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ORIGINAL PAPER

A novel *FAD2-1 A* allele in a soybean plant introduction offers an alternate means to produce soybean seed oil with 85% oleic acid content

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Abstract The alteration of fatty acid profiles in soybean to improve soybean oil quality has been a long-time goal of soybean researchers. Soybean oil with elevated oleic acid is desirable because this monounsaturated fatty acid improves the nutrition and oxidative stability of soybean oil compared to other oils. In the lipid biosynthetic pathway, the enzyme fatty acid desaturase 2 (FAD2) is responsible for the conversion of oleic acid precursors to linoleic acid precursors in developing soybean seeds. Two genes encoding FAD2-1A and FAD2-1B were identified to be expressed specifically in seeds during embryogenesis and have been considered to hold an important role in controlling the seed oleic acid content. A total of 22 soybean plant introduction (PI) lines identified to have an elevated oleic acid content were characterized for sequence mutations in the *FAD 2-1A* and *FAD2-1B* genes. PI 603452 was found to contain a deletion of a nucleotide in the second exon of *FAD2-1A*. These important SNPs were used in developing molecular marker genotyping assays. The assays appear to be a reliable and accurate tool to identify the *FAD 2-1A* and *FAD2-1B* genotype of wild-type and mutant plants. PI 603452 was subsequently crossed with PI 283327, a soybean line that has a mutation in *FAD2-1B*. Interestingly, soybean lines carrying both homozygous insertion/deletion mutation (indel) *FAD2-1A* alleles and mutant *FAD2-1B* alleles have an average of 82–86% oleic acid content, compared to 20% in conventional soybean, and low levels of linoleic and linolenic acids. The newly identified indel mutation in the *FAD2-1A* gene offers a simple method for the development of high oleic acid commercial soybean varieties.

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Introduction

Soybean (*Glycine max* (L.) Merr.) oil is one of the most economically important products of soybean. Due to its neutral flavor and a competitive price, soybean oil is used extensively in food industry and has been the most consumed vegetable oil in the world (Soystats 2010). It is reported to account for more than 70% of the total edible fat and oil consumption in the USA from 2001to 2009, and three-quarters of this amount is used as cooking oil, margarine, and baking and frying fat (Soystats 2010). Therefore, soybean oil is almost ubiquitously present in daily diets as a hidden ingredient in processed foods, snacks, and fast foods or directly consumed as vegetable oil. As a result, enhancement of soybean oil quality is desirable.

Soybean oil is composed mostly of triacylglycerol, which is produced by the esterification of fatty acids to glycerols. Besides inherent antioxidant properties, the nutrition and utilization values of soybean oil ultimately

depend on the seed fatty acid composition (Ensminger and Ensminger 1993). Commodity soybean oil typically contains 11% palmitic acid (16:0), 4% stearic acid (18:0), 25% oleic acid (18:1), 52% linoleic acid (18:2), and 8% linolenic acid (18:3) (Fehr 2007). The high linoleic acid (also known as ω-6) content of soybean oil is potentially nutritionally negative, because diets high in linoleic acid content may reduce the nutritionally positive effects of the health-beneficial ω-3 fatty acids such as eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA n−3), and docosahexaenoic acid (DHA) in tissue (Blasbalg et al. 2011; Clark et al. 1992; Friesen and Innis 2010). In addition, the high concentration of linoleic acid and linolenic acid is disadvantageous for food production due to the fact that the oil is oxidized easily and the foods go rancid quickly (Warner and Gupta 2005; Yadav et al. 1993). Thus, to improve the quality of soybean oil and processed foods, chemical hydrogenation has been employed to increase oleic acid content to nearly 50% and reduce the amount of the polyunsaturated fatty acids (Yusem and Pintauro 1992). Oil hydrogenation generates *trans* fat that has been linked to heart disease and stroke, high levels of "bad" cholesterol (low-density lipoproteins), and a higher risk of developing type 2 diabetes (Hu et al. 1997; Mozaffarian et al. 2006). Another effective approach to improve soybean oil functionality without hydrogenation is to genetically increase the oleic acid content in soybean seeds at the expense of linoleic and linolenic acids.

In the lipid biosynthetic pathway, the delta-12 fatty acid desaturase 2 enzyme (FAD2) is responsible for the conversion of oleic acid (18:1) precursors to linoleic acid (18:2) precursors (Okuley et al. 1994; Schlueter et al. 2007b). In developing soybean seeds, among FAD2 genes identified, the two microsomal FAD2-1 desaturases *FAD2-1A* (Glyma10g42470) and *FAD2-1B* (Glyma20g24530) have the highest expression levels (Schlueter et al. 2007a; Tang et al. 2005). Thus, *FAD2-1A* and *FAD2-1B* are considered to play an important role in controlling the oleic acid level in developing soybean seeds and were used as targets or candidate genes to produce high oleic acid soybean. Two approaches were used to generate soybeans with oleic acid content over 80% of the total oil, including genetic engineering and candidate gene-based molecular breeding (Buhr et al. 2002; Hoshino et al. 2010; Pham et al. 2010). Transgenic high oleic acid soybeans were produced using ribozyme-terminated antisense to down-regulate *FAD2-1* gene transcript levels (Buhr et al. 2002). Though the transgenic high oleic acid soybeans had the desirable fatty acid profile, production and importation of these transgenic soybeans will have to overcome regulatory hurdles in some important markets such as Europe and Asia. On the other hand, we used a candidate gene approach to identify a mutant *FAD2-1B* gene in PI 283327 (a missense mutation causing a change of P137R in the amino acid sequence) and combine this gene with existing mutant *FAD2-1A* genes from two soybean lines, M23 (genomic deletion of 160 kb including *FAD2-1A*) and 17D (an S117N missense mutation) to produce a stable, non-transgenic high oleic acid (>80%) content in soybean seed oil (Bolon et al. 2011; Dierking and Bilyeu 2009; Pham et al. 2010) The usefulness of this approach to produce high oleic soybeans was confirmed by another research group, although different sources of mutant *FAD2-1A* (KK21, a single nucleotide deletion) and *FAD2-1B* genes (from two EMS mutant soybean lines with missense mutations) were used (Hoshino et al. 2010).

