Genetic Regulation of Linolenic Acid Concentration in Wild Soybean *Glycine soja* Accessions

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ABSTRACT: Soybean [*Glycine max* (L.) Merr.] oil from current commercial cultivars typically contains *ca.* 8% linolenic acid. Inheritance studies have shown that linolenic acid concentration in soybean seed is determined by at least two genes which govern activity of the predominant ω-6 and ω-3 desaturases. Selection of germplasm exhibiting homozygous recessive alleles that encode these desaturases has enabled development of soybeans having less than 3.0% linolenic acid. However, accessions of the wild ancestor of modern soybean cultivars, *Glycine soja* (Sieb. and Zucc.), have oils containing twice the highest linolenic acid concentration found in normal *G. max* cultivars. Although little is known about inheritance of linolenic acid in wild soybean, it would appear that additional or alternative forms of genes may govern its synthesis. To test this hypothesis, cultivated soybean germplasm was hybridized with wild soybean genotypes having significant differences in linolenic acid concentration. Seed of F_3 progeny from these *G. max* × *G. soja* populations exhibited distinct segregation patterns for relative estimates of ω-6 and ω-3 desaturase activity. Frequency class distribution analyses of the segregation patterns, and linear relations between median ω-6 or ω-3 desaturation estimates and corresponding linolenic acid concentration among allelic classes from these populations suggested the high-linolenic acid trait in wild soybean genotypes was determined by a set of desaturase alleles that were different from corresponding alleles in *G. max*. Introgression of these alternative alleles in *G. max* germplasm opens a new avenue of research on the genetic regulation of linolenic acid, and may lead to the production of highly polyunsaturated soybean oils for various industrial applications.

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The bulk of 18:3 found in soybean [*Glycine max* (L.) Merr.] oil is synthesized by ω-6 and ω-3 desaturases that respectively catalyze desaturation of 18:1 esterified to phosphatidylcholine (PC) to 18:2-PC, and 18:2-PC to 18:3-PC (1). Two decades ago, research to determine the genetic regulation of 18:3 in soybean was initiated by three different breeding programs. The goal of each program was to develop germplasm which expressed lower than normal levels of 18:3 (*ca.* 8% of crude oil) for improved soybean oil quality. Wilson *et al.* (2) and Burton *et al.* (3) used recurrent selection to develop soybean germplasm lines N78-2245, N87-2120-3, and N87-2122-4, which contained *ca.* 4.2% 18:3. Chemical mutagenesis by ethyl methanesulfonate (EMS) was used by Hammond and Fehr (4) to develop A5, a germplasm that contained 3.3 to 4.2% 18:3. EMS also was used by Wilcox and Cavins (5) to develop C1640, a germplasm that exhibited *ca.* 3.5% 18:3. Subsequent genetic and biochemical studies showed that N78-2245, N87-2122-4, and A5 have a gene in the homozygous recessive state which appears to govern ω-6 desaturase activity; and PI 123440, N87-2120-3, and C1640 have another gene in the homozygous recessive state which appears to govern ω-3 desaturase activity (6,7). The latter allele was designated as the *fan* locus (8). N85-2176, an inbred line derived from N78-2245 × PI 123440, exhibited a double homozygous recessive genotype for these alleles and yielded a crude oil containing *ca.* 3.3% 18:3 (9–11). This work demonstrated that at least two independently segregating alleles determined 18:3 concentration in *G. max*.

Genes for several ω-6 and ω-3 desaturases have been cloned from various plant species. Three genes appear to regulate desaturation of 18:1-PC and 18:2-PC in the cytoplasm of soybean seed (12). Two of those genes, designated *Fad2-1* and *Fad2-2*, encode ω-6 desaturases, and are located on different chromosomes (13,14). The predominant 18:1-PC desaturase activity in soybean seed appears to be associated with *Fad2-1*. When expressed in the antisense orientation in transgenic soybean embryos, the resultant *anti-Fad2-1* phenotype exhibited nearly 80% 18:1 (15). The third gene, *Fad3*, encodes an ω-3 desaturase (16). When this gene is expressed in antisense orientation in transgenic soybean embryos, the *anti-Fad3* phenotype exhibited high-18:2 and very low-18:1 (15). Although direct association of these genes with the alleles that determine the trait in low-18:3 soybean germplasm has not been shown, molecular genetic manipulation of desaturase activities has confirmed the original concepts established through plant breeding. These technologies have advanced understanding of genetic regulation of biological processes that affect improved soybean oil quality. Indeed, genetic control of polyunsaturated fatty acid synthesis also may lead to new high-18:3 industrial oils. In that regard, over-

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expression of the *Fad2-2* gene is reported to affect elevated 18:3 (14).

