Increasing erucic acid content in the seed oil of rapeseed (*Brassica napus* L.) by combining selection for natural variation and transgenic approaches

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Ujjal Kumar Nath

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D7

Referee: Prof. Dr. Heiko C. Becker

Co-referee: Prof. Dr. Elke Pawelzik

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The following manuscripts are part of this Dissertation:

I. Early, non-destructive selection of microspore-derived embryo genotypes in oilseed rape (*Brassica napus* L.) by molecular markers and oil quality analysis. Published in Mol Breed (2007) 19, 285-289.

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

III. Increasing erucic acid content in high erucic acid rapeseed (*Brassica napus* L.) through combination with mutant genes for low polyunsaturated fatty acids content and with *Ld-LPAAT-Bn-fae*1 transgenes.

1. Introduction

Oilseed rape is an important source of vegetable oil and regarding world oilseed production it is now the second largest oilseed crop after soybean (FAO 2007). Rapeseed has gained acceptance worldwide largely because of major improvements in the seed oil and meal quality. World vegetable oil markets are highly competitive requiring a steady improvement in oil quality to increase market prospects. The objective of modifying oil quality is to develop oils with enhanced nutritional and functional properties and which require if possible no further processing for specific end-use markets. The market for rapeseed oil is primarily for human consumption, but also for a range of industrial applications (Craig and Millam 1995). Presently, different types of rapeseeds with a modified fatty acid composition are available for different purposes (Möllers 2004).

In traditional *Brassica* oilseeds, the occurrence of erucic acid is considered as anti-nutritional factor for human consumption. Therefore, it was minimized by breeding and finally developed Canola- or ‘00’-quality (Lühs and Friedt 1994, Przybylski and Mag 2002). High Erucic Acid Rapeseed (HEAR) cultivars are regaining interest for industrial purposes. Erucic acid (*cis*-13-docosenoic acid, 22:1) a very long chain fatty acid having 22 carbon atoms with one double bond at the cis-13 position of the carbon chain. Genetic studies show that in rapeseed, which arose from a spontaneous cross between *B. rapa* (A-genome) and *B. oleracea* (C-genome), 22:1 content is controlled by the two gene loci, *E1* (*Bn-fae*1.1) and *E2* (*Bn-fae*1.2), which have additive effects (Harvey and Downey 1964, Stefansson 1983, Lühs et al. 1999). Studies of *Arabidopsis thaliana* mutants deficient in very long chain fatty acids (VLCFAs) showed that the fatty acid elongase (*fae*1) gene product is required in the seeds for the elongation from 18:1 to 22:1 (Kunst et al. 1992, James et al. 1995). The development of seed oils bearing a high percentage of erucic acid for industrial applications is a subject of research (Taylor et al. 1995, Kott et al. 1996) because it is a very excellent renewable raw material used in plastic film manufacture, in the synthesis of nylon and in the lubricant and emollient industries (Leonard 1994, Sonntag 1995, Murphy 1996). A rapeseed line containing high proportions of erucic acid would significantly reduce the processing costs.

Attempts are being made by conventional breeding to increase the erucic acid content. This has so far resulted in breeding lines with up to 60% erucic and eicosenoic acid. These fatty acids are found in the *sn*-1 and the *sn*-3 position of the triacylglycerols but are excluded from the *sn*-2 position. This *sn*-2 exclusion limits the erucic acid content to a total of 66% and prevents the synthesis of trierucin (Cao et al. 1990, Frentzen 1993, Katavic et al. 2001). The gene of an erucoyl-CoA preferring *sn*-2 acyltransferase from *Limnanthes douglasii* (*Ld-LPAAT*) has been successfully cloned and over expressed in rapeseed (Brown et al. 1995, Hanke et al. 1995, Lassner et al. 1995, Brough et al. 1996, Friedt and Lühs 1998). However, the overall proportions of 22:1 in the seed oil did not increase. In a next step, interest focussed on the fatty acid elongation mechanism from oleic acid to eicosenoic acid and then to erucic acid. This elongation is the result of two cycles of a four-step mechanism, in which 18:1-CoA and 20:1-CoA are used as substrates. The first step, the initial condensation reaction of these fatty acids with malonyl-CoA is catalysed by the ß-ketoacyl-CoA synthase (KCS). It is believed that this initial reaction is the rate-limiting step (Cassagne et al. 1994). The *fae*1 gene encoding the KCS, has been cloned from a range of plant species and has been over expressed under control of a seed specific promoter in HEAR. However, only very minor increases in 22:1 content were reported (Katavic et al. 2001, Han et al. 2001). Even in combination with the *Ld-LPAAT* no substantial increase in 22:1 content has been found (Han et al. 2001).

It seems that there are other bottlenecks in the pathway, such as the pool of oleic acid available for elongation to eicosenoic and erucic acid. To study the question if availability of 18:1-CoA is limiting for 22:1 synthesis, Sasongko and Möllers (2005) crossed HEAR (cv. Maplus) to high oleic acid rapeseed (HOAR, Schierholt et al. 2001) to recombine the genes for high 22:1 with those for high 18:1 (i.e. low content of polyunsaturated fatty acids). However, the recombinant line HELP (High Erucic and Low Polyunsaturated fatty acid) did not show a significant change of 22:1 acid content, indicating that in this material the ß-ketoacyl-CoA synthase (KCS) activity may be limiting.

To test this hypothesis, two separate approaches were followed involving two transgenic lines over expressing the *fae*1 gene in combination with the *Ld-LPAAT* gene. Those lines were crossed to the line 6575-1 HELP. Segregating populations were studied for the inheritance of erucic acid content and other traits as outlined in Figure 1.

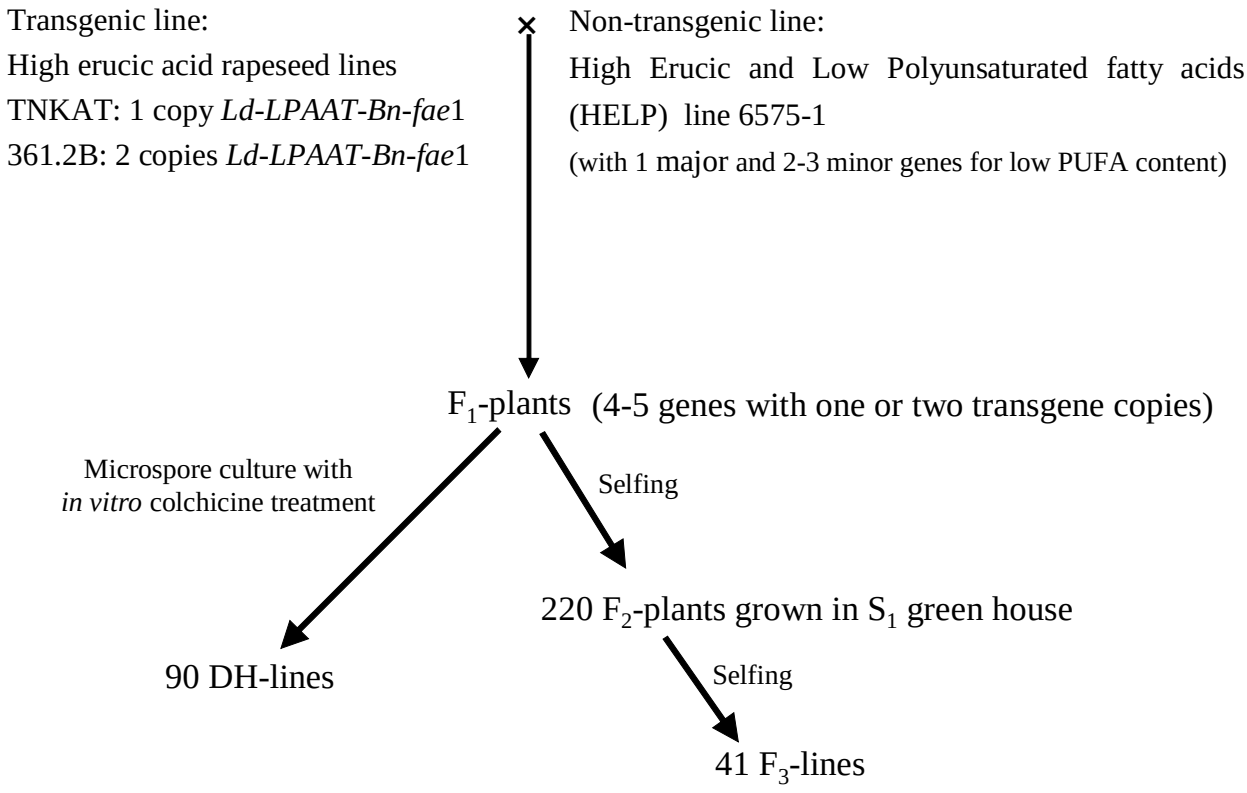

Figure 1 Schematic diagram of the cross between transgenic and non-transgenic parents with probable number of genes segregating in the DH and F_2/F_3-generations developed and used in the experiment.

The doubled haploid (DH) population was developed from the cross TNKAT x 6575-1 HELP and segregating F_2- and F_3-populations were developed from the cross 361.2B x 6575-1 HELP. The DH population and selected high erucic acid F_3-lines were tested in a replicated green house experiment (Figure 1).

The main objective of the present experiments was to develop rapeseed lines with erucic acid content beyond that so far reported in the literature. Besides that, other objectives were:

- to optimize a suitable protocol for *in vitro* selection of microspore derived embryos
- to study the inheritance of erucic acid content and other seed quality traits
- to develop locus and allele specific PCR primers to follow the segregation of the erucic acid alleles at the endogenous *fae*1.1 (*Brassica rapa* L.) and the *fae*1.2 (*Brassica oleracea* L.) loci

- to determine the effect of a low polyunsaturated fatty acids (PUFA) content on erucic acid content in transgenic oilseed rape
- to increase 22:1 content through combination of alleles of endogenous erucic acid and low polyunsaturated fatty acid loci (HELP) with β-ketoacyl-CoA synthase (KCS) over expressing and lysophosphatidic acid acyltransferase (LPAAT) from *Limnanthes douglasii* expressing rapeseed genotypes.

2. Literature Review

2.1 Importance of rapeseed and erucic acid

The oleiferous *Brassica* represented by rapeseed and mustard play an important role for vegetable oil production of the world. Oil plays an important role in our daily diet. From nutritional point of view, fats and oils in our daily life are mostly needed for calories and fat soluble vitamins absorbent. At present, rapeseed is the second most important oilseed in the world, after soybean and is used for both nutritional and industrial purposes (FAO 2007). The past forty years have seen significant growth in rapeseed production due to the introduction of food rapeseed (canola), low in erucic acid (22:1; *cis*-13 docosenoic acid) and glucosinolates (Lühs and Friedt 1994, Przybylski and Mag 2002).

In traditional *Brassica* oilseeds, the occurrence of 22:1 distinguishes from other major oilseeds (Lühs and Friedt 1994). This component is considered as anti-nutritional for human. However, 22:1 and its derivatives are important renewable raw materials used in plastic film manufacture, in the synthesis of nylon 13, 13 and in the lubricant and emollient industries (Leonard 1994, Sonntag 1995). A rapeseed line containing high proportions of 22:1 would significantly reduce processing costs and could meet the demand for high 22:1 oil as a renewable environmentally friendly industrial feedstock (Sonntag 1991, Murphy 1996). The term ‘industrial rapeseed’ traditionally referred to any rapeseed variety producing oil with 22:1 content higher than about 45% (high erucic acid rapeseed, HEAR; Piazza and Foglia 2001). Erucic acid is in turn subjected to a number of simple chemical transformations to produce useful materials. Catalytic hydrogenation gives behenic acid; esterification with methanol or higher alcohols gives erucate esters (Piazza and Foglia 2001). The probable market prospects of 22:1, behenic acid and their derivatives are presented in Table 1.

Table 1 Estimated world erucic and behenic acid markets (Sonntag 1995; cited in Piazza and Foglia 2001).

Application	Erucic acid	Behenic acid
	Units*	
Surfactants	1.38	0.94
Detergents	0.75	2.38
Plastic additives	7.96	3.15
Recording materials	-	0.34
Food additives	0.77	0.03
Cosmetics	1.76	1.56
Pharmaceuticals	0.59	0.36
Personal care products	0.84	1.70
Ink additives	0.30	0.57
Textiles	0.94	1.02
Lubricants	0.50	0.09
Fuel additives	0.24	0.11

* Equivalent × 10^3 metric tons of 90% erucic acid and 10^3 metric tons 85% behenic acid.

2.2 Occurrence of Very Long Chain Fatty Acids (VLCFAs) in rapeseed

Very-long-chain fatty acids with more than 18 carbons are widely distributed in nature. In plants, they are mainly found as components or precursors of epicuticular waxes and in the seed oil of certain plant species (Harwood 1980, Post-Beittenmiller 1996). But erucic acid (22:1) is found only in the seed oil and not in membrane lipids. Erucic acid accounts for 45-60% of the total fatty acid mixture in traditional *B. napus* cultivars (Frentzen 1993).

Genetic (Kunst et al. 1992) and biochemical (Cassagne et al. 1994, Domergue et al. 1998) studies have suggested the existence of several elongase activities in *Brassica*. In the presence of labeled malonyl-CoA, VLCFAs could be synthesized from acyl-CoAs (acyl-CoA elongation) of the microsomal lipids; there is an absolute requirement for ATP (ATP-dependent elongation; Domergue et al. 1999). In the case of acyl-CoA elongation, Lessire et al. (1985) demonstrated that successive additions of C2 units to stearoyl-CoA (18:0-CoA) were responsible for the sequential synthesis of arachidonoyl-CoA (20:0-CoA), behenoyl-

CoA (22:0-CoA) and lignoceroyl-CoA (24:0-CoA). In the case of developing rapeseed, the situation appears to be more complex.

Several studies suggested that two biochemical steps are critical for improvement of erucic acid production in rapeseed: membrane bound fatty-acid elongation and lysophosphatidic acid acyltransferase (LPAAT) activity leading to the biosynthesis of trierucin. The gene of an erucoyl-CoA preferring acyltransferase (*Ld-LPAAT*) has been successfully cloned from meadowfoam (*Limnanthes douglasii* L.) and over expressed in rapeseed (Brown et al. 1995, Hanke et al. 1995, Lassner et al. 1995, Brough et al. 1996). However, the overall proportions of 22:1 in the seed oil did not increase. Zou et al. (1997) have confirmed that the yeast (*Saccharomyces cerevisiae*) *SLC1-1* gene encodes *sn*-2-acyltransferase capable of acylating *sn*-1-oleoyl-lysophosphatidic acid using a range of acyl-CoA thioesters, including 22:1-CoA. However, neither the meadowfoam nor the yeast-*LPAAT* transgene approach were successful in achieving high trierucin content in HEAR *B. napus* seed oil. Weier et al. (1997) suggested that the level of trierucin depends not only on the activity of the introduced *sn*-2-acyltransferase but also on other biosynthesis or incorporation steps. It is possible that the levels of erucoyl-CoA in the seed acyl-CoA pool may be too low to allow high levels of trierucin biosynthesis. If this is the case, then over expression of genes regulating VLCFAs biosynthesis may be required to boost very long-chain acyl-CoA availability for incorporation into seed triacylglycerols (TAGs).

2.3 Biosynthesis of erucic acid in rapeseed

The seed reserve materials which consists mainly lipids and protein are produced during seed development. They play an important role in germination and early phases of development. During seed development, seeds may accumulate fatty acids with different amount of carbon chain lengths as well as degree of saturation (Slabas et al. 2001).

The first product of fatty acid synthethase in rapeseed is palmitic acid (16:0), elongated by palmitoyl-ACP elongase to stearoyl-ACP (18:0, Figure 2). Due to Δ9 stearoyl-ACP fatty acid desaturase enzyme, 18:0 is then desaturated to 18:1. Palmitic, stearic and oleic acid may also be released by an acyl-ACP thioesterase and reesterified on the chloroplast envelop to Coenzyme-A (16:0-CoA, 18:0-CoA and 18:1-CoA; Downey 1987). In rapeseed 18:1 is further desaturated by Δ12-desaturase to form linoleic acid (18:2) and in the

presence of Δ15-desaturase 18:2 is desaturated to linolenic acid (18:3; Arondel et al. 1992, Okuley et al. 1994).

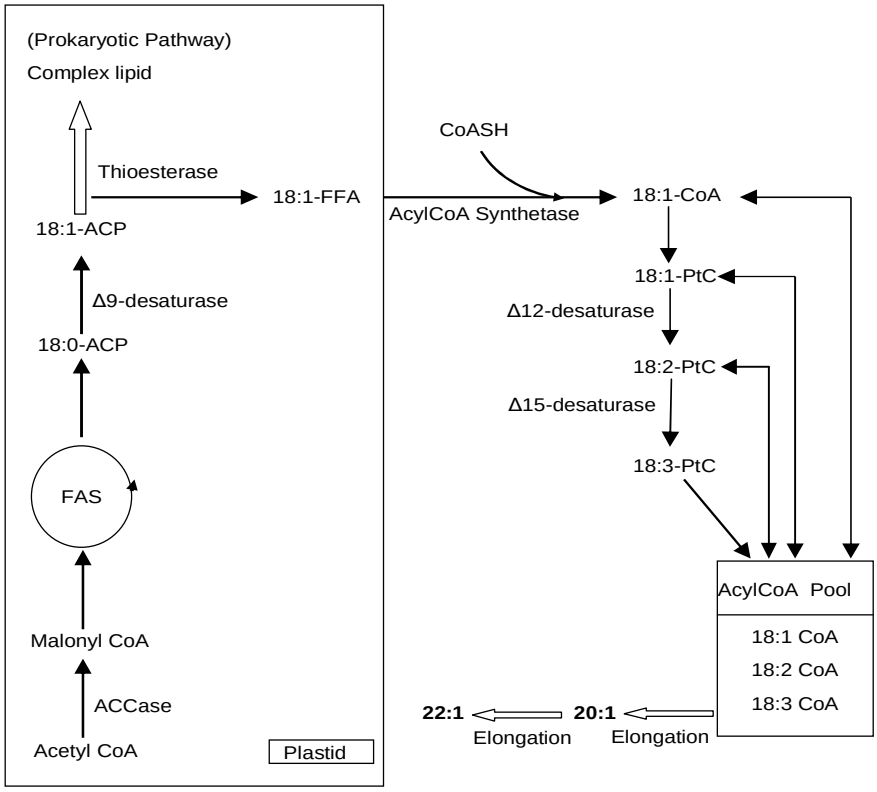

Figure 2 Biosynthesis of fatty acids in *B. napus* utilising ACP substrates in the plastid and subsequent reactions on CoA substrates occuring in the cytoplasm (adapted from Slabas et al. 2001). ACCase = Acetyl-CoA carboxylase; ACP = Acyl Carrier Protein; FAS = Fatty acid synthetase; FFA = Free fatty acid; PtC = Phosphatidyl choline and CoASH = Activated CoA.

In rapeseed, very long chain fatty acids (VLCFAs) elongation takes place in the cytosol (Downey 1987, Barret et al. 1998). The initial substrate for elongation is oleic acid, synthesised in the plastids. Erucic acid is synthesized from oleoyl-CoA and malonyl-CoA by a membrane-bound enzyme complex called acyl-CoA elongase (Cassagne et al. 1994). From the identification of the intermediates of the elongation process (Fehling and Mukherjee 1991, Lassner et al. 1995, Puyaubert et al. 2005), it has been shown that fatty acid elongation is achieved by the sequential addition of 2 carbon moieties donated by malonyl-CoA to a long chain acyl-CoA primer. Each round of elongation involves four enzymatic reactions catalyzed by the fatty acid elongase (FAE) complex, a protein complex

localized in the microsomal fraction: i) malonyl-CoA and oleoyl-CoA are condensed by 3-ketoacyl-CoA synthase or condensing enzyme; ii) resulting 3-ketoacyl-CoA is then reduced by the action of 3-ketoacyl-CoA reductase, resulting in the synthesis of 3-hydroxyacyl-CoA; iii) the latter is transformed in the third step into enoyl-CoA by 3-hydroxyacyl-CoA dehydratase and iv) a second reduction catalyzed by 2,3-enoyl-CoA reductase yields the acyl-CoA elongated by two carbons (Figure 3).

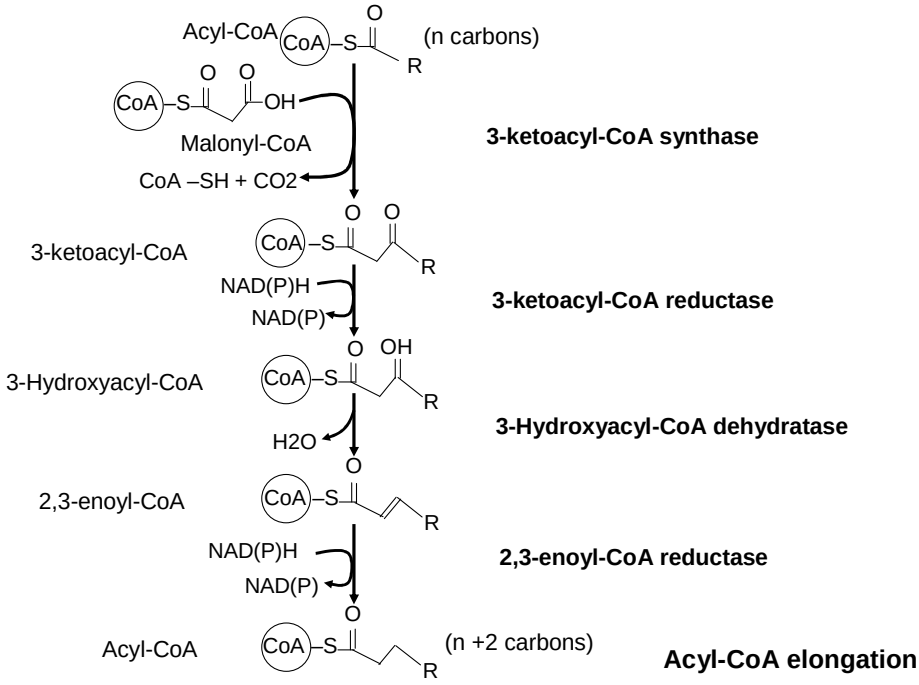

Figure 3 Different steps and intermediate enzymatic reactions involved in acyl-CoA elongation, adapted from Puyaubert et al. (2005).

