Toward Increasing Erucic Acid Content in Oilseed Rape (*Brassica napus* L.) Through the Combination with Genes for High Oleic Acid

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ABSTRACT: Erucic acid (22:1) is a valuable renewable resource that has several applications in the oleochemical industry. High 22:1 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), PUFA (18:2 + 18:3), and saturated FA (16:0 + 18:0) contents. In the present experiment, HEAR was crossed to high oleic acid rapeseed (*ca.* 85% 18:1) with the hypothesis that a combination of the involved genes should lead to a reduced 18:1 desaturation and to an increased availability of oleoyl-CoA, which should result in enhanced 22:1 synthesis. A NIR spectroscopic calibration for 22:1 was developed for single seeds, and the calibration was used to select, in a nondestructive manner, F_2 seeds high in 22:1. Selected F_2 seeds were sown in the field and F_3 seeds were harvested. The results of the FA analysis showed recombinant genotypes with increased total monounsaturated FA (22:1 + 20:1 + 18:1) of up to 89% and decreased PUFA (<8%) and saturated FA content (<3.5%). There was no significant difference in 22:1 content, but a 3 to 5% increase in 20:1 content was observed in comparison to the HEAR parental cv. Maplus. Results were confirmed following cultivation of selected plant material a second year in the field. The present study revealed that there are other biochemical limitations than the pool of available oleoyl-CoA that restrict FA elongation to 22:1 in rapeseed. The generated high 22:1 plant material with an increased 18:1 content may be useful in further studies to identify these limitations.

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Rapeseed cultivars with a high content of erucic acid (22:1) in the seed oil are cultivated in Europe and in North America for industrial utilization of the oil. Erucic acid is a valuable and renewable resource that has several applications in the oleochemical industry for the production of such things as plastics, lubricants, slip and coating agents, soaps, printing inks, and surfactants (1,2). High erucic acid rapeseed (HEAR) cultivars contain around 50% 22:1 in the seed oil. Increasing the 22:1 content would considerably reduce the processing costs of the oil and hence increase the profitability of growing the crop. A reduction of the eicosenoic acid (20:1, around 8% in HEAR) content is also of interest, because this FA is difficult to separate from 22:1 by fractional distillation, which is the main industrial process applied to separate erucic acid from the other by-product FA (3). Furthermore, a decrease of the PUFA and the saturated FA content of the HEAR oil is expected to increase the oxidative stability and the flow properties of the oil at low ambient temperatures, respectively.

Several approaches have been pursued to increase the erucic acid content, however, with only limited success so far. In the amphidiploid species *Brassica napus*, 22:1 content is inherited by two genes that act in an additive manner. In HEAR oil, 22:1 is exclusively esterified at the *sn*-1 and *sn*-3 positions of the glycerol backbone owing to the specificity of the *B. napus sn*-2-acyltransferases, which exclude erucic acid from the *sn*-2 position of TAG. Therefore, 22:1 content is limited to 67% (4). By expressing chimeric erucoyl-CoA-specific lysophosphatidate acyltransferase (LPAAT) genes in developing seeds of transgenic HEAR lines, 22:1 was directed to all three positions of the glycerol backbone, allowing the synthesis of trierucoyl glycerol. However, the overall proportion of erucic acid in the seed oil did not increase (5–7).

Erucoyl-CoA is synthesized from oleoyl-CoA by a microsomal multienzyme elongase system and involves sequential additions of two carbon moieties derived from malonyl-CoA in a series of reactions that are similar to *de novo* FA biosynthesis (8). In the initial β-ketoacyl-CoA synthase (KCS) reaction, malonyl-CoA condenses with oleoyl-CoA of a cytosolic pool, which is maintained by plastidial FA synthesis (9). Subsequently, the condensation product undergoes reduction, dehydration, and a further reduction to remove the keto group from the elongated acyl chain. *Via* a further elongation cycle, eicosenoyl-CoA is converted to erucoyl-CoA and becomes available to seed oil synthesis. The *fae1*-gene encoding the KCS has been cloned and has been overexpressed under control of the seed-specific napin promoter in combination with the above-mentioned *LPAAT*-gene in HEAR (10,11). However, the results obtained were disappointing, because only a minor increase in the 22:1 content was observed. These results indicate that there are other limitations restricting an enhanced 22:1 synthesis. As stated above, oleoyl-CoA is one of the substrates needed for elongation to 22:1. The 18:1 content in the seed oil of HEAR is, at around 13%, already quite low compared with the 60% of '00'-quality/canola oil. This could be indicative of a low cytosolic pool of available oleoyl-CoA, which may limit

