Cloning of *Fad2* and *Fad3* Genes and Development of Gene-Specific Markers for High Oleic and Low Linolenic Acids in Canola (*Brassica napus* L.)

Xueyi Hu[1], Mandy L. Sullivan[1], Manju Gupta[1] and Steven A. Thompson[1]
[1]Dow AgroSciences *LLC*, 9330 Zionsville Road, Indianapolis, IN 46268

ABSTRACT

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 thermostability and nutritional value of oil. Thus, development of canola cultivars with increased oleic acid and reduced linolenic acid is highly desirable for canola oil production. Chemical mutagenesis with ethyl methanesulphonate (EMS) has been successfully used to induce fatty acid mutations in rapeseed and other oilseed crops. In this study, we have sequenced genomic clones of fatty acid desaturase-2 (*fad2*) and fatty acid desaturase-3 (*fad3*) genes amplified from an EMS-induced mutant (>70% oleic acid and <3% linolenic acid) and a wild-type canola cultivars. A comparison of the mutant and wild type allele sequences of the *fad2* and *fad3* genes revealed single nucleotide mutations in each of the genes. Detailed sequence analyses have 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 *fad2* and *fad3* gene mutations, were developed. These markers will be highly useful for direct selection of desirable *fad2* and *fad3* alleles during marker-assisted trait introgression and breeding.

Key words: *Brassica napus*, *fad2*, *fad3*, oleic acid, linolenic acid, fatty acid desaturase

INTRODUCTION

The quality of canola oil is determined by its constituent fatty acids. Reduced levels of the polyunsaturated fatty acids, such as linolenic acid (C18:3), and increased levels of the monounsaturated oleic acid (C18:1) are associated with higher oxidative stability. Substantial efforts have been made by both private and public breeding programs to develop canola varieties with high C18:1 and low C18:3 content. High C18:1 and low C18:3 mutants have been produced through mutagenesis (Rakow, 1973; Auld et al. 1992; Lanuza and Sernyk, 2001).

Marker-assisted selection can be an important tool for selection of genes controlling the fatty acid contents, and thereby leading to genetic improvement of canola oil quality. In this study, we have sequenced genomic clones of fatty acid desaturase-2 (*fad2*) and desaturase-3 (*fad3*) genes from the wild-type and HOLL (High Oleic and Low Linolenic) canola lines and discovered single nucleotide mutations in each of the genes. Two single nucleotide polymorphism (SNP) markers corresponding to the *fad2* and *fad3* gene mutations were developed, and they proved to be very useful for marker-assisted selection of HOLL traits.

MATERIALS AND METHODS

Plant Material: Canola line DMS100 (mutant type) and variety Quantum (wild-type) were used in this study for cloning of *fad2* and *fad3* alleles. DMS100 is a Dow AgroSciences (DAS) proprietary canola line containing seed oil with ~ 77% oleic acid and ~3% linolenic acid. It was derived from AG019, subject to a US patent (Lanuza and Sernyk, 2001). The altered fatty acid profile of AG019 traces back to sources derived from EMS mutagenesis. Quantum is a commercial variety and contains low oleic acid (~66%) and high linolenic acid (~7%) contents. A double haploid (DH) mapping population consisting of 183 DH lines derived from the cross of Quantum and DMS100 was kindly provided by Dr. Steve Webb, DAS, Canada and was used in this study.

PCR amplification and cloning of *fad2* and *fad3* alleles: DNA of both parental lines and 183 DH lines was extracted from the leaves of 2-week-old greenhouse-grown plants using Qiagen

DNeasy 96 Plant Test Kit. PCR amplification reactions contained 20-30 ng of genomic DNA, 0.25 μM 10-mer primer, 2.5 mM $MgCl_2$, 0.2mM of each dNTP, 1X PCR buffer and 0.6 unit of *Taq* DNA polymerase. Amplifications were performed in a GeneAmp PCR System 9700 programmed for 35 cycles of 45 sec at 94 ^{0}C, 30 sec at 55-60 ^{0}C, 1 min at 72 ^{0}C and ending with 7 min at 72 ^{0}C. The *fad2* and *fad3* fragments amplified from each of the two lines were cloned using a TA-cloning kit (Invitrogen Corp., San Diego, California) per manufacturer's instructions. The positive clones containing the insert were subsequently sequenced by Sequetech Corporation (Mountain View, California).

Invader assay: Invader Assay kits specific to *fad2* and *fad3* gene mutations were developed through Third Wave Technologies (Madison, Wisconsin). Invader Assay was done in 96-well plates per manufacturer's instruction.

Sequence and data analyses: The sequences were analyzed and aligned by using a web-based, SeqWeb (version 2) sequence analysis software, present in GCG software package (University of Wisconsin). The genetic linkage map was generated with JoinMap V2.0 computer software (Stam and Van Ooijen, 1995). Putative QTL regions associated with C18:1 and C18:3 were located by an interval mapping MapQTL V3.0 software (Van Ooijen and Maliepaard, 1996).

RESULTS AND DISCUSSION

Mutations in *fad2* and *fad3* genes

The C18:1 content in canola is influenced by the level of endoplasmic delta-12 oleate desaturase enzyme, responsible for the desaturation of oleic acid (C18;1) to linoleic acid (C18:2). The *fad2* gene encodes this enzyme (Ohirogge and Browse, 1995). The genomic fragments corresponding to the *fad2* gene were amplified from the mutant line DMS100 and wild-type line Quantum using the primers based on the published *B. rapa fad2* gene sequence (Tanhuanpää et al. 1998). The amplified sequences were then cloned followed by sequencing of the 9 DMS100 clones and 10 Quantum clones. The sequence analysis and alignment of these clones identified a single nucleotide mutation, C to T, that consistently occurred in the *fad2* gene sequences of all DMS100 clones but not Quantum clones. Further analysis indicated that this single nucleotide mutation occurred in the coding sequence (exon) of the *fad2* gene. The mutation of the C to T created a stop codon that will cause early termination of the polypeptide chain during translation. This could mean that the truncated polypeptide may not function as an active desaturase for the desaturation of oleic acid to linoleic acid, and therefore, will result in the accumulation of oleic acids in the seeds of the mutant line. This explains why the mutant line DMS100 has significantly higher C18:1 content (77%) than the wild type line Quantum (66%).

The *fad3* gene encodes for endoplasmic delta-15 linoleate desaturase which is responsible for the desaturation of linoleic acid (C18:2) to linolenic acid (C18:3). Genomic DNA fragments corresponding to the *fad3* gene were amplified from DMS100 and Quantum using the primers based on the published *B. napus fad32* gene sequence (Brunel et al. 1999, GenBank Accession AF056570). The amplified fragments were cloned and then sequenced. Six DMS100 clones and 6 Quantum clones were sequenced. The sequence alignment revealed a single nucleotide mutation, G to A, at the first base of 5' splice site of the third intron.

Plant introns contain highly conserved 5' splice site (exon/intron junction- AG/GTAAG) and 3' splice site (intron/exon junction - TGCAG/G). The first two nucleotides in the 5' splice site intron junction sequence, +1G and +2T, have shown 100% and 99% conservation respectively among over 1000 *Arabidopsis* introns studied (Brown, 1996; Lorkovic, 2000). The accuracy of splicing depends on the mechanisms of intron signal recognition and the correct selection of 5' and 3' splice sites. The mutation of +1G to +1A at the 5' splice site in our finding can either abolish splicing or lead to exon skipping, i.e., splicing of the affected exon together with the downstream intron (intron 6). Such exon skipping could lead to early termination of translation and synthesis of a shorter polypeptide for *fad3*. This will occur because the intron contains stop codons in all 3 possible reading frames and hence, exons downstream the mutation site will remain untranslated. The incomplete translation of the *fad3* gene 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.

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

The single nucleotide mutations present in the *fad2* and *fad3* genes can be used as SNP markers for selection of high C18:1 and low C18:3 in canola breeding. We have developed two such allele-specific PCR-based markers, FAD2GM and FAD3cGM. The primers specific to *fad2* amplified a polymorphic band that was present in DMS100 but absent in Quantum (Figure 1). We tested this gene-specific marker on a DH population derived from the cross of Quantum and DMS100 and found that the allele distribution was highly correlated to high C18:1 (Table 1). The *fad3* 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 tightly linked to low C18:3 (Table 1).

Table 1. The correlation of the mutant allele-specific markers and fatty acid content of 184 DH lines derived from the cross of Quantum and DMS100

Marker	Trait	No. of Lines tested		Average fatty acid content		*t*-value
		Presence of marker	Absence of marker	Presence of marker	Absence of marker	
FAD2GM	C18:1	85	98	75.67	64.23	15.49**
FAD3cGM	C18:3	74	99	2.81	5.42	13.13**

