

GENETIC MAPPING OF SOYBEAN RESISTANCE GENES TO FROGEYE LEAF
SPOT IN FIVE CHINESE PLANT INTRODUCTIONS AND EFFICIENCY OF
EARLY GENERATION SELECTION FOR LOW PHYTATE SOYBEAN LINES

by

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(Under the Direction of H. Roger Boerma)

ABSTRACT

Frogeye leaf spot (FLS), caused by *Cercospora sojina*, is a disease found on soybean (*Glycine max*) in many areas of the world. The *Rcs3* resistance gene was previously discovered in the soybean cultivar Davis and genetically maps to chromosome 16 (*Gm16*;Lg-J) near Satt244. *Rcs3* is currently resistant to all known isolates of *C. sojina*. Five plant introductions (PIs) from China (PI594619, PI594661, PI594662A, PI594774, and PI594891) were found to have broad resistance to FLS. The objective of this study was to determine if these FLS-resistant PIs contain unique genes for resistance to FLS. Results indicate resistance genes in PI594619 and PI594662A were found near Satt501 on *Gm18*(Lg-G) and near Satt547 and Satt244 on *Gm16*(Lg-J), respectively. A resistance gene in PI594661 was found near Satt244 on *Gm16*(Lg-J). The resistance gene in PI594774 and PI594891 mapped to *Gm13*(Lg-F) near Satt114. These results indicate that four of the plant introductions (PI594619, PI594662A, PI594774, and PI594891) contain unique resistance genes. Phosphorus stored in the seeds of soybean is primarily stored as phytic acid, a form unavailable to monogastric animals. The

objectives of the current research were (i) to determine the effect of soybean maturity on the effectiveness of single-plant selection for inorganic phosphorus levels in soybean seeds containing alleles from CX1834-1-2, a low phytate, high inorganic P soybean line, and (ii) to determine effects of backcross generations on levels of inorganic P in soybean seeds. There were weak, but significant ($P = 0.05$) correlations between temperature 15, 30, and 45 d prior to maturity and Pi levels in the Boggs ($r = -0.14$ to 0.18) and Benning ($r = -0.13$ to -0.21) populations in 2008, but not 2009. In 2009 the lines in the high Pi classes of Boggs (1228 vs. 203 ng g⁻¹ P), Benning (1445 vs. 87 ng g⁻¹ P), and Prichard (1359 vs. 103 ng g⁻¹ P) averaged significantly ($P = 0.05$) higher in Pi than the lines in the low Pi class. Using single plants in a single environment may not be the most efficient means of selection when making selections for inorganic phosphorus in a breeding program.

INDEX WORDS: Frogeye leafspot, SSR, *Glycine max*, marker-assisted selection, soybean, phytic acid, low phytate soybean, disease resistance, *Cercospora sojina*, single-plant selection

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DEDICATION

To my loving wife Sarah.

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CHAPTER I

INTRODUCTION

History and Uses of Soybean

Soybean (*Glycine max*) is an annual legume that originated in China and has been cultivated for many centuries. Historical evidence suggests domestication of soybean occurred during the Shang dynasty in China circa 1500-1100 B.C. or perhaps earlier. During the first century A.D. it is believed the soybean reached south China as well as Korea. From the first century until approximately the 15 to 16th century it is believed that soybean was introduced into several Asian countries which include: Japan, Indonesia, Phillipines, Nepal, and India. In the 16th and 17th centuries, Europeans became familiar with the uses of soybean for food when they would travel to China and Japan. Soybean was introduced into North America by Samuel Bowen in 1765. The soybean was planted by Henry Yonge, who was the surveyor of the General Colony of Georgia. In 1766, Samuel Bowen planted soybean on his plantation near Savannah, Georgia. Mr. Bowen used the soybean that he grew to manufacture soy sauce and vermicelli for which he received a patent for his inventions (Hymowitz, 2004).

Soybean is grown in over 50 countries worldwide. In 2007 nearly 95 million hectares of soybean were harvested in the world. The United States of America has been the leading producer of soybean for the past 50 years, and in 2007 harvested over 30.5 million hectares. Brazil and Argentina harvested over 20 and 16 million hectares, respectively, in South America, while China was the largest producer in Asia harvesting nearly 9 million hectares (Anonymous, 2008b).

On average, accessions in the USDA soybean germplasm collection contain about 42% protein and 20% oil. Soybean is important in that it produces more edible protein per hectare of arable land than any other major crop and also accounts for 35% of the world total oilseed production (Lusas, 2004; Wilcox, 2004). Protein from soybean is the closest plant source to the optimum dietary essential amino acids profile for human and animal nutrition. Soybean meal is currently the number one source of feed protein in the world. Oil that has been extracted from soybean seed can be used for edible uses such as; salad or cooking oil, baking or frying, and as margarines and spreads. Soybeans oil can also be used in industrial applications. Some non-edible uses of soybean oil include; soap, resins, plastics, inks, paints, varnishes, and biodiesel (Lusas, 2004).

Breeding Goals

Soybean seed yield is the number one goal of soybean breeding programs as it has the greatest effect on producers' net income. Other traits that are also important for an improved soybean cultivar include; disease, nematode, and insect resistance. Important agronomic traits that are essential for soybean cultivars are resistance to lodging and shattering and high levels of oil and protein. In the past 10 years, soybean cultivars have been genetically modified to tolerate applications of glyphosate herbicide. Herbicide tolerance has become an important trait in soybeans grown worldwide as herbicide tolerant soybeans were grown on 58.6 million hectares worldwide in 2006 (ISAAA, 2008). Genetically modified soybeans also have been developed that are resistant to insects, initially using a cry1Ac gene from *Bacillus thuringiensis*, to provide resistance to Lepidoptera insects (Walker et al., 2000).

Wilcox (2001) estimates soybean seed yield has increased 60% by the breeding efforts of public breeders over the past 60 years. Through genetic modification this yield potential can be protected by incorporating resistance genes to important soybean pathogens (Grau, 2004). In 2002, it was estimated that soybean production was suppressed by nearly 9.7 metric tons due to diseases that were prevalent in soybean producing regions. This equates to approximately \$2 billion dollars (216/mega tons) in lost revenue to soybean producers in the USA. Soybean cyst nematode (SCN) caused the greatest economic damage in 2002. Losses due to SCN alone were over \$780 million. Other diseases that caused significant yield loss were phytophthora root and stem rot, Sclerotinia stem rot and charcoal rot (Wrather et al., 2003).

Modification of the oil profile in soybean has occurred for more than 50 years. The goals of this modification were to develop oils that could better meet the demands of the end users (Fehr, 2007). This has allowed for the creation of niche markets for specialty soybeans. The average composition of the five major fatty acids in soybean oil are: 11% palmitic (16:0), 4% stearic (18:0), 22% oleic (18:1), 54% linoleic (18:2), and 10% linolenic (18:3). Lowering the levels of 18:2 and 18:3 concentrations in soybean seed has decreased the use of catalytic hydrogenation and resulted in improved oxidative stability for soybean. This has allowed for lower levels of trans-fats in soybean oil (Wilson, 2004).

Germplasm Resources

Before written records were kept Chinese farmers exploited genetic diversity in soybean found in the wild to produce the domesticated soybean that is grown today. During the domestication of soybean, farmers developed many phenotypically distinct

landraces. By the 20th century Chinese farmers were raising 20,000 to 45,000 distinct types of soybean. Soybean researchers have archived much of the genetic diversity from around the world into soybean germplasm collections. Globally there are over 150,000 accessions of *Glycine max* housed in over 20 countries. The Institute of Crop Germplasm Resources in China houses over 23,000 of these accessions and the USA currently has over 18,000 soybean accession housed in the USDA Soybean Germplasm Collection at Urbana, IL (Carter, 2004).

Unadapted germplasm presents many problems for plant breeders in that it usually possesses undesirable agronomic traits such as; vining, lodging susceptibility, lack of complete leaf abscission, seed shattering, black seedcoat, and small seed (Carpenter and Fehr, 1986). When soybean breeders have attempted to use exotic germplasm to enhance elite cultivars, it has been with limited success (Concibido et al., 2003).

Glycine soja (Sieb. And Zucc.) is thought to be the ancestor of domestic soybean and a potential source of desirable alleles that can contribute to *G. max* (Wang et al., 2001). Concibido et al. (2003) demonstrated the potential of an unadapted germplasm line's ability to enhance yield in soybean when they crossed *G. soja* plant introduction (PI) 407305 to 'HS-1'. A yield enhancing quantitative trait locus (QTL) was discovered and mapped to linkage group (LG) B2 of the soybean genetic linkage map. Progeny which contained the haplotype at the QTL locus demonstrated a 9.4% yield advantage. *G. soja* has also been used as sources of novel resistance to SCN. Wang et al. (2000) mapped QTLs that provided resistance to SCN in *G. soja* PI 468916. The QTLs for resistance mapped to locations on the genome where SCN resistance genes had not previously been

identified. Luzzi, et al. (1987) found PI 96354 to be highly resistant to *Meloidogyne incognita* (Kofoed & White). When field microplots were inoculated at the highest levels, PI96354 was found to contain 62% fewer J2 nematodes in roots compared to other resistant genotypes (Herman et al., 1990). They concluded that PI96354 would be useful in a soybean breeding program to improve the level of resistance to *M. incognita*.

Hyten et al. (2006) evaluated the impacts of genetic bottlenecks on soybean genome diversity. Soybean has undergone several bottlenecks. These include domestication in Asia, introduction to North America, and selective breeding over the past 75 years. The researchers concluded that modern cultivars have retained 72% of the sequence diversity present in Asian landraces.

The objectives of this research were: 1) to genetically map resistance to frogeye leafspot in five germplasm lines from China, 2) determine maturity and backcross generation effects on the phytate composition of soybean seed.

REFERENCES

- Anonymous. 2008b. Food and Agricultural Organization of the United Nations FAO Statistical Tables [Online]
<http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#ancor>.
- Carpenter, J.A., and W.R. Fehr. 1986. Genetic Variability for Desirable Agronomic Traits in Populations Containing Glycine soja Germplasm. *Crop Science* 26:681.
- Carter, T.E., R. L. Nelson, C. H. Sneller, Z. Cui. 2004. Genetic Diversity in Soybean. Soybeans: Improvement, production, and uses. 3rd ed. Agron. Monogr:303-416.
- Concibido, V., B. La Vallee, P. McLaird, N. Pineda, J. Meyer, L. Hummel, J. Yang, K. Wu, and X. Delannay. 2003. Introgression of a quantitative trait locus for yield from Glycine soja into commercial soybean cultivars. *TAG Theoretical and Applied Genetics* 106:575-582.
- Fehr, W.R. 2007. Breeding for Modified Fatty Acid Composition in Soybean. *Crop Sci* 47:S-72-87.
- Grau, C.R., A.E. Dorrance, J. Bond, and J.S. Russin. . 2004. Fungal diseases. *In* H.R. Boerma and J.E. Specht (ed) Soybeans: Improvement, production, and uses. 3rd ed. Agron. Mongr. 16, ASA, CSSA, and SSSA, Madison, WI.:679-763.
- Herman, M., R.S. Hussey, and H.R. Boerma. 1990. Response of resistant soybean plant introductions to *Meloidogyne incognita* in field microplots. *Journal of Nematology* 22:237-241.
- Hymowitz, T. 2004. Speciation and Cytogenetics. Soybeans: Improvement, Production, and Uses. 3rd edition. Ed by Boerma, HR and Specht, JE American Society of Agronomy, Inc. Madison, Wisconsin, USA:97-136.

- Hyten, D.L., Q. Song, Y. Zhu, I.Y. Choi, R.L. Nelson, J.M. Costa, J.E. Specht, R.C. Shoemaker, and P.B. Cregan. 2006. Impacts of genetic bottlenecks on soybean genome diversity. *Proceedings of the National Academy of Sciences* 103:16666.
- ISAAA. 2008. Global Status of Commercialized Biotech/GM Crops: 2006 [Online] <http://www.isaaa.org/RESOURCES/PUBLICATIONS/BRIEFS/35/EXECUTIVE SUMMARY/default.html>
- Lusas, E. 2004. Soybean Processing and Utilization. *Soybeans: Improvement, Production, and Uses*. 3rd edition. Ed by Boerma, HR and Specht, JE American Society of Agronomy, Inc. Madison, Wisconsin, USA 949-1045.
- Luzzi, B.M., H.R. Boerma, and R.S. Hussey. 1987. Resistance to Three Species of Root-Knot Nematode in Soybean1. *Crop Sci.* 27:258-262.
- Walker, D.R., J.N. All, R.M. McPherson, H.R. Boerma, and W.A. Parrott. 2000. Field Evaluation of Soybean Engineered with a Synthetic cry1Ac Transgene for Resistance to Corn Earworm, Soybean Looper, Velvetbean Caterpillar (Lepidoptera: Noctuidae), and Lesser Cornstalk Borer (Lepidoptera: Pyralidae). *Journal of Economic Entomology* 93:613-622.
- Wang, D., B.W. Diers, P.R. Arelli, and R.C. Shoemaker. 2001. Loci underlying resistance to Race 3 of soybean cyst nematode in Glycine soja plant introduction 468916. *TAG Theoretical and Applied Genetics* 103:561-566.
- Wilcox, J.R. 2001. Sixty Years of Improvement in Publicly Developed Elite Soybean Lines. *Crop Sci* 41:1711-1716.

- Wilcox, J.R. 2004. World distribution and trade of soybean. Soybeans: Improvement, Production, and Uses. 3rd edition. Ed by Boerma, HR and Specht, JE American Society of Agronomy, Inc. Madison, Wisconsin, USA:1-14.
- Wilson, R.F. 2004. Seed Composition. Soybeans: Improvement, production, and uses. 3rd ed. Agron. Monogr:621-677.
- Wrather, J.A., S.R. Koenning, and T.R. Anderson. 2003. Effect of diseases on soybean yields in the United States and Ontario (1999 to 2002). Plant Health Progress 25.

CHAPTER II

LITERATURE REVIEW

Frogeye Leaf Spot

Frogeye leaf spot (FLS) is caused by the fungus *Cercospora sojina* Hara. Frogeye leaf spot was first discovered on soybean in Japan in 1915 and in the United States in 1924 (Phillips, 1999). Estimated soybean yield loss in the USA in 2005 due to frogeye leaf spot were over 178 thousand tones (Wrather and Koenning, 2006). *C. sojina* primarily infects leaves but can also infect stems, pods, and seeds of the soybean (Phillips 1999). Lesions on soybean leaves are circular to angular spots with diameters ranging from less than 1 mm to 5 mm. The lesions begin as dark water soaked spots in which lighter centers may become visible. These spots ultimately turn into brown spots surrounded by slender dark reddish brown margins. Lesions may coalesce to cover more than 30% of the leaf area causing a blighting phase which results in leaf wither and premature defoliation. Stem lesions appear late in the growing season and are less common. Lesions can be up to four times as wide as they are long and encircle half the stem. Pod lesions are reddish brown in color and are circular to elongate and slightly sunken. Seeds infected by the fungus develop noticeable light to dark brown areas that can range from specks to large blotches that can cover the entire seed coat. The more discoloration that appears on the seed, the lesser the germination. The seed coats can often crack or flake as a result of the fungal infection (Phillips 1999).

The fungus over-winters as mycelium in infected seeds and infested soybean residues. There have not been any confirmed cases of this pathogen infecting other

crop or weed hosts. Seedlings that germinate from infected seeds tend to be weak and stunted with lesions on the cotyledons. Young leaves may become infected from spores produced on the lesions of the cotyledons. On plants infected by the fungus, lesions can appear in 8 to 12 days. Sporulation in the lesions is most abundant when weather conditions are warm and humid. Young expanding leaves are most susceptible to infection by the fungus, as leaves mature they are less likely to become infected (Phillips 1999).

Frogeye leaf spot is typically found in warm humid growing conditions associated with southern soybean producing states. However in the past several years it has been found as far north as Ohio and Iowa (Mengistu et al., 2002; Yang et al., 2001). Yang et al. (2001) evaluated 80 soybean cultivars in Central Iowa and observed that all entries showed some level of disease. This would indicate that northern germplasm lacks genes for resistance to frogeye leaf spot. This combined with the adoption of conservation tillage practices, which is designed to leave residue on the soil surface, may be responsible for the spread of the disease northward.

In the USA yield loss caused by frogeye leaf spot on susceptible soybean cultivars have been reported as high as 30% if extensive leaf blighting occurs (Phillips 1999). In Nigeria susceptible cultivars yielded 65% less than the same cultivar that was treated with a fungicide. The application of fungicide increased soybean yields by reducing disease severity. (Dashiehl and Akem, 1991). Fungicides should be applied during late flowering through beginning seed (R2-R5) stages of development (Fehr, 1977) to protect against infection of *C. sojae* (Grau et al. 2004). In Nigeria, yield loss was as high as 31% with each 2-week delay in planting,

indicating that planting a soybean crop as early as possible would lead to an increase in soybean yields (Akem and Dashiell, 1994). Deep plowing of soybean residue to bury infested soybean plants and planting high quality disease-free seed are also useful cultural practices to reduce frogeye leaf spot on soybean (Phillips 1999).

Cultural practices provide a means of controlling frogeye leaf spot but genetic resistance provides the most effective management option for managing the disease (Grau, 2004). A single dominant resistance gene *Cscs* (now *Rcs₁* *rsc₁*) was found to provide resistance to race 1 of the pathogen *C. sojae* (Probst, 1965). This gene provided resistance until the late 1950s when the soybean cultivars Clark and Wabash, previously known to be resistant were found to be heavily infected. Athow et al. (1962) discovered the disease outbreak to be caused by race 2 of *C. sojae*. An additional single dominant gene for resistance was found in the soybean cultivar Kent and was designated *Rcs₂*. In the 1960s races 3 and 4 of the pathogen were located in North Carolina (Ross, 1968). An additional race of *C. sojae* was detected in the late 1970s and was classified as race 5 (Phillips and Boerma, 1981). Many elite cultivars were found to be susceptible to race 5 but resistance was available in some cultivars. *Rcs₃*, a single dominant gene found in the cultivar Davis was responsible for resistance to race 5 and is also believed to condition resistance to all known races of *C. sojae* in the USA (Phillips and Boerma, 1981).

