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Proximate composition of enhanced DGAT high oil, high protein soybeans

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ABSTRACT

Developing new soybean (*Glycine max*) varieties with greater amounts of oil and protein enhances soybean as a renewable source of food and fuel. Soybean expressing highly active ACYL-COA: Diacylglycerol acyltransferase (DGAT) from *Vernonia galamensis* had oil increased by 4% with no protein reduction. To assess other seed composition changes, seeds of two independent transgenic (VgD) soybean lines and controls were obtained from two field locations in multiple years. Many analyses addressed what seed component(s) was/were reduced to compensate for the oil increase, including carbohydrate analysis (structural and nonstructural), NDF (Neutral detergent fiber), main minerals and proximate analyses. In Princeton, oil increased by 4% for the high oil lines in 2016 and 2017, with no significant reduction in protein, this was slightly different in Spindletop with an increase in oil by 1.6–1.7 %, and an increase in protein by 2.6–2.4 % in comparison to the control, as an average for both high oil lines in 2016 and 2017. Soluble carbohydrate was reduced in VgD by 1.2 % in Princeton location for the high oil lines in 2016 and 2017, whereas only VgD1-2 was significantly reduced in Spindletop in comparison to Jack on 2016 with 2%, Oil in the VgD seeds was greater than in control seeds without a decrease in seed protein in both locations, suggesting that the trait was maintained across environments.

1. Introduction

Soybean (*Glycine max*) seeds are an affordable source of oil and protein for use as food and as animal feed (Brumm, 2004). Rich in both protein (35–45%) and oil (20–25%), soybean seeds are higher in protein and oil compared to other cereal and legume species (Salunkhe et al., 1983). The 2014/2015 global soybean production was 48 million metric tons of oil and 88 million metric tons of soybean meal, which accounted for about 27% and 68% of world plant oil and protein meal requirements (USDA, 2018b). The United States, Brazil and Argentina are the three leading soybean producing countries, contributing approximately 82% of world soybean production in 2018 (USDA, 2018a)Increasing seed (and corresponding oil and protein) yield per unit land area using conventional breeding has continued to progress (USDA, 2018b). This is often with little or no increased inputs making renewable oil production from plants less expensive over time and progressively more competitive with petroleum as an industrial chemical feedstock. Oil and protein are the most valuable components of soybean seeds (Salunkhe et al., 1983) and it is desirable to increase their percentage relative to other, less valuable seed components (Hatanaka et al., 2016). However, historically, neither conventional breeding nor quantitative trait locus mapping approaches for increasing soybean oil while maintaining protein content have been successful, because an increase in one of these components is associated with a decrease in the other (Eskandari et al., 2013). Soybean oil is mainly composed of triacylglycerol (TAG), which is fueled by sucrose delivered from leaves and other photosynthetic tissues (Allen et al., 2009; Hildebrand, 2010, 2011; Lonien and Schwender, 2009; Ohlrogge and Browse, 1995; Weselake et al., 2009). Assimilates move through the vascular connection from the mother plant into the funiculus, and the integuments, but then into developing embryo tissue across the apoplastic space. Different strategies have been used to increase storage lipids (Eskandari et al., 2013; Feng et al., 2004; Sonah et al., 2015). There are three main approaches leading to higher TAG accumulation: increased flux of hydrocarbon into fatty acid

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biosynthetic pathways (Push),optimizing TAG assembly (Pull) and reducing the degradation of the resulting oil bodies (Protect) (Vanhercke et al., 2014b). One approach to increase oil content illustrated by several studies is via the elevated expression of endogenous or transgenic TAG biosynthetic genes (Lardizabal et al., 2008; Rao and Hildebrand, 2009; Taylor et al., 2009; Vyacheslav et al., 2009). Elevated expression of regulatory genes that up-regulate multiple enzymes for fatty acid biosynthesis also can result in augmented oil amounts (Andrianov et al., 2010). Co-expression of the transcription factor *WRINKLED1* (*WRI1*) with ACYL-COA: diacylglycerol acyltransferase (*DGAT1*) is shown to have a synergistic effect on TAG biosynthesis in plants (Sanjaya et al., 2011; van Erp et al., 2014; Vanhercke et al., 2014a, 2014b). Expression of a *Tropaeolum majusDGAT1*, *TmDGAT1*, was reported to increase oil content, seed size and yield in Arabidopsis (*Arabidopsis thaliana*). Similar increases in oil content and seed yield were reported with greater expression of a *Brassica napus* (canola) *DGAT1*, *BnDGAT1*, in *B. napus* (Siloto et al., 2009). Enhanced drought tolerance of this transgenic canola was also reported. Lardizabal et al. (2008) reported a 1.5% increase in oil amounts of transgenic soybeans expressing a fungal (*Umbelopsis ramanniana*) *DGAT2*. Over expression of *DGAT1* from *Sesamum indicum* insoybean in a controlled environment also increased oil content by 1.8 and 1.4 percent in T2 and T3 transgenic lines (Wang et al., 2014). Breeding has produced lines with improved protein content and enhanced the amino acid composition, but this has been impeded by a negative correlation between seed yield and protein content of the seeds, in addition to the deficiency in sulfur-containing amino acids in the seed storage protein, (Hill et al., 2005; Liener, 1994; Peregrine et al., 2008). The amino acids for seed protein synthesis, including storage proteins, arrives from leaves mainly in the form of the N-rich amino acids asparagine (Asn) and glutamine (Gln)(Bradstreet, 1954). In soybean, fixed nitrogen is exported from nodules as ureides which do not directly enter developing seeds. Rather ureides are converted into amino acids in leaves before being sent to the sink tissues (viz. developing seeds). Seed protein and oil contents are controlled by the relative amounts of amino acids and sucrose arriving at the developing soybean apoplastic space (Allen et al., 2009). Sucrose is cleaved into hexose sugars by acid invertase in the wall, and the hexoses can be phosphorylated prior to uptake into the symplast where they can be cleaved into triose phosphates. Triose phosphates can be reduced to glycerol-3-phosphate, the backbone for glycerolipids or oxidized to 3-phosphoglycerate that can re-arrange to phosphoenolpyruvate, which can be dephosphorylated to pyruvate. Intermediates from hexose phosphates to pyruvate and pyruvate itself can be translocated into plastids of developing seeds and pyruvate converted (decarboxylated) into acetyl-CoA (2:0-CoA). The acetyl-CoA then provides hydrocarbons for fatty acid biosynthesis in plastids and oil (TAG) synthesis in the Endoplasmic Reticulum (ER).