Another approach toward a more complete understanding of the genetic regulation of 18:3 synthesis may involve the wild ancestor of cultivated soybean. *Glycine soja* Sieb and Zucc. accessions in the USDA, ARS Soybean Germplasm Collection, which contain as much as 23% 18:3, may be a useful genetic resource for identifying additional genes that govern expression of 18:3 (17). While little is known of the genetic mechanisms that govern 18:3 in wild soybean, it is proposed that a different complement of genes may condition expression of the very high-18:3 trait. To test that hypothesis, inheritance of 18:3 was investigated among inbred progeny of four *G. max* × *G. soja* populations. Results indicated that separate genes from each parent plant were recombined and acted, possibly in an additive genetic manner, to affect higher and lower 18:3 concentrations. Thus, transfer of these genes to *G. max* germplasm could establish the foundation for development of highly polyunsaturated soybean oils that have application in the manufacture of lubricants and drying oils.

MATERIALS AND METHODS

Interspecific crosses were made between *G. max* lines (N87-2120-3 or N87-2122-4) and *G. soja* accessions (PI 342434 or PI 424031). N87-2120-3 was a F_6-derived line from N79-2077 × PI 123440; N87-2122-4 was a F_6-derived line from N78-2245 × N79-2077 (3). N87-2120-3 had a homozygous recessive allele that appears to govern ω-3 desaturase activity, and a homozygous allele governing normal ω-6 desaturase activity. N87-2122-4 exhibited a homozygous recessive allele that appears to govern the predominant ω-6 desaturase activity in soybean seed, and a homozygous allele governing normal ω-3 desaturase activity (10). PI 342434 and PI 424031 were obtained from the USDA, ARS Wild Soybean Collection (17). These *G. soja* plant introductions exhibited significant differences in relative 18:1- and 18:2-desaturation, which are practical estimates of ω-6 and ω-3 desaturase activities. Relative 18:1-desaturation was calculated as: %[(18:2 + 18:3)/(18:1 + 18:2 + 18:3)]; relative 18:2-desaturation was calculated as: %[18:3/(18:2 + 18:3)], (10). Four populations were formed from the hybridizations: N87-2120-3 × PI 342434 (Population I), N87-2120-3 × PI 424031 (Population II), N87-2122-4 × PI 342434 (Population III), and N87-2122-4 PI 424031 (Population IV). F_1 and F_2 plants were selfed under greenhouse conditions. The original parents of each population plus a random sample of F_3 seed derived from an equal representation of F_2 plants were grown under field conditions at the Central Crops Research Station (Clayton, NC), and the Sandhills Research Station (Jackson Springs, NC). Seed from individual F_3 plants and parental lines were harvested at maturity. Data were consistent and variances were homogeneous over the two locations. Therefore, data were reported as a combined analysis.

Oil was extracted from crushed seed with a mixture of chloroform/hexane/methanol (8:5:2, vol/vol/vol). Fatty acid methyl esters were prepared by transmethylation with sodium methoxide (1). Fatty acid composition was determined by gas chromatography using a Hewlett-Packard 5890-II (Palo Alto, CA) equipped with a model 7673 auto sampler, dual flame-ionization detectors, and a 0.53 mm × 30 m AT-Silar capillary column (Alltech Associates, Inc., Deerfield, IL). Operating conditions were: carrier, He (3 mL/min); 25:1 (vol/vol) split injection; injection temperature, 250°C; detector temperature, 275°C; and column temperature, 190°C. Lipid analyses were reported as means of three replications.

