Characterization of Soybean Germplasm with Modified Phosphorus and Sugar Composition

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ABSTRACT

The development of soybean [*Glycine max* (L.) Merr.] cultivars with modified phosphorus (P) composition has nutritional and environmental benefits, but poor seed germination and emergence presents challenges for commercial production. Different genetic mutations in two sources of germplasm, CX1834 and V99-5089, decrease the phytate and increase the inorganic phosphorus (Pi) content of seed. In V99-5089, a mutation in the D-*myo*-inositol 3-phosphate synthase 1 gene (*MIPS1*) also results in elevated sucrose content with a concomitant decrease in raffinose and stachyose content, further improving the nutritional value of soybean meal. Prior to the release of V99-5089-derived germplasm, germplasm with the *MIPS1* mutation was characterized and compared to CX1834-derived germplasm to determine the effects of this mutation on agronomic and seed composition traits in multiple environments.

The correlations between P and sugar seed composition traits were favorable for improving the nutritional composition of soybean. Lack of genotype × environment interaction for sugar traits allows for selection in one growing environment. Despite the significant genotype × environment interaction for phytate and Pi, lines with the MIPS1 gene could readily be distinguished from normal phytate lines, even in unfavorable environments. Phenotypic selection for seed Pi content was more effective than marker assisted selection with the Satt453 marker. The CX1834-derived lines were lower for phytate and higher for Pi content compared to the V99-5089-derived lines. The use of subtropical winter nursery environments for population development resulted in significant reductions in emergence of low phytate genotypes, skewing segregation ratios and prohibiting the analysis of agronomic traits. Emergence was significantly

affected by genotype, environment, and the genotype × environment interaction in three emergence tests of advanced low phytate lines. Emergence of modified lines was reduced but some were in a range that would not prohibit commercialization of P modified cultivars. Yields of the best emerging lines were not significantly different from the control cultivars. The results of this study indicate that the development of commercial cultivars with the V99-5089-derived *MIPS1* mutation is possible but breeders and producers must focus attention on emergence during population development and seed production to emphasize selection of lines with high emergence potential.

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ATTRIBUTIONS

Several colleagues have assisted with the completion of the research in several chapters of this dissertation. The following is a brief description of their involvement and contribution.

Katy Martin Rainey - Crop and Soil Environmental Sciences Department

Dr. Rainey served as the major adviser and provided expert advice and guidance during the implementation and completion of the research project and dissertation.

M. Luciana Rosso - Crop and Soil Environmental Sciences Department

As a member of the soybean research group, Dr. Rosso assisted with data collected for chapters II, III, and IV by providing laboratory assistance to quantify sugar, phytate, and molecular data. Dr. Rosso also assisted with the preparation of the materials and methods section of the manuscript and provided valuable feedback during the editing process.

Chao Shang - Crop and Soil Environmental Sciences Department

As a senior research associate, Dr. Shang provided the high pressure ion chromatography protocol used in chapters III and IV to determine phytate concentration.

I. Introduction

The development of soybeans [*Glycine max* (L.) Merr.] with modified composition is an ongoing objective in the soybean research community. Recently, attention has focused on improving the nutritional quality of soybean meal through the genetic modification of phosphorus (P) and sugar composition in soybeans. Although, the development of soybean with modified composition has nutritional and environmental benefits, the poor emergence associated with the trait presents challenges for commercial production. The commercialization of soybean cultivars with modified seed composition depends on grower acceptance of agronomic traits. Adoption by the feed industry depends on uniformity and stability of modified composition traits as expressed over environments. Therefore, in the genetic improvement of soybeans, it is important to understand the impact of modified composition traits on agronomic traits and the stability of modified composition when produced in multiple environments.

The major storage form of P in seeds comprising about 70% of the total P in mature soybean seeds is phytic acid (*myo*-inositol 1,2,3,4,5,-hexa*kis*phosphate) (Raboy et al., 1984). However, the P in phytate, the mixed cation salt of phytic acid, cannot be digested by monogastric animals resulting in the need to modify feed to improve P nutrition. The development of soybean lines with a decrease in phytate content results in a concomitant increase in nutritionally available inorganic phosphorus (Pi) content (Hitz et al., 2002; Wilcox et al., 2000; Yuan et al., 2007). Therefore, the commercial acceptance of soybean lines with modified P composition could improve the value, nutrition, and sustainability of soybean meal.

Soybean seeds consist of approximately 33% carbohydrates; the primary sugars in soybeans are sucrose, raffinose, and stachyose. Sucrose is a beneficial component of soybeans because it is readily digested by monogastric animals (Karr-Lilienthal et al., 2005). Conversely,

the presence of raffinose and stachyose in soybean meal is detrimental because they are not easily digested by monogastric animals and decrease the metabolizable energy that can be obtained from soybean meal (Karr-Lilienthal et al., 2005). The development of soybean lines with modifications to sugar content by increasing sucrose levels and decreasing raffinose and stachyose would improve the metabolizable energy of soybean meal (Parsons et al., 2000).

Multiple sources of soybean germplasm with modified P and sugar composition have been identified. Soybean breeders have used the publicly available CX1834 germplasm to develop cultivars with modified P content. Low phytate in the CX1834 germplasm line developed by Wilcox et al. (2000) is the result of two recessive genes (Gillman et al., 2009; Oltmans et al., 2004; Walker et al., 2006). Modifications of both P and sugar composition simultaneously in soybean are the result of mutations in the D-*myo*-inositol 3-phosphate synthase 1 (*MIPS1*) gene. Three soybean lines with different mutations in the *MIPS1* gene have been discovered including LR33 (Hitz et al., 2002; Sebastian et al., 2000), *Gm-lpa*-TW-1 (Yuan et al., 2007), and V99-5089 (Saghai Maroof and Buss, 2008). Mutations in the *MIPS1* gene result in increased levels of Pi and sucrose and reduced levels of phytate, raffinose, and stachyose. However, reduced emergence has been reported in all modified P lines (Oltmans et al., 2005; Sebastian et al., 2000; Yuan et al., 2007) and the seed production environment has been implicated as a contributing factor for reduced emergence (Anderson and Fehr, 2008; Meis et al., 2003; Yuan et al., 2007).

Extensive research has been completed to evaluate emergence in CX1834-derived germplasm and the effect of the LP trait on yield (Anderson and Fehr, 2008; Hulke et al., 2004; Oltmans et al., 2005; Spear and Fehr, 2007; Trimble and Fehr, 2010). However, limited data is available regarding the stability of phytate and Pi content across multiple environments. The

evaluation of CX1834-derived lines in a multi-location trial to evaluate P composition provides information for the acceptance of modified P composition soybeans by the feed industry. Additionally, comparisons between CX1834- and V99-5089-derived germplasm in multiple environments provide additional information in the selection of a germplasm source to use in the modification of P composition.

Limited multi-location data is available for the characterization of V99-5089 germplasm which contains a novel mutation in the *MIPS1* gene and is the only *MIPS1* mutation in the public sector. In V99-5089, 46% of total P was in the form of phytate compared to 76% for Essex (Gao et al., 2008). Huhn (2003) reported a 3:1 segregation ratio for the high to low stachyose phenotype in two V99-5089 populations indicating a single recessive gene. In the evaluation of F_2-derived lines by Saghai Maroof and Buss (2008), correlation values were highly significant between phytate and the sugar components of sucrose (-0.87), raffinose (0.85) and stachyose (0.88). In addition, a simple sequence repeat (SSR) marker (Satt453) was shown to identify the low stachyose genotype of V99-5089 when evaluated in F_2-derived lines. The characterization of V99-5089-derivded germplasm for agronomic and seed composition traits provides needed information prior to public release and commercial acceptance.

Objectives

The characterization of the V99-5089 germplasm is necessary to assist plant breeders who will use it in their breeding programs and to provide information for adoption by the commercial sector. Ultimately, incorporating *MIPS1* mutations into commercial cultivars depends on the ease of phenotypic and genotypic selection, the stability of the seed composition traits, and the effect of the mutation on agronomic traits. To facilitate development of commercial germplasm with the *MIPS1* mutant gene the objectives were to: (i) correlate seed composition traits; (ii) estimate heritability for Pi content; (iii) validate Satt453 for marker assisted selection; (iv) evaluate environmental effects on P and sugar composition; (v) determine the stability of P and sugar composition; (vi) evaluate agronomic traits; and (vii) determine the relationships between seed composition and agronomic traits. In the comparison of advanced lines derived from CX1834 and V99-5089 germplasm the objectives were to (i) evaluate the effects of genotype, environment, and genotype × environment interaction on P composition, emergence, and yield; (ii) determine the stability of phytate and Pi composition across multiple environments; and (iii) evaluate the effect of temperate seed production environment on emergence of low phytate lines. The research questions addressed will assist with understanding the associated effects of the *MIPS1* mutant germplasm on agronomic and seed composition traits in multiple growing environments.

II. Environmental Effects and Stability of Soybean with Modified Phosphorus and Sugar Composition

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Abbreviations: HPLC, high-performance liquid chromatography, *MIPS1*, D-*myo*-inositol 3-phosphate synthase 1 gene; mips, *myo*-inositol phosphate synthase mutant lines with modified seed composition; MAS, marker assisted selection; P, phosphorus; Pi, inorganic phosphorus; RIL, recombinant inbred line; RFOs, raffinose family oligosaccharides; SSR, simple sequence repeat; WT, wild-type lines with normal seed composition.

ABSTRACT

Development of soybeans [*Glycine max* (L.) Merr.] with modified phosphorus (P) and sugar composition adds value to soybean meal. The identification and release of the soybean line V99-5089 with a mutation in the D-*myo*-inositol 3-phosphate synthase 1 (*MIPS1*) gene provides a germplasm source that dually enhances both P and sugar composition of soybeans. An evaluation of the recombinant inbred line population from the cross of V99-5089 × Essex was completed at three locations in two years. Phenotypic selection for modified composition was slightly more effective than marker assisted selection with the Satt453 marker which was 87% efficient. In the mutant lines, a significant genotype × environment interaction was shown for inorganic phosphorus (Pi) content but not sugar content. Mutant lines with high inorganic phosphorus (Pi) and sucrose content were average for trait stability. Correlations for improving P and sugar composition were favorable, and with the exception of emergence there were no strong correlations with agronomic traits. The correlation of emergence with Pi was significant (r = -0.59), indicating reduced emergence due to the mutation. Reduced emergence is a problem in low phytate germplasm generally and further research is necessary to genetically improve emergence of low phytate lines.

INTRODUCTION

Breeding soybean [*Glycine max* (L.) Merr.] cultivars with modified phosphorus (P) and sugar composition can improve the nutritional quality of soybean meal. However, for commercialization of soybeans with modified seed composition it is important to develop cultivars with acceptable agronomic traits including emergence and yield in combination with consistent seed composition across multiple environments. Therefore, it is important to understand the genotypic, environmental, and genotype × environment interaction effects on seed with modified P and sugar composition.

The major seed storage form of P comprising about 70% of total P (Raboy et al., 1984) in conventional soybean seed is phytate, a mixed cation salt of phytic acid (*myo*-inositol 1,2,3,4,5,6-hexa*kis*phosphate). Phytate is considered antinutritional because it binds essential mineral nutrients, reducing their availability. In addition, phytate is not easily digested by monogastric animals resulting in decreased available P in diets and increased P in the form of phytate released into the environment. To solve the problem of phytate, producers supplement feed with additional inorganic phosphorus (Pi) to increase the available P, or add phytase enzyme to improve digestion of intrinsic phytate in soybeans. Both solutions increase feed costs, and the addition of Pi to feed may intensify environmental degradation from continued presence of phytate in animal waste. The development of soybean lines with a concomitant increase in Pi and decrease in phytate could improve the value, nutrition, and sustainability of soybean meal.

In the analysis of breeding lines with conventional P content, Israel et al. (2006) reported significant genotypic, environmental, and genotype × environment interaction effects for Pi content, but only genotypes were significant in the analysis of phytate. Raboy and Dickinson (1993) studied twelve lines in three environments and concluded that only the main effects of

environment and genotype were significant for phytic acid, and no effects were significant for non-phytic acid P. Increases in phytate and Pi occurred when lines with modified P content were grown in subtropical conditions but the factors influencing P composition were unclear (Anderson and Fehr, 2008). In growth chamber experiments, total P content increased as temperatures increased (Thomas et al., 2003) whereas, phytic acid remained unchanged when grown in high temperatures (Ren et al., 2009). Increases in external P supply increased Pi but did not influence phytic acid in lines with modified P content (Israel et al., 2007) indicating higher trait stability for phytic acid.

Soybean seeds consist of approximately 33% carbohydrates; the carbohydrates of interest are the soluble sugars consisting of sucrose and the raffinose family oligosaccharides (RFOs) including raffinose and stachyose. Sucrose is a beneficial component of soybeans because it is 100% digestible by monogastric animals including humans, poultry, and swine (Karr-Lilienthal et al., 2005). Conversely, the presence of RFOs decrease the metabolizable energy that can be obtained from soybean meal because they are poorly digested by monogastric animals (Karr-Lilienthal et al., 2005). The development of soybean lines with an increase in sucrose and a concomitant decrease in RFOs improves the feeding efficiency of soybean meal.

Multiple reports have shown non-significant genotype × environment interactions for individual sugar components including sucrose, raffinose, and stachyose (Cicek et al., 2006; Hymowitz et al., 1972; Openshaw and Hadley, 1981; Wilcox and Shibles, 2001). However, these studies reported significant genotypic and environmental effects on sugar content in soybean. In growth chamber experiments, environmental conditions affect sugar composition with increases in temperature generally causing decreases in sugar components (Ren et al., 2009; Thomas et al., 2003; Wolf et al., 1982).

Soybeans modified for P content are a dual benefit for the feed industry because there is an inverse relationship between Pi and phytate (Gao et al., 2008; Hitz et al., 2002; Wilcox et al., 2000; Yuan et al., 2007). Multiple sources of soybean germplasm with modified P or sugar composition, or both P and sugar composition, have been identified. Low phytate in the CX1834 germplasm line developed by Wilcox et al. (2000) is the result of two recessive genes (Gillman et al., 2009). Reduced seedling emergence has been associated with the low phytate trait in this germplasm (Anderson and Fehr, 2008; Oltmans et al., 2005) but a recent publication indicates that genetic improvement of seedling emergence is possible (Spear and Fehr, 2007). A novel unknown mutation in the low phytate soybean *Gm-lpa*-ZC-2 was created by mutagenesis in which the emergence percentage of the mutant line was similar to its progenitor (Yuan et al., 2007). Sebastian et al. (2000) identified PI200508 with reduced levels of raffinose and stachyose content with normal emergence (Neus et al., 2005; Sebastian et al., 2000). Although these germplasm sources are beneficial for improving soybean seed composition, the combined modification of P and sugar composition offers more value to the feed industry.

Modifications of both P and sugar composition simultaneously in soybean are the result of mutations in the D-*myo*-inositol 3-phosphate synthase 1 (*MIPS1*) gene, and have been discovered in three soybean lines, LR33 (Hitz et al., 2002; Sebastian et al., 2000), *Gm-lpa*-TW-1 (Yuan et al., 2007), and most recently V99-5089 (Saghai Maroof and Buss, 2008). Mutations in the *MIPS1* gene result in increased levels of Pi and sucrose and reduced levels of phytate, raffinose, and stachyose. Reduced emergence has been reported in these lines (Sebastian et al., 2000; Yuan et al., 2007) and the seed production environment has been implicated as a contributing factor for reduced emergence (Meis et al., 2003; Yuan et al., 2007).

Line V99-5089 contains a novel mutation in the *MIPS1* gene which results in seed composition similar to those reported for LR33 and *Gm-lpa*-TW-1, and is the only *MIPS1* mutation in the public sector. In V99-5089, 46% of total P was in the form of phytate compared to 76% for Essex (Gao et al., 2008). Huhn (2003) reported a 3:1 segregation ratio for the high to low stachyose phenotype in two V99-5089 populations indicating a single recessive gene. In the evaluation of F_2-derived lines by Saghai Maroof and Buss (2008), correlation values were highly significant between phytate and the sugar components of sucrose (-0.87), raffinose (0.85) and stachyose (0.88). In addition, a simple sequence repeat (SSR) marker (Satt453) was shown to identify the low stachyose genotype of V99-5089 when evaluated in F_2-derived lines.

Incorporating *MIPS1* mutations into commercial cultivars depends on the ease of phenotypic and genotypic selection, the stability of the seed composition traits, and the effect of the mutation on agronomic traits. To facilitate development of commercial germplasm with the *MIPS1* mutant gene conditioning the high Pi, low phytate, high sucrose, low raffinose, and low stachyose phenotype, the objectives were to characterize recombinant inbred lines (RIL) developed from V99-5089 to (i) evaluate Satt453 for marker assisted selection, (ii) evaluate environmental effects on P and sugar composition, (iii) determine the stability of seed composition, and (iv) evaluate the relationships between seed composition and agronomic traits.

MATERIALS AND METHODS

Field Experiment

The RIL population was developed from a cross between V99-5089 and Essex. V99-5089 is a Virginia Tech experimental line with high Pi, high sucrose, low phytate and low raffinose oligosaccharide composition due to a novel mutation in the *MIPS1* gene (Saghai Maroof and Buss, 2008) while Essex is a commercial cultivar with normal seed composition (Smith and Camper, 1973). Both parental lines are of mid-V maturity. The RIL population was developed from different F_2 plants by single seed descent (SSD) method with a total of 178 RILs consisting of two subpopulations with 85 and 93 RILs. The second subpopulation was started one year later to expand the initial population. The SSD method was used until the F_4 (93 RILs) and F_5 (85 RILs) generations and in 2006 seed for the 2008 experiment were harvested in bulk from each F_4 and F_5 row in Blacksburg, VA (M.A. Saghai Maroof, personal communication, 2010).

The 178 RILs and both parents were planted in a randomized complete block design with two replications at Blacksburg, VA and an early and late planting at Warsaw, VA, in 2008 and 2009. In Blacksburg, the experiment was planted on 5 June 2008 and 23 May 2009, at the Virginia Tech Kentland College Farm. In Blacksburg, VA the plots were fertilized with a 0-60-60 fertilizer in 2008 and a 40-100-100 fertilizer in 2009. Two planting dates each year in Warsaw were considered as separate environments. At the Eastern Virginia Agricultural Research and Extension Center in Warsaw, the first planting date was during the normal planting season (Warsaw early) on 28 May 2008 and 20 May 2009 and the second planting date was later in the season (Warsaw late) on 1 July 2008, and 2009. In Warsaw, VA the plots were fertilized with a 0-80-80 fertilizer in 2008 and a 0-90-60 fertilizer in 2009. Each parent and RIL was

grown in a single row plot, 0.9 m long with 0.8 m row spacing. In 2008, 10 seeds of each RIL were planted and in 2009, 20 seeds were planted using 2008 Warsaw early environment seed.

At Warsaw, data were collected on all plots for seedling emergence, maturity, plant height, lodging, seed size, seed quality, and Pi, sucrose, raffinose, and stachyose content. Seedling emergence, maturity, seed size, and seed quality were collected for all plots from Blacksburg. Based on seed composition data collected from Warsaw, a subset of 10 *myo*-inositol phosphate synthase mutant RILs with modified seed composition (mips) and 10 wild-type RILs with normal seed composition (WT) were randomly selected for Pi, sucrose, raffinose, and stachyose determination from the Blacksburg environments.

Percent seedling emergence was determined approximately 21 days after planting. Maturity was recorded as days after planting when 95% of the pods in a plot had reached their mature color. At maturity, both plant height from the soil surface to tip of the main stem and lodging on a scale of 1 (all plants erect) to 5 (all plants prostrate) were measured. All plants in each row were bulk harvested by hand and threshed with a stationary thresher in the field. Seed size was recorded as the weight of 100 random whole seeds from each plot. Seed quality was rated on a scale of 1 (seed surface smooth with no discoloration) to 5 (seed wrinkled with severe discoloration). For seed composition analysis, a 25 g subsample from each plot was ground in an Udy cyclone mill (U.D. Corp., Boulder, CO) to pass through a 1.0 mm screen.

Seed Composition Analysis

Inverse relationships between phytate and Pi have been reported for three low phytate germplasm sources in soybean (Hitz et al., 2002; Wilcox et al., 2000; Yuan et al., 2007). The experimental line V99-5089 is low phytate with normal levels of total P (Gao et al., 2008) so an inverse relationship was assumed between phytate and Pi. A modified version of the

colorimetric assay developed by Raboy et al. (2000) adapted from the assay by Chen et al. (1956) was used to determine Pi concentration. The extraction and quantification methods for Pi were as described by Scaboo et al. (2009) with Pi concentrations determined using a BioTek Synergy HT plate reader (BioTek Instruments, Winooski, VT) set at a wavelength of 882 nm. The final Pi content of each plot was calculated from an average of three subsamples using the initial weight of each sample to calculate the Pi concentration on a dry weight basis with results reported as micrograms per gram.

Sucrose, stachyose, and raffinose content were determined by high performance liquid chromatography (HPLC) as described by Cicek et al. (2006). Extracted sugar samples were stored in the freezer at -20 °C until HPLC could be completed. As an internal check, V99-5089 was included with each set of samples during extraction and quantification. The final concentration of each sugar was calculated on a dry weight basis.

DNA Extraction and Marker Analysis

One set of young trifoliolate leaves from three randomly selected plants of each RIL and parental line was sampled from one replication of the Warsaw early environment in 2008 and the Blacksburg environment in 2009. DNA extraction was performed using Plant DNeasy Mini Kit (Qiagen, Valencia, CA). The samples were screened with the SSR FAM-labeled marker Satt453 (LG B1) developed by Cregan et al. (1999). PCR reactions for the SSR assay were performed in a total volume of 12.5 µl in a Bio-Rad C1000 (Bio-Rad, Hercules, CA) thermal cycler. The initial denaturing step, 5 min at 95 °C, was followed by 40 cycles of 30 s at 94 °C, 40 s at 47 °C, 30 s at 72 °C, and then by a final extension step for 7 min at 72 °C. Genotypes were visualized by a 3130x*l* Genetic Analyzer (Applied Biosystems, Carlsbad, CA). The software Mapmaker 3.0 was used to construct a linkage map between Satt453 marker and the *MIPS1* gene using the

maximum likelihood method to estimate the recombination distance and marker order with a log-likelihood threshold of 3.0 (Lander et al., 1987).

Statistical Analysis

Preliminary data analysis showed no significant difference between the two RIL subpopulations (data not shown) therefore subpopulation was not included in the analyses. RILs heterozygous at the Satt453 locus were removed from the dataset because we were interested in the stability and effects of genotype and environment on homozygous genotypes.

