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Identification of *Primula* fatty acid Δ^6-desaturases with *n*-3 substrate preferences[1]

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Abstract Fatty acid Δ^6-desaturation, the first committed step in C_{20} polyunsaturated fatty acid biosynthesis, is generally considered not to discriminate between *n*-3 and *n*-6 substrates. We previously identified higher plant species that showed preferential Δ^6-desaturation of *n*-3 C_{18} fatty acid substrates. A polymerase chain reaction-based approach was used to isolate 'front-end' cytochrome b_5 fusion desaturases from *Primula vialii* Franchet and *Primula farinosa* L. Functional analysis in *Saccharomyces cerevisiae* identified fatty acid Δ^6-desaturases with a strong specificity for the *n*-3 substrate α-linolenic acid (18:3$\Delta^{9,12,15}$). These results indicate that the accumulation of octadecatetraenoic acid (18:4$\Delta^{6,9,12,15}$) in planta is due to the activity of a novel *n*-3-specific fatty acid Δ^6-desaturase.

Key words: Cytochrome b_5 fusion; Desaturase; γ-Linolenic acid; Polyunsaturated fatty acid

1. Introduction

Polyunsaturated fatty acids (PUFAs) perform many important functions in the cellular metabolism of mammals. They are key components of cell membranes and affect both membrane fluidity and the properties of a range of membrane-bound receptors, enzymes and channels [1]. They also help to regulate oxygen and electron transport in the processes of oxidation and energy production, and maintain growth, vitality and mental state in mammals. Perhaps the most crucial role of PUFAs is in the biosynthesis of eicosanoids, serving as precursors for molecules such as prostaglandins, thromboxanes and leukotrienes that influence a wide range of functions, including inflammation, blood clotting, development of allergies and regulation of cholesterol metabolism [1,2]. PUFAs are widely distributed throughout the animal and plant kingdoms as well as in micro-organisms, though C_{20} PUFAs are not normally present in higher plants [3].

PUFAs can be classified into the *n*-6 and *n*-3 families, derived from the two essential fatty acids (EFA), linoleic acid (LA, 18:2$\Delta^{9,12}$; *n*-6) and α-linolenic acid (ALA, 18:3$\Delta^{9,12,15}$; *n*-3) respectively [3,4]. These two fatty acids are crucial for human health and nutrition since they cannot be synthesised in the body and hence must be provided by the diet. In mammals, LA and ALA are metabolised to arachidonic acid (20:4$\Delta^{5,8,11,14}$; *n*-6) and eicosapentanoic acid (20:5$\Delta^{5,8,11,14,17}$; *n*-3), respectively, by a series of desaturation (and a C_2-elongation) reactions [3,4]. It is generally considered that the two families of fatty acids (*n*-3, *n*-6) are not inter-convertible, and their metabolites are functionally distinct with opposing physiological functions [1]. γ-Linolenic acid (GLA, 18:3 $\Delta^{6,9,12}$) is the first intermediate of the *n*-6 series, formed through the Δ^6-desaturation of LA. Octadecatetraenoic acid (OTA; 18:4$\Delta^{6,9,12,15}$) is also synthesised by the same enzyme activity (via Δ^6-desaturation of ALA) and is the first intermediate of the *n*-3 metabolic pathway [3]. As the initial step in EFA metabolism, the fatty acid Δ^6-desaturase is clearly a key enzyme in *n*-3/*n*-6 PUFA homeostasis. The first example of the microsomal Δ^6-desaturase was cloned from the plant borage (*Borago officinalis*) [5] and shown to define a new class of cytochrome b_5 fusion lipid desaturases. Subsequently, further Δ^6-desaturase orthologues were identified from the nematode worm *Caenorhabditis elegans* [6], mammals [7,8], fungi [9], mosses [10,11] and higher plants [12]. Functional characterisation of these desaturases in yeast indicated no distinct preference for *n*-6 substrates compared to *n*-3 substrates [10,11,13]. This has been seen as evidence for the balance of flux through the *n*-3/*n*-6 pathway being defined by substrate availability, rather than substrate preference of enzymes [2,4,13].