Mian et al. (1998) evaluated four resistant and susceptible near isolines (NILs) in the southern USA to determine the effect that *Rcs₃* had on seed yield loss due to frogeye leaf spot. They found that all four NILs containing *Rcs₃* were resistant to the common races of frogeye leaf spot in all environments tested. When the disease was

present significant yield loss occurred in the absence of *Rcs₃*, up to 31% compared to the NILs that contained *Rcs₃*. It was also discovered that the effect of frogeye leaf spot on seed yield was dependent on the progression of the disease during the growing season not just solely on the level of disease infestation at the end of the growing season (Mian et. al 1998).

Proper characterization of pathogen isolates is necessary for genetic studies to be conducted. *C. sojae* lacks a universally accepted system to identify or designate races of the pathogen on soybean differentials. Mian et al. (2008) have proposed a race designation for proper characterization of *C. sojae* using 12 soybean differential cultivars. Research revealed 11 *C. sojae* isolates designated race 5 to 15 that will allow for advances in characterization and identification of additional FLS resistance genes in soybean.

Marker assisted selection is a tool that plant breeders can use to improve the efficiency of selection. Through the use of biotechnology, molecular markers have been developed that allow plant breeders to screen single traits, shorten the breeding cycle, evaluate germplasm, and protect cultivars. Plant breeders can now screen single traits that are associated with agronomic traits of interest such as disease resistance. DNA based markers such as restriction fragment length polymorphism (RFLPs), random amplified polymorphic DNA (RAPD), simple sequence repeats (SSR), and single nucleotide polymorphisms (SNPs) have provided an alternative to phenotyping traits for characterizing cultivars (Acquaah 2007).

SSR markers have many attributes that make them desirable for use in soybean breeding including genetic map development and genotype identification.

SSR markers are polymerase chain reaction (PCR)-based and are highly polymorphic in nature. SSR markers target tandemly repeated two to five nucleotide DNA core sequences such as (CA)_n, (ATT)_n, or (AGAT)_n of varying length (Akkaya, 1992; Akkaya, 1995). The DNA sequences outside the SSR regions are usually conserved amongst individuals of the same species allowing PCR primers to be selected that will amplify the SSR in all genotypes. Another positive aspect of SSR markers is primers are designed to produce only one PCR product avoiding the difficulty in interpreting multiple banding patterns such as in RFLP markers. SSR markers are codominant and evenly spaced throughout the soybean genetic map (Cregan, 1998). Consensus genetic maps of soybean have been developed with over 1000 SSR markers. These maps contain marker positions and genetic distances based on multiple soybean mapping populations (www.soybase.org).

SNPs (single nucleotide polymorphisms), which are single DNA base differences between homologous DNA fragments, have been shown to be the most abundant source of DNA polymorphisms in humans, with SNPs in plants beginning to receive attention in the early 2000s (Zhu Y. L., 2003). Due to the fact that SNPs are more plentiful than SSRs chances are better for achieving results in positional cloning, association analysis, QTL mapping, and determination of genetic relationships among individuals (Choi I. K., 2007). Choi et al. (2007) were able to add 1141 sequence-based markers to the existing soybean genetic map. Through the use of SNP markers they were able to locate genes in the gaps of previous SSR based-genetic maps of soybean.

Bulked segregant analysis (BSA) is a technique that can be utilized to identify markers linked to any specific gene or genomic region of a plant. In this method two pools of DNA are created from a segregating population originating from a single cross. Each pool consists of individuals that are identical for the trait or gene of interest but are random for all other genes (Michelmore et al., 1991). The use of BSA has been shown to be useful in mapping traits in different species (Liu, 2007; Mian, 1999)

Much work has been accomplished in the past several years through the use of molecular breeding to map the *Rcs₃* gene that confers resistance to FLS. Mian et al. (1999) mapped the *Rcs₃* through the use of molecular markers to aid in the breeding for resistance to frogeye leaf spot. The *Rcs₃* was mapped to a previously known disease resistance cluster in soybean on linkage group *Gm16*(Lg-J). The gene was closely linked to SSR markers Satt244 and Satt547 (Mian, 1999). Missaoui et al. (2007b) confirmed these two SSR markers and their association with *Rcs₃* in 64 cultivars and breeding lines from the ancestors and descendants of Davis. SSR markers are not ideal for high throughput genotyping because they are based on repeat length variants and detection requires electrophoresis. The use of SNP technologies allows for high-throughput genotyping. Missaoui et al. (2007a) developed a SNP marker to be used for marker assisted selection for the detection of *Rcs₃*.

Phytic Acid in Soybean

Phosphorus (P) is classified as a macronutrient and is considered essential to plant growth. P is required in the plant for energy transfer and storage. P is also

important in forming structural components in the plant such as; nucleic acids, nucleotides, phosphoproteins, and phospholipids (Tisdale, 1993). P is required in soybean (*Glycine max* L.) for nodule development. Uptake of P from the soil is greatest two to three months after planting (deMOOY, 1973). Phytic acid (myoinositol hexaphosphate) is the most common organic form of phosphate in the soil comprising up to 50% of total organic P in soil (Tisdale, 1993). Phytic acid serves as the chief storage form of phosphate (>60% total P) and inositol in mature seeds of oilseed crops (Erdman, 1979; Lei and Porres, 2003). Phytic acid accumulation in soybean seeds has been shown to begin early in embryogenesis and continue through seed development (Raboy and Dickinson, 1987).

Animal food supplies that contain high phytate levels have been shown to lead to poor bioavailability of minerals such as zinc, calcium, magnesium, phosphorus, and possibly iron. Non-ruminant animals such as poultry and swine have little or none of the phytase enzyme needed to digest phytate P rendering it unavailable to them from major food sources such as soybean meal (Erdman, 1979). Poultry and swine rations are typically supplemented with phytase, which results in the removal of phosphate from phytate, or with inorganic phosphate which results in increased P availability to the livestock (Adeola, 1995; Lei and Porres, 2003). The phytate phosphorus that goes unutilized gets excreted by the animals and becomes an environmental pollutant in areas of intensive agriculture. The excessive phosphorus in soils runs off into lakes and seas and causes eutrophication (Lei and Porres, 2003).

Ethyl methanesulfonate (EMS) has been used successfully to create low phytate mutants in barley (*Hordeum vulgare* L; (Larson et al., 1998), maize (*Zea*

mays (Raboy et al., 2000) and soybean (Wilcox et al., 2000). Soybean lines CX1834-1-2 and CX1834-1-6 are two low phytate soybean lines generated from the cross of 'Athow' x M153-1-4-6-14. M153-1-4-6-14 is a low phytate mutant line that was generated from soybean breeding line CX1515-4 after being treated with EMS. Seed from M153-1-4 showed an 80% reduction in phytic acid-P (PA-P) compared with its non mutant sibling. This reduction in PA-P corresponded to an equivalent increase in inorganic P (Wilcox et al., 2000). Researchers have developed a transgenic soybean line (CAPPA) in which an *Escherichia coli* periplasmic phytase gene was expressed in the developing cotyledons. The CAPPA soybean line resulted in high levels of phytase expression and showed greater than 90% reduction in seed PA. It was also shown that soymeal from CAPPA could be used to convert PA from cornmeal to inorganic P, thus eliminating the need for additional P, or phytase added to animal rations (Bilyeu et al., 2008).

Inorganic P (P_i) and PA-P can be measured in soybean seeds. Researchers have examined several different processes in which to quantify the amount of PA-P that is in soybean seeds (Thompson and Erdman, 1982). Thompson et al. (1982) concluded that precipitate analysis or supernatant difference method were both acceptable methods to determine the amount of PA-P in soybean seeds. Another method that has shown utility in determining PA-P was a modified colorimetric method. This method obtains results similar to high performance liquid chromatography (HPLC), anion exchange column (AEC), and ^{31}P nuclear magnetic resonance (^{31}P -NMR), but is much less expensive and more useful for screening a large number of soybean lines (Gao et al., 2007). P_i can also be measured in soybean

seeds by colorimetric testing. A modified version of Chen's (Chen Jr et al., 1956) procedure has been well documented to accurately portray the PA-P levels in the seed through an inverse relationship with P_i (Oltmans et al., 2004; Scaboo et al., 2009; Walker et al., 2006). Oltmans et al. (2004) evaluated 11 F_3 seeds that were grown in a single replication in a row that was 0.76 m long. In other tests to evaluate dominance and maternal effects of phytate, researchers used a seed chip from a single seed of a F_1 plant (Oltmans et al., 2004). Walker et al (2006) randomly selected eight seeds from an F_2 plant and crushed the seed and determined P_i . Scaboo et al (2009) used approximately 30 g of soybean seed that were harvested from progeny rows that were either 3.05 or 6.10 m long. The varying sample sizes utilized shows that selection for low phytate can be accomplished using a number of different selection units.

The importance of genotype by environment interactions effects on phytate levels differs for wild-type and mutant low-phytate soybean lines. External P supplies have been shown to influence the phytic acid P concentrations in wild-type soybean seed. Six soybean cultivars showed an average four-fold increase in phytic acid P as external P supplied to plants increased from 2 to 50 mg L⁻¹. Increases in phytic acid accounted for nearly all of the increase in total P in the seed (Raboy and Dickinson, 1993).

When evaluating populations that contained low phytate mutant line CX1834-1-2 as a parent, researchers found phytate levels remained constant as soil phosphorus levels increased from deficient to excessive. This suggests that crosses which contain

the mutant line as a parent should produce the low phytic acid phenotype across a wide range of P levels in the soil (Israel et al., 2007).

Reduced germination of soybean lines that have low phytate levels have been well documented (Hulke et al., 2004; Meis et al., 2003; Oltmans et al., 2005). Meis et al. (2003) found that germination of soybean lines containing a recessive *mips* allele were three times greater when seed sources were from a temperate environment in Iowa compared to seed produced in a subtropical environment of Mexico. Oltmans et al. (2005) evaluated the germination of soybean lines that were homozygous recessive for *pha1* and *pha2* that conditioned for low phytate (LP). They found that seedling emergence of LP soybean lines was 45% compared to 68% for normal phytate (NP) lines. Anderson and Fehr (2008) evaluated lines in Iowa and Puerto Rico for the effects on seed emergence. Research showed that field emergence of LP lines in Iowa was 77.6% while field emergence of lines harvested in Puerto Rico was dependent on when the harvest occurred. LP lines harvested in January had a field emergence of 70.1% while May harvested seed had a field emergence of 25.4%. This corresponded to changes in phytate P levels in the seed as well. Phytate levels from Iowa produced seed were 0.18 mg g⁻¹, while seed produced in Puerto Rico was 0.41 mg g⁻¹ and 0.61 mg g⁻¹ for seed harvested in January and May respectively. These results show that the environment where the seed is produced can have a major impact on the phytate P levels of seed and on emergence levels. Research conducted in Virginia also suggests that there is a genotype by environment interaction that results in reduced emergence of low phytate soybean lines (Gao et al., 2008). In 2007, experiments planted in two locations resulted in varying emergence patterns.

At one location there were not any differences in emergence between the NP and the LP lines, whereas at another location the NP lines had higher emergence than the LP lines (Gao et al., 2008).

Two separate research studies have found that backcrossing CX1834 to a recurrent parent results in higher field emergence ratings (Gao et al., 2008; Spear and Fehr, 2007). Spear et al. (2007) found 18 out of 36 BC₃F₄ lines had a mean field emergence that was greater than CX1834 and not significantly different than B019, the recurrent parent. The mean phytate and inorganic P content of all of the backcross lines was not significantly different from CX1834. Gao et al (2008) also found that when LP lines were backcrossed to a recurrent parent that field emergence improved. However, they also concluded that the improved emergence did not appear to be stably inherited and that further studies were needed to determine the nature of the genotype by environment interaction that was occurring.

Scaboo et al. (2009) evaluated the heritability of the low phytate trait in lines that were derived from cross of 5601T x CX1834-1-2. Heritability was calculated using the variance component method. The heritability estimate was conducted in a recombinant inbred line population that was grown in three environments each with three replications. The heritability estimate for P_i was 0.97. A heritability estimate this high would suggest that selection for the low phytate genotype from the CX1834-1-2 line could be easily accomplished (Scaboo et al., 2009).

Simple sequence repeat (SSR) markers were discovered to be linked to the low phytate quantitative trait loci (QTL) (Walker et al., 2006). Two loci were mapped to two different linkage groups *Gm03*(Lg-N) and *Gm19*(Lg-L). Mapping the

genes for low phytate was completed in a cross of ‘Boggs-RR’ (Normal Phytate-NP) x CX1834-1-2 (Low Phytate-LP). A total of 226 F₂ individuals from six separate F₁ plants were used. Three different approaches were used to locate the low phytate loci: i) bulked segregant analysis (BSA) (Michelmore et al., 1991), ii) a modified BSA, and iii) whole genome scan of a subset of 94 individuals. In the BSA and modified BSA methods, 180 SSR markers were used to test for polymorphisms between the two parents. In the whole genome scan procedure 318 SSR markers were used to test for polymorphisms. Polymorphic markers were spaced every 15 to 20 cM along each LG according to the integrated linkage maps of Cregan et al (1999) and Song et al (2004). Marker data was then used to locate areas of the genome previously found to be associated with differences in P_i. SSR marker Satt237 on *Gm03*(Lg-N) was found to be associated with seed P_i levels. Multiple regression analysis was used to determine the existence of a single phytate locus at this location. The highest LOD scores 25.5 obtained from interval mapping (IM) confirmed that the low phytate locus was near Satt 237 and most likely in the area between Satt339 and Satt237 (Walker et al., 2006). A second phytate QTL was located on *Gm19*(Lg-L) near Satt527 and Satt561. The IM approach indicated an LOD of 6.4. Composite interval mapping did not detect any additional loci associated with phytic acid. These two loci were confirmed in two separate populations. A population from the Univ. of Tennessee ‘5601T’ x CX1834-1-2 and a population from the Univ. of Georgia ‘Benning-RR’ x CX1834-1-2 were used. The two populations that were used confirmed the previous results. Homozygous CX1834-1-2 alleles present at Satt237 on *Gm03*(Lg-N) or Satt527 on *Gm19*(Lg-L) achieved the highest P_i concentrations.

The two QTL mapped in this study are believed to be the *pha1* and *pha2* loci designated by conventional segregation analyses (Oltmans et al., 2004; Walker et al., 2006).

The recessive alleles of *pha1* and *pha2* QTLs are required to have phytate levels equivalent to the low phytate soybean line CX1834-1-2. Walker et al. (2006) found that the QTL on *Gm03*(Lg-N) and *Gm19*(Lg-L) acted epistatically. They found that if the Boggs allele was present at Satt237 on *Gm03*(Lg-N) then the genotype at the *Gm19*(Lg-L) locus had little to no effect on seed P_i levels. Substituting a CX1834-1-2 allele at the *Gm19*(Lg-L) locus when Satt237 was either homozygous or heterozygous for the CX1834-1-2 allele resulted in an increase in P_i levels. When both loci (Satt 237, Satt561) were homozygous for the CX1834-1-2 allele, P_i levels were similar to the CX1834-1-2 parent, while heterozygosity at either locus resulted in intermediate levels of P_i (Walker et al., 2006). These same results were confirmed in studies conducted at the Univ. of Tennessee in the cross of 5601T x CX1834-1-2 and S97-1688 x CX1834-1-2 (Scaboo et al., 2009).

The two QTL found in soybean that condition for the low phytate have been examined in three different studies.(Gao et al., 2008; Scaboo et al., 2009; Walker et al., 2006). The mapping population Boggs-RR x CX1834-1-2 showed that SSR marker Satt237 on *Gm03*(Lg-N) accounted for 40% of the variation in seed P_i while the SSR marker Satt561 on *Gm19*(Lg-L) accounted for 11% of variation in seed P_i , and the interaction of the two markers on *Gm03*(Lg-N) and *Gm19*(Lg-L) accounted for an additional 8% of variation in seed P_i . In the Benning-RR x CX1834-1-2 confirmation population these markers were found to account for 81% of variation in

seed Pi content when the markers were fixed for the CX1834-1-2 parent genotype (Walker et al., 2006). In experiments conducted at the Univ. of Tennessee a cross of 5601T x CX1834-1-2 was evaluated for P_i content in the seed. SSR markers Satt237 on *Gm03*(Lg-N) and Satt561 on *Gm19*(Lg-L) were both found to be associated with seed P_i levels. Satt237 was found to account for 30% of variation in seed P_i while Satt561 was shown to account for 8.2% (Scaboo et al., 2009). In a study conducted at Virginia Tech Univ. the low phytate QTLs were validated in a cross of CX1834-1-6 x V99-3337. SSR markers Satt527 and Satt561 on *Gm19*(Lg-L) were found to contribute 12% of the total phytate variation while another SSR marker Satt237 and Satt339, accounted for 28% of the total variation in phytate content (Gao et al., 2008). Gao et. al. (2008) confirmed the interaction between the two QTLs reported by Walker et al. (2006) on *Gm03*(Lg-N) and *Gm19*(Lg-L) that accounted for approximately 12.9% total phytate variation in their population.

REFERENCES

- Adeola, O. 1995. Digestive utilization of minerals by weanling pigs fed copper-and phytase-supplemented diets. *Can. J. Anim. Sci* 75:603-610.
- Akem, C.N., and K.E. Dashiell. 1994. Effect of planting date on severity of frogeye leaf spot and grain yield of soybeans. *Crop Protection* 13:607-610.
- Akkaya, M.S., A. A. Bhagwat and P. B. Cregan. 1992. Length Polymorphisms of Simple Sequence Repeat DNA in Soybean. *Genetics* 132:1131-1139.
- Akkaya, M.S., R. C. Shoemaker, J. E. Specht, A. A. Bhagwat, and P. B. Cregan. 1995. Integration of Simple Sequence Repeat DNA Markers into a Soybean Linkage Map. *Crop Sci.* 35:1439-1445.
- Anderson, B.P., and W.R. Fehr. 2008. Seed source affects field emergence of low-phytate soybean lines. Submitted.
- Bilyeu, K.D., P. Zeng, P. Coello, Z.J. Zhang, H.B. Krishnan, A. Bailey, P.R. Beuselinck, and J.C. Polacco. 2008. Quantitative Conversion of Phytate to Inorganic Phosphorus in Soybean Seeds Expressing a Bacterial Phytase. *Plant Physiol.* 146:468-477.
- Chen Jr, P.S., T.Y. Toribara, and H. Warner. 1956. Microdetermination of Phosphorus. *Analytical Chemistry* 28:1756-1758.
- Choi I. K., D.L.H., L. K. Matukumalli, Q. Song, J. M. Chaky, C. V. Quigley, K. Chase, K. G. Lark, R. S. Reiter, M. Yoon, E. Hwang, S. Yi, N. D. Young, R. C. Shoemaker, C. P. van Tassell, J. E. Specht and P. B. Cregan. 2007. A Soybean Transcript Map: Gene Distribution, Haplotype and Single-Nucleotide Polymorphism Analysis. *Genetics* 176:685-696.