Carbohydrates are the third main component of soybean seeds and accounts for ca. 35% of the dry seed weight. Approximately half of the total carbohydrates in soybean s are structural carbohydrates. Structural carbohydrates are cell-wall polysaccharides (cellulose, hemicellulose, and pectin), whereas non-structural carbohydrates include sucrose and other mono-, di- and oligo-saccharides (Karr-Lilienthal et al., 2005b). soybean seeds are reported to have 6% (dry weight; DW) crude fiber and 27% nitrogen free extract (NFE)(Medic et al., 2014). The sugars galactose, glucose and fructose were found in 10 soybean cotyledons ranging between 0.7 and 4.0,1.2–4.7 and 1.1–4.7 mg/g DW (Grieshop et al., 2003). Sucrose is the primary soluble sugar in soybean seeds and ranged between 37.1 and 72.5 mg/g of dry seed weight in 18 different enhanced oil soybean lines. However the sucrose concentration in 20 enhanced protein soybean lines was less and ranged between 21.8 and 48.9 mg/g DW (Hartwig et al., 1997). The other half of the soybean carbohydrate is made up of structural polysaccharides. This includes dietary fiber that is comprised of cellulose, pectin, and hemicelluloses, along with mannans, galactans, and xyloglucans. The neutral detergent fiber (NDF) concentration of 36 soybean samples grown in the US ranged from 11.26% to 18.52% of DW whereas this range was from 11.5% to 17.1% of DM for 48 soybean samples grown in Brazil and was 12.2–14.4% of DM for 49 samples collected from China (Grieshop and Fahey, 2001; Karr-Lilienthal et al., 2005a).

Soybean fiber ranges between 4 and 8 % seed DW (Medic et al., 2014). It has been reported, however, that crude fiber analysis recovers (captures) only 20 % of hemicellulose and 50–80 % of cellulose on average, since the other method to determine fiber such as neutral detergent fiber (NDF), underestimates pectins, a soluble fiber fraction (Jung, 1997). Transgenic soybean with enhanced DGAT (Roesler et al., 2016) has been reported to contain greater oil without protein reduction and this relationship has been explained by the tradeoff between oil and soluble sugars. A3.5 percent increase in seed storage oil and protein was accompanied by a 1.9 percent reduction in soluble carbohydrate. The increase in seed oil and protein was not entirely explained by the reduction in the soluble sugars (Roesler et al., 2016).

The objective of this paper is to address the question of what seed component was reduced, commensurate with the increase in oil without reduction in protein using high oil transgenic soybean lines (Hatanaka et al., 2016). This was addressed by a detailed examination of the composition of enhanced oil soybean seeds relative to the parental control.

2. Material and methods

2.1. Seed collection

Four lines were used in the current study, VgD1-1, VgD1-2, Jack (control) and an empty vector control (VC). Seeds were planted in Princeton, KY (37.098,749–87.868,730) and Lexington, KY (38.125,835 N, -84.496781W) in 2015–2016. Both VgD lines used in the study have highly active acyl-co a: diacylglycerol acyltransferase (DGAT) from *Vernonia galamensis* (VgDGAT1A).

2.2. Lipid analysis

Ten mg of seeds were broken using a mortar, pestle, and spiked with 17:0 to quantify oils. Oil was extracted from seed chips using 500 μL of diethyl either with 0.0001% BHT (butylated hydroxytoluene) twice and dried. Once dry, 500 μL sodium methoxide was added and shaken for 10 min with 1 mL isooctane containing 0.001% BHT added after 200 μL was pulled off the upper layer and transferred into a gas chromatograph (GC) vial with an additional 1 mL of isooctane added. GC vials were then run on a Varian CP-3800 GC using a 25m x 0.25 mm ID fused silica column with a Varian (chrompack) CP=Select CB for FAME, with a film thickness of 0.25 μm. The temperature program ran from 90 C to 250 °C with a 25 °C ramp for a total of an 8-min run time with a constant column flow mode of 0.9 mL/min utilizing a splitless injection. Quantification was performed by using a flame ionization detector with peaks quantified using Star Chromatography Workstation Version 6.00 software with peak area being used to calculate relative percentages of FAMEs (Fatty Acid Methyl ester) (Hatanaka et al., 2016).

Oil extraction was performed according to AOCS Method Am 2–93 using petroleum ether as the extraction solvent and defatted, oven dried paper towels in substitution for thimbles. Approximately 2 g of seeds ground in a burr mill were used for extraction in a Soxhlet extraction apparatus for at least 30 cycles (Soxhlet, 1879).

2.3. Nitrogen and phosphorus analysis

Samples were prepared for analysis by weighing 100 mg of dried material into 25x200 mm Pyrex glass ignition tubes marked at 50 mL (Bradstreet, 1954). Five mL of concentrated sulfuric acid containing 0.05 g of salicylic acid/mL were added and the samples allowed to react for 1 h at room temperature. This step caused any inorganic nitrate present in the sample to form nitrosalicylic acid. Next, 0.5 g of sodium

thiosulfate was added, and the samples were placed in a Technicon BD-40 block digester set at 180 °C for 1 h. This resulted in a reduction of the nitrosalicylic acid to the less refractory compound amino salicylic acid. Then 1.8 g of potassium sulfate and boiling chips were added and the digestion was continued for 2.5 more hours at 360 °C. All forms of nitrogen were converted to ammonia and all forms of phosphorus were converted to orthophosphate during this process. The samples were allowed to cool at room temperature then diluted to 50 mL with deionized water. After mixing, the samples were poured into polystyrene cups for analysis.

The instrument used for colorimetric determination of total nitrogen and total phosphate was a dual Technicon System II Autoanalyzer which was configured to perform both analyses simultaneously. The wavelength was 660 nm for each procedure. The method for ammonia was a modification of the Berthelot reaction developed by Chaney and Marbach (1962). Two reagents were used, one containing 0.5% sodium hydroxide 0.042% sodium hypochlorite in deionized water, the other containing1.0% phenol and 0.02% sodium nitroprusside in deionized water, both were introduced before adding the sample into a bubble-segmented stream. The reaction took place inside the instrument, and the blue indophenol formed was passed through a colorimeter for final determination of ammonia concentration. The original manual method was modified to speed up the reaction rate in order to make it compatible with the constraints of the automated system. It was necessary to increase the nitroprusside catalyst concentration to four times the recommendation and pass the reaction stream through a 60 °C heating bath, though the protocol was otherwise similar to the manual method.

