

## ABSTRACT

RUDDLE II, PAUL ANDREW. Molecular and Agronomic Analysis of Alleles Governing Elevated Stearic Acid in Soybean. (Under the direction of Dr. Lilian Miranda.)

Soybean [*Glycine max* (L.) Merr.] oil with increased stearic acid would be valuable for baking and other solid fat applications because of its physicochemical properties and nutritional profile. Most soybean cultivars possess seed stearic acid of 2%-5% but lines with increased stearic acid have been identified. Unfortunately, the agronomic consequences of the elevated stearic acid alleles in these lines are poorly understood and, in many cases, the genetic basis of the elevated stearic acid phenotype is unknown. This study aims to characterize the genetic basis of elevated seed stearic acid in three lines developed via mutagenesis, evaluate the agronomic effects of elevated stearic acid alleles, and determine the feasibility of developing an elevated stearic acid, high oleic acid cultivar.

Two populations, FA-G and FA-H, were developed by bi-parental crosses between an induced mutant of 'Holladay' with elevated stearic acid, TCJWB03-806-7-19, and two lines, LLL-05-01 and LLL-05-14, carrying the same mutation (*fas<sub>nc</sub>*) in a seed-specific isoform of a  $\Delta 9$ -stearoyl-acyl carrier protein-desaturase (*SACPD-C*). Fatty acid and oil analysis was performed on F<sub>2</sub>-derived lines grown in two environments. The three known isoforms of *SACPD* (*A*, *B* and *C*) were sequenced in TCJWB03-806-7-19 and a single nucleotide deletion was observed in exon 3 of *SACPD-B*. The FA-G and FA-H populations were genotyped for the known *SACPD-C* mutation and the novel *SACPD-B* single nucleotide deletion using allele specific primers. Additive effects and R<sup>2</sup> for stearic acid were +3.3 and .55 for *SACPD-C* and +1.9 and .19 for *SACPD-B*. Average stearic acid in lines homozygous for

both mutations was 14.6%. This *SACPD-B* mutation represents a novel elevated stearic acid allele which has been designated *fas2<sub>nc</sub>*.

Fifty F<sub>2</sub>-derived lines homozygous at the *SACPD-B* and *SACPD-C* loci were randomly selected from the FA-G and FA-H populations and evaluated in a growth chamber and two field environments. *SACPD-B* mutants suffered from a decreased seedling growth rate, were 15% shorter at maturity, had seed 4% smaller and yielded 6% less than the wild-type lines. *SACPD-C* mutants were agronomically indistinguishable from the wild-type lines and yielded as well. The different agronomic performance between *SACPD-C* and *SACPD-B* mutants was attributed to altered fatty acid composition in vegetative tissues of the *SACPD-B* mutants.

The FA-I and FA-J populations were developed by crosses between FAM94-41-3, a line with the *fas<sub>nc</sub>* mutation, and two elevated stearic acid induced mutants of ‘Holladay’, TCHM08-1087 and TCHM08-755. Both populations were grown in four field environments and fatty acid composition and oil content were evaluated. Sequencing of *SACPD* isoforms in TCHM08-1087 and TCHM08-755 revealed a deletion of at least one megabase encompassing the *SACPD-C* locus in both lines. Lines were genotyped for the *SACPD-C* point mutation from FAM94-41-3 and the *SACPD-C* deletions from TCHM08-1087 and TCHM08-755. The additive effect for stearic acid was +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%. The *SACPD-C* deletions in TCHM08-1087 and TCHM08-755 have been designated *fas<sub>ncdel1</sub>* and *fas<sub>ncdel2</sub>* respectively.

LLL-05-14 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<sub>1</sub> plants were grown in a greenhouse and individual F<sub>2</sub> seed were genotyped and phenotyped. No interaction was observed between either *FAD2-1A* and *FAD2-1B* and either of the *SACPD-C* mutant alleles. Seed homozygous mutant for the *SACPD-C fas<sub>nc</sub>* mutation/*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. Also, it was determined that an unknown allele from LLL-05-14 was affecting stearic acid levels.

Molecular and Agronomic Analysis of Alleles Governing Elevated Stearic Acid in Soybean

by  
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## BIOGRAPHY

Paul Ruddle II was born in Valdosta, Georgia and his interest in agriculture began in large part through his middle school agriculture education course work and participation in the Future Farmers of America. During high school he conducted photosynthesis research and participated in numerous science and agriscience research competitions where he developed a desire to work in the field of plant breeding. After graduation from Lowndes High School in 2005 he attended the University of Georgia where he earned a B.S. Applied Biotechnology in Plant Sciences and a B.S. Agriculture in Biological Sciences. While at UGA, he worked in the Plant Biology laboratory of Dr. Susan Wessler as an undergraduate researcher studying transposable elements in *Medicago truncatula*. In the summer of 2008, Paul also had the opportunity to work as a maize breeding intern for Pioneer Hi-Bred International in Princeton, Illinois under the mentorship of Dr. Michael Jines. He also studied agricultural and ecological practices abroad as part of his undergraduate scholarship program, traveling to Australia, Costa Rica, Egypt, and Ecuador. Following graduation, Paul served as a missionary for The Church of Jesus Christ of Latter-day Saints in Guatemala City and Palin, Guatemala. Upon return to the United States, he worked as a Technical Analyst with Syngenta Biotechnology in Research Triangle Park, North Carolina before joining the soybean breeding program of Dr. Lilian Miranda at North Carolina State University in 2011 as a graduate research assistant. Following graduation with his M.S in Crop Science, Paul will continue his graduate studies at NCSU in the Institute for Advanced Analytics.

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**CHAPTER 1: Effect of a Novel Mutation in a  $\Delta 9$ -Stearoyl-ACP-Desaturase on Soybean Seed Oil Composition**

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## ABSTRACT

Soybean [*Glycine max* (L.) Merr.] oil typically contains 2-4% stearic acid. Seed oil with 20% stearic acid would be useful for solid fat applications, both for its cooking properties and health benefits. Breeding lines with high stearic acid have been developed, but many suffer from agronomic problems. This study identifies a new source of high stearic acid, determines its relationship with another high stearic locus and presents molecular markers for its use in breeding. TCJWB03-806-7-19, a 'Holladay' mutant with high stearic acid, was crossed to two FAM94-41-derived lines that contained a point mutation in a seed-specific isoform of a  $\Delta 9$ -stearoyl-acyl carrier protein-desaturase (*SACPD-C*). Fatty acid analysis was performed over two growing seasons with F<sub>2</sub>-derived lines and transgressive segregation for stearic acid content was observed. Sequencing of *SACPD* isoforms in TCJWB03-806-7-19 revealed the deletion of an 'A' nucleotide in exon 3 of *SACPD-B* which results in a protein whose final 28 amino acids are predicted to differ from Williams 82 *SACPD-B*. Sorting Intolerant From Tolerant (SIFT) analysis revealed that this frameshift mutation may affect *SACPD-B* protein function. Allele-specific genotyping for the *SACPD-C* point mutation and *SACPD-B* nucleotide deletion was performed in both populations. Additive effects and R<sup>2</sup> for stearic acid were +3.3 and .55 for *SACPD-C* and +1.9 and .19 for *SACPD-B*. Average stearic acid in lines homozygous for both mutations was 14.6%. This *SACPD-B* mutation represents a novel high stearic allele.

Abbreviations: 18:1-ACP TE, 18:1-acyl carrier protein thioesterase; KASII, 3-ketoacyl-acyl carrier protein synthase II; KASPar, KBiosciences Competitive Allele Specific PCR; NEB, New England Biolabs; SACPD,  $\Delta^9$ -stearoyl-acyl carrier protein-desaturase; SIFT, Sorting Intolerant From Tolerant; TSAP, thermosensitive alkaline phosphatase

## INTRODUCTION

Soybean [*Glycine max* (L.) Merr.] is the most widely produced oil seed crop in the world, accounting for over half of worldwide oil crop production (USDA-Economic Research Service 2011). An oxidatively stable oil with a relatively high melting temperature is necessary for solid fat applications (Clemente and Cahoon 2009). Soybean seed oil naturally high in saturates would be suitable for this end use (List and Pelloso 2007; Clemente and Cahoon 2009). Palmitic (16:0) and stearic acid (18:0) are the two saturated fatty acids present in soybean oil. Typical palmitic and stearic acid contents of soybean oil are 11% and 4% respectively (Hildebrand et al. 2008).

Short chain saturated fatty acids, such as palmitic acid, are undesirable because their consumption results in an unfavorable lipoprotein profile in blood serum (Mensink and Katan 1990). In contrast, stearic acid appears to have no cholesterolemic effects in humans (Kris-Etherton and Yu 1997) and exhibits similar thrombogenic effects as oleic and linoleic acids (Thijssen et al. 2005).

Stearic acid content in soybean typically represents only 2-5% of total fatty acids (ILSI 2010; USDA-ARS 2012); however, several germplasm lines have been developed with increased stearic acid. All high stearic soybean germplasm lines have been developed using mutagenesis, with the exception of FAM94-41 (Pantalone et al. 2002). FAM94-41 (9% stearic acid) has a spontaneously occurring mutation in the *SACPD-C* gene, a seed-specific isoform of a  $\Delta^9$ -stearoyl-acyl carrier protein-desaturase (SACPD), which gives rise to the elevated stearic phenotype (Zhang et al. 2008). SACPD is responsible for the desaturation of stearic acid to oleic acid (Ohlrogge and Browse 1995). The stearic acid QTL on linkage

group B2 identified by Spencer et al. (2003) is likely due to the *SACPD-C* mutation in FAM94-41, as FAM94-41 was a parent in the mapping population used and the most closely linked marker identified in the study, Satt070, is 0.2 cM from *SACPD-C*. The elevated stearic acid phenotype in A6 (28% stearic acid; Hammond and Fehr 1983) is due to the deletion of *SACPD-C* (Zhang et al. 2008) and is allelic with the mutations in A81-606085 (19% stearic acid) and FA41545 (16% stearic acid; Graef et al. 1985). RG-7 (12% stearic acid) and RG-8 (10.6% stearic acid) both have a point mutation in *SACPD-C* and are presumably allelic with the A6 and FAM94-41 mutations (Boersma et al. 2012). ST1 (29% stearic acid), ST3 (23% stearic acid), and ST4 (23% stearic acid) also have high stearic mutations allelic to the *SACPD-C* deletion in A6; however, the mutation in ST2 (28% stearic acid) has been hypothesized to be at a different locus (Bubeck et al. 1989). Other elevated stearic germplasm lines have also been developed with unknown causative loci (Rahman et al. 1995; Hudson 2012).

Two other isoforms of *SACPD* have been identified in soybean, *SACPD-A* and *SACPD-B* (Byfield et al. 2006), but to date neither has been associated with an elevated stearic acid phenotype. *SACPD-A* and *SACPD-B* transcripts have been detected in developing seed, root, leaf, and flower tissues while *SACPD-C* transcript has been primarily detected in developing seed (Byfield et al. 2006; Zhang et al. 2008; Kachroo et al. 2008).

3-keto-acyl-acyl carrier protein synthase II (*KASII*) and 18:1-acyl carrier protein thioesterase (18:1-ACP TE) are other genes in which a mutation or differential expression could lead to elevated stearic acid content (Pantalone et al. 2002). *KASII* is responsible for

the elongation of palmitic acid to stearic acid (Ohlrogge and Browse 1995). A mutation in KASII has been found to increase palmitic acid in soybean (Aghoram et al. 2006).

