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**IDENTIFICATION AND CHARACTERIZATION OF SOYBEAN CYST
NEMATODE RESISTANCE GENES USING DNA MARKERS**

**A THESIS
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA**

BY

VERGEL CIERTE CONCIBIDO

**IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY**

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ABSTRACT

Vergel Cierte Concibido
326 words

A major partial soybean cyst nematode (SCN; *Heterodera glycines*) resistance locus, common among various sources of SCN resistance, was identified on linkage group 'G' of the soybean [*Glycine max* (L.) Merrill] molecular linkage map using restriction fragment length polymorphisms (RFLPs). The 'G' locus explains 51.4% of the total phenotypic variation in SCN disease response in PI 209332, 52.7% in PI 90763, 40% in PI 88788 and 28% in 'Peking' and appears to be additive to partially recessive in gene action. It was also observed that the ability to detect minor SCN resistance loci was complicated by the presence of strong phenotypic effects due to the locus on 'G'. In one experiment, the 'G' locus appeared to be race specific. Marker-assisted selection, based on the 'G' locus, was shown to be 92% accurate in selecting SCN resistant soybean lines compared to 81% obtained with phenotype-based selection. A targeted comparative mapping strategy was employed resulting in a sevenfold increase in marker density within the 14.7 centimorgan (cM) region of interest, which initially had a low density of markers. Qualitative scoring of SCN, using a recombinant inbred soybean population, made it possible to identify recombinants between the two flanking markers (Bng122 and Bng 173) and the SCN resistance locus. Since cloning a gene by map position is dependent on the ability to uncover tightly linked recombinants around the target gene, the strategy that is described in this thesis facilitated this process. By combining mapping information from four distinct populations, an integrated map of the linkage group 'G' region was created, placing the SCN locus 2.1 cM from the nearest RFLP marker (B53). This thesis demonstrates how various molecular mapping strategies, such as RFLP mapping, comparative genome and integrated mapping, together with qualitative scoring of a quantitative trait, can provide the necessary tools for developing a high resolution map around a quantitative trait locus (QTL) for eventual map-based cloning.

INTRODUCTION

The soybean, *Glycine max* (L.) Merrill, is one of the major economic crops grown worldwide as a primary source of vegetable oil and protein (Sinclair and Backman, 1989). The growing demand for low cholesterol and high fiber diets has also increased soybean's importance as a health food. As soybean production has expanded worldwide, soybean diseases have also increased in number and severity (Sinclair and Backman, 1989).

The soybean cyst nematode (SCN) *Heterodera glycines* Ichinohe is perhaps one of the most important soybean diseases throughout the United States. (Noel, 1992). Soybean yield losses due to SCN vary from negligible up to 90% (Riggs, 1977; Sinclair and Backman, 1989). Furthermore, SCN has been expanding its range to other soybean growing regions including Minnesota (Stienstra and MacDonald, 1990). To date, the most cost effective control measure is the use of host plant resistance and breeders have been generally successful in developing SCN resistant soybean lines. However, breeding for resistance is difficult and time-consuming due to the complex and oligogenic nature of resistance (Anand and Rao-Arelli, 1989). In addition, a considerable amount of genetic variability exists among field populations of SCN (McCann *et al*, 1982). It has also been observed that many resistant soybean varieties carry a significant yield penalty when planted in the absence of SCN.

With the advent of molecular markers, such as restriction fragment length polymorphisms (RFLPs), it is now possible to tag agriculturally important genes, such as SCN resistance. Identification and characterization of SCN resistance genes will ultimately accelerate the breeding process, since selection can be based on genotype rather than on phenotype. Hence, a more deterministic approach to breeding for SCN resistance called "marker-assisted selection" (MAS) can be employed (Tanksley *et al*, 1989).

This dissertation contains three sections, written in *Crop Science* format, that demonstrate the utility of DNA markers in the genetic mapping and characterization of soybean cyst nematode resistance. Chapter 1 describes a preliminary search for putative DNA marker loci associated with genetic resistance to soybean cyst nematode. The results of this study were published in *Crop Science* and were the first to describe this complex host-parasite interaction at the molecular genetic level. In the process, three important resistance loci were uncovered and one on linkage group 'G' was found to be especially important. The second chapter highlights efforts to characterize the major partial SCN resistance locus by assessing its race specificity. Knowledge about the effectiveness of this major partial SCN resistance gene against different races of the cyst nematode will be useful in gene deployment and pyramiding strategies to effectively manage host plant resistance. This chapter also demonstrates the practical application of DNA markers in SCN resistance breeding in soybean. The third chapter focuses on efforts to construct a high resolution genetic map around the major partial SCN resistance gene. This chapter also demonstrates how comparative DNA mapping, an especially powerful application of DNA markers that utilizes mapping information from one population to predict the properties of other populations, can be used to increase marker density near genes of interest. Another strategy in developing high density maps that will be described in this chapter is integrated or "join" mapping. "Join" mapping allows the integration of linkage maps that were developed in independent populations. This mapping approach facilitates the placement of genes that do not segregate in one mapping population onto a unified map by combining information from separate mapping populations. These two strategies aim at increasing marker density around the genomic region of interest and are important steps towards the ultimate goal of cloning the gene.

OBJECTIVES

Primary objectives:

- To identify and characterize genomic regions associated with SCN resistance using DNA markers.
- To construct a high resolution map of the genomic region containing the major partial SCN resistance gene on linkage group 'G' and assess its race specificity.

Secondary objectives:

- To assess the feasibility of marker-assisted selection for SCN resistance breeding.
- To develop unique recombinant inbred lines that will be useful in characterizing SCN disease response as a qualitative rather than a quantitative trait.

LITERATURE REVIEW

Overview of the soybean-soybean cyst nematode interaction

SCN symptomatology

The disease caused by soybean cyst nematode (SCN), *Heterodera glycines* Ichinohe, was first observed as early as 1881 in Japan (Hori, 1915). Field symptoms characterized by round patches of stunted, yellow soybean were attributed to a disease called "moon night" or "yellow dwarf" disease and was first thought to be caused by sugar beet cyst nematode (*Heterodera schachtii* Schmidt) (Katsufugi, 1919; Ito, 1921). Damage due to SCN is dependent on the nematode population density, response of the cultivar, presence of other microorganisms, and environmental factors, such as temperature, oxygen, duration of the growing season, soil fertility and soil physical properties

(Zirakparvar, 1985). In a review article by McGawley (1992), it was reported that SCN can predispose soybeans to fungi, bacteria, viruses, insects, weeds, and other nematodes. Since SCN symptoms can be easily confused with other foliar diseases, mineral deficiencies and abiotic stresses, positive identification of SCN by visual examination of the above ground symptoms is very unreliable. Thus, diagnosis of the disease must be based on the presence of white to yellow females and brown cysts, that erupt from the roots of infected soybean plants.

Life cycle

The cysts of *H. glycines* are brown, tough, lemon-shaped bodies filled with 100 or more eggs. Young females are white and turn yellow at maturity. When young females die, their body walls harden and turn into dark brown cysts (Riggs and Schmitt, 1989). This process, known as the quinone tanning process, protects the larvae that may remain in the egg and cyst for as long as 11 years (Ellenby, 1946). *H. glycines* has an egg stage, four juvenile stages and an adult stage. First stage juveniles develop within the egg and undergo one molt; second stage juveniles emerge at hatching. In the soil, second stage juveniles are attracted toward roots by plant exudates, invade the roots, and establish a feeding site. Within 48 hours after penetration of the soybean root, perforations of the cell walls occur at the feeding site, marking the beginning of syncytium development (Gipson *et al*, 1971). This syncytium, which becomes a sink where nutrients accumulate, is the most common feeding structure induced in plants by sedentary parasites such as SCN (Endo, 1964; Gipson *et al*, 1971; Jones and Dropkin, 1975a, 1975b; Kim *et al*, 1987). It is a result of the transformation of host cells into a "giant cell" (syncytium) through cell wall dissolution, and break-up of the central vacuoles into many small vacuoles (Gipson *et al*, 1971). The establishment and maintenance of a syncytium determine the growth and development of the nematode once it enters the root (Young, 1992).

H. glycines can survive up to 11 years in the soil and its long-term survival may be attributed to protection by the cyst wall and, in part, to a genetically controlled diapause (Ellenby, 1946; Riggs and Schmitt, 1989). The persistence of SCN in the soil may also be related to its broad host range (Riggs, 1992). SCN can move only a few inches per year, but any movement of soil by wind, water, seed and machinery can disseminate cysts or eggs locally (Riggs, 1977). Long distance spread of SCN is accomplished through passage of cysts, viable juveniles and eggs through digestive tracts of blackbirds (Epps, 1971).

Physiological variability and the concept of race in SCN

Extensive physiologic variation among SCN populations has presented a challenge for soybean breeders and nematologists since the nematode was first described in the U.S. (Schmitt and Shannon, 1992). Ross (1962) was the first to report the occurrence of physiologic variation within *H. glycines*. After widespread discoveries of SCN infestations and reports of physiological and morphological variability among populations (Miller, 1966, 1967; Riggs *et al.*, 1988; Sugiyama *et al.*, 1968), nematologists and breeders convened in 1969 and designated the infraspecific forms of *H. glycines* as "races". As a result of this meeting, Golden *et al.* (1970) established a standardized system to differentiate SCN races (Table 1). The assignment of a race for a *H. glycines* isolate was based on a pattern of positive and negative disease reactions induced on a set of four host differentials: 'Pickett', 'Peking', Plant Introduction (PI) 90763 and PI 88788. A negative (-) reaction indicated that the number of females and cysts that was recovered was less than 10% of the number on a susceptible check, while a positive (+) reaction indicated that the number of females and cysts recovered was greater than or equal to 10% of the number on the susceptible check (Riggs and Schmitt, 1989). This disease reaction has been referred to as host "reaction" (Riggs, 1988), "female index" (Riggs *et al.*, 1988), "index of parasitism" (Triantaphyllou, 1987), "cyst index" (Price *et al.*, 1977), "mature female index" (Hancock

et al, 1987), "reproductive index" (Riggs, 1982), or simply an "index" (Riggs *et al*, 1981; Riggs and Schmitt, 1991). However, the existing race classification scheme is not without controversy. Using this scheme, SCN races 1, 2, 3, and 4 (Golden *et al*, 1970), race 5 (Inagaki, 1979), race 7 (Chen *et al*, 1987) and race 14 (Hartwig, 1985) have been isolated from field and greenhouse soil samples. Despite the characterization of most races (Riggs and Schmitt, 1988), races 11, 12, 13 and 16 have not been found. Schmitt and Shannon (1992) suggested that it would be surprising to find these races since they are all defined by a positive reaction on Peking and a negative reaction on Pickett (Table 1). Yet Pickett is a progeny line of Peking and probably did not inherit all the resistance genes from its parent (Anand and Shumway, 1984; Riggs, 1988). Thus, it is very unlikely that Pickett would have a negative disease response when Peking has a positive one.

Thomas *et al* (1975) criticized the race designation for using host differentials based solely on chronological discovery and not an indication of how these races evolved. Price *et al* (1977) also warned that the ability to discriminate the wide range of parasitic variabilities in SCN is a function of the host differentials or index combination used. In support of this, Riggs *et al* (1981) tested 38 *H. glycines* populations collected from 13 U.S. states and Japan. Depending on the number of differentials or the rating system used, they could differentiate from six up to 36 races. Likewise, Miller (1970) could distinguish 11 biotypes of SCN using several legumes as differentials. Furthermore, the present race classification system lacks stability and consistency due to its often unachievable dependence on inoculum uniformity and SCN assay (Riggs *et al*, 1981). For example, Riggs *et al* (1981) conducted SCN race determination tests using differential host lines in Arkansas, Illinois and North Carolina to assess uniformity of test results. Results indicated that the tests were highly variable. Isolates previously designated as race 1 tested either as race 1 or 3. They attributed the observed discrepancies to inoculation and cyst recovery techniques, and impurities of host differentials used. Thus, it is imperative to include pure

and defined differentials, as well as adequate experimental controls, in any test where race designation is crucial to ensure that the proper race is reported with the results (Riggs *et al.*, 1981).

Another major issue is the use of the 10% rule in defining resistance and susceptibility to SCN. Populations that give reactions slightly more or less than 10% are difficult to classify with confidence. A statistical approach using confidence intervals was proposed to resolve this issue (Young, 1989). Schmitt and Shannon (1992) also questioned the relevance of the index of parasitism on the actual field performance of cultivars. Niblack (1992) explained that the race concept in *H. glycines* is actually a population concept and thus cannot account for either the within-race variability of the nematode, the variability within soybean differential lines used in race classification, or the interaction between the two. Despite the many criticisms of the present race scheme in SCN, it is still very useful in communication among breeders and nematologists.

The issue of race in SCN is further complicated by the high level of genetic variability that exists among field populations of SCN. Female nematodes can have multiple matings with various males (Schmitt and Shannon, 1992) and males can inseminate several females (Sipes, 1992). Inbreeding among relatives are common in SCN with frequent sib and half-sib matings (Sipes, 1992).

Control of SCN

Control of SCN can be accomplished by chemicals (Rodriguez-Kabana, 1992), cultural management (Wrather *et al.*, 1992), biological agents (Kim and Riggs, 1992) and host plant resistance (Caviness, 1992). It has been suggested that any strategy to control SCN should be aimed at reducing nematode populations below economic thresholds. However, the long-term control of SCN necessitates the integration of control practices, including crop rotation, use of resistant cultivars, and good crop management (Stienstra and MacDonald, 1990). While the following subsections may not be directly relevant to

the goals of this dissertation, they are still helpful in putting the various strategies for SCN control into perspective.

A. Chemicals. In the past, nematicides were an integral part of SCN control strategy. Fumigants such as DBCP (1,2-dibromo-3-chloropropane) and EDB (ethylene dibromide) were considered as effective nematicides (Weaver *et al*, 1988). The success of nematicides in soybean is also dependent on the level of resistance (or tolerance) available in soybean cultivars. However, the combination of pesticide decertification and the relatively low current market value of soybean has resulted in a rapid decline in the use of nematicides as an SCN management strategy (Rodriguez-Kabana, 1992).

B. Biological control. The use of natural enemies to control nematodes is not a new concept; in fact, it has been studied for many years (Tribe, 1977, 1980; Morgan-Jones and Rodriguez-Kabana, 1987; Kerry, 1988; Carris and Glawe, 1989). Despite an intense search for appropriate biological agents, only a handful have shown promise for practical application. *Pasteuria penetrans* Sayre & Starr, a bacterial parasite, has always been considered as an excellent candidate due to its effectiveness, ability to withstand adverse conditions, and host specificity (Kim and Riggs, 1992). However, the potential usefulness of this obligatory parasite is limited by the inability to culture it on artificial media (Kim and Riggs, 1992). Extensive studies have also been conducted on six species of fungi: *Catenaria auxiliaris*, *Cylindrocarpon destructans*, *Fusarium oxysporum*, *Paeciliomyces lilacinus*, *Nematophthora gynophila*, and *Verticillium chlamydosporium* and one unidentified fungus, ARF18 (Kerry, 1988; Kim and Riggs, 1991). To date, no effective biological control agent has been found for SCN. Kim and Riggs (1992) have suggested that novel approaches, such as combinations of multiple agents or improvement of formulations, might have considerable potential for biological control of SCN.

C. Cultural management. Another strategy to manage SCN populations is the use of sound cultural practices, such as crop rotation, tillage, altered planting dates, irrigation,

fertilization and weed control. Hussey and Boerma (1989) suggested that the most effective crop rotation scheme involves a varying number of years of a nonhost crop, a resistant soybean cultivar, and a susceptible soybean cultivar. The number of years that a soybean field needs to be devoted to nonhost crop prior to a susceptible soybean crop is dependent on initial inoculum density in the soil, edaphic factors, location, and environment (Wrather *et al*, 1992). Tillage can also influence the population dynamics of *H. glycines* (Wrather *et al*, 1992). Cyst numbers were reported to be lower in no-till than in conventional-till soybean fields (Tyler *et al*, 1983; Koenning *et al*, 1990; Lawrence *et al*, 1990), although in some cases, SCN levels did not go down under reduced tillage conditions. Perhaps, the beneficial effects of reduced tillage require several years to be realized (Niblack, 1995). There has been some indication that late planting of soybeans lowers SCN populations (Hussey and Boerma, 1983; Koenning and Anand, 1991). However, the population level of *H. glycines* at harvest is sometimes higher in late-planted than in early-planted soybean (Hill and Schmitt, 1989). Despite the dearth of understanding on the effect of SCN infestation on plant nutrient deprivation, there has been some indication that adequate soil fertility can alleviate yield losses due to SCN (Ross, 1959; Luedders *et al*, 1979). Likewise, adequate soil moisture has also been recommended to reduce SCN damage (Young *et al*, 1986; Barker and Koenning, 1989). Johnson *et al* (1993) reported that SCN response depends on the water status of the soil and the level of infestation. For example, they reported that in years when rainfall is abundant during the growing season in Arkansas, no or little effect of SCN on soybean could be detected.

D. Tolerance. Tolerance in soybean to SCN is defined as minimal depression in yield and intolerance as high yield depression, whereas resistance to SCN denotes low nematode reproduction and susceptibility as high nematode reproduction when planted in SCN-infested fields (Cook, 1974). Boerma and Hussey (1984) and Anand and Koenning (1986) recommended the use of SCN-tolerant cultivars as an additional strategy to limit

soybean losses in SCN-infested fields. Some of the SCN-tolerant soybean lines that are available include: 'Coker 156', PI 97100, 'S79-8059' (Anand and Koenning 1986), and 'Wright' (Reese *et al*, 1986). Boerma *et al* (1986) proposed that the combination of high levels of tolerance and nematode resistance could increase the longevity and stability of a new cultivar against SCN. They added that tolerant cultivars are advantageous over resistant cultivars since they limit yield losses due to SCN in the absence of selection pressure on the nematode, pressures that can potentially lead to the development of new and more aggressive races. In addition, tolerance to SCN in soybean appeared to be race-independent (Anand and Koenning, 1986; Reese *et al*, 1986). For tolerance to be an effective control strategy, however, it must be integrated into a management program that complements crop rotation, resistance, sound cultural practices and biological control (Luedders, 1987).

Resistance to SCN

Just after the discovery of SCN on soybean in the USA, sources of resistance to a field population of SCN in North Carolina were sought and identified by Ross and Brim (1957). Some of these lines were immediately incorporated into breeding programs and Peking became the most widely used in crosses due to a lack of agronomically undesirable traits. Pickett, a selection from Peking, was the first SCN resistant cultivar released. Soon it became apparent that certain SCN populations could overcome resistant soybean cultivars, leading to the establishment of the race classification scheme (Golden *et al*, 1970). Higher levels of resistance to the new races of SCN were sought by Epps and Hartwig (1972); Thomas *et al* (1975); Anand and Gallo (1984); Hartwig (1985); Anand *et al* (1985, 1988); Hancock *et al* (1987) and Young (1990). The soybean genotype PI 437654 has drawn a lot of attention because of its remarkable degree of resistance to almost all SCN isolates tested. This soybean genotype may be potentially useful in cases where

changes in gene frequency of the nematode population (race shifts or breaks in resistance) occur in the field.

Genetics and inheritance of resistance. Classical genetic studies have demonstrated that inheritance of SCN resistance is oligogenic and complex. Early studies indicated that resistance appeared to be conditioned by at least five major genes: one dominant and four recessive genes (Caldwell *et al*, 1960; Matson and Williams, 1965; Ross and Brim, 1957; Hartwig and Epps, 1970; Thomas *et al*, 1975). The dominant gene, *Rhg4*, was found to be closely linked to the seed coat color locus (*i*) (Matson and Williams, 1965). The *i* locus in Peking was also reported to be linked with a recessive gene for resistance to SCN in Japan (Sugiyama and Katsumi, 1966).

Resistance against race 3 in particular, appears to be controlled by one dominant and two recessive genes in soybean lines Peking and PI 90763 (Rao-Arelli *et al*, 1992), whereas in PI 88788 resistance seems to be conditioned by one recessive and two dominant genes.

Resistance to race 4 in PI 90763 also appears to be conditioned by one dominant and two recessive genes (Thomas *et al*, 1975). In some crosses, resistance to race 4 has also been reported to be conditioned by a single dominant gene, whereas a single recessive gene was responsible for resistance to "Bedford Biotype" of SCN (Hancock *et al*, 1985). In studying the inheritance of resistance to the so-called 'race X' (later described as race 2), Hancock *et al* (1987) found that a single recessive gene appears to condition resistance in PI 90763. Myers *et al* (1988) reported that resistance to SCN race 5 in PI 437654, PI 90763 and Peking seems to be conditioned by two dominant and two recessive genes.

Analysis of genetic relationships has also revealed that resistance genes may be shared among sources of resistance to SCN race 3 (Rao-Arelli and Anand, 1988). Peking was found to have genes in common with PI 90763 and PI 438489, while PI 90763 has genes in common with PI 438489B, PI 404166 and PI 404198A (Rao-Arelli and Anand,

1988). Furthermore, Peking and PI 90763 appeared to have the same genes for resistance to race 3. Triantaphyllou (1975) stated that race 3 had no genes for parasitism of resistant cultivars, so resistance to race 3 appears to be necessary for resistance to any of the other races. This is further supported by the studies of Anand *et al* (1985, 1988) who found all lines resistant to race 4 or 5 were also resistant to race 3. However, Young (1990) reported that race 3 was able to parasitize three PIs that were highly resistant to race 5. Thus, it is likely that race 3 may actually have genes for parasitism of some resistant cultivars, contrary to the previous conclusion (Triantaphyllou, 1975).

Peking, PI 90763 and PI 437654 differ by at least one gene for resistance (Anand and Rao-Arelli, 1989) against race 5. However, one recessive and two dominant genes seemed to condition resistance in crosses between PI 437654 and PI 88788.

Results of this dissertation further supports the theory that resistance genes may be shared among sources of SCN resistance. In this study, a major partial SCN resistance locus on linkage group 'G' has been found to be common in PI 209332, PI 88788, PI 90763 and Peking.

Some researchers believe that more genes condition SCN resistance than have been described (Luedders, 1989). The reduced gene number identified to this point has been attributed to the heterogeneous cyst populations used in most investigations and inaccuracy in race identification brought about by the 10% rule (Caviness, 1992).

However, results of this dissertation indicated that inheritance of SCN resistance might not be as complex as most researchers believed. The identification of a major partial SCN resistance locus with a relatively large genetic effect on SCN disease response does not fit the polygenic theory of inheritance of SCN resistance. In addition, the relatively high estimates of heritability obtained in various studies, which will be discussed in the next subsection, does not fit a complex model of inheritance.

Estimates of heritability of SCN resistance. The heritability of resistance to SCN in soybeans has not received much attention. There have been only a few studies done to estimate heritability of this trait. Hancock *et al* (1987) estimated narrow sense heritability based on F₂:F₃ regression of female index means. Estimates were relatively uniform with heritability values from 61 to 64%. Reese *et al* (1988) reported that heritability estimates of tolerance to SCN race 3 averaged 19% and 26%. Mansur *et al* (1993) estimated the genetic effects in SCN race 3 resistance using six generation means (P₁, P₂, F₁, F₂, BCP₁, and BCP₂) involving three crosses with resistance from PI 88788 and Peking. The results indicated that an additive genetic model was sufficient to explain most of the variations with some degree of dominance. Results indicated that no more than four genes were involved in the inheritance of the trait. Broad sense heritability estimates on a single plant basis were relatively high, from 48% to 81%, indicating the possibility of selecting genotypes with intermediate level of resistance.

Breakdown of resistance and race shifts. One major problem with the continued use of resistant cultivars in agriculture is the strong selection pressure on SCN populations, potentially leading to an increase in the frequency of genetic variants that can overcome resistant cultivars (Zirakparvar and Norton, 1981; McCann *et al*, 1982; Young, 1984). These changes in the ability of SCN populations to reproduce on resistant soybean lines are assumed to be accompanied by changes in gene frequencies for parasitism (Young, 1984). To prevent widespread race shifts in SCN populations, researchers in the area recommend that growers employ genes from various sources of resistance (Rao-Arelli *et al*, 1989). One way to accomplish this is to rotate soybean cultivars with different genes for resistance, potentially reducing the risk of developing new virulent SCN populations, but also helping to maintain high yields (Luedders, 1983; Young, 1984).

Mechanisms of resistance. Proposed mechanisms of plant resistance to SCN include: inhibition of syncytium development (Acedo *et al*, 1984) and the formation of cell

wall thickenings to seal off the syncytium (Riggs *et al*, 1973). In addition, early syncytial degeneration (Endo, 1964) and necrosis of cells surrounding the underdeveloped juvenile nematodes in the absence of syncytial formation (Riggs *et al*, 1973) have also been proposed. However, Acedo *et al* (1984) found various degrees of necrosis in the compatible and incompatible soybean cultivars, suggesting that the necrotic reaction was not the sole barrier to parasite development. In other host plants infected by other species of cyst nematodes, the mechanisms of resistance also varied and depended on host-parasite associations (Cook, 1974; Wyss *et al*, 1984; Yu and Steele, 1980).

Genetic markers and gene mapping

Conventional markers and mapping theory

Gene mapping is the process of determining a gene's position relative to other genes and genetic markers through linkage analysis. The basic principle for linkage mapping is that the closer together two genes are on the chromosome, the more likely they are to be inherited together (Rothwell, 1988). The process is relatively straightforward. Briefly, a cross is made between two genetically compatible but divergent parents relative to traits under study. Genetic markers are then used to follow the segregation of traits under study in the progeny from the cross (often a backcross, F₂, or recombinant inbred population). Linkage analysis is based on the level at which markers and genes are co-inherited (Rothwell, 1988). Simple statistical tests like chi-square analysis can be used to test the randomness of segregation or linkage (Kochert, 1989). In linkage mapping, the proportion of recombinant individuals out of the total mapping population provides the information for determining the genetic distance between the loci (Young, 1994).

Classical mapping studies utilize easily observable, visible traits also known as naked eye polymorphisms (NEPs). These traits can be morphological like plant height, fruit size, shape and color or physiological, like disease response, photoperiod sensitivity

or crop maturity. NEPs are very useful and are still in use because they represent actual phenotypes and are easy to score without any specialized lab equipment. By contrast, the other types of genetic markers are important only as arbitrary loci for use in linkage mapping and often not associated to specific plant phenotypes (Young, 1994). However, most morphological markers cause such large effects on phenotype that they are undesirable in breeding programs. Many other NEPs have the disadvantage of being developmentally regulated (i.e. expressed only certain stages; or at specific tissues and organs). Oftentimes, NEPs mask the effects of linked minor genes making it nearly impossible to identify desirable linkages for selection (Tanksley *et al*, 1989).

Restriction fragment length polymorphisms (RFLPs) and quantitative trait loci (QTLs)

Although a number of important agronomic characters are controlled by loci having major effects on phenotype, most economically important traits, such as yield, quality and some forms of disease resistance, are quantitative in nature. This type of phenotypic variation in a trait is characterized by continuous, normal distribution of phenotypic values in a particular population (Beckmann and Soller, 1986). Such traits are governed by a large number of loci often termed as "quantitative trait loci" (QTL), each of which can make a small positive or negative effect on the final phenotypic value of the trait (Beckmann and Soller, 1986). Loci contributing to such genetic variation are often termed, 'minor genes', as opposed to major genes with large effects that follow a Mendelian pattern of inheritance. Polygenic traits are also predicted to follow a Mendelian type of inheritance, however the contribution of each locus is expressed as an increase or decrease in the final trait value. Thus, it is often not possible to determine how many loci are involved nor distinguish their individual effects (Beckmann and Soller, 1986). More importantly, it is generally not possible to distinguish the effect of genetic factors from those of environmental factors.

