Genetic Regulation of Elevated Stearic Acid Concentration in Soybean Oil

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ABSTRACT: Soybean [*Glycine max* (L.) Merr.] oil from commercial cultivars typically contains *ca.* 3% stearic acid (18:0). However, germplasm carrying different mutations at the locus governing stearic acid (*Fas*) may contain 3% to about 35% 18:0. Among these germplasm, a newly developed line, FAM94-41 (9% 18:0), carries a serendipitous natural mutation that is temporarily designated as the recessive fas_{nc} allele, and the germplasm A6 (26% 18:0) carries the recessive fas^a allele. Mendelian genetic analysis of progeny from FAM94-41 × A6 revealed that fas_{nc} and fas^a are allelic to each other and represent different mutations in the same structural gene. However, the gene products (enzymes) produced by these alleles are unknown. The observation that 18:0 concentrations among progeny from FAM94-41 × A6 increased primarily at the expense of unsaturated C_{18} FA suggests that *fas* alleles may reduce either 18:0-acyl carrier protein (AcP) desaturase or 18:1-ACP thioesterase activity. However, it also is conceivable that elevated 18:0 concentrations may result from increased 3-keto-acyl-ACP synthetase (KAS) II activity. To test the latter possibility, a population was created that segregated for the fas_{nc} and the fap_2 alleles (the latter of which is associated with reduced KAS-II activity). Mendelian genetic analysis showed that these alleles represent independent genes at different gene loci and interact in an additive genetic manner to increase the total saturate concentration in this population. Based on this finding, we speculate that *fas* alleles probably encode 18:0-ACP desaturase or 18:1-ACP thioesterase in soybeans.

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KEY WORDS: Developing seed, *fas* alleles, genetics, glycerolipid composition, *Glycine max*, inheritance, oil, palmitic acid, saturated fat, stearic acid.

The stearic acid (18:0) concentration in soybeans typically averages about 3% of crude oil (1). However, this phenotypic trait may be genetically altered by mutations at gene loci, designated *Fas,* which typically result in elevated 18:0 concentrations. Nearly all of these variants have been induced by chemical or X-ray mutagenesis, with the exception of a newly developed line, FAM94-41. FAM94-41 (9% 18:0) is the only known soybean germplasm that carries a serendipitous natural mutation, presumably at *Fas*, which now is temporarily designated as the recessive fas_{nc} allele. Prior to the discovery of fas_{nc}, five germplasm lines were reported to carry homozygous recessive alleles that influenced 18:0 concentrations in soybean oil: fas^a [A6 (2)], fas^b [FA41545 (3)], *fas* [A81-606085 (3)], st_1 [KK-2 (4)], and or st_2 [M25 (4)]. As reported (3), fas^a (30% 18:0), fas^b (15% 18:0), and *fas* (19% 18:0) are allelic to each other and presumably represent different mutations in the same gene. In an F_3 population segregating for st_1 and st_2, the observation of progeny with 18:0 concentrations above and below the respective parental means provides evidence for two independently inherited genes governing higher 18:0 concentrations in soybeans. The proposed $st_1\ st_1\ st_2\ st_2$ genotype may elevate the 18:0 concentration beyond 35% in crude oil (4). However, it is unknown whether st_1 or st_2 is allelic to fas^a, fas^b, or *fas*. Mendelian genetic studies for uniqueness and allelism among these *fas* and *st* alleles have not been conducted. Still, other evidence supports the hypothesis that genes at different *Fas* loci may contribute to a very high 18:0 phenotype. In the mating of A6 and ST2 (another line derived from chemical mutagenesis), the F_1 progeny mean 18:0 content was greater than that of either parent (5). Based on this information, it is highly probable that mutations in at least two independent genes determine the activity of one or more enzymes involved in 18:0 synthesis in soybeans.

