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ORIGINAL PAPER

Increasing erucic acid content through combination of endogenous low polyunsaturated fatty acids alleles with *Ld-LPAAT* + *Bn-fae*1 transgenes in rapeseed (*Brassica napus* L.)

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Abstract High erucic acid rapeseed (HEAR) oil is of interest for industrial purposes because erucic acid (22:1) and its derivatives are important renewable raw materials for the oleochemical industry. Currently available cultivars contain only about 50% erucic acid in the seed oil. A substantial increase in erucic acid content would significantly reduce processing costs and could increase market prospects of HEAR oil. It has been proposed that erucic acid content in rapeseed is limited because of insufficient fatty acid elongation, lack of insertion of erucic acid into the central *sn*-2 position of the triaclyglycerol backbone and due to competitive desaturation of the precursor oleic acid (18:1) to linoleic acid (18:2). The objective of the present study was to increase erucic content of HEAR winter rapeseed through over expression of the rapeseed fatty acid elongase gene (*fae*1) in combination with expression of the lysophosphatidic acid acyltransferase gene from *Limnanthes douglasii* (*Ld-LPAAT*), which enables insertion of erucic acid into the *sn*-2 glycerol position. Furthermore, mutant alleles for low contents of polyunsaturated fatty acids (18:2 + 18:3) were combined with the transgenic material. Selected transgenic lines showed up to 63% erucic acid in the seed oil in comparison to a mean of 54% erucic acid of segregating non-transgenic HEAR plants. Amongst 220 F_2 plants derived from the cross between a transgenic HEAR line and a non-transgenic HEAR line with a low content of polyunsaturated fatty acids, recombinant F_2 plants were identified with an erucic acid content of up to 72% and a polyunsaturated fatty acid content as low as 6%. Regression analysis revealed that a reduction of 10% in polyunsaturated fatty acids content led to a 6.5% increase in erucic acid content. Results from selected F_2 plants were confirmed in the next generation by analysing F_4 seeds harvested from five F_3 plants per selected F_2 plant. F_3 lines contained up to 72% erucic acid and as little as 4% polyunsaturated fatty acids content in the seed oil. The 72% erucic acid content of rapeseed oil achieved in the present study represents a major breakthrough in breeding high erucic acid rapeseed.

Dedicated to professor Gerhard Röbbelen on the occasion of his 80th birthday.

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Introduction

World vegetable oil markets are highly competitive requiring a steady improvement in oil quality to maintain or increase market shares. Genetic improvement of oilseeds has the objective of increasing oil yields with a uniform fatty acid composition for nutritional, pharmaceutical and industrial purposes (Roscoe 2005). Oil from traditional rapeseed (*Brassica napus* L.) differs significantly from most

other vegetable oils by its high contents of erucic acid (22:1) and eicosenoic acid (20:1; Jönsson 1977; Becker et al. 1999). They are the major very long chain fatty acids (VLCFAs) in the seed oil, together accounting for 45–60% of the total fatty acid mixture. High levels of erucic acid in cooking and salad oil extracted from rapeseed have been associated with health problems (Beare et al. 1963). Following identification of a spontaneous rapeseed mutant with low erucic acid content, genetic studies have shown that this trait is under control of two loci that act in an additive manner (Harvey and Downey 1964; Kondra and Stefansson 1965). Major efforts in the 1960s led to the development of low erucic acid rapeseed (LEAR) varieties. Nowadays, double zero (00) or Canola quality cultivars with a low erucic acid content in the seed oil and a low glucosinolate content in the seeds are predominantly being grown in most parts of the world.

However, High Erucic Acid Rapeseed (HEAR) types retained some importance. HEAR cultivars are presently cultivated to a small extent in Europe (up to 40,000 hectares in 2006/2007) and in USA/Canada as an identity preserved crop (Möllers 2004; Scarth and Tang 2006). Erucic acid and its derivatives are important renewable raw materials used in plastic film, nylon, lubricant, cosmetic and emollient industries (Leonard 1994; Sonntag 1995; Piazza and Foglia 2001). Currently available HEAR cultivars contain only about 50% erucic acid in the seed oil. A substantial increase of the erucic acid content of the rapeseed oil would significantly reduce processing costs and could increase market prospects. However, possibilities to increase erucic acid by classical breeding are limited, because in rapeseed and related *Brassica* species erucic acid is inserted only in the first (*sn*-1) and third (*sn*-3) position of the glycerol backbone. This limits erucic acid content to a maximum of 67%. The reason for this limitation lies in the specificity of the *B. napus sn*-2 acyltransferase (LPAAT—lysophosphatidic acid acyltransferase), which does not accept erucoyl-CoA as a substrate (Cao et al. 1990; Frentzen 1993). To overcome this limitation, the gene for an erucoyl-CoA preferring *sn*-2 acyltransferase was isolated from *Limnanthes* species. Expression of this gene in transgenic rapeseed altered seed oil *sn*-2 proportions of erucic acid, but did not lead to an increase in erucic acid content of the seed oil (Lassner et al. 1995; Brough et al. 1996; Weier et al. 1997).

