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Mapping of a gene determining linolenic acid concentration in rapeseed with DNA-based markers

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Abstract Rapeseed ranks third in world oil production. An important breeding objective to improve oil quality in this crop is to lower linolenic acid concentration in the seeds. Previous reports indicate that the concentration of this acid in *Brassica napus* is determined by two or three nuclear genes. Using DNA-based markers, we have successfully mapped a gene determining linolenic acid concentration in an F_2 population derived from crossing the cultivar 'Duplo' and a low linolenic acid line, 3637-1. Linolenic acid concentration in this population ranged from 2.1% to 10.5% with a mean of 6.2%. A RAPD marker, K01-1100, displayed significantly different frequencies between two subpopulations consisting of either high or low linolenic acid concentration individuals sampled from the two extremes of the F_2 distribution. Marker K01-1100 segregated in a codominant fashion when used as an RFLP probe on DNA from individuals of this F_2 population. The linolenic acid concentration means for the three resulting RFLP genotypes in the F_2 population were 4.8% (homozygous 3637-1 allele), 6.4% (heterozygous), and 7.5% (homozygous 'Duplo' allele), respectively. It is estimated that this marker accounts for 26.5% of the genetic variation of linolenic acid concentration in this population.

Key words RAPD markers · Rapeseed · *Brassica napus* Linolenic acid

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Introduction

High linolenic acid (LA) concentration is an undesirable trait affecting flavor and quality of rapeseed oil (Galliard 1980). Therefore, lowering the LA concentration is an important objective for quality improvement of rapeseed oil. Since LA plays an essential role in photosynthesis, it is virtually impossible to eliminate LA from oilseeds. It has been reported that in crucifers, LA concentration ranges from 6% to 12%, most of the variation being caused by environmental factors such as temperature (Thies 1971). However, the above range was dramatically widened by chemical mutagenesis in two species: *Arabidopsis thaliana* mutants with 1.2–22.5% LA (James and Dooner 1990; Lemieux et al. 1990) and *Brassica napus* (rapeseed) mutants with 2–20% LA (Robbelen and Nitsch 1975) were obtained. Interspecific hybridization has also been employed for further development of high LA/low LA rapeseed germplasm (Roy and Tarr 1986). The creation of these materials has laid the foundation for the development of improved rapeseed cultivars with low LA concentration. However, because LA concentration is influenced by environmental factors and shows a low response to selection based on phenotype, progress in lowering LA in rapeseed oil has been slow in the past two decades. The development of DNA-based markers for identifying the mutant alleles would increase selection efficiency for this trait.

Fatty acids are synthesized by fatty acid synthetase in the chloroplast or other plastids (Browse and Somerville 1991). The first intermediate of triacylglycerols and other storage lipids is palmitoyl-acyl carrier protein , or 16:0-ACP. Most of the 16:0-ACP is elongated to stearoyl-ACP, or 18:0-ACP, and then desaturated by a desaturase closely associated with fatty acid synthetase to form oleoyl-ACP, or 18:1-ACP (Nagai and Bloch 1968). LA can be synthesized by sequential desaturation of stearate in two distinct pathways in plants. The prokaryotic pathway occurs in the chloroplast and utilizes 16:0 and 18:1 fatty acids as substrates to synthesize monogalactosyldiglyceride, or MGD, which can be further desaturated into 18:2 and 18:3 fatty

acids (Roughan and Slack 1982). The eukaryotic pathway occurs predominantly in the endoplasmic reticulum and utilizes the 16:0 and 18:1 fatty acids exported from the chloroplasts to synthesize phosphatidic acid (PA), phosphatidylglycerol (PG), diacylglycerol (DAG) , phosphatidylcholine (PC), and tricylglycerol (TAG). Linoleoyl-PC (18:2-PC) and linolenoyl-PC (18:3-PC) are synthesized by specific desaturases (Miquel and Browse 1992; Roughan 1985). LA and choline are released from 18:3-PC by a phosphatase.

Genetic analysis of LA concentration in rapeseed *B. napus* has been carried out by several laboratories using different approaches, such as progeny testing (Brunklaus-Jung and Robbelen 1987), comparison of reciprocal crosses (Diepenbrock and Wilson 1987), diallel analysis (Pleines and Friedt 1989), and observation of segregation ratios in microspore-derived populations (Chen and Bevesdorf 1990). These studies have shown that LA concentration is determined mainly by the genotype of the embryo, involving maternal effects and the interaction of two or three loci with some influence from environmental factors.