It has been shown that the more severe the mutations in either *FAD2-1A* or *FAD2-1B*, the higher and more stable is the oleic acid content in the soybean oil, due to residual enzymatic activity of FAD2. For instance, high oleic acid soybeans with the 17D-derived mutant *FAD2-1A* alleles containing a missense mutation showed a larger reduction of oleic acid content (more flux through the desaturase pathway) when grown in cooler environments, compared to those that had the M23-derived *FAD2-1A* null alleles, which are completely deleted (Anai et al. 2008; Pham et al. 2010). However, M23-derived lines have been shown to have reductions in yield that appear to be linked to the deleted portion of chromosome 10 (Taylor et al. 2002). Therefore, identifying natural mutations involving single base pair deletions/mutations of these two candidate genes in soybean lines in the public germplasm collection and combining them by marker-assisted breeding appears to be a promising strategy to produce high and more stable oleic acid content with less effect on yield. Fortunately, a set of soybean plant introductions (PIs) in the US soybean germplasm collection was identified to have elevated oleic acid contents ranging from 30 to 50% of the total fatty acid in seed oil. Fifteen soybean lines with the highest oleic acid content among those were evaluated for the stability of the trait in multi-location field trials (Lee et al. 2009). These PIs are promising sources of novel mutant *FAD2-1A* and *FAD2-1B* alleles. Therefore, we initiated a project to: (1) screen for mutations in candidate *FAD2-1* genes that may be responsible for the elevated oleic acid content in these PIs; and (2) produce high oleic soybean lines using the identified mutant alleles of *FAD2-1A* and *FAD2-1B*.

Materials and methods

DNA isolation and sequencing of *FAD2-1A* and *FAD2-1B*

Primers specific for *FAD2-1A* and *B* soybean genes and PCR amplifications were described by Pham et al. (2010).

PCR products were obtained and validated using agarose gel electrophoresis to confirm the products' authenticity. Sequencing reactions were performed at the DNA Core Center, University of Missouri. Sequence alignment and analysis were accomplished using Multiple Sequence Alignment by CLUSTALW (http://align.genome.jp/). Variant nucleotides between 'Williams 82' reference (http://www.phytozome.net/soybean) and the PIs were identified. Protein translation was conducted using ExPaSy (http://ca.expasy.org/tools/dna.html) and protein alignment was done using Multiple Sequence Alignment.

Population development

PI 603452 was crossed with two different lines that contain the P317R *FAD2-1B* allele derived from PI 283327. In cross 1, generating population 1, PI 603452 was crossed to a soybean (Jake × PI283327)-derived line that contained the mutant *FAD2-1B* allele P137R; for cross 2, generating population 2, PI 603452 was crossed to a (17D × PI283327)-derived line that contained the same mutant allele of *FAD2-1B* and wild-type alleles of *FAD2-1A*. True F_1 seeds were confirmed using a molecular marker assay of *FAD2-1B* from PI 283327 (Pham et al. 2010).

F_1 and F_2 seeds of population 1 were grown in Costa Rica in the fall of 2009 and spring of 2010, and F_3 seeds were sown in field conditions in Columbia, MO, USA and Portageville, MO, USA in summer 2010. F_1 seeds of the cross 2 were planted in a growth chamber in spring 2010 and F_2 seedlings were transplanted to the field at the Bradford Research and Extension Center, Columbia, MO, USA in summer 2010, adjacent to the blocks of the population 1 lines.

FAD2-1A allele-specific molecular marker assay

SimpleProbe assay for the deletion in the *FAD2-1A* of PI 603452 was developed based on the SimpleProbe protocol described by Pham et al. 2010. The Probe contained 5′-fluorescein-SPC-CCTCTAGG**A**AGGGCTGTTTCTC T-phosphate-3′ (the deleted nucleotide is indicated by bold font and underlining). Primers used to generate template for Simpleprobe genotyping assay were designed by aligning the *FAD2-1A* and *FAD2-1B* region containing the SNPs. Primers were selected to be as close as possible to the SNPs while differing in at least three nucleotides between the two genes to specifically amplify the targeted region in *FAD2-1A*. Genotyping reactions were performed with a 5:2 asymmetric mix of primers (5′-CCAAGGTTG CCTTCTCACTGGT-3′ at 2 μM final concentration, and 5′-TAGGCCACCCTATTGTGAGTGTGAC-3′ at 5 μM final concentration). Reactions were carried out in 20 μl, containing template, primers, 0.2 μM final concentration of SimpleProbe, buffer (40 mM Tricine-KOH [pH 8.0] 16 mM $MgCl_2$, 3.75 μg ml^{-1} BSA,), 5% DMSO, 200 μM dNTPs, and 0.2X Titanium Taq polymerase (BD Biosciences, Palo Alto, CA, USA). Genotyping reactions were performed using a Lightcycler 480 II real time PCR instrument (Roche), using the following PCR parameters: 95°C for 5 min followed by 40 cycles of 95°C for 20 s, 65°C for 20 s, 72°C for 30 s, and then a melting curve from 50 to 68°C. PI 603452 and all soybean lines with an identical *FAD2-1A* allele genotype have a characteristic peak at 56°C, while Williams 82 (wild-type *FAD2-1A*) lines have a peak at 62°C. Heterozygous individuals' genotype showed two peaks at 56 and 62°C.