Pi, sucrose, raffinose, and stachyose data were logarithmically transformed by $\ln = \log_e$ to normalize distribution of residuals and remove heterogeneity of error variances. The SAS procedure PROC MIXED (SAS, 2009) was used to conduct analysis of variance for Pi, sucrose, raffinose, and stachyose content. The RILs separated into two distinct phenotypic classes and a separate analysis of variance was computed for each phenotypic class in which genotype, environment, and the interaction were considered fixed effects and replication within environment was considered a random effect. Pairwise comparisons of means were made using Tukey's multiple means comparison method. Phenotype correlation coefficients among traits were calculated with the RIL means across environments using the PROC CORR procedure in SAS (SAS, 2009). Analysis of variance was completed to determine differences for emergence between phenotype classes. Phenotype, environment, and phenotype × environment interaction were considered fixed effects and replication within environment was considered a random effect. Finlay and Wilkinson's (1963) regression analysis was completed with the SAS procedure PROC REG (SAS, 2009). Regression coefficients (b) were derived by a linear regression of mean of each RIL on the average mean of all genotypes at each environment.

RESULTS AND DISCUSSION

Based on Pi, sucrose, raffinose, and stachyose data from four Warsaw environments, 153 RILs were classified into two distinct phenotypic classes: *myo*-inositol phosphate synthase mutant lines (mips) with modified seed composition and wild-type lines (WT) with normal seed composition (Table 1). All RILs within the mips class were high Pi (2139 µg g^{-1}) and high sucrose (9.1%), compared to WT RILs with low Pi (365 µg g^{-1}) and low sucrose (5.2%) content. The mips RILs were lower in raffinose (0.33%) and stachyose (0.26%) content than WT RILs (0.75% and 3.58%, respectively). The ability to clearly distinguish RILs into distinct bimodal phenotypic classes with either Pi or sugar composition data demonstrates the effectiveness of phenotypic selection for improved seed composition within *MIPS1* germplasm.

Marker Assisted Selection

The Satt453 marker was previously shown to differentiate the low stachyose phenotype in F_2-derived lines from V99-5089 × Essex (Saghai Maroof and Buss, 2008) and we evaluated Satt453 in RILs to determine the feasibility of marker-assisted selection (MAS). Selection efficiency is determined by the percentage of mips lines selected with the V99-5089 allele at the Satt453 marker. In our study, marker analysis identified 76 RILs homozygous for the V99-5089 mutant allele at Satt453, but 10 of those RILs were categorized as WT with phenotypic data, resulting in a marker selection efficiency of 87% (Table 1). MAS did not identify all possible low phytate lines because 10 RILs were characterized as mips by phenotype but were identified as homozygous for the Essex allele at Satt453 with MAS. Additionally, the *MIPS1* gene mapped 8.1 centimorgans from Satt453. These results indicate that recombination between the *MIPS1* gene and the SSR marker was responsible for the reduced selection efficiency. In the validation of two SSR markers in two CX1834 populations by Scaboo et al. (2009), MAS identified all low

phytate individuals but was only 50% accurate because half the lines identified with MAS were not compositionally low phytate. Molecular marker assays to directly select for the mutant alleles in CX1834 germplasm have been developed (Gillman et al., 2009) and similar perfect markers are needed for V99-5089.

Genotype × Environment Interaction

To determine the effect of genotype, environment, and the interaction on Pi, sucrose, raffinose, and stachyose, a full analysis was completed with 146 RILs grown in four environments including Warsaw early 2008 and 2009 (WE2008 and WE2009) and Warsaw late 2008 and 2009 (WL2008 and WL2009). Of the original 153 RILs, seven mips RILs were removed from the dataset due to non-estimable lsmeans for environment because of missing values due to poor emergence in 2008.

Variation among genotypes, environments, and the genotype × environment interaction were significant for Pi content in mips RILs (Table 2a). Pi content in the mips RILs was the only seed composition trait in which the genotype × environment interaction was significant. The interaction, although statistically significant appears less important than the environmental or genotypic effects. Pi content in all mips RILs in the WE2008 environment was significantly lower than other environments (Table 3). The three remaining environments were not significantly different and genotypes within those environments were generally similar. The factors influencing Pi content in the different environments are not immediately clear. In the evaluation of CX1834 germplasm, Israel et al. (2007) reported increases in Pi with increasing P availability. Although, tests for soil P availability were not completed, differences between WE2008 and the WE2009 and WL2009 environments could be the result of P availability. However, the WE2008 and WL2008 experiments were in the same field so the factors reducing

Pi content in WE2008 are unclear. Regardless of environmental effects on Pi content, phenotypic selection in one environment will accurately identify lines with the mips phenotype.

The genotype × environment interaction was not significant for sugar composition (Table 2a), which agrees with previous work on various germplasm types (Cicek et al., 2006; Hymowitz et al., 1972; Openshaw and Hadley, 1981). Environmental effects were significant for all sugars in the mips RILs, and significant only for sucrose and raffinose for WT RILs. The WE2008 environment was the lowest for sucrose content for both mips and WT RILs which is in agreement with Pi content (Table 3). Differences between environments for raffinose and stachyose content were not clearly defined. The WE2008 environment was the highest for raffinose and stachyose content in the mips RILs, although differences were not statistically significant in all other environments.

Genotypic effects were significant for all traits in both the mips and WT analyses, with the exception of stachyose in the mips analysis (Table 2a). Lack of significance for stachyose in mips lines is expected because the *MIPS1* mutation reduces stachyose content significantly and the stachyose range for genotypes was only 0.13% to 0.48%, as compared to the WT genotypes which ranged from 2.97% to 4.50%. Our results support previous reports that genotypic and environmental effects were significant for sugar composition traits including sucrose, raffinose and stachyose (Cicek et al., 2006; Hymowitz et al., 1972; Wilcox and Shibles, 2001).

A subset of 10 mips and 10 WT RILs were analyzed from six environments with two additional environments, Blacksburg in 2008 (BB2008) and 2009 (BB2009) to further evaluate the environmental effect on Pi, sucrose, raffinose, and stachyose content. For Pi composition, the analysis of variance results (Table 2b) were similar to the full RIL analysis in four environments (Table 2a). For the mips lines, the two Blacksburg environments were similar to

each other for Pi content in the mips analysis, the BB2008 environment was equivalent to the high Pi environments (WE2009, WL2008, and WL2009), and the BB2009 environment was not significantly different from the low WE2008 environment (Table 4). Differences were reported by Anderson and Fehr (2008) in which increases in phytate and Pi were observed in CX1834 seeds produced in subtropical environments implicating conditions of the seed production environment as a factor influencing Pi and phytate content. There were no obvious relationships between Pi content with temperature or precipitation for the six environments in our analysis (Table 4). Increasing levels of external P availability was shown by Israel et al. (2007) to increase the Pi content of a CX1834-derived low phytate line. Therefore, it is likely that environmental differences observed in the mips lines are due to differences in soil P availability.

In the analysis of sugar composition for the WT RILs, the analysis of variance results were similar for the full analysis of 146 RILs in four environments and the subset analysis of 20 RILs in six environments (Table 2). However, in the evaluation of mips RILs there were differences between the full and subset analysis for sugar composition. In the full analysis of mips RILs, genotypic and environmental effects significantly affected raffinose content. This differs from the subset analysis of mips lines in which there were no significant effects for raffinose content (Table 2b). In the full analysis of mips RILs, the environmental effects significantly affected stachyose content which differs from the subset analysis in which environmental effects were not significant (Table 2). Additionally, in the full analysis of mips RILs, genotypic differences were not significant but in the subset analysis genotypic differences were significant for stachyose content. This significant result for the genotypic effect was due to the presence of one RIL with a mean stachyose content of 0.54% compared to the remaining nine RILs with a range of 0.22-0.35% stachyose content. The significant environmental effects in the

full analysis of raffinose and stachyose content in the mips RILs were due to the WE2008 environment. The WE2008 was highest for raffinose and stachyose content making it less favorable for production of soybeans with modified sugar composition. In the overall evaluation of raffinose and stachyose content, the mips RILs remained distinct from the WT lines regardless of environmental effects allowing for selection of lines with improved raffinose and stachyose content in one environment.

In the analysis of sucrose content in mips RILs, the genotypic and environmental effects were significant in both the full and subset analysis (Table 2). The significant variation among genotypes allows for selection of lines with higher sucrose content from within the mips RILs. In the subset analysis of mips RILs in six environments, a general trend was observed in which the environmental means of sucrose content decreased as the mean maximum and minimum temperatures in the 30 days before maturity increased (Table 4). The WE2008 environment was the lowest for sucrose content and the highest for mean maximum temperature in the 30 days prior to maturity. In contrast, the Blacksburg environments characterized by cooler mean maximum and minimum temperatures in the 30 days before maturity produced mips and WT lines with higher sucrose content. Our results agree with previous research in which sucrose content decreased with increased temperatures (Thomas et al., 2003; Wolf et al., 1982).

Stability

Stability analysis for Pi and sucrose content was calculated using the regression coefficient (*b*) for the subset of 20 RILs in six environments (Table 5). Lines with low regression coefficients and high R^2 values are the most stable for seed composition. According to Finlay and Wilkinson (1963), a *b* value less than 1 is unresponsive to different environments, equal to 1 indicates average stability, and greater than 1 is responsive to favorable environments.

The characterization of a line as unresponsive to different environments results in an above average stability whereas a line responsive to favorable environments is below average for stability. To better differentiate RILs for trait stability, they were characterized as above average if *b* was less than 0.7, average if *b* was between 0.7 and 1.3, and below average if *b* was greater than 1.3 for stability (Lin and Binns, 1985).

For the WT RILs, stability analysis was not informative because Pi content did not vary due to environment (Table 2, 3, and 4) or genotype (Table 5) indicating an overall stability. In the analysis of mips RILs for Pi stability, R^2 values were high for most genotypes with six RILs significant ($P < 0.05$) for stability (Table 5). Four lines were average for stability with *b* values ranging from 0.77 – 1.20 and two lines were below average for stability with *b* values of 1.60 and 1.66. There was no relationship between stability and mean Pi content. RILs 1675 and 1739 were close to the 0.7 classification of above average stability and had the lowest *b* values (0.79 and 0.77, respectively) but differed for Pi content. RIL 1675 was ranked highest for Pi content, demonstrating the possibility of selecting lines with high Pi and average stability in mips germplasm.

In the analysis of sucrose content, R^2 values were high and all genotypes were significant for stability analysis regardless of phenotype (Table 5). All WT RILs were considered average or above average for stability with *b* values ranging from 0.63 – 1.28, demonstrating the overall stability of sucrose levels in WT germplasm. Nine mips RILs were classified as average for stability with regression values from 0.79 to 1.32, and only one RIL classified as below average for stability. The mips RILs all had high sucrose content and the identification of 9 of 10 RILs as average for stability is beneficial for selection. The RIL 1675 had average stability of sucrose content ($b = 1.04$) and also was highest for Pi content with average stability, indicating that

selection of a mips line with combined high performance and high stability in two composition traits is possible.

Correlations of Seed Components, Quality, and Agronomic Traits

Phenotypic correlation values were all significant between the seed composition traits of Pi, sucrose, raffinose, and stachyose for 153 RILs grown in four environments and 20 RILs grown in six environments (Table 6). Significant positive correlations were reported between Pi and sucrose, which were both negatively correlated with raffinose and stachyose. The correlation values for seed composition in this study are higher than those reported for V99-5089 F_2-derived lines evaluated by Saghai Maroof and Buss (2008). In other mips germplasm, Hitz et al. (2002) did not specifically estimate correlation coefficients for LR33 but reported increases in sucrose and decreases in RFOs. Our results are in agreement with the evaluation of PI200508 germplasm with reduced raffinose and stachyose content (Neus et al., 2005), and in contrast to studies of germplasm with typical sugar content. In the evaluation of 241 plant introductions, Hou et al. (2009) reported significant correlations for sucrose with stachyose (-0.68) and raffinose (0.66) and between raffinose and stachyose (0.68). The positive correlations of sucrose with raffinose and raffinose with stachyose, would make using the plant introductions for genetic improvement of all three components difficult. In the V99-5089-derived material all seed composition correlations were favorable for progress with plant breeding to improve seed composition with increases in Pi and sucrose and decreases in phytate, raffinose, and stachyose.

Correlations between seed composition and all agronomic traits were either not significant or significant but sufficiently low to allow for the development of agronomically acceptable mips lines, with the notable exception of emergence (Table 6). Our results are consistent with studies of CX1834 germplasm in which agronomic traits were not affected by the

low phytate trait and low phytate lines with acceptable agronomic traits can be developed (Oltmans et al., 2005; Scaboo et al., 2009; Spear and Fehr, 2007). The correlations of emergence with Pi (-0.59) and sucrose (-0.50) were significantly negative but not so high as to obviate selection for high Pi and sucrose content. The reduced emergence in V99-5089-derived mips germplasm is similar to results reported for other low phytate germplasm including the LR33 mips germplasm (Meis et al., 2003; Sebastian et al., 2000) and the CX1834 low phytate germplasm (Anderson and Fehr, 2008; Oltmans et al., 2005).

Emergence

There was a significant difference among phenotypes (mips and WT), environments, and the phenotype × environment interaction for emergence percentage in the analysis of 153 RILs grown in six environments (data not shown). Overall emergence for all environments was low for both phenotypes with 51% for mips RILs and 63% for WT RILs. Examination of the phenotype × environment means revealed that the mips lines were significantly lower for emergence as compared to the WT lines only in 2008 environments (Table 7). At all three environments in 2009 mips RILs were not significantly different from WT RILs. The lowest environment for emergence with the mips lines was 2008 at Blacksburg (29%). The 2008 seed source was from 2006, suggested that deterioration in seed quality over time reduced emergence. Additionally, WT RILs in WE2008 were the highest for emergence of all environments. These results suggest that mips germplasm, or low phytate germplasm generally, is more susceptible to seed deterioration.

The seed source for all environments in 2009 was WE2008, so long term seed storage was not a factor in 2009 emergence. However, emergence differences among 2009 environments were significant and the emergence at Warsaw early was extremely low with only

46% emergence for both phenotypes. Overall emergence of both mips and WT lines were higher in Blacksburg and Warsaw late with mips lines emerging 69% at Blacksburg and 71% at Warsaw late. These results suggest the impact of an environmental factor on emergence at the WE2009 environment. The amount of rainfall in the 14 days after planting was reduced in WE2009, which received 1.1 mm compared to 7.3 mm for WE2008, it is likely that the reduced rainfall contributed to the overall reduction in emergence for WE2009.

Although differences between WT and mips lines for emergence were not consistent from 2008 to 2009, we believe there is a residual problem with emergence for low phytate lines. The reduction in emergence for mips lines is likely due to the low phytate trait or another seed component modified by the mutation rather than the low raffinose and stachyose composition. Analyses of PI200508 germplasm have not reported low emergence problems (Dierking and Bilyeu, 2009; Neus et al., 2005), and Dierking and Bilyeu, (2009) concluded that raffinose oligosaccharides including raffinose and stachyose were not an essential source of energy for soybeans during seed germination. Problems with reduced emergence have been reported for all low phytate germplasm sources with both normal (Anderson and Fehr, 2008; Oltmans et al., 2005) and reduced levels of raffinose and stachyose (Meis et al., 2003; Sebastian et al., 2000). For low phytate soybeans to be acceptable agronomically, research is needed to determine the cause of reduced emergence and to genetically improve emergence of low phytate lines.

CONCLUSIONS

This study has provided an evaluation of the seed composition and agronomic traits of RILs from a novel *MIPS1* mutant germplasm source (V99-5089) with improved nutritional composition. The selection efficiency for the Satt453 marker was sufficiently high to be useful in MAS; however, the development of a perfect marker would eliminate the misclassifications due to recombination between the marker and the *MIPS1* mutant allele observed in this experiment. Phenotypic selection was consistently effective at differentiating lines, and strong correlation between Pi and sucrose allows for selection to be based on Pi assay, which is quick and easy compared to the HPLC sugar assay. Lack of genotype × environment interaction for sugar traits allows for selection in one growing environment, and although the genotype × environment interaction was significant for Pi, selection in one environment is possible because RILs did not change their relative phenotype when grown in an unfavorable environment. In addition, we have demonstrated the potential to further improve seed composition across environments by selecting for seed composition trait stability within *MIPS1* mutant germplasm. With the exception of emergence, there were no strongly significant correlations with agronomic traits, which is beneficial for line development. The problem of reduced emergence observed generally in low phytate germplasm will be a barrier in the development of commercial soybean lines with the *MIPS1* mutant gene. Additional research and germplasm development is necessary to overcome low emergence, allowing commercialization of *MIPS1* mutant germplasm.

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Table 1. Mean inorganic phosphorus (Pi), sucrose, raffinose, and stachyose content in four environments[†] for 153 recombinant inbred lines (RILs) of V99-5089 × Essex.

	Phenotype[‡]											Marker
	mips					Wild-type						Selection
	RILs	Pi	Sucrose	Raffinose	Stachyose	RILs	Pi	Sucrose	Raffinose	Stachyose	RILs	Efficiency
Marker Genotype[§]	(n)	µg g^{-1}	---------- % ----------			(n)	µg g^{-1}	---------- % ----------			(n)	%
5089/5089	66	2139	9.0	0.33	0.27	10	376	5.0	0.75	3.55	76	87
Essex/Essex	10	2139	9.1	0.33	0.26	67	354	5.3	0.75	3.62	77	87
Total / Average	76	2139	9.1	0.33	0.26	77	365	5.2	0.75	3.58	153	

† Environments included two planting dates (early and late) at Warsaw, VA in 2008 and 2009.

‡ Based on seed composition, mips = *myo*-inositol phosphate synthase mutant RILs with modified seed composition, Wild-type = RILs with normal seed composition.

§ Satt453 genotype, 5089/5089 = homozygous for V99-5089 mutant allele, Essex/Essex = homozygous for Essex WT allele.

Table 2. Analysis of variance of V99-5089 × Essex recombinant inbred line (RIL) population for seed composition[†] including inorganic phosphorus (Pi), sucrose, raffinose, and stachyose content a) full analysis of 146[‡] RILs grown at four environments[§] and b) subset analysis of 10 *myo*-inositol phosphate synthase mutant (mips) RILs and 10 wild-type (WT) RILs grown at six environments[¶].

		mips		WT		Pi		Sucrose		Raffinose		Stachyose	
		Num.[#]	Den.[#]	Num.	Den.	mips	WT	mips	WT	mips	WT	mips	WT
Analysis	Source of Variation	df	df	df	df	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*
a) Full	Genotype (G)	68	253	76	301	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0886	<0.0001
	Environment (E)	3	4	3	4	0.0003	0.5716	0.0063	0.0016	0.0261	0.0182	0.0186	0.0578
	G × E	204	253	228	301	<0.0001	0.7996	0.5572	0.3959	0.0587	0.1227	0.7413	0.6915
b) Subset	Genotype (G)	9	53	9	54	<0.0001	0.0367	0.0119	<0.0001	0.1719	<0.0001	0.0004	<0.0001
	Environment (E)	5	6	5	6	0.0009	0.4040	0.0054	0.0016	0.1753	0.0464	0.1155	0.1759
	G × E	45	53	45	54	<0.0001	0.3877	0.7967	0.4982	0.0650	0.3668	0.6448	0.4951

† All seed composition data were ln transformed for analysis.

‡ 146 RILs were analyzed separately by phenotypic class with 69 *myo*-inositol phosphate synthase mutant (mips) RILs and 77 wild-type (WT) RILs.

§ Environments included two planting dates (early and late) at Warsaw, VA in 2008 and 2009.

¶ Environments included one planting date in Blacksburg, VA in 2008 and 2009 and two planting dates (early and late) in Warsaw, VA in 2008 and 2009.

Numerator degrees of freedom = Num. df and Denominator degrees of freedom = Den. df

Table 3. Environment means† for seed composition‡ including inorganic phosphorus (Pi), sucrose, raffinose, and stachyose for 69 *myo*-inositol phosphate synthase mutant (mips) recombinant inbred lines (RILs) and 77 wild-type (WT) RILs from the V99-5089 × Essex population.

		Pi	Sucrose	Raffinose	Stachyose
Phenotype	Environment	µg g^{-1}	---------- % ----------		
mips	Warsaw Early 2008	1595b	7.5b	0.46a	0.37a
	Warsaw Early 2009	2281a	9.3a	0.31ab	0.22b
	Warsaw Late 2008	2391a	9.2a	0.25b	0.25ab
	Warsaw Late 2009	2450a	10.3a	0.32ab	0.24b
WT	Warsaw Early 2008	344ns	4.7b	0.78a	3.18ns
	Warsaw Early 2009	359ns	4.9b	0.68b	3.69ns
	Warsaw Late 2008	361ns	5.6a	0.74ab	3.69ns
	Warsaw Late 2009	366ns	5.9a	0.79a	3.91ns

ns, Not significant at the 0.05 significance level.

† Within each phenotype and seed composition type, environment means followed by the same letter are not significantly different according to Tukey's pairwise comparison at P=0.05.

‡ All seed composition data were ln transformed for analysis and back transformed for table.

Table 4. Environment means† for seed composition‡ including inorganic phosphorus (Pi) and sucrose for subset of 10 *myo*-inositol phosphate synthase mutant (mips) recombinant inbred lines (RILs) and 10 wild-type (WT) RILs of the V99-5089 × Essex population and weather data§ from six growing environments.

Phenotype	mips	WT	mips	WT	Mean Temperature		Precipitation
	Pi		Sucrose		Maximum	Minimum	Total
Environment	------- µg g^{-1} -------		-------- % --------		---------- °C ----------		mm
Blacksburg 2008	2123ab	394ns	11.9a	6.9a	20	6	140
Blacksburg 2009	1784bc	334ns	11.2a	6.4ab	22	11	218
WarsawEarly 2008	1560c	357ns	7.5b	4.4d	26	14	77
WarsawEarly 2009	2301a	351ns	9.3ab	5.0cd	26	15	77
WarsawLate 2008	2516a	371ns	9.1ab	5.4bcd	23	10	54
WarsawLate 2009	2505a	362ns	10.4a	5.9abc	23	12	111

ns, Not significant at the 0.05 significance level.

† Within each phenotype and seed composition type, environment means followed by the same letter are not significantly different according to Tukey's pairwise comparison at P=0.05.

‡ All seed composition data were ln transformed for data analysis and back transformed for table.

§ Mean temperatures and total precipitation for each growing environment were calculated for 30 days prior to average maturity of all lines at each environment.

Table 5. Stability parameters (*b*) and coefficient of determination (R^2) for mean inorganic phosphorus (Pi) and sucrose content of 10 *myo*-inositol phosphate synthase mutant (mips) recombinant inbred lines (RILs) and 10 wild-type (WT) RILs from V99-5089 × Essex population from six environments[†].

