

Determination of fatty acid composition in seed oil of rapeseed (*Brassica napus* L.) by mutated alleles of the FAD3 desaturase genes

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Abstract One of the goals in oilseed rape programs is to develop genotypes producing oil with low linolenic acid content (C18:3, $\leq 3\%$). Low linolenic mutant lines of canola rapeseed were obtained *via* chemical mutagenesis at the Plant Breeding and Acclimatization Institute – NRI, in Poznan, Poland, and allele-specific SNP markers were designed for monitoring of two statistically important single nucleotide polymorphisms detected by SNaPshot analysis in two FAD3 desaturase genes, *BnaA.FAD3* and *BnaC.FAD3*, respectively. Strong negative correlation between the presence of mutant alleles of the genes and linolenic acid content was revealed by analysis of variance. In this paper we present detailed characteristics of the markers by estimation of the additive and dominance effects of the FAD3 genes with respect to particular fatty acid content in seed oil, as well as by calculation of the phenotypic variation of seed oil fatty acid composition accounted by particular allele-specific marker. The obtained percentage of variation in fatty acid composition was considerable only for linolenic acid content and equaled 35.6% for *BnaA.FAD3* and 39.3% for *BnaC.FAD3*, whereas the total percentage of variation in linolenic acid content was 53.2% when accounted for mutations in both genes simultaneously. Our results revealed high specificity of the

markers for effective monitoring of the wild-type and mutated alleles of the *Brassica napus* FAD3 desaturase genes in the low linolenic mutant recombinants in breeding programs.

Keywords *Brassica napus* L. · FAD3 desaturase · Fatty acid composition · Genetic effects · Seed oil · SNaPshot analysis

Introduction

Oilseed rape (*Brassica napus* L.) is the second-most important oil crop of the moderate climate zone. Seeds of the double low varieties (canola, '00', with very low glucosinolates content in seed meal and with no erucic acid in seed oil) produce oil containing, on average: 7% of saturated fatty acids – palmitic (C16:0) and stearic (C18:0), and polyunsaturated fatty acids – oleic (C18:1, 61%), linoleic (C18:2, 20%), linolenic (C18:3, 10%) and eicosenoic fatty acid (C20:1, 1%). Due to the food- and non-food use of the oil, rapeseed breeding forms characterized by diversified seed oil fatty acid content are of great value on the oil crop market, and one of the breeding goals is to develop genotypes producing oil with low linolenic acid content (C18:3, $\leq 3\%$). Such oil is desirable for deep frying, as low content of polyunsaturated linolenic acid prevents oxidation and rancidation of the oil, which is crucial for healthy food production. In addition, oil with low linolenic acid content makes an important source of raw material for biofuel production, due to high stability of such oil. Genetic analyses revealed that in *B. napus* low linolenic acid content was controlled by the two fatty acid desaturase 3 genes (FAD3), named *BnaA.FAD3* and *BnaC.FAD3*, according to the standardized gene nomenclature for the *Brassica* genus (Østergaard and King 2008). The genes are located in the A and C genomes of *B. napus*

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Table 1 Regression analysis of the mutations in the FAD3 genes on particular fatty acid content in seed oil; C16:0, palmitic, C18:0, stearic, C18:1, oleic, C18:2, linoleic, C18:3, linolenic, C20:1, eicosenoic acid; SNP – genotyping of allelic forms of the FAD3

desaturase genes with the use of the SNaPshot assay, in the *BnaA.FAD3* gene (FAD3A) and in the *BnaC.FAD3* gene (FAD3C), respectively

