Identification of a cDNA encoding a novel C18-Δ⁹ polyunsaturated fatty acid-specific elongating activity from the docosahexaenoic acid (DHA)-producing microalga, *Isochrysis galbana*[1]

Baoxiu Qi[a], Frédéric Beaudoin[b], Tom Fraser[a], A. Keith Stobart[a,*], Johnathan A. Napier[b], Colin M. Lazarus[a]

[a] *School of Biological Sciences, University of Bristol, Woodland Road, Bristol BS8 1UG, UK*
[b] *Institute of Arable Crops Research-Long Ashton Research Station, Department of Agricultural Sciences, University of Bristol, Long Ashton, Bristol BS41 9AF, UK*

Received 10 October 2001; revised 8 November 2001; accepted 9 November 2001

First published online 10 December 2001

Edited by Richard Cogdell

Abstract ***Isochrysis galbana*, a marine prymnesiophyte microalga, is rich in long chain polyunsaturated fatty acids such as docosahexaenoic acid (C22:6n-3, $\Delta^{4,7,10,13,16,19}$). We used a polymerase chain reaction-based strategy to isolate a cDNA, designated *IgASE1*, encoding a polyunsaturated fatty acid-elongating activity from *I. galbana*. The coding region of 263 amino acids predicts a protein of 30 kDa that shares only limited homology to animal and fungal proteins with elongating activity. Functional analysis of *IgASE1*, by expression in *Saccharomyces cerevisiae*, was used to determine its activity and substrate specificity. Transformed yeast cells specifically elongated the C18-Δ^9 polyunsaturated fatty acids, linoleic acid (C18:2n-6, $\Delta^{9,12}$) and α-linolenic acid (C18:3n-3, $\Delta^{9,12,15}$), to eicosadienoic acid (C20:2n-6, $\Delta^{11,14}$) and eicosatrienoic acid (C20:3n-3, $\Delta^{11,14,17}$), respectively. To our knowledge this is the first time such an elongating activity has been functionally characterised. The results also suggest that a major route for eicosapentaenoic acid (C20:5n-3, $\Delta^{5,8,11,14,17}$) and docosahexaenoic acid syntheses in *I. galbana* may involve a Δ^8 desaturation pathway.** © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: *Isochrysis galbana*; Polyunsaturated fatty acids (PUFAs); cDNA; Fatty acid elongating activity

1. Introduction

In humans, ω6 (n-6) and ω3 (n-3) long chain polyunsaturated fatty acids (PUFAs) are obtained directly from the diet or synthesised from dietary linoleic acid (LA, C18:2n-6, $\Delta^{9,12}$) and α-linolenic acid (ALA, 18:3n-3, $\Delta^{9,12,15}$), respectively. Long chain PUFAs such as arachidonic acid (AA, C20:4n-6, $\Delta^{5,8,11,14}$), eicosapentaenoic acid (EPA, C20:5n-3, $\Delta^{5,8,11,14,17}$) and docosahexaenoic acid (DHA, C22:6n-3, $\Delta^{4,7,10,13,16,19}$) are considered important as structural components of membrane glycerolipids and as precursors of the eicosanoids, including the biologically active prostaglandins and leukotrienes [1]. AA and DHA are of special importance in the brain and blood vessels, and are considered essential for pre- and post-natal brain and retinal development [2]. Certain human medical conditions may be related to an imbalance in the intake and/or metabolism of the ω3 and ω6 PUFAs resulting in some dysfunction in eicosanoid metabolism [3]. 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 fish oils and other marine organisms, and their consumption has decreased significantly in recent years [3]. There is therefore interest in obtaining them from other sources and particularly in genetically engineering the potential to synthesise such products in agronomically important oil seed species.

The conversion of dietary LA and ALA to long chain PUFAs requires further desaturation and elongation reactions, possibly as depicted in Fig. 1. The common route is that both LA and ALA are first desaturated by a Δ^6 desaturase, then a chain-elongating system adds two carbons to the hydrocarbon chains, followed by a further desaturation to yield AA and EPA, respectively [4,5]. These two pathways are referred to here as the ω6 (Δ^6) and ω3 (Δ^6) pathways, since the first step involves Δ^6 desaturation. However, recent evidence suggests that alternative modes of synthesis may exist in some organisms. For example, the production of long chain PUFAs could involve a Δ^8 desaturation step, and a Δ^8 desaturase gene has been isolated from *Euglena* [6]. Such biosynthetic pathways would require the initial elongation of 18:2n-6 and 18:3n-3, rather than desaturation, producing eicosadienoic acid (EDA, C20:2n-6, $\Delta^{11,14}$) and eicosatrienoic acid (ETrA, C20:3n-3, $\Delta^{11,14,17}$), respectively. Δ^8 desaturase activity would then yield dihomo-γ-linolenic acid (DGLA, C20:3n-6, $\Delta^{8,11,14}$) and eicosatetraenoic acid (ETA, C20:4n-3, $\Delta^{8,11,14,17}$) for subsequent conversion to AA and DHA (Fig. 1). To distinguish these modes of synthesis from the previous ones, they are referred to here as the ω6 (Δ^8) and ω3 (Δ^8) pathways.

*Corresponding author. Fax: (44)-117-9257374.
E-mail address: a.k.stobart@bristol.ac.uk (A.K. Stobart).

[1] The nucleotide sequence reported in this paper has been submitted to the GenBank℠/EBI Data Bank with accession number AF390174.

Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; DGLA, dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; EDA, ω6-eicosadienoic acid; EPA, eicosapentaenoic acid; ETA, eicosatetraenoic acid; ETrA, ω3-eicosatrienoic acid; GLA, γ-linolenic acid; LA, linoleic acid; STA, stearidonic acid; GC, gas chromatography; MS, mass spectroscopy; PUFA, polyunsaturated fatty acid

PII: S0014-5793(01)03247-1

Desaturases involved in PUFA production have been the subject of intensive study in recent years, and many desaturase genes have been isolated [7]. In contrast, only a few genes encoding PUFA-elongating activities have been characterised, and all appear to be related to the *ELO* gene family of *Saccharomyces cerevisiae* [8]. To identify *GLELO*, which encodes an activity involved in elongating the two Δ^6-desaturated C18 PUFAs γ-linolenic acid (GLA, C18:3n-6, $\Delta^{6,9,12}$) and stearidonic acid (STA, C18:4n-3, $\Delta^{6,9,12,15}$), Parker-Barnes et al. [9] constructed a *Mortierella alpina* cDNA library in a yeast expression vector and screened yeast clones for the ability to elongate GLA to DGLA (see Fig. 1). The other genes encoding PUFA-specific elongating activities, *HELO1* from *Homo sapiens* [5] and *F56H11.4* from *Caenorhabditis elegans* [10], were identified via their sequence homology to yeast *ELO2*, which is involved in the elongation of saturated fatty acids [8]. Zhang et al. [11] have recently reported a human retinal photoreceptor-specific gene, *ELOVL4*, which may be involved in one of the three elongation steps required for DHA biosynthesis [12,13]. A 5-bp deletion in the coding region of this gene may be responsible for two related forms of autosomal dominant macular dystrophy [11].

The fatty acid elongation system of both plants and animals is generally considered to comprise a rate-limiting condensing enzyme together with two reductases and one dehydrase; the latter three components are thought to be present constitutively or induced by the condensing enzyme [14,15]. It is therefore considered that it is the condensing enzyme that determines the substrate specificity in terms of chain length and the degree of desaturation of fatty acid substrates. In support of this concept, constitutive expression of the normally seed-specific condensing enzyme encoded by the fatty acid elongation 1 gene (*FAE1*) in transgenic *Arabidopsis* was sufficient to bring about the synthesis of C20 and C22 fatty acids in non-seed tissues [15].

In order for pathways involving Δ^8 desaturation to operate (Fig. 1) it is possible that specific elongases for Δ^9-desaturated PUFAs may exist. Here we describe the isolation and characterisation of a cDNA encoding a protein with such an elongating activity from the DHA-rich microalga, *Isochrysis galbana*.

2. Materials and methods

2.1. Cultivation of I. galbana

I. galbana CCAP 927/1 was obtained from the Culture Collection of Algae and Protozoa, Centre for Coastal and Marine Sciences, Dunstaffnage Marine Laboratory, Oban, Argyll, UK. The algal cultures were grown in f/2 medium [16] in an orbital incubator at 100 rpm and 14°C with continuous white light of intensity of about 30 μmol/m^2/s.

2.2. Isolation of total and poly(A)$^+$ RNA from I. galbana

Algal cultures were harvested by centrifugation at 3000×*g* for 5 min. The cell pellet was ground to a fine powder under liquid nitrogen with a pestle and mortar before being suspended in 5 ml of TriPure® Isolation Reagent (Roche). Total RNA was then isolated following the manufacturer's protocol. Poly(A)$^+$ RNA was prepared with an mRNA isolation kit (Amersham Pharmacia Biotech).

2.3. cDNA library construction

Double-stranded, end-adapted cDNA synthesised using a cDNA synthesis kit (Stratagene) was passed through a Sephacryl S-400 Spun Column (Amersham Pharmacia Biotech) to remove adapters and small cDNA molecules. cDNA eluted from the column was phenol-extracted, ethanol-precipitated and ligated to the arms of the Uni-Zap vector (Stratagene) before packaging into λ phage using the Ready-To-Go Lambda Packaging Kit (Amersham Pharmacia Biotech). A primary library of 1×10^6 pfu was obtained with the majority of the inserts examined ranging from 0.4 to 2 kb. The library was subsequently amplified.

2.4. PCR amplification and cloning of overlapping segments of a putative elongase cDNA

A degenerate primer $EloR_2$ was designed based on the conserved motif MYXYYF/GL in *ELO*-like open reading frames (ORFs) (Fig. 2) and was used in touchdown PCR in conjunction with a universal T3 promoter primer (5′-AATTAACCCTCACTAAAGGG-3′) using an aliquot of the amplified *I. galbana* cDNA library as template. The PCR conditions were: one initial denaturation step of 94°C for 3 min; four cycles of 94°C for 15 s, 52°C for 30 s and 72°C for 45 s; 10 cycles of 94°C for 15 s, 52°C (decreasing by 1°C in each successive cycle) for 30 s, 72°C for 45 s; 25 cycles of 94°C for 15 s, 42°C for 30 s