Fatty acid, protein and oil determination

Fatty acid profiles of all samples were obtained using the method of gas chromatography of total fatty acid methyl esters of extracted oil (Beuselinck et al. 2006). The individual fatty acid contents are reported as the relative percentages of palmitic, stearic, oleic, linoleic, and linolenic acids in the extracted oil. In population 1 and 2, five whole crushed individual seeds were used as samples to determine the fatty acid content of each soybean line or plant. The genotypes of four homozygous combinations of mutant *FAD2-1A* and *FAD2-1B* alleles are represented as AABB, AAbb, aaBB, and aaBB from now onward with the lowercase allele designation always specifying the mutant allele and the capital case specifying the wild-type allele.

Protein and oil contents were determined for seeds of high oleic lines aabb_1-1, aabb_1-2, aabb_2, ***aa***bb_M23 (soybean line with "null" mutant *FAD2-1A* alleles derived from M23 and mutant *FAD2-1B* P137 alleles derived from PI 283327), the parental lines, and Williams 82 using NIR spectroscopy. Fifty seeds from each line were used for the analysis (Hartwig and Hurburgh 1990).

Population genotyping using SimpleProbe assay

Populations were genotyped using SimpleProbe assays designed specifically for each mutant allele (Pham et al. 2010). For population 1, DNAs of F_1 and F_2 plants were collected for PCR reactions using Whatman FTA card protocol BD05 (http://www.whatman.com). For population 2, seeds were chipped to get a small portion for fatty acid determination. The remaining portion with hypocotyl was germinated in germination packages to collect DNA on Whatman FTA cards.

Design of field experiments

Seeds of two soybean lines aabb_1-1 and aabb_1-2 were grown in Columbia and Portageville in summer 2010

together with two parents (Jake × PI283327)/(17D × PI 283327), PI 603452 (maturity group (MG) III), and various checks including Williams 82 (MG III), LG04-6863 (MG mid group IV), 5002T (MG IV late to V early), N98-4445A (MG IV), M23 (MG V), AP09INCR1, and AP09INCR2 (80% oleic lines from M23 × PI 283327). There were three replications for each location, and randomized complete block design was used for setting up the experiment. In both locations, plantings were made in rows spaced 76 cm apart. Within the rows, ten seed of each soybean line was planted in a hill plot, spaced 51 cm apart. After seedling emergence, hills were thinned to five plants from which all data were collected for each soybean line. In Columbia, seeds were planted on 28 May and harvested on 21 September for aabb_1-1 and 23 September for aabb_1-2, which were similar to Williams 82 with MG group III. In Portageville, seeds were planted on 2nd June and harvested on 24th September for aabb_1-1and 21st September for aabb_1-2, which were also similar to Williams 82. Seeds from five plants were bulked and five seeds from each line were used for fatty acid analysis.

For population 2, one F_2 seedling plant of genotype AABB and aabb_2 and two seedling plants for each of the genotypes, aaBB, AAbb were transplanted into the field. These seedlings were grown 10 cm apart from each other in a hill plot right behind population 1. All of the plants were transplanted on 9th June and aabb_2 seeds were harvested on 28th September, which was considered to be in MG III. Each plant in the experiment was harvested and threshed separately, and five seeds from each plant were individually analyzed for fatty acid content of the oil, except for aabb_2, for which ten individual seeds were analyzed.

Statistical analysis

The data and statistical analysis presented for the replicated experiment (two genotype aabb_1-1 and aabb_1-2, aabb_M23, two parental lines and Williams 82 in population 1) was generated using proc mixed procedure, SAS® 9.2 Enhanced Logging Facilities, Cary, NC: SAS Institute Inc., 2008 where the standard deviation is derived from the means of the three replicates (Table 3). The mean and standard deviation presented for the unreplicated lines (three genotypes of population 1 including AABB_1, AAbb_1 and aaBB_1, and four genotypes of population 2 including AABB_2, AAbb_2, aaBB_2, and aabb_2) were generated using fatty acid values of two lines used for phenotypic analysis, five seeds per line with standard deviations derived from the set of all individual seed values (Table 3). One exception was with AABB_2 in which only seeds from one line were analyzed. No statistics were conducted on population 2 and the three genotypes of population 1 that were planted without replication.

Results

Identification of novel alleles of *FAD2-1A* and *FAD2-1B* in soybean plant introductions

A set of 22 soybean lines was selected for the sequencing of *FAD2-1A* and *FAD2-1B* genes (Table 1). These PIs have mean oleic acid contents in the range of 27–49% of the total fatty acid content in seed oil. Lines with identifier number 1–15 were evaluated in 3 years, 2005–2007, in Portageville, MO, USA (J. G. Shannon, unpublished data), while lines 16–22 were evaluated in 16 environments during 2005–2007 (Lee et al. 2009).

FAD2-1A

Among 22 PIs, 17 lines have a wild-type *FAD2-1A* identical to the Williams 82 reference sequence. Four soybean lines (KLG11028, PI 404160B, PI 506885, PI 507420) contained a missense mutation (g64c in coding sequence) resulting in a change from G22R in protein sequence and a silent mutation of t990c. To predict the potential effect of the amino acid changes to soybean FAD2-1A enzyme function, the program PolyPhen was used to analyze the potential severity of each amino acid change (Ramensky et al. 2002). In addition, the relative conservation for each amino acid position in the enzyme was evaluated visually using Weblogo after alignment of 100 FAD2 protein sequences present in the National Center for Biotechnology Information database (Crooks et al. 2004). G22R was classified by Polyphen as a benign substitution (score $0.6 < 1$), indicating that it is likely to have no phenotypic effect. Additionally, alignment in Weblogo also showed that the position 22 in the FAD2-1A protein was extremely variable, indicating that modification of the glycine residue at this position would not necessarily affect the enzyme's function (Fig. 1a).

Notably, there was a deletion mutation in the *FAD2-1A* gene of PI 603452. A deletion of a single adenosine at position 544/545 in an exon region of this gene resulted in a frameshift of the translation and premature termination of the peptide after 191 amino acids.

FAD2-1B

A total of 12 SNPs were identified in *FAD2-1B* gene for 22 PIs. Because some of the SNPs are shared among the PIs, and each PI contains a unique combination of these SNPs, the results of the SNP discovery in the 22 PIs are summarized and presented in Table 2. Among the 12 SNPs, five are missense mutations and seven are silent mutations in the *FAD2-1B* sequence of these 22 PIs.

Of the five missense mutations, four were reported previously and only one is new (Pham et al. 2010).

Table 1 Twenty-two soybean lines selected for cloning and sequencing of *FAD2-1A* and *FAD2-1B* genes

Identifier number	Name	Maturity group	Origin	Oleic acid[a] (% of total oil content)
1	PI 561315	I	China	36.4
2	PI 437593 B	II	China	46.0
3	PI 567155 B	II	Japan	47.3
4	PI 603452	III	China	36.7
5	PI 592974	III	China	42.0
6	PI 578451	IV	Vietnam	28.7
7	KLG11028	ND[1]	Korea	30.9
8	KLG10926	ND	South Korea	35.0
9	Kwangankong	ND	South Korea	30.8
10	PI 416908	V	Japan	29.2
11	PI 210179	V	Taiwan	29.4
12	PI 458238	V	South Korea	31.3
13	PI 506885	VI	Japan	27.0
14	PI 467310	II	China	45.4
15	PI 561338A	II	China	48.3
16	PI 196165	III	Japan	37.6
17	PI 417054	III	Japan	49.1
18	PI 567205	IV	Germany	32.8
19	PI 458044	V	South Korea	30.0
20	PI 507307	V	Japan	30.1
21	PI 507420	V	Japan	31.0
22	PI 404160B	III	Russian Federation	47.0

[a] Oleic acid contents of lines #1–6 were obtained from Dr. Grover Shannon, unpublished data. Oleic acid contents of lines #7–9 were obtained from the paper by Dhakai et al. 2006. Oleic acid contents of lines 9–22 were obtained from the paper by Lee et al. 2009

Interestingly, P137R in PI 210179 and I143T in PI 578451 are identical to the mutations in PI 283327 and PI 567189A, respectively, and these pairs of lines have identical *FAD2-1B* alleles and origins (Pham et al. 2010). S86F and M126V were found in most of the PIs, and they were determined as being benign in Polyphen and not associated with the oleic acid phenotype (Pham et al. 2010).

The only novel missense mutation found in *FAD2-1B* gene is L242M in soybean line KLG10926. However, this mutation was classified by Polyphen as a benign substitution (score 0.2 < 1), indicating that it likely does not affect the enzyme's functionality. This conclusion was supported by other evidence including the variability of the position 242 amino acid residue in the FAD2 protein sequence among 100 FAD2 aligned protein sequences (Fig. 1b), and

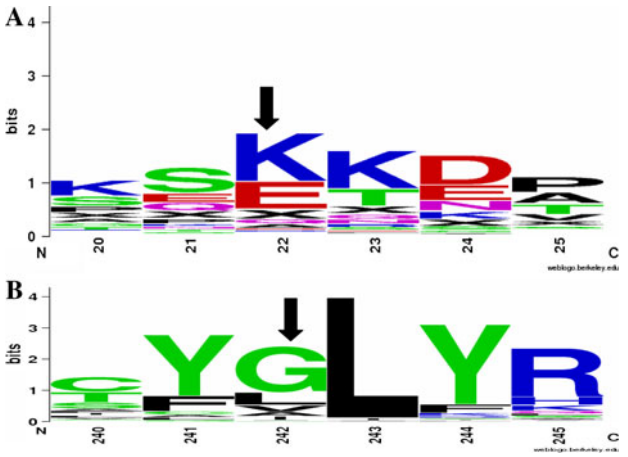

Fig. 1 Weblogo output of the amino acid conservation FAD2 enzyme as part of the BLINK feature at NCBI using GI number 197111722. The top 100 best matched sequences were aligned and used as input for sequence LOGO http://weblogo.berkeley.edu/logo.cgi. The logo consists of stacks of *symbols*, one stack for each position in the amino acid sequence. The overall height of the stack indicates the sequence conservation at that position, while the height of *symbols* within the stack indicates the relative frequency of each amino acid at that position. **a** *Arrow* indicates residue changed due to the Q22R *FAD2-1A* mutation in KLG11028, PI404160B, PI506885, and PI507420. **b** Also, *arrow* indicates residue changed due to the L242M *FAD2-1B* mutation in KLG10926

the similarity of chemical properties between methionine and leucine. As a result, we predict that this missense mutation is not causative for the elevated oleic acid content of this soybean line.

Combinations of novel mutant alleles of *FAD2-1A* with mutant *FAD2-1B* alleles produce high oleic acid levels in the seed oil

Two independent studies report that the combination of mutant *FAD2-1A* and mutant *FAD2-1B* alleles resulted in soybean lines with more than 80% oleic acid (Pham et al. 2010; Hoshino et al. 2010). We hypothesized that the newly identified mutant *FAD2-1A* gene of PI 603452 when combined with a mutant *FAD2-1B* gene would also result in progeny with 80% oleic acid. To prove this, PI 603452 was crossed to two different lines that contain the *FAD2-1B* P137R gene derived from PI 283327. In cross 1, PI 603452 was crossed to a (Jake × PI283327)-derived soybean line to produce population 1, and for cross 2, PI 603452 was crossed to a different (17D × PI283327)-derived soybean line to generate population 2. The association of oleic acid phenotype and four combinations of the novel PI 603452 *FAD2-1A* alleles and P137R *FAD2-1B* alleles were analyzed using field-produced F_3 and F_4 soybean seeds developed from these two crosses.

Table 2 Variants in DNA sequences of FAD2-1B of 24 tested soybean lines

Soybean lines	Nucleotide position									
	66	105	257 (S86F)	376 (M126 V)	410 (P137R)	426 (I143T)	636	657/ 669/682	724 (L242 M)	918
Williams 82 (control) PI437593B, Kwangankong	G	A	C	A	C	T	C	CTT	T	A
PI 467310, PI 404160B, PI 561338A, PI 561315, PI 603452, PI 417054				G				TCC		G
KLG10926				G			T	TCC	A	G
PI 567155 B			T	G				TCC		G
KLG11028			T	G						G
PI 592974, PI 196165, PI 416908, PI 458044		G		G						G
PI 578451		G	T	G		C		TCC		G
PI 210179		G	T	G	G			TCC		G
PI 567205	A									
PI 458238	A	G		G						G
PI 506885, PI 507307	A		T	G				TCC		G
PI 507420	A	G		G				TCC		G

In the first row, numbers with amino acid abbreviation are single nucleotide polymorphism (SNPs) positions that caused amino acid changes specified in the bracket; numbers without amino acid abbreviations are SNP positions that have no effect to protein sequences

Two soybean lines, aabb_1-1 and aabb_1-2, from population 1 and soybean line aabb_2 from population 2 were identified to possess both mutant *FAD2-1A* and *FAD2-1B* alleles (*FAD2-1* aabb) and to have similar maturity. There were significant differences for average palmitic acid and linolenic acid contents between aabb_1-1 and aabb_1-2 from population 1 (Table 3). Hence, the data for these two soybean lines will be presented separately in Table 3. In addition, the contents of palmitic, oleic, linoleic and linolenic acids of these two lines were significantly different between the two production environments, but were similar in stearic acid contents ($P = 0.12$ for stearic acid, $P < 0.001$ for others). Data from soybean lines with different combinations of genotypes for *FAD2-1*: aaBB, AAbb and AABB from population 1 also showed the same trend (Table 3). Therefore, data from population 1 and the two parental lines, an M23-derived high oleic acid soybean line (designated as ***aa***bb_M23, this line has 80% oleic acid content) and Williams 82 (control) were presented by location (Table 3). Data for population 2 were presented only for the Columbia location where all the plants were in the F_2 generation (Table 3).

In both of the populations, transgressive segregation for oleic acid content was observed for the lines that inherited the *FAD2-1* AABB and aabb genotypes. In population 1, lines with the genotype *FAD2-1* AABB had an average oleic acid content significantly higher compared to that of the control Williams 82 in both locations (Table 3) ($P < 0.0001$ for Portageville and $P = 0.008$ for Columbia, MO, USA). Likewise, lines with either homozygous mutant *FAD2-1A* or *FAD2-1B* alleles (aaBB or AAbb) had average oleic acid contents significantly higher than the oleic acid content of either PI 603452 or (Jake × PI 283327) line in both locations (Table 3, $P < 0.0001$ for all of the comparisons).

Two individual lines aabb_1-1 and aabb_1-2 had oleic acid contents not significantly different from each other ($P = 0.1$ in Columbia and 0.9 in Portageville), but significantly higher than that of the soybean line with genotype ***aa***bb_M23 in both locations ($P < 0.0001$ for both locations) (Table 3).

In population 2, lines with the genotype *FAD2-1* AABB had an average oleic acid content lower than that of the control Williams 82 (Table 3). Soybean line aabb_2 had an oleic acid content of 82.4%, which was similar to that of ***aa***bb_M23, but lower than those of aabb_1-1 and aabb_1-2.

Full fatty acid profiles and total oil and protein content

The full fatty acid profiles of the seeds of contrasting *FAD2-1* genotypic classes produced from two populations in this study revealed changes in palmitic acid, linoleic acid, and linolenic acid content (Table 3). As expected for a major increase in the accumulation of oleic acid, the linoleic acid and linolenic acid contents were dramatically reduced in the high oleic *FAD2-1A* and *FAD2-1B* homozygous mutant lines. The linoleic acid contents of aabb_1-1, aabb_1-2, and ***aa***bb_M23 in both locations were not significantly different from each other but significantly lower than those from other homozygous combinations and parental lines. Linoleic acid content was reduced to 1% for

Table 3 Fatty acid profiles and protein and oil contents for homozygous mutant *FAD2-1A and FAD2-1B* genotypes of population 1in field trials in Portageville and Columbia in summer 2010

