Development of New and Alternative Resources for Breeding Low Phytate Soybeans

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ABSTRACT

Soybean (*Glycine max* (L.) Merr) cultivars with genetic reduction in seed phytate concentration will be a valuable resource to livestock producers both nutritionally and economically, as well as in the preservation of the environment. However, due to association with reduced seed germination and field emergence potential, low phytate (LP) soybean cultivars are not being advanced to commercialization. Soybean breeders of LP lines must adjust their techniques and resources in order to efficiently select LP germplasm. Reduced seed germination and field emergence potential associated with LP germplasm eliminates many individuals with the desired LP trait during early generation population development, making improvement of agronomic performance among LP lines difficult. Thus, development of resources for improved phenotyping, genotyping, and early generation population development will facilitate the commercialization of LP cultivars.

Discovery of variation for field emergence potential among F_2-derived LP families suggests the potential for using a pedigree method for early generation population development in LP germplasm, as it preserves diversity and allows selection for improved field emergence. Integration of markers into a pedigree method will be useful to breeder. An example is BARCSOYSSR_11_1495 with 90-93% selection efficiency in various V99-5089-derived populations. Another resource for improved LP selection is a time-efficient, high-throughput modified iron (Fe) colorimetric phytate assay that does not suffer from inorganic phosphorus (P) concentration interference. Together these tools will provide breeders more accurate selection of LP lines.

DEDICATION

I dedicate the work in this thesis to my mom, Sue, my dad, Ron, and my brother, Tommy. Their hard work and dedication on our farm inspired me to pursue an education and career in crop improvement. Everything I have learned about hard work, I learned from their example and am eternally grateful for those lessons and so much more. Without their continuing love and support I would never have achieved the accomplishments that I have.

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ATTRIBUTIONS

Below is a brief summary of the roles of several people that contributed significantly to the completion of the research in this thesis, by chapter.

II. Improvement of Emergence in Low-Phytate Soybean Early Generation Populations with Alternative Selection Methods.

Laura Maupin – former PhD candidate of Department of Crop and Soil Environmental Sciences, now Breeder with Monsanto Company in Payette, ID. Dr. Maupin contributed germplasm used in this experiment, and previous genotypic and phenotypic data used to classify families.

Sarah Burleson – Masters degree candidate in Department of Crop and Soil Environmental Sciences. Ms. Burleson prepared and planted seed, took and analyzed stand count data, and ran statistical analysis with help of Laboratory for Interdisciplinary Statistical Analysis (LISA) team collaboration.

III. Development of Breeder-Friendly SSR Markers for Selection of *MIPS1* Mutants

M. Luciana Rosso – Research Associate in the Department of Crop and Soil Environmental Sciences. Dr. Rosso conducted genotyping runs, allele-calling, and genetic analysis in Mapmaker, and contributed to the preparation and editing of the manuscript represented in this chapter.

Laura Maupin – Dr. Maupin contributed advice and expertise in data collection and handling.

Katy Martin Rainey – Dr. Rainey provided expertise and advice and contributed to preparation of the manuscript represented this chapter.

Sarah Burleson – Ms. Burleson conducted DNA extraction and amplification with primers and contributed to the preparation and editing of the manuscript represented in this chapter

IV. A High-Throughput Colorimetric Method for Selection of Soybean Phytate Concentration

Chao Shang – Senior research associate in the Department of Crop and Soil Environmental Sciences. Dr. Shang developed the modified indirect-Fe colorimetric assay method, and provided feedback in the editing of the manuscript represented in this chapter.

M. Luciana Rosso –Dr. Rosso assisted with developing the high-throughput protocol and assisted with colorimetric data collection, and provided previous HPIC data, and contributed to the preparation and editing of the manuscripts represented in this chapter.

Laura Maupin – Dr. Maupin contributed the samples.

Katy Martin Rainey – Dr. Rainey provided expertise and advice and contributed to preparation of the manuscript represented this chapter.

Sarah Burleson – Ms. Burleson modified the method provided by Dr. Shang to make it more high-throughput and completed each run of the method, collecting and interpreting the colorimetric data for this experiment. She also contributed to preparation of the manuscript represented this chapter.

TABLE OF CONTENTS

LIST OF FIGURES

I. Introduction

II. Improvement of Field Emergence Potential in Low Phytate Soybean through Early Generation Populations with Alternative Selection Methods

III. Development of Breeder-Friendly SSR Markers for Selection of *MIPS1* Mutants

IV. A High-Throughput Colorimetric Method for Selection of Soybean Phytate Concentration

LIST OF TABLES

II. Improvement of Field Emergence Potential in Low Phytate Soybean through Early Generation Populations with Alternative Selection Methods

III. Development of Breeder-Friendly SSR Markers for Selection of *MIPS1* Mutants

IV. A High-Throughput Colorimetric Method for Selection of Soybean Phytate Concentration

I. INTRODUCTION

Phytate and Its Role in Animal Feeds

Soybean (*Glycine max* L. Merrill) is a widely and diversely used crop. Currently, soybean meal serves as a prominent feed source for livestock in Virginia and throughout North America. While soybean meal is an important source of protein, its potential to provide energy and minerals is not fully reached in non-ruminant animals due to their inability to digest certain compounds such as phytate (Sebastian et al., 2000). Phytate (*myo-inositol* 1,2,3,4,5,6-hexakisphosphate), also known as phytic acid, is the main form of phosphorus (P) storage in seeds, composing over 75% of the total seed P (Raboy et al., 2001). In addition to being indigestible for non-ruminants, this negatively charged compound chelates minerals from the diet such as calcium, zinc, and iron (Raboy et al., 2001). This results in both a lack of available P for animals' nutrition and excretion of the unused P in the waste. This P-rich waste, whether stored or used as crop-land fertilizer, makes its way into waterways causing environmental pollution such as eutrophication. Thus, phytate causes problems for the producer and the environment, presenting a growing problem especially for the Chesapeake Bay watershed.

Phytate composes as much as 1.5% of soybean seed dry weight and serves as a storage compound, signal transduction molecule, osmoprotectant, and cell wall constituent (Erdman, 1979). Additionally, phytate in the seed when broken down by the enzyme phytase becomes inorganic phosphate and *myo-inositol*, which are then available for seedling growth (Erdman, 1979). Phytate is formed from glucose by a series of reactions, the first of which is catalyzed by several enzymes including D-*myo-inositol*(3)-phosphate synthase, also known as MIPS (Raboy et al., 2001). This transformation occurs along a two part pathway with byproducts such as raffinose and stachyose. In the first part of the pathway, glucose becomes *myo-inositol* and the

second part of the pathway forms phytic acid from *myo-inositol*. *Myo-inositol* is also combined with UDPgalactose to produce galactinol, the compound necessary to form raffinose and stachyose (Figure 1). Therefore, without the MIPS enzyme, *myo-inositol* cannot be formed and without *myo-inositol*, raffinose, stachyose, and phytic acid cannot be formed in this pathway (Hegeman et al., 2001; Obendorf et al., 2009). P not bound in the form of phytate remains as free P or inorganic P (Pi) in the seed, forming an inverse relationship between phytate and Pi content which allows the use of Pi assays to phenotype low phytate (LP) lines (Scaboo et al., 2009).

Genetic Reduction of Phytate in Soybean

The economic, digestive, nutritional, and environmental problems associated with phytate in animal feed are reduced by improving availability of P through additions to the feed and modifications to soybean seed composition. One approach involves genetically adding phytase to the animal's diet, either through transformation of the soybean or to the animal's saliva (Denbow et al., 1998; Golovan et al., 2001). It has also been noted that growing a normal soybean in a P-starved environment produces seed with one-third the normal phytate content and no germination problems (Hegeman et al., 2001). In addition to these methods of reducing soybean seed phytate content, a variety of genetic alterations controlling the biochemical pathways producing phytate have also produced different LP lines (Larson et al., 1998; Maupin et al., 2011a; Pilu et al., 2005; Raboy et al., 2000; Rasmussen and Hatzack, 1998; Saghai Maroof and Buss, 2008; Sebastian et al., 2000; Yuan et al., 2007).

Low Phytate Mutants in Other Species

Two different mutations expressing the LP phenotype have been discovered in maize and barley (Larson et al., 1998; Pilu et al., 2005; Raboy et al., 2000; Rasmussen and Hatzack, 1998). In maize, phytate composes as much as 80% of the P in the seeds and is found predominantly in the germ and aleurone (Raboy et al., 2000). Two mutations are the cause of LP phenotypic expression in maize: low phytic acid 1 (*lpa1*) and *lpa2*. Both mutations were found to be generally recessive and located on different arms of the 1S maize chromosome (Raboy et al., 2000). The *lpa* mutations are reduced-function or loss of function mutations because of the effect their presence has on the function of the phytic acid pathway. *Lpa1-1* (named such as it is the first of *lpa1* mutations discovered in maize) is involved in the reduced function of the first step of the phytic acid synthesis pathway, while *lpa2-1* influences the metabolism of *myo-inositol* later in the phytic acid pathway (Raboy et al., 2000). The *lpa1-1* mutation was caused by chemical mutagenesis of pollen grains used in creation of the maize lines studied by Raboy et al. (2000). This *lpa1* mutation was shown to produce a 66% reduction in phytate and a corresponding molar equivalent increase in Pi. *Lpa2-1* was thought to have occurred spontaneously, and lines with the *lpa2-1* mutation show a 50% decrease in phytate and an increase in Pi (Raboy et al., 2000). Raboy et al. (2000) hypothesized that levels of cellular Pi may have a part in starch synthesis and accumulation as well. In other studies of the effects of LP mutation, Pilu et al. (2005) reported a decrease in temperature stress tolerance in seedlings containing the *lpa*241 mutation, which is either closely linked to or an allele of the *lpa1* mutation. Shi et al. (2007) observed that the *lpa1* mutation in maize controls a multidrug resistance-associated protein (MRP) ATP-binding cassette transporter. It was also determined

that the *lpa1* mutation is not a MIPS mutation because the *lpa1* mutation increases *myo-inositol* concentration (Shi et al., 2007).

Similar *lpa* mutations have been studied in barley, but have not yet been classified as homologous to the mutations in maize (Rasmussen and Hatzack, 1998; Larson et al., 1998; Raboy et al., 2000). In barley, 60-75% of the seed P is stored as phytate, also in the aleurone and germ tissues (Larson et al., 1998). Rasmussen and Hatzack (1998) identified the two distinct loci of the *lpa* mutations: *lpa1-1* and *lpa2-1*(named such because these are the first *lpa1* and *lpa2* mutations discovered in barley) and state that both decrease phytate content and increase free phosphates. The *lpa2* barley mutation is thought to be orthologous to that in maize, each controlled by one gene and mostly recessive to the non-mutant type (Larson et al., 1998). The *lpa1-1* mutation in barley has not been proven to be orthologous to that in maize (Larson et al., 1998). It is also noted that the mutant lines show a greater decrease in phytate content than using environmental methods of reducing the compound such as reducing the P in the soil (Larson et al., 1998).

Both maize and barley *lpa* mutants have been reported to reduce germination and field emergence potential (Hatzack et al., 2001; Larson et al., 1998; Pilu et al., 2005; Raboy et al., 2000). Pilu et al. (2005) noted that in the *lpa*241 derived lines there was a pleiotropic negative effect on germination and other agronomic traits such as seedling growth rate and corn ear size. Raboy et al. (2000) reported no germination problems but did observe a decrease in yield that is attributed to an effect of the LP mutation. Similar to findings in maize, barley *lpa2-1* mutation studies showed negative germination effects (Larson et al., 1998). Also in barley, homozygous *lpa2-1* mutants showed germination reduction of more than 10% compared to the *lpa1-1* type mutants, causing a skew in segregation ratios of F_2 and F_3 genotypes (Larson et al., 1998).

Low Phytate Mutations in Soybean

In soybean, there are two main sources of mutations that condition the LP phenotype. The two sources are: the mutations in lines derived from the cultivar CX-1834 and lines with a mutation in the *MIPS1* gene. CX-1834 was derived from a line known as M153 that contains an *lpa* mutation (Wilcox et al., 2000). CX-1834 derived lines have low-phytic acid content and corresponding high Pi content but have been shown, in some studies, to have reduced germination and field emergence potential (Anderson and Fehr, 2008; Maupin et al., 2011a; Oltmans et al., 2005).

Both sources of mutation, have been mapped and confirmed to be separate (Chappell et al., 2006; Gillman et al., 2009; Saghai Maroof et al., 2009). Chappell et al. (2006) confirmed that a mutation in the *MIPS1* gene is not the mutation responsible for the CX-1834 LP phenotype, based on the fact that phytic acid is reduced while other products of the pathway such as raffinose are not affected. Saghai Maroof et al. (2009) also confirmed independence of the CX-1834 derived mutations from any MIPS family mutation in mapping the four MIPS genes.. Two genes (*lpa1* and *lpa2*) are responsible for the mutant phenotype in the CX-1834 derived lines and are located on Gm19 (LG N) and Gm3 (LG L), respectively, the first having more influence on the phenotype (Gao et al., 2008; Saghai Maroof et al., 2009; Walker et al., 2006). Simple sequence repeat (SSR) markers Satt237 and Satt561 were confirmed to be associated with *lpa1* and *lpa2*, respectively (Gillman et al., 2009). As a homolog to the maize *lpa1* mutant gene, the nonsense mutation in soybean on Gm3 in combination with the missense mutation on Gm19 were found to have a similar function in disruption of an MRP, producing the LP phenotype (Gillman et al., 2009; Saghai Maroof et al., 2009). These reduced phytate lines make approximately 75% of the total seed P available to non-ruminant animals (Walker et al., 2006).

On the basis of weight, these lines were shown to have about 1.9 g/kg phytate, compared to the wild type which has normal phytate levels of about 4.3 g/kg (Wilcox et al., 2000).

Mutations in the *MIPS1* gene are another source of the LP phenotype, separate from that of CX-1834 derived lines and are found in three germplasm sources: LR33 (Hitz et al., 2002), Gm-lpa-TW-1 (Yuan et al., 2007), and V99-5089 (Saghai Maroof and Buss, 2008). There are four differentially expressed MIPS genes on various chromosomes that influence the biochemical pathway that produces phytic acid and these genes are expressed at the cotyledonary stage of seed development (Chappell et al., 2006; Hegeman et al., 2001; Saghai Maroof et al., 2009). The *MIPS1* gene is located on Gm11 (LGB1) between map intervals 58 and 64 cM (which contains the quantitative trait locus for phytate) which is considered a low diversity area of the soybean genome (Saghai Maroof and Buss, 2008; Saghai Maroof et al., 2009; Song et al., 2004). In addition to LP, *MIPS1* mutations convey high sucrose, low stachyose, and low raffinose content. The mutation in the *MIPS1* gene that produces the LP phenotype in LR33 is a single nucleotide base change in exon 10, the codon for the amino acid residue 396, which is located at position 1,188 from the start codon (Hitz et al., 2002; Saghai Maroof and Buss, 2008; Saghai Maroof et al., 2009; Sebastian et al., 2000). LR33 shows a phytic acid decrease of 50% as well as a decrease in raffinosaccharides and an increase in sucrose levels (Hitz et al., 2002).

