MOLECULAR APPROACHES TO FACILITATE MARKER-ASSISTED SELECTION FOR THE SOUTHERN ROOT-KNOT NEMATODE AND RENIFORM NEMATODE IN SOYBEAN

by

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(Under the Direction of H.R. Boerma)

ABSTRACT

This study was conducted to enhance marker-assisted selection for southern root-knot nematode [*Meloidogyne incognita* (Kofoid and White) Chitwood] (Mi) and reniform nematode (*Rotylenchulus reniformis* Linford and Oliveira) in soybean [*Glycine max* (L.) Merr.]. The objective of the first study was to determine the frequency of elite Mi-resistant cultivars that inherited the major resistance QTL (*Rmi1*) on LG-O and identify the ancestral source of the Mi-resistance allele at this locus in elite U.S. soybean cultivars. Forty-eight soybean genotypes, including ancestral, Mi-susceptible, and Mi-resistant genotypes, were evaluated for Mi-gall formation in a greenhouse in a randomized complete block experimental design with six replications. These genotypes were genotyped at six SSR loci (Satt358, Sat_132, Satt487, Satt500, Satt492, and Satt445) that flank the major Mi QTL on LG-O. Codescent analysis of markers and phenotypes across six cycles of breeding showed that Mi-resistant cultivars inherited a 200-bp band at Satt358 and a 238-bp band at Sat_132 from ‘Palmetto’.

The objective of the second study was to identify SNPs linked to the Mi resistant QTL on LG-O and LG-G and to optimize SNP genotyping conditions using Luminex 100 flow cytometer. We discovered four SNPs in Satt358 allele sequences located near a major Mi-resistant QTL on LG-O and 4 SNPs in Satt199 allele sequences located near a minor Mi-resistant QTL on LG-G between PI 96354 (Mi resistant) and Bossier (Mi susceptible). Using a direct hybridization assay, the genotypes of the SNP358 marker that targets two SNPs in Satt358 allele sequences were identical to the genotypes of the SSR marker Satt358 among 94 $F_{2:3}$ lines of PI 96354 × Bossier. The genotypes of SNP199 marker which targets a SNP in Satt199 allele sequences also showed 100% congruence with the genotypes of the SSR marker Satt199.

The objective of the third study was to map soybean QTL conferring resistance to reniform nematode in a population of 228 recombinant-inbred lines (RILs) derived from a cross between susceptible 'BSR101' and resistant PI 437654 . A major resistance QTL (R^2=21%) conditioning reniform reproductive index was found on LG-L flanked by Sat_184 and Satt513. Two other QTL were identified. One was located in the interval from Satt359 to Satt484 on LG-B1 (R^2=16%) and the other on LG-G (R^2=8%) linked to Sat_168. By screening the 'Prichard' × S94-1956 RILs, we confirmed that QTL on LG-G and -B1 would enhance reniform nematode resistance.

INDEX WORDS: Soybean, *Glycine max*, *Meloidogyne incognita*, Southern root-knot nematode, *Rotylenchulus reniformis*, Reniform nematode, QTL, SSR, Mapping, Nematode resistance, Pedigree, SNP, Direct hybridization, Luminex

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CHAPTER I

INTRODUCTION

Soybean [*Glycine max* (L.) Merr.] is a primary source of vegetable protein and oil. In 2000, soybean provided 67% of the world's protein meal and 29% of the vegetable oil. More soybeans are produced in the USA than any other country in the world. Soybean was planted on a record 74.5 million acres (30.2 million hectares) in 2000, producing 2.7 billion bushels (75.4 million metric tons) of seed. At the average price paid to farmers, $4.4 per bushel ($162 per metric ton), the total value of the 2000 U.S. soybean crop was $12.2 billion. In 2000, soybean provided 56% of world oilseed production, and 45% of those soybeans were produced in the USA. Fully 54% of the world's soybean trade originated from the USA (United Soybean Board, 2001).

However, a variety of pests have devastating effects on the agronomics and economics of soybean production, affecting yield and quality of grain and seed. Soybean diseases are primarily caused by four kinds of organisms or pathogens: (1) viruses, (2) bacteria, (3) fungi, and (4) nematodes. Plant parasitic nematodes are a serious threat for crop production worldwide because of their broad distribution, extensive host ranges, and involvement with fungi, bacteria, and viruses in disease complexes (Sasser, 1980). On a worldwide scale, losses to parasitic nematodes of all species are estimated to be $78 billion per year for the major food and fiber crops, and about $8 billion per year in the

USA (Barker et al., 1994).

Nematodes, the most abundant multicellular organisms on earth, are found in all habits and ecosystems of the biosphere. They occur in soil, decaying organic matter, and in all forms of plant life and most animals, including both domesticated and wild species (Norton, 1978). Nematodes are defined as microscopic, roundworm invertebrates with a body cavity and complete digestive tract (Hirschmann, 1971; Poinar, 1983).

Many types of nematodes are associated with plants, including crop plants growing in the field, garden, orchard, turf, and the diverse plant species that occur in natural ecosystems. Plant-parasitic nematodes represent one of the most important groups of organisms that affect the growth and yield of crop plants. Some plant parasitic nematodes are ectoparasites, living outside their host. These species cause severe root damage and can be important virus vectors (Brown et al., 1995). Other species spend much of their lives inside roots as migratory or sedentary endoparasites. Migratory parasites move through the root, causing massive cellular necrosis. However, it is the sedentary endoparasites, e.g., *Meloidogyne*, *Globodera*, *Heterodera*, and *Rotylenchulus* species, that cause the most economic damage worldwide (Williamson et al., 1996; Sijmons et al., 1994).

Current methods of limiting crop yield loss in fields infested with nematodes include crop rotation, resistant cultivars, and use of nematicides (Kinloch, 1980). However, due to the expense of nematicides and few profitable rotation crops, resistant cultivars are the most extensively used and cost-effective method. Therefore, breeders have been intensifying their efforts to develop improved nematode resistant cultivars. One of the challenges in the past has been that screening for resistant plants was tedious,

time-consuming, and variable (Boerma and Hussey, 1992). Recently, breeders have explored the use of genetic markers in place of traditional phenotypic screening. The essential requirements for marker-assisted selection in a crop breeding program are that marker should co-segregate or be closely linked with desired trait, the availability of an efficient means of screening large breeding populations, and the screening technique should be economical to use (Gupta et al., 1999; Francia et al., 2005).

The objectives of this research include: (1) identify the ancestral source of the *Rmil* gene on LG-O and determine the frequency of the resistance allele in elite southern root-knot nematode resistant cultivars, (2) develop SNP assays for high-throughput marker-assisted selection of southern root-knot nematode resistance in soybean, and (3) map soybean QTL conditioning resistance to reniform nematode.

CHAPTER II

REVIEW OF LITERATURE

Root-knot nematodes

Root-knot nematodes (*Meloidogyne* spp.) are economically important plant parasites that cause extensive crop losses all over the world (Sasser, 1977). Root-knot nematodes are widely distributed pathogens that establish a complex and long-lasting parasitic relationship with more than 2000 plant species (Sasser, 1980). To complete their life cycles, infective second-stage juveniles (J2) penetrate roots of susceptible hosts behind the root cap (Hussey, 1985) and migrate intercellularly to the developing protoxylem in the vascular cylinder (Wyss et al., 1992). Initial feeding activities by J2 induce localized modification of host cells to form a feeding site consisting of several multinucleate sites each containing several multinucleate giant-cells (Huang, 1985; Jones and Northcote, 1972). These giant-cells are larger than normal cells with multiple nuclei, thickened walls with extensive ingrowths, and dense cytoplasm (Bird, 1974; Jones, 1981). After 10 to12 d in roots of susceptible plants, the J2 ceases feeding and molts three times over a 48 h period. Subsequently, the female feeds and grows considerably larger than the giant-cells. The increases in nutrient demands and giant-cell growth correspond to egg production by the female (Jones, 1981). After completion of its life cycle, the female nematode dies and the giant-cells degenerate (Bird, 1962). The life cycle is complete in

approximately 25 d when the temperature remains around 27°C (Agrios, 1997).

Typical symptoms of nematode injury can involve both above-ground and below-ground plant parts. Below-ground symptoms include the swollen, knotted roots. The swellings become large and easy to see on some hosts such as soybean. However, above-ground symptoms exhibited by infected plants are similar to those produced on any plants having a damaged and malfunctioning root system (Hussey, 1985). These include: i) suppressed shoot growth and accompanying decreased shoot-root ratio; ii) nutritional deficiencies showing in the foliage, particularly chlorosis; iii) temporary wilting during periods of mild water stress or during mid-day, when temperatures are high even though adequate soil moisture is available, and iv) suppressed plant yields. The magnitude of these symptoms is often related to the number of juveniles penetrating and becoming established within the root tissue of young plants. The common explanation for the production of above-ground symptoms on diseased plants is that *Meloidogyne* infection affects water and nutrient absorption and upward translocation by the root system (Hussey, 1985).

Root-knot nematodes often interact with other soil-inhabiting plant pathogens to form disease complexes in which the resulting disease is much more severe than the individual components of the complex would cause alone. Powell (1971) showed that roots of mature tobacco (*Nicotiana tabacum* L.) plants infected by *M. incognita* were predisposed to infection by *Pythium ultimum* and *Rhizoctonia solani*, soil-inhabiting fungi that normally only parasitize roots of seedlings. Another significant role *Meloidogyne* spp. have in disease complexes involves altering the resistance of a host to the secondary pathogen. Tomato (*Lycopersicon esculentum* Mill.) and tobacco plants

resistant to *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) Snyder & Hansen and *Phytophthora parasitica* var. *nicotianae* (Breda de Haan) Tucker, respectively, become susceptible hosts for these fungi following infection by *M. incognita* (Powell and Nusbaum, 1960; Sidhu and Webster, 1977).

Root-knot nematodes in soybean

The three main species of *Meloidogyne* that cause damage in soybean are *M. incognita* (Kofoid and White) Chitwood (Mi), *M. arenaria* (Neal) Chitwood (Ma), and *M. javanica* (Treub) Chitwood (Mj) (Riggs and Schmitt, 1987). Root-knot nematode disease of soybean in the USA is largely confined to the southeastern states because of the warm climate and sandy soils (Walters and Barker, 1994). Thirty-five percent of soybean fields in North Carolina (Schmitt and Barker, 1988) and 28% in South Carolina (Lewis et al., 1993) were infested with root-knot nematodes. Garcia and Rich (1985) found that 70% of 103 soybean fields sampled in five north-central Florida counties were infested with Mi, 24% with Mj, and 6% with Ma. Kinloch (1974) indicated that yields of susceptible soybean cultivars were reduced 53 to 90% and yields of resistant cultivars reduced 32 to 40% in a Mi-infested field in Florida. Yield losses in the southern USA due to root-knot and ectoparasitic nematodes were estimated at $79.50 million in 1996 (Pratt and Wrather, 1998).

The most effective tactics currently available to limit soybean losses on root-knot nematode-infested land include crop rotation, resistant cultivars, and use of nematicides. Most *Meloidogyne* populations decline 90 to 95% between growing seasons in the

absence of a host (Bergeson, 1959). A 1-year rotation to cotton (*Gossypium hirsutum*) was shown to decrease subsequent population densities of Ma race 1 on peanut (*Arachis hypogaea* L.) by 43% (Rodriguez-kabana et al, 1987). However, cotton, a host for Mi, cannot be used in rotation with soybean for managing Mi. For Ma race 2, peanut and cotton are nonhosts and can be grown in rotation with soybean for management of this nematode (Kinloch and Dunavin, 1993). The wide and variable host range of the various *Meloidogyne* spp. requires that a considerable emphasis be placed on frequent assays for soil nematode species and population densities. In addition, the usefulness of crop rotation is limited by the lack of alternative high-value crops and other nematode species may build up in the soil (Riggs and Schmitt, 1987). Nematicides, such as soil fumigation with 1,2-dibromo-3-chloropropane (DBCP) and ethylene dibromide (EDB) have been shown to control the three *Meloidogyne* species (Kinloch, 1974; Minton and Parker, 1979). However, use of these chemicals has been prohibited because of environmental concerns (Anonymous, 1978; Ruckelshaus, 1983). In addition, their relatively high cost means that they are generally not economical for soybean production. The present lack of effective, inexpensive nematicides, the potential hazards of pesticides in the environment, and the wide host ranges of the *Meloidogyne* spp. make using resistant cultivars a critical management tactic.

Soybean cultivars resistant to *Heterodera glycines* (soybean cyst nematode) yield 10 to 50% more than susceptible cultivars in infested soil (Hartwig, 1981; Young and Hartwig, 1988). Planting a single resistant cultivar increased profits for soybean producers over $400 million during a 6-yr period (Bradley and Duffy, 1982). Resistant cultivars without nematicide treatment often yield as much as high-yielding susceptible

cultivars treated with nematicides (Epps et al., 1981). In Florida, yields from soybean cultivars resistant to Mi were five times greater than yields from highly susceptible cultivars (Kinloch et al., 1985). Use of resistant cultivars has the following advantages: i) suppresses nematode reproduction, ii) reduces need for toxic chemicals, iii) shortens length of rotations, iv) does not require use of specialized equipment, v) maintains cost of seed generally equal to that of susceptible cultivars, and vi) may limit disease complexes associated with nematodes (Boerma and Hussey, 1992; Mai and Abawi, 1987).

Resistance to root-knot nematodes

In plant nematology, host resistance is defined as the ability of host plants to restrict or prevent nematode reproduction. A highly resistant genotype allows only limited reproduction, whereas a partially resistant genotype supports an intermediate level of reproduction compared to a susceptible genotype that allows the nematode to reproduce freely. Resistance to root-knot nematodes does not protect the plants against nematode invasion, but once the nematode is inside the root the induction of the feeding site is either inhibited or initially established feeding structures disintegrate in early stages of nematode development (Jung and Wyss, 1999). The most common postinfectional resistance to *Meloidogyne* spp. involves hypersensitivity, in which necrosis occurs in giant cells or plant cells adjacent to these feeding sites (Cook, 1991). Mi-mediated resistance, which also confers resistance to several root-knot nematode species in tomato, is characterized by a localized necrosis of host cells near the invading nematode (Dropkin, 1969). Also, hypersensitive responses have been associated with resistance to Mi in

soybean resistance cultivars (Kaplan et al., 1979; Veech and Endo, 1970).

In a modern soybean breeding program screening protocol used to identify root-knot nematode resistant breeding lines should be capable of readily and reliably evaluating thousands of genotypes (Hussey and Boerma, 1981). This requirement is best fulfilled in a greenhouse environment that permits tests to be conducted throughout the year and it eliminates non-uniformity of nematode infestations that occur in fields. Hussey and Boerma (1981) reported a positive correlation between greenhouse and field Mi-gall number on a number of soybean genotypes.

During 1983 to 1989, 139 soybean cultivars adapted to the southern USA were evaluated for resistance to Mi in a greenhouse (Hussey et al., 1991). Seventy-one of the cultivars were identified with a moderate or high level of resistance to Mi. However, even cultivars with a high level of resistance to Mi sustain significant yield loss when nematode population levels are high (Kinloch, 1974). Luzzi et al. (1987) screened soybean genotypes (2370) in the USDA Southern Germplasm Collection (Maturity Group V, VI, VII, and VIII) under greenhouse conditions to identify the highest levels of resistance to Mi, Ma, and Mj based on root galling and nematode reproduction. Several soybean genotypes were identified with higher levels of resistance to Mi (Amredo, PI 96354, PI 408088, and PI 417444), Ma (PI 200538 and PI 230977), and Mj (PI 230977) than selected available in cultivars. Moura et al. (1993) showed that Mi parasitizing PI 96354 had the slowest developmental rate and produced 99% fewer eggs per egg mass than females on either ‘Forrest’ (partially resistant) or ‘Bossier’ (susceptible). In addition, Pedrosa et al. (1994) reported that there were fewer Ma galls, less Ma reproduction, and less yield loss on PI 200538 and PI 230977 than on ‘Jackson’, a partially resistant cultivar.

The resistant genotypes, G93-9009, G93-9106, and G93-9223, provided a higher level of resistance to Mi (Luzzi et al., 1996a), Ma (Luzzi et al., 1996b), and Mj (Luzzi et al., 1997) than exist in current commercial cultivars. The genotypes also have multiple pest resistance and acceptable agronomic characteristics including seed yield.

Inheritance of root-knot nematode resistance

The mode of inheritance of nematode resistance is important for the design of the most efficient breeding strategy to incorporate the resistance into commercial cultivars. Resistance genes can be classified based on their effects on resistance expression (major verses minor genes), their mechanism or durability (horizontal, race-nonspecific, and durable versus vertical, race-specific, and nondurable), and mode of inheritance (monogenic, oligogenic, and polygenic) (Roberts, 1990).

Once the source of resistance is identified, the breeder is interested in the number of genes conditioning the resistance. Several recent studies have examined soybean resistance to Mi (Luzzi et al., 1994b), Ma (Luzzi et al., 1995a), and Mj (Luzzi et al., 1995b) and found that the resistance was quantitatively inherited with moderate to high heritability. However, Luzzi et al. (1994a) reported that in a cross of Forrest and Bossier, partial resistance to Mi from Forrest was inherited as a single additive resistance gene, *Rmi1*. Variance component heritability estimates for Mj ranged from 0.48 to 0.76 on an entry-mean basis, for Ma it ranged from 0.74 to 0.83, and for Mi it ranged from 0.73 to 0.93. These moderate to high variance component heritabilities suggest that the resistance to three species of root-knot nematodes is controlled by a relatively few genes.

Additionally, resistance genes in many economically important crop plants to a range of *Meloidogyne* spp. species have been identified. The *Rk* locus in cowpea [*Vigna unguiculata* (L.) Walp.] confers resistance to three root-knot nematode species. Fery and Dukes (1980) characterized this gene as being a single, completely dominant nuclear gene with a major effect that expressed resistance to Mi, Mj, and Ma. Also, the *Mi* gene of tomato, that confers resistance to Mi, Ma, and Mj, was characterized as a single, completely dominant gene (Gilbert and McGuire, 1956; Bost and Triantaphyllou, 1982).