Two additive alleles, designated fas_2 and fas_x, have been shown to determine lower 18:0 concentrations in *N*-nitroso-*N*-methylurea-treated sunflower (*Helianthus annuus* L.) (6). Also, two alleles in ethylmethane-sulfonate-treated *Arabidopsis* have been shown to elevate 18:0 concentrations in both seed and leaf tissue (7). These alleles have been identified as $fab_{2\text{-}1}$ (20% 18:0) and $fab_{2\text{-}2}$ (6% 18:0) and represent mutations at loci designated *Fab* in *Arabidopsis*. A striking feature of the latter research is the observation that both $fab_{2\text{-}1}$ and $fab_{2\text{-}2}$ can cause up to a fivefold reduction in plant size. This finding is interesting in view of reports that the yielding ability of soybeans is severely depressed by fas^a, fas^b, or *fas* alleles (8,9). Poor yielding ability apparently is a reason for slow progress in the development of acceptable commercial soybean cultivars with higher 18:0 contents. Our experience with FAM94-41 suggests that this problem may be overcome. The pedigree of FAM94-41, Brim × {Brim × [Brim × (N87-2117 × Brim)]}, has four occurrences of the high-yielding maturity group VI cultivar Brim in its genetic background (10). Thus, FAM94-41 is an agronomically robust high-18:0 line, and may become invaluable in reversing the apparent yield depression intrinsic to material

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developed with the fas^a, fas^b, or *fas* alleles. Before testing such a hypothesis, however, it is necessary to determine whether the inheritance of fas_{nc} is independent of or allelic to fas^a. In addition, the interaction of *fas* with other alleles that influence FA composition in a segregating population often provides important clues to help guide the identification of the probable metabolic site(s) within the FA synthetic pathway that are subject to these alleles.

MATERIALS AND METHODS

Plant material. Soybean lines exhibiting homozygous genes at *Fap* and *Fas* loci were grown at the Central Crops Research Station in Clayton North Carolina in 1996. These lines included C1727 ($fap_2fap_2\ Fas_{nc}Fas_{nc}$), A6 ($Fap_2Fap_2\ fas^afas^a$), and FAM94-41 ($Fap_2Fap_2\ fas_{nc}fas_{nc}$). The cultivar Dare ($Fap_2Fap_2\ Fas_{nc}Fas_{nc}$) was grown to establish a normal soybean FA composition. The stage of seed development was reported in days after flowering (DAF). Cross-pollinations were made between FAM94-41 and A6, and between FAM94-41 and C1727. Populations were developed to examine the inheritance of 18:0 in FAM94-41. The F_1 hybrid plants from both crosses were grown under controlled environmental conditions in a greenhouse with a randomized complete block design. The average day/night temperature throughout plant development was 30/18°C. Single F_2 seeds from FAM94-41 × A6 were harvested at maturity and subjected to FA analysis. Homozygous parental types and homozygous or heterozygous recombinant types were classified on the basis of variability and SD of the F_2 mean for 18:0 concentration. Similarly, single F_2 seeds were harvested at maturity from FAM94-41 × C1727. In this cross, phenotypic classes were based on variability and SD of the respective F_2 means for 16:0 and 18:0 concentrations. Chi-square analyses were used to determine whether the respective phenotypic classifications were consistent with genetic ratios for alleles at the same or two independent loci.

Tissue analyses. Representative dry mass and oil content were determined in seed (20 to 30 g fresh weight) harvested at 70 DAF. Oil concentration was measured with a Maran pulsed NMR instrument (Resonance Instruments Ltd., Whitney, Oxfordshire, United Kingdom). Oil was extracted from crushed seed with 10 mL chloroform/hexane/methanol (7:5:2, by vol). FAME were prepared from total lipids (TL) by heating with 1 mL 5% (vol/vol) sulfuric acid in methanol at 80°C for 90 min. After cooling to ambient temperature, the reaction mixture was vortexed with 1.5 mL 1.5% NaCl plus 1 mL hexane and held at −20°C to allow phase separation. The hexane phase (top) was removed, dried under nitrogen at 55°C, and resuspended in 100 µL 2:1 (vol/vol) chloroform/methanol prior to analysis by GC. FA composition was determined with a Hewlett-Packard model 5890-II gas chromatograph (Palo Alto, CA) equipped with a model 7673 auto sampler, dual FID detectors, and dual 0.53 mm × 30 m AT-Silar capillary columns (Alltech Associates Inc., Deerfield, IL). Operating conditions were as follows: carrier, He (3 mL/min); 25:1 (vol/vol) split injection; injection temperature, 250°C; detector temperature, 275°C; column temperature, 200°C.

