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Very long chain and hydroxylated fatty acids in offspring of somatic hybrids between *Brassica napus* and *Lesquerella fendleri*

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Abstract Offspring of somatic hybrids between the zero-erucic acid rapeseed cv Hanna and *Lesquerella fendleri* were analysed regarding their fatty acid profiles. In the first back-cross generation one plant was found that produced a seed containing up to 16.5% erucic acid and 15% eicosaenoic acid (Line 1), as well as a seed having 4.3% ricinoleic acid (Line 2). This was interpreted as due to a contribution of elongase and hydroxylase genes from the *L. fendleri* genome since these two fatty acids are not produced in the recipient rapeseed cultivar Hanna. Crosses between Line 1 and cv Hanna resulted in the production of seeds with 35% erucic acid (F_2). Furthermore, crosses between the F_2 plants and the rapeseed cultivar Gulle, producing 35% erucic acid in the seeds, resulted in F_3 seeds with 48% erucic acid. The highest amount of erucic acid, 61.5%, was found in the F_6 generation after crossing Line 1 with a high erucic acid rapeseed line, HEAR, followed by self-fertilisation for two generations. When performing Southern-blot analysis on the F_6 plants, seven of the nine analysed plants hybridised with the *L. fendleri* species-specific repetitive probe. The presence of the hydroxylase gene was also observed in the F_6 generation of Line 1 according to Southern-blot analysis. Hybridisation with a hydroxylase probe was seen although no hydroxy fatty acids could be detected in any of the F_6 plants. In parallel, Line 2 was crossed with HEAR cv Gulle and self fertilised. No hydroxy fatty acids were detected in the F_2 generation of Line 2 and no specific hybridisation patterns could be found in the Southern-blot analysis.

Key words *Brassica napus* · *Lesquerella fendleri* Somatic hybrids · Erucic acid · Hydroxy fatty acids

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Introduction

A group of valuable fatty acids which it would be of great interest to produce in rapeseed or other highly domesticated oilcrops, for example linseed, are the hydroxy fatty acids. Currently, hydroxy fatty acids are predominantly extracted from seeds of castor bean, *Ricinus communis*, which due to the toxic lectin produced in its endosperm causes problems when they are isolated and purified (Atsmon 1989). The hydroxy fatty acids are utilised for the production of polymers such as nylons as well as plastics, textile dyes, printing ink, leather and lubricants (Töpfer et al. 1995; Murphy 1996). The interest in utilising hydroxy fatty acids other than ricinoleic acid as industrial raw materials has stimulated breeders to investigate different species. The genus *Lesquerella* contains several species that accumulate hydroxy fatty acids, mainly lesquerolic, densipolic or auricolic acids, in their seeds (Reed et al. 1997). *Lesquerella fendleri* is one of the main candidates for breeding purposes (Muuse et al. 1992; Dierig et al. 1993) since its oil contains high amounts of hydroxy fatty acids, about 60% lesquerolic acid and 1.5% densipolic acid (Barclay et al. 1962).

Other technically important fatty acids besides hydroxy fatty acids are the long chain fatty acids, especially erucic acid, which has many industrial uses,

e.g. the production of polymers such as nylons, plastizisers, surfactants, paints, detergents, cosmetics and lubricants (Sonntag 1995; Töpfer et al. 1995). Erucic acid can be found in storage lipids in the family, *Brassicaceae* and old varieties of *Brassica napus* contain high amounts of this fatty acid. Erucic acid is nutritionally detrimental to mammals and breeding efforts in the late 1950s resulted in cultivars practically free from erucic acid (Stefansson et al. 1961). Apart from breeding for zero-erucic acid rapeseed cultivars, breeders also have an interest in producing rapeseed cultivars with very high erucic acid contents for technical uses. Lines containing about 59% erucic acid have been reported in winter rape (Appelqvist and Jönsson 1970). However, to give an economically worthwhile production the content should preferably reach levels of 90% (Murphy 1996).

