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Molecular Marker Assisted Backcross Development and Evaluation of an Environmentally Friendly, Commercially Acceptable Low Seed Phytate Soybean

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To the Graduate Council:

I am submitting herewith a thesis written by Jeffrey David Boehm Jr. entitled "Molecular Marker Assisted Backcross Development and Evaluation of an Environmentally Friendly, Commercially Acceptable Low Seed Phytate Soybean." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Plant Sciences.

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Abstract

Soybean [*Glycine max* (L). Merrill] is the world's top oilseed crop. High protein soymeal is a primary soybean use and staple food of livestock and fish. The storage form of phosphorus (P) in the seed is phytic acid, or phytate [myo-inositol 1,2,3,4,5,6 hexa*kis*phosphate] which binds and chelates key nutritional cations forming stable salts that are non-digestible. Livestock waste laden with phytate P is an environmental concern, and a source of nonpoint P pollution causing eutrophication and surface water quality deterioration. The primary objective of this research was the development of a commercially acceptable low phytate (LP) soybean line which would enhance nutritional qualities of soymeal and sustainably lessen P pollution. The LP soybean lines evaluated in this study were twelve BC_5 [5^{th} generation backcross] derived lines from their recurrent parent '5601T', a high yielding University of Tennessee developed cultivar. The presence of the two LP loci in all twelve BC_5 derived lines was confirmed using perfect SNP [single nucleotide polymorphism] molecular markers near the confirmed QTL [quantitative trait loci] *cqPha-001* and *cqPha-002.*

In 2012, a field trial using a randomized complete block design with two replications was grown at two locations in two row plots to evaluate the agronomic performance of 10 BC_5 LP lines in comparison to their parents 5601T, TN09-239, and high yield check cultivars; the study was repeated in 2013, evaluating 12 BC_5 LP lines in four row plots in two locations and in two row plots in a third location with three replications per location. Data analyzed using SAS version 9.3 revealed that the yields of four BC_5 LP lines, 56CX-1273 (4107 kg ha^{-1} [kilograms per hectare]), 56CX-1274 (4137 kg ha^{-1}), 56CX-1277 (4112 kg ha^{-1}) and 56CX-1282 (4160 kg ha^{-1}), were not significantly different ($p>0.05$) than that of 5601T (4227 kg ha^{-1}) and also were numerically

better than the yield of current USDA Maturity Group V check ‘Osage’ (4089 kg ha^{-1}). These results indicate that LP trait introgression to those four BC_5 LP lines did not have detrimental effects on yield and one of the lines may be considered for a cultivar or germplasm release.

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Part 1

Introduction and Literature Review

Introduction

Soybean [*Glycine max* (L). Merrill] is the world's top oilseed crop and is considered to be one of the most important agricultural cash crops grown in the United States. Soybeans were harvested on 31.2 million hectares in 2012, producing 82 million metric tons of soybeans with the crop value estimated at $43.1 billion dollars (www.nass.usda.gov). The United States exported 36.6 million metric tons of soybeans and soy products in 2012, with China being the largest customer. In 2012, the United States also produced 34.9 million metric tons of soybean meal, which was used primarily as feed in the livestock sector. Tian et al. (2010) estimated that soybeans provide more than a quarter of the world's food and animal feed. Domestically, soybeans are the second largest cash crop in sales and second most planted crop in hectares next to corn, but soybeans are the top exported crop for the U.S. According to the U.S. Environmental Protection Agency (EPA), the U.S. produces over 50% of the world's soybeans. In 2012 the top producing states were Iowa (3784 thousand hectares planted producing 11.2 million metric tons), Illinois (3662 thousand hectares planted producing 10.4 million metric tons), Minnesota (2873 thousand hectares planted producing 8.1 million metric tons), Nebraska (2044 thousand hectares planted producing 5.6 million metric tons), Indiana (2084 thousand hectares planted producing 6 million metric tons), Ohio (1862 thousand hectares planted producing 5.6 million metric tons), Missouri (2185 thousand hectares planted producing 4.2 million metric tons), South Dakota (1922 thousand hectares planted producing 3.8 million metric tons), Arkansas (1295 thousand hectares planted producing 3.7 million metric tons), North Dakota (1922 thousand hectares planted producing 4.3 million metric tons) and Kansas (1619 thousand hectares planted producing 2.2 million metric tons). As for Tennessee, soybeans were planted on 510 thousand hectares in 2012 which produced 1.2 million metric tons valued at over

$480 million dollars (Soy Stats, 2012; www.nass.usda.gov). The yearly average commodity trading price for soybeans in 2012 was $536/metric ton. In 2013, U.S. soybeans were still in high demand. According to the United Soybean Board (USB), worldwide demand for U.S. soybeans in 2013 hit record levels, as over 46.3 million metric tons of soybeans were exported in 2013, with meal from 12.35 million metric tons adding to the total. Domestically, 30 million metric tons of soymeal from 34.29 million metric tons of soybeans was used primarily for broiler chickens (*Gallus domesticus*, 12.95 million metric tons), hogs (*Sus domesticus*, 11.15 million metric tons), laying hens (*Gallus domesticus*, 2.28 million metric tons), turkeys (*Meleagris gallopavo*, 2.04 million metric tons), and combined other feed uses (5.9 million metric tons). According to the USB study, the U.S. soybean industry is reliant on the annual demand strength coming from the U.S. animal agriculture industrial sector.

Soybean belongs to the Fabaceae family, which is commonly called the legume, pea or bean family. The center of origin, where soybean was first cultivated, is China. The subfamily classification is Papilionoideae; the tribe is Phaseoleae and the genus is *Glycine*. The *Glycine* genus has two subgenera: *Glycine* and *Soja*. The cultivated soybean, *Glycine max,* and its wild annual relative, *Glycine soja*, belong to the subgenus *Soja*. The subgenus *Glycine* is comprised of 16 wild perennial species (Mishra and Verma, 2010). The annual soybean and some of the wild perennial species have 2n=40 chromosomes. The annual *Soja* subgenus is most widespread in Northern China while the perennial subgenus *Glycine* is found mostly in Australia. *Glycine* is thought to be an ancient autotetraploid having x=10 chromosomes, but according to Mishra and Verma (2010), behave cytologically like diploids with 2n=40 chromosomes. In China, the wild *Glycine soja* was first domesticated and used for agriculture over 5000 years ago. One can find wild soybeans, semi-wild soybeans, and highly evolved cultivated soybeans growing in China

today. All of the soybean land races, germplasm lines, and modern cultivars, collectively and with varying degrees of evolution and selection pressure, have adapted to grow in many different natural environments, and the utilization requirements have been diverse depending on the needs and cultural conditions being met. When *Glycine max* is crossed with its wild relative *Glycine soja*, the seed size, plant height, lodging and many other traits are inherited as quantitative traits which demonstrate that the two groups have accumulated major or minor variants of the same underlying genes (Mishra and Verma, 2010). It is understood that the evolution of soybean has undergone a continuous accumulation of minor variations to quantitative variation and that artificial selection has further promoted the differentiation of these traits into the soybean that's extensively grown on farms today (Mishra and Verma, 2010). For example, most agricultural communities have selected for seed quality traits, and over time have focused on selecting larger seeds; that selection pressure has affected other traits. As a result, the seed size and the pod size has increased, but the plant height has decreased, and the stem has gotten thicker. The reduced plant height and thickened stem have been favorable agricultural traits, which demonstrate the soybean's progression from the viny type *Glycine soja* to the erect type *Glycine max*, with the latter plant being better suited for agricultural management practices. Joshi et al. (2013) identified 425 genes that are unique to *Glycine max* yet unavailable in *Glycine* soja, with twelve of the identified genes involving seed development, three involving oil and six involving protein concentration. Agricultural management has favored the selection for strong, lodging-resistant, agronomic type plants over time.

Today, plant breeders continue to select plants that demonstrate superior agronomic traits. This study will focus on phytate, myo-inositol-1,2,3,4,5,6-hexakisphosphate, which is the main source of phosphorus (P) in soybean seeds (Erdman, 1979). Soybean breeders have

endeavored to develop low phytate (LP) cultivars, to enhance the mineral nutrition and P availability in soymeal, while at the same time sustainably lessening P pollution from livestock waste runoff. The backcross (BC) development and selection of the LP plants in this study, much like the selections of our agricultural ancestors, were made to satisfy current agricultural production demands. One such production demand from the animal agricultural sector is the creation of a high yielding LP cultivar.

The soybean lines being used in this study are twelve BC_5 derived LP lines from their recurrent parent (RP) '5601T' and TN09-239, our LP, indeterminate donor parent, whose LP trait was introgressed from the indeterminate germplasm CX 1834-1-2 (Shu, 2009). A BC plant breeding strategy was utilized to introgress the LP trait from the donor parent TN09-239 into the favored genetic background of RP 5601T. The LP genes are recessive, thus the process required generations of backcrossing and self-pollination to develop and test double homozygote recessive genotypes using an inorganic P (Pi) assay. However, the use of molecular markers to test for the presence of the desired LP trait at each backcross eliminated the need for selfing for progeny testing until the final BC stage (Vogel 2009). The twelve BC_5 LP genotypes in this study are theoretically expected to be 98.4% genetically similar on average to RP 5601T, a high yielding University of Tennessee released cultivar.

The objectives of this thesis project were to:

(1) Evaluate the agronomic performance of twelve new LP BC_5 lines in comparison to their parents 5601T, TN09-239, and USDA high yield check cultivars.

(2) Test whether seeds produced by each of the LP lines differ significantly from each other or from their recurrent parent 5601T for Pi content.

(3) Test whether seeds produced by each of the LP lines differ significantly from each other or from their recurrent parent 5601T for seed protein concentration, oil concentration, amino acid composition of the protein and fatty acid composition of the oil.

(4) Document stable transfer of the two LP QTL, Pha-001 and Pha-002, using gene sequence based SNP technology.

(5) Break the genetic linkage between the Pha-002 locus on Gm 19 (LG L) and the nearby Dt1 locus to develop a BC_5 LP line with determinate stem termination.

Literature Review

The origin and discovery of phytic acid (myo-inositol 1,2,3,4,5,6 hexa*kis*phosphate) stems from research conducted by Theodor Hartig, a German botanist and biologist, who conducted experiments from 1855-1856 and first isolated small, non-starch grains from various plant seeds and considered the grains to be providing essential reserves for seed germination and plant growth (Reddy et al., 1989). In 1872, another German scientist named Wilhelm Pfeffer, was able to separate these grains into three groups: (1) crystals of calcium oxalate, (2) a protein substance, and (3) a compound giving no reactions for protein, fat or inorganic salts (Reddy et al., 1989). He characterized the last group as having rounded surfaces with spheroidal shapes which were found in all of the 100 seeds of that group that he examined. They were free of nitrogen (N), but contained calcium (Ca), magnesium (Mg) and P, and he named them globoids (Reddy et al., 1989). Globoids of soybean seeds are a proteinaceous matrix of protein bodies found in the cotyledons of the seeds (Reddy et al., 1989). In 1903, Posternak extensively experimented with the globoid particles which were earlier classified as "inosite phosphoric

acid" by Winterstein in 1897, and in 1903 Posternak was given credit for being the first to describe and discover phytic acid which he called phytin (Mullaney and Ullah, 2005; Reddy, 1989). However, scientists could not agree as to the exact chemical structure of phytic acid, as both Neuberg in 1908 and Anderson in 1912 submitted proposed structural models. Modern chemical analysis and data now confirm that the model submitted by Anderson in 1912 to be the accurate depiction, showing the form of phytic acid found in plant seeds and grains (Reddy et al., 1989). (Figure 1.1)

We know today that legume seeds like soybean contain large amounts of phytic acid which are localized in the crystalline globoid inclusions within protein bodies of soybean seeds (Wilson, 1987). Phytate refers to the mixed cation salt of phytic acid or the mixed salt form of phytic acid found in seeds (Gao et al., 2007; Reddy et al., 1989). Phytic acid refers to free acid in the seed, and phytin refers to a Ca-Mg salt (Reddy, 1989). Researchers commonly use the terms phytic acid and phytate interchangeably. The chemical construction of the globoids contains phytate, potassium (K), Ca, and Mg (Reddy, 1989). Typically phytic acid is present in seeds as phytin salts of K and Mg, but the salts deposited may also contain other cations such as iron (Fe), zinc (Zn) and Ca (Raboy, 2002). Roughly 75% of the total P in conventional soybean seed is phytate (Oltmans et al., 2005; Lott et al., 2007), but phytate can account for as little as 50% or as much as 85% of the total P (Reddy, 1989; Raboy, 2002). Phytates are considered to be the primary form of inositol and phosphate in most seeds and the most abundant inositol phosphate in nature (Erdman, 1979; Raboy, 2009). Phytic acid is utilized during germination and early seedling growth and aids in other metabolic functions. Phytic acid is also a key component of numerous developmental and signaling processes, which occur in the cytoplasm and plastids, in the nucleus in pathways crucial to deoxyribonucleic acid (DNA) repair, in

chromatin remodeling, in ribonucleic acid (RNA) editing and in the regulation of gene expression (Raboy, 2009). Phytic acid also assists in signal transduction molecules, osmoprotectants, and cell wall constituents (Hegeman et al. 2001). Phytate located in the protein bodies of soybean seeds can be found in the both water-soluble form and water-insoluble forms (Prattley et al., 1982; Powers et al., 2013; Reddy, 1989). Phytates are known to be heat stable, and can withstand high temperatures, rough field and weather conditions and various storage environments lending to their mobility in watersheds (Sathe and Reddy, 2002). However, phytic acid is degraded during seed germination by a specific group of enzymes called the phytases (Brinch-Pedersen et al., 2002). Phytase (myo-inositol hexaphosphate phosphohydrolase) is also present in developing and germinating seed and catalyzes the hydrolysis of phytic acid to myo-inositol and Pi (Wilson, 1987) (Figure 1.2). Phytase is so effective in breaking down the phytate molecule that it is commonly added to soybean meal to improve the digestibility and bioavailability of dietary P (Traylor et al., 2001). It is approximated that nearly 80% of the poultry and 60% of the pig feeds manufactured world-wide contain an exogenous phytase to improve availability and digestibility of P, Ca, Zn, Sodium (Na), Mg and amino acids (Walk, 2012).

Phytic acid is synthesized from the phosphorylation of myo-inositol (Brinch-Pedersen et al., 2002). The carbocyclic structure of phytate leading to phytic acid biosynthesis is myo-inositol (Loewus, 2002). Free myo-inositol is a ubiquitous constituent of plant and animal cells, and is involved in many plant metabolic functions, such as ion uptake, cell wall biogenesis, and the formation of myo-inositol polyphosphates including phytic acid (Loewus, 2002). Once phytic acid is synthesized, phytate will quickly accumulate in seeds during the period of ripening or period of maturation (Reddy, 1989). Yao et al. (1983) observed phytate levels increasing from

0.87 to 1.26% during soybean maturation on a dry-weight basis (Sathe and Reddy, 2002). Five physiological roles have been suggested for phytate in seeds and grains: (1) as a P store, (2) as an energy store, (3) as a source of cations, (4) to initiate dormancy and (5) as a source of myo-inositol (Reddy, 1989). As mentioned above, the P reserves are used to support germination, early seedling growth and development, and are an essential nutrient required by all life (Raboy, 2002). Phytate offers myo-inositol, the macro-nutrients P, K, and Mg and micro-nutrients Ca, Fe, Zn, and manganese (Mn) to growing seedlings and phytic acid synthesis plays an integral role in the seed storage processes for minerals like K, Zn, Fe and Mg (Reddy, 1989; Raboy, 2002). Typically Pi is taken up by the plant in the form of phosphoric acid (H_3PO_4), then translocated to the developing seed and synthesized to become the storage form of P which is phytic acid (Raboy, 2002). Many factors can influence seed phytate levels, such as environmental fluctuations, growing locations, irrigation conditions, fertilizer applications, soil types, growing conditions, cultivar and time of year the plant is grown (Reddy, 1989).

Soybean seeds contain approximately 40% protein and 20% oil on a dry weight basis (Singh, 2010). Typically the protein quality, quantity, nutrient content and caloric value of soybeans are very good (Erdman, 1979). The soybean fatty acids are commonly identified with their systematic abbreviations such as 16:0, with the first number representing the number of carbon atoms in the molecule and the second number indicating the number of unsaturated carbon-carbon double bonds in the molecule. The average fatty acid composition of soybean oil is roughly 10% palmitic acid (16:0), 4% stearic acid (18:0), 22% oleic acid (18:1), 54% linoleic acid (18:2), and 10% linolenic acid (18:3) (Wilson, 1987). The soybean's plentiful oil concentration led to the consumption of 6.4 million kg of its edible fats and oils in the United States in 2011 (Soy Stats, 2012). After the oil is extracted, the de-hulled soybeans are cracked,

heated and flaked thereby producing soymeal. The high protein soymeal is used domestically to feed ruminant and non-ruminant livestock such as cattle (*Bos primigenius*), hogs (*Sus domesticus*), poultry (*Gallus domesticus*), sheep (*Ovis aries*), and fish (*Ichthyoid spp.*). In 2012, over 27 million metric tons of soybean meal was used domestically to feed livestock. In 2013, those numbers increased, as 1.26 billion bushels of soybeans were used to produce 30 million metric tons of soybean meal (Soystats 2013). According to a United Soybean Board (USB) study, the U.S. soybean industry is reliant on the annual demand strength coming from the U.S. animal agriculture industry.

Livestock producers use the high protein soymeal as a staple food but the animals also digest large amounts of phytate present in the soymeal (Raboy and Dickinson 1984; Erdman, 1979). Chemically, the structure of the phytate molecule acts as a binding or chelating agent to mineral cations forming salts that are non-digestible, which essentially removes the mineral bioavailability from the seeds by forming the more complex phytate molecule (Erdman, 1979; Maenz et al., 1999) (Figure 1.1). Many of the phytate mineral complexes that are formed are insoluble and therefore unavailable for absorption (Reddy, 1989). The bound cations, such as Ca, Fe, Mg, Mn and Zn, are nutritional minerals that become non-digestible insoluble phytate salts (Erdman, 1979; Raboy, 2002; Reddy, 1989). The interference with intestinal absorption of the mineral complexes, such as Ca, Fe, K, Mg, P and Zn, may lead to mineral deficiencies in animals and humans earning phytate the label of "anti-nutrient" (Reddy, 1989; Lott et al., 1995). However, the antioxidant functions can have beneficial medical applications (Graf and Eaton, 1990). The chelation activity of phytic acid is pH dependent (Prattley et al. 1982; Wilson, 1987). The phytic acid is not broken down in the intestinal tract of monogastric animals because they possess little to no phytase activity in their digestive tracts, which is why phytic acid is not

efficiently utilized (Brinch-Pedersen et al., 2002). For the phytic acid to be efficiently utilized, animal feeds must be supplemented with a phytase enzyme to free cations and other nutrients bound by phytate P-complexes. In addition, the animal feed may be supplemented with digestible Pi for the animals to receive the optimum amount of P needed for animal growth and skeletal development (Khalid et al., 2013; Shi, 2007; Walk, 2012).

Because animals lack the phytase enzyme in their digestive tracts necessary to break down the stable phytate molecule, Powers (2006) found that the excess P in the form of phytic acid in the animal waste is both insoluble and water soluble, whose relative abundance changed with the dietary treatments (Brinch-Pedersen et al., 2002; Jendza, 2009). The non-digestible, stable phytate salts present in soybeans and soymeal are therefore excreted by animals that consume them (Erdman, 1979; Raboy, 2002; Raboy, 2009; Bilyeu et al., 2008; Gillman et al., 2009; Wilcox et al., 2000). The excess P in the excretion then becomes a P management concern, because high levels of P in the form of phytic acid present in manure is often applied as fertilizer to agricultural lands (Hegeman et al., 2001). Soil bacteria break the chelate bonds allowing Pi to become mobile in watersheds, resulting in accelerated eutrophication and surface water quality deterioration in ponds, lakes, streams and estuaries. The P laden animal waste is a major point source (Carpenter, et al., 2010) and non-point source of P pollution detrimental to the environment (Sharpley et al. 1994, Carpenter et al., 2010, Walker et al., 2006). The creation of a LP soybean cultivar to be used for soymeal would ease environmental concerns and provide benefits to livestock nutrition. Lowering the phytate concentration in the seed would serve to enhance the dietary nutrition of soymeal and also increase the metabolic energy available to ruminant and non-ruminant livestock by increasing P bioavailability (Oltmans et al., 2004, Shu, 2009). The increased P bioavailability would allow non-ruminant livestock to digest the Pi

present in the soymeal thereby removing the P from the waste stream and greatly reducing eutrophication in aquatic environments (Brinch-Pedersen, Sorensen and Holm, 2002; Sharpley et al., 1994). The combined benefits would lead to more effective and sustainable P nutrient management on farms for crop and livestock production. In addition, livestock producers would also save costs in relation to the reduced need to purchase and add phytase amendments to soybean meal. In summary, modifying the P in soybean seed by lowering the phytate level would enable livestock producers to enhance animal nutrition and would sustainably lessen P pollution from animal agricultural waste runoff. These improvements are the rationale behind this research.

As Mellinger (2012) noted, there are several genetic sources of LP in soybean: the Gm-lpha-TW-1 allele, the Gm-lpa-ZC-2 allele, the MIPS allele and the combination of the LP alleles on Gm03 and Gm19 totaling five alleles that have been reported for LP in soybean (Wilcox et al., 2000). This study focused on the combination of the LP alleles on Gm03 and Gm19, which are two independent recessive loci that govern LP confirmed by Maroof et al. (2009), Gao et al. (2008), Oltmans et al. (2005), and Walker et al. (2006). Oltmans et al. (2005) analyzed segregation ratios of LP plants and determined that the recessive alleles exhibit duplicate dominant epistasis making it necessary for both recessive alleles to be homozygous before a plant can express the LP trait. The DNA sequence of the two LP mutant alleles (*lpa1* and *lpa2*) is known and perfect molecular markers have been constructed to detect their presence which breeders can use to make selections (Bilyeu et al., 2009). In our study we used single nucleotide polymorphism (SNP) technology, specifically perfect molecular markers at cqPha-001 and cqPha-002, to ensure and confirm that all twelve BC_5 plant progenies in this study were double homozygous recessive for the two alleles that express LP concentration in soybean seeds (Shu,

2009; Scaboo et al., 2009). Seed phytate content and Pi content have an inverse relationship (Bilyeu et al., 2008; Chen, 1956; Gao et al., 2008; Raboy, 2000; Shi et al., 2007; Wilcox et al., 2000; Oltmans et al., 2005; Spear and Fehr 2007). Low phytate lines have demonstrated elevated levels of Pi and a concomitant decrease in phytic acid as total seed P does not change (Gillman et al. 2009; Scaboo et al. 2009). Inorganic P is digestible and does not chelate nutritional mineral cations. The line CX 1834-1-2 was derived from mutated line M153 which was created by Wilcox et al. (2000) through EMS (ethyl methanesulfonate) mutagenesis (Maroof, et al., 2009). Plants typically produce more phytic acid than is needed for growth and development and reducing the phytic acid has not inhibited peak agronomic performance (Scaboo et al. 2009; Gillman et al. 2009; Bilyeu et al., 2008; Shi et al., 2007; Spear and Fehr 2007). Raboy (1984) tested and evaluated 163 soybean lines for phytic acid content and found that the soybean lines tested averaged 6.9 g Kg^{-1} seed total P and 17.6 g Kg^{-1} phytic acid, with seed total P and phytic acid being highly correlated (r = 0.99) with the percentage of seed total P as phytic acid ranging from 51 to 57%. Wilcox et al. (2000) compared the phytic acid content of 'Athow' 15.33 g Kg^{-1} (normal seed phytate) versus the LP mutant lines and found the phytic acid mean value of the LP mutants to be much lower (5.42 g Kg^{-1}). The LP genotypes in this study are derived from the mutated lines developed by Wilcox et al. (2000).

We presently speculate that donor parent TN09-239 is segregating for the *Dt1* locus on Gm19 (LG L), where the dominant form of that locus has the indeterminate growth habit (Tian et al., 2010). The position of the *Dt1* locus on Gm19 is positioned less than 20 cM from the LP locus, which indicates genetic linkage. Despite being at the 5^{th} stage of the BC strategy, many of the BC_5 LP lines, as well as BC_4 line and donor parent TN09-239, were still exhibiting the indeterminate growth habit even though RP 5601T is determinate. Indeterminacy in a maturity

group (MG) V soybean would lead to excessive plant height and contribute to lodging which could reduce yield. In 2012, Dr. Kristen Bilyeu (USDA-ARS, Columbia, MO) utilized this research to create a new gene sequence based SNP for the stem termination locus (*Dt1*). We used the new *Dt1* R166W SNP to analyze all of our BC_5 LP lines, and the results indicate that eight BC_5 56CX LP lines have been classified homozygous recessive (*dt1 dt1*), expressing the desired MG V determinate growth habit. Thus we have been successful in breaking the Gm19 genetic linkage in over 50% of the BC_5 LP lines being evaluated which was a major research objective.

References

Anderson, B. and W. Fehr. 2008. Seed source affects field emergence of low-phytate soybean lines. Crop Sci. 48**:** 929-932.

Anderson, R.J. 1912. Phytin and phosphoric acid esters of inosite. J.Biol. Chem. 43:117.

Bilyeu, K.D., Zeng, P., Coello, P., Zhang, Z.J., Krishnan, H.B., Bailey, A., Beuselinck, P.R., and Polacco, J.C. 2008. Quantitative Conversion of Phytate to Inorganic Phosphorus in Soybean Seeds Expressing a Bacterial Phytase. Plant Physiology. 146: 468-477.

Brinch-Pedersen, H., Sorensen, L.D., and Holm, P.B. 2002. Engineering crop plants: getting a handle on phosphate. Trends in Plant Science. 7:3.

Carpenter, S.R., Caraco, N.F., Correll, D.L., Howarth, R.W., Sharpley, A.N., and Smith, V.H. Nonpoint Pollution of Surface Waters With Phosphorus And Nitrogen. 1998. Ecological Applications. 8: 559-568.

Chen Jr. P, T. Toribara, and H. Warner. 1956. Microdetermination of P. Anal. Chem. 28(11): 1756–1758.

Cichy, Karen, and Victor Raboy. "Evaluation and Development of Low-Phytate Crops." 2009. Modification of Seed Composition to Promote Health and Nutrition. Ed. Hari Krishnan. Madison, WI: American Society of Agronomy, Crop Science Society of America, Soil Science Society of America. 177-200.

Daniel, T.C., A.N. Sharpley, D.R. Edwards, R. Wedepohl, and J.L. Lemunyon. 1994. Minimizing surface water eutrophication from agriculture by phosphorus management. J. Soil Water Conserv. 49: 30-38.

Erdman, J.W. 1979. Oilseed Phytates: Nutritional Implications. Department of Food Sciences. Urbana, Illinois.

Fehr, W.R. and Caviness, C.E. 1977. Soybean Stages of Development. Ames, IA. Cooperative Extension Service.

Gao, Y., Biyashev, M.A., Maroof, S., Glover, N.M., Tucker, D.M., and Buss, G.R. 2008. Validation of Low Phytate QTLs and Evaluation of Seedling Emergence of Low-Phytate Soybeans. Crop Sci. 48: 1355-1364.

Gillman, Jason D., Vincent R. Pantalone, and Kristin Bilyeu. 2009. The low phytic acid phenotype in soybean line CX1834 is due to mutations in two homologs of the maize low phytic acid gene. Plant Genome. 2: 179-190.

Graf, E. and Eaton, J.W. 1990. Antioxidant Functions of Phytic Acid. Free Radical Biology and Medicine. 8:61-69.

Hegeman, C.E., Good, L.L., and Grabau, E.A. 2001. Expression of d-myo-Inositol-3-Phosphate Synthase in Soybean. Implications for Phytic Acid Biosynthesis. Plant Physiology. 125: 1941-1948.

Jendza, J.A., and Adeola, O. 2009. Water-soluble phosphorus excretion in pigs fed diets supplemented with microbial phytase. Animal Science Journal. 80:296-304.

Joshi, T., Valliyodan, B., Wu, J., Lee, S., Xu, D., and Nguyen, H.T. 2013. Genomic Differences between cultivated soybean, *G. max* and its wild relative *G. soja*. From the Eleventh Asia Pacific Bioinformatics Conference. Vancouver, Canada.

Khalid, M.F., Hussain, M., Rehman, A.U., Shahzad, M.A., Sharif, M. and Rahman, Z.U. 2013. Broiler Performance in Response to Phytate and Supplemented Phtyase. Iranian Journal of Applied Science. 3:1-12.

Loewus, F.A. 2002. Biosynthesis of Phytate in Food Grains and Seeds. Food Phytates. Boca Raton, FL: CRC Press, Inc.

Lott, J.N.A., Ockenden, I., Raboy, V., and Batten, G.D. 2000. Phytic Acid and Phosphorus in crop seeds and fruits: a global estimate. Seed Science Research. 10:1: 11-33.

Maenz, D. D., Engele-Schaan, C.M., Newkirk, R.W., and Classen, H.L. 1999. The effect of minerals and mineral chelators on the formation of phytase-resistant and phytase-succeptible forms of phytic acid in solution and in a slurry of canola meal. Animal Feed Science and Technology. 81: 177-192.

Maroof, S.M., Glover, N.M., Biyashev, R.M., Buss, G.R. and Grabau, E.A. 2009. Genetic Basis of the Low-Phytate Trait in the Soybean Line CX1834. Crop Science. 49:69-76.

Mellinger, S., 2012. Molecular Marker Assisted Development and Stability Analysis of Low Phytate, High Inorganic Phosphorus Soybean [*Glycine max* (L.) Merrill]. University of Tennessee, MS Thesis.

Mishra, S.K. and Verma, V.D. 2010. The Soybean: Botany, Production and Uses. Cambridge, MA: CAB International.

Mullaney, E.J. and Ullah, A.H.J. 2005. Phytases: Attributes, Catalytic Mechanisms, and Applications. Commodity Utilization Research.

Oltmans, S. E., W. R. Fehr, G. A. Welke, and S. R. Cianzio. 2004. Inheritance of low-phytate phosphorus in soybean. Crop Sci. 44: 433-435.

Oltmans, S. E., W. R. Fehr, G. A. Welke, V. Raboy, and K. L. Peterson. 2005. Agronomic and seed traits of soybean lines with low-phytate phosphorus. Crop Sci. 45: 593-598.

Pantalone, V.R., F.L. Allen, and D. Landau-Ellis. 2003. Registration of Soybean Cultivar 5601T. Crop Sci. 43:1123.

Powers, W. J., E.R. Fritz, W. Fehr, and R. Angel. 2006. Total and water-soluble phosphorus excretion from swine fed low-phytate soybeans. J. Animal Sci. 84: 1907-1915.

Prattley, C.A., Stanley, D.W., Smith, T.K., Van De Voort, F.R. 1982. Protein-Phytate Interactions in Soybean. J. Food Biochem. 6:4:273-282.

Raboy, V., Hudson, S.J., and Dickson, D.B. 1984. Reduced Phytic Acid Does Not Have an Adverse Effect on Germination of Soybean Seeds. Plant Physiology. 79: 323-325.

Raboy, Victor. 2002. Progress in breeding low phytate crops. Nutrition. 2002:503S-505S.

Raboy, V., D. B. Dickinson, and F. E. Below. 1984. Variation in seed total phosphorus, phytic acid, zinc, calcium, magnesium, and protein among lines of Glycine max and G. soja. Crop Sci. 24: 431-434.

Raboy, V. 2002. Impacts of Agriculture on Human Health and Nutrition. EOLSS Publishers, Ltd. Oxford, UK.

Reddy, N. R., M. D. Pierson, S. K. Sathe, and D. K. Salunkhe. 1989. Phytates in Cereals and Legumes. Boca Raton, FL: CRC Press, Inc.

SAS Institute. 2011. The SAS system for Windows. Release 9.3. SAS Inst., Cary, NC.

Sathe, S.K. and Reddy, N.R. 2002. Food Phytates. Boca Raton, FL. CRC Press.

Scaboo, A.M., V.R. Pantalone, D.R. Walker, H.R. Boerma, D.R. West, F.R. Walker, and C.E. Sams. 2009. Confirmation of molecular markers and agronomic traits associated with seed phytate content in two soybean RIL populations. Crop Sci. 49: 426-432.

Sharpley, A., B. Foy, and P. Withers. 2000. Practical and innovative measures for the control of agricultural phosphorus losses to water: and overview. J. Environ. Qual. 29.1:1-9.

Shi, J., Wang, H., Schellin, K., Li, B., Faller, M., Stoop, J.M., Meeley, R.B., Ertl, D.S., Ranch, J.P., and Glassman, K. 2007. Embryo-specific silencing of a transporter reduces phytic acid content of maize and soybean seeds. Nature Biotechnology. 25:8.

Shu, Q.Y. 2009. Induced Plant Mutations in the Genomics Era. Food and Agriculture Organization of the United Nations, Rome: 316-318.

Soy Stats. 2012. United States Soy Crop Statistics. The American Soybean Association. <http://www.soystats.com/2012/Default-frames.htm>

Spear, J. D., and W. R. Fehr. 2007. Genetic improvement of seedling emergence of soybean lines with low phytate. Crop Sci. 47:1354-1360.