RESULTS AND DISCUSSION

Two soybean germplasm lines, A6 and FAM94-41, which exhibit higher levels of 18:0, were mated to evaluate the inheritance of the alleles responsible for this trait. A6 typically contains about six times the 18:0 concentration commonly found in soybean oil; whereas FAM94-41 germplasm contains a two-fold increase in 18:0 concentration (Table 1). In comparing the compositions of these lines, the gain in 18:0 concentration appears to have come at the expense of the sum of all unsaturated C_{18} FA (total 18:0), more so than 16:0. As a result, the high-18:0 trait contributed to significantly elevated total saturates in the oil. In A6, 18:0 accounted for about 76% of total saturates (TS). Analysis of seed from FAM94-41 × A6 showed that the respective calculated midparent values were within one SD of the F_2 progeny mean for the measured traits. This result suggests that maternal and cytoplasmic effects were not important factors in the genetic control exerted by fas_{nc} or fas^a on 18:0 concentrations in soybeans.

The frequency distribution for 18:0 concentrations among F_2 progeny, segregating for fas_{nc} and fas^a, is given in Figure 1. These data show a wide range of values, from 6 to 26% 18:0. Within reasonable limits of certainty, the variation in 18:0 concentrations among all of these progeny falls between that of the two parents. Thus, the possibility that the observed phenotypic variation arises from the combination of complementary or independently inherited genes is remote because

Table 1
FA Composition of Soybean Germplasm Exhibiting Genetic Variation at Gene Loci Designated as *Fas*[a]

		% Total lipid at maturity				
Line	Genotype	C16:0	C18:0	Other[b]	TS[c]	C18:0/TS
Dare (normal)	*Fas Fas*	10.5	4.0	85.5	14.5	27.6
FAM94-41	$fas_{nc}\ fas_{nc}$	10.1	8.8	81.1	18.9	46.6
A6	$fas^a\ fas^a$	8.1	26.0	65.9	34.1	76.3
FAM94-41 × A6	Midparent	9.0	17.4	73.6	26.4	65.9
	F_2 mean	8.7	15.3	76.0	24.0	63.8
SD		0.8	3.8	7.5	3.5	7.0

[a]Other: C18:1 + C18:2 + C18:3. TS, total saturates: 16:0 + 18:0.

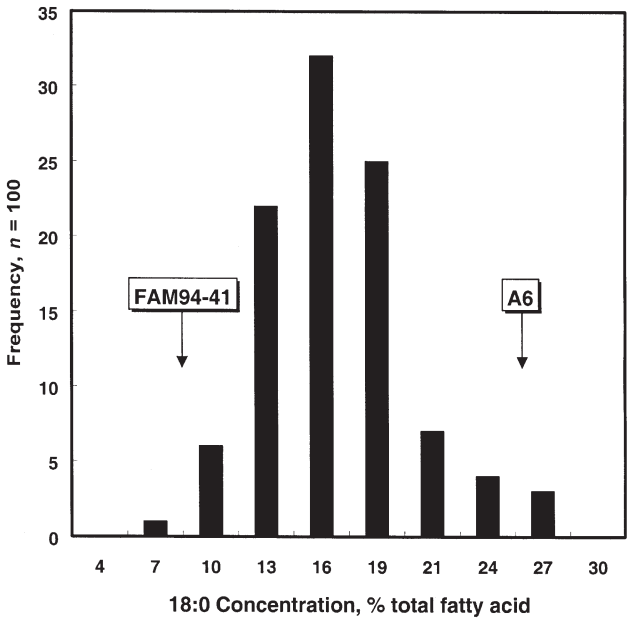

FIG. 1. Frequency distribution of 18:0 concentration in total lipids of mature seed from an F_2 population of FAM94-41 × A6. Parental lines were FAM94-41 ($fas_{nc}fas_{nc}$) and A6 (fas^afas^a). The lack of transgressive segregants among the progeny of this population indicated that fas_{nc} was allelic to fas^a.

