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ORIGINAL PAPER

# Mapping of the loci controlling oleic and linolenic acid contents and development of *fad2* and *fad3* allele-specific markers in canola (*Brassica napus* L.)

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**Abstract** The quality of canola oil is determined by its constituent fatty acids such as oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3). Most canola cultivars normally produce oil with about 55–65% oleic acid and 8–12% linolenic acid. High concentrations of linolenic acid lead to oil instability and off-type flavor, while high levels of oleic acid increase oxidative stability and nutritional value of oil. Therefore, development of canola cultivars with increased oleic acid and reduced linolenic acid is highly desirable for canola oil quality. In this study, we have mapped one locus that has a major effect and one locus that has a minor effect for high oleic acid and two loci that have major effects for low linolenic acid in a doubled haploid population. The major locus for high C18:1 was proven to be the fatty acid desaturase-2 (*fad2*) gene and it is located on the linkage group N5; the minor locus is located on N1. One major QTL for C18:3 is the fatty acid desaturase-3 gene of the genome C (*fad3c*) and it is located on N14. The second major QTL resides on N4 and is the *fad3a* gene of the A genome. We have sequenced genomic clones of the *fad2* and *fad3c* genes amplified from an EMS-induced mutant and a wild-type canola cultivar. A comparison of the mutant and wild-type allele sequences of the *fad2* and *fad3c* genes revealed single nucleotide mutations in each of the genes. Detailed sequence analyses suggested mechanisms by which both the mutations can cause altered fatty acid content. Based on the sequence differences between the mutant and wild-type alleles, two single nucleotide polymorphism (SNP) markers, corresponding to the *fad2* and *fad3c* gene mutations, were developed. These markers will be highly useful for direct selection of desirable *fad2* and *fad3c* alleles during marker-assisted trait introgression and breeding of canola with high oleic and low linolenic acid.

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## Introduction

The quality of canola oil is determined by its constituent fatty acids. Reduced levels of polyunsaturated fatty acids such as linolenic acid and increased levels of monounsaturated oleic acids are associated with higher oxidative stability. The standard canola oil contains about 60% oleic acid (C18:1), 20% linoleic acid (C18:2) and 10% linolenic acid (C18:3). The high level of linolenic acid in canola oil is an undesirable trait because linolenic acid is highly unsaturated and can be easily oxidized to cause off-flavor and rancidity to the oil, thus resulting in a shortened shelf life. The high oleic oil can be heated to a higher temperature without smoking, making it more suitable as cooking oil. Breeding canola varieties with high oleic (HO) acid and low linolenic (LL) acid in oilseeds is one of major objectives for many canola breeding programs.

High C18:1 and low C18:3 mutants have been produced through mutagenesis (Rakow 1973; Auld et al.

1992). Genetic analysis of a population derived from the cross of "Stellar" that is low in C18:3 content (3%) and "Drakkar" with a "normal" C18:3 level (9–10%) indicated that low C18:3 was controlled by two major loci with additive effects designated L1 and L2 (Jourdren et al. 1996b). The two major loci controlling C18:3 content were found to correspond to the two *fad3* (*fatty acid desaturase 3*) genes, one is located in the A genome and the other is in the C genome (Jourdren et al. 1996a; Barret et al. 1999). The *fad3* gene encodes for endoplasmic delta-15 linoleate desaturase which is responsible for the desaturation of linoleic acid (C18:2) into linolenic acid (C18:3). Thormann et al. (1996) mapped two quantitative trait locus (QTLs) that explained 60% of the variance for the linolenic content, while Somers et al. (1998) identified three QTLs that collectively explained 51% of the phenotypic variation of C18:3 content. A three locus additive model was also reported by Chen and Beversdorf (1990). Whereas Rücker and Röbbelen (1996) indicated that probably several minor genes are involved in the desaturation steps. C18:1 content was controlled by a major locus called *fatty acid desaturase 2 (fad2)* gene which encodes the enzyme (endoplasmic delta-12 oleate desaturase) responsible for the desaturation of C18:1–C18:2 (Tanhuanpää et al. 1998; Schierholt et al. 2001). Scheffler et al. (1997) mapped four loci of the *fad2* gene on four different linkage groups, two of A- (*B. rapa*) and two of C-genome (*B. oleracea*) origin. All of the functional gene copies of *fad2* that have been reported and mapped to date are located on the A-genome linkage group N5 (Scheffler et al. 1997; Schierholt et al. 2000). Environmental conditions such as temperature and moisture often affect the seed fatty acid content (Canvin 1965; Deng and Scarth 1998), making phenotypic selection of plants less reliable.

The molecular markers tightly linked to the genes controlling C18:1 and C18:3 contents can be used in canola breeding programs to facilitate selection of plants carrying genes for high C18:1 and low C18:3. Different types of molecular markers, such as random amplified polymorphic DNA (RAPD) (Tanhuanpää et al. 1995; Hu et al. 1995; Rajcan et al. 1999; Jourdren et al. 1996b), restriction fragment length polymorphism (RFLP) (Thormann et al. 1996), and sequence characterized amplified region (SCAR) (Hu et al. 1999), have been identified to be associated with low C18:3 levels in *B. napus*. Allele-specific markers were developed for *fad3* in *B. rapa* ssp. *oleifera* (Tanhuanpää and Schulman 2002). Molecular markers have also been identified for high C18:1 content. A RAPD marker linked to the QTL affecting oleic acid content was identified in spring turnip rape (*B. rapa* ssp. *oleifera*) and was later converted into a SCAR marker (Tanhuanpää et al. 1996). Schierholt et al. (2000) identified three amplified fragment length polymorphism (AFLP) markers linked to a high oleic mutation in winter oilseed rape (*B. napus* L.). Tanhuanpää et al. (1998) developed an allele-specific PCR marker for oleic acid by comparing the wild-type and high-oleic allele of the *fad2* gene locus in spring turnip rape (*B. rapa* ssp. *oleifera*). However, most of these markers are low-throughput markers such as RAPD, AFLP and RFLP and are not suitable for large scale screening through automation. The objectives of this study were to (1) identify and map the genes responsible for oleic and linolenic acid synthesis in canola; (2) identify the mutations in the *fad2* and *fad3* gene sequences that show altered fatty acid contents in the mutant canola line, and (3) develop high-throughput markers linked to high oleic- and low linolenic acid content to facilitate the selection of these traits in canola trait introgression and breeding.

