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Development of ultra-high erucic acid oil in the industrial oil crop *Crambe abyssinica*

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Summary

Erucic acid (22 : 1) is a major feedstock for the oleochemical industry. In this study, a gene stacking strategy was employed to develop transgenic *Crambe abyssinica* lines with increased 22 : 1 levels. Through integration of the *LdLPAAT*, *BnFAE1* and *CaFAD2-RNAi* genes into the crambe genome, confirmed by Southern blot and qRT-PCR, the average levels of 18 : 1, 18 : 2 and 18 : 3 were markedly decreased and that of 22 : 1 was increased from 60% in the wild type to 73% in the best transgenic line of T4 generation. In single seeds of the same line, the 22 : 1 level could reach 76.9%, an increase of 28.0% over the wild type. The trierucin amount was positively correlated to 22 : 1 in the transgenic lines. Unlike high erucic rapeseed, the wild-type crambe contains 22 : 1 in the seed phosphatidylcholine and in the *sn-2* position of triacylglycerols (5% and 8%, respectively). The transgenic line with high 22 : 1 had decreased 22 : 1 level in phosphatidylcholine, and this was negatively correlated with the 22 : 1 level at the *sn-2* position of TAG. The significances of this study include (i) achieving an unprecedented level of 22 : 1 in an oil crop; (ii) disclosing mechanisms in the channelling of a triacylglycerol-specific unusual fatty acid in oil seeds; (iii) indicating potential limiting factors involved in the erucic acid biosynthesis and paving the way for further increase of this acid and (iv) development of an added value genetically modified oil crop having no risk of gene flow into feed and food crops.

Introduction

Erucic acid (22 : 1) and its derivatives, mainly erucamide, are important industrial feedstocks in manufacturing plastics, nylon13-13 and high temperature lubricants (Leonard, 1994; Piazza and Foglia, 2001; Sonntag, 1991). Erucic acid is currently mainly derived from high erucic acid rapeseed (HEARs with ca. 50% of 22 : 1 in its seed oil). However, HEARs cross readily with the existing food oil rapeseed, and its seeds can be inadvertently mixed with food qualities, thus complicating the commercial production and supply of HEARs. This has severely hampered the development of HEAR in many countries, for example, the harvested acreage of HEARs in the United States declined from 19 400 acres in 1987/88 to 2400 in 1995/96. Recently, a genetically modified (GM) rapeseed with a significant increase in 22 : 1 has been reported (Nath *et al.*, 2009). However, the use of GM industrial rapeseed imposes an additional barrier in EU because of its strict legislation on GM crops. There is a zero tolerance for GM products for industrial uses to be present in feed and food. There is a maximal allowed level of 22 : 1 in food oil in most countries (5% in EU). It will be practically impossible under the current legislation to cultivate high 22 : 1 GM rapeseed in EU where food quality rapeseed is grown.

It is estimated that with every 10% increase in 22 : 1 of total fatty acids (FAs) in the seed oil, the production cost of erucamide is reduced to half. Breeding for varieties rich in 22 : 1 in the oilseed crops has thus been extensively conducted in the past. However, the success with conventional breeding has been very limited. In the *Brassicacae species (spp.),* this is supposed to be partly because of the fact that the erucoyl moiety cannot be incorporated in the *sn*-2 position of the triacylglycerols (TAG) because of the inability of the endogenous lysophosphatidic acid acyltransferase (LPAAT) to use erucoyl-CoA as an acyl donor (Cao *et al.*, 1990; Frentzen, 1993). The exclusion of 22 : 1 from the *sn*-2 position would set up a threshold of 22 : 1 up to 67% in the seed oil, and hybridization among the existing materials within *Brassica spp.* cannot break this limitation, thus not being an efficient method for generating new high 22 : 1 *Brassica* varieties.

As a fast and more precise breeding approach, genetic engineering enables to modify plant oil qualities suitable for a wide range of applications (Cahoon *et al.*, 2007; Lu *et al.*, 2010; Napier and Graham, 2010). Considering the inability of native LPAATs to incorporate erucoyl moieties into the *sn*-2 position of TAG, the first attempts for high 22 : 1 were mainly focused on identification of novel *LPAAT* genes originated from other species (Löhden and Frentzen, 1992; Oo and Huang, 1989) including *LPAATs* from *Limnanthes alba* and *L. douglasii*. These enzymes have been proved to be efficient in incorporating 22 : 1 into the *sn*-2 position and yield trierucin in transgenic plants (Brough *et al.*, 1996; Cao *et al.*, 1990). However, the 22 : 1 content in the transgenic plant was not increased, suggesting that the ability of LPAAT to incorporate 22 : 1 into the *sn*-2 position is not the only factor limiting the production of 22 : 1 (Brough *et al.*, 1996; Lassner *et al.*, 1995). These results were generally interpreted as that either the activity of LdLPAAT

is not strong enough (Frentzen, 1998; Nath *et al.*, 2009) or the production of erucoyl-CoA is too low to support higher levels of 22 : 1 in the oil (Lassner *et al.*, 1995).

Further attempts to increase 22 : 1 involved the *BnFAE1* (fatty acid elongase) gene from *Brassica napus*, encoding the β-ketoacyl-CoA synthase, responsible for elongation of FAs from oleic acid (18 : 1) to eicosenoic acid (20 : 1) and further to 22 : 1 (Barret *et al.*, 1997). However, overexpression of *BnFAE1*, either alone or in combination with *LdLPAAT*, resulted in an increase in 22 : 1 in certain HEAR lines only (Han *et al.*, 2001; Nath *et al.*, 2009; Wilmer *et al.*, 2003). Another well-characterized gene regulating 22 : 1 is *FAD2*, encoding a fatty acid desaturase catalysing the desaturation of 18 : 1 to linoleic acid (18 : 2), which can be further converted to linolenic acid (18 : 3) by *FAD3*. Both co-suppression and antisense approaches of *FAD2* were tested in *B. carinata*, and the transgenic plants did show decreased levels of 18 : 2 and 18 : 3 and significantly increased levels of 18 : 1 and 22 : 1. However, none of them surpassed 66% of 22 : 1, indicating again that blockage in incorporation of 22 : 1 in the *sn*-2 position was a major bottleneck (Jadhav *et al.*, 2005).

