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IMPACT OF A HIGH OIL AND PROTEIN ON AGRONOMIC TRAITS AND OVERALL SEED COMPOSITION IN SOYBEAN

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IMPACT OF A HIGH OIL AND PROTEIN ON AGRONOMIC TRAITS AND OVERALL
SEED COMPOSITION IN SOYBEAN

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DISSERTATION

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A dissertation submitted in partial fulfillment of the requirements for the degree
of Doctor of Philosophy in the College of Agriculture, Food and Environment at
the University of Kentucky

By
Maythem AL-Amery

Lexington, Kentucky

Director: Dr. David Hildebrand, Professor of Plant and Soil Sciences

Lexington, Kentucky

2017

ABSTRACT OF DISSERTATION

IMPACT OF A HIGH OIL AND PROTEIN ON AGRONOMIC TRAITS AND
OVERALL SEED COMPOSITION IN SOYBEAN

New soybean lines have been developed with significantly higher oil, protein + oil and
higher meal protein. These soybeans contain a VgD1 gene (highly active acyl-
CoA:diacylglycerol acyltransferase, DGAT from *Vernonia galamensis* (VgDGAT1A)
produces much higher oil synthesis and accumulation activity in soybean. Soybean with
active DGAT from *Vernonia galamensis* (VgDGAT1A) has active TAG biosynthesis
relative to other DGATs including from soybeans and Arabidopsis. DGATs catalyze the
final step of TAG synthesis: DAG (diacylglycerol) + acyl-CoA $\rightarrow$ TAG + CoASH
(Coenzyme A is notable for its role in the synthesis and oxidation of fatty acids, and the
oxidation of pyruvate in the citric acid cycle). A thorough analysis of the major
components in VgD1 lines, especially those of nutritional or anti-nutritional value
including what else changed (decreased); and what remained at normal levels was
conducted. A field study was conducted in Spindletop and Princeton KY, reviled no
reduction in yield nor protein, and about 4 % (DW) more oil was obtained in Princeton
and 2% (DW) in Spindeltop. No consistent reduction in the other seed
composition.VgDGAT1A soybean lines indicated noticeably early maturation compared
to the parental line. This is associated with higher expression of the flowering genes FT2
(*FLOWERING LOCUS T2*) and FT5 (*FLOWERING LOCUS T5*), for the high oil lines. A
single recessive mutation in soybean (MIPS) *myo-inositol 1-phosphate synthase*, confers
a seed phenotype of increase inorganic phosphate (Pi) crossed with high oil lines
expressing a DGAT from *Vernonia galamensis* (VgDGAT1A) (VgD). The oil and
protein were maintained compart to VgD. VgD X MIPS (VM), had 21.2, and 22 % oil in
2015, and 23.3 and 24.0 oil in 2016, and protein 46, 49 in 2015, and 37 and 39 % in

2016. Phosphate results suggesting the cross MV is still segregating for MIPS and more selection and planting are needed.

Measurement of seed phosphate levels is an established technique for screening for low phytate mutants but to date, it has not been performed non-destructively from single soybean seeds. A protocol was developed greatly reducing the sample size thereby reducing the cost and time and saving a generation in the selection of low phytate mutant seeds based on the high Pi phenotype.

Genotyping single seeds are useful in breeding and genetics while maintaining high germination rates. Nondestructive single-seed genomic DNA extraction protocols using 12 mg cotyledon tissue with a modified cetyl trimethyl ammonium bromide (CTAB) technique and a commercial seed DNA extraction kit using 1 mg cotyledon tissue were developed for dry soybean seeds and cross-verified with leaf DNA analysis.

Keywords: diacylglycerol acyltransferase, triacylglycerides, MIPS, Flowering genes, DNA extraction

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 November 13, 2017

IMPACT OF A HIGH OIL AND PROTEIN ON AGRONOMIC TRAITS AND
OVERALL SEED COMPOSITION IN SOYBEAN

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November 13, 2017

*This thesis is dedicated to the memory of my father **Mohsen Ali AL-Amery***

A brave man whom I still miss every day.

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Chapter 1: INTRODUCTION

Global warming is a main feature of climate change and can be caused by excessive use of fossil fuel. Plant breeders and geneticists have worked to understand and provide plants varieties with high oil composition that can be an alternative by providing carbon neutral fuel source for sustainable energy use. Plants constitute a primary resource of carbon, vitamins, minerals, protein, essential fatty acids, and utilizable energy for human food production. Protein is the main component of soybean seeds and an essential component of the diet needed on a daily basis for animals and humans. The main goal of this research is to evaluate compositional changes of high oil and protein soybeans and investigate if MIPS mutants can provide more photosynthate for oil biosynthesis.

Soybeans (*Glycine max* L) are a valuable agriculture commodity with a high seed protein level (35-45%) and a moderately high level of oil (20-25%) compared to cereals and other legume species (Salunkhe et al., 1983). Soybeans are the world's largest source of animal protein and second largest source of vegetable oil, a little behind palm oil. The United States, Brazil and Argentina are the leading soybean producers and exporters (USDA, 2016).

The increase in human population, and meat consumption throughout the world, in addition renewable fuel sources, adding pressure for increased soybean production. The negative relationship between oil and protein among soybean genotypes is well documented in literature (Bellaloui et al., 2009; Rajkumar et al., 2010) and effected by both maternal and seed genetic background (Hayati et al., 1996; Hernández-Sebastià et al., 2005; Pandurangan et al., 2012).

A thorough understanding and processes for manipulating and regulating the oil and protein levels is not complete (Schwender and Hay, 2012). Increasing soybean seed protein levels while also improving the amino acids profile has been a major target for breeders worldwide. High protein soybean lines can be found in the USDA germplasm collection with up to 58% protein on a seed dry weight basis. These genotypes have very low oil percentages (Hill et al., 2005a). Therefore, a goal is to maintain the protein level and while increasing oil levels further.

Triacylglycerol biosynthesis and production has studied thoroughly, as well as the enzymes controlling the process. The two precursors for TAG biosynthesis are acyl-CoA and G3P (glycerol-3 phosphate). Overexpression of G3P phosphate dehydrogenase in canola led to increase in seed oil about 40% (Vigeolas et al., 2007). Sucrose is the main molecule transported to different sinks, such as developing seeds, and the major carbon supply by the maternal plant for oil and protein biosynthesis during seed development

(Baud and Lepiniec, 2010). Once it reaches seeds it is converted into starch, in the first 30-40 days of seed development, however normal soybean seed has less than 1% starch at physiological maturity (Medic et al., 2014). Starch starts accumulating after the 8th week of development and reaches around 14% and drops to < 1% at harvest by the 10th week (Saldivar et al., 2011). Oil accumulation starts at week 8 and keeps accumulating until week 10. Possible that the high oil soybean cultivars were more efficient in term of using starch near the end of seed filling period, and probably using more galactooligosaccharides , which are undesirable components (Medic et al., 2014b), In addition, targeting anti nutritional components such as oligosaccharides and phytate in soybean seed is one approach to increase oil while increasing the nutrition value of soybean seeds.

The assimilate supply (carbon) form the photosynthesis process is the main supply for TAG, and it is under competition with other storage composition, the conversion of phospho*enol*pyruvate (PEP) by PEP carboxylase (PEPC) or pyruvate kinase (PK). PEPC is important in supplying carbon to amino acid synthesis and its high activity is related with high protein content (Smith et al., 1989; Sugimoto et al., 1989).

Fatty acids are mainly produced in the chloroplast and the endoplasmic reticulum from the precursor acetyl-CoA. Acetyl CoAs are converted to malonyl-CoA by acetyl-CoA carboxylase, the next step is convesion into malonyl-ACP. Two carbon units are added one at the time from malonyl-ACP by KAS enzymes leading to 16:0, 18:0 and 18:1, which can be translocated to the endoplasmic reticulum for further desaturation into 18:2 and 18:3 (Sidorov and Tsydendambaev, 2014). This process is termed the Kennedy pathway which combines fatty acids to G3P (glycerol-3-phosphate) via G3P acyltranferase, forming lysophosphatidic acid (LPA). The second acyl-CoA transfer is catalyzed by lypophosphatidic acid acyltransferase (LPAT) with the formation of PA (Phosphatidic acid), and release of the phosphate group forming diacylglycerol (DAG). Finally DAG is converted to (TAG) by DAG acyltranferase (DGAT) which is the main step to TAG and releasing CoA (Sidorov and Tsydendambaev, 2014).

Over-expression of DGAT1 in *Tropaeolum majus* led to increased oil levels, in addition to seed size and final yield (Xu et al., 2008b). Similar results were found by (Weselake et al., 2008) from over-expression of *BnDGAT1* back in *Brassica napus* (canola). They also

reported that over expression of DGAT enhanced drought tolerance in canola plants. (Lardizabal et al., 2008a) also reported an increase in seed yields in four out five locations in the US and Argentina although these yield increases were not significant, when using transgenic soybean expressing a fungal DGAT, and the oil increased by 1.5 % dry weight.

In some studies protein levels are negatively correlated with yield. For instance the NC6202 germplasm line produced seeds with 45.7% protein content and a 10% reduction in grain yield compared to the control, NC-Roy (Carter et al., 2010). Contrasting results were found with TN03-350 and TN04-5321, which have 43.1-43.9% protein levels without yield reduction.

The temperature during seed filling, can possibly effect the seed oil composition. High temperature effect on fatty acid profile, by increasing oleic acid (18:1) and less (18:2, 18:3), however low temperature enhanced the unsaturated fatty acids such as 18:2 and 18:3. (Rennie and Tanner, 1989), when tempreature has such effect on fatty acid profile , this can also applied on the oil concentration, which could possibly increase with high temperature increase, since delay planting reduce oil and increase protein, while early planting increase oil because the high temperature during seed fill duration (Kane et al., 1997b).

Early maturing genotypes often have higher protein contents (399-476 g/kg^{-1}). This increase is accompanied with high air temperature during seed filling (Vollmann et al., 2000). Similar results were found by (Dardanelli et al., 2006; Kane et al., 1997b; Maestri et al., 1998; Piper and Boote, 1999). They found that early planted soybean was subjected to increase in oil percentage, and late planting increase protein content, this could explaine by the temperature during seed filling.

In the current study, we are using two soybean genotypes that have unique composition characteristics MIPS *myo*-inositol 1-phosphate synthase, converts glucose-6-phospate into *myo*-inositol 1-phosphate which is a key precursor to both phytic acid or phytate and the raffinosaccharides, raffinose and stachyose. These MIPS mutants have as much as 10% more hydrocarbon to be "pulled" into oil by our high activity oil biosynthesis genes. VgDGAT1A, which have higher expression acyl-CoA:diacylglycerol acyltransferase DGAT from *Vernonia galamensis,* soybean lines expression VgDGAT1A

showed an increase 4 percent point in oil without reduction in seed protein or yield. This study both genotypes are tested in in field and greenhouse condition and evaluating the cross, in addition the environment by genotype interaction.

Chapter 2: EFFECT OF ENHANCED DGAT OIL/PROTEIN SOYBEANS ON YIELD, CARBOHYDRATES LEVELS AND SEED GROWTH

Abstract

Seed growth and development is an important part of the yield production process. The objective of this study is to investigate the effect of modified oil transgenic soybean with highly active acyl-CoA: diacylglycerol (DGAT) from *Vernonia galamensis* (VgDGAT1A) on growth, and accumulation of the carbohydrate level during seed growth rate. The experiment was conducted in two years in Lexington KY, the modified oil lines (VgD) had earlier R7 (beginning of maturity). Seed growth rate was lower in both years of study. The reduction in seed growth rate (SGR) was accompanied by an increase in the effective filling period in one year, and increase in a number of days to R1 (beginning of flowering). Pod dry weight was inconsistency over the years of study, at maturity the transgenic lines gave the lowest dry weight compared to control. Sucrose concentration was lower in almost all VgD lines, but stachyose was higher. This information suggesting that VgD lines have a consistent effect on seed growth rate with clear early maturation. In addition, VgD varieties have higher undesirable carbohydrates, they are good candidates for further increase in oil.

KEYWORDS. Seed fill period, sucrose and stachyose, high oil and protein, yield, FT genes

Introduction

Soybean is a major source of high quality of protein and oil (Grieshop and Fahey, 2001). The negative relationship between oil and protein is well documented in the literature (Bellaloui et al., 2009; Rajkumar et al., 2010). New soybean varieties with highly active acyl-CoA:diacylglycerol acyltransferase (DGAT), which can increase the hydrocarbon flux without a reduction in protein content. The DGAT is inserted form *Vernonia galamensis* (VgDGAT1A) which can produce much higher oil synthesis and accumulation activity in soybean (Hatanaka et al., 2016). These new lines can produce 3-5 percentage point more oil compare to control. Since Eskandari et al (2013), found a negative correlation between oil and maturity, which was agreed with Bellaloui et al (2009) that finding a negative relationship between oil and maturity in soybean variety Clark (R^2=0.75, 0.63) for 2004 and 2005.the current experiment was conducted to test this hypothesis on new high oil soybeans.

Considering crop productivity depends partially on solar radiation, especially when the temperature is suitable for growth (Egli, 1994). Part of the productivity depends on how well is the crop can efficiently utilize the assimilate supply over time. In short season, crop must mature before decreasing in temperature, whereas in the long season the suitable environment may be available for a longer growth cycle, but it is not necessarily reflected in more yield (Egli, 1993).

The effect of the environment and maturity groups (MG) on soybean seed composition have been studied. Dardanelli (2006) evaluated six maturity groups (II,III, IV, V,VI,VII and VIII-IX) in 12-14 different environments, and found all environment produced high oil in cultivars belong to MG II-III and IV, whereas MGII-III yielded more protein than others did. The high oil can explain by the high temperature during seed fill in earlier MG.

Yield is also a function of seed size, which depends on seed growth rate (SGR)(Smith and Nelson, 1986), even though a genetics background could play an important role in seed growth process (Pfeiffer and Egli, 1988). In addition, seed fill duration is positive correlation with the date of maturity (Pfeiffer and Egli, 1988).

Egli (1993) found seed fill duration for MG00, was shorter however, no changes have seen in seed fill duration in the other maturity groups. This results in agreement with

(Kane et al., 1997a) that vegetative growth period increased with the increase in MG, but the seed fill duration showed no effect.

Flowering step refers to the transition from vegetative to reproductive stage. From agronomy standpoint this transition is important for grain crop production (Cockram et al., 2007).Therefore understanding flowering gene influences on maturation is important, since it will enable more efficient breeding program (Kim et al., 2012). The early flowering is related to flowering genes (FT), and flowering stimulated by accumulating FT and PC (Phosphatidylcholine) in the shoot apical meristem, and suggesting that FT binds to diurnally changing molecular species of PC to promote flowering (Nakamura et al., 2014).

Soybean contains approximately 33% carbohydrates (Hymowitz and Collins, 1974), and it consists of 4-5 % sucrose,1 to 2 % raffinose, and 3.5 to 4.5% stachyose (percentage point) (Wilson, 2008). The relationship between oil, protein, and sugars has been studied. Hymowitz et al (1972), found a positive correlation between sucrose and oligosaccharide (Raffinose), and negative correlation with protein, these results included 60 different soybean lines. Smiler results found by Saldivar et al (2011), studying five soybean lines, in addition, they found a small accumulation of oligosaccharide during early stages of seed growth, and start accumulating at the end of seed maturation.

Achieving a significant increase in oil and protein in soybean without a reduction in yield is the breeder's goal. Since it will increase the value of the crop substantially, this approach is feasible by altering the carbon partitioning, since the majority of soybean seed weight contains fiber and indigestible oligosaccharides (Padgette et al., 1996). However, conventional breeding could not reach this goal, because of the negative correlation between oil and protein. Diacylglycerol acyltransferase (DGAT) catalyze the final step in triacylglycerol (TAG), or oil production. In addition, several attempts have been done to increase oil percentage by over expression DGAT, in *Brassica napus* (Taylor et al., 2009b), in maize (Oakes et al., 2011), and soybean (Lardizabal et al., 2001). Compared to other plant DGATs, a DGAT from *Vernonia galamensis* (VgDGAT1A) produces much higher oil synthesis and accumulation soybean. Soybean lines expressing VgDGAT1A show a 4 percentage point (dry weight) increase in oil

content without reductions in seed protein contents or yield per unit land area (Hatanaka et al., 2016). In addition, VgDGAT1A, showed early maturation in greenhouse condition. The aim of the study is to 1- Evaluate the effect of enhanced DGAT oil/protein soybean on flowering and maturation time. 2-Determine the influence of maturation on seeds and pods growth rate in field conditions. 3-Evaluate the effect of enhanced DGAT oil/protein soybean on photosynthate level in seeds and pods in field condition.

Materials and Methods

Seed Collection

Four lines were used in the field experiment VgD1-1, VgD1-2 and Jack, both VgD lines used in the study have highly active acyl-CoA: diacylglycerol acyltransferase (DGAT) from *Vernonia galamensis* (VgDGAT1A) (Hatanaka et al., 2016). The field studies were established at Spindletop research farm in Lexington, KY (38.125835 N, -84.496781W) in 2015 and 2016. Soil type at this location was a Loradale Silt Loam (Fine, mixed, active, mesic Typic Argiudoll). Planting occurred in mid-May in 2015 and late May to early June in 2016 in Spindletop KY. Seeding rate was 100000 seeds ha^{-1} for both seasons. Plots were maintained weed-free, and irrigation when needed for the entire growing season in both locations.

Stage development for soybean was measured, 10 planed were marked in the two middle rows and the plants were staging within a week interval. Reproductive stages growth stages (Fehr and Caviness, 1977) of all lines were taken at weekly intervals (2-3 d (days)) intervals as growth stage R7 approach) on 10 consecutive plants in a border row. The date of R1 was determinate by linear interpolation and R7 was defined as the date when 70 % of the plants had reached R7.

Sample preparation

Individual pods at the same stage of development, regardless of nodal position, were identified by marking fully developed pods with acrylic paint when the seeds were just starting to swell. The pods were marked 3-6 d before plants reached growth stage R5. The marked pods were in bordered rows and ten marked pods were harvested from each plot at 5 to7 d intervals. The pods were placed on ice in the field and taken immediately to the laboratory where approximately five seeds were removed from the pods in a cold room (10 to 8 °C). The seed coat and axis were removed and the samples were freeze-dried and grind (mortar and pestle) for carbohydrate analysis. Seed dry weight determined dry weights (after drying at 65 8 °C) of the remaining seeds (Egli and Bruening, 2001).

Sugar analysis and Quantification

To extract sugars (*myo*-inositol, sorbitol, sucrose, raffinose, stachyose and verbascose) three replications of five dehydrated soybean seeds per replication were weighed, ground in a mortar and pestle and then pulverized in liquid nitrogen using a mortar and pestle. One aliquot of 1 mL 70% (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. 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, diluted to 25mL with distilled, deionized water. The supernatant was recovered (5 EtOH + 25 HOH = 30 mL) and 1/3 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 eight hours. 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 using a 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 standards. Reintegration of the peak start- and stop-times, baseline identification, and areas under the curve were performed using Chromeleon software (Version 6.8; Dionex Corp). Estimates of sugar amounts per mg/tissue were adjusted for losses during processing by comparing external standard 2-DG quantities with the recovery of 2-DG added during extraction (internal standard) (Downie and Bewley, 2000; Nosarzewski et al., 2012).

Flowering gene expression and staging

To determine the early maturation in VgD, the expression of two genes were tested flowering locus 2 and 5 (Flowering locus T2 (FT2) and Flowering locus T5 (FT5)), in both seed and pod. Five whole pods and leaves were collected from each replication at growth stage R5. Samples were stored at -80°C.

RNA isolation

1- Weight out 50 mg from e freeze (-80 °C), after that put the sample in a mortar and pestle and freeze and grind the samples with liquid nitrogen to a fine powder. Add 10 mL of Trizol to the frozen tissue, mixing well until liquid the powder is thawed. It is possible could take few minutes for the slurry of frozen tissues to liquefy in the presence of trizol. None of the tissues should be allowed to thaw without being incorporated into the Trizol solution.

2- Allow the thawed Trizol/leaf/pod tissues at room temperature for 5 minutes. Add 0.2 mL chloroform per ml Trizol used in step 1. Shake the tube vigorously for 15 seconds, followed by incubation at room temperature for 3 minutes.

3- Centrifuge at 12,000x g for 15 minutes.

4- Carefully transfere the upper aqueous layer to a fresh tube. Add 0.5 mL isopropyl alcohol per ml of transferred aqueous layer. Store at -20°C overnight.

5- Centrifuge at 7500x g for 5 minutes.

6- Discard the supernatant and air-dry the pellets for 5-10 minutes.

