Fujikawa, M., Hamazaki, T., Yano, S. & Shono, T. Comparison of the conversion rates of α-linolenic acid (18:3n-3) and stearidonic acid (18:4n-3) to longer polyunsaturated fatty acids in rats. *Biochim. Biophys. Acta* **1123**, 18–26 (1992).
12. Sayanova, O. *et al.* Expression of a borage desaturase cDNA containing an N-terminal cytochrome b5 domain results in the accumulation of higher levels of Δ^6-desaturated fatty acids in transgenic tobacco. *Proc. Natl. Acad. Sci. USA* **94**, 4211–4216 (1997).
13. Cho, H.P., Nakamura, M.T. & Clarke, S.D. Cloning, expression, and nutrition regulation of the mammalian Δ^6 desaturase. *J. Biol. Chem.* **274**, 471–477 (1999).
14. Napier, J.A., Hey, S.J., Lacey, D.J. & Shewry, P.R. Identification of a *Caenorhabditis elegans* Δ^6-fatty acid-desaturase by heterologous expression in *Saccharomyces*

cerevisiae. *Biochem. J.* **330**, 611–614 (1998).
15. Girke, T., Zähringer, U., Lerchl, J. & Heinz, E. Identification of a novel Δ^6-acyl-group desaturase by targeted gene disruption in *Physcomitrella patens*. *Plant J.* **15**, 39–48 (1998).
16. Sperling, P. *et al.* A bifunctional Δ^6-fatty acyl acetylenase/desaturase from the moss *Ceratodon purpureus*. A new member of the cytochrome b5 superfamily. *Eur. J. Biochem.* **267**, 3801–3811 (2000).
17. Huang, Y.S. *et al.* Cloning of delta12- and delta6-desaturases from *Mortierella alpina* and recombinant production of gamma-linolenic acid in *Saccharomyces cerevisiae*. *Lipids* **34**, 649–659 (1999).
18. Sakuradani, E., Kobayashi, M. & Shimizu, S. Delta6-fatty acid desaturase from an arachidonic acid-producing Mortierella fungus. Gene cloning and its heterologous expression in a fungus, *Aspergillus*. *Gene* **238**, 445–453 (1999).
19. Beaudoin, F. *et al.* Heterologous reconstitution in yeast of the polyunsaturated fatty acid biosynthetic pathway. *Proc. Natl. Acad. Sci. USA* **97**, 6421–6426 (2000).
20. Parker-Barnes, J.M. *et al.* Identification and characterization of an enzyme involved in the elongation of n-6 and n-3 polyunsaturated fatty acids. *Proc. Natl. Acad. Sci. USA* **97**, 8284–8289 (2000).
21. Zank, T.K. *et al.* Cloning and functional characterization of an enzyme involved in the elongation of Delta6-polyunsaturated fatty acids from the moss *Physcomitrella patens*. *Plant J.* **31**, 255–268 (2002).
22. Cho, H.P., Nakamura, M.T. & Clarke, S.D. Cloning, expression, and fatty acid regulation of the human Δ^5 desaturase. *J. Biol. Chem.* **274**, 37335–37339 (1999).
23. Leonard, A.E. *et al.* cDNA cloning and characterization of a human Δ^5-desaturase involved in the biosynthesis of arachidonic acid. *Biochem. J.* **347**, 719–724 (2000).
24. Watts, J.L. & Browse, J. Isolation and characterization of a Δ^5-fatty acid desaturase from *Caenorhabditis elegans*. *Arch. Biochem. Biophys.* **362**, 175–182 (1999).
25. Michaelson, L.V. *et al.* Functional identification of a fatty acid Δ^5-desaturase gene from *Caenorhabditis elegans*. *FEBS Lett.* **439**, 215–218 (1998).
26. Michaelson, L.V., Lazarus, C.M., Griffiths, G., Napier, J.A. & Stobart, A.K. Isolation of a Δ^5-fatty acid desaturase gene from *Mortierella alpina*. *J. Biol. Chem.* **273**, 19055–19059 (1998).