Theoretically, overexpression or a mutation of KASII could lead to elevated stearic acid.

18:1-ACP TE catalyzes the hydrolyzation of 18:1-ACP and 18:0-ACP to release fatty acid in order to facilitate their transport to the cytoplasm (Jones et al. 1995). Increased 18:1-ACP TE activity has been shown to increase stearic acid content in sunflower (Cantisán 2000).

Stearic acid QTL not associated with any known enzyme in the fatty acid pathway have also been reported. In addition to the QTL associated with *SACPD-C*, twelve additional stearic acid QTL have been reported, on eight different linkage groups (Diers and Shoemaker 1992; Hyten et al. 2004; Panthee et al. 2006; Reinprecht et al. 2006; Li et al. 2011).

Agronomic problems such as lower seed yield, poor germination, and reduced seedling growth rate have been associated with elevated levels of stearic acid (Lundeen et al. 1987; Rahman et al. 1997; Wang et al. 2001a). The effect varies depending on the causative allele, and not every allele results in a yield decrease (Lundeen et al. 1987). The identification of new high stearic QTL and/or novel mutations in known isoforms of *SACPD*, *KASII*, or 18:1 ACP TE may provide alternatives to overcome these constraints, and also may lead to a greater ability to finely-tune fatty acid composition (Cardinal 2008). The objectives of this research were to genetically characterize a new source of elevated stearic acid identified in a population developed by mutagenesis, to determine its relationship to other known high stearic loci, and to develop molecular markers for use in breeding for improved fatty acid composition.

## **MATERIALS AND METHODS**

### **Plant Materials**

Two populations were developed by bi-parental crosses performed in 2009 in Clayton, NC. FA-G is a population of 209 F<sub>2</sub>-derived lines from the cross LLL-05-1 x TCJWB03-806-7-19. FA-H is a population of 117 F<sub>2</sub>-derived lines from the cross LLL-05-14 x TCJWB03-806-7-19. LLL-05-1 (12.0% stearic acid) and LLL-05-14 (11.7% stearic acid) are maturity group V F<sub>5</sub>-derived selections from the cross FAM94-41-3 x N98-4445A. LLL-05-14 matures three days later than LLL-05-1 and both lines differ in pubescence color. FAM94-41-3 (8.8% stearic acid; Pantalone et al. 2002) is a selection from an elevated stearic germplasm line with a natural mutation in *SACPD-C* (Zhang et al. 2008). N98-4445A (Burton et al. 2006) is a mid-oleic germplasm line. TCJWB03-806-7-19 is a maturity group V elevated stearic selection from a mutagenized population of the cultivar ‘Holladay’ (Burton et al. 1996) that was developed by exposing seed to 250 grays (Gy) of gamma radiation from a Gammacell 220 (MDS Nordion Inc., Ottawa, Ontario, Canada). TCJWB03-806-7-19 has 7% stearic acid compared to the 4% stearic acid of ‘Holladay’ (unpublished data).

### **Field Evaluation**

In 2010, F<sub>2</sub> plants were grown in Clayton, NC and harvested individually. F<sub>2</sub>-derived lines were grown in 3.7 m long one-row plots in 2011. Soils at Clayton, NC were Norfolk Loamy Sands for both years. In 2011, flower color, pubescence color, and maturity date at the R8 reproductive stage (Fehr and Caviness 1977) were recorded.

### **Seed Oil Analysis**

Fatty acid methyl ester analysis was performed on a twenty seed sample from each F<sub>2</sub> plant in 2010 and from each F<sub>2:3</sub> line in 2011. Seed samples were crushed and approximately 1 g was extracted for about 24 hours in 3 mL of solvent (chloroform: hexane: methanol, 8:5:2 v/v/v) in stoppered glass test tubes. Fatty acid methyl esters of the lipid extracts were prepared and analyzed as outlined by Burkey et al. (2007).

Oil content was measured from a ten gram seed sample from each F<sub>2</sub> plant in 2010 and each F<sub>2:3</sub> line in 2011. 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 the three parental lines and Holladay. Primers used for amplification and sequencing are listed in Tables 1 and 2.

Amplification reactions were carried out in 1X New England Biolabs (NEB, Ipswich, Massachusetts, USA) standard reaction buffer with MgCl<sub>2</sub> (final MgCl<sub>2</sub> 1.5 mM), 1.4 units NEB Taq polymerase, 208 uM dNTPs, 6 pmol forward primer, 6 pmol reverse primer, and 3 uL of 10 ng/uL DNA for a reaction volume of 15 uL. A touchdown PCR reaction was performed with the following parameters – 94°C for 2 minutes – 13 cycles of: 94°C for 32s, 63°C for 30s, -1°C/cycle, 72°C for 2 minutes – 22 cycles of: 94°C for 32s, 50°C for 30s, and 72°C for 2 minutes – 72°C for 7 minutes. PCR products were cleaned with a

thermosensitive alkaline phosphatase (TSAP)/Exol enzyme mix prepared as follows: 10.4 uL TSAP, 10.4 uL Exol, 187.2 uL H<sub>2</sub>O. Two uL of the TSAP/Exol mix was used per 8 uL of PCR product and incubated at 37°C for 45 minutes followed by inactivation at 80°C for 15 minutes. Samples were submitted to GeneWiz (Research Triangle Park, NC) with 5 uM of the sequencing primer.

### **Molecular Marker Analysis**

A single leaf was collected at random from twenty plants from each F<sub>2</sub>-derived line in 2011. A single punch was taken from each leaf using a cork borer and the punches for each F<sub>2</sub>-derived line were bulked for DNA extraction. DNA was extracted using a CTAB method (Allen et al. 2006). Primers were designed based on the *SACPD-B-1114* SNP and *SACPD-C-229* SNP (Zhang et al. 2008) for use in the KBiosciences Competitive Allele Specific PCR (KASPar) SNP genotyping system (KBiosciences, Herts, UK) (Table 3).

The DNA samples were diluted to 2 ng/uL and 3 ul from each sample were transferred to a 384-well plate and dried down at 55°C for 30 minutes in an incubator. Total volume per reaction was 4 ul, which consisted of 2 ul 2X KASP Reaction Mix, 0.11 ul 0.5X Assay Mix, 0.072 ul 50 mM MgCl<sub>2</sub>, and 1.818 ul H<sub>2</sub>O. The 0.5X Assay Mix was prepared to a total volume of 20 ul which consisted of 1.2 uL 100 uM of each allele-specific primer, 3 ul 100 uM common reverse primer, and 14.6 uL 10 mM Tris pH 8.3. Thermocycling parameters were – 94°C incubation for 15 minutes – 10 cycles of: 94°C for 20s, 65°C for 60s, -0.8°C/cycle – 30 cycles of: 94°C for 20s and 57°C for 60s. Endpoint fluorescence

reading was performed using a Roche LightCycler<sup>®</sup> 480 (Penzberg, Germany). Allele calling was performed using Version 1.5 of the Roche LightCycler<sup>®</sup> 480 software

### **Statistical Analysis**

Chi-square tests were conducted to determine if the *SACPD-C* and *SACPD-B* loci were segregating according to the expected ratios in each population (Snedecor and Cochran 1956). Analysis of variance (ANOVA) for all fatty acids and total oil was conducted using SAS PROC GLM (SAS Institute, Cary, NC, 2009). Means were calculated for each F<sub>2</sub>-derived line, using the years as the replicates, and linear regression analysis of maturity versus fatty acid concentration was conducted using PROC REG to determine whether maturity had an effect on fatty acid concentration.

For each population, the F<sub>2</sub>-derived line means were used in a PROC GLM ANOVA for the *SACPD-B* and *SACPD-C* loci and least squares means were calculated for each genotypic class. Since both populations share the same male parent and the female parents are sister lines, a combined ANOVA for both populations was also conducted. In this combined ANOVA, population and genotypic class by population were also considered as factors. Dunnett pairwise comparison was conducted on the genotypic class least square means to determine if the mutant alleles resulted in a different phenotype than the wild type. Contrast statements were used to estimate the additive and dominant effects of each locus and the additive by additive, additive by dominant, dominant by additive and dominant by dominant epistatic interactions between the *SACPD-B* and *SACPD-C* loci (Holland 2001). Additive and additive by additive effects were estimated using the genotypic class least

squares means from both years of data while dominant, additive by dominant, dominant by additive, and dominant by dominant effects were estimated with only the data from F<sub>2</sub> plants.

## RESULTS

### Sequence analysis of *SACPD* isoforms

The sequencing results were compared to the Williams 82 reference sequence for each isoform (Schmutz et al. 2010). The *SACPD-A* (glyma07g32850) reference sequence was found to be identical to the Williams 82 reference sequence in all three parents and Holladay.

A silent mutation was identified at nucleotide 930 in *SACPD-C* (glyma14g27990) in LLL-05-01, LLL-05-14, TCJWB03-806-7-19, and Holladay. In addition, the SNP previously identified in this locus at nucleotide 229 in FAM94-41 (Zhang et al.2008) was also found in LLL-05-01 and LLL-05-14.

The *SACPD-B* (glyma02g15600) coding sequence had a silent mutation at nucleotide 76 in TCJWB03-806-7-19 and Holladay. TCJWB03-806-7-19 had a deletion of the A at nucleotide 1114 (Figure 1a) that is predicted to alter the location of the stop codon and result in a longer protein whose final 28 amino acids are predicted to differ from those of the *SACPD-B* protein in Williams 82 (Figure 1b) and the *SACPDs* examined by Byfield et al. (2006). The TCJWB03-806-7-19 *SACPD-B* sequence is available as GenBank no. JQ993842.

To further examine the conservation of this position, the Sorting Intolerant From Tolerant (SIFT) algorithm as described by Ng and Henikoff (2001) was used. The Williams 82 *SACPD-B* reference coding sequence was used as the query sequence and the TCJWB03-

806-7-19 amino acid substitutions caused by the nucleotide deletion were the substitutions of interest. The “UniProt-TrEMBL 2009 Mar” protein database was used, with median conservation of sequences specified as 3.00 and sequences more than 90 percent identical to the query sequence were removed. One hundred thirty five related sequences were retrieved by the algorithm and twelve of the twenty amino acid carboxyl-terminus substitutions due to the nucleotide deletion were predicted to affect protein function.

### **Genetic Analysis of the *SACPD-C* and *SACPD-B* mutations**

No segregation distortion was detected for the *SACPD-C* locus or *SACPD-B* locus in either population (Table 4). There was also no segregation distortion for flower or pubescence color (data not shown). Since the results for both populations were very similar, only the results from the combined analysis are presented. Also, results for F<sub>2</sub> single plants were similar to those observed in F<sub>2:3</sub> lines. Maturity differed by 16 days among F<sub>2</sub>-derived lines and was not a significant factor in the regression analysis; therefore, it was not included as a covariate in the statistical analysis. No significant additive by dominant, dominant by additive, nor dominant by dominant epistatic effects were detected. The mutant forms of both genes were associated with an increase in stearic acid and a decrease in oleic acid content in all the analyses (Table 5). The *SACPD-C* nucleotide 229 mutation had a larger positive additive effect (+3.3) on stearic acid levels than the *SACPD-B* nucleotide 1114 deletion (+1.9), while both mutations had comparable negative additive effects on oleic acid (Table 6). Significant epistatic additive by additive gene action of effect +3.3 on stearic acid was also detected. The *SACPD-C* mutation exhibited a dominance genetic effect (-0.8) on stearic acid levels

while the *SACPD-B* mutation did not (Table 7). Both mutations were associated with a small decrease in palmitic acid and a small decrease in total oil (Table 6). Interestingly, both mutations were associated with opposite significant effects on linolenic acid concentration, a decrease of -0.2 with the mutant form of *SACPD-C* and an increase of 0.4 with the mutant form of *SACPD-B*. R-square values for the *SACPD-C* and *SACPD-B* loci are indicated in Table 6.