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the elongation (9,12). Additional evidence for a FA elongation-limiting effect of oleoyl-CoA is provided by mutation experiments performed in *B. carinata* (13). A mutant line with a reduced content of linoleic acid (8.0 vs. 20.6%) and correspondingly increased 18:1 showed an increased 22:1 content (52.0 vs. 43.5% in the original breeding line).

An alternative way to study the question of whether the availability of oleoyl-CoA is limiting 22:1 synthesis is to cross high oleic acid rapeseed (HOAR) to HEAR so as to recombine the genes for high 22:1 with those for high 18:1. The development of HOAR has been reported in several previous studies and has been accomplished either through antisense inhibition (14) of the 18:1 desaturase *fad*2-gene or through mutagenesis (15).

In the present study, HOAR rapeseed lines (*ca*. 85% 18:1) were crossed to the HEAR cv. Maplus (*ca*. 50% 22:1). Since 22:1 content is inherited by two genes and the high-18:1 trait in the present material is inherited by one major and an additional two or three minor genes (15), a large number of F_2 seeds had to be analyzed to identify the desired recombinants. This was done in the present study by using a NIR spectroscopic (NIRS) calibration for prediction of the 22:1 content of single seeds (16).

EXPERIMENTAL PROCEDURES

Four high 18:1 and low linolenic acid (18:3) sister doubled haploid lines ('00'-quality, winter rapeseed, derived from the cross F4 7-65/94 NPZ × ((453 × Hk 102) × Gö9) were crossed to the high 22:1 winter rapeseed cv. Maplus ('+0', 50% 22:1). The four doubled haploid lines had similar 18:1 (82–86%), 18:2 (2.7–4.7%), and 18:3 (2.6–4.3%) contents. F_1 plants were grown in the greenhouse and selfed to obtain F_2 seeds. From one cross DH XXII D9 × cv. Maplus, 1333 single F_2 seeds were analyzed for their FA composition by gas chromatography (17) to study the segregation in F_2 seeds. F_2 seeds comprising a subsample from all four crosses (n = 867) were scanned by NIRS (Foss 6500) using a single seed adapter as described in Sasongko (18) and analyzed afterward for their FA composition. A calibration for the 22:1 content was developed using WinISI 1.04 (19). This had a coefficient of determination in cross-validation of 0.58 and a SE in cross-validation of 6.5%. The calibration was used to select out of 3200–3600 scanned seeds from each of the four F_2 populations those 200 seeds with the highest 22:1 content.

Selected seeds were sown in 2001 in rows (25 seeds per row) together with the parental cv. Maplus (every 10th row) in the field at the experimental station Reinshof, Göttingen, Germany. After selfing, seeds from the F_2 plants and of cv. Maplus (3 plants per row) were harvested in 2002. GC was used to analyze 200 mg of the seeds as well as single seeds for their FA composition (17). F_3 seeds from selected F_2 plants with a high 22:1 and a low PUFA content were again sown in the field, together with cv. Maplus as in the year before (Reinshof, Göttingen, 2002). After selfing, seeds of the F_3 plants and of cv. Maplus (3 plants per row) were harvested in 2003. Harvested F_4 seed samples were analyzed by GC for their FA composition. Since in both years the means for the different FA of cv. Maplus were not significantly different between the rows, the data of the selfed Maplus plants were combined in each year for further analysis. Spearman rank correlations were calculated using Plabstat (20); means were analyzed for significant difference using the Tukey–Kramer test (Superanova, v. 1.1, 1989; Abacus Software, Grand Rapids, MI).

