Theor Appl Genet (2012) 125:503–515
DOI 10.1007/s00122-012-1849-z

ORIGINAL PAPER

Combinations of mutant *FAD2* and *FAD3* genes to produce high oleic acid and low linolenic acid soybean oil

Anh-Tung Pham · J. Grover Shannon · Kristin D. Bilyeu

Received: 12 July 2011 / Accepted: 10 March 2012 / Published online: 4 April 2012

Abstract High oleic acid soybeans were produced by combining mutant *FAD2-1A* and *FAD2-1B* genes. Despite having a high oleic acid content, the linolenic acid content of these soybeans was in the range of 4–6 %, which may be high enough to cause oxidative instability of the oil. Therefore, a study was conducted to incorporate one or two mutant *FAD3* genes into the high oleic acid background to further reduce the linolenic acid content. As a result, soybean lines with high oleic acid and low linolenic acid (HOLL) content were produced using different sources of mutant *FAD2-1A* genes. While oleic acid content of these HOLL lines was stable across two testing environments, the reduction of linolenic acid content varied depending on the number of mutant *FAD3* genes combined with mutant *FAD2-1* genes, on the severity of mutation in the *FAD2-1A* gene, and on the testing environment. Combination of two mutant *FAD2-1* genes and one mutant *FAD3* gene resulted in less than 2 % linolenic acid content in Portageville, Missouri (MO) while four mutant genes were needed to achieve the same linolenic acid in Columbia, MO. This study generated non-transgenic soybeans with the highest oleic acid content and lowest linolenic acid content reported to date, offering a unique alternative to produce a fatty acid profile similar to olive oil.

Communicated by I. Rajcan.

A.-T. Pham
Division of Plant Sciences, University of Missouri,
110 Waters Hall, Columbia, MO 65211, USA

J. G. Shannon
Division of Plant Sciences, University of Missouri-Delta
Research Center, University of Missouri,
Portageville, MO 63873, USA

K. D. Bilyeu (✉)
USDA/ARS, Plant Genetics Research Unit,
University of Missouri, 110 Waters Hall,
Columbia, MO 65211, USA
e-mail: kristin.bilyeu@ars.usda.gov; bilyeuk@missouri.edu

Introduction

Soybean is the largest oilseed crop worldwide with over half of the total oilseed production, and 38 % of the soybeans were produced in the US (Oilseeds: World Markets and Trade 2010). Accordingly, soybean is an important provider of oil globally and in the US, representing 24 % of the total vegetable oil consumption in the world and approximately 70 % total fat and oil consumption in the US in 2010 (Soystats 2010). The majority of soybean oil was used for edible products, including salad and cooking oil (50 %), baking and frying fat (25 %) and margarine (4 %) (Soystats 2010).

Oil extracted from commodity soybeans contains 11 % palmitic acid (16:0), 4 % stearic acid (18:0), 23 % oleic acid (18:1), 54 % linoleic acid (18:2), and 8 % linolenic acid (18:3) (Wilson 2004). The high concentration of polyunsaturated fatty acids (PUFA) such as linoleic acid and linolenic acid in soybean oil was demonstrated to be responsible for the low oxidative and frying stability of soybean oil, resulting in rancidity, rapid decrease in optimum flavor, and shortened storage time of manufactured food products (Warner and Fehr 2008). To overcome this disadvantage, soybean oil is usually hydrogenated to reduce the PUFA contents and increase the content of oleic acid, which was shown to be more resistant to oxidation and has many health benefits (Mattson and Grundy 1985; Perez-Jimenez et al. 1995). However, an unwanted outcome of the hydrogenation process is the creation of

10–40 % *trans* fatty acids, for which consumption has been linked to a number of health problems including higher risk of obesity and coronary heart disease (Hu et al. 1997; Mounts et al. 1994; Mozaffarian et al. 2006; Zaloga et al. 2006). Hence, there have been numerous efforts to increase the oxidative stability of soybean oil without hydrogenation by means of producing soybean with high oleic acid and low polyunsaturated fatty acid concentration.

In soybean, one trait is often controlled by several homologous genes, and this presents a major challenge for breeding soybean lines with multiple traits (Chi et al. 2011; Schmutz et al. 2010). Initiated by USDA-ARS in 1952, traditional breeding has been successful in identifying loci responsible for oleic acid and linolenic acid contents, but selection was primarily based on phenotype because of limited knowledge about the genes and molecular markers associated with the trait (Fehr 2007). Genes that control the oleic acid content and PUFA content in soybean seed oil have been characterized (Bilyeu et al. 2003; Heppard et al. 1996; Schlueter et al. 2007; Schmutz et al. 2010). The two oleate desaturase genes *FAD2-1A* (Glyma10g42470) and *FAD2-1B* (Glyma20g24530) and the three linoleate desaturase genes *FAD3A* (Glyma14g37350), *FAD3B* (Glyma02g39230) and *FAD3C* (Glyma18g06950) were unambiguously assigned a chromosomal position. Mutations identified in either *FAD2-1A* or *FAD2-1B* resulted in elevated oleic acid contents in the range of 27–50 % (Anai et al. 2008; Dierking and Bilyeu 2009; Pham et al. 2010). Mutations in *FAD3* resulted in lower linolenic acid content from 7 to 10 % in lines containing wild-type alleles to approximately 4 % for lines containing mutations in *FAD3A* (Chappell and Bilyeu 2007). Combinations of mutations in *FAD3A* with either *FAD3B* or *FAD3C* lower linolenic acid to approximately 3 % of the seed oil, while mutations in *FAD3A*, *FAD3B*, and *FAD3C* result in 1 % linolenic acid (Bilyeu et al. 2005, 2006, 2011; Reinprecht et al. 2009).

Although utilization of the mutant alleles of the *FAD2-1* and *FAD3* genes was successful in creating either high oleic acid or low linolenic acid soybean, respectively, these soybean lines still have some disadvantages. Soybeans with low linolenic acid content (3–4 %) were shown to be influenced by temperature with lower linolenic acid content often seen with higher temperature (Rennie and Tanner 1989; Wilcox and Cavins 1992). In addition, Warner and Fehr (2008) showed that oils having 1 % linolenic acid content had higher stability than those prepared with normal soybean oil, but are significantly less stable than high oleic acid oil (80 %). The authors reasoned that this result might be due to the wild-type oleic acid and linoleic acid content in the oils with 1 % linolenic acid content. On the other hand, high oleic acid soybean (80 % oleic acid) generated by the combination of mutant *FAD2-1A* and *FAD2-1B* genes had the linolenic acid content reduced to only half of the linolenic acid content in commercial soybean oil (4–6 %), which may result in hydrogenation requirement for high oleic oils (Hoshino et al. 2010; Pham et al. 2010). Moreover, the linoleic acid content is usually equal or lower than the linolenic acid content in the high oleic acid soybean lines (Hoshino et al. 2010; Pham et al. 2010). It was suggested that the linoleic acid content (which was lower than the linolenic acid content) in the high oleic acid oil is partially responsible for the deep fried flavor of the foods (Warner and Gupta 2005). Therefore, in order to have soybean oils with high stability but without adverse effects on the food flavors, it is rational to introduce mutant *FAD3* genes into the high oleic acid background to reduce the linolenic acid content at the expense of the linoleic acid content to potentially improve the functional properties of the oil.

One question addressed in this study was to determine the number of mutant *FAD3* genes needed to be combined with two mutant *FAD2-1* genes in high oleic soybean lines to lower linolenic acid content to less than 3 %, which is the current industry standard for a low linolenic acid soybean oil. Because the amount of linoleic acid in 80 % high oleic acid soybean oil is only 3–5 % compared to 55 % in commercial soybeans, we hypothesized that less than three mutant *FAD3* genes would be needed to produce the target linolenic acid content in the high oleic acid background.

The *FAD3A* gene was shown to have a greater impact on linolenic acid level in soybean seed than *FAD3B* and *FAD3C*, consistent with higher expression of *FAD3A* in developing seeds (Bilyeu et al. 2003, 2005). We hypothesized that crossing an 80 % high oleic soybean line carrying two mutant *FAD2-1* genes to a low linolenic soybean line carrying two mutant *FAD3A* and *FAD3C* genes will produce in the progeny soybeans with oleic acid content more than 80 % and linolenic acid content lower than 3 %. Although this may require incorporation of four genes into one soybean line and a larger effort to develop populations, it may be possible to achieve this goal using only a single *FAD3* mutation in combination with two *FAD2* mutations. The objectives of this study were to : (1) combine two mutant *FAD2-1* genes with one or two mutant *FAD3* genes to produce high oleic acid (>75 %) low linolenic acid (<3 %) soybean (HOLL); (2) examine the stability of the HOLL lines in appropriate environments to select the most stable phenotype/genotype combination.

