Cloning and sequencing of FAD-3 desaturase gene from winter oilseed rape (*Brassica napus* L.) lines characterized by differentiated content of linolenic acid

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ABSTRACT

One of the selection goals in breeding programmes is to obtain oilseed rape genotypes producing oil of natural stability, which is characterized by higher content of oleic acid (over 75%) and lowered content of linolenic acid (below 3%). Low-linolenic genotypes (about 2%) were obtained, among others, *via* chemical mutagenesis in the Plant Breeding and Acclimatization Institute in Poznań, Poland. In order to search for the obtained mutation as well as to design specific DNA markers, which could enable effective and environment independent selection of plants at different stages of development, genomic DNA was isolated from a double-low (00) winter oilseed rape line as well as from a low-linolenic mutant inbred line (LLMut). A and C alleles of FAD-3 desaturase, an enzyme involved in linolenic acid synthesis, were amplified with the use of degenerated primers and then cloned, sequenced and analyzed. Obtained results reveal differences in nucleotide sequences of 00 – and mutant line genes. Other clones will be sequenced and SNP markers will de designed in order to enable selection of low-linolenic mutant genotypes. In addition, possible changes in the 00 and mutant FAD-3 desaturase genes expression were the subject of research.

Key words: winter oilseed rape (*Brassica napus* L.), FAD-3 desaturase, DNA markers

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 [Mikołajczyk and 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 [Fitzpatrick and Scarth, 1998; Scarth and 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 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 Krzymański, 19983]. DNA markers appear as an accurate and environment independent tool to be used for breeding of the low-linolenic oilseed rape cultivars [Snowdon and Friedt, 2004]. There are several breeding organizations in the world having low-linolenic oilseed rape cultivars in development and production [Scarth and McVetty, 1999; Rakow and Raney, 2003]. In Poland, in the Plant Breeding and Acclimatization Institute in Poznań stable inbred lines of about 3% of linolenic acid have been obtained as a result of crosses between double-low winter oilseed rape lines with the low-linolenic spring cultivars Stellar and Apollo. In addition, chemical mutagenesis was performed on double-low winter oilseed rape line and resulted in low-linolenic mutant plants (about 2% of linolenic acid) which were further used in recombinant breeding programmes [Spasibionek *et al.*, 2000]. Significant improvement of the efficiency in breeding process could be achieved with the use of specific DNA markers; FAD-3 desaturase gene, an enzyme catalysing the third unsaturated bound synthesis in linoleic acid, seems to be a target for possible mutation [Jourdren *et al.*, 1996; Barret *et al.*, 1999; Hu *et al.*, 2003]. In allotetraploid *B.napus* genome (AACC genome), coming from *B.campestris* (AA genome) and *B.oleracea* (CC genome), there are two alleles of FAD-3 desaturase – A and C.

The main goal of this work was to search for the obtained mutation as well as to design specific DNA markers, which could enable effective and environment independent selection of plants at different stages of development.

MATERIALS AND METHODS

Plant Material: Winter oilseed rape (*Brassica napus* L.) plants were used, as follows:

- 00 type PN 1775/02 line developed at the Plant Breeding and Acclimatization Institute, Poznań Branch
- low-linolenic inbred line developed from M-681 mutant (LLMut) obtained *via* chemical mutagenesis (Tab. 1) [Spasibionek *et al*., 2000]

DNA Isolation: Genomic DNA was isolated from 10-days old leaves with the use of the method described by Doyle [1990].

DNA Electrophoresis: Genomic DNA as well as PCR reaction products were analysed by 0,8% and 2,0% agarose gel electrophoresis in 1×TBE buffer, respectively.

DNA Cloning and Sequencing: FAD-3 desaturase genes were PCR amplified with the use of degenerated primers [Jourdren *et al*., 1996]. Reaction products referring to A and C alleles of FAD-3 (Fig. 1) were cloned by means of TOPO T/A Invitrogen cloning system. Plasmid DNA of positive clones was isolated with the use of Qiagen kit (Fig. 2). Sequencing reaction was performed authomatically on both strands with the use of DTCS (Beckman Coulter) i BigDye v3.1 (Applied Biosystems) reagents as well as CEQ2000XL i ABI Prism 3130XL sequencers. Nucleotide sequences obtained were further analysed using NCBI BLASTN alignment tools.

RNA Isolation and RT/PCR Analysis: Total RNA was isolated from both 10-days leaves and developing seeds – four (mutant plants) or six weeks (00 type plants) after pollination, with the use of Qiagen RNeasy plant mini kit with the DNase digestion step. UV measurments as well as agarose gel electrophpresis were done for estimation of quality and quantity of the obtained RNA samples (Fig. 3). Reverse transcription reaction (RT) was done with the use of Fermentas Revert Aid First Strand cDNA Synthesis Kit, using oligo(dT) as a primer, according to the manual. Aliqouts of 1 µg of total RNA were used for RT and the RNA amount was controlled by RT/PCR for ß-actin gene. Obtained cDNA was PCR amplified with the use of 5' and 3' primers specific for the cloned *B.napus* FAD-3 desaturase gene (Fig. 4).

Table 1. Fatty acid content [%] of winter oilseed rape genotypes: PN 1775/02 – a 00-type inbred line and PN 1712/02 – a 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 1775/02	**OO**	4,6	1,6	64,0	18,1	**9,5**	2,3	0
PN 1712/02	**LLMut**	3,6	1,8	65,7	25,1	**1,7**	2,1	0

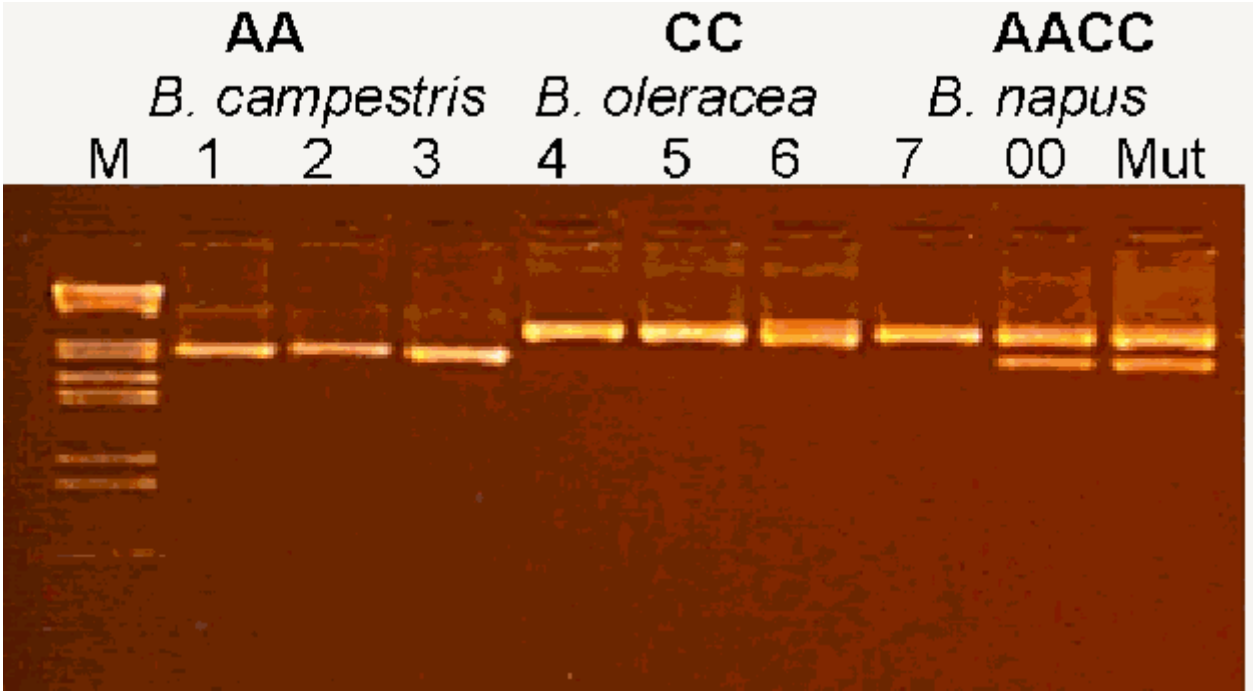
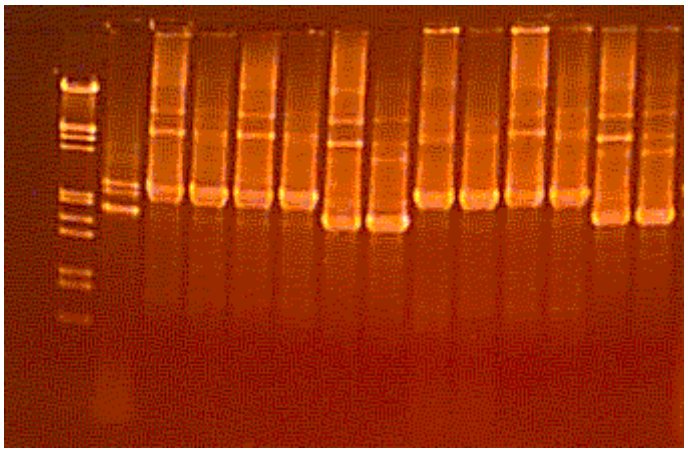


Figure 1. Agarose gel electrophoresis of DNA amplification products obtained with the use of primers specific for linoleic acid desaturase (FAD-3); DNA isolated from plants of: *B.campestris* – AA genome, *B.oleracea* – CC, *B.napus* – AACC; 00 – PN 1775/02 inbred line, LLMut – low-linolenic mutant obtained from 00 line; M – size marker: λ phage DNA hydrolised by endonucleases *Eco RI* and *Hind III*.

RESULTS AND CONCLUSIONS

1. Genomic DNA was isolated from a double-low winter oilseed rape line (00) as well as from the low-linolenic mutant inbred line (LLMut) obtained from the 00 line (Tab. 1). *B.napus* FAD-3 A and C alleles were PCR amplified (Fig. 1) and cloned (eighteen clones) (Fig. 2).
2. Up till now, four clones – one for each allele, have been sequenced. Alignment of the obtained sequences with those of NCBI database revealed that the clones contain alleles of FAD-3 desaturase and that there are differences between sequences of 00 and LLMut lines.
3. The other clones are sequenced and analysed in order to design SNP markers specific for the low-linolenic mutant.
4. Comparison of FAD-3 genes expression level between non-mutated and mutant plants by means of RT/PCR analysis showed that it was increased in developing seeds of 00 plants whereas it remains at the same – lower level in mutants (Fig. 4). a) **M k 1 2 3 4 5 6**



