INVESTIGATION

Genotyping-by-Sequencing-Based Investigation of the Genetic Architecture Responsible for a Sevenfold Increase in Soybean Seed Stearic Acid

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ABSTRACT Soybean oil is highly unsaturated but oxidatively unstable, rendering it nonideal for food applications. Until recently, the majority of soybean oil underwent partial chemical hydrogenation, which produces trans fats as an unavoidable consequence. Dietary intake of trans fats and most saturated fats are conclusively linked to negative impacts on cholesterol levels and cardiovascular health. Two major soybean oil breeding targets are: (1) to reduce or eliminate the need for chemical hydrogenation, and (2) to replace the functional properties of partially hydrogenated soybean oil. One potential solution is the elevation of seed stearic acid, a saturated fat which has no negative impacts on cardiovascular health, from 3 to 4% in typical cultivars to > 20% of the seed oil. We performed QTL analysis of a population developed by crossing two mutant lines, one with a missense mutation affecting a stearoyl-acyl-carrier protein desaturase gene resulting in ~11% seed stearic acid crossed to another mutant, A6, which has 24–28% seed stearic acid. Genotyping-by-sequencing (GBS)-based QTL mapping identified 21 minor and major effect QTL for six seed oil related traits and plant height. The inheritance of a large genomic deletion affecting chromosome 14 is the basis for largest effect QTL, resulting in ~18% seed stearic acid. This deletion contains SACPD-C and another gene(s); loss of both genes boosts seed stearic acid levels to ≥ 18%. Unfortunately, this genomic deletion has been shown in previous studies to be inextricably correlated with reduced seed yield. Our results will help inform and guide ongoing breeding efforts to improve soybean oil oxidative stability.

KEYWORDS Glycine max soybean fatty acid composition genotyping-by-sequencing QTL mapping

Soybean [Glycine max (L.) Merr.] is the source of the most widely consumed edible oil in the US, representing 55% of total oil consumption (http://soystats.com/2015-soystats/). Soybean oil and its derivatives are ubiquitous ingredients in packaged foods, especially in cookies and other snack foods (Van Camp et al. 2012). Soybean oil from a "typical" cultivar is comprised of five main fatty acids: palmitic, stearic, oleic, linoleic, and linolenic (11, 3–4, 25, 52, and 8%, respectively) (Fehr 2007). Due to the high proportion of polyunsaturated fatty acids, which are oxidatively unstable (Frankel 1991), the majority of soybean oil is chemically hydrogenated in order to increase stability and shelf life. Unfortunately, the process of hydrogenation produces trans fats, which have been tied to increased risk of heart disease and elevated low-density lipoprotein (LDL) levels in blood serum (Hunter et al. 2009). In 2003, the Food and Drug Administration (FDA) mandated labeling of packaged foods containing trans fats in amounts exceeding 0.5 g per serving (Van Camp et al. 2012), culminating in the removal of the “Generally Regarded As Safe” status for partially hydrogenated foods, due to the presence of trans fats. (http://www.fda.gov/Food/IngredientsPackagingLabeling/FoodAdditivesIngredients/ucm449162.htm accessed 08-24-2016).

For these reasons, a major soybean breeding target has been modification of soybean oil composition, so as to reduce or eliminate the need for chemical hydrogenation. Achievement of this goal would potentially translate to decreased trans fat intake for consumers, as well as fewer steps in processing for manufacturers. Biotechnological and traditional breeding have independently increased the oleic acid content of soybean oil and thereby increased oxidative stability (Bilyeu et al. 2011; Buhr et al. 2002; Pham et al. 2010). Soybean oil high in oleic acid (> 70%) has been shown to be more chemically stable than typical soybean oil (Warner and Gupta 2005). However, both conventional and high oleic acid
soybean oil have very low melting points, and for many applications fully hydrogenated oil is more suitable (Ribeiro et al. 2009). For instance, edible oil high in saturated fats is desirable for solid fat baking applications (Renzyaeva 2013; Tarancón et al. 2013).

Interestingly, new research has shown that stearic acid (C18:0), a long-chain saturated fatty acid, does not have the same cholesterolemic effects in humans as shorter acyl-chain saturated fats (Hunter et al. 1995); it is effectively “heart-neutral.” Unfortunately, soybean oil generally contains only ~3–4% stearic acid (Wilson 2004), and >20% must be achieved to meet current market demands (K. Whiting, Smith-Bucklin/United Soybean Board, personal communication).

Elevated stearic acid is an apparently rare trait; out of 21,849 USDA-GRIN soybean collection entries that have been evaluated for oil composition, only a single entry has stearic acid > 9% (https://npgswb.ars-grin.gov/gringlobal/descriptors.aspx, accessed 08-19-2016). It is known that soybean stearic acid content is determined largely by alterations in the Stearoyl-Acyl Carrier Protein Desaturase-C (SACPD-C) gene (Glyma14g27990). SACPD-C is a soluble enzyme that selectively desaturates stearic acid precursors (C18:0) to oleic acid precursors (C18:1) (Zhang et al. 2008). It has been previously reported that SACPD-C is a major determinant of seed stearic acid levels (Ruddle et al. 2013a,b, 2012; Gillman et al. 2014).

