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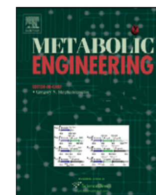

Metabolic engineering of omega-3 long-chain polyunsaturated fatty acids in plants using an acyl-CoA Δ6-desaturase with ω3-preference from the marine microalga *Micromonas pusilla*

James R. Petrie [a,1], Pushkar Shrestha [a,1], Maged P. Mansour [b], Peter D. Nichols [b], Qing Liu [a], Surinder P. Singh [a,*]

[a] *Food Futures National Research Flagship, CSIRO Plant Industry, PO Box 1600, Canberra, ACT 2601, Australia*
[b] *Food Futures National Research Flagship, CSIRO Marine and Atmospheric Research, GPO Box 1538, Hobart, Tasmania 7000, Australia*

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ABSTRACT

Long-chain ($\geq C_{20}$) polyunsaturated fatty acids (LC-PUFA) EPA and DHA ($20{:}5^{\Delta 5,8,11,14,17}$ and $22{:}6^{\Delta 4,7,10,13,16,19}$) have well-documented health benefits against coronary heart disease, rheumatoid arthritis and other disorders. Currently, the predominant sources of these fatty acids are marine fish and algal oils, but research is being conducted to ensure that a sustainable, land-based production system can be developed. We here describe the metabolic engineering of an artificial pathway that produces 26% EPA in leaf triacylglycerol using a newly-identified Δ6-desaturase from the marine microalga *Micromonas pusilla.* We also demonstrate that this enzyme appears to function as an acyl-CoA desaturase that has preference for ω3 substrates both *in planta* and in yeast. Phylogenetic analysis indicates that this desaturase shares highly conserved motifs with previously described acyl-CoA Δ6-desaturases.

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1. Introduction

Long-chain ($\geq C_{20}$) polyunsaturated fatty acids (LC-PUFA) including the omega-3 (ω3) eicosapentaenoic acid (EPA, $20{:}5^{\Delta 5,8,11,14,17}$) and docosahexaenoic acid (DHA, $22{:}6^{\Delta 4,7,10,13,16,19}$) are essential to human health and development. Several studies have indicated that deficiencies in these fatty acids increase the risk of cardiovascular disease (von Schacky, 2006), hypertension (Ueshima et al., 2007), inflammatory diseases, rheumatoid arthritis (Kremer et al., 1995; Simopoulos, 2002; Nagel et al., 2003) and neuropsychiatric disorders including dementia and depression (Parker et al., 2006; Schaefer et al., 2006; Freeman et al., 2006). Humans, particularly infants, are unable to synthesise DHA and other LC-PUFA to any great extent and must therefore obtain them through their diet. Unfortunately, many western countries, in particular, have median intakes of EPA and DHA that are far below the recommended or suggested intake amounts (Meyer et al., 2003). Recent concern about the ability of wild fisheries to meet an ever-increasing global demand for LC-PUFA (Myers and Worm, 2003; Brunner et al., 2009; Bimbo, 2007; Tocher, 2009) has encouraged research into the production of alternative, land-based sources of these oils.

One of the primary producers of LC-PUFA are marine microalgae, and these are capable of converting C_{18} fatty acids linoleic acid (LA, $18{:}2^{\Delta 9,12}$) and α-linolenic acid (ALA, $18{:}3^{\Delta 9,12,15}$) to arachidonic acid (AA, $20{:}4^{\Delta 5,8,11,14}$) and EPA and DHA, respectively, by a series of aerobic desaturations and elongations (Fig. 1). Both LA and ALA are found in many crop plants such as canola, linseed and soybean and provide a good starting point for transgenic conversion to LC-PUFA. Transgenic aerobic LC-PUFA production begins with either a Δ6-desaturation or a Δ9-elongation as the first committed steps of two separate pathways that lead to LC-PUFA. These enzymes can often act on ALA or LA equally well, resulting in parallel pathways, which yield both ω3 PUFA and potentially less desirable ω6 PUFA products including AA. It is important to note that the ω6 LC-PUFA product AA will only be converted to EPA, and thus DHA, by a Δ17-desaturation (Wu et al., 2005). These pathways are clearly a