In a next step, interest focussed on the fatty acid elongation mechanism from oleic acid to erucic acid. This elongation is the result of two cycles of a four-step mechanism, in which first oleoyl-CoA and then eicosenoyl-CoA serve as substrates (Puyaubert et al. 2005). In the first step, the β-ketoacyl-CoA synthase (KCS; *fae*1 gene) catalyses the condensation reaction of oleoyl-CoA or eicosenoyl-CoA with malonyl-CoA. It is believed that this initial reaction is the rate-limiting step of the four-step mechanism (Cassagne et al. 1994). The *fae*1 genes from *Arabidopsis* and from rapeseed were cloned and over expressed under control of a seed specific promoter in transgenic HEAR (Katavic et al. 2001; Han et al. 2001). However, this led only to a minor increase in erucic acid content. Even the combination with the expression of the *Ld-LPAAT* gene from *Limnanthes douglasii* did not result in a substantial increase of the erucic acid content in transgenic HEAR (Han et al. 2001).

There is some evidence that the cytosolic pool of available oleoyl-CoA or malonyl-CoA may limit fatty acid elongation (Bao et al. 1998; Domergue et al. 1999). Crossing conventional HEAR with oilseed rape 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 activity (*fae*1.1 and *fae*1.2 genes) was too low to allow for enhanced erucic acid synthesis.

The first objective of the present study was to repeat the approach of Han et al. (2001) to increase erucic acid content by expressing the *Ld-LPAAT* gene and over expressing the *Bn-fae*1 gene in transgenic HEAR. The second objective was to study in this material, the effect of genetically reduced polyunsaturated fatty acids content on erucic acid content.

Materials and methods

Plant material

6575-1 HELP is a winter rapeseed F_4 line with 27% oleic acid, 7% linoleic and linolenic acid and 50% erucic acid content obtained from a cross between the winter rapeseed cultivar Maplus and the high oleic acid doubled haploid winter rapeseed line DH XXII D9 (for details see Sasongko and Möllers 2005). BGRV2 is a UK high erucic acid winter oilseed rape breeding line from Nickerson UK Ltd with about 52% erucic acid in the seed oil (Wilmer et al. 2003). This line was used in the transformation experiments to produce transgenic line 361.2B (see below). F_1 plants were obtained by crossing transgenic line 361.2B with line 6575-1 HELP. Segregating F_2 and F_3 plant generations were produced by growing the plants in the greenhouse and self pollination of F_1 and F_2 plants, respectively.

Performance of greenhouse experiments

In August 2005, 220 randomly selected F_2 seeds along with eight seeds from each parent were sown for producing the

F_2 plant population. F_2 seeds were sown in a multipot tray containing T-soil (Fruhstorfer Erde; pH 5.9). F_2 plants were allowed to grow for three weeks in the greenhouse. For vernalisation, plantlets were transferred to 4°C temperature with 8 h light for 8 weeks. Afterwards, plantlets were transferred to 9 cm diameter pots containing normal compost soil. Plantlets were cultivated in the greenhouse providing 16 h light by using 400 W sodium-steam lamps. Temperature varied during the day from 20 to 25°C and during the night from 10 to 15°C. Hakaphos fertilizer containing N:P:K (15:11:15) + 0.2% trace minerals was added at two week intervals to the top soil (100 mg) of each pot until the plants matured. Insecticide was applied when aphid and thrips attack was recognized. Sulphur vapour supply was constant during the whole experiment to avoid fungal diseases. Self-fertilisation was ensured by covering the flowers of the main raceme with micropore plastic bags. F_3 seeds were harvested at maturity from the main raceme in February 2006 and stored at 4°C.

During the period October 2006 to April 2007, the greenhouse experiment was repeated with F_3 seeds (from sowing to harvest). For this experiment 41 F_2 plants with an erucic acid content higher than the mean of parent 361.2B (>62.5%) were selected. Five F_3 seeds from each of the F_2 plants and of the parents were sown. The greenhouse experiment was conducted as described above, following a randomized complete block design. The five F_3 plants from each F_2 plant and the parents were considered as five replicates. Five separate tables inside the green house represented the complete blocks consisting of all genotypes (F_3 plants and parents). Following self-fertilisation F_4 seeds were harvested from main raceme.