of a lack of discernible transgressive segregants (progeny with 18:0 concentrations above the high-parent value or below the low-parent value) in this population. Furthermore, chi-square analyses of the two-gene models, including epistatic models, failed to support the inheritance of two different genes. Therefore, this frequency pattern is indicative of allelic segregation among progeny and suggests that the elevated 18:0 phenotype in both FAM94-41 and A6 is governed by the same gene. In addition, chi-square analysis of these data supported a 3:1 progeny ratio ($\chi^2 = 1.33$) that did fit a single-gene model for differing alleles between FAM94-41 and A6 (Table 2). Hence, fas_{nc} and fas^a are allelic to each other. In other words, they represent different mutations at the *fas* locus. A similar conclusion was reported from the inheritance pattern for fas^a, fas^b, and *fas* alleles (3). In that study, the fas^a allele was dominant over the *fas* allele but not dominant over fas^b. The current genetic analysis suggests that the fas_{nc} allele in FAM94-41 is different and recessive to the fas^a allele in A6. Thus, all five of these *fas* alleles probably contribute major genetic effects to the activity of the same enzyme in the FA or glycerolipid synthetic pathway.

Although the gene product (enzyme) of these *fas* alleles is unknown, further interpretation of the population segregating for fas_{nc} and fas^a provides useful information regarding the nature of these genetic effects. As shown in Figure 2, a strong negative correlation (r^2, 0.96) was found between total 18:1 and 18:0 concentrations, whereas only a weak negative correlation (r^2, 0.28) was seen between 16:0 and 18:0. This suggests that increases in 18:0 concentration primarily were achieved at the expense of all C_{18} unsaturated FA (total 18:1). In fact, the decline in total 18:1 accounted for 89% of the increase in 18:0 within these F_2 progeny. Among all enzymes in the FA and glycerolipid pathways, it generally is believed that the enzyme activities of 3-keto-acyl-ACP (acyl carrier protein) synthetase II (KAS-II), 18:0-ACP desaturase (Δ9DES), or 18:1-ACP thioesterase (18:1-ACP TE) have the highest probability of effecting changes in 18:0 concentration in oilseeds and other plant tissues. KAS-II catalyzes the elongation of 16:0-ACP to 18:0-ACP, Δ9DES desaturates 18:0-ACP to 18:1-ACP, and 18:1-ACP TE initiates conversion of 18:0-ACP and 18:1-ACP (with lesser activity on 18:0-ACP) to their respective acyl-CoA derivatives (11). In view of the apparently strong negative relation between 18:0 and total 18:1 concentrations in this population, it is likely that the high-18:0 trait arises from *fas* gene action, which reduces either Δ9DES or perhaps 18:1-ACP TE activity. However, it is conceivable that the mutations resident in fas_{nc} and fas^a might effect increased conversion of 16:0 to 18:0, as may be envisioned by greater KAS-II activity.

A new population (FAM94-41 × C1727) was created to test the theory that *fas* alleles influence KAS-II activity. The C1727 germplasm carries a homozygous recessive mutation (fap_2) at a gene locus designated *Fap* (12). The fap_2 mediates an oil phenotype with about 18% 16:0 and 3% 18:0 and appears to effect reduced KAS-II activity (13). Given that assumption, if fas_{nc} encodes greater KAS-II activity, the segregating population should produce no progeny with a 16:0 plus 18:0 concentration greater than the original parents owing to opposing genetic effects on KAS-II activity. Conversely, if fas_{nc} mediates either reduced Δ9DES or perhaps reduced 18:1-ACP TE activity, a small proportion of the progeny from

TABLE 2
Frequency Class Distribution of Alleles at *Fas* in F_2 Seed of FAM94 41 × A6 Soybeans[a]

	% Total lipid at maturity (mean ± SD)		Number of individuals		
Genoypic class	C16:0	C18:0	Observed	Expected	χ^2
fas_{nc} fas_{nc}	9.4 ± 0.8	10.5 ± 1.5	25	25	0.00
fas^a fas_{nc}	8.5 ± 0.7	15.2 ± 1.5	54	50	0.32
fas^a fas^a	8.3 ± 0.7	20.8 ± 2.6	21	25	0.64
Total			100	100	0.96

[a]χ^2 calculated = 0.96 is less than χ^2 (0.05, 2) = 5.99 for two DF, supporting the fit of three genotypic classes in a 1:2:1 single-gene inheritance ratio.

