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W. Ecke · M. Uzunova · K. Weißleder

Mapping the genome of rapeseed (*Brassica napus* L.). II. Localization of genes controlling erucic acid synthesis and seed oil content

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Abstract A F_1 microspore-derived DH population, previously used for the development of a rapeseed RFLP map, was analysed for the distribution of erucic acid and seed oil content. A clear three-class segregation for erucic acid content could be observed and the two erucic acid genes of rapeseed were mapped to two different linkage groups on the RFLP map. Although the parents of the segregating DH population showed no significant difference in seed oil content, in the DH population a transgressive segregation in oil content was observed. The segregation closely followed a normal distribution, characteristic of a quantitative trait. Using the program MAPMAKER/QTL, three QTLs for seed oil content could be mapped on three different linkage groups. The additive effects of these QTLs explain about 51% of the phenotypic variation observed for this trait in the DH population. Two of the QTLs for oil content showed a close association in location to the two erucic acid genes, indicating a direct effect of the erucic acid genes on oil content.

Key words *Brassica napus* · RFLP · Erucic acid genes · Oil content · QTL mapping

Introduction

Seed oils from many *Brassica* species differ from most other vegetable oils in containing substantial amounts of the long chain monoenoic fatty acids, eicosenoic acid ($C_{20:1}$) and erucic acid ($C_{22:1}$), that are derived from oleic acid ($C_{18:1}$) by the addition of one and two C_2 units, respectively. Feeding experiments with animals indicated that erucic acid may pose a health risk in human consumption (Thomasson 1955; Beare et al. 1959; Roine and Uksila 1959). Recognizing these results, major efforts were made in the sixties to elucidate the genetic control of erucic acid synthesis in *Brassica napus* and *Brassica rapa* and to reduce the erucic acid content in the seed oils of these plants. In rapeseed (*Brassica napus* L.) it was found that erucic acid synthesis is controlled by two genes with additive effects (Harvey and Downey 1964; Stefansson and Hougen 1964; Siebels and Pauls 1989) and that these genes also control the synthesis of eicosenoic acid, although not in an additive manner (Kondra and Stefansson 1965; Jönsson 1977). The successful breeding of rapeseed varieties with seed oil virtually free of erucic acid led to a dramatic increase in the production of rapeseed oil, making rapeseed one of the major oil crops world-wide. In recent years, however, a growing interest again emerged in rapeseed oil with a high amount of erucic acid for technical applications. High erucic acid oils are excellent lubricants and the fatty acid can be modified in numerous ways to produce water repellents, plasticizers, waxes, and surface-active agents (Lühs and Friedt 1993).

In the initial breeding of erucic acid-free rapeseed varieties, a significant reduction in seed oil content was observed. Due to the complex genetic control of oil content it was not possible to distinguish whether this reduction was due to a direct effect of the erucic acid genes or had to be attributed to the genetic background of the new varieties.

In many agronomically important plant species, including rapeseed, the present availability of large numbers of RFLP markers has led to the development of dense linkage maps (Landry et al. 1991; Pillen et al. 1992; Tanksley et al. 1992; Kishimoto et al. 1993; Kleinhofs et al. 1993; Ferreira et al. 1994). These RFLP linkage maps have proved to be very useful tools for localizing genes of interest, for studying genome structure and evolution, and for comparing the genome organization and gene order of different species (Bonierbale et al. 1988; Tanksley et al 1988; Barone et al. 1990; Hosaka et al. 1990; Jung et al. 1990; Klein-Lankhorst et al. 1991). Furthermore, using bulked segregant analysis it

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W. Ecke (✉) · M. Uzunova · K. Weißleder
Institute of Agronomy and Plant Breeding, University of Göttingen, Von-Siebold-Strasse 8, D-37075 Göttingen, Germany

has been possible to specifically select markers linked to genes controlling simply inherited characters (Giovannoni et al. 1991; Michelmore et al. 1991). In the analysis of traits showing quantitative variation new approaches to identify and localize the genetic factors contributing to these traits and to estimate their effects have become available through the use of RFLP linkage maps (Lander and Botstein 1989; Haley and Knott 1992).

