Mineral evaluation and Quantitative Trait Loci mapping in a soybean (*Glycine max* (L.) Merr.) population developed for iron deficiency chlorosis resistance

by

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DEDICATION

This work is dedicated to the memory of Annie Bell King. She was the matriarch of the King family, but more importantly she was my rock throughout my academic career, who had her going home celebration in my first summer here at Iowa State University. She is still dearly missed, but I know she looking down upon me and proud of her grandson.

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ABSTRACT

The identification of the causal gene(s) of disease resistance is paramount in plant breeding. Nutrient analysis in plant tissues can lead to the identification of these gene(s). The objectives of this research were 1) to map quantitative trait loci (QTL) for seed and leaf iron and zinc concentration in the soybean population Anoka x A7, 2) to determine if QTL for iron and zinc accumulation co-localized to the same regions of the genome, 3) to determine if any of newly identified QTL for Fe and/or Zn concentration correlate with QTL previously identified for Fe efficiency, 4) to determine the amount of variation of total phosphorus in a population developed for iron deficiency chlorosis resistance, and 5) to map total phosphorus as a QTL. Iron, zinc, and total phosphorus concentration was determined in 92 $F_{2:4}$ lines from the Anoka x A7 population grown on non-calcareous soil grown in 2008 and 2009. Dry ashing was the method used to determine iron and zinc, and total phosphorus was determined through an overnight digestion. Nutrients in each set of samples were quantified using inductively coupled plasma-optical emission spectrometry (ICP-OES). The genetic map was integrated with the map of Lin et al. (1997) which consisted of a total of 150 markers and used to locate QTL.

For the iron and zinc study, one significant QTL for iron concentration in soybean seed was mapped on chromosome 20 in the combined data. One marker in this QTL interval, pa_*515-1*, previously mapped an Fe efficiency QTL. This result presented evidence of a genetic link between Fe efficiency and iron concentration in soybean.

In the total phosphorus study, one significant QTL was mapped on chromosome 12 for phosphorus concentration in the combined data. Candidate genes in this marker interval, *S12_0711-S12_1103,* are involved in the P storage and homeostasis pathways and mediate the transport of phosphate. The results of this study indicated that total phosphorus uptake and transport could be modified.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

Iron deficiency chlorosis (IDC) is a major disease of soybean (*Glycine max* (L.) Merr.) and commonly observed in plants grown on calcareous soils, which are common in the upper Midwestern United States. Worldwide, calcareous soil is estimated to make up about 30% of the world's arable land (Mori, 1999). The disease is often observed when the supply of iron to the developing plant is interrupted, which results in stunted plants with interveinal pale green or yellow-to-nearly-white leaves with green veins (Franzen, et al., 2004). Ultimately, the expression of IDC results in a reduction in yield and in extreme cases plant death (Froehlich and Fehr, 1981). Consequently, identifying resistant genotypes is paramount to reducing IDC expression.

Previous research by Lin et al. (1997) suggested the genetic mechanisms that control IDC. These were a single major gene with modifiers proposed by Cianzio and Fehr (1980), and classic polygenic inheritance proposed by Cianzio and Fehr (1982). Through this work Fe efficiency quantitative trait loci (QTL) were identified for chlorophyll a/b content and IDC score in two mapping populations, Pride B216 x A15 and Anoka x A7, with the major gene being identified in the latter population. The most recent work was the development of the publically available lines AR2 and AR3. Both lines were developed using selection with molecular marker Satt481, which has been shown to be significantly associated with Fe efficiency scores across multiple environments (Cianzio et al., 2006a, 2006b; Charlson et al., 2003).

Although progress in breeding for resistance to IDC has been made in recent years with the release of these lines, there is still a lack of genotypes with complete resistance to the disease. Development of completely resistant genotypes will require identification of novel gene combinations that have not yet been achieved. Determining mineral concentration in leaves or seed is an approach to selecting iron efficient genotypes that has not yet been fully explored in soybean. Furthermore, the variability in Fe concentration across generations and genotypes needs to be better understood in order to deploy Fe-enhanced varieties for the field (Qu et al., 2005). It is known that the amount of Fe in leaves and in the seed is related to the mobility and redistribution of Fe from vegetative tissue via the phloem (Garnett and Graham, 2005). In beans and peas, researchers have found evidence of good remobilization. Zhang et al. (1995) reported this in mature bean leaves, and Grusak (1995) found that as much as 75% of total shoot Fe was ultimately found in the seeds.

Determining the concentration of Fe in seeds may aid in breeding for tolerance/resistance to IDC, because micronutrient reserves in seeds represent a significant source of mineral elements for early seedling establishment in nutrient-limited growing conditions (Bityutskii et al., 2002). The total Fe balance between seeds and leaves is relatively unknown, but Fe translocation from leaves to seeds may be very important in determining Fe accumulation in seeds (Qu et al., 2005). There is relatively little knowledge about the diversity in Fe accumulation in the soybean germplasm pool. Research by Tiffin et al. (1973) and Moraghan and Helms (2005) provided information on the range of Fe concentrations in soybean seed in various genotypes. The authors even speculated that Fe

efficient genotypes could potentially have higher seed Fe concentrations than Fe-inefficient genotypes. Currently, there is a no known genetic link between Fe efficiency and Fe accumulation in soybean.

The purposes of this study were: to map QTL for seed and leaf iron and zinc concentration in the soybean population Anoka x A7, to determine if QTL for iron and zinc accumulation co-localized to the same regions of the genome, to determine if any newly identified QTL for Fe and/or Zn concentration correlated with QTL previously identified for Fe efficiency, to determine the amount of variation of total phosphorus in a population developed for iron deficiency chlorosis resistance, and to map total phosphorus as a QTL.

Dissertation Organization

This dissertation is organized into 6 chapters. After the current chapter, Chapter 2 provides background information as it pertains to this study as well as the research objectives and is followed by a literature review. The review includes genetics of the iron deficiency chlorosis response, iron efficiency and concentration in plant tissue, seed iron concentration studies, breeding for iron deficiency chlorosis resistance, and quantitative trait mapping of other minerals. Chapters 3 and 4 are written in journal article format and will be submitted for publication. Chapter 5 summarizes the conclusions from this study.

Chapter 3 of this dissertation is a journal article prepared for submission to the *Journal of Plant Nutrition* is *in press* and entitled "Mapping of iron and zinc quantitative trait loci in soybean (*Glycine max* (L.) Merr.) for association to iron deficiency chlorosis

resistance." This chapter evaluates iron and zinc concentration in seed and leaves to determine if there is a genetic link between iron efficiency and mineral accumulation in an Anoka x A7 population. This population was originally developed to map QTL for iron deficiency chlorosis resistance. Metals such as zinc and iron can be transported via the same pathways and genes, consequently, co-localization of iron and zinc QTLs is evaluated. The author contributions for this manuscript are: Keith E. King performed the research and writing related to phenotyping, SSR genotyping, and QTL analysis under the supervision of Randy Shoemaker. Gregory Peiffer helped in the field layout, leaf tissue collection for DNA analysis, SSR genotyping, and harvesting mature seeds. Manju Reddy provided incite and technical advice for the methods used to determine iron concentration. Nick Lauter provided the research software license and guidance for QTL mapping. Shun Fu Lin performed original work to develop the first genetic map in this population. Silvia Cianzio provided guidance on the research and provided the population for evaluation.

Chapter 4 of this dissertation is a journal article prepared for submission to the *Euphytica* and is entitled, "Evaluation and QTL mapping of total P in Soybean." This chapter evaluates the total phosphorus variation among genetic lines of the Anoka x A7 population, and the trait is mapped as a QTL. Total phosphorus uptake, transport, and storage candidate genes are identified, and their functions are discussed. The author contributions for this manuscript are: Keith E. King performed the research and writing related to phenotyping and QTL analysis under the supervision of Randy Shoemaker. Nick Lauter provided the research software license and guidance for QTL mapping. Shun Fu Lin performed original work to

develop the first genetic map in this population. Paul Scott provided technical assistance and guidance with phosphorus extraction protocols. Following these two research papers are General Conclusions and References chapters. Works cited in the General Introduction and Conclusions are listed in the final References section.

CHAPTER 2. LITERATURE REVIEW

Introduction

Iron's availability in the environment and its importance to plants

Iron (Fe) is classified as a micronutrient in plants. However, Fe is the fourth most abundant element in the earth's crust. Fe is very important to all biological organisms because of its physicochemical properties (Grotz and Guerinot, 2006; Briat, 2005). Fe is one of the most versatile metals in biology, functioning in two ionic forms created in the reversible redox reaction of ferrous (Fe^{2+}) and ferric (Fe^{3+}) iron. Iron also has the ability to form octahedral complexes with various ligands and to vary its redox potential in response to different ligand environments (Hell and Stephan, 2003).

In plants, a continuous supply of Fe is required for cellular processes such as electron transport in photosynthesis, respiration, and cell division. In legumes, Fe also plays a very important role in nitrogen fixation within the nodules. Although Fe is one of the nutrients that is most often a limiting factor in growth and development, in over-accumulation it can be toxic. A deficiency can be equally detrimental when plants cannot acquire enough iron for biological processes (Colangelo and Guerinot, 2004; Briat et al., 2009; Schikora and Schmidt, 2001). This makes Fe uptake, utilization, and storage central to maintaining homeostasis (Connolly et al., 2003; Motta et al., 2001).

Iron Deficiency Chlorosis in Soybean

Even though abundant in the earth's crust, Fe is often unavailable for plants to utilize because it forms insoluble ferric hydroxide complexes in aerobic environments at neutral or basic pH (Connolly et al., 2003). Soil pH has a very strong influence on Fe availability and solubility. For every one unit drop in pH, Fe becomes a thousand-fold more soluble (Grotz and Guerinot, 2006). Fe in the soil is almost exclusively present in the oxidized form Fe^{3+} rather than in the Fe^{+2} form which is required for the plants (Krouma et al., 2006). The resulting limited availability of Fe because of the low solubility of Fe^{3+} causes what is known as Iron Deficiency Chlorosis (IDC) in plants. IDC is characterized in soybean (*Glycine max* (L.) Merr.) by stunted plants with interveinal pale green or yellow-to-nearly-white leaves with green veins. IDC is not usually observed until the first trifoliate leaf emerges, since prior to this developmental stage, Fe from the cotyledon is translocated to new growth. After the first trifoliate emerges, the soybean must rely on soil availability for its supply since the younger leaves cannot acquire iron from the older leaves. Chlorosis develops when insufficient Fe is supplied to leaves. IDC may be so severe that necrosis and death of the leaves or entire plant occurs (Franzen, et al., 2004; Zheng et al., 2009).

Iron deficiency generally occurs in plants grown on calcareous soils, which are common in the upper Midwestern United States. Worldwide, calcareous soil is estimated to make up 30% of the world's arable land (Lin et al., 1997; Mori, 1999). Poor aeration in soil has also been linked to Fe deficiencies, but the mechanism of this interaction is not known. It has been hypothesized that one of the reasons may be the relation between poor aeration

and decreased root development, which may result in increased ethylene concentrations in the rhizosphere (Mortvedt, 1991). Other soil factors that may combine to hinder iron uptake include temperature, $CaCO^{-3}$ content, water content, and the concentration of HCO^{-3} in the soil solution. Early in the growing season, cool, wet soil conditions or poorly drained soils help to intensify IDC expression in calcareous regions where Fe deficiencies are common (Franzen et al., 2004).

Plants, as oxygenic photosynthetic organisms, are faced with two challenges; one is to acquire Fe from an inorganic environment, and the second is to make this Fe available in bound organic form for the basic processes of growth and development. In order for this to occur, plants have developed two different strategies for iron uptake by roots (Hell and Stephan, 2003). Graminaceous plants utilize a Strategy II response that relies on chelation of Fe (III) rather than reduction. The response is mediated by the synthesis and secretion of natural Fe chelators from the mugineic acid family of phytosiderophores, allowing the solubilization of Fe^{3+} in the rhizosphere and re-absorption into root cells (Kobayashi et al., 2005). Soybeans and other dicotyledonous plants utilize a Strategy I response to overcome Fe deficiency. This response occurs because several physiological mechanisms act to increase solubility and uptake of Fe from soils. Strategy I plants release from their roots reductants or chelators in the rhizosphere, thereby enhancing proton excretion into the rhizosphere to acidify the soil, increasing their ferric reduction capacity at the root surface, and transporting Fe^{2+} ions through the Fe^{2+} transporter in the plasma membrane (Dasgan et al., 2004; Mori, 1999). The Strategy I response is also influenced by root morphology changes, which include

root hair, transfer cell developments, and an increase in citrate concentration in the phloem (Hell and Stephan, 2003). Genotypes that have a faster response to Fe deficient conditions are considered to be Fe efficient.

Genes Involved in the Fe Efficiency Response

In recent times, the genes and gene families that are involved in Fe uptake under Fe deficient conditions have been identified and further characterized. Plants that are Fe efficient are able to respond to the Fe-limited condition and delay the onset or minimize the impact of IDC. However, inefficient genotypes respond slower or lack the capability to respond. In non-graminaceous plants such as soybean, the key gene inducer in the Fe deficiency response is FRO2 (Ferric-Chelate Reductase) that reduces Fe at the root-soil interface (Briat, 2005; Walker and Connolly, 2008). FRO2 belongs to an eight-member gene family and is primarily expressed in roots, with other members of the family being expressed in shoots. IRT1 (Iron Regulated Transporter) has been established as the major iron uptake system in response to Fe deficiency and is responsible for transporting the reduced Fe across the plasma membrane of root epidermal cells. IRT1 has also been shown to be responsible for uptake of other metals including Zn, and is a member of the ZRT IRT-like Protein family (ZIP) (Grotz and Guerinot, 2006; Curie and Briat, 2003; Hell and Stephan; 2003).

Further work has shown that the induction of the FRO2/IRT1 system is controlled by FIT (FER-Like Iron Deficiency Induced Transcription Factor), a functional ortholog of transcription factor FER that functions in the Fe deficiency signaling pathway. Along with

FIT, basic helix-loop-helix (bHLH38/39) is increased under Fe deficient conditions, where FIT heterodimerizes with either bHLH38 or bHLH39 to induce the transcription of FRO2 and IRT1 at the protein level in the root cells. The heterodimers of FIT/bHLH38 and FIT/bHLH39 directly activate or enhance the expression of the iron uptake genes like FRO2 and IRT1 for effective Fe acquisition. Even though FIT can interact with itself in the plant cell and form a homodimer, the FIT dimer itself is not involved in controlling the transcription of FRO2 and IRT1 (Yuan et al., 2008; Walker and Connolly, 2008; Grotz and Guerinot, 2006). The expression of bHLH38/39 is induced in roots and shoots of plants grown in Fe deficient conditions; however no or very low expression is detected in the leaves and roots of plants grown under Zn or Mn deficiency conditions (Yuan et al., 2008).

Fe Efficiency and Fe Concentration in Plant Tissue

Iron status in leaves as well as leaf Fe concentration could be very important factors in developing crops, such as soybean, that are resistant to IDC. Determining Fe concentration is not a new concept, as several studies have evaluated leaf Fe concentration in different crops. Early on, the assumption was that leaves that exhibited chlorosis should have less Fe than green leaves. Chlorotic leaves were found to actually contain more iron than green leaves (Oserkowsky, 1933; Bennett, 1945). Several decades later in 1975, additional research showed that Fe concentration in the same plant species can vary up to 500% depending on the environment in which the plants are grown. Not surprisingly however, levels of other plant constituents also vary widely as the plant responds to the environment,

with some such as phosphate being important in Fe absorption (Wien et al., 1975).