Segregation patterns for relative estimates of ω-6 desaturase activity (%18:1-desaturation) and ω-3 desaturase activity (%18:2-desaturation) were determined for each population. The following general descriptors were assigned to designate putative genotypes of each parental line based on homozygous dominant or recessive alleles governing ω-6 (A_n/a_n) and ω-3 (B_n/b_n) desaturase activity: N87-2120-3 ($A_1A_1b_1b_1$), N87-2122-4 ($a_1a_1B_1B_1$), PI 342434 ($A_2A_2B_2B_2$), and PI 424031 ($A_3A_3B_3B_3$). To test the hypothesis that each *G. soja* parental line contributed a different complement of alleles governing expression of 18:3 to each population, progeny phenotypic classes were constructed (assuming a confidence interval of two standard deviations about the mean value of the *G. max* parent) from the frequency distribution of 18:1- and 18:2-desaturation estimates within each population. A contingency table was used to establish the independence of each frequency distribution (18). A chi-square analysis also was performed to compare class frequencies for a given set of putative alleles within each population against expected frequencies for inheritance of a single gene (5:3 phenotypic ratio) or two independent genes exhibiting duplicate action (55:9 phenotypic ratio) in the F_3 generation. In addition, linear regression was performed on median 18:1- and 18:2-desaturation estimates among allelic classes from populations having a common *G. max* parent.

RESULTS AND DISCUSSION

Glycine max and *G. soja* germplasm selected for in this investigation were distinguished by statistically significant differences in relative 18:1- and 18:2-desaturation values, which indicate the genetic basis for the observed 18:3 concentration in each genotype (Table 1). In *G. max* lines, 18:1-desaturation values lower than *ca*. 64% and 18:2-desaturation values lower than *ca.* 9% are associated with recessive alleles encoding the respective desaturases; whereas, values equal to or higher than those limits denote dominant alleles (6,10). Assuming those conditions, N87-2120-3 carried a pair of recessive alleles for the ω-3 desaturase, and N87-2122-4 carried a pair of recessive alleles governing expression of the ω-6 desaturase (3). Although little is known about inheritance or genetic regulation of 18:3 concentration in *G. soja* germplasm, the relative estimates of 18:1- and 18:2-desaturation in the plant introductions used in this study were significantly greater than those typically expected for corresponding dominant genes in *G. max*. This suggested a different complement or perhaps additional copies of genes that determine 18:3 concentration in these *G. soja* accessions. To test that hypothesis, inheritance of these desat-

TABLE 1
Fatty Acid Composition of Parental Lines Used to Develop Four *Glycine max* × *Glycine soja* Populations

	(% total lipid)					(%)	
Line	16:0	18:0	18:1	18:2	18:3	18:1-D[a]	18:2-D[b]
N87-2120-3	6.6	4.0	29.7	56.2	3.5	66.8	5.9
N87-2122-4	5.6	3.6	45.8	40.6	4.4	49.6	9.8
PI-342434	11.6	3.6	19.1	54.7	11.0	77.5	16.8
PI-424031	14.8	3.3	12.0	55.2	14.7	85.3	21.1
$LSD_{0.05}$[c]	1.9	0.2	5.7	2.7	2.4	6.0	3.2

[a]18:1-Desaturation, %[(18:2 + 18:3)/(18:1 + 18:2 + 18:3)]; relative estimate of ω-6 desaturase activity.
[b]18:2-Desaturation, %[18:3/(18:2 + 18:3)]; relative estimate of ω-3 desaturase activity.
[c]LSD, least significant difference.

urase activities was examined in F_3 progeny derived from four *G. max* × *G. soja* populations.

Interspecific hybridization of N87-2120-3 with PI 342434 (Population I) or PI 424031 (Population II) revealed a wide range of segregation for 18:1-desaturation among F_3 progeny of these *G. max* × *G. soja* populations (Fig. 1). Although these populations lacked recessive alleles associated with low ω-6 desaturase activity, these data showed a skewed distribution typical of the segregation pattern observed for 18:1-desaturation in progeny of *G. max* lines that exhibited allelic differences for genes governing ω-6 desaturase activity (10). However, if the putative A_2 and A_3 alleles contributed by these *G. soja* accessions were identical to the putative A_1 allele in N87-2120-3, these frequency distributions for Populations I and II should overlap. Contingency table analyses established that these data sets were distinct or independent from each other (calculated $\chi^2 = 148.5$; tabular value, $\chi^2_{0.05\,(14)} = 23.7$). A *t*-test indicated that Population II had significantly greater ($P < 0.01$) mean ω-6 desaturase activity than Population I. Transgressive segregants in both populations, where 18:1-desaturation values exceed those of the *G. soja* parents, also suggested that different alleles were present. These observations were supported by chi-square analyses which showed the A_1A_1 and A_2__ progeny class frequencies in Population I and the A_1A_1 and A_3__ progeny class frequencies in Population II each fit the phenotypic distribution expected for a single gene in the F_3 generation (Table 2). This evidence suggested PI 342434 and PI 424031 carry alternative alleles governing ω-6 desaturase activity.