Abbreviations: AA, arachidonic acid, $20{:}4^{\Delta 5,8,11,14}$; ALA, α-linolenic acid, $18{:}3^{\Delta 9,12,15}$; DGLA, di-homo-γ-linolenic acid, $20{:}3^{\Delta 8,11,14}$; DHA, docosahexaenoic acid, $22{:}6^{\Delta 4,7,10,13,16,19}$; DPA, docosapentaenoic acid, $22{:}5^{\Delta 7,10,13,16,19}$; EPA, eicosapentaenoic acid, $20{:}5^{\Delta 5,8,11,14,17}$; ETA, eicosatetraenoic acid, $20{:}4^{\Delta 8,11,14,17}$; GC, gas chromatography; GC–MS, gas chromatography–mass spectrometry; GLA, γ-linolenic acid, $18{:}3^{\Delta 6,9,12}$; LA, linoleic acid, $18{:}2^{\Delta 9,12}$; LC, long chain ($\geq C_{20}$); NL, neutral lipids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SDA, stearidonic acid. $18{:}4^{\Delta 6.9.12.15}$; TAG, triacylglycerol; TLC, thin layer chromatography; PUFA, long-chain polyunsaturated fatty acids; ω3, omega-3; ω6, omega-6

* Corresponding author. Fax: +61 2 62464950.
E-mail address: surinder.singh@csiro.au (S.P. Singh).

[1] These authors contributed equally.

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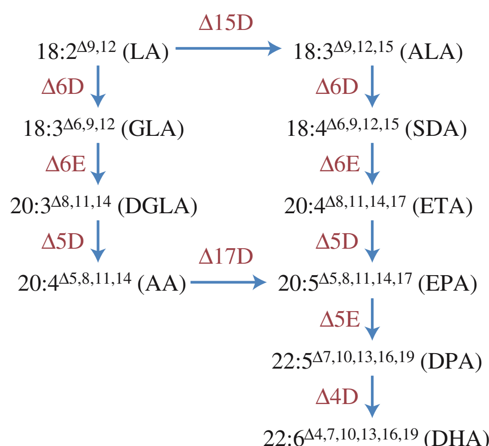

Fig. 1. The parallel ω6 (left with the exception of OA) and ω3 (right) Δ6-desaturase LC-PUFA biosynthesis pathways. 'D' refers to desaturase and 'E' refers to elongase. The Δ12- and Δ15-desaturases are endogenous to *Nicotiana benthamiana*. Whilst the Δ17-desaturase step is included in the figure, this activity was not utilised in this study. Fatty acids are OA, oleic acid; LA, linoleic acid; GLA, γ-linolenic acid; DGLA, di-homo-γ-linolenic acid; AA, arachidonic acid; ALA, α-linolenic acid; SDA, stearidonic acid; ETA, eicosatetraenoic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid and DHA, docosahexaenoic acid.

complex challenge from a metabolic engineering perspective and whilst many examples of each of these desaturases and elongases have been isolated (Robert, 2006; Napier, 2007; Truksa et al., 2009 and references therein), there is still great interest in identifying highly efficient genes, and more particularly combinations of these genes, which effectively increase the metabolic flow to DHA in transgenic crops. One of the major bottlenecks in the accumulation of high levels of LC-PUFA is the relatively low rate of elongation in transgenic plants (Truksa et al., 2009), mostly due to substrate dichotomy (Napier, 2007) caused by the fact that elongases function on acyl-CoA thioesters whereas the substrates for these enzymes are generally produced by lipid-linked (acyl-PC) desaturases and must be transferred between the acyl pools by acyltransferase enzymes before elongation can occur.