Very long chain fatty acids (VLCFA) are synthesised from oleic acid by successive additions of 2-carbon units and the process is regulated by the fatty acid elongase (FAE1) complex. This is composed of four different enzymes (Fehling and Mukherjee 1991) and the expression of the *FAE1* gene is thought to be the rate-limiting component in the elongation process (Millar and Kunst 1997). Harvey and Downey (1964) proved that the elongation process, as well as the erucic acid content, is affected by the number of elongase gene copies present in the plant and that each gene copy contributes about 9–10% erucic acid. The *FAE1* gene and the E1 and E2 loci, controlling the erucic acid content (Harvey and Downey 1964), were shown to co-segregate. This indicates that *FAE1* controls the erucic acid content in rapeseed (Fourmann et al. 1998). Since *B. napus* is an amphidiploid species (U 1935) it can contain a maximum of four elongase gene copies if the two loci are homozygous for the gene. An extra gene copy introduced in rapeseed would therefore open up the possibility to obtain an even higher content of erucic acid in the oil. By traditional breeding methods, homozygous lines would give a maximum erucic acid content of 40% and if an additional homozygous locus from, for example, *L. fendleri* were introduced it might be possible to reach 60% erucic acid.

To investigate whether elongase and hydroxylase genes could be transferred from *L. fendleri* to rapeseed, offspring from somatic hybrids (Skarzhinskaya et al. 1996) between the two species were analysed. An asymmetric somatic hybrid back-cross with the zero-erucic acid rapeseed cultivar Hanna produced one seed with about 16.5% erucic acid (F_1) as well as one seed containing up to 4.3% ricinoleic acid. Successive crosses were performed in order to increase the erucic acid and hydroxy fatty acid contents as well as to obtain the longer hydroxy fatty acids produced, such as lesquerolic and auricolic acids.

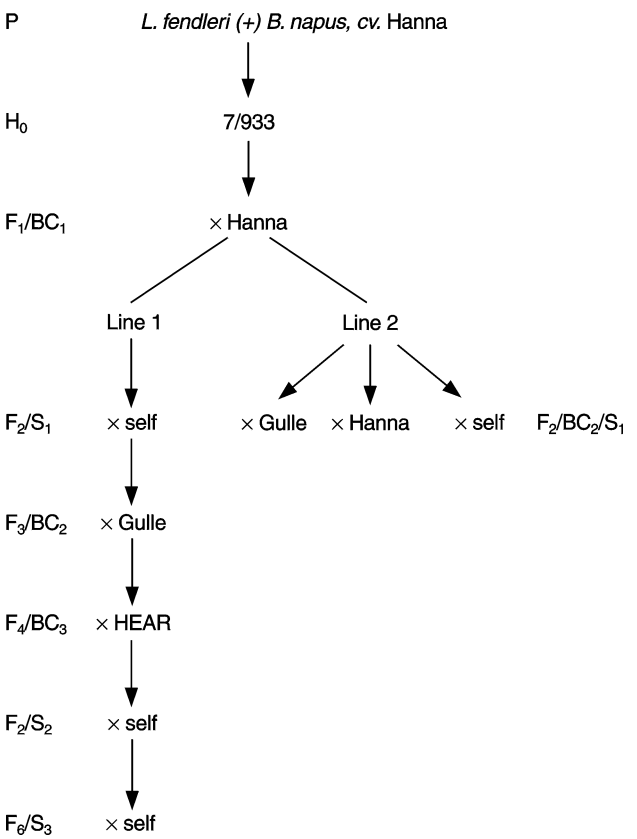

Fig. 1 A schema of the plant material used in this study. Two lines with an interesting fatty acid composition in their seeds were obtained after back-crossing (BC) an asymmetric somatic hybrid plant, 7/933, between *B. napus* and *L. fendleri* with the rapeseed cv Hanna. The erucic acid-containing Line 1 was first selfed, S_1, and then subsequently back-crossed with the rapeseed cv Gulle (BC_2), followed by crossing with the rapeseed line HEAR (BC_3) and then further self-fertilised again for two generations. In each generation only the individual seed with the highest content of erucic acid was used for the next sexual cross. The ricinoleic acid-containing Line 2 was back-crossed with cv Gulle and cv Hanna (BC_2) and self-fertilised (S_1) in the F_2 generation