Tian, Z., Wang, X., Lee, R., Li, Y., Specht, J.E., Nelson, R.L., McClean, P.E., Qiu, L., and Ma, J. 2010. Artificial Selection for determinate growth habit in soybean. PNAS. 107:19:8563-8568.

Traylor, S.L., Cromwell, G.L., Lindemann, M.D., and Knabe, D.A. 2001. Effects of level of supplemental phytase on ileal digestibility of amino acids, calcium, and P in dehulled soybean meal for growing pigs. Animal Science. 79: 2634-2642.

United States Environmental Protection Agency. Ag 101: Major Crops Grown in the United States. Web 29 Aug 3013. <http://www.epa.gov/agriculture/ag101/cropmajor.html>

United States Department of Agriculture. National Agricultural Statistics Service. Crops and Plants. Field Crops. Beans. Web 29 Aug 2013. <www.nass.usda.gov>

Vogel, K.E. 2009. Methods in Molecular Biology: Transgenic Maize, vol. 526. Humana Press. 14: 161-169.

Walk, C.L., Bedford, M.R. and McElroy, A.P. 2012. Infleunce of limestone and phytase on broiler performance, gastrointestinal pH, and apparent ileal nutrient digestibility. Poult Sci. 91(6):1371-8.

Walker, D.R., A.M. Scaboo, V.R. Pantalone, J.R. Wilcox, and H.R. Boerma. 2006. Genetic mapping of loci associated with seed phytic acid content in CX1834-1-2 soybean. Crop Sci. 46:390-397.

Wilcox, J. R., G. S. Premachandra, K. A. Young, and V. Raboy. 2000. Isolation of high seed inorganic P, low-phytate soybean mutants. Crop Sci. 40:1601-1605.

Wilson, R.F. Soybeans: Improvement, Production and Uses. 1987. Agronomy. 16: 643-686.

Yao, J.J. Wei, L.S. and Steinberg, M.P. 1983. Effect of maturity on chemical composition and storage stability of soybeans. J. Amer. Oil Chem. Soc. 60:1245-1249.

Appendix I

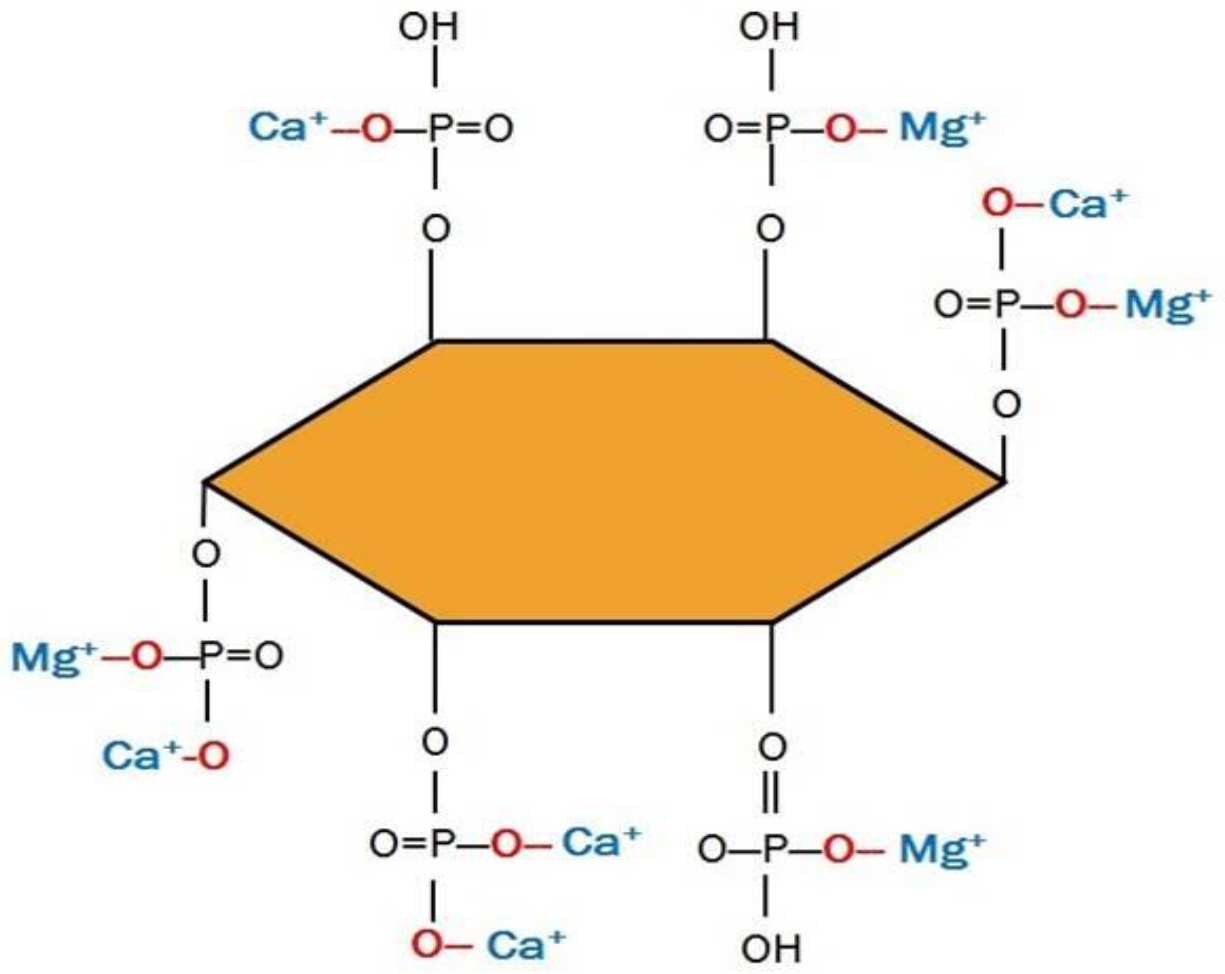


Figure 1.1

Phytic acid molecule showing P at each ring which bonds positively charged nutrients as chelates.

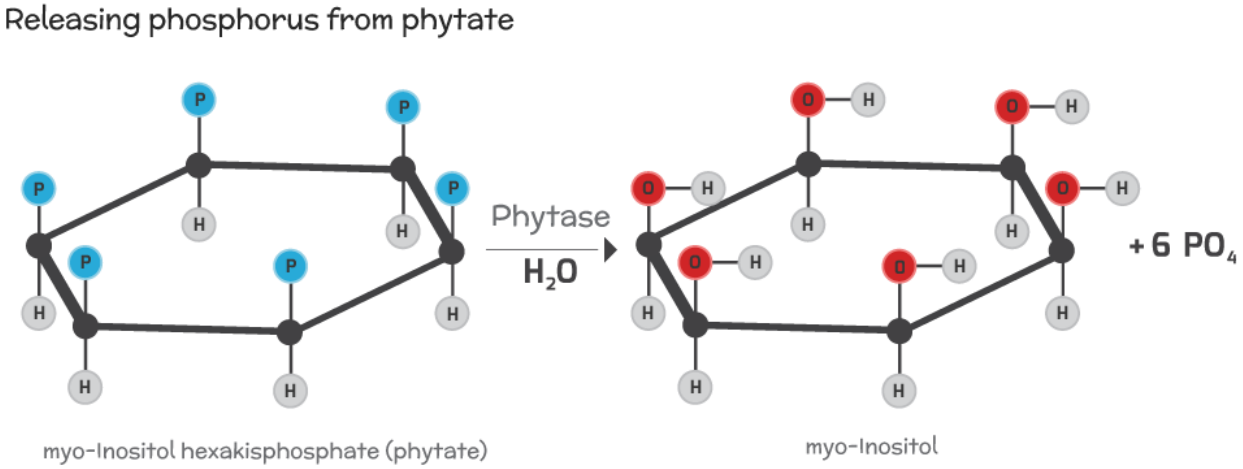


Figure 1.2

Release of P from the phytate molecule via phytase.
Image borrowed from: http://phytate.info/phytases.

Part 2

Development of a Maturity Group V Low Phytate Soybean Line for Tennessee using Molecular Backcrossing for Trait Introgression

Abstract

Soybean [*Glycine max* (L). Merrill] is the world's top oilseed crop. High protein soymeal is a primary soybean use and staple food of livestock and fish. The storage form of phosphorus (P) in the seed is phytic acid, or phytate (myo-inositol 1,2,3,4,5,6 hexa*kis*phosphate) which binds and chelates many key nutritional cations forming stable salts that are non-digestible. Livestock waste laden with phytate P is an environmental concern, and a source of nonpoint P pollution causing accelerated eutrophication and surface water quality deterioration. The primary objective of this research was the development of a commercially acceptable, determinate low phytate (LP) soybean line which would enhance nutritional qualities of soymeal for livestock and sustainably lessen P pollution from animal agricultural waste runoff.

The LP soybean lines developed in this study were twelve BC_5 derived lines from their recurrent parent '5601T', a high yielding University of Tennessee developed cultivar. The line TN09-239 was the LP donor parent. The presence of the two LP alleles in the homozygous state in all twelve BC_5F_3 derived lines was verified using perfect SNP molecular markers at the confirmed QTL loci *cqPha-001* and *cqPha-002*. Thus the twelve BC_5 LP lines have been confirmed to double homozygous recessive for the LP trait. In 2012, a field experiment with a randomized complete block design with two replications was grown at two locations in two row plots in order to evaluate the height and inorganic phosphorus expression of 10 BC_5 LP lines in comparison to their parents 5601T, TN09-239, and high yield check cultivars; the study was repeated in 2013, with the addition of two more BC_5 lines, and tested in four row plots in two locations and in two row plots in a third location with three replications per location. Single nucleotide polymorphism (SNP) analysis conducted on each BC_5F_3 LP line utilizing the new

stem termination locus (*Dt1)* SNP developed by Dr. Kristin Bilyeu (USDA-ARS, Columbia, MO) revealed that eight BC_5 LP lines were determinate (*dt1 dt1*) and four other BC5 LP lines were indeterminate or segregating (*Dt1 Dt1, Dt1 dt1*). Analyzed data revealed that all 12 BC_5 LP lines showed elevated levels of inorganic P. The height of the eight determinate LP lines (87.9 cm) was significantly lower ($p<0.05$) than the four indeterminate LP lines (138.2 cm) which matched the stem termination genotyping. These results indicate that the eight determinate BC_5 LP lines have broken the genetic linkage (<20 cM) between the *Dt1* locus and the *pha-002* locus on Gm19 confirming that they are triple homozygous recessive at all three loci (*pha-001*, *pha-002* and *dt1*).

Introduction

Domestically in 2013, 34.32 million metric tons of soymeal was used in livestock feed for broiler chickens (*Gallus domesticus*, 12.95 million metric tons), hogs (*Sus domesticus*, 11.15 million metric tons), laying hens (*Gallus domesticus*, 2.28 million metric tons), turkeys (*Meleagris gallopavo*, 2.04 million metric tons), and combined other feed uses (5.9 million metric tons) (Soy Stats 2013). According to the United Soybean Board (USB), the U.S. soybean industry is reliant on annual demand from the U.S. animal agricultural sector. Livestock producers use the high protein soymeal as a staple feed, but the animals ingest large amounts of phytate which are typically present in the soymeal (Raboy and Dickinson 1984; Erdman, 1979).

Chemically, the structure of the phytate molecule acts as a binding or chelating agent to mineral cations forming salts that are non-digestible, which essentially removes the mineral bioavailability from the seeds by forming the more complex phytate molecule (Erdman, 1979; Maenz et al., 1999) (Figure 1.1). Many of the phytate mineral complexes that are formed are insoluble and therefore unavailable for absorption (Reddy, 1989). The bound cations, such as Ca, Fe, Mg, Mn and Zn, are nutritional minerals that become non-digestible insoluble phytate salts (Erdman, 1979; Raboy, 2002; Reddy, 1989). The interference with intestinal absorption of the mineral complexes, such as Ca, Fe, K, Mg, P and Zn, may lead to mineral deficiencies in animals and humans earning phytate the label of "anti-nutrient" (Reddy, 1989; Lott et al., 1995). However, the antioxidant functions can have beneficial medical applications (Graf and Eaton, 1990).

The chelation activity of phytic acid is pH dependent (Prattley et al. 1982; Wilson, 1987). The phytic acid is not broken down in the intestines of monogastric animals because they possess

little to no phytase activity in their digestive tracts, which is why phytic acid is not efficiently utilized (Brinch-Pedersen et al., 2002). For the phytic acid to be efficiently utilized, animal feeds must be supplemented with the phytase enzyme to free cations and other nutrients bound by phytate P-complexes or the animal feed may be supplemented with digestible Pi for the animals to receive the optimum amount of P needed for animal growth and skeletal development (Khalid et al., 2013; Shi, 2007) (Figure 1.2). The non-digestible phytate is present in the livestock waste, as livestock manures typically contain 4.3% N, 1.4% P and 2.2% K on average (Daniel et al., 1994).

Powers (2006) found that the excess P in the form of phytic acid in the animal waste is both insoluble and water soluble, the relative abundance of which varies with the dietary treatment (Brinch-Pedersen et al., 2002; Jendza, 2009). The non-digestible, stable phytate salts present in soybeans and soymeal are excreted by animals that consume them (Erdman, 1979; Raboy, 2002; Raboy, 2009; Bilyeu et al., 2008; Gillman et al., 2009; Wilcox et al., 2000). The excess P excretion then becomes a P management concern; high levels of P in the form of phytic acid present in manure are often applied as fertilizer to agricultural lands as a common agricultural production practice (Hegeman et al., 2001). Nitrogen is usually the only macronutrient being monitored; typically P is not. Phytates are known to be heat stable, and can withstand high temperatures, rough field and weather conditions and various storage environments lending to their mobility in watersheds (Sathe and Reddy, 2002). The mobile P in watersheds can cause accelerated eutrophication and surface water quality deterioration in ecosystems of ponds, lakes, streams and estuaries. The P laden animal waste is a major point source and non-point source of P pollution detrimental to the environment and often responsible for fish kills and aquatic dead zones (Sharpley et al. 1994, Carpenter et al., 2010, Walker et al.,

2006). The creation of a LP soybean cultivar to be used for soymeal would ease environmental concerns and serve to enhance the dietary nutrition and metabolic energy of the soymeal for livestock. The combined benefits would lead to more effective and sustainable P nutrient management on farms for crop and livestock production.

As Mellinger (2012) noted, there are several genetic sources of LP in soybean: the Gm-lpha-TW-1 allele, the Gm-lpa-ZC-2 allele, the MIPS allele and the combination of the LP alleles on Gm03 and Gm19 totaling five alleles that have been reported for LP in soybean (Wilcox et al., 2000). This study focused on the combination of the LP alleles on Gm03 and Gm19 which are two independent recessive loci that govern the LP trait as confirmed by Maroof et al. (2009), Gao et al. (2008), Oltmans et al. (2005), and Walker et al. (2006). Oltmans et al. (2005) analyzed segregation ratios of LP plants and determined that the recessive alleles exhibit duplicate dominant epistasis making it necessary for both recessive alleles to be homozygous before a plant can express the LP trait. Scaboo et al. (2009) confirmed that the LP trait is controlled by two independent quantitative trait loci (QTL), and the gene symbols cqPha-001 and cqPha-002 were approved for the SoyBase depository. The loci are referred to as *pha1* (Gm03) and *pha2* (Gm19) with the desired genotype being *pha1 pha1 pha2 pha2*. The line CX 1834-1-2 was derived from mutated LP line M153 (Wilcox et al., 2000) and contains both of the alleles in the homozygous recessive state necessary for expression of the LP trait, validating its use as the original LP donor parent in this study (Figure 2.3). By selecting progeny containing SSR markers Satt237 and Satt561 in the development of LP lines, Mellinger (2012) noted higher concentrations of P than in lines containing just one simple sequence repeat (SSR) marker or the other. With both loci together, Walker et al. (2006) found Satt237 and Satt561 can predict approximately 63% of the variation of phytate in the seed by using those molecular markers.

Gillman et al. (2009) found that the *pha1* allele on Gm03 accounted for 41% of the LP expression and the *pha2* allele on Gm19 accounted for 11% (Mellinger, 2012). Thus the LP locus on Gm03 is classified as a major locus and the LP locus on Gm19 is classified as a major locus as well, but with lower additive effect (Scaboo, 2009; Mellinger, 2012). The DNA sequences of the two LP mutant alleles (*lpa1* and *lpa2*) are known and perfect molecular markers have been constructed from gene sequence data to detect their presence (Bilyeu et al., 2009). In this study we used SNP technology to guide LP selections, specifically those perfect molecular markers at cqPha-001 and cqPha-002 developed by Gillman et al. (2009).

Seed phytate content and Pi content have an inverse relationship (Bilyeu et al., 2008; Chen, 1956; Gao et al., 2008; Raboy, 2000; Shi et al., 2007; Wilcox et al., 2000; Oltmans et al., 2005; Spear and Fehr, 2007). The LP lines have demonstrated elevated levels of Pi and a concomitant decrease in phytic acid as total seed P does not change (Gillman et al., 2009; Scaboo et al., 2009). The Pi is digestible by livestock and does not chelate nutritional mineral cations. Plants typically produce more phytic acid than is needed for growth and development and reducing the phytic acid and increasing the Pi has not inhibited peak agronomic performance (Scaboo et al., 2009; Gillman et al., 2009; Bilyeu et al., 2008; Shi et al., 2007; Spear and Fehr, 2007). However original donor parent CX 1834-1-2 is a poor agronomic line that is also indeterminate in growth habit. The indeterminate trait is not suitable for growing MG V soybeans in the southern U.S., as it can lead to excessive plant height which contributes to lodging, thereby reducing yields. Unfortunately, the LP donor parent, BC_4 line TN09-239, inherited the indeterminate growth habit from CX 1834-1-2.

We presently speculate that TN09-239 is either homozygous dominant or segregating for the *Dt1* allele on Gm19 (LG L), where the dominant form of that locus has the indeterminate

growth habit (Tian et al., 2010). Thus the *Dt1* allele would signify an indeterminate plant growth habit and the homozygous recessive (*dt1 dt1*) form of the allele must be present for the plant to be determinate. The *Dt1* locus on Gm19 is positioned less than 20 cM from the LP *pha2* locus, which indicates genetic linkage (Figure 2.10). This was not anticipated to be a concern until we noted that many BC_4 lines and donor parent TN09-239 still exhibited the non-desirable indeterminate *Dt1* growth habit. The RP 5601T is determinate (*dt1 dt1*). Determinate phenotypic selections were made at the BC_5F_2 stage (Table A.1; Table A.2) and a large population of BC_5F_1 hybrid crosses were attempted in order to secure at a 95% confidence interval at least one plant capable of producing LP lines with the determinate growth habit in the F_2 generation (Table 2.2). The indeterminate $BC_5F_{3:5}$ and $BC_5F_{3:6}$ LP lines still present in our study can be attributed to the genetic linkage and inheritance of the indeterminate, dominant *Dt1* allele from CX 1834-1-2. With the help of molecular geneticist Dr. Kristen Bilyeu (USDA-ARS, Columbia, MO), who used genetic materials from this research to develop a new R166W SNP marker based on gene sequence of the stem termination *Dt1* locus, we were able to analyze BC_5 derived LP lines to help us make selections for the triple recessive determinate BC_5 LP lines (*pha1 pha1*, *pha2 pha2* and *dt1 dt1*) that occurred at low frequency. The objectives of Part 2 were to:

(1) Test whether seeds produced by each of the LP lines differed significantly from each other or from their RP 5601T and donor parent TN09-239 for Pi content and height.
(2) Document stable transfer of the two LP alleles, *lpa1* and *lpa2*, from donor parent TN09-239 into all BC_5 LP lines using gene sequence based SNP technology.
(3) Break the genetic linkage between the *Pha-002* locus on Gm19 (LG L) and the nearby *Dt1* locus to develop a BC_5 LP line with determinate (*dt1 dt1*) stem termination.

Materials and Methods

The LP soybean lines used in this study were twelve BC_5 derived lines from their RP 5601T, a high yielding University of Tennessee cultivar (Pantalone et al., 2003) and former USDA maturity group V check. The line TN09-239 was our LP, donor parent, whose LP trait was originally introgressed from the donor line CX 1834-1-2. TN09-239 and 5601T were included in the study as well as Osage, a current USDA maturity group V check cultivar developed by the University of Arkansas (Chen, 2007) and 'Ellis' (Pantalone, 2013), a new high yielding University of Tennessee cultivar whose pedigree includes 5601T as its female parent.

Germplasm: The Backcross (BC) Parents

The cultivar 5601T is an F_6 derived line from the cross of 'Hutcheson' (Buss et al., 1988) x TN89-39 (a Tennessee experimental line). 5601T is resistant to stem canker, soybean mosaic virus (SMV), southern root-knot nematode and moderately resistant to peanut root-knot nematode (Pantalone et al., 2003). The recurrent parent of TN09-239 was 5601T, thus the pedigree of TN09-239 is 5601T[4] × CX 1834-1-2.

The pedigree of the BC_4 donor line, TN09-239 can be traced back to the original cross pollination reported by Scaboo et al. (2009) in 2000 between 5601T and the LP line CX 1834-1-2 which was developed by Wilcox et al. (2000) through EMS mutagenesis from mutated line M153. The cross of 5601T and CX1834-1-2 created the recombinant inbred line (RIL) designated as 56Cx-284 among the population of 313 RIL (Scaboo et al., 2009).

In summer 2004, 5601T was crossed with 56Cx-284 which produced the approximate genetic equivalent of BC_1F_1 seed. At the time, simple sequence repeat (SSR) markers for LP were available (Walker et al., 2006) at the University of Georgia Boerma Lab. Our BC_1F_1

material was found by the Boerma Lab to be heterozygous for the LP allele at two QTL loci [Pha-001 (Gm03) and Pha-002 (Gm19)] using three SSR markers: Satt237 (Gm03, linkage group (LG) N), Satt561 (Gm19, LG L) and nearby Satt527 (Gm19, LG L)]. The BC_1F_1 seed was planted at the USDA Tropical Agricultural Research Station (TARS) winter nursery in Isabela, Puerto Rico during the winter of 2004-2005. When the plants reached R2 flowering stage in Puerto Rico during the winter 2004-2005, recurrent parent 5601T was backcrossed with the BC_1F_1 plant designated TN05-PR-069 producing BC_2F_1 seed. The BC_2F_1 seeds were subsequently planted in Knoxville in late spring 2005. Leaves of the individual BC_2F_1 plants were collected and tested for the presence of the SSR phytate markers in the Pantalone lab at the University of Tennessee, and the presence of the heterozygous alleles were detected at phytate loci *Pha-001* and *Pha-002* using SSR markers Satt237 and Satt561, respectively. Recurrent parent 5601T was then crossed with pollen from BC_2F_1 plant 452-4-01 to create BC_3F_1 seed.

In late spring 2006, the BC_3F_1 seeds were planted in A×B row 90 in the conventional crossing block at the East Tennessee Research and Education Center (ETREC) at the University of Tennessee in Knoxville, TN, and each plant in row 90 was tested for SSR markers Satt237 and Satt561. Plant 23 was confirmed to be heterozygous at both Satt237 and Satt561 loci, and 5601T was crossed with pollen from plant #90-23 to create BC_4F_1 seed.

During the winter 2006-2007 the BC_4F_1 seeds were grown at USDA-TARS in Isabela, Puerto Rico and also at the University of Tennessee greenhouse. The BC_4F_2 seeds were harvested in Isabela in April 2007 from hill VP07-072, and subsequently planted at ETREC in rows 20,083-20,089 during the summer 2007. One of the single BC_4F_2 plants (#50) was harvested amongst others in fall 2007. In late spring 2008, the $BC_4F_{2:3}$ seed from plant #50 was planted at ETREC in Knoxville in row 30,356 and the row was bulk harvested. The $BC_4F_{2:4}$ seed

of row 30,356 was designated as line TN09-239 the subsequent year in a 2009 preliminary yield trial.

Development of "56CX" BC_5 Lines

In 2009, TN09-239 (male) serving as the LP trait donor was planted in the conventional crossing block in Knoxville, TN at ETREC and crossed with 5601T (female) serving as the recurrent parent to produce BC_5F_1 seeds (cross 09-15). The BC_5F_1 seeds were grown in Isabela, Puerto Rico at USDA-TARS during the winter 2009-2010 and DNA extracted from individual BC_5F_1 plants was screened for SSR phytate markers Satt237 and Satt561 at the University of Tennessee. DNA samples confirmed that seven of the plants in Puerto Rico were double heterozygous and were harvested as individual plants with their assigned spring 2010 Puerto Rico plant number (VP10-124, VP10-125, VP10-133, VP10-141, VP10-143, VP10-145 and VP10-146). (Table A.1)

In summer of 2010 in Knoxville, TN at ETREC, the seeds from the seven BC_5F_1 plants were planted as BC_5F_2 plant rows as follows: VP10-124, rows 20,335-20,338; VP10-125, rows 20,339-20,442; VP10-133, rows 20,365-20,368; VP10-141, rows 20,392-20,393; VP10-143, rows 20,398-20,401; VP10-145, rows 20,406-20,409; and VP10-146, rows 20,410-20,413. All 100 rows (approximately 10,000 plants) were visually evaluated and 616 individual BC_5F_2 plants were selected and pulled to harvest those whose phenotypes appeared to exhibit a determinate (dt_1 dt_1) growth habit. (Table A.2) The BC_5F_2 plants were tagged and labeled VP10-124-11, VP10-124-18, VP10-125-11, VP10-133-5, VP10-141-8, VP10-143-32, VP10-145-58, and VP10-146-14. (Table A.1)

During the winter 2010-2011, $BC_5F_{2:3}$ single plants were grown in the greenhouse in Knoxville, TN from the $BC_5F_{2:3}$ seed source. Single plants were confirmed to be LP by the Pi assay and assigned labels by their 96 well plate position used in the assay. These $BC_5F_{2:3}$ single plants were harvested from the greenhouse and their $BC_5F_{3:4}$ seeds were planted as $BC_5F_{3:4}$ plant rows at ETREC in Knoxville, TN during the summer of 2011: A79-02, row 40,002 (from plant VP10-124-11), A79-05, row 40,004 (from plant VP10-124-11), A86-02, row 40,006 (from plant VP10-124-18), B05-02, row 40,012 (from plant VP10-125-11), B05-04, row 40,014 (from plant VP10-125-11), C66-02, row 40,024 (from plant VP10-133-5), E69-01, row 40,036 (from plant VP10-141-8), F04-04, row 40,044 (from plant VP10-143-32), F79-03, row 40,047 (from plant VP10-145-58), G70-02, row 40,049 (from plant VP10-146-14), G70-04, row 40,051 (from plant VP10-146-14), and G70-05, row 40,052 (from plant VP10-146-14). In the fall of 2011, all fifteen $BC_5F_{3:4}$ plant rows were harvested from the field. The harvested $BC_5F_{3:4}$ plant rows included those derived from the greenhouse BC_5F_3 plants that were confirmed to be homozygous recessive using SNPs at phytate loci cq-Pha-001 and cq-Pha-002. (Table A.1)

During the winter of 2011-2012, $BC_5F_{3:5}$ seeds were grown in the winter nursery in Homestead, FL. A sample of seeds harvested from 2011 ETREC BC_5 $F_{3:4}$ plant rows were assigned as seed increase rows at Homestead, FL as follows: VH12-1273 from 2011 ETREC row 40,002, VH12-1274 from 2011 ETREC row 40,004, VH12-1275 from 2011 ETREC row 40,006, VH12-1276 from 2011 ETREC row 40,012, VH12-1277 from 2011 ETREC row 40,014, VH12-1279 from 2011 ETREC row 40,024, VH12-1280 from 2011 ETREC row 40,036, VH12-1282 from 2011 ETREC row 40,044, VH12-1283 from 2011 ETREC row 40,047, VH12-1284 from 2011 ETREC row 40,049, VH12-1286 from 2011 ETREC row 40,051 and VH12-1287 from 2011 ETREC row 40,052. Eight plants were tagged from each row and leaf samples from

each plant were sent back to the University of Tennessee Soybean Molecular Marker Lab for analysis. DNA from the leaf samples was used to conduct the SNP assays for seed phytate to check for the presence of the homozygous recessive LP alleles in the tagged plants. The $BC_5F_{3:5}$ rows at Homestead, FL were individually bulk harvested and sent back to Knoxville, TN along with the eight tagged plants which were pulled separately. Seeds were harvested by hand from the tagged plants and eight seeds subsampled from the $BC_5F_{3:5}$ plants were used to complete the assay for Pi. (Table A.1)

In the spring of 2012, total seed weights of the current BC_5 LP lines were measured from the $BC_5F_{3:4}$ harvested rows (2011 ETREC 40,000's) and from the $BC_5F_{3:5}$ harvested rows (2012 spring Homestead, FL VH12#s) to determine which source, either the remnants retained from Knoxville 2011 ($BC_5F_{3:4}$) or their seed increase from Homestead, FL ($BC_5F_{3:6}$ seed), had sufficient seed to plant the replication for the first year (2012) of the study. (Table A.1)

Field Experiment 2012 and Field Experiment 2013

A randomized complete block design (RCBD) was used for the study. In 2012, 160 seeds per row were planted in two row plots with two replications in two locations [ETREC and Research and Education Center at Milan, TN (MILAN)]. Data were evaluated using a mixed model analysis of variance in SAS version 9.3 (Glimmix procedure, SAS Institute, Cary, NC) and least square means were compared using Tukey's protected LSD at the 5% significance level. The random factors were the location, the G × E or genotype by location interaction and the replications nested within location. The fixed term or treatments were the genotypes. The dependent variables being evaluated for the genotypes were Pi and height. The following statistical model was employed:

$$Y_{ijk} = \mu + L_i + R(L)_{k(i)} + G_j + (GL)_{ji} + e_{ijk}$$

Where Y_{ijk} was the specific measured value of genotype *j* in rep *k* within location *i*; μ was the mean overall; L_i was the effect of location *i*; $(GL)_{ji}$ was the interaction between genotype *j* and location *i*, and Y_{ijk}'s error term was e_{ijk}. The designation "56CX" was assigned as a prefix to all genotypes denoting that the LP lines originated from the 5601T × CX 1834-1-2 population. That population abbreviation was followed by a four digit number denoting the 2011-2012 FL row number. Ten LP genotypes were tested in the 2012 field trial: 56CX-1273 (BC_5 $F_{3:6}$), 56CX-1276 (BC5 $F_{3:6}$), 56CX-1277 (BC_5 $F_{3:6}$), 56CX-1279 (BC_5 $F_{3:5}$), 56CX-1280 (BC_5 $F_{3:5}$), 56CX-1282 (BC_5 $F_{3:5}$), 56CX-1283 (BC_5 $F_{3:5}$), 56CX-1284 (BC_5 $F_{3:5}$), 56CX-1286 (BC_5 $F_{3:5}$) and 56CX-1287 (BC_5 $F_{3:6}$). The RP, cultivar 5601T, and LP donor parent, BC_4 line TN09-239, were also included as check entries in the study. In addition, five other lines from the same BC_5 population were grown for evaluation in single rows, as they did not have sufficient seed to make it as entries in the replicated field study: 56CX-1274 (BC_5 $F_{3:5}$), 56CX-1275 (BC_5 $F_{3:5}$), 56CX-1278 (BC_5 $F_{3:5}$), 56CX-1281 (BC_5 $F_{3:5}$) and 56CX-1285 (BC_5 $F_{3:5}$). Plants in row 56CX-1274 and plants in row 56CX-1275 visually exhibited a determinate growth habit and were confirmed to be determinate (*dt1 dt1*) using the *Dt1* R166W SNP marker developed by Dr. Bilyeu, and those lines were added to the 2013 replicated field trial.

In 2013, a RCBD was again used for the study. A sample of 200 seeds per row were planted in four row plots with three replications in three locations [ETREC, MILAN, and at the Highland Rim Research and Education Center at Springfield, TN (HRREC)]. Data were evaluated using a mixed model analysis of variance in SAS version 9.3 (Glimmix procedure, SAS Institute, Cary, NC) and least square means compared using Tukey's protected LSD at the

5% significance level using the same statistical model employed in 2012 and evaluating the same dependent variables. Lines 56CX-1274 and 56CX-1275 and USDA MG V check Osage and recently released University of Tennessee cultivar Ellis, now a new USDA high yielding check, were added to the 2013 study. The null hypothesis was tested to see whether seeds produced by each of the LP lines differed significantly from each other or from their recurrent parent 5601T and donor parent TN09-239 for Pi concentration and whether growth habit (determinate vs. indeterminate) contributed to measurable differences in plant height.

DNA Preparation and Isolation

Fresh leaf samples were collected in the field or greenhouse from plants and placed in eight chamber plastic octagons and then placed inside portable coolers to preserve the DNA in the leaf samples while en route to the lab. At the University of Tennessee Soybean Molecular Lab, a small trifoliate leaf from each sample collected was pressed onto a Whatman FTA Classic Plant Card for DNA extraction (Whatman Intl. Ltd., Maidstone, Kent, UK). The DNA sample of each genotype in the study was then ready for melting curve SNP analysis to validate the presence of the recessive forms of the *lpa1* and *lpa2* alleles.