7- Dissolve the RNA in 50-200 µL of RNase-free water. Incubate at 55-60°C for 10 minutes. To completely dissolve the RNA, it may be necessary to pipette RNA solutions following the 10-minutes heating at 55-60°C.

cDNA synthesis

1- Mix 14 µL of purified RNA with 1 µL of the biotinylated reserve transcription primer (100 pmol/ µL) heat to 65°C and incubate for 5 minutes in heat block, chill on ice for 2 minutes.

2- Add 5 µL 5x RevetAid reverse transcriptase first strand buffer 2.5 µL 10 x dNTPs for reverse transcription,1 µL 100 Mm DTT, and 0.5 µL RNase inhibitor to the tube from step 1. Bring the tube to 42°C in the thermocycler with heated lid. Then add 1 µL RevertAid reverse transcriptase and incubate for 2 h at 42°C.

3- Heat the mixture to 70 °C for 5 minutes. Add 1 µL RNase H and RNase A/T1 (this combination of the ribonucleases exceeded the inhibitory capacity of the RNAse inhibitor added to the reverse transcription reaction by more than 10-fold. This treatment eliminates the RNA (free hydrolyzed to *cDNA*) Allowing unfettered access of the *cDNA* to DNA primers and reducing the possibility of amplification of RNA by Taq polymerase that may possess low level of reverse transcriptase activity under some conditions). Incubate at 37°C for 1 h.

4- Purify the *cDNA* using the PCR purification kit, elute into 50 µL of the elution buffer provided with the PCR purification kit (Hunt and Li, 2016).

RT-PCR and quantitative RT-PCR analyses

RT-PCR of GmFT2a and GmFT5a and Tubulin (as an internal control) was, conducted using *cDNA* synthesized from RNA. PCR condition were as follow:

The quantitative RT-PCR mixture was prepared by mixing 12.5 µL of 5 mL of SYBR® Premix SsoAdvanced ™ Universal and water to a final volume of 25 µL. 1 µL of *cDNA*, 1 µL selective primer and 10.5 Milli-Q water. The PCR cycling conditions were as

follows: 95°C for 1 min, 95°C for 15 s, 57°C for 40 s, 72°C for 30 s, This cycle was repeated 39 times (Kong et al., 2010).

Statistical analysis

The measurement of sucrose, stachyose and pods dry weight at different growth stages qualifies as a repeated measures experimental design. Seed dry weight was analyzed separately a bilinear with plateau model was fitted to the response of seed dry weight (Y) and days after pod painting (X).

$$y = a + bx$$

Where (a) is the intercept, b the slope at the response part of the curve. The second step is to use the slope in randomize complete plot designed, The main effects of variety and location environment, the environment x year interaction were considered fixed effects while replication was considered a random effect in the ANOVA for each environment. LSD comparison was used to separate means, if significant, at a critical level of $P \leq 0.05$.

Results and discussion

Evaluate the effect of enhanced DGAT oil/protein soybean on flowering, maturation time and seed and pod growth rate

Number of days to R1 (beginning bloom) was significant in both years, in 2015 VgD1-2 gave the lowest days to R1, however, it was similar to VgD1-1 in 2016, and lower compared to Jack. Seed growth rate (SGR) was significantly lower in both years of study. The VgD1-1 performance was consistent, whereas VgD1-2 was lower in 2015 only. Soybean seed dry weight accumulation follows a certain pattern starting with a short phase of exponential pattern, and a constant growth afterward, then a final phase with decreasing in seed weight, until the final seed weight at physiological maturity (Egli, 1975; Egli and Leggett, 1976). The reduction in SGR reduction in seed growth rate in VgD1-1 was accompanied with a numeric increase in seed filling period, however no reduction was found in SFP in 2016. Instead of beginning of flowering (R1) was prolonged in VgD1-1f compare to jack (Table 2-1). In 2016 VgD1-2 shows no significance in seed growth rate, but was lower in seed filling period compare to jack.

The number of days to R7 (Beginning of maturity) was significant in both years of study. VgD1-1 was about seven days earlier in 2015 and five days earlier in 2016 compart to the jack. VgD1-2 showed no differences in a number of days to the beginning of maturity (R7) compare to jack (Table 2-1). Temperature differences were small (Table 2-5), that they were unlikely to make an effect on seed duration, these results are in agreement with (Egli, 1994). Oil g/100g was numerically higher in 2015 and significantly higher in 2016. No significant differences were found in yield, seed number and seed weight for both years of study (Table 2-2) however the modified oil lines were numerically lower in seed weight and higher in seed number for both years of study. Yield depends on two components seed size and seed number in addition, seed size has two components as well (SGR and SFD). SGR is influenced by the environment and genetic background. Since the temperature was stable as we mentioned earlier, the genetic background could be the main reason for the reduction in SGR in the current study (Table 2-1 and 2-2). This reduction is associating in compensatory changes with seed number with no effect on yield (Egli, 2017). This result in agreement with (White, 1981), when he conducted his experiment on determine and non-determine beans. Different study conducted by (Egli and Leggett, 1976), mentioned that the SGR is not closely related to the photosynthesis proses, and the carbohydrate in the seed works as a buffer between seed growth rate and the photosynthesis process. Unlike SGR, seed fill is directly related to yield in soybean (Smith and Nelson, 1986) which mean even if long seasonal duration available, and short seed filling, the final yield will not improve. (Egli, 1993) found no difference in yield between MG I, III and V, under an irrigated condition, these responses were consistent across years and cultivars. This results in agreement with (Swank et al., 1987), finding the variation in final seed size is associated with either the variation in SGR or SFD. *FLOWERING LOCUS T* (FT), is a systemic inducer of flowering that is expressed in the companion cell of the phloem and transported to the shoot apex (Nakamura et al., 2014). FT genes are not only induce flowering but also promote maturity since both *E3* and *E3* encodes copies of PHYTOCHROME A protein, as well as *GmFT2a* and *GmFT5a* as homologues of *FLOWERING LOCUS T* coordinately promote flowering in soybean (Xia et al., 2012). Figure 2-1 and Figure 2-2, showed a high expression for *FT2* and *FT5* in seeds for VgD1-1 which showed early maturation compare to another VgD line, but no

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expression was found in VgD1-2, and no expression was found also for both gene in pods.

The trend for pod dry weight (mg/pod) Table 2-3 did not show significant differences, at the early and late stages, for each line individually, however, the differences between Jack and the modified oil lines was noticeable for both years both VgD were smaller in final pod weight to the control. Pod dry weight was inconsistence across year in the current study, and the modified oil lines gave the lowest pod weight at maturity, (Egli and Leggett, 1976) found by creating strong sink by removing pods from the plant, that pods serving as a buffer toward the photosynthesis and seed growth to maintain the assimilate supply. This could be possible in VgD lines in the current study, since they have over expression DGAT which create a strong sink, therefore the pod dry matter was variable to maintain the assimilate supply to the seed. What support this theory, that the final pod weight for VgD lines was lower compared to the control (Table 2-3).

Effect of enhanced DGAT oil/protein soybean on photosynthate levels in seeds and pods in field conditions

Sucrose and the oligosaccharides stachyose and raffinose are the most common free sugars present in soybean (Nonogaki, 2013). The rate of sucrose was increasing at the early stages of cotyledon development and then mostly decreases toward seed maturation (Table 2-4). On the other hand, pod sucrose (Table 2-5) was variable but decreased significantly at maturation. The modified oil lines gave the lowest g/100 g weight compare to the control. Stachyose, starts accumulating at mid-point of seed development. Stachyose, was higher in the modified oil seed compart to the control.

Soybean sucrose accumulation in pod was increasing close to seed maturation and then decline. The carbohydrates (sucrose and stachyose) differences were variable during seed and pod development among the studied lines. The reduction in sucrose suggested that the accumulation of oil in VgD lines, is limited by source strength and sensitive to any manipulation in cytosolic sucrose supply. (Roesler et al., 2016b), however that stachyose increase compart to the control. in agreement with (Phillips et al., 1984) finding sucrose begins to accumulate prior to mid-pod fill as a function of elevated inverses and sucrose synthase activity. The early maturation apparently did not have a noticeable effect on the modified oil lines. this results agree with Hymowitz (1974) , they found a significant

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variability in soluble carbohydrate when they evaluated 195 soybean cultivars from maturity group 00-IV, in addition Hymowitz (1972) found that total sugars and oil concentration are correlated positively, and the maturity and sugar could possibly influenced by the environment and genetic background. In reference to that temperature could possibly be a limited factors in sucrose accumulation, since sucrose dropped drastically when the temperature increase, (Wolf et al., 1982) that we did not experienced in the current study.

Sucrose concentration in pod mg/100g declined during seed growth rate, reaching the lowest concentration at maturity. This could possibly support the idea that pod sucrose is consider as a buffer to maintain the photosynthesis assimilate to the seeds (Swank et al., 1987), even though the sugar in pod is much lower compare to seeds. That could be suggest the compensatory relationship between pods and seed the products of photosynthesis in seed composition, however, it did not effect on the final yield. Similar results was found (Egli, 1993; Schweitzer and Harper, 1985), which suggested changing the growth cycle for soybean did not necessary effect the final yield.

Conclusions

The growth of high oil soybean lines (VgD) are clearly influenced seed growth rate. The high active acyl-CoA: diacylglycerol (DGAT) from *Vernonia galamensis* (VgDGAT1A) had of lower seed growth rate, accompanied with fewer days to the beginning of maturity (R7). Seed filling period was numerically higher in one year and significantly higher in the other. Days to R5 (begging of seed) was significantly lower in both years suggesting the high oil lines has shorter reproductive stage, considering not much change in days to flowering (R1).

Carbohydrate were variable, lower sucrose and higher stachyose at maturity. These results suggesting carbohydrate concentration may not be the limiting factor in seed growth. The increase in oligosaccharide suggesting expression of DAGT with combination of reducing Raffinose oligosaccharide may allow even more oil to be provided.

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Table 2- 1 Seed growth characteristics of soybean lines with modified seed oil level.

Component	2015			2016		
	Jack	VgD1-1	VgD1-2	Jack	VgD1-1	VgD1-2
Seed growth rate (mg/seed/day)	3.4 [a‡]	2.7 [a]	2.4 [b]	4.2 [a,c]	3.4 [b]	3.9 [c]
Effective filling period (Days)	36.3 [N.S]	40.5	41.5	30.0 [a,b]	30.7 [b]	29.0 [c]
Days to R1	33.0 [a]	33.0 [a]	31.3 [b]	40.0 [a]	43.0 [b]	42.0 [b]
Days to R7	97.3 [a]	90.0 [b]	94.0 [b,a]	96.0 [a]	91.0 [b]	93.0 [b]
Oil g/100 g	21.0	22.2	23.0	22.3 [a]	24.0 [b]	24.0 [b]

‡ Values followed by the same letter within each variety and seed component (protein, oil and fatty acids) are not statistically different at P< 0.05.*The results represent the average of two years in one location(Spindletop).

Table 2- 2. Yield and yield components, of soybean lines with modified seed oil level

Component	2015			2016		
	Jack	VgD1-1	VgD1-2	Jack	VgD1-1	VgD1-2
Yield kg/ha^{-1}	2109 [N.S]	1930	1867	2150	2096	2096
Seed weight mg/seed	111	100	100	119	112	115
Seed Number	1901	1925	1867	1808	1873	1819

NS, no significant different

Table 2- 3 Changes in pod weight (mg/pod) of soybean lines with modified oil level during seed development for 2015 and 2016 Spindletop KY.

		2015			2016	
		Pod			Pod	
Genotype	Days	mg/Pod	±SE	Days	mg/Pod	±SE
Jack	0	109.7 [a,b]‡	11.2	0	105.9 [a]	11.2
	7	96.8 [b]	9.5	7	113.4 [b]	9.5
	13	89.7 [b]	11.8	12	105.1 [c]	12
	21	96.2 [b]	11.6	18	113.5 [b]	12
	25	100.8 [b]	12.4	23	129.5 [b]	12.4
	35	104.7 [a,b]	13.8	27	125.3 [b]	14
VgD1-1	0	61.1 [a]	11.2	0	89.5 [a]	5.0
	7	86.5 [a,b]	9.5	7	111.5 [a]	3.3
	13	98.8 [b]	11.9	12	109.5 [a,b]	1.7
	21	97.1 [b]	11.6	18	117.4 [b]	14.6
	25	85.1 [a,b]	12.4	23	113.5 [b]	7.3
	35	86.1 [a,b]	14.0	27	103.5 [a]	3.5
VgD1-2	0	71.3 [a,b]	11.2	0	84.3 [a]	7.0
	7	97.0 [b]	9.5	7	83.6 [a]	6.9
	13	89.6 [a,b]	12.0	12	97.4 [a]	1.8
	21	116.0 [a,b]	12.0	18	107.5 [a]	2.6
	25	116.3 [b]	12.4	23	115.3 [a]	1.6
	35	96.9 [b]	14.0	27	115.5 [a]	16.3

‡ Values followed by the same letter within each variety and seed component (protein, oil and fatty acids) are not statistically different at P< 0.05.*The results represent the average of two years in one location(Spindletop).

Table 2- 4. Changes in cotyledon sucrose concentration during seed development of soybean lines with modified oil level for 2015 and 2016 Spindletop KY

		2015			2016	
Genotype	Days	Cotyledon-Sucrose (g/100g)	±SE	Days	Cotyledon-Sucrose (g/100g)	±SE
Jack	0	3.8 [a‡]	0.6	0	4.7 [a]	0.6
	7	5.4 [b]	0.4	7	5.5 [b]	0.4
	13	5.3 [b]	0.3	12	5.0 [a,b]	0.3
	21	4.3 [a,b,c]	0.4	18	4.0 [a]	0.4
	25	3.3 [d]	0.2	23	2.2 [c]	0.2
	35	2.1 [d]	0.2	27	2.2 [c]	0.2
VgD1-1	0	4.3 [a,c]	0.6	0	5.2 [a]	0.6
	7	5.6 [b]	0.4	7	5.7 [a]	0.41
	13	5.2 [a,b]	0.3	12	4.2 [b]	0.3
	21	4.0 [c]	0.4	18	4.0 [b]	0.4
	25	2.6 [c,d]	0.2	23	2.0 [c]	0.2
	35	1.5 [d]	0.2	27	1.5 [c]	0.2
VgD1-2	0	3.2 [a]	0.6	0	5.6 [a]	0.6
	7	5.4 [b]	0.4	7	5.5 [a]	0.4
	13	4.4 [b,c]	0.3	12	4.6 [b]	0.3
	21	3.5 [a,b]	0.4	18	4 .0 [b]	0.4
	25	2.5 [a,d]	0.2	23	3.0 [c]	0.2
	35	1.7 [d]	0.2	27	2.5 [c]	0.2

‡ Values followed by the same letter within each variety and seed component (protein, oil and fatty acids) are not statistically different at P< 0.05.*The results represent the average of two years in one location(Spindletop).

Table 2- 5. Changes in pod sucrose concentration during seed development of soybean
lines with modified oil level for 2015 and 2016 Spindletop KY

		2015			2016	
		Pod-Sucrose			Pod-Sucrose	
Genotype	Days	(mg/100g)	±SE	Days	(mg/100g)	±SE
Jack	0	336.1 [c‡]	51.5	0	769.8 [a,b]	48.4
	7	400.9 [b,c]	69.4	7	715.1 [a,b]	55.6
	13	560.7 [a]	60.5	12	597.4 [a,b,c]	71.0
	21	519.3 [a]	47.5	18	239.0 [c]	46.1
	25	112.1 [d]	13.6	23	273.9 [d]	46.1
	35	47.4 [d]	11.6	27	21.8 [d]	7.4
VgD1-1	0	372.6 [b,c]	45.4	0	605.9 [a]	45.1
	7	393.3 [b,c]	48.4	7	689.2 [a]	48.4
	13	513.5 [a,b,c]	52.0	12	858.9 [b]	55.6
	21	401 [a,b,c]	71.0	18	677.4 [a]	70.8
	25	103.2 [d]	46.1	23	208.4 [c]	45.0
	35	24.3 [d]	7.3	27	26.5 [d]	7.3
VgD1-2	0	329.5 [a,b]	45.1	0	675.7 [a]	50.8
	7	377.4 [b,e]	48.4	7	735.0 [a]	48.4
	13	495.3 [a,c,e]	51.5	12	736.4 [a]	51.5
	21	387.2 [a,c]	71.0	18	617.4 [a]	70.8
	25	106.6 [d]	46.1	23	178.7 [b]	46.1
	35	26.1 [d]	7.4	27	13.7 [c]	7.4

‡ Values followed by the same letter within each variety and seed component (protein, oil
and fatty acids) are not statistically different at P< 0.05.*The results represent the average
of two years in one location(Spindletop).
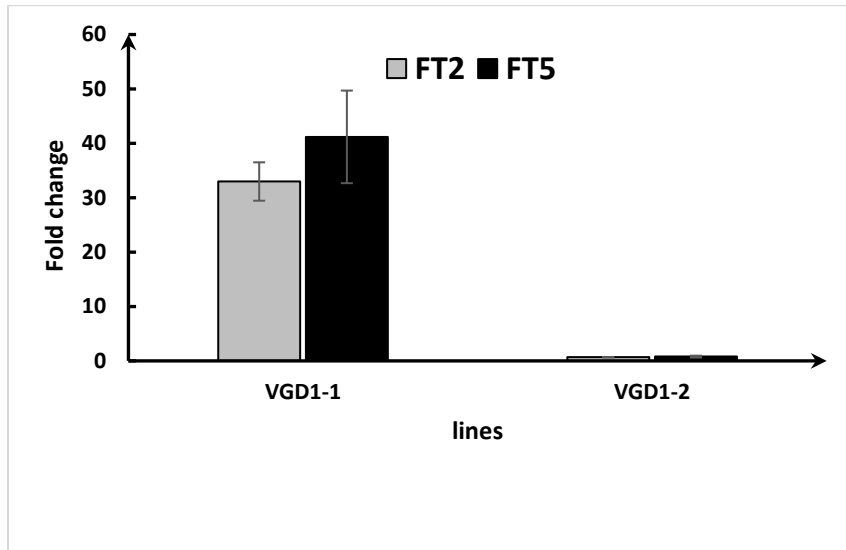
Figure 2- 1. Gene expression (FT2 and FT5) for VgD lines compare to Jack in leaves.
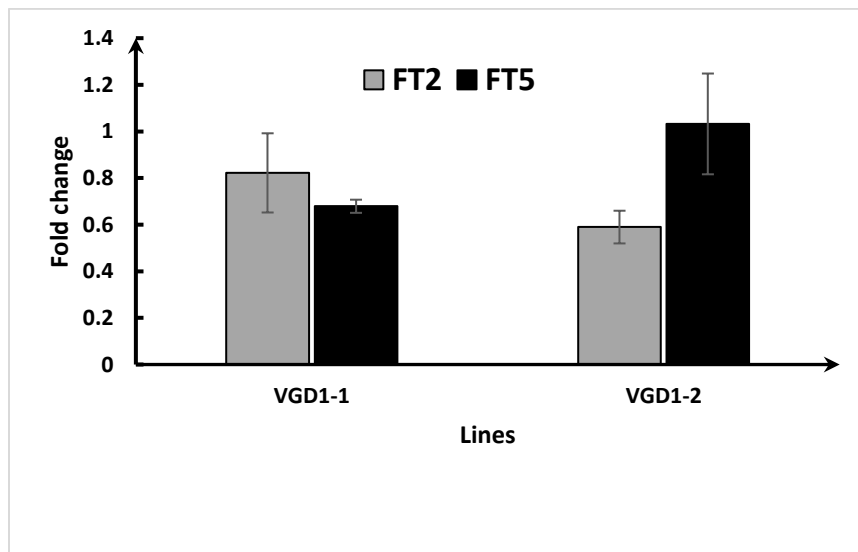
Figure 2- 2. Gene expression (FT2 and FT5) for VgD lines compare to Jack in Pods.

Chapter 3: Proximate composition of enhanced DHAT high oil/protein soybeans

Abstract

Developing new soybean (*Glycine max*) varieties with greater levels of both oil and protein will further enhance soybean as a valuable, renewable source of food and fuel. However, breeding for greater oil content in soybean usually results in lower protein. Using a highly active acyl-CoA: diacylglycerol acyltransferase (DGAT) from *Vernonia galamensis* (VgD), expressed in transgenic soybean, it was possible to achieve an increase of 4% in oil, relative to oil recovery from parental and empty vector controls, without a reduction in protein. To determine how oil increased (while maintaining protein content) was, two independent *VgD* transgenic soybean lines and relevant controls were grown in two field locations in two different years. Proximate analysis was performed to address what seed component(s) was/were reduced to compensate for the oil increase. The seed oil in the VgD transgenic seeds was greater than in empty vector control seeds in both locations. The VgD maintained wild type protein levels in one location and increased protein content in the other, suggesting that the trait was maintained across two environments. The soluble carbohydrate (sucrose) fraction was reduced in the VgD lines one location but this trend was not statistically significant in the other. The VgD lines did not show significant yield differences vs. the control. This study demonstrated that engineering soybeans with a highly active DGAT could increase oil levels, at the expense of carbohydrate components, while at least maintaining protein amounts but this approach could not explain all the oil increase.

KEYWORDS: Proximate analysis, Soybean, Oil and DGAT (Diacylglycerol acyltransferase).

Introduction

Soybean are an affordable source of oil and protein for use as food and as animal feed (Brumm, 2004). The composition of the seeds are rich in protein (35-45%) and contain a relatively high level of oil (20-25%) compared to other cereal and legume species (Salunkhe et al., 1983).

As a globally produced crop, the 2014/2015 soybean yield reached 48 million metric ton of oil and 88 metrics tons of soybean meal, which accounts for about 27% and 68% of world plant oil and protein meal requirements (www.fas.usda.gov/data/oilseeds). The United States, Brazil, and Argentina are the three top soybean producing countries, contributing approximately 82% of world soybean production in the 2014 season (http://www.soystats.com/2014). Increasing oil yield per unit land area using conventional breeding has continued to progress (Egli, 2008). 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. Theoretically, an increase in the compositional value of soybeans via altered carbon partitioning should be plausible, since a portion of the soybean dry weight is composed of less valuable fiber and the low digestible oligosaccharides (Padgette et al., 1996), which could be diverted into oil or protein. However, neither conventional breeding nor quantitative trait locus mapping approached for increasing soybean oil while maintaining Protein content have been successful, because increase in one of these component associate with decrease in the other. Soybean oil is composed of triacylglycerol (TAG), which is fueled by sucrose delivered from the maternal plant leaves and other photosynthetic tissues (Allen et al., 2009; Hildebrand, 2010; Hildebrand, 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 further vascular connections in the integuments and 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 has been three main steps leading to higher TAG, increased flux of hydrocarbon into fatty acid biosynthesis 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., 2008b; Rao and Hildebrand, 2009; Taylor et al., 2009a; Vyacheslav et al., 2009). This is particularly rational considering seed oil is biosynthesized during the second main stage of seed maturation (Goldberg et al., 1989; Harwood and Page, 1994; Le et al., 2007), at which time the relevant biosynthetic enzymes are highly expressed. TAG biosynthesis requires the acylation of glycerol 3 phosphate (G3P) to three free fatty acids (FFAs). Phosphatidyl choline (PC) has been identified as an intermediate in oil biosynthesis and plays a central role in the production of polyunsaturated fatty acids by serving as a substrate for Δ-12, and ω-3 desaturases (Jackson et al., 1998; Lu et al., 2009a). The final acylation step is catalyzed by diacylglycerol (DAG) acyltransferase (DGAT) (Saha et al., 2006).The nature of the acyl composition of the TAG is dependent on the availability of the fatty acids from the acyl-CoA substrate pool as well as the selectivity of the acyltransferases of the Kennedy pathway (Harwood, 1997; Harwood and Page, 1994) and possibly transacylase. Elevated expression of regulatory genes that up-regulate multiple enzymes for fatty acid biosynthesis also can result in higher oil levels (Andrianov et al., 2010). Co-expression of the transcription factor WRI1 with 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; Vanhercke et al., 2014b). Expression of a *Tropaeolum majus* DGAT1, TmDGAT1, was reported to increase oil content, seed size and yield in Arabidopsis (Xu et al., 2008a). Similar increases in oil content and seed yield were reported with higher expression of a *Brassica napus* (canola) DGAT1, BnDGAT1, back in *B. napus* (Siloto et al., 2009). They also reported enhanced drought tolerance of this transgenic canola with increased DGAT expression. Lardizabal et al. (2008a) reported a 1.5% increase in oil levels of transgenic soybeans expressing a fungal DGAT2. Over expression of DGAT1 from *Sesamum indicum* in soybean 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 attempts have produced lines with high protein content and enhanced the amino acid composition, but this has been impeded by the negative correlation between yield and protein content of the seeds and the deficiency in sulfur amino acids, which lower digestibility of the legume protein (Hill et al., 2005b; Liener, 1994;

Peregrine et al., 2008). The amino acids for seed protein synthesis, including storage proteins, arrive from leaves mainly in the form of the N rich amino acids asparagine (asn) and glutamine (gln). In soybeans, fixed nitrogen is exported from nodules as ureides, which are reconverted into amino acids in leaves, and ureides do not directly enter developing seeds. In conventional breeding, seed protein and oil content is 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 sugar monomers and hexose phosphates 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-arranged to phosphoenolpyruvate, which can 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 hydrocarbon for fatty acid biosynthesis in plastids and oil (TAG) synthesis in the Endoplasmic Reticulum (ER).

Carbohydrates are the third abundant component in soybean seeds and account for 35% of the dry seed weight; approximately half of the total carbohydrates in soybeans are structural carbohydrates, whereas the other half are nonstructural. Structural carbohydrates are cell-wall polysaccharides (cellulose, hemicellulose, and pectin), whereas non-structural carbohydrates include sucrose and other mono-, di- and oligosaccharides (Karr-Lilienthal et al., 2005b). Soybean seeds has 6% (DW) crude fiber and 27% nitrogen free extract (NFE). The sugars found essentially at the cotyledon of soybean seeds, free galactose, glucose, and fructose was found in 10 soybean samples ranged between 0.7-4.0,1.2-4.7 and 1.1-4.7 mg/g (DW), (Grieshop et al., 2003). Sucrose is the primary sugars found in soybean seeds and that ranged between 37.1-72.5 mg/g on dry seed, these results represented 18 different high oil soybean lines, however sucrose concentration in 20 high protein soybean lines were lower and ranged between 21.8-48.9 mg/g DW (Hartwig et al., 1997). The other half of the SB (soybean) and SBM(soybean meal) carbohydrate is made up of structural polysaccharides. This includes dietary fiber that is comprised of cellulose, pectin, and hemicelluloses, along with mannons, galactans, and xyloglucans. The neutral detergent fiber (NDF) concentration of

36 SB samples grown in the US ranged from 11.26% to 18.52% of DM, whereas this range was from 11.5% to 17.1% of DM for 48 SB 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 dry weight (Medic et al., 2014a). 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 (NDF) neutral detergent fiber, underestimate pectins, a soluble fiber fraction (Jung, 1997). Transgenic soybean with DGAT have been reported with higher oil without protein reduction and this relationship explained by the tradeoff between oil and soluble sugars. Since 3.5 increase in seed, storage composition (oil and protein) is accompanied by 1.9 percentage point reduction in soluble sugar, there were also small absolute sugar fraction were reduced as well (Fructose and glucose. This shift in seed composition cannot entirely explain by the reduction in the soluble sugars (Roesler et al., 2016c). Among the major minerals components in soybean potassium is found in the highest concentration followed by phosphorus, magnesium, sulfur, calcium, chloride and sodium. The content of these minerals ranged from 0.2-2.1% on average values (Lui, 1977), whereas (Medic et al., 2014a)account 4-5 percentage for total Ash in soybean seed. New soybean lines have been developed with significantly higher oil and protein (3-5 higher) and higher protein meal.in different environment, compare to 3.5 percentage point increase in seed oil+ protein achieved by (Roesler et al., 2016a) Such soybeans have higher nutritional and economic value, the new DGAT lines has been advanced to field trials to determine the effect of different environment on seed composition.

Materials and Methods

Seed Collection

Four varieties were used in the field experiment, (VgD1-1, VgD1-2, Jack (control) and empty vector (VC), Both VgD lines used in the study, have highly active acyl-CoA:diacylglycerol acyltransferase (DGAT) from *Vernonia galamensis* (VgDGAT1A) (Hatanaka et al., 2016). The field studies were established at two locations in Kentucky during 2015 and 2016. One site was located at the Spindletop Research Farm in

Lexington, KY (38.125835 N, -84.496781W) and the experiment was tillage, soil type at this location was a Loradale Silt Loam (Fine, mixed, active, mesic Typic Argiudoll). The other site was located on Princeton, KY (37.098749,-87.868730) the soil type was crider sily loam (fine –silty, mixed, active, mesic Typic Palueudalf). Transgenic soybean (*Glycine max* L.) seeds with highly active acyl-CoA:diacylglycerol acyltransferase (DGAT) from *Vernonia galamensis* (VgDGAT1A) that can produce 4 % more oil than controls (Hatanaka et al., 2016). This material was used at both locations in both years. Planting occurred in mid-May in 2015 and late- May to early June in 2016.   All plots were seeded in 0.38 m row spacing and at a seeding rate 100000 seeds ha^{-1}. The herbicides Authority xl (Sulfentrazone:Chorimuron) full rate 474.80 mL /ha^{-1} and Dual II magnum (S-metolachlor) 1500 mL/ha^{-1} helped reduce weed in the plots in both locations and growing seasons.

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. Five mL of concentrated sulfuric acid containing 0.05 g of salicylic acid/mL were added and the samples allowed reacted for one hour at room temperature (Bradstreet, 1965). 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 one hour. This resulted in 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 cooled 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 to 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, one containing 0.5% sodium hydroxide and 0.042% sodium hypochlorite in deionized water, the other containing 1.0% phenol and 0.02% sodium nitroprusside in deionized water. The sample was introduced into a bubble-segmented stream followed by the reagents. 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) and is the same as 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 molar sulfuric acid was reacted with the samples in the segmented stream to form a heteropoly phosphomolbdate 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.

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 hours at 110 °C in 6N HCl, in accordance with AOAC procedure 994.12. In order to determine methionine content, ~50 mg of ground sample underwent performic acid oxidation procedures, as described in AOAC method 994.12, prior to the acid hydrolysis procedures. Following hydrolysis procedures, 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%.

Mineral Analysis

Samples were sieved in 0.45μm sieve, and run on ultimate proximate analyze, The Proximate analysis (Moisture, Ash, Volatile material, Fixed Carbone) were analyzed using a LECO TGA 701, Ultimate analysis (Nitrogen and total sulfur) were analyzed using a LECO CHN 628 and the elemental analysis was analyzed using a Varian 720-ES Spectrometer. Fixed Carbone was analyzed at 950 °C in O_2, Total sulfur at 1350 °C, Moisture at 107 °C, Volatile material at 950 and Ash at 750 °C.

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 hours. Samples were then hydrolyzed in 67% sulfuric acid for 1 hr. The glucose content was then determined using the anthrone method. Here, 20uL of sulfuric acid hydrolyzed sample was mixed with 500 μL water to 1ml 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 cellulose polymer (Updegraff, 1969).

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 twice and dried. Once dry, 500 μL sodium methoxide was added and shaken for 10 minutes with 1 mL isooctane containing 0.001% BHT added after 200 μL was pulled off the upper layer and transferred into a GC vial with an additional 1 mL of isooctane

added. GC vials were then run on a Varian CP-3800 Gas Chromatograph 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 um. The temperature program ran from 90 C to 250 °C with 25 °C ramp for a total of an 8-minute 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 and peaks quantified using Star Chromatography Workstation Version 6.00, with peak area being used to calculate relative percentages of FAMEs (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).

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. 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, diluted to 25mL with distilled, deionized water. The supernatant was recovered (5 EtOH + 25 HOH = 30 mL) and 1/3 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 eight hours. 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 using a 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 standards. Reintegration of the peak start- and stop-times, baseline identification, and areas under the curve 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 with the recovery of 2-DG added during extraction (internal standard) (Downie and Bewley, 2000; Nosarzewski et al., 2012).

NDF (Neutral Detergent Fiber)

Sample dry weight for NDF analysis was 0.5 g., which 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 for. The method to determines Neutral detergent fiber (NDF) which is the residue remaining after digesting the sample in detergent solution. The fiber residue are predominantly hemicellulose, cellulose and lignin. (Vogel et al., 1999).

Starch

Starch analysis was conducted using Kit provided by Bioassay system kit (EnzyChrom™ starch assay kit), but the starch level was undetectable for this we did not include the data.

Statistical analysis

1. Statistical analysis was performed using the SAS 9.2 statistics package (SAS Institute Inc., Cary, NC). We combine years over locations, since each location has different environmental attributes and soil types, also we have planted tillage in Spindletop and no-till in Princeton locations. The main effects of variety and location environment, the environment x year interaction were considered fixed effects while replication was considered a random effect in the ANOVA for each environment (Supplement 1 and 2). LSD comparison was 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.

2. 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. 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.

Result and discussion

Seed oil content shifts in transgenic DGAT alleles

In the current study, we compared the level of the key nutritional and anti-nutritional components in (VgD) soybean lines compare to the wild type (Jack) and conclude that targeting engineering of the *DGAT* gene is a satisfactory mechanism to enhance oil content in soybean and results in compensatory metabolic shifts. In fact, 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 regardless of location ($P \leq 0.05$). Seed oil was 4 % greater at the Princeton location and 1.6 % greater at Spindletop (Table 3-1) and this increase accompanied with no reduction on protein with different environment, this finding is consistent with (Hatanaka et al., 2016). DGAT1A from *Vernonia galamensis* exhibits high activity compare to orthologous DGATs, and is a good candidate for increasing

renewable oil production. A similar approach was used in a yeast system and gave the same results (Lardizabal et al., 2008b; Xu et al., 2008a).

Shifts within oil composition related to genotype

Interestingly, a trend was observed whereby 18:1 fatty acids increased in VgD1-1 and VgD1-2 lines at both locations. Here, we measured an 11 and 17-% increase for Princeton, and 21 and 19 % increase at Spindletop compared to the control. This increase was accompanied by a reduction in 18:2 by 8 and 16 % in Princeton and 16 and 15 % in Spindletop for both lines. Empty vector (VC) showed significant differences but not in all fatty acids (Table 3-1). This alteration in fatty acid profiles could possible affect the high oil soybean line uses. 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 Kennedy pathways. Improved DGAT with increase affinity for 18:1 CoA will compete efficiently to produce 18:1 CoA, resulting in more 18:1 fatty acid acyl groups going directly to TAG, without going to PC, thus avoiding additional desaturation (Roesler et al., 2016c).

Increased expression of foreign and native DGATs have been found to increase TAG oleoyl (18:1) levels in species as diverse as maize (*Zea mays*), olive (*Genus species*) (Banilas et al., 2010), Arabidopsis (*Arabidopsis thaliana*) (Jako et al., 2001) and soybean (Hatanaka et al., 2016) consistent with the data in this study.

Carbohydrate profiling in transgenic versus control

Carbohydrate profiles were measured and showed a statistically significant trend in one location. Cellulose was also not uniform across locations. The cellulose content was significantly lower for the high oil lines grown at Princeton, but no significant differences were seen at Spindletop except for VC (Table 3-2). Sucrose levels showed differences in Princeton only, similar trends were shown for stachyose and total sugars (Table 3-2). Neutral detergent fiber (NDF) did not change among varieties and control when examined at both locations. However, in Spindletop NDF increased compared to Jack (control) in the high oil lines, whereas no significant difference was seen between the

high oil lines and the VC. This finding is not similar with (Lardizabal et al., 2008b) finding a 15% decrease (on percentage dry weight basis) in the transgenic positive relative to the control. Roesler et al (2016c) reported high oil GmDGAT1b-MOD soybean that displays 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 concluded that the high oil trait is accompanied by significant reduction in the insoluble fiber fraction namely pectin derived from galactose, which we did not include in our study. Rather, we observed shifts in cellulose content on a location basis. However, raffinose oligosacchrides still represented $\geq$ 1.5% of the transgenic seed weight implying that combination of DGAT genes with methods of decreasing raffinose oligosaccharides could be one approach to increase oil content.

Protein analyses
We measured protein amounts to assess whether a compensation occurred, which has been shown to influence fitness (REF). Protein amount showed no significance among studied varieties in Princeton except for VC; whereas in Spindletop it was greater in high oil lines compare to control (Table 3-1), VC gives the greatest protein quantities in Princeton 42.2 %, while it did not show differences from the VgD lines in Spindeltop 44.0 %. In addition no statistical significance was observed in protein Kg.ha^{-1} , except for VC that gave 551 Kg.ha^{-1}, and the lowest protein yield 216.4 Kg.ha^{-1}. The high oil lines showed no significance compared to Jack (Table 3-5). Princeton location acted aliitle different, Jack gave higher peotein Kg.ha^{-1} and it was no different than VC and VgD1-2 Most amino acids were significantly higher for VgD1-2 in Princeton compare to Jack, but the trend is not consistant in Spindletop (Table 3-3). Protein quality (amino acid composition) is more important than total protein content in soybeans for animal prospective, Animal feed needs to have a balanced amino acid composition to meet the animal's requirements (Medic et al., 2014a). Amino acids are the "building block" organic compounds linked in various combinations to form unique proteins. In human diets, amino acids are supplied by the variety of plant and animal proteins ingested (Miller-Garvin and Naeve, 2015). The essential amino acids that humans and monogastric animals cannot synthesize are phenylalanine, valine, threonine, tryptophan,

methionine, leucine, isoleucine, lysine, and histidine. High oil lines showed different responses in different location, except for leucine, isoleucine and lysine which did not show significant differences across locations. Sulfur amino acids as a part of the essential amino acids in soybean are the key element in livestock and poultry production, since soybean used as a high protein feed. (Krishnan, 2005) The sulfur-containing amino acid methionine, significantly varied for both locations. Significant reduction was shown in Princeton however, in Spindletop VgD1-1 line gave higher methionine level compare to Jack Glycinin also distinct from a nutritional standpoint, since it contains more sulfur-containing amino acids (Medic et al., 2014a) . No significant difference was found in Princeton in glycinin, however VC gave the lowest concentration in Spindletop (Table 3-3). Thus, our data conclude that no major change in protein content resulted from expression of either VgD lines in soybean compared with controls these results will enhance meal protein level without significant changes in amino acids levels, which could make the defatted meal more valuable for animal feed, food and many industrial applications.

Mineral analysis

Studying minerals composition in soybean has been done while ago and, and since then the genetics of soybean and the agronomic practices have exceptionally changed, and whether these modification could possibly change the mineral composition (Batal et al., 2010). Significant differences were found in minerals concentration by different locations, Zn, Na and Mg, were significant in Princeton, whereas Na, P, and K were significant in Spindeltop. Ash was significant only in Spindletop (Table 3-4). In addition, no significant differences was found in volatile matter, total sulfur and fixed carbon did not show significance over locations, whereas nitrogen was significant in Princeton and the line that expressing the empty vector (VC) was significantly higher 6.7 percent compare to the high oil lines and Jack. Volatiles matter and moisture content are part of the (ISO) The International Standard Organization Committee. (Pritchard, 1983), volatile matters constitutes those compounds which are driven-off as volatiles by heating while fixed carbon, refers to the remaining constituents after the releases of volatiles, excluding ash and moisture content (Lu et al., 2009b).

Yield analysis

Yield is the result of seed weight and seed size, yield was not different between Jack and VgD lines at Spindletop, seed weight was not significant, however VgD lines gave lower seed weight and compensate the reduction with the increase in seed number. At Princeton, the yield was significantly lower, seed number and seed size followed a similar trend, (Table 3-5). Plant height was not significant in Spindletop and Princeton. The oil yield Kg. ha^{-1} does not show significance between varieties in Spindletop, however in Princeton locations, soybean expressing empty vector (VC) gives 216.4 Kg.ha^{-1} (Table3-5). Maintaining agronomic practices are essential for high yield, low population density and weed presser could explain low yield for VgD lines compare to wild type (Jack) in Princeton (Gulluoglu et al., 2017). In addition, different planting methods were used tillage in Spindletop and no tillage in Princeton.

These results are consistent with (Lardizabal et al., 2008b) findings, where their results showed no significant changes in the content of protein or carbohydrates. In mature seed of the transgenic plants relative to controls, by using a codon-optimized version of a *DIACYLGLYCEROL ACYLTRANSFERASE 2A* from the soil fungus *Umbelopsis* (formerly Mortierella) *ramanniana* in soybean seed growing for three years and 63 locations. No major impact on protein and yield was found, and 1.5 percent point increase in oil was maintained inn their study, which indicated a stability of oil phenotype for soybean plants expression UrDGAT2A.

On the other hand Roesler et al (2016c) mentioned that the high oil trait in soybean GmDGAT1b-MOD, with no reduction in protein content was built on the tradeoff between oil and soluble sugars, but adding that the compositional shift cannot completely be explained even when we have a reduction in one component and increase in the other. They mentioned also the high oil trait is accompanied with significant reduction in insoluble fiber fraction namely pectin derived galactose.

Principal Components Analysis

To further test whether DGAT overexpression affects the biochemical composition of soybeans, we conducted a principal components analysis. 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, indicating that DGAT overexpression did not cause major compositional changes in our samples. Instead, sampling year and location were the primary sources of variation among samples. Along PC1, there was clear separation between samples collected in year 1 vs year 2, while along 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 the primary drivers of compositional differences in our samples. An inspection of the principal component loadings indicates that the separation along PC1 is primarily a result of increased 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) but higher levels of NDF.

