Identification of fad2 mutations and development of Allele-Specific Markers for High Oleic acid content in rapeseed (*Brassica napus* L.)

Falentin Cyril, Brégeon Michel, Lucas Marie-Odile, Deschamps Max, Leprince Françoise, Fournier Marie-Thérèse, Delourme Régine, Renard Michel

UMR INRA-Agrocampus Rennes, Amélioration des Plantes et Biotechnologies Végétales, BP35327, F-35650 Le Rheu Cedex, France Email: regine.delourm@rennes.inra.fr

Abstract

The quality of rapeseed oil is determined by its constituent fatty acids such as oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3). Most winter rapeseed cultivars normally produce oil with about 60% oleic acid. Development of winter rapeseed cultivars with increased oleic acid is highly desirable for new food and non food markets (better resistance to oxidation…). In this study, we sequenced genomic clones of the *B. napus fad2* genes (originating from *B. oleracea* (*fad2C*) and *B. rapa* (*fad2A*)) amplified from double cycled EMS-induced mutants and wild-type rapeseed cultivars. A comparison of the mutant and wild-type allele sequences of the *fad2* genes revealed single nucleotide mutations in each of the genes (*fad2C* and *fad2A*). Detailed sequence analyses suggested mechanisms by which both mutations can cause altered fatty acid contents in the mutants. Based on the sequence differences between the mutant and wild-type alleles, two single nucleotide polymorphism (SNP) markers, corresponding to the *fad2C* and *fad2A* gene mutations, were developed. Six doubled haploid populations have been used to characterize the effect of both *fad2C* and *fad2A* mutated alleles on oleic acid content. These new molecular markers will be highly useful for direct selection of desirable *fad2C* and *fad2A* alleles during marker-assisted trait introgression and breeding of high oleic rapeseed.

Key words: rapeseed, Brassica napus, high oleic acid content, SNP, marker-assisted selection

Introduction

High oleic acid content of vegetable oils is a desirable trait both because of the health benefits and of the stability to oxidation and heat of oleic acid. In particular, it has been shown that oleic acid is effective in lowering plasma cholesterol levels (Bonanome *et al.*, 1988; Liu *et al*., 2002). Furthermore, its single insaturated bond makes oleic acid (C18:1) a much less vulnerable fatty acid than its multiply insaturated counterparts. For instance, the rate of oxidation of linolenic acid (C18:3) is 100 times that of oleic acid (Debruyne, 2004).

One of the most promising paths towards such plants is to select plants essentially deprived of FAD2 activity. Indeed, FAD2 (delta12 oleate desaturase) catalyses the transformation of oleic acid (C18:1) to linoleic acid (C18:2); plants having lowered FAD2 activity thus have higher oleic acid content thanks to limited catabolism of the latter.

Brassica napus (rapeseed) is an amphidiploid which contains the genomes of two diploid ancestors, *B. rapa* (the A genome) and *B. oleracea* (the C genome) (U, 1935). The oleic acid content is 61 % for the traditional oil of rapeseed (Stan Skrypetz, 2005).

The present study notably arises from the obtention of five previously unrecognised mutations in the *fad2* genes of *Brassica napus* plants. These mutations can be used in plant breeding programs by marker-assisted selection.

Material and Methods

Plant Material

Winter rapeseed lines 'LOR1#S007' (wild type), 'LOR1#PR-2601' (wild type), 'HOR1#S005′ (mutant type), 'HOR1#B005′ (mutant type), 'HOR1#NPZ-12' (mutant type), 'HOR2' (mutant type), 'HOR3′ (mutant type) and 'HOR4' (mutant type) were used in this study for cloning of *fad2* (fatty acid desaturase-2) alleles.

'HOR1#S005′, 'HOR1#B005′ and 'HOR1#NPZ-12' have been obtained by crossing an ethyl methanesulphonate (EMS) mutant line 'HOR1' with three classical rapeseed lines (S005, B005 and NPZ-12); these three mutant lines have an oleic acid content at about 80 to 90%.

'LOR1#S007' and 'LOR1#PR-2601' are rapeseed lines with oleic acid content at about 60%.

'HOR2', 'HOR3′ and 'HOR4' are three other independent EMS mutant lines with oleic acid content at about 75 to 85%.

