



# Mutations in SACP-D Result in a Range of Elevated Stearic Acid Concentration in Soybean Seed

Militza Carrero-Colón<sup>1</sup>, Nathan Abshire<sup>2</sup>, Daniel Sweeney<sup>2</sup>, Erik Gaskin<sup>2</sup>, Karen Hudson<sup>1\*</sup>

**1** Crop Production and Pest Control Research Unit, Agricultural Research Service (ARS), United States Department of Agriculture (USDA), West Lafayette, Indiana, United States of America, **2** Department of Agronomy, Purdue University, West Lafayette, Indiana, United States of America

## Abstract

Soybean oil has a wide variety of uses, and stearic acid, which is a relatively minor component of soybean oil is increasingly desired for both industrial and food applications. New soybean mutants containing high levels of the saturated fatty acid stearate in seeds were recently identified from a chemically mutagenized population. Six mutants ranged in stearate content from 6–14% stearic acid, which is 1.5 to 3 times the levels contained in wild-type seed of the Williams 82 cultivar. Candidate gene sequencing revealed that all of these lines carried amino acid substitutions in the gene encoding the delta-9-stearoyl-acyl-carrier protein desaturase enzyme (SACP-D) required for the conversion of stearic acid to oleic acid. Five of these missense mutations were in highly conserved residues clustered around the predicted di-iron center of the SACP-D enzyme. Co-segregation analysis demonstrated a positive association of the elevated stearate trait with the SACP-D mutation for three populations. These missense mutations may provide additional alleles that may be used in the development of new soybean cultivars with increased levels of stearic acid.

**Citation:** Carrero-Colón M, Abshire N, Sweeney D, Gaskin E, Hudson K (2014) Mutations in SACP-D Result in a Range of Elevated Stearic Acid Concentration in Soybean Seed. PLoS ONE 9(5): e97891. doi:10.1371/journal.pone.0097891

**Editor:** Girdhar K. Pandey, University of Delhi South Campus, India

**Received:** November 7, 2013; **Accepted:** April 24, 2014; **Published:** May 20, 2014

This is an open-access article, free of all copyright, and may be freely reproduced, distributed, transmitted, modified, built upon, or otherwise used by anyone for any lawful purpose. The work is made available under the Creative Commons CC0 public domain dedication.

**Funding:** Funding for this work was provided through the USDA-ARS Current Research Information System 3602-21000-006-00D. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

\* E-mail: hudson@ars.usda.gov

## Introduction

Stearic acid is one of the component fatty acids in soybean oil, comprising 2–4% of the total oil fraction. Stearic acid has a neutral effect on blood serum LDL cholesterol concentration and is therefore a desirable constituent of oils for food use [1]. Stearic acid confers a high melting temperature and oxidative stability to oils destined for end use in baking fats. Previously, to increase the proportion of stearic acid in soybean oil, the oil was subjected to hydrogenation. However, genetic manipulation of stearic acid level is more efficient and reduces the trans-fats that may be introduced by the hydrogenation process [2].

Three soybean genes have been characterized with homology to delta-9-stearoyl-acyl carrier protein desaturases (SACPDs) which are required for the conversion of stearic acid to oleic acid [3]. These genes are delimited SACPD-A, SACPD-B, and SACPD-C. SACPD-C encodes the seed-specific isoform of this enzyme, where SACPD-A and SACPD-B transcripts accumulate in all soybean tissues [1,4].

Soybeans with mutations in the SACPD-C and SACPD-B genes have been described. FAM94-41 is a spontaneously occurring change in the SACPD-C gene and results in plants with levels of stearic acid in the seed of ~9% [5]. Deletion of the SACPD-C gene in the A6 germplasm line results in up to 28% stearic acid in the seed, but the size of this deletion is uncharacterized [4,6]. Additional SACPD-C mutants have been described with a range of 10–16% stearic acid in the seeds [4,7]. SACPD-B mutants have recently been reported to contain ~10% stearic acid [8]. No mutations have been described for the SACPD-A gene. Some high stearate mutants have previously been associated with poor

germination and low seed yield [9,10] however recently it was demonstrated that missense mutations in *SACP-D* are not associated with poor agronomic characteristics [11]. Additional sources of germplasm carrying novel mutations in the *SACP-D* gene, or novel loci which influence seed stearic acid levels are needed to circumvent this issue to enable the production of soybeans with elevated levels of stearic acid to meet the demands of the food-oil market.

## Materials and Methods

### Plants and growth conditions and fatty acid analysis

For screening, plants were grown in the field in West Lafayette, Indiana, as described in reference [12]. Field location GPS coordinates are latitude 40.468 degrees north, longitude minus 86.991 degrees west. Soybeans described in this study are non-transgenic, therefore no specific permits were required for growth. Fatty acid composition analysis was performed as previously described [13].

### Sequencing and Genotyping

Three segments of the *SACP-D* (Glyma14g27990) coding region were amplified and sequenced using the primers in Table S1. DNA sample preparation for sequencing was performed using the CTAB method [14] and sample preparation for genotyping was as previously described [13]. dCAPS genotyping [15] was performed using standard protocols with the assays developed specifically for the *SACP-D* mutants provided in Table S1. To evaluate the position of substitutions, mutations were overlaid on

**Table 1.** Elevated stearic acid levels in *SACPD-C* mutants.

	Palmitic Acid (16:0)	Stearic Acid (18:0)	Oleic Acid (18:1)	Linoleic Acid (18:2)	Linolenic Acid (18:3)	n
W82	10.0±0.2	4±0.1	20.6±0.5	57.0±0.5	8.3±0.2	6
15073 <i>SACPD-C</i> <sub>G224E</sub>	8.1±0.4 <i>1.3e<sup>-6</sup>*</i>	12.1±1.2 <i>1.8e<sup>-8</sup>*</i>	15.9±0.6 <i>4.6e<sup>-8</sup>*</i>	55.0±1.3 <i>0.0055*</i>	8.8±0.6 <i>0.13</i>	6
14197 <i>SACPD-C</i> <sub>Y211C</sub>	8.8±0.4 <i>1.25e<sup>-4</sup>*</i>	12.1±3.5 <i>5.3e<sup>-4</sup>*</i>	20.0±2.9 <i>0.62</i>	51.6±0.7 <i>2.4e<sup>-6</sup>*</i>	7.6±0.6 <i>0.0044*</i>	3
18190 <i>SACPD-C</i> <sub>A218E</sub>	8.5±0.6 <i>4.2e<sup>-4</sup>*</i>	13.5±0.6 <i>1.8e<sup>-9</sup>*</i>	14.8±0.2 <i>2.6e<sup>-7</sup>*</i>	53.8±0.4 <i>1.8e<sup>-5</sup>*</i>	9.3±0.3 <i>5.0e<sup>-4</sup>*</i>	3
18948 <i>SACPD-C</i> <sub>H223R</sub>	10.1±0.5 <i>0.75</i>	9.5±1.3 <i>4.4e<sup>-6</sup>*</i>	17.4±0.6 <i>1.3e<sup>-5</sup>*</i>	54.9±1.1 <i>0.0034*</i>	8.1±0.4 <i>0.26</i>	4
18610 <i>SACPD-C</i> <sub>A239T</sub>	9.45±0.6 <i>0.032*</i>	6.1±1.2 <i>0.0021*</i>	22.2±1.2 <i>0.0093*</i>	54.1±2.0 <i>0.0057*</i>	8.1±0.9 <i>0.57</i>	6
21084 <i>SACPD-C</i> <sub>R329I</sub>	8.4±0.3 <i>0.79</i>	8.7±0.8 <i>5.3e<sup>-4</sup>*</i>	27.8±1.8 <i>0.016</i>	48.6±1.4 <i>0.51</i>	6.5±0.8 <i>0.77</i>	4

Fatty acid levels were averaged for n homozygous M<sub>4</sub> or M<sub>5</sub> lines for each mutation, and averages and standard deviations are shown. *p*-value (in italics) was calculated from a two-tailed, type 2 t-test for the average fatty acid level in each mutant relative to the Williams-82 (W82) wild type control. Single asterisk indicates *p*-values that are significant at the *p*<0.05 level.