The advent of DNA markers, such as restriction fragment length polymorphisms (RFLPs) and random amplified polymorphic DNAs (RAPDs), has allowed the resolution

of complex, multigenic traits into their individual Mendelian components (Paterson *et al*, 1988). RFLP analysis is based on DNA fragment length differences between individuals after their total DNA is cut with restriction enzymes. Changes in the location of restriction sites between individuals, either due to base pair substitutions or insertions or deletions, provide alternate alleles or polymorphisms that can be used in segregation analysis for gene mapping (Tanksley *et al*, 1989). These polymorphisms are detected by separating the DNA fragments by gel electrophoresis, transferring the fragments onto a nylon membrane and hybridizing a radioactively-labeled probe to the specific DNA fragment of interest (Southern, 1975). RFLPs are particularly useful as genetic markers in breeding and linkage mapping because of their important properties of codominance, absence of pleiotropy and the lack of environmental influence (Walton and Helentjaris, 1988). In addition, RFLP probes can detect variation in both coding and non-coding DNA sequences, and thus have the potential to provide virtually unlimited numbers of polymorphisms (Soller and Beckmann, 1988). A number of applications of RFLPs have been suggested for plant breeding. Among the potential applications for RFLPs in plant breeding include: varietal identification (Soller and Beckmann, 1983; Tanksley *et al*, 1989), QTL mapping (Edwards *et al*, 1987; Nienhuis *et al*, 1987; Osborn *et al*, 1987; Romero-Severson *et al*, 1989; Young *et al*, 1988; Martin *et al*, 1989; Sarfatti *et al*, 1989; Tanksley *et al*, 1989; Barone *et al*, 1990; Jung *et al*, 1990; Keim *et al*, 1990a, 1990b; Paterson *et al*, 1990; Martin *et al*, 1991; Messeguer *et al*, 1991; Michelmore *et al*, 1991; Ottaviano *et al*, 1991; Yu *et al*, 1991; Diers *et al*, 1992a, 1992b, 1992c; Doebley *et al*, 1990), screening genetic resource strains for useful quantitative trait alleles and introgression of these alleles into commercial varieties (Beckmann and Soller, 1983; Tanksley *et al*, 1989), marker-assisted selection (Tanksley *et al*, 1989) and map-based cloning (Tanksley *et al*, 1989). In addition, DNA markers can be used to obtain information about: (1) the number, effect, and chromosomal location of each gene affecting a trait; (2) effects of multiple copies of

individual genes (gene dosage); (3) interaction between/among genes controlling a trait (epistasis); (4) whether individual genes affect more than one trait (pleiotropy); and (5) stability of gene function across environments (G x E interactions) (Allen, 1994).

QTL mapping in soybean and other crops

Early studies using DNA markers to analyze QTL, primarily focused on agronomic and morphological characters in plants. In maize (*Zea mays* L.), QTLs contributing to heterosis in several quantitative traits have been mapped (Stuber *et al*, 1992), as well as QTLs for heat tolerance (Ottaviano *et al*, 1991) and morphological characters distinguishing maize from teosinte (*Zea mays* ssp. *mexicana*) (Doebley *et al*, 1990). In tomato (*Lycopersicum esculentum* Mill.), RFLPs have been used in locating and determining effects of QTLs associated with fruit size, pH, soluble solids (Paterson *et al*, 1990) and water use efficiency (Martin *et al*, 1989). In soybean, QTLs for seed size, seed weight, seed oil, protein and fatty acid content, maturity, hard seededness (Keim *et al*, 1990a, 1990b; Diers and Shoemaker, 1992; Diers *et al*, 1992b; Mansur *et al*, 1993), iron deficiency (Diers *et al*, 1992c), and supernodulation (Landau-Ellis *et al*, 1991) have also been identified using molecular markers.

In the past, the use of DNA genetic markers in studying plant disease resistance has mainly focused on single locus resistance genes such as the *Tm-2* (Young *et al*, 1988) and *Tm-1* (Levesque *et al*, 1990) resistance loci to tomato mosaic virus, *Pto* locus for *Pseudomonas syringae* pv. tomato Okabe (Martin *et al*, 1991), *I₂* (Sarfatti *et al*, 1989) loci for *Fusarium oxysporum* Schlect f. sp. *lycopersici* Sacc., *Sm* locus for *Stemphylium* (Behare *et al*, 1990), *Cf-2* and *Cf-9* for *Cladosporium fulvum* resistance loci in tomato (Jones *et al*, 1991). Other resistance loci include *Dm* (*Bremia lactucae* Regel) in lettuce (*Lactuca sativa* L.) (Michelmore *et al*, 1991), leaf blast (*Magnaporthe grisea* Barr) resistance in rice (*Oryza sativa* L.) (Yu *et al*, 1991) and *Rps* for *Phytophthora sojae* (Kauf. and Gende.) resistance in soybean (Diers *et al*, 1992a). Similarly, single gene resistances

for nematode pests have been identified such as the *Mi* resistance gene to the nematode *Meloidogyne incognita* (Kofoid and White) in tomato (Messeguer *et al*, 1991), *Heterodera schachtii* (Schmidt) in sugar beets (*Beta vulgaris L.*) (Jung *et al*, 1990) and *Globodera rostochiensis* (Woll) in potato (*Solanum tuberosum L.*) (Barone *et al*, 1990).

Now, the use of DNA markers has been expanded to studies of oligogenic or polygenic forms of resistance in plants. Studies that have been carried out include: powdery mildew (*Erysiphe polygoni* D.C.) resistance in mungbean [*Vigna radiata* (L.) R. Wilcz.] (Young *et al*, 1993), glandular trichomes, an insect resistance trait in tomato (Nienhuis *et al*, 1987), maize dwarf mosaic virus resistance in maize (Romero-Severson *et al*, 1989), bacterial wilt (*Pseudomonas solanacearum* E.F. Smith) resistance in tomato (Danesh *et al*, 1994) and SCN (Concibido *et al*, 1994) in soybean. DNA markers have also been used in mapping genes underlying complete and partial resistance to rice blast (*Pyricularia oryzae* Cavara) (Wang *et al*, 1994) and in assessing race specificity against late blight (*Phytophthora infestans*) in potato (Leonards-Schippers *et al*, 1994). Contrary to the general belief that partial resistance is race nonspecific (Vanderplank, 1968), Leonards-Schippers *et al* (1994) identified race-specific QTLs conferring partial resistance against *P. infestans*. In support of this, race-specific QTLs for partial resistance to SCN were also found in this study and will be described later in Chapter 2 of this dissertation.

High-density maps

With the goal of physical mapping and ultimately cloning of QTLs, high-density RFLP and allozyme linkage map development are imperative (Darvasi *et al*, 1993). The success of map-based positional cloning of the *Pto* gene in tomato (Martin *et al*, 1993) and the *RPS2* gene in *Arabidopsis* (Bent *et al*, 1994) has been realized through the development of high density physical-genetic maps for these two species.

In studying disease resistance genes, high-resolution RFLP mapping is important for two reasons: (1) molecular markers flanking such genes can be used to increase the efficiency of simultaneous introgression of several targeted chromosome segments, (2) RFLP markers, tightly linked to genes of interest, can be used as starting points for physical mapping and chromosome walking aimed at cloning the gene (Sarfatti *et al*, 1991). Both of these applications require precise mapping of the factors responsible for resistance.

The construction of high density maps is typically constrained by the lack of informative markers in the genomic region near the QTL, in part, because of the limited DNA polymorphism. In the next section, the use of comparative mapping, one of the more powerful applications of DNA markers that utilizes mapping information from one plant taxon to predict the properties of another, will be described. This strategy can be used to potentially increase marker density near genomic regions of interest.

Comparative genome mapping

The process of genome evolution suggests that large chromosomal blocks have been reshuffled during speciation to reconstitute the current set of chromosomes found in present-day species (Beckmann and Soller, 1989). Despite the evolutionary reshuffling, much of the genomic structure is likely to be basically conserved. This means that the genes that are close to each other in one species are more likely to have maintained this relationship in other species as well (Beckmann and Soller, 1989). This is likely to apply even for the relatively plastic plant genomes, as evidenced by the synteny reported in tomatoes and potatoes (Bonierbale *et al*, 1988). Furthermore, the extensive gene duplication and gene order conservation of the maize genome (Helentjaris *et al*, 1988) can also be taken as suggestive evidence for the maintenance of synteny despite the scrambling forces acting upon plant genomes (Tanksley *et al*, 1992).

The use of DNA markers allows the comparison of homology and marker order in reproductively isolated plant taxa. When co-segregating homologous DNA sequences

(markers) are also in the same order in two distinct taxa, this suggests that the chromosomal segment defined by those markers has been partially or fully conserved since the divergence of the taxa (Young, 1994). This approach allows map construction in a related species by utilizing a common set of molecular markers as hybridization probes. Using this approach, many studies in plant genome organization and evolutionary relationships in several plant species have been conducted (Song *et al*, 1990; Gawel *et al*, 1992; McGrath and Quiros, 1992; Wang *et al*, 1992; Wilde *et al*, 1992). For example, in the Gramineae, large genomic segments of rice and maize are nearly identical with respect to gene order based on single copy sequences (Ahn and Tanksley, 1993). Likewise, parallel results have been obtained in comparative mapping studies of maize and sorghum (Hulbert *et al*, 1990; Pereira *et al*, 1993; Whitkus *et al*, 1992). In the Solanaceae, Tanksley *et al* (1992) reported that except for five inversions, the genomes of potato and tomato are nearly identical. Prince *et al* (1992) reported that in pepper and tomato, many loci have been found on conserved linkage groups, although significant rearrangements in gene order have been detected. Frequent chromosomal rearrangement and conserved loci on common linkage groups has also been observed in species of *Brassica* (Kianian and Quiros, 1992; Slocum *et al*, 1990). Comparative mapping between mungbean and cowpea [*V. unguiculata* (L.) Walp.] revealed that nucleotide sequences are conserved but copy number can vary and several rearrangements in linkage occurred since the divergence of the two species (Menancio-Hautea *et al*, 1993). Furthermore, entire linkage groups were not conserved, but several large linkage blocks were maintained in both genomes (Menancio-Hautea *et al*, 1993). Boutin *et al* (1995) assessed linkage conservation and collinearity among genomes of mungbean, common bean (*Phaseolus vulgaris* L.) and soybean using 219 DNA clones. A high degree of linkage conservation and synteny was observed between mungbean and common bean, which are both members of the subtribe Phaseolinae. By contrast, only short and scattered linkage blocks were conserved between

soybean and common bean, as well as between soybean and mungbean. However, it may be noteworthy that the seemingly small conserved blocks from soybean in the mungbean or common bean genomes occasionally represented very large segments in soybean (Boutin *et al*, 1995).

Knowledge about genome conservation among different plant species can potentially be used to increase marker density in regions of interest. One can possibly utilize the markers that are made available in one species with a high density of markers in a region of interest to populate the map of a species with relatively low density of markers in the same region (Boutin *et al*, 1995). This approach will be demonstrated in Chapter 2 of this dissertation, wherein additional DNA markers originating from mungbean and common bean (based on the study by Boutin *et al*, 1995) were used to increase marker density near a major partial SCN locus.

Integrated mapping

Another strategy to develop high density maps within the same species is to integrate linkage maps that were developed independently. This mapping approach allows genes that do not segregate in a single mapping population to be placed onto a single map by combining information from multiple crosses. At present, separate RFLP maps developed by various laboratories are available for some well-studied organisms such as maize (Coe *et al*, 1990), *Arabidopsis thaliana* (Chang *et al*, 1988; Nam *et al*, 1989) and soybean (Shoemaker and Olson, 1993). The number of markers on these maps is increasing at a very fast rate (Stam, 1993). However, in most cases, generating many different detailed maps from different crosses is neither necessary nor practical. A more efficient approach is to develop a detailed standard map from one or a few crosses and then superimpose small detailed regions generated from other crosses onto a standard map (Stam, 1993). Realizing the need to integrate maps to support the current global efforts in genome mapping, Stam (1993) developed a computer software, *JoinMap*, that enables the

construction of integrated genetic maps. *JoinMap* can handle data from F₂, backcross and recombinant inbed line populations. The key factor in the integration of distinct maps is the availability of markers that are common among the maps (Stam, 1993). Despite the availability of common markers, problems such as variability in the source and type of mapping information among data sets are often encountered in the alignment of distinct maps (Stam, 1993; Shoemaker and Specht, 1995). Thus, the ability to come up with the best joint map is limited by the statistical approaches available (Stam, 1993).

Marker-Assisted Selection (MAS)

One very powerful application of DNA markers is the indirect selection for traits which are expensive to score, difficult to evaluate phenotypically, or highly affected by environmental factors (Nienhuis *et al*, 1987). This deterministic type of selection, which is based on genotype rather than phenotype, is known as "marker-assisted selection" (MAS). The concept of MAS is not new. As early as 1923, Sax (1923) used a seed coat color locus to predict variation in seed size, a quantitative trait. He then proposed the identification and selection for minor genes by linkage with major genes, which are easily scored. MAS can dramatically increase genetic progress by increasing accuracy of selection, reducing generation interval, and by breaking undesirable linkages (Soller, 1978; Smith and Simpson, 1986). This can be accomplished by positive selection for markers tightly linked to the gene of interest and negative selection against marker alleles that come from elsewhere on the donor parent genome (Tanksley *et al*, 1989). The potential efficiency of MAS on a single trait using a combination of molecular and phenotypic information compared to standard methods of phenotypic selection depends on the heritability of the trait, the proportion of additive genetic variance associated with the marker loci, and the selection strategy (Lande and Thompson, 1990). However, the effectiveness of MAS is greatly reduced by lack of polymorphism at the marker locus (Dekkers and Dentine, 1991). This problem can be ameliorated in a breeding program by

occasional hybridization of genetically differentiated lines. Finally, for MAS to be practical, it should be less expensive or tedious than direct selection of the trait (Diers *et al*, 1992a). In Chapter 2 of this dissertation, the efficiency and cost-effectiveness of DNA marker-based selection was compared with phenotype-based selection in breeding SCN resistant soybean lines.

Physical mapping

Although the goal of this dissertation is not map-based cloning (Tanksley *et al*, 1989), it is still valuable to discuss the key steps involved in this cloning strategy. First, DNA markers tightly linked to a gene of interest are identified. Once a high density genetic map is developed, the next step in map based cloning is physical mapping. Despite the vital information provided by segregation analysis on the orientation and genetic distance between RFLP markers in terms of recombination frequency (i.e. centimorgans), it is still imperative to know the actual physical distance between RFLPs flanking a target gene in terms of nucleotides (Young, 1994; Wu and Tanksley, 1993). The importance of physical mapping is exemplified by the fact that RFLPs that appear to be very close to a gene in terms of genetic distance may still be physically far away (Ganal *et al*, 1989). Thus, it is essential to know the magnitude of the physical distance between RFLPs before starting chromosome walking and map-based cloning. It was only recently that detailed characterization of large DNA molecules for map-based cloning has been possible. Normal agarose gel electrophoresis can only separate DNA molecules up to 50 kilobases (kb), while map-based cloning requires distances up to one megabase (Mb) or more. Pulsed field gel electrophoresis now provides a means to separate DNA molecules up to 10 Mb in size (Anand, 1986; Schwartz and Cantor, 1984). In contrast to genetic mapping, physical mapping is independent of fragment length or primer site polymorphisms (Schwartz and Cantor, 1984). With physical mapping, the precise order of DNA markers can be determined in the absence of recombination events among them (Botstein *et al*, 1980),

although the mapping of phenotypic markers on a physical map still requires recombination data. Physical mapping of large genomic regions using closely-linked RFLP markers has been extensively carried out in mammalian systems (Kenrick *et al*, 1987; Lawrence *et al*, 1987). PFGE now provides a powerful tool for studies of genome organization and in physical isolation of specific genomic regions for map-based cloning in plants (Ganal *et al*, 1989; Honeycutt *et al*, 1992; Funke *et al*, 1993; Kleine *et al*, 1993; Wu and Tanksley, 1993; Funke and Kolchinsky, 1994; Xiaozhu *et al*, 1995; Danesh *et al*, 1995). In soybean, PFGE analysis has been used to examine various chromosomal regions including: nodulin regions (Honeycutt *et al*, 1992), genomic regions containing duplicated sequences (Funke *et al*, 1993), and the SCN disease resistance region (Danesh *et al*, 1995).

Complexity of the soybean genome

Despite the availability of powerful tools for map-based cloning, the rather unusual characteristic of the soybean genome may complicate efforts toward gene mapping, especially QTLs. To understand the complexity of the soybean genome, it is imperative to review its evolutionary development.

Soybean belongs to the genus *Glycine* Wild, family Leguminosae, subfamily Papilionoidae and tribe *Phaseoleae*. It has been estimated that for a haploid (1n) DNA content, soybean contains from 1.29×10^9 base pairs (bp) (Gurley *et al*, 1979) to 1.81×10^9 bp (Goldberg, 1978). The genome consists of about 40-60% repetitive sequences (Goldberg, 1978; Gurley *et al*, 1979), the majority of which contain short period interspersion with single copy sequences of 1.1-1.4 kb alternating with repetitive sequence elements of 0.3-0.4 kb (Gurley *et al*, 1979). Unlike most genera of the Phaseoleae with a genome complement of $2n = 22$, soybean has a diploid chromosome number of $2n = 40$. This unusual genome of soybean led Lackey (1980) to theorize that *Glycine* was probably derived from a diploid ancestor ($n = 11$) that had an aneuploid loss ($n = 10$). Subsequent

polyploidization resulted to the current $2n = 2x = 40$ genome size (Palmer and Kilen, 1987). Genetic evidence of gene duplication emphasizes suggestions that soybean is a polyploid (Buttery and Buzzell, 1976; Palmer and Kilen, 1987). Thus, soybean should be regarded as a diploidized tetraploid (Hymowitz and Singh, 1987; Apuya *et al.*, 1988). This is further supported by many examples in soybean of qualitative traits controlled by two loci (Zobel, 1983; Palmer and Kilen, 1987). Despite this evidence, Keim *et al* (1989) suggested that the duplicated nature of RFLP loci observed in soybean falls into a pattern inconsistent with a tetraploid origin. They postulated that the duplications may be caused by mechanisms other than polyploidy. For example, some sets of duplicated loci reside on the same linkage group. This would be consistent with 'gene clustering' such as that observed in mammalian globin genes (Maniatis *et al.*, 1980).

Regardless of the origin and cause of duplicity in the soybean genome, this situation poses many difficulties in gene mapping and cloning. For example in comparing gene synteny between species, the high proportion of duplicated genes creates a lot of difficulty in the assignment of orthologous loci (i.e. correct or proper order and position of marker-loci among species). In most cases, it would not be clear which fragments represent the same gene in different species and which fragments were duplicated prior to speciation. In addition, the unusually large genome size and the high degree of repetitive sequences present in soybean (Bennet *et al.*, 1982; Goldberg, 1978) further complicate efforts to accomplish map-based cloning.

Summary and Perspectives

The increasing threat of SCN in soybean production has led to a tremendous amount of research aimed at better understanding this complex host-parasite interaction. Several control strategies are currently used singly or in combination to effectively manage

SCN populations in the field. By far, host plant resistance is still the most widely-used due to its cost-effectiveness. Despite many attempts to better understand SCN resistance using conventional strategies, oftentimes, results have been confusing and ambiguous due to the complex genetics in both the host and the pathogen. Thus, our knowledge about this complex host-parasite interaction is still fragmentary.

The development of molecular tools offers novel approaches that can improve our understanding, not only of host plant resistance but also parasitism in the pathogen, of the soybean-SCN system. For example, the identification and characterization of a major QTL for partial resistance to SCN, described in this dissertation, can accelerate the process of breeding SCN resistant varieties since selection can now be based on genotype rather than phenotype. The prospect of MAS in SCN resistance breeding can potentially eliminate the need for tedious and difficult SCN tests. In addition, DNA markers, such as sequence-tagged sites (STSS) (Olson *et al*, 1989) and RAPDs, can also be used to better characterize SCN races and potentially replace the controversial SCN race scheme. The successful map-based cloning of disease resistance genes in *Arabidopsis* (Bent *et al*, 1994) and tomato (Martin *et al*, 1993) provides inspiration for the eventual cloning of the major partial SCN resistance gene. The ultimate cloning of this gene might require a slightly different approach because of basic problems, such as the lack of DNA polymorphism near the region of interest and the complexity of the soybean genome. However, the availability of various gene mapping strategies, including comparative genome analysis and integrated mapping, can help overcome these limitations, as described in Chapter 3 of this dissertation. In conclusion, advances in molecular technology will further enhance our understanding of this complex host-parasite interaction and will open up opportunities towards the study of other intriguing aspects of SCN research.

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Table 1. Classification of races of *Heterodera glycines* by means of host differentials
 (Riggs and Schmitt, 1989).

Race	Pickett	Peking	PI 88788	PI 90763
1	-	-	+	-
2	+	+	+	-
3	-	-	-	-
4	+	+	+	+
5	+	-	+	-
6	+	-	-	-
7	-	+	-	-
8	-	-	-	+
9	+	+	-	-
10	+	-	-	+
11	-	+	+	-
12	-	+	-	+
13	-	-	+	+
14	+	+	-	+
15	+	-	+	+
16	-	+	+	+

†+ = Number of females and cysts recovered is greater than or equal to 10% of the number on the susceptible check; - = number of females and cysts recovered is less than 10% of the number on the susceptible check.

CHAPTER 1

DNA MARKER ANALYSIS OF LOCI UNDERLYING RESISTANCE TO SOYBEAN CYST NEMATODE

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ABSTRACT

Inheritance of resistance to soybean cyst nematode (SCN; *Heterodera glycines* Ichinohe) is oligogenic and complex. DNA markers, such as restriction fragment length polymorphisms (RFLPs), can be used to identify loci associated with complex genetic characters, including SCN resistance. To uncover these associations, 56 F₂ lines from a cross between a susceptible (M83-15) and a resistant (M85-1430) soybean [*Glycine max* (L.) Merrill] inbred line were mapped for DNA marker polymorphisms. F₃ lines derived from these F₂ individuals were assayed for SCN disease response in the greenhouse to a field isolate of SCN from Minnesota that tested as race 3. The F₂ genotypic classes for each of 43 DNA markers were then contrasted with SCN disease response to identify marker loci associated with SCN resistance. Two RFLP markers, A85 and B32, were found to be significantly associated with SCN disease response, together accounting for 51.7% of total phenotypic variation. Based on RFLP analysis, individual F_{2:3} lines that retained either one or both resistance loci were identified. Lines that possess both resistance loci can be valuable in developing resistant soybean lines free of linkage drag, while lines that have just one of the resistance loci may be useful as single-gene differentials for SCN race determination.

INTRODUCTION

The soybean cyst nematode (SCN), *Heterodera glycines*, is one of the most destructive pests of soybean [*Glycine max* (L.) Merrill]. To date, host plant resistance has been the most effective control measure, but is oligogenic in nature and genetically complex. Classical genetic studies indicate that resistance appears to be conditioned by at least five major genes: one dominant and four recessive genes, each with multiple alleles (Caldwell *et al*, 1960; Matson and Williams, 1965; Ross and Brim, 1957; Thomas *et al*, 1975). In particular, resistance against race 3 appears to be controlled by one dominant and two recessive genes in soybean lines 'Peking' and Plant Introduction (PI) 90763 (Rao-Arelli *et al*, 1992). Analysis of genetic relationships has also revealed that resistance genes may be shared among sources of resistance to SCN race 3 (Rao-Arelli and Anand, 1988). Breeding for SCN resistant soybean lines is further complicated by the high level of genetic variability that exists among field populations of SCN, along with the difficulty involved in screening soybean germplasm and in SCN race determination. Thus, conventional breeding for SCN resistance is time-consuming and difficult.

Advances in biotechnology now make it possible to map agriculturally important genes, including SCN resistance genes, with DNA markers such as restriction fragment length polymorphisms (RFLPs). Using DNA markers, it will be possible to rapidly identify resistant soybean lines based on genotype instead of phenotype. This would eliminate actual nematode tests and may enable the development of lines with a minimum of linkage drag. Potentially, individual lines containing a combination of different types of SCN resistance genes could also be created.

In the past, the use of DNA markers to study quantitative trait loci (QTL) has primarily focused on agronomic and morphological characters in plants. RFLPs have been utilized in locating and determining effects of QTLs associated with fruit size, pH, soluble

solids (Paterson *et al*, 1990) and water use efficiency (Martin *et al*, 1989) in tomato (*Lycopersicum esculentum* Mill.). QTLs contributing to heterosis in several quantitative traits have been mapped in maize (*Zea mays* L.) (Stuber *et al*, 1992), as have QTLs for heat tolerance (Ottaviano *et al*, 1991) and morphological characters distinguishing maize from teosinte (*Zea mays* ssp. *mexicana*) (Doebley *et al*, 1990). In soybean, QTLs underlying several reproductive and morphological traits (Keim *et al*, 1990b) and hard-seededness (Keim *et al*, 1990a) have also been identified using molecular markers.

However, in studying disease resistance in plants, the use of DNA genetic markers has been primarily focused on single locus resistance genes such as *Tm2* (tomato mosaic virus), *Pto* (*Pseudomonas syringae* pv. tomato Okabe), and *I2* (*Fusarium oxysporum* Schlect f. sp. *lycopersici* Sacc.) resistance loci in tomato (Young *et al*, 1988; Martin *et al*, 1991; Sarfatti *et al*, 1989), *Dm* (*Bremia lactucae* Regel) resistance loci in lettuce (*Lactuca sativa* L.) (Michelmore *et al*, 1991), leaf blast (*Magnaporthe grisea* Barr) resistance in rice (*Oryza sativa* L.) (Yu *et al*, 1991) and the *Rps* loci for *Phytophthora sojae* (Kauf. and Gende.) resistance in soybean (Diers *et al*, 1992). Similarly, single gene resistances for nematode pests have been identified such as *Mi* resistance gene to the nematode *Meloidogyne incognita* (Kofoid and White) in tomato (Messeguer *et al*, 1991), *Heterodera schachtii* (Schmidt) in sugar beets (*Beta vulgaris* L.) (Jung *et al*, 1990) and *Globodera rostochiensis* (Woll) in potato (*Solanum tuberosum* L.) (Barone *et al*, 1990).

The use of DNA markers has not been widely utilized in studies of oligogenic or polygenic forms of resistance in plants. A few of these studies that have been carried out include: powdery mildew (*Erysiphe polygoni* D.C.) resistance in mungbean [*Vigna radiata* (L.) R. Wilcz.] (Young *et al*, 1993), glandular trichomes, an insect resistance trait in tomato (Nienhuis *et al*, 1987), and maize dwarf mosaic virus resistance in maize (Romero-Severson *et al*, 1989).

This chapter demonstrates the utility of RFLP genetic markers in identifying genomic regions contributing partial resistance to SCN and considers the implications of DNA markers in genetic dissection and marker-assisted breeding for SCN resistance in soybean.