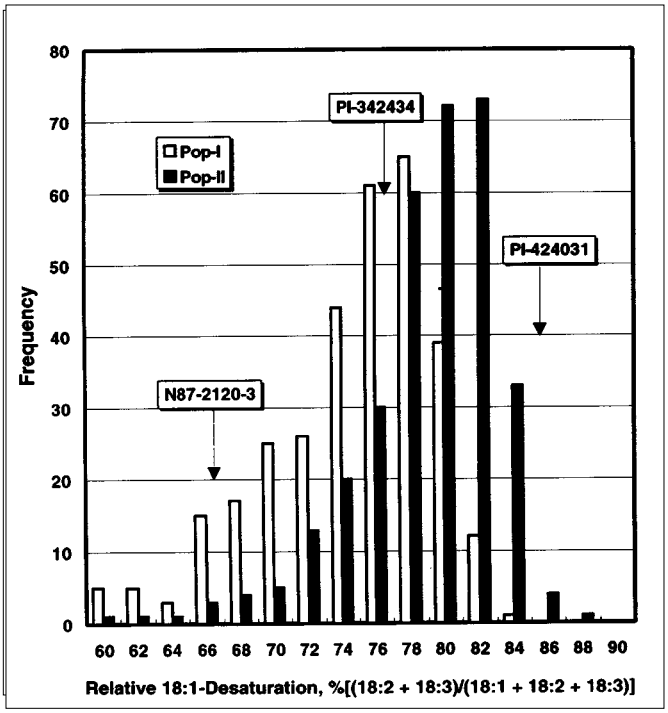

FIG. 1. Frequency distribution of relative estimates of 18:1-desaturation in seed from F_3 progeny of *Glycine max* × *G. soja* populations exhibiting alternative alleles governing ω-6 desaturase activity. Population I, N87-2120-3 × PI 342434 ($A_1A_1b_1b_1 \times A_2A_2B_2B_2$), $n = 318$; Population II, N87-2120-3 × PI 424031 ($A_1A_1b_1b_1 \times A_3A_3B_3B_3$), $n = 321$.

Interspecific hybridization of N87-2122-4 with PI 342434 (Population III) or PI 424031 (Population IV), which lacked recessive alleles associated with low ω-3 desaturase activity, exhibited a wide range of segregation for 18:2-desaturation among *G. max* × *G. soja* progeny in the F_3 generation (Fig. 2). Each population gave a normal distribution without transgressive segregants. However, these segregation patterns for Populations III and IV should overlap if the putative B_2 and B_3 alleles contributed by the *G. soja* lines were identical to the putative B_1 allele in N87-2122-4. Contingency table analyses established that these data sets were distinct or independent from each other (calculated $\chi^2 = 143.9$; tabular value, $\chi^2_{0.05\,(16)} = 26.3$). A *t*-test indicated that Population IV had significantly greater ($P < 0.01$) mean ω-3 desaturase activity than Population III. Thus, dissimilar distributions between these data again suggested that different alleles were present. These observations were confirmed by chi-square analyses which showed the B_1B_1 and B_2__ progeny class frequencies in Population III and the B_1B_1 and B_3__ progeny class frequencies in Population IV each fit the phenotypic distribution expected for a single gene in the F_3 generation (Table 2). Thus, apparent genetic differences between these populations supported the theory that PI 342434 and PI 424031 also contributed alternative alleles governing ω-3 desaturase activity.

Regression of median desaturation estimates for each allelic class against corresponding 18:3 concentrations in crude oil of populations derived from a common *G. max* parent provided

TABLE 2
Frequency Classs Distribution and Expectation of Alleles That Determine 18:3 Concentration in Seed of F_3 Progeny of *Glycine max* × *G. soja* Populations