In another experiment, two LP lines, Gm-lpa-TW-1 and Gm-lpa-ZC-2, were developed using artificial mutagenesis (Yuan et al., 2007). In the line Gm-lpa-TW-1 the *MIPS1* gene is mutated at the third exon by a 2 nucleotide deletion (Saghai Maroof et al., 2009). Similarly to other *MIPS1* gene mutations, the Gm-lpa-TW-1 line proved to have reduced emergence when seed was produced in a subtropical environment (Yuan et al., 2007). The Gm-lpa-TW-1 line showed a 66.6% decrease in phytate as well as corresponding increase in Pi (Yuan et al., 2007).

Soybean line V99-5089, with a naturally occurring gene for LP, was discovered at Virginia Tech in the soybean breeding and genetics program (Saghai Maroof and Buss, 2008). The V99-5089 LP phenotype is controlled by a mutation in the *MIPS1* gene discovered to be a single nucleotide polymorphism (SNP) of nucleotide base C changed to nucleotide base G on Gm11 within the same region as the LR33 mutation, but not the same position (Saghai Maroof and Buss, 2008). An SSR marker, Satt453, was also identified as linked to the trait (Saghai Maroof and Buss, 2008). The causal SNP mutation can also be assayed for and used to genotype lines.

Phenotyping Low Phytate in Soybean

The LP phenotype can be selected using assays for phytate and Pi concentration but most are time-inefficient and suffer from inaccuracies due to environmental and chemical influence. To more quickly and conveniently develop LP soybean lines, breeders need more resource efficient methods for assaying phytate concentration of lines in conjunction with genotypic selection methods.

Published methods for seed composition analysis have been used to accurately phenotype or quantify concentrations of Pi, phytate, and raffinosaccharides in soybean seed. The colorimetric Pi assay has been used as an indirect measurement of phytate that is more efficient than phytate quantification methods (Chen et al., 1956; Hitz et al., 2002; Oltmans et al., 2005; Raboy et al., 2000; Scaboo et al., 2009; Wilcox et al., 2000). Pi assays are suitable for phenotyping and quick selection of LP lines but are not reliable across environments since environment has had been shown to have a significant effect on Pi concentration (Maupin et al., 2011a). High performance liquid chromatography and high performance ion chromatography are two accurate methods for direct quantification of phytate concentration in ground soybean,

however, both are labor, time, and resource intensive (Gao et al., 2007; Maupin et al., 2011a). Other methods for measuring phytate are extensively reviewed by Gao et al. (2007) such as anion exchange column and Solution ^{31}P Nuclear Magnetic Resonance Analysis assays. These other methods are included as comparison for development of a modified colorimetric phytate assay that employs colorimetric determination of phytate content based on the decoloration of the pink Fe^{3+}–sulfosalicylate complex (known as Wade's reagent) by phytate. This colorimetric assay is time and labor intensive and its selectivity is negatively affected by interference from Pi that causes an inaccurate measurement of phytate in high Pi samples (Gao et al., 2007).

Reduced Seed Germination and Field Emergence Potential

Reduced seed germination and field emergence potential in LP lines is an obstacle that breeders need to be aware of in order to successfully develop LP lines. Accurate tests for seed germination and field emergence potential provide breeders methods for assessing reduced germination and emergence before it is experienced in the field, avoiding an unexpected shift in genotype and loss of genetic material due to natural selection in the field.

LP lines have been associated with reduced emergence, particularly seed produced in subtropical environments. For example, Meis et al. (2003) performed germination tests on LR33 derived lines in comparison to wild type lines using both subtropical and temperate seed source environments. The LP lines had 63% germination when seeds were produced in temperate environment, versus 8% germination for seeds from the subtropical environment. The wild type lines had 77% and 83% germination in the temperate and subtropical environments, respectively. Meis et al. (2003) were able to use an accelerated aging test to effectively differentiate the potential for emergence between LP and wild type lines. Thus, there was a significant line by seed source environment interaction effect occurring. Similar results were observed for V99-

5089 derived *MIPS1* mutants (Maupin et al., 2011a). Maupin et al. (2011a) report significantly lower emergence rates for LP lines derived from both V99-5089 and CX-1834, especially in seeds coming from a subtropical seed source environment. Maupin et al. (2011a) also noted *MIPS1* LP lines with acceptable levels of emergence across different environments, suggesting that genetic diversity does exist among lines containing a *MIPS1* gene mutation.

In studying emergence in CX-1834 derived lines, Trimble and Fehr (2010) determined that an increase in seed phytic acid P and corresponding decrease in Pi are not the cause of better emergence, therefore other factors must be contributing to the emergence problem. Emergence for progeny of a cross of a LP line with a wild type was compared with emergence of the parents from various environments from different seed sources and it was determined that emergence is a factor controlled by presence of favorable or unfavorable alleles. It was also concluded that both parents contributed to germination and that better emergence was not a result of increased phytate P content or of reduced Pi content when compared to other LP lines (Trimble and Fehr, 2010). In a similar study, Oltmans et al. (2005) studied CX1834-1-6 derived lines and found that the LP lines had lower emergence than wild type lines and that this difference also changed over environments.

Reduced seed germination and field emergence potential have been detrimental factors in development of LP soybean lines. In order for LP soybean lines to be commercially acceptable, germination and field emergence potential must be improved. Germination and field emergence potential play the first vital role in development of the plant and have a significant effect on yield. Tests have been conducted to find an effective method of testing seed germination and field emergence potential in the laboratory. Johnson and Wax (1978) stated that cold germination testing is more correlated to field results than warm germination testing and that a

range of different germination tests should be conducted to accurately assess potential performance over a variety of environments. It is suggested that vigor tests also be used as a method of determining seed quality as reduced seed vigor is often responsible for loss in seed viability (Johnson and Wax, 1978). The vigor test used by Johnson and Wax (1978) was a measurement and rating of hypocotyl length of the seedlings that germinated from the cold test conducted in the experiment. More recently, Trimble and Fehr (2010) conducted germination experiments with LP lines that incorporated an extended cold germination test. In this extended cold test, the seedlings were kept at 10 degrees C for 21 days because Iowa fields in which the field emergence would be measured do not experience as sharp an increase in temperature as called for in other cold germination tests. The authors found the extended cold germination test to have a strong correlation to actual field emergence in tests performed in Iowa and that it can be used to remove lines with consistently reduced emergence. When used in studies in the Mid-Atlantic, however, the extended cold germination test was found to be a less accurate indicator of emergence which could be due to differences in soil temperatures in the two regions (Maupin et al., 2011b).

Factors Affecting Germination and Field Emergence Potential

Breeders also need to be familiar with the avoidable environmental and mechanical factors that cause damage to seeds. Diminishing factors affecting germination and field emergence potential will reduce the loss of LP lines with genetic disposition for acceptable emergence caused by unfavorable environmental and handling conditions in earlier generations.

Some post-planting environmental factors that affect seedling emergence include moisture, temperature, and pathogens. Wuebker et al. (2001) investigated the effects of flooding and temperature on germination and found that as little as one hour of flooding greatly harms

seed viability and that soybean seedlings subjected to water-soaked conditions have more reduced germination at higher temperatures (25-30 °C) than seedlings flooded at lower temperatures. Mechanical damage of seed at harvest also plays a large role in germination success, especially when damaged seed goes through temperature stress (Wuebker et al., 2001).

Before the seed is harvested, stress factors during seed development and maturation can reduce the probability of acceptable germination and field emergence potential. High temperatures and humidity during seed fill lower viability of the soybean seed and make them susceptible to infection from *Phomopsis longicolla* (Egli et al., 2005). This has become a concern because many breeding programs have winter nurseries in subtropical environments where high temperatures and humidity are prolonged. High temperatures and humidity are also becoming a problem in the Southeast U.S. (Smith et al., 2008). Such environmental conditions while the seed is maturing promote seed coat wrinkling and can cause hard-seededness, both of which are factors responsible for reduced seed germination and field emergence potential. These conditions disrupt seed development processes and cause a reduction in germination and vigor (Egli et al., 2005). Additionally, field weathering of the seed in the pod compounds the reduction in germination and field emergence potential. Egli et al. (2005) found that seedling vigor was harshly affected by higher air temperatures during seed filling stages. High air temperatures were specifically a detrimental factor for seed germination in the soybean cultivar Hutcheson even when seeds infected with *Phomopsis longicolla* were removed. Vigor is also a trait to consider as a line could express good germination and emergence but still have a reduced stand if seedling vigor is poor. The specific stages at which higher temperatures are most detrimental are still unknown, but high temperatures and humidity during seed filling and maturing are stressors that contribute to reduced germination in soybeans. In studies on effects

of high temperatures on soybean seeds, Ren et al. (2009) found that excessive heat changed seed storage and stress responding proteins as well as caused a decrease in sugars. Interestingly, Blackman et al. (1992) note that a fast dry down of seeds in pods causes a decreased tolerance to seed desiccation and that stachyose plays a key role in protecting the seed from desiccation. This could be important when considering LP, low stachyose lines with reduced field emergence potential grown in subtropical environments. Smith et al. (2008) stated the concern that today's cultivars do not have sufficient genetic variation for use in developing improved germination in high temperature environments, suggesting that further research incorporating genetic germination factors will be beneficial.

Thus, previous studies have shown that both environmental and genetic factors contribute to seed quality, vigor, and germination. Some of these factors can be reduced, such as mechanical damage, and if the seed production environment can be chosen, an environment might be selected with moderate temperatures to favor seed fill. Also, genetic factors can be studied to determine lines containing favorable alleles for germination. There are also predictive methods for testing germination that identify lines that contain better genetics for germination and emergence to breed into lines of interest such as LP lines.

Objectives

Meal from soybean with reduced phytate will be valuable to livestock and crop producers, especially within the Chesapeake Bay watershed and other sensitive watershed areas. Without improvement of agronomic traits such as seed germination and field emergence potential, LP germplasm will not be commercialized. Plant breeders need access to convenient, high-throughput, accurate, and time and resource efficient methods for phenotypic and genotypic selection of the LP trait and lines. This need is compounded by the problem of reduced seed germination and field emergence because LP segregates that do not emerge in early generations cause a loss in genetic diversity as well as a potential allele shift in germplasm to favor the wild type. Thus, the overall objective of these experiments is to facilitate the development of LP soybean germplasm containing the V99-5089-derived *MIPS1* mutation using molecular markers, seed composition assays, and selection methods to improve emergence and agronomic performance. Specifically, objectives included measurement of field emergence in F_2-derived LP families to make inferences about improvements to potential early generation population development methods; to identify breeder-friendly molecular markers for *MIPS1* mutant LP line selection; and to develop and demonstrate the use of a cost and time effective colorimetric assay for direct phenotypic selection of phytate.

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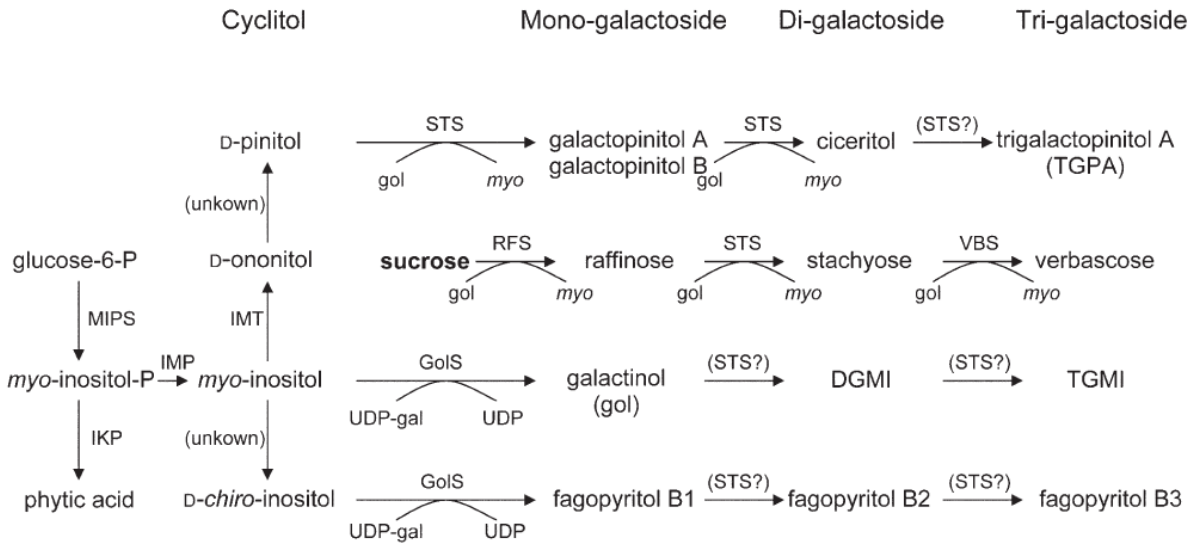
Figure 1. From Obendorf et al., 2009. Proposed pathways for production of phytic acid and other sugars from glucose.



II. Improvement of Emergence in Low-Phytate Soybean Early Generation Populations with Alternative Selection Methods

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Abbreviations: LP, low phytate; *lpa*; low phytic acid; *MIPS1*, D-*myo*-inositol 3-phosphate synthase 1; PI, plant introduction; SSD, single seed descent; WT, wild type for P composition.

ABSTRACT

Low phytate (LP) soybean lines suffer from reduced seed germination and field emergence problems which are amplified by growing environments experiencing extreme heat and humidity such as a subtropical climate. To examine seed germination and field emergence potential in V99-5089-derived *MIPS1* mutant families, emergence was studied on 20 F_2-derived families of LP and wild type P (WT) composition from four different generations. It was hypothesized that this study would reveal families with consistently better emergence that could be selected for and used to improve field emergence potential. Harsh natural selection events experienced in seed source environments were expected to improve field emergence over successive generations as lines with genetic disposition for poor emergence would theoretically be eliminated. However, significant improvements in field emergence over generations were not observed. No statistically significant differences in emergence among LP families were found. However, some LP families had better emergence than others, suggesting that the pedigree selection method could be used in LP line selection to improve field emergence potential.