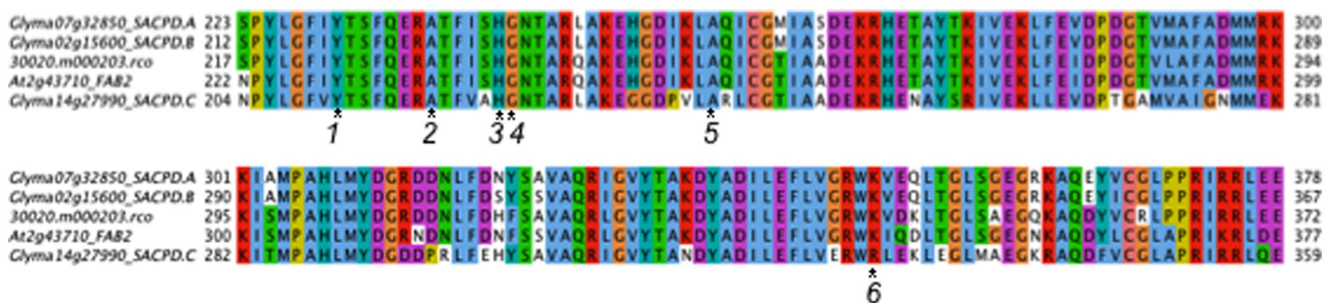
doi:10.1371/journal.pone.0097891.t001

the protein structure PDB ID 1AFR using the program Cn3D v. 4.3 [16]. Mutant *SACPD-C* sequences are deposited in GenBank with accession numbers KJ522450-KJ522455.