Whilst the majority of plant species do not synthesise Δ^6-desaturated fatty acids, a few families such as Saxifragaceae, Primulaceae and Boraginaceae contain species which accumulate these lipids [14]. We have previously shown that the distribution of *n*-3 and *n*-6 Δ^6-desaturated fatty acids in the Primula family does not correlate with substrate availability [15]. Here we report the isolation of several cytochrome b_5 fusion desaturases from two *Primula* species, *Primula farinosa* L. (syn. *Aleuritia farinosa* L.) and *Primula vialii* Franchet (syn. *Muscarioides vialii* Franchet) and their functional expression in *Saccharomyces cerevisiae*. This has allowed us to identify

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[1] The sequences identified in this study have been deposited in GenBank, with the accession numbers AY234124, AY234125, AY234126 and AY234127.

Abbreviations: ALA, α-linolenic acid; EFA, essential fatty acid; GLA, γ-linolenic acid; LA, linoleic acid; LCB, long chain base; ORF, open reading frame; OTA, octadecatetraenoic acid; PUFA, polyunsaturated fatty acid

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fatty acid Δ^6-desaturases with distinct specificity towards *n*-3 substrates.

2. Materials and methods

2.1. Plant material

Seeds were obtained from Chiltern Seeds, Cumbria, UK.

2.2. Nucleic acid manipulation

Total RNA was isolated from young leaves of *P. vialii* Franchet and *P. farinosa* L. using a RNAeasy kit (Qiagen, Crawley, UK). Poly(A)$^+$ RNA was purified from total RNA using oligo(dT) cellulose according to standard methods [16] and was reverse transcribed with the SMART RACE cDNA Amplification kit (BD-Clontech, Basingstoke, UK) according to the manufacturer's instructions. Single-stranded cDNAs prepared from these poly(A)$^+$ RNAs were used for polymerase chain reaction (PCR)-based cloning. Genomic DNA was isolated using the GenElute Plant Genomic DNA kit (Sigma, Poole, UK).

2.3. PCR-based cloning

cDNAs were amplified as previously described [5] with degenerate primers designed to conserved amino acid sequences of plant b_5-fusion desaturases: G-H-D-S-G-H-Y (5′-GGICA(T/C)GA(T/C)(T/A/G)(C/G)IGGICA(T/C)TA-3′), W-E-A-L-(N/H)-T-H-G (5′-CC(A/G)TCIGT(A/G)T(T/G)IA(A/G)IGC(T/C)TCCCA-3′). PCR amplification products were cloned into pGEM-T Easy (Promega, Southampton, UK) and sequenced to identify candidate desaturase clones for further analysis. The 5′ ends of putative desaturases were amplified with the SMART RACE cDNA system (BD-Clontech).

The derived 5′ cDNA sequence data were used to amplify full-length copies of the four putative desaturases from cDNAs of *P. farinosa* and *P. vialii*. Gene-specific primers were designed to the 5′ and 3′ ends of the coding regions of the corresponding desaturase sequences, with restriction sites to facilitate cloning into the yeast expression vector pYES2 (Invitrogen, Paisley, UK). The following pairs of forward/reverse primers were used (restriction sites *Kpn*I and *Eco*RI are indicated in bold):

far6: 5′-**ggtacc**atggctaacaaatctcccacc-3′
5′-**gaattc**tcacccgagagttttaagagct-3′
far8: 5′-**ggtacc**atggctgatccaccaccaa-3′
5′-**gaattc**ctacccatgggtgttcacagc-3′
vial6: 5′-**ggtacc**atggctaacaaatctcccacc-3′
5′-**gaattc**ttagccgtgtgtgtggacggctt-3′
vial8: 5′-**ggtacc**atggctgatccaccaccaa-3′
5′-**gaattc**ctacccatgggtgttcacagc-3′

The amplified PCR products were gel-purified, restricted and cloned into the corresponding restriction sites behind the galactose-inducible GAL1 promoter of pYES2 to yield the plasmids pYFar6, pYVial6, pYFar8 and pYVial8. Genomic PCR products were generated via the same specific primers using the Expand Fidelity PCR System (Roche Diagnostics, Lewes, UK) and genomic DNA as a template.