Materials and methods

Population development

The soybean line designations and genotype descriptions used here are provided in Table 1. The two crosses made for

Table 1 Gene combination of high oleic low linolenic acid (HOLL), parental, and control lines

Genotype	Gene				
	FAD2-1A	*FAD2-1B*	*FAD3A*	*FAD3B*	*FAD3C*
HO(Δ)**LL4ac**	Δ[a]	P137R[d]	Splice site ($G^{810}A$)[e]	WT	G128E[g]
HO(Δ)**LL3a**	Δ	P137R	Splice site ($G^{810}A$)	WT	WT
HO(Δ)**LL3c**	Δ	P137R	WT	WT	G128E
Parent: HO(Δ)	Δ	P137R	WT	WT	WT
Parent: MO(Δ)LLac	Δ	WT	Splice site ($G^{810}A$)	WT	G128E
HO(S117N **LL4ac**	S117N[b]	P137R	Splice site ($G^{810}A$)	WT	G128E
HO(S117N) **LL3a-1**	S117N	P137R	Splice site ($G^{810}A$)	WT	WT
HO(S117N) **LL3a-2**	S117N	P137R	Splice site ($G^{810}A$)	WT	WT
HO(S117N) **LL3a-3**	S117N	P137R	Splice site ($G^{810}A$)	WT	WT
HO(S117N)LL**3c**	S117N	P137R	WT	WT	G128E
Parent: HO(S117N)	S117N	P137R	WT	WT	WT
Parent: NOLLac	WT[c]	P137R	Splice site ($G^{810}A$)	WT	G128E
B1-52abc	WT	WT	Splice site ($G^{810}A$)	Splice site ($G^{466}A$)[f]	G128E
Williams 82	WT	WT	WT	WT	WT

[a] *FAD2-1A* allele derived from M23 (Sandhu et al. 2007)

[b] *FAD2-1A* allele derived from 17D (Dierking and Bilyeu 2009)

[c] A wild-type allele of the gene

[d] *FAD2-1B* allele derived from PI 283327 (Pham et al. 2010)

[e] *FAD3A* allele derived from CX1512-44 (Bilyeu et al. 2005)

[f] *FAD3B* allele derived from A29 (Bilyeu et al. 2006)

[g] *FAD3C* allele derived from CX1512-44 (Bilyeu et al. 2005)

this study were: Cross 1 = HO(Δ)LL, the high oleic soybean line S08-1692 [designated HO(Δ): homozygous for mutant M23 *FAD2-1A*Δ allele and mutant PI 283327 *FAD2-1B* P137R allele] was crossed to soybean line KB07-1 #123, a mid-oleic low linolenic acid [designated MO(Δ)-LLac: homozygous for mutant M23 *FAD2-1A*Δ allele, homozygous for mutant CX1512-44 *FAD3A* and *FAD3C* alleles] (Bilyeu et al. 2005). The mutant *FAD2-1A*Δ alleles were fixed in this population while the mutation in *FAD2-1B* is the P137R missense mutation from PI 283327, and the *FAD3A* splice-site mutation and *FAD3*C G128E misense mutation are derived from CX1512-44 (Bilyeu et al. 2005; Pham et al. 2010). Cross 2 = HO(S117N)LL, an early flowering high oleic F_2 parent 17D/PI 283327 #67 or #92 [designated HO(S117N): homozygous for mutant 17D *FAD2-1A* S117N alleles and PI 283327 *FAD2-1B* P137R alleles], was crossed to 10–73, a normal oleic, 3 % linolenic acid line (designated NOLLac: wild-type for *FAD2-1A* and *FAD2-1B*, homozygous for mutant *FAD3A* and *FAD3C* alleles from CX1512-44). The two mutant *FAD2-1* alleles (*FAD2-1A* S117N and *FAD2-1B* P137R) as well as mutant *FAD3A* and *FAD3C* were segregating in this population. The mutant *FAD2-1B*, *FAD3A* and *FAD3C* genes used in this research are identical, while two mutant *FAD2-1A* alleles were evaluated: the *FAD2-1A* alleles donated from either M23 (Δ) or 17D (S117N) (Dierking and Bilyeu 2009; Pham et al. 2010; Sandhu et al. 2007).

Naming convention: the combination of four mutant genes: *FAD2-1A*, *FAD2-1B*, *FAD3A* and *FAD3*C herein is represented as either HO(Δ)**LL4ac** or HO(S117N)**LL4ac**, to distinguish the null *FAD2-1A* allele donated from either M23 (Δ) or 17D (S117N) (Dierking and Bilyeu 2009; Sandhu et al. 2007). The three mutant gene combination of *FAD2-1A*, *FAD2-1B* and *FAD3A* is designated as HO(Δ or S117N) **LL3a**, while the three mutant gene combination of *FAD2-1A*, *FAD2-1B* and *FAD3C* is designated as HO(Δ or S117N)**LL3c**. An additional control line included the 1 % linolenic acid line B1-52abc with wild-type *FAD2-1* genes and mutant *FAD3A*, *FAD3B*, and *FAD3C* genes (splice site mutant *FAD3B* alleles derived from A29 (Bilyeu et al. 2006). The reference cultivar Williams 82 with wild-type alleles of the *FAD2* and *FAD3* genes was also used as a check.

True F_1 seeds were planted in the field to produce F_2 seeds, which were germinated and genotyped for three mutant genes [*FAD2-1B*, *FAD3A* and *FAD3C* in Cross 1's population (HO(Δ)LL)] or four mutant genes [Cross 2's population (HO(S117N)LL)], respectively.

For the Cross 1, nine F_2 plants with the genotype *FAD2-1 (aabb) FAD3(AaCc)* and two plants for each of the three genotypes *FAD2-1(aabb) FAD3(aacc)* = HO(Δ)**LL4ac**,

FAD2-1(*aabb*) *FAD3*(*aaCC*) = HO(Δ)**LL3a** or *FAD2-1*(*aabb*) *FAD3*(*AAcc*) = HO(Δ)**LL3c** were identified. For this cross, only F_2 plants with the desirable gene combinations *FAD2-1(aabb) FAD3(aaCC/AAcc/aacc)* were selected and advanced to $F_{2:3}$ generation in Sears growth chamber, University of Missouri, Columbia, MO. $F_{2:3}$ seeds were harvested, bulked by genotype [HO(Δ)**LL4ac**, HO(Δ)**LL3c** or HO(Δ)**LL3c**] and were used for stability test at the Bradford Research and Extension Center (BREC) in Columbia, MO and Delta Research Center, Portageville, MO. Data from field produced F_4 seeds were used for statistical analysis.

For the Cross 2 [HO(S117N)LL], ten true F_1 plants were identified and produced seed at a winter nursery center near Upala, Costa Rica. The F_2 populations were planted at the winter nursery in February 2010, and DNA samples from 289 individual F_2 plants were collected on Whatman FTA (Whatman, Clifton, NJ, USA) cards for genotyping assays conducted in our lab at the University of Missouri, Columbia, MO in March 2010. After genotyping, one F_2 plant with the genotype *FAD2-1*(aabb) *FAD3*(AaCc), and two plants with genotype *FAD2-1*(aabb) *FAD3*(aaCC) were advanced to $F_{2:3}$ seeds in the nursery center. A total of 120 F_3 seeds were harvested from the F_2 plant with genotype *FAD2-1*(aabb) *FAD3*(AaCc) in May 2010, shipped to Columbia, MO and germinated there for another round of genotyping. Subsequently, three F_3 soybean plants with genotype *FAD2-1*(aabb) *FAD3C*(aacc) = HO(S117N) **LL4ac**, four with genotype *FAD2-1(aabb)FAD3(aaCC)* = HO(S117N)**LL3a-3**, and one with genotype *FAD2-1*(aabb) *FAD3C*(AAcc) = HO(S117N)**LL3c** were identified and transplanted to the field at BREC in Columbia, MO for seed production. Approximately two hundred F_3 seeds were harvested from each of the two F_2 plants in the winter nursery with genotype *FAD2-1*(aabb) *FAD3*(aaCC), designated as HO(S117 N)**LL3a-1** and HO(S117N)**LL3a-2**, and F_3 seeds from each of the two F_2 plants with genotype *FAD2-1*(aabb) *FAD3*(aaCC) were also directly planted in a row at the same time as the transplants in the same field in Columbia, MO for seed increase. F_3 plants were single plant threshed for F_4 seeds, and three random individual plants for either HO(S117N)**LL3a-1** or HO(S117N)**LL3a-2** genotype were used for fatty acid analysis together with seeds from plants with genotypes HO(S117N)**LL4ac**, HO(S117N)**LL3a-3** and HO(S117N)**LL3c**.