		Pi					Sucrose					
		---------- µg g^{-1} ----------					---------- % ----------					Mean[¶]
Phenotype	RIL	Mean[‡]	Rank	*b*[§]	*P*	R^2	Mean[‡]	Rank	*b*[§]	*P*	R^2	Rank
mips	1736	2389ab	2	0.89	0.1064	0.52	10ab	3	0.88	0.0180	0.79	2.5
	1822	2213abcd	4	1.66	0.0047	0.89	11.2a	1	1.06	0.0433	0.68	2.5
	1675	2419a	1	0.79	0.0040	0.90	9.5ab	7	1.04	0.0022	0.92	4.0
	1693	2148bcd	6	0.94	0.0621	0.62	9.8ab	5	0.79	0.0041	0.90	5.5
	1809	1956de	9	1.60	0.0002	0.98	10.6ab	2	0.83	0.0029	0.91	5.5
	1769	2268abc	3	0.50	0.0742	0.59	9.3b	10	1.03	0.0019	0.93	6.5
	1739	2098bcd	7	0.77	0.0223	0.77	9.8ab	6	0.99	0.0060	0.88	6.5
	1740	2192abd	5	0.90	0.0003	0.97	9.7ab	9	1.07	0.0071	0.87	7.0
	1823	1813e	10	1.20	0.0092	0.85	9.8ab	4	1.32	0.0035	0.90	7.0
	1792	2072cd	8	0.64	0.1798	0.40	9.5b	8	1.09	0.0074	0.86	8.0
WT	1788	404ns	1	2.15	0.0030	0.91	5.6bcd	6	1.17	0.0145	0.81	3.5
	1826	399ns	2	1.68	0.3465	0.22	5.6bcd	7	1.05	0.0055	0.88	4.5
	1856	360ns	5	2.31	0.0697	0.60	5.8abcd	5	0.87	0.0150	0.81	5.0
	1713	351ns	8	1.59	0.1050	0.52	6.4ab	2	1.00	0.0250	0.75	5.0
	1723	358ns	7	0.97	0.2826	0.28	6.2abc	4	1.06	0.0072	0.86	5.5
	1832	328ns	10	-0.36	0.7030	0.04	6.5a	1	0.63	0.0047	0.89	5.5
	1744	341ns	9	0.92	0.4675	0.14	6.2abc	3	1.09	0.0108	0.84	6.0
	1720	390ns	3	0.29	0.5230	0.11	4.6e	10	1.14	0.0079	0.86	6.5
	1733	377ns	4	0.93	0.1839	0.39	5.1de	9	1.28	0.0026	0.92	6.5
	1804	359ns	6	-0.47	0.5405	0.10	5.3cde	8	0.70	0.0400	0.69	7.0

Table 5. Continued.

† Environments included one planting date in Blacksburg, VA in 2008 and 2009 and two planting dates (early and late) in Warsaw, VA in 2008 and 2009.

‡ Within each phenotype and seed composition type, genotype means followed by the same letter are not significantly different according to Tukey's pairwise comparison at P=0.05.

§ Stability coefficients were calculated on ln transformed Pi and sucrose data.

¶ Mean rank based on the average rank of Pi and sucrose means.

Table 6. Phenotypic correlation coefficients of seed composition and agronomic traits for V99-5089 × Essex population in the full set of 153 recombinant inbred lines (RILs) grown at four environments[†] (above diagonal) and a subset of 20 RILs grown at six environments[‡] (below diagonal).

Trait	Pi	Sucrose	Raffinose	Stachyose	Seed Size	Quality[§]	Emergence	Maturity	Height	Lodging[¶]
	µg g^{-1}	---------- % ----------			g 100^{-1}	1-5	%	DAP[#]	cm	1-5
Pi	-	0.94***	-0.92***	-0.97***	-0.18*	0.23**	-0.59***	0.18*	ns	ns
Sucrose	0.95***	-	-0.87***	-0.94***	ns	0.26**	-0.50***	0.21*	ns	ns
Raffinose	-0.94***	-0.90***	-	0.92***	0.16*	-0.25**	0.53***	-0.21**	ns	ns
Stachyose	-0.97***	-0.96***	0.92***	-	ns	-0.25**	0.56***	-0.19*	ns	ns
Seed Size	ns	ns	ns	ns	-	ns	ns	0.31***	0.32***	ns
Quality	ns	ns	ns	ns	ns	-	-0.28**	0.23**	ns	0.18*
Emergence	-0.57**	-0.49*	0.49*	0.52*	0.50*	-0.59**	-	ns	0.36***	ns
Maturity	ns	ns	ns	ns	ns	ns	ns	-	0.70***	0.37***
Height	NA	NA	NA	NA	NA	NA	NA	NA	-	0.33***
Lodging	NA	NA	NA	NA	NA	NA	NA	NA	NA	-

* Significance at the 0.05 probability level.

** Significance at the 0.01 probability level.

*** Significance at the 0.001 probability level.

ns, Not significant at the 0.05 significance level.

† Four environments included two planting dates (early and late) at Warsaw, VA in 2008 and 2009.

‡ Six environments included four Warsaw environments and one planting date in Blacksburg in 2008 and 2009.

§ Quality rating from 1 to 5: 1 = seed surface smooth with no discoloration to 5 = seed wrinkled with severe discoloration.

Table 6. Continued.

¶ Lodging rating from 1 to 5: 1 = all plants erect to 5 = all plants prostrate.

Days after planting.

Table 7. Percent emergence for 76 *myo*-inositol phosphate synthase mutant (mips) recombinant inbred lines (RILs) and 77 wild-type (WT) RILs from V99-5089 × Essex population grown at six environments.

	Phenotype[†]	
Environment	mips	WT
Blacksburg 2008	29e	46d
Blacksburg 2009	69b	68b
WarsawEarly 2008	58c	77a
WarsawEarly 2009	46d	46d
WarsawLate 2008	33e	66b
WarsawLate 2009	71ab	77a

† Phenotype means followed by the same letter are not significantly different according to Tukey's pairwise comparison at P=0.05.

III. Evaluation of Emergence and Phosphorus Content of Soybean with Modified Phosphorus and Sugar Composition

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Abbreviations: MAS, marker assisted selection; *MIPS1*, D-*myo*-inositol 3-phosphate synthase 1 gene; mips, *myo*-inositol phosphate synthase mutant lines with modified seed composition; P, phosphorus; Pi, inorganic phosphorus; SSR, simple sequence repeat; WT, wild-type lines with normal seed composition.

ABSTRACT

Reduced seedling emergence is a challenge in developing new soybean [*Glycine max* (L.) Merr.] lines with low phytate seed composition. Previous research with the soybean line V99-5089 with a novel mutation in the D-*myo*-inositol 3-phosphate synthase 1 (*MIPS1*) gene reported low but significant correlations between phytate content and emergence. Our objectives were to determine relationships among seed composition traits, validate the Satt453 marker in V99-5089-derived germplasm, determine heritability of inorganic phosphorus content, and evaluate agronomic traits associated with the mutant phenotype. Evaluation of an F_2 population and F_2-derived lines from the cross between V01-1693, a low linolenic fatty acid line and V03-5901, a V99-5089-derived line, was completed in three growing environments. Correlations between seed composition traits were favorable without significantly affecting fatty acid content. The use of subtropical winter nursery environments for population development resulted in significant reductions in emergence for both F_2 and $F_{2:4}$ generations. In the F_2 generation, poor emergence limited the number of lines identified with modified seed composition (mips), skewing segregation ratios and preventing validation of the Satt453 marker. In the $F_{2:4}$ generation, poor stands prevented analysis of agronomic traits. The heritability estimate was 0.67 for inorganic phosphorus content. However, because low emergence is likely to skew segregation ratios, large F_2 populations are necessary to allow for selection of high-yielding mips lines from segregating populations.

INTRODUCTION

Improvement of the feed efficiency of soybean [*Glycine max* (L.) Merr.] meal via modifications of phosphorus (P) and/or sugar composition is a recent focus for genetic improvement. Phytic acid (*myo*-inositol 1,2,3,4,5,-hexa*kis*phosphate) is the major storage form of P in seeds comprising about 70% of the total P in mature soybean seeds (Raboy et al., 1984). The P in phytate, the mixed cation salt of phytic acid, cannot be digested by monogastric animals resulting in the rational for modifying feed to improve P nutrition. Sucrose, raffinose, and stachyose are the primary sugars in soybeans and the presence of raffinose and stachyose in soybean meal is detrimental because they are not easily digested. The modification of sugar content in soybeans by increasing sucrose levels and decreasing raffinose and stachyose improves the metabolizable energy of soybean meal (Parsons et al., 2000).

Three germplasm sources with different mutations in the D-*myo*-inositol 3-phosphate synthase 1 (*MIPS1*) gene have been reported in which both the P and sugar content of soybean are modified (Hitz et al., 2002; Saghai Maroof and Buss, 2008; Yuan et al., 2007). For P modification, the recessive *MIPS1* mutant gene results in a phenotype (mips) characterized by increases in inorganic phosphorus (Pi) and decreases in phytate which is a dual benefit for P availability. The mutation also modifies sugar content with simultaneous increases in sucrose content and decreases in raffinose and stachyose content. Reports of reduced soybean emergence in mips germplasm hinders the application of this germplasm to modify seed composition (Meis et al., 2003; Sebastian et al., 2000; Yuan et al., 2007). Reduced emergence has also been reported for CX1834 (Anderson and Fehr, 2008; Oltmans et al., 2005), an additional germplasm source with only modified P composition. The reduced emergence of *MIPS1* mutant and CX1834 germplasm was more pronounced when seeds were produced in

tropical and subtropical environments (Anderson and Fehr, 2008; Meis et al., 2003; Yuan et al., 2007). The development and commercialization of lines with modified P and sugar composition is dependent on improving the emergence to commercially acceptable levels.

A single recessive gene for low stachyose was reported in V99-5089 (Huhn, 2003), a new *MIPS1* mutant line in which correlations between Pi, phytate, sucrose, raffinose, and stachyose were all significant (Saghai Maroof and Buss, 2008), facilitating the development of soybeans with higher feed efficiency. The simple sequence repeat (SSR) marker Satt453 was associated with the *MIPS1* mutant phenotype of V99-5089 (Saghai Maroof and Buss, 2008). Recently, Maupin et al. (2010) reported an 87% marker selection efficiency in a recombinant inbred line (RIL) population and concluded that phenotypic selection was more effective for *MIPS1* mutant alleles. However, validation of the association between the Satt453 marker and the mips seed composition phenotype in advanced lines derived from V99-5089 is important for continued mips germplasm development.

In the evaluation of a V99-5089 RIL population by Maupin et al. (2010), reduced emergence was observed in mips lines as compared to WT lines, but only in year one of the experiment. These differences were likely due to more rapid deterioration of seed quality in mips lines as compared to WT lines during the two years of seed storage before year one of the experiment. Previous reports indicate significant reductions in the emergence of mips and CX1834 germplasm when seed was produced in a subtropical environment (Anderson and Fehr, 2008; Meis et al., 2003; Sebastian et al., 2000; Yuan et al., 2007). In the evaluation of CX1834-derived germplasm, Anderson and Fehr (2008) indicated that the production of segregating populations in a subtropical winter nursery environment could decrease the frequency of low phytate genotypes in successive generations. Since soybean breeders use winter nurseries for

population advancement, it is important to further evaluate the impact of those conditions on the development of new mips germplasm by following population development in successive generations.

Working within a population developed from the advanced line V03-5901, a derivative of the original V99-5089 mutant, our objectives were to (i) establish the correlation between Pi and phytate in mips germplasm, (ii) correlate other seed composition traits, including fatty acid content, (iii) validate the association of Satt453 marker with the mips seed composition phenotype, (iv) estimate heritability for Pi content and, (v) evaluate agronomic traits in mips germplasm. In addition, a comprehensive description of low phytate population development in successive generations is presented to understand the use of winter nursery and the associated effects of reduced emergence on population development in a soybean breeding program for mips germplasm.

MATERIALS AND METHODS

Field Experiment

In 2007, a low linolenic acid line, V01-1693, was crossed with V03-5901, a line with low phytate, high Pi, high sucrose, and low raffinose oligosaccharide content. Both parents are experimental lines of the Virginia Agricultural Experiment Station and are of mid-V maturity. V01-1693 was developed from [(Hutcheson (2) × N94-199) × (Hutcheson (2) × RR experimental line)]. Line N94-199, developed by Dr. Joseph Burton (USDA-ARS, Raleigh, NC), provided the low linolenic (3.8% 18:3) fatty acid trait. The line V03-5901 was developed from the cross between V99-5089, an experimental line with a novel mutation in the *MIPS1* gene (Saghai Maroof and Buss, 2008) and the high yielding cultivar Essex (Smith and Camper, 1973).

During the winter of 2007-2008, six F_1 seeds were planted in Costa Rica and each F_1 plant was harvested individually. The F_2 seeds were planted 11 June 2008 at the Virginia Tech Kentland College Farm near Blacksburg, VA. A honeycomb planting design was used with rows spaced 76 cm apart, plots within rows spaced 88 cm apart and every other row offset by 44 cm. The design consisted of 162 F_2 plots and three check plots of each parent. For each plot, two seeds were planted with the intention of thinning the plots to one seedling per plot. Seedling emergence data were collected approximately 21 days after planting and emergence throughout the entire planting was reduced. Therefore, the F_2 plots were not thinned to increase the total number of plants available for segregation analysis. The F_2 plants were confirmed as progeny from F_1 hybrids by flower color. Maturity was recorded as the number of days after planting when 95% of the pods reached mature color. At maturity, plant height was measured from the soil surface to the apex of the main stem. Each F_2 and parent check was harvested and threshed individually with a stationary thresher in the field.

During the winter of 2008-2009, 30 seeds of both parents and 135 $F_{2:3}$ lines (only 20 seeds of six lines due to seed limitations) were sent to Costa Rica for increase. Emergence data were collected approximately 30 days after planting in Costa Rica. Each of the $F_{2:3}$ rows were harvested separately to provide seeds for a replicated field test in 2009.

The field experiment with two replications of $F_{2:4}$ lines arranged in a randomized complete block design was planted 27 June 2009 at Virginia Tech Kentland College Farm near Blacksburg, VA. Each plot consisted of two rows with a total of 200 seeds per plot planted into rows 3 m long with 0.76 m row spacing. Each replication of the experiment included a total of 135 $F_{2:4}$ plots, three plots of each parent (V01-1693 and V03-5901) and one plot of Glenn, a high-yielding control cultivar. Plants in each plot were counted approximately 21 days after planting. Reduced emergence was observed in all $F_{2:4}$ plots, however, $F_{2:4}$ plots derived from F_2 lines with modified composition (mips) were severely reduced, preventing analysis of agronomic traits. Plots in replication two were bulk harvested with a plot combine on 20 November 2009.

Seed Composition Analysis

Seeds from F_2 plants grown in Blacksburg in 2008 (BB2008), $F_{2:3}$ rows grown in Costa Rica in 2009 (WN2009), and $F_{2:4}$ rows grown in Blacksburg in 2009 (BB2009) were evaluated for seed size, quality, and Pi content. Seed size was recorded as the weight of 100 random whole seeds from each plot. Seed quality was rated on a scale of 1 (seed surface smooth with no discoloration) to 5 (seed wrinkled with severe discoloration).

Fatty acid content and sugars including sucrose, raffinose, and stachyose were determined for $F_{2:3}$ seed of all individual F_2 plants. Phytate content was determined from 22 F_2 plants to establish the correlation between Pi and phytate. For seed composition analysis including Pi, phytate, and sugar content, a 15 g subsample of $F_{2:3}$ seed from 135 F_2 plants was

ground in an Udy cyclone mill (U.D. Corp., Boulder, CO) to pass through a 1.0 mm screen. A 25 g subsample was ground for Pi analysis using $F_{2:4}$ seed from Costa Rica and $F_{2:5}$ seed from Blacksburg. Based on the combination of Pi seed composition data from 2008 and 2009, F_2-derived lines were characterized into three phenotypic classes including: wild-type lines (WT) with normal seed composition, heterozygous lines with intermediate seed composition and *MIPS1* mutant lines (mips) with modified seed composition.

A modified version of the colorimetric assay developed by Raboy et al. (2000) adapted from the assay by Chen et al. (1956) was used to determine the Pi content. The extraction and quantification methods for Pi were as described by Scaboo et al. (2009) with Pi concentrations determined using a BioTek Synergy HT plate reader (BioTek Instruments, Winooski, VT) set at a wavelength of 882 nm. The final Pi content of each plot was calculated from an average of three subsamples using the initial weight of each sample to calculate the Pi concentration on a dry weight basis with results reported as micrograms per gram.

Sucrose, raffinose, and stachyose content was determined by high performance liquid chromatography (HPLC) as described by Cicek et al. (2006). Extracted sugar samples were stored in the freezer at -20 °C until HPLC could be completed. As an internal control, V99-5089 was included with each set of samples during extraction and quantification. The final concentration of each sugar was calculated on a dry weight basis and results are reported as a percent.

For fatty acid (FA) analysis, a 10 g sample from each F_2 plant was submitted to the USDA National Center for Agricultural Utilization Research (Peoria, Illinois). Three seeds were randomly selected for FA analysis from the 10 g sample, placed into an envelope and manually crushed with a hammer. Each sample of crushed seeds was transferred to a small vial and mixed

with 1.5 mL of chloroform/hexane/methanol (8:5:2, by vol) solution. After 4 h, 0.1 mL of oil sample was transferred to an autosampler vial and mixed with 0.1 mL sodium methoxide solution (4 g sodium in 500 mL of methanol). Analysis of FA content was completed on an Agilent 6890 series gas chromatograph with a FID detector and an Agilent J&W GC column (30 m x 0.25 mm id x 0.25 μm film thickness). Data were reported as the area percent of the peaks associated with C16:0, C18:0, C18:1, C18:2 and C18:3 content in which peaks were normalized for the total area percent to equal 100%.

High performance ion chromatography (HPIC) was used to determine phytate concentration. For phytate extraction, approximately 0.5 g of ground soybean seed was shaken in 10.0 mL of 0.5 M HCl in a 16 mL centrifuge tube for 4h at room temperature. At the end of shaking, 1.5 mL of sample was placed into a 2.0 mL microcentrifuge tube and centrifuged at 12,000 g, for 15 min at 10 °C. One mL of the supernatant was mixed with 1.0 mL of 20% NaCl in a 2.0 mL microcentrifuge tube. The extract was mixed by inversion 10 times and the salt-treated samples were placed in the refrigerator for 3 h before being centrifuged at 12,000 g for 10 min. This treatment removes the proteins and starch in the sample, which can potentially interfere with the ion chromatography elution. The supernatant was then diluted two times and filtered through a 0.2 μm PTFE membrane (IC Millex®-LG, Fisher, PA) into a 10-mL Dionex (Dionex, Sunnyvale, CA) sample vial for ion chromatography (IC) analysis. IC separation of phytate was performed on a Dionex ICS 3000 unit, equipped with an UltiMate 3000 VW detector, using Dionex AS7 analytical column and AG7 guard column. The elution was a 10-min isocratic process using 0.25 M HNO_3 at 1 mL per min. (Phillippy et al., 2003) with an injection volume of 25 μL. Phytate in effluent was detected colorimetrically using a post-column reaction taking place at room temperature in a reaction coil of 300 cm long and 0.5 mm

I.D. The post-column reagent was 0.1% $Fe(NO_3)_3$ in 2% $HClO_4$ and pumped at 1 mL per min using a HPLC pump. The column effluent and color reagent met in a Tee connector, connected with the reaction coil. The UV absorption at 290 nm was recorded (Phillippy et al., 2003). The final phytate content of each F_2 plant was calculated from an average of two samples using the initial weight of each sample to calculate the phytate concentration on a dry weight basis with results reported as micrograms P per gram.

DNA Extraction and Marker Analysis

One set of young trifoliolate leaves from each F_2 plant and parental line was sampled from Blacksburg in 2008. DNA extraction was performed using Plant DNeasy Mini Kit (Qiagen, Valencia, CA). The samples were screened with the SSR FAM-labeled marker Satt453 (Chr11-B1) developed by Cregan et al. (1999). PCR reactions for SSR assay were performed in a total volume of 12.5 µl in a Bio-Rad C1000 (Bio-Rad, Hercules, CA) thermal cycler. The initial denaturing step, 5 min at 95 °C, was followed by 40 cycles of 30 s at 94 °C, 40 s at 47 °C, 30 s at 72 °C, and then by a final extension step for 7 min at 72 °C. Genotypes were visualized by a 3130x*l* Genetic Analyzer (Applied Biosystems, Carlsbad, CA). Two genotypes did not amplify and were dropped from the genotypic analysis. Genotypes were separated into three classes based on marker analysis including: homozygous for the V01-1693 allele (1693/1693), heterozygous (1693/5901), and homozygous for the V03-5901 allele (5901/5901). The software Mapmaker 3.0 was used to construct a linkage map between Satt453 marker and the *MIPS1* gene using the maximum likelihood method to estimate the recombination distance and marker order with a log-likelihood threshold of 3.0 (Lander et al., 1987).

Statistical Analysis

The Pi and stachyose data were not normally distributed and logarithmic transformation by ln = $\log_e$ was successful to normalize distribution of residuals and remove heterogeneity of error variances. However, analysis of variance of both transformed and original data resulted in the same results therefore, the original data were analyzed. Chi-square analysis was used to determine the goodness of fit for single gene segregation of phenotypic (3:1) data and genotypic (1:2:1) data using PROC FREQ in SAS (SAS, 2009). A t-test compared seed quality scores of mips and WT lines from the WN2009 environment with PROC TTEST in SAS (SAS, 2009).

To evaluate differences between Pi, emergence, and stachyose, a new classification variable (phenotype + marker genotype class) was created based on the combination of phenotype and marker genotype classes with the purpose of separating means among lines. Individual F_2 plants and F_2-derived lines were assigned to one of the eight phenotype + marker genotype classes for further analysis. No lines were observed with the mips phenotype and the 1693/1693 genotype class. Only one line was observed in the WT phenotype + 5901/5901 genotype class and was dropped from the analysis. Differences for the fixed effect phenotype + marker genotype class were evaluated using the SAS procedure PROC GLIMMIX (SAS, 2009). A separate analysis was completed for replicated emergence data from BB2009 using PROC GLIMMIX (SAS, 2009) with phenotype + marker genotype class and pedigree within the phenotype + marker genotype class as fixed effects and replication as a random effect. Pairwise comparisons of phenotype + marker genotype class means were made using Tukey's multiple means comparison method. Phenotype correlation coefficients among traits were calculated using the PROC CORR procedure in SAS (SAS, 2009).

RESULTS AND DISCUSSION

Seed Composition

Inverse relationships between phytate and Pi have been reported for three low phytate germplasm sources in soybean (Hitz et al., 2002; Wilcox et al., 2000; Yuan et al., 2007). An inverse relationship was assumed between phytate and Pi in the experimental line V99-5089, which was characterized as low phytate with normal levels of total P (Gao et al., 2008). In this study we established that relationship in the low phytate line, V03-5901, which is derived from V99-5089. The linear relationship was significant between phytate and Pi content with an R^2 of 0.87 (Figure 1). Our results for mips germplasm are similar to CX1834-derived germplasm, which was reported by Scaboo et al. (2009) to have a significant linear regression with an R^2 of 0.84. This relationship indicates the Pi assay accurately selects the low phytate phenotype from *MIPS1* mutant germplasm. This is efficient for plant breeders because Pi determination is simpler, faster, and less expensive than phytate determination with HPIC.

Differences between V01-1693 and V03-5901 for phosphorus and sugar composition are distinct (Table 1) and the correlations between Pi and sugar composition of F_2 plants were strong and significant (Table 2). Correlations between Pi with sucrose, raffinose, and stachyose facilitate the improvement of P and sugar composition simultaneously. The correlation values reported for F_2 plants were slightly lower than those based on an advanced RIL population with V99-5089 (Maupin et al., 2010), but they are high enough to allow for phenotypic selection based on $F_{2:3}$ seed in an early generation selection program.

