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Mapping a high oleic acid mutation in winter oilseed rape (*Brassica napus* L.)

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Abstract Two winter oilseed rape mutant lines, 7488 and 19661, with a high oleic (HO) acid content in the seed oil were characterized phenotypically. In both mutant lines the HO trait was monogenically inherited. Segregation analysis in an F_2 population derived from a cross between 7488 and 19661 showed the two mutations to be allelic. From a comparison of seed, leaf and root fatty acid composition it was concluded that *fad2*, the endoplasmic oleic acid desaturase, is affected by the mutation. In a bulked segregant analysis three AFLP markers linked to this mutation were detected and localized on the genetic map of *Brassica napus*. The markers mapped near the locus of one copy of the *fad2* gene in the rapeseed genome.

Key words *Brassica napus* L. · Molecular marker · AFLP · *fad2* · High oleic acid · Fatty acids · Plant lipids

Introduction

Brassica napus L. is the most important oilseed crop grown in the temperate climate. Rapeseed cultivars usually produce oil with about 60% oleic acid (C18:1), 20% linoleic acid (C18:2) and 10% linolenic acid (C18:3). Shifts in the fatty acid composition of *Brassica napus* have played an important role in rapeseed breeding. An improved rapeseed oil with high oleic (HO) acid content, like HO sunflower (Fernandez-Martinez et al. 1989) and HO soybean oil (Takagi and Rahmann 1996) would be of great interest for industrial as well as for nutritional purposes. HO oil is less susceptable to oxidative changes during refining, storage and frying, and it can be heated to a higher temperature without smoking (Miller 1987). A diet incorporating a high content of oleic acid may reduce the content of low-density lipoprotein cholesterol in blood plasma, and it seems that mono-unsaturated fatty acids are superior to poly-unsaturated fatty acids in preventing or treating arteriosclerosis (Chang and Huang 1998).

Since no substantial genetic variation for oleic acid content is known in the *Brassica napus* germplasm, an ethyl methanesulphonate mutagenesis program was set up, and several HO mutants were derived from the winter oilseed rape cultivar Wotan (Rücker and Röbbelen 1995). The oleic acid content in the different mutants varied from 70% to 81%, C18:2 content was reduced while C18:3 content remained nearly stable.

In breeding programs for plant oils with specific fatty acid patterns the seed fatty acids are usually determined by gas chromatography. The oleic acid content can be strongly influenced by environmental effects (Harwood 1996), and it can be difficult to differentiate between several HO alleles in HO genotypes or between plants homo- or heterozygous for the HO mutation. Therefore, a selection by linked molecular markers would be a better and more reliable method.

Here we report (1) the development of amplified fragment length polymorphism (AFLP) markers for the HO locus in the mutant lines 7488 and 19661 by bulked segregant analysis and (2) the localization of these markers in the linkage map of *Brassica napus* L. (Uzunova et al. 1995). Also discussed is whether the mutation might be in the endoplasmic oleic acid desaturase gene (*fad2*).

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Materials and methods

Plant material

Two F_2 mapping populations, 7488 × DH 11.4 Samourai, and 19661 × DH 11.4 Samourai were developed from crosses between the M3 generation of the HO mutants 7488 and 19661 with DH 11.4 Samourai. The two mutants were selected in an EMS mutagenesis program of the winter rapeseed cultivar Wotan (Rücker and Röbbelen 1995). For mutant 19661 it had been shown that the

HO trait is determined by one gene (Rücker and Röbbelen 1997; Schierholt et al. unpublished results). The doubled haploid (DH) line 11.4 was extracted from the canola-quality winter rapeseed variety Samourai (Uzunova et al. 1995). F_1 plants were selfed, and F_2 plants were cultivated. Between 14 and 16 F_3 progenies of 91 and 66 F_2 plants arising from the crosses with 7488 and 19661, respectively, were grown to determine the oleic acid genotype of the F_2 individuals. Seeds of selfed F_3 plants were harvested, and the fatty acid composition of a bulked seed sample of each plant was determined. In addition, the mutants 7488 and 19661 were crossed, and 9 F_1 plants were cultivated together with the parental lines for an analysis of the allelic relationship of the mutants.

Plant cultivation

Plants for seed fatty acid analysis were grown and selfed in the greenhouse with exception of the mapping population 19661 × DH 11.4 Samourai, for which F_3 progenies were grown in the field.

Plant material for the analysis of the leaf and root fatty acid composition was obtained from 20-day-old plantlets grown in sand culture in a climate chamber (day: 16 h; 25°C; light: 90 µmol/s^{-1} m^{-2}; night: 8 h; 20°C). Genotypes were tested in two replications. Samples were taken from 20 bulked plants per line and replication. Plant material was freeze-dried before analysis.

Fatty acid analysis

The fatty acid composition of single seeds, bulked seed samples (200 mg) and leaf and root material was analyzed by gas chromatography according to Thies (1971). Components of the oil are reported as percentages of total fatty acids. The oleic acid desaturation ratio (ODR) was calculated following Pleines and Friedt (1988). The desaturation ratio estimates the proportion of substrate which is desaturated. ODR = (% C18:2 + % C18:3) / (% C18:1 + % C18:2 + % C18:3).

DNA preparation and marker- and linkage analysis

DNA of both the parental lines and the F_2 individuals was isolated from leaf material of 10-week old plants according to Uzunova et al. (1995). For the bulked segregant analysis (Michelmore et al. 1991) DNA of 9–10 F_2 plants with either high or low C18:1 content was pooled to form the bulks. Selection of F_2 plants for the bulks was based on the seed oil analysis of their F_3 progenies. A bulked segregant analysis with AFLP markers was carried out using the AFLP Core Reagent Kit and AFLP Starter Primer Kit (Life Technologies) to test all of the 64 *Eco*RI/*Mse*I primer combinations provided. AFLP analysis was carried out according to the manufacturer's instructions with minor modifications: primer labeling was done with only half of the recommended volume of *Eco*RI-primer per polymerase chain reaction (PCR) set; gels were loaded with 6 µl of sample instead of 2–3 µl. Amplified PCR products were separated by electrophoresis on denaturing 8% polyacrylamide gels. Primer combinations which produced polymorphic bands between bulks and between parental lines were then mapped in the mapping populations 7488 × DH 11.4 Samourai (58 F_2 plants) and 19661 × DH 11.4 Samourai (36 F_2 plants). Only F_2 individuals that could be assigned unequivocally to one of the three possible genetic classes, based on the phenotypic analysis of the F_3 families, were used for genetic mapping. For linkage analysis the program MAPMAKER/EXP version 3.0b (Lander et al. 1987) was used.

Results

Genetic analysis

Seeds of the mutant lines 7488 and 19661 contained about 15% more C18:1 than the wildtype Wotan, but the fatty acid composition of the leaves and roots were only slightly different (Table 1). The seed oil of the bulked seed samples of F_1 plants of a cross between 7488 and 19661 showed the same fatty acid combination as that of the parental lines. In the single-seed analysis no F_2 seeds with a C18:1 content in the range of wildtype Wotan could be found (Table 2). This lack of segregation indicates that the mutants are allelic for the HO locus and that the HO trait in 7488 is inherited monogenically as in 19661. To determine the HO genotype of the F_2 plants of the mapping populations 7488 × DH 11.4 Samourai and 19661 × DH 11.4 Samourai, we phenotypically characterized F_3 progenies for mean values and standard deviations of seed C18:1 content (Fig. 1). Most of the F_3 families segregated into the three expected groups: two groups with high and low mean oleic acid content, respectively, and small standard deviations and a third group with intermediate oleic acid content and a high standard deviation. F_2 plants whose F_3 families could not be assigned unequivocally to one of the three groups were excluded from further analyses. The mean oleic acid content of the F_3 families in the first two groups corresponded to the range of oleic acid contents observed in the parental lines of the two crosses. For genetic analysis the F_2 plants were grouped into homozygous wildtype and mutant, and heterozygous classes on the basis of the phenotype of the respective F_3 families.