The structure and functioning of the acyl-CoA elongase complex is poorly understood because of the difficulty in purifying functional membrane proteins to homogeneity. The acyl-CoA elongase complex has been partially purified from developing rapeseed embryos and has resulted in the enrichment of four proteins between 54 and 67 kDa in size (Créach and Lessire 1993). The β-ketoacyl-CoA synthase (KCS) was purified from jojoba embryos by Lassner et al. (1996). The corresponding cDNA, homologous to the Arabidopsis *fatty acid elongation*1 (*fae*1) gene (James et al. 1995), was used to transform rapeseed plants. Subsequent KCS activity in developing embryos of Low Erucic Acid Rapeseed (LEAR) plants resulted in an enrichment (up to 33.5% by weight) of the seed oil with VLCFAs, thereby demonstrating that KCS activity had been restored.

2.4 Inheritance of erucic acid in rapeseed

The erucic acid content of seeds of the amphidiploid species *Brassica napus* (2n=38) has been shown to be governed by two genes which act in an additive fashion (Downey and Craig 1964, Harvey and Downey 1964, Siebel and Pauls 1989). Digenic inheritance of 22:1 was confirmed in *B. juncea* (Kirk and Hurlstone 1983) and in *B. carinata* (Getinet et al. 1997). Jönsson (1977), Pourdad and Sachan (2003) reported that in rapeseed (*B. napus*) 22:1 content is controlled by alleles at one, one or two and two loci leading to 5-10%, 10-35% and more than 35% 22:1, respectively. The two elongation steps from oleoyl-CoA to 22:1 are each controlled by alleles at two loci (Harvey and Downey 1964, Stefansson 1983). Ecke et al. (1995), Jourdren et al. (1996) mapped the two loci determining erucic content in rapeseed population using random fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) markers, respectively. Assignment of the two loci to independent linkage groups was confirmed via a quantitative trait locus (QTL) approach by Thormann et al. (1996). However, two loci do not contribute equally to erucic acid content. It has also been determined that multiple alleles occur at each locus (Stefansson and Hougen 1964, Jönsson 1977). At least five alleles govern the erucic acid in *Brassica*, including; e, E^a, E^b, E^c and E^d. Therefore, levels of erucic acid can be fixed at a large number of values ranging from < 1% to >60% (Jönsson 1977).

2.5 Development of rapeseeds with high erucic acid

The qualitative manipulation of seed oils involves the modification of its fatty acid composition. In order to make rapeseed oil more competitive in various segments of the food and industrial oil markets, modification of the fatty acid composition has been an important objective of plant breeding (Friedt and Lühs 1998). A maximum content of the desired fatty acid will not only decrease the amount of waste, but can also result in considerable savings in downstream processing costs. Erucic acid for example is subjected to number of simple chemical transformations to produce useful materials for many industrial applications.

Beside the standard breeding aims for ‘00’ quality rapeseed (Becker et al. 1999), there are additional specific breeding aims for the development of HEAR. These specific breeding aims depend on whether the high erucic acid oil is used either as an oil for lubrication purposes or to isolate erucic acid for further chemical modifications. If the oil is used for

lubrication, a major improvement can be achieved through the reduction of the polyunsaturated and saturated fatty acids (Metz et al. 2001, Sasongko and Möllers 2005), because these fatty acids negatively affect the stability and properties of the oil at low ambient temperatures, respectively. If the high erucic acid oil is used for the extraction of erucic acid, the major breeding aim is to increase erucic acid content and to reduce 20:1 because this fatty acid can not be easily separated from 22:1 by distillation as it is currently applied (Möllers 2004).

High erucic acid rapeseed (HEAR) oil with a greater than 80% 22:1 level is desired to reduce the cost of producing this fatty acid and its derivatives as a renewable, environment friendly industrial feedstock (Leonard 1994, Taylor et al. 2001, Mietkiewska et al. 2004). Existing high erucic acid rapeseed (HEAR) cultivars have less than 1% 22:1 incorporated into the central position (*sn*-2) of the glycerol backbone because of the poor affinity of the rapeseed LPAAT activity for very long chain fatty acids, including 22:1 (Brough et al. 1996, Frentzen and Wolter 1998, Lühs et al. 1999). This restricts the level of 22:1 in the existing HEAR seed oil to the theoretical limit of 66% while the maximum expression is closer to 50% in commercially produced winter HEAR cultivars (McVetty and Scarth 2002). Even the total 22:1 level in seed oil did not increase by over expression *Ld-LPAAT* gene together with *Bn-fae*1 gene in rapeseed (Katavic et al. 2001, Han et al. 2001, Taylor et al. 2001, Mietkiewska et al. 2004).

The failure to significantly increase the 22:1 level by engineering *Ld-LPAAT* could be due to a limitation in the acyl-CoA pool in the cytosol, which is required to support high levels of trierucin synthesis (Lühs et al. 1999, Sasongko and Möllers 2005). This hypothesis was supported by the increase to 22:1 levels between 48 and 53% in transgenic Hero plants expressing the yeast *fae*1 compared with the wild-type control lines average of 43% 22:1 (Katavic et al. 2000). Similar results were obtained with the expression of *Arabidopsis* and *B. napus fae*1 in rapeseed (Katavic et al. 2001, Han et al. 2001, Wilmer et al. 2003). On the basis of these studies, the proportion of 22:1 in rapeseed oil is limited by both 22:1 synthesis and its subsequent incorporation into TAG (Katavic et al. 2000). HEAR oil could eventually be produced by combining these and other genetic modifications.

3. Materials and Methods

3.1 Materials

Two different experiments were performed in the laboratory and safety 1 (S1) green house during December 2004 to July 2007 in the Department of Crop Sciences, Division Plant Breeding, Georg-August University, Göttingen. First experiment had two parts, in part I, microspore derived embryos (MDE) were produced using the F_1-plants following microspore culture technique (Iqbal et al. 1994, Fletcher et al. 1998). The F_1-plants derived from the cross between transgenic resynthesised high 22:1 rapeseed line TNKAT (chimeric one transgene copy *Ld-LPAAT-Bn-fae*1; Han et al. 2001) and non-transgenic High Erucic acid and Low Polyunsaturated fatty acid (HELP) line 6575-1 (Sasongko and Möllers 2005), in the following called 6575-1 HELP. Six weeks after microspore culture, embryos were well developed and a single cotyledon was dissected. The dissected cotyledon was used for fatty acid and DNA extraction and rest of the MDE with single cotyledon was transferred to solid medium for plantlet regeneration, for details see manuscript I.

In part II of the first experiment, ninety doubled haploid (DH) lines derived from the cross (TNKAT x 6575-1 HELP) along with their parents were grown in randomized complete block design (RCBD) in the green house with three replicates. Selfed seeds were harvested from the main raceme only and used for analyzing oil, protein, trierucin and fatty acids content, for details see manuscript II.

For the second experiment F_1-plants were produced from the cross between transgenic 361.2B (two chimeric transgene copies *Ld-LPAAT-Bn-fae*1; Wilmer et al. 2003) and non-transgenic line 6575-1 HELP (see above). F_2-generation (F_3-seeds) was produced from randomly selected 220 selfed F_2-plants. F_3-seeds from selected 41 F_2-plants having high erucic acid content along with their parents were grown following RCBD with five replicates. Seeds obtained after selfing the plants, were used for analyzing oil, protein, trierucin and fatty acids content, for details see manuscript III.

3.2 Methods

Microspore culture to obtain doubled haploid line

F_1-plants were obtained after crossing TNKAT x 6575-1 HELP. They were used as donors for microspore culture, which was performed according to a protocol described by (Iqbal et al. 1994, Fletcher et al. 1998), for details see manuscript I.

DNA isolation and PCR amplification of the transgene *Ld-LPAAT*

DNA was isolated from the dissected cotyledon of MDE after lipid extraction and was used for PCR amplification. Multiplex-PCR for the *fad*2 and the *Ld-LPAAT* gene was performed as described in Nath et al. (2007), for details see manuscript I.

Oil and protein analysis by NIRS

Seed oil and protein content, expressed on seed dry matter basis were determined by using Near-Infrared-Reflectance Spectroscopy (NIRS) with the calibration equation raps2001.eqa (Tillmann 2007). Values obtained using a 14 mm PVC adapter were adjusted to standard ring values using regression equations, for details see manuscript II and III.

Trierucoylglycerol (Trierucin) analysis by HT-GLC

Trierucin (C_{69}; EEE) content of the seed samples was determined by high temperature gas liquid chromatography (HT-GLC) analysis of fatty acid esters according to the method described by Möllers et al. (1997). Silicon capillary column RTX-65TG (Restek no. 17005) 15 m x 0.25 mm i.d. (0.1 μm film thickness) was used for HT-GLC. Trierucin is expressed as % of the sum of all triglycerides, for details see manuscript II and III.

Fatty acid analysis by GLC (Gas liquid chromatography)

Lipids were isolated from dried cotyledons of MDE, and fatty acids were trans-esterified with sodium methylate and used for gas chromatographic analysis described by Albrecht et al. (1995). Fatty acids composition of half-seed and bulk-seed samples was analysed by gas liquid chromatography according to Thies (1971), Rücker and Röbbelen (1996). Individual fatty acids, such as palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), linolenic (18:3), eicosenoic (20:1), erucic acid (22:1) and nervonic acid (24:1) were determined by GLC and expressed as % of total fatty acids, for details see manuscript II and III.

Fatty acid analysis at central (*sn*-2) position of triacylglycerol by GLC

The identification of fatty acids esterified in the *sn*-2 position of the triacylglycerols (TAGs) was conducted by taking 15 mg seed samples. Seed oil of the samples were emulsified and digested with 25 µl (250 units) lipase from *Rhizopus arrhizus* (*SIGMA-ALDRICH*) in 500 µl buffer (50 mM KH_2PO_4- pH 7.2 with 0.5% Triton X-100). After incubation at 30ºC for 1 hr, lipids were extracted, *sn*-2 monoacylglycerol was separated by thin layer chromatography (TLC; F 1500/LS 254, 20 × 20 cm) in the eluent diethylether/petroleumether (3:1). The fatty acids were converted into FAMEs by transmethylation as described in the previous section and analysed by GLC, for details see manuscript III.

4. Results and Discussion

4.1 *In vitro* selection of microspore derived embryo in rapeseed (Manuscript I)

It is necessary that the microspore-derived embryo (MDE) have a relatively large size to isolate lipid and DNA in sufficient quantities from single detached MDE cotyledon. This was achieved in the present study by sub-culturing the MDE after 3 to 4 weeks of culture initiation in fresh culture medium at a reduced density. Germination of the embryos during this period was prevented by maintaining a high osmotic potential of the culture medium with 13% sucrose. About six weeks after culture initiation, large embryos which have accumulated sufficient amounts of storage lipids were obtained (Albrecht et al. 1995). Figure 4 shows the development of MDE from week 2 to 6 after culture initiation and their relative size in comparison to a single rapeseed kernel. The large size of the MDE allows easy dissection of a single cotyledon under aseptic conditions.

The dry weight of the detached MDE cotyledons compares favorably to the 1 to 3 mg dry weight of single dissected cotyledons of germinated rapeseeds of segregating F_2-seed populations, which are routinely analysed for their fatty acid composition when breeding for oil quality traits. In the present study, 4 to 10 mg of a MDE cotyledon was enough to extract a sufficient amount of oil for a reliable and reproducible fatty acid analysis with good peak resolution using a gas chromatograph equipped with an auto sampler (see in manuscript I Table 1). The storage lipids isolated from the cotyledons of the MDE showed clear differences in the content of erucic acid and polyunsaturated fatty acids.

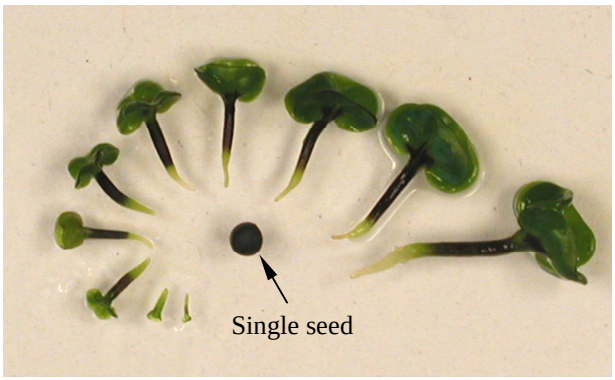

Figure 4 Development of microspore derived embryos from week 2 to week 6 after culture initiation and relative size in comparison to a seed of oilseed rape (*Brassica napus* L.).

Figure 5a-b shows that among the segregating MDE there are some MDE which have a higher 22:1 and an equal 18:1, but a reduced PUFA content compared to the transgenic parental MDE (TNKAT). This indicates that the reduction in PUFA content may have led to an increased 22:1 content.

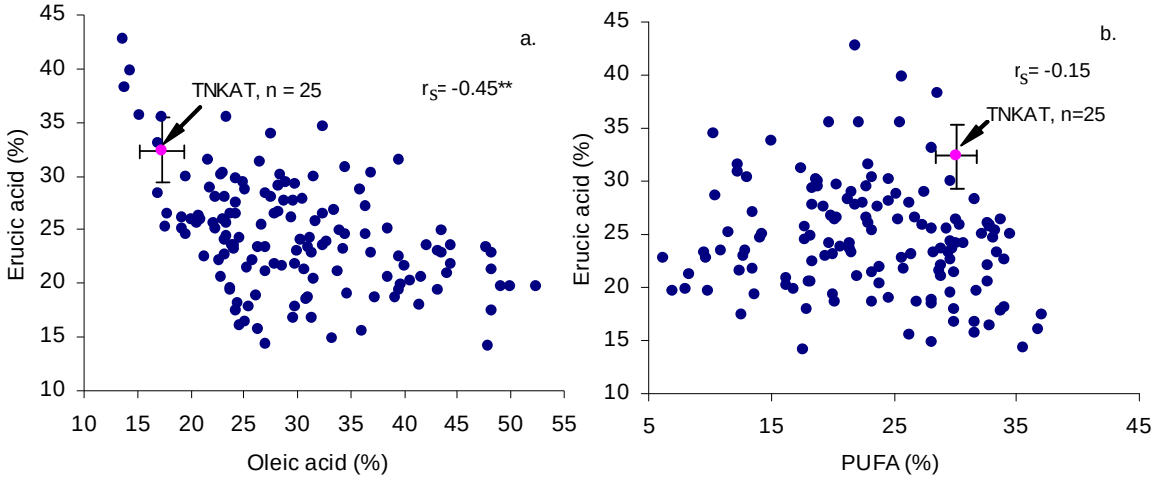

Figure 5a-b Fatty acid compositions of MDE derived from a cross between transgenic (TNKAT) and non-transgenic (6575-1 HELP) high erucic acid rapeseed lines along with the mean of MDE from TNKAT parent (bar showing standard deviation). ** indicates significance at P= 0.01 probability.

DNA was isolated from the single dissected MDE cotyledon after oil extraction and subsequent PCR amplification. DNA extraction was insufficient for PCR amplification when 6M NaI buffer was used. By reducing the molarity of NaI to 4M and 2M in the extraction buffer, the consistency of DNA amplification was improved with 2M NaI giving consistent results. However, DNA isolation was not successful in all cases (19 out of 20 MDE; = 95%), indicating the necessity of an internal control system. This was realized in the present study by performing multiplex PCR (Polymerase Chain Reaction), including PCR primers for the resident, single copy *fad*2 locus (oleic acid desaturase) of the *Brassica* A-genome. This allowed the identification of samples which did not contain DNA in sufficient quantity or quality for successful PCR amplification. To follow the segregation pattern of transgene *Ld-LPAAT* for the DH- (doubled haploid) lines used in experiment 2 (manuscript II), a higher number of segregating MDE genotypes were investigated. In the segregating MDE population the transgene was amplified only in 50% of the genotypes,

confirming upon chi-square test the expected 1:1 segregation of a single copy transgene in a doubled haploid MDE population (Table 2). If two or three genes are segregating, then only 25% and 12.5% of the MDE are expected to carry the positive alleles, respectively. Considering the polygenic inheritance of many agronomic and seed quality traits, the early detection of those MDE genotypes having positive alleles by marker assisted selection enables their early identification and preferential regeneration to plantlets.

Table 2 Segregation of the *Ld-LPAAT* gene in single detached MDE cotyledons in two different F_1-plants (represented by number in bracket) derived from the cross TNKAT x 6575-1 HELP.

Cross Combination	Total MDE	*Ld-LPAAT* (positive)	*Ld- LPAAT* (negative)	χ^2-Value	Remark
TNKAT × HELP (10)	107	57	50	0.46	fit in 1:1 ratio
TNKAT × HELP (13)	31	20	11	2.60	
Total	138	77	61	1.85	

4.2 Early selection of segregating DH and F_2-generation for erucic acid content in the rapeseed breeding programme (Manuscript II, III)

There are different methods to select desirable genotypes with specific fatty acid content. Some methods require a relatively long time, others are quicker. The F_1-plants used in the present experiments were derived from a cross between transgenic and non-transgenic high 22:1 rapeseed lines. They differed in two to three major genes that segregate in succeeding generations (F_2-, F_3- and so on). Therefore, it was necessary to investigate a larger number of individuals to select a homozygous line from the segregating generation. An overview on number of individuals necessary to investigate to identify a specific homozygous genotype in F_2 in comparison to DH is presented in Table 3. It is laborious and requires large space in the green house to maintain and propagate the segregating transgenic lines. To overcome these limitations, DH-population had been produced. Due to restrictions in time and availability of material DH-lines were produced only from F_1-plants of the cross TNKAT x 6575-1 HELP, although the line 361.2B contained two transgene copies. DH technology is presently used in breeding of a number of crop species. This method enables breeders to develop completely homozygous genotypes from heterozygous parents in one single generation, although it is costly.

Table 3 Minimum population size of F_2 and DH for the selection of a specific homozygous genotype for a trait in the case of unlinked loci (α=0.95; adapted from Jansen 1992).

∑Gene	F_2-ratio	F_2-plants	DH-ratio	DH-plants
1	1:3	11	1:1	5
2	1:15	63	1:3	11
3	1:63	191	1:7	23
4	1:255	766	1:15	47
5	1:1023	3067	1:31	95

DH lines in oilseed rape are commonly produced from F_1-plants by microspore culture (Chen and Beversdorf 1990). Presently, efficient genetic investigations can be carried out on specific characters from segregating generations by saving time as DH-methods. The routine methods that could be used for early selection are presented in Figure 6.

Rapid and non-destructive analyses for quality traits like oil, protein and fatty acids in samples are prerequisite for quality breeding programs. Near-infrared-reflectance spectroscopy (NIRS) has been reported as an excellent tool for analyzing such traits in comparison with gas liquid chromatography (GLC) or high pressure liquid chromatography (HPLC). It is faster and reliable, even in small quantity of samples. Velasco and Becker (1998) noted similar results (RSQ) by using smaller amount of samples (300 mg seeds and 60 mg seeds), to that of 3 g samples. NIRS technique could be used for estimating fatty acid composition in single seed of rapeseed (Sato et al. 1998, Niewitetzki et al. 2007). This allows of selection for high erucic acid content in segregating F_2-seed population as indicated in Figure 6

Gas liquid chromatography (GLC) could also be used for early selection of genotypes using half-seed (Thies 1971) and half-MDE cotyledon technique (Nath et al. 2007). GLC technique helps to know the absolute value of all fatty acids of the sample, but it is laborious and costly. NIRS works only with the major fatty acids and requires precise and reliable equations, but it is much faster and non-destructive. However, NIRS cannot be applied to segregating MDE populations.

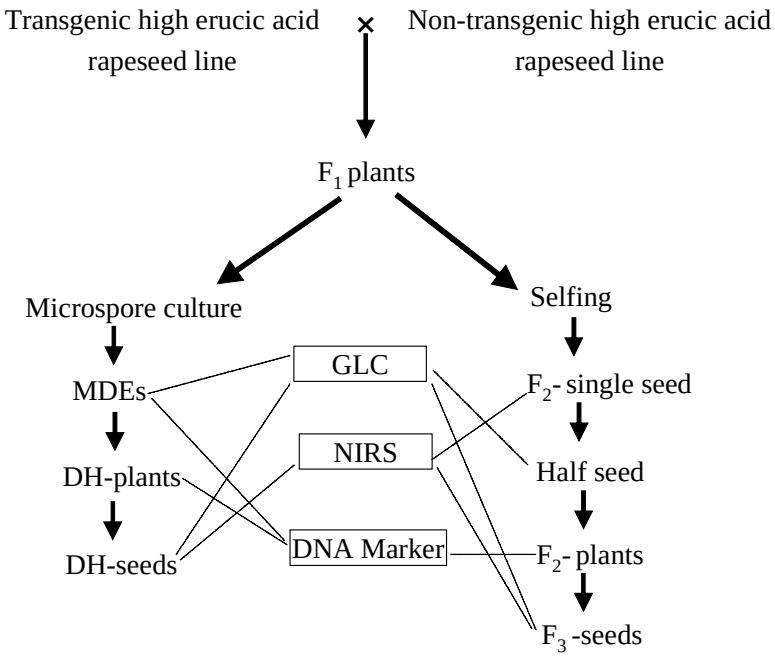

Figure 6 Possible methods of early selection for high erucic acid genotypes in rapeseed breeding programme. Thin lines indicate the possible analytical methods (adapted from Schierholt 2000).

Applying the half-cotyledon technique to the segregating MDE population derived from the cross TNKAT x 6575-1 HELP in the present study did not allow an effective selection for high erucic acid MDE genotypes. No significant correlation was found between the erucic acid content of MDE cotyledons and of the seeds obtained from regenerated DH-lines (Figure 7a). The scatter plots for 22:1 F_2-half seeds vs. F_2-population (F_3-seeds) showed significant positive correlation ($r_s = 0.57^{**}$; Figure 7b) revealed effective selection of high 22:1 plants based on half seed analysis.

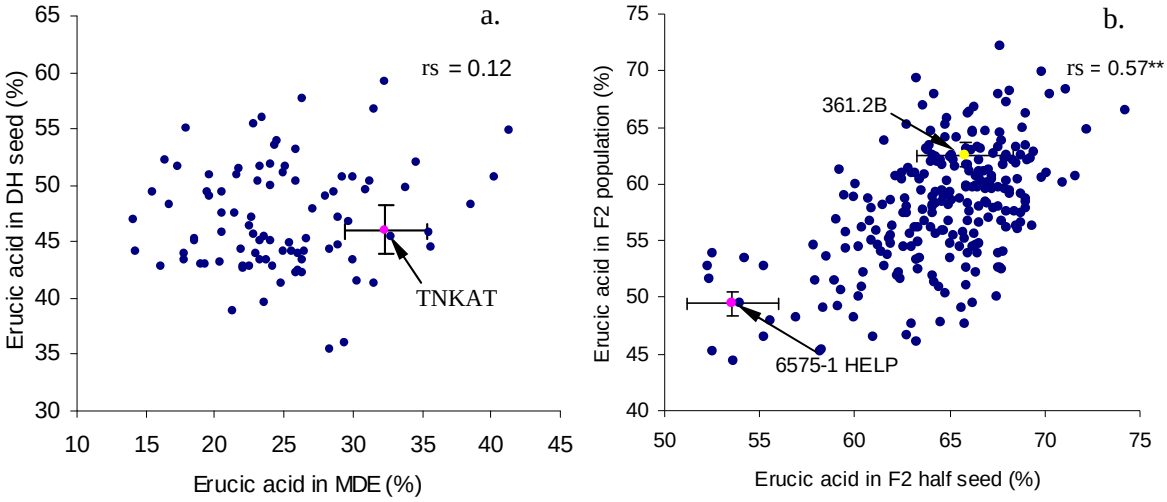

Figure 7a-b Correlation for erucic acid content between MDE and DH-seeds (mean of three plants) and F_2-half seeds and F_2-population (F_3-seeds). ** indicates significance at P= 0.01 probability and bar showing standard deviation.

4.3 Effect of polyunsaturated fatty acid on erucic acid content (Manuscript II, III)

Spearman's rank correlation coefficient analysis showed strong significant negative correlation between erucic acid (22:1) and polyunsaturated fatty acid content for both, the segregating DH (r_s = -0.40**) and the F_2-population (r_s = -0.50**; Figure 8a-b). The regression equations shown in Figure 8 indicate that a reduction of 10% PUFA lead to an increase of 4.2% 22:1 content in the segregating DH-population, whereas a 6.5% increase in 22:1 content was found for the segregating F_2-population. The larger effect in the F_2-population may be due to higher activities of the KCS and LPAAT enzyme of the transgene. The F_3-line III-G-7 with the highest erucic acid content of 72.3% had a by 9.6% lower PUFA content, which is 9.1% more erucic acid compared to 361.2B (Table 4). However, according to the regression equation (Figure 8b) one would have expected only a 6.3% increase in erucic acid content, suggesting the presence of other genetic factors in this line.

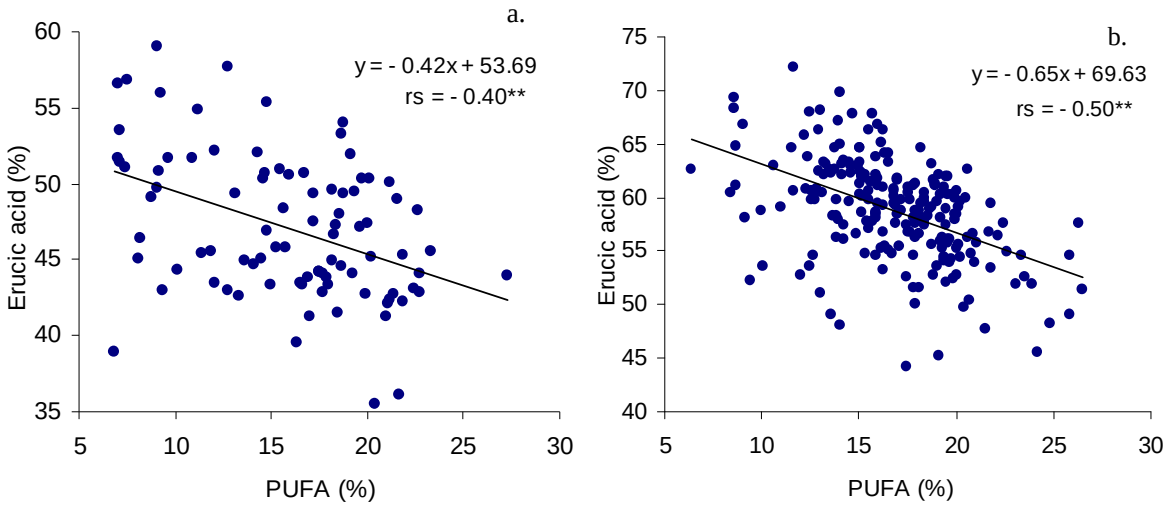

Figure 8a-b Relationship between erucic acid and PUFA content in (a) the segregating DH-population (n = 90) and (b) the segregating F_2-population (n = 220) derived from the cross between transgenic and non-transgenic high erucic acid rapeseed lines.

4.4 Effect of the *Ld-LPAAT* gene on the fatty acid composition at the *sn*-2 triacylglycerol position (Manuscript II, III)

To determine the effect of the *Ld-LPAAT* gene the fatty acid composition of the the *sn*-2 position of the triacylglycerols (TAGs) of selected DH- and F_3- (F_4-seeds) lines were analysed along with their parents. Seed oil was emulsified and digested by lipase enzyme from *Rhizopus arrhizus*, thereby lipids were extracted and *sn*-2 monoacylglycerol (MAG) was separated by thin layer chromatography (TLC; see an example in Figure 9). Fatty acids were extracted from MAG and analysed by GLC after transmethylation.

Seed oil from non-transgenic parent (6575-1 HELP) predominantly (73.3%) contained oleic acid at the *sn*-2 position, while very long chain fatty acid (22:1) was detectable in trace amount only. On the other hand, the oil from the transgenic parents (TNKAT and 361.2B) and the selected best DH- and F_3-lines (F_4-seeds) contained higher amount of 22:1 and correspondingly lower proportion of 18:1 (Table 4). The highest amount 22:1 at *sn*-2 position was found in F_3-line (III-G-7), followed by DH (IV-10-F-6) with the values 65.3% and 40.3%, respectively. These *sn*-2 compositions of the transgenic seed oils correlated with the 22:1-CoA specificity of the expression of *Ld-LPAAT* gene from *L. douglasii*. This result is an agreement with the observation of Weier et al. (1997), Han et al. (2001). Hence, lipid analyses revealed that the introduced *Ld-LPAAT* gene effectively competes with the

endogenous rapeseed enzyme and preferentially incorporates 22:1 into the *sn*-2 position of the glycerol backbone. However, considerable amounts of oleic acid were also detected at the *sn*-2 position, indicating that endogenous *Bn-LPAAT* activity may be limiting for achieving higher erucic acid content at the *sn*-2 of *Ld-LPAAT* over expressing rapeseed lines.

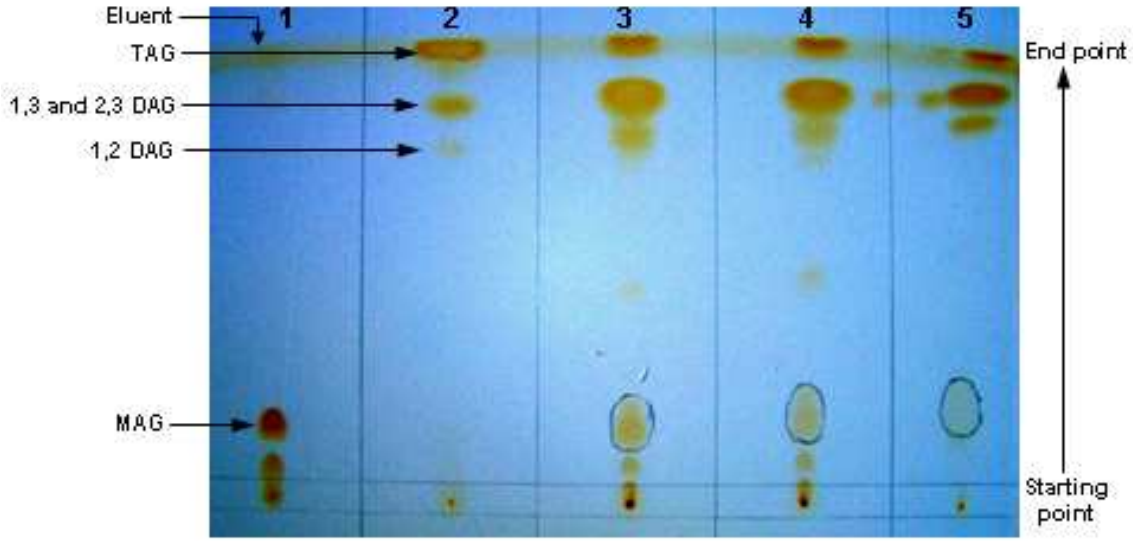

Figure 9 Thin layer chromatography (TLC) plate with different components of triacylglyceride after treatment with (Lanes 3-5) and without (Lane 2) lipase enzyme from *Rhizopus arrhizus* (Lane 1 is standard monoacylglycerol; MAG). DAG: diacylglycerol, TAG: triacylglycerol.

Table 4 Fatty acid composition of seed triacylglycerols and at the *sn*-2 position of one DH line derived from the cross TNKAT x 6575-1 HELP and of one F_3-line (F_4-seeds) derived from the cross 361.2B x 6575-1 HELP along with their parents.

Genotype	Fatty acid composition (%) at *sn*-2						Fatty acid composition (%) in seed oil					
	SFA	18:1	PUFA	20:1	22:1	MUFA	SFA	18:1	PUFA	20:1	22:1	MUFA
TNKAT	3.1	45.9	26.8	1.1	20.8	67.8	4.1	15.9	20.8	10.0	46.1	72.0
361.2B	2.1	34.2	28.3	0.0	31.6	65.8	2.9	8.3	15.3	4.3	63.2	75.8
HELP	4.7	73.3	19.8	0.0	0.5	73.8	3.3	26.5	5.6	11.2	49.6	87.3
DH (IV-10-F-6)	2.4	34.3	17.6	2.8	40.3	77.4	2.6	17.5	9.1	7.8	59.1	84.4
F_3 (III-G-7)	1.2	25.3	6.5	0.0	65.3	90.6	1.9	12.1	5.7	5.0	72.3	89.4

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

4.5 Additional transgenic approaches to increase erucic acid

i. ATP-citrate lyase (ACL):

During oilseed development, significant amounts of sugar provided by photosynthesis are converted to storage triacylglycerols by the developing seed in rapeseed. In oilseed plants, at which the pathway commits the fixed carbon to lipid biosynthesis is the conversion of acetyl-CoA (Ratledge et al. 1997). The acetyl-CoA to be used in fatty acid biosynthesis must be synthesized in the subcellular compartment in which it is to be used, since subcellular membranes are impermeable to acetyl-CoA (Fatland et al. 2005). Recent studies indicate that the acetyl-CoA pool required for de novo fatty acid biosynthesis is primarily generated by the plastidic isoform of the pyruvate dehydrogenase complex (Ke et al. 2000). The fatty acid elongation is cytosolic processes, and cytosolic ATP-citrate lyase (ACL) generates the required acetyl-CoA precursor. The temporal distribution of ATP-citrate lyase (ACL) activity in developing seeds of rapeseed closely paralleled both that of acetyl-CoA carboxylase (ACCase) in the cytosol and the overall rate of lipid biosynthesis (Fatland et al. 2002). In the cytosol, acetyl-CoA can be carboxylated by ACCase to form malonyl-CoA and hence is converted to long chain fatty acids (Figure 10). Therefore, over expression of ATP-citrate lyase (ACL) gene will help to produce more acetyl-CoA in the cytosol from mitochondria, which might have possibility to produce necessary malonyl-CoA for long chain fatty acid biosynthesis.

ii. Cytosolic acetyl-CoA carboxylase (ACCase):

In cytosol, plant fatty acids are synthesized ultimately from the conversion of acetyl-CoA to malonyl-CoA by the action of acetyl-CoA carboxylase (ACCase). Cytosolic acetyl-CoA is metabolized via one of three mechanisms: carboxylation, condensation, or acetylation. In the cytosol, acetyl-CoA can be carboxylated by acetyl-CoA carboxylase to form malonyl-CoA (Figure 10; Fatland et al. 2002). Cytosolic malonyl-CoA is required for the biosynthesis of long chain fatty acids like erucic acid (22:1). Therefore, over expression of cytoplasmic acetyl-CoA carboxylase (ACCase) and ATP-citrate lyase in transgenic High Erucic and Low Polyunsaturated fatty acid (HELP) rapeseed line could help to further increase erucic acid content in the seed oil of rapeseed.

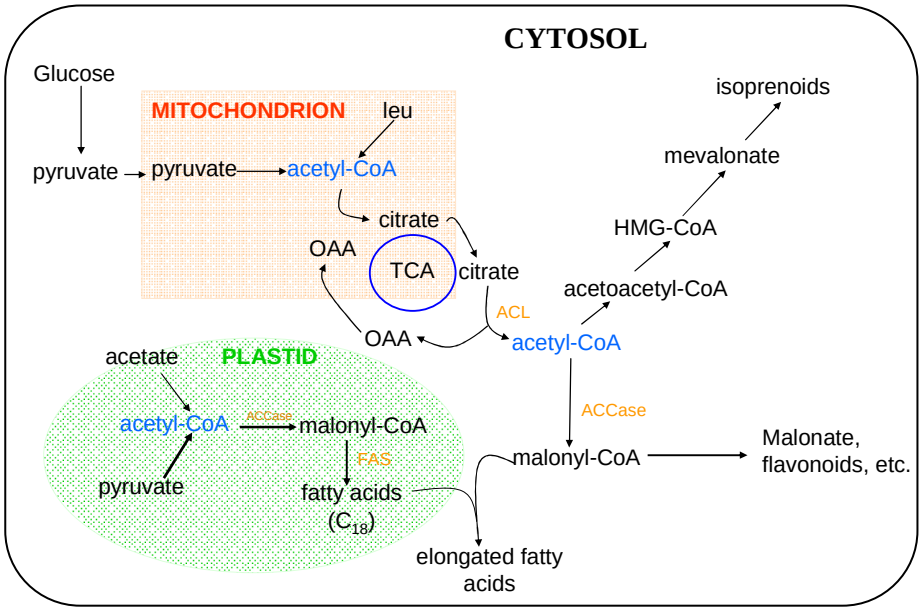

Figure 10 Scheme of putative fatty acids and different metabolites (isoprenoids, malonate, flavonoids, etc.) biosynthetic pathways in plants (adapted from Fatland et al. 2002). ACL is depicted together with a postulated citrate cycle that would provide citrate from the mitochondria. In the cytosol, acetyl-CoA can be carboxylated by ACCase to form malonyl-CoA and hence converted to long chain fatty acids (22:1). FAS: Fatty acid synthase; TCA: tricarboxylic acid cycle; leu: Leucine; ACL: ATP-citrate lyase; ACC: acetyl-CoA carboxylase.

iii. *Brassica napus* lysophosphatidic acid acyltransferase (*Bn-LPAAT*) antisense:

In the course of the glycerolipid synthesis three distinct acyltransferases are responsible for the sequential transfer of acyl groups from acyl thioesters to the glycerol backbone forming triacylglycerol (TAG; Ohlrogge and Browse 1995). Among them lysophosphatidic acid acyltransferase (LPAAT) catalyzes the second acylation reaction so that the central position (*sn*-2) in the biosynthesis of the various glycerolipids, is formed. Therefore, LPAAT substrate specificities are decisive for establishing the fatty acid pattern of TAG. In conventional rapeseed the microsomal LPAAT has a pronounced specificity for 18:1 over other fatty acids. Therefore, the microsomal pathway results in the formation of glycerolipids in which the *sn*-2 position is specifically esterified with oleic acid (Frentzen 1998). The enzyme activity of the endogenous rapeseed LPAAT (*Bn-LPAAT*) competes with the activity of the erucoyl-CoA specific *Ld-LPAAT* from *Limnanthes douglasii*. Down regulation of endogenous *Bn-LPAAT* gene by antisense technique or mutation could help to increase 22:1 content at *sn*-2 position as well as in the seed oil.

5. Summary

Erucic acid (22:1) obtained from the seed oil of high erucic acid rapeseed (HEAR) is of interest for the oleochemical industry. It is used in the plastic film, nylon, lubricant and emollient industries. Currently available conventional HEAR cultivars contain 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. 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 22:1 content compared to the parental HEAR genotype. This indicated that the β-ketoacyl-CoA synthase (KCS; *fae*1 gene) activity, the enzyme responsible for the fatty acid elongation from oleic acid (18:1) to eicosenoic acid (20:1) and to erucic acid, may be limiting. Furthermore, the rapeseed native lysophosphatidic acid acyltransferase (*Bn-LPAAT*) does not accept erucic acid as a substrate for insertion into the central *sn*-2 position of the triacylglycerol backbone. Here, the LPAAT enzyme from *Limnanthes douglasii* (*Ld-LPAAT*) has been found to preferentially insert erucic acid into the *sn*-2 position.

The main objective of the present study was to increase 22:1 content in the seed oil of rapeseed through the genetic combination of alleles from a HELP rapeseed form with transgenic rapeseed over expressing the *Bn-fae*1 and expressing the *Ld-LPAAT* gene and to study trait inheritance in segregating populations.

To this end, two separate experiments were conducted using HELP line and two different sources of transgenic rapeseed. The two transgenic sources were: TNKAT, a resynthesised high 22:1 rapeseed line carrying one transgene copy of *Ld-LPAAT-Bn-fae*1, and 361.2B, a winter rapeseed line carrying two transgene copies. In both cases the *Ld-LPAAT* and the *Bn-fae*1 gene were under control of the seed specific napin promoter. In the first experiment, F_1-plants derived from the cross between TNKAT and 6575-1 HELP were used to produce microspore derived embryos (MDE). Six weeks after microspore culture initiation, a single cotyledon was dissected from each MDE and used for fatty acid analysis and DNA extraction. The remaining parts of the embryos were regenerated to plantlets. Those were transferred to the green house. Seeds were obtained after colchicine treatment and self-

pollination of the plants. A green house experiment was performed with ninety doubled haploid (DH) lines and the parental genotypes in a complete randomized block design with three replicates. At onset of flowering, plants were bagged to secure self-pollination. Seeds harvested from individual plants were analysed by gas liquid chromatography for fatty acid composition and trierucoylglycerol (trierucin) and by Near-Infrared-Reflectance Spectroscopy (NIRS) for oil and protein content. The fatty acid elongase (*fae*1) genes were amplified by PCR and sequenced from a diverse collection of *Brassica rapa* (*fae*1.1; A-genome) and of *Brassica oleracea* (*fae*1.2; C-genome) to develop locus specific primers. Those were then used to separately amplify and sequence the *fae*1.1 and *fae*1.2 alleles from TNKAT and 6575-1 HELP. Detected single nucleotide polymorphisms among the two *fae*1.1 alleles were used to develop *fae*1.1 allele specific primers. Those primers were used to follow the segregation of the *fae*1.1 alleles in the DH population.

In the second experiment F_1-plants derived from the cross between transgenic 361.2B and 6575-1 HELP were used to produce F_2-seeds. 220 randomly chosen F_2-seeds were sown in the green house and F_3-seeds were harvested from the selfed F_2-plants. F_3-seeds were analyzed for quality traits as described above. F_3-seeds from 41 F_2-plants with the highest erucic acid content in the seed oil along with the parental lines were tested in a green house experiment in a randomized complete block design with five replicates. F_4-seeds obtained after selfing were analyzed for seed quality traits as described above.

The results of the first experiment showed that from single detached cotyledons from six weeks old MDE, fatty acids and DNA can be extracted for gas liquid chromatographic and PCR analyses, respectively. This allows for the early identification of valuable MDE genotypes in segregating populations at an early stage of development in the Petri-dish and their preferential regeneration to plants. PCR analyses using specific primers for the *Ld-LPAAT-Bn-fae*1 transgene confirmed the presence of a single transgene copy by its 1: 1 segregation in the MDE population. However, results from fatty acid analysis did not reveal a pronounced effect of the transgene on the erucic acid content of the MDE and were not correlated with the erucic acid contents of the seeds obtained from the corresponding DH-plants in the green house. Testing of the ninety doubled haploid plants showed surprisingly that the presence of the transgene had a negative effect on erucic acid content. The 54 DH-lines with the transgene had a by 2.3% lower mean erucic acid content than the 36 DH-lines

lacking the transgene. A strong negative correlation was found between erucic acid and polyunsaturated fatty acid content (18:2 + 18:3; r_s = -0.40**). A separation of the DH-population into half according to their PUFA (Polyunsaturated fatty acid) content revealed that the DH-lines with a lower PUFA content (mean = 11.4%) had a by 3.7% higher erucic acid content than the DH-lines with a high PUFA content (mean = 19.5%). The best DH-line had 59% erucic acid in the seed oil. This was 9% more than the higher erucic acid parent 6575-1 HELP. The development of locus and subsequently allele specific PCR primers for the two endogenous *Bn-fae*1 genes allowed distinguishing the *fae*1.1 alleles (*Brassica* A-genome) of TNKAT and 6575-1 HELP in the DH-population. However, no significant difference of the *fae*1.1 alleles from the two parents on erucic acid content was found.