Materials and methods

Plant material

Asymmetric somatic hybrids between *B. napus* cv Hanna and *L. fendleri* were produced by protoplast fusion (Skarzhinskaya et al. 1996). In the F_1 generation two lines with different fatty acid profiles, Line 1 and Line 2, were derived from the hybrid 7/933 after a back-cross with the cultivar Hanna (Fig. 1). These lines were crossed in different ways in order to increase the levels of the fatty acids of interest. Line 1 was crossed both with the spring rapeseed cv Gulle containing about 35% erucic acid and a high erucic acid line, HEAR, containing about 50% erucic acid, as well as being self- fertilised. Line 2 was crossed with cv Gulle, cv Hanna and also selfed (Fig. 1).

The F_1–F_3 generations of Line 1 were grown under greenhouse conditions at approximately 20°C, the F_4 generation at 27°C/22°C, and the F_5–F_6 generations and Line 2 at 20°C/17°C, with a light/darkness regime of 16/8 h.

Up to 50 seeds per plant were analysed for their fatty acid composition in the H_0 generation. In the following generations approximately 150 seeds per plant were analysed. One to three plants from each generation derived from Line 1, having the highest amount of erucic acid, were crossed further according to Fig. 1.

Southern-blot hybridisation

Twentysix F_1 plants and eight F_6 plants from Line 1 and one F_2 plant from Line 2 were analysed by Southern-blot analysis. The plants were chosen on the basis of their fatty acid composition.

Total DNA was prepared according to Sharpe et al. (1995), digested with *Hin*dIII or *Eco*RI and separated on 0.7% agarose gels. Southern-blot hybridisation was performed according to Sharp et al. (1988) with the following modifications: DNA was blotted onto Hybond N^+ nylon membranes; Pipes and gelatine were excluded in the hybridisation solutions; 0.2% BSA and 0.12 M TRIS, pH 8, were added to both the pre-hybridisation and hybridisation solutions; 1% dextran sulphate was added to the pre-hybridisation solution and 10% dextran sulphate to the hybridisation solution; 50 µg/ml of autoclaved herring testes DNA were added to the pre-hybridisation solution and 5 µg/ml of herring testes DNA to the hybridisation solution.

Filters were hybridised with a *L. fendleri* species-specific repetitive DNA sequence (Skarzhinskaya et al. 1996), a hydroxylase gene-specific fragment from *L. fendleri* (kindly received from Pierre Broun) and two *FAE1* gene fragments originating from *B. napus* and *A. thaliana* (kindly received from Ljerka Kunst). The hydroxylase gene was purified on agarose gels as a 1.5-kb *Eco*RI fragment and the *A. thaliana*-specific *FAE1* probe was prepared as a 1.5 kb *Xba*I fragment from the plasmid pT3 *FAE1* (Millar and Kunst 1997). The rapeseed-specific probe was prepared as a 1.6-kb *Xba*I/*Sac*I fragment from a *B. napus FAE*1-homologous sequence cloned in the plasmid pT7T3 18 U.

DNA fragments were radiolabelled with (α-^{32}P)dCTP (Amersham) using the High Prime Kit from Boehringer Mannheim (Germany) or the Oligolabelling Kit from Pharmacia Biotech (Sweden), and filters were exposed to Kodak X-Omat AR X-ray film (Eastman Kodak Company) for 3–5 days at −70°C using intensifying screens.

Northern-blot hybridisation

Developing seeds with embryos at the late cotyledon stage were harvested from four F_6 plants and stored at −70°C. Total RNA was prepared from 50 developing seeds according to Verwoerd et al. (1989), separated on 1% formaldehyde gels (Lehrach et al. 1977) and transferred to nylon membranes; hybridisation was done according to Sambrook et al. (1989). The same *FAE1* gene fragments as in the Southern-blot analysis were used for hybridisation.