The double mutant genotype resulted in average stearic acid of 14.6% versus the wild type average of 4.3%. This occurred largely at the expense of oleic acid where lines with both mutations had 14.8% oleic acid versus 25.4% oleic acid for the wild type. The double mutant genotype also resulted in a small, but significant decrease in total oil, 19.6% compared to 20.8% for the wild type (Table 8).

## DISCUSSION

The effect of the *SACPD-C* nucleotide 229 mutation on fatty acid composition was confirmed in FAM94-41-derived lines and a new functional mutation in *SACPD-B* was identified. The effect of this *SACPD-C* mutation on stearic acid in these populations is in agreement with the mapping study performed by Spencer et al. (2003); however, this study provides stronger evidence of a dominance genetic effect, likely because of a larger population size and the use of perfect markers. Boersma et al. (2012) recently reported a line with a *SACPD-C* mutation whose resulting protein product is predicted to be only 63 amino acids long and a separate line with a point mutation resulting in a predicted proline → leucine substitution at amino acid 286. Interestingly, those two lines, FAM-94-41 and the FAM-94-

41-derived lines in this study all exhibit comparable levels of stearic acid. This suggests that all characterized elevated stearic mutations involving *SACPD-C* are due to a complete, rather than partial, loss of function and that one or more additional loci are probably involved in the 28% stearic acid phenotype observed in A6 (Hammond and Fehr 1983; Zhang et al. 2008). The nucleotide 1114 deletion in *SACPD-B* is the first functional mutation in this isoform reported in soybean. This deletion causes a frameshift and is predicted to result in a longer protein whose 28 final amino acids are predicted to differ from Williams 82 *SACPD-B* (glyma02g15600). The SIFT analysis provides support that this frameshift results in a functional mutation and that the altered fatty acid composition is due to this mutation and not due to a mutation at a linked locus; however, it is unknown whether the *SACPD-B* nucleotide 1114 deletion results in a complete or partial loss of enzymatic function. This mutation has a similar effect on phenotype to that observed with the *SACPD-C* nucleotide 229 mutation, which is associated with increased stearic acid, primarily at the expense of oleic acid, and small decreases in palmitic acid and total oil. Additionally, the *SACPD-B* nucleotide 1114 deletion is associated with a small increase in linolenic acid while the *SACPD-C* mutation is associated with a small decrease in linolenic acid in these populations. This difference could be due to a low linolenic acid QTL inherited from N98-4445A (Bachlava et al. 2009) being linked to the *SACPD-C* mutation in the LLL-05-1 and LLL-05-14 parental lines. Although *SACPD-B* expression is not seed specific (Byfield et al. 2006) and *SACPD-B* is not as strongly expressed in seed as *SACPD-C* (Kachroo et al. 2008), this research demonstrates that mutations in this gene do affect seed fatty acid composition and total oil accumulation.

There is also significant evidence for an additive by additive epistatic interaction between the *SACPD-C* and *SACPD-B* loci. Biologically, this could be due to the wild type enzyme of one isoform compensating for the mutant enzyme of another isoform, therefore, the observed additive effect of *SACPD-C* is larger when *SACPD-B* is mutant than when this loci is wild type .

These mutations bring us closer to the 20% stearic acid necessary for high stearic oil for use in solid fat applications, but another elevated stearic locus will be needed. Combining these two mutations with a *SACPD-A* mutation may reduce 18:0 to 18:1 desaturation activity sufficiently to achieve this goal. Alternatively, these mutant genes could be combined with other mutant genes in the fatty acid metabolic pathway that could result in elevated stearic acid concentration, such as *KASII* or *18:1-ACP TE*. Also, if the *SACPD-B* nucleotide 1114 deletion only results in a partial loss of function, a mutation with a complete loss of function could also be a candidate for use in developing a high stearic acid soybean oil.

High stearic acid oil, also high in oleic acid, is another oil desired by industry (Clemente and Cahoon 2009) for its baking qualities and health properties. These data suggest that obtaining both high stearic acid and high oleic acid from a single cultivar may prove difficult due to the increase in stearic acid being obtained at the expense of desaturation to oleic acid. Developing high stearic acid oil and blending it with high oleic acid oil may be a more realistic goal.

Since *SACPD-B* is expressed in a variety of plant tissues, a mutation in it may have agronomic implications not realized in mutations observed in seed-specific desaturases. Silencing all three known isoforms of *SACPD* in soybean has been shown to result in adverse morphological differences (Kachroo et al. 2008). Soybean seed with elevated stearic acid has been shown to have increased triacylglycerol and phospholipid melting temperatures (Wang 2001b). Changes in membrane fluidity are known to affect plant metabolic processes (Quinn and Williams 1978; Yamamoto et al. 1981). Decreased membrane fluidity could be responsible for the reduction in total seed oil accumulation associated with these *SACPD-C* and *SACPD-B* mutations. Because the non-seed specific *SACPD-B* had a larger effect on total oil accumulation, the authors propose that metabolic processes occurring in vegetative tissues, rather than seed, are driving this reduction in total seed oil.

It would be of great value to evaluate this new *SACPD-B* mutation for agronomic effects, such as seed yield, emergence, and cold tolerance to determine if it would be a viable candidate for use in breeding a high-yielding, elevated stearic soybean. The KASPar markers developed in this study would be useful for marker assisted backcrossing in crosses involving these *SACPD-B* and *SACPD-C* mutations to develop a cultivar with elevated stearic acid content.

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Table 1. Amplification primers for *SACPD* isoforms in *Glycine max*

Primer	Direction	Sequence (5'->3')
<i>SACPD-A</i> exon 1	Forward	ACACTTGCTCCTCCTCTCTC
<i>SACPD-A</i> exon 1	Reverse	CACACACAAATGAAAGC
<i>SACPD-A</i> exons 2 and 3	Forward	CTATGATGCCATGCAAACCTT
<i>SACPD-A</i> exons 2 and 3	Reverse	CTCCACAGACATCACTACC
<i>SACPD-B</i> exon 1	Forward	CCTTCTTCTTCTGCCTTACATC
<i>SACPD-B</i> exon 1	Reverse	TTAGACACTCAACCACAC
<i>SACPD-B</i> exons 2 and 3	Forward	CTTCTACCAATGATGCCT
<i>SACPD-B</i> exons 2 and 3	Reverse	CTCACATGTTAACGTAGCG
<i>SACPD-C</i> exons 1 and 2	Forward	CCAACCTCTCCACAGTTCCAC
<i>SACPD-C</i> exons 1 and 2	Reverse	GCTAAGCTAATATGCAAGCC

Table 2. Sequencing primers for *SACPD* isoforms in *Glycine max*

Exon Sequenced	Direction	Sequence (5'->3')
<i>SACPD-A</i> exon 1	Forward	CTCCTCCTCTCTCCTTCTAC
<i>SACPD-A</i> exon 2	Forward	CTGTGTTTCATGTAATGGTG
<i>SACPD-A</i> exon 3	Forward	CATTCATATTCACACGAGTG
<i>SACPD-B</i> exon 1	Forward	GCCTTACATCACATACTTC
<i>SACPD-B</i> exon 2	Reverse	CATAAATGGGTAGCAAGAG
<i>SACPD-B</i> exon 3	Forward	GAATACGATGACACTTTCC
<i>SACPD-C</i> exon 1	Forward	CTCTCCACAGTTCCACTC
<i>SACPD-C</i> exon 2	Forward	GGGCAGACTTAAATTGAAC

Table 3. KASPar genotyping primers for *SACPD-B* snp 1114 and *SACPD-C* snp 229

Primer	Fluor	Direction	Sequence (5'->3')
<i>SACPD-B</i> snp 1114 Wild type	FAM	Forward	GAAGGTGACCAAGTTCATGCTAAGTGTTGACGACTCCTTGACTCTT
<i>SACPD-B</i> snp 1114 Mutant	VIC	Forward	GAAGGTCTGGAGTCAACGGATTGTGTTGACGACTCCTTGACTCTG
<i>SACPD-B</i> snp 1114 Common	none	Reverse	AGAAGGTTGGAGGAGAGAGCTCAA
<i>SACPD-C</i> snp 229 Wild type	FAM	Forward	GAAGGTGACCAAGTTCATGCTGGGAGATATGGTCACCGAGG
<i>SACPD-C</i> snp 229 Mutant	VIC	Forward	GAAGGTCTGGAGTCAACGGATTGTGGGAGATATGGTCACCGAGA
<i>SACPD-C</i> snp 229 Common	none	Reverse	GTTGATCATGGTCTGGTAAGTGGGAA

Table 4. Segregation ratios for *SACPD-C* and *SACPD-B* among F<sub>2</sub>-derived lines in the LLL-05-01 x TCJWB03-806-7-19 (FA-G) and LLL-05-14 x TCJWB03-806-7-19 (FA-H) populations

Population	Gene	Mutant	Heterozygous	Wildtype	Total	$\chi^2$ (1:2:1)	P-value
FA-G	<i>SACPD-C</i>	44	111	54	209	1.77	0.41
	<i>SACPD-B</i>	40	108	61	209	4.45	0.11
FA-H	<i>SACPD-C</i>	27	53	37	117	2.74	0.25
	<i>SACPD-B</i>	21	62	34	117	3.31	0.19

Table 5. Fatty acid and total oil least squares means (%) for each genotypic class in the two-year combined analysis of the F<sub>2</sub>-derived populations LLL-05-01 (*SACPD-C* mutant) x TCJWB03-806-7-19 (*SACPD-B* mutant) and LLL-05-14 (*SACPD-C* mutant) x TCJWB03-806-7-19, segregating for *SACPD-C* snp 229 and *SACPD-B* snp 1114

Genotypic Class	Palmitic Mean	Stearic Mean	Oleic Mean	Linoleic Mean	Linolenic Mean	Total Oil Mean
<i>SACPD-C</i> Homozygous Mutant	11.3***	11.9***	18.1***	52.0	6.8*	20.2***
<i>SACPD-C</i> Heterozygous	11.6***	7.9***	20.7***	52.7	7.1	20.5
<i>SACPD-C</i> Homozygous Wild Type	12.1	5.3	22.8	52.5	7.2	20.5
<i>SACPD-B</i> Homozygous Mutant	11.5**	10.4***	17.8***	52.8	7.5***	19.9***
<i>SACPD-B</i> Heterozygous	11.7	8.2***	20.9***	52.3	6.9	20.5***
<i>SACPD-B</i> Homozygous Wild Type	11.8	6.5	22.8	52.1	6.7	20.8

\*, \*\*, \*\*\*Mean different from Homozygous Wild Type for a Dunnett pairwise comparison, p<.05, p<.01, p<.001