- Cregan, P.B., T. Jarvik, A. L. Bush, R. C. Shoemaker, K. G. Lark, A. L. Kahler, N. Kaya, T. T. VanToai, D. G. Lohnes, J. Chung, and J. E. Specht. 1998. An Integrated Genetic Linkage Map of the Soybean Genome. *Crop Sci.* 39.
- Cregan, P.B., T. Jarvik, A. L. Bush, R. C. Shoemaker, K. G. Lark, A. L. Kahler, N. Kaya, T. T. VanToai, D. G. Lohnes, J. Chung, and J. E. Specht. 1999. An Integrated Genetic Linkage Map of the Soybean Genome. *Crop Sci.* 39:1464-1490.
- Dashiell, K.E., and C.N. Akem. 1991. Yield losses in soybeans from frogeye leaf spot caused by *Cercospora sojina*. *Crop Protection* 10:465-468.
- deMOOY, C.J., J. Pesek, E. Spaldon. 1973. Mineral Nutrition, p. 285-291, *In* B. E. Caldwell, ed. SOYBEAN: Improvement, Production, and Uses.
- Erdman, J.W. 1979. Oilseed phytates: Nutritional implications. *Journal of the American Oil Chemists' Society* 56:736-741.
- Fehr, W.R., and C.E. Caviness. 1977. Stages of soybean growth and development. *Iowa Coop. Ext. Serv. Spec Rep.* 80.
- Gao, Y., R.M. Biyashev, M.A.S. Maroof, N.M. Glover, D.M. Tucker, and G.R. Buss. 2008. Validation of Low-Phytate QTLs and Evaluation of Seedling Emergence of Low-Phytate Soybeans. *Crop Sci* 48:1355-1364.
- Gao, Y., C. Shang, M.A.S. Maroof, R.M. Biyashev, E.A. Grabau, P. Kwanyuen, J.W. Burton, and G.R. Buss. 2007. A Modified Colorimetric Method for Phytic Acid Analysis in Soybean. *Crop Sci* 47:1797-1803.
- Grau, C.R., A.E. Dorrance, J. Bond, and J.S. Russin. . 2004. Fungal diseases. *In* H.R. Boerma and J.E. Specht (ed) Soybeans: Improvement, production, and

- uses. 3rd ed. Agron. Mongr. 16, ASA, CSSA, and SSSA, Madison, WI.:679-763.
- Hulke, B.S., W.R. Fehr, and G.A. Welke. 2004. Agronomic and Seed Characteristics of Soybean with Reduced Phytate and Palmitate. *Crop Science* 44:2027.
- Israel, D.W., P. Kwanyuen, J.W. Burton, and D.R. Walker. 2007. Response of Low Seed Phytic Acid Soybeans to Increases in External Phosphorus Supply. *Crop Science* 47:2036.
- Larson, S.R., K.A. Young, A. Cook, T.K. Blake, and V. Raboy. 1998. Linkage mapping of two mutations that reduce phytic acid content of barley grain. *TAG Theoretical and Applied Genetics* 97:141-146.
- Lei, X.G., and J.M. Porres. 2003. Phytase enzymology, applications, and biotechnology. *Biotechnology Letters* 25:1787-1794.
- Liu, X., Q. Yang, F. Lin, L. Hua, C. Wang, L. Wang, Q. Pan. . 2007. Identification and fine mapping of Pi39(t), a major gene conferring the broad-spectrum resistance to *Magnaporthe oryza*. *Epub* 278:403-410.
- Meis, S.J., W.R. Fehr, and S.R. Schnebly. 2003. Seed Source Effect on Field Emergence of Soybean Lines with Reduced Phytate and Raffinose Saccharides. *Crop Science* 43:1336.
- Mengistu, A., N.C. Kurtzweil, and C.R. Grau. 2002. First Report of Frogeye Leaf Spot (*Cercospora soja*) in Wisconsin. *Plant Disease* 86:1272-1272.
- Mian, M.A., T. Wang, D.V. Phillips, J. Alvernaz, and H.R. Boerma. 1999. Molecular Mapping of the *Rcs3* Gene for Resistance to Frogeye Leaf Spot in Soybean. *Crop Sci.* 39:1687-1691.

- Mian, M.A.R., A.M. Missaoui, D.R. Walker, D.V. Phillips, and H.R. Boerma. 2008. Frogeye Leaf Spot of Soybean: A Review and Proposed Race Designations for Isolates of *Cercospora sojina* Hara. *Crop Science* 48:14.
- Michelmore, R.W., I. Paran, and R.V. Kesseli. 1991. Identification of Markers Linked to Disease-Resistance Genes by Bulk Segregant Analysis: A Rapid Method to Detect Markers in Specific Genomic Regions by Using Segregating Populations. *Proceedings of the National Academy of Sciences of the United States of America* 88:9828-9832.
- Missaoui, A.M., B.K. Ha, D.V. Phillips, and H.R. Boerma. 2007a. Single Nucleotide Polymorphism Detection of the *Rcs3* Gene for Resistance to Frogeye Leaf Spot in Soybean. *Crop Sci.* 47:1681-1690.
- Missaoui, A.M., D.V. Phillips, and H.R. Boerma. 2007b. DNA Marker Analysis of 'Davis' Soybean and Its Descendants for the *Rcs3* Gene Conferring Resistance to *Cercospora sojina*. *Crop Sci.* 47:1263-1270.
- Oltmans, S.E., W.R. Fehr, G.A. Welke, and S.R. Cianzio. 2004. Inheritance of Low-Phytate Phosphorus in Soybean. *Crop Sci* 44:433-435.
- Oltmans, S.E., W.R. Fehr, G.A. Welke, V. Raboy, and K.L. Peterson. 2005. Agronomic and Seed Traits of Soybean Lines with Low-Phytate Phosphorus. *Crop Sci* 45:593-598.
- Phillips, D.V. 1999. Frogeye Leaf Spot, *In* G. L. Hartman, et al., eds. *Compendium of Soybean Diseases*, 4th ed. American Phytopathological Society, St. Paul, MN.
- Phillips, D.V., and H.R. Boerma. 1981. *Cercospora sojina* Race 5: A Threat to Soybeans in the Southeastern United States. *Phytopathology* 71:334-336.

- Probst, A.H., K. L. Athow, and F. A. Laviolette. 1965. Inheritance of Resistance to Race 2 of *Cercospora sojina* in Soybeans. *Crop Sci.* 5:332.
- Raboy, V., and D.B. Dickinson. 1987. The Timing and Rate of Phytic Acid Accumulation in Developing Soybean Seeds. *Plant Physiol* 85:841-844.
- Raboy, V., and D. Dickinson. 1993. Phytic Acid Levels in Seeds of *Glycine max* and *G. soja* as Influenced by Phosphorus Status. *Crop Sci.* 33:1300-1305.
- Raboy, V., P.F. Gerbasi, K.A. Young, S.D. Stoneberg, S.G. Pickett, A.T. Bauman, P.P.N. Murthy, W.F. Sheridan, and D.S. Ertl. 2000. Origin and Seed Phenotype of Maize low phytic acid 1-1 and low phytic acid 2-1. *Plant Physiol.* 124:355-368.
- Ross, J.P. 1968. Additional Pysiological Races of *Cercospora sojina* on Soybeans in North Carolina. *Phytopathology* 58:708-709.
- Scaboo, A.M., V.R. Pantalone, D.R. Walker, H.R. Boerma, D.R. West, F.R. Walker, and C.E. Sams. 2009. Confirmation of Molecular Markers and Agronomic Traits Associated with Seed Phytate Content in Two Soybean RIL Populations. *Crop Sci* 49:426-432.
- Song, Q.J., L.F. Marek, R.C. Shoemaker, K.G. Lark, V.C. Concibido, X. Delannay, J.E. Specht, and P.B. Cregan. 2004. A new integrated genetic linkage map of the soybean. *TAG Theoretical and Applied Genetics* 109:122-128.
- Spear, J.D., and W.R. Fehr. 2007. Genetic Improvement of Seedling Emergence of Soybean Lines with Low Phytate. *Crop Sci* 47:1354-1360.
- Thompson, D.B., and J.W. Erdman. 1982. Phytic Acid Determination in Soybeans. *Journal of Food Science* 47:513-517.

- Tisdale, S.L., W. L. Nelson, J. D. Beaton, and J. L. Havlin. 1993. Soil Fertility and Fertilizers. Fifth ed. Prentice-Hall.
- Walker, D.R., A.M. Scaboo, V.R. Pantalone, J.R. Wilcox, and H.R. Boerma. 2006. Genetic Mapping of Loci Associated with Seed Phytic Acid Content in CX1834-1-2 Soybean. *Crop Science* 46:390.
- Wilcox, J.R., G.S. Premachandra, K.A. Young, and V. Raboy. 2000. Isolation of High Seed Inorganic P, Low-Phytate Soybean Mutants. *Crop Sci* 40:1601-1605.
- Wrather, J.A., and S.R. Koenning. 2006. Estimates of disease effects on soybean yields in the United States 2003 to 2005. *Journal of Nematology* 38:173.
- Yang, X.B., M.D. Uphoff, and S. Sanogo. 2001. Outbreaks of Soybean Frogeye Leaf Spot in Iowa. *Plant Disease* 85:443-443.
- Zhu Y. L., Q.J.S., D. L. Hyten, C. P. Van Tassell, L. K. Matukumalli, D. R. Grimm, S. M. Hyatt, E. W. Fickus, N. D. Young, and P. B. Cregan. 2003. Single-Nucleotide Polymorphisms in Soybean. *Genetics* 163:1123-1134.

CHAPTER III

**GENETIC MAPPING OF FROGEYE LEAFSPOT RESISTANCE IN FIVE
SOYBEAN PLANT INTRODUCTIONS¹**

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ABSTRACT

Frogeye leaf spot (FLS), caused by *Cercospora sojina*, is a disease found on soybean (*Glycine max*) in many areas of the world. Soybean yield loss attributed to FLS has been shown to be up to 60% under hot, humid conditions. Resistant cultivars have proven to be the most reliable control methods of FLS. The *Rcs3* resistance gene was previously discovered in the soybean cultivar Davis and genetically maps to chromosome 16 (*Gm16*;Lg-J) near Satt244. *Rcs3* is currently resistant to all known isolates of *C. sojina*. Five plant introductions (PIs) from China (PI594619, PI594661, PI594662A, PI594774, and PI594891) were found to have broad resistance to FLS. The objective of this study was to determine if these FLS-resistant PIs contain unique genes for resistance to FLS. F₂ populations created from the PIs crossed to Davis segregated 15 resistant to 1 susceptible, indicating the PIs resistance was different than *Rcs3*. F₂ populations were created from the crosses of 'Blackhawk' to the five resistant PIs and screened for FLS resistance in the greenhouse. Bulk segregant analysis (BSA) was utilized and analyzed using the 1,536 SNP GoldenGate[®] assay. Results indicate resistance genes in PI594619 and PI594662A were found near Satt501 on *Gm18* (Lg-G) and near Satt547 and Satt244 on *Gm16* (Lg-J), respectively. A resistance gene in PI594661 was found near Satt244 on *Gm16* (Lg-J). The resistance gene in PI594774 and PI594891 mapped to *Gm13* (Lg-F) near Satt114. These results indicate that four of the plant introductions (PI594619, PI594662A, PI594774, and PI594891) contain unique resistance genes. Further studies will need to be conducted to determine if the resistance genes that mapped near the *Rcs3* locus in PI594619, PI594661, and PI594662A are different from *Rcs3*.

INTRODUCTION

Frogeye leaf spot (FLS), caused by *Cercospora sojina*, is a disease of economic importance worldwide in soybean, *Glycine max*, producing countries. Frogeye leaf spot is typically found in warm humid growing conditions associated with southern U.S. soybean producing states, but has recently been discovered in the midwestern USA (Athow and Probst, 1952; Mengistu et al., 2002; Yang et al., 2001). Frogeye leaf spot symptoms usually develop on foliage but can also infect seeds, pods, and stems (Phillips, 1999). Lesions are circular to angular spots on the leaves that vary in size from 1 to 5 mm in diameter. Lesions begin as water soaked spots with or without lighter centers and develop into brown spots with dark reddish brown margins. Multiple lesions can coalesce and to form larger irregular spots. A blighting phase can occur when lesions are numerous enough to cover about 30% of the leaf area, and leaves can wither and fall prematurely (Phillips, 1999).

Soybean seed yield loss caused by FLS is caused primarily through the reduction of photosynthetic leaf area caused by necrotic lesions and premature leaf defoliation. Yield losses have been reported to have been as high as 60% in Nigeria (Dashieil and Akem, 1991). In the southeastern USA yield losses of up to 30% have been observed in studies in which four pairs of FLS susceptible vs. resistant near-isogenic lines were evaluated (Mian, 1998). In Indiana, plots that contained FLS yielded 17 to 21% lower than plots which were disease free (Laviolette, 1970).

The Soybean Genetics Committee currently recognizes three single genes that condition resistance to *C. sojina*. *Rcs1* was discovered in soybean cultivar 'Lincoln' and was shown to be resistant to *C. sojina* race 1 (Athow and Probst, 1952). *Rcs2* resistance

gene was found in 'Kent' and conditioned resistance to race 2 (Athow, 1962). *Rcs3* from the cultivar Davis was found to condition resistance to race 5 and all other known races of *C. sojae* in the USA (Boerma, 1983; Phillips and Boerma, 1981) as well as to all known Brazilian isolates (Yorinori, 1992).

The use of DNA markers has been shown to be a useful in detecting single loci that confer resistance to economically important soybean diseases in the USA (Soybase; <http://www.soybase.org>). Recently, the *Rpp3* locus that provides resistance to Asian soybean rust (*Phakopsora pachyrizi* Syd.) was detected using the 1,536 single nucleotide polymorphism (SNP) GoldenGate assay and bulked segregant analysis (BSA) (Michelmore et al., 1991; Hyten et al., 2009). The *Rcs3* gene identified in Davis was genetically mapped to *Gm16* (Lg-J) (Mian, 1999). DNA markers have also been utilized to locate quantitative trait loci (QTL) for diseases in soybean. Examples include soybean cyst nematode (SCN) (*Heterodera glycines* Ichinohe), (Concibido et al., 2004; Concibido et al., 1994; Cregan et al., 1999; Webb et al., 1995) and reniform nematode (*Rotylenchus reniformis* Linford and Oliveira) (Ha et al., 2007). Webb et al. (1995) detected three QTL that conferred resistance to SCN race 3 in the resistant soybean line PI 437654. Ha et al. (2007) detected three QTLs that conditioned resistance to reniform nematode in the same soybean line PI 437654.

The use of cultivars that contain genetic resistance to FLS have been found to be the most cost-effective and environmentally friendly method for controlling this disease. However the effectiveness of deployed resistance genes depends on the races of the pathogen that are present. Due to the pathogen's ability to overcome single resistance genes (R-genes), the deployment of single resistance genes can select for prevalence of

new pathogenic races (Browning and Frey, 1969; Horsfall, 1972). *C. soja* is a pathogen that has been able to overcome host resistance genes. In the late 1950s *C. soja* race 2 appeared (Athow, 1962), in the mid 1960s races 3 and 4 appeared (Ross, 1968) and race 5 appeared in the late 1970s (Phillips and Boerma, 1981). The appearance of these new fungal races demonstrates the need for multiple resistance genes.

In the late 1990s, 608 germplasm lines from the southern provinces of the People's Republic of China were obtained and made available to researchers by Dr. R.L. Nelson (curator of USDA-ARS Soybean Germplasm collection, Urbana, IL) (Harris, 2003). These 608 lines were screened by Dr. D.V. Phillips with multiple isolates of *C. soja*. From this research PI594619, PI594661, PI594662A, PI594774, and PI594891 were found to possess resistance to a broad spectrum of *C. soja* isolates. The objectives of this research were to: (i) determine if resistance genes in these five PIs were different from *Rcs3*, and (ii) genetically map the location of the resistance genes in the five Chinese germplasm lines.

MATERIALS AND METHODS

Mapping Population Development

Five Chinese plant introductions (PI594619, PI594661, PI594662A, PI594774, and PI594891) were selected from 608 soybean accessions based on their resistant reaction to 15 unique *C. sojae* isolates contained in the Univ. of Georgia collection (Mian et al., 2008). These five PIs were then crossed as male parents to the FLS resistant cultivar Davis in 2002 and FLS susceptible cultivar Blackhawk in 2003 at the Univ. of Georgia Plant Science Farm near Watkinsville, GA. F₂ seeds from the F₁ plants were produced in a greenhouse on the Univ. of Georgia campus in Athens, GA in 2003 or 2004.

FLS Greenhouse Screen

PI594619, PI594661, PI594662A, PI594774, PI594891, Davis, Blackhawk, and F₂ plants from crosses of the five Chinese PIs and Blackhawk (Table 3.1) were evaluated in a greenhouse at the Univ. of Georgia Griffin campus as described previously (Mian, 1999; Mian et al., 2008; Missaoui, 2007; Phillips and Boerma, 1981). Plants were grown in 10-cm pots on a greenhouse bench. Seedlings were inoculated at the V2 to V3 stage of development (Fehr, 1977). A single trifoliolate of each plant was inoculated with various races of *C. sojae* (Table 3.1) by atomizing a conidial suspension of approximately 6.0×10^3 spores mL⁻¹. Inoculated plants were placed into a clear plastic bag for 48 h to maintain a high relative humidity. Disease ratings were made 14 d after inoculation. The FLS reaction was scored as a qualitative trait (i.e. susceptible vs. resistant) for all populations except PI594662A. This population was rated as susceptible, intermediate, or resistant. Plants were classified as susceptible when lesions were predominately large and spreading with light centers and dark margins. Plants were classified as resistant

when they showed no lesions or predominately small lesions without clearly defined centers (Mian et al., 2008). Some F_2 plants (primarily the susceptible F_2 plants) were transplanted to 25-cm pots and grown to maturity. At maturity seed from these plants were harvested to create $F_{2,3}$ lines (F_3 families).