Failure in substantially increasing 22 : 1 in oilseed crops reflects the limitation of approaches where the target genes were modified individually. Recent studies have shown that engineering of oil quality needs to modify multiple factors, such as the substrate pool size, substrate preference of different enzymes as well as transcription factors. Therefore, a more rational design of genetic engineering should include simultaneous modification of multiple genes involved in the oil biosynthesis (Ohlrogge, 1994; Puyaubert *et al.*, 2005). This gene stacking strategy has been applied in rapeseed where 22 : 1 in a F_3 line could reach 72% by crossing a transgenic line expressing *LdLPAAT* and *BnFAE1* with a mutant line having low 18 : 2 and 18 : 3 (Nath *et al.*, 2009).

Crambe (*Crambe abyssinica* Hochst.), belonging to the Brassicaceae family, is a suitable oilseed crop for genetic engineering for high 22 : 1 as it contains already 60% 22 : 1 in its seed oil. Most importantly, crambe does not outcross with any existing food oilseed crops, thereby eliminating the problem of gene flow into such crops. Crambe is already commercially cultivated on a small scale, and novel varieties can yield the same amount of oil per hectare as spring rapeseed does (Temple-Heald, 2004), implying that GM crambe could be easily adopted in regular agriculture practices.

In this study, we have used the gene stacking strategy to increase 22 : 1 in crambe through overexpressing *LdLPAAT* and *BnFAE1* and down-regulating *FAD2* and obtained lines with substantial increases in 22 : 1 compared with the wild type. This achievement is an important step in our efforts to develop crambe into an industrial oil crop platform using biotechnology (http://icon.slu.se/ICON/). Moreover, we have evaluated the individual functions of these genes in lipid biosynthesis in crambe through using constructs with one or two target genes.

Results

Transformation, inheritance and expression of the transgenes

To evaluate the individual functions of the target genes, three constructs were used for transformation, harbouring: (i) *LdLPAAT* and *BnFAE1*genes; (ii) *CaFAD2- RNAi*; (iii) *LdLPAAT, BnFAE1* and *CaFAD2- RNAi*. More than 30 transgenic lines were recovered from each construct. Southern blot analysis confirmed the stable integration of the transgenes into in the crambe genome and inheritance in all generations analysed (Figure S1).

qRT-PCR analysis on two T3 lines (3G7-6-7 and 3G7-6-13) with the three target transgenes was carried out as these lines showed relatively higher stability of 22 : 1. All three target genes were expressed as expected in the transgenic lines. It should be noted that the PCR primers also amplified the cDNA from the endogenous crambe *FAE* (Figure 1).

Erucic acid content and FA composition in seed oil of transgenic lines with *LdLPAAT* and *BnFAE1*

Fifty-nine transgenic lines harbouring the *LdLPAAT* and *BnFAE1* genes were preliminarily screened using ten pooled-seed samples. On the basis of FA analysis, two lines (PH11 and PH15) having higher 22 : 1 were chosen for further evaluation, and partial results are listed in Table 1. A stable increase in the average 22 : 1 content, accompanied with a significant reduction in 18 : 1, was observed from T1 to T3 generations, indicating a stable integration and expression of two target transgenes and an effective selection of elite transgenic lines. The maximum 22 : 1 content in single seeds could reach 70.4% in T3 generation compared with 62% in the wild type, with the average level of 67.5% compared with 60% in the wild type, demonstrating that a combination of *LdLPAAT and BnFAE1* could effectively increase the conversion of 18 : 1 into 22 : 1 and channel this FA into TAG.

It should be noticed that, unlike the results stated above, 18 : 1 in some transgenic lines with the same gene construct was increased dramatically, accompanied by a drastic decrease in 22 : 1 and some changes in 18 : 2 and 18 : 3. This effect that could be stably maintained in the subsequent generations (Table S1) is probably due to the co-suppression of endogenous *FAE* genes with the integration of the *BnFAE1* gene. Further studies are needed to confirm this.

FA composition in seed oil of transgenic lines with *FAD2-RNAi*

From 34 transgenic lines containing *FAD2-RNAi*, 19 lines were chosen for preliminary screening using pooled seeds. Two lines (WG4 and WG19) with lower 18 : 2 and 18 : 3 were further

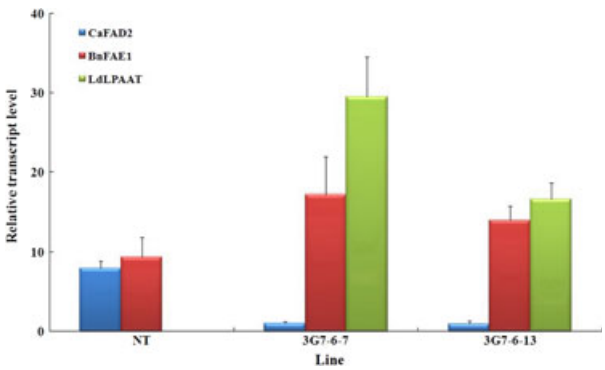

Figure 1 Relative transcript levels of the *BnFAE1*, *LdLPAAT* and *CaFAD2* genes in immature seeds of the transgenic line 3G7-6-7 and 3G7-6-13 in comparison with the non-transgenic control (NT). Results are means with standard error bars from three biological replicates of one immature seed per line.

evaluated (Table 2). A clear segregation of 18 : 1 among individuals was observed in T1, but the variation becomes smaller in T2 and T3 generations, especially in T3 generation, indicating a stable transmission of the gene silencing effect in the subsequent generations. It is particularly noteworthy that the significant increase in 18 : 1 did not lead to an clear increase in 22 : 1 (Table 2), demonstrating that increasing the pool size of 18 : 1 alone is not sufficient for raising 22 : 1 in the seed oil.