Mapping of root-knot resistance genes

Several RFLP markers associated with quantitative trait loci (QTL) conferring soybean resistance to Mi (Tamulonis et al., 1997c), Ma (Tamulonis et al., 1997b), and Mj (Tamulonis et al., 1997a) were identified. For Mi resistance, PI 96354, a plant introduction with a high level of resistance to Mi (Luzzi et al., 1987), was crossed with Bossier, a highly susceptible cultivar, and 110 F_2 plants were produced and mapped with 121 RFLP markers. Two QTL, a major resistance QTL (R^2=31%) linked to maker G248A-1 on LG-O of the USDA/ARS-Iowa State Univ. (USDA/ARS-ISU) soybean RFLP Map (Shoemaker and Specht, 1995) and a second minor QTL (R^2=14%) located on LG-G in the interval from K493H-1 to Cs008D-1, were identified. Together, the two QTL explained 39% of the variation in Mi galling in a multiple QTL regression model. Marker G248A-1 was reported to be closely linked to the *Rmi*1 gene (Tamulonis et al., 1997c).

For Ma resistance, a F_2 population from a cross between PI 200538 (Ma resistant) and 'CNS' (Ma susceptible) was mapped with 130 RFLPs (Tamulonis et al.,

1997b). Two QTL conferring resistance to Ma were identified. One QTL was mapped at RFLP marker B212V-1 on LG-F, and accounted for 32% of the variation in gall number. Another QTL was mapped in the interval of B212D-2 to A111H-2 on LG-E, and accounted for 16% of the variation in gall number. The two QTL accounted for 51% of the variation in gall number in a two-QTL multiple regression model.

For Mj resistance, 84 F_2 progeny from a cross between CNS and PI 230977 were used to create a linkage map that included 86 RFLP markers and three morphological traits. Two QTL with the resistance alleles derived from PI 230977 were identified on two separate linkage groups. Marker B212-1 on LG-F accounted for 46% of the variation in Mj gall number, whereas A725-2 on LG-D1 accounted for 13%. The additive model best fit the data, and together the two markers accounted for 54% of the variation in gall number.

The results from the RFLP mapping of QTL conditioning Ma and Mj resistance and the phylogenetic analysis of *Meloidogyne* spp. show the existence of co-evolution between Ma and Mj. Phylogenetic analyses show a closer relationship between Ma and Mj than Ma and Mi (Hyman and Powers 1991; Baum et al., 1994). Results from the QTL mapping of Ma and Mj identified the same major QTL on LG-F and different minor QTL. Different minor-resistance QTL for Ma and Mj resistance would support the segregation in gall number observed in the cross of PI 200538 × PI 230977 when inoculated with Ma (Luzzi et al. 1995a). PI 200538 and PI 230977 are resistant to both Ma and Mj (Pedrosa et al. 1994; Luzzi et al. 1987). As a result, RFLP mapping of Ma resistance and Mj resistance reveals that the major resistance QTL for Ma and Mj could be the same gene (Tamulonis et al., 1997b).

Clustering of disease resistance genes

In addition to the Ma- and Mj-resistance QTL on LG-F, five other disease resistance QTL are known to reside in the same 10-cM region of LG-F. A soybean corn earworm, *Helicoverpa zea* Boddie, resistance QTL (CEW2-1) was detected in the intervals between markers B212-1 and A757-1 on LG F (Rector et al., 1999). The *Rpg1* locus conferring resistance to *Pseudomonas syringae* pv. *glycinea* is on LG F, flanked by the marker K644-1 and B212-1 (Ashfield et al., 1998). The *Rps3* loci conferring resistance to phytophthora root rot, caused by *Phytophthora megasperma* var. *sojae*, in soybean was linked with RFLP marker R045-1 on LG F (Diers er al., 1992; Shoemaker and Specht, 1995). The *Rsv1*, a gene conferring resistance to soybean mosaic virus (SMV) (Yu et al., 1994) and the *Rpv1* locus conferring resistance to peanut mottle virus (Palmer and Kilen, 1987) are closely linked to RFLP marker A186-1 on LG F. The clustered QTL confer resistance to a diverse group of pathogens from three kingdoms (animal, monera, and fungi) and two viruses.

This region of clustered genes could relate to an evolutionary association of development and divergence of resistance factors that share some significant homology, probably through some process of gene duplications. The soybean genome has undergone one or possibly several rounds of duplication during its evolution (Zhu et al., 1995; Shoemaker et al., 1996). This suggests that the resistance genes may have been duplicated as well. Duplication followed by mutation, genetic divergence, and specificity could account for the clustered resistance genes. Alternatively, a gene may confer

resistance to more than one pathogen.

Complex resistance loci are known for other plant pathosystems that conform to gene-for-gene interactions. Resistance to the fungus *Cladosporium fulvum* (*Cf-2* and *Cf-5*) and the nematode *M. incognita* are closely linked in tomato (Dixon et al., 1995). The barley, *Hordeum vulgare* L., *Mla* locus for resistance to the powdery mildew, caused by the fungus *Erysiphe graminis* f. sp. *Hordei*, has dual attributes of a complex R gene locus. Currently 28 characterized alleles and another 16 race-specific powdery mildew R genes closely linked in coupling to named alleles at the *Mla* locus provide a total of 44 mildew resistance genes at or near locus *Mla* (Jorgensen, 1994). The maize, *Zea mays* L., *Rp1* locus for resistance to the rust fungus *Puccinia sorghi* has been characterized as a complex R gene locus, composed of at least 14 alleles and genes within about 1 cM of *Rp1* and an additional three loci within 1 to 3 cM of *Rp1* (Hulbert et al., 1993; Sudupak et al., 1993).

Reniform nematode

Reniform nematodes (*Rotylenchulus reniformis* Linford & Oliveira) are semi-endoparasites of roots and occur commonly in tropical and subtropical regions (Robinson et al., 1997). The distribution extends over more than 38 countries in South America, the southern Atlantic Gulf-coast of the USA, Africa, the Middle East, South-East Asia, China, Japan, and Australia (Gaur and Perry, 1991). The term “reniform” refers to kidney-shape of the body of the sedentary mature female (Linford and Oliveira, 1940). *Rotylenchulus reniformis* has a wide host range with at least 314 hosts out of 364 species evaluated,

including soybean and cotton (Robinson et al., 1997), and has been reported in Alabama, Arkansas, Florida, Georgia, Louisiana, Mississippi, South Carolina, and Texas in the USA (Minton and Hopper, 1959; Robbins, 1982; Bird et al, 1973; Neal, 1954; Fassuliotis and Raw, 1967; Birchfield et al., 1966; Heald and Robinson, 1990).

The life cycle of reniform nematode is usually shorter than 3 wk depends on soil temperature (Robinson et al., 1997). However, it can survive at least 2 yr in the absence of a host in dry soil through anhydrobiosis, a survival mechanism without water (Radewald and Takeshita, 1964). Eggs hatch 1 to 2 wk after being laid. The first-stage juvenile molts within the egg and produces the J2 that emerges from the egg. The J2 and following juvenile stages (J3 and J4) are not parasitic and retain the cuticles of the previous stages after molting (Robinson et al., 1997). The final molt gives rise to approximately equal numbers of vermiform parasitic females and nonparasitic males (Bird, 1983). In the presence of host roots, vermiform females develop feeding sites in the pericycle or deep cortex by interacting with the host in the formation of a syncytium. The syncytium consists of dense granular cytoplasm, dissolution of cell walls and coalescing of the cytoplasm of adjacent cells, thickening of cell walls by deposition of polysaccharides, hypertrophy of cells and enlargement of nuclei and nucleoli (Rebois et al., 1975). During the 10 to 20 d after feeding begins, depending on temperature, the posterior of the female swells into a characteristic kidney shape and 40 to 200 (usually about 60) eggs are laid in a sticky gelatinous matrix (Robinson et al., 1997). In most populations reproduction is amphimictic with numerous (40-60%) males, but a few populations are parthenogenetic with very few or no males (Sivakumar and Seshadri, 1971; Nakasono, 1977).

Reniform nematode in soybean

Recently, reniform nematode has been recognized to be a major problem on soybean in the southern USA (Robbins et al., 1994a). Though females induce the development of a syncytium at the feeding site, the root does not swell in response to feeding as with root-knot nematode. However, reniform nematode causes a root decay, unthrifty growth, empty been pods, and up to 10% yield reduction of susceptible cultivars (Birchfield et al., 1971; Williams and Birchfield, 1974). Parasitized plants exhibit varying degrees of stunting and chlorosis. Initial field infestations may exhibit patchy areas of poor growth. Significant to heavy yield loss was observed in both Louisiana (Birchfield et al., 1971) and South Carolina (Fassuliotis and Raw, 1967). Rebois and Johnson (1973) reported that an initial population of 6579 nematodes per L of soil consistently reduced yields on both moderately resistant and susceptible cultivars by an average of 33%, and reduced the content of phosphorus and potassium in harvested seeds.

The reniform nematode is normally managed by use of resistant cultivars, nematicides, and crop rotation. Since nematicides and/or rotation to a non-host crop are usually not economical for soybean producers, genetic resistance is the most desirable control method (Harville et al., 1985). Rebois et al. (1968) found that the soybean cultivars Pickett and Dyer were resistant to the reniform nematode. Also Rebois et al. (1970) reported that resistance to reniform nematode could be expected in genotypes with resistance to the soybean cyst nematode (SCN). This was related to similarities in the female feeding site and the associated tissue responses for both the reniform and cyst

nematodes (Rebois et al., 1970). Recent studies have shown that SCN-resistant soybean cultivars that derive resistance from 'Peking' (e.g. Forrest and 'Centennial'), or PI 437654 (e.g. 'Hartwig'), or PI 90763 (e.g. 'Cordell') are also resistant to *R. reniformis*, whereas those that derive SCN resistance from PI 88788 are not resistant to *R. reniformis* (Robbins et al., 1994a,b; Davis et al., 1996; Robbins and Rakes, 1996). Also, Robbins and Rakes (1996) found that of 45 soybean lines reported to be resistant to soybean cyst nematode, 16 were resistant to reniform nematode whereas 29 were susceptible.

Resistance to reniform nematode

The genetics of resistance against *R. reniformis* has been studied in soybean (Williams et al., 1981; Harville et al., 1985). Williams et al. (1981) reported that in a cross with Forrest (resistant) and 'Ransom' (susceptible), reniform nematode resistance was recessive and controlled by alleles at one locus. In other soybean populations, resistance in soybean was controlled by two loci with unequal effects (Harville et al., 1985). The proposed genotypes of the four parental cultivars were susceptible soybean cultivar Davis Rn_1 Rn_1 Rn_2 Rn_2, moderately susceptible soybean cultivar Bragg Rn_1 Rn_1 rn_2 rn_2, moderately resistant soybean cultivar Dare rn_1 rn_1 Rn_2 Rn_2, and resistant soybean cultivar Pickett 71 rn_1 rn_1 rn_2 rn_2.

Many studies using DNA markers have concentrated on QTL that control parasitic nematodes in soybean. Southern root-knot nematode [*M. incognita* (Kofoid and White) Chitwood] (Tamulonis et al., 1997c; Li et al., 2001), peanut root-knot nematode [*M. arenaria* (Neal) Chitwood] (Tamulonis et al., 1997b) and javanese root-knot

nematode [*M. javanica* (Treub) Chitwood] resistance QTL (Tamulonis et al., 1997a) have been identified. In addition, DNA markers have been used to dissect quantitatively inherited resistance to the SCN (Concibido et al. 1994; Webb et al. 1995; Cregan et al., 1999). Webb et al. (1995) mapped three major QTL to linkage groups (LG) A2, G and M, respectively, that confer resistance to SCN race 3 in a cross between PI 437654, resistant to SCN and reniform nematode, (Robbins and Rakes, 1996) and 'BSR101'. All SCN resistant genotypes, PI 209332, PI 88788, PI 90763, and Peking, had a major SCN resistance QTL, *rhg1*, on LG-G (Condibido et al., 1996, 1997; Chang et al., 1997). Peking and PI 437654 both had a resistance gene *Rhg4* which maps near the *I* locus on LG-A2 (Chang et al., 1997; Webb et al., 1995). Additionally, PI 90763 and PI 209332 were found to have a resistance gene mapping to the same location on LG-J (Concibido et al., 1997). Currently, Cregan et al. (1999) reported that marker-assisted selection (MAS) using Satt309, located 1 to 2 cM from *rhg1* on LG-G should be effective to select for SCN resistance at *rhg1* derived from Peking, PI 90763, and PI 437654 in crosses with most SCN-susceptible soybeans genotypes. In a direct comparison, genotypic selection with Satt309 was 99% accurate in predicting lines that were susceptible in subsequent greenhouse assays (Mudge et al., 1997; Cregan et al., 1999).

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CHAPTER III

PEDIGREE ANALYSIS OF A MAJOR QTL CONDITIONING SOYBEAN RESISTANCE TO SOUTHERN ROOT-KNOT NEMATODE[1]

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ABSTRACT

The southern root-knot nematode [*Meloidogyne incognita* (Kofoid and White) Chitwood] (Mi) is a serious pest of soybean [*Glycine max* (L.) Merr.] in the southern USA. Many soybean cultivars with Mi resistance and high productivity have been developed in the USA during the past few decades. DNA markers have been used to identify a major quantitative trait locus (QTL) near the top of Linkage Group O (LG-O) conferring resistance to Mi. The objectives of this study were to determine the frequency of elite Mi-resistant cultivars that inherited the major Mi resistance QTL on LG-O and determine the ancestral source of the Mi-resistance allele at this QTL. Forty-eight soybean lines, including ancestral, Mi-susceptible, and Mi-resistant genotypes were analyzed at six simple sequence repeat (SSR) loci that flank the major Mi QTL on LG-O. Codescent analysis of markers and phenotypes across six cycles of breeding showed that Mi-resistant cultivars inherited a 200-bp band at Satt358 and a 238-bp band at Sat_132 from 'Palmetto'. The tight linkage of both Satt358 and Sat_132 to the Mi QTL on LG-O indicates that selection for the Mi-resistant parent's allele at either of these markers should be highly effective in identifying Mi-resistant plants or lines.

INTRODUCTION

Root-knot nematodes (*Meloidogyne* spp.) are economically important plant pathogens that cause extensive crop losses all over the world (Sasser, 1977). They have extensive host ranges, their distribution is worldwide, and they interact with fungi in disease complexes. In the southeastern USA, the southern root-knot nematode (Mi) is the most common root-knot nematode. In a Florida field infested with Mi, soybean yields of susceptible cultivars were reduced by 53 to 90% (Kinloch, 1974). Yield losses in the southern USA due to root-knot nematode were estimated at $80 million in 1996 (Pratt and Wrather, 1998).

The development and use of nematode-resistant soybean cultivars result in reduced yield losses and increased grower profits (Boerma and Hussey, 1992). Thus, many soybean cultivars with root-knot nematode resistance and high productivity have been developed in the USA druing the past few decades. The Mi-resistant soybean 'Bragg' (Hinson and Hartwig, 1964) originated from the cross 'Jackson' × D49-2491 and the Mi-resistant 'Forrest' (Hartwig and Epps, 1973) from the cross 'Dyer' × Bragg. The Mi-resistant 'Maxcy' (Shipe et al., 1994) and 'Doles' (Boerma et al., 1994b) were released in the 1990s. Both cultivars share Forrest as a common parent. Forrest was shown to possess a single additive Mi-resistance gene, *Rmi1* (Luzzi et al., 1994). In a cross of PI 96354, which possesses a high level of resistance to Mi (Luzzi et al., 1987), and 'Bossier' (Mi susceptible), a major QTL was identified which mapped to LG-O of the public soybean genetic linkage map (Tamulonis et al., 1997). With SSR markers, Li et al. (2001) found the major Mi QTL on LG-O was indicated in the Satt492 and Satt358

interval and located 3.1 cM from Satt492. Tamulonis et al. (1997) speculated, based on segregation for gall number in a population of Forrest × PI 96354 and the level of resistance in Forrest, that the Mi-resistance QTL on LG-O was the *Rmi1* gene.

The availability of pedigree data and molecular linkage maps provides an opportunity to track genomic regions through the breeding process (Shoemaker et al., 1992). King et al. (1999) tracked *Vf* for scab (*Venturia inaequalis*) resistance in apple (*Malus*) accessions and assessed linkage drag through a genome scan of regions flanking the introgression site. Narvel et al. (2001a, 2001b) used a molecular pedigree analysis to track the *rxp* gene for bacterial pustule (*Xanthomonas campestris* pv. *Glycines*) resistance in elite North American soybean cultivars and to monitor introgression of soybean insect resistance in 15 resistant genotypes.

The initial applications of marker-assisted selection in soybean improvement were for traits conditioned by QTL with relatively large phenotypic effects and in which the positive alleles were rare in the ancestoral germplasm (Orf et al., 2004). In these situations, breeders are usually aware of the QTL or genes conditioning the selected trait in their segregating populations and the parental source of the positive alleles at these QTL. In this study, we determine the frequency of elite Mi-resistant cultivars that inherited the major LG-O resistance QTL (*Rmi1*) and determine the ancestral source of the Mi-resistance allele at this QTL in elite U.S. soybean cultivars.

MATERIALS AND METHODS

Forty-eight soybean genotypes, including ancestral, Mi-susceptible, and Mi-resistant genotypes, were chosen on the basis of their potentially informative pedigree and published resistance to Mi. Seed for these cultivars were obtained from the USDA Soybean Germplasm Collection maintained at the Univ. of Illinois (Urbana, IL). The 48 genotypes were evaluated for Mi-gall formation in a greenhouse in a randomized complete block experimental design with six replications. Three seed of each genotype were planted into individual 20.6-cm Ray Leach Single Cell Cone-tainers (Stuewe & Sons, Inc., Corvallis, Oregon) containing approximately 15.6 cm^2 of methyl-bromide-fumigated Pacolet sandy loam soil, a member of the fine, kaolinitic, thermic family of Typic Kanhapludults, amended with methyl-bromide-fumigated sand to a texture of 73% sand, 16% silt, and 11% clay (by weight). Cones were placed in every other row of a Ray Leach 98-cone tray. Resistant and susceptible checks were planted in each tray to develop a gall index. The checks included 'Bryan' (highly resistant), 'Perrin' (resistant), and 'GaSoy 17' (susceptible).