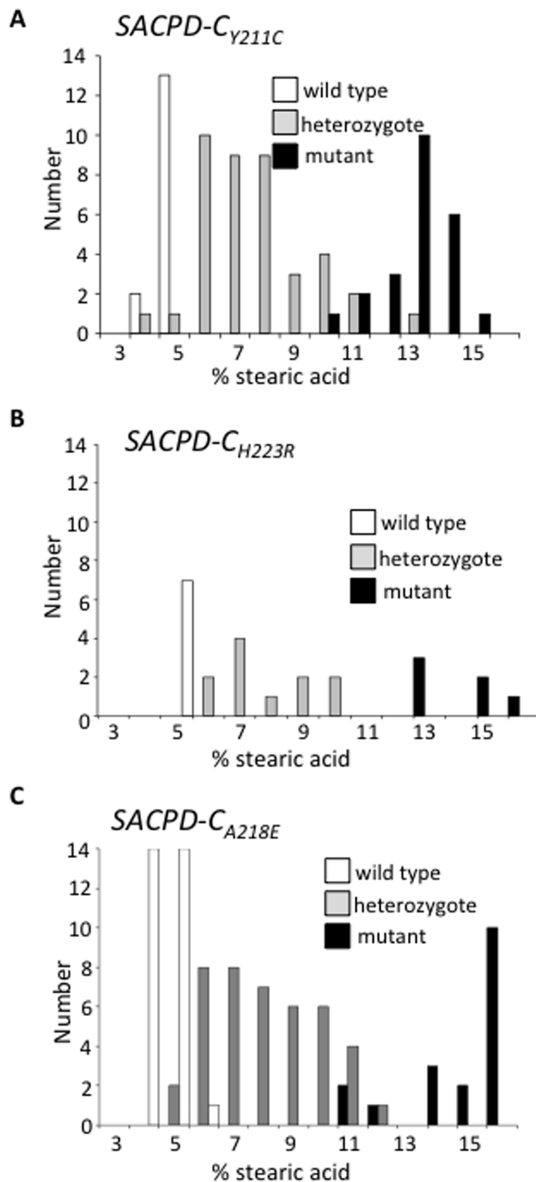
## Results and Discussion

Mutant plants with high levels of stearic acid in seeds were identified in an ongoing screen for soybean seed with altered fatty acid composition (reference [12], and unpublished data) and six lines were chosen for further characterization. These mutants were obtained from an NMU-mutagenized population in the Williams-82 genetic background [17]. Levels of stearic acid in the seed of the mutant lines ranged from 6–13% (Table 1), with the highest levels in line 18190. Line #18948 corresponds to line #14, line 18190 corresponds to #16, and line 18610 corresponds to #17 in reference [12] while the isolation of lines 15073, 14197, and 21084 has not been previously described. Line 14197 and 18948 were isolated as heterozygous M<sub>3</sub>, as revealed by segregating types of M<sub>4</sub> seed, while the remaining lines showed a consistent level of stearic acid across multiple M<sub>4</sub> individuals and were presumed to be homozygous isolates (Table S2 and [12]).

Sequencing of the *SACPD-C* gene from these mutant lines revealed that each carries a distinct and independent missense mutation in the coding region of *SACPD-C* (Figure 1 and Table 1). The sequence of the *SACPD-C* gene in the Williams-82 accession that serves as the germplasm background for this mutant is consistent with the previously described soybean *SACPD-C* sequence (Genbank #EF113911) [4] but not the Williams-82 sequence within the Phytozome database (version 9.1, www.phytozome.net) which carries a frameshift mutation early in the first exon. The exons of the *SACPD-A* and *SACPD-B* genes (Glyma07g32850 and Glyma02g15600, respectively) were also sequenced in these lines and were found to be identical to the Williams-82 sequence (not shown). As line 21084 and 18610 M<sub>3</sub> and M<sub>4</sub> individuals produced seed with a marginal increase in stearic acid content from ranging from 5–8%, to verify that these lines were homozygous the *SACPD-C* gene was sequenced from M<sub>4</sub> plants (Table S2). All of these M<sub>4</sub> individuals were homozygous for their respective mutations. This suggests that the mutations in these lines are less damaging to enzyme structure or function and the resultant plants display a marginal increase in stearic acid.



**Figure 1. Mutations in the *SACPD-C* enzyme.** Multiple sequence alignment for soybean *SACPD-C* with close sequence homologs showing the position of mutations in conserved locations. Other proteins shown are *SACPD-A* and *SACPD-B* from soybean, *FAB2* from Arabidopsis (At3g02610/DES2), and the castor bean *SACPD* (30020.m000203.rc0). Mutations described in this work are located at the following positions: 1. *SACPD-C*<sub>Y211C</sub> 2. *SACPD-C*<sub>A218E</sub> 3. *SACPD-C*<sub>H223R</sub> 4. *SACPD-C*<sub>G224E</sub> 5. *SACPD-C*<sub>A239T</sub> 6. *SACPD-C*<sub>R329I</sub>. doi:10.1371/journal.pone.0097891.g001



**Figure 2. Cosegregation of SACPDC mutations and elevated stearic acid phenotype.** Individuals from segregating populations were analyzed for stearic acid content and genotyped for A. SACPDC<sub>Y211C</sub> (78 individuals) B. SACPDC<sub>H223R</sub> (24 individuals), and C. SACPDC<sub>A218E</sub> (89 individuals). White bars indicate wild-type individuals, grey bars indicate heterozygous individuals, and black bars indicate homozygous mutants. doi:10.1371/journal.pone.0097891.g002

Visualization of the location of the affected amino acids in the determined crystal structure for the SACPDC enzyme from *Ricinus communis* reveals that five of the six mutations are located in the enzyme channel domain which is composed of several alpha helices, the function of this channel is to position the hydrocarbon chain of the fatty acid so that it can be oxidized (Figure S1) [18]. SACPDC<sub>Y211C</sub>, SACPDC<sub>A218E</sub>, SACPDC<sub>G224E</sub>, SACPDC<sub>H223R</sub> are located on alpha helix six, and SACPDC<sub>A239T</sub> is located on alpha helix seven. The amino acid substitutions introduce either changes in side chain charge or size into this region of the enzyme. These mutations may reduce the ability of a fatty acid chain to be correctly positioned within the channel

domain of the enzyme. Proximity of mutations to the di-iron core and increases in polarity also correlate with greater reduction of enzymatic function [18]. SACPDC<sub>R329I</sub> is located on a loop residue after helix ten, and this mutation results in a less dramatic increase in stearic acid level. We speculate that this residue may be involved in correct folding of the active site or possibly in interaction with other proteins.