	Fatty acid					Oil	Protein
	16:0	18:0	18:1	18:2	18:3		
Portageville							
AABB_1[1] (n = 10)[IS]	11.4 ± 0.6[2]	3.3 ± 0.6	30.2 ± 5.9	48.6 ± 3.7	7.5 ± 1.5	15.9[3]	39.5
aaBB_1(n = 10)[IS]	10.4 ± 0.5	3.4 ± 0.2	50.2 ± 7.2	29.1 ± 6.3	6.8 ± 0.8	–[4]	–
AAbb_1(n = 10)[IS]	10.7 ± 1.3	3.2 ± 0.5	45.4 ± 6.7	34.5 ± 4.6	6.3 ± 1.3	–	–
aabb_1-1 (n = 15)[R]	7.3 ± 0.5a[5]	2.4 ± 0.1a	84.8 ± 0.1ab	2.0 ± 0.4a	3.2 ± 0.3b	17.8 ± 2.3bc	42.3 ± 2.3a
aabb_1-2 (n = 15)[R]	7.4 ± 0.4a	2.8 ± 0.3a	85.3 ± 0.3a	1.6 ± 0.4a	2.5 ± 0.1a	18.1 ± 0.1b	41.1 ± 1.1a
aabb_M23 (n = 15)[R]	7.2 ± 0.2a	4.4 ± 0.3c	82.6 ± 0.4c	2.4 ± 0.1a	3.2 ± 0.2b	19.4 ± 0.4a	38.6 ± 0.3b
PI 603452aa (n = 15)[R]	11.5 ± 0.4b	2.9 ± 0.1ab	34.5 ± 2.6d	41.5 ± 1.8b	6.5 ± 0.6c	17.1 ± 1.0c	38.5 ± 1.0b
PI 283327bb (n = 15)[R]	11.0 ± 0.5b	4.7 ± 0.3e	28.6 ± 2.2e	41.2 ± 1.6b	9.2 ± 0.4d	17.6 ± 0.1bc	38.0 ± 0.9b
Williams 82 (n = 15)[R]	11.0 ± 0.2b	3.7 ± 0.3d	21.8 ± 2.7f	56.1 ± 2.0c	6.9 ± 0.6c	19.4 ± 0.1a	35.9 ± 0.1c
Columbia							
AABB_1(n = 10)[IS]	10.2 ± 0.4	3.5 ± 0.4	25.3 ± 4.0	53.3 ± 2.7	7.7 ± 1.4	16.6	38.3
aaBB_1(n = 10)[IS]	8.8 ± 0.5	3.2 ± 0.2	34.5 ± 5.0	25.4 ± 4.3	7.0 ± 0.5	–	–
AAbb_1(n = 10)[IS]	10.9 ± 0.8	3.5 ± 0.5	55.7 ± 6.0	44.0 ± 3.5	7.2 ± 2.0	–	–
aabb_1-1 (n = 15)[R]	7.5 ± 0.3b	2.7 ± 0.1a	83.9 ± 0.5ab	1.8 ± 0.4a	4.0 ± 0.3b	17.0 ± 0.3b	41.1 ± 0.5a
aabb_1-2 (n = 15)[R]	6.9 ± 0.6a	2.9 ± 0.4ab	86.4 ± 0.3a	1.0 ± 0.5a	2.9 ± 0.1a	17.4	40.9
aabb_M23 (n = 15)[R]	7.3 ± 0.1b	3.6 ± 0.2b	82.7 ± 1.4c	2.3 ± 1.0a	4.2 ± 0.5b	18.9 ± 0.5a	38.3 ± 0.6b
PI 603452aa (n = 15)[R]	11.1 ± 0.2c	3.2 ± 0.1b	31.9 ± 1.7d	46.2 ± 1.3b	7.6 ± 0.8c	16.9 ± 0.8b	36.9 ± 1.9b
PI 283327bb (n = 15)[R]	10.8 ± 0.1c	4.3 ± 0.3d	23.6 ±2.3e	50.0 ±1.6b	11.3 ±1.0d	16.9 ± 0.1b	37.4 ± 0.4b
Williams 82 (n = 15)[R]	10.8 ± 0.1c	3.8 ± 0.1bc	21.0 ±0.5e	57.1 ±0.9b	7.4 ± 0.3c	19.5 ± 0.2a	33.8 ± 0.6c
AABB_2 (n = 5)[IS]	11.4 ± 0.8	3.7 ± 0.2	17.3 ± 0.7	57.3 ± 1.0	10.4 ± 0.6	17.7	36.8
AAbb_2 (n = 10)[IS]	11.3 ± 0.4	3.6 ± 0.2	26.8 ± 4.1	50.4 ± 3.3	8.0 ±1.0	–	–
aaBB_2 (n = 10)[IS]	10.6 ± 0.6	3.5± 0.2	36.8± 4.7	41.6 ± 3.9	7.5± 0.9	–	–
aabb_2 (n = 10)[IS]	7.8 ± 0.5	3.4 ± 0.2	82.4 ± 1.9	2.3 ± 0.9	4.1 ± 0.9	18.3	39.3

[1] AA = wild-type *FAD2-1A* alleles, aa = mutant indel *FAD2-1A* alleles derived from PI 603452, BB = wild-type *FAD2-1B* alleles, bb = mutant missense *FAD2-1B* P137R alleles derived from PI 283327, ***aa*** = null *FAD2-1A* allele from M23, which is a deletion of 160 kb in the genome including the *FAD2-1A* gene, AABB_1 means that this genotype AABB is from population 1, AABB_2 means that this genotype is from population 2. These two populations were not planted on the same date. aabb_1-1 and aabb_1-2 are soybean lines with the mutant indel *FAD2-1A* alleles derived from PI 603452 and missense mutant *FAD2-1B* P137R alleles derived from PI 283327. PI 603452aa is a soybean line with indel mutant *FAD2-1A*. PI 283327bb is a (Jake × PI283327)-derived line with mutant *FAD2-1B* P137R alleles and wild-type *FAD2-1A* alleles. ***aa***bb_M23 = soybean line with "null" mutant *FAD2-1A* alleles derived from M23 and mutant *FAD2-1B* P137 alleles derived from PI 283327. Williams 82 is the control line with normal oleic acid content and wild-type *FAD2-1*