2.4. Functional expression in yeast

The pYFar6, pYVial6, pYFar8 and pYVial8 plasmids and pYES2 vector were transformed into *S. cerevisiae* by a lithium acetate method [17]. Cultures were grown at 22°C in the presence of 2% (v/v) raffinose and expression of the transgenes was induced by the addition of galactose to 2% (w/v) in the presence of 0.5 mM of the corresponding fatty acid and 1% (w/v) tergitol–Nonidet P-40 (Sigma) as described [6].

2.5. Fatty acid analysis

Total fatty acids extracted from yeast cultures were analysed by gas chromatography (GC) of methyl ester derivatives [5]. Fatty acids were identified by comparison with the retention times of fatty acid methyl ester (FAME) standards (Sigma) and by GC-mass spectrometry (MS) [5,6].

2.6. Long chain base (LCB) analysis

Induced yeast cells were subject to strong alkaline hydrolysis and the extracted LCB were converted to dinitrophenyl derivatives and analysed by reversed-phase high-performance liquid chromatography using previously described methods [18,19].

3. Results and discussion

3.1. Isolation of cytochrome b_5 fusion desaturases from Primula species

Comparison of the deduced amino acid sequences from previously cloned plant cytochrome b_5 fusion desaturases [20–22] revealed a high level of identity between the different members of this family of proteins (which includes the sphingolipid LCB Δ^8-desaturase [18], as well as the fatty acid Δ^6-desaturase). Based on this amino acid similarity, two degenerate primers for PCR were synthesised. The forward primer was designed to the first histidine box and the reverse primer to the complement of the actual C-terminus of plant cytochrome b_5-fused desaturases. The primers were used in PCR reactions in which single-stranded cDNAs derived from two *Primula* species, *P. farinosa* and *P. vialii*, were used as template; GC analysis of material from these plants previously indicated high levels of OTA in seed and vegetative tissues [15]. PCR products of the expected length (870 bp) were cloned and sequenced. The clones isolated from the two *Primula* species encoded polypeptide sequences of the same length but with distinct sequences, both with the expected similarity to fatty acyl Δ^6- and sphingolipid Δ^8-desaturases. One class of sequences from *P. farinosa* and *P. vialii* showed slightly higher identity to the borage Δ^6 fatty acid desaturase and were therefore designated far6 and vial6, while a second class of products from the same two species showed higher homology to the family of plant sphingolipid LCB Δ^8-desaturases [18] and were designated far8 and vial8. These four partial sequences were used to design 5′-RACE primers to amplify the 5′ ends of four putative desaturases using a SMART RACE Kit. The resulting PCR products, corresponding to the four partial *Primula* cDNA clones, were cloned and sequenced. Full-length open reading frames (ORFs) for these four putative desaturases were amplified from the appropriate cDNA template and then cloned and resequenced to confirm the initial identification.

