

# The role of $\Delta 6$ -desaturase acyl-carrier specificity in the efficient synthesis of long-chain polyunsaturated fatty acids in transgenic plants

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## Summary

The role of acyl-CoA-dependent  $\Delta 6$ -desaturation in the heterologous synthesis of omega-3 long-chain polyunsaturated fatty acids was systematically evaluated in transgenic yeast and *Arabidopsis thaliana*. The acyl-CoA  $\Delta 6$ -desaturase from the picoalga *Ostreococcus tauri* and orthologous activities from mouse (*Mus musculus*) and salmon (*Salmo salar*) were shown to generate substantial levels of  $\Delta 6$ -desaturated acyl-CoAs, in contrast to the phospholipid-dependent  $\Delta 6$ -desaturases from higher plants that failed to modify this metabolic pool. Transgenic plants expressing the acyl-CoA  $\Delta 6$ -desaturases from either *O. tauri* or salmon, in conjunction with the two additional activities required for the synthesis of C20 polyunsaturated fatty acids, contained higher levels of eicosapentaenoic acid compared with plants expressing the borage phospholipid-dependent  $\Delta 6$ -desaturase. The use of acyl-CoA-dependent  $\Delta 6$ -desaturases almost completely abolished the accumulation of unwanted biosynthetic intermediates such as  $\gamma$ -linolenic acid in total seed lipids. Expression of acyl-CoA  $\Delta 6$ -desaturases resulted in increased distribution of long-chain polyunsaturated fatty acids in the polar lipids of transgenic plants, reflecting the larger substrate pool available for acylation by enzymes of the Kennedy pathway. Expression of the *O. tauri*  $\Delta 6$ -desaturase in transgenic *Camelina sativa* plants also resulted in the accumulation of high levels of  $\Delta 6$ -desaturated fatty acids. This study provides evidence for the efficacy of using acyl-CoA-dependent  $\Delta 6$ -desaturases in the efficient metabolic engineering of transgenic plants with high value traits such as the synthesis of omega-3 LC-PUFAs.

**Keywords:** desaturases, fatty acids, omega-3, polyunsaturated fatty acids, stearidonic acid, transgenic plants.

## Introduction

Fatty acid desaturases from vertebrates are generically classified as acyl-CoA desaturases (Okayasu *et al.*, 1981; Sprecher and Chen, 1999), based on the assumption that these enzymes use CoA-linked fatty acids as their substrate. This substrate preference is assumed for both the stearate  $\Delta 9$ -desaturase SCD1 and also the 'front-end' cytochrome b5-fusion desaturases involved in the synthesis of long-chain polyunsaturates. However, the only example in the literature of the biochemical *in vitro* characterization of a front-end desaturase is for the rat  $\Delta 6$ -desaturase (Okayasu *et al.*, 1981). This enzyme was purified to homogeneity from rat liver microsomes and was used in detergent-containing assays in the presence of additional cytochrome b5 and cytochrome b5 reductase to show that linoleoyl-CoA was the reaction substrate. Surprisingly, the desaturase activity was absolutely dependent on the addition of exogenous cytochrome b5, although the subsequent cloning of the gene encoding the rat  $\Delta 6$ -desaturase (and other animal orthologs) revealed that the open reading frame contained an N-terminal cytochrome b5 domain expected to function as immediate electron donor (Cho *et al.*, 1999; Marquardt *et al.*, 2000). This sole study suggests that mammalian  $\Delta 6$ -desaturases use acyl-CoA substrates.

Biochemical studies with plants and fungi indicate a preference for lipid-linked substrates. Ground-breaking studies using alkenyl ether glycerolipids and tomato cell cultures demonstrated that

purified plant microsomal  $\Delta 12$ - and  $\Delta 15$ -desaturases act on (phospho)lipid-linked substrates (Sperling *et al.*, 1993). *In vitro* enzyme assays with microsomes from the fungus *Mucor circinelloides* and from borage (*Borago officinalis*) indicated that  $\Delta 6$ -desaturases also act on the *sn*-2 position of phosphatidylcholine (PC), converting linoleate at the *sn*-2 position of complex lipids to  $\gamma$ -linolenic acid (GLA, 18:3 $\Delta 6,9,15$ ; n-6) (Stymne and Stobart, 1986; Griffiths *et al.*, 1988; Jackson *et al.*, 1998). Because the purification of enzymatically active membrane-bound desaturases is notoriously difficult, the functional characterization of most front-end desaturases has been based on heterologous expression in various host organisms (usually *S. cerevisiae*) followed by appropriate fatty acid analysis. Using such approaches, it was shown (for example) that all  $\Delta 6$ -desaturases had similar specificity towards different fatty acid substrates, but that enzymes from different organisms differed with respect to the acyl-carrier (CoA vs. phospholipids) they use as substrate (Domergue *et al.*, 2003; Napier *et al.*, 2003; Sato *et al.*, 2004). These observations indicate approaches to engineer biosynthesis of omega-3 long-chain polyunsaturated fatty acids (LC-PUFAs) in plants (Drexler *et al.*, 2003; Napier *et al.*, 2004; Hoffmann *et al.*, 2008; Petrie *et al.*, 2010).

For example, detailed biochemical analyses of transgenic linseed expressing genes of the conventional  $\Delta 6$ -pathway ( $\Delta 6$  and  $\Delta 5$ -desaturases of the marine alga and  $\Delta 6$ -elongase from moss) indicated that successful production of non-native C20 LC-PUFAs in plants required efficient exchange of acyl groups

between phospholipids and the acyl-CoA pool (Abadi *et al.*, 2004), due to the so-called substrate dichotomy arising from phospholipid-dependent desaturases and the acyl-CoA-dependent elongase that was predicted to generate a metabolic bottleneck (Domergue *et al.*, 2005a; Napier, 2007; Robert, 2006). Approaches suggested to overcome this blockade (Damude and Kinney, 2008; Cheng *et al.*, 2010; Petrie *et al.*, 2010) include the use of acyl-CoA-dependent desaturases, thus allowing the synthesis of LC-PUFAs to proceed exclusively in the acyl-CoA pool. As noted earlier, mammalian desaturases have such substrate preference but the differences in codon-usage between plants and animals precluded the efficient expression of native animal sequences in transgenic plants. However, Domergue *et al.* (2005b) identified an acyl-CoA-dependent  $\Delta 6$ -desaturase from the picoalga *Otrococcus tauri* (OtD6). They noted the appearance of  $\Delta 6$ -desaturation products in the acyl-CoA pool prior to their presence in total fatty acids, consistent with acyl-CoA desaturation followed by incorporation of the CoA-linked  $\Delta 6$ -desaturation product into phospholipids.

Here, we report on the efficacy of several different front-end  $\Delta 6$ -desaturases to produce LC-PUFAs in transgenic plants and yeast. Our data also indicate the unexpected consequences of changes to the acyl-CoA pool through the actions of acyl-CoA-dependent desaturases.

## Results and discussion

### Systematic expression and analysis of $\Delta 6$ -desaturases in yeast

We set out to test whether the use of acyl-CoA-dependent desaturases in the biosynthesis of omega-3 LC-PUFAs can overcome the problem of substrate dichotomy. First, we examined the kinetics of *in vivo* desaturation for a number of different  $\Delta 6$ -desaturases previously shown to be active in yeast. The coding sequences for  $\Delta 6$ -desaturases from *O. tauri* OtD6 (Domergue *et al.*, 2005b); mouse, MmD6 (Cho *et al.*, 1999); salmon, SsD6 (Zheng *et al.*, 2005); parasitic protozoa *Trypanosoma brucei*, TbD6 (Tripodi *et al.*, 2006); borage, BoD6 (Sayanova *et al.*, 1997) and *Primula luteola*, PID6 (Sayanova *et al.*, 2006) were cloned into the yeast expression vector pYES2, and the accumulation of the  $\Delta 6$ -desaturation product stearidonic acid (SDA; 18:4 $\Delta 6,9,12,15$ ; n-3) was determined after a 24-h induction period (to allow the expression of the non-native desaturase) and the subsequent addition of the substrate fatty acid  $\alpha$ -linolenic acid (ALA; 18:3 $\Delta 9,12,15$  n-3). Aliquots of the yeast cultures were taken at time points previously defined by Domergue *et al.* (2003), and trans-methylated and total fatty acid methyl esters (FAMES) were analyzed and quantitated by GC-FID. As can be seen in Figure S1, all six desaturases showed activity towards ALA, directing the synthesis of SDA in yeast. Of the six enzymes tested, the *O. tauri*  $\Delta 6$ -desaturase displayed the highest conversion (substrate to product) rate, followed by the mouse and salmon activities. Markedly lower conversion rates were observed for the trypanosome  $\Delta 6$ -desaturase and the two higher plant forms (Table 1). We detected SDA within 5 min for all of the non-higher plant enzymes (i.e. *O. tauri*, *T. brucei*, salmon and mouse) expressed in either our laboratory haploid yeast strain (W303) or the diploid INVSc1 (Invitrogen, Paisley, UK) strain used by Domergue *et al.* (2003, 2005b), who observed the accumulation of  $\Delta 6$ -desaturated fatty acids only after longer incubation.