Methods

Binary plasmid construction

The *fae1-1* (C-genome) and *fae1-2* (A-genome) genes were amplified from BGRV2 using primers BnFAE-F (5′ CCTC ATGACGTCCATTAACGTAAAGCTCC 3′) and BnFAE-R (5′ GTGAGCTCTTATTAGGACCGACCGTTTGGG 3′), cloned into pBluescript II (KS+) (Stratagene), and sequenced. Next, the *fae* genes were transferred as *Rca*I–*Eco*ICRI fragment into pAR4 (Biogemma UK) *Nco*I–*Sma*I sites to place the gene between the *Pnapin* and CHS polyA sequences. The expression cassettes were then lifted out as a *Sal*I–*Sac*I fragment and ligated into pT7Blue2 *Sal*I–*Sac*I sites, before transfer of an *Eco*RI–*Eco*ICRI fragment into the binary vector pSCVnos144 (Biogemma UK) *Eco*RI–*Sma*I adjacent to a similar Pnapin::lat2 LPAAT::CHS poly A cassette (Brough et al. 1996) to create pEW13 and pEW14 (Wilmer et al. 2001).

Agrobacterium tumefaciens transformation and generation of T1-plants

Agrobacterium tumefaciens strain C58 pMP90 carrying above described binary plasmid pEW13 or pEW14 were used to transform rapeseed line BGRV2 essentially following the protocol of Moloney et al. (1989). Ten transgenic plants (T_1) carrying pEW13 and 18 transgenic plants with pEW14 were regenerated and oil composition was analysed following self pollination of T_1 and T_2 plants. Erucic acid content in these lines varied between less than 30 and about 65%. Line 361.2B, transformed with pEW14—*fae1-2*, was selected as it had the highest erucic acid content in T_3 seeds (Wilmer et al. 2001). T_4 seeds of 361.2B were used in the crossing experiment described above.

DNA isolation and PCR analysis to follow transgene segregation in the F_2 population

DNA was isolated from leaf material frozen in liquid nitrogen (−196°C) following the protocol of Ishizawa et al. (1991) with some modifications as described in Nath et al. (2007). Multiplex-PCR was performed using specific primers for the *Ld-LPAAT* gene and locus specific primers for the endogenous single copy *Bn-fad*2 gene. Amplification of the single copy *Bn-fad*2 gene served as positive control for the presence of DNA in sufficient quantity and quality. PCR primers, PCR conditions and gel electrophoresis were as described in Nath et al. (2007).

Seed quality analysis

Trierucin analysis

Seed samples (150 mg) obtained from F_2 and F_3 plants were analysed for trierucin (C_{69}; EEE) content by high temperature gas liquid chromatography (HT-GLC) analysis according to the method described by Möllers et al. (1997). The analysis was done using silicon capillary column RTX-65TG (Restek no. 17,005) 15 m × 0.25 mm i.d. (0.1 µm film thickness). Trierucin is expressed as percent of the sum of all triglycerides.

Analysis of total fatty acid composition

Following trierucin analysis the remaining part of each sample was transferred to a new tube and left on a hot plate at 37.5°C over night to evaporate. The fatty acid profile was determined by analysis of methyl esters by gas liquid chromatography according to Rücker and Röbbelen (1996). Fatty acids are expressed as percent of the sum of all fatty acids. 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 acids (SFA; 16:0 + 18:0), polyunsaturated fatty acids (PUFA; 18:2 + 18:3) and monounsaturated fatty acids (MUFA; 18:1 + 20:1 + 22:1) were calculated from the contents of individual fatty acids.

Analysis of the fatty acid composition at the sn-2 position of triacylglycerols

A total of 15 mg mixed seed samples from 5 replicates of the F_3 population (F_4 seeds) were collected in a 5 ml plastic tube. A measure of 0.5 ml iso-octane:iso-propanol (9:1) was added to each sample and seeds were homogenised with a steel rod. The supernatant was transferred to a new tube and the solvent evaporated by using a stream of warm air. The dried oil residue was mixed with 500 µl buffer (50 mM KH_2PO_4-, pH 7.2 with 0.5% Triton X-100) and kept in a supersonic bath for 2 min. The mixture was incubated at 30°C for 1 h by adding 25 µl (250 units) lipase from *Rhizopus arrhizus* (Sigma-Aldrich). Then 200 µl petroleum ether-70:iso-propanol (3:2) was added and the supernatant was collected in a 1 ml glass tube by centrifugation at 150 × G (1,000 rpm) for 5 min. The extraction was repeated twice and the supernatants were merged and evaporated by using a stream of warm air. 25 µl petroleum ether-70:iso-propanol (3:2) was added and mixed well by vortex. A measure of 20 µl from this mixture was taken and transferred to a thin layer chromatography (TLC) plate (F 1,500/LS 254, 20 × 20 cm) using a 20 µl syringe. TLC plates were placed in the eluent diethylether/petroleumether (3:1) and allowed to run for 30 min. Afterwards dry TLC plates were stained by subjecting them to iodine vapour. Monoacylglycerol spots (representing the fatty acids at the *sn*-2 position) were marked with a pencil. Following evaporation of the iodine, the marked areas were scraped out using a scalpel and transferred for fatty acid extraction in a separate 1 ml glass tube. The scrape was mixed with 250 µl iso-octane, incubated for 20 min and the supernatant was collected by centrifugation in a new 1 ml glass tube. This procedure was repeated twice. Iso-octane from the collected supernatant was evaporated and then the fatty acids were extracted and analysed as described above.

Analysis of seed oil and protein content

Oil and protein content of seed samples were determined by Near-Infrared-Reflectance-Spectroscopy (NIRS) using the equation raps2001.eqa (Tillmann 2007). Spectra were recorded using a standard ring cup equipped with a 14 mm PVC adapter. Values were adjusted using previously developed regression equations. Oil and protein contents are expressed on seed dry matter basis.

Statistical analysis

Spearman's rank correlation coefficients were calculated using the Plant Breeding Statistical Program PLABSTAT (Version 2N; Utz 2007). Multiple mean comparisons were made with Fisher's least significant difference (LSD) procedure using Stat Graphics Plus for Windows 3.0 (Statistical Graphics Corp., Rockville, USA). Hypotheses for transgene copy number segregation in the F_2 population were tested using Chi-square (χ^2) test as described by Gomez and Gomez (1976).

Results

Variation of seed quality traits and segregation of the transgenes in the F_2 population

The F_2 population derived from the cross between the transgenic line 361.2B and the high erucic acid, low polyunsaturated fatty acid line 6575-1 HELP showed a large quantitative variation for erucic acid content and other traits (Table 1). Erucic acid (22:1) was the most prominent fatty acid, accounting for 58.8% of the total fatty acid content.

Table 1 Variation in oil, protein, fatty acids and trierucin content (%) in 220 F_2 plants (F_3 seeds) derived from the cross 361.2B × 6575-1 HELP in comparison to the parental lines (each parent with $n = 8$)

	Item	Oil content	Protein content	Fatty acid contents (%)						
				SFA	18:1	PUFA	20:1	22:1	MUFA	Trierucin
361.2B	Range	46–54	24–26	2–5	8–11	16–24	3–7	61–64	72–78	9–18
	Mean	49.9	24.1	3.2	8.3	19.1	4.6	62.5	75.4	15.2
6575-1 HELP	Range	45–51	22–27	4–6	26–31	7–9	7–11	49–52	83–89	0.0–0.0
	Mean	49.5	23.9	4.9	27.8	8.1	8.9	49.4	86.1	0.0
F_2 population	Range	37–54	19–33	2–9	5–26	6–26	2–10	44–72	64–87	0–25
	Mean	47.7	24.8	4.0	11.0	16.8	5.2	58.8	75.0	12.6

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

Its content ranged from 44 to 72% and showed large transgressive segregation compared to the higher parent 361.2B. Frequency distribution of the erucic acid content showed a continuous variation indicating polygenic inheritance (Fig. 1). Polyunsaturated fatty acid content (PUFA; 18:2 + 18:3) varied from six to 26%, in some F_2 plants thereby being as low as in the 6575-1 HELP parent. Trierucoylglycerol (Trierucin) content showed a discontinuous variation from 0.0 to 25% (Table 1 and Fig. 2), indicating segregation and functioning of the *Ld-LPAAT* transgene. From the 220 F_2 plants analysed, 211 contained trierucin (Fig. 2), suggesting the presence of two transgene copies in transgenic parent 361.2B and a 15:1 segregation in the F_2 population (Chi-square $\chi^2 = 2.52$). Using PCR primers directed against the *Ld-LPAAT* transgene, four out of 57 randomly selected F_2 plants were negative in PCR (see an example in Fig. 3), again confirming 15:1 segregation of two transgene copies in the F_2 population (Chi-square $\chi^2 = 0.06$). All F_2 plants positive in PCR for the *Ld-LPAAT* gene contained trierucin, whereas those negative in PCR did not contain trierucin. The clearly separated group of segregating non-transgenic F_2 plants (Fig. 2) lacking the *Ld-LPAAT* + *Bn-fae1* transgenes contained between 45 and 57% erucic acid in the seed oil (see Fig. 4), with a mean of 53.6%. Parent 361.2B contained 62.5% erucic acid in the seed oil, indicating that the two transgenes in the homozygous state in this population increased erucic acid content by 8.9%.