Our goal is to map the genes responsible for LA concentration in *B. napus* with DNA-based markers. These markers will be used in rapeseed improvement to increase selection efficiency and in plant molecular biology to investigate the organization and regulation of these genes. We report here the use of RAPD markers in mapping a gene determining LA concentration in a segregating *B. napus* population.

Materials and methods

A cross was made between the German rapeseed cultivar, 'Duplo' and a low LA line, 3637–1, derived from a cross between two low LA lines, 'Mutant Oro' (Robbelen and Nitsch 1975) and 'IXLIN' (Roy and Tarr 1986). LA concentration was determined on 132 plants from the F_2 populations grown in Germany, following the method of Thies (1971). Seeds from each individual F_2 plant of these populations were grown at Davis, California, for the gene mapping experiments.

DNA was extracted from 3- to 4-week-old F_3 seedlings following the procedure of Kianian and Quiros (1992). Approximately similar amounts of tissue from 8 to 10 plants of each F_3 family were used for DNA extraction in order to determine the genotype of their maternal F_2 plant.

The procedure for random amplified polymorphic DNA (RAPDs) reported by Hu and Quiros (1991)was followed. RAPD primers (Operon Technologies) were used to amplify the DNA samples from the parents and the F_1 population for polymorphism screening.

For RFLP analysis, 3–5 µg DNA samples prepared from each family were digested with the restriction endonuclease *Eco*RI and electrophoresed in 0.8% agarose gel in 1×TAE buffer, and transferred onto Zeta-Probe GT (Bio-Rad) nylon membranes. About 50 ng of probe DNA was labeled with the Multiprimer DNA labeling kit (Amersham). Hybridization was performed in a hybridization incubator (Robbins Scientific) at 65°C overnight. Post-hybridization washes and autoradiography were carried out by following the manufacturer's protocols.

For preliminary screening of polymorphism between the parents, DNA samples from the parents and reciprocal F_1s were digested with *Eco*RI, *Bam*HI, and *Hin*dIII. Probes used were from the *B. napus* cDNA library that have been mapped in *B. oleracea* (Kianian and Quiros 1992). Southern blots were made with *Eco*RI-digested DNA samples from each of the F_3 families for testing the segregation of promising markers.

Because of the low level of polymorphism between the two parents of the segregating population, we adapted the trait-based approach of Lebowitz et al. (1987). Twelve F_3 families at each end of the distribution (12 lowest, 2.1–4.4%, and 12 highest, 8.1–10.5%, LA concentration), two parental lines and the F_1 were used in the screening for markers associated with LA concentration. Only the RAPD primers previously tested as being polymorphic between the parents were used in the screening. Selected markers were scored for segregation in the population. Regression analysis was used to test the association of markers and LA concentration in the segregating population (Haley and Knott 1992).

Bulked segregant analysis (Michelmore et al. 1991) was used to attempt the identification of other markers tightly linked with K01-1100. We bulked equal amounts of DNA samples from five families with respect to LA concentration as well as K01-1100 genotypes. Only homozygotes confirmed by restriction fragment length polymorphism (RFLP) analysis were used. Two bulked tubes for each genotype and the two parental tubes were amplified with 140 RAPD primers not used in the preliminary screening.

Results and discussion

The LA concentrations for 'Duplo', 3637-1 and the F_1 hybrid were 10.4%, 2.2% and 4.7%, respectively. The F_2 LA concentration exhibited a slightly skewed continuous distribution (Fig. 1), thus making discrete classification of the individuals impossible. Maternal effect, partial dominant action of the genes involved, and environmental influence may be responsible for the deviation between F_2 and the mid-parent value (6.3%) and the F_2 skewness. We concluded that LA concentration was mainly determined by nuclear genes since the F_2 mean (6.2%) was practically identical to the mid-parent value (6.3%), which is in agreement with previous reports (Brunklaus-Jung and Robbelen 1987; Diepenbrock and Wilson 1987; Pleines and Friedt 1989; Chen and Bevesdorf 1990).

