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ORIGINAL PAPER

Effect of Δ9-stearoyl-ACP-desaturase-C mutants in a high oleic background on soybean seed oil composition

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Abstract
Key message **Two new sources of elevated seed stearic acid were identified and the feasibility of an elevated stearic acid, high oleic acid germplasm was studied.**
Abstract Soybean [*Glycine max* (L.) Merr.] oil typically contains 2–4 % stearic acid. Oil with at least 20 % stearic acid is desirable because of its improved baking properties and health profile. This study identifies two new sources of high stearic acid and evaluates the interaction of high stearic and oleic acid alleles. TCHM08-1087 and TCHM08-755, high stearic acid 'Holladay' mutants, were crossed to FAM94-41-3, a line containing a point mutation in a seed-specific isoform of a Δ9-stearoyl-acyl carrier protein-desaturase (*SACPD-C*). F_2-derived lines were evaluated for fatty acid content in four field environments. Sequencing of *SACPDs* in TCHM08-1087 and TCHM08-755 revealed distinct deletions of at least one megabase encompassing *SACPD-C* in both lines. After genotyping, the additive effect for stearic acid was estimated at +1.8 % for the *SACPD-C* point mutation and +4.1 % for the *SACPD-C* deletions. Average stearic acid in lines homozygous for the deletions was 12.2 %. A FAM94-41-3-derived line and TCHM08-1087-11, a selection from TCHM08-1087, were crossed to S09-2902-145, a line containing missense mutations in two fatty acid desaturases (*FAD2-1A* and *FAD2-1B*). F_1 plants were grown in a greenhouse and individual F_2 seed were genotyped and phenotyped. No interaction was observed between either *FAD2-1A* or *FAD2-1B* and any of the *SACPD-C* mutant alleles. Seed homozygous mutant for *SACPD-C/FAD2-1A/FAD2-1B* contained 12.7 % stearic acid and 65.5 % oleic acid while seed homozygous for the *SACPD*-C deletion and mutant for *FAD2-1A* and *FAD2-1B* averaged 10.4 % stearic acid and 75.9 % oleic acid.

Abbreviations

18:1-ACP TE	18:1-Acyl carrier protein thioesterase
FAD	Fatty acid desaturase
KASII	3-Ketoacyl-acyl carrier protein synthase II
KASPar	KBiosciences competitive allele specific PCR
SACPD	Δ9-Stearoyl-acyl carrier protein-desaturase

Introduction

Soybean [*Glycine max* (L.) Merr.] is extensively produced worldwide, comprising over half of all oilseed production (USDA-Economic Research Service 2012). The baking industry requires an oxidatively stable oil with a relatively high melting temperature (Clemente and Cahoon 2009). One way that this oil is produced is by blending soybean oil with another vegetable oil high in the sixteen-carbon saturated fatty acid palmitate. However, palmitic acid is undesirable for human food use because its consumption results in an unfavorable lipoprotein profile in blood serum (Mensink and Katan 1990). In contrast, stearic acid, an eighteen-carbon saturated fatty acid, has been shown to not be associated with the same negative health effects (Kris-Etherton and Yu 1997). An ideal soybean oil for use in solid fat applications would consist of 20 % stearic acid

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and would have more desirable chemical and nutritional properties than the oils it would replace (Kok et al. 1999; Hunter et al. 2010). Also valuable would be a soybean oil high in both stearic acid and oleic acid because of its oxidative stability, relatively high melting temperature, and favorable nutritional profile (Clemente and Cahoon 2009).

Most soybean germplasm has a stearic acid content between two and five percent of total fatty acids (ILSI 2010; USDA-ARS 2013); however, several lines have been identified with higher amounts of stearic acid. Most sources of identified variation for stearic acid content in soybean oil involve mutations in the Δ9-stearoyl-acyl carrier protein-desaturase (*SACPD)* genes (Ohlrogge and Browse 1995). These enzymes desaturate stearoyl-ACP to oleoyl-ACP in the plastid. Three isoforms of *SACPD* have been identified in soybean (Byfield et al. 2006; Zhang et al. 2008). Two of the isoforms, *SACPD-A* and *SACPD-B*, are active in both vegetative and reproductive tissues while the third, *SACPD-C*, is primarily expressed in developing seed and the embryonic axle of germinating seed (Byfield et al. 2006; Zhang et al. 2008; Kachroo et al. 2008). One *SACPD-B* mutant has been linked to an elevated stearic acid phenotype (Ruddle et al. 2012) and several *SACPD-C* elevated seed stearic acid mutants have been identified (Hammond and Fehr 1983; Zhang et al. 2008; Boersma et al. 2012). In addition, a number of lines have been reported with elevated stearic acid whose causal loci are unknown or unverified (Graef et al. 1985; Bubeck et al. 1989; Rahman et al. 1995; Hudson 2012).

Mutations in the *SACPD-C* locus have been shown to have higher additive effects on stearic acid composition than the only identified mutation in *SACPD-B* (Ruddle et al. 2012). This mutation in the non-seed-specific *SACPD-B* isoform resulted in agronomically inferior plants and ultimately in reduced seed yield while the *SACPD-C* mutation examined in the same study had no associated adverse agronomic effects (Ruddle et al. 2013).