FA composition in seed oil of transgenic lines with *LdLPAAT*, *BnFAE1* and *FAD2-RNAi*

Thirty individual transgenic lines harbouring all three target transgenes were firstly analysed with pooled-seed samples. One line (3G7) with a significant increase in 22 : 1 was further evaluated (Tables 3 and 4). A stable increase in 22 : 1 in the subsequent generations was observed, for example, the number of seeds with 22 : 1 higher than 70% accounted for 33% of all seeds in T1 generation, while it was 92.5% in T4 generation, indicating that all three target transgenes had been stably inherited in the subsequent generations, and the homozygous lines with high 22 : 1 could be obtained through recurrent selection for high 22 : 1 content. The average 22 : 1 content reached 72.9% in the best line (Table 3). FA composition of the selected individual seeds from different generations showed

Table 1 The average, minimum (min) and maximum (max) contents (%) of 18 : 1, 18 : 2, 18 : 3 and 22 : 1 in the seed oil from different generations of transgenic crambe lines with *LdLPAAT* and *BnFAE1* genes

Line	Seed no.	FA level	18 : 1	18 : 2	18 : 3	22 : 1
Control	20	Mean	14.5 ± 1.3a	7.6 ± 0.6bc	6.2 ± 0.6bc	60.1 ± 0.8d
		Min	12.0	6.7	5.2	58.0
		Max	16.4	9.3	7.6	61.6
T1						
PH11	32	Mean	13.2 ± 3.1ab	6.5 ± 0.6d	5.6 ± 1.5c	62.8 ± 3.4c
		Min	10.2	5.3	3.0	52.0
		Max	20.0	7.4	8.2	70.0
PH15	32	Mean	12.3 ± 3.4abc	8.1 ± 0.9ab	5.8 ± 1.5c	63.5 ± 2.8bc
		Min	5.9	6.9	2.4	56.0
		Max	20.6	9.8	8.4	68.8
T2						
PH11	32	Mean	10.5 ± 2.2cd	6.7 ± 1.3d	5.7 ± 1.3c	64.0 ± 3.9bc
		Min	6.9	4.7	4.3	51.2
		Max	14.8	8.9	8.1	68.6
PH15	32	Mean	11.3 ± 2.5bcd	8.9 ± 1.3a	6.6 ± 1.3abc	64.2 ± 2.7bc
		Min	7.1	5.9	3.3	55.6
		Max	17.1	11.7	8.4	69.1
T3						
PH11	20	Mean	9.2 ± 2.6d	6.8 ± 1.0cd	7.7 ± 0.7a	67.5 ± 1.6a
		Min	6.3	4.9	6.7	64.1
		Max	13.0	9.5	9.8	70.4
PH15	20	Mean	10.1 ± 3.3cd	8.2 ± 1.4ab	7.0 ± 0.9ab	65.0 ± 3.1b
		Min	5.5	6.2	5.4	56.0
		Max	15.3	9.7	8.6	70.3

T1, T2 and T3 are generations of transgenic lines. Means ± SD followed by the different letters are statistically different at $P = 0.05$. Turkey's test was conducted among different lines for the same fatty acid.

Table 2 Average, minimum (min) and maximum (max) contents (%) of 18 : 1, 18 : 2, 18 : 3 and 22 : 1 in the seed oil from different generations of transgenic crambe lines with the *FAD2-RNAi* gene

Line	Seed no.	FA level	18 : 1	18 : 2	18 : 3	22 : 1
Control	20	Mean	14.5 ± 1.3e	7.6 ± 0.6a	6.2 ± 0.6a	60.1 ± 0.8ab
		Min	12.1	6.7	5.2	58.0
		Max	16.4	9.3	7.6	61.6
T1						
WG4	32	Mean	25.7 ± 1.7b	2.0 ± 1.4cd	2.3 ± 0.6d	61.9 ± 2.0a
		Min	17.3	1.2	1.4	54.7
		Max	27.9	9.1	4.5	64.1
WG19	32	Mean	28.1 ± 1.9a	1.5 ± 1.0d	2.4 ± 0.9d	58.4 ± 4.3b
		Min	23.8	0.8	1.8	48.4
		Max	33.3	5.1	5.4	64.5
T2						
WG4-1	20	Mean	20.8 ± 1.5d	3.8 ± 0.7b	4.2 ± 0.5b	60.3 ± 2.4ab
		Min	18.7	3.2	3.6	59.7
		Max	22.5	4.8	4.7	62.7
WG4-2	20	Mean	22.2 ± 1.5cd	2.3 ± 0.5bcd	3.6 ± 0.5bc	61.6 ± 0.6ab
		Min	20.9	1.8	2.8	60.8
		Max	25.4	3.1	4.0	63.1
T3						
WG4-1-1	20	Mean	22.9 ± 0.2bc	3.1 ± 0.9bc	3.7 ± 0.4bcd	60.6 ± 1.7ab
		Min	21.0	2.3	2.7	58.8
		Max	24.2	4.6	4.6	63.1
WG4-1-2	20	Mean	24.9 ± 0.8bc	1.9 ± 0.4bcd	2.5 ± 0.4cd	60.0 ± 0.7ab
		Min	23.6	1.5	2.1	59.3
		Max	25.7	2.4	3.1	60.7

T1, T2 and T3 are generations of transgenic lines. Means ± SD followed by the different letters are statistically different at $P = 0.05$. Turkey's test was conducted among different lines for the same fatty acid.

Table 3 The average, maximum (max) and minimum (min) levels of erucic acid (22 : 1) content in the seed oil from different generations of transgenic crambe lines with *LdLPAAT, BnFAE1* and *FAD2-RNAi* genes

Line	Seed no.	Max (%)	Min (%)	Mean (%)	Seed no. with 22 : 1 ≥ 70%	Percentage of seeds with 22 : 1 ≥ 70%
Control	20	61.6	58.0	60.1 ± 0.8c	0	0
T1						
3G7	97	75.9	60.1	69.0 ± 3.9b	32	33.0
T2						
3G7-6	61	75.5	55.5	69.1 ± 4.8b	26	42.6
3G7-16	30	74.5	58.1	70.0 ± 4.8b	18	60.0
T3						
3G7-6-7	64	76.8	65.4	72.7 ± 2.3a	59	92.2
3G7-6-13	64	75.4	67.4	72.2 ± 1.8a	58	90.6
T4						
3G7-6-13-2	80	76.9	64.9	72.9 ± 2.7a	74	92.5

T1, T2, T3 and T4 are generations of transgenic lines. Means ± SD followed by the different letters are statistically different at $P = 0.05$. Turkey's test was conducted among different lines for the same fatty acid.

Table 4 Oil composition (%) of some selected T1, T2, T3 and T4 seeds with increased 22 : 1 levels from transgenic crambe lines with *LdLPAAT, BnFAE1* and *FAD2-RNAi* genes

Line	18 : 1	18 : 2	18 : 3	20 : 1	22 : 1	24 : 1	VLCFA*
Control	14.5	7.6	6.2	1.6	60.1	1.7	68.7
T1							
3G7-6	8.3	3.7	0.9	5.0	75.7	1.6	81.4
3G7-40	7.6	1.2	2.1	5.2	75.9	1.4	85.6
T2							
3G7-6-7	9.7	1.0	2.1	4.5	75.5	1.6	85.6
3G7-6-21	6.1	0.8	2.7	4.8	75.1	2.3	88.8
T3							
3G7-6-7-1	5.9	2.1	4.2	1.7	76.7	1.8	85.4
3G7-6-7-13	6.2	1.9	4.7	2.2	75.3	1.7	85.3
T4							
3G7-6-13-22	4.4	1.2	4.2	1.1	76.9	3.0	87.5
3G7-6-13-28	4.6	1.0	3.4	2.3	76.6	2.9	87.9

*VLCFA, fatty acids longer than C18, including the levels of 20 : 0, 22 : 0, 22 : 2, 22 : 3 and 24 : 0 (minor component not shown in the table).

that the increase in 22 : 1 was closely accompanied by a significant reduction in 18 : 1, 18 : 2 and 18 : 3. Some single seeds showed levels of 22 : 1 over 76%, with a highest recorded level of 76.9% in T4 generation (Table 4). Progeny of this line is currently under further selection.

It should be noticed that FA composition from this construct varied a lot among different generations. Most seeds showed markedly decreased contents in either 18 : 1 or 18 : 2 and 18 : 3, whereas the level of 22 : 1 was unchanged. For some seeds, the reduction in 18 : 1, 18 : 2 and 18 : 3 levels did not lead to increase in 22 : 1, instead the 20 : 1 content was increased (data not shown). These results suggest that to increase biosynthesis of 22 : 1, the target genes have to work optimally and simultaneously to maintain a maximal 18 : 1 substrate pool for elongation (regulated by *FAD2-RNAi*), an improved flux from 18 : 1 to 22 : 1 (promoted by *BnFAE1*) and a strong 22 : 1 sink (enhanced by the activity of *LdLPAAT*).

FA composition in hybrid seeds

High 22 : 1 transgenic lines with *LdLPAAT* and *BnFAE1* of T2 generation and low 18 : 2 and 18 : 3 transgenic lines with *FAD2-RNAi* of T2 generation were crossed to produce F1, F2 and F3 seeds. In total, 336 F1, 108 F2 and 129 F3 seeds were analysed using the half-seed method. At the individual seed level, the highest 22 : 1 content could reach up to 76% in F1 and F2 and 74% in F3 (Figure S2), a similar level to that of the three-gene construct transgenic lines. In general, the average level of 22 : 1 content increased steadily with each generation. For F3 generation, the majority of the seeds had 22 : 1 levels of 69 to 70%, and about 50% of seeds contained 22 : 1 higher than 70% (Figure S2). However, this ratio is much lower than that of transgenic lines obtained with the three-gene vector, in which 92% of T3 seeds have 22 : 1 higher than 70%. Hence, our results show that both methods are effective in developing transgenic lines with increased 22 : 1 contents, but direct transformation with three genes is more efficient than crossing.

Trierucin level and positional distribution of erucic acid in relation to erucic acid level

Trierucin analysis was carried out on some transgenic seeds with increased 22 : 1 contents of T2 generation. As expected, the positive correlation (r^2 = 0.64) between amount of trierucin and the total 22 : 1 content in seed oil was observed (Figure 2), indicating that the *LdLPAAT* gene is functional in incorporating the erucoyl moiety into the *sn*-2 position of TAG in crambe. In this study, the highest content of trierucin was 22.5% and this seed had 75% of 22 : 1. Analysis of the distribution of 22 : 1 on the outer positions (*sn*-1 + *sn*-3) and *sn*-2 position of pooled seeds from two transgenic lines of T3 generation revealed that although substantial 22 : 1 was found at the *sn*-2 position in the transgenic lines (52% and 58%), it was appreciably lower than in the outer positions (Table 5). Assuming an equal distribution of 22 : 1 between *sn*-1 and *sn*-3 positions and a glycerol backbone acylation that is largely independent of the molecular species of the acyl acceptor, the amount of trierucin would in these transgenic lines be 32%, much higher than actually found (Figure 2). These data suggest that the endogenous GPAT and DAGAT effectively compete with the introduced LdLPAAT for 22 : 1-CoA, whereas the endogenous LPAAT competes with LdLPAAT for the acyl acceptor erucoyl-glycerol-3-phosphate. Unlike LdLPAAT, the crambe LPAAT, however, directs primarily C18 acyl groups to the *sn*-2 position, thus preventing trierucin formation. As discussed below, further mechanisms might be involved as well. It should be noted here that the two transgenic lines analysed came from the same transformation event and stemmed from the same T1 seed (3G7-6). Although the total level of 22 : 1 was nearly the same in the two lines, the percentage of 22 : 1 at the *sn*-2 position differed, indicating that small genotypic differences could exert substantially phenotypic differences in the effects of the transgenes.

Surprisingly, we found that the wild type crambe differs from all other reports about the 22 : 1 distribution in TAGs in *Brassicaseae spp* (Cao *et al.*, 1990; Frentzen, 1993), in which it contains a significant amount of 22 : 1 (8%) in the *sn*-2 position (Table 5). A calculated level of trierucin species in the wild-type crambe would be 6%, provided the acylation of acyl acceptor species was non-selective and the distribution of 22 : 1 between *sn*-1 and *sn*-3 was similar. However, no trierucin species at all was found in the seeds of the wild type (data not shown). The percentage distribution of VLCFAs between *sn*-1 and *sn*-3 cannot differ too much in the wild type seeds because

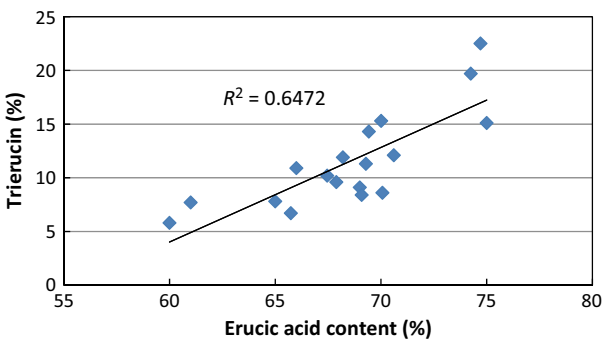

Figure 2 Correlation of erucic acid (22 : 1) and trierucin contents in the selected transgenic crambe lines of T2 generation. The analysis was carried out on 18 single seeds of the transgenic line 3G7-6.