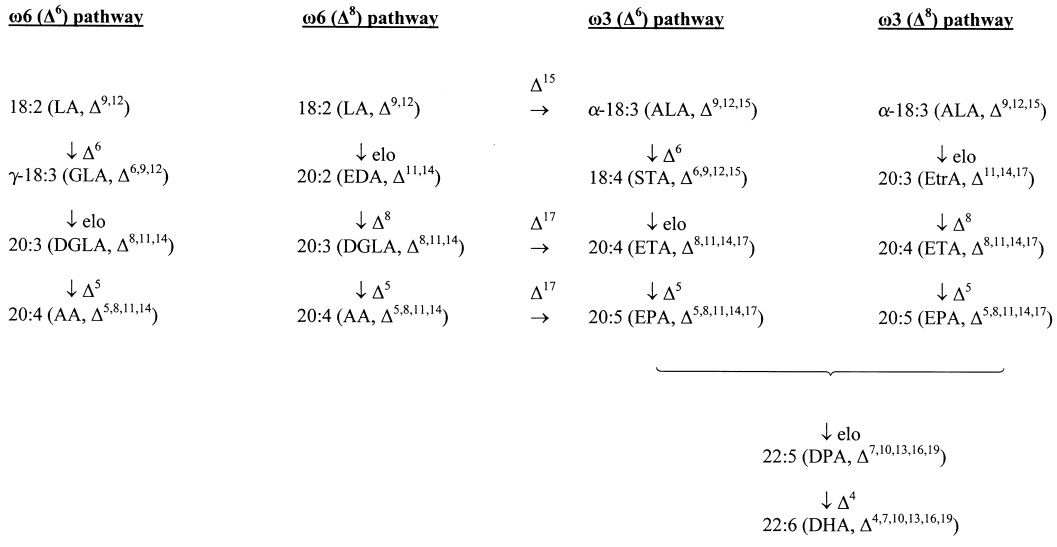

Fig. 1. Possible modes of ω3 and ω6 long chain PUFA biosynthesis. The ω6 (Δ^6) and ω3 (Δ^6) pathways for the synthesis of very long chain PUFAs start with Δ^6 desaturation of the C18 fatty acids, LA and ALA, followed by 2-carbon elongation and then further desaturation and elongation steps. The ω6 (Δ^8) and ω3 (Δ^8) pathways start with chain elongation followed by Δ^8 desaturation, then further desaturation and elongation steps to produce the final products AA and DHA, respectively.

and 72°C for 45 s (increasing by 3 s in each successive cycle); one final extension step of 72°C for 6 min. The resultant putative elongase 5′ end fragment of about 650 bp was gel-purified and cloned into plasmid pCR2.1-TOPO using the TOPO TA cloning system (Invitrogen). The insert of one recombinant plasmid was sequenced and the gene-specific (sense) primer $IgEloF_1$ (5′-ACTCGAAGCTCTTCACATGG-3′) synthesised for use in a further library PCR reaction with a universal M13 forward primer (5′-GTAAAACGACGGCCAGT-3′) under the following conditions: one initial denaturation step of 94°C for 3 min; 10 cycles of 94°C for 15 s, 55°C for 30 s and 72°C for 90 s; 20 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 93 s (increasing by 3 s in each successive cycle); one final extension step of 72°C for 6 min. The resultant putative elongase 3′ end fragment of about 850 bp was gel-purified, cloned and sequenced.

2.5. Functional analysis of the IgASE1 ORF by expression in yeast

The entire *IgASE1* coding region was amplified from the *I. galbana* cDNA library with primers IgEloBeg 5′-GGTACC**ATG**GCCCTCG-CAAACGA-3′ (ORF start codon indicated by bold type; underlined sequence is an added *Kpn*I site) and IgEloEnd 5′-TAAGGACATC-CACAATCCAT-3′ (primes 60 bp downstream of the ORF stop codon). The Expand High Fidelity PCR System (Roche) was employed to minimise potential PCR errors. The resultant 860-bp product was cloned in pCR2.1-TOPO (as above) and recombinant plasmids checked for insert orientation. The insert from a selected clone was excised with *Kpn*I and *Sac*I and ligated into the corresponding restriction sites of yeast expression vector pYES2 (Invitrogen), downstream of the *GAL1* promoter. The ligation mixture was used to transform *Escherichia coli* TOP10 cells (Invitrogen), from which the recombinant plasmid designated pY2ASE1 was isolated and used to transform *S. cerevisiae* strain W303-1A to uracil independence by the lithium acetate method [17]. Expression of *IgASE1* was induced by the addition of galactose to 2% (w/v) to cultures grown on raffinose as described [10,18]. After induction, the cultures were grown for 48 h at 22°C in selective medium with individual fatty acid substrates added to 0.1 M and Tergitol Type NP-40 (Sigma) added to 1%.

2.6. Fatty acid analysis

Microalgal or yeast cells were pelleted, washed and dried under a stream of N_2. Total fatty acids were extracted and transmethylated with methanolic HCl. Fatty acid methyl esters were analysed by gas chromatography (GC) using heptadecanoic acid as an internal standard on a 30 m × 0.25 mm DB-23 capillary column (J and W Scientific).

2.7. DNA and protein sequence analysis

DNA sequences were determined using an ABI 377 automatic sequencer (Perkin Elmer). The sequence was translated into amino acid sequence using DNAMAN Sequence Analysis Software (Lynnon Biosoft), and the output transferred to BlastP for homology searches.

Table 1
Composition of the major fatty acids of *I. galbana*

Fatty acid	Mol% total
14:0	21
16:0	9.3
16:1n-7	2.6
18:1n-9	18.1
18:2n-6	4.0
18:3n-3	11
18:4n-3	11.5
20:1n-9	8.8
20:5n-3	1.6
22:6n-3	12.0

The microalgae were harvested after 1 month of growth in culture as described in Section 2. Fatty acids were extracted and converted to their methyl esters before analysis by GC. Each peak was identified by the retention time compared to known standards; values represent the average of determinations made from three independent cultures.