INTRODUCTION

Reduced germination and field emergence has been reported in low phytate (LP) soybeans, in CX-1834-derived LP lines as well as V99-5089-derived D-*myo*-inositol 3-phosphate synthase 1 (*MIPS1*) LP mutants. This reduction in emergence is particularly pronounced in seeds produced in subtropical environments (Anderson and Fehr, 2008; Maupin 2010; Meis et al., 2003; Scaboo et al., 2009; Sebastian et al., 2000; Yuan et al., 2007). Maupin and Rainey (2011) reported that in over twelve Southern US environments, V99-5089-derived *MIPS1* mutant lines and CX1834-derived mutant lines containing the low phytic acid 1 and low phytic acid 2 (*lpa)* genes display reduced germination and field emergence potential as compared to WT lines. Anderson and Fehr (2008) also found certain seed source environments to significantly reduce field emergence in CX-1834-derived lines.

High temperatures and humidity during seed fill, such as those in subtropical environments, cause seed damage that directly affects seed germination and field emergence potential (Egli et al., 2005; Maupin, 2010; Smith et al., 2008). Heat and humidity reduce seed germination and make seeds susceptible to infection from *Phomopsis longicolla,* as well as promote seed coat wrinkling and hard-seededness, which are both responsible for reduced seed germination and field emergence potential (Egli et al., 2005). Ren et al. (2009) found that excessive heat during seed development changed seed storage and stress responding proteins and resulted in a decrease in sugars. In other studies of the effects of high heat environments, Blackman et al. (1992) noted that a fast dry down of seeds in pods causes a decreased tolerance to seed desiccation. Heat and humidity are not only problematic in extremes such as in subtropical environments, but even moderately high heat and humidity such as that experienced in the Southeast U.S. may be detrimental to seed germination and field emergence potential

(Smith et al., 2008). The hot and humid environmental conditions that are experienced by developing seeds in the subtropics should be taken into consideration when advancing breeding populations, especially in developing a trait that is prone to reduced seed germination and field emergence potential such as LP.

Soybean line V99-5089, with a naturally occurring gene for LP, was discovered at Virginia Tech in the soybean breeding and genetics program (Saghai Maroof and Buss, 2008). A single nucleotide polymorphism in the *MIPS1* gene on Gm11 that changed nucleotide base C to nucleotide base G in the region of position 1188 from the start codon, controls the LP phenotype (Saghai Maroof and Buss, 2008). A simple sequence repeat marker, Satt453, was also identified as linked to the trait (Saghai Maroof and Buss, 2008). The *MIPS1* mutant phenotype includes low levels of seed inositol and phytate (Hitz et al. 2002), while soybean *lpa* mutants have normal levels of seed inositol and lower phytate content than *MIPS1* mutants (Israel et al. 2011). Maupin (2010) found reduced seed germination and field emergence potential in both CX-1834-derived as well as V99-5089-derived LP lines. Therefore, low inositol levels as caused by *MIPS1* mutants specifically, may not be the direct cause of reduced germination and field emergence, but the LP trait itself may be interfering with seed germination and field emergence. Also, Bilyeu et al. (2008) showed that obtaining the LP phenotype through soybean transformation with bacterial phytase caused reduced seed germination, thus further suggesting that reduced seed germination and field emergence is not caused by solely *MIPS1* mutations.

In early generation population development, breeders inbreed lines from generations F_1 to around F_4 to increase homozygosity so that subsequently selected lines will be genetically stable. Soybean breeding programs typically employ an inbreeding selection method of either single-seed descent (SSD), bulk method, or a modification of one or both in early generation population

development. The SSD method is conducted by harvesting one seed from a large number of F_2 plants and planting them the following season to again harvest one seed from each plant. This process continues for several generations to maintain maximum genetic diversity without selection until the superior progeny are identified and selected (Sleper and Poehlman, 2006). A bulk method of line development is carried out by harvesting all seed of the F_2 generation in bulk, then planting them the next year and bulking all of the progeny again. This process continues for several generations allowing natural selection to influence genetic composition of populations, until around F_5 to F_6 before the breeder begins selection of lines for desired traits (Sleper and Poehlman, 2006).

With both methods natural selection is the only selection occurring in early generations. When reduced seed germination and field emergence potential is conditioned by the trait being studied such as with LP, natural selection will eliminate individuals during inbreeding. Populations from crosses involving the LP trait might have few to no individuals with the desired phenotype after several generations (Maupin, 2010). Therefore, commonly used breeding methods are not suitable for development of LP germplasm as they result in selection pressure against the trait of interest.

Natural selection acting in early generation population development has been noted to cause a shift in the population ratio of WT to LP in both CX-1834-derived lines as well as V99-5089-derived *MIPS1* mutant lines (Maupin, 2010). Natural selection favors proliferation of WT individuals over LP individuals as WT lines have better seed germination and field emergence potential, especially from low quality seed source environments, and therefore the population shifts to a lower than expected frequency of the LP allele (Maupin, 2010). Soybean breeders have developed populations under the assumption that natural selection acts as a favorable

selection factor when it comes to traits such as field emergence potential, because it is assumed that genotypes with improved field emergence potential will survive differentially. Thus, breeders assume that reduced seed germination and field emergence potential is resolved by natural selection during early-generation population development. However, in populations segregating for LP genes, natural selection against reduced emergence also selects against the LP genotypes. Thus, current assumptions should be modified and alternative methods should be explored to ensure the survival of LP lines.

The pedigree method of early generation population development entails selecting individuals with the desired phenotypes in the F_2 generation and then planting the seed of each plant in a separate row. Selections are made again in subsequent generations for the desired phenotype, propagating individuals from only the superior families and maintaining the pedigrees separately so that the breeder knows from which original F_2 the current plant was derived. This allows different suites of genes to be monitored (Sleper and Poehlman, 2006). This method reduces germplasm loss to natural selection by ensuring the lines have the desired trait from the earliest generations and advancing only the individuals with the trait of interest as well as agronomic traits superior to other individuals of the cross. For LP early generation population development, the pedigree method would allow selection of families and lines with highest field emergence so that agronomic evaluation of advanced lines is focused on the best emerging LP lines.

Rationale

Natural selection may improve seed germination and field emergence potential in LP soybean germplasm but no previous studies have reported such an observation. Planting seed of successive generations from the same LP pedigree into one environment will potentially allow detection of the effects natural selection may have on seed germination and field emergence potential in LP germplasm. The pedigree method of early generation population development may allow selection of LP lines with better seed germination and field emergence potential, however, genetic variation for seed germination and field emergence potential must exist within the LP populations to select from. To determine whether variation for seed germination and field emergence potential exists within V99-5089-derived *MIPS1* mutant families, emergence of families must be compared in the same environment.

It is hypothesized that F_2 LP families from a cross will show differences for field emergence. In addition, more advanced generations of LP families and LP families that have undergone more harsh natural selection environments should be improved for field emergence.

Objectives

Objectives of this experiment include: i) measuring field emergence among LP F_2 families of the same pedigree, and ii) measuring field emergence of LP families in successive generations of inbreeding.

MATERIALS AND METHODS

Germplasm

Germplasm used in this experiment is derived from populations developed and characterized by Maupin (2010), beginning with the cross of V03-5901 × V01-1693 in 2007. V03-5901 is a LP line developed from the cross between V99-5089 and the high-yielding cultivar Essex (Smith and Camper, 1973). V01-1693 is a low linolenic fatty acid line, with the pedigree [(Hutcheson (2) × N94-199) × (Hutcheson (2) × RR experimental line)]. N94-199, developed by Dr. Joseph Burton (USDA-ARS, Raleigh, NC), is the source of the low linolenic (3.8% 18:3) fatty acid trait. In late 2007, six F_1 seeds of this cross were sent to Semillas Olson, a winter nursery operation in Costa Rica. There were 162 plots of two seeds per plot of the F_2 generation planted in Blacksburg, VA in 2008, then harvested individually (Maupin, 2010). From this harvest, 135 $F_{2:3}$ families were planted in the 2008-2009 winter nursery and each family was harvested in bulk. $F_{2:4}$ seeds were planted in Blacksburg, VA in 2009 and seeds were harvested in bulk from each $F_{2:4}$ family. Remnant $F_{2:4}$ seed and $F_{2:5}$ seed were planted in the 2009-2010 winter nursery. $F_{2:4}$ seeds were harvested to create the $F_{2:5B}$ experimental generation and $F_{2:5}$ seeds were harvested to create the $F_{2:6}$ experimental generation. Remnant $F_{2:5}$ seed from the Blacksburg 2009 planting became the $F_{2:5A}$ experimental generation, and remnant $F_{2:4}$ seed from the winter nursery in 2008-2009 became the $F_{2:4}$ experimental generation. Each generation underwent different levels of natural selection pressure dependent on the environmental conditions of the seed source and planting environment in combination with the number of selection events a generation had experienced.

Twenty of the 135 F_2-derived families of each experimental generation ($F_{2:4}$ to $F_{2:6}$) of the V03-5901 × V01-1693 cross (ten LP families and ten WT families) were chosen along with three

checks and both parents to be planted in Blacksburg, VA in 2010. The three checks consisted of two plant introductions (PI), PI 588030 and PI 588026A with previously reported high germination rates (Smith et al., 2008) and the adapted, high yielding cultivar Glenn, which was developed at Virginia Tech. The parents and checks were not harvested from the same seed source as the generation with which they were planted, they were added from a composite of available seed harvested from various tests in Blacksburg, VA, Warsaw, VA, and the winter nursery. The twenty F_2-derived V03-5901 × V01-1693 families were phenotyped for LP in a previous study using the colorimetric assay for inorganic phosphorus concentration. The F_2 families were also genotyped for LP in a previous study, using the Satt453 marker to select homozygous *MIPS1* mutants and WT families (Maupin, 2010). Seed from each family was represented in the four different generations, $F_{2:4}$, $F_{2:5A,}$ $F_{2:5B}$, and $F_{2:6}$ (Table 1, Figure 1).

Experimental Design

The four different seed source generations were chosen to represent different levels of natural selection on each F_2-derived family from the winter nursery. Thus, 20 families from each generation were planted in Blacksburg in 2010 with four replications totaling 400 rows when including parents and checks. Seed was planted at a rate of 75 seeds per row unless a generation/family combination did not provide 75 seed, in which case the maximum number of seed available up to 75 was planted. Generations $F_{2:5B}$ and $F_{2:6}$ had entries missing for a total of ten families and 31 missing plots, assumed to be due to weather and other harsh conditions of winter nursery causing low yield. Each replication was given 75 seed before continuing to fill the next replication, therefore if insufficient seed was available for all four replications, the replications were filled in consecutive order. All missing plots were substituted with parental or check lines as place holders in the field and stand count data for the substitutions was not

recorded, the data for these plots were considered missing. Each plot consisted of one row that was 3 m long and spaced 0.76 m from the next row. There were 400 plots total, including four replications of 20 families, plus two parents and three checks were included for all four seed source generations.

Seed Quality and Missing Plots

The heat and humidity of the Costa Rican climate is hypothesized to have a negative impact on both emergence within the given growing environment and the seeds developing within the pods, decreasing seed quality for seed germination and field emergence potential in future plantings. Seed of all generations was given a visual quality rating based on seed color, uniformity, coat condition, texture, and evidence of disease. Seed of generations returning from winter nursery was all given a low visual quality rating and only the generation produced in Blacksburg in 2009 ($F_{2:5A}$) was given a good quality score before planting. The seed of the $F_{2:5A}$ generation was the only seed considered to have good quality. Low seed yield due to harsh environmental conditions in the winter nursery resulted in too little seed to plant the necessary replications in the $F_{2:5B}$ and $F_{2:6}$ generations. Upon return from winter nursery the amount of seed from the $F_{2:6}$ generation was insufficient in two different families, totaling seven missing plots for this generation, and the $F_{2:5B}$ generation was lacking in seed for eight different families, totaling 24 missing plots for the $F_{2:5B}$ generation.

Data Collection and Statistical Analysis

Stand count data were taken for each plot by recording the number of plants that stood in the row at the V3 stage of development. This value was then divided by the number of seeds per row planted to obtain a comparable percentage of emergence for each plot. Data were plotted

and analyzed and LS Means calculated with SAS JMP and the SAS procedure PROC GLIMMIX (SAS 2010). PROC GLIMMIX was employed for analysis of variance as it is useful in analyzing data that is not normally distributed. Significant differences among families and environments were determined by pairwise comparisons of means using Tukey-Kramer's multiple mean comparison method. Replication by generation interaction was considered a random effect. Generation, family, and the interaction of family with generation were fixed effects tested for significance. In order for the model to compare families and genotypes across sources, all missing data had to be imputed using a single imputation procedure. Imputation is the process of using the observed values to assign plausible estimated values to the missing samples that will not affect the means, so that all data points are present to be statistically compared. Without imputation of missing data, statistical inferences could not be made due to many missing data points.

RESULTS

Analysis of stand count data for all plots showed that overall, field emergence of seedlings was greatly reduced: no LP families reached 80% emergence, which is considered the lowest commercially acceptable emergence rate (Keith and Delouche, 1999), and only 27 plots overall reached or exceeded 80% (Figure 2). This overall reduction in emergence limits the scope of the conclusions that can be made from the data. Table 2 represents overall mean emergence percentages by factors of: families over generations, overall families by generation, genotypes (WT and *MIPS1* mutant) over generations, and genotypes overall. Table 2 shows that check did not reach mean emergence of 80%. The $F_{2:5A}$ generation showed the best overall field emergence with an overall mean of 64.6%. The WT families showed a mean emergence of 32.3%, higher (not statistically significantly) than the 18.8% for LP families but still reduced when compared to the 80% cutoff.

Thirty-one missing data points had to be statistically imputed to compare significance among factors. Fixed effects of generation, family, and the family-generation interaction (excluding checks) were significant effects to the $p < 0.0001$ level (Table 3), the $F_{2:5A}$ generation was significantly different from the $F_{2:5B}$ and $F_{2:6}$ generations at the $p < 0.0001$ level (Table 4).

Figure 3 compares emergence rates among LP families from the $F_{2:5B}$ and $F_{2:6}$ generations, as theoretically, the latter should have undergone the most natural selection pressure for emergence of all generations. The $F_{2:6}$ generation did not show improved field emergence over the $F_{2:5B}$ that was produced in the same environment, and showed slight but not statistically significant emergence improvement over the original $F_{2:4}$ (Figure 3). Despite a lack of statistically significant difference among LP families, field emergence of some families were higher than others overall (Table 2) and within the generation with the best emergence (Figure

4). Variation for emergence among LP families allows for selection among families based on the pedigree method of early generation population development.