MATERIALS AND METHODS

Plant materials

The segregating F₂ population was constructed by crossing two contrasting soybean lines, M85-1430 carrying resistance originally from PI 209332 (Anand and Brar, 1983; Anand and Gallo, 1984), and the susceptible line M83-15, which has no known resistance in its background, and advancing the cross to the F₂ generation. Relative to 'Essex', the susceptible parent in this cross had nearly the same level of susceptibility (Table 1). Briefly, M85-1430 is a selection from L77-906 (a selection from 'Williams' x PI 209332) x L78-189 (a selection from 'Corsoy'(8) x 'Kingwa'). The susceptible parent, M83-15, was derived from the cross between 'A2' and 'Hodgson 78'. Fifty-six F₂ individuals, together with the parents, were grown in a greenhouse in St. Paul, Minnesota and used as source of leaf tissue for DNA extraction and DNA marker analysis. Plants were allowed to recover and set F₃ seeds, which were saved for SCN disease assay. Data were also collected from each F₂ plant for mean 30-seed weight and hilum color.

DNA clones

The source of putative RFLP markers was a soybean genomic library, prepared by digestion of total genomic DNA with the methylation-sensitive restriction enzyme, *Pst*I, and ligated to the bacterial plasmid, pBS+ (Stratagene, La Jolla, CA). This library was provided by Dr. Randy Shoemaker (Iowa State University, Ames, IA).

Plant DNA extraction, restriction digestion, electrophoresis and blotting

Plant DNA was extracted by the modified method of Dellaporta *et al* (1983) and analyzed following the technique of Young *et al* (1992). First, the best restriction enzyme for each DNA clone was determined. Individual clones were probed against "Parental survey" blots which consisted of parental DNA digested with fifteen different restriction enzymes (*Ban*I, *Bam*H I, *Bc*II, *Bst*nI, *Dde*I, *Dra*I, *Eco*RI, *Eco*RV, *Hae*III, *Hind*III, *Hinf*I, *Rsa*I, *Sca*I, *Taq*I, and *Xba*I) (New England Biolabs, Beverly, MA; Promega, Madison WI; Gibco/BRL, Gaithersburg, MD), followed by 1% agarose gel electrophoresis, and transfer onto Hybond N+ membranes (Amersham Corporation, Arlington Heights, IL) using a method adapted from Southern (1975). The best clone/enzyme combination was used for segregation analysis. This involved probing F₂ "progeny" blots containing digested DNA from all 56 F₂ individuals with the corresponding DNA clones.

DNA hybridizations

Cloned DNA inserts were amplified by the polymerase chain reaction (Saiki *et al*, 1988) for use in radiolabeling reactions and nucleic acid hybridization as described by Young *et al* (1992). Briefly, lysates from colonies of individual DNA clones were prepared from individual bacterial colonies and a sample of 2 µl of the supernatant was then used in a polymerase chain reaction to amplify the insert sequence using oligonucleotide primers flanking the insert sequence. About 40 to 50 ng of the amplified product was then radiolabeled by the random hexamer reaction (Feinberg and Vogelstein, 1983) and incubated with either a "Parental" survey or F₂ "progeny" blot as described by Young *et al* (1992).

Random amplified polymorphic DNA (RAPD) analysis

The RAPD analysis was carried out by a method adapted from Williams *et al* (1990). First, primers that give clear, polymorphic, and unambiguous bands were determined. This involved amplifying 25 ng of DNA from the parents and five F₂ lines in a 25 µl solution of 1 mM deoxynucleotide triphosphates (dNTPs), 50 mM KCl, 1.5 mM MgCl₂, 0.5% TWEEN-20, 15 ng of a 10-base primer (Operon Technologies, Alameda, CA) and 1.25 units Taq DNA Polymerase (Promega, Madison, WI) per reaction. Individual reactions were thoroughly mixed, overlaid with mineral oil and placed in a Coy thermal cycler (Coy Laboratory Products, Inc., Ann Arbor, MI). The thermal cycle profile consisted of an initial soak at 94 °C (45 sec) and then followed by 45 thermal cycles of 94 °C (45 sec), 35 °C (1 min) and 72 °C (90 sec). The RAPD products were resolved on 1.4% agarose gels, stained with ethidium bromide and visualized with ultraviolet light. The best primers were then used to amplify DNA samples for all 56 individuals to generate segregation data for mapping analysis.

Soybean cyst nematode assay

F₃ seedlings were germinated and transplanted either to pots on greenhouse bench or to Ray Leach cone-tainers (Stuewe & Sons, Inc., Portland, OR) in a waterbath. A total of 25 F₃ seedlings for each F₂ line was assayed, 13 by the bench method and 12 in the waterbath. Each plant was inoculated with 1,000 eggs at transplanting and again three days later of an SCN isolate originally isolated in a field in southern Minnesota in the summer of 1990. This isolate was maintained in soil in 6-inch pots that were continuously planted to susceptible checks, Evans, and Essex. Cysts and white females were then collected every 28 days, stored in sand at 4 °C and later used for inoculations.

Race determination tests were regularly conducted on this same isolate to check the possibility of race shifts. In a series of eight experiments done on the same isolate,

including the F₂ population nematode assay, the same isolate tested as race 3 in all experiments (Table 1). According to the present race determination scheme, a susceptible host reaction (+) is declared if the number of females and cysts recovered is greater than or equal to 10% of the number on the susceptible check; a resistant reaction (-) if the number of females and cysts recovered is less than 10% of the number on the susceptible check (Riggs and Schmitt, 1989).

For this study, the parents, as well as the soybean differential lines (Peking, 'Pickett', PI 90763, and PI 88788), and susceptible checks (Evans and Essex) were included in both the bench and waterbath tests. The experiment was laid out in a completely randomized design. Soil temperatures were maintained at 28 °C with 16-hour daylength for 28 days. On the 28th day, individual plants were uprooted and cysts were collected by blasting the roots with pressurized water and collecting the cysts on sieves. The total number of cysts from individual plants was counted under a dissecting microscope and converted to an index by dividing this number by the average total number of cysts on the susceptible parent. For each F₂ line, SCN indices of the twenty-five F₃ plants were averaged to estimate mean SCN index for that line.

Mapping quantitative resistance loci

To uncover genomic regions associated with SCN resistance, the F₂ genotypic classes for each DNA marker were contrasted with SCN disease response using regression analysis and analysis of variance (ANOVA). These analyses were performed using Statview-II and SuperAnova (Abacus Concepts, Berkeley, California). A significant association between a DNA marker and SCN disease response was declared if the probability was equal or less than 0.002 to minimize the detection of false positives experiment-wide (Lander and Botstein, 1989). Data were also analyzed by Mapmaker-QTL (Lander *et al*, 1987). Putative QTLs were inferred whenever the LOD (\log_{10} of the

odds ratio) score exceeded 2.5. Statview-II and SuperAnova were also used to perform two-factor analysis of variance of all marker loci tested in this study in order to determine if there were significant interaction among loci. A significance level of $P < 0.001$ was used to uncover potential interactions.

RESULTS AND DISCUSSION

Status of soybean RFLP linkage map

A total of 126 DNA clones and 60 RAPD primers were surveyed for polymorphisms, but only 36 DNA clones gave clear restriction fragment length polymorphisms and seven RAPD primers gave polymorphic amplified products in the segregating population. Thiry-two RFLP and five RAPD markers mapped to 14 coherent linkage groups (Figure 1) based on the existing soybean RFLP map (Keim *et al*, 1990b). In theory, the polymorphisms that were observed should either correspond to linkage blocks of introgressed DNA material from PI 209332 or polymorphisms between M83-15 (the susceptible parent) and the cultivated soybean background of M85-1430. Assuming that all the observed DNA polymorphisms were due to introgressed material from PI 209332, an estimate of the amount of introgressed regions from PI 209332 into M85-1430 was approximately 20% (Figure 1). After two crosses away from the ancestral donor parent (PI 209332) to generate M85-1430, it is estimated that about 25% of the donor parent's genome should have been retained in this line (Briggs and Knowles, 1967).

Frequency distribution of SCN disease response among F_2 lines

The SCN disease response among F_2 plants showed an approximately normal, continuous distribution, although there is some indication of peaks at both extremes (Figure 2). Negative transgressive segregation was observed among F_2 plants based on mean

SCN index (Figure 2). A number of positive transgressive segregants were also observed based on individual F_3 mean SCN indices. However, since these individuals represent only single F_3 plants, it is difficult to determine if the observed variations are genetic in nature or due to environmental effects. Nonetheless, the possibility of identifying positive transgressive segregants from this population is being investigated. If such lines are identified, they may be useful as starting points for developing resistant soybean cultivars.

Correlation analysis showed a significant association between the bench and the waterbath method ($r = 0.79$, $P < 0.0001$), indicating a high degree of homogeneity between the two tests, allowing SCN indices for both tests to be pooled. This was further confirmed by tests of homogeneity (paired t-test, ANOVA) between data sets to compare the effects of inoculation method on SCN disease response, which were found to be not significant (data not shown).

DNA markers associated with SCN disease response

The strategy used in locating potential partial resistance loci to SCN in this study was to detect significant associations between F_2 genotypic classes for each DNA marker and SCN disease response using regression analysis and ANOVA. Significant association between a DNA marker and SCN disease response was declared if the probability was equal or less than 0.002 to minimize the detection of false positives experiment-wide (Lander and Botstein, 1989). Fifty-six plants is a relatively small population to potentially uncover all genes controlling SCN disease response. With a population of this size, only those partial resistance loci with moderately high effects would be expected to be uncovered (Lander and Botstein, 1989). Thus, minor loci involved in SCN disease response were probably overlooked in this study.

Two unlinked RFLP loci showed significant effects on SCN disease response. Together, these two loci explained 51.7% of the total variation in SCN disease response.

The combined effects of the two RFLP markers are illustrated in Figures 3a and 3b. In Figure 3a, the segregation patterns and corresponding genotypic scores for the parental lines and 16 randomly chosen F₂ individuals are shown for the two RFLP markers, A85 and B32, which showed significant associations with SCN disease response. In Figure 3b, a bar chart showing the corresponding SCN indices for the same F₂ individuals is shown. Note that individuals that retained both resistant (M85-1430) alleles for both markers generally had considerably lower SCN indices than individuals that have retained only one or none of the resistant alleles. By contrast, individuals with both the susceptible alleles for A85 and B32 had significantly higher SCN indices.

RFLP marker A85 alone accounted for 15% of the total variation, with P = 0.0015. Homozygotes and heterozygotes for the M85-1430 allele for this marker were, on average, 32% more resistant than homozygous individuals for the M83-15 allele. Since there was no significant difference between homozygotes and heterozygotes for the M85-1430 alleles, A85 appeared to be completely dominant in effect (Table 1, Figure 4).

This marker, A85, tentatively located on linkage group 'A' of the soybean RFLP map, was linked to hilum color at a distance of 10.9 centimorgans (cM) (Figure 1). Previously, this character was reported to be tightly linked to a dominant SCN resistance gene, called *Rhg4* (Matson and Williams, 1965; Weiss, 1970). Additional RFLP markers reported to map on linkage group 'A' were also surveyed for polymorphisms. Only A111 was polymorphic and linked to A85 at a distance of 29.6 cM (Figure 1). However, A111 did not show a significant effect on SCN disease response, although it was also linked to hilum color at a distance of 18.7 cM. Hilum color was also linked to SCN resistance, although at a level that did not reach statistical significance ($r^2 = 0.10$, P = 0.0130) (data not shown).

It is possible that the resistance locus uncovered on linkage group 'A' in the current study is the same as the locus described by Weismann *et al* (1992). In their study, they

found two molecular markers, BLT24 and BLT65, to be associated with SCN resistance gene *Rhg4*, although they did not perform a direct assay for nematode resistance and the association was based solely on tight linkage of the two markers to the *i* locus (seed coat color).

A second marker, B32, tentatively located on linkage group 'K' of the soybean RFLP map, showed a stronger influence on SCN disease response. This RFLP marker accounted for 38% of the total variation ($p < 0.0001$). Homozygotes and heterozygotes for the M85-1430 allele for B32 were 48% and 34% more resistant than the homozygous individuals for the M83-15 allele, respectively. Thus, B32 appears to be partially additive in its effect (Table 2, Figure 4). The genomic region where B32 was located was also defined by three other RFLP markers, K472, K401, and K417 (Figure 1). Of these, K417 also showed a significant effect on SCN response ($r^2 = 0.16$; $P = 0.0048$).

The assignment of B32 to linkage group 'K' is tentative because this RFLP was previously mapped to two different soybean linkage groups ('J' and 'K') (Keim *et al.*, 1990b). However, one of the other RFLPs it is linked to, K401, has also been previously mapped to linkage group 'K' (Keim *et al.*, 1990b). Moreover, only a single pair of hybridizing bands were observed on the autoradiograph used to map K401, strongly suggesting it maps to a single locus only. The other RFLP markers in this linkage block, K417 and K472, were previously mapped to different soybean linkage groups (Keim *et al.*, 1990b). Both of these RFLPs hybridized to multiple bands, so it is likely, therefore, that alternative loci on linkage group 'K' have been mapped for these RFLP probes. However, after this paper was published, later experiments have placed B32 on linkage group 'J', based on its linkage with markers that were known to map only to the locus on 'J' (Chapter 2 of this dissertation).

Mapmaker-QTL analysis identified the same genomic regions (A85 and B32) as containing major QTLs for SCN resistance, explaining 21.4 and 40.2 % of the total SCN

disease response, respectively (Table 1). Two-way ANOVA did not indicate any interactions observed among the partial resistance loci and all the other DNA markers that were analyzed in this study (data not shown). In addition, a third genomic region defined by RFLP K69 was found to be associated with SCN resistance ($r^2 = 0.16$, $P = 0.0026$), but at a significance level slightly over the cut-off of $P = 0.002$ used in this study. The RFLP K69 was previously reported to be associated with SCN resistance in a study using isogenic soybean lines (Boutin *et al.*, 1992). Based on the current genetic map, this K69 locus appears to be located on linkage group 'G' (Randy Shoemaker, 1992, pers. comm.). Later, the K69 locus was mapped with an enzyme-probe combination that yielded banding patterns that were a lot easier to score than the one used at the time this paper was published, thus increasing the locus' statistical significance ($r^2 = 0.36$, $P = 0.0001$). Mapmaker-QTL scan of the K69 region indicated that this locus can explain a higher proportion of the total phenotypic variation (43.2%, LOD = 5.67) than B32 (40.2%, LOD = 6.07) (Table 2). This led to the discovery of other markers tightly linked with K69, uncovering a major partial resistance locus on the chromosome arm of linkage group 'G'. The genomic region on linkage group 'G', containing a major partial SCN resistance gene, is the main focus of this dissertation described in the next two chapters.

Implications for genetic dissection and marker-assisted breeding

Now that the utility of DNA markers in characterizing partial resistance loci for SCN resistance has been demonstrated, lines that have retained all the major resistance loci with minimum linkage drag can be identified by DNA marker genotype. This will not only accelerate the breeding of SCN-resistant soybean lines, but may also minimize the need for nematode tests, which are tedious and very difficult. These putative partial resistance loci can also be used to determine the genetic relationships among different sources of resistance. In the past, genetic relationships of known sources of SCN resistance were

estimated using the classical genetic approach (Rao-Arelli and Anand, 1988, Anand and Rao-Arelli, 1989). Using DNA markers, it may be possible to determine the genetic relationships among different sources of SCN resistance with greater accuracy by selecting on the basis of the putative genomic regions described in this study.

Through genetic dissection, it is also possible to evaluate the effects of individual resistance locus in detail. Using DNA markers, lines that are homozygous for all putative resistance loci except one can be identified or developed. For example, a line that is segregating for B32 alleles and fixed for resistance in all other markers can be challenged with a SCN race. The resistance locus in question would then be allowed to segregate and its individual effect on SCN disease response evaluated without the influence of the other putative resistance loci. Experiments with this goal in mind are now underway for the genomic regions around RFLP markers B32 and A85.

The high level of genetic variability that exists among SCN field populations complicates breeding SCN resistant soybean cultivars (Cloud *et al*, 1988). Therefore, it is imperative to have a reliable race determination test. At present, race determination, which is based on parasitism of a given isolate on a set of four soybean differentials, is not entirely clear-cut. However, in the F₂ population, used in this study, two lines that contain just one of the two major genomic regions described in this study have been identified. For example, one of the lines identified is homozygous for the resistance alleles of B32, and homozygous for the susceptibility alleles of A85. It will be possible to evaluate the ability of this line in differentiating known races of SCN. Hence, single-gene differentials that can be utilized in SCN race determination tests may be potentially developed. Lastly, the use of DNA markers in genotypic selection offers the potential to combine different sources of SCN resistance into a common soybean background by pyramiding genes.

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Table 1. Race determination tests by means of host differentials using the same source of inoculum for both the F₂ greenhouse assay and for seven experiments conducted on separate test dates.

Differential Cultivars	Host Reaction (% of susceptible check) [†]	
	Differential Test 1 [‡]	Differential Test 2 [§]
Pickett	3.66±0.89	7.21±1.40
Peking	0.80±0.76	0.54±0.34
PI 88788	2.84±0.52	3.24±0.71
PI 90763	1.34±0.38	2.77±0.63
PI 209332	2.81±0.50	-
Essex	100.00	100.00
M83-15	-	102.34±12.84
M85-1430	-	10.74±3.34

[†]Differential host reaction is expressed as percentage of the number of females and cysts recovered on the susceptible check 'Essex' (Riggs and Schmitt, 1989).

[‡]Means of differential cultivars based on seven greenhouse experiments conducted on seven separate test dates.

[§] Means of differential cultivars assayed simultaneously with the F₂ segregating population in the greenhouse.

Table 2. RFLP markers and linkage groups showing significant associations with SCN disease response.

Locus	Linkage	r^2	F-test	LOD [†]	d/a [‡]	%variation
	Group	(p-value)	(p-value)	score		
A85	A	0.15 (0.0041)	7.42 (0.0015)	2.88	1.1	21.4
B32	J	0.38 (0.0001)	17.80 (0.0001)	6.07	0.4	40.2
K69	G	0.36 (0.0001)	16.01 (0.0001)	5.677	0.5	43.2

[†]LOD is the log₁₀ of the odds ratio that supports evidence for the presence of a QTL at the locus.

[‡]is the ratio of dominance to additivity, 0 indicates complete additivity, 1 indicates complete dominance.

Figure 1. RFLP map of soybean (Keim *et al*, 1990b) showing locations of putative soybean cyst nematode (SCN) resistance loci. Introgressed regions that have been retained from the resistant ancestor (PI 209332) in this cross are shown as thick lines in the chromosomes. Locations of putative SCN resistance loci corresponding to intervals of LOD scores within 1.0 and 2.0 log units of the peak are indicated by broad and intermediate bars adjacent to chromosomes, respectively. RFLP markers showing an association significant at $P < 0.002$ ($\text{LOD} > 2.5$) are in bold letters.

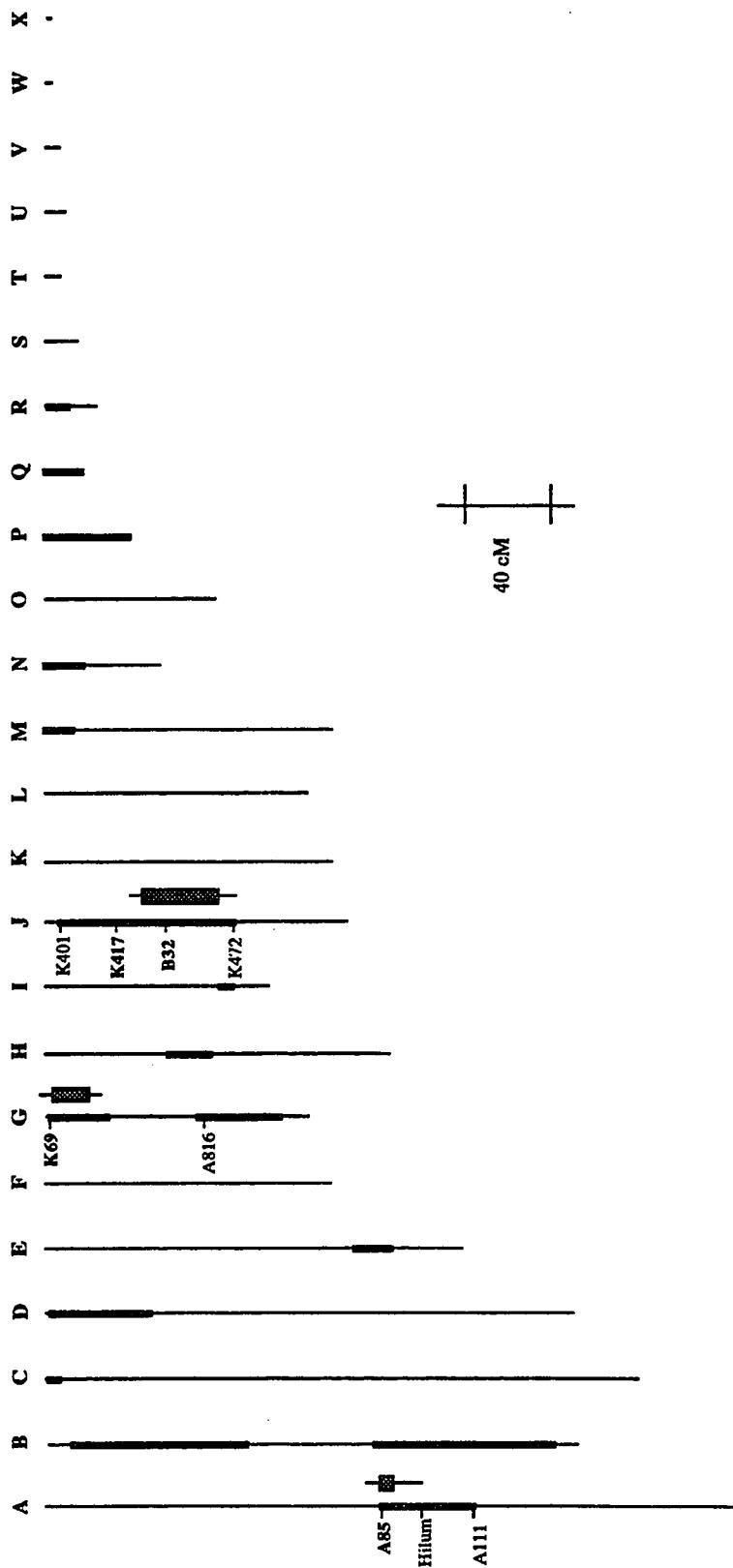


Figure 2. Frequency distribution of 56 F₂ lines for mean SCN disease response, averaged from results of bench and waterbath methods. Vertical axis shows the number of individuals for a given disease score. Horizontal axis represents the corresponding SCN indices for all 56 lines. The mean SCN index of all F₂ lines, as well as the two parents, are also shown.

Figure 2

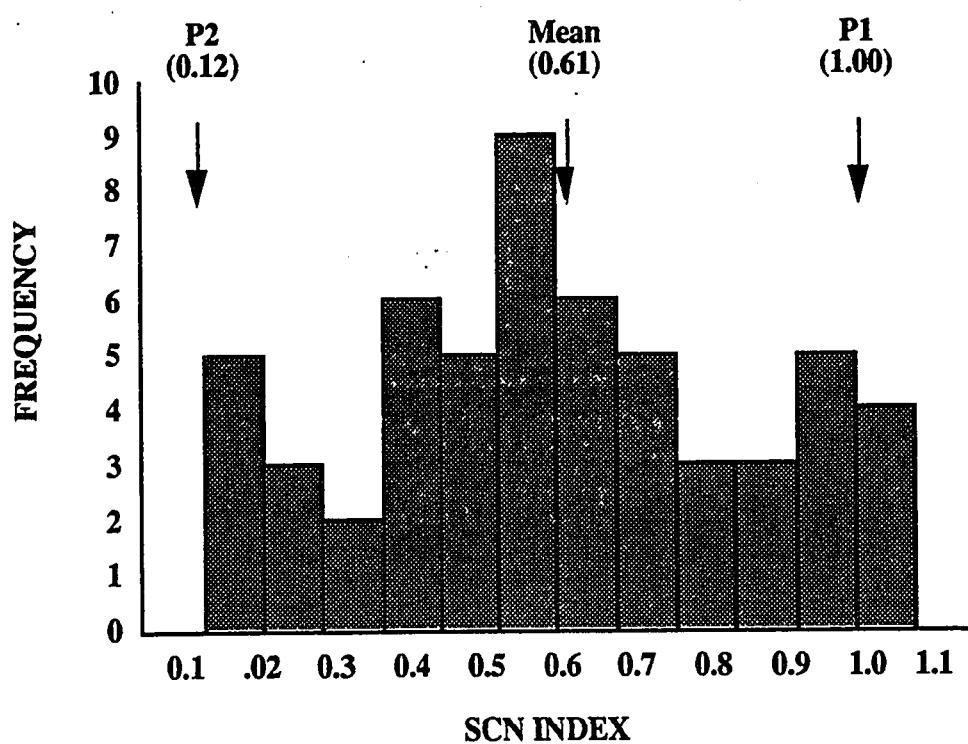


Figure 3a. F₂ segregation analysis of RFLP markers B32 and A85. Fifty-six F₂ progeny, derived from a cross between a resistant (M85-1430) and a susceptible (M83-15) soybean inbred line, were analyzed for DNA marker polymorphism. On the left, autoradiographs of the two parents showing the RFLP patterns for markers B32 and A85 are shown; on the right, the RFLP patterns for 16 F₂ progeny plants. Below the RFLP patterns, the corresponding genotypic score for each plant is shown. "1" indicates homozygous for M83-15 (susceptible parent) alleles; "2" indicates heterozygous; and "3" indicates homozygous for M85-1430 (resistant parent) alleles.

Figure 3b. Bar chart illustrating the combined effects of RFLP markers B32 and A85 on the mean SCN indices of the 16 F₂ progeny shown in Figure 3a. Vertical axis shows the mean SCN index for each individual plant. The first two bars on the left of the horizontal axis corresponds to the two parents. The remaining bars correspond to the 16 F₂ progeny. The average genotypic score (based on the results of RFLP markers B32 and A85) is shown below the bars.

