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ORIGINAL PAPER

Enhanced Oleic Acid Content in the Soybean Mutant M23 Is Associated with the Deletion in the *Fad2-1a* Gene Encoding a Fatty Acid Desaturase

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Abstract Oleic acid is one of the three major unsaturated fatty acids in soybean oil. Expression of the embryo-specific *Fad2-1* gene correlates with fatty acid biosynthesis and oil deposition in developing seeds. Elevated levels of oleate in the X-ray-induced mutant line M23 have been shown to be associated with the deletion of a *Fad2-1* gene. In soybean, two homologs of the *Fad2-1* gene, termed *Fad2-1a* (L43920) and *Fad2-1b* (AY611472), presumably encode functional embryo-specific fatty acid desaturase. The objectives of this investigation were to determine which copy of the *Fad2-1* gene is associated with the increased oleate content in the mutant line and what is the relative transcript abundance of these two embryo-specific genes. PCR and DNA blot analyses showed that increased oleate content in M23 mutant was associated with the deletion of *Fad2-1a*. These results were further validated using five independent soybean populations developed by crossing mid-oleate and normal-oleate parents. Investigation of the soybean expressed sequence tag database and reverse transcription PCR analyses revealed that *Fad2-1b* is the predominantly transcribed copy of the *Fad2-1* gene. We hypothesize that null mutation in *Fad2-1b* in the *fad2-1a* mutant background should further elevate the oleic contents of soybean oil.

Keywords *Fad2-1* · M23 · Oleic acid · Oil · Soybean

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Introduction

Soybean is one of the most important oilseed crops in the world. The quality of soybean oil depends on its fatty acid composition. Polyunsaturated fatty acids in soybean seed reduce the stability of oil and lead to off-flavors. Chemical hydrogenation improves oxidative stability, but results in the formation of *trans* fatty acids that are undesirable for cardiovascular health. Oleic acid is one of the most stable of the three unsaturated fatty acids in soybean oil [1].

In Arabidopsis, a single gene, fatty acid desaturase-2 (*Fad2*), metabolizes oleic into polyunsaturated fatty acids such as linoleic acid and α-linolenic acid [2]. In soybean, two genes, *Fad2-1* and *Fad2-2*, encode ω-6 fatty acid desaturase. *Fad2-1* is embryo-specific, whereas *Fad2-2* is a constitutively expressed gene [3]. They share 73% identity at the amino acid level, suggesting that *Fad2-1* and *Fad2-2* are very closely related and functionally redundant genes. They were subfunctionalized; one for embryo or cotyledon and the other, most likely, for all organs [3]. The soybean gene *Fad2-2* showed higher sequence identity than *Fad2-1* to the Arabidopsis *Fad2* gene; therefore, soybean *Fad2-2* most likely is the ortholog of Arabidopsis *Fad2*. In complementation assays, however, both soybean genes complemented the Arabidopsis *fad2* mutation [3].

In soybean, variation in linolenic acid content has been correlated with the deletion of the *ω-3 desaturase* gene [4]. Deletion of *Fad2-1* in the X-ray-induced mutant M23 resulted in the elevation of oleic acid contents [5–7]. A recent

study revealed that there are two copies of the *Fad2-1* gene [8]. These two copies were designated as *Fad2-1a* (L43920) and *Fad2-1b* (AY611472) [3, 8, 9]. Complementary DNAs (cDNAs) for these two genes show 94% nucleic acid identity. The objectives of this study were to determine (1) which copy of the *Fad2-1* gene is associated with increased oleic acid content in the M23 mutant line and (2) which transcripts of which copy of the gene are most abundant. Our results suggest that the steady-state transcript level of *Fad2-1b* is fourfold greater than that of *Fad2-1a* and, therefore, mutation of *Fad2-1b* in M23 mutant should further increase the oleic acid contents of soybean oil.

Experimental Procedures

Materials Used

An F_2 population was developed from a cross between the mutant line M23 and the cultivar Archer [5]. DNA was isolated from leaves of individual F_2 plants. The F_2 plants were harvested individually. Eleven individual F_3 seeds from each of 69 F_2 plants were analyzed for fatty ester content [10].

Five segregating populations were developed at Iowa State University by crossing mid-oleate, 1% linolenate lines related to M23 to normal-oleate, 1% linolenate lines. The mid-oleate, 1% linolenate parents were the F_3-derived lines AX18895-6, AX18896-2, AX18894-1, and AX18894-6. AX18894-1 and AX18894-6 are experimental lines from the cross IA2064 × AX18434. AX18895-6 is an experimental line from the cross IA3017 × AX18434. AX18896-2 is an experimental line from the cross A15647B039 × AX18434. AX18434 is the population from the cross A97-553017 × M23. A15647B039 and A97-553017 are normal-oleate, 1% linolenate experimental lines. IA2064, IA2073, IA3017, and IA3024 are normal-oleate, 1% linolenate cultivars.

The parents were crossed in July 2004 at the Agricultural and Agronomy Research Center near Ames, IA, USA. The cross IA3024 × AX18895-6 was designated AX19716, IA3024 × AX18896-2 was designated AX19717, IA2073 × AX18894-1 was designated AX19721, IA2073 × AX18895-6 was designated AX19722, and IA2073 × AX18894-6 was designated AX19723. The F_1 seeds from each cross were planted during October 2004 at the Illinois Crop Improvement Association (ICIA) research station at Ponce, Puerto Rico. The plants were grown under artificial light to extend the day length to enhance seed production. The soil type is a fine-loamy, mixed, superactive, isohyperthermic Cumulic Haplustolls. The F_1 plants from each population were harvested individually, and ten individual seeds were analyzed from each plant. Plants with seeds that segregated for oleate content were considered to be hybrids. The F_2 seed from the F_1 plants of the same population were bulked together.

A random sample of 125 F_2 seeds of AX19716 and AX19717 and 84 F_2 seeds of AX19721, AX19722, and AX19723 were used for fatty ester analysis [10]. Each seed was cut into two parts with a razor blade. The third of the seed that did not contain the embryonic axis was used for fatty ester analysis and other two thirds of the seed with the embryonic axis was planted in January 2005 at the ICIA research station at Ponce, Puerto Rico. The F_2 plants from split seeds were grown under artificial light to extend the day length to enhance seed production. One leaf was harvested from each F_2 plant and DNA from the leaf was tested for the deletion conferred by M23 [5]. A total of 125 F_2 plants from all the populations produced 38 or more seeds that were required for analysis and progeny testing. The plants were harvested individually in May 2005, and two five-seed bulks were analyzed from each plant for fatty ester content.

In May 2005, one set of 130 entries was grown at two locations. The set consisted of the F_3 progeny of the 125 F_2 plants harvested in Puerto Rico; two mid-oleate lines, FA22 and N98-4445A; two 1% linolenate cultivars, IA2073 and IA3024; and one conventional cultivar, IA3203. The set was grown in two replications of a randomized complete-block design at the Agronomy Farm and the Burkey Farm of the Agricultural and Agronomy Research Center near Ames, IA, USA. The soil type for both farms is a Nicollet loam (fine-loamy, mixed, superactive, mesic Aquic Hapludoll). For each entry, up to 20 seeds were planted in rows 76-cm long, with 102 cm between rows. There was a 107-cm alley between the ends of the plots. If an entry did not have 80 seeds, its seeds were divided among the four replications.

At harvest, the first five plants were harvested from each plot of the $F_{2:3}$ lines. Two five-seed bulks from each plant were analyzed for fatty ester content. The fatty ester contents of the two-five seed bulks were averaged to obtain a mean for each plant. All F_3 plants from an $F_{2:3}$ line were averaged to obtain the mean fatty ester content of the line.

DNA Analysis

Genomic DNA was prepared for DNA gel blot and PCR analyses [11]. Ten micrograms of genomic DNA was digested with the restriction enzyme *Eco*RI and electrophoretically separated on 0.8% agarose gels [12]. DNA was blotted from the gel onto a nylon membrane (Zeta Probe, Bio-Rad Laboratories, Hercules, CA, USA) using capillary action with 0.4 M NaOH overnight at room temperature.

Probe Preparation, Hybridization, and Autoradiography

About 80 ng DNA was labeled with 50 μCi of [α^{32}P]-dATP [13]. Hybridization was performed in 10 ml of hybridization buffer (50% formamide, 1% sodium dodecyl sulfate, SDS, 1 M NaCl, 5× Denhardt's solution, 100 µg/ml herring sperm DNA), incubated at 42 °C for 16–18 h in a hybridization rotisserie oven. Blots were washed with 2× saline/sodium citrate (SSC) for five min at 42 °C followed by 0.2× SSC, 0.1% SDS solution at 65 °C for 45 min, and once more at 65 °C with 0.1× SSC, 0.1% SDS for 45 min. Blots were exposed to X-ray films.