Allele-specific molecular marker assays

A SimpleProbe assay for the mutation in the *FAD2-1A* of 17D was developed based on the SimpleProbe protocol described by Pham et al. (2010). The Probe contained 5′-Fluorescein-SPC-GTACTTGCTGAAGGCATGGTGA-Phosphate-3′ [the underlined base is mutated to T in the *FAD2-1A* (S117N) allele]. 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 5:1 asymmetric mix of primers (5′-CCAAGGTTG CCTTCTCACTGGT-3′ at 0.5 μM final concentration, and 5′-TAGGCCACCCTATTGTGAGTGTGAC-3′ at 0.1 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 0.2× Titanium Taq polymerase (BD Biosciences, Palo Alto, CA, USA). Genotyping reactions were performed using a Lightcycler 480 II real time PCR instrument (Roche Applied Sciences, Indianapolis, IN, USA), 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. 17D and all soybean lines with an identical mutant *FAD2-1A* allele had a characteristic peak at 54 °C, while Williams 82 (wild-type *FAD2-1A*) had a peak at 62 °C. Heterozygous individuals showed both peaks.

Similarly, SimpleProbe assays were developed for the *FAD2-1B* (P137R) allele from PI 283327. The *FAD2-1B* probe consisted of 5′-Fluorescein-AGTCCCTTATTTC TCATGGAAAATAAGC-Phosphate-3′ [C>G mutation (P137R allele from PI 283327) is underlined]. Genotyping reactions were performed with a 5:2 asymmetric mix of primers (5′-GGTTCTCCAAGGTTGCATTCTTACT-3′ at 0.2 μM final concentration, and 5′-AGGGTTGTTCAGGT ACTTGGTGT-3′ at 0.5 μM final concentration). Reactions were carried out as described above, using the following PCR parameters: 95 °C for 5 min followed by 40 cycles of 95 °C for 20 s, 60 °C for 20 s, 72 °C for 20 s, and then a melting curve from 50 to 70 °C. PI 283327 and all soybean lines with an identical *FAD2-1B* allele had a characteristic peak at 56.5 °C, wild-type *FAD2-1B* samples had a peak at 62.5 °C. Heterozygous individuals showed both peaks.

When genotyping the F_2 seeds of the Cross 1, seeds were chipped to get a small portion for fatty acid determination (data not shown). The remaining portion with embryo was germinated in germination packages to collect unifoliate leaf tissue for DNA on Whatman FTA cards, and the DNA was collected for PCR according to the manufacturer's instructions. PCR templates were prepared by using a hammer to pound a folded leaflet enclosed in the FTA card, excision of the template sample with a 1.2-mm micropunch, and two rounds each of 5 min washes with 100 μl FTA reagent (Whatman) and TE^{-1} (10 mM Tris–HCl, 0.1 mM EDTA, pH 8.0). F_2 seeds of the Cross 2 were

not chipped and DNAs were collected from the F_2 plants, which were grown in the field in our nursery center near Upala, Costa Rica.

For Cross 1, genotyping assays were conducted to select genotypes of interest in the F_2 generation while for Cross 2, genotyping assays were conducted to select desirable genotypes in F_2 and F_3 generations. SimpleProbe assays of *FAD3A* and *FAD3C* alleles were conducted as described (Bilyeu et al. 2011).

Analysis of fatty acid, oil and protein contents

Fatty acid profiles of individual F_4 seeds were determined by gas chromatography of total fatty acid methyl esters of extracted oil (Beuselinck et al. 2006). For the field produced seeds from the Cross 1-derived lines, seeds from five plants in one replication of a genotype were bulked and five seeds from each bulk were assayed individually for fatty acid composition. For the field produced seeds from the HO(S117N)LL-derived lines, seeds from individual F_3 plants of each genotype were bulked and five F_4 seeds from each bulk were assayed individually for fatty acid composition.

Oil and protein contents were determined by near infrared reflectance (NIR) spectroscopy (NIR System Model 6500; Silver Springs, MD, USA) on whole seed samples, as described (Hartwig and Hurburgh 1990; Choung et al. 2001).

Stability of high oleic acid and low linolenic acid soybean

F_3 seeds of three genotypes HO(Δ)**LL4ac**, HO(Δ)**LL3a** and HO(Δ)**LL3c** from the Cross 1 were planted in Columbia, MO on Mexico silt loam soil on May 28th 2010 and in Portageville, MO on Dundee silt loam soil on June 2nd 2010 together with the parents, MO(Δ)LLac and a soybean line with the same *FAD2* genotype as S08-1692 HO(Δ), and checks including Williams 82 [Maturity Group (MG) III with wild-type *FAD2-1* and *FAD3* genes], B1-52 (1 % linolenic acid content controlled by three mutant *FAD3* genes). There were three replications for each location utilizing Randomized Complete Block Design. 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 5 cm apart. After seedling emergence, hills were thinned to five plants from which all data were collected for each soybean line.

For the Cross 2 (HO(S117N)LL), at the F_3 generation when the mutants *FAD2-1A* and *FAD2-1B* were fixed, the plants containing wild-type and mutant alleles of *FAD3A* and *FAD3C* were selected based on seedling genotype: HO(S117N)**-** (two F_3 plants, with mutant *FAD2-1* genes and wild-type *FAD3A* and *FAD3C* genes), HO(S117N)**LL4ac**- (three F_3 plants), HO(S117N)**LL3a-3**- (four F_3 plants) and HO(S117N)**LL3c**- (one F_3 plant). These selected genotyped seedlings were transplanted 10 cm apart from each other in a hill plot adjacent to the experiment for the population derived from Cross 1 at the BREC on June 9th, 2010. F_3 seed rows from each of two lines with either HO(S117N)**LL3a-1** or HO(S117N)**LL3a-2** genotype were grown in the same field. Ten seeds each for the two parents and control lines were also planted in the field at the same time.

Data were determined from F_4 seeds for Cross 2 with genotypes HO(S117N)**LL4ac**, HO(S117N)**LL3a**, and HO(S117N)**LL3c** derived from one F_2 high oleic acid plant heterozygous for *FAD3*A and *FAD3*C and two additional HO(S117N)**LL3a** lines derived from two independent F_2 high oleic acid plants that were genotyped to be homozygous for mutant *FAD3*A and wild-type *FAD3*C. The three HO(S117N)**LL3a** lines are designated as HO(S117N)**LL3a-1**, HO(S117N)**LL3a-2**, HO(S117N)**LL3a-3**.

Statistical analysis

The normal distribution of the data of each fatty acid species for each genotype in both populations was examined using Kolmogorov–Smirnov test in SAS 9.2 software (SAS Institute Inc. 2008). Because the data of the Cross 1 [HO(Δ)LL] showed normal distribution for all of the five fatty acid species or for each of the four or three gene combination of interest, the genotype and environmental interactions (G × E) for each fatty acid species of Cross 1-derived lines were evaluated using proc mixed procedure in SAS 9.2. Due to the G × E interaction found for stearic acid and linolenic acid, the fatty acid data of each genotype from two locations were not combined, and the comparison of fatty acid composition of any pair of genotypes in the Cross 1 was conducted with proc mixed procedure for each of the two testing environments. For the Cross 2 [HO(S117N)LL], HO(S117N)**LL4ac** and HO(S117N)**LL3a-1** were not normally distributed while data of the other genotypes were. Therefore, comparisons of significant differences between any two soybean HO(S117N)LL lines or between a HO(S117N)LL and a parental line or a control line in Cross 2 were conducted using Wilcoxon rank-sum test in SAS 9.2 program with significant level $\alpha = 0.05$ (Wilcoxon 1945).

Results

We developed two populations of high oleic acid soybean lines containing the same mutant *FAD2-1B* alleles, but that differed in their mutant *FAD2-1A* alleles, with the lines derived from Cross 1 containing the deletion alleles

[*FAD2-1A*(Δ)] from M23, and the lines from Cross 2 containing the missense alleles from 17D [*FAD2-1A* (S117N)]. To determine the allele combinations necessary to produce HOLL oil, lines were identified with different combinations of functional and mutant *FAD3A* and *FAD3C* alleles in the high oleic acid background in both populations and evaluated for fatty acid profile. Lines derived from Cross 1 were evaluated in two locations, while lines derived from Cross 2 were evaluated in one location.

