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ORIGINAL PAPER

Towards the production of high levels of eicosapentaenoic acid in transgenic plants: the effects of different host species, genes and promoters

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Abstract Eicosapentaenoic acid (EPA, 20:5n-3) plays an important role in many aspects of human health. In our efforts towards producing high levels of EPA in plants, we investigated the effects of different host species, genes and promoters on EPA biosynthesis. Zero-erucic acid *Brassica carinata* appeared to be an outstanding host species for EPA production, with EPA levels in transgenic seed of this line reaching up to 25%. Two novel genes, an 18-carbon ω3 desaturase (CpDesX) from *Claviceps purpurea* and a 20-carbon ω3 desaturase (Pir-ω3) from *Pythium irregulare*, proved to be very effective in increasing EPA levels in high-erucic acid *B. carinata*. The conlinin1 promoter from flax functioned reasonably well in *B. carinata*, and can serve as an alternative to the napin promoter from *B. napus*. In summary, the judicious selection of host species and promoters, together with the inclusion of genes that enhance the basic very long chain polyunsaturated fatty acid biosynthetic pathway, can greatly influence the production of EPA in plants.

Keywords Eicosapentaenoic acid · *Brassica carinata* · *B. juncea* · Transformation

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Introduction

Very long chain polyunsaturated fatty acids (VLCPUFAs) such as arachidonic acid (20:4n-6, AA), eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) are essential for human health and well-being. Increased levels of EPA and DHA in the human diet can result in improved brain and retinal health, and can reduce the risks of coronary heart disease and type II diabetes (Simopoulos 2003). Currently, the main dietary sources of VLCPUFAs are fish and other seafood, or commercially available fish oil capsules. However, in the long term, theses sources may not be able to meet the demands of the ever-growing human population, because global wild

fish stocks are becoming over-exploited (Myers and Worm 2003) and aquaculture can not compensate for the increasing shortage (Naylor et al. 2000). Thus, there is an urgent need to find a sustainable and affordable alternative source of VLCPUFAs. Recently, the metabolic pathways involved in the synthesis of VLCPUFAs have been elucidated and the relevant genes have been identified and cloned, leading to great interest in genetic engineering approaches to producing VLCPUFAs in various oilseed crops.

There are two main desaturation/elongation pathways (△6 desaturase/elongase and △9 elongase/△8 desaturase) for the biosynthesis of VLCPUFAs. In the △6 pathway (Fig. 1), the first step is the △6 desaturation of linoleic (LA, 18:2n-6) and linolenic (ALA, 18:3n-3) acids to produce γ-linolenic acid (GLA) and stearidonic acid (SDA), respectively. VLCPUFAs synthesized by this pathway include arachidonic acid, which is produced from GLA by △6 elongation followed by △5 desaturation, and EPA, which is produced by △6 elongation and △5 desaturation of SDA or by ω3 desaturation of AA. The △6 desaturase/elongase pathway has been reconstituted in a number of different plant species. Abbadi et al. (2004) reported levels of only 1.5% AA and 1.0% EPA in transgenic flax seeds containing △6 and △5 desaturases and a △6 elongase. However, in soybean, the introduction of a △6 desaturase, a △5 desaturase and a △6 elongase, together with △15 and △17 desaturases, resulted in the production of up to 20% EPA in somatic embryos (Kinney et al. 2004). Wu et al. (2005) used the oilseed species *Brassica juncea* as the host plant for biosynthesis of VLCPUFAs. Production of up to 25% AA and 15% EPA was observed in transgenic seeds containing 5 and 9 structural genes, respectively. Robert et al. (2005) reported the production of 3.2% EPA and 1.6% AA in transgenic *Arabidopsis* using a construct including a dual-purpose △5/△6 desaturase that acts on acyl-CoA substrates. Taken together, these results indicate that both the specific gene combinations used and the choice of host plant are important for VLCPUFA production.

Wu et al. (2005) reported that the zero-erucic acid species *B. juncea* serves as a good host for VCLPUFA production since its seed oil contains high levels of linoleic acid (LA) (40%) and α-linolenic acid (ALA) (15%). The potential of other oilseed *Brassica* species such as *B. carinata* to serve as host species has not yet been investigated. *B. carinata* seed contains significant amounts of the precursor fatty acids LA (16.5%) and ALA (16.0%) that are required for VCLPUFA production, although it has approximately 35% erucic acid. *B. carinata* germplasm lacking erucic acid and containing high LA (40%) and ALA (20%), has been developed using traditional breeding methods at the Saskatoon Research Center, Agriculture and Agri-Food Canada.