SNP	Trait	Estimates of regression coefficients	P-values	Percentage variation accounted	Standard error of observations	Heritability
FAD3A	C16:0	0.1355	<0.001	3.0	0.550	0.50
FAD3C	C16:0	0.065	0.051	0.6	0.557	
FAD3A, FAD3C	C16:0	0.13, 0.02	<0.001, 0.662	2.8	0.551	0.72
FAD3A	C18:0	-0.1548	<0.001	5.9	0.448	
FAD3C	C18:0	-0.1213	<0.001	4.1	0.452	
FAD3A, FAD3C	C18:0	-0.12, -0.07	<0.001, 0.011	7.0	0.445	0.56
FAD3A	C18:1	-0.587	0.027	0.9	4.140	
FAD3C	C18:1	0.402	0.106	0.4	4.150	
FAD3A, FAD3C	C18:1	-0.92, 0.75	0.002, 0.005	2.3	4.100	
FAD3A	C18:2	-0.98	<0.001	3.4	3.730	0.79
FAD3C	C18:2	-1.91	<0.001	15.5	3.490	
FAD3A, FAD3C	C18:2	-0.17, -1.85	0.490, <0.001	15.4	3.490	0.88
FAD3A	C18:3	1.606	<0.001	35.6	1.580	
FAD3C	C18:3	1.5738	<0.001	39.3	1.540	
FAD3A, FAD3C	C18:3	1.10, 1.15	<0.001, <0.001	53.2	1.350	0.44
FAD3A	C20:1	-0.0186	0.129	0.3	0.191	
FAD3C	C20:1	-0.0128	0.264	0.1	0.191	
FAD3A, FAD3C	C20:1	-0.02, -0.001	0.245, 0.588	0.1	0.191	

(AACC amphidiploid genome) (Barret et al. 1999; Jourden et al. 1996), and were mapped by Hu et al. (2006) on the N4 (A4) and N14 (C4) linkage groups, respectively.

Low linolenic mutant lines of canola rapeseed were obtained *via* chemical mutagenesis at the Plant Breeding and Acclimatization Institute – NRI, in Poznan, Poland (Spasibonek 2006). Two statistically important single nucleotide polymorphisms were detected in two FAD3 desaturase genes: (1) a C to T substitution in the third position of the sixth codon of the seventh exon in the *BnaA.FAD3* gene and (2) a G to A transition in the 5' splice donor site of the sixth intron in the *BnaC.FAD3* gene (Mikolajczyk et al. 2010). Allele-specific SNP markers were designed involving a two-step detection of the wild-type and mutant alleles in both loci by SNaPshot analysis comprising an independent and specific PCR amplifications of *B. napus BnaA.FAD3* and *BnaC.FAD3* gene fragments comprising the mutation sites, and detection of the mutant alleles analysis using locus-specific primers (Mikolajczyk et al. 2010). Strong negative correlation between the presence of mutated alleles in the A and C genomes and linolenic acid content was revealed by analysis of variance (Mikolajczyk et al. 2010).

In this paper we report detailed characteristics of the developed allele-specific markers by estimation of the additive and dominance effects of the FAD3 genes with respect to particular fatty acid content in seed oil. In addition,

percentage of phenotypic variation of seed oil fatty acid composition accounted by particular allele-specific marker was calculated for wild-type and mutated alleles of *BnaA.FAD3* and *BnaC.FAD3*, respectively.

Materials and methods

Plant material consisted of 450 winter oilseed rape plants: 36 canola-type (00, 7-10% of linolenic acid in seed oil) cultivars and 26 doubled haploid (DH) mutant low linolenic (LL, $\leq 3\%$

Table 2 Additive (a) and dominance (d) effects of the *BnaA.FAD3* (FAD3A) i *BnaC.FAD3* (FAD3C) alleles on seed oil composition; particular fatty acid abbreviations (in the 'Trait' column) as in Table 1

Trait	FAD3A		FAD3C	
	a	d	a	d
C16:0	0.099 ^a	-0.251 ^b	0.059	-0.255 ^b
C18:0	-0.157 ^b	-0.002	-0.121 ^b	0.025
C18:1	-0.382	1.419 ^b	0.436	1.551 ^b
C18:2	-1.136 ^b	-0.945 ^a	-1.935 ^b	-1.117 ^b
C18:3	1.595 ^b	-0.225	1.571 ^b	-0.180
C20:1	-0.016	0.019	-0.013	0.007

^a significant at 0.01 level; ^b significant at 0.001 level

of linolenic acid) lines, as well as 26 DH lines developed from F₁ hybrid obtained as a result of crosses between 00 and LL lines in addition to 362 plants of F₂ and F₃ segregating lines obtained from crosses of 00 and LL lines, from 2 to 10 plants of each line. Field experiments (2007/ 2008 and 2008/ 2009) were conducted in Karzniczka, Lagiewniki and Poznan, Poland (Mikolajczyk et al. 2010).