The problems of variability in Fe concentration across generations and genotypes need to be better understood before Fe-enhanced varieties can be used in the field (Qu et al., 2005). The Fe content of plants varies considerably, with the range in normal plants being 60-300 ppm, and the range in Fe-deficient plants being 10-30 ppm. In contrast, during Fe-excessive conditions plants can have levels between 400-1000 ppm. Interpretation of these levels of Fe in plants can be complicated because total plant Fe content is only a general guide to the plant's nutritional status (Vose, 1982). Fertilizer application and rate can also have an impact on the amount of Fe in leaves of plants. Mottaghian et al. (2008) evaluated micronutrient accumulation in soybean cultivars after application of organic and chemical fertilizers. The authors found that there was a significant interaction, with cultivar and fertilizer producing discernible levels of Fe content. The highest level was obtained in the treatment consisting of municipal compost enriched with a chemical fertilizer.

Plant roots are essential to micronutrient uptake throughout the growth and development of the plant (Garnett and Graham, 2005). However, Mengel (1994) was able to show, in young roots and leaves of green and chlorotic plants grown on calcareous soil that high Fe concentration in roots did not necessarily corresponded with adequate Fe supply to the rest of the plant. In the experiment, chlorotic plants were found to have high Fe concentration in roots. On this basis it was concluded that chlorosis could be related both to the mobilization of root Fe and its translocation to upper plant parts and to the efficiency of leaf use of Fe. The amount of Fe in leaves and eventually the seed is related to the mobility

and redistribution of Fe from vegetative tissue via the phloem (Garnett and Graham, 2005). In beans and peas, researchers have found evidence of good remobilization. Zhang et al. (1995) reported this in mature bean leaves, and Grusak (1995) found that as much as 75% of total shoot Fe was ultimately found in the seeds.

Fe content in leaves and chlorosis has been commonly measured as chlorophyll concentration in the leaves. However, Mengel (1994) reported that numerous research findings have shown there is no correlation between Fe concentration in leaves and chlorophyll concentration of the same leaves. Therefore, chlorophyll concentration in leaves is not always a good indicator of the degree of Fe deficiency. Norvell and Adams (2006) found that concentrations of chlorophyll and Fe in soybean leaves were strongly influenced by cultivar and Fe supply. They found that A7, an IDC-resistant line, generally contained the highest concentration of leaf Fe of any treatment. This was evident even in the low Fe treatment, in which A7 accumulated roughly 50% more leaf Fe than the least resistant cultivar.

Seed Fe and the Fe Efficiency Response

Seeds are heterotrophic organs, which makes them totally dependent on nutrients from the parent plant for their growth and development. Nutrient loading of seeds not only influences seed number at seed set, but also determines final seed size (Zhang et al., 2007). The concentration of Fe in seeds can help in breeding for tolerance/resistance to IDC, because micronutrient reserves of seeds represent a significant source of mineral elements for

early seedling establishment in nutrient-limited growing conditions (Bityutskii et al., 2002).

Additionally, although the total Fe balance between seeds and leaves is relatively unknown, Fe translocation from leaves to seeds may be very important in determining Fe accumulation in seeds (Qu et al., 2005). One would think that the amount of Fe in the soil would dictate how much Fe is taken up, utilized by the plant, and eventually redistributed to the seed. However, in wheat, Garnett and Graham (2005) reported that increased Fe availability did not increase Fe content in the grain. The implication here is that increasing Fe level by root uptake or foliar application would not be effective in increasing Fe content in grain.

Several studies have been carried out on Fe concentration in the seed. Some of the research evaluated germplasm lines and others evaluated transgenic seeds. Bityutskii et al. (2002) evaluated 25 genotypes to determine the distribution of Fe as well as that of several other micronutrients in mature maize (*Zea mays* L.) seed grains. The authors reported the highest concentration of micronutrients was in the seed coats and the scutellum, with lowest level measured in the endosperm. Among the 25 genotypes, Fe concentration in the scutella varied from 103 to 188 µg g^{-1}, while in the endosperm it ranged from 32 to 47 µg g^{-1} on a dry weight basis. However, variation in micronutrient concentration in the endosperm was insignificant. Also important was the observation that removal of seed coats did not influence dynamics of micronutrients in grains and seedlings. Shen et al. (2002) evaluated genotypic differences in seed Fe content and early responses to IDC in 26 cultivars of wheat (*Triticum aestivum* L.). The authors found significant differences in Fe content among the cultivars,

with the difference in seed Fe content mainly attributed to genotypic differences rather than to seed size. Also, seed high in Fe had better seedling vigor, greater chlorophyll concentration in fresh, fully expanded leaves, and greater Fe concentration in both roots and shoots compared with plants from low-Fe seed under a no-Fe treatment. The authors suggested that seed Fe content should be evaluated when comparing genotypes for resistance to Fe deficiency, particularly at early growth stages.

When Tiffin et al. (1973) evaluated the translocation of Fe from soybean cotyledons, they found that the mean Fe in the seed coat was 29.9% of the seed Fe, which indicated that the Fe concentration in the seed coat was five times greater than that of the embryos on a dry weight basis. The authors reported a range in Fe content of 44 to 74 µg Fe/g in seeds and other tissues. Moraghan and Helms (2005) evaluated seed Fe in 27 soybean genotypes differing in seed size. The authors stated that there is relatively little knowledge about diversity in seed Fe accumulation in the soybean germplasm pool. They also speculated that Fe efficient soybean cultivars possibly have a higher seed Fe concentration than Fe inefficient genotypes. Genotypes differed in seed Fe concentration, having a range of 48-81 µg Fe g^{-1}. As in the maize grain experiment, in which distribution of dry matter between the seed coat and embryo were compared, soybean seed Fe was much more concentrated in the seed coat fraction than the embryo for all genotypes. Seed Fe content in soybean ranged from 4.6-14.3 µg Fe g^{-1} seed, with the variability in seed Fe content mainly due to differences in seed weights of genotypes. The genotype that had the highest average seed Fe concentration also had the highest seed Fe concentration at individual locations. Conversely,

the genotype with the lowest seed Fe concentration also had the lowest concentration at each individual location. This provides evidence that Fe efficient plants can produce seed with higher Fe concentration than Fe inefficient plants. Interestingly enough, Tiffin et al. (1973) stated that Fe deficient seedlings, export 90% of the Fe located in the cotyledons. This suggests that iron export is an adaptive response for the plant to provide physiologically important Fe to developing cells remote from the storage site. Moraghan and Helms (2005) concluded that the use of seed high in Fe to reduce early-season IDC in relatively Fe efficient soybean genotypes would likely necessitate that the parent seed be grown on soils high in available Fe, which are generally acid soils. Wiersma (2007) found that the cultivar 'Corona' possibly had an inherent genetic ability to acquire, transport, and/or remobilize Fe to a greater extent than the less Fe efficient cultivars 'Jim' and 'Daksoy,' an effect observed across treatments. Even though seed Fe concentration appeared to be regulated by genotype, in the less efficient varieties seed Fe concentration was increased by increasing seeding density or reducing the severity of Fe deficiency.

Bioavailability-Issues with Phytate and Phosphorous

Another concern in developing seed high in Fe content is the bioavailability of the Fe in the end product. In soybean and cereal seeds, phytate, the naturally occurring form of phytic acid, is one of the major inhibitors or Fe bioavailability (Drakakaki et al. 2005). Interestingly, Prom-u-thai et al. (2006) stated that phytate concentration was not consistently related to the Fe concentration and bioavailability in unpolished, polished rice grain, and bran

fractions, but this could have been due to the limited range of variability in phytate among the five genotypes they tested. According to Wilcox et al. (2000), conventional soybean contains about 4.3 g kg^{-1} phytate and 0.7 g kg^{-1} inorganic P. Raboy et al. (1984) reported phytic acid varied from 67 to 77% of total P among seeds of soybean. Consequently, phytic acid P is typically highly and positively correlated with seed total P, with correlation coefficients (r) > + 0.90, and influenced by the supply of phosphorus to the developing seed (Raboy et al. 2001). Identification of Fe efficient varieties with significantly lower phytate levels would be ideal. In 2000, Wilcox et al. developed a low-phytate soybean line (CX1834-1-6) through chemical mutagenesis, and since that time several studies have evaluated seed traits as well as the inheritance of the trait within the line. Oltmans et al. (2005) found that lines that had the low phytate genotype had reduced seedling emergence. The authors also determined that the low phytate trait was controlled by recessive alleles (*pha*1 and *pha*2) at two independent loci that exhibited duplicate dominant epistasis. Walker et al. (2006) concluded that the inheritance of seed phytic acid content was quantitative and that loci on LGs N and L are associated with variation in seed phytic acid content, which was also inversely related to seed inorganic P in the low phytate mutants. Their marker data indicated that a locus near Satt237 on LG N and a locus near Satt527 and Satt561 were associated with seed inorganic P levels. The authors also stated, "according to data reported by Shoemaker (2004), that loci detected on the mentioned linkage groups also appeared to be located in duplicated regions of the soybean genome, since RFLP probes B162 and A535 annealed to sequences flanking the estimated location of loci on both linkage groups." Of interest to this

research is whether QTL associated with Fe efficiency map close to the region associated with seed phosphorus concentration. According to Lee et al. (2004) and Lin et al. (1997) QTLs associated with salt tolerance and IDC have mapped to both the region of LG N and to the region of the phytate locus on LG L.

Challenges to Managing IDC and Identifying Resistance

Several studies have been performed to evaluate the best management practice to decrease IDC occurrences in the field. Longnecker and Welch (1990) stated that the best long-term solution to IDC is to breed resistant cultivars, but the lack of a clear understanding of the physiology of resistance to Fe deficiency has been an impediment in breeding programs. Brown and Holmes (1955) concluded from their results that the inability of the plant to absorb Fe from the soil, the inactivation of Fe in the plant, and the effect of Cu on the utilization of Fe, may be co-participants with the fundamental physiological and biochemical difference between plants species and varieties. They also concluded that having a clear understanding of these differences in plant metabolism would probably lead to an understanding of the causative factors for IDC. This understanding would lead to efficient breeding and selection programs.

The high level of temporal and spatial variability in chlorosis expression in the field is a challenging factor in studying and developing genotypes resistant to Fe deficiency. In some years, chlorosis symptoms are expressed during early growth stages and disappear as the plants mature. In severe cases, chlorosis can persist throughout the entire season. Chlorosis

symptoms generally occur in patchy areas of fields and frequently, but not always, in low areas. Even more confusingly, chlorotic patches have not been shown to occur in patterns consistent with changes in soil type (Hansen et al., 2003). Due to this patchiness, Fe deficiency problems are frequently localized in sporadic areas making treatment applications very challenging (Vose, 1982).

Over a half century has been dedicated to identifying soybean genotypes resistant to Fe deficiency. This in turn, has led to understanding the Fe deficiency response used by soybean. Spatial field variation complicates screening resistant genotypes. There is a wide variation in susceptibility to Fe deficiency among different soybean varieties (Hansen et al. 2003). Goos and Johnson (2000, 2001) evaluated several methods for reducing Fe deficiency in soybean and concluded that cultivar selection was the most effective tool for reducing chlorosis. The authors also found that increasing the seeding rate reduced chlorosis of all cultivars evaluated, although for growers this would not be the most cost-effective method. An interpretation of this result is that increasing seed planting rates may increase exudate concentration, which would have a positive impact on the available Fe (Lingenfelser et al., 2005). Other researchers have come to similar conclusions. Wiersma (2005) and Mortvedt (1991) stated that cultivars tolerant to IDC are considered to be the most economical and practical means to profitable production on chlorosis-prone soils, although even the best cultivars can suffer yield losses when harsh conditions prevail.

Lingenfelser et al. (2005) in Kansas stated that the use of resistant genotypes proved to be the most effective treatment in reducing chlorosis scores but resulted in a considerable

yield penalty. The authors also found that Fe sulfate fertilizers were not more effective than the untreated controls at controlling IDC and increasing yield and should not be recommended as a treatment for soybean in the state. They also stated that Fe foliar sprays and Fe chelate seed treatments should not be recommended for Kansas growing conditions. Increasing soil fertility through the addition of plant residue was also not a recommended practice, because this may also cause increased disease. On the contrary, Mortvedt (1991) stated that foliar sprays remained the predominant method of correcting IDC of field crops although some iron sources may be rather ineffective even as foliar sprays, usually requiring more than one spraying application for complete correction.

All the mentioned factors related to soil and symptom expression impose limitations on research for evaluating soybean genotypes under field conditions. To overcome these limitations hydroponic systems have been used for genotype evaluations. Coulombe et al. (1984) established the chemical composition of the nutrient solution and the basic hydroponics system for IDC evaluations. Since then, the system has been used to study chlorosis in controlled environments where iron is the only limiting factor to plant growth. Advantages of screening in greenhouse or controlled conditions include the fact that screening can be done year-around, each cycle of evaluation can be completed in a relatively short period of time, higher levels of chlorosis severity can be induced, and the problems of spatial and temporal variations observed on calcareous soils and under general field screening conditions can be avoided (Lin et al., 1998). It is important to note, that Jessen et al. (1988) reported high rank correlations between field and nutrient solution evaluations

when eight soybean cultivars with a varying range of chlorosis ratings were used.

Genetics and Breeding for IDC Resistance

Several studies have reported that various genes control IDC resistance. The first report was by Weiss (1943) who showed that the difference in efficiency of Fe utilization between susceptible and resistant soybeans was conditioned by a single recessive gene. Bejiga et al. (1996) evaluated kabuli chickpea (*Cicer arietinum* L.) for resistance to IDC and concluded that recessive genes control IDC. The authors also recommended negative selection to discard susceptible genotypes from breeding material as an effective breeding strategy. In peppers (*Capsicum* spp.), Fe deficiency is controlled by a single dominant gene, and in dry beans (*Phaseolus* spp.), two complimentary, dominant genes control iron deficiency tolerance (Cianzio, 1991; Shifriss and Eidelman, 1983; Coyne et al., 1982). However, Cianzio and Fehr (1980, 1982) determined that a major gene and several modifying genes controlled iron deficiency tolerance in soybeans or that there was a quantitative trait locus (QTL) with additive gene action.

Even though many years have been dedicated to identifying IDC-resistant cultivars, success in breeding efficient/resistant soybean cultivars has been limited. One of the earliest populations (AP9) developed was by Fehr and Cianzio (1980), and several years later through recurrent selection A7 was released as an Fe-efficient germplasm line (Fehr et al., 1984). Another germplasm line, A15, which also has been used to develop a population to identify the genetic mechanism of resistance, was released in 1988 (Jessen et al., 1988). In

most recent times, AR2 and AR3 have been released for Fe efficiency; these were developed with molecular marker Satt481, which has been shown to be significantly associated with Fe efficiency across multiple environments (Cianzio et al., 2006a, 2006b; Charlson et al., 2003).

QTL Mapping and Markers for Iron Deficiency Chlorosis

Studies designed to map genes responsible for IDC have resulted in the identification of multiple QTL, however all genes involved in the efficiency response have yet to be identified. Lin et al., (1997) confirmed that depending on the parents used to develop the population, IDC was controlled by one of two genetic mechanisms: a major gene with modifying genes, or a polygene mechanism with each gene having a minor effect. The two populations evaluated were Pride B216 x A15 and Anoka x A7. Four QTLs were found to be responsible for 21.6% of the phenotypic variation in the Pride population. In the Anoka x A7 population two QTLs were responsible for 75.3% of the variation and a major QTL mapped to linkage group N. However, these QTLs did not prove useful in marker-assisted selection (MAS) because flanking markers of the QTLs polymorphic in one population were absent in the other (Lin et al., 2000). Previous work had also demonstrated that nutrient solution and field tests identify similar QTLs controlling IDC in soybean (Lin et al., 1998).

