Increasing erucic acid content through combination of endogenous low polyunsaturated fatty acids alleles with \( Ld-LPAAT + Bn-fae1 \) 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 triacylglycerol 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 rape- seed through over expression of the rapeseed fatty acid elongase gene (\( fae1 \)) 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.

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; fae1 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 fae1 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) F3 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 (fae1.1 and fae1.2 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-fae1 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 F1 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). F1 plants were obtained by crossing transgenic line 361.2B with line 6575-1 HELP. Segregating F2 and F3 plant generations were produced by growing the plants in the greenhouse and self pollination of F1 and F2 plants, respectively.

Performance of greenhouse experiments

In August 2005, 220 randomly selected F2 seeds along with eight seeds from each parent were sown for producing the
**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 (T1) carrying pEW13 and 18 transgenic plants with pEW14 were regenerated and oil composition was analysed following self pollination of T1 and T2 plants. Eruvic 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 T3 seeds (Wilmer et al. 2001). T4 seeds of 361.2B were used in the crossing experiment described above.

DNA isolation and PCR analysis to follow transgene segregation in the F2 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-fad2 gene. Amplification of the single copy Bn-fad2 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 F2 and F3 plants were analysed for trierucin (C65, 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 Rückert and Röbelen (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

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**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 ATGACGTCCATTAAACGTAAAGCTCC 3’) and BnFAE-R (5’ GTGAGCTCTTATTAGGACC GACC TGTTGGG 3’), cloned into pBluescript II (KS+) (Stratagene), and sequenced. Next, the fae genes were transferred as RcaI–EcoIICRI fragment into pAR4 (Biogemma UK) Neocl–Smal sites to place the gene between the Pnapin and CHS polyA sequences. The expression cassettes were then ligated as Sall–SacI fragment and ligated into pT7Blue2 Sall–SacI sites, before transfer of an EcoRI–EcoIICRI fragment into the binary vector pSCVnos144 (Biogemma UK) EcoRI–Smal adjacent to a similar Pnapin::lat2 LPAAT::CHS poly A cassette (Brough et al. 1996) to create pEW13 and pEW14 (Wilmer et al. 2001).
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 F3 population (F4 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 KH2PO4, 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 F2 population were tested using Chi-square ($\chi^2$) test as described by Gomez and Gomez (1976).

Results

Variation of seed quality traits and segregation of the transgenes in the F2 population

The F2 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.

| Item           | Oil content | Protein content | Fatty acid contents (%) | Trierucin |
|----------------|-------------|-----------------|-------------------------|-----------|
|                |             |                 | SFA 18:1 PUFA 20:1 22:1 MUFA |           |
| 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       |
| F2 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. Trierucglycerol (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 /afii9851 = 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 /afii9851 = 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%.

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. 2). 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...
Theor. Appl. Genet. (2009) 118:765–773

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-fae1+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 F2 population the segregation of two transgene copies. Comparing erucic acid contents of 361.2B with the mean erucic acid content of non-transgenic F2 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-fae1 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 F2 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 F2 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 | $-0.86^b$ | $-0.21^b$ | $-0.23^b$ | $0.27^b$ | $0.07$ | $0.16^a$ | $-0.41^b$ | $-0.12$ |
| SFA | $0.17^a$ | $0.02$ | $0.27^b$ | $0.08$ | $0.18^b$ | $0.40^b$ | $-0.12$ | $-0.40^b$ |
| 18:1 | $0.16^a$ | $0.08$ | $0.02$ | $0.18^b$ | $0.27^b$ | $-0.41^b$ | $-0.41^b$ | $-0.40^b$ |
| PUFA | $0.11$ | $-0.05$ | $-0.19^b$ | $-0.31^b$ | $-0.21^b$ | $-0.41^b$ | $-0.41^b$ | $-0.40^b$ |
| 20:1 | $0.35^b$ | $-0.21^b$ | $-0.39^b$ | $-0.41^b$ | $-0.50^b$ | $-0.45^b$ | $0.54^b$ | $0.56^b$ |
| Trierucin | $0.08$ | $0.06$ | $-0.33^b$ | $0.40^b$ | $-0.03^b$ | $0.40^b$ | $-0.93^b$ | $0.14^a$ |
| 22:1 | $0.17^b$ | $0.35^b$ | $-0.39^b$ | $-0.41^b$ | $-0.50^b$ | $-0.45^b$ | $0.54^b$ | $0.56^b$ |

$^a$ 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
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 \(fad2\) 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 con-
tent leads to a 6.5% increase in erucic acid content. Nath
(2008) reported similar results for a high erucic acid dou-
bled haploid winter rapeseed population segregating for
PUFA content. Furthermore, environmental effects, differ-
et effective alleles at the two endogenous erucic acid
loci \(fae1.1\) (A-genome, \(B. rapa\)) and \(fae1.2\) (C-genome,
\(B. oleracea\)) as well as other unknown genetic factors may
have contributed to the variation found in the \(F_2\) popula-
tion (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 gen-
eration 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 trans-
genetic 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 green-
house (compare results in Tables 1 and 3). Furthermore,
parent 6575-1 HELP was tested previously in field experi-
ments and likewise had about 50% erucic acid in the seed
oil (Sasongko and Möllers 2005).

The formation of trierucoylglycerol (Trierucin, \(C_{60}\))
proved that the \(Limnanthes douglasii\) lysophosphatidic acid
acyltransferase (\(Ld-LPAAT\)) functioned in transgenic oil-
seed rape. Amongst the six \(F_3\) lines with the highest erucic
acid content, line VI-D-9 showed the highest trierucin con-
tent (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 distribu-
tion of erucoyl-CoA to all three triacylglycerol positions,

![Fig. 5 Scatter plot for erucic acid content (%) of the selected 41 F
2 individuals and derived F3 lines (F4 seeds; mean of five plants) along
with the parents (bar showing standard deviation). Double asterisk
indicates significance at \(P = 0.01\) probability](image)

| Table 3 Comparison for the traits oil, protein, fatty acids and trierucin content (%) of six selected high erucic acid F3 lines (F4 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 18:2 + 18:3 | MUFA 18:1 + 20:1 + 22:1 | 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)

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one would expect a trierucin content of 36% for F₃ 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 F₃ 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 faded gene into the same T-DNA as the Ld-LPAAT and the Bn-fae1 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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