DISCUSSION

The overall low emergence results are due to compounded environmental factors such as a drought in Blacksburg during the 2010 growing season in combination with low seed quality from three sources due to winter nursery conditions. These data further confirm the negative effect that subtropical environments have on seed quality and field emergence potential, and how winter nursery compounds reduced seed germination and field emergence potential in LP families. The statistical significance of the genotype-generation interaction effect shows the detrimental effect the winter nursery has on LP individuals. Confirmation of reduced seed germination and field emergence potential due to LP and winter nursery environment interaction is useful for breeders developing LP lines, as it will be beneficial to advance LP populations in a less harsh winter nursery environment, such as one that is not in a subtropical or high-heat climate.

Natural selection was not observed to significantly improve seed germination and field emergence potential in LP families. Seed from generations that underwent more harsh selection conditions, and more inbreeding, such as the $F_{2:6,}$ were hypothesized to have better emergence as they had more natural selection for field emergence potential, however this did not occur consistently (Figure 3). While results were not as expected, emergence was extremely lower than expected overall. Therefore, the data are not adequate to draw any conclusions relative to changes in seed emergence potential over generations of increase. The 31 missing data points, due to problems with seed production in the winter nursery environment, also made the data as a whole difficult to analyze and interpret, and further reduce the clarity of data for drawing strong conclusions. However weak the conclusions, it is clear that environmental factors play a major role in seed quality.

Emergence variation among families, despite lack of statistical significance, suggests that a pedigree selection method may be successfully applied in early generation population development to improve emergence of LP soybeans and that potential for improvement in emergence exists. While emergence was low overall, some LP families achieved higher emergence than others and these data can be compared to stand count data from previous years and used to select families with higher emergence rates as families from which to select improved lines. These results suggest that a pedigree method of line selection could be employed to develop LP lines with better seed germination and field emergence potential. It is important for breeders to consider the use of a pedigree method when developing LP lines because it avoids loss of germplasm to unforeseen environmental selection that would likely occur when using a more common method such as a bulk or SSD method.

CONCLUSIONS

This is the first report of variation for seed germination and field emergence potential in V99-5089-derived *MIPS1* mutant LP families and supports the use of the pedigree selection method of early generation population development for breeding LP soybeans. Unavoidable environmental conditions such as drought and complications in winter nursery compounded the levels of reduced seed germination and field emergence that were expected. While the first objective of observing effects of natural selection on field emergence remains unachieved, the second objective of observing genetic variation for seed germination and field emergence potential among LP families was successful.

If a future study with similar objectives, suggested improvements would include incorporation of more LP families and more generations to potentially capture a greater expanse of diversity. In hindsight, more appropriate parents of the cross from which the populations used in this experiment were derived could have been chosen. The WT parent, V01-1693, was not analyzed for field emergence potential before it was selected as a parent for the LP cross but in the future it might be helpful to select a WT parent on the basis of consistently high germination and field emergence potential data. Also, confounding effects contributed by seed source environment could be reduced by removing use of winter nursery and using seed of different generations that was all produced in the same seed source environment, potentially of a less harsh climate. If applied in future studies, these changes could improve results to complete objectives related to field emergence observation and comparison.

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Table 1. Seed source environment and quality information for seed from each generation.

Seed Source	Seed Quality†	Generation
Winter Nursery 2008/2009	Low	$F_{2:4}$
Blacksburg 2009	High	$F_{2:5\ A}$
Winter Nursery 2009/2010 (seed source: remnant Winter Nursery 2008/2009)	Low	$F_{2:5\ B}$
Winter Nursery 2009/2010 (seed source: remnant Blacksburg 2009)	Low	$F_{2:6}$

† Seed quality was determined based on visual quality rating data taken for each generation before planting. Rating was based on visual assessment of seed color, uniformity, coat condition, texture, and evidence of disease.

Table 2. Overall mean % emergence, standard deviation, minimum, and maximum for: families over generations, generations over families, genotype† by generation, and genotypes overall.

Genotype	Family	Generation	Mean	Std dev	Min	Max	n
Families and Checks over all Generations							
MIPS1 mutant	1	Overall	22.1	28.9	0	73.3	13
MIPS1 mutant	2	Overall	21.2	28.7	0	78.7	16
MIPS1 mutant	3	Overall	18.5	26.6	0	61.3	13
MIPS1 mutant	4	Overall	15.7	23.7	0	56	13
MIPS1 mutant	5	Overall	18.4	18.1	0	48	13
MIPS1 mutant	6	Overall	29.3	30.3	0	72	9
MIPS1 mutant	7	Overall	19.5	26.8	0	61.3	13
MIPS1 mutant	8	Overall	17.6	21.7	0	61.3	13
MIPS1 mutant	9	Overall	10.3	15.5	0	45.3	16
MIPS1 mutant	10	Overall	20	26.9	0	74.7	13
WT	11	Overall	32.8	29.9	5.3	89.3	16
WT	12	Overall	40.3	28.5	6.7	85.3	16
WT	13	Overall	33.6	27.9	9.3	84	16
WT	14	Overall	27.1	28.8	0	86.7	16
WT	15	Overall	39.4	27.1	8	88	16
WT	16	Overall	36.2	23.3	1.3	69.3	16
WT	17	Overall	32	25.6	6.7	84	15
WT	18	Overall	26.8	32.4	1.3	89.3	16
WT	19	Overall	23.8	27.6	0	78.7	15
WT	20	Overall	31.6	25.7	4	80	16
V01-1693	V01-1693	Overall	31.5	32.3	0	82.7	16
V03-5901	V03-5901	Overall	19.2	28.5	0	70.7	16
Glenn	Glenn	Overall	76.7	8.1	60	89.3	16
PI 588030	PI 588030	Overall	77.1	7.9	56	86.7	16
PI 588026A	PI 588026A	Overall	69.8	9.6	50.7	86.7	16
Generation over all Families							
Overall	1-20 and parents	F2:4	10.4	16.9	0.0	76.0	88
Overall	1-20and parents	F2:5 A	64.6	14.5	25.3	89.3	86
Overall	1-20 and parents	F2:5 B	14.4	11.4	0.0	46.7	64
Overall	1-20 and parents	F2:6	11.4	12.0	0.0	56.0	81

Table 2. Continued.

Genotype	Family	Generation	Mean	Std dev	Min	Max	n
Genotype by each Generation							
MIPS1 mutant	1-10	F2:4	0.2	0	4	0.3	44
MIPS1 mutant	1-10	F2:5A	55	25.3	78.8	2.6	44
MIPS1 mutant	1-10	F2:5B	8.6	0	26.7	1.7	20
MIPS1 mutant	1-10	F2:6	4.7	0	20	1.3	40
WT	11-20	F2:4	20.7	0	76	4.8	44
WT	11-20	F2:5A	18	56	89.3	8.5	42
WT	11-20	F2:5B	17	2.7	46.7	3.6	44
WT	11-20	F2:6	74.6	0	20	1.4	41
Genotype over all Generations							
MIPS1 mutant	1-10	Overall	18.8	79.7	0.0	78.7	148
WT	11-20	Overall	32.3	27.8	0.0	89.3	171

†Genotype referred to as *MIPS1* mutant refers to the V99-5089-derived D-*myo*-inositol 3-phosphate synthase 1 gene mutation, and WT genotype refers to wild type for P composition.

Table 3. Analysis of variance of percent emergence of four generations of soybean grown at Kentland Farm, Blacksburg, VA 2010. Each generation represented different levels of natural selection. Sources of variation were considered fixed.

Type III Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
REP	3	9	2.24	0.1525
Generation	3	9	43.92	<.0001
Family	19	228	16.06	<.0001
Family*Generation	57	228	8.15	<.0001

Table 4. Pairwise comparison of percent emergence of four generations of F_2 derived soybean families.

Differences of Generation Least Squares Means Adjustment for Multiple Comparisons: Tukey-Kramer							
Generation	**Generation**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Adj P**
F2:5A	**F2:6**	3.2	0.2	9	10.64	<.0001	<.0001
F2:5A	**F2:4**	9.4	58.7	9	0.16	0.8767	0.9984
F2:5A	**F2:5B**	2.8	0.3	9	9.73	<.0001	<.0001
F2:6	**F2:4**	6.2	58.7	9	0.11	0.9181	0.9995
F2:6	**F2:5B**	-0.3	0.3	9	-1.07	0.3121	0.7145
F2:4	**F2:5B**	-6.5	58.7	9	-0.11	0.9139	0.9995

Figure 1. Map of generations and the environments each experienced before being planted into this experiment in Blacksburg, VA in 2010.

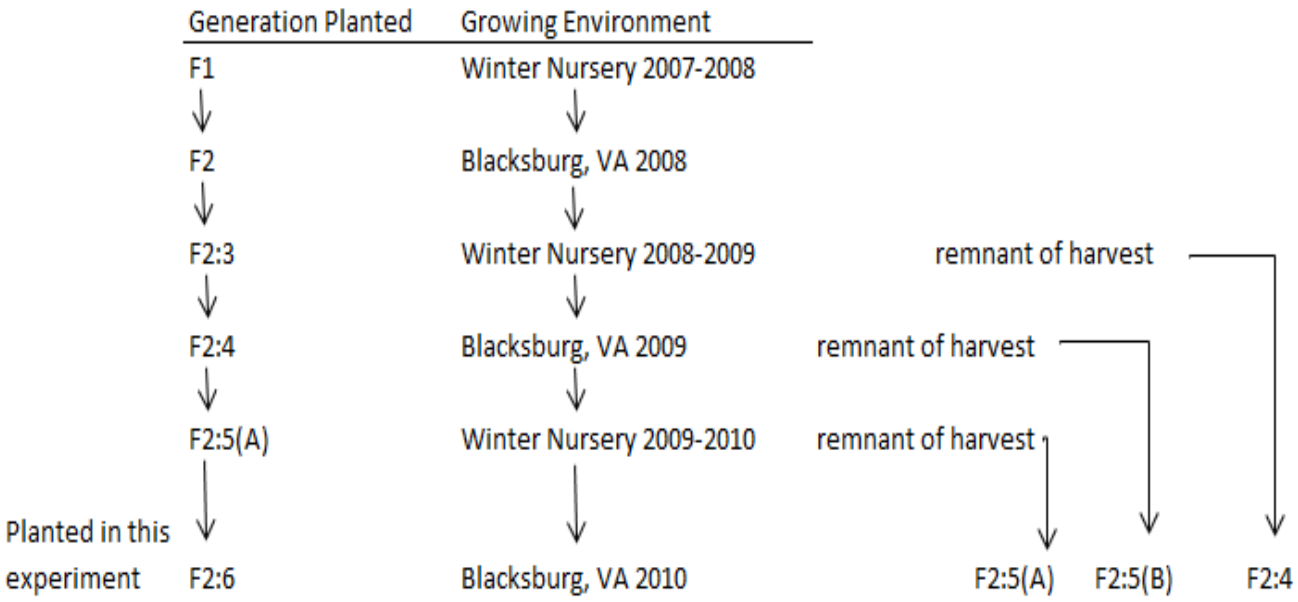


Figure 2. Percent emergence for each of 400 plots by generation and genotype, including checks. Plots were grown on Kentland Farm in Blacksburg, VA in 2010.

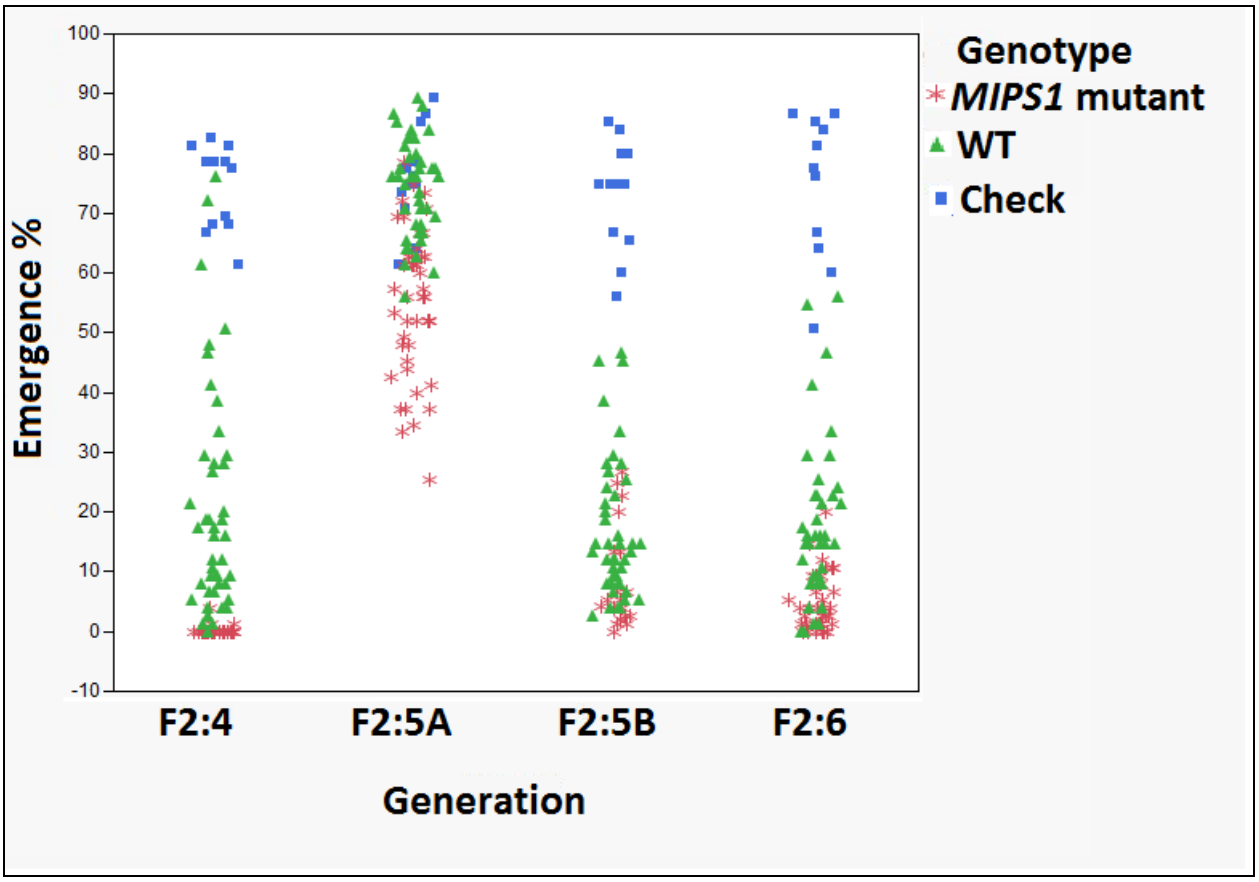


Figure 3. Percent emergence for individuals from each low phytate family over three generations for comparison of the two most distant generations ($F_{2:4}$ and $F_{2:6}$) and the two that underwent the harshest events for natural selection and came from the same seed source environment ($F_{2:5B}$ and $F_{2:6}$). Plots were grown on Kentland Farm in Blacksburg, VA in 2010.