The phosphorus technique was based on the method of Fiske and Subbarow (1925) (Fiske and Subbarow, 1925) and is the same as the Technicon Industrial Method 348 R 6-3 1-5, except the dialysis step was not necessary for digested samples. A solution of ammonium molybdate (7.5 g/L) in 1.92 M sulfuric acid was reacted with the samples in the segmented stream to form a heteropolyphosphomolbdate complex. This compound was then reduced by adding a solution containing 150 g of sodium bisulfite, 5.0 g sodium sulfite and 2.5 g 1-amino-2-naphthol-4--sulfoninc acid in 1 L deionized water and heating the reagent stream to 95 °C in an oil bath. The reaction resulted in the formation of an intense blue color proportional to the phosphate concentration. Four standards and a blank were run before and after each set of 15 samples to minimize baseline drift.

2.4. Sugar analysis and quantification

To extract sugars (*myo*-inositol, sucrose, raffinose, stachyose and verbascose) three replications of five dehydrated soybean seeds per replication were weighed, ground in an electric coffee grinder and then pulverized in liquid nitrogen using a mortar and pestle. One aliquot of 1 mL 80% (v/v) ethanol containing 1 mmol L^{-1} 2-deoxyglucose (2-DG), used as internal standard, was added to the seed powder, ground to produce a slurry, and transferred to a 15-mL polypropylene tube on ice. The process was repeated four times with 1 mL aliquots of 70% (v/v) ethanol (no internal standard after the first aliquot). Each time, after grinding the slurry, the 1 mL was transferred to the same tube. This 5 mL homogenate was centrifuged at 15,000 x g for 20 min at 4 °C, the supernatant collected, and diluted to30 mL with distilled, deionized water. One third volume of each sample (10 mL) was added to 10 mL of water in 50 mL polypropylene tubes. After freezing at −80 °C, the samples were lyophilized to dryness and reconstituted in 1 mL distilled, deionized water by vortexing the samples (kept on ice) every hour for 8 h. After transferring the sample to 1.5 mL microtubes and centrifuging (16,000 x g for 30 min at 4 °C), the supernatants were collected and stored overnight at −20 °C. Once filtered (CoStar Spin-X HPLC 0.45 μm Nylon filter, Corning Incorporated, Corning, NY, USA), the samples were diluted 10 times with water prior to analysis.

To identify and quantify the sugars, the diluted extracts were injected onto a Carbo-Pac PA1 with guard column ona BioLC HPLC system with pulsed electrochemical detection (HPLC-PED) (ED50 detector and PeakNet software (Version 6.0); Dionex Corp, Palo Alto, CA, USA). The separation via anion exchange used isocratic conditions of 19 mmol L^{-1} NaOH at 1 mL min^{-1}. Sugars were identified and quantified by comparing their retention times and peak areas with that of known external standards. Reintegration of the peak start- and stop-times, baseline identification, and calculations of areas under the peaks were performed using Chromeleon software (Version 6.8; Dionex Corp). Estimates of sugar amounts per seed fresh weight were adjusted for losses during processing by comparing external standard 2-DG quantities (2.5 nmol) with the recovery of 2-DG added during extraction (internal standard adjusted for dilutions) (Downie and Bewley, 2000; Nosarzewski et al., 2012).

2.5. Cellulose analysis

Cellulose was measured colorimetrically according to Updegraff (1969). Briefly, exactly 20 mg samples of dried seed tissue were weighed (n = 4) and boiled in acetic-nitric acid reagent (acetic acid: nitric acid: water 8:1:2) for 30 min. The remaining material was washed three times with 8 mL water and 4 mL of acetone and dried under a vacuum for 48 h. Samples were then hydrolyzed in 67% v/v sulfuric acid for 1h. The glucose content was then determined using the anthrone method in which 20uL of sulfuric acid hydrolyzed sample was mixed with 500 μL water and 1 mL 0.3% anthrone in concentrated sulfuric acid on ice. The absorbance of samples was measured using a Bio-Mate Thermos Scientific spectrophotometer (Thermos Fisher, Waltham, MA, USA) set at OD 620 nm. The cellulose content was calculated by multiplying the measured glucose concentration of each sample by the total volume of the assay and then by hydration correction factor of 0.9 to correct for the water molecules added during hydrolysis of the cellulose polymer (Young and Evans, 1973).

2.6. NDF (Neutral Detergent Fiber)

For NDF analysis 0.5 g samples were weighed and put in the filter bags (constructed from chemically inert and heat resistant filter media, capable of being heat sealed and able to retain 25-μm particles while permitting solution penetration (F57 ANKOM Technology). The Marking pen solvent and acid resistant was used from (F08, ANKOM Technology). The filter bags with the samples were seeped in acetone for 5 min and then dried on a paper towel, after which the samples were dried for 22 h at 100 °C before weighing to obtain pre-extraction dry weights. The same drying procedure was used to obtain post-extraction dry weights. For the filter bag system, the samples were weighed into individual pre-weighed and numbered filter bags which were then heat-sealed, and final undigested residue weights were determined after drying the samples 72 h at 60 °C.An ANKOM 200 fiber analyzer (ANKOM Technologies, Macedon, NY, USA) was used to determine Neutral detergent fiber (NDF), the reagents consist of (Neutral Detergent Solution) prepared by adding 30 g sodium dodecyl sulfate 18.61 g Ethylenediaminetetraacetic acid disodium salt (dehydrated), 6.81 g sodium borate, 4.56 g sodium phosphate dibasic (anhydrous), and 10 mL triethylene glycol to 1L distilled H_2O (premixed chemical solution available from ANKOM Technology). The pH was then checked to make sure it was in the range 6.9–7.1 and the mixture was heated and agitated to ensure dissolution of reagents. Next was added alpha-amylase-Heat-stable bacterial alpha-amylase: activity = 17,400 Liquefon Units/mL (One Liquefon Unit is the measure of digestion time required to produce a color change with iodine solution indicating a definite stage of dextrinization of starch substrate under specified conditions), (FAA, ANKOM Technology). Finally, anhydrous sodium sulfite Na_2SO_3, (FSS, ANKOM Technology) was added. The residue remaining after digesting in a detergent solution is mainly fiber and the fiber residues were predominantly hemicellulose, cellulose and lignin (Vogel et al., 1999).