Marker Analysis

For PCR analysis, 30 ng genomic DNA was used as the template in 10 µl reaction mixtures containing 1× buffer [10 mM tris(hydroxymethyl)aminomethane–HCl, 50 mM KCl, pH 8.3], 2.0 mM $MgCl_2$, 0.25 µM of each primer, 200 µM deoxyribonucleoside triphosphates and 0.25 units of Biolase DNA polymerase (Bioline USA, Boston, MA, USA). The forward and reverse PCR primers were 5′-GGG CCA TAG TGG GAG TTA TGG AAG-3′ and 5′-GCT ATA AGC AGA ACA CTT TCC ACA T-3′, respectively (Fig. 1). The PCR conditions were as follows: initial cycle of 2 min at 94 °C followed by 35 cycles with denaturation at 94 °C for 30 s, primer annealing at 58 °C for 30 s, and extension at 72 °C for 1 min. A single cycle of 8 min at 72 °C for extension was provided at the end of the amplification reactions. The amplification products were size-separated on a 4% agarose gel.

Sequence Analysis

NCBI BLASTN and BLASTX programs were used for identification and analyses of soybean *Fad2-1*-specific expressed sequence tags (ESTs). ClastalW and BLAST2 programs were used in identifying individual ESTs of the *Fad2-1a* and the *Fad2-1b* genes. The sequence of *Fad2-1b* was assembled from two ESTs and a cDNA sequence by applying ClastalW and BLAST2 programs.

Reverse Transcription PCR Analysis

For expression analysis embryos were collected at three developmental stages from the cultivar Williams 82. Average weights were 14, 41, and 79 mg, for stage 1, 2, and 3 embryos, respectively. Tissues were frozen quickly in liquid nitrogen and ground to fine powder using a mortar and pestle. Total RNA was extracted from tissue powder using the Qiagen RNeasy Plant Mini kit (Qiagen, Valencia, CA, USA). RNA concentration was determined using a Unico UV-2000 spectrophotometer (Unico, Dayton, NJ, USA). RNA was treated with deoxyribonuclease I for 30 min at 37 °C to eliminate any contaminating DNA. Reverse transcription (RT) was conducted with gene-specific PCR primers (primer sequence mentioned in ''Marker Analysis'') using a RT-PCR kit (Qiagen). To determine the extent of amplification from contamination, a control reaction lacking reverse transcriptase was conducted for each RNA sample. Primers specific to actin gene GmACT1 (gi 18531) were spiked in the PCRs in order to determine the variation in RNA amounts among the samples. RT was conducted at 50 °C for 30 min. The samples were then incubated at 95 °C for 15 min to initiate the hot start DNA polymerase enzyme. Twenty-five cycles with an initial melting temperature at 94 °C for 30 s, primer annealing at 52 °C for 30 s, and extension at 72 °C for 1 min, were followed by a 10-min extension at 72 °C. PCR products were electrophoresed in 3% agarose gel for separating PCR products of the *Fad2-1a* and *Fad2-1b* genes and also on 0.8% agarose gels to resolve the GmACT1 band. A 100-bp DNA ladder (Life Technologies, Rockville, MD, USA) was used as a DNA marker. Band quantification was done using a GS-800 calibrated densitometer (Bio-Rad, Hercules, CA, USA).

Results and Discussion

Soybean *Fad2* Homologs

The *Fad2* gene was first cloned from Arabidopsis through T-DNA mutagenesis experiments [2]. *Fad2* encodes fatty acid desaturase that is involved in desaturation of oleic acid into polyunsaturated fatty acids. An NCBI database search identified three soybean cDNA sequences (L43920, L43921, and AY611472) with high similarity to the Arabidopsis *Fad2* gene. One cDNA sequence (L43921) represents the transcript of the *Fad2-2* gene. The other two cDNAs (AY611472 and L43920) share very high nucleic acid sequence identity (94%) and most likely represent duplicated copies of the *Fad2-1* gene [3, 9]. These two paralogous genes were designated recently by Tang et al. [8] as *Fad2-1a* and *Fad2-1b*. Sequence comparison between these two closely related genes revealed that there is an 18-bp deletion at the 3′ untranslated region of *Fad2-1b* (Fig. 1).