## Materials and methods

### Plant materials and DNA extraction

A doubled haploid (DH) population consisting of 604 lines was developed by microspore culture from the F1 plants of the cross between canola lines Quantum and DMS100, and was used in this study. DMS100 is a high Oleic and Low Linolenic (HOLL) canola line containing seed oil with ~77% oleic acid and ~3% linolenic acid and was developed through ethyl methane sulfonate (EMS) mutagenesis and subsequent breeding. Quantum is a commercial variety and contains low oleic acid (~66%) and high linolenic acid (~7%) contents. The DH population was developed by Dow AgroSciences (DAS) canola breeding program at Saskatoon, Canada. Of the 604 DH lines, 183 were randomly selected for marker analysis and mapping. A complete fatty acid analysis of the seeds of the DH lines and their parents was implemented by using gas chromatography (Thies 1971). Components of the oil were reported as percentages of total fatty acids.

DNA of both parental lines and 183 DH lines was extracted from the leaves of 2-week-old greenhouse grown plants. Six DNA bulks for C18:1 and C18:3 were constructed by using equal amount of DNA from each of the five or ten DH lines representing distribution tails for high and low C18:1 and C18:3 contents (Table 1).

**Table 1** DNA bulks for high oleic (C18:1) and low linlenic (C18:3) acid contents

| Bulk | Trait | | Bulk size (no. of lines) |
|---|---|---|---|
| | C18:1 | C18:3 | |
| B1 | | High | 10 |
| B2 | | Low | 10 |
| B3 | Low | High | 5 |
| B4 | High | Low | 5 |
| B5 | High | | 10 |
| B6 | Low | | 10 |

## SSR and AFLP analyses

Microsatellite or simple sequence repeat (SSR) markers were developed by Saskatoon Research Center, Agriculture and Agri-Food Canada (AAFC) for the *Brassica* Microsatellite Consortium. SSR forward primers were labeled either with 6-FAM, HEX or NED fluorescent tags and synthesized by Applied Biosystems (Foster City, CA). PCR was performed in 96 or 384-well PCR plates, with each reaction containing 20–30 ng of genomic DNA, 0.25 μM of each forward and reverse primer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1× PCR buffer and 0.6 units of *Taq* DNA polymerase. Amplifications were performed in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). Amplification program was 30 cycles of 30 s at 94°C, 30 s at 55°C, 30 s at 72°C and ending with 30 min at 72°C. The PCR products of three SSR markers with different fluorescent labels were then pooled together and separated in 6% denatured polyacrylamide gels using an ABI Prism 377 or 3700 DNA Sequencer (Applied Biosystems, Foster City, CA). Gel images were analyzed with either GeneScan (ABI Prism 377) or Data Collection (ABI Prism 3700) software (PE Applied Biosystems). The Genotyper software (PE Applied Biosystems) was used for allele characterization and for automated data output.

For AFLP analysis, the genomic DNA was digested with restriction enzyme *Hind* III and *Mse* I and the digested DNA fragments were ligated to *Hind* III and *Mse* I adapters, respectively. The ligated DNA was amplified with AFLP primers with the sequences complementary to the adapters. Marker data were generated using standard protocols reported in literature (Vos et al. 1995) with minor modifications.

## Cloning of *fad2* and *fad3* alleles

The *fad2* fragments of parental lines DMS100 (mutant type) and Quantum (wild-type) were amplified by using the primers homologous to the *Arabidopsis fad2* gene sequences (Tanhuanpää et al. 1998). The *fad2* fragments amplified from each of the parents by primers 2 and 6 of Tanhuanpää et al. (1998) were cloned and sequenced. The DNA sequences of the *fad31* and *fad32* loci of *B. napus* were retrieved from NCBI-GenBank database (AF056569 and AF056570, respectively). Three pairs of primers for each *fad31* and *fad32* loci were designed by using Primer Express™ 1.0 (PE Applied Biosystems, Foster City, CA) primer designing software. The *fad31* fragments amplified by the primers BNFD31-CF and BNFD31-CR and the *fad32* fragments amplified by the primers BNFD32-CF and BNFD32-CR from each of the parents were cloned and sequenced.

BNFD31-CF: GAGGCTTGGACGACCACTTG
BNFD31-CR: GACTGGACCAACGAGGAATG
BNFD32-CF: CAAGAATTTGTCCCACAGTACAC
BNFD32-CR: CAACTGTTGTTAATCCTCCACG

The PCR amplification products of the *fad2* and *fad3* fragments were resolved by agarose-gel electrophoresis, and the bands of interest were excised from the gel. The excised bands were placed in a microfuge tube containing 50 μl sterilized water and heated for 5 min in boiling water. The dissolved DNA was amplified by PCR with the corresponding primer pairs. The amplified products were ligated to PCR 2.1-TOPO cloning vector using a TA-cloning kit (Invitrogen Corp, San Diego, CA, USA) per manufacturer's instructions. The positive clones containing the insert were sequenced by Sequetech Corporation (Mountain View, CA) or Lark Technologies (Houston, TX). The sequences were analyzed and aligned by using SeqWeb (version 2) sequence analysis software present in GCG software package (University of Wisconsin).