Preliminary screening with RFLP probes and RAPD primers revealed low levels of polymorphism between the

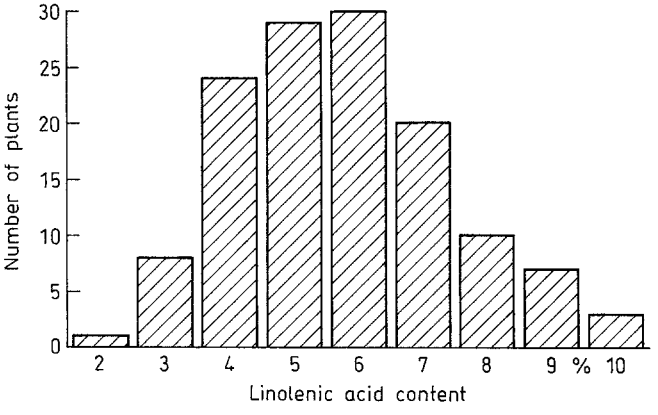

Fig. 1 Histogram of linolenic acid concentration in the 'Duplo'× 3637-1 F_2 population containing 132 plants

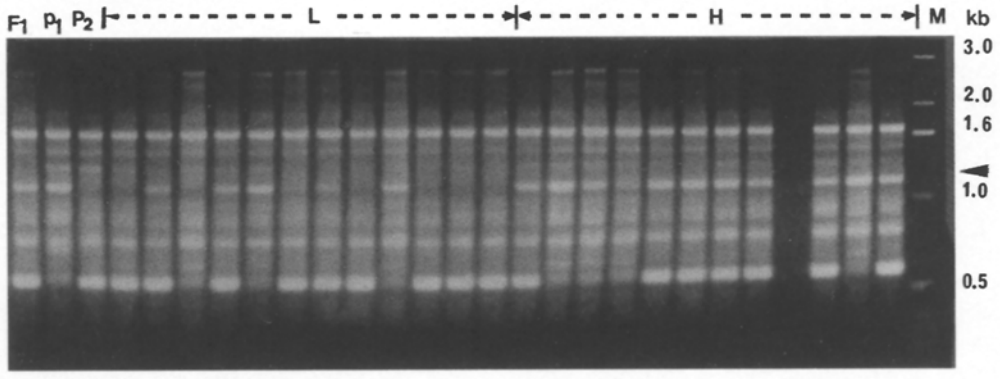

Fig. 2 Profile of the amplified products with primer K01 on the parents, F_1, and 11 high LA and 12 low LA F_3 families during the initial screening for association between markers and LA concentration. K01-1100 is indicated by *arrow*. (*H* high LA concentration families, *L* low LA concentration families, P_1 'Duplo', P_2 3637-1, *M* DNA size marker 1 kb ladder from BRL

parents. For RFLPs, about 10% of the probes (4 of 38) detected polymorphic bands on the three enzyme-digested DNA blots. For RAPDs, 1 primer amplified 6–8 scorable bands in either parent, less than 50% of the primers (71 out of 160) amplified polymorphic markers. Based on this observation, we adapted the trait-based approach of Lebowitz et al. (1987), which has been proposed as an alternative to the marker-based analysis, when interest is centered in a quantitative trait and the cost of scoring markers is high. In this approach, markers associated with LA concentration can be identified by their frequencies in the two sub-populations sampled from the two extremes of the F_2 distribution. We scored 82 polymorphic markers amplified by 71 RAPD primers in the 12 families at each end of the F_2 distribution. Of these, 81 segregated in both high and low groups regardless of LA concentration. Only 1 marker, K01-1100, was present in 11 of the 12 high LA families and only in 5 of the 12 low LA families (Fig. 2), indicating that it could be associated to LA concentration.

We examined 30 of the 82 polymorphic markers amplified by 17 of the 71 primers in the 132 F_3 families representing the F_2 population. Twenty-six markers fitted the expected 3:1 ratio, and 4 deviated from the expected Mendelian segregation (Table 1). A significant regression coefficient was found only between the presence of K01-1100 and high LA concentration (r=0.357***); the values of the regression coefficient between LA concentration and other markers were all below the significant level (Table 1). On the basis of presence or absence of K01-1100, the F_2 population could be divided in two groups with respect to LA concentration (Fig. 3). Comparison of the LA means in the two groups (101 F_2 plants with K01-1100, 6.5%; and 31 F_2 plants without K01-1100, 5.2%) revealed a statistically significant difference (t=4.06, P<0.001). The individuals having a homozygous recessive genotype (lacking K01-1100 band) occupied the tail of low LA concentration of the distribution. The variance in LA concentration within this genotype suggests the involvement of other genes and environmental effects on the expression of this trait. However, the association of K01-1100 to LA concentration is clear, indicating that one of the genes governing LA is linked to this marker. Regression analysis estimated that this marker explains about 12.8% of the genetic variation of LA concentration in this population.

