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Stepwise engineering to produce high yields of very long-chain polyunsaturated fatty acids in plants

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Very long chain polyunsaturated fatty acids (VLCPUFAs) such as arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are valuable commodities that provide important human health benefits[1–5]. We report the transgenic production of significant amounts of AA and EPA in *Brassica juncea* seeds via a stepwise metabolic engineering strategy. Using a series of transformations with increasing numbers of transgenes, we demonstrate the incremental production of VLCPUFAs, achieving AA levels of up to 25% and EPA levels of up to 15% of total seed fatty acids. Both fatty acids were almost exclusively found in triacylglycerols, with AA located preferentially at *sn-2* and *sn-3* positions and EPA distributed almost equally at all three positions. Moreover, we reconstituted the DHA biosynthetic pathway in plant seeds, demonstrating the practical feasibility of large-scale production of this important ω-3 fatty acid in oilseed crops.

Plants have the capacity to serve as green factories for the production of novel industrial materials, nutritionally enhanced foods or pharmaceuticals, via metabolic engineering[6–8]. One goal of plant metabolic engineering is the production of high levels of VLCPUFAs in oilseed plants[9,10], which would provide a novel and cost-effective source of these fatty acids. Several pathways for the biosynthesis of VLCPUFAs exist in nature[11]. To produce VLCPUFAs in seeds, we followed the alternating desaturation/elongation pathways of n-6 and n-3 fatty acids. The two routes commence with linoleic acid (18:2n-6, LA) and α-linolenic acid (18:3n-3, ALA), respectively, followed by sequential Δ6 desaturation, Δ6 elongation and Δ5 desaturation, leading to the synthesis of arachidonic acid (20:4n-6, AA) in the n-6 and eicosapentaenoic (20:5n-3, EPA) in the n-3 pathway. The two pathways can be interconnected by a ω3 desaturase that converts AA into EPA. Further Δ5 elongation and Δ4 desaturation reactions lead to the synthesis of docosapentaenoic (22:5n-3, DPA) and finally docosahexaenoic acid (22:6n-3, DHA). The *B. juncea* breeding line 1424 was chosen as a host plant for biosynthesis of VLCPUFAs because of its high LA content and lack of erucic acid. The constructs for VLCPUFA production in seeds carried three to nine structural genes, with each gene under the control of the seed-specific napin promoter (**Fig. 1**).

The first construct (BJ3) introduced into *B. juncea* contained a Δ6 desaturase from *Pythium irregulare*[12], a Δ5 desaturase from *Thraustochytrium* sp.[13] and a Δ6 fatty acid elongase from *Physcomitrella patens*[14]. This represents the minimal set of transgenes required for the synthesis of AA and EPA from endogenous LA and ALA. RT-PCR indicated that all three genes were highly expressed in the developing seeds (data not shown). Several new fatty acids were detected in BJ3 seeds (**Fig. 2**). The most abundant was γ-linolenic acid (GLA), the Δ6 desaturation product of LA, with an average value of 27.7% of total seed fatty acids. AA, the Δ5 desaturation product of dihomo-γ-linolenic acid (20:3n-6, DGLA), ranged from 5.0% to 8.5% (average 7.3%), whereas stearidonic acid (SDA), the Δ6 desaturation product of ALA, averaged 3.1%; several other minor new fatty acids, such as 18:2n-9 (1.7%), were also present (**Table 1**). Consequently, LA content dropped dramatically from 45.2% in the untransformed control to 13.7% in transgenic seeds. Thus, the Δ6 and Δ5 desaturases functioned well, with conversion rates of 68.3% and 94.2%, respectively. The Δ6 elongase performed less efficiently, with a conversion rate of only 23.6%. The n-6 pathway appeared to be much more effective in VLCPUFA biosynthesis, perhaps not surprisingly, given that *B. juncea* oil is characterized by high LA (45.2%) and low ALA (9.7%).

To increase LA and concurrently reduce the side-product 18:2n-9, we added a Δ12 desaturase gene from *Calendula officinalis*[15] to the triple construct, producing the construct BJ4. The addition of this desaturase resulted in only a slight decrease (0.5%) of 18:2n-9. Enhanced conversion of oleic acid (OA) to LA was evident from the decrease in OA content. Although the GLA content remained similar to that in BJ3 plants, the average level of AA increased from 7.3% to 12.0%, with the highest level reaching 17.7%. EPA also increased from 0.8% to 1.3% (**Table 1**).