To date, there is only one known naturally occurring source of elevated stearic acid, FAM94-41, which contains a missense mutation in SACPD-C and ~13% seed stearic acid (Zhang et al. 2008). All other lines with elevated stearic acid are due to mutagenesis (Bolon et al. 2011; Gillman et al. 2014; Rahman et al. 1995, 1997). The majority of lines contain 8–12.5% seed stearic acid, but one notable exception is mutant line A6, which has been reported to have arisen by sodium azide-induced mutagenesis (Hammond and Fehr 1983), but which contains several large genomic deletions more consistent with radiation-induced mutagenesis. A6 seeds exhibit a dramatic increase in seed stearic acid levels (~24–28%, as compared to the wild-type 4%). A6 bears several large genomic deletions, including a large portion of chromosome 14 (~6,221,000 bp) corresponding to ~1/8 of the chromosome (Gillman et al. 2014). Regrettably, A6 is also extremely agronomically deficient, exhibiting poor germination, low seed yield, reduced seed quality, extreme early maturity (MG 0), and short plant stature when alleles for elevated stearic acid are present (Lundeen et al. 1987; Hammond and Fehr 1983). Some of the defects are likely due to one (or more) of the significant genomic deletions (Gillman et al. 2014).

We identified multiple SACPD-C deletion and missense mutation lines from multiple genetic backgrounds and, except for line A6, loss of functional SACPD-C per se can only elevate seed stearic acid levels to ~13%, regardless of mutation or deletion (Gillman et al. 2014). The identification of multiple mutants with genetically controlled variation in elevated stearic acid content presented an opportunity to investigate the genetic basis for the extremely elevated stearic acid content (~24%) in line A6. To that end, a recombinant inbred line (RIL) population was developed by crossing a line with ~11% stearic acid (194D) to a line with ~24–28% stearic acid (A6). We then employed a GBS method (Elshire et al. 2011), which is routine in maize, but remains less utilized in soybean. GBS is a simple, repeatable, and robust method for identifying single nucleotide polymorphisms (SNPs) via sequencing of reduced representation libraries produced with methylation-sensitive restriction enzymes, which bias against highly repetitive and gene-poor genomic regions (Poland et al. 2012).

A dense genetic linkage map was constructed from 2977 high-quality GBS markers. Using phenotypic data from 173 RILs over three

| Chromosome | Marker # | Length (cM) | Average Spacing (cM) | Maximum Spacing (cM) |
|------------|----------|-------------|----------------------|----------------------|
| 1          | 123      | 67.5        | 0.6                  | 15.5                  |
| 2          | 190      | 131.8       | 0.7                  | 13.1                  |
| 3          | 115      | 125.7       | 1.1                  | 73.7                  |
| 4          | 197      | 136.0       | 0.7                  | 52.5                  |
| 5          | 145      | 117.8       | 0.8                  | 19.2                  |
| 6          | 233      | 141.1       | 0.6                  | 16.4                  |
| 7          | 61       | 66.0        | 1.1                  | 18.0                  |
| 8          | 95       | 162.7       | 1.7                  | 51.0                  |
| 9          | 196      | 62.6        | 0.3                  | 6.9                   |
| 10         | 219      | 122.6       | 0.6                  | 12.7                  |
| 11         | 13     | 111.9       | 3.0                  | 65.6                  |
| 12         | 49       | 125.5       | 2.6                  | 62.0                  |
| 13         | 180      | 126.2       | 0.7                  | 33.0                  |
| 14         | 151      | 85.9        | 0.6                  | 30.3                  |
| 15         | 203      | 107.4       | 0.5                  | 25.9                  |
| 16         | 102      | 118.8       | 1.2                  | 62.0                  |
| 17         | 165      | 45.3        | 0.3                  | 8.1                   |
| 18         | 190      | 126.0       | 0.7                  | 49.8                  |
| 19         | 191      | 105.4       | 0.6                  | 27.8                  |
| 20         | 134      | 115.2       | 0.9                  | 16.9                  |
| Overall    | 2977     | 2201.4      | 0.7                  | 73.7                  |

| Trait          | Source of Variation | MS   | F   | h² |
|----------------|---------------------|------|-----|----|
| Plant height   | Location            | 16050.15 | 84.85 | *** | 0.54 |
|                | Line                | 387.27   | 2.05 | *** |
|                | Location × line     | 208.22   | 1.10 | *** |
|                | Rep                 | 874.84   | 4.62 | **  |
|                | Error               | 189.16   |      |     |
| Total seed oil | Location            | 146.55   | 88.55 | **  | 0.28 |
|                | Line                | 3.07     | 1.86 | *** |
|                | Location × line     | 2.57     | 1.55 | *** |
|                | Rep                 | 1.97     | 1.19 | *** |
|                | Error               | 1.66     |      |     |
| Palmitic       | Location            | 12.83    | 34.25 | *** | 0.89 |
|                | Line                | 2.39     | 6.38 | *** |
|                | Location × line     | 0.33     | 0.89 | *** |
|                | Rep                 | 0.17     | 0.46 | *** |
|                | Error               | 0.38     |      |     |
| Stearic        | Location            | 300.94   | 48.50 | *** | 0.88 |
|                | Line                | 58.78    | 9.47  | *** |
|                | Location × line     | 9.37     | 1.51  | *** |
|                | Rep                 | 0.84     | 0.14  | *** |
|                | Error               | 6.20     |      |     |
| Oleic          | Location            | 1278.44  | 216.52 | *** | 0.81 |
|                | Line                | 42.49    | 7.20  | *** |
|                | Location × line     | 9.95     | 1.69  | *** |
|                | Rep                 | 15.35    | 2.60  | *** |
|                | Error               | 5.90     |      |     |
| Linoleic       | Location            | 925.85   | 112.96 | *** | 0.73 |
|                | Line                | 53.09    | 6.48  | *** |
|                | Location × line     | 16.93    | 2.07  | *** |
|                | Rep                 | 10.57    | 1.29  | *** |
|                | Error               | 8.20     |      |     |
| Linolenic      | Location            | 337.56   | 666.36 | *** | 0.72 |
|                | Line                | 2.27     | 4.48  | *** |
|                | Location × line     | 0.79     | 1.55  | *** |
|                | Rep                 | 1.31     | 2.58  | *** |
|                | Error               | 0.51     |      |     |

* P < 0.05; ** P < 0.01; *** P < 0.001. MS, mean sum of squares; F, value; h², broad sense heritability.
environments, 21 QTL were identified for seven traits in soybean, including four QTL for seed stearic acid content.

MATERIALS AND METHODS

Plant material and population development

Two RIL populations were developed, derived from the cross between A6 and 194D (designated population 2), and the reciprocal cross between 194D and A6 (designated population 3). Population 2 consisted of 100 individuals in 2014 and 2015, and population 3 consisted of 95 individuals in 2014 and 94 individuals in 2015. Walter Fehr generously provided seeds from the high stearic acid line, A6. 194D is a midstearic line produced seeds from the high stearic acid line, A6. 194D is a midstearic line (dimension 97 · 55 mm). The NIRS reflectance (R) spectra were collected at 2 nm intervals in the NIRS region of 400–2500 nm at room temperature. Calibrations previously developed by FOSS were used to estimate moisture content. In 200 samples from multiple plots stored in this manner, seed moisture content was found to be minimally variable (7.7 ± 0.4% SD).

Seed samples were also analyzed for seed fatty acid composition using the derivatized lipid gas chromatography method (Bilyeu et al. 2011). In 2014, two samples of three seeds each per plot were analyzed, and in 2015, four or five individual seeds per plot were analyzed. Seeds were crushed then extracted in 1 ml chloroform-hexane-methanol (8:5:2, v/v/v) overnight. Derivatization of 150 µl extracted oils was done with 75 µl methylating reagent (0.25 M methanolic sodium methoxide-petroleum ether-ethyl ether, 1:5:2, v/v/v). Seed samples were then diluted with hexane to 1 ml. An Agilent (Palo Alto, CA) series 6890 capillary gas chromatograph fitted with a flame ionization detector (275°C) was used with an AT-Silar capillary column (Alltech Associates, Deerfield, IL). Standard fatty acid mixtures (Animal and Vegetable Oil Reference Mixture 6, AOACS) were used as reference standards.

Quantification of field traits

Plant height was measured in centimeters on three separate plants within each plot (three plots per genotype) when 95% of the pods showed mature pod color.
Parental allele assignment and imputation were performed using the DNeasy Plant Mini kit (QIAGEN, Valencia, CA) according to the manufacturer’s instructions. DNA samples from 190 lines from populations 2 and 3 were submitted to the Institute for Genomic Diversity (IGD) at Cornell University. GBS libraries were prepared as previously described (Elshire et al. 2011; Swarts et al. 2014) using the ApeKI enzyme, DNA ligase, and appropriate Illumina adapters. The IGD staff performed the library construction, read mapping, and downstream SNP calls. A total of 455,924,779 reads were produced, 428,671,333 of which were used for downstream analysis. A total of 61.5% of the reads were successfully aligned to unique positions in the Wm82.a2.v1 Williams 82 reference sequence (Schmutz et al. 2010, http://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Gmax) using the BWA 0.7.8-r455 program (Li and Durbin 2009). SNPs were called using the TASSEL 5.0 pipeline (Bradbury et al. 2007) resulting in 33,728,018 tags for all 190 samples.

A total of 27,672 SNPs were identified, with an average SNP coverage of 10.98-fold. SNPs were filtered using TASSEL 5.0 to exclude those with > 20% missing data, and to restrict allele frequency to between 0.2 and 0.8, bringing the SNP count down to 5423. Lastly, a chi-square test was performed to remove any severely distorted markers (at F45), resulting in a final count of 2977 high-quality markers for mapping.

Heritability was calculated in the broad-sense ($h^2$) as follows:

$$h^2 = \frac{\sigma_g^2}{\sigma_g^2 + (\sigma_{ge}^2/n) + (\sigma^2/rn)}$$

where $h^2$ = heritability, $\sigma_g^2$ = genotypic variance among RILs, $\sigma_{ge}^2$ = genotype x environment (location), $\sigma^2$ = error variance, $r$ = number of reps, and $n$ = number of environments.