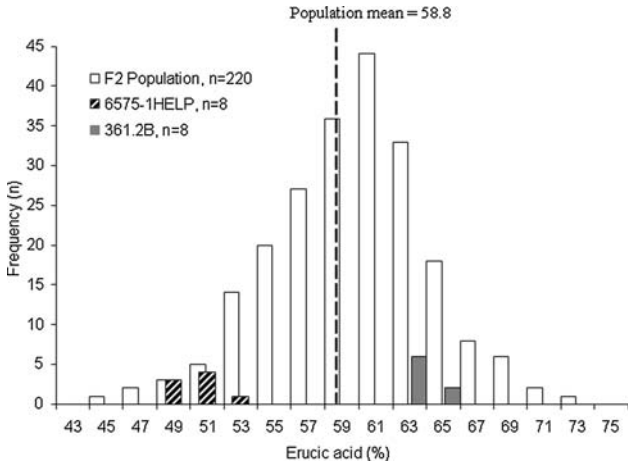

Fig. 1 Frequency distribution of erucic acid content in the F_2 population (F_3 seeds) derived from a cross between 361.2B × 6575-1 HELP rapeseed lines

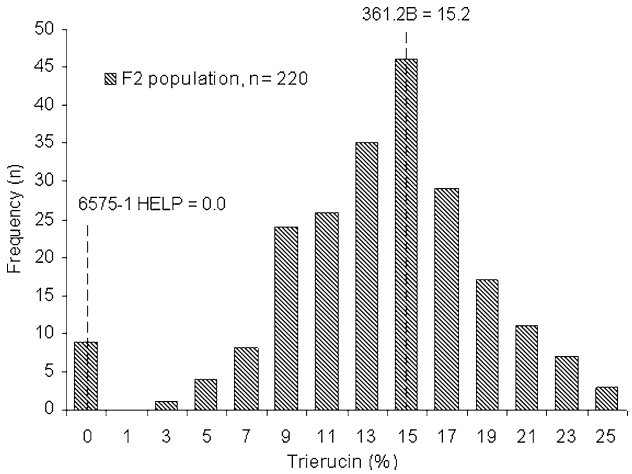

Fig. 2 Frequency distribution of trierucin (C_{69}; EEE) content in F_2 population (F_3 seeds) of the cross 361.2B × 6575-1 HELP with mean of the parents ($n = 8$)

Correlations between traits in the F_2 population

Spearman's rank correlation coefficient analysis of the F_2 population (F_3 seeds) showed negative correlations between erucic acid and protein, SFA, oleic acid, PUFA and eicosenoic acid content (Table 2). Correlations between erucic acid content and oil, trierucin and MUFA content were positive (see also Fig. 4). The calculation of the regression between erucic acid content and PUFA content ($y = -0.65x + 69.63$) showed that a reduction of 10% in PUFA content led to an increase of 6.5% in erucic acid content.

Performance of selected F_3 lines

From the F_2 population, 41 plants showed a higher erucic acid content (>62.5%) than the mean of parent 361.2B. Five F_3 plants from each of those 41 F_2 plants were cultivated in the greenhouse and F_4 seeds were harvested. In the F_3 population, erucic acid content varied from 50 to 72% (Fig. 5) with the mean of 64.8%. The scatter plot for the mean erucic acid content of F_3 lines (F_4 seeds) versus F_2 plants (F_3 seeds) shows a significant positive correlation ($r_s = 0.57^{**}$; Fig. 5), proving effective selection of high erucic acid plants amongst single plants of the F_2 population. The fatty acid composition of the six F_3 lines (F_4 seeds) with the highest erucic acid contents is shown in Table 3. F_3 line III-G-7 contained with 72.3% the highest amount of erucic acid. Four of the F_3 lines had an equal to or lower PUFA content than parent 6575-1 HELP, indicating homozygosity of the genes causing low PUFA content. All six F_3 lines showed higher trierucin contents compared to transgenic parent 361.2B.