Fad2-1a Is Deleted in the M23 Mutant Line

The 18-bp deletion in *Fad2-1b* was used to distinguish this gene from *Fad2-1a* through PCR analysis (Figs. 1, 2). PCR-amplified fragments generated using primers that flank the deletion were expected to be 184 bp in *Fad2-1a* and 163 bp in *Fad2-1b*. When these primers were used to investigate the parental lines, M23 and Archer, a single

Fig. 1 Development of a PCR marker that distinguishes the *Fad2-1a* and *Fad2-1b* genes. The sequence for the *Fad2-1b* gene was assembled from two expressed sequence tags, AI748158 and AI735881, and a complementary DNA sequence, AY611472. Sequences used for developing the primers are *underlined*. ATG and TAG codons are shown in *bold*. *Fad2-1b* shows an 18-bp deletion

DNA fragment was amplified in M23, whereas two were amplified in Archer. In M23, the *Fad2-1a*-specific 184-bp PCR fragment was absent, suggesting that *Fad2-1a* was deleted in this mutant. This PCR-based marker was used in conjunction with DNA gel blotting to analyze 69 F_2 plants for determining the association between deletion and elevated oleic acid content (Fig. 2). In all 69 F_2 plants, the *Fad2-1b*-specific 163-bp fragment was amplified. Segregation of the *Fad2-1a*-specific 184-bp fragment was observed among the F_2 progenies.

On the basis of DNA gel blot analyses, F_2 plants were placed into three classes: (1) normal homozygous for *Fad2-1a* (two copies), (2) heterozygous for *Fad2-1a* (one copy), and (3) homozygous for the deletion (no copy). Plants carrying the deletion in homozygous conditions lacked a 4.6-kb *Eco*RI fragment, as determined from the gel blot analysis (Fig. 2c). On the basis of the PCR data, we concluded that this 4.6-kb *Eco*RI fragment is *Fad2-1a*-specific. The *Fad2-1b*-specific *Eco*RI fragment is about 2.1 kb and is about half the intensity of the 4.6-kb fragment

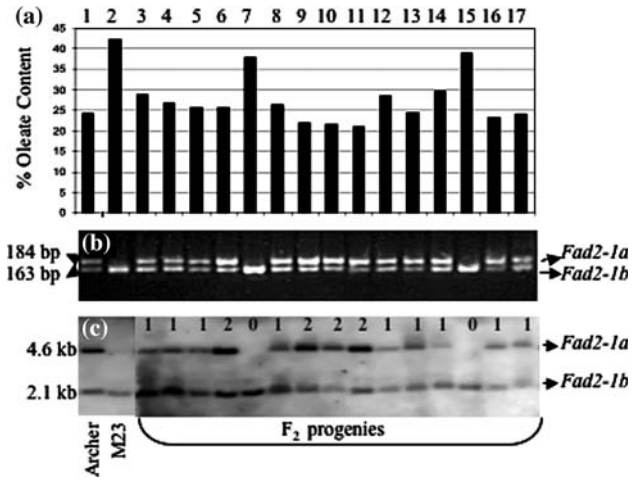

Fig. 2 The deletion in *Fad2-1a* is associated with the increased oleic acid content among F_2 segregants obtained from the cross M23 x Archer. **a** The oleic acid contents of 15 F_2 plants. Segregation of *Fad2-1a* among these 15 F_2 plants is shown following PCR (**b**) and DNA blot analysis (**c**). The primers shown in Fig. 1 were used for the PCR analysis and *Fad2-1b* was used as the probe for the DNA blot analysis. In the DNA blot, copy numbers of *Fad2-1a* are shown at *top* of the gel (**c**). The genotypes with either one copy or two copies were distinguished on the basis of the intensities of the *Fad2-1a* band compared with that of the homozygous *Fad2-1b* 2.1-kb band