Other enzymes in the fatty acid biosynthetic pathway have also been proposed as targets for developing a high stearic acid soybean. Two of those enzymes are 3-keto-acyl-acyl carrier protein synthase II (KASII) and 18:1-acyl carrier protein thioesterase (18:1-ACP TE; Pantalone et al. 2002). KASII elongates palmitoyl-ACP to stearoyl-ACP in the step immediately prior to SACPD's desaturation of stearoyl-ACP to oleoyl-ACP (Ohlrogge and Browse 1995). 18:1-ACP TE catalyzes the hydrolyzation of 18:1-ACP and 18:0-ACP prior to export out of the chloroplast (Jones et al. 1995). Increased activity of either enzyme could lead to enhanced levels of stearic acid, as has been reported in other crops (Cantisán et al. 2000).

Transgressive segregation for stearic acid levels was previously observed when a line containing a mutation in *SACPD-C* was crossed with the mid-oleic germplasm line N98-4445A (Zhang et al. 2008). It is unknown whether this further increase in stearic acid levels was due to increased oleic acid levels per se or to an unknown locus or multiple loci. The mid-oleic trait in N98-4445A is controlled by several QTL with relatively small effects (Bachlava et al. 2008), but mutations in major genes (*FAD2-1A* and *FAD2-1B*) that result in elevated oleic acid phenotypes in seed oil have also been reported by several authors (Dierking and Bilyeu 2009; Pham et al. 2010). The FAD2 enzymes desaturate oleoyl moieties to linoleoyl moieties (Ohlrogge and Browse 1995) and several isoforms of genes coding for these enzymes have been identified (Schlueter et al. 2007).

The combination of high oleic and high stearic mutations will greatly improve the oxidative stability of soybean oil, but the possible interactions between these loci are unknown.

A previous study suggested that obtaining oil high in both stearic acid and oleic acid may be difficult because increases in stearic acid due to a mutant SACPD-C enzyme occur largely at the expense of oleic acid (Ruddle et al. 2012). The objectives of this research were to genetically characterize two new sources of elevated stearic acid identified in a population developed by mutagenesis and to establish if combinations of mutant *SACPD-C*s and *FAD2*s could result in an elevated stearic acid, high oleic acid soybean oil.

Materials and methods

Plant materials

Two high stearic populations were developed from crosses performed in 2009 in Clayton, NC. FA-I is a population of 188 F_2-derived lines from the cross FAM94-41-3 × TCHM08-1087. FA-J is a population of 184 F_2-derived lines from the cross FAM94-41-3 × TCHM08-755. FAM94-41-3 (8 % stearic acid; Pantalone et al. 2002) is a maturity group VI selection from an elevated stearic acid germplasm line with a natural mutation in *SACPD-C* (Zhang et al. 2008) which has been designated fas_{nc}. TCHM08-1087 and TCHM08-755 are maturity group V elevated stearic acid selections from a mutagenized population of the cultivar 'Holladay' (Burton et al. 1996) that was developed by exposing seed to 200 grays (Gy) of gamma radiation from a Gammacell 220 (MDS Nordion Inc., Ottawa, ON, Canada (personal communication, Thomas Carter, Jr. Jan 20, 2012). The $M_{1:2}$ rows of TCHM08-1087 and TCHM08-755 that were used as male parents contained 7 % stearic acid compared to the 4 % stearic acid of 'Holladay' (unpublished data). Because the M_1 plants would have been hemizygous or heterozygous for any induced mutations, the $M_{1:2}$ rows were segregating for the high stearic trait.

To study the interaction between high stearic and high oleic loci, two additional populations were developed from crosses performed in 2012 in Clayton, NC. The High Stearic, High Oleic 1 (HSHO-1) population was developed from the cross between S09-2902-145 (homozygous mutant for *FAD2-1A* and *FAD2-1B*) and LLL-05-14 (homozygous mutant for *SACPD-C*). S09-2902-145 (3 % stearic acid, 79 % oleic acid; unpublished data) has the pedigree (S05-11482 × 17D) × S07-14788. S05-11482 is a maturity group V F_5-derived line from the cross S99-2281 × S00-9985-03. Both S99-2281 and S00-9985-03 are breeding lines from Missouri. 17D (Dierking and Bilyeu 2009) carries a missense mutation in *FAD2-1A*. S07-14788 has the pedigree Jake × PI 283327. Jake (Shannon et al. 2007) is a maturity group V cultivar. The PI 283327 carries three missense mutations in *FAD2-1B* (Pham et al. 2010). LLL-05-14 (12 % stearic acid, 28 % oleic acid; Ruddle et al. 2012) is a maturity group V F_5-derived selection from the cross FAM94-41-3 × N98-4445A. N98-4445A (Burton et al. 2006) is a mid-oleic germplasm line. The High Stearic, High Oleic 2 (HSHO-2) population was developed from the cross S09-2902-145 × TCHM08-1087-11. TCHM08-1087-11(13 % stearic acid, 22 % oleic acid) was selected as a single M_3 plant from a TCHM08-1087 bulk and is presumably homozygous for the unknown elevated stearic locus.

Field evaluation

In 2010, F_2 plants from FA-I and FA-J were grown in Clayton, NC and harvested individually. F_2-derived lines were grown in 3.7 m long single-row plots in 2011. Soils at Clayton, NC were Norfolk Loamy Sands in both years. In 2012, F_2-derived lines were grown in Clayton, NC and Kinston, NC, with two replicates of each line grown at each location. In 2011 and 2012 flower color at the R2 reproductive stage and maturity date at R8 (Fehr and Caviness 1977) were recorded.