From the six F_3 lines with the highest erucic acid content and the parental lines, the fatty acid composition at the *sn*-2 position of the triacylglycerols was analysed. In the F_3 lines the erucic acid content at the *sn*-2 position varied from 36.8 to 65.3%, which compares favourably to the 31.6% of transgenic parent 361.2B (Table 3). Erucic acid at *sn*-2 position was only detected in case of the presence of

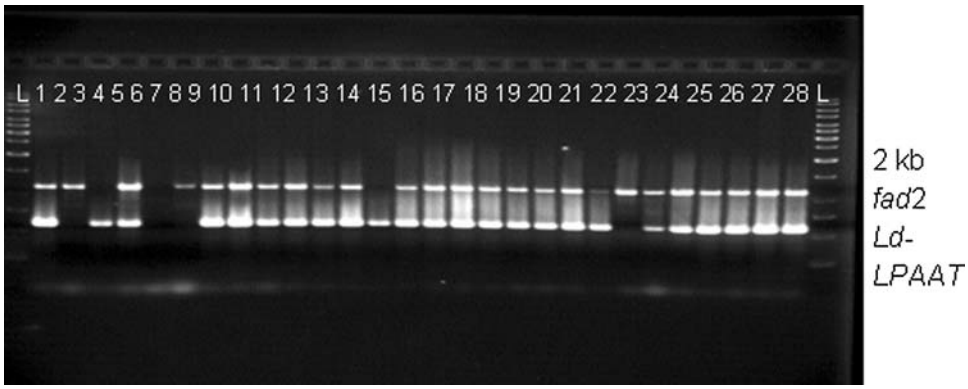

Fig. 3 Multiplex-PCR of a 603 bp fragment of the *Ld-LPAAT* gene together with internal control amplification of the 1.1 kb *fad*2 gene sequence using DNA extracted from 26 F_2 plants segregating for the *Ld-LPAAT* gene (Lanes 3–28 F_2, lanes 1 and 2: 361.2B and 6575-1 HELP as control)

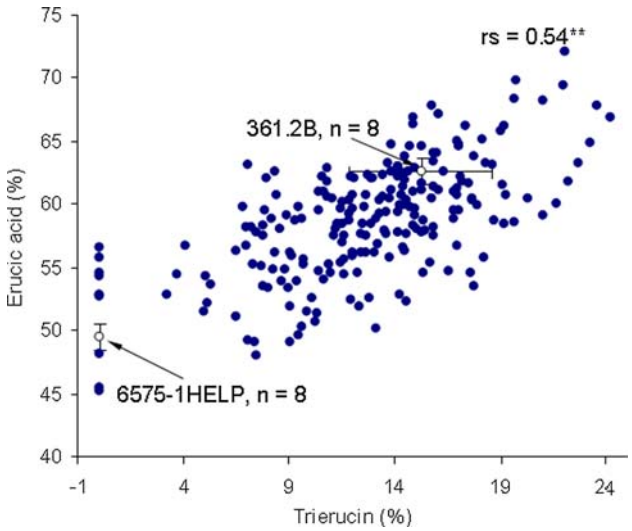

Fig. 4 Correlation between erucic acid and trierucin content of the F_2 population (F_3 seeds) derived from the cross 361.2B × 6575-1 HELP in comparison with the parental genotypes (*bar* showing standard deviation). *Double asterisk* indicates significance at $P = 0.01$ probability

Ld-LPAAT transgene. No, or only in one case very little, eicosenoic acid (20:1) was detected at the *sn*-2 position (data not shown).

Discussion

Transformation of the winter rapeseed breeding line BGRV2 with the *Bn-fae*1+*Ld-LPAAT* construct resulted in the regeneration of 18 primary transgenic lines, from which 361.2B was selected in preliminary experiments as the transgenic line with the highest erucic acid content. Crossing of 361.2B to 6575-1 HELP revealed in the F_2 population the segregation of two transgene copies. Comparing erucic acid contents of 361.2B with the mean erucic acid content of non-transgenic F_2 plants segregating in the population showed that, the two transgene copies led to an increase in erucic acid content from 53.6 to 62.5%, i.e. 8.9% (Fig. 4). This result is in contrast to the results of Han et al. (2001), who did not observe a significant increase in erucic acid content following transformation of a resynthesized high erucic acid line with principally the same construct. It could be that the *Bn-fae*1 gene in the transgene construct of Han et al. (2001) was not functional (Nath 2008).