To demonstrate that changes in the SACPDC-C gene cause the elevated stearic acid phenotype in these mutant lines, cosegregation analysis was performed in segregating populations. In each case the single nucleotide polymorphism introduced by the mutation was used to develop a codominant dCAPs marker. Lines 18948 (SACPDC<sub>H223R</sub>) and 14197 (SACPDC<sub>Y211C</sub>) were isolated as heterozygous mutants for SACPDC-C mutations. M<sub>4</sub> plants segregating for these SNPs were grown in the field during the 2011 growing season, and M<sub>5</sub> seed was genotyped for the SNP and phenotyped for fatty acid content. Figure 2A shows association of the SACPDC<sub>Y211C</sub> mutation with elevated levels of stearic acid in the seed. Figure 2B shows cosegregation of SACPDC<sub>H223R</sub> with elevated levels of stearic acid in the seed. Line 18190 (SACPDC<sub>A218E</sub>), which has the highest levels of stearate, was crossed to the cultivar Prize, allowed to self-pollinate, and F<sub>2</sub> plants were grown in the field (during the 2012 growing season) and genotyped. F<sub>3</sub> seed from F<sub>2</sub> individuals was analyzed for stearic acid content. Figure 2C shows the cosegregation of SACPDC<sub>A218E</sub> with elevated levels of stearate. In the SACPDC<sub>A218E</sub> x Prize segregating population, a number of homozygous mutant individuals were observed which contained stearic acid levels greater (>20% stearate) than those observed in the homozygous mutant isolate in the Williams-82 background (approximately 13%). This may be due to the segregation of a second, modifying locus distinct from that of the mutated SACPDC-C gene in the SACPDC<sub>A218E</sub> line. This genetic modifier may be in the outcross parent (Prize) genetic background, or it may be a second-site mutation in the heavily mutagenized background of the SACPDC<sub>A218E</sub> parent line. It is estimated that the mutation frequency in the parent of SACPDC<sub>A218E</sub> parent is on the order of 100 genic mutations per individual [17]. The seed stearate level in the Prize parent grown in parallel was 4%, similar to Williams-82. As the other SACPDC genes would be good candidates to act additively with SACPDC<sub>A218E</sub>, the SACPDC-A, -B, (and -C) genes were sequenced in the Prize parent and found to encode predicted proteins with an amino acid sequence identical to the published reference sequence [4].

There is evidence that other, unknown loci in addition to SACPDC-C impact stearic acid levels in soybean seed [8]. The strong mutations in SACPDC-C described here are comparable to the defects observed in RG7 and RG8 which are also chemically induced point mutants in SACPDC-C [7] and may therefore serve as a basis for germplasm to enhance stearic acid levels. Weaker alleles such as SACPDC<sub>R329I</sub> and SACPDC<sub>A239T</sub> may be useful when combined with other genes to increase stearic acid while maintaining high oleic acid levels. In addition, identification of the factors that enable the >20% stearic acid levels observed in association with SACPDC<sub>A218E</sub> may suggest to further increase stearic acid levels for a soybean oil with enhanced functionality.

## Supporting Information

### Figure S1 Position of mutations in SACPDC-C crystal structure.

(TIFF)

### Table S1 Primer sequences.

(PDF)

**Table S2 M<sub>3</sub>/M<sub>4</sub> phenotypes and genotypes.**  
(PDF)

## Acknowledgments

Disclaimer: Product names are necessary to report factually on available data. However the USDA neither guarantees nor warrants the standard of the product, and use of the names implies no approval of the product to the exclusion of others that may also be suitable.