Polymerase Chain Reaction (PCR) and SNP Analysis

Single Nucleotide Polymorphism analysis was performed with the isolated DNA extracted from the leaf samples using the Simple Probe Melting Curve Assay on the Roche 480 Light Cycler instrument (Roche Scientific, Basel, Switzerland). We used the Melting Curve Genotyping Protocols, including probe and primer sequences developed by the Boerma lab at the University of Georgia, using information reported by Gillman et al. (2009). The melting curve

obtained for the wild type alleles from RP 5601T, the two LP mutant alleles inherited from CX-1834-1-2, and the melting curve for a heterozygote on both Gm03 and Gm19 are displayed in Figures 2.1, 2.2, 2.3, 2.4, 2.5, and 2.6. Two 96 well plates were used for each analysis run, with each plate containing a representative punch from the same leaf press of each genotype being evaluated in the study.

Procedure for Inorganic P (Pi) Assay

Pi concentrations were determined using a modified version of a colorimetric assay developed by Raboy et al. (2000) which was an adaption of the assay described by Chen et al. (1956)(Table A.3). Reactions were expected to range from nearly clear for normal soybeans to dark blue for LP soybeans (Figure 2.9). The concentration of Pi was estimated from reflectance values using a Bio-Tek Powerwave XS microplate spectrophotometer plate reader set at 882 nm with the reflectance readings of samples compared to those of a standards table (Figure 2.10). In both 2012 and 2013, the Pi assay was conducted on three samples per replication per location to quantify the seed Pi in each BC_5 LP line. Images of the results were taken using a Canon EOS Rebel T3i digital SLR camera (Canon, Melville, NY) (Figure 2.9).

Soil Core Sample Analysis

LP plants produce seeds that have normal levels of total P but usually greatly reduced levels of phytic acid P with a concomitant increase in Pi (Raboy 2009, Raboy 2002). The mutations do not affect the plant's ability to take up P from the soil and transport it to developing seed, but instead block the plant's ability to synthesize P into phytic acid P. Because most P research addresses soil P root uptake, we obtained soil core samples at a depth of six inches from

every plot in every location using a chrome plated Lamotte Soil Core Sampler 1016 to determine if there was any correlation between existing levels of soil P from each sample and the seed levels of Pi expressed by the LP plants compared with the parents and checks used in this study. Soil core samples were placed in soil collection boxes and sent to Ellington Plant Sciences Complex in Nashville, TN, where the Mehlich-1 soil test was conducted on each core sample to measure total P per plot.

DNA Preparation and Isolation for analysis and development of the Dt1R166W SNPMarker

The dominant *Dt1* allele on Gm19 is genetically linked (<20cM) in repulsion phase to the recessive allele at the Pha-002 locus, and that linkage configuration has continued to thwart our efforts to create a MG V determinate LP conversion line of 5601T. Prior to the planting of the first replicated field trial in 2012, four seeds harvested from $BC_5F_{3:5}$ LP single plants collected in Homestead, FL, whose stem termination was unknown, were planted in flats and grown in the University of Tennessee Central Greenhouse. In addition, several other genotypes and cultivars, whose stem termination was known, were also selected to be planted in the flats to assist in the development of the new *Dt1* R166W SNP marker being developed by Dr. Bilyeu (USDA-ARS, Columbia, MO). The following BC_5 single plants pulled from the Homestead, FL winter nursery were grown in the Central Greenhouse with the 4 digit number representing their FL row number: 4 seeds from single plants (SPs) 1273-1 and 1273-2; 4 seeds from SPs 1274-3 and SPs 1274-5; 4 seeds from SPs 1275-1 and 1275-7; 4 seeds from SPs 1276-3 and 1276-7; 4 seeds from SPs 1277-6 and 1277-8; 4 seeds from SPs 1278-1 and 1278-5; 4 seeds from SPs 1279-3 and 1279-4; 4 seeds from SPs 1280-7 and 1280-8; 4 seeds from SPs 1281-3 and 1281-6; 4 seeds from SPs 1282-2 and 1282-6; 4 seeds from SPs 1283-1 and 1283-3; 4 seeds from SPs 1284-1 and

1284-2; 4 seeds from SPs 1285-7 and 1285-8; 4 seeds from SPs 1286-1 and 1286-6, and 4 seeds from SPs 1287-5 and 1287-8. The following other cultivars and genotypes were also grown in the Central Greenhouse: 6 seeds from UT cultivar 5002T (Pantalone et al., 2004) (determinate); 16 seeds from RP 5601T (determinate); 8 seeds from CX 1834-1-2 (indeterminate); 16 seeds from cultivar 'Essex' (Buss, 1973 (determinate); 4 seeds from genotype #N33 (determinate); 4 seeds from genotype #N34 (determinate); 4 seeds from genotype #N39 (determinate); 4 seeds from genotype #N02 (determinate); 4 seeds from genotype #O32 (indeterminate); 4 seeds from genotype #O51 (indeterminate); 4 seeds from genotype #P24 (indeterminate); 16 seeds from LP donor parent TN09-239 (unknown); 9 seeds from cultivar 'Williams' (Bernard, 1982) (indeterminate); 5 seeds from genotype #31018 (indeterminate); 5 seeds from genotype #31022 (determinate); 5 seeds from genotype #31028 (determinate), and 6 seeds from genotype #31029 (indeterminate). Fresh leaf samples were collected in the greenhouse from each single plant and taken to the University of Tennessee Soybean Molecular Lab, where a small trifoliate leaf from each sample collected was pressed onto a Whatman FTA Classic Plant Card for DNA extraction (Whatman Intl. Ltd., Maidstone, Kent, UK). Then the DNA sample of each genotype in the study was shipped to Dr. Bilyeu in Columbia, MO for further analysis. All of the single plants were hand planted in S range at ETREC prior to the planting of the 2012 replicated field trial for further evaluation.

Results and Discussion

There are two independent recessive loci that govern the LP trait: *pha1* on Gm03 (formerly linkage group N) and *pha2* on Gm19 (formerly linkage group L) (Gao et al. 2008, Oltmans et al., 2005). The presence of the two recessive loci was confirmed in all 12 BC_5F_3 derived lines using SNP technology, specifically with perfect SNP molecular markers at the confirmed QTL cqPha-001 and cqPha-002 (Scaboo et al., 2009). Melting curve analysis of the LP alleles from DNA collected from the leaves of all BC_5F_3 plant progenies analyzed in this experiment revealed the inherited CX mutant alleles melting at approximately 62° C on Gm03 (cq-Pha-001) (Figure 2.3) and at approximately 54° C on Gm 19 (cq-Pha-002) (Figure 2.1). The SNP markers confirmed that all 12 BC_5 LP plant progenies in this study were double homozygous recessive for the two alleles that express LP concentration in soybean seeds. Documentation of the stable transfer of the LP alleles in all BC_5 plant progenies was a primary goal of this research.

The LP trait was further quantified by conducting an assay of Pi in 2012 and 2013 on three samples of seeds harvested from all plots in all replications from all locations of the twelve BC_5 LP lines being evaluated in the study. Seed phytate content and Pi content have an inverse relationship (Bilyeu et al., 2008; Chen, 1956; Gao et al., 2008; Raboy, 2000; Shi et al., 2007; Wilcox et al., 2000; Oltmans et al., 2005; Spear and Fehr 2007). The LP lines have demonstrated elevated levels of Pi and a concomitant decrease in phytic acid, but the total seed P does not change (Gillman et al. 2009; Scaboo et al. 2009). Elevated levels of Pi were confirmed for all BC_5 LP lines in this study, indicating that all BC_5 LP lines were also low in expression of phytate, however no direct analytical measurements of phytic acid were made. When the mean value of Pi for the BC_5 LP lines (1816.5 µg Pi g^{-1}) was compared to that of recurrent parent

5601T (221.2 µg Pi g^{-1}) the results were highly significant ($p<0.0001$) (Figure 2.2). The fifth backcross lowered the phytate level further, as the mean value of Pi for the BC_5 LP lines (1816.5 µg Pi g^{-1}) was also significantly higher ($p<0.001$) than that of BC_4 LP donor parent line TN09-239 (1516.2 µg Pi g^{-1}) (Figure 2.11). There was no significant difference ($p>0.05$) in Pi between the mean of the determinate (1799.8 µg Pi g^{-1}) and indeterminate (1850.0 µg Pi g^{-1}) BC_5 LP lines (Figure 2.8), a strong indication that we had successfully broken the genetic linkage between *Dt1* and *lpa2* on Gm19. To our knowledge, this is the first report of the development of a triple recessive progeny expressing low phytate and determinate growth habit.

Regression analysis was processed to test whether Mehlich-1 measured levels of soil P from each plot core sample could explain Pi content present in the harvested seeds of each genotype, and a correlation analysis was conducted to validate the strength of the relationship. Regression results indicated that existing levels of soil P ($p<0.01$) can help to explain Pi content in the seeds, but the R square value (0.0485) indicates that existing levels of soil P from the core samples were only explaining very little (~5%) of the variation in seed Pi content, indicating a weak relationship.

A primary focus of this study was to verify the stability of the LP trait when evaluated across multiple growing environments. When expression of Pi was compared between the individual environments in TN, the quantified levels of Pi were not stable and varied significantly. Despite the lack of stability, the expression of the LP trait expressed via quantified seed levels of Pi in the BC_5 lines was still about an order of magnitude greater than the quantified levels of Pi in 5601T, Ellis and Osage. Regression was used to test if the Pi concentrations for each genotype fit a linear equation (Figure 2.15). While each genotype was tested for an expected slope of zero, only 5601T (0.05), Ellis (-0.01), Osage (0.05) and 56CX-1279 (0.7) had

slopes that were not significantly different from zero ($p>0.05$), while the rest of the BC_5 LP lines had slopes significantly different from zero ($p<0.05$). Low phytate line 56CX-1279 was the only BC_5 genotype whose slope (0.7) was not significantly different than zero indicating it was more stable than the other eleven BC5 LP lines for the low phytate trait when grown in these three Tennessee environments. The means for each genotype, the equations explaining their linear trend line and the environmental index for each genotype are outlined in Table 2.1. The slopes for all BC_5 LP lines averaged greater than a 1.20 µg Pi g^{-1} unit change in Pi for each unit change in environmental index (deviation from the mean level of Pi), which further indicated that all BC_5 genotypes were not stable for the LP trait when evaluated across the three TN environments (ETREC, HRREC and MILAN) (Figure 2.12). Pi is normally taken up by the plant in the form of phosphoric acid (H_3PO_4), then translocated to the developing seed and synthesized to become the storage form of P which is phytic acid (Raboy, 2002). However, many other factors and conditions can influence seed phytate levels, such as environmental fluctuations, growing locations, irrigation conditions, fertilizer applications, soil types, soil temperature, growing conditions, cultivar used and time of year the plant is grown (Reddy, 1989).

Another important goal of the study was to develop a determinate MG V BC_5 LP line suitable for growing in TN or the mid-South region. As early as the BC_5F_2 stage, single plants were pulled from plant rows in 2010 that were phenotypically exhibiting a determinate growth habit (Table A.1; Table A.2). According to Sedcole (1977), to be 95% confident in recovering at least one plant expressing the desired recessive (*dt1 dt1*) determinate genotype and the double homozygous recessive LP genotype in a BC_5 population of F_2 plants, at least 951 plants would need to have been evaluated in order to produce one triple homozygous recessive plant (Table A.2). This became even more difficult due to the genetic linkage (<20cM) of the *Pha-002* locus

and the *Dt1* locus on Gm19. In 2010 at ETREC, 10,000 plants in 100 rows were evaluated, and 616 single plants were pulled from those rows that were phenotypically exhibiting a determinate growth habit. Those 616 plants have now been screened with molecular markers. Data analyzed from the 2012 replicated yield trial indicated that the genetic linkage between the stem termination growth habit *Dt1* locus on Gm 19 and the *Pha-002* locus on the same chromosome was broken through the development of eight of our $BC_5F_{3:5}$ LP lines (Table A.2). Overcoming the genetic linkage has been validated molecularly using the new stem termination R166W SNP marker which revealed the homozygous recessive (*dt1 dt1*) allelic form controlling the determinate stem type in 56CX-1273, 56CX-1274, 56CX-1275, 56CX-1276, 56CX-1277, 56CX-1279, 56CX-1282 and 56CX-1283. All eight lines have been confirmed to be LP possessing the double homozygous recessive alleles on Gm03 and Gm19. Therefore, we did successfully recover eight triple homozygous recessive low phytate, determinate plants from the BC_5F_2 generation. These eight lines have also been quantified as expressing elevated levels of Pi (Figure 2.8). Molecular genetic analysis also revealed that four other BC_5 LP genotypes expressed the dominant (*Dt1 Dt1*) allelic form or were segregating (*Dt1 Dt1, Dt1 dt1, dt1 dt1*) for stem termination: 56CX-1280, 56CX-1284, 56CX-1286, and 56CX-1287. Visual field evaluations and measurements confirming the determinate phenotypes with appropriate plant height, plant architecture, and elite line appearance were noted, and there were highly significant differences ($p<0.0001$) in plant height between the determinate (88 cm) and indeterminate (138 cm) BC_5 LP lines (Figure 2.1). The *Dt1* R166W SNP results also revealed that the stem termination genotype of donor parent TN09-239 was indeterminate (*Dt1 Dt1*) in 15 out of 16 individual plant DNA samples tested by Dr. Bilyeu or were segregating (*Dt1 Dt1, Dt1 dt1, dt1*

dt1) in one DNA sample. The new R166W SNP marker will be an effective tool that soybean breeders can use in the future to guide selections.

References

Bilyeu, K.D., Zeng, P., Coello, P., Zhang, Z.J., Krishnan, H.B., Bailey, A., Beuselinck, P.R., and Polacco, J.C. 2008. Quantitative Conversion of Phytate to Inorganic Phosphorus in Soybean Seeds Expressing a Bacterial Phytase. Plant Physiol. 146: 468-477.

Brinch-Pedersen, H., Sorensen, L.D., and Holm, P.B. 2002. Engineering crop plants: getting a handle on phosphate. Trends in Plant Science. 7:3.

Buss, 1988. Registration of 'Hutcheson' Soybean. Crop Science. 28:6.

Carpenter, S.R., Caraco, N.F., Correll, D.L., Howarth, R.W., Sharpley, A.N., and Smith, V.H. Nonpoint Pollution of Surface Waters With Phosphorus And Nitrogen. 1998. Ecological Applications. 8: 559-568.

Chen Jr. P, T. Toribara, and H. Warner. 1956. Microdetermination of P. Anal. Chem. 28(11): 1756–1758.

Chen, P. 2007. Registration of 'Osage' Soybean. J. of Plant Registrations: 1:89-92.

Cichy, Karen, and Victor Raboy. 2009. Evaluation and Development of Low-Phytate Crops. Hari Krishnan. Madison, WI: 177-200.

Eberhart and Russell, 1966. Stability Parameters for Comparing Varieties. Crop Sci.6:36-41.

Erdman, J.W. 1979. Oilseed Phytates: Nutritional Implications. Department of Food Sciences. Urbana, Illinois.

Fehr, W.R. and Caviness, C.E. 1977. Soybean Stages of Development. Ames, IA. Cooperative Extension Service.

Gao, Y., Biyashev, M.A., Maroof, S., Glover, N.M., Tucker, D.M., and Buss, G.R. 2008. Validation of Low Phytate QTLs and Evaluation of Seedling Emergence of Low-Phytate Soybeans. Crop Science. 48: 1355-1364.

Gillman, Jason D., Vincent R. Pantalone, and Kristin Bilyeu. 2009. The low phytic acid phenotype in soybean line CX1834 is due to mutations in two homologs of the maize low phytic acid gene. Plant Genome. 2: 179-190.

Graf, E. and Eaton, J.W. 1990. Antioxidant Functions of Phytic Acid. Free Radical Biology and Medicine. 8:61-69.

Hart, E.B., and Tottingham, W.E. 1909. The Nature of the Acid Soluble Phosphorus Compounds of Some Important Feeding Materials. Agricultural Chemical Laboratory, Wisconsin Agricultural Experiment Station.

Hegeman, C.E., Good, L.L., and Grabau, E.A. 2001. Expression of d-myo-Inositol-3-Phosphate Synthase in Soybean. Implications for Phytic Acid Biosynthesis. Plant Physiology. 125: 1941-1948.

Jendza, J.A., and Adeola, O. 2009. Water-soluble phosphorus excretion in pigs fed diets supplemented with microbial phytase. Animal Science Journal. 80:296-304.

Khalid, M.F., Hussain, M., Rehman, A.U., Shahzad, M.A., Sharif, M. and Rahman, Z.U. 2013. Broiler Performance in Response to Phytate and Supplemented Phtyase. Iranian Journal of Applied Science. 3:1-12.

Lott, J.N.A., Ockenden, I., Raboy, V., and Batten, G.D. 2000. Phytic Acid and Phosphorus in crop seeds and fruits: a global estimate. Seed Science Research. 10:1: 11-33.

Maenz, D. D., Engele-Schaan, C.M., Newkirk, R.W., and Classen, H.L. 1999. The effect of minerals and mineral chelators on the formation of phytase-resistant and phytase-succeptible forms of phytic acid in solution and in a slurry of canola meal. Animal Feed Science and Technology. 81: 177-192.

Maroof, S.M., Glover, N.M., Biyashev, R.M., Buss, G.R. and Grabau, E.A. 2009. Genetic Basis of the Low-Phytate Trait in the Soybean Line CX1834. Crop Science. 49:69-76.

Mellinger, S., 2012. Molecular Marker Assisted Development and Stability Analysis of Low Phytate, High Inorganic Phosphorus Soybean [*Glycine max* (L.) Merrill]. MS thesis, University of Tennessee.

Oltmans, S. E., W. R. Fehr, G. A. Welke, and S. R. Cianzio. 2004. Inheritance of low-phytate phosphorus in soybean. Crop Sci. 44: 433-435.

Oltmans, S. E., W. R. Fehr, G. A. Welke, V. Raboy, and K. L. Peterson. 2005. Agronomic and seed traits of soybean lines with low-phytate phosphorus. Crop Sci. 45: 593-598.

Pantalone, V.R., F.L. Allen, and D. Landau-Ellis. 2003. Registration of Cultivar 5601T. Crop Sci. 43:1123.

Pantalone, Vincent R. 2013. Release of Ellis soybean cultivar. TN Agric. Exp. Stn.

Powers, W. J., E.R. Fritz, W. Fehr, and R. Angel. 2006. Total and water-soluble phosphorus excretion from swine fed low-phytate soybeans. J. Animal Sci. 84: 1907-1915.

Prattley, C.A., Stanley, D.W., Smith, T.K., Van De Voort, F.R. 1982. Protein-Phytate Interactions in Soybean. Journal of Food Biochemistry. 6:4:273-282.

Raboy, V. 2002. Impacts of Agriculture on Human Health and Nutrition. EOLSS Publishers, Ltd. Oxford, UK.

Raboy, V., Hudson, S.J., and Dickson, D.B. 1984. Reduced Phytic Acid Does Not Have an Adverse Effect on Germination of Soybean Seeds. Plant Physiology. 79: 323-325.

Raboy, Victor. 2002. Progress in breeding low phytate crops. Journal of Nutrition. 2002:503S-505S.

Raboy, V., D. B. Dickinson, and F. E. Below. 1984. Variation in seed total phosphorus, phytic acid, zinc, calcium, magnesium, and protein among lines of Glycine max and G. soja. Crop Sci. 24: 431-434.

Reddy, N. R., M. D. Pierson, S. K. Sathe, and D. K. Salunkhe. 1989. Phytates in Cereals and Legumes. Boca Raton, FL: CRC Press, Inc.

SAS Institute. 2011. The SAS system for Windows. Release 9.2. SAS Inst., Cary, NC.

Sathe, S.K. and Reddy, N.R. 2002. Food Phytates. Boca Raton, FL. CRC Press.

Scaboo, A.M., V.R. Pantalone, D.R. Walker, H.R. Boerma, D.R. West, F.R. Walker, and C.E. Sams. 2009. Confirmation of molecular markers and agronomic traits associated with seed phytate content in two soybean RIL populations. Crop Sci. 49: 426-432.

Sedcole, 1977. Number of plants necessary to recover a trait. Crop Sci. 17: 667-668.

Sharpley, A., B. Foy, and P. Withers. 1994. Practical and innovative measures for the control of agricultural phosphorus losses to water: and overview. J. Environ. Qual. 29.1:1-9.

Shi, J., Wang, H., Schellin, K., Li, B., Faller, M., Stoop, J.M., Meeley, R.B., Ertl, D.S., Ranch, J.P., and Glassman, K. 2007. Embryo-specific silencing of a transporter reduces phytic acid content of maize and soybean seeds. Nature Biotechnology. 25:8.

Shu, Q.Y. 2009. Induced Plant Mutations in the Genomics Era. Food and Agriculture Organization of the United Nations, Rome: 316-318.

Spear, J. D., and W. R. Fehr. 2007. Genetic improvement of seedling emergence of soybean lines with low phytate. Crop Sci. 47:1354-1360.

Tian, Z., Wang, X., Lee, R., Li, Y., Specht, J.E., Nelson, R.L., McClean, P.E., Qiu, L., and Ma, J. 2010. Artificial Selection for determinate growth habit in soybean. PNAS. 107:19:8563-8568.

"United States Soy Crop Statistics." Soy Stats 2013. The American Soybean Association , 2012. Web. 29 Aug 2013. <http://www.soystats.com/2012/Default-frames.htm>

Walker, D.R., A.M. Scaboo, V.R. Pantalone, J.R. Wilcox, and H.R. Boerma. 2006. Genetic mapping of loci associated with seed phytic acid content in CX1834-1-2 soybean. Crop Sci. 46:390-397.

Wilcox, J. R., G. S. Premachandra, K. A. Young, and V. Raboy. 2000. Isolation of high seed inorganic P, low-phytate soybean mutants. Crop Sci. 40:1601-1605.

Wilson, R.F. Soybeans: Improvement, Production and Uses. 1987. Agronomy. 16: 643-686

Appendix II

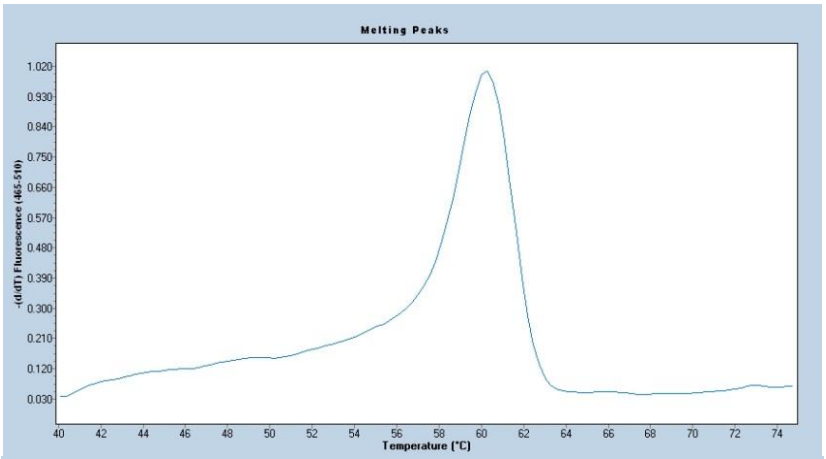
Table 2.1

Stability analysis parameters of inorganic phosphorus content for all 12 BC_5F_3 derived low phytate soybean lines, 5601T, TN09-239, Ellis and Osage grown in three TN environments from 2012-2013: ETREC (Knoxville, TN), HRREC (Springfield, TN) and MILAN (Milan, TN).

	Inorganic Phosphorus Content	Stability Parameters				Slope Different than Zero	Slope Different than One
Genotype	(µg Pi g^{-1})	CV†	b_i	sd_i	R^2	P value	P value
56CX-1287	1857.1	9.0	1.2	166.9	0.72	<0.001	p>0.05
56CX-1286	1843.9	13.3	1.6	246.0	0.67	<0.01	p>0.05
56CX-1284	1835.0	13.1	0.8	240.2	0.37	<0.05	p>0.05
56CX-1280	1775.2	9.4	0.9	166.6	0.61	<0.01	p>0.05
56CX-1283	1636.2	15.4	0.9	251.6	0.38	<0.05	p>0.05
56CX-1282	1800.1	7.7	1.2	137.9	0.78	<0.0001	p>0.05
56CX-1279	1612.2	14.0	0.7	226.2	0.32	>0.05	p>0.05
56CX-1277	1661.4	8.7	1.0	145.3	0.70	<0.001	p>0.05
56CX-1276	1670.4	8.5	1.0	142.8	0.70	<0.001	p>0.05
56CX-1275	1945.9	9.5	1.6	183.9	0.84	<0.01	p>0.05
56CX-1274	2049.5	9.2	1.6	189.1	0.84	<0.01	p>0.05
56CX-1273	1887.1	13.6	1.9	256.0	0.73	<0.001	p<0.05
TN09-239	1494.3	8.8	0.7	131.8	0.55	<0.01	p>0.05
5601T	216.0	13.2	0.1	28.4	0.17	>0.05	<0.0001
Ellis	238.8	13.6	0.0	32.5	0.02	>0.05	<0.0001
Osage	206.7	13.6	0.1	28.1	0.20	>0.05	<0.0001

CV = coefficient of variability is calculated by 100*std. deviation/mean and measures the amount of variability in the data relative to the mean.
bi = regression coefficient that measures the response of genotype i to varying environments.
sdi = a genotype's deviation from the regression line.
R^2 = coefficient of determination, determined for each genotype to measure the percentage of the total variance explained by the regression model.
†CV is calculated for individual genotype mean (Eberhart and Russell, 1966).

A



B

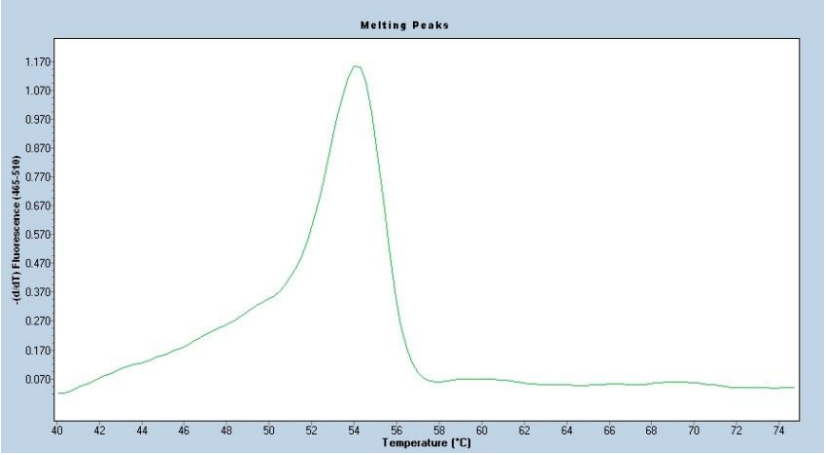
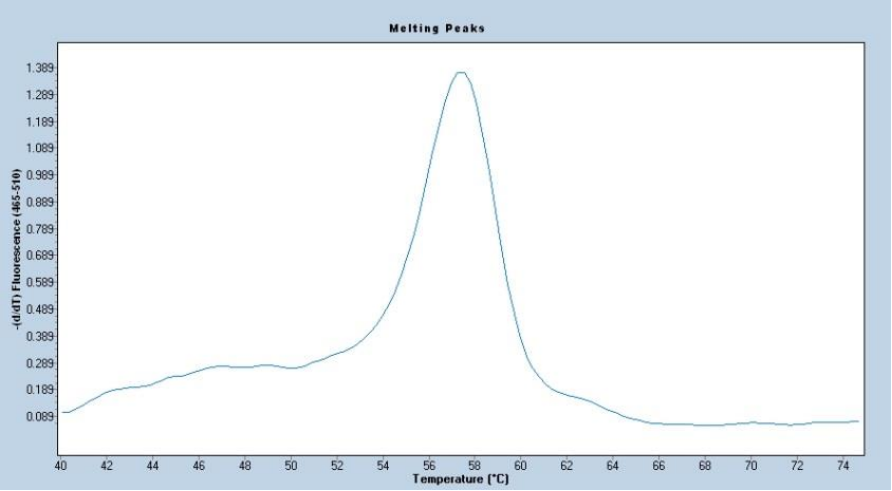


Figure 2.1

A. Single Nucleotide Polymorphism (SNP) melting curve assay for a wild type allele on Gm19, which melts at approximately 60º C indicating a normal phytate, homozygous dominant genotype.

B. Single Nucleotide Polymorphism (SNP) melting curve assay for *cqPha-002* allele on Gm19, which melts at approximately 54º C indicating a low phytate homozygous recessive genotype.

A



B

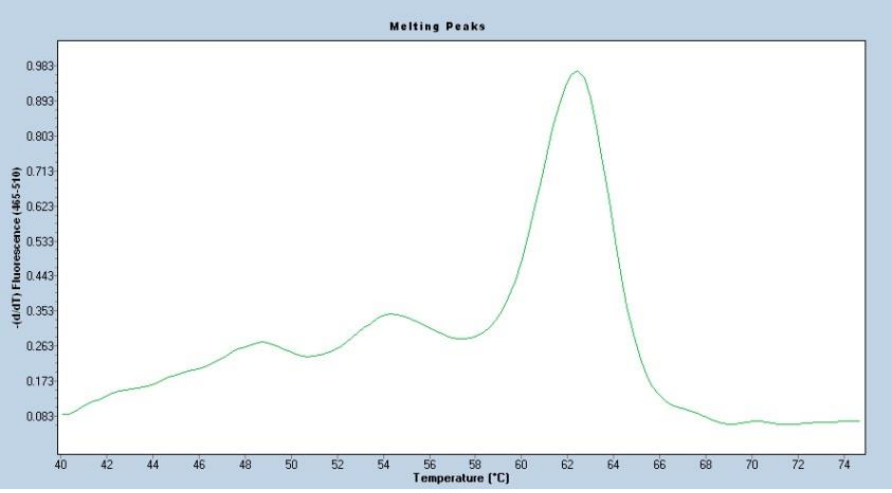


Figure 2.2

A. Single Nucleotide Polymorphism (SNP) melting curve assay for a wild type allele on Gm03, which melts at approximately 57º C indicating a normal phytate homozygous dominant genotype.

B. Single Nucleotide Polymorphism (SNP) melting curve assay for *cqPha-001* allele on Gm03, which melts at approximately 62º C indicating a low phytate homozygous recessive genotype.

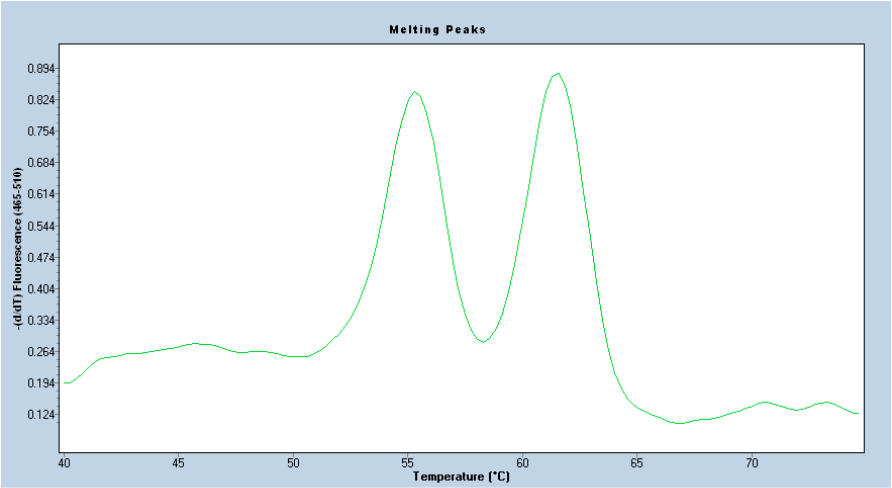


Figure 2.3

Single Nucleotide Polymorphism (SNP) melting curve assay on Gm19. The double melting peaks indicate a heterozygote genotype with melting peaks at approximately 54ºC and 61°C.

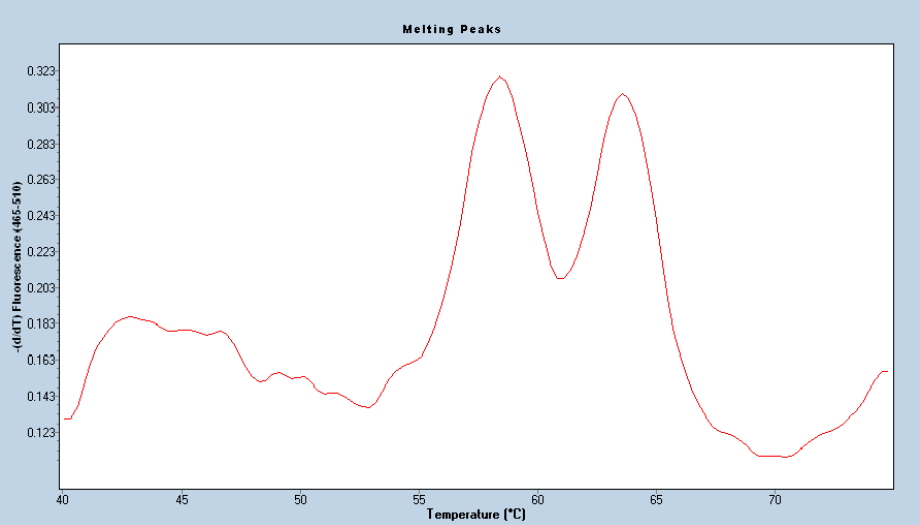


Figure 2.4

Single Nucleotide Polymorphism (SNP) melting curve assay on Gm03. The double melting peaks indicate a heterozygote genotype with melting peaks at approximately 58ºC and 63°C.