2.7. Starch

Starch analysis was conducted using an EnzyChrom™ starch assay kit (BioAssay Systems, Hayward, CA). Starch was not detectable so the data was not included.

2.8. Amino acid analysis

Seed samples defatted with petroleum ether were ground to a fine powder using a burr mill on the finest setting. To determine the amino acid composition of the seeds, 100 – 200 mg of defatted, whole ground samples were mixed with 300 µL of the internal standard 100 mM norleucine, and the samples were hydrolyzed for 24 h at 110 °C in 6N HCl, in accordance with AOAC procedure 994.12. In order to determine the methionine content, ~50 mg of ground sample underwent performic acid oxidation as described in AOAC method 994.12, prior to the acid hydrolysis. Following hydrolysis, samples were converted to their phenylisothiocyanate derivatives and analyzed using reverse phase HPLC (3.9 X 300 mm PICO-TAG reverse phase column; Waters Corporation, Milford, MA) (Cohen and Strydom, 1988). All samples were run in duplicate, with an inter-sample variation of less than 10%.

2.9. Mineral analysis

Samples were sieved through a 0.45 µm sieve and run on an Ultimate Proximate analyzer. The Proximate analysis (moisture, and ash) were analyzed using a LECO TGA 701. Nitrogen and total sulfur were analyzed using a LECO CHN 628 and the elemental analysis was achieved using a Varian 720-ES Spectrometer. Total sulfur at 1350 °C, moisture at 107 °C and ash at 750 °C.

2.10. Pyrolysis Gas chromatography

Analyses were performed using a Pyroprobe Model 5200 (CDS Analytical, Inc.) connected to an Agilent 7890 GC with an Agilent 5975C MS detector. The pyroprobe was run in direct mode under a He atmosphere. Pyrolysis was conducted at temperature 400–650 °C (1000 °C/s heating rate) for 20 s in order to check for major compositional differences between samples. The valve oven and transfer lines were maintained at 325 °C. The column used in the GC was a DB1701 (60 m (length)×0.25 mm (film)×0.25 µm (inner diameter)), and the temperature program was as follows: 45 °C for 3 min, ramped to 125 °C at 4 °C/min, ramped to 160 °C at 2 °C/min, ramped to 280 °C at 4 °C/min, held for 5 min. The flow rate was set to 1 mL/min using He as the carrier gas. The inlet and auxiliary lines were both maintained at 300 °C, and the MS source was set at 1906 eV. Ca. 1 mg of the ground biomass samples were analyzed in a quartz cell packed with quartz wool. Samples were heated to 100 °C for 10 s in the probe prior to analysis to remove any residual water. Prior to sample analysis, blank experiments were performed in order to validate the cleanliness of the system. After sample analysis, methanol was run as a sample to remove any condensed products inside the pyroprobe. Methanol and blank experiments were repeated as necessary until the system was clean. Each sample was run in triplicate (Supplement file).

2.11. QUICKEXTRACT™ seed DNA extraction kit

After defatting, one to two mg of ground seed particles was placed in a 200 µL PCR tube with 50 µL of the QuickExtract™ Seed DNA Extraction Solution and vortexed for 10 s, followed by heating first at 65 °C for 6 min and then at 95 °C for 2 min. The samples were kept on ice if analyzed immediately, or clear supernatants were obtained by centrifugation at 2000 g for 5 min to be stored in a −20 °C freezer (AL-Amery et al., 2016).

2.12. PCR verification

The primers for specific amplification of VgDGAT1 was used in the current experiment with the expected size of 472 bp (Li et al., 2010) and a primer pairs (forward primer 5′-GAAAGGAGACTTTTTA-TATGCGATTGAG-3′ and reverse primer 5′-ACCAAGAATGCTGAA GAAGCACCAG-3′).

The PCR was carried out in a final volume of 15 µL containing 0.5 µL genomic DNA, 0.27 mM each dNTP, 0.27 µM primer pair, and 0.5 units of Bio Ready rTaq DNA polymerase (Bulldog Bio, Rochester, NY) using a T100 Thermal Cycler (Bio-Rad, Hercules, CA). The PCR conditions consisted of the initial denaturing at 95 °C for 10 min; then a two-temperature thermal cycle consisting of denaturation at 95 °C for 15 s, followed by annealing and extension at 60 °C for 1 min, totally 40 cycles. The PCR products were separated via 1% (w/v) agarose gel electrophoresis and visualized using ethidium bromide staining and UV light (Li et al., 2010), (Fig. 1).

3. Statistical analysis

Statistical analysis was performed using the SAS 9.2 statistics package (SAS Institute Inc., Cary, NC, U.S.A.). Years were separated for each location. The main effects of variety were considered to be fixed effects while replication was considered a random effect. ANOVA RCBD (Random Complete Block Design) was used with three replications, least significant differences (LSD) were used to separate means, if significant, at a critical level of $P \leq 0.05$. The P-value represented in the tables is the interaction between year and varieties (ANOVA table included in the Supplemental file).

For principal components analysis, missing data were imputed by taking the median of that particular variable across all samples. Principal components analysis was conducted in R 3.4.1 using the prcomp function with scale = T (Team, 2014). To visualize the relatedness among samples, we plotted scores for the first two principal components, which together explained >45% of the variation in the dataset, (Fig. 2).

4. Result and discussion

4.1. Oil and protein content in high oil lines expressing VgDGAT1A

In the current study, we compared the amount of the key nutritional and anti-nutritional components in (VgD) a highly active ACYL-COA: Diacylglycerol acyltransferase (DGAT) from *Vernonia galamensis* transformed into soybeans (Hatanaka et al., 2016). The transgenic varieties tested were significantly different for most of the parameter evaluated for each location ($P \leq 0.05$). VgD lines compared to control (Jack) showed consistent increases in seed oil content was greater by ($P \leq 0.05$). In Princeton, oil increased by 4% for the high oil lines in 2016 and 2017, with no significant reduction in protein, this was slightly different in Spindletop with an increase in oil by 1.6–1.7 %, and an

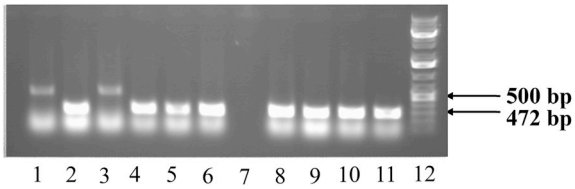

Fig. 1. Amplification of soybean (VgDGAT1A). Seed DNA was extracted using a QuickExtract™seed DNA extraction kit. The expected product sizes were 472 bp.
The negative and positive control were 1 and 2 lanes, VgDGAT1A for lanes M2,4,5,6,8,9,10 and 11 the negative was 3 and no template in 7 lanes. See methods for the PCR protocol.