Greenhouse evaluation

In 2012–2013, F_1 plants from HSHO-1 and HSHO-2 were grown in the greenhouse. Individual plants were harvested and F_2 seeds were chipped for genotyping and collected in 1.2 mL strip tubes with two steel grinding balls per tube. The remaining portion of the seed was used for fatty acid analysis.

Seed oil analysis

Fatty acid methyl ester (FAME) analysis was performed on a twenty seed sample from each F_2 plant in 2010 and from each F_2-derived line in 2011 and 2012 for the FA-I and FA-J populations. FAME analysis was performed on individual F_2 seeds from HSHO-1 and HSHO-2 harvested under greenhouse conditions in 2013. Seed samples were crushed and approximately 1 g was extracted for about 24 h in 3 mL of solvent (chloroform:hexane:methanol, 8:5:2 v/v/v) in stoppered glass test tubes. Extracted lipids were transesterified using sodium methoxide. Operating conditions for the gas chromatography analyses were as described by Burkey et al. (2007). Oil content was measured from a 10 g seed sample from each F_2 plant in 2010 and each F_2-derived line in 2011 and 2012 for FA-I and FA-J. Oil content was determined by pulsed proton nuclear magnetic resonance (NMR) using a Maran pulsed NMR (Resonance Instruments, Witney, Oxfordshire, UK) by the Field Induction Decay-Spin Echo procedure (Rubel 1994).

SACPD gene isoforms sequencing

Sequencing was performed for the three known *SACPD* gene isoforms (A, B and C) in FAM94-41-3, Holladay, and on M_3 high stearic selections from TCHM08-1087 to TCHM08-755. Amplification reactions were carried out as described by Ruddle et al. (2012) and samples were submitted to GeneWiz (Research Triangle Park, NC) for sequencing.

Deletion size determination

Simple sequence repeat (SSR) markers from the *Glycine max* Consensus Map 4.0 (Hyten et al. 2010) on chromosome 14 were used to delimit the deletions encompassing the *SACPD-C* locus in TCHM08-1087 and TCHM08-755. SSR markers evaluated were Satt601, Satt318, Satt474, Sat_189, Satt070, Satt556, and Satt020. SSR marker reaction mix consisted of 0.4 μL H_2O, 0.8 μL 10× PCR Buffer, 1.6 μL 15 μM $MgCl_2$, 0.6 μL 3.12 mM dNTPs, 0.2 μL *Taq* polymerase, 0.7 μL 5 μM forward primer, 0.7 μL 5 μM reverse primer, and 3 μL of 5 ng/μL DNA, for a total volume of 8 μL. Polymerase chain reactions were performed in a 384-well PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, MA). Amplification conditions were 95 °C for 120 s and 40 cycles of 92 °C for 30 s, 49 °C for 30 s, and 68 °C for 45 s, followed by a final extension at 72 °C for 5 min. The amplification products were resolved on 4.5 % superfine resolution agarose gels (Amresco, Solon, OH) with ethidium bromide staining in 1× Tris–Borate–EDTA buffer. TCHM08-1087 and TCHM08-755 were evaluated relative to 'Holladay' to determine the presence or absence of the expected amplification product.

Molecular marker analysis

In 2012, tissue collection and DNA extraction were conducted as described by Ruddle et al. (2012) for each

Table 1 KASPar genotyping primers for *FAD2-1A* snp 354 and *FAD2-1B* snp 410

Primer	Fluorophore[a]	Direction	Sequence (5′ ≥ 3′)
FAD2-1A snp 354 wild type	FAM	Forward	GAAGGTGACCAAGTTCATGCTAGTGTGGTCACCATGCCTTCAG
FAD2-1A snp 354 mutant	VIC	Forward	GAAGGTCGGAGTCAACGGATTGAGTGTGGTCACCATGCCTTCAA
FAD2-1A snp 354 common	None	Reverse	CACAACATCATCAACCCATTGGTA
FAD2-1B snp 410 wild type	FAM	Forward	GAAGGTGACCAAGTTCATGCTGTTCACTCAGCACTTTTAGTCCC
FAD2-1B snp 410 mutant	VIC	Forward	GAAGGTCGGAGTCAACGGATTGTTCACTCAGCACTTTTAGTCCG
FAD2-1B snp 410 common	None	Reverse	CGATGGCTTRTTTTCCATGAGA

[a] Flourescence dye type

F_2-derived line in the FA-I and FA-J populations. The *SACPD-C*-229 SNP (Zhang et al. 2008) was genotyped in both populations using the KBiosciences competitive allele specific PCR (KASPar) SNP genotyping system (KBiosciences, Herts, UK), as described by Ruddle et al. (2012). The SSR marker Satt020 (Cregan et al. 1999) was also used to further differentiate the *SACPD-C*-229 SNP homozygote class from the *SACPD-C*-deletion/*SACPD-C*-229 SNP heterozygote class. SSR marker reactions were carried out as described in the previous section.