## Allele-specific PCR

Allele-specific PCR was performed in 96 well PCR plates, with each reaction containing 20–30 ng of genomic DNA, 0.1 μM of each forward and reverse primers, 2.5 mM $MgCl_2$, 0.1 mM of each dNTP, 1× PCR buffer and 0.6 units of *Taq* DNA polymerase. The amplification program for allele-specific marker FAD2GM was similar to that for SSR except that annealing temperature was set at 62°C. A "touchdown" PCR program was used for the amplification of the allele-specific marker FAD3GM. This program included a denaturing step of 30 s at 94°C and an extension step of 30 s at 72°C. The initial annealing was done for 30 s at 62°C and the annealing temperature was subsequently

reduced by 0.7°C every cycle until a final temperature of 52°C was reached, which was maintained for the remaining 20 cycles of amplification. The PCR amplification products were separated in 1.5% agarose gels with 1× TBE buffer and visualized under UV light after staining with ethidium bromide.

### Invader® assay

Invader® assay kits specific to *fad2* and *fad3* gene mutations were developed through Third Wave Technologies (Madison, WI). The concentration of DNA samples for Invader® assay was normalized to 15 ng/μl using QiaGen Bio-Robot 3000 (Valencia, CA). Invader® assay was done in 96-well plates per manufacturer's instruction. In brief, DNA samples were denatured at 95°C for 10 min, 7 μl of the denatured DNA (15 ng/μl) and 8 μl of the reaction mix (3 μl oligo mix and 5 μl of 24 mM $MgCl_2$) were added into each well of 96-well Invader® assay plates. Then, each reaction was overlaid with 15 μl of mineral oil and the plates were incubated in the BioOven III from St John Associates, Inc (Beltsville, MD) at 63°C for 4 h. The reaction plates were read using a Spectra Max GEMINIS XS microplate fluorometer from Molecular Devices (Sunnyvale, CA) for fluorescent signals. Percent signal over background for the mutant allele was divided by the percent signal for wild-type allele for each sample to calculate the ratio. Individual genotypes were determined based on the calculated ratio of the signal strengths from mutant and wild-type alleles.

### Data analysis

The genetic linkage map was generated with JoinMap V3.0 computer software (Van Ooijen and Voorrips 2001) using a minimum LOD of 3.0. Map distance was converted to centiMorgans (cM) using the Kosambi function (Kosambi 1944). Linkage association between the markers and HOLL traits were first determined by the analysis of variance (ANOVA) and subsequently confirmed by QTL mapping. Putative QTL regions associated with the C18:1 and C18:3 were located by interval mapping using the MapQTL V 4.0 software (Van Ooijen et al.2002).

## Results

### Phenotypic distribution of C18:1 and C18:3 contents

Six hundred and four DH lines derived from the F1 of the cross Quantum × DMS 100 were distributed in two distinct groups for C18:1 (Fig. 1a), suggesting that C18:1 content was controlled by a major gene. However, the DH population appeared to be normally distributed for C18:3 (Fig. 1b), suggesting that C18:3 content was controlled by more than one gene.

### Sequence analysis of *B. napus fad2* and *fad3 genes*

The genomic fragments corresponding to the *B. napus fad2* gene were amplified from the mutant line DMS100 and wild-type line Quantum using the primers homologous to *Arabidopsis fad2* gene sequence (Tanhuanpää et al. 1998). The amplified fragments were then cloned followed by sequencing of 9–10 clones of each type. The sequence alignment of the 10 DMS100 and 9 Quantum sequences revealed that the 19 sequences were classified into two major groups based on the sequence similarities (data not shown). The two groups were distinct from each other at several positions and could represent two different gene copies of *fad2*. As found in *Arabidopsis* (Okuley et al. 1996) and *B. rapa fad2* (Tanhuanpää et al. 1998), no introns were found in the *B. napus fad2* either. The sequence analysis and alignment of these clones identified a single nucleotide mutation, C to T, which consistently occurred in one of low copies of the *fad2* gene

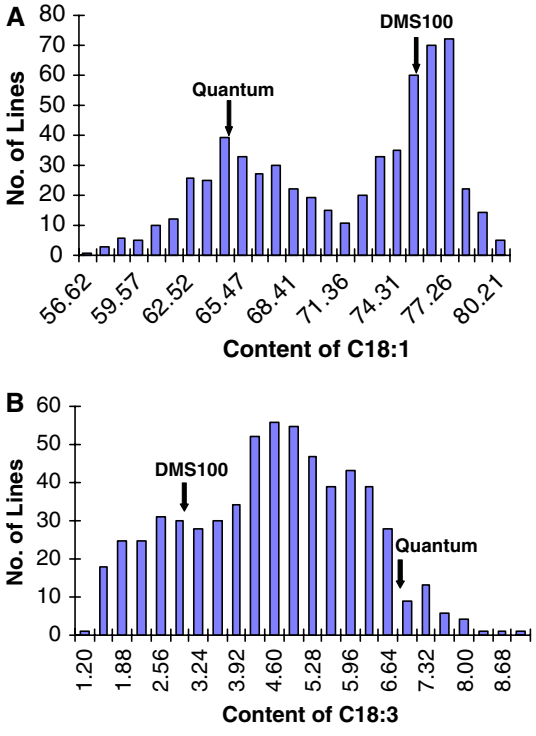


**Fig. 1** Distribution of oleic acid (C18:1) (**a**) and linolenic acid (C18:3) (**b**) of 604 doubled haploid lines derived from the cross of Quantum and DMS100. C18:1 and C18:3 values for the parents relative to the progeny are indicated

sequences of all DMS100 clones. Further analysis indicated that the mutation of the C to T created a stop codon (TAG) that will result in premature termination of the open reading frame (Fig. 2).