In our ongoing effort to map genes and QTLs controlling agronomically important traits of rapeseed we have used an RFLP map of the rapeseed genome to localize the two erucic acid genes and three QTLs influencing seed oil content. By comparing the map positions of these genes new insights into the relationship between erucic acid synthesis and oil content were obtained.

Materials and methods

Plant materials and RFLP map

The erucic acid genes and the QTLs for oil content were mapped in a segregating DH population using an RFLP map that had been previously established in the same population. The segregating DH population consists of 151 F_1 microspore-derived doubled haploid lines from a cross between DH lines from the winter rapeseed varieties 'Mansholt's Hamburger Raps' and 'Samourai'. 'Mansholt' is an old landrace with high contents of erucic acid and glucosinolates. 'Samourai' is a new French variety of canola quality.

The RFLP map consists of 205 genetic markers distributed across 19 linkage groups with a total length of 1441 cM (Kosambi 1944). In addition to 201 RFLP markers the map includes three RAPD markers and one phenotypic marker (Uzunova et al. 1995).

Estimation of seed oil and erucic acid content

In 1992/1993 the parental DH lines, the F_1, and the lines of the segregating DH population were grown at Göttingen-Reinshof in separate but neighbouring blocks in the field. Plots consisted of two rows, 2.5 m in length. The distance between rows was 0.33 m and between plants within rows 0.12 m. Seed samples of 4–5 g harvested separately from 1 to 12 plants of each double-row were used to measure the seed oil content by near-infrared reflection spectroscopy (NIRS; Reinhardt 1992). Erucic acid content in the seed oil was estimated by gas chromatography in single seeds from the DH lines according to the method of Thies (1971). For each DH line two seeds were analysed.

Bulked segregant analysis

Bulked segregant analysis was performed according to Michelmore et al. (1991) and Giovannoni et al. (1991), using two bulks of 8 or 12 genotypes each for RFLP and RAPD markers, respectively. DNA isolation, RFLP, and RAPD analysis have been described previously (Uzunova et al. 1995).

Mapping of genes and QTLs

Deviation from free segregation between individual markers and the erucic acid genes was tested by χ^2 analysis using 2×2 tables (contingency tables; Mather 1957) setting out marker classes vs erucic acid phenotypes (erucic acid-free and erucic acid-containing). Interval mapping was performed on framework maps of highly informative and well spaced markers, representing the 19 linkage groups of the RFLP map.

For interval mapping of the erucic acid genes two genetic models were applied. In the first (model I) the two genes were assumed at fixed positions in two marker intervals on two different linkage groups. In the second (model II) free segregation between the genes and the flanking markers of the intervals was assumed. For both models the likelihood to observe the actual population was estimated (Mather 1957) and the LOD score was derived as the $\log_{10}$ from the quotient of the two likelihoods $[\log_{10}(\text{likelihood}_{\text{model II}}/\text{likelihood}_{\text{model III}})]$. To map the genes the two assumed positions were independently moved in steps of 0.5 cM through all marker intervals of two linkage groups, resulting in a LOD surface with each point representing the log-likelihood ratio for the presence of the two erucic acid genes at the corresponding positions of the linkage groups.

QTLs were mapped by interval mapping using the computer program MAPMAKER/QTL. A LOD score threshold of 2.8 was used, giving approximately a 5% chance of falsely declaring a QTL to exist anywhere on the map (Lander and Botstein 1989). The QTLs were first localized by scanning the linkage groups in 2-cM steps. When using models with fixed QTLs, the LOD score attributable to the putative QTL at the scanned position was calculated as the difference between the total LOD score and the LOD score derived from the fixed QTLs only. MAPMAKER/QTL's "map" command was used to precisely determine the most likely positions of QTLs and to estimate the additive effects of the mapped QTLs.