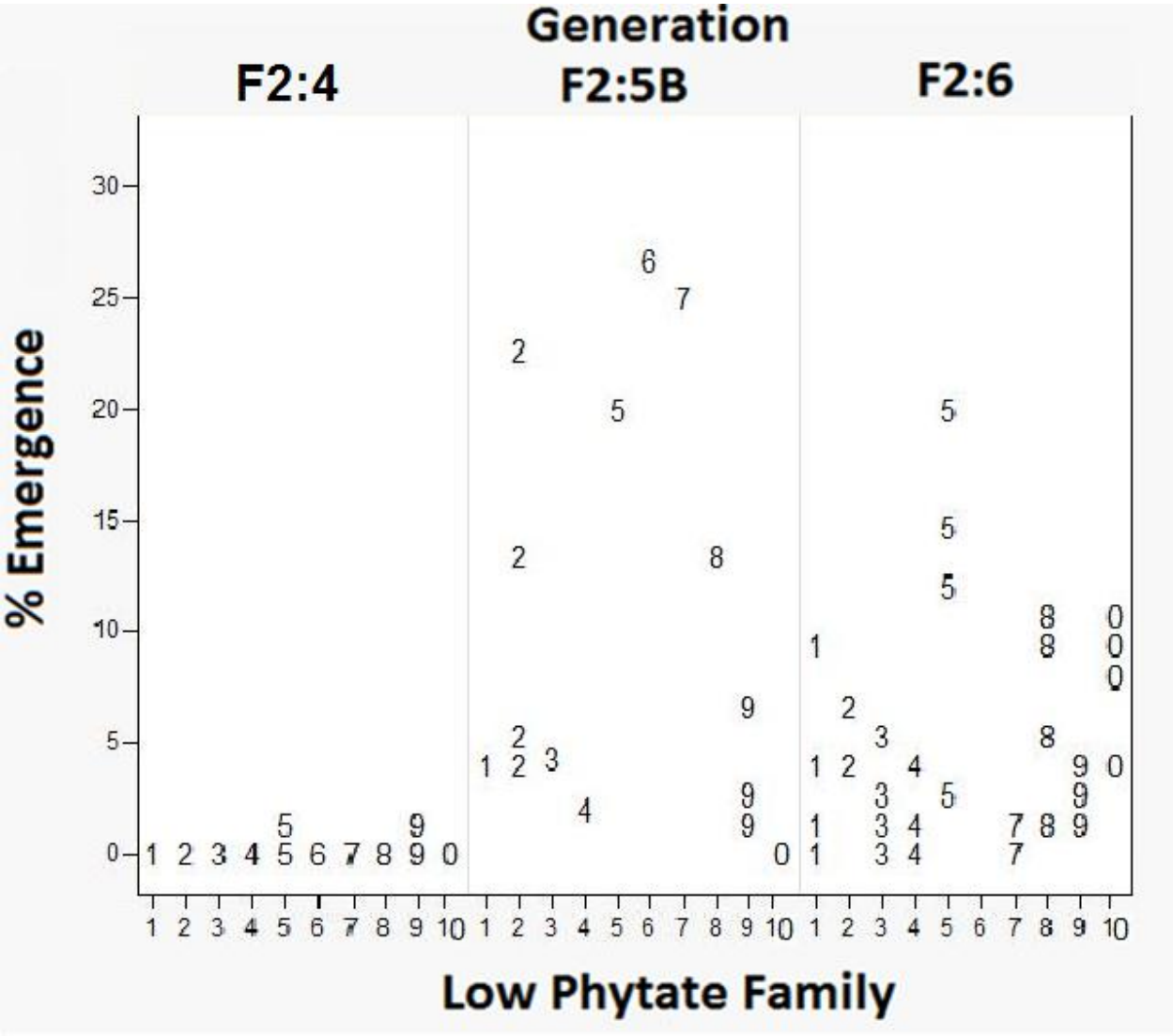
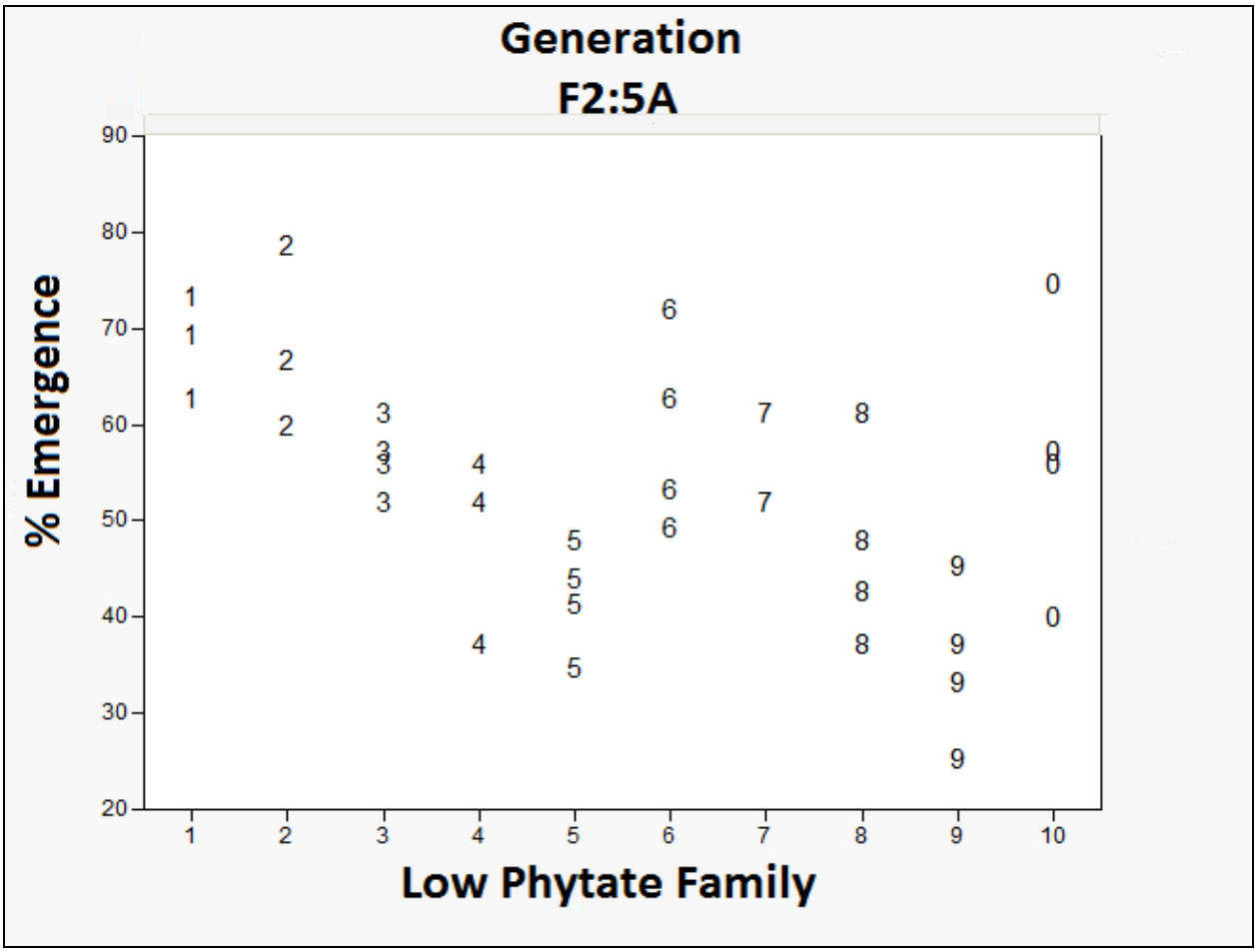


Figure 4. Percent emergence of each plot of each low phytate family from the highest emerging generation ($F_{2:5A}$). Plots were grown on Kentland Farm in Blacksburg, VA in 2010.



III. Development of Breeder-Friendly SSR Markers for Selection of *MIPS1* Mutants

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Abbreviations: bp, base pair*;* hetero, heterozygous; LP, low phytate; *lpa*, low phytic acid; Pi, inorganic Phosphorus; *MIPS1*, D-*myo*-inositol 3-phosphate synthase 1 gene; SNP, single nucleotide polymorphism; SSR, simple sequence repeat; WT, wild type for P composition.

ABSTRACT

Breeding soybean lines is simplified by the employment of molecular markers, especially in low phytate (LP) germplasm whose phenotype is conditioned by a mutation in the D-*myo*-inositol 3-phosphate synthase 1 gene (*MIPS1*) in soybean (*Glycine max* (L.) Merr.). LP soybean line V03-5901, descendent from soybean line V99-5089, which contains a unique *MIPS1* mutation, is a parental line in the segregating population used in this study to develop breeder-friendly markers. Ten candidate simple sequence repeat (SSR) markers located on Gm11, from the new BARCSOYSSR_1.0 database were screened in the segregating population and four were polymorphic. The marker BARCSOY_11_1495 was 93% selection efficient in the V03-5901-derived segregating population and 90% effective when validated in 87 advanced lines from nine diverse V99-5089 descended pedigrees.

INTRODUCTION

BARCSOYSSR1.0 is a new database resource of 33,065 potential SSR markers predicted from the soybean genome sequence Glyma1.01 by Song et al. (2010) that are available on SoyBase (http://soybase.org). The BARCSOYSSR1.0 database can be used to locate SSR markers within certain proximity to any known sequence or locus on the soybean genome. The mutation in the *MIPS1* gene that conditions a LP phenotype is located on Gm11 (LGB1), which is considered a low diversity area of the soybean genome (Saghai Maroof et al., 2009; Song et al., 2004). Soybean line V99-5089, with a naturally occurring gene for LP, was discovered at Virginia Tech in the soybean breeding and genetics program (Saghai Maroof and Buss, 2008). A single nucleotide polymorphism (SNP) in the *MIPS1* gene that changed nucleotide base C to nucleotide base G in the region of the position 1188 from the start codon, controls the LP phenotype (Saghai Maroof and Buss, 2008). The discovery of the locus of the SNP gives a position from which to search the BARCSOYSSR1.0 database for polymorphic SSR markers. Previous studies also identified and screened a SSR marker (Satt453) that was shown to identify the V99-5089-derived *MIPS1* mutation in F_2-derived V99-5089 RIL population lines (Saghai Maroof and Buss 2008) with 87% marker selection efficiency (Maupin et al. 2011). Maupin et al. (2011) attribute the reduced selection efficiency to the 8.1 cM distance between Satt453 and *MIPS1* allowing recombination to occur.

LP soybeans containing the *MIPS1* gene mutation have reduced seed germination and field emergence potential (Anderson and Fehr, 2008; Maupin and Rainey, 2011; Meis et al., 2003; Sebastian et al., 2000; Yuan et al., 2007). The *MIPS1* mutant phenotype includes low levels of seed inositol and phytate (Hitz et al. 2002), while soybean *lpa* mutants have normal levels of seed inositol and lower phytate content than *MIPS1* mutants (Israel et al. 2011). Maupin and

Rainey (2011) demonstrated that both V99-5089-derived *MIPS1* mutants and CX1834-derived low phytic acid (*lpa)* mutants display reduced germination and field emergence potential as compared to wild type (WT) lines when produced under a range of environmental conditions and germination assays. Therefore, low inositol levels may not be responsible for reduced germination and field emergence potential so further development and selection of LP lines with acceptable germination and field emergence may be possible. Regardless of the cause, reduced seed germination and field emergence of LP lines necessitates convenient and accurate molecular markers to select LP lines.

Rationale and Objectives

Maupin (2010) observed that advanced homozygous *MIPS1* mutant populations will shift towards normal phytate concentrations over generations if the WT allele reproduces at a higher rate, due to reduced germination and field emergence of V99-5089-derived *MIPS1* mutants. Therefore, molecular markers are an additional tool for breeding LP germplasm to ensure maintenance of the *MIPS1* mutant allele within advanced lines. Access to different markers for the gene of interest is useful to breeders because some markers select more efficiently in different genetic backgrounds. Also, for the purpose of introducing the V99-5089-derived *MIPS1* mutation into new soybean germplasm, markers positioned near the gene are useful for isolation of the mutant allele in a recurrent genetic background. Markers that are of most interest to breeders are markers with a high selection efficiency in varying genetic backgrounds and thus have close physical position to the gene of interest, that allow efficient, and inexpensive screening analysis. The objective of this work is to develop breeder-friendly markers using the BARCSOYSSR1.0 database and V99-5089 derived germplasm.

MATERIAL AND METHODS

Phenotypic Analysis of Germplasm

The germplasm used to screen candidate markers includes 142 individuals from an $F_{2:3}$ population of the 2007 cross of the low linolenic acid line, V01-1693, and the LP line, V03-5901, grown in Blacksburg, VA in 2008 (Maupin, 2010). V03-5901 is a LP line developed from the cross between V99-5089 and the high-yielding cultivar Essex (Smith and Camper, 1973). To estimate phytate concentration, each sample was assayed using a modified version of the inorganic P (Pi) colorimetric assay developed by Raboy et al. (2000) which was adapted from the assay by Chen et al. (1956) and described by Maupin (2010). A sample of approximately 20 g of seed from each line was ground using a UDY cyclone mill grinder, with a 0.1 mm screen (UDY Corporation, Fort Collins, CO). Between 0.06 and 0.1 g of samples were weighed into 1.5 ml micro-centrifuge tubes. The samples were soaked overnight in 1ml of extraction buffer (12.5% trichloroacetic acid and 25mM $MgCl_2$) at 4^oC, and vortexed once during this incubation period. Samples were vortexed a final time at the end of the incubation period, allowed to sit for 30 minutes and centrifuged for 4 minutes at 4000 rpms. Ten µl of each sample were plated in three replications onto 96 Well Optical Nunc Flat bottom Plates, (Nalgene Nunc International, Rochester, NY), each plate including LP, WT, and blank checks. Ninety µl of ddH_2O and 100µl of Chen's reagent (one part 6N H_2SO_4, one part 2.5% Ammonium molybdate, one part 10% Ascorbic acid, and two parts ddH_2O) were then added to each plate well. Upon completion of one hour's incubation at room temperature, the plates were read using a BioTek Synergy HT plate reader at a wavelength of 882 nm. The concentrations of each replication were then averaged for analysis. Pi concentration is presented as mg kg^{-1} dry weight. Phytate concentration is inversely related to Pi concentration, thus phenotypic classification as LP or WT

is inferred from measurement of Pi concentration (Raboy et al., 2000). The individual F_2's were classified as WT/normal Pi phenotype or *MIPS1* mutant/high Pi phenotype using seed from plants grown in Blacksburg, VA in 2008. Individuals with a Pi concentration of >900 mg kg^{-1} were classified as having the *MIPS1* mutant phenotype based on previous data that showed the LP line V03-5901 to have a Pi concentration of approximately 900 mg kg^{-1} or higher. Individuals with Pi concentration of <600 mg kg^{-1} were assigned a WT phenotype and those with a Pi concentration between 900 and 600 mg kg^{-1} were considered phenotypically heterozygous.