Partial genomic DNA fragments corresponding to the *fad31* and *fad32* genes were amplified from DMS100 and Quantum lines using the primers designed from the *B. napus fad31* and *fad32* gene sequences (GenBank Accession AF056569 and AF056570). The *fad31* fragments amplified by the primers BNFD31-CF and BNFD31-CR and *fad32* fragments amplified by the primers BNFD32-CF and BNFD32-CR were cloned and sequenced. Seven DMS100 clones and six Quantum clones of *fad31* and 6 DMS100 clones and six Quantum clones of *fad32* were sequenced. Sequence analysis and alignment revealed no sequence difference between DMS100 and Quantum for the *fad31* sequence (data not shown). However, sequence alignment revealed a single nucleotide mutation, G to A, which consistently occurred in all six DMS100 clones. A comparison of the *fad32* sequence with the *B. rapa* (Tanhuanpää 2002) and *Arabidopsis fad3* (Nishiuchi et al. 1994) gene sequences, especially at the conserved exon/intron splice site junctions, AG/GTAAG, (Lorkovic et al. 2000), revealed that the G to A mutation was in the first 5′ splice site base of the third intron in the *fad32* sequence (Fig. 3). This intron corresponds to the intron six of the *fad3* gene *in B. rapa* (Tanhuanpää 2002) *and Arabidopsis* (Nishiuchi et al. 1994). The *fad3* genes of *B. rapa* and *Arabidopsis* each contains eight exons and seven introns. Our sequence covers exons 4 (partial), 5, 6 and 7 (partial) and introns 4, 5 and 6. Our exon/intron interpretation was supported by the fact that the deduced exon sequences of *fad32* in our study formed an open reading frame that encoded an amino acid sequence highly homologous to the amino acid sequences of the *B. rapa* and *Arabidopsis fad3* genes. Although the cDNA sequence of *B. napus fad3* was not cloned to confirm our exon/intron interpretation in this study, it is well known that the *fad3* gene sequences are highly conserved among *Brassica* species and *Arabidopsis*.

## Mutant allele-specific markers for *fad2* and *fad3* genes

The single nucleotide mutations present in the *fad2* and *fad32* genes were used to develop allele-specific

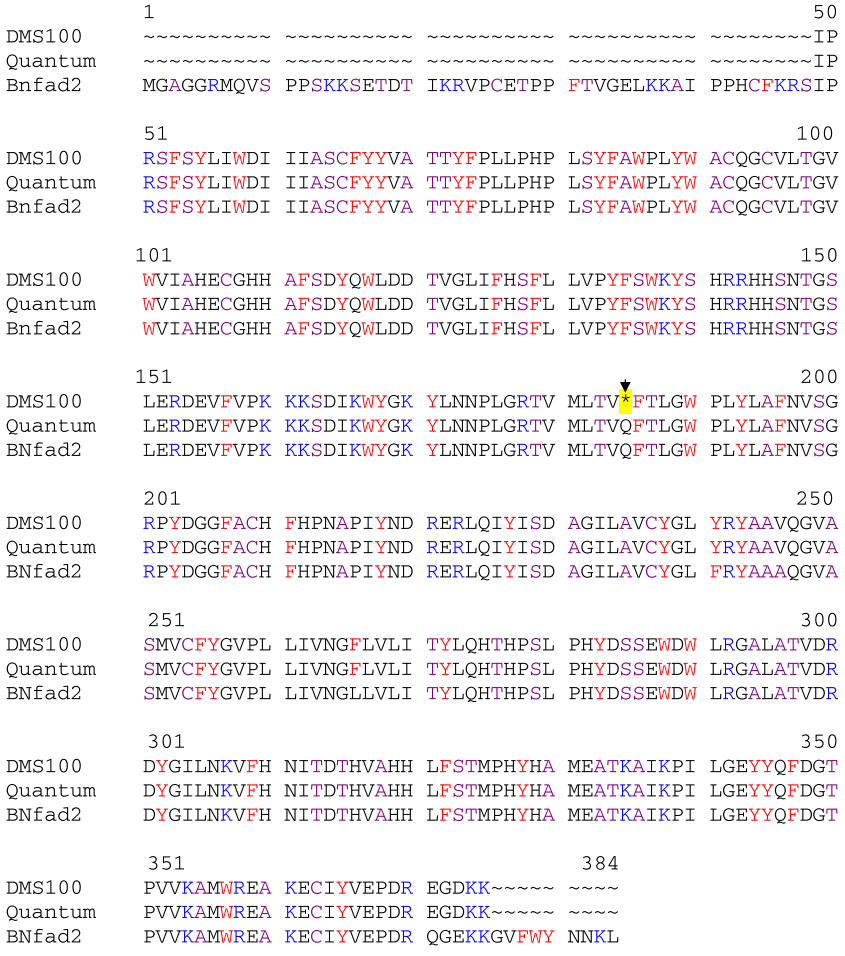


**Fig. 2** Comparison of the partial amino acid sequences of the *B. napus fad2* gene, cloned from DMS100, Quantum and from published *B. napus fad2* gene (BNfad2) (GenBank Accession AF243045). *Arrowhead* indicates the position of the stop codon resulting from a single nucleotide mutation (C to T) in DMS100

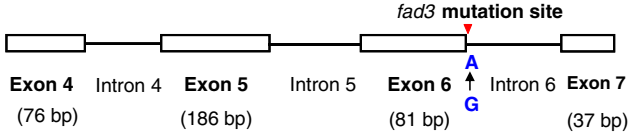


**Fig. 3** Schematic representation of partial genomic nucleotide sequences of *B. napus fad3c* gene cloned from DMS100 and Quantum. *Arrowhead* indicates a single nucleotide mutation of G to A at the first nucleotide of intron 6

markers to tag the *fad2* and *fad32* genes. The mutant allele-specific primer for each mutation of the *fad2* and *fad32* genes were designed such that the mutated base was at the 3′ end of one of the primers for allele-specific PCR amplification as shown below. The boxed bases, T, at the 3′ ends of the FAD2GM forward primer and FAD3GM reverse primer are the mutated bases in the *fad2* and *fad32* genes, respectively .

| | | |
|---|---|---|
| FAD2GM | Forward primer: | CGCACCGTGATGTTAACGGTT**T** |
| | Reverse primer: | ACAGGTGATGCGCCACGTGCGT |
| FAD3GM | Forward primer: | CAAGAATTTGTCCCACAGTACAC |
| | Reverse primer: | ATAAATAATGTTGATCTACTTA**T** |

