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ORIGINAL PAPER

Enhancing the accumulation of omega-3 long chain polyunsaturated fatty acids in transgenic *Arabidopsis thaliana* via iterative metabolic engineering and genetic crossing

Noemi Ruiz-López · Richard P. Haslam · Mónica Venegas-Calerón · Tianbi Li · Joerg Bauer · Johnathan A. Napier · Olga Sayanova

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Abstract The synthesis and accumulation of long chain polyunsaturated fatty acids such as eicosapentaenoic acid has previously been demonstrated in the seeds of transgenic plants. However, the obtained levels are relatively low, indicating the need for further studies and the better definition of the interplay between endogenous lipid synthesis and the non-native transgene-encoded activities. In this study we have systematically compared three different transgenic configurations of the biosynthetic pathway for eicosapentaenoic acid, using lipidomic profiling to identify metabolic bottlenecks. We have also used genetic crossing to stack up to ten transgenes in Arabidopsis. These studies indicate several potential approaches to optimize the accumulation of target fatty acids in transgenic plants. Our data show the unexpected channeling of heterologous C20 polyunsaturated fatty acids into minor phospholipid species, and also the apparent negative metabolic regulation of phospholipid-dependent Δ6-desaturases. Collectively, this study confirms the benefits of iterative approaches to metabolic engineering of plant lipid synthesis.

Keywords Arabidopsis · Desaturase · Elongase · Omega-3 · Polyunsaturated fatty acids · Transgenic plants

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N. Ruiz-López · R. P. Haslam · M. Venegas-Calerón · T. Li · J. A. Napier (✉) · O. Sayanova
Department of Biological Chemistry, Rothamsted Research, Harpenden, Herts AL5 2JQ, UK
e-mail: johnathan.napier@rothamsted.ac.uk

M. Venegas-Calerón
Instituto de la Grasa, CSIC, Av. Padre Garcia Tejero 4, 41012 Seville, Spain

J. Bauer
BASF Plant Sciences, Limburgerhof, Germany

J. Bauer
Metanomics GmbH, Tegeler Weg 33, 10589 Berlin, Germany

Introduction

The nutritional value of omega-3 long chain polyunsaturated fatty acids (LC-PUFAs) in reducing risk of cardiovascular disease is now widely appreciated, and validated by epidemiological, genetic and dietary intervention studies (reviewed by Saravanan et al. 2010). Equally, the decline in global fish stocks, which represent the primary dietary source of these fatty acids, is also well-established and compounded by the increased use of fish oils in aquaculture and also increases in human populations and the associated consumption of animal-derived products (Cressey 2009; Garcia and Rosenberg 2010). Collectively, these factors serve as strong drivers for the development of alternative sustainable sources of these

"marine" oils. For several years, we and others have suggested that transgenic oilseeds metabolically engineered with the heterologous capacity to synthesis omega-3 LC-PUFAs represents a novel (terrestrial) production system which could substitute for some or all of the applications currently using fish oils (Domergue et al. 2005a; Napier et al. 2004; Robert 2006). Recently, proof-of-concept studies have indicated the viability of such an approach (reviewed in Venegas-Calerón et al. 2010; Napier and Graham 2010) yet it is also clear that some endogenous metabolic bottlenecks need to be overcome to enable transgenic plants to synthesize and accumulate omega-3 LC-PUFAs such as eicosapentaenoic acid (20:5 $\Delta^{5,8,11,14,17}$; abbreviated to EPA) and docosahexaenoic acid (22:6 $\Delta^{4,7,10,13,16,19}$) to levels equivalent to those found in the marine environment (Abbadi et al. 2004; Qi et al. 2004; Sayanova and Napier 2004).

Several different metabolic engineering rationales to increase omega-3 LC-PUFA production in transgenic seed oils have been suggested. One approach is to identify superior desaturases that have desirable substrate specificity and/or can efficiently use acyl-CoA substrates. For example, Δ6-desaturases from liverwort, *Marchantcia polymorpha* and microalgae *Ostreococcus tauri*, *Mantoniella squamata* and *Micromonas pusilla* have been suggested to act as acyl-CoA-dependent desaturases, potentially bypassing the "substrate-dichotomy" acyl-exchange bottleneck (Kajikawa et al. 2004; Domergue et al. 2005b; Graham et al. 2007; Hoffmann et al. 2008; Petrie et al. 2010). Alternatively, to favor the production of omega-3 LC-PUFAs over omega-6 LC-PUFAs, ω3-desaturases that are able to specifically convert omega-6 substrates to their omega-3 forms could be used (Wu et al. 2005; Cheng et al. 2010) (represented schematically in Fig. 1a). A second iteration to enhance levels of omega-3 substrates for conversion to LC-PUFAs is to use bi-functional Δ12- and Δ15-desaturases, such as Δ12/Δ15-desaturases from *Fusarium moniliforme* and *Acanthamoeba castellanii* (Damude et al. 2006; Sayanova et al. 2006a, b). In order to investigate the impact of different metabolic configurations on accumulation of LC-PUFAs, Arabidopsis plants were transformed with a number of recombinant binary plasmids, expressing a range of different genes from a variety