Table 5 Fatty acid distribution (area%) at different *sn*-positions of triacylglycerols (TAG) and in phosphatidylcholine in wild type (NT) and two transgenic very high 22 : 1 crambe lines of T3 generation (n.d, not detectable)

Line	Position	16 : 0	18 : 1	18 : 2	18 : 3	20 : 0	20 : 1	22 : 0	22 : 1	24 : 0	24 : 1
NT	Sn-1 + sn 3 in TAG	2.0	0.9	0.5	0.3	0.6	2.2	2.3	85.8	0.8	2.8
	Sn-2 in TAG	2.1	34.3	19.8	12.8	0.2	0.8	0.4	8.1	0.2	0.7
	Calculated % in TAG	2.0	12.0	6.9	4.5	0.5	1.7	1.7	59.9	0.6	2.1
	Actual % in TAG	1.6	13.3	8.3	5.4	0.5	1.8	1.9	59.8	0.5	1.5
	Phosphatidylcholine	7.5	49.0	23.0	5.1	n.d	3.3	n.d	4.7	n.d	n.d
3G7-6-13	Sn-1 + sn 3 in TAG	2.2	2.8	0.5	0.9	0.5	7.2	2.0	74.8	0.7	3.4
	Sn-2 in TAG	0.9	15.6	7.9	12.0	0.6	1.2	0.6	57.9	0.0	0.5
	Calculated % in TAG	1.8	7.1	3.0	4.6	0.5	5.2	1.5	69.2	0.5	2.4
	Actual % in TAG	1.6	7.6	3.4	5.9	0.4	4.9	1.6	70.5	0.5	1.9
	Phosphatidylcholine	6.8	55.0	11.4	14.1	n.d	4.3	n.d	2.4	n.d	n.d
3G7-6-7	Sn-1 + sn 3 in TAG	2.6	1.4	0.3	0.4	0.6	5.5	2.1	78.5	0.6	3.0
	Sn-2 in TAG	0.5	18.1	8.2	11.9	0.2	0.3	0.2	52.2	0.1	0.5
	Calculated % in TAG	1.9	7.0	2.9	4.2	0.5	3.8	1.5	69.7	0.4	2.2
	Actual % in TAG	1.7	7.6	3.6	5.9	0.5	2.3	1.3	69.7	0.4	1.6
	Phosphatidylcholine	6.6	53.2	11.7	13.7	n.d	5.7	n.d	3.7	n.d	n.d

the two positions together have over 94% of VLCFA (Table 5). Thus, as demonstrated both in non-transgenic and transgenic plants, there is clear a bias against the formation of trierucin species in crambe that is not because of inefficient acylation of 22 : 1 at the *sn*-2 position *per se*. It can be speculated that this might be due to steric hindrances in the erucoyl acylation of either or both of erucoyl–lysophosphatidic acid and di-erucoyl-glycerol, catalysed by the LPAAT and DGAT enzymes, or that it might be largely caused by the properties of the crambe LPAAT, which can use the 22 : 1-CoA as acyl donor better when the acyl acceptor is carrying a C18 or C16 acyl group.

FA composition of phosphatidylcholine in seeds

Erucic acid is a TAG-specific FA largely excluded from membrane lipids of seeds from *Brassicaceae spp* (Kunst *et al.*, 1992). We therefore analysed the FA composition of phosphatidylcholine (PC) in seeds from the transgenic and non-transgenic crambe (Table 5). The 20 : 1 FA was found in PC in both lines and increased from 3.3% in the wild type up to 5.7% in the transgenic line. An increase in 20 : 1 was also observed in the TAGs of transgenic lines compared to the wild type. The wild type also contained 4.7% of 22 : 1 in PC. Most interestingly, the content of 22 : 1 decreased by 49% in the transgenic line having 58% of 22 : 1 in the *sn*-2 position, but by 20% in the line having 52% of 22 : 1 in the *sn*-2 position (Table 5). It should be emphasized that the two transgenic lines had nearly the same amount of 22 : 1 in the TAGs.

Discussion

In oil seeds, the biosynthesis of TAG is mainly via the Kennedy pathway, in which three acyl moieties are esterified at the *sn*-1, *sn*-2 and *sn*-3 positions of the glycerol backbone by the *sn*-glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidate acyltransferase (LPAAT) and 1, 2-diacylglycerol acyltransferase (DGAT), respectively (Stymne and Stobart, 1987). Of the three acyltransferases involved in the Kennedy pathway, GPAT and DGAT from diverse seed species have been demonstrated to be flexible in using various acyl-CoAs, whereas LPAATs have pronounced specificities towards certain acyl-CoAs (Snyder *et al.*, 2009). Different specificities of GPAT, DGAT and LPAATs suggest that the acyl moieties in the *sn*-1 and *sn*-3 positions of TAG depend largely on the availability of acyl-CoAs in the intracellular pool, while that in the *sn*-2 position relies mainly on the acyl preference of LPAAT (Cao *et al.*, 1990*).* These preferences of enzymes have in fact determined the setup of genetic engineering for high 22 : 1 levels in oilseed crops. Not only should the introduced genes enable the production of a given moiety, for example the introduction of extra *FAE* expression to create more substrate 22 : 1, but also ensure effective incorporation of 22 : 1 into all three *sn*-positions of the glycerol backbone by introducing the proper LPAAT. A strategy with modification of multiple genes is thus required to combine these functions. This has been confirmed in this study, in which all transgenic lines with single or double transgenes showed a limited increase in 22 : 1, while the combination of all three transgenes resulted in higher 22 : 1 levels.

The prevailing hypothesis on the biosynthesis of 22 : 1 in developing seeds is that 18 : 1, produced in the plastid, is activated to oleoyl-CoA. This oleoyl moiety is then elongated to 22 : 1 in the ER or in oil body-associated membranes through successive additions of two carbons derived from malonyl-CoA. However, Hlousek-Radojcic *et al.* (1995) indicated that oleoyl-CoA is not the immediate substrate for elongation. Bao *et al.* (1998) showed the complexity in the biosynthesis of 22 : 1, namely the supply of oleoyl groups for chain elongation is a combination of the release of 18 : 1 from a large intermediate lipid pool, probably PC, and the direct provision of newly synthesized 18 : 1 from the plastid. Jadhav *et al.* (2005) proposed that the oleate availability was a rate-limiting factor in the biosynthesis of 22 : 1. All these results and assumptions suggest that the availability of oleoyl-CoA in the cytosol is crucial to the increase of 22 : 1. As 18 : 1 in the developing seeds can be converted to 18 : 2 through *FAD2*, down-regulation of the respective genes through RNAi is expected to increase the concentration of 18 : 1 moiety and thus increase 22 : 1 (Nath *et al.*, 2009; Stoutjesdijk *et al.*, 2002). However, in this study, although *FAD2-RNAi* could result in significant reduction in 18 : 2 and 18 : 3 and increase in 18 : 1, 22 : 1 did not increase, suggesting that the pool size of 18 : 1 is not by itself