To confirm the FLS reactions of the F_2 plants, the corresponding F_3 families were also evaluated for FLS reactions. The screening of F_3 families was accomplished in the same manner as the individual F_2 plants, except that a minimum of 8 plants were evaluated for each family when available. The F_2 plant FLS reactions were confirmed based on the reaction of the corresponding F_3 family. An F_2 plant producing progeny with all susceptible plants was classified as susceptible, if a F_2 plant produced F_3 progeny with all resistant plants or a mixture of resistant and susceptible plants that F_2 plant was classified as resistant.

Mapping of Resistance Genes

DNA was extracted from the leaves of a single trifoliolate from the F_2 plants that were evaluated in the greenhouse. Leaves were placed in desiccators for 3 d and DNA was extracted using a modified CTAB (hexadecyltrimethylammonium acid) protocol (Keim, 1988). Susceptible DNA bulks were created as described previously (Hyten et al., 2009; Michelmore et al., 1991) with each bulk containing approximate equal amounts of DNA from 10 to 12 F_2 plants. The inclusion of only susceptible plants in a bulk was based on the assumption that the resistance gene in the resistant Chinese PIs was dominant (Table 3.1). When possible, multiple susceptible bulks were created and analyzed to provide replication of the BSA assay (Table 3.1).

A total of 1,536 SNP markers were analyzed on the susceptible bulks, Blackhawk, and the appropriate resistant PI with the Universal Soybean Linkage Panel 1.0 (USLP 1.0) (Hyten et al., 2010), using the GoldenGate Assay on the Illumina BeadStation 500G (Illumina, San Diego, CA) (Hyten et al., 2008). The analysis of these markers resulted in the identification of candidate regions harboring the PI's resistance gene(s).

Simple Sequence Repeat (SSR) markers developed at the Beltsville Agricultural Research Center (BARC) (Choi et al., 2007; Song, 2010) were evaluated in the genomic region surrounding the candidate genomic region identified by BSA to discover SSR markers which were polymorphic between Blackhawk and the resistant PI. SSR markers that were polymorphic in the candidate region were assayed on DNA from individual F₂ plants in each mapping population (Table 3.1). SSR genotyping was performed as described by Cregan et al. (1999) using a 96-well Bio-Rad Tetrad 2 thermocycler (Bio-Rad, Hercules, Ca) for DNA amplification. The size of the DNA amplicons were determined using an ABI 3730 DNA analyzer (Applied Biosystems, Foster City, CA). SNP markers were also identified in the candidate region using the soybean genome sequence (Schmutz et al., 2010) (www.phytozome.net) and Soybase, an USDA-sponsored soybean database (www.soybase.org). SNP markers were assayed by melting curve analysis as described previously (Ha, 2008) using a LightCycler 480 instrument (Roche Applied Science, Indianapolis, IN).

JoinMap 4 (Van Ooijen, 2006) was used with the Kosambi mapping function to create genetic linkage maps and to map the location of the resistance gene(s) in the five resistant PIs. Single-factor analysis of variance (SF-ANOVA) was used to determine the significance of each SSR or SNP marker using PROC GLM. PROC MEANS was also

used to determine SSR or SNP (SAS, 2004) genotypic class means. For this analysis the resistance reaction was given a value of zero and the susceptible reaction a value of one. For the Blackhawk x PI594662A population, where an intermediate phenotype was found the resistant reaction was scored as 1.0, the intermediate reaction as 2.0, and the susceptible reaction as 3.0.

RESULTS

Blackhawk x PI594619

All 26 Blackhawk plants were susceptible to race 8 of *C. sojae* while all 26 PI594619 and Davis plants were resistant. The 157 F₂ plants of the population of Blackhawk x PI594619 segregated 144 resistant and 13 susceptible plants which fit a 15:1 (resistant:susceptible) ratio ($P = 0.34$). These results indicate two genes were conditioning resistance to FLS in PI594619 which exhibited duplicate dominant epistasis (Fehr, 1987). Evaluations of F₃ families from 13 individual susceptible F₂ plants confirmed the susceptible phenotypes of the original F₂ plants.

The bulk DNA sample that was used for BSA included DNA from 11 different susceptible F₂ plants. The results from the 1,536 SNP GoldenGate assay identified 10 SNPs with similar alleles in the susceptible bulk and Blackhawk that were different from the resistant parent PI594619. These SNP markers were located on two different chromosomes. On *Gm16* (Lg-J), three SNPs were identified by BSA, between 44 and 71 cM on the USDA Soybean Consensus Map 4.0 (Hyten et al., 2010). On *Gm18* (Lg-G), seven SNPs were identified by BSA, between 38 and 47 cM on the USDA Soybean Consensus Map.

Seven polymorphic SSR markers and one polymorphic SNP were mapped in the F₂ population on *Gm16* (Lg-J) while five polymorphic SSRs and two polymorphic SNPs were mapped on *Gm18* (Lg-G). The SF-ANOVA for the FLS phenotype and the marker genotypes on *Gm16* (Lg-J) placed the FLS resistance gene, *Rcs3*_(PI594619) between Satt547 and SNP 045099 (Fig. 3.1a). Based on SF-ANOVA between the FLS phenotype and genotypes of markers on *Gm18* (Lg-G), the *Rcs*_(PI594619) resistance gene was

mapped between S4_3180K (Ha, et al., 2010) and Satt501 (Fig. 3.1b). The linkage map created by the F₂ mapping population of Blackhawk x PI594619 is in general agreement with the USDA Soybean Consensus Map for marker order and recombination distances (Fig. 3.1a and 3.1b)

Blackhawk x PI594661

All 26 Blackhawk plants were susceptible to race 11 of *C. sojae* while 24 out of 24 plants of PI594661 and all 26 Davis plants were resistant to the fungus. The 127 F₂ plants of the cross of Blackhawk x PI594661 segregated 95 resistant to 32 susceptible and fit a 3 resistant to 1 susceptible model ($P = 0.95$; Table 1). Evaluations of 43 F₃ families found four F₂ plants that were misclassified in their initial scoring.

Two susceptible bulk DNA samples were created for BSA with each containing DNA from 12 different susceptible F₂ plants. The results from the GoldenGate assay of the two susceptible bulks and parents identified three SNPs markers on *Gm16* (Lg-J) where the susceptible bulk and Blackhawk had a different base than PI594661, the resistant parent. These SNPs were located between 73 and 88 cM on *Gm16* (Lg-J) of the USDA Soybean Consensus Map 4.0 (Hyten et al., 2010).

Five polymorphic SSR markers on *Gm16* (Lg-J) were mapped in the F₂ population in the 73 to 88-cM region of *Gm16*(Lg-J). The dominant resistant gene, Rcs3_(PI594661) was mapped on *Gm16* (Lg-J) between SSR marker BARC16_1189 and Satt431 (Fig. 3.2).

Blackhawk x PI594662A

All 24 Blackhawk plants that were inoculated with race 17 of *C. sojae* were susceptible while all 26 plants each of PI594662A and Davis were resistant. The F₂ population of

Blackhawk x PI594662A segregated 59 resistant, 42 intermediate, and 38 susceptible plants when inoculated with *C. sojae* race 17. Evaluations of the plants in F₃ families resulted in nine F₂ plants identified as being misclassified. This resulted in a final F₂ segregation of 68 resistant, 42 intermediate, and 29 susceptible F₂ plants (Table 3.1).

The BSA of the two susceptible bulks, consisting of DNA from 10 and 11 different F₂ plants, and parents did not show any definitive results for this population. As a second approach, we ran markers that were located near FLS resistance loci identified in other populations. Six polymorphic SSR markers and one polymorphic SNP marker were used to create the genetic linkage map for *Gm18* (Lg-G) while four polymorphic SSR markers were used for *Gm16* (Lg-J). Some map expansion was observed between SSR markers Sat_131 and Satt501 on *Gm18* (Lg-G). The region was expanded by approximately 12 cM when compared to the consensus map.

We found associations of FLS reactions with markers on *Gm18* (Lg-G) and *Gm16* (Lg-J). These resistance loci were near positions similar to that in the Blackhawk x PI594619 population (Fig. 3.1a and 3.1b). The SF-ANOVA using the DNA markers as independent variables and assigning values of 1.0 for resistance, 2.0 for intermediate, and 3.0 for susceptible as dependent variables showed the most likely location of the Rcs3_(PI594662A) gene on *Gm16* (Lg-J) was between Sat_366 and Satt244. Analysis of *Gm18* (Lg-G) markers found the most likely location of Rcs_(594662A) was near SNP marker S4-3180K. Additional analysis showed additive gene effects for Satt244 on *Gm16* (Lg-J) and S4_3180K on *Gm18* (Lg-G) (Table 3.2). Further analysis showed that there was an additive by additive marker effect for these two loci (data not shown). Means of the marker genotypes show that when the homozygous PI594662A alleles are

present at S4_3180K complete resistance is observed (Table 3.2). When only Blackhawk alleles are present at the Satt244 and S4_3180K loci all plants were found to be susceptible (Table 3.2).

Blackhawk x PI594774

Race 17 of *C. sojae* was used to inoculate the 195 plants of the F₂ population of Blackhawk x PI594774. Blackhawk (34 plants) was susceptible and PI594774 (34 plants) and Davis (34 plants) were resistant. After correcting the F₂ segregation ratio for the nine F₂ plants that were misclassified (based on F₃ family reactions) the F₂ population of Blackhawk x PI594774 fit a 3:1 ratio (139 resistant : 56 susceptible) (Table 3.1). The results from the analysis of DNA from the parents and two susceptible bulks (12 plants each) using the 1,536 GoldenGate assay identified nine SNPs being similar for the two susceptible bulks and Blackhawk on *Gm13* (Lg-F) between 50 and 75 cM on the USDA Soybean Consensus Map 4.0 (Hyten et al., 2010).

Seven polymorphic SSR markers and one polymorphic SNP marker on *Gm13* (Lg-F) were used for genotyping the population. The Rcs_(PI594774) resistance locus was mapped as a dominant gene with these SSR and SNP markers. Genetic mapping places the Rcs_(PI594774) gene for resistance between SSR markers Satt663 and Satt114 (Fig. 3.4). The genetic map created using the F₂ population expanded by approximately 15 cM between Satt663 and Satt114.

Blackhawk x PI594891

Race 17 of *C. sojae* was used to inoculate the F₂ and F_{2,3} population of Blackhawk x PI594891. A total of 36 plants of Blackhawk were susceptible while PI594891 (36 plants) and Davis (36 plants) were resistant to race 17. After correcting the

F₂ segregation ratio for the one F₂ plant that was found misclassified based on evaluation of F₃ families, the F₂ population of Blackhawk x PI594891 segregated 87 resistant to 23 susceptible plants (3 resistant :1 susceptible ratio) when the plants were inoculated with the race 17 (Table 3.1).

The results from the BSA with the GoldenGate analysis were inconclusive. SSR markers that were found to be significant in other populations were ran on this population and significant areas were identified by SF-ANOVA as described above for the Blackhawk x PI594662a population on *Gm13* (Lg-F).

SSR markers that were linked to the resistance locus Rcs_(PI594774) on *Gm13* (Lg-F) in the Blackhawk x PI594774 population were found to be significant in this population. Six SSR markers were found to be polymorphic on *Gm13*(Lg-F). The Rcs_(PI594891) resistance locus was mapped as a dominant gene between SSR markers Satt114 and Sct_033 (Fig. 3.5).

DISCUSSION

Rcs3 has shown to be resistant to all known races of the pathogen *C. sojae* (Missaoui, 2007; Phillips and Boerma, 1982). The possibility of *Rcs3* being ineffective against new *C. sojae* races underscores the need for additional sources of resistance to FLS (Mian et al., 2008). The five selected Chinese PIs all provided broad resistance to 15 isolates from the *C. sojae* collection at the Univ. of Georgia. The 15 *C. sojae* isolates used to identify these five PIs were collected from North America, South America, and Asia and represented a wide range of the diversity of the fungus (D.V. Phillips, personal communication). Previous unpublished data found F₂ populations of Davis x each of these five resistant PIs (Table 3.1) segregated 15 resistant to 1 susceptible, indicating that the source of resistance from the Chinese PIs contain resistance genes other than *Rcs3*. Our results support these findings for PI594619, PI594662A, PI594774, and PI594891. The results from this study for the F₂ population of Blackhawk x PI594661 place the resistance gene in the same genomic region as the previously reported *Rcs3* locus. It is possible the susceptible F₂ plants from the Davis x PI594661 were misclassified. The phenotypes of these susceptible F₂ plants were not confirmed in the F₃ generation.

Identification of putative genomic positions for resistance genes has previously been successful using the GoldenGate assay in combination with BSA (Hyten et al., 2009). In this experiment the use of the GoldenGate assay along with BSA identified putative genomic locations of these new FLS resistance loci in three of the five populations evaluated.

The resistance genes *Rcs*_(PI594774) and *Rcs*_(PI594891) that mapped to *Gm13* (Lg-F) and *Rcs*_(PI594619) and *Rcs*_(PI594662A) that mapped to *Gm18* (Lg-G) provide unique sources of resistance to FLS compared to what has been previously described (Mian et al., 2008). The FLS resistance gene(s) from PI594619, PI594661, and PI594662A mapped to *Gm16* (Lg-J) near *Rcs3* (Mian, 1999). The 15 *C. sojae* isolates used for phenotyping the Chinese PIs did not distinguish differences between the sources of resistance. Thus, these resistance genes may be *Rcs3*, a new allele at *Rcs3*, or a tightly linked resistance locus.

The pedigrees of the selected germplasm lines are unavailable therefore the relationships among the five FLS resistant PIs are unknown. However, PI594619, PI594661, and PI594662A are from the Guizhou province of China, while PI594774 is from Guangxi, and PI594891 is from the Yunnan province. This geographical information along with the results from this study would indicate the possibility of the genes for FLS resistance on *Gm16* (Lg-J) and *Gm18* (Lg-G) in PI594619 and PI594662A being the same along with and the resistance genes on *Gm13* (Lg-F) in PI594774 and PI594891.

The *Rcs3*_(PI594662A) resistance gene that was mapped to *Gm16* (Lg-J) is likely not *Rcs3* or the same resistance gene that is found in PI594619 [*Rcs3*_(PI594619)] or in PI594661 [*Rcs3*_(PI594661)]. The PI594662A allele at the *Rcs3*_(PI594662A) locus on *Gm16* (Lg-J) does not provide for disease resistance unless in combination with the *Rcs*_(PI594662A) allele on *Gm18* (Lg-G) (Table 3.2). When the PI594619, PI594661 (data not shown), or the Davis alleles at the *Rcs3* locus (Mian, 1999) were either homozygous or heterozygous at the Satt244 or Satt547 markers all plants were found to

be resistant to FLS. The resistance allele(s) on *Gm16* (Lg-J) from Davis, PI594619, or PI594661 would be more beneficial sources of FLS resistance than the allele from PI594662A since they provide greater levels of resistance to the fungus than that found in PI594662A.

The Rcs_(PI594774) and Rcs_(PI594891) resistance genes mapped to the same genomic region on *Gm13* (Lg-F). The Rcs_(PI594774) gene mapped approximately 13 cM above Satt114, while the Rcs_(PI594891) gene mapped 3 cM below Satt114. It is possible that misclassifications of marker genotypes or FLS phenotypes resulted in the different genomic locations of these genes.

Expansion of the genetic linkage maps was observed in the PI594662A and PI594774 populations. In the PI594774 population map expansion was observed both when the genetic markers were mapped alone or when the FLS resistance locus and DNA markers were mapped (Fig. 3.4). Individual markers were removed from the analysis to determine if incorrect scoring of an individual marker occurred. There was no single marker responsible for the map expansion occurring in these two populations. There is little information known about PI594662A and PI594774, therefore limited conclusions can be drawn from the data presented. The presence of chromosomal arrangements in PI594662A and PI594774 that are not present in the susceptible parent Blackhawk is one possibility. Another possibility would be that there are genes present in PI594662A and PI594774 that would increase the genomic recombination observed in these regions. The data presented indicates that the genotypic or phenotypic scores that were recorded are not the likely causes of the map expansion.

Multiple QTL for pest resistance have been mapped on *Gm13* (Lg-F) between Sat_297 and Sct_033 (43-56 cM, Map 4.0) (Soybase; <http://www.soybase.org>). Resistance to two root-knot nematode species, *Meloidogyne arenaria* (Ma 1-1, 1-2, 1-3, 1-5, and 2-1) and *M. javanica* (Mj 1-3, 1-4, 1-5, 1-6, 1-7, and 2-1), and one fungal disease, Sclerotinia stem rot (Sclero 2-12, 3-9, 4-4, 5-6, 5-7, 6-5, and 6-6), have been reported. Also, QTL for resistance to corn earworm (CEW 2-1) have also been mapped to this region. On *Gm16* (Lg-J) several resistance QTL have been mapped between Sat_350 and Satt547 (60-75 cM, Map4.0). Disease resistance QTL include, brown stem rot (BSR 6-1-6-10) and the *Rcs3* gene for resistance to FLS. Resistance QTL for soybean cyst nematode (SCN 1-2, 5-2, 28-2, 28-4, 29-2, and 29-6) are also located in this area. A cluster of disease resistance QTL is also located on *Gm18* (Lg-G) from Sat_290 to Satt427 (30 cM to 50 cM, Map 4.0). These QTL have been found to provide resistance to soybean cyst nematode (SCN 17-4, 18-5, and 19-4) and Sclerotinia stem rot (Sclero 2-13, 4-5, and 5-8).

Further research will be required to determine if the resistance genes that were mapped near *Rcs3* on *Gm16* (Lg-J), *Rcs3*_(PI594619) and *Rcs3*_(PI594661), are at the *Rcs3* locus or are tightly linked to *Rcs3*. As mentioned above, the PI594662A allele at the *Rcs3*_(PI594662A) locus on *Gm16* (Lg-J) appears unique from the Davis allele at *Rcs3* and the PI594619 and PI594661 alleles at or near the *Rcs3* locus. In addition, it is unclear if the FLS resistance alleles mapped on *Gm13* (Lg-F), *Rcs*_(PI594774) and *Rcs*_(PI594891), or the resistance allele(s) at *Rcs*_(PI594619) and *Rcs*_(PI594662A) on *Gm18* (Lg-G) are unique.