DNA was extracted from individual F_2 seed chips for the HSHO-1 and HSHO-2 populations as follows. Fifty μL of sterile water was added to each seed chip and 96-well blocks of seed chips were placed in a shaker incubator for 90 min at 60 °C and 200 rpm. Seeds were then homogenized in a grinder with 400 μL per well of extraction buffer containing 100 mM Tris pH 8.0, 20 mM EDTA, 0.5 M NaCl and 0.5 % sodium dodecyl sulfate. Ground seed chips were incubated and shaken for 20 min at 60 °C and 200 rpm. Sixty-eight μL of 5 M potassium acetate was added to each well. Following centrifugation at 2,800 relative centrifugal force, supernatants were transferred to new tubes and an equal volume of 75 % isopropanol-2.5 M ammonium acetate was added to precipitate DNA. Blocks were subsequently centrifuged, supernatants discarded and 70 % ethanol was added to each tube. Following centrifugation, the ethanol was poured off and pellets were allowed to air-dry over night before resuspension in 1× TE buffer. KASPar primers designed for the *FAD2-1A* SNP 354 (Dierking and Bilyeu 2009) and *FAD2-1B* SNP 410 (Pham et al. 2010) (Table 1) were used to genotype the HSHO-1 and HSHO-2 populations. The *SACPD-C*-229 SNP was genotyped as described above in the HSHO-1 population. The SSR marker Satt020 was used to determine if the deleted allele from Holladay or the wild type allele from S09-2902-145 was inherited at the *SACPD-C* locus for the HSHO-2 population.

Statistical analysis

For the FA-I and FA-J populations, Chi-squared tests were conducted to determine if the expected genotypic ratios were present in both populations (Snedecor and Cochran 1956). Since the rows used as male parents for both populations were segregating for the high stearic locus, the expected 1:2:1 ratio was tested considering the following genotypic classes: homozygous for the fas_{nc} mutation (25 %), heterozygous for fas_{nc}/wild type or fas_{nc}/*SACPD-C* deletion (50 %) and homozygous for *SACPD-C* deletion or wild type (25 %). Mean values for fatty acid, maturity, and total oil were determined for each F_2-derived line by first calculating means over replications for each environment, then averaging over environments.

For FA-I and FA-J, a general linear model was fit using the GLM procedure in SAS 9.2 (SAS Institute, Cary, NC, 2009) with *SACPD-C* genotypic class as the sole factor. Using the F_2-derived line means, least squares means were determined for each *SACPD-C* genotypic class, for each fatty acid and total oil. As both populations had a common female parent and almost identical male parents, an analysis considering both populations was also conducted. In this combined analysis, population and the genotypic class by population interaction were also considered as factors. Dunnett's test, with the fas_{nc}/fas_{nc} genotypic class as the control, was conducted on least squares means to determine if either *SACPD-C* locus deletion resulted in a different phenotype than the fas_{nc} mutation. Additive effects of all *SACPD-C* alleles were estimated using contrast statements with the wild type allele as the reference allele. Multivariate analysis of variance was conducted using the MANOVA procedure in GLM to determine if maturity date and fatty acid composition were correlated, after accounting for the other effects in the model.

For the HSHO-1 and HSHO-2 populations, Chi-squared tests were conducted to determine if the expected genotypic ratios were present in both populations. A general linear model was fit with the *SACPD-C*, *FAD2-1A*, and *FAD2-1B* genotypic classes, and their interactions as fixed effects, while F_1 plant and the appropriate interactions with fixed effects were considered as random effects; least square means for each genotypic class were calculated. Dunnett's test, with the *SACPD-C* mutant/*FAD2-1A* wild type/*FAD2-1B* wild type genotypic class as the control, was conducted

Table 2 Presence of expected amplification products relative to 'Holladay' in the Holladay mutants TCHM08-1087 and TCHM08-755 around the *SACPD-C* locus on chromosome 14 of *Glycine max*

Locus	Start (base pairs)	End (base pairs)	TCHM08-1087	TCHM08-755	Holladay
Satt601	27131235	27131413	Present	Present	Present
Satt318	28809608	28809872	Present	Present	Present
Satt474	33076539	33076797	Absent	Absent	Present
Sat_189	33180317	33180429	Absent	Absent	Present
Satt070	34228964	34229137	Absent	Absent	Present
SACPD-C	34322853	34325575	Absent	Absent	Present
Satt122	34445176	34445301	Absent	Absent	Present
Satt556	39579290	39579452	Present	Absent	Present
Satt020	42022252	42022366	Present	Present	Present

Table 3 Segregation ratios for *SACPD-C* among F_2-derived lines in the FAM94-41-3 (*SACPD-C* allele:fas_{nc}) × TCHM08-1087 (*SACPD-C* deletion/wild type) (FA-I) and FAM94-41-3 × TCHM08-755 (*SACPD-C* deletion/wild type) (FA-J) populations

Population	FAM-94-41-3 genotype	Heterozygous	TCHM08-1087/755 genotype	Total	χ^2 (1:2:1)	*p* value
FA-I	60	108	20	188	21.19	<0.001
FA-J	58	88	38	184	2.96	0.23

on least squares means to determine how alleles affected fatty acid composition. The additive and dominant effects of each locus and the additive by additive epistatic interactions between each locus were estimated using contrast statements as described by Holland (2001).

Results

Sequence analysis of *SACPD* isoforms

The sequencing results were compared to the Williams 82 reference sequence for each *SACPD* isoform (Schmutz et al. 2010). The *SACPD-A* (glyma07g32850) reference sequence was found to be identical to the Williams 82 reference sequence in FAM94-41-3, TCHM08-1087, TCHM08-755 and Holladay. The *SACPD-B* (glyma02g15600) coding sequence had a silent mutation at nucleotide 76 in FAM94-41-3, TCHM08-1087, TCHM08-755, and Holladay. A silent mutation was identified at nucleotide 930 in *SACPD-C* (glyma14g27990) in FAM94-41-3 and Holladay. In addition, the SNP previously identified in this locus at nucleotide 229 in FAM94-41 (Zhang et al. 2008) was confirmed. Primers designed to amplify the *SACPD-C* locus failed to produce amplification products in TCHM08-1087 and TCHM08-755.