Approximately 7 to 10 d after planting, the plants were thinned to one plant per cone and then inoculated with 4000 Mi eggs. The Mi inoculum was propagated on 'Marion' tomato (*Lycopersicon esculentum* Mill.) and egg inoculum was collected according to the procedure described by Hussy and Barker (1973). The number of eggs per milliliter of inoculum was adjusted so that the desired inoculum density was applied in a volume of 3 to 5 mL. The inoculum was placed at a soil depth of 2 to 3 cm with an

ARTEK Systems Corporation digital dispensing pump (Asteck Systems Corp., Farmingdale, NY). Each plant was fertilized weekly with 6 mg N, 3 mg P, and 5 mg K. Fifteen hours of supplemental light was provided by 400-watt Multivapor metal halide lamps (Westinghouse Electric Corp., Lamp Division, Bloomfield, NJ) which were suspended 1.4 m above the greenhouse bench. The greenhouse was maintained at 28°C±5°. The plants were irrigated twice a day by a mechanical overhead irrigation system with water that was heated to 36°C±3°. The experiment was terminated 30 d after inoculation when galls had developed on the susceptible checks. Following removal of plants from the cones, the roots were excised, washed free of soil, and evaluated for gall number. The number of galls on the resistant and susceptible standards was used to develop a gall index, where 1 ≤ 10 galls per plant, 2 = 11 to 20, 3 = 21 to 30, 4 = 31 to 40, and 5 >40 galls. Data for gall number were analyzed by ANOVA with SAS (SAS Institute, 1992).

Soybean DNA was extracted from seeds of each genotype according to modified procedures of Kang et al. (1998), quantified by a FL600 Microplate Fluorescence Reader (Bio-Tek Instruments, Winooski, VT), and diluted to 20ng μL^{-1} for the polymerase chain reaction (PCR). Seven seeds from each cultivar were ground with coffee grinder (Braun KSM2, Boston, MA), and 0.1g of each homogenate was transferred to a new 1.5-mL tube containing 500 µL extraction buffer [200 m*M* Tris pH 8, 200 m*M* NaCl, 25 m*M* ethylenediamine tetraacetic acid (EDTA), 0.5% sodium dodecyl sulfate], 10 µL Proteinase K (20 mg mL^{-1}). The samples were incubated in a water bath at 50°C for 1h and then 500 µL CTAB solution (2% cetyltrimethylammonium bromide, 100 m*M* Tris-

HCl pH 8, 20 m*M* EDTA pH 8, 1.4 *M* NaCl) was added. After shaking, the samples were spun at 12 000 rpm (Beckman Microfuge E, Beckman Instruments, Carlsbad, CA) for 10 min. The supernatant was transferred to a new 1.5-mL tube, and then one volume chloroform/isoamyl alcohol (24:1, v/v) was added. After shaking for 1 min at room temperature, the samples were spun at 12 000 rpm for 10 min. The supernatant was transferred to a new 1.5-mL tube, and then 80% volume of isopropyl alcohol was added to precipitate DNA. The supernatant was decanted and the pellets were washed with 70% (v/v) ethanol. The DNA pellets were then dried and dissolved in 100 μL of tris-EDTA buffer.

On the basis of the integrated genetic linkage map of soybean (Cregan et al., 1999), six SSR markers (Satt358, Satt487, Satt500, Satt492, Satt445, and Sat_132) located near Mi-resistance QTL on LG-O were chosen (Li et al., 2001). These six markers span approximately 15-cM region of LG-O. The primer sequences for each SSR were obtained from SoyBase, a USDA-sponsored genome database (http://soybase.org/ssr.html; verified 11 July 2006). Fluorescent-labeled forward primers and nonlabeled reverse primers were obtained from PE-ABI (Foster City, CA). Polymerase chain reactions were prepared on the basis of the protocol of Diwan and Cregan (1997), with slight modifications. The 10-μL reaction mix contained 2 μL of 40 ng template DNA, 1.0 × PCR buffer, 2.5 m*M* $MgCl_2$, 100 μ*M* of each dNTP, 0.2 μ*M* each of forward and reverse primers, and 0.5 unit of Taq DNA polymerase. The reactions were performed in a dual 384-well GeneAmp PCR System 9700 (Perkin Elmer, Norwalk, CT). The PCR amplicons were analyzed on an ABI-Prism 377 DNA sequencer (PE-ABI,

Foster City, CA) with a 4.8% acrylamide to bisacrylamide (19:1) gel at 750 V for 2 h. Marker data were collected with DNA Sequencer Collection software v.2.5. The marker fragments were analyzed with GeneScan software v.3.0 and scored with Genotyper software v.2.1 (PE-ABI, Foster City, CA).

RESULTS AND DISCUSSION

The published pedigrees and Mi reactions of the 48 genotypes included in this study are shown in Table 3.1. Seed was not available for two parental breeding lines, R66-1517 and N70-2173 and Bossier was included in the experiment as the susceptible check. As reported by Hussey et al. (1991), a gall index of ≤ 1.5 indicated a high level of Mi-resistance, an index of ≥ 1.6 to ≤ 2.5 was considered to indicate a moderate level of Mi-resistance, and a gall index of ≥ 2.6 was considered susceptible. After screening with an inoculum density of 4000 Mi eggs per plant, 24 genotypes were identified resistant to Mi (Table 3.2). Also, Bragg, 'Wright', and 'Cook' had moderate resistance to Mi. However, 'Sharkey', Dyer, and FC 33243 produced a large number of galls which is inconsistent with their previously reported Mi reactions (Hartwig and Epps, 1968; Caviness et al., 1975; Hartwig et al., 1988). The susceptible reaction of Sharkey is consistent with the result of Hussey et al. (1991). In addition, 'Pharaoh' was identified resistant to Mi in this study, but Schmidt et al. (1993) reported Pharaoh as moderately susceptible. We also screened unreported genotypes to Mi reaction. Palmetto was resistance to Mi, but Volstate, Tokyo, and PI54610 were identified as susceptible to Mi.

This results indicate that the source of the Mi-resistance in Jackson which originated from the cross Volstate (2) × Palmetto was most likely Palmetto (Table 3.1).

The ability to detect the major Mi-resistance QTL on LG-O through SSR marker analysis was tested by genotyping 48 soybean lines, including ancestral, Mi-susceptible, and Mi-resistant cultivars (Table 3.2). At Satt358, three alleles, 160, 192, and 200 bp, were detected across the 48 genotypes. The 200-bp band of Satt358 was present in all the Mi-resistant genotypes with the exception of 'Lee 74', which will be discussed in greater detail later is this section. All the Mi-susceptible genotypes possessed a 192-bp band at this marker except FC 33243. The cultivars Gregg and Maxcy were resistant to Mi, but were heterogeneous for the two bands at Satt358 (Table 3.2).

Six different allele sizes were found among the 48 genotypes at Sat_132 (Table 3.2). All of the Mi-resistant genotypes with the exception of Maxcy had a 238-bp band at Sat_132. The Mi-resistant Gregg was heterogeneous for the 238- and 252-bp bands. Maxcy possesses a 236-bp band at Sat_132, which was also present in the Mi-susceptible genotypes 'Hampton', 'Coker 338', 'Johnston', and 'Coker 488'. This band was not present in any of the other genotypes in our study, and all the other Mi-susceptible genotypes possess a 246-, 248-, 250-, or 252-bp band at this marker. It is interesting that the Mi-resistant Maxcy and the Mi-susceptible Coker 338 and Coker 488 share Hampton as a common ancestor (Table 3.1). Maxcy appears to possess a cross-over between Sat_132 and the Mi QTL. The alleles in the 48 genotypes at markers Satt487, Satt445, and Satt500 were not as strongly associated with Mi-resistance as Satt358 or Sat_132 (Table 3.2). Satt492 had a same allele in all genotypes except Young and Gasoy 17.

In the cases where we found a Mi reaction that was different from that reported in the literature, the Satt358 marker data supported our classification. For example, Pharaoh, which was previously reported as moderately susceptible to Mi but was resistant in our study, had the 200-bp band at Satt358 (Tables 3.1 and 3.2). Sharkey and Dyer, which were previously reported as resistant to Mi but found to be susceptible in our study, possess the 192-bp band at Satt358, which is present in all the other Mi-susceptible genotypes. When Pharaoh, Dyer, and Sharkey were evaluated for their Mi reaction in a common experiment with 45 other genotypes, their Mi reaction was predicted on the basis of the presence or absence of the 200-bp allele for Mi resistance at Satt358.

Lee 74 originated from the cross 'Lee 68' × R66-1517. The breeding line R66-1517 was selected from a backcross population of 'Lee' (5) × FC 33243. The donor parent of Lee 74's Mi resistance was reported to be FC 33243 (Caviness et al., 1975). The 160-bp band at Satt358 from FC 33243 was present in the Mi-resistant Lee 74. The problem with use of these data to delineate the association of Satt358 and the Mi QTL on LG-O is that FC 33243 was susceptible to Mi in our study (Table 3.2). Lee and its parents 'S100' and 'CNS' were also susceptible. During our Mi screening experiment, we noticed that FC 33243 possessed a greater amount of plant–to-plant variation than the other genotypes. To verify this observation, we determined the Mi reaction on an additional 40 individual FC 33243 plants. After counting the number of galls on these 40 plants, we transplanted seven plants with the fewest galls (9 to 19 galls) and the six plants with the most galls (73 to 90 galls). The progeny from these plants produced either a low or high number of galls similar to the gall number of their progenitor. It is assumed that one of the FC 33243 Mi-resistant plants was the source of the Mi resistance in Lee 74. Thus, FC

33243 possesses phenotypic heterogeneity for its Mi reaction and is homogenous for the 160-bp band at Satt358 and homogeneous for the other five SSR markers we evaluated in this region of LG-O. Since the FC 33243 resistant and susceptible selections possessed the 160-bp band at Satt358, it is possible that Lee 74 possesses a Mi-resistance gene other than *Rmi1*. Another explanation would be a mutation in the *Rmi1* gene in the Mi-susceptible FC 33243 plants.

The results of the Mi screening and SSR marker genotyping for the Satt358 and Sat_132 markers were integrated in Figure 3.1. Genotypes in nonshaded boxes are Mi susceptible, genotypes in gray-shaded boxes are Mi resistant and inherited a 200-bp band at Satt358 and a 238-bp band at Sat_132, and genotypes in dark-shaded boxes are Mi resistant and inherited a 160-bp band at Satt358 and a 248-bp band at Sat_132. On the base of these results, we can conclude that there are two ancestral sources of Mi resistance in elite southern U.S. soybean cultivars. One is Palmetto and the other is FC 33243. Palmetto is likely the source of Mi-resistance gene (*Rmi1*) that was found in Forrest (Luzzi et al., 1994), and most elite Maturity Group V, VI, VII, and VIII cultivars with Mi resistance contain the *Rmi1* gene.

Our study provides strong evidence of the co-segregation of the 200-bp band at Satt358 and Mi resistance (*Rmi1*). This co-segregation has been maintained across approximately six cycles of breeding (crossing and inbreeding). The apparent tight linkage of Satt358 and Sat_132 and the major QTL for Mi resistance on LG-O, the major phenotypic effect of this QTL on Mi gall number, and the relatively rare occurrence of the resistance allele at this QTL among the ancestors of southern U.S. elite cultivars suggest that these markers could be effectively employed in breeding for Mi resistance.

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Table 3.1. Pedigrees and published southern root-knot nematode [*Meloidogyne incognita* (Kofoid and White) Chitwood] (Mi) reactions of 48 soybean genotypes.

Genotype	Pedigree	Year of release	Mi react-ion[†]	Source
D49-2491	S-100×CNS	-	-	
PI 54610		-	-	
'Tokyo'	Variety name assigned directly to or variety Selected from PI 8424	1907	-	
'Palmetto'	Variety name assigned directly to or variety Selected from PI 71587	1936	-	
'Volstate'	Tokyo × PI 54610	1942	-	
S-100	Selected from 'Illini' in 1938 by Lee Mumford, farmer, Rutledge, MO. Reselected in 1942 by the Missouri Agric. Exp. Stn.	1945	-	
'Pharaoh'	Forrest × V71-480	1989	MS	Schmidt et al. (1993)
FC 33.243	A rogue selected from Lincoln by H.J. Anderson, Calamus, IA, in 1954	-	R	Caviness et al. (1975)
'Jackson'	Volstate(2)[‡] × Palmetto	1953	R	Johnson (1958)
'Bragg'	Jackson × D49-2491 (S-100×CNS)	1963	R	Hinson and Hartwig (1964)
'Dyer'	Hill × ('Lee' (2) × Peking)	1967	R	Hartwig and Epps (1968)
'Forrest'	Dyer × Bragg	1972	R	Hartwig and Epps (1973)
'Hutton'	F55-822 (Jackson×D49-2491) × (Roanoke×CNS-4)	1972	R	Hinson (1973)
'Cobb'	F57-735×D58-3358 (Jackson(4) ×D49-	1973	R	Hartwig and Lappas

	2491)			(1980)
‘Lee 74’	‘Lee 68’ × R66-1517 (Lee(5) × FC 33.243)	1974	R	Caviness et al. (1975)
‘Centennial’	D64-4636 (Hill×(D49-2491(4) ×Jackson) × a tawny pubescent ‘Pickett 71’ type)	1977	R	Hartwig and Epps (1977)
‘Govan’	Bragg × Semmes	1977	R	Hartwig et al. (1978)
‘Braxton’	F59-1505 × (Bragg(3) ×D60-7965)	1979	R	Hussey et al. (1991)
‘Wright’	Bragg × Lee	1979	R	Boerma et al. (1980)
‘Gregg’	Bragg × Pickett 71	1983	R	Harville et al. (1988)
‘Gordon’	Forrest × Pickett 71	1984	R	Boerma et al. (1985)
‘Coker 6738’	Braxton × Coker 368	1987	R	Hartwig and Edwards (1989)
‘Perrin’	Coker 488 × Braxton	1988	R	Shipe et al. (1990)
‘Lamer’	‘Tracy-M’ × selection (Centennial × D75-10169)	1989	R	Hartwig et al. (1990)
‘Hagood’	Centennial × Young	1990	R	Shipe et al. (1992)
‘Walters’	Forrest × Narow	1990	R	Caviness et al. (1991)
‘Cook’	Braxton × Young	1991	R	Boerma et al. (1992)
‘Hartwig’	Forrest (3) × PI 437654	1991	R	Anand (1992)
‘Manokin’	L70L-3408 × D74-7824 (Forrest × D70-3001)	1991	R	Kenworthy et al. (1996)
‘Maxcy’	D76-9665 (Forrest × Centennial) ×Johnston	1992	R	Shipe et al. (1994)
‘Doles’	D74-7741 (Forrest × D70-3001) × Young	1993	R	Boerma et al. (1994b)
‘Haskell’	Johnston × Braxton	1993	R	Boerma et al. (1994a)
‘Dillon’	Centennial × Young	1995	R	Shipe et al. (1997)
‘Benning’	Hutcheson × Coker 6738	1996	R	Boerma et al. (1997)
‘Sharkey’	Tracy × Centennial	1987	R	Hartwig et al. (1988)

			S	Hussey et al. (1991)
‘CNS’	Selected from 'Clemson'	1943	S	Hussey et al. (1991)
‘Lee’	S-100 × CNS	1954	S	Johnson (1958)
‘Bossier’	A late mutant in Lee	1958	S	Hussey et al. (1991)
‘Hampton’	Majos × Lee	1962	S	Webb and Hicks (1965)
‘Ransom’	(N55-5931 × N55-3818) × D56-1185	1970	S	Brim and Elledge (1973)
‘Pickett 71’	'Pickett' × phytophthora rot-resistant Lee type	1971	S	Hartwig et al. (1971)
‘Coker 338’	Hampton 266 × Bragg	1973	S	Hartwig and Lappas (1980)
‘Coker 488’	Hampton 266 × Bragg	1977	S	Hartwig and Lappas (1980)
‘Gasoy 17’	Bragg × Hood	1977	S	Baker and Harris (1979)
‘Johnston’	N70-2173 (Hampton × Ransom) × Hutton	1983	S	Burton et al. (1987a)
‘Narow’	R66-873 (Jackson × Semmes) × Mack	1984	S	Caviness et al. (1985)
‘Young’	‘Davis’ × ‘Essex’	1984	S	Burton et al. (1987b)
‘Hutcheson’	V68-1034 (York × PI 71506) × Essex	1987	S	Buss et al. (1988)

† R = Mi resistant, S = Mi susceptible, and MS = moderately susceptible to Mi.

‡ The number in parenthesis indicates the total number of backcrosses.

Table 3.2. Mean southern root-knot nematode [*Meloidogyne incognita* (Kofoid and White) Chitwood] (Mi) gall index and band size at six simple sequence repeat (SSR) markers that flank the major Mi quantitative trait loci (QTL) on Linkage Group O (LG-O) for 48 soybean genotypes.