b) *Eco RI Xba I*

M k 1 2 3 4 5 6 1 2 3 4 5 6

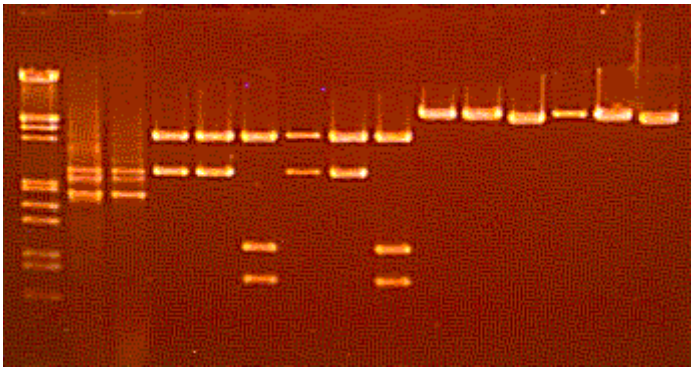


Figure 2. Agarose gel electrophoresis of plasmid DNAs containing inserts of *B.napus* FAD-3 desaturase A and C alleles; a) PCR/FAD-3 amplification products, b) restricion endonucleases *Eco RI* and *XbaI* hydrolysis products*;* 1, 2 – C alleles of LLMut, 3 – A allele of LLMut; 4, 5 – C alleles of 00 line, 6 – A allele of 00 line*;* M – size marker: λ phage DNA hydrolised by endonucleases *Eco RI* and *Hind III*; k – PCR/FAD-3 amplification products of *B.napus* genomic DNA

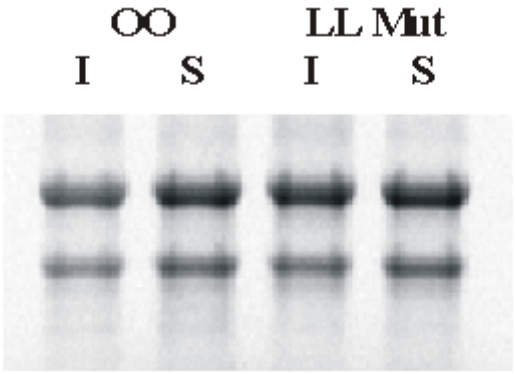


Figure 3. 1% agarose gel electrophoresis of total RNA; 00 – PN 1775/02 line, LLMut – PN 1712/02 mutant line; l – ten days old leaves, s – developing seeds, four (LLMut) or six weeks after pollination

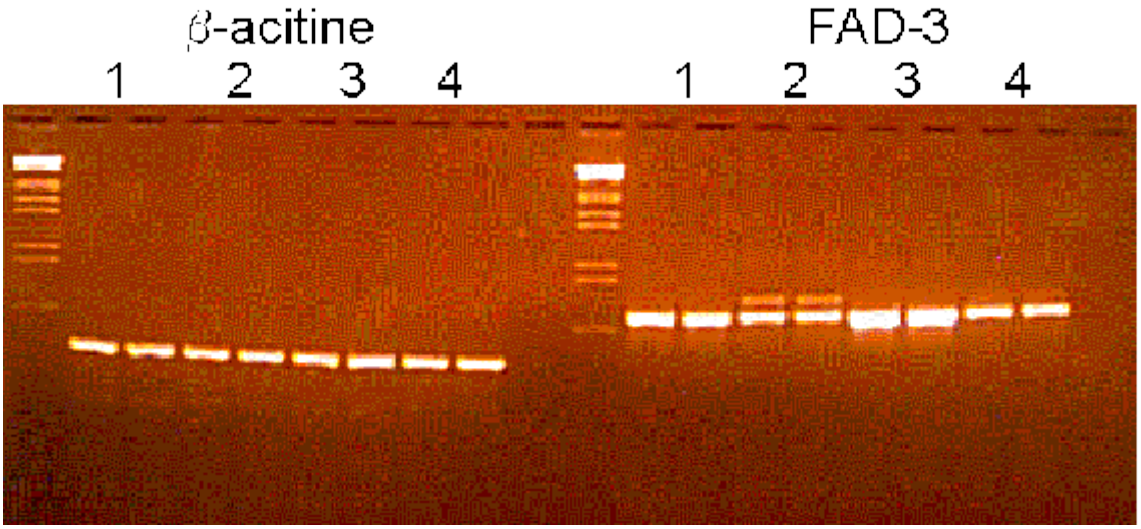


Figure 4. RT/PCR analysis of FAD-3 desaturase gene expression; 1 – 00 young leaves, 2 – LLMut young leaves, 3 – 00 developing seeds, 4 – LLMut developing seeds

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