	ω-6 Desaturase (% 18:1-desaturation)				ω-3 Desaturase (% 18:2-desaturation)			
Population	Allele	Observed	Expected	Sum χ^2	Allele	Observed	Expected	Sum χ^2
I	A_1A_1	107	119[a]	1.93[c]	b_1b_1	52	45[b]	1.27[c]
	A_2—	211	199		B_2—	266	273	
II	A_1A_1	102	120[a]	4.31[c]	b_1b_1	54	45[b]	2.09[c]
	A_3—	219	201		B_3—	267	276	
III	a_1a_1	35	33[b]	0.14[c]	B_1B_1	106	88[a]	5.90[c]
	A_2—	199	201		B_2—	128	146	
IV	a_1a_1	33	44[b]	3.20[c]	B_1B_1	101	117[a]	5.90[c]
	A_3—	278	267		B_3—	210	194	

[a]Phenotypic distribution (5:3 ratio) expected for a single gene in the F_2 generation.
[b]Phenotypic distribution (55:9 ratio) expected for two independent genes in the F_3 generation.
[c]$\chi^2_{0.01(1)} = 6.63$; smaller sum χ^2 values indicate agreement with the expected ratio.

further evidence that these *G. soja* accessions contributed different alleles to the respective populations. A very strong linear relation among median 18:1-desaturation values and 18:3 concentrations for A_1A_1, A_2— and A_3— classes between Populations I and II indicated these alleles were related but different from each other (Fig. 3). A similar pattern was found between a_1a_1, A_2— and A_3— classes in Populations III and IV. The strength of these associations suggested possible additive genetic effects between the *G. soja* alleles and *G. max* alleles that mediate normal or low 18:1-desaturation. Similarly, a strong linear relation was found among median 18:2-desaturation values and 18:3 concentrations for B_1B_1, B_2— and B_3— classes between Populations III and IV; and b_1b_1, B_2— and B_3— classes between Populations I and II (Fig. 4). Again, these data suggested possible additive effects of superior alternative alleles that determine high-18:3 concentration in *G. soja*.

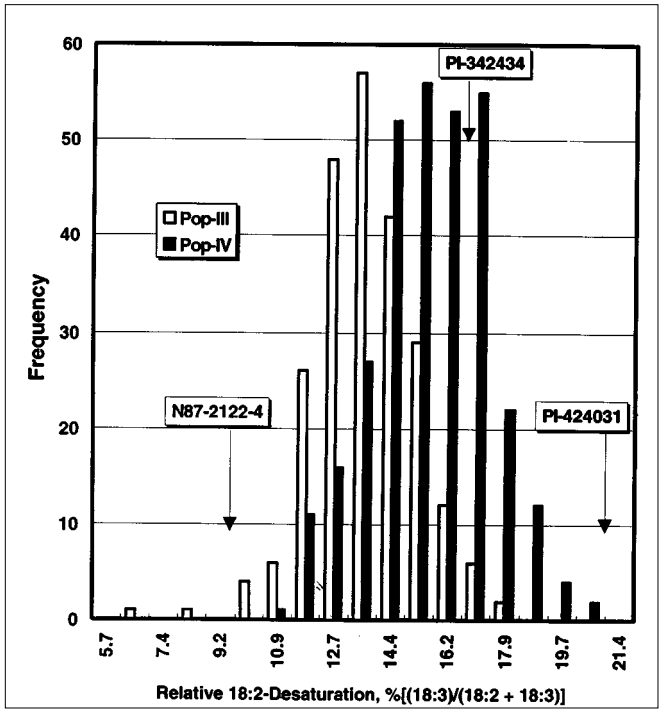

FIG. 2. Frequency distribution of relative estimates of 18:2-desaturation in seed from F_3 progeny of *Glycine max* × *G. soja* populations exhibiting alternative alleles governing ω-3 desaturase activity. Population III, N87-2122-4 × PI 342434 ($a_1a_1B_1B_1 \times A_2A_2B_2B_2$), $n = 234$; Population IV, N87-2122-4 × PI 424031 ($a_1a_1B_1B_1 \times A_3A_3B_3B_3$), $n = 311$.