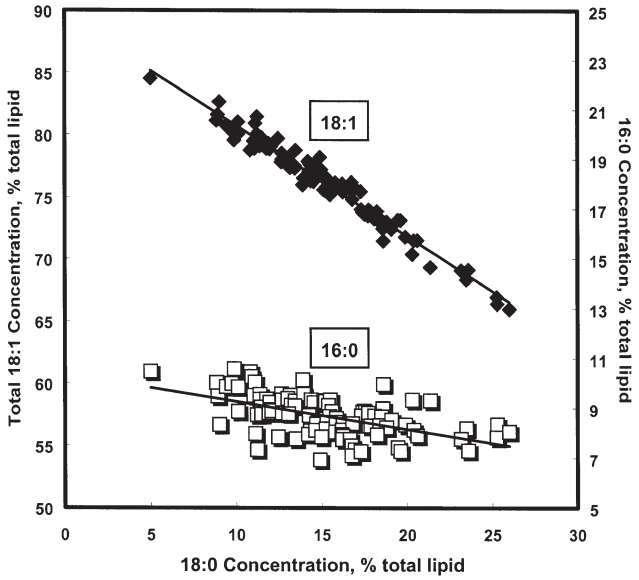

FIG. 2. Effect of *fas* alleles on FA composition of mature seed from an F_2 population of FAM94-41 × A6. A strong negative correlation (R^2, 0.96) was found between 18:0 concentration and total C18 unsaturated FA (total 18:1) concentration. A very weak negative correlation (R^2, 0.28) was found between 18:0 and 16:0 concentration in progeny segregating at *Fas*.

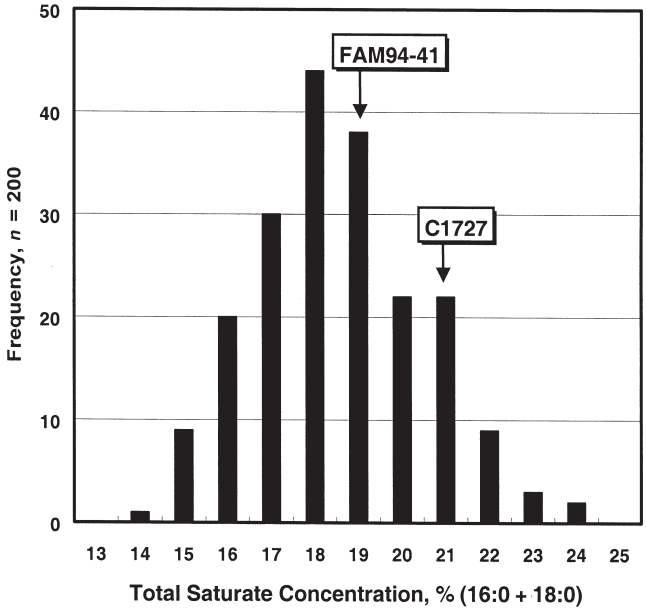

FIG. 3. Frequency distribution of total saturated FA concentration in oil of mature seed from an F_2 population of FAM94-41 × C1727. Parental lines were the high-18:0 line FAM94-41 ($fas_{nc}fas_{nc}$) and the high-16:0 line C1727 (fap_2fap_2). Independently, the fas_{nc} allele segregated in a 3:1 ratio for low-18:0 concentration, and the fap_2 allele segregated in a 3:1 ratio for low-16:0 concentration. In combination, these alleles fit a complete dominance model (9:3:3:1 ratio) for two independently segregating alleles at different loci. Progeny carrying one or more Fap_2, and Fas_{nc} alleles represented transgressive segregants for lower total saturated FA concentration. Recombinants having homozygous recessive $fas_{nc}fas_{nc}$ alleles represented transgressive segregants for higher total saturate content.

this population should represent transgressive segregants with a high-16:0 plus high-18:0 phenotype.