The frequency distribution for the erucic acid content of the F_2 population showed a large and continuous variation as expected for a polygenic inherited trait (Fig. 1). Erucic acid content varied from 44 to 72%. In addition to the effect

Table 2 Spearman's rank correlation coefficients (r_s) amongst different seed quality traits (%) in the segregating F_2 population derived from the cross 361.2B × 6575-1 HELP ($n = 220$)

Traits	Oil	Protein	SFA	18:1	PUFA	20:1	Trierucin	22:1
Protein	−0.86[b]							
SFA	0.17[a]	−0.21[b]						
18:1	−0.23[b]	0.27[b]	0.07					
PUFA	−0.11	−0.02	0.16[a]	−0.41[b]				
20:1	−0.16[a]	0.08	0.18[b]	0.40[b]	−0.12			
Trierucin	0.11	−0.05	−0.19[b]	−0.31[b]	−0.21[b]	−0.47[b]		
22:1	0.35[b]	−0.21[b]	−0.39[b]	−0.41[b]	−0.50[b]	−0.45[b]	0.54[b]	
MUFA	0.08	0.06	−0.33[b]	0.40[b]	−0.93[b]	0.14[a]	0.17[b]	0.56[b]

[a] Significant at 0.05 probability

[b] Significant at 0.01 probability

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

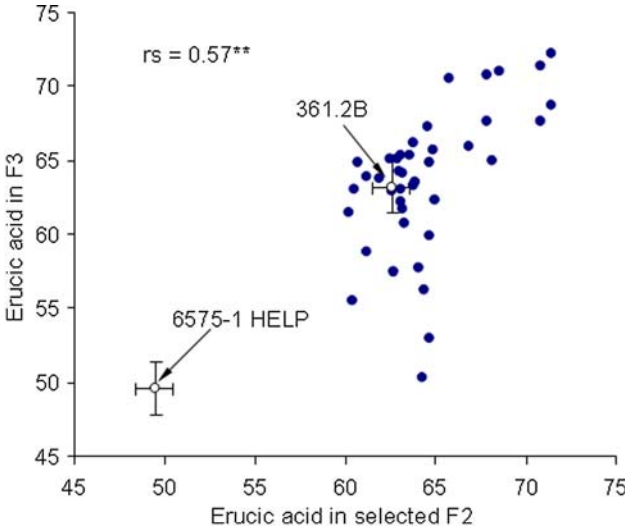

Fig. 5 Scatter plot for erucic acid content (%) of the selected 41 F_2 individuals and derived F_3 lines (F_4 seeds; mean of five plants) along with the parents (*bar* showing standard deviation). *Double asterisk* indicates significance at $P = 0.01$ probability

caused by the two transgene copies, this variation may be due to segregation of loci of parent 6575-1 HELP responsible for low contents of polyunsaturated fatty acids (PUFA). The low PUFA content in parent 6575-1 HELP is caused by a mutation in the oleic acid desaturase *fad*2 gene and by two to three other unknown genes having minor effects (Schierholt et al. 2001; Sasongko and Möllers 2005). According to the regression, a 10% reduction in PUFA content leads to a 6.5% increase in erucic acid content. Nath (2008) reported similar results for a high erucic acid doubled haploid winter rapeseed population segregating for PUFA content. Furthermore, environmental effects, different effective alleles at the two endogenous erucic acid loci *fae*1.1 (A-genome, *B. rapa*) and *fae*1.2 (C-genome, *B. oleracea*) as well as other unknown genetic factors may have contributed to the variation found in the F_2 population (Stefansson and Hougen 1964; Jönsson 1977; Ecke et al. 1995; Zhao et al. 2008).

Selection for erucic acid content amongst segregating F_2 plants proved to be efficient as shown by comparing the erucic acid contents of F_2 plants with those of the derived F_3 plants (Fig. 5). F_2 plants with about 70% erucic acid content in the seed oil were confirmed in the F_3 generation (F_4 seeds, Table 3). It is likely that the F_3 plants are already homozygous for the two transgene copies and for the endogenous two erucic acid genes. However, F_4 plant generation need to be tested to confirm this. Some of the high erucic acid lines had PUFA contents as low as in parent 6575-1 HELP (Table 3), indicating homozygosity for the genes causing low PUFA content.

The F_3 line III-G-7 had with 72.3% the highest erucic acid content in the seed oil as a mean of five plants (Table 3). This is about 9 and 23% more than that of transgenic parent 361.2B and non-transgenic parent 6575-1 HELP. The 72.3% erucic acid content can be regarded as relatively stable, because the two parental lines had similar erucic acid contents at two different seasons in the greenhouse (compare results in Tables 1 and 3). Furthermore, parent 6575-1 HELP was tested previously in field experiments and likewise had about 50% erucic acid in the seed oil (Sasongko and Möllers 2005).