The distribution of F_2 plants across the three classes in the F_2 populations 7488 × DH 11.4 Samourai and 19661 × DH 11.4 Samourai did not differ significantly from the 1:2:1 segregation ratio (χ^2 = 4.89 and 0.84, respectively) expected for a monogenic inheritance of the HO trait.

Table 1 Fatty acid composition and ODR in bulked seed samples, leaves and roots of HO mutants 7488 and 19661, wildtype cv. Wotan and DH 11.4 Samourai. Individual fatty acids are given as percentages of total fatty acids

Seeds	C18:1	C18:2	C18:3	ODR
Wotan (wildtype)	60.3	19.2	9.6	0.33
7488	74.7	9.1	8.4	0.19
19661	75.0	8.4	8.4	0.18
DH 11.4 Samourai	58.0	20.1	9.2	0.33
Leaves	**C18:1**	**C18:2**	**C18:3**	**ODR**
Wotan (wildtype)	4.4	15.0	48.2	0.93
7488	6.5	13.0	48.3	0.91
19661	5.7	13.9	48.4	0.92
Roots	**C18:1**	**C18:2**	**C18:3**	**ODR**
Wotan (wildtype)	17.8	24.6	27.8	0.74
7488	19.6	17.1	36.5	0.73
19661	22.6	19.2	33.5	0.69

Table 2 Mean and standard deviation (SD) of seed oleic acid contents of bulked seeds of the mutants 19661 and 7488 and of F_1 plants of a cross between these mutants. Variation between single seeds (n = 100) was determined for Wotan and F_2 seeds of F_1 plants 7488 × 19661

	Bulked seeds			Single seeds		
	Number of plants	C18:1		C18:1		
		Mean	SD	Mean	Minimum	Maximum
19661	7	74.7	1.03	–	–	–
7488	7	75.0	1.02	–	–	–
F_1 7488 × 19661	9	75.6	0.80	75.3	70.7	80.3
Wotan	10	60.3	1.30	60.5	54.8	64.0

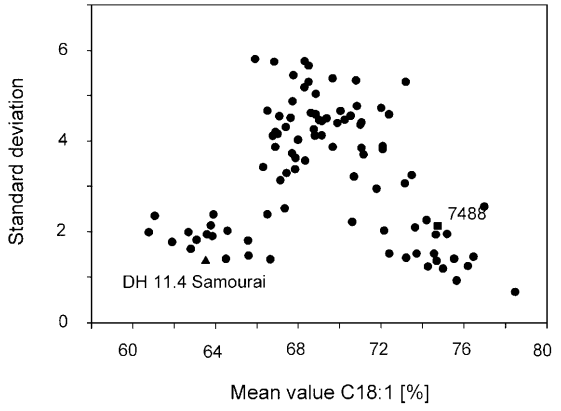

Fig. 1 Distribution of means and standard deviations of seed oleic acid contents in 91 F_3 families derived from the F_2 population 7488 × DH 11.4 Samourai. Mean parental values are shown as a *triangle* (DH 11.4 Samourai) and *square* (7488). C18:1 is calculated in percentage of total fatty acids