Determination of fatty acid profiles

Seeds were germinated on wet filter paper and after 2 days one cotyledon from each seedling was excised and stored at −70°C until analysis. Lipids were methylated by incubating the cotyledons at 85°C for 45 min according to Mancha and Stymne (1997). Methyl esters were extracted by adding 0.2 ml of *n*-hexane and 0.5 ml of water. Aliquots of 0.15 ml from all samples were either transferred to micro-vials and injected into the gas chromatograph (GC) by a Shimadzu AOC-9 automatic sample injector according to Wiberg et al. (1991) or, alternatively, injected manually into a Shimadzu GC-14 A using the same conditions. The relative molar percentage was calculated as an area percentage by comparing the peak areas. The relative amounts of palmitic acid, stearic acid, oleic acid, linoleic acid, linolenic acid, eicosanoic acid, eicosenoic acid, erucic acid, ricinoleic acid, densipolic acid, lesquerolic acid and auricolic acid, were also determined.

The methylesters from samples putatively containing hydroxy fatty acids were analysed by GC through a 50 m × 0.32 mm CP-Wax 58-CB fused-silica column (Chrompack, Middelburg, The Netherlands).

Determination of hydroxy fatty acids by thin-layer chromatography and gas chromatography

Samples were applied to silica-gel TLC plates (silica 60; Merck) and separated in *n*-hexane : diethylether : acetic acid (50 : 50 : 1 v : v). Hydroxy fatty acid methyl esters were eluted by adding 3.75 ml of methanol : chloroform (2 : 1 v : v) after which 1 ml of acetic acid, 1 ml of water and 1.25 ml of chloroform were added and the mixture subsequently vortexed and centrifuged. The chloroform phase was evaporated under N_2(g) and methyl esters were dissolved in *n*-hexane. Samples were injected manually using a capillary column as described previously.

Results

Identification of hybrid offspring with an altered fatty acid composition

From 20 primary hybrids (H_0 plants), 1–50 seeds were screened by gas chromatography to determine the fatty acid composition. The number of seeds investigated from each hybrid differed due, in a large extent, to the difference in fertility of the H_0 plants. Some H_0 plants, such as 7/933, had to be back-crossed with cv Hanna in order to obtain seeds. From the cross between Hanna and the H_0 plant 7/933, two F_1 seeds were found that produced up to 16.5% erucic acid and 15% eicosenoic acid (Line 1), as well as one seed having 4.3% ricinoleic acid and trace amounts of a fatty acid tentatively identified as densipolic acid (Line 2). These two lines were selfed and crossed to different cultivars according to Fig. 1 in order to increase the amounts of the desired fatty acids. In the F_2 generation of Line 2 the erucic acid content was doubled (35%) and the eicosenoic acid content was equal to that in the F_1 generation, i.e. 15%.

Increase of erucic acid in Line 1 by sexual crossing

In the F_2–F_6 generations approximately 150 seeds were screened for the presence of erucic acid. Only those seeds with the highest content of erucic acid were planted and used for further crossings. Subsequent crosses with Line 1 raised the erucic acid content from 16.5% in the F_1 generation to 61.5% in the F_6 generation (Fig. 2). Simultaneously, the oleic acid content decreased from 35% in the F_1 generation to less than 7% in the F_6 generation. In the eight tested F_6 plants, the average content of erucic acid ranged from 50% to 58%. In the plant with the highest average content of erucic acid, 2-19, ten of the 52 tested seeds contained more than 60% erucic acid and two of these contained 61.2% and 61.5%, respectively. In addition, the sum of all fatty acids longer than 20-carbons units, VLCFAs, increased from 32% of the total fatty acids in the F_2 generation to 65% in the F_6 generation (Fig. 3).