a limiting factor to the production of 22 : 1 in crambe. Because we showed that over 94% of all FAs at *sn*-1 + *sn*-3 are VLCFA, any further significant increase in 22 : 1 in TAGs has to come from incorporation of 22 : 1 in the *sn*-2 position. Thus, introducing the *LdLPAAT* gene capable to acylate 22 : 1 at the *sn*-2 position in combination with overexpressing the *BnFAE* gene effectively increased the 22 : 1 content without increasing the total amount of monounsaturated FAs. It was further shown that in these transgenic plants, the 18 : 1 pool became the limiting factor in the production of 22 : 1 because introduction of an *FAD2-RNAi* together with the two other transgenes further increased the 22 : 1 content.

Although we have developed transgenic crambe lines with over 70% of 22 : 1, further increase of the 22 : 1 level would still be desirable. Erucamide, the main industrial product derived from 22 : 1, is usually sold as slipping agent in plastic manufacturing at the purity of 90% or above. If 90% of 22 : 1 can be obtained directly in the crambe seed oil, there will be no need for purification, thus greatly reducing the cost for erucamide production. The results obtained in this study together with the present knowledge of plant lipid biochemistry provide some indications on how this might be achieved. The pathways involved in the plant storage lipid biosynthesis with common FAs have been well documented. Besides the Kennedy pathway, the acyl groups can also be channelled into PC via the activity of lysophosphatidylcholine acyltransferase (LPCAT) and thereafter transferred from PC to TAG directly via the activity of phospholipid : diacylglycerol acyltransferase (PDAT) or indirectly by interconversion with DAG, presumably catalysed by a phosphatidylcholine : diacylglycerol cholinephospho transferase (PDCT) (Lu *et al.*, 2009). It is evident that PC plays a key role in TAG synthesis because the majority of *de novo* synthesized FAs (mainly 18 : 1 and 16 : 0) are, at least in soybean and Arabidopsis seeds, first incorporated into PC and that the DAG used for TAG synthesis is mostly derived from PC (Bates and Browse, 2011; Bates *et al.*, 2007, 2009; Cassagne *et al.*,1994). Clearly, any manipulation of FA composition of the oil has thus to take into account the flow of acyl groups through PC in TAG synthesis. PC is also the site where 18 : 1 is further desaturated to 18 : 2 and 18 : 3 as well as the site for the synthesis of a number of unusual FAs by desaturase-like enzymes (Carlsson *et al.*, 2011). The 18 : 1, 18 : 2 and 18 : 3 from PC can then be either transferred into the acyl-CoA pool, mainly used in the Kennedy pathway or entered into DAG and TAG via PDCT and PDAT catalysed reactions, also called non-acyl-CoA-mediated pathway (Lu *et al.*, 2009, 2010).

The production of high amount of unusual FA in seed TAGs is not well understood. A feature common for most of these FAs, including 22 : 1, is that they are more or less excluded from the seed phospholipids (Carlsson *et al.*, 2011). Besides 22 : 1, the main FAs in the transgenic crambe seeds having around 76% of 22 : 1 are 18 : 2 and 18 : 3 that together make up about 6.5% and 18 : 1 around 6%. It is reasonable to assume that 18 : 2 and 18 : 3 are produced on PC and channelled to TAG either via the acyl-CoA pool or via DAG or directly by PDAT catalysed acylation of DAG. Because acyl groups acylated to TAG by PDAT will be at the *sn*-3 position and the outer positions *sn*-1 + *sn*-3 in the transgenic crambe have together only 0.7%–1.4% of these FAs (Table 5), we can exclude that this is a major route for the incorporation of the polyunsaturated FAs. We can also postulate that it is not a major route for 18 : 1 incorporation into TAG either because the levels of 18 : 1 in the outer positions are 1.4%–2.8% (Table 5). If we assume that all the 18 : 1 + 18 : 2 + 18 : 3 in the outer positions of TAG are in the *sn*-3 position and that they are incorporated via PDAT, PDAT could maximally contribute to 25% ($100 \times 4.2/16.9$, Table 5) of the C18 unsaturated FAs found in TAGs in the transgenic seeds with very high 22 : 1 contents. The main part of the C18 unsaturated FAs is in the *sn*-2 position, and this could be derived from acylated DAG produced from PC via PC : DAG interconversion catalysed by PDCT. Because the 22 : 1 in PC is low, PDCT contribution would mainly result in the flow of C16 and C18 PC species into DAG and TAG. However, our positional analysis indicates that such TAG species with C16- C18 FAs in both *sn*-1 and *sn*-2 position will be very rare in the transgenic lines. However, Arabidopsis mutated in PDCT showed elevated levels of 20 : 1 in its seed TAGs (Lu *et al.*, 2009), which might be explained by increased flux of 18 : 1 from PC out in the acyl-CoA pool for elongation when the flux of this FA from PC to DAG is blocked.

In this context, it is interesting to discuss the mechanism by which 22 : 1 is excluded from PC. The major flow of FAs from DAG to TAG in oil seeds like soybean and Arabidopsis goes through PC via DAG : PC interconversion (Bates and Browse, 2011; Bates *et al.*, 2009). Because the vast majority of the DAG molecules synthesized by the wild type crambe seeds will have 22 : 1 in the *sn*-1 position, the low content of 22 : 1 in PC might be achieved by a selection against 22 : 1 containing DAG species by PDCT or a selective acylation of this species by DGAT, or a combination of both mechanisms. The 22 : 1 content in PC in transgenic lines producing 70% of 22 : 1 was substantially reduced, and this reduction was greater in the line that had more 22 : 1 in the *sn*-2 position of DAG (Table 5). Thus, it can be expected that DAGs with two erucoyl groups are even more efficiently excluded by PDCT and/or more efficiently acylated by DGAT.