In the second experiment the results from the fatty acid analysis of the F_2-plants (F_3-seeds) showed a large variation in erucic content ranging from 44 to 72%. The frequency distribution of the F_2-population showed a normal distribution without any separable classes. Results from PCR and trierucin analysis confirmed the presence and segregation of two transgene copies in the F_2-population. A strong significant negative correlation between erucic acid (22:1) and PUFA content (r_s = -0.50**) was found. F_3-lines (F_4-seeds) were identified which contained up to 72% erucic acid (mean) in the seed oil. This compares favourably with the 63.2% of the transgenic parent 361.2B and 49.6% of the non-transgenic 6575-1 HELP parents. The best F_3-lines had a PUFA content of only 5 to 6%, which is about 10% lower than the PUFA content of parent 361.2B. Results from regression analysis of the F_2-population indicated that reduction in PUFA content by 10% led to a 6.3% increase in erucic acid content. The 72% erucic acid content achieved in the present study mark a major breakthrough in breeding high erucic acid rapeseed. This material is valuable for future approaches to increase erucic acid content in rapeseed beyond the levels currently obtained. Additional promising transgenic approaches include over expression of ATP-citrate lyase (ACL) and cytosolic acetyl-CoA carboxylase (ACCase) as well as antisense expression of *Brassica napus* lysophosphatidic acid acyltransferase (*Bn-LPAAT*).

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Manuscript I

Short communication

Early, non-destructive selection of microspore derived embryo genotypes in oilseed rape (*Brassica napus L.)* by molecular markers and oil quality analysis

Ujjal Kumar Nath[1], Mohammed C. M. Iqbal[2], Christian Möllers[1]*

[1] Department of Crop Sciences, Plant Breeding, Von Siebold Str. 8, D-37075 Göttingen, *cmoelle2@gwdg.de

[2] Plant Reproductive Biology, Institute of Fundamental Sciences, Hantane Road, Kandy, Sri Lanka

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UKN performed the experiments, analysed the data and wrote the manuscript. All authors conceived the experiments and improved the manuscript.

Abstract

In oilseed rape (*Brassica napus* L.) breeding, microspore culture is frequently applied for the immediate regeneration of homozygous doubled haploid plants. From the regenerated microspore derived embryos (MDE) usually only a smaller subset of around 200 are used for plantlet regeneration and cultivation in the green house until seed harvest, without there being any knowledge their quality traits and agronomic performance. The random selection of MDE implies that valuable rare recombinant genotypes may be discarded at an early stage of *in vitro* culture. We report here on the development of a simple protocol for simultaneously extracting lipids (for oil quality analysis) and the isolation of DNA (for marker assisted selection) from single cotyledons dissected from MDE under aseptic conditions, thus keeping the rest of the embryo *in vitro* for plantlet regeneration. Neither the fatty acid extraction nor the transmethylation with sodium methylate at high pH did interfere with subsequent DNA isolation. The feasibility of the protocol was tested using MDE from a cross segregating for two linked transgenes, *fae*1 and plsC, affecting the fatty acid composition. Multiplex-PCR was performed with specific PCR-primers for the *pls*C-gene and with locus specific primers for a resident single copy *fad*2 gene. The amplification of the *fad*2 gene provided a control for the presence of DNA in sufficient quantity and quality, whereas the amplification of the *pls*C gene showed a 1:1 segregation expected for a single copy transgene in a segregating doubled haploid population. The early identification of the 50% MDE genotypes carrying the desired transgenes along with a high expression of the trait allows their early selection for plantlet regeneration.

Since the development of the isolated microspore culture technique for the production of homozygous doubled haploid (DH) lines in oilseed rape (*Brassica napus* L.) by Lichter in 1982, this method has gained considerable importance in rapeseed breeding programmes. The method has been optimised variously (Zaki and Dickinson 1991, Iqbal et al. 1994, Chen et al. 1994, Möllers et al. 1994). Presently, hundreds to thousands of microspore derived embryos (MDE) can be obtained from a single microspore preparation. Usually only small fractions of 200-300 of these MDE are sub-cultured *in vitro* to regenerate plantlets which are then transferred to the green house for seed production. However, regeneration of *in vitro* plantlets, their transfer to soil and cultivation in the green house until seed harvest is quite laborious, time consuming and requires much of the often limited green house space.

The subset of MDE genotypes used for plantlet regeneration represents a random sample of the total number of regenerated MDE, without there being any knowledge about their quality traits and agronomic performance. Thus, many undesired genotypes go through plantlet regeneration and the costly green house process. It also implies that valuable rare recombinant genotypes may be discarded at an early stage of *in vitro* culture. Any method that could be applied to determine useful agronomic or seed quality traits at an early stage of *in vitro* culture would definitely increase the frequency of valuable genotypes among the total number of regenerated MDE. Hence, marker-assisted selection (MAS) at the *in vitro* stage would screen a larger population of MDE and reduce green house costs.

Selection *in vitro* for seed oil quality traits is possible in segregating populations of MDE (Albrecht et al. 1995). Fatty acid composition was determined by gas liquid chromatography (GLC) from single cotyledons, dissected from MDE. The rest of the MDE were maintained *in vitro* and regenerated to plantlets. Unequivocal selection for absence of, intermediate and high erucic acid (Albrecht et al. 1995) and oleic acid (Möllers et al. 2000) was possible in segregating MDE populations. However, the application of this early *in vitro* selection system is limited to those traits that can be rapidly and cost effectively analysed and for which a close correlation between the MDE and the seeds from the regenerated plants has been shown. Furthermore, phenotypic results may be confounded by MDE genotype x environment interactions, i.e. *in vitro* culture conditions may differently affect the fatty acid composition of storage lipids in individual MDE genotypes. Such shortcomings are overcome if MAS is applied concomitantly.

We report here on the simultaneous isolation of storage lipids and DNA from a single dissected MDE cotyledon of oilseed rape, suitable for fatty acid analysis and PCR amplification and regeneration of the rest embryo, allowing selection in segregating MDE populations.

To this end, the homozygous transgenic resynthesized high erucic acid rapeseed line RS306, carrying a single T-DNA with two chimeric genes (Han et al. 2001) was crossed to the high erucic acid winter rapeseed line 6575-1 (Sasongko and Möllers 2005). In the transgenic RS306, the first chimeric gene was the *Bn-fae*1.1 gene (accession number AF274759) and the second was the *pls*C gene from *Limnanthes douglasii* (X83266), both under control of the seed specific napin promotor. F1-plants from the above-mentioned cross were used for microspore culture as described in Iqbal et al. (1994). Three weeks after microspore culture, MDE were transferred to fresh medium at a density of 10 - 15 embryos in petri dishes (9 cm) with 17 ml of NLN medium containing 13% sucrose to allow storage lipid accumulation and to prevent germination. Three weeks after transfer, embryos were well developed and a single cotyledon was dissected under aseptic conditions. The remaining embryo was cultured for plantlet regeneration. The dissected cotyledon was dried overnight at 40° C in 1.5 ml Eppendorf tubes. Lipids were isolated from dried cotyledons or part, fatty acids were transesterified with sodium methylate and used for gas chromatographic analysis (Albrecht et al. 1995). DNA was isolated from the oil extracted residue of the cotyledons by homogenising extraction buffer containing NaI (2M, 4M or 6M) and 0.5% sodium N-lauroyl sarcosine (Ishizawa et al. 1991). RAPD assay and multiplex PCR for the two chimeric genes were performed on DNA from the single MDE cotyledons. PCR primers for the *plsC*-gene (*Ld-LPAAT*) from *Limnanthes douglasii* were LPAAT-F: 5′-CCGCAACAGGAGACAACTAAA-3′ (Genbank: X83266) and LPAAT-R 5′-TATTGGGAGATGTGACTGAAG-3′ (www.lag-gentechnik.de/dokumente/ok_laurical_raps.pdf; site last visited 08.05.2006). 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 2003). Primer sequences were FAD2A-F 5′-ATGGGTGCAGGTGGAAGAATG-3′ and FAD2A-R 5′-CAGTTTCTTCTTTGCTTCATAAC-3′.

Results and Discussion

High osmotic potential culture medium prevented precocious MDE germination and allowed the development of large embryos. These contained sufficient amounts of DNA and lipids in a single cotyledon of 20 - 30 mg, of which however only 4 - 18 mg were used for lipid and DNA extraction. In gas liquid chromatography the total peak area values for the sum of fatty acids ranged between 27 and 231 (Table 1), allowing good peak resolution and accurate determination of the fatty acid composition.

Table 1 Effect of the seed specific expression of the *pls*C gene and the *fae*1 gene on the erucic acid content of single detached MDE cotyledons of the cross RS306 x 6575-1

MDE Genotype	N	Total fatty acid area		22:1 (%)	
		Range	Mean	Range	Mean
*pls*C+*fae*1 positive	73	27-228	80	14.1 - 42.7	25.3a
*pls*C+*fae*1 negative	65	29-231	88	14.7 - 30.0	23.5b

a,b – means are significantly different (t-test)

The dried pellet obtained after oil extraction and transesterification was used for DNA extraction and subsequent PCR amplification. Adequate DNA quantity and quality were demonstrated by amplification using the RAPD primer OPAK-14. The rapid DNA isolation method of Edwards et al. (1991) developed for minute amounts of tissue did not provide consistent results with tissues of MDE. However, the method described by Ishizawa et al. (1991) proved to be as simple and gave good results following some minor modifications. Ishizawa et al. (1991) used a high concentration of NaI (6M) in the extraction buffer. Using the dried and oil extracted pellet from the MDE cotyledons, the DNA extraction with 6M NaI buffer was insufficient and PCR amplification was inconsistent (Table 2).

By reducing the molarity of NaI to 4M and 2M in the extraction buffer, the consistency of DNA amplification was improved with 2M NaI giving consistent results. The high molarity of the original Ishizawa et al. (1991) protocol prevented pelleting of cell debris even following extended centrifugation. However, single cotyledons from germinated seeds (mean dw 1 - 1.5 mg), produced good and consistent PCR amplification with 6M NaI buffer

(results not shown), indicating that the high dry matter of the MDE (ca. 10mg) may have affected DNA extraction. Sakamoto et al. (2000) similarly reported successful DNA amplification with single seeds (3 - 5 mg) and young leaves (5 - 10 mg) of *Brassica* with the method of Ishizawa et al. (1991). The method is also much simpler than the one developed for DNA extraction from MDE by Horn and Rafalski (1992). In the present study, it was shown that neither the fatty acid extraction nor the transmethylation with sodium methylate at high pH did interfere with the subsequent DNA isolation. PCR amplification of DNA isolated from the cotyledons with and without fatty acid extraction did not show any differences. The DNA isolation from MDE cotyledons by this method does not require fresh or liquid nitrogen frozen material: oven drying at 40˚ C for 24 hours proved sufficient.

Table 2 Consisteny of PCR amplification of DNA by different extraction methods using single dissected cotyledons of microspore derived embryos of *B. napus*

Extraction buffer	Cotyledons from MDE		
	Mean dw (mg)	Range (mg)	Consistency of amplification
6 M	9.9	6 - 15	6/18
4 M	6.8	4 - 10	10/18
2 M	9.5	5 - 18	19/20
Edwards buffer	9.5	4 - 18	4/23

Application of rapid DNA extraction protocols to minute amounts of tissue are necessary for efficient handling of large number of samples. However, as shown in Table 2 this may lead in some cases to inconsistent results, which may be due to lack of template DNA in the PCR or due to impurities affecting the polymerase activity. It is therefore recommended to perform two amplifications, in addition including primers for a gene naturally present in the genome (‘Multiplex PCR’). For obvious reasons this internal control should preferentially be a single copy gene (Mannerlöf and Tenning 1997). In the present study we have used PCR primers for the *fad*2 gene, which specifically amplify the single copy *fad*2 alleles of the A-genome of the amphidiploid oilseed rape. To test the applicability of the ‘Multiplex PCR’ we used a MDE population derived from a F1-plant of a cross between a homozygous singly copy transgenic and a non-transgenic *Brassica napus* line. Following fatty acid and subsequent DNA extraction according to the above-described method, the *fad*2 gene was

amplified in all samples except sample 4, indicating the reliability of the DNA-extraction method (Fig. 1). The *pls*C gene was amplified in only 50% of the genotypes, confirming the expected 1:1 segregation for a single copy transgene in a doubled haploid MDE population.

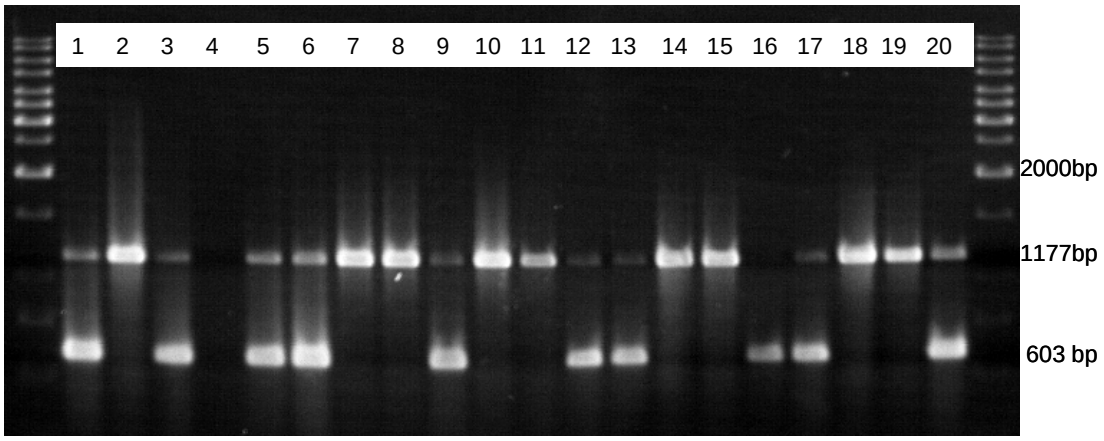

Fig. 1 Multiplex PCR of a 603bp fragment of the *pls*C gene together with internal control amplification of the 1.2kb *fad*2 gene sequence using DNA extracted from 20 MDE (Lanes 1-20) segregating for the *pls*C gene

The expression of the *pls*C+*fae*1 genes under control of the napin promotor has previously been shown to cause only a small increase in the erucic acid content (22:1) of the seed oil of transgenic rapeseed plants (Han et al. 2001). In the present study this has been confirmed using the MDE system. A relatively small, but significant 22:1 increase was observed for the group of MDE carrying the *pls*C+*fae*1 genes (Table 1). However, in the *pls*C+*fae*1 positive MDE group, the range of 22:1 content was much larger. The *pls*C+*fae*1 positive MDE genotypes and especially those with a very high 22:1 content are preferentially regenerated to plantlets and transferred to the green house so that trait expression in seeds derived from those plants can be studied. The detection of the 50% MDE genotypes carrying the desired transgenes in combination with a high expression of the trait enables their early selection for plantlet regeneration. Hence, much labour and green house space are saved during plantlet transfer to soil and their cultivation in the green house.

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Manuscript II

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

ABSTRACT

Erucic acid (22:1) is a valuable renewable resource that has several applications in the oleochemical industry. High erucic acid rapeseed (HEAR) contains around 50% 22:1. For its technical use it is desirable to increase the 22:1 content and to decrease the eicosenoic acid (20:1), the polyunsaturated fatty acids content (PUFA, 18:2 + 18:3), and saturated fatty acids (16:0 + 18:0) content. In the present study, the transgenic resynthesised rapeseed line TNKAT, over expressing the fatty acid elongase (*fae*1) gene in combination with *Ld-LPAAT* gene from *Limnanthes douglasii* was crossed with the high erucic acid winter rapeseed line 6575-1 HELP (High Erucic and Low Polyunsaturated fatty acid) having low content of polyunsaturated fatty acids (50% 22:1, 7% PUFA). The hypothesis was that combination of the involved genes should lead to a reduced oleic acid (18:1) desaturation and to an increased availability of oleoyl-CoA, which should result in enhanced 22:1 synthesis. Microspores from F_1-plants were cultured and doubled haploid (DH) plants were obtained. The inheritance and variation of 22:1 in seed oil were studied in DH-lines with 3 replicates in the green house. Erucic acid content varied from 35-59% in the segregating DH-lines, 46-48% in TNKAT and 49-52% in 6575-1 HELP parental lines. Segregation pattern suggested that inheritance of erucic acid content was controlled by one major locus following 1:1. Transgene *Ld-LPAAT-Bn-fae*1.1 showed a 1:1 segregation confirming the presence of a single transgene copy. Transgenic DH-lines produced up to 8% trierucolyglycerol (trierucin), however significant negative effect on the 22:1 content was found in the DH-population. This result indicates that the ectopic *fae*1.1 gene may not be functional. Dividing the DH-population into half according to their PUFA content showed that an 8.1% reduction in PUFA content resulted in a 3.7% increase in erucic acid content. Locus specific PCR primers were developed for the two resident *fae*1.1 (A-genome) and *fae*1.2 genes (C-genome) of *Brassica napus*. Sequencing of the *fae*1.2 gene of TNKAT and of 6575-1 HELP revealed no polymorphisms. However, two single nucleotide polymorphisms were found for the *fae*1.1 gene. Allele specific *fae*1.1 PCR primers allowed following the segregation of the TNKAT and the 6575-1 HELP *fae*1.1 allele in the DH-population. However, presence of neither *fae*1.1 allele from TNKAT nor 6575-1 HELP did leave to a difference in erucic acid content. The best DH-line had with 59% erucic acid

content a more than 9% higher value than parent 6575-1 HELP. Together, the results indicate that over expression of a functional chimeric *Bn-fae*1.1 gene could further enhance erucic acid content in seed oil of this DH-line.

1. INTRODUCTION

Modifying the fatty acid composition of *Brassica* seed oil to increase its value as nutritional or as industrial oil has been a major objective in *Brassica* breeding programs worldwide. The conventional approach to fatty acid modification has explored natural variations or induced mutations occurring in the same plant species or close relatives within the *Brassica* genus (Scarth and Tang 2006). There is interest in developing germplasm in the Brassicaceae to provide a source of high erucic acid rapeseed oil for use as an industrial feedstock. Several types of *Brassica* oil with altered levels of the long chain fatty acid, erucic acid (22:1) have been developed (McVetty et al. 1999, Sasongko and Möllers 2005). However, in *B. napus* and most members of the Brassicaceae, the content of erucic acid in the seed oil is limited by the fact that erucoyl moieties are typically excluded from the *sn*-2 position of the triacylglycerol molecule. At least part of this limitation has been attributed to the specificity of the *sn*-2 acyltransferase LPAAT (Lysophosphatidic acid acyltransferase) in *B. napus*, which is incapable of utilizing erucoyl-CoA as an acyl donor (Taylor et al. 1992). This *sn*-2 exclusion limits erucic acid and eicosenoic acid (20:1) content to a total of 66% and prevents the synthesis of trierucin (Frentzen 1993, Katavic et al. 2001).

To overcome this limit, combined efforts in plant breeding and genetic engineering have been undertaken. The gene of an erucoyl-CoA preferring *sn*-2 acyltransferase *Ld-LPAAT* from meadowfoam (*Limnanthes douglasii*) has been successfully cloned and over expressed in rapeseed (Brown et al. 1995, Hanke et al. 1995, Lassner et al. 1995, Brough et al. 1996, Friedt and Lühs 1998). However, this achievement was not accompanied by an increase in erucic acid content in the seed oil. It is believed that the initial reaction of the four steps elongation process is rate-limiting in the biosynthesis of erucic acid (Cassagne et al. 1994). β-ketoacyl-CoA synthase (KCS) has been proposed as a candidate gene for explaining the erucic acid level in rapeseed. Studies of *Arabidopsis thaliana* mutants deficient in very long chain fatty acid (VLCFA) showed that the fatty acid elongase (*fae*1) gene product was required in the seeds for the elongations from 18:1 to 22:1 (Kunst et al. 1992, James et al. 1995). The two genes *Bn-fae*1.1 and *Bn-fae*1.2 mapped in the *B. napus* representing the

parental species *B. rapa* (A-genome) and *B. oleracea* (C-genome) fatty acid elongase (*fae*1) gene, respectively, showed polymorphism (Fourmann et al. 1998). The *fae*1 gene encoding the KCS (ß-ketoacyl-CoA synthase) has been cloned from a range of plant species and has been ectopically expressed in high erucic acid rapeseed (HEAR), which, however, showed only a minor increase in 22:1 content (Katavic et al. 2001, Han et al. 2001). Even in combination with the expression of the *Ld-LPAAT* no substantial increase in the 22:1 content has been found (Han et al. 2001).

There is some evidence that the cytosolic pool of available oleoyl-CoA or malonyl-CoA may limit the elongation (Bao et al. 1998, Domergue et al. 1999). Sasongko and Möllers (2005) combined the genes for high erucic acid content (cv. Maplus) with those for high oleic acid/low polyunsaturated fatty acid content (Schierholt et al. 2001) in order to increase the pool of oleoyl-CoA available for fatty acid elongation. However, no significant change in the erucic acid content was observed. Based on these results, it was assumed that the KCS activity may be limiting the erucic acid synthesis in those rapeseed forms.