Recently, our group has identified a number of novel microbial genes that are involved in the biosynthesis of VLCPUFAs. Meesapyodsuk et al. (2007) isolated an 18-carbon ω3 desaturase gene, CpDesX, from *Claviceps purpurea*. Yeast transformation experiments indicated that CpDesX catalyzed △12, △15 and ω3 types of desaturation, with ω3-type activity predominating. The ω3 function of CpDesX resulted in the conversion of ω6- fatty acids to their ω3

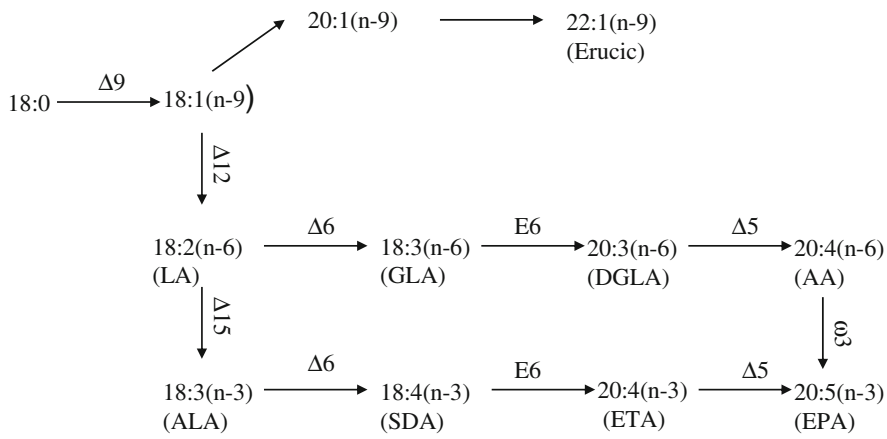

Fig. 1 Biosynthetic pathways of erucic acid, arachidonic acid (*AA*) and eicosapentaenoic acid (*EPA*). *LA* linoleic acid, *ALA* α-linolenic acid, *GLA* γ-linolenic acid, *SDA* stearidonic acid, *DGLA* dihomo- γ-linolenic acid, *ETA* eicosatetraenoic acid, *AA* arachidonic acid, *EPA* eicosapentaenoic acid

counterparts, making CpDesX a potentially valuable gene for PUFA production. A new 20-carbon $\omega 3$ desaturase gene, Pir-$\omega 3$, was isolated from *Pythium irregulare* (Bauer et al. 2008). In yeast, this gene catalyzed the desaturation of dihomo-γ-linolenic acid (DGLA) to eicosatetraenoic acid (ETA) and AA to EPA at conversion levels of 9.7 and 40.8%. A similar $\omega 3$ desaturase gene from *Phytophytora infestans* (Pi-$\omega 3$) effectively converted AA to EPA in plants (Wu et al. 2005), but the alternative source $\omega 3$ desaturase from *P. irregulare* has not yet been tested in plants.

The napin promoter from *B. napus* has proven to be very effective for seed-specific expression in different *Brassica* species (e.g., Wahlroos et al. 2004; Wang et al. 2005; Wu et al. 2005). However, it would be advantageous to have additional promoters available for the coordinated, seed-specific expression of multiple genes. Truksa et al. (2003) isolated the promoter for the gene encoding the flax 2S storage protein conlinin1 (cnl1), and demonstrated that this promoter conditioned seed-specific gene expression in flax plants.

The objectives of our experiments were to investigate the influence of three factors on EPA production: (1) the use of *B. carinata* versus *B. juncea* as host species; (2) the addition of two novel $\omega 3$ desaturase genes, CpDesX and Pir-$\omega 3$, to transformation constructs, and (3) the use of the napin versus the cnl1 promoter for the expression of transgenes.