RESULTS AND DISCUSSION

In the F_2 seed population, a segregation for 22:1 approximates a 1:4:6:4:1 pattern, as expected for a digenic inherited trait (Fig. 1). However, the different classes could not be separated, and the high-22:1 class appeared much too small. Among the 1333 single seeds analyzed by GC, there was not a single one having a 22:1 content higher than 48%. The scatter plot showing 18:1 vs. 22:1 content in the single seeds indicated the presence of high 22:1 F_2 seeds with a considerable variation in 18:1 content (Fig. 2). It also showed the segregation of F_2 seeds with an 18:1 content up to 87%. Highly significant negative correlations were found between 18:1 and 22:1 and 20:1 + 22:1 (Table 1). Correlations between 18:2 + 18:3 and 22:1 and 20:1 + 22:1 were negative but relatively low. A significantly negative correlation was also found between the saturated FA and the monounsaturated FA (MUFA) content ($r_S = -0.53$**, where ** indicates significance at the 99% probability level). The correlation between 22:1 and 20:1 + 22:1 and with 20:1 obviously deviated from a linear relationship (not shown). Maximal 20:1 contents were found for genotypes with an intermediate 22:1 and 20:1 + 22:1 content, respectively.

From each of the four different crosses, the 200 F_2 seeds with the highest 22:1 content as predicted by the NIRS calibration developed in our laboratory (data not shown) were selected and sown in the field. Owing to unfavorable weather conditions in the summer of 2002, F_3 seeds were obtained from only 351 F_2 plants. The F_2 plants of the four different crosses were not significantly different for 22:1 or 18:1 content (data not shown). Hence, the data of the four different crosses were combined for further evaluation. Figure 3A shows that following

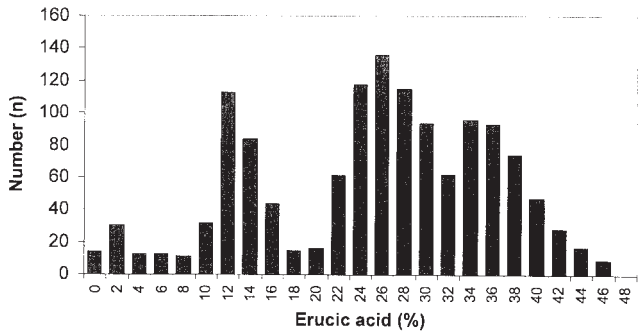

FIG. 1. Frequency distribution of the erucic acid (22:1) content in F_2 seeds derived from a cross between high erucic acid and high oleic acid rapeseed (n = 1333).

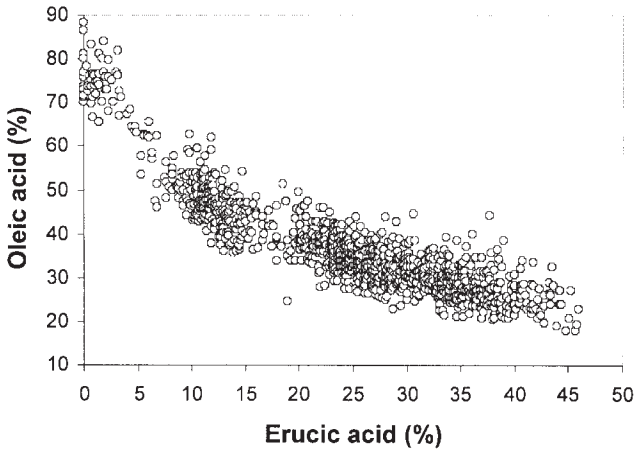

FIG. 2. Oleic acid (18:1) and erucic acid (22:1) content of F_2 seeds derived from a cross between high erucic acid and high oleic acid rapeseed (n = 1333).