Genotypic Analysis of Candidate Markers in Screening Germplasm

Ten candidate SSR's (Table 1) were selected from the BARCSOYSSR1.0 database by locating the position of Satt453, a SSR marker discovered to be linked to the V99-5089-derived *MIPS1* mutation by Saghai Maroof and Buss (2008), using the whole genome sequence browser on SoyBase (http://soybase.org). Available SSR's upstream and downstream from the Satt453 locus were then evaluated to select the ten candidate SSR's. DNA was isolated using a new protocol for tissue maceration. From each F_2 plant, one young trifoliate leaf was placed in individual 1.27 × 2.54 cm polycarbonate vials with one .95 cm stainless steel grinding ball (Spex Sample Preparation, USA). Tubes were placed at -80 °C overnight. Frozen tissue was ground using a 2000 Geno/Grinder (Spex Sample Preparation, USA) at 200 strokes for 15 s. Genomic DNA was extracted using a Plant DNAeasy Mini Kit (Qiagen, USA). DNA concentration was quantified using a ND-100 NanoDrop spectrophotometer (Thermo Scientific, USA) and adjusted to 30ng/µl for SSR marker analysis. PCR reactions for the BARCSOYSSR assays were performed in a total volume of 12.5 µl. 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. All ten candidate

markers were screened and analyzed in both parent lines prior to the entire $F_{2:3}$ population to determine if the markers would be polymorphic in the population. Those markers found to be polymorphic (Table 1) were then screened in all samples of the $F_{2:3}$ population. Mapmaker V3.0 was used to determine genetic linkage and genetic distances between BARCSOYSSR1.0 markers and the *MIPS1* gene and to construct a linkage map (Lander et al., 1987). The type of band for each sample as determined by the 3130x*l* Genetic Analyzer, either LP, heterozygous, or wild type, was entered into the Mapmaker V3.0 program along with coordinating phenotypic data for the sample. The maximum likelihood method was used to estimate the recombination distance and marker order was determined at a log-likelihood threshold of 3.0 (Lander et al., 1987). Selection efficiency for each polymorphic marker was calculated by number of marker-selected individuals with the desired LP phenotype (as determined by Pi assay) divided by total number of marker-selected individuals, multiplied by 100 (Table 2).

Validation of Markers in Separate Germplasm

Upon development of a plausible marker, V99-5089-derived *MIPS1* mutant germplasm was used to validate the polymorphic marker BARCSOYSSR_11_1495. Composite samples from two reps of 82 F_4-derived lines with V99-5089 in their pedigree were selected from 2008 preliminary line tests which were grown in Warsaw, VA. Nine different pedigrees were included and five check cultivars were also selected (Table 3). Phenotypic analysis of the validation germplasm was conducted using the same Pi assay procedure as described above for the $F_{2:3}$ population. The individuals of the validation germplasm were assigned a phenotypic class based on the same scale of Pi concentration (Table 3) as described for the $F_{2:3}$ population. Genotypic analysis of the validation germplasm for marker BARCSOYSSR_11_1495 was completed and

selection efficiency calculated (Table 4), using the same procedures detailed above for the $F_{2:3}$ population.

RESULTS

Four primers from the BARCSOYSSR1.0 database: BARCSOYSSR_11_1468, BARCSOYSSR_11_1480, BARCSOYSRR_11_1495, and BARCSOYSR_11_1469 were polymorphic out of the ten candidate marker primers screened (Table 1) in the $F_{2:3}$ population. BARCSOYSSR _11_1495, of the four polymorphic markers, is positioned closest (Figure 1) to the *MIPS1* gene locus on Gm 11 at an estimated 4.1 cM away (Table 1). Selection efficiencies in the $F_{2:3}$ V01-1693 × V03-5901 derived population for each marker were calculated (Table 2). BARCSOYSSR_11_1495 was determined to have the highest selection efficiency of the four polymorphic BARCSOYSSR primers, with 93%. Although highly efficient, BARCSOYSSR_11_1495 failed to select one phenotypically LP individual.

When BARCSOYSSR_11_1495 was screened against the 87 lines of nine different pedigrees for validation, it showed 90% efficiency (Table 4). BARCSOYSSR_11_1495 left nine LP lines unselected out of the 82 lines and five checks used for validation (Table 3), and there were nineteen lines that amplified no fragments and were thus classified as undetermined genotypes.

DISCUSSION

Of the polymorphic markers screened, BARCSOYSSR_11_1495 is estimated by input of phenotypic and genotypic results into the Mapmaker V3.0 program to be positioned 4.1 cM from the *MIPS1* gene and is the best available marker. While BARCSOYSSR_11_1495 is the closest it did not select all individuals with the LP phenotype, because it is not a perfect marker as it is not the mutation responsible for the LP phenotype. The SNP for the V99-5089-derived *MIPS1* mutation is useful as a perfect marker, but it requires expensive technology that is not convenient or easily accessible to all breeders and will not select other *MIPS1* mutations. Accurate SSR markers will be useful to breeders developing lines with other *MIPS1* mutations as well as breeders using backcrossing to incorporate the *MIPS1* mutation into their genetic material. The high efficiency of BARCSOYSSR_11_1495 in different backgrounds including the V01-1693 × V03-5901 population as well as all nine of the different V99-5089 pedigrees screened in this experiment makes it a breeder-friendly marker that can be used to select LP individuals for population development and LP line advancement. The remaining three polymorphic BARCSOYSSR_1.0 markers, BARCSOYSSR_11_1468, BARCSOYSSR_11_1480, and BARCSOYSR_11_1869, identified and screened in this study have potential to be useful resources to breeders for marker assisted selection in other LP populations, as their efficiency may be increased in other crosses. Access to accurate and breeder-friendly markers will provide more resources to LP breeders for overcoming seed germination and field emergence potential problems and development of LP lines toward commercialization.

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Table 1. Ten BARCSOYYSSR_1.0 primers on Gm11, corresponding presence or absence of polymorphism based on screening in first the parents of and then the $F_{2:3}$ V01-1693 × V03-5901 derived population, and distance from the *MIPS1* gene.

BARCSOYSSR primers	**Position (cM)**	**PCR product size (bp)**	**Polymorphic**	**Distance from *MIPS1*(cM)†**	**Citation**
1373	93.137	178	No		Cregan et al. (1999)
1467		182	No		
1468	108.409	234	Yes	32.3	Cregan et al. (1999)
1469	110.731	267	Yes	25.3	Song et al. (2004)
1474		163	No		
1480		257	Yes	22.6	
1481		241	No		
1487		273	No		
1495		245	Yes	4.1	
1501		291	No		Shoemaker et al. (2008)

† *MIPS1* is estimated to be located at position 135 cM of chromosome Gm11, based on known positions of SSR's listed in the table above.

Table 2. Selection efficiency† of four BARCSOYSSR_1.0 markers polymorphic for *MIPS1* alleles in a V99-5089-derived F_2 population.

Phenotype	n	Genotypic class (n)			
		BARCSOYSSR_11_1495	BARCSOYSSR_11_1480	BARCSOYSSR_11_1469	BARCSOYSSR_11_1468
MIPS1 mutant	16	15	28	29	31
Heterozygous	-	89	59	77	75
WT	122 ‡	32	35	36	34
undetermined	4	6	20	0	2
Selection efficiency†		93%	50%	48%	42%
Number of high Pi individuals not selected by marker		1	2	2	3

†Selection efficiency = number of marker-selected individuals with the high Pi mutant phenotype ÷ total number of marker-selected individuals × 100.

‡ WT phenotypes consist of wild type and heterozygous genotypes.

Table 3. Phenotypic and genotypic classification for 87 advanced lines derived from crosses of nine various Virginia Agricultural Experiment Station lines, used to validate the BARCSOYSSR_11_1495 marker. Pi concentration is reported in mg kg^{-1}.

Entry #	Pedigree	Pi Conc.	Phenotype†	BARSOYSSR_11_ 1495 Genotype‡
08T1211-1	(V99-5089 × V99-8060) × (V99-8097 × GP2608)	552	WT	WT
08T12-11-2	V97-9003 × (V99-5089 × MN 1401)	621	Hetero	WT
08T12-11-3	Macon	636	Hetero	Hetero
08T12-11-4	V99-5089 × Mn1401	778	Hetero	WT
08T12-11-5	SS RT3860	1024	*MIPS1* mutant	WT
08T12-11-6	V99-5089 × Mn1401	1207	*MIPS1* mutant	Hetero
08T12-11-7	(V99-5089 × V97-7158) × (V99-8097 × GP2608)	473	WT	WT
08T12-11-8	(V99-5089 × V99-8060) × (V99-8097 × GP2608)	1633	*MIPS1* mutant	*MIPS1* mutant
08T12-11-9	V99-5089 × S97-1688	578	WT	Hetero
08T12-11-10	(V99-5089 × V97-7158) × (V99-8097 × GP2608)	664	Hetero	WT
08T12-11-11	V99-5089 × S97-1688	529	WT	undetermined
08T12-11-12	V99-5089 × S97-1688	2767	*MIPS1* mutant	undetermined
08T12-11-13	(V99-5089 × V97-7158) × (V99-8097 × GP2608)	728	Hetero	WT
08T12-11-14	V97-9003 × (V99-5089 × V99-8060)	552	WT	undetermined
08T12-11-15	V99-5089 × S97-1688	609	Hetero	WT
08T12-11-16	(V99-5089 × V97-7158) × (V99-8097 × GP2608)	664	Hetero	WT
08T12-11-17	V99-5089 × S97-1688	517	WT	WT
08T12-11-18	V99-5089 × S97-1688	494	WT	WT
08T12-11-19	V99-5089 × S97-1688	452	WT	WT
08T12-11-20	(V99-5089 × V99-8060) × (V99-8097 × GP2608)	2667	*MIPS1* mutant	*MIPS1* mutant
08T12-11-21	V99-5089 × S97-1688	614	Hetero	WT
08T12-11-22	(V99-5089 × V97-7158) × (V99-8097 × GP2608)	495	WT	WT
08T12-11-23	V99-5089 × Mn1401	1111	*MIPS1* mutant	Hetero
08T12-11-24	(V99-5089 × V97-7158) × (V99-8097 × GP2608)	747	Hetero	WT
08T12-11-25	(V99-5089 × V97-7158) × (V99-8097 × GP2608)	458	WT	WT
08T12-11-26	V99-5089 × S97-1688	2269	*MIPS1* mutant	*MIPS1 m*utant
08T12-11-27	(V99-5089 × V97-7158) × (V99-8097 × GP2608)	727	Hetero	WT
08T12-11-28	(V99-5089 × V99-8060) × (V99-8097 × GP2608)	573	WT	WT
08T12-11-29	V99-5089 × Mn1401	540	WT	WT
08T12-11-30	(V99-5089 × V97-7158) × (V99-8097 × GP2608)	506	WT	Hetero

Table 3. Continued.

Entry #	Pedigree	Pi Conc.	Phenotype†	BARSOYSSR_11_1495 Genotype‡
08T12-12-1	5002T	612	Hetero	WT
08T12-12-2	V99-5089 × S97-1688	521	WT	WT
08T12-12-3	(V99-5089 × V97-7158) × (V99-8097 × GP2608)	756	Hetero	WT
08T12-12-4	V99-5089 × Mn1401	526	WT	undetermined
08T12-12-5	V99-5089 × S97-1688	490	WT	WT
08T12-12-6	V97-9003 × (V99-5089 × MN 1401)	525	WT	WT
08T12-12-7	V99-5089 × S97-1688	631	Hetero	*MIPS1* Mutant
08T12-12-8	V97-9003 × (V99-5089 × V99-8060)	604	Hetero	WT
08T12-12-9	V99-5089 × S97-1688	700	Hetero	WT
08T12-12-10	V97-9003 × (V99-5089 × V99-8060)	657	Hetero	WT
08T12-12-11	V99-5089 × Mn1401	617	Hetero	undetermined
08T12-12-12	V99-5089 × Mn1401	543	WT	undetermined
08T12-12-13	V97-9003 × (V99-5089 × MN 1401)	540	WT	WT
08T12-12-14	V99-5089 × Mn1401	771	Hetero	undetermined
08T12-12-15	V99-5089 × S97-1688	599	WT	WT
08T12-12-16	V99-5089 × Mn1401	589	WT	undetermined
08T12-12-17	V99-5089 × Mn1401	872	Hetero	undetermined
08T12-12-18	V97-9003 × (V99-5089 × MN 1401)	700	Hetero	WT
08T12-12-19	V97-9003 × (V99-5089 × V99-8060)	661	Hetero	WT
08T12-12-20	V99-5089 × S97-1688	580	WT	WT
08T12-12-21	V99-5089 × S97-1688	629	Hetero	WT
08T12-12-22	V99-5089 × S97-1688	1336	*MIPS1* mutant	WT
08T12-12-23	Teejay	899	Hetero	WT
08T12-12-24	V99-5089 × Mn1401	543	WT	undetermined
08T12-12-26	V97-9003 × (V99-5089 × MN 1401)	467	WT	WT
08T12-12-27	V99-5089 × S97-1688	734	Hetero	undetermined
08T12-12-28	V99-5089 × S97-1688	694	Hetero	WT
08T12-12-29	V99-5089 × Mn1401	588	WT	undetermined
08T12-12-30	V94-2800 × (V95-7456 × V99-5089)	589	WT	WT
08T12-14-1	V99-5089 × S97-1688	381	WT	undetermined
08T12-14-2	V94-2800 × (V95-7456 × V99-5089)	444	WT	WT
08T12-14-3	V97-9003 × (V99-5089 × V99-8060)	1333	*MIPS1* mutant	*MIPS1* mutant
08T12-14-4	V99-5089 × S97-1688	1615	*MIPS1* mutant	Hetero
08T12-14-5	V97-9003 × (V99-5089 × V99-8060)	501	WT	WT
08T12-14-6	V97-9003 × (V99-5089 × MN 1401)	1702	*MIPS1* mutant	*MIPS1* mutant

Table 3. Continued.