Figure 3a

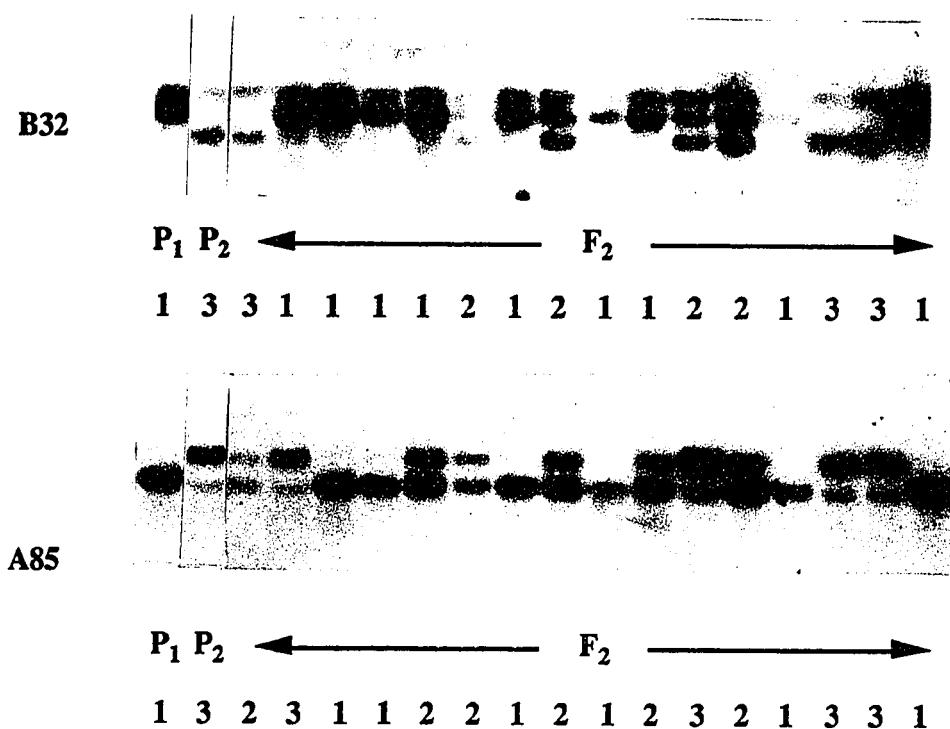


Figure 3b

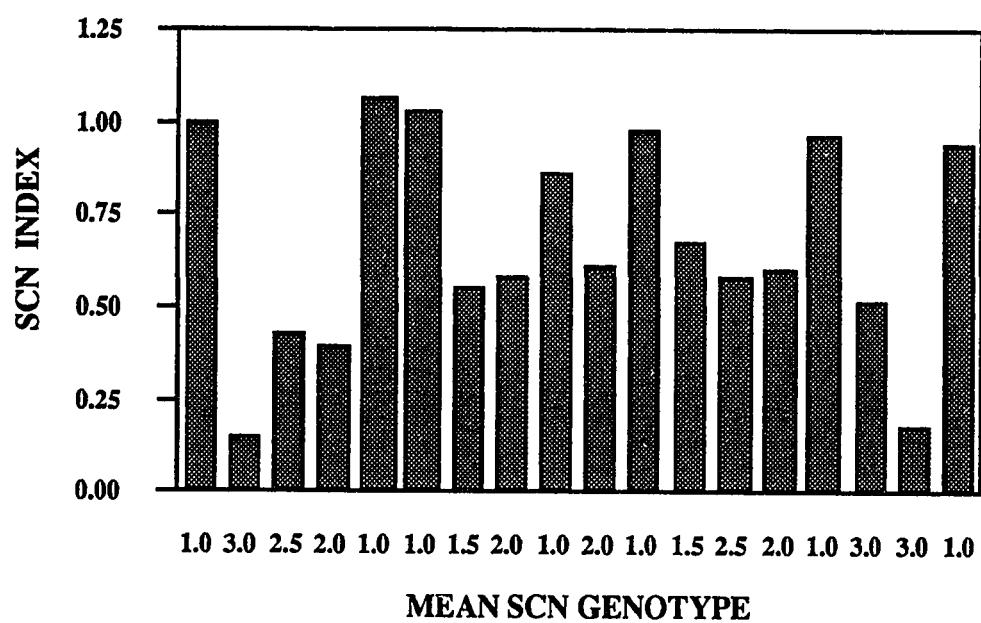
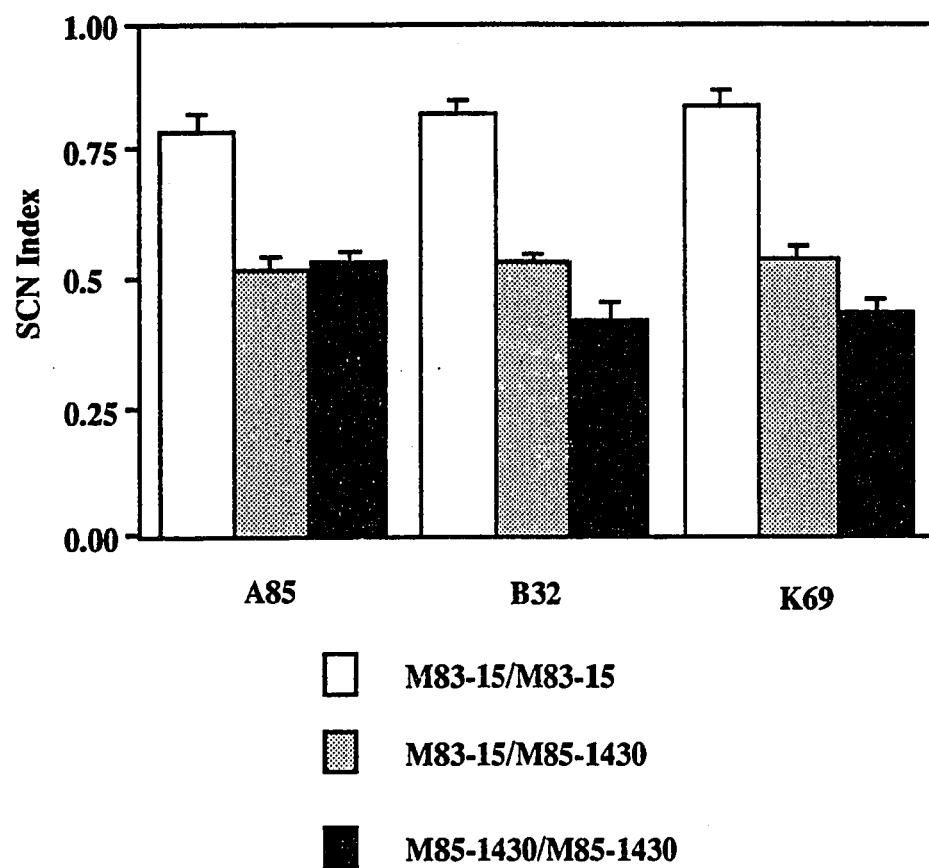


Figure 4. Comparison of mean SCN indices for different genotypic classes of RFLP markers A85, B32 and K69 . Average SCN indices for the three genotypic classes [M83-15/M83-15 (\square), M83-15/M85-1430 (\blacksquare), and M85-1430/M85-1430 (\blacksquare)], including the standard errors of means, are shown.

Figure 4



CHAPTER 2

RACE-SPECIFICITY AND MARKER-ASSISTED SELECTION OF SOYBEAN CYST NEMATODE PARTIAL RESISTANCE GENES

Parts of this chapter to be submitted to *Crop Science* as: V.C. Concibido, R.L. Denny, D.A. Lange, J.H. Orf, and N.D. Young. 1995. Race specificity and marker-assisted selection of soybean cyst nematode partial resistance genes.

ABSTRACT

Using DNA markers, a major partial soybean cyst nematode (SCN; *Heterodera glycines* Ichinohe) resistance locus on linkage group 'G' of the soybean [*Glycine max* (L.) Merrill] molecular linkage map was identified in two related populations ($F_{2:3}$ and $F_{5:6}$) derived from a cross between 'Evans' and Plant Introduction (PI) 209322. Race specificity of this locus was also assessed with various races of SCN predominant in Minnesota. In the $F_{2:3}$ population, the 'G' locus was equally effective against two distinct populations of race 3 and explained 47% to 54% ($P = 0.0001$) of the total phenotypic variation in SCN disease response but was less effective against a race 1 population and accounted only for 17% ($P = 0.0011$) of the total variation. By contrast, in the $F_{5:6}$ population, the 'G' locus was equally effective against all three SCN race populations evaluated and explained 35% ($P = 0.0001$) of the total phenotypic variation in SCN disease response against race 1, 50% ($P = 0.0001$) against race 3, and 54% ($P = 0.0001$) against a race 6 population. Additional partial SCN resistance loci were uncovered at $P = 0.02$ on linkage groups 'E', 'I', 'J' and 'K' in the $F_{2:3}$ population and on linkage groups 'D' and 'F' in the $F_{5:6}$ population. It was also observed that the ability to detect additional minor loci was complicated by the presence of strong phenotypic effects due to the locus on 'G'. In the absence of the effects of the locus on 'G', additional minor loci associated with SCN resistance were detected. Discrepancies among partial resistance loci detected between the two populations may be attributed to variability in the SCN races and the possible variability in the conditions during the separate SCN assays. Marker-assisted selection (MAS) for the locus on linkage group 'G' was shown to be 92% accurate in selecting SCN resistant soybean lines compared to 81% obtained with phenotype-based selection. Furthermore, MAS potentially shortens the time required for conventional SCN phenotypic scoring and has the potential to screen a larger number of plants.

INTRODUCTION

The extensive physiologic variation that exists among soybean cyst nematode (SCN; *Heterodera glycines*) populations has always been a challenge for soybean [*Glycine max* (L.) Merrill] breeders and nematologists (Schmitt and Shannon, 1992). The occurrence of physiologic variation within *H. glycines* was first reported by Ross (1962). Later, widespread discoveries of SCN infestations and reports of physiological and morphological variability among populations were documented (Miller, 1966, 1967; Riggs *et al.*, 1988; Sugiyama *et al.*, 1968). To cope with the pressing need to differentiate SCN races, a race classification system was established by Golden *et al.* (1970), which has proven to be immensely valuable to breeders, pathologists, and extension agents over the years. However, the existing race classification scheme is very controversial. The present race scheme lacks stability and consistency as a result of genetic relationships among differential lines, and the often unachievable requirement for inoculum uniformity and SCN assay procedure (Riggs *et al.*, 1981). In addition, the concept of race in *H. glycines* is population-based, so it cannot account for either the variability within a race of the nematode, the variability within differential soybean lines used in race classification, or the interaction between the two (Niblack, 1992).

Early genetic studies demonstrated that inheritance of resistance to SCN is oligogenic and complex (Anand and Rao-Arelli, 1989; Caviness, 1992). Genetic control of resistance also varies among SCN races and sources of resistance. For example, resistance against race 3 appears to be controlled by one dominant and two recessive genes in soybean lines Peking and PI 90763, whereas in PI 88788, resistance seems to be conditioned by one recessive and two dominant genes (Rao-Arelli *et al.*, 1992). Against other SCN races, the genetics of resistance is equally complex (Caviness, 1992). One dominant and two recessive genes (Thomas *et al.*, 1975), as well as a single dominant gene (Hancock *et al.*,

1985) have been reported conditioning resistance to race 4 in PI 90763. A single recessive gene has been reported to control resistance to "Bedford Biotype" (Hancock *et al*, 1985) and also to the so-called "race X" in PI 90763 (Hancock *et al*, 1987), later shown to be race 2. Resistance to SCN race 5 in PI 437654, PI 90763 and Peking appeared to be conditioned by two dominant and two recessive genes (Myers *et al*, 1988).

Analysis of genetic relationships has also revealed that resistance genes may be shared among sources of resistance to SCN race 3 (Rao-Arelli and Anand, 1988). Peking and PI 90763 appeared to have the same genes for resistance to race 3. Peking, PI 90763 and PI 437654 differ by at least one gene for resistance against race 5, (Anand and Rao-Arelli, 1989).

Just as soybean resistance to SCN has been found to be genetically complex, so has pathogenicity in the nematode. Studies indicate that parasitism is inherited in a dominant or partially dominant fashion (Price *et al*, 1977; Triantaphyllou, 1987) and separate genes are involved in parasitism against different resistant genotypes (Luedders, 1983; Price *et al*, 1977). In addition, the genetics of SCN is further complicated by the fact that SCN population structures can be genetically complex. This is brought about by sexual promiscuity, a distinctive trait of SCN (Thorne, 1962).

Due to the complex genetics of inheritance of resistance in soybean and pathogenicity in the nematode, proper identification and characterization of SCN races is imperative for effective deployment and pyramiding of SCN resistance genes.

The advent of DNA markers such as restriction fragment length polymorphisms (RFLPs) allows the characterization and analysis of multigenic traits into simple Mendelian components (Paterson *et al*, 1988). One very powerful application of DNA markers is the indirect selection for traits which are expensive or difficult to evaluate phenotypically, or highly affected by environmental factors (Nienhuis *et al*, 1987). Marker-assisted selection (MAS), is a deterministic type of selection based on genotype rather than phenotype

(Tanksley *et al*, 1989). MAS can dramatically increase genetic progress by increasing the accuracy of selection, reducing generation interval and by breaking undesirable linkages (Soller, 1978; Smith and Simpson, 1986; Young and Tanksley, 1989). This can be accomplished by positive selection for markers tightly linked to the gene of interest and negative selection against marker alleles that come from elsewhere on the donor parent genome (Tanksley *et al*, 1989).

This chapter describes the utility of DNA markers in the identification and localization of a major partial SCN resistance gene and efforts towards assessment of its effectiveness against three races of SCN. It also describes how MAS can dramatically increase the efficiency of selecting SCN resistant soybean lines.

MATERIALS AND METHODS

Plant materials

A. F₂ population

An F₂ population consisting of 76 individuals was constructed by crossing two contrasting soybean lines, PI 209332, a known source of SCN resistance (Anand and Brar, 1983; Anand and Gallo, 1984) as the male parent, and the susceptible cultivar 'Evans', as the maternal parent.

B. Recombinant inbred line (RIL)population

An F_{5:6} recombinant inbred line population was constructed by advancing the 76 lines, plus an additional 22 sibling lines from the original F₂ population, to the F₅ generation by single seed descent both in the greenhouse and in the field. Seventy-five seeds from each line were row-planted in Rosemount, St. Paul, MN and used as source of bulk leaf tissue for DNA extraction and analysis. Plants were allowed to recover and set F₆ seeds, which were bulked and saved for SCN disease assay.

RFLP probes

Cloned DNA fragments from soybean, mungbean [*Vigna radiata* (L.) R. Wilcz.], cowpea [*V. unguiculata* (L.) Walp.], and common bean (*Phaseolus vulgaris* L.) were used as probes for RFLP analysis. These probes were prepared from *PstI* random genomic libraries as described previously (Keim *et al.*, 1989; Chase *et al.*, 1990; Young *et al.*, 1992). The soybean clones were provided by Dr. Randy Shoemaker (Iowa State University, Ames, IA) and the common bean clones were from Dr. Edward C. Vallejos (University of Florida, Gainesville, FL). Briefly, the following protocol was followed in preparing these libraries: DNA was digested with *PstI*, separated according to size by sucrose gradient centrifugation, and the fraction between 500 and 3,000 base pairs ligated into pUC18 (Gibco/BRL, Gaithersburg, MD) by standard molecular methods (Sambrook *et al.*, 1989).

DNA extraction and Southern analysis

Plant DNA was extracted by the modified method of Dellaporta *et al* (1983) and analyzed following the technique of Young *et al* (1992). First, the best restriction enzyme for each DNA clone was determined. Individual clones were probed against "Parental survey" blots containing parental DNA digested with fifteen different restriction enzymes: *BanI*, *BamHI*, *BcII*, *BstnI*, *DdeI*, *DraI*, *EcoRI*, *EcoRV*, *HaeIII*, *HindIII*, *HinfI*, *RsaI*, *ScalI*, *TaqI*, and *XbaI* (New England Biolabs, Beverly, MA; Promega, Madison WI; Gibco/BRL, Gaithersburg, MD). Digested DNA was electrophoresed on 1% agarose gel and transferred onto Hybond N+ membranes (Amersham Corporation, Arlington Heights, IL) using a method adapted from Southern (1975), consisting of blotting in an alkaline solution of 0.5M NaOH and 0.5 M NaCl. The best clone/enzyme combination was then used for segregation analysis. DNA from all 76 F₂ or 98 F₅ individuals was digested and blotted in a similar manner to produce F₂ and F₅ "progeny" blots, respectively. Each "progeny" blot contained DNA from all F₂ or F₅ individuals and their parents digested with

a single restriction enzyme. In this way, each RFLP clone could be hybridized against F₂ or F₅ DNA that had been digested with the appropriate restriction enzyme for that clone, based on the results of the corresponding parental survey.

DNA hybridizations

Cloned DNA inserts were amplified by the polymerase chain reaction (PCR) (Saiki *et al*, 1988) for use in radiolabeling reactions and nucleic acid hybridization as described by Young *et al* (1992). Briefly, individual bacterial colonies were grown overnight in suspension culture, centrifuged for 5 min at 2000 x g, rapidly frozen and thawed, and centrifuged at 2000 x g and the supernatants used as plasmid templates for the reactions. Two microliters of the supernatant was then used in a PCR reaction to amplify the insert sequence using the M13-forward and -reverse sequence (Gibco/BRL, Gaithersburg, MD) as primers. About 40 to 50 ng of the amplified product was then radiolabeled by the random hexamer reaction (Feinberg and Vogelstein, 1983).

The radiolabeled sequence was then incubated with either a "Parental survey", F₂ or F₅ "progeny" blot for 16-24 hr at 60 °C in a hybridization solution of 5X saline sodium citrate (SSC), 0.1 M phosphate buffer, pH 7.5, 1X Denhardt's solution (2% each of Ficoll, polyvinylpyrrolidone and bovine serum albumin fraction V), 0.1% sodium dodecyl sulfate (SDS), and 5% dextran sulfate. After incubation, blots were washed three times for 15 min each at 60 °C at medium stringency (2X, 1X and 0.5X SSC with 0.1% SDS). After washing, blots were placed against Kodak X-AR film and stored at -80 °C for one to seven days to produce autoradiographs. Blots were re-used up to seven times after stripping the previous probe by a series of washes in 0.1N NaOH, 0.25 M TRIS-HCL, pH 7.5 with 2X SSC and 0.1% SDS and 0.5X SSC with 0.1% SDS at room temperature. Blots were then used immediately or blot-dried for later use.

Soybean cyst nematode assay

F_3 seeds were germinated in sand-filled Ray Leach cone-tainers (Stuewe & Sons, Inc., Portland, OR) placed in sand-filled, 10-liter buckets in a controlled temperature waterbath. A total of 10 F_3 plants were assayed in the waterbath per SCN race evaluated. Each plant was inoculated with 2,000 eggs three days after they were germinated with either race 1a, race 3a or race 3b population in three separate assays (Tables 1-3). These races were chosen for the tests since they are the most common in Minnesota. Inocula from each race were maintained on susceptible checks, Evans or 'Essex', and on susceptible differential soybean lines. For example, race 1a, which can successfully develop on PI 88788 only (Table 1), was maintained both on PI 88788 and susceptible checks; races 3a and 3b on Essex or Evans; and race 6, one of the race populations used to assay the RIL population, was grown on Pickett and Evans. Cysts and white females from each isolate were then collected every 28 days, stored in sand at 4 °C and later used, for inoculations. Race determination tests were regularly conducted on the isolates to check the possibility of race shifts (Table 3).

At the time of inoculations, cysts and white females were recovered from sand, ground in a tissue grinder to release the eggs, and the inoculum was standardized at about 2,000 eggs per plant using a hemacytometer. The parental lines, the soybean differential cultivars (Peking, Pickett, PI 90763 and PI 88788), and susceptible checks (Evans and Essex) were included in all tests. Waterbath temperatures were maintained at 28 °C with 16-hour daylength for 28 days. On the 28th day, individual plants were uprooted and cysts were collected by blasting the roots with pressurized water and cysts collected on sieves. The total number of cysts from individual plants was counted under a dissecting microscope and converted to an index by dividing this number by the average total number of cysts on the susceptible parent. For each F_2 line, SCN indices of the 10 F_3 plants were averaged to estimate mean SCN index for that line. Exactly the same procedure was later

used for the RIL population, with race 1b, race 3a, and race 6 populations used as inocula (Tables 1-3).

Linkage mapping

RFLP maps for the F₂ and RIL mapping population were constructed by conventional linkage analysis. To accomplish this, a total of 258 genomic clones were analyzed by hybridization with "parental survey" blots, and of these clones, 133 were subsequently hybridized against F₂ or F₅ "progeny" blots for segregation analysis (Table 3). Segregation for each clone was coded into a numeric form and analyzed by two-way contingency table analysis with the statistics software Statview II (Abacus Concepts, Berkeley, California), and for maximum likelihood by Mapmaker II (Lander *et al*, 1987). Linkage between RFLP markers was inferred if the probability of observing a chi-square value was less than 0.001 or if the "LOD" score exceeded 3.0. A LOD score is the log₁₀ of the ratio between the odds of the null hypothesis (that the markers are linked) against an alternative hypothesis (that they are not linked). To determine the order of markers, a multipoint analysis was used in which the favored order of markers exceeded other possible orders by a LOD of 3.0 or greater.

Mapping quantitative resistance loci

To uncover genomic regions associated with SCN resistance, F₂ or F₅ genotypic classes for each DNA marker were contrasted with SCN disease response using regression analysis and one-way analysis of variance (ANOVA). These analyses were performed using Statview-II and SuperAnova (Abacus Concepts, Berkeley, California) and SAS (SAS Institute). A significance level of P ≤ 0.002 was used to uncover potential association between DNA markers and SCN disease response (Lander and Botstein, 1989). However, marker-loci significant at P ≤ 0.02 were also reported (Tables 6 and 7),

although they were not analyzed in detail. Data were also analyzed by Mapmaker-QTL (Lander and Botstein, 1989). Although Mapmaker-QTL is not designed for analysis of RIL populations, it is sufficiently robust to detect putative QTLs in this type of population (Wang *et al*, 1994). Putative QTLs were inferred whenever the LOD score exceeded 2.7. Loci significant at $P \leq 0.02$ were fitted into a multivariate linear regression analysis to determine their combined phenotypic effects on SCN disease response. Interactions among putative loci were not evaluated in this study since the population sizes are too small to conduct meaningful tests. In the case of distributions that showed some deviations from normality, statistical analyses using arc-sin transformation and non-parametric procedures (Kruskal-Wallis, Spearman Rank Correlation) were also performed. However, no meaningful differences have been observed among the results obtained from various analyses, so the data presented in this chapter are all based on untransformed data.

Race specificity determination

To assess the race specificity of loci associated with resistance, tests of homogeneity among slopes (Steele and Torrie, 1980), derived from significant regression analyses between marker-loci and SCN disease response for each race evaluated, were performed using t-tests. Race specificity of a locus was declared if heterogeneity was detected among slopes obtained from the regression analyses between marker-locus and SCN disease responses. A locus was also declared race specific if it showed significant association with SCN disease response against one race population but not in another at $P \leq 0.02$.

Assessment of marker-assisted selection in SCN resistance breeding

The accuracy of F_2 marker-genotype in selecting soybean lines to advance in later generations was compared with selection based on conventional SCN phenotypic scoring.

For phenotype-based selection, F_{2:3} lines that had SCN indices greater than 30% were rated "susceptible" and plants with indices less than 30% were rated "resistant" as defined by Schmitt and Shannon (1992). Resistant lines were given a numerical code of 2 and susceptible lines were given a numerical code of 1. Likewise, the SCN indices of corresponding F_{5:6} lines were also converted into the same numerical codes. Thus, lines given a score of 2 (resistant) in the F_{2:3} generation were predicted to be resistant also in the F_{5:6} generation. By contrast, selection based on DNA marker genotype was accomplished based on the genotypic scores of RFLP marker C6 and the interval between C6 and Bng122 (the markers flanking the most important SCN region) (Concibido, 1995; Chapter 3 of this dissertation). For RFLP C6, the genotypic scoring was accomplished by giving an individual F_{2:3} line that inherited the banding pattern of the susceptible parent, Evans, a numerical score of 1, and a numerical score of 2 if it inherited the banding pattern of the resistant parent, PI 209332. Heterozygous individuals or plants that inherited the banding patterns of both parents were not included in the analysis since they were expected to segregate in later generation and would definitely diminish the efficiency of selection. The interval between RFLP markers C6 and Bng122 was estimated by comparing the numerical scores for each marker. Every time the same scores were obtained for both markers, that score was retained and each time the scores differed, the line was treated as a missing value. To estimate the accuracy of each selection, the relationships of the F_{2:3} genotypic and phenotypic scores with the F_{5:6} phenotypic scores were analyzed using regression and contingency table analyses using Statview-II and SuperAnova (Abacus Concepts, Berkeley, California).

RESULTS AND DISCUSSION

Status of soybean RFLP linkage map

The published soybean molecular map contains nearly 490 RFLP and random amplified polymorphic DNA (RAPD) markers placed onto 24 molecular linkage groups spanning a recombinational distance of about 3000 cM (Shoemaker and Olson, 1993). A total of 258 RFLP markers were surveyed in the current study, and of these, 177 were *G. max*, 65 were *P. vulgaris*, 12 were *V. radiata* and four *V. unguiculata* genomic clones (Table 4). Of the 258 markers, 140 (54.3%) were polymorphic and 98 (70%) of these polymorphic clones were chosen and mapped with the RIL population (Table 4). The degree of polymorphism in soybean observed in this study (57.1%) was higher than most of the estimates previously reported (Apuya *et al.*, 1988; Keim *et al.*, 1990; Diers *et al.*, 1992; Shoemaker and Specht, 1995). The higher degree of polymorphism observed could have been due to a larger number of enzymes (15) evaluated in this study. It is also possible that the soybean parental lines (Evans and PI 209332) used in this study are more genetically diverse than the ones used in previous studies.

About 56.1% of the markers mapped to the same location as previously published (Shoemaker and Olson, 1993), 39.8% mapped to locations other than previously reported (Shoemaker and Olson, 1993), while 4.1% failed to segregate when mapped (Tables 4 and 5). Based on mapping results from the F_{2:3} population (data not shown), polymorphic clones used for mapping in the RIL population were chosen based on the following criteria: markers that previously mapped to the right (published) location, the density of markers on a particular genomic region, and the importance of a genomic region relative to SCN resistance. Based on these criteria, several polymorphic markers were not used in developing the linkage map for the RIL population. Eighteen of the 24 (75%) linkage groups contained at least one marker, with some linkage groups having as many as 12

marker-loci (Figures 1a-1c). Based on the size of the soybean genome and the regions spanned by the markers in this study, approximately 58% genome coverage was obtained with the 98 markers placed onto this map.

In theory, after five generations of selfing, the RIL population should be approximately 97% homozygous towards the adapted parent, Evans (Briggs and Knowles, 1967). However, it was estimated that about 10% of the genome was still heterozygous in this population. It is likely that certain linkages favoring the heterozygous condition were present in the RIL population. In self-pollinated crops like soybean, it has been suggested that heterozygosity imparts some advantage that allows increased seed production in subsequent generations (Brigg and Knowles, 1967).

Frequency distributions of SCN disease response in the F_{2:3} and RIL populations

Figures 2a-2c show the approximately normal distributions of SCN disease response against races 1a, 3a and 3b, respectively, in the F_{2:3} population. Figures 2a and 2c show positively skewed SCN disease response distributions towards lower SCN indices for both races 1a and 3b. By contrast, the distribution for race 3a had the most symmetric distribution with many extreme values spread over a wide range (Figure 2b). The SCN distributions in the RIL population show bimodal peaks indicative of the approach towards homozygosity at both extremes, especially with race 3a and race 6 (Figures 3a-3c).

The degree of relatedness of the different SCN races used to assay each population was determined using regression analysis. The SCN disease response of the F_{2:3} population to race 1a appeared to be more divergent than those of either races 3a or 3b (Figures 4a-4c). The SCN disease response of the RIL population against the race 1b, race 3a and race 6 did not vary significantly (Figures 5a -5c). As much as 82% similarity was

observed between the SCN disease responses of the RIL population to race 3a and race 6 (Figure 5c). Based on the race identification assays, the three race populations tested as three distinct races (Table 3). However, the high degree of relatedness observed among the three races suggests that PI 209332 could not differentiate races 1, 3, and 6. Conversely, race 1a and 1b were both classified as race 1 using the race identification scheme but the two differed significantly in the two tests (Table 3). In the F_{2:3} SCN assay, race 1a had a cyst index of 10.4% on PI 88788, the only differential soybean line that is successfully parasitized by race 1 (Table 3). By contrast, race 1b had a cyst index of 20.8% in the F_{5:6} SCN assay (Table 3), indicative of increased aggressiveness of this isolate. This prevented the parallel comparison of the two populations in their reactions to race 1. These results show the inability of the present race scheme to fully characterize and distinguish SCN race populations.