Table 6. Additive effect, additive by additive effect estimates, and R<sup>2</sup> associated with *SACPD-C* snp 229 and *SACPD-B* snp 1114 in the two-year combined analysis of the F<sub>2</sub>-derived populations, LLL-05-01 (*SACPD-C* mutant) x TCJWB03-806-7-19 (*SACPD-B* mutant) and LLL-05-14 (*SACPD-C* mutant) x TCJWB03-806-7-19

Gene and Parameter Estimated	Palmitic	Stearic	Oleic	Linoleic	Linolenic	Total Oil
<i>SACPD-C</i> Additive Effect	-0.4***	3.3***	-2.4***	NS	-0.2**	-0.2***
<i>SACPD-B</i> Additive Effect	-0.2**	1.9***	-2.5***	NS	0.4***	-0.4***
<i>SACPD-C</i> Additive x <i>SACPD-B</i> Additive	NS	3.3***	NS	-2.8**	NS	NS
<i>SACPD-C</i> R <sup>2</sup>	.12	.55	.20	<.01	<.01	.04
<i>SACPD-B</i> R <sup>2</sup>	.01	.19	.22	<.01	.03	.18

\*, \*\*, \*\*\*Estimate significance, p<.05, p<.01, p<.001

Table 7. Dominance effect estimates of *SACPD-C* snp 229 and *SACPD-B* snp 1114 in one year analysis of F<sub>2</sub> populations, LLL-05-01 (*SACPD-C* mutant) x TCJWB03-806-7-19 (*SACPD-B* mutant) and LLL-05-14 (*SACPD-C* mutant) x TCJWB03-806-7-19

Gene and Parameter Estimated	Palmitic	Stearic	Oleic	Linoleic	Linolenic	Total Oil
<i>SACPD-C</i> Dominance Effect	-0.2*	-0.8***	NS	NS	NS	NS
<i>SACPD-B</i> Dominance Effect	NS	NS	NS	NS	NS	NS

\*, \*\*, \*\*\*Estimate significance, p<.05, p<.01, p<.001

Table 8. Fatty acid and total oil least squares means (%) of two F<sub>2</sub>-derived populations, LLL-05-01 (*SACPD-C* mutant) x TCJWB03-806-7-19 (*SACPD-B* mutant) and LLL-05-14 (*SACPD-C* mutant) x TCJWB03-806-7-19 at *SACPD-C* snp 229 and *SACPD-B* snp 1114

<i>SACPD-C</i>	<i>SACPD-B</i>	Palmitic Mean	Stearic Mean	Oleic Mean	Linoleic Mean	Linolenic Mean	Total Oil Mean
Homozygous Mutant	Homozygous Mutant	11.1***	14.6***	14.8***	52.1	7.3	19.6***
	Heterozygous	11.4***	11.9***	19.0***	51.3	6.6	20.3**
	Homozygous Wild Type	11.6**	9.1***	20.4***	52.5	6.4	20.7
Heterozygous	Homozygous Mutant	11.4***	9.9***	18.6***	52.7	7.4	20.0***
	Heterozygous	11.7**	7.7***	20.8***	52.8*	7.0	20.6
	Homozygous Wild Type	11.7*	6.1***	22.7***	52.6	6.8	21.0
Homozygous Wild Type	Homozygous Mutant	12.1	6.6***	20.0***	53.6**	7.7*	20.1**
	Heterozygous	12.2	5.0*	23.0**	52.7	7.1	20.7
	Homozygous Wild Type	12.1	4.3	25.4	51.2	6.9	20.8

\*, \*\*, \*\*\*Mean different from Homozygous Wild Type/Homozygous Wild Type for a Dunnett pairwise comparison, p<.05, p<.01, p<.001

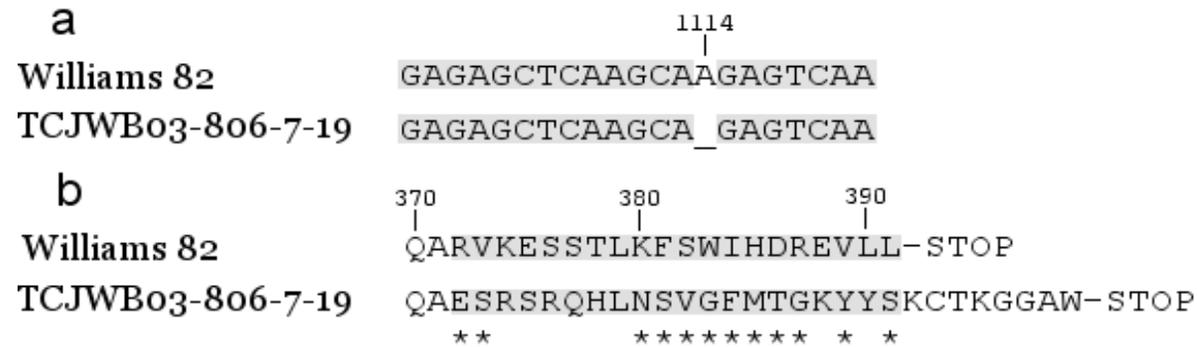


FIGURE 1. Frameshift mutation in the *SACPD-B* gene of TCJWB03-806-7-19. **a** Nucleotide sequence comparison of the *SACPD-B* genes from 'Williams 82' (Glyma02g15600) and TCJWB03-806-7-19 (GenBank no. JQ993842) in the region surrounding the 'A' deletion at position 1114 (relative to the start codon). **b** Amino acid comparison showing the frameshift mutation, starting at position 372 (relative to the start amino acid) and predicted altered location of the stop codon in the *SACPD-B* enzyme from TCJWB03-806-7-19. Amino acids with an asterisk are substitutions predicted to affect enzymatic function by sorting intolerant from tolerant (SIFT) analysis

## **CHAPTER 2: Agronomic Effects of Mutations in Two Soybean $\Delta 9$ -Stearoyl-ACP-Desaturases**

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### Abbreviations

AOSA- Association of Seed Analysts

FAMEs- Fatty acid methyl esters

SACPD-  $\Delta 9$ -stearoyl-acyl carrier protein-desaturase

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**ABSTRACT**

Soybean [*Glycine max* (L.) Merr.] oil normally contains 2-4% stearic acid. Oil with higher levels of stearic acid is desired for use in the baking industry, for both its chemical properties and human health benefits. Several lines with increased stearic acid have been identified; however, the agronomic consequences of high seed stearic acid are poorly understood. This study evaluates the effects of high stearic acid mutant alleles for two different  $\Delta 9$ -stearoyl-acyl carrier protein-desaturase genes, *SACPD-B* and *SACPD-C*, on agronomic performance. Fifty  $F_2$ -derived lines developed in a previous study were evaluated in a germination chamber and two field environments. Lines were either homozygous for the *SACPD-C* *fas<sub>nc</sub>* allele previously identified in FAM94-41, homozygous for the *SACPD-B* *fas<sub>2nc</sub>* allele from the induced 'Holladay' mutant TCJWB03-806-7-19, homozygous for both mutant genes or homozygous wild-type for both genes. *SACPD-C* mutant lines were agronomically indistinguishable from the wild-type lines and yielded as well (3534 kg ha<sup>-1</sup> vs. 3414 kg ha<sup>-1</sup>). *SACPD-B* mutant lines suffered from a decreased seedling growth rate, were 15% shorter at maturity, had a 4% seed size reduction and yielded 6% less than the wild-type lines. The different agronomic performance between *SACPD-C* and *SACPD-B* mutant lines was attributed to altered fatty acid composition in vegetative tissues of the *SACPD-B* mutant lines.

## INTRODUCTION

Soybean [*Glycine max* (L.) Merr.] accounts for over half of worldwide oilseed production (USDA-Economic Research Service, 2012) and is widely utilized by the feed and food industries. Soybean oil with increased saturated fatty acids would be useful for solid fat applications in the baking industry due to its increased oxidative stability and relatively high melting temperature (List and Pelloso, 2007; Clemente and Cahoon, 2009). Palmitic and stearic acid are the two major saturated fatty acids present in commodity soybean oil, at 11% and 4% of total fatty acids respectively (Hildebrand et al., 2008).

An increase of palmitic acid in fat intended for human use would be undesirable as its consumption results in an unfavorable lipoprotein profile in blood serum (Mensink and Katan, 1990). In contrast, consumption of stearic acid does not adversely affect human health compared to the trans- and shorter chain saturated fats that it would replace (Hunter et al., 2010).

Stearic acid variation in soybean is primarily governed by variants in the  $\Delta 9$ -stearoyl-acyl carrier protein-desaturase (*SACPD*) genes. *SACPD* enzymes desaturate steaoryl-acyl carrier protein to oleoyl-acyl carrier protein in the fatty acid biosynthetic pathway (Ohlrogge and Browse, 1995). Three *SACPD* isoforms have been identified: *SACPD-A*, *SACPD-B* and *SACPD-C* (Byfield et al., 2006; Zhang et al., 2008). *SACPD-A* and *SACPD-B* are expressed in a wide variety of plant tissues while *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). However, these studies differ as to whether *SACPD-C* is expressed in root tissue. Several *SACPD-C* mutations and one *SACPD-B* mutant have been linked to

elevated seed stearic acid phenotypes (Hammond and Fehr, 1983; Zhang et al., 2008; Boersma et al. 2012; Ruddle et al., 2012). Several elevated stearic acid germplasm lines have also been identified with unknown or unverified causal loci (Graef et al., 1985; Bubeck et al., 1989; Rahman et al., 1995; Hudson, 2012).

Elevated stearic acid results in an increase in melting transition temperature in the triacylglycerols and phospholipids (Wang et al., 2001a). Lipid composition is known to govern membrane fluidity and affect plant metabolic processes (Quinn and Williams, 1979; Yamamoto et al., 1981), thus, increased stearic acid, especially in vegetative tissues, could adversely affect agronomic performance.

The agronomic effects of elevated seed stearic acid in soybean and other crops have been studied before but previous research did not link agronomic performance to specific genes nor evaluated levels of stearic acid in vegetative tissues. Lundeen et al. (1987) reported lower seed yield associated with increased seed stearic acid in one of three populations evaluated. Another study noted poor germination in seeds with over 300 g kg<sup>-1</sup> stearic acid (Rahman et al., 1997); however, in *Brassica* sp. the poor germination associated with elevated stearic acid has been attributed to low total oil, and normal oil with elevated stearic acid was not associated with low germination (Knuzton et al., 1992). Wang et al. (2001b) observed a negative correlation between lipid saturates and both germination and seedling growth rate. Primomo et al. (2002) noted that RG6 and RG8, two elevated stearic acid mutants developed by induced mutagenesis with ethyl methanesulfonate, yielded lower than the check cultivars, while another elevated stearic acid mutant developed in the same

manner, RG7, yielded similarly to the check cultivars. A greenhouse study utilizing viral-induced silencing of *SACPD-A/B* in soybean revealed increased levels of stearic acid in vegetative tissues (Kachroo et al., 2008). These plants suffered from dwarfing and necrotic leaf lesions. Levels of gene transcripts associated with defense response pathways and disease resistance were also affected.