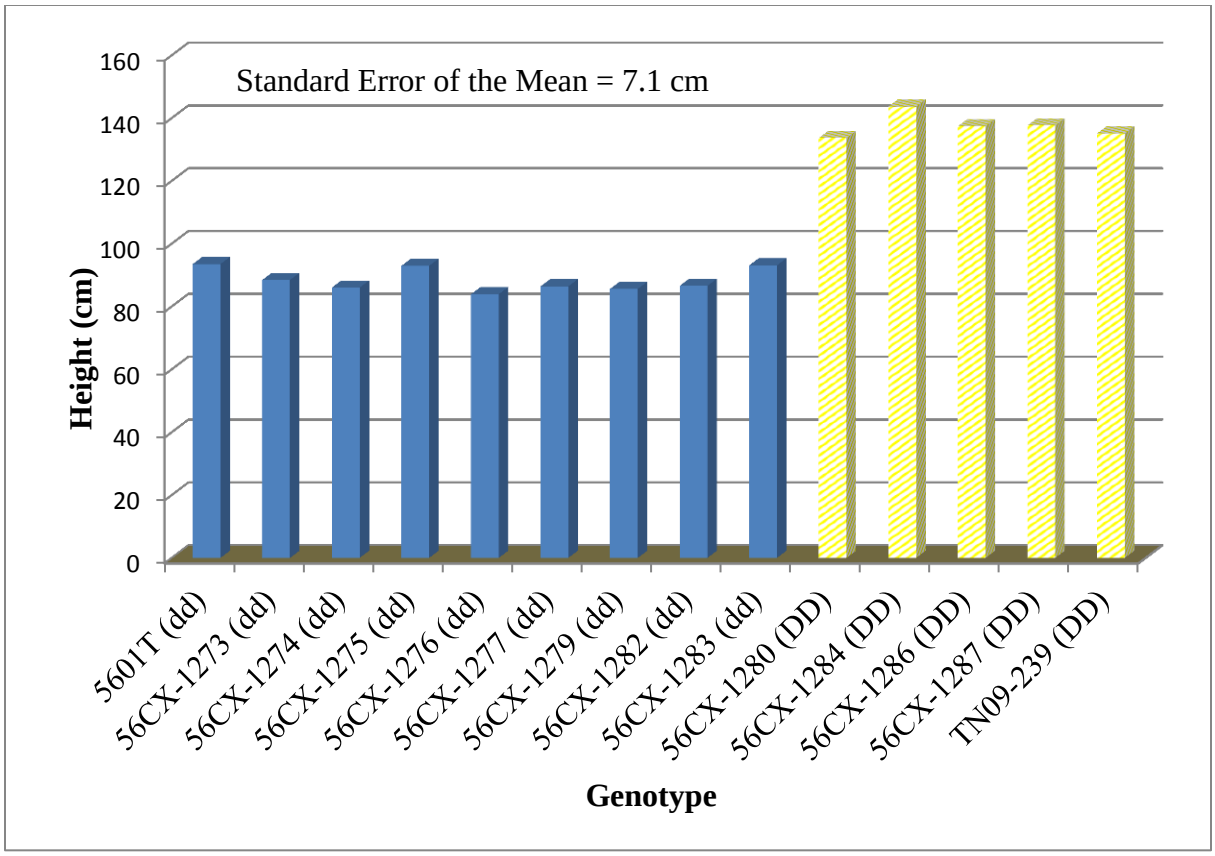


Figure 2.5

Significant differences ($p<0.001$) in plant height (cm) were found between the mean of determinate (*dt1*) and the mean of indeterminate (*Dt1*) BC_5 low phytate lines. Donor parent TN09-239 was indeterminate (*Dt1)* but recurrent parent 5601T was determinate (*dt1*).

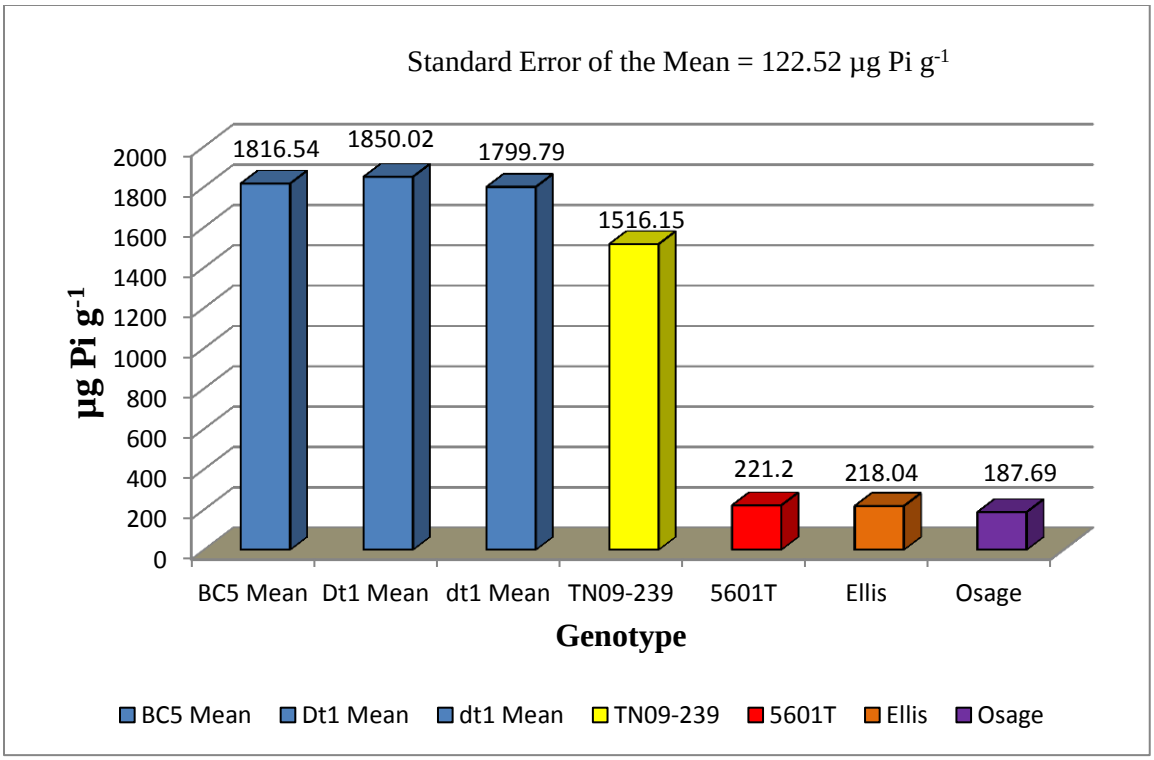


Figure 2.6

The BC_5 determinate (*dt1*) and indeterminate (*Dt1*) low phytate lines exhibited highly significant ($p<0.0001$) elevated levels of Pi in comparison to recurrent parent 5601T, donor parent TN09-239 and high yielding checks Ellis and Osage.

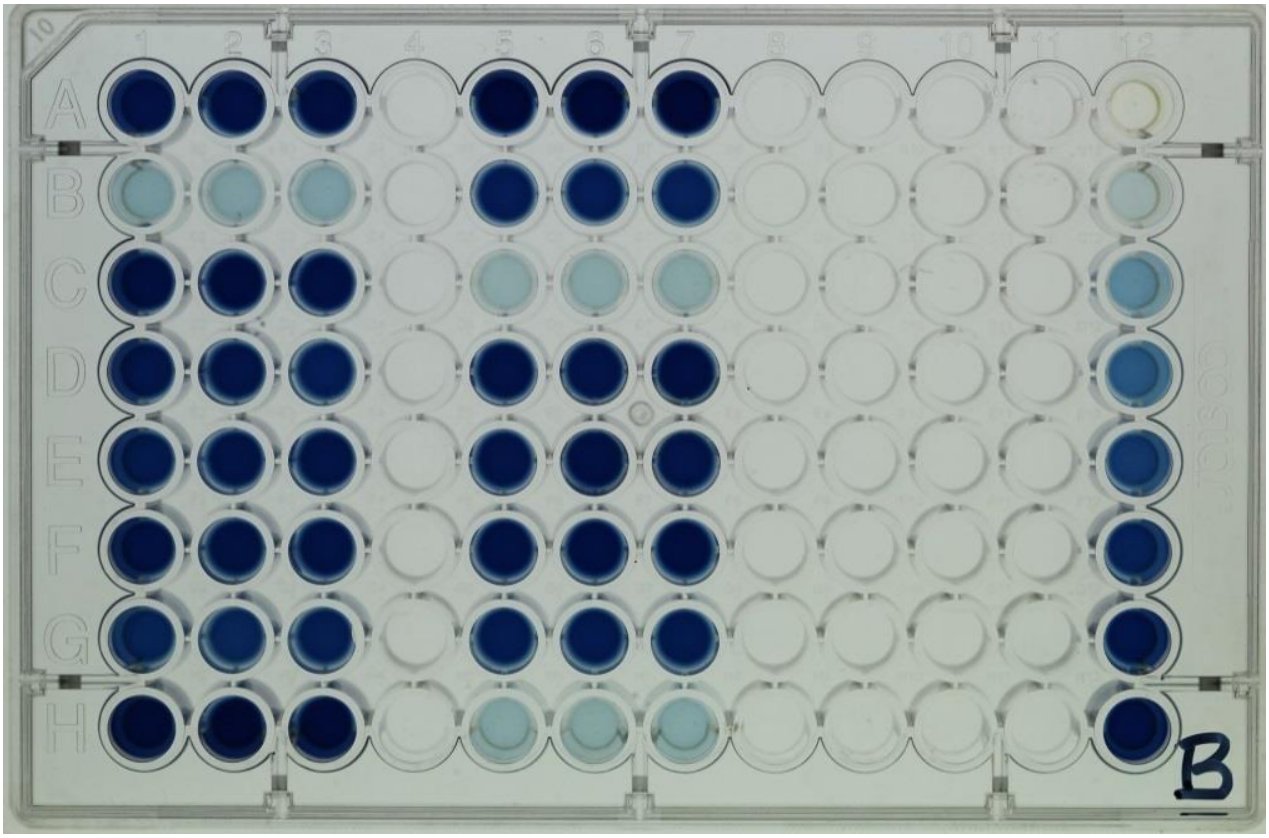
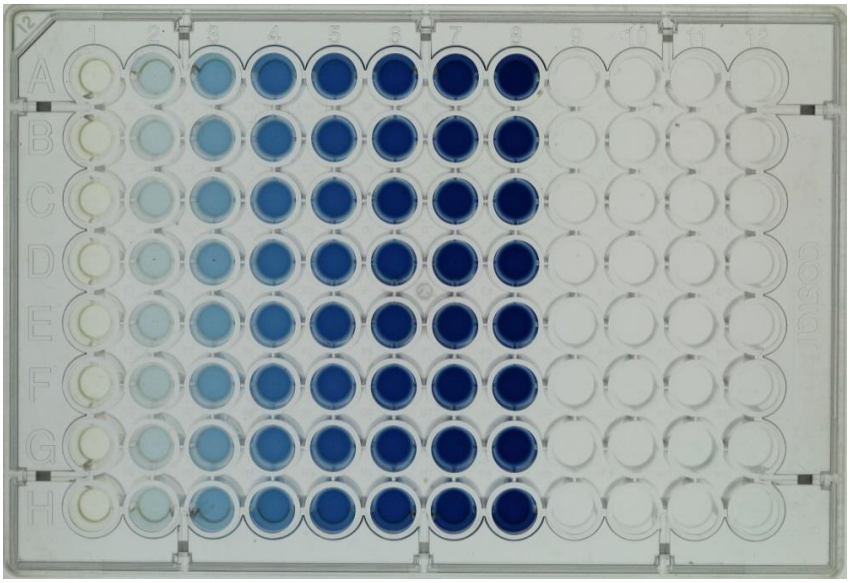


Figure 2.7

Inorganic phosphorus assay for soybean samples grown at the Eastern Tennessee Research & Education Center, Rep 3, Plots 7533 – 7548 [well positions A1, A2, A3 (plot 7533) to well positions H5, H6 & H7(plot 7548)]. One set of Pi standards were run per plate (far right). The well positions of the three samples for Ellis plot 7534 (B1, B2 and B3), Osage plot 7543 (C5, C6 & C7) and recurrent parent 5601T plot 7548 (H5, H6 & H7) can easily be distinguished from the other BC_5 low phytate lines by density of blue pigmentation. The Pi standards values and their well positions are as follows: well position (WP) A12 = 0 µg Pi g^{-1}; WP B12 = 155 µg Pi g^{-1}; WP C12 = 465 ng Pi µL; WP D12 = 930 ng Pi µL; WP E12 = 1395 ng Pi µL; WP F12 = 1860 µg Pi g^{-1}; WP G12 = 2325 µg Pi g^{-1}, and WP H12 = 2635 µg Pi g^{-1}.

A



B

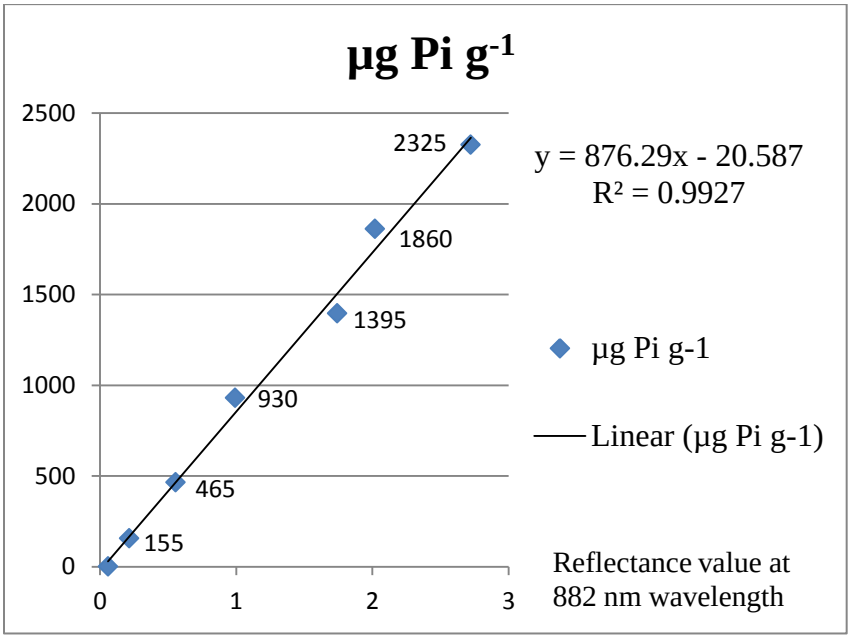


Figure 2.8

A. A standards plate used to estimate seed Pi. The average value of the eight spectrophotometer reflectance readings within a lane at 882 nm was used to create a standards curve (B) to quantify seed levels of Pi in the BC_5 low phytate lines. Lane 1 (0 µg Pi g^{-1}), Lane 2 (155 µg Pi g^{-1}), Lane 3 (465 µg Pi g^{-1}), Lane 4 (930 µg Pi g^{-1}), Lane 5 (1395 µg Pi g^{-1}), Lane 6 (1860 µg Pi g^{-1}), Lane 7 (2325 µg Pi g^{-1}), and Lane 8 (2635 µg Pi g^{-1}).

B. Inorganic phosphorus standard curve created for the Bio-Tek Powerwave XS microplate spectrophotometer plate reader at 882nm.

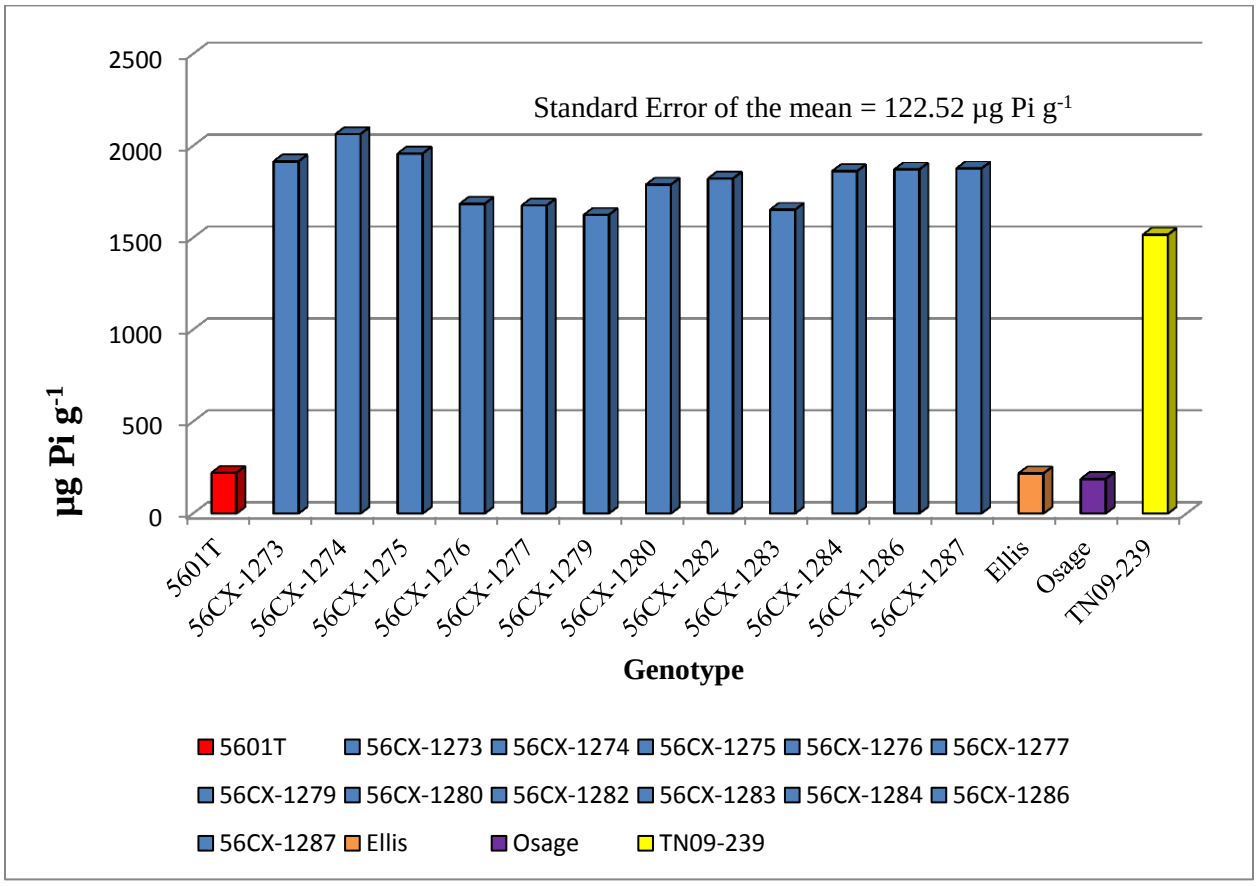


Figure 2.9

Two year mean Pi levels (µg Pi g^{-1}) of 12 BC_5 low phytate lines (blue) compared with recurrent parent 5601T (red), high yield check cultivars Ellis (brown) and Osage (purple), and BC_4 donor parent TN09-239 (yellow).

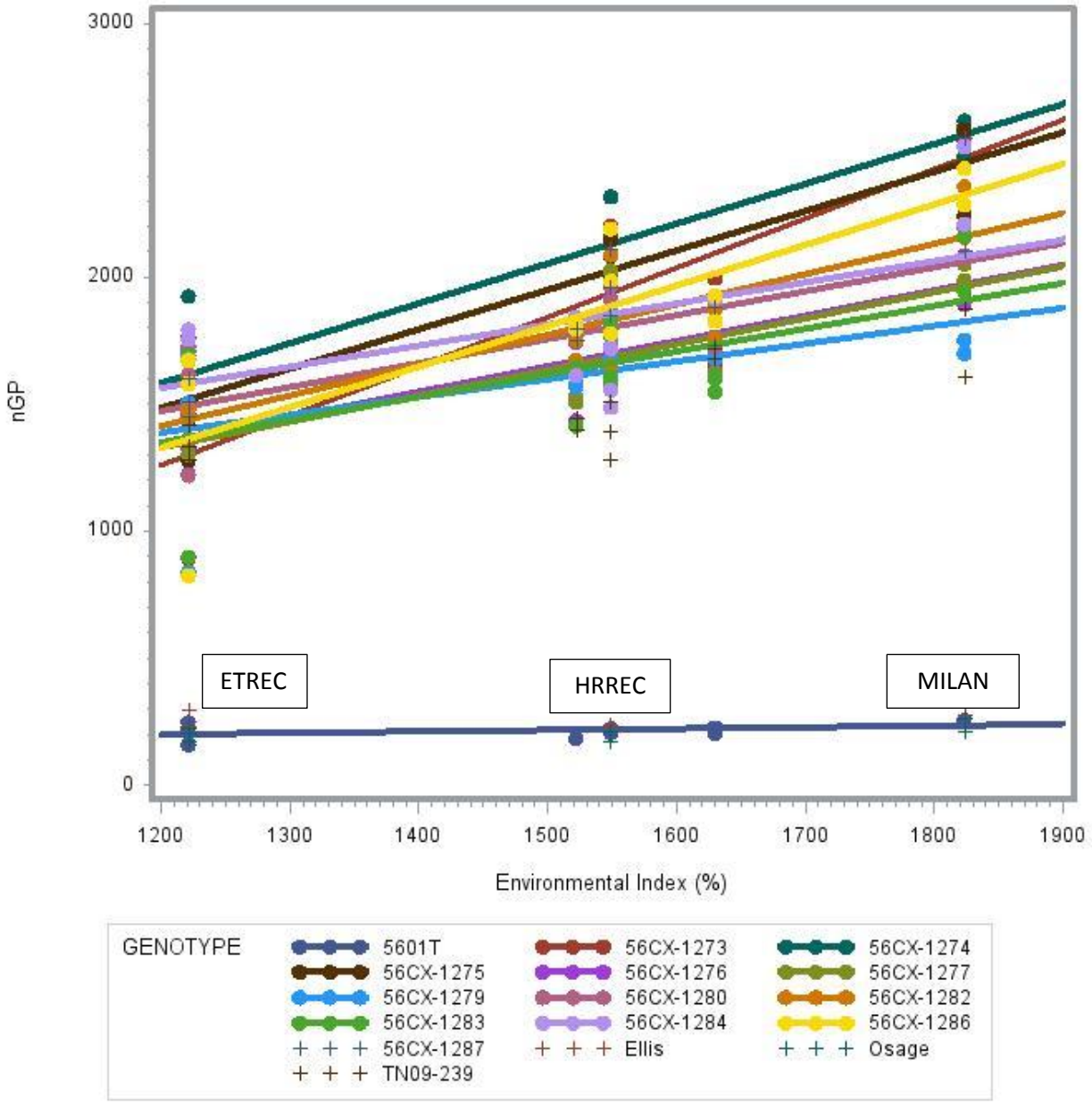


Figure 2.10

Environmental index linear regression with Pi for 12 BC_5 low phytate lines, donor parent, recurrent parent, and high yielding cultivars Ellis and Osage. Slopes for 56CX-1279 (0.7), Ellis (-0.01), Osage (0.05) and recurrent parent 5601T (0.05) were not significantly different than zero ($p>0.05$) and were stable for the low phytate trait when grown across three locations in TN in 2013. The slopes of donor parent TN09-239 (0.7) and the eleven other BC_5 low phytate line were significantly greater than zero ($p<0.0001$) and thus indicative of environmental sensitivity for the low phytate trait. The y axis value of "nGP" is equivalent to µg Pi g^{-1} in seed dry weight.

Part 3

Evaluation of Agronomic Traits, Seed Quality Traits, and Commercial Performance of Twelve New BC_5 Low Phytate Lines in Comparison to Their Parents 5601T, TN09-239, and USDA High Yield Check Cultivars

Abstract

Soybean [*Glycine max* (L). Merrill] is the world's top oilseed crop. High protein soymeal is a primary soybean use and staple food of livestock and fish. The storage form of phosphorus (P) in the seed is phytic acid, or phytate (myo-inositol 1,2,3,4,5,6 hexa*kis*phosphate) which binds and chelates many key nutritional cations forming stable salts that are non-digestible. Livestock waste laden with phytate P is an environmental concern, and a source of nonpoint P pollution causing accelerated eutrophication and surface water quality deterioration. The primary objective of this research was the development of a commercially acceptable low phytate (LP) soybean line which would enhance nutritional qualities of soymeal for livestock and sustainably lessen P pollution from animal agricultural waste runoff. The LP soybean lines used in this study were twelve BC_5 derived lines from their recurrent parent '5601T', a high yielding University of Tennessee developed cultivar. The line TN09-239 was our LP, donor parent. The presence of the two LP alleles in all twelve BC_5 derived lines was confirmed using perfect SNP molecular markers near the confirmed QTL *cqPha-001* and *cqPha-002*. In 2012, a field trial using a randomized complete block design (RCBD) with two replications was grown at two locations in two row plots to evaluate the agronomic performance of 10 BC_5 LP lines in comparison to their parents 5601T, TN09-239, and high yield check cultivars. The study was repeated in 2013, adding two additional BC_5 lines which were evaluated in four row plots in two locations and in two row plots in a third location with three replications per location. Data analyzed using SAS version 9.3 revealed that the yields of four $BC_5F_{3:5}$ LP lines, 56CX-1273 (4107 kg ha^{-1}), 56CX-1274 (4137 kg ha^{-1}), 56CX-1277 (4112 kg ha^{-1}) and 56CX-1282 (4160 kg ha^{-1}), were not significantly different ($p>0.05$) than that of 5601T (4227 kg ha^{-1}) and also slightly bested the yield of current USDA Maturity Group V check 'Osage' (4089 kg ha^{-1}). The lodging results of

the determinate BC_5 lines (1.8) were significantly better ($p<0.05$) than RP 5601T (2.9). The protein content of the determinate BC_5 lines (439 g kg^{-1}) was not significantly different ($p>0.05$) than the high protein content of 5601T (436 g kg^{-1}) which qualifies five of the determinate BC_5 LP lines for 50% ultra-high protein soymeal. These results indicate that the agronomic performance of the determinate LP lines was comparable to high yield MG V check cultivars and therefore one of the lines may be considered for a cultivar release.

Introduction

Domestically in 2013, 34.32 million metric tons of soymeal was used in livestock feed for broiler chickens (*Gallus domesticus*, 12.95 million metric tons), hogs (*Sus domesticus*, 11.15 million metric tons), laying hens (*Gallus domesticus*, 2.28 million metric tons), turkeys (*Meleagris gallopavo*, 2.04 million metric tons), and combined other feed uses (5.90 million metric tons) (Soy Stats 2013). According to the United Soybean Board, the U.S. soybean industry is reliant on the annual demand strength coming from the U.S. animal agricultural sector. Livestock producers use the high protein soymeal as a staple food but the animals fail to digest large amounts of phytate present in the soymeal (Raboy and Dickinson, 1984; Erdman, 1979). Chemically, the structure of the phytate molecule acts as a binding or chelating agent to mineral cations forming salts that are non-digestible, which removes the mineral bioavailability from the seeds by forming the more complex phytate molecule (Erdman, 1979; Maenz et al., 1999). Many of the phytate mineral complexes that are formed are insoluble and therefore unavailable for absorption (Reddy, 1989). The bound cations, such as Ca, Fe, Mg and Zn, are nutritional minerals that become non-digestible insoluble phytate salts (Erdman, 1979; Raboy, 2002; Reddy, 1989). The interference with intestinal absorption of the mineral complexes, such as Ca, Fe, K, Mg, P and Zn, may lead to mineral deficiencies in animals and humans earning phytate the label of "anti-nutrient" (Reddy, 1989; Lott et al., 1995). The phytic acid is not broken down in the intestinal tract of monogastric animals because they possess little to no phytase activity in their digestive tracts, which is why phytic acid is not efficiently utilized (Brinch-Pedersen et al., 2002). For the phytic acid to be efficiently utilized, animal feeds must be supplemented with phytase to free cations and other nutrients bound by phytate P-complexes or the animal feed may be supplemented with digestible Pi for the animals to receive the

optimum amount of P needed for animal growth and skeletal development (Khalid et al., 2013; Shi, 2007).

Because animals lack the phytase enzyme in their digestive tracts necessary to break down the stable phytate molecule, Powers (2006) found that the excess P in the form of phytic acid in the animal waste is both insoluble and water soluble, the relative abundance of which varies with the dietary treatments (Brinch-Pedersen et al., 2002; Jendza, 2009). The non-digestible, stable phytate salts present in soybeans and soymeal are therefore excreted by animals that consume them (Erdman, 1979; Raboy, 2002; Raboy, 2009; Bilyeu et al., 2008; Gillman et al., 2009; Wilcox et al., 2000). This is especially a concern near concentrated animal production areas where the P runoff from livestock manure is often great. The excess P in the excretion then becomes a P management concern, because high levels of P in the form of phytic acid present in the manure are often applied as fertilizer to agricultural lands as a common agricultural production practice (Hegeman et al., 2001). The P becomes mobile in watersheds and can cause accelerated eutrophication and surface water quality deterioration in freshwater ecosystems such as ponds, lakes and streams. Eutrophication resulting from algal blooms is worsened by excess P pollution and can be damaging to aquatic ecosystems as low levels of oxygen in the water depleted by decaying algae has been responsible for fish kills and hypoxic dead zones. The P laden animal waste is a major point source (Carpenter, et al., 2010) and non-point source of P pollution detrimental to the environment which is often not monitored (Sharpley et al. 1994, Carpenter et al., 2010, Walker et al., 2006). The creation of a high yielding, LP soybean cultivar to be used for soymeal would ease environmental concerns and provide other benefits.

First, lowering the phytate concentration in the seed would serve to enhance the dietary nutrition of soymeal and increase the metabolic energy available to ruminant and non-ruminant

livestock by increasing P bioavailability (Oltmans et al., 2004, Shu, 2009). The increased P bioavailability would allow non-ruminant livestock to digest the Pi present in the soymeal thereby removing the P from the waste stream and greatly reducing eutrophication in aquatic environments (Brinch-Pedersen et al., 2002; Sharpley et al., 1994). The combined benefits would lead to more effective and sustainable P nutrient management on farms for crop and livestock production. In addition livestock producers would also save costs annually in relation to reduced need to purchase phytase amendments placed in soybean meal. In summary modifying the P in soybean seed by lowering the phytate level would enable livestock producers to enhance animal nutrition and sustainably lessen P pollution from animal agricultural waste runoff. These improvements have been the rationale behind this research and for creating a commercially acceptable LP cultivar.

Creating a commercially acceptable LP cultivar has proven to be difficult as no high yielding LP cultivars exist to date despite the efforts of many plant breeders. Even though the total P content in the seed remains unchanged in LP lines, lowering the phytate and increasing the Pi content in the seeds has been suggested to lower the germination rate of seeds, an outcome that would not be commercially acceptable. Germination losses could be due in part to the fact that phytate is used physiologically as a P store and an energy store supporting germination, early seedling growth and development (Reddy, 1989; Raboy, 2002). Phytate offers myo-inositol, macro-nutrients P, K, and Mg and micro-nutrients Ca, Fe, Zn, and Mn to growing seedlings and phytic acid synthesis plays an integral role in the seed storage processes for minerals like K, Zn, Fe and Mg (Reddy, 1989; Raboy, 2002). Typically Pi is taken up by the plant in the form of phosphoric acid (H_3PO_4), then translocated to the developing seed and synthesized to become the storage form of P, which is phytic acid (Raboy, 2002). Many factors

can influence seed phytate levels, such as environmental fluctuations, growing locations, irrigation conditions, fertilizer applications, soil types, soil temperature, growing conditions, cultivar used and time of year the plant is grown (Reddy, 1989). Plants typically produce more phytic acid than is needed for growth and development and reducing the phytic acid has not inhibited peak agronomic performance (Scaboo et al., 2009; Gillman et al., 2009; Bilyeu et al., 2008; Shi et al., 2007; Spear and Fehr, 2007). The objectives of Part 3 were to:

(1) Evaluate the agronomic performance of twelve new BC_5 LP lines by testing the null hypotheses of no significant differences in seed yield, plant lodging, plant height, seedling field emergence, plant maturity and seed germination between the twelve new BC_5 LP lines and their recurrent parent 5601T, their donor parent TN09-239 and high yield MG V check cultivars Ellis and Osage.

(2) Test the null hypotheses of no significant differences between twelve BC_5 LP lines and recurrent parent 5601T for seed protein concentration, seed oil concentration, seed amino acid concentration (cysteine, lysine, methionine, threonine and tryptophan), and seed fatty acid composition of the oil (stearic, palmitic, oleic, linoleic, and linolenic).

Materials and Methods

The LP soybean lines used in this study were twelve BC_5 derived lines from their RP 5601T, a high yielding cultivar developed by the University of Tennessee that was a former USDA MG V check (Pantalone et al., 2003). The line TN09-239 was our LP, donor parent,

whose LP trait was originally introgressed from the donor line CX 1834-1-2. TN09-239 and 5601T were included in the study as well as Osage, a current USDA maturity group V check cultivar developed by the University of Arkansas and Ellis, a new high yielding University of Tennessee cultivar and new USDA check whose pedigree includes 5601T.