Conclusions

VgDGAT1A transgenic soybeans showed consistently enhanced oil content without protein reduction under field condition but we could not fully address to what extent seed composition accounts for the oil increase in the new soybean lines The reduction in composition was inconsistent, across locations. In Princeton oil increase was higher and partially explained by the reduction in total sugar (Table 2), yet this reduction did not explain all increase in oil in Princeton location. Other composition could possibly be the missing composition associated with the oil increase such as insoluble fiber fraction such as pectin derived from galactose (Roesler et al., 2016c). In the current study, the oil was higher in both locations, this increased accompanied with increase in raffinose oligosaccharide which could consider as a carbohydrate pool for future increase in oil.

Table 3- 1. Oil, Protein and fatty acid composition (gm/100g) of soybean seed from VgD, empty vector and parental lines planted in two locations in KY.

Princeton	Jack	VgD1-1	VgD1-2	VC
Oil	21.0 [b]‡ *	25.0 [a]	25.0 [a]	18.0 [c]
Protein	40.2 [b]	39.3 [b]	41.0 [b]	43.2 [a]
Oil + Protein	61.2 [b]	64.0 [a]	65.3 [a]	61.2 [b]
16:0	10.0 [d]	10.4 [b]	10.3 [c]	11.5 [a]
18:0	4.8 [c]	5.1 [b]	6.0 [a]	4.5 [d]
18:1	25.0 [c]	28.3 [b]	30.4 [a]	25.0 [c]
18:1d	1.1 [b]	1.1 [b]	1.0 [c]	1.2 [a]
18:2	52.6 [a]	48.5 [c]	45.1 [d]	52.5 [b]
18:3	6.5 [b]	6.4 [b]	7.5 [a]	7.7 [a]
Spindletop	Jack	VgD1-1	VgD1-2	VC
Oil	21.5 [a]‡	23.1 [a,b]	23.4 [b]	17.0 [c]
Protein	40.0 [b]	42.0 [a]	42.3 [a]	44.0 [a]
Oil + Protein	61.4 [b]	65.1 [a]	66.0 [a]	61.0 [b]
16:0	10.1 [c]	11.0 [b]	11.0 [b]	12.0 [a]
18:0	5.0 [b]	6.0 [a]	6.0 [a]	5.0 [b]
18:1	26.1 [b]	33.1 [a]	32.5 [a]	25.0 [b]
18:1d	1.2 [b,a]	1.2 [b,c]	1.12 [c]	1.2 [a]
18:2	51.6 [a]	44.2 [b]	44.7 [b]	50.4 [a]
18:3	6.2 [b]	5.2 [c]	5.3 [c]	7.2 [a]

‡ Values followed by the same letter within each variety and seed component (protein, oil and fatty acids) are not statistically different at P< 0.05.*The results represent the average of two years in each location.

Table 3- 2. Structural and soluble carbohydrate composition (gm/100 gm) of Soybean Seeds from VgD, empty vector and parental lines planted in two locations in KY.

Princeton				
Component	Jack	VgD1-1	VgD1-2	VC
Structural carbohydrate				
Cellulose	4.6 [b]‡ [*]	3.2 [c]	4.4 [b,c]	6.5 [a]
NDF	26.1 [N.S]	26.4	26.0	23.4
Soluble carbohydrate				
myo-inositol	0.2 [a]	0.1 [b]	0.1 [b]	0.2 [a,b]
Sucrose	2.4 [a]	1.2 [b]	1.1 [b]	2.3 [a]
Raffinose	0.6 [N.S]	0.3	0.3	0.4
Stachyose	2.0 [a]	2.0 [a]	0.4	2.6 [b]
Verbascose	0.1 [N.S]	0.2	0.2	0.3
Total Sugars	5.0 [a]	3.1 [b]	3.0 [b]	6.0 [a]
Total carbohydrate (Estimated)	35.0 [a,b]	32.0 [b,c]	31.0 [c]	35.3 [a]
Spindletop				
Component	Jack	VgD1-1	VgD1-2	VC
Structural carbohydrate				
Cellulose	7.6 [N.S]	8.5	8.7	5.7
NDF	25 .0 [b]	28.2 [a]	28.4 [a]	27.3 [a,b]
Soluble carbohydrate				
myo-inositol	0.2 [N.S]	0.1	0.2	0.1
Sucrose	2.3 [N.S]	2.3	2.0	2.0
Raffinose	0.4 [N.S]	0.4	0.3	0.3
Stachyose	2.3 [N.S]	2.3	2.0	2.0
Verbascose	0.03 [N.S]	0.02	0.01	0.01
Total Sugars	5.1 [a]	5.0 [a,b]	4.0 [b]	4.0 [b]
Total carbohydrate (Estimated)	34.1 [a,b]	31.1 [b]	31.4 [b]	36.0 [a]

‡ Values followed by the same letter within each variety and seed component (structural and soluble carbohydrate) are not statistically different at P< 0.05. NS, no significant different. *The results represent the average of two years in each location.

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Table 3- 3. Amino acid composition (gm/100g) of soybean seeds from VgD, empty vector

Components	Princeton				Spindletop			
	Jack	VgD1-1	VgD1-2	VC	Jack	VgD1-1	VgD1-2	VC
Asp+Asn	3.1 [N.S]	3.4	4.0	4.0	4.0 [N.S]	4.0	4.0	4.3
Glutamic acid + Glutamine	5.3 [N.S]	6.0	7.0	6.1	6.2 [N.S]	6.5	6.4	7.2
Serine	1.5 [b‡]*	2.0 [a,b]	2.0 [a]	2.0 [a,b]	2.0 [b]	2.0 [b]	2.0 [a,b]	2.0 [a]
Glycinin	1.0 [N.S]	1.1	1.3	0.2	1.2 [b]	1.3 [b]	1.3 [b]	2.0 [a]
Histidine	1.0 [N.S]	1.0	1.0	1.0	1.0 [N.S]	1.0	1.0	1.0
Arginine	2.2 [N.S]	2.4	3.0	3.0	3.0 [b]	0.8 [b]	3.0 [b]	3.2 [a]
Threonine	1.0 [N.S]	1.1	1.2	1.2	1.2 [b]	1.2 [b]	1.2 [b]	1.4 [a]
Alanine	1.1 [b]	1.2 [a,b]	1.3 [a]	1.3 [a,b]	1.3 [b]	1.3 [b]	1.3 [b]	2.0 [a]
Proline	1.4 [N.S]	2.0	2.0	1.7	2.0 [a,b]	2.0 [a,b]	2.0 [a,b]	2.1 [a]
Tryosine	1.0 [N.S]	1.1	1.2	1.1	1.1 [N.S]	1.2	1.2	1.4
Valine	2.0 [N.S]	2.0	2.0	2.0	2.0 [a,b]	2.0 [a]	2.0 [b]	2.0 [a]
Isoleucine	1.0 [N.S]	1.0	1.0	1.0	2.0 [N.S]	2.0	1.4	2.0
Leucine	2.0 [N.S]	2.0	2.3	2.1	3.0 [N.S]	3.0	3.0	3.0
Phenylalanin	2.0 [N.S]	2.0	2.4	2.0	2.0 [b]	2.0 [a,b]	2.0 [b]	2.0 [a]
Lysine	2.0 [N.S]	2.0	2.0	2.0	2.1 [N.S]	2.2	2.1	3.0
Methionine	0.9 [a]	0.5 [c]	0.7 [b]	0.5 [c]	0.4 [b]	0.7 [a]	0.6 [a,b]	0.5 [b]
Norleucine	3.0 [N.S]	3.0	3.0	3.0	3.0 [N.S]	3.0	3.0	3.0
Total amino acids	30.0 [N.S]	31.0	35.0	33.1	34.2 [b]	36.2 [a,b]	35.0 [b]	40.1 [a]

‡ Values followed by the same letter within each variety and seed component (amino acids) are not statistically different at P< 0.05. NS, no significant different.
*The results represent the average of two years in each location.

Table 3- 4. Proximate composition of soybean seed from VgD, empty vector and parental Lines, planted at two locations in KY.

Components	Princeton				Spindletop			
	Jack	VgD1-1	VgD1-2	VC	Jack	VgD1-1	VgD1-2	VC
Ash	4.2 [a‡]	4.2 [a,b]	4.0 [b]	4.1 [a,b]	4.5 [a]	4.6 [a]	3.3 [b]	3.5 [b]
Moisture	7.0 [a,b]	6.1 [b]	7.2 a	7.4 [a]	5.7 [b]	7.0 [a,b]	7.8 [a]	8.0 [a]
Nitrogen	7.0 [b]	7.0 [b]	6.4 [b]	6.7 [a]	6.4 [b]	6.4 [a,b]	6.7 [a]	7.0 [a,b]
Total Sulfur	0.3 [N.S]	0.3	0.3	0.3	0.2 [N.S]	0.2	0.2	0.3
Volatile material	81.3 [a,b]	82.0 [a]	81.0 [a,b]	80.0 [b]	83.0 [N.S]	83.0	82.0	82.0
Fixed Carbon	8.7 [a]	8.5 [a,b]	7.9 [b]	8.7 [a,b]	6.4 [N.S]	6.3	7.5	6.8
Micro-Minerals								
Fe	7.0 [N.S]	6.0	5.4	5.2	8.0 [c]	8.1 [c]	8.7 [b]	9.7 [a]
Cu	1.1 [N.S]	1.1	1.1	1.1	0.9 [N.S]	0.9	0.9	1.1
Zn	3.7 [c]	4.2 a,b	4.1 [b]	4.4 [a]	4.3 [N.S]	4.2	4.4	4.3
Al	7.0 [N.S]	8.1	8.0	8.6	12.0 [N.S]	12.5	13.6	15.0
Na	37.0 [b]	40.4 [b]	39.0 [b]	57.4 [a]	71.0 [a]	70.6 [a]	70.4 [a]	60.5 [b]
Mg	221 [a]	218.0 [a,b]	212.2 [b,c]	207.0 [c]	215.0	211.0	212.0	213.0
Pb	0.9 [N.S]	0.9	0.9	0.9	1.0 [N.S]	0.9	0.9	1.0
Macro-Minerals								
P	0.7 [N.S]	0.7	0.7	0.71	0.7 [a,b]	0.7 [a,b]	0.7 [b]	0.7 [a]
Ca	0.3 [a]	0.24 [a,b]	0.20 [b]	0.3 [a,b]	0.2 [N.S]	0.2	0.2	0.2
K	1.3 [N.S]	1.3	1.3	1.3	1.2 [a,b]	1.2 [c]	1.2 [b,c]	1.3 [a]

‡Values followed by the same letter within each variety and seed component (amino acids) are not statistically different at P< 0.05. NS, no significant different.
*The results represent the average of two years in each location.

Table 3- 5. Yield and its components of soybean seed from VgD, empty vector and parental lines, planted at two locations in KY.

Component	Princeton				Spindletop			
	Jack	VgD1-1	VgD1-2	VC	Jack	VgD1-1	VgD1-2	VC
Yield (Kg.ha^{-1})	1841.0 [a]‡ *	1341.1 [b]	1539.0 [a,b]	1727.0 [a]	2129.6 [a]	2013.3 [a]	1981.8 [a]	1266.7 [b]
Oil (Kg.ha^{-1})	385.5 [N.S]	332.3	377.3	310.2	457.0 [a]	464.1 [a]	464.0 [a]	216.4[b]
Protein (Kg.ha^{-1})	741.3 [a]	526.9 [b]	627.0 [a,b]	747.4 [a]	851.0 [a]	850.0 [a]	837.3	550.7 [b]
Height (cm)	73.4	67	70.2	72.3	74.0 [a]	67.5 [a,b]	69.3 [a,b]	64.0 [b]
Seed weight (mg)	115.0 [b]	109.0 [b]	109.0 [b]	141.0 [a]	115.0 [b]	106.0 [b]	108.0 [b]	130.0 [a]
Seed Number (Seed.m^2)	1631.9 [b]	1239 [b]	1418.8 [a,b]	1243.2 [b]	1854.5 [a]	1898.9 [a]	1842.5 [a]	992.5 [b]

‡Values followed by the same letter within each variety are not statistically different at P< 0.05. NS, no significant different.

*The results represent the average of two years in each location.
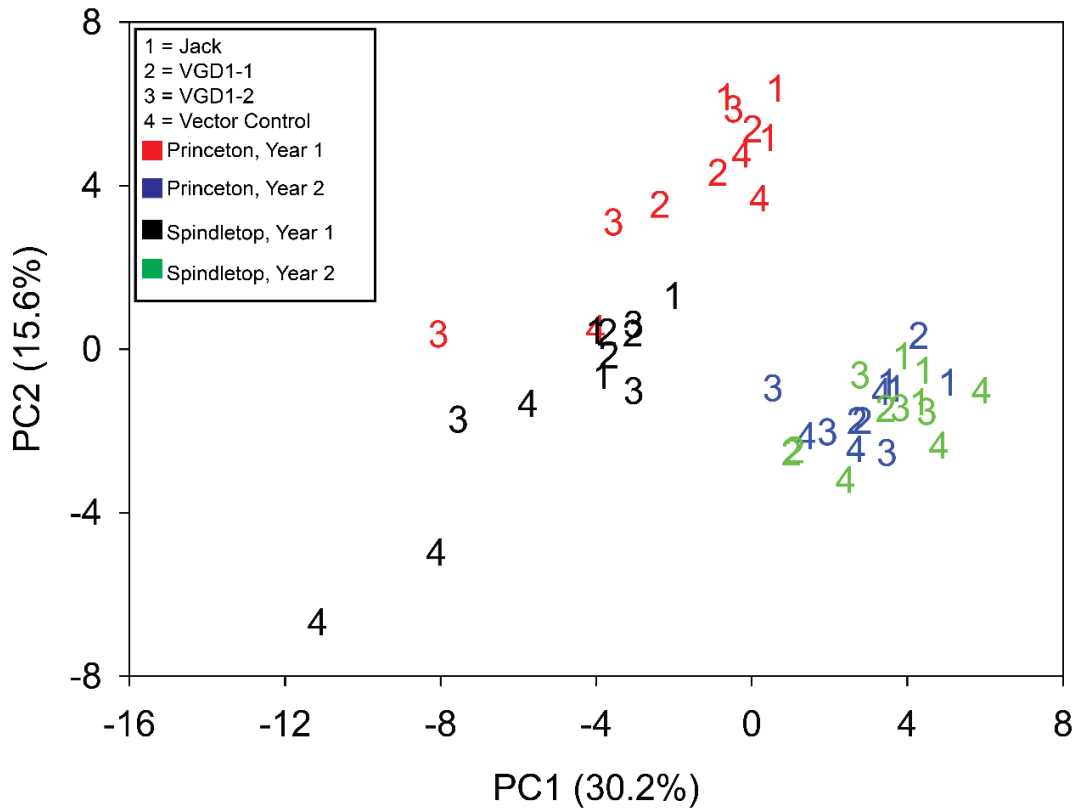
Figure 3- 1 .Plot of principal component scores along first two principal components axes. The four lines are indicated by numbers 1-4, while the symbols are color-coded by sampling year and location. There is clear cluster of year 2 samples, irrespective of line, indicating that year-to-year variation is the primary source of variance in our dataset.

Chapter 4: Impact of combining DGAT1 (diacylglycerol acyltransferase-1) soybean with a MIPS (*myo-insitol-1-phosphate synthase*) mutant

Abstract

Soybean (*Glycine max Merrill*) meal is a very important protein source for humans and is commonly used in animal feed worldwide. A single recessive mutation in soybean (MIPS) *myo*-inositol *1-phosphate synthase* confers a seed phenotype of increased inorganic phosphate (Pi), crossed with high oil lines expressing a DGAT from *Vernonia galamensis* (VgD). The late generation (each generation was confirmed by DNA, and Pi analysis) showed promising results, even though the oil and protein were maintained compared to VgD. VgD X MIPS (VM), had 21.2, and 22 % oil in 2015, and 23.3 and 24.0 oil in 2016, and 46, 49 % protein in 2015, and 37 and 39 % in 2016. Phosphate results suggesting the cross MV is still segregating for MIPS and more selection and planting are needed. Taiwan 75 (T75), gave the highest seed weight, in greenhouse and field conditions, yield g/plant, was similar in greenhouse condition, and T75 was higher in the field giving 57.2 g/ plant.

Sucrose was reduced in VgD, whereas stachyose was reduced in 2016. Pi was higher in MIPS line as expected followed by MV giving 5 g/100g Pi, followed by MV about 2 mg/100g. Phytate was reduced in MV compared to other lines except for MIPS line. This finding provides a soybean variety with enhanced Pi level for nutritional value for human and animal, as well as providing more available carbohydrate for further engineering into more oil or protein.

Introduction

Soybean is an important source for protein and oil, for human and it is regularly used in animal feed worldwide. The majority of phosphorus (P) in the seeds is in the form of phytic acid (60-80%) (Raboy et al., 1984), soybean seed composition has about 4.3g/kg^{-1} phytic acid (PA), and around 0.6 g /kg^{-1} inorganic P (Pi) (Wilcox et al., 2000). Phytic acid PA is unavailable to mono-gastric animals such as pigs, poultry and fish and the negative charge of P in phytic acid can strongly bind to metallic such as Ca, Fe, Mg and Zn and make them unavailable nutritionally (Cromwell, 2005; Cromwell and Coffey, 1991). To overcome this problem the addition of Pi or microbial phytase (E.C.3.1.3.8) to feed is commonly practiced to increase available P to animals (Yuan et al., 2012). Excretion of PA can lead to accumulation of P in soil and water, and subsequently to eutrophication of fresh water streams and near coastal seawaters (Bohn et al., 2008). Low-PA mutant seeds can potentially reduce these problems. (Yuan et al., 2012) found a new low phytate and high phosphate mutant, with a reduction of 67% in phytate and a six-fold increase in inorganic P. To divert hydrocarbon from the undesirable raffinose saccharides and phytate a key enzyme for their synthesis, *myo-inositol 1-phosphate synthase* (MIPS), has been down-regulated. MIPS converts glucose-6-phospate into *myo*-inositol 1-phosphate which is a key precursor to both phytic acid and phytate and the raffinose saccharides, raffinose and stachyose (Hitz et al., 2002; Yuan et al., 2007) Increasing oil yield per unit land area by using conventional breeding has continued to progress 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. Theoretically, an increase in the compositional value of soybeans via altered carbon partitioning should be feasible approach, since a portion of the soybean dry weight is composed of less valuable fiber and the low digestible oligosaccharides (Padgette et al., 1996), which could be diverted into oil or protein. This approach has been done by manipulating a highly active acyl-CoA:diacylglycerol acyltransferase (DGAT) the hydrocarbon flux to oil in oilseeds can be increased without reducing the protein component. Compared to other plant DGATs, a DGAT from *Vernonia galamensis* (VgDGAT1A) produces much higher oil synthesis and accumulation activity in soybean. Soybean lines expressing VgDGAT1A show a 4%(

percentage point) increase in oil content without reductions in seed protein contents or yield per unit land area (Hatanaka et al., 2016).

In the present study the high oil line (VgDGAT1A) that developed with additional oil biosynthetic genes (Rao and Hildebrand, 2009), was crossed with low phytate and oleigosucchride mutant (MIPS) *myo-inositol 1-phosphate synthase*. VgDGAT1A, with active acyl-CoA DGAT form *Vernonia galamensis* oil line will used to divert hydrocarbon from the undesirable oligosaccharide and phytate. These MIPS mutants have as much as 10% more hydrocarbon to be "pulled" into oil by VgDGAT1A high activity oil biosynthesis genes this should result in considerable higher oil levels without affecting total protein levels but increasing meal protein levels further. The hypothesis of the current study is combining VgD lines with MIPS mutant could provide more carbohydrates for oil synthesis. The objective is to determine the impact of that cross of the current study is evaluating the cross VgDGAT1A, and MIPS, on oil, protein and carbohydrate level.

Materials and Methods

Seed Material

Five lines were used in these studies, VgD, Jack, Taiwan75, (MIPS) *myo-inositol 1-phosphate synthase* and VgD X MIPS (VM), and grown in a Greenhouse in 2015 and field in 2016.The VgD line used in the study has a highly active acyl-CoA:diacylglycerol acyltransferase DGAT from *Vernonia galamensis* (VgDGAT1A) (Hatanaka et al., 2016).The MIPS and Taiwan75 lines were provided by Dr. Yuan, F, Jie, AEA-Zhejiang University Collaborating Center, Institute of Nuclear Agricultural Sciences, Zhejiang University, Hangzhou, China. MIPS and VgD lines were crossed and the plants that containing the VgD and MIPS were selected for several generation. The greenhouse experiment conducted in a University of Kentucky Greenhouse, Lexington, Kentucky 2015 and the field experiment was located at the Spindletop Research Farm in Lexington, KY (38.125835 N, -84.496781W) in 2016.