The HOLL soybean lines with null *FAD2-1A*(Δ) alleles derived from M23 (Cross 1)

Cross1: combination of four mutant genes (FAD2-1aabb FAD3aacc)

Because interaction of genotype and environment was found significant for stearic and linolenic acids (data not shown), all the data of the cross 1 were presented by location (Table 2). At Portageville, MO, except for stearic and oleic acids, the contents of the other three fatty acids of HO(Δ)**LL4ac** were significantly different from those of HO(Δ)**LL3a** or HO(Δ)**LL3c** (Table 2). The oleic acid contents of HO(Δ)**LL4ac** in both locations were approximately 85 % on average, and not significantly different from those of HO(Δ)**LL3a** or HO(Δ)**LL3c**. The linolenic acid content of HO(Δ)**LL4ac** was 1.5 %, significantly higher than that of B1-52abc, the low linolenic acid control soybean line (1.2 %) and lower than that of HO(Δ)**LL3c** (2 %), but not significantly different from that of HO(Δ)**LL3a** (1.8 %) (Table 2). In contrast, at Columbia, MO the linolenic acid content of HO(Δ)LL**4ac** was 1.9 %, significantly higher than that of B1-52abc (1.3 %), but significantly lower than those of HO(Δ)**LL3a** or HO(Δ)**LL3c**, which were 2.6 and 2.5 %, respectively (Table 2). In this location, stearic acid, oleic and linoleic acid contents of the four mutant gene combination were also significantly different from those of the three mutant gene combinations while no difference was seen for palmitic acid contents. Stearic acid content of HO(Δ)**LL4ac** in Columbia, MO was significantly lower than that of HO(Δ)**LL3a**, but not different from that of HO(Δ)**LL3c**. Oleic acid content of HO(Δ)**LL4ac** in Columbia, MO was significantly lower than that of HO(Δ)**LL3c**, but not different from that of HO(Δ)**LL3a**. Linoleic acid content of HO(Δ)**LL4ac** was significantly higher than that of both HO(Δ)**LL3a** and HO(Δ)**LL3c**, which reflects less enzymatic activity for two mutant *FAD3* genes combined compared to one mutant *FAD3* gene in HO(Δ)**LL3a** and HO(Δ)**LL3c**. Location-wise, palmitic, oleic, and linoleic

Table 2 Fatty acid profiles and protein and oil contents for high oleic low linolenic acid [HO(Δ)LL] soybeans with M23 *FAD2-1A* allleles in field trials in Portageville and Columbia, MO in summer 2010

	Fatty acid					Seed component	
	16:0	18:0	18:1	18:2	18:3	Oil	Protein
	Percent of total fatty acid					Percent of seed at 13 % moisture	
Genotype			Portageville, MO				
HO(Δ)**LL4ac**[a]	7.9 ± 0.2c[b]	2.7 ± 0.2a	85.3 ± 0.9a	2.5 ± 0.6b	1.5 ± 0.1b	18.1 ± 0.1	39.3 ± 0.4
HO(Δ)**LL3a**	7.6 ± 0.2b	2.9 ± 0.3a	85.9 ± 1.0a	1.9 ± 0.4a	1.8 ± 0.4bc	18.4 ± 0.3	39.8 ± 0.3
HO(Δ)**LL3c**	7.5 ± 0.0b	2.8 ± 0.1a	85.9 ± 0.4a	1.8 ± 0.2a	2.0 ± 0.2c	18.1 ± 0.3	39.8 ± 0.9
Parent: HO(Δ)	7.2 ± 0.2a	4.4 ± 0.3c	82.8 ± 0.4b	2.4 ± 0.1b	3.2 ± 0.2e	19.4 ± 0.4	38.6 ± 0.3
Parent: MO(Δ)LLac	10.8 ± 0.4d	3.8 ± 0.1b	29.6 ± 2.0c	53.1 ± 1.9c	2.6 ± 0.2d	19.1 ± 0.4	36.8 ± 1.5
B1-52abc	10.7 ± 0.6d	3.9 ± 0.3b	28.1 ± 5.7c	56.2 ± 5.3d	1.2 ± 0.1a	20.0 ± 0.3	33.9 ± 0.6
Williams82	11.2 ± 0.2e	3.7 ± 0.2b	22.5 ± 2.7d	56.1±2.0d	6.5 ± 0.6f	19.4 ± 0.1	35.9 ± 0.1
Genotype			Columbia, MO				
HO(Δ)**LL4ac**	7.5 ± 0.1b	2.9 ± 0.1a	84.5 ± 0.7b	3.3 ± 0.6c	1.9 ± 0.2b	17.5 ± 0.5	39.5 ± 0.5
HO(Δ)**LL3a**	7.5 ± 0.2b	3.3 ± 0.2b	84.4 ± 0.6b	2.2 ± 0.3b	2.6 ± 0.2c	17.9 ± 0.6	39.1 ± 0.7
HO(Δ)**LL3c**	7.5 ± 0.1b	2.8 ± 0.1a	85.9 ± 0.5a	1.4 ± 0.4a	2.5 ± 0.1c	17.6 ± 0.3	39.8 ± 0.3
Parent: HO(Δ)	7.3 ± 0.1a	3.5 ± 0.2b	82.7 ± 1.4b	2.0 ± 1.0ab	4.2 ± 0.6d	19.1 ± 0.2	37.8 ± 0.5
Parent: MO(Δ)LLac	10.1 ± 0.0c	3.8 ± 0.1c	33.6 ± 1.9c	49.8 ± 1.8d	2.6 ± 0.1c	18.7 ± 0.2	36.1 ± 0.3
B1-52abc	10.2 ± 0.1c	4.2 ± 0.1d	24.9 ± 0.9d	59.4 ± 0.8f	1.3 ± 0.1a	20.1 ± 0.1	35.2 ± 0.6
Williams 82	10.8 ± 0.1d	3.8 ± 0.1c	21.0 ± 0.5e	57.1 ± 0.9e	7.4 ± 0.3e	19.5 ± 0.2	33.8 ± 0.6

[a] Combination of *FAD2* and *FAD3* genes were described in Table 1

[b] 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; two values with same letter are not statistically different at $\alpha = 0.05$

acid contents of HO(Δ)**LL4ac** lines were significantly different between two testing locations.

Compared to its parents, HO(Δ)**LL4ac** had significantly higher palmitic acid, and linoleic acid contents, and lower stearic acid and linolenic acid contents than those of the high oleic parent HO(Δ) in both Columbia, MO and Portageville, MO. However, in Portageville, MO the linolenic acid content was not significantly different for HO(Δ)**LL4ac** from that of the high oleic acid parent, but the oleic acid content was significantly higher for HO(Δ)**LL4ac** from that of the high oleic acid parent. HO(Δ)**LL4ac** also had significantly lower linolenic acid contents than those of the mid oleic low linolenic parent MO(Δ)**LLac** in both locations. Compared to the control cultivar 'Williams 82', there was a significant change in the fatty acid profile of HO(Δ)LL**4ac** soybeans as it was observed a significantly higher oleic acid content and a significant reduction in contents of all of the other fatty acids (Table 2). It was reported that there is often a reduction in palmitic acid content but not stearic acid content in high oleic acid soybean (80 %) compared to the commodity soybean cultivar Williams 82 (Hoshino et al. 2010; Pham et al. 2010). The HO(Δ)**LL4ac** soybean line developed in this study showed a significant increase in palmitic acid content and a significant reduction in stearic acid content compared to the high oleic parent line, and a significant reduction in both palmitic and stearic acid contents compared to those of Williams 82 (Table 2). The smallest significant change observed was for stearic acid, which was about a 25 % reduction in the high oleic acid lines compared to the wild-type stearic acid content of Williams 82 (from 3.6 to 2.7 %). Connected to the dramatic change in oleic acid content in the high oleic acid lines was a reduction in linoleic acid content, which was equivalent to a 97 % reduction compared to the wild-type linoleic acid content of Williams 82 (from 56 % to less than 3 %).

Cross1: combination of three mutant genes (FAD2-1aabb FAD3aaCC or FAD2-1aabb FAD3AAcc)

There were no significant differences in fatty acid contents between HO(Δ)**LL3a** and HO(Δ)**LL3c** in Portageville, MO. In average, both HO(Δ)**LL3a** and HO(Δ)**LL3c** have about 86 % oleic, 2 % linoleic and 2 % linolenic acid. However, in Columbia, MO these two genotypes have statistically different contents of stearic, oleic, and linoleic acid but not the contents of palmitic and linolenic acid. Location-wise, only the linolenic acid content of the three mutant gene combination is influenced by the planting location with linolenic acid levels in Columbia, MO significantly higher than those of Portageville, MO (P value <0.0001 for linolenic and >0.05 for other four fatty acid species).