The fatty acid composition of mature seeds was determined by gas liquid chromatography of the methyl esters (Byczynska and Krzymanski 1969) using an Agilent Technologies 6890N gas chromatograph (DB 23 30 m, ID 025, 0.25 µm layer capillary column) equipped with a Chemstation integrator.

Allelic forms of the *BnaA.FAD3* and *BnaC.FAD3* genes were identified by independent and specific PCR amplifications of the gene fragments comprising mutation sites and detection of the mutant alleles by SNaPshot analysis using locus-specific primers (Mikolajczyk et al. 2010). Regression analysis was done for the mutations in the *FAD3* genes on fatty acid composition in seed oil. The analysis was performed both – 1) individually, as well as 2) simultaneously for the two genes. The *FAD3* genes observations were treated as independent variables and considered in individual models (the first case), as well as in multiple regression model (the second case). The additive and dominance effects of the presence or absence of mutations in the *FAD3* genes for particular fatty acid content in seed oil were estimated. The additive effect of each allele was estimated as half of the difference between homozygotes and we tested null hypothesis that the difference between mean values for homozygotes is equal to zero by two-sample *t*-test. The dominance effect was estimated as deviation of mean value for heterozygotes from grand mean. We tested a null hypothesis that mean value for heterozygotes is equal to grand mean by two-sample *t*-test. The broad-sense heritability coefficients were calculated (Falconer and Mackay 1996). All calculations were carried out using GenStat v. 7.1 (Payne et al. 2003).

Results and conclusions

The presence of the mutated *BnaA.FAD3* allele was associated with seed oil fatty acid composition with statistical significance, except for eicosenoic acid content (C20:1) ($P=0.129$) – possibly due to little content of eicosenoic acid in oilseed rape oil. At the same time, the mutant form of the *BnaC.FAD3* gene was associated with stearic (C18:0) ($P<0.001$), linoleic (C18:2) ($P<0.001$) and linolenic acid (C18:3) ($P<0.001$) content (Table 1). The percentage of variation in fatty acid composition accounted

for the presence of mutations in the *BnaA.FAD3* and *BnaC.FAD3* genes by the SNaPshot analysis was considerable only for the linolenic acid content and equaled 35.6% and 39.3%, respectively (Table 1), whereas the total percentage of variation in linolenic acid content was 53.2% when accounted for mutations in both genes simultaneously (Table 1). At the same time, the oleic acid content was markedly influenced by the presence of the mutated alleles of the *FAD3* genes (Table 1) and it was not the case for the presence of the mutated *BnaC.FAD3* alone (Table 1).

Additive effects of the mutated alleles of the *BnaA.FAD3* and *BnaC.FAD3* genes were significant for stearic, linoleic and linolenic acid content and the signs of the effect values were the same for both genes (Table 2). Moreover, additive effect of the mutated allele of the *BnaA.FAD3* gene was significant for the palmitic acid content (Table 2).

Dominance effects assessed for the presence of the mutated alleles were significant for palmitic, oleic and linoleic acid content (Table 2). The absolute values of the dominance effects were larger than the absolute values of the additive effects for palmitic and oleic acids content. However, it was not the case for stearic, linoleic and linolenic acids content.

Additive effects are fixed in the population as the number of homozygotes increases in successive generations (Bocianowski and Krajewski 2009) and insignificant dominance effects at early generations indicate high selectivity of the markers, which proved very useful for monitoring wild-type and mutant alleles of *FAD3* desaturase. The markers will be further applied in oilseed rape breeding programs concerning diversification of seed oil fatty acid composition.

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