RFLP markers associated with SCN disease response

To identify potential partial resistance loci to SCN, the genotypic classes for each marker were correlated with corresponding SCN disease responses for each race assayed. A potentially significant association between a DNA marker and SCN disease response was declared only if the probability was equal or less than 0.02. However, to minimize the detection of false positives experiment-wide (Lander and Botstein, 1989), only significant associations at $P \leq 0.002$ were studied in detail. It was expected that with relatively small population sizes, only those partial resistance loci with moderately high effects would be uncovered at this cut-off level (Lander and Botstein, 1989). Thus, looking at markers significantly associated with SCN disease response at $P \leq 0.02$ might have decreased the probability of overlooking minor, but still biologically relevant loci in this study, although it could also lead to false positives.

In the F_{2:3} population, a total of five independent partial resistance loci associated with SCN disease response in at least one race were uncovered at P ≤ 0.02 (Table 6). Five additional partial resistant loci with minor effects on SCN disease response were also detected at P < 0.05 (data not shown). Among loci detected at P ≤ 0.02, only two (C6 and A315) had significant associations with SCN disease response in all three races (Table 6) and the same two markers had significant associations in at least one of the races at P ≤ 0.002. One of these markers, A315, which is linked to three other markers on linkage group 'K' (Figure 1), accounted for 8.9 to 14.4% of the total phenotypic variation in SCN disease response (Table 6). The other marker, RFLP C6, which is linked to seven other markers on linkage group 'G' (Figure 1), appeared to be the most important locus, explaining 16.9 to 54.5% of the total phenotypic variation in SCN disease response (Table 6). It also appears that the ability to detect other loci associated with SCN resistance and the magnitude of their effects is conditional upon the magnitude of the phenotypic effects of the locus on 'G'. Wang *et al* (1994) also described a similar situation in rice, where the ability to detect QTLs for partial resistance to rice blast (*Pyricularia oryzae* Cavara) in rice is conditional upon the presence of QTLs for complete resistance. For example, in race 1a, where C6 had the least effect on SCN resistance, three additional loci were detected, whereas only two additional loci were detected in race 3a and one in race 3b, where the effects of C6 were much stronger (Table 6). In addition, except for A386, the phenotypic effects of the other putative resistance loci showed a generally decreasing trend as the effect of C6 increased (Table 6).

A total of three independent partial resistance loci associated with SCN disease response in at least one of the races tested were identified in the RIL population at P ≤ 0.02 (Table 7). Three additional partial resistant loci with minor effects on SCN disease response were also detected at P < 0.05 (data not shown). Among loci detected at P ≤ 0.02, only one locus, (C6), was found to be associated with resistance in all three races at

$P \leq 0.0002$ (Table 7). Consistent with the results from the $F_{2:3}$ population, C6 also appeared to be the most important locus in the RIL population and explained 35 to 53% of the total phenotypic variation in SCN disease response (Table 7). The lower number of partial resistant loci detected in the RIL population could also be due to stronger phenotypic effects of C6 in this population.

Except for C6, the small r^2 values observed among the other markers detected in this study may represent either QTLs with minor effects, or QTLs with larger effects but loosely linked to marker loci (Edwards *et al*, 1987). In addition, the phenotypic effects of a particular allele is dependent upon the genetic background (Tanksley and Hewitt, 1988) and the environmental conditions (Burton, 1987). Thus, 'minor' loci described in this study could also have major importance in other soybean genetic backgrounds or environments or in other SCN races that were not evaluated in this study and should not be ignored.

The total phenotypic effects of individual partial resistance loci detected at $P \leq 0.02$ in both populations could account only for up to 65% of the total phenotypic variation in SCN disease response (Tables 6 and 7). This suggests that additional loci with minor effects were probably overlooked in this study since only 58% of the genome was covered with markers. Although it is also possible that variability due to non-genetic factors might have underestimated the phenotypic effects measured among loci (Edwards *et al*, 1987). Furthermore, when significant loci were analyzed using multivariate linear regression analysis, their combined phenotypic effects were less than the sum of their individual effects, especially in the $F_{2:3}$ population (Tables 6 and 7). It is possible that the other markers are explaining the same variation due to chance collinearity in the data set (Keim *et al*, 1990) or some type of interactions exist among significant SCN loci. However, the relatively small sizes of the $F_{2:3}$ (76 genotypes) and the RIL (98 genotypes) populations prevented a more detailed analysis of possible interactions among loci. Because of the relatively small population sizes, certain genotypic combinations would most likely be

unrepresented in the analysis, which would diminish the validity and biological significance of the results.

Despite the consistency of RFLP C6 between the populations, the observed discrepancy among other partial SCN resistance loci uncovered between the two populations is noteworthy and warrants some discussion. One possibility is that differences might have been due to the variability of SCN isolates used in the study. For example, A315 was detected to be most effective against race 1a, which was tested only in the F_{2:3} population. Likewise, A64 was found to be most effective against race 6, an isolate used only in the RIL population. However, since the same isolate of race 3 was used in both populations, one would expect that this isolate would result in the same degree of parasitism on the two loci rather than the differential effects exhibited against race 3a by RFLPs A64 and A315. To further illustrate this, A315 was found to be effective against race 3a ($r^2 = 0.09$, $P = 0.0090$) in the F_{2:3} but not in the RIL population where it showed an r^2 of 0.007 ($P = 0.4465$). On the other hand, A64 was detected to be significant against race 3a ($r^2 = 0.07$, $P = 0.0090$) in the RIL but not in the F_{2:3} population where it showed an r^2 of 0.0002 ($P = 0.9078$). A possible explanation is that the two race 3a isolates used in the separate assays were not identical. Table 3 shows that the race 3a used in the RIL population had a higher and more variable index on Pickett (7.2 ± 3.0) compared to the one used in the F_{2:3} population (3.8 ± 0.6). Although the two populations of race 3a were not significantly different from each other, this isolate of race 3 still tested as race 6 in some replications in the RIL population, potentially explaining why A64 was detected at a lower level of significance, but A315 was not.

Differences in resistance loci against similar fungal races were also detected in *Phytophthora infestans* in potato upon dissection of the disease distributions of the two races into QTLs (Leonards-Schippers *et al.*, 1994). Discrepancies observed among SCN resistance loci detected in both populations may also be attributed to the large phenotypic

effects due to the locus on 'G'. Wang *et al* (1994) observed that in rice, the presence of genes for partial resistance to rice blast could affect the classification of lines in relation to the presence or absence of genes for complete resistance to the disease. Although the major locus on 'G' does not confer complete resistance to SCN, it has a considerable effect on SCN disease response that can potentially affect the detection of loci with minor effects. With this in mind, the F_{5:6} population was re-evaluated for additional QTLs that were probably missed due to the presence of the strong genetic effects due to the resistance locus on 'G'. First, the effects of the resistance locus on 'G' were isolated by excluding all lines that were homozygous and heterozygous for the resistance alleles at this locus. Then, regression analyses between DNA markers and the SCN disease response were performed. This analysis was performed only in the F_{5:6} population because there were not enough lines left in the F_{2:3} population after lines carrying the resistance alleles at the 'G' locus were excluded.

In the process, a total of six more markers associated with SCN resistance were uncovered in the F_{5:6} population (Table 8). The locus on linkage group 'L' accounted for 23% of the total phenotypic variation in SCN disease response against race 1, while the locus on 'N' effective against race 6 was also found to be associated with SCN resistance against race 6 in PI 90763 and Peking (Young *et al*, in preparation) (Table 8). It is interesting to note that B32 on linkage group 'J' was found to be significantly associated with SCN resistance against race 3 at $P = 0.0002$ in the absence of the 'G' resistance locus, but was undetected when the effects of the 'G' locus was present. It barely passed the cut-off for significance against race 1a when tested at the F_{2:3} generation. The same locus has been shown to be strongly associated with SCN resistance to race 3 in PI 90763 (Young *et al*, in preparation) and was previously identified to be an important partial resistance locus to the same race in PI 209332 (Concibido *et al*, 1994). In that study, three unlinked RFLP loci associated with SCN resistance were identified, A85 on linkage group A, B32 on

linkage group J and K69 on linkage group 'G' (Concibido *et al*, 1994). Mapmaker analysis showed that 21.4% (LOD = 2.88) of the total phenotypic variation in SCN disease response could be attributed to A85, 40.2% (LOD = 6.07) due to B32, and 43.2% (LOD = 5.67) due to K69. At that time the phenotypic variation than can be attributed to the locus on linkage group 'G' was relatively low, which allowed the detection of B32. The availability of new markers on linkage group 'G' provided by the results of the comparative mapping study by Boutin *et al* (1995) uncovered a major partial SCN resistance locus on linkage group 'G'. The presence of the major effects of the locus on 'G' appeared to have masked the effects of other loci associated with SCN resistance and rendered them undetectable in this study. RFLP A85, however, was not found to be associated with SCN resistance in this study, even in the absence of the major effects of 'G'. Despite the common source of SCN resistance (PI 209332) in both studies, it is possible that the PI 209332 progenitor of M85-1430, the resistant parent in the previous study (Concibido *et al*, 1994) was genetically divergent from the PI 209332 used as the resistant parent in this study. In addition, M85-1430 is two crosses away from its ancestral PI 209332 parent and only about 25% of the donor parent's genome has been theoretically retained in this line. Thus, differences in the genetic background between the mapping populations used in the studies, variability of the sources of PI 209332 germplasm and, experimental error might account for the discrepancy in detecting the same QTLs.

Race specificity of loci associated with SCN resistance

The strategy to assess the race specificity among loci associated with SCN resistance was to detect significant differences among slopes derived from the regression analyses between marker-loci and SCN disease response for each race evaluated using paired t-tests (Steele and Torrie, 1980). Since the slope of the regression line between the genotypic classes and SCN disease response represents the phenotypic effect of an allele at

a given locus, then differences detected among slopes represent varying degrees of effectiveness of a marker-locus against different SCN races. With this in mind, race specificity of a locus was declared if heterogeneity was detected among slopes obtained from the regression analyses between marker-locus and SCN disease responses. A locus was also declared race specific if it showed significant association ($P \leq 0.02$) with SCN disease response against one race population but not in another.

In the F_2 population, the locus on 'G' appeared to be equally effective against race 3a and race 3b but had a statistically significant smaller effect against race 1a (Table 9). Each additional dose of the resistant allele at C6 accounted for 8%, 18% and 24% fewer cysts than individuals with the susceptibility allele against race 1a, race 3a and race 3b, respectively (Table 9). Thus, homozygous resistant individuals at C6 had 48% fewer cysts than homozygous susceptible individuals against race 3b. By contrast, RFLP A315 on linkage group 'K' behaved as race nonspecific in the F_2 population with equal effectiveness against three races (Table 9). Additional RFLP markers on linkage groups 'T' (A102) and 'J' (B32) showed race specificity for race 1a, while a marker on linkage group 'E' (A386) was effective only to race 3a. It is noteworthy that the locus on 'E' had alleles originating from the donor parent (PI 209332) that were associated with increased susceptibility to SCN. For example, individuals with additional dose of the PI 209332 allele at A386 had about 7% more cysts than individuals with the Evans allele when tested against race 3a. This genomic region is an example of deleterious genes coming from the donor parent that are often undetected in a conventional plant breeding program. Conversely, it also demonstrates an example where a resistant allele appears to come from the susceptible parent. In a recent study to uncover QTLs for sudden death syndrome (SDS) in soybean, Hnetkovsky *et al* (1995) also described two resistance alleles originating from the susceptible parent, Essex.

In the RIL population, RFLP C6 appears to be race nonspecific against races 1b, 3a and 6. On average, individuals carrying the resistant alleles for C6 had nearly 40% fewer cysts than the susceptible ones when challenged with any of the three races. In the RIL population, the slopes represent the phenotypic effects of two doses of an allele of a given locus because only homozygous individuals (representing 90% of the population) were included in the analysis. An additional locus, RFLP A64 on linkage group 'D' was found to be race specific against races 3a and 6, but not 1b. This locus also had susceptibility alleles to SCN originating from the resistant parent, PI 209332 (Table 9). On average, individuals with an additional dose of PI 209332 allele tended to have 15% more cysts than individuals with the Evans allele. With the availability of DNA markers, it is possible to select against susceptibility alleles (e.g A64 and A386) from the donor parent, PI 209332, and select for resistance alleles coming from the susceptible parent, Evans.

The identification of race-specific partial SCN resistance loci in this study is contradictory to the popular belief that quantitative resistance is race nonspecific (Vanderplank, 1968). However, QTL studies in rice blast (Wang *et al*, 1994) and late blight in potato (Leonards-Schippers *et al*, 1994) have also indicated the presence of race-specific components of partial resistance. Results of this study have serious implications for SCN resistance breeding, especially in the spatial and temporal gene deployment strategies to counter race-shifts in field populations of SCN (Anand and Koenning, 1986). Vanderplank (1968) has suggested that the durability of resistance genes is dependent upon the presence of race nonspecific components that minimize selection pressure on the pathogen population. Now, that DNA markers have identified race-specific and race nonspecific partial SCN resistance loci, marker-assisted selection can be employed to develop favorable gene combinations that might lead to durable SCN resistance.

Practical application of MAS in SCN breeding

To be useful in breeding, it is essential that the accuracy of genetic markers in predicting plant performance be compared with conventional phenotypic scoring. The availability of two related populations ($F_{2:3}$, $F_{5:6}$ RIL) three generations apart, allowed the comparison between RFLP-based and phenotypic-based selections performed on F_2 lines to predict the SCN disease response of their F_6 descendants. It should be noted, however, that only those lines with complete data sets for both genotypic and phenotypic scores in the $F_{2:3}$ and their corresponding $F_{5:6}$ phenotypic scores were included in the analysis. This requirement led to discrepancies in the number of lines that were included in the analysis, which involved two parts. First, the mean SCN indices of five plants from each $F_{2:3}$ line were regressed against the corresponding mean SCN indices of their $F_{5:6}$ RIL descendants. The five plants represent the first five replications of the $F_{2:3}$ SCN assay against race 3a, which is the usual number of plants used in screening F_2 lines for SCN resistance in typical breeding programs. At the same time, the F_2 genotypic classes for RFLP C6 were similarly regressed against $F_{5:6}$ SCN indices. The results of the regression analysis between the $F_{2:3}$ and $F_{5:6}$ SCN indices were compared with the results of the regression between F_2 RFLP genotype and $F_{5:6}$ SCN indices. Using phenotype-based selection, 32% of the total phenotypic variation in the $F_{5:6}$ SCN disease response could be explained by the $F_{2:3}$ SCN indices (Table 10). By contrast, in the RFLP genotype-based selection, 46% of the total phenotypic variation in the $F_{5:6}$ SCN disease response could be explained by RFLP C6 genotype (Table 11). Furthermore, the interval between RFLP markers C6 and Bng122 explained 71% of the total phenotypic variation in SCN disease response at the $F_{5:6}$ generation (Table 12). The use of the interval between the two markers flanking the major partial SCN resistance locus on linkage group 'G' provides a more accurate estimate of its genetic effect since interval scoring can minimize the probability of misclassifying recombinants between the two markers (Lander and Botstein, 1989).

As a second way to assess the practical usefulness of marker-assisted selection in breeding SCN resistant soybean varieties, a contingency table analysis was conducted using the F_{2:3} SCN indices and the RFLP genotypic classes as predictors in selecting resistant F₂ lines to advance in later generations. In the phenotype-based selection, the following criterion was used. F_{2:3} lines that had SCN indices greater than 30% were rated susceptible and plants with indices less than 30% were rated resistant (Schmitt and Shannon, 1992). Based on RFLP C6 genotype, individuals that inherited the alleles of the resistant parent (PI 209332) were classified as "resistant" and those that inherited the susceptible parent's (Evans) alleles were classified as "susceptible". Individuals that inherited the alleles of both parents (heterozygotes) were not included in the analysis since they were expected to segregate in later generations. The ability of MAS to uncover heterozygotes is one of its advantages over conventional breeding strategies, especially in traits governed by dominant gene action. In traits governed by dominant gene action, deleterious recessive forms of the gene "hidden" in heterozygous individuals are unknowingly selected together with homozygous dominant individuals, thus slowing down the approach to homozygosity and decreasing genetic gain in a conventional breeding strategy (Fehr, 1987). Using the F_{2:3} SCN indices as the criterion, correct decisions on which plants to discard (susceptible) and which plants to advance (resistant) in later generations were made 55 out of 68 times (81%) (Table 13). By contrast, 84% (26 out of 31) accuracy was obtained with selection based solely on the genotype of RFLP marker C6 (Table 13). Furthermore, the accuracy of marker-assisted selection was further increased up to 92% (22 out of 24) when the interval between RFLP markers C6 and Bng122 was used as the predictor (Table 14).

However, it is cautioned that the discrepancies between the number of lines used in the phenotypic and genotypic-based selections brought about by the inability of the phenotypic-based selection to detect heterozygous individuals might have favored

genotypic-based selection. With this in mind, a more parallel and unbiased comparison using the 12 most resistant and 13 most susceptible F_{2:3} lines was performed. Again, the F₃ SCN indices of these 25 lines were regressed against their corresponding F_{5:6} SCN indices. Likewise, a contingency table analysis, described earlier, was also performed. Using the most extreme F_{2:3} phenotypes, the predictability of phenotype-based selection improved from 32 (Table 10) to 55% with regression analysis. However, results of the contingency table analysis using the 25 most extreme F_{2:3} phenotypes gave the same accuracy (80%) (data not shown) as the analysis using all 67 phenotypes (81%, Table 13). These results further reaffirmed the advantage of MAS over conventional selection strategy.

Since the efficiency of a breeding strategy is usually measured in terms of genetic progress per unit of time, or genetic gain (Fehr, 1987), as well as the related cost associated with each strategy (Ragot and Hoisington, 1993), a comparison was made between phenotype and genotype-based selections. In this study, it was estimated that using RFLPs in genotyping 100 soybean lines would cost about \$2.00 per data point. In addition, the entire process of DNA analysis, including DNA extraction, restriction digest, electrophoresis, Southern blotting, and autoradiography, would take one person about eight days to accomplish. This estimate, based on a single use of Southern blots for genotyping RFLP markers C6 and Bng122, is relatively close to previous estimates (Beckmann and Soller, 1983; Ragot and Hoisington, 1993). By contrast, conventional SCN screening costs about \$1.00 per data point. Furthermore, SCN phenotypic screening is a tedious and difficult process, which consists of seed preparation, inoculation, water bath incubation and cyst counting. The entire process involves two people and typically takes more than 30 days to accomplish. Thus, one can genotype up to three times as many plants within the time period required to assay 1000 plants (100 lines, 10 replications) using conventional SCN screening procedure. The development of polymerase chain reaction (PCR)-based markers, such as sequence-tagged sites (STSs) (Olson *et al*, 1989),

offers ways to further cut down the time and the cost of genotyping plants in the long run. It was estimated that PCR-based markers are expected to cost about \$1.50 per data point (based on the present cost of *Taq* DNA polymerase, the most expensive reagent in PCR) and take about two days to genotype 100 plants. This estimate was also very close to a previous study by Ragot and Hoisington (1993). Furthermore, the use of PCR-based markers offer a simpler and safer (i. e. non-radioactive) way of genotyping plants.

Implications for SCN resistance breeding

This work has demonstrated the utility of DNA markers in the localization and characterization of quantitative trait loci associated with SCN disease response. The locus on 'G' appears to contain a major partial SCN resistance loci with some degree of race specificity to SCN race 3, but less so to race 1. Additional race nonspecific partial resistance loci were also found on linkage group 'K' and on linkage group 'D'. Additional minor partial resistance loci with race-specific effects were also found on linkage groups 'E', 'F', 'T', and 'J'. It was also observed that the ability to detect additional minor loci was conditional upon the presence of the strong phenotypic effects due to the locus on 'G'. In the absence of the effects of the locus on 'G', additional minor loci associated with SCN resistance were detected. These results are very important in gene deployment strategies against field populations of SCN, as well as in pyramiding SCN resistance genes for more robust SCN resistant soybean cultivars. For example, various gene combinations can be developed in a common or distinct soybean genetic background to prolong the useful life of these resistance genes through spatial and temporal gene deployment (Anand and Koenning, 1986). Defeated gene combinations due to race shifts in SCN field populations can then be replaced with a more appropriate novel gene combination for more effective gene deployment strategy.

The results demonstrate the powerful, yet simple application of marker-assisted selection, in which tremendous gains in selection can be attained using DNA markers compared to conventional breeding. Despite the lower cost involved in conventional SCN screening compared with RFLPs, it is still less appealing due to the time-consuming and tedious nature of screening soybean germplasm. The entire process, consisting of seed preparation, seedling inoculations and many hours of counting cysts recovered on individual test plants, takes more than 30 days to accomplish with a limited number of plants that can be handled. By contrast, RFLP genotyping currently costs about twice as much, but can handle three times as many plants within the time period required to accomplish conventional SCN screening. In addition, DNA markers provide a more deterministic selection strategy with a minimum of undesirable linkages, possible only by many generations of backcrossing by traditional breeding. For example, the susceptibility alleles for SCN resistance, originating from PI 209332, can now be easily selected against using RFLP markers. Furthermore, using marker-assisted selection, one can develop soybean lines containing only the beneficial alleles for SCN resistance and possibly break unwanted linkages with yield depression normally associated with resistance genes (Anand and Koenning, 1986).

However, the use of markers in breeding is still limited by the cost and the time needed for genotyping plants (De Verna and Alpert, 1990). The development of polymerase chain reaction (PCR)-based markers is imperative to further cut down the time and more likely the cost of genotyping plants in the long run, as well as simplify and make the genotyping procedures safer by using non-radioactive protocols.

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Table 1. Classification of races of *Heterodera glycines* by means of host differentials.

<u>Reaction of Host Differentials</u>					
Race	Pickett	Peking	PI 88788	PI 90763	PI 209332
1†	-‡	-	+	-	-
2	+	+	+	-	-
3	-	-	-	-	-
4	+	+	+	+	-
5	+	-	+	-	+
6	+	-	-	-	-
7	-	+	-	-	•
8	-	-	-	+	•
9	+	+	-	-	•
10	+	-	-	+	•
11	-	+	+	-	•
12	-	+	-	+	•
13	-	-	+	+	•
14	+	+	-	+	•
15	+	-	+	+	•
16	-	+	+	+	•

† - Races highlighted in bold were the ones used in this study.

‡ + - Indicates a susceptible reaction when the number of females and cysts recovered is greater than or equal to 10% of the number on the susceptible check; and - indicates a resistant reaction if the number is less than 10% than the susceptible check (Riggs and Schmitt, 1989). PI 209332 is not an official differential soybean line but is included in all the race determination tests because of its importance in this dissertation. • indicates that there is no information available on the disease response of PI 209332 on a particular race.

Table 2. Sources and characteristics of SCN isolates used in the study.

Isolate	Designation in this study	State of origin	Race
UMN1	Race 3a	Minnesota	Race 3
UMN2	Race 3b	Missouri	Race 3
UMN11	Race 6	Minnesota	Race 6
UMN14	Race 1a	Minnesota	Race 1
UMN30	Race 1b	North Carolina	Race 1

Table 3. SCN races used in the study and their mean SCN indices on host differentials.

Differential Lines [‡]	Host Reaction (% of susceptible check) [†]					
	Race 1a	F ₂ :F ₃ population		Race 3b	F ₅ :F ₆ RII population	
	Race 1a	Race 3a	Race 3b	Race 1b	Race 3a	Race 6
Pickett	2.8 ± 1.2	3.8 ± 0.6	2.6 ± 0.5	0.3 ± 0.1	7.2 ± 3.0	16.0 ± 2.3
Peking	1.6 ± 0.7	1.1 ± 0.4	0.8 ± 0.2	2.9 ± 1.7	0.0 ± 0.0	1.5 ± 0.5
PI 88788	10.4 ± 1.6	6.5 ± 1.6	1.2 ± 0.4	20.8 ± 3.5	4.7 ± 1.1	3.5 ± 0.7
PI 90763	0.4 ± 0.2	2.0 ± 0.4	0.2 ± 0.2	0.4 ± 0.1	2.0 ± 0.9	0.7 ± 0.3
PI 209332	7.0 ± 2.4	2.7 ± 1.2	2.8 ± 1.0	5.9 ± 1.1	3.5 ± 1.0	3.2 ± 0.7
Evans	100.00	100.00	100.00	100.00	100.00	100.00

† - Based on the present SCN race classification scheme (Riggs and Schmitt, 1989), an individual is considered susceptible (+ reaction) if the number of females and cysts recovered on it is greater than or equal to 10% of the number on the susceptible check; and resistant (- reaction) if the number of females and cysts recovered on it is less than 10% of the number on the susceptible check.

‡ - PI 209332 is not an official differential soybean line but is included in all the race determination tests because of its importance in this dissertation.

Table 4. Number of polymorphic clones, clones that mapped to previously published locations (anchors), and clones that mapped onto other locations (duplicate loci) in *G. max*.

Source	Number of clones surveyed	Polymorphic clones (%)	Clones Mapped	Anchors†	Duplicate loci	No segregation
<i>G. max</i>	177	101 (57%)	75	41	30	4
<i>P. vulgaris</i>	65	31 (48%)	18	12	6	0
<i>V. radiata</i>	12	5 (42%)	4	4	0	0
<i>V. unguiculata</i>	4	3 (75%)	1	1	0	0
Total	258	140 (54%)	98	55	39	4

†-Clones that mapped exactly to locations previously published (Shoemaker and Olson, 1993).

Table 5. List of clones that mapped to locations other than previously published
 (Shoemaker and Olson, 1993).

Clone†	Published	Duplicate	Clone	Published	Duplicate
	Linkage Group	Locus		Linkage Group	Locus
A36	H	B	A644	I	M
A64	M	D	A806‡	F	C
A77	D	N	A810	H	F
A83‡	R	B	A885	G	B
A95	D	A	A947	D	Q
A111	A	E	A955	I	M
A122	U	A	B151	G	R
A130	H	N	K286‡	R	G
A132	B,L,H	J	K401	K	A
A136	A	E	K644	F,I	F
A235	G,D	K	K647	Q	B
A329	B	R‡	Bng27	E	K
A398	D	B	Bng62	O	K
A401	F	D	Bng119	Q	B
A489	L	K	Bng145	H	B
A517	E	F	Bng161‡	F	C
A567	S	K			
A584	G	M			
A632	B	G			

† - Clones designated by A, B, and K are *G. max* and Bng indicates *P. vulgaris* genomic clones.

‡ - Linked to one or more markers with undetermined location.

Table 6. RFLP loci found to be associated with SCN resistance in F_{2:3} population.

Locus	<u>Percent of variation explained‡</u>		
	Race 1a	Race 3a	Race 3b
A386 (E)†	1.5 (0.3249) ^{NS}	7.8 (0.0147)*	8.4 (0.0203) ^{NS}
C6 (G)	16.9 (0.0011)**	46.8 (0.0001)***	54.5 (0.0001)***
A102 (I)	10.7 (0.0131)*	7.4 (0.0245) ^{NS}	2.6 (0.2380) ^{NS}
B32 (J)	9.7 (0.0114)*	6.4 (0.0270) ^{NS}	0.5 (0.5776) ^{NS}
A315 (K)	14.4 (0.0018)**	8.9 (0.0090)*	10.1 (0.0107)*
Total§	51.7	63.5	64.6
Combined Model††	35.1 (0.0001)***	51.5 (0.0001)***	55.5 (0.0001)***

† - Letters in parentheses following the RFLP loci represent their corresponding linkage groups.

‡ - Based on regression analysis between the marker locus and the SCN disease response, the P values of the regression analyses are enclosed by parentheses.

§ - The sum of the individual effects of partial SCN resistance loci detected at P = 0.02.

†† - The combined effects of partial SCN resistance loci detected at P = 0.02 based on a multivariate linear regression analysis.

* - significant at P = 0.02, ** - significant at P = 0.002, *** - significant at P = 0.0002, and ^{NS} - not significant.

Table 7. RFLP loci found to be associated with SCN resistance in the F_{5:6} RIL population.

Locus†	<u>Percent of variation explained‡</u>		
	Race 1b	Race 3a	Race 6
A64 (D)	4.8 (0.0388) ^{NS}	7.1 (0.0112)*	10.9 (0.0014)**
A186 (F)	6.6 (0.0162)*	4.3 (0.0537) ^{NS}	2.4 (0.1559) ^{NS}
C6 (G)	34.8 (0.0001)***	49.9 (0.0001)***	54.0 (0.0001)***
Total§	41.4	57.0	64.9
Combined Model††	40.3 (0.0001)***	55.5 (0.0001)***	61.5 (0.0001)***

† - Letters in parentheses following the RFLP loci represent their corresponding linkage groups.

‡ - Based on regression analysis between the marker locus and the SCN disease response, the P values of the regression analyses are enclosed by parentheses.

§ - The sum of the individual effects of partial SCN resistance loci detected at P = 0.02.

†† - The combined effects of partial SCN resistance loci detected at P = 0.02 based on a multivariate linear regression analysis.

* - significant at P = 0.02, ** - significant at P = 0.002, *** - significant at P = 0.0002, and ^{NS} - not significant.

Table 8. Additional RFLP loci associated with SCN resistance in the absence of the effects of 'G' locus[†] in F_{5:6} population.

Locus	<u>Percent of variation explained[§]</u>		
	Race 1b	Race 3a	Race 6
A89 (B) [‡]	14.3 (0.0161)*	0.04 (0.9056)NS	8.4 (0.0203)NS
A23 (L ₁)	22.7 (0.0016)**	0.1 (0.8434)NS	0.9 (0.5601)NS
K644 (I)	10.7 (0.0131)*	3.8 (0.2217)NS	0.1 (0.8591)NS
B32 (J)	2.5 (0.3239)NS	29.4 (0.0002)***	15.8 (0.0100)*
A586 (G ₂)	7.3 (0.1577)NS	7.2 (0.1600)NS	23.8 (0.0072)*
A77 (N)	0.4 (0.7003)NS	7.6 (0.0807)NS	16.1 (0.0093)*

[†] - The genomic region on linkage group 'G' containing a major partial resistance locus defined by seven RFLP markers.

[‡] - Letters in parentheses following the RFLP loci represent their corresponding linkage groups. Subscript following the linkage group indicates two independent loci on the same linkage group.

[§] - Based on regression analysis between the marker locus and the SCN disease response, the P values of the regression analyses are enclosed by parentheses.

* - significant at P = 0.02, ** - significant at P = 0.002, *** - significant at P = 0.0002, and NS - not significant.

Table 9. Race specificity of loci associated with SCN resistance.

Slope of the regression line between marker-locus and SCN disease response									
Race	F _{2:3} population					F _{5:6} RIL population			
	C6G†	A386E	A102I	B32J	A315K	C6G	A64D	A186F	
Race 1a	-0.080 ^a	NS	-0.062	-0.059	-0.076 ^c	-	-	-	
Race 1b	-‡	-	-	-	-	-0.383 ^d	NS	+0.153	
Race 3a	-0.180 ^b	+0.070	NS	NS	-0.081 ^c	-0.396 ^d	+0.145 ^e	NS	
Race 3b	-0.243 ^b	NS	NS	NS	-0.109 ^c	-	-	-	
Race 6	-	-	-	-	-	-0.368 ^d	+0.158 ^e	NS	

† - Letters following the RFLP loci represent their corresponding linkage groups.

‡ - Indicates that SCN disease response against the race was not evaluated for the marker-locus.

Slopes of a marker-locus followed by a common letter are not significantly different from each other at P = 0.05, while slopes not indicated by any letter are significantly associated with only one SCN race and could not be statistically compared with the non-significant slopes detected in the other races.

NS - regression analysis between the marker and SCN disease response was not significant at P = 0.02.

Table 10. Regression analysis between the F_{2:3} and F_{5:6} SCN indices

Source	Degrees of freedom	Sum of squares	Mean Square	F-test	P - value
Regression	1	1.536	1.536	31.154	0.0001
Residual	66	3.254	0.0499		
Total†	67	4.789			
r ²		0.321			

† - In this analysis, out of the possible 76 F_{2:3} lines assayed for SCN resistance at the F₃ generation, only 67 had corresponding F_{5:6} SCN indices due to the unavailability of seed among several lines for SCN assay in the F₆.

Table 11. Regression analysis between the F₂ RFLP C6 genotype and F_{5:6} SCN indices

Source	Degrees of freedom	Sum of squares	Mean Square	F-test	P - value
Regression	1	1.225	1.225	24.77	0.0001
Residual	29	4.085	0.049		
Total†	30	7.355			
r ²		0.461			

† - Based on the analysis of 30 homozygous resistant and susceptible lines for RFLP C6 genotype. Heterozygous individuals were not included in the analysis.

Table 12. Regression analysis between the F₂ RFLP C6-Bng122 genotype[†] and F_{5:6} SCN indices.

Source	Degrees of freedom	Sum of squares	Mean Square	F-test	P - value
Regression	1	1.447	1.447	54.803	0.0001
Residual	22	0.581	0.026		
Total‡	23	2.027			
r ²	0.714				

† - Based on the genotypic scores of the RFLP C6-Bng122 interval of individual F₂ plants.

Plants were predicted to be "resistant" in the F₆ generation if they inherited the banding pattern of the resistant parent, PI 209332, and were given a numerical score of 2. Plants were rated "susceptible" if they inherited the banding pattern of the susceptible parent, Evans, and were given a score of 1. The interval between RFLP markers C6 and Bng122 (Lander and Botstein, 1989) was estimated by comparing the numerical scores for each marker. Every time the same score was given for both markers, that score was retained and each time the scores differed, it was treated as a missing value. Heterozygous individuals or plants that inherited the banding patterns of both parents were not included in the analysis.

‡ - Only the lines that had identical genotypic scores for Bng122 and C6 were included in the analysis.

Table 13. Comparison of the reliability of predicting SCN disease response in the F₅:F₆ generation between F₂:F₃ SCN indices and F₂ RFLP C6 genotype.

Observed [†]	F ₂ phenotype prediction [‡]			F ₂ genotype prediction [§]		
	Resistant	Susceptible	Total	Resistant	Susceptible	Total
Resistant	12	11	23	10	2	12
Susceptible	2	43	45	3	16	19
Total	14	54	68	13	18	31
x ²		21.21			13.78	
		(P = 0.0001)			(P = 0.0002)	

[†] - Based on mean SCN indices of 10 F₆ plants; plants were rated "resistant" if the number of cysts recovered were less than 30% than those recovered from the susceptible check and "susceptible" if the number of cysts recovered exceeded 30%.

[‡] - Based on mean SCN indices of five F_{2:3} plants, which is a typical number of plants used in most breeding programs. Plants were predicted to be "resistant" in the F₆ generation if the averaged number of cysts recovered on five F_{2:3} plants were less than 30% than those recovered from the susceptible check and "susceptible" if the number of cysts recovered exceeded 30%.

[§] - Based on RFLP C6 genotype of individual F₂ plants; plants were predicted to be "resistant" in the F₆ generation if they inherited the banding pattern of the resistant parent, PI 209332, and "susceptible" if they inherited the banding pattern of the susceptible parent, Evans. Heterozygous individuals or plants that inherited the banding patterns of both parents were not included in the analysis.

Table 14. Predicting SCN response in the F_{5:6} generation using the genotypic scores of the RFLP C6-Bng122 interval.

Observed†	Prediction based on F ₂ C6-Bng122 genotype‡		
	Resistant	Susceptible	Total
Resistant	8	0	8
Susceptible	2	14	16
Total	10	14	24
χ^2			16.8
		(P = 0.0001)	

† - Based on mean SCN indices of 10 F₆ plants; plants were rated resistant if the number of cysts recovered were less than 30% than those recovered from the susceptible check and susceptible if the number of cysts recovered exceeded 30%.

‡ - Based on the genotypic scores of the RFLP C6-Bng122 interval of individual F₂ plants. Plants were predicted to be "resistant" in the F₆ generation if they inherited the banding pattern of the resistant parent, PI 209332, and were given a numerical score of 2. Plants were rated susceptible if they inherited the banding pattern of the susceptible parent, Evans, and were given a score of 1. The interval between RFLP markers C6 and Bng122 (Lander and Botstein, 1989) was estimated by comparing the numerical scores for each marker. Every time the same score was given for both markers, that score was retained and each time the scores differed, it was treated as a missing value. Heterozygous individuals or plants that inherited the banding patterns of both parents were not included in the analysis.

Figure 1a-1c. RFLP linkage map of soybean(based on Shoemaker and Olson, 1993).

Horizontal lines represent linkage groups, tick marks represent mapped RFLP markers. Markers preceded by the letters A, B, C, K, L and P are soybean genomic clones, Bng are common bean clones, and Mng are mungbean clones. Seed coat indicates the locus for seed coat color.

Figure 1a

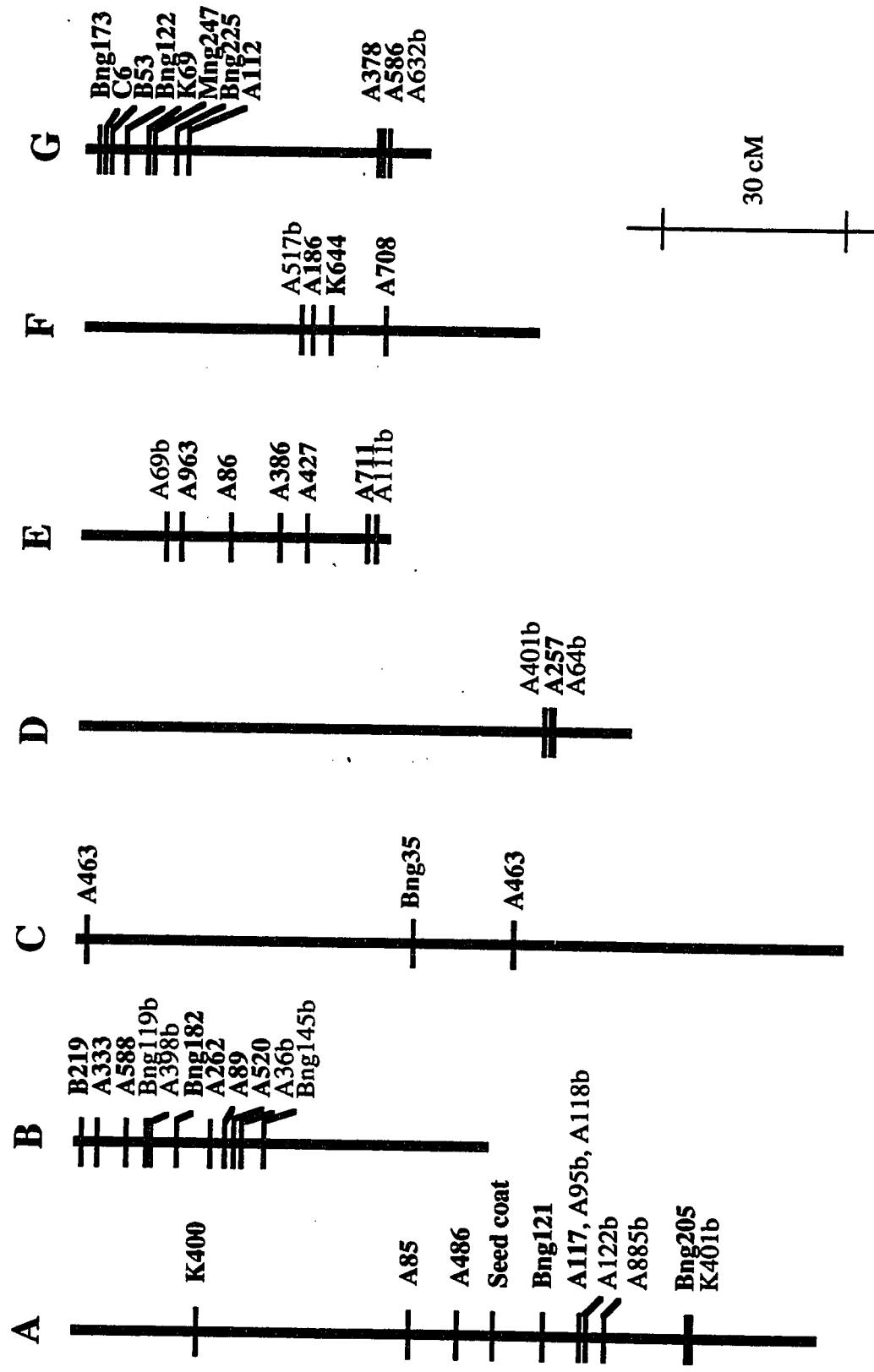
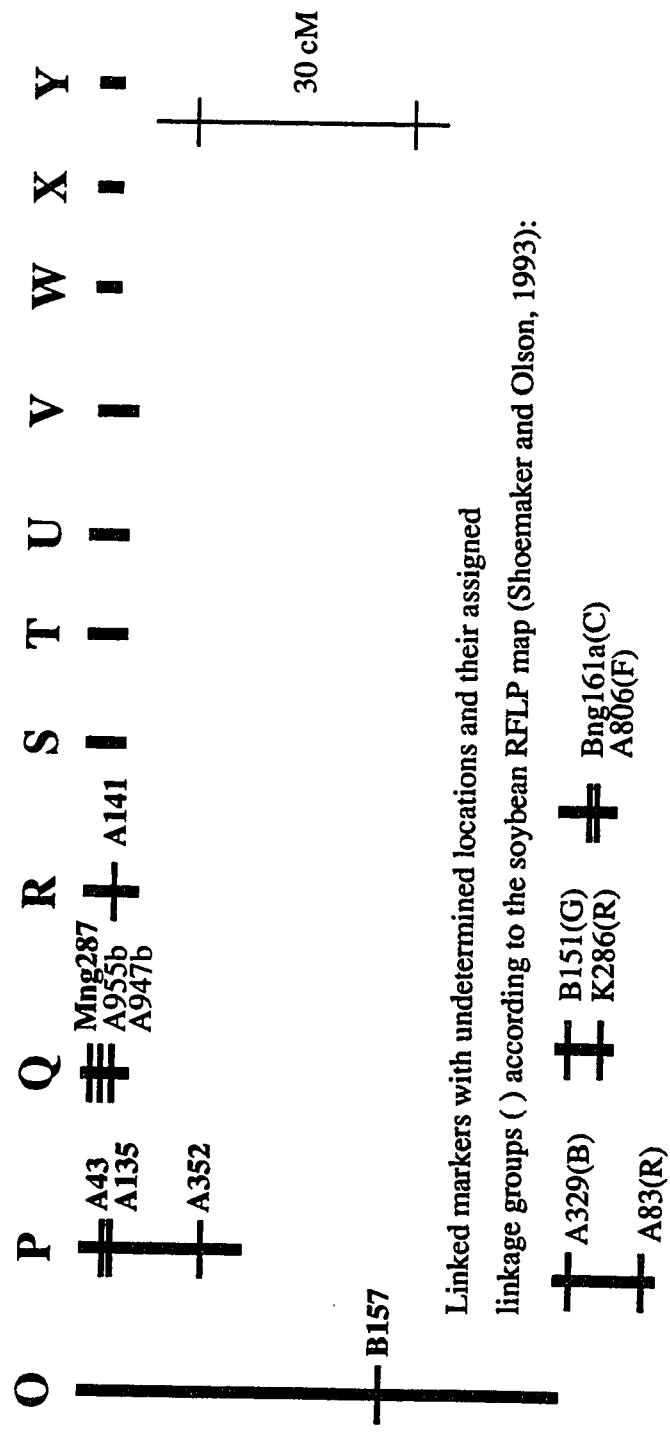


Figure 1b



Figure 1c



Linked markers with undetermined locations and their assigned linkage groups () according to the soybean RFLP map (Shoemaker and Olson, 1993):

⊕ A329(B) ⊕ B151(G)
⊕ A83(R) ⊕ K286(R) ⊕ Bng161a(C)
⊕ A806(F)

Unlinked markers that were not able to place onto this map and their assigned linkage groups () according to the soybean RFLP map (Shoemaker and Olson, 1993):

A136(A)
A162(H)
B151b(G)
Bng161c(C).

Figure 2a-2c. Frequency distributions of 76 F₂ lines for mean SCN disease response to race 1a (2a), race 3a (2b), and race 3b (2c). Vertical axes show the number of individuals with a given disease index. Horizontal axes represent the corresponding SCN indices for all 76 lines.

Figure 2a

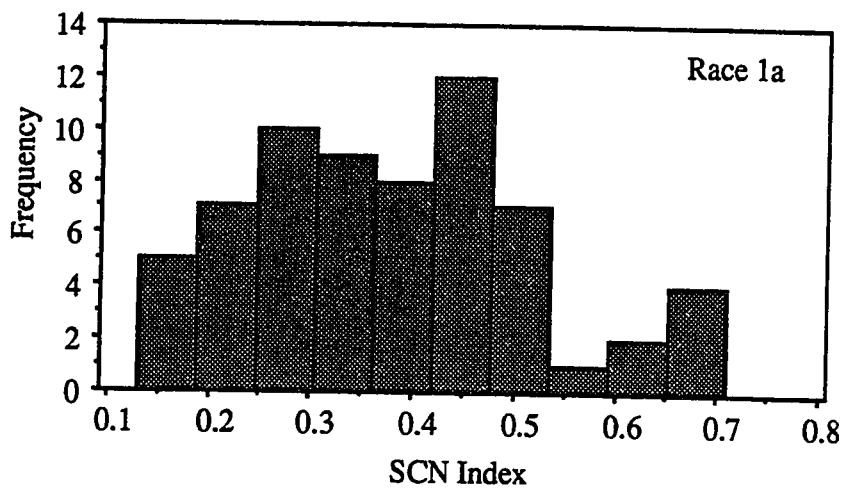


Figure 2b

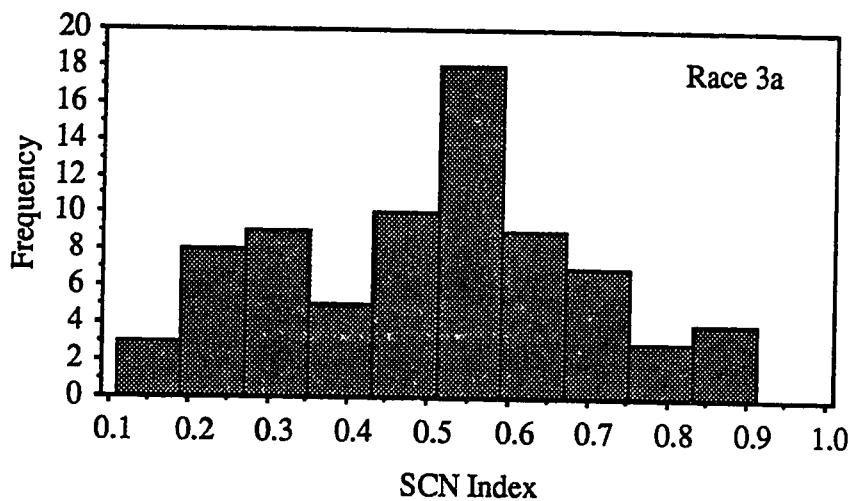


Figure 2c

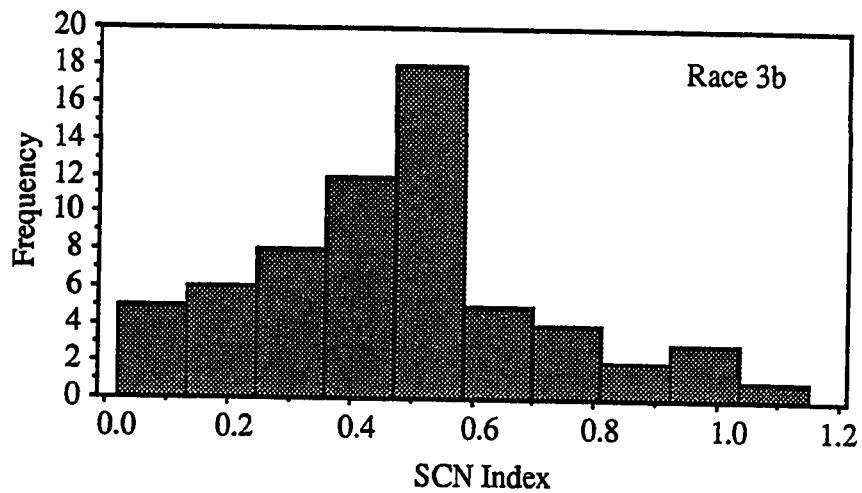


Figure 3a-3c. Frequency distributions of 98 F₆ RIL lines for mean SCN disease responses to race 1b (3a), race 3a (3b), and race 6 (3c). Vertical axes show the number of individuals for a given disease index. Horizontal axes represent the corresponding SCN indices for all 98 lines.

Figure 3a

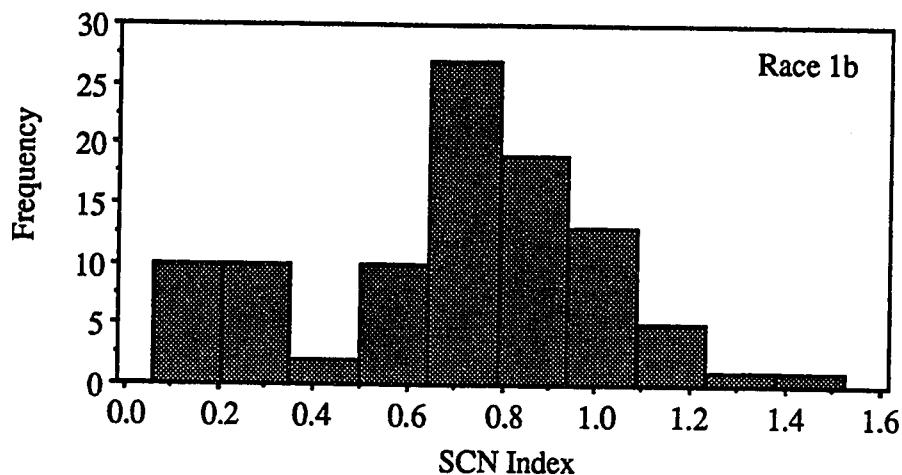


Figure 3b

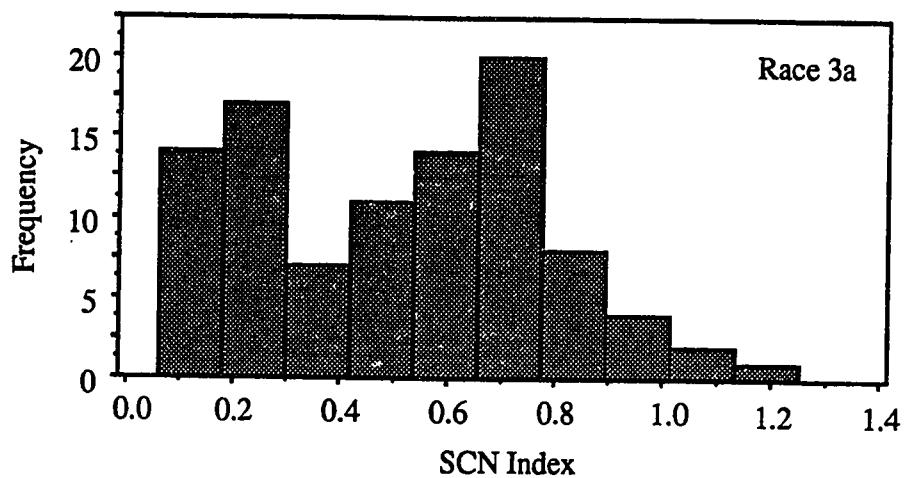


Figure 3c

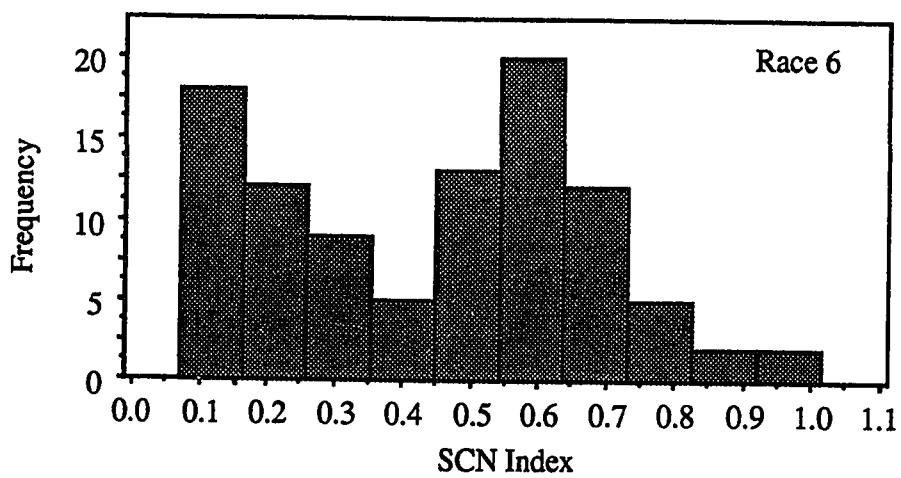


Figure 4a-4c. Scattergrams showing the relationships of SCN disease responses of 76 F₂ lines against three SCN races. Figure 4a shows the relationship between race 1a (x-axis) and race 3a (y-axis). Figure 4b shows the relationship between race 1a (x-axis) and race 3b (y-axis). Figure 4c shows the relationship between race 3a (x-axis) and race 3b (y-axis). The equation above each scattergram is the equation for best-fitting line for that scattergram.