Nitrogen and Phosphorus analysis

Samples were prepared for analysis by weighing 100 mg of grind material into 25x200 mm Pyrex glass ignition tubes marked at 50 mL. Five mL of concentrated sulfuric acid containing 0.05 g of salicylic acid/mL were added and the samples reacted for one hour at room temperature (Bradstreet, 1965). 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 one hour. This resulted in 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. Two reagents, one containing 0.5% sodium hydroxide and 0.042% sodium hypochlorite in deionized water, the other containing 1.0% phenol and 0.02% sodium nitroprusside in deionized water. The sample was introduced into a bubble-segmented stream followed by the reagents. 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. (Chaney and Marbach, 1962). The phosphorus technique was based on the method of (Fiske and Subbarow, 1925) the dialysis step was not necessary for digested samples. A solution of ammonium molybdate (7.5 g/L) in 1.92 molar sulfuric acid was reacted with the samples in the segmented stream to form a heteropoly phosphomolbdate 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.

Pi analysis

A new technique (Al-Amery et al., 2015) was tested using 1 to 2 mg samples (nondestructively), 96-well plates Selection of low phytate mutant seeds based on the high Pi level measured via a colorimetric assay based on a reaction of extracted Pi with ammonium molybdate forming a blue color.

DNA analysis

A commercial seed DNA extraction kit using 1 mg cotyledon tissue were developed for dry soybean seeds and cross-verified with leaf DNA analysis (AL-Amery et al., 2016). One mg tissue was used in this study for PCR analysis.

Mineral analysis

For the elements Ca, Mg, K, Cu, Fe, Mn , soybean seeds were ground to a fine powder, ashed in a muffle furnace for 4 hours at 450°C, then digested using a 14:5:1 ratio of water:hydrochloric acid:nitric acid, with final acid concentration of 4% HCl and 1% of HNO_3. All values listed are reported in mg/kg. Samples were acid digested in duplicate, and analyzed using AOAC method 968.08 with a Varian Vista Pro ICP-OES.

For B, samples were ground to a fine powder and 100 mg sub-samples were digested in 1 mL of a 3:1 mixture concentrated trace-metal grade HNO_3 and 20% H_2O_2. The samples were heated in metal-free polypropylene centrifuge tubes to 110°C over 30 min and refluxed at that temperature for 10 min using a temperature-controlled microwave reaction system (MARS Xpress, CEM, Matthews, NC, and USA). Standard reference materials (SRM 1573a, tomato leaves, National Institute of Standards and Technology, Gaithersburg, MD, USA) reagent blanks and duplicate digestions for randomly selected samples were included with each digestion set. The samples were then brought to 15 mL with de-ionized water (DI). Aliquots were spiked with an internal standard mixture to

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achieve 1 ug/L of Sc, Ge, In, Tb and Bi. Randomly selected samples were fortified with the calibration standard to determine spike recovery. The samples were analyzed using an inductively coupled plasma mass spectrometer equipped (ICP-MS; 7500cx, Agilent Technologies, Santa Clara, CA, USA) operating in standard mode for B. Where possible, multiple isotopes were used for quantification to check for spectral interferences. Calibration was performed by serial dilution of a certified reference standard in a matched matrix (5% ultrapure HNO_3 and 5% n-butanol in DI) and was verified by analyzing a dilution of an independent certified standard of a different lot number (Inorganic Ventures, Christiansburg, VA, USA).

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 twice and dried. Once dry, 500 µL sodium methoxide was added and shaken for 10 minutes with 1 mL isooctane containing 0.001% BHT added after 200 µL was pulled off the upper layer and transferred into a GC vial with an additional 1 mL of isooctane added. GC vials were then run on a Varian CP-3800 Gas Chromatograph 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 um. The temperature program ran from 90 C to 250 °C with 25 C ramp for a total of an 8-minute 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 and peaks quantified using Star Chromatography Workstation Version 6.00, with peak area being used to calculate relative percentages of FAMEs (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).

Oil quantification

Oil contents analysis was performed by nondestructive means on a bulk basis using near-infrared spectroscopy (NIRS). Briefly, the instrument collects spectra on a seed as it falls

through a short length of illuminated glass tubing. Optic fibers are aligned with the tube axis and connected to a spectrometer for collection of seed spectra. The instrument is designed for large sample sorting and processes seeds at about 3 per second, although seeds were hand fed for this study to preserve identity. Seed and oil mass were measured and converted to a dry basis oil percentage using a moisture content estimated from room equilibrium moisture conditions.

Sugar analysis and quantification

To extract sugars (*myo*-inositol, sucrose, raffinose, and stachyose) 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. 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, diluted to 25mL with distilled, deionized water. The supernatant was recovered (5 EtOH + 25 HOH = 30 mL) and 1/3 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 eight hours. After transferring the sample to 1.5-mL micro tubes 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 using a 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 standards. Reintegration of the peak start- and stop-times,

baseline identification, and areas under the curve 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 with the recovery of 2-DG added during extraction (internal standard) (Downie and Bewley, 2000; Nosarzewski et al., 2012).

Results and discussion

In field condition 2016, VgD and VgD X MIPS (VM), gave the highest oil (24.0 and 22.1 g/100g dry weight). (Figure 4-1), followed by Jack, MIPS and Taiwan75. However, in greenhouse (GH), MIPS was slightly higher in oil 23 g/100g dry weight (DW) and no differences was found between VM, and VgD. All lines gave similar protein composition in GH except for Taiwan 75 was lower 35.1 %; whereas in field condition VgD, MIPS and Taiwan 75 were slightly higher in protein g/100g dry weight with 40.1 g/100g dry weight (Figure 4-2).

Fatty acid profile showed interesting results (Figure 4-3 and 4-4), VM and VgD, were not different in 18:1 and 18:2, whereas Taiwan 75 gave the highest 18:1 and Jack was higher in 18:2 in greenhouse condition. VM and VgD preformed similarly in field condition, and jack and MIPS gave the highest 18:2. The soluble sugars analysis was run for field location only (Figure 4-5), and the results showed reduction in sucrose in VgD, however it was maintained in VM, stachyose reduced in VM, while remains high in VgD. Taiwan 75, acted similarly to VgD line.

The phosphate analysis (Figure 4-6) revealed an interested information even though it presented for field location only. MIPS gave the highest Phosphate (Pi) in seed 4.5 mg/100g seed dry weight, followed by VM, while VgD, Taiwan 75 and Jack were the lowest in this trait. Phytate analysis was lower in VM, compared to VgD, Taiwan75 and Jack, whereas MIPS was lowest in phytate concentration (Figure 4-6).

Mineral analysis, preformed only for one year 2016, MIPS was lowest in K (g/kg dry weight), and VM gave the highest level in Fe, compart to the other line in the study. MIPS gave the highest level of available phosphate in seed (Figure 4-7A and 4-7B) 4.5 g/kg, flowed by VM, 1.5 g/kg, whereas Jack. Taiwan 75 and VgD were the lowest.

Taiwan 75 gave the highest seed weight followed by MIPS (Figure 4-8 and 4-9), whereas VM, VgD, and Jack were similar in this trait and the differences between them were small.

Yield per plant, generally was higher in 2016, and VM gave 23 g, jack 20 g, MIPS 32 g /Plant, whereas Taiwan75 was the highest with57 g/plant, followed by VgD 40.6 g/plant. In 2015 not much different noticed in VM, Taiwan75, VgD and MIPS, giving (18, 16, 17 and 19.2 g/plant), while Jack was the lowest with only 10 g/plant.

The key enzyme for MIPS *myo*-inositol 1-phosphate synthase synthesis, has been down regulated. MIPS converts glucose-6-phospate into *myo*-inositol 1-phosphate, which is a key precursor to both phytic acid or phytate and the raffinose saccharides, raffinose and stachyose.

VgD high oil + protein lines have been crossed with MIPS mutants low in phytate and oligosaccharides and the first objective was to evaluate the cross. By crossing with the high oil line VgD, the hydrocarbon for oil synthesis is being diverted from the undesirable soybean seed components, raffinose, and stachyose. This cross presumably will push hydrocarbon flux away from less desirable seed components and into oil without affecting seed protein levels. This theory was not applying in in oil and protein g/100g (DW), since they both did not change in MV and VgD, as well as fatty acid profile acted the same, and that could possibly accept the same explanation for VgD. Which 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 Kennedy pathways. Improved DGAT with increase affinity for 18:1 CoA will compete efficiently to produce 18:1 CoA, resulting in more 18:1 fatty acid acyl groups going directly to TAG, without going to PC, thus avoiding additional desaturation (Roesler et al., 2016c).

The results showed a reduction in stachyose (Figure 4-5) which support that theory, but no reduction found in *myo*-inositiol, in fact a little increase in *myo*- inositol found in VM. The results in figure 4-5, showed minimal concentration of stachyose in VM and MIPS compare to the studied lines. Whereas *myo*-inositol showed no reduction, this in agreement with Hitz et al.(2002) that *myo*- inositol in single seed bases is variable in low

phytate soybean mutant. There is a clear substantial pool of *myo*-inositol, but it did not apparat to be reduced in the VM, neither in sucrose. In contrast sucrose was reduces in VgD, and this results is not in agreement with Lardizabal et al(2008b) finding no reduction in carbohydrate (on percentage dry weight basis) in the transgenic positive relative to the control in soybean with overexpression DGAT from of *Umbelopsis ramanniana*, even though the increase in oil was 1.5 percentage point.

Phosphate level was higher in MIPS and phytic acid was lower, followed by VM. VM has enhanced phosphate level and lower phytic acid, but still not compared to MIPS, suggesting the line is still heterozygous and more planting and selection needed.

Seed size and weight was smiler between VM and VgD, and smaller compare to MIPS and Taiwan 75 (VM backgrounds). Seed size can be possible used as a parameter to predict the final yield, even though this is not an easy task, since seed size controlled by genetic an environment. And it is represented seed growth rate (SGR) and seed fill duration (SFD) (Egli, 2017). The differences in seed size found in the current study could possible explained by the relationship between SGR and SFD, and the genetic differences between the lines or the environmental effect. Larger seed size is not necessary resulted for high yield and vice versa. White and Gonzalez (1990) Found no significant correlation between seed size and variety.

The VM line consider to be a promising line, because of the addition of phosphate, which will enhance the nutrition value for soybean meal for human, and animal.

Conclusions

We did not see an increase in oil and protein in multiple environment condition between VM, and VgD. The environmental effect was noticeable; similarly, the yield per plant and seed weight (mg) was not different, but the carbohydrate level reduction in stachyose and maintain the same level of sucrose is considered to be promising. In addition, the level of phosphate and Fe are increased, which will enhance the nutrition value for the soybean meal compare to all lines in the current study, as well as no noticeable change in sucrose concentration, which providing carbohydrates pool for further composition enhancement. More planting and selection are required to obtain a homozygous soybean cross.
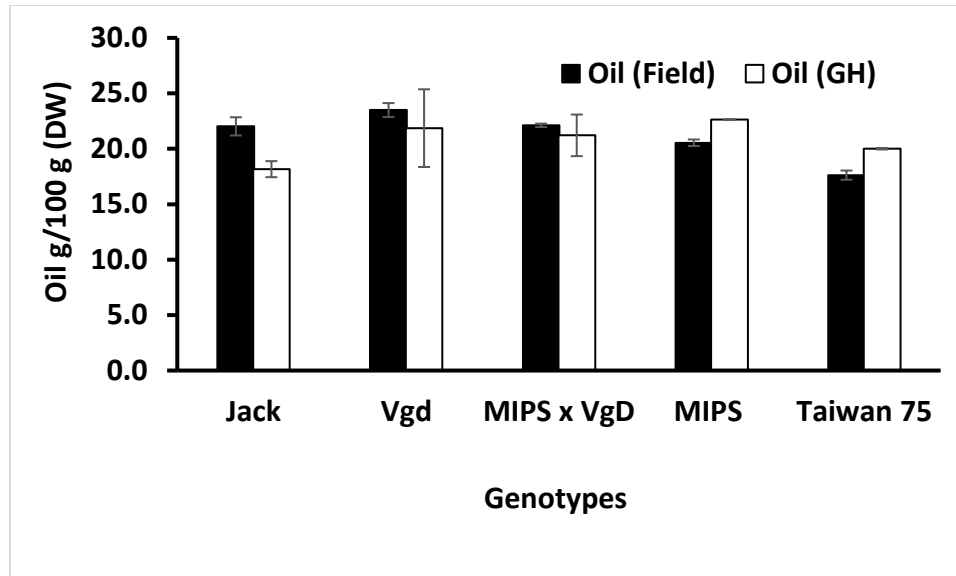
Figure 4- 1. Oil g/100 g (DW) for studied soybean lines in greenhouse 2015, and 2016
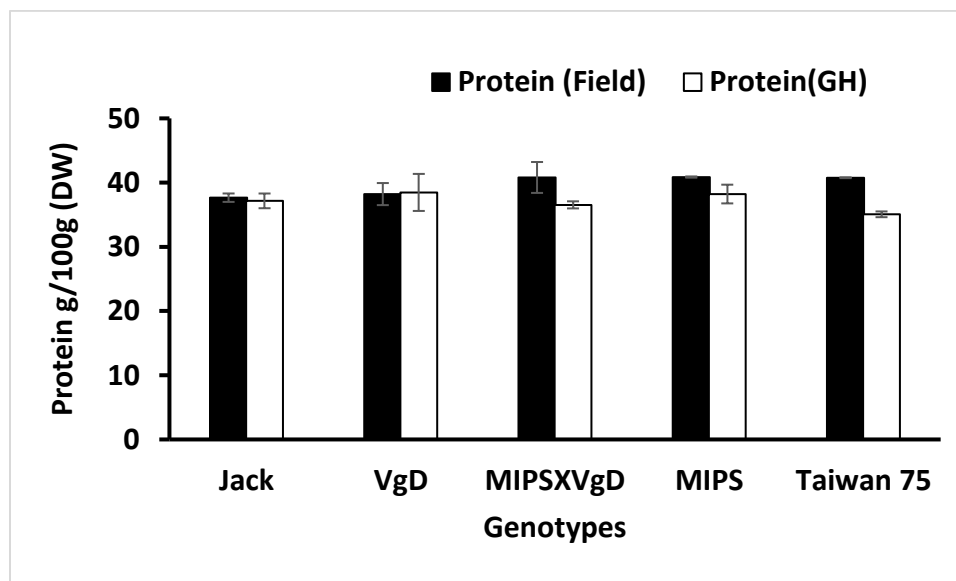
Figure 4- 2. Protein g/100 g (DW) for studied soybean lines in 2015 and 2016
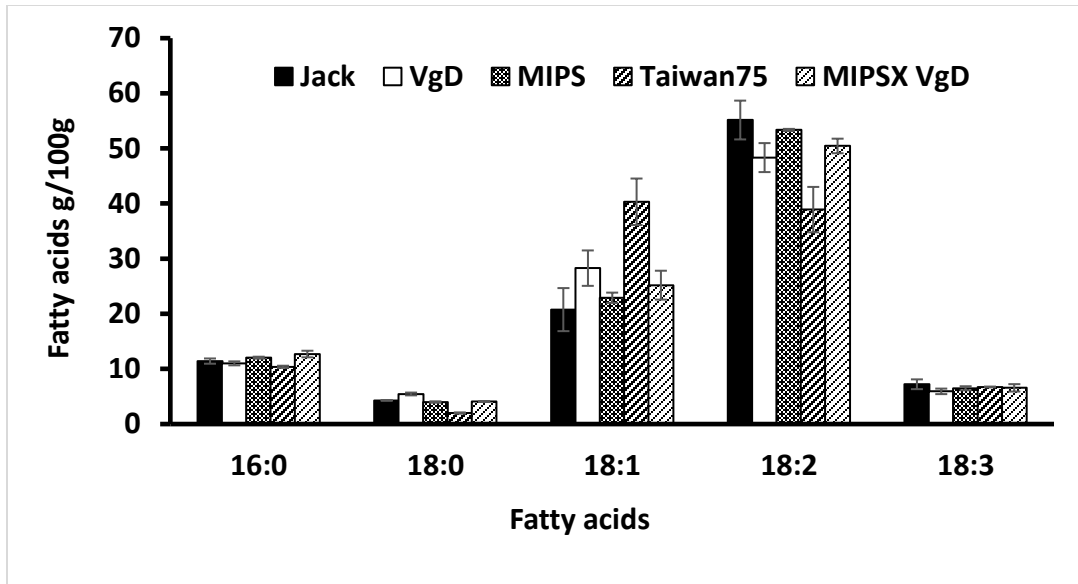
Figure 4- 3. Fatty acids profile in soybean liness in 2015
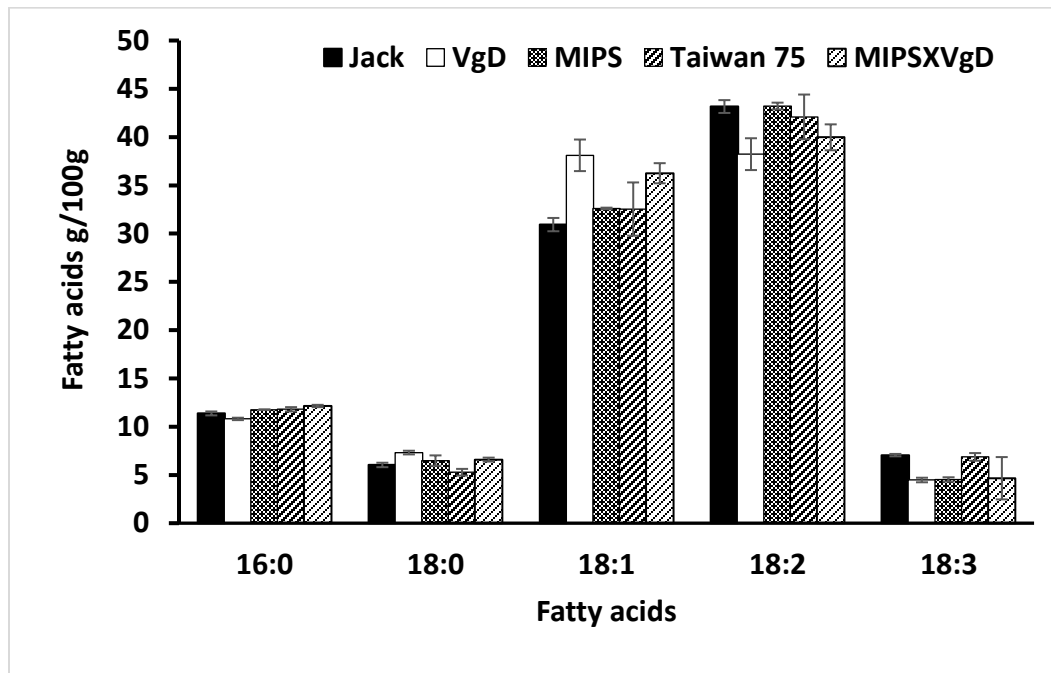
Figure 4- 4. Fatty acids profile in soybean lines in field 2016
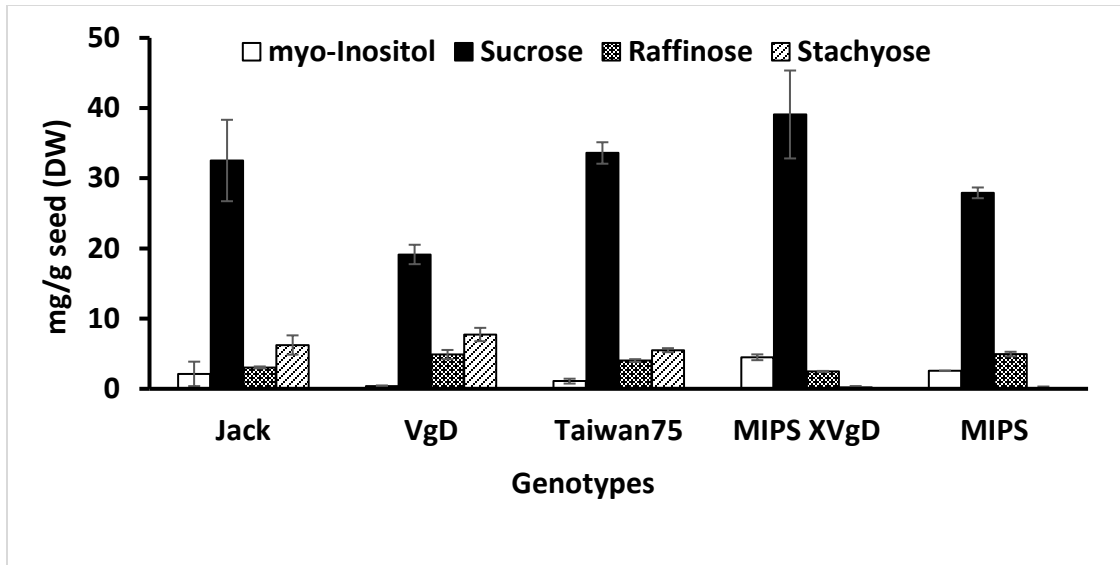
Figure 4- 5. Carbohydrate profile in soybean lines in 2016.
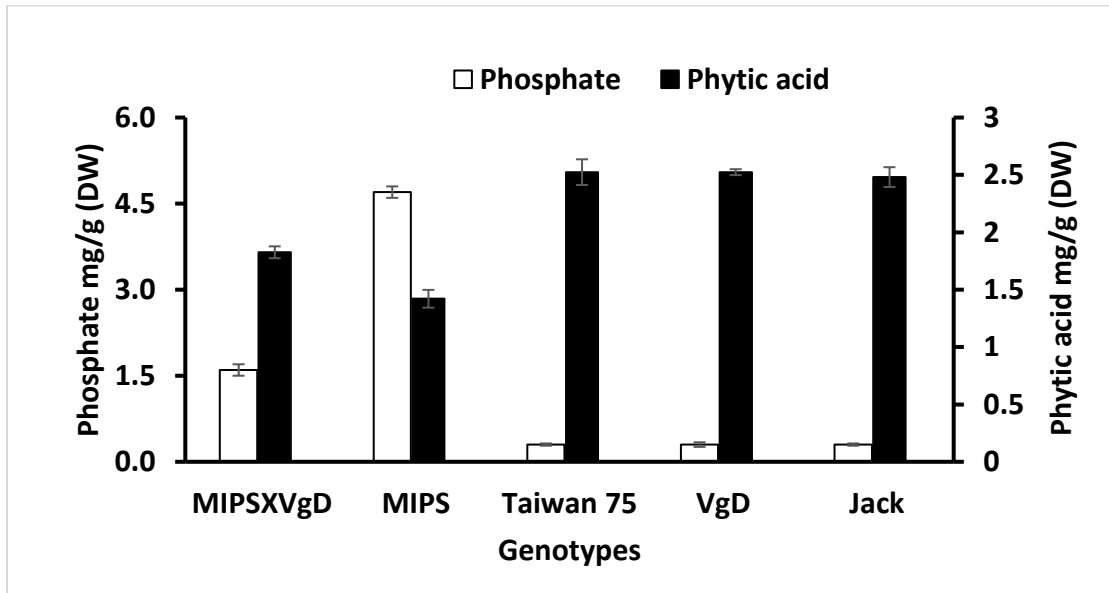
Figure 4- 6. Phosphate and phytic acid concentration µg/mg in soybean lines in 2016

A.