HO(Δ)**LL3a** or HO(Δ)**LL3c** lines had significantly higher oleic acid contents in Portageville, MO compared to that of the high oleic acid parent (Table 2). In Columbia, MO the oleic acid content of the HO parent was significantly lower than that of HO(Δ)**LL3c**, but not significantly different from that of HO(Δ)**LL3a.** These two genotypes had significantly lower linolenic acid content in both locations compared to the linolenic acid content of the high oleic acid parent. In addition, the linolenic acid content of HO(Δ)**LL3a** and HO(Δ)**LL3c** was significantly lower in Portageville, MO but was not significantly different to that of the MO(Δ)LL parent in Columbia, MO. Compared to Williams 82, HO(Δ)**LL3a** or HO(Δ)**LL3c** lines not only had higher oleic and lower linolenic acid content as targeted but also had significantly lower content of palmitic, stearic and linoleic acids. The largest difference was seen for linoleic acid content with a reduction from 57 % in Williams 82 to under 3 % in both HO(Δ)LL3 lines and the least difference was seen for stearic acid content with reduction from 3.7–3.8 % in Williams 82 to 2.8–3.3 % in both HO(Δ)LL3 lines.

HOLL soybean lines with S117N missense *FAD2-1A* alleles derived from 17D (Cross 2)

Because Cross 2 involved the segregation of all four candidate genes, an alternate strategy was employed to obtain enough plants with the selected genotypes for the evaluation. This strategy involved the identification of two homozygous plants in the F_2 generation that were advanced as F_3 lines. In addition, the remainders of the tested F_3 plants were siblings that were selected for contrasting *FAD3* genotypes derived from a single F_2 plant that was heterozygous for both *FAD3A* and *FAD3C*.

Cross 2: combination of four mutant genes (FAD2-1aabb FAD3aacc)

HO(S117N)**LL4ac** had significantly lower palmitic and linolenic acid contents, higher linoleic content and similar stearic and oleic acid contents compared to HO**LL3a-1** (Table 3). Compared to HO**LL3a-2**, HO(S117N)**LL4ac** had significantly higher palmitic content and lower linolenic acid content, and similar stearic, oleic and linoleic acid contents. Fatty acid composition of HO(S117N)**LL4ac** was not significantly different from that of HO(S117N)**LL3a-3**, except for linolenic acid content. Linolenic acid content of HO(S117N)**LL4ac** (2.5 %) was significantly lower than that of HO(S117N)**LL3a-3** (3.5 %).

The fatty acid composition of HO(S117N)**LL4ac** was significantly different from that of HO(S117N)**LL3c** except for stearic and oleic acids. The palmitic and linolenic acid contents of HO(S117N)**LL4ac** were significantly

Table 3 Fatty acid profiles and protein and oil contents for high oleic low linolenic acid [HO(S117N)LL] soybeans with 17D *FAD2-1A* alleles in field trials in Columbia, MO in summer 2010

		Fatty acid					Seed component	
	N	16:0	18:0	18:1	18:2	18:3	Oil	Protein
		Percent of total fatty acid					Percent of seed at 13 % moisture	
Genotype			Columbia, MO					
HO(S117N)**LL4ac**[a]	15[b]	7.0 ± 0.2b[c]	3.6 ± 0.5ab	80.2 ± 1.5ab	6.7 ± 1.3b	2.5 ± 0.7b	19.4 ± 0.1	38.2 ± 0.0
HO(S117N)**LL3a**-1	12	7.4 ± 0.3c	3.7 ± 0.5b	80.8 ± 0.8a	4.9 ± 0.8a	3.1 ± 0.5c	20.2 ± 0.6	36.6 ± 0.2
HO(S117N)**LL3a**-2	15	6.4 ± 0.2a	3.6 ± 0.1b	79.7 ± 1.2ab	7.0 ± 0.8b	3.3 ± 0.3cd	20.1 ± 0.1	36.4 ± 0.6
HO(S117N)**LL3a**-3	20	7.0 ± 0.3b	3.6 ± 0.4ab	79.5 ± 2.1ab	6.3 ± 1.4b	3.5 ± 0.5d	19.9 ± 0.1	37.3 ± 0.3
HO(S117N)**LL3c**	15	7.7 ± 0.4d	3.3 ± 0.1a	80.1 ± 2.3ab	4.3 ± 2.0a	4.5 ± 0.5e	19.4 ± 0.2	37.4 ± 0.5
HO(S117N)	10	7.1 ± 0.2b	3.4 ± 0.2a	80.1 ± 1.7ab	4.3 ± 0.8a	5.3 ± 0.7f	19.7 ± 0.5	36.7 ± 1.4
Parent: HO(S117N)	4	7.6 ± 0.4cd	3.3 ± 0.4a	80.1 ± 2.2ab	4.0 ± 1.0a	4.9 ± 1.0ef	19.4 ± 0.7	37.8 ± 1.5
Parent: NOLLac	10	11.6 ± 0.4g	4.2 ± 0.3c	21.8 ± 3.0d	59.9 ± 2.8d	2.5 ± 0.1b	20.4 ± 0.3	33.6 ± 0.2
B1-52abc	15	10.2 ± 0.2e	4.2 ± 0.3c	24.9 ± 3.0c	59.4 ± 2.9d	1.3 ± 0.1a	20.1 ± 0.1	35.2 ± 0.6
Williams 82	15	10.8 ± 0.3f	3.8 ± 0.3b	21.0 ± 1.6d	57.1 ± 1.4c	7.4 ± 0.6g	19.5 ± 0.2	33.8 ± 0.6

[a] Combination of *FAD2* and *FAD3* genes were described in Table 1

[b] Number of seeds used for fatty acid analysis

[c] Mean value ± standard deviation was obtained by averaging fatty acid values of all seeds used for fatty acid analysis for each genotype, five seeds for each plant with the genotype of interest, except for HO(S117N)LL**3c** in which all the seeds were from one plant only. Letters of significance are based on *P* values of Wilcoxon rank-sum tests. Two values with same letter are not statistically different at $\alpha = 0.05$

lower than those of HO(S117N)**LL3c**, while the linoleic acid content was significantly higher than that of HO(S117N)**LL3c** (Table 3).

The oleic acid content of HO(S117N)**LL4ac** (80.2 %) was not significantly different from that of the high oleic parent HO(S117N) but significantly higher than that of Williams 82 (Table 3). Its linolenic acid content was not significantly different from that of 10–73, the normal oleic low linolenic acid parent NOLLac with similar mutants *FAD3A* and *FAD3C*, but was significantly lower than those of the HO(S117N) parent and Williams 82 (Table 3). The palmitic acid content of HO(S117N)**LL4ac** was significantly lower than that of the HO(S117N) parent, the NOLLac parent and Williams 82. The stearic acid content of this genotype was not different from those of the HO(S117N) parent and Williams 82, but significantly lower than that of the NOLLac parent. The linoleic acid content of HO(S117N)**LL4ac** showed a significant reduction compared to those of the NOLLac parent and Williams 82, but a significant increase compared to that of the HO(S117N) parent.

Cross 2: combination of three mutant genes genes (FAD2-1aabb FAD3aaCC or FAD2-1aabb FAD3AAcc)

In contrast to the situation with HO(Δ)LL lines, there was a marked difference between fatty acid composition of seeds of HO(S117N)**LL3a** and HO(S117N)**LL3c**. HO(S117N)**LL3c** soybean lines had significantly higher palmitic and linolenic acid contents, lower stearic and linoleic content and similar oleic acid content compared to the three HO(S117N)**LL3a** lines (Table 3). There were two exceptions in which HO(S117N)**LL3c**'s stearic acid content was not significantly different from that of HO(S117N)**LL3a-3**, and the linoleic acid content of this genotype was not significantly different from that of HO(S117N)**LL3a-1**. Three HO(S117N)**LL3a** lines had stearic and oleic acid contents not significantly different from each other with approximately 3.7 % stearic and 80 % oleic acid. Their palmitic, linoleic acid and linolenic acid contents varied depending on the individual line. HO(S117N)**LL3a-2** had the lowest palmitic acid content (6.4 %) and highest linoleic acid content (7 %) among three HO(S117N)**LL3a** lines (Table 3). The linolenic acid content of HO(S117N)**LL3a-1** was significantly lower than that of HO(S117N)**LL3a-3**, and the linolenic acid content of HO(S117N)**LL3a-2** is in between these two values and not different from that of either HO(S117N)**LL3a-1** or HO(S117N)**LL3a-3**. The oleic acid contents of all the HO(S117N)**LL3a** or HO(S117N)**LL3c** were not significantly different from that of the HO(S117N) parent, but significantly higher than those of the NOLLac parent and Williams 82. In addition, their linolenic acid contents were significantly lower than that of Williams 82, but higher than that of the NOLLac parent. The palmitic acid contents of HO(S117N)**LL3a-2** and HO(S117N)**LL3a-3**, but not

HO(S117N)**LL3a-1** and HO(S117N)**LL3c**, were significantly lower than those of the two parents and Williams 82.