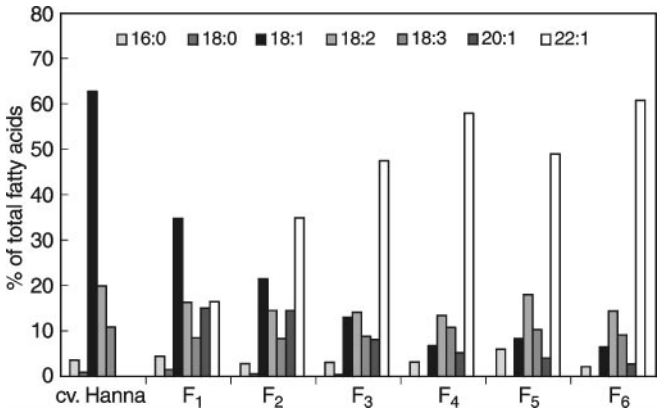

Fig. 2 Fatty acid profiles in the offspring of Line 1 from generation F_1 to F_6

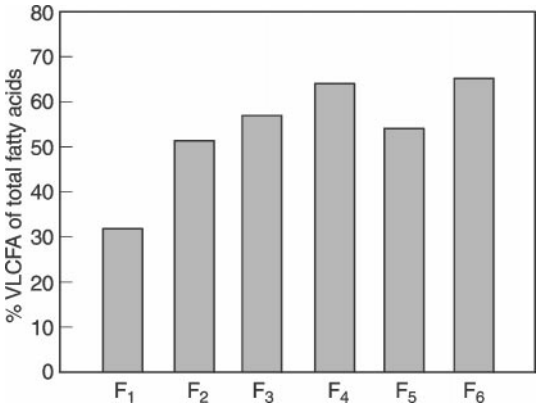

Fig. 3 Total amounts of fatty acids longer than the 20-carbons units, VLCFAs, in the F_1 to F_6 generations of Line 1

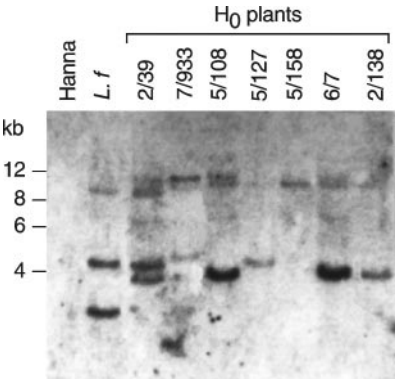

Fig. 4 Southern-blot analysis of seven different H_0 plants. Total DNAs were digested with *Eco*RI and filters were hybridised with the hydroxylase gene-specific probe. DNA from the parental material, *B. napus* cv Hanna and *L. fendleri*, are used as controls. Sizes in kb are shown to the left of the panel

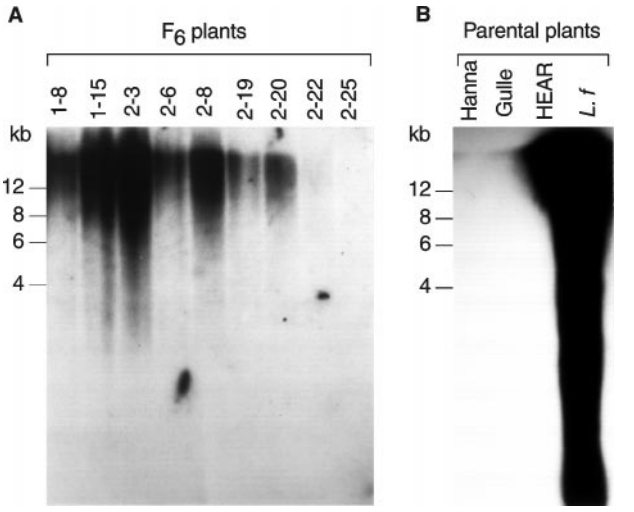

Fig. 5 Southern-blot analysis of **A** nine different F_6 plants and **B** parental plants using the *L. fendleri* species-specific repetitive probe. Total DNA was digested with *Hin*dIII. Sizes in kb are shown to the left of the panel

The presence and expression of *L. fendleri* genes in Line 1

The presence of the *L. fendleri* hydroxylase gene in the asymmetric hybrids was examined in 26 H_0 plants by hybridisation with a hydroxylase gene probe. Bands corresponding to the hydroxylase gene were observed in 11 of the analysed H_0 plants (Fig. 4). Inheritance of the hydroxylase gene in the following generations was studied in nine F_6 plants from Line 1. One F_6 plant, 2-6, was identified as having hybridised to a band that was interpreted as a hydroxylase gene. The presence of *L. fendleri* DNA was confirmed in seven of the nine analysed F_6 plants after hybridisation with the *L. fendleri* species-specific repetitive sequence (Fig. 5).

Southern- and Northern-blot analysis using the *FA1*/*B. napus*- and the *FA1*/*A. thaliana*-specific probes did not reveal any *L. fendleri*-specific hybridisation patterns (data not shown).

Analysis of hydroxylated fatty acids in Line 2

Small amounts of ricinoleic acid were found in the F_1 generation of Line 2. To confirm this result, methyl esters from the hydroxy fatty acid fraction were purified from TLC silica-gel plates and analysed through a capillary column by gas chromatography. A comparison between Line 2 and methylated castor bean endosperm (*R. communis)* suggests that ricinoleic acid was present in the hybrid since the retention times were the same in the purified sample as in the control. However, in contrast to Line 1, Line 2 did not contain erucic acid although the seed was obtained from the same original H_0 plant.

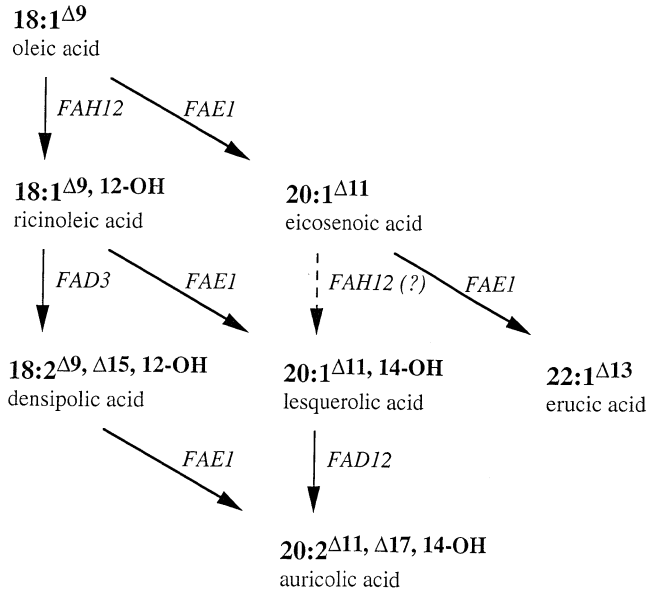

Fig. 6 Proposed biosynthetic pathway for the synthesis of hydroxylated fatty acids, modified after Broun and Sommerville (1997) and Reed et al. (1997)

In order to increase the content of ricinoleic acid and to produce hydroxylated fatty acids with a longer carbon chain, such as lesquerolic acid (Fig. 6), Line 2 was selfed and crossed with the high-erucic acid rapeseed cv Gulle (Fig. 1). As a control, crosses with cv Hanna were made. Fifty seeds were screened from the selfed offspring and the back-cross with cv Hanna, respectively, and 30 seeds were analysed from the cross with cv Gulle. Hydroxylated fatty acids were not found in progeny from either the crossed or selfed populations of Line 2. In accordance with this result, Southern-blot analysis of Line 2 using the hydroxylase gene probe did not show any hybridisation pattern that was indicative for the presence of a *L. fendleri* hydroxylase gene.

