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Inheritance and variation of erucic acid content in a transgenic rapeseed (*Brassica napus* L.) doubled haploid population

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Abstract Erucic acid (22:1) is a valuable renewable resource for the oleochemical industry. Currently available high erucic acid rapeseed cultivars contain only about 50% erucic acid in the seed oil. A substantial increase of the erucic acid content of the rapeseed oil could increase market prospects. The transgenic line TNKAT, over expressing the rapeseed fatty acid elongase gene (*fae*1) and expressing the *Ld-LPAAT* gene from *Limnanthes douglasii* was crossed with the line 6575-1 HELP (high erucic and low polyunsaturated fatty acid). A from the F_1 plants produced population of 90 doubled haploid (DH) lines was tested in a greenhouse with three replicates. Parental lines TNKAT and 6575-1 HELP contained 46 and 50% erucic acid in the seed oil, respectively. In the DH population the erucic acid content ranged between 35 and 59%. The *Ld-LPAAT* + *Bn-fae*1.1 transgene showed a 1:1 segregation. The transgenic DH lines contained up to 8% trierucolyglycerol, but surprisingly had a by 2.3% lower erucic acid content compared to the non-transgenic segregants. Results indicated that the ectopically expressed *fae*1.1 gene may not be functional. The DH population also showed a large quantitative variation for PUFA content ranging from 6 to 28% (TNKAT: 21%, 6575-1 HELP: 8%). Regression analysis showed that in the DH population a 10% reduction in PUFA content led to a 4.2% increase in erucic acid content. Development of locus specific PCR primers for the two resident erucic acid genes *fae*1.1 (A-genome) and *fae*1.2 genes (C-genome) of rapeseed allowed sequencing of the respective alleles from TNKAT and 6575-1 HELP. Single nucleotide polymorphisms were only found for the *fae*1.1 gene. Use of allele specific *fae*1.1 PCR primers, however, did not reveal a significant effect of the *fae*1.1 allele from either parent on erucic acid content. The high erucic acid low polyunsaturated fatty acid DH lines and the *fae*1 locus specific primers developed in the present study should be useful in future studies aimed at increasing erucic acid content in rapeseed.

Keywords *Brassica napus* · Erucic acid · Oil quality · Fae1 · LPAAT

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Introduction

Erucic acid (22:1) obtained from the seed oil of high erucic acid rapeseed (HEAR) is a sought-after raw material used by the oleochemical industry (Scarth and Tang 2006). It is used in the production of plastic films, nylon, lubricants and emollients. Currently available conventional HEAR cultivars contain only about 50% 22:1 in the seed oil. A substantial increase of the 22:1 content of the rapeseed oil would

significantly reduce processing costs and could increase market prospects. However, in *Brassica napus* and related *Brassica* species, the content of erucic acid in the seed oil is limited by the fact that erucoyl moieties are typically excluded from the central *sn*-2 position of the triacylglycerol molecule. This limitation is due to the specificity of the rapeseed lysophosphatidic acid acyltransferase (*Bn*-LPAAT), which is incapable of utilizing erucoyl-CoA as an acyl donor (Taylor et al. 1992). The exclusion of erucic acid from the *sn*-2 position implies a threshold of 66% erucic acid in the seed oil (Frentzen 1993; Katavic et al. 2001). However, as indicated above, in reality only much lower erucic acid contents in the seed oils are achieved, which indicates that the *β*-ketoacyl-CoA synthase (KCS; *fae*1 gene) activity, the first enzyme responsible for the fatty acid elongation from oleic acid (18:1) to eicosenoic acid (20:1) and to erucic acid (Cassagne et al. 1994) may be limiting erucic acid content in the seed oil. In rapeseed there are two *fae*1 genes, the *Bn-fae*1.1 and the *Bn-fae*1.2, which correspond to the *fae*1 gene of the parental species *B. rapa* (A-genome) and *B. oleracea* (C-genome), respectively (Ecke et al. 1995; Fourmann et al. 1998). Multiple alleles at the two *fae*1 loci have been shown to contribute to a different degree to the total erucic acid content in the seed oil, which may vary from <1 to >60% (Stefansson and Hougen 1964; Jönsson 1977).

In a first approach to increase erucic acid content in rapeseed, the LPAAT enzyme from meadowfoam (*Limnanthes douglasii*; *Ld*-LPAAT) has been found to preferentially insert erucic acid into the *sn*-2 position. The *Ld-LPAAT* gene was cloned and expressed under control of a seed specific promoter in rapeseed. However, this achievement was not accompanied by an increase in erucic acid content in the seed oil (Brown et al. 1995; Hanke et al. 1995; Lassner et al. 1995; Brough et al. 1996). In a second approach, the *fae*1 gene has been cloned from *Arabidopsis* (James et al. 1995) and subsequently from rapeseed. Over expression of the *fae*1 gene in HEAR, also in combination with expression of the *Ld-LPAAT* gene, however, was also not successful. Transgenic plants showed only a minor increase in eicosenoic and erucic acid content (Katavic et al. 2001; Han et al. 2001).

There is some evidence that the cytosolic pool of oleoyl-CoA available for elongation may be limiting erucic acid biosynthesis (Bao et al. 1998; Domergue et al. 1999). Crossing of conventional HEAR to rapeseed with reduced contents of linoleic acid (18:2) and linolenic acid (18:3) did result in recombinant high erucic low polyunsaturated fatty acid (HELP) F_3 plants which, however, did not show an increased erucic acid content compared to the parental HEAR genotype (50% erucic acid; Sasongko and Möllers 2005). An obvious explanation for this result was that in the HELP material the activity of the *β*-ketoacyl-CoA synthase (*fae*1.1 and *fae*1.2 genes) may be too low to allow for enhanced erucic acid synthesis.

To test this hypothesis, a resynthesised transgenic HEAR line, expressing a single copy of the *Brassica napus fae*1 gene (*Bn-fae*1) in combination with an erucoyl-CoA specific lysophosphatidic acid acyltransferase from *Limnanthes douglasii* (*Ld-LPAAT*) gene, both under control of the strong seed specific napin promoter (Han et al. 2001), was crossed with the non-transgenic line 6575-1 HELP (Sasongko and Möllers 2005). Thereby, the low content of polyunsaturated fatty acids of 6575-1 HELP is inherited by one major and probably two to three minor genes (Schierholt et al. 2001; Sasongko and Möllers 2005). This indicates that a large number of F_2 plants need to be cultivated in the greenhouse to identify a specific homozygous genotype with a high probability. As an alternative way to overcome these problems, DH lines may be produced through microspore culture (Lichter 1982).

In the present study, F_1 plants of the above mentioned cross were used to produce DH lines. Those were then grown along with parental lines in a replicated experiment in the greenhouse to study the inheritance of erucic acid content and other seed quality traits. Locus and allele specific PCR primers were developed to follow the segregation of the erucic acid alleles at the endogenous *fae*1 loci.

Materials and methods

Plant materials

TNKAT

A resynthesised transgenic winter rapeseed line (RS306) derived from an interspecific cross between Yellow Sarson (*Brassica rapa*) and cauliflower (*Brassica oleracea* sp. *capitata*) cv. Super Regama

(Lühs and Friedt 1994) was used for *Agrobacterium* mediated transformation to produce TNKAT line carrying a single transgene copy (T-DNA contained the chimeric *Bn-fae*1.1 and the *Ld-LPAAT* gene, both under control of the seed specific napin promoter; see Han et al. 2001). TNKAT seeds were provided by Dr. Margrit Frentzen, University of Aachen, Germany.

6575-1 HELP (high erucic and low polyunsaturated fatty acid)

This line was F_4 seed generation of winter rapeseed with 27% 18:1, 8% 18:2 + 18:3 and 50% 22:1 content (Sasongko and Möllers 2005) obtained from a cross between the winter rapeseed cv. Maplus and the high oleic acid DH winter rapeseed line DH XXII D9 (for details see Sasongko and Möllers 2005).

Doubled haploid plant population

F_1 plants were obtained after crossing TNKAT × 6575-1 HELP. They were used as donors for microspore culture, which was performed according to a protocol described by Fletcher et al. (1998).

Methods

Greenhouse experiment

A greenhouse experiment was performed during the period August 2006 to April 2007 (from sowing to harvest). Ninety DH lines derived from the above mentioned cross along with their parents were sown in multipot trays containing T-soil (Fruhstorfer Erde; pH 5.9) with each three replicates, and allowed to grow for 3 weeks. For vernalisation, seedlings were then transferred to 4°C temperature with 8 h light for 8 weeks. Following vernalization plantlets were transferred to 9 cm diameter pots containing normal compost soil. Greenhouse experiment was conducted following a randomized complete block design with three replicates for each DH-line and parents. Each steel bench inside the greenhouse represented a complete block consisting of all genotypes (DH and parents). Plantlets were allowed to grow in the greenhouse providing 16 h day-light by using additional 400 W sodium-steam lamp. Temperature varied during the day from 20 to 25°C and during the night from 10 to 15°C, respectively. Hakaphos fertilizer containing N:P:K 15:11:15 + 0.2% trace minerals was added at fortnight on the top soil (100 mg) of each pot until maturation of the plants. Insecticide was applied when aphid and thrips attack was recognized. Sulfur vapor supply was constant during the whole experiment to avoid fungal diseases. Self-fertilization was imposed to the plants by covering the flowers of the main raceme with crisp-plastic bags. Selfed-seeds were harvested from main raceme only, when they were mature.

Data collection

Seed filling period

Begin of flowering (first open flower) and maturity (most of siliques turned to brown) was scored and from these data seed filling period (days) was calculated for each individual plant.

Seed quality analysis

Seed samples (150 mg) from each individual replicate were analyzed for trierucoylglycerol content (C_{69}; EEE = trierucin) by high temperature gas liquid chromatography analysis (HT-GLC) according to the method described in Möllers et al. (1997). Following trierucin analysis remaining parts of the oil samples were transferred to a new tube and left over night on a hot plate at 37.5°C to evaporate the solvent. Fatty acids composition were analyzed by GLC according to Rücker and Röbbelen (1996). Trierucin and fatty acids are expressed as % of the sum of all triglycerides and fatty acids, respectively. The following fatty acids were determined: palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), linolenic acid (18:3), eicosenoic acid (20:1), erucic acid (22:1) and nervonic acid (24:1). Saturated fatty acid content (SFA) was calculated as the sum of 16:0 + 18:0, polyunsaturated fatty acids content (PUFA) was calculated as the sum of 18:2 + 18:3 and monounsaturated fatty acid content (MUFA) was calculated as the sum of 18:1 + 20:1 + 22:1 + 24:1. Seed oil and protein content was determined by near-infrared-reflectance spectroscopy (NIRS) using the equation raps2001.eqa (Tillmann 2007) and are expressed on seed dry matter basis.

DNA isolation and PCR amplification of the transgene Ld-LPAAT

Hundred milligram fresh leaf sample was quickly frozen in liquid nitrogen. DNA was isolated following a modified protocol of Ishizawa et al. (1991). To have a positive control for the presence of DNA in the PCR mix, a locus-specific primer pair for the *fad*2 gene of the *Brassica napus* A-genome (*B. rapa*) was used (Spiekermann 2005). Multiplex-PCR for the *fad*2 and the *Ld-LPAAT* gene was performed as described in Nath et al. (2007). To the PCR reaction products, 5 µl of loading buffer was added and run on a 1.5% agarose gel containing TE buffer. After electrophoresis at 90 V for 5 h the gel was stained with ethidium bromide for 20 min and subsequently washed in a water bath for 20 min and photographed on UV trans-illuminator to detect DNA bands.

Development of fae1 locus and allele specific PCR markers

Previously published sequences of the *fae*1.1 promoter (AF275254.1) and coding sequence (AF274750.1) of the winter rapeseed cultivar Askari (*B. napus*; Han et al. 2001) were used to develop locus and allele specific PCR Primers. PCR primers were picked using Primer3 software at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi (Rozen and Skaletsky 2000). The *fae*1 specific PCR primers (Primers No. 19 and 20; Table 1) were used to amplify part of the *fae*1 3′ promoter region and 5′ coding sequence from a genetically diverse collection of *B. rapa* and *B. oleracea* genotypes (Table 2). Amplicons were sequenced on a capillary electrophoresis ABI 3100 following a standard protocol. Multiple sequences were aligned using the ClustalW 1.8 program at http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html. Sequencing of the PCR products from −91 to 690 bp revealed few differences among the *B. rapa* and the *B. oleracea* genotypes (data not shown). Consistent differences between the *B. oleracea* and *B. rapa* sequences were only found at 63 and 72 bp (Table 2). These differences were used to design locus specific forward and corresponding reverse primers (Primers No. 24/25 and No. 26/27; Table 1). Using the *Brassica* collection shown in Table 2, the primers proved to be species specific (data not shown). The locus specific primers were

Table 1 Locus and allele specific *fae*1 PCR primers

No.	Forward primer 5′→3′	No.	Reverse primer 5′→3′	*T* (°C)	Size	Description (forward/reverse)
19	GGCACCTTTCATCGGACTAC	20	CGTTCGAACCGTGTGAACTA	60.0	1,070	*fae*1 promotor/*fae*1 coding sequence
24	TCAACCTTTGCTTCTTTCCG	25	GAGAAACATCGTAGCCATCAAA	55.0	1,537	*fae*1.1 locus specific/*fae*1 coding sequence
26	TCAACCTTTGTTTCTTTCCA	27	AGAACACCATTGCATTCTTT	50.0	1,572	*fae*1.2 locus specific/*fae*1 coding sequence
43	TGTTTCATATATTTAGCCCTGTTCA	44	ATCGCCGTTAACGGAAAGAAG	56.5	529	*fae*1 promotor/*fae*1.1 locus specific
43	TGTTTCATATATTTAGCCCTGTTCA	45	TCGCCGTTAATGGAAAGAAA	56.5	528	*fae*1 promotor/*fae*1.2 locus specific
46	GAGACAGAAATCTAGACTCTTTATTTG	44	ATCGCCGTTAACGGAAAGAAG	61.0	432	*fae*1.1 6575-1 HELP specific/*fae*1.1 locus specific

Table 2 Consistent differences between *fae*1 sequences of different *B. rapa* and *B. oleracea* genotypes and the *fae*1.1 sequence of *B. napus* cv. Askari (AF AF274750.1)

	1 ... 46	61	76	90
Br1 Opava (winter turnip)	ATG ...	AACCTTTTCAACCTT	TGCTTCTTTCCGTTA	ACGGCGATCGTCGCC
Br2 Yellow Sarson China Fu**	ATG ...	AACCTTTTCAACCTT	TGCTTCTTTCCGTTA	ACGGCGATCGTCGCC
Br3 Chinese cabbage cv. Storkin*	ATG ...	AACCTTTTCAACCTT	TGCTTCTTTCCGTTA	ACGGCGATCGTCGCC
Br4 ssp. *pekinensis* China Fu**	ATG ...	AACCTTTTCAACCTT	TGCTTCTTTCCGTTA	ACGGCGATCGTCGCC
Br5 Arktus (winter turnip)	ATG ...	AACCTTTTCAACCTT	TGCTTCTTTCCGTTA	ACGGCGATCGTCGCC
Br6 Yellow Sarson CR2232, IPKG	ATG ...	AACCTTTTCAACCTT	TGCTTCTTTCCGTTA	ACGGCGATCGTCGCC
Br7 Perko (winter turnip, tetraploid)	ATG ...	AACCTTTTCAACCTT	TGCTTCTTTCCGTTA	ACGGCGATCGTCGCC
Br8 Yellow Sarson S1 cv. Sampad§	ATG ...	AACCTTTTCAACCTT	TGCTTCTTTCCGTTA	ACGGCGATCGTCGCC
Br9 Brown Sarson CR2347-99a, IPKG	ATG ...	AACCTTTTCAACCTT	TGCTTCTTTCCGTTA	ACGGCGATCGTCGCC
Bn1 Askari *fae*1.1 (AF274750.1)	ATG ...	AACCTTTTCAACCTT	TGCTTCTTTCCGTTA	ACGGCGATCGTCGCC
Bo1 Green cabbage cv. Rustico*	ATG ...	AACCTTTTCAACCTT	TGTTTCTTTCCATTA	ACGGCGATCGTCGCC
Bo2 Brussels sprouts cv. Roger*	ATG ...	AACCTTTTCAACCTT	TGTTTCTTTCCATTA	ACGGCGATCGTCGCC
Bo3 Cauliflower cv. Alverda*	ATG ...	AACCTTTTCAACCTT	TGTTTCTTTCCATTA	ACGGCGATCGTCGCC
Bo4 Savoy cabbage cv. Salarite*	ATG ...	AACCTTTTCAACCTT	TGTTTCTTTCCATTA	ACGGCGATCGTCGCC
Bo5 Broccoli cv. Montop*	ATG ...	AACCTTTTCAACCTT	TGTTTCTTTCCATTA	ACGGCGATCGTCGCC
Bo6 White cabbage cv. Stardon*	ATG ...	AANCTTTTCAACCTT	TGTTTCTTTCCATTA	ACGGCGATCGTCGCC
Bo7 Stem cabbage cv. Express Forcer*	ATG ...	AACCTTTTCAACCTT	TGTTTCTTTCCATTA	ACGGCGATCGTCGCC

Br = *Brassica rapa*, Bn = *Brassica napus*, Bo = *Brassica oleracea*, IPKG = accessions obtained from the genebank IPK Gatersleben

Template for *fae*1.1 locus specific reverse PCR primer: CTTCTTTCCGTTAACGGCGAT (Primer no. 44; Table 1)

Template for *fae*1.2 locus specific reverse PCR primer: TTTCTTTCCATTAACGGCGA (Primer no. 45; Table 1)

Br1, Br5, Br7: *B. rapa* subsp. *oleifera*, Br2, Br6, Br8: *B. rapa* var. *trilocularis*, Br3: *B. rapa* var. *pekinensis*, Br9: *B. rapa* subsp. *sarson*

Bo1: *Brassica oleracea* subsp. *acephala* Bo2: *Brassica oleracea* subsp. *capitata* var *gemmifera*, Bo3: *B. oleracea* subsp. *botrytis*, Bo4: *B. oleracea* var. *sabauda*, Bo5: *B. oleracea* subsp. *botrytis* var. *cymosa*, Bo6: *B. oleracea* subsp. *capitata*, Bo7: *B. oleracea* subsp. *gongyloides*

* Seeds were obtained from Syngenta Seeds, Kleve, Germany; ** seeds were obtained from Prof. Fu, Huazhong Agricultural University, Wuhan, China § seeds were obtained from Dr. Rahman, Danisco, DK

used to amplify and sequence the *fae*1.1 and the *fae*1.2 alleles from the *B. napus* genotypes TNKAT and 6575-1 HELP. Downstream sequencing did not reveal differences between the TNKAT and 6575-1 HELP sequences (data not shown). Hence, sequence differences at 63 bp (from ATG) and 72 bp were used to design locus specific reverse primers which were used in combination with a forward primer located in the *fae*1 5′ promoter region (Primers No. 43/44 for *fae*1.1 and No. 43/45 for *fae*1.2). *Fae*1.2 sequences of TNKAT and 6575-1 HELP did not show any differences (data not shown). However, *fae*1.1 sequences of TNKAT and 6575-1 HELP showed at two positions single nucleotide polymorphisms (Table 3). The first polymorphism (G for 6575-1 HELP, C for TNKAT) was used to design primer No. 46 which in combination with primer 44 proved to specifically amplify the 6575-1 HELP *fae*1.1 allele. No amplification occurred with DNA from cauliflower cv. Super Regama (C-genome donor of TNKAT; seeds were obtained from gene bank Gatersleben, Accession number BRA 1381) nor with TNKAT. Multiplex-PCR was performed with DNA extracted from the 90 DH lines including primers no. 46/44 and *fad*2 specific primers as described above and in Nath et al. (2007) using annealing temperature at 61°C for 60 s.

Statistical analysis

Analysis of variance (ANOVA), calculation of heritability and Spearman's rank correlation coefficients were performed using the Plant Breeding Statistical Program (PLABSTAT, Version 2N, Utz 2007). In the ANOVA, the replicates were considered as random variable. Multiple mean comparisons were made with Fisher's least significant difference (LSD) procedure using StatGraphics Plus for Windows 3.0 (Statistical Graphics Corp., Rockville, USA). The number of transgene copy segregating in the DH population was

Table 3 Sequences and sequence difference between *fae*1.1 (A-genome) of 6575-1 HELP and TNKAT

6575-1 HELP Primer 43/44

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CGCATTTTTATTTAAAATTTGTAAACTTTTTTGGTCAAAGAACATTTTGTAATTAGAGACAGAAATCTAGACTCT
    -323
TTATTTGGAATAATAATAATAAAGAAGATATTTTGGGCAATGAATTTATGATGTTATGTTTATATAGTTTATTTC
ATTTTAAATTGAAAAGCATTATTTTTATCGAAATGAATCTAGTATACAATATATTTGTTTTTTTTCATCAGATACT
TTCCTATTTTTTGGCACCTTTCATCGGACTACTGATTTATTTCAATGTGTATGCATGCATGAGCATGAGTATACA
CATGTCTTTAAAAATGCATGTAAAGTGTAACGGACCACAAAAGAGGATCCATACAAATACATCTCATCGCTTCCA
                                1
TTACTATTCTCCGACACACACACTGAGCAATGACGTCCGTTAACGTAAAGCTCCTTTACCATTACGTCATAACCA
ACCTTTTCAACCTTTGCTTCTTTCCGTTAACGGCGAT
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TNKAT Primer 43/44

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CTTTTTATTTAAAATTTGTAAACTTTTTTGGTCAAAGAACATTTTGTAATTAGAGACAGAAATCTAGACTCTTTA
  -323
TTTCGAATAATAATAATAAAGAAGATATTTTGGGCAATGAATTTATGATGTTATGTTTATATAGTTTATTTCATT
TTAAATTGAAAAGCATTATTTTTATCGAAATGAATCTAGTATACAATATATATGTTTTTTTTCATCAGATACTTTC
CTATTTTTTGGCACCTTTCATCGGACTACTGATTTATTTCAATGTGTATGCATGCATGAGCATGAGTATACACAT
GTCTTTAAAAATGCATGTAAAGTGTAACGGACCACAAAAGAGGATCCATACAAATACATCTCATCGCTTCCACTA
                              1
CTATTCTCCGACACACACACTGAGCAATGACGTCCGTTAACGTAAAGCTCCTTTACCATTACGTCATAACCAACC
TTTTCAACCTTTGCTTCTTTCCGTTAACGGCGAT
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Position of Forward Primer 46: GAGACAGAAATCTAGACTCTTTATTTG
Position of Reverse Primer 44: CTTCTTTCCGTTAACGGCGAT
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calculated using χ^2 test for a fixed ratio hypothesis described by Gomez and Gomez (1976).

The effect of transgene and non-transgene, low and high PUFA and *fae*1.1 genes of 6575-1 HELP on different traits was analyzed by LSD (least significant difference) by comparing two pair of treatment means considering different number of observations of the treatments following the formula proposed by Gomez and Gomez (1976):

$$\text{LSD} = t_{(0.05)} \times \sqrt{s^2\left(1/ri + 1/rj\right)}$$

where LSD is the least significant difference of the two treatments at 0.05 probability, $t_{(0.05)}$ is the tabular value of t at 0.05 probability and with error degree of freedom, s^2 is the error mean square from the analysis of variance, *ri* and *rj* are the number of observations of *i*-th and *j*-th treatments, respectively.

Direct and indirect path coefficients of the path coefficient analysis were calculated as described in Lynch and Walsh (1998):

$$r_{yi} = P_{yi} + \sum_{\substack{i'=1 \\ i' \neq i}}^{k} r_{ii'} P_{yi'} \quad \text{for } i \neq 1$$

where r_{yi} is the correlation coefficient between the *i*-th causal variable (X_i) and effect variable (y), $r_{ii'}$ is the correlation coefficient between the *i*-th and i'-th causal variables, P_{yi} is the path coefficient (direct effect) of the *i*-th causal variable (X_i), $r_{ii'}P_{yi'}$ is the indirect effect of the *i*-th causal variable via the i'-th causal variable. To determine the direct effect, square matrices of the correlation coefficients between independent traits in all possible pairs were inverted and multiplied by the correlation coefficients between the independent and dependent traits. Path coefficient analysis was performed for erucic acid content as effect variables and SFA, 18:1, PUFA, 20:1 and trierucin content were considered as causal variables.