Comparison between Cross 1 and Cross 2 lines in Columbia, MO

The total content of linoleic and linolenic acid in Cross 1 lines was approximately 5 % lower than those in Cross 2 lines (Fig. 1). On the other hand, the oleic acid contents of Cross 1 lines were 5 % higher than those of Cross 2 lines (Tables 2, 3). Unlike Cross 1 lines, which showed an increase in palmitic acid content and a reduction in stearic acid content compared to those of their high oleic acid parental line, Cross 2 lines generally had an equal or significantly reduced content of palmitic acid and an equal or significantly increased content of stearic acid content compared to those of their high oleic acid parent.

Agronomic characteristics and oil and protein content of HOLL lines

The soybean lines used in this study responded similarly to the photoperiod requirement for flowering time and maturity, with maturity equivalent to those of MG III. The plants from population 1 were short, with thick green leaves, and appeared to have inherited a dominant gene for determinate growth habit (Tian et al. 2010). Seed weight of these soybean lines with *FAD2-1A* derived from either M23 or 17D was not different from that of the parents (data not shown).

In Columbia, MO, average days from planting to harvesting of HO(Δ)**LL4ac** and HO(Δ)**LL3c** were 111 days, of HO**LL**(Δ)**3a** was 118 days, which were similar to Williams 82 with MG III (114 days). In Portageville, MO, average days from planting to harvesting of HO(Δ)**LL4ac** were 110, of HO(Δ)**LL3a** was 106, of HO(Δ)**LL3c** was 111, which were also similar to Williams 82 (112 days). In Columbia, MO, average days from planting to harvesting of HO(S117N)**LL4ac** was 116 days, of HO(S117N)**LL3a** and HO(S117N)**LL3c** was around 120 days.

The lines HO(Δ)**LL4ac**, HO(Δ)**LL3a** and HO(Δ)**LL3c** had reduced oil (approximately 1 %) compared to those of their Cross 1 parents and Williams 82, but this was not observed for Cross 2 lines (Tables 2, 3). The protein contents of the Cross 1 lines were approximately 1 % higher than those of the parents and 3–4 % higher than that of Williams 82. While the oil contents of Cross 2 lines, in general, were similar to those of the parents and Williams 82, the protein contents of these lines were 3–5 % higher than those of the parents and Williams 82. The oil and protein contents of HO**LL4ac** were not significantly different from those of HO**LL3a** and HO**LL3c** independent of the source of the mutant *FAD2-1A* alleles.

Discussion

Conventional breeding and genetic engineering have been employed to combine elevated oleic acid and low linolenic acid in one soybean line (Brace et al. 2011; Rahman et al. 2001). The mid oleic acid low linolenic acid soybean developed by Rahman et al. (2001) had approximately 55 % oleic acid and 4.2 % linolenic acid and possesses three mutant genes: *FAD2-1A*(Δ) from M23, mutant

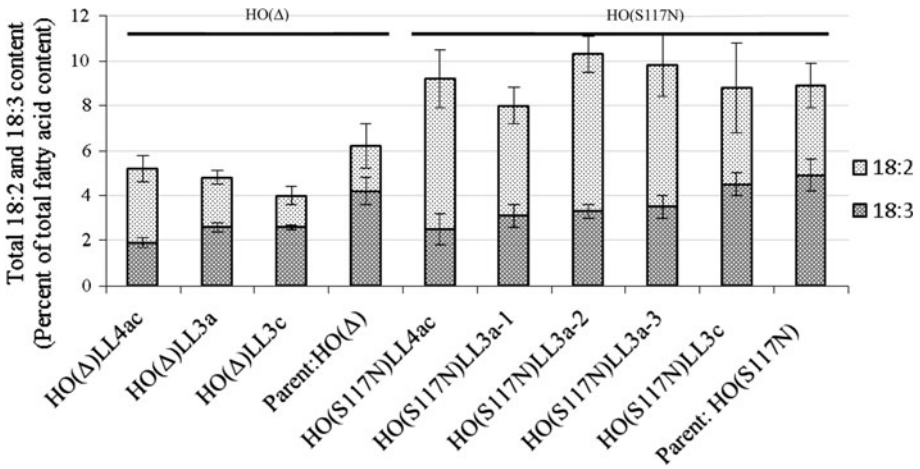

Fig. 1 Seed linoleic (18:2) and linolenic acid (18:3) content of HOLL soybean lines developed with either M23 or 17D *FAD2-1A* alleles in Columbia summer 2010. Linoleic and linolenic acid phenotype data of each genotype are the mean of linoleic and linolenic acid content as a percentage of the total fatty acid content of the oil. *Error bars* for the HO(Δ) lines represent plus and minus one standard deviation from the mean from three replicates; standard deviation for HO(S117N) lines was obtained by averaging fatty acid values of all seeds used for fatty acid analysis for each genotype, five seeds for each plant with the genotype of interest, except for HO(S117N)LL**3c** in which all the seeds were from one plant only

FAD3A from M24 and *FAD3B* from M5 (Anai et al. 2005; Rahman et al. 2001). By combination of a transgenic event which silences transcriptional activities of *FAD2-1A* and *FAD2-1B* genes and two mutant *FAD3A* (C1640) and *FAD3B* (RG10) genes, HOLL soybeans with oleic acid content in the range of 77–79 % and linolenic acid content in the range of 2.1–2.8 % have been produced by Brace et al. (2011). The novelty of our study is the production of non-transgenic HOLL lines conditioned by three or four mutant genes with similar fatty acid profiles to that of transgenic HOLL. In this study, the four mutant gene combination with null *FAD2-1AΔ* or *FAD2-1A* S117N alleles produced higher oleic acid contents and lower linolenic acid contents compared to those of the transgenic HOLL lines developed by Brace et al. (2011). However, this difference may be due to the relative maturity difference between soybean lines and/or temperature differences between testing locations. Because our testing locations are at lower latitudes compared to those of Brace et al. (2011), we predict that our HOLL lines with four mutant genes may have a fatty acid profile similar to that of the transgenic HOLL soybeans reported by Brace et al. (2011) when the genes are incorporated into the appropriate maturity group for planting in locations in more northern latitudes.

Our study indicates that in two testing environments in Missouri, with M23 mutant *FAD2-1AΔ* contributing to the high oleic acid background, only one mutant *FAD3* gene (either *FAD3A* or *FAD3C)* is needed to lower the linolenic acid content to less than 3 %. The HO(S117N)LL with either mutant *FAD3A* or *FAD3C* had 3–5 % linolenic acid content in the Columbia, MO location and was not tested in the Portageville, MO location. In the Columbia, MO environment it requires four mutant genes with the 17D *FAD2-1A* missense allleles contributing to the high oleic background to produce less than 3 % linolenic acid content consistently, and combining four genes could be more challenging for soybean breeders. Recently, our group has generated another source of high oleic acid soybeans with nearly 85 % oleic acid and less than 3 % linolenic acid content with a combination of a mutant *FAD2-1A* allele containing a single base deletion resulting in a frameshift and premature translation termination from PI 603452 and the missense mutant *FAD2-1B* gene from PI 283327 (Pham et al. 2011). We anticipate that with this high oleic acid soybean background, similar to the situation with the null *FAD2-1* alleles from M23, only one mutant *FAD3* gene will be needed to lower the content of linolenic acid to below 3 % in Missouri production environments, and possibly two mutant *FAD3* genes will be necessary to further reduce linolenic acid content to 1 % in cooler environments.

Oleic acid and linolenic acid contents were demonstrated to be influenced greatly by temperature and modifier genes in several studies (Chapman et al. 1976; Dornbos and Mullen 1992; Graef et al. 1988; Hyten et al. 2004; Wilcox et al. 1993). It is shown clearly in this study that when two mutant *FAD3A* and *FAD3C* genes were incorporated into a high oleic acid background to reduce the enzymatic activity of FAD3, the linolenic acid content of HOLL soybeans was still affected by environmental factors while the oleic acid content showed a reduction in the Columbia, MO location compared to Portageville, MO that was statistically insignificant. Although the oleic acid content was not reduced significantly in a cooler environment, the stability of the fatty acid composition of the oil of HOLL soybean needs further evaluation by conducting experiments in a wider scale, with more diverse environments, and across different years. The instability of linolenic acid content across environments was possibly due to the influence of temperature on the enzymatic activity of the wild-type FAD3B enzyme in the HOLL lines, because mutant *FAD3A* and *FAD3C* genes from 10–73 used in this study would not produce enzymes with proper function. A study demonstrated that FAD2 and FAD3 enzymatic activities in soybean seeds cultured in vitro greatly declined when the temperature increased from 20 to 25 °C (100-fold for FAD2 and 60-fold for FAD3, and were almost inactive at 35 °C for both of the enzymes) (Cheesbrough 1989). To date, it was demonstrated that it is not the change of mRNA level but actually the regulation at the protein level (phosphorylation or degradation by a combination of *cis*-acting degradation signals and the ubiquitin–proteasome pathway) that regulate the response of FAD2 and FAD3 to cold temperature (O'Quin et al. 2010; Somerville 1995; Tang et al. 2005). In addition to the environmental effect, modifier genes may also play a role in controlling the linolenic content in the HOLL lines developed in this study. We identified three HO(S117-N)**LL3a** lines with the same gene combination that had significantly different linolenic acid contents when planted in Columbia, MO. Because they were planted close to each other in the same environment, the FAD3 enzymes in each plant in each line would have received the same environmental signals and cues, and therefore should respond in the same fashion. However, the existing variation for linolenic acid content between these HO(S117N)LL lines is significant, indicating more complicated regulatory mechanisms that may involve other genes besides the *FAD3* genes for linolenic acid content.

The changes of saturated fatty acid contents, oil and protein contents of HO(Δ)LL compared to those of high oleic acid parents were different from HO(S117N)LL lines. HO(Δ)LL lines showed a small but significant increase in palmitic acid content and a small but significant decrease in stearic acid content compared to those of the high oleic acid parent, while it was a reduction for palmitic acid and

an increase for stearic acid for HO(S117N)LL compared to its high oleic acid parent. The change in the saturated acid content of HO(Δ)LL is more valid because it was shown in both of the testing locations, while data for HO(S117N)LL were obtained from individual plants grown only in Columbia, MO. In addition, few but not all of the HO(S117N)LL lines showed the described changes. The reverse changes in the palmitic and stearic acid content of these HOLL lines resulted in no overall change in the total content of saturated fatty acids compared to that of the high oleic acid parent; the changes in the HOLL lines resulted in significantly lower saturated fatty acid content compared to Williams 82. Therefore, this change should not affect the usage values of the HOLL soybean lines. The increase in protein content at the expense of a reduction in oil content of the HO(Δ)LL lines with M23-derived null *FAD2-1A* alleles is in agreement with the report by Brace et al. (2011). In contrast, the HO(S117N)LL lines with 17D mutant *FAD2-1A* alleles maintained the oil content equivalent to those of the high oleic parent (19–20 %), and had higher protein contents compared to those of the two parents and Williams 82 in the Columbia, MO location. The small reduction in oil content of the HO(Δ)LL lines, however, can be improved when they are incorporated into an elite background to enhance agronomic traits, including oil and protein content. Although the HOLL transgenic lines in the study of Brace et al. (2011) showed a small but significant reduction (<5 %) in yield compared to soybeans with the normal fatty acid composition, we are not pessimistic about the impact of HOLL content to soybean yield. Neither high oleic acid nor low linolenic acid content has been shown to negatively influence yield and other agronomic traits of soybean and rapeseed (Graef et al. 2009; Rücker and Röbbelen 1996). Therefore, the stability and the effect of HOLL content generated in our study to yield and other agronomic traits should be evaluated after three or four mutant genes are incorporated in an elite background.

The new high oleic acid and low linolenic acid oils produced from our HOLL soybeans need to be tested for their effect on flavor of foods. Although the oils extracted from HOLL soybeans may have the highest oxidative stability of soybean oil to date, the influence on characteristics of food products including taste, flavor, and textures must be carefully evaluated considering that 75 % of soybean oils is being used for food preparation and production. Warner and Gupta (2005) have shown that although having the highest oxidative and frying stability, HOLL oil with 85 % oleic, 1.3 % linoleic and 2 % linolenic had the lowest sensory scores compared to those of low linolenic acid oil (2 %) and the 1:1 mixture of the high oleic acid and low linolenic oils (50 % oleic, 2 % linolenic). They also suggested that as the linolenic acid contents were the same in the three tested types of oil, the low sensory scores of high oleic soybean oil may be due to the extremely low content of linoleic acid; the low linolenic acid oil which had the highest linoleic acid content also had the highest sensory scores (Warner and Gupta 2005). However, in another study, only the potato chips that were freshly fried with high oleic low linolenic sunflower oil (8 % saturated fatty acids, 78 % oleic acid, 12 % linoleic, 0.1 % linolenic) had lower flavor scores compared to those fried in other oils. During the storage time up to 6 months, the flavor scores of food prepared with HOLL sunflower oil were still lower than those with other oils but they were not significantly different (Warner et al. 1997). It is hoped that by combining mutant *FAD3* gene with two mutant *FAD2-1* genes, the linolenic acid content of the HO soybean would be reduced and become less than the linoleic acid content so that the flavor issue can be evaluated. It was reported in three studies in human infants, rat and chicken that the most beneficial ratio of linoleic: linolenic acid content to improve health is equal or higher than 4:1 (Clark et al. 1992; Puthpongsiriporn and Scheideler 2005; Yehuda et al. 1996). Olive oil, which has 15 % saturated fatty acid, 75 % oleic, 9 % linoleic and 1 % linolenic acid has been long claimed to be one of the most healthy natural vegetable oils (White 2007). Among the HOLL lines we created, seeds of HO(S117N)**LL4ac** soybean lines with 17D *FAD2-1A* (S117N) alleles had the fatty acid profile that is close to that of olive oil with 11 % saturated fatty acids, 80 % oleic, 7 % linoleic and 2 % linolenic, and the ratio of linoleic is as follows: linolenic acid content was 3.5:1. It is believed that with this olive-like fatty acid composition, high oleic acid low linolenic acid soybean can offer more applications for industrial purposes and can be used as a less expensive but equally functional alternative to olive oil.

Acknowledgments The authors wish to acknowledge excellent technical assistance provided by Paul Little, Christine Cole and Stewart Selves. Dr. Jeong-Dong Lee provided F_1 seeds for Cross 1. Mention of a trademark, vendor, or proprietary product does not constitute a guarantee or warranty of the product by the USDA and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

References

Anai T, Yamada T, Kinoshita T, Rahman SM, Takagi Y (2005) Identification of corresponding genes for three low-[alpha]-linolenic acid mutants and elucidation of their contribution to fatty acid biosynthesis in soybean seed. Plant Sci 168:1615–1623

Anai T, Yamada T, Hideshima R, Kinoshita T, Rahman S, Takagi Y (2008) Two high-oleic-acid soybean mutants, M23 and KK21, have disrupted microsomal omega-6 fatty acid desaturase, encoded by *GmFAD2-1a*. Brd Sci 58:447–452

Beuselinck PR, Sleper DA, Bilyeu KD (2006) An assessment of phenotype selection for linolenic acid using genetic markers. Crop Sci 46:747–750

Bilyeu KD, Palavalli L, Sleper DA, Beuselinck PR (2003) Three microsomal omega-3 fatty-acid desaturase genes contribute to soybean linolenic acid levels. Crop Sci 43:1833–1838

Bilyeu K, Palavalli L, Sleper D, Beuselinck P (2005) Mutations in soybean microsomal omega-3 fatty acid desaturase genes reduce linolenic acid concentration in soybean seeds. Crop Sci 45:1830–1836

Bilyeu K, Palavalli L, Sleper D, Beuselinck P (2006) Molecular genetic resources for development of 1% linolenic acid soybeans. Crop Sci 46:1913–1918

Bilyeu KD, Gillman JD, LeRoy AR (2011) Novel *FAD3* mutant allele combinations produce soybeans containing 1% linolenic acid in the seed oil. Crop Sci 51:259–264

Brace RC, Fehr WR, Schnebly SR (2011) Agronomic and seed traits of soybean lines with high oleate concentration. Crop Sci 51:534–541

Chapman G, Robertson J, Burdick D, Parker M (1976) Chemical composition and lipoxygenase activity in soybeans as affected by genotype and environment. JAOCS 53:54–56

Chappell AS, Bilyeu KD (2007) The low linolenic acid soybean line PI 361088B contains a novel *GmFAD3A* mutation. Crop Sci 47:1705–1710

Cheesbrough TM (1989) Changes in the enzymes for fatty acid synthesis and desaturation during acclimation of developing soybean seeds to altered growth temperature. Plant Physiol 90:760–764

Chi X, Yang Q, Lu Y, Wang J, Zhang Q, Pan L, Chen M, He Y, Yu S (2011) Genome-wide analysis of fatty acid desaturases in soybean (*Glycine max*). Plant Mol Bio Report 29:769–783

Choung MG, Baek IY, Kang ST, Han WY, Shin DC, Moon HP, Kang KH (2001) Determination of protein and oil contents in soybean seed by near infrared reflectance spectroscopy. Korean J Crop Sci 46:106–111

Clark KJ, Makrides M, Neumann MA, Gibson RA (1992) Determination of the optimal ratio of linoleic acid to linolenic acid in infant formulas. J Pediatr 120:S151–S158

Dierking E, Bilyeu K (2009) New sources of soybean seed meal and oil composition traits identified through TILLING. BMC Plant Bio 9:89

Dornbos D, Mullen R (1992) Soybean seed protein and oil contents and fatty acid composition adjustments by drought and temperature. JAOCS 69:228–231

Fehr WR (2007) Breeding for modified fatty acid composition in soybean. Crop Sci 47:S-72–S-87

Graef GL, Fehr WR, Miller LA, Hammond EG, Cianzo SR (1988) Inheritance of fatty acid composition in a soybean mutant with low linolenic acid. Crop Sci 28:55–58

Graef G, LaVallee B, Tenopir P, Tat M, Schweiger B, Kinney A, Gerpen J, Clemente T (2009) A high-oleic-acid and low-palmitic-acid soybean: agronomic performance and evaluation as a feedstock for biodiesel. Plant Biotech J 7:411–421

Hartwig RA, Hurburgh CR (1990) Near-infrared reflectance measurement of moisture, protein and oil content of ground crambe seed. JAOCS 67:435–437

Heppard E, Kinney A, Stecca K, Miao G (1996) Developmental and growth temperature regulation of two different microsomal [omega]-6 desaturase genes in soybeans. Plant Physiol 110:311–319

Hoshino T, Takagi Y, Anai T (2010) Novel *GmFAD2-1b* mutant alleles created by reverse genetics induce marked elevation of oleic acid content in soybean seeds in combination with *GmFAD2-1a* mutant alleles. Breed Sci 60:419–425

Hu FB, Stampfer MJ, Manson JE, Rimm E, Colditz GA, Rosner BA, Hennekens CH, Willett WC (1997) Dietary fat intake and the risk of coronary heart disease in women. N Engl J Med 337:1491–1499

Hyten D, Pantalone V, Saxton A, Schmidt M, Sams C (2004) Molecular mapping and identification of soybean fatty acid modifier quantitative trait loci. JAOCS 81:1115–1118

Mattson FH, Grundy SM (1985) Comparison of effects of dietary saturated, monounsaturated, and polyunsaturated fatty acids on plasma lipids and lipoproteins in man. J Lipid Res 26:194–202

Mounts TL, Warner K, Usta GR, Neffa WE, Wilson RF (1994) Low linolenic acid soybean oils—alternatives to frying oils. J AOCS 71:495–499

Mozaffarian D, Katan MB, Ascherio A, Stampfer MJ, Willett WC (2006) Trans fatty acids and cardiovascular disease. N Engl J Med 354:1601–1613

O'Quin JB, Bourassa L, Zhang D, Shockey JM, Gidda SK, Fosnot S, Chapman KD, Mullen RT, Dyer JM (2010) Temperature-sensitive post-translational regulation of plant omega-3 fatty-acid desaturases is mediated by the endoplasmic reticulum-associated degradation pathway. J Bio Chem 285:21781–21796

Oilseeds: World Markets and Trade FAS, USDA (2010) http://www.fas.usda.gov/oilseeds_arc.asp

Perez-Jimenez F, Espino A, Lopez-Segura F, Blanco J, Ruiz-Gutierrez V, Prada J, Lopez-Miranda J, Jimenez-Pereperez J, Ordovas J (1995) Lipoprotein concentrations in normolipidemic males consuming oleic acid-rich diets from two different sources: olive oil and oleic acid-rich sunflower oil. Am J Clin Nutr 62:769–775

Pham A-T, Lee J-D, Shannon JG, Bilyeu K (2010) Mutant alleles of *FAD2-1A* and *FAD2-1B* combine to produce soybeans with the high oleic acid seed oil trait. BMC Plant Bio 10:195

Pham A-T, Lee JD, Shannon JG, Bilyeu K (2011) A novel *FAD2-1A* allele in a soybean plant introduction offers an alternate means to produce soybean seed oil with 85% oleic acid content. Theor Appl Genet 123:793–802

Puthpongsiriporn U, Scheideler S (2005) Effects of dietary ratio of linoleic to linolenic acid on performance, antibody production, and in vitro lymphocyte proliferation in two strains of leghorn pullet chicks. Poult Sci 84:846–857

Rahman SM, Kinoshita T, Anai T, Takagi Y (2001) Combining ability in loci for high oleic and low linolenic acids in soybean. Crop Sci 41:26–29

Reinprecht Y, Luk-Labey SY, Larsen J, Poysa VW, Yu K, Rajcan I, Ablett GR, Pauls KP (2009) Molecular basis of the low linolenic acid trait in soybean EMS mutant line RG10. Pl Brdg 128:253–258

Rennie B, Tanner J (1989) Fatty acid composition of oil from soybean seeds grown at extreme temperatures. J AOCS 66:1622–1624

Rücker B, Röbbelen G (1996) Impact of low linolenic acid content on seed yield of winter oilseed rape (*Brassica napus* L). Plt Brdg 115:226–230

Sandhu D, Alt J, Scherder C, Fehr W, Bhattacharyya M (2007) Enhanced oleic acid content in the soybean mutant M23 is associated with the deletion in the *Fad2-1a* gene encoding a fatty acid desaturase. JAOCS 84:229–235

SAS Institute Inc. (2008) SAS® 9.2 enhanced logging facilities. SAS Institute Inc., Cary, NC

Schlueter JA, Vasylenko-Sanders IF, Deshpande S, Yi J, Siegfried M, Roe BA, Schlueter SD, Scheffler BE, Shoemaker RC (2007) The *FAD2* gene family of soybean:Insights into the structural and functional divergence of a paleopolyploid genome. Crop Sci 47(S1):S14–S26

Schmutz J, Cannon SB, Schlueter J, Ma J, Mitros T, Nelson W, Hyten DL, Song Q, Thelen JJ, Cheng J, Xu D, Hellsten U, May GD, Yu Y, Sakurai T, Umezawa T, Bhattacharyya MK, Sandhu D, Valliyodan B, Lindquist E, Peto M, Grant D, Shu S, Goodstein D, Barry K, Futrell-Griggs M, Abernathy B, Du J, Tian Z, Zhu L, Gill N, Joshi T, Libault M, Sethuraman A, Zhang X-C, Shinozaki K, Nguyen HT, Wing RA, Cregan P, Specht J,

Grimwood J, Rokhsar D, Stacey G, Shoemaker RC, Jackson SA (2010) Genome sequence of the palaeopolyploid soybean. Nature 463:178–183

Somerville C (1995) Direct tests of the role of membrane lipid composition in low-temperature-induced photoinhibition and chilling sensitivity in plants and cyanobacteria. PNAS 92:6215–6218

Soystats (2010) http://www.soystats.com/

Tang G, Novitzky W, Griffin H, Huber S, Dewey R (2005) Oleate desaturase enzymes of soybean: evidence of regulation through differential stability and phosphorylation. Plant J 44:433–446

Tian Z, Wang X, Lee R, Li Y, Specht JE, Nelson RL, McClean PE, Qiu L, Ma J (2010) Artificial selection for determinate growth habit in soybean. PNAS 107:8563–8568

Warner K, Fehr W (2008) Mid-oleic/ultra low linolenic acid soybean oil: a healthful new alternative to hydrogenated oil for frying. JAOCS 85:945–951

Warner K, Gupta M (2005) Potato chip quality and frying oil stability of high oleic acid soybean oil. Food Sci 70:395–400

Warner K, Orr P, Glynn M (1997) Effect of fatty acid composition of oils on flavor and stability of fried foods. JAOCS 74:347–356

White PJ (2007) Fatty acid in oilseeds (vegetable oils). In: Chow CK (ed) Fatty acids in foods and their health implications. CRC press, Marcel Dekker, Inc., New York, pp 210–263

Wilcox JR, Cavins JF (1992) Normal and low linolenic acid soybean strains: response to planting date. Crop Sci 32:1248–1251

Wilcox JR, Nickell AD, Cavins JF (1993) Relationships between the fan allele and agronomic traits in soybean. Crop Sci 33:87–89

Wilcoxon F (1945) Individual comparisons by ranking methods. Biometrics Bull 1:80–83

Wilson R (2004) Seed composition. In: Boerma HR, Specht JE (eds) Soybeans: improvement, production, and uses. ASA, Madison, pp 621–677

Yehuda S, Brandys Y, Blumenfeld A, Mostofsky DI (1996) Essential fatty acid preparation reduces cholesterol and fatty acids in rat cortex. Int J Neurosci 86:249–256

Zaloga GP, Harvey KA, Stillwell W, Siddiqui R (2006) Trans fatty acids and coronary heart disease. Nutr Clin Pract 21:505–512