Figure 4a

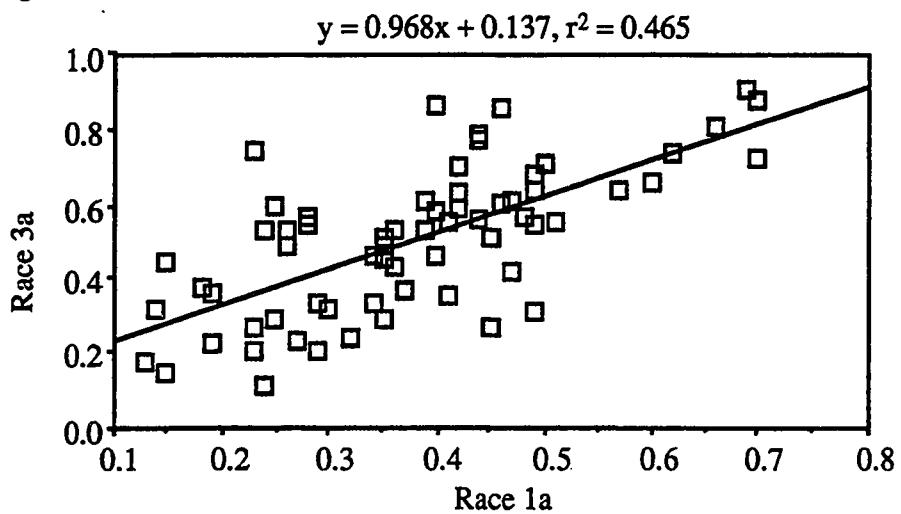


Figure 4b

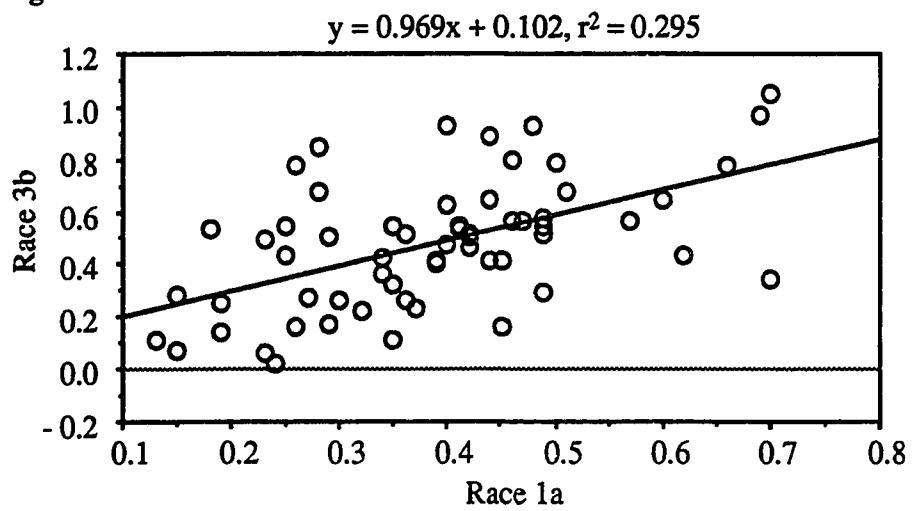


Figure 4c

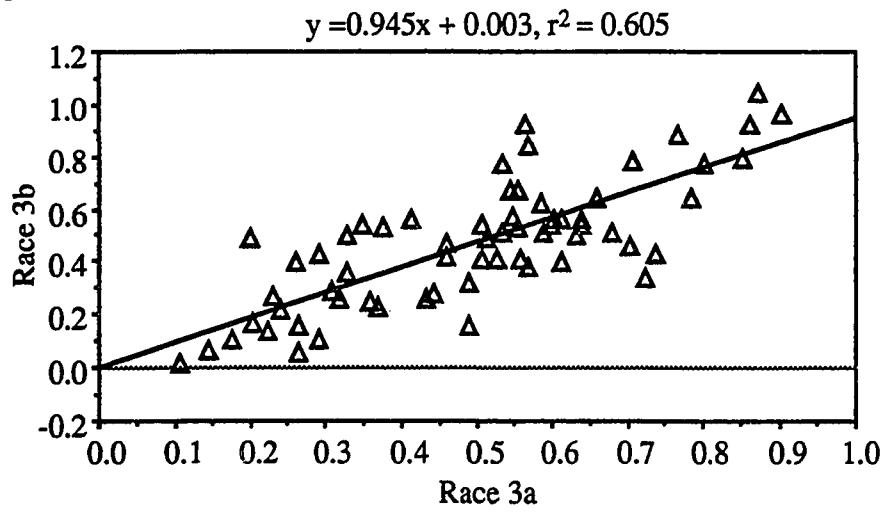


Figure 5a-5c. Scattergrams showing the relationships of SCN disease responses of 98 F₆ RIL lines against three SCN races. Figure 4a shows the relationship between race 1b (x-axis) and race 3a (y-axis). Figure 4b shows the relationship between race 1b (x-axis) and race 6 (y-axis). Figure 4c shows the relationship between race 3a (x-axis) and race 6 (y-axis). The equation above each scattergram is the equation for best-fitting line for that scattergram.

Figure 5a

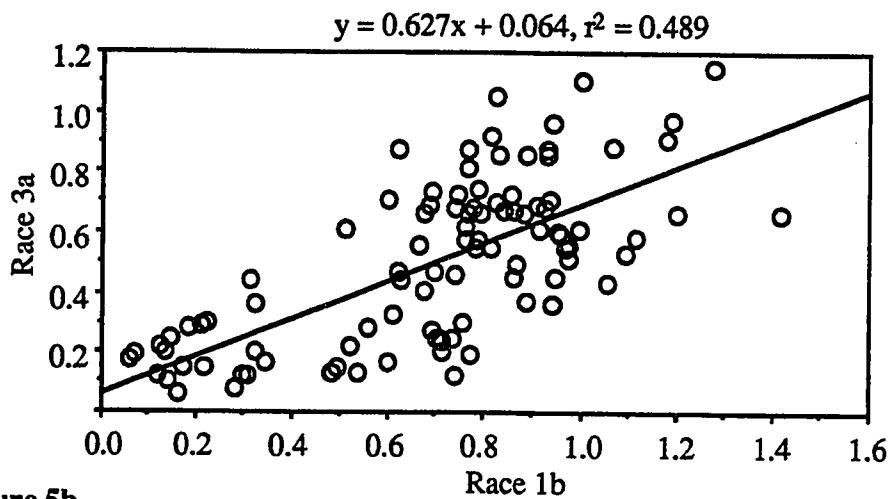


Figure 5b

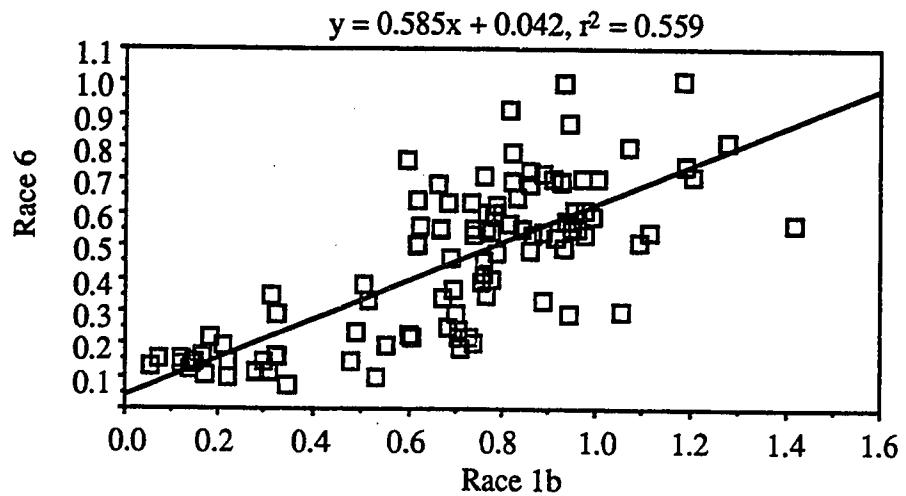
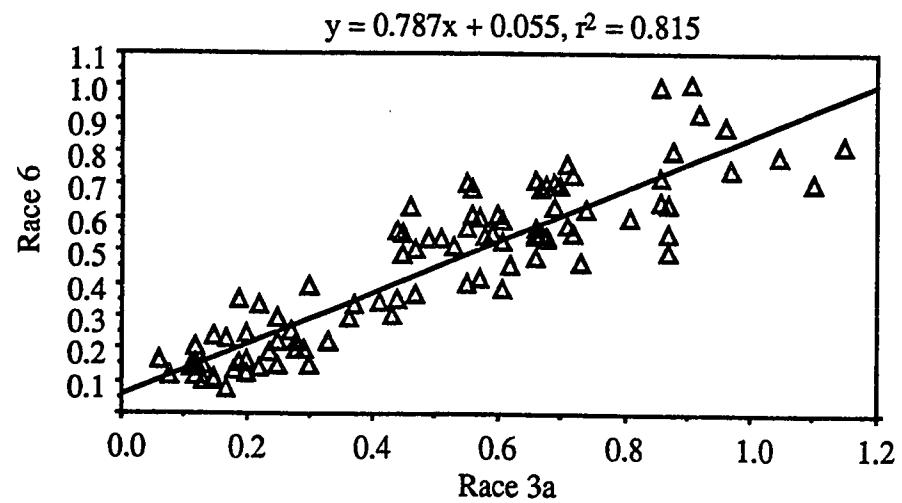


Figure 5c



CHAPTER 3

**TARGETED COMPARATIVE GENOME ANALYSIS AND QUALITATIVE
MAPPING OF A MAJOR PARTIAL RESISTANCE GENE FOR SOYBEAN
CYST NEMATODE**

ABSTRACT

Using restriction fragment length polymorphisms (RFLPs), a major partial resistance locus to soybean cyst nematode (SCN; *Heterodera glycines* Ichinohe) was identified on the chromosome arm of linkage group 'G' of the soybean [Glycine max (L.) Merrill] molecular linkage map. This locus explains 51.4% (LOD = 10.35) of the total phenotypic variation in SCN disease response in PI 209332, 52.7% (LOD = 15.58) in PI 90763, 40% (LOD = 10.50) in PI 88788, and 28.1% (LOD = 6.94) in 'Peking'. Initially, the region around the major partial SCN resistance was poorly populated in terms of DNA markers. To increase the density of markers in this region, first random, and later targeted comparative mapping with mungbean [*Vigna radiata* (L.) R. Wilcz.] and common bean (*Phaseolus vulgaris* L.) RFLPs was used to increase the marker density sevenfold to one RFLP every 1.8 centimorgans (cM). Even with this high marker density, the inability to resolve SCN disease response into discrete Mendelian categories posed a major limitation in the localization of this trait to a specific genomic region. Thus, qualitative scoring of SCN disease response was carried out in a recombinant inbred population derived from 'Evans' x Plant Introduction (PI) 209332 using a 30% cut-off for resistance. The strategy described in this study made it possible to rapidly identify recombinants between the two markers flanking the resistance locus in PI 209332 (Bng122 and Bng173). Using the computer software *JoinMap*, an integrated map of the region of interest was created, placing the SCN locus 2.1 cM from B53 and 6.1 cM from Bng122. This study demonstrates how various molecular mapping strategies, such as RFLP mapping, comparative genome and integrated mapping, together with qualitative scoring of a quantitative trait, can provide the necessary tools for developing a high resolution map around a quantitative trait locus (QTL) as a basis for eventual map-based cloning.

INTRODUCTION

Many economically important traits in plants, including some forms of disease resistance, are quantitative in nature. These traits are characterized by complex inheritance patterns and continuous distribution of phenotypic values in segregating populations. Thus, breeding for quantitative traits is difficult and time-consuming. The advent of DNA markers, such as restriction fragment length polymorphisms (RFLPs), allows the resolution of complex, multigenic traits into their individual Mendelian components (Paterson *et al*, 1988), paving the way for cloning complex disease resistance genes and other QTLs.

The success in cloning qualitative plant resistance genes can be attributed, in part, to the development of molecular tools required for insertional mutagenesis and map-based cloning (Staskawicz *et al*, 1995). In the past, insertional mutagenesis using transposons to identify and isolate genes have been widely used in many species including plants. As a result, the first plant resistance gene (*Hm1*) was successfully cloned in maize (*Zea mays* L.) (Johal and Briggs, 1992). Similar successes have been attained using transposons in cloning the *N* gene against tobacco mosaic virus (TMV) in tobacco (*Nicotiana sylvestris* L) (Whitham *et al*, 1994), *Cf-9* gene against *Cladosporium fulvum* in tomato (*Lycopersicum esculentum* Mill.) (Jones *et al*, 1994), and the *L⁶* gene against *Melampsora lini* in flax (*Linum usitatissimum* L.) (Ellis *et al*, 1995).

By contrast, the *Pto* gene for *Pseudomonas syringae* in tomato (Martin *et al*, 1993) and the *RPS2* gene for *P. syringae* pvs. tomato and *maculicola* in *Arabidopsis* (Bent *et al*, 1994) are the only two examples of successful map-based positional cloning of disease resistance genes. The development of high density physical-genetic maps and the relatively small genome sizes of *Arabidopsis* (150 megabases, Mb) and tomato (950 Mb) have facilitated the positional cloning of these resistance genes (Staskawicz *et al*, 1995). In

addition, both of these species have a relatively small number of repeated sequences present in their genomes (Martin *et al*, 1993; Bent *et al*, 1994). In more complex and larger plant genomes, such as soybean [*Glycine max* (L.) Merrill] (Bennet *et al*, 1982; Goldberg, 1978), map-based positional cloning can be more challenging. Furthermore, gene duplication due to soybean's ancestral tetraploid origin (Buttery and Buzzell, 1976; Palmer and Kilen, 1987; Hymowitz and Singh, 1987; Apuya *et al*, 1988) and the lack of polymorphism in genomic regions of interest can further complicate efforts towards map-based positional cloning in soybean. In the present study, quantitative trait loci (QTLs) for partial resistance against soybean cyst nematode, *Heterodera glycines* Ichinohe (SCN), a serious pest of soybean, have been identified using RFLPs (Concibido *et al*, 1994). Despite the availability of these markers, efforts towards developing a high density genetic map near the SCN region has been hampered by the limited number of polymorphisms available (Concibido *et al*, 1995) and the inability to characterize SCN as a qualitative trait.

The development of other powerful applications of DNA markers, such as comparative genome (Tanksley *et al*, 1988) and integrated mapping strategies (Stam, 1993), offer potential ways to increase marker density near genomic regions of interest; a crucial step towards map-based positional cloning. Comparative genome mapping, a strategy that utilizes mapping information from one population to predict the properties of other populations, can be used to increase marker density near genomic regions of interest. Likewise, integrated or "Join" mapping (Stam, 1993) allows the development of high density maps by integrating linkage maps that were developed independently. This mapping approach allows genes that do not segregate in one mapping population to be placed onto a joint map by combining information from different mapping populations.

This chapter describes the use of comparative genome and integrated mapping strategies in combination with the resolution of a partial resistance gene as a qualitative

character to develop a high density genetic map near the major partial SCN resistance region.

MATERIALS AND METHODS

Mapping populations

Four F₂ segregating soybean populations and an F_{5:6} recombinant inbred line (RIL) population were used in the study. The F₂ populations were constructed by crossing the maternal parent, 'Evans' (a SCN-susceptible variety) with the following sources of SCN resistance to race 3 as male parents: Plant Introduction (PI) 209332 (Anand and Brar, 1983; Anand and Gallo, 1984), PI 90763 (Anand *et al.*, 1985; Hartwig, 1985), PI 88788 (Anand *et al.*, 1985; Hartwig, 1985) and 'Peking' (Anand *et al.*, 1985; Hartwig, 1985). The Evans x PI 209332 population consisted of 76 lines, 115 lines for the Evans x PI 90763, 110 lines for the Evans x Peking and 106 for the Evans x PI 88788 population.

The F_{5:6} RIL population was constructed by advancing the 76 lines from the Evans x PI 209332 F₂ population, plus an additional 22 sibling lines, to the F₅ generation by single seed descent both in the greenhouse and in the field. Seventy-five seeds from each line were row-planted in Rosemount, St. Paul, MN and used as source of bulk leaf tissue for DNA extraction and analysis. Plants were allowed to recover and set F₆ seeds, which were bulked and saved for SCN disease assay.

RFLP probes

Cloned DNA fragments from soybean, mungbean [*Vigna radiata* (L.) R. Wilcz.], cowpea [*V. unguiculata* (L.) Walp.], and common bean (*Phaseolus vulgaris* L.) were used as probes for RFLP analysis. These probes were prepared from *Pst*I random genomic libraries as described previously (Keim *et al.*, 1989; Chase *et al.*, 1990; Young *et al.*, 1992).

The soybean clones were provided by Dr. Randy Shoemaker (Iowa State University, Ames, IA) and the common bean clones were from Dr. Edward C. Vallejos (University of Florida, Gainesville, FL). Briefly, the following protocol was followed in preparing these libraries: DNA was digested with *Pst*I, separated according to size by sucrose gradient centrifugation, and the fraction between 500 and 3,000 base pairs ligated into pUC18 (Gibco BRL, Gaithersburg, MD) by standard molecular methods (Sambrook *et al*, 1989).

DNA extraction and Southern analysis

Plant DNA was extracted by the modified method of Dellaporta *et al* (1983) and analyzed following the technique of Young *et al* (1992). First, the best restriction enzyme for each DNA clone was determined. Individual clones were probed against "Parental survey" blots containing parental DNA digested with fifteen different restriction enzymes: *Ban*I, *Bam*HI, *Bcl*II, *Bst*nI, *Dde*I, *Dra*I, *Eco*RI, *Eco*RV, *Hae*III, *Hind*III, *Hinf*I, *Rsa*I, *Sca*I, *Taq*I, and *Xba*I (New England Biolabs, Beverly, MA; Promega, Madison WI; Gibco/BRL, Gaithersburg, MD). Digested DNA was electrophoresed on 1% agarose gel and transferred onto Hybond N+ membranes (Amersham Corporation, Arlington Heights, IL) using a method adapted from Southern (1975), consisting of blotting in an alkaline solution of 0.5M NaOH and 0.5 M NaCl. The best clone/enzyme combination was then used for segregation analysis. DNA from all 76 F₂ or 98 F₅ individuals was digested and blotted in a similar manner to produce F₂ and F₅ "progeny" blots, respectively. Each "progeny" blot contained DNA from all F₂ or F₅ individuals and their parents digested with a single restriction enzyme. In this way, each RFLP clone could be hybridized against F₂ or F₅ DNA that had been digested with the appropriate restriction enzyme for that clone, based on the results of the corresponding parental survey.

DNA hybridizations

Cloned DNA inserts were amplified by the polymerase chain reaction (PCR) (Saiki *et al*, 1988) for use in radiolabeling reactions and nucleic acid hybridization as described by Young *et al* (1992). Briefly, individual bacterial colonies were grown overnight in suspension culture, centrifuged for 5 min at 2000 x g, rapidly frozen and thawed, and centrifuged at 2000 x g and the supernatants used as plasmid templates for the reactions. Two microliters of the supernatant was then used in a PCR reaction to amplify the insert sequence using the M13-forward and -reverse sequence (Gibco/BRL, Gaithersburg, MD) as primers. About 40 to 50 ng of the amplified product was then radiolabeled by the random hexamer reaction (Feinberg and Vogelstein, 1983).

The radiolabeled sequence was then incubated with either a "Parental survey", F₂ or F₅ "progeny" blot for 16-24 hr at 60 °C in a hybridization solution of 5x saline sodium citrate (SSC), 0.1 M phosphate buffer, pH 7.5, 1x Denhardt's solution (2% each of Ficoll, polyvinylpyrrolidone and bovine serum albumin fraction V), 0.1% sodium dodecyl sulfate (SDS), and 5% dextran sulfate. After incubation, blots were washed three times for 15 min each at 60 °C at medium stringency (2X, 1X and 0.5X SSC with 0.1% SDS). After washing, blots were placed against Kodak X-AR film and stored at -80 °C for one to seven days to produce autoradiographs. Blots were re-used up to seven times after stripping the previous probe by a series of washes in 0.1N NaOH, 0.25 M TRIS-HCL, pH 7.5 with 2X SSC and 0.1% SDS and 0.5X SSC with 0.1% SDS at room temperature. Blots were then used immediately or blot-dried for later use.

Soybean cyst nematode assay

Each population was assayed for SCN resistance on separate occasions. F₃ seeds were germinated in sand-filled Ray Leach cone-tainers (Stuewe & Sons, Inc., Portland, OR) placed in sand-filled 20 liter buckets in a waterbath. Ten F₃ seedlings from each F₂

line were assayed in the waterbath. Each plant was inoculated with 2,000 eggs three days after germination with SCN race 3. The SCN race 3 isolate used in the assays was maintained in cone-tainers planted to susceptible soybean hosts 'Essex' or Evans. Cysts and white females from each isolate were then collected every 28 days, stored in sand at 4 °C and later used for inoculations. Race determination tests were regularly conducted on the isolate to check the possibility of race shifts.

At the time of each inoculation, cysts and white females were recovered from sand, ground in tissue grinder to release the eggs, and inoculum was standardized using a hemacytometer. In each assay, the parental lines, the soybean differential cultivars (Peking, Pickett, PI 90763 and PI 88788), and the susceptible check, Essex, were included in all tests. Waterbath temperatures were maintained at 28 °C with 16-hour daylength for 28 days. On the 28th day, individual plants were uprooted and cysts were collected by blasting the roots with pressurized water and cysts were collected on sieves. The total number of cysts from individual plants was counted under a dissecting microscope and converted to an index by dividing this number by the average total number of cysts on the susceptible parent. For each F₂ line, SCN indices of the 10 F₃ plants were averaged to estimate mean SCN index for that line. Exactly the same procedure was used for the SCN assay of the RILs.

Linkage analysis and QTL mapping

A comprehensive RFLP map was constructed by linkage analysis in the Evans x PI 209332 population, where the most mapping information is available. To accomplish this, a total of 258 genomic clones were analyzed by hybridization with "parental survey" blots and, of these clones, 133 were subsequently hybridized against F₂ and F₅ "progeny" blots for segregation analysis. Segregation for each clone was coded into a numeric form and analyzed by two-way contingency table analysis with the statistics software Statview II

(Abacus Concepts, Berkeley, California), and by Mapmaker (Lander *et al*, 1987). Linkage between RFLP markers was inferred if the probability of observing a chi-square value was less than 0.001 or if the "LOD" score exceeded 3.0. A LOD score is the \log_{10} of the ratio between the odds of the null hypothesis that the markers are linked against an alternative hypothesis that they are not linked. To determine the order of markers, a multipoint analysis was used in which the favored order of markers exceeded other possible orders by a LOD of 3.0 or greater. The resulting RFLP map contained 98 RFLP markers distributed throughout linkage groups.

At the same time, the search for genomic regions associated with SCN resistance was conducted. Briefly, the F₂ genotypic classes for each DNA marker were contrasted with SCN disease response using regression analysis and analysis of variance (ANOVA). These analyses were performed using Statview-II and SuperAnova (Abacus Concepts, Berkeley, California) and SAS (SAS Institute). A significance level of P < 0.002 was used to uncover association between a DNA marker and SCN disease response to minimize the detection of false positives experiment-wide (Lander and Botstein, 1989). Data were also analyzed by Mapmaker-QTL (Lander *et al*, 1987). Putative QTLs were inferred whenever the LOD score exceeded 2.7. Interaction among loci were not evaluated in this study since the population sizes of both populations were too small to conduct meaningful tests. As soon as the major partial SCN resistance region on linkage group 'G' was identified in the Evans x PI 209332 (Concibido *et al*, 1994; Concibido *et al*, 1995), detailed QTL analyses of linkage group 'G' were also performed on the Evans x PI 88788, Evans x PI 90763, and Evans x Peking populations.

Comparative genome mapping

To increase marker density near the region of interest, specifically the locus on linkage group 'G', which has been identified as a major partial SCN resistance gene

(Boutin *et al*, 1992; Concibido *et al*, 1994; Concibido *et al*, 1995), DNA clones from common bean and mungbean were randomly chosen as probes for linkage mapping in soybean. The results of Boutin *et al* (1995) on the comparative genome mapping in three cultivated species of the legume tribe Phaseoleae: *P. vulgaris*, *V. radiata* and *G. max*, demonstrated that many genomic segments were conserved between these species, including the region on linkage group 'G' near SCN resistance in *G. max*. This information was then used in a targeted comparative mapping strategy to further increase marker density near the locus on 'G'. Briefly, *V. radiata* and *P. vulgaris* markers in the relevant conserved regions were hybridized with soybean F₂ and F₅ blots and placed onto the soybean RFLP map.

"Join" mapping

JoinMap (Stam, 1993), a computer software program that allows integration of genetic linkage maps from distinct mapping populations, was used to create a consensus map of linkage group 'G' based on mapping information from the RIL and the four F₂ populations. In this way, markers that did not segregate in one mapping population could be placed onto a single map by combining information from other populations. The presence of common markers among the populations, a requirement for map integration, facilitated the development of a consensus map of linkage group 'G'. Briefly, *JoinMap* first pooled the segregation data of the four mapping populations, recalculated the pairwise recombination frequencies and LOD scores, and then created a joint linkage map with the best-fitting arrangement with an LOD of 3.0 (Stam, 1993; Hauge *et al*, 1993).

"Qualitative" mapping of SCN

In an attempt to resolve SCN disease response into its simple Mendelian components, a "qualitative" mapping of SCN was conducted in the RIL population.

Briefly, lines that had SCN indices greater than 40% were rated "susceptible" and given a numerical score of 1, and plants with indices less than or equal to 30% were rated "resistant" and given a numerical score of 2 (Schmitt and Shannon, 1992). Lines that had indices of 31-39% were not included in the analysis since they cannot be classified with high degree of certainty. This range was chosen because the SCN distribution of the 98 RIL lines started to break into two distributions at this point (Figure 1). The extra caution in scoring individual lines falling in this range was taken since misclassification of these lines can drastically bias the estimation of map distances between the flanking markers and the qualitative SCN scoring. Assigning qualitative disease scores was only possible in the RIL population (Figure 1), as the distribution of SCN indices was not sufficiently discrete in the other mapping populations analyzed (data not shown).

RESULTS AND DISCUSSION

Identification of a major partial SCN resistance locus

Previously, several genomic regions significantly associated with partial resistance to SCN race 3 were uncovered in PI 209332 using RFLP markers (Concibido *et al.*, 1994). Of all the genomic regions identified, the locus on 'G', which is common among various sources of SCN resistance, appeared to be the most important partial resistance locus. The 'G' locus explained up to 51.4% of the total phenotypic variation in SCN disease response in PI 209332, 52.7% in PI 90763, 40% in PI 88788 and 28% in Peking (Table 1). In all populations, this locus appeared to behave in an additive to partially recessive manner, indicated by the relatively low dominance to additivity ratios observed in all populations (Table 1). To determine whether the genetic effects of the locus on 'G' was largely additive, dominant or recessive among mapping populations, the relative likelihood of models restricting the locus on 'G' to specific types of gene action was evaluated following

the method of Paterson *et al* (1988). Briefly, using Mapmaker-QTL, the locus on 'G' was tested for fitness to a purely additive model by forcing the dominance term $d = 0$, a dominant model by forcing $d = a$ (additivity), and a recessive model by forcing $d = -a$. For example, if the recessive and dominant models were at least one LOD unit (ten-fold) less likely than the additive model, the other two models were rejected in favor of the additive model (Paterson *et al*, 1988). Using this analysis, except for Evans x Peking population, which tested to be partially additive to partially recessive, the locus on 'G' was largely additive in gene action in the other four mapping populations.

Prior to this dissertation, the only marker near the partial SCN resistance locus on linkage group 'G' was RFLP K69 (Boutin *et al*, 1992). More detailed analysis of the genomic region around this locus resulted in the identification of RFLP markers with stronger associations with SCN disease response. In particular, the interval with RFLP markers C6 and Bng122 was identified as explaining a large percentage of the total phenotypic variation in SCN disease response (Chapter 2 of this dissertation). This locus is likely the basic gene for SCN resistance to race 3 postulated by Triantaphyllou (1975). He suggested that resistance to race 3 appears to be necessary for resistance to any of the other races, since race 3 had no genes for parasitism of resistance genes.