The primers specific to the *fad2* mutant allele amplified a polymorphic band that was present in DMS100 and DNA bulks for high C18:1 but absent in Quantum and the DNA bulks for low C18:1 (Fig. 4). We tested this mutant allele-specific marker on the DH population derived from the cross of Quantum and DMS100 and found that the mutant allele distribution was highly correlated to high C18:1. The mean seed C18:1 content (75.67%) in the 85 DH lines with the presence of the mutant allele was significantly higher than that (64.23%) of 98 DH lines with the presence of the wild-type allele (*P<.001*). The *fad32* mutant allele-specific primers also amplified a polymorphic fragment that was present in DMS100 but absent in Quantum (data not shown). The analysis with the DH population indicated that this allele-specific marker was significantly correlated to low C18:3. The mean seed C18:3 content (2.81%) in the 74 DH lines with the presence of the mutant allele was significantly lower than that (5.42%) of 99 DH lines with the presence of the wild-type allele ($P<0.001$).

## Invader® assay

The allele-specific PCR detected the *fad2* and *fad32* SNPs (Single Nucleotide Polymorphims) for the presence or absence of the mutant allele, i.e., in a dominant manner, but could not detect heterozygous genotypes. In addition, a detection method based on gel electrophoresis of a large number of samples can be tedious and inefficient for high-throughput screening. Therefore, we developed high-throughput Invader® assays through third wave technologies (TWT) to detect SNPs generated from the *fad2* and *fad32* gene mutations. Using Invader® assays, we were able to clearly detect homozygous mutant, homozygous wild-type and heterozygous genotypes (Fig. 5).

## Mapping of genes controlling C18:1 and C18:3 contents

SSR and AFLP markers that were polymorphic between Quantum and DMS 100 were first screened on the six DNA bulks (Table 1). The SSR and AFLP markers that showed polymorphisms between the bulks were then tested on 183 DH lines derived from the cross of Quantum and DMS100 for linkage analysis and mapping. Linkage between the markers and HOLL traits were established by comparing C18:1 and C18:3 means in two groups of the DH lines carrying DMS100 and Quantum alleles. Statistical significance of the differences in the C18:1 and C18:3 means between the two groups was determined by analysis of variance (AVOVA). Molecular mapping was performed by analyzing segregation data of the 183 DH lines with JoinMap V3.0 computer software (Van Ooijen and Voorrips 2001). The markers that were identified to be linked to either C18:1 or C18:3 by ANOVA


**Fig. 4** PCR products amplified from the mutant allele-specific marker for the *fad2* gene. M, 100 bp DNA ladder; *Lane 1*, DMS100; *lane 2*, Quantum; lanes 3–27, DH lines from the cross of Quantum and DMS100. The PCR products were separated by electrophoresis in a 1.5% agarose gel and stained with ethidium bromide

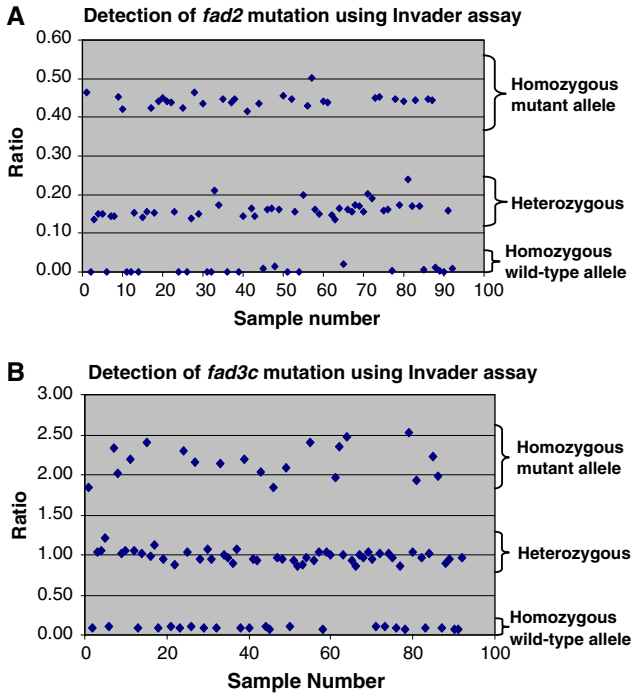


**Fig. 5** Invader® assays of the *fad2* (**a**) and *fad3c* (**b**) single nucleotide mutations. Invader® assays were carried on a segregating population, demonstrating a clear detection of the homozygous mutant alleles, heterozygous and homozygous wild-type alleles for the *fad2* (**a**) and *fad3c* (**b**) genes. Individual genotypes were determined by calculating a ratio of the signal strength from mutant and wild-type alleles

were mapped to four different linkage groups. The markers linked to C18:1 were assigned to two linkage groups (N1 and N5), while the markers linked to C18:3 were assigned to the other two distinct linkage groups (N4 and N14). The *fad2* and *fad32* mutant allele-specific markers were mapped in linkage groups N5 and N14, respectively. These results suggested that, in addition to N5 and N14, linkage groups N1 and N4 could potentially contain other loci for C18:1 and C18:3. Since only a few markers were initially assigned to each of these four linkage groups, additional SSR markers located on linkage groups N1, N4 N5 and N14 were selected and screened on the 183 DH lines for developing a better map. The final map was composed of 12 markers covering 134 cM in linkage group N1, 8 markers covering 58 cM in N5 (Fig. 6), 10 markers covering 85 cM in N4, and 18 markers covering 102 cM in N14 (Fig. 7).