On the basis of the above considerations, we believe that most of the 18 : 1, 18 : 2 and 18 : 3 found in TAGs in the transgenic high erucic crambe lines come from FAs channelled from PC into the acyl-CoA pool and then incorporated into TAG via the Kennedy pathway. The mechanisms whereby the acyl groups from PC are entering the acyl-CoA pool are unclear, but a strong candidate enzyme is LPCAT catalysing the exchange of FAs between the acyl-CoA pool and PC. There are two genes encoding LPCAT in Arabidopsis (Ståhl *et al.*, 2008), and knockout of one of these genes led to a significant increase in 20 : 1 on the expense of mainly 18 : 2 in Arabidopsis seed lipids (Zou *et al.*, 2011). It is possible that the inhibition of LPCAT activity shifts the flow of 18 : 1 exported from the plastid away from incorporation into PC and promotes its elongation to VLCFA and their incorporation into TAG via the Kennedy pathway. Lower LPCAT activities would also inhibit the flow of polyunsaturated FAs from PC into the acyl-CoA pool. The down-regulation of *LPCAT* gene expression in crambe might thus in both cases yield a larger 18 : 1-CoA pool for elongation to 22 : 1. However, the endogenous LPAAT in crambe will compete with the FAE for the 18 : 1-CoA substrate and thereby limiting its conversion into 22 : 1.

Another aspect is the timing of the expression of the enzymes involved in 22 : 1 accumulation during the seed development. At very early stages of the seed development, crambe seeds have TAGs with much lower 22 : 1 contents (A. Banas, pers. commun.). However, because mature seeds of the wild type contain 94% of VLCFA at the outer positions of TAG, the

deposition of TAG with lower amount of 22 : 1 at early stages can only slightly influence the total amount of 22 : 1 found in the mature seed, at least in the wild type crambe.

Other bottlenecks for higher 22 : 1 might be the availability of cytosolic malonyl-CoA for carbon chain elongation. It has been shown that *B. napus* seeds have 40-times higher expression of cytosolic acetyl-CoA carboxylase genes than seeds lacking VLCFAs in its oil (Troncoso-Ponce *et al.*, 2011). It remains to clarify whether there is also a limitation in the supply of cytosolic malonyl-CoA and/or the hydratase and the reductase activities of the elongase complex that limit achievements of producing even higher amount of 22 : 1 than here reported in crambe seeds.

In conclusion, the previous efforts focused mainly on single gene modification have constrained the progress of studies on producing high 22 : 1 in oilseed crops. The complexities of 22 : 1 biosynthesis require a gene stacking strategy for creating and keeping a large substrate pool, an intensive flux and a strong sink for the synthesized 22 : 1. Through overexpression of *LdLPAAT* and *BnFAE1* combined with down-regulation of *CaFAD2,* we have developed transgenic crambe lines with up to 76.9% of 22 : 1 in its seed oil, which represents the highest level reported up to now. This achievement has demonstrated the effectiveness of gene stacking strategy for seed oil modification. Our results could help to understand the limiting factors involved in the 22 : 1 biosynthesis. Furthermore, as the first successful *C. abyssinica* engineering attempt to modify oil qualities, this study has confirmed the feasibility of developing *C. abyssinica* as a competitive industrial oil crop platform and also provided valuable transgenic crambe material for further efforts in achieving even higher 22 : 1 levels.

Experimental procedures

Plant material and *in vitro* growing conditions

Crambe abyssinica Hochst. cv. Galactica was used in this study. All *in vitro* materials were grown in a chamber with 16 h photoperiod, 33 μmol/m^2/s light intensity and 25/18 °C temperature (day/night).

Transformation vectors and plant transformation

The vectors used in this study include pHAN (PH), harbouring the *LdLPAAT* and *BnFAE1*genes (Han *et al.*, 2001), pWatergate (WG) (CSIRO, Australia, http://www.pi.csiro.au/rnai/vectors.htm#pHANpKAN), harbouring *CaFAD2-RNAi,* and three-gene construct (3G), modified based on WG, harbouring *LdLPAAT, BnFAE1* and *CaFAD2-RNAi*. The *FAD2* sequence used in the RNAi construct was 460 bp, located in the middle of the gene (when compared with the sequences from *Brassica rapa* (JN859550) and *Brassica napus* (DQ767949) as no sequence of crambe *FAD2* available). The schematic representation of T-DNA of the three-gene (3G) vector is presented in Figure 3. All the target genes are under napin promoter. The plant selection marker is *nptII* (*neomycin phosphotransferase II*). Crambe transformation was performed according to Li *et al.* (2010). *In vitro* transgenic lines were maintained under conditions stated above.

Southern blot analysis

In vitro cultures or leaves of greenhouse- or biotron-grown plants, depending on generation, were used for Southern blot analysis according to Zhu *et al.* (2001). The Southern blot was performed on the *nptII* gene, which is located close to the left border of T-DNA (Figure 3). The probe was PCR-synthesized and DIG-labelled. The primers used were: 5′-GCCCTGAATGAACTGCAGGACGAGGC-3′ and reverse 5′-GCAGGCATCGCCATGGGTCACGACGA-3′.

Figure 3 Schematic representation of T-DNA of the 3G vector containing 3 target genes, *CaFAD2-RNAi*, *LdLPAAT* and *BnFAE1* as well as the *nptII* gene. All the 3 target genes are under napin promoter (N-promoter). LB and RB are left and right borders.

Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted from immature seeds collected at 30–40 days after flowering using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Because of the non-complete homozygous nature of the transgenic lines, each seed was used as one sample with three biological samples for each line and four technical replicates for each qRT-PCR. Residual genomic DNA was removed through DNase treatment using TURBO DNA-free (Ambion, Austin, TX). First-strand cDNA was synthesized from 400 ng of total RNA in 20 μL with RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). The cDNA was diluted four times, and 2 μL was used for each 20 μL qRT-PCR using Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) with Platinum SYBR Green qPCR SuperMix-UDG module (Invitrogen, Carlsbad, CA). The PCR programme was 50 °C for 2 min and 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. Melting curve analysis was performed to confirm the product specificity. The primers for *CaFAD2*, *BaFAE1* and *LdLPAAT* were selected from several sets of primers tested in preliminary analyses. Gene expression was normalized to the reference gene *UBC21* (Table 6) as no sequence information of reference genes from crambe is available. The partial sequence of crambe ubiquitin gene has shown that the primers used for qRT-PCR are homologous between Arabidopsis and crambe (data not shown).