Entry #	Pedigree	Pi Conc.	Phenotype†	BARSOYSSR_11_1495 Genotype‡
08T12-14-7	V94-2800 × (V95-7456 × V99-5089)	488	WT	WT
08T12-14-8	V99-5089 × S97-1688	2658	*MIPS1* mutant	*MIPS1* mutant
08T12-14-9	V95-7456 × V99-5089	578	WT	undetermined
08T12-14-10	V97-9003 × (V99-5089 × V99-8060)	2659	*MIPS1* mutant	*MIPS1* mutant
08T12-14-11	V97-9003 × (V99-5089 × V99-8060)	551	WT	WT
08T12-14-13	V97-9003 × (V99-5089 × MN 1401)	1393	*MIPS1* mutant	Hetero
08T12-14-15	V95-7456 × V99-5089	453	WT	undetermined
08T12-14-17	V99-6364 × V99-5089	625	Hetero	WT
08T12-14-18	V97-9003 × (V99-5089 × V99-8060)	605	Hetero	undetermined
08T12-14-19	V94-2800 × (V95-7456 × V99-5089)	417	WT	WT
08T12-14-20	V94-2800 × (V95-7456 × V99-5089)	362	WT	WT
08T12-14-21	V97-9003 × (V99-5089 × MN 1401)	2767	*MIPS1* mutant	*MIPS1* mutant
08T12-14-22	V99-5089 × S97-1688	1000	*MIPS1* mutant	Hetero
08T12-14-23	R03-410	447	WT	WT
08T12-14-24	V99-6364 × V99-5089	429	WT	undetermined
08T12-14-25	V99-5089 × S97-1688	1199	*MIPS1* mutant	Hetero
08T12-14-26	V99-5089 × S97-1688	2454	*MIPS1* mutant	*MIPS1* mutant
08T12-14-27	V95-7456 × V99-5089	512	WT	Hetero
08T12-14-28	V97-9003 × (V99-5089 × V99-8060)	483	WT	Hetero
08T12-14.29	V99-6364 × V99-5089	647	Hetero	undetermined
08T12-14-30	V99-5089 × S97-1688	794	Hetero	WT
08T12-14-31	V95-7456 × V99-5089	343	WT	undetermined

†Phenotype classified as '*MIPS1* mutant' refers to the expression of the V99-5089-derived *MIPS1* gene mutation, and was assigned based on a Pi concentration of >900mg kg^{-1}; 'WT' phenotypes refer to expression of the wild type allele for P content and were classified based on a Pi concentration of <600 mg kg^{-1}, and the expression of a heterozygous phenotype (Hetero) is a Pi concentration between 900 and 600 mg kg^{-1}.

Table 3. Continued.

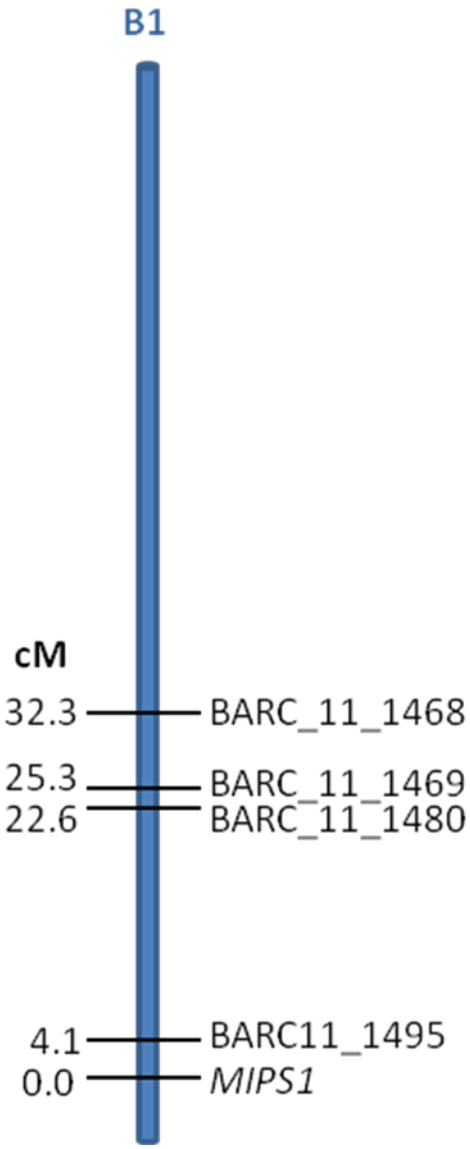
‡Genotypes were determined by fragment length allele call and classified as either: '*MIPS1* mutant', referring to the V99-5089-derived *MIPS1* gene mutant allele genotype; 'WT', referring to the wild type allele; or 'Hetero', referring to detection of the heterozygous genotype. Classification as 'undetermined' refers to lines for which bands from the BARCSOYSSR_11_1495 could not be accurately determined.

Table 4. Selection efficiency† of marker BARCSOYSSR_11_1495, polymorphic for *MIPS1* alleles in 82 V99-5089-derived lines.

Phenotype	n	Genotypic class (n)
		BARCSOYSSR_11_1495
MIPS1 mutant	18	10
Heterozygous	29	11
WT	40	47
undetermined	-	19
Selection efficiency†		90%
Number of high Pi individuals not selected by marker		9

†Selection efficiency = number of marker-selected individuals with the high Pi mutant phenotype ÷ total number of marker-selected individuals × 100.

Figure 1. Map produced by Mapmaker V3.0 to indicate distance (cM) of each marker from *MIPS1* gene based on comparison of phenotypic and genotypic classifications of individuals of the $F_{2:3}$ V01-1693 × V03-5901 derived population.



IV. A High-Throughput Colorimetric Method for Selection of Soybean Phytate Concentration

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Abbreviations: HPIC, high performance ion chromatography; HPLC, high performance liquid chromatography; LP, low phytate genotype; *lpa*, *lpa1* and *lpa2* mutant lines with modified seed composition; *MIPS1*, D-myo-inositol 3-phosphate synthase 1 gene; Pi, inorganic phosphorus; WT, wild-type lines with normal seed composition

ABSTRACT

Determining seed phytate concentration in soybeans allows for screening of germplasm and for selection of modified phosphorus (P) lines with stable low phytate expression. Accurate quantitative phytate assays and simple colorimetric assays have been developed; however, a high-throughput colorimetric phytate assay is needed. The objectives of this study were to develop a cost and time effective modified Fe colorimetric phytate assay, and to demonstrate that the assay can be used for direct selection of phytate concentration in soybean seeds. The new assay was tested on 192 samples from eight lines from three germplasm sources (normal P composition, and two different modified P composition mutants) produced in twelve environments. Using this modified Fe colorimetric method approximately 100 samples can be assayed per day. Compared to other quantitative and colorimetric methods, this assay is faster, less expensive, simpler, and can assay a larger number of samples, which meets the needs of breeders for selection among modified P soybean lines and screening of germplasm over environments.

INTRODUCTION

Phytate, (phytic acid or *myo-inositol* 1,2,3,4,5,6-hexakisphosphate), is the main form of phosphorus (P) storage in the soybean seed, composing over 75% of the total seed P (Raboy et al., 2001). The seed of most grain crops have a phytic acid concentration of approximately 10 g kg^{-1} of seed dry weight which is equivalent to 65-85% of total seed P (Raboy et al., 1990; Raboy et al., 1997; Raboy et al., 2001). Phytate is indigestible by non-ruminant animals and chelates minerals such as calcium, zinc, and iron, resulting in both a lack of available P for the animals' nutrition and excretion of the biounavailable P (Raboy et al., 2001). When the waste P ends up in waterways it contributes to environmental problems such as eutrophication. These problems for the producer, the animal's nutrition, and the environment can be reduced by genetically reducing the phytate concentration in the soybean meal being fed.

Two types of genetic mutations are responsible for modifications of phytate production pathways to produce modified P soybean seeds: mutations in the myo-inositol phosphate synthase (*MIPS1*) gene, or the combination of the *lpa1* and *lpa2* mutant genes. *MIPS1* mutations 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 V99-5089 (Saghai Maroof and Buss, 2008), and they convey a 50% lower phytate and 80% lower raffinosaccharide concentration in seeds (Hitz et al., 2002). The combination of the *lpa1* and *lpa2* mutations (referred to as *lpa*) is the source of the modified P phenotype in crop species including maize and barley, and the soybean line CX-1834 (Larson et al., 1998; Raboy et al., 2000; Rasmussen and Hatzack, 1998; Shi et al., 2007; Wilcox et al., 2000). In soybean, *lpa* mutants produce seed with increased inorganic P of 45 to 55% (over non-mutant seed) and 80% reduced phytate but with normal levels of raffinosaccharides (Bilyeu et al., 2008; Wilcox et al., 2000). It is simple and cost-effective to select mutant modified P lines

using orthophosphate P, or inorganic P, (Pi) assays, molecular marker assays, or both. An estimate of phytate concentration can be obtained through measurement of Pi concentration, due to the inverse relationship between phytate concentration and Pi concentration in soybean seeds observed in both *MIPS1* and *lpa* mutant soybean lines (Maupin et al., 2011; Scaboo et al., 2009).

Maupin (2010) studied three lines each derived from the V99-5089 and CX1834-modified P germplasm sources, and two normal P soybean lines, in twelve environments throughout the Southeast. The *MIPS1* mutant lines showed a mean phytate concentration of 1992 mg P kg^{-1}, compared to the mean of 1091 mg P kg^{-1} in the CX1834-derived lines. Within the modified P germplasm, environments were significantly different for Pi concentration but not phytate, and genotype × environment effects were significant for both Pi and phytate. The three CX1834-derived lines had different genetic backgrounds, and were significantly different for phytate concentration. Lines from both germplasm sources displayed differential stability for phytate concentration. All mutant lines were relatively more stable for phytate concentration compared to Pi, and the opposite was true for normal P composition lines. Other studies have shown that normal P composition soybean lines grown in variable P conditions respond with changes to phytate concentration but remain constant for Pi concentration (Israel et al., 2006; Raboy and Dickinson, 1984, 1993). Therefore, determining P allocation to Pi and phytate in seed from various environments may be necessary for genetic improvement of low phytate (LP) soybeans and to select cultivars with stable phytate composition. Seed phytate concentration is more informative than Pi concentration for screening non-mutant germplasm and for selection of LP lines with stable phytate expression. Additionally, Maupin and Rainey (2011) observed that modified P composition lines from both germplasm sources often displayed less than 80% field emergence in the twelve-environment study described above. However, seed of mutant lines

with low overall phytate + Pi concentration emerged well, suggesting that a LP soybean with low total seed P may offer improved germination and field emergence potential, and could be the target of germplasm screening and would require determining both Pi and phytate seed concentration.

Phytate assays involving high performance liquid chromatography (HPLC), anion exchange column, and colorimetric assays are extensively reviewed by Gao et al. (2007). High performance ion chromatography (HPIC) is an additional accurate method for quantifying phytate concentration of soybean seed (Maupin, 2010); however, this analytical method requires specialized equipment and expertise. The HPIC method provides an accurate measure of phytate concentration as it is a direct measure of phytate that uses an analytical column and comparison of samples to three standards of exact known phytate concentrations. Gao et al. (2007) developed a modified colorimetric phytate assay that employs colorimetric determination of phytate content based on the decoloration of the pink Fe^{3+}–sulfosalicylate complex (known as Wade's reagent) by phytate, for simpler screening of soybean lines with modified phytate concentration based on the method by Vaintraub and Lapteva (1988). In this method Fe^{3+} is removed by phytate from the Wade's reagent; this step allows Pi to interfere with precipitation and therefore lowers selectivity for LP samples which are higher in Pi. The interference from Pi results in a misrepresentative range of phytate concentration values which is the limitation of the method.

Haug and Lantzsch (1983) described a Fe colorimetric method that avoids Pi interference by incorporating excess Fe^{3+} under acidic conditions (0.5 M HCl) to create a ferric complex of phytate that has lower solubility than Pi under acidic and high salt conditions (Anderson, 1962), and should allow higher selectivity. Removal of Fe^{3+}-phytate precipitate in the Fe colorimetric

method is critical to the selectivity and accuracy of the method since formation of ferric phytate does not strictly follow a chemical stoichiometry and the precipitate coagulation is slow in most media. According to Anderson (1962), temperature, salt concentration, acidity, and salt type all affect the recovery of the precipitate; of all the factors tested, a boiling treatment is most effective for the purpose. However, this method is not preferred because the boiling treatment is inconvenient. Simplification of the Fe colorimetric method would include: eliminating the boiling treatment and replacing the original 2-2' bipyridine Fe^{3+} assay with the 1-10 phenanthroline-Fe^{3+} assay, and using ethanol in the ferric phytate precipitation (Haug and Lantzsch, 1983).

Objectives

The objectives of this study were to (i) develop and demonstrate an Fe colorimetric method that provides a cost and time effective option for assaying phytate concentration without interference from Pi concentration, and (ii) to demonstrate that the assay can be used for direct selection of phytate composition in soybean seeds.

MATERIALS AND METHODS

Preliminary Study

In the preliminary study for determining which method of precipitation to incorporate into the development of the modified Fe colorimetric phytate method, twenty ground soybean seed samples with varying phytate concentrations that had been well quantified were used. HPIC separation was performed on the same extracts to compare the phytate values obtained by three variations of the Fe method to (Table 1).

Germplasm

For demonstration of the selectability of the modified Fe colorimetric phytate assay, seed from eight soybean lines produced in twelve environments from the experiment described previously (Maupin, 2010) were assayed in this study. Two lines had normal P composition, referred to as WT genotype (Essex and 5601T), and six had modified P concentration: three of the modified P lines were derived from the line CX-1834, referred to as *lpa* genotype (lines 04-05N32, LP-5601T-BC1, and S04-053-05), and three were derived from the line V99-5089, referred to as the *MIPS1* mutant genotype (V03-5900, V03-5901, and V03- 5906). Seed of these eight lines was grown in six locations (MD, MO, NC, TN, and two in VA) in 2008 and 2009.

Colorimetric Assay Method and HPIC

Colorimetric phytate concentration of two plots of each line from each environment (location and year combinations) was determined in this study using the modified Fe colorimetric method. P composition of the same 192 samples was previously determined with the modified Pi colorimetric assay and the HPIC phytate assay (Maupin, 2010). For phytate extraction, each sample was ground using a UDY cyclone mill grinder, with a 0.1 mm screen

(UDY Corporation, Fort Collins, CO). 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 4 h 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 2 h before being centrifuged at 12,000 *g* for 15 min. Supernatant was then diluted five times with water. One mL of Ferric Iron solution (0.5 mM FeCl3 in 0.75 M HCl and 80% EtOH) and 0.5 mL of the diluted phytate sample were placed into a 2.0-mL microcentrifuge tube, and then mixed by inversion. The precipitate was incubated for 2 h at room temperature before being centrifuged at 12,000 *g* for 15 min. Then, 1 mL of the supernatant and 3 mL of the color reagent (1, 10-phenanthroline, 80 mg L^{-1}; hydroxylamine hydrochloride, 2.7 g L^{-1}; ammonium acetate, 0.27 M) were placed in 5-mL plastic vials and the contents mixed and then incubated for 30 min at room temperature.

The phytate concentration was determined using a BioTek Synergy HT plate reader (BioTek Instruments, Winooski, VT) set at a wavelength of 510 nm. The final phytate concentration of each sample was calculated from an average of three subsamples using the initial weight of each sample to calculate the phytate concentration on a dry weight basis with results reported as micrograms per gram. To establish a calibration curve, the absorbance of seven standards was plotted against the phytate concentration. Concentrations were 0, 3, 6, 12, 18, 24, and 30 mg P L^{-1}. Standard concentration was determined based on the concentration of the phytate stock solution from which it was prepared, which was determined by an inductively coupled plasma atomic emission spectrometer (Spectro ARCOS ICP Model FHS16 with CETAC Autosampler Type: ASX-520, Spectro Analytical Instruments, Inc., Mahwah, NJ). Phytate

concentration (mg P L^{-1}) of each sample was determined from the calibration curve. Phytate concentration of samples as determined by HPIC was quantified in a previous study (Maupin, 2010).

Statistical Analysis

Analysis of variance and least square means for phytate concentration among lines and environments, and among methods tested, were computed using the SAS procedure PROC GLIMMIX (SAS, 2010). Replication was considered a random effect and fixed effects of line, environment, and line by environment interaction were tested for significance. Significant differences among lines and environments were determined by pairwise comparisons of means using Tukey's multiple mean comparison method. The SAS procedure PROC CORR (SAS, 2010) was used to calculate correlation coefficients between phytate concentrations determined by the modified Fe colorimetric method and HPIC.

RESULTS

In the preliminary study for development of the modified Fe colorimetric phytate method, all three Fe methods produced excellent linear calibration in the range of 0 – 30 mg P L^{-1}; the boiling treatment of Haug and Lantzsch (1983) gave the highest sensitivity ($\left|\text{slope}\right| = 0.0203$), and the ethanol precipitation was slightly more sensitive than the methanol precipitation (Table 1). The procedure including 53% ethanol in the ferric phytate precipitation substantially simplified the laboratory operations for phytate assay compared to the original version reported by Haug and Lantzsch (1983). The precipitation medium for ferric phytate with 53% methanol was equally useful but required a longer incubation time during precipitation in order to achieve the same efficiency as 53% ethanol for certain samples (data not shown). Use of high-concentration salts medium did not produce meaningful results due to low efficiency of ferric phytate precipitation. Data from the three Fe colorimetric methods were highly correlated with the HPIC data (Table 1). All three methods yielded statistically significant lower phytate values than HPIC (Table 1). The underestimation of phytate by the three modified Fe colorimetric methods suggests an unknown matrix effect is present in soybean extracts that interferes with the ferric phytate formation and precipitation, and is absent in phytate calibration standards. The ethanol method of precipitation was chosen to incorporate into the modified Fe colorimetric phytate assay based on its accuracy in comparison to HPIC, relative sensitivity, and reduced time requirements as compared to the methanol and boiling procedures.

The modified Fe colorimetric phytate assay was then evaluated in 192 soybean samples produced in a previous study (Maupin, 2010). Overall phytate concentration means in mg P kg^{-1} for the modified Fe colorimetric method reported by genotype are: 943±347 for *lpa*, 1842±425 for *MIPS1* mutants, and 2676±597 for WT; and by line are: 800±238 for S04-053-05, 921±275

for 04-05N32, 1109±435 for 5601T BC1, 1785±419 for V03-5906, 1879±482 for V03-5900, 1862±381 for V03-5901, 2595±589 for 5601T, and 2757±607 for Essex. There were significant differences for phytate concentration among lines and environments as determined by the modified Fe colorimetric method (Table 2). Line, environment, and line by environment interaction were significant factors affecting the phytate concentration as measured by the modified Fe colorimetric method; while environment was not previously found to be a statistically significant factor when phytate concentration was quantified using HPIC (Maupin, 2010). Overall mean phytate concentrations by factor were similar to, but lower than, values obtained from HPIC (Table 2). Phytate concentration ranking by line was consistent between the modified Fe colorimetric method and HPIC except for a switch in the 5th and 6th ranked lines (Table 2), although the mean difference between the two lines was 17 mg P kg^{-1} which is much less than one standard deviation for both lines. Phytate concentration ranking among environments changed slightly between the modified Fe colorimetric method and HPIC, but was limited to intermediate values.

Data from the modified Fe colorimetric method and HPIC for the 192 samples were strongly correlated (r^2 = 0.85). The overall means by each factor indicate the modified Fe colorimetric method underestimates the phytate concentration for the majority of samples (Figure 1). However, the modified Fe colorimetric method also overestimated phytate concentration in some samples (data not shown). Therefore, the difference in phytate concentration between the modified Fe colorimetric method and HPIC is not predictable and cannot be estimated on a per sample basis which did not differ significantly for the modified Fe colorimetric method.

DISCUSSION

After assessment of this method in the previously quantified in 192 samples of three different genotypes, it was determined that this modified Fe colorimetric method is faster and more efficient than previously described methods for determining phytate concentration in soybean seeds. The authors estimate that 100 samples can be processed per day. The elimination of orthophosphate interference as compared to the previously published colorimetric method using Wade's reagent has increased the phytate quantification range making it ideal for selection of low phytate lines and germplasm screening. The incorporation of ethanol in the precipitating medium replaces the boiling treatment in the original method which increases efficiency. The method described here uses a plate reader for easy assay of sample replications and for quantification of samples simultaneously.

While the modified Fe colorimetric method does not match the accuracy of HPIC in phytate quantification, the strong correlation with HPIC and the maintenance of mean phytate concentration rank for lines indicates that the method can be used to select low phytate mutant lines. An estimate of stability of phytate concentration over environments among mutant lines may be possible through selection for rank. In combination with determination of Pi concentration, this method can be used to screen soybean germplasm for modified P composition**.**

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Table 1. The performance of three Fe colorimetric methods for analyzing phytate concentration in soybean seeds, as compared to HPIC, in the preliminary study (n = 20).

Method[†]	Phytate concentration mg P kg^{-1} Range	Mean[§]	Calibration (0 – 30 mg P L^{-1}) Equation	Correlation[¶] Equation	r^2
EtOH-Fe-Phen[‡]	701-4466	2503a	y = -0.015x + 0.94	y = 0.893x - 0.94	0.99
MeOH-Fe-Phen	794-4475	2580b	y = -0.014x + 0.94	y = 0.836x + 0.47	0.99
Boiling-Fe-Bipyridine	957-4697	2594b	y = -0.020x + 1.03	y = 0.863x - 0.22	0.99
HPIC	929-6071	3095c			

[†]EtOH-Fe-Phen = 53% ethanol present in ferric phytate precipitation medium followed by the phenanthroline-Fe^{3+} assay; MeOH-Fe-Phen = 53% methanol present in ferric phytate precipitation medium followed by the phenanthroline-Fe^{3+} assay; Boiling-Fe-Bipyridine = ferric phytate precipitation under boiling condition, followed by the bipyridine-Fe^{3+} assay; HPIC = high performance ion chromatography.

[‡]n=18.

[§]Means followed by the same letter are not significantly different according to Tukey's pairwise comparison at p=0.05.

[¶]Correlation of each colorimetric method with HPIC.

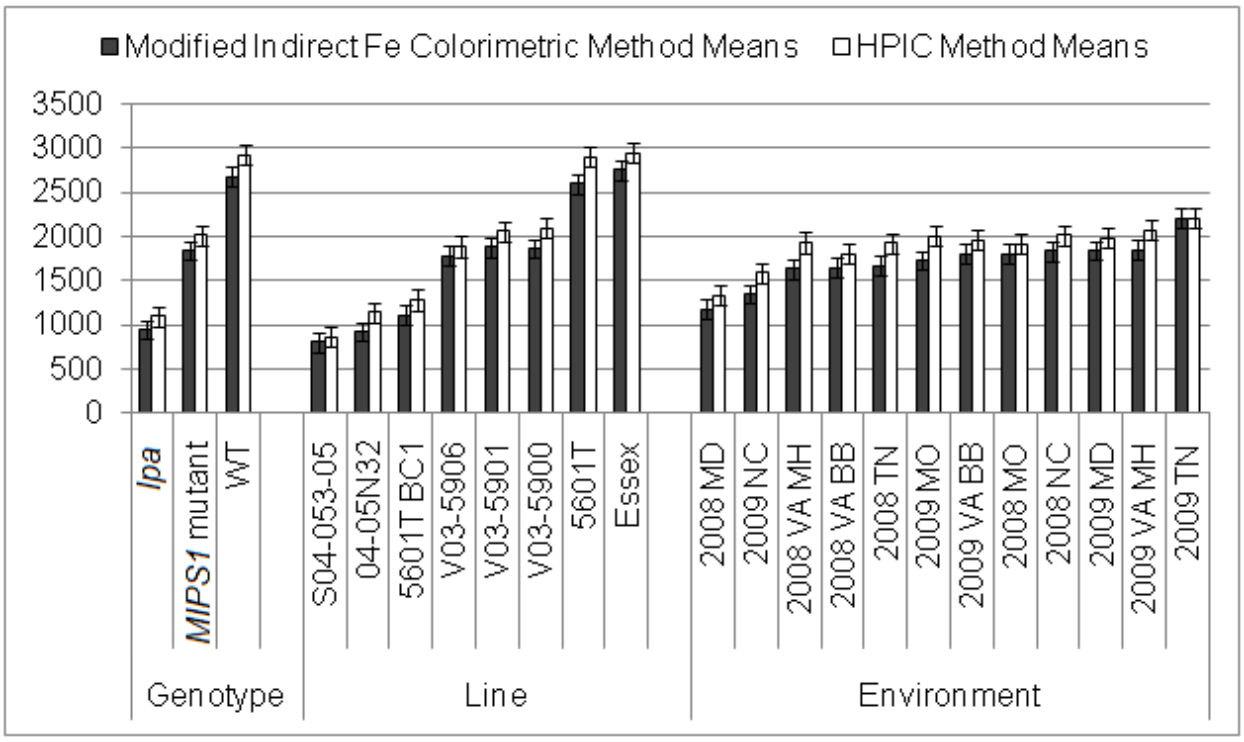
Table 2. Line means[†] and rankings[‡] and environments means and rankings for phytate concentration (mg P kg^{-1}) quantified with the modified indirect Fe colorimetric method and HPIC for eight lines grown in 12 environments.

	Modified Fe colorimetric method		HPIC	
Line	Mean	Rank	Mean	Rank
S04-053-05	800a	1	860a	1
04-05N32	921ab	2	1137b	2
5601T-BC1	1109b	3	1283b	3
V03-5906	1785c	4	1888c	4
V03-5900	1862c	5	2093c	6
V03-5901	1879c	6	2058c	5
5601T	2595d	7	2898d	7
Essex	2757d	8	2947d	8
Environment				
MD-2008	1178a	1	1331a	1
NC-2009	1348ab	2	1588ab	2
VAMH-08	1630abc	3	1932ab	6
VABB-2008	1650bc	4	1803ab	3
TN-2008	1673bc	5	1925ab	5
MO-2009	1733bc	6	2010ab	9
VABB-2009	1805bcd	7	1960ab	7
MO-2008	1806bcd	8	1915ab	4
NC-2008	1838cd	9	2018ab	10
MD-2009	1843cd	10	1985ab	8
VAMH-2009	1846cd	11	2076b	11
TN-2009	2211d	12	2203b	12

[†]Line and environment means followed by the same letter are not significantly different according to Tukey's pairwise comparison at p=0.05.

[‡]Rankings for each method are based on relative order of phytate concentration from lowest to highest, by method.

Figure 1. Overall mean phytate concentration by factor in mg P kg^{-1} for eight genotypes grown in 12 environments.



V. CONCLUSIONS

Developing a soybean cultivar with a low phytate (LP) trait will be useful to livestock producers from both a nutritional and economical standpoint and will in turn benefit the environment. For breeders to commercialize a LP cultivar, reduced seed germination and field emergence potential must be overcome by adjusting all aspects of the breeding program to address and select for improvement. After observing the effects of natural selection on the reduced frequency of LP lines in segregating populations we determined that alternative methods of line selection should be explored. Selection methods previously assumed by breeders to be acceptable such as single seed descent and bulk methods, may cause loss of both genetic diversity and the subsequent potential to select advanced LP lines with improved field emergence potential. A method of early generation population development that could improve reduced seed germination and field emergence potential such as the pedigree method should be adopted for overcoming the loss of LP types in early population development. Pedigree selection will reduce such losses and allow the breeder to choose LP families with the best emergence to advance.

Integration of marker assisted selection within early generation population development, using accurate simple sequence repeat (SSR) markers such as BARCSOYSSR_11_1495 that was found to be 90-93% efficient in over 200 lines of different genetic backgrounds, will improve efficiency of line advancement by ensuring the lines being advanced contain the LP trait. Genotypic confirmation of the LP trait in advanced progeny will eliminate possible skew in genotypic ratios caused by elimination of LP lines during emergence. Access to accurate SSR markers is useful to breeders. The identification of the single nucleotide polymorphism (SNP) for the V99-5089-derived D-*myo*-inositol 3-phosphate synthase 1 (*MIPS1*) mutation (Saghai

Maroof and Buss, 2008), but SNP marker detection technology is expensive and not readily available to all breeders. Other markers such as BARCSOYSSR_11_1468, BARCSOYSSR_11_1480, and BARCSOYSSR_11_1869 could also be useful to the breeder if backcrossing the LP trait from a LP donor parent into superior germplasm was a suitable approach for the program objectives.

Once the elite LP families have been advanced several generations, incorporation of an accurate, time-efficient, and high-throughput method of determining phytate concentration and stability will be useful to select superior lines for further advancement. The modified Fe colorimetric phytate assay can be used to screen LP lines efficiently and without interference from inorganic P concentrations that has been experienced in other methods. Application of an effective method of phenotypic evaluation will also allow breeders to track the stability of phytate concentration in advanced lines.

Thus, the studies described in these works are beneficial to breeders working with LP soybean lines because they address improvements for line development including early generation population development methods, molecular markers, and phenotypic assays. The integration of the resources studied in this research including closely linked molecular markers with improved-efficiency phenotypic assays will allow breeders to advance lines with phenotypic and genotypic confirmation of the LP trait, without question of inaccuracies due to Pi concentration. Additionally, integration of improved molecular markers and phenotypic selection methods with use of the pedigree line selection method will ensure that progeny being advanced were not skewed to the WT phenotype by natural selection. Improved markers and selection methods will also allow breeders to more accurately identify and incorporate the LP trait into lines with higher emergence in attempt to improve seed germination and field

emergence potential in LP lines and commercialize a LP cultivar for aiding livestock producers as well as the environment.