Discussion

Asymmetric somatic hybridisation between *B. napus* and *L. fendleri* made it possible to modify the fatty acid composition in seeds of the hybrid progeny. The presence of the novel ricinoleic acid and higher amounts of both erucic and eicosenoic acids were detected in seeds from one progeny derived from the first back-cross with the same rapeseed cultivar as used in the hybridisation experiment. The original F_1 plant was previously analysed in respect of its chromosome constitution (Skarzhinskaya et al. 1998). By genome in situ hybridisation, GISH, it was not possible to detect the presence of complete chromosomes or large chromosome fragments originating from *L. fendleri*. However, it has been shown by Southern-blot analysis, both earlier (Skarzhinskaya et al. 1998) and in this study, that this plant indeed contained DNA from *L. fendleri*. To test whether the translocated *L. fendleri* DNA was stably integrated in the *B. napus* genome, and if the fatty acid contents could be improved, subsequent crosses were performed. Crosses with different rapeseed cultivars and self-fertilisations resulted in F_6 plants predominantly containing the rapeseed genome.

The F_1 generation of Line 1 produced 16.5% erucic acid and 15% eicosenoic acid, which suggests a contribution of a new elongase gene copy from *L. fendleri*. After selfing F_2 plants, individuals were found producing up to 35% erucic acid and 15% eicosenoic acid. The rapeseed elongase gene has earlier been shown to contribute 9–10% erucic acid per gene copy (Harvey and Downey 1964). Our result indicates that the *L. fendleri* elongase gene contributes more erucic acid per gene copy than the rapeseed elongase gene. Moreover, *L. fendleri* produces mainly 20-carbon fatty acids in its storage lipids. However, in the hybrid plants 22-carbon fatty acids are primarily produced, which suggests that there are more factors determining carbon-chain length in *B. napus* than just the elongase gene. In offspring of the *B. napus* and *L. fendleri* hybrids, the origin of the *FAE1* gene copies could not be determined when using the *B. napus* homologous sequence and the *A. thalina FAE1* gene as probes. These sequences were not specific enough for the *L. fendleri FAE1* gene.

After a series of crosses and selfings, one F_6 plant was produced which generated seeds containing slightly more than 61% erucic acid. This result can be compared with the highest levels of 57% reported in resynthesised rapeseed lines made by protoplast hybridisation (Heath and Earle 1995). Re-synthesised rapeseed lines made by interspecific sexual crosses between *B. oleracea* and *B. rapa* have also been produced containing about 60% erucic acid (Lühs and Friedt 1994). The theoretical upper limit of erucic acid and eicosenoic acid that can be produced in *B. napus* is 66% since its lysophosphatidic acyltransferase (LPAAT) lacks the ability to incorporate these acyl groups at the *sn*-2 position in the triacylglycerol backbone (Bernerth and Frentzen 1990; Frentzen 1993). This implies that our result, reaching 65% of the total content of the VLCFAs, is very close to the upper limit. In another case the gene coding for LPAAT from *Limnanthes alba* and *L. douglasii* has been cloned and transferred to rapeseed with the purpose of increasing erucic acid levels even further (Lassner et al. 1995; Brough et al. 1996). Transgenic plants that posessed the LPAAT gene also had erucic acid at the *sn*-2 position in its triacylglycerols; however, the total erucic acid content was not increased (Brough et al. 1996). Since the *Limnanthes* LPAAT shows specificity for both oleic and erucic acids it may not be specific enough for producing higher amounts of trierucoylglycerol when transformed into rapeseed (Löhden and Frentzen 1992). Hypothetically, the rate-limiting step in the process is the amount of erucoyl-CoA produced.

A higher amount of available erucoyl-CoAs from a transgenic rapeseed line with an extra *FAE1* gene could force the *Limnanthes* LPAAT to produce more trierucoylglycerols. One way to obtain more erucoyl-CoAs would be by over-expression of the *FAE1* gene. This could be made possible by transfer of the gene to rapeseed using transformation or, as in our study, by somatic hybridisation. To test this hypothesis, our high erucic acid line would be an interesting starting material for transformation with the *Limnanthes* LPAAT gene. This approach may be a way to produce seed oils reaching towards the 90% erucic acid level which is deemed necessary for competing economically with fossil oils (Murphy 1996).