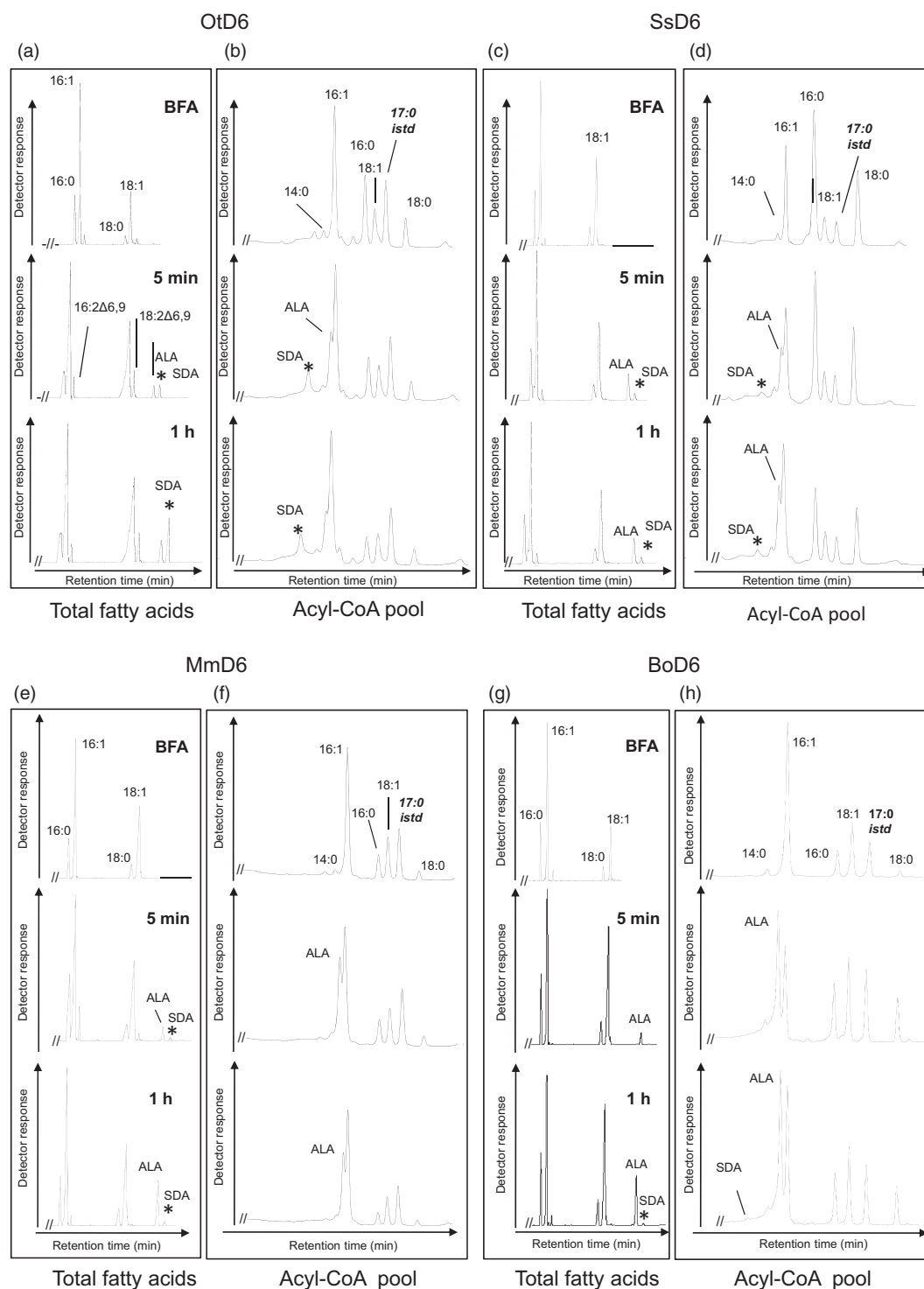
**Table 1** Desaturation efficiency of the  $\Delta 6$ -desaturases

Construct	ALA	SDA	Conversion rate (%)
OtD6			
5'	2.6 ± 0.5	2.6 ± 0.1	50.0
1 h	4.6 ± 0.8	7.7 ± 1.0	62.6
24 h	13.6 ± 1.5	36.5 ± 2.1	72.8
SsD6			
5'	2.1 ± 0.4	0.6 ± 0.1	22.2
1 h	13.2 ± 1.0	4.3 ± 0.8	24.6
24 h	36.8 ± 1.4	22.9 ± 2.9	38.3
MmD6			
5'	2.6 ± 0.6	0.3 ± 0.1	10.3
1 h	8.7 ± 1.2	2.1 ± 0.7	19.4
24 h	18.1 ± 0.8	12.1 ± 3.2	40.2
TbD6			
5'	4.4 ± 0.9	0.3 ± 0.1	6.3
1 h	15.4 ± 2.1	1.2 ± 0.2	7.2
24 h	49.1 ± 4.2	7.2 ± 1.1	13.0
BoD6			
5'	3.6 ± 0.5	0.2 ± 0.1	5.2
1 h	11.3 ± 0.6	0.5 ± 0.2	4.0
24 h	50.5 ± 3.6	3.6 ± 0.5	7.0
PID6			
5'	3.2 ± 0.5	0.0	–
1 h	8.7 ± 0.9	0.2 ± 0.1	2.2
24 h	50.5 ± 3.8	3.6 ± 0.3	5.2

Yeast transformed with respective constructs were grown for 24 h at 30 °C in the presence of galactose before being supplemented with 250  $\mu$ M  $\alpha$ -linolenic acid (ALA). Time points were taken 5 min, 1 and 24 h after substrate was supplied to the medium. The ratios were calculated as (products/educt) using values corresponding to per cent of total fatty acids. Each value is the mean  $\pm$  SD from three independent experiments.

### Acyl-CoA profiling of $\Delta 6$ -desaturase activities in yeast

To better understand the dynamics of acyl desaturation in yeast, we also determined the acyl-CoA pool composition for the same time points. As can be seen in Figure 1, the *O. tauri*  $\Delta 6$ -desaturase generated substantial levels of SDA-CoA after just five minutes, although this was not directly proportional to the (higher) levels observed in total lipids at this time-point. Two other predicted acyl-CoA-dependent  $\Delta 6$ -desaturases, from mouse and salmon, also directed the conversion of ALA-CoA to SDA-CoA within the first time-point (5 min), although again there was no obvious relationship between the levels of non-native fatty acids in the acyl-CoA pool vs. the total fatty acids. In similar analysis of the phospholipid-dependent borage  $\Delta 6$ -desaturase, only very minor levels of SDA were detected in either the acyl-CoA pool or total fatty acids after 1 h, despite the presence of high levels of substrate ALA. Thus, the early appearance of the  $\Delta 6$ -desaturation product SDA in the acyl-CoA pool is, in agreement with the studies of Domergue *et al.* (2003, 2005b), indicative of an acyl-CoA-dependent desaturase. Conversely, the later detection of SDA-CoA in yeast cells expressing BoD6 is commensurate with a lipid-dependent enzyme carrying out desaturation on fatty acids esterified to phospholipids, with these desaturation products then subsequently undergoing acyl-exchange and entering the CoA pool. As noted earlier, several putative acyl-CoA-dependent