Six doubled haploid (DH) populations were developed by microspore culture (Coventry *et al.*, 1988) from F1 plants of crosses between:

(1) 'LOR1#S007' and 'HOR1#S005′ rapeseed lines,
(2) 'LOR1#PR-2601' and 'HOR1#NPZ-12' rapeseed lines,
(3) 'LOR1#S007' and 'HOR1#B005′ rapeseed lines,
(4) 'LOR1#S007' and 'HOR1#NPZ-12' rapeseed lines,

(5) 'LOR1#PR-2601' and 'HOR1#B005′ rapeseed lines and
(6) 'LOR1#PR-2601' and 'HOR1#S005′ rapeseed lines.

In 2003, the oleic acid content was assessed in greenhouse on plants of each DH line. In 2005, 399 DH lines and their respective parents were experimented in the field in a randomised trial with three replications. A complete fatty acid analysis of the seeds of each plot was implemented by using gas chromatography. All the 399 DH lines were used for marker and statistical analysis. No DH population has been produced with 'HOR2', 'HOR3′ and 'HOR4' mutant lines.

Genomic DNA extraction and quantification :

DNA of both parental lines and 399 DH lines was extracted from leaves of 2-week-old field grown plants using a CTAB-based method modified from Dellaporta *et al.* (1983). Absorbance at 260 nm was used for DNA quantification. In a UV-microtiter plate, 195µl of ultrapure sterile water were added into each well and then 5µl of each DNA sample were added. The plate was then briefly agitated and read using the Spectra Max M2 microplate spectrophotometer from Molecular Devices.

PCR amplification

PCR amplification reactions used for *fad2* alleles cloning contained 3-4 ng/µl of genomic DNA, 1.25µM of each primer, 2.5mM $MgCl_2$, 0.3mM of each dNTP, 1 × PCR buffer and 0.12U/µl of *Taq* DNA polymerase. Amplifications were performed in a PTC-225 MJResearch PCR system programmed for 30 cycles of 30 sec at 94°C, 30 sec at 60°C, 30 sec at 72°C and ending with 5 min at 72°C.

PCR amplification reactions used for genotyping the 399 DH lines contained 3-4 ng/µl of genomic DNA, 0.625µM of each primer, 1.5mM $MgCl_2$, 0.3mM of each dNTP, 1 × PCR buffer and 0.06U/µl of *Taq* DNA polymerase. Amplifications were performed in a PTC-225 MJResearch PCR system programmed for 30 cycles of 30 sec at 94°C, 30 sec at 60°C, 10 sec at 72°C and ending with 5 min at 72°C.

Identification of mutations in fad2 genes :

Primers designed with Primer 3 software on *fad2* gene sequence from GenBank Accession AY577313 were used to amplify genomic DNA fragments of the *fad2* gene from *B. napus* lines 'LOR1#S007', 'LOR1#PR-2601', 'HOR1#S005′, 'HOR1#B005′, 'HOR1#NPZ-12', 'HOR2', 'HOR3′ and 'HOR4'. The primer pair FAD2BnF1 : AGTGTCTCCTCCCTCCAAAAA and FAD2BnR1 : TCTTCTCACCTTGCCTGTCC amplified a *fad2* fragment of the same length (1100 bp) from each of the six parents. The amplified fragments were then cloned and sequenced to investigate the sequence differences of *fad2* gene between the five parents.

Cloning of fad2 alleles :

The *fad2* fragments of parental lines 'LOR1#S007', 'LOR1#PR-2601', 'HOR1#S005′, 'HOR1#B005′, 'HOR1#NPZ-12', 'HOR2', 'HOR3′ and 'HOR4' were amplified by using primers designed with Primer 3 software on *fad2* gene sequence from GenBank Accession AY577313. The *fad2* fragments amplified from each of the parents by the primers FAD2BnF1 and FAD2BnR1 were ligated to pGEM®-T Easy cloning vector using a pGEM®-T Easy Vector System kit (Promega Corp., Madison, USA) per manufacturer's instructions. The ligated products were transformed into competent cells and the cells plated on LB-agar plates containing ampicillin, X-GAL and IPTG to enable white/blue selection. White colonies in the transformation plates were picked and identification of the cloned PCR products were verified by PCR using universal M13 Forward and Reverse primers flanking the insert fragment. PCR revealed the insert fragment of the expected size. The positive clones containing the insert were sequenced by Genome Express (Meylan, France).