Materials and methods

Vector construction

For building the constructs Napin-3, Napin-4 and Napin-5, a triple cassette containing three napin promoters, three different multiple cloning site linkers and three octopine synthase (OCS) terminators was constructed in the plasmid pUC19. A three-gene construct was built by inserting Pi$\triangle$6, a $\triangle$6 desaturase gene from *P. irregulare* (Hong et al. 2002), Tc$\triangle$5, a $\triangle$5 desaturase gene from *Thraustochytrium sp*. ATCC 26185 (Qiu et al. 2001a) and TpElo, an elongase gene from *Thalassiosira pseudonana* (Meyer et al. 2004) into this cassette. For the four-gene construct Napin-4, an XhoI/SalI fragment containing the desaturase gene CpDesX from *C. purpurea* (Meesapyodsuk et al. 2007) linked to a napin promoter and OCS terminator was removed from a single-gene construct and subcloned into the triple gene construct. For the five-gene construct Napin-5, the same approach was used to add the $\omega 3$ desaturase gene Pir-$\omega 3$ from *P. irregulare* (Bauer et al. 2008). Finally these 3-, 4-, and 5-gene constructs were removed from pUC19 by digestion with AscI, and cloned into the binary vector pSUN2 (Fig. S1 in Electronic supplementary material). A similar approach was used for building the Cnl1-5 construct, except that each gene was under the control of the cnl1 promoter (Fig. S1 in Electronic supplementary material). The construct Napin-A (Fig. S1 in Electronic supplementary material), which contained the same $\triangle$5 and $\triangle$6 desaturases as described above, plus the $\triangle$6 elongase gene TcElo from *Thraustochytrium sp*. (Vrinten et al., unpublished data), the $\omega 3$ desaturase gene Pi-$\omega 3$ from *Phytophytora infestans* (Wu et al. 2005) and a $\triangle$12 desaturase from *Calendula officinalis* (Qiu et al. 2001b) was constructed as described by Wu et al. (2005). The Napin-A construct used for transforming *B. carinata* also contained two additional genes under the control of new promoters; however, these promoters were inactive (unpublished data), therefore the construct was functionally identical to the Napin-A five-gene construct with each gene under the control of the napin promoter. Binary vectors were transferred into *Agrobacterium tumefaciens* strain GV3101 (pMP90) by electroporation.

Plant materials and transformation methods

The high-erucic line C90-1163 and the zero-erucic line 10H3 of *B. carinata* and the zero-erucic line 1424 of *B. juncea* were used to study the effects of host species on EPA production. The high-erucic acid *B. carinata* line C90-1163 was used as the host plant in experiments investigating the effects of the two novel genes (CpDesX and Pir-$\omega 3$) and the cnl1 promoter on EPA biosynthesis, because abundant seed of this line was available for carrying out transformation experiments.

The protocol described by Babic et al. (1998) was used for *B. carinata* transformation. Cotyledon petioles from 5 to 6 day old seedlings were excised and inoculated with *A. tumefaciens* strain GV3101 containing the desired gene construct and co-cultured for 2 days at 22°C before being transferred to the

regeneration medium containing 25 mg/l kanamycin. For *B. juncea* transformation, the protocol described by Radke et al. (1992) was followed. Hypocotyls from 5 to 6 day old seedlings were cut into 4–6 mm lengths and used as explants for inoculation with *A. tumefaciens* strain GV3101 containing the desired gene construct. The inoculated explants were co-cultured for 3 days before being transferred to the regeneration medium, which contained 15 mg/l kanamycin. The putative transgenic plants obtained from all experiments were validated by PCR analysis, and PCR–positive plants were bagged for the production of self-pollinated T_1 seeds.

Fatty acid analysis

Single seeds were crushed in 12 ml glass culture tubes with a glass rod, followed by the addition of 2 ml of 1% (v/v) H_2SO_4 in methanol. The tube containing the mixture was sealed and heated at 60°C for 1 h. After cooling to room temperature, 0.5 ml of 0.9% NaCl and 2 ml of hexane were added, and after vortexing, phases were separated by centrifuging at 2,500 rpm for 2 min. The hexane layer was transferred to a clean tube and dried under nitrogen. About 100 µl of hexane was then added to the tube and the resulting fatty acid methyl esters (FAMES) were analyzed using a gas chromatograph (6890N, Agilent Technologies) equipped with a DB-23 fused silica column (30 m × 0.25 mm inside diameter, 0.25-µm film thickness; J&W Scientific, Folsom, CA) with a temperature program of 160°C for 1 min, increasing 4°C/min to 240°C, and holding for 10 min.

Statistical analysis

Statistical analyses of fatty acid quantifications were conducted using a minimum of two T_1 positive seeds harvested from each of at least four different transgenic plants. The Student's *t*-test was used to assess the differences between means.

Results

The effects of host species on EPA production

The construct Napin-A (Fig. S1 in Electronic supplementary material) was used to investigate the effects of host species (*B. carinata* versus *B. juncea*) on EPA production. With the zero-erucic acid *B. juncea* line, nine transgenic plants carrying the Napin-A construct were obtained, and seeds of all these plants produced the expected novel fatty acids (Table 1). The amount of GLA averaged 19.9% of total seed fatty acids (range 1.9–29.0%), SDA averaged 2.2% (range 0.3–3.0%), and AA averaged 4.3% (range 0.3–7.6%). The highest level of EPA found in an individual seed was 10.8%, with the average level being 5.0%. The elongase from *Thraustochytrium sp*. can elongate both △6 and △5 desaturated substrates, and thus is able to elongate EPA to docosapentaenoic acid (DPA) (Vrinten et al. unpublished data), explaining the production of DPA (average 0.4%) in the transgenic seeds. The conversion levels [product/(substrate + product)] of △6 and △5 desaturases were 57.7 and 80.8%, respectively, while the △6 elongase had a conversion level of 35.2%.