B.
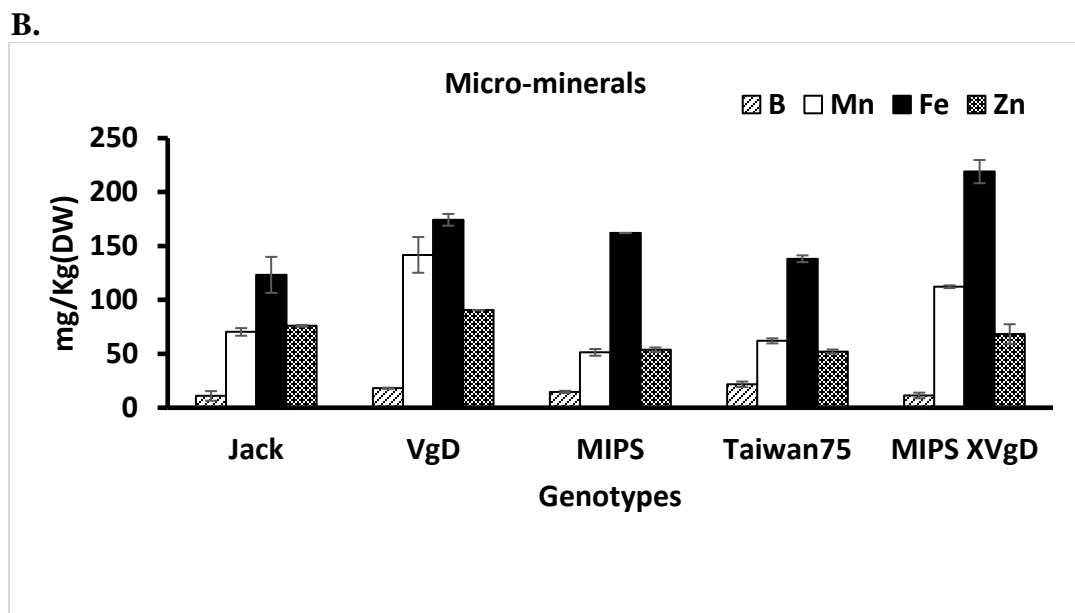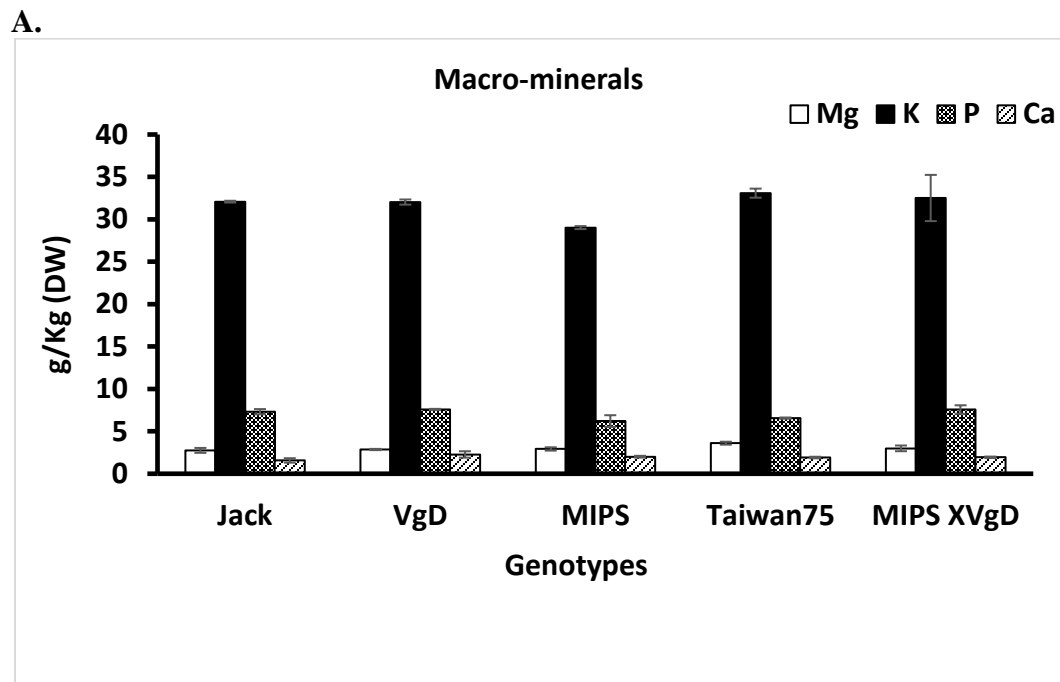
Figure 4- 7. Summary statistics for the inorganic/mineral analysis of components in soybean lines, Macro minerals, B. Micro minerals in 2016
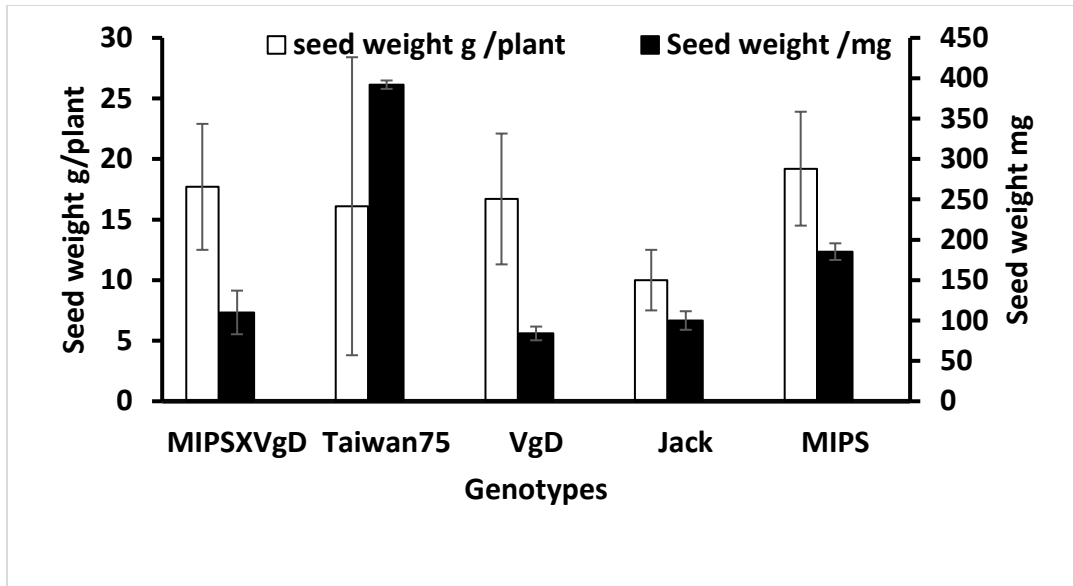
Figure 4- 8 . Seed weight/mg and plant/seed g, in soybean lines in 2015
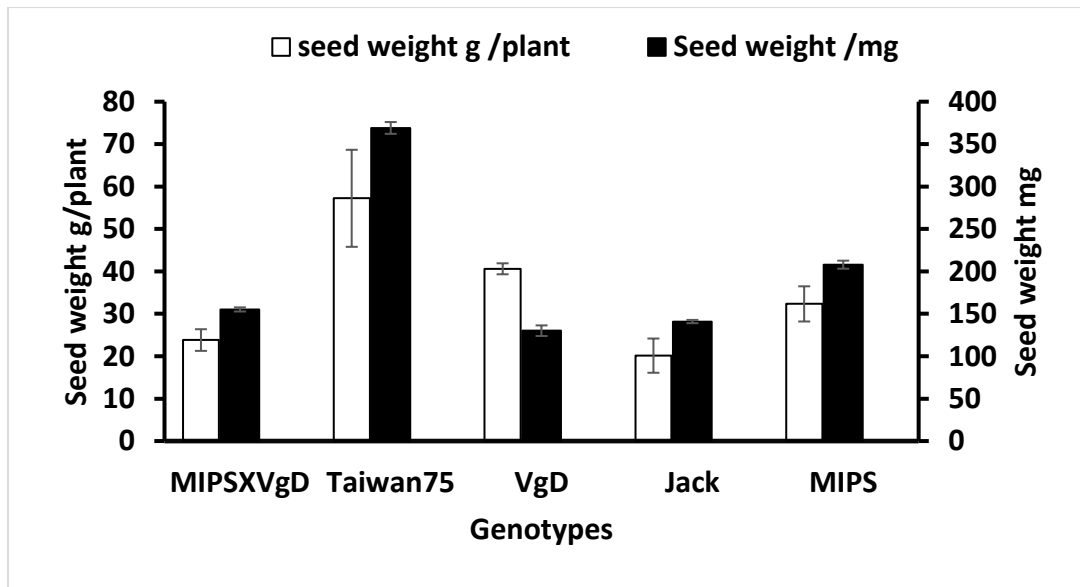
Figure 4- 9. Seed weight/mg and plant/seed g in soybean lines, 2016

Chapter 5: Single seed selection for low phytate lines

Abstract

Most seed phosphorus (P) is bound in phytate which is unavailable to monogastric animals depriving Them of P and causing eutrophication from P in animal waste. It is valuable to reduce the phytate levels of seeds used for food and feed. Low phytate mutant seeds such as soybean [*Glycine max* (L.) Merr.] MIPS (D-*myo-inositol 3-phosphate synthase*) mutants have correspondingly increased inorganic phosphate (Pi). Measurement of seed Pi levels is an established technique for screening for low phytate mutants but to date it has not been performed nondestructively from single seed samples. A protocol was developed greatly reducing the sample size thereby reducing the cost and time and saving a generation in the selection of low phytate mutant seeds based on the high Pi phenotype. Pi was extracted using 12.5 % trichloroacetic acid. Pi reacts with ammonium molybdate in acidic solution to form phosphomolybdic acid, which upon reduction with ascorbic acid produces an intensive blue color. This new technique was tested using 1–2 mg samples, 96-well plates with 8 MIPS mutants (GM-*lpa*-TW1), 8 wild-type and 8 seeds segregating for the MIPS mutation. Selection of low phytate mutant seeds based on the high Pi level measured via a colorimetric assay based on a reaction of extracted Pi with ammonium molybdate forming a blue color. This was confirmed by DNA analysis of seed chips. This protocol is applicable to many other crop seeds including corn, wheat, rice and sorghum.

KEYWORDS: Soybeans, *Glycine max,* · Phosphate, · Feed quality, and · Nutrition

Introduction

Phytate represents a mixture of different cationic salts of phytic acid (PA) [*myo*-inositol hexakis-(dihydrogen phosphate)] and is the main storage form of phosphorous (P) in seeds and grains, accounting up to 85 % of the total seed P, and as much as several percent of the seed dry weight (Lott, 1984) .

In soybean seeds, the phytate P ranges from 51 to 77 % of the total P (Raboy et al., 1984; Su-Cheng et al., 1990). In addition, the phosphate level in normal soybean seeds is ~0.6 g/kg^{-1} (Wilcox et al., 2000); whereas in the low phytate mutant the Pi level can increase to 1.5–4 g/kg^{-1} (Yuan et al., 2007).Grains are major ingredients in animal diets worldwide. However the phytate P is unavailable to monogastric animals such as pigs, poultry and fish and the negative charge of PA can strongly bind to metallic cations such as Ca, Fe, Mg and Zn and make them unavailable nutritionally (Cromwell and Coffey, 1991; Vohra et al., 1965).

Furthermore, undigested phytate P is excreted and results in P pollution in manure and the environment. To reduce such problems, animal diets can be supplemented with phytase (Cromwell, 2005) or low phytate (LP) seeds also can be used. LP mutations fall into two general categories: (1) Mutations in the PA biosynthesis pathway or (2) in transport/compartmentalization of PA. The former involves one of the several enzymes such as *myo*-inositol phosphate synthase (MIPS) (Hitz et al., 2002) , *myo*-inositol kinase (Shi et al., 2005) and *myo*-inositol phosphate kinases (IPK) (Shi et al., 2003). A recessive *mips* mutation is responsible for the reduction of phytate P and raffinose saccharide content in soybean (Hitz et al., 2002; Sebastian et al., 2000). Yuan et al (2007; 2012) also generated and characterized a *mips1* (Gm-*lpa*-TW-1) mutant and an *ipk1* (Gm-*lpa*-ZC-2) mutant in soybean with both LP phenotypes. The first two LP soybean mutants generated by Wilcox et al(2000) turned out to be mutations in one or two of the phytate-specific ABC transporters, *lpa1* (*pha1*) and *lpa2* (*pha2*), of the second category of LP mutations similar to the *lpa1* mutant of maize(Gillman et al., 2009; Shi et al., 2007) .The present technique is a less expensive and faster Pi measurement than has been deployed for LP screening. Rockstein and Herron (1951) who used ferrous sulfate as a reducing agent reported measurement of Pi in microgram quantities. This method is based on the reaction between phosphate and ammonium molybdate to form phosphomolybdic acid,

which is then reduced by FeSO4 in a weak acid solution with maximum absorbance at 720 nm. This reaction is complete within a few seconds and the intensity of the color remains stable up to 2 h standing at room temperature. The use of ascorbic acid as a reducing agent was first reported by Ammon and Hisburg (Ammon and Hinsberg, 1936) and further improved by Chen *et al*. (1956) for the greater sensitivity. Yuan *et al*. (2007) measured Pi from one of the two soybean seedling cotyledons using Chen's protocol modified by Wilcox *et al*. (2000). Scaboo *et al*. (2009) modified the protocol utilized by Raboy *et al*.(2000) to evaluate Pi in 100 mg of soybean dry seed flour. Chiangmai *et al*. (2011) also adopted Chen's protocol to evaluate Pi of seeds of inbred parents of F1 hybrids of corn (*Zea mays* L.) grown in the rainy season in Thailand using 0.5 g seed samples. This new method will save a generation and reduce samples that need to be analyzed in breeding programs by analyzing a small part of a seed and planting the same seed subsequently. This protocol can be applied to non-destructive phytate determination in many other crop seeds including corn, wheat, rice and sorghum. This protocol is a simple method for screening breeding populations and supporting genetic studies for LP mutants. This protocol was confirmed by DNA genotyping.

Materials and Methods

For Pi analysis about 1–2 mg seed chips was taken from 24 dried seeds comprising 8 seeds containing the GM-*lpa*-TW1 mutant gene, 8 non-mutant (wild-type cv. 'Jack') seeds and 8 seeds segregated from the cross of 'Jack' x GM-*lpa*-TW1. A total of 72 subsamples, 3 extractions from each seed chip, were taken for the colorimetric phenotype analyses. The QuickExtract™ Seed DNA Extraction Solution was obtained from Epicentre, Madison, WI. The chemicals used in the extraction and Chen' reagent [10 % ascorbic acid solution and 3 M sulfuric acid and 2.5 % ammonium molybdate and water (1:1:1:2)] are from Fisher Scientific. The sodium phosphate dibasic heptahydrate used for the standard curve preparing the concentration of 0, 0.17, 0.34, 0.85, 1.70, 3.40 µg Pi, Chempure™ Ultra, is from Curtin Matheson Scientific Inc.

Genotyping samples

A 2 bp deletion is reported in the third exon of the Gm-lpa-TW-1 MIPS1 gene compared to the wild-type (WT) allele. The PCR primer sets were developed to differentiate the mutant and WT alleles of MIPS1 gene by Yuan et al. (2007). Theprimer sequences, matching either the WT or mutant allele and expected size of amplification are 121 bp for MIPS1_WT and 400 bp for MIPS1_M2 Yuan et al. (2007). Four seeds were used in this analysis, two seeds for GM-lpa-TW1 and two seeds for the wild type (Figs. 1 and 2). One mg seed slices were shaved off and placed in 1.7-mL microfuge tubes, then 100 μL of the QuickExtract™ Seed DNA Extraction solution (Epicentre,Chicago, IL) was added. The samples were mixed by vortexing for 10 s, and then heated at 65 ℃ for 6 min then98 ℃ for 2 min to denature the enzymes in the solution and placed on ice until use. The PCR reaction was carried out in a final volume of 15 μL containing 1 μL genomic DNA, 1.5 μL 10X RED Taq PCR reaction buffer, 0.3 μL dNTP mix (10 mM ea.), 0.3 μL Primer mix (10 μM ea.), and 0.7 μL Red Taq PCR reaction mix 0.7 μL (one unit = 1 μL incorporates 10 nmol of total deoxyribonucleoside-triphosphates into acid precipitable DNA in 30 min at 74 ℃; Sigma-Aldrich, St.Louis, MO). The PCR conditions were 94 ℃ for 2 min, 94 ℃ for 1 min, 50 ℃ for 1 min, 72 ℃ for 30 s and repeated for 34 cycles, with the final extension of 72 ℃ for 4 min, then holding at 8 ℃ until the samples were removed.

Pi measurement

Single seed samples are obtained by shaving off a cotyledon slice opposite to the embryonic axis (Fig. 1). After the seed coat is discarded, 1–2 mg of the samples are used (Fig. 3) and 50 μL of an extraction buffer (25 mM MgCl2 and 12.5 % trichloroacetic acid) is added. Samples are incubated at 37 °C with gentle shaking for 14–16 h. The mixtures are centrifuged for 3 min at 100 g. Three subsamples of 10 μL are taken from the supernatants and loaded into 96-well plates, and diluted with 90 μL water. Then 100 μL of Chen's reagent (Chen et al., 1956) is added to each sample, mixed by pipetting several times and incubated at 37 °C with medium shaking for 1 h. The absorbance of the samples in the plates is read at 882 nm (Chen et al., 1956) using a plate reader (e.g. Bio Teck-Synergy HT Plate reader Module) after shaking the samples for 5 s using the built-in shaker. The individual components of Chen's Reagent do not need to be made every day. The 10 % ascorbic acid solution can be kept for several weeks at 2 °C and 3 M

sulfuric acid and 2.5 % ammonium molybdate can be kept at room temperature (Chen et al., 1956) In the modified method of this paper the ammonium molybdate and sulfuric acid solutions can be kept at room temperature for 2 months and are still effective for preparing Chen's reagent but for ascorbic acid the color changed after 3 weeks keeping it at 4 ℃. The components of Chen's Reagent should be mixed together within hours of Pi determination of extracts. Pi extracts in the extraction buffer should be stable for a few days at 4 °C or can be frozen for long periods before thawing and reacting with Chen's Reagent (Scaboo et al., 2009).