Increased saturate (palmitate + stearate) content in low phytate lines derived from CX1834 germplasm may restrict the development of low phytate lines with improved fatty acid content (Hulke et al., 2004). In our experiment, the variation of individual fatty acids in F_2

individuals was small with the exception of linolenic, which ranged from 4.0 to 12.5% (data not shown). The range of linolenic acid content was 4.5-12.0% in the mips lines with four lines below 6.0% linolenic acid (data not shown). The 4.5% level is not low enough for commercialization but the ability to identify lines with modified P, sugar, and linolenic acid composition supports the potential for further population improvement to reduce linolenic acid content in mips germplasm. In addition, there were transgressive segregates for lower saturate (palmitic acid + stearic acid) content within the F_2 plants and specifically within the mips lines (data not shown) allowing for development of mips lines with lower saturate content which is in contrast to the results reported by Hulke et al. (2004). There were a few significant but low correlations between P and sugar content with individual fatty acids, which does not negatively affect the development of mips cultivars (Table 2). It should be possible to develop mips lines with modified fatty acid profiles by crossing with modified fatty acid lines to further improve soybean seed composition.

Inheritance of *MIPS1* Mutant and Marker

Correlations between reduced seedling emergence and low phytate content have been reported (Oltmans et al., 2005; Sebastian et al., 2000; Yuan et al., 2007), which could reduce the number of low phytate plants in a population. Reduced emergence in the F_2 generation of this population affected observed frequencies of the *MIPS1* mutant allele and the associated Satt453 marker. In 2008, a total of 324 F_2 seeds originating from six F_1 plants were planted but emergence was reduced with only 47% of seeds emerging to produce F_2 plants. Average emergence for the parental lines, V01-1693 and V03-5901 were 83% and 67%, respectively (Table 1).

The most distinct trait for phenotype classification was stachyose content (Table 1) and at harvest the characterization of the 135 F_2 plants resulted in 118 WT plants and 17 mips plants (Table 3). The observed ratio was significantly different from the expected ratio of 3:1 for a trait controlled by a single gene based on chi-square analysis. Huhn (2003) evaluated two V99-5089 populations and reported a 3:1 segregation ratio for the high to low stachyose phenotype indicating a single recessive gene. However, our results did not agree due to the differential emergence of phenotypic classes in 2008 reducing the number of mips individuals, which skewed segregation ratios.

The SSR marker Satt453 was previously reported to be associated with the mutant phenotype of V99-5089 (Saghai Maroof and Buss, 2008). In the RIL population (V99-5089 × Essex), a marker selection efficiency of 87% was reported, allowing for the use of marker assisted selection (MAS) in developing populations (Maupin et al., 2010). Confirmation of Satt453 in derived germplasm and diverse genetic backgrounds is important for application of MAS in plant breeding programs. In the chi-square analysis of the Satt453 marker segregation ratios, the observed values were not significantly different from the expected 1:2:1 ratio (Table 3) indicating normal marker segregation. The linkage analysis between Satt453 and the mips phenotype showed there was 32.3 cM between the SSR and *MIPS1* gene. However, a total of 32 plants with homozygous V03-5901 marker genotype were selected with the Satt453 marker, which was an increase over the 17 identified in the phenotypic analysis. The mean of stachyose values for the 32 homozygous V03-5901 marker genotypes was 1.99%, which is higher than the mean of 0.29% observed in the 17 mips phenotypes. Marker selection efficiency can be determined by calculating the number of marker-selected individuals with the desired mips phenotype divided by the total marker-selected individuals. In the phenotypic evaluation of the

32 homozygous V03-5901 marker genotypes only 13 were actually low for stachyose content resulting in a 41% marker selection efficiency. A similarly low marker selection efficiency (50%) was reported by Scaboo et al. (2009) in the analysis of the two recessive genes and their associated SSR markers in CX1834 low phytate germplasm.

One of the initial objectives was to determine heritability of Pi content in mips germplasm with parent offspring regression using data from F_2 single plants and derived $F_{2:4}$ lines. The heritability estimate was determined by the slope of the regression line which was 0.67 with an R^2 = 0.83 (Figure 2). This high heritability estimate supports our previous conclusion that selection for the mips phenotype (high Pi) in early generation populations will produce advanced lines with the mips phenotype.

MIPS1 Mutant Population Development

In the evaluation of a trait controlled by a single recessive gene, phenotypic data is not typically used to distinguish heterozygous phenotypes from homozygous WT phenotypes. However, the classification of individuals into heterozygous phenotypes was possible with this germplasm, using data collected in three growing seasons (BB2008, WN2009, and BB2009) (Table 4). In the initial classification for the chi-square analysis (Table 3), stachyose content separated individuals with WT and mips phenotypes. Using BB2008 data, individual plants with a heterozygous phenotype were not easily separated from homozygous WT lines based on stachyose and/or Pi data. However, upon evaluation of Pi and emergence data for lines in subsequent generations, the individuals with a heterozygous (WT/mips) phenotype were apparent and this information could be used to understand population development with mips germplasm.

In WN2009, emergence of all F_2-derived lines was high with a mean of 89% (Table 1). The subtropical winter nursery environment in Costa Rica represented a favorable environment allowing emergence of mips seeds within both heterozygous and homozygous mips lines. Therefore, no significant differences were observed between phenotype classes for emergence (Table 4). Mutations in *MIPS1* result in a strong phenotypic expression significantly increasing Pi and decreasing stachyose content, producing clear bimodal distributions in segregating populations. Similar emergence of mips and WT seeds in heterozygous $F_{2:3}$ rows was apparent in the harvested bulk seed, as the lines originating from heterozygous individuals were significantly higher than homozygous WT lines for Pi content, and significantly lower than homozygous mips lines.

The favorable emergence environment in Costa Rica did not produce seeds with high emergence potential. The emergence of $F_{2:3}$ lines was below commercially acceptable levels when planted in Blacksburg in 2009 (Table 4). The seed source environment can effect emergence of low phytate lines, with subtropical environments significantly reducing emergence as compared to temperate environments (Anderson and Fehr, 2008; Meis et al., 2003). Severely reduced emergence of homozygous mips lines in BB2009 resulted in the failure of the agronomic trial as yield would be meaningless from rows with emergence levels from 1-18% (Table 4).

The seed source environment also reduced emergence in heterozygous lines as compared to homozygous WT $F_{2:3}$ lines, the parental line (V01-1693), and Glenn, the high yielding control cultivar (Table 4). The Pi data indicate a change in allele frequencies in the population caused by the failure of some of the mips seeds to emerge within the heterozygous lines. The heterozygous lines form a group within the $F_{2:3}$ WN2009 Pi data which is significantly lower than the homozygous mips lines but significantly higher than the homozygous WT lines. In

BB2009, the heterozygous $F_{2:4}$ lines are no longer significantly different from the homozygous WT lines for Pi content. The range of Pi content for heterozygous lines in the $F_{2:4}$ was 374-701 µg g^{-1}. This intermediate value provides evidence that mips seeds did emerge but not in equal numbers to WT seeds. These data indicate that the heterozygous lines within the population are shifting towards the WT phenotype when mips seeds emerge less frequently than WT seeds over generations. The differential emergence will ultimately decrease the frequency of mips lines for selection during population development. Therefore, population development of mips germplasm should account for reduced emergence and large populations should be generated to allow for selection of mips lines with superior agronomic performance.

Similarly, breeders should consider that advanced homozygous *MIPS1* mutant lines will shift towards normal phytate levels over generations if contaminated with a low frequency of the WT allele. Therefore, phenotypic selection for low phytate lines should be done in very advanced generations ($>F_{4-5}$) and released lines should be pure to ensure homozygosity of the mutant allele. Further, despite the ease of phenotypic selection using the Pi assay, molecular markers are a necessary additional tool for breeding low phytate germplasm to ensure purity of advanced lines. Similar recommendations would apply to other low phytate germplasm sources.

The significant differences between emergence of WT and mips lines demonstrated the impact of a subtropical winter nursery environment on mips germplasm. The use of winter nursery environments may be detrimental to population development because changes in allele frequency may prevent selection of mips lines in later generations, or at least truncate diversity among homozygous mips lines. Another consideration is that the winter nursery environment may function as a tool for natural selection in which those mips seeds that germinated after a winter nursery growing environment develop into a population of lines with a higher emergence

potential. We are currently conducting experiments testing the natural selection theory for population improvement for emergence of mips lines.

Seed Source Environment

Our experiment was not designed to evaluate the effect of seed production environments on emergence of mips germplasm. However, observations indicated that environmental conditions of the winter nursery were influencing both P content and emergence. The environment means for Pi content of mips lines ranged from 1571 µg g^{-1} in seed from BB2009 and 2011 µg g^{-1} in seed from BB2008 to a high Pi content of 2622 µg g^{-1} from seed grown in WN2009 in Costa Rica (Table 1). Additionally, the correlation of -0.86 for Pi content in WN2009 to seedling emergence in BB2009 was significant. In the evaluation of CX1834 germplasm, Anderson and Fehr (2008) reported significantly higher Pi and phytate content in seed produced in a subtropical environment as compared to seed from a temperate environment. In theory, the higher phytate content should contribute to higher emergence but that is not the case in the CX1834 germplasm and likely the V99-5089 germplasm. It is unclear if the significantly higher Pi content observed in seed grown in subtropical winter nursery environments is responsible for the decreased emergence.

Pi and phytate content may not be the only cause of reduced emergence. The winter nursery environment is compounding the emergence problems in low phytate germplasm and other seed attributes that reduce emergence may be magnified by the winter nursery environment. Potential factors could include additional disease pressures, high temperatures and humidity, and differences in daylength of the subtropical winter nursery environments.

Previous research has demonstrated the effect of high temperature on seed quality and emergence (Egli et al., 2005; Keigley and Mullen, 1986). The high temperatures of the winter

nursery environment may be reducing seed quality, contributing to the decreased emergence. In all environments, the quality of V03-5901 was slightly poorer (higher value) than V01-1693 (Table 1). The good seed quality from the BB2008 environment for both the mips (2.1) and WT (2.0) lines were not significantly different. Additionally, there were no significant differences for emergence in WN2009 (Table 4). However, in the WN2009 environments, the seed quality was reduced as compared to the BB2008 environment. The seed quality scores of the 17 mips lines were significantly ($P < 0.001$) poorer than WT lines in WN2009 (mean of 3.2 vs. 2.7 Table 1). The reduced seed quality of the mips lines in WN2009 may contribute to the reduced emergence observed in BB2009 with a -0.61 correlation. Additionally, the emergence of mips lines with poor WN2009 seed quality scores (3.5 and 4.0) was 3.4% for emergence in BB2009 compared to 9.9% emergence of mips lines with superior quality scores of 2.5 and 3.0. The evaluation of the 17 mips lines indicates that selection of mips lines with good seed quality could improve emergence potential. Therefore, to develop mips germplasm with improved emergence it may be beneficial to select parental lines with high seed quality and good emergence in high temperature environments as identified by Smith et al. (2008).

CONCLUSIONS

The ability to evaluate agronomic traits associated with mips lines was hindered by reduced emergence in two growing seasons. Reduced emergence in the F_2 generation decreased the number of lines with the *MIPS1* mutant allele resulting in skewed segregation ratios and decreased marker selection efficiency. The use of winter nursery for population advancement resulted in significant reductions in emergence of mips lines in the subsequent planting. In heterozygous lines, decreased emergence of mips plants caused the population to shift towards WT seed composition reducing the ability to identify mips lines in successive generations. Winter nursery environments should probably be avoided for mips or any low phytate population development. Seed quality was indicated as a potential factor in emergence of mips lines and efforts are underway to select lines with high seed quality as parents in breeding populations. Reduced emergence is the primary challenge to development of mips germplasm, therefore, soybean breeders need to emphasize the selection of lines with good seed quality and high emergence.

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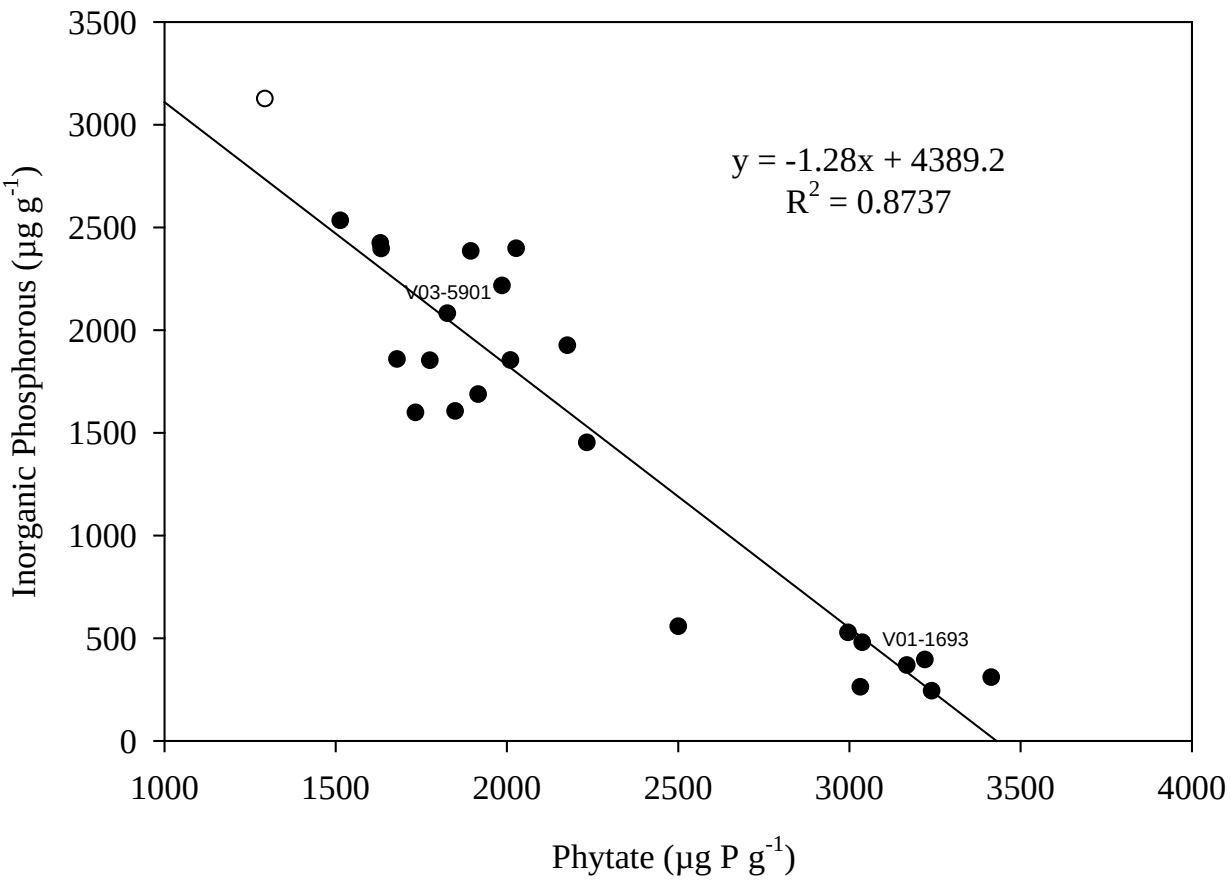

Figure 1. Linear regression for seed phytate and inorganic phosphorus (Pi) content of 22 F_2 individual plants and each parent from the cross V01-1693 × V03-5901.

Table 1. Descriptive statistics for seed composition and agronomic traits in 135 F_2, $F_{2:3}$, and $F_{2:4}$ derived lines from the cross V01-1693 × V03-5901 from three growing environments in 2008 and 2009.

	Parents				F_2 Progeny[†]					
	V01-1693		V03-5901		WT		Heterozygous		mips	
Trait	Mean	StDev[‡]	Mean	StDev	Mean	StDev	Mean	StDev	Mean	StDev
Maturity, DAP[§]										
BB2008	141	2	140	6	140	6	141	5	142	4
Pi, µg g^{-1}										
BB2008	356	134	1947	250	318	61	649	111	2011	500
WN2009	425	23	2617	124	384	41	1133	244	2622	372
BB2009	338	58	1534	148	367	38	509	71	1571	419
Seed Quality, 1-5[¶]										
BB2008	2.0	0.0	2.2	0.3	2.0	0.3	2.0	0.4	2.1	0.2
WN2009	2.7	0.3	3.0	0.9	2.7	0.5	2.8	0.4	3.2	0.4
BB2009	2.4	0.1	2.8	0.5	2.3	0.3	2.5	0.4	2.7	0.4
Seed Size, g 100^{-1}										
BB2008	11.0	1.2	15.2	0.5	13.4	1.4	13.7	1.5	13.7	1.6
WN2009	17.6	0.6	21.0	1.8	20.0	1.3	19.8	1.4	19.8	1.2
BB2009	11.3	0.4	13.8	0.7	12.9	1.1	12.7	1.0	12.3	1.3
Emergence, %										
BB2008	83	29	67	14	NA		NA		NA	
WN2009	87	12	81	14	87	10	90	9	89	8
BB2009	61	3	4	3	61	8	37	10	7	5
Sugar BB2008, %										
Sucrose	5.4	0.4	10.3	0.7	6.4	1.1	7.6	0.9	11.5	1.2
Raffinose	0.65	0.02	0.20	0.08	0.70	0.1	0.64	0.1	0.28	0.1
Stachyose	3.91	0.23	0.29	0.13	4.20	0.7	3.11	0.4	0.29	0.1
FA BB2008, %										
Palmitic (16:0)	11.7	0.8	11.1	0.3	11.6	0.7	11.7	0.7	11.6	0.7
Stearic (18:0)	4.2	0.7	4.4	0.3	3.9	0.4	4.0	0.6	4.3	0.5
Oleic (18:1)	17.6	1.3	18.9	0.6	19.2	1.3	18.8	1.3	18.7	1.4
Linoleic (18:2)	61.6	1.4	54.3	0.7	57.7	1.8	57.9	1.9	56.9	1.9
Linolenic (18:3)	5.0	0.4	11.3	0.1	7.6	1.6	7.6	2.0	8.5	2.4
Number of Lines					27		91		17	

Table 1. Continued.

† Based on phenotype, progeny include F_2 individual plants from Blacksburg in 2008 (BB2008), $F_{2:3}$ lines from winter nursery in Costa Rica in 2009 (WN2009), and $F_{2:4}$ lines from Blacksburg in 2009 (BB2009), WT = wild-type lines with normal seed composition, Heterozygous = heterozygous for seed composition, mips = *myo*-inositol phosphate synthase mutant lines with modified seed composition.

‡ Standard Deviation.

§ Days after planting.

¶ Seed quality rating from 1 to 5: 1 = seed surface smooth with no discoloration to 5 = seed wrinkled with severe discoloration.

Table 2. Phenotypic correlation coefficients of seed composition traits from 135 F_2 plants from the cross V01-1693 × V03-5901 grown in Blacksburg, VA in 2008.

Trait	Sucrose	Raffinose	Stachyose	Palmitic	Stearic	Oleic	Linoleic	Linolenic
Pi	0.78***	-0.68***	-0.88***	ns	0.18*	ns	ns	ns
Sucrose		-0.42***	-0.66***	-0.19*	ns	ns	-0.22*	ns
Raffinose			0.79***	ns	ns	ns	ns	ns
Stachyose				ns	-0.21*	ns	ns	ns
Palmitic					-0.43***	-0.48***	0.23**	ns
Stearic						0.45***	ns	-0.32**
Oleic							-0.39***	-0.25**
Linoleic								-0.74***

* Significance at the 0.05 probability level.

** Significance at the 0.01 probability level.

*** Significance at the 0.001 probability level.

ns, Not significant at the 0.05 significance level.

Table 3. Descriptive statistics and chi-square analysis[†] of stachyose content for 135 F_2 plants from the cross V01-1693 × V03-5901 grown in Blacksburg, VA in 2008.

	Stachyose (%)							
Phenotype	Mean	StDev[‡]	Minimum	Maximum	Observed	Expected	Chi-Square	*P*
WT	3.35	0.67	2.39	6.59	118	101	11.07	0.0009
mips	0.29	0.08	0.18	0.44	17	34		
Satt453 Marker Genotype[§]								
1693/1693	3.77	0.65	2.65	5.28	29	33	1.05	0.5930
1693/5901	3.09	0.87	0.20	6.59	72	67		
5901/5901	1.99	1.46	0.18	3.83	32	33		

† Chi-square analysis based on 3:1 ratio for phenotype and 1:2:1 ratio for genotype analysis.

‡ Standard Deviation.

§ 1693/1693, homozygous for V01-1693 marker allele; 1693/5901, heterozygous for V01-1693 and V03-5901 marker alleles; 5901/5901, homozygous for V03-5901 marker allele.

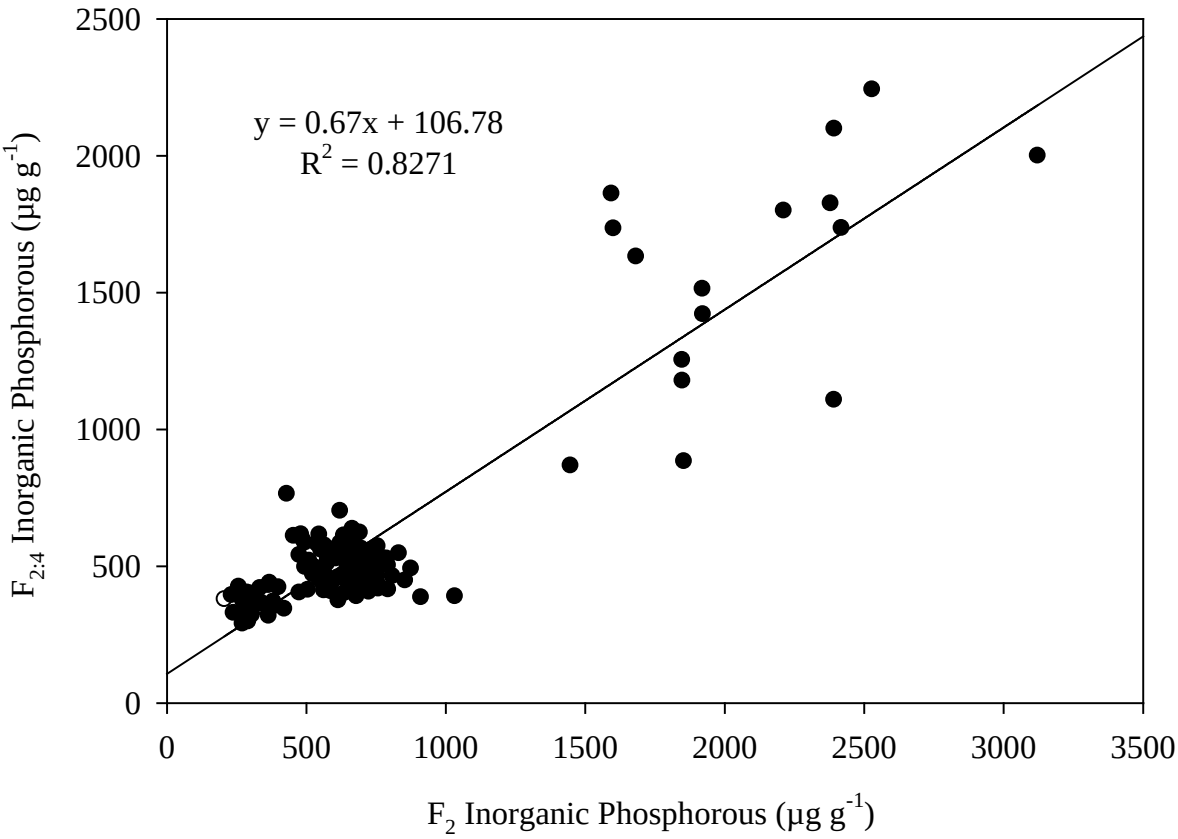

Figure 2. Parent-offspring regression of inorganic phosphorus content of seeds from F_2 single plants against seeds from $F_{2:4}$ rows for V01-1693 × V03-5901 population.