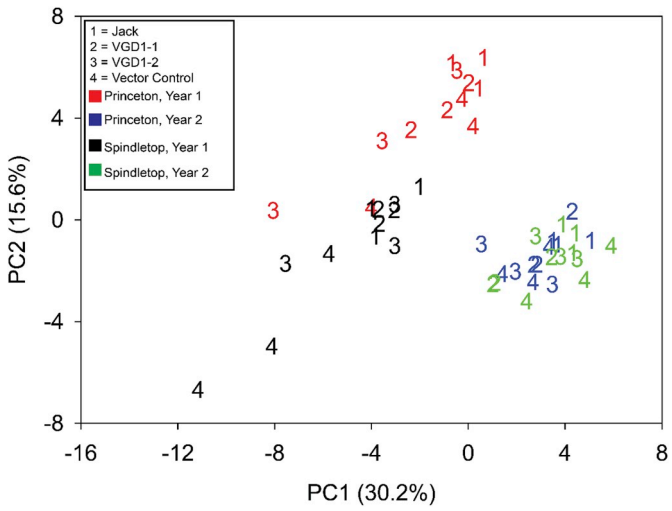

Fig. 2. Plot of principal component scores along the first two principal components axes. The four genotypes are indicated by numbers 1–4, while the symbols are color-coded by sampling year and location. There is clear cluster of year 2 samples, irrespective of genotype, indicating that year-to-year variation is the primary source of variance in our dataset. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

increase in protein by 2.6–2.4 % in comparison to Jack, as an average for both high oil lines in 2016 and 2017 (Table 1). *DGAT1A* from *Vernonia galamensis* has resulted in higher TAG accumulation compared to orthologous DGATs. Increased expression of other DGATs has also been shown to enhance TAG accumulation (Lardizabal et al., 2008; Xu et al., 2008).

Protein amounts were not significantly different among the lines over locations and years of the study except for VC in Princeton which was 43.8 and 43% for 2016 ad 2018; whereas at Spindletop it was greater by 44.1 % for both years of the study (Table 1). These results are consistent with the findings of Lardizabal et al. (2008) their results showing no significant changes in the content of protein in mature seed of transgenic plants relative to controls using a codon-optimized version of a Diacylglycerol acyltransferase *2A* from the soil fungus *Umbelopsis* (formerly *Mortierella*) *ramanniana* in soybean seed grown for three different years and at 63 locations. No major impact on protein and yield was found, and 1.5 percent point increase in oil was maintained in their study, indicating the stability of the enhanced oil phenotype for soybean plants expression *UrDGAT2A*.

Roesler et al. (2016) mentioned that the enhanced oil trait in soybean *GmDGAT1b-MOD*, with no reduction in protein content was built on the tradeoff between oil and soluble sugars but added that the compositional shift cannot be completely explained as the reduction in one component does not sufficient offset the increase in the other.

Thus, current data indicate that no major changes in protein content resulted from over expression of VgD in either soybean lines compared with controls. This attribute will result in enhanced meal protein amounts while oil + protein increased in Princeton by 2.3, 2.7, 3.4, and 5.3 % for 2016 and 2017 and by 3.1, 4.8, 4.3 and 3.7% in Spindletop for the same years. (Table 1). This increase was acquired without a significant change in amino acids amounts (Table 2), which could make the defatted meal more valuable for animal feed, food and many industrial applications.

4.2. Fatty acid composition in enhanced oil lines

Consistent with previous studies, the 18:1 fatty acid content was increased in VgD1-1 and VgD1-2 lines by 0.6, 3.6, 5.8 and 7.0% in 2016 and 2017 at Princeton, and by 6.4, 6.7, 7.8 and 6.7 % at Spindletop for both high oil lines and both years in comparison to Jack. This increase was accompanied by a reduction in 18:2 by 3, 8, 6, and 7 % at Princeton for 2016 and 2017, and 2, 7, 8 and 7 % at Spindletop for both lines and years. The empty vector (VC) showed significant differences but not in all fatty acids (Table 1). This alteration in fatty acid profiles for the high oil lines is consistent compared to controls (Hatanaka et al., 2016) and it is reproducible in the current study in multiple environments. This modification could possibly be explained because most newly produced 18:1 fatty acid acyl groups are initially transferred from the acyl-CoA pool to phosphatidylcholine (PC), where they undergo further desaturation to produce 18:2 and 18:3 before becoming incorporated into TAG by the Kennedy pathway (Orlowski et al., 2017). Greater in vivo DGAT activity can compete with desaturases for 18:1 resulting in more 18:1 fatty acid acyl groups going directly to TAG, without desaturation (Roesler et al., 2016). Increased over expression of foreign and native DGATs have been found to increase TAG oleoyl (18:1) amounts in species as diverse as maize (*Zea mays*), olive (*Olea europaea*) (Banilas et al., 2010), Arabidopsis (Jako et al., 2001) and soybean (Hatanaka et al.,

Table 1
Oil, Protein and fatty acid composition (g/100 g) of soybean seed from VgD, empty vector and parental lines planted at two locations in KY.

