Development of allele-specific SNP markers for the new low-linolenic mutant of winter oilseed rape

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Abstract

Genotypes of oilseed rape characterized by lowered linolenic acid content in seed oil are under development and investigation worldwide due to the non-food use of rapeseed oil for biofuel biocomponents production. The low linolenic mutant (LLMut), characterized by about 2% of linolenic acid in seed oil, was obtained as a result of chemical mutagene treatment of double low (00) winter oilseed rape breeding line (about 10% of linolenic acid in seed oil) at the Plant Breeding and Acclimatization Institute in Poznan, Poland. The aim of this work was to develop allele-specific DNA markers for the obtained genotype. Genomic DNA was isolated from the low-linolenic mutant and from non-mutated (00) breeding line. *Fad3* desaturase gene alleles of LLMut and 00 genotypes were PCR amplified, cloned and sequenced. Two significant mutation changes in nucleotide sequences were identified and allele-specific SNP markers were designed. DH lines population was developed from F_1 hybrid obtained as a result of 00 × LLMut crossing. SNP genotyping of the DH lines was done and correlation coefficient (r) was calculated, indicating a strong negative correlation between linolenic acid content in seed oil and the presence of the mutations in *fad3* gene.

Key words: *Brassica napus*, fad3 desaturase, linolenic acid, SNP

Introduction

Rapeseed oil is used not only for human nutrition but also as a raw material – in industry and technology (Tőpfer *et al*., 1995; McDonell *et al.,* 1999, Altin *et al.*, 2001). Differentiated fatty acids composition is required due to the means of oilseed rape oil application (Friedt & Lühs, 1999; Mikolajczyk & Bartkowiak-Broda, 2003]. One of the main advantages of the oil, while used as nutrition product, is the presence of polyunsaturated - linoleic and linolenic fatty acids which makes it a valuable source of essential for human health exogenic fatty acids (Scarth & McVetty, 1999, Simopoulos, 2000, Leckband *et al*., 2002). However, this characteristic could be a disadvantage, provided such oil was applied for industrial and technological purposes because polyunsaturated fatty acids cause its flexibility and oxidative rancidity. Different approaches have been performed for introducing of the low-linolenic acid trait into rapeseed genotypes. However, the breeding process is complicated by the fact that the trait has a complex genetic inheritance being highly influnced by the environment (Bartkowiak-Broda and Krzymanski, 1983). DNA markers appear as an accurate and environment independent tool to be used for breeding of the low-linolenic oilseed rape cultivars (Snowdon & Friedt, 2004). There are several breeding organizations in the world having low-linolenic oilseed rape cultivars in development and production (Scarth & McVetty, 1999, Rakow & Raney, 2003). In Poland, at the Plant Breeding and Acclimatization Institute, Poznan Branch, chemical mutagenesis was applied for changing of seed fatty acids content of a double-low winter oilseed rape line and resulted, among others, in low-linolenic mutant plants, characterized by about 2% of linolenic acid in seed oil (Spasibionek, 2006). Low-linolenic genotypes were further applied for breeding programmes. Significant improvement of the efficiency in breeding process could be achieved with the use of specific genetic markers. And genes coding for fad3 desaturase, an enzyme involved in the synthesis of linolenic acid, seem to be targets for mutation (Jourdren *et al.*, 1996, Barret *et al.*, 1999, Hu *et al*., 2003).
The aim of this work was to search for the mutation and to develop allele-specific SNP markers for the mutant genotype which could enable effective selection of plants at different stages of development.

Material and Methods

Plant Material: (1) 00 winter oilseed rape (*Brassica napus* L.) PN 1775 line developed at the Plant Breeding and Acclimatization Institute in Poznan, Poland, (2) low-linolenic inbred line (LLMut) developed from the M-681 mutant (Tab. 1) (Spasibionek, 2006) and DH lines developed from LLMut inbred line with the use of the isolated microspores method (Cegielska-Taras *et al*., 2002), (3) DH lines population developed from F_1 hybrid obtained as a result of 00 × LLMut crossing.

DNA isolation and analysis: genomic DNA was isolated from 10-days old leaves with the use of the method described by Doyle (1990) or with the use of the Qiagen Plant DNeasy Kit, according to manufacturer's protocols. Genomic DNA as well as PCR reaction products were analysed by 0.8% or 2.0% agarose gel electrophoresis in 1xTBE buffer.