[2] The standard deviations from unreplicated lines (IS) are derived from each sample analzyed for the line; the standard deviations from the replicated experiment lines (R) are derived from the three replicate means

[3] The data have no standard deviation because either the genotype was planted without replication or the genotype was planted with replication, but only one replication had enough seeds for oil and protein analysis

[4] No data were collected for these soybean lines

[5] Letters of significance. Two values with the same letter are not statistically different at $\alpha = 0.05$. Lines with letters were analyzed using the data of three replications

[IS] This means that plants that have this genotype were grown without replication in the field. Therefore, this genotype was excluded from statistical analysis in SAS. Mean value ± standard deviation was obtained by averaging fatty acid values of ten seeds used for fatty acid analysis, five seeds per line, two lines for each genotype, except for aabb_2 for which ten seeds from one plant were analyzed

[REP] This means that this soybean line was grown with replication in the field and was included in statistical analysis in SAS. Mean value ± standard deviation was obtained by averaging means of three replications, which were averaged from fatty acid values of five individual seeds per replication. Standard deviation was calculated using mean values of three replications

line aabb_1-2 in Columbia, which was approximately 50-fold lower than soybean lines with wild-type *FAD2-1* alleles (Table 3). The linolenic acid contents of aabb_1-1, and the ***aa***bb_M23 line were significantly higher than that of aabb_1-2, but significantly lower than those of other homozygous combinations of *FAD2-1A* and *FAD2-1B*

alleles and parental lines in both locations (Table 3). The trend for aabb_2 was most similar to that of ***aa***bb_M23 for fatty acid profile. The greatest reduction in linolenic acid content was once again observed for line aabb_1-2 with less than 3% linolenic acid contents in both locations, compared to ~7–11% in the parental lines.

Interestingly, aabb_1-1 and aabb_1-2 had significantly lower stearic acid levels compared to those of the contrasting *FAD2-1* genotypes, the parental lines, ***aa***bb_M23 in both locations (Table 3). Consistent with previous studies in which mutant *FAD2-1A* and *FAD2-1B* genes were combined by Pham et al. (2010) and Hoshino et al. (2010), all of the three *FAD2-1* aabb mutant lines produced lower palmitic acid levels than lines with the *FAD2-1* AABB genotype with the most dramatic reduction recorded for aabb_1-1 and aabb_1-2. The content of palmitic acid was approximately 7% for the *FAD2-1* aabb mutant lines compared to 10–11% for the *FAD2-1* AABB lines.

To evaluate the impact of the enhanced oleic acid content on the total oil and protein profiles of the seeds, we analyzed protein and oil contents for the field-produced F_4 seeds of aabb_1-1 and aabb_1-2 harvested from Columbia and Portageville, aabb_2 from Columbia, their parental lines and Williams 82 (Table 3). The oil content of the high oleic acid soybean line aabb_1-1 was not significantly different from those of their parents ($P = 1$ for all of the comparisons) in both Columbia and Portageville. However, the protein content of this line was significantly higher than the protein contents of the parental lines ($P < 0.05$ for all of the comparisons). In addition, line aabb_1-1 had significantly lower oil content and higher protein content compared to Williams 82 ($P < 0.001$ for both oil and protein content), and it is note-worthy that the oil contents of both of its parental lines were also lower compared to Williams 82 (Table 3). In Portageville, the oil and protein content of aabb_1-2 were not statistically different from those of aabb_1-1. In Columbia, because only one data point was obtained for either oil or protein content of each of aabb_1-2 and aabb_2, the data are presented in Table 3 but not used for statistics analysis.

Discussion

Plant introductions from the national germplasm collections are excellent sources of natural mutations and genetic variation that can be exploited to enrich the means to produce high oleic acid soybeans. Our study identified one novel indel allele of *FAD2-1A* in PI 603452 and confirmed two PIs that have the same mutations in *FAD2-1B*, which we previously reported (Pham et al. 2010). Using this novel indel *FAD2-1A* allele, three soybean lines were developed to have more than 82% oleic acid content in two testing environments including Columbia and Portageville, Missouri. Among those, aabb_1-1 and aabb_1-2 are two soybean lines that had oleic acid content up to 86% and total linolenic and linoleic content less than 5% in two production environments. These combinations produce the highest oleic acid content and lowest polyunsaturated fatty acid and saturated fatty acid contents that have been reported to date. For the first time, stearic acid contents were recorded to be lower than those of the parents and Williams 82, the control cultivar. Combinations of mutant *FAD2-1A* and mutant *FAD2-1B* created in the two studies by Pham et al. (2010) and Hoshino et al. (2010) did not have any reduction in stearic acid content compared to the parental lines or wild-type soybean lines. The significantly low levels in the saturated fatty acids (palmitic + strearic) and the reduced levels in linoleic and linolenic acids of the high oleic acid genotypes compared to the parents and the typical cultivar offer the opportunity to develop soybean oils with higher oxidative stability than M23 and 17D-derived high oleic acid soybean oils. Moreover, because the prematurely terminated FAD2-1A enzyme from PI 603452 will likely not produce a functional enzyme, the high oleic acid content in soybean lines containing the indel *FAD2-1A* alleles has the potential to be more stable across environments compared to lines developed with the missense mutation FAD2-1A enzyme from 17D (Dierking and Bilyeu 2009; Pham et al. 2010). However, it is necessary that these mutant *FAD2-1* alleles are incorporated into various maturity groups and tested in additional environments for stability of the high oleic acid trait, particularly in the northern USA where temperatures at seed fill are cooler.