Given the usefulness of an oil high in stearic acid, it would be important to determine if a soybean variety high in stearic acid could have acceptable agronomic performance, and to be able to assign any detrimental effect to specific high stearic alleles in order to inform breeding decisions. The objectives of this research were to study the effects of two *SACPD* mutations on germination, seedling growth rate, seedling fatty acid profile, seed emergence, maturity, plant height, 100 seed weight, total oil, seed fatty acid profile, and yield.

## **MATERIALS AND METHODS**

### **Plant Materials**

Fifty F<sub>2</sub>-derived lines homozygous for one of two alleles at the *SACPD-B* and *SAPCD-C* loci were randomly selected from the FA-G and FA-H populations previously described by Ruddle et al. (2012). The FA-G and FA-H populations were developed by biparental crosses between the FAM-94-41-derived lines LLL-05-01 and LLL-05-14 (Ruddle et al., 2012) and TCJWB03-806-7-19. FAM-94-41 (8.8% stearic acid; Pantalone et al., 2002) carries an *SACPD-C* allele designated *fas<sub>nc</sub>* which contains a spontaneously arising point mutation (Zhang et al., 2008). TCJWB03-806-7-19 (7% stearic acid; Ruddle et al., 2012) is an induced mutant of the maturity group V cultivar Holladay (Burton et al., 1996) carrying an

*SACPD-B* allele designated *fas2<sub>nc</sub>* which contains a single nucleotide deletion. Both of these mutant alleles have been previously shown to result in elevated seed stearic acid content compared to the wild-type alleles (Pantalone et al., 2002; Ruddle et al., 2012).

The distribution of selected lines was as follows: twelve lines homozygous wild-type for *SACPD-B* and *SACPD-C*; fourteen lines homozygous wild-type for *SACPD-B* and homozygous *fas<sub>nc</sub>* for *SACPD-C*; fourteen lines homozygous *fas2<sub>nc</sub>* for *SACPD-B* and homozygous wild-type for *SACPD-C*; ten lines homozygous *fas2<sub>nc</sub>* for *SACPD-B* and homozygous *fas<sub>nc</sub>* for *SACPD-C*. Twenty-six lines were from the FA-G population and twenty-four lines were from the FA-H population. Distribution of lines was balanced between populations with the exception that eight homozygous wild-type for *SACPD-B* and homozygous *fas<sub>nc</sub>* for *SACPD-C* lines came from the FA-G population and the remaining six from the FA-H population. ‘Holladay’, ‘NC-Roy’, TCJWB03-806-7-19, and the FAM-94-41-derived lines LLL-05-1 and LLL-05-14 were included as check lines for yield and maturity. NC-Roy is a maturity group VI cultivar (Burton et al., 2005) and LLL-05-1, LLL-05-14, and TCJWB03-806-7-19 are all maturity group V lines (Ruddle et al., 2012). Seed for all lines was harvested from the same field in Clayton, NC in 2011. This seed was used both for germination and yield tests in 2012.

### **Germination Chamber Experiments**

A standard germination test was conducted and scored in accordance with the Association of Seed Analysts’ (AOSA) *Rules for Testing Seeds* (1998) and *Seedling Evaluation Handbook*

(1992). Standard weight germination paper towels were the testing substratum and the germination chamber parameters were 25°C with relative humidity of 95%. Lots of fifty seed per line were tested in the chamber at once and the test was performed four times.

Following each replicate of the germination test, seedlings rated as normal had their cotyledons excised and the “Seeding Growth Rate Test-Dry Weight” was conducted in accordance with the AOSA’s *Seed Vigor Testing Handbook* (2009). Seedlings were dried at 80°C for 24 hours.

Dried seedlings, excepting cotyledons, were subsequently evaluated for fatty acid content via acid methanolysis (Browse et al., 1986). Dried, homogenized seedlings were heated at 85°C for 90 minutes in 3mL of 5% HCl/methanol (v/v) solution. Samples were cooled to room temperature, 1 mL of 1% aqueous NaCl (w/v) was added and the fatty acid methyl esters (FAMES) were partitioned into 1 mL hexane and transferred to 2mL vials for gas chromatography analysis. FAMES were analyzed by gas chromatography as outlined by Burkey et al. (2007).

### **Field Evaluation**

The fifty lines were randomly divided into two sets for field evaluation. The previously described check lines were also included in each set. Two replications of each set were grown in 2012 in Plymouth, NC (planted May 23) and Kinston, NC (planted May 29). Lines were randomized within each replication and within each location. Each set was planted as a randomized complete block. Soils were Portsmouth fine sandy loam and Lumbee sandy

loam, respectively. Plots were three rows wide (0.97 m between rows) and 4.3 m long. Four hundred seed were planted in each plot. Emergence was determined by counting the number of plants in each row of a three-row plot at the V2-V3 stage (Fehr and Caviness, 1977) and averaging the three rows. Plots were end trimmed to 3.0 m and only the center row was harvested for yield and 100-seed weight determination. Flower color, pubescence color, plant height and maturity date at the R8 reproductive stage were recorded.

### **Seed Oil Analysis**

FAME analysis was performed on a twenty seed sample from each harvested plot. Oil content was measured on a ten gram seed sample from each harvested plot. Analyses were conducted as previously described (Ruddle et al. 2012).

### **Statistical Analysis**

For germination chamber experiments, a mixed model was fit using the MIXED procedure in SAS 9.2 (SAS Institute, Cary, NC, 2009). The *SACPD-B* and *SACPD-C* loci, population, and their interactions were considered as fixed effects. Line (nested within *SACPD-B*, *SACPD-C*, and population), replicate, and the appropriate interactions with fixed effects were considered as random effects. Dunnett's test, with the homozygous wild type at both the *SACPD-B* and *SACPD-C* loci as the control group, was conducted on the genotypic class least squares means to determine if the mutant alleles were associated with different phenotypes than the wild-type alleles. Satterthwaite's approximation (Satterthwaite, 1941) was used to determine appropriate degrees of freedom for the means separation procedure.

Contrast statements were used to determine if an additive by additive epistatic interaction between the *SACPD-B* and *SACPD-C* loci was present for each trait (Holland 2001).

A similar mixed model was also used to analyze the data from field experiments, with each location considered separately. The *SACPD-B* and *SACPD-C* loci, population and their interactions were considered as fixed effects. Line (nested within *SACPD-B*, *SACPD-C*, population and set), replicate (nested within set), set, and the appropriate interactions with fixed effects were considered as random effects. Dunnett's test was conducted and epistatic interactions were tested as described above. A separate model considering both locations together, with location as a fixed effect, was also analyzed. Location was considered fixed because only two locations were trialed. Multivariate regression analysis was conducted using the MANOVA procedure in GLM to determine if maturity and yield were correlated, after accounting for the fixed and random effects in the model.

Yield data for each line was compared to the cultivars NC-Roy and Holladay, to determine if lines in these studies yielded as high as the highest yielding check cultivar for each location. A mixed model was fit where entry was considered as a fixed effect while replicate was considered as a random effect. A separate model was fit for each location. Dunnett's test was conducted with the highest yielding check cultivar for each location as the control.

## RESULTS

### Germination Chamber Experiments

The statistical analyses showed no significant interaction between population and genotypic class, thus the results for both populations combined are presented. All differences presented were significant in a Dunnett's pairwise comparison with the wild-type ( $p < 0.05$ ). Neither the *SACPD-C* nor *SACPD-B* mutation affected germination (Table 1). Reduced seedling dry weight was associated with the *SACPD-B* mutation (32.2 mg vs. 34.4 mg) and when both the *SACPD-C* and *SACPD-B* mutations were combined (30.8 mg). The only significant seedling fatty acid changes observed due to solely the *SACPD-C* mutation were a small increase in stearic acid (76 g kg<sup>-1</sup> vs. 62 g kg<sup>-1</sup>) and a small decrease in oleic acid (37 g kg<sup>-1</sup> vs. 44 g kg<sup>-1</sup>). The *SACPD-B* mutation resulted in an increase in stearic acid (116 g kg<sup>-1</sup> vs. 62 g kg<sup>-1</sup>) and decreases in oleic (33 g kg<sup>-1</sup> vs. 44 g kg<sup>-1</sup>) and linoleic acid (336 g kg<sup>-1</sup> vs. 361 g kg<sup>-1</sup>) in seedlings. Both mutations combined were associated with decreases in palmitic acid (221 g kg<sup>-1</sup> vs. 274 g kg<sup>-1</sup>) and oleic acid (28 g kg<sup>-1</sup> vs. 44 g kg<sup>-1</sup>) and an increase in stearic acid (144 g kg<sup>-1</sup> vs. 62 g kg<sup>-1</sup>) in seedlings. Significant epistatic additive by additive gene action was detected for seedling palmitic acid (-2.40 g kg<sup>-1</sup>) and seedling stearic acid (+1.50 g kg<sup>-1</sup>). No other significant additive by additive epistasis was observed.

### Field Evaluation

The statistical analyses showed no significant interaction between population and genotypic class nor set and genotypic class, thus the results for both populations and sets combined are presented. All differences presented were significant in a Dunnett's pairwise comparison

with the wild-type ( $p < 0.05$ ), unless otherwise indicated. Yield and maturity were determined not to be correlated; furthermore, no differences were detected in mean maturity between genotypic classes. Mean maturity date was three days later than the maturity group V cultivar Holladay. No lines matured later than the check variety NC-Roy. No differences were detected between the *SACPD-C* mutants and the wild-type entries in any of the evaluated traits. The combination of both mutations was associated with a small, significant, decrease in emergence at one location (91% emergence vs. 94% emergence; Table 2). Plant height at maturity was reduced at both locations for *SACPD-B* mutants (63 cm vs. 74 cm) and double mutants (62 cm). *SACPD-B* mutants produced smaller seed when both locations are considered jointly (14.3 g 100 seed<sup>-1</sup> vs. 14.9 g 100 seed<sup>-1</sup>;  $p = 0.06$ ) as did double mutants (13.8 g 100 seed<sup>-1</sup>). A significant epistatic interaction of -0.9 g 100 seed<sup>-1</sup> was detected when both locations are considered together. Yield was lower in *SACPD-B* mutants (3317 kg ha<sup>-1</sup> vs. 3514 kg ha<sup>-1</sup>;  $p = 0.06$  at Plymouth,  $p = 0.13$  at Kinston and  $p < 0.05$  when both locations are considered jointly) and for double mutants at both locations (3134 kg ha<sup>-1</sup>). An additive by additive negative epistatic effect on yield (-384 kg ha<sup>-1</sup>) was observed at Plymouth only. Seed fatty acid profiles and total seed oil for each genotypic class are detailed in Table 3. A significant increase in stearic acid and decrease in oleic acid was observed at both locations when compared to the wild-type genotypic class.

Among the top five highest yielding lines from each genotypic class, one wild-type line and one *SACPD-C* mutant line yielded higher than the highest yielding check cultivar at Plymouth (NC-Roy); however, the differences were not statistically significant (Table 4). At

Kinston, three wild-type lines and two *SACPD-C* mutant lines yielded higher than the highest yielding check cultivar for this location (Holladay) but none of the differences were statistically significant.