single seed selection in F_2 for high 22:1 content by NIRS, only F_2 plants with about 20% or more 22:1 are obtained. Some of the high 22:1 F_2 plants had a 15% increased 18:1 content compared with the high 22:1 parent cv. Maplus (Fig. 3A), which corresponded to a reduced 18:2 + 18:3 content (Fig. 3B). However, this did not lead to an enhanced 22:1 content. The high 22:1 F_2 plants with an elevated 18:1 content tended to have a higher 20:1 content (Fig. 3C). A wide variation for MUFA content, ranging from 65 to 87%, was also observed (Fig. 3D). A negative correlation between the monounsaturated erucic acid and the sum of palmitic acid and stearic acid was found (r_S = –47**; data not shown). An even closer negative correlation was found between MUFA the sum of palmitic acid and stearic acid (r_S = –059**; Fig. 3D) with levels of saturated FA as low as 3%. Such a negative correlation has been reported before for high oleic acid rapeseed (10,21,22). Since 18:1 is the precursor for 20:1 and 22:1, the sum of these FA should be equivalent to the 18:1 content in the HOAR parental line used in the present crosses. Möllers and Schierholt (23) developed a hypothesis to explain this negative correlation.

From the plant material shown in Figure 3, five high-22:1 F_2 plants with an elevated 18:1 content were selected for further analysis. From these, single F_3 seeds were analyzed to see whether there was further segregation in their FA composition. The results obtained confirmed that the F_2 plants had a 22:1 content equal to cv. Maplus, but a 3 to 5% significantly increased 20:1 content (Table 2). Their content of PUFA was reduced to around 7 to 10% (see Table 2). Furthermore, MUFA content was 85 to 88% as high as the 18:1 content of the high-18:1 parental lines. SD for 22:1 and the other FA were small and in the range of cv. Maplus, suggesting homozygosity of the genes involved in the relevant FA pathway. Therefore, further changes in the FA composition of the present material are unlikely to occur in later generations. The five 22:1 F_2 plants with an elevated 18:1 content were tested as F_3 lines a second year in the field (Table 3). Compared with the first year, the 22:1 contents were found to be slightly higher. Again, there was no difference in the 22:1 content between cv. Maplus and the selected F_3 lines. The tested F_3 plants proved to have a significantly reduced saturated FA content compared with cv. Maplus. Their 18:1 content was doubled and their 18:2 + 18:3 content was reduced to values well below 10%. The MUFA content ranged from 85 to 88%. Table 3 also shows that the plants with a high MUFA content had 48 to 50% 22:1, 11 to 12% 20:1, and 26 to 27% 18:1.

In the present experiments, the genes for high-22:1 and an elevated 18:1 content have successfully been recombined; however, this has not led to an increase of the 22:1 content in the recombinant lines (Tables 2 and 3). These results are in contrast with those of Velasco *et al.* (13), who observed in *B. carinata* that following mutagensis a reduced 18:2 content was associated with a significant increase in 22:1 content. The present results are rather in line with the results of Lemieux *et al.* (21) and Auld *et al.* (22), who reported that *Arabidopsis* and *B. rapa* mutant lines with a reduced 18:1 desaturation showed only a minor increase of 4 to 7% in the 20:1 and 22:1 content, respectively. It is not known whether, in the present high-22:1 plant material with an elevated 18:1 content, KCS activity is limiting 22:1 biosynthesis. This needs to be analyzed in crosses with transgenic lines overexpressing the *fae1*-gene (11).

If the aim is to extract 22:1 for oleochemical utilization, the high-22:1 plant material with an elevated 18:1 content generated in the present study does not represent any improvement over the available HEAR cultivars. However, the economic value of the by-products is an important factor to consider (3). Following 22:1 extraction from conventional HEAR oil, remaining FA are sold as a by-product at a comparatively low

TABLE 1
Spearman's Rank (r_s) Correlations[a] of FA Contents in F_2 Seeds of the Cross DH XXII D9 × cv. Maplus[a] (n = 1333)

FA	16:0 + 18:0	18:1	18:2 + 18:3	20:1	22:1	20:1 + 22:1
18:1	0.27**	—				
18:2 + 18:3	0.34**	–0.23**	—			
20:1	–0.09**	0.23**	–0.22**	—		
22:1	–0.45**	–0.89**	–0.12**	–0.33**	—	
20:1 + 22:1	–0.50**	–0.86**	–0.22**	–0.16**	0.97**	—
MUFA[b]	–0.53**	0.18**	–0.95**	0.26**	0.18**	0.28**

[a]**Significant at $\alpha < 0.01$.
[b]MUFA, monounsaturated FA (18:1 + 20:1 + 22:1).