Results

Phenotypic variation among DH lines

In the greenhouse experiment, the parental line 6575-1 HELP had a higher erucic acid and oleic acid content and a lower polyunsaturated fatty acid (PUFA) content compared to the parent TNKAT (Table 4). The DH population showed highly significant differences among the DH lines for all recorded traits. Large

differences were found for seed filling period, oil and protein content as well as for the fatty acid composition of the seed oil (Table 4). Erucic acid was the most prominent fatty acid, accounting for 47.1% of the total fatty acid content. It ranged from 34.6% to 59.1% and showed large transgression compared to the higher parent. Variance components for the effect of genotypes were in general large compared to the effect of error. Heritabilities were rather high for all traits, ranging from 0.79 to 0.96. The frequency distribution of erucic acid content was continuous, but appeared to involve two phenotypic classes (Fig. 1). Separating the classes at 49% erucic acid, χ^2 test showed that the segregation pattern was consistent with 1:1 hypothesis ($\chi^2_{(0.05)} = 1.6$ NS), indicating that this trait may be controlled by alleles at one locus.

Table 4 Means, ranges, variance components and heritabilities (h^2) of seed filling period (days), oil, protein and different fatty acids content (%) of 90 DH lines derived from the cross between TNKAT × 6575-1 HELP, along with parental lines

Item	Seed filling period	Oil content	Protein content	Fatty acid content (%)						
				SFA	18:1	PUFA	20:1	22:1	24:1	MUFA
Mean	67	40.5	29.6	4.0	19.7	15.9	9.9	47.1	1.2	76.7
Minimum	50	28.3	20.6	2.4	9.1	5.6	3.9	34.6	0.2	62.0
Maximum	96	50.7	37.5	5.9	36.5	27.9	18.9	59.1	2.3	88.1
TNKAT	75.0	40.8	30.9	4.1	15.9	20.8	10.0	46.1	1.1	73.1
HELP	62.0	46.4	25.1	3.8	26.8	8.4	7.9	50.4	0.8	85.9
σ^2_G	38.2**	12.5**	5.9**	0.4**	17.9**	22.8**	5.9**	19.2**	0.05**	27.8**
σ^2_R	2.7	0.12	0.1	0.0	0.1	0.2	0.0	0.1	0.0	0.1
σ^2_E	16.4	7.7	3.7	0.23	4.9	2.2	1.9	7.5	0.04	3.2
h^2	0.87	0.82	0.82	0.83	0.91	0.96	0.90	0.88	0.79	0.96

SFA = 16:0 + 18:0; PUFA = 18:2 + 18:3; MUFA = 18:1 + 20:1 + 22:1 + 24:1

σ^2_G genetic variance, σ^2_R variance for replication, σ^2_E error variance and h^2 heritability

** Significant at $P = 0.01$, F-test in analysis of variance

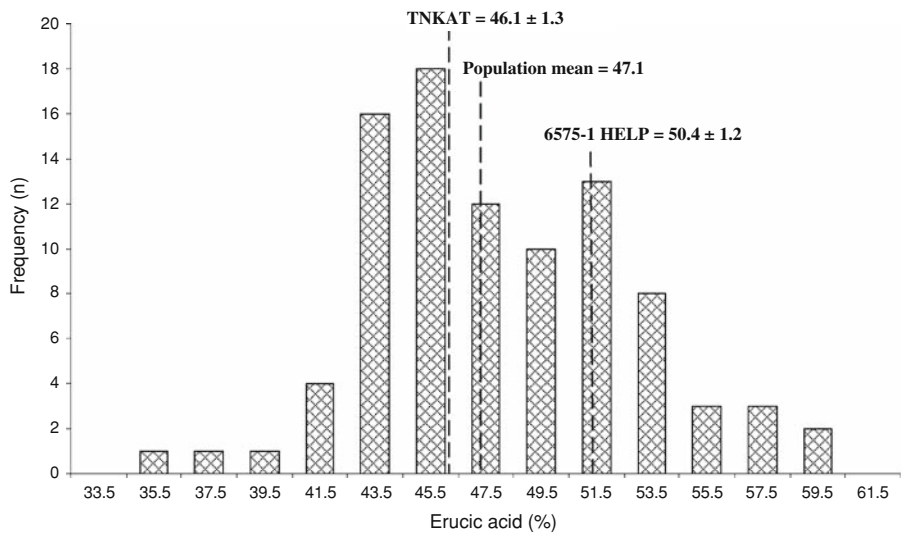

Fig. 1 Distribution of erucic acid content in the DH population derived from a cross between transgenic high erucic acid (TNKAT) and non-transgenic (6575-1 HELP) rapeseed lines in comparison to parents. Parental values are the mean ± standard deviation

Effect of the *Ld-LPAAT* + *Bn-fae*1 transgene on erucic acid content and other traits

Based on the presence or absence of the transgenes as confirmed by PCR amplification of the *Ld-LPAAT* gene and by estimating trierucin content, the DH lines were subdivided into a non-transgenic and a transgenic group (Table 5). Fifty four DH lines proved to be *Ld-LPAAT* positive, the remaining 36 were negative in PCR and trierucin analysis. χ^2 test confirmed 1:1 segregation for a single transgene copy in the DH population ($\chi^2_{(0.05)} = 3.6$ NS). The effect of the transgenes was investigated by comparing the means of the two groups. Results showed that the transgenic group had a by 2.3% significant lower erucic acid content. However, among others this group also had a significantly lower oleic acid and a significantly higher PUFA and eicosenoic acid content. The *Ld-LPAAT* positive group contained 3.4% trierucin, whereas no trierucin was detected in the *Ld-LPAAT* negative group.