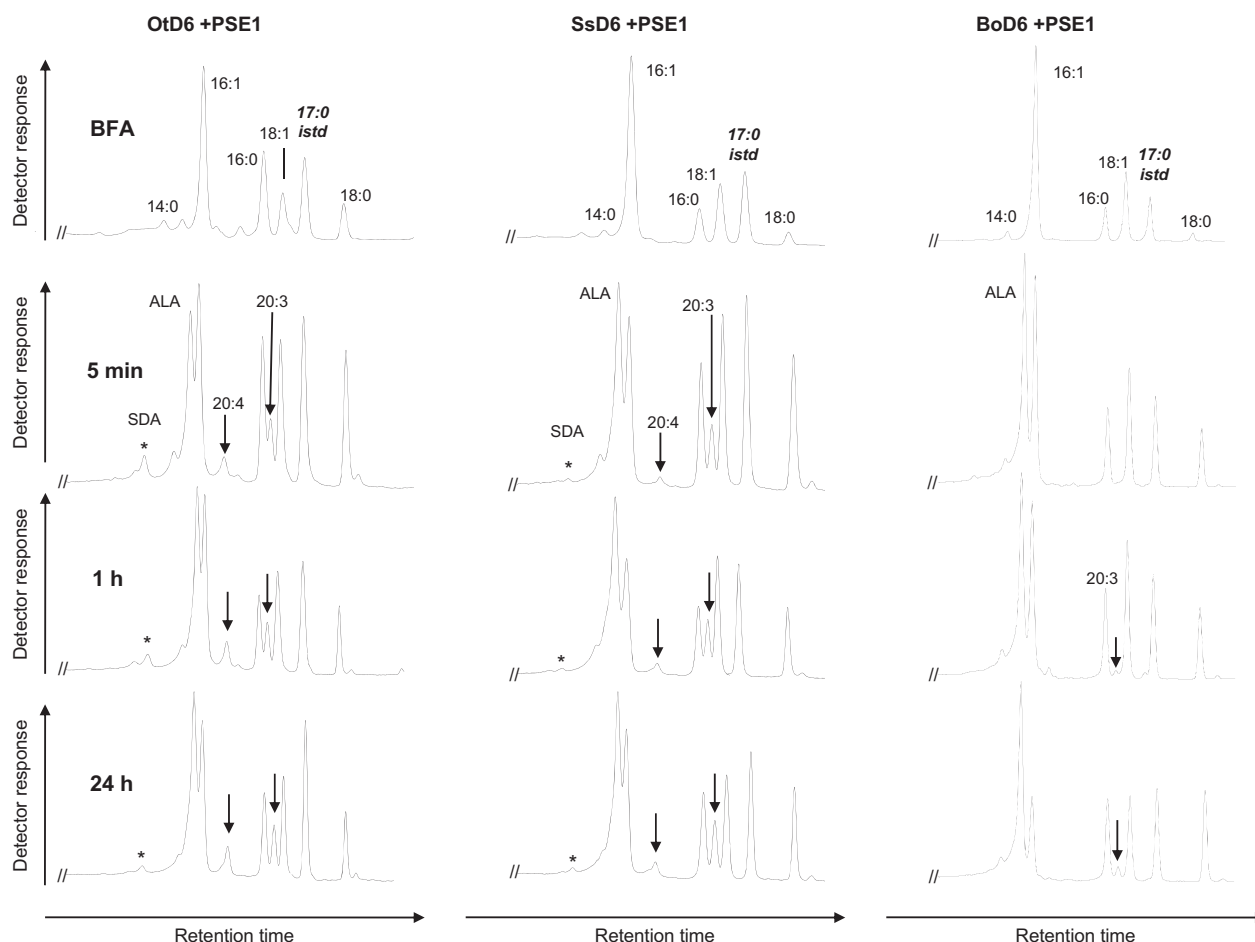
**Figure 1** Kinetic analysis of fatty acid profiles in lipids and acyl-CoAs of yeast expressing  $\Delta 6$ -desaturases in the presence of  $\alpha$ -linolenic acid (ALA). Cultures of yeast transformed with OtD6, SsD6, MmD6 and BoD6 were grown for 24 h at 30 °C in the presence of galactose before being supplemented with 250  $\mu$ M ALA. The fatty acid composition as well as the acyl-CoA pool composition of the transgenic yeast was analyzed before adding ALA (BFA = before addition) as well as 5 min and 1 h after substrate was supplied to the medium. The desaturated product stearidonic acid (SDA) is marked by a star. Data shown are representative for three independent experiments, with chromatographic resolution occurring against time (X-axis). Detector response (Y-axis) is derived from FID (a) or FLD with the excitation wavelength was set to 230 nm and the emission wavelength to 420 nm. (a) Fatty acid composition of yeast cells expressing OtD6 (a, b), SsD6 (c, d), MmD6 (e, f) and BoD6 (g, h) in the presence of ALA. (b) Acyl-CoA profiling of yeast cells expressing OtD6, MmD6, SsD6 and BoD6 in the presence of ALA. The internal standard ( $I_{std}$ ) is 17:0 acyl-CoA.

desaturases directed the accumulation of SDA in total fatty acids after only 5 min. Given that the acyl-CoA pool is a very small metabolic pool (in the pmol range), we suggest that the observed accumulation represents the acyl-CoA-dependent incorporation of SDA into glycerolipids. This exchange between the acyl-CoA pool and phospholipids involves several enzymes, including the acyl-CoA-dependent enzymes of the Kennedy pathway, acyl-CoA generating activities such as phospholipases and also the reverse reaction of LPCAT (reviewed in Napier and Graham, 2010). The latter has been suggested as a mechanism by which the metabolic bottleneck of substrate dichotomy is overcome *in vivo* (Abbadì *et al.*, 2004; Venegas-Calero *et al.*, 2010).

### Reconstitution of LC-PUFA synthesis in yeast

To further explore the nature of these desaturases and better determine their acyl-substrate preferences, we co-expressed them with a microsomal acyl-CoA-dependent ketoacyl-CoA synthase (PSE1) from *Physcomitrella patens*, which has previously been shown to specifically elongate C18  $\Delta 6$ -unsaturated acyl-CoAs to C20 forms (Zank *et al.*, 2002). Here, we studied the desaturases from *O. tauri* (algal lower eukaryote acyl-CoA-dependent enzyme) salmon (vertebrate acyl-CoA enzyme) and

borage (higher plant phospholipid-dependent enzyme). As the product of an acyl-CoA-dependent desaturase will remain esterified to CoA, the *in vivo* coupling of desaturation and elongation has previously been used as an (indirect) method by which to differentiate desaturase substrate preference (CoA vs. phospholipids) (Domergue *et al.*, 2003). Co-expression in yeast, induction and kinetic analysis of total fatty acids and acyl-CoAs was carried out as described earlier. As can be seen in Figure 2, when OtD6 was co-expressed with PSE1, substantial levels of both the  $\Delta 6$ -desaturation product SDA and the subsequent C20 elongation product eicosatetraenoic acid (ETA; 20:4n-3) were detected in the acyl-CoA pool after only 5 min, whereas they comprised <2% of the yeast total fatty acids at this time (Table 2). The absolute levels of SDA-CoA and ETA-CoA did not substantially increase over 24 h for OtD6 + PSE1, whereas their percentage of total fatty acids (tfa) increased markedly (Figure 2, Table 2). Co-expression of SsD6 with PSE1 generated modest but detectable levels of SDA-CoA and ETA-CoA (Figure 2), and the tfa profile showed slower accumulation of SDA with lower levels of ETA (Table 2). As predicted for a phospholipid-dependent desaturase, BoD6 + PSE1 did not generate any appreciable SDA-CoA or ETA-CoA, although both these non-native fatty acids were present in tfas.



**Figure 2** Kinetic analysis of fatty acid changes in acyl-CoAs of yeast co-expressing  $\Delta 6$ -desaturases and PSE1. Cultures of yeast co-transformed with one of the  $\Delta 6$ -desaturases (OtD6, SsD6 or BoD6) and PSE1 were grown for 24 h at 30 °C in the presence of galactose before being supplemented with 250  $\mu\text{M}$   $\alpha$ -linolenic acid (ALA). The acyl-CoA pool composition of the transgenic yeast was analyzed before adding ALA (BFA) and as well as 5 min, 1 and 24 h after substrate was supplied to the medium. The desaturated product stearidonic acid (SDA) is marked by a star, and elongated products are indicated with arrows. Data shown are representative for three independent experiments. The internal standard ( $I_{std}$ ) is 17:0 acyl-CoA.

**Table 2** Kinetic analysis of fatty acid changes of yeast co-expressing  $\Delta 6$ -desaturases and PSE1

Construct	18:3 n-3 (ALA)	18:4 n-3 (SDA)	20:4 n-3 (ETA)
<b>OtD6 + PSE1</b>			
5'	1.4 ± 0.4	1.2 ± 0.3	<0.5
1 h	4.2 ± 1.0	1.9 ± 0.2	1.0 ± 0.3
24 h	17.7 ± 2.5	8.2 ± 0.9	6.2 ± 0.7
<b>SsD6 + PSE1</b>			
5'	1.0 ± 0.2	–	–
1 h	6.5 ± 1.1	0.4 ± 0.1	0.7 ± 0.2
24 h	32.0 ± 4.6	6.1 ± 1.0	1.3 ± 0.3
<b>BoD6 + PSE1</b>			
5'	2.1 ± 0.5	–	–
1 h	10.2 ± 2.2	0.2 ± 0.1	0.3 ± 0.1
24 h	40.6 ± 4.4	0.5 ± 0.2	1.5 ± 0.6

Cultures of yeast co-transformed with one of the  $\Delta 6$ -desaturases (OtD6, SsD6 or BoD6) and PSE1 were grown for 24 h at 30 °C in the presence of galactose before being supplemented with 250  $\mu$ M ALA. The fatty acid composition of the transgenic yeast was analyzed 5 min, 1 and 24 h after substrate was supplied to the medium. Each value is the mean  $\pm$  SD from three independent experiments.

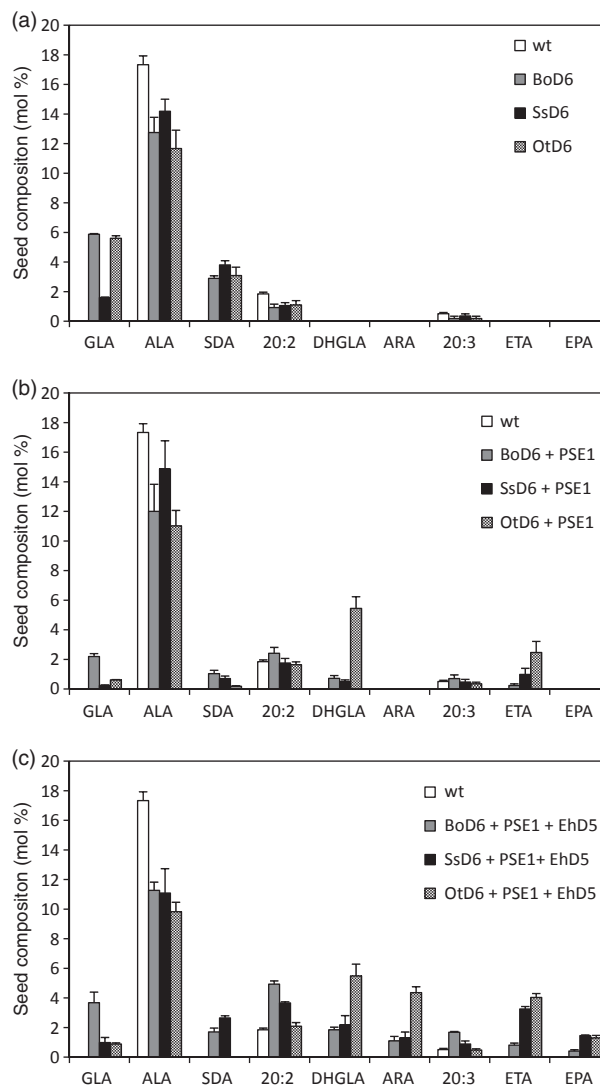
ALA,  $\alpha$ -linolenic acid; SDA, stearidonic acid; ETA, eicosatetraenoic acid.

Interestingly, an abundant acyl-CoA identified as 20:3n-3 CoA was present in all samples in which ALA had been exogenously supplied (Figure 2), indicating that PSE1 can utilize additional C18 substrates beyond just SDA and GLA. In view of the promiscuous activity of the PSE1  $\Delta 6$ -elongase, which potentially could deplete the substrate pool of ALA-CoA, we repeated the co-expression experiments with a second, different  $\Delta 6$ -elongase, CeElo6 from *Caenorhabditis elegans* (Beaudoin *et al.*, 2000; Napier and Michaelson, 2001; Robert *et al.*, 2005), which is specific for C18  $\Delta 6$ -unsaturated substrates. Desaturation and elongation products were almost undetectable in the acyl-CoA pool of yeast cells expressing the borage desaturase and CeElo6 (Table S1).

Domergue *et al.* (2005b) suggested the asynchronous appearance of desaturation products in the acyl-CoA pool prior to detection in tfa extracts (as they reported for the *O. tauri*  $\Delta 6$ -desaturase) as a diagnostic hallmark of a *bona fide* acyl-CoA-dependent desaturase. Our results indicate that this is not a reliable indicator. Also, the presence or absence of desaturation products in total lipid extracts is not in itself likely to prove a reliable marker for enzyme substrate preference. Some small differences between our present study and that of Domergue *et al.* (2003, 2005b), including the detection of acyl-CoA intermediates for OtD6 + PSE1 and SsD6 + PSE1 reactions, likely reflect improvements in chromatographic resolution of acyl-CoAs. Collectively our data support the classification of OtD6 and SsD6 as acyl-CoA-dependent activities.

### $\Delta 6$ -desaturase activities in transgenic plants

Previously, no systematic comparative analysis of the performance of different  $\Delta 6$ -desaturases in transgenic plants has been carried out, even though the use of acyl-CoA-dependent  $\Delta 6$ -desaturases has been widely suggested as a means to overcome the metabolic bottleneck of substrate dichotomy, and hence, enhance the accumulation of valuable C20+ long-chain



**Figure 3** Total fatty acid composition of seeds from transgenic Arabidopsis lines. FAMES were analyzed from wild type (wt), single-transgenic lines expressing  $\Delta 6$ -desaturases (a), double-transgenic lines co-expressing  $\Delta 6$ -desaturases and PSE1 (b) and triple-transgenic lines of Arabidopsis, co-expressing  $\Delta 6$ -desaturases, PSE1 and EhD5 (c). Each value represents the mean  $\pm$  SD of 10 independent lines. In all cases, the SD was <1.

polyunsaturated fatty acids. We therefore generated transgenic Arabidopsis plants that expressed OtD6, SsD6 or BoD6 desaturases under the control of the conglycinin promoter. As shown in Figure 3, all three desaturases independently directed the synthesis and accumulation of  $\Delta 6$ -unsaturated fatty acids in mature seeds from homozygous T2 progeny. SDA accumulated to broadly similar levels for all three desaturases (3.5% tfa, BoD6, 4.7% tfa, SsD6; 3.8% tfa, OtD6), whereas plants expressing SsD6 accumulated less GLA (1.8% tfa) compared with those expressing BoD6 or OtD6 (6.0 and 5.8% tfa). These data indicate that the salmon desaturase has a strong substrate preference for omega-3 fatty acids. Petrie *et al.* (2010) recently reported that expression of an acyl-CoA-dependent  $\Delta 6$ -desaturase from *Micromonas pusilla* yielded GLA + SDA levels ranging from 8% to 18% tfa in the seeds of transgenic Arabidopsis.