## DISCUSSION

The effects of the *SACPD-B* (*fas2<sub>nc</sub>*) and *SACPD-C* (*fas<sub>nc</sub>*) mutations on seed fatty acid composition were confirmed and adverse agronomic effects associated with the *SACPD-B* mutation were identified. Seed fatty acid profiles and total seed oil for each genotypic class were consistent with those previously reported (Ruddle et al., 2012) – the primary effect of both the *SACPD-B* and *SACPD-C* mutations on seed oil composition was an increase in stearic acid and a decrease in oleic acid. Unlike previous studies (Rahman et al. 1997; Wang et al. 2001b), the high seed stearic acid levels due to these two mutations did not significantly reduce germination. Seedling fatty acid composition was affected primarily by the *SACPD-B* mutation, with the effect of nearly doubling seedling stearic acid content. This finding is consistent with previous transcription and phenotypic analysis of vegetative tissues (Byfield et al. 2006; Zhang et al. 2008; Kachroo et al. 2008); however, the further increase of seedling stearic acid when both mutations are present suggests that both loci, in addition to the *SACPD-A* locus, can affect fatty acid biosynthesis in the developing soybean plant. Increased *SACPD-C* expression may partially compensate for a deficient *SACPD-B* enzyme, thus, the observed additive effect of a deficient *SACPD-B* enzyme is larger when the *SACPD-C* locus is also mutant than when the *SACPD-C* locus is wild-type. Such an epistatic interaction has been previously noted in seed stearic acid phenotypes in populations

segregating for these *SACPD-C* and *SACPD-B* mutations (Ruddle et al., 2012). Seedlings with the *SACPD-B* mutation had a six percent decrease in dry weight and seedlings with both mutations had a ten percent decrease in dry weight compared to the wild-type seedlings while seedlings with only the *SACPD-C* mutation were comparable to the wild-type. This suggests that the altered seedling fatty acid profile is responsible for the decrease in seedling growth rate and any reduced viability in elevated stearic seeds may be more accurately attributed to elevated seedling stearic acid than elevated seed stearic acid per se. It would be of value to see if this assertion holds true for other germplasm with elevated seed stearic acid and unknown causal loci.

Mature *SACPD-B* mutants were 15% shorter, had a 4% seed size reduction, and yielded 6% less while *SACPD-C* mutants were indistinguishable from the wild-type on the basis of these characteristics; furthermore, on an individual line basis, the top yielding *SACPD-C* mutants outperformed the top yielding *SACPD-B* mutants. Likely as a result of the absence of a compensating effect from the *SACPD-C* locus, the detrimental effects of the *SACPD-B* mutation were heightened in the presence of the *SACPD-C* mutation, and a negative epistatic interaction for yield was observed at one location. These observations are consistent with the growth reductions observed in seedlings and support the previous assertion that altered fatty acid composition in vegetative tissues is responsible for the reduced growth.

Given the reduction in yield associated with the *SACPD-B* mutation, mutations in this gene may not be useful for developing a high-yielding, high-stearic acid soybean cultivar.

The lack of adverse agronomic effects associated with solely the *SACPD-C* mutation underscores the utility of this source of high stearic acid for breeding purposes, however, this mutation alone is not sufficient to achieve the 20% stearic acid sufficient for solid fat applications. Since *SACPD-A* is also expressed in vegetative tissues, it, along with *SACPD-B*, should be avoided if a high-yielding soybean cultivar is also the end goal. A previous study with a presumed *SACPD-C* null mutant revealed a larger increase in stearic acid when crossed with germplasm containing low levels of palmitic acid (Boersma et al., 2012). Another study realized enhanced stearic acid levels when a *SACPD-C* mutant was crossed to a mid-oleic line (Zhang et al., 2008). Neither cross achieved the requisite 20% stearic acid but both provide insights on how such an oil could be realized. A null *SACPD-C* enzyme combined with mutant genes that result in a high oleic phenotype, such as those identified by Pham et al. (2010), may result in lines with the desired levels of stearic acid.

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Table 1. Least squares means of germinated seedlings, dry weight per seedling and whole seedling fatty acid of fifty lines from the F<sub>2</sub>-derived populations, LLL-05-01 (SACPD-C mutant: *fas<sub>nc</sub>*) x TCJWB03-806-7-19 (SACPD-B mutant: *fas<sub>2nc</sub>*) and LLL-05-14 (SACPD-C mutant: *fas<sub>nc</sub>*) x TCJWB03-806-7-19

<i>SACPD-C</i>	<i>SACPD-B</i>	Germinated Seedlings (%)	Dry Weight Per Seedling (mg)	Palmitate (g kg <sup>-1</sup> )	Stearate (g kg <sup>-1</sup> )	Oleate (g kg <sup>-1</sup> )	Linoleate (g kg <sup>-1</sup> )	Linolenate (g kg <sup>-1</sup> )
Wild Type	Wild Type	96	34.4	274	62	44	361	257
Wild Type	Mutant	98	32.2**	245***	116***	33***	336**	270
Mutant	Wild Type	96	34.9	274	76**	37***	366	248
Mutant	Mutant	95	30.8***	221***	144***	28***	349	260

\* significantly different from Wild Type at the 0.05 probability level (Dunnett's pairwise comparison)

\*\* significantly different from Wild Type at the 0.01 probability level (Dunnett's pairwise comparison)

\*\*\* significantly different from Wild Type at the 0.001 probability level (Dunnett's pairwise comparison)

Table 2. Least squares means of emerged seedlings, height at R8 reproductive stage, maturity at R8 reproductive stage, 100 seed weight, and yield of fifty lines from the F<sub>2</sub>-derived populations, LLL-05-01 (SACPD-C mutant: *fas<sub>nc</sub>*) x TCJWB03-806-7-19 (SACPD-B mutant: *fas<sub>2nc</sub>*) and LLL-05-14 (SACPD-C mutant: *fas<sub>nc</sub>*) x TCJWB03-806-7-19

<i>SACPD-C</i>	<i>SACPD-B</i>	Emergence (%)			Height (cm)			Maturity†			100 Seed Weight (g 100 seed <sup>-1</sup> )			Yield (kg ha <sup>-1</sup> )		
		P	K	B	P	K	B	P	K	B	P	K	B	P	K	B
Wild Type	Wild Type	94	95	94	75	73	74	11	13	12	14.6	15.2	14.9	3788	3239	3514
Wild Type	Mutant	95	95	95	64***	62***	63***	11	13	12	14.0‡	14.7	14.3§	3575‡	3066¶	3317*
Mutant	Wild Type	93	93	93	76	75	76	12	13	13	14.8	15.5	15.2	3815	3256	3534
Mutant	Mutant	91*	94	92*	61***	62***	62***	11	13	12	13.4***	14.3*	13.8**	3232***	3035#	3134***

\* significantly different from Wild Type at the 0.05 probability level (Dunnett's pairwise comparison)

\*\* significantly different from Wild Type at the 0.01 probability level (Dunnett's pairwise comparison)

\*\*\* significantly different from Wild Type at the 0.001 probability level (Dunnett's pairwise comparison)

P- Plymouth, NC; K- Kinston, NC; B-Both Locations

† Days after September 31

‡ p=0.13

§ p=0.06

¶ p=0.10

# p=0.09

Table 3. Least squares means of seed fatty acid ( $\text{g kg}^{-1}$ ) and seed total oil ( $\text{g kg}^{-1}$ ) of fifty lines from the  $F_2$ -derived populations, LLL-05-01 (SACPD-C mutant: *fas<sub>nc</sub>*) x TCJWB03-806-7-19 (SACPD-B mutant: *fas<sub>2nc</sub>*) and LLL-05-14 (SACPD-C mutant: *fas<sub>nc</sub>*) x TCJWB03-806-7-19

<i>SACPD-C</i>	<i>SACPD-B</i>	Palmitate		Stearate		Oleate		Linoleate		Linolenate		Arachidate		Total Oil	
		P	K	P	K	P	K	P	K	P	K	P	K	P	K
Wild-Type	Wild Type	115	115	38	41	247	267	523	505	73	67	5	5	199.6	200.0
Wild-Type	Mutant	116	117	59 <sup>***</sup>	57 <sup>**</sup>	203 <sup>***</sup>	228 <sup>***</sup>	531	515	85 <sup>*</sup>	76 <sup>**</sup>	6 <sup>***</sup>	6 <sup>***</sup>	193.3 <sup>**</sup>	194.3 <sup>**</sup>
Mutant	Wild Type	111	111 <sup>*</sup>	86 <sup>***</sup>	95 <sup>***</sup>	208 <sup>***</sup>	221 <sup>***</sup>	519	500	68	65	8 <sup>***</sup>	9 <sup>***</sup>	198.5	197.9
Mutant	Mutant	105 <sup>***</sup>	106 <sup>***</sup>	138 <sup>***</sup>	139 <sup>***</sup>	149 <sup>***</sup>	177 <sup>***</sup>	515	493	82	74	11 <sup>***</sup>	11 <sup>***</sup>	190.7 <sup>***</sup>	189.5 <sup>***</sup>

\* significantly different from Wild Type at the 0.05 probability level (Dunnett's pairwise comparison)

\*\* significantly different from Wild Type at the 0.01 probability level (Dunnett's pairwise comparison)

\*\*\* significantly different from Wild Type at the 0.001 probability level (Dunnett's pairwise comparison)

P- Plymouth, NC; K- Kinston, NC

Table 4. Top five lines for each SACPD-C/SACPD-B genotypic class from the F<sub>2</sub>-derived populations, LLL-05-01 (SACPD-C mutant: *fas<sub>nc</sub>*) x TCJWB03-806-7-19 (SACPD-B mutant: *fas2<sub>nc</sub>*) and LLL-05-14 (SACPD-C mutant: *fas<sub>nc</sub>*) x TCJWB03-806-7-19, and their comparison to the highest yielding check cultivar, by location

SACPD-C	SACPD-B	Line	Plymouth			Kinston			
			Yield (kg ha <sup>-1</sup> )	% of Check Variety Yield	Maturity†	Line	Yield (kg ha <sup>-1</sup> )	% of Check Variety Yield	Maturity†
Wild Type	Wild Type	N11-3749	4409	108	12	N11-3943	3476	101	17
		N11-3726	3996	98	4	N11-3761	3471	101	15
		N11-4035	3962	97	18	N11-3723	3443	100	10
		N11-4084	3917	96	16	N11-3751	3434	100	11
		N11-4009	3899	95	3	N11-3980	3365	98	17
	Mutant	N11-4105	3910	96	17	N11-3949	3406	99	11
		N11-3817	3814	93	12	N11-3798	3325	97	9
		N11-3738	3748	92	7	N11-4105	3321	97	18
		N11-4132	3696	90	17	N11-3986	3258	95	17
		N11-3949	3690	90	9	N11-3817	3245	94	14
Mutant	Wild Type	N11-3755	4187	102	10	N11-4078	3618	105	18
		N11-3991	4025	98	19	N11-3991	3471	101	19
		N11-3730	4015	98	7	N11-3748	3393	99	10
		N11-3748	3951	97	6	N11-4053	3377	98	22
		N11-3978	3934	96	14	N11-3755	3335	97	10
	Mutant	N11-3895	3769	92	6	N11-4129	3246	94	22
		N11-4119	3658	89	18	N11-3820	3171	92	9
		N11-3897	3557	87	7	N11-4118	3161	92	15
		N11-4118	3351	82	18	N11-4095	3148	92	15
		N11-4075	3112**	76	16	N11-3926	3083	90	10
	Roy	4093	N/A	20	Holladay	3438	N/A	11	
	Holladay	3842	N/A	7	Roy	3296	N/A	22	
	LLL-05-14	3613	N/A	16	LLL-05-14	3201	N/A	17	
	LLL-05-01	3602	N/A	9	TCJWB03-806-7-19	3085	N/A	9	
	TCJWB03-806-7-19	3517	N/A	8	LLL-05-01	3027	N/A	10	

\* significantly different from highest yielding check cultivar at the 0.05 probability level (Dunnett's pairwise comparison)

\*\* significantly different from highest yielding check cultivar at the 0.01 probability level (Dunnett's pairwise comparison)

\*\*\* significantly different from highest yielding check cultivar at the 0.001 probability level (Dunnett's pairwise comparison)

†Days after September 31

**CHAPTER 3: Effect of a  $\Delta 9$ -Stearoyl-ACP-Desaturase-C Mutants in a High Oleic  
Background on Soybean Seed Oil Composition**

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**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  $\Delta^9$ -stearoyl-acyl carrier protein-desaturase (*SACPD-C*). F<sub>2</sub>-derived lines were evaluated for fatty acid content in four field environments. Sequencing of *SACPDs* in TCHM08-1087 and TCHM08-755 revealed a deletion 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<sub>1</sub> plants were grown in a greenhouse and individual F<sub>2</sub> seed were genotyped and phenotyped. No interaction was observed between either *FAD2-1A* and *FAD2-1B* nor either 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. The *SACPD-C* deletions in TCHM08-1087 and TCHM08-755 shall be designated *fas<sub>ncdel1</sub>* and *fas<sub>ncdel2</sub>* respectively.