Development of DNA-based markers is important for selection and improvement of varieties and hybrids in plant breeding programs (Van et al., 2005), and marker-assisted selection (MAS) has emerged as a possible tool in breeding for IDC resistance. Charlson et al. (2003) found simple sequence repeat (SSR) markers, which were genetically linked to

previously identified QTL for IDC resistance. These were tested on two parents not used in previous research, P9254 and A97-770012, and were evaluated for their efficiency in selecting IDC resistance in soybean. After testing 103 SSR markers, 23% of the markers were identified as polymorphic between the parents. Of these 16 markers were examined for their association with field data from the population obtained in 2001. Several markers (Satt211, Satt181, Satt292, Satt448, Satt481, and Satt237) were found to be significantly associated with chlorosis scores. It was concluded that it may be possible to develop molecular markers as effective tools for identifying IDC-resistant lines within breeding populations. These markers for selection to IDC resistance will decrease the time required for evaluation and screening for resistance.

QTL Mapping for Efficiency of Other Minerals in Soybean and Other Plants

In the limited success of breeding for IDC resistance, there is still a need to develop more effective strategies for identification and selection of Fe-efficient genotypes. Currently, according to the Soybase website (http://www.soybase.org), there are 36 QTL associated with Fe efficiency, with the last update for these QTL in 2003. There are also two QTL for flood tolerance, one for salt tolerance, and 17 for seed nitrogen. However, there is a lack of information on QTL that are associated with macro and micronutrients in the seed that could be associated with mineral efficiency. QTL analysis for mineral accumulation could also identify genes encoding, for example, transporters, chelators, or chelator biosynthesis enzymes, in addition to regulatory factors such as protein kinases, membrane receptors, or

transcription factors (Vreugdenhil et al., 2004).

Success has been realized in mapping QTL for mineral contents of Zn, Fe, Cu, Mn, and P in wheat, rice, *Arabidopsis thaliana*, *Brassica* napus, maize (Zhou et al., 2010; Garcia-Oliveira et al., 2009; and Ding et al., 2010). Tiwari et al., (2009) mapped QTL for both Fe and Zn concentration in wheat. They identified two QTL for grain Fe and 1 QTL for grain Zn. Vreugdenhil et al. (2004) evaluated content of several minerals in *A. thaliana* and identified QTL for Fe, Mn, Zn, K, Na, Mg, and P. More importantly, some of the QTL seemed to co-localize with each other, four of which were K-Ca QTL. There was also one QTL where co-localization occurred with K-Ca-Mn, which was also in the region where there is a strong QTL for phosphate and phytate accumulation in seeds and leaves. The authors stated that this co-localization could possibly be coincidental or may rely on two or more different and closely linked genes, especially since maintaining cation homeostasis requires a network of metal uptake, transportation, trafficking, and sequestering mechanisms. Willems et al. (2010) also mapped QTL for mineral concentrations in the progeny of the cross of *A. halleri* and *A. lyrata petraea* that was grown on soil contaminated with cadmium. The authors identified four QTLs for Zn accumulation on four linkage groups, which explained 54.7% of the genetic variance. One of these QTL was a major QTL in the soil with normal Zn concentrations. They also identified 3 QTLs for Fe on three separate groups, which explained 35.3% of the phenotypic variance. There was also one suggestive Fe QTL contributing to 5.5% of the phenotypic variance.

At present, research has identified QTL for Fe efficiency in soybean, and it has been

proven that molecular markers, although very limited, can be used in marker-assisted selection to improve Fe efficiency. However, breeding of completely IDC resistant genotypes has not been as successful. Some genotypes that are thought to be tolerant or resistant still exhibit symptoms of IDC when the conditions are extreme. This expression results in a negative impact on yield, which costs the producer. Fe efficiency is a quantitative trait, and other traits can be evaluated to better determine efficiency and to identify resistant genotypes. Mineral contents such as Fe and Zn have been successfully mapped as QTL in several plant species. This has yet to be done in soybean, but more importantly there is lack of information correlating mineral efficiency with mineral content in soybean. Furthermore, populations that have been used for mapping QTL for Fe efficiency have yet to be characterized for mineral content and lack QTL corresponding to these Fe and Zn. Also, in general, QTL for mineral content in soybean is very limited. This research aims to identify QTL for Fe and Zn content and determine the relationship of Fe-efficiency QTL in a soybean population developed for Fe efficiency; this would allow the selection of Fe-efficiency genotypes with increased mineral content. IDC often is observed early in the growing season, when less Fe-efficient genotypes cannot obtain Fe from the soil. Elevated Fe could possibly delay the onset of IDC until the plant is able to obtain Fe from the soil.

CHAPTER 3. MAPPING OF IRON AND ZINC QUANTITATIVE TRAIT LOCI IN SOYBEAN (*Glycine max* (L.) Merr.) FOR ASSOCIATION TO IRON DEFICIENCY CHLOROSIS RESISTANCE

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Abstract

Iron deficiency chlorosis (IDC) is a nutritional disease of soybean (*Glycine max* (L.) Merr.) that when left unchecked can result in a severe yield penalty or even death in the most extreme cases. In order to curb these effects, resistance to the disease is needed. Breeding for resistance has been somewhat successful but no cultivar has complete resistance. Mineral content of the soybean could be an indicator of the ability of the plant to withstand the effects of IDC. Iron and zinc concentration in soybean seed and leaves was evaluated as an estimator of mineral deficiency resistance in early growing stages, when symptoms are commonly observed. The progeny ($F_{2:4}$) of the cross Anoka x A7, previously used to map Fe efficiency QTL, were analyzed to quantify Fe and Zn concentration for QTL mapping and determine if there was a relation to previously identified Fe efficiency quantitative trait loci

QTL. Significant differences in Fe and Zn concentration were observed in the progeny population. The range for seed Fe concentration was 67.6 – 92.7 µg/g, and for seed Zn the range was 3.5 – 4.0 µg/g. One hundred-fifty SSR, RFLP, and BARCSOYSSR markers completed the linkage map used for (QTL) mapping of Fe and Zn concentrations. The QTL analysis in the combined data identified one major QTL for seed Fe accumulation that mapped to chromosome 20 and explained 21.5 % of the variation. Three suggestive QTL were mapped to chromosomes 1 and 12 with chromosome 12 having two peaks. These QTL explained 10.6-12.7 % of the variation. No significant QTL for seed Zn concentration were identified, however there were two suggestive QTL explaining 18.5 and 23.4 % of the phenotypic variation. The significant QTL for Fe concentration identified was in the marker interval *pa_515-1-Satt239*. Marker *pa_515-1* was previously used to map Fe efficiency QTL and thus provides the first evidence of a potential genetic link between Fe efficiency and Fe accumulation in the soybean seed.

Introduction

Iron deficiency chlorosis (IDC) affects plants grown on calcareous soils with a high soil pH, which is a common soil characteristic in the upper Midwestern United States. IDC is observed in the interveinal tissue of new leaves. This phenotype is a direct result of the plant's inability to utilize available iron from the rhizophere (Froehlich and Fehr, 1981). IDC in soybean (*Glycine max* (L.) Merr.) results in stunted plants with interveinal pale green or yellow to nearly white leaves, shortened internodes, and in the most extreme cases necrosis

and death. Ultimately, plants surviving any expression of IDC will experience a reduction in yield (Franzen, et al., 2004; Zheng et al., 2009; Niebur and Fehr, 1981).

Strategies to alleviate IDC symptoms have included increasing seed density, seed treatments with Fe-EDDHA, and foliar application of Fe-EDTA. Each method has garnered limited success and requires an added cost to the producer (Wiersma, 2007; Goos and Johnson, 2000; 2001). A more practical and cost effective approach is cultivar selection based on genetic tolerance/resistance to IDC. Seed Fe concentration has been proposed as a selection factor for breeding for tolerance/resistance to IDC, because micronutrient reserves of seeds represents a significant source of mineral elements for early seedling establishment in nutrient-limited growing conditions (Bityutskii et al., 2002).

Research in several plant species has been done to determine Fe concentration in the seed (Garnett and Graham, 2005; Bityutskii et al., 2002; Cichy et al., 2009; Silva et al., 2003). However there has been little reported on the direct relationship between IDC tolerance/resistance and Fe concentration in soybean. Shen et al. (2002) showed that wheat seed high in Fe had better seedling vigor, and greater chlorophyll concentration in fresh fully expanded leaves. Moraghan and Helms (2005) evaluated seed Fe in 27 soybean genotypes differing in seed size. Genotypes differed in seed Fe concentration from 48 to 81 µg Fe g^{-1}. Seed Fe content ranged from 4.6 to 14.3 µg Fe g $seed^{-1}$. Variability in seed Fe content was mainly due to differences in seed weights of genotypes, whereas seed Fe concentration was not correlated with individual seed weight. The authors were able to provide evidence that Fe-efficient plants produce seed with higher Fe concentration than Fe-inefficient plants.

Earlier, Tiffin et al. (1973) reported that Fe-deficient seedlings export 90% of the Fe located in the cotyledons. This suggests that iron export is an adaptive response for the plant to provide physiologically important Fe to developing cells remote from the storage site.

Quantitative trait loci for IDC have been mapped. However, many genes involved in the iron stress response/adaptation have yet to be identified. Parental selection can influence the genetic mechanism for response to IDC. Lin et al. (1997) confirmed that IDC is a complex trait, controlled by either a major gene with modifying genes, or by a polygenic mechanism with each gene having a minor effect. The populations evaluated were Pride B216 x A15 and Anoka x A7. Four QTLS were found to be responsible for 21.6% of the phenotypic variation in the Pride B216 X A15 population. In the Anoka x A7 population two QTLs were responsible for 75.3% of the variation with the major QTL mapped to linkage group N (chromosome 3) and accounted for 72.2 % of the variation.

Charlson et al. (2003) reviewed simple sequence repeat (SSR) markers which were genetically linked to resistance and significantly associated with chlorosis scores. They determined that genotypic selection with resistance-linked markers could increase the potential of selecting resistant lines in the field. The markers that were most promising were in regions of Fe efficiency QTL. A total of 36 QTL have been associated with Fe efficiency (http://www.soybase.org). A recent study using more than two-hundred genotypes and association mapping identified additional IDC-related loci (Mamidi et al. 2011). However, there is a lack of information on QTL that are associated with the macro- and micronutrients that are loaded into the seed and leaves and which could be associated with mineral

efficiency. QTL analysis for mineral accumulation could also identify genes encoding, for example, transporters, chelators or chelator biosynthesis enzymes in addition to regulatory factors such as protein kinases, membrane receptors, or transcription factors (Vreugdenhil et al., 2004).

For instance, the IRT1 (iron regulated transporter), a member of the ZIP (Zinc Regulated Transport IRT-like protein family), is involved in the response to Fe stress, but it is also involved in the transport of Zn (Grotz and Guerinot, 2006). Zn availability and uptake is important to plant growth and development and especially to tolerance for both biotic and abiotic stress, as well as electron transport in photosynthesis (Kirkby and Romheld, 2004). Like Fe, the environmental and genetic components to Zn deficiency have yet to be fully characterized. However, Zn is also a common crop deficiency observed in soils high in pH as with Fe deficiency (Broadly et al., 2007; Wissuwa et al., 2006). Both Fe and Zn utilize similar mechanisms for mineral transport as well as response to mineral limited environments.

The objectives of this research were to 1) identify seed and leaf QTL for iron and zinc concentration in the soybean population Anoka x A7, the same population previously used to identify a major gene and modifying gene mechanism QTL for IDC efficiency; 2) to determine if QTL for iron and zinc accumulation co-localized to the same regions of the genome; and 3) to determine if any of newly identified QTL for Fe and/or Zn concentration correlate with QTL previously identified for Fe efficiency. If the QTL correlate it would suggest that genotype selection based upon iron and/or zinc composition in seed or leaves

may be an indirect selection method for IDC resistance.

Material and Methods

Plant Material

The plant material used for mapping of the QTL for soybean seed and leaf Fe and Zn concentration included 92 $F_{2:4}$ lines from Anoka x A7. This population was previously used to confirm a major gene with modifying gene action mechanism affecting IDC (Lin et al., 1997). A7 was the Fe efficient parent developed through a recurrent selection program, and Anoka was determined to be Fe inefficient (Fehr et al., 1984; Cianzio and Fehr, 1980). Inefficient/susceptible checks used in the study were Pride B216, Williams 82, and PI 547430, and efficient/resistant checks were A15 and Clark.

Phenotypic Evaluations

The 92 F2-derived lines and the checks were planted at the Bruner Farm, near Ames, IA on non-calcareous soil in 2008 and 2009. This soil has a Clarion-Nicollet loam soil type (fine-loamy, mixed, superactive, mesic Typic Hapludoll and fine-loamy, mixed, superactive, mesic Aquic Hapludoll). The experiment was set up as a randomized complete block design (RCBD) with three replications. Forty seeds were planted for each line/genotype with no thinning performed. The plots were 1.52 m long and 0.91 m in distance from adjacent plots. Leaf samples were taken from each line/genotype from the second fully expanded trifoliate 3-4 weeks after planting. Leaf tissue was then stored in liquid nitrogen, before being

lyophilized for analysis and DNA extraction. Upon maturity, seed from each plot was harvested in bulk for Zn and Fe analysis.

Nutrient analysis followed the procedure of Westerman (1990). Briefly, five grams of seed from each line/genotype were ground using a Foss Cyclotec mill (1093 Sample Mill, Foss, Eden Prairie, MN) equipped with a 1-mm screen. A 0.5 g subsample was taken from each genotype and dry-ashed in a muffle furnace in the following sequence: 200 ° C for 1 hour, 350 °C for 1 hour, and 500 ° C for a minimum of 4 hours but not more than 8 hours. Samples were then allowed to air cool for a minimum of 2 hours. Once cooled, they were digested in a dilute acid solution (300 ml HCl, 100 ml HNO3 in 1 L of ddH2O) and brought to a final volume of 10 ml for analysis. Correspondingly, a 0.5 g subsample of leaf tissue ground with mortar and pestle was dry-ashed and digested using the previous procedure. Fe and Zn concentrations along with standards of 0.00, 0.25, 0.50, 1.0, 2.0, 4.0, and 10.0 ppm Fe/Zn were quantified at the Iowa State Agronomy Soil and Plant Testing Facility using the Inductive coupled plasma-optical emission spectroscopy (ICP-OES) and computed as parts per million.

Statistical Analysis

For this experiment, all effects were considered random. Data was analyzed using standard ANOVA procedures with the JMP statistical package (JMP, Version 8. SAS Institute Inc., Cary, NC, 1989-2007). Broad sense heritability (h_b^2) was estimated on an entry

mean basis using expected mean squares from the combined ANOVA (Fehr, 1987) as follows:

$$h_b^2 = \frac{F_g^2}{\left(\frac{F_e^2}{r \times e} + \frac{F_{ge}^2}{e} + F_g^2\right)}$$

where F_g^2= genetic variance, F_{ge}^2= genotype x environment interaction variance, F_e^2= experimental error variance, r= number of replications, and e= number of environments. Expected mean square estimates that were negative were treated as a zero value. Zinc values were log transformed to normalize the data. Pearson's correlation coefficients were calculated for seed Fe and Zn concentration and leaf Fe and Zn concentration to evaluate the correlation, if any, between Fe and Zn accumulation in soybean seed and leaves.