The *M. pusilla*  $\Delta 6$ -desaturase appears to have a preference for omega-3 substrate, unlike the OtD6 enzyme that has a clear omega-6 bias.

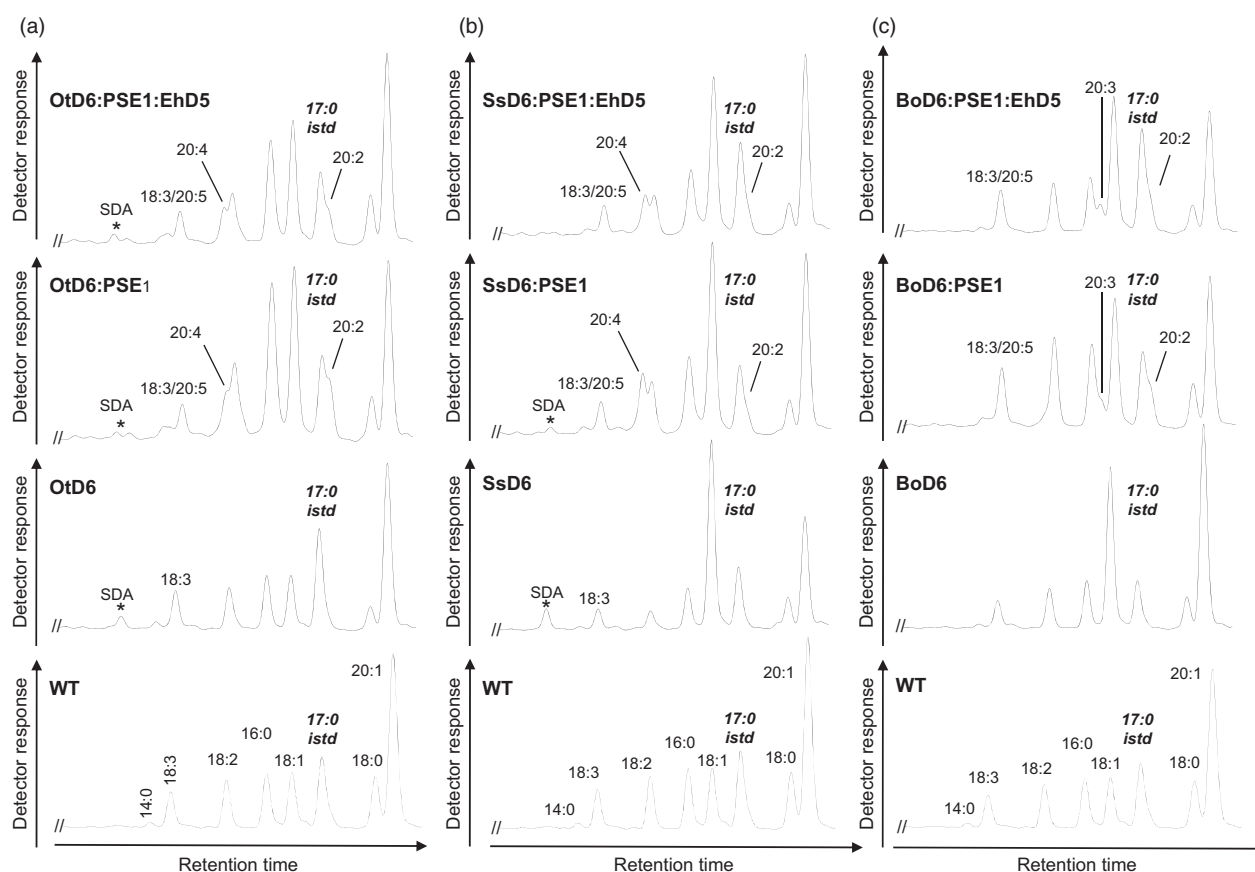
In a second iteration, the individual desaturases were co-expressed with the PSE1  $\Delta 6$ -elongase, also under the control of the seed-specific conglycinin promoter. Similar analysis fatty acid composition in mature T2 seeds confirmed our observations in yeast that the phospholipid-dependent BoD6 desaturase did not facilitate the subsequent elongation of  $\Delta 6$ -unsaturated fatty acids, with only low levels of C20 products being detected (1.0% 20:3n-6, 0.4% 20:4n-3). Seed-specific expression of SsD6 + PSE1 yielded 0.8% 20:3n-6 and 1.5% 20:4n-3, and OtD6 + PSE1 generated 5.8% 20:3n-6 and 3.0% 20:4n-3, indicative (most clearly in the latter case) of a more efficient flux of substrate between the desaturation and elongation reactions.

In a third iteration, we aimed to reconstruct the biosynthetic pathway for C20 LC-PUFAs by co-expressing the  $\Delta 6$ -desaturases and the PSE1  $\Delta 6$ -elongase with a downstream C20  $\Delta 5$ -desaturase, thus reconstructing the biosynthetic pathway for C20 LC-PUFAs. We placed the recently characterized *Emiliania huxleyi*  $\Delta 5$ -desaturase (EhD5) (Sayanova et al., 2011). Low levels of EPA were obtained in mature T2 seeds (0.5% tfa for BoD6 + PSE1 + EhD5; 1.4% tfa for OtD6 + PSE1 + EhD5; 1.5% tfa for SsD6 + PSE1 + EhD5). Interestingly, the omega-6 LC-PUFA arachidonic acid (ARA, 20:4n-6) accumulated to 4.7% tfa in the OtD6-containing construct, compared with 1.2% tfa in the

BoD6-containing construct and 1.2% in the SsD6-containing version, perhaps indicative of an omega-6 substrate preference with this enzyme.

### Targeted lipidomic analysis of transgenic plants expressing $\Delta 6$ -desaturases

To better define the biochemical basis of these results, detailed analysis was carried out on the acyl-CoA pool and glycerolipids of these Arabidopsis seeds. As can be seen in Figure 4 and confirming previous observations, the acyl-CoA pool is altered by the expression of heterologous desaturases and elongase. In the case of the transgenic lines solely expressing a  $\Delta 6$ -desaturase, acyl-CoA profiles for OtD6 and SsD6 contained an additional peak identified as SDA-CoA, whereas this peak was absent in profiles for BoD6, which was essentially the same as WT (not shown). Co-expression of the PSE1 elongase with the  $\Delta 6$ -desaturase confirmed the promiscuous nature of the PSE1 activity, as a peak identified as 20:2n-6 was present in all three double constructs. In the case of BoD6 + PSE1, a small peak identified as 20:3 (n-3 or n-6; it is not possible to resolve regioisomers using chromatography) was detected. This peak was not seen in the OtD6 + PSE1 or SsD6 + PSE1 double constructs (Figure 4). In the case of the OtD6 and SsD6 double constructs, an additional acyl-CoA identified as 20:4-CoA was interpreted as the elongation product of SDA-CoA. In support of this, the levels of SDA-CoA were seen to decrease in these samples. For seeds derived from the triple construct, in which a  $\Delta 6$ -desaturase and



**Figure 4** Acyl-CoA profiles of transgenic Arabidopsis. Acyl-CoA profiles of developing seeds of wild type (wt) and single-, double- and triple-transgenic Arabidopsis lines, expressing OtD6 (a), SsD6 (b) and BoD6 (c). The internal standard (*I*<sub>std</sub>) is 17:0 acyl-CoA. The positions of transgene-derived non-native fatty acids are indicated.

PSE1 were combined with EhD5, the acyl-CoA profiles were essentially the same as those observed with the double construct (Figure 4), with an apparent conversion of heterologous SDA-CoA to 20:4-CoA. Again, due to the limitations of the chromatography used to resolve acyl-CoAs, it was not possible to determine whether this 20:4-CoA was ARA (20:4n-6, from the elongation and  $\Delta$ 5-desaturation of GLA) or 20:4n-3 (the elongation product of SDA).