3. Results

3.1. Isolation of a cDNA encoding a PUFA-elongating activity

We selected *I. galbana* as the source organism from which to isolate genes involved in EPA and DHA production. Fatty acid analysis of *I. galbana* cultivated as described above showed that it contained some 12% DHA (Table 1). Other major unsaturated fatty acids present were 18:1n-9, Δ^9, 18:3n-3, $\Delta^{9,12,15}$, 18:4n-3, $\Delta^{6,9,12,15}$ and 20:1n-9, Δ^{11}. It is noteworthy that small amounts of 18:2n-6, $\Delta^{9,12}$ and 20:5n-3, $\Delta^{5,8,11,14,17}$ were also present.

The strategy used to isolate a gene encoding a PUFA-elongating activity was to construct and amplify a directional cDNA library and screen it by PCR using degenerate primers based on single conserved motifs and universal primers located in flanking vector sequences. The degenerate primer $EloR_2$ was designed as the reverse complement of the conserved motif MYXYYF/GL in *ELO*-like ORFs [5,9,11, 10,19]. An alignment of the sequences on which the primer design was based is shown in Fig. 2. The primer combination $EloR_2$/T3 amplified a 650-bp product from the library; sequence analysis of the cloned PCR product revealed a continuous ORF extending from a putative start codon to the MYXYYF/GL motif. The 5′ region of the cDNA consisted of a single G upstream of the putative start codon. A further 16 bases of putative 5′ untranslated region were determined by 5′ rapid amplification of cDNA ends (data not shown). However, the data do not exclude the possibility that the first ATG codon of the cDNA encodes an internal methionine residue.

To amplify the 3′ region of the cDNA from the library the gene-specific sense primer IgEloF1 was used with the universal M13 forward primer. A PCR product of about 850 bp was cloned and sequenced; the 5′ and 3′ PCR product sequences overlapped by 235 bp, confirming that they were ultimately derived from a single gene (*IgASE1*). The presence of 17 bases of poly(A) tail indicated that the cDNA was complete at the 3′ end, and that the 3′ untranslated region of the *IgASE1* mRNA was 253 bases.

Assuming the coding region of the cDNA to be full length, *IgASE1* encodes a protein of 263 amino acids with a molecular mass of 30 kDa. The deduced amino acid sequence of IgASE1 aligned with other proteins with elongating activity is shown in Fig. 2. Hydropathy analysis predicts multiple membrane-spanning regions, and dilysine residues at −3 and −6, relative to the C-terminus, probably locate it in the endoplasmic reticulum [20,21]. A further characteristic of all Elo-like proteins described so far is the presence of the histidine box motif HXXHH, which was also found in IgASE1, albeit with glutamine (Q) substituted for the first histidine.

The amino acid sequence of IgASE1 exhibits only limited similarity to the sequences of *ELO*-type genes (Fig. 2), with at best only 27% identity with GLELO from *M. alpina*, which elongates specifically the two Δ6-desaturated C18 PUFAs, GLA and STA [9]. The second highest identity of 26% was found with Ssc1, which, together with Ssc2 (21% identity) and Cig30 (20% identity), is a member of a mouse gene family involved in the elongation of the very long chain fatty acids that become esterified to sphingolipids [22,23]. Ssc1 could replace Elo3 to synthesise C26:0 for sphingolipid synthesis in a yeast mutant, whilst Cig30 restored levels of C20–C24 fatty acids in an Elo2-deficient mutant. IgASE1 shows 26% identity