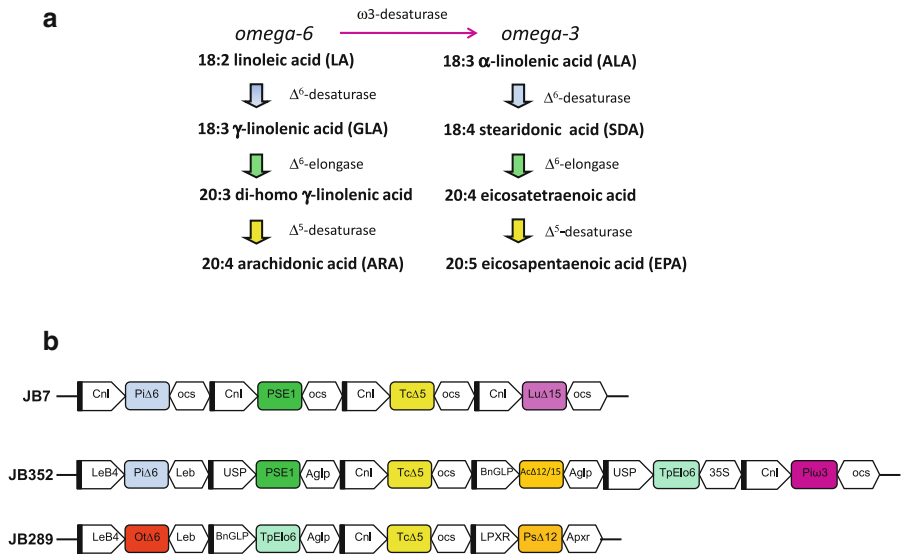

Fig. 1 Pathways and constructs. **a** Schematic representation of LC-PUFA biosynthesis. Different activities are identified with *different colours*, also used in (**b**). **b** Simplified maps of the vector constructs used for Arabidopsis transformation. Cnl, conlinin 1promoter for the gene encoding the flax 2S storage protein conlinin; LeB4, promoter of the legumin gene from *Vicia faba*; USP, promoter region of the unknown seed protein of *V. faba*; BnGLP, promoter of the globulin-like protein gene of *Brassica napus*; LPXR, promoter of peroxiredoxin gene from *Linum usitatissimum*; PiΔ6 and OtΔ6 represent Δ6-desaturases from *P. irregulare* and *O. taurii* respectively; TcΔ5- a Δ5-desaturase from *Thraustochytrium* sp.; LuΔ15- a Δ15-desaturase from *L. utitatissimum*; AcΔ12/15- a bi-functional Δ12/15 desaturase from *A. castellanii*; Pi ω3- an ω3 desaturase from *P. infestans*; PsΔ12- a Δ12-desaturase from *P. sojae*; PSE1 and TpElo6- represent Δ6-elongases from *P. patens* and *T. pseudonana*; OCS, Leb, Aglp, 35S, Apxr – represent terminators

of organisms under control of seed-specific promoters. In addition, the flexibility of using Arabidopsis as a model system allowed the further stacking of transgenes through conventional sexual crosses, resulting in transgenic lines containing up to ten independent transcription units in two unlinked T-DNA loci.

Here we demonstrate the reconstitution of arachidonic acid (20:4$\Delta^{5,8,11,14}$; abbreviated to ARA) and EPA biosynthesis in transgenic Arabidopsis and discuss the advantages of expressing an acyl-CoA dependent Δ6-desaturase activity instead of phospholipid-dependent Δ6–desaturases due to the circumvention of the well-documented "substrate- dichotomy" bottleneck. In addition our data indicates that the accumulation in triacylglycerols of non-native omega-3 LC-PUFAs is primarily via the PC-to-DAG route (Bates et al. 2007), analogous to that recently shown for hydroxylated fatty acids (Bates and Browse 2011). This has consequences for the efficient accumulation of EPA synthesised by the acyl-CoA pathway (Venegas-Calerón et al. 2010).

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana, Columbia (Col-0) ecotype, were grown for analyses in a controlled environment chamber at 23°C day/18°C night, 50–60% humidity, and kept on a 16-h, 250 μmol $m^{-2}s^{-1}$, photoperiod (long day).

Generation of transgenic plants

Transgenic Arabidopsis lines were generated as previously described (Sayanova et al. 2006a).

Vector construction

To build the first four-gene construct named as JB7 a similar approach was used as described in Cheng et al. (2010). The JB7 contained PiΔ6, a Δ6-desaturase from *Pythium irregular* (Hong et al. 2002), TcΔ5, a Δ5-desaturase from *Thraustochytrium* sp. (Qiu et al. 2001), PSE1, a Δ6 fatty acid elongase from *Physcomitrella patens* (Zank et al. 2002) and LuΔ15, a Δ15-desaturase gene from *Linum usitatissimum* (Vrinten et al. 2005). Each gene was under control of the conlinin1 promoter (Cnl) (Cheng et al. 2010) and linked to a terminator region of OCS, octopin synthase gene of *A. tumefaciens* (Fig. 1b).

The construct JB352 containing six gene cassettes was built using the Gateway® recombination system (Invitrogen). Respective genes were inserted as *Nco*I/*Pac*I or *Asc*I/*Pac*I fragments into the promoter/terminator cassettes and three pENTR vectors A, B and C were constructed to receive the promoter/terminator cassettes containing the gene of interest, with insertion mediated by conventional restriction endonuclease digestion and ligation of specific target sites introduced into both vectors. Specifically, pENTRY-A vector was built by inserting two cassettes containing (1) BnGLP promoter +, a Δ12/15 bi-functional desaturase gene from *Acanthamoeba castellanii* (AcΔ12/15, Sayanova et al. 2006a) linked to the AtGLP terminator and (2) Cnl promoter + ω3-desaturase gene from *Phytophtora infestans* (Piω3, Wu et al. 2005) linked to the OCS terminator. The pENTR-B vector consisted of two cassettes containing (1) USP promoter + the Δ6-elongase gene from *Thalassiosira pseudonana* (TpElo6, Meyer et al. 2004), linked to the 35S terminator, and (2) Cnl promoter + *Thraustochytrium* Δ5-desaturase (TcΔ5), linked to the OCS terminator (Qiu et al. 2001). pENTRY C vector contained two cassettes comprising (1) Legumin LeB4 promoter + the *P. irregulare* Δ6-desaturase (PiΔ6) (Hong et al. 2002), linked to the LeB terminator and (2) the USP promoter + the Δ6-elongase from *P.patens* (PSE1, Zank et al. 2002), linked to the AtGLP terminator.