The same seed samples for the single and double constructs were also subject to detailed analysis of glycerolipids (GL) and glycerophospholipids (Tables 3 and 4). In the cases of the individual desaturase constructs, this revealed the widespread distribution of  $\Delta$ 6-desaturation products amongst polar and neutral lipids, with GLA and SDA being present, at varying levels, in triacylglycerol (TAG), diacylglycerol (DAG) and phospholipids (PL). Interestingly, the ratio of GLA in neutral lipids (NL) vs. phospholipids (PL) was higher from a lipid-dependent desaturase BoD6 (5.6% in NL and 4.0% in PL), than from SsD6 (1.6% in NL and 3.7% in PL) or OtD6 (5.4% in NL and 10.9% in PL) (Figure 5). Unexpectedly for acyl-CoA-dependent desaturases OtD6 and SsD6, GLA was accumulated mainly in polar lipids [phosphoethanolamine (PE); phosphatidylcholine (PC)]. SDA was present in

NL (2.6%) and PL (1.25%) for BoD6-expressing seeds, at 3.2% in both NL and PL for OtD6 and higher in NL (3.7%) than PL (2.5%) for SsD6, thus demonstrating that salmon  $\Delta$ 6-desaturase has preference for n-3 substrates. Similar analysis was also carried out for the double constructs described earlier. Here, the combination of the OtD6 desaturase and PSE1 elongase resulted in the specific enrichment (9.8% tfa) of DHGLA in PC, compared with 4.8% tfa in DAG and 3.4% for PI (Table 4). A similar distribution (albeit at a lower baseline) was also observed for SsD6 + PSE1, with notable accumulation of DHGLA in PC and to a lesser extent in PE. In contrast, DHGLA generated by BoD6 + PSE1 accumulated more in all phospholipids compared with neutral lipids, although to an overall lower level.

### Expression of the acyl-CoA-dependent $\Delta$ 6-desaturase OtD6 in transgenic *Camelina sativa*

To compare with our observations in *Arabidopsis*, we also engineered *Camelina sativa* to express the OtD6 acyl-CoA desaturase (using a similar construct to that used in *Arabidopsis*). *C. sativa* is a (re-)emerging oilseed crop, grown extensively in North America as a biofuel crop due to its modest requirements for nitrogen

**Table 3** Fatty acid composition (mol%) of neutral lipids isolated from seeds of wild type (wt), single- and double-transgenic *Arabidopsis* lines

Mol%	16:0	18:0	18:1	LA	GLA	ALA	SDA	20:1	DHGLA	ETA	Others
Total neutral lipids											
wt	7.1	3.5	16.3	27.4	0.0	17.2	0.0	21.2	0.0	0.0	7.2
BoD6	8.4	3.9	23.0	21.5	5.6	12.2	2.6	18.3	0.0	0.0	4.4
SsD6	8.6	3.9	20.2	27.3	1.6	14.3	3.7	15.2	0.0	0.0	5.2
OtD6	8.5	3.4	17.1	26.3	5.4	12.1	3.2	18.8	0.0	0.0	5.1
BoD6 + PSE1	8.8	3.6	17.9	28.5	2.3	12.6	1.1	17.2	0.8	<0.5	7.1
SsD6 + PSE1	8.5	3.8	17.8	29.6	<0.5	15.6	0.8	15.9	0.5	1.1	6.3
OtD6 + PSE1	9.2	3.8	15.5	28.8	0.6	11.4	<0.5	16.6	5.8	2.8	5.5
TAG											
wt	8.2	3.5	16.4	27.3	0.0	17.4	0.0	20.3	0.0	0.0	6.9
BoD6	8.2	3.8	22.3	21.6	5.8	13.0	3.0	17.8	0.0	0.0	4.6
SsD6	8.6	3.8	20.3	27.2	1.6	14.3	3.7	15.3	0.0	0.0	5.2
OtD6	8.5	3.3	16.8	26.4	5.5	12.4	3.4	18.5	0.0	0.0	5.2
BoD6 + PSE1	9.0	3.6	18.0	28.7	2.3	12.7	1.1	17.1	0.7	<0.5	6.9
SsD6 + PSE1	8.7	3.8	18.4	29.6	<0.5	14.9	0.7	16.3	0.5	1.0	6.1
OtD6 + PSE1	9.2	3.8	15.8	28.9	0.6	11.3	<0.5	16.8	5.6	2.6	5.5
DAG											
wt	7.8	3.6	17.1	36.1	0.0	14.4	0.0	14.7	0.0	0.0	6.2
BoD6	8.7	3.8	24.8	24.5	5.1	11.1	2.4	15.2	0.0	0.0	4.3
SsD6	8.6	3.7	19.3	32.2	2.2	12.4	4.4	12.1	0.0	0.0	5.0
OtD6	8.7	3.3	19.2	28.8	6.6	10.3	3.4	14.6	0.0	0.0	5.1
BoD6 + PSE1	10.3	3.8	18.5	30.9	1.8	11.1	0.9	13.9	1.2	0.5	7.1
SsD6 + PSE1	8.7	3.5	17.8	33.5	<0.5	14.6	1.3	11.8	0.7	1.4	6.8
OtD6 + PSE1	9.9	3.9	18.5	31.2	0.7	9.7	<0.5	12.8	4.8	2.2	6.4
Free fatty acids											
wt	9.5	8.5	15.5	22.1	0.0	12.7	0.0	21.7	0.0	0.0	9.9
BoD6	9.9	7.2	18.4	17.1	5.4	12.3	3.2	20.6	0.0	0.0	6.0
SsD6	10.3	7.3	15.5	18.8	1.7	16.0	5.9	17.1	0.0	0.0	7.4
OtD6	10.2	6.3	14.0	21.0	4.6	12.2	3.5	21.5	0.0	0.0	6.8
BoD6 + PSE1	13.1	6.9	15.2	21.7	2.1	9.5	0.9	20.6	0.7	<0.5	9.3
SsD6 + PSE1	11.8	6.8	14.5	22.2	0.6	14.8	1.0	17.9	0.7	1.4	8.4
OtD6 + PSE1	11.2	6.3	11.9	23.5	0.9	11.6	<0.5	18.5	5.6	2.8	7.6

Values shown are means derived from independent lines ( $n = 5$ ). In all cases, SD was <1.

GLA,  $\gamma$ -linolenic acid; ALA,  $\alpha$ -linolenic acid; SDA, stearidonic acid; ETA, eicosatetraenoic acid.

**Table 4** Fatty acid composition (mol%) of polar lipids isolated from seeds of wild type (wt), single- and double-transgenic Arabidopsis lines

