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**Short Communication**

# G-to-A mutation at a 5′ splice site of *fad3c* caused impaired splicing in a low linolenic mutant of canola (*Brassica napus* L.)

Xueyi Hu[1,*], Mandy Sullivan-Gilbert[1], Manju Gupta[1], Steven A. Thompson[1]

[1] Dow AgroSciences, LLC, 9330 Zionsville Road, Indianapolis, IN 46268-1054, USA
* E-mail: xhu@dow.com Tel: +1-317-337-3460 Fax: +1-317-337-5989

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**Abstract** Two genes (*fad3a and fad3c*) encode for endoplasmic delta-15 linoleate desaturase, which is responsible for the desaturation of linoleic acid (C18:2) into linolenic acid (C18:3) in canola (*Brassica napus*). The canola mutant line DMS100 carries a G-to-A base substitution at the 5′ splice site, located at +1 G, of *fad3c* intron 6 and contains reduced C18:3 content in oil seeds. Reverse transcription (RT) PCR analysis was used to amplify part of the *fad3c* transcript spanning intron 5, exon 6 and intron 6 using the primers specific to the exon 5 and 7 to investigate whether the mutation caused any abnormal splicing. The RT-PCR amplified a fragment of larger size in the *fad3c* mutant than in wild-type. Sequencing of the RT-PCR fragments revealed that the mutation activated an impaired splicing which retained the entire intron 6 in the mature *fad3c* transcript of the mutant. The impaired splicing could result in early termination of translation and synthesis of a shorter *fad3c* polypeptide because the intron 6 contained stop codons in all three possible reading frames. The incomplete translation could produce an inactive enzyme which blocked the desaturation of linoleic acid (C18:2) to linolenic acid (C18:3), resulting in the decrease of C18:3 accumulation in canola seeds. This is consistent with the observation that the *fad3c* mutant has significantly lower C18:3 content (<3%) than the wild type (~7%).

**Key words:** *Brassica napus*, *fad3c*, pre-mRNA splicing.

The plant *fad3* (*F*atty *A*cid *D*esaturase 3) gene encodes an endoplasmic delta-15 linoleate desaturase which is responsible for the desaturation of linoleic acid (C18:2) to linolenic acid (C18:3). Two *fad3* genes (*fad3a* and *fad3c*) were reported to control linolenic content in *Brassica napus*, with *fad3a* located on linkage group N4 and *fad3c* on N14 (Barret et al. 1999; Brunel et al. 1999; Hu et al. 2006). The high level of linolenic acid in canola oil is undesirable because linolenic acid is highly unsaturated and easily oxidized to cause off-flavor and rancidity of the oil, thus resulting in a shortened shelf life. Breeding canola varieties with low linolenic acid in oilseeds is one of major objectives for many canola breeding programs. Low C18:3 mutants have been produced through mutagenesis (Rakow 1973; Auld et al. 1992). The development of canola varieties with fatty acid profile of C18:1 above 70% and C18:3 below 3.0% in oilseeds, for example, Natreon varieties, is a major objective of the canola breeding program in Dow AgroSciences. A single nucleotide mutation of +1G to +1A at the 5′ splice site of intron 6 of the *B. napus fad3c* gene was previously identified by comparing the wild-type and high oleic mutant allele of the locus (Hu et al. 2006). This mutant was developed through ethyl methanesulphonate (EMS) mutagenesis and contains reduced C18:3 content in oil seed. An allele-specific PCR marker diagnostic to the mutation was developed. Linkage analysis with 183 doubled haploid lines derived from the cross of Quantum and DMS100 indicated that this allele-specific marker was significantly correlated to low C18:3. A high-throughput Invader® assay detecting the SNP generated from the *fad3c* gene mutation clearly differentiated the homozygous mutant, homozygous wild-type and heterozygous genotypes, thus allowing specific selection of the *fad3c* alleles conferring low C18:3 content (Hu et al. 2006).