	Princeton 2016				Princeton 2017			
Seed component/line	Jack	VgD1-1	VgD1-2	VC	Jack	VgD1-1	VgD1-2	VC
Oil	21.3 ^{b‡}	25.8 ^{a}	24.8 ^{a}	17.3 ^{c}	20.6 ^{a}	23.6 ^{b}	24.6 ^{b}	18.6 ^{a}
Protein	40.6 ^{b}	39 ^{b}	40 ^{b}	43.8 ^{a}	39.8 ^{b}	40.0 ^{b}	41.3 ^{b}	43.0 ^{a}
Oil + Protein	62.0 ^{ns}	64.3	64.7	64.7	60.5 ^{cb}	63.9 ^{ab}	65.8 ^{a}	61.3 ^{b}
16:0	9.8 ^{c}	10.2 ^{a}	9.8 ^{c}	11.0 ^{b}	10.0 ^{c}	10.6 ^{b}	10.7 ^{b}	11.9 ^{a}
18:0	5.2 b	4.9 ^{c}	6.0 ^{a}	4.8 ^{c}	4.5 ^{b}	5.4 ^{a}	5.4 ^{a}	4.2 ^{b}
18:1	24.4 ^{bc}	25.0 ^{b}	28.0 ^{a}	23.3 ^{c}	26.0 ^{b}	31.8 ^{a}	33.0 ^{a}	25.8 ^{b}
18:1d	1.2 ^{b}	1.3 ^{a}	1.0 ^{c}	1.2 ^{b}	1.1 ^{b}	1.0 ^{c}	1.0 b ^{c}	1.2 ^{a}
18:2	53.0 ^{a}	50.3 ^{b}	45.0 ^{c}	50.3 ^{b}	52.4 ^{a}	46.6 ^{b}	45.5 ^{b}	52.4 ^{a}
18:3	7.0 ^{c}	8.3 ^{b}	11.0 ^{a}	9.3 ^{b}	6.3 ^{a}	4.6 ^{b}	4.3 ^{c}	6.1 ^{a}
	Spindletop 2016				Spindletop 2017			
Seed component/line	Jack	VgD1-1	VgD1-2	VC	Jack	VgD1-1	VgD1-2	VC
Oil	22.3 ^{a}	24.0 ^{a}	23.8 ^{a}	15.4 ^{b}	22.3 ^{a}	23.9 ^{a}	23.8 ^{a}	15.4 ^{b}
Protein	39.6 ^{b}	42.2 ^{ab}	42.2 ^{ab}	44.1 ^{a}	39.5 ^{b}	42.2 ^{ab}	41.6 ^{ab}	44.1 ^{a}
Oil + Protein	61.0 ^{ns}	64.1	65.8	61.6	61.8 ^{b}	66.1 ^{a}	65.5 ^{a}	59.5 ^{b}
16:0	9.7 ^{b}	10.5 ^{ab}	10.2 ^{ab}	11.2 ^{b}	10.4 ^{c}	10.8 ^{bc}	10.9 ^{b}	11.8 ^{a}
18:0	5.2 ^{ns}	5.8	5.8	5.1	4.4 ^{b}	5.5 ^{a}	5.6 ^{a}	4.7 ^{b}
18:1	24.4 ^{ns}	30.6	30.9	24.5	27.6 ^{b}	35.4 ^{a}	34.3 ^{a}	24.8 ^{b}
18:1d	1.2 ^{ns}	1.1	1.2	1.3	1.2 ^{a}	1.1 ^{b}	1.1 ^{b}	1.2 ^{a}
18:2	52.9 ^{a}	54.8 ^{b}	46.2 ^{a}	50.9 ^{ab}	50.2 ^{a}	42.6 ^{b}	43.2 ^{b}	49.8 ^{a}
18:3	6.5 ^{ab}	6.0 ^{b}	6.0 ^{b}	7.0 ^{a}	5.9 ^{b}	4.5 ^{c}	4.7 ^{c}	7.4 ^{a}

Table 2
Amino acid composition (gm/100) of soybean seeds from VgD, empty vector and parental lines planted in two locations and years in KY.

	Princeton 2016				Princeton 2017			
Component/line	Jack	VgD1-1	VgD1-2	VC	Jack	VgD1-1	VgD1-2	VC
Asp + Asn	3.4 ns‡	4.0	4.0	4.0	2.9 ns	3.0	3.5	3.3
Glutamic acid + Glutamine	5.4 ns	6.2	7.1	6.4	5.2 ns	5.4	6.0	5.8
Serine	1.5 ns	1.7	2.0	1.7	1.5 ns	1.5	1.6	1.5
Glycine	1.0 ns	1.2	1.2	1.2	1.0 ns	1.1	1.2	1.2
Histidine	0.8 ns	0.8	1.0	0.8	0.7 ns	0.7	0.8	0.8
Arginine	2.2 ns	2.6	2.7	2.9	2.1 ns	2.2	2.4	2.5
Threonine	1.0 ns	1.1	1.4	1.1	1.1 ns	1.0	1.1	1.2
Alanine	1.1 ns	1.3	1.4	1.3	1.1 ns	1.1	1.2	1.2
Proline	1.4 ns	1.6	1.9	1.7	1.5 ns	1.5	1.6	1.7
Tryosine	1.0 ns	1.1	1.1	1.2	1.0 ns	0.9	1.1	1.1
Valine	1.3 ns	1.4	1.6	1.3	1.7 ns	1.6	2.0	2.0
Isoleucine	0.1 ns	0.5	1.0	0.6	1.2 ns	1.1	1.1	1.3
Leucine	1.2 ns	1.6	2.3	1.7	2.4 ns	2.4	2.4	2.5
Phenylalanine	2.3 ns	2.3	2.3	2.3	1.4 ns	1.5	2.4	1.5
Lysine	1.8 ns	1.9	2.1	2.0	2.0 ns	2.0	2	2.1
Methionine	1.4 a	0.8 c	1.2 b	0.7 c	0.2 ns	0.3	0.2	0.3
Norleucine	2.2 ns	2.3	2.2	2.2	3.2 ns	3.2	3.2	3.1
Total amino acids	29.3 ns	31.1	36.2	32.9	20.0 ns	25.5	24.4	25.7
	Spindletop 2016				Spindletop 2017			
Component/line	Jack	VgD1-1	VgD1-2	VC	Jack	VgD1-1	VgD1-2	VC
Asp + Asn	4.4 b	4.3 b	4.5 b	5.7 a	3.2 ns	3.4	3.0	3.0
Glutamic acid + Glutamine	7.2 b	7.1 b	7.5 b	9.5 a	5.2 ns	5.8	5.2	5.0
Serine	1.8 b	1.9 b	2.1 b	2.5 a	1.4 ns	1.4	1.5	1.3
Glycine	1.4 b	1.4 b	1.5 b	1.8 a	1.1 ns	1.1	1.1	1.1
Histidine	1.0 b	1.0 b	1.1 b	1.4 a	0.7 ns	0.7	0.9	0.6
Arginine	2.9 b	3.0 b	3.2 b	4.1 a	0.7 ns	0.9	0.7	0.6
Threonine	1.3 b	1.3 b	1.4 b	1.8 a	2.2 ns	2.6	2.3	2.3
Alanine	1.5 b	1.4 b	1.5 b	1.9 a	1.0 ns	1.1	1.0	1.0
Proline	1.9 b	2.1 b	2.1 b	2.7 a	1.1 ns	1.2	1.1	1.1
Tryosine	1.3 ns	1.4	1.5	1.8	1.4 ns	1.6	1.6	1.5
Valine	1.7 ns	1.6	1.6	2.2	1.0 ns	1.1	1.0	1.0
Isoleucine	1.8 ns	1.6	1.7	2.3	1.2 b	1.5 a	1.5 b	1.3 ab
Leucine	2.9 ns	2.8	2.9	3.7	2.3 ns	2.6	2.4	2.3
Phenylalanine	1.9 ns	1.9	2.0	2.5	1.2 b	1.8 a	1.2 b	1.5 a
Lysine	2.2 b	2.2 b	2.4 b	3.0 a	1.9 ns	2.2	2.0	2.0
Methionine	0.7 b	1.1 a	0.9 ab	0.9 ab	0.2 ns	0.3	0.2	0.3
Norleucine	2.2 ns	2.2	2.2	2.2	3.1 ns	3.2	3.1	3.2
Total amino acids	38.5 b	38.3 b	40.2 b	50.1 a	30.0 ns	34.1	30.0	30.1