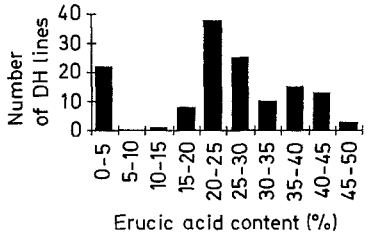
Results

Mapping of the erucic acid genes

The erucic acid content was analysed in 135 lines from the segregating DH population used for the construction of the rapeseed RFLP map. In agreement with the segregation of two genes with equal effects, three classes could be distinguished in the distribution of erucic acid contents between these lines (Fig. 1). As the two classes with high and medium erucic acid content were not clearly separated, for all of the following genetic studies the DH lines were partitioned into only two classes, erucic acid-free and erucic acid-containing. For two genes with equal effects a segregation ratio of 1 : 3 is then expected in a segregating DH population. With only 22 DH lines free from erucic acid a significant shortage of erucic acid-free genotypes was observed ($\chi^2 = 5.45$; $df = 1$; $P < 0.025$), indicating a skewed segregation of at least one of the erucic acid genes.

Since the erucic acid content in rapeseed is controlled by two genes with additive effects, standard algorithms for genetic mapping of genes or QTLs as implemented in programs like Linkage, MAPMAKER or MAP-

Fig. 1 Distribution of erucic acid content in the segregating DH population



MAKER/QTL could not be used for mapping of these genes. Therefore, three independent approaches were used to localize the erucic acid genes.

In a first approach markers putatively linked to the genes were identified by bulked segregant analysis. Simultaneously with the construction of the rapeseed RFLP map, the mapped RFLP markers were tested with two DNA pools derived from eight DH lines with high erucic acid content and eight DH lines free of erucic acid, respectively. Of the 19 markers positive in bulked segregant analysis, a majority of 14 markers was located on linkage groups 6 and 12, representing a high proportion (5 of 7 and 9 of 16, respectively) of the total number of markers on these linkage groups. The remaining five markers were distributed on three additional linkage groups.

To find more markers linked to the erucic acid genes, the bulked segregant analysis was extended to RAPD markers. From a total of 260 decamer primers, 103 that had been proved to be polymorphic between 'Mansholt' and 'Samourai', were tested with two DNA pools from 12 DH lines each, resulting in nine primers that revealed ten polymorphisms between the bulks. Nine of these polymorphisms could be mapped. With one exception, all of these RAPD markers were again located on linkage groups 6 and 12.

In a second approach, the segregation between the markers mapped on the rapeseed RFLP map and the erucic acid genes was tested by χ^2 analysis. Of a total of 214 markers 24 markers showed a significant deviation from free segregation ($P \leqslant 0.05$). With the exception of two markers on linkage groups 9 and 11 all of these markers were found to be on linkage groups 6 and 12.

The results from both approaches clearly place the erucic acid genes on linkage groups 6 and 12. The distribution of markers positive in bulked segregant and linkage analysis on these linkage groups is shown in Fig. 2. On linkage group 6 positive markers are distributed across the total length of 63 cM of this linkage group. On linkage group 12 the positive markers span an equally extended region of 69 cM, representing the lower half of this larger linkage group.

In a third approach, interval mapping was used to improve the localization of the erucic acid genes. Figure 2 shows the resulting LOD score curves. Using confidence intervals of 3.0 LOD units, the positions of the erucic acid genes could be confined to intervals of 22 cM and 14 cM on linkage groups 6 and 12, respectively, with the highest LOD scores at marker RP841.H1 on linkage group 6 and about 2 cM above marker RP1544.E1 on linkage group 12.