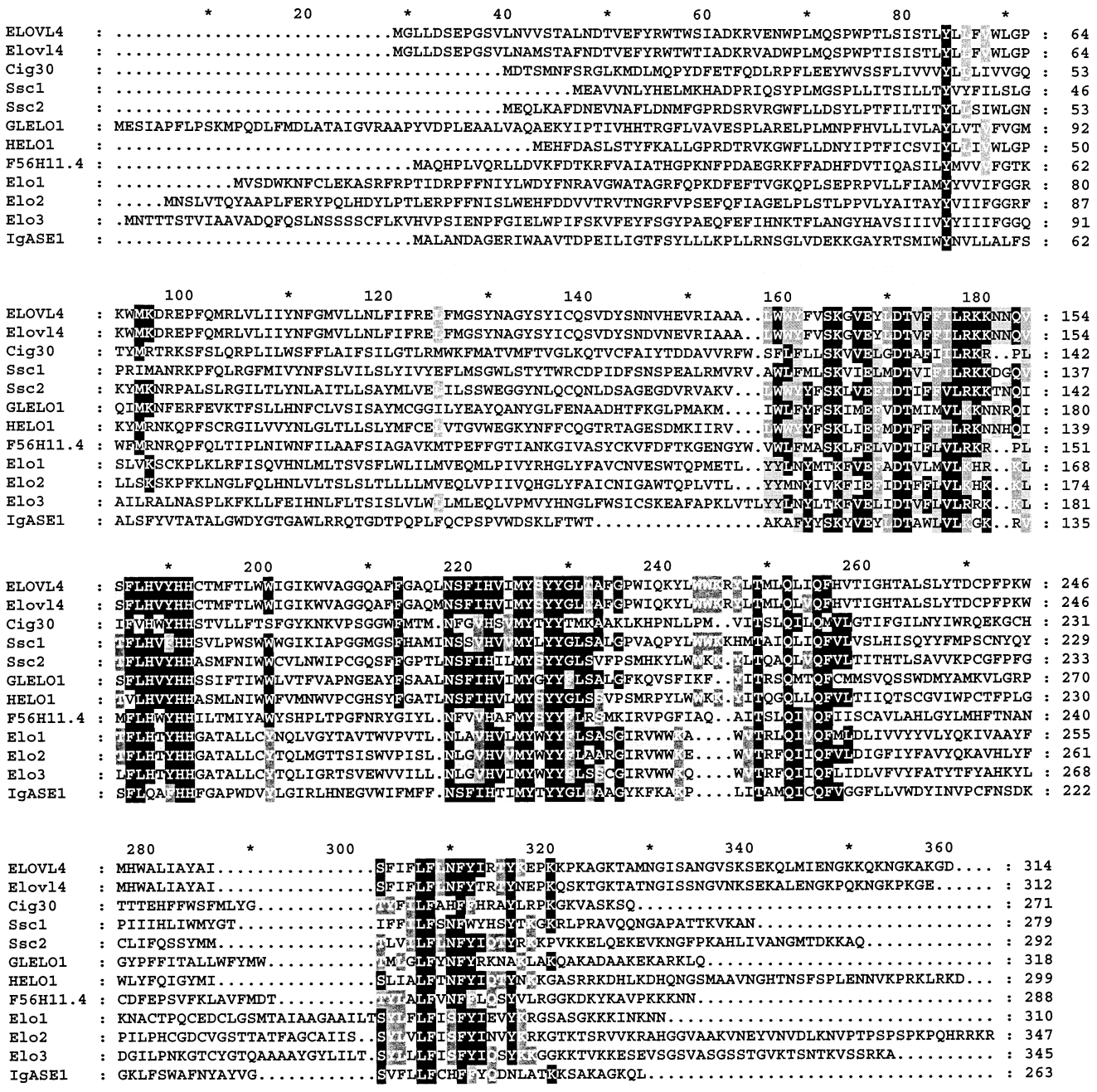

Fig. 2. Alignment of the predicted protein sequence of *I. galbana IgASE1* with sequences predicted from other known elongating activity genes. *ELOVL4* [11] and *HELO1* [5] are from human; *Elovl4* [11], *Cig30*, *Ssc1* and *Ssc2* [22,23] are from mouse; *GLELO1* [9] is from *M. alpina*; *F56H11.4* [10] is from *C. elegans*; *Elo1, Elo2* and *Elo3* [8,19] are from yeast.

with human ELOVL4 and 24% identity with the mouse equivalent Elovl4 [11], and 24%, 23% and 21% identity, respectively, with the Elo3, Elo2 and Elo1 proteins from yeast [8]. The lowest identity value of 19% was found with F56H11.4 from *C. elegans* [10]. There is no obvious similarity between IgASE1 and the plant condensing enzyme FAE1, which is required for the synthesis of the mono-unsaturated fatty acid, erucic acid (C22:1n-9, Δ^{13}) in *Arabidopsis* [15].

3.2. Functional characterisation of IgASE1 in yeast

Further characterisation of *IgASE1* was achieved by expression in yeast. The complete ORF was amplified from the cDNA library and cloned in pCR2.1-TOPO. Recombinant plasmids were screened for orientation of the insert to allow subsequent excision with the 5′ primer-encoded *Kpn*I site and the *Sac*I site in the flanking vector sequence. The 5′ *Kpn*I site also provided an A base at −3 with respect to the start codon for efficient translation initiation in yeast cells. The *IgASE1* ORF was inserted into the yeast expression vector pYES2 to create pY2ASE1, and this was transferred to the yeast strain, W303-1A.