Table 3
Structural and soluble carbohydrate composition (gm/100 gm) of Soybean Seeds from VgD, empty vector and parental lines planted in two locations and years in KY.

	Princeton 2016				Princeton 2017			
Component/line	Jack	VgD1-1	VgD1-2	VC	Jack	VgD1-1	VgD1-2	VC
Structural carbohydrate								
Cellulose ‡	7.3 a	4.6 b	5.3 ab	8.8 a	1.8 c	1.5 c	2.5 b	3.2 a
NDF	29.6 ns	31.0	32.0	28.9	20.0 a	25.5 b	24.4 b	25.7 b
Soluble carbohydrate								
myo-inositol	0.12 ns	0.10	0.10	0.10	0.3 a	0.1 b	0.14 b	0.3 a
Sucrose	2.3 ns	1.5	1.3	1.8	2.5 a	0.9 b	0.9 b	3.0 a
Raffinose	0.4 ns	0.4	0.3	0.3	0.8 ns	0.2	0.2	0.6
Stachyose	2.2 a	2.1 ab	1.8 b	2.1 ab	0.9 ns	0.7	1.2	3.1
Verbascose	0.14 ns	0.41	0.25	0.43	0.20 ns	0.1	0.12	0.14
Total Sugars	5.2 a	4.5 ab	3.2 b	4.1 ab	4.1 ns	2.0	2.7	6.9
	Spindletop 2016				Spindletop 2017			
Component/line	Jack	VgD1-1	VgD1-2	VC	Jack	VgD1-1	VgD1-2	VC
Structural carbohydrate								
Cellulose	11.3 ns	8.7	7.6	7.8	3.1 b	7.1 a	8.5 a	3.0 b
NDF	25.0 ns	28.2	26.3	24.1	27.2 ns	24.6	25.0	22.6
Soluble carbohydrate								
myo-inositol	0.12 ns	0.10	0.10	0.10	0.18 ns	0.16	0.23	0.22
Sucrose	2.3 ns	1.5	1.3	1.9	2.3 ns	3.1	2.1	1.8
Raffinose	0.4 ns	0.4	0.3	0.3	0.4 ns	0.5	0.4	0.3
Stachyose	2.2 ns	2.1	1.8	2.2	2.3 ns	2.4	2.0	2.0
Verbascose	0.1 b	0.3 ab	0.2 ab	0.4 a	0.03 ns	0.02	0.01	0.01
Total Sugars	5.0 ns	4.0	3.0	3.0	5.2 ns	6.2	5.0	4.2

2016; Roesler et al., 2016)consistent with the data in this study. Further analysis comparing VgD and Jack via Pyrolysis Gas chromatography-mass spectrometry indicated that the chemical fingerprint between lines is equivalent (see Supplemental file).

4.3. Carbohydrate profiling in transgenic versus control soybean lines v

Carbohydrate and cellulose profiles were variable. The cellulose content was significantly lower for the VgD1-1 in 2016 at Princeton, whereas cellulose was significantly higher in 2017 at Spindletop (Table 3). Sucrose amounts significantly lower in high oil lines at Princeton in 2017 only whereas no significant differences were shown in 2016 in Spindletop for both years of the study (Table 3).

Neutral detergent fiber (NDF) did not change among varieties and control. However, at Peregrine et al., 2008 NDF increased significantly compared to Jack (control) in the high oil lines, whereas no significant differences were seen between the high oil lines and the VC. This finding disagreed with Lardizabal et al. (2008) who reported a 15% decrease in NDF in the transgenic positive line relative to the control. Roesler et al. (2016) reported the high oil *GmDGAT1b-MOD* soybean also had no reduction in protein content. This result was built on the tradeoff between oil and soluble sugars. They note that the compositional shift cannot rationally be explained through carbon reshuffling alone and suggest other factors are at play. They also report that the high oil trait is accompanied by a significant reduction in pectin derived from galactose, which we did not include in our study. Rather, we observed a reduction in cellulose at one location. Raffinose oligosacchrides still represented ≥1.5% of the transgenic seed weight implying that a combination of introducing genes encoding more efficient DGAT enzymes with methods of decreasing raffinose oligosaccharides could be one approach to increase oil content.

4.4. Amino acid composition in enhanced oil lines

Protein quality (amino acid composition) may bemore important than total protein content in soybeans for animal feed. Animal feed needs to have a balanced amino acid composition to meet the animal's nutritionalrequirements (Medic et al., 2014). The essential amino acids that humans and monogastric animals cannot synthesize are phenylalanine, valine, threonine, tryptophan, methionine, leucine, isoleucine, lysine, and histidine. In Princeton, enhanced oil lines, and VC were significantly lower in Methionine compared to Jack for 2016 only, whereas in Spindletop Methionine was significantly higher in VgD1-1 only in 2016, on the other hand VC was significantly higher in most of the amino acids in 2016 only, (Table 2). The differences in amino acids was minimal between enhanced oil lines and Jack, and the differences were inconsistence.

4.5. Mineral analysis

The mineral composition of soybeans was reported many years ago, and since then the genetics of soybeans and agronomic practices have

Table 4
Proximate composition of soybean seed from VgD, empty vector and parental lines, planted at two locations and years in KY.