The two resistance genes that were mapped onto *Gm13* (Lg-F) and *Gm18* (Lg-G) are previously unmapped sources of resistance to FLS. These genes have been shown in greenhouse studies to be effective with a broad range of *C. sojae* isolates (15 isolates) contained in the Univ. of Georgia *C. sojae* collection from the USA, Brazil, and China. Soybean breeders can pyramid the newly identified resistance genes on *Gm13* (Lg-F) and *Gm18* (Lg-G) with the Davis allele at the *Rcs3* locus in elite breeding material. The newly discovered resistance gene(s) on *Gm13* (Lg-F) will be the most beneficial new sources of resistance. The single gene identified in PI594774 and PI594891 provided the same level of resistance as compared to the two genes that were discovered in PI594619 and PI594662A. This should provide a broad-based resistance to *C. sojae* and help to prevent the fungus from overcoming a single source of resistance.

REFERENCES

- Athow, K.L., and A.H. Probst. 1952. The inheritance of resistance to frogeye leaf spot of soybeans. *Phytopathology* 42:660-662.
- Athow, K.L., A. H. Probst, C. P. Kurtzman, and F. A. Laviolette. 1962. A Newly Identified Physiological Race of *Cercospora sojina* on Soybean. *Phytopathology* 52:712-714.
- Boerma, H.R., and D.V. Phillips. 1983. Genetic Implication of the Susceptibility of Kent Soybean to *Cercospora sojina*. *Phytopathology* 74:1666-1668.
- Browning, J.A., and K.J. Frey. 1969. Multiline cultivars as a means of disease control. *Annual Review of Phytopathology* 7:355-382.
- Choi, I.Y., D.L. Hyten, L.K. Matukumalli, Q. Song, J.M. Chaky, C.V. Quigley, K. Chase, K.G. Lark, R.S. Reiter, and M.S. Yoon. 2007. A soybean transcript map: gene distribution, haplotype and single-nucleotide polymorphism analysis. *Genetics* 176:685.
- Concibido, V.C., B.W. Diers, and P.R. Arelli. 2004. A Decade of QTL Mapping for Cyst Nematode Resistance in Soybean. *Crop Science* 44:1121-1131.
- Concibido, V.C., R.L. Denny, S.R. Boutin, R. Hautea, J.H. Orf, and N.D. Young. 1994. DNA marker analysis of loci underlying resistance to soybean cyst nematode (*Heterodera glycines* Ichinohe). *Crop Science* 34:240.
- Cregan, P.B., J. Mudge, E.W. Fickus, D. Danesh, R. Denny, and N.D. Young. 1999. Two simple sequence repeat markers to select for soybean cyst nematode resistance conditioned by the *rhg1* locus. *TAG Theoretical and Applied Genetics* 99:811-818.

- Cregan, P.B., T. Jarvik, A. L. Bush, R. C. Shoemaker, K. G. Lark, A. L. Kahler, N. Kaya, T. T. VanToai, D. G. Lohnes, J. Chung, and J. E. Specht. 1999. An Integrated Genetic Linkage Map of the Soybean Genome. *Crop Sci.* 39:1464-1490.
- Dashiell, K.E., and C.N. Akem. 1991. Yield losses in soybeans from frogeye leaf spot caused by *Cercospora sojina*. *Crop Protection* 10:465-468.
- Fehr, W.R. 1987. Principles of cultivar development. v. 1. Theory and technique Macmillan, New York.
- Fehr, W.R., and C.E. Caviness. 1977. Stages of soybean growth and development. Iowa Coop. Ext. Serv. Spec Rep. 80.
- Ha, B.K., R.T. Robbins, F. Han, R.S. Hussey, J.F. Soper, and H.R. Boerma. 2007. SSR mapping and confirmation of soybean QTL from PI 437654 conditioning resistance to reniform nematode. *Crop Science* 47:1336.
- Ha, B.K., and H.R. Boerma. 2008. High-Throughput SNP Genotyping by Melting Curve Analysis for Resistance to Southern Root-knot Nematode and Frogeye Leaf Spot. *J. Crop Sci. Biotech.* 11:91-100.
- Harris, D.K., H. R. Boerma, R. S. Hussey, and S. L. Finnerty. 2003. Additional Sources of Soybean Germplasm Resistant to Two Species of Root-Knot Nematode. *Crop Sci.* 43:1848-1851.
- Horsfall, J.G. 1972. Genetic vulnerability of major crops. National Academy of Sciences.
- Hyten, D.L., J.R. Smith, R.D. Frederick, M.L. Tucker, Q. Song, and P.B. Cregan. 2009. Bulk Segregant Analysis Using the GoldenGate Assay to Locate the *Rpp3* Locus that Confers Resistance to Soybean Rust in Soybean. *Crop Sci* 49:265-271.

- Hyten, D.L., I.Y. Choi, Q. Song, J.E. Specht, T.E. Carter Jr, R.C. Shoemaker, E.Y. Hwang, L.K. Matukumalli, and P.B. Cregan. 2010. A High Density Integrated Genetic Linkage Map of Soybean and the Development of a 1536 Universal Soy Linkage Panel for Quantitative Trait Locus Mapping. *Crop Science* 50:960-968.
- Hyten, D.L., Q. Song, I.Y. Choi, M.S. Yoon, J.E. Specht, L.K. Matukumalli, R.L. Nelson, R.C. Shoemaker, N.D. Young, and P.B. Cregan. 2008. High-throughput genotyping with the GoldenGate assay in the complex genome of soybean. *TAG Theoretical and Applied Genetics*:1-8.
- Keim, P., T.C. Olson, and R.C. Shoemaker. . 1988. A rapid protocol for isolating soybean DNA. . *Soybean Genet. Newsl.* 15:150-152.
- Laviolette, F.A., K. L. Athow, A.H. Probst, J.R. Wilcox, and T.S. Abney. 1970. Effect of bacterial pustule and frogeye leafspot on yield of Clark soybean. *Crop Sci* 10:418-419.
- Mengistu, A., N.C. Kurtzweil, and C.R. Grau. 2002. First Report of Frogeye Leaf Spot (*Cercospora sojina*) in Wisconsin. *Plant Disease* 86:1272-1272.
- Mian, M.A., T. Wang, D.V. Phillips, J. Alvernaz, and H.R. Boerma. 1999. Molecular Mapping of the *Rcs3* Gene for Resistance to Frogeye Leaf Spot in Soybean. *Crop Sci.* 39:1687-1691.
- Mian, M.A.R., A.M. Missaoui, D.R. Walker, D.V. Phillips, and H.R. Boerma. 2008. Frogeye Leaf Spot of Soybean: A Review and Proposed Race Designations for Isolates of *Cercospora sojina* Hara. *Crop Science* 48:14.

- Mian, M.A.R., H. R. Boerma, D. V. Phillips, M. Mm. Kenty, G. Shannon, E. R. Shipe, A. R.. Soffes Blount, D. B. Weaver. 1998. Performance of Frogeye Leaf Spot-Resistant and -Susceptible Near-Isolines of Soybean. *Plant Disease* 82:1017-1021.
- Michelmore, R.W., I. Paran, and R.V. Kesseli. 1991. Identification of Markers Linked to Disease-Resistance Genes by Bulk Segregant Analysis: A Rapid Method to Detect Markers in Specific Genomic Regions by Using Segregating Populations. *Proceedings of the National Academy of Sciences of the United States of America* 88:9828-9832.
- Missaoui, A.M., D.V. Phillips, and H.R. Boerma. 2007. DNA Marker Analysis of 'Davis' Soybean and Its Descendants for the *Rcs3* Gene Conferring Resistance to *Cercospora sojina*. *Crop Sci.* 47:1263-1270.
- Phillips, D.V. 1999. Frogeye Leaf Spot, *In* G. L. Hartman, et al., eds. *Compendium of Soybean Diseases*, 4th ed. American Phytopathological Society, St. Paul, MN.
- Phillips, D.V., and H.R. Boerma. 1981. *Cercospora sojina* Race 5: A Threat to Soybeans in the Southeastern United States. *Phytopathology* 71:334-336.
- Phillips, D.V., and H.R. Boerma. 1982. Two Genes for Resistance to Race 5 of *Cercospora sojina* in Soybeans. *Phytopathology* 72:764-766.
- Ross, J.P. 1968. Additional Pysiological Races of *Cercospora sojina* on Soybeans in North Carolina. *Phytopathology* 58:708-709.
- SAS. 2004. SAS/Stat® 9.1 User's Guide, Cary, NC.
- Schmutz, J., S.B. Cannon, J. Schlueter, J. Ma, T. Mitros, W. Nelson, D.L. Hyten, Q. Song, J.J. Thelen, and J. Cheng. 2010. Genome sequence of the palaeopolyploid soybean. *Nature* 463:178-183.

- Song, Q., Gaofeng Jia, Youlin Zhu, David Grant, Rex T. Nelson, Eun-Young Hwang, David L. Hyten, Perry B. Cregan. 2010. Abundance of SSR Motifs and development of Candidate Polymorphic SSR Markers (BARCSOYSSR_1.0) in Soybean. Crop Sci. In Press.
- Van Ooijen, J.W. 2006. JoinMap® 4, Software for the calculation of genetic linkage maps in experimental populations. Kyazma BV, Wageningen, Netherlands.
- Webb, D.M., B.M. Baltazar, A.P. Rao-Arelli, J. Schupp, K. Clayton, P. Keim, and W.D. Beavis. 1995. Genetic mapping of soybean cyst nematode race-3 resistance loci in the soybean PI 437.654. TAG Theoretical and Applied Genetics 91:574-581.
- Yang, X.B., M.D. Uphoff, and S. Sanogo. 2001. Outbreaks of Soybean Frogeye Leaf Spot in Iowa. Plant Disease 85:443-443.
- Yorinori, J.T. 1992. Management of foliar fungal diseases in Brazil, p. 185-193, *In* I. L. G. C. e. a. (ed.), ed. Pest management in Soybean. Elsevier Applied Science, London.

Table 3.1. Segregations ratios for F₂ plants from five FLS-resistant plant introductions crossed with FLS-susceptible Blackhawk.

Resistant plant introduction	<i>C. soja</i> race*	Resistant [#] plants no.	Intermediate plants no.	Susceptible plants no.	X ^{2#} Probability	No. of bulks created no.
PI594619	8	144	-	13	0.34 ^{ns†}	1
PI594661	11	95	-	32	0.95 ^{ns‡}	2
PI594662A	17	68	42	29	-	2
PI594774	17	139	-	56	0.23 ^{ns‡}	2
PI594891	17	87	-	23	0.32 ^{ns‡}	2

[†] Chi square statistic to determine if population fit a 15 resistant to 1 susceptible ratio.

[‡] Chi square statistic to determine if population fit a 3 resistant to 1 susceptible ratio.

*Mian et al. (2008).

[#] Number of plants and Chi-Square test represent data after the FLS ratings obtain from the F₂ derived lines.

Table 3.2. Soybean DNA markers associated with frogeye leaf spot resistance score in the F₂ population of Blackhawk x PI594662A.[†]

Satt244 (<i>Gm16</i>)	S4 3180K (<i>Gm18</i>)			Mean
	P/P	P/B	B/B	
	score [‡]			
P/P	1.00	1.33	1.60	1.31
P/B	1.00	1.51	1.91	1.47
B/B	1.00	2.00	3.00	2.00
Mean	1.00	1.61	2.17	1.59

[†] P/P = homozygous for the allele from PI594662A, B/B = homozygous for the allele from Blackhawk, and P/B = heterozygous for alleles from PI594662A and Blackhawk.

[‡] Score: 1.0 = resistant, 2.0 = intermediate lesions, and 3.0 = susceptible lesions.

Figure 3.1. Genetic linkage maps of soybean regions containing a FLS resistance locus. Interval distances and location of resistance locus are based on segregation of F₂ plants derived from the cross of Blackhawk x PI594619. Interval distances (cM) were generated using the Kosambi's mapping function. A segment of the associated consensus map is shown on the left, the right map contains linkage map created with DNA markers. Striped bar indicates area where R² values were greater than 15% and likely location of Rcs3_(PI594619) (Fig. 3.1a), Rcs_(PI594619) (Fig. 3.1b), single lines indicate markers which were shown to be significant for resistance to FLS as indicated by single factor analysis of variance.

Figure 3.1a)

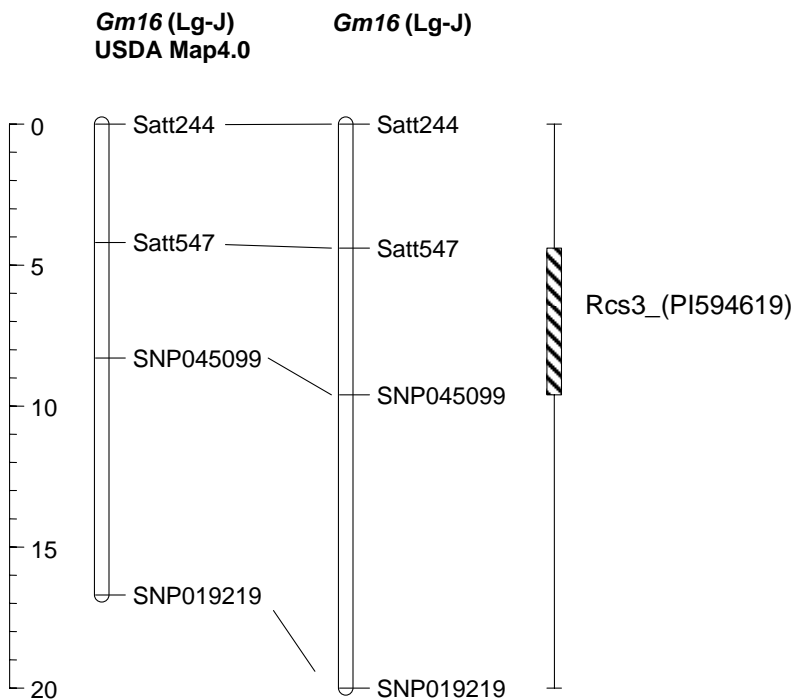
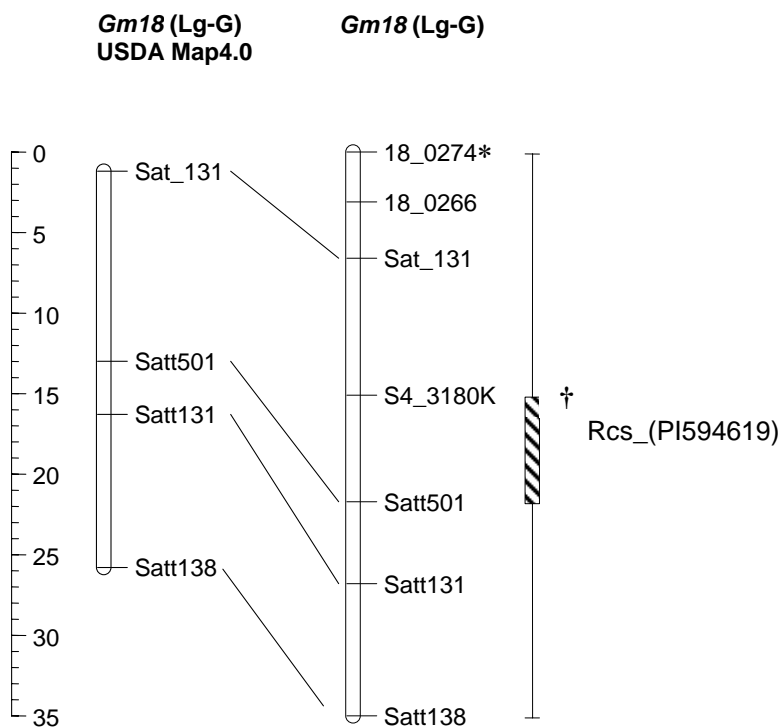


Figure 3.1b)



*Markers prefix is BARCSSR_

† Striped bar indicates area where R^2 values were greater than 20%.

Figure 3.2. Genetic linkage maps of soybean regions containing a FLS resistance locus. Interval distances and location of resistance locus are based on segregation of F₂ plants derived from the cross of Blackhawk x PI594661. Interval distances (cM) were generated using the Kosambi's mapping function. A segment of the associated consensus map is shown on the left, the middle map contains the linkage map created with DNA markers, the map on the right shows the linkage map with the DNA markers and the resistance gene Rcs3_(PI594661).

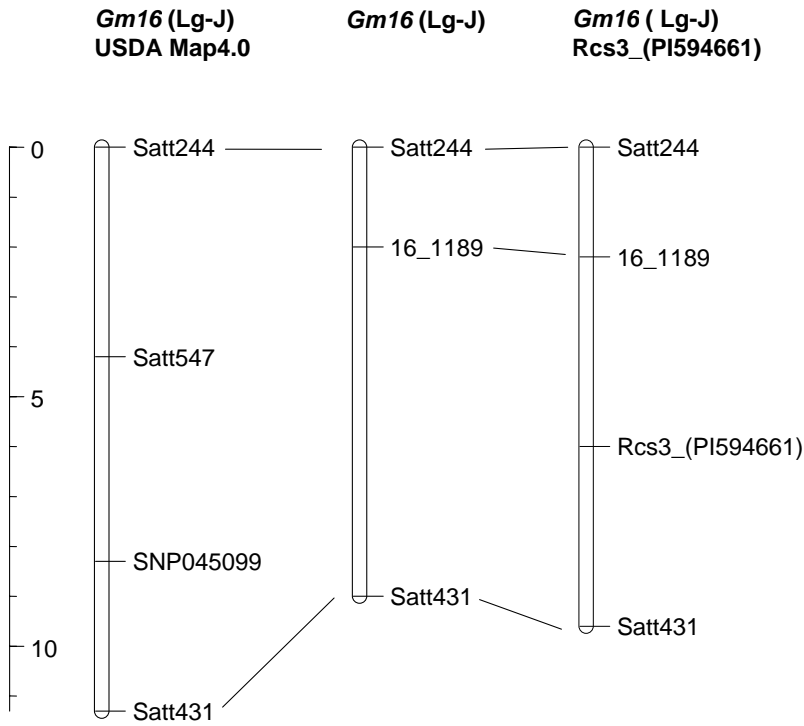


Figure 3.3. Genetic linkage maps of soybean regions containing a FLS resistance locus. Interval distances and location of resistance locus are based on segregation of F₂ plants derived from the cross of Blackhawk x PI594662A. Interval distances (cM) were generated using the Kosambi's mapping function. A segment of the associated consensus map is shown on the left, the right map contains linkage map created with DNA markers. Striped bar indicates area where R² values were greater than 15% and likely location of Rcs3_(PI594662A) (Fig. 3.3a), Rcs_(PI594662A) (Fig. 3.3b), single lines indicate markers which were shown to be significant for resistance to FLS as indicated by single factor analysis of variance.