Quantitative trait locus analysis with MapQTL 4.0 (Van Ooijen et al. 2002) was performed to determine the putative locations of the regions (loci) controlling C18:1 and C18:3 content. One major locus (LOD=33.5) was detected for C18:1 (Fig. 6) and was mapped in the linkage group N5 where the functional *fad2* gene was located (Schierholt 2000). It explained 76.3% of the variation in the C18:1 content. The C18:1 QTL was located at the *fad2* gene-specific marker and therefore, was confirmed to correspond to the *fad2* gene. Two other markers, one SNP (H17DM1) and one SSR (BN0801), flanked the *fad2* locus. H17DM1 and BN0801 were 12 and 8.5 cM, respectively on either side of the *fad2* locus. H17DM1 was developed from the SCAR marker H17 (Tanhuanpää et al. 1996), which was monomorphic in our mapping population. In addition, one minor QTL (LOD=3.6) was also identified in the linkage group N1 (Fig. 6), which explained 9.4% of the variation in the C18:1 content.

Two major regions QTLs were associated with the C18:3 content; one in the linkage group N4 and another in the linkage group N14 (Fig. 7). The N4 and N14 correspond to the A and C genomes, respectively (Parkin et al. 1995; Udall et al. 2005). The QTL in the linkage group N4 (LOD=11.4) explained 25.2% of the variation in C18:3 content, while the QTL in the linkage group N14 (LOD=28.0) explained 52.4% of the variation in C18:3 content. The two QTL together explained a total of 77.6% of the variation in C18:3 content. The major QTL on the linkage group N14 was flanked by the SSR marker BN0802 and the SCAR marker D08-1310, which was developed from the RAPD marker D08-1310 (Jourdren et al. 1996b), and was located exactly at the *fad32* allele-specific marker and the SSR marker BN0869A and BN1237 (Fig. 7). The result indicated that our *fad32* sequence belonged to the *fad3c* gene (*fad3* gene in C genome) and this QTL was the *fad3c* locus. The C18:3 QTL on the linkage group N4 was confirmed to be *fad3a* (*fad3* in A genome), because the *fad3a* allele-specific marker developed by Saskatoon Research Center, Agriculture and Agri-Food Canada (SRC-AAFC), was mapped at this QTL in our mapping population (Fig. 7). Two SSR markers (BN0804 and BN0869B) were located closely at this locus.

## Discussion

C18:1 content in *B. napus* is influenced by the *fad2* gene that encodes an enzyme, endoplasmic delta-12 oleate desaturase, responsible for the desaturation of oleic acid (C18:1) into linoleic acid (C18:2). It has been reported that a single nucleotide mutation in the *fad2* gene of *B. rapa* (Tanhuanpää et al. 1998) caused an increase in the C18:1 content. This mutation substituted leucine with proline. By cloning and sequencing the *fad2* genomic fragment from the mutant line DMS100 and wild-type line Quantum of *B. napus*, we

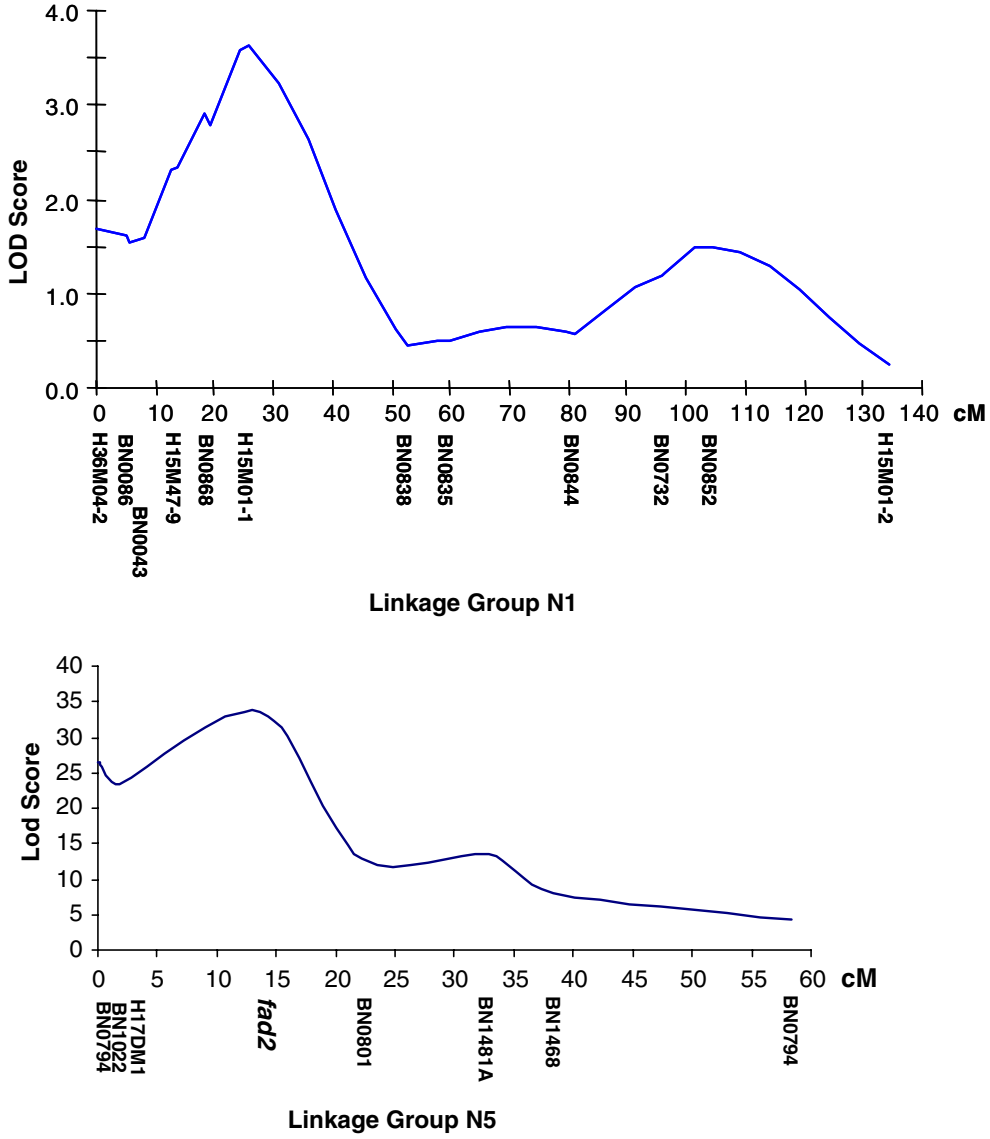


**Fig. 6** QTL likelihood maps for oleic acid content in the DH population from the cross of Quantum and DMS100. The linkage maps were constructed by using JoinMap V3.0 and LOD scores were computed using MapQTL V4.0 computer programs