With the high-erucic acid *B. carinata* line, six transgenic plants carrying the Napin-A construct were obtained. Two of these plants produced only a low level (≤1% of total fatty acids) of GLA in seed oil, while the remaining plants produced the expected new fatty acids (Table 1). In these plants, GLA and SDA averaged 18.2 and 3.2% of total fatty acids in seed, respectively. The amount of AA ranged from 0.4–4.3% (average 2.8%), while EPA reached an average level of 9.3%, with the highest value observed being 13.7%. The average amount of DPA was 1.4%. Both the △6 and △5 desaturases functioned very well, with conversion levels of 81.8 and 87.7%, respectively. The △6 elongase showed a conversion level of 41.9%, which was higher than that observed in transgenic *B. juncea*. The average EPA level (9.3%) produced in transgenic high erucic *B. carinata* was significantly higher than in zero-erucic *B. juncea* (5.0%) ($t = 2.53$, $P < 1\%$), suggesting that *B. carinata* serves as a better host species for the production of EPA.

The superiority of *B. carinata* as a host species was confirmed by results with the zero-erucic acid *B. carinata* line. Four independent transgenic plants carrying the Napin-A construct were obtained, and the seeds contained the expected novel fatty acids at various levels (Table 1; Fig. 2a). The amounts of GLA and SDA averaged 26.9% (range 22.2–31.7%) and 5.4% (range 3.0–8.1%) of total fatty acids, respectively. AA had an average level of 5.7% (range 4.1–6.9%), while EPA had an average level of 20.4%

Table 1 Total fatty acid composition of oilseeds from wild-type and transgenic plants of zero-erucic *B. juncea* line 1424, and high-erucic line C90-1163 and zero-erucic line10H3 of *B. carinata* (area %)

Fatty acid	Zero-erucic *B. juncea* line 1424		High-erucic *B. carinata* line C90-1163		Zero-erucic *B. carinata* line 1ine 10H3	
	Wild type ($N = 2$, $n = 6$)	Transgenic ($N = 9$, $n = 18$)	Wild type ($N = 2$, $n = 10$)	Transgenic ($N = 4$, $n = 8$)	Wild type ($N = 2$, $n = 6$)	Transgenic ($N = 4$, $n = 11$)
16:0	5.8 ± 1.2	6.6 ± 0.9	5.3 ± 0.7	6.3 ± 1.2	7.1 ± 0.6	6.2 ± 1.2
18:0	1.9 ± 0.3	2.2 ± 0.3	1.2 ± 0.2	1.5 ± 0.4	1.7 ± 0.3	2.7 ± 0.8
18:1n-9 (OA)	32.8 ± 6.3	22.3 ± 7.3	7.3 ± 0.7	5.5 ± 3.9	18.9 ± 8.0	4.4 ± 2.9
18:1n-7	3.7 ± 0.4	3.1 ± 0.5	1.5 ± 0.2	1.7 ± 0.2	3.5 ± 0.3	3.9 ± 0.6
18:2n-6 (LA)	39.2 ± 4.4	18.8 ± 8.6	16.5 ± 0.9	5.8 ± 4.2	44.1 ± 3.6	4.2 ± 1.2
18:3n-6 (GLA)	0.0	19.9 ± 8.9	0.0	18.2 ± 4.2	0.0	26.9 ± 3.0
		(1.9–29.0)		(8.9–23.3)		(22.2–31.7)
18:3n-3 (ALA)	12.6 ± 1.3	6.2 ± 3.9	16.0 ± 1.3	2.4 ± 2.1	21.0 ± 6.4	2.0 ± 0.8
18:4n-3 (SDA)	0.0	2.2 ± 0.8	0.0	3.2 ± 0.6	0.0	5.4 ± 1.7
		(0.3–3.0)		(2.8–4.5)	0.0	(3.0–8.1)
20:1n-9	0.0	0.0	6.2 ± 1.0	4.5 ± 0.6	0.0	0.0
20:1n-7	0.0	0.0	1.4 ± 0.1	1.7 ± 0.3	0.0	0.0
20:3n-6 (DGLA)	0.0	1.8 ± 0.7	0.0	0.9 ± 0.2	0.0	2.2 ± 0.8
20:4n-6 (AA)	0.0	4.3 ± 2.1	0.0	2.8 ± 1.5	0.0	5.7 ± 1.1
		(0.3–7.6)		(0.4–4.3)	0.0	(4.7–6.9)
20:4n-3 (ETA)	0.0	0.5 ± 0.3	0.0	1.0 ± 0.4	0.0	2.5 ± 1.1
20:5n-3 (EPA)	0.0	5.0 ± 3.1	0.0	9.3 ± 4.4	0.0	20.4 ± 3.3
		(0.7–10.8)		(1.4–13.7)	0.0	(15.3–25.0)
22:5n-3 (DPA)	0.0	0.4 ± 0.3	0.0	1.4 ± 0.9	0.0	4.0 ± 1.6
Erucic	0.0	0.0	36.1 ± 2.4	25.7 ± 3.6	0.0	0.0
Other	4.0 ± 1.1	6.7 ± 2.1	8.5 ± 1.5	8.2 ± 1.8	3.7 ± 0.7	9.4 ± 1.5