**DNA isolation and gGBS**

DNA was isolated from ~40 mg of lyophilized leaf tissue using the DNeasy Plant Mini kit (QIAGEN, Valencia, CA) according to the manufacturer’s instructions. DNA samples from 190 lines from populations 2 and 3 were submitted to the Institute for Genomic Diversity (IGD) at Cornell University. GBS libraries were prepared as previously described (Elshire et al. 2011; Swarts et al. 2014) using the ApeKI enzyme, DNA ligase, and appropriate Illumina adapters. The IGD staff performed the library construction, read mapping, and downstream SNP calls. A total of 455,924,779 reads were produced, 428,671,333 of which were used for downstream analysis. A total of 61.5% of the reads were successfully aligned to unique positions in the G. max Wm82.a2.v1 Williams 82 reference sequence (Schmutz et al. 2010, http://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Gmax) using the BWA 0.7.8-r455 program (Li and Durbin 2009). SNPs were called using the TASSEL 5.0 pipeline (Bradbury et al. 2007) resulting in 33,728,018 tags for all 190 samples.

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**Statistical analyses**

ANOVA was performed using the “anova” and “aov” functions in R (R Foundation for Statistical Computing, Vienna, Austria 2008) to determine if there were significant differences between any locations or blocks. Pearson’s correlation coefficients between phenotypes were determined using PROC CORR in SAS (SAS Institute Inc., 2002).

Best linear unbiased predictors (BLUPs) were calculated using the lme4 package in R (R Foundation for Statistical Computing). All effects were considered random. Heritability was calculated in the broad-sense ($h^2$) as follows:

$$h^2 = \frac{\sigma_g^2}{\sigma_g^2 + (\sigma_{ge}^2/n) + (\sigma^2/rn)}$$

where $h^2$ = heritability, $\sigma_g^2$ = genotypic variance among RILs, $\sigma_{ge}^2$ = genotype x environment (location), $\sigma^2$ = error variance, $r$ = number of reps, and $n$ = number of environments.

**Linkage map construction**

Linkage map construction was performed in R/qtl (R Foundation for Statistical Computing) using the 2977 polymorphic, high-quality SNP markers obtained from GBS (Supplemental Material, Figure S1, File S1, File S2, and Table 1). Genetic distances between markers were determined using the est.map function, with an estimated genotyping error rate of 0.01. Iterative evaluation of chromosomes with excessive map distances (> 200 cM) was performed using droponemarker and est.map functions. Finally, all chromosomes were evaluated for correct marker order using the ripple function, using five markers at a time. No better order was identified than that assigned by the position in the Wm82.a2.v1 assembly.

**Covariate analysis and QTL mapping**

BLUP phenotypes were used for a one-dimensional QTL scan using the SIM method to determine the most significant marker associated with seed stearic acid for use in covariate analysis. Marker c14.loc58 (chromosome 14 @ 58 cM, Gm14:42206409) was identified as significantly associated with stearic acid and was subsequently designated as an interactive covariate using the pull.genre and chinb functions in R/qtl. Further analysis using this interactive covariate was performed using BLUPs values and the SIM method through the “scanone” function.

QTL analysis was performed using the qtl package in R (Broman and Sen 2009). Because R/qtl assumes an F2, the cross file was first converted into an F4 using the BC0F1 tool (Shannon et al. 2013). Conditional genotypic probabilities were calculated using an error probability rate of 0.01, due to the inherent likelihood of genotyping errors of the GBS method (Elshire et al. 2011). Single QTL analysis was run using the Standard Interval mapping method as described in Broman and Sen (2009). Significance thresholds were calculated using 1000 permutation tests for each phenotype, but the extremely large effects from the sacpd-c deletion resulted in substantially elevated LOD scores for oil related traits; in general, the only QTL above threshold was the sacpd-c deletion.

As a result, we set a LOD significance threshold of LOD = 4. BLUPs, best linear unbiased predictors; LOD, logarithm of the odds; QTL, quantitative trait loci; SIM, standard interval mapping.
Confidence intervals for QTL locations are presented as 1.5-LOD intervals. QTL were evaluated for overlap with selected literature (listed in results) and historical QTL using genetic/physical map tools available online (http://soybase.org/search/qtllist_by_symbol.php, accessed 01-01-2016 through 05-30-2016). The effect of each QTL was estimated in R/QTL using averaged phenotypic data over the three study locations via the effectplot command, following sim.geno with 1000 draws and an error probability of 0.01.

Data availability
The authors state that all data necessary for confirming the conclusions presented in the article are fully represented either within the article or within Supplemental Materials.

RESULTS

Phenotypic distributions
Analysis of variance showed that there were no significant differences between population 2 and population 3, so we conclude that there is no significant cytoplasmic effect on any of the traits evaluated in this study. Full results of the statistical analysis are presented in Table 2. There was significant genetic variation among RILs for all traits (Table 2), as well as a significant location effect for all traits (P < 0.05). The two parents, A6 and 194D, showed significant differences in all traits examined except oleic acid (P < 0.05). There were highly significant line and environment effects for all traits (P < 0.001) and a genotype × environment interaction was significant for all traits except palmitic acid and plant height (P < 0.01). Heritability estimates were moderately high for the five main fatty acid species, the highest value being $R^2 = 0.89$ for palmitic acid.