Analysis of *SACPD-C* deletion size

No amplification products were observed for microsatellite markers Satt474, Sat_189, Satt070 and Satt122 in either TCHM08-755 or TCHM08-1087. The expected amplification products (those observed in Holladay) were present at Satt601, Satt318, and Satt020 in both lines, but TCHM08-755 lacked the expected product at Satt556. Based on these results, it was concluded that each line possessed a unique deletion of at least one megabase encompassing the *SACPD-C* locus (Table 2).

Genetic analysis of the *SACPD-C* deletions

Segregation distortion was detected towards the fas_{nc} allele at the *SACPD-C* locus in the FA-I population and no segregation distortion was detected for this locus in the FA-J population (Table 3). No segregation distortion was observed for flower color in either population (data not shown). As both male parents possessed a deletion in the same gene, and the results for both populations were similar, only the analysis with both populations combined is presented. Lines differed by 15 days for maturity and maturity was a significant factor in the multivariate analysis comparing variance for maturity vs. stearic acid, oleic acid, linoleic acid, linolenic acid, and total oil. However, utilizing maturity as a covariate was found to not significantly increase statistical power (i.e., the significance values for all fatty acids remained the same), thus it was excluded from the analyses. A small ($R^2 < 0.01$), but significant ($p < 0.05$) *SACPD-C* by environment interaction was observed for stearic and oleic acid.

Additive effect estimates are presented in Table 4. Both *SACPD-C* deletions had over double the additive effect on stearic acid content when compared to the fas_{nc} allele (+4.1 vs. +1.8 %). The deletion also had a larger negative effect on oleic acid (−2.0 vs. −1.1 %), linoleic acid (−1.9

Table 4 Additive effect estimates (% of total fatty acid) and R^2 associated with the *SACPD-C* alleles fas_{nc} and a *SACPD-C* deletion in the four environment combined analysis of the F_2-derived populations FA-I = FAM94-41-3 (*SACPD-C* allele:fas_{nc}) × TCHM08-1087 (*SACPD-C* deletion/wild type) and FA-J = FAM94-41-3 × TCHM08-755 (*SACPD-C* deletion/wild type)

Gene and parameter estimated	Palmitate	Stearate	Oleic	Linoleate	Linolenate	Total oil
SACPD-C deletion additive effect	−0.3***	4.1***	−2.0***	−1.9***	0.2*	−0.4***
SACPD-C fas_{nc} additive effect	−0.3***	1.8***	−1.1***	−0.6**	NS	−0.1*
SACPD-C R^2	0.12	0.74	0.32	0.40	0.04	0.22

*,**,*** Estimate significance, $p < 0.05$, $p < 0.01$, $p < 0.001$

Table 5 Fatty acid and total oil least squares means (%) for each genotypic class in the four environment combined analysis of the F_2-derived populations FAM94-41-3 (*SACPD-C* allele:fas_{nc}) × TCHM08-1087 (*SACPD-C* deletion/wild type) and FAM94-41-3 × TCHM08-755 (*SACPD-C* deletion/wild type)

Genotypic class	Palmitate mean	Stearate mean	Oleate mean	Linoleate mean	Linolenate mean	Total oil mean
SACPD-C deletion/deletion	11.0	12.2***	17.7***	50.6***	8.4*	19.8***
SACPD-C deletion/fas_{nc}	11.1	9.4***	18.9***	52.5***	8.1	20.1***
SACPD-C fas_{nc}/fas_{nc}	11.1	7.7	19.6	53.4	8.1	20.4
SACPD-C fas_{nc}/wild type	11.3***	5.6***	20.9***	54.2***	8.0	20.5
SACPD-C wild type/wild type	11.7***	4.0***	21.8***	54.5*	8.0	20.6

*,**,*** Mean different from *SACPD-C* fas_{ns}/fas_{ns} for a Dunnett pairwise comparison, $p < 0.05$, $p < 0.01$, $p < 0.001$

Table 6 Segregation ratios for *SACPD-C*, *FAD2-1A*, and *FAD2-1B* among F_2 seed in the S09-2902-145 × LLL-05-14 (HSHO-1) and S09-2902-145 × TCHM08-1087-11 (HSHO-2) populations

Population	Gene	Mutant	Heterozygous	Wildtype	Total	χ^2 (1:2:1)	*P* value
HSHO-1	*SACPD-C*	113	240	99	452	2.60	0.27
	FAD2-1A	88	245	119	452	7.45	0.02
	FAD2-1B	124	224	104	452	1.81	0.40
HSHO-2	*SACPD-C*	109	238	120	467	0.69	0.71
	FAD2-1A	120	237	110	467	0.53	0.77
	FAD2-1B	103	250	114	467	2.85	0.24

vs. −0.6 %), and total oil (−0.4 vs. −0.1 %) than the fas_{nc} allele. A small positive additive effect was detected for linolenic acid due to the *SACPD-C* deletion (+0.2 %), but no significant effect was observed due to the fas_{nc} allele. Both mutations had a similar effect on palmitic acid (−0.3 %). *R* squared values for the *SACPD-C* locus are also indicated in Table 4. The homozygous *SACPD-C* deletion genotype resulted in mean stearic acid of 12.2 %, compared to the homozygous fas_{nc} average of 7.7 % and the homozygous wild type average of 4.0 % (Table 5).