3.2. Sequence analysis of 'front-end' desaturases from the Primulaceae

The *Primula* cDNA clones encode very similar polypeptides of 453 (Far6 and Vial6) and of 452 (Far8 and Vial8) amino acids (Fig. 1). Visual inspection and database searches confirmed that all four polypeptides contain N-terminal cytochrome b_5 domain fused to the presumptive desaturase domain. Similarly, the cytochrome b_5 domains of all four *Primula* proteins contained the eight invariant residues characteristic for the cytochrome b_5 superfamily and H-P-G-G haem-binding motif which has been shown to be essential for enzymatic activity [20,22,23]. The desaturase domains of Far6, Far8, Vial6 and Vial8 have three conserved histidine boxes that are characteristic of all membrane-bound desaturases [24], with the third histidine box containing a glutamine for histidine substitution ([H→Q]-x-x-H-H) which is a conserved feature of all 'front-end' acyl (Δ^4, Δ^5, Δ^6, Δ^8) and sphingolipid Δ^8-desaturases characterised so far [20–22]. Site-directed mutagenesis of the variant third histidine box demonstrated that this glutamine is essential for enzyme activity of the borage Δ^6-desaturase and histidine is not able to substitute for this residue [25]. The deduced amino acid sequences of the Far6 and Vial6 proteins had 92% identity to each other and 66% identity to the borage fatty acid Δ^6-desa-

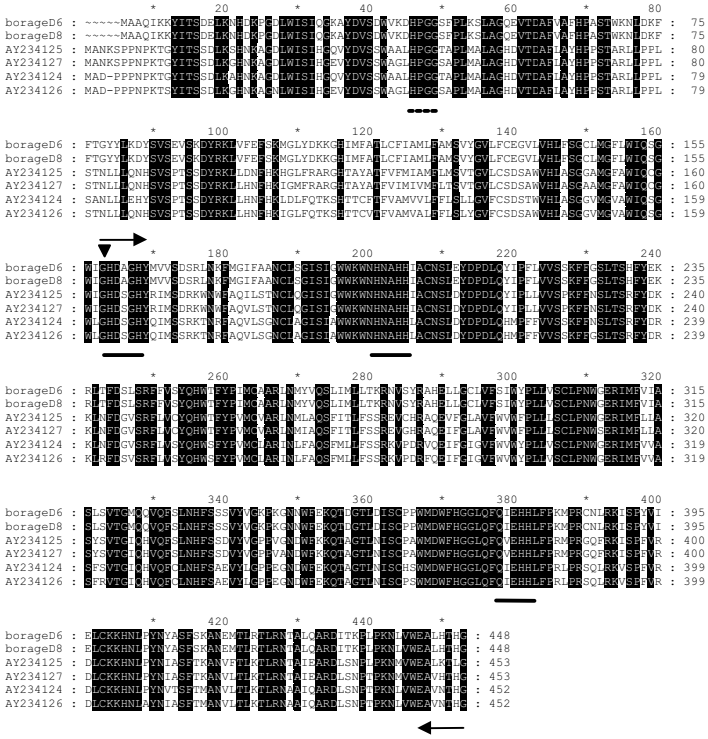

Fig. 1. Sequence comparisons of *Primula* desaturases. Comparison of amino acid sequences of *Primula* cytochrome b_5 fusion desaturases Far8 (AY234124), Far6 (AY234125), Vial8 (AY234126) and Vial6 (AY234127) with related sequences (borage fatty acid Δ^6- and sphingolipid Δ^8-desaturases). The position of conserved motifs is underlined either with solid lines (histidine boxes) or a broken line (cytochrome b_5 haem-binding motif). Arrows indicate the positions of the PCR primers. The position of the intron in the *P. vialii* Δ^6-desaturase (AY234127) is indicated by a solid triangle. Numbers refer to GenBank accessions for the *Primula* desaturases.

turase. Similarly, the two predicted sequences for putative sphingolipid desaturases Far8 and Vial8 had 97% amino acid identity with each other and 63% identity with the borage Δ^8-sphingolipid desaturase [26]. Whilst there is clearly a high degree of similarity between the candidate *Primula* sphingolipid Δ^8-desaturases and the fatty acid Δ^6-desaturases (80% identity) it is not possible to identify any precise amino acids which might serve as determinants of either substrate or regio specificity. Previous attempts to alter the activity of the borage Δ^6-desaturase via chimeric domain swaps with the borage sphingolipid Δ^8-desaturase failed to identify any one of eight different regions as being functionally dominant [27]. This may imply that the determinants of enzymatic specificity are dispersed throughout the polypeptide sequence. Intriguingly, the four *Primula* ORFs display their highest level of conserved amino acids throughout the N-terminal cytochrome b_5 domain, though this region is not a determinant of substrate [27].

'Front-end' cytochrome b_5 fusion desaturase genes from mammals, *C. elegans* and moss contained multiple introns interspersed throughout their coding sequence, with their conserved distribution indicated a role for gene duplication in enzyme evolution [28]. In contrast, little is known about the distribution of introns in plant genes corresponding to either the fatty acyl Δ^6- or sphingolipid Δ^8-desaturases. We therefore cloned genomic sequences from the two *Primula* species corresponding to the four desaturases identified above. Genomic PCR resulted in products of identical length to that obtained with the same primers on cDNA templates for Far6, Far8 and Vial8, indicating the absence of introns in these genes. However, the genomic PCR product for the *P. vialii* Vial6 desaturase was 221 bp longer than the corresponding cDNA sequence, indicative of presumptive intron sequences. Sequencing of this genomic PCR product positioned a single intron just before the first histidine box (see Fig. 1). This putative intron did not have an obvious consensus splice site at either the 5′ or 3′ junctions but contained an identical 10 bp sequence at both junctions, resembling a mobile genetic element rather than a 'consensus' intron.

3.3. *Functional characterisation of* Primula *cytochrome* b_5 *fusion desaturases*

As described above, the two *Primula* cDNA sequences designated Far8 and Vial8 appeared to be orthologous to functionally characterised sphingolipid LCB Δ^8-desaturases from other plant species. To confirm that these *Primula* cytochrome b_5 fusion proteins were involved in sphingolipid, but not fatty acyl, desaturation, the ORF from each cDNA was cloned into the yeast expression vector pYES2 and characterised as previously described [18,19,26]. This indeed confirmed that both these cytochrome b_5 fusion desaturases functioned as sphingolipid desaturases by introducing a (stereo-unselective) double bond at the Δ^8 position of the LCB (data not shown).

The two full-length cDNAs corresponding to the two *Primula* ORFs predicted to encode fatty acyl Δ^6-desaturases were cloned into pYES2 to give constructs designated pYFar6 and pYVial6. These plasmids or the empty vector were transformed into *S. cerevisiae* strain W303-1A; transformed cells were grown in a minimal medium containing raffinose as the carbon source and induced with 2% galactose. Since *S. cere-*