Construction of the Genetic Linkage Maps

The parental lines Anoka and A7 were surveyed for polymorphism with 916 SSR markers available from the Soybase website (http://soybase.agron.iastate.edu). Most likely orders of and recombination rates among markers were estimated with Mapmaker 3.0 (Lander et al., 1987). Linkage groups were determined with the "group" command using a LOD of 4.0 and maximum Haldane distance of 50 centiMorgans (cM). Map order was determined using the "three point" command followed by "order", "framework", and "place." Previous markers that formed linkage groups for this population were integrated into the current map (Lin et al., 1997). Additionally, 303 BARCSOYSSR markers were downloaded from the Soybase website and surveyed for polymorphism to increase marker density on chromosomes with larger marker intervals (Song et al., 2010).

QTL Mapping

Linkage maps were imported into MapQTL6 (van Ooijen, 2009), and QTL positions and effects for soybean seed and leaf Fe and Zn were determined using interval mapping (IM) and multiple-QTL mapping (MQM) (Lander and Botstein, 1989; Jansen, 1993; Jansen, 1994). The significant threshold logarithm of the odds (LOD) scores for detection of the QTL were calculated based on 1,000 permutations at $P \leq 0.05$ (Churchill and Doerge, 1994). Both the genome wide and chromosome wide thresholds were determined in order to detect minor QTL effects that would be excluded under the genome wide threshold (Churchill and Doerge, 1994). As described by Liang et al. (2010), the IM method was used to determine locations of putative QTL for the traits. Subsequently, MQM was performed to eliminate inference from background markers. In order to reduce residual variance, background markers closest to the LOD peak were selected as cofactors. The mapping step size was 1.0 cM with the maximum number of neighboring markers five, and maximum number of iterations set to 200.

Results

Construction of Genetic Linkage Maps

The original genetic map of the Anoka x A7 used by Lin et al. (1997) which consisted of 82 RFLP, 14 SSR, and one morphological marker, was integrated into the current genetic map. Informative marker data were available for 146 SSRs and were assigned to the 20 linkage groups. One hundred-six SSR markers completed the linkage map along with the 12

informative BARCSOYSSRs. The complete linkage map consisted of a total of 150 markers. Using Haldane's mapping function and summing over all linkage groups, we obtained a total of 2722.09 cM flanked by linked markers. The average length of the linkage groups was 136.10 cM, with a range from 44.71 cM on chromosome 10 to 201.41 cM on chromosome 18. The average length of the marker intervals was 18.15 cM. The average number of markers per chromosome was 7.5, with a range from 3 to 14 (Figure 1).

Zn and Fe Concentration

The progeny in the population exhibited segregation for both seed and leaf Fe and Zn (Table 1 and Figure 2). Each of the traits exhibited a significant year effect ($P < 0.01$) (Table 2*)*. A significant genotype effect was detected for seed Zn and Fe, as well as leaf Zn. Seed Fe and leaf Zn exhibited a significant genotype x environment interaction effect. The trait with the highest broad sense heritability was seed Zn (0.47), and seed Fe followed with a broad sense heritability of 0.30 (Table 1.). Leaf mineral concentration had considerably lower broad sense heritability estimates than that of the seed minerals. Leaf Zn had no detectable heritability in this study and leaf iron was 0.14.

To evaluate the effect of environment on traits in the mapping population, Pearson's correlation coefficient (*r*) was determined for 2008 and 2009 and then over combined data (Tables 3 and 4). The highest correlations were between seed Fe 2008 and seed Zn 2008 (0.63) (Table 3.). This was followed by leaf Fe 2009 and leaf Fe 2008 (0.52), and leaf Zn 2009 and seed Fe 2008 (0.51). With the exception of four correlation coefficients, all were

significant at the P <0.01 level. The coefficient for seed Fe 2009 and seed Zn 2009 (0.12) was weak and non significant, which was different from that for 2008. With the exception significant correlations between leaf Zn 2009 with seed Zn 2008 and 2009 (0.46; 0.22), the rest of the coefficients were mostly significantly negatively correlated with leaf mineral concentration. Combined over years, seed Fe and seed Zn concentrations had a significantly high correlation (0.72), indicating that concentrations for each mineral may be controlled by the same loci for mineral loading (Table 4.). Leaf Fe and seed Fe had a small significant correlation, which could indicate that the same genes involved in iron uptake and translocation in the leaves are involved in the Fe loading phase of pod development. The rest of the coefficients for Zn minerals were negative and significant. This would indicate selecting for leaf Zn concentration could have a negative impact on the amount of Fe or Zn accumulated in the seed, which with leaf Zn having no detectable heritability could be expected.

QTL Analysis for Seed Zn and Fe Concentration

The data for 2008 and 2009 and the average across years were used for detection and mapping of QTL controlling seed and leaf Zn and Fe concentrations. For seed Fe concentration, two suggestive QTL were detected in 2008 (Table 5), whereas, one significant QTL and one suggestive QTL was detected in 2009. Suggestive QTL were those QTL that had peaks that did not exceed the genome wide threshold, but on the chromosome level were close to or significant at P = 0.05 (Tiwari et al., 2009; Willems et al., 2010). Those four QTL

were each on different chromosomes, with the significant QTL on chromosome 1 in the marker interval *Satt295-Satt383*. This QTL had a LOD score of 4.3, and represented 21.2 % of the variation for the trait. The three suggestive QTL ranged in LOD scores from 2.5-3.0 and were on chromosomes 7, 12, and 17. These were in the marker intervals *pk_417H-pk_70T*, *Satt635-pa_132*, and *Satt528-Satt488*, respectively. The R^2 values for these QTL were 20.1, 25.7, and 14.3. When data was combined over years, one significant QTL was detected and three suggestive QTL for seed Fe concentration (Figure 3). The significant QTL mapped to chromosome 20 in the marker interval *pa_515-1-Satt239* with a LOD score of 4.7 and representing 21.5 % of the variation. The suggestive QTL were on chromosome 1 (one) and on chromosome 12 (two). The LOD scores for these QTL were 3.2, 3.4, and 2.8, respectively. They had R^2 values of 10.6, 12.3, and 12.7 and were in the marker intervals *Satt532-Satt321*, *Satt635-pa_132H*, and *Sat_334-S12_0711*. Marker *S12_0711I* is a BARCSOYSSR marker used to increase marker density on chromosome 12 (Song et al., 2010). A7, the efficient parent in the population, contributed the positive allele for the major QTL using the combined data. Two of the suggestive QTL were attributed to the heterozygote class in the population indicating overdominance gene action for these loci. The other QTL on chromosome 12 with the LOD of 3.4 received the positive allele from the inefficient parent Anoka, which was similar to what Lin et al. (1997) observed for visual scoring of IDC on chromosome 20.

There were no significant QTL identified for Zn concentration in 2008, 2009, nor in the combined data. However two suggestive QTL were identified in 2008, one in 2009, and

two in the data set combined over years. The suggestive QTL in 2008 were on chromosomes 12 and 19 and had LOD scores of 3.7 and 3.0, and which 21.2 % and 16.7 % of the variation, respectively. They were identified in the marker intervals *Sat_334-S12_0711* and *Satt694-Satt143*. In 2009, the suggestive QTL was in the marker interval *Satt175-pK_417H*, and it had a LOD score of 2.8 and represented 19.0 % of the variation. Combined over years, the suggestive QTL were on chromosomes 7 and 18 with LOD scores of 3.0 and 2.9. These QTL were in the marker intervals *pk_417H-pk_70T* and *pa_890V-K_493H* and represented 23.4 % and 18.5 % of the variation. Even though these QTL were not significant (LOD= 3.7; P=0.95), the marker *pk_417H* was consistent in 2009 and in the combined data. The positive allele for the QTL on chromosome 7 was associated with the inefficient parent Anoka, and on chromosome 18, the heterozygote.

QTL Analysis Leaf Zn and Fe Concentration

There were no significant QTL for leaf Fe concentration detected in this study. There were, however, five suggestive QTL detected in 2008, 2009, and in the combined data (Table 6). Only one suggestive QTL was detected in 2008. This QTL was on chromosome 20 and had a LOD score of 2.1 which represented 13.9 % of the variation. This QTL was in the marker interval *Satt292-S20_1142* with the positive allele being contributed by the efficient parent A7. In 2009, two suggestive QTL were detected on chromosomes 6 and 18. The QTL on chromosome 6 has a LOD score of 3.1, which represented 21.8 % of the variation, and was in the marker interval *Sat_263-Satt708*. The positive allele for this QTL was contributed

by Anoka the inefficient parent. On chromosome 18, the QTL had a LOD score of 3.0 and represented more of the variation at 15.4 %. The marker interval was *pk_69I-Satt394* and in this case the positive allele was associated with A7. In the combined years, two suggestive QTL were detected. One QTL on chromosome 16 had a LOD score of 3.0 representing 15.3% of the variation. This QTL was in the marker interval *pk_375H-pA_233D*. On chromosome 18, the QTL had a LOD score of 2.6, which represented 28.9 % of the variation. In both cases of these QTL, the positive allele came from the inefficient parent Anoka.

Similar results were obtained for leaf Zn in 2008 with no significant QTL detected. However, one suggestive QTL was detected in 2009 and two in the combined data. The suggestive QTL in 2009 was on chromosome 18 and had a LOD score of 2.4. The marker interval was *Satt309-pk*_69T with the positive allele coming from A7. The two QTL in the combined data were on chromosomes 1 and 8 and had LOD scores of 2.7 and 3.8. They were in the marker intervals *Satt502-Satt532* and *pa_111H-pa_hilu*. These QTL represented 13.9 and 18.2 % of the variation with the positive allele on chromosome 1 coming from Anoka and A7 on chromosome 8.

Discussion

Iron deficiency chlorosis has been studied for more than fifty years and is still a major problem in areas where crops are produced on calcareous soil. There has been progress made in developing genetically resistant lines, however the release of agronomically suitable cultivars with IDC resistance and high yields has been limited (Weiss, 1943; Fehr and

Cianzio, 1980; Fehr et al., 1984; Jessen et al., 1988). Although there is a wealth of knowledge of the genetic mechanisms contributing to iron homeostasis in plants as well as knowledge of QTL associated with Fe efficiency, there is still limited information on the genetic mechanisms associated with mineral accumulation in the seed and leaves and how that might ultimately relate to iron homeostasis (Weiss, 1943; Cianzio and Fehr (1980, 1982); Lin et al., 1997; Briat, 2008). Several reports are available on mapping QTL for seed or grain Zn and Fe concentrations and contents in various species (Zhou et al., 2010; Garcia-Oliveira et al., 2009; Ding et al., 2010; Tiwari et al., 2009). These reports identified as few as one significant QTL for Fe and Zn in wheat grain, and as many as five Zn and four Fe QTL in *Arabidopsis halleri* (Tiwari et al., 2009; Willems et al., 2010). We report here, the first QTL for soybean seed Fe and Zn concentration, and their relationship with Fe efficiency.

One major QTL was identified in 2009 as well as over combined years for seed Fe concentration. The major QTL on chromosome 1 for 2009 was in the interval *Satt295-Satt383*. There are no other QTL for mineral concentration on the Soybase website, nor have either one of those markers been associated with QTL for Fe efficiency. This may suggest a novel gene(s) for Fe accumulation in soybean seed at this locus. There are, however, three Fe efficiency QTL on chromosome 1 that have been mapped previously with the marker *K647_1* in the A81356022 x PI468916 population (Diers et al., 1992). This QTL is in the position of 93.90-95.50 cM compared to 55.22 and 56.57 for the markers in our QTL interval (httt://www.soybase.org). The significant QTL for Fe concentration over combined years mapped to chromosome 20, which Lin et al. (1997) identified as a linkage group with Fe

efficiency QTL in the Anoka x A7 population. Our QTL mapped in the interval of *pa_515-Satt239*. More importantly, the marker *pa_515* was a marker used in the Lin et al. (1997) paper that was associated with and mapped to the Fe efficiency QTL in the Anoka x A7 population for visual score at the V2 stage of 1993 and chlorophyll concentration at V4 stage of 1993 and 1994. The positive allele comes from A7 with the genotypic average 80.70 [Fe] μg/g as opposed to 75.11 [Fe] μg/g for the inefficient parent Anoka. This is the first evidence of a genetic link for QTL of Fe efficiency being associated to QTL for Fe accumulation in soybean.

Consistently mapping QTL over locations and years with significant effects continues to be a major hindrance to QTL mapping and marker assisted selection (MAS) as it is related the genetic diversity among parents, population size, and the number of markers tested (Zhou et al., 2010; Diers et al., 1992; Lin et al., 1997; Brondani, et al., 2002). Zhou et al. (2010) mapped QTL for Zn, Fe, Cu, and Mg contents in maize, and detected five QTL in 2007 and nine QTL in 2008; however, they were unable to detect the same QTL over the two years when the data were combined. In another population evaluated, they identified 12 QTL in 2007 and six in 2008, but only two QTL were significant over both years. In rice grain, Garcia-Oliveira et al. (2009) identified a total of 31 QTL for mineral accumulation, but only 17 were observed over both years of the experiment. Similarly, Lin et al. (1997) detected a QTL for visual scores on LG I, now chromosome 20, with the marker *K644* in common in independent years at both V2 and V4 stages. However, when the data were combined over the years in the V4 stage, there were no QTL detected on that linkage group. In contrast, the

QTL that was detected on chromosome 20 for chlorophyll concentration at the V4 stage combined over the years 1993 and 1994 was not detected in independent years or at the different stages evaluated.

We obtained similar results here for the major QTL detected and suggestive QTL for Fe concentration. The major QTL was not detected in separate years, but in the combined data for 2008 and 2009. For 2008, the suggestive QTL was on chromosome 12 and in the combined data 2008 and 2009. In both cases, the QTL was in the same marker interval *Satt635-pa_132H*, however the positions of the peak of the QTL were different. In 2008, the position of the QTL was at 7.0 cM, but in the combined data, it was at 0.0 cM. In both years, the allele from Anoka was positive with the greatest contribution observed in the combined data. A similar occurrence was observed in 2009 and combined over years with the QTL being detected on chromosome 1. However, the QTL was detected at two different positions on the chromosome. In 2009, that position was at 49.9 cM and in the combined data it was 22.1 cM, suggesting that two separate loci may have been identified. Regardless of whether the QTL are due to two loci or a single locus, the positive allele in 2009 was attributed to the inefficient parent Anoka, and over the combined data the genetic affect was additive as well and associated with Anoka.

Although no significant QTL for Zn concentration was detected, a similar result of identification of suggestive QTL over years as observed in Fe concentration occurred. The QTL detected in 2008 were not observed in 2009 or in combined data. The QTL detected in 2009 on chromosome 7 was also detected in combined data, however the QTL was in a

slightly different marker interval with marker *pk_417H* in common between the QTL. Interestingly only one of the QTL detected had the positive allele coming from the A7 parent, and it was the QTL on chromosome 19, detected in 2008. This could indicate that chromosome 7 contains genes that influence Zn accumulation in soybean seed and that the major region on the chromosome is located near *pk_417H*.

Of importance to this research, was to determine if QTL for seed and leaf Zn and Fe concentration co-localize to the same regions in the soybean genome, which would suggest that the same genetic mechanisms are involved in their accumulation and transport. A major impediment to drawing strong conclusions was the lack of consistency between years and in the combined data. Chromosomes 7 and 12 were the only chromosomes for which QTL for Fe and Zn concentration were both mapped. However in 2008 the QTL on chromosome 12 for Fe concentration mapped to position 7.0 cM, but the QTL for Zn concentration mapped to position 105.6. When only the combined data for Fe concentration is taken into account, the interval of markers *Sat_334-S12_0711* are the same as for Zn concentration for 2008. This could indicate that chromosome 12 may have genes that are involved in both Fe and Zn accumulation in the seed. A similar occurrence was observed for chromosome 7 where in 2008 a suggestive QTL for Fe concentration was detected. However, in 2009 and in the combined data a suggestive QTL was detected for Zn concentration. The positions for each QTL detected were not the same, but marker *pk_417H* was in common with all three. Even though, the QTL for Zn and Fe concentration were not detected in the same years this provides evidence of a possible connection between Zn and Fe accumulation, controlled by

the same genes at this locus. If this is the case, consistent identification of these QTL for mineral accumulation could allow for the increase in accumulation of both minerals simultaneously.