Transgenic: plants carried the Napin-A construct. *N* and *n* are the number of independent transgenic plants and the total number of T_1 positive seeds analyzed, respectively. Wild type represents untransformed plants. Each value represents the mean ± SE. The values in brackets indicate the ranges of GLA, SDA, AA and EPA among seeds

and reached 25.0% in an individual seed. The difference in EPA levels between zero-erucic and high-erucic *B. carinata* lines was highly significant ($t = 6.0$, $P < 0.05\%$). DPA content averaged 4.0% in the zero-erucic acid line, and the aggregate amount of novel fatty acids reached 67.1% of total fatty acids. Both the △6 and △5 desaturases functioned very well, with conversion levels of 91.5 and 86.5%, respectively. The △6 elongase showed a conversion level of 51.9% in zero erucic acid *B. carinata*, which was higher than levels observed in either the high-erucic acid *B. carinata* line or the zero-erucic acid *B. juncea* line. The level of 22-carbon PUFAs produced by transgenic zero-erucic acid *B. carinata* represents one of the highest levels reported to date, and this was achieved without the use of an elongase specific for △5-desaturated products. Taken together with the EPA levels observed here (up to 25% of total fatty acids), it appears that zero-erucic acid *B. carinata* has outstanding potential as a host species for VLCPUFA production.

The effects of two novel desaturases on EPA production in high-erucic acid *B. carinata*

A minimal set of three genes, comprising a △5 desaturase, a △6 desaturase and a △6 elongase, is required for the synthesis of AA and EPA from endogenous LA and ALA. In order to quantify the contributions of the two novel desaturase genes, CpDesX and Pir-ω3, to EPA production in transgenic plants, we needed to know the base levels of AA and

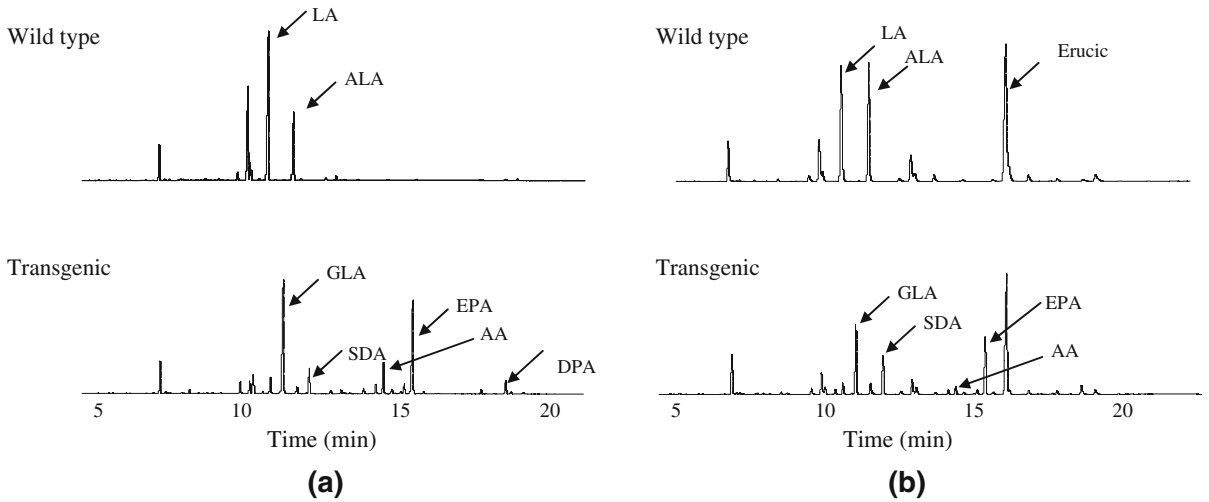

Fig. 2 **a** Gas chromatography analysis of seed fatty acid methyl esters from *B. carinata* (line 10H3) non-transgenic seeds and transgenic seeds containing the five-gene construct Napin-A. **b** Gas chromatography analysis of seed fatty acid methyl esters from *B. carinata* (line C90-1163) non-transgenic seeds and transgenic seeds containing the five-gene construct Napin-5. *LA* linoleic acid, *ALA* α-linolenic acid, *GLA* γ-linolenic acid, *SDA* stearidonic acid, *AA* arachidonic acid, *EPA* eicosapentaenoic acid, *DPA* docosapentaenoic acid

EPA in plants carrying the minimal set of transgenes. For this purpose, the gene construct Napin-3 (Fig. S1 in Electronic supplementary material) containing a △6 desaturase from *P. irregulare*, a △5 desaturase from *Thraustochytrium sp*., and a △6 elongase from *T. pseudonana* (Meyer et al. 2004) was introduced into the high-erucic *B. carinata* line. In transgenic seeds of plants carrying Napin-3, GLA and SDA averaged 17.6 and 4.3% of total fatty acids, respectively (Table 2). The amount of AA reached 12.2% in an individual seed (average 8.4%), and EPA had an average level of 2.3% (range 0.8–3.5%). The △6 and △5 desaturases functioned very well, with substrate conversion levels of 86.0 and 85.6%, respectively, while the conversion level of the △6 elongase was 36.3%. The total amount of novel ω6 fatty acids (GLA, DGLA and AA) represented 27.5% of seed fatty acids, while novel ω3 fatty acids (SDA, ETA and EPA) represented 6.9%, indicating that the ω6 pathway was operating more effectively than the ω3 pathway.

In experiments in yeast, the 18-carbon ω3 desaturase gene CpDesX was able to convert LA to ALA, and GLA to SDA (Meesapyodsuk et al. 2007). We felt that an increase in the initial ω3 substrate ALA and/or the conversion GLA to SDA might lead to higher EPA production. To determine if CpDesX could increase the level of ω3 fatty acids in plants, this gene was added to the three-gene construct Napin-3, producing the four-gene construct Napin-4. Eight independent transgenic plants were obtained, but two of these plants produced only GLA as a novel fatty acid at very low levels (≤1%). The remaining six plants produced the expected new fatty acids at various levels (Table 2). With the expression of CpDesX substantially increasing the levels of ω3 fatty acids, SDA reached 9.4% in an individual seed and the average level (6.3%) was significantly higher than in the transgenic seeds carrying Napin-3 (4.3%; $t = 2.90$, $P < 1\%$). The average EPA level was 4.2%, which was significantly higher than the 2.3% found in seeds from plants transformed with Napin-3 ($t = 3.96$, $P < 1\%$). The total amounts of novel ω6 and ω3 fatty acids averaged 19.0 and 10.9%, respectively, indicating an increase of flux through the ω3 pathway due to the activity of the CpDesX desaturase. This increase of ω3 fatty acids suggests that CpDesX can make an important contribution to EPA production in transgenic plants.

To test the utility of the new 20-carbon ω3 desaturase Pir-ω3 for producing EPA in plants, this gene was added to the Napin-4 construct. Twelve independent transgenic plants were obtained, but five of these plants produced seeds containing only GLA (≤2.9%) as a novel fatty acid. The seeds of the remaining plants contained the expected novel fatty acids at various levels (Table 2; Fig. 2b). Expression

Table 2 Total fatty acid composition of seeds from wild-type and transgenic high-erucic *B. carinata* (line C90-1163) plants (area %)