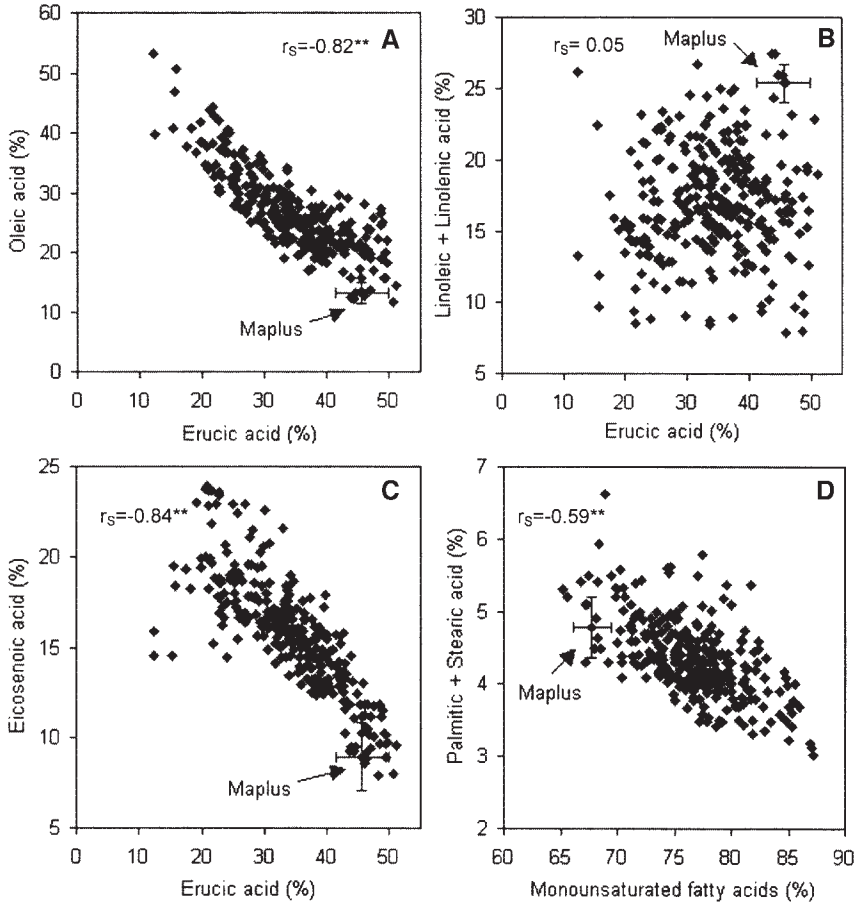

FIG. 3. FA composition of F_2 plants (F_3 seeds) derived from crosses between high erucic acid (22:1) and high oleic acid (18:1) rapeseed, preselected for a high erucic content (n = 351), in comparison with cv. Maplus (n = 51, mean ± SD).

price (Westfechtel, A., Cognis, Düsseldorf, Germany; personal communication). To date, whether the modified composition of the by-product FA of the present material represents any value-added improvement. However, 18:1 is an important FA in the oleochemical industry, and Warwel (24) stated in 1993 that plant breeders should try to breed 'Combi-Raps' with an oil consisting of 50% 18:1 and 50% 22:1. The plant material generated within this study does represent some progress toward this aim (see Table 3). If high 22:1 oil is used as a biodegradable lubricant or hydraulic oil, the high-MUFA oil generated in the present study could have some advantage over the conventional high-22:1 oil. Technical testing of the oil is necessary to obtain solid data.