	16:0	18:0	18:1	LA	GLA	ALA	SDA	20:1	DHGLAA	ETA	Others
Total polar lipids											
wt	11.0	2.7	14.5	49.2	0.0	12.3	0.0	5.0	0.0	0.0	5.3
BoD6	14.5	2.6	24.0	39.3	4.0	8.3	1.2	4.2	0.0	0.0	1.8
SsD6	15.2	2.9	17.3	43.7	3.7	7.5	2.5	3.9	0.0	0.0	3.3
OtD6	14.2	2.4	17.9	36.7	10.9	7.3	3.2	4.9	0.0	0.0	2.4
BoD6 + PSE1	14.1	2.4	19.1	47.1	0.8	8.1	<0.5	3.3	2.0	<0.5	3.0
SsD6 + PSE1	12.6	3.2	16.0	41.0	1.9	8.2	2.5	6.8	1.9	1.4	4.3
OtD6 + PSE1	13.7	2.4	19.2	40.4	<0.5	6.3	<0.5	4.1	8.5	1.8	3.6
PC											
wt	8.8	7.7	15.6	41.1	0.0	11.3	0.0	6.7	0.0	0.0	8.7
BoD6	9.8	2.2	27.5	39.6	3.9	8.8	1.2	4.6	0.0	0.0	2.3
SsD6	10.2	2.3	20.4	45.7	3.7	7.8	2.7	4.2	0.0	0.0	2.9
OtD6	10.0	2.1	20.9	37.4	11.4	7.3	3.3	4.9	0.0	0.0	2.7
BoD6 + PSE1	11.1	2.5	20.2	44.6	0.7	9.0	<0.5	5.1	2.0	0.5	4.3
SsD6 + PSE1	10.5	2.4	19.6	45.9	<0.5	9.2	<0.5	4.4	2.1	1.3	4.8
OtD6 + PSE1	10.5	2.1	20.7	38.6	<0.5	6.7	<0.5	5.0	9.8	2.2	4.3
PE											
wt	13.3	2.5	12.0	53.4	0.0	10.5	0.0	2.9	0.0	0.0	5.3
BoD6	16.9	2.1	19.0	44.2	5.0	7.6	1.2	2.6	0.0	0.0	1.5
SsD6	16.2	2.7	15.4	46.6	4.2	7.0	2.1	3.4	0.0	0.0	2.5
OtD6	17.5	2.2	14.9	40.8	11.5	6.2	2.3	2.6	0.0	0.0	2.0
BoD6 + PSE1	18.6	2.0	14.7	48.9	1.2	7.4	<0.5	2.2	2.1	0.5	2.4
SsD6 + PSE1	17.8	2.2	13.5	50.3	<0.5	8.3	<0.5	2.4	1.6	1.1	2.8
OtD6 + PSE1	19.1	2.2	14.2	44.6	<0.5	5.9	0.0	2.5	7.3	1.8	2.4
PI											
wt	24.9	5.2	8.7	38.5	0.0	16.1	0.0	1.9	0.0	0.0	4.8
BoD6	28.9	4.5	12.3	32.3	3.2	8.8	1.3	5.5	0.0	0.0	3.1
SsD6	29.8	4.5	9.6	34.5	2.5	8.7	2.4	4.9	0.0	0.0	3.1
OtD6	30.5	4.0	9.6	30.7	7.6	7.4	2.2	4.8	0.0	0.0	3.2
BoD6 + PSE1	29.0	5.1	10.9	36.6	0.9	8.0	<0.5	3.1	2.3	0.9	3.3
SsD6 + PSE1	29.3	4.6	9.9	35.4	<0.5	10.0	<0.5	3.8	0.9	1.0	5.1
OtD6 + PSE1	30.8	4.8	10.7	34.9	0.8	7.2	<0.5	3.9	2.5	1.0	3.4

Values shown are means derived from independent lines ( $n = 5$ ). In all cases, SD was <1.

GLA,  $\gamma$ -linolenic acid; ALA,  $\alpha$ -linolenic acid; SDA, stearidonic acid; ETA, eicosatetraenoic acid; DHGLA = di-homo- $\gamma$ -linolenic acid.

(Gehring *et al.*, 2006) but also used in Europe as a high omega-3 vegetable oil for human nutrition. As shown in Table 5, we recovered 12 transgenic *C. sativa* lines, the seeds of which were shown to contain substantial levels of GLA (~18% tfa) and SDA (~12% tfa). The level of non-native  $\Delta 6$ -desaturated fatty acids (30% of seed oil) was about twice that achieved in Arabidopsis using the same construct (Figure 3a) and did not compromise *C. sativa* seed development or germination.

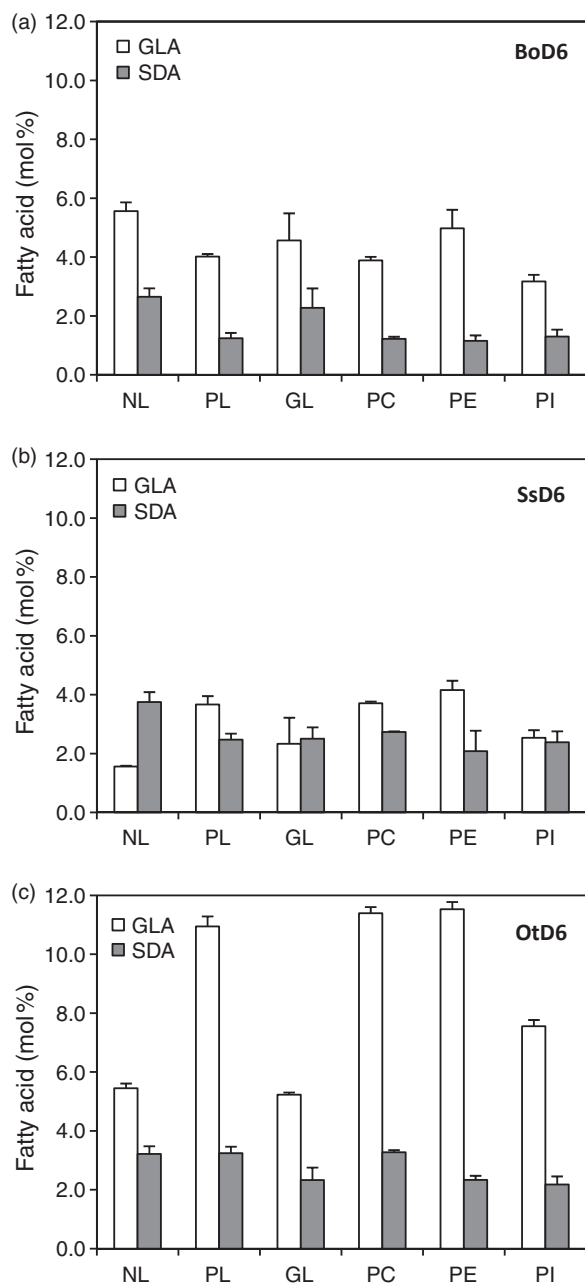
The results obtained with *C. sativa* for the acyl-CoA-dependent OtD6 desaturase were compared well to previous attempts to synthesize these fatty acids in transgenic soybean (Sato *et al.*, 2004; Eckert *et al.*, 2006) and *Brassica juncea* (Hong *et al.*, 2002) using phospholipid-dependent  $\Delta 6$ -desaturase activities. Compared with the accumulation of GLA and SDA in transgenic Arabidopsis seeds, the levels achieved in *C. sativa* with the same acyl-CoA-dependent OtD6 are notably higher (cf. Figure 3a – maximal total levels of  $\Delta 6$ -desaturated fatty acids = ~15%). In this current study, the levels of ALA are markedly reduced in *C. sativa* lines accumulating GLA and SDA, whereas LA levels are unchanged, even though the LA metabolite GLA is at a higher level than the ALA metabolite SDA (Table 5). This could indicate that LA is under stronger homeostatic regulation compared

with ALA and may have implications for any future attempts to utilize *C. sativa* as a transgenic host for heterologous lipid metabolism. Given that any transgene-derived activities will have to efficiently interact with endogenous lipid synthesis, further biochemical characterization of *C. sativa* fatty acid metabolism is required. However, our initial results on the accumulation of  $\Delta 6$ -desaturated fatty acids in this crop support the suggestion of Lu *et al.*, 2011 that *C. sativa* is an amenable host for the accumulation of non-native fatty acids, including nutritionally important ones such as SDA.

## Conclusions

The results obtained in this study confirm the value of using an acyl-CoA-dependent  $\Delta 6$ -desaturase in attempts to synthesize C20 LC-PUFAs in transgenic plants. We have demonstrated that the use of either algal or vertebrate acyl-CoA  $\Delta 6$ -desaturases can enhance the flux of substrate acyl chains through the LC-PUFA biosynthetic pathway, leading to increased levels of target C20 PUFAs and reduced accumulation of undesirable intermediates such GLA. Conversely, the presence of an acyl-CoA-dependent  $\Delta 6$ -desaturase leads to the more uniform distribution of C20





**Figure 5** Distribution of GLA and stearidonic acid (SDA) within the different lipid fractions of transgenic *Arabidopsis* seeds, expressing different  $\Delta 6$ -desaturases (a, BoD6; b, SsD6; c, Otd6). Mature *Arabidopsis* seeds were used for lipid analysis. The ratios were calculated as (products/educt) using values corresponding to per cent of total fatty acids. The values are the mean  $\pm$  SD (<1) of three independent lines.

PUFAs in phospholipids and neutral lipids, commensurate with the elevated levels of C20 acyl-CoAs available as substrates for forward acylation reactions associated with the Kennedy pathway.