	Princeton 2016				Princeton 2017			
Line	Jack	VgD1-1	VgD1-2	VC	Jack	VgD1-1	VgD1-2	VC
Ash‡	4.6 [b]	4.6 [b]	4.5 b	4.7 a	4.1 [ns]	4.0	3.2	3.4
Moisture	7.5 [ns]	7.5	7.6	7.8	3.4 [ns]	4.6	6.8	6.8
Nitrogen	6.5 [ab]	6.3 b	6.3 b	6.6 a	6.4 [ns]	6.6	6.5	6.6
Total Sulfur	0.24 [ns]	0.25	0.27	0.25	0.3 [ns]	0.3	0.3	0.3
Micro-Minerals (mg/kg)								
Fe	7.3 [ns]	5.7	4.2	4.3	6.3 [ns]	6.2	6.5	6.0
Cu	1.0 [ns]	1.0	1.0	1.0	1.1 [ns]	1.1	1.1	1.1
Zn	2.8 [a]	3.4 b	3.3 b	4.0 c	4.6 [ns]	5.1	4.8	4.8
Al	2.0 [ns]	2.3	3.5	4.7	11.2 [ns]	14	11.9	12.4
Mg	205.0 [ns]	208.0	200.0	201.0	236.7 [a]	227.3 [ab]	224.8 [ab]	212.4 [b]
Pb	0.9 [ns]	0.9	1.0	0.8	0.9 ns	0.8	0.8	1.0
Macro-Minerals (g/kg)								
P	0.7 [ns]	0.6	0.7	0.7	0.7 [ns]	0.7	0.7	0.7
Ca	0.2 [a]	0.2 [a]	0.2 [b]	0.2 [a]	0.3 [ns]	0.2	0.3	0.3
Na	0.6 [ns]	0.6	0.6	0.5	0.7 [ns]	0.7	0.7	0.7
K	1.2 [ns]	1.2	1.3	1.3	1.4 [ns]	1.4	1.4	1.3
	Spindletop 2016				Spindletop 2017			
Line	Jack	VgD1-1	VgD1-2	VC	Jack	VgD1-1	VgD1-2	VC
Ash	4.6 [ns]	4.4	4.5	4.5	4.5 [ab]	4.7 [a]	2.5 [b]	2.5 [b]
Moisture	4.5 [ns]	4.4	4.5	4.5	4.1 [ns]	6.0	8.8	7.7
Nitrogen	7.4 [ns]	7.5	6.7	8.3	6.5 [b]	6.5 [b]	6.7 [a]	6.7 [a]
Total Sulfur	0.2 [ns]	0.2	0.2	0.2	0.2 [ns]	0.2	0.3	0.3
Micro-Minerals (mg/kg)								
Fe	6.3 [b]	6.5 [b]	7.5 [b]	9.1 [a]	9.3 [c]	9.9 [b]	10.0 [ab]	10.3 [a]
Cu	0.8 [ns]	0.83	0.9	1.1	1.1 [ns]	1.1	1.1	1.1
Zn	4.2 [ns]	4.0	4.2	4.1	4.5 [ns]	4.4	4.5	4.5
Al	1.0 [ns]	10.7	12.8	10.5	13.9 [ns]	14.2	14.4	19.3
Mg	200.9 [ns]	197.5	196.5	209.9	228.4 [ns]	223.8	227.2	216.6
Pb	1.0 [ns]	0.8	0.8	1.0	1.0 [ns]	1.0	1.0	1.0
Macro-Minerals (g/kg)								
P	0.7 [ns]	0.7	0.7	0.7	0.7 [ns]	0.7	0.7	0.8
Ca	0.2 [ns]	0.2	0.2	0.2	0.3 [ns]	0.2	0.2	0.2
Na	0.7 [ns]	0.7	0.7	0.7	0.7 [ns]	0.7	0.7	0.7
K	1.2 [ns]	1.2	1.2	1.3	1.3 [ns]	1.2	1.2	1.3

changed significantly (Batal et al., 2010).

The sodium content of high oil lines VgD1-1 and VgD1-2 was varied. At Princeton Zinc was higher in 2016 in high oil lines by 1, 0.5 % compare to Jack. Iron showed significant difference in Spindletop only, VgD1-1 was slightly higher compare to Jack (5.6, 7.5, 9.9 and 10 mg/kg), in 2016 and 2017, but the increase was significant in 2017 only. Empty vector (VC) was significantly higher in Fe, compared to the other lines in this location, at 9.1, and 10.3 mg/kg for both years of the study. No significant differences were found in Cu and Al in either location (Table 4). Ash was variable in both locations, at Princeton the high oil lines varied between 4.6, and 4.5 in 2016 and was not significant in 2017. VgD1-1 was not significantly different compared to Jack, while VgD1-2 was numerically lower compared to Jack at Spindletop in 2017 only. In addition, no significant differences were found in total sulfur between the locations and years.

5. Principle component analysis (PCA)

To further test whether DGAT overexpression affects the biochemical composition of soybeans, principal component analysis was conducted. The first two principal components explained >45% of the variation in the dataset. Along these principal components, there was no clear clustering of samples by genotype. Sampling year and location were the primary sources of variation among samples. For PC1 there was clear separation between samples collected in year 1 vs. year 2, while for PC2 there was moderate separation between the Princeton year 1 samples and the rest of the samples. Thus, it appears temporal and spatial variation in environmental conditions are major drivers of compositional differences in these samples. An inspection of the principal component loadings indicates that the separation for PC1 is primarily a result of altering amino acid levels in the year 1 samples, while the separation along PC2 is largely driven by Princeton Year 1 samples having lower levels of certain amino acids (Leu, Ile, and Val) and minerals (Na, Zn, and Al) and higher levels of NDF, but no environmental effects were noticed on oil and protein across two years in each location (Fig. 2).

6. Conclusions

The soybean high oil trait maintained greater oil amounts without a reduction in protein amounts under field conditions under different environment. VgDGAT1A transgenic soybeans showed consistently enhanced oil content without protein reduction. The reduction in other major components was inconsistent and varied across locations and years. At Princeton the oil increase was greater and was partially explained by a reduction in total sugar (Table 3). Similarly, the total ash amount was significantly less in one high oil line, VgD1-2, compared to the control at both locations and years. However, the reduction was not consistent with the other high oil line, VgD1-1. Yet this reduction did not explain all of the increase in oil from the Princeton location. Other seed components could contribute in to the oil increase such as an insoluble fiber fraction (e.g. pectin) which is in agreement with (Roesler et al., 2016).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101303.

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