****** significant at $t = 0.01$.

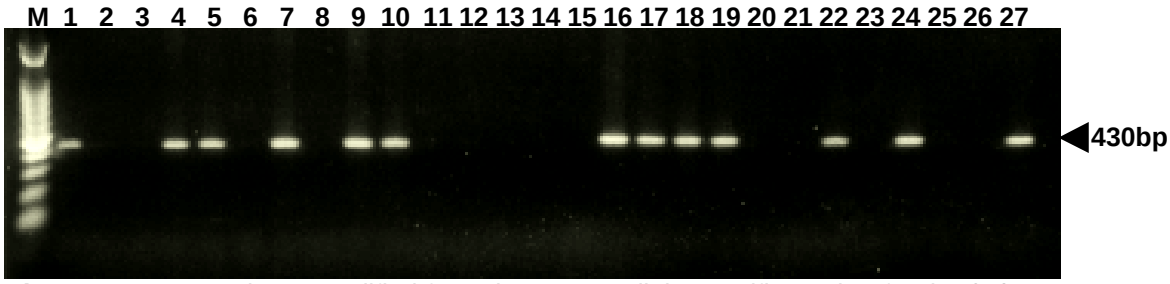

Figure 1. PCR products amplified from the mutant allele-specific marker for the *fad2* gene. M, 100 bp DNA ladder; Lane1, DMS100; lane 2, Quantum; lane 3-27, DH lines from the cross of Quantum and DMS100. The PCR products were separated by electrophoresis in a 1.5% agarose gel and stained with ethidium bromide.

Genetic and QTL mapping with the DH population indicated that the *fad2* mutant allele-specific marker was located exactly at the location of a major QTL for C18:1 on the linkage group N5. It supported 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 *fad2* gene is located on the linkage group N5 (Schierholt, 2000). The location of the *fad3* 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). It supported the conclusion that this QTL is the *fad3*c (*fad3* on C genome) gene.

The Invader technology, developed by Third Wave Technologies (TWT), provides a robust tool for high throughput genotyping of SNPs. We have developed Invader assays through TWT to detect SNPs generated from the *fad2* and *fad3* gene mutations. Using Invader assays, we were able to clearly detect the mutant and wild type alleles, thus allowing specific selection of *fad2* and *fad3* alleles conferring high C18:1 and low C18:3 content (Figure 2). The two mutant allele-specific markers in combination with Invader assay will provide a high throughput tool for direct selection of desirable *fad2* and *fad3* alleles during marker-assisted trait introgression and breeding.

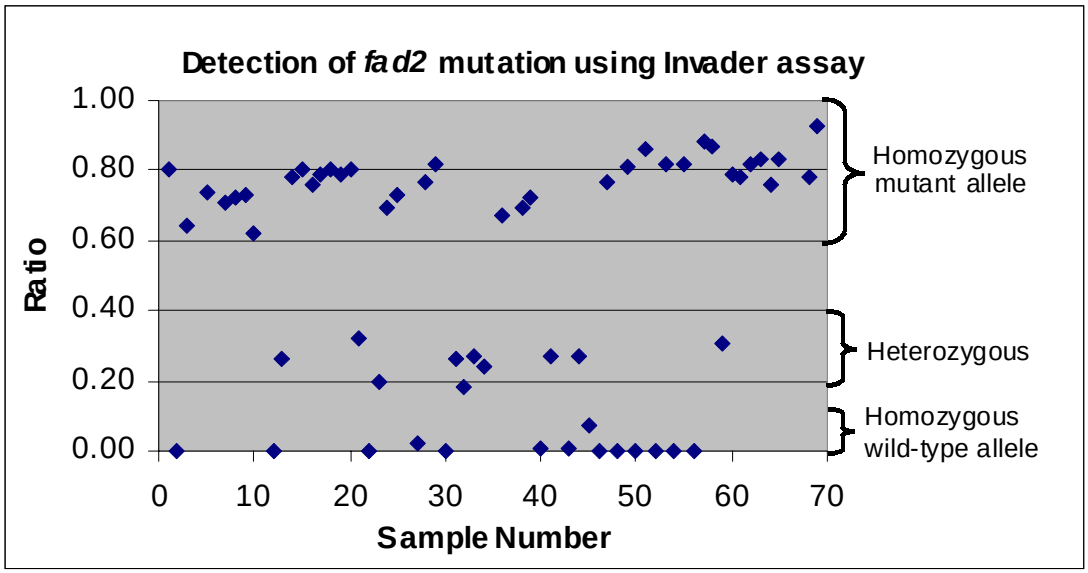

Figure 2. Allele-specific *fad2* Invader assays were carried on a segregating population, demonstrating a clear detection of the homozygous mutant allele, heterozygous and homozygous wild-type allele for the *fad2* gene. Individual genotypes were determined by calculating a ratio of the signal strength from mutant and wild type alleles.

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