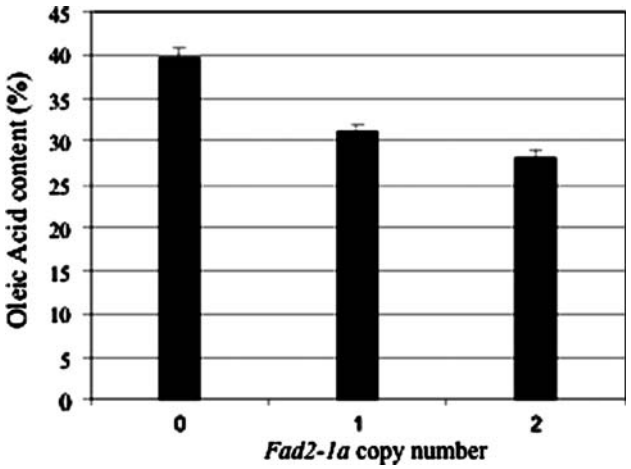

Fig. 3 Oleic acid contents of individuals carrying varying copy numbers of *Fad2-1a*. Standard errors are represented by *error bars*. Of the 69 plants, 16 had two copies, 41 had one copy, and 12 had no copy

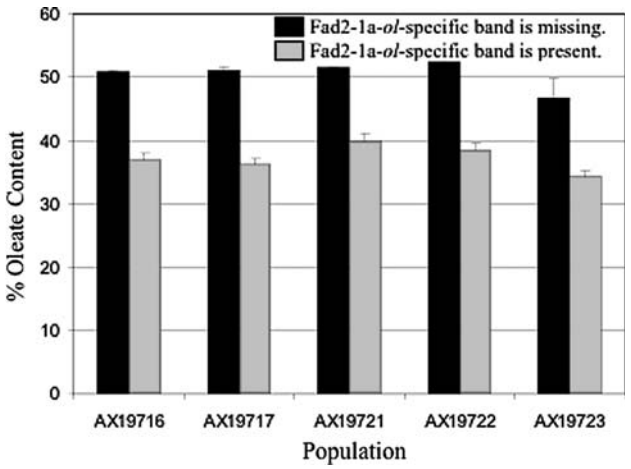

Fig. 4 Segregation analyses of five independent populations using the Fad2-1a-ol marker. The mean oleate contents of lines from five populations for which the Fad2-1a-ol marker band was absent (*black bars*) or present (*gray bars*). Standard errors are represented by *error bars*

on DNA blots (Fig. 2c). The copy number of *Fad2-1a* was estimated by comparing both bands of an individual genotype. If the 4.6-kb band was about double the intensity of the 2.1-kb band then this meant there were two copies of *Fad2-1a*, and if both bands were of similar intensities, then there was a single copy of *Fad2-1a*. On the basis of DNA gel blot analyses, 16 out of 69 F_2 plants were shown to contain two copies of *Fad2-1a*, 41 had one copy, and 12 plants had no copy. Plants containing one *Fad2-1a* copy showed modest increases in oleic acid contents compared with the increase in wild-type plants containing two copies (Fig. 3). Plants with no *Fad2-1* copies showed significantly higher oleic acid contents compared with the other two classes (Fig. 3). Twelve plants were homozygous for the deletion in both PCR and DNA gel blot analyses (Fig. 2b, c). These 12 plants showed elevated levels of oleic acid content. The PCR marker was named as Fad2-1a-ol. The marker can identify genotypes that are homozygous for the deletion, and will be a useful tool for introgressing the deletion into soybean lines in order to improve the oleic acid contents.

Segregation Analysis

To validate our results, we tested the Fad2-1a-ol marker on lines developed from five independent populations segregating for oleate content (see ''*Experimental Procedures*''). The oleate content of $F_{2:3}$ lines missing the Fad2-1a-ol marker band was statistically higher than that in the lines with the Fad2-1a-ol band present (Fig. 4). The mean oleate content varied from 46.6 to 52.4% in $F_{2:3}$ lines where the Fad2-1a-ol marker was missing and varied from 34.2 to 39.9% where the Fad2-1a-ol marker was present (Fig. 4). The results for the five populations were in agreement.