have identified a single nucleotide mutation, C to T, in the gene. This particular mutation created a stop codon (TAG) leading to premature termination of the peptide chain during translation. As a result, only 185 amino acids were incorporated into the polypeptide instead of all 384 amino acids representing the full-length polypeptide. This could mean that the truncated polypeptide may not function as an active desaturase for the desaturation of C18:1 to C18:2 and therefore, will result in the accumulation of C18:1 in the seeds of the mutant line. Hence, significantly higher C18:1 content (77%) in the mutant line DMS100 compared to the wild-type line Quantum (66%) could be explained due to this mutation. Furthermore, significant correlation of the *fad2* mutant-specific marker to HO content confirmed that the high oleic content in the mutant line DMS100 was caused by this single nucleotide mutation. Genetic and QTL mapping with the DH population indicated that the *fad2* mutant allele-specific marker was located at the location of the major QTL for C18:1 (Fig. 6), supporting the fact that this QTL corresponds to the functional *fad2* gene that is affected by the mutation in DMS100. This is consistent with the previous studies that functional *fad2* gene is located on the linkage group N5 (Schierholt et al. 2000). However, the number of functional *fad2* genes remains unknown (Scheffler et al. 1997; Schierholt et al. 2000). The putative QTL (LOD=3.6) of minor effect identified in the linkage group N1 (Fig. 6) could represent a duplicate copy of the *fad2* gene with minor effects on C18:1

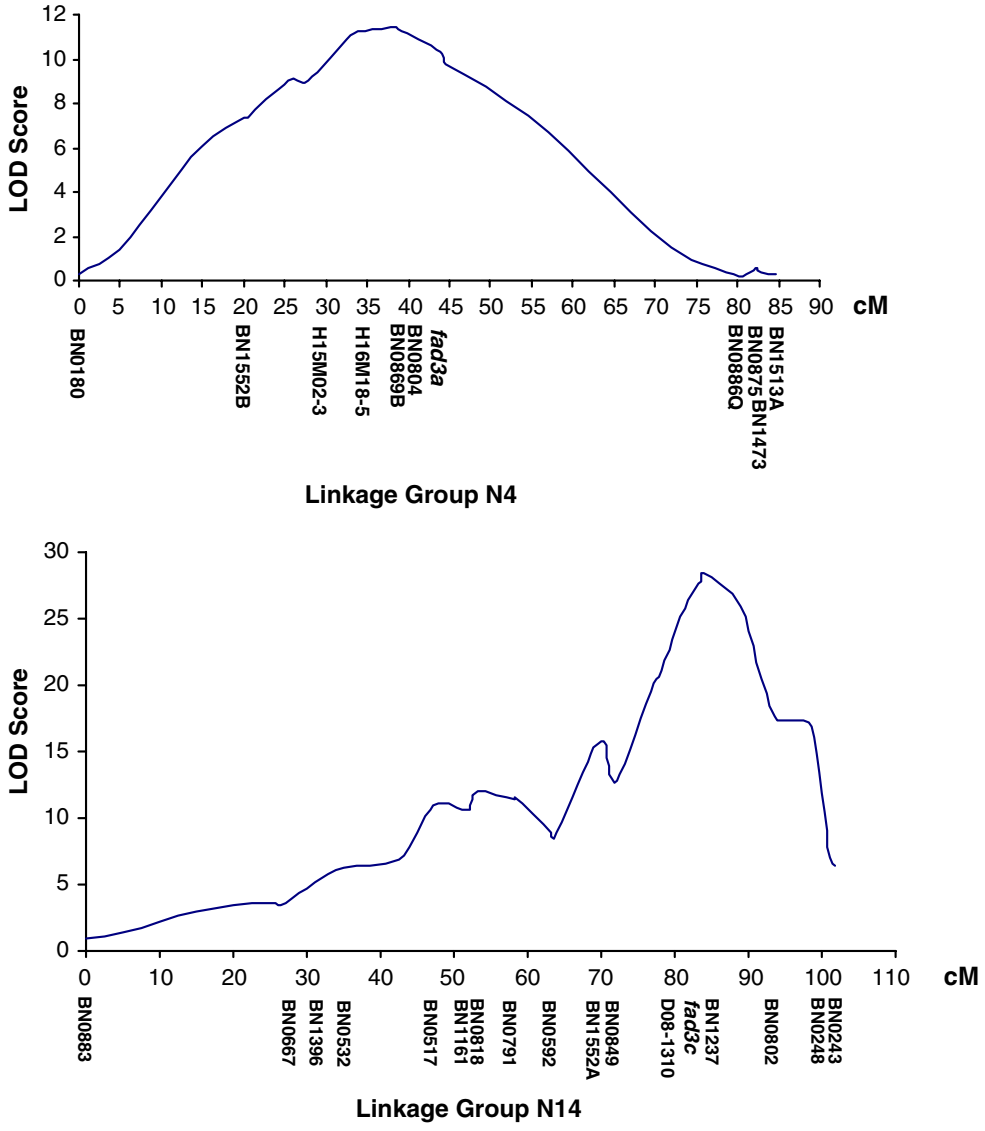


**Fig. 7** QTL likelihood maps for linolenic acid content in the DH population from the cross of Quantum and DMS100. The linkage maps were constructed by using JoinMap V3.0 and LOD scores were computed using MapQTL V4.0 computer programs

content. The duplicate copies of the *fad2* gene were previously identified in the linkage groups N1, N5, N11 and N15 (Scheffler et al. 1997). However, since LOD score of this putative QTL is low, whether it is a true QTL or whether it corresponds to the locus mapped on N1 by Scheffler et al. (1997) remains to be verified.