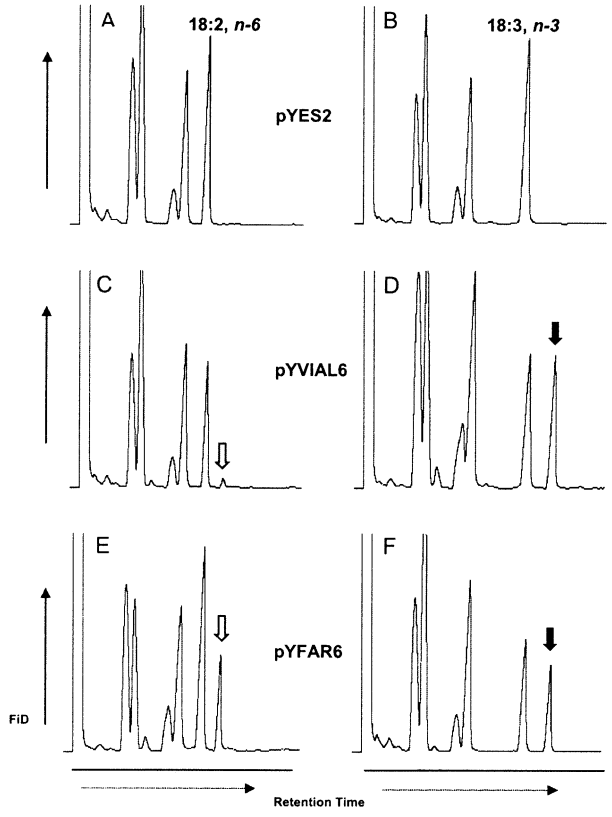

Fig. 2. Identification of Δ^6-desaturated fatty acids in transgenic yeast. FAMEs of total lipids were prepared from yeast cells grown under inducing conditions (+galactose) in the presence of LA (*n*-6; panels A,C,E) or ALA (*n*-3; panels B,D,F). Yeast transformed with empty vector pYES2 (control) (panels A,B). Yeast transformed with the Δ^6-desaturase from *P. farinosa* (pYFar6) (panels C,D). Yeast transformed with the Δ^6-desaturase from *P. vialii* (pYVial6) (panels E,F). Arrows indicate additional peaks, corresponding to Δ^6-desaturation products, GLA (*n*-6; open arrow) and OTA (*n*-3; solid arrow).

visiae contains only saturated and monoenoic fatty acid substrates, but not the C_{18} di- and trienoic fatty acids which serve as substrates for Δ^6-desaturation, the growth medium was supplemented with either LA or ALA in the presence of 1% tergitol–Nonidet P-40 [6]. After 48 h of growth, total yeast fatty acids were extracted in methanol–HCl and the resulting FAMEs analysed by GC-MS.

GC analysis of these FAMEs revealed that yeast cells transformed with pYFar6 or pYVial6 plasmids produced an additional fatty acid, commensurate with their predicted role of Δ^6-desaturase (Fig. 2). This assignment of function was confirmed by co-migration of authentic fatty acid standards and GC-MS of the novel products, which identified them as the tri- and tetraenoic fatty acids $18{:}3\Delta^{6,9,12}$ (GLA) and $18{:}4\Delta^{6,9,12,15}$ (OTA) as previously described [5,6,25] (data not shown). Cells transformed with pYES2 empty vector did not produce any additional desaturated fatty acids (Fig. 2A,B). However, whilst both the *Primula* Δ^6-desaturases were able to desaturate either exogenously supplied $18{:}2\Delta^{9,12}$ or $18{:}3\Delta^{9,12,15}$ fatty acids, substrate preference clearly varied between the two enzymes (Fig. 2C–F). In particular, whilst the *P. farinosa* enzyme desaturated both LA and ALA, the *P. vialii* enzyme appeared to use only ALA as a substrate.

To better compare the substrate specificity of *Primula* plant fatty acid Δ^6-desaturases, yeast cultures expressing pYFar6 or pYVial6 were exogenously supplied with equimolar amounts of both LA and ALA substrates. Similar analysis was also carried out with the borage Δ^6-desaturase, which served as a (non-selective) control. GC analysis of the resulting desaturation products indicated that the borage Δ^6-fatty acid desaturase displayed only a slight preference for the *n*-6 substrate (LA), whereas the *Primula* Δ^6-desaturases displayed a distinct preference for the *n*-3 substrate ALA (Table 1). However, whilst the *P. farinosa* Δ^6-desaturase displayed only a moderate preference for ALA over LA, in the case of the *P. vialii* enzyme this preference was very pronounced. Based on the data given in Table 1, the ratio of *n*-3/*n*-6 Δ^6-desaturated fatty acids produced by the *P. vialii* enzyme was 4.8, whereas the ratios for the *P. farinosa* and borage desaturases were 1.5 and 0.7, respectively. It is also clear that the increased *n*-3/*n*-6 ratio is a result of a strong decrease in the levels of Δ^6-desaturase activity towards LA (*n*-6), as opposed to an enhanced preference for ALA (*n*-3). Neither of the *Primula* fatty acid Δ^6-desaturases showed activity on exogenously supplied C_{20} substrates, in agreement with previous observations with the borage Δ^6-desaturase [25]. Furthermore, neither enzyme introduced a Δ^6 double bond into endogenous (Δ^9-unsaturated) palmitoleic acid present in the yeast cells used for heterologous expression. This differed from the results obtained with the borage Δ^6-desaturase, which was capable of using this monounsaturated fatty acid as a substrate for Δ^6-desaturation [25]. Closer inspection of the deduced amino acid sequences of the *P. vialii* and *P. farinosa* Δ^6-desaturases revealed only 14 differences between the two enzymes (see Fig. 1). Thus, it seems likely that these variations define the differences in substrate selectivity between the two desaturases. It remains an open question as to whether the presence of an intron in the *P. vialii* Δ^6-desaturase has any particular significance in the evolution of *n*-3-specific substrate preferences. However, mutations proximal to the histidine boxes of other desaturase enzymes have been shown to alter substrate specificity [29].