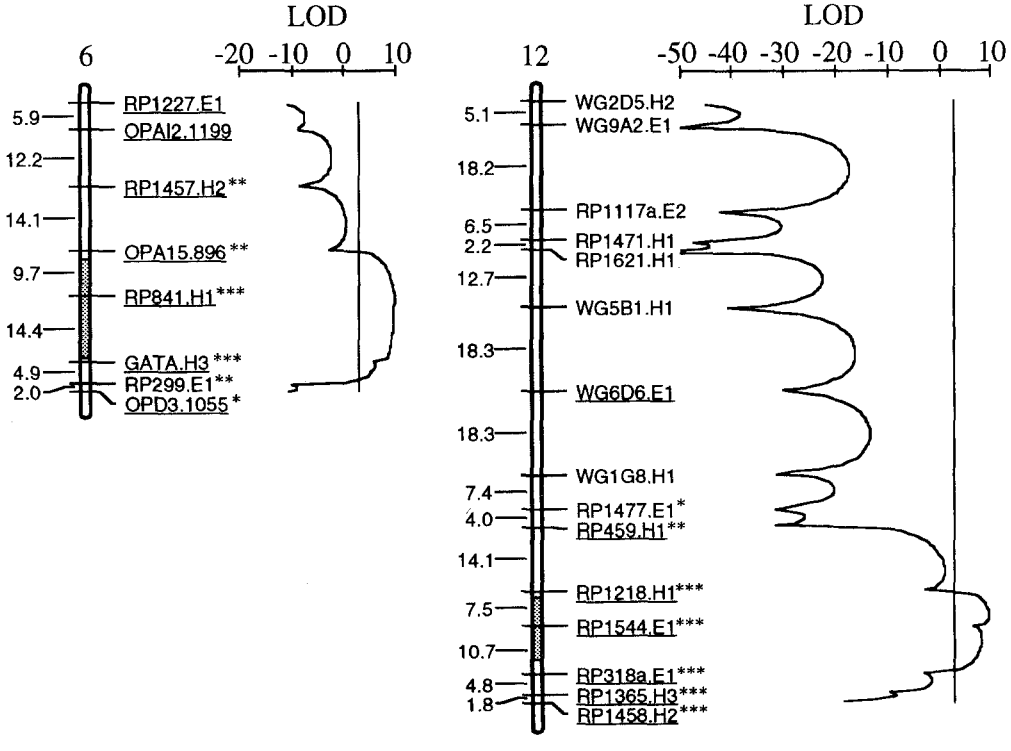


Fig. 2 Mapping of the erucic acid genes. The framework maps of linkage groups 6 and 12 are shown. Distances between markers are given in cM (Kosambi 1944). *Underlined* markers have been positive in bulked segregant analysis for erucic acid content. Markers with significant deviation at $P \leqslant 0.05$, 0.02 and 0.001 from free segregation to the erucic acid genes are indicated by *, **, and ***, respectively. The LOD curves represent right-angled sections of the LOD score surface resulting from the interval mapping of the erucic acid genes on linkage groups 6 and 12. The sections include the LOD score maximum and are parallel to the linkage groups, representing the log-likelihood ratios for the presence of a erucic acid gene at the corresponding positions of the respective linkage group when fixing the erucic acid gene on the second linkage group at the most likely position. The *stippled areas* in the linkage groups represent confidence intervals of 3 LOD score units around the LOD score maximum

Localization of QTLs for oil content

Table 1 shows the average seed oil content of different generations of the cross 'Mansholt' × 'Samourai'. Although there is no significant difference in oil content between the parents of the cross, in the F_1-derived DH population a range of 37–46% seed oil content was found, with a variance exceeding the mean variance of the parental generations and the F_1 by a factor of two. This indicates a strong genetic component in the observed segregation for oil content, especially since the values for the DH lines were obtained as means from up to 12 plants, thereby significantly reducing environmental variance. With a skewness of 0.07 and a kurtosis of −0.26 the distribution of oil content in the DH population closely followed a normal distribution; no distinct classes were discernible.

Table 1 Average seed oil content in different generations of the cross 'Mansholt's Hamburger Raps' × 'Samourai'

Generation	n[a]	Average oil content [%]	Variance	t-Test[b]
Mansholt	121	41.8	2.07	
Samourai	88	42.7	1.82	
F_1	141	43.1	1.46	c
DH population[c]				
Average	115	41.6	3.61	b
Maximum	1	46.4		
Minimum	1	37.0		
Mid parent value		42.1		a

[a] Parental lines, F_1: number of plants analysed; DH population: number of DH lines analysed
[b] Comparison of generation means: different letters indicate significant differences at $P \leq 0.05$
[c] Values for individual DH lines were obtained as a mean from up to 12 plants

The QTLs underlying this transgressive segregation in oil content were mapped by interval mapping using MAPMAKER/QTL. Initially, frameworks for all linkage groups of the rapeseed RFLP map were scanned, using a model without fixed QTLs. LOD scores exceeding the significance threshold of LOD = 2.8 were found on linkage groups 6 and 10 (Fig. 3) indicating the presence of two QTLs for oil content on these linkage groups.