Consistency was a problem with leaf Zn and Fe concentration as well. Of the five suggestive QTL identified for leaf Fe concentration. Only chromosome 18 had QTL for 2009 and in the combined data; however the positions for the QTL were different with one QTL at position 32.8 cM in 2009 and at position 156.6 cM in the combined data. Interestingly enough, both of the intervals for these QTL are in regions of previously mapped Fe-efficiency QTL in different populations. Marker *pk_69I* is associated with the Fe efficiency QTL 1-1, 1-2, 1-3, and 1-4 in the A81356022 x PI468916 population (Diers et al., 1992). Marker *pa_890V*, on the Williams physical map, is at position 67.7 cM. There are four IDC QTL in the region of 66.6-73.3 cM. Furthermore, three additional Fe efficiency QTL are at the position 73.0-76.00 cM, all of which were identified in the Pride B216 x A15 population (Lin et al. 1997). The interval for the Fe concentration QTL encompasses these QTL. Although, the IDC QTL were mapped in different populations than that used in this study it seems likely that the QTL region in all populations contain genes involved in iron accumulation and iron efficiency. No QTL for leaf Zn were detected that occurred on the same chromosome.

Iron homeostasis in plant tissues during growth and development is the result of an integrated regulation of expression of various genes encoding proteins acting in the transport, storage, and utilization of iron (Briat, 2008). Ding et al. (2010) concluded that mineral

accumulation in seed of *Brassica napus* is controlled by multiple genes. Furthermore, common physiological and molecular mechanisms could be involved in the accumulation of multiple mineral elements. With the genomic locations of molecular markers used in this study known, we queried the intervals of the marker positions associated with the identified QTL against the whole soybean genome assembly (Gmax_109) (Schmutz et al., 2010; http://www.phytozome.net) to identify candidate genes which could be related to mineral accumulation and transport and to further our understanding of the genetic basis for Fe and Zn accumulation in soybean seed and leaf tissue. We identified metal-related genes in those intervals on each chromosome that could potentially provide a genetic basis for mineral accumulation in soybean seed and leaves (Appendix 1). On chromosome 20, several candidate genes were identified that are involved with metal transport and uptake. One gene is an *Aluminum activated malate transporter*, which is normally involved in Al toxicity/stress tolerance and is a part of the multi-drug and toxic compound extrusion (MATE) family (Liu et al., 2008; Sasaki et al., 2004). Other genes within the QTL intervals that have been shown to be involved in Fe stress responses were: *Myb-like DNA-binding domain*, *ABC1 family*, *F-box domain*, *Leucine Rich Repeat protein* (Zheng et al., 2009). Several genes are of interest on chromosome 7 on which mapped QTL for both Fe and Zn seed concentration: *Universal stress protein family*, *Ferric reductase like transmembrane component*, *Ferric reductase NAD binding domain*, *Metallothionein*, *Ctr copper transporter family*, *Ferritin-like domain*, *Auxin response factor*, *AUX/IAA family*, *2OG-Fe(II) oxygenase superfamily* and *Cation transport protein*. *bZIP* transcription factors are found on chromosomes 12 and 18. Members

of this family of transcription factors have been shown to be involved in leaf and seed formation, energy homeostasis, and abiotic and biotic stress responses (Guedes et al., 2008). Additionally on chromosomes 12 and 18, there were genes associated with mineral transport and photosynthesis. On chromosome 12, there was a *Heavy-metal associated domain*, and on chromosome 18 a *ferritin-like domain*, *MATE*, and *ZIP Zinc transporter* was identified.

Conclusions

This research has presented the first evidence of a link between Fe efficiency and Fe concentration in soybean through QTL mapping of Fe concentration on an integrated soybean genetic linkage map developed from an Anoka x A7 population. Through the use of previous markers used to screen the population and the addition of new SSR markers, the data identified a significant QTL for Fe concentration that co-localized with markers previously used to map Fe efficiency (IDC) QTL. In 2009 as well as in combined data, these QTL represent QTL for Fe accumulation that has not previously been mapped. Both of these QTL and the genes within the region can be targeted in MAS for improving soybean genetic breeding for IDC resistance through development of lines with elevated Fe concentration. The power to detect QTL is paramount to QTL mapping. Suggestive QTL were identified as being significant on the chromosome level, which potential identified genes that would have gone undetected. These minor effects of the suggestive QTL identified could possibly aide in improving Fe efficiency through a modifying polygene mechanism that has been described by Lin et al. (1997). However, these QTL effects were more than likely in regions involved

in Fe stress response and loading. Due to the experiment being grown in non-calcareous soil, a non-stressed environment, detection of a major effect was minimal. This would indicate, because the same chromosomal regions were identified for both Fe and Zn concentration, that there are similar physiological processes in sink to source for both Fe and Zn (Garcia-Oliveira et al., 2009). Candidate genes identified in these QTL regions could be possibly involved in mineral uptake, transport, and loading, which could potentially aid in breeding crops that are Fe efficient and have higher iron concentration. Ultimately, these genes may be in the pathways involved in mineral accumulation in soybean. This research provided evidence of QTL for Zn and Fe accumulation in soybean with a strong indication that Fe and Zn have loci in common. Furthermore, Fe efficiency and Fe accumulation appears to be governed by similar genes. This research has provided information to gaining further understanding of the genetic complexity of iron homeostasis, transport, and mineral accumulation in soybean.

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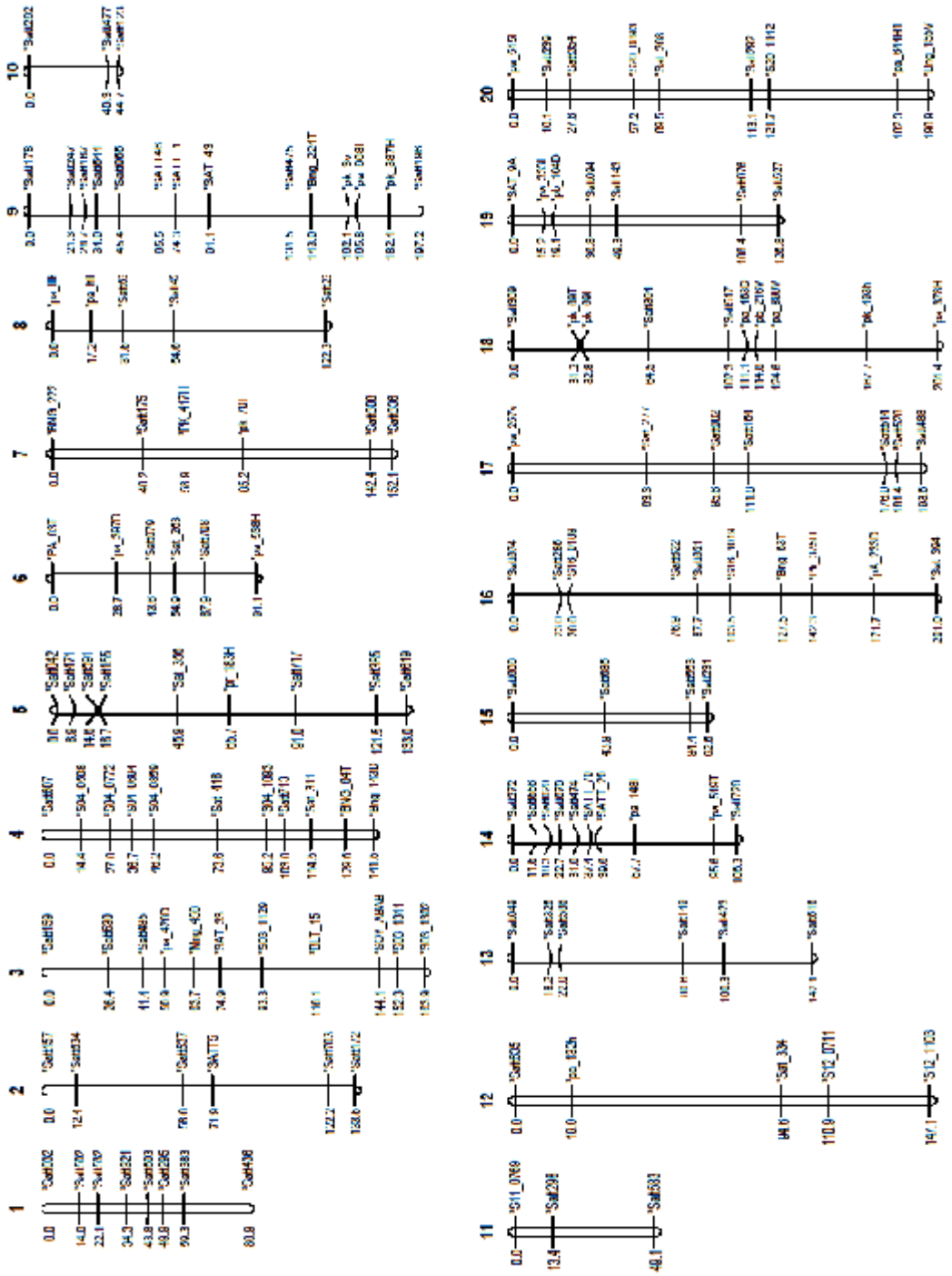

Figure 1**.** The genetic linkage map of the Anoka x A7 population.

Table 1. Mean (standard deviation), range, and heritability estimates for the four traits grown 2008 and 2009 and combined over years.

Trait (μg/g)	2008	2009	Combined	Range	h^2
Seed Zn	3.5 ± 0.1	3.9 ± 0.1	3.7 ± 0.1	3.5 ~ 4.0	0.47
Seed Fe	59.5 ± 4.5	97.0 ± 7.2	78.3 ± 4.4	67.6 ~ 92.7	0.30
Leaf Zn	56.1 ± 4.3	10.7 ± 3.0	33.4 ± 2.5	26.5 ~ 40.9	0.00
Leaf Fe	515.0 ± 115.5	681.9 ± 153.8	600.0 ± 101.5	379.9 ~ 1089.7	0.14

Zn: Zinc; Fe: Iron

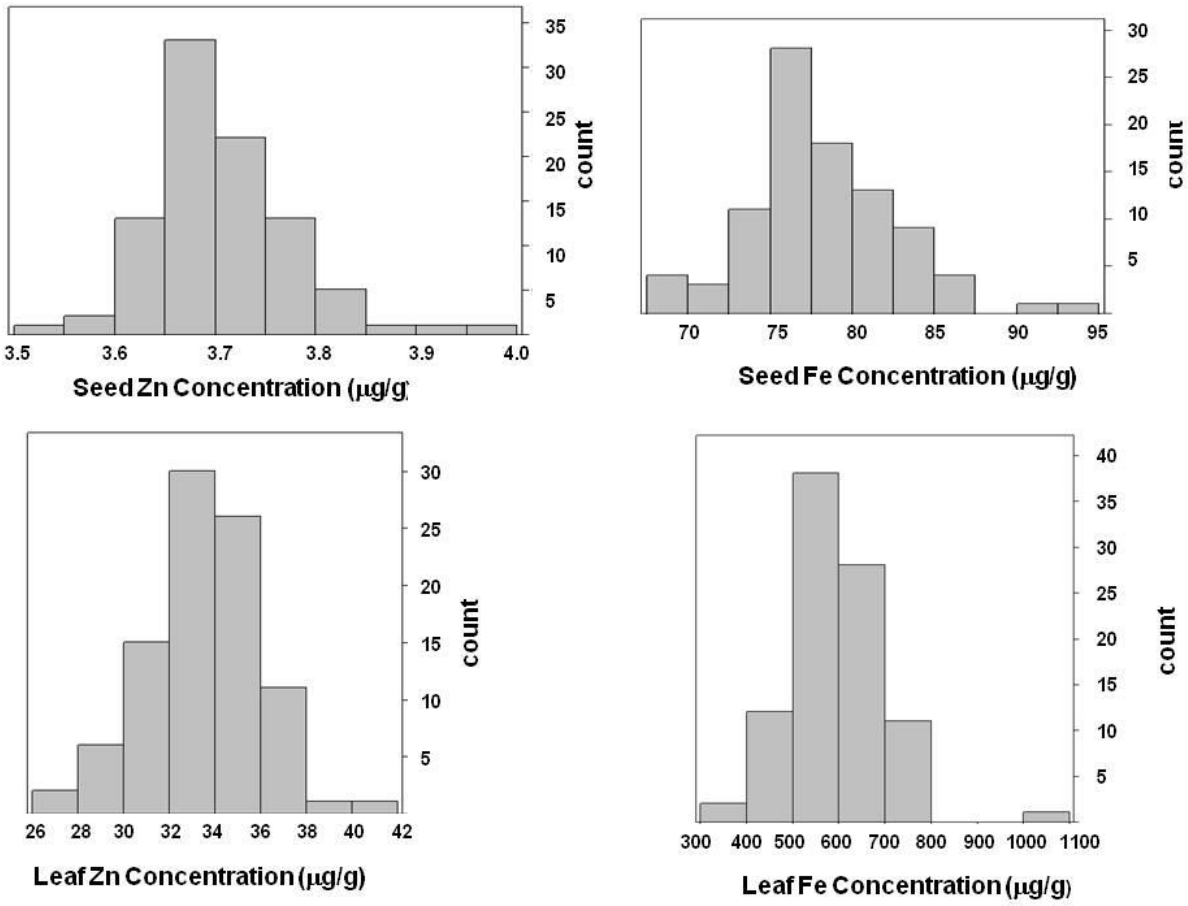

Figure 2. Distributions of the Fe and Zn concentrations in the Anoka x A7 population in seed and leaf tissue.