To determine the substrate specificity of the *IgASE1* prod-

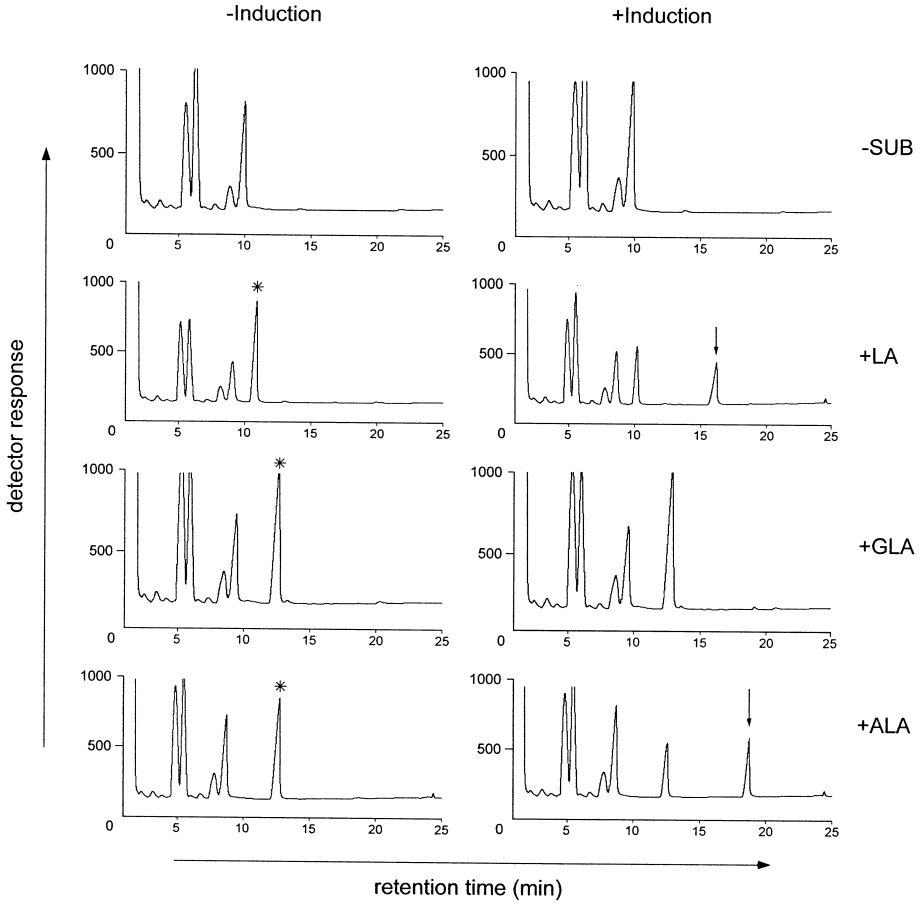

Fig. 3. Gas chromatograms of fatty acid methyl esters extracted from transformed yeast containing pY2ASE1. Yeast cultures were grown in the absence (−SUB) or presence (+LA, +GLA, +ALA) of exogenous fatty acid substrates. Exogenous fatty acids (in the form of their sodium salts) were linoleic acid (18:2n-6, $\Delta^{9,12}$, +LA), γ-linolenic acid (18:3n-6, $\Delta^{6,9,12}$, +GLA) and α-linolenic acid (18:3n-3, $\Delta^{9,12,15}$, +ALA); peaks corresponding to added substrates are indicated by asterisks in the GC traces of uninduced cultures. Expression of *IgASE1* was induced by the addition of galactose to 2%. Yeast cells were harvested after 48 h and fatty acids extracted and assayed as their methyl esters using standard methods. Peaks were identified by co-migration with known standards. Arrows in induced +LA and +ALA traces indicate novel peaks, which were confirmed by GC-MS as EDA (20:2n-6, $\Delta^{11,14}$) and ETrA (20:3n-3, $\Delta^{11,14,17}$), respectively.

uct, the transformed yeast cells were grown in minimal medium with raffinose as the carbon source and supplemented with a range of individual long chain PUFAs. The results (Fig. 3 and Table 2) show that when LA (18:2n-6, $\Delta^{9,12}$) and ALA (18:3n-3, $\Delta^{9,12,15}$) were present in the medium, EDA (20:2n-6, $\Delta^{11,14}$) and ETrA (20:3n-3, $\Delta^{11,14,17}$) accumulated to 9.1 and 8.4 mol% of total fatty acids, respectively. This represented about 45% conversion [product/(product+

Table 2
Fatty acid elongation of different substrates supplied to yeast cells transformed with pY2ASE1

Fatty acid	Mol% of fatty acid							
	−substrate		+LA (18:2n-6)		+ALA (18:3n-3)		+GLA (18:3n-6)	
	+gal	−gal	+gal	−gal	+gal	−gal	+gal	−gal
16:0	28.7	30.2	27.0	28.9	26.6	28.9	30.3	31.0
16:1n-9	41.6	42.4	30.7	25.4	30.1	26.4	24.3	24.6
18:0	6.8	6.1	5.7	5.8	6.3	6.3	6.8	6.2
18:1n-9	22.9	21.3	16.5	13.4	18.4	16.6	14.7	13.4
18:2n-6*	–	–	11.0	26.5	–	–	–	–
18:3n-6*	–	–	–	–	–	–	24.2	24.8
18:3n-3*	–	–	–	–	10.2	21.8	–	–
20:2n-6	–	–	9.1	–	–	–	–	–
20:3n-3	–	–	–	–	8.4	–	–	–
% elongation	0	0	45.3	–	45.2	–	0	–

Exogenous fatty acids supplied as substrates for elongation are indicated by asterisks. The values given are expressed as mol% of total fatty acid methyl esters identified by GC and flame ionization detection. In the case of elongated substrates, this is also expressed as % elongation (product/(product+substrate)×100). Expression of the *IgASE1* transgene was induced by the addition of galactose. Only C18 substrates with a double bond at the Δ^9 position were elongated by the IgASE1. All values are the means of triplicates from three separate experiments.

substrate)×100] of both C18 fatty acid substrates into C20 fatty acids after 48 h growth of the transformed yeast cells. GLA (18:3n-6, $\Delta^{6,9,12}$) was not elongated in the transformed yeast (Fig. 3). Other fatty acids, such as 16:1n-7, Δ^{9}, 18:1n-9, Δ^{9}, 18:4n-3, $\Delta^{6,9,12,15}$, 20:5n-3, $\Delta^{5,8,11,14,17}$ and 22:5n-3, $\Delta^{7,10,13,16,19}$, were also found to be inactive, however, the substrates were always recovered in lipid extracts of the cells (data not shown). Although in these experiments the incorporation of putative fatty acid substrates into membrane components was not investigated, it is noteworthy in the functional characterisation of other fatty acid genes using the yeast system [5,18,25] that the predicted fatty acid conversions have always been observed. These data clearly demonstrate that *IgASE1* encodes a C18-Δ^{9}-specific PUFA-elongating activity.

4. Discussion

We have used PCR in combination with a cDNA library construction to isolate a sequence encoding a C18-Δ^{9}-specific PUFA-elongating activity from the DHA-producing microalga, *I. galbana*. The predicted polypeptide sequence shares only limited identity (up to 27%) with other proteins characterised as having related activity, such as GLELO from *M. alpina*, human HELO1 and the protein encoded by F56H11.4 from *C. elegans*. Other pair-wise alignments of Elo-like proteins (data not shown) produce higher percentage identities, but these probably reflect more the sources of the genes rather than any higher degree of functional relatedness. Hence alignments of the yeast proteins Elo1, Elo2 and Elo3 produce identities in the range 45–56%, while the mammalian proteins HELO1, ELOVL4/Elovl4, Ssc1 and Ssc2 generate identity values in the range 41–57%. The lower identity values arising from comparisons across taxonomic groupings (such as IgASE1 with all the others) result from averaging relatively high degrees of conservation over a limited 'core region' of the proteins, with very little conservation over the large stretches comprising the rest of the sequences. An effect of this is to maintain various consensus motifs identified in known examples of this category of protein, such as KXXEXXDT, HXXMYXYY and TXXQXXQ, while FHXXHH is modified to FH/QXXHH. This last motif is interesting because H/QXXHH also comprises one of the three histidine boxes identified in almost all membrane desaturases and other dioxy iron cluster proteins [7], and deviation from the consensus of HXXHH for the third histidine box to QXXHH is also apparent in various so-called front-end desaturases, including Δ^{5} desaturases from *M. alpina* [18,24] and *C. elegans* [25], Δ^{6} desaturases from *Anabaena* [26] *Borago* [27] and *C. elegans* [28], and the Δ^{8} desaturase from *Euglena gracilis* [6]. Histidine boxes in desaturases and other dioxy iron protein clusters have been implicated in the binding of di-iron [7], but it remains to be determined whether they also play this role in IgASE1 and other proteins with elongating activity.