A similar approach was used for the construct JB289. For that purpose two pENTRY vectors were designed containing two cassettes. The pENTRY-A2 construct contained (1) LXPR promoter + the Δ12-desaturase gene from *Phytophtora sojae* (PsΔ12), linked with Atpxr terminator and (2) Legumin LeB4 promoter + the *Ostreococcus tauri* Δ6-desaturase (OtΔ6) (Domergue et al. 2005b), linked to the the LeB terminator. The pENTRY-B2 construct comprised (1) BnGLP promoter + the Δ6-elongase TpElo6 gene from *T. pseudonana* (Zank et al. 2002), linked to the AtGLP terminator and (2) Cnl promoter + *Thraustochytrium* Δ5-desaturase (TcΔ5), linked to the OCS terminator (Qiu et al. 2001).

Final T-plasmids were obtained by recombination of the respective pENTRY vectors with the bespoke destination vector pSUN2. All binary vectors contained the *NPT*II gene with the NOS promoter as a

selection marker. Binary vectors were transferred into *Agrobacterium tumefaciens* strain GV3101 by electroporation.

Lipid extraction and separation

Three hundred milligrams of seeds were heated for 10 min at 95°C in 1 mL of isopropanol and homogenized using a mortar and pestle. The homogenate was centrifuged, supernatant collected, and the pellet re-extracted with isopropanol : chloroform (1:1, v/v). Both extracts were pooled, evaporated, and dissolved in chloroform: acetic acid (100:1, v/v). The lipid extract was loaded on a Sep-pack column and prefractionated into neutral lipids, glycolipids, and phospholipids adding chloroform : acetic acid (100:1, v/v), acetone : acetic acid (100:1), and methanol respectively. These fractions were further resolved on thin-layer chromatography silica gel plates, thickness 0.25 mm. Neutral lipids were developed with hexane : ethyl ether : formic acid (75:25:1, by volume), and polar lipids with chloroform : methanol : ammonia : water (70:30:4:1, by volume). The individual lipid classes were identified under UV light after a primuline spray (0.05% [w/v] in acetone : water, 80:20, v/v), scraped from the plate, and used directly for methylation or extracted for further analysis.

Fatty-acid analysis

Fatty acids were extracted and methylated as described (Sayanova et al. 1997). Methyl ester derivatives of total fatty acids (FAMEs) extracted were analysed by GC and GC–MS. Data presented as representative numbers derived from replicated analysis.

Acyl-CoA profiling

Twenty-milligrams of developing (15 days after flowering) seed material were collected, frozen in liquid nitrogen and extracted for subsequent quantitative analysis of fluorescent acyl-etheno-CoA derivatives by HPLC. HPLC (Agilent 1100 LC system; Phenomenex LUNA 150 × 2 mm C18(2) column) was performed using the methodology and gradient conditions described previously (Larson and Graham 2001). For the purpose of identification, standard acyl-CoA esters with acyl chain lengths from C14 to C20 were purchased from Sigma as free acids or lithium salts.

Results and discussion

Reconstitution of omega-3 LC-PUFA biosynthesis in transgenic arabidopsis

Three constructs, JB7, JB352 and JB289 (described in detail below and also represented schematically in Fig. 1b) were used to transform Arabidopsis plants via floral dip and the resulting progeny were identified by selection of seedlings on kanamycin-containing agar plates. Mature seeds from such Kan^R-plants were collected and samples from individual lines analysed by GC-FID for total fatty acid composition. Fatty acid analysis of transgenic Arabidopsis seeds confirmed that transgenic plants expressing these constructs (JB7, JB352 and JB289) accumulated significant levels of LC-PUFAs in the form of ARA and EPA (Table 1). Depending on the combination of transgenes used, we could identify lines with appreciable ARA (5.2%, line JB7) or EPA (2.5%, line JB352) content.

The first construct, designated JB7, contained the minimal set of genes (PiΔ6, PSE1 and TcΔ5) required to direct the production of omega-3/omega-6 LC-PUFAs via the conventional Δ6-desaturase pathway. To increase the available substrates for the synthesis of EPA, a Δ15-desaturase gene from *Linum usitatissimum* (Vrinten et al. 2005; designated LuΔ15) was also included in this construct (Fig. 1a, b). All the lipid biosynthetic genes in construct JB7 were under the control of individual seed-specific promoters (*conlinin1*). Twenty T_1 Arabidopsis lines for JB7 were taken forward and several new fatty acids were detected in seeds. The fatty acid analysis of the T_2 seeds from four representatives of these T_1 plants showed that the most abundant non-native species was the Δ6-desaturated C18 omega-6 fatty acid γ-linolenic acid ($18{:}3\Delta^{6,9,12}$; abbreviated to GLA) with an average value of 20% of total seed fatty acids (Table 1)—this fatty acid is completely absent from wildtype Arabidopsis and represents the first committed product of the Δ6-pathway (Fig. 1a). Other non-native fatty acids detected in these transgenic seeds were the C18 omega-3 stearidonic acid ($18{:}4\Delta^{6,9,12,15}$; abbreviated to SDA) (~6.1%), C20 omega-6 ARA (5.2%) and the C20 omega-3 EPA averaged 1.3% of total fatty acids. The inclusion of the LuΔ15 FAD3 C18 ω3-desaturase in construct JB7 would be predicted to have generated higher levels of α-linolenic acid ($18{:}3\Delta^{9,12,15}$;