Growth conditions and plant management

The transgenic lines and control plants were planted in the biotron (T1, T3, F1 and F3 generations) or greenhouse (F2, T2 and T4 generations). The conditions in the biotron were 16 h photoperiod, 250 μmol/m^2/s light intensity, 21/18 °C temperature (day/night) and 60% humidity, and those in the greenhouse were 16 h photoperiod and 21/13 °C temperature (day/night). The plantlets were watered regularly with fertilizer

Table 6 The sequences of the primers used for qRT-PCR

Gene	Forward primer (5′–3′)	Reverse prime (5′–3′)
AtUBC21	TGCGACTCAGGGAATCTTCT	TCATCCTTTCTTAGGCATAGCG
CaFAD2	CCGTGAACGTCTCCAGATAT	CGTTGACTATCAGAAGCCGA
BnFAE1	CCTCCCCGGAAGACTTTTG	CATGCTTGAGTTCACCACAAG
LdLPAAT	AAGTAAACGCCCATCTCTCG	GGCTGCGGCTATTCAGTCTA

(N : P : K = 21 : 3 : 10). The harvested seeds were kept at 4 °C before oil composition analysis.

Hybridization of transgenic lines

Apart from the transgenic approach, the transgenic T2 lines harbouring *LdLPAAT* and *BnFAE1* were crossed with the T2 lines harbouring *FAD2-RNAi* to assure the obtainment of best transgenic lines with all target genes. For crossing, 5–10 normal-sized and unopened flowers from each shoot were emasculated under microscope and covered with paper bags. Hand pollination was performed 1 day later, and the pollinated flowers were covered with paper bags for 2 weeks. Mature and well-developed hybridized seeds were collected for fatty acid analysis.

Oil composition analysis

Half-seed technique

This technique was used for all oil analyses unless otherwise stated. Seeds without siliques were surface-sterilized using 15% calcium hypochlorite for 20 min and rinsed thoroughly with sterile water before being placed on the germination medium (Li *et al.*, 2010) in the chamber. After about 15 h, the seed coats were removed, and the larger outer cotyledon was excised for FA analysis, while the rest were maintained on the culture medium until planted in pots after FA analysis in greenhouse or biotron depending on season.

Fatty acid composition analysis by GC

The excised cotyledons were first grounded in mortars with 500 μL hexane followed by an addition of 500 μL hexane for complete homogenization of the tissues. The extraction solution was passed through a Pasteur pipette with a glass wool plug to remove seed residues. Of the flow through, 100 μL was pipetted into a GC vial for intact TAG analyses, and the rest was dried under nitrogen stream, thereafter 2 mL methylation solution (2% H_2SO_4 in water-free methanol) was immediately added and methylated at 95 °C for 45 min. After methylation, 0.5 mL hexane and 2 mL water were added. After brief vortexing and centrifugation at 350 ***g*** for 3 min, the hexane phase containing FA methyl esters (FAMEs) was transferred into a GC vial for FA composition analysis using Shimadzu GC-17A gas chromatograph with WCOT Fused Silica CP-Wax 58 with a FID detector (Shimadzu Corporation, Kyoto, Japan). Peaks were identified according to their retention time in comparison with a standard FAME mixture, and results are expressed as area percentage of the erucic acid methyl esters in total detectable peak areas.

Trierucin analysis

For trierucin analysis, 100 μL of the initial hexane extract as described above was directly transferred into a GC vial and analysed using Agilent GC machine (model 7890 A with a FID detector: Agilent Technologies, Böblingen, Germany) with the column Rtx-65TG 15 m × 0.25 mm × 0.1 μm at the constant temperature 370 °C. The trierucin was identified through comparing the sample retention time with that of the standard trierucin (Larodan, Malmö, Sweden) and expressed as area percentage in the total peak areas.

Positional analysis of triacylglycerols and fatty acid composition of phosphatidylcholine

About 30 mature seeds of T3 generation from wild type and two T3 transgenic lines were homogenized with Ultraturrax® (IKA Laboritechnik, Stauden, Germany) in methanol : chloroform (2 : 1 by vol.), and lipids were extracted into chloroform according to Bligh and Dyer (1959). The lipids were then separated on silica gel TLC plates (Silica 60; Merck, Darmstadt, Germany) in n-heptane : diethylether : acetic acid (70 : 30 : 1), and gel areas corresponding to reference TAG areas were scraped off. The TAGs were eluted from the gel with methanol : chloroform (2 : 1) and extracted into chloroform. An aliquot of the chloroform phase was methylated and analysed on GC as described above to give the actual FA composition of the TAGs, whereas the rest was evaporated under nitrogen and residue dissolved in 0.5 mL diethyelether. One millilitre of 60 mM HEPES buffer at pH 7.3 with 10 mM $CaCl_2$ containing 15 μL (300 units) of Rizomucor miehei lipase (Sigma L4277, St Louis, MO) was added to the diethylether solution, and the mixture was vigorously vortexed for 5 min at 25 °C after which 20 μL of 20% trichloroacetic acid and 80 μL 100 mM EDTA were added to terminate the lipase activity. The diethylether was removed under a stream of nitrogen, and the lipids were extracted into chloroform and separated on silica gel TLC plates in n-heptane : diethylether : acetic acid (60 : 40 : 1), and gel areas corresponding to free FAs (*sn*-1 and *sn*-3 positions) and monoacylglycerols (*sn*-2 position) reference lipids were removed, methylated on the gel by adding 2 mL of 2% H_2SO_4 in water-free methanol and analysed by GC as described above. Calculated FA composition of the TAGs was based on the FA composition of *sn*-1 + *sn*3 and *sn*-2 (% ((*sn*-1 + *sn*-3)*2 + *sn*-2)/3). FA composition of calculated and methylated intact TAG gave a good agreement (Table 5), demonstrating that the lipase was hydrolysing the different TAG molecular species non-selectively.

An aliquot of the seed extract was applied on the silica TLC plates and developed in chloroform : methanol : acetic acid : -water (90 : 15 : 10 : 3 by vol). PC spots were identified by migration relative to standard PC after light staining with iodine. The gel areas containing PC were removed, and remaining iodine was removed in a stream of nitrogen, and the lipids were methylated on the gel and FA composition determined as described above.

Statistical analysis

Data of oil analysis were statistically analysed with ANOVA and Turkey's test using the Statgraphics program.

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Supporting information

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

Figure S1 Results of Southern blot analysis of some transgenic crambe lines.

Figure S2 Frequency distribution of erucic acid levels in the seed oil from F1 (PH15-10 × WG4-32), F2 and F3 seeds containing different levels of 22 : 1 in the seed oil.

Table S1 Oil composition (%) of selected T1, T2 and T3 single seeds of transgenic crambe lines with *LdLPAAT* and *BnFAE1* genes showing an increase in 18 : 1 content.

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