To test this hypothesis a resynthesised transgenic high 22:1 rapeseed line, expressing a single copy of the *Brassica napus fae*1 gene (*Bn-fae*1) in combination with a 22:1-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 a non-transgenic High Erucic acid and Low Polyunsaturated fatty acid (HELP) line 6575-1 (Sasongko and Möllers 2005). Thereby, the low content of polyunsaturated fatty acids is inherited by one major and probably two to three minor genes (Schierholt et al. 2001, Sasongko und Möllers 2005). This indicates that a large number of F_2-plants need to be cultivated in the green house to identify a specific homozygous genotype with a high probability. As an alternative way to overcome these problems, doubled haploid (DH) lines could be produced through microspore culture (Lichter 1982, Fletcher et al. 1998).

In the present study, F_1-plants of the above mentioned cross was used to produce DH-lines. Those were then grown along with parental lines in a replicated experiment in the green house to study the inheritance of erucic acid content and other seed quality traits. Another objective was to develop locus and allele specific PCR primers to follow the segregation of the erucic acid alleles at the endogenous *fae*1.1 (*Brassica rapa* L.) and the *fae*1.2 (*Brassica oleracea* L.) loci.

2. MATERIALS AND METHODS

2.1 Plant materials

TNKAT: A resynthesised transgenic winter rapeseed line (RS306) was derived from an interspecific cross between Yellow Sarson (*Brassica rapa*) and cauliflower (*Brassica oleracea* sp. *capitata*) cv. Super Regama as outlined by Lühs and Friedt (1994). RS306 was used for *Agrobacterium* mediated transformation to produce TNKAT line carrying a single transgene copy (chimeric *Bn-fae*1.1 with *Ld-LPAAT*, both control under 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, 7% 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 doubled haploid 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 x 6575-1 HELP. They were used as donors for microspore culture, which was performed according to a protocol described by Fletcher et al. (1998). First generation DH-lines were produced during the period December 2004 to June 2005.

2.2 Methods

2.2.1 Growing of the plants for green house experiment

A green house experiment was performed during the period August 2006 to April 2007 (from sowing to harvest). Ninety doubled haploid (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 five replications, and allowed to grow for 3 weeks. For vernalisation, seedlings were then transferred to 4ºC temperature with 8 hours (hrs) light for 8 weeks.

2.2.2 Conducting the green house experiment

Following vernalisation plantlets were transferred to 9 cm diameter pots containing normal compost soil. Green house experiment was conducted following a randomized complete block design with 3 replications using 3 plantlets for each DH-line and parents. Each steel

bench (table) inside the green house represented a complete block consisting of all genotypes (DH and parents). Plantlets were allowed to grow in the green house providing 16 hrs day-light by using additional 400 Watt Sodium-steam lamp. Temperature during the day was varied from 20ºC to 25ºC and night was from 10ºC to 15ºC, 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 vapour supply was constant during the whole experiment to avoid fungal diseases. Self-fertilisation 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.

2.2.3 Data collection

i. 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 were calculated for each individual plant.

ii. Trierucin and fatty acids analysis and determination of oil and protein content

Bulked seed samples (150 mg) were analysed for trierucin (C_{69}; EEE) content of the DH seed samples by high temperature gas liquid chromatography (HT-GLC) analysis of fatty acid esters according to the method described by Möllers et al. (1997). The analysis was done using silicon capillary column RTX-65TG (Restek no. 17005) 15 m x 0.25 mm i.d. (0.1 μm film thickness). Remaining part of the sample after taking for trierucin analysis was transferred to a new tube and left on a hot plate at 37.5°C over night to evaporate. Fatty acids composition was analysed using the samples by gas liquid chromatography 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 from the sum of 16:0 + 18:0, polyunsaturated fatty acids content (PUFA) was calculated from the sum of 18:2+18:3 and monounsaturated fatty acid content (MUFA) was calculated from 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). Values were adjusted for 14 mm PVC adapter using regression equations and expressed on seed dry matter basis.

iii. DNA isolation and PCR amplification of the transgene *Ld-LPAAT*

100 mg fresh leaf sample was quickly frozen by dipping in liquid nitrogen (-96ºC) and homogenised with the plastic pestle in 300 µl of extraction buffer. The extraction buffer consisted of 2M NaI, 13 mM EDTA, 0.5% sodium N-laurolyl sarcosine, 26 mM Tris-HCl, pH 8.0 (modified; Ishizawa et al. 1991). To have a positive control for the presence of DNA in the PCR mix, a locus-specific primer pair was used for the *fad*2 gene of the *Brassica napus* A-genome (*B. rapa*) (Spiekermann 2003). 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 90V for 5 hours 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.

iv. Development of *fae*1 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 bp 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 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 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 sec.

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	1070	*fae*1 promotor / *fae*1 coding sequence
24	TCAACCTTTGCTTCTTTCCG	25	GAGAAACATCGTAGCCATCAAA	55.0	1537	*fae*1.1 locus specific / *fae*1 coding sequence
26	TCAACCTTTGTTTCTTTCCA	27	AGAACACCATTGCATTCTTT	50.0	1572	*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
* 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.

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*

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

6575-1 HELP Primer 43/44

CGCATTTTTATTTAAAATTTGTAAACTTTTTTGGTCAAAGAACATTTTGTAATTAGAGACAGAAATCTAGACTCT
-323
TTATTTGGAATAATAATAATAAAGAAGATATTTTGGGCAATGAATTTATGATGTTATGTTTATATAGTTTATTTC
ATTTTAAATTGAAAAGCATTATTTTTATCGAAATGAATCTAGTATACAATATATTTGTTTTTTTCATCAGATACT
TTCCTATTTTTTGGCACCTTTCATCGGACTACTGATTTATTTCAATGTGTATGCATGCATGAGCATGAGTATACA
CATGTCTTTAAAAATGCATGTAAAGTGTAACGGACCACAAAAGAGGATCCATACAAATACATCTCATCGCTTCCA
1
TTACTATTCTCCGACACACACACTGAGCA**ATG**ACGTCCGTTAACGTAAAGCTCCTTTACCATTACGTCATAACCA
ACCTTTTCAACCTTTGCTTCTTTCCGTTAACGGCGAT

TNKAT Primer 43/44

CTTTTTATTTAAAATTTGTAAACTTTTTTGGTCAAAGAACATTTTGTAATTAGAGACAGAAATCTAGACTCTTTA
-323
TTTCGAATAATAATAATAAAGAAGATATTTTGGGCAATGAATTTATGATGTTATGTTTATATAGTTTATTTCATT
TTAAATTGAAAAGCATTATTTTTATCGAAATGAATCTAGTATACAATATATATGTTTTTTTCATCAGATACTTTC
CTATTTTTTGGCACCTTTCATCGGACTACTGATTTATTTCAATGTGTATGCATGCATGAGCATGAGTATACACAT
GTCTTTAAAAATGCATGTAAAGTGTAACGGACCACAAAAGAGGATCCATACAAATACATCTCATCGCTTCCACTA
1
CTATTCTCCGACACACACACTGAGCA**ATG**ACGTCCGTTAACGTAAAGCTCCTTTACCATTACGTCATAACCAACC
TTTTCAACCTTTGCTTCTTTCCGTTAACGGCGAT

Position of Forward Primer 46: GAGACAGAAATCTAGACTCTTTATTTG
Position of Reverse Primer 44: TCTTTCCGTTAACGGCGAT

2.2.4 Statistical analysis

Analysis of variance was performed using the Plant Breeding Statistical Program (PLABSTAT, Version 2N, Utz 2007) with the following model:

$$Y_{ij} = g_i + r_j + \varepsilon_{ij}$$

where: Y_{ij} was observation of genotype *i* in replicate *j*; g_i and r_j were the effects of genotype *i* and replicate *j*, respectively and ε_{ij} was the residual error of genotype *i* in replicate *j*. 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 heritability was calculated from the variance components using following formula:

$$h^2 = \frac{\acute{o}^2_G}{\acute{o}^2_G + \acute{o}^2_E / r}$$

where, h^2 = heritability; σ^2_G = genetic variance; σ^2_E = error variance and r = number of replicates.

The number of transgene copy segregated among the DH-lines was calculated using χ^2 test for a fixed ratio hypothesis described by Gomez and Gomez (1976) following the formula:

$$\chi^2 = \sum_{i=1}^{p} \frac{(\text{observed value} - \text{expected value})^2}{\text{expected value}} \text{ with } (p\text{-}1) \text{ degree of freedom}$$

Where, *p* is the total number of observed classes.

The effect of transgene and non-transgene, low and high PUFA and *fae*1.1 genes of 6575-1 HELP on different traits was analysed 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):

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

Where, LSD is the least significant difference of the two treatments pair at 0.05 probability of significance, $t_{(0.05)}$ is the tabular value of *t* at 0.05 probability of significance 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.

Spearman's rank correlation coefficients were calculated using PLABSTAT software version 2N (Utz 2007). Direct and indirect path coefficients of the path coefficient analysis were calculated as described in Lynch and Walsh (1998):

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

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 affect variables and SFA, 18:1, PUFA, 20:1 and trierucin content were considered as causal variables.

3. RESULTS

3.1 Phenotypic variation among DH-lines

The DH-population showed highly significant variations for all traits; seed filling period, oil, protein and different fatty acids content in seed oil (Table 4). Erucic acid was the most prominent fatty acid, accounting for 47.1% of the total fatty acid content, followed by oleic acid (19.7%), PUFA (15.9%), eicosenoic acid (9.9%) and SFA (4%). Nervonic acid (24:1) content was only 1.2% in the DH-population. A considerable quantitative variation was found for 22:1, 18:1, PUFA and 20:1 content (Table 4). Large variations were also found for seed filling period, oil and protein content. Large and highly significant variance component of the genotypes were found for all traits. Variance components for the effect of replication were small. However, variance component of error was comparatively large for seed filling period, oil, protein, oleic and erucic acid content. Nevertheless, high heritabilities were found for all traits ranging from 0.79 to 0.96. The parental line 6575-1 HELP had a higher erucic acid and oleic acid and a lower PUFA content compared to TNKAT (Table 4). The frequency distribution of erucic acid content appeared to involve two phenotypic classes (Figure 1); however, the classes were not distinct. Chi square test ($\chi^2_{(0.05)}$ = 1.6 NS) showed that the segregation pattern was consistent with 1:1 model, indicating that this trait was controlled by alleles at one locus.

Table 4 Means, ranges, variance components and heritabilities (h^2) of seed filling period, 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	Oil	Protein	Fatty acid content (%)						
	period	content	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

** significant at p=0.01, F-Test in analysis of variance
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

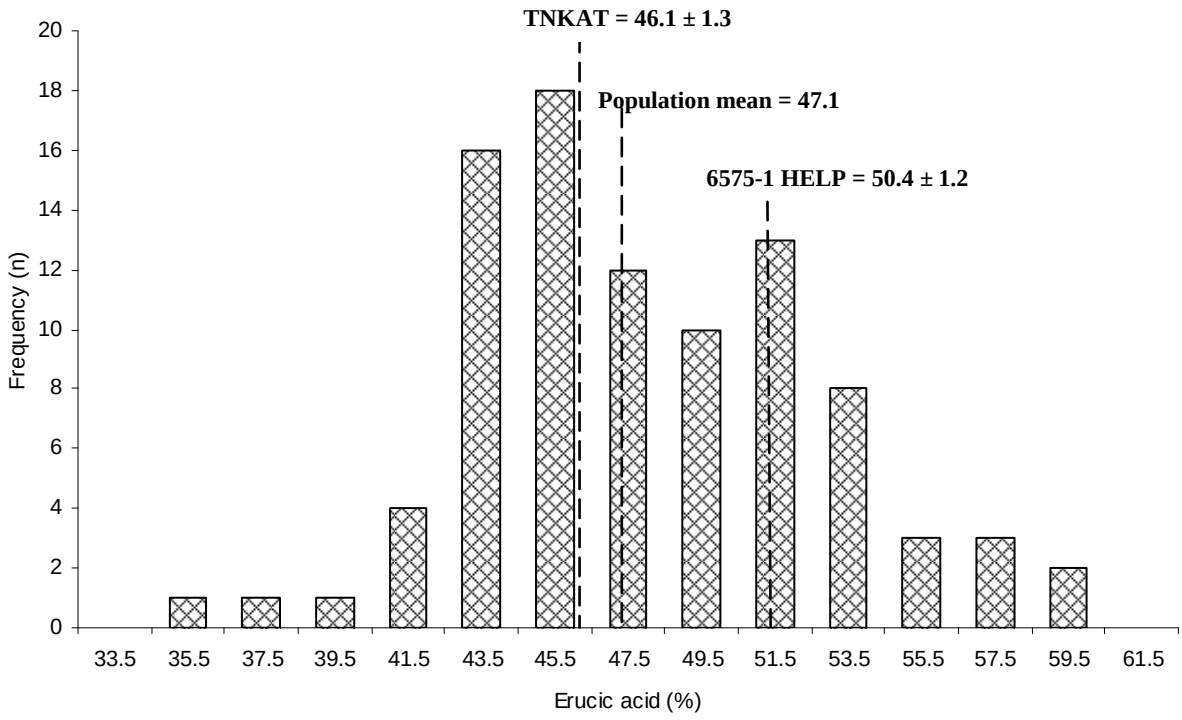

Figure 1 Distribution of erucic acid content in the DH-population derived from a cross between transgenic high erucic acid (TNKAT) and non-transgenic (6575-1 HELP) rapeseed lines in comparison to parents. Parental values are the mean ± standard deviation.

3.2 Effect of the *Ld-LPAAT* gene on erucic acid content and other traits

All the DH-lines of the population were grouped into two categories on the basis of the presence and absence of *Ld-LPAAT* transgene confirmed by PCR amplification (Nath et al. 2007) and by estimating trierucin (C_{69}; EEE) content in case of *Ld-LPAAT* positive. Among the DH-lines, *Ld-LPAAT* positive and *Ld-LPAAT* negative were 54 and 36 lines, respectively (Table 5). The segregation pattern of *Ld-LPAAT* gene was investigated by chi-square (χ^2) test for 1:1 segregation, the calculated value of chi-square was 3.6, which is non-significant, indicating that the DH-population segregated for one copy *Ld-LPAAT* transgene. The effect of transgene was investigated by comparing the mean between two groups of DH-lines using least significant difference (LSD) test. The mean difference indicated that the transgene had significant negative effect of -2.3% on erucic acid content. On the other hand, *Ld-LPAAT* positive plants had a 3% and 3.4% higher PUFA and trierucin content, respectively (Table 5). Frequency distribution along with the mean values of the two groups of DH-lines for 22:1 content have been presented as an example to see the effect of *Ld-LPAAT* gene, the DH-lines were randomly distributed in both of the groups (Figure 2). The values of mean difference of two groups indicated that transgene had significant negative effect on seed filling period, oil, oleic acid and MUFA content. Significant positive differences were observed for *Ld-LPAAT* positive plants in case of PUFA and 20:1 content. There were no statistical differences for protein, SFA and 24:1 content (Table 5).

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 x 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

* significant at p=0.05

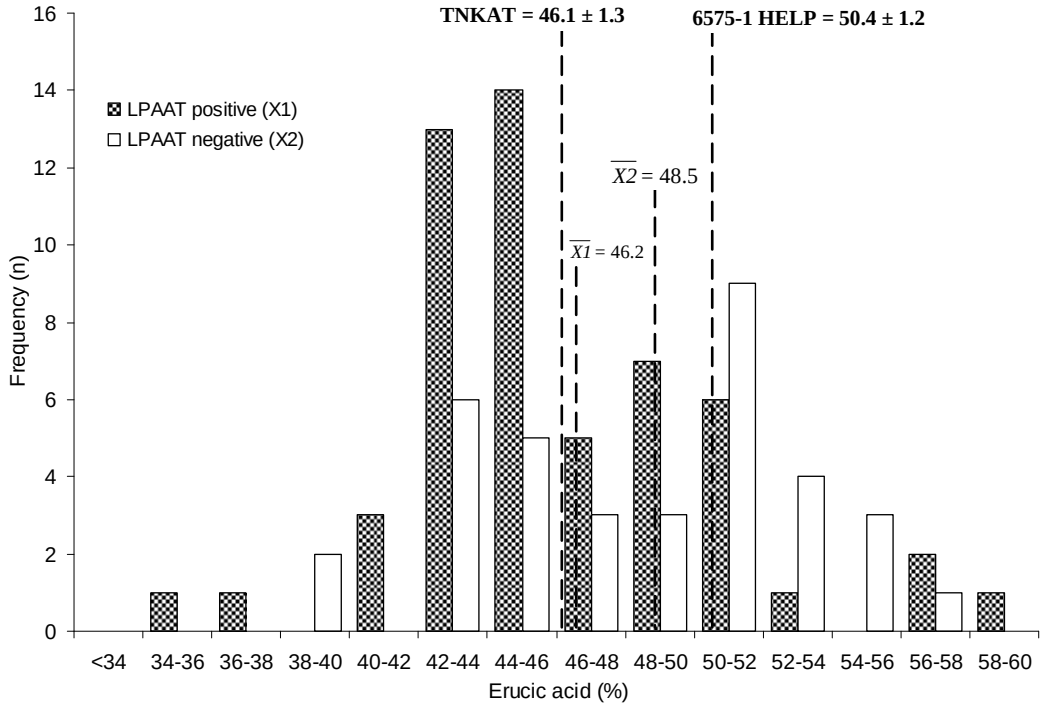

Figure 2 Frequency distribution of erucic acid content of two groups of DH-lines (*Ld-LPAAT* positive and *Ld-LPAAT* negative) in the DH-population derived from a cross between TNKAT x 6575-1 HELP along with parental means. Parental values are the mean ± standard deviation.

3.3 Effect of PUFA content on erucic acid content and other traits

Since, the *Ld-LPAAT* transgene did not show any considerable effect on erucic acid content, the question arose how individual doubled haploid line achieved up to 59% erucic acid content, although both parental lines had much lower values (Table 4). Therefore, the effect of PUFA content on erucic acid as well as on other traits was investigated. DH-population was grouped into two classes based on the mean of the PUFA content (Table 6). Among the DH-lines, 41 lines were low (below average; ≤15.9%) and 49 were high (above average; >15.9%) in PUFA content, respectively. The low PUFA class had 3.7% higher erucic acid content compared to the high PUFA class (Table 6). The mean difference of two groups indicated that low PUFA genes also had positive effect on oil (+2.2%) and oleic acid (+5.6%) content. Significant negative differences were observed for low PUFA class in case of protein (-1.6%), SFA (-0.7%) and PUFA (-8.1%) content. There were no statistical differences for seed filling period, 20:1, 24:1 and MUFA content (Table 6).

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 x 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

* significant at p=0.05
SFA = 16:0 + 18:0; PUFA = 18:2 + 18:3; MUFA = 18:1 + 20:1 + 22:1 + 24:1

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

Since, the cv. Maplus as well as thereof the derived low PUFA line 6575-1 HELP had higher erucic acid content than TNKAT, it was presumed that this difference may be due to effect of the alleles of the endogenous erucic acid genes *fae*1.1 (*B. rapa*) and *fae*1.2 (*B. oleracea*). Therefore, *fae*1.1 and *fae*1.2 locus specific primers were developed (Table 1 and 2). It was assumed that there may be allelic differences of the *fae*1 genes from A- and C-genome in the parental lines TNKAT and 6575-1 HELP, which could segregate in the DH-population. TNKAT parent was a resynthesised line derived from inter-specific cross between Yellow Sarson (*B. rapa*) for A-genome (*fae*1.1) and the cauliflower variety Super Regama for C-genome (*fae*1.2). On the other hand, 6575-1 HELP parental line contained alleles for both *fae*1 genes from the high erucic acid winter rapeseed line cv. Maplus, with unknown A- and C-genome donor. Differences between the sequences were only found for the *fae*1.1 alleles from 6575-1 HELP and TNKAT (Table 3) but not between the *fae*1.2 alleles. The DH-lines were categorized into two groups on the basis of the presence and absence of the *fae*1.1 allele from 6575-1 HELP line i.e. Maplus by PCR amplification using allele specific primers (Table 1 and 3; for results see example in Figure 3). Among the DH-lines 54 lines were found having *fae*1.1 allele from 6575-1 HELP (cv. Maplus) and 36 DH-

lines had *fae*1.1 allele from Yellow Sarson of parental line TNKAT. There was no significant difference observed in case of the presence of *fae*1.1 allele of 6575-1 HELP for erucic acid content in DH-population, although it contributed 1% more 22:1 (Table 7) compared to the allele for *fae*1.1 gene from TNKAT. Significant positive difference was observed in case of MUFA (+1.7%) content and significant negative difference observed in case of PUFA (-1.5%) content. All other recorded traits exhibited non-significant difference in case of absence or presence of *B. rapa* (A-genome) *fae*1.1 allele (Table 7).

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 x 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

*significant at p=0.05
SFA = 16:0 + 18:0; PUFA = 18:2 + 18:3; MUFA = 18:1 + 20:1 + 22:1 + 24:1

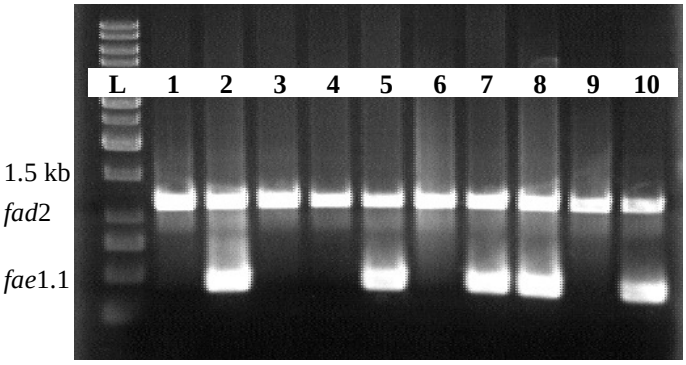

Figure 3 Multiplex-PCR of a 432bp fragment of the *fae*1.1 gene together with internal control amplification of the 1.1kb *fad*2 gene sequence using DNA extracted from 8 DH plants segregating for the *fae*1.1 gene of 6575-1 HELP from A-genome of *B. rapa* (Lanes 3-10 DH, lanes 1 and 2 are TNKAT and 6575-1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

3.5 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 showed significant positive correlations with oleic acid, eicosenoic acid and MUFA and negative correlation with protein, SFA, PUFA and trierucin content. Protein content also showed positive significant correlations with SFA and PUFA content and significant negative correlations with oleic, eicosenoic acid and MUFA content. PUFA showed strong positive correlation with SFA and strong negative with oleic acid. The variation in MUFA content is caused by the variation in SFA (r_s= - 0.75**), PUFA (r_s= -0.99**), oleic acid (r_s= 0.74**) and erucic acid (r_s= 0.43**) content and not by the variation in eicosenoic acid and 24:1 content (Table 8). The scatter plot for 22:1 vs. trierucin showed two distinct groups and no correlation was observed with a considerable variation in trierucin content (Figure 4).

