ENHANCING RESISTANCE TO SOUTHERN STEM CANKER AND SOUTHERN ROOT-KNOT NEMATODE IN SOYBEAN

by

ZACHARY PHILLIP SHEARIN

(Under the Direction of Roger Boerma)

ABSTRACT

Southern stem canker, caused by the fungus *Diaporthe phaseolorum* f. sp. *meridionalis* and Southern root-knot nematode (*Meloidogyne incognita*) (RKI), are two economically important diseases of soybean (*Glycine max*) in the southeastern USA. The stem canker resistance genes *Rdc1*, *Rdc3*, and *Rdc?* have been mapped using linkage analysis of genotypic data from simple sequence repeat (SSR) DNA markers and phenotypic data from greenhouse resistance screens. A new RKI resistance quantitative trait locus (QTL) in the soybean germplasm line G93-9009 has been linked to the *T*-locus for pubescence color using greenhouse resistance screens of two advanced backcross populations, BenningRR(5) x G93-9009 and BoggsRR(6) x G93-9009, that continued to segregate for pubescence color after phenotypic selection for RKI resistance of individual $F_2$ plants following each cycle of backcrossing. Replicated field experiments were used to determine that RKI resistance from G93-9009 has been introgressed into these populations without yield reduction through linkage drag.

INDEX WORDS: *Glycine max*, soybean, *Diaporthe phaseolorum*, stem canker, *Meloidogyne incognita*, root-knot nematode, MAS, SSR, QTL, bulked segregant analysis, backcross, *T*-locus, pubescence color

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# DEDICATION

I would like to thank my wife Katie and the rest of my family and friends for their constant patience and support. I would also like to thank every person in my past that has believed in me and given me an opportunity to succeed, especially Bill and Roseann Novitzky, Dr. Tommy Carter, and Dr. Roger Boerma. This thesis is dedicated to everyone involved in American agriculture and its improvement.

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# CHAPTER 1

# INTRODUCTION

Soybean (*Glycine max* L. Merr.) is a leguminous species native to eastern Asia. Domestication of soybean most likely occurred in China during the Zhou Dynasty (1122 – 256 BCE) after it was introduced from northeast, perhaps from the Manchurian Plain (Ho, 1977). Centuries later, Samuel Bowen introduced soybean to North America when he asked Henry Yonge, the Surveyor General of the Colony of Georