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Phenotypic and Molecular Analysis of Oleate Content in the Mutant Soybean Line M23

Jessie L. Alt, Walter R. Fehr,* Grace A. Welke, and Devinder Sandhu

ABSTRACT

Soybean [*Glycine max* (L.) Merr.] oil with elevated oleate content would be useful for food and industrial applications that require increased oxidative stability. The first objective of this study was to determine if molecular selection for the *Fad2-1* deletion associated with the *ol* allele in the mid-oleate mutant line M23 could be used to identify mid-oleate individuals in a breeding program. The second objective was to determine if modifying genes affect phenotypic expression of oleate content in individuals homozygous for the deletion from a cross between M23 and Archer, a cultivar with normal oleate content. The segregation among 88 F_2 plants from the cross satisfactorily fit a ratio of 1:2:1 homozygous normal (*OlOl*)/heterozygous (*Olol*)/homozygous (*olol*) for the *Fad2-1* deletion on the basis of Southern analysis. A PCR-based marker Fad2-1-*ol* identified the same *olol* individuals as the Southern analysis. The PCR-based marker would be a more rapid and less labor intensive method for molecular selection of *olol* individuals than Southern analysis. The *olol* individuals had the highest mean oleate content, the *Olol* individuals were intermediate, and the *OlOl* individuals had the lowest mean oleate content. There was significant variation among the *olol* individuals and their distribution overlapped that of the *OlOl* and *Olol* individuals, which indicated that modifying genes had an important influence on the trait. It would be necessary to test the fatty acid profile of *olol* individuals to select those with the highest oleate content.

Vegetable oils with increased oleate have greater oxidative stability during high temperature heating and frying than normal oleate oils (Warner et al., 1994; Warner and Knowlton, 1997; Warner et al., 1997). Oleate has been demonstrated to lower total and low-density lipoprotein cholesterol, which is beneficial in reducing cardiovascular disease in humans (Kris-Etherton, 1999).

The mid-oleate mutant soybean line M23 was developed by X-ray irradiation of dry seeds of Bay, a cultivar with normal oleate content (Rahman et al., 1994). M23 had an oleate content of 461 g kg^{-1} as an M_2 plant compared with 223 g kg^{-1} oleate for Bay. Rahman et al. (1994) hypothesized that X-ray irradiation caused a mutation that partially blocked the desaturation pathway from oleate to linoleate. Takagi and Rahman (1996) proposed a single locus with two alleles, *Ol* controlling normal oleate and *ol* controlling mid-oleate.

Linoleate is formed from oleate by desaturation at the ω-6 position mediated by the enzyme ω-6 fatty acid desaturase. Okuley et al. (1994) isolated the cDNA encoding the microsomal ω-6 fatty acid desaturase from *Arabidopsis thaliana* (L.) Heynh. as the *Fad2* gene. Heppard et al. (1996) found two clones encoding ω-6 fatty acid desaturase in soybean that they designated *Fad2-1* and *Fad2-2*. Heppard et al. (1996) found *Fad2-1* to be strongly expressed in developing seeds while *Fad2-2* was expressed in both vegetative tissue and developing seeds.

Kinoshita et al. (1998) used the cDNA of *Fad2-1* and *Fad2-2* as probes to screen 40 F_2 plants from the cross of Bay × M23. The population was analyzed using restriction fragment length polymorphisms with the restriction enzyme *Eco*RI. The *Fad2-1* probe detected a 4.6-kbp fragment in Bay, but not in M23. The population screened with *Fad2-1* satisfactorily fit the 1:2:1 segregation for the *ol* locus as proposed by Takagi and Rahman (1996). Kinoshita et al. (1998) concluded that the deletion in the *Fad2-1* gene gave rise to the *ol* allele in M23.

Genetic analyses of soybean mutants with major genes for altered fatty ester content have demonstrated that modifying genes can influence the segregation observed when a mutant line is crossed with conventional cultivars (Graef et al., 1988; Horejsi et al., 1994). The possible role of modifying genes in the oleate content of *olol* individuals has not been reported.

One objective of this study was to determine if molecular selection for the deletion in *Fad2-1* could be used to identify lines with mid-oleate content in a breeding program. A second objective of this study was to determine the role of modifying genes in the segregation of progeny from the cross of M23 and a soybean cultivar with normal oleate content.

MATERIALS AND METHODS

Population Development

The mutant line M23 developed by Rahman et al. (1994) was crossed to Archer, a cultivar of maturity group I with normal oleate content developed by Iowa State University and the Puerto Rico Agricultural Experiment Station (Cianzio et al., 1991). The cross was made during July 2002 at the Iowa State University Agricultural Engineering and Agronomy Research Center near Ames, IA. The F_1 seeds and seeds of each parent were planted during October 2002 at the Iowa State University–University of Puerto Rico soybean breeding nursery at Isabela, PR, on a Coto clay (very-fine, kaolinitic, isohyperthermic Typic Eutrustox). Hybrid F_1 plants were confirmed using the simple sequence repeat marker Satt173 developed by Cregan et al. (1999).

A random sample of 300 F_2 seeds and 24 seeds of each parent were planted during February 2003 at Isabela. A single

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677 S. Segoe Rd., Madison, WI 53711 USA

Abbreviations: bp, base pair; kbp, kilobase pair.

trifoliolate leaf of each F_2 and parent plant was harvested for DNA extraction. Genomic DNA was isolated by the method described by Anderson et al. (1992).

The F_2 plants were harvested individually to obtain $F_{2:3}$ lines. There were 88 F_2 plants with sufficient DNA to perform Southern analysis. From 88 F_2 plants and 10 plants of each parent, 11 individual seeds were split with a razor blade and the identity of each seed was maintained for analysis and planting. The one-third of the seed without the embryonic axis was analyzed for fatty ester content. The remaining part of the seed was saved for planting. All of the fatty ester analyses for the study were conducted by the method described by Hammond (1991). The fatty ester content of each F_2 plant used for statistical analysis was the average fatty ester content of its 11 split F_3 seeds.

In May 2003, the 88 selected $F_{2:3}$ lines and 10 entries of each parent were planted in a randomized complete-block design with two replications at the Iowa State University Research Center near Ames, IA, on a Nicollet loam (fine-loamy, mixed, superactive, mesic Aquic Hapludoll). Two replications also were planted at the University of Missouri– Columbia Delta Research Center near Portageville, MO, on a Tiptonville silt loam (fine-silty, mixed, superactive, thermic Oxyaquic Argiudoll). The Portageville location was used because M23 is of maturity group V and was likely not to mature before frost at Ames. The split seeds were planted in one of the replications at the Iowa State University Research Center. The remaining plots were planted with random whole seeds. The plots at Ames were a single row 0.76 m long with 1.02 m between rows and 1.07 m between the ends of plots. For the hill plots at Portageville, there was 0.91 m between plots within a row and 0.76 m between rows. The seeding rate was 11 seeds in all plots. Maturity was taken when 95% of pods on the main stem were mature. After the plants matured naturally or were killed by frost, they were harvested and threshed individually. The identity of each plant originating from a split seed was maintained. A five-seed bulk from each F_3 plant was analyzed for fatty ester content.

Southern Analysis

The cDNA for *Fad2-1* was obtained from Richard Dewey, USDA-ARS, Raleigh, NC. The probe was amplified to obtain sufficient quantity for Southern analysis using the forward primer 5′-ATG GGT CTA GCA AAG GAA AC-3′ from Kinoshita et al. (1998) and the reverse primer 5′-GAG CAA CCA ATG GGC CAT AG-3′. The reverse primer was designed from the published cDNA sequence of *Fad2-1*. The final polymerase chain reaction (PCR) volume was 50 μL consisting of 50 ng *Fad2-1* DNA, 2 m*M* $MgCl_2$, 200 μ*M* dNTPs, 1.25 U of BIOLASE DNA polymerase, 1× BIOLASE 10× NH_4 buffer, and 0.25 μ*M* of each primer (Bioline USA Inc., Boston, MA). PCR was conducted on programmable thermal controllers (MJ Research Inc., Waltham, MA) model PTC-100. The PCR procedure was 94°C for 2 min, 11 cycles starting at 60°C for 30 s (−1°C per cycle) and 72°C for 2 min, 35 cycles of 94°C for 30 s, 48°C for 30 s, and 72°C for 2 min followed by a final extension of 8 min at 72°C.

From the 88 F_2 plants and each parent, a 5-μg sample of genomic DNA was digested using the restriction enzyme *Eco*RI. Probe preparation and Southern hybridization were performed according to the method described by Sandhu et al. (2001). All of the F_2 plants and parents had a 2.1-kbp band. There was segregation for a 4.6-kbp band associated with *Fad2-1*. The F_2 plants were placed into three classes based on visual evaluation of the 4.6-kbp band intensities: similar to Archer (double intensity), heterozygous (single intensity), or similar to M23 (absent).

Analysis of the PCR-Based Marker Fad2-1-*ol*

Fad2-1-*ol* is a PCR-based marker developed by D. Sandhu (unpublished data, 2005). The 88 F_2 plants and parents were evaluated to determine if using a PCR-based marker would be a more efficient method of selecting for the *Fad2-1* deletion in the *ol* allele of M23. The forward primer was 5′-GGG CCA TAG TGG GAG TTA TGG AAG-3′ and the reverse primer was 5′-GCT ATA AGC AGA ACA CTT TCC ACA T-3′. The final PCR volume was 20 μL consisting of 25 ng DNA, 0.25 μ*M* of each primer, 200 μ*M* dNTPs, 2 m*M* $MgCl_2$, 0.5 U BIOLASE DNA polymerase, 1× BIOLASE 10× NH_4 Buffer (Bioline USA Inc., Boston, MA). The PCR procedure was 94°C for 2 min, 6 cycles starting at 55°C for 30 s (−1°C per cycle) and 72°C for 2 min, 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 2 min followed by a final extension of 8 min at 72°C. All plants had a 162-bp band. Segregation occurred for the 183-bp band. The plants were scored as the same as Archer (presence of 183-bp band) or M23 (absence of the 183-bp band).

Statistical Analysis

The 88 F_2 plants used for Southern analysis and their corresponding $F_{2:3}$ lines were used for statistical analysis of fatty ester content. The effect of environments, genotypes, and the genotype × environment interaction was determined for oleate using $F_{2:3}$ lines by the general linear model (PROC GLM) of SAS software (SAS Institute Inc., 1999). Environments, replications, and genotypes were considered random effects.

To determine significance among lines within the three genotypic classes, the data were analyzed using general linear model (PROC GLM) by SAS software (SAS Institute Inc., 1999). Environments and replications were considered random and genotypes were considered fixed. Contrasts were used to determine if there was a significant difference between the genotypic classes.

Phenotypic correlation coefficients were calculated for fatty esters between $F_{2:3}$ lines, between environments, and between F_2 plants and $F_{2:3}$ lines using the correlation procedure (PROC CORR) by SAS software (SAS Institute Inc., 1999).

Single-seed heritability estimates were obtained by regressing the fatty ester content of each F_3 plant from the single replication grown at Ames on the corresponding split F_3 seed from which it was derived using the regression procedure (PROC REG) by SAS software (SAS Institute Inc., 1999) and adjusting for inbreeding according to Nyquist (1991). Single-plant heritability estimates were obtained by regressing the oleate content of the $F_{2:3}$ lines grown at Ames and Portageville on the oleate content of the F_2 plants grown at Isabela using the regression procedure (PROC REG) by SAS software (SAS Institute Inc., 1999). Heritability estimates on a plot and entry-mean basis were calculated using variance components determined from the analysis of the $F_{2:3}$ lines at Ames and Portageville (Hallauer and Miranda, 1988).

RESULTS AND DISCUSSION

The *ol* allele conditioned by the *Fad2-1* deletion was segregating in the Archer × M23 F_2 population on the basis of Southern analysis. The segregating *Eco*RI fragment was approximately 4.6-kbp, which was consistent with the results observed by Kinoshita et al. (1998). The F_2 plants segregated into three molecular classes on the

basis of band intensity: 24 were *OlOl* (double intensity), 49 were *Olol* (single intensity), and 15 were *olol* (no band) (Table 1). The segregation satisfactorily fit a 1:2:1 ratio ($P > 0.23$), which was consistent with the single-gene model for inheritance of oleate in M23 proposed by Takagi and Rahman (1996).

After Southern analysis was completed, the PCR-based marker Fad2-1-*ol* developed by D. Sandhu (unpublished data, 2005) was used to evaluate the 88 F_2 plants for the *Fad2-1* deletion in M23. All of the plants had a 162-bp band associated with a homoeologous *Fad2-1* sequence (Sinha et al., 2004). Segregation among plants occurred for a 183-bp band associated with the *Fad2-1* deletion in M23. Plants with the *Fad2-1* deletion (*olol*) were expected to lack the 183-bp band that would be present for individuals with either the *OlOl* or *Olol* genotype. The 15 F_2 plants classified as *olol* by the Southern analysis were classified the same with the PCR-based marker, and the same 73 plants identified by the Southern analysis as *OlOl* or *Olol* had the expected 183-bp band present. The PCR-based marker was faster and less labor intensive than the Southern analysis for identifying *olol* individuals in a segregating population.

Southern analysis and the PCR-based marker were used to evaluate two other soybean germplasm sources of mid-oleate. FA22 is a mutant line with mid-oleate content developed by Iowa State University. N98-4445A is a mid-oleate line developed by the United States Department of Agriculture and North Carolina State University (Wilson, 2004). Neither of the lines had the *Fad2-1* deletion exhibited by M23 and could not be distinguished from Archer by Southern analysis or the PCR-based marker. The results indicated that mid-oleate content in FA22 and N98-4445A was not conditioned by the *Fad2-1* deletion as in M23.

The *olol* F_2 plants and their $F_{2:3}$ lines had the greatest mean oleate content and the *OlOl* individuals had the least oleate (Table 2). The two genotypic classes were different ($P < 0.01$) on the basis of entry means across environments. The mean oleate of the *Olol* individuals was greater than that of the *OlOl* individuals ($P < 0.1$) and less ($P < 0.01$) than that of the *olol* individuals on the basis of entry means. The mean of the *Olol* individuals was less than the midpoint between the means of the *OlOl* and *olol* individuals, which indicated that the *Ol* allele exhibited partial dominance. Partial dominance for lower oleate in crosses with M23 also was reported by Takagi and Rahman (1996).

Table 1. Genotypic classification determined by Southern analysis of 88 soybean F_2 plants from the cross Archer (*OlOl*) × M23 (*olol*) using a single-recessive gene model.

F_2 genotype	Expected	Observed†
	Number	
OlOl	**22**	**24**
Olol	**44**	**49**
olol	**22**	**15**

† Observed genotypic frequency fit the expected 1:2:1 ratio based on the chi-square test ($\chi^2 = 2.98$; $P > 0.23$).

There were significant differences for oleate content among $F_{2:3}$ lines within the *OlOl* ($P < 0.01$), *Olol* ($P < 0.01$), and *olol* ($P < 0.05$) classes on the basis of the combined analysis of variance of data from Ames and Portageville. The range of oleate among the 15 *olol* F_2 plants and $F_{2:3}$ lines overlapped the range of the 49 *Olol* individuals and 24 *OlOl* individuals at all environments (Table 2). Of the 10 F_2 plants with the greatest oleate at Isabela, 5 had the *olol* genotype. Nine of the 10 $F_{2:3}$ lines with the greatest oleate at Ames, 4 of the 10 highest oleate lines at Portageville, and 2 of the 10 highest oleate lines on the basis of the mean of the two environments had the *olol* genotype. The results indicated that modifying genes had a significant influence on the oleate content of *olol* individuals that are homozygous for *Fad2-1* deletion in M23. If molecular analysis is used to identify individuals homozygous for the *Fad2-1* deletion, it will be necessary to determine the fatty acid profile of the selected individuals to identify those with the highest oleate content.

Narrow-sense heritability estimates for oleate based on the 88 F_2 individuals were 0.33 on an F_3 seed basis and 0.44 on an F_2 plant basis. Broad-sense heritability estimates were 0.46 on a plot basis at Ames, 0.37 on a plot basis at Portageville, and 0.82 on an entry-mean basis. The heritability estimates were lower than those

Table 2. Mean and range of oleate content for 88 F_2 soybean plants and $F_{2:3}$ lines compared with the parents grown in three environments.

	OlOl†		*Olol*		*olol*	
Environment	$\bar{X}$‡	**Range**	$\bar{X}$	**Range**	$\bar{X}$	**Range**
	g kg^{-1}					
Isabela, PR						
F_2 plants	**278 ± 99**	**206–369**	**300 ± 119**	**180–449**	**397 ± 123**	**320–532**
Archer	**187 ± 28**	**168–212**				
M23					**604 ± 37**	**569–625**
Ames, IA						
$F_{2:3}$ lines	**243 ± 58**	**195–320**	**271 ± 58**	**216–361**	**360 ± 48**	**317–390**
Archer	**221 ± 12**	**213–233**				
M23					**357 ± 27**	**336–374**
Portageville, MO						
$F_{2:3}$ lines	**334 ± 92**	**246–412**	**364 ± 109**	**272–547**	**428 ± 68**	**381–512**
Archer	**262 ± 35**	**246–301**				
M23					**489 ± 124**	**335–551**
Entry Mean						
$F_{2:3}$ lines	**289 ± 71**	**220–357**	**317 ± 70**	**252–424**	**394 ± 51**	**350–451**
Archer	**242 ± 22**	**232–265**				
M23					**423 ± 45**	**349–450**

† Genotype determined by Southern analysis. There were 24 individuals of the *OlOl* genotype, 49 of the *Olol* genotype, and 15 of the *olol* genotype.
‡ Mean ± 2 standard deviations or 2 standard errors of the means for 88 F_2 plants and $F_{2:3}$ lines and 8 entries of each parent.

reported by Hawkins et al. (1983) who analyzed 20 experimental lines with normal oleate at Ames and Isabela. They reported broad-sense heritability estimates for oleate of 0.50 on a seed basis, 0.52 on a plant basis, 0.58 on a plot basis, and 0.92 on an entry-mean basis.

The genotype × environment interaction for oleate between Ames and Portageville was significant ($P < 0.01$) for the 88 $F_{2:3}$ lines. The phenotypic correlation for oleate between the 88 $F_{2:3}$ lines at Ames and Portageville was 0.70 ($P < 0.01$) and the correlation for the 15 *olol* lines at Ames and Portageville was 0.60 ($P < 0.05$). The significant correlations occurred even though there were major differences in maturity between M23 and Archer, which resulted in a broad segregation for the trait among the *olol* lines. At Ames, M23 and 11 of the *olol* lines did not mature before frost; whereas M23 and all the *olol* lines matured before frost at Portageville. Failure of M23 and some of the $F_{2:3}$ lines to mature at Ames was considered responsible for the lower mean oleate at that environment (Table 2). Thomas et al. (2003) found that oleate concentration in soybean increased with higher temperatures during seed development. Nevertheless, four of the five *olol* lines with the greatest oleate at Ames were in the top five lines at Portageville. These results and the magnitude of the phenotypic correlation among the two environments suggested that initial selection for oleate in a limited number of environments may be possible. Additional research with lines adapted to appropriate production environments and from multiple crosses should be conducted to further evaluate alternative selection strategies for oleate content in *olol* lines.

Oleate content of the 88 $F_{2:3}$ lines was significantly negatively correlated ($P < 0.01$) with palmitate (−0.47), stearate (−0.38), linoleate (−0.97), and linolenate (−0.45) on the basis of entry means across Ames and Portageville. Hawkins et al. (1983) reported significant negative correlations ($P < 0.01$) of oleate with stearate (−0.57), linoleate (−0.95), and linolenate (−0.84), but no correlation with palmitate (0.00). As oleate increases among lines, all of the other fatty esters are likely to decrease, particularly linoleate. The importance of the reductions in the other fatty esters will depend on the oil that is desired for a particular end use.

The results of the study indicated that the deletion of *Fad2-1* at the *ol* locus of M23 segregated as a single gene in the Archer × M23 F_2 population. Use of the PCR-based marker should facilitate molecular selection of segregates with the *olol* genotype in populations derived from M23. The range of oleate among *olol* genotypes indicated that modifying genes have an important influence on oleate content of *olol* individuals, and that analysis of fatty ester content of the seed will be necessary to identify *olol* individuals with the highest oleate content.

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