Phenotypic distributions and parent values are shown in Figure S2 and Table 3. All traits were approximately normally distributed. Transgressive segregation was noted for all traits of interest, particularly fatty acid traits, which suggested the contribution of multiple genes and/or multiple genes of small effect.

Correlations between traits
Significant correlations (P < 0.01) were found between several traits with the strongest being between the five fatty acid species (P < 0.0001). Pearson’s correlation coefficients and associated P-values are presented in Table S1. Significant coefficients between traits ranged between 0.10 and $-0.81$. The majority of significant correlations were detected in multiple environments for each trait. Stearic acid was significantly associated with all fatty acid traits, including total seed oil, in at least one environment.

Results using BLUPs
In order to attempt to lower the residual phenotypic variance even further, we chose to use the most significant marker on chromosome 14 as a covariate in all subsequent analyses. Using the same BLUPs as described above, we ran the SIM method in order to pick out the most significant marker associated with stearic acid on chromosome 14, (Figure 1). Once this marker was determined, the SIM was rerun with marker c14.loc58 as an interactive covariate. Using this method, the LOD scores and $R^2$ values were the highest, and an additional QTL was identified on chromosome 2 that no other method had identified previously (Figure 2). The 1.5-LOD intervals were also much narrower using this method than with SIM alone (the largest interval was only 5 cM wide for the QTL on chromosome 4) (Table 4).

QTL analysis
A total of 21 QTL were detected for seven traits on 9 of the 20 chromosomes. We observed colocation of QTL for several traits. The same significant region on chromosome 14 was detected for all oil traits examined (total seed oil, palmitic, stearic, oleic, linoleic, linolenic, and linolenic; see Discussion). The same QTL was detected on chromosome 2 for palmitic, stearic, and linolenic acids. A region on chromosome 4 was detected for stearic, oleic, linoleic, and linolenic acids. QTL were considered to be colocated when there was significant overlap between the 1.5-LOD intervals for each QTL. Phenotypic variation explained by each QTL ranged from 6.12% ($q.4s$) to 56.76% ($q.14s$) (Table 5). A6 alleles contributed to an increase in phenotype for 12 out of 21 QTL, while 194D alleles contributed to an increase in phenotype for 11 out of 21 QTL.

Total seed oil QTL
Two QTL were detected for total seed oil, one of minor effect on chromosome 9, accounting for 14.43% of the phenotypic variation, and one major effect QTL on chromosome 14, which accounted for 20.58% of the variation. For both loci, individuals homozygous for the 194D allele showed the highest total seed oil content. Mansur et al. (1993) detected an association between markers on chromosome 9 and total seed oil whose QTL intervals overlapped with $q.9oi$. There are several QTL published for total seed oil on chromosome 14 within 10 cM of $q.14oi$ on the soybean consensus map (Chen et al. 2007; Csandai et al. 2001; Eskandari et al. 2012; Liang et al. 2010; Qi et al. 2011).

Palmitic acid (C16:0) QTL
Three minor QTL were detected for palmitic acid. QTL on chromosome 2, 5, and 10 each accounted for 10–13% of the phenotypic variation. For all three loci, homozygosity for the 194D allele increased palmitic acid content. Q2p mapped to the same genomic region as fatty acid desaturase gene FAD3-B (Glyma02g39230) (Bilyeu et al. 2003; Gillman and Bilyeu 2012). Two QTL for palmitic acid have been published in this region (Panthee et al. 2006; Reinprecht et al. 2006). Two QTL for palmitic acid have also been published in the same region of chromosome 5 as $q.5p$ (Li et al. 2002; Wang et al. 2012). $Q.5p$ mapped to the

Table 4 SIM and SIM + covariate analysis for stearic acid-related QTL using BLUPs

| Trait         | Method | Chromosome | SNP Marker Nucleotide | Position | LOD  | $R^2$ | 1.5 Interval |
|---------------|--------|------------|-----------------------|----------|------|-------|--------------|
| Stearic acid  | SIM    | 2          | Gm2:15552879          | 78.00    | 4.38 | 4.4   | 44–111       |
|               |        | 4          | Gm4:18312993          | 76.00    | 4.08 | 4.1   | 72–83        |
|               |        | 14         | Gm14:42206409         | 49.00    | 34.25| 53.2  | 48–54        |
| Stearic acid  | SIM + Cov | 2          | Gm2:5946912           | 39.00    | 14.63 | 13.75 | 38–42        |
|               |        | 2          | Gm2:15552879          | 79.79    | 9.05 | 7.9   | 78–80        |
|               |        | 4          | Gm4:18312993          | 75.90    | 7.23 | 6.1   | 73–78        |
|               |        | 14         | c14.loc58             | 58.00    | 40.84| 56.8  | 57–59        |

LOD, logarithm of the odds; SIM, SIM, standard interval mapping; Cov, covariate analysis.
same region as *FAT1*-A (Glyma05g26110), a keto-acyl ACP synthase gene (Cardinal et al. 2007). The map location of q.14p corresponded to SACPD-C (Glyma14g27990), a stearyl-acyl carrier protein desaturase gene. No positional confirmation exists for this particular region; however, two QTL have been reported that are within 10 cM (Diers and Shoemaker 1992).