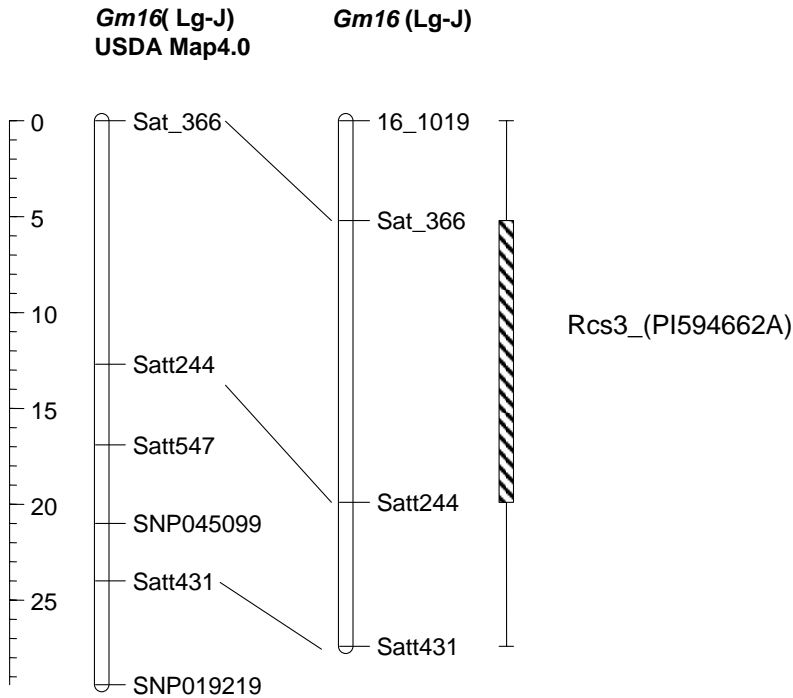


Figure 3.3b)

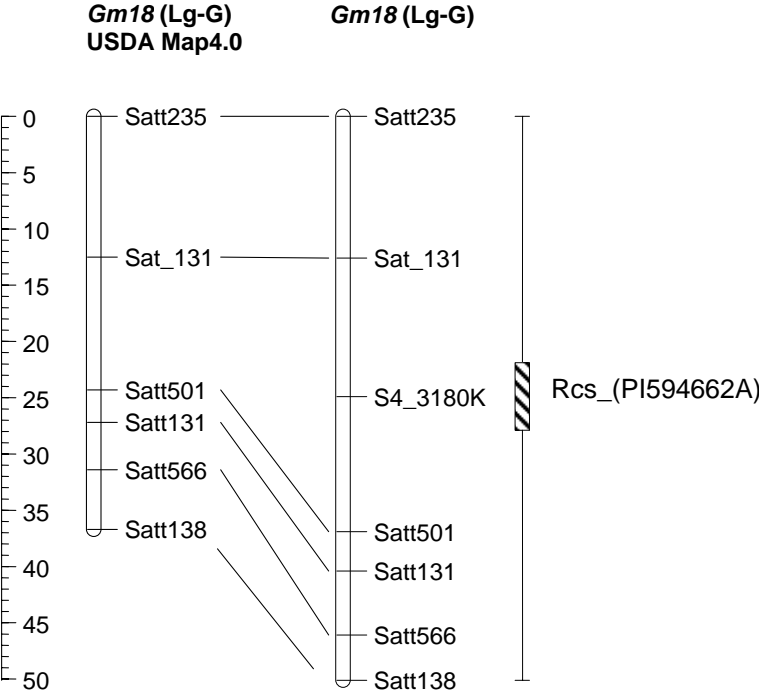


Figure 3.4. Genetic linkage maps of soybean regions containing a FLS resistance locus. Interval distances and location of resistance locus are based on segregation of F₂ plants derived from the cross of Blackhawk x PI594774. Interval distances (cM) were generated using the Kosambi's mapping function. A segment of the associated consensus map is shown on the left, the middle map contains the linkage map created with DNA markers, the map on the right shows the linkage map with the DNA markers and the resistance gene Rcs3_(PI594774).

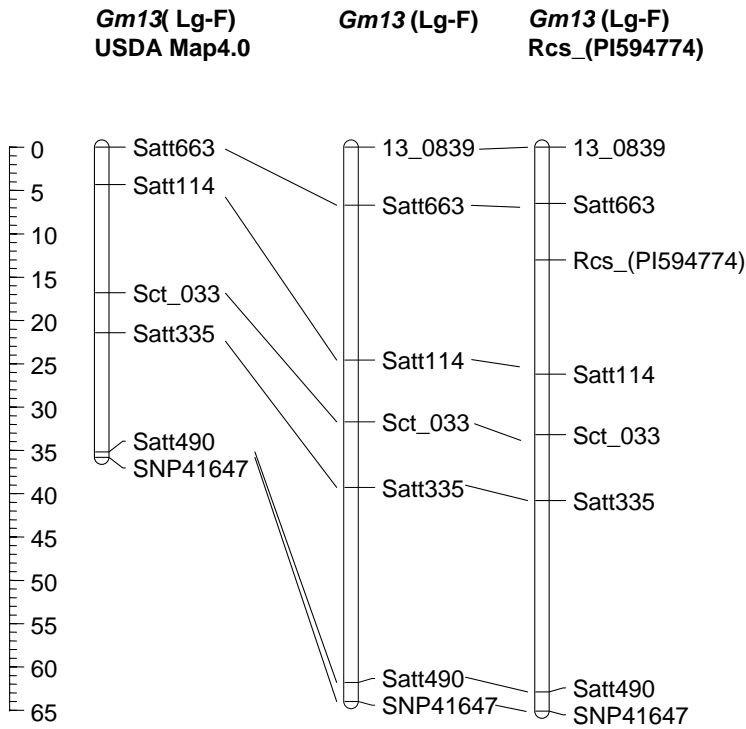
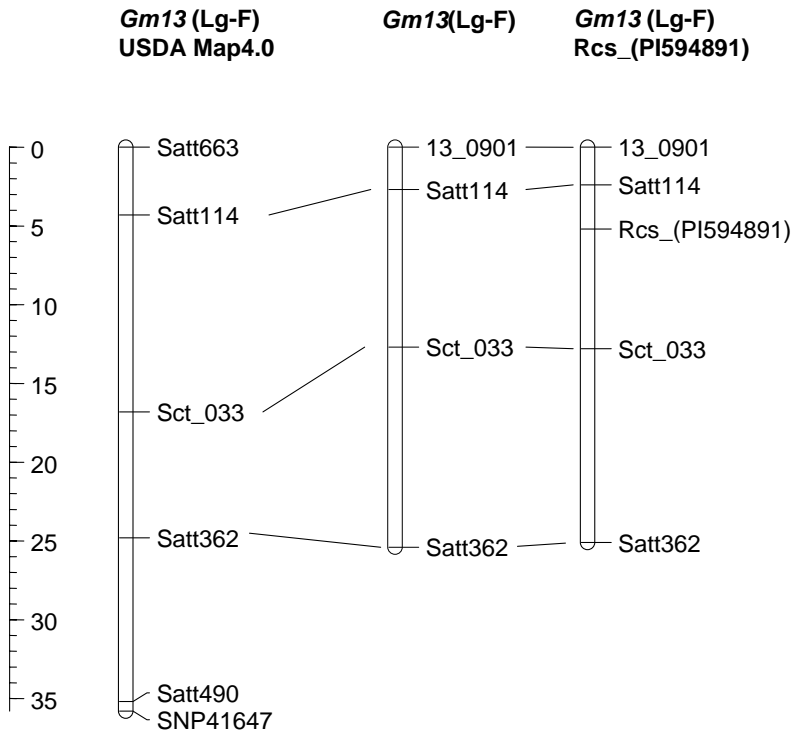


Figure 3.5. Genetic linkage maps of soybean regions containing a FLS resistance locus. Interval distances and location of resistance locus are based on segregation of F₂ plants derived from the cross of Blackhawk x PI594891. Interval distances (cM) were generated using the Kosambi's mapping function. A segment of the associated consensus map is shown on the left, the middle map contains the linkage map created with DNA markers, the map on the right shows the linkage map with the DNA markers and the resistance gene *Rcs3*_(PI594891).



CHAPTER IV

PHENOTYPIC SELECTION FOR LOW PHYTATE IN SOYBEAN¹

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ABSTRACT

Phosphorus is stored in the seeds of soybean (*Glycine max* (L.) Merr.) primarily as phytic acid, a form unavailable to monogastric animals. A low phytic acid soybean would increase inorganic P (Pi) availability to these animals and also help to alleviate the negative environmental impact of normal phytic acid soybean. Development of soybean cultivars that contain decreased levels of phytic acid have become an important objective in soybean breeding programs. The objectives of the current research were (i) to determine the effect of soybean maturity on the effectiveness of single-plant selection for Pi levels in soybean seeds containing low phytate alleles from CX1834-1-2, a low phytate, high inorganic P (Pi) soybean line, and (ii) to determine effects of backcross generations on levels of inorganic P in soybean seeds. There were weak, but significant ($P = 0.05$) correlations between temperature 15, 30, and 45 d prior to maturity and Pi levels in the 'Boggs' ($r = -0.14$ to -0.18) and 'Benning' ($r = -0.13$ to -0.21) populations in 2008, but not 2009. In 2009, the lines selected for high Pi in 2008 in the Boggs ($1228 \text{ ng g}^{-1} \text{ P}$), Benning ($1445 \text{ ng g}^{-1} \text{ P}$), and Prichard ($1359 \text{ ng g}^{-1} \text{ P}$) populations averaged significantly ($P = 0.05$) higher in Pi than the lines selected for low Pi (203 , 87 , and $103 \text{ ng g}^{-1} \text{ P}$, respectively). This was expected given the relatively high heritabilities of Pi level in all three populations ($h^2 = 0.54$ to 0.89). Correlations between Pi level and maturity were not significant ($P > 0.05$) in any of the backcross generations.

INTRODUCTION

Phosphorus (P) is classified as a macronutrient and is considered essential to plant growth. Phytic acid (myoinositol hexaphosphate) is the most common organic form of phosphate in the soil comprising up to 50% of total organic P in soil (Tisdale, 1993). Phytic acid serves as the chief storage form of phosphate (>60% total P) and inositol in mature seeds of oilseed crops (Erdman, 1979; Lei and Porres, 2003). Animal food that contains high phytate levels have been shown to lead to poor bioavailability of minerals such as zinc, calcium, magnesium, phosphorus, and possibly iron. Non-ruminant animals such as poultry and swine have little or none of the phytase enzyme needed to digest phytate P rendering it unavailable from major food sources such as soybean meal (Erdman, 1979). Poultry and swine rations are typically supplemented with phytase, which results in the removal of phosphate from phytate, or with inorganic phosphate which results in increased P availability to the livestock (Adeola, 1995; Lei and Porres, 2003). The phytate phosphorus that goes unutilized gets excreted by the animals and becomes an environmental pollutant in areas of intensive agriculture. The excessive phosphorus in soils runs off into lakes and seas and causes eutrophication (Lei and Porres, 2003).

Ethyl methanesulfonate (EMS) has been used successfully to create low phytate mutants in barley (*Hordeum vulgare* L; (Larson et al., 1998), maize (*Zea mays* (Raboy et al., 2000), and soybean (Wilcox et al., 2000). Soybean lines CX1834-1-2 and CX1834-1-6 are two low phytate soybean lines generated from the cross of Athow x M153-1-4-6-14. M153-1-4-6-14 is a mutant line that was selected from soybean breeding line CX1515-4 after being treated with EMS. Seed from M153-1-4 showed an 80% reduction in phytic

acid-P (PA-P) compared with its wild type sibling, and this reduction in PA-P corresponded to an equivalent increase in Pi (Wilcox et al., 2000). Experiments conducted by Oltmans et al. (2004) concluded that two independent recessive loci exhibiting duplicate dominant epistasis controlled the low phytate phenotype from the line CX1834-1-6. Walker et al. (2006), genetically mapped the low phytate loci in CX1834-1-2. They discovered two QTLs were responsible for the low phytate phenotype, one on soybean chromosome *Gm03* (Lg-N) which accounted for 42% of the variation, and another on *Gm19* (Lg-L) which explained 11% of variation in soybean seed Pi levels. The interaction of these two loci on *Gm03* (Lg-N) and *Gm19* (Lg-L) accounted for an additional 8 to 11% of the phenotypic variation in seed Pi levels. Independent studies confirmed these two QTL in additional genetic backgrounds (Gao et al., 2008; Scaboo et al., 2009).

The first gene shown to encode a transport function important to phytic acid synthesis or accumulation was the *lpa1* gene in maize. The *lpa-1* gene is a multi-drug resistance gene associated protein (MRP), a member of the ATP-Binding Cassette (ABC) transporter gene family (Shi et al., 2007). The exact function of the ABC transporter in accumulation of phytic acid is not known. Utilizing the soybean genome sequence (Schmutz et al., 2010), researchers were able to determine the two recessive mutations responsible for the low phytate phenotype in soybean were homologs of the maize ABC transporter (Gillman et al., 2009; Maroof et al., 2009). These genes were located near the previously reported genomic locations of QTL responsible for the low phytate phenotype in soybean (Walker et al., 2006).

Research has shown that phytate accumulation in soybean seeds increased linearly after flowering until maturity (Raboy and Dickinson, 1987). In low phytate crops, reductions in phytate P are often matched by increases in Pi, so that total P remains unchanged (Raboy, 2009). Saneoka et al. (2006) reported that phytate concentrations increased rapidly in soybean seeds from 30 to 60 days after anthesis (DAA) while Pi decreased during this same time period.

The objectives of this research were (i) to determine the effect of soybean maturity on the effectiveness of single-plant selection for Pi levels in soybean seeds containing alleles from CX1834-1-2, a low phytate, high Pi soybean line, and (ii) to determine effects of backcross generations on levels of Pi in soybean seeds.

MATERIALS AND METHODS

Field experiments were conducted at the Univ. of Georgia Plant Sciences Research Farm near Watkinsville, GA in 2008 and 2009. Three F_2 populations derived from the crosses of the glyphosate tolerant backcross-derived lines of ‘Boggs’, Maturity Group (MG) VI, ‘Benning’ (MG VII), and ‘Prichard’ (MG VIII) to CX1834-1-2 and various backcross F_2 populations with these three glyphosate tolerant lines as recurrent parents were included in this study (Table 4.1; Boerma et al., 1997; 2000; and 2001). The populations were identified as Boggs, Benning, and Prichard rather than Boggs-RR, Benning-RR, and Prichard-RR. The soybean line CX1834-1-2 (Wilcox et al., 2000) was the donor parent for the low phytate-high Pi phenotype.

On 18 June 2008 the F_2 generation of each initial cross, the various F_2 backcross generations, and the recurrent parents were planted as single rows 3.65 m long with spacing of 0.78 m between each row (Table 4.1). The number of plants per population and backcross generation varied from 54 F_2 plants of the BC_1 F_2 population of Benning to 687 plants of the BC_3 F_2 population of Benning.

Maturity of each plant was recorded on a weekly basis when 95% of pods on an individual plant reached R8 and recorded as days after 1 September (Fehr, 1977). Plants were cut at the soil surface, removed from the field, and threshed individually.

The Pi level was measured using a modified colorimetric assay developed by Raboy et al. (2000), which was adapted from the assay described by Chen et al. (1956), on 8 seeds/plant in 2008. The seeds were ground in a Wiley mill (Model 4, Philadelphia, Pa) for approximately 12 s and allowed to pass through a 2-mm sieve. The assay was described previously with slight modifications (Walker et al., 2006). A total of 100 mg

of ground seed tissue was measured into the well of a 96-well plate. Ground seed samples were mixed with an extraction buffer [12.5% trichloroacetic acid (TCA) and 25 mM MgCl₂] at a ratio of 10 µL buffer per 100 mg of tissue, and incubated overnight (~15 h) at 4° C. The samples were then vortexed and allowed to settle for 30 min before aliquots of extract solution were collected. A 10 µL of sample extract was diluted with 90 µL of H₂O. The diluted samples were then mixed with 100 µL Chen's Reagent, which consists of 1 volume 6N H₂SO₄: 1 volume 0.02 M ammonium molybdate: 1 volume 10% ascorbic acid: 2 volumes H₂O (Chen Jr et al., 1956). After the Chen's reagent was added, the reaction was allowed to proceed at ambient temperatures for 1 h, after which the samples were rated visually for amount of blue color. Eight P standards (0.0, 155, 465, 930, 1395, 1860, 2325, and 2635 ng P) were used as controls and corresponded to the rating scale. The darkest blue color produced was equivalent to 2635 ng P. Using this rating scale made it possible to use the original sample dilution ratio (10 µL solution + 90 µL H₂O) as described previously (Raboy et al., 2000).

Selections of the F_{2:3} lines for the 2009 experiment were made based on Pi levels recorded on the 2008 harvested seed from each F₂ or backcross-derived F₂ plant. For the backcross generations 10% of high Pi plants and 10% of low Pi plants were selected. Plants that had inorganic P levels of 465 ng g⁻¹ P or less were classified as "low Pi" while plants that had inorganic P levels of 930 ng g⁻¹ or higher were classified as "high Pi". The selected low Pi and high Pi F₂ plants within each of the initial crosses of Boggs, Benning, and Prichard by CX1834-1-2 were further classified as "early" if they matured before 7 October or "late" if they matured after 7 October. This resulted in four selected

groups (early/low Pi, late/low Pi, early/high Pi, and late/high Pi) within each cross (Fig. 4.1).

In 2009, plots were planted in hill plots on 17 June as a split plot design with cultivar/recurrent parent populations as the main plot and the selections within each of the cultivar/recurrent parent populations as a sub-plot. Spacing between each hill was 0.78 m, with 10 seeds per hill. Maturity was recorded as the date when 95% of the plants reached their mature pod color. Plots were harvested at maturity and threshed in a plot combine. In 2009 a total of 25 seeds were sampled from each hill plot. The Pi levels for seed from each hill plot were determined as described for 2008.

The SAS version 9.1 PROC CORR procedure (SAS, 2004) was used for Pi levels for F_2 plants and their respective $F_{2:3}$ lines to calculate standard unit heritability. Analysis of variance was conducted using SAS PROC GLM for Pi levels and maturity to determine differences between the various maturity/ Pi classes.