Table 1 Total fatty acids composition of seeds from transgenic Arabidopsis lines

	Wt N = 4	JB7 (F2) N = 4, n = 8	JB352 (F2) N = 5, n = 18	JB289 (F2) N = 4, n = 16	JB352xJB7 (F1) N = 86, n = 90	JB289xJB7 (F1) N = 28, n = 30
16:0	6.2 ± 0.2	6.6 ± 0.4	8.8 ± 0.7	6.6 ± 0.6	6.8 ± 0.6	6.2 ± 0.4
18:0	3.3 ± 0.2	3.5 ± 0.3	3.6 ± 0.3	3.7 ± 0.3	4.0 ± 0.2	3.6 ± 0.2
18:1	14.5 ± 0.5	13.0 ± 1.3	16.4 ± 1.1	16.6 ± 0.6	15.7 ± 1.3	13.0 ± 1.4
18:2	27.9 ± 0.6	6.5 ± 1.2	17.2 ± 3.0	29.3 ± 1.7	10.3 ± 3.3	26.7 ± 1.5
GLA	–	20.0 ± 4.0	13.3 ± 3.5	1.0 ± 0.3	17.7 ± 1.9	1.7 ± 0.3
ALA	15.7 ± 0.4	11.2 ± 2.1	11.0 ± 1.7	13.9 ± 0.9	8.1 ± 0.7	12.9 ± 1.1
SDA	–	6.1 ± 2.1	3.1 ± 0.6	0.6 ± 0.2	3.8 ± 0.3	0.8 ± 0.1
20:1	20.5 ± 0.2	19.1 ± 0.9	17.8 ± 0.9	17.6 ± 0.9	20.7 ± 0.9	18.5 ± 1.1
DHGLA	–	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	0.3 ± 0.2	0.2 ± 0.1
ARA	–	5.2 ± 0.2	1.0 ± 0.2	2.8 ± 0.6	2.1 ± 0.9	6.2 ± 1.5
EPA	–	1.3 ± 0.4	2.5 ± 0.6	2.0 ± 0.5	4.1 ± 1.0	4.0 ± 0.8
Others	11.9 ± 0.4	7.3 ± 1.0	5.1 ± 0.5	5.8 ± 0.6	6.4 ± 0.6	6.2 ± 1.2

The mean fatty acid composition (expressed as mol. %) is shown, along with standard errors

N the number of independent transgenic lines

n the number of positive transgenic plants analysed

abbreviated to ALA) through the Δ15-desaturation of linoleic acid (18:2$\Delta^{9,12}$; abbreviated to LA), which in turn would undergo Δ6-desaturation to SDA. However, although T2 progeny of JB7 showed a dramatic decrease of LA levels (from the average 27.7% in WT to 6.5% in transgenic lines) there was no statistically significant positive effect on the levels of ALA and the modest SDA levels displaying a stoichiometric relationship with the decrease in LA. The accumulation of omega-6 fatty acids in JB7 was more efficient than for omega-3 products, despite the presence of the LuD15 FAD3 desaturase (Table 1). Overall levels of new omega-6 fatty acids were much higher than that of omega-3 fatty acids (ratio of 3.4:1).

In an attempt to engineer the conversion of these omega-6 fatty acids into the more desirable omega-3 form, and also to test available seed-specific regulatory elements (e.g. promoters and terminators) in combinations with different desaturase and elongase genes, we designed a second construct, BJ352 (Fig. 1b). Previous studies have indicated that a ω3-desaturase from *Phytophora infestans* (Piω3) (Hong et al. 2002; Wu et al. 2005.) efficiently converted ARA to EPA in *B. juncea*. Similarly, a Δ6-elongase from the diatom *Thaliossira pseudonana* (TpElo6) was shown to have activity only towards C18-PUFA substrates in yeast (Meyer et al. 2004). Bi-functional desaturases, such as Δ12/Δ15-desaturase from *A. castellanii*, and *F. moniliforme* have been shown to have significant potential to increase the production of omega-3 substrates through the "skewing" of the LC-PUFA biosynthetic pathway (Damude et al. 2006; Sayanova et al. 2006a). Therefore, to the core LC-PUFA biosynthetic pathway set of transgenes (PiΔ6, TcΔ5 and PSE1; Fig. 1a, b) we added the ω3 desaturase from *P. infestans, a* bi-functional Δ12/15 desaturase from *A. castellanii and* a second Δ6-elongases from *T. pseudonana* to generate expression cassette JB352. Fatty acid analysis of transgenic plants revealed that a decrease of LA levels was not as pronounced as observed in the JB7 plants (most likely due to the stronger Δ12-desaturase activity of bi-functional AcΔ12/15 [Sayanova et al. 2006a]) and averaged 17.2% while the ALA levels remained the same (11%) as seen in JB7 plants (Table 1). The addition of a second Δ6-elongase seemingly contributed to the reduced levels of GLA and SDA (from 20 and 6.1% in JB7 lines to 13.3 and 3% in JB352 plants). Most strikingly, the presence of the Piω3 activity resulted in the efficient conversion of C20 omega-6 fatty acids to the omega-3 form, generating a decrease of ARA from 5.2% in JB7 plants to 1.0% in JB352 plants and consequently, an increase in EPA levels from 1.3 to 2.5%. The expression of new activities had a positive effect on the omega-6/omega-3 ratio (reducing it to 2.5/1). However, both JB7 and JB352 lines accumulated very high levels of omega-6 pathway intermediates such as GLA (up to 20% for JB7).