In view of the results from the BJ3 and BJ4 plants, where poor elongation from 18- to 20-carbon fatty acids limited the metabolic flux, we attempted to enhance elongation by adding a second Δ6 elongase from *Thraustochytrium* sp. When expressed in yeast, this elongase showed activity with both 18-carbon and 20-carbon fatty acids, but elongated GLA and SDA much more efficiently than AA and EPA (data not shown). In transgenic plants carrying this construct, named BJ5, a slight, but still significant increase in GLA elongation occurred. This in turn resulted in an increase in AA from an average of

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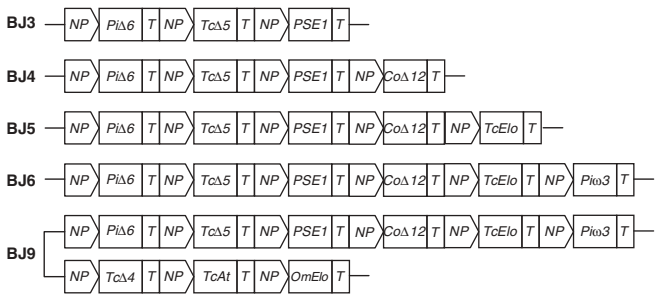

Figure 1 Simplified maps of the binary vector constructs used for plant transformation. BJ3, BJ4, BJ5, BJ6 and BJ9 represent the three-, four-, five-, six- and nine-gene constructs used for *B. juncea* transformation. *PiΔ6*, *TcΔ5*, *PSE1*, *CoΔ12*, *TcElo*, *Piω3*, *TcΔ4*, *TcAt* and *OmElo* represent a Δ6 desaturase from *P. irregulare*, a Δ5 desaturase from *Thraustochytrium* sp. 26185, an elongase from *P. patens*, a Δ12 desaturase from *C. officinalis*, an elongase from *Thraustochytrium* sp. 26185, an ω3 desaturase from *P. irregulare*, a Δ4 desaturase from *Thraustochytrium* sp. 26185, a lysophosphatidyl acyltransferase from *Thraustochytrium* sp. 26185 and an elongase from *O. mykiss,* respectively. *NP*, napin promoter; *T*, terminator OCS.

12.0% in BJ4 to 13.7% in BJ5 seeds, with the highest value observed being 25.8% (**Table 1**). Elongation of n-3 fatty acids also increased slightly, such that the overall elongation rate of both pathways increased from 34.0% in BJ4 to 38.3% in BJ5 seeds.

The high metabolic flux via the n-6 pathway in *B. juncea* resulted in the accumulation of considerable amounts of n-6 fatty acids such as GLA and AA. To use these n-6 fatty acids for the production of n-3 fatty acids, we included a ω3 desaturase from *Phytophtora infestans* in the construct BJ6. In yeast, this desaturase introduced an n-3 double bond specifically into AA (data not shown). The ω3 desaturase also effectively converted AA into EPA in transgenic seeds. As a result, the EPA content increased significantly, from an average of 1.4% in BJ5 to 8.1% in BJ6 plants, with a concurrent decrease in AA (**Table 1**).

After achieving substantial production of AA and EPA in plant seeds, the next logical step was to produce DHA. Therefore, three more genes were added to BJ6, creating the nine-gene construct BJ9. One of these genes encodes an elongase from *Oncorhynchus mykiss* that can elongate both 18- and 20-carbon fatty acids in yeast[16], whereas the second gene encodes a Δ4 desaturase from *Thraustochytrium* sp.[13]. The third gene, also from *Thraustochytrium* sp., represents a putative lysophosphatidic acid acyltransferase. We reasoned that this enzyme from a VLCPUFA-rich organism might improve trafficking of very long chain fatty acyls among lipid pools. Transcripts from all nine genes were detected in transgenic plants (data not shown). A fatty acid with a retention time identical to that of DHA was present in BJ9 seeds (**Fig. 2**), and gas chromatography/mass spectrometry (GC/MS) analysis confirmed that this fatty acid was indeed DHA (data not shown). The average yield of DHA was 0.2% of total fatty acids, whereas the highest observed value was 1.5%. BJ9 transgenic plants also produced slightly higher levels of EPA, with the highest observed level reaching 15.0% of total fatty acids. Whether the lysophosphatidyl acyltransferase or the third elongase contributes to the improvement in EPA production remains to be determined.