27. Knutzon, D.S. *et al.* Identification of Δ^5-desaturase from *Mortierella alpina* by heterologous expression in bakers' yeast and canola. *J. Biol. Chem.* **273**, 29360–29366 (1998).
28. Qi, B. *et al.* Identification of a cDNA encoding a novel C18-Δ^9-polyunsaturated fatty acid-specific elongating activity from the docosahexaenoic acid (DHA)-producing microalga, *Isochrysis galbana*. *FEBS Lett.* **510**, 159–165 (2002).
29. Wallis and Browse. The Δ8-desaturase of *Euglena gracilis*: an alternate pathway for synthesis of 20-carbon polyunsaturated fatty acids. *Arch. Biochem. Biophys.* **365**, 307–316 (1999).
30. Zhang, J.Y., Yu, Q.T., Liu, B.N. & Huang, Z.H. Chemical modification in mass spectrometry IV – 2-alkenyl-4,4-dimethyloxazolines as derivatives for the double bond location of long-chain olefinic acids. *Biomed. Environ. Mass Spectrom.* **15**, 33–44 (1988).
31. Berdeaux, O. & Wolff, R.L. Gas-liquid chromatography-mass spectrometry of the 4,4-dimethyloxazoline derivatives of Δ^5-unsaturated polymethylene-interrupted fatty acids from conifer seed oils. *J. Am. Oil Chem. Soc.* **73**, 1323–1326 (1996).
32. Qi, B. *et al.* The variant 'his-box' of the C18-Δ^9-PUFA-specific elongase IgASE1 from *Isochrysis galbana* is essential for optimum enzyme activity. *FEBS Lett.* **547**, 137–139 (2003).
33. Larson, T.R. & Graham, I.A. Application of a new method for the sensitive detection and quantification of acyl-CoA esters in *Arabidopsis thaliana* seedlings and mature leaves. *Biochem. Soc. Trans.* **28**, 575–577 (2000).
34. Domergue, F. *et al.* Acyl carriers used as substrates by the desaturases and elongases involved in very long-chain polyunsaturated fatty acids biosynthesis reconstituted in yeast. *J. Biol. Chem.* **278**, 35115–35126 (2003).
35. Stymne, S. & Stobart, A.K. Evidence for the reversibility of the acyl-CoA: lysophosphatidylcholine acyltransferase in microsomal preparations from developing safflower cotyledons and rat liver. *Biochem. J.* **223**, 305–314 (1984).
36. Stymne, S. & Stobart, A.K. Triglycerol Biosynthesis. in *The Biochemistry of Plants: A Comprehensive Treatise* (ed. Stumpf, P.K.) 175–214 (Academic Press, NY, 1988).
37. Millar, A.A., Wrischer, M. & Kunst, L. Accumulation of very-long-chain fatty acids in membrane glycerolipids is associated with dramatic alterations in plant morphology. *Plant Cell* **10**, 1889–1902 (1998).
38. Qi, B. *et al.* Expression of the Isochrysis C18-Δ9 polyunsaturated fatty acid specific elongase component alters *Arabidopsis* glycerolipid profiles. *Plant Physiol.* (in the press) (2004).
39. Xiang, C., Han, P., Lutziger, I., Wang, K. & Oliver, D.J. A mini binary vector series for plant transformation. *Plant Mol. Biol.* **40**, 711–717 (1999).
40. McCormac, A.C., Elliott, M.C. & Chen, D.-F. pBECKS. A flexible series of binary vectors for Agrobacterium-mediated plant transformation. *Mol. Biotechnol.* **8**, 199–213 (1997).
41. Clough, S.J. & Bent, A.F. Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735–743 (1998).
42. Browse, J., McCourt, J. & Somerville, C.R. Fatty acid composition of leaf lipids determined after combined digestion and fatty acid methyl ester formation from fresh tissue. *Anal. Biochem.* **152**, 141–145 (1986).