Table 4. Descriptive statistics and Tukey's separation of means† for stachyose and inorganic phosphorus (Pi) of F_2 plants grown in Blacksburg, VA in 2008, emergence and Pi of $F_{2:3}$ lines grown in Costa Rica in winter 2009, and emergence and Pi of $F_{2:4}$ lines from a replicated test grown in Blacksburg, VA in 2009.

			F_2				$F_{2:3}$				$F_{2:4}$			
	Satt453		Stachyose		Pi		Emergence		Pi		Emergence		Pi	
	Marker		------- % -------		------- µg g^{-1} -------		------- % -------		------- µg g^{-1} -------		------- % -------		-------- µg g^{-1} --------	
Phenotype	Genotype‡	N	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
WT	1693/1693	18	4.12a	2.86-5.28	328c	208-475	87a	63-100	377c	319-478	61a	42-70	362d	289-429
	1693/5901	7	4.45a	3.73-6.59	286c	232-319	88a	67-100	406c	342-443	63a	49-71	379cd	361-402
	5901/5901§	1	3.73‡	-	333‡	-	90‡	-	380‡	-	56‡	-	364‡	-
	V01-1693	3	3.91ab	3.64-4.07	356bc	271-511	87a	77-100	425c	403-448	61a	58-65	338d	292-402
	Glenn	1	-	-	-	-	-	-	-	-	68a	61-75	365‡	-
Heterozygous	1693/1693	11	3.24b	2.65-4.15	649b	369-1034	90a	83-100	1179b	731-1950	37b	18-60	480cd	386-615
	1693/5901	61	3.07b	2.39-4.44	653b	455-876	90a	50-100	1139b	716-1598	37b	17-56	514c	374-701
	5901/5901	18	3.10b	2.58-3.83	645b	512-749	89a	70-100	1074b	694-1370	39b	21-63	498cd	401-585
mips	1693/1693	0	-	-	-	-	-	-	-	-	-	-	-	-
	1693/5901	4	0.33c	0.20-0.44	1878a	1008-2530	84a	77-90	2578a	2140-2844	6c	1-9	1975a	1824-2241
	5901/5901	13	0.28c	0.18-0.42	2052a	1448-3123	90a	70-100	2636a	1878-3266	7c	2-18	1478b	867-2098
	V03-5901	3	0.29c	0.19-0.44	1947a	1685-2184	81a	67-93	2617a	2474-2693	4c	2-7	1534b	1445-1705

† Within each trait, phenotype + marker genotype class means followed by the same letter are not significantly different according to Tukey's pairwise comparison at P=0.05.

‡ 1693/1693, homozygous for V01-1693 marker allele; 1693/5901, heterozygous for V01-1693 and V03-5901 marker alleles; 5901/5901, homozygous for V03-5901 marker allele.

§ Phenotype + marker genotype class not included in analysis of variance.

IV. Genotype × Environment Interaction and Stability of Phosphorus Content across Twelve Environments in Two Soybean Germplasm Sources with Modified Phosphorus Composition

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Abbreviations: LP, low phytate; *MIPS1*, D-*myo*-inositol 3-phosphate synthase 1 gene; NP, normal phytate; Pi, inorganic phosphorus; P, phosphorus.

ABSTRACT

Two sources of soybean [*Glycine max* (L.) Merr.] germplasm, CX1834 and V99-5089, improve the nutritional composition of soybeans due to different genetic mutations resulting in decreases in phytate and increases in inorganic phosphorus (Pi) content. It is important to understand the effect of environmental conditions on phytate and Pi content because the commercial production of cultivars with these germplasm sources depends on the stability of phytate and Pi content under diverse growing environments. We analyzed phytate and Pi content from eight genotypes grown in 12 environments to evaluate the genotypic and environmental effects and to determine trait stability. Two lines with normal composition and six lines modified for phosphorus composition (LP), including three CX1834-derived lines from different genetic backgrounds and three sister lines derived from V99-5089, were evaluated in this study. The effects of genotype and genotype × environment interaction were significant for phytate and Pi content. In addition, the environment significantly affected Pi content but not phytate content. The CX1834-derived lines were lower for phytate and higher for Pi content compared to the V99-5089-derived lines. Trait stability of the LP lines was higher for phytate content and lower for Pi content compared to the control cultivars. Five LP lines were average or above average for phytate stability which will allow LP cultivars to be produced commercially in a range of environments.

INTRODUCTION

Phytate, a mixed cation salt of phytic acid (*myo*-inositol 1,2,3,4,5,6-hexa*kis*phosphate) comprises up to 70% of total phosphorus (P) in conventional soybean [*Glycine max* (L.) Merr.] seed (Raboy et al., 1984). Phytate is an undesirable component of soybeans used to feed monogastric animals because phytate is not easily digested. Soybean germplasm with modified P content is available in which a reduction in phytate content and an increase in inorganic phosphorus (Pi) content are dually beneficial for the development of soybeans with improved nutritional profiles. Currently, low phytate (LP) soybeans are not commercially available, but future commercialization will require stability of the trait when grown in a wide variety of environments. Therefore it is important to evaluate LP genotypes in multiple environments to determine environmental effects and trait stability of P content.

Two of the four germplasm sources of soybeans with modified P composition are CX1834 (Wilcox et al., 2000) and V99-5089 (Saghai Maroof and Buss, 2008). Reduced seedling emergence has been reported for lines from both sources (Maupin et al., 2010; Oltmans et al., 2005) and commercial production will depend on acceptable emergence of LP cultivars. Soybean breeders have used the publicly available CX1834 germplasm to develop cultivars with two independent recessive mutations resulting in a LP phenotype (Gillman et al., 2009; Oltmans et al., 2004; Walker et al., 2006). Recently, the experimental line, V99-5089, with a single recessive mutation in the D-*myo*-inositol 3-phosphate synthase 1 gene (*MIPS1*) was described by Saghai Maroof and Buss (2008). In V99-5089, the *MIPS1* mutation not only modifies P composition but also results in elevated sucrose content with a concomitant decrease in raffinose and stachyose content. Gao et al. (2008) reported a similar percent of total P as phytate P for CX1834-1-6 and V99-5089. However, CX1834-1-6 (8.6 ± 0.4 mg g^{-1}) was significantly lower in

phytate content than V99-5089 (9.9 ± 0.5 mg g^{-1}). The evaluation (has not previously been completed) using advanced lines derived from both germplasm sources in multiple environments to determine trait stability of phytate and Pi content.

Inconsistent results have been obtained from studies of total P allocation to phytic acid in conventional soybeans with normal P composition grown in a range of external P concentrations. As a result of increases in external P concentrations, the seed total P increased due to increases in phytic acid P, whereas the non-phytic acid P remained constant (Raboy and Dickinson, 1984; 1993). Contrasting results have been reported for the genotype × environment effect on P composition of conventional soybeans with normal P composition (Israel et al., 2006; Raboy and Dickinson, 1993). In a field study by Raboy and Dickinson (1993), significant cultivar and environment effects, but not cultivar × environment interaction, were reported for phytic acid P concentrations. The environmental variation for available soil P resulted in a positive linear response for phytic acid concentrations in seeds. In contrast, non-phytic acid P content (including Pi) was not affected by the cultivar or environment. In the evaluation of breeding lines with normal composition from southern germplasm, Israel et al. (2006) reported that genotype, environment, and genotype × environment interaction were significant for Pi content but only genotypes were significant for phytic acid P content. As compared to Raboy and Dickinson (1993), soil P was not limiting in this experiment and they concluded that phytic acid P was stable over environments for a specific genotype with sufficient soil P. The effects of genotype × environment interaction have not been reported for soybeans with modified P composition in field environments.

The response of a CX1834-derived LP line to increases in external P supply was evaluated in controlled environmental chambers by Israel et al. (2007). Increasing P supply

increased the total seed P concentration similarly in both the normal phytate (NP) and LP lines. In the NP line, increases in external P supply increased phytic acid P content but did not change Pi content. In contrast, only Pi content increased while phytic acid P remained unchanged as a result of increases in the external P supply in the LP line. They concluded that the CX1834-derived line was stable for phytate content regardless of the external P supply indicating that the LP phenotype will be expressed when lines were grown in a variety of P soil conditions.

This study is unique because we included two LP germplasm sources in different genetic backgrounds and evaluated them for phytate and Pi content in a number of diverse field environments including different years and locations. The objectives of this research were (i) to evaluate the effects of genotype, environment, and genotype × environment interaction on phytate and Pi composition and (ii) to determine the stability of phytate and Pi composition across 12 environments for 8 genotypes with normal and modified P composition.

MATERIALS AND METHODS

Germplasm

Eight soybean lines were evaluated in this study, including three lines derived from CX1834 germplasm (04-05N32, LP-5601T-BC1, and S04-053-05), three lines derived from V99-5089 germplasm (V03-5900, V03-5901, and V03-5906), and two control cultivars (5601T and Essex). The genotype 04-05N32 is an F_4 derived lined developed by single seed descent from a single backcross population, NC-Roy × (CX1834-1-6 × NC-Roy), by Dr. Joseph Burton (USDA-ARS in Raleigh, NC). At the University of Tennessee, Dr. Vince Pantalone developed a LP derivative of 5601T from a single backcross: 5601T × (5601T × CX1834-1-2). The genotype of LP-5601T-BC1 was verified using the confirmed QTL cqPha001 and cqPha002 (Scaboo et al., 2009). The genotype S04-053-05 was developed from S03-4359 × S02-3934 at the University of Missouri, by Dr. Grover Shannon. The line S03-4359 contributing the LP trait was derived from (S99-2461RR × CX1834-1-2) and S02-3934 was derived from (DP5960RR × Anand). The three V99-5089-derived lines (V03-5900, V03-5901, and V03-5906) are sister lines developed from the cross between V99-5089, an experimental line with a novel mutation in the *MIPS1* gene, and the high yielding cultivar Essex. The two maturity group V control cultivars with normal seed P composition were 5601T (Pantalone et al., 2003) and Essex (Smith and Camper, 1973).

Field Experiment

The experiment was completed at six locations (Queenstown, MD; Portageville, MO; Plymouth, NC; Knoxville, TN; Blacksburg, VA and Mount Holly, VA) in 2008 and 2009 (Table 1). Each location and year combination was an environment. The experimental design was a randomized complete block design with three replications. The latitudes, elevations, soil types,

fertilizer applications, planting and harvest dates, mean temperatures and precipitation amounts for each location are presented in Table 1. Three locations were irrigated with either overhead irrigation (TN and VAMH) or furrows (MO) and the remaining locations were not irrigated (Table 1). The Mount Holly, VA location was a double crop production system in which the plots were planted following barley harvest into a field without soil tillage. All plots were planted based on the standard yield trial methods of the individual locations following standard agronomic practices for soybean production (Table 1).

Maturity data was recorded when 95% of the pods in a plot had reached their mature color and the data was converted to days after planting for each location. At maturity, plant height from the soil surface to tip of the main stem and lodging on a scale of 1 (all plants erect) to 5 (all plants prostrate) were measured. Seed size was recorded as the average weight of 100 random whole seeds from two samples of each plot. Seed quality was rated on a scale of 1 (seed surface smooth with no discoloration) to 5 (seed wrinkled with severe discoloration). For seed composition analysis, a 25 g subsample from each plot was ground in an Udy cyclone mill (U.D. Corp., Boulder, CO) to pass through a 1.0 mm screen.

Seed Composition Analysis

A modified version of the colorimetric assay developed by Raboy et al. (2000) adapted from the assay by Chen et al. (1956) was used to determine Pi content. The extraction and quantification methods for Pi were as described by Scaboo et al. (2009) with Pi concentrations determined using a BioTek Synergy HT plate reader (BioTek Instruments, Winooski, VT) set at a wavelength of 882 nm. The final Pi content of each plot was calculated from an average of three subsamples using the initial weight of each sample to calculate the Pi concentration on a dry weight basis with results reported as micrograms per gram.

High performance ion chromatography was used to determine phytate concentration. For phytate extraction, approximately 0.5 g of ground soybean seed was shaken in 10.0 mL of 0.5 M HCl in a 16 mL centrifuge tube for 4h at room temperature. At the end of shaking, 1.5 mL of sample was placed into a 2.0 mL microcentrifuge tube and centrifuged at 12,000 g, for 15 min at 10 °C. One mL of the supernatant was mixed with 1.0 mL of 20% NaCl in a 2.0 mL microcentrifuge tube. The extract was mixed by inversion 10 times and the salt-treated samples were placed in the refrigerator for 3 h before being centrifuged at 12,000 g for 10 min. This treatment removes the proteins and starch in the sample, which can potentially interfere with the ion chromatography elution. The supernatant was then diluted two times and filtered through a 0.2 µm PTFE membrane (IC Millex®-LG, Fisher, PA) into a 10-mL Dionex (Dionex, Sunnyvale, CA) sample vial for ion chromatography (IC) analysis. IC separation of phytate was performed on a Dionex ICS 3000 unit, equipped with an UltiMate 3000 VW detector, using Dionex AS7 analytical column and AG7 guard column. The elution was a 10-min isocratic process using 0.25 M HNO_3 at 1 mL per min. (Phillippy et al., 2003) with an injection volume of 25 µL. Phytate in effluent was detected colorimetrically using a post-column reaction taking place at room temperature in a reaction coil of 300 cm long and 0.5 mm I.D. The post-column reagent was 0.1% $Fe(NO_3)_3$ in 2% $HClO_4$ and pumped at 1 mL per min using a HPLC pump. The column effluent and color reagent met in a Tee connector, connected with the reaction coil. The UV absorption at 290 nm was recorded (Phillippy et al., 2003). Only plots from replication one and two of all environments were assayed for phytate concentration. The final phytate concentration was calculated using the initial weight of each sample to calculate the phytate concentration on a dry weight basis with results reported as micrograms P per gram.

Statistical Analysis

Pi and phytate data were logarithmically transformed by ln = $\log_e$ to normalize distribution of residuals and remove heterogeneity of error variances. The SAS procedure PROC MIXED (SAS, 2009) was used to conduct analysis of variance for Pi and phytate content. Genotype, environment, and their interaction were considered fixed effects and replication within environment was considered a random effect (Table 2). Pairwise comparisons of means were made using Tukey's multiple means comparison method. Genotype × environment least square means were evaluated using the slice=genotype option to determine environmental differences for each genotype. Phenotype correlation coefficients among traits were calculated with the means across environments using the PROC CORR procedure in SAS (SAS, 2009). Finlay and Wilkinson's (1963) regression analysis was completed with the SAS procedure PROC REG (SAS, 2009). Regression coefficients (*b*) were derived by a linear regression of mean of each genotype on the average mean of all genotypes at each environment. To evaluate stability, genotypes with a *b* value below 1 were considered above average for stability because they did not respond to different environments, genotypes with a *b* value of 1 were considered average for stability, and genotypes with a *b* value above 1 were considered below average for stability due to their response to different environments.

RESULTS AND DISCUSSION

This research is unique because we evaluated two sources of LP soybean germplasm in 12 diverse field environments to assess environmental effects on Pi and phytate content. The evaluation of advanced lines from two LP germplasm sources in multiple environments has not been reported. Trait stability for phytate and Pi content was also determined and trait stability between the two germplasm sources was compared. Trait stability of phytate and Pi content has not been reported for either germplasm source.

Seed Traits

The soybean genotypes were significantly different for seed traits including seed size and quality (Table 3). For seed size, there were significant ($P < 0.0001$) correlations with phytate (r = -0.63) and Pi (r = 0.71). Seed size of the CX1834-derived line, LP-5601T-BC1 was significantly larger than all other entries including the parental line, 5601T. The three V99-5089-derived lines had significantly larger seeds than the parental line, Essex. Genotypes were significantly different for maturity ranging from 136 days after planting to 144 days after planting (Table 3). There were no significant correlations between Pi and phytate with seed quality, but differences between genotypes for seed quality were significant. The CX1834-derived line, 04-05N32 was significantly better than all other lines for seed quality with a mean of 1.7 over all environments. The seed quality score of LP-5601T-BC1 was not as good as 5601T, but differences were not significant. Only V03-5906 was significantly worse than the control cultivars for seed quality. The quality scores of two V99-5089-derived lines (V03-5900 and V03-5901) were not significantly different from Essex. In both germplasm sources, quality scores were not affected by the modification in P composition which is beneficial for commercialization. In previous research (chapter III) evaluating V99-5089-derived germplasm,

quality scores were inferior in LP genotypes, which may have contributed to reduced emergence associated with the LP genotypes (Maupin, 2010). Therefore, during line development quality scores are important for selection both to maintain quality acceptable for commercialization of LP cultivars and to potentially protect emergence potential.

Genotype × Environment Interaction

There was significant variation for phytate and Pi content due to genotypes (Table 2). As expected, the control cultivars with normal P composition, 5601T and Essex, were highest for phytate and lowest for Pi content (Table 3). As a group, the three CX1834-derived lines were lowest for phytate content and highest for Pi content. Genotype S04-053-05 was significantly lowest for phytate content (Figure 1A) and significantly highest for Pi content (Figure 1B) in all environments. For both phytate and Pi content, the lines 04-05N32 and LP-5601T-BC1 were significantly different from each other but changed rank in the two P composition types (Table 3). Genetic variation has been reported for phytate and Pi content in conventional soybean lines (Israel et al., 2006) and parental choices could impact P composition when using mutant germplasm in a breeding program for modified P composition. The different genetic backgrounds of the parental lines contributed to the differences in phytate and Pi content between the three CX1834-derived lines.

The lines derived from V99-5089 were significantly higher for phytate content and significantly lower for Pi content in comparison to the CX1834-derived lines (Table 3). There were no significant differences among the V99-5089-derived lines for phytate content when evaluated in all environments. For Pi content, V03-5906 was significantly higher than both V03-5900 and V03-5901 which showed no significant difference. The V99-5089-derived lines are sister lines, therefore it is not surprising that they were similar for phytate and Pi content. In

comparing the two germplasm sources, our results agree with Gao et al. (2008), who reported that phytate content in CX1834-1-6 was significantly lower than V99-5089.

The environments were significantly different for Pi ($P < 0.0001$) but not phytate ($P = 0.0520$) content (Table 2). A few environments were distinct for high and low levels of Pi (Table 1) and in Figure 1 the environments are presented in order from left to right based on the environmental means for Pi content. The Pi content of the MD-2008 environment was significantly lower than all other environments followed by NC-2009 and MO-2009 which were not significantly different (Table 1). VAMH-2009 was the highest environment for Pi content and was not significantly different from the VAMH-2008 environment. The remaining environments were average for Pi content with less variation between the environments. Although the environments were not significantly different for phytate content, the correlation between phytate and Pi content for environmental means was positive ($r = 0.63$) and significant ($P = 0.0267$). As with Pi content, the MD-2008 environment was the lowest for phytate content followed by the NC-2009 environment. There were no obvious relationships between environmental means of phytate and Pi content with mean temperatures or precipitation levels at each environment (Table 1). Soil tests at each environment were not completed, but farm managers indicated that high/adequate levels of P are maintained to prevent P limitations. However, differences in soil P availability likely contributed to the significant differences between environmental means. It appears that the environmental differences for Pi and the lack of environmental differences for phytate content are dependent on the genotypic response over environments, therefore the significance of the G × E interaction must be considered.

There was significant variation due to the genotype × environment interaction for phytate ($P = 0.0017$) and Pi ($P < 0.0001$) content (Table 2), although the variation did not result in rank

changes between the LP and control cultivars. The control cultivars, 5601T and Essex, were the highest for phytate content (Figure 1A) and the lowest for Pi content (Figure 1B) in each environment. For phytate content, the control cultivars responded to the changes in environmental means including differences in rank in the different environments. In comparing Pi content in all environments, 5601T and Essex were constant with very little variation and only small differences between the two cultivars. Therefore, the control cultivars did not respond to the increasing environmental means for Pi content. These results are in agreement with previous research of genotypes with normal P composition, in which changes in P nutrition increased total P in mature soybeans by increasing the phytic acid P content with no detectable effects on non-phytic acid P including Pi content (Raboy and Dickinson, 1984).

The three V99-5089-derived lines were intermediate for both phytate (Figure 1A) and Pi content (Figure 1B) and the V99-5089-derived lines were distinct from the three CX1834-derived lines in any environment. Interactions including changes in rank among the V99-5089-derived lines were observed for phytate and Pi content across the 12 environments. Within the CX1834-derived lines, S04-053-05 was lowest for phytate content in all environments. The lines, 04-05N32 and LP-5601T-BC1, were not as consistent for phytate content but in most environments 04-05N32 was lower than LP-5601T-BC1. For Pi content, there were no crossover interactions between the CX1834-derived lines; S04-053-05 was highest followed by LP-5601T-BC1 which was intermediate to 04-05N32 which was the lowest.

The relationship between Pi and phytate content was strong and negatively correlated ($r = -0.82$, $P < 0.0001$) for all genotypes evaluated in the twelve environments. All LP lines remained relatively constant for phytate content in the different environments which is contrary to the response of the control cultivars. While the G × E interaction was significant, three LP

lines including 04-05N32 (P = 0.1274), LP-5601T-BC1 (P = 0.0626), and V03-5900 (P = 0.2337) were not significantly different in the slice analysis for phytate content in the 12 environments indicating stability for phytate content. As a group, the Pi content of the LP lines responded similarly to changes in mean Pi content across environments. The Pi means of each line increased in relation to the increases in the environmental mean which is in contrast to the normal P composition control cultivars which remained stable. The response of both LP germplasm sources was similar to the response of the CX1834-derived line evaluated by Israel et al. (2007) in which Pi content increased and phytate content remained stable with increasing P nutrition.

The correlation between phytate and Pi for the LP genotype means was strong (r = -0.97, $P < 0.0001$) indicating the effectiveness of the mutations in altering P content. The mutation(s) in both germplasm sources reduced the production of phytate, as a result mutant plants grown in soils with increased P availability responded with an accumulation of Pi content. This is beneficial for commercialization because LP soybean cultivars will remain low in phytate content regardless of the growing environment.