Although both iterations (JB7, JB352) demonstrated the successful biosynthesis of EPA using

combinations of different genes and promoters, the levels of this target fatty acid resulting from either construct were modest (<3%), though an improvement on earlier studies in Arabidopsis (Hoffmann et al. 2008). Biochemical studies on the lower eukaryote front–end desaturases which underpin LC-PUFA biosynthesis indicate that these enzymes utilize glycerolipid-linked substrates, which can (in transgenic hosts) generate the metabolic bottleneck between desaturation and elongation known as "substrate dichotomy" (Domergue et al. 2003; Abbadi et al. 2004; Napier et al. 2004; Domergue et al. 2005a). The use of acyl-CoA dependent desaturases is proposed to circumvent this limitation (Hoffmann et al. 2008; Petrie et al. 2010; Sayanova et al. 2012) and can result in the significant reduction in the accumulation of C18 Δ6-desaturated intermediates such as GLA and SDA. However, such studies failed to observe a notable concomitant increase in EPA (Robert 2006; Hoffmann et al. 2008; Sayanova et al. 2012). To determine if acyl-CoA–dependent EPA biosynthesis in plants could be optimized we designed an expression cassette containing the *O. tauri* Δ6-desaturase (Domergue et al. 2005b) which we have recently demonstrated to act as an acyl-CoA-dependent Δ6-desaturase in transgenic plants (Sayanova et al. 2012), in combination with TpElo6 and TcΔ5 under control of independent seed-specific promoters. To enhance the levels of LA-CoA (as substrate for the OtD6 enzyme) we also included a Δ12-desaturase from *Phytophthora sojae* (PsD12), generating the construct JB289 (Fig. 1b). In agreement with previous results obtained with the expression of acyl-CoA Δ6-desaturases in transgenic plants, only minor accumulation of Δ6-desaturated fatty acids was observed (1% of GLA and 0.6% of SDA). However, the content of ARA was higher than in JB352 construct (2.8%), most likely due to the absence of a C20 ω3 specific desaturase and reflecting the bias in Arabidopsis seeds towards the synthesis of omega-6 fatty acids. The average EPA content in JB289 plants was 2.0% of total seed fatty acids and was similar to those observed in JB352 plants. Interestingly, unlike JB7 and JB352, the levels of LA in JB289 were not reduced, and remained similar to those in WT (Table 1). From the perspective of trying to engineer plant seed oils with a composition similar to that found in fish oils, the observation that construct JB289 produced similar levels of C20 LC-PUFAs, but no C18 pathways intermediates such as GLA or SDA is relevant. The basis of this improvement was likely the presence of the *O. tauri* acyl-CoA dependent Δ6-desaturase generating substrates for the elongase which are rapidly converted to C20 forms, thus avoiding becoming trapped in metabolic dead-ends.

Stacking of metabolic pathways via genetic crossing

In order to study the effect of additional enzyme activities on the expression of LC-PUFAs pathway in Arabidopsis plants lines JB7, 352 and 289 were used in genetic crossing experiments. Transgenic Arabidopsis events JB352 and JB289 were used as males in crosses with event JB7—in all cases the parental lines were homozygous for the transgene, based on 100% resistance to kanamycin in T3 of selected lines. The F1 progeny of a cross between JB352 and JB7 had reduced levels of LA compared to JB352 (10.3% of total fatty acids), probably due to activity of LuΔ15 Fad3 and reduced levels of ARA compared to JB7 (2.1%) which is more likely due to the presence of Piω3, ω3-specific desaturase from *P. infestans* (Table 1). Fatty acid analysis of F1 seeds detected elevated levels of EPA (4.1%). The levels of accumulation of Δ6-desaturated intermediates like GLA and SDA were similar to parental lines. The F2 progeny derived from self-fertilization of several F1 plants were grown and the fatty acid composition determined from seeds of 40 plants. The Mendelian inheritance pattern and the co-segregation of reduced LA with reduced ARA but increased EPA in the F1/F2 populations indicate that the altered fatty acid composition is caused by the presence of new LuΔ15 and Piω3 activities.

The fatty acid analysis of F1 seeds derived from ♀JB7xJB289♂ cross showed that ARA was most abundant among the newly produced fatty acids, with a mean of 6.2% (Table 1). EPA levels were increased in comparison with parental lines to 4.0% of total fatty acids, most likely as a result of the ω3-desaturase present in JB7. Conversely, the very low levels of Δ6-unsaturated intermediates detected in this cross were synonymous with the fatty acid composition of the parental JB289 line. Collectively, these data indicate that in general, the acyl-CoA Δ6–desaturase and ω3-desaturase were identified as major influencing factors on flux to EPA in Arabidopsis.

Lipid analysis of transgenic Arabidopsis accumulating omega-3 LC-PUFAs

In order to gain further insight into the synthesis and channeling of transgene-derived fatty acids in Arabidopsis, lipid analyses was carried out on developing (17 daf) and mature seeds of JB7, JB352 and JB289 lines. FAMEs were prepared from the neutral, phospholipid and glycolipid fractions, with the different lipid classes separated by TLC. Three main fractions of neutral lipids corresponding to triacylglycerols (TAG), diacylglycerols (DAG) and free fatty acids (FFA) and the main fractions of phospholipids, phosphatidylcholine (PC), phosphatidylethanolamine (PE) and a fraction designated PI&PS containing both phosphatidylinositol (PI) and phosphatidylserine (PS) were separated, methylated and resolved by GC-FID. The comprehensive fatty acid compositions of each individual fraction were analyzed and the results are presented in Supplementary Tables 1–3, with the accumulation and distribution of non-native GLA, ARA and EPA highlighted graphically in Fig. 2a, b, and c, respectively.

Accumulation of GLA (Fig. 2a) and SDA in JB7 and JB352 lines was spread across all lipid classes with no obvious precursor-product relationship visible in neither line nor developmental stages. The highest percentage of Δ6-desaturated fatty acids occurred in the neutral lipids of mature seeds of line JB7 (23.6% [= the sum of GLA and SDA] of total fatty acids) (Supplementary Table 1) and in PE fraction of mature seeds in line JB352 (22.5%) (Supplementary Table 2). High levels of Δ6-desaturated fatty acids were also present in the TAGs of mature seeds (JB7 = 23.5%, JB352 = 21.6%) and also the neutral lipid intermediate DAG (JB7 = 19.3%, JB352 = 20.8%) (Supplementary Table 3). For line JB289, accumulation of GLA and SDA in all lipid classes was very low (< 1.8% for GLA and <0.6% for SDA), as would be predicted for this acyl-CoA-dependent desaturase (Supplementary Tables 2, 3).

Further analysis of the accumulation of the non-native fatty acids in these transgenic lines provided some unexpected insights into their distribution. For example, in lines JB7 and JB289 the C20 omega-6 ARA accumulated predominantly in phospholipids, especially in PI&PS fractions of mature seeds (JB7 = 11.3%; JB289 = 7.2%) (Supplementary Table 2; Fig. 2a). The mechanistic basis for this

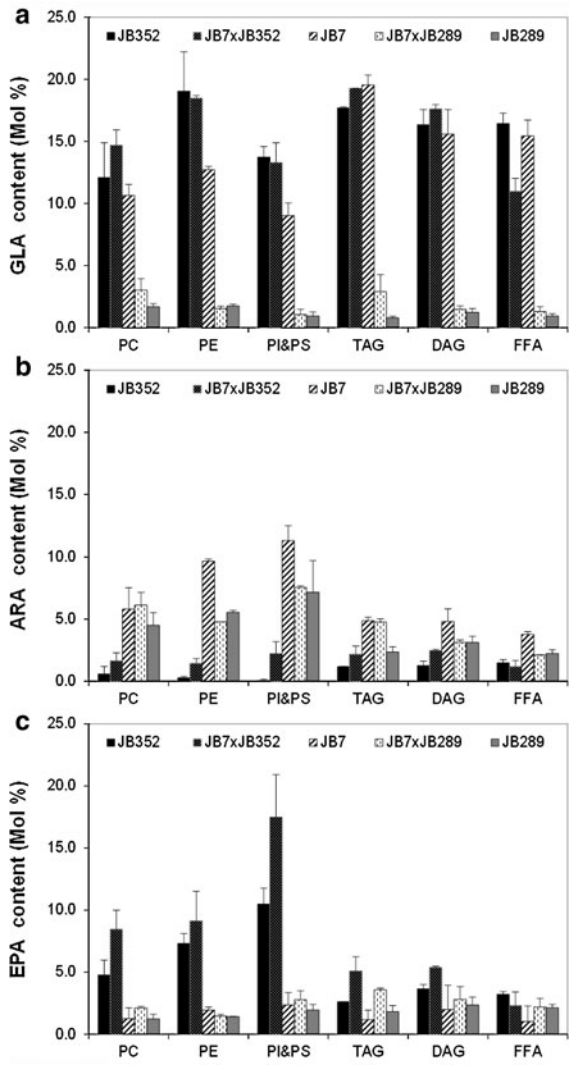

Fig. 2 Distribution of non-native fatty acids within the lipid fractions of transgenic Arabidopsis seeds. The distribution and accumulation of GLA (**a**), ARA (**b**) and EPA (**c**) in the mature seeds of the three primary transgenic lines (JB7, JB289, JB352) and crosses (♀JB7xJB289♂, ♀JB7xJB352♂) is shown for PC, PE, PI&PS, TAG, DAG and free fatty acids (FFA). These non-native fatty acids are absent from WT Arabidopsis

phenomena is currently unknown but may reflect the accumulation of endogenous (saturated and monounsaturated) very long chain fatty acids in these phospholipids, meaning that the biosynthetic enzymes are more amenable to accepting C20 substrates. Similarly, we observed that EPA in all three lines was present in greater proportions in mature seeds and concentrated in polar lipids (mainly in PE and PI&PS fractions—Fig. 2c). In the PI&PS fraction of mature seeds of

JB352 levels of EPA reached 10.5% of total fatty acids (Supplementary Table 2), a threefold enrichment to the levels of this fatty accumulating in the neutral lipids of this line (Supplementary Table 1). Interestingly, lipid analysis of neutral classes demonstrated that EPA was more abundant in DAG as opposed to TAG (Supplementary Table 2). Analysis of glycolipids in mature seeds of the JB7 and JB352 lines indicated moderate levels of GLA (8.5%; 5.3%) and lower levels of ARA (2.8%; 0.4%) and EPA (1.0–2.3%) in these lipids (Supplementary Table 1). Analysis of JB289 lines revealed a similar picture for the accumulation of ARA and EPA (1.4 and 1.3%). However, GLA was again almost absent from the glycolipids.

In terms of the endogenous fatty acid composition, the most striking alteration was observed in the phospholipids of lines JB7 and JB352, in which not only were significant levels of Δ6-unsaturated fatty acids accumulated, but also a pronounced reduction in the levels of LA, counter-balanced by an increase in oleic acid ($18{:}1\Delta^9$; abbreviated to OA) (Supplementary Table 2). This pattern (↑OA, ↓LA) was seen in both PC and PE for both JB7 and JB352, and was coincident with the accumulation of non-native GLA in these lipids. Notably, this was not seen in the JB289 lines, ruling out the cause of the perturbation as due to the synthesis of C20 LC-PUFAs (Fig. 3). A more logical explanation is that the phospholipid-dependent Δ6-desaturase present in JB7 and JB352 generates high levels of the non-native fatty acid GLA at the sn-2 position of PC, as previously described (Domergue et al. 2003). The accumulation (and lack of acyl-exchange) of this fatty acid would result in a reduced flux of substrate for the endogenous FAD2 oleate Δ12-desaturase, generating a decrease in LA (product) and increase in OA (substrate). Thus, in this study, the pattern (↑OA, ↓LA) is similar to that observed for transgene-derived activities of other phospholipid-dependent acyl-modifying enzymes, including the FAD2-like family involved in the synthesis of unusual fatty acids (Cahoon et al. 2006; Napier 2007; Dyer et al. 2008).