The present study has revealed that other yet unknown biochemical limitations restrict the elongation to 22:1 in rapeseed (for discussion see, e.g., Ref. 25). The plant material generated in the present study may be useful in further studies to identify these limitations. As indicated in the introduction, several molecular approaches to increase the 22:1 content have already been tested, however, a major breakthrough has not yet been achieved. A combination of the above-mentioned transgenic plants (11) with the high 22:1 and increased 18:1 plant material

TABLE 2
Mean FA Content and SD of Selected High-Erucic (22:1) F_2 Plants with an Increased Oleic Acid (18:1) Content and of the Parental cv. Maplus[a] (analysis of single F_3 seeds; field year 2001/2002)

Plant	*n* (seeds)	16:0 + 18:0	18:1	18:2 + 18:3	20:1	22:1	MUFA
6575-1	20	3.2 ± 0.3^b	30 ± 1.5^b	6.7 ± 0.8^a	12.4 ± 1.3^b	47 ± 2.8^a	89 ± 1.1^b
6586-3	19	3.6 ± 0.6^b	28 ± 2.3^b	8.0 ± 2.3^a	11.1 ± 1.6^b	48 ± 4.4^a	87 ± 3.3^b
6585-1	25	3.3 ± 0.3^b	26 ± 3.1^b	9.7 ± 3.1^a	12.2 ± 1.4^b	48 ± 1.9^a	86 ± 3.3^b
6596-6	23	3.5 ± 0.4^b	27 ± 2.7^b	8.7 ± 1.1^a	10.4 ± 1.6^b	49 ± 3.8^a	86 ± 1.2^b
6620-1	25	3.5 ± 0.5^b	26 ± 5.0^b	10.6 ± 4.2^a	10.5 ± 1.8^b	49 ± 3.3^a	84 ± 4.6^b
Maplus	19	5.0 ± 0.6^a	14 ± 2.9^a	25.7 ± 0.6^b	7.2 ± 1.8^a	47 ± 4.6^a	68 ± 3.1^a

[a]Superscript letters a,b indicate significantly different at $P < 0.05\%$, Tukey-Kramer. For abbreviation see Table 1.

TABLE 3
Mean FA Composition of F_3 Plants (F_4 seeds) of Selected High-Erucic (22:1) F_3 Plants with an Increased Oleic Acid (18:1) Content and of the Parental cv. Maplus (analysis of 200 mg seed samples; field year 2002/2003)

Plant	*n* (plants)	16:0 + 18:0	18:1	18:2 + 18:3	20:1	22:1	MUFA
6575-1	23	3.1 ± 0.2[b]	27 ± 2.8[b]	6.9 ± 0.3[a]	10.9 ± 2.7[b]	50 ± 1.8[a]	88 ± 2.9[b]
6586-3	14	3.4 ± 0.2[b]	27 ± 1.2[b]	8.1 ± 0.2[a]	11.2 ± 1.1[b]	49 ± 1.6[a]	87 ± 1.3[b]
6585-1	15	3.4 ± 0.2[b]	26 ± 2.4[b]	9.5 ± 2.1[a]	11.1 ± 1.7[b]	48 ± 2.4[a]	85 ± 2.2[b]
6596-6	25	3.6 ± 0.2[b]	26 ± 1.2[b]	8.7 ± 1.0[a]	10.6 ± 1.5[b]	50 ± 2.2[a]	86 ± 1.1[b]
6620-1	6	3.3 ± 0.2[b]	26 ± 1.0[b]	8.0 ± 0.7[a]	12.0 ± 1.4[b]	48 ± 2.4[a]	87 ± 0.7[b]
Maplus	23	4.7 ± 0.4[a]	13 ± 1.2[a]	22.9 ± 0.9[b]	8.0 ± 1.4[a]	50 ± 2.0[a]	71 ± 1.0[a]

[a]Superscript letters a,b indicate significantly different at $P < 0.05\%$, Tukey-Kramer. For abbreviation see Table 1.

generated in the present study by classical breeding could be useful to achieve some enhancement in 22:1 biosynthesis.

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