Development of "56CX" BC_5 LP Lines and Pedigree

The same backcrossing strategy and procedures were used as was documented in Part 2 of this thesis to develop all 12 BC_5 LP lines. Briefly, the presence of the LP alleles on Gm03 and Gm19 has been validated using perfect SNP molecular markers at loci cqPha-001 and cqPha-002 meaning that all 12 BC_5 LP lines in this study have been confirmed to be double homozygous recessive for the two alleles that express LP concentration in soybean seeds (Scaboo et al., 2009) (Figure 2.1 & Figure 2.3).

Data Analysis of Field Experiment 2012 and Field Experiment 2013

A randomized complete block design (RCBD) was used for the study. In 2012, 160 seeds per row were planted in two row plots with two replications in two locations [ETREC and Research and Education Center at Milan, TN (MILAN)]. Data were evaluated using a mixed model analysis of variance in SAS version 9.3 (Glimmix procedure, SAS Institute, Cary, NC) and least square means were compared using Tukey's protected LSD at the 5% significance level. The random factors in the statistical model were the location, the genotype by location (G × E) interaction and the replications nested within location. The fixed terms or treatments in the statistical model were the genotypes. The dependent variables being evaluated for the genotypes were seed yield, plant height, plant lodging, plant maturity, seed field emergence, seed

germination, 100 seed weights, seed palmitic acid content, seed stearic acid content, seed oleic acid content, seed linoleic acid content, seed linolenic acid content, seed cysteine content, seed methionine content, seed lysine content, seed threonine content, seed tryptophan content, and seed protein and seed oil content on a dry weight basis. The following statistical model was employed:

$$Y_{ijk} = \mu + L_i + R(L)_{k(i)} + G_j + (GL)_{ji} + e_{ijk}$$

Where Y_{ijk} is the specific measured value of genotype *j* in rep *k* within location *i*; μ is the mean overall; L_i is the effect of location *i*; $(GL)_{ji}$ is the interaction between genotype *j* and location *i*, and Y_{ijk}'s error term is e_{ijk}. The designation "56CX" was assigned as a prefix to all genotypes denoting that the lines originated from the 5601T × CX 1834-1-2 population. That population abbreviation was followed by a four digit number denoting the 2011-2012 FL row number. Ten LP genotypes were tested in the 2012 field trial: 56CX-1273 (BC_5 $F_{3:6}$), 56CX-1276 (BC5 $F_{3:6}$), 56CX-1277 (BC_5 $F_{3:6}$), 56CX-1279 (BC_5 $F_{3:5}$), 56CX-1280 (BC_5 $F_{3:5}$), 56CX-1282 (BC_5 $F_{3:5}$), 56CX-1283 (BC_5 $F_{3:5}$), 56CX-1284 (BC_5 $F_{3:5}$), 56CX-1286 (BC_5 $F_{3:5}$) and 56CX-1287 (BC_5 $F_{3:6}$). The RP, cultivar 5601T, and LP donor parent, TN09-239, were also included as check entries in the study. In addition, five other lines from the same BC_5 population were grown for evaluation in single rows, as there was not a sufficient seed amount to include them as entries in the 2012 replicated field study: 56CX-1274 (BC_5 $F_{3:5}$), 56CX-1275 (BC_5 $F_{3:5}$), 56CX-1278 (BC_5 $F_{3:5}$), 56CX-1281 (BC_5 $F_{3:5}$) and 56CX-1285 (BC_5 $F_{3:5}$). Plants from row 56CX-1274 and plants from row 56CX-1275 visually exhibited a determinate (*dt1*) growth habit in 2012 and were confirmed to be determinate using the *Dt1* R166W SNP marker developed by Dr. Bilyeu

(USDA-ARS, Columbia, MO), and as a result those two lines were added to the 2013 replicated field trial.

In 2013, a RCBD was again used for the study. A sample of 200 seeds per row were planted in four row plots with three replications in three locations [ETREC, MILAN, and at the Highland Rim Research and Education Center at Springfield, TN (HRREC)]. Data were evaluated using a mixed model analysis of variance in SAS version 9.3 (Glimmix procedure, SAS Institute, Cary, NC) and least square means compared using Tukey's protected LSD at the 5% significance level using the same statistical model employed in 2012 and evaluating the same dependent variables. The ten LP lines tested in 2012, plus 56CX-1274 and 56CX-1275, along with their parents and new University of Tennessee high yielding cultivar Ellis, a new USDA high yield check, and current USDA MG V check Osage were evaluated in the 2013 replicated field trial.

Phenotypic Traits Observed

Flower color (white with the exception of the purple flowers for Osage added in 2013) was observed and recorded when more than 95% of all plants in the plot were at the R2 stage in full bloom (Fehr et al., 1977). Pod pubescence color (gray for all genotypes) and days to maturity from planting date were noted when 95% of the pods showed their mature color. Lodging score was assigned on a one to five scale at time of maturity, with one being a plot with erect plants and five being a plot whose plants were prostrate to the ground (Gillen and Shelton, 2012). The average plant height of all plants in the plot was measured and recorded at time of maturity, and seed yield was measured at harvest and adjusted to a 13% moisture basis.

Field Emergence

The seedling emergence percentage was determined for all plots in 2012 and 2013 by counting the number of emerged plants, dividing by the number of seeds planted in each plot, and multiplying the quotient by 100. Plants were counted at the V3 stage when there were three nodes on the main stem with fully developed leaves (Fehr and Caviness, 1977). We tested the null hypothesis to see if significant differences existed in field emergence between the LP lines and the recurrent parent 5601T, donor parent TN09-239 and high yield check cultivars Ellis and Osage.

Germination Test

In 2014, a germination test was conducted using seeds harvested in 2013 from all twelve BC_5 LP lines, the recurrent parent 5601T, the donor parent TN09-239, and check lines Osage and Ellis. For the germination test, three replications of 25 seeds of each genotype (one replication each of 2013 seeds harvested from ETREC, HRREC and MILAN) were placed on wetted germination sheets (Seedburo, Des Plaines, IL). The germination sheets were rolled up, sealed with an outer layer of wax paper (Packaging Converters, Hudson, WI) with two rubber bands fastened at each end to seal in the moisture and labeled by genotype. The "rag dolls" of the 25 wetted seeds of each genotype were then placed upright in moistened tubs (one replication per tub) which were left untouched in the greenhouse for three consecutive days. After three days, the rag dolls were unfastened, carefully unrolled, and the number of germinated seeds counted and scored based on radicle growth (Figure 3.1 and 3.2). The number of germinated seeds were then counted and multiplied by four to obtain a germination percentage rate for each genotype at each location.

Procedure for Inorganic P (Pi) Assay

An assay for Pi was conducted on all BC_5 lines, RP 5601T, and donor parent TN09-239 in both 2012 and 2013 as was detailed in the materials and methods and results and discussion sections of Part 2.

Near Infrared Reflectance (NIR) Spectroscopy analysis of Protein and Oil Concentration

Approximately 25 g of harvested soybean seeds from this study was ground in a water-cooled (25°C) Knifetec 1095 Sample Mill (FOSS Tecator, S-26321, Hogana, Sweden) for 20 sec. This setting produced whole ground soybean seed powder with uniformity of particle size. In 2012, protein, oil, and amino acid dry weight (DW) concentrations were measured using a Foss 6500 NIR instrument using ISIscan™ software IS-2200 and the amino acid values converted to express as amino acids per gram DW crude protein. The instrument was routinely left on and a diagnostics test, a performance test and a Check Cell scan were run each day to ensure that the instrument was working properly. A room dehumidifier was present throughout the analysis, with the humidity set at 40% and the room temperature set at 20 ºC. Approximately 236 ml of the ground soybean samples were placed in the glass and metal chamber and scanned. Seed samples from all harvested plots were analyzed for dry weight protein and oil concentration, and for five essential amino acids in feed rations for livestock nutrition (methionine, cysteine, lysine, threonine and tryptophan). In 2013, the FOSS 6500 NIR instrument would not pass routine diagnostics tests due to a faulty detector. As a result, ground samples harvested from both 2012 and 2013 were labeled with barcodes provided by the University of Minnesota's ZM 400 Barcode Printer and sent to the University of Minnesota's NIR lab for analysis. The ground samples (12.5 g per sample) were then analyzed using a Perten

DA 7250 Near Infrared Analyzer capable of 900-1650 nm wavelength readings. The research lab uses a climate controlled environment and a JET air filtration system. The protein, oil, and seed amino acid concentration values provided by the University of Minnesota for both 2012 and 2013 were analyzed for this research.

Gas Chromatography

Because 5601T has a good combination of seed protein and oil concentrations that is sufficient to produce high protein meal (>47.5% protein in soymeal, after processor oil extraction), it was desirable to develop a LP line with measurable seed qualities and appropriate traits similar to those of the recurrent parent. A gas chromatograph was used to measure the five predominant fatty acids found in soybean oil which are palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3). A sample of five seeds harvested from each plot was crushed using a metal cylinder, hammer and metal plate. The same protocols were used as reported by Mellinger (2012). Each sample was placed into a test tube and 3 mL of an 8:5:2 (v/v/v) ratio of chloroform:hexane:methanol solvent was added. The samples were then capped and allowed to sit overnight. After extraction was completed, 100 µL of the extract from each sample was transferred to its respective vial. Seventy-five µL of methylation reagent [8:4:2 (v/v/v) ratio of petroleum ether: ethyl ether: sodium methoxide/methanol] and 0.75 mL of hexane were added to each vial before it was capped with a crimper. The fatty acid compositions were determined with the gas chromatograph (6890 series; Agilent/Hewlett Packard, Palo Alto, CA) using a model 7683 auto sampler and a model 7673 flame ionization detector, and an immobilized 30 mm x 0.53 mm inner diameter, Alltech AT-Silar capillary column with 0.5 µm

fused stationery phase. Analysis was conducted under the following operating conditions: carrier, Helium (20 ml/min); 20:1(v/v) split injection; injection temperature 250 °C; detector temperature 275 °C, and column temperature 230 °C. The RM-1 standard (appropriate for measuring soybean oil) was used in order to calibrate and determine the relative fatty acid content of the experimental samples according to Mellinger (2012).

Results and Discussion

Improving the agronomic traits and performance of elite soybean cultivars is a common goal shared by soybean breeders, especially for new lines that possess a unique seed quality trait such as LP. In this study, the goal was to maintain the elite agronomic and seed quality traits of RP 5601T in selected BC_5 LP lines while at the same time adding the LP seed quality trait. The least square means averaged over both years, all environments and replications are reported.

There were no significant differences ($p>0.05$) in mean seed yield between all BC_5 LP lines (3945 kg ha^{-1}) and recurrent parent 5601T (4221 kg ha^{-1}), or the high yield check cultivars Ellis (4185 kg ha^{-1}) and Osage (4083 kg ha^{-1}). However, when contrasting the mean yield (3903 kg ha^{-1}) of the indeterminate BC_5 lines (56CX-1280, 56CX-1284, 56CX-1286 and 56CX-1287) to that of 5601T (4221 kg ha^{-1}), there were significant differences ($p<0.05$) which emphasized the need to break the linkage between the *Dt1* locus and *pha 002* locus on Gm 19 due to observed loss in yield. The yield means of determinate BC_5 LP lines 56CX-1273 (4107 kg ha^{-1}), 56CX-1274 (4131 kg ha^{-1}), 56CX-1277 (4111 kg ha^{-1}) and 56CX-1282 (4155 kg ha^{-1}) were not significantly different than RP 5601T but were numerically higher than the mean yield of current USDA MG V high yielding check cultivar Osage (4083 kg ha^{-1}), with 56CX-1282 recording the highest overall mean yield of any BC_5 LP line (Table 3.3). A contrast statement comparing the

yield means of the four determinate BC_5 LP lines 56CX-1273, 56CX-1274, 56CX-1277 and 56CX-1282 (4129 kg ha^{-1}) to that of BC_4 donor parent TN09-239 (3688 kg ha^{-1}) revealed significant differences ($p<0.01$) which justified having made the fifth backcross. The mean yield of 5601T (4221 kg ha^{-1}) was also significantly higher ($p<0.01$) than that of TN09-239 (3688 kg ha^{-1}). Highly significant differences ($p<0.0001$) were found for 100 seed weight between 5601T (13.2 g) and the mean of all BC_5 LP lines (15.8 g). It was observed that the 100 seed weight of the high yield checks 5601T, Ellis (12.0 g) and Osage (11.4 g) was significantly lower than the mean for all BC_5 lines. Moreover, two high yielding BC_5 lines, 56CX-1273 (15.0 g) and 56CX-1283 (15.4 g) also had significantly lower ($p<0.05$) 100 seed weights than some of the other BC_5 LP lines, so a regression procedure was used to see if seed weight could help to explain yield results. Regression results ($p<0.05$) determined that 100 seed weight explained very little (3.6%) of the variation in yield, indicating a weak positive relationship.

There were significant differences for height amongst the BC_5 LP lines, with the mean height (138.0 cm) of the indeterminate genotypes (56CX-1280, 56CX-1284, 56CX-1286 and 56CX-1287) being significantly different ($p<0.0001$) when contrasted against the mean height (87.8 cm) of the determinate genotypes (56CX-1273, 56CX-1274, 56CX-1275, 56CX-1276, 56CX-1277, 56CX-1279, 56CX-1282 and 56CX-1283). The mean height of donor parent TN09-239 (135.1 cm) was significantly higher ($p<0.001$) than that of the determinate RP 5601T (93.5 cm). There was no significant difference ($p>0.05$) between the height of TN09-239 and the mean height of the indeterminate BC_5 genotypes which suggests that TN09-239 is indeterminate (*Dt1*) in stem termination.

There were highly significant differences ($p<0.0001$) for lodging scores. The mean lodging score of the indeterminate BC_5 genotypes (3.5) was higher (worse) than that of the

determinate BC_5 genotypes (1.8). Furthermore the mean lodging score of the determinate genotypes (1.8) was also significantly better ($p<0.0001$) than RP 5601T (2.9) and donor parent TN09-239 (3.3). There was no significant difference ($p>0.05$) between the lodging scores of 5601T and TN09-239. A contrast statement also revealed that there was no significant difference ($p<0.05$) between the mean of the determinate BC_5 LP lines (1.8) and the mean of elite USDA check cultivars Ellis and Osage (1.5).

A regression procedure was used to see if height of the BC_5 LP lines could help explain the lodging scores, and the regression results were significant ($p<0.0001$). The R square value (0.5167) indicated that plant height could explain just over 50% of the variation in the lodging scores indicating a moderate positive relationship between plant height and lodging scores. A regression procedure was also used to see if the BC_5 LP mean lodging scores or plant height could help to explain seed yield, and regression results indicated that both lodging score ($p<0.0001$) and plant height ($p<0.01$) were significantly associated with yield. The R square values for lodging (0.1213) and for height (0.0597) each indicated a weak positive relationship with seed yield.

Highly significant differences ($p<0.001$) were found in relative maturity. For example, the mean of the indeterminate genotypes 56CX-1280, 56CX-1284, 56CX-1286 and 56CX-1287 (134.5 d) was significantly different ($p<0.0001$) when contrasted with that of the determinate genotypes 56CX-1273, 56CX-1274, 56CX-1275, 56CX-1276, 56CX-1277, 56CX-1279, 56CX-1282 and 56CX-1283 (133.7 d), indicating that the determinate BC_5 genotypes mature about a day earlier. The maturity for 5601T (134.9 d) was significantly later ($p<0.0001$) than the overall mean of the determinate genotypes (133.7 d) but was no different than that of the indeterminate

genotypes (134.5 d). The earliest maturing BC_5 LP lines were 56CX-1273 (133.3 d) and 56CX-1282 (133.3 d), which were two of the higher yielding determinate BC_5 lines.

There were no significant differences ($p>0.05$) in seedling field emergence between any of the BC_5 LP lines and 5601T, TN09-239, Ellis or Osage. 5601T (74%) did have the highest percentage of seedlings emerge in the plots which was slightly numerically higher than 56CX-1283 (73%), 56CX-1275 (72%), 56CX-1283 (73%), 56CX-1287 (71%) and 56CX-1282 (70%).

The results of the germination test conducted in 2014 from seeds harvested in 2013 also revealed no significant differences ($p>0.05$) between the germination rates for any of the BC_5 LP lines and 5601T, TN09-239, Ellis or Osage. The mean germination rate of all of the BC_5 LP lines (93%) was statistically equivalent to that of RP 5601T (96%), Osage (99%), Ellis (96%) and TN09-239 (97%). Pantalone (2012) also noted high germination (100%) in LP line TN09-239.

An important seed quality trait of 5601T is the high protein value which qualifies it as a 48% meal source. Maintaining seed protein levels of the BC_5 LP lines was an important consideration of this research. There were highly significant differences ($p<0.0001$) for seed protein content among the BC_5 LP lines: 56CX-1286 (429.0 g kg^{-1}) was significantly lower ($p<0.05$) than 56CX-1273 (441.7 g kg^{-1}) and 56CX-1274 (443.6 g kg^{-1}). Single degree of freedom contrasts between the mean seed protein content in RP 5601T (436 g kg^{-1}) and the mean seed protein content in the determinate BC_5 LP genotypes (439 g kg^{-1}) and between the indeterminate genotypes (433 g kg^{-1}) and RP 5601T (436 g kg^{-1}) were not significantly different ($p>0.05$). However, a single degree of freedom contrast between the seed protein content of the indeterminate BC_5 LP genotypes was significantly lower ($p<0.0001$) (433 g kg^{-1}) than the mean seed protein content of the determinate LP genotypes (439 g kg^{-1}). Protein and oil contents in soybean can fluctuate depending on various G × E interactions; typical values are approximately

400 g kg^{-1} protein and 200 g kg^{-1} oil (Wilson, 2004). It is well documented that soybean protein and oil also share an inverse relationship, where it is estimated that a 1% reduction in seed oil content will increase the seed protein content by 2% (Clemente and Calhoun, 2009). The seed oil concentration of the indeterminate genotypes (203 g kg^{-1}) was significantly higher ($p<0.0001$) than that of the determinate genotypes (200 g kg^{-1}). The seed oil concentration of RP 5601T (208 g kg^{-1}) was significantly higher ($p<0.0001$) than the seed oil concentration of both the determinate and indeterminate genotypes, yet was not significantly different ($p>0.05$) than that of 56CX-1286 (206 g kg^{-1}), 56CX-1276 (205 g kg^{-1}), 56CX-1274 (204 g kg^{-1}), 56CX-1280 (204 g kg^{-1}) and 56CX-1275 (203 g kg^{-1}).

When comparing the means of the five predominant fatty acids found in soybean oil between RP 5601T and all other BC_5 LP lines, every BC_5 line was significantly different ($p>0.05$) than 5601T for each fatty acid with a few exceptions. For palmitic acid, only 56CX-1274 (141 g kg^{-1}) and 56CX-1275 (140 g kg^{-1}) were not significantly different ($p>0.05$) than 5601T (134 g kg^{-1}). Every other BC_5 LP line displayed significantly higher ($p<0.05$) levels of palmitic acid. For stearic acid concentration, 56CX-1276 (39 g kg^{-1}), 56CX-1277 (39 g kg^{-1}), 56CX-1279 (39 g kg^{-1}), 56CX-1282 (39 g kg^{-1}), 56CX-1284 (40 g kg^{-1}), and 56CX 1286 (40 g kg^{-1}) were not significantly different ($p>0.05$) than 5601T (38 g kg^{-1}). All other BC_5 LP lines had significantly higher ($p<0.05$) levels of stearic acid. For oleic acid concentration, 56CX-1275 (359 g kg^{-1}) and 56CX-1284 (327 g kg^{-1}) were significantly higher ($p<0.05$) than 5601T (294 g kg^{-1}) but 56CX-1277 (280 g kg^{-1}) and 56CX-1282 (280 g kg^{-1}) had concentrations that were significantly lower ($p<0.05$). The oleic acid concentrations for the remaining BC_5 lines were not significantly different ($p>0.05$) than that of 5601T. For linoleic acid concentration, 56CX-1275 (395 g kg^{-1}) and 56CX-1284 (420 g kg^{-1}) were significantly lower ($p<0.05$) than RP

5601T (448 g kg^{-1}). For linolenic acid, only BC_5 LP lines 56CX-1276 (80 g kg^{-1}), 56CX-1277 (82 g kg^{-1}) and 56CX-1282 (81 g kg^{-1}) were not significantly different ($p>0.05$) than 5601T (85 g kg^{-1}). The rest of the BC5 LP lines had significantly lower ($p<0.05$) levels of linolenic acid with 56CX-1275 (65 g kg^{-1}) having the lowest. Two single degree of freedom contrasts between the mean of the determinate BC_5 genotypes and RP 5601T and indeterminate BC_5 genotypes and RP 5601T revealed significant differences ($p<0.05$) for palmitic, stearic, linoleic and linolenic acids. For oleic acid, the mean of the determinate BC5 genotypes (306 g kg^{-1}) was not significantly different than 5601T (294 g kg^{-1}) but the mean of the indeterminate genotypes (312 g kg^{-1}) was significantly higher ($p<0.05$).

Hulke et al. (2004) noted that breeding for desirable fatty acid levels and LP appeared to be problematic for targeted low saturated fat soybeans, because there may be modifiers for elevated saturated fat content in the CX 1834 donor that may be linked to the *pha* alleles or that one or both of the *pha* alleles that may have a pleiotropic effect on the fatty acid content. Fatty acid modifier QTL for all five fatty acids were detected on Gm19 (LG L) (Hyten et al. 2004). The modifier QTL were located within 26.5 cM (palmitic), 4.4 cM (stearic), 19.9 cM (oleic), 11.9 cM (linoleic), and 12.0 cM (linolenic) of the Gm19 *pha2* (Hyten et al., 2004). Furthermore Hulke et al. (2004) reported that to meet the FDA labeling regulations for low saturates, a product may have 1.25 g or less of total saturated fatty acids in a 14 g serving (89 g kg^{-1} of saturated fat), which can be rounded down to 1 g and the oil can be labeled as low in saturated fat. Soybean breeders consider lines to be low in total saturates when the sum of stearic and palmitic acid content is less than 70 g kg^{-1} (Pantalone, personal communication). The mean total saturate content of these 12 BC_5 LP lines for stearic acid (41 g kg^{-1}) and palmitic acid (144 g kg^{-}

^{1}) sums to 184 g kg^{-1} which is more than twice that amount and would fail to meet that low saturates cutoff.

For the monounsaturated and polyunsaturated fats, Hulke et al. (2004) found that the mean oleic acid content of the LP lines in their study was significantly greater than the normal phytate lines and that the linoleic acid and linolenic acid content were significantly lower for the LP lines. In our study, the BC_5 LP mean for seed oleic acid content (308 g kg^{-1}) was significantly higher than the mean of the normal phytate lines, which included 5601T, Ellis and Osage (291 g kg^{-1}). As for seed linoleic acid and seed linolenic acid content, the BC_5 mean for linoleic acid (432 g kg^{-1}) and linolenic acid (75 g kg^{-1}) was significantly lower than the mean of 5601T, Ellis and Osage (448 g kg^{-1} for linoleic acid and 83 g kg^{-1} linolenic acid) which supports similar results reported by Hulke et al. (2004).

The five essential amino acids most important to the livestock feed sector were evaluated in this study: cysteine, lysine, methionine, threonine and tryptophan. For the basis of this study, the quantified levels of amino acids were converted to units of crude protein, which are being reported in g kg^{-1}, which was the same conversion used by Fallen (2013). There were no significant differences ($p>0.05$) found between the overall BC_5 mean levels of crude protein per unit for cysteine (15.29 g kg^{-1}), lysine (14.19 g kg^{-1}), or tryptophan (9.94 g kg^{-1}) and the mean levels of crude protein of RP 5601T for cysteine (15.39 g kg^{-1}), lysine (14.32 g kg^{-1}) or tryptophan (9.96 g kg^{-1}). However, a single degree of freedom contrast revealed that the mean crude protein level per unit of tryptophan in the seed of the indeterminate genotypes (9.98 g kg^{-1}) was significantly higher ($p<0.05$) than the mean crude protein level per unit of tryptophan in the seed of the determinate BC5 LP genotypes (9.92 g kg^{-1}). Moreover, there were also significant differences ($p<0.05$) found in the crude protein levels per unit of methionine between the

determinate (61.81 g kg^{-1}) and indeterminate (61.91 g kg^{-1}) genotypes, between the overall BC5 mean (61.84 g kg^{-1}) and RP 5601T (61.67 g kg^{-1}), and between the indeterminate genotypes (61.91 g kg^{-1}) and RP 5601T (61.67 g kg^{-1}). Yet the mean crude protein level per unit of methionine in the determinate genotypes (61.81 g kg^{-1}) was not significantly different ($p<0.05$) than RP 5601T (61.67 g kg^{-1}). For the seed crude protein levels per unit of threonine, there were significant differences ($p<0.01$) detected between the overall BC5 mean (36.60 g kg^{-1}) and RP 5601T (36.77 g kg^{-1}), between the determinate genotypes (36.56 g kg^{-1}) and RP 5601T (36.77 g kg^{-1}), and between the determinate (36.56 g kg^{-1}) and indeterminate genotypes (36.67 g kg^{-1}). Although the overall seed protein content was significantly lower ($p<0.0001$) in the mean of the indeterminate genotypes (433 g kg^{-1}) when compared to the mean of the determinate genotypes (439 g kg^{-1}), the mean levels of methionine, threonine and tryptophan per unit of crude protein were actually significantly higher ($p<0.05$) for the indeterminate genotypes.

The fifth backcross successfully introgressed the LP trait into the elite agronomic genome of cultivar 5601T. The yield of four determinate $BC_5F_{3:5}$ lines, 56CX-1273 (4107 kg ha^{-1}), 56CX-1274 (4137 kg ha^{-1}), 56CX-1277 (4112 kg ha^{-1}) and 56CX-1282 (4160 kg ha^{-1}) was not significantly different ($p>0.05$) than that of 5601T (4227 kg ha^{-1}) and was numerically higher than the yield of current USDA Maturity Group V check Osage (4089 kg ha^{-1}) (Table 3.3). Furthermore there were no significant differences ($p>0.05$) found between 5601T and these four BC_5 LP lines for seedling emergence in the field or for germination % conducted in the greenhouse, which has been a documented concern for plant breeders in the development of LP cultivars. The lodging scores of 56CX-1273 (1.9), 56CX-1274 (1.3), 56CX-1277 (1.6) and 56CX-1282 (1.7) were significantly better ($p<0.05$) than 5601T (2.9), which was another indication of progress. Combining the LP trait with the high seed protein content of RP 5601T

would be a winning combination for soymeal to be used in the livestock sector, and the combined seed protein and oil content also qualify 56CX-1273 (50.6%), 56CX-1274 (51%), 56CX-1277 (49.8%) and 56CX-1282 (49.9%) as ultra-high protein meal candidates (Table 3.3). Results indicate that these four BC_5 LP lines have high seed yield, lodging resistance, and seedling field emergence and germination rates comparable to high yield check cultivars. Therefore, one of these BC_5 LP lines may well be considered for a cultivar or germplasm release.

References

Anderson, B. and W. Fehr. 2008. Seed source affects field emergence of low-phytate soybean lines. Crop Sci. 48**:** 929-932.

Anderson, R.J. 1912. Phytin and phosphoric acid esters of inosite. J.Biol. Chem. 43:117.

Bilyeu, K.D., Zeng, P., Coello, P., Zhang, Z.J., Krishnan, H.B., Bailey, A., Beuselinck, P.R., and Polacco, J.C. 2008. Quantitative Conversion of Phytate to Inorganic Phosphorus in Soybean Seeds Expressing a Bacterial Phytase. Plant Physiology. 146: 468-477.

Brinch-Pedersen, H., Sorensen, L.D., and Holm, P.B. 2002. Engineering crop plants: getting a handle on phosphate. Trends in Plant Science. 7:3.

Carpenter, S.R., Caraco, N.F., Correll, D.L., Howarth, R.W., Sharpley, A.N., and Smith, V.H. 1998. Nonpoint Pollution of Surface Waters With Phosphorus And Nitrogen. Ecological Applications. 8: 559-568.

Chen Jr. P, T. Toribara, and H. Warner. 1956. Microdetermination of P. Anal. Chem. 28(11): 1756–1758.

Cichy, Karen, and Victor Raboy. 2009. "Evaluation and Development of Low-Phytate Crops." Modification of Seed Composition to Promote Health and Nutrition. Ed. Hari Krishnan. Madison, WI: American Society of Agronomy, Crop Science Society of America, Soil Science Society of America. 177-200.

Clemente and Cahoon. 2009. Soybean Oil: Genetic Approaches for Modification of Functionality and Total Content. Plant Physiol. 151(3): 1030-1040.

Eberhart and Russell, 1966. Stability Parameters for Comparing Varieties. Crop Sci.6:36-41.

Erdman, J.W. 1979. Oilseed Phytates: Nutritional Implications. Department of Food Sciences. Urbana, Illinois.

Fehr, W.R. and Caviness, C.E. 1977. Soybean Stages of Development. Ames, IA. Cooperative Extension Service.

Gao, Y., Biyashev, M.A., Maroof, S., Glover, N.M., Tucker, D.M., and Buss, G.R. 2008. Validation of Low Phytate QTLs and Evaluation of Seedling Emergence of Low-Phytate Soybeans. Crop Science. 48: 1355-1364.

Gillen, A.M., and Shelton, G.W. 2013. USDA Uniform Soybean Tests, Southern States: p.14.

Gillman, Jason D., Vincent R. Pantalone, and Kristin Bilyeu. 2009. The low phytic acid phenotype in soybean line CX1834 is due to mutations in two homologs of the maize low phytic acid gene. Plant Genome. 2: 179-190.

Graf, E. and Eaton, J.W. 1990. Antioxidant Functions of Phytic Acid. Free Radical Biology and Medicine. 8:61-69.

Hart, E.B., and Tottingham, W.E. 1909. The Nature of the Acid Soluble Phosphorus Compounds of Some Important Feeding Materials. Agricultural Chemical Laboratory, Wisconsin Agricultural Experiment Station.

Hegeman, C.E., Good, L.L., and Grabau, E.A. 2001. Expression of d-myo-Inositol-3-Phosphate Synthase in Soybean. Implications for Phytic Acid Biosynthesis. Plant Physiology. 125: 1941-1948.

Hulke, B. S., Fehr, W.R. and Welke, G. A. 2004. Agronomic and Seed Characteristics of Soybean with Reduced Phytate and Palmitate. Crop Sci. 44: 2027-2031.

Jendza, J.A., and Adeola, O. 2009. Water-soluble phosphorus excretion in pigs fed diets supplemented with microbial phytase. Animal Science Journal. 80:296-304.

Joshi, T., Valliyodan, B., Wu, J., Lee, S., Xu, D., and Nguyen, H.T. 2013. Genomic Differences between cultivated soybean, *G. max* and its wild relative *G. soja*. From the Eleventh Asia Pacific Bioinformatics Conference. Vancouver, Canada.

Khalid, M.F., Hussain, M., Rehman, A.U., Shahzad, M.A., Sharif, M. and Rahman, Z.U. 2013. Broiler Performance in Response to Phytate and Supplemented Phtyase. Iranian Journal of Applied Science. 3:1-12.

Loewus, F.A. 2002. Biosynthesis of Phytate in Food Grains and Seeds. Food Phytates. Boca Raton, FL: CRC Press, Inc.

Lott, J.N.A., Ockenden, I., Raboy, V., and Batten, G.D. 2000. Phytic Acid and Phosphorus in crop seeds and fruits: a global estimate. Seed Science Research. 10:1: 11-33.

Maenz, D. D., Engele-Schaan, C.M., Newkirk, R.W., and Classen, H.L. 1999. The effect of minerals and mineral chelators on the formation of phytase-resistant and phytase-succeptible forms of phytic acid in solution and in a slurry of canola meal. Animal Feed Science and Technology. 81: 177-192.

Maroof, S.M., Glover, N.M., Biyashev, R.M., Buss, G.R. and Grabau, E.A. 2009. Genetic Basis of the Low-Phytate Trait in the Soybean Line CX1834. Crop Science. 49:69-76.

Mellinger, S., 2012. Molecular Marker Assisted Development and Stability Analysis of Low Phytate, High Inorganic Phosphorus Soybean [*Glycine max* (L.) Merrill]. University of Tennessee, (Trace).

Mishra, S.K. and Verma, V.D. 2010. The Soybean: Botany, Production and Uses. Cambridge, MA: CAB International.

Mullaney, E.J. and Ullah, A.H.J. 2005. Phytases: Attributes, Catalytic Mechanisms, and Applications. Commodity Utilization Research.

Oltmans, S. E., W. R. Fehr, G. A. Welke, and S. R. Cianzio. 2004. Inheritance of low-phytate phosphorus in soybean. Crop Sci. 44: 433-435.

Oltmans, S. E., W. R. Fehr, G. A. Welke, V. Raboy, and K. L. Peterson. 2005. Agronomic and seed traits of soybean lines with low-phytate phosphorus. Crop Sci. 45: 593-598.