RESULTS AND DISCUSSION

The cultivars selected as the normal phytate parents Boggs, Benning and Prichard all had Pi levels of 0 in 2008 while in 2009 their Pi levels varied from 0 to 155 ng g⁻¹ P (Table 4.2, 4.3, and 4.4). The low phytate parent CX1834-1-2 had Pi levels that ranged from 2325 to 2635 ng g⁻¹ P in 2008 and 2009. Progeny in all generations of the three genetic backgrounds segregated for Pi. Levels of Pi in 2008 differed with cultivar/recurrent parent and generation. For the Boggs populations Pi ranged from 0 to 2635 ng g⁻¹ P for the F₂ generation and 0 to 2325 ng g⁻¹ P for the BC₁F₂ generation (Table 4.2). The F₂ populations that contained Benning as the parent/recurrent parent ranged from 0 to 2635 ng g⁻¹ P for the F₂, BC₁F₂, and BC₃F₂ populations, whereas the BC₂F₂ ranged from 0 to 2325 ng g⁻¹ P (Table 4.3). The BC₄F₂ and BC₅F₂ populations ranged from 0 to 1860 ng g⁻¹ P and 0 to 930 ng g⁻¹ P, respectively. All generations of plants that contained Prichard as the cultivar/recurrent parent ranged from 0 to 2325 ng g⁻¹ P. Previously studies have reported that Pi has an inverse relationship with phytate and Pi is a reliable indicator of the relative level of phytate contained in soybean seed (Scaboo et al., 2009; Wilcox et al., 2000). Pi was measured in this experiment, utilizing the assay to measure Pi in soybean seeds described by Raboy et al. (2000) which is a much simpler, quicker, and less expensive assay than measuring phytate.

In 2008, CX1834-1-2 matured 24 d after 1 Sept. while Boggs, Benning, and Prichard, matured 45, 57, and 66 d after 1 Sept. respectively (Table 4.2, 4.3, and 4.4). In 2009, CX1834-1-2 matured 25 d after 1 Sept. while Boggs matured 57, Benning 56, and Prichard 65 d after 1 Sept.. The later maturity of Boggs in 2009 is likely related to the lower average high temperature in 2009 when compared to 2008 (Fig 4.2). The F₂

populations that contained Boggs as the parent or recurrent parent ranged from 24 to 52 d after 1 Sept. in 2008 and 25 to 59 d after 1 Sept. in 2009 (Table 4.2), while Benning populations in 2008 ranged from 24 to 59 d after 1 Sept. in 2008 and 25 to 63 d after 1 Sept. in 2009 (Table 4.3). Prichard populations ranged from 24 to 66 d after 1 Sept. in 2008 while in 2009 they ranged from 28 to 66 d after 1 Sept. (Table 4.4).

Although the selection scheme imposed on the F_2 plants will provide some bias in calculating heritability estimates, we used the standard unit heritability method as described by Frey et al. (1957) to provide a comparison across various populations. Heritability for Pi was high ($h^2 = 0.80$ to 0.83) when all generations were combined within each cultivar/recurrent parent population (Table 4.6). A high heritability estimate for Pi in soybean seed would indicate that the variability within Pi would be under major genetic control, indicating that environment would have only moderate effect on Pi in the soybean seed.

When plants in all generations were combined across the three cultivar/recurrent parent populations, the heritability for maturity was 0.54, 0.66, and 0.86 for Boggs, Benning, and Prichard populations, respectively. The overall lower heritability estimates for maturity than Pi levels in the Boggs and Benning populations would indicate that maturity was more affected by the environment in which the plants were grown than Pi levels and that genotypes ranked differently in maturity in 2008 and 2009 in both the Boggs and Benning populations. An indication of these types of interactions was evident considering that Boggs matured 12 days earlier than Benning in 2008 and 1 day later than Benning in 2009 (Tables 4.2 and 4.3). The cooler temperature recorded during the

growing season in 2009 compared with 2008 could be responsible for this type of differential response (Fig. 4.2).

Maturity/Pi Selection Results

In 2008, the selected F₂ plants in the low Pi class from Boggs averaged 216 ng g⁻¹ P compared to 1344 ng g⁻¹ P for the high Pi plants from Boggs across both maturity classes (Table 4.5). Across the low and high Pi classes from Boggs the early class averaged 10.8 d earlier in maturity than the late selections. The selected high Pi F₂ plants for Benning and Prichard exceeded the selected low Pi by an average of 1193 and 1073 ng g⁻¹ P, respectively, when averaged across the two maturity classes. The selected late F₂ plants from Prichard averaged 9.6 d later in maturity while the late Benning selections averaged 11.2 d later in maturity than the plants in the early class when averaged across the Pi classes.

There were no significant differences ($P = 0.05$) in average Pi levels between early and late maturity classes for any of the three populations (Table 4.5). In the Boggs population the early/high Pi lines averaged 4.2 d later than the early/low Pi lines and the late/high Pi lines averaged 5.7 days later than the late/low Pi lines. These maturity differences between the high and low Pi class were not predicted based on the mean maturities of the low and high Pi F₂ selections in 2008. The mean maturities averaged for the high and low Pi classes were similar for the Benning and Prichard populations.

In 2009 the lines in the high Pi classes of Boggs (1228 vs. 203 ng g⁻¹ P), Benning (1445 vs. 87 ng g⁻¹ P), and Prichard (1359 vs. 103 ng g⁻¹ P) averaged significantly ($P = 0.05$) higher in Pi than the lines in the low Pi class. This was expected given the high heritability of Pi levels in all three populations.

There were weak, but significant ($P = 0.05$) correlations between temperature 15, 30, and 45 d prior to maturity and Pi levels in the Boggs ($r = -0.14$ to -0.18) and Benning ($r = -0.13$ to -0.21) populations in 2008, but not 2009 (Table 4.7). The higher temperatures observed in 2008 than 2009 during the time the seeds were developing could be the reason for this effect (Fig. 4.2). In these populations, higher temperatures 15 to 45 days prior to maturity resulted in lower levels of seed Pi. In the Boggs population there was a negative correlation at 15 and 30 days before maturity while in the Benning population there was a significant negative correlation for 15, 30, and 45 days before maturity. Since Prichard is a later maturing cultivar the cooler temperatures during seed maturation could have resulted in this lack of association. There were not any significant correlations between days before maturity and levels of seed Pi observed in the backcross generations regardless of the recurrent parent (data not shown).

Segregation was observed for both Pi levels and maturity among the lines within the four maturity/Pi classes in the backcross generations. The Pi levels in the low Pi BC₁F_{2,3} lines of the Boggs population for the selected low Pi class ranged from 0 to 930 ng g⁻¹ P while the lines in the high Pi class ranged from 0 to 2635 ng g⁻¹ P (Table 4.2). The low Pi class in backcross populations that contain Benning as the recurrent parent ranged from 0 to 1395 ng g⁻¹ P while, the lines grouped in the high Pi category ranged from 155 to 2635 ng g⁻¹ P (Table 4.3). In the Prichard backcross populations the low Pi selections ranged from 0 to 930 ng g⁻¹ P and the high Pi selections ranged from 155 to 2635 ng g⁻¹ P in 2009 (Table 4.4).

As mentioned earlier, the correlation between Pi and maturity was not found to be significant in any of the backcross generations in 2008 and the backcross generations

were not subdivided into early and late groups. The maturity ranged from 32 to 59 d after 1 Sept. for the Boggs BC₁F₂ population (Table 4.2). The maturity of the backcross generations that contained Benning as the recurrent parent ranged from 28 to 59 d after 1 Sept. and the Prichard backcross populations ranged from 42 to 66 d after 1 Sept..

The ranges in the Pi seed content in all of the recurrent parent and backcross generation populations resulted in plants being misclassified for Pi levels from 2008 to 2009. This was evident when the progeny of F₂ plants that were classified as high Pi in 2008 produced seed that contained 465 ng g⁻¹ P or less in 2009. All high Pi groups in 2009 regardless of recurrent parent or backcross generation contained lines that produced seed Pi levels of 465 ng g⁻¹ P or lower (Table 4.2, 4.3, and 4.4). The later maturity of the F₂ plants in the backcross generations compared with the F₂ populations could be the cause of the lack of association between maturity and Pi seed levels in these backcross-derived plants in 2008 (Table 4.7)

Previous studies on the low phytate line CX1834-1-2 as the donor parent found that there was no association between maturity and Pi levels in the progeny (Oltmans et al., 2005; Scaboo et al., 2009). In the study conducted by Oltmans et al (2005) when low phytate lines were selected a normal phytate line of the same maturity was selected to minimize maturity effects on the Pi trait. Scaboo et al. (2009) also found that there was not an association between Pi and maturity in recombinant inbred line (RIL) population that had a range in maturities of 38 d. The lack of affect of maturity could be due to cooler temperatures as was observed in 2009. Our data does not agree with a study which evaluated temperature effects on Pi levels (Anderson and Fehr, 2008). The experiment was conducted in three different environments, one in Iowa and two harvest

dates in Puerto Rico, and found significant differences in Pi levels and seed germination depending on the environment. The Iowa environment had the coolest average daily high temperature during seed fill (27.9° C) and produced seed with the highest germination rates, but had the lowest Pi levels (2210 ng g⁻¹). There were two harvest dates in the warmer Puerto Rico environment, one in January and one in May. The May harvest dates had the highest average daily temperature (32.4° C) and Pi (4380 ng g⁻¹), but had the lowest germination. The results of the current study and the research conducted by Anderson et al. (2008) indicate that factors other than temperature are likely responsible for the changes in Pi levels.

Studies have examined the accumulation of phytate in soybean seeds. Raboy et al. (1987) found that phytate accumulation begins 21 d after flowering while Saneoka et al. (2006) found that phytate accumulation began 30 d after anthesis. However, these studies were conducted with wild type phytate soybean lines that do not contain the mutant alleles in CX1834-1-2. They also did not measure how temperature impacts the accumulation of phytate in soybean seeds. Future studies would be beneficial to measure the affects of temperature in the CX1834-1-2 lines.

The environment in which the soybean plants are grown in has been shown to have an impact on Pi accumulation in soybean seed and could result in ineffective selection. Although the results are not consistent between studies or years the, temperature in which the plants are grown for selection could result in plants being selected that will not contain the levels of Pi that are desirable when the plants are grown in the target environment. The association between molecular markers and the low phytate trait have been well documented (Gao et al., 2008; Scaboo et al., 2009; Walker et

al., 2006) The use of marker assisted selection (MAS) for the low phytate alleles from CX1834-1-2 in soybean seeds would negate any environmental factors on selection (Gillman et al., 2009; Walker et al., 2006) and would allow for the identification of desirable alleles to be selected for regardless of the environment in which the plants are grown.

REFERENCES

- Adeola, O. 1995. Digestive utilization of minerals by weanling pigs fed copper-and phytase-supplemented diets. *Can. J. Anim. Sci* 75:603-610.
- Anderson, B.P., and W.R. Fehr. 2008. Seed Source Affects Field Emergence of Low-Phytate Soybean Lines. *Crop Sci* 48:929-932.
- Chen Jr, P.S., T.Y. Toribara, and H. Warner. 1956. Microdetermination of Phosphorus. *Analytical Chemistry* 28:1756-1758.
- Erdman, J.W. 1979. Oilseed phytates: Nutritional implications. *Journal of the American Oil Chemists' Society* 56:736-741.
- Fehr, W.R., and C.E. Caviness. 1977. Stages of soybean growth and development. Iowa Coop. Ext. Serv. Spec Rep. 80.
- Gao, Y., R.M. Biyashev, M.A.S. Maroof, N.M. Glover, D.M. Tucker, and G.R. Buss. 2008. Validation of Low-Phytate QTLs and Evaluation of Seedling Emergence of Low-Phytate Soybeans. *Crop Sci* 48:1355-1364.
- Gillman, J.D., V.R. Pantalone, and K. Bilyeu. 2009. The Low Phytic Acid Phenotype in Soybean Line CX1834 Is Due to Mutations in Two Homologs of the Maize Low Phytic Acid Gene. *The Plant Genome* 2:179-190.
- Larson, S.R., K.A. Young, A. Cook, T.K. Blake, and V. Raboy. 1998. Linkage mapping of two mutations that reduce phytic acid content of barley grain. *TAG Theoretical and Applied Genetics* 97:141-146.
- Lei, X.G., and J.M. Porres. 2003. Phytase enzymology, applications, and biotechnology. *Biotechnology Letters* 25:1787-1794.

- Maroof, M.A.S., N.M. Glover, R.M. Biyashev, G.R. Buss, and E.A. Grabau. 2009. Genetic Basis of the Low-Phytate Trait in the Soybean Line CX1834. *Crop Sci* 49:69-76.
- Oltmans, S.E., W.R. Fehr, G.A. Welke, and S.R. Cianzio. 2004. Inheritance of Low-Phytate Phosphorus in Soybean. *Crop Sci* 44:433-435.
- Oltmans, S.E., W.R. Fehr, G.A. Welke, V. Raboy, and K.L. Peterson. 2005. Agronomic and Seed Traits of Soybean Lines with Low-Phytate Phosphorus. *Crop Sci* 45:593-598.
- Raboy, V. 2009. Approaches and challenges to engineering seed phytate and total phosphorus. *Plant Science* 177:281-296.
- Raboy, V., and D.B. Dickinson. 1987. The Timing and Rate of Phytic Acid Accumulation in Developing Soybean Seeds. *Plant Physiol* 85:841-844.
- Raboy, V., P.F. Gerbasi, K.A. Young, S.D. Stoneberg, S.G. Pickett, A.T. Bauman, P.P.N. Murthy, W.F. Sheridan, and D.S. Ertl. 2000. Origin and Seed Phenotype of Maize low phytic acid 1-1 and low phytic acid 2-1. *Plant Physiol.* 124:355-368.
- Saneoka, H., T. Hosokawa, A. Yorizane, N. Nakayama, T. Itani, and K. Fujita. 2006. Changes in the composition of phytate, phosphorus and other cations in soybean seeds with maturity. *Grassland Science* 52:99-104.
- SAS. 2004. SAS/Stat® 9.1 User's Guide, Cary, NC.
- Scaboo, A.M., V.R. Pantalone, D.R. Walker, H.R. Boerma, D.R. West, F.R. Walker, and C.E. Sams. 2009. Confirmation of Molecular Markers and Agronomic Traits Associated with Seed Phytate Content in Two Soybean RIL Populations. *Crop Sci* 49:426-432.

- Schmutz, J., S.B. Cannon, J. Schlueter, J. Ma, T. Mitros, W. Nelson, D.L. Hyten, Q. Song, J.J. Thelen, and J. Cheng. 2010. Genome sequence of the palaeopolyploid soybean. *Nature* 463:178-183.
- Shi, J., H. Wang, K. Schellin, B. Li, M. Faller, J.M. Stoop, R.B. Meeley, D.S. Ertl, J.P. Ranch, and K. Glassman. 2007. Embryo-specific silencing of a transporter reduces phytic acid content of maize and soybean seeds. *Nature biotechnology* 25:930-937.
- Tisdale, S.L., W. L. Nelson, J. D. Beaton, and J. L. Havlin. 1993. *Soil Fertility and Fertilizers*. Fifth ed. Prentice-Hall.
- Walker, D.R., A.M. Scaboo, V.R. Pantalone, J.R. Wilcox, and H.R. Boerma. 2006. Genetic Mapping of Loci Associated with Seed Phytic Acid Content in CX1834-1-2 Soybean. *Crop Science* 46:390.
- Wilcox, J.R., G.S. Premachandra, K.A. Young, and V. Raboy. 2000. Isolation of High Seed Inorganic P, Low-Phytate Soybean Mutants. *Crop Sci* 40:1601-1605.

Table 4.1. F₂ populations evaluated for Pi levels and maturity.

Female parent	Male parent	Generation	Plants harvested 2008 no.	Hills planted 2009 no.
Boggs	CX1834-1-2	F ₂	217	68
Boggs	Boggs-RR (1) x CX1834-1-2	BC ₁ F ₂	357	49
Benning	CX1834-1-2	F ₂	226	47
Benning	Benning-RR x CX1834-1-2	BC ₁ F ₂	54	12
Benning	Benning-RR (2) x CX1834-1-2	BC ₂ F ₂	273	56
Benning	Benning-RR (3) x CX1834-1-2	BC ₃ F ₂	687	58
Benning	Benning-RR (4) x CX1834-1-2	BC ₄ F ₂	177	35
Benning	Benning-RR (5) x CX1834-1-2	BC ₅ F ₂	196	35
Prichard	CX1834-1-2	F ₂	212	38
Prichard	Prichard-RR (1) x CX1834-1-2	BC ₁ F ₂	267	51
Prichard	Prichard-RR (2) x CX1834-1-2	BC ₂ F ₂	299	56
Prichard	Prichard-RR (4) x CX1834-1-2	BC ₄ F ₂	619	68

Figure 4.1. Established selection groups and number of F₂ plants and selected F_{2:3} lines based on 2008 Pi and maturity date for three populations. [†] CX1834 = CX1834-1-2.