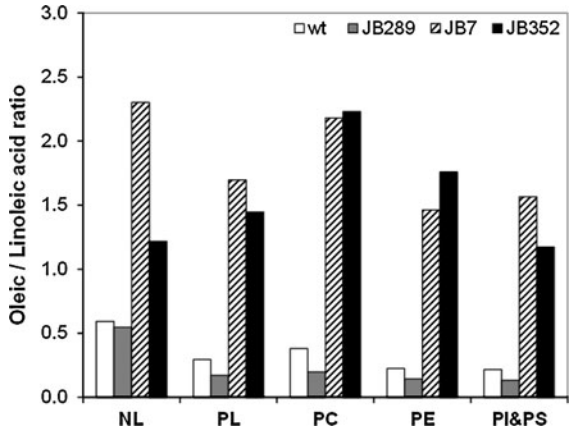

Fig. 3 The ratio of oleic acid to linoleic acid within different lipid fractions of transgenic Arabidopsis seeds. The absolute ratio of oleic acid and linoleic acid present in different lipid fractions is shown for the three primary transgenic lines (JB7, JB289, JB352), compared with WT. Lipid classes are NL (neutral lipids), PL (phospholipids), PC (phosphatidylcholine), PE (phosphatidylethanolamine) and PI&PS (phosphatidylinositol and phosphatidylserine)

Lipid analysis of genetic crosses

Having established the acyl-composition of lipids from the three parental lines, similar analysis was also carried out on the different classes of lipids from mature seeds of the ♀JB7xJB352♂ and ♀JB7xJB289♂ crosses. Accumulation of GLA was maximal in the neutral lipids fraction (20.8% of total fatty acids) of the JB7xJB352 cross (Supplementary Table 1), indicating an additive effect most likely arising from the presence of two phospholipid-dependent Δ6-desaturases. Surprisingly, GLA was almost absent from the neutral and phospholipid fractions of JB7xJB289 in comparison with parental lines (Supplementary Tables 1–3)—this phenomena is discussed below.

In terms of target C20 PUFAs, levels of the omega-6 ARA were generally markedly decreased and equally distributed among all main lipid fractions arising from the JB7xJB352 cross, reflecting the presence of the ω3-desaturase in the JB352 parent. A concomitant increase in the levels of EPA was seen in the JB7xJB352 cross, with maximal levels of 17.5% being detected in the PI&PS fraction (Supplementary Table 2). In contrast, cross JB7xJB289 displayed a maximal level of 5.4% EPA in DAGs (Supplementary Table 3), although this was higher than that observed in either parental line.

Thus, the presence of phospholipid-dependent Δ6-desaturases (JB7, JB352) can drive target levels of EPA higher, compared to the acyl-CoA-dependent Δ6-desaturase. However, this C20 PUFA appears to accumulate in unexpected lipids, specifically the

PI&PS fraction (which represents a minor pool of total seed lipids). In addition, the presence of a phospholipid-dependent desaturase results in the high level accumulation of undesired biosynthetic intermediates such as GLA and SDA. In contrast, these intermediates are present at only very low levels in the lines containing the acyl-CoA-dependent Δ6-desaturase.

Determination of acyl-CoA pool composition

The composition of the acyl-CoA pool was determined for the Arabidopsis JB7, JB352 and JB289 transgenic lines and crosses JB7xJB352 and JB7x289 at mid-stage of seed development. As shown in Fig. 4, the acyl-CoA profiles from the developing seeds of WT Arabidopsis and JB7 and JB352 lines expressing the lipid-dependent *P. irregular* Δ6-desaturase show no significant difference in acyl-CoA composition, except for SDA, which was present as a very minor component in the acyl-CoA pool of both lines. In contrast, SDA-CoA was clearly present in JB289, as was ARA-CoA and also 20:2-CoA. Unfortunately, GLA-CoA co-migrates with ALA-CoA on this chromatographic system, so it was not possible to determine the levels of that Δ6-desaturation product—however, the presence of SDA-CoA is entirely consistent with the action of the *O. tauri* acyl-CoA Δ6-desaturase (Domergue et al. 2005b; Sayanova et al. 2012).

Acyl-CoA analysis of the two crosses revealed that progeny of the JB7xJB289 cross contained elevated levels of ARA-CoA, compared to that observed in the parental JB289 (JB7 contained no ARA-CoA). Interestingly, the levels of the C18 Δ6-desaturation product, SDA-CoA, were unchanged in the JB7xJB289 compared with the JB289 parent, whereas the levels of 20:2-CoA were reduced to levels similar to that seen in the JB7 parent (Fig. 4). Thus, the acyl-CoA profile of this cross clearly indicates the contribution of both parents to their progenies' metabolic composition, generating a profile which is distinct from that of either parent or WT. This is in contrast to the situation for the JB7xJB352 cross which contains only phospholipid-dependant desaturases and does not result in any significant perturbation to the acyl-CoA profile compared to the two parental lines, which themselves are very similar to WT (Fig. 4). In that respect, the presence of C18 Δ6-desaturated fatty acids in the acyl-CoA pool of the JB7xJB289 may provide an explanation for the apparent dominance of the *O. tauri* Δ6-desaturase over the phospholipid-dependent *P. irregulare* Δ6–desaturase: we hypothesis that the presence of SDA-CoA and GLA-CoA act as inhibitors of the latter enzyme, most likely via metabolic feedback, resulting in the absence of any significant Δ6-desaturation products present in the phospholipids or neutral lipids of this cross.