Fad2-1b is the Predominantly Expressed Embryo-Specific Desaturase Gene

Fad2-1a is a functional desaturase because increased oleic acid content was associated with the *Fad2-1a* deletion. To

determine the relative role of the two *Fad2-1* genes in oleic acid metabolism, we investigated the steady-state transcript levels of *Fad2-1a* and *Fad2-1b*. The Genbank was searched for *Fad2-1*-like sequences and 49 ESTs were obtained. All these ESTs originated from cDNA libraries that were constructed from developing embryos or seeds (Table S1), confirming a previous finding that the expression of *Fad2-1* is embryo-specific [3]. *Fad2-1a* and *Fad2-1b* share 94% sequence identity. Among the 49 ESTs, approximately 80% originated from *Fad2-1b* and only approximately 20% were from *Fad2-1a* (Table S1).

To confirm the differences in expression levels between the two embryo-specific genes observed in EST database analyses, we conducted an RT-PCR experiment using the RNA samples prepared from embryos. Three developmental stages of embryos were considered for preparing RNAs (see ''Experimental Procedures''). RT-PCR products specific to *Fad2-1a* and *Fad2-1b* were quantified along with that of the GmACT1 gene using a densitometer (Bio-Rad, Hercules, CA, USA) and RT-PCR products of *Fad2-1a* and *Fad2-1b* were normalized against that of the GmACT1 gene. The results showed that the expression levels of *Fad2-1b* were about fourfold higher than those of *Fad2-1a* (Fig. 5). These results are consistent with the results obtained from the analyses of *Fad2-1*-specific ESTs (Table S1).

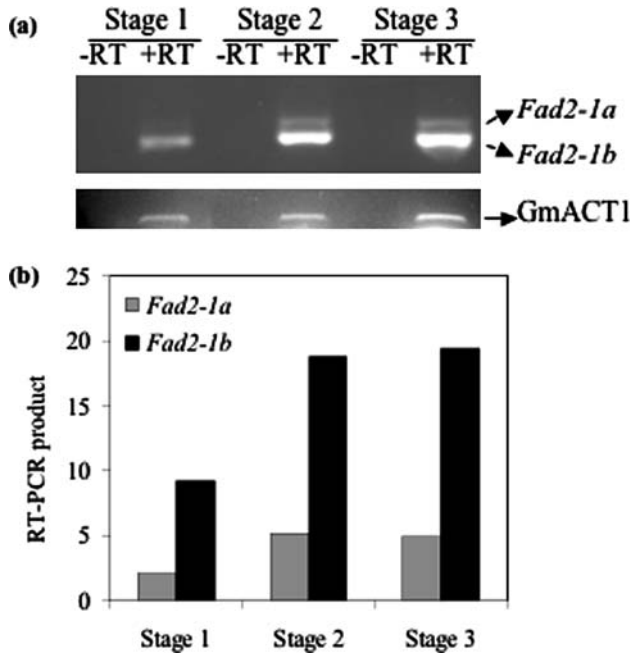

Fig. 5 *Fad2-1b* is the predominantly expressed embryo-specific desaturase gene. **a** Reverse transcription PCR (*RT-PCR*) products from three developmental stages of embryos. *Fad2-1*-specific products were resolved in a 3% agarose gel, whereas that of the GmACT1 gene was resolved in a 0.8% gel. All three genes were PCR-amplified in a single PCR. **b** The relative amounts of RT-PCR products for *Fad2-1a* and *Fad2-1b* for three developmental stages of embryos. The ratios of *Fad2-1b*-specific and *Fad2-1a*-specific RT-PCR products were 4.42, 3.68, and 3.98 for stage 1, stage 2, and stage 3, respectively. *+RT* with reverse transcriptase enzyme, *–RT* without reverse transcriptase enzyme

The range of oleic acid levels varied between 30 and 60% in the lines homozygous for the deletion in *Fad2-1a*, derived from the cross between M23 and Archer [5]. Expression of Fad2-1 protein in yeast revealed that at higher growth temperatures Fad2-1b is more stable than Fad2-1a [8]. This may be one of the reasons for the observed variation in the oleic acid content in a previous study [5]. An earlier study showed that silencing of the *Fad2-1* gene in transgenic soybean lines elevated the oleic acid content to 82% [14]. Presumably, both copies of *Fad2-1* were silenced in that study because of the high similarities observed between the two paralogous genes. Much higher oleic acid contents in transgenic lines compared with that in M23, presumably resulting from silencing of both *Fad2-1* copies, also suggests that *Fad2-1b* encodes a functional desaturase. Knocking out the *Fad2-1b* gene together with *Fad2-1a* should increase the oleic acid content to much higher levels.

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