**Stearic acid (C18:0) QTL**

Four QTL were detected for seed stearic acid content; three minor effect QTL, two on chromosome 2, and one on chromosome 4, as well as one major effect QTL on chromosome 14. For the QTL on chromosomes 2 and 14 (Figure 3), homozygosity for the A6 allele contributed to elevated stearic acid content. Q.14s had an overwhelming major effect, which contributed 56.76% of the total variation in seed stearic acid content. Q.14s also mapped to the same genomic region as SACPD-C, which is also the same result we saw when examining palmitic acid. The two QTL identified on chromosome 2 accounted for 13.75 and 7.86% of the variation in seed stearic acid content. QTL associated with stearic acid have been previously reported on chromosome 2 (Diers and Shoemaker 1992; Kim et al. 2010). Q.4o and q.4e on chromosome 4 were effectively identical to q.4s for seed stearic acid and, like stearic acid, no QTL for oleic or linoleic acid have been previously published on this chromosome.

**Linolenic acid (C18:3) QTL**

Six QTL were detected for linolenic acid content on chromosomes 2, 4, 8, 10, 13, and 14, more than were detected for any other trait. All six QTL had minor effects, ranging from 7.97 to 12.16% of the phenotypic variation explained (60.6% total). Two of the QTL, q.8n and q.13n interacted epistatically (Table 6), and accounted for an additional 7.45% of the variation in linolenic acid content. Though all the QTL were of minor effect, we saw some positional overlap with published QTL for seed linolenic acid. *Li et al.* (2011) identified an association between *Sat537* and seed linolenic acid content on chromosome 2 at nearly the same map position as q.2n (within 5 cM). In the same study, a QTL was identified on chromosome 4, however it was not in the same region as q.4n. One QTL for linolenic acid has been published on chromosome 8 within 5 cM of q.8n (Bachlava et al. 2009). A QTL on chromosome 7 was reported (Shibata et al. 2008) in an adjacent region to q.10n on chromosome 10, not likely associated with *FAD2-1a*, as our interval was >20 cM away on the genetic map. Lastly, a single QTL was previously reported on chromosome 13, however it was on the opposite side of the chromosome as q.13n (Hyten et al. 2004). Several markers have been reported to be associated with alterations in seed linolenic acid on chromosome 14, however they were all greater than 10 cM away from SACPD-C based on the consensus map (Bachlava et al. 2009; Reinprecht et al. 2006; Spencer et al. 2004; Xie et al. 2011).

**Plant height QTL**

QTL of major and minor effect for plant height were detected on chromosomes 10 and 19, respectively. Q.10h accounted for 20.90% of

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**Table 5 Summary of QTL results for seven traits in soybean A6 × 194D cross**

| Trait           | QTL  | Chr | SNP | Marker Nucleotide | Position (cM) | 1.5 Interval | LOD | R² (%) | QTL Effect |
|-----------------|------|-----|-----|-------------------|---------------|--------------|-----|-------|------------|
| Palmitic acid   | q.2p | 2   | Gm2 | 7138451           | 44.58         | 38–128       | 5.55 | 10.49 | +0.36%     |
|                 | q.5p | 5   | Gm5 | 1420686           | 2.48          | 0–8          | 6.79 | 13.06 | +0.44%     |
|                 | q.14p| 14  | Gm14 | 12506615         | 44.91         | 42–58        | 6.89 | 13.26 | +0.44%     |
| Stearic acid    | q.2.1s| 2  | Gm2 | 5946912           | 39            | 38–42        | 14.63 | 13.75 | −1.16%     |
|                 | q.2.2s| 2  | Gm2 | 15552879          | 79.79         | 78–80        | 9.05 | 7.86  | −0.52%     |
|                 | q.4s | 4   | Gm4 | 18312993          | 75.99         | 73–78        | 7.23 | 6.12  | +1.24%     |
|                 | q.14s| 14  | Gm14 | 42206409         | 58            | 57–59        | 40.84 | 56.76 | −5.00%     |
| Oleic acid      | q.4o | 4   | Gm4 | 19496796          | 76.38         | 74–80        | 6.57 | 12.17 | +1.37%     |
|                 | q.14o| 14  | Gm14 | 322990452       | 51            | 49–54        | 13.7 | 28.05 | +2.9%      |
| Linoleic acid   | q.4e | 4   | Gm4 | 32351007          | 78.88         | 73–84        | 6.74 | 14.75 | −2.31%     |
|                 | q.14e| 14  | Gm14 | 34918500         | 54            | 45–63        | 4.48 | 9.49  | +2.02%     |
| Linolenic acid  | q.2n | 2   | Gm2 | 15552879          | 79.79         | 78–81        | 7.05 | 10.4  | +0.33%     |
|                 | q.4n | 4   | Gm4 | 27332180          | 78.12         | 76–84        | 7.04 | 10.39 | −0.21%     |
|                 | q.8n | 8   | Gm8 | 4116750           | 150           | 148–152      | 6.93 | 10.2  | −0.15%     |
|                 | q.10n| 10  | Gm10| 45310798          | 89.68         | 81–98        | 8.13 | 12.16 | −0.42%     |
|                 | q.13n| 13  | Gm13| 41141355          | 118.19        | 108–125      | 6.48 | 9.48  | −0.17%     |
| Plant height    | q.10h| 10  | Gm10| 44639359          | 90            | 88–94        | 9.79 | 20.9  | −6.54 cm   |
|                 | q.19h| 19  | Gm19| 49398020          | 100.51        | 91–104       | 5.2  | 10.42 | +5.03 cm   |
| Oil             | q.9oi| 9   | Gm9 | 6801513           | 16            | 15–17        | 6.85 | 14.43 | +0.28%     |
|                 | q.14oi| 14  | Gm14 | 42206409        | 62            | 49–68        | 9.43 | 20.58 | +0.71%     |