Genotype	Gall		Band size					
	index[†]	no.	Satt358	Sat_132	Satt487	Satt500	Satt492	Satt445
			---------- bp ----------					
High level of Mi-resistance[‡]								
'Gregg'	1.0a[§]	0.8	200/192	238/252	196/205	297	232	205/208
'Gordon'	1.0a	2.7	200	238	196	297	232	205
'Braxton'	1.0a	3.0	200	238	196	297	232	205
'Perrin'	1.0a	3.4	200	238	196	297	232	205
'Benning'	1.0a	3.5	200	238	196	297	232	199
'Haskell'	1.0a	4.0	200	238	196	297	232	205
'Govan'	1.0a	4.2	200	238	196	297	232	205
'Dillon'	1.0a	4.3	200	238	196	297	232	208
'Forrest'	1.0a	4.3	200	238	196	297	232	205
'Centennial'	1.0a	4.7	200	238	196	297	232	208
'Cobb'	1.0a	5.2	200	238	196	297	232	205
'Maxcy'	1.0a	5.8	200/192	236	196	297	232	208
'Hutton'	1.2ab	5.0	200	238	196	297	232	205
'Lamer'	1.2ab	5.0	200	238	196	297	232	205
'Manokin'	1.2ab	5.2	200	238	196	297	232	208

‘Doles’	1.2ab	5.2	200	238	196	297	232	205
‘Walters’	1.2ab	5.5	200	238	196	297	232	205/208
‘Hagood’	1.2ab	5.7	200	238	196	297	232	205
‘Hartwig’	1.2ab	5.8	200	238	196	297	232	205
‘Palmetto’	1.2ab	6.0	200	238	196	297	232	205
‘Pharaoh’	1.2ab	6.5	200	238	196	297	232	205
‘Coker6738’	1.2ab	7.0	200	238	196	297	232	208
‘Jackson’	1.2ab	7.5	200	238	196	297	232	205
‘Lee 74’	1.4ab	10.6	160	248	208	297	232	208
Moderate level of Mi-resistance								
‘Bragg’	1.7b	10.5	200	238	196	297	232	205
‘Wright’	1.7b	13.5	200	238	196	297	232	205
‘Cook’	2.5c	19.5	200	238	196	297	232	205
Mi-susceptible								
‘Hampton’	3.6d	34.0	192	236	196	300	232	205
‘Tokyo’	4.5e	44.5	192	250	202	297	232	208
FC 33243	4.7e	56.0	160	248	208	297	232	181
‘Coker 338’	4.6e	48.0	192	236	196	300	232	205
‘Volstate’	4.8e	51.8	192	246	202	288	232	208
‘Young’	4.8e	55.8	192	246	202	288	235	208
‘Johnston’	4.8e	57.0	192	236	196/202	288/300	232	208
‘Ransom’	5.0e	66.8	192	246	202	288/297	232	208
‘Coker 448’	5.0e	67.0	192	236	196	300	232	205

‘Dyer’	5.0e	70.0	192	250	205	297	232	208
D49-2491	5.0e	70.7	192	252	205	297	232	208
‘Gasoy 17’	5.0e	71.0	192	246	202	288	235	208
S-100	5.0e	72.5	192	250	202	300	232	211
‘Narow’	5.0e	72.5	192	252	205	297	232	208
‘Hutcheson’	5.0e	75.2	192	246	202	297	232	199
PI 54610	5.0e	76.0	160	248	202	297	232	208
‘Sharkey’	5.0e	76.7	192	252	202	297	232	208
‘Pickett 71’	5.0e	76.8	192	252	205	297	232	208
‘Bossier’	5.0e	77.5	192	250	205	297	232	208
‘CNS’	5.0e	79.2	192	252	205	297	232	208
‘Lee’	5.0e	79.7	192	252	205	297	232	208

† Gall index = 1 (few galls) to 5 (many galls).

‡ Based on Hussey et al. (1991).

§ Values in columns followed by different letters are significantly (P=0.05) different according to Duncan’s multiple range test.

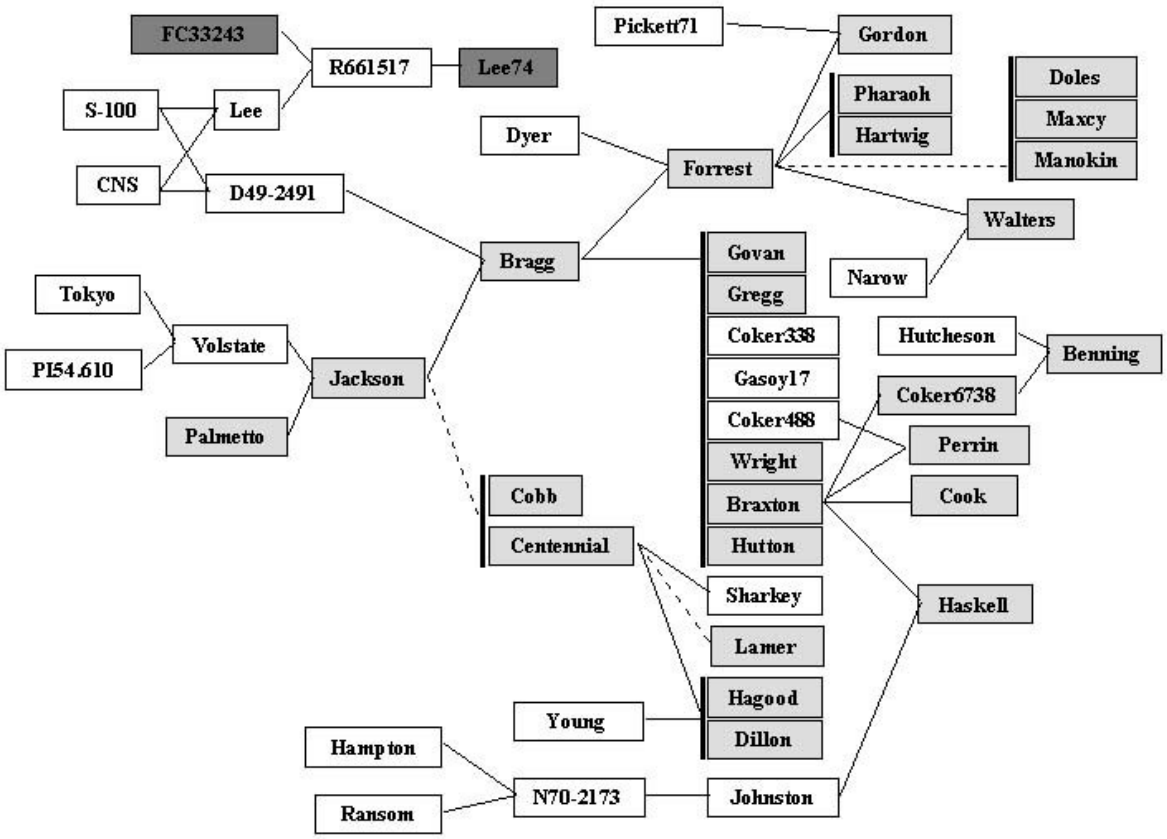

Figure 3.1. A representation of the pedigree relationships and the simple sequence repeat (SSR) bands at two SSR markers near the major southern root-knot nematode [*Meloidogyne incognita* (Kofoid and White) Chitwood] (Mi) quantitative trait loci (QTL) on Linkage Group O (LG-O) among Mi-resistant and Mi-susceptible cultivars. Note: FC 33243 was found to possess both susceptible and resistant plants. Solid lines indicate a direct parent and progeny relationship and broken lines indicate an indirect parent and progeny relationship. No shading indicates high Mi galls; gray shading indicates low Mi galls, 200-bp band at Satt358, and 238-bp band at Sat_132; and dark shading indicates low Mi galls, 160-bp band at Satt358, and 248-bp band at Sat_132.

CHAPTER IV

DEVELOPMENT OF SNP ASSAYS FOR HIGH-THROUGHPUT MARKER ASSISTED SELECTION OF SOUTHERN ROOT-KNOT NEMATODE RESISTANCE IN SOYBEAN[2]

[2] Ha, B.K., R.S. Hussey, and H.R. Boerma. To be submitted to Crop Science.

ABSTRACT

Single nucleotide polymorphism (SNP) markers could enhance the efficiency of marker-assisted selection (MAS) for southern root-knot nematode (*Meloidogyne incognita*; Mi) resistance in soybean [*Glycine max* (L.) Merr.] populations. Bacterial artificial chromosome (BAC) ends and simple sequence repeat (SSR)-containing genomic DNA clones were used to develop SNP markers linked to two soybean Mi resistance quantitative trait locus (QTL). Eight SSR markers, located near a major QTL for Mi-resistance on linkage group O (LG-O) (most likely *Rmi1*) and a minor QTL for Mi-resistance on LG-G, were used to select BAC ends and SSR-containing clones in GenBank. A total of 16 BAC-end sequences and eight SSR flanking regions were used to design primers to amplify genomic fragments of PI 96354 (Mi resistant) and 'Bossier' (Mi susceptible). The polymerase chain reaction (PCR) products were directly sequenced. The sequence data from 24 amplicons of PI 96354 and Bossier were analyzed with ClustalW 1.8. A total of 13 SNPs, including seven single-base changes and six small insertions and deletions (Indels), were identified in 7.7 kbp of sequence. We discovered four SNPs in Satt358 allele sequences located near a major Mi-resistant QTL on LG-O and 4 SNPs in Satt199 allele sequences located near a minor Mi-resistant QTL on LG-G. Using a direct hybridization assay detected on a Luminex 100 flow cytometer, the genotypes of the SNP358 marker that targets two SNPs in Satt358 allele sequences were identical to the genotypes of the SSR marker Satt358 among 94 $F_{2:3}$ lines of PI 96354 × Bossier. The genotypes of SNP199 marker which targets a SNP in Satt199 allele sequences also showed 100% congruence with the genotypes of the SSR marker Satt199.

SNP genotyping with 24 known Mi-resistant or Mi-susceptible cultivars showed that SNP358 and SNP199 markers should be highly effective in marker-assisted selection for the Mi-resistance QTL on LG-O and LG-G.

INTRODUCTION

Root-knot nematodes (*Meloidogyne* spp.) are a major soybean pest that causes severe yield losses in the southern USA. Soybean breeders have been intensifying their efforts to develop improved root-knot nematode resistant cultivars. One of the challenges in the past has been that screening for resistant plants was tedious, time-consuming, and variable (Boerma and Hussey, 1992). Recently, breeders have explored the use of genetic markers in place of traditional phenotypic screening once the marker/QTL relationship has been established and confirmed. Marker-assisted selection (MAS) has many advantages when compared to phenotypic screening. Selection can be performed in early segregating populations and at early stages of plant development. MAS can be used to pyramid genes. With MAS, breeders can conduct many cycles of selection in a year without depending on the natural occurrence or the necessity of inoculum maintenance of the pest or pathogen (Mohan et al., 1997). However, a successful application of MAS in a plant breeding program depends on several factors; i) marker should co-segregate or be closely linked with desired trait, ii) the availability of an efficient means of screening large breeding populations, and iii) the screening technique should be economical to use (Gupta et al., 1999; Francia et al., 2005).

Molecular markers have been used to map the genomic location of QTL for southern root-knot nematode [*Meloidogyne incognita* (Kofoid and White) Chitwood] (Mi) resistance. Using RFLP markers, Tamulonis et al. (1997) mapped a major QTL on LG-O that explained 31% of the total variation in gall number and a minor QTL on LG-G that accounted for 14% of variation. In addition, Li et al. (2001) mapped the region of the

major and minor QTL with SSR markers. MAS using SSR markers flanking the two QTL was effective to predict the Mi phenotypes (Li et al., 2001). Also, Ha et al. (2004) identified an SSR marker Satt358 that cosegregated with *Rmi1*, a Mi resistance gene, using a molecular pedigree analysis in southern U.S. elite soybean cultivars.

The use of either RFLP or SSR markers in MAS has some limitations. They need gel-based assay and are therefore time-consuming and expensive. Recently single nucleotide polymorphism (SNP) markers have been considered as the marker of choice because of their high frequency, widespread distribution throughout the genome, and their suitability for high-throughput, automated genotyping (Schork et al., 2000). In humans, Wang et al. (1998) reported the frequency of SNP was 1.4 per kilobase in a survey of 10 individuals. Lindblad-Toh et al. (2000) found that the frequency of SNP was 0.95 per kilobase in seven inbred mouse lines. Also, SNPs are highly abundant and distributed throughout the genome in plants such as *Arabidopsis thaliana* (Drenkard et al., 2000), *Oryza sativa* (Nasu et al., 2002), and *Zea mays* (Batley et al., 2003). In soybean, Grimm et al. (1999) estimated the frequency of SNP as 3.4 per kilobase in approximately 18,000 bases of DNA sequence obtained from 18 genotypes that are the ancestors of modern North American soybean cultivars, which is about two times the frequency in human. Recently, Zhu et al. (2003) found a total of 280 SNPs in 76-kb of sequence from 25 different soybean genotypes. This study also reported that nucleotide diversity in random non-coding genomic sequence from bacterial artificial chromosome (BAC) clones and SSR flanking regions was higher that that of the genomic DNA associated with coding regions. This suggests that the BAC-end sequence and SSR flanking regions provide a good source of data for SNP discovery.

Several SNP genotyping techniques are available. The SNP genotyping methods are based on hybridization methods, allele-specific PCR, primer extension, oligonucleotide ligation, and endonuclease cleavage (Gupta et al., 2001; Syvänen, 2001). For plant improvement applications, SNP genotyping assays containing single-base extension (SBE), allele-specific primer extension (ASPE), oligonucleotide ligation (OL), allele-specific oligonucleotide (ASO) hybridization, and direct hybridization (DH) were compared using the Luminex 100 flow cytometer platform (Lee et al., 2004). On the basis of cost, simplicity, and speed, DH was considered the most economical assay for MAS. The DH assay tests for single base mismatch with two short ASO probes. The probes are allowed to pair with the target DNA that contains the SNP at conditions in which only perfectly matched probe-target hybrids are stable, and hybrids that contain a mismatch are unstable (Syvänen, 2001). DH assays with the Luminex 100 flow cytometer was used in several studies to screen for mutations in the cystic fibrosis transmembrane conductance regulator gene (Dunbar and Jacobsen, 2000), genotype β–globin variants (Colinas et al., 2000), and haplotype the *NAT2* gene (Hurley et al., 2004).

The objectives of this study were to identify SNPs linked to the Mi resistant QTL on LG-O and LG-G and to optimize SNP genotyping conditions using Luminex 100 flow cytometer for automated high-throughput MAS.

MATERIALS AND METHODS

Plant materials

Ninety four F_2 plants from the cross of PI 96354 × Bossier were used in this study. PI 96354 was identified with the highest level of resistance to Mi for both gall formation and nematode reproduction and Bossier as highly susceptible (Luzzi et al., 1987). This population was utilized in the RFLP mapping (Tamulonis et al., 1997) and SSR mapping (Li et al., 2001) of the QTL conferring Mi resistance. $F_{2:3}$ lines were evaluated for Mi galling in the greenhouse (Tamulonis et al., 1997). DNA was extracted from individual parental genotypes and individual field-grown F_2 plants using the modified CTAB procedure of Keim et al. (1988), and diluted to 20 ng μL^{-1}.

SNP identification

Based on the integrated genetic linkage map of soybean (Song et al., 2004), four SSR markers (Satt358, Sat_132, Satt487, and Satt492) located near a major Mi-resistance QTL on LG-O and four SSR markers (Satt199, Satt505, Satt400, and Satt012) located near a minor Mi-resistance QTL on LG-G were chosen (Li et al., 2001). These SSR markers were used to identify BAC clones from GenBank. Primers were designed with BAC end sequence data using OLIGO primer design software (Molecular Biology Insights, Inc., Cascade, CO).

All PCR primers were used to amplify genomic DNA of PI 96354 and Bossier. PCR amplification used total 10-μL reaction mix containing 2 μL of 40 ng template DNA,

1.0 × PCR buffer, 2.5 m*M* $MgCl_2$, 100 μ*M* of each dNTP, 0.2 μ*M* each of forward and reverse primers, and 0.5 unit of Taq DNA polymerase. PCR cycling conditions were 30 s DNA denaturation step at 94°C, a 30 s annealing step at 50°C, and a 1 min extension step at 72°C for 35 cycles on a MJ Tetrad thermocycler (MJ Research, Watertown, MA). The products were analyzed on a 1.5% agarose gel stained with ethidium bromide. Those primer sets that produced what appeared to be a single product were selected for further testing. Those that produced no products or multiple products were further examined using lower annealing temperatures or higher Mg^{2+} (those giving no products) or higher annealing temperature or lower Mg^{2+} (those giving multiple products) (Zhu et al., 2003).

After the initial determination that a set of PCR primers appeared to produce a single amplicon from genomic DNA, the PCR product was directly sequenced using one of the PCR primers with a BigDye Terminator v3.1 Cycle Sequencing Kit (PE-ABI, Foster City, CA), and these were analyzed on an ABI 377 automated sequencer (PE-ABI, Foster City, CA) with a 36-cm 4.5% polyacrylamide gels (29:1) gel, 6M Urea. The sequence data from amplicons of PI 96354 and Bossier were analyzed with ClustalW 1.8 (Thompson et al., 1994).

Microsphere-direct hybridization with biotinylated upstream primer

Microsphere-direct hybridization was performed according to Dunbar and Jacobson (2000) with slight modifications. The upstream primer for amplification of the SNP-containing fragment was labeled at the 5' terminus with biotin (BioSource Int, Camarillo, CA). Targets were amplified and used in a total 10-μL reaction mix containing

2 μL of 40 ng template DNA, 1.0 × PCR buffer, 2.5 m*M* $MgCl_2$, 100 μ*M* of each dNTP, 0.5 μ*M* each of 5'-biotinylated primer and an unmodified reverse primer, and 0.5 unit of Taq DNA polymerase. PCR cycling conditions were 30 s DNA denaturation step at 94°C, a 30 s annealing step at 50°C, and a 30 s extension step at 72°C for 35 cycles on a MJ Tetrad thermocycler (MJ Research, Watertown, MA).

Allele-specific oligonucleotides (ASOs) specific for each genotype sequence (PI 96354 and Bossier) were synthesized with a 5' amine Uni-Link modification (BioSource Int, Camarillo, CA). The ASOs were coupled to carboxylated microspheres using a carbodiimide coupling method (Fulton et al., 1997). For each ASO and microsphere set combination, 5×10^6 carboxylated microspheres were suspended in 50 μL 0.1*M* 2-(N-Morpholino) ethanesulfonic acid, pH 4.5 (MES). One nmol of amine-substituted ASO was added, followed by addition of 25 μg N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide [EDC] and incubation in the dark for 30 min. The EDC addition and incubation were repeated and the microspheres were washed once with 0.02% Tween-20 and once with 0.1% SDS. Coupled microspheres were stored in MES at 4°C in the dark.