The molecular characterization of mutations in splicing site sequences in *Arabidopsis* provides valuable information on plant pre-mRNA splicing. As in other organisms, plant genes 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 (Lorkovic et al. 2000; Brown 1996). The accuracy of splicing depends on the mechanisms of intron signal recognition and the correct selection of 5′ and 3′ splice sites. Mutations in splice sites can abolish splicing or lead to exon skipping, i.e., the affected exon and both flanking introns are removed in a single splicing event (Lorkovic et al. 2000; Simpson et al.

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Figure 1. Partial genomic nucleotide sequences of the exon 6 and inton 6 of *B. napus fad3c* gene cloned from DMS100 and Quantum, indicating a single nucleotide mutation, G to A, at 5′ splicing site of the intron 6 in the mutant line DMS100.

Table 1. Primers for RT-PCR analysis

| Primer name | Orientation | Length (bp) | Primer sequence |
|---|---|---|---|
| FD3C-34 | Forward | 26 | CAGTCACAGTTCTAAAAGTCTATGGT |
| FD3C-55 | Forward | 22 | ATCATTCCTCGTTGGTCCAGTC |
| FD3C-76 | Forward | 22 | TGTAATGTGGTTGGACGCTGTC |
| FD3C-84 | Forward | 22 | TCGATCGTGTTGGCCACTCTTG |
| FD3C-90 | Forward | 22 | TCAACTACTTGCTGGTCGATCG |
| FD-3C-106 | Forward | 22 | TGCAACTTCAACTACTTGCTGG |
| BNFD32-CR | Reverse | 22 | CAACTGTTGTTAATCCTCCACG |

1998). The mutation in splice sites could also block splicing at the normal splice site and activate cryptic splice sites at different positions, which can cause cryptic splicing of the affected exon together with the downstream intron (McCullough et al. 1993). It has been shown that a G-to-A base substitution at the 5′ splice site +1 G nucleotide of intron 1 of *PHYB* (phytochrome B) caused impaired splicing which retained the entire intron in the mature *PHYB* transcript of the mutant *phyB-103* in *Arabidopsis* (Bradley et al. 1995).

To investigate whether the G-to-A base substitution located at +1 G of the 5′ splice site of *fad3c* intron 6 in our finding (Figure 1) caused exon skipping, cryptic splicing or impaired splicing, we implemented reverse transcription (RT) PCR analysis of the total RNA isolated from the developing seeds of the mutant and wild-type lines using the primers (Table 1) homologous to the exon 5 and 7. Seeds of DMS100 (~77% oleic acid and ~3% linolenic acid) and Quantum (~66% oleic acid and ~7% linolenic acid) were planted in 10 inch plastic pots containing peat soil. Five plants (one plant per pot) of each line were grown in a greenhouse under natural greenhouse lighting supplemented with high pressure sodium lamps with a 16 h photoperiod at a temperature ranged from 15.8°C (night) to 25.2°C (day). Individual flowers were tagged for flowering dates in order to track the development stage of the seeds. Seed samples at five developing stages, 20, 25, 29, 30, 35 and 40 days after flowering (DAF), were collected from both DMS100 and Quantum and were immediately frozen in liquid nitrogen and stored at −80°C for RNA extraction. Total RNA was isolated from the developing seeds of DMS100 and Quantum at 20, 25, 29, 35 and 40 DAF using RioboPure™ RNA isolation kit (Ambion Inc., Austin, Texas). Four primers (FD3C-55, FD3C-76, FD3C-84 and FD3C-106) were first designed to be paired with the reverse primer BNFD32-CR for RT-PCR analysis. However, although these primers amplified polymorphic bands between DMS100 and Quantum, they all amplified multiple fragments when they were paired with the reverse primer BNFD32-CR, suggesting they are not *fad3c* specific (data not shown). Two new primers (FD3C-34 and FD3C-90) were then designed to pair with the reverse primer BNFD32-CR, and first tested on the genomic DNA of *B. napus* lines DMS100, Quantum, Nex710 and a *B. rapa* line, Reward, to make sure the primers were functional and could amplify the expected fragments before they were used for RT-PCR. The primer FD3C-34 when paired with the reverse primer BNFD32-CR amplified a single fragment of the expected size from all four line genomic DNA as visualized on a 4% agarose gel. However, FD3C-90 when paired with BNFD32CR amplified a band only from DMS100, Quantum and Nex710 but not from Reward at an annealing temperature of 60°C (data not shown). Since Reward is a *B. rapa* line and contains only the A genome, the result suggested that the primer FD3C-90 should be specific to *fad3c*. Therefore, the primer FD3C-90 was selected to be paired with BNFD32-CR for RT-PCR analysis. The RT-PCR was conducted on total RNA isolated from the developing seeds of Quantum and DMS100 at 20, 25 and 29 DAF using SuperScript™ One-Step RT-PCR System (Invitrogen Corp., San Diego, California) per manufacturer's instructions. In brief, each RT-PCR reaction, in a total volume of 50 μL, contained 500 ng of total RNA, 1X reaction mix containing 0.2 mM of each dNTP and 1.2 mM $MgSO_4$, 0.2 μM of each forward and reverse primer, 0.6% polyvinylpyrrolidone (PVP), and 1.0 μL of SuperScript™ II RT/Platinum® *Taq* mix (RT/Platinum® *Taq* mix), which contained a mix of Transcriptase SuperScript™ II Reverse Transcriptase and