Erucic acid content can also be affected by external factors such as growth temperature. Erucic acid, together with oleic acid, are the two fatty acids that respond most to climatic conditions. It has been found that at low temperatures the content of erucic acid is increased whilst the content of oleic acid is decreased (Canvin 1965; Wilmer et al. 1996). In *B. oleracea* an increase in temperature from about 20°C to about 30°C has resulted in a dramatic drop of erucic acid content from about 63% to 43% (Doug Heath, personal communication). In our study, plants in the F_4 generation were grown in a day/night regime of 27°C/22°C and produced F_5 seeds containing up to 51% erucic acid. This result can be compared with both the F_3 and the F_5 generations which were grown at lower temperatures. These F_3 plants produced seeds containing more than 58% erucic acid, and in the F_5 generation more than 61%. Since the genetic constitutions of the F_4 and F_5 plants are rather similar, the high temperature is most likely the cause of the lower content of erucic acid in the seeds derived from the F_4 plants.

Although *L. fendleri* primarily synthesises lesquerolic acid, Line-2 seeds produced mainly ricinoleic acid. The rapeseed cultivar Hanna that was used for somatic hybridisation is a zero-erucic acid cultivar and lacks the enzymes involved in the elongation of oleic acid to longer fatty acids. Thus, it is reasonable to expect that the hydroxylated fatty acid produced would be ricinoleic acid, rather than hydroxy fatty acids with longer carbon chains, if an elongase gene was not simultaneously transferred to the hybrid from the *L. fendleri* genome (Reed et al. 1997). Similar results were achieved in transgenic rapeseed plants by expressing the *L. fendleri* and *R. communis* hydroxylase genes (Broun et al. 1998). The transgenic rapeseed plants produced mainly ricinoleic acid which is as expected since the rapeseed cultivar employed was zero-erucic acid variety.

The subsequent sexual crosses performed with Line 2 were designed with the purpose of transferring the elongase gene from cv Gulle, or to produce similar or greater amounts of ricinoleic acid by selfing. However, no hydroxy fatty acids were produced in the following generation and the gene could not be detected by Southern-blot analysis. This points to an unstable integration of this gene in the rapeseed genome and was also observed in the same generation of Line 1. Restriction fragments corresponding to a putative hydroxylase gene were, on the other hand, observed in 11 of the 26 analysed H_0 plants and in one F_6 plant of Line 1, 2-6. The instability of this gene expression may be due to gene silencing by methylation of the promoter regions (Matzke et al. 1989), or by damage to the gene by the X-irradiation treatment (Vizir et al. 1996) which was used before protoplast fusion.

In conclusion, this study shows that elongase and hydroxylase genes can be transferred to rapeseed and expressed in the offspring of asymmetric somatic hybrids between *B. napus* and *L. fendleri*. By successive crosses to lines and cultivars containing two and four copies of the *FAE1* gene it has been possible to raise the level of erucic acid to more than 61%.

Acknowledgements The *FAE1* clones from *A. thaliana* and the *FAE1* homologue from *B. napus* were kindly donated by Ljerka Kunst, University of British Columbia, Canada, while the hydroxylase gene fragment from *L. fendleri* came from Chris Somerville, Stanford University, USA. We thank Svalöf Weibull AB, Svalöv, Sweden, for kindly providing *B. napus*, cv Hanna, cv Gulle and HEAR seeds, Kristin-Sophie Mellsjö for help with the crossings, and Ingrid Eriksson for excellent work with the Southern and Northern blots. This investigation was supported by grants from the Swedish Research Council for Engineering Sciences, the Swedish Council for Forestry and Agricultural Research, the Nilsson-Ehle foundation, Sweden, the EU-INTAS programme 94-2019, the SLU TEMA-programme in Plant Biotechnology, and the Swedish Foundation for Strategic Research.

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