Pantalone, V.R., F.L. Allen, and D. Landau-Ellis. 2003. 5601T. Crop Sci. 43:1123.

Powers, W. J., E.R. Fritz, W. Fehr, and R. Angel. 2006. Total and water-soluble phosphorus excretion from swine fed low-phytate soybeans. J. Animal Sci. 84: 1907-1915.

Prattley, C.A., Stanley, D.W., Smith, T.K., Van De Voort, F.R. 1982. Protein-Phytate Interactions in Soybean. Journal of Food Biochemistry. 6:4:273-282.

Raboy, V., Hudson, S.J., and Dickson, D.B. 1984. Reduced Phytic Acid Does Not Have an Adverse Effect on Germination of Soybean Seeds. Plant Physiology. 79: 323-325.

Raboy, Victor. 2002. Progress in breeding low phytate crops. Journal of Nutrition. 2002:503S-505S.

Raboy, V., D. B. Dickinson, and F. E. Below. 1984. Variation in seed total phosphorus, phytic acid, zinc, calcium, magnesium, and protein among lines of Glycine max and G. soja. Crop Sci. 24: 431-434.

Raboy, V. 2002. Impacts of Agriculture on Human Health and Nutrition. EOLSS Publishers, Ltd. Oxford, UK.

Reddy, N. R., M. D. Pierson, S. K. Sathe, and D. K. Salunkhe. 1989. Phytates in Cereals and Legumes. Boca Raton, FL: CRC Press, Inc.

SAS Institute. 2011. The SAS system for Windows. Release 9.2. SAS Inst., Cary, NC.

Sathe, S.K. and Reddy, N.R. 2002. Food Phytates. Boca Raton, FL. CRC Press.

Scaboo, A.M., V.R. Pantalone, D.R. Walker, H.R. Boerma, D.R. West, F.R. Walker, and C.E. Sams. 2009. Confirmation of molecular markers and agronomic traits associated with seed phytate content in two soybean RIL populations. Crop Sci. 49: 426-432.

Sharpley, A., B. Foy, and P. Withers. 2000. Practical and innovative measures for the control of agricultural phosphorus losses to water: and overview. J. Environ. Qual. 29.1:1-9.

Shi, J., Wang, H., Schellin, K., Li, B., Faller, M., Stoop, J.M., Meeley, R.B., Ertl, D.S., Ranch, J.P., and Glassman, K. 2007. Embryo-specific silencing of a transporter reduces phytic acid content of maize and soybean seeds. Nature Biotechnology. 25:8.

Shu, Q.Y. 2009. Induced Plant Mutations in the Genomics Era. Food and Agriculture Organization of the United Nations, Rome: 316-318.

Spear, J. D., and W. R. Fehr. 2007. Genetic improvement of seedling emergence of soybean lines with low phytate. Crop Sci. 47:1354-1360.

Tian, Z., Wang, X., Lee, R., Li, Y., Specht, J.E., Nelson, R.L., McClean, P.E., Qiu, L., and Ma, J. 2010. Artificial Selection for determinate growth habit in soybean. PNAS. 107:19:8563-8568.

Traylor, S.L., Cromwell, G.L., Lindemann, M.D., and Knabe, D.A. 2001. Effects of level of supplemental phytase on ileal digestibility of amino acids, calcium, and P in dehulled soybean meal for growing pigs. Animal Science. 79: 2634-2642.

United States Environmental Protection Agency. Ag 101: Major Crops Grown in the United States. Web 29 Aug 3013. <http://www.epa.gov/agriculture/ag101/cropmajor.html>

United States Department of Agriculture. National Agricultural Statistics Service. Crops and Plants. Field Crops. Beans. Web 29 Aug 2013. <www.nass.usda.gov>

"United States Soy Crop Statistics." Soy Stats 2012. The American Soybean Association , 2012. Web. 29 Aug 2013. <http://www.soystats.com/2012/Default-frames.htm>

Vogel, K.E. 2009. Methods in Molecular Biology: Transgenic Maize, vol. 526. Humana Press. 14: 161-169.

Walker, D.R., A.M. Scaboo, V.R. Pantalone, J.R. Wilcox, and H.R. Boerma. 2006. Genetic mapping of loci associated with seed phytic acid content in CX1834-1-2 soybean. Crop Sci. 46:390-397.

Wilcox, J. R., G. S. Premachandra, K. A. Young, and V. Raboy. 2000. Isolation of high seed inorganic P, low-phytate soybean mutants. Crop Sci. 40:1601-1605.

Wilson, R.F. Soybeans: Improvement, Production and Uses. 1987. Agronomy. 16: 643-686.

Yao, J.J. Wei, L.S. and Steinberg, M.P. 1983. Effect of maturity on chemical composition and storage stability of soybeans. J. Amer. Oil Chem. Soc. 60:1245-1249.

Appendix III

Table 3.1

Summary of the seed quality trait means of four promising BC_5 determinate (*dt1*) low phytate genotypes in comparison to recurrent parent (RP) 5601T.

BC_5 Line	Pi (µg Pi g-1)	Protein (g kg-1)	Oil (g kg-1)	Protein Meal (%)
56CX-1273	1915.4	441.7	200.2	50.6%
56CX-1274	2064.3	443.5	203.9	51%
56CX-1277	1676.1	434.1	202.3	49.8%
56CX-1282	1822.5	436.9	196.8	49.9%
5601T (rp)	221.2	435.8	207.6	50.3%
LSD (0.05)	364.9	†NS	4.7	†NS

†NS = no significant difference detected using Tukey's protected LSD at the 5% significance level.

Table 3.2

Comparison of the concentrations of fatty acids of the indeterminate (Dt1) BC_5 low phytate (LP) genotypes and the determinate (dt1) BC_5 LP genotypes revealed no significant differences (p>0.05).

LP Genotype	Palmitic Acid (g kg^{-1})	Stearic acid (g kg^{-1})	Oleic acid (g kg^{-1})	Linoleic acid (g kg^{-1})	Linolenic acid (g kg^{-1})
Dt1	143	41	312	429	75
dt1	144	41	306	433	76
$LSD_{(0.05)}$	†NS	†NS	†NS	†NS	†NS

†NS = no significant difference detected using Tukey's protected LSD at the 5% significance level.

Table 3.3

Summary of the agronomic trait means of four promising BC_5 determinate (*dt1*) low phytate lines in comparison to recurrent parent (RP) 5601T.

BC_5 Line	Yield (kg ha^{-1})	Lodging (score)	Height (cm)	Field Emergence (%)	Germination Test (%)	Maturity (d)
56CX-1273	4107	1.98	88.4	65.9	95	133.3
56CX-1274	4137	1.34	86.0	69.5	87	133.7
56CX-1277	4112	1.63	86.4	67.8	95	134.2
56CX-1282	4160	1.68	86.5	70.4	97	133.3
5601T (rp)	4227	2.90	93.5	73.9	96	134.9
$LSD_{(0.05)}$	†NS	1.04	†NS	†NS	†NS	1.2

†NS = no significant difference detected using Tukey's protected LSD at the 5% significance level.

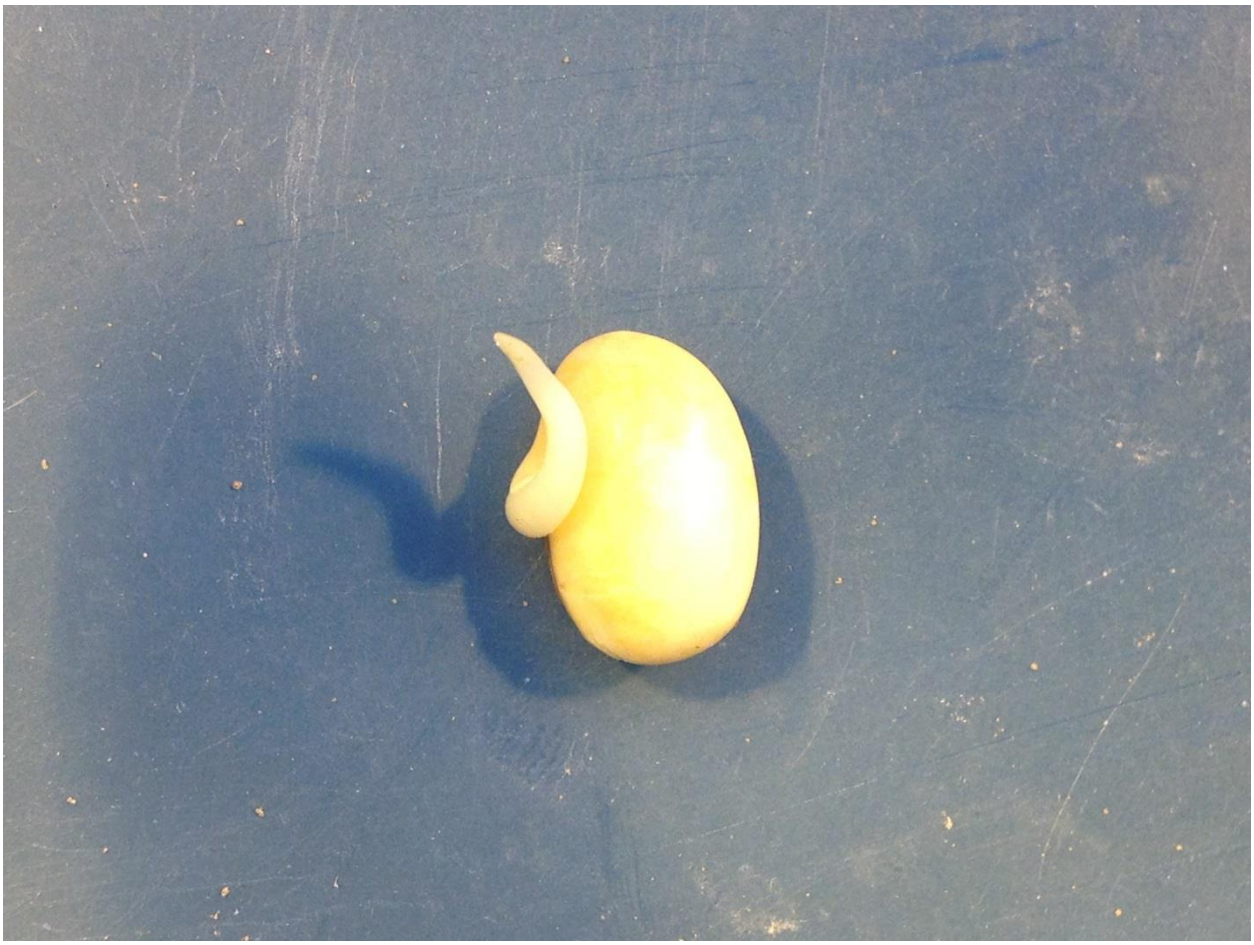


Figure 3.1

Seeds were scored as "germinated" in the 2014 germination test if the radicle (pictured) had emerged from the low phytate seed.

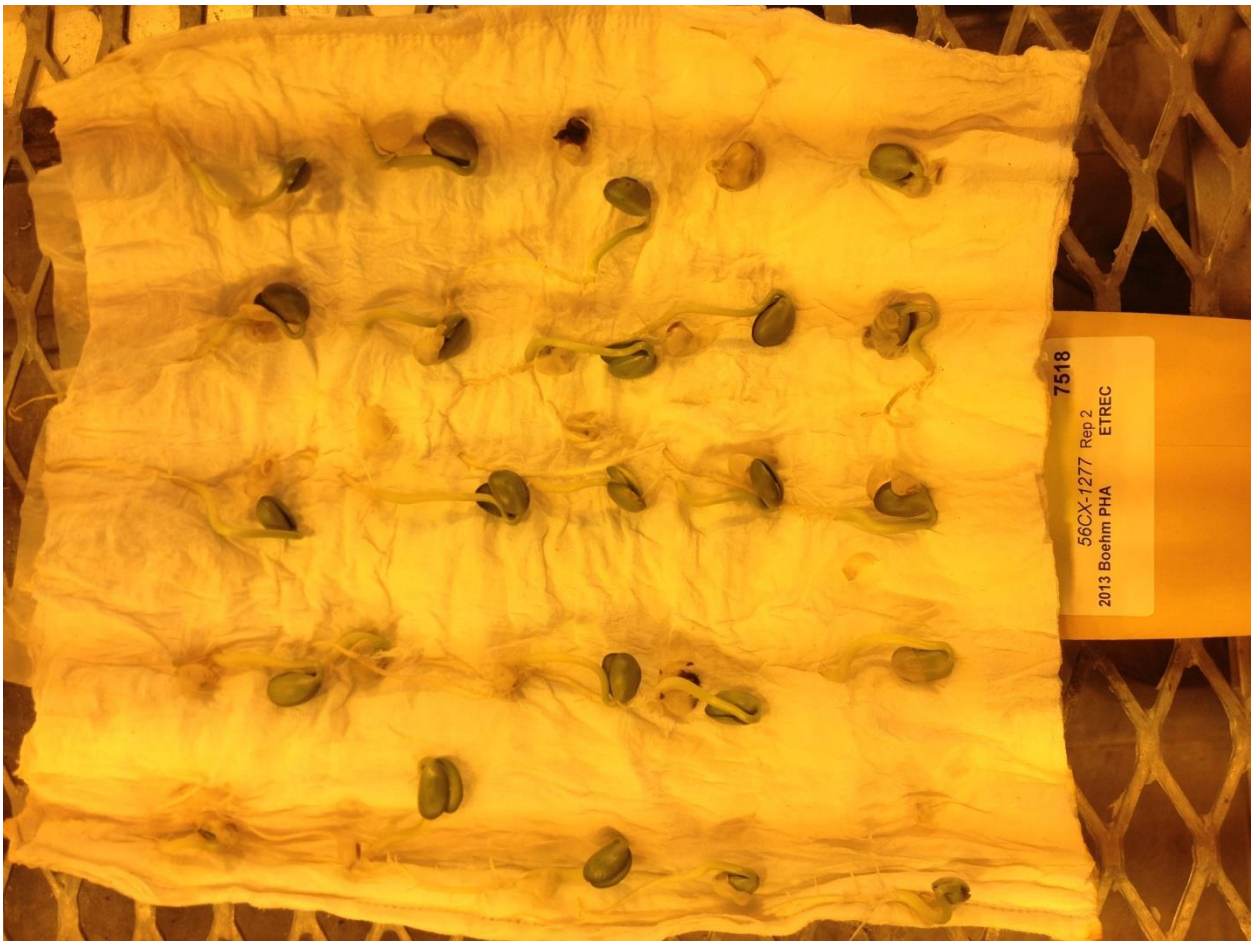


Figure 3.2

Unrolled "ragdoll" of 56CX-1277 displaying germinated seeds from the 2014 germination test conducted on low phytate seeds harvested in 2013.

Part 4

Agronomic Traits, Performance and Seed Inorganic Phosphorus Stability Evaluated Across Six Southern U.S. Environments for Two Low Phytate Lines and Two USDA MG V Check Cultivars

Abstract

Soybean [*Glycine max* (L). Merrill] is the world's top oilseed crop. High protein soymeal is a primary soybean use and staple food of livestock and fish. The storage form of phosphorus (P) in the seed is phytic acid, or phytate (myo-inositol 1,2,3,4,5,6 hexa*kis*phosphate) which binds and chelates many key nutritional cations forming stable salts that are non-digestible. Livestock waste laden with phytate P is an environmental concern, and a source of nonpoint P pollution causing accelerated eutrophication and surface water quality deterioration. The two LP soybean lines used in this study were two BC_5 derived lines from their recurrent parent '5601T', a high yielding University of Tennessee developed cultivar. The presence of the two LP loci in both BC_5 derived lines was confirmed using perfect SNP molecular markers near the confirmed QTL *cqPha-001* and *cqPha-002*. In 2013, a field trial using a randomized complete block design (RCBD) with two replications was grown at seven locations in four row plots in the United Soybean Board Quality Traits MG V test to evaluate agronomic performance, seed quality traits, and seed inorganic phosphorus stability of two BC_5 derived LP lines, 56CX-1273 and 56CX-1283, and two current USDA MG V high yielding check cultivars, 5002T and Ellis, developed by the University of Tennessee. A regression procedure was used to see if the seed Pi concentration for each genotype fit a linear equation. Each genotype was tested for the expected slope of zero and regression results were significant ($p<0.05$) and revealed the slopes of 56CX-1273 (2.04 µg g^{-1} unit change in Pi for each unit change in environmental index) and 56CX-1283 (1.94 µg g^{-1} unit change in Pi for each unit change in environmental index) were significantly different from zero ($p<0.0001$) whereas both 5002T and Osage fit that slope ($p>0.05$). The expression of Pi was not stable for the two BC_5 LP lines when grown across multiple southern U.S. environments but the quantified levels of Pi were about an order of magnitude greater and

significantly higher ($p<0.0001$) than the expression of the Pi for the 5002T and Osage. Data analyzed using SAS version 9.3 also revealed that the seed yields of 56CX-1273 (3886 kg ha^{-1}) and 56CX-1283 (3822 kg ha^{-1}) were not significantly different ($p>0.05$) than both 5002T (4212 kg ha^{-1}) and Osage (4360 kg ha^{-1}). This study confirmed that the two BC_5 LP lines showed significantly elevated Pi compared to the normal phytate check cultivars, regardless of location, and documents for the first time that a LP line can produce seed yields equivalent to high yielding check cultivars.

Introduction

Domestically in 2013, 34.32 million metric tons of soymeal was used in livestock feed for broiler chickens (*Gallus domesticus*, 12.95 million metric tons), hogs (*Sus domesticus*, 11.15 million metric tons), laying hens (*Gallus domesticus*, 2.28 million metric tons), turkeys (*Meleagris gallopavo*, 2.04 million metric tons), and combined other feed uses (5.90 million metric tons) (Soy Stats 2013). According to the United Soybean Board, the U.S. soybean industry is reliant on the annual demand strength coming from the U.S. animal agricultural sector.

Livestock producers use the high protein soymeal as a staple food but the animals also digest large amounts of phytate present in the soymeal (Raboy and Dickinson 1984; Erdman, 1979). Chemically, the structure of the phytate molecule acts as a binding or chelating agent to mineral cations forming salts that are non-digestible, which removes the mineral bioavailability from the seeds by forming the more complex phytate molecule (Erdman, 1979; Maenz et al., 1999). Many of the phytate mineral complexes that are formed are insoluble and therefore unavailable for absorption (Reddy, 1989). The bound cations, such as Ca, Fe, Mg and Zn, are nutritional minerals that become non-digestible insoluble phytate salts (Erdman, 1979; Raboy, 2002; Reddy, 1989). The interference with intestinal absorption of the mineral complexes, such as Ca, Fe, K, Mg, P and Zn, may lead to mineral deficiencies in animals and humans earning phytate the label of "anti-nutrient" (Reddy, 1989; Lott et al., 1995). The phytic acid is not broken down in the intestinal tract of monogastric animals because they possess little to no phytase activity in their digestive tracts, thus phytic acid is not efficiently utilized (Brinch-Pedersen et al., 2002). For the phytic acid to be better utilized, animal feeds must be supplemented with phytase to free cations and other nutrients bound by phytate P-complexes or

the animal feed may be supplemented with digestible inorganic phosphorus for the animals to receive the optimum amount of phosphorus needed for animal growth and skeletal development (Khalid et al., 2013; Shi, 2007).

Because animals lack the phytase enzyme in their digestive tracts necessary to break down the stable phytate molecule, Powers (2006) found that the excess P in the form of phytic acid in the animal waste is both insoluble and water soluble, the relative abundance of which varies with the dietary treatments (Brinch-Pedersen et al., 2002; Jendza, 2009). The non-digestible, stable phytate salts present in soybeans and soymeal are therefore excreted by animals that consume them (Erdman, 1979; Raboy, 2002; Raboy, 2009; Bilyeu et al., 2008; Gillman et al., 2009; Wilcox et al., 2000). This is especially a concern near concentrated animal production areas where the P runoff from livestock manure is often great. The excess P excretion then becomes a P management concern, because high levels of P in the form of phytic acid present in manure is often applied as fertilizer to agricultural lands as a common agricultural production practice (Hegeman et al., 2001). The P becomes mobile in watersheds and can cause accelerated eutrophication and surface water quality deterioration in freshwater ecosystems such as ponds, lakes and streams. Eutrophication resulting from algal blooms which is worsened by excess P pollution can be damaging to freshwater ecosystems as low levels of oxygen in the water depleted by decaying algae has been responsible for fish kills and hypoxic dead zones in many ecosystems. The P laden animal waste is a major point source (Carpenter, et al., 2010) and non-point source of P pollution which is detrimental to the environment (Sharpley et al. 1994, Carpenter et al., 2010, Walker et al., 2006). The creation of a high yielding, low phytate (LP) soybean cultivar to be used for soymeal would ease environmental concerns and provide other animal nutritional benefits.

First, lowering the phytate concentration in the seed would serve to enhance the dietary nutrition of soymeal and also increase the metabolic energy available to ruminant and non-ruminant livestock by increasing P bioavailability (Oltmans et al., 2004, Shu, 2009). The increased P bioavailability of LP soybean would allow non-ruminant livestock to digest the Pi present in the soymeal thereby removing the P from the waste stream and greatly reducing eutrophication in aquatic environments (Brinch-Pedersen, et al., 2002; Sharpley et al., 1994). The combined benefits would lead to more effective and sustainable P nutrient management on farms for crop and livestock production. In addition, livestock producers would also save costs relative to annual phytase amendments placed in soybean meal. In summary, modifying the composition of P in soybean seed without changing total P by lowering the phytate level and increasing the seed inorganic phosphorus (Pi) concentration would enable livestock producers to enhance animal nutrition and would sustainably lessen P pollution from animal agricultural waste runoff. These improvements have been the rationale behind creating a commercially acceptable LP cultivar.

However, creating a commercially acceptable LP cultivar has proven to be difficult as no high yielding LP cultivars exist to date despite the efforts of many plant breeders. Even though the total P content in the seed remains unchanged in LP lines, lowering the phytate and increasing the Pi content in the seeds has been suggested to lower the germination percentage of seeds, a result which is not commercially acceptable. This could be in part because phytate is used physiologically as a P store and an energy store (Reddy, 1989) supporting germination, early seedling growth and development (Reddy, 1989; Raboy, 2002). Phytate offers myo-inositol, macro-nutrients [P, potassium (K), and magnesium (Mg)] and micro-nutrients [calcium (Ca), iron (Fe), zinc (Zn), and manganese (Mn)] to growing seedlings and phytic acid synthesis

plays an integral role in the seed storage processes for minerals like K, Zn, Fe and Mg (Reddy, 1989; Raboy, 2002). Typically Pi is taken up by the plant in the form of phosphoric acid (H_3PO_4), then translocated to the developing seed and synthesized to become the storage form of P which is phytic acid (Raboy, 2002). Many factors can influence seed phytate levels, such as environmental fluctuations, growing locations, irrigation conditions, fertilizer applications, soil types, cultivar and planting time of year (Reddy, 1989). Plants typically produce more phytic acid than is needed for growth and development and reducing the phytic acid has not inhibited peak agronomic performance (Scaboo et al. 2009; Gillman et al. 2009; Bilyeu et al., 2008; Shi et al., 2007; Spear and Fehr 2007). The objectives of Part 4 were to:

1. Test the null hypothesis of no significant differences in measured values for seed yield, plant lodging, seed size, plant maturity, plant height, seed protein and oil content, seed amino acid concentration (cysteine, lysine, methionine, threonine and tryptophan) seed fatty acid concentration (palmitic, stearic, oleic, linoleic and linolenic) and seed Pi between two new BC_5 LP lines, 56CX-1273 and 56CX-1283, in comparison to two current MG V USDA high yielding check cultivars Osage and 5002T.
2. Evaluate the environmental stability of the LP trait in BC5 LP lines 56CX-1273 and 56CX-1283 when grown in six southern U.S. environments of the 2013 United Soybean Board (USB) MG V Quality Traits Test.

Materials and Methods

Development of BC_5 Low Phytate Lines 56CX-1273 and 56CX-1283

The backcrossing strategy and procedures used was documented in Part 2 of this thesis to develop the two BC_5 LP lines used in this study. Briefly, the presence of the LP alleles has been validated using perfect SNP molecular markers at loci *cqPha-001* (Gm03) and *cqPha-002* (Gm19) meaning that both lines in this study have been confirmed to be double homozygous recessive for the two alleles that express LP concentration in soybean seeds (Scaboo et al., 2009) (Figures 2.1 & 2.3).

Data Analysis and Field Experiment 2013

A randomized complete block design (RCBD) was used to evaluate four lines – two BC_5 LP lines 56CX-1273 and 56CX-1283 and two high yielding check cultivars 5002T and Osage as entries to the United Soybean Board (USB) Quality Traits (QT) MG V test, which was grown in two replications at the following locations: Knoxville, TN; Warsaw, VA; Blacksburg, VA; Keiser, AR; Portageville, MO (clay) and Portageville, MO (loam). Data were evaluated using a mixed model analysis of variance in SAS version 9.3 (Glimmix procedure, SAS Institute, Cary, NC) and least square means were compared using Tukey's protected LSD at the 5% significance level. The random factors in the model were location, the genotype by location (G × E) interaction and the replications nested within location. The fixed terms or treatments were the four lines. The dependent variables being evaluated were seed yield, plant height, plant lodging, plant maturity, seedling field emergence, seed fatty acid concentration (palmitic, stearic, oleic, linoleic and linolenic), seed amino acid concentration (cysteine, methionine, lysine, threonine

and tryptophan), seed Pi, seed weight, and seed protein and oil concentration. The following statistical model was employed:

$$Y_{ijk} = \mu + L_i + R(L)_{k(i)} + G_j + (GL)_{ji} + e_{ijk}$$

Where Y_{ijk} is the specific measured value of genotype *j* in rep *k* within location *i*; μ is the mean overall; L_i is the effect of location *i*; $(GL)_{ji}$ is the interaction between genotype *j* and location *i*, and Y_{ijk}'s error term is e_{ijk}.

Stability of Pi content was calculated using analysis of variance and least square means estimation, similar to the procedure described by Fallen et al. (2012) and by Mellinger (2012). In order to estimate the stability of Pi content across locations, the REG procedure was used in SAS v. 9.3 (SAS Institute, Cary, NC). The stability parameters for this method were the regression coefficient (b_i) and deviation from regression (sd_i) (Eberhart and Russell, 1966). The model used for estimating the linear regression was:

$$Y_{ij} = \mu + b_i I_j + \delta_{ij}$$

Where Y_{ij} is the mean of genotype *i* within location *j*; μ is the mean of genotype *i* over all locations; b_i is the regression coefficient that measures genotype *i*'s response to varying locations; *Ij* is the environmental index (defined as the mean deviation of all genotypes at a given location from the overall mean), and δ_{ij} is the deviation from the regression for genotype *i* in location *j*. Additionally, coefficients of determination (R^2) for the genotypes were estimated to

measure the percent of total variance explained by the regression model (Eberhart and Russell 1966).

Phenotypic Traits Observed

Plant maturities were noted when 95% of the pods showed their mature color. Days to maturity was computed by subtracting maturity date from planting date. Lodging score was assigned on a one to five scale at time of maturity, with one being a plot with erect plants and five being a plot whose plants were prostrate to the ground. The average plant height of all plants in the plot was recorded at maturity, and seed yield and moisture content were measured at harvest and adjusted to 13% moisture basis.

Field Emergence

For this study, the seedling emergence percentage was determined for all plots in 2013 by counting the number of emerged plants, dividing by the number of seeds planted in each plot, and multiplying the quotient by 100. Plants were counted at the V3 stage when there were three nodes on the main stem with fully developed leaves (Fehr and Caviness, 1977). The null hypothesis was tested to see if there were no significant differences for field emergence between 56CX-1273 and 56CX-1283 and the high yielding cultivars 5002T and Osage.

Procedure for Inorganic P (Pi) Assay

Inorganic phosphorous (Pi) concentrations were determined using a modified version of a colorimetric assay developed by Raboy et al. (2000) which was an adaption of the assay described by Chen et al. (1956) (Table A.3). Reactions were expected to range from nearly

clear for normal soybeans to dark blue for LP soybeans (Figure 2.9). The concentration of Pi was estimated from reflectance values using a Bio-Tek Powerwave XS microplate spectrophotometer plate reader (Bio-Tek, Winooski, VT) set at 882#nm with the reflectance readings of samples compared to those of a standards table (Figure 2.10). In 2013, the Pi assay was conducted on three samples per location per replication to quantify the seed Pi. Images of the results were taken using a Canon EOS Rebel T3i (Canon, Melville, NY) digital SLR camera.

Near Infrared Reflectance (NIR) Spectroscopy analysis of Protein and Oil Content

Approximately 25 g of harvested soybean seeds from this study was ground in a water-cooled (25°C) Knifetec 1095 Sample Mill (FOSS Tecator, S-26321, Hogana, Sweden) for 20 seconds. This setting produced ground soybean seed powder with uniformity of particle size. Ground samples were then labeled with barcodes provided by the University of Minnesota's ZM 400 Barcode Printer (Zebra Technologies, Lincolnshire, IL) and sent to the University of Minnesota's NIR lab for analysis. The ground samples (12.5g per sample) were then analyzed using a Perten DA 7250 Near Infrared Analyzer (Perten Instruments, Springfield, IL) capable of 900-1650 nm wavelength readings. The Minnesota research lab used a climate controlled environment and a JET (Jet Tools, LaVergne, TN) air filtration system. Seed samples from all harvested replications and locations were analyzed for protein and oil concentration, and for the five essential amino acids critical to the poultry (*Gallus domesticus*) and swine (*Sus domesticus*) livestock sector (methionine, cysteine, lysine, threonine and tryptophan). NIR analyses of protein and oil concentration and amino acid composition were used to test whether seeds produced by each of the LP lines differed significantly from each other or from high yield check cultivars 5002T and Osage for those traits.

Gas Chromatography

A Hewlett Packard 6890 series Agilent Gas Chromatograph (Palo Alto, CA) was used to measure the five predominate fatty acids found in soybean oil which are palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3). A sample of five seeds harvested from each plot was crushed using a metal cylinder, hammer and metal plate. The same protocols were used as described by Mellinger (2012) as the sample was placed into a test tube and 3 mL of an 8:5:2 (v/v/v) ratio of chloroform:hexane:methanol solvent was added. The samples were then capped and allowed to sit overnight. After extraction was completed, 100 µL of the extract from each sample was transferred to its respective vial. Seventy-five µL of methylation reagent [8:4:2 (v/v/v) ratio of petroleum ether:ethyl ether: sodium methoxide/methanol] and 0.75 mL of hexane was added to each vial before it was capped with a crimper. The fatty acid compositions were determined by a Hewlett Packard HP 6890 series gas chromatograph (Palo Alto, CA) system set using a model 7683 auto sampler and a model 7673 flame ionization detector, and an immobilized 30 mm x 0.53 mm inner diameter Alltech AT-Silar capillary column with 0.5 µm fused stationery phase. Analysis was conducted under the following operating conditions: carrier, helium (20 ml/min); 20:1(v/v) split injection; injection temperature 250 °C; detector temperature 275 °C, and column temperature 230 °C. The RM-1 (Supelco, Bellefonte, PA) standard (appropriate for measuring soybean oil) was used in order to calibrate and determine the relative fatty acid concentration in the lipid of the experimental samples.

Results and Discussion

Improving the agronomic traits and performance of elite soybean cultivars is a common goal shared by soybean breeders. That goal can become challenging, especially for developing new lines that possess a unique seed quality trait such as LP. No LP cultivars exist to date despite the efforts of many plant breeders. When two new promising determinate BC_5 LP lines, 56CX-1273 and 56CX-1283, were evaluated in comparison to current USDA MG V checks for agronomic traits in the 2013 USB MG V QT test, there were no significant differences ($p>0.05$) in seed yield when comparing the means of 56CX-1273 (3886 kg ha^{-1}), 56CX-1283 (3822 kg ha^{-1}), Osage (4360 kg ha^{-1}) and 5002T (4212 kg ha^{-1}). There were also no significant differences ($p>0.05$) found for lodging between the LP lines and the high yielding cultivars. Significant differences ($p<0.05$) did exist for height, as 56CX-1283 (88.9 cm) and 56CX-1273 (81.3 cm) were taller than Osage (72.6 cm) and 5002T (67.3 cm).

Spear and Fehr (2007) and Raboy (2009) have reported that backcrossing seems to be successful for developing LP lines. Despite the initial study of Raboy et al. (1984), poor germination has been reported for many LP lines, so it was thought that modifying the phytic acid reserves in the seed could negatively affect early seed metabolic processes such as germination and early seedling growth (Raboy 2009). Gao et al. (2008) found no significant differences between LP and normal phytate lines for field emergence but agreed with Spear and Fehr (2007) that backcrossing could continue to improve seedling emergence of LP lines derived from CX 1834. Anderson and Fehr (2008) reported that the growing environment of the seed source can affect the germination performance of LP cultivars. For example, seed stock produced in Puerto Rico showed significantly lower germination than seed stock of the same genotype produced in Iowa. Regardless, rating the germination performance of LP cultivars remains a

priority. There were no significant differences ($p>0.05$) when contrasting the means of the BC_5 LP lines (67.0) and the means of the high yielding cultivars (66.4). However, significant differences were found for field emergence, as the emergence rate of 5002T (70.9) was significantly higher ($p<0.05$) than Osage (61.9), but not significantly higher than 56CX-1283 (69.5) and 56CX-1273 (64.6). Significant differences ($p<0.05$) were found for plant maturities as 5002T (133 d) was significantly earlier ($p<0.05$) to maturity compared with 56CX-1283 (139 d) and Osage (141 d), but not significantly earlier ($p>0.05$) than 56CX-1273 (137 d). Significant differences ($p<0.0001$) were observed for 100 seed weights, as the 100 seed weight of Osage (13.4 g) was significantly less than that of 5002T (16.0 g), 56CX-1273 (16.7 g) and 56CX-1283 (16.9 g). A summary of the comparison of agronomic traits is presented in Table 4.1.