## References

1. Byfield GE, Xue H, Upchurch RG (2006) Two Genes from Soybean Encoding Soluble  $\Delta 9$  Stearoyl-ACP Desaturases. *Crop Sci* 46: 840–846.
2. Clemente TE, Cahoon EB (2009) Soybean Oil: Genetic Approaches for Modification of Functionality and Total Content. *Plant Physiology* 151: 1030–1040.
3. Ohlrogge J, Browse J (1995) Lipid biosynthesis. *Plant Cell* 7: 957–970.
4. Zhang P, Burton JW, Upchurch RG, Whittle E, Shanklin J, et al. (2008) Mutations in a Delta(9)-Stearoyl-ACP-Desaturase Gene Are Associated with Enhanced Stearic Acid Levels in Soybean Seeds. *Crop Science* 48: 2305–2313.
5. Pantalone VR, Wilson RF, Novitzky WP, Burton JW (2002) Genetic regulation of elevated stearic acid concentration in soybean oil. *Journal of the American Oil Chemists Society* 79: 549–553.
6. Hammond EG, Fehr WR (1983) Registration of A6-Germplasm Line of Soybean. *Crop Science* 23: 192–193.
7. Boersma JG, Gillman JD, Bilyeu KD, Ablett GR, Grainger C, et al. (2012) New Mutations in a Delta-9-Stearoyl-Acyl Carrier Protein Desaturase Gene Associated with Enhanced Stearic Acid Levels in Soybean Seed. *Crop Sci* 52: 1736–1742.
8. Ruddle P, 2nd, Whetten R, Cardinal A, Upchurch RG, Miranda L (2013) Effect of a novel mutation in a Delta9-stearoyl-ACP-desaturase on soybean seed oil composition. *Theor Appl Genet* 126: 241–249.
9. Lundeen PO, Fehr WR, Hammond EG, Cianzio SR (1987) Association of Alleles for High Stearic-Acid with Agronomic Characters of Soybean. *Crop Science* 27: 1102–1105.
10. Rahman SM, Takagi Y, Kinoshita T (1997) Genetic control of high stearic acid content in seed oil of two soybean mutants. *Theoretical and Applied Genetics* 95: 772–776.
11. Ruddle P, Cardinal A, Upchurch RG, Arellano C, Miranda L (2013) Agronomic Effects of Mutations in Two Soybean  $\Delta 9$ -Stearoyl-Acyl Carrier Protein-Desaturases. *Crop Sci* 53: 1887–1893.
12. Hudson K (2012) Soybean Oil-Quality Variants Identified by Large-Scale Mutagenesis. *International Journal of Agronomy* 2012: Article ID 569817, 7.
13. Head K, Galos T, Fang Y, Hudson K (2012) Mutations in the soybean 3-ketoacyl-ACP synthase gene are correlated with high levels of seed palmitic acid. *Molecular Breeding* 30: 1519–1523.
14. Richards E, Reichardt M, Rogers S (2001) Preparation of Genomic DNA from Plant Tissue. *Current Protocols in Molecular Biology*: John Wiley & Sons, Inc.
15. Neff MM, Neff JD, Chory J, Pepper AE (1998) dCAPS, a simple technique for the genetic analysis of single nucleotide polymorphisms: experimental applications in *Arabidopsis thaliana* genetics. *Plant J* 14: 387–392.
16. CN3D <http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml>
17. Cooper JL, Till BJ, Laport RG, Darlow MC, Kleffner JM, et al. (2008) TILLING to detect induced mutations in soybean. *BMC Plant Biol* 8: 9.
18. Lindqvist Y, Huang W, Schneider G, Shanklin J (1996) Crystal structure of delta9 stearoyl-acyl carrier protein desaturase from castor seed and its relationship to other di-iron proteins. *EMBO J* 15: 4081–4092.

The authors wish to thank Dr. K Bilyeu, USDA-ARS, Columbia, MO and the Functional Foods Research Unit (USDA-ARS, Peoria, IL) for performing fatty acid analysis.

## Author Contributions

Conceived and designed the experiments: KH. Performed the experiments: MC NA DS EG. Analyzed the data: MC KH DS NA. Contributed reagents/materials/analysis tools: KH. Wrote the paper: KH NA.