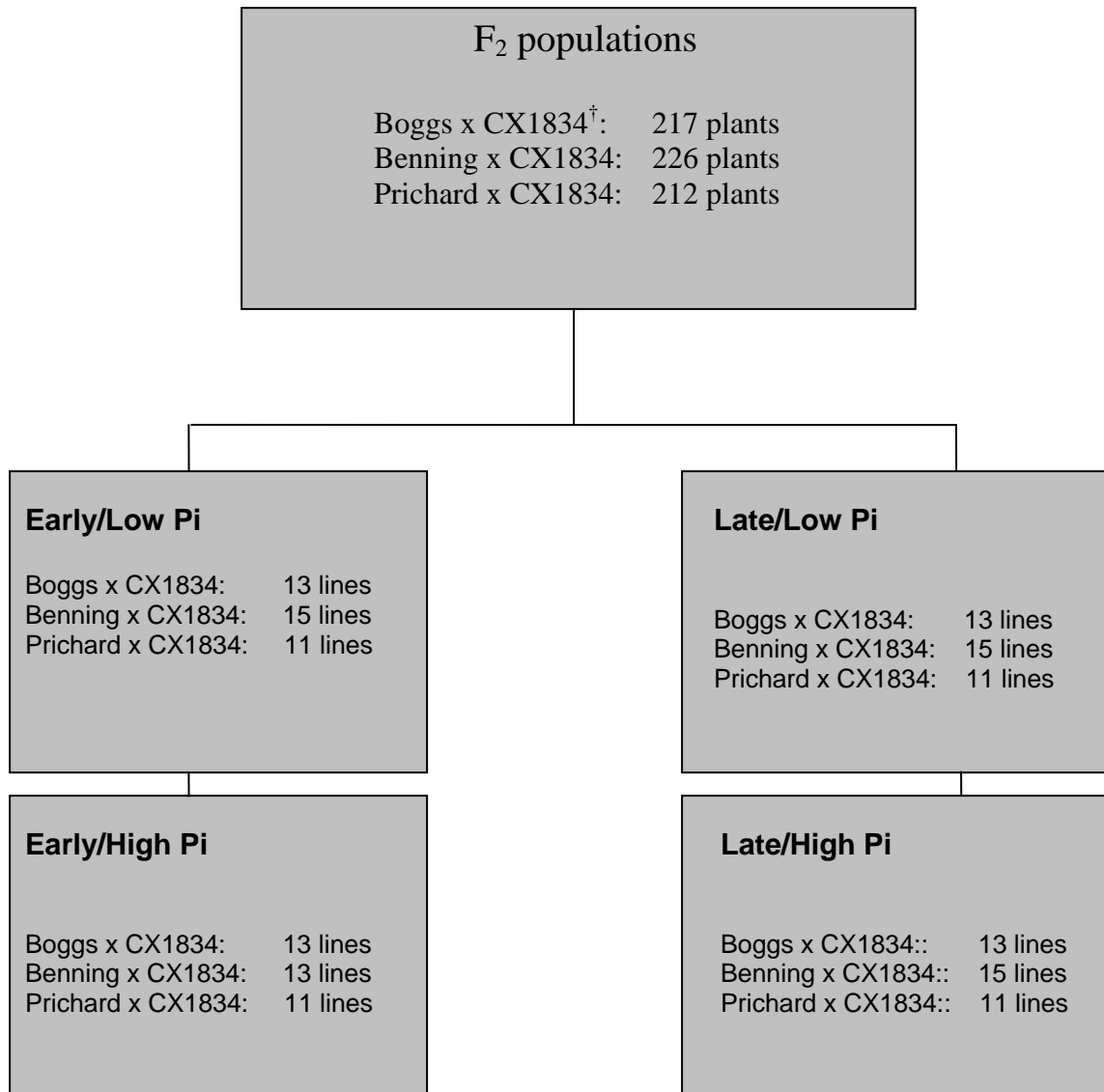


Figure 4.2. Temperature averages for 2008, 2009, and 10-year (2001-2009) average at the Univ. of Georgia Plant Sciences Farm near Watkinsville, GA.

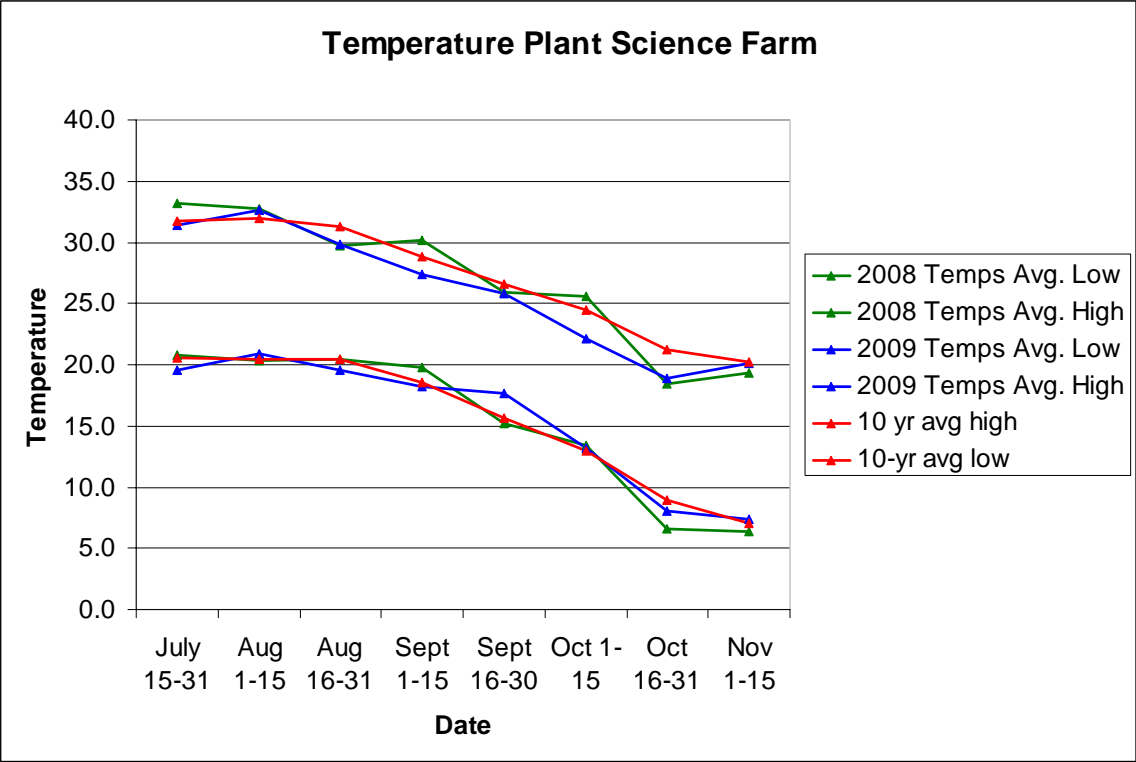


Table 4.2. Mean maturity and inorganic phosphorus (Pi) for the Boggs x CX1834-1-2 F₂ and Boggs backcross F₂ populations in 2008 and selected F_{2:3} lines 2009.

Generations	2008		2009	
	Mean \pm SE	Range	Mean \pm SE	Range
<u>Maturity</u> (d after 1 Sept.)				
All generations	41 \pm 0.2	24-52	46 \pm 0.6	25-59
F ₂	38 \pm 0.5	24-52	40 \pm 0.9	25-55
BC ₁ F ₂	42 \pm 0.2	24-52	50 \pm 0.6	32-59
Boggs	45 \pm 0.0	45	57 \pm 0.3	56-58
CX1834-1-2	24 \pm 0.0	24	25 \pm 0.0	25
<u>Pi (ng P)</u>				
All generations	346 \pm 21	0-2635	644 \pm 46	0-2635
F ₂	516 \pm 41	0-2635	650 \pm 79	0-2635
BC ₁ F ₂	243 \pm 23	0-2325	640 \pm 56	0-2635
Low			178 \pm 30	0-930
High			1108 \pm 71	0-2635
Boggs	0 \pm 0	0	71 \pm 16	0-155
CX1834-1-2	2635 \pm 0	2635	2440 \pm 28	2325-2635

Table 4.3. Mean maturity and inorganic phosphorus (Pi) for the Benning x CX1834-1-2 F₂ and Benning backcross F₂ populations in 2008 and selected F_{2:3} lines 2009.

Generation	2008		2009	
	Mean \pm SE	Range	Mean \pm SE	Range
<u>Maturity</u> (d after 1 Sept.)				
All generations	48 \pm 0.2	24-59	49 \pm 0.4	25-63
F ₂	39 \pm 0.4	24-59	42 \pm 1.0	25-56
BC ₁ F ₂	44 \pm 0.5	38-59	45 \pm 1.6	29-55
BC ₂ F ₂	46 \pm 0.2	31-59	50 \pm 0.6	28-57
BC ₃ F ₂	51 \pm 0.2	31-59	51 \pm 0.5	36-58
BC ₄ F ₂	50 \pm 0.4	38-59	51 \pm 0.7	40-58
BC ₅ F ₂	53 \pm 0.4	38-59	54 \pm 0.6	45-59
Benning	57 \pm 2.3	52-59	56 \pm 0.1	55-57
CX1834-1-2	24 \pm 0.0	24	25 \pm 0.0	25
<u>Pi (ng P)</u>				
All generations	293 \pm 12	0-2635	665 \pm 36	0-2635
F ₂	395 \pm 39	0-2635	797 \pm 86	0-2635
BC ₁ F ₂	399 \pm 90	0-2635	872 \pm 215	0-2635
Low			324 \pm 124	0-1395
High			1627 \pm 95	465-2635
BC ₂ F ₂	341 \pm 32	0-2325	730 \pm 78	0-2635
Low			99 \pm 20	0-465
High			1398 \pm 96	465-2635
BC ₃ F ₂	329 \pm 21	0-2635	855 \pm 83	0-2635
Low			90 \pm 22	0-930
High			1558 \pm 90	155-2635
BC ₄ F ₂	162 \pm 24	0-1860	600 \pm 75	0-2325
Low			137 \pm 33	0-930
High			1090 \pm 97	155-2325
BC ₅ F ₂	77 \pm 14	0-930	662 \pm 79	0-2635
Low			340 \pm 73	0-1395
High			1048 \pm 120	0-2635
Benning	0 \pm 0	0	25 \pm 12	0-155
CX1834-1-2	2635 \pm 0	2635	2440 \pm 28	2325-2635

Table 4.4. Mean maturity and inorganic phosphorus (Pi) for the Prichard x CX1834-1-2 F₂ and Prichard backcross F₂ populations in 2008 and selected F_{2:3} lines 2009.

Gen	2008		2009	
	Mean \pm SE	Range	Mean \pm SE	Range
<u>Maturity</u> (d after 1 Sept.)				
All generations	61 \pm 0.2	24-66	58 \pm 0.4	28-66
F ₂	43 \pm 0.4	24-52	43 \pm 0.9	28-61
BC ₁ F ₂	56 \pm 0.3	38-66	59 \pm 0.5	42-65
BC ₂ F ₂	66 \pm 0.0	66-66	59 \pm 0.3	40-64
BC ₄ F ₂	66 \pm 0.0	66-66	64 \pm 0.1	59-66
Prichard	66 \pm 0.0	66	64 \pm 0.1	64-66
CX1834-1-2	24 \pm 0.0	24	25 \pm 0.0	25
<u>Pi (ng P)</u>				
All generations	316 \pm 13	0-2325	815 \pm 41	0-2635
F ₂	383 \pm 35	0-2325	673 \pm 73	0-2325
BC ₁ F ₂	330 \pm 30	0-2325	747 \pm 78	0-2635
Low			163 \pm 37	0-930
High			1354 \pm 98	155-2635
BC ₂ F ₂	236 \pm 25	0-2325	870 \pm 89	0-2635
Low			105 \pm 19	0-465
High			1635 \pm 102	155-2635
BC ₄ F ₂	326 \pm 20	0-2325	900 \pm 80	0-2635
Low			191 \pm 32	0-930
High			1710 \pm 91	465-2635
Prichard	0 \pm 0	0	58 \pm 15	0-155
CX1834-1-2	2635 \pm 0	2635	2440 \pm 28	2325-2635

Table 4.5. Inorganic phosphorus (Pi) of selected F₂ plants and resultant F_{2:3} lines for three populations (Boggs, Benning, and Prichard by CX1834-1-2) in 2008 and 2009.

Gen	Boggs x CX1834-1-2				Benning x CX1834-1-2				Prichard x CX1834-1-2			
	Maturity		Pi		Maturity		Pi		Maturity		Pi	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
	d after 1 Sept		(ng P)		d after 1 Sept		(ng P)		d after 1 Sept.		(ng P)	
2008 [†]												
Early/Low	34	24-38	167	0-465	34	24-38	117	0-465	36	24-38	146	0-465
Early/High	35	24-38	1259	930-2635	34	24-38	1210	930-2635	36	31-38	1225	930-2325
Late/Low	45	45-52	265	0-465	46	45-59	128	0-465	46	45-52	125	0-465
Late/High	45	45-45	1428	930-2635	45	45-52	1420	930-2635	46	45-52	1192	930-2325
LSD(0.05)	1.8		139		1.8		141		1.4		130	
2009 (Selection Results)												
Early/Low	35	28-45	191	0-1395	34	25-51	78	0-465	36	25-51	109	0-465
Early/High	40	25-52	1268	155-2635	36	25-51	1381	930-2635	40	28-51	1292	930-2635
Late/Low	42	26-51	215	0-930	50	37-56	96	0-465	50	41-61	97	0-465
Late/High	48	28-55	1188	155-2635	48	36-56	1509	930-2635	49	39-58	1426	930-2325
LSD(0.05)	4.2		310		4.6		270		6.4		255	

[†] Early or Late maturity; Low or High Pi.

Table 4.6. Standard unit heritability estimates for inorganic phosphorous (Pi) and maturity in the F₂ generation of three crosses.

Population	Boggs x CX1834-1-2	Benning x CX1834-1-2	Prichard x CX1834-1-2
	h^2	h^2	h^2
<u>Maturity</u>			
All	0.54***	0.66***	0.86*
F ₂	0.50***	0.77***	0.73***
BC ₁ F ₂	0.20*	0.26 ^{ns}	0.62***
BC ₂ F ₂		0.38***	n/a
BC ₃ F ₂		0.34***	
BC ₄ F ₂		0.41***	n/a
BC ₅ F ₂		0.55***	
<u>Pi</u>			
All	0.83***	0.82***	0.80***
F ₂	0.86***	0.81***	0.54***
BC ₁ F ₂	0.80***	0.78***	0.78***
BC ₂ F ₂		0.85***	0.88***
BC ₃ F ₂		0.89***	
BC ₄ F ₂		0.79***	0.88***
BC ₅ F ₂		0.71***	

*Significant at the 0.05 probability level.

**Significant at the 0.01 probability level.

***Significant at the 0.0001 probability level.

^{ns}non-significant

Table 4.7. Pearson's correlation coefficients between Pi and temperature 15, 30, and 45 days before maturity in 2008 and 2009 in three F₂ populations.

<u>Year/days</u>	<u>Boggs x CX1834-1-2</u>	<u>Benning x CX1834-1-2</u>	<u>Prichard x CX1834-1-2</u>
<u>2008</u>			
15	-0.14*	-0.15*	-0.05 ^{ns}
30	-0.18**	-0.21**	-0.07 ^{ns}
45	-0.12 ^{ns}	-0.13*	-0.03 ^{ns}
<u>2009</u>			
15	-0.19 ^{ns}	0.02 ^{ns}	-0.00 ^{ns}
30	-0.10 ^{ns}	0.002 ^{ns}	-0.02 ^{ns}
45	-0.15 ^{ns}	0.01 ^{ns}	-0.01 ^{ns}

*Significant at the 0.05 probability level.

**Significant at the 0.01 probability level.

***Significant at the 0.0001 probability level.

^{ns} non-significant

CHAPTER V

SUMMARY

Improvement of soybean cultivars in the USA has focused on higher seed yield. Other improvements have included incorporating disease resistance and altering the chemical composition of the soybean seed to provide healthier oil profiles or greater nutrient absorption by animals. Soybean yield losses to plant disease in the USA are greater than a billion dollars a year. The genetic yield potential of a soybean cultivar needs to be protected from disease by incorporating genes for resistance.

My research has discovered novel sources of resistance to FLS that will help growers to protect yield potential of newly developed soybean cultivars. We identified at least two new sources of resistance on two chromosomes of soybean where FLS resistance genes were not previously known. These new genes were discovered in PI594619, PI594662A, PI594774, and PI594891 that were obtained from China. These plant introductions were previously found to be resistant to 15 isolates of *Cercospora sojina* maintained in the Univ. of Georgia collection. These new resistance genes can be pyramided with the *Rcs3* resistance gene to provide a broader level of protection and lower the probability that *C. sojina* can overcome the resistance of soybean cultivars containing these multiple resistance genes. Greenhouse studies combined with new DNA marker technologies such as the Golden Gate Assay by Illumina found the genomic location of a novel *Rcs* gene in PI594774 and PI594662A on *Gm13* (Lg-F), while the genes in PI594619 and PI594662A were found to be on *Gm18* (Lg-G). Resistance genes were also found near the *Rcs3* locus on *Gm16* (Lg-J) in PI594619, PI594661, and

PI594662A. Further studies will need to be conducted to determine if the *Rcs* genes located on *Gm16* (Lg-J) from these PIs are different than *Rcs3*.

Low phytate soybean seeds have been found to be more beneficial to the environment. When monogastric animals are fed low phytate soybean meal they absorb more phosphorus from the ration and less phosphorous is excreted to contaminate the environment. Two QTL conditioning the low phytate trait from the mutant soybean line CX1834-1-2 were previously identified and named cqPha-001 and cqPha-002. In this study we examined the efficiency of selecting for the low phytate phenotype on a single plant basis and the effects of backcross generations on the phenotype.

Field studies revealed that temperature may have an effect on the inorganic phosphorus (Pi) levels and the effectiveness of single plant selection for Pi levels. There were weak, but significant ($P = 0.05$) correlations between temperature 15, 30, and 45 d prior to maturity and Pi levels in the Boggs ($r = -0.14$ to 0.18) and Benning ($r = -0.13$ to -0.21) populations in 2008, but not 2009. In 2009, the lines selected for high Pi in 2008 in the Boggs ($1228 \text{ ng g}^{-1} \text{ P}$), Benning ($1445 \text{ ng g}^{-1} \text{ P}$), and Prichard ($1359 \text{ ng g}^{-1} \text{ P}$) populations averaged significantly ($P = 0.05$) higher in Pi than the lines selected for low Pi (203 , 87 , and $103 \text{ ng g}^{-1} \text{ P}$, respectively). This was expected given the relatively high heritabilities of Pi level in all three populations ($h^2 = 0.54$ to 0.89). Correlations between Pi and maturity were not significant ($P > 0.05$) in any of the backcross generations. These results indicate that earlier maturing soybean plants that mature when temperature are warmer may produce in lower levels of Pi. This temperature/maturity effect can result in reducing the efficiency of selection for phytate levels in soybean populations segregating for maturity.

New technologies have been introduced that have greatly accelerated the rate at which novel genes can be discovered. After discovery of the genes has occurred plant breeders can incorporate the positive alleles at these loci into the breeding program and select improved cultivars at a more rapid rate than in the past. Using molecular markers that are linked to the newly discovered *Rcs* genes can help to incorporate these genes into elite breeding lines, while eliminating the deleterious alleles that are often found in plant introductions. Using the SNP markers that have been associated with the CX1834-1-2 alleles may be a more effective method for identifying low phytate plants than selection based on Pi levels of field-grown single plants.