Fatty acid	Wild type ($N = 3$, $n = 10$)	Napin-3 ($N = 5$, $n = 11$)	Napin-4 ($N = 6$, $n = 12$)	Napin-5 ($N = 7$, $n = 15$)	Cnl1-5 ($N = 4$, $n = 10$)
16:0	5.3 ± 0.7	7.2 ± 1.5	6.6 ± 1.5	6.4 ± 1.1	7.1 ± 1.7
18:0	1.2 ± 0.2	1.4 ± 0.4	1.3 ± 0.4	1.2 ± 0.2	1.4 ± 0.4
18:1n-9 (OA)	7.3 ± 0.7	8.2 ± 3.8	7.8 ± 2.5	6.3 ± 2.3	6.1 ± 1.1
18:1n-7	1.5 ± 0.2	2.2 ± 0.7	1.8 ± 0.5	1.6 ± 0.2	2.0 ± 0.8
18:2n-9	0.0	2.0 ± 1.6	0.9 ± 0.6	1.5 ± 0.9	0.8 ± 0.4
18:2n-6 (LA)	16.5 ± 0.9	3.6 ± 6.9	4.0 ± 3.0	3.7 ± 3.3	3.3 ± 1.3
18:3n-6 GLA)	0.0	17.6 ± 3.8	11.8 ± 1.6	11.1 ± 2.4	14.2 ± 1.9
		(13.7–24.2)	(9.0–15.6)	(4.8–15.3)	(12.3–18.6)
18:3n-3 (ALA)	16.0 ± 1.3	2.0 ± 0.6	3.2 ± 1.3	3.4 ± 2.9	3.2 ± 1.0
18:4n-3 (SDA)	0.0	4.3 ± 1.3	6.3 ± 2.0	6.2 ± 1.8	7.2 ± 1.6
		(2.4–6.3)	(4.1–9.4)	(2.9–10.9)	(5.7–9.7)
20:1n-9	6.2 ± 1.0	4.8 ± 1.3	4.6 ± 1.1	4.2 ± 1.2	3.6 ± 1.1
20:1n-7	1.4 ± 0.1	1.8 ± 0.5	1.8 ± 0.7	2.1 ± 0.4	2.0 ± 0.6
20:3n-6 (DGLA)	0.0	1.5 ± 0.9	0.7 ± 0.3	0.6 ± 0.3	0.6 ± 0.3
20:4n-6 (AA)	0.0	8.4 ± 2.5	6.5 ± 1.9	1.3 ± 0.4	1.2 ± 0.4
		(4.3–12.2)	(1.8–9.5)	(0.6–1.8)	(0.7–1.9)
20:4n-3 (ETA)	0.0	0.3 ± 0.4	0.4 ± 0.2	0.6 ± 0.2	0.8 ± 0.2
20:5n-3 (EPA)	0.0	2.3 ± 0.8	4.2 ± 1.4	9.7 ± 3.4	7.4 ± 2.3
		(0.8–3.5)	(1.0–5.6)	(1.5–15.5)	(4.3–10.8)
Erucic acid	36.1 ± 2.4	25.3 ± 7.3	31.2 ± 5.3	31.1 ± 3.8	29.8 ± 3.4
Other	8.5 ± 1.5	7.1 ± 1.0	6.9 ± 2.4	9.0 ± 1.6	9.3 ± 1.7

Napin-3, Napin-4 and Napin-5 represent transgenic plants transformed with the three-, four- and five-gene constructs, with each gene under the control of the napin promoter. Cnl1-5 represents transgenic plants transformed with the five-gene construct, with each gene under the control of the conlinin promoter. *N* and *n* are the number of independent transgenic plants and the total number of positive T_1 seeds analyzed, respectively. The values in parentheses indicate the ranges of GLA, SDA, AA and EPA among seeds. Wild type represents untransformed plants. Values represent the mean ± SE

of the Pir-ω3 desaturase appeared to result in a large increase in EPA levels. With the addition of this gene, EPA reached 15.5% in an individual seed and averaged 9.7%, which was significantly higher than the 4.2% found in the transgenic seeds carrying Napin-4 ($t = 5.6$, $P < 1\%$). The level of novel ω6 and ω3 fatty acids averaged 13.0 and 16.5%, respectively. The high levels of EPA achieved in transgenic seeds carrying the Napin-5 construct can be attributed to the activities of the two novel desaturase genes (CpDesX and Pir-ω3), underlining the value of these genes for VLCPUFA production in plants.

The effect of the cnl1 promoter on EPA production in *B. carinata*

To determine the utility of the flax seed-specific promoter cnl1 for EPA production in *B. carinata* seed, a construct (Cnl1-5) carrying the same genes as the Napin-5 construct, but with each gene under the control of the cnl1 promoter, was introduced into the high-erucic acid *B. carinata* line. Out of ten transgenic plants, six produced seeds containing low amounts of GLA (0.3–7.5%) and SDA (0.0–4.6%) as the only novel fatty acids, indicating that only the $\triangle$6 desaturase was expressed. The transgenic seeds of the remaining four plants produced the expected novel fatty acids (Table 2). The amount of GLA in seeds averaged 14.2%. The average amount of SDA was 7.2%, which was comparable to the amounts in transgenic seeds carrying the Napin-5 construct ($t = 1.45$, $P > 10\%$). The EPA content of seeds (average 7.4%) was not significantly lower than that (9.7%) found in Napin-5 transgenic plants ($t = 2.0$, $P > 5\%$). This suggested that the cnl1 promoter functions reasonably well in *B. carinata* and can be

used in this species, although the napin promoter may be somewhat more effective.

Discussion

In the experiments conducted here, the choice of host species had the largest effect on EPA levels, with *B. carinata* appearing to out-perform *B. juncea*. In particular, the use of a zero-erucic acid *B. carinata* line appeared to be most successful for VLCPUFA production, with novel fatty acids reaching 67.1% of total seed fatty acids. The higher VLCPUFA levels observed in the zero-erucic *B. carinata* line as compared to the high-erucic line may be influenced by several factors. In the zero-erucic acid line, 18:1n-9 is not directly elongated; therefore, higher levels of the desaturation products 18:2n-6 and 18:3n-3 are produced and can be used as substrates for VLCPUFA synthesis. Additionally, in zero-erucic acid *B. carinata*, the endogenous condensing enzyme (elongase) responsible for the production of erucic acid is missing or inactive, but the other endogenous enzymes involved in the four-step elongation process are unaffected. These enzymes are presumably also used by the transgenic elongases, and the absence of the native elongase may increase their availability, as has previously been suggested by Wu et al. (2005).

In experiments using the high erucic acid *B. carinata* line, all transgenic seeds had lower amounts of erucic acid than did non-transgenic seeds. While the erucic acid content averaged 36.1% in non-transgenic seeds, the average level in transgenic seeds carrying Napin-3 was 25.3% (Table 2). This decrease in erucic acid content could be due to the competition for substrate between the erucic acid and VLCPUFA biosynthetic pathways. Both pathways utilize 18:1n-9 as a starting substrate, as indicated in Fig. 1. The use of this substrate in AA and EPA production may result in less 18:1n-9 being available for erucic acid synthesis.

Highly variable transgene expression levels were observed among independent transgenic plants in all experiments. For example, certain plants transformed with the constructs Napin-4, Napin-5 and Cnl1-5 produced only low levels of GLA in seed oil, indicating that only the △6 desaturase was being expressed, although PCR testing indicated that all transgenes were present in these plants (data not shown). Transgenic plants carrying the Napin-5 construct had EPA levels varying from 1.5 to 15.5%. The variability in transgene expression can be attributed to a number of factors, including position effects and transgene copy number (Kooter et al. 1999; Matzke and Matzke 1998).

In both *B. carinata* and *B. juncea*, the inefficiency of the △6 elongation step was evidenced by the high levels of GLA and SDA remaining in transgenic seeds. This inefficient elongation is thought to be a major limiting factor for the production of AA and EPA. Abbadi et al. (2004) suggested that △6 elongation may be inefficient because lipid-linked substrates are used by most desaturases, and the desaturated products must then be channeled to the acyl-CoA pool for elongation to occur. The use of the alternative △9 elongase /△8 desaturase pathway, where the elongation of endogenous fatty acids occurs prior to desaturation, might help to alleviate this problem. The use of genes from the alternative pathway led to the accumulation of 7% ARA and 3% EPA in *Arabidopsis* leaf tissue (Qi et al. 2004). We have transformed flax with the △9 elongase and △8 desaturase genes from *Euglena gracilis*, with each gene under the control of the cnl1 promoter. The △9 elongation products represented up to 13% of the total fatty acids in transgenic flax seeds (Cheng and Vrinten unpublished data), although 20-carbon PUFAs represented only about 5% of fatty acids in flax seed transformed with genes from the △6 desaturase/ △6 elongase pathway (Abbadi et al. 2004). Therefore, simultaneous introduction of the two pathways (△6 desaturase/ △6 elongase and △9 elongase/△8 desaturase) into plants might lead to further increases in EPA levels.

The activity of the Pir-ω3 desaturase from *P. irregulare* in plants seems to be at least comparable to that of Pi-ω3 from *P. infestans* used in our earlier work. Here, the transgenic high erucic acid *B. carinata* plants carrying Pir-ω3 produced 9.7% EPA, while Wu et al. (2005) found that *B. juncea* plants transformed with a similar construct carrying Pi-ω3 produced 8.1% EPA. However, these results are not directly comparable since different host species were used.

Overall, we have demonstrated that the specific promoter used to control transgene expression, the presence of one or more ω3 desaturase genes in constructs, and particularly, the choice of host species, can all make substantial contributions to the level of EPA produced in transgenic plants.

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