Marker saturation by comparative genome mapping

Initially, the soybean map had a low density of markers in the region near the major partial SCN resistance gene (Figure 2). Within the 55.2 cM spanning the genomic region around the SCN resistance locus, there were only five markers available for mapping when the study began (Figure 2). Among the five markers available, RFLP C6 was found to be the closest to the resistance locus at that time. To increase marker density around this region, *P. vulgaris* and *V. radiata* clones were randomly tested as probes that might potentially map to the region of interest based on the study of Boutin *et al* (1995). Using

this approach, three *P. vulgaris* clones were mapped onto the region, increasing marker density from one marker every 11 cM to one every seven cM (Figure 2). Consequently, the comparative genome mapping study among three cultivated species of the legume tribe Phaseoleae: *P. vulgaris*, *V. radiata* and *G. max* by Boutin *et al* (1995), suggested several other *P. vulgaris* and *V. radiata* markers might be tightly linked to SCN resistance in *G. max*. Using this information, three more *P. vulgaris* clones and a *V. radiata* clone mapped to the region further increasing marker density to one marker every five cM (Figure 2). Within the 14.7 cM region (Bng173-Bng30 interval) containing the major partial SCN resistance gene, final marker density now reaches one RFLP every 1.8 cM. In addition, three *P. vulgaris* markers Bng189, Bng113 and Bng83 are not yet linked by segregation analysis in soybean (Figure 2), but are known to be tightly linked in *P. vulgaris* (Vallejos *et al*, 1992). These three markers have also been shown by pulsed field gel electrophoresis to be physically linked in soybean (Danesh *et al*, 1995). Thus, marker density within the 14.7 cM region of interest is now as high as one marker every 1.2 cM. These results demonstrate how the degree of conservation among these three related species can accelerate mapping research in soybean.

Integrated map of linkage group 'G' containing a major partial SCN resistance gene

Despite the 15 potential RFLPs available in the region near the major partial SCN resistance gene, (ten of which were uncovered by comparative mapping), the lack of DNA polymorphism in the mapping populations in this study, particularly in Evans x PI 209332, which is the most characterized, impeded progress towards the development of a high resolution map in this genomic region. Out of the possible 15, twelve markers could be mapped onto the Peking population, eight in PI 209332 and PI 90763, and seven in PI 88788 (Table 2). Markers Bng173, Mng247, A112, L156, C6 and Bng122 were

polymorphic in all populations, markers Bng225 and K69 were polymorphic in three populations, and marker Bng171 was polymorphic only in two populations (Table 2). However, the polymorphism observed in marker Mng247 in Peking, markers A112 and L156 in PI 88788 and Peking populations were different enzyme-probe polymorphisms than previously published (Shoemaker and Olson, 1993). It is possible that these markers could map onto different locations other than linkage group 'G'. Thus, there were three markers (A112, L156 and Mng247) that were mapped only in PI 209332 and three unique markers in Peking (Bng30, Bng126 and B53) (Table 2). From the standpoint of map integration, markers that were common among mapping populations were key towards the creation of a joint map. However, the placement of the unique markers onto an integrated map provided a significant amount of information about the order and arrangement of markers that could not be mapped in any one population due to the lack of DNA polymorphism. Using the information from the five mapping populations, a consensus map with all available twelve markers was generated by *JoinMap* analysis. Since markers that were common among populations were mapped using the same restriction enzymes, the final placement and order of markers on the joint map of linkage group 'G' were similar to the maps of the five mapping populations (Figure 3). Although it was not encountered in this study, differing enzyme-probe combinations could pose some difficulties in determining whether a segregating marker mapped with a particular restriction enzyme in one mapping population is identical with the same segregating marker mapped with a different restriction enzyme in another mapping population (Shoemaker and Specht, 1995). The placement of C6 and Bng171 on the same locus next to Bng173 on the joint map differed from the maps of the five mapping populations, where Bng171 was positioned between C6 and Bng173. Using mapping information from the four F₂ populations alone, Bng171 was also placed between C6 and Bng173. However, the integration of the RIL map forced the placement of C6 and Bng171 onto a single locus next to Bng173. It is

Bng171 on a single locus next to Bng173 was due the collinearity between the Evans x PI 209332 F₂ and its derivative F_{5:6} RIL population. In addition, both populations were not polymorphic with Bng171, which probably forced *JoinMap* to place C6 closer to Bng173 since there was no mapping information available for Bng171 in these two populations. The development of a consensus map of linkage group 'G' now provides a high density map around the SCN resistance region, a significant step towards the eventual positional cloning of the major partial SCN resistance gene.

"Qualitative" scoring of SCN resistance and its implications in map-based cloning

Despite the enormous potential of quantitative resistance loci in agriculture, their usefulness is constrained by how difficult they are to study and manipulate. Molecular markers have greatly enhanced our ability to resolve complex quantitative traits into simple Mendelian components. In an attempt to resolve SCN disease response into its simple Mendelian components, a "qualitative" mapping of SCN was conducted in the RIL population. Figure 1 shows the bimodal distribution of SCN indices for the RIL population, indicative of the approach to homozygosity at both extremes. It is for this reason that qualitative disease scores was done only in the RIL population, as the distribution of SCN indices was not sufficiently discrete in the other mapping populations analyzed (data not shown). The strategy was based on the use of a modified 30% cut-off for resistance (Schmitt and Shannon, 1992). Briefly, the SCN indices were categorically scored either as a susceptible (Evans) or resistant (PI 209332). Using this strategy, the SCN locus was localized into a genomic interval between markers Bng122 and C6 facilitated by the identification of recombination events between the qualitative SCN scoring and the two flanking markers. Eight recombinants on the side of C6 and ten recombinants on the side of Bng122 were found with respect to the SCN locus.

With the availability of the "qualitative" SCN scoring obtained from the RIL population and the mapping information from four F₂ populations, an integrated map of linkage group 'G' spanning 55.2 cM was created by *JoinMap*. Based on this joint map, the SCN locus was placed 2.1 cM from B53 and 6.1 cM from Bng122 (Figure 3). In addition, Mapmaker-QTL scans of the region around the major partial SCN resistance in PI 209332, PI 90763 and Peking populations indicate that the "qualitative" scoring of SCN was within one LOD unit of the peak in all three populations (Figure 4). In the PI 209332 and PI 90763 populations the qualitative SCN locus was placed 0.2 cM to the right of their peaks of the LOD scans; whereas in Peking population, the SCN locus was placed 0.1 cM to the right of the peak (Figure 4). Since there were only three markers mapped in the PI 88788 population, the QTL scan for this population is not included.

Perspectives

The inability to resolve SCN into a qualitative trait is perhaps one of the most important road blocks towards the ultimate goal of cloning the SCN resistance gene. In addition, the extremely difficult and time-consuming nature of conventional SCN assays limit the number of plants that one can assay to look for recombinants around the region of interest. Since cloning a gene by map position is dependent on the ability to uncover tightly linked recombinants around the target gene, the strategy described in uncovering recombinants in this thesis can facilitate this process, thus placing the SCN locus on a specific genomic interval.

However, it is recognized that the strategy described in this study is not the best approach to score SCN disease response qualitatively. To rapidly uncover recombinants between the markers flanking the major partial SCN resistance region, one strategy is to develop a near-isogenic (NIL) soybean line population, which is homozygous for all

putative resistance loci but still segregating with respect to the markers flanking the major partial SCN resistance region (i.e. Bng122 and C6). In this way, the major partial resistance locus would then be allowed to segregate in a truly Mendelian fashion and its individual effect on SCN disease response can be evaluated qualitatively without the influence of the other putative SCN resistance loci. Recombinants between the flanking markers and SCN locus can then be rapidly identified by first genotyping and later phenotyping only the progeny of the recombinants. This would dramatically reduce the number of plants needed to be assayed with SCN. A similar strategy was used by DeScenzo *et al* (1994) in developing a high resolution map of the *Hor1/Mla/Hor2* region on chromosome 5S in barley..

The localization of the gene of interest into a particular well-defined genomic interval, is an initial step for chromosome walking (Bender *et al*, 1983) or chromosome landing (Tanksley *et al*, 1992) to pinpoint the exact location of the gene for cloning. However, even with the present marker density around the major partial SCN locus, it is still imperative to place more markers in this region prior to physical mapping. Although there are no strict rules on how dense a map should be, DeScenzo *et al* (1994) suggested that it is advantageous to have markers 0.1 cM apart prior to physical mapping. Further increase in marker density in the genomic region of interest can be potentially accomplished by bulk segregant analysis (Michelmore *et al*, 1991) with PCR-based markers, such as amplified fragment length polymorphisms (AFLPs) (Caetanno-Anolles *et al*, 1991) or simple sequence repeats (SSRs) (Cregan and Akkaya, 1993), using a pair of NILs. One of the NILs should be homozygous resistant and the other homoyzygous recessive with respect to the markers flanking the major partial SCN resistance region, while all other putative SCN resistance loci are fixed for the alleles of the susceptible parent. Despite the relatively low marker density, Danesh *et al* (1995) has initiated physical mapping of the

region around the major partial SCN locus with current markers that are available to resolve recombination sites between closely linked loci using pulse-field gel electrophoresis.

The 'G' locus identified in this study was assumed to be identical in the different mapping populations to facilitate its placement onto a unified map of linkage group 'G'. However, it is possible that this genomic interval contains distinct genes in a cluster in each of the populations evaluated. Since the existence of gene clusters have been documented in several plant species (Martin *et al*, 1993; Islam and Shepherd, 1991; Sudupak *et al*, 1993; Paran *et al*, 1991) and the fact that four independent and unrelated soybean accessions have all been found to have SCN resistance genes in the same region of the genome, it is therefore possible that a gene cluster does exist. The clustering of SCN resistance genes on linkage group 'G' can be tested by crossing a pair of RILs derived from different sources of SCN resistance such as (Evans x PI 209332) x (Evans x Peking) that are both homozygous for the resistant parents' alleles on 'G' but homozygous susceptible at other putative SCN loci. F₂ progeny from this cross can then be genotyped to identify recombinants for the flanking markers around the SCN resistance region as described earlier. This would involve challenging a large number of progeny from the recombinant individuals with various SCN races. The key would be the identification of novel resistance phenotypes distinct from parental RIL lines, that would indicate the presence of multiple resistance genes at the SCN locus as demonstrated for *Mla1* in barley (DeScenzo *et al*, 1994) and *Rp1* locus in maize (Sudupak *et al*, 1993). If a gene cluster does exist, the same novel resistance phenotypes can be used to study in detail key aspects about genetic and cellular mechanisms of SCN disease resistance, such as race specificity.

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Table 1. Percent of variation in SCN disease response explained at the peak of the resistance locus on linkage group 'G' in four mapping populations.

Population	Approximate location [†]	Percent of variation	LOD Scores [‡]	d/a [§]
Evans x PI 209332	C6-Bng122	51.4	10.35	-0.095
Evans x PI 90763	C6-Bng122	52.7	15.58	-0.073
Evans x PI 88788	Bng171-B53	40.0	10.50	-0.422
Evans x Peking	C6-122	28.1	6.94	-0.506

[†]- interval where the LOD score is approximately at its peak.

[‡] - \log_{10} of the odds ratio that supports evidence for the presence of a QTL at the locus.

[§] - the ratio of dominance over additivity, 0 indicates complete additivity, +1 indicates complete dominance, -1 indicates completely recessive.

Table 2. RFLP markers on linkage group 'G' in various mapping populations.

Available markers	PI 209332 (F ₂ , RIL)	PI 90763	PI 88788	Peking
Bng173	Bng173	Bng173	Bng173	Bng173
Bng171	-†	-	Bng171	Bng171
Bng83	-	-	-	-
C6	C6	C6	C6	C6
B53	-	-	-	B53
Bng122	Bng122	Bng122	Bng122	Bng122
Bng126	-	-	-	Bng126
Bng30	-	-	-	Bng30
Bng189	-	-	-	-
Bng113	-	-	-	-
K69	K69	K69	-	K69
Mng247	Mng247	Mng247‡	Mng247‡	Mng247§
Bng225	Bng225	Bng225	-	Bng225‡
A112	A112	A112‡	A112‡	A112§
L156	L156	L156‡	L156‡	L156§

† - Marker locus is not polymorphic.

‡ - Marker locus is polymorphic but have not been mapped.

§ - Marker locus is polymorphic but with a different enzyme-probe combination and has not been mapped.

Figure 1. Frequency distribution of 98 F₆ RIL lines from the cross Evans x PI 209332 for mean SCN disease response to race 3. Vertical axis shows the number of individuals for a given disease index. Horizontal axis represents the corresponding SCN indices for all 98 lines.

Figure 1

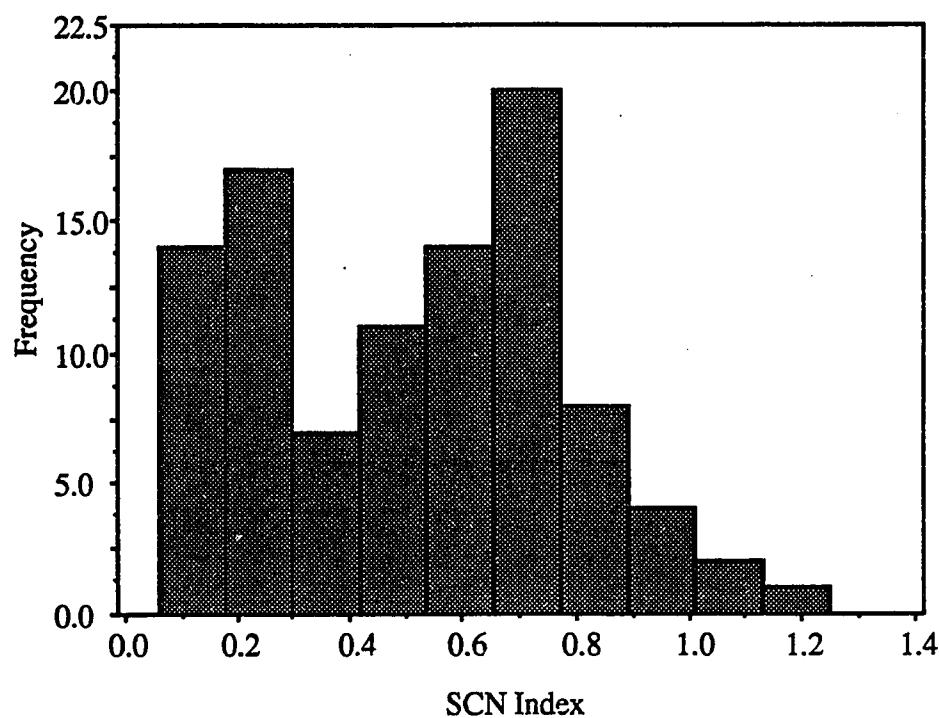


Figure 2. Increase in marker density near the SCN resistance locus mapping clones from *P. vulgaris* and *V. radiata*, in *G. max*. Vertical lines represent the 'G' chromosome, tick marks represent the locations of marker loci. Soybean clones are in plain text, *P. vulgaris* markers are in bold and *V. radiata* marker is in outline form. Underlined markers are yet to be placed on the map using RFLP segregation analysis but are known to reside in this region based on pulsed field gel electrophoresis analysis (Danesh *et al.*, 1995).

Figure 2

Marker Saturation Through Comparative Genome Mapping

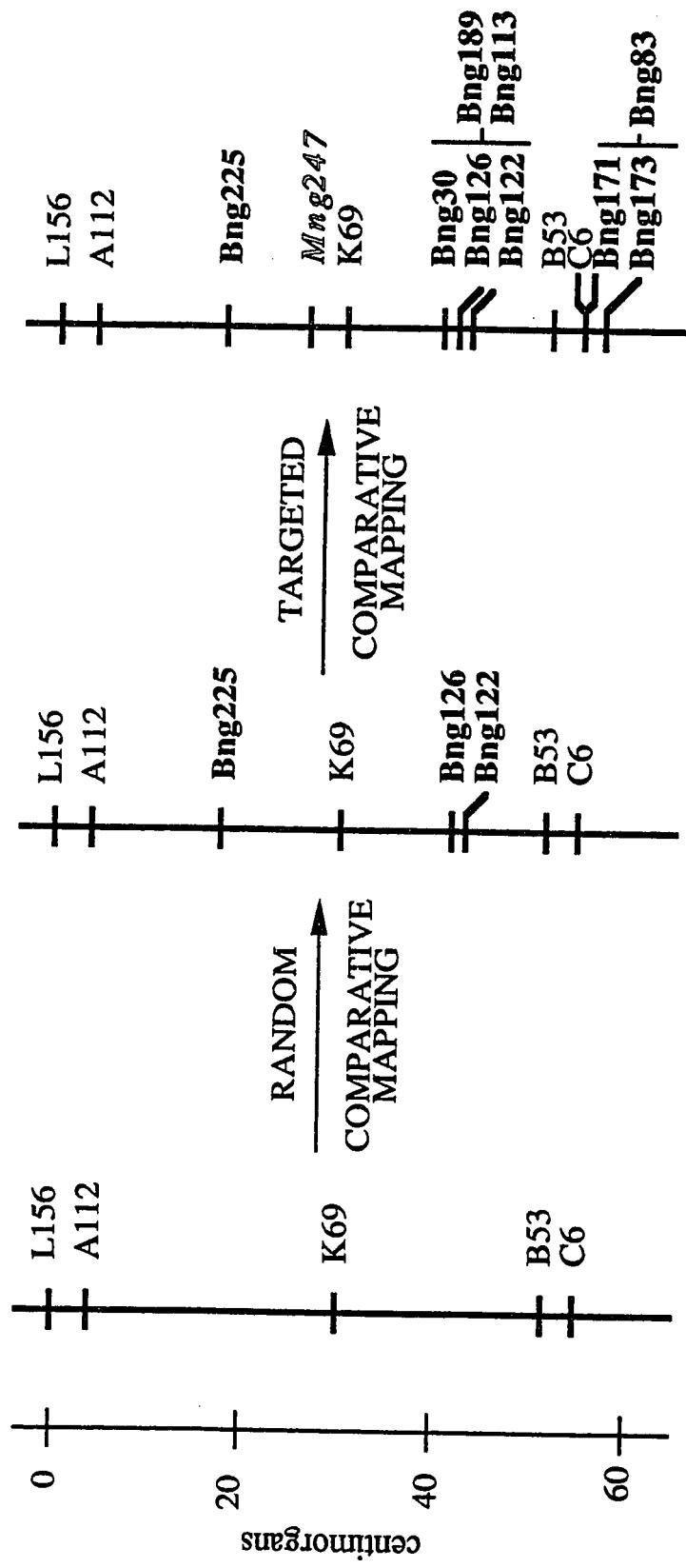


Figure 3. Genomic region on top of linkage group 'G' containing a major partial SCN resistance locus. The figure on the left represents the entire linkage group 'G' of the soybean RFLP map (Shoemaker and Olson, 1993). Vertical line represents the linkage group, tick marks represents mapped marker loci, dashed line indicate region of the linkage group with no markers analyzed in this study. The figure on the right shows a close-up of the region containing the major partial SCN resistance locus. Markers preceded by Bng are common bean clones, markers preceded by the letters A, B, K, L and P are soybean clones and the marker preceded by the letter Mng is a mungbean clone.

Figure 3

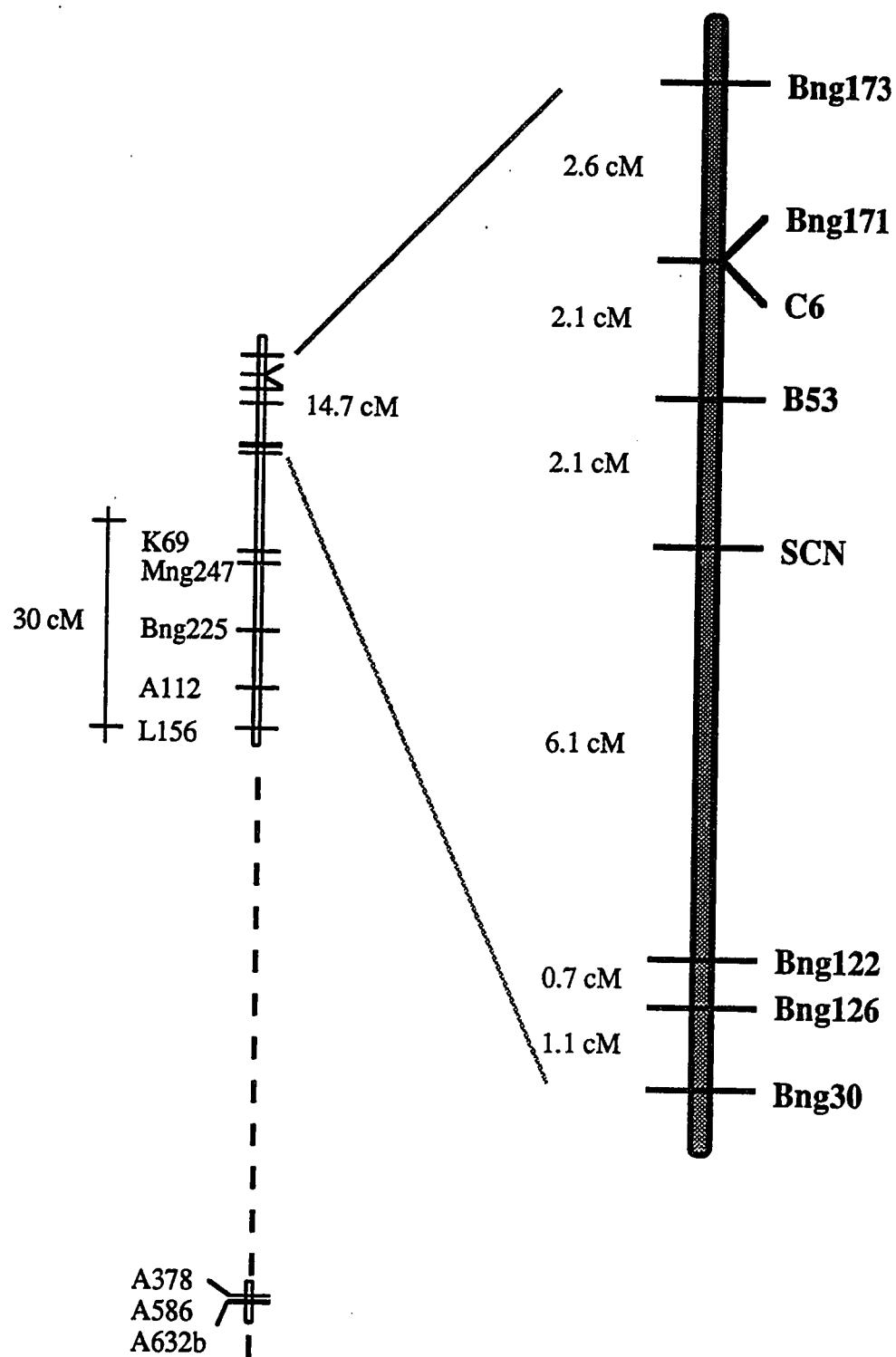
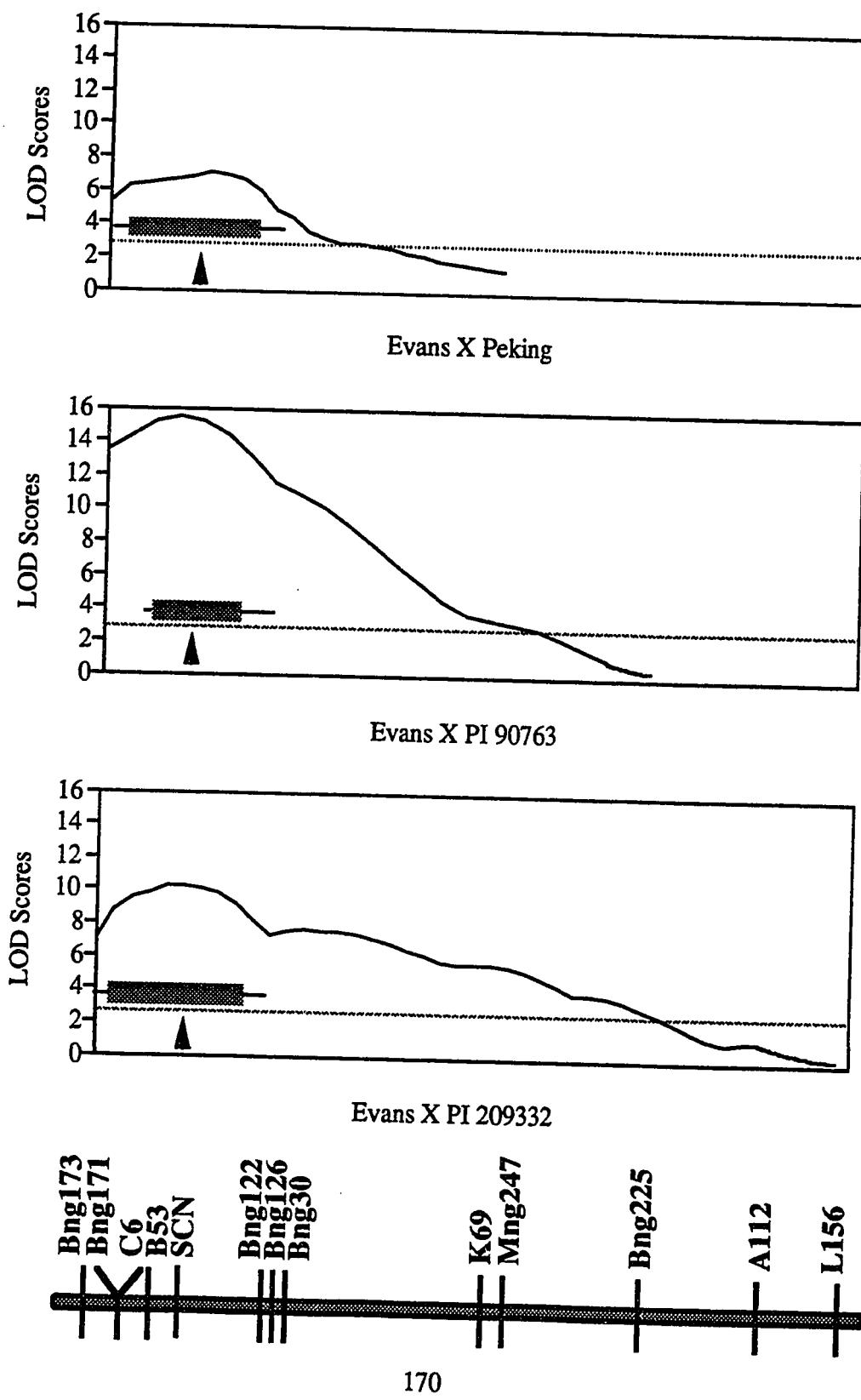


Figure 4. Mapmaker-QTL scans of the genomic region on linkage group 'G' containing a major partial SCN resistance locus in three mapping populations. Horizontal axes correspond to the genetic map for linkage group 'G' (shown at the bottom); vertical axes represents LOD scores of a QTL at the corresponding position on the chromosome. The dotted line at LOD = 2.7 indicates the required significance level for declaring the presence of a QTL. Near the bottom of each graph, a horizontal line indicates the most likely location for a QTL, with the thicker portion indicating the chromosomal segment where the LOD score drops one unit from the peak of the curve and the thinner portion indicating the segment where LOD scores drops by two units. The arrow underneath the horizontal line correspond to the putative map location of the qualitative scoring of SCN disease response.

Figure 4





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6 June 1995

Mr. Vergel C. Concibido
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University of Minnesota
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Dear Mr. Concibido

Please consider this letter is our permission for you to use in your Ph.D. thesis the paper titled "DNA Marker Analysis of Loci Underlying Resistance to Soybean Cyst Nematode (*Heterodera glycines* Ichinohe)". The paper was published in the January-February 1995 issue of *Crop Science*, volume 34.

Sincerely

A handwritten signature in black ink, appearing to read "David M. Kral".

David M. Kral
Associate Executive Vice President