Repeating the scan with the QTL on linkage group 6 fixed at its most likely position resulted in additional LOD scores exceeding the significance threshold on linkage group 12 (Fig. 3), indicating the presence of a third QTL for oil content on this linkage group. On the other hand, fixing the QTL on linkage group 6 reduced all LOD scores on linkage group 10 to values below 2.8. The presence of a QTL for oil content on this linkage group could be confirmed using a model with both QTLs on linkage groups 6 and 12 fixed. This model again resulted in LOD scores well above the significance threshold on linkage group 10 (Fig. 3).

Table 2 presents the most likely positions and additive effects of the three mapped QTLs for oil content estimated with a model regarding all three QTLs simultaneously. The additive effects of the three QTLs sum up to 4.8% of oil content for homozygous genotypes and explain about 51% of the phenotypic variation observed in the DH population. In accordance with the trans-

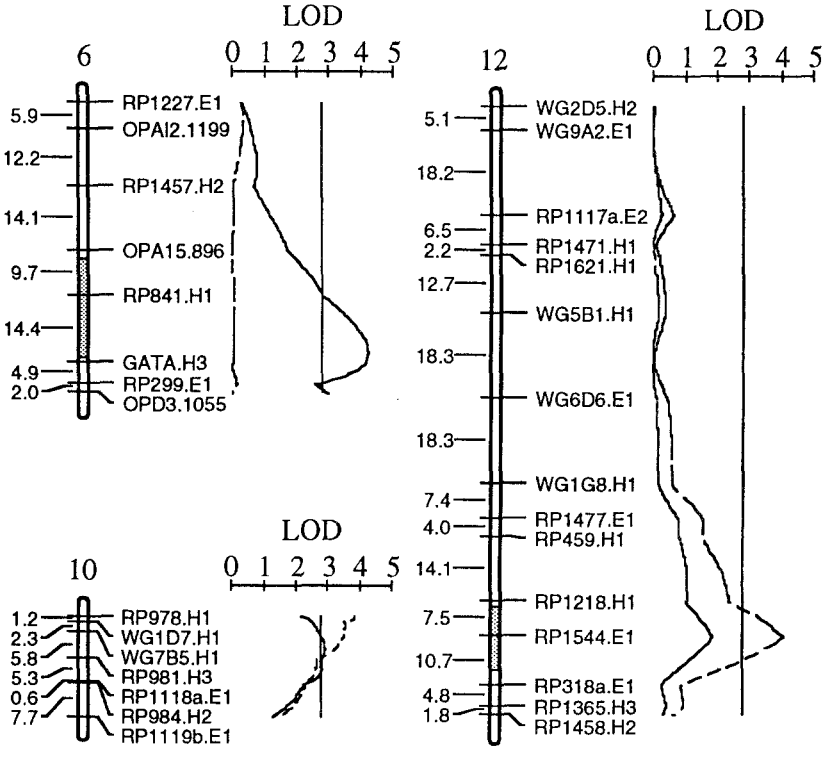


Fig. 3 Mapping of QTLs for seed oil content. The framework maps of linkage groups 6, 10 and 12 are shown. Distances between markers are given in cM (Kosambi 1944). The LOD curves represent the log-likelihood ratios for the presence of a QTL at the corresponding positions of the linkage groups (*1*) without fixed QTLs (continuous line), (*2*) with the QTL on linkage group 6 fixed at its most likely position (*dotted line* on linkage groups 6 and 12), and (*3*) with both the QTLs on linkage groups 6 and 12 fixed (*dotted line* on linkage group 10). The *vertical lines* indicate the significance threshold of LOD = 2.8. The *stippled areas* in linkage groups 6 and 12 represent the confidence intervals of 3 LOD score units around the most likely positions of the erucic acid genes

Table 2 Additive effects and most likely positions of the mapped QTLs for seed oil content

Linkage group	Marker interval	QTL position [a] [cM]	Additive effect [b] (% oil content)
6	RP841.H1–GATA.H3	9.3	0.90
10	RP978.H1–WG1D7.H1	0.4	−0.65
12	RP1544.E1	0.0	0.85

[a] Distance to the first marker of the indicated interval
[b] Estimated for the substitution of a 'Samourai' allele by an allele of 'Mansholt's Hamburger Raps'

gressive segregation, the additive effects of the three mapped QTLs have different directions. While for the QTLs on linkage groups 6 and 12 the 'Mansholt' alleles increase seed oil content, on linkage group 10 the allele from this parent has a negative effect on the trait.