Elongation of EPA appears to be a serious bottleneck in DHA synthesis. The elongation rate from EPA to DPA was only 4%; consequently, only a low level of DHA was produced in BJ9 seeds. This might be due to limitations in the host plant's ability to release EPA into the acyl-CoA pool. Alternatively, the heterologous elongase may not cooperate efficiently with the endogenous elongation complex.

It should be noted that the fatty acid profiles and the derived conversion rates reflect only the situation in the whole cell, and disregard the possible existence of unavailable fatty acid pools. For instance, in transgenic *B. juncea* GLA accumulated to a relatively stable level (27.1 to 29.4%) regardless of the construct used. These GLA molecules could be incorporated into certain lipid classes, such as triacylglycerols, where they become unavailable for further modification. Nonelongated GLA seems to remain at a constant level, with amounts exceeding this threshold apparently becoming available for elongation. This is best illustrated in BJ4 plants, where the addition of a Δ12 desaturase led to a 7.8% decrease in OA, and the consequent increase in substrate flux led to a 5.9% increase of 20-carbon VLCPUFAs, rather than in the further accumulation of 18-carbon fatty acids.

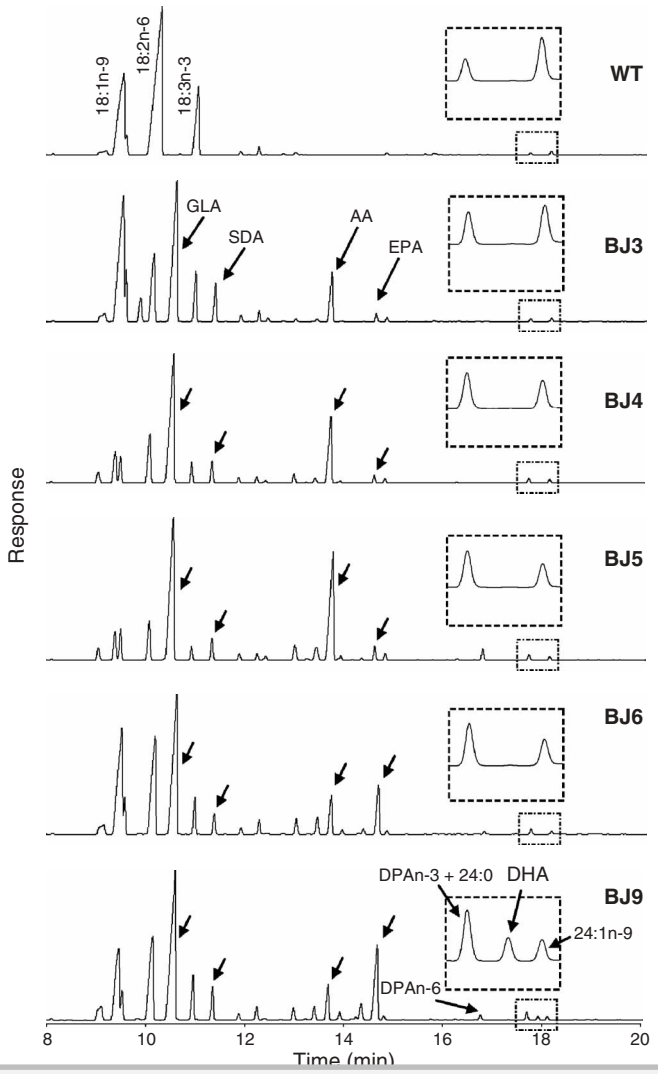

Figure 2 GC analysis of seed fatty acid methyl esters from wild-type and transgenic *B. juncea* plants. The constructs used for transformation are described in **Figure 1**. GLA, γ-linolenic acid; SDA, stearidonic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic

Table 1 Total fatty acid composition of oilseeds from the wild-type and transgenic *B. juncea* plants (wt%)