"QTL Effect" represents the impact of converting homozygous A6 alleles to homozygous 194D alleles. QTL, quantitative trait loci; Chr, chromosome; SNP, single nucleotide polymorphism; LOD, logarithm of the odds.
DISCUSSION

QTL mapping method selection in the soybean literature

The ability to detect QTL is entirely dependent on population structure and the level of genetic diversity within that population, as well as the number and quality of markers used in the analysis. Considering the multitude of available mapping strategies and software, as well as usage of individualized linkage maps of differing genetic lengths, it is difficult to compare historical mapping results with our study. However, several of our QTL in this study were positionally confirmed. In the public genetic database for soybean, there are very few QTL mapping studies published that analyzed seed stearic acid, in relation to the number of QTL published for other oil composition traits (www.soybase.org, accessed 05-01-2016). Four of the studies utilized the CIM method and successfully detected associations between markers and the stearic acid trait (Hyten et al. 2004; Panthee et al. 2006; Reinprecht et al. 2006; Wang et al. 2012). All other stearic acid QTL published in the database were determined by simple marker regression (Diers and Shoemaker 1992; Xie et al. 2011).

Difficulties in detecting QTL

A major confounding effect in our study was the extreme differences between the parent line A6, which exhibits a remarkably large deletion on chromosome 14 encompassing the entirety of the SACPD-C gene (as well as ~142 other genes), and the missense mutation in 194D (Gillman et al. 2014). The extreme phenotype values paired with the large deletion swelled the LOD score for the QTL on chromosome 14; LOD = 40.84 for stearic acid, which is extraordinarily high in the context of quantitative trait mapping. A QTL of such major effect makes it extremely difficult to detect other, relatively minor effect QTL. In addition, because of the genomic deletion on chromosome 14, any markers on chromosome 14 that showed a significant association with fatty acid traits flank the deletion. This experiment is the first to conduct QTL mapping using SACPD-C mutants, and as SACPD-C is a known factor in determining seed stearic acid levels, we can safely make the association between QTL on chromosome 14 the same as discussed for palmitic, stearic, oleic, and linoleic acids, and is almost certainly due to the differences between the missense SACPD-C in 194D and the deletion in A6. Q.14p, q.14s, q.14o, q.14e, q.14n, and q.14oi correspond to SACPD-C (Glyma14g27990).

Ultimately, we chose to continue with the final method described, SIM with covariate analysis using BLUPs for our analysis. By calculating BLUPs, we were able to take environmental variance into account, which strengthened the association between each marker and the stearic acid phenotype. We sought to further reduce the residual phenotypic variation by conducting covariate analysis. When a locus of strong effect exists in a population, it can mask the associations between any small effect loci and confound understanding of genetic architecture behind the phenotype (Xu 2003; Mackay 2001; Broman and Sen 2009). Using this method, we successfully identified an additional 20 QTL in our study, and strengthened the association of QTL with phenotypes. In this way, we were able to dampen the effects of the chromosome 14 QTL, thereby strengthening the associations between other markers and each trait.

We noted a pronounced effect of the A6 chromosomal deletion on seed stearic acid; ~18% for all lines with the chromosome 14 deletion, + ~5% stearic acid relative to lines with the 194D missense mutation. This difference was greater than expected based on our previous studies with other SACPD-C genomic deletions of smaller size (Gillman et al. 2014). Although unproven, our results strongly suggest that the A6 genomic deletion, which encompasses SACPD-C, also contains another gene (or genes) whose loss also elevates stearic acid content from ~12.5 to ~18%. Identification of such gene(s) is impossible using our mapping population and, as the A6 chromosomal deletion contains at least 142 additional genes, it is a daunting task for reverse genetic approaches.
Minor effect QTL for other fatty acid traits and plant height

We detected QTL for several annotated and molecularly characterized fatty acid biosynthetic genes as well as one major plant height/maturity gene. Although the significant marker may differ slightly for each trait due to maximum LOD optimization in model selection, for the sake of discussion, the QTL on chromosome 2 for palmitic, stearic, and linolenic acids can be considered to be identical because their support intervals overlap very significantly. Q.2p may be near the map position of FAD3-B (Glyma02g39230), which participates in the desaturation of C18:2 (linoleic acid) to C18:3 (linolenic acid) (Bilyeu et al. 2003; Gillman and Bilyeu 2012); however, our interval for this particular QTL was quite wide so the association is tenuous. This same level of overlap was seen with the chromosome 14 QTL found for all five fatty acid species, as well as for total seed oil. We expected to see this high level of colocation because of the close genetic association among fatty acid traits due to genes that participate in the fatty acid biosynthetic pathway (l|v a l u e s ranging from 0.23 to 0.81). This level of overlap is also highly indicative of the strength and reliability of our QTL results.