Effect of PUFA content on erucic acid content and other traits

To study the effect of a genetically modified PUFA content on erucic acid content and on other traits, the DH population was subdivided into two classes based on the mean PUFA content (15.9%; Table 4). Forty nine DH lines had an above average PUFA content (mean 19.5%), the remaining 41 ones a below average content (mean 11.4%; Table 6). The low PUFA class had a by 3.7% higher erucic acid content than the high PUFA class. Significant positive and negative effects of PUFA content on other traits were also observed (Table 6). Regression analysis between PUFA and erucic acid content ($y = -0.42x + 53.69$) revealed that a reduction of 10% in PUFA content led

Table 5 Comparison between two groups of DH-lines for different traits with segregation pattern of transgene in the DH-population derived from the cross TNKAT × 6575-1 HELP

Line	*n*	Mean value of the traits										
		Seed filling period	Oil content	Protein content	SFA	18:1	PUFA	20:1	22:1	24:1	MUFA	Trierucin
Ld-LPAAT positive	54	67	39.8	29.7	4.1	18.2	17.1	11.0	46.2	1.2	76.6	3.4
Ld-LPAAT negative	36	69	41.6	29.4	3.9	21.9	14.1	8.3	48.5	1.2	79.9	0.0
Mean difference		−2*	−1.8*	0.3	0.2	−3.7*	3.0*	2.8*	−2.3*	0.0	−3.3*	3.4*
LSD (0.05)		1.7	1.2	0.8	0.2	0.9	0.6	0.6	1.2	0.1	0.8	1.8

SFA = 16:0 + 18:0; PUFA = 18:2 + 18:3; MUFA = 18:1 + 20:1 + 22:1 + 24:1

* Significant at $P = 0.05$

Table 6 Comparison between low (≤15.9%) and high (>15.9%) PUFA content of DH-lines for different traits in the DH-population derived from the cross between TNKAT × 6575-1 HELP

Line	*n*	Mean value of the traits									
		Seed filling period	Oil content	Protein content	SFA	18:1	PUFA	20:1	22:1	24:1	MUFA
DH with low PUFA	41	67.4	41.7	28.7	3.6	22.8	11.4	9.7	49.2	1.2	82.9
DH with high PUFA	49	68.0	39.5	30.3	4.3	17.2	19.5	10.1	45.5	1.2	73.9
Mean difference		−0.6	2.2*	−1.6*	−0.7*	5.6*	−8.1*	−0.4	3.7*	0.0	9.0
LSD (0.05)		1.7	1.2	0.8	0.2	0.9	0.6	0.6	1.1	0.1	0.8

SFA = 16:0 + 18:0; PUFA = 18:2 + 18:3; MUFA = 18:1 + 20:1 + 22:1 + 24:1

* Significant at $P = 0.05$

to a 4.2% increase in erucic acid content in the DH population.

Effect of the *fae*1.1 allele from TNKAT and 6575-1 HELP on erucic acid content and other traits

Differences in erucic acid content between the parental lines 6575-1 HELP and TNKAT and among the DH lines may be as well due to differences in the efficiency of the endogenous *fae*1 alleles at the erucic acid loci *fae*1.1 (*B. rapa*) and *fae*1.2 (*B. oleracea*). To address this, *fae*1.1 and *fae*1.2 locus specific primers were developed and they proved to be species specific using a genetic divergent collection of *B. rapa* and *B. oleracea* genotypes (Tables 1 and 2). However, using those primers for sequencing the *fae*1.1 and *fae*1.2 alleles of the parental lines revealed single nucleotide polymorphism only for the alleles of the *fae*1.1 locus (Table 3). 6575-1 HELP *fae*1.1 allele specific primers (Tables 1 and 3) allowed following the segregation of this allele in the DH population (for results see example in Fig. 2). Among the DH population 54 lines had the *fae*1.1 allele from 6575-1 HELP and 36 DH lines carried the *fae*1.1 allele from TNKAT. However, only a non-significant difference of 1% erucic acid content was found between these two groups (Table 7).

Correlations between traits and path coefficient analysis in the DH population

Spearman's rank correlation coefficients analysis showed strong negative correlations between erucic acid and SFA ($r_s = -0.64$**), PUFA ($r_s = -0.40$**) and eicosenoic acid content ($r_s = -0.71$**; Table 8). Oil content was significant positive correlated with oleic acid, eicosenoic acid and MUFA and negatively correlated with protein, SFA, PUFA and trierucin content. Only a low positive correlation of erucic acid and trierucin content was found for the whole population. However, the *Ld-LPAAT* transgenic DH plants formed a distinct trierucin containing group and within this group there was a close correlation between erucic acid and trierucin content ($r_s = 0.84$**). Although oleic acid as oleoyl-CoA is the primary precursor for erucic acid biosynthesis, only a very weak negative correlation between the two traits were observed ($r_s = -0.11$). The question arose whether erucic content is directly affected by oleic acid or other fatty acids, or indirectly via other fatty acids, which showed strong correlations with oleic acid content. Therefore, path coefficient analysis was used to partition the correlations into direct and indirect effects, considering oil content and those fatty acids as causal variables, which are directly involved in erucic acid biosynthesis (Table 9). Path coefficient analysis showed strong direct negative effects of oleic acid (−0.59), PUFA (−0.79) and eicosenoic acid content (−0.65) on erucic acid content. There were only negligible direct effects of oil (0.13) and trierucin content (0.08) on erucic acid content. There were indirect effects on erucic acid content via other fatty acids, especially in case of oleic acid via PUFA content and PUFA and trierucin via oleic acid content (Table 9).

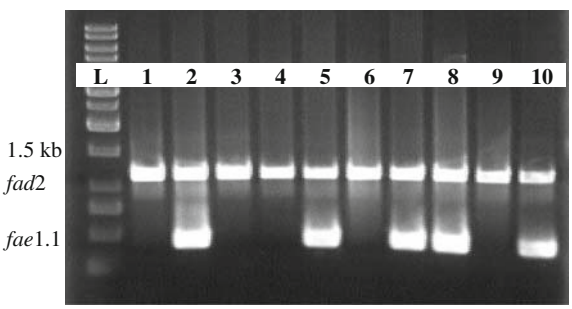

Fig. 2 Multiplex-PCR of a 432 bp fragment of the *fae*1.1 gene together with internal control amplification of the 1.1 kb *fad*2 gene sequence using DNA extracted from 8 DH plants segregating for the *fae*1.1 gene of 6575-1 HELP from A-genome of *B. rapa* (Lanes 3-10 DH, lanes 1 and 2 are TNKAT and 6575-1 HELP as control)

Characterization of the DH lines with the highest erucic acid content

Some of the DH lines had considerable higher erucic acid contents in the seed oil compared to both parental lines (cf. Fig. 1). Multiple mean comparisons between three DH lines with the highest erucic acid contents and the parental lines confirmed significant differences (Table 10). DH line IV-10-F-6 contained with 59.1% the highest amount of erucic acid, which is about 9% more than parent 6575-1 HELP. In this line trierucin content was also significantly higher compared to parental line TNKAT. In two of the three DH lines PUFA content was as low and MUFA content as high as in parent 6575-1 HELP. Significant differences between the DH lines and the parents were also found for the other traits, e.g. oil and protein content and seed filling period.

Table 7 Effect of fatty acid elongase (*fae*1.1) gene from different sources on different traits in the DH population derived from the cross between TNKAT × 6575-1 HELP

Line	*n*	Mean value of the traits									
		Seed filling period	Oil content	Protein content	SFA	18:1	PUFA	20:1	22:1	24:1	MUFA
DH with *fae*1.1 allele of HELP	54	67.6	41.0	29.2	4.0	20.0	15.3	9.9	47.5	1.2	78.6
DH with *fae*1.1 allele of TNKAT	36	68.1	39.8	30.0	4.1	19.2	16.8	10.0	46.5	1.1	76.9
Mean difference		−0.5	1.2	−0.8	−0.1	0.8	−1.5*	−0.6	1.0	0.1	1.7*
LSD (0.05)		1.7	1.2	0.8	0.2	0.9	0.6	0.6	1.2	0.1	0.8

SFA = 16:0 + 18:0; PUFA = 18:2 + 18:3; MUFA = 18:1 + 20:1 + 22:1 + 24:1

* Significant at $P = 0.05$

Table 8 Spearman's rank correlation coefficients (r_s) among different traits in the DH population derived from the cross between TNKAT × 6575-1 HELP ($n = 90$)

Traits	Seed filling period	Oil content	Protein content	SFA	18:1	PUFA	20:1	22:1	Trierucin[a]	24:1
Oil	−0.07									
Protein	0.09	−0.78**								
SFA	0.04	−0.33**	0.36**							
18:1	−0.11	0.35**	−0.28**	−0.34**						
PUFA	0.11	−0.43**	0.45**	0.69**	−0.76**					
20:1	−0.09	0.27*	−0.29**	0.26*	0.15	0.05				
22:1	−0.03	0.04	−0.09	−0.64**	−0.11	−0.40**	−0.71**			
Trierucin[a]	−0.11	−0.27*	0.04	0.03	−0.41**	0.15	0.27**	0.06		
24:1	0.16	−0.20	0.15	−0.27**	−0.23*	−0.02	−0.52**	0.47**	0.02	
MUFA	−0.10	0.45**	−0.47**	−0.75**	0.74**	−0.99**	−0.05	0.43**	−0.14	0.03

SFA = 16:0 + 18:0; PUFA = 18:2 + 18:3; MUFA = 18:1 + 20:1 + 22:1 + 24:1

*, ** Significant at $P = 0.05$ and $P = 0.01$, respectively

[a] Correlations are calculated considering complete population

Table 9 Direct and indirect effects of different fatty acids and oil content on erucic acid content in the DH population derived from a cross between TNKAT × 6575-1 HELP

Trait	Indirect effect on erucic acid via					Direct effect on erucic acid
	Oil	18:1	PUFA	20:1	Trierucin	
Oil	–	−0.21	0.34	−0.18	−0.04	0.13
18:1	0.04	–	0.60	−0.10	−0.06	−0.59
PUFA	−0.05	0.45	–	−0.03	0.02	−0.79
20:1	0.03	−0.09	−0.04	–	0.04	−0.65
Trierucin	−0.03	0.24	−0.12	−0.18	–	0.15
Residual effect = 0.34						

Discussion

In F_2 populations of rapeseed (*Brassica napus* L.) derived from crosses between genotypes with high and low erucic acid contents, erucic acid content has been shown to be inherited by two major genes that act in an additive manner (Harvey and Downey 1964; Kondra and Stefansson 1965; Jönsson 1977). In the present study, two high erucic acid lines were crossed and in the DH population derived from F_1 plants a

Table 10 Mean comparisons among parents and the three DH lines selected for high erucic acid content from DH population derived from the cross TNKAT × 6575-1 HELP for different traits after multiple range test

Line	Seed filling period	Oil content	protein content	Fatty acid composition (%)							
				SFA	18:1	PUFA	20:1	22:1	24:1	MUFA	Trierucin
TNKAT	75.0 b	40.8 b	30.9 a	4.1 a	15.9 d	20.8 a	10.0 a	46.1 d	1.1 bc	73.1 c	6.1 b
HELP	62.0 c	46.4 a	25.1 b	3.8 b	26.8 a	8.4 cd	7.9 b	50.4 c	0.8 c	85.9 a	–
IV-10-F-6	84.0 a	36.6 b	31.3 a	2.6 e	17.5 c	9.1 c	7.8 b	59.1 a	1.9 a	86.3 a	8.0 a
XI-10-D-6	77.0 b	42.2 a	27.7 b	3.4 c	14.5 d	12.8 b	7.9 b	57.6 ab	1.4 b	81.4 b	5.7 b
IX-10-C-8	63.0 c	43.6 a	26.8 b	3.1 d	23.5 b	7.0 d	6.4 c	56.6 b	1.4 b	87.9 a	3.9 b
$LSD_{(0.05)}$	6.5	4.4	3.1	0.7	3.5	2.3	2.2	4.4	0.3	2.8	1.8

SFA = 16:0 + 18:0; PUFA = 18:2 + 18:3; MUFA = 18:1 + 20:1 + 22:1 + 24:1

Mean values with different letters indicate significant differences at $P = 0.05$ (Fisher's LSD)

large transgressive quantitative variation was found for erucic acid content which ranged from 34.6 to 59.1% (Table 4; Fig. 1). This quantitative variation may be explained by the segregation of the *Ld-LPAAT* + *Bn-fae*1 single copy transgene and by the segregation of one major *fad*2 mutant gene plus additional minor genes affecting oleic acid desaturation to linoleic acid. In addition, differently effective alleles at the two endogenous erucic acid loci (*fae*1.1 and *fae*1.2), other yet unknown factors and environmental effects may have contributed to the observed variation in erucic acid content. Using a DH population derived from a cross between two high erucic acid genotypes, Zhao et al. (2008) also found a quantitative variation in erucic acid content and identified eight quantitative trait loci (QTL). Only one of the QTL for erucic acid content was probably caused by segregation at one of the two erucic acid loci (*fae*1), whereas the remaining seven QTL represent other, yet unknown factors influencing erucic acid content in the seed oil.