Table 1 Segregation of 30 RAPD markers and the regression coefficients between these markers and linolenic concentration in 'Duplo'×3637-1 F_2 population (n=132)

Markers	Present	Absent	χ^2 (3:1)	r
A07-1400	107	25	2.27	– 0.144
A07-480	102	30	0.25	– 0.035
A07-780	118	14	13.83***	– 0.04
A07-800	108	24	2.92	– 0.08
A08-1100	105	27	1.22	0.063
A08-700	96	36	0.25	0.028
A18-1000	104	28	0.82	0.104
A18-1600	105	27	1.22	– 0.007
A18-550	102	30	0.25	– 0.097
B06-1300	91	41	2.27	– 0.124
B06-1700	96	36	0.25	0.01
B09-2800	106	26	1.71	0.02
B11-1100	110	22	4.45*	0.039
B11-1700	114	18	8.49***	– 0.085
B15-850	92	40	1.71	– 0.045
B15-900	91	41	2.27	– 0.082
C01 1800	100	32	0.01	0.112
C01 800	101	31	0.09	– 0.092
C04-1750	99	33	0.01	– 0.039
C09-1400	91	41	2.27	– 0.126
C09-2500	97	35	0.09	0.03
C15-2000	82	50	11***	0.008
E09-900	98	34	0.01	0.043
K01-1100	101	31	0.09	0.357***
N02-760	97	35	0.09	0.134
N02-800	89	43	3.65	– 0.078
N08-800	95	37	0.49	0.054
O04-1000	97	35	0.09	– 0.075
O13-1200	99	33	0.01	– 0.136
O13-1700	93	39	1.22	0.055

*, *** Significant at 0.05 and 0.01 probability levels, respectively

When the amplified fragment, K01-1100, was used as an RFLP probe on a Southern blot containing *Eco*RI-digested F_2 genomic DNA, four bands were produced. Two of these (0.7 and 1.3 kb) were monomorphic, while the other two (6.5 and 7.5 kb) were allelic and segregated in a 1:2:1 ratio (22:57:18, χ^2=3.3, and P>0.1). The 6.5-kb band was contributed by the low LA concentration parent, 3637–1, and the 7.5-kb band by 'Duplo', the high LA concentration parent. The heterozygotes displayed both parental bands as expected (Fig. 4). The histogram of these three

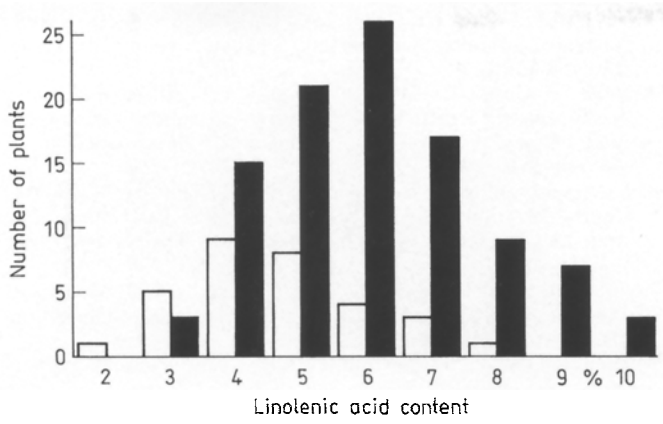

Fig. 3 Histogram of LA concentration of the two genotypes revealed with RAPD marker K01-1100 in the 'Duplo'×3637-1 F_2 containing 132 plants. ▭ Plants without K01-1100, ■ plants containing K01-1100

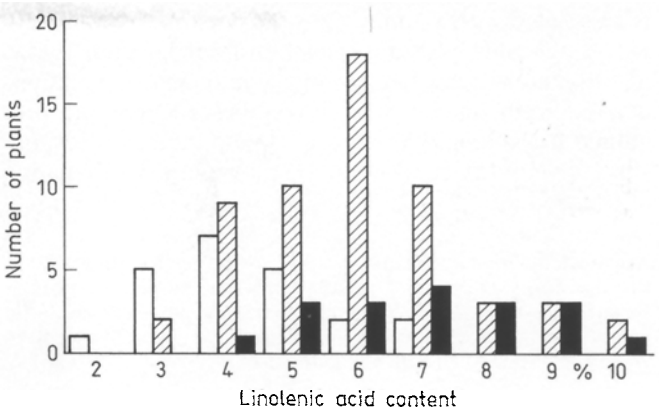

Fig. 5 Histogram of linolenic acid concentration of the three genotypes revealed with RFLP analysis of K01-1100 in the 'Duplo' ×3637-1 F_2 containing 97 plants. ▭ Plants with low LA parental genotype, ▨ plants with heterozygote genotype, ■ plants with high LA parental genotype