After PCR amplification, genotyping was performed in a 50 μL hybridization reaction containing 10 μL unpurified PCR product, and 2500 of each ASO-coupled microsphere set in 1 × TMAC buffer (4.5 *M* tetramethylammonium chloride, 75 m*M* Tris-HCl (pH 8.0), 6 m*M* EDTA (pH 8.0), 0.15% Sarkosyl). Reactions were denatured at 95°C for 2 min and incubated at 55°C for 30 min. The beads were pelleted by microcentrifugation, and the supernatant was removed. The beads were labeled with 50 μL of freshly made 4 μg mL^{-1} streptavidin-phycoerythrin in 1 × TMAC at 55°C for 20 min. Reactions were then analyzed using the Luminex 100 instrument (Luminex

Corporation, Austin, TX). The data collection software was set to analyze 100 beads from each set and the mean fluorescence intensity (MFI) was used for analysis.

The genotype score was calculated: $\log_{10}$ [(fraction of PI 96354 allele signal + 0.01) / (fraction of Bossier allele signal + 0.01)], which results in heterozygotes having values close to 0, homozygotes for PI 96354 allele close to +1, and homozygotes for Bossier allele close to –1 (a score of ±1 corresponds that MFI of the positive allele is 10 × higher than MFI of the negative allele) (Hirschhorn et al., 2000).

Microsphere-direct hybridization with biotinylated sequence-specific oligonucleotide

Another microsphere-direct hybridization method was achieved by the method of Hurley et al. (2004). This method used a biotinylated sequence-specific oligonucleotide (SSO) that recognizes a sequence in the target amplicon that is devoid of SNPs. Targets were amplified in a 10-μL reaction mix containing 2 μL of 40 ng template DNA, 1.0 × PCR buffer, 2.5 m*M* $MgCl_2$, 100 μ*M* of each dNTP, 0.5 μ*M* each of unmodified forward and reverse primers, and 0.5 unit of Taq DNA polymerase. PCR cycling conditions were 30 s DNA denaturation step at 94°C, a 30 s annealing step at 50°C, and a 30 s extension step at 72°C for 35 cycles on a MJ Tetrad thermocycler (MJ Research, Watertown, MA).

After PCR amplification, genotyping was performed in a 40 μL hybridization reaction containing 10 μL unpurified PCR product, 100 n*M* biotinylated Detector SSO, and 2000 of each ASO-coupled microsphere set in 1 × TMAC buffer (4.5 *M* tetramethylammonium chloride, 75 m*M* Tris-HCl (pH 8.0), 6 m*M* EDTA (pH 8.0), 0.15%

Sarkosyl). Reactions were denatured at 95°C for 2 min and incubated at 53°C for 30 min. A 10 μL of freshly made 50 μg mL^{-1} streptavidin-phycoerythrin in 1 × TMAC was added and the reaction was incubated at 53°C for 20 min. Reactions were then analyzed using the Luminex 100 instrument (Luminex Corporation, Austin, TX). The data collection software was set to analyze 100 beads from each set and the mean fluorescence intensity (MFI) was used for analysis.

Confirmation of SNP genotyping with soybean cultivars

Seed for Mi-resistant genotypes and Mi-susceptible genotypes were obtained from the USDA Soybean Germplasm Collection maintained at the Univ. of Illinois (Urbana, IL). Soybean DNA was extracted from seeds of each genotype according to modified procedures of Kang et al. (1998) and diluted to 20 ng μL^{-1}. SSR genotyping was followed by the protocol of Diwan and Cregan (1997), with slight modifications. The PCR amplicons were analyzed on an ABI-Prism 377 DNA sequencer (PE-ABI, Foster City, CA). The SNP genotyping followed the protocol of microsphere-direct hybridization with biotinylated sequence-specific oligonucleotide as described earlier.

RESULTS AND DISCUSSION

SNP discovery for Mi resistance

In soybean, it is difficult to discover SNPs at defined positions in the genome because soybean physical map is not complete. However, existing DNA sequence data from SSR flanking regions and RFLP probes, both of which are already positioned on the soybean linkage map, provides an excellent source of data for SNP discovery throughout soybean genome (Cregan, 1999). Furthermore, the same SSR primers or RFLP probes can be used to identify BAC clones in existing libraries from which end sequences or subclone sequences can be obtained to discover SNPs.

Based on the integrated genetic linkage map of soybean (Song et al., 2004), four SSR markers (Satt358, Sat_132, Satt487, and Satt492) located near the major Mi-resistance QTL (*Rmi1*) on LG-O and four SSR markers (Satt199, Satt505, Satt400, and Satt012) located near minor Mi-resistance QTL on LG-G were chosen to locate SNPs (Li et al., 2001). Sequence data were obtained from SSR flanking regions from GenBank. Sequences from the SSR markers also were used to identify BAC clones from GenBank. Ten BACs were identified and six out of 10 BACs contained forward and reverse BAC-end sequence data. A total of 7.7 kbp sequence data were obtained from 24 PCR products of PI 96354 and Bossier. A total of 13 SNPs including seven single-base changes and six indels were identified from sequences alignment of each PCR fragment (Table 4.1). Five indels were derived from different alleles between PI 96354 and Bossier at Satt358, Satt492, Satt199, Satt505, and Satt012, respectively. In our study, the SNP frequency

(1.68 SNPs per kbp) was lower than the previous estimate of 3.24 SNPs per kbp among 25 soybean genotypes reported by Zhu et al. (2003). The lower SNP frequency was probably related to the use of only two soybean genotypes.

In the Satt358 flanking region, three SNPs (A/T, G/A, and G/T) were identified at bp 83, 91, and 190, respectively (Figure 4.1). Two haplotypes were found. Haplotype AG at the 83 and 91 bp positioned for the Bossier genotype (susceptible to Mi), and haplotype TA at the 83 and 91 bp positioned for the PI 96354 genotype (resistant to Mi).

On LG-G we also identified two SNPs, G/A and C/G, in the Satt199 flanking region and one SNP, G/A, in the Satt012 flanking region. Previous research has shown that MAS based on Satt358, Satt199, and Satt012 located near Mi-resistance QTL on LG-O and -G enhanced the level of Mi resistance (Li et al., 2001). Therefore, SNPs in targeted for Satt358, Satt199, and Satt012 were evaluated for high-throughput genotyping assay development.

SNP genotype analysis for Mi resistance

The Luminex 100 flow cytometer has the capacity for conducting multiplex analyses in a single reaction tube by using reaction-specific microspheres that fluoresce at different frequencies (Dunbar, 2006; Lee et al., 2004). On the basis of cost, simplicity, and speed, we choose the direct hybridization (DH) assay. Until now, two DH methods have been reported depending on the location of biotinylated oligonucleotide. One method used a biotinylated upstream primer (Dunbar and Jacobson, 2000) and the other used biotinylated sequence-specific oligonucleotide (SSO) (Hurley et al., 2004).

To test an assay using a biotinylated upstream primer, we used SNP358 marker developed from Satt358 allele sequences on LG-O. Allele-specific oligonucleotide (ASO) probes corresponding to each PI 96354 and Bossier allele were designed such that the two polymorphic bases at the 83 and 91 bp in Satt358 allele sequences were positioned in the center of either a 23-mer or 19-mer in length to determine which probe length provided the best clustering pattern (Table 4.2). The ASO358-TA probe was specific for the PI 96354 allele and the ASO358-AG probe was specific for the Bossier allele. A clustering method was used to score the genotypes, with heterozygotes having values close to 0 and homozygotes generally close to ±1 (Hirschhorn et al., 2000).

To optimize the assay, three hybridization temperatures and two lengths of ASOs were tested (Figure 4.2). Thirty minute hybridization at 50°C, 53°C, and 55°C temperatures were conducted and the same temperatures were used to incubate the reaction containing streptavidin for 20 min. As seen in Figure 4.2, the 19-mer ASOs gave more unambiguous data compared to the 23-mer ASOs under all three temperature hybridization conditions across PI 96354, Bossier, and 94 $F_{2:3}$ lines of the cross from PI 96354 × Bossier. As hybridization temperature increased, the value of the mean fluorescence intensity (MFI) decreased. In hybridization at 50°C temperatures, we observed a few ambiguous data points. Based on the clustering pattern and signal intensity, 53°C hybridization temperature and 19-mer ASOs of SNP359 marker gave the best clustering pattern to score these genotypes. These results were consistent with previously reports that the effect of a mismatch on hybridization was greater with decreasing probe length and increasing hybridization temperature (Armstrong et al., 2000; Dunbar, 2006). The SNP genotypes obtained on a flow cytometer were extremely

accurate, being 100% congruent with genotypes previously obtained by Satt358 analysis across the 94 $F_{2:3}$ lines of the cross from PI 96354 × Bossier (Li et al., 2001).

Herley et al. (2004) reported a new DH method using biotinylated sequence-specific oligonucleotide (SSO). This method doesn't require centrifugation for bead washing, and thus is more amendable to high-throughput applications. We tested two SNP markers (Figure 4.3). One was SNP358 marker with the 19-mer ASOs as described earlier. The other was the SNP199 marker which targeted a SNP in the Satt199 allele sequences on LG-G. The ASO199-G probe was specific for PI 96354 allele and the ASO199-C probe was specific for Bossier allele (Table 4.2). The original ASOs of SNP199 marker were designed such that one SNP locus was positioned in the center of a 19-mer with same length, but MFI of the two alleles was not large enough to effectively score the genotypes. The SNP199 ASOs were redesigned with different length as shown in Table 4.2. Figure 4.3 showed that clear clustering patterns with approximately 10:1 ratio of the two alleles were obtained for the two SNP markers. Compared with the method of biotinylated upstream primer in Figure 4.2, the value of the MFI was lower with the SSO method, but the clustering pattern was not different. In addition, the SNP199 marker that has one SNP difference in the ASOs showed a similar clear clustering pattern to the SNP358 marker that has two SNPs in the ASOs. These results indicate that designing of effective ASOs could increase the efficiency of the DH assay with other SNP loci.

In addition, we tested genotyping of the SNP012 marker which targets a G/A SNP in Satt012 allele sequences on LG-G. However, SNP012 marker did not produce a clear clustering pattern with good signal strength. This may have resulted from the low

GC content of the ASOs (less than 25%).

Confirmation of SNP genotyping in elite U.S. soybean cultivars

SNP genotypes were compared with SSR genotypes using Mi-resistant and Mi-susceptible cultivars (Table 4.3). To genotype the SNP358 marker among cultivars, a 19-mer ASOs, a biotinylated SSO, and the 53°C hybridization temperature were used. The relationship between Mi reaction and Satt358 genotypes were previously described by Ha et al. (2004). The study reported that Mi-resistant cultivars inherited a 200-bp band and Mi-susceptible cultivars inherited a 192-bp band at Satt358. Table 4.3 showed that all Mi-resistant cultivars carrying the 200-bp allele at Satt358 had approximately 10 × higher positive signal for the ASO358-TA probe specific to the PI 96354 allele than the ASO358-AG probe specific to the Bossier allele. Gregg, which is heterogeneous for the 192/200 bp band at Satt358, showed positive signal for both ASO358-TA and ASO358-AG. All Mi-susceptible cultivars carrying the 192-bp band at Satt358 had positive signal for the Bossier-specific ASO358-AG probe. However, FC33243 with a 160-bp band at Satt358 showed low MFI at both ASO358-TA and ASO358-AG. To verify this observation, we sequenced the PCR product of FC33243. The sequence showed that FC33243 has a unique AA haplotype at SNP358. Therefore, SNP358 has three SNP haplotypes TA, AG, and AA.

At the Satt199 SSR marker on LG-G, four alleles, 159, 162, 170, and 200 bp, were detected among the cultivars (Table 4.3). PI 96354 that contained a Mi-resistance QTL linked to Satt199 possessed the 200-bp band. However, the 200-bp band of Satt199

was also present in Cobb, FC33243, Gasoy17, and Volstate that likely do not possess the Mi-resistance QTL on LG-G. In genotyping of SNP199 marker, all cultivars carrying 200-bp band at Satt199 had about 10 × higher positive signal at the ASO199-G specific probe for the PI 96354 allele than at ASO199-C specific for the Bossier allele. The other cultivars carrying 159, 162, and 170-bp band at Satt199 had positive signal for the ASO199-C probe. We sequenced the PCR product of cultivars carrying 159, 162, and 170-bp Satt199 alleles. The results showed that all these cultivars contained same SNP allele (C). Therefore, the SNP199 marker had two different alleles (G and C). Although SNP199 marker didn't co-segregate with the Mi-resistance QTL on LG-G perfectly, SNP199 marker could be used for marker-assisted selection in populations known to contain PI 96354 in their pedigree and that show polymorphism between parents for SNP199. In our study, SNP loci were mostly bi-allelic across cultivars. However the SNPs identified should provide an effective means for high throughput marker screening for the LG-O and LG-G Mi-resistance QTL from PI 96354.

MAS generally involves the genotyping of small number of markers across thousands of individuals, and as such, requires low cost/genotype, short assay time, and simplicity. In this study, we have developed the resources for a high-throughput method for determining Mi-reaction by assaying SNP markers using the Luminex 100 flow cytometry platform. Microspheres with 100 unique color codes provides for multiplexing in a single tube and reduces the materials cost. In our study, the cost per data point for SNP genotyping was approximately 66% of that for SSR genotyping.

Time for genotyping with the SNP markers requires 4 hours including 2 h for

PCR, 1 h for hybridization, 1 h for assaying a Luminex instrument. In contrast, time for genotyping with the SSR markers requires 5 hours including 2 h for PCR, 1 h for gel preparation, and 2 h for gel electrophoresis. SSR marker analysis requires gel electrophoresis that uses moderately hazardous reagents, is difficult to automate, and requires a highly skilled staff to run the gels and for data analysis (Meksem et al., 2001). The SNP assay using direct hybridization eliminates the need for gel electrophoresis, manual gel tracking, and data input, and therefore allows accurate and high speed detection of polymorphism in thousands of individuals.

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Table 4.1. GenBank accessions used in the study and SNP polymorphisms between PI 96354 and Bossier.

LG	GenBank Accession no.	Description	Bases	SNPs
O	BH126547	Satt358 containing genomic DNA clone	439	3 and 1 indel
O	BH758946	Gm_UMb001_162_O20_F UMN Soybean BAC identified by Satt358	331	-
O	BH919737	Gm_UMb001_162_O20_R UMN Soybean BAC identified by Satt358	218	-
O	BH126302	Sat_132 containing genomic DNA clone	372	-
O	BH001230	Gm_UMb001_133_O11_F UMN Soybean BAC identified by Sat_132	395	-
O	BH001231	Gm_UMb001_133_O11_R UMN Soybean BAC identified by Sat_132	273	-
O	BH001176	Gm_ISb001_021_G03_F ISU Soybean BAC identified by Sat_132	338	-
O	BH001177	Gm_ISb001_021_G03_R ISU Soybean BAC identified by Sat_132	162	-
O	BH126658	Satt487 containing genomic DNA clone	608	-
O	BH126663	Satt492 containing genomic DNA clone	290	1 indel
O	BH001241	Gm_ISb001_008_H04_F ISU Soybean BAC identified by Satt492	305	1
O	BH001242	Gm_ISb001_008_H04_R ISU Soybean BAC identified by Satt492	221	-
G	BH126406	Satt199 containing genomic DNA clone	326	2 and 2 indel

G	BH126676	Satt505 containing genomic DNA clone	244	1 indel
G	AQ989375	Gm_UMb001_088_M13F UMN Soybean BAC identified by Satt505	203	-
G	AQ989441	Gm_UMb001_088_M13R UMN Soybean BAC identified by Satt505	392	-
G	BH126583	Satt400 containing genomic DNA clone	260	-
G	AQ989160	Gm_ISb001_090_C06F ISU Soybean BAC identified by Satt400	403	-
G	AZ044670	Gm_ISb001_090_C06.R ISU Soybean BAC identified by Satt400	377	-
G	BH146213	Satt012 containing genomic DNA clone	226	1 and 1 indel
G	BH919777	Gm_UMb001_110_C20_F UMN Soybean BAC identified by Satt012	378	-
G	BH919806	Gm_UMb001_163_M15_R UMN Soybean BAC identified by Satt012	269	-
G	BH919790	Gm_UMb001_131_C20_F UMN Soybean BAC identified by Satt012	294	-
G	BH854483	Gm_ISb001_102_O17_R ISU Soybean BAC identified by Satt012	420	-
		Total	7744	13

```
PI96354 GAATTTTATTTTGAACGCCTTTCTGAAGATTCATTAACTTCCATTCTGGCACCAGAAATC 60
BOSSIER GAATTTTATTTTGAACGCCTTTCTGAAGATTCATTAACTTCCATTCTGGCACCAGAAATC 60
        ************************************************************
PI96354 ATTATAAATGCTATCCTTTAATTCTTAGCTATGCGCTTTATGTAACAATACGATTTCTAT 120
BOSSIER ATTATAAATGCTATCCTTTAATACTTAGCTGTGCGCTTTATGTAACAATACGATTTCTAT 120
        ********************** ******* ****************************
PI96354 TATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTAT 180
BOSSIER TATTATTATTATTATTATTATTATTATTATTATTATTATTATTAT---------TAT 171
        ***********************************************         ***
PI96354 TATATTTTTTCCTATTTTTGGAAATATATTTCTATTTTCAAAATAATATACTTTTTATTT 240
BOSSIER TATATTTTTGCCTATTTTTGGAAATATATTTCTATTTTCAAAATAATATACTTTTTATTT 231
        ********* **************************************************
PI96354 TAATTATATATTTTAAGTTTAATTACTCCGCACTCTGCTTTTACTAAAATTTTTAATTAG 300
BOSSIER TAATTATATATTTTAAGTTTAATTACTCCGCACTCTGCTTTTACTAAAATTTTTAATTAG 291
        ************************************************************
PI96354 TTCTCTAAATTTTTTTAAGTAAGTTTGGATTCTAGAACTTTACATTAAATTCATCTAACT 360
BOSSIER TTCTCTAAATTTTTTTAAGTAAGTTTGGATTCTAGAACTTTACATTAAATTCATCTAACT 351
        ************************************************************
PI96354 CAATTCACAAGCATCCTCTGAATATATCATTATTTAAAGTATTCAGTAAAAAAAACAAGT 420
BOSSIER CAATTCACAAGCATCCTCTGAATATATCATTATTTAAAGTATTCAGTAAAAAAAACAAGT 411
        ************************************************************
PI96354 CTTATTTAAAGTACTGCCA 439
BOSSIER CTTATTTAAAGTACTGCCA 430
        ******************
```

Figure 4.1. Sequence alignment of PCR fragments derived from the Satt358 containing genomic DNA clone between PI 96354 and Bossier.