Fatty acid	Wild type ($n = 14$)	BJ3 ($N = 10$; $n = 19$)	BJ4 ($N = 7$; $n = 20$)	BJ5 ($N = 4$; $n = 28$)	BJ6 ($N = 3$; $n = 12$)	BJ9 ($N = 8$; $n = 30$)
16:0	5.6 ± 0.2	5.7 ± 0.2	5.8 ± 0.1	5.7 ± 0.1	5.6 ± 0.1	5.1 ± 0.2
18:0	1.7 ± 0.1	2.1 ± 0.1	2.2 ± 0.1	2.5 ± 0.1	2.4 ± 0.1	3.0 ± 0.2
18:1n-9 (OA)	33.2 ± 0.7	26.5 ± 0.4	18.7 ± 0.5	15.8 ± 0.9	18.4 ± 1.0	18.9 ± 1.0
18:1n-7	3.4 ± 0.1	3.5 ± 0.1	3.2 ± 0.1	3.3 ± 0.1	2.8 ± 0.10	2.4 ± 0.1
18:2n-9		1.7 ± 0.2	1.2 ± 0.2	0.5 ± 0.1	0.2 ± 0.04	0.2 ± 0.1
18:2n-6 (LA)	45.2 ± 0.5	13.7 ± 0.3	14.2 ± 0.4	15.4 ± 0.7	16.9 ± 0.5	16.0 ± 0.5
18:3n-6 (GLA)		27.7 ± 0.5	29.4 ± 0.9	28.6 ± 0.8	27.1 ± 0.8	27.3 ± 0.7
18:3n-3 (ALA)	9.7 ± 0.2	4.6 ± 0.3	4.1 ± 0.1	3.1 ± 0.2	4.2 ± 0.5	3.0 ± 0.1
18:4n-3 (SDA)		3.1 ± 0.1	2.7 ± 0.1	2.2 ± 0.1	2.4 ± 0.1	2.2 ± 0.1
20:3n-6 (DGLA)		0.5 ± 0.1	1.2 ± 0.1	2.1 ± 0.1	2.0 ± 0.1	1.9 ± 0.1
20:4n-6 (AA)		7.3 ± 0.4 (5.0–8.5)	12.0 ± 0.3 (8.2–17.7)	13.7 ± 0.7 (8.8–25.8)	5.4 ± 0.3 (4.0–7.4)	4.0 ± 0.2 (2.0–7.3)
20:4n-3 (ETA)				0.5 ± 0.1	0.8 ± 0.1	1.1 ± 0.1
20:5n-3 (EPA)		0.8 ± 0.1 (0.1–1.1)	1.3 ± 0.2 (0.9–1.7)	1.4 ± 0.1 (0.9–2.7)	8.1 ± 0.4 (6.4–11.0)	8.1 ± 0.4 (2.8–15.0)
22:5n-3 (DPA)						0.1 ± 0.02
22:6n-3 (DHA)						0.2 ± 0.03 (0–1.5)
Other	1.2 ± 0.2	2.8 ± 0.3	4.0 ± 0.2	5.2 ± 0.4	3.7 ± 0.4	6.5 ± 0.4

BJ3, BJ4, BJ5, BJ6 and BJ9 represent the transgenic plants generated from the three-, four-, five-, six- and nine-gene constructs. *N* and *n* are the number of independent transgenic plants and the total number of positive seeds analyzed, respectively. Each value represents the mean ± s.e.m. The values in brackets indicate the fatty acid ranges for AA, EPA and DHA. Other fatty acids include 14:0, 16:1n-7, 20:0, 20:1n-9, 20:2n-6, 22:0, 24:0 and others.

Transgenic BJ9 plants were phenotypically normal, as were plants carrying other constructs, and seeds containing substantial amounts of VLCPUFAs showed no obvious germination problems (data not shown). Thus, transgenic *B. juncea* seeds might be able to use the newly synthesized VLCPUFAs to support initial growth.