Comparison of the positions of the QTLs for oil content on linkage groups 6 and 12 with the estimated positions of the erucic acid genes shows a tight association between these genes. The region on linkage group 6 with significant LOD scores for oil content strongly overlaps with the confidence interval for the presence of the erucic acid gene on this linkage group. On linkage group 12 the equivalent region is completely enclosed in the confidence interval for the erucic acid gene.

Discussion

By using three independent approaches we have been able to localize the erucic acid genes of rapeseed on two different linkage groups of the rapeseed RFLP map. None of the methods used for mapping these genes gave evidence for additional genetic factors involved in the control of erucic acid synthesis, confirming earlier investigations showing that erucic acid content in rapeseed oil is determined by the additive action of only two genes (Harvey and Downey 1964; Stefansson and Hougen 1964; Siebels and Pauls 1989). During the construction of the RFLP map, a high percentage of markers with disturbed segregations has been found. The majority of these markers defined seven exclusive genomic regions on seven different linkage groups (Uzunova et al. 1995). The erucic acid gene on linkage group 12 maps within one of these regions showing skewed marker segregations in favor of 'Mansholt' alleles, indicating a similar deviation from the expected segregation ratio for the erucic acid gene. This result explains the significant shortage of genotypes free of erucic acid in the DH population analysed. As the markers surrounding the second erucic acid gene on linkage group 6 show no significant deviations from expected segregation ratios a direct effect of the erucic acid genes on allelic segregation can be excluded. Most likely the erucic acid gene on linkage group 12 is only linked to a genetic factor causing the disturbed segregations in this part of the rapeseed genome.

In the mapping of the erucic acid genes bulked segregant analysis was used as a first approach to identify markers putatively linked to these genes. The rather large number of five false positives from 19 positive RFLP markers may be due to the small number of genotypes pooled for each bulk. In the RAPD analysis, using bulks of 12 genotypes each, only one out of nine markers proved to be false positive.

Bulked segregant analysis was originally proposed to be used for the identification of markers linked to simply-inherited traits (Michelmore et al. 1991) or to rapidly identify additional markers located in previously mapped genomic regions (Giovannoni et al. 1991). The successful identification of markers linked to either one of the two erucic acid genes, using pools from plants phenotypically selected for high and zero erucic acid content, respectively, shows that it is also possible to apply bulked segregant analysis to traits controlled by more than one unlinked gene. This opens up the possibility to "multiplex" bulked segregant analysis, using bulks from plants selected for more than one monogenically inherited trait. The actual number of genes or genomic regions that can be tagged simultaneously in bulked segregant analysis will depend on the size of the population that can be screened for the respective traits, as only certain genotypes can be pooled.

The above results revealed a close association between the erucic acid genes and genetic factors strongly affecting seed oil content. This association may be due to a close linkage between the erucic acid genes and otherwise independent QTLs for seed oil content. On the other hand, during the elongation of oleic acid to erucic acid the molecular mass of the individual fatty acid molecule is increased by about 20%. If all other parameters of fatty acid synthesis and storage, especially the number of stored fatty acid chains, remains unaffected by the elongation process, a significant effect of erucic acid synthesis on seed oil content can be expected. With a mean oil content of 41.6% in the DH population and an average of 24.6% of erucic acid in the seed oil this effect would amount to an increase in oil content of 1% per erucic acid allele of 'Mansholt'. The estimated additive effects of the QTLs for oil content on linkage groups 6 and 12 are close to this value, indicating a direct effect of the erucic acid genes on seed oil content. This effect explains the reduction in oil content that had been observed with the first rapeseed varieties free of erucic acid. On the other hand, recent rapeseed varieties of canola quality have seed oil contents equal to or exceeding that of older erucic acid containing varieties, indicating the successful selection of more efficient alleles for oil content at gene loci different from the erucic acid genes by breeding efforts during the last two decades. One of these additional loci may be the QTL on linkage group 10. This QTL with its large additive effect may be the main factor giving 'Samourai' a seed oil content similar to 'Mansholt'.

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