Because the recurrent parent (RP) of 56CX-1273 and 56CX-1283 (5601T) has a favorable combination of seed protein and oil concentration that is sufficient to produce high protein meal (>47.5% protein in soymeal, after processor oil extraction), it was desirable RP choice to develop a LP line. There were significant differences for seed protein concentration ($p<0.0001$) and seed oil concentration ($p<0.0001$) between the two LP BC_5 lines and high yield check cultivars. For seed protein concentration, Osage had the highest seed protein (451 g kg^{-1}), which was not significantly different ($p>0.05$) than 56CX-1273 (445 g kg^{-1}) or 56CX-1283 (444 g kg^{-1}) but was significantly higher than 5002T (423 g kg^{-1}). Osage is noted for its higher protein concentration (Chen, 2007) and it is notable that our two new BC_5 LP lines also have protein concentration that is not significantly different ($p>0.05$) than Osage. For seed oil concentration, 5002T had the highest seed oil concentration (210 g Kg^{-1}), which was significantly higher than Osage (198 g kg^{-1}), 56CX-1273 (194 g kg^{-1}), and 56CX-1283 (193 g kg^{-1}). The protein and oil concentration for each of the lines was used to calculate soybean meal quality. High protein

soymeal (>47.5% meal) or ultra-high soymeal (>49.5% meal) is desired for feed in the livestock sector (Table 4.2). Adding the LP trait to high quality soymeal would make it a desirable commodity for livestock producers. Both 56CX-1273 (50.6% meal) and 56CX-1283 (50.4% meal) meet the ultra-high protein meal standards used by the United Soybean Board to assess soymeal quality. A summary of seed protein, oil, inorganic phosphorus and the protein meal is presented in Table 4.2.

For the basis of this study, the quantified levels of amino acids being evaluated (cysteine, lysine, methionine, threonine and tryptophan) were converted to units of crude protein, which are being reported in g kg^{-1}, which was the same conversion used by Fallen (2013). No significant differences ($p>0.05$) were detected in amino acid composition per units of crude protein between the two BC_5 LP lines and Osage for cysteine, lysine, methionine, threonine or tryptophan. However there were significant differences ($p<0.05$) detected for all five amino acids per units of crude protein between 56CX-1273, 56CX-1283, Osage and 5002T, as 5002T had significantly lower levels of all five amino acids being evaluated. The mean seed amino acid content per unit of crude protein for each entry is displayed in Table 4.3.

Significant differences ($p<0.0001$) were found to exist between the high yielding cultivars and the BC_5 LP lines for saturated fats, monounsaturated fats, and polyunsaturated fats. For the saturated fats, the palmitic acid content of 56CX-1273 (140.3 g kg^{-1}) and 56CX-1283 (143.3 g kg^{-1}) was significantly higher ($p<0.0001$) than that of 5002T (120.3 g kg^{-1}) or Osage (132.7 g kg^{-1}). Significant differences ($p<0.0001$) were also found for stearic acid, but the mean level of 56CX-1273 (43.4 g kg^{-1}) was lower than the mean level of Osage (44.6 g kg^{-1}), 5002T (48.1 g kg^{-1}) and 56CX-1283 (47.5 g kg^{-1}). Hulke (2004) noted that breeding for desirable fatty acid levels and LP appeared to be problematic for targeted low saturated fat soybeans, because

there may be modifiers for elevated saturated fat content in the CX 1834 donor that may be linked to the *pha* alleles or that one or both of the *pha* alleles may have a pleiotropic effect on the fatty acid content. Fatty acid modifier QTL for all five fatty acids were detected on Gm19 (LG L) (Hyten et al., 2004). The modifier QTL (Hyten et al., 2004) were located within 26.5 cM (palmitic), 4.4 cM (stearic), 19.9 cM (oleic), 11.9 cM (linoleic), and 12.0 cM (linolenic) of the Gm19 *pha2*. Furthermore Hulke et al. (2004) reported that to meet the FDA labeling regulations for low saturates, a product may have 1.25 g or less of total saturated fatty acids in a 14 g serving (89 g kg^{-1} of saturated fat), which can be rounded down to 1 g and the oil can be labeled as low in saturated fat. Data from our study indicate that none of the LP lines or cultivars would meet the 7% low saturate standard. Hulke et al. (2004) also found that the mean oleate content of the LP lines in their study was also significantly greater than the normal phytate lines and that the linoleate and linolenate content of the LP lines were significantly lower. In this study, the mean oleic content of 56CX-1273 (313.8 g kg^{-1}) and 56CX-1283 (293.6 g kg^{-1}) was not significantly different ($p>0.05$) than Osage (311.0 g kg^{-1}) but was significantly lower ($p<0.05$) than 5002T (374.7 g kg^{-1}). The mean linoleic content of 56CX-1273 (426.4 g kg^{-1}) and 56CX-1283 (436.6 g kg^{-1}) was not significantly different ($p<0.05$) than Osage (428.9 g kg^{-1}) but was significantly higher than 5002T (387.6 g kg^{-1}). The mean linolenic acid content of 5002T (69.1 g kg^{-1}) was significantly lower ($p<0.05$) than 56CX-1273 (76.0 g kg^{-1}), which was significantly lower than 56CX-1283 (78.8 g kg^{-1}) and Osage (82.7 g kg^{-1}). The fatty acids are summarized for all four entries in Table 4.4.

A primary focus of this study was to evaluate the stability of the LP trait when LP lines were grown across multiple southern U.S. environments. As expected, Pi assays conducted on the LP lines and the high yielding cultivars revealed significant differences ($p<0.0001$) for mean

seed Pi. The means for 56CX-1273 (2084.7 µg Pi g^{-1}) and 56CX-1283 (1744.4 µg Pi g^{-1}) were about an order of magnitude greater than Osage (185.7 µg Pi g^{-1}) or 5002T (228.0 µg Pi g^{-1}) (Figure 4.1).

When concentration of Pi was compared between the individual environments, the quantified levels of Pi were not stable and varied greatly for 56CX-1273 and 56CX-1283. For 56CX-1273, the highest amount of Pi quantified was in Blacksburg, VA (2509 µg Pi g^{-1}) and the lowest level of Pi was found at Keiser, AR (1529 µg Pi g^{-1}). For 56CX-1283, the highest level of Pi quantified was in Warsaw, VA (2198 µg Pi g^{-1}) and the lowest level Pi was found at Keiser, AR (1251 µg Pi g^{-1}). However, the relative difference in expression of Pi when comparing only the two LP lines was fairly constant over the six environments as the slopes of the lines of 56CX-1273 and 56CX-1283 were nearly parallel (Figure 4.2). When comparing the LP lines to the normal phytate lines for the expression of Pi in the worst environment for Pi expression (Keiser, AR), the difference in expression of Pi for 56CX-1283 (1251 µg Pi g^{-1}) was significantly greater ($p<0.0001$) than 5002T (208 µg Pi g^{-1}). Regression was used to test if the Pi concentration for each genotype fit a linear equation (Figure 4.2). There was no change in the rank of the genotypes for Pi across environments between LP lines or cultivars. Breeders would have no difficulty recognizing a LP line versus a cultivar for Pi regardless of environment, however the magnitude of the differences in response of LP vs normal varied across environments. The stability parameters are summarized in Table 4.5. While each genotype was tested for the hypothesized slope of zero, both 5002T and Osage fit that slope ($p>0.05$) but neither 56CX-1273 nor 56CX-1283 had slopes that were significantly different from zero ($p<0.0001$). The means for each genotype, the equations explaining their linear trend line and the environmental index for each genotype are presented in Table 4.5. The slopes of 56CX-1273 (2.04 µg Pi g^{-1}unit change

for each unit change in environmental index) and 56CX-1283 (1.94 µg Pi g^{-1}unit change for each unit change in environmental index) indicate that both genotypes were not stable for the LP trait across the six southern environments. However, many factors can influence seed phytate levels, such as environmental fluctuations, growing locations, irrigation conditions, fertilizer applications, soil types, growing conditions, cultivar and time of year the plant is grown (Reddy 1989). The various southern testing locations varied for environmental factors which likely influenced the expression of the LP trait and the G × E interaction of the Pi in 56CX-1273 and 56CX-1283.

Despite environmental influences, this study showed that two new LP lines clearly expressed significantly elevated seed Pi compared to the normal check cultivars, regardless of growing location. Moreover, this study documents (for the first time) that new LP lines 56CX-1273 and 56CX-1283 produce seed yields equivalent to check cultivars. That ultra-high protein soymeal (>49.5%) can be produced from either of these new high yielding LP lines warrants consideration for releasing one as a cultivar.

References

Anderson, B. and W. Fehr. 2008. Seed source affects field emergence of low-phytate soybean lines. Crop Sci. 48**:** 929-932.

Bilyeu, K.D., Zeng, P., Coello, P., Zhang, Z.J., Krishnan, H.B., Bailey, A., Beuselinck, P.R., and Polacco, J.C. 2008. Quantitative Conversion of Phytate to Inorganic Phosphorus in Soybean Seeds Expressing a Bacterial Phytase. Plant Physiology. 146: 468-477.

Brinch-Pedersen, H., Sorensen, L.D., and Holm, P.B. 2002. Engineering crop plants: getting a handle on phosphate. Trends in Plant Science. 7:3.

Carpenter, S.R., Caraco, N.F., Correll, D.L., Howarth, R.W., Sharpley, A.N., and Smith, V.H. Nonpoint Pollution of Surface Waters With Phosphorus And Nitrogen. 1998. Ecological Applications. 8: 559-568.

Chen Jr. P, T. Toribara, and H. Warner. 1956. Microdetermination of P. Anal. Chem. 28(11): 1756–1758.

Cichy, Karen, and Victor Raboy. "Evaluation and Development of Low-Phytate Crops." 2009. Modification of Seed Composition to Promote Health and Nutrition. Ed. Hari Krishnan. Madison, WI: American Society of Agronomy, Crop Science Society of America, Soil Science Society of America. 177-200.

Eberhart and Russell, 1966. Stability Parameters for Comparing Varieties. Crop Sci.6:36-41.

Erdman, J.W. 1979. Oilseed Phytates: Nutritional Implications. Department of Food Sciences. Urbana, Illinois.

Fallen, B. D., Hatcher, C.N., Allen, F.L., Kopsell, D.A., Saxton, A.M., Chen, P., Kantartzi, S.K., Cregan, P.B., Hyten, D.L., and Pantalone, V.R. 2013. Soybean Seed Amino Acid

Content QTL Detected Using the Universal Soy Linkage Panel 1.0 with 1,536 SNPs. Journal of Plant Genome Science (3): 68-79.

Fehr, W.R. and Caviness, C.E. 1977. Soybean Stages of Development. Ames, IA. Cooperative Extension Service.

Gao, Y., Biyashev, M.A., Maroof, S., Glover, N.M., Tucker, D.M., and Buss, G.R. 2008. Validation of Low Phytate QTLs and Evaluation of Seedling Emergence of Low-Phytate Soybeans. Crop Science. 48: 1355-1364.

Gillman, Jason D., Vincent R. Pantalone, and Kristin Bilyeu. 2009. The low phytic acid phenotype in soybean line CX1834 is due to mutations in two homologs of the maize low phytic acid gene. Plant Genome. 2: 179-190.

Hegeman, C.E., Good, L.L., and Grabau, E.A. 2001. Expression of d-myo-Inositol-3-Phosphate Synthase in Soybean. Implications for Phytic Acid Biosynthesis. Plant Physiology. 125: 1941-1948.

Hulke, B. S., Fehr, W.R. and Welke, G. A. 2004. Agronomic and Seed Characteristics of Soybean with Reduced Phytate and Palmitate. Crop Sci. 44: 2027-2031.

Jendza, J.A., and Adeola, O. 2009. Water-soluble phosphorus excretion in pigs fed diets supplemented with microbial phytase. Animal Science Journal. 80:296-304.

Khalid, M.F., Hussain, M., Rehman, A.U., Shahzad, M.A., Sharif, M. and Rahman, Z.U. 2013. Broiler Performance in Response to Phytate and Supplemented Phtyase. Iranian Journal of Applied Science. 3:1-12.

Lott, J.N.A., Ockenden, I., Raboy, V., and Batten, G.D. 2000. Phytic Acid and Phosphorus in crop seeds and fruits: a global estimate. Seed Science Research. 10:1: 11-33.

Maenz, D. D., Engele-Schaan, C.M., Newkirk, R.W., and Classen, H.L. 1999. The effect of minerals and mineral chelators on the formation of phytase-resistant and phytase-succeptible forms of phytic acid in solution and in a slurry of canola meal. Animal Feed Science and Technology. 81: 177-192.

Mellinger, S., 2012. Molecular Marker Assisted Development and Stability Analysis of Low Phytate, High Inorganic Phosphorus Soybean [*Glycine max* (L.) Merrill]. University of Tennessee, (Trace).

Oltmans, S. E., W. R. Fehr, G. A. Welke, and S. R. Cianzio. 2004. Inheritance of low-phytate phosphorus in soybean. Crop Sci. 44: 433-435.

Raboy, V., Hudson, S.J., and Dickson, D.B. 1984. Reduced Phytic Acid Does Not Have an Adverse Effect on Germination of Soybean Seeds. Plant Physiology. 79: 323-325.

Raboy, Victor. 2002. Progress in breeding low phytate crops. Journal of Nutrition. 2002:503S-505S.

Raboy, V., D. B. Dickinson, and F. E. Below. 1984. Variation in seed total phosphorus, phytic acid, zinc, calcium, magnesium, and protein among lines of Glycine max and G. soja. Crop Sci. 24: 431-434.

Raboy, V. 2002. Impacts of Agriculture on Human Health and Nutrition. EOLSS Publishers, Ltd. Oxford, UK.

Reddy, N. R., M. D. Pierson, S. K. Sathe, and D. K. Salunkhe. 1989. Phytates in Cereals and Legumes. Boca Raton, FL: CRC Press, Inc.

SAS Institute. 2011. The SAS system for Windows. Release 9.3. SAS Inst., Cary, NC.

Scaboo, A.M., V.R. Pantalone, D.R. Walker, H.R. Boerma, D.R. West, F.R. Walker, and C.E. Sams. 2009. Confirmation of molecular markers and agronomic traits associated with seed phytate content in two soybean RIL populations. Crop Sci. 49: 426-432.

Sharpley, A., B. Foy, and P. Withers. 2000. Practical and innovative measures for the control of agricultural phosphorus losses to water: and overview. J. Environ. Qual. 29.1:1-9.

Shi, J., Wang, H., Schellin, K., Li, B., Faller, M., Stoop, J.M., Meeley, R.B., Ertl, D.S., Ranch, J.P., and Glassman, K. 2007. Embryo-specific silencing of a transporter reduces phytic acid content of maize and soybean seeds. Nature Biotechnology. 25:8.

Shu, Q.Y. 2009. Induced Plant Mutations in the Genomics Era. Food and Agriculture Organization of the United Nations, Rome: 316-318.

Spear, J. D., and W. R. Fehr. 2007. Genetic improvement of seedling emergence of soybean lines with low phytate. Crop Sci. 47:1354-1360.

"United States Soy Crop Statistics." Soy Stats 2013. The American Soybean Association , 2012. Web. 29 Aug 2013. <http://www.soystats.com/2012/Default-frames.htm>

Walker, D.R., A.M. Scaboo, V.R. Pantalone, J.R. Wilcox, and H.R. Boerma. 2006. Genetic mapping of loci associated with seed phytic acid content in CX1834-1-2 soybean. Crop Sci. 46:390-397.

Wilcox, J. R., G. S. Premachandra, K. A. Young, and V. Raboy. 2000. Isolation of high seed inorganic P, low-phytate soybean mutants. Crop Sci. 40:1601-1605.

Appendix IV

Table 4.1

Summary of Agronomic Traits of 56CX-1273 and 56CX-1283 in comparison to cultivars Osage and 5002T grown in six environments in the 2013 United Soybean Board MG V Quality Traits Test.

Genotype	Yield (kg ha^{-1})	Lodging (score)	Height (cm)	Emergence (%)	Maturity (d)	Seed Wgt. (g)
56CX-1273	3886	1.6	81	64.6	137	16.68
56CX-1283	3822	1.9	89	69.5	139	16.88
5002T	4212	2.2	67	70.9	133	16.03
Osage	4360	1.5	73	61.8	141	13.41
LSD (0.05)	612	0.8	14	8.5	5	1.2

Table 4.2

Seed protein, seed oil and seed Pi concentration and soymeal protein of two BC_5 low phytate soybean lines 56CX-1273 and 56CX-1283 in comparison to two high yield check cultivars Osage and 5002T grown in six southern environments of the 2013 United Soybean Board MG V Quality Traits Test.

Line	Protein ($g\ kg^{-1}$)	Oil ($g\ kg^{-1}$)	Meal ($g\ kg^{-1}$)	Pi ($\mu g\ g^{-1}$)
56CX-1273	444.8	194.2	506	2085
56CX-1283	444	192.6	504	1744
5002T	423.2	210.3	490	228
Osage	450.9	198.4	515	186
LSD (0.05)	8.3	5.8	n/a	351

Table 4.3

Seed amino acid concentration per unit of crude protein for two low phytate soybean BC_5 lines 56CX-1273 and 56CX-1283 in comparison to two USDA high yield check cultivars Osage and 5002T from the 2013 United Soybean Board MG V Quality Traits Test.

Genotype	Cysteine (g kg-1)	Lysine (g kg-1)	Methionine (g kg-1)	Threonine (g kg-1)	Tryptophan (g kg-1)
56CX-1273	34.4	22.5	32.4	83.7	141.7
56CX-1283	35.0	22.6	33.1	84.3	142.7
Osage	36.2	22.5	33.2	83.2	140.3
5002T	32.0	19.9	29.9	74.7	125.5
LSD (0.05)	2.2	0.8	1.4	3.0	5.5

Table 4.4

Fatty Acid content of Low Phytate soybean lines 56CX-1273 and 56CX-1283 in comparison to cultivars 5002T and Osage grown in the 2013 Quality Traits Test.

Fatty Acid	Palmitic Acid ($g\ kg^{-1}$)	Stearic Acid ($g\ kg^{-1}$)	Oleic Acid ($g\ kg^{-1}$)	Linoleic Acid ($g\ kg^{-1}$)	Linolenic Acid ($g\ kg^{-1}$)
5002T	120.3	48.1	374.7	387.5	69.1
56CX-1273	140.3	43.4	313.8	426.4	76.0
56CX-1283	143.3	47.5	293.6	436.6	78.8
Osage	132.7	44.6	311.0	428.9	82.6
LSD (0.05)	5.2	2.5	26.3	17.8	5.9

Table 4.5

Stability Parameters for inorganic phosphorus of two low phytate lines and two high yield check cultivars grown across six Southern U.S. environments in the 2013 United Soybean Board MG V Quality Traits Test.

Genotype	Inorganic Phosphorus Content (µg Pi g^{-1})	Stability Parameters				Slope Different than Zero	Slope Different than One
		CV†	b_i	sd_i	R^2	P value	P value
56CX-1273	2084.7	1.8	2.0	37.8	0.99	<0.0001	<0.0003
56CX-1283	1744.4	2.4	1.9	42.4	0.99	<0.0001	<0.0007
Osage	185.7	9.5	0.0	17.6	0.06	>0.05	<0.0001
5002T	228.0	6.2	0.0	14.2	0.12	>0.05	<0.0001

CV = coefficient of variability is calculated by multiplying the standard deviation by 100 then dividing by the mean and measures the amount of variability in the data relative to the mean.
bi = regression coefficient that measures the slope response of genotype i to varying environments.
sdi = a genotype's deviation from the regression line.
R^2 = coefficient of determination, determined for each genotype to measure the amount of the total variance explained by the regression model.
†CV is calculated for individual genotype mean (Eberhart and Russell 1966).

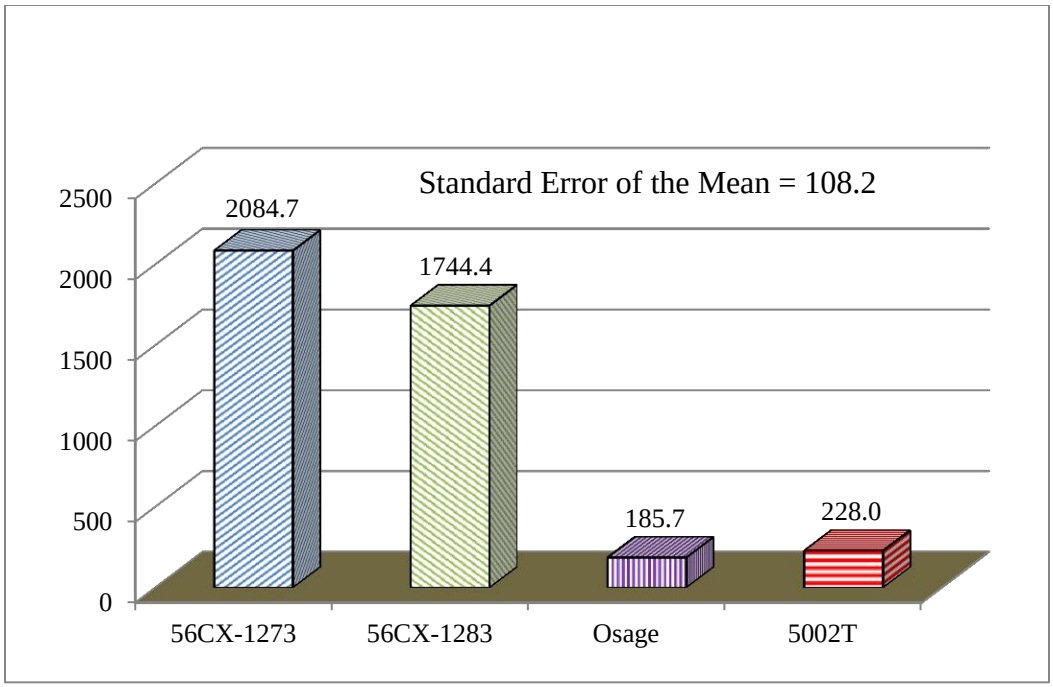


Figure 4.1

Inorganic phosphorus levels for BC_5 low phytate soybean lines 56CX-1273 and 56CX-1283 as compared to normal phytate cultivars Osage and 5002T grown in six southern U.S. environments in the 2013 United Soybean Board MG V Quality Traits Test.

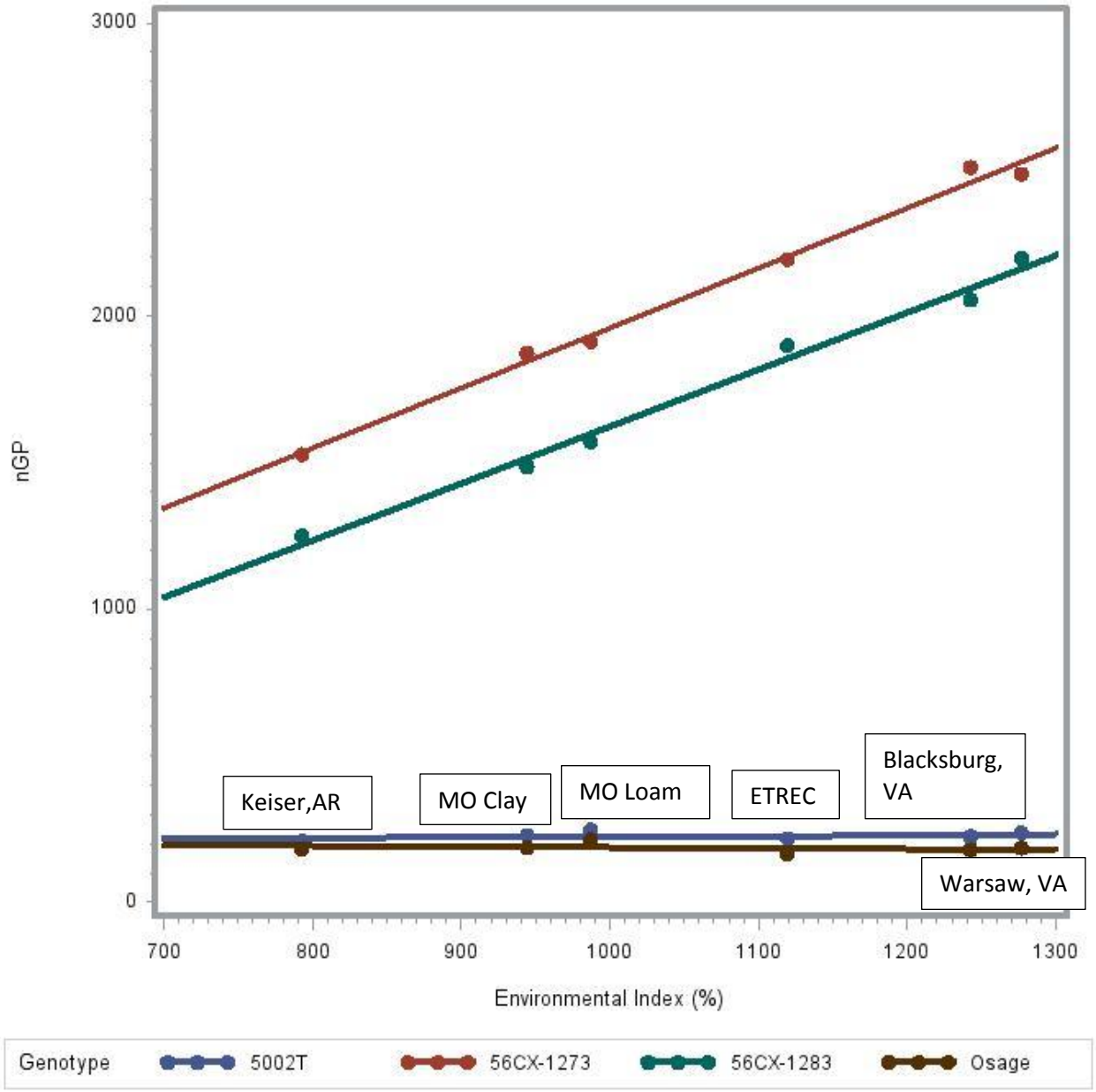


Figure 4.2

Genotype by environmental (G × E) index linear regression with seed Pi concentration for two BC_5 Low Phytate lines 56CX-1273, 56CX-1283 and two high yielding check cultivars 5002T and Osage. Slopes for 56CX-1273 (2.04) and 56CX-1283 (1.94) were significantly different than zero ($p<0.0001$) and were not stable for the low phytate trait when grown across six southern U.S. environments in 2013.

Part 5

Future Research with the BC_5 Low Phytate Lines

The molecular backcross strategy utilized for this plant breeding research to introgress the double homozygous recessive low phytate (LP) alleles into the genome of high yielding recurrent parent (RP) 5601T was successful. Every BC_5 LP line evaluated in this study displayed seed yields that were not significantly different ($p>0.05$) than 5601T when grown in multiple replications and in three distinctly different Tennessee environments over two years. Furthermore, eight BC_5 lines have now been classified as homozygous recessive (*dt1 dt1*) for stem termination, which is the growth habit type essential for growing MG V soybeans in southern U.S. Each BC_5 LP line has inherited the high concentration of seed protein from RP 5601T; six BC_5 LP lines have been qualified as having soymeal protein concentration greater than 49%, and six other BC_5 LP lines as having greater than a 50% soymeal protein. Much progress has been achieved. However, the fatty acid levels of the BC_5 LP lines could be improved. One BC_5 LP line, 56CX-1275, might be a suitable candidate for a germplasm release and for future breeding projects targeting fatty acids. Another BC_5 LP line may be a candidate for a cultivar release.

The high saturated fat content (sum of palmitic acid and stearic acid) of every BC_5 LP line was greater than 180 g kg^{-1} (RP 5601T had 172 g kg^{-1}). Saturated fats quantified at this level do not meet the <7% cutoff established for healthy, low saturated fat soybean oil, however that is true for nearly *all* soybean cultivars. The creation of a high protein, low phytate, *and* low saturated fat soybean line would be a winner. Soybean oil that is low in saturated fats is in high demand, due to the increased attention placed on healthy vegetable oils and consumer awareness of health issues surrounding saturated fats (Clemente and Cahoon, 2009). The development of a low saturated fat, low phytate, and high protein soybean line might be possible if a large enough

population of recombinant inbred lines were developed and tested from the low saturate BC_5 LP crosses.

The BC_5 LP line 56CX-1275 would be a suitable crossing candidate with a high oleic line, as its seed oleic acid concentration (358 g kg^{-1}) was significantly ($p<0.05$) the highest of any BC_5 line and was over 60 g kg^{-1} higher than recurrent parent (RP) 5601T. A high oleic, high protein, LP soybean line would have desirable seed quality characteristics. While high oleic acid is desirable in soybean oil, so is low linolenic acid, with soybean breeders targeting less than 3% linolenic acid to be classified as low-lin and less than 2% linolenic acid to be classified as ultra-low-lin (Clemente and Cahoon 2009). Line 56CX-1275 was quantified to have significantly the lowest ($p<0.05$) linolenic acid concentration (65 g kg^{-1}) of any BC_5 line, and may be a good candidate to develop a low phytate, high protein, high oleic, low-lin line.

Another possibility for future research might entail further yield trial testing of the eight determinate BC_5 LP lines. One consideration might be the inclusion of one or more of the top four yielding lines from the 2012-2013 BC_5 LP study into the 2014 or 2015 Uniform Test (UT), which is grown in replicated yield trials in many southern environments. In that test, the BC_5 LP lines would be evaluated for soybean pathogens like soybean cyst nematode (*Heterodera glycines* Ichinohe) and sudden death syndrome (caused by *Fusarium solani*) amongst others to see what type of disease resistance is present in the BC_5 LP lines. RP 5601T is known to be resistant to stem canker (caused by *Diaporthe phaseolorum*), soybean mosaic virus, southern root-knot nematode (*Meloidogyne incognita*) and moderately resistant to peanut root-knot nematode [*Meloidogyne* arenaria (Neal) Chitwood] (Pantalone et al., 2003). One of the top yielding LP performers from the UT could then be considered for a cultivar release

recommendation that included new information about their disease resistance and field tolerance to soybean pathogens.

A final suggestion for future research would be the establishment of livestock feeding trials, where the low phytate soymeal could be tested and compared to normal phytate soymeal feed often amended with phytase and inorganic phosphorus. The feed combination of low phytate and ultra-high protein soymeal may be able to benefit poultry (*Gallus domesticus*) producers, because it is hypothesized that the enhanced nutrition and metabolic energy obtained from the low phytate soymeal may lessen the amount of feed necessary to raise mature, healthy chickens which may improve upon existing margins used in the poultry business. In addition, poultry producers may be able to save millions of dollars annually in inorganic P and phytase amendments to soymeal, which would be unnecessary given the enhanced nutrition and high amounts of inorganic phosphorus in low phytate feed. Furthermore, the greater environmental benefits could be measured if the manure was collected from these trials and evaluated and compared for non-point phosphorus pollution potential. Now that we have finally developed a high yielding, low phytate cultivar suitable for growing in MG V where billions of chickens are raised annually, it is time to proceed with further testing targeting livestock feed and environmental sustainability.

References

Clemente and Cahoon. 2009. Soybean Oil: Genetic Approaches for Modification of Functionality and Total Content. Plant Physiol. 151(3): 1030-1040.

Pantalone, V.R., F.L. Allen, and D. Landau-Ellis. 2003. Registration of Soybean Cultivar 5601T. Crop Sci. 43:1123.

Appendix V

Table 5.1

Summary of assigned breeder numbers of BC_5 LP soybean lines from inception of first backcross in 2009 to BC_5F_3 derived lines in 2013.