Although overall elongation rates in transgenic *B. juncea* appear low, they are actually much higher than those observed in flax and tobacco[10]. Accordingly, transgenic *B. juncea* accumulated higher amounts of VLCPUFAs in seeds. This could be due to the contribution of endogenous enzymes in *B. juncea* that are directly involved in the elongation process. The elongation complex includes four enzymes[17], and although the condensing enzyme (elongase) is critical in determining the substrate specificity of the elongation process, the remaining three enzymes may also play important roles in the overall elongation efficiency. Whereas erucic acid represents approximately 45% of total seed fatty acids in traditional *B. juncea* lines, the line used here contains only a trace amount of erucic acid. This might be due to a mutation in a particular condensing enzyme, as was observed in low erucic acid *Brassica napus*[18]. The remaining three enzymes in the elongation complex may thus be free to co-act with the transgenic elongase, resulting in higher elongation rates than in flax or tobacco. Alternatively, in *B. juncea* the shuffling of fatty acyls between the phospholid and acyl-CoA pools during the biosynthesis of VLCPUFAs may be more efficient. Indeed, in transgenic flax and tobacco, the low rate of elongation of Δ6-desaturated 18-carbon fatty acids to their 20-carbon counterparts was associated with a low level of Δ6-desaturated 18-carbon fatty acids in the acyl-CoA pool[10].

The VLCPUFAs produced in *B. juncea* seeds are almost exclusively present as triacylglycerols. For example in BJ4 and BJ9 seed, 93.9–98.6% of total AA and 96.0–98.1% of total EPA was found in triacylglycerols. Other lipid classes contain only very small amounts of these fatty acids. This is not unexpected, given the high levels to which AA and EPA accumulated in the seeds.

Positional analysis of BJ4 and BJ9 phospholipids (**Fig. 3a**) showed that GLA and AA were mainly located at the *sn-2* position, which appears to be the main site of Δ5 and Δ6 desaturation[19]. However, EPA, which is also a Δ5-desaturation product, was not predominantly located at the *sn-2* position. For most fatty acids, distribution at the *sn-1* and *sn-2* positions of triacylglycerol reflected the distribution pattern in phospholipids (**Fig. 3b**). The majority of AA was located at the *sn-2* and *sn-3* positions and EPA was almost equally distributed at all three positions of triacylglycerol. Preliminary analyses of developing BJ4 seeds (30 days after flowering) indicated that the fatty acid compositions of phospholipids and triacylglycerols were similar to those in mature seeds (data not shown). This suggests that the relative AA content in phospholipids is not dramatically reduced during seed desiccation, in contrast to what has been observed for medium-chain fatty acids[20,21].

A recent study reconstituted the conventional Δ6 desaturase/elongase pathway in seeds of transgenic flax and tobacco[10]. While the transgenic seeds accumulated high levels of Δ6 desaturated fatty acid, amounts of AA and EPA were only in the range of 1–2%. In work with *Arabidopsis thaliana*[9], somewhat higher levels of AA and EPA accumulated in leaves of plants carrying genes from the alternative Δ9 elongase/Δ8 desaturase pathway. This suggested that using the alternative pathway might overcome the problems associated with relatively poor elongation rates in the conventional pathway[9,22,23]. In fact, our preliminary experiments showed that the constitutive expression of the Δ6 elongase pathway in *B. juncea* resulted in even higher EPA levels in leaves than those observed in seeds (data not shown), implying that the particular host plant and targeted tissue have major effects on the efficiency of individual systems.

The synthesis of VLCPUFAs in plant seeds is an intricate biochemical process, requiring the sequential activity of multiple transgenic enzymes. The use of stepwise metabolic engineering provides an opportunity to observe the effects of individual genes in the biosynthetic pathway and offers insights into the intermediate steps of this complex process. Using this stepwise engineering, we increased the AA from 8.5% in BJ3 plants to 25.8% in BJ5 plants (maximum observed levels). The highest EPA level observed in BJ3 seeds was 1.1%, which increased to 15.0% in BJ9 plants. Since these measurements were taken from segregating populations, the highest observed value demonstrates the potential of lines to produce specific fatty acids.

The cloning of the first Δ4 desaturase from *Thraustochytrium* sp.[13] implied the existence of a simple pathway for DHA biosynthesis and suggested the possibility of producing this important fatty acid in

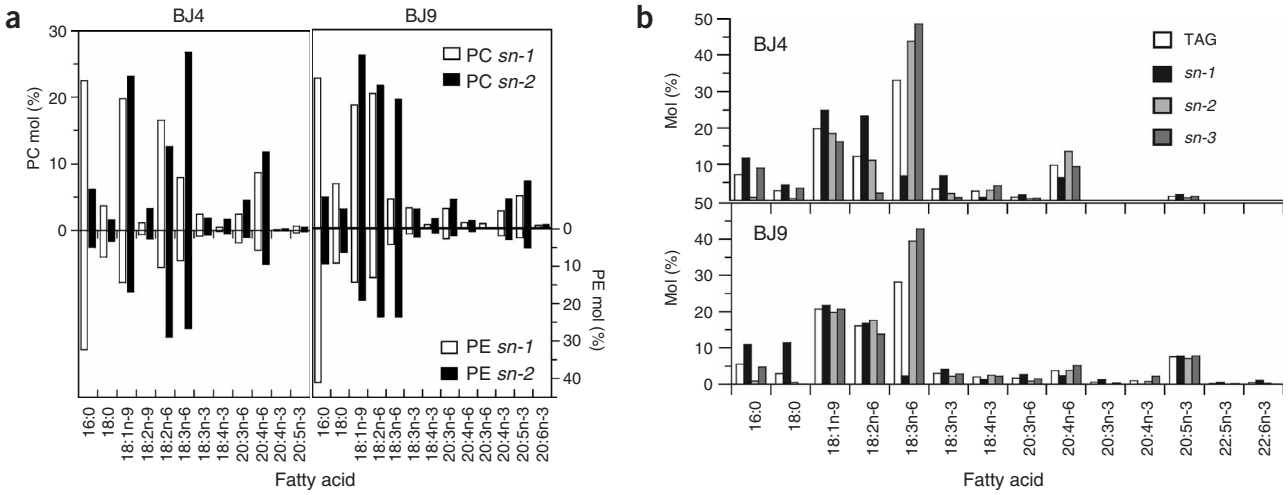

Figure 3 Stereospecific analysis of phospholipids and triacylglycerols. (**a**) The positional distribution of phosphatidylcholine (upper panels) and of phosphatidylethanolamine (lower panels). The distribution of selected fatty acids between the *sn-1* and *sn-2* positions are shown for BJ4 (left) and BJ9 (right) seeds. Fractionated phospholipids from mature seeds were digested with phospholipase A2 and the products were resolved by TLC, transmethylated and analyzed by GC. (**b**) The positional distribution of fatty acids in triacylglycerols from mature BJ4 and BJ9 seeds. Triacylglycerols were partially deacylated using ethyl magnesium bromide and the purified α,β-diacylglycerols were used in the synthesis of phosphatidylcholine. The distribution of fatty acids at the *sn-1* and *sn-2* positions was analyzed by digesting the resulting PC with phospholipase A2 and the *sn-3* position was calculated as described in the text. PC, phosphatidylcholine; PE, phosphatidylethanolamine; TAG, triacylglycerols.

plants. Here we described the reconstitution of the entire DHA biosynthetic pathway in plants. Achieving DHA synthesis in seeds, albeit at low levels, is a basis for further optimization to attain commercially viable levels, as has been demonstrated here for AA and EPA.

METHODS

Vector construction and plant transformation. A triple cassette containing three napin promoters[24], three different multiple cloning site linkers and three octopine synthase (OCS) terminators was prepared in the plasmid pUC19. A three-gene construct (BJ3) was built by inserting *Pi*Δ*6*, a phospholipid-acyl Δ6 desaturase gene from *P. irregulare*[12], *Tc*Δ*5*, a phospholipid-acyl Δ5 desaturase gene from *Thraustochytrium* sp.[13], and *PSE1*, an acyl CoA elongase gene from *P. patens*[14], into the multiple cloning sites (**Fig. 1**). For the four-gene construct (BJ4), an *Xho*I/*Sal*I fragment containing a phospholipid-acyl Δ12 desaturase gene from *C. officinalis* (*Co*Δ*12*)[15] linked to a napin promoter and OCS terminator was removed from a one-gene construct and subcloned into the three-gene construct. The same approach was applied to make the five- and six-gene constructs (BJ5 and BJ6) by adding an elongase gene from *Thraustochytrium* sp. (*TcElo*) and a phospholipid-acyl ω3 desaturase gene from *P. infestans* (*Piω3*), respectively. Finally these three-, four-, five- and six-gene constructs were removed from pUC19 by digestion with *Asc*I, and cloned into the binary vectors pGPTV or pSUN2. For the nine-gene construct (BJ9), a three-gene construct containing the *TcΔ4* phospholipid-acyl Δ4 desaturase gene from *Thraustochytrium* sp.[13], an elongase, *OmElo*, from the fish *O. mykiss*[16] and a lysophosphatidyl acyltransferase, *TcAt*, from *Thraustochytrium* sp., was constructed and transferred into the six-gene binary vector using the Gateway system (Invitrogen). All binary vectors used the *NPT*II gene with the NOS promoter as a selection marker. Binary vectors were transferred into *Agrobacterium tumefaciens* strain GV3101 (pMP90) by electroporation.