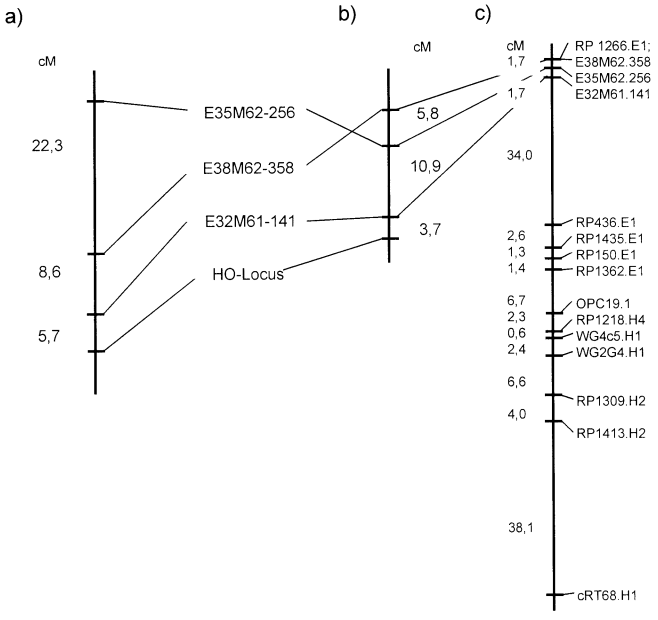

Fig. 2a–c Alignment of the linkage groups with the HO mutation of the mapping populations: **a** 7488 × DH 11.4 Samourai, **b** 19661 × DH 11.4 Samourai, **c** the corresponding LG15 of the mapping population DH 11.4 Samourai × DH Mansholts Hamburger Raps of Uzunova et al. (1995) with integrated AFLP markers (Ecke et al. unpublished results)

AFLP marker development and mapping

The AFLP analysis revealed about 30–40 scorable bands per primer combination, with about 20–25% of these bands proving to be polymorphic between the parental lines of the mapping populations. Accordingly, the number of polymorphic loci tested in the bulked segregant analysis ranged between 380 and 640.

The bulked segregant analysis with DNA pools derived from the population 7488 × DH 11.4 Samourai revealed three primer combinations (E32M61, E38M62, E35M62) showing one polymorphic band each between parental lines as well as between the bulks. The three AFLP markers (E32M61–141, E38M62–358, E35M62–256) were mapped in the F_2 population, where they proved to be linked with the HO mutation (Fig. 2a). The visible alleles of the three AFLP markers were all in coup-ling phase with the HO mutant allele. The mapping of the same AFLP markers in the second F_2 population, 19661 × DH 11.4 Samourai, showed these markers again to be linked with the HO mutation (Fig. 2b). The most likely marker orders differed in the two F_2 populations (Fig. 2a,b) with log likelihood differences of 3.74 and 0.61 between the alternative orders in 7488 × DH 11.4 Samourai and 19661 × DH 11.4 Samourai, respectively.

The AFLP markers linked with the HO mutation had previously been mapped in a DH mapping population derived from the cross between Mansholts Hamburger Raps and DH 11.4 Samourai (Ecke, Crispin and Pelemann, unpublished data) and located on linkage group 15 of the rapeseed genetic map of Uzunova et al. (1995). The marker loci are located at the end of the linkage group in a genomic region with a low marker density (Fig. 2c). The order of the marker loci here corresponds to the order determined in the F_2 population 19661 × DH 11.4 Samourai, with a LOD support of 1.72 with reference to the alternative order, but marker distances are much smaller than in the two F_2 populations.

Discussion

Genetic analysis and candidate gene hypothesis

The 1:2:1 segregation pattern in the F_2 populations of the crosses with the mutant lines 7488 and 19661 indicate that the trait ‘increased seed C18:1 content’ is inherited monogenically, as already shown by Rücker and

Röbbelen (1997) and Schierholt et al. (unpublished data). Furthermore, the fatty acid analysis of single F_2 seeds of a cross between both mutants showed no segregation for C18:1 content, clearly indicating that the mutants are allelic at this locus.

There are several genes involved in plant lipid metabolism that influence the oleic acid contents in seeds, leaves and roots and which could be affected by the HO mutation. The fatty acid composition of the mutants with significantly increased seed oleic acid content, decreased ODR values but stable linolenic acid content indicates that oleic acid desaturation might be affected. Two parallel oleic acid desaturation pathways exist, one is located in the membranes of the endoplasmic reticulum and the other in the plastid. Oleic acid is desaturated by an oleic acid desaturase to linoleic acid by insertion of a *cis*-double bond in the delta 12 (omega 6) position (Scheffler et al. 1997). The endoplasmic and plastidial desaturation enzymes are named accordingly delta 12 (*fad2*) and omega 6 (*fad6*) oleic acid desaturase, respectively.