Table 4.2. Sequences of target amplification primers, allele-specific oligonucleotide (ASO) probes, and sequence-specific oligonucleotides (SSO) for SNP genotyping assays.

Name		Primer sequence
SNP358	F	5'-GAA TTT TAT TTT GAA CGC CTT TC
	R	5'-GCA GAG TGC GGA GTA ATT AAA CT
ASO358-AG	23-mer	5'-AMINE-AGC GCA **C**AG CTA AG**T** ATT AAA GG
ASO358-TA	23-mer	5'-AMINE-AGC GCA **T**AG CTA AG**A** ATT AAA GG
ASO358-AG	19-mer	5'-AMINE-GCG CA**C** AGCTAA G**T**A TTA A
ASO358-TA	19-mer	5'-AMINE-GCG CA**T** AGC TAA G**A**A TTA A
SSO358	20-mer	5'-biotinylated-CTG GTG CCA GAA TGG AAG TT
SNP199	F	5'-TAT AAA ATA GGC AAA ACA CC
	R	5'-GAA AAC CTT TAT TCA TAC GCT
ASO199-G	18-mer	5'-AMINE-CAA AAT GTC **G**AC AGT AGT
ASO199-C	16-mer	5'-AMINE-CAA AAT GTC **C**AC AGT A
SSO199	21-mer	5'-biotinylated-GGC AAA ACA CCG TAT GAA AAA

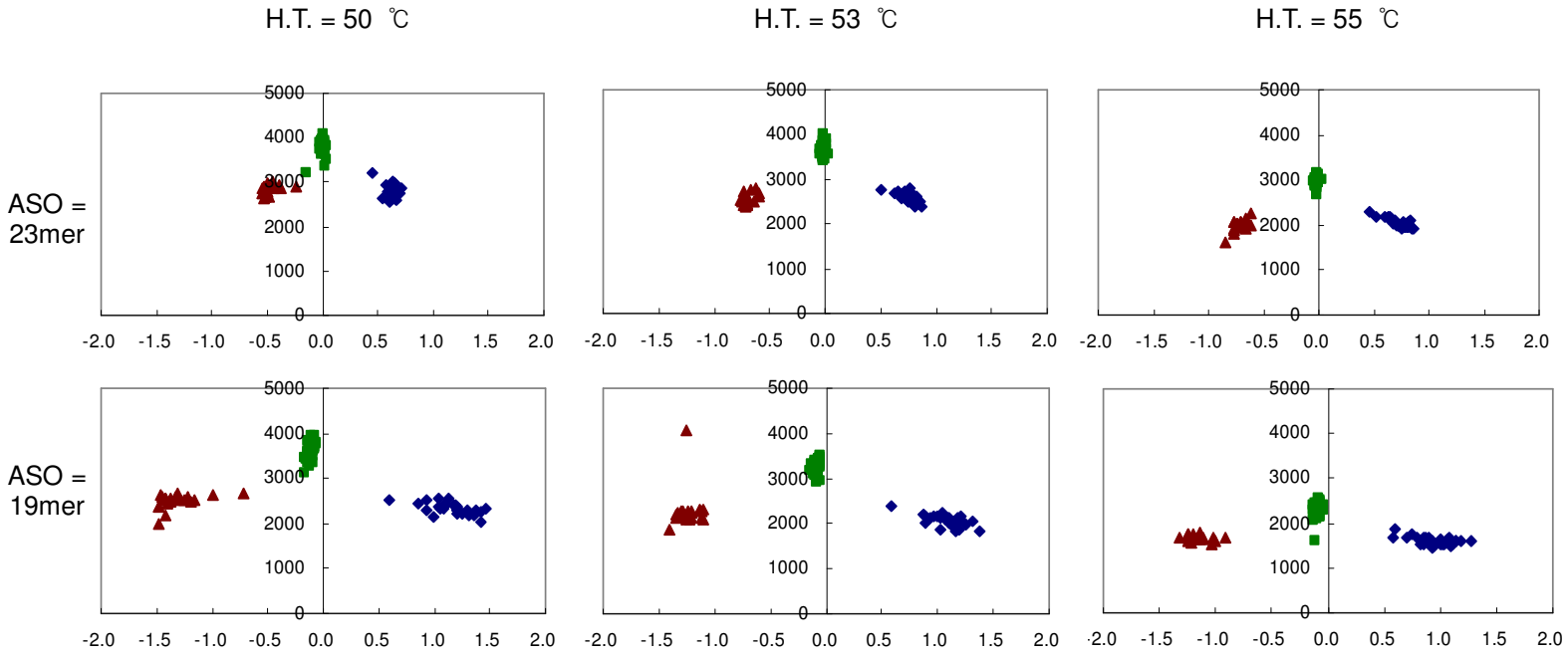

Figure 4.2. Optimization of direct hybridization (DH) assay using biotinylated upstream primer. SNP358 marker was genotyped across the $F_{2:3}$ population from the cross of PI 96354 × Bossier using different length of probes and different hybridization temperature conditions. Data are presented as the $\log_{10}$ [(fraction of PI 96354 allele signal + 0.01) / (fraction of Bossier allele signal + 0.01)] versus total mean fluorescence intensity (MFI) of two alleles (Hirschhorn et al., 2000). Genotypes are represented by homozygotes for PI 96354 allele (♦), heterozygotes allele (■), and homozygotes for Bossier allele (▲) determined by the band sizes of the Satt358 SSR marker. (H.T. = hybridization temperature; ASO = allele-specific oligonucleotide)

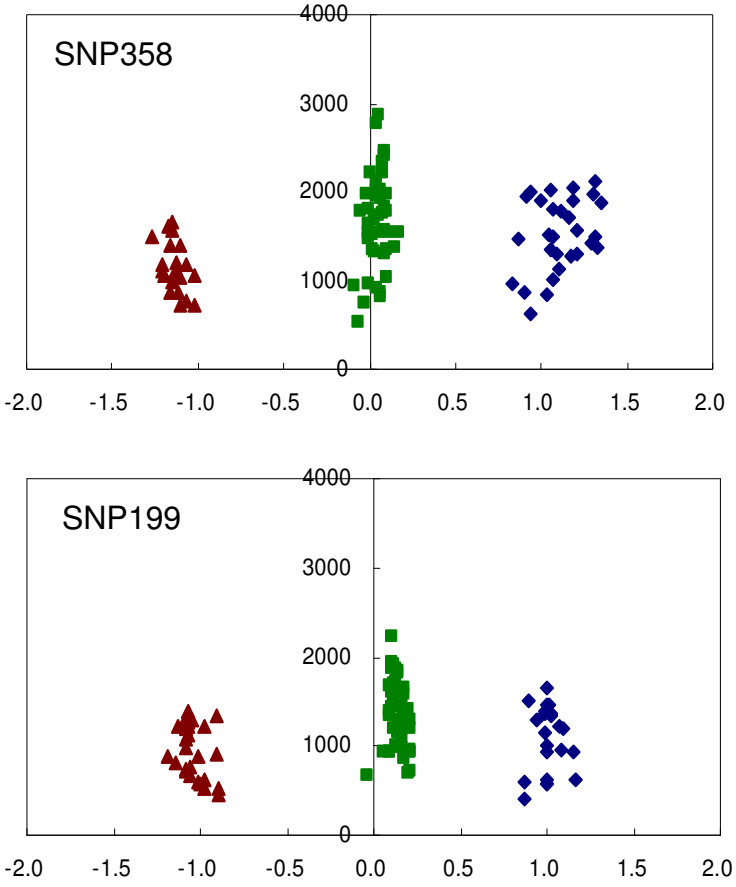

Figure 4.3. Results of SNP genotyping across the $F_{2:3}$ population from the cross of PI 96354 × Bossier using biotinylated sequence-specific oligonucleotide. Data are presented as the $\log_{10}$ [(fraction of PI 96354 allele signal + 0.01) / (fraction of Bossier allele signal + 0.01)] versus total mean fluorescence intensity (MFI) of two alleles (Hirschhorn et al., 2000). Genotypes are represented by homozygotes for PI 96354 allele (♦), heterozygotes allele (■), and homozygotes for Bossier allele (▲) determined by the band sizes of the SSR marker Satt358 (SNP358) and SSR marker Satt199 (SNP199), respectively.

Table 4.3. Comparison between SSR markers and SNP markers on LG-O and LG-G in the Mi-resistant and Mi-susceptible cultivars.

Genotype	Mi reaction	Satt358 (bp)	SNP358		Satt199 (bp)	SNP199	
			ASO358 -TA	ASO358 -AG		ASO199 -G	ASO199 -C
PI 96354	HR[†]	200	1272[‡]	88	200	1006	91
Centennial	R	200	1100	85	159	76	647
Gregg	R	200/192	802	1171	159	69	679
Jackson	R	200	1248	107	170	78	863
Palmetto	R	200	1292	125	170	63	730
Bragg	R	200	1284	122	159	42	631
Benning	R	200	1228	92	159	58	708
Cobb	R	200	1240	123	200	991	121
Lamer	R	200	1181	95	159	51	525
Forrest	R	200	1347	104	159	64	847
Hartwig	R	200	1305	116	159	66	911
Perrin	R	200	1274	117	159	67	698
Tokyo	S	192	43	1214	159	53	789
FC33243	S	160	155	275	200	1144	104
Johnston	S	192	35	721	159	75	861
Young	S	192	57	1201	159	115	996
Lee	S	192	72	1482	159	74	796
Dyer	S	192	69	1146	159	65	698

Gasoy17	S	192	90	1458	200	686	92
CNS	S	192	70	1022	162	47	645
Hutcheson	S	192	97	1493	159	57	605
S-100	S	192	81	1584	159	84	995
Volstate	S	192	100	1448	200	1021	139
Bossier	S	192	88	1682	159	85	1108

† HR: highly resistant to Mi, R: resistant to Mi, S: susceptible to Mi

‡ Mean fluorescence intensity values.

CHAPTER V

SSR MAPPING AND CONFIRMATION OF SOYBEAN QTL FROM PI 437654 CONDITIONING RESISTANCE TO RENIFORM NEMATODE[3]

[3] Ha, B.K., R.T. Robbins, F. Han, R.S. Hussey, J.F. Soper, and H.R. Boerma. To be submitted to Crop Science.

ABSTRACT

Reniform nematode (*Rotylenchulus reniformis* Linford & Oliveira) is a semi-endoparasite of roots, and occurs commonly in tropical and subtropical regions. Recently, reniform nematode has been recognized to be a major problem on cotton (*Gossypium hirsutum* L.) and soybean [*Glycine max* (L.) Merr.] in the southern USA. Planting reniform nematode-resistant soybean cultivars is the most cost effective control method. DNA marker-assisted breeding could improve the development of reniform nematode-resistant cultivars. With RFLP markers, Pioneer Hi-Bred International, Inc. had previously identified a quantitative trait loci (QTL) on LG-B1 and another on LG-L associated with reniform nematode resistance in a cross between 'BSR101' and PI 437654. Recent studies have shown that soybean cyst nematode (*Heterodera glycines* Ichinohe) resistant cultivars derived from PI 437654 are also potentially resistant to reniform nematode. In this study, we validated existing QTL and identified an additional QTL conditioning resistance to reniform nematode in a population of 228 recombinant-inbred lines (RILs) from a cross of BSR101 × PI 437654. A QTL (R^2=21%) conditioning reniform reproductive index (RI) was found on linkage group L (LG-L) and was flanked by Sat_184 and Satt513. Two other QTL were identified, one on LG-B1 (R^2=16%) and the other on LG-G (R^2=8%). These QTL acted in an epistatic manner with lines homozygous for PI 437654 alleles at both QTL providing the lowest reniform RI. The 31 RILs from BSR101 × PI 437654 that were homozygous for the PI 437654 alleles at Satt513, Satt358, and Sat_168 averaged 1077 reniform eggs and juveniles (RI=0.63), while the 33 RILs homozygous for the BSR101 alleles averaged 10606 (RI=6.24). By

screening the RILs population of ‘Prichard’ × ‘Anand’ we confirmed that the QTL on LG-G and -B1 conditioned reniform RI. These results support the genetic linkage between soybean resistance to the reniform nematode and the soybean cyst nematode.

INTRODUCTION

The reniform nematode (*Rotylenchulus reniformis* Linford & Oliveira), a semi-endoparasite of roots (Robinson et al., 1997), is recognized to be a major problem on soybean in the southern USA (Robbins et al., 1994a). The reniform nematode induces a feeding site, called a syncytium, and causes root decay, unthrifty growth, empty pods, and significant yield reduction in susceptible soybean cultivars (Birchfield et al., 1971; Williams and Birchfield, 1974). Parasitized plants exhibit varying degrees of stunting and chlorosis and initial field infestations may exhibit patchy areas of poor growth. Moderate to heavy soybean yield loss was observed from damage caused by reniform nematode in both Louisiana (Birchfield et al., 1971) and South Carolina (Fassuliotis and Rau, 1967). Rebois and Johnson (1973) reported that an initial population of 6579 reniform juveniles and preadult females per L of soil consistently reduced yields on both moderately resistant and susceptible cultivars by an average of 33%, and reduced the content of phosphorus and potassium in harvested seeds.

Since nematicides and/or rotation to a non-host crop are usually not economical for soybean producers, genetic resistance is the most desirable control method (Harville et al., 1985). Rebois et al. (1968) found that the soybean cultivars Pickett and Dyer were resistant to the reniform nematode. Also Rebois et al. (1970) reported that resistance to reniform nematode could be expected in genotypes with resistance to the soybean cyst nematode (SCN) (*Heterodera glycines* Ichinohe) due to the similarities in the feeding sites. However, Caviness and Riggs (1976) reported that 'Mack' was resistant to SCN and susceptible to reniform nematode. Recent studies have shown that SCN-resistant soybean

cultivars that derive resistance from Peking (e.g. 'Forrest' and 'Centennial'), PI 437654 (e.g. 'Hartwig'), or PI 90763 (e.g. 'Cordell') are potentially resistant to *R. reniformis*, whereas those that derive SCN resistance form PI 88788 are not resistant to *R. reniformis* (Robbins et al., 1994a,b; Davis et al., 1996; Robbins and Rakes, 1996). The phenotypic relationship suggests that common genes and/or linked genes are responsible for resistance to both soybean cyst and reniform nematode in soybean.

The genetics of resistance to *R. reniformis* has been studied in soybean (Williams et al., 1981; Harville et al., 1985). Williams et al. (1981) reported that in a cross of Forrest (resistant) and 'Ransom' (susceptible), reniform nematode resistance was recessive and controlled by alleles at one locus. In other soybean populations, resistance in soybean was controlled by two loci with unequal effects (Harville et al., 1985). The proposed genotypes of the four parental cultivars were: i) susceptible soybean cultivar Davis Rn_1 Rn_1 Rn_2 Rn_2, ii) moderately susceptible soybean cultivar Bragg Rn_1 Rn_1 rn_2 rn_2, iii) moderately resistant soybean cultivar Dare rn_1 rn_1 Rn_2 Rn_2, and iv) resistant soybean cultivar Pickett 71 rn_1 rn_1 rn_2 rn_2. In 1999, Pioneer Hi-Bred International, Inc. identified two quantitative trait loci (QTL) for reniform nematode resistance in a cross between 'BSR101' and PI 437654 with RFLP markers covering whole linkage groups (unpublished). One QTL on LG-L explained 20% of the total variation in reniform reproductive index (RI), and another QTL on LG-B1 accounted for 11% of variation.

Many studies using DNA markers have concentrated on QTL that control parasitic nematodes in soybean. Southern root-knot nematode [*M. incognita* (Kofoid and White) Chitwood] (Tamulonis et al., 1997c; Li et al., 2001), peanut root-knot nematode [*M. arenaria* (Neal) Chitwood] (Tamulonis et al., 1997b) and javanese root-knot nematode

[*M. javanica* (Treub) Chitwood] resistance QTL (Tamulonis et al., 1997a) have been identified. In addition, DNA markers have been used to dissect quantitatively inherited resistance to the SCN (Concibido et al. 1994, 2004; Webb et al. 1995; Cregan et al., 1999). Webb et al. (1995) mapped three major QTL to linkage groups (LG) A2, G, and M, respectively, that confer resistance to SCN race 3 in a cross between PI 437654, resistant to SCN and reniform nematode, (Robbins and Rakes, 1996) and 'BSR101'. Cregan et al. (1999) reported that marker-assisted selection (MAS) using Satt309, located 1 to 2 cM from *rhg1* on LG-G should be effective to select for SCN resistance at *rhg1* derived from Peking, PI 90763, and PI 437654 in crosses with most SCN-susceptible soybean genotypes. In a direct comparison, genotypic selection with Satt309 was 99% accurate in predicting lines that were susceptible in subsequent greenhouse assays (Mudge et al., 1997; Cregan et al., 1999).

The objectives of this study were to use simple sequence repeat (SSR) markers to: i) validate existing, but unpublished QTL and identify an additional QTL conferring resistance to reniform nematode ii) determine the magnitude of resistance of each QTL, and iii) evaluate the potential interaction of identified QTL.

MATERIALS AND METHODS

Mapping population

A population of 228 RILs from a cross of BSR101 × PI 437654 was used in this study. This population was maintained and increased in Iowa by Pioneer Hi-Bred

International, Inc. PI 437654 is a plant introduction that originated from China but it was received in the USA from the Russian Federation in 1979 and placed in the USDA Soybean Germplasm Collection (Nelson et al., 1988). It is Maturity Group III and is resistant to reniform nematode (Davis et al., 1996; Robbins and Rakes, 1996). BSR101 is a Maturity Group I cultivar developed at Iowa State Univ. and is susceptible to reniform nematode (Tachibana et al., 1987).