Although there was a quantitative variation in erucic acid content in the DH population, there was some indication for the presence of two classes, suggesting an underlying 1:1 segregation of a single gene (Fig. 1). For the *Ld-LPAAT* + *Bn-fae*1 transgene the presence of a single copy was confirmed, but the subgroup carrying the transgene in the homozygous form surprisingly did show a by 2.3% significantly reduced erucic acid content compared to the non-transgenic subgroup of DH lines (Table 5). The reduction in erucic acid content was, however, counterbalanced by an increase in eicosenoic acid content. Furthermore, the two subgroups differed in PUFA content, which also influenced erucic acid content (Table 6). Nevertheless, performing a multiple linear regression analysis considering erucic acid content as dependent variable and PUFA content, presence of the *Ld-LPAAT* gene and of the *fae*1.1 6575-1 HELP allele as independent variables confirmed the negative effect of the *Ld-LPAAT* gene on erucic acid content (−1.1% erucic acid), which was, however, not significant (data not shown). The presence of trierucin in the seed oil proved functioning of the *Ld-LPAAT* transgene (Table 5). Weier et al. (1997) also reported non-significant change of erucic acid content following expression of only the *Ld-LPAAT* gene in transgenic rapeseed. Therefore, it seems that the fatty acid elongase (*fae*1) gene in the chimeric construct may not be functional or that this step is not limiting erucic acid biosynthesis is this cross. This hypothesis is controversial to the observation of Han et al. (2001), who reported 30% erucic acid content in low erucic acid cultivar Drakkar following transformation with the same *Ld-LPAAT* + *Bn-fae*1 transgene construct as present in the TNKAT line used in this study. However, Han et al. (2001) also reported a very minor increase in erucic acid content in resynthesised rapeseed line 'RS306' following transformation with the same *Ld-LPAAT* + *Bn-fae*1 transgene construct. Even though the three DH lines with the highest erucic acid contents were transgenic, it seems that the *Bn-fae*1 transgene in the chimeric construct is not capable of increasing erucic acid content in the present material.

Comparing the two groups of DH lines with below average and above average PUFA content (Table 6) as well as the negative correlation between erucic acid and PUFA content (Table 8) showed that reduced PUFA contents leads to increased erucic acid contents. According to the regression, a 10% decrease in PUFA content

leads to a 4.2% increase in erucic acid content. This confirms the initial hypothesis that erucic acid content can be increased by genetically lowering PUFA content.

It was further hypothesized that the quantitative variation in erucic acid content in the DH population may have been caused by differently effective alleles at the two endogenous *fae*1 loci in rapeseed. The development of locus and subsequently allele-specific PCR primers allowed following the segregation of the *fae*1.1 allele from 6575-1 HELP parent (Tables 1–3; Fig. 2). However, presence of the *fae*1.1 allele from 6575-1 HELP instead of the one from TNKAT resulted only in a non-significant increase of 1% erucic acid (Table 7). Although almost the complete coding and the promoter sequence of the *fae*1.2 locus were analyzed, no differences were found between the TNKAT and 6575-1 HELP alleles. Therefore, at present, there is no evidence that the *fae*1.2 alleles of the two parents have different effects on erucic acid content. The *fae*1.1 and *fae*1.2 locus specific PCR primers (forward and reverse) developed in the present study proved to be species specific using a collection of genetically diverse *B. rapa* and *B. oleracea* genotypes. Those primers should be useful in future studies aimed at genetic mapping the *fae*1 genes or exploring the allelic diversity at these two loci (marker assisted selection, TILLING and Eco-TILLING; Gilchrist and Haughn 2005).

Closest negative correlations were found between erucic acid content and eicosenoic acid ($r_s = -0.71$), SFA ($r_s = -0.64$) and PUFA content ($r_s = -0.41$; Table 8). The close negative correlation between erucic acid and SFA content is not directly understandable, but has been reported before (Sasongko and Möllers 2005; Zhao et al. 2008) and a hypothesis to explain the effect has been developed earlier (Möllers and Schierholt 2002). Although oleic acid (18:1) is the primary precursor for erucic acid biosynthesis, only a low non-significant negative correlation between these two traits were found ($r_s = -0.11$). This may be explained by the intermediate position of oleoyl-CoA in elongation, desaturation and incorporation into storage lipids. However, results from path coefficient analysis (Table 9) confirmed the strong negative direct effect of oleic acid on erucic acid content.

The three best DH lines had between 6 and 9% more erucic acid in the seed oil than parental line 6575-1 HELP (Table 10). 6575-1 HELP has been tested earlier in field experiments and was found to have an equivalent amount of erucic acid in the seed oil as in the present greenhouse experiments, i.e. around 50% erucic acid (Sasongko and Möllers 2005). Hence, it seems likely that the 57–59% erucic acid content found for the three DH lines may also be reproducible in field experiments. Since the difference in PUFA content between 6575-1 HELP and two of the DH lines is negligible, the increase in erucic acid must be due to other yet unknown genetic factors. The allelic composition at the *fae*1.2 locus may be only one of them (cf. Zhao et al. 2008). The trierucin content of the three DH lines was with 4–8% trierucin in the range of that of transgenic parent TNKAT (6% trierucin). Considering the 59% erucic acid in DH line IV-10-F-6 this seems low, because following a random distribution of erucoyl-CoA to the three triacylglycerol positions one would have expected 20% trierucin. The low trierucin content could be indicative for an insufficient *Ld*-LPAAT activity, also considering the presence of competing native *Bn*-LPAAT activity in rapeseed.

Future approaches to increase erucic acid content in rapeseed could include an antisense strategy to reduce the native *Bn*-LPAAT activity. Also, an ectopically inserted functional *fae*1 transgene should boost up erucic acid content in seed oil by efficiently utilizing oleoyl-CoA for elongation, if desaturation to linoleic acid is blocked by mutation or other ways. Highest contents of erucic acid may be achieved through introgression of alleles that further reduce PUFA content in combination with transgenic approaches aimed at over expressing cytosolic acetyl-CoA carboxylase (ACC) and ATP-citrate lyase (Fatland et al. 2005).

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