Trait Stability

Trait stability was evaluated with the regression coefficient (b) to determine if phytate and Pi content were stable in different environments (Table 3). Although the genotype × environment interaction was significant for phytate and Pi content, four LP lines exhibited above average stability for phytate and two LP lines exhibited above average stability for Pi content. The control cultivars were variable in the stability of phytate content and above average for Pi stability. The cultivar 5601T was average for phytate stability with a *b* value of 1.01, but was the most stable for Pi content with the lowest *b* value (0.41) of all lines. Essex was below average

and the least stable for phytate content. However, for Pi content, Essex was above average for stability and ranked second to 5601T. These results agree with Raboy and Dickinson (1984; 1993) and Israel et al. (2007) in which normal P composition soybean lines grown in variable P conditions respond with changes in phytate content but remain constant for Pi content.

Four out of six LP lines were above average for phytate stability including 04-05N32, S04-053-05, V03-5900, and LP-5601T-BC1 (Table 3). The two CX1834-derived genotypes (S04-053-05 and 04-05N32) with the lowest phytate content were also the most stable for phytate content. The above average stability of 04-05N32, LP-5601T-BC1, and V03-5900 agrees with the genotype × environment interaction analysis, in which the three lines were not significantly different in the 12 environments. The line V03-5901 was average for phytate stability and V03-5906 with a b value of 1.15 was below average for phytate stability. The overall stability of the LP lines was due to the mutations, which reduce the accumulation of phytate in seeds regardless of the growing environment. Slight differences in stability may allow breeders to select lines with above average stability to ensure consistency in phytate levels from different environments.

Pi content was more variable in the LP lines as compared to the control cultivars (Table 3). Additionally, within the LP lines, Pi content was not as stable as phytate content. Of the CX1834-derived line, S04-053-05 and 04-05N32 were above average for Pi stability and LP-5601T-BC1 was average for stability. The three V99-5089-derived lines with b values ranging from 1.18 to 1.51 were below average for Pi stability. The additional variation in Pi levels in the LP lines was similar to results with CX1834 germplasm by Israel et al. (2007). In the P nutrition experiment by Israel et al. (2007), the LP CX1834-derived line responded to increased P nutrition with increases in Pi content. In our experiment, the LP lines also responded to higher

environmental P means with increases in Pi content. Mutations in CX1834 and V99-5089 prevent significant increases in phytate; therefore the response to environmental changes is expressed with elevated Pi content. The results indicated that the environmental variation associated with soil P levels was expressed in the Pi content of modified P lines and the phytate content of conventional P composition lines.

The lower stability for V99-5089-derived lines as compared to CX1834-derived lines was unexpected and may be due to the differences in the amount of P content modified by the mutations. Previously, Gao et al. (2008) reported lower levels of phytate for CX1834-1-6 as compared to V99-5089 and our experiment confirmed those results (Table 3). In addition, we have confirmed that CX1834-derived lines are higher for Pi content than V99-5089-derived lines, which was previously assumed. The strength of the mutations in CX1834 may be contributing to the increased stability for phytate and Pi content, preventing additional variation in P composition. In contrast, the mutation in the *MIPS1* gene in V99-5089 germplasm is intermediate for phytate and Pi content between CX1834-derived germplasm and normal P germplasm, and the V99-5089 phenotype has intermediate stability for both phytate and Pi content. The V99-5089-derived lines are more variable for phytate content as a result of environmental P availability (similar to the control cultivars). Also, intermediate levels of Pi content results in a phenotype that is responsive to the environmental variation as with CX1834-derived lines. In V99-5089-derived lines, the phytate content was not as low or as stable as CX1834-derived lines and the Pi content was not as high or as stable as the normal P control cultivars.

In considering the commercial development of LP cultivars, the levels of phytate necessary for market acceptance remains unknown but both germplasm sources are beneficial for

improving P composition of soybeans. In plant breeding, the ease of gene integration could impact germplasm selection for breeding LP cultivars. The single gene mutation in V99-5089 would be preferred over the two recessive genes in CX1834. However, the recent development of molecular marker assays that directly select for the mutant alleles of CX1834 (Gillman et al., 2009) reduces the difficulty of integrating the two recessive genes in the CX1834 germplasm. The significant correlation between P composition and sugar components including sucrose, raffinose, and stachyose are additional benefits associated with the V99-5089 germplasm (Maupin et al., 2010; Saghai Maroof and Buss, 2008), and may be a reason to consider the two LP germplasm sources as distinct niches. The resulting modification of P content based on crossing CX1834 and V99-5089 germplasm is unknown and segregating populations are currently being evaluated, but our preliminary data suggest very LP phenotypes may be obtained (unpublished data). The impacts of mutations resulting in modification of P composition on emergence was evaluated in this experiment and are reported by Maupin and Rainey (2010). Ultimately, the reduced emergence associated with LP germplasm will be a factor in the commercialization of LP cultivars, and further research to compare both germplasm types for emergence will assist with germplasm selection.

CONCLUSIONS

Advanced lines from two sources of soybean germplasm with modified P composition were evaluated to determine the effects of genotype, environment, and genotype × environment interaction on phytate and Pi content. The genotypes were significantly different for phytate and Pi content and the CX1834-derived lines were lower for phytate and higher for Pi content compared to the V99-5089-derived lines. The environment significantly affected Pi content but not phytate content. The genotype × environment interaction was significant for both phytate and Pi content. However, there were no rank changes between the low phytate (LP) lines and control cultivars, allowing LP cultivars to be produced commercially in a range of environments. Lines with modified P composition accumulate more Pi rather than phytate content which contributed to the higher stability for phytate content but lower stability for Pi content when evaluated over 12 environments. However, five of the six LP genotypes were average or above average for phytate stability demonstrating the effectiveness of the mutations in reducing phytate levels regardless of the growing environment.

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Table 1. Description of locations including environmental conditions during the growing season† and means‡ for phytate and inorganic phosphorus (Pi) composition§ of eight soybean genotypes evaluated in 12 environments.

Location	Latitude and Elevation	Soil type	N-P-K Fertilizer	Irrigation	Planting date	Harvest date	Code	Mean Max. Temp	Mean Min. Temp	Precip	Phytate	Pi
								----- °C -----		mm	µg P g^{-1}	µg g^{-1}
Queenstown, MD	38.97 N	Matapeake silt	15-40-150	None	30 May	30 Oct	MD-2008	27	16	434	1333ns	612e
	0 m	loam	10-30-100		28 May	9 Nov	MD-2009	25	15	652	1799ns	1249bc
Portageville, MO	36.39 N	Tiptonville silt	0	Furrow	30 Apr	11 Oct	MO-2008	30	18	349	1780ns	1185c
	82 m	loam	0		22 Apr	19 Oct	MO-2009	28	18	748	1887ns	994d
Plymouth, NC	35.85 N	Portsmouth fine	0	None	22 May	31 Oct	NC-2008	29	17	297	1832ns	1306bc
	3 m	sandy loam	0		27 May	6 Nov	NC-2009	28	17	645	1628ns	993d
Knoxville, TN	35.89 N	Sequatchie silt	0-40-40	Overhead	22 May	20 Oct	TN-2008	29	16	399	1754ns	1320b
	325 m	loam	0-40-40		14 May	22 Oct	TN-2009	27	16	834	2051ns	1290bc
Blacksburg, VA	37.23 N	Wheeling silt	0-60-60	None	1 Jun	10 Nov	VABB-2008	25	11	321	1629ns	1338b
	540 m	loam	40-100-100		22 May	26 Oct	VABB-2009	25	13	436	1721ns	1326b
Mount Holly, VA	38.05 N	State fine sandy	0-60-100	Overhead	13 Jun	20 Nov	VAMH-2008	27	15	488	1741ns	1379ab
	22 m	loam	0-50-70		16 Jun	6 Nov	VAMH-2009	27	16	532	1853ns	1490a

† Mean maximum temperature, mean minimum temperature and precipitation based on planting and harvest dates for each location.

‡ Within each trait, environment means followed by the same letter are not significantly different according to Tukey's pairwise comparison at P=0.05.

§ All phosphorus composition data were ln transformed for analysis and back transformed for table.

Table 2. Analysis of variance for phytate and inorganic phosphorus composition[†] for eight genotypes in 12 environments[‡].

	Phytate				Inorganic Phosphorus			
	---------- µg P g^{-1} ----------				---------- µg g^{-1} ----------			
	Fixed Effects							
Source of Variation	Numerator DF	Denominator DF	*F*	*P*	Numerator DF	Denominator DF	*F*	*P*
Genotype	7	81	434.6	<0.0001	7	168	2732.1	<0.0001
Environment	11	12	2.7	0.0520	11	24	130.6	<0.0001
Genotype × Environment	77	81	1.9	0.0017	77	168	6.1	<0.0001
	Random Effects							
Source of Variation	Estimate	Standard Error	*Z*	*P*	Estimate	Standard Error	*Z*	*P*
Replication (Environment)	0.0068	0.0033	2.1	0.0201	0.0003	0.0004	0.7	0.2472
Residual	0.0102	0.0016	6.4	<0.0001	0.0084	0.0009	9.2	<0.0001

† All seed composition data were ln transformed for analysis.

‡ Environments included six locations in Queenstown, MD, Portageville, MO, Plymouth, NC, Knoxville, TN, Blacksburg, VA, and Mount Holly, VA in 2008 and 2009.

Table 3. Genotype means[†] for phosphorus composition[‡], maturity, seed size, and quality and stability parameters for phosphorus composition[‡] for eight genotypes grown in 12 environments[§].

	Phytate						Inorganic Phosphorus						Maturity[¶]	Seed Size	Quality[#]
	---------- µg P g^{-1} ----------						---------- µg g^{-1} ----------						DAP	g 100^{-1}	1 - 5
Genotype	Mean	Rank	*b*	r^2	*P*	Stability Rank	Mean	Rank	*b*	r^2	*P*	Stability Rank	Mean	Mean	Mean
S04-053-05	878e	1	0.76	0.35	0.0444	2	2473a	1	0.91	0.92	<0.0001	3	143ab	18.3b	2.3bc
04-05N32	1126d	2	0.73	0.43	0.0204	1	2002c	3	0.97	0.94	<0.0001	4	144a	15.9d	1.7d
LP-5601T-BC1	1269c	3	0.94	0.62	0.0025	4	2198b	2	1.02	0.94	<0.0001	5	137f	18.9a	2.4abc
V03-5906	1935b	4	1.15	0.74	0.0003	7	1525d	4	1.34	0.91	<0.0001	7	139de	16.8c	2.5a
V03-5901	1970b	5	1.03	0.69	0.0008	6	1416e	5	1.18	0.92	<0.0001	6	139e	15.4e	2.3abc
V03-5900	2071b	6	0.89	0.74	0.0003	3	1388e	6	1.51	0.97	<0.0001	8	140cd	15.6de	2.4ab
5601T	2924a	7	1.01	0.45	0.0178	5	344f	7	0.41	0.35	0.0407	1	142bc	14.1f	2.2c
Essex	2933a	8	1.62	0.63	0.0021	8	334f	8	0.66	0.58	0.0039	2	136f	13.9f	2.2bc

† Within each trait, genotype means followed by the same letter are not significantly different according to Tukey's pairwise comparison at *P*=0.05.

‡ All phosphorus composition data were ln transformed for analysis and back transformed for table.

§ Environments included six locations in Queenstown, MD, Portageville, MO, Plymouth, NC, Knoxville, TN, Blacksburg, VA, and Mount Holly, VA in 2008 and 2009.

¶ Maturity based on 10 environments excluding MD in 2009 and NC in 2008 and reported as days after planting.

Quality rating from 1 to 5: 1 = seed surface smooth with no discoloration to 5 = seed wrinkled with severe discoloration.

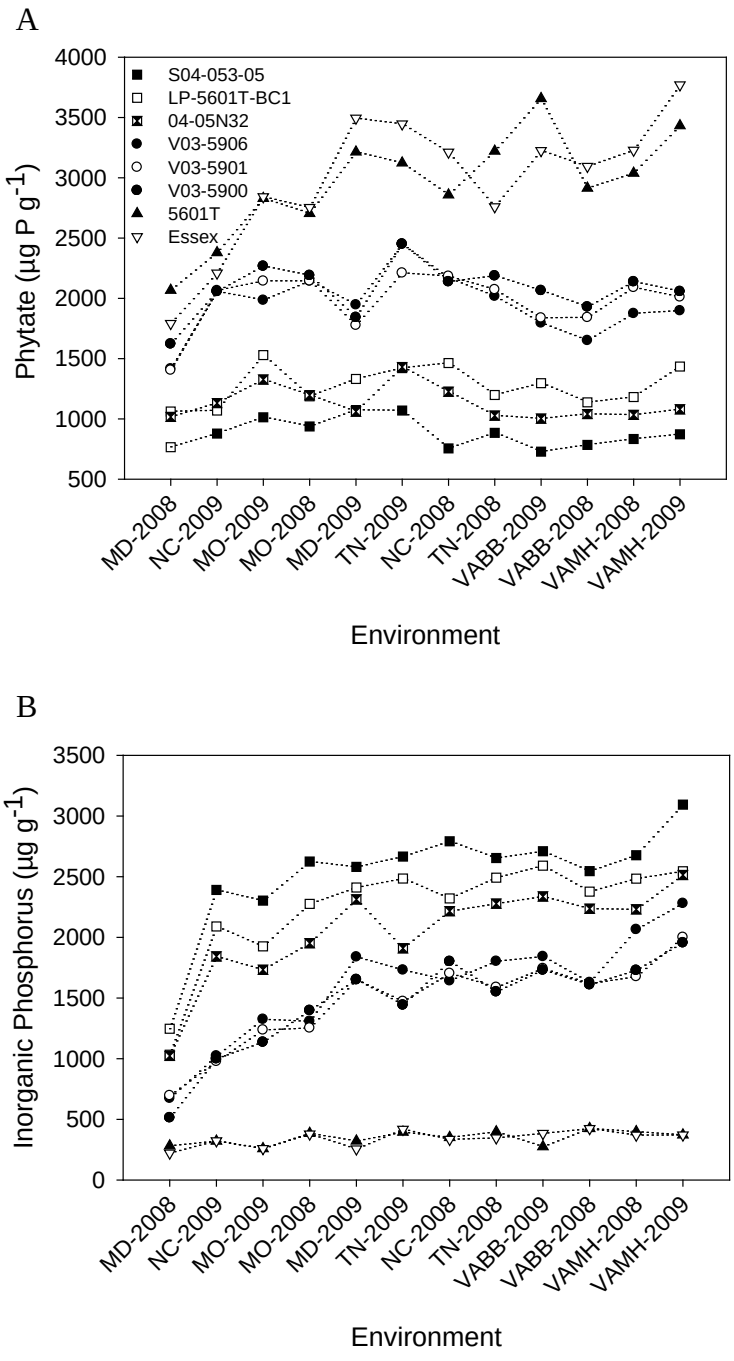

Figure 1. Mean A) phytate and B) inorganic phosphorus composition of eight soybean genotypes in 12 environments in 2008 and 2009. MD = Queenstown, MD, MO = Portageville, MO, NC = Plymouth, NC, TN = Knoxville, TN, VABB = Blacksburg, VA, and VAMH = Mount

Figure 1. Continued.

Holly, VA. Symbols are squares for CX1834-derived lines, circles for V99-5089-derived lines, and triangles for control cultivars.

V. Seedling Emergence of Two Soybean Germplasm Sources with Modified Phosphorus Composition

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Abbreviations: LP, low phytate genotype; *MIPS1*, D-*myo*-inositol 3-phosphate synthase 1 gene; NP, normal phytate genotype; P, phosphorus; Pi, inorganic phosphorus.

ABSTRACT

The development of soybeans [*Glycine max* (L.) Merr.] with reduced phytate and increased inorganic phosphorus levels has nutritional and environmental benefits, but poor seedling emergence presents challenges for commercial production. Two low phytate germplasm sources in the public sector are CX1834 and V99-5089. This experiment evaluated emergence of three advanced lines derived from the CX1834 low phytate germplasm, three advanced lines derived from V99-5089 low phytate germplasm, and two normal phytate control cultivars at six locations in 2008, 2009, and 2010. In 2010, seed from the eight lines grown in 12 environments was evaluated in an extended cold germination test (ECGT) and in a replicated field emergence test in Warsaw, VA. Genotype, environment, and the genotype × environment interaction were significant ($P < 0.001$) for emergence in the agronomic trial (2009 and 2010), ECGT, and field emergence test in 2010. One CX1834-derived line (04-05N32) and two V99-5089-derived lines were not significantly different from the two control cultivars for emergence in 12 environments. Yields of the best emerging lines were not significantly different from the control cultivars. The ECGT and field emergence test were significantly correlated ($r = 0.78$) but the ECGT severely reduced germination as compared to the field emergence test. In the ECGT, all the low phytate lines emerged significantly less than the control cultivars, but in the field emergence test, one low phytate line was not significantly different from the check cultivars. The ECGT was successful in separating lines based on emergence. However, poor emergence in the ECGT did not always translate to poor emergence under field conditions, preventing the use of ECGT as a selection tool or in determining seeding rates for the Southeastern and Mid-Atlantic regions.

INTRODUCTION

The commercial production of low phytate (LP) soybean [*Glycine max* (L.) Merr.] cultivars will be nutritionally and environmentally beneficial. However, the reduced seedling emergence associated with LP cultivars has been a barrier for commercialization (Anderson and Fehr, 2008; Hulke et al., 2004; Meis et al., 2003; Oltmans et al., 2005; Spear and Fehr, 2007; Trimble and Fehr, 2010; Yuan et al., 2007). Multiple sources of soybean germplasm with modified phosphorus (P) content have been identified in which the phytate content is reduced and the inorganic phosphorus (Pi) content is increased (Saghai Maroof and Buss, 2008; Sebastian et al., 2000; Wilcox et al., 2000; Yuan et al., 2007). The CX1834 LP germplasm developed by Wilcox et al. (2000) has been used by public and commercial soybean breeders to develop LP cultivars. The LP soybean line V99-5089 with a mutation in the D-*myo*-inositol 3-phosphate synthase 1 (*MIPS1*) gene not only modifies P composition but also results in increased levels of sucrose and reduced levels of raffinose and stachyose (Saghai Maroof and Buss, 2008). In a related report evaluating P content in 12 environments, the CX1834 germplasm was lower for phytate and higher for Pi content as compared to the V99-5089 germplasm (Maupin et al., 2010b).

Reduced emergence has been reported in lines from both germplasm sources and the subtropical winter nursery environment for seed production has been implicated as a contributing factor for reduced emergence. In the evaluation of LR33-derived germplasm with a mutation in the *MIPS1* gene developed by Pioneer Hi-Bred International, Meis et al. (2003) compared six subtropical environments and four temperate environments in which the LR33-derived lines were significantly lower for emergence than the normal phytate (NP) lines for all seed sources. However, emergence reductions of LR33-derived lines were more severe with seed produced in

subtropical winter nursery environments (8%) as compared to seed from temperate environments (63%). In three VA environments, the emergence percentages for V99-5089 were 52%, 81%, and 12% (Gao et al., 2008). In a V99-5089 RIL population, the correlation for emergence with Pi was significant (r = -0.59), indicating reduced emergence due to the mutation (Maupin et al., 2010a). However, when the seed was produced the previous season in the temperate environment of Warsaw, VA, emergence between LP and NP lines was not significantly different in three environments. In a separate population of the V99-5089-derived germplasm, the winter nursery environment significantly reduced emergence of LP lines, skewing segregation ratios and preventing analysis of agronomic traits (Maupin, 2010).

Extensive research has been completed to evaluate emergence in CX1834-derived germplasm and the effect of the LP trait on yield (Anderson and Fehr, 2008; Hulke et al., 2004; Oltmans et al., 2005; Spear and Fehr, 2007; Trimble and Fehr, 2010). In the evaluation of CX1834-derived genotypes with reduced palmitate, Hulke et al. (2004) reported significantly reduced emergence of LP lines as compared to NP lines in three Iowa locations. Although the LP lines were lower for plant density, the yield of the LP lines was not significantly different from the NP lines when averaged over the three Iowa locations. Additionally, Oltmans et al. (2005) reported significant differences in emergence among three Iowa environments for LP and NP lines in which the LP lines were lower for emergence in all of the environments. In all populations, the LP lines were lower than NP lines for yield but true yield differences were difficult to assess due to the reduced emergence and plant density in LP lines. In a study backcrossing the CX1834 trait into a NP line to improve the emergence of LP lines, Spear and Fehr (2007) reported significant differences in emergence among the backcross lines with 47% to 75% emergence. Additionally, eight lines that were significantly higher for emergence than

CX1834 and not significantly different from the NP parent were further evaluated for yield in which none were significantly different from the NP parent. Non-significant correlations between Pi concentrations and yield reported by Scaboo et al. (2009) further demonstrated that the LP trait derived from CX1834 does not negatively impact yield. Although yield was not affected by the LP trait, each report indicated reduced emergence for LP lines limiting the commercialization of LP lines. Therefore, additional research will be necessary to develop LP lines with consistent emergence.

The use of laboratory germination tests including warm germination, cold vigor, and accelerated aging have been evaluated as a means of predicting field emergence in LP lines. A study by Meis et al. (2003) focused on seed produced in subtropical winter nursery environments to evaluate the seed source effect on field emergence of lines derived from the proprietary LR33 germplasm. In addition, they evaluated the ability of three laboratory germination tests to differentiate emergence potential in LP and NP lines. For the temperate sources, the warm germination and cold vigor tests were not consistent in predicting the observed differences for field emergence between the LP and NP genotypes. However, they determined that the accelerated aging test was effective in differentiating field emergence of both LP and NP lines produced in all source environments and concluded that the accelerated aging test would be effective at identifying the emergence potential of LR33-derived lines regardless of the seed source. In the evaluation of CX1834-derived germplasm, Spear and Fehr (2007) evaluated 36 backcross lines for field emergence and selected eight high and seven low lines for additional laboratory germination testing. The phenotypic correlations for field emergence with warm germination ($r = 0.49$) were not significant but correlations with cold vigor and accelerated aging were significant (0.82 and 0.69, respectively). They determined that the cold vigor and

accelerated aging test were beneficial for discarding poor emerging lines but were not sufficient to replace field emergence testing to identify the best emerging lines.

Recently, Trimble and Fehr (2010), developed the extended cold germination test (ECGT) to evaluate LP lines. In the ECGT, seeds are placed in 10 °C for 21 days (d) as compared to the standard cold test in which seeds are subjected to 10 °C for 7 d followed by 25 °C for 5 to 7 d. Results from an experiment in three IA locations with seed produced in Puerto Rico using the CX1834 germplasm were not useful due to reduced emergence in all LP lines (Trimble and Fehr, 2010). Seed from the Carlisle, IA location was used in the subsequent year to plant five IA locations which were evaluated for emergence. The emergence of NP and LP seed from the Carlisle, IA seed source was low when evaluated across the five IA locations. They developed the ECGT to evaluate the lines from the original three IA locations and results indicated that the Carlisle, IA source was low for emergence in the ECGT as well. The correlation between ECGT emergence of the Carlisle, IA seed source and the field emergence of the five IA locations planted with the Carlisle seed source for all lines was 0.95 ($P < 0.01$). The high correlation indicates that the ECGT is useful for assessing the emergence potential of LP lines in IA field environments. They determined that the ability of the ECGT to consistently identify lines with poor emergence regardless of the temperate seed source environment was beneficial for discarding lines with poor emergence.