Phytate phosphate determination

Packing the column

Weight 0.5 gm BioRad Resin (Anion) unto weight boat, and carefully add water to the weight boat while pouring into column; washing resin into column.
Fill column with ddH_2O and let drip into backer. Refill once or twice until Anion packs to the bottom of the column. Leave the dripping from the backer. When not in use, screw stopper into bottom, fill column with ddH_2O, and put cap on top. Resin must be kept wet with ddH2O when not in use. Resin can not be used up to 4 times. Pack up to 24 columns at a time, this will allow you to test 12 samples. Proximate test time 12 samples in 4 hours.

Procedure for phytate analysis

Weight approximately 1 gm sample in to 50 ml Erlenmeyer flasks. Weight the night before and recorded weight and number flask accordingly. Run samples in duplicate.
Add 20 ml of 2.4 %HCL solution. Shack (low) for 1 hour on shaker.
Empty samples into 50 ml centrifuge tube. Centrifuge for 15 minutes at 2500 rpm. Sample may look cloudy and /or have varying colors. It all depends on nature of sample. Pipette 1 ml of the supernatant into numbered and clean 50 ml Erlenmeyer flasks filled with 24 ml ddH_2O.Shacke to mix. Pipette 10 ml of the dilutedsample solution (5 ml at a time) in to anion column and remains in column. Elute with 15 ml (5 ml at a time) of 0.1M NaCl solution. Discard elute. A low molar solution elutes those molecules that have a lower affinity for the resin than phytate. Phytate remains. Collect samples Elute with 10

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ml (5 at a time) of 0.7M NaCl solution into clean and numbered 50 ml flask or equivalent container. Cap and shake. Immediately (within 1 h) pipette 3 ml of the unknown into test tube. Add 1 ml of Wade's reagent. The mixture is now stable. Vortex for 5 seconds. Centrifuge 10 minutes at 3000 rpm. Read on spectrophotometer at 500 nm. Blank is ddH$_2$O (Latta and Eskin, 1980).

Results and discussion

Polymerase Chain Reaction (PCR)

The results in Figure 3, shows the PCR reaction results, the amplification of the soybean MIPS1 gene by using two primers MIPS1_M2 and MIPS1_WT. The seeds with the MIPS mutant gene have a band at 400 bp using the MIPS1_M2 primer, whereas the wild type has a 121 bp band using the MIPS1_WT primer. These results are the same as reported by Yuan et al. (2007).

Phosphate colorimetric analysis

Pi was extracted using 12.5 % trichloroacetic acid. Pi reacts with ammonium molybdate in acidic solution to form phosphomolybdic acid, which upon reduction with ascorbic acid produces an intensive blue color (Fig. 5-2) (Su-Cheng et al., 1990). WT x lpa progenies with LP phenotype were confirmed to contain the mips1 mutation by seed DNA analysis (Fig. 5-3). Phytate is reported in many seeds in varying levels with high levels in sesame (39.3–57.2 mg/g), lower levels in rice (1.2–3.7 mg/g) and wheat (1.1–3.3 mg/g) and intermediate levels in maize (9.8–21.3 mg/g), buckwheat (9.2–16.2 mg/g), peanuts (9.2–19.7 mg/g) and soybeans (12.2–19.3 mg/g)(Bilyeu et al., 2008; Greiner and Konietzny, 2006; Kim et al., 2002) . Figure 5-3 shows that seeds with the GM-lpa-TW1 gene (columns 1, 2 and 3) have high Pi and become dark blue when reacted with Chen's reagent, whereas normal wild type seeds (column 4, 5 and 6) have considerably lower Pi and remain very light blue to clear. Segregating seeds (columns 7, 8 and 9) shows light blue, clear and a strong blue color. GMTW mutant seeds Pi Ranges from 2.20 to 3.12 mg/g and the wild type seeds Pi ranges from 0.33 to 0.52 mg/g (Table 5-1).
A small group of ABC transporters are involved in the transportation of phytate into vacuoles(Shitan and Yazaki, 2013) . All known soybean LP mutations except Gm-lpa-

ZC-2 have been reported to be associated with reduced seedling emergence (Meis et al., 2003; Oltmans et al., 2004; Yuan et al., 2007). Different methods were developed to evaluate phytate or PA and analyze them quantitatively, starting with basic methods of precipitation as insoluble ferric phytate in acid solution. Techniques involving many instrumental methods including colorimetric, synchronous fluorescence, isotachophoresis, high-performance ion chromatography and high-performance liquid chromatography have been reported (Burleson et al., 2012; Wu et al., 2009). However, the reduction in phytate plus other inositol phosphates is usually accompanied by an increase in Pi maintaining the total P levels (Raboy et al., 2000; Scaboo et al., 2009). In this study, we modified Chen's technique (Chen et al., 1956), and adopted it for screening of soybean MIPS mutant seeds obtained by Yuan et al (2007) (Yuan et al., 2007)for high Pi and reduced the sample size down to 1–2 mg. This can be done non-destructively on single dry seeds using only ~1 % of the total seed allowing the present generation to be phenotype by the high Pi phenotype. Prior techniques used multiple seeds or seedlings not allowing the direct identification of single seed phenotypes of segregating seeds. This method is very stable, and eliminates the necessity of reading samples immediately after the suggested color formation time, which is 1 h at 37 °C (Chen et al., 1956). Using a plate reader provides an easy and accurate assay of small seed samples This protocol is applicable to many other single seeds such as rice(Kim et al., 2008; Larson et al., 2000), corn (Latta and Eskin, 1980), peanuts (Burbano et al., 1995) , lupine, lentils, beans, chickpeas and fava beans which utilize sample sizes, e.g., 5 g, requiring many seeds (Lorenz et al., 2008; Raboy et al., 2000; Vaintraub and Lapteva, 1988). Eliminating the opportunity to plant the seeds afterward as we have shown with this protocol. The applied method in this paper can reduce costs and maintain selected low phytate seed for future grow-out.

Conclusions

Measurement of seed Pi levels is an established technique for screening for low phytate mutants but to date it has not been performed non-destructively from single seed samples. A protocol was developed greatly reducing the sample size (about 1–2 mg) thereby reducing the cost and time and saving a generation in the selection of low phytate mutant

seeds based on the high Pi phenotype. The present technique was confirmed by DNA analysis of seed chips and is applicable to many other crop seeds including corn, wheat, rice and sorghum.
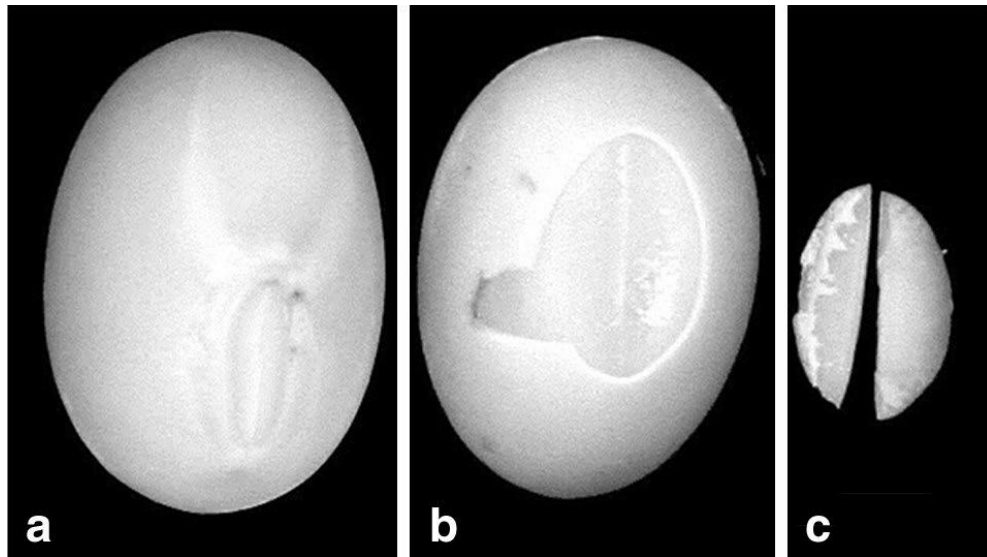
Figure 5- 1 a A soybean seed with the embryonic axis above the hilum at the *top* of the seed depicted, b a soybean seed with the side opposite the embryonic axis shown (rotated 180° *vs*. Figure. 5-1A) with a chip removed, c a typical chip of a size used in the protocol described herein.
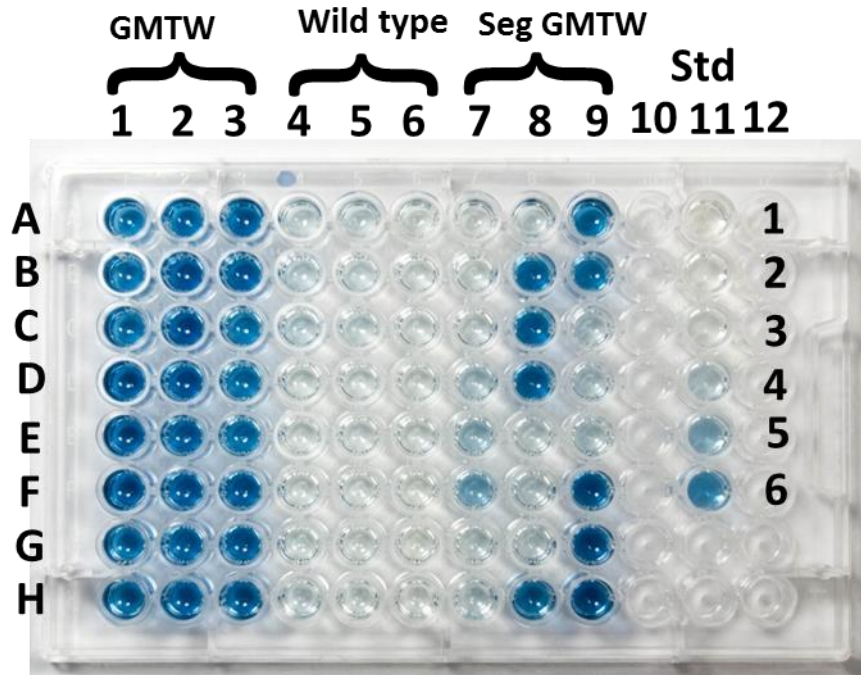
Figure 5- 2. Determination of phosphate of normal and low phytate (LP) seeds. This figure shows a test of 24 samples between 1 and 2 mg single seed chips from soybean lines with three subsamples from each seed, 72 total samples were tested. Columns 1–3 contain extracts of MIPS1 mutant seeds with the GM-*lpa*-TW1 gene that have high Pi levels, whereas normal wild-type seeds (columns 4, 5 and 6) have considerably lower Pi levels. Columns 7–9 represent segregating seeds from GM-*lpa*-TW1 × wild-type. Column 11 represents the standard curve (*1*—blank, *2*—0.17 µg Pi, *3*—0.34 µg Pi, *4*—0.85 µg Pi, *5*—1.70 µg Pi, *6*—3.40 µg Pi).
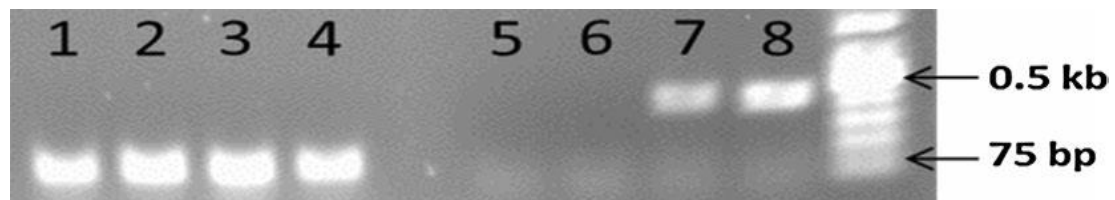
Figure 5- 3. Amplification of soybean D-myo-inositol 3-phosphate synthase (MIPS1 EC 5.5.1.4) mutant (GM-*lpa*-TW1) and wild type genes. Lanes 1–2 and 5–6 represented DNA extracted from the wild type seeds, and lanes 3–4 and 7–8 represented DNA extracted from low phytate MIPS1 mutant seeds with the GM-*lpa*-TW1 gene. Two sets of PCR primers were used MIPS-WT (wild type, lanes 1–4) and MIPS-M2 (gene, lanes 5–8). PCR products were separated on a 1.5 % agarose gel.

Table 5- 1 .Pi mg/g seed, of GM-*lpa*-TW1 seeds corresponding to the colorimetric results shown in the 96 well plate (Figure. 5-2).

GM-*lpa*-TW1 Pi (mg g^{-1})	Wild type Pi (mg g^{-1})	Segregating seeds Pi (mg g^{-1})
2.2±0.08*	0.4± 0.1	0.4± 0.03
3.1± 0.1	0.3± 0.06	0.4± 0.1
2.4± 0.1	0.4± 0.09	0.3± 0.09
2.4± 0.01	0.3± 0.1	3.0± 0.03
2.6± 0.1	0.5± 0.1	0.3± 0.05
2.6± 0.1	0.4± 0.1	0.7± 0.1
3.1±0.07	0.3± 0.06	0.6± 0.2
2.3± 0.1	0.4± 0.2	2.1± 0.1

*Each reading represents the average of three subsamples, which were either GM-*lpa*TW1, Wild type or segregating (Seg.) for GM-*lpa*-TW1.

Chapter 6: Nondestructive DNA extraction techniques for soybeans (*Glycine max* L.) seeds.

Abstract

Genotyping single seeds, while maintaining high germination rates, is useful in breeding and genetics. Non-destructive single-seed genomic DNA extraction protocols using 12 mg cotyledon tissue with a modified cetyl trimethyl ammonium bromide (CTAB) technique and a commercial seed DNA extraction kit using 1 mg cotyledon tissue were developed for dry soybean seeds and cross-verified with leaf DNA analysis. The DNA extracted by these methods was sufficient for polymerase chain reaction (PCR) and the results were reproducible for several endogenous genes and transgenes in D-*myo*-inositol 3-phosphate synthase (MIPS) mutant seeds (GM-*lpa*-TW1). This single-seed DNA-analysis method is amenable to rapid high-throughput genotype screening and verifying the genetic purity of seed stocks.

KEYWORDS: CTAB; DNA extraction; high throughput genotyping; seed chips; molecular breeding.

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Introduction

While seed DNA-extraction techniques have been developed for a handful of species, non-destructive techniques are even rarer, with seed size and sample requirements causing issues with embryo preservation, resulting in total destruction of individual seeds. In soybeans, several non-destructive DNA-extraction techniques are available, but they have limitations. For example, a technique published by (Kamalay et al., 1990) resulted in low DNA quality and low germination percentage (40%) of the chipped seeds. The protocol of Kamiya and Kiguchi (2003) requires NP-40, a reagent with limited availability because of environmental concerns in the developed world, including North America and Europe. Demeke et al. (2012) used three different methods of DNA extraction; two of which required commercial extraction and 30 min to complete, and a third, sodium dodecyl sulfate (SDS) method, required 63 min to complete. Longer requirements for DNA extraction (130 min) have also been reported for soybean (King et al., 2014). Randhawa and Firke (2006) were able to amplify DNA from 100 mg soybean seed flour using the protocol of Krishna and Jawali (1997), though such a large amount would result in the destruction of single seeds.(Rogers and Bendich, 1985) Rogers and Bendich (1985) used a modified CTAB protocol to isolate 620 ng of DNA from 11.2 mg of soybean embryos and embryo axes, which could be digested with EcoRI, though they did not verify whether the DNA could be amplified via PCR. (Kamiya and Kiguchi, 2003) used an electric drill to collect a small amount of cotyledon powder from soybean seed, being careful to avoid destroying the hypocotyl. In that study, 10-30 mg seed powder was used to extract DNA with an extraction buffer containing SDS, proteinase K (Keller and Manak, 1989), Tween 20 and NP-40 (Kamalay et al., 1990).

Phytate represents a mixture of different cationic salts of phytic acid (PA) [myo-inositol hexakis-(dihydrogen phosphate)] and is the main storage form of phosphorous (P) in seeds and grains, up to 85 % of the total seed P (Lott, 1984). In soybean seeds, the phytate ranges from 51 to 77 % of the total P (Raboy et al., 1984; Su-Cheng et al., 1990), and the phosphate level in normal soybean seeds is ~0.6 g kg^{-1} (Wilcox et al., 2000); whereas in the low-phytate mutant, the Pi level can increase to 1.5–4 g kg^{-1} (Yuan et al., 2007). However, the phytate P is unavailable to monogastric animals, such as pigs, poultry and fish and the negative charge of PA can strongly bind to metallic cations, such

as Ca, Fe, Mg and Zn, and make them unavailable nutritionally (Cromwell and Coffey, 1991; Vohra et al., 1965). Furthermore, undigested phytate P is excreted, which causes environmental pollution. To reduce such problems, animal diets can be supplemented with the enzyme phytase or low phytate (LP) seeds also can be used (Al-Amery et al., 2015).

The objective of the current study was to develop a quick and efficient bench technique for extraction of PCR-ready DNA cotyledon chips based on CTAB buffer (Murray and Thompson, 1980) and readily available commercial kit [Quick Extract™ Seed DNA Extraction Solution (Epicenter, Madison, WI)] while maintaining a good seed-germination rate.

Materials and Methods

Low phytate gene-mutant (mips1) seeds were kindly provided by Dr. Qing-Yao Shu (Institute of Crop Science, Zhejiang University).The Quick Extract™ Seed DNA Extraction Solution was provided by Epicenter, Madison, WI. The BioReady rTaq and REDTaq DNA polymerase were obtained from BulldogBio Inc, Portsmouth, NH and SIGMA- ALDRICH®., St. Louis, MO. The chemicals used in the CTAB extraction were obtained from Fisher Scientific, Pittsburgh, PA.

 CTAB extraction

Using a razor blade, 6-12 mg of seed samples were obtained by shaving off a cotyledon slice opposite to the embryonic axis. After discarding the seed coat, the seeds were ground and extracted twice with 500 µL of diethyl ether (EE) [or methyl *tert*-butyl ether (MTBE)] + 0.001% butylated hydroxytoluene (BHT) in glass tubes using glass rods to grind the samples in the tubes. The defatted particles were air-dried and 4 to 6 mg of these samples was treated with 150 µL of CTAB extraction buffer (50 mM Tris-HCl, pH8.0, 10 mM EDTA, 0.7 M NaCl, 1% CTAB and 0.2% 2-mercaptoethanol, freshly added and pre-heated at 65°C) and incubated at 65°C for 20 min with intermittent mixing. After cooling at room temperature for 5 min, 150 µL of chloroform: isoamylalcohol (24:1) was mixed by inversion and then centrifuged at 7,500g for 5 min. The supernatant (100 to 140 µL) was collected and mixed with an equal volume of CTAB precipitation buffer (50 mM Tris-HCl,pH8.0, 10mM EDTA and 1%CTAB) and incubated at 65°C for

30 min and centrifuged at 2,000 g for 5 min. The supernatants were discarded and the clear (frequently invisible) pellets were re-suspended in 50 µL high salt TE buffer (10 mM Tris-HCl, pH8.0, 1 mM EDTA and 1 M NaCl) and incubated at 65°C for 2-3 min. An equal volume of iso-propanol was added and mixed by inversion and incubated at room temperature (~ 22°C) for 10 min. It was then centrifuged at 2,000g for 5 min and the supernatants were discarded. The pellets were washed with 500 µL of 70% ethanol and centrifuged at 2,000 g for 5 min. The pellets were air-dried for 5 min and dissolved in 50 µL of the TE buffer (10 mM Tris-HCl, pH 8.0 and 1.0 mM EDTA).

QuickExtractTMSeedDNA extraction kit

After defatting, 1 to 2 mg of ground seed particles were placed in a 200 µL PCR tube with 50 µL of the QuickExtract™ Seed DNA Extraction Solution and vortexed for 10 sec, 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 2,000 g for 5 min to be stored in a -20°C freezer.

PCR verification

We used an MIPS2_M2 primer pair with the expected size of 400 bp to detect an *mips1* mutation identified by Yuan et al. (2007) and a primer pair (forward primer 5'-ACATTTGATGCTGTTGATGGGAAG-3' and reverse primer 5'-TGTAATTGCAGATAT-TAACCACCACC-3') to detect amino-alcohol phosphotransferase (GmAAPT1, Glyma12g08720) with the expected size of 351 bp. The PCR primers were obtained from Integrated DNA Technologies Inc. (Coralville, IA). 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 BioReady 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 94°C for 2 min, 35 cycles of 94°C for 30 sec, 50°C (MIPS_M2) or 58°C (GmAAPT1) for 30 sec, and 72°C for 30 sec, followed by the final extension at 72°C for 4 min. This protocol was found to work with various DNA polymerases, especially REDTaq. REDTaq worked routinely with or without defatting the seed material, whereas BioReady rTaq was most effective when

seed chips were first defatted before performing the DNA extraction. The PCR products were separated via 1% (w/v) agarose gel electrophoresis and visualized using ethidium bromide staining and UV light.