Abbreviations: 18:1-ACP TE, 18:1-acyl carrier protein thioesterase; FAD, fatty acid desaturases; KASII, 3-ketoacyl-acyl carrier protein synthase II; KASPar, KBiosciences Competitive Allele Specific PCR; SACPD,  $\Delta^9$ -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 this oil is produced is by blending soybean oil with another vegetable oil high in the sixteen carbon saturated fatty acid palmitate. 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 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  $\Delta^9$ -stearoyl-acyl carrier protein-desaturase (*SACPD*) genes. *SACPD* enzymes desaturate stearoyl-ACP to oleoyl-ACP in the plastid (Ohlrogge and Browse 1995). 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). Also, a number of lines have been identified 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).

*SACPD-C* mutations have been shown to have higher additive effect on stearic acid composition than the one identified *SACPD-B* mutant (Ruddle et al. 2012). Additionally, mutations in the non-seed specific *SACPD-B* have been shown to result in adverse agronomic consequences 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 stearyl-ACP in the step immediately prior to SACPD's desaturation of stearyl-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 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.

FAD2 enzymes desaturate oleoyl moieties to linoleoyl moieties (Ohlrogge and Browse 1995) and multiple isoforms of genes coding for these enzymes have been identified (Schlueter et al. 2007). Mutations in *FAD2-1A* and *FAD2-1B* have been found to result in elevated oleic acid phenotypes in seed oil (Dierking and Bilyeu 2009; Pham et al. 2010).

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 *SACDPC* 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-Cs* and *FAD2s* could result in an elevated stearic acid, high oleic acid soybean oil.

## **MATERIALS AND METHODS**

### **Plant Materials**

Two populations were developed by crosses performed in 2009 in Clayton, NC. FA-I is a population of 188 F<sub>2</sub>-derived lines from the cross FAM94-41-3 x TCHM08-1087. FA-J is a population of 184 F<sub>2</sub>-derived lines from the cross FAM94-41-3 x TCHM08-755. FAM94-41-3 (8% stearic acid; Pantalone et al. 2002) is a selection from an elevated stearic acid germplasm line with a natural mutation in *SACPD-C* (Zhang et al. 2008) which has been

designated *fas<sub>nc</sub>*. 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, Ontario, Canada). The M<sub>1:2</sub> 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<sub>1</sub> plants would have been hemizygous or heterozygous for any induced mutations, the M<sub>1:2</sub> rows were segregating for the high stearic trait.

Two additional populations were developed by crosses performed in 2012 in Clayton, NC. High Stearic, High Oleic 1 (HSHO-1) is a population from the cross S09-2902-145 (homozygous mutant for *FAD2-1A* and *FAD2-1B*) x LLL-05-14 (homozygous mutant for *SACPD-C*). S09-2902-145 (3% stearic acid, 79% oleic acid) is a maturity group V F<sub>5</sub> line from the cross [S05-11482 x 17D] x S07-14788. S05-11482 is a maturity group V F<sub>5</sub>-derived line from the cross S99-2281 x S00-9985-03. Both S99-2281 and S00-9985-03 are breeding lines from Missouri. 17D (Dierking and Bilyeu 2009) is a mutagenized line with a missense mutation in *FAD2-1A*. S07-14788 is a selection from the cross Jake x PI 283327. Jake (Shannon et al. 2007) is a maturity group V cultivar. PI 283327 is a maturity group V plant introduction with 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<sub>5</sub>-derived selection from the cross FAM94-41-3 x N98-4445A. N98-4445A (Burton et al. 2006) is a mid-oleic germplasm line. High Stearic, High Oleic 2 (HSHO-2) is a population from the

cross S09-2902-145 x TCHM08-1087-11. TCHM08-1087-11(13% stearic acid, 22% oleic acid) is a M<sub>3</sub>-derived selection from TCHM08-1087 presumably homozygous for the unknown elevated stearic locus.

### **Field Evaluation**

In 2010, F<sub>2</sub> plants from FA-I and FA-J were grown in Clayton, NC and harvested individually. F<sub>2</sub>-derived lines were grown in 3.7 m long one-row plots in 2011. Soils at Clayton, NC were Norfolk Loamy Sands in both years. In 2012, F<sub>2</sub>-derived lines were grown in Clayton, NC and Kinston, NC, with two replicates of each line grown at each location. Soils at Clayton, NC and Kinston, NC were Varina Loamy Sand and Lumbee Sandy Loam, respectively. In 2011 and 2012 flower color and maturity date at the R8 reproductive stage (Fehr and Caviness 1977) were recorded.

### **Greenhouse Evaluation**

In 2012-2013, F<sub>1</sub> plants from HSHO-1 and HSHO-2 were grown in the greenhouse. Individual plants were harvested. Individual F<sub>2</sub> seed 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<sub>2</sub> plant in 2010 and from each F<sub>2</sub>-derived line in 2011 and 2012 for the FA-I and FA-J populations. FAME analysis was performed on individual F<sub>2</sub> seeds from HSHO-1 and

HSHO-2 harvested under greenhouse conditions in 2013. Oil content was measured from a ten gram seed sample from each F<sub>2</sub> plant in 2010 and each F<sub>2</sub>-derived line in 2011 and 2012 for FA-I and FA-J. Analyses were conducted as described by Ruddle et al. (2012).

### **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<sub>3</sub> high stearic selections from TCHM08-1087 and 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 markers (SSRs) 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. SSRs evaluated were Satt601, Satt318, Satt474, Sat\_189, Satt070, Satt556, and Satt020. SSR reaction mix consisted of 0.4uL H<sub>2</sub>O, 0.8uL 10 x PCR Buffer, 1.6 uL 15 uM MgCl<sub>2</sub>, 0.6 uL 3.12 mM dNTPs, 0.2 uL *taq* polymerase, 0.7 uL 5uM forward primer, 0.7 uL 5uM reverse primer, and 3 ul of 5 ng/ul DNA, for a total volume of 8 uL. 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 1x 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 Ruddle et al. (2012) for each F<sub>2</sub>-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 reactions were carried out as described in the previous section.

DNA was extracted from individual F<sub>2</sub> seed chips for the HSHO-1 and HSHO-2 populations as follows. Fifty uL of sterile water was added to each seed chip and 96-well blocks of seed chips were placed in a shaker incubator for 90 minutes at 60°C and 200 rpm. Seed were then homogenized in a grinder with 400 uL 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 uL of 5M potassium acetate was added to each well. Following centrifugation at 2800 relative centrifugal force, supernatants were transferred to new tubes and an equal volume of 75% isopropanol-2.5M 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 overnight before resuspension in 1 X 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 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<sub>nc</sub>* mutation (25%), heterozygous *fas<sub>nc</sub>*/wild type or *fas<sub>nc</sub>*/*SACPD-C* deletion (50%) and homozygous *SACPD-C* deletion or wild type (25%). Mean values for fatty acid, maturity, and total oil were determined for each F<sub>2</sub>-derived line by first calculating means over replications for each environment, then averaging environmental means.

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<sub>2</sub>-derived line means, least squares means were determined for each *SACPD-C* genotypic class, for each fatty acid and total oil. As both populations have female parent in common and similar 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<sub>nc</sub>/fas<sub>nc</sub>* 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<sub>nc</sub>* mutation. Additive effects of all *SACPD-C* alleles were estimated using contrast statements. Multivariate regression analysis 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<sub>1</sub> 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 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 (Holland 2001).

## **RESULTS**

### **Sequence analysis of *SACPD* isoforms**

The sequencing results were compared to the Williams 82 reference sequence for each 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. TCHM08-1087 and TCHM08-755 were found to lack the *SACPD-C* locus.

#### **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 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 1 megabase encompassing the *SACPD-C* locus (Table 2).

#### **Genetic analysis of the *SACPD-C* deletions**

Segregation distortion was detected towards the *fas<sub>nc</sub>* 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 are similar, only the analysis with both populations combined is presented. Lines differed by 15 days for maturity and maturity was a significant factor in linear

regression for maturity versus 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, thus it was excluded from the analyses.

Both *SACPD-C* deletions had over double the additive effect on stearic acid composition when compared to the *fas<sub>nc</sub>* allele (+4.1% versus +1.8%; Table 4). The deletion also had a larger negative effect on oleic acid (-2.0% versus -1.1%), linoleic acid (-1.9% versus -0.6%), and total oil (-0.4% vs. -0.1%) than the *fas<sub>nc</sub>* 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<sub>nc</sub>* allele. Both mutations had a similar effect on palmitic acid (-0.3%). R-square values for the *SACPD-C* locus are indicated in Table 4. The *SACPD-C* deletion/deletion genotype resulted in mean stearic acid of 12.2%, compared to the homozygous *fas<sub>nc</sub>* average of 7.7% and the wild-type/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<sub>nc</sub>* allele had a larger additive effect on stearic acid (+3.3%; Table 7) and oleic acid (-3.3%) in the HSHO-1 population than in the FA-I/FA-J populations (+1.8%, -1.1%). The *fas<sub>nc</sub>* 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%; Table 8), stearic, (+3.8%) and linolenic acid (+0.2%) in the HSHO-2 population 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 for palmitic (-1.2% additive vs -0.9%, +0.5% dominant vs not significant), oleic (+13.2% additive 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 epistasis 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 epistasis was observed in either population between *SACPD-C* and *FAD2-1A* nor *FAD2-1B*.

In the HSHO-1 population, the *SACPD-C* *fas<sub>nc</sub>*/*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<sub>nc</sub>*/*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 found to not be significantly different while 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<sub>nc</sub>*/*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 but the oleic acid levels were significantly different. 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.