S. cerevisiae possesses multiple fatty acid elongation systems [8,19], and so by providing the ubiquitous elongase components the yeast expression system has proved a valuable testing ground for characterising the function of putative fatty acid-elongating condensing components from various sources [9,10]. IgASE1 was strictly selective and could only elongate the Δ^{9}-desaturated C18 (ω3 or ω6) PUFAs, LA (18:2n-6, $\Delta^{9,12}$) and ALA (18:3n-3, $\Delta^{9,12,15}$) to yield EDA (20:2n-6, $\Delta^{11,14}$) and ETrA (20:3n-3, $\Delta^{11,14,17}$), respectively. For both fatty acid substrates some 45% was converted to products in the transformed yeast cells.

Some PUFA-elongating activities are active on a range of substrates; human HELO1, for example, can elongate both C18 and C20 to C20 and C22 PUFAs along both the ω3 and ω6 pathways [5]. In contrast, GLELO1 from *M. alpina* showed substrate discrimination, acting specifically on the two products of Δ^{6} desaturation, 18:3n-6 ($\Delta^{6,9,12}$) and 18:4n-3 ($\Delta^{6,9,12,15}$) [9].

It is noteworthy that the gene from *Euglena* encoding a Δ^{8} desaturase activity [6] is specific for the elongation products of IgASE1, ETrA and EDA. This implies that the ω3 (Δ^{8}) desaturase pathway for the synthesis of EPA and DHA is present in *I. galbana* (Fig. 1). It is uncertain, however, whether this coexists with the ω3 (Δ^{6}) desaturase pathway (Fig. 1). Fatty acid analysis of *I. galbana* (Table 1) showed the presence of 18:4n-3, $\Delta^{6,9,12,15}$, a fatty acid known to be specifically synthesised from ALA through the activity of a Δ^{6} desaturase [28–30]. Providing 18:4n-3, $\Delta^{6,9,12,15}$ can be elongated to ETA then this suggests that both ω3 pathways may operate in *I. galbana* for the production of long chain PUFAs.

The fact that the product of *IgASE1* elongates 18:2 to EDA (Table 2 and Fig. 3) and that the *Euglena* Δ^{8} desaturase can act upon this product to produce 20:3n-6, $\Delta^{8,11,14}$ [6] is interesting and implies that the ω6 (Δ^{8}) pathway (Fig. 1) should also operate in *I. galbana*. Although no EDA, GLA or AA could be detected in *I. galbana* it is possible that the enzymes catalysing the synthesis of these intermediates and products are relatively highly active and that there is a rapid flux through these parts of the pathway. Such a flux is also evident in the ω3 pathways in that the intermediates ETrA and ETA were also not detected in fatty acid analyses.

If an ω6 (Δ^{8}) pathway can operate in *I. galbana* then what happens to the products 20:3n-6 and 20:4n-6? Work with *M. alpina* [31] suggested the existence of a desaturase introducing a double bond at the Δ^{17} position of AA to produce EPA, and Spychalla et al. [32] have isolated a *C. elegans* glycerolipid desaturase with ω3 activity on a range of ω6 fatty acid substrates, including DGLA and AA. It is possible that a similar desaturase activity is present in *I. galbana*, rapidly channelling ω6 (Δ^{8}) desaturase pathway products into the ω3 (Δ^{6}) and ω3 (Δ^{8}) pathways for EPA and DHA production (Fig. 1). The presence of 18:4n-3, $\Delta^{6,9,12,15}$ in *I. galbana* implies Δ^{6} desaturase activity (see above). Δ^{6} desaturases are generally non-specific for ω3 and ω6 C18 fatty acids, and can utilise both LA and ALA [29,30]. If this were the case in *I. galbana*, one might anticipate that the ω6 (Δ^{6}) pathway is also active, again with the products rapidly transferred to ω3 fatty acid synthesis via Δ^{17} desaturation (Fig. 1). It is of course possible that the Δ^{6} desaturase is specific for ALA and hence dedicated to ω3 fatty acid production. *I. galbana*, therefore, provides an excellent experimental system for investigating ω3 fatty acid biosynthesis, the interactions between the Δ^{6} and Δ^{8} desaturase pathways and the possible involvement of ω6 metabolism in the production of EPA.

The isolation and characterisation of a novel PUFA elongating activity from *I. galbana*, described in this paper, should make a significant contribution to the determination of structure–function relationships in this class of protein. IgASE1 appears to be less closely related to the Elo-like proteins described hitherto than they are to each other. It is distinct in

having clear specificity for Δ^9-desaturated C18 PUFAs, and this may prove to be of biotechnological as well as biochemical significance.

Acknowledgements: Institute of Arable Crops Research receives grant-aided support from the Biotechnology and Biological Sciences Research Council.