Analysis of seed from FAM94-41 × C1727 showed that the respective calculated midparent values were within one SD of the F_2 progeny mean of the measured traits. This result suggests that maternal and cytoplasmic effects were not important factors in the genetic control exerted by fas_{nc} or fap_2 on saturated FA concentrations in soybeans. Although data are not shown, the frequency distribution for 18:0 concentrations among F_2 progeny segregating for fas_{nc} (ranging from 3 to 10.4% 18:0) fit a 1:2:1 progeny ratio for a single-gene model. The frequency distribution for 16:0 concentrations in this population (ranging from 10 to 17.9% 16:0) also fits a 1:2:1 progeny ratio (data not shown). Additional evidence suggesting that fas_{nc} and fap_2 segregate as independent genes at different loci is shown in Figure 3. These data represent a wide range of values, from 13.8 to 23.2% TS, where transgressive segregants occur above the high-parent value and below the low-parent value in this population. This type of phenotypic variation may be expected with the combination of independently inherited genes. Furthermore, chi-square analyses of these data fit a 9:3:3:1 ratio, which supports a two-gene model (Table 3). Thus, recombinants of fas_{nc} and fap_2 may be represented by the phenotypic classes Fap_2—Fas_{nc}—, Fap_2— $fas_{nc}fas_{nc}$, fap_2fap_2 Fas_{nc}—, and fap_2fap_2 $fas_{nc}fas_{nc}$. Based on these data, the elevated TS phenotype in

TABLE 3
Frequency Class Distribution of Alleles at *Fas* and *Fap* in F_2 Seed of FAM94 41 × C1727 Soybeans[a]

	% Total lipid at maturity (mean ± SD)		Number of individuals		
Phenotypic class	C16:0	C18:0	Observed	Expected	χ^2
Fap_2—Fas_{nc}—	12.8 ± 1.2	4.0 ± 0.6	118	112	0.32
Fap_2—$fas_{nc}fas_{nc}$	11.9 ± 1.2	7.1 ± 1.2	46	38	1.93
fap_2 fap_2 Fas_{nc}—	16.3 ± 0.7	4.0 ± 0.6	31	38	1.13
$fap_2fap_2fas_{nc}fas_{nc}$	16.2 ± 1.0	6.2 ± 0.8	5	12	4.08
Total			200	200	7.46

[a]χ^2 calculated = 7.46 is less than χ^2 (0.05, 2) = 7.81 for three DF, supporting the fit of four phenotypic classes in a 9:3:3:1 ratio of two independent genes.

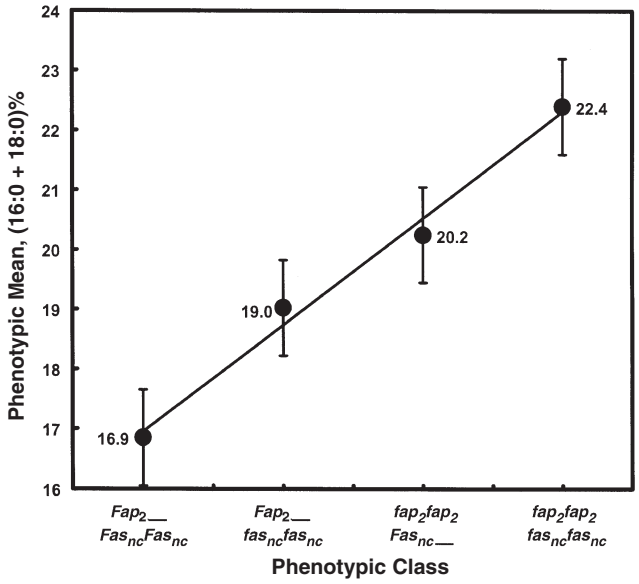

FIG. 4. Influence of gene combinations governing 18:0 and 16:0 concentrations on total saturates in the oil of mature seed from an F_2 population of FAM94-41 × C1727. The linear relation among mean estimates of total saturated FA concentration within a genotypic class indicates additive genetic interaction; R^2, 0.962.

this population is governed by two different genes, and the genetic interaction of these alleles appears to be additive in nature (Fig. 4). Therefore, we may reject the hypothesis that *fas* alleles encode or regulate the KAS-II enzyme.

Given that conclusion, and based on what is known about the enzymes for this pathway from the literature, reduced Δ9DES and perhaps reduced 18:1-ACP TE activity become the leading candidates for the causal mechanism for elevated 18:0 concentrations in soybeans. Researchers may now focus attention on characterization of the Δ9DES and 18:1-ACP TE genes, and on evaluation of their respective enzyme activities in soybean germplasm carrying fas_{nc} or fas^a alleles.

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