CROSS	GENOTYPE	Winter PR WN 2009-2010	Summer ETREC 2010 F2 ROW	PEDIGREE 5601T BC5 5601T X TN09-239 Single Plants from 2010-20335-20338	Winter GH WN 2011 F3 SPs	Summer ETREC 2011 F3:4 ROWS	Winter FLA WN 2011-2012	Summer GENERATION 2012	Summer GENERATION 2013
09-**15**	**56CX-1273**	VP10-124	20335-20338	VP10-124-11	A79-02	40,002	VH12-1273	BC5 3:6	BC5 F3:7
09-**15**	**56CX-1274**	VP10-124	20335-20338	VP10-124-11	A79-05	40,004	VH12-1274	BC5 3:5	BC5 F3:6
09-**15**	**56CX-1275**	VP10-124	20335-20338	VP10-124-18	A86-02	40,006	VH12-1275	BC5 3:5	BC5 F3:6
09-**15**	**56CX-1276**	VP10-125	20339-20342	VP10-125-11	B05-02	40,012	VH12-1276	BC5 3:6	BC5 F3:7
09-**15**	**56CX-1277**	VP10-125	20339-20342	VP10-125-11	B05-04	40,014	VH12-1277	BC5 3:6	BC5 F3:7
09-**15**	**56CX-1279**	VP10-133	20365-20368	VP10-133-5	C66-02	40,024	VH12-1279	BC5 3:5	BC5 F3:6
09-**15**	**56CX-1280**	VP10-141	20392-20393	VP10-141-8	E69-01	40,036	VH12-1280	BC5 3:5	BC5 F3:6
09-**15**	**56CX-1282**	VP10-143	20398-20401	VP10-143-32	F04-04	40,044	VH12-1282	BC5 3:5	BC5 F3:6
09-**15**	**56CX-1283**	VP10-145	20406-20409	VP10-145-58	F79-03	40,047	VH12-1283	BC5 3:5	BC5 F3:6
09-**15**	**56CX-1284**	VP10-146	20410-20413	VP10-146-14	G70-02	40,049	VH12-1284	BC5 3:5	BC5 F3:6
09-**15**	**56CX-1286**	VP10-146	20410-20413	VP10-146-14	G70-04	40,051	VH12-1286	BC5 3:5	BC5 F3:6
09-**15**	**56CX-1287**	VP10-146	20410-20413	VP10-146-14	G70-05	40,052	VH12-1287	BC5 3:6	BC5 F3:7

Table 5.2

Total Number of F2 Plants Needed to Grow in Order to Obtain at Least One Triple Homozygous Recessive Plant with Desired Genes in the F2 generation.

1. Decide on an acceptable confidence level, ***P***, of success (95%).

2. Determine the expected proportion of (***X***) individuals that inherit the trait from the particular generation of inbreeding [eg. 0.25 for a recessive trait in the F2 generation]. The LP trait is double homozygous recessive, and the determinate trait is also homozygous recessive, thus we were looking to recover a triple homozygous recessive genotype:

AA BB dt1 dt1 (5601T, female) × aa bb Dt1 Dt1 (TN09-239, male)

F1: Aa Bb Dt1 dt1 (triple het)
(0.25)a * (0.25)b * (0.25)dt1 = (0.015625)

Probability of triple recessive F2 genotype: 0.015625

3. Solve the equation for ***N***, as the minimum number of plants needed to grow to find at least one F2 plant inheriting the traits of interest:

$$(1 - X)^N = (1 - P)$$
$$(1 - 0.015625)^N = (1 - 0.95)$$
$$N \text{ Log } (0.984375) = \text{Log } (0.05)$$
$$N = \text{Log}(0.05)/\text{Log}(0.984375)$$
$$N = -1.3010299 / -0.0068394$$

$$N = 190.225$$

4. To factor in genetic linkage, N is then divided by the cM distance between the two traits. In our case, the Dt1 locus and the pha002 locus on Gm19 are 20 cM apart:

$$190.225/0.20 = 951.125$$

Thus, ***952*** plants would have been needed to have been grown and evaluated from the cross of RP 5601T and donor parent TN09-239 to recover at least one triple homozygous recessive genotype (plant) in the BC_5F_2 generation. In Winter 2009-2010, 30 BC_5F_1 plants were grown (VP10-121 – VP10-150) and harvested seeds from those 30 SPs were planted in rows 20,325 – 20,425 at 100 seeds/row (ETREC, Summer 2010). Thus, 10,000 BC_5F_2 LP plants were evaluated, and 616 SPs were pulled that phenotypically exhibited a determinate (*dt1*) growth habit. Of those 616 plants screened with molecular markers, only eight turned out to be triple homozygous recessive for *pha-001*, *pha-002* and *dt1* (Sedcole 1977).

Table 5.3

Assay Procedure for quantifying Pi with a spectrophotometer plate reader.

1. 25g of soybean seed was ground in a Knifetec (Foss, Eden Prairie, MN) grinder with cool water circulating through the grinder. A subsample (0.1 g) will be put into a 1.5mL centrifuge tube. 1ml of Pi extraction buffer was added [12.5% trichloroacetic acid (TCA) and 25 mM $MgCl_2$] (10µl per mg of ground sample). This volume was adjusted if weight was different than 1g.
2. Each individual tube was vortexed well.
3. Ground seed tissue was allowed to soak 6 hours (or overnight) at 4° to soften the tissue.
4. Each individual tube was vortexed again to mix extraction buffer and seed tissue. This was repeated once or twice during the incubation period, which can be from 4 hours at room temperature to overnight at 4°C.
5. Samples were vortexed one more time and tissue was allowed to settle for 10 - 30 min. Chen's reagent (Appendix Table B.3) was prepared while waiting. The recipe for the Reaction Buffer is provided in Appendix Table B.4. Ten ml Chen's reagent will be needed for each set of 96 samples.
6. Tubes were centrifuged at 4000rpm for 4 min 100 µL of this supernatant extract was transferred to a fresh tube.
7. → 90 µL of dH_2O and then 10 µL of above supernatant extract was pipetted from each sample into the corresponding well of the microtitre plate. The flat bottomed Corning Costar plates (cat. # 3997 FISHER 09-761-145) were used.
8. 100 µL Chen's reagent was added to each well and was incubated at room temperature for *exactly* one hour. The total volume was 200 µL.
9. Inorganic P concentrations were measured using a Bio-Tek Powerwave XS microplate spectrophotometer, at wavelength 882nm. Concentrations are mean values averaged across sub-samples. See Table 2.10 for the standards plate that was used to create the linear equation for the standard curve that quantified inorganic P for all samples.

Table 5.4

Summary of genotype trait means by location for 2012.

2012 Genotype Means by Location

Location	GENOTYPE	Yield	Maturity	Lodging	Height	Emerge	Protein	Oil	GCPalmitic	GCStearic	GCOleic	GCLinoleic	GCLinolenic	µg Pi g	SdWt	Cysteine CP	Lysine CP	Methionine CP	Threonine CP	Tryptophan CP
ETREC	5601T	62.7	138.5	2.6	35	85	42.3	20.7	13.6	3.7	27.7	45.7	9.3	185	13.4	14.9	14.2	62.3	36.9	10.2
ETREC	56CX-1273	56.9	136	1.5	34	70	42.7	20.0	14.1	4.5	28.7	44.1	8.6	1784	14.8	15.3	14.2	62.4	36.6	10.1
ETREC	56CX-1276	46.6	136.5	1.5	32	56	42.4	20.1	14.7	4.0	26.1	45.7	9.4	1590	16.8	15.2	14.2	62.3	36.8	10.1
ETREC	56CX-1277	54.7	137	1.7	33	63	42.2	20.0	14.9	4.0	24.9	46.7	9.5	1520	16.1	15.8	14.5	62.4	36.9	10.1
ETREC	56CX-1279	51.9	136.5	1.6	33	58	42.6	19.5	14.5	3.9	26.1	46.0	9.7	1576	15.9	15.7	14.6	62.5	36.8	10.0
ETREC	56CX-1280	53.5	136.5	2.5	52	58	42.4	20.1	14.6	4.2	28.6	43.9	8.8	1638	16.1	14.8	14.2	62.6	36.7	10.1
ETREC	56CX-1282	53.3	136.5	1.55	33	55	42.3	19.4	15.1	4.0	26.7	45.3	8.9	1714	16.3	14.8	13.8	62.5	36.8	10.1
ETREC	56CX-1283	56.9	136.5	1.7	34	67	43.0	19.7	14.2	4.8	27.5	44.5	9.0	1532	15.9	14.3	14.1	62.4	36.7	9.9
ETREC	56CX-1284	44.3	137.5	2.6	52	63	43.0	19.8	14.5	4.2	29.1	43.8	8.5	1729	16.8	15.0	14.0	62.4	36.7	10.0
ETREC	56CX-1286	53.6	137	2.5	54	55	42.4	20.2	14.2	4.1	28.8	44.2	8.7	1808	16.3	14.7	13.9	62.3	36.6	10.2
ETREC	56CX-1287	55.5	137	2.45	54	57	42.5	20.0	14.4	4.2	29.5	43.5	8.5	1773	16.7	15.1	13.9	62.5	36.7	10.1
ETREC	TN09-239	52.9	137	3.3	54	59	42.9	20.3	14.1	4.0	27.5	45.7	8.7	1421	16.0	15.4	14.2	62.3	36.6	10.2
MILAN	5601T	75.7	132	4	38	83	42.4	20.5	13.3	3.7	27.2	46.4	9.4	216	13.1	15.2	62.2	14.3	36.8	10.2
MILAN	56CX-1273	77.0	130.5	3.25	41	81	43.0	20.1	14.5	4.1	29.2	44.2	8.0	1832	15.8	14.1	62.3	13.7	36.6	10.3
MILAN	56CX-1276	68.5	131	2	40	74	41.9	20.6	14.5	3.7	27.5	46.0	8.4	1701	17.4	14.4	62.5	14.1	36.9	10.2
MILAN	56CX-1277	67.0	131.5	2	36	76	41.9	20.3	14.8	3.8	25.1	47.4	9.0	1662	16.8	14.8	62.3	14.2	36.8	10.4
MILAN	56CX-1279	67.7	131	3	37	79	42.3	19.8	14.7	4.0	27.4	45.6	8.3	1705	16.7	14.2	62.5	14.1	36.7	10.2
MILAN	56CX-1280	72.6	132.5	3.75	64	85	43.0	19.9	14.2	4.0	29.0	44.6	8.2	1746	15.8	15.0	62.2	13.9	36.6	10.2
MILAN	56CX-1282	76.8	130.5	3	39	81	42.6	19.7	14.5	3.9	25.7	47.1	8.8	1820	16.4	15.5	62.3	14.2	36.6	10.1
MILAN	56CX-1283	76.7	132	3.5	41	85	43.2	19.4	14.6	4.4	29.6	44.0	7.4	1578	16.0	15.5	62.1	14.4	36.6	10.2
MILAN	56CX-1284	73.6	131	4	73	81	43.2	19.8	13.9	3.7	31.5	43.5	7.4	1918	16.4	14.7	62.1	13.9	36.6	10.2
MILAN	56CX-1286	70.2	131.5	4	65	83	41.9	20.4	14.0	3.8	30.3	44.2	7.8	1879	16.1	14.7	62.4	14.3	36.8	10.2
MILAN	56CX-1287	77.5	131.5	4	67	83	42.3	19.8	14.4	3.8	28.3	45.2	8.3	1807	15.8	15.6	62.1	14.6	36.8	10.2
MILAN	TN09-239	71.9	131.5	4	66	76	42.5	20.4	14.1	3.8	30.1	44.3	7.7	1700	15.9	15.3	62.2	14.2	36.7	10.4

Table 5.5

Summary of genotype trait means by location for 2013.

2013 Genotype Means by Location

Location	GENOTYPE	Yield	Maturity	Lodging	Height	Emerge	Protein	Oil	GCPalmitic	GCStearic	GCOleic	GCLinoleic	GCLinolenic	µg Pi g	SdWt	Germ	Cysteine CP	Lysine CP	Methionine CP	Threonine CP	Tryptophan CP
ETREC	5601T	51.2745	124	1	35	70.3	44.1	21.3	13.6	3.9	32.1	42.5	7.9	255	12.8	96	15.55	14.15	61.3	36.75	10
ETREC	56CX-1273	48.75996	123	1	30	55.6	46.2	20.1	14.2	4.4	36.0	39.1	6.3	2569	15.0	96	15.4	13.95	61.35	36.15	9.75
ETREC	56CX-1274	47.24836	124	1	28	60.6	45.8	20.5	13.7	4.4	38.3	37.5	6.2	2548	15.5	88	15.95	14	61.3	36.3	9.7
ETREC	56CX-1275	48.64896	123	1	31	61.7	44.2	20.8	14.0	4.4	37.2	38.2	6.1	2412	15.4	96	15.75	14.1	61.75	36.4	9.85
ETREC	56CX-1276	40.63597	122	1	25	51.6	44.1	21.2	14.2	4.2	32.8	41.7	7.1	1927	14.6	100	15.85	14.05	61.3	36.6	9.95
ETREC	56CX-1277	43.55735	123	1	28	53.6	44.3	20.8	14.6	4.1	31.7	42.5	7.0	2022	15.6	100	15.5	14.1	61.35	36.6	9.8
ETREC	56CX-1279	43.95542	124	1	28	42.2	44.5	20.4	14.8	3.9	34.4	40.3	6.6	1728	15.0	96	14.95	14.15	61.45	36.5	9.9
ETREC	56CX-1280	46.19807	124	2.5	42	51.7	42.2	21.1	15.0	3.9	30.2	43.5	7.4	2140	14.6	100	15.5	14.6	61.85	36.95	10.1
ETREC	56CX-1282	52.34838	122	1	30	62.8	44.6	20.0	14.7	3.8	31.6	42.6	7.2	2258	16.0	100	15.35	14.2	61.75	36.4	9.85
ETREC	56CX-1283	51.26812	124	1.5	35	65.5	45.3	19.9	14.9	4.7	34.6	39.4	6.3	2055	15.1	92	15.2	14	61.6	36.2	9.95
ETREC	56CX-1284	45.75978	123	2.5	45	48.0	42.9	21.0	14.6	4.1	33.5	40.7	7.0	2363	14.9	96	15.15	14.35	61.7	36.55	10.1
ETREC	56CX-1286	46.42948	124	3.5	46	54.2	42.6	21.2	14.5	4.2	32.8	41.5	7.1	2358	14.8	88	15.8	13.95	61.65	36.8	10.1
ETREC	56CX-1287	51.02761	124	3	43	60.5	42.6	20.8	14.3	4.2	32.8	41.7	7.0	2325	14.5	100	15.3	14.1	62.05	36.8	9.85
ETREC	Ellis	48.30971	122	1	24	58.9	42.5	20.7	12.9	4.3	31.5	43.6	7.8	243	11.8	92	15.3	14.6	61.95	36.95	9.85
ETREC	Osage	45.51255	123	1	27	58.6	45.4	20.6	13.8	4.4	32.9	41.6	7.4	235	10.8	100	15.45	14.1	61.15	36.45	9.8
ETREC	TN09-239	46.40649	123	2.5	44	59.1	43.4	21.1	14.9	3.8	29.4	44.0	7.8	1741	14.4	100	15.9	14.25	61.45	36.75	10
HRREC	5601T	66.2	144	3.7	42	60.3	45.1	20.0	13.0	3.7	29.9	45.0	8.4	211	14.2	92	15.90	14.50	61.17	36.63	9.70
HRREC	56CX-1273	69.1	142	2.3	37	60.0	45.3	19.4	13.6	4.0	34.6	41.5	6.3	1318	15.7	96	15.43	14.23	61.50	36.37	9.87
HRREC	56CX-1274	63.2	143	1.3	38	69.7	45.2	19.8	13.6	4.0	32.2	43.2	7.0	1608	16.7	80	15.93	14.43	61.23	36.47	9.83
HRREC	56CX-1275	58.9	143	2.0	40	69.0	45.5	19.6	13.1	4.2	39.2	37.9	5.6	1497	17.3	84	15.90	14.23	61.53	36.27	9.80
HRREC	56CX-1276	63.1	144	1.3	38	64.0	44.6	19.7	13.9	3.8	28.9	45.6	7.8	1336	17.2	100	15.87	14.43	61.27	36.60	9.93
HRREC	56CX-1277	74.4	144	2.0	40	69.1	44.7	19.3	14.0	3.8	29.3	44.9	8.0	1372	18.6	88	16.30	14.70	61.43	36.70	9.67
HRREC	56CX-1279	57.7	145	2.3	38	66.8	45.3	18.7	13.7	3.8	32.2	42.9	7.5	1352	16.8	88	15.80	14.63	61.33	36.43	9.70
HRREC	56CX-1280	60.4	145	4.3	50	67.6	44.9	19.8	14.0	4.1	32.6	42.1	7.2	1522	16.2	80	15.50	14.17	61.43	36.50	9.80
HRREC	56CX-1282	62.9	143	1.3	38	68.0	45.1	19.0	14.3	3.8	28.1	45.8	8.0	1481	17.1	92	15.47	14.33	61.47	36.53	9.67
HRREC	56CX-1283	53.4	143	3.0	38	66.6	46.0	19.0	14.2	4.5	31.6	42.9	6.8	1428	15.8	88	15.40	13.83	61.33	36.23	9.70
HRREC	56CX-1284	57.9	144	4.7	56	64.6	45.2	19.2	13.5	4.0	36.0	40.2	6.3	1744	16.6	80	15.60	14.13	61.60	36.40	9.80
HRREC	56CX-1286	56.9	145	4.0	50	68.3	43.9	20.0	13.6	4.0	33.2	42.0	7.1	1360	16.6	100	16.03	14.73	61.63	36.70	9.77
HRREC	56CX-1287	64.0	144	5.0	52	65.0	44.2	19.6	13.3	4.1	34.8	41.1	6.7	1520	15.9	92	15.23	14.63	61.73	36.63	9.73
HRREC	Ellis	65.6	143	1.3	33	63.0	43.8	19.1	13.0	4.2	28.8	45.7	8.4	250	11.9	96	15.77	14.57	61.73	36.80	9.67
HRREC	Osage	59.1	143	1.3	35	61.7	45.9	19.3	13.4	4.2	31.4	43.3	7.7	199	10.8	96	15.83	14.50	61.47	36.30	9.80
HRREC	TN09-239	49.2	144	4.0	49	66.4	45.5	19.6	13.9	4.0	34.3	41.1	6.6	1343	15.7	92	15.60	14.43	61.37	36.43	9.73
MILAN	5601T	59.4	136	2.8	35	79.2	44.0	21.3	13.7	4.0	30.2	44.3	7.8	216	12.7	100	15.37	14.47	61.47	36.80	9.87
MILAN	56CX-1273	58.3	135	1.8	33	67.8	43.8	20.5	15.1	4.1	29.9	44.0	6.9	2108	14.1	92	14.93	14.20	61.87	36.63	9.90
MILAN	56CX-1274	63.7	135	1.7	30	77.8	44.3	21.1	15.0	4.2	29.0	44.4	7.4	2159	15.3	92	15.10	14.13	61.50	36.63	9.77
MILAN	56CX-1275	54.2	135	2.3	33	81.4	44.4	20.9	14.8	4.2	34.9	40.0	6.0	2084	14.6	100	15.47	14.40	61.73	36.60	9.87
MILAN	56CX-1276	60.3	135	1.8	31	79.9	43.8	21.2	15.2	3.9	29.2	44.4	7.3	1867	14.9	92	15.75	14.05	61.25	36.70	9.90
MILAN	56CX-1277	62.4	135	1.5	33	76.3	44.0	20.8	15.1	4.1	28.6	44.9	7.4	1805	15.7	96	15.60	14.47	61.47	36.77	9.80
MILAN	56CX-1279	64.1	135	2.0	33	87.3	44.3	20.2	14.9	4.0	30.0	43.9	7.2	1758	14.1	96	15.63	14.17	61.53	36.70	9.70
MILAN	56CX-1280	57.8	136	3.0	56	85.1	44.5	20.9	14.6	4.2	31.1	43.0	7.1	1896	14.8	96	14.83	13.97	61.50	36.57	9.83
MILAN	56CX-1282	63.7	135	1.7	31	84.1	43.9	20.3	15.1	4.0	28.4	44.9	7.6	1859	14.9	100	15.33	14.50	61.63	36.80	9.80
MILAN	56CX-1283	48.4	135	2.8	36	83.7	44.8	20.1	14.8	4.6	30.8	43.0	6.7	1674	14.2	92	14.97	14.03	61.33	36.60	9.77
MILAN	56CX-1284	58.7	136	3.2	57	83.1	44.1	20.8	14.5	3.9	32.8	42.2	6.6	1589	15.4	92	14.75	14.10	61.75	36.65	9.85
MILAN	56CX-1286	59.6	136	3.7	57	86.6	43.7	21.2	14.8	4.1	29.6	44.0	7.4	1986	15.1	92	15.10	14.03	61.47	36.83	9.93
MILAN	56CX-1287	56.8	135	3.5	55	89.3	43.4	20.9	15.3	4.2	29.2	44.1	7.1	1971	14.5	100	16.07	14.43	61.63	36.80	9.83
MILAN	Ellis	63.3	135	1.8	30	77.5	41.5	21.5	13.8	4.2	27.3	46.4	8.3	225	11.6	100	15.53	14.47	62.27	37.17	9.90
MILAN	Osage	67.8	135	2.2	33	81.7	44.9	20.5	13.9	4.3	30.7	43.2	7.9	195	11.6	100	15.70	14.85	61.40	36.55	9.80
MILAN	TN09-239	56.0	135	2.7	54	86.4	44.9	21.1	14.8	4.2	30.2	43.9	6.9	1393	14.5	100	15.30	13.77	61.20	36.57	9.80

Table 5.6

Summary of genotype trait means by location for 2012-2013.

2012-2013 Genotype Trait Means by Location																				
Location	GENOTYPE	Yield	Maturity	Lodging	Height	Emerge	Protein	Oil	GCPalmiti	GCStearic	GCOleic	GCLinolei	GCLinolen	µg Pi g	SdWt	Cysteine C	Lysine CP	Methionine CI	Threonine	Tryptopha
ETREC	5601T	57.8938	131.25	1.89463	34.3136	77.8826	43.2075	21.0075	13.6266	3.83205	29.8622	44.1061	8.5731	219.82	13.09	15.225	14.175	61.8	36.825	10.075
ETREC	56CX-1273	53.2423	129.5	1.22903	31.5557	63.2966	44.4675	20.0475	14.1668	4.48475	32.3385	41.5598	7.45268	2176.43	14.5918	15.35	14.05	61.85	36.375	9.9
ETREC	56CX-1274	50.1414	130.332	0.97766	30.3822	59.8223	45.1282	20.1782	13.6203	4.41539	35.8314	39.0744	7.08633	2323.24	16.0421	15.7817	14	61.7023	36.3395	9.7516
ETREC	56CX-1275	51.542	129.332	1.10691	33.3423	64.1565	43.5932	20.4332	14.0105	4.44129	34.7199	39.8125	7.04367	2187.57	15.9671	15.5817	14.1	62.1523	36.4395	9.9016
ETREC	56CX-1276	43.6255	129.25	1.25	28.125	53.67	43.2775	20.6325	14.4663	4.10157	29.4696	43.7038	8.25877	1758.34	15.7	15.525	14.1	61.775	36.675	10.025
ETREC	56CX-1277	49.1262	130	1.35	30.25	58.3575	43.2225	20.4025	14.7412	4.0472	28.338	44.5822	8.29396	1770.81	15.8725	15.625	14.275	61.85	36.725	9.95
ETREC	56CX-1279	47.9277	130	1.3	30.25	49.995	43.5575	19.9775	14.6322	3.89257	30.2518	43.1028	8.11815	1651.5	15.4125	15.3	14.35	61.95	36.625	9.925
ETREC	56CX-1280	49.8465	130	2.5	47	54.84	42.3025	20.625	14.7989	4.04591	29.3777	43.6835	8.09399	1888.98	15.3475	15.125	14.375	62.2	36.825	10.075
ETREC	56CX-1282	52.8192	129.25	1.275	31	58.9025	43.4775	19.6625	14.933	3.87148	29.1822	43.9502	8.0631	1985.89	16.12	15.05	14	62.125	36.575	9.95
ETREC	56CX-1283	54.0816	130.25	1.6	34.375	66.3225	44.13	19.7675	14.5531	4.77193	31.0684	41.9575	7.65158	1793.17	15.51	14.75	14.05	62	36.425	9.925
ETREC	56CX-1284	45.0424	130.25	2.55	48.5	55.5425	42.955	20.4225	14.5614	4.13929	31.2946	42.2575	7.7472	2046.19	15.815	15.075	14.15	62.025	36.6	10.05
ETREC	56CX-1286	49.9997	130.5	3	49.625	54.84	42.5425	20.7125	14.3249	4.16447	30.8031	42.8411	7.86888	2082.57	15.505	15.25	13.925	61.975	36.7	10.125
ETREC	56CX-1287	53.2688	130.25	2.725	48.25	58.5125	42.5225	20.4	14.3218	4.18399	31.1411	42.5991	7.754	2049.23	15.5825	15.175	14	62.25	36.75	9.975
ETREC	Ellis	51.2027	128.832	1.10691	26.8423	61.3465	41.8382	20.3182	12.8452	4.30403	28.984	45.2047	8.68994	18.93	12.3271	15.1317	14.6	62.3523	36.9895	9.9016
ETREC	Osage	48.4056	129.832	1.10691	29.8423	61.0315	44.7382	20.2282	13.7444	4.38038	30.3693	43.1795	8.35412	10.85	11.4071	15.2817	14.1	61.5523	36.4895	9.8516
ETREC	TN09-239	49.6507	130	2.9	48.75	58.9825	43.1425	20.6725	14.4821	3.90777	28.4841	44.8441	8.28197	1580.94	15.165	15.65	14.2	61.85	36.675	10.075
HRREC	5601T	66.1793	144.333	3.66667	42.3333	60.31	45.0633	19.96	13.026	3.72406	29.9422	44.9573	8.35039	211.34	14.1967	15.9	14.5	61.1667	36.6333	9.7
HRREC	56CX-1273	65.248	142	2.33333	37.3333	59.9933	45.34	19.3733	13.6144	4.01338	34.6275	41.4512	6.29359	1317.76	15.6767	15.4333	14.2333	61.5	36.3667	9.86667
HRREC	56CX-1274	63.2398	142.667	1.33333	38	69.6833	45.2367	19.81	13.5519	4.00835	32.2453	43.2284	6.96606	1608.3	16.7233	15.9333	14.4333	61.2333	36.4667	9.83333
HRREC	56CX-1275	58.94	142.667	2	40	68.9567	45.4933	19.6467	13.0819	4.20641	39.2218	37.8506	5.63925	1496.89	17.2933	15.9	14.2333	61.5333	36.2667	9.8
HRREC	56CX-1276	63.137	144	1.33333	37.6667	63.9533	44.6	19.73	13.9143	3.82846	28.8503	45.6117	7.79526	1336.08	17.2	15.8667	14.4333	61.2667	36.6	9.93333
HRREC	56CX-1277	74.4254	144.333	2	40.3333	69.06	44.7333	19.32	14.037	3.79753	29.2897	44.8819	7.99389	1372.34	18.56	16.3	14.7	61.4333	36.7	9.66667
HRREC	56CX-1279	57.7487	145	2.33333	38	66.77	45.3367	18.7133	13.7163	3.77023	32.187	42.863	7.46351	1352.35	16.78	15.8	14.6333	61.3333	36.4333	9.7
HRREC	56CX-1280	60.3585	144.667	4.33333	50	67.6	44.86	19.75	13.9863	4.12038	32.5883	42.0668	7.23829	1521.87	16.2167	15.5	14.1667	61.4333	36.5	9.8
HRREC	56CX-1282	62.9214	142.667	1.33333	38.3333	68.0133	45.0667	19.0133	14.2909	3.82054	28.139	45.7692	7.98037	1480.82	17.1067	15.4667	14.3333	61.4667	36.5333	9.66667
HRREC	56CX-1283	49.5815	143	3	38.3333	66.56	45.9867	19.0333	14.1783	4.47085	31.617	42.9029	6.83093	1428.29	15.7933	15.4	13.8333	61.3333	36.2333	9.7
HRREC	56CX-1284	57.9067	144	4.66667	56.3333	64.58	45.2467	19.1733	13.4958	4.00249	36.05	40.1721	6.27964	1743.73	16.5933	15.6	14.1333	61.6	36.4	9.8
HRREC	56CX-1286	56.9021	145	4	49.6667	68.33	43.8667	20.0367	13.6094	4.02061	33.1726	42.0492	7.14812	1360.48	16.55	16.0333	14.7333	61.6333	36.7	9.76667
HRREC	56CX-1287	64.0098	144.333	5	52.3333	64.9933	44.2167	19.6233	13.33	4.06114	34.8001	41.0871	6.72167	1520.21	15.93	15.2333	14.6333	61.7333	36.6333	9.73333
HRREC	Ellis	65.6301	142.667	1.33333	33.3333	63.0133	43.7733	19.1133	12.9552	4.18215	28.7666	45.7393	8.3568	249.56	11.8567	15.7667	14.5667	61.7333	36.8	9.66667
HRREC	Osage	59.0526	142.667	1.33333	34.6667	61.6667	45.94	19.3433	13.4286	4.17791	31.405	43.2953	7.69316	199.39	10.8	15.8333	14.5	61.4667	36.3	9.8
HRREC	TN09-239	49.1825	144	4	49.3333	66.35	45.5333	19.56	13.9282	4.02115	34.3487	41.1072	6.59471	1343.24	15.74	15.6	14.4333	61.3667	36.4333	9.73333
MILAN	5601T	67.4083	133.815	3.3981	36.8638	81.0981	43.2132	20.936	13.4794	3.82133	28.8614	45.2898	8.56618	216.66	12.9154	15.2792	14.3779	0.62162	36.8	10.0139
MILAN	56CX-1273	67.3688	132.798	2.51454	36.9824	73.8436	43.3187	20.302	14.8528	4.08025	29.4762	44.1661	7.44173	1979.47	14.9352	14.5332	13.9952	0.60562	36.6	10.0754
MILAN	56CX-1274	70.5813	133.051	2.06801	34.1924	77.2832	43.5403	20.7266	14.7962	4.07367	28.2816	45.0164	7.87728	2153.35	16.013	14.9461	14.1333	0.61476	36.6326	9.9465
MILAN	56CX-1275	61.1556	133.051	2.73467	37.1924	80.7932	43.627	20.4966	14.5999	4.09775	34.1359	40.661	6.5506	2078.88	15.2897	15.3128	14.4	0.62809	36.5993	10.0465
MILAN	56CX-1276	64.741	133.008	1.93234	35.4421	77.2033	42.86	20.8975	14.8654	3.78604	28.3545	45.1407	7.86783	1790.26	16.1014	15.0812	14.075	0.6025	36.7997	10.0305
MILAN	56CX-1277	65.1775	133.218	1.75426	34.5036	76.3466	42.9706	20.534	14.8995	3.91863	27.0333	46.0363	8.12853	1738.59	16.2797	15.2362	14.3574	0.61762	36.76	10.0601
MILAN	56CX-1279	66.4544	133.008	2.48714	34.9634	83.5039	43.3037	19.942	14.8029	3.96275	28.7929	44.7057	7.7511	1734.01	15.3247	14.9933	14.1394	0.61162	36.68	9.9389
MILAN	56CX-1280	65.151	134.025	3.3707	59.5725	85.1797	43.7521	20.42	14.4065	4.1011	30.0941	43.777	7.63587	1826.83	15.2887	14.8912	13.9394	0.61162	36.58	9.9947
MILAN	56CX-1282	70.2629	132.798	2.30906	34.9824	82.948	43.2218	20.012	14.7838	3.952	27.1981	45.8783	8.2049	1841.34	15.6548	15.3912	14.3771	0.62162	36.72	9.9331
MILAN	56CX-1283	61.737	133.621	3.16522	38.1932	84.2834	44.0447	19.754	14.7115	4.47771	30.1817	43.5552	7.09028	1629.78	15.0335	15.1845	14.163	0.62362	36.58	9.9774
MILAN	56CX-1284	66.0742	133.395	3.57618	64.4304	82.1465	43.605	20.3	14.1979	3.81414	32.156	42.8643	6.98035	1744.06	15.8869	14.7562	14	0.61	36.6247	10.0305
MILAN	56CX-1286	65.0443	133.605	3.8433	60.9326	84.896	42.7964	20.828	14.4492	3.94204	29.7198	44.2141	7.69107	1936.69	15.6262	14.8889	14.1221	0.60362	36.82	10.0523
MILAN	56CX-1287	66.7586	133.412	3.75426	61.0014	86.6188	42.809	20.372	14.9209	4.02657	28.7204	44.6629	7.68301	1895.36	15.1673	15.8483	14.4811	0.61962	36.8	9.9947
MILAN	Ellis	70.2255	132.718	2.23467	34.1924	76.9365	40.6703	21.0966	13.5683	4.09509	26.5773	46.9795	8.825	219.79	12.2797	15.3794	14.4667	0.58809	37.1659	10.0798
MILAN	Osage	74.728	133.051	2.56801	36.5257	81.1032	44.077	20.145	13.6306	4.20591	29.9291	43.8458	8.43382	189.81	12.3297	15.5973	14.85	0.65309	36.5482	9.9915
MILAN	TN09-239	63.8222	133.218	3.30906	59.6413	81.961	43.7881	20.748	14.4788	4.01031	30.0492	44.1959	7.2836	1537.41	15.1709	15.2852	13.9442	0.61162	36.6	10.0601

Vita

Jeffrey D. Boehm Jr. was born in Chattanooga, TN on June 20, 1973. He attended the Baylor School in Chattanooga, TN, graduating in 1991, and received a Bachelor of Arts Degree from the University of Georgia in 1996.

In the fall of 2012, Jeff started a Master of Science program at the University of Tennessee, Knoxville in Plant Sciences, with a concentration in plant breeding. Jeff plans to receive his Master of Science degree in May of 2014. Upon completion of this MS degree, Jeff will continue his research and graduate studies in plant breeding at Washington State University as a PhD student.