The formation of trierucoylglycerol (Trierucin, C_{69}) proved that the *Limnanthes douglasii* lysophosphatidic acid acyltransferase (*Ld-LPAAT*) functioned in transgenic oilseed rape. Amongst the six F_3 lines with the highest erucic acid content, line VI-D-9 showed the highest trierucin content (23.5%; Table 3). Variation amongst the lines might be due to differences in homozygosity of the transgenes and in the availability of erucoyl-CoA. Assuming random distribution of erucoyl-CoA to all three triacylglycerol positions,

Table 3 Comparison for the traits oil, protein, fatty acids and trierucin content (%) of six selected high erucic acid F_3 lines (F_4 seeds) of the cross 361.2B × 6575−1 HELP along with their parents (values are means of five plants)

Line	Oil content	Protein content	Fatty acid content (%)							
			SFA	18:1	PUFA	20:1	22:1	MUFA	Trierucin	22:1 (*sn*-2)
361.2B	50.2 b	24.4 c	2.9 b	8.3 f	15.3 a	4.3 de	63.2 c	75.8 d	11.4 d	31.6
HELP	51.1 b	21.4 de	3.3 a	26.5 a	5.6 c	11.2 a	49.6 d	87.3 b	–	0.5
IV-D-3	54.2 a	21.9 de	2.1 cd	14.2 b	5.3 c	7.1 b	68.7 b	90.0 a	13.1 cd	38.5
II-B-2	47.9 c	28.5 a	2.3 c	10.7 de	7.7 b	4.4 de	70.5 ab	85.6 c	14.7 c	36.8
II-G-8	49.8 bc	24.4 c	2.2 cd	9.3 ef	8.5 b	3.9 e	70.7 ab	83.9 c	18.7 b	40.3
IV-F-6	53.6 a	22.9 cd	2.3 c	14.0 b	3.9 d	6.1 bc	70.9 ab	91.0 a	13.1 cd	41.3
VI-D-9	55.1 a	20.7 e	2.0 cd	13.2 bc	4.7 cd	5.3 cd	71.4 a	89.9 a	23.5 a	61.3
III-G-7	49.7 bc	26.7 b	1.9 d	12.1 cd	5.7 c	5.0 cd	72.3 a	89.4 a	22.2 a	65.3

SFA = 16:0 + 18:0, PUFA = 18:2 + 18:3, MUFA = 18:1 + 20:1 + 22:1, 22:1 (*sn*-2) erucic acid content at the *sn*-2 position

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

one would expect a trierucin content of 36% for F_3 line VI-D-9 with 71.4% erucic acid content. However, this line showed only 23.5% trierucin content, indicating that *Ld-LPAAT* activity may not be strong enough in comparison to the resident rapeseed *Bn-LPAAT* activity, which has a strong preference for oleic acid (Frentzen 1998). The *sn*-2 fatty acid mixture of the seed oils of the F_3 lines contained up to 65% erucic acid (Table 3). However, lines with similar erucic acid content contained very different amounts of erucic acid in the *sn*-2 position, indicating that beside the *Ld-LPAAT* gene there may be other genes involved in the expression of this trait. No, or very limited amounts of erucic acid at *sn*-2 position were found in non-transgenic parental line 6575-1 HELP (Table 3). Similar results were also reported by Weier et al. (1997) and Han et al. (2001) in *Ld-LPAAT* expressing transgenic rapeseed lines. The lack of significant amounts of eicosenoic acid at the *sn*-2 position confirms the pronounced preference of the *Ld-LPAAT* enzyme for erucoyl-CoA (Han et al. 2001).

The around 72% erucic acid content achieved in the present experiments represents a milestone in the breeding of high erucic acid oilseed rape. Further increases in erucic acid content can be expected from progress in reducing the contents of the remaining fatty acids, mainly oleic acid, polyunsaturated fatty acids and eicosenoic acid. Reduction in PUFA content probably could be achieved more easily following an RNAi antisense approach (Stoutjesdijk et al. 2002). Integration of the antisense *fad*2 gene into the same T-DNA as the *Ld-LPAAT* and the *Bn-fae*1 gene would greatly simplify the genetics and breeding of high erucic acid cultivars. The material developed in the present study should be of interest for the oleochemical industry but also for further studies aimed at identifying other physiological limitations in erucic acid biosynthesis.

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