## DISCUSSION

Two new alleles were identified at the *SACPD-C* locus, which shall be designated *fas<sub>ncdel1</sub>* and *fas<sub>ncdel2</sub>*. Both alleles represented a deleted region on chromosome 14 of *G. max*, encompassing the *SACPD-C* locus. The deletions were found to have over twice the additive effect on seed stearic acid composition compared to the single nucleotide mutation present in the *fas<sub>nc</sub>* 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<sub>nc</sub>* 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. 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, as the original source ‘Holladay’ is a high yielding maturity group V cultivar. While a previous study (Ruddle, 2013) observed no deleterious effects associated with a partial loss of function *SACPD-C* allele, *fas<sub>nc</sub>*, it will be necessary to determine what, if any, agronomic effects are associated with these *SACPD-C* deletions.

The previously observed multiplicative effect of high stearic alleles at the *SACPD-C* locus (Zhang et al. 2008) in a higher than normal oleic background was confirmed. The *fas<sub>nc</sub>* allele had nearly twice the additive effect on seed stearic acid in the [FAM94-41-3 x N98-4445A] x S09-2902-145 (HSHO-1) population than in the FAM94-41-3 x TCHM08-1087 (FA-I) and FAM94-41-3 x TCHM08-755 (FA-J) populations; however, the *fas<sub>ncdel1</sub>* allele had similar additive effects on stearic acid in both the TCHM08-1087-11 x S09-2902-145 (HSHO-2) population and the FA-I population. Also, 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<sub>nc</sub>* 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 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 depress 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 while 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<sub>nc</sub>* with unknown interacting locus, nor *fas<sub>ncdel1</sub>* alleles 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 of stearic acid. It would also be of benefit 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%.

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

Primer	Fluor	Direction	Sequence (5'->3')
<i>FAD2-1A</i> snp 354 Wild type	FAM	Forward	GAAGGTGACCAAGTTCATGCTAGTGTGGTCACCATGCCTTCAG
<i>FAD2-1A</i> snp 354 Mutant	VIC	Forward	GAAGGTCCGAGTCAACGGATTGAGTGTGGTCACCATGCCTTCAA
<i>FAD2-1A</i> snp 354 Common	none	Reverse	CACAACATCATCAACCCATTGGTA
<i>FAD2-1B</i> snp 410 Wild type	FAM	Forward	GAAGGTGACCAAGTTCATGCTGTTCACCTCAGCACTTTTAGTCCC
<i>FAD2-1B</i> snp 410 Mutant	VIC	Forward	GAAGGTCCGAGTCAACGGATTGTTCACCTCAGCACTTTTAGTCCG
<i>FAD2-1B</i> snp 410 Common	none	Reverse	CGATGGCTTRTTTTCCATGAGA

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 (basepairs)	End (basepairs)	TCHM08-1087	TCHM08-755
Satt601	27131235	27131413	Present	Present
Satt318	28809608	28809872	Present	Present
Satt474	33076539	33076797	Absent	Absent
Sat_189	33180317	33180429	Absent	Absent
Satt070	34228964	34229137	Absent	Absent
<i>SACPD-C</i>	34322853	34325575	Absent	Absent
Satt122	34445176	34445301	Absent	Absent
Satt556	39579290	39579452	Present	Absent
Satt020	42022252	42022366	Present	Present

Table 3. Segregation ratios for *SACPD-C* among F<sub>2</sub>-derived lines in the FAM94-41-3 (*SACPD-C* allele: *fas<sub>nc</sub>*) x TCHM08-1087 (*SACPD-C* deletion/wild-type) (FA-I) and FAM94-41-3 x TCHM08-755 (*SACPD-C* deletion/wild-type) (FA-J) populations

Population	FAM-94-41-3 genotype	Heterozygous	TCHM08-1087/755 genotype	Total	$\chi^2$ (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

Table 4. Additive effect estimates (% of total fatty acid) and R<sup>2</sup> associated with the *SACPD-C* alleles *fas<sub>nc</sub>* and a *SACPD-C* deletion in the four environment combined analysis of the F<sub>2</sub>-derived populations FAM94-41-3 (*SACPD-C* allele: *fas<sub>nc</sub>*) x TCHM08-1087 (*SACPD-C* deletion/wild-type) and FAM94-41-3 x TCHM08-755 (*SACPD-C* deletion/wild-type)

Gene and Parameter Estimated	Palmitate	Stearate	Oleic	Linoleate	Linolenate	Total Oil
<i>SACPD-C</i> deletion Additive Effect	-0.3***	4.1***	-2.0***	-1.9***	0.2*	-0.4***
<i>SACPD-C fas<sub>nc</sub></i> Additive Effect	-0.3***	1.8***	-1.1***	-0.6**	NS	-0.1*
<i>SACPD-C</i> R <sup>2</sup>	0.12	0.74	0.32	0.40	0.04	0.22
*, **, ***Estimate significance, p<.05, p<.01, p<.001						

Table 5. Fatty acid and total oil least squares means (%) for each genotypic class in the four environment combined analysis of the F<sub>2</sub>-derived populations FAM94-41-3 (*SACPD-C* allele: *fas<sub>nc</sub>*) x TCHM08-1087 (*SACPD-C* deletion/wild-type) and FAM94-41-3 x TCHM08-755 (*SACPD-C* deletion/wild-type)

Genotypic Class	Palmitate Mean	Stearate Mean	Oleate Mean	Linoleate Mean	Linolenate Mean	Total Oil Mean
<i>SACPD-C</i> deletion/deletion	11.0	12.2***	17.7***	50.6***	8.4*	19.8***
<i>SACPD-C</i> deletion/ <i>fas<sub>nc</sub></i>	11.1	9.4***	18.9***	52.5***	8.1	20.1***
<i>SACPD-C fas<sub>nc</sub>/fas<sub>nc</sub></i>	11.1	7.7	19.6	53.4	8.1	20.4
<i>SACPD-C fas<sub>nc</sub>/wild type</i>	11.3***	5.6***	20.9***	54.2***	8.0	20.5
<i>SACPD-C</i> wild type/wild type	11.7***	4.0***	21.8***	54.5*	8.0	20.6

\*, \*\*, \*\*\*Mean different from *SACPD-C fas<sub>ns</sub>/fas<sub>ns</sub>* for a Dunnett pairwise comparison, p<.05, p<.01, p<.001

Table 6. Segregation ratios for *SACPD-C*, *FAD2-1A*, and *FAD2-1B* among F<sub>2</sub> seed in the S09-2902-145 x LLL-05-14 (HSHO-1) and S09-2902-145 x TCHM08-1087-11 (HSHO-2) populations

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

Table 7. Additive, dominant, and additive by additive effect estimates (% of total fatty acid) and  $R^2$  associated with *SACPD-C* allele *fas<sub>nc</sub>*, *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<sub>nc</sub>*) x S09-2902-145 (*FAD2-1A* SNP 254, *FAD2-1B* SNP 410) (HSHO-1)

Gene and Parameter Estimated	Palmitate	Stearate	Oleate	Linoleate	Linolenate
<i>SACPD-C fas<sub>nc</sub></i> Additive Effect	-0.4***	3.3***	-3.3***	NS	NS
<i>SACPD-C fas<sub>nc</sub></i> Dominant Effect	NS	-0.9***	1.8*	NS	NS
<i>FAD2-1A</i> SNP 254 Additive Effect	-0.9***	NS	9.0 ***	-7.5***	-0.3*
<i>FAD2-1A</i> SNP 254 Dominant Effect	NS	NS	-2.0*	1.5*	0.3**
<i>FAD2-1B</i> SNP 410 Additive Effect	-0.5***	NS	7.7***	-7.0***	-0.2*
<i>FAD2-1B</i> SNP 410 Dominant Effect	NS	NS	-2.2***	1.8*	0.5***
<i>SACPD-C</i> x <i>FAD2-1A</i> Additive x Additive Effect	NS	NS	NS	NS	NS
<i>SACPD-C</i> x <i>FAD2-1B</i> Additive x Additive Effect	NS	NS	NS	NS	NS
<i>FAD2-1A</i> x <i>FAD2-1B</i> Additive x Additive Effect	-2.0***	NS	20.3***	-17.1***	-1.8**
<i>SACPD-C</i> $R^2$	0.02	0.32	0.02	<0.01	<0.01
<i>FAD2-1A</i> $R^2$	0.08	<0.01	0.13	0.12	0.02
<i>FAD2-1B</i> $R^2$	0.02	<0.01	0.10	0.11	0.03
<i>FAD2-1A</i> * <i>FAD2-1B</i> $R^2$	0.02	<0.01	0.03	0.03	0.06

\*, \*\*, \*\*\* Estimate significance, p<.05, p<.01, p<.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) x S09-2902-145 (*FAD2-1A* SNP 254, *FAD2-1B* SNP 410) (HSHO-2)

Gene and Parameter Estimated	Palmitate	Stearate	Oleate	Linoleate	Linolenate
<i>SACPD-C fas<sub>nc</sub></i> Additive Effect	-0.4***	3.8***	-3.2***	NS	0.2*
<i>SACPD-C fas<sub>nc</sub></i> Dominant Effect	0.3**	-2.2***	2.1***	NS	NS
<i>FAD2-1A</i> SNP 254 Additive Effect	-1.2***	NS	13.2***	-11.4***	-0.6***
<i>FAD2-1A</i> SNP 254 Dominant Effect	0.5***	0.4*	-6.9***	5.6***	0.5***
<i>FAD2-1B</i> SNP 410 Additive Effect	-0.9***	NS	11.2***	-9.7***	-0.6***
<i>FAD2-1B</i> SNP 410 Dominant Effect	0.5***	NS	-4.7***	3.6***	0.6***
<i>SACPD-C</i> x <i>FAD2-1A</i> Additive x Additive Effect	NS	NS	NS	NS	NS
<i>SACPD-C</i> x <i>FAD2-1B</i> Additive x Additive Effect	NS	NS	NS	NS	NS
<i>FAD2-1A</i> x <i>FAD2-1B</i> Additive x Additive Effect	-3.6***	NS	39.5***	-31.2***	-4.8**
<i>SACPD-C</i> $R^2$	0.03	0.51	0.02	<0.01	0.01
<i>FAD2-1A</i> $R^2$	0.19	<0.01	0.31	0.32	0.06
<i>FAD2-1B</i> $R^2$	0.12	<0.01	0.23	0.24	0.07
<i>FAD2-1A</i> * <i>FAD2-1B</i> $R^2$	0.10	<0.01	0.12	0.11	0.15

\*, \*\*, \*\*\* Estimate significance, p<.05, p<.01, p<.001

Table 9. Fatty acid least square means (%) for the lines homozygous at the *SACPD-C*, *FAD2-1A*, and *FAD2-1B* loci in the F<sub>2</sub> population LLL-05-14 (*SACPD-C* allele: *fas<sub>nc</sub>*) x S09-2902-145 (*FAD2-1A* SNP 254, *FAD2-1B* SNP 410) (HSHO-1)

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

\*, \*\*, \*\*\*Mean different from Mutant/Wild Type/Wild Type for a Dunnett pairwise comparison, p<.05, p<.01, p<.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<sub>2</sub> population TCHM08-1087-11 (*SACPD-C* deletion) x S09-2902-145 (*FAD2-1A* SNP 254, *FAD2-1B* SNP 410) (HSHO-2)

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

\*, \*\*, \*\*\*Mean different from Mutant/Wild Type/Wild Type for a Dunnett pairwise comparison, p<.05, p<.01, p<.001