References

[1] Harwood, J.L., Cryer, A., Gurr, M.I. and Dodds, P. (1994) in: The Lipid Handbook (Gunstone, F.D., Harwood, J.H. and Padley, F.B., Eds.), pp. 665–683, Chapman and Hall, London.
[2] Crawford, M.A. (2000) Am. J. Clin. Nutr. 71, 275–284.
[3] Gill, I. and Valivety, R. (1997) Trends Biotechnol. 15, 401–409.
[4] Napier, J.A., Michaelson, L.V. and Stobart, A.K. (1999) Curr. Opin. Plant Biol. 2, 123–127.
[5] Leonard, A.E., Bobik, E.G., Dorado, J., Kroeger, P.E., Chuang, L.T., Thurmond, J.M., Parker-Barnes, J.M., Das, T., Huang, Y.S. and Mukerji, P. (2000) Biochem. J. 350, 765–770.
[6] Wallis, J.G. and Browse, J. (1999) Arch. Biochem. Biophys. 365, 307–316.
[7] Shanklin, J. and Cahoon, E.B. (1998) Annu. Rev. Plant Physiol. Plant Mol. Biol. 49, 611–641.
[8] Toke, D.A. and Martin, C.E. (1996) J. Biol. Chem. 271, 18413–18422.
[9] Parker-Barnes, J.M., Das, T., Bobik, E., Leonard, A.E., Thurmond, J.M., Chuang, L., Huang, Y.S. and Mukerji, P. (2000) Proc. Natl. Acad. Sci. USA 97, 8284–8289.
[10] Beaudoin, F., Michaelson, L.V., Hey, S.J., Lewis, M.J., Shewry, P.R., Sayanova, O. and Napier, J.A. (2000) Proc. Natl. Acad. Sci. USA 97, 6421–6426.
[11] Zhang, K., Kniazeva, K., Han, M., Li, M., Yu, W., Yang, Z., Li, Z., Metzker, Y., Allikmets, R., Zack, D.J., Kakuk, L.E., Lagali, P.S., Wong, P.W., MacDonald, I.M., Sieving, P.A., Figueroa, D.J., Austin, C.P., Gould, R.J., Ayyagari, R. and Petrukhin, K. (2001) Nature Genet. 27, 89–93.
[12] Sprecher, H., Luthria, D.L., Mohammed, B.S. and Baykousheva, S.P. (1995) J. Lipid Res. 36, 2471–2476.
[13] Luthria, D.L., Mohammed, B.S. and Sprecher, H. (1996) J. Biol. Chem. 271, 16020–16025.
[14] Cinti, D.L., Cook, L., Nagi, M.H. and Suneja, S.K. (1992) Prog. Lipid Res. 31, 1–51.
[15] Millar, A. and Kunst, L. (1997) Plant J. 12, 121–131.
[16] Guillard, R.R.L. and Ryther, J.H. (1962) Can. J. Microbiol. 8, 229–239.
[17] Elble, R. (1992) BioTechniques 13, 18–22.
[18] Michaelson, L.V., Lazarus, C.M., Griffiths, G., Napier, J.A. and Stobart, A.K. (1998) J. Biol. Chem. 273, 19055–19059.
[19] Oh, C., Toke, A.D., Mandala, S. and Martin, C.E. (1997) J. Biol. Chem. 272, 17376–17384.
[20] Jackson, M.R., Nilsson, T. and Peterson, P.A. (1990) EMBO J. 9, 3153–3162.
[21] Jackson, M.R., Nilsson, T. and Peterson, P.A. (1993) J. Cell Biol. 121, 317–333.
[22] Tvrdik, P., Asadi, A., Kozak, L.P., Nedergaard, J., Cannon, B. and Jacobsson, A. (1997) J. Biol. Chem. 272, 31738–31746.
[23] Tvrdik, P., Westerberg, R., Silve, S., Asadi, A., Jacobsson, A., Cannon, B., Loison, G. and Jacobsson, A. (2000) J. Cell Biol. 149, 707–717.
[24] Knutzon, D.S., Thurmond, J.M., Huang, Y.S., Chaudhary, S., Bobik, E.G.Jr., Chan, G.M., Kirchner, S.J. and Mukerji, P. (1998) J. Biol. Chem. 273, 29360–29366.
[25] Michaelson, L.V., Napier, J.A., Lewis, M., Griffiths, G., Lazarus, C.M. and Stobart, A.K. (1998) FEBS Lett. 439, 215–218.
[26] Reddy, A.S., Nuccio, M.L., Gross, L.M. and Thomas, T.L. (1993) Plant Mol. Biol. 27, 293–300.
[27] Sayanova, O., Smith, M.A., Lapinskas, P., Stobart, A.K., Dobson, G., Christie, W.W., Shewry, P.R. and Napier, J.A. (1997) Proc. Natl. Acad. Sci. USA 94, 4211–4216.
[28] Griffiths, G., Jackson, F., Brechany, E.Y., Christie, W.W., Stymne, S. and Stobart, A.K. (1996) Phytochemistry 203, 381–386.
[29] Cho, H.P., Nakamura, M.T. and Clarke, S.D. (1999) J. Biol. Chem. 274, 471–477.
[30] Sayanova, O.V., Shewry, P.R. and Napier, J.A. (1999) Plant Physiol. 121, 641–646.
[31] Shimizu, S., Kawashima, H., Akimoto, K., Shinmen, Y. and Yamada, H. (1989) Appl. Microbiol. Biotechnol. 32, 1–4.
[32] Spychalla, J.P., Kinney, A.J. and Browse, J. (1997) Proc. Natl. Acad. Sci. USA 94, 1142–1147.