Table 2. ANOVA table for the seed and leaf iron and zinc for 2008 and 2009 with p-values from the F-test

		Sd Zn	Sd Fe	Lf Zn	Lf Fe
Source	DF	Prob > F	Prob > F	Prob > F	Prob > F
Year	1	<.0001*	<.0001*	<.0001*	<.0001*
Replication[Year]	4	<.0001*	<.0001*	<.0001*	<.0001*
Genotype	91	0.012*	<.0001*	0.0391*	0.3225
Year*Genotype	91	0.503	0.0054*	0.0026*	0.4989

* Significant at *P* <0.01

Table 3. Pearson correlation coefficients between seed and leaf Fe and Zn concentrations in the Anoka x A7 population in 2008 and 2009

	Log(Sd Zn (μg/g)) 08	Log(Sd Zn (μg/g)) 09	Sd Fe (μg/g) 08	Sd Fe (μg/g) 09	Lf Zn (μg/g) 08	Lf Zn (μg/g) 09	Lf Fe (μg/g) 08
Log(Sd Zn (μg/g)) 08	1						
Log(Sd Zn (μg/g)) 09	0.25*	1					
Sd Fe (μg/g) 08	0.63*	0.17*	1				
Sd Fe (μg/g) 09	-0.32*	0.12	-0.21*	1			
Lf Zn (μg/g) 08	0.03	-0.05	-0.18*	0.14*	1		
Lf Zn (μg/g) 09	0.46*	0.22*	0.51*	-0.29*	-0.23*	1	
Lf Fe (μg/g) 08	-0.47*	-0.16*	-0.38*	0.30*	0.21*	-0.26*	1
Lf Fe (μg/g) 09	-0.55*	-0.27*	-0.40*	0.33*	0.05	-0.42*	0.52*

* Significant at $P < 0.01$

Table 4. Pearson correlation coefficients between seed and leaf Fe and Zn concentrations in the Anoka x A7 population combined over years 2008 and 2009

	Log(Sd Zn (μg/g))	Sd Fe (μg/g)	Lf Zn (μg/g)
Log(Sd Zn (μg/g))	1		
Sd Fe (μg/g)	0.72*	1	
Lf Zn (μg/g)	-0.65*	-0.88*	1
Lf Fe (μg/g)	-0.07	0.26*	-0.26*

* Significant at $P < 0.01$

Table 5. Summary of Quantitative Trait Loci (QTL) detected for seed iron and zinc concentrations in the Anoka x A7 population

Chromosome	Marker Interval	Position (cM)	LOD	R^2	Means of QTL genotypic classes			
					A1A1	A1A2	A2A2	Additive Effects
QTL for Soybean Seed Fe Concentration								
2008								
12/H	Satt635-pa_132H	7.0	3.0	25.7	59.67	61.81	56.39	1.64
7/M	pk_417H-pk_70T	69.9	2.5	20.1	58.47	60.30	55.42	1.52
GW LOD Threshold			4.6					
Chromosome 7 Threshold			3.0					
Chromosome 12 Threshold			3.3					
2009								
1/D1a	Satt295-Satt383	49.9	4.3	21.2	96.61	95.89	88.67	3.97
17/D2	Satt528-Satt488	193.4	3.0	14.3	93.36	97.90	91.92	0.72
GW LOD Threshold			3.7					
Chromosome 17 Threshold			2.3					
2008 and 2009								
20/I	pa_515-1-Satt239	4.0	4.7	21.5	75.11	76.90	80.70	-2.79
1/D1a	Satt532-Satt321	22.1	3.2	10.6	79.09	80.49	76.79	1.15
12/H	Satt635-pa_132H	0.0	3.4	12.3	80.12	78.08	75.76	2.18
12/H	Sat_334-S12_0711	100.6	2.8	12.7	76.48	80.55	77.64	-0.58
GW LOD Thresholds			3.7					
Chromosome 1 Threshold			2.2					
Chromosome 12 Threshold			2.2					
QTL for Soybean Seed Zn Concentration								
2008								
12/H	Sat_334-S12_0711	105.6	3.7	21.2	3.55	3.55	3.48	0.04
19/L	Satt694-Satt143	42.8	3.0	16.7	3.47	3.52	3.56	-0.04
GW LOD Threshold			3.8					
Chromosome 12 Threshold			2.4					
Chromosome 19 Threshold			2.2					
2009								
7/M	Satt175-pK_417H	52.2	2.8	19.0	3.93	3.84	3.83	0.05
GW LOD Threshold			3.4					
Chromosome 7 Threshold			2.1					

Table 5. (Continued).

Chromosome	Marker Interval	Position (cM)	LOD	R^2	Means of QTL genotypic classes			
					A1A1	A1A2	A2A2	Additive Effects
2008 and 2009								
7/M	pk_417H-pk_70T	65.9	3.0	23.4	3.72	3.66	3.65	0.04
18/G	pa_890V-pK_493H	124.6	2.9	18.5	3.68	3.73	3.67	0.00
GW LOD Threshold			3.7					
Chromosome 7 Threshold			2.3					
Chromosome 18 Threshold			2.4					

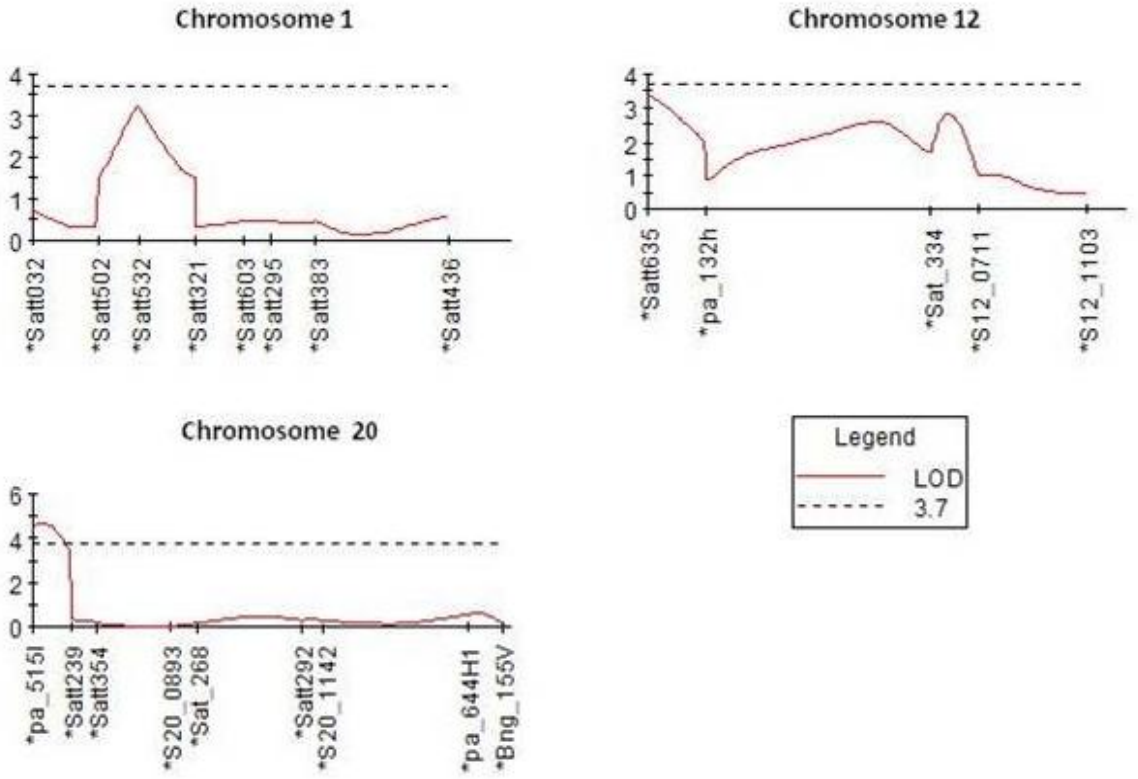

Figure 3. Seed Fe concentration QTL mapped in the combined 2008 and 2009 data with the significant QTL on chromosome 20 with dashed (---) line showing the genomewise threshold level of significance.

Table 6. Summary of Quantitative Trait Loci (QTL) detected for leaf iron and zinc concentrations in the Anoka x A7 population grown on non-calcareous soil

Chromosome	Marker Interval	Position (cM)	LOD	R^2	Means of QTL genotypic classes			
					A1A1	A1A2	A2A2	Additive Effects
QTL for Soybean Leaf Fe Concentration								
2008								
20/I	Satt292-S20_1142	115.1	2.1	13.9	538.08	463.87	570.61	-16.27
GW LOD Threshold			3.5					
Chromosome 20 Threshold			2.3					
2009								
6/C2	Sat_263-Satt708	59.9	3.1	21.8	739.89	855.54	679.81	30.04
18/G	pk_69I-Satt394	32.8	3.0	15.4	711.53	609.17	746.27	-17.37
GW LOD Threshold			3.7					
Chromosome 6 Threshold			2.3					
Chromosome 18 Threshold			2.6					
2008 and 2009								
16/J	pk__375H-pA_233D	142.3	3.0	15.3	717.63	601.38	641.44	38.10
18/G	pa_890V-pk_493H	156.6	2.6	28.9	755.35	610.91	654.34	50.50
GW LOD Threshold			3.4					
Chromosome 16 Threshold			2.4					
Chromosome 18 Threshold			2.1					
QTL for Soybean Leaf Zn Concentration								
2008								
No QTL								
2009								
18/G	Satt309-*pk_69T	6.0	2.4	20.4	11.25	9.38	12.53	-0.64
GW LOD Threshold			3.9					
Chromosome 18 Threshold			2.4					

Table 6. (Continued)

Chromosome	Marker Interval	Position (cM)	LOD	R^2	Means of QTL genotypic classes			
					A1A1	A1A2	A2A2	Additive Effects
2008 and 2009								
1/D1a	Satt502-Satt532	15.0	2.7	13.9	34.59	32.03	33.46	0.57
8/A2	pa_111H-pa_hilu	13.0	3.0	18.2	32.48	33.47	35.78	-1.65
GW LOD Threshold			3.8					
Chromosome 1 Threshold			2.2					
Chromosome 8 Threshold			2.2					

CHAPTER 4. EVALUATION AND QTL MAPPING OF TOTAL P IN SOYBEAN

A paper to be submitted to *Euphytica*

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Abstract

Phosphorus (P) is an essential macronutrient required for many biological and metabolic plant functions. Phosphate is the form of P used by plants. Reducing the amount of P in the seed can further aide breeding efforts to reduce the amount of P released into the environment due to the indigestibility of the complexes formed with metal ions. Analysis of the variation of phosphorus concentration in soybean seed under non-stressed conditions revealed that phosphorus ranged from 3948.1 μg/g to 5695.8 μg/g total phosphorous (TP) in combined years. The averages for independent years were significantly different from one another. Quantitative trait loci (QTL) analysis of TP was performed to identify candidate gene(s) that is (are) involved in P accumulation in soybean seed. One putative QTL region was identified on chromosome 12 in the combined data that contained a phosphate transporter gene. Two additional suggestive QTL were identified on chromosomes 7 and 17 with chromosome 7 having both a phosphate transport gene and a ZIP transporter gene in the region of the QTL. There were additional genes in these regions that are involved in phosphate metabolism and transport. These three QTL had the positive allele from an Fe

inefficient parent suggesting that an increase in phosphate in the plant could negatively impact the Fe efficiency response.

Introduction

Phosphorus (P) is an essential macronutrient for all living organisms (Poirier and Bucher 2002). In plants, it is present both as a constituent of such compounds as nucleic acids, phospholipids and ATP, and as a metabolite involved in energy transfer, the activation of proteins and the regulation of metabolic processes (Marschner 1995). Plants take up P in the form of orthophosphate (Pi). Growth and development can be limited by the availability of Pi in most natural ecosystems (Smith et al. 2003). The availability of Pi is often limited due to it being in the soil in complex, insoluble, inorganic, and organic forms which cannot be acquired directly by the plant (Chiou et al., 2001; Raghothama 1999).

In legumes such as soybean (*Glycine max* (L.) Merr.), phytate (myo- inositol 1,2,3,4,5,6 hexakisphosphate, Ins P_6) is the major storage form of phosphorus (Reddy et al. 1989; Raboy 1997). Phytate makes up ~75% of the total phosphorus (TP) in the seed and about 1-2% of the seed composition. This composition would make TP a good measurement criterion to establish an estimate of the PA status in a population (Raboy et al. 1984; Raboy 1997; Bilyeu et al. 2008). Ins P_6 represents a major pool or bottle neck in the flux of P in the world's agricultural ecology because it covalently binds to cations such as K^+, Ca^{2+}, Mg^{2+}, Fe^{2+}, and Zn^{2+}. This binding results in a reduction of bioavailability of P and the cations that are complexed with it (Raboy et al. 2001; Tsao et al. 1997; Schlemmer et al. 1995). Ultimately this presents a problem because Ins P_6 is not readily digested by monogastric animals (Maupin et al. 2011). This underutilized Ins P_6 is then excreted in manure leading to

an increase of P pollution and fouling of groundwater and eutrophication of inland bodies of water (Sharpley and Withers 1994; Correll 1998; Raboy 2001).

Exploiting natural variation in a population is the hallmark of plant breeding. A survey of mineral content and their interactions can provide information for breeding efforts directed toward increasing the concentration of some minerals and decreasing others. The population in this study, Anoka x A7, had been previously characterized to have a major QTL for Fe efficiency on chromosome 3/LG N (Lin et al. 1997). Although total phosphorus doesn't change in a genotype, rather the fractions of P, the objective of this research was to determine the extent of variation of total P in the Anoka x A7 mapping population in order to determine the efficacy of using total P as a selection factor in breeding and to map total P concentration as a QTL. Understanding the extent of variation in this population could potentially aide in the selection of genotypes that are more efficient in nutrient uptake and transport.

Material and Methods

Plant Material

The plant material used for QTL mapping soybean seed total phosphorus included 92 $F_{2:4}$ lines from Anoka x A7, which was previously used to identify a major QTL for Fe efficiency (Lin et al. 1997). A7 was the Fe efficient parent developed through a recurrent selection program, and Anoka was an Fe inefficient cultivar (Cianzio 1984; Cianzio and Fehr 1980).

Phenotypic Evaluations

The 92 F_2-derived lines and checks were planted at Bruner Farm, near Ames, IA on non-calcareous (Clarion-Nicollet loam soil type (fine-loamy, mixed, superactive, mesic Typic Hapludoll and fine-loamy, mixed, superactive, mesic Aquic Hapludoll)) soil in 2008 and 2009. The experiment was set up as a randomized complete block design (RCBD) with three replications. Each line/genotype was planted in plots of forty seeds and no thinning performed upon emergence. The plots were 1.52 m long and separated from adjacent plots by 0.91 m. Leaf samples were taken from each line/genotype from the second fully expanded trifoliate, about 3-4 weeks after planting. Leaf tissue was then stored in liquid nitrogen, before being lyophilized for DNA extraction and marker analysis. Upon maturity, seed from each plot was harvested in bulk for analysis. Total phosphorus was determined using the slightly modified procedures of Gao et al. (2007) and Zheng et al. (2009). Briefly, five grams of seed from each line/genotype were ground using a Foss Cyclotec mill (1093 Sample Mill, Foss, Eden Prairie, MN) equipped with a 1-mm screen. A 0.5 g subsample of soybean powder was taken from each genotype and vortexed with 10 ml of 2.4% HCl in 14-ml falcon tubes until a milky-white solution could be observed. Tubes were stacked in tube racks, the racks placed on their side, and tubes and rack secured using laboratory tape. The tube samples were digested overnight (~16 hours) and shaken at 220 rpm on a bench top shaker (Daigger orbital shaker, Vernon Hills, Ill.) at room temperature. To obtain a crude extract, tubes were then centrifuged at 2900 g at 10^{0} C for 15 minutes (Beckman Coulter, Avanti-J26XPI), and 500 µl of the solution phase of each was pipetted into a 2 ml microcentrifuge tube. Additionally, 500 µl of 20% NaCl solution was added to the top of each sample. This

crude extract was precipitated at room temperature for one to two hours and then centrifuged @ 16,100 g for 15 min on a Labnet International, Inc. Spetrafuge 24D microcentrifuge to obtain supernatant. An aliquot of 400 μL of each supernatant was then pipetted into a 14-ml falcon tube pre-filled with 9.6 ml of double-distilled water (ddH_2O) for a dilution of 25 times. Calibration standards followed as such containing 0.00, 1.12, 2.24, 3.36, 5.6, 7.84, 8.96, and 11.2 ppm P from phytic acid sodium salt hydrate from rice (Sigma, St. Louis, MO.) and the same concentration of NaCl and HCl as the samples. Phosphorus concentrations were measured using Inductive coupled plasma-optical emission spectroscopy (ICP-OES) (Spectro Ciros CCD; Spectro Analytical Instruments) in the Iowa State Agronomy Soil and Plant Testing Facility. The samples were submitted as a 10 ml sample with a 400 μl aliquot of the supernatant in 9.6 ml of ddH_2O along with the calibration standards. Data were returned in total phosphorus (TP) ppm and converted to μg/g TP by multiplying by the dilution factors 25, 10, and 2.