These data show that we have identified and functionally characterised fatty acid Δ^6-desaturases with *n*-3 preferences, this substrate specificity being very pronounced in the case of the *P. vialii* enzyme. We have previously shown that both *Primula* species had a high ratio of *n*-3/*n*-6 fatty acids in

Table 1
Fatty acid composition (%) of yeast cells expressing either the empty vector control (pYES2), borage Δ^6-desaturase (pYBdes6), *P. farinosa* Δ^6-desaturase (pYFar6) or *P. vialii* Δ^6-desaturase (pYVial6)

Fatty acid	Desaturase expression construct			
	pYes2	pYBdes6	pYFar6	pYVial6
16:0	24.8	20	23.2	20.3
$16{:}1\Delta^9$	22.5	20	18.2	21.7
$16{:}2\Delta^{6,9}$	0	0.2	0	0
18:0	6.1	6.2	5.9	4.7
$18{:}1\Delta^9$	17.1	17.1	14.9	15.8
$*18{:}2\Delta^{9,12}$	13.8	11.5	12.8	15.3
$18{:}3\Delta^{6,9,12}$	0	5.5	5	1.4
$*18{:}3\Delta^{9,12,15}$	15.6	15.5	12.2	13.9
$18{:}4\Delta^{6,9,12,15}$	0	3.9	7.7	6.8

Yeast cells were cultured in the presence of equimolar amounts of both LA and ALA (substrates for the Δ^6-desaturase, marked with an asterisk) to determine substrate preference. Total fatty acids were extracted after extensive washing of cells, with FAMEs being analysed and quantified by GC.

planta for these Δ^6-desaturation products [15]. In contrast, heterologous expression in yeast shows that the *P. vialii* enzyme has a much greater preference for *n*-3 substrates than the *P. farinosa* desaturase. This apparent discrepancy may be due to the additional activity in planta of a Δ^{15}-desaturase, which in the case of *P. farinosa* would convert GLA to OTA. However, simple GC analysis of leaf fatty acids is not able to distinguish between the synthesis of OTA by Δ^6-desaturation of ALA or Δ^{15}-desaturation of GLA. In that respect, the heterologous expression system described in this study has the advantage of defining the precise enzymatic reactions within the C_{18} Δ^6-PUFA biosynthetic pathway. Moreover, it has allowed the unambiguous identification of a Δ^6-desaturase with clear preferences for *n*-3 substrates.

4. Conclusion

We have identified and functionally characterised several cytochrome b_5 fusion desaturases from *Primula* spp. This includes a fatty acid Δ^6-desaturase from *P. vialii* that has a very strong preference for the *n*-3 substrate ALA compared with the *n*-6 substrate LA. Given the high degree of identity between the *n*-3-specific *P. vialii* enzyme and the less selective *P. farinosa* Δ^6-desaturase, it may be possible to identify the precise amino acid residues responsible for such substrate preference. The identification of a fatty acid Δ^6-desaturase with *n*-3 selectivity also has great potential in the heterologous production of *n*-3 PUFAs.