For plant transformation, hypocotyls from 5 to 6 day old seedlings of the 0% erucic acid *B. juncea* breeding line 1424 were used as explants for inoculation with *A. tumefaciens* containing the binary constructs described above. Transformation of *B. juncea* was performed as described[25].

Fatty acid and lipid analyses. Fatty acid analyses of seeds and yeast cultures were performed by GC as described previously[15]. Individual fatty acids were identified by comparing the GC peaks with authentic fatty acid standards and/or by GC/MS.

If seeds were to be used for more detailed lipid analyses, individual seeds were first heated for 10 min at 95 °C in 1 ml of isopropanol, and after homogenization, 50-μl aliquots were removed and analyzed by GC to identify the segregating transgenic and nontransgenic seeds. The isopropanol extracts of transgenic seeds were then pooled (12 seeds per sample), centrifuged, the supernatant collected and the pellet reextracted with isopropanol/chloroform 1:1 (vol/vol). The two extracts from each sample were combined, evaporated and dissolved in chloroform. T2 seeds were processed directly without GC screening. The resulting lipid extract was prefractionated into neutral lipids, glycolipids and phospholipids on a silica PrepSep column (Fisher Scientific)[26]. These fractions were further resolved on silica G-25 thin layer chromatography (TLC) plates (Macherey-Nagel). Neutral lipids were developed with hexane/diethyl ether/acetic acid (70:30:1, vol/vol/vol), glycolipids with chloroform/methanol/ammonia (65:25:4, vol/vol/vol) and phospholipids with chloroform/methanol/ammonia/water (70:30:4:1, vol/vol/vol/vol). The individual lipid classes were identified under UV light after a primuline spray (0.05% (wt/vol) in acetone/water, 80:20, vol/vol; Sigma), removed from the plate by scraping, and used for direct transmethylation or extracted by an appropriate solvent for further analysis. The diacylglycerols were extracted and rerun on a boric acid–containing silica TLC plate with chloroform/acetone (96:4, vol/vol) before GC analysis.

Positional analysis of triacylglycerols and phospholipids. Separated and extracted phospholipid classes were dissolved in 0.5 ml of borate buffer (0.5M, pH 7.5, containing 0.4 mM $CaCl_2$). After a brief sonication, 5U of phospholipase A2 from venom of *Naja mossambica* (Sigma P-7778) and 2 ml diethyl ether were added and samples were vortexed for 2 h at 22 °C. The ether phase was evaporated, the digestion was stopped with 0.3 ml 1M HCl, and the reaction mixture was extracted with chloroform/methanol (2:1, vol/vol). The digested phospholipids were separated by TLC in chloroform/methanol/ammonia/water (70:30:4:2, vol/vol/vol/vol) and spots corresponding to released free fatty acids and lysophospholipids were removed by scraping and directly transmethylated.

Fatty acid profiles of triacylglycerol stereopositions were determined by partial chemical deacylation of 20–30 mg of TLC-purified triacylglycerol, as described previously[10], with some modifications. α,β-diacylglycerol was purified by TLC on boric acid-treated silica plates (chloroform/acetone, 96:4, vol/vol), extracted and used for phosphatidylcholine synthesis[27]. The mixture of

phosphatidylcholine molecules with head groups at *sn-1* and *sn-3* positions was extracted from silica after TLC (chloroform/methanol/ammonia/water, 70:30:4:1, vol/vol/vol/vol) and digested with phospholipase A2 as described above for phospholipids. The fatty acid profile of the resulting lysophosphatidylcholine represented the triacylglycerol *sn-1* position, and the released free fatty acids, the *sn-2* position. The remaining *sn-3* position was calculated according to the formula $sn\text{-}3 = 3 \times \text{TAG} - (sn\text{-}1 + sn\text{-}2)$.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Biotechnology* website for details).

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