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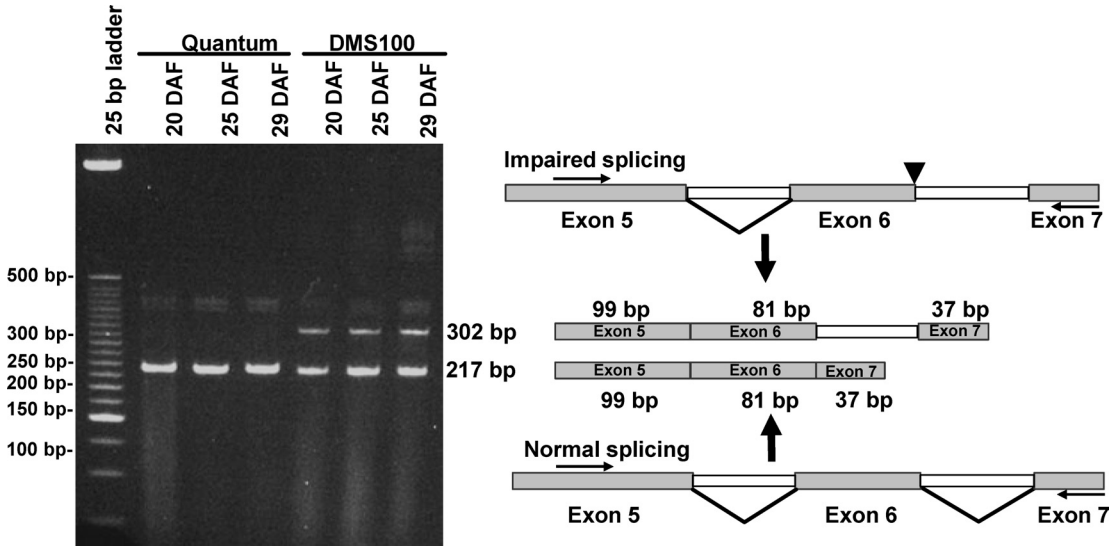


Figure 2. RT-PCR splicing analysis of the *fad3c* mutation and schematic representation of normal splicing and impaired splicing that was caused by the G to A mutation at the 5′ splicing site of the intron 6 of the *fad3c gene*. Exons are shown as filled blocks and introns as unfilled blocks. The arrow head indicates the mutation site. Arrows above and below exons 5 and 7 show the positions of the forward (FD3C-90) and reverse (BNFD32-CR) primers used for RT-PCR analysis, respectively. The RT-PCR products were separated in a 6% acrylamide gel and stained with ethidium bromide.