Reports for reduced emergence of CX1834 have focused on seed produced in subtropical winter nursery environments. However, seed produced in the temperate environment such as IA and VA were not exempt from reductions in emergence (Gao et al., 2008; Maupin et al., 2010a; Oltmans et al., 2005; Trimble and Fehr, 2010). Therefore, CX1834 and V99-5089 advanced lines produced in VA for emergence in 12 environments throughout the Mid-Atlantic and

Southeastern US were evaluated. Yield was evaluated because extensive multi-location yield data for advanced *MIPS1* mutant lines was not available. In addition, advanced LP lines from the two germplasm sources have not been compared for emergence and yield in a combined experiment in multiple environments. We also focused on the effect of temperate seed production environments on emergence of LP lines by evaluating the seed produced in the 12 Mid-Atlantic and Southeastern environments in the ECGT and a field emergence test.

MATERIALS AND METHODS

Germplasm

Eight soybean lines were evaluated in this study, including three lines derived from CX1834 germplasm, three lines derived from V99-5089 germplasm, and two control cultivars. The genotype 04-05N32 is an F_4 derived lined developed by single seed descent from a single backcross population, NC-Roy × (CX1834-1-6 × NC-Roy), by Dr. Joseph Burton (USDA-ARS in Raleigh, NC). At the University of Tennessee, Dr. Vince Pantalone developed a LP derivative of 5601T from a single backcross: 5601T × (5601T × CX1834-1-2). The genotype of LP-5601T-BC1 was verified using the confirmed QTL cqPha001 and cqPha002 (Scaboo et al., 2009). The genotype S04-053-05 was developed from S03-4359 × S02-3934 at the University of Missouri, by Dr. Grover Shannon. The line S03-4359 contributing the LP trait was derived from (S99-2461RR × CX1834-1-2) and S02-3934 was derived from (DP5960RR × Anand). The three V99-5089-derived lines (V03-5900, V03-5901, and V03-5906) are sister lines developed from the cross between V99-5089, an experimental line with a novel mutation in the *MIPS1* gene, and the high yielding cultivar Essex. The two maturity group V controls with normal seed P composition were 5601T (Pantalone et al., 2003) and Essex (Smith and Camper, 1973).

Agronomic Trial

The experiment was completed at six locations (Queenstown, MD; Portageville, MO; Plymouth, NC; Knoxville, TN; Blacksburg, VA and Mount Holly, VA) in 2008, 2009, and 2010. Each location and year combination was an environment with environments abbreviated as the state initials and the year. The two VA locations are designated VABB for Blacksburg, VA and VAMH for Mount Holly, VA. The experimental design was a randomized complete block design with three replications. Three locations were irrigated with either overhead irrigation (TN

and VAMH) or furrows (MO) and the remaining locations were not irrigated (Table 1). The Mount Holly, VA location was a double crop production system in which the plots were planted following barley harvest into a field without soil tillage. Emergence data were collected in 2008, 2009 and 2010. In 2008, seed of each genotype was provided by individual breeding programs and differences in seed source production environments prevented comparisons between genotypes for emergence. In 2009 and 2010, all locations were planted with seed harvested from VAMH in 2008 and 2009, respectively.

Prior to planting in 2009 and 2010, a standard (warm) germination test was completed on seed from VAMH-2008 and VAMH-2009. SGS Mid-West Seed Services, Inc. completed the warm germination test on a 400 seed sample at 25 °C for 7 d. Germination was evaluated based on standards provided by the Association of Official Seed Analysts (AOSA, 2009). For each genotype, the number of seeds planted per plot at each location was increased to achieve 100% live seed planted. Plot dimensions differed at each location therefore the number of seeds planted per plot were adjusted based on both the warm germination test and the planting density recommended by each location. All plots were planted based on the standard agronomic trial methods of the individual locations following standard agronomic practices for soybean production.

In three planting seasons (2008-2010), seedling emergence data were collected in each plot when plants were between the V2 – V5 stage (Fehr and Caviness, 1977). Due to differences in seed source environments in 2008, only seedling emergence data from 2009 and 2010 were included in the statistical analysis. Seed from BB-2008 was evaluated for sucrose, stachyose, and raffinose content by high performance liquid chromatography (HPLC) as described by Cicek

et al. (2006). During the 2008 and 2009 seasons, seed yield (kg ha^{-1}) was collected for all plots at all locations but only 2009 yield data were analyzed due to differences in emergence in 2008.

Seed Source Experiments: Extended Cold Germination Test and Field Emergence Test

To determine the effect of seed source and the LP trait, seed harvested from all locations in 2008 and 2009 were evaluated in an ECGT and in a field emergence test. Both tests were completed in the spring 2010 and the 2008 seed was stored in cold storage at -8 °C and 0% humidity to prevent seed deterioration prior to testing.

The ECGT was developed by Trimble and Fehr (2010) to determine seedling emergence of LP lines. Seed samples grown at the six locations in 2008 and 2009 were evaluated at the Iowa State University Seed Science Center in Ames, IA. The experiment was completed as a split plot with six replications in which the seed source environment was the whole plot and genotype was the subplot. A sample consisted of 100 seeds of each genotype from each environment. The protocol as described by Trimble and Fehr (2010) was followed in which seeds were covered with 4:1 ratio of sand-soil mixture and placed in a cold room at 10 °C for 21 d at which point seedling emergence was evaluated.

To evaluate field emergence, seed from 12 environments (six locations from 2008 and 2009) was planted 21 May 2010 in Warsaw, VA. A split plot experiment with six replications evaluated seed source as the whole plot and genotype as the subplot. Each plot was two rows with 50 seeds planted per 3 m row with 0.76 m row spacing. Emergence data were collected 30 d after planting.

Statistical Analysis

The SAS procedure PROC MIXED (SAS, 2009) was used to conduct analysis of variance. For 2009 and 2010 field emergence data, genotype, environment, and the interaction

were considered fixed effects and replication within environment was considered a random effect. For yield data from 2009, each location was analyzed separately with genotypes as fixed effects and replications as random. In the analysis of ECGT and field emergence test in 2010, genotype, environment, and the interaction were considered fixed effects and replication was considered a random effect. Pairwise comparisons of means were made using Tukey's multiple means comparison method. Genotype × environment least square means were evaluated using the slice=environment option in SAS to determine differences between genotypes within each environment (SAS, 2009). Phenotype correlation coefficients among traits were calculated using the PROC CORR procedure in SAS (SAS, 2009).

RESULTS AND DISCUSSION

Agronomic Trial

In the subsequent results and discussion, seedling emergence percentages of 80% or greater are considered commercially acceptable. There were differences for emergence due to genotypic and environmental effects in 2009 and 2010 (Table 1). Rank changes and magnitude differences for a few genotypes resulted in a significant genotype × environment interaction (Table 1 and 2). However, the majority of genotypes responded similarly in each environment highlighting the greater influence of the environmental conditions at planting on emergence, rather than the seed production environment or genotype.

In the 12 environments, there were significant differences for emergence with environmental means ranging from 45% in MO-2009 to 96% in NC-2009 (Table 2). In five environments, emergence was unacceptably low for all genotypes including both control cultivars and LP lines. These reductions are likely due to the environmental conditions during planting and emergence. The significant reductions in emergence for MO-2009 were due to two significant rain events with 40 and 42 mm of rainfall within 10 d after planting. In VAMH, poor planting conditions as a result of unusually dry conditions in 2010 (43 mm of rainfall in the 20 d prior to planting) reduced emergence to 60% compared to 85% emergence in 2009 in which there was 173 mm of rainfall in the 20 d prior to planting. Reductions in emergence of both LP and control cultivars were similar in the environments with reduced emergence indicating that environmental conditions were responsible for emergence reductions rather than germplasm type or the seed source environment. Conversely, emergence in seven environments was greater than 80% including three environments with greater than 90% emergence (MD-2009, MD-2010, and NC-2009). The same seed source was used in environments with both high and low emergence

percentages in each year, again indicating that planting environment is a greater factor reducing emergence than seed production environment.

In the evaluation of genotypes, 04-05N32 with 84% emergence was the highest for emergence averaged over all locations (Table 2). Line 04-05N32 was not significantly different from the two control cultivars which averaged 82% emergence. The V99-5089-derived lines, V03-5900 and V03-5901, were lower for emergence with 80% and 78%, respectively. However, V03-5900 was not significantly different from 04-05N32 or the control cultivars and V03-5901 was not significantly different from the control cultivars. Emergence for the remaining LP lines including LP-5601T-BC1 (76%), V03-5906 (74%), and S04-053-05 (72%) was significantly lower than the control cultivars. The development of LP commercial cultivars has been in question due to previous reports of reduced emergence. The emergence potential of 04-05N32 was high in the majority of environments and further evaluations should be completed to determine the factors responsible for superior emergence. The seed quality of 04-05N32 was previously reported by Maupin et al. (2010b) to be significantly better than all other LP lines and control cultivars. In this study, non-significant differences between the control cultivars and three LP lines (04-05N32, V03-5900, and V03-5901) demonstrate that LP lines can be developed with acceptable emergence, which will facilitate commercial production of LP cultivars.

Yield was evaluated because the commercialization of LP cultivars depends on comparable yields to commercial cultivars. In 2008, the seed source environment of each line was different preventing comparisons between lines for emergence. Additionally, due to significant differences between lines within each location for emergence (data not shown), analyses to compare yield of control cultivars and LP lines in 2008 was prohibited.

In 2009, evaluation of yield differences between lines was possible because in three locations (NC, TN, and VAMH) lines were not significantly different (using genotype × environment slice analysis) from each other for emergence (Table 2). In the yield analysis of NC-2009, the control cultivars 5601T and Essex were highest for yield but four of the six LP lines were not significantly different from the control cultivars (Table 3). Additionally, in TN-2009 and VAMH-2009, lines were not significantly different for yield. Emergence was significantly different in MO-2009 and VABB-2009, preventing comparisons between lines for yield (Table 2). In the evaluation of MD-2009, emergence was significantly different for lines; however, yield was not significantly different among lines (Table 3). Yield equivalence between control cultivars and LP genotypes in three environments is favorable for the development of commercial LP cultivars. Additionally, in all 2009 environments, yield and emergence were not correlated with Pi and/or phytate content. Our results are in agreement with previous research on CX1834 germplasm showing that the LP trait does not negatively impact soybean yield (Hulke et al., 2004; Oltmans et al., 2005; Scaboo et al., 2009; Spear and Fehr, 2007). In evaluating two LP germplasm sources we have shown that low phytate *per se* does not negatively impact soybean yields.

Seed Source Experiments

The environmental conditions at the time of planting were the primary reason for reductions in emergence in the five environments with poorest emergence in 2009 and 2010. However, emergence remains a concern for LP lines due to the reduced emergence of some LP lines in this experiment (Table 2). The seed source environment, specifically subtropical winter nursery environments, has been implicated as a factor in the reduced emergence of LP lines (Anderson and Fehr, 2008; Maupin, 2010; Meis et al., 2003) but the impact of temperate

growing environments of the Mid-Atlantic and Southeastern regions on emergence of LP lines has not been evaluated. To assess the impact of seed production environment on germination and emergence of LP germplasm, seed of LP lines (CX1834- and V99-5089-derived) and control cultivars from six different locations and two years (2008 and 2009) were evaluated in an ECGT and a field emergence test in 2010.

Extended Cold Germination Test

The ECGT resulted in significant genotypic, environmental, and genotype × environment interaction effects (Table 4). The genotype × environment interaction was significant due to changes in rank of individual lines and to changes in the magnitude of the differences among lines (Table 5). Germination was variable for genotypes and environments ranging from 1% for V03-5906 in seed from NC-2008 to 92% for seed of 5601T (MD-2008 and VAMH-2008) and Essex (MD-2008). Additionally, there was a significant difference for genotypes within each of the 12 seed source environments based on the slice analysis of the genotype × environment interaction.

Germination in the ECGT was significantly affected by the seed source environment (Table 4). Seeds from only one environment, MD-2008, averaged greater than 80% germination for all genotypes (Table 5). In the six environments with mean germination from 63% to 79%, the control cultivars germinated well ranging from 80% to 93%. There were five environments (TN-2008, MO-2008, NC-2008, NC-2009, and TN-2009) which produced seed that averaged less than 50% germination. In TN-2008 (46%) and NC-2009 (47%), the germination of the control cultivars was slightly lower than 80%. Therefore, in these environments the overall reduced emergence was a result of lower germination by the LP genotypes. In the three environments (NC-2008, MO-2008, and TN2009) with the lowest germination, the two control

cultivars also germinated poorly indicative of an overall environmental cause for reductions in germination. In seed from NC-2008 and MO-2008, all lines were low for germination but the LP lines were responsible for the much lower emergence, which is in contrast to the TN2009 environment in which germination for all lines including the control cultivars was significantly reduced. In the environments with lower emergence, the LP lines were primarily responsible for the reduced emergence potential. These results suggest increased sensitivity of LP lines to potential stressors present in seed production environments, as expressed in unfavorable emergence conditions.

The two environments with the poorest seedling emergence were from 2008 raising concern that seed storage was solely responsible for reducing emergence; however the environment with the highest emergence percentage was also from 2008 indicating that seed storage was not the primary reason for reduced emergence (Table 5). The mean maximum in-season temperature in the top five environments for emergence was either 25 °C or 27 °C, as compared to the two environments with the lowest emergence, NC-2008 and MO-2008 with mean maximum temperatures of 29 °C and 30 °C, respectively. Egli et al. (2005) reported that reductions in seed germination and vigor were related to increases in mean daily maximum temperatures. However, the temperatures that decreased germination were higher than those reported in our experiment. A report by Spears et al. (2005) reported that increased temperatures decreased the germination of high-oleate soybeans but moderate temperatures also reduced seed germination and quality. Our results suggest that moderately high temperatures in the seed production environment reduces emergence of LP lines.

However, temperature was not the only factor reducing germination in the ECGT. The mean maximum temperature was 27 °C in both TN-2009, in which seed was low for

germination, and MD-2008, which produced seed with the highest overall germination (Table 5). Seed quality was different in the two environments: seed produced in TN-2009 was significantly poorer in quality (3.1) as compared to seed from MD-2008 (2.2). In addition, sudden death syndrome (*Fusarium virguliforme*) was observed in TN-2009 (Ben Fallen, personal communication, 2009), which has been reported to reduce seed quality (Njiti et al., 1998) and to reduce germination and vigor (Leitz et al., 1995; Wintizer et al., 1991). Seed quality and mean maximum temperatures influence germination in the ECGT based on significant correlations with quality (r = -0.51) and mean maximum temperatures (r = -0.52). Overall, the seed production environment was a major influence on emergence in the ECGT but genotypes also were significantly different in the ECGT (Table 4).

Averaged over all seed production environments, all genotypes had less than 76% germination in the ECGT (Table 5). In the majority of environments, the control cultivars 5601T and Essex were the best for germination and averaged the highest overall germination with 76% and 71%, respectively. The germination of LP lines was significantly reduced in comparison to the control cultivars but there was considerable variation for germination in the LP lines derived from both CX1834 and V99-5089 germplasm. There was no distinct pattern for the three CX1834-derived genotypes for germination in the 12 environments. Line 04-05N32 was significantly higher than all other LP lines with 63% emergence followed by LP-5601T-BC1 and S04-053-05 that were not significantly different with 57% and 52% emergence, respectively. The three CX1834-derived genotypes were intermediate as compared to the V99-5089-derived genotypes which were lowest for germination from most environments. The CX1834 lines were lower for phytate and higher for Pi than the V99-5089 lines; therefore, P content was not directly

responsible for the reduced emergence observed in V99-5089 lines compared to the CX1834 lines (Maupin et al., 2010b).

Of the V99-5089-derived lines, only V03-5900 was not significantly different from the lowest CX1834-derived line (Table 5). V03-5906 was the line with the lowest emergence over all environments (35%). The poor performance of the V99-5089-derived lines may be due to the *MIPS1* mutation which also reduces raffinose and stachyose content. Stachyose and raffinose composition are associated with desiccation tolerance and longevity of seeds during storage (Horbowicz and Obendorf, 1994). The longevity of seeds has been correlated with the ratio of sucrose to total raffinose family oligosaccharide content, in which lower ratios indicated longer storability (Horbowicz and Obendorf, 1994). Sugar composition was determined for the BB-2008 environment in order to calculate the ratio between sucrose and raffinose plus stachyose content. The ratio for the control cultivars and CX1834-derived genotypes ranged from 1.1 to 1.5 as compared to 10.9, 13.5, and 14.2 for the V99-5089-derived genotypes, indicating reduced storability for the V99-5089-derived genotypes. Reduced storability might explain the single digit emergence percentages observed in the ECGT for all V99-5089 genotypes from MO-2008 and NC-2008 but it does not account for the higher emergence of the V99-5089-derived genotypes in the MD-2008 environment. Therefore, a synergistic effect between the higher mean maximum temperatures of MO-2008 and NC-2008 and the 2-year storage period may have caused the extremely low emergence of the V99-5089-derived lines. Multiple factors influence the emergence potential of all LP lines but the V99-5089-derived lines are especially sensitive when evaluated for germination in the ECGT.

Although emergence of V99-5089-derived lines was significantly reduced in the ECGT, previous research on germplasm with modified sugar content has indicated that modifications to

sugar composition does not affect emergence. Low emergence problems have not been reported for PI200508 which has increased sucrose content and reduced raffinose and stachyose content (Dierking and Bilyeu, 2009; Neus et al., 2005). Additionally, Dierking and Bilyeu (2009) concluded that raffinose oligosaccharides, including raffinose and stachyose, were not an essential source of energy for soybeans during seed germination. Previously we concluded that the reduction in emergence in a V99-5089 population was due to the LP trait rather than the low raffinose and stachyose content (Maupin et al., 2010a) because problems with reduced emergence have been reported for all LP germplasm sources with both normal (Anderson and Fehr, 2008; Oltmans et al., 2005) and reduced levels of raffinose and stachyose (Meis et al., 2003; Sebastian et al., 2000). However, additional seed components are elevated or reduced in mutant germplasm that may contribute to emergence potential. Obendorf et al. (2009) reported that lines from LR33-derived germplasm were lower in galactosyl cyclitols during maturation as compared to normal composition lines and lines with reduced raffinose and stachyose. Research is necessary to determine why the mutation in the *MIPS1* gene further reduces germination and emergence in the ECGT.

The prolonged period of 21 d in 10 °C of the ECGT may create a stressful environment for germination in which the V99-5089-derived genotypes were less tolerant. An experiment by Obendorf et al. (2008) demonstrated that lines with reduced phytate, raffinose, and stachyose were sensitive to imbibitional chilling but control lines and lines with reduced raffinose and stachyose were tolerant. In the chilling treatment, seeds were imbibed at 5 °C for 36 h, transferred to 25 °C for 4 d and evaluated for axis length and percent germination. The axis length of the low phytate, raffinose, and stachyose lines was significantly reduced showing sensitivity to the imbibitional chilling treatment. Lines derived from CX1834 with only reduced

phytate were not evaluated in the study by Obendorf et al. (2008) so it is uncertain if they are sensitive to imbibitional chilling. The sensitivity of *MIPS1* mutants when subjected to cold temperatures during imbibition may be related to the reduced emergence of V99-5089-derived lines in the ECGT.

The ECGT was successful in separating genotypes for their emergence potential. However, field emergence is a crucial consideration. Trimble and Fehr (2010) reported significant correlations (r = 0.95 and 0.92) between ECGT and field emergence for Iowa locations. Therefore, we evaluated the eight genotypes from 12 seed source environments for field emergence in 2010 at Warsaw, VA to determine the reliability of the ECGT in predicting field emergence.

Field Emergence Test

The field emergence test in 2010 at Warsaw, VA was completed to understand the impact of seed production environments on field emergence for LP genotypes. Genotypic, environmental and the interaction effects were significant for emergence (Table 4). Genotype × environment interaction was significant due to changes in rank and differences in magnitude (Table 5). Overall the field emergence of all genotypes from all environments was higher than germination in the ECGT. The planting conditions in Warsaw, VA in 2010 were ideal, allowing for a uniform assessment of emergence in a Mid-Atlantic environment.

In seed from five environments, the genotypes were not significantly different (using genotype × environment slice analysis) indicating less genotypic influence on emergence potential (Table 5). Emergence was high for seed from these source environments indicating superior and equivalent emergence for both LP lines and control cultivars. The five environments with the lowest emergence percentages were the same in both the ECGT and field

emergence test with slight differences in order but large differences in the magnitude of reduction. Emergence was correlated (r = -0.65) with quality scores. Quality of seed from the TN-2009 (3.1) and NC-2008 (3.2) indicated these locations were the worst environments for quality and emergence. In seed from the five seed production environments with the lowest emergence, emergence ranged from 77% in seed from MO-2009 to 41% for seed from TN-2009. Seed from all genotypes grown in TN-2009 (SDS environment) emerged at significantly reduced rates. In the other four environments with reduced emergence, the control cultivars were above 80% and the LP genotypes were responsible for reducing emergence. The mean maximum temperature in these seed production environments was 28 to 30 °C reinforcing that moderate temperature increases during seed production reduces the emergence potential of LP lines. Within these seed source environments, genotypes were significantly different because emergence was reduced and more variable in the LP genotypes.

From most environments, the control cultivars (5601T or Essex) were the highest for emergence, and both were high in every environment with the exception of TN-2009 (Table 5). Overall, 5601T and Essex both averaged 83% emergence for seed from all source environments. The CX1834-derived line, 04-05N32 (83% emergence) was not significantly different from the control cultivars. Seed of 04-05N32 produced in 9 of the 12 environments emerged greater than 80% with reduced emergence occurring in seed from VABB-2008 (79%), MO-2009 (77%), and TN-2009 (69%). Emergence of seed was low from the TN-2009 environment, but 04-05N32 was actually the highest for emergence from that environment.

Four LP lines, V03-5900, V03-5901, LP-5601T-BC1, and S04-053-05, with emergence ranging from 76% to 71% were not significantly different from each other (Table 5). Overall emergence was low in these lines because of performance of seed from poor seed production

environments. Environmental conditions during the growing season impacted the emergence of these lines (but not 04-05N32 or the control cultivars) suggesting an increased sensitivity to potential stressors. In seed from the environments with high emergence percentages, these LP lines averaged equal to or greater than 80% emergence demonstrating good emergence from multiple seed source environments. Although S04-053-05 was not significantly different when averaged over all environments, seed from eight environments emerged at less than 80% demonstrating more variation for emergence. The lowest line in most environments was V03-5906 which averaged 63% emergence over all environments with low emergence in 10 environments and especially low emergence in seed from TN-2009 (22%) and NC-2008 (20%). Four LP lines (V03-5900, V03-5901, LP-5601T-BC1, and S04-053-05) demonstrated commercially acceptable emergence levels from the majority of seed source environments in the Southeastern and Mid-Atlantic regions. Therefore, production of commercial LP seed for planting in the Mid-Atlantic region should be possible.

Emergence Tests Comparison

The warm germination test of VAMH 2008 and 2009 source seed used to plant the agronomic trials in 2009 and 2010 overestimated field emergence in the majority of agronomic trial environments (Table 2). The only exception occurred in the two environments in 2009 with the highest emergence percentage, in which field emergence was higher than the warm germination test. The warm germination test was correlated to field emergence in two high emerging environments with correlations of 0.74 and 0.82 for NC-2009 and MD-2010, respectively (Table 2). Although the correlation of 0.89 was significant for the MO-2010 environment, the warm germination test was not accurate for predicting field emergence because it overestimated the field emergence by a mean of 26 percentage units. In 2010, the warm

germination percentages for seed from VAMH-2009 were high and no genotypes emerged at a higher percentage in the six field environments. However, the MD-2010 environment was the best for emergence in 2010 demonstrating the accuracy of the warm germination test when field emergence conditions are favorable. In environments with high emergence conditions, the warm germination test was an accurate test for predicting emergence of LP and NP genotypes in the Southeast and Mid-Atlantic region. If planting conditions are not ideal, the warm germination test does not accurately predict emergence and seeding rates should be adjusted above the warm germination results to overcome poor planting conditions.

For the source seed from VAMH 2008 and 2009, the warm germination test was not correlated to the ECGT or 2010 field emergence test. However, in comparing the germination and emergence of the VAMH source seed only, the warm germination results were a better predictor of field emergence than ECGT, especially for the LP lines (Table 2 and 5). The VAMH seed source from 2008 and 2009 was used to plant the six environments in 2009 and 2010, respectively. Therefore, the performance of the VAMH source seed in the ECGT was compared with emergence in each field environment of the agronomic trial throughout the Southeast and Mid-Atlantic in 2009 and 2010 (Table 2 and 5).

There were five environments in 2009 and two environments in 2010 with high (≥ 80%) field emergence (Table 2). In those environments, the LP lines and the control cultivars generally emerged equally or higher in the field than predicted based on the ECGT. In the lowest emerging environment (MO) in 2009, all lines were lower for emergence as compared to the ECGT with one exception. In the four lowest environments in 2010, most lines were lower for field emergence than predicted by the ECGT results. The performance of the lines 04-05N32 and V03-5906 were two exceptions to this trend. The line 04-05N32 was the highest emerging

over all 12 environments in the 2009 and 2010 agronomic trials (Table 2) but was low with 73% emergence in the ECGT (Table 5) for the agronomic trial source seed (VAMH-2009). In 2010, emergence of 04-05N32 was higher in five of the six environments compared to the ECGT. The line V03-5906 emerged better in the field as compared to the 11% (VAMH-2008) and 70% (VAMH-2009) reported in the ECGT. Overall V03-5906 had poor emergence but the ECGT was especially severe compared to field emergence.

In 2009, a significant correlation ($r = 0.80$) between ECGT and field emergence occurred in MO-2009 demonstrating the ability of the ECGT to more accurately predict emergence in low emerging environments (Table 2). In contrast, the ECGT underestimated the field emergence in environments with greater than 80% emergence. The magnitude of the reductions in the ECGT on the LP lines, especially the V99-5089-derived lines, underestimated the emergence potential in the field specifically when the environmental conditions are favorable for emergence. Overall, ECGT was not consistent in predicting the field emergence at locations throughout the Southeast and Mid-Atlantic region.

The field emergence of 04-05N32 was superior to the control cultivars when evaluated in 12 Southeast and Mid-Atlantic environments. In the ECGT, it was superior to the other LP lines with 63% emergence but significantly lower than the control cultivars while in the field emergence test it was not significantly different from the control cultivars. The combined results of the agronomic trial and field emergence test are favorable for the selection of 04-05N32 as a high emerging LP cultivar. Although the results of the ECGT were low for 04-05N32, in comparison to the other LP lines, this line was superior in emergence potential. Therefore, the high emergence potential of 04-05N32 suggests that LP germplasm can be bred to have superior emergence.

In contrast, V03-5906 was extremely low for emergence in the ECGT with a mean of 35% (Table 5). However, in the field emergence test, in which V03-5906 averaged 63% from all seed sources, seed produced in two environments had commercially acceptable germination and emergence (Table 5). Additionally, V03-5906 had significantly lower field emergence than all other lines in the 12 environments but emergence was acceptable in six environments (Table 2). Although the field results are more promising, the combined emergence results for V03-5906 would prohibit commercialization of this line. This is in contrast to the sister lines V03-5900 and V03-5901. If ECGT was used as the only selection tool, the magnitude of the reductions caused by the ECGT on the V99-5089-derived germplasm may have prevented advancement of V03-5900 and V03-5901. However, in the agronomic trial and field emergence trial, V03-5900 and V03-5901 demonstrated good emergence potential (Table 2 and 5). The field performance of V03-5900 and V03-5901 demonstrates that lines with commercially acceptable emergence can be developed from the V99-5089-derived germplasm.

The ECGT and field emergence test were significantly correlated ($r = 0.78$) but the correlation was lower than those ($r = 0.95$ and 0.92) reported by Trimble and Fehr (2010). The similarity between the conditions of the ECGT and the planting environments of IA likely contributed to the higher correlation. Similarly, the differences between the ECGT conditions and the planting environment of the VA location decreased the correlation in this experiment. Soil temperatures during planting in the Southeast and Mid-Atlantic are usually higher as compared to the Mid-West, resulting in more favorable planting conditions. The severity of the reductions in emergence caused by the ECGT was not observed in the field emergence test. Although there were differences in magnitude between the ECGT and field emergence test, genotypes with inferior emergence were identified in both tests. Poor emergence in the ECGT

does not guarantee that field emergence will also be poor in the Southeast and Mid-Atlantic regions. Therefore, in breeding LP germplasm adapted to the Southeast and Mid-Atlantic, ECGT can be used for additional confirmation of emergence potential, but not to discard lines with inferior germination before yield testing.

Low Phytate Soybean Production Environments

In experiments using seed produced in IA, the field emergence of CX1834-derived germplasm was generally below 60% when planted in IA environments (Oltmans et al., 2005; Trimble and Fehr, 2010). In contrast, seed produced in VAMH of CX1834-derived lines and V99-5089-derived lines emerged greater than 80% in six environments throughout the Southeast and Mid-Atlantic. In addition, field emergence of LP lines above 80% was observed in Warsaw, VA with seed produced from seven environments throughout the Southeast and Mid-Atlantic. The cool germination conditions of the ECGT severely reduced emergence of the LP lines. Therefore, commercial production of LP soybeans in the Southeast and Mid-Atlantic regions may be favorable due to higher soil temperatures at planting. However, seed production of LP cultivars for commercial planting should be in environments with moderate temperatures.

CONCLUSIONS

Emergence is a dynamic trait in which the interaction between the genotype, including the mutation type and background genetics, and the environmental conditions, including both the seed production environment and planting environment, are responsible for emergence. The warm germination test predicted field emergence in ideal planting conditions but overestimated field emergence in poor planting environments. The ECGT was a mediocre predictor for emergence of control cultivars and was not an accurate predictor of emergence for LP lines in 12 environments throughout the Southeast and Mid-Atlantic regions. The correlation between the ECGT and field emergence was significant and can be used to separate genotypes for emergence potential, but not as a selection tool for discarding genotypes. The severe germination reductions in the ECGT do not allow the test to be used to determine seeding rates or predict field emergence for commercial production. A laboratory germination test with better correlations to the planting conditions of the Southeast and Mid-Atlantic is needed for discarding lines with poor emergence potential prior to line advancement into preliminary yield trials. The superior performance of 04-05N32 demonstrates the potential to develop LP lines with improved emergence. The performance of V03-5900 was also acceptable in multiple environments. However, the majority of LP lines need improvement to be consistently good at emerging in multiple environments.

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Table 1. Analysis of variance for field emergence (%) for eight genotypes in 12 environments[†].

	Fixed Effects			
Source of Variation	Numerator DF	Denominator DF	*F*	*P*
Genotype	7	164	14.9	<0.0001
Environment	11	24	54.8	<0.0001
Genotype × Environment	77	164	2.8	<0.0001
	Random Effects			
Source of Variation	Estimate	Standard Error	*Z*	*P*
Replication(Environment)	6.7	3.4	2.0	0.0242
Residual	38.4	4.2	9.1	<0.0001

† Environments are locations in Queenstown, MD, Portageville, MO, Plymouth, NC, Knoxville, TN, Blacksburg, VA, and Mount Holly, VA in 2008 and 2009.

Table 2. Warm germination (%) for Mount Holly, VA (VAMH) source seed, field emergence (%) of eight genotypes grown in 12 environments[†], slice analysis[‡] of field emergence (%) for genotype × environment interaction, and correlations of warm germination, extended cold germination, and field emergence of VAMH source seed with field emergence in each of the 12 environments.

	Warm Germination	2009 Agronomic Trial Field Emergence						Warm Germination	2010 Agronomic Trial Field Emergence						
	%							%							
Genotype	VAMH 2008 Seed	NC	MD	VAMH	TN	VABB	MO	VAMH 2009 Seed	MD	NC	VABB	TN	MO	VAMH	Mean[§]
5601T	92	98	96	83	88	90	47	98	95	87	85	75	79	56	82ab
Essex	84	95	95	88	82	89	53	97	93	86	82	74	76	69	82ab
04-05N32	94	97	93	89	91	88	69	98	96	85	76	75	78	67	84a
LP-5601T-BC1	83	95	91	91	85	70	50	95	88	82	69	68	56	66	76cde
S04-053-05	88	95	71	86	80	58	42	96	94	82	72	77	65	46	72e
V03-5900	83	96	96	89	87	86	39	97	97	83	80	72	75	56	80abc
V03-5901	87	97	93	79	84	87	32	98	97	85	75	75	71	68	78bcd
V03-5906	84	95	91	80	83	80	30	97	95	76	71	66	72	51	74de
Mean[§]		96a	91abc	85bcd	85bcd	81cde	45g		94ab	83cd	76de	73e	71e	60f	
G × E Slice Analysis															
F		0.1	5.5	1.6	1.1	9.9	12.4		0.6	0.9	2.5	1.1	4.9	5.0	
P		ns	***	ns	ns	***	***		ns	ns	*	ns	***	***	
Correlation															
Warm Germination															
r		0.74	-0.05	-0.11	0.56	0.22	0.54		0.82	0.43	0.61	0.42	0.89	0.20	
P		*	ns	ns	ns	ns	ns		**	ns	ns	ns	**	ns	
Extended Cold Germination Test															
r		0.34	0.16	0.56	0.36	0.20	0.80		0.21	0.63	0.66	0.77	0.33	-0.18	
P		ns	ns	ns	ns	ns	*		ns	ns	ns	*	ns	ns	
Field Emergence Test															
r		0.53	0.45	0.40	0.49	0.57	0.57		0.09	0.74	0.69	0.40	0.27	0.39	
P		ns	ns	ns	ns	ns	ns		ns	*	ns	ns	ns	ns	

* Significance at the 0.05 probability level.

** Significance at the 0.01 probability level.

Table 2. Continued.

*** Significance at the 0.001 probability level.

ns, Not significant at the 0.05 significance level.

† Environments are locations in Queenstown, MD, Portageville, MO, Plymouth, NC, Knoxville, TN, Blacksburg, VA (VABB), and Mount Holly, VA (VAMH) in 2008 and 2009.

‡ Slice analysis based on genotype × environment least square means using the slice=environment option in PROC MIXED procedure in SAS.

§ Genotype and environment means followed by the same letter are not significantly different according to Tukey's pairwise comparison at *P*=0.05.

Table 3. Yield (kg ha^{-1}) means[†] and yield rank for eight genotypes grown at six environments[‡] in 2009.

	VABB-2009		MD-2009		VAMH-2009		TN-2009		MO-2009		NC-2009		Genotype	Mean
Genotype	Mean	Rank	Mean	Rank	Mean	Rank	Mean	Rank	Mean	Rank	Mean	Rank	Mean	Yield Rank
5601T	4611a	1	4128ns	2	4164ns	2	4192ns	1	3550a	1	3177a	1	3970	1.3
Essex	4265ab	2	3637ns	7	3841ns	3	3203ns	2	3017abc	4	3006ab	2	3495	3.3
LP-5601T-BC1	3943bc	4	3816ns	4	3792ns	4	3060ns	4	3277ab	2	2856abc	4	3457	3.7
V03-5900	3825bc	5	3765ns	5	3357ns	5	2904ns	5	2775bcd	6	2877ab	3	3250	4.8
04-05N32	3765bc	6	3657ns	6	4251ns	1	2663ns	7	3015abc	5	2782abc	5	3355	5.0
S04-053-05	3629c	8	4168ns	1	3355ns	6	2344ns	8	3057abc	3	2284c	8	3140	5.7
V03-5901	4066bc	3	3635ns	8	3334ns	8	3149ns	3	2504cd	7	2774abc	6	3244	5.8
V03-5906	3718c	7	3881ns	3	3344ns	7	2696ns	6	2195d	8	2440bc	7	3046	6.3
Mean	3978		3836		3680		3026		2924		2774			
CV[§]	8.7		7.3		13.5		30.9		15.1		12.0			

ns, Not significant at the 0.05 significance level.

† Genotype means followed by the same letter within an environment are not significantly different according to Tukey's pairwise comparison at P=0.05.

‡ Environments are locations in Queenstown, MD, Portageville, MO, Plymouth, NC, Knoxville, TN, Blacksburg, VA (VABB), and Mount Holly, VA (VAMH).

§ Coefficient of Variation.

Table 4. Analysis of variance for extended cold germination test (%) and field emergence test (%) for seed of eight genotypes produced in 12 environments[†].

	Extended-Cold Germination Test				Field Emergence Test			
	Fixed Effects							
Source of Variation	Numerator DF	Denominator DF	*F*	*P*	Numerator DF	Denominator DF	*F*	*P*
Genotype	7	35	195.3	<0.0001	7	35	33.1	<0.0001
Environment	11	55	107.7	<0.0001	11	55	54.0	<0.0001
Genotype × Environment	77	383	14.7	<0.0001	77	367	5.5	<0.0001
	Random Effects							
Source of Variation	Estimate	Standard Error	*Z*	*P*	Estimate	Standard Error	*Z*	*P*
Replication	10.7	8.1	1.3	0.0934	4.9	4.3	1.1	0.1272
Replication × Genotype	0.0	.	.	.	1.8	2.2	0.8	0.2044
Replication × Environment	17.0	4.9	3.5	0.0002	9.3	3.8	2.4	0.0075
Residual	66.4	4.6	14.5	<0.0001	80.5	5.9	13.5	<0.0001

† Environments included seed produced from locations in Queenstown, MD, Portageville, MO, Plymouth, NC, Knoxville, TN, Blacksburg, VA, and Mount Holly, VA in 2008 and 2009.

Table 5. Means[†] and slice analysis[‡] for genotype × environment interaction in the extended cold germination test (%) and field emergence test (%) for seed from eight genotypes produced in 12 environments[§] with mean maximum temperature[¶] and seed quality[#] for each environment.

	Seed Source Environment												
	2008						2009						
	MD	VAMH	VABB	TN	MO	NC	VAMH	VABB	NC	MD	MO	TN	
Mean Max. Temp., °C	27	27	25	29	30	29	27	25	28	25	28	27	
Quality, 1-5	2.2	1.5	2.0	2.3	2.0	3.2	1.9	1.9	2.1	1.8	2.6	3.1	
Genotype	Extended Cold Germination Test, %												Mean
5601T	92	92	82	80	63	51	88	83	76	93	86	28	76a
Essex	92	89	80	76	42	47	85	80	67	86	80	33	71b
04-05N32	83	85	51	76	50	51	73	59	31	72	62	58	63c
LP-5601T-BC1	90	76	72	55	17	15	71	71	48	84	68	11	57d
S04-053-05	82	59	75	41	21	24	86	70	34	72	42	23	52de
V03-5900	78	47	64	16	8	2	78	67	56	69	74	31	49ef
V03-5901	77	45	62	11	3	3	80	69	54	66	59	23	46f
V03-5906	64	11	57	13	2	1	70	60	16	66	52	13	35g
Mean	82a	63d	68cd	46e	26f	24f	79ab	70bcd	47e	76abc	66d	27f	
G × E Slice Analysis													
F	7.8	70.2	10.3	82.2	48.3	45.2	4.3	6.6	34.3	9.2	19.1	19.8	
P	***	***	***	***	***	***	***	***	***	***	***	***	
Genotype	Field Emergence Test, %												Mean
5601T	92	86	88	89	88	82	91	83	81	84	87	45	83a
Essex	89	88	88	87	84	83	89	91	86	91	80	37	83a
04-05N32	90	87	79	86	87	83	82	86	84	88	77	69	83a
LP-5601T-BC1	92	80	80	79	62	71	85	88	80	83	77	19	75b
S04-053-05	89	78	80	65	57	72	83	83	74	69	61	45	71b
V03-5900	91	86	84	69	61	51	86	83	81	87	87	46	76b
V03-5901	91	84	83	74	54	48	90	90	87	90	81	45	76b
V03-5906	81	70	78	63	41	20	81	76	77	78	69	22	63c
Mean	89a	82abc	82abc	76c	67d	64d	86ab	85abc	81abc	84abc	77bc	41e	
G × E Slice Analysis													
F	0.7	2.8	1.0	8.1	20.5	35.8	0.9	1.8	1.3	3.9	5.8	16.3	
P	ns	**	ns	***	***	***	ns	ns	ns	***	***	***	

Table 5. Continued.

** Significance at the 0.01 probability level.

*** Significance at the 0.001 probability level.

ns, Not significant at the 0.05 significance level.

† Genotype and environment means followed by the same letter are not significantly different according to Tukey's pairwise comparison at *P*=0.05.

‡ Slice analysis based on genotype × environment least square means using the slice=environment option in PROC MIXED procedure in SAS.

§ Environments included seed produced from locations in Queenstown, MD, Portageville, MO, Plymouth, NC, Knoxville, TN, Blacksburg, VA (VABB), and Mount Holly, VA (VAMH) in 2008 and 2009.

¶ Mean maximum temperature based on planting and harvest dates for each location.

Quality rating from 1 to 5: 1 = seed surface smooth with no discoloration to 5 = seed wrinkled with severe discoloration.

VI. Conclusions

In this study, we evaluated three types of populations derived from V99-5089 with a mutation in the D-*myo*-inositol 3-phosphate synthase 1 gene (*MIPS1*) gene that enhances both the phosphorus (P) and the sugar content of soybeans.

An evaluation of the recombinant inbred line population from the cross of V99-5089 × Essex was completed at three locations in two years. Phenotypic selection for modified seed composition was more effective than marker assisted selection (MAS) but the selection efficiency for the Satt453 marker was sufficiently high (87%) to be useful in MAS. However, the development of a perfect marker is needed due to recombination between the marker and the *MIPS1* mutant allele observed in this experiment. In the *myo*-inositol phosphate synthase mutant lines with modified seed composition (mips), a significant genotype × environment interaction was shown for inorganic phosphorus (Pi) content but not sugar content. Selection based on Pi content in one environment would be possible because lines did not change their phenotype when grown in an unfavorable environment. Additionally, there was variation for trait stability but mips lines with average stability for Pi and sucrose content were identified. Significant positive correlations were reported between Pi and sucrose, which were both negatively correlated with raffinose and stachyose. These correlations are favorable for improving seed composition, and with the exception of emergence there were no strong correlations with agronomic traits. The correlation for emergence with Pi was significant ($r = -0.59$) indicating reduced emergence due to the mutation.

The evaluation of an F_2 population and F_2-derived lines from the cross between V01-1693, a low linolenic fatty acid line and V03-5901, a V99-5089-derived line, was completed in three growing environments. Correlations between seed composition traits were favorable

without significantly affecting fatty acid content. The use of subtropical winter nursery environments for population development resulted in significant reductions in emergence and winter nursery environments should probably be avoided for low phytate population development and seed production. In the F_2 generation, poor emergence limited the number of lines identified with modified seed composition (mips) skewing segregation ratios. The marker selection efficiency was 41% preventing the efficient use of Satt453 in MAS. In the F2:4 generations, emergence was severely reduced in homozygous mips lines, preventing the analysis of agronomic traits. Additionally, in heterozygous lines of the $F_{2:4}$ generation, decreased emergence of mips plants caused the population to shift towards wild-type lines with normal seed composition (WT) reducing the ability to identify mips lines in successive generations. The heritability estimate between F_2 individual plants and $F_{2:4}$ lines was 0.67 for inorganic phosphorus content, allowing for early generation selection based on Pi content. However, because low emergence is likely to skew segregation ratios, large F_2 populations are necessary to allow for selection of high-yielding mips lines from segregating populations.

Advanced lines from two sources of soybean germplasm with modified P composition were evaluated including three CX1834-derived lines from different genetic backgrounds and three sister lines derived from V99-5089. In 12 environments, the effect of genotype, environment, and genotype × environment interaction on emergence, phytate and Pi content were determined.

The genotypes were significantly different for phytate and Pi content and the CX1834-derived lines were lower for phytate and higher for Pi content compared to the V99-5089-derived lines. The environment significantly affected Pi content but not phytate content. The genotype × environment interaction was significant for both phytate and Pi content. However, there were no

rank changes between the low phytate (LP) lines and control cultivars, allowing LP cultivars to be produced commercially in a range of environments. Lines with modified P composition accumulate more Pi rather than phytate content which contributed to the higher stability for phytate content but lower stability for Pi content when evaluated over 12 environments. However, five of the six LP genotypes were average or above average for phytate stability demonstrating the effectiveness of the mutations in reducing phytate levels regardless of the growing environment.

Genotypes were significantly different for emergence but one CX1834 line (04-05N32) was significantly higher and two V99-5089 lines were not significantly different than the two control cultivars for emergence in 12 environments. The conditions of the planting environment significantly altered emergence but in favorable environments the LP lines were agronomically competitive with the control cultivars. Additionally, seed quality was indicated as a potential factor in emergence of modified P composition lines and selection of lines with high seed quality could preserve emergence potential. The evaluation of yield demonstrated that the low phytate trait does not reduce yield when emergence is not significantly different.

The extended cold germination test (ECGT) and field emergence test demonstrated the significant affects of the seed production environment on emergence potential. The ECGT and field emergence test were significantly correlated ($r = 0.78$) allowing it to be used as a tool for separating genotypes for emergence potential. However, the ECGT severely reduced germination as compared to the field emergence preventing the use of the ECGT to determine seeding rates. Emergence is a dynamic trait in which the interaction between the genotype including the mutation type and background genetics and the environmental conditions including both the seed production environment and planting environment are responsible for emergence.

The development of commercial cultivars with modified P composition is possible but attention should be focused on selecting lines with high emergence potential during population development.

In considering the commercial development of LP cultivars, the levels of phytate necessary for market acceptance remains unknown. The lower levels of phytate in CX1834-derived genotypes may offer an advantage over V99-5089-derived genotypes but both germplasm sources are beneficial for improving P composition of soybeans. In plant breeding, the ease of gene integration could impact germplasm selection for breeding LP cultivars. The single gene mutation in V99-5089 would be preferred over the two recessive genes in CX1834. However, the development of molecular marker assays that directly select for mutant alleles reduces the difficulty of integrating recessive genes. The significant correlation between P composition and sugar components including sucrose, raffinose, and stachyose are additional benefits associated with the V99-5089 germplasm. Ultimately, reduced emergence due to the sensitivity of LP germplasm to environmental conditions of the seed production environment will be a factor in the commercialization of LP cultivars.

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