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

Traits	Seed filling period	Oil content	Protein content	SFA	18:1	PUFA	20:1	22:1	Trierucin^{+}	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^{+}	-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

*, ** significant at p=0.05 and p=0.01, respectively
SFA = 16:0 + 18:0; PUFA = 18:2 + 18:3; MUFA = 18:1 + 20:1 + 22:1 + 24:1
$^{+}$ correlations are calculated considering complete population

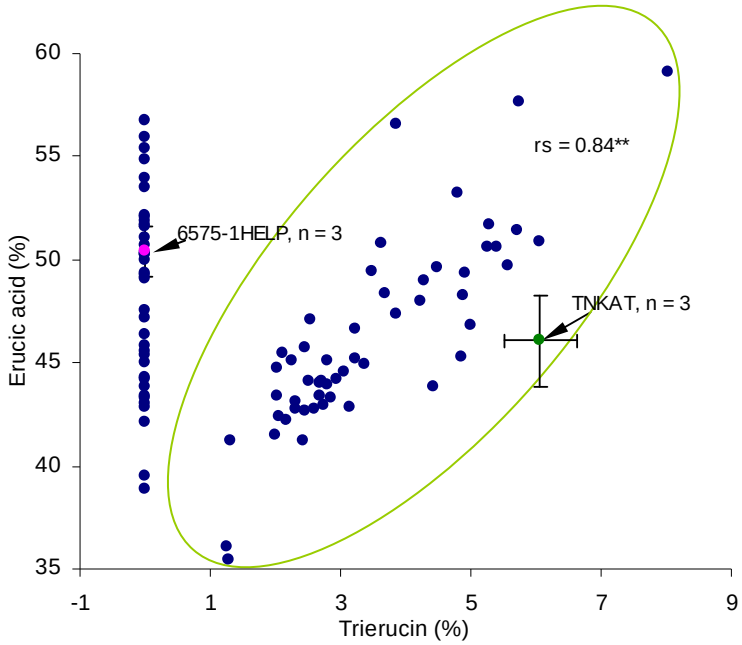

Figure 4 Scatter plot of erucic acid and trierucin content of 90 DH-lines compared with their parents (each point is the mean of 3 plants) derived from a cross between TNKAT x 6575-1 HELP. NS = Non-significant.

Oleic acid (18:1) is the primary precursor for erucic acid biosynthesis, however only a very weak negative correlation between the two traits were observed (r_s = - 0.11). The question arose whether erucic content was directly affected by oleic acid or other fatty acids, or indirectly via other fatty acids, which showed strong correlations with 18:1 content. Therefore, a path coefficient analysis was used to partition the correlations into direct and indirect effects considering those fatty acids which seem to be primary components for erucic acid biosynthesis (Table 9). Path coefficient analysis showed a strong direct negative effects of oleic acid (-0.57), PUFA (-0.65) and eicosenoic acid (-0.55) on erucic acid content. There was also negative direct effect of SFA (-0.25) on the content of erucic acid in the DH-population. Trierucin showed negligible direct effect (0.08) on 22:1 content. There were indirect effects on erucic acid content via other fatty acids, especially in case of SFA and 18:1 via PUFA content and PUFA and trierucin via 18:1 content (Table 9).

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

Trait	Indirect effect on erucic acid via					Direct effect on erucic acid
	SFA	18:1	PUFA	20:1	Trierucin	
SFA	-	0.20	-0.45	-0.14	0.00	-0.25
18:1	0.08	-	0.49	-0.08	-0.03	-0.57
PUFA	-0.17	0.44	-	-0.03	0.01	-0.65
20:1	-0.06	-0.09	-0.03	-	0.02	-0.55
Trierucin	-0.01	0.24	-0.10	-0.15	-	0.08
Residual effect = 0.34						

3.6 Comparisons of mean among parents and selected best DH-lines in the DH-population

Comparisons were done among the means of three selected DH-lines for high 22:1 content with the means of parents to find out whether the best DH-lines were significantly different from their parents for different phenological and quality traits. DH-line IV-10-F-6 contained the highest amount of erucic acid (59.1%), MUFA (86.3%) and trierucin (8%) with lower amount of SFA, 18:1 and PUFA. The other two DH-lines XI-10-D-6 and IX-10-C-8 contained 57.6% and 56.6% erucic acid respectively, which were also statistically higher than both of the parents TNKAT and 6575-1 HELP, respectively (Table 10). DH-line IX-10-C-8 showed the highest oil (43.6%) and MUFA (87.9%) content that were statistically different from the parent TNKAT and equal to the 6575-1 HELP parent (Table 10). The best DH-line, IV-10-F-6 exhibited mean values for seed filling period (84 days), oil (36.6%) and protein (31%) which content were similar to the transgenic parent TNKAT (Table 10). Only the DH-line IV-10-F-6 showed higher amount of trierucin (8%) content compared to transgenic parent TNKAT and other DH-lines.

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 x 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)

4. DISCUSSION

The first intention of the present study was to study the inheritance of erucic acid content and other seed quality traits in the segregating recombinant DH-population. Another objective was to develop locus and allele specific PCR primers to follow the segregation of the erucic acid alleles at the endogenous *fae*1.1 (*B. rapa* L.) and the *fae*1.2 (*B. oleracea* L.) loci. Ninety microspore derived DH-lines were produced from the F_1-plants of the cross between resynthesised transgenic high erucic acid rapeseed line TNKAT and a high erucic and low polyunsaturated winter rapeseed line 6575-1 HELP. This material was tested with three replicates along with the parental lines in a green house experiment.

The analysis of variance showed highly significant difference for different phenological and quality traits (Table 4). Microspore derived doubled haploid (DH) lines offer a quick means of getting homozygous segregating generation for the traits through chromosome doubling. Efficient genetic investigations could be carried out on specific trait, based on the assumption that microspore-derived populations reflect a random sampling of segregating F_1-gametes (Chen and Beversdorf 1990). The amount of variation in different phenological and quality traits of the segregating DH-population of cross (TNKAT x 6575-1 HELP) was due to recombination of the alleles of parental lines.

A quantitative variation was found for erucic acid content which ranged from 34.6% to

59.1%. This variation may be explained by differently effective alleles responsible for erucic acid (Jönsson 1977) and by other factors modifying the erucic acid content. The lower value of the range might be due to the combination of weak alleles for erucic acid from both of the parents. Whereas, higher value of the range might be due to the combination of stronger alleles for erucic acid from both of the parents. Multiple alleles were suggested for erucic acid content (Dorrell and Downey 1964, Stefansson and Hougen 1964, Jönsson 1977) in *Brassica*, including; e, E^a, E^b, E^c and E^d therefore, levels of erucic acid can be fixed at a large variation ranging from < 1% to >60% (Jönsson 1977). Zhao et al. (2008) identified eight quantitative trait loci (QTL) for erucic acid content in the segregating doubled haploid (DH) population derived from a cross between two high erucic acid rapeseed cultivars. One QTL was one of the two erucic acid genes (*fae*1) and other seven were not related to *fae*1 gene, but contributing in 22:1 content.

Distribution of erucic acid content showed two classes, but not distinct, following a 1:1 segregation (Figure 1). Even by shifting the value of the classes by 0.5%, a similar distribution occurred. These result indicated that this trait was controlled by alleles of one locus. This locus might be either the transgene *Ld-LPAAT-Bn-fae*1 or the low PUFA locus (*fad*2 mutant).

The DH-lines were grouped into two classes based on the presence and absence of *Ld-LPAAT* transgene confirmed by PCR using *Ld-LPAAT* specific primer (Nath et al. 2007). Significant negative effect for the traits seed filling period, oil, oleic acid, MUFA and erucic acid content in the *Ld-LPAAT* positive group indicated that the transgene *Ld-LPAAT* adversely affected those traits (Table 5). However, reduction of erucic acid content in DH *Ld-LPAAT* positive group was unexpected; it was presumed that 22:1 could be increased due to the presence of transgene *Ld-LPAAT*. Weier et al. (1997) also reported non-significant change of 22:1 content by over expressing only *Ld-LPAAT* gene in rapeseed. It seems that the fatty acid elongase (*fae*1) gene in the chimeric construct may not be functional. This result is controversial from the observation of Han et al. (2001), who have found 30% increase in 22:1 content in low erucic acid cultivar Drakkar but non-significant increased in 22:1 content in resynthesised line ‘RS306’ due to over expression of the same transgene construct. Although the best selected three DH-lines for high 22:1 were transgenic, it seems that transgene (*fae*1) cannot increase the 22:1 content but interacting

with other factors like low PUFA genes play a vital role to increase it by reducing PUFA content (Table 6).

Since, the transgene did not show any effect to increase the erucic acid content, it was presumed that the 59% erucic acid of the best selected DH-line was achieved by other factors. The difference of two groups (low PUFA and high PUFA content) suggested that PUFA genes had effect to increase 22:1 content up to 3.7% (Table 6). The mean of the low PUFA group exhibited still more PUFA content than of selected highest erucic acid lines suggesting the probability to increase the erucic acid content more than the mean difference observed. Low PUFA genes could increase erucic acid content by 3.7%, but they do not explain the 59% erucic acid content in the best selected line, that was 9% more than the non-transgenic parent 6575-1 HELP.

It was hypothesised that there are allelic differences of the fatty acid elongase (*fae*1) gene in the DH-population. Therefore, DH-population was grouped into two based on PCR results using *fae*1.1 allele specific primers (Tables 1 and 3 and Figure 3). Comparison between the *fae*1.1 allele from 6575-1 HELP and TNKAT showed only 1% increase of 22:1 content, but non-significant in the DH-population (Table 7). This result suggested that the *fae*1.1 allele for A-genome of 6575-1 HELP was stronger than that of TNKAT parent for fatty acid elongation from oleoyl-CoA to erucic acid. There were no differences found between TNKAT and 6575-1 HELP for *fae*1.2 locus analysing part of the promoter and coding region in sequencing.

In the DH-population erucic acid showed a high negative correlation with SFA, PUFA and eicosenoic acids (Table 8). Earlier studies indicated that eicosenoic acid and erucic acid content are controlled by the same genes, the alleles involved have an additive effect on erucic acid, but a dominant effect on eicosenoic acid (Kondra and Stefansson 1965). Therefore, the negative correlation between the two acids was expected to be highly significant (Table 8), which agrees with the results of Jönsson (1977) and Chen and Beversdorf (1990). Negative correlation for 22:1 with SFA and 22:1 with PUFA are indicating their significant role to increase the 22:1 content. Such negative correlations have been reported before by Sasongko and Möllers (2005), when combined the high erucic and high oleic acid genes together. Erucic acid had highly significant positive correlation with MUFA, Sasongko and Möllers (2005) also found such type of relationship with erucic acid

and MUFA in their study. Positive correlations for oil with 18:1 and with MUFA were observed. Such a positive correlation was also reported for a doubled haploid winter rapeseed population segregating for oleic acid content (Möllers and Schierholt 2002).

Oleic acid (18:1) showed non-significant negative correlation with 22:1 (r_s = - 0.11). The question arose whether erucic acid content was directly affected by oleic acid or other fatty acids, or indirectly via other fatty acids which showed strong correlations with 18:1 content. Therefore, a path coefficient analysis was used to partition the correlations into direct and indirect effects considering those fatty acids which seems to components for erucic acid biosynthesis (Table 9). The path coefficient analysis showed strong direct negative effect of 18:1, PUFA and 20:1 content on 22:1 content are congruent with the negative correlation between 22:1 with the traits. The residual effect of the path coefficient analysis indicates that there are additional traits effecting erucic acid content beyond those considered in path analysis. Zhao et al. (2008) identified in total eight quantitative trait loci (QTL) for erucic acid content, among them seven were not related to *fae*1 gene in the segregating doubled haploid (DH) population derived from a cross between two high erucic acid rapeseed cultivars.

The three DH-lines with higher erucic acid content were compared with their parents by multiple comparison test at 95% LSD. The selected best DH-lines showed statistically higher amount of 22:1 content than their parents (Table 10). DH-line IV-10-F-6 was the best among the selected DH-lines. It exhibited 59% erucic acid content which was about 9% and 13% more than the content of non-transgenic 6575-1 HELP and transgenic TNKAT parental lines, respectively. Other fatty acids content were drastically decreased in the best line compared to their parents, such as SFA was much lower than both of the parents and PUFA was equal to the 6575-1 HELP parent. These results indicate that an ectopic inserted functional *fae*1 transgene could boost up the erucic acid content in seed oil by utilizing the oleic acid for elongation if the desaturation activities are blocked by mutation or other ways. It could help to increase the available oleoyl-CoA in the cytosolic pool for elongation, which was one of the limiting factors (Bao et al. 1998, Domergue et al. 1999).

Trierucin (EEE; C_{69}) content helps to know the efficiency of lysophosphatidic acid acyltransferase (LPAAT) activity. Only DH-line IV-10-F-6 showed significant differences in trierucin content (8%) compared to transgenic TNKAT line (Table 10). However, three

selected DH-lines and TNKAT contained same *Ld-LPAAT* transgene copy for trierucin production. Therefore, the variations exhibited among them might be due to availability of 22:1 content in acyl-CoA pool. The best DH-line had 59% erucic acid content, which had probability to produce 20% trierucin by randomly esterified in each position of the triacylglycerol molecule with 59% 22:1. However, it showed only 8% of trierucin content compared to 20% indicating the limited LPAAT activities in the DH-population. This limited activity might be due to the presence of native *Bn-LPAAT* gene from rapeseed, even in more than one copy.

From the above results it could be concluded that highest amount of erucic acid might be achieved through introgression of alleles that further reduce PUFA content in combination with stronger endogenous or ectopically expressed *fae*1 alleles with the suppression of normal *LPAAT* gene of rapeseed either by mutation or by using anti-sense technique.

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Manuscript III

Increasing erucic acid content in high erucic acid rapeseed (*Brassica napus* L.) through combination with mutant genes for low polyunsaturated fatty acids content and with *Ld-LPAAT-Bn-fae*1 transgenes

Abstract

High erucic acid rapeseed (HEAR) cultivars are regaining interest for industrial purposes because erucic acid (22:1) and its derivatives are important renewable raw materials for the oleochemical industry. For oleochemical use, it is desirable to increase 22:1 content from now around 50% to 80% and more. This would significantly reduce processing costs and would increase market prospects of HEAR oil. The objective of the present study was to increase 22:1 content of HEAR through combination with mutant genes for low polyunsaturated fatty acids content and with *Ld-LPAAT-Bn-fae*1 transgenes. In the present experiment, the transgenic winter rapeseed genotype 361.2B (63% 22:1, 15% 18:2+18:3) over expressing the *Bn-fae*1 gene from rapeseed in combination with a *Ld-LPAAT* gene from *Limnanthes douglasii* was crossed with the winter rapeseed line 6575-1 having High Erucic acid and Low Polyunsaturated fatty acid (HELP; 50% 22:1, 7% 18:2+18:3). 220 F_2-plants derived from this cross were grown in the green house and bagged at flowering. F_3-seeds were harvested and analysed for fatty acid composition and trierucoylglycerol (trierucin) content. The F_2-plants showed a large variation ranging from 44-72% 22:1. The frequency distribution of F_2-population showed a normal distribution without any separable classes. The quantitative variation in 22:1 content were caused by the segregation of two transgene copies and by segregation of genes responsible for low polyunsaturated fatty acid content. The results of selected F_2-plants were also 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% 22:1 and as little as 4% polyunsaturated fatty acids content (18:2 + 18:3) in the seed oil. The 72% erucic acid content of rapeseed oil achieved in the present study represents a major progress in breeding high erucic acid rapeseed.

1. 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 content of erucic acid (22:1) and eicosenoic acid (20:1; Jönsson 1977). They are the major very long chain fatty acids (VLCFAs) in the seed oil, accounting for 45-60% of the total fatty acid mixture. Genetic studies show that in rapeseed the 22:1 content is controlled by the two genes loci *E1* and *E2*, which have additive effects (Harvey and Downey 1964, Stefansson 1983, Lühs et al. 1999). High levels of 22:1 in the cooking and salad oil extracted from rapeseed have been associated with health problems (Beare et al. 1963). A major effort was made in the 1960s to develop low erucic acid rapeseed (LEAR) varieties. Nowadays, double zero ('00') or canola quality type rapeseed is predominantly being grown in the world.

However, High Erucic Acid Rapeseed (HEAR) types retained some importance for utilization in the oleochemical industry. HEAR cultivars are presently cultivated to a smaller extent in Europe (up to 40.000 hectares in 2006/2007) and USA/Canada as an identity preserved crop (Möllers 2004). Erucic acid and its derivatives are important renewable raw materials used in plastic film manufacture, in the synthesis of nylon, lubricant, cosmetic and emollient industries (Leonard 1994, Sonntag 1995, Piazza and Foglia 2001). Currently available HEAR cultivars contain a maximum of 50% erucic acid and around 8% eicosenoic acid. A strategic goal is to genetically increase the content of erucic acid in rapeseed, because separation of erucic acid from the remaining fatty acids by distillation is expensive. Theoretically, the highest level of erucic acid that can be achieved through classical breeding is 66%. The reason for this limitation lies in the specificity of the *B. napus sn*-2 acyltransferase, which excludes erucic acid from *sn*-2 position of triacylglycerols (Cao et al. 1990, Frentzen 1993). To overcome this limit, the gene for an erucoyl-CoA preferring *sn*-2 acyltransferase *LPAAT* (lysophosphatidic acid acyltransferase) has been cloned from *Limnanthes* spp. and used successfully to alter seed oil *sn*-2 proportions of erucic acid in rapeseed (Lassner et al. 1995, Brough et al. 1996, Weier et al. 1997). However, this achievement was not accompanied by an increase in erucic acid content in the seed oil.

In a next step, interest focussed on the fatty acid elongation mechanism. This elongation is the result of 2 cycles of a four-step mechanism, in which oleoyl (18:1)-CoA is used as substrate (Puyaubert et al. 2005). The first step, the initial condensation reaction of 18:1-CoA with malonyl-CoA is catalysed by the KCS (ß-ketoacyl-CoA synthase). It is believed that this initial reaction is the rate-limiting step (Cassagne et al. 1994). The fatty acid elongase (*fae*1) gene encoding the ß-ketoacyl-CoA synthase has been cloned from a range of plant species and has been over expressed under control of a seed specific promoter in transgenic HEAR. The results of these experiments showed that there was only a minor increase in erucic acid content (Katavic et al. 2001, Han et al. 2001). Even in combination with the expression of the *Ld-LPAAT* gene from *Limnanthes douglasii*, no substantial increase in the erucic acid content has been found in HEAR (Han et al. 2001). On the other hand Wilmer et al. (2003) reported an increase of 22:1 content to 68 mol% in seed oil by ectopically expressing the *Ld-LPAAT* gene from *Limnanthes douglasii* in combination with *B. napus fae*1.1 (*Ld-LPAAT-Bn-fae*1.1) gene in rapeseed.

There are some evidences that the cytosolic pool of available oleoyl-CoA or malonyl-CoA may limit the elongation (Bao et al. 1998, Domergue et al. 1999). Sasongko and Möllers (2005) combined the genes for high erucic acid with those for high oleic acid in order to increase the pool of oleoyl-CoA available for fatty acid elongation. However, no significant change in the erucic acid content was observed. In comparison to the high erucic acid parental line (Maplus, 50% 22:1), they found reduced contents of polyunsaturated fatty acids (7% PUFA). Therefore, this material was named as High Erucic acid and Low Polyunsaturated fatty acid - HELP - rapeseed line. The objective of the present study was to increase 22:1 content through combination of alleles of endogenous erucic acid and low polyunsaturated fatty acid loci (HELP) with β-ketoacyl-CoA synthase (KCS) and lysophosphatidic acid acyltransferase (LPAAT) over expressing rapeseed genotypes.

2. Materials and methods

2.1 Plant Materials

The plant materials used in the present study were F_1- and segregating generations (F_2-, F_3- and F_4-seeds) with their parental lines. The F_1- and segregating generations were produced from a cross between a high erucic acid transgenic winter rapeseed line 361.2B over

expressing *Ld-LPAAT-Bn-fae*1.1 chimeric gene (Wilmer et al. 2003) and non-transgenic high erucic acid winter rapeseed line 6575-1 HELP (Sasongko and Möllers 2005) having high erucic and low polyunsaturated fatty acid alleles.

361.2B: a high erucic acid transgenic winter rapeseed line with 63% 22:1 and 15% 18:2+18:3 produced from BGRV2 (a HEAR line from UK) by transformation using the construct *Ld-LPAAT-Bn-fae*1 (Wilmer et al. 2003). The seed materials were provided by the company Biogemma, UK Ltd.

6575-1 HELP (High Erucic and Low Polyunsaturated fatty acid): This line was F_4-seed generation of winter rapeseed with 27% 18:1, 7% 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 doubled haploid winter rapeseed line DH XXII D9 (for details see Sasongko and Möllers 2005).