Analysis of high stearic acid, high oleic acid populations

Segregation distortion was detected towards the wild type allele of *FAD2-1A* in the HSHO-1 population (Table 6). No other single-locus segregation distortion was detected in HSHO-1 and HSHO-2. The fas_{nc} allele had a larger additive effect on stearic acid (+3.3 %; Table 7) and oleic acid (−3.3 %; Table 7) in the HSHO-1 population than in the FA-I/FA-J populations (+1.8, −1.1 %). The fas_{nc} allele had a similar effect on palmitic acid levels (−0.4 %) and on linoleic acid (not significant) in the HSHO-1 and FA-I/FA-J populations (−0.3 %, not significant). No significant effect was detected for linoleic acid levels. Dominant effects were observed for stearic (−0.9 %) and oleic acid (+1.8 %).

The *SACPD-C*-deletion had similar additive effects on palmitic (−0.4 %), stearic, (+3.8 %) and linolenic acid (+0.2 %) in the HSHO-2 population (Table 8) as the deletions in the FA-I/FA-J populations (−0.3, +4.1, +0.2 %). A larger additive effect was detected for oleic acid (−3.2 %) than in the FA-I/FA-J populations. The *SACPD-C* deletion did not have a significant effect on linoleic acid accumulation. Dominant effects were detected for palmitic (+0.3 %), stearic (−2.2 %) and oleic acid (−3.2 %).

The *FAD2-1A* mutation had larger additive and dominant effects in the HSHO-2 population than in the HSHO-1 population (Table 8) for palmitic (−1.2 additive vs. −0.9 %, +0.5 % dominant vs. not significant), oleic (+13.2 additive

Table 7 Additive, dominant, and additive by additive effect estimates (% of total fatty acid) and R^2 associated with *SACPD-C* allele fas_{nc}, *FAD2-1A* SNP 254 mutation from 17D, and *FAD2-1B* SNP 410 mutation from PI 283327 in the F_2 population LLL-05-14 (*SACPD-C* allele:fas_{nc}) × S09-2902-145 (*FAD2-1A* SNP 254, *FAD2-1B* SNP 410) (HSHO-1)

Gene and parameter estimated	Palmitate	Stearate	Oleate	Linoleate	Linolenate
SACPD-C fas_{nc} additive effect	−0.4***	3.3***	−3.3***	NS	NS
SACPD-C fas_{nc} dominant effect	NS	−0.9***	1.8*	NS	NS
FAD2-1A SNP 254 additive effect	−0.9***	NS	9.0 ***	−7.5***	−0.3*
FAD2-1A SNP 254 dominant effect	NS	NS	−2.0*	1.5*	0.3**
FAD21-B SNP 410 additive effect	−0.5***	NS	7.7***	−7.0***	−0.2*
FAD21-B SNP 410 dominant effect	NS	NS	−2.2***	1.8*	0.5***
SACPD-C × *FAD2-1A* additive × additive effect	NS	NS	NS	NS	NS
SACPD-C × *FAD2-1B* additive × additive effect	NS	NS	NS	NS	NS
FAD2-1A × *FAD2-1B* additive × additive effect	−2.0***	NS	20.3***	−17.1***	−1.8**
SACPD-C R^2	0.02	0.32	0.02	<0.01	<0.01
FAD2-1A R^2	0.08	<0.01	0.13	0.12	0.02
FAD2-1B R^2	0.02	<0.01	0.10	0.11	0.03
FAD2-1A × *FAD-21B* R^2	0.02	<0.01	0.03	0.03	0.06

*, **, *** Estimate significance, $p < 0.05$, $p < 0.01$, $p < 0.001$

Table 8 Additive, dominant, and additive by additive effect estimates (% of total fatty acid) and R^2 associated with an *SACPD-C* deletion from TCHM08-1087-11, *FAD2-1A* SNP 254 mutation from 17D, and *FAD2-1B* SNP 410 mutation from PI 283327 in the F_2 population TCHM08-1087-11 (*SACPD-C* deletion) × S09-2902-145 (*FAD2-1A* SNP 254, *FAD2-1B* SNP 410) (HSHO-2)

Gene and parameter estimated	Palmitate	Stearate	Oleate	Linoleate	Linolenate
SACPD-C fas_{nc} additive effect	−0.4***	3.8***	−3.2***	NS	0.2*
SACPD-C fas_{nc} dominant effect	0.3**	−2.2***	2.1***	NS	NS
FAD2-1A SNP 254 additive effect	−1.2***	NS	13.2***	−11.4***	−0.6***
FAD2-1A SNP 254 dominant effect	0.5***	0.4*	−6.9***	5.6***	0.5***
FAD2-1B SNP 410 additive effect	−0.9***	NS	11.2***	−9.7***	−0.6***
FAD21-B SNP 410 dominant effect	0.5***	NS	−4.7***	3.6***	0.6***
SACPD-C × *FAD2-1A* additive × additive effect	NS	NS	NS	NS	NS
SACPD-C × *FAD2-1B* additive × additive effect	NS	NS	NS	NS	NS
FAD2-1A × *FAD2-1B* additive × additive effect	−3.6***	NS	39.5***	−31.2***	−4.8**
SACPD-C R^2	0.03	0.51	0.02	<0.01	0.01
FAD2-1A R^2	0.19	<0.01	0.31	0.32	0.06
FAD2-1B R^2	0.12	<0.01	0.23	0.24	0.07
FAD2-1A × *FAD-21B* R^2	0.10	<0.01	0.12	0.11	0.15