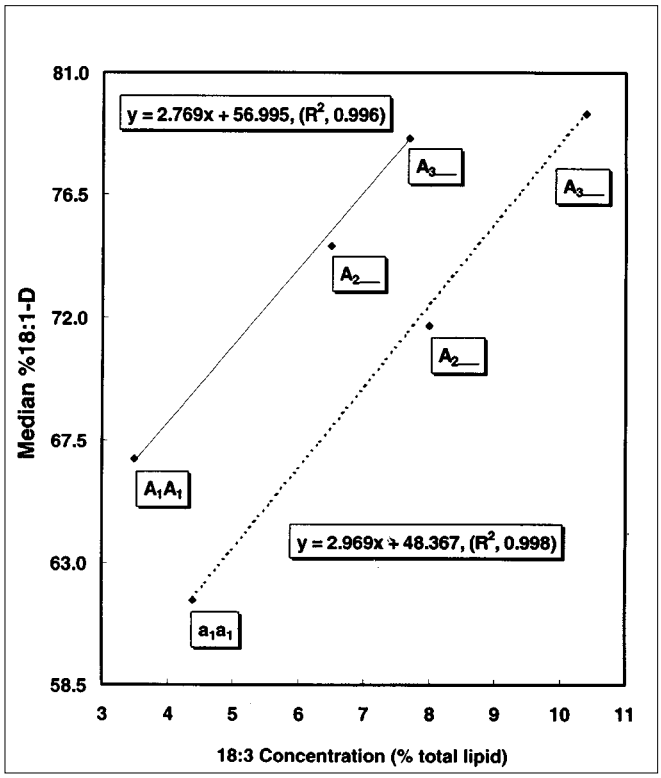

FIG. 3. Relation of allelic classes governing ω-6 desaturase activity in F_3 seed among progeny of four *Glycine max* × *G. soja* populations. Median %18:1-desaturation estimates were regressed against corresponding 18:3 concentrations among interspecific populations with the *G. max* parents N87-2120-3 (————) or N87-2122-4 (·····).

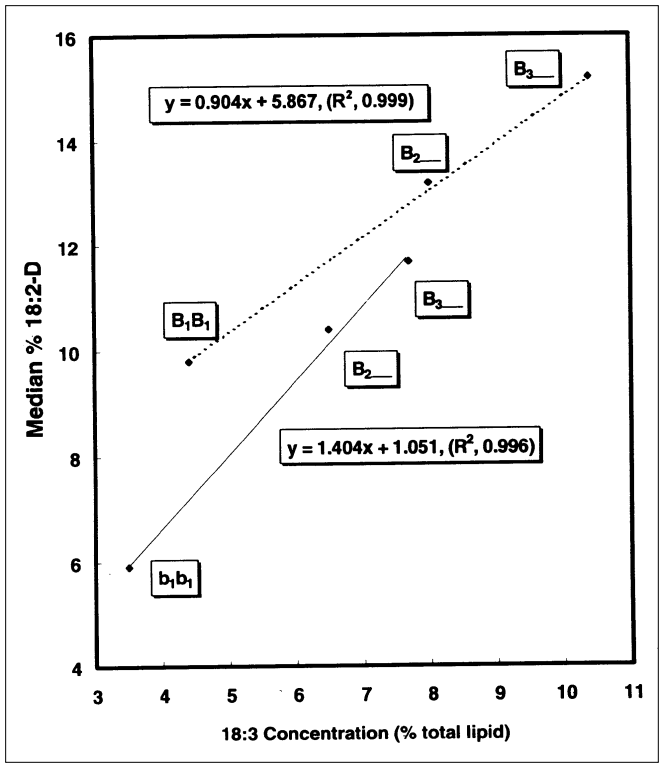

FIG. 4. Relation of allelic classes governing ω-3 desaturase activity in F3 seed among progeny of four *Glycine max* × *G. soja* populations. Median %18:2-desaturation estimates were regressed against corresponding 18:3 concentrations among interspecific populations with the *G. max* parents N87-2120-3 (————) or N87-2122-4 (·····).

In conclusion, discovery of putative alternative alleles which augment expression of ω-6 and ω-3 desaturase activities in *G. soja* opens a new avenue of research on genetic regulation of 18:3 concentration in cultivated soybean. Breeding and molecular genetic approaches have advanced the development of low-18:3 cultivars for general purpose edible applications. Now it appears that the wild ancestor of *G. max* will provide the genetic resources needed to develop highly polyunsaturated oils for industrial uses such as paints, ink carriers, and biodiesel fuel. Although knowledge of inheritance and number of genes governing polyunsaturated fatty acid composition in wild soybeans is limited, this investigation demonstrates that alternative alleles inherent to specific *G. soja* germplasm may be transferred relatively easily to *G. max* to develop new high-18:3 soybean lines. Continuation of these research efforts should not only broaden the genetic base for *G. max*, but also lead to new opportunities that may extend the utility of soybean in existing and emerging markets for industrial oils.

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