2.2 Methods

2.2.1 Crossing of the parental plants and production of F_2-seed generation

Crossing was performed in the direction of using transgenic (361.2B) line as seed parent and the non-transgenic (6575-1 HELP) line as a pollen parent. The F_1-seeds were harvested when the seeds were matured, characterized by changing the siliques colour to brown. F_1-seeds from the above mentioned cross were analysed for fatty acids content by half-seed gas liquid chromatography (GLC; Thies 1971) to confirm the F_1-seed character by the presence of low polyunsaturated fatty acids content. The embryos having one cotyledon were transferred in multipot tray containing T-soil (Fruhstorfer Erde; pH 5.9) and placed in the green house. Three weeks later, seedlings were transferred to 4ºC temperature with 8 hours light for 8 weeks. Afterwards, plantlets from multipot tray were transferred to 9 cm diameter pots containing normal compost soil in the S1 green house. Self-fertilisation was imposed to the plants by covering the flowers of the main raceme with crisp-plastic bags. F_2-seeds were harvested at maturity and stored in cool room.

2.2.2 Selection of F_2-seeds for F_2-population

In August 2005, 220 F_2-single seeds along with seeds from parents were randomly selected for producing F_2-population. The randomly selected single seeds were analysed by half-seed GLC (Thies 1971). The embryos having one cotyledon were transferred in multipot trays containing T-soil (Fruhstorfer Erde; pH 5.9) and placed in the green house. Growing conditions and vernalisation were same as described above. Afterwards, plantlets were transferred to 9 cm diameter pots containing normal compost soil in the S1 green house. Plantlets were allowed to grow in the green house providing 16 hrs day-light by using additional 400 Watt sodium-steam lamp. Temperature varied during the day from 20ºC to 25ºC and during the night from 10ºC to 15ºC. 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. Fungicides and insecticides were sprayed whenever necessary. Self-fertilisation was imposed to the plants by covering the flowers of the main raceme with crisp-plastic bags. F_3-seeds were harvested from 220 F_2-plants of the F_2-population in February 2006 after complete maturity and used for analysis of oil, protein fatty acids and trierucin content.

2.2.3 Green house experiment with selected F_3-seeds

A green house experiment was performed from October 2006 to April 2007 (from sowing to harvest). Five F_3-seeds from each of the 41 selected F_2-plants having high erucic acid content along with their parents as control were sown in multipot trays containing T-soil (Fruhstorfer Erde; pH 5.9) and allowed to grow for 3 weeks. Growing conditions and vernalisation were same as described for the F_1-seedlings. Following vernalisation, plantlets were transferred to 9 cm diameter pots containing normal compost soil. Green house experiment was conducted in RCBD (Randomized complete block design). The 5 F_3-plants from each F_2-plant were considered as five replicates. Five separate tables inside the green house represented the complete blocks consisting of all genotypes (F_3- and parental lines plants). Growing conditions and plant protection measures inside the green house were the same as described in F_2-population. Self-fertilisation was imposed to the plants by covering the flowers of the main raceme with crisp-plastic bags. F_4-seeds were harvested from main raceme only, when they were mature.

2.2.4 Trierucin and fatty acids analysis and determination of oil and protein content

Bulked seed samples (150 mg) were analysed for trierucin (C_{69}; EEE) content of the seed

samples (F_3- and F_4-seeds) by high temperature gas liquid chromatography (HT-GLC) analysis of fatty acid esters according to the method described by Möllers et al. (1997). The analysis was done using silicon capillary column RTX-65TG (Restek no. 17005) 15 m x 0.25 mm i.d. (0.1 µm film thickness). Remaining part of the sample after taking for trierucin analysis was transferred to a new tube and left on a hot plate at 37.5°C over night to evaporate. Fatty acids composition was analysed using the samples by gas liquid chromatography 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 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. Seed oil and protein content was determined by Near-Infrared-Reflectance-Spectroscopy (NIRS) using the equation raps2001.eqa (Tillmann 2007) and values were adjusted for 14 mm PVC adapter using regression equations. Values are expressed on seed dry matter basis.

2.2.5 Fatty acid analysis at central (*sn*-2) position of triacylglycerol by GLC

15 mg mixed seed samples from 5 replicates of F_3-population (F_4-seeds) were taken in a 5 ml plastic tube. 0.5 ml iso-octane : iso-propanol (9:1) was added for each sample and seeds were homogenised with a steel rod and evaporated the iso-octane 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) by supersonic bath for 2 min. The mixture was incubated at 30ºC for 1 hr by adding 25 µl (250 units) lipase from *Rhizopus arrhizus* (*SIGMA-ALDRICH*). In each time 200 µl petroleum ether-70 : iso-propanol (3:2) was added and collected the supernant in a 1 ml glass tube by centrifugation at 150xG (1000 rpm) for 5 min by repeating two times. The collected supernant was evaporated by using a stream of warm air. 25 µl petroleum ether-70 : iso-propanol (3:2) was added and mixed well by vortex. 20 µl from this mixture was taken and apply on thin layer chromatography (TLC) plate (F 1500/LS 254, 20 x 20 cm) by 20 µl syringe gently. TLC plates were placed in the eluent diethylether/petroleumether (3:1) and allowed to run for 30 minutes. Afterwards dry TLC plates were stained with iodine by placing in iodine vapour. Interesting points of monoacylglycerol were marked with pencil and kept them in light to disappear of the iodine stain. Marked area with pencil for each

sample was scrapped out by scalpel and used for fatty acid extraction in a separate 1 ml glass tube. The scrap was mixed with 250 µl iso-octane and incubated for 20 min and collected the supernant by centrifugation in another 1 ml glass tube. This procedure was repeated two times. Iso-octane from the collected supernant was evaporated by using a stream of warm air and then the fatty acids were extracted and analysed using the samples by gas liquid chromatography according to Rücker and Röbbelen (1996).

2.2.6 Segregation pattern of transgene in F_2-population by PCR

*DNA extraction and multiplex-PCR for the Ld-LPAAT and the Bn-fad*2 *gene:* 100 mg fresh leaf sample was taken in the Eppendorf tube and quickly frozen by dipping in liquid nitrogen (-96ºC). Sample was homogenised with the plastic pestle in 300 µl of extraction buffer. The extraction buffer consisted of 2M NaI, 13 mM EDTA, 0.5% sodium N-laurolyl sarcosine, 26 mM Tris-HCl, pH 8.0 (modified; Ishizawa et al. 1991). The homogenate was incubated in a water-bath at 60°C for 15 min and centrifuged at 10.000 g for 5 min. From the supernatant 200 µl was transferred to a new eppendorf tube to which an equal volume of iso-propanol (100%) was added, shaken and left at room temperature for 15 min. This was centrifuged at 10.000 g for 5 min to precipitate the DNA. The supernatant was discarded and 1 ml of 40% iso-propanol added to the pellet and vortexed. This was centrifuged at 10.000 g for 5 min and the supernatant discarded. The pellet was dissolved in 100 µl TE buffer containing RNAase (1µg/µl) and incubated at 60°C for 20 min. To this, 100 ml of ethanol (70%) was added to again precipitate the DNA and centrifuged at 10.000 g for 5 min. The supernatant was discarded and the pellet dried at room temperature and dissolved in 10 µl of TE buffer. Suitable PCR primers for multiplex-PCR of the *Ld-LPAAT* and the Bn-*fad*2 gene, PCR reaction and conditions and gel electrophoresis were as described by Nath et al. (2007).

2.2.7 Statistical analysis

Analysis of variance was performed using the Plant Breeding Statistical Program (PLABSTAT, Version 2N, Utz 2007) with the following model:

$$Y_{ij} = g_i + r_j + \varepsilon_{ij}$$

where: Y_{ij} was observation of genotype i in replicate j; g_i and r_j were the effects of genotype i and replicate j, respectively and ε_{ij} was the residual error of genotype i in replicate j. 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 heritability was calculated following formula described by Hill et al. (1998):

$$h^2 = \frac{R}{i\sqrt{V_P}}$$

where, h^2 = heritability; R = response to selection; i = the intensity of selection for erucic acid and $\sqrt{V_P}$ = phenotypic standard deviation.

The number of transgene copy segregated in the segregating F_2-population was calculated using χ^2 test for a fixed ratio hypothesis described by Gomez and Gomez (1976) following the formula:

$$\chi^2 = \sum_{i=1}^{p} \frac{(\text{observed value} - \text{expected value})^2}{\text{expected value}} \text{ with } (p\text{-}1) \text{ degree of freedom}$$

Where, *p* is the total number of observed classes.

Spearman's rank correlation coefficients were calculated using PLABSTAT software version 2N (Utz 2007). Direct and indirect path coefficients of the path coefficient analysis were calculated as described in Lynch and Walsh (1998):

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

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 affect variables and SFA, 18:1, PUFA, 20:1 and trierucin content were considered as causal variables.

3. Results

3.1 Phenotypic variation for different traits in the F_2-population

Mean values and ranges of oil, protein and different fatty acids and trierucin content are shown in Table 1 for F_2-population derived from the cross between the transgenic high erucic acid winter rapeseed line 361.2B and the non-transgenic high erucic and low polyunsaturated winter rapeseed line 6575-1 HELP. Frequency distribution of erucic acid (22:1) content varied from 44% to 72 % with a mean of 58.8 % in segregating F_2-population (Figure 1). The F_2-population (F_3-seeds) showed a continuous variation for erucic acid (22:1) content in the seed oil. There were no separable classes as expected for a polygenic inherited trait. There were a considerable number of F_2-plants having a higher 22:1 content compared to the parental line 361.2B. Oil content varied from 37% to 54% with a mean of 47.7% and protein content ranged from 19% to 33% with a mean of 24.8% for F_2-population. Polyunsaturated fatty acids (PUFA; 18:2 + 18:3) and monounsaturated fatty acid (MUFA; 18:1 + 20:1 + 22:1) content varied from 6% to 26% and from 64% to 87%, respectively. Large variations were also observed for SFA, 18:1, 20:1 and trierucin content in the F_2-population compared to both of the transgenic 361.2B and non-transgenic 6575-1 HELP parental lines (Table 1).

Table 1 Variation in oil, protein, different fatty acids and trierucin content (%) in F_2-population (F_3-seeds) of derived from the cross between 361.2B x 6575-1 HELP in compare to parents

	Item	Oil	Protein	Fatty acid contents (%)						
		content	content	SFA	18:1	PUFA	20:1	22:1	MUFA	Trierucin
361.2B*		49.9	24.1	3.2	8.3	19.1	4.6	62.5	75.4	15.2
6575-1 HELP*		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	0-24.5
	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
* Mean of n = 8 plants

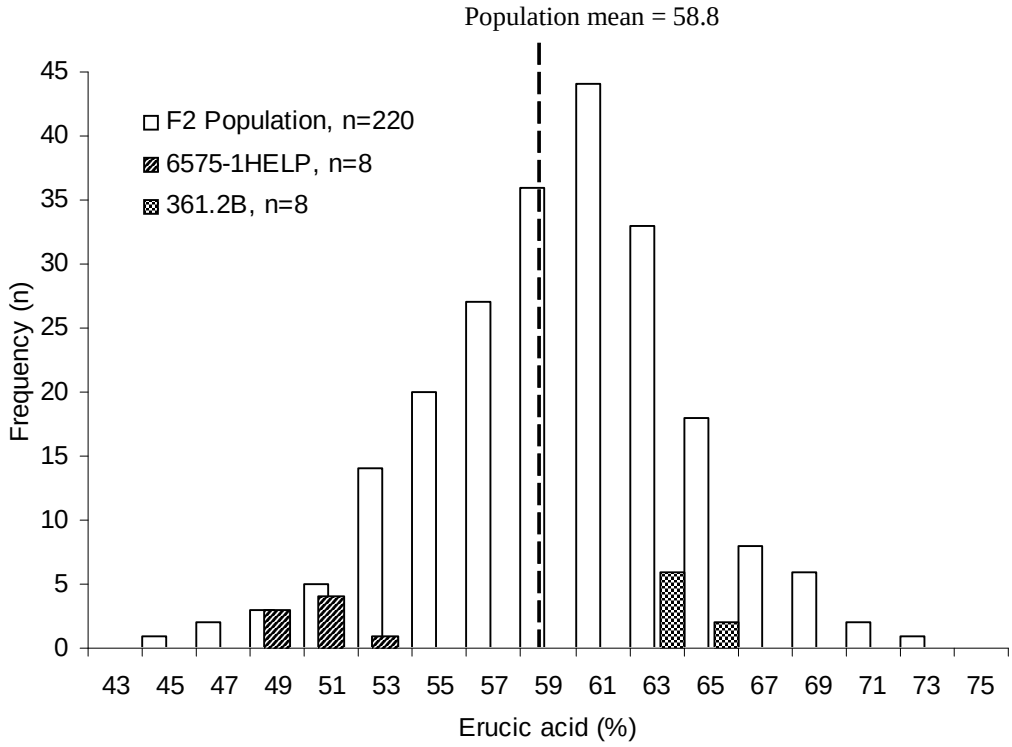

Figure 1 Frequency distribution of erucic acid content in the F_2-population (F_3-seeds) derived from a cross between 361.2B x 6575-1 HELP rapeseed lines

3.2 Segregation of transgene in F_2-population

Two methods were applied to investigate the segregation pattern of the transgene in the segregating F_2-population. One of them was PCR (Polymerase Chain Reaction) technique and another was trierucin analysis of the seed oil triglyceride. All F_2-plants which were positive in PCR for the *Ld-LPAAT* gene contained trierucin, whereas those which were negative in PCR did not have trierucin. Four out of 57 randomly selected F_2-plants were negative in the PCR reaction for the *Ld-LPAAT* transgene (see an example in Figure 2) upon chi-square ($\chi^2 = 0.06$) test, confirming that the transgenic parent 361.2B had two transgene copies which led to a 15:1 segregation in F_2-population. This result was also found when looking at the segregation of the trierucin phenotype; in the F_2-population 9 F_2-individuals out of 220 were free from trierucin production (Figure 3). Chi-square ($\chi^2 = 2.52$) test also confirmed the presence of two transgene copies in transgenic parent which led to 15:1 segregation in the F_2-population.

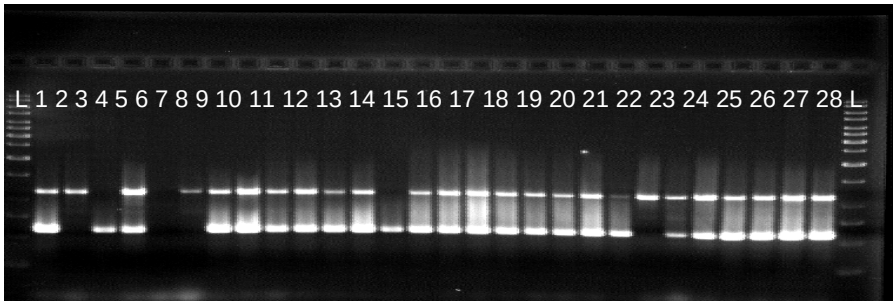

Figure 2 Multiplex-PCR of a 603bp fragment of the *Ld-LPAAT* gene together with internal control amplification of the 1.1kb *fad*2 gene sequence using DNA extracted from 26 F_2-plants segregating for the *Ld-LPAAT* gene (Lanes 3-28 F_2, lanes 1 and 2: 361.2B and 6575-1 HELP as control)

The frequency distribution of trierucin content showed two distinct groups, one group without trierucin and the other group with trierucin. The group with no trierucin represented the group lacking the *Limnanthes douglasii* lysophosphatidic acid acyltransferase (*Ld-LPAAT*) gene in the individuals of the F_2-population. The group with trierucin showed a continuous variation as one would expect for a polygenic trait (Figure 3). Erucic acid content varied from 45% to 57% for the zero trierucin plants and in the trierucin group it varied from 48% to 72% in the segregating F_2-population (see Figure 4c).

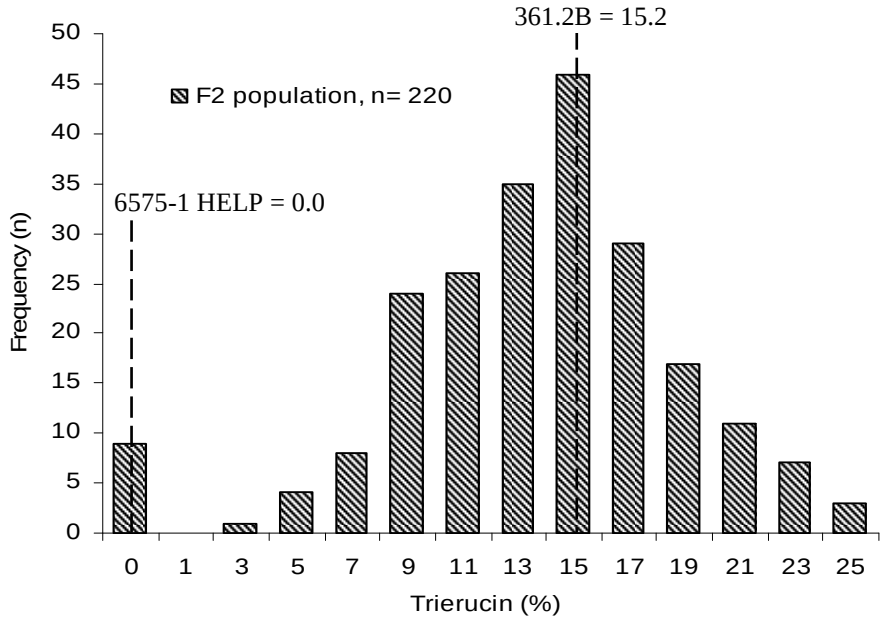

Figure 3 Frequency distribution of trierucin (C_{69}; EEE) content in F_2-population (F_3-seeds) of the cross 361.2B x 6575-1 HELP with mean of the parents (n = 8)

3.3 Correlations between traits and path coefficient analysis in the F_2-population

Spearman's rank correlation coefficient analysis in the F_2-population showed strong positive correlations between erucic acid content and oil ($r_s = 0.35^{**}$; Figure 4d), trierucin ($r_s = 0.54^{**}$) and MUFA content ($r_s = 0.56^{**}$). Negative correlations were observed between erucic acid and protein, SFA, oleic acid ($r_s = -0.41^{**}$), PUFA ($r_s = -0.50^{**}$) and eicosenoic acid ($r_s = -0.45^{**}$) content (Table 2). The scatter plot in Figure 4b showed that among the F_2-population there are some F_2-plants (F_3-seeds) that had a higher 22:1 and a reduced PUFA content compared to the transgenic parent 361.2B. These F_2-plants had an oleic acid (18:1) content which was about equal to the transgenic parent 361.2B (Figure 4a), suggesting that the reduction in PUFA content has led to an increased 22:1 content in the range of 5-10%. In some of the high erucic acid segregants, the PUFA content was as low as in the 6575-1 HELP parental line, indicating homozygosity for the genes causing low PUFA content. The analysis of trierucin revealed a positive correlation between 22:1 and trierucin content and effect of transgene on 22:1 content (Figure 4c). Furthermore, oil content showed significant negative correlations with protein ($r_s = -0.86^{**}$) oleic acid and eicosenoic acid and positive correlation with SFA content. Protein content also showed significant positive correlations with oleic acid content and significant negative correlations with SFA content. Oleic acid content showed significant positive correlation with eicosenoic acid and MUFA content. Significant negative correlations were observed between oleic acid and PUFA ($r_s = -0.41^{**}$) and trierucin content. PUFA showed negative correlations with trierucin and MUFA content. Trierucin content showed positive correlation with MUFA content (Table 2).