The significant contribution of this study is the identification of a natural indel allele of *FAD2-1A* in PI 603452 that when combined with mutant alleles of *FAD2-1B* produced the highest oleic acid content in soybean to date. Prior to this discovery, there were only three sources of mutant *FAD2-1A* genes in soybean including M23, KK21, and 17D to combine with four reported mutant *FAD2-1B* alleles for generation of high oleic acid soybean. The M23 null *FAD2-1A* alleles have been shown to contribute to higher and more stable oleic acid content compared to 17D and KK21 when combined with soybean lines carrying mutant *FAD2-1B* genes (Hoshino et al. 2010; Pham et al. 2010). In this study, we observed that using the indel *FAD2-1A* alleles of PI 603452 resulted in significantly higher oleic acid content compared to those derived from M23, although the difference may be due to a relative maturity effect. Temperature during seed fill has long been correlated with differences in fatty acid profile, with cooler temperatures producing generally lower oleic acid contents (Heppard et al. 1996; Lee et al. 2009; Oliva et al. 2006). For both testing locations, the two PI 603452-derived lines

were harvested 1 month earlier than the M23-derived lines, therefore they experienced warmer temperature during seed filling periods (the differences in minimum temperatures between 30-day before-harvesting periods of two high oleic acid sources were approximately 2–3°C in both locations), resulting in the record oleic acid contents observed for high oleic acid soybean lines grown in two testing locations. Because PI 603452 is available for public use, it is a valuable resource of the mutant *FAD2-1A* allele for the production of a stable, non-transgenic high oleic acid soybean.

The impact of seed oil with high oleic acid content on agronomic traits including yield, and eventually the flavor and stability of the high oleic acid oil in foods, requires further evaluation. Whether or not high oleic acid content in the seed oil will have an effect on soybean variety performance and yield has not yet been resolved. Field trials of transgenic high oleic acid soybeans with 80% oleic acid content from three studies showed conflicting results. While Kinney (1996) and Graef et al. (2009) found no adverse impact of high oleic acid content on yield, Brace et al. (2011) identified a small but statistically significant reduction in both oil content and yield (less than 5% compared to those of the control soybean line with normal fatty acid composition) in high oleic acid soybeans. Taken together, these results seem to indicate that the high oleic acid trait alone will not necessarily reduce agronomic performance or yield. In this work, donation of at least the genomic segment containing the indel *FAD2-1A* allele in initial populations developed with the high oleic acid trait will also lead to the inheritance of potentially negative agronomic characteristics from the PI 603452. Donation of the *FAD2-1B* allele from PI 283327 or derived lines will lead to a similar situation for the incorporation of potentially negative agronomic characteristics in the development of high oleic acid soybean lines. Therefore, the incorporation of the two mutant genes into an elite background is necessary before commencing yield trials.

In addition, whether the new high oleic acid oils produced from our study will create a satisfactory flavor and texture in foods also needs to be tested. Warner and Gupta (2005) showed that potato chips fried with a high oleic acid soy oil with very similar fatty acid composition to those described in this work had some flavor problems. Except for the flavor-related issue, the high oleic acid soy oil showed a better oxidative stability and lifetime compared to oils with normal fatty acid composition or oils with low linolenic acid content used in the study. More breeding efforts with additional genes controlling the fatty acid profile may be required to develop an oil with the most functionality.

Our sequencing data indicate that the cause of the elevated oleic acid contents in the majority of the plant introductions used in this experiment is not due to changes in DNA coding for FAD2-1 enzymes. We hypothesize that the mechanisms or factors that cause the increased oleic acid content in these soybean lines can happen at a regulatory, post-transcriptional or post-translational level. Recently, a transcriptional factor and one of its partners in the transcriptional activation apparatus of *FAD2* gene were found to be needed to activate the FAD2 enzyme in sesame (Kim et al. 2007, 2010), whereas a mutation in a DGAT gene was identified to cause high oleic acid content in maize (Zheng et al. 2008). Also, some PIs with mid-oleic acid contents were reported to have down-regulated expression levels of *FAD2-1A*, *FAD2-1B* and oleate-ACP thioesterase (*GmFATB1a*) genes and/or up-regulated expression levels of delta-nine stearoyl acyl carrier protein desaturase A, B and C (*GmSACPD*) genes (Reinprecht et al. 2009). These genes are good targets for a candidate gene-based approach to identify the cause of the elevated oleic acid contents in the PIs. In addition, mapping approach can be used to identify additional genes that control the increased oleic acid content in these PIs.

It is interesting to observe that though PI 603452 and M23 carry similar types of presumably non-functional mutant alleles of *FAD2-1A* in which the former has a deletion of a single nucleotide in the coding region of the gene while the latter has a large section of the genome containing *FAD2-1A* gene completely deleted, the oleic acid contents in these two soybean lines are statistically different. In both locations, the oleic acid contents of PI 603452 were approximately 15% less than those of M23 (data not shown). In this case, the difference in oleic acid content between these two lines would not be explained by maturity, because PI 603452 is in MG (maturity group) III while M23 is in MG V. Additionally, crossing PI 603452 to two soybean lines containing the PI 283327 *FAD2-1B* P137R allele in either Jake or the 17D background with the same maturity resulted in different levels of high oleic acid content, and it is the first time to observe that in one of the populations soybean progeny with the parental genotype had higher oleic acid contents compared to those of the parents. This implies that there are modifying genes with small effects or other complicated mechanisms regulating the oleic acid accumulation in soybean seed oil that are yet to be discovered. It is likely that these mechanisms may be not only determined by the genetic factor, but also influenced by the environmental factor or the interaction of both.

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Conflict of interest None.

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