*, **, *** Estimate significance, $p < 0.05$, $p < 0.01$, $p < 0.001$

vs. +9.0 %, −6.9 dominant vs. −2.0 %), linoleic (−11.4 additive vs. −7.5 %, +5.6 dominant vs. +1.5 %) and linolenic acid (−0.6 additive vs. −0.3 %, +0.5 dominant vs. +0.3 %). The same trend was observed with the *FAD2-1B* mutation in both populations. The only significant effect of either gene on stearic acid was a small dominant effect of +0.4 % due to *FAD2-1A* in HSHO-2. Significant additive by additive epitasis was observed between *FAD2-1A* and *FAD2-1B* for palmitic, oleic, linoleic, and linolenic acid in the HSHO-1 and HSHO-2 populations. No significant additive by additive epitasis was observed in either population between *SACPD-C* and *FAD2-1A* or *FAD2-1B*.

In the HSHO-1 population, the *SACPD-C* fas_{nc}/*FAD2-1A* wild type/*FAD2-1B* wild type genotypic class (control for Dunnett's test) averaged 11.5 % stearic acid and 32.7 % oleic acid while the sole *SACPD-C* fas_{nc}/*FAD2-1A* mutant/*FAD2-1B* mutant genotypic class member recovered possessed 12.7 % stearic acid and 65.5 % oleic acid (Table 9). Those stearic acid levels were not significantly different, whereas the oleic acid levels were different for Dunnett's test ($p < 0.05$ for all comparisons presented). The *SACPD-C* wild type/*FAD2-1A* mutant/*FAD2-1B* mutant genotypic class averaged 4.0 % stearic acid and 78.1 % oleic acid, which was significantly different from the *SACPD-C* fas_{nc}/*FAD2-1A* wild type/*FAD2-1B* wild type genotypic class for both stearic acid and oleic acid.

For the HSHO-2 population, the *SACPD-C* deletion/*FAD2-1A* wild type/*FAD2-1B* wild type genotypic class (control for Dunnett's test) averaged 11.9 % stearic acid and 21.9 % oleic acid and the *SACPD-C* deletion/*FAD2-1A* mutant/*FAD2-1B* mutant averaged 10.4 % stearic acid and 75.9 % oleic acid (Table 10). These stearic acid levels were not significantly different, whereas the oleic acid levels were. The *SACPD-C* wild type/*FAD2-1A* mutant/*FAD2-1B* mutant genotypic class averaged 3.8 % stearic acid and 81.8 % oleic acid, which was significantly different from the *SACPD-C* deletion/*FAD2-1A* wild type/*FAD2-1B* wild type genotypic class for both stearic acid and oleic acid.

Table 9 Fatty acid least square means (%) for the lines homozygous at the *SACPD-C*, *FAD2-1A*, and *FAD2-1B* loci in the F_2 population LLL-05-14 (*SACPD-C* allele:fas_{nc}) × S09-2902-145 (*FAD2-1A* SNP 254, *FAD2-1B* SNP 410) (HSHO-1)

SACPD-C	*FAD2-1A*	*FAD2-1B*	*N*	Palmitate mean	Stearate mean	Oleate mean	Linoleate mean	Linolenate mean
Homozygous wild type	Homozygous wild type	Homozygous wild type	4	14.0	4.0***	34.4	41.4	6.2
		Homozygous mutant	3	13.6	4.2***	42.9	33.2	6.0
	Homozygous mutant	Homozygous wild type	5	12.1	4.1***	49.6***	28.9	5.3
		Homozygous mutant	8	10.3***	4.0***	78.1***	3.4***	4.1**
Homozygous mutant	Homozygous wild type	Homozygous wild type	9	12.8	11.5	32.7	36.8	6.2
		Homozygous mutant	7	12.5	11.6	41.7	28.2	6.1
	Homozygous mutant	Homozygous wild type	7	11.9*	10.3	39.0	32.5	6.3
		Homozygous mutant	1	9.7	12.7	65.5***	8.5***	3.6

*, **, *** Mean different from homozygous mutant/homozygous wild type/homozygous wild type for a Dunnett pairwise comparison, $p < 0.05$, $p < 0.01$, $p < 0.001$

Table 10 Fatty acid least square means (%) for the lines homozygous at the *SACPD-C*, *FAD2-1A*, and *FAD2-1B* loci in the F_2 population TCHM08-1087-11 (*SACPD-C* deletion) × S09-2902-145 (*FAD2-1A* SNP 254, *FAD2-1B* SNP 410) (HSHO-2)

SACPD-C	*FAD2-1A*	*FAD2-1B*	*N*	Palmitate mean	Stearate mean	Oleate mean	Linoleate mean	Linolenate mean
Homozygous wild type	Homozygous wild type	Homozygous wild type	5	13.9	3.5***	23.5	51.5	7.6
		Homozygous mutant	6	13.0	3.8***	35.0***	40.6	7.4
	Homozygous mutant	Homozygous wild type	9	13.0	3.7***	35.3***	40.1*	8.0
		Homozygous mutant	8	8.7***	3.8***	81.8***	2.2***	3.5***
Homozygous mutant	Homozygous wild type	Homozygous wild type	8	12.8	11.9	21.9	46.3	7.0
		Homozygous mutant	7	12.7	9.8	24.8	43.7	8.9
	Homozygous mutant	Homozygous wild type	8	11.6	10.5	30.1**	39.7*	8.1
		Homozygous mutant	6	7.9***	10.4	75.9***	1.5***	4.3**