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

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

* and ** significant at 0.05 and 0.01 probability, respectively
SFA = 16:0 + 18:0, PUFA = 18:2 + 18:3, MUFA = 18:1 + 20:1 + 22:1

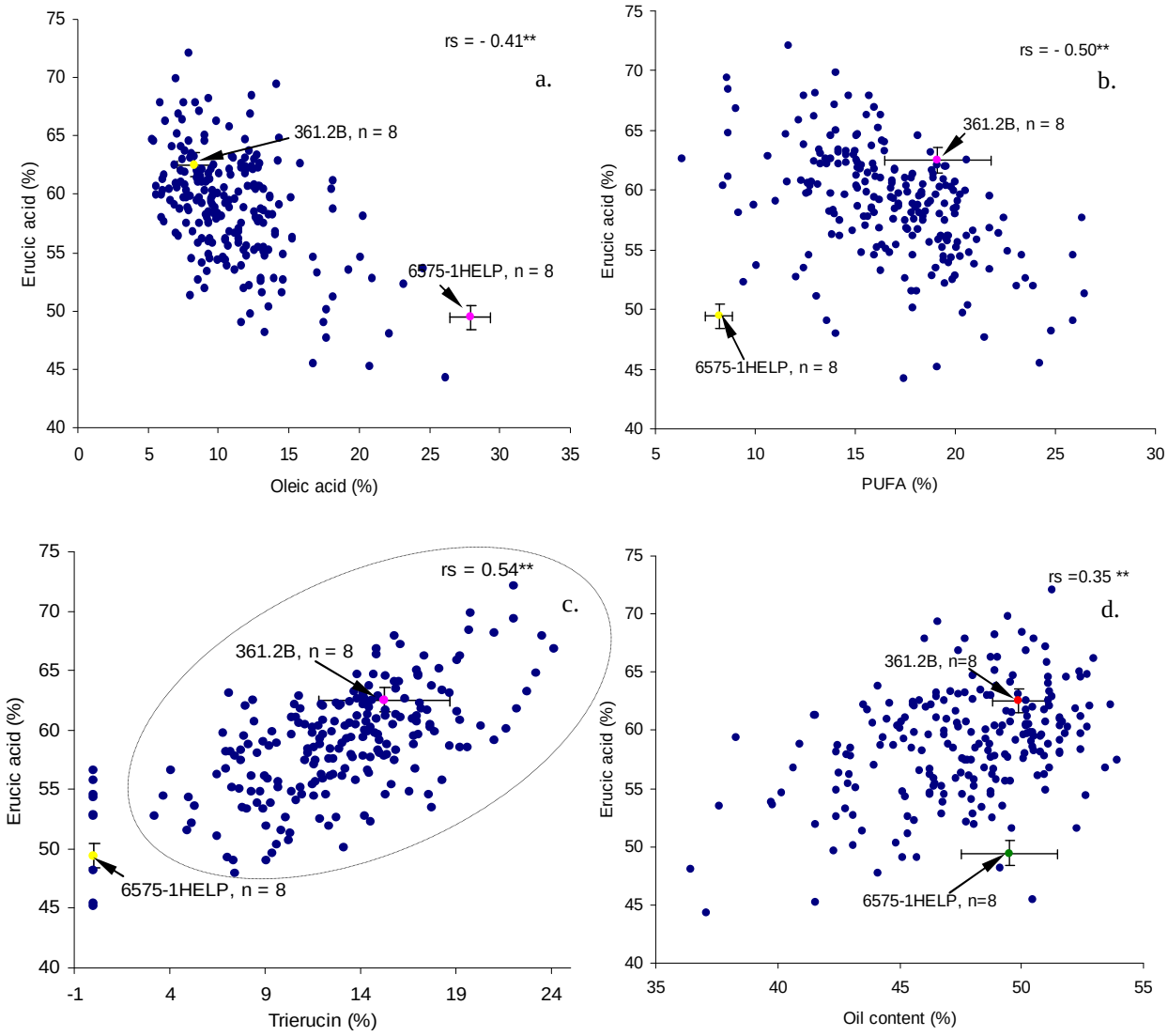

Figure 4a-d Correlation for fatty acid compositions and trierucin content and the relationship for erucic acid and oil content of F_2-plants (F_3-seeds) compare with their parents (bar showing standard deviation) derived from a cross 361.2B x 6575-1 HELP. ** indicates significance at P= 0.01 probability

Since, trierucin content was positively correlated with erucic acid content ($r_s = 0.54^{**}$) the question arose whether erucic acid content was directly affected by trierucin content or other fatty acids which were assumed as primary substrates for erucic acid biosynthesis. Therefore, a path coefficient analysis was used to partition the correlations into direct and indirect effects (Table 3). The results of the path coefficient analysis showed little direct effect of trierucin content on erucic acid (0.06). Strong negative direct effects of oleic acid and PUFA content on erucic acid content (-0.58 and -0.73) were observed. Other fatty acids such as SFA and 20:1 also showed little direct effect on erucic acid content. Positive

indirect effects were observed for oleic acid and PUFA content via PUFA and oleic acid on erucic acid content. Eicosenoic acid showed indirect negative effect via oleic acid as much as direct effect on erucic acid content. Trierucin content also showed larger positive indirect effect via 18:1, PUFA and 20:1 content on erucic acid than direct effect (Table 3).

Table 3 Direct and indirect effects of other fatty acids and trierucin on erucic acid content in the segregating F_2-population derived from a cross 361.2B x 6575-1 HELP

Trait	Direct effect on erucic acid	Indirect effect on erucic acid via				
		SFA	18:1	PUFA	20:1	Trierucin
SFA	-0.18	-	-0.04	-0.12	-0.04	-0.01
18:1	-0.58	-0.01	-	0.30	-0.10	-0.02
PUFA	-0.73	-0.03	0.24	-	0.03	-0.01
20:1	-0.25	-0.03	-0.23	0.09	-	-0.03
Trierucin	0.06	0.03	0.18	0.15	0.12	-
Residual effect = 0.43						

3.4 Performance of the selected F_3-population

Five F_3-seeds from each of the 41 selected F_2-plants having high erucic acid content were grown in the green house to produce the F_3-population. Analysis of variance (ANOVA) showed large and significant variations among the F_3-lines (F_4-seeds) in the population for all traits. Large and highly significant variance components of the genotypes were found for all traits. Variance components for the effect of replication were small. However, variance component of error was also relatively larger for all the traits. Erucic acid content varied from 50% to 72% with the mean of 64.8% (Table 4). The mean for 22:1 exhibited in the F_3-population (F_4-seeds) was 6% more compared to the mean of F_2-population, indicating the response to selection. Medium heritability (h^2 = 0.73) was observed for 22:1, calculated based on the response to selection, phenotypic standard deviation of F_2-population (4.7) and selection intensity (1.76). The scatter plot for 22:1 F_2-individuals (F_3-seeds) vs. 22:1 F_3-lines (F_4-seeds) showed significant positive correlation ($r_s = 0.57^{**}$; Figure 5), proving effective selection of high 22:1 plants based on single F_2-plant (F_3-seeds) selection for F_3-population.

Table 4 Variability in oil, protein, different fatty acids and trierucin content (%) of 41 F_3-lines (F_4-seeds) grown in replicated trial derived from the cross 361.2B x 6575-1 HELP

Item	Oil content	Protein content	Fatty acid content (%)						
			SFA	18:1	PUFA	20:1	22:1	MUFA	Trierucin
Range	49-55	21-29	3-5	7-27	4-17	4-11	50-72.3	76-91	0-24
Mean	51.6	23.6	2.6	11.4	11.3	5.8	64.8	82.0	11.2
σ^2_G	2.04**	3.33**	0.3**	11.68**	10.43**	1.43**	13.2**	15.03**	14.1**
σ^2_R	0.08	0.04	0.01	0.07	0.07	0.06	0.17	0.06	0.14
σ^2_E	5.2	3.33	0.3	4.69	5.65	1.25	10.5	8.29	7.81

** significant at p=0.01, F-Test in analysis of variance
SFA = 16:0 + 18:0; PUFA = 18:2 + 18:3; MUFA = 18:1 + 20:1 + 22:1
σ^2_G genetic variance, σ^2_R variance for replication and σ^2_E error variance

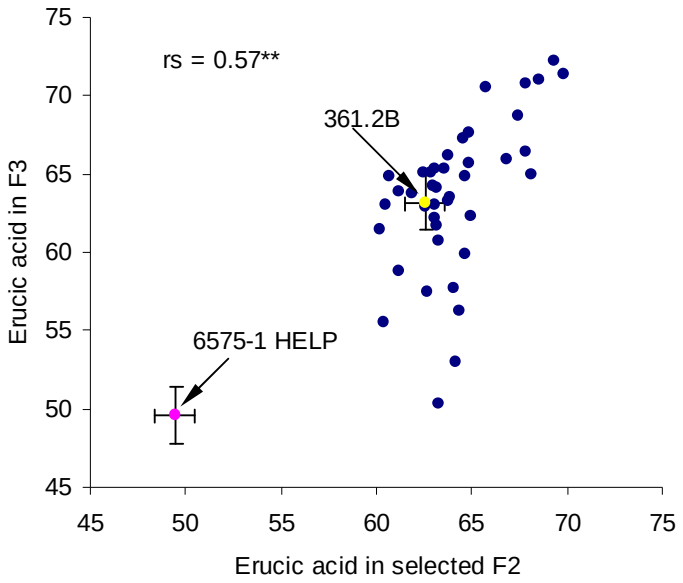

Figure 5 Scatter plot for erucic acid content (%) of the selected 41 F_2-individuals and derived F_3-lines (F_4-seeds; mean of five plants) along with the parents (bar showing standard deviation). ** indicates significance at P= 0.01 probability

The mean values were the mean of five plants from each F_3-line. The fatty acid contents as well as some other traits are shown in Table 5 for the six F_3-lines with the highest 22:1 content, along with the results of the parental lines 361.2B and 6575-1 HELP. The F_3-line III-G-7 contained the highest amount of erucic acid (72.3%). The F_3-lines IV-F-6, VI-D-9,

IV-D-3 and III-G-7 had lower or equal PUFA content ranging from 3.9 to 5.7% compared to 6575-1 HELP parental line. Oleic acid content was still higher in the all selected F_3-lines compared to transgenic 361.2B line ranging from 9.3 to 14.2%. All of the selected F_3-lines exhibited lower content of SFA (1.9 - 2.3%) compared to both of the parents. Eicosenoic acid content (3.9 - 7.1%) in the selected F_3-lines was lower than the parental line 6575-1 HELP. MUFA content varied from 83.9 to 91% which was higher than the parental line 361.2B. The F_3-lines IV-F-6, IV-D-3, VI-D-9 and III-G-7 were observed to have a higher MUFA content compared to both of the parental lines and there was no statistical difference among them. All the selected F_3-lines exhibited higher trierucin content (13.1 - 23.5%) compared to transgenic parental line 361.2B. The F_3-line VI-D-9 contained the highest amount of trierucin which was statistically identical to the line III-G-7. The F_3-lines VI-D-9, IV-D-3 and IV-F-6 showed higher oil content than both the parental lines. The F_3-line II-B-2 had the highest protein content (28.5%) among all the lines (Table 5).

Table 5 Comparison for the traits oil, protein, fatty acids and trierucin content (%) of 6 selected high erucic acid F_3-lines (F_4-seeds) of the cross 361.2B x 6575-1 HELP along with their parents. (Values are mean of 5 plants)

Line	Oil content	Protein content	Fatty acid content (%)						
			SFA	18:1	PUFA	20:1	22:1	MUFA	Trierucin
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
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	-
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
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
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
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
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
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

SFA = 16:0 + 18:0, PUFA = 18:2 + 18:3, MUFA = 18:1 + 20:1 + 22:1
Mean values with different letters indicate significant differences at p=0.05 (Fisher's LSD)

3.5 Fatty acid composition at *sn*-2 position

From the selected 6 F_3-lines with the highest erucic acid content the fatty acid composition at the *sn*-2 position of the triacylglycerols was analysed. The erucic acid content at the *sn*-2 position varied from 36.8% to 65.3%, which compares favorably to the 31.6% of the

transgenic parental line 361.2B (Table 6). The F_3-lines III-G-7 contained the highest amount of erucic acid at the *sn*-2 position followed by the line VI-D-9. Erucic acid at *sn*-2 position was only detected in case of the presence of *Ld-LPAAT* transgene. No eicosenoic acid (20:1) was detected at the *sn*-2 position except for the F_3-line VI-D-9, which showed very little 20:1 (1.1%) at the *sn*-2 position.

Table 6 Erucic (22:1) and eicosenoic acid (20:1) content in the total and *sn*-2 specific fatty acid mixtures of seed oils of the parental lines and of 6 selected F_3-lines (values are mean of 5 plants) having a high erucic acid content harboring the chimeric transgene

Line	Total fatty acid mixture (%)			*sn*-2 fatty acid mixture (%)	
	20:1	22:1	Trierucin	20:1	22:1
361.2B	4.3	63.2	11.4	0.0	31.6
HELP	11.2	49.6	-	0.0	0.5
IV-D-3	7.1	68.7	13.1	0.0	38.5
II-B-2	4.4	70.5	14.7	0.0	36.8
II-G-8	3.9	70.7	18.7	0.0	40.3
IV-F-6	6.1	70.9	13.1	0.0	41.3
VI-D-9	5.3	71.4	23.5	1.1	61.3
III-G-7	5.0	72.3	22.2	0.0	65.3

4. Discussion

High erucic acid rapeseed cultivars regain interest because erucic acid and their derivatives are environmentally friendly and renewable raw materials for the oleochemical industry. Therefore, combined efforts of biotechnology and plant breeding techniques are imposed to increase the erucic acid content in the seed oil. To increase the 22:1 content a transgenic high erucic acid winter rapeseed line 361.2B was crossed with the non-transgenic high erucic acid and low polyunsaturated winter rapeseed line 6575-1 HELP. The segregation showed a large variation for erucic acid content ranging from 44 to 72% in F_2-population (Table 1). The frequency distribution of F_2-population showed a continuous variation and there were no separable classes as expected for a polygenic inherited trait (Figure 1). Therefore, the variation for 22:1 content may be explained by different effective alleles segregating in the F_2-population.

In conventional high erucic acid rapeseed the 22:1 content is inherited by two genes and in crosses with low erucic acid genotypes, a 1:4:6:4:1 segregation can be expected in F_2 (Stefansson 1983, Lühs et al. 1999). However, the contribution of the two genes and their alleles to the total 22:1 content may be different. In addition to this, the quantitative variation in 22:1 content in the present population is caused by the segregation of two transgene copies and likely by the segregation of genes responsible for the low PUFA (18:2+18:3) content. The low PUFA content in parent 6575-1 HELP is caused by a mutation in the oleic acid desaturase *fad*2 gene which causes a reduction of 18:2 content by around 15% and by 2 to 3 other unknown genes having minor effects (Schierholt et al. 2001). Zhao et al. (2008) found eight quantitative trait loci (QTL) for erucic acid content, one QTL probably representing an erucic acid gene and another seven, affecting erucic acid content in a segregating doubled haploid (DH) population derived from a cross between two high erucic acid rapeseed cultivars.

The segregation pattern of transgene in F_2-population suggested a 15:1 segregation which was confirmed upon chi-square (χ^2) test using PCR and trierucin analyses data. Four out of 57 randomly selected F_2-plants were negative in the PCR reaction for the *Ld-LPAAT* transgene (see an example in Figure 2), confirming that the transgenic parent 361.2B had two transgene copies which led to a 15:1 segregation in F_2. This result was also found when looking at the segregation of the trierucin phenotype. Wilmer et al. (2003) also confirmed the presence of two transgene copies in the same material by southern blotting. The continuous variation in the trierucin positive group (Figure 3) may be explained by the number of segregating transgene copies (two) and availability of 22:1 for trierucin production.

The scatter plot in Figure 4b shows that among the F_2-population there are some F_2-plants (F_3-seeds) that have a higher 22:1 and a reduced PUFA content compared to the transgenic parent 361.2B. These F_2-plants had an oleic acid (18:1) and eicosenoic acid (20:1) content which was about equal to the transgenic parent 361.2B, suggesting that the reduction in PUFA content has also led to an increased 22:1 content in the range of 5-10%. In some of the high erucic acid segregants, the PUFA content was as low as in the 6575-1 HELP parent line, indicating homozygosity for the genes causing low PUFA content.

Erucic acid content was negatively correlated with the contents of polyunsaturated fatty acids, oleic acid and eicosenoic acid (Table 2). Sasongko and Möllers (2005) found highly significant negative correlations among 18:1 and 22:1 and 20:1+22:1 in F_2-segregating population derived from a cross between high erucic and high oleic acid winter rapeseed lines. Oil content was positively correlated with erucic acid content ($r_s = 0.35^{**}$; Figure 4d). This result is the agreement with the observations of Ecke et al. (1995) who found three QTL for oil content in *B. napus* and Cheung and Landry (1998) identified two QTL for oil content in *B. juncea*. In both studies, two of these QTL showed the same genes for erucic acid, indicating positive effect of erucic acid on oil content. In individual F_2-plants up to 25% trierucin was detected in the seed oil (Figure 4c). The capability of forming trierucin depends on the transgenic character of plants, expressing the *Ld-LPAAT* transgene. The high 22:1 F_2-plants with an elevated 18:1 and 20:1 content tended to have a higher MUFA content. Therefore, positive correlations between MUFA with oleic acid, 20:1 and 22:1 are expected. A negative correlation was found between MUFA and SFA (Table 3). Such a negative correlation has been reported before for high erucic (Sasongko and Möllers 2005) and high oleic acid rapeseed (Katavic et al. 2001, Auld et al. 1992). Möllers and Schierholt (2002) developed a hypothesis to explain this negative correlation in case of high oleic acid rapeseed population.

Path coefficient analysis revealed that trierucin content contributed directly only to a small extent to erucic acid content. On the other hand major direct effects by oleic acid (-0.58) and PUFA (-0.73) content are congruent with the highly significant negative correlation between erucic acid and these traits. Direct negative effects, but small, of SFA (-0.18) and 20:1 (-0.25) are also due to the negative correlations for 22:1 with SFA and 20:1. These results show that 18:1 and PUFA content are the major contributors for erucic acid biosynthesis. The residual effect of the path coefficient analysis indicates that there are additional traits effecting erucic acid content beyond those considered in path analysis.

The best six F_3-lines (F_4-seeds) were selected from the population based on high erucic acid content and were compared for different quality traits with their parents. The selected best 6 F_3-lines showed significantly higher amount of 22:1 content than their parents (Table 5). The F_3-line III-G-7 was the best among the selected lines with 72% erucic acid content which was about 9% and 23% more than the content of transgenic 361.2B and non-transgenic 6575-1 HELP parental lines, respectively. Other fatty acids content were

drastically decreased in the best line compared to their parents, such as SFA (16:0 + 18:0) was lower than both of the parents, PUFA (18: + 18:3) was less or equal to the 6575-1 HELP parent suggesting the homozygosity of low PUFA genes for low PUFA content. Compared to the transgenic parent 361.2B, the best selected F_3-line had lower PUFA content which helps also to increase 22:1 content. The best F_3-line contained 5% eicosenoic acid, which has an advantage to use in oleochemical industries, because eicosenoic acid is more difficult to separate from erucic acid than the other fatty acids (Möllers 2004).

Trierucin (C_{69}; EEE) content allows estimating the efficiency of the lysophosphatidic acid acyltransferase (LPAAT) activity leading to the biosynthesis of trierucin. Among the six best F_3-lines, line VI-D-9 showed the highest trierucin content (23.5%). It is not clear whether the selected F_3-lines contained the two *Ld-LPAAT* transgene copies in the homozygous form or not. Therefore, the variation exhibited among the lines might be due to dosage effect of transgene and availability of erucoyl-CoA in the acyl-CoA pool for trierucin production. The best F_3-line had 72% erucic acid content, with the theoretical probability to produce 37% trierucin, when all erucoyl molecules are randomly esterified to all three triacylglycerol positions. However, this line showed only 23.5% trierucin content, indicating that *Ld-LPAAT* activity may be still too weak. Alternatively, endogenous rapeseed *Bn-LPAAT* activity with a strong preference for oleic acid (Frentzen 1998) may be too strong.

The *sn*-2 fatty acid mixture of the seed oils the F_3-lines contained up to 65% 22:1. From this observation it is clear that proportion of erucic acid content at *sn*-2 position depends on the total 22:1 content in the seed oil. Those lines showed higher amount of 22:1 content at *sn*-2 position and had at the same time higher amounts of total erucic acid in their seed oil. Furthermore, erucic acid at *sn*-2 position only found in case of the presence of *Ld-LPAAT* transgene. No, or very limited amounts of erucic acid (0.5%) at *sn*-2 position were found in non-transgenic parental line 6575-1 HELP (Table 6). Similar results were also reported by Weier et al. (1997), Han et al (2001) in *Ld-LPAAT* expressing transgenic rapeseed lines. On the other hand, 20:1 was not detectable in the *sn*-2 fatty acid mixtures although the total fatty acid mixture contained both 20:1 and 22:1. This indicates that the *Ld-LPAAT* gene has a pronounced preference for 22:1-CoA than 20:1-CoA. These results together with the observation of Han et al. (2001) provide further evidence that the *Ld-LPAAT* gene of

Limnanthes douglasii has a pronounced preference for 22:1-CoA in comparison to 20:1-CoA.

From the above results, it can be concluded that the around 72% erucic acid achieved in the present experiments represent a large progress in breeding high erucic acid rapeseed. Further increases in erucic acid content can be expected from progress in reducing the remaining PUFA content from now 5% to values of 2-3%. However, this has so far not been achieved by mutagenesis or transgenic approaches. When the intermediate elongation product 20:1 and very long chain fatty acid nervonic acid (24:1) are taken into account the total amount of very long chain fatty acids (VLCFAs) reaches a maximum 79% in the present material. 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 VLCFAs biosynthesis.

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I apologize if any one feels her/his name should have been mentioned here.

Curriculum Vitae

Personal data

Name: Ujjal Kumar Nath

Date of birth: 01 December 1973

Place of birth: Barisal, Bangladesh

Nationality: Bangladeshi (By birth)

Education and trainings

2004 - date	Studying at the Department of Crop Sciences, division- Plant Breeding, Georg-August University, Göttingen, Germany for the awarded of Ph.D. degree in Plant Breeding
2000 - 2001	Studied at Bangladesh Agricultural University, Mymensingh, Bangladesh and awarded M.S. degree in Genetics and Plant Breeding
1992 - 1995	Studied at Bangladesh Agricultural University, Mymensingh, Bangladesh and awarded B.Sc. degree in Agricultural Science
1989 - 1991	Studied at Govt. Bakerganj College for Higher Secondary (H.S.C.) education, Bakerganj, Barisal, Bangladesh
1979-1989	Attended primary school and secondary school (S.S.C.) education at Bakerganj J.S.U. high school, Bakerganj, Barisal, Bangladesh
2003	Attended a training on “facilitating learning” at Graduate training institute (GTI), Bangladesh Agricultural University, Mymensingh, Bangladesh and Olds College, Canada
2003	Attended a training on “Scientific Report writing” at GTI, Bangladesh Agricultural University, Mymensingh, Bangladesh
2002	Attended a training on “Teaching Methods and Techniques” at GTI, Bangladesh Agricultural University, Mymensingh, Bangladesh
2001	Attended training on “Various common Laboratory equipments” arranged by Network of Instrument Technical personnel and User scientists of Bangladesh (NITUB)
2001	Participated a 3 months “English Language course” in English Society, Bangladesh Agricultural University, Mymensingh, Bangladesh
1999	Attended a training on “Administration, Office management and Communication” at GTI, Bangladesh Agricultural University, Mymensingh, Bangladesh

Professional career

2004- date	Assistant Professor, Department of Genetics and Plant Breeding, Bangladesh Agricultural University, Mymensingh, Bangladesh
2001-2004	Served as Lecturer, Department of Genetics and Plant Breeding, Bangladesh Agricultural University, Mymensingh, Bangladesh