Reniform nematode screening

Reniform nematode resistance screening was conducted in a greenhouse at the Univ. of Arkansas. Seeds of each genotype were germinated in vermiculite and as the cotyledons where starting to open the seedlings were transplanted into 10-cm-diam. clay pots (one seedling/pot) containing 500 cm^3 of pasteurized fine sandy loam soil (ca. 91% sand, 5% silt, 4% clay, <1% O.M.). One day after transplanting each pot was inoculated with 1700 vermiform reniform nematodes (i.e., initial population; Pi). The reniform population used was from a culture originating in Jefferson County Arkansas and maintained on 'Braxton' soybean. The experimental design was a randomized complete block with five replications. All pots were watered at least twice daily and other times, if needed, and fertilized each week with 20-20-20 (N-P-K) fertilizer.

Thirteen weeks after inoculation, the number of reniform nematode eggs and vermiform nematodes contained in egg masses on the roots and the number of vermiform nematodes in the soil of each pot were determined. The eggs and vermiform nematodes in the egg masses on roots were collected with 0.525% NaOCl (Hussey and Barker, 1973)

and counted. To calculate the final reniform nematode soil population, a 100-cm^3 aliquot of well-mixed soil from each pot was suspended in water and poured through nested 850- and 38-μm-pore sieves to remove plant debris and collect the nematodes. Nematodes caught on the 38-μm-pore sieve were separated from soil by sucrose centrifugal-flotation (Jenkins, 1964), counted, and multiplied by 5 to give the total soil number per pot. The total number of reniform nematode eggs and vermiform nematodes per pot was calculated by adding the number from the soil to the number from the roots. A reproductive index (RI), defined as the number of eggs + vermiform nematodes at test termination (Pf) / initial infestation level (Pi), was calculated for each pot. Because of an association between the mean and variance of a genotype, the RI of each pot was transformed to log ratio [Log_{10}(RI+1)] values for statistical analysis and presented as antilogs.

SSR marker data collection

Soybean DNA was extracted from seeds of the 228 RILs according to modified procedures of Kang et al. (1998), quantified using a Perkin-Elmer Lambda 2 UV/VIS Spectrometer (PerkinElmer Life and Analytical Sciences, Shelton, CT), and diluted to 20 ng μL^{-1} for the PCR reaction. Ten seeds from each line were ground with coffee grinder (Braun KSM2, Boston, MA), and 0.1 g of each homogenate was transferred to a new 1.5-mL tube containing 500 μL extraction buffer [200 m*M* Tris pH 8, 200 m*M* NaCl, 25 m*M* ethylenediamine tetraacetic acid (EDTA), 0.5% sodium dodecyl sulfate] and 10 μL Proteinase K (20mg m L^{-1}). The samples were incubated in a water bath at 50°C for 1 h

and then 500 μL CTAB solution (2% cetyltrimethylammonium bromide, 100 m*M* Tris-HCl pH 8, 20 m*M* EDTA pH 8, 1.4 *M* NaCl) was added. After shaking, the samples were spun at 12000 rpm (Beckman Microfuge E, Beckman Instruments, Carlsbad, CA) for 10 min. The supernatant was transferred to a new 1.5-mL tube, and then one volume chloroform/isoamyl alcohol (24:1, v/v) was added. After shaking for 1 min at room temperature, the samples were spun at 12000 rpm for 10 min. The supernatant was transferred to a new 1.5-mL tube, and then 80% volume of isopropyl alcohol was added to precipitate DNA. The supernatant was decanted and the pellets were washed with 70% (v/v) ethanol. The DNA pellets were then dried and dissolved in 100 μL of tris-EDTA buffer.

On the basis of relationship with SCN and reniform nematode resistance, 96 SSR markers were chosen. These markers were near previously reported QTL for SCN resistance and were located on five linkage groups (A2, B1, G, L, and M) (Concibido et al., 2004). The primer sequences for each SSR were obtained from SoyBase, a USDA-sponsored genome database (http://soybase.org/ssr.html). Fluorescent-labeled forward primers and nonlabeled reverse primers were obtained from PE-ABI (Foster City, CA). Polymerase chain reactions were prepared based on the protocol of Diwan and Cregan (1997) with slight modifications. The 10-μL reaction mix contained 2 μL of 40 ng template DNA, 1.0 × PCR buffer, 2.5 m*M* $MgCl_2$, 100 μ*M* of each dNTP, 0.2 μ*M* each of forward and reverse primers, and 0.5 unit of Taq DNA polymerase. The reactions were performed in a MJ Research PTC-225 thermal cycler (MJ Research, Inc., Reno, NV). The PCR amplicons were analyzed on an ABI-Prism 377 DNA sequencer (PE-ABI, Foster City, CA) with a 4.8% acrylamide to bisacrylamide (19:1) gel at 750 V for 2 h. The

marker fragments were analyzed with GeneScan software v.3.0 and scored with Genotyper software v.2.1 (PE-ABI, Foster City, CA).

Linkage map and QTL analysis

Initially, 134 randomly selected RILs were evaluated for the association of SSR marker alleles and reniform nematode RI. To further increase precision, 94 additional RILs were included. A broad sense heritability estimate based on variance component was calculated on entry-mean basis with the following formula: $h^2 = \sigma^2_{RILs} / [\sigma^2_{RILs} + (\sigma^2_e / r)]$ (Fehr, 1987), where σ^2_{RILs} is the genotypic variance component for reniform RI per plant among RILs, σ^2_e is the error variance, and r is the number of replications.

Single factor analysis of variance (SF-ANOVA) was used to determine the significance (P = 0.05) among SSR genotypic class means by use of General Linear Model (GLM) (SAS Institute, 2001). A multiple regression model with a FORWARD option was used for identifying the independent markers linked to the QTL within and among linkage groups at the 5% significance level.

Genetic linkage maps were constructed in MAP MANAGER QTX Version b16 with the Kosambi map function (Manly et al., 2001). QTL analyses of RI were conducted using interval mapping implemented in the program MAP MANAGER QTX (Manly et al., 2001). Genome-wide threshold values for declaring the presence of QTL were determined by 1000 permutations for reniform RI, as implemented by MAP MANAGER QTX. The additive effects (α) for individual QTL were estimated by MAP MANAGER QTX.

Multilocus QTL analyses were performed with three genetic marker loci in mixed linear models (Littel et al., 1996; Tang et al., 2006). Statistical analyses were performed by SAS PROC MIXED, where genotype (G) effects were fixed and RIL nested within genotype (RIL:G), and replications (R) were random. Variance components were estimated by the REML method. Type III sums of squares and *F* statistics were estimated for genotype effects, and least square means were estimated for genotypes. Additive (α), additive × additive ($\alpha \times \alpha$), and additive × additive × additive ($\alpha \times \alpha \times \alpha$) effects were estimated by ESTIMATE statements.

QTL confirmation

To evaluate the effect of identified QTL in a different genetic background, F_7-derived RILs from the cross of Prichard × Anand were evaluated for reniform RI. Anand originated from the cross 'Holladay' × Hartwig and was reported as resistant to reniform nematode (Anand et al., 2001; Robbins et al., 1999). Hartwig was developed from the cross of Forrest (3) × PI 437654 (Anand, 1992). Prichard originated from the cross of Co82-622 × 'Howard' and was susceptible to reniform nematode (Boerma et al., 2001). Both Howard and Co82-622 include Forrest in their pedigree. A total of 353 RILs were genotyped using the SSR markers Satt513 on LG-L, Satt359 on LG-B1, and Sat_168 on LG-G. The 51 plants representing the eight possible homozygous classes for three markers were evaluated for reniform nematode resistance in a greenhouse as described earlier. The screening was conducted in a randomized complete block experimental design with five replications. Each plant was inoculated with 1644 vermiform reniform

nematode (Pi). Final nematode population (Pf) for each pot was determined only from the soil.

RESULTS AND DISCUSSION

Phenotypic variation and heritability

The mean reniform RI and total number of eggs + vermiform nematode (in parentheses) was 0.20 (336) for the resistant parent PI 437654 and 3.51 (5971) for the susceptible parent BSR101. The mean RI for the 228 RILs ranged from 0.14 to 15.05 per plant (Figure 5.1). Log transformed reniform RI data for the 228 RILs were used to determine heritability. The heritability on entry-mean basis (mean of five replications) was 0.78, suggesting that there was a major genetic component conditioning reniform nematode resistance in this population.

QTL conditioning reniform nematode resistance

To map reniform nematode resistance, we examined five linkage groups that had been previously associated with resistance to SCN. Ninety-six previously mapped SSR markers on LG-A2, -B1, -G, -L, and -M (Song et al., 2004) were screened for polymorphism between the parental genotypes. Sixty-one of these 96 markers (64%) were polymorphic. Among these, 53 SSR markers were used to genotype 134 of the 228 RILs. Based on the association of the reniform RI phenotypes and the SSR marker alleles

for those 53 markers by SF-ANOVA, we putatively identified three QTL associated with reniform nematode RI on LG-B1, -G, and -L (data not shown).

To further increase the precision for QTL mapping, 94 additional RILs were genotyped with 28 SSR markers on LG-B1, -L, and -G. Data from these 94 RILs were combined with the 134 original RILs. When the marker and phenotypic data from the 228 RILs were analyzed by interval mapping with Map Manager QTX, a QTL mapped to a 25.3-cM interval between Sat_184 and Satt513 on LG-L and explained 21% of the variation in reniform RI (Figure 5.2). The QTL peak was 8 cM from Satt513 (LOD = 11.8). Another QTL mapped to a 14.5-cM interval between Satt359 and Satt484 on LG-B1 and explained 16% of the phenotypic variation (LOD = 8.8). The QTL peak was 5 cM from Satt359. A third QTL, accounting for 8% of the phenotypic variation in reniform RI (LOD = 4.1), was linked to marker Sat_168 on LG-G (Figure 5.2).

The three single-linkage group multiple regression analyses that included the SSR markers found significantly associated with reniform RI based on SF-ANOVA on LG-B1 (7 markers), -G (6 markers), and –L (4 markers) retained Satt359 on LG-B1, Sat_168 on LG-G, and Satt513 and Sat_184 on LG-L (Table 5.1). Satt513 on LG-L accounted for the most variation (18.1%) in reniform RI across linkage groups. Marker Sat_184 on LG-L explained an addition 3.2% of the variation in reniform RI. Given a single QTL peak was identified by interval mapping of LG-L, it is likely Satt513 and Sat_184 are flanking a single LG-L reniform RI QTL.

Satt359 on LG-B1 accounted for the 17.3% and Sat_168 on LG-G accounted for 8.5% of phenotypic variation in renifrom RI. RILs homozygous for PI 437654 alleles had significantly lower reniform RI than RILs homozygous for BSR101 alleles. When all four

significant markers from LG-B1, -L, and -G were included in a multiple regression analysis, all four SSR markers were retained in the model, and collectively explained 36% of the phenotypic variation in reniform RI (Table 5.1).

QTL effects on reniform nematode resistance

Three QTL conferring reniform RI showed a significant ($P < 0.05$) additive main effect (Table 5.1). Also, significant ($P < 0.01$) additive × additive epistasis was detected between Satt359 and Sat_168 (Table 5.2). The LG-B1 QTL effect was greater than the LG-G QTL effect (Table 5.1 and 5.2). However, RILs homozygous for PI 437654 alleles at Satt359 and Sat_168 had a reniform RI of 0.79 which was significantly lower than the mean RI (RI = 4.5) for the other three genotypes (Table 5.2).

The mean reniform RI for RILs homozygous for PI 437654 alleles at Satt513, Satt359, and Sat_168 was 0.63 and was not significantly different from the resistant parent PI 437654 (RI = 0.20), whereas RILs homozygous for BSR101 alleles had a mean RI of 6.24, which was significantly greater than susceptible parent BSR 101 (RI = 3.51) (Table 5.3). The LG-L QTL had a larger effect on RI than the LG-B1 or LG-G QTL. The combined effect on RI of the two LG-B1 and LG-G QTL was very similar to the combined effect of the three LG-L, LG-B1, and LG-G QTL. RILs homozygous for PI 437654 alleles at the QTL on LG-B1 and LG-G averaged a RI of 1.49 compared to a RI of 0.63 for the RILs that were homozygous for the PI 437654 alleles at all three QTL. These data suggest that having a resistance allele at only one of the three QTL would not produce a high level of resistance in a commercial soybean cultivar.

The three individual markers, Satt359 (LG-B1), Sat_168 (LG-G), and Satt513 (LG-L) associated with QTL for reniform RI, the three two-way interaction, and one three-way interaction among marker loci were entered into multilocus QTL analyses performed by SAS PROC MIXED. The main effects of Satt359, Satt513, Sat_168, and the Satt359 × Sat_168 interaction were significant ($P = 0.01$) in the model and explained 60% of the phenotypic variation in reniform RI. With the heritability estimate of 78% for reniform RI in this population, the multilocus model explained 76% (60/78) of the genotypic variation.

Of the 228 RILs in this population, 206 RILs were homozygous and without missing data for the SSR markers Satt513 on LG-L, Satt359 on LG-B1, and Sat_168 on LG-G. For the eight possible homozygous classes of the three markers, we expected an equal distribution of lines in each class (~26 lines per class). The actual number of lines in these marker classes was significantly different ($\chi^2 = 28.25$, $P < 0.01$) from expectations (Table 5.3). One genotypic class that had the BSR101 (B) allele at Satt513 and PI 437654 (P) alleles at the Satt359 and Sat_168 included only seven lines. Also, the B/B/P and B/P/B classes for Satt513, Satt359, and Sat_168, respectively, contained fewer than the 26 expected lines. Apparently, the combination of alleles from BSR101 in the region of Satt513 on LG-L with alleles from PI 437654 in the region of Satt359 on LG-B1 and the combination of alleles from BSR101 in the region of Satt513 on LG-L with alleles from PI 437654 in the region of Sat_168 on LG-G were deleterious to survival or were unintentionally selected against during population development. A distortion in marker classes also had been reported in another study that mapped SCN resistance using the same BSR101 × PI 437654 population that was also maintained and increased in Iowa

by Pioneer Hi-Bred International, Inc. (Webb et al., 1995). Results of that study suggested that the distortion in allele frequencies was attributed to natural selection associated with particular genotypes on specific linkage groups prior to the maturation of seed.

Confirmation of the QTL in a different genetic background

Prior to the application of marker assisted selection it is desirable to confirm QTL in the same population with a different sample of lines or in a different genetic background (Fasoula et al., 2004). A population of 353 RILs derived from the cross of Prichard × Anand was evaluated to confirm the previously described QTL for reniform RI.

Anand and its ancestors (Holladay, Hartwig, Forrest, and PI 437654) were screened with SSR markers flanking QTL on LG-L, B1, and G (Table 5.4). Anand inherited the allele at the reniform QTL on LG-B1 from Hartwig (via Forrest), the allele at the reniform QTL on LG-G from Hartwig (via PI 437654 or Forrest), and the allele at the QTL on LG-L from Holladay. Putatively, Anand appeared to possess resistance alleles at the LG-B1 and LG-G QTL, but not at the LG-L QTL.

In greenhouse screening, Anand averaged a reniform RI of 5.9 while Prichard averaged 19.7. Resistant checks, PI 437654, Forrest, and Hartwig, averaged an RI of 1.7, 6.4, and 4.7, respectively, and susceptible check, Braxton, averaged an RI of 38.5. These data indicate that Hartwig did not inherit all the reniform resistance alleles present in PI 437654 and that Prichard may possess partial reniform nematode resistance. A group of 51 RILs that were homozygous for Prichard or Anand alleles at Satt513 (LG-L), Satt359

(LG-B1), and Sat_168 (LG-G) were selected (Table 5.5). Eight RILs homozygous for the Prichard alleles at Satt513, Satt359, and Sat_168 averaged a RI of 26.8, while two RILs homozygous for the Anand alleles averaged 5.5. Only Sat_168, near the reniform QTL on LG-G, appeared to impact reniform RI. Given the presence of the susceptible Holladay allele in Anand at LG-L QTL, the lack of difference between RILs homozygous for the Anand and Prichard alleles at this QTL was predicted based on the QTL mapping study. The lack of a difference in reniform RI for RILs homozygous for either Anand or Prichard alleles at the reniform QTL on LG-B1 was unexpected and suggest that both Anand and Prichard possess a reniform resistance allele at this QTL. If correct, this would result in all the RILs in this population possessing a reniform resistance allele at the LG-B1 QTL and explain the lower reniform RI in Prichard (RI = 19.7) compared to Braxton (RI = 38.5). The fixation of a reniform resistance allele at the LG-B1 QTL would also explain the large reduction in reniform RI shown by RILs homozygous for the Anand allele compared to the Prichard allele at the LG-G QTL (Table 5.5). Prichard could have inherited an allele for reniform resistance from either of its parents, Co82-622 or Howard, since the parents of Prichard include Forrest in their pedigrees. While the lack of segregation of a resistance allele at the reniform QTL on LG-L precluded its confirmation in this population, our results serve to confirm the reniform resistance conditioned by the combination of reniform resistance alleles at QTL on LG-G and LG-B1.

The confirmation population also showed segregation distortion in marker classes. One genotypic class that was homozygous for the Anand alleles at Satt513, Satt359, and Sat_168 contained only two lines out of 353 RILs evaluated (Table 5.5). Also, one class

that had the Anand alleles at Satt513 and Satt359 and the Prichard allele at Sat_168 had only one line out of 353 RILs evaluated. The lines in this population were selected for late maturity and there are known maturity QTL in the same region of the genome as the three reniform QTL. Maturity gene *E3*, a major locus conferring photoperiod-insensitivity in soybean, is located between Satt006 and Satt373 on LG-L (Tasma and Shoemaker, 2003). Pod maturity QTL are also located near Satt359 on LG-B1 and near Satt309 on LG-G, respectively (Lee et al., 1996; Brucker et al., 2005). Therefore due to genetic linkage of maturity and reniform RI QTL, it is likely the Prichard alleles would be more frequent in the lines from this population near markers Satt513 and Satt359.