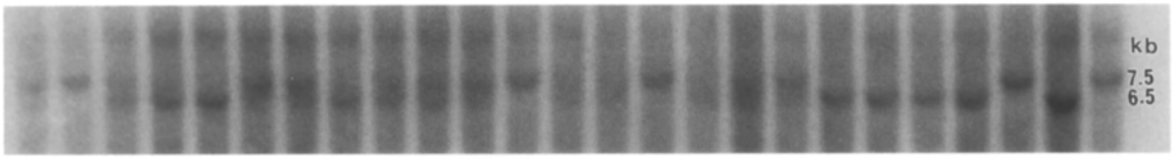

Fig. 4 Sample of the autoradiogram produced by K01-1100 on the Southern blots containing *Eco*RI-digested genomic DNA from F_3 families. The two segregating bands, about 7.5 kb and 6.5 kb, are contributed by 'Duplo' and 3637-1, respectively

genotypes with respect to LA concentration is shown in Fig. 5. The LA concentration means were 4.8% for the low LA parental marker genotype, 6.4% for the heterozygous, and 7.5% for the high LA parental marker genotype. However, there was enough variation in LA concentration to result in a slight overlapping of the two homozygous genotypes and a wide range distribution for the heterozygotes. This variation could be attributed to environmental effects, the interaction between the genotypes of the embryo and the maternal plant, and possible other genes governing this trait. The regression coefficient estimated when using the RFLP-derived codominant K01-1100 marker increased to r=0.515, explaining now about 26.5% of the genetic variation of LA concentration in this population. In any case, the fact that the two extremes of the F_2 distribution are occupied by the homozygous individuals of the two parental genotypes confirms that K01-1100 is co-segregating with a major gene determining LA concentration. An accurate estimate of the degree of linkage was unattainable mainly because of (1) the continuous distribution of LA, which makes it impossible to classify the individuals discretely, and (2) the small number of markers available. However, we can guess that K01-1100 is tightly linked with one of the major genes determining LA. This surmise is based on the observation that no recombinant was found between the marker and the trait for the two homozygous marker genotypes, which were continuously distributed at either side.

Our results clearly demonstrate that combining the trait-based approach and RAPD technique can be used for mapping genes of agronomic importance. Since most of the RAPD markers are dominant, it is worthwhile to convert the dominant marker into codominant markers to provide greater power in detecting the locus responsible for quantitative genetic variation. In the present study, for the same marker, only 12.8% of the genetic variation of LA concentration was explained when it was scored as dominant; the value was more than doubled when it was converted into a codominant marker. The association between K01-1100 and LA concentration was established with regression analysis, which is based on least squares. The use of 2 flanking markers for interval mapping with maximum-likelihood analysis of the data would provide much more accurate parameter estimates. However, none of the other markers scored in the population was linked to K01-1100. Our attempt to identify other markers tightly linked to K01-1100 with bulked segregant analysis (Michelmore et al. 1991) was unsuccessful: all four bulked tubes (two low and two high LA) amplified the 65 polymorphic markers detected between the parents with 140 primers. We are now adding more RAPD and RFLP markers to the population in order to construct a linkage map and localize K01-1100 and other markers affecting LA in the population.

LA synthesis involves sequential enzymatic desaturation of oleic acid (Browse and Somerville 1991); therefore, the genetic determination of LA concentration in *B. napus* is expected to be complex, especially because of its allotetraploid nature. Since 26.5% of the genetic variation for LA concentration in this population could be attributed to RFLP marker K01-1100, we believe that marker K01-1100

is associated with a prominent gene determining this trait. K01-1100 could be useful in marker-aided selection for the identification of low LA genotypes among the populations derived from mutant 3637-1 in rapeseed breeding programs. It also could be used to isolate the genes responsible for low LA concentration and genetic manipulation of this trait.

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