Plant introns contain highly conserved 5′ splice site (exon/intron junction-AG/GTAAG) and 3′ splice site (intron/exon junction-TGCAG/G). The accuracy of splicing depends on the mechanisms of intron signal recognition and the correct selection of 5′ and 3′ splice sites. The first two nucleotides in the 5′ splice site intron junction sequence,+1G and +2T, have shown 100 and 99% conservation, respectively among over 1,000 *Arabidopsis* introns studied (Lorkovic et al. 2000; Brown 1996). Mutations in any of these bases can abolish normal splicing leading to the accumulation of impaired splicing products. In this study, we identified a single nucleotide mutation of +1G to +1A at the 5′ splice site of the *B. napus fad3c* gene by comparing the wild-type and high oleic mutant allele of the locus. The mutation in our finding could lead to either exon skipping, i.e., the affected exon (exon 6 in this study) (Fig. 3) and both flanking introns are removed in a single splicing event (Lorkovic et al. 2000; Simpson et al. 1998), or block splicing at the normal 5′ splice site and activate cryptic splice sites at different positions (Brown 1996; McCullough et al. 1993). The exon skipping could lead to synthesis of a polypeptide missing the amino acids encoded by the exon 6 of the *fad3c*

gene, while the activation of cryptic splice sites at different positions could cause cryptic splicing of the affected exon together with the downstream intron i.e., intron 6 in this study. Such cryptic splicing could lead early termination of translation and synthesis of a shorter polypeptide for delta-15 linoleate desaturase encoded by *fad3c*. This will occur because the intron contains stop codons in all three possible reading frames and hence, exons 7 and 8 will remain untranslated. The incomplete translation of the *fad3c* can inactivate the enzyme and block the desaturation of linoleic acid (C18:2) to linolenic acid (C18:3), resulting in the decrease of C18:3 accumulation in canola seeds. Evidence for exon skipping and cryptic splicing in plant pre-mRNA splicing has been obtained by characterizing the genes carrying splice site mutations in *Arabidopsis* (Simpson et al. 1998; Brown 1996) and pea (McCullough et al. 1993). Further investigation of the expression of the *fad3c* mRNA is underway to determine whether the mutation causes exon skipping or cryptic splicing. The location of the *fad3c* mutant allele-specific marker matches exactly with the mapped location of one of the major QTL loci for C18:3 on the linkage group N14 (C genome), supporting the conclusion that this QTL is the *fad3*c (*fad3* on C genome) gene that was affected by the second mutation in DMS100. The second major locus responsible for C18:3 content was identified in linkage group N4, which belongs to A genome (Parkin et al. 1995; Udall et al. 2005). This locus was confirmed to be the *fad3a* (*fad3* in A genome) by mapping the *fad3a* allele-specific marker of SRC-AAFC in our population. Two loci were previously identified to control linolenic acid content in *B. napus* (Jourdren et al. 1996a; Thormann et al. 1996). The locus in N4 in our study could correspond to the L1 locus of Jourdren et al. (1996a), which was confirmed later to be the *fad3a* gene of the A genome, derived from *B. rapa* (Jourdren et al. 1996b). The *fad3c* gene mapped in N14 in our study corresponds to the L2 locus of Jourdren et al. (1996a) because the RAPD marker D08-1310 that was mapped close to the L2 locus was also mapped close to the *fad3c* locus in N14 in our study. The L2 locus of Jourdren et al. (1996a) was later shown to correspond to the *fad3c* gene (Barret et al. 1999).

The single nucleotide mutations present in the *fad2* and *fad3c* genes can be used as SNP markers to tag the *fad2* and *fad3c* genes for selection of high C18:1 and low C18:3 in canola trait introgression and breeding. Different alleles can be selectively amplified using mismatch at the 3′ end of the primer (Wu et al. 1989; Tanhuanpää et al. 1998). However, the allele-specific PCR is not suitable for high-throughput genotyping of SNPs because running gel electrophoresis of a large number of PCR products can be tedious and inefficient. The PCR based on a single base mismatch requires stringent conditions and may not be stable. The Invader® technology developed by Third Wave Technologies (Madison, WI) provides a robust tool for high throughput genotyping of SNPs (Lyamichev and Neri 2003; de Arruda et al. 2002). The Invader® assay offers a simple diagnostic platform to detect single nucleotide changes with high specificity and sensitivity from unamplified genomic DNA. Using Invader® assays, we were able to clearly detect the mutant and wild-type alleles, thus allowing specific selection of *fad2* and *fad3c* alleles conferring high C18:1 and low C18:3 contents (Fig. 5) in a large scale. Invader® assay will provide a high throughput tool for direct selection of desirable *fad2* and *fad3c* alleles during marker-assisted trait introgression and breeding.

In conclusion, we mapped one major locus in the linkage group N5 and one minor locus in linkage group N1 for oleic acid content and two major loci for linolenic acid content in the linkage groups N4 and N14. The major locus for oleic acid content is corresponding to the functional copy of the *fad2* gene and the major locus in the linkage group N14 for linolenic acid content is corresponding to the *fad3c* gene and the locus on N4 is *fad3a*. A single nucleotide mutation was identified in each of the *fad2* and *fad3c* genes. Sequence and linkage analyses indicated that these single nucleotide mutations were responsible for the increase of oleic acid content and the decrease of linolenic acid content in the mutant line DMS100. The *fad2* and *fad3c* mutant allele-specific markers for high oleic- and low linolenic acids were successfully developed. Invader® assay has been developed for the two mutant allele specific markers for high throughput screening of high oleic and low linolenic acid content. These markers along with the SSR markers that are tightly linked to high oleic and low linolenic acids will provide very useful tools for marker-assisted selection of the two traits in canola trait introgression and breeding.

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