DNA Cloning and Sequencing: fad3 desaturase genes were PCR amplified with the use of degenerated primers and the

reaction products (Fig. 1) were cloned by means of TOPO T/A Invitrogen system. Plasmid DNA of positive clones was isolated with the use of Qiagen kit. Sequencing reaction was performed automatically on both strands with the use of DTCS (Beckman Coulter) i BigDye v3.1 (Applied Biosystems) reagents as well as CEQ2000XL and ABI Prism 3130XL sequencers. And, nucleotide sequences were analysed using NCBI BLASTN alignment tools.

Table 1. Fatty acid content [%] of winter oilseed rape genotypes: PN 1775 – 00 inbred line and PN 1712 – low-linolenic mutant (LLMut) inbred line; $C_{16:0}$ – palmitic acid, $C_{18:0}$ – stearic acid, $C_{18:1}$ – oleic acid, $C_{18:2}$ – linoleic acid, $C_{18:3}$ – linolenic acid, $C_{20:0}$ – eicosenoic acid, $C_{22:1}$ – erucic acid.

Line	Type	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:1}$	$C_{22:1}$
PN 1775	00	4.6	1.6	64.0	18.1	9.5	2.3	0
PN 1712	LLMut	3.6	1.8	65.7	25.1	1.7	2.1	0

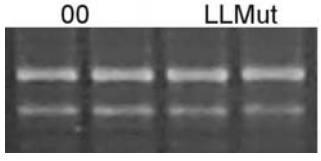

Figure 1. Agarose gel electrophoresis of PCR amplification products of *B. napus fad3* desaturase genes; 00 - PN 1775 inbred line, LLMut – low-linolenic mutant line.

Seed fatty acids content was analysed with the use of fatty acids methyl esters method (Byczynska & Krzymanski, 1969) and Agillent Technologies 6890N gas chromatograph (DB 23 30m, ID 025, 0.25µm layer capillar column) equipped with Chemstation integrator was applied.

Results

NCBI alignment revealed that the sequenced 00 and LLMut *B. napus* genomic regions were homologous to *A. thaliana fad3* desaturase gene, comprising fragments from the second to the seventh exon. The amplified *B. napus fad3* genes of 00 and LLMut were highly homologous to each other (96% of identity) revealing several point mutations, and two of them were statistically significant. The first one changed a codon, thus leading to posible changes of physical properties of the encoded aminoacid, and the second – changed an intron, leading to disruption of a donor splicing site.

In order to develop allele-specific SNP markers, *fad3* genomic sequences in the vicinity of mutation sites were PCR amplified in independent reactions, with the use of designed specific primers. The specificity of amplificartion was checked by sequencing. In order to detect mutations, microsequencing was performed with the use of fluorescent-labelled site-specific primers. SNP analysis results were obtained in GeneMapper programme (Applied Biosystems). The results of 00, LLMut and DH lines population SNP genotyping were compared with biochemical analyses of seed oil fatty acids composition. 00 – non-mutated and LLMut - mutated homozygotes in both sites were characterized by 6.0 – 7.2% and 1.4 – 1.9% of linolenic acid content in seed oil, respectively. Out of 122 DH lines developed from F_1 hybrid obtained as a result of 00 × LLMut crossing only 27 produced seeds of quality and quantity appropriate for biochemical analyses. The DH lines revealed genotypes, as follows: non-mutated homozygous alleles in both sites, mutated - in one site, mutated in another site and mutated in both sites, being characterized by 7.5 – 1.7% of linolenic acid content in seed oil. The DH lines were grouped in respect of the presence of mutated alleles and three groups - '0', '1' and '2' of 6, 8 and 6 lines, respectively, were distinguished. Each group was characterized by different average linolenic acid content in seed oil, as follows: group '0' – by 6.1% (sd = 1.16), '1': 3.22% (sd = 0.85) and '2': 2.3% (sd = 0.51). And, correlation coefficient (r = -0.856) was stated between the number of mutated homozygous alleles in both sites and linolenic acid content in seed oil, indicating a strong negative correlation between linolenic acid content in seed oil and the presence of the identified mutations in *fad3* gene, thus making the new SNP marker a powerful tool to be applied for marker assisted selection in breeding programmes.

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