Multi-Location Field Testing New Environmentally Friendly, High Yielding, Low Phytate Soybean Lines Developed Via Molecular Breeding

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ABSTRACT

Soymeal is the primary food source for poultry, swine, and fish; however it contains phytic acid or phytate (myo-inositol 1,2,3,4,5,6 hexakisphosphate). Phytate chelates key nutrients making them less available in the diet. Phytate accumulates in animal waste and causes nonpoint phosphorous (P) pollution leading to eutrophication. Two confirmed QTL exist for seed phytate cqPha-001(Gm03) and cqPha-002 (Gm19). Our program developed high yielding maturity group V soybean cultivar 5601T, whose soymeal is excellent for poultry nutrition. The goal of this study was to produce a high yielding, low phytate (LP) backcross (BC) derived progeny of 5601T. However, the genetic linkage between the stem termination locus (*Dt1*) and the low phytate *pha-002* locus prevented progress until we were able to use SNP technology to assist in breaking the genetic linkage.

In 2012, a field trial using a randomized complete block was grown at two locations to evaluate the agronomic performance of 10 BC_5 LP lines in comparison to recurrent and donor parents 5601T and TN09-239 and high yield check cultivar Osage. In 2013, the study was grown at three locations with the addition of two BC_5 LP lines. The yields from four BC_5 LP lines were excellent: 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}), and were not significantly different ($p>0.05$) than that of recurrent parent 5601T (4227 kg ha^{-1}) or Osage (4089 kg ha^{-1}). SNP analysis revealed that these four BC_5 LP lines were determinate (*dt1 dt1*) having broken the linkage between *Dt1* and *pha-002* on Gm19. These results indicated that LP trait introgression is possible with high yield.

INTRODUCTION

In 2013 over 76 million acres of soybeans were harvested in the US, producing $41 billion dollars in revenue (*http://www.nass.usda.gov*). Approximately 36.3 million metric tons of these soybeans were processed into soymeal as a feed source for livestock in 2013 (*http://www.soystats.com*). In soybean seeds there are large concentrations of phytic acid (Erdman 1979). Phytate myo-inositol-1,2,3,4,5,6-hexakisphosphate (**figure 1**), is the result of mixed cation salt of phytic acid, with chelating properties (Wilson 1987). Phytic acid is utilized in the early germination process of the seed and is phosphoralated by phytase during germination by hormones produced in the plant (Raboy, 2002, Hegeman 2001).

Monogastric animals cannot properly digest phytate (Maenz 1999), and furthermore many animals suffer from gastric degradation due to phytatse's chelating properties that make key mineral nutrients unavailable to livestock (Kemme 1997, Miyamoto 2002). The feedstock industry integrates nutrients into feed sources to counter problems with phytate digestibility (Reddy, 1989). This adds additional cost to production and increases the phosphate waste production from animal feces.

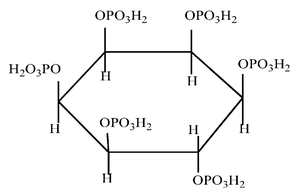


Figure 1
Phytic acid molecule showing P at each ring.

Runoff from farmland laden with livestock waste is highly phosphoric and causes problems to the environment. Agricultural runoff has contributed to massive algae blooms that cause a rapid decrease in oxygen content in the water, thus quickly killing off many fish and other aquatic life in streams and estuaries (Carpenter ,1998). Eutrophication has caused widespread environmental and economic costs for many industries (UNEP).
There is an additional cost in fertilizers to many farmers who lose precious phosphorus due to runoff and seepage. Many swine and chicken producers add phytase enzyme as well as additional mineral supplements to increase mineral availability to livestock. Key nutrients are thereby made more available to livestock, however digestible intake is improved by only about by 20±45% in poultry and swine (Maenz et al. 1999). The long term goal of our project is to provide high yielding soybean cultivars with low seed phytate that would produce soymeal more effective for livestock producers. The goal is for the low phytate meal to catalyze more nutrients in the feed source, and ultimately reduce the phosphate found in animal waste for environmental protection.

MATERIALS AND METHODS

Backcrossing Strategy to introgress the low phytate trait:

Our germplasm source for the low phytate trait (CX1834-1-2) was developed by the USDA-ARS and Purdue University through ethyl methanesulfonate (EMS) mutagenesis (Gillman et al., 2009). This germplasm carried the two low phytate genes but was not adapted to Tennessee and was not agronomically desirable (Oltmans, et al.,2004). The purpose of our backcrossing program was to develop a commercially acceptable, superior quality, high yielding soybean cultivar with low seed phytate for the southern region of USA.

The cultivar 5601T, developed from the cross of 'Hutcheson' (Buss et al. 1988) x TN89-39 is a high yielding cultivar and former USDA maturity group V check. (Pantalone et al., 2003). 5601T is resistant to several plant pathogens, such as; stem canker, soybean mosaic virus (SMV), southern root-knot nematode and moderately resistant to peanut root-knot nematode. Moreover 5601T produces higher seed protein content than typical cultivars and its soymeal has been found to be near ideal for broiler chick diets. Because of these desirable traits, 5601T was chosen as the recurrent parent for the low phytate introgression project (Landau-Ellis, 2009).

TN09-239 (5601T[4] × CX 1834-1-2), was the low phytate BC_4 donor used to create our BC_5F_1 seed (cross 09-15). This was accomplished at the East Tennessee Research and Education Center (ETREC), Knoxville, TN in the summer of 2009. In the summer of 2010 seed from these seven BC_5F_1 plants were planted as BC_5F_2 plant rows. Plants selected by visual appearance for determinate (dt1dt1) growth habit were evaluated using the inorganic phosphorus (Pi) assay (**figure 2**). These BC_5F_2 seed were grown in Isabela, Puerto Rico at USDA-TARS during the winter of 2009-2010.

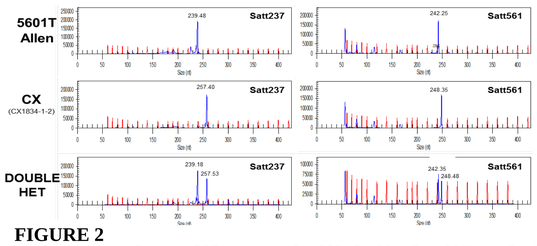
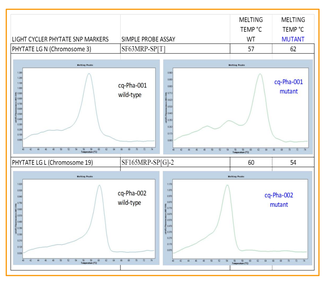


FIGURE 2
Simple Sequence Repeat (SSR) markers Satt237 (Gm03 cqPha-001) and Satt561 (Gm 19 cqPha-002) were used to confirm seven double heterozygotes.



SNP Melting Curves of the two low phytate alleles and two wild type alleles. The presence of the two low phytate loci in all BC_5 derived lines was confirmed using perfect SNP molecular markers designed from DNA sequence data (Gillman et al., 2009) at the confirmed QTL cqPha-001 and cqPha-002 to ensure that the BC_5 low phytate plant progenies were double homozygous recessive for the two alleles that express low phytate concentration in soybean seeds.

FIGURE 4

Reactions were expected to range from nearly clear (5601T, Column 4) for normal soybeans to dark blue (CX 1834-1-2, column 1) for low phytate soybeans. The concentration of Pi was estimated from reflectance values using a Bio-Tek Powerwave XS microplate spectrophotometer plate reader set at 882nm with the reflectance readings of samples compared to those of a standards table (Column 12, **Figure 3**). Eight seeds were tested from each genotype in the study.

During the winter of 2010-2011, BC_5F_3 plants were grown in the greenhouse and screened for the two low phytate alleles. Plants that were confirmed to be homozygous recessive using SNPs at phytate loci cqPha-001 and cqPha-002 (**Figure 4**) were planted in $BC_5F_{3:4}$ rows at ETREC in the summer of 2011. 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 assessed as seed increase rows at Homestead, FL. 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 low phytate CX alleles in the tagged plants. The $BC_5F_{3:5}$ rows at Homestead, FL were individually bulk harvested and sent back to Knoxville, TN. This was the seed source for replicated field trials in 2012 and 2013

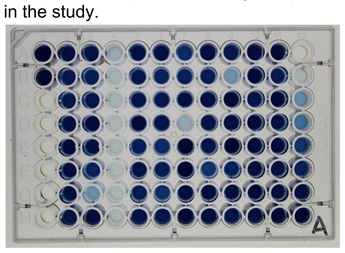


FIGURE 3
Inorganic phosphorous (Pi) concentrations were determined using a modified version of a colorimetric assay developed by Raboy et al. (2000) which was an adaption of the assay described by Chen et al. (1956)

RESULTS

The Dt1 Locus linkage to the cqPha-002 allele on Gm19:

One of the primary goals of this study was the development of an agronomically superior and commercially acceptable low phytate cultivar because none exist to date, despite the efforts of many plant breeders. In our BC_5 derived material, the indeterminate stem termination trait was inherited from line CX 1834-1-2 through the original low phytate plant mutation M153 created by Wilcox et al. (2000). However, our choice of adapted recurrent parent (RP) is the determinate MG V cultivar 5601T. The indeterminate trait is not desirable for Southern USA production in a MG V genetic background because indeterminate MG V plants continue to grow very tall and usually lodge easily which lowers yield. With our newly developed 56CX lines at the BC_5 stage, it is theoretically expected that over 98% of the genome should be consistent with that of the recurrent parent 5601T. At the previous backcross generation, the BC_4 stage, our low phytate line TN09-239 appeared to have an undesirable indeterminate growth habit (**Figure 5**). The dominant Dt1 allele on Gm19 is linked (<20cM) to the recessive phytate allele cqPha-002, which continued to thwart our efforts to create a MG V determinate low phytate conversion line of 5601T . Dr. Bilyeu (unpublished), designed a new Dt1 SNP marker based on DNA sequence data of the cloned Dt1 gene which enabled us to test and confirm which individual BC_5F_3 single plants contained the recessive (dt1dt1) genotype. All of the low phytate lines in this study were screened with that new Dt1 locus SNP marker to guide selections of MG V determinant plants that break the genetic linkage between the Gm19 phytate locus and the Dt1 stem termination locus.

Figure 5
Breaking the Linkage: Donor parent TN09-239 exhibiting an indeterminate (Dt1Dt1) growth habit (132 cm) on the right contrasted with BC_5 line 56CX-1274 exhibiting a determinate (dt1dt1) Growth habit (76 cm) on the left.

Table 1. Four highest performing BC_5 LP lines compared with high yield parent 5601T

BC5 Line	Yield kg ha^{-1}	Lodging (score)	Height (cm)	Field Emergence (%)	Germ 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	4227	2.90	93.5	73.9	96	134.9
LSD α = .05	† NS	1.04	† NS	† NS	† NS	1.2

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

Evaluation of Agronomic Traits and Seed Quality Traits of Twelve New BC5 Low Phytate Lines In Comparison with Their Parents 5601T and TN09-239 with High Yield Check Cultivars ***(Table 1)***

Yield: 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).

Lodging and Height: The *determinate* BC_5 genotypes had a mean lodging score of (1.8) which was also significantly better ($p<0.0001$) than both the RP 5601T (2.9) and donor parent TN09-239 (3.3). 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). This provided initial evidence that the determinate trait improved agronomic performance. The data for recorded plant **height** was a clear indication of which low phytate $BC_{5\ line}$ was determinate or indeterminate: the mean for the indeterminate plants (152 cm) was significantly taller ($p<0.05$) than the mean of the determinate plants (91 cm) (Figure 5).

Field Emergence: While seed germination rates are an indicator of seed viability, the ability to germinate and emerge through the soil surface is critical for stand establishment in the field. 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%) was slightly higher than 56CX-1283 (73%), 56CX-1275 (72%), 56CX-1283 (73%), 56CX-1287 (71%) and 56CX-1282 (70%).

Germination Rates: The results of the germination test conducted in 2014 from seeds harvested in 2013 also revealed no significant differences ($p>0.05$) between any of the BC5 LP lines and 5601T, TN09-239, Ellis or Osage. The mean germination rate of all of the BC5 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.

Maturity: 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 BC5 LP lines were 56CX-1273 (133.3 d) and 56CX-1282 (133.3 d), which were two of the higher yielding determinate BC5 lines.

Protein Content:
An important seed quality trait of 5601T is its seed protein concentration value which typically is higher than average cultivars and routinely qualifies 5601T as exceeding 48% protein in its soymeal. 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 BC5 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}) were not significantly different ($p>0.05$) an indication that our new low phytate lines will produce favorable high protein soymeal.

Table 2
Summary of the seed quality trait means of four promising BC5 determinate (*dt1*) low phytate genotypes in comparison to recurrent parent (RP) 5601T

BC5 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	51.0%
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.

This study showed that new LP lines clearly expressed significantly elevated seed Pi compared to the normal check cultivars, regardless of growing location **(Table 2)**. Moreover, this study documents (for the first time) that new LP lines 56CX-1273 and 56CX-1283 produce seed yields (4107 kg ha-1 equivalent to check cultivars. Our goal would be to produce ultra-high protein soymeal (>50%) from new high yielding LP lines.

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