*, **, *** Mean different from homozygous mutant/homozygous wild type/homozygous wild type for a Dunnett pairwise comparison, $p < 0.05$, $p < 0.01$, $p < 0.001$

Discussion

Two new deletions were identified at the *SACPD-C* locus. Although they differ in size, both include a deleted region on chromosome 14 of *G. max*, encompassing the *SACPD-C* locus. The deletions were found to have more than twice the additive effect on seed stearic acid composition compared to the single nucleotide mutation present in the fas_{nc} allele. The *SACPD-C* deletions also had a larger additive effect on oleic acid, linoleic acid, linolenic acid, and total oil composition. This finding reveals that the single nucleotide mutation present in the fas_{nc} allele of *SACPD-C* only results in a partial, rather than a complete, loss of function. These two deletions would be expected to have a similar effect on stearic acid accumulation as the previously characterized null *SACPD-C* allele from RG7 (Boersma et al. 2012) and the deletion encompassing the *SACPD-C* locus in A6 (Zhang et al. 2008). Differences in reported stearic acid values between these lines could be attributed to maturity group and growing conditions, as stearic acid levels have been shown to differ between environments largely because of temperature differences (Primomo et al. 2002) The observed segregation distortion in the *SACPD-C* locus for one of the populations carrying the TCHM08-1087 deletion (FA-I) could indicate some reduction in fitness due to this deletion, but no distortion was observed when this line was crossed to the high oleic germplasm. While a previous study (Ruddle et al. 2013) observed no deleterious effects associated with a partial loss of function *SACPD-C* allele, fas_{nc}, it will be necessary to determine if any adverse agronomic characteristics are associated with these *SACPD-C* deletions.

The previously observed larger effect of the fas_{nc} allele in a higher than normal oleic background (Zhang et al. 2008) was confirmed. The fas_{nc} allele had nearly twice the additive effect on seed stearic acid in the (FAM94-41-3 × N98-4445A) × S09-2902-145 (HSHO-1) population than in the FAM94-41-3 × TCHM08-1087 (FA-I) and FAM94-41-3 × TCHM08-755 (FA-J) populations; however, the *SACPD-C* deletion allele had similar additive effects on

stearic acid in both the TCHM08-1087-11 × S09-2902-145 (HSHO-2) population and the FA-I population. In addition, no epistatic interactions were observed between the *SACPD-C* locus and the *FAD2-1A* or *FAD2-1B* loci in the HSHO-1 and HSHO-2 populations. Thus, the enhanced additive effect of fas_{nc} on stearic acid in the HSHO-1 population is not due to high oleic acid per se nor due to mutations at the *FAD2-1A* or *FAD2-1B* loci, but most likely due to an unknown locus from the mid-oleic germplasm line N98-4445A. Previous studies have identified several QTL for increased oleic acid content associated with alleles from N98-4445A (Bachlava et al. 2008; Monteros et al. 2008), however, neither study involved populations with high stearic acid alleles.

This study determined that it could be possible to develop an elevated stearic acid variety that also has high levels of oleic acid. As in previous studies which focused on either elevated stearic acid (Zhang et al. 2008; Ruddle et al. 2012) or high oleic acid (Dierking and Bilyeu 2009; Pham et al. 2010), it was determined that increases in stearic acid due to mutant *SACPD-C*s occurred primarily at the expense of decreases in oleic acid, and to a lesser extent decreases in palmitic acid, and that mutant *FAD2* genes were able to greatly increase oleic acid, especially when combined with each other. The combination of the mutant *FAD2* genes in conjunction with a mutant *SACPD-C* did not greatly reduce the additive effects of any of the three genes; however, small reductions of oleic acid were observed in the *SACPD-C/FAD2-1A/FAD2-1B* triple mutants compared to the *SACPD-C* wild type/*FAD2-1A* mutant/*FAD2-1B* mutant in both the HSHO-1 and HSHO-2 populations. The *SACPD-C/FAD2-1A/FAD2-1B* triple mutant from HSHO-1 possessed 12.7 % stearic acid and 65.5 % oleic acid whereas the average of the *SACPD-C/FAD2-1A/FAD2-1B* triple mutants from HSHO-2 was 10.4 % stearic acid and 75.9 % oleic acid. While this is a useful finding, neither the fas_{nc} with the unknown interacting locus from N98-4445A nor the deletions provided the requisite 20 % stearic acid. It would be of great benefit to determine the causative locus that is interacting with the *SACPD-C* locus in HSHO-1 to produce a greater than expected increase in stearic acid. It would also be helpful to subsequently combine that novel allele with a non-functional or deleted *SACPD-C*, such as that from RG7, A6, or TCHM08-1087-11 and to determine if the resulting stearic acid levels reach the desired 20 %. In conclusion, these deletions of *SACPD-C* in TCHM08-1087 and TCHM08-755 could be of great use in developing a high-yielding, elevated stearic acid cultivar in the southeastern United States, because in contrast with all the previously reported early maturing germplasm lines carrying *SACPD-C* deletions (Hammond and Fehr 1983; Boersma et al. 2012) they are derived from the cultivar 'Holladay' which is well adapted to this environment. Nonetheless, additional research is needed to determine their agronomic potential and their interactions with other high stearic loci.

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Ethical standard All experiments performed for this publication comply with the current laws of the United States of America.

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