Statistical Analysis

For this experiment, all effects were considered random. Data were analyzed using standard ANOVA procedures with the JMP statistical package (Anon. 2009). Broad sense heritability (h^2) for TP was estimated on an entry mean basis using expected mean squares from the combined ANOVA (Fehr 1987) as follows:

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where = genetic variance, = genotype x environment interaction variance, = experimental error variance, r= number of replications, and e= number of environments.

Construction of the Genetic Linkage Maps

The parental lines Anoka and A7 were surveyed for polymorphism with 916 SSR markers available from SoyBase website (http://soybase.agron.iastate.edu). Most likely orders of and recombination rates among markers were estimated with Mapmaker 3.0 (Lander et al. 1987). Linkage groups were determined with the "group" command using a LOD of 4.0 and maximum Haldane distance of 50 centiMorgans (cM). Map order was determined using the "three point" command followed by "order", "framework", and "place." Previous markers that formed linkage groups for this population (Lin et al. 1997) were integrated into the SSR map. Additionally, 303 BARCSOYSSR markers (Song et al. 2010) were surveyed for polymorphism to increase marker density on chromosomes with larger marker intervals.

QTL Mapping

Linkage maps were imported into MapQTL6 (van Ooijen 2009), and QTL positions and effects for soybean seed total phosphorus concentration for independent years and over combined years were determined using interval mapping (IM) and multiple-QTL mapping (MQM) (Lander and Botstein 1989; Jansen 1993; Jansen 1994). The significant threshold logarithm of the odds (LOD) scores for detection of the QTL was calculated based on 1,000 permutations at $P \leq 0.05$ (Churchill and Doerge 1994). As described by Liang et al. (2010), the IM method was used to determine locations of putative QTL for the total P. Subsequently, MQM was performed to eliminate inference from background markers. In order to reduce residual variance, background markers closest to the LOD peak were selected

as cofactors. The mapping step size was 1.0 cM with the maximum number of neighboring markers five, and maximum number of iterations set to 200.

Results

Construction of Genetic Linkage Maps

The current map was integrated with the original genetic map of the Anoka x A7 population generated by (Lin et al. 1997). The original map contained of 82 RFLP, 14 SSRs, and one morphological marker. Informative marker data was obtained for an additional 146 SSRs and assigned to 20 linkage groups. One hundred six SSR markers completed the linkage map along with the 12 previously unmapped informative BARCSOYSSRs (Song et al. 2010). Using Haldane's mapping function and summing over all linkage groups, there was a total of 2722.09 cM flanked by linked markers. The average length of the linkage groups was 136.10 cM ranging from 44.71 cM on chromosome 10 to 201.41 cM on chromosome 18. The average length of the marker intervals was 18.15 cM. The average number of markers per chromosome was 7.5, with a range from 3 to 14 (Figure 1).

Total P Determination

Using ICP-OES, we determined TP for all lines comprising the Anoka x A7 mapping population. Segregation for TP was observed (Table 1 and Figure 2 A, B, and C). The means varied between years, with 2009 having a higher average mean than in 2008. For overall average the mean was 4759.4 μg/g TP. The distribution of the combined data was slightly negatively skewed and had low kurtosis indicating a normal distribution and that TP was inherited quantitatively. The ANOVA indicated a significant year and rep within year effect with most of the variation being observed in the year effect indicating that most of variation

observed is due to environmental effects. This significant year effect could be attributed to the rainy conditions observed in 2009 and hail damage received eight weeks after planting in 2008. The genotype effect was not significant at the P = 0.05 level, nor was the G x E interaction, indicating that one year of testing would be sufficient for evaluation of genotypes with superior genetic potential (Fehr 1987) (Table 2). The heritability estimate for TP was (0.41), indicating that about 60 % of the variation is due to non genetic factors.

Total P QTL Analysis

QTL detection for loci controlling TP accumulation was determined for 2008 and 2009 and the average across years. In 2008, three suggestive QTL were detected on chromosomes 10, 18, and 20 (Table 3). Suggestive QTL were those QTL possessing peaks in LOD scores that did not exceed the genome wide threshold, but on the chromosome level were at or exceeded significant P = 0.05 (Liu et al. 2007; Tiwari et al. 2009; Willems et al. 2010). The LOD scores of these QTL ranged from 2.6-2.9 and the variation explained was 11.9- 19.4 %. The positive allele for the QTL on chromosome 10 and 18 was attributed to the A7 parent, whereas the positive allele on chromosome 20 was attributed to Anoka. In 2009, there were no QTL detected that were significant at the genome wise or chromosome wise thresholds.

With the TP averages combined over years, one significant and two suggestive QTL were detected. The major QTL was on chromosome 12 in the marker interval S12_0711-S12_1103 and had a LOD of 3.9 (Figure 3). This QTL had a R^2 of 29.9 %, and its position was at 118.9 cM. The two suggestive QTL were on chromosomes 7 and 17. Markers *S12_0711* and S12_1103 are previously unmapped BARCSOYSSR markers used to increase

marker density on chromosome 12 (Song et al. 2010). On chromosome 7, the marker interval was pk_70T-Satt308, and the QTL had LOD score of 2.4 with a R^2 of 18.4 %. The QTL on chromosome 17 was in the marker interval pa_257-Sat_277. The LOD score of this QTL was 2.3 and represented 16.3 % of the variation.

Discussion

In modern breeding programs, QTL mapping has become an increasingly important first step to marker-assisted selection and gene discovery (Price 2006). In this study, TP was evaluated in soybean seed powder using ICP-OES to determine the variation within the population, and the trait was mapped as a QTL. Total P ranged between 3948.1 μg/g to 5695.8 μg/g with an average of 4759.4 μg/g in the combined data. TP was not significant within this population, which was similar to a finding of Oltmans et al. (2005). However, there is still the possibility that there are significant differences in levels of PA (Raboy et al. 1984). Variation in TP is influenced by genetic and non-genetic factors. Consequently, the PA is influenced by the supply of TP to the developing seed. Identifying these genetic factors gives a better understanding of P status and its importance to P homeostasis in the normal function of major metabolic pathways (Raboy et al. 2001).

In this study, one significant QTL for TP was mapped in the combined data. Additionally, five suggestive QTL for TP in 2008 and in the combined data were identified. In the combined data, the additive effects for the suggestive and significant QTL show that the positive allele was attributed to Anoka, the Fe inefficient parent. This could potentially indicate that elevated TP could impact Fe inefficiency through the supply of TP absorbed and loaded in the seed. The effect of the elevated TP then could reduce the availability of seed Fe

to the plant because of the binding effect associated with the PA form of P. The identification of only one significant QTL for TP was similar to that of other research. In *Arabidopsis* for instance, only three QTL were identified for P content (Vreugdenhil et al. 2004). Furthermore, two different studies in *Phaseolus vulgaris* L. identified a similar number of QTL for TP concentration. In one study under medium and high P fertilizer applications, one QTL for each treatment was identified (Blair et al. 2009). In the other, several QTL were identified in differing years, however no more than three were identified per year per high or low P treatment with the R^2 ranging from 11% to 40% (Cichy et al. 2009).

Once QTL regions have been identified, the next step is to identify the gene(s) or causal mechanism(s) responsible for the observed phenotype (Paran and Zamir 2003). The evaluation of mineral accumulation in soybean seed for QTL mapping may offer insight into genes encoding, for example, transporters, chelators or chelator biosynthesis enzymes (Vreugdenhil et al. 2004). The genomic locations of molecular markers on the soybean genome sequence assembly (Gmax_109) were determined to obtain the intervals of the marker positions associated with the identified QTL (Schmutz et al. 2010; http://www.phytozome.net). This allowed for the identification of phosphate related genes. In this study two suggestive QTL were identified on chromosomes 10 and 20 (Figures 1 and 3). The QTL region on chromosome 20 encompasses the Pi transporter gene (*Glyma20g34610*) reported recently by Wu et al. (2011) (Appendix 2). Additionally, one more gene in the QTL interval is associated with phosphate transport, Glyma20g34620. These low-affinity transporter genes belong to the phosphate: H+ symporter (PHS) transporter of the major facilitator super family (Pao et al., 1998). The QTL interval on chromosome 10 is just outside

of the location of the Pi transporter gene reported by Wu et al. (2011). This 'off-set' positioning could potentially be due to the paucity of polymorphic markers on this chromosome. Chromosome 10 had only three polymorphic markers that only spanned 44.7 cM. Extensive screening of public SSRs failed to identify additional markers which could potentially map the QTL more precisely. Because the QTL reported in this study were identified in a non-stressed environment without any P treatments, it may be possible that these QTL represent genic regions not only involved in phosphate transport but also seed phosphate loading. The non-stress environment could further explain the lack of a strong QTL effect observed in this work. These two chromosomes are known to be highly homeologous and possessing many duplicate loci and would explain the high sequence similarity of the coding and protein sequences that was observed (Wu et al. 2011; http//www.Soybase.org).

The QTL detected for TP over combined years occurred in a manner similar to that reported by Lin et al. (1997) using the same population to study iron stress. The QTL were noted in separate years and during different growth stages. Here, the significant and two suggestive QTLs were not detected in independent years, but were detected in combined data. The QTL regions in this study, however, do contain genes that are involved in phosphorus transport and utilization. The significant QTL mapped to chromosome 12. The interval for the markers *S12_0711-S12_1103* contains several genes that are involved in mineral transport or phosphate metabolism. Genes Glyma12g16410, Glyma12g30070, and Glyma12g30100, are all a part of the ABC transporter family, which is involved in the transport of molecules through membranes (Appendix 2.; (Schulz and Kolukisaoglu 2006)).

Other genes in this QTL region that could have a role in TP accumulation, synthesis, and transport are Glyma12g22680, Glyma12g23920, and Glyma12g29790. Glyma12g22680 is a member of the *Inositol monophosphatase family*. The enzyme activity of this gene is essential for the de novo synthesis of *myo*-Inositol (Ins) and for the recycling of Ins into Ins $(1,4,5)P_3$. This enzyme is a potential regulatory point for all pathways that utilize free Ins (Fu et al. 2008). Glyma12g23920 and Glyma12g29790 are both members of the *Triose-phosphate Transporter family*, which is in the P storage and homeostasis pathways and mediates the transport of Pi between the cytosol and the stroma of the chloroplast (Raboy et al. 2001; Flugge 1999; Flügge et al. 1989).

Several genes in the suggestive QTL regions play a role in P metabolism. The most important was Glyma07g34870, which is a part of the major facilitator super family, and has a KOG annotation as an *Inorganic phosphate transporter* like that of those transporters on chromosome 10 and 20 (Tatusov et al. 2003). This gene, on chromosome 7, would have a strong potential to be involved in P transport and storage and has previously gone undetected in other studies. Also in the QTL marker interval is Glyma07g34030, a *Phosphatidylinositol-4-phosphate 5-Kinase* (PIP5K), which plays an essential role in coordinating plant growth, especially in response to environmental factors (Lou et al. 2007). It also catalyzes the phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP_2) to form phosphatidylinositol-4-phosphate (PIP) (Divecha and Irvine 1995). Glyma07g32190 is a solute carrier of *Glucose-6-phosphate/phosphate* which is the source of *Ins* before being converted to D-*Ins* 3-phsopate, the precursor of all remaining *Ins*-containing compounds (Loewus and Loewus 1983; Raboy et al. 2001). Finally, there was a *ZIP Zinc transporter*

(Glyma07g34930) in the QTL interval, which transports iron as well. Along with the Pi transporter and this gene in the interval, this could be a potential region of interest for the beginning of mineral chelation.

Similar candidate genes identified in the other QTL intervals in this study were also identified in the QTL interval on chromosome 17. These included two *triose-phosphate transporter family* genes, which also were *Glucose-6-phosphate/phosphate transporter*, two *AUX/IAA* genes, eight *ABC transporter* genes, one *Ins 5-Phosphatase*, and one *PIP5K* gene. Even though, the QTL on chromosome 17 was only suggestive, the several genes in the interval are related to P metabolism and homeostasis, and contributed small effects to this QTL being detected. As with chromosome 10, the marker interval for chromosome 17 spanned ~60.0 cM with the peak occurring at 7.0 cM. An increase in marker density on this chromosome would also help to confirm this QTL as well as to narrow the list of genes actually involved in P uptake and utilization.

In summary, the objectives of this study were to determine the natural variation of total phosphorus concentration for use as selection criteria for breeding and determine the plausibility of mapping the trait as a quantitative trait locus. The variation observed amongst genotypes was not significant but allowed the mapping of QTL for TP concentration in seeds. We mapped one putative QTL as well as several suggestive QTL. One of these suggestive QTL intervals had genes previously identified to be phosphate transporters, and additional QTL newly reported on chromosome 7. The lack of higher marker density on chromosome 10 did not allow the detection of the other phosphate transporter as it was just outside the marker interval. Additionally, it was determined that a ZIP Fe/Zn transporter gene

was in a QTL interval, which in previous work (King et al. 2011; In Review) identified suggestive QTL for both Fe and Zn accumulation and could potentially be a site of metal chelation. There is still work to be done in order to validate these TP QTL regions as well as the genes involved. Furthermore, this research did not rely on the population being grown in a stressed environment, but rather determined that there is minor natural variation with minor gene effects observed. A research program designed with different rates of available P could potentially elucidate the genetic effects and differentiate which genes are involved in P uptake, transport and homeostasis in this population.

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Table 1. Mean (standard deviation), kurtosis, skewness, and range, in 2008 and 2009 and combined over years for the Anoka x A7 mapping population

	Mean			Kurtosis	Skewness	Range		
2008 Total P (μg/g)	4334.2	±	392.8	0.6	-0.5	3179.3	~	5204.2
2009 Total P (μg/g)	5184.7	±	505.9	1.9	0.5	4134.5	~	7176.8
Total P (μg/g)	4759.4	±	343.5	0.3	-0.2	3948.1	~	5695.8

Table 2. Total P ANOVA table for 2008 and 2009 with p-values from the F-test[†]

Source	DF	MSE	F Ratio	Prob > F
Year	1	99811670	181	<.0001
Replication[Year]	4	7629599	13.8	<.0001
Genotype	91	707752	1.28	0.0586
Year*Genotype	91	522986	0.95	0.6144

[†] h^2=0.41 for the data combined over years

Table 3. Summary of Quantitative Trait Loci (QTL) detected for Total Phosphorus concentrations in the Anoka x A7 population

Chromosome	Marker Interval	Position (cM)	LOD	R^2	Means of QTL genotypic classes			
					A1A1	A1A2	A2A2	Additive Effects
QTL for Soybean seed Total P								
2008								
10/O	Satt477-Satt123	40.3	2.7	12.3	4466.7	4179.3	4467.3	-0.3
18/G	pk_493H-pa378H	167.7	2.6	11.9	4281.6	4404.6	4652.4	-185.4
20/I	S20_1142-pa_644H	132.7	2.9	19.4	4667.3	4487.6	4193.8	236.8
GW LOD Threshold			3.8					
Chromosome 10 Threshold			1.9					
Chromosome 18 Threshold			2.5					
Chromosome 20 Threshold			2.3					
2009								
No Detectable QTL								
2008 and 2009								
7/M	Pk_70T- Satt308	133.7	2.4	18.4	4957.7	4992.0	4649.1	154.3
12/H	S12_0711-S12_1103	118.9	3.9	29.9	4995.5	4551.0	4723.0	136.2
17/D2	pa_257-Sat_277	7.0	2.7	16.3	5018.9	4677.7	4682.4	168.3
GW LOD Threshold			3.8					
Chromosome 7 Threshold			2.3					
Chromosome 12 Threshold			2.2					
Chromosome 17 Threshold			2.3					

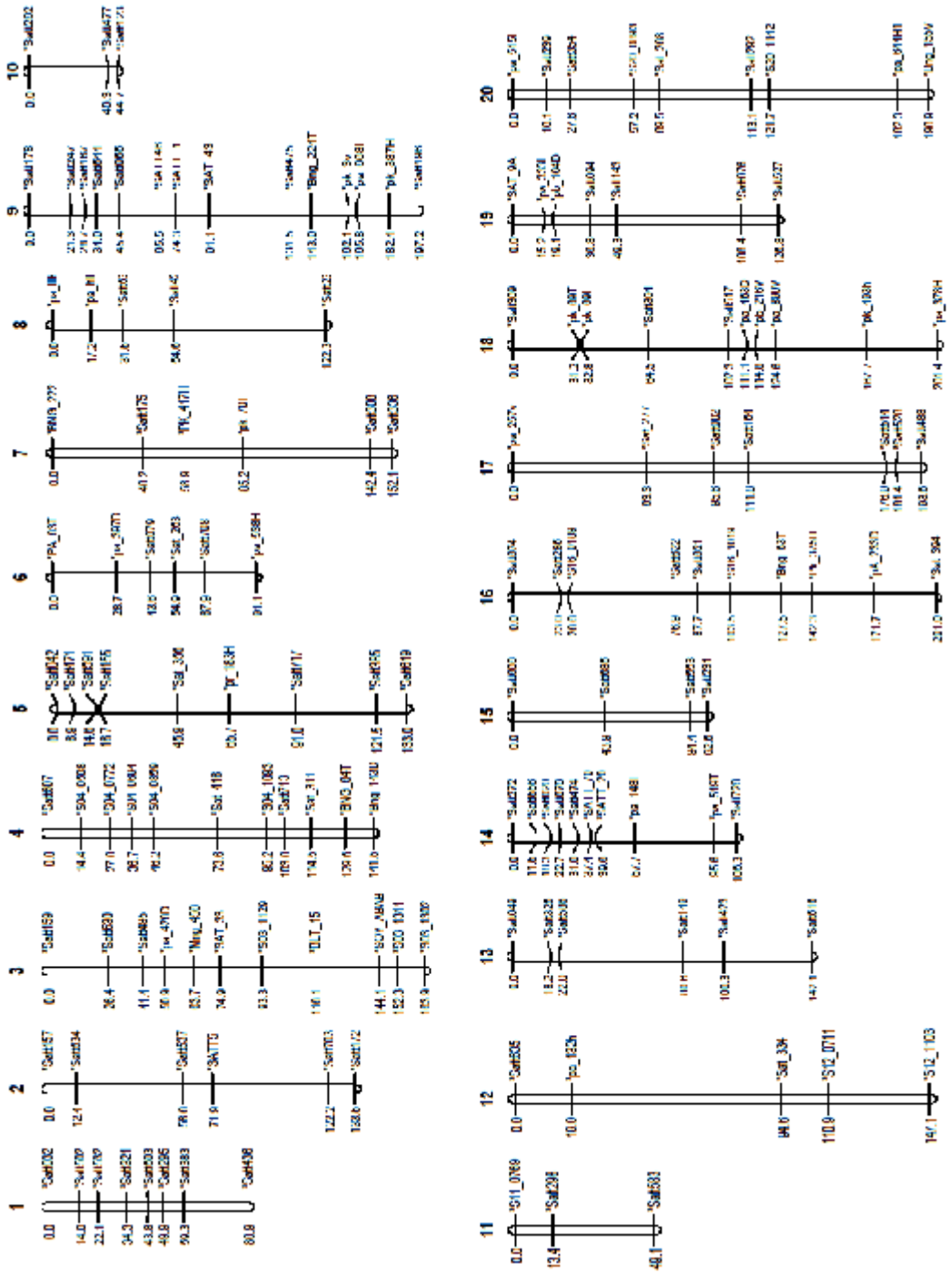

Figure 1. Anoka x A7 population genetic map

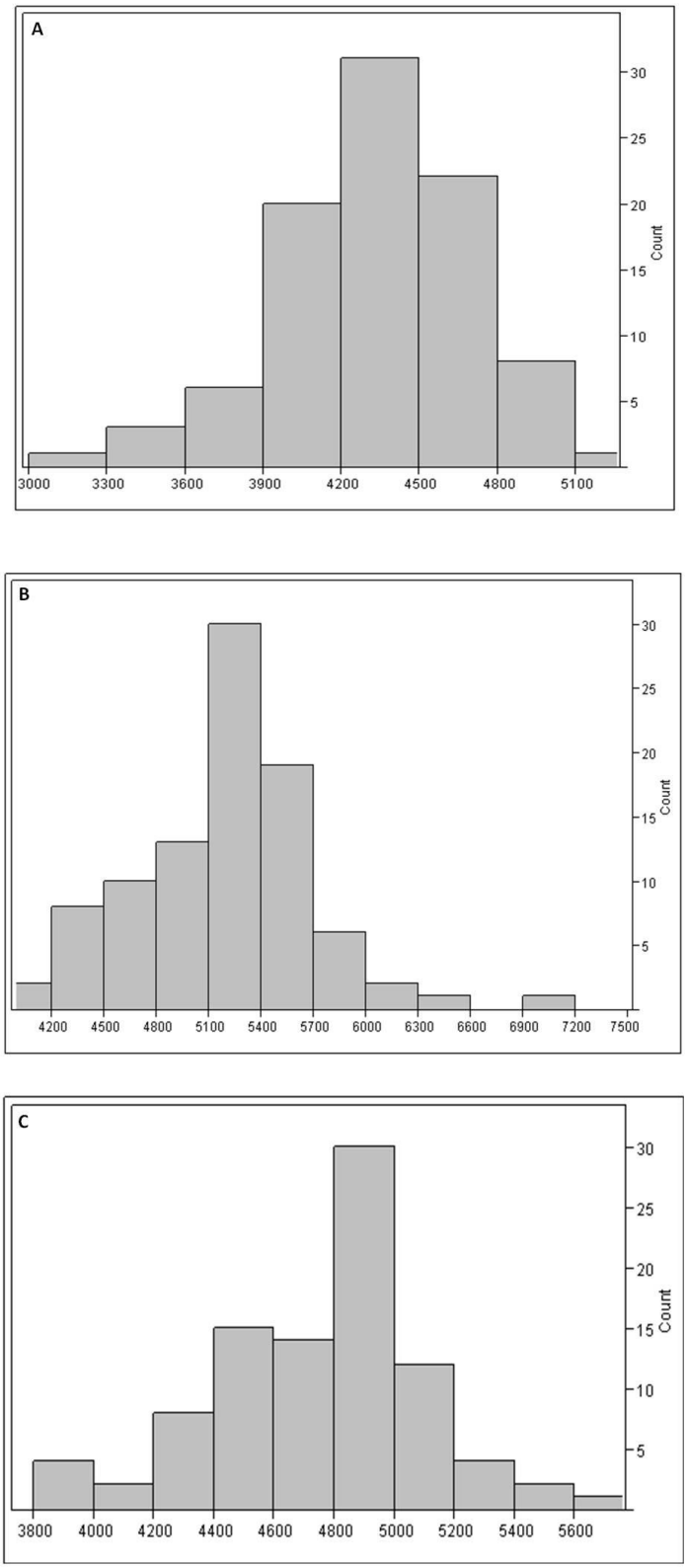

Figure 2. Total P Distribution in the Anoka x A7 population (A-2008; B-2009, C-Combined)

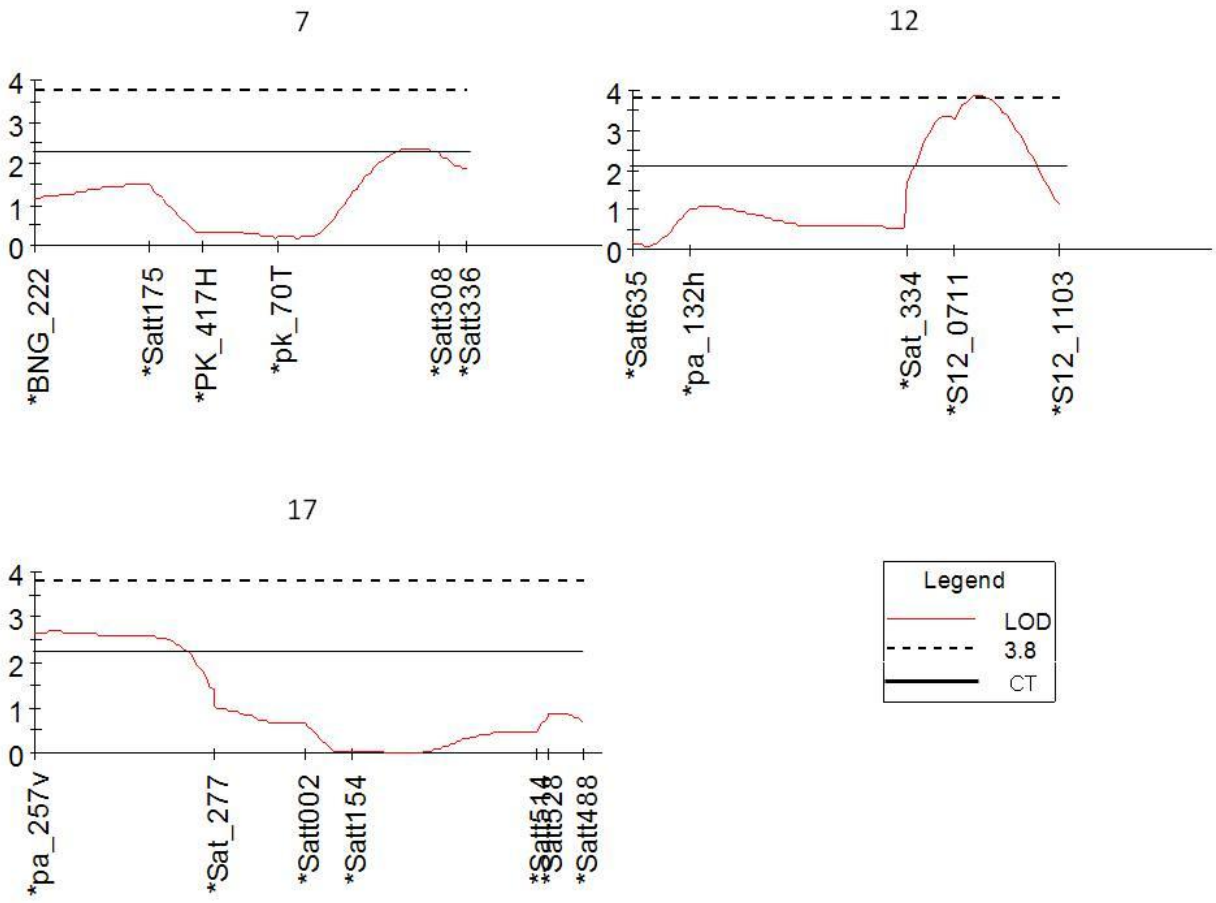

Figure 3. TP QTL mapping in combined data with the significant QTL on Chromosome 12. Solid line represents the chromosome-wide threshold and the dashed line represents the genome-wide threshold levels of significance P = 0.05.

CHAPTER 5. GENERAL CONCLUSIONS

The objectives of this research were to identify seed and leaf quantitative trait locus (QTL) for iron and zinc concentration in the soybean population Anoka x A7, to determine if QTL for iron and zinc accumulation co-localized to the same regions of the genome, to determine if any of newly identified QTL for Fe and/or Zn concentration correlate with QTL previously identified for Fe efficiency, to determine the amount of variation of total phosphorus in a population developed for iron deficiency chlorosis resistance, and to map total phosphorus as a QTL.

The Anoka x A7 population was used in this study because previous work identified a major iron efficiency QTL on chromosome 3 (Lin et al., 1997). The population was planted in 2008 and 2009 in order to determine Zn and Fe concentrations in leaf and mature seed tissue. Leaf tissue was collected at the second trifoliate leaf stage for mineral determination and DNA analysis. Additionally, total phosphorus was determined in mature soybean seed. To perform QTL mapping of these traits, the original genetic map constructed from the Anoka x A7 population, and consisting of 82 RFLP, 14 SSR, and one morphological marker, was integrated into the current genetic map. The complete linkage map consisted of a total of 150 markers that included 12 previously unmapped BARCSOYSSRs. These BARCSOYSSRs were used to increase marker density in sparse intervals.

In the first paper, major and suggestive QTL were identified for Fe and Zn concentration in independent and combined years. The one major QTL identified in the combined data was on chromosome 20. It had a LOD of 4.7 and represented 21.5 % of the variation. This QTL region also had previously been identified to contain an Fe efficiency

QTL. The left flanking marker of the Fe concentration QTL, pa_515-1, also identified the Fe efficiency QTL (Lin et al., 1997). Furthermore the additive effects for this significant QTL showed that alleles associated with elevated Fe concentrations were attributed to the Fe efficient parent. This was the first study to show that Fe efficiency QTL may be related to Fe accumulation in soybean seed. In the combined data, three suggestive QTL were identified on chromosomes 1 and 12, with two QTL identified on chromosome 12. These QTL had LOD scores of 2.8-3.4 with R^2s explaining 10.6-12.7% of the variation.

There were no significant QTL identified for Zn concentration in the seed, or for leaf Fe or Zn. However, suggestive QTL were identified, and the marker intervals of these suggestive QTL contained genes involved in mineral uptake, transport, and loading. The most important of these occurring on chromosome 7, which had *Ferric reductase, Ferritin-like* domain, and *Cation transport protein*. Chromosome 18 had a *ZIP Zinc transporter*, which is also involved in the transport of other minerals such as Fe. These candidate genes will need further evaluation to confirm if they are truly involved in mineral uptake and transport in this population.

This was the first study to show a genetic marker correlation for Fe efficiency and Fe accumulation in an IDC mapping population. The other QTL in this study was only suggestive, which could be due to the fact that the population was planted on non-calcareous soil. These results would suggest that the regions that have been identified are not necessarily involved in the IDC efficiency response, but rather are involved in Fe homeostasis and uptake, with the QTL on chromosome 20 being of major interest for Fe efficiency and accumulation. Stressing the lines within this population through planting on calcareous soil

could elucidate these effects. With the previous identification of Fe efficiency QTL in this population, and now Fe accumulation QTL, Fe efficient genotypes with elevated Fe levels can now be selected.

In the second paper, the genotypic variation for total phosphorus was not significant P = 0.0586. However, one significant QTL and five suggestive QTL were identified for total phosphorus concentration. This significant QTL was identified on chromosome 12 in the marker interval *S12_0711-S12_1103*, had a LOD of 3.9, and represented 29.9 % of the variation. The additive effects determined that Anoka contributed the positive allele for total phosphorus accumulation, which could indicate that an increase in total phosphorus could have an impact on the availability of iron to the developing plant. It was further determined that genes in this marker interval are involved in mineral transport or biochemical pathways that involve phytic acid phosphate (Raboy et al., 2001).

Intervals in the suggestive QTL regions also contained genes that are involved in phosphate transport and metabolism. For instance, the suggestive QTL regions of chromosomes 7 and 20 harbored an inorganic phosphate transporter gene. On chromosome 20, this gene was identified in a previous study to be a low-affinity phosphate transporter (Wu et al., 2011). The phosphate transporter on chromosome 7 was newly identified in the QTL region, although its specific function is yet to be determined. This research identified regions of interest for phosphorus uptake and utilization in soybean even though the population was not stressed for phosphorus availability.

This dissertation has presented information that iron, zinc, and phosphorus concentration can be mapped as quantitative traits. Furthermore, there are genetic factors

involved in the accumulation in these minerals; breeding for elevated iron and zinc with a decrease in total phosphorus was discussed.

CHAPTER 6. REFERENCES (for Introduction, Literature Review, and Conclusions chapters)

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