## Experimental procedures

### Expression in yeast

The different yeast expression constructs used in this study are listed in Table S2. The *S. cerevisiae* strains W303-1A and INVSc1

from Invitrogen were transformed with plasmid DNA using a lithium acetate method. Cultures were grown at 22 °C in the presence of 2% (v/v) raffinose for 48 h, and expression of the transgenes was induced by addition of galactose to 2% in the presence of 0.5 mM of ALA and 1% (w/v) tergitol-Nonidet p40 (Sigma, St Louis, MO, USA) as described (Tonon *et al.*, 2005).

For kinetic experiments, transgenic yeast cultures were grown for 24 h at 30 °C in the presence of galactose to a 1.5 OD<sub>600</sub> for permanent expression of  $\Delta 6$ -desaturases before the start of kinetic analysis by adding 250  $\mu$ M ALA as a substrate Domergue *et al.*, 2003). Just before supplementing ALA (BFA) as well as 5 min, 1 and 24 h after substrate addition, the fatty acid composition of the transgenic yeast cells and of their acyl-CoAs were determined.

### Acyl-CoA profiling

For acyl-CoA analysis of yeast, 20 mL of liquid cultures was harvested and frozen in liquid nitrogen for subsequent extraction and quantitative analysis of fluorescent acyl-etheno-CoA derivatives by HPLC (Agilent 1100 LC system; Phenomenex LUNA 150  $\times$  2 mm C18(2) column) as described previously (Larson and Graham, 2001).

For acyl-CoA analysis of plants, 20 mg of developing (15 days after flowering) seeds was collected, frozen in liquid nitrogen and analyzed as described earlier.

### Fatty acid analysis

Fatty acids were extracted and methylated as described (Sayanova *et al.*, 1997). Methyl ester derivatives of total fatty acids extracted were analyzed by GC and GC-MS.

### Lipid analysis

Three-hundred milligrams of seeds was 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 pre-fractionated 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.

### 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  $\mu$ mol/m<sup>2</sup>/s, photoperiod (long day). *Camelina sativa* was grown under similar conditions.

### Plant transformation constructs

Open reading frames encoding Otd6, SsD6, EmD5 and PSE1 were used as templates to chemically synthesize (Genscript Corporation, NJ) codon-optimized nucleotide sequences for expression in *Arabidopsis*. All coding regions used were placed under

**Table 5** Accumulation of  $\Delta 6$ -desaturated fatty acids in transgenic *Camelina sativa* via seed-specific expression of Otd6

		Fatty acids (Mol%)								
Line ID		16 : 0	18 : 0	18 : 1	18 : 2	GLA	ALA	SDA	20 : 1	Others
Mean	WT	7.0	3.3	16.2	20.8	0.0	29.5	0.0	13.6	9.4
SD		0.1	0.5	0.7	0.4	0.0	0.4	0.0	1.2	0.4
Mean	# 48	10.3	6.8	5.5	19.1	18.8	8.3	12.5	8.0	10.8
SD		0.3	0.6	0.2	0.3	1.0	0.8	0.5	0.4	0.9
Mean	#75	9.7	9.2	6.2	20.9	17.5	7.5	10.5	7.0	11.6
SD		0.1	0.3	0.0	0.6	0.7	0.1	0.6	0.1	0.3
Mean	#2	10.1	8.1	6.0	20.0	18.7	8.2	11.6	6.5	10.8
SD		0.3	0.5	0.1	0.1	0.1	0.5	0.3	0.3	0.7
Mean	#206	8.8	7.1	6.7	22.0	16.4	9.8	10.4	8.0	10.9
SD		0.4	0.4	0.3	0.5	0.8	0.8	0.2	0.2	0.4
Mean	#244	8.8	7.4	5.6	19.5	16.6	10.3	12.3	7.9	11.5
SD		0.5	0.1	0.4	0.8	0.7	0.6	0.6	0.3	0.2
Mean	#40	10.8	6.7	5.4	19.0	19.0	9.0	13.4	6.9	9.8
SD		0.4	0.4	0.4	0.4	0.6	0.3	0.1	0.3	1.0
Mean	#53	9.9	7.9	5.7	19.4	18.7	8.3	12.2	7.1	10.8
SD		0.4	0.2	0.2	0.8	0.3	0.8	0.6	0.1	0.2
Mean	#232	9.7	9.0	6.2	20.5	17.4	7.8	10.5	7.4	11.5
SD		0.2	0.3	0.3	0.6	1.1	0.3	0.7	0.4	1.1
Mean	#200	8.9	8.1	6.4	19.9	17.9	8.6	11.5	7.6	11.1
SD		0.2	0.1	0.1	0.2	0.4	0.3	0.0	0.1	0.6
Mean	#102	9.4	8.0	5.7	19.3	18.0	8.7	12.1	7.7	11.1
SD		0.2	0.4	0.0	0.3	0.4	0.6	0.5	0.1	0.3
Mean	#55	9.7	8.9	5.8	20.6	18.0	7.9	11.3	7.2	10.6
SD		0.3	0.4	0.0	0.3	0.2	0.3	0.3	0.3	0.4
Mean	#215	9.3	6.9	5.5	19.5	17.0	10.5	13.0	8.0	10.4
SD		0.1	0.8	0.3	0.9	0.5	0.8	0.9	0.1	0.2

Total seed fatty acids from transgenic *C. sativa* lines (T3) expressing the Otd6 desaturase under the glycinin promoter are shown, compared with wild type controls. For each line, the figure given is the mean of three biological replicates (5 seeds/rep) for each line. SD is shown. The predominant fatty acids are shown.

GLA,  $\gamma$ -linolenic acid; ALA,  $\alpha$ -linolenic acid; SDA, stearidonic acid.

the control of the glycinin-1 promoter. The coding sequences for  $\Delta 6$  desaturases from *B. officinalis* (Sayanova et al., 1997) and both *Salmo salar* (Zheng et al., 2005) and *O. tauri* (Domergue et al., 2005a, 2005b) were inserted as *XbaI-XhoI* and *EcoRI-XhoI* fragments, respectively, into the binary vector RS-3GSeedDSred, kindly provided by Dr Ed Cahoon (University of Nebraska). The coding regions of  $\Delta 5$  desaturase from *E. huxleyi* (EmD5, Sayanova et al., 2011) and  $\Delta 6$ -elongase PSE1 from *P. patens* (Zank et al., 2002) were introduced as *NotI-NotI* fragments into pKMS3 promoter-nos terminator expression cassettes. The double-gene constructs were built by removing glycinin-1::PSE1 cassette from pKMS3 by digestion with *AscI* and cloning it into *MluI* site of the binary vector RS-3GSeedDSred containing one of the three  $\Delta 6$ -desaturases (BoD6, SsD6 or Otd6). The glycinin-1::EmD5 expression cassette was excised with *AscI* and inserted into the binary vector pKAN as previously described (Sayanova et al., 2006). The orientation of expression cassettes was unidirectional within the T-DNA vector.

### Generation of transgenic plants

Transgenic *Arabidopsis* plants were generated as previously described (Sayanova et al., 2006). Single-transgenic plants expressing  $\Delta 6$ -desaturases (BoD6, SsD6 or Otd6) and double-

transgenic plants all showing positive for visual selection of the DsRed marker were self-fertilized to T3. Single-transgenic plants expressing EmD5 were kanamycin resistant and were germinated after two rounds of self-fertilization. Triple-transgenic plants were produced by transforming the double-transgenic homozygous lines with the highest C20 fatty acid content with a T-DNA containing EmD5 and a kanamycin-resistance gene. Red seeds from kanamycin (50  $\mu\text{g}/\text{mL}$ ) resistant plants were selected and grown to obtain homozygous T3 transformants.

Transgenic *Camelina sativa* plants were generated, selected and grown as described by Lu and Kang (2008). Samples analyzed in this study were T3 generation.

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## Supporting information

Additional Supporting information may be found in the online version of this article:

**Figure S1** Kinetic analysis of fatty acid profiles of yeast expressing  $\Delta 6$ -desaturases in the presence of 18:3 n-3.

**Table S1** Kinetic analysis of fatty acid profiles in lipids and acyl-CoAs of yeast co-expressing  $\Delta 6$ -desaturases and CeElo6 in the presence of ALA.

**Table S2** Description of yeast strains used in this study.

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