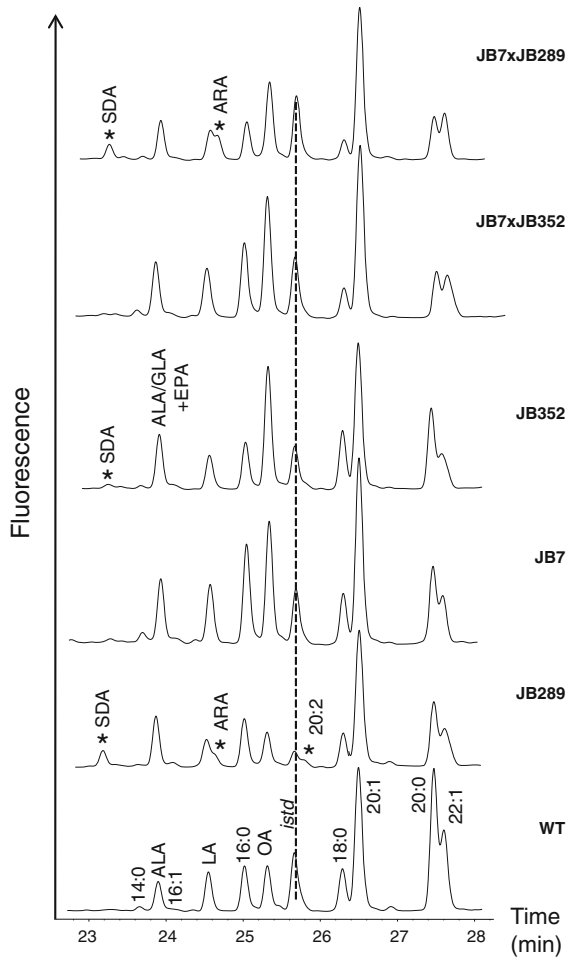

Fig. 4 Acyl-CoA profiles of developing seeds from transgenic Arabidopsis lines. Acyl-CoAs were extracted, derivatised, resolved and identified by HPLC-FLD as previously described. The acyl-CoA profiles for all the different lines described in this study are shown (WT, JB7, JB289, JB352, ♀JB7xJB289♂, ♀JB7xJB352♂). The identify of peaks is shown, and the presence of non-native products of the LC-PUFA biosynthetic pathway indicated (*). The internal standard (istd) was 17:0-CoA

Conclusions

In this study we have adopted an iterative approach to optimizing the accumulation of C20 omega-3 LC-PUFAs in the seeds of transgenic Arabidopsis plants. These studies re-affirmed very recent studies which showed the benefits of using the *O. tauri* acyl-CoA dependent Δ6-desaturase to avoid the accumulation of unwanted C18 intermediates (such as GLA and SDA) without significantly reducing the final level of target C20 LC-PUFAs. When the *O. tauri* Δ6-desaturase was co-expressed with the phospholipid dependent *P. irregulare* Δ6-desaturase, the former activity was dominant, with no obvious evidence of phospholipid-depedent Δ6-desaturation. In transgenic plants expressing only the *P. irregulare* Δ6-desaturase, not only did we detect the accumulation in phospholipids of products GLA and SDA, we also observed a strong reduction in LA and accumulation of OA specifically in these lipids. This lipid "fingerprint" may therefore serve as a convenient marker for phospholipid-dependent front-end desaturases, similar as to that seen for FAD2-like enzymes. We also observed, in the case of the *P. irregulare* Δ6-desaturase activity, indirect evidence of the desaturation products fluxing from PC into TAG via DAG, as indicated by the relative accumulation of GLA in these lipids (10.7% in PC, 15.6% in DAG, 19.3% in TAG; Supplementary Tables 2, 3, [JB7]). Thus, for the phospholipid-dependent Δ6-desaturase, GLA-containing PC (i.e. the site of synthesis for this Δ6-unsaturated fatty acid) would undergo remodeling (most likely by the reverse reaction choline-phosphotransferase or specific phospholipases) to generate GLA-containing DAG, which is then acylated at the sn-3 position to generate a GLA-containing TAG species. Such a scenario would agree with the recent models of Bates (Bates et al. 2007; Bates and Browse 2011) for the accumulation of hydroxyl-fatty acids in Arabidopsis seed oil, in which the authors used metabolic labeling to demonstrate that the bulk of extraplastidial fatty acids enter TAG via PC and DAG, as opposed to a direct acylation route via the Kennedy pathway (reviewed in Venegas-Calerón et al. 2010). Thus, our observations have important implications for subsequent attempts to engineer LC-PUFA accumulation via phospholipid-dependent desaturases. Similarly, the enrichment of target fatty acids (EPA) in minor phospholipid pools (such as PI&PS) indicates that multiple activities likely generate competing fluxes to direct the accumulation of non-native fatty acids into different sinks. In this particular case, it may be that since these phospholipid species are known to contain endogenous VLCFAs, their biosynthetic acyltransferases have an increased preference for non-native LC-PUFAs.

In the case of the acyl-CoA dependent Δ6-desaturase, no such widespread accumulation of either biosynthetic intermediates or end products was observed, indicative of the more minor role for direct acylation of fatty acid-CoAs as an entry point for flux to TAG (Bates and Browse 2011). However, as noted above, the benefits of using such a desaturase likely outweigh the lower rate of TAG synthesis by this pathway. In addition, it is clear that whilst a PC→DAG→TAG pathway is predominant in Arabidopsis seeds, this may well not be the case for some other oilseeds (Bates and Browse 2011). Thus, further work needs to be done to optimize the synthesis of omega-3 LC-PUFAs in transgenic plants, and this will require a detailed analysis of TAG synthesis, most likely on a species-by-species basis.

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