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References

[1] Gill, I. and Valivety, R. (1997) Trends Biotechnol. 15, 401–409.
[2] Sprecher, H. (2002) Prostaglandins Leukot. Essent. Fatty Acids 67, 79–83.
[3] Napier, J.A. and Michaelson, L.V. (2001) J. Sci. Food Agric. 81, 883–888.
[4] Broun, P., Gettner, S. and Somerville, C. (1999) Annu. Rev. Nutr. 19, 197–216.
[5] Sayanova, O.V., 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.
[6] Napier, J.A., Hey, S.J., Lacey, D.J. and Shewry, P.R. (1998) Biochem. J. 330, 611–614.
[7] Cho, H.P. and Nakamura, M.T. (1999) J. Biol. Chem. 274, 4711–4717.
[8] D'andrea, S., Guillou, H., Jan, S., Catheline, D., Thibault, J.N., Bouriel, M., Rioux, V. and Legrand, P. (2002) Biochem. J. 364, 49–55.
[9] Huang, Y.S., Chaudhary, S., Thurmond, J.M., Bobik Jr., E.G., Yuan, L., Chan, G.M., Kirchner, S.J. and Mukerji, P. (1999) Lipids 34, 649–659.
[10] Girke, T., Schmidt, H., Zahringer, U., Reski, R. and Heinz, E. (1998) Plant J. 15, 39–48.
[11] Sperling, P., Lee, M., Girke, T., Zahringer, U., Stymne, S. and Heinz, E. (2000) Eur. J. Biochem. 267, 3801–3811.
[12] Garcia-Maroto, F., Garrido-Cardenas, J.A., Rodriguez-Ruiz, J., Vilches-Ferron, M., Adam, A.C., Polaina, J. and Alonso, D.L. (2002) Lipids 37, 417–426.
[13] de Antueno, R.J., Knickle, L.C., Smith, H., Elliot, M.L., Allen, S.J., Nwaka, S. and Winther, M.D. (2001) FEBS Lett. 509, 77–80.
[14] Aitzetmüller, K. and Tsevegsüren, N. (1994) J. Plant Physiol. 143, 538–543.
[15] Sayanova, O., Napier, J.A. and Shewry, P.R. (1999) Phytochemistry 52, 419–422.
[16] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Plainview, NY.
[17] Elble, R. (1992) BioTechniques 13, 18–20.
[18] Sperling, P., Zahringer, U. and Heinz, E. (1998) J. Biol. Chem. 273, 28590–28596.
[19] Michaelson, L.V., Longman, A.J., Stobart, A.K. and Napier, J.A. (2002) Biochem. Soc. Trans. 30, 1073–1075.
[20] Napier, J.A., Sayanova, O., Stobart, A.K. and Shewry, P.R. (1997) Biochem. J. 328, 717–720.
[21] Napier, J.A., Sayanova, O.V., Sperling, P. and Heinz, E. (1999) Trends Plant Sci. 4, 2–4.
[22] Sperling, P. and Heinz, E. (2001) Eur. J. Lipid Sci. Technol. 103, 158–180.
[23] Sayanova, O., Shewry, P.R. and Napier, J.A. (1999) Plant Physiol. 121, 641–646.
[24] Shanklin, J., Whittle, E. and Fox, B.G. (1994) Biochemistry 33, 12787–12794.
[25] Sayanova, O.V., Beaudoin, F., Libisch, B., Castel, A.., Shewry, P.R. and Napier, J.A. (2001) J. Exp. Bot. 52, 1581–1585.
[26] Sperling, P., Libisch, B., Zahringer, U., Napier, J.A. and Heinz, E. (2001) Arch. Biochem. Biophys. 388, 293–298.
[27] Libisch, B., Michaelson, L.V., Lweis, M.J., Shewry, P.R. and Napier, J.A. (2000) Biochem. Biophys. Res. Commun. 279, 779–785.
[28] Napier, J.A. and Michaelson, L.V. (2001) Lipids 36, 761–766.
[29] Broun, P., Shanklin, J., Whittle, E. and Somerville, C. (1998) Science 282, 1315–1317.