This study was initiated based on the observation that there was a genetic linkage between SCN and reniform nematode resistance. Many studies reported that SCN-resistant soybean cultivars that derive resistance from Peking, PI 437654, or PI 90763 are also resistant to reniform nematode (Robbins et al., 1994a,b; Davis et al., 1996; Robbins and Rakes, 1996). The SCN-resistant genotype PI 437654 has major SCN QTL on LG-G (*rhg1*) and LG-A2 (*Rhg4*). The SSR marker Satt309, located 1 to 2cM from *rhg1* has been shown to be useful for marker-assisted selection for SCN resistance (Cregan et al., 1999). Concibido et al. (2004) reported that QTL on LG-A1, -B1, -C1, -J, and -M conditioned SCN resistance in PI 437654. Interestingly, the QTL for SCN resistance linked to RFLP marker A006 on LG-B1 was reported by Vierling et al. (1996) to explain more than 90% of the phenotypic variation in a cross between 'Williams 82' and Hartwig. Hartwig was derived from backcrossing SCN resistance from PI 437654 into Forrest (Anand, 1992). Also, a SCN-resistant QTL linked to an RFLP marker (A567a) on LG-B1

was found in a cross from BSR101 × PI 437654, according to a patent claim by Webb (2003).

Harville et al. (1985) suggested that reniform nematode resistance was quantitatively controlled by two genes with unequal effects. By contrast, we found a QTL on LG-L and two epistatically interacting QTL on LG-B1 and LG-G. The reniform resistance QTL on LG-G with its resistance allele derived from PI 437654 was located near SSR marker Sat_168 which is closely linked to SCN resistance gene *rhg1*. Also, the reniform QTL on LG-B1 located between Satt359 and Satt484 is positioned between RFLP markers A006 and A567a, which were reported to be linked to SCN QTL (Vierling et al., 1996; Webb, 2003). Therefore, our results support linkage between certain SCN and reniform resistance QTL and provide confirmation of the interaction of QTL for reniform RI on LG-G and LG-B1.

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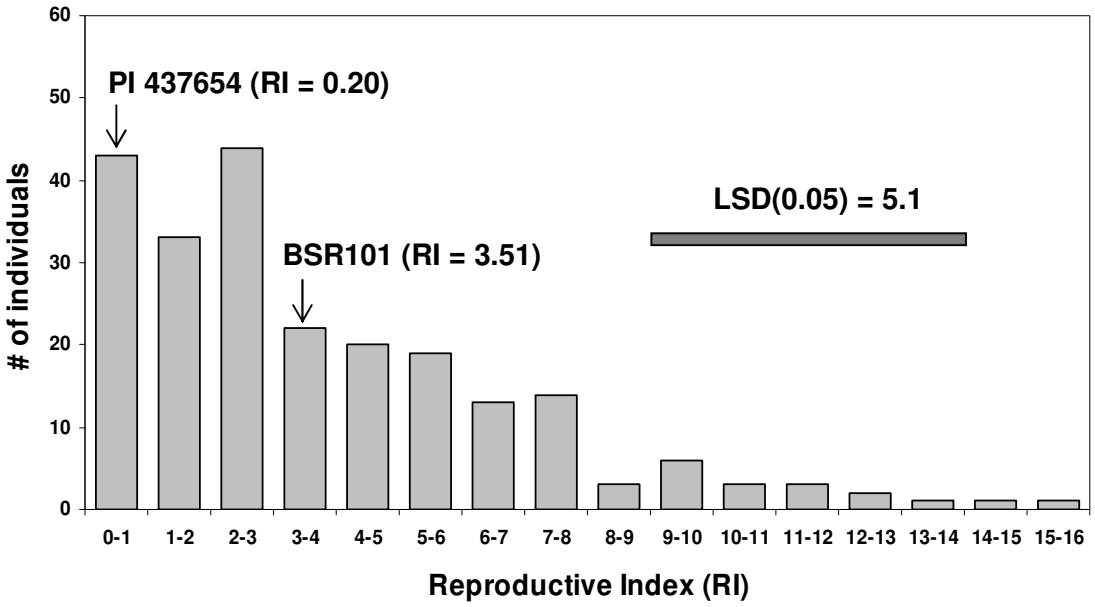

Figure 5.1. Distribution of reniform reproductive index (RI) for 228 recombinant-inbred lines (RILs) from BSR101 × PI 437654.

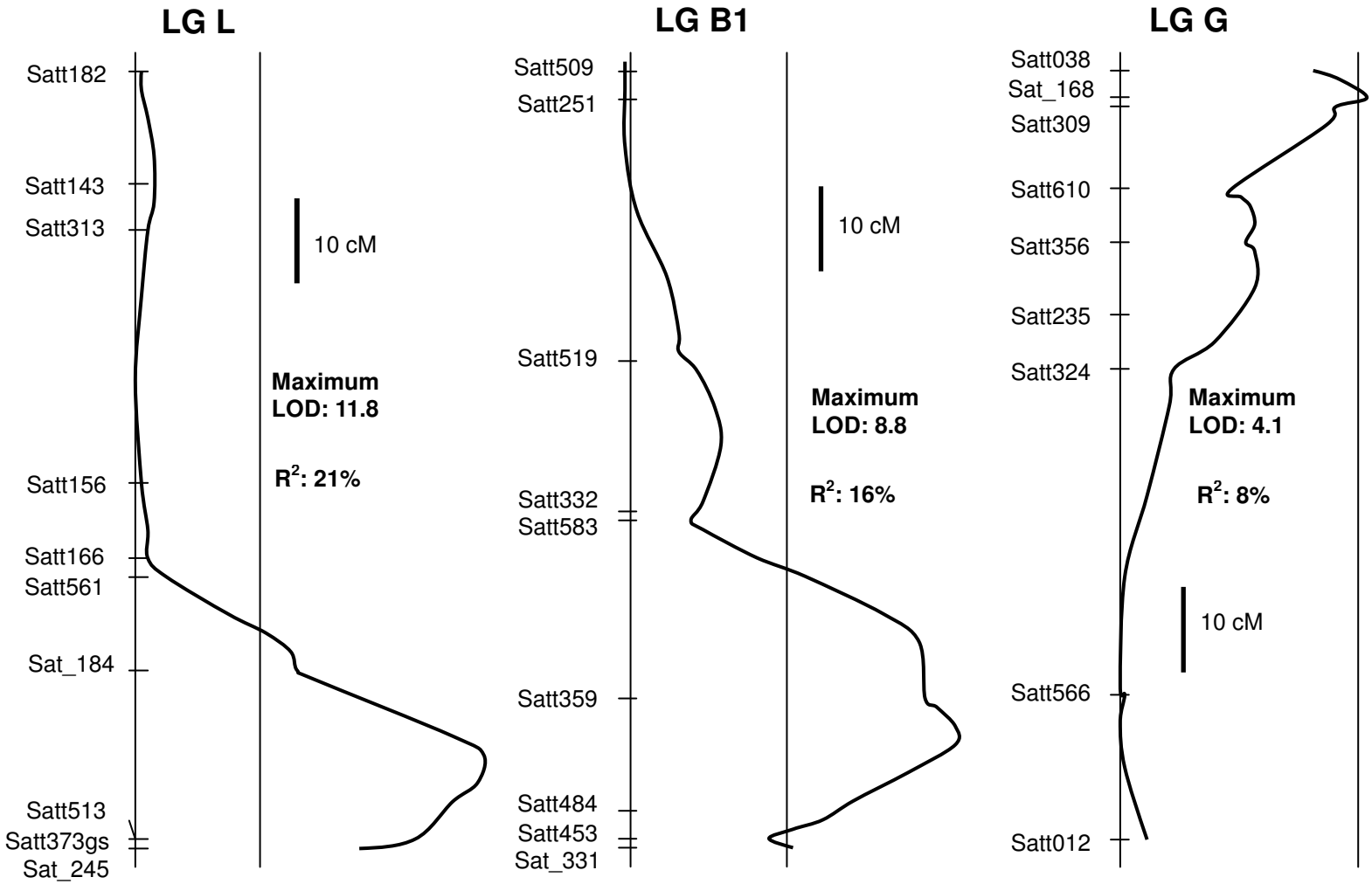
LG L
Satt182
Satt143
Satt313
Satt156
Satt166
Satt561
Sat_184
Satt513
Satt373gs
Sat_245
10 cM
Maximum
LOD: 11.8
R2: 21%
LG B1
Satt509
Satt251
Satt519
Satt332
Satt583
Satt359
Satt484
Satt453
Sat_331
10 cM
Maximum
LOD: 8.8
R2: 16%
LG G
Satt038
Sat_168
Satt309
Satt610
Satt356
Satt235
Satt324
Satt566
Satt012
Maximum
LOD: 4.1
R2: 8%
10 cM

Figure 5.2. Maximum likelihood plots indicating genomic regions of quantitative trait loci (QTL) associated with reniform RI in 228 recombinant-inbred lines (RILs) from BSR101 × PI 437654. Lines parallel to the linkage groups indicate the genome-wide $\alpha = 0.001$ significance threshold (LOD = 3.8).

Table 5.1. Soybean simple sequence repeat (SSR) markers associated with reniform nematode reproductive index in 228 recombinant-inbred lines (RILs) from BSR101 × PI 437654.

Marker	LG	SLG-reg[†]		MLG-reg[‡]		d^{ξ}
		P value	Partial R^2 (%)	*P* value	Partial R^2 (%)	
Satt359	B1	<0.001	17.3	<0.001	10.3	-0.98
Sat_184	L	0.003	3.2	0.003	3.3	-0.99
Satt513	L	<0.001	18.1	<0.001	16.8	-1.41
Sat_168	G	<0.001	8.5	<0.001	5.4	-0.56
				Total R^2:	35.8	

[†] SLG-reg: Multiple regression analysis including significant ($P < 0.05$) markers within a single linkage groups.

[‡] MLG-reg: Multiple regression analysis including significant ($P < 0.05$) markers across linkage groups

[ξ] Additive genetic effect on reniform reproductive index = $(y_{PI\ 437654} - y_{BSR101})/2$, $y_{PI\ 437654}$ is the PI 437654 homozygote mean, and y_{BSR101} is the BSR101 homozygote mean.

Table 5.2. Main effects and interaction of two reniform reproductive index (RI) QTL in the recombinant-inbred lines (RILs) from BSR101 × PI 437654.

Satt359 (LG-B1)	Sat_168 (LG-G)		Mean
	B/B[†]	PI/PI	
	Reproductive index (RI)[‡]		
B/B	4.48 ab[ξ]	5.12 a	4.80
PI/PI	4.00 b	0.79 c	2.40[***]
Mean	4.24	2.96[***]	

[†] B/B: Homozygous for allele from BSR101; P/P: Homozygous for allele from PI 437654.

[‡] Reproductive index = the number of eggs + vermiform nematodes at test termination (Pf) / initial infestation level (Pi).

[ξ] Numbers followed by the same letters are not significantly different at the 0.05 probability level.

[***] Significantly different from its counterpart at the 0.001 probability level based on analysis of variance.

Table 5.3. The effect on reniform nematode reproductive index for QTL at Satt513 (LG-L), Satt359 (LG-B1) and Sat_168 (LG-G) in 228 recombinant-inbred lines (RILs) from BSR101 × PI 437654.

Satt513	Satt359	Sat_168	N	Reproductive index (RI)[‡]	
LG-L	LG-B1	LG-G		Mean	Std Error
B/B[†]	B/B	B/B	33	6.24	0.56
B/B	B/B	P/P	19	6.48	0.76
B/B	P/P	B/B	19	5.62	0.56
B/B	P/P	P/P	7	1.49	1.14
P/P	B/B	B/B	39	2.99	0.37
P/P	B/B	P/P	27	4.16	0.55
P/P	P/P	B/B	31	3.01	0.41
P/P	P/P	P/P	31	0.63	0.12

† B/B: Homozygous for allele from BSR101; P/P: Homozygous for allele from PI 437654.

‡ Reproductive index = the number of eggs + vermiform nematodes at test termination (Pf) / initial infestation level (Pi).

Table 5.4. Comparison of band sizes of simple sequence repeat (SSR) markers linked to conditioning reniform nematode reproductive index (RI) quantitative trait loci (QTL) for Anand, its ancestors, and Prichard.

SSR marker	LG	Prichard	Anand	Holladay	Hartwig	Forrest	PI 437654	BSR101
					base pairs			
Satt513	L	167	143	143	118	118	173	143
Satt373gs	L	89	100	100	160	160	86	124
Satt359	B1	162	195	180	195	195	198	180
Satt484	B1	304	304	304	304	304	294	304
Sat_168	G	176	162	162	162	162	162	176
Satt309	G	127	136	149	136	136	136	127

Table 5.5. The effect on reniform nematode reproductive index of three QTL linked to markers Satt513 (LG-L), Satt359 (LG-B1) and Sat_168 (LG-G) in the recombinant-inbred lines (RILs) from Prichard × Anand.

Satt513	Satt359	Sat_168	N	Reproductive index[‡]	
LG-L	LG-B1	LG-G		Mean	Std Error
P/P[†]	P/P	P/P	8	26.79	3.06
P/P	P/P	A/A	8	7.32	0.87
P/P	A/A	P/P	8	23.80	3.56
P/P	A/A	A/A	8	8.45	0.85
A/A	P/P	P/P	8	26.69	1.69
A/A	P/P	A/A	8	6.99	1.24
A/A	A/A	P/P	1	43.86	.
A/A	A/A	A/A	2	5.53	3.18

[†] P/P: Homozygous for allele from Prichard; A/A: Homozygous for allele from Anand.

[‡] Reproductive index = the number of vermiform nematodes at test termination (Pf) / initial infestation level (Pi).

CHAPTER VI

SUMMARY

The southern root-knot nematode [*Meloidogyne incognita* (Kofoid and White) Chitwood] (Mi) is a major pest and reniform nematode (*Rotylenchulus reniformis* Linford & Oliveira) has been recently recognized as an increasing serious problem for soybean [*Glycine max* (L.) Merr.] production in the southern USA. Soybean breeders have been intensifying their efforts to develop highly productive, nematode resistant soybean cultivars. One of the challenges in the past has been that screening for resistant plants was tedious, time consuming, and variable. In this study, molecular approaches were applied to facilitate marker-assisted selection (MAS) for the southern root-knot nematode and reniform nematode in soybean.

The objective of the first study was to determine the frequency of elite Mi-resistant cultivars that inherited the major resistance QTL (*Rmi1*) on LG-O and identify the ancestral source of the Mi-resistance allele at this locus in elite U.S. soybean cultivars. Forty-eight soybean genotypes, including ancestral, Mi-susceptible, and Mi-resistant genotypes, were evaluated for Mi-gall formation in a greenhouse in a randomized complete block experimental design with six replications. These genotypes were genotyped at six SSR loci (Satt358, Sat_132, Satt487, Satt500, Satt492, and Satt445) that flank the major Mi resistance QTL on LG-O. Codescent analysis of markers and

phenotypes across six cycles of breeding showed that Mi-resistant cultivars inherited a 200-bp band at Satt358 and a 238-bp band at Sat_132 from 'Palmetto'. Palmetto is likely the source of the Mi-resistance allele (*Rmi1*) that was found in most elite Mi-resistant Maturity Group V, VI, VII, and VIII cultivars. The apparent tight linkage of Satt358 and Sat_132 to the major QTL for Mi resistance on LG-O suggest that these markers should be effectively employed in breeding for Mi resistance.

SSR markers, which are highly polymorphic, abundant, and distributed throughout the genome, have been considered a major tool for the marker-assisted selection in soybean. However, SSR marker assays involve gel electrophoresis that is moderately hazardous and difficult to automate. Recently, SNPs have been extensively used as the marker of choice because of their high frequency and suitability for high-throughput, automated genotyping. The objective of the second study was to identify SNPs linked to the Mi resistant QTL on LG-O and LG-G and to optimize SNP genotyping conditions using Luminex 100 flow cytometer for automated high-throughput MAS. Eight SSR markers, located near a Mi-resistance QTL on LG-O (most likely *Rmi1*) and LG-G, were used to select BAC ends and SSR-containing genomic DNA clones in GenBank. Sixteen BAC-end sequences and eight SSR flanking regions were used to design primers to amplify genomic fragments of PI 96354 (Mi resistant) and Bossier (Mi susceptible). We discovered four SNPs in Satt358 allele sequences located near a major Mi-resistant QTL on LG-O and 4 SNPs in Satt199 allele sequences located near a minor Mi-resistant QTL on LG-G. Using a direct hybridization assay detected on a Luminex 100 flow cytometer, the genotypes of SNP358 marker that targets two SNPs in Satt358 allele sequences were identical to the genotypes of the SSR marker Satt358 across 94 $F_{2:3}$ lines

of PI 96354 × Bossier. The genotypes of SNP199 marker which targets a SNP in Satt199 allele sequences also showed 100% congruence with the genotypes of SSR marker Satt199. Also we compared SNP genotypes with SSR genotypes using 24 known Mi-resistant cultivars or Mi-susceptible cultivars. These results showed that the SNP-marker assay using direct hybridization allowed accurate and high speed detection of polymorphism in the LG-O and LG-G genomic regions near the Mi resistance QTL.

The objective of the third study was to identify soybean QTL conferring resistance to reniform nematode, to determine the magnitude of resistance of each QTL, and to evaluate the potential interaction of identified QTL. A total of 228 recombinant-inbred lines (RILs) derived from a cross between susceptible 'BSR101' and resistant PI 437654 were used to map QTL governing reproductive index (RI) and number of reniform eggs and juveniles. The reniform nematode screening was conducted in a greenhouse in a randomized complete block experimental design with five replications. A QTL (R^2=21%) conditioning RI was found on Linkage Group L (LG-L) flanked by Sat_184 and Satt513. Two other QTL were identified. One was located in the interval from Satt359 to Satt484 on LG-B1 (R^2=16%) and the other on LG-G (R^2=8%) linked to Sat_168. These QTL acted in an epistatic manner with lines homozygous for PI 437654 alleles at both QTL providing the lowest reniform RI. By screening the Prichard × S94-1956 RILs, we confirmed that QTL on LG-G and -B1 would enhance reniform nematode resistance. These results are useful in developing strategies for using resistance QTL in marker-assisted selection to improve the efficiency of selection for reniform nematode resistance in soybean. In addition, these results support the genetic linkage between certain SCN and reniform resistance QTL.