The Δ6-desaturases found in vertebrates are generally considered to be capable of functioning on acyl-CoA substrates and whilst these may seem an attractive source of genes, there are potential difficulties with their use in transgenic plants. First, there may be considerable difficulty in gaining market acceptance for a food produced *in planta* by animal genes and, second, it has not yet been confirmed that such desaturases can function optimally in plants, given that these divergent proteins would need to rely on plant-derived cofactors. The first acyl-CoA Δ6-desaturases isolated from plant-like organisms were from a liverwort, *Marchantia polymorpha* (Kajikawa et al., 2004), and microalgae *Ostreococcus tauri* (Domergue et al., 2005), which the authors characterised as being able to produce acyl-CoA thioester SDA in yeast. Publication of another plant-like acyl-CoA Δ6-desaturase followed in 2008 with the in-depth biochemical characterisation of Δ6-desaturase from *Mantoniella squamata* (Hoffmann et al., 2008). The authors were able to demonstrate function in both yeast and *Arabidopsis* with small amounts of EPA produced *in planta*. However, a pathway including this gene failed to produce 0.1% EPA in *Arabidopsis* making it difficult to judge the effectiveness of the desaturase in a pathway context. Identification of this gene was, however, important in that it was the first putativev acyl-CoA Δ6-desturase described with strong ω3 substrate preference.

In this study, we use *Nicotiana benthamiana* assay (Wood et al., 2009) as well as yeast and *Arabidopsis* transformations to characterise the new Δ6-desaturase obtained from the marine microalga *M. pusilla*. We demonstrate that this enzyme is highly efficient in an EPA pathway context due to its ω3-preference and probable ability to act for acyl-CoA substrates. We also confirm that the preference for ω3 substrates is displayed in *Arabidopsis* seed.

2. Materials and methods

2.1. Expression is *S. cerevisiae*

The yeast expression construct was built by cloning a blunt-ended fragment containing *M. pusilla* Δ6-desaturase gene coding region (Genbank accession EEH58637) into the *Pvu*II site of pYES2 (Invitrogen, Carlsbad, CA, USA). *S. cerevisiae* strain INVSc1 (Invitrogen, genotype *his3Δ1/his3Δ1 leu2/leu2 trp1-289/trp1-289 ura3-52/ura3-52*) was then transformed with plasmid DNA by heat shock and transformants were selected on yeast minimal medium (YMM) plates containing 2% raffinose as the sole carbon source. A clonal inoculum culture was established in liquid YMM with 2% raffinose as the sole carbon source. Experimental cultures of 50 mL were inoculated from these in YMM+1% NP-40 and grown to an initial OD_{600} of ~0.1 at 30 °C with shaking (~60 rpm). At this point, galactose was added to a final concentration of 2% and precursor fatty acids were added to a final concentration of 0.25 mM. Cultures were incubated at 24 °C with shaking for a further 24 h (for FAME preparation) or 48 h (lipid fractionation) prior to harvesting by centrifugation. Cell pellets were washed with 1% NP-40, 0.5% NP-40 and water to remove any unincorporated fatty acids from the surface of the cells.

2.2. Binary expression constructs for transient expression in plants

With the exception of *Echium plantagineum* Δ6-desaturase (kindly donated by Dr Xue-Rong Zhou; Zhou et al., 2006) all PUFA synthesis genes were codon optimised for plant expression (Geneart, Regensburg, Germany) and delivered in cloning vectors. Blunt-ended fragments containing the gene coding regions from the *M. pusilla* and *O. tauri* Δ6-desaturases, *P. salina* Δ5-desaturase (Genbank accession ABL96295) and *Pyramimonas cordata* Δ6-elongase (Genbank accession GQ202034) were all cloned into the *Eco*RV-*Sma*I site of Cauliflower Mosaic Virus (CaMV)35 S-pORE04 (Wood et al., 2009). A CaMV 35S-driven version of the P19 viral suppressor was kindly donated by Dr Peter Waterhouse.

2.3. *N. benthamiana* infiltration

N. benthamiana plants were grown and infiltrated with mixtures of *Agrobacterium tumefaciens* cultures harbouring single-gene CaMV 35S-driven binary vectors as previously described (Wood et al., 2009). In order to decrease silencing effects, all infiltrations were performed in the presence of P19 protein and *Arabidopsis thaliana* DGAT1 (Bouvier-Nave et al., 2000) was included to increase the amount of TAG in the leaf (Wood et al., 2009). Biological variation was reduced by performing infiltrations on either side of the mid-vein on the same leaf, thus allowing for direct comparisons of infiltrations.

2.4. Expression in *Arabidopsis*

A plant expression vector was constructed by cloning a *Swa*I fragment containing the coding region of *M. pusilla* Δ6-desaturase into another blunt-end restriction site, *Sma*I, which was flanked

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