While the mutation in 7488 and 19661 caused a significant increase in the oleic acid content of seeds, the fatty acid composition of neither the leaves nor roots differed much from the wildtype Wotan. Browse et al. (1986) observed in *Arabidopsis* that more than 70% of the leaf membrane lipids are located in the chloroplast. A change in the plastidial C18:1 desaturation should consequently lead to an increase in C18:1 content in the leaves. In contrast, a change in the microsomal C18:1 desaturation would have a much smaller influence on leaf fatty acid composition (Lemieux et al. 1990) but a profound effect on seed oil composition. Mutations in the *fad2* gene – and the resulting HO types – have been reported for *Arabidopsis* (Lemieux et al 1990; Miquel and Browse 1992; Okuley et al. 1994), *Brassica rapa* (Tanhuanpää et al. 1998) and *Helianthus annuus* (Hongtrakul et al. 1998). We conclude from these data that the microsomal oleic acid desaturase *fad2*, and not the plastid located *fad6*, is affected in the HO mutants 7488 and 19661.

Marker analysis

The AFLP markers E32M61–141, E38M62–358 and E35M62–256 were identified in a bulked segregant analysis. These marker loci were linked in both mapping populations with the HO mutation. This is in accordance with the F_2 single-seed analysis results from the cross between the two mutants, proving the HO locus of the mutants to be allelic.

The change in the order of the markers and the varying distances between the marker loci in the different mapping populations (Fig. 2) might be caused by the different sizes of the mapping populations or by the different population structures (F_2 versus DH population). Furthermore it is possible that scoring errors in the phenotypic or marker data might have contributed to these shifts.

In the F_2 populations, the most closely linked AFLP marker E32M61–141 was observed to be located 3.7 cM or 5.7 cM from the HO locus. E32M61–141 could be used as a molecular selection marker for this trait, especially if marker distances should be even smaller than 3.7 cM, as indicated in the DH population.

Despite the high number of polymorphic loci tested in the bulked segregant analysis, only three markers linked to the HO locus could be identified. This is in accordance with the genetic map of LG15 (Fig. 2c), where the genomic region around the HO locus shows a low marker density.

fad2 in *Brassica* genomes

Mapping of the AFLP markers in the already existing genetic map of *Brassica napus* (Uzunova et al. 1995) localized the markers at the end of linkage group 15 (LG15; Fig. 2). In a linkage group alignment of different genetic maps of *Brassica napus* L. (Sharpe, Osborn, Ecke and Lydiate unpublished data) LG15 of Uzunova et al. (1995) was found to correspond to linkage group N5 of Parkin et al. (1995) and LG2 of Ferreira et al. (1994).

This linkage group alignment supports the conclusion that *fad2* is the gene which is affected in the HO mutants 7488 and 19661. Scheffler et al. (1997) have mapped loci of *fad2* (microsomal oleic acid desaturase) in the *Brassica napus* linkage map of Parkin et al. (1995) using a cloned *fad2* gene as a probe in a restriction fragment length polymorphism (RFLP) analysis. They estimated the copy number of the *fad2* gene in the *Brassica napus* genome to be four to six and localized four of these copies on four different linkage groups, two of A- (*Brassica rapa*) and two of C-genome (*Brassica oleracea*) origin. Scheffler et al. (1997) reported that one copy of the *fad2* gene is located at one end of the *Brassica rapa*-originated linkage group N5, flanked by the RFLP markers pN215aNP and pO123cNP. According to the linkage group alignment the three AFLP markers linked with the oleic acid desaturase (HO) locus are located in the same region. The *fad2* mapping data of Scheffler et al. (1997) support our hypothesis, that *fad2* is the gene which is affected in the HO mutants 19661 and 7488.

It is not yet known, which of the four to six loci hybridizing to the *fad2* probe are functional *fad2* genes (Scheffler et al. 1997). Our results indicate that at least the locus on LG15 (N5) represents a functional gene. This view is in accordance with Tanhuanpää et al. (1998), who located a functional gene copy of *fad2* on linkage group 6 of *Brassica rapa*. This linkage group corresponds to LG2 of the *Brassica napus* map of Ferreira et al. (Teutonico and Osborn 1994), which is equivalent to LG15 of Uzunova et al. (1995).

All of the functional gene copies of *fad2* in *Brassica* genomes that have been reported and mepped to date are located on the A-genome-originated linkage group 15 (N5). This indicates that the locus on LG15 is one (or the) active copy of *fad2* in *Brassica napus*.

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