Sequence and data analysis :

The sequences were analysed and aligned by using Clustal W (Kyoto University Bioinformatics Center) and Genedoc Software (Nicholas *et al.*, 1997). Linkage association between the markers and high oleic trait was determined by ANOVA and regression analyses.

Results

In this study, several clones were sequenced. The sequence alignment of these clones with *B. rapa* and *B. oleracea fad2* sequences found in public databases identified:

(1) two *fad2* genes originating from *B. rapa* (*fad2A*) and *B. oleracea* (*fad2C*) for 'HOR1' lines,
(2) one *fad2* gene originating from *B. rapa* (*fad2A*) for 'HOR2' line,
(3) one *fad2* gene originating from *B. rapa* (*fad2A*) for 'HOR3′ line and
(4) one *fad2* gene originating from *B. rapa* (*fad2A*) for 'HOR4' line.

For 'HOR1' lines, the sequence analysis identified a single nucleotide mutation in each *fad2* gene (*fad2A* and *fad2C*) resulting in amino acid substitutions.

For 'HOR2' line, the sequence analysis identified a single nucleotide mutation in the *fad2A* gene resulting in an amino acid substitution.

For 'HOR3′ and 'HOR4' lines, the sequence analysis identified a single nucleotide mutation in the *fad2A* gene creating a stop codon that causes early termination of the polypeptide chain during translation.

Molecular tests were developed for genotyping the 6 doubled haploids populations.

After genotyping all the 399 DH lines, it was found that the alleles distribution was highly correlated to C18:1 content (Fig. 1).

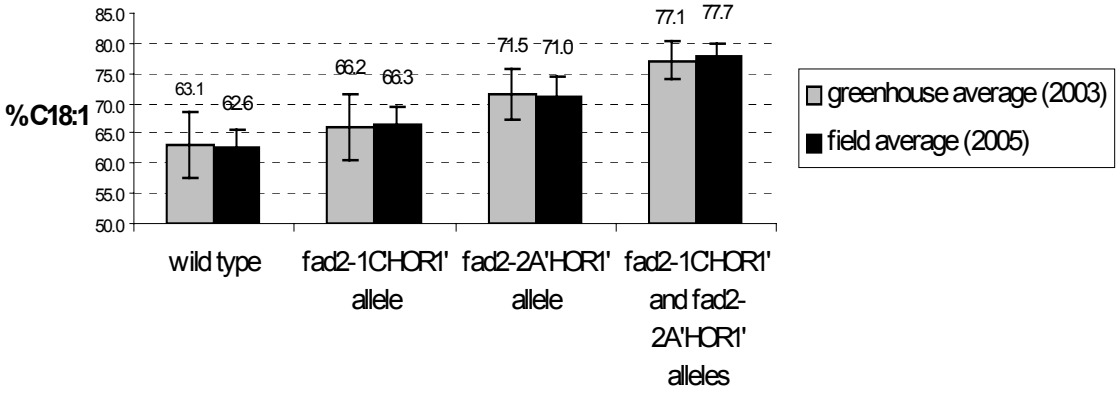

Fig. 1. Histogram showing the relation between the presence of the mutant allele-specific markers and oleic acid content in field (2005) and greenhouse (2003).

Discussion

The analysis of *fad2* nucleic sequences obtained after cloning and sequencing showed that there are two copies of the *fad2* gene in rapeseed : one from *B. rapa* (*fad2A*) and one from *B. oleracea (fad2C)*. The preliminary results of correlation of the oleic acid content with the molecular genotyping highlight the cumulative effect of the transferred alleles *fad2A* and *fad2C* on the oleic acid content. Thus, 77% of oleic acid content could be obtained on average when the two mutated alleles *fad2A* and *fad2C* were cumulated in the genotypes. The maximum values obtained in greenhouse and in the field were higher than 80%.

Conclusions

This study highlights new SNP mutations in *fad2* genes of 4 different mutants. These new molecular markers will be highly useful for direct selection of desirable *fad2C* and *fad2A* alleles during marker-assisted trait introgression and breeding of high oleic rapeseed. All information concerning these molecular markers was the subject of a patent filling. For more information, please contact INRA : cyril.falentin@rennes.inra.fr

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