Q.5p is near the genomic region of FATB1-A (Glyma05g12300), which catalyzes the conversion of 16:0-ACP to C16:0 (palmitic acid) (Cardinal et al. 2007).

Q.19h is almost certainly E3 (Glyma19g224200), which affects flowering time in soybean in combination with the other “E” genes to determine flowering time/plant maturity (Watanabe et al. 2009). In the context of dissection of genetic architecture of complex traits, these results are purely confirmatory, i.e., they serve to provide confidence in our ability to detect QTL. Environmental influence and heritability of fatty acid composition

It is a strongly accepted fact that seed oil traits are highly dependent on environmental conditions during seed fill, particularly temperature (Kumar et al. 2006). A6’s stearic acid phenotype was much lower in the three Missouri environments we studied (23.4 ± 4.4, 20.9 ± 2.9, and 22.3 ± 5.5%) as compared with the original report of 28.1% stearic acid (Hammond and Fehr 1983; Lundeen et al. 1987), and this difference may be due to different environmental conditions in Northern Missouri compared to Ames, Iowa during seed fill/maturation. Fatty acid traits are relatively simple from a genetic perspective, and the target of ~20% stearic acid is almost reached with three QTL from A6 (Figure 3), although we noted relatively large environmental variation (Table 2). However, heritability values (h²) for fatty acid traits in our study were quite strong (0.72 < h² < 0.89), and twelve out of the 17 lines examined exhibited stearic acid levels > 20% consistently across environments, which suggests that the target may be achievable, at least in certain environments.

Impact of the chromosome 14 deletion on yield, seed traits, and plant morphology

Early studies have definitively demonstrated that A6 alleles for elevated stearic acid are also associated with decreased seed yield and unacceptably agronomic performance (Lundeen et al. 1987), presumably due to the large genomic deletion responsible for elevated stearic acid. In our recent work, we demonstrated that loss of SACPD-C activity is inextricably correlated with nodule morphological abnormalities, early nodule senescence, and an impaired ability to fix nitrogen (Gillman et al. 2014; Krishnan et al. 2016). Although we did not measure seed yield in our present study, we noted a decrease in seed oil content in lines that inherited the A6 chromosome 14 deletion (Table 5).

We saw no correlation between plant height and seed stearic acid content in this study, and our goal was a rapid QTL analysis for seed composition rather than a true seed yield study. Nevertheless, it is expected that the large chromosome 14 deletion will result in an unavoidable yield penalty as has been previously described (Lundeen et al. 1987). In the continued effort to develop a high stearic acid soybean variety that meets the current breeding target (> 20% stearic acid), the genomic deletion present in A6 provided valuable genetic information, but deletions/mutations from A6 per se will almost certainly not be of direct use in development of germplasm or cultivars with > 20% seed stearic acid and acceptable seed yield.

Identification of minor effect QTL for stearic acid

We identified three additional minor effect seed stearic acid QTL (Table 5), which do not appear to overlap with deletions identified in our previous work (Gillman et al. 2014), and it is tempting to report genes within the other QTL intervals whose annotations would suggest they may be associated with oil biosynthesis. However, the confidence intervals for our QTL are still quite large and the effects are relatively small. Further research would be needed to fine map the minor effect QTL. Moreover, the vast majority of gene annotation in soybean is almost completely inferred based on protein similarity to Arabidopsis genes (http://soybase.org/genomeannotation/, accessed 05-01-2016), and even in Arabidopsis only about 40% of enzyme and transporter genes have strong evidence for function (Niehaus et al. 2015)

Conclusions

We evaluated the genetic architecture for extremely elevated stearic acid (> 20%) through GBS/QTL analysis of RILs produced by reciprocal crosses between two mutant lines. Numerous minor effect QTL were identified for soybean fatty acids, total oil, and for plant height. In contrast to a priori expectation, a major effect QTL for stearic acid corresponded to a large genomic deletion from line A6, which explained ~57% of the phenotypic variance for seed stearic acid. However, the loss of SACPD-C activity per se boosts seed stearic acid from ~3% stearic acid to ~12.5%. These results suggest that the large genomic deletion in A6 contains both SACPD-C and an unidentified gene; the loss of both in line A6 increases seed stearic acid content to ~18% of seed oil. A combination of the major QTL and two minor effect QTL was able to boost seed stearic acid to above 20% of seed oil and meet the market target, but this elevation is expected to occur at a significant cost to seed yield. These genetic results will help guide and inform breeding efforts to increase soybean oil oxidative stability.

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