DNA analysis

Genomic DNA preparations were analyzed spectrophotometrically by measuring absorbance at 230, 260 and 280 nm wavelengths (Scientific, 2011). Typically, DNA preps were diluted 100–fold using deionized water. Similarly, diluted extraction buffers/solutions were used to determine the baseline for each wavelength.

Phosphate (Pi) evaluation

Dry cotyledon without seed coat (1-2 mg) was placed in 50 µL of an extraction buffer (25 mM $MgCl_2$ and 12.5% trichloroacetic acid and samples were incubated at 37°C with gentle shaking for 14-16 h. The mixtures were centrifuged for 3 min at 100 g. Three sub-samples of 10 µL each were taken from the supernatants and loaded into 96-well plates and diluted with 90 µL of water. Chen's reagent (100 µL) (Chen et al., 1956) was added to each sample, mixed by pipetting several times and incubated at 37°C with medium shaking for 1 h. The absorbance of the samples in the plates was read at 882 nm using a plate reader (e.g., Bio Teck-Synergy HT Plate-reader Module) after shaking the samples for 5 sec using the built-in shaker. According to (Chen et al., 1956), the individual components of Chen's Reagent do not need to be made every day. The 10 % ascorbic acid solution can be kept for several weeks at 4°C, and 3M sulfuric acid and 2.5% ammonium molybdate can be kept at room temperature. In the modified method used in this study, the ammonium molybdate and sulfuric acid solutions, kept at room temperature for 2 months, were still effective for preparing Chen's Reagent, but for ascorbic acid, the color changed after 3 weeks at 4 °C. It is highly recommended that the components of Chen's Reagent be mixed together within one hour of Pi determination of extracts. Pi extracts in the extraction buffer are stable for up to 14 days at 4 °C, or can be frozen for long periods before thawing and reacting with Chen's Reagent. A standard curve was prepared using sodium phosphate dibasic heptahydrate at 0, 0.17, 0.34, 0.85, 1.70, 3.40 µg. The volume was made to 100 µL H_2O, then 100 µL of Chen's Reagent,

which consists of 1 volume of 3 M H_2SO_4, 1 volume of 0.02 M ammonium molybdate, 1 volume of 10% ascorbic acid, and 2 volumes of water (Chen et al., 1956), was added to each sample, mixed by pipetting several times and incubated at 37°C with medium shaking for 1 h. The absorbance of the samples in the plates was read at 882 nm using a plate reader (e.g., Bio Teck-Synergy HT Plate reader Module) after shaking the samples for 5 sec using the built-in shaker (Scaboo et al., 2009).

Germination test

 Germination test was performed should be 'performed' not 'preformed' using 'Jack' soybean cultivar. From this seed stock, one mg, 20 mg seed chips, and unchipped seeds were used with four replications. Each replication contained 25 seeds individually potted (one seed per pot). After 8 days, seedlings which had reached the true two-leaf stage were counted from each replication, and scored as successfully germinated (Table 6-3).

Cost analysis

Four important cost factors were assessed for the two protocols: reagent cost, time of completion, labor cost, and total monetary cost. Reagent cost included the cost of consumable chemicals, such as Tris HCl and NaCl, Labor costs were based on minimum wage for undergraduate students and up to $14.00 per hour for a skilled technician, a typical wage in the USA. Labor and time were not factored in for seed-chip preparation and lipid extraction, amount of time, which were nearly identical for each procedure.

While the two techniques produced the same results; costs, labor and amount of time varied significantly (Table 6-2). The cost estimate and time required for Xin and Chen's protocol (Xin and Chen, 2012) are also included in Table 6-2 column 5. The reagent cost for the wet bench protocol was about one-fifth of the cost for the commercial kit we tested and about one-third of the cost for Xin and Chen's protocol.

Results

We performed many PCRs with various primer pairs and templates obtained with our method and validated the PCR readiness of genomic DNA preparations obtained via our

protocol. One such example is shown in Figure 1. We obtained similar results from the QuickExtract™ Seed DNA Extraction Solution (Figure 2).

Clear and reproducible PCR results were obtained with DNA isolated with the kit (see results shown in Figure 2 and Table 6-4). *Gm-lpa-TW-1* gene was amplified using the same primers and PCR conditions as described herein with the DNA extraction kit protocol and those samples were confirmed by high phosphate colorimetric protocol (Pi). The variation between high inorganic phosphate GM-*lpa*-TW1 and wild type (Jack) lines is shown in Table 6-4. The highest phosphate concentration was 2.6 µg/mg of seeds, whereas the wild type was 0.5 µg/mg, which means the *lpa* line had 5 times more Pi (phosphate) compared with the wild type. The mutant line (GM-*lpa*-TW1) used in this comparison was produced by a 2 bp deletion in the *MIPS1* gene, causing the *lpa* phenotype of Gm-*lpa*-TW-1. This deletion caused a frame-shift mutation in the mRNA and internal stop codons, which consequently could lead to the dysfunction of the *MIPS1* gene in Gm-*lpa*-TW-1 (Yuan et al., 2007).

Yields of genomic DNA using our protocol varied a little between batches based on several factors, such as the seed-particle size and seed condition (i.e., varieties, ages and storage conditions) (see Table 6-1). The A_{260}/A_{280} ratio of DNA was sufficient for PCR amplification. However, the crude extracts obtained by QuickExtract^{TM} Seed DNA Extraction Solutions contained large amounts of compounds with substantial UV light absorbance, such as degraded polysaccharides (low A_{260}/A_{230}), which obscured DNA quantification via spectrophotometry. In spite of this, endogenous Taq polymerase inhibitors were neutralized enough to obtain PCR products. These DNA yields were comparable to recently established techniques, including that of Xin and Chen (2012). However, the technique reported by Xin and Chen (2012) requires at least 300 mg seed material, eliminating the possibility of making this amenable to non-destructive seed chipping with medium-size seeds, such as soybeans (Demeke et al., 2012; Kamalay et al., 1990; King et al., 2014).

Discussion

While both the techniques yield sufficient quantity and quality of DNA for PCR reactions, different labs may find one more useful than the other. Perhaps most

importantly, there are significant differences in time and labor costs. It would take six to nine times longer to complete 96 samples on a plate compared with the commercial kit we tested; it took only about half of the time required for Xin and Chen's protocol. Reagent costs were much lower for the wet chemical technique, though labor and time costs were much higher. A technician can be trained quickly to perform seed kit protocol, with fewer steps than required for CTAB protocol. Therefore, the kit would most likely come as an advantage to many breeding labs with a strong agronomic emphasis but which may not have many of the CTAB reagents on hand, whereas molecular biology labs may be more interested in the CTAB protocol since they have all reagents already available, especially if low-cost labor (i.e., graduate students, undergraduate researchers) is available.

Lipid extraction was found not to be necessary for clear PCR results, but the protocol may be more reproducible with lipid removal prior to DNA extraction. All reported methods require significantly more time for extraction compared with the kit, which requires 1 mg of dry cotyledon and only eight minutes for extraction. Furthermore, seeds analyzed with the method reported herein showed differences in germination rate compared with intact seeds. The 20 mg seed chip size (Table 6-3) reduced the germination by 15% and the 1 mg chip size resulted in an 8 % reduction in germination. Compared with other reported chipping techniques, this technique described herein has the lowest reduction in germination. Both the CTAB and extraction kit protocols could be useful depending on the geneticists' or breeders' facilities and interests. For example, the CTAB protocol (see Method 2-1) requires many reagents commonly found in molecular biology and biochemistry labs, meaning it would cost these labs little to adapt this technique. If they also have a fume hood available to them for the chloroform: isoamyl alcohol extraction, it would cost little to utilize this technique. In addition, these chemicals can be stored at room temperature and are widely available. In contrast, breeding labs with a heavy agronomic emphasis are less likely to have most of these reagents on hand or a fume hood available, so ordering them to startup could come at a significant cost. These labs would probably be much more interested in using the seed kit, which, in addition to ease of use, has much lower space requirements; and there is no

need to purchase the many chemicals required for the wet-bench technique. Of course, each lab will need to assess the resources they have on hand before making a decision.

Conclusions

The development of a non-destructive seed technique for DNA extraction in soybean, with little effect on germination rate, has many implications. For example, breeders can perform genotyping on a chip and do not have to grow out plants to the two-leaf stage. Depending on the size of the breeding program, this can invoke significant space, time and labor costs. Breeders can discard unwanted seeds easily and grow out chipped seeds at a time convenient to them.
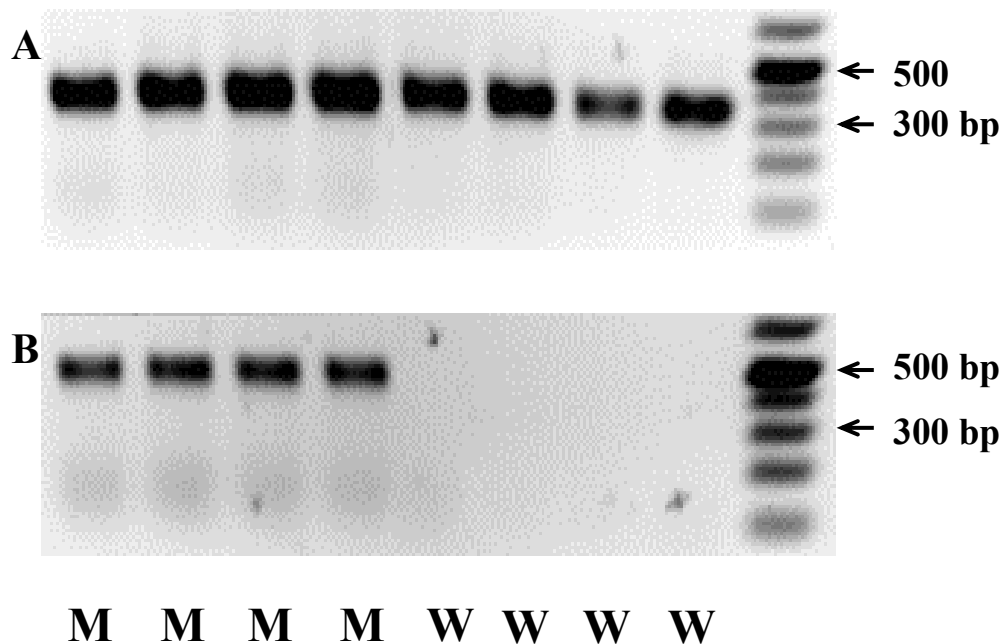
Figure 6- 1. Amplification of soybean aminoalcoholphosphotransferase (GmAAPT1) and a myo-inositol phosphate synthase mutant (GmMIPS1). Seed DNA was extracted using CTAB extraction technique. The expected product sizes for A) GmAAPT1 and B) GmMIPS1 are 351 and 400 bp, respectively. Templates used were Jack for lanes W1 - 4 and Gm-lpa-TW-1 (mips1) for lanes M1 - 4. See methods for the PCR protocol.

A

1 2 3 4 5 6 7 8 9 10 11 12

← 300 bp
← 500 bp

B

1 2 3 4 5 6 7 8 9 10 11 12

← 300 bp
← 500 bp
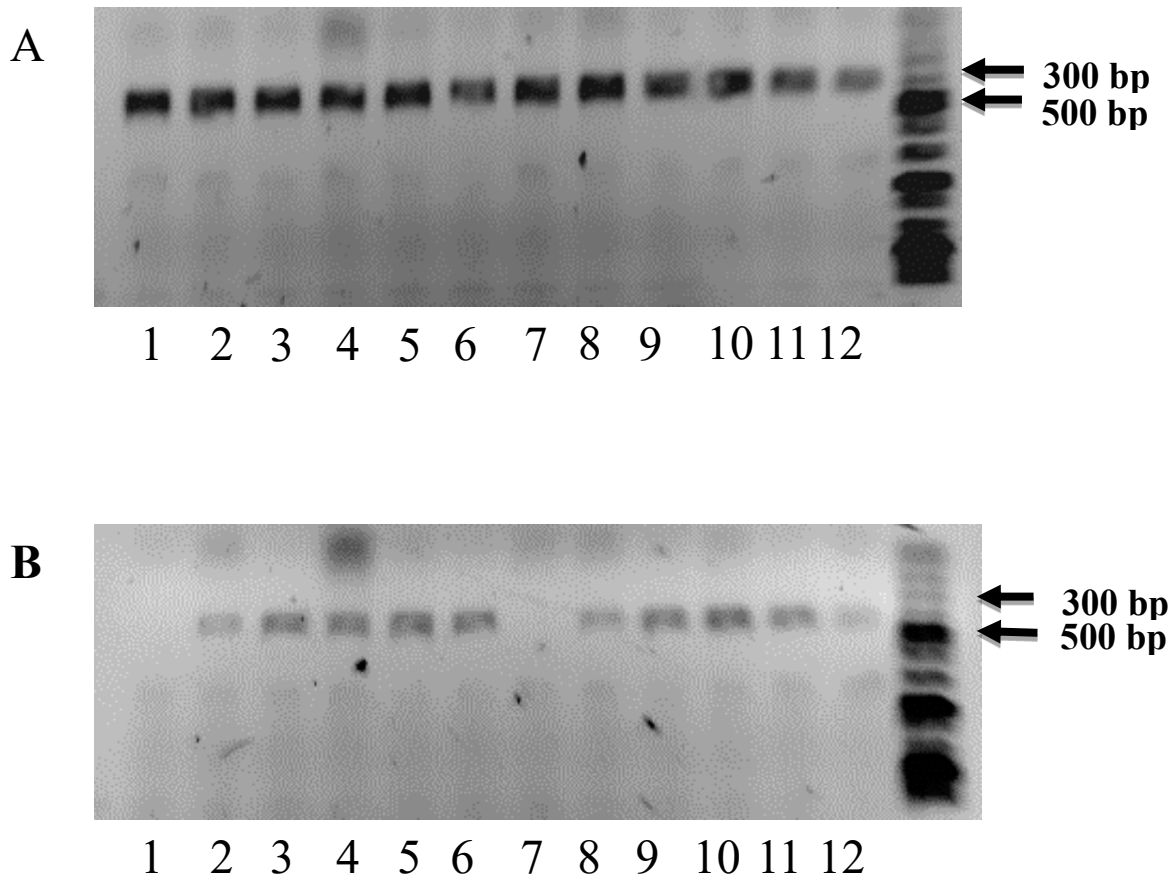
Figure 6- 2. Amplification of low phytate soybean population aminoalcoholphosphotransferase (GmAAPT1) and a *myo*-inositol phosphate synthase mutant (GmMIPS1). Seed DNA was extracted using Quick Extract™ seed DNA extraction kit. The expected product sizes for A) GmAAPT1 and B) GmMIPS1 are 351 and 400 bp, respectively. The negative and positive controls are represented by 1 and 2, respectively. See methods for the PCR protocol.

Table 6- 1. Recovery rates and quality of DNA isolated via our protocol and a commercial extraction buffer analyzed spectrophotometrically.

Method	Starting material (mg)	Final volume (µL)	DNA conc. (µg/mL)	DNA recovery (µg/mg)	A_{260}/A_{280}	A_{260}/A_{230}
†CTAB 1	4.6 ± 0.1	$50^{\ddagger}$	136 ± 5.2	1.5 ± 0.04	1.8 ± 0.02	1.4 ± 0.05
†CTAB 2	4.4 ± 0.04	50	246 ± 14.7	2.8 ± 0.2	1.7 ± 0.01	1.4 ± 0.03
Kit	1.2 ± 0.05	$50^{\S}$	¶	¶	0.98 ± 0.01	0.29 ± 0.01

†The CTAB1 and CTAB2 groups are from different soybean genotypes.
‡Smaller sub-samples were obtained from the same defatted soybean particles used for the CTAB extraction group #1.
§The number is the volume added; the recovered volume was lower, but not measured.
¶Since the A_{260}/A_{230} ratio was low, DNA concentrations were not calculated.

Table 6- 2. Cost analysis of each technique. Reagent cost and estimated time to extract 12 seed DNA samples are based on extraction by a technician experienced at each protocol. The labor-cost range is based on a wage of $7.25 to $14.00 per hour.

Technique	Reagent Cost	Estimated Time	Labor Cost	Total Cost
Wet Chemistry	$0.68	120 min	$14.50 to $28.00	$15.18 to $28.68
Seed Kit	$3.06	20 min	$2.39 to $4.67	$5.45 to $7.73
Xin and Chen's	$2.17	230 min[†]	$27.79 to $53.67	$29.96 to $55.84

Table 6- 3. Germination of chipped and un-chipped Jack soybean seeds

Treatment	Germination %	SE±
Un-chipped	90	3.46
1 mg chip	82	4.16
20 mg chip	75	4.12

Table 6- 4. Phosphate concentration (µg/mg) in seed for GM-*lpa*-TW1 corresponding to the amplifications in Figure 2B.

PCR lanes	Pi µg/mg	SE±
1	0.37	0.09
2	3.1	0.13
3	2.4	0.12
4	2.4	0.01
5	2.6	0.09
6	2.6	0.07
7	0.33	0.06
8	2.3	0.06
9	3.0	0.03
10	2.1	0.11
11	3.1	0.07
12	2.2	0.08

Chapter 7: Conclusion and further directions

VgDGAT1A Increases Oil and Oleic Fatty Acid composition in Soybeans in multiple Environments

In field environments, the high oil soybean trait maintained the higher oil level without protein reduction. VgDGAT1A transgenic soybeans showed consistently enhanced oil content without protein reduction, but we could not fully address to what extent seed composition accounts for the oil increase in the new soybean lines. The reduction in composition was variable across locations. In Princeton, the oil content was greater and partially explained by the reduction in total sugar. The reduction of the insoluble fiber fraction, namely pectin derived from galactose, (Roesler et al., 2016c) may account for some of the carbon which is redistributed to produce increased oil content. While the increase in oil content is valuable, little change was seen in anti-nutrient components such as oligosaccharides, which are the ideal carbon source for redistribution to oil. In addition to increased oil and no change in the protein, VgDGAT1A line has early maturation compared to the parental lines, which may also influence seed fill and growth rates. The increase in oleic acid could be due to the increase of oil accumulation rate, making the fatty acid unavailable metabolically to desaturases and eventually will decrease the amount of linolenic fatty acid in soybean oil.

Soybeans with high oleic acid consider being healthier than conventional soybean oil. In addition, soybean with high oleic acid (omega-6 fatty acid) has high oxidative stability, since it reduces the level of polyunsaturated fatty acids. Soybean with high oleic acid is desirable for coating applications, such as paint, ink, and varnishes.

The increasing need for food supplies from populations in developing countries, while trying to prevent increases in land use, will inevitably lead to areas of traditional cultivation utilizing new multiple cropping systems. A variety such as this, which has a shorter growing season without a reduction in yield and quality, will be an ideal candidate for such as system (Egli, 2017).

VgDGAT1A crossed with MIPS (*myo*-inositol-1-phosphate synthase)

New low phytate and high phosphate mutant (MIPS) with a reduction of 67% (dry weight) in phytate and a six-fold increase in inorganic P (Pi). When crossed with high oil

soybean line VgDGAT1A, the hypothesis is to divert the hydrocarbon from undesirable raffinose saccharides into oil. MIPS converts glucose-6-phosphate into *myo-inositol* 1-phosphate which is a key precursor to both phytic acid or phytate and the raffinose saccharides, raffinose and stachyose (Hitz et al., 2002; Yuan et al., 2007).

The cross (VgDGAT1A X MIPS) showed no increase in oil and protein compare to VgD, but the carbohydrate showed an interesting profile, since the stachyose level was reduced and the cross maintained sucrose concentration, with an increase in phosphate concentration and a corresponding reduction in phytate.

Providing high nutrition value in soybean and protecting our natural resources by reducing phytate runoff into the ground water causing eutrophication are important goals in environment and food production. The cross between MIPS and VgD is promising, still more research is still needed to improve field emergence of soybean cross and to explore the possibilities of using this cross in soymeal-based feeds in nutrition markets.

Different techniques for screening high oil/phosphate soybean lines

Most seed phosphorus (P) is bound in phytate which is unavailable to monogastric animals depriving them of P and causing eutrophication from P in animal waste. It is valuable to reduce the phytate levels of seeds used for food and feed.

Measurement of seed Pi levels and DNA extraction both are an established technique for screening for low phytate mutants but to date, it has not been performed non-destructively from single seed samples. A protocol was developed greatly reducing the sample size (about 1–2 mg) thereby reducing the cost and time and saving a generation in the selection of low phytate mutant seeds based on the high Pi phenotype. Both techniques are applicable to many other crop seeds including corn, wheat, rice, and sorghum. This can save significant space, time, and labor costs. Breeders can discard unwanted seeds easily and grow out chipped seeds at a time convenient to them.

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