Platinum® *Taq* DNA polymerase. Reactions were incubated at 55°C for 30 min for cDNA synthesis, followed by pre-denaturation at 94°C for 2 min and PCR amplification for 30 cycles of 30 s at 94°C, 30 s at 60°C, 30 s at 72°C and ending with 7 min at 72°C. Amplifications were performed in a GeneAmp PCR System 9700 (PE Applied Biosystems, Foster City, California). RT-PCR failed without PVP and was significantly improved by adding 0.6% PVP in the reactions. RT-PCR with the primer pair FD3C-90 and BNFD32-CR amplified a single fragment of expected size (217 bp) in the wild-type line but two fragments (217 and 302 bp) in the mutant line (Figure 2). The upper band (302 bp) in the mutant might represent the fragment produced by abnormal splicing which could have retained either partial or entire intron 6 in the mature *fad3c* transcript. The lower band in the mutant could correspond to the normal splicing product of *fad3a*. Although the primer FD3C-90 used for the RT-PCR analysis were designed to be specific to *fad3c* and demonstrated to amplify only *fad3c* at a high stringent annealing temperature (60°C) when paired with BNFD32CR, it could amplify a non-specific fragment due to the mismatch at a lower annealing temperature since only one base at the 3′ end of the primer differentiated *fad3c* from *fad3a* at the primer annealing site. Non-specific amplification was observed in the *B. rapa* line Reward (A genome) when the annealing temperature was set at 58°C (data not shown). Since RT-PCR was incubated at 55°C for 30 min for cDNA synthesis, the mismatch could occur during the cDNA synthesis. We cloned and sequenced the lower bands of the mutant and found that the fragment contained only exon sequences which corresponded to the normal splicing products of *fad3a*.

To confirm whether the upper bands represent the *fad3c* mRNA fragments produced by abnormal splicing and contain partial or entire intron 6, we isolated the upper bands of DMS100 (mutant) from the gel and re-amplified, followed by cloning the PCR products using TOPO TA cloning kit. A total of 15 clones representing the developing seed stages at 20, 25 and 29 DAF were selected and sent to Lark Technologies (Huston, Texas) for sequencing. Sequencing of the upper bands from the mutant revealed that the entire intron 6 was retained in the *fad3c* transcript (Figure 3), indicating that the single nucleotide G-to-A mutation at 5′ splicing site of the intron 6 caused impaired splicing of the *fad3c* transcript. This impaired splicing could lead to incomplete translation of *fad3c* because the intron 6 contains stop codons in all 3 possible reading frames. The predicted protein of the mutant consists of the product of the first 6 exons of *fad3c*, followed by a region composed of 13 novel amino acid residues from intron 6 sequence. The amino acid residues encoded by exon 7 and 8 were missing. The incomplete translation of the *fad3c* could lead to an inactive enzyme and block the desaturation of C18:2 to C18:3, resulting in decreased C18:3 accumulation in canola seeds. This is consistent with the observation that the *fad3c* mutant has significantly lower C18:3 content (<3%) than the wild type (~7%) and also confirms the previous linkage analysis result that the mutation could cause altered fatty acid content (Hu et al. 2006). In conclusion, the single nucleotide mutation at

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Figure 3. Consensus sequence of the *fad3c* RT-PCR clones of DMS100. The RT-PCR was performed on the total RNA isolated from the developing seeds at 20, 25 and 29 DAF, by using primers FD3C-90 and BNFD32-CR. The asterisk indicates the junction of exon 5 and 6 and the shaded sequences represent intron 6. The positions and sequences of the primers FD3C-90 and BNFD32-CR are underlined.

the 5′ splicing site of the *fad3c* gene caused impaired splicing, leading to a defective enzyme and blocking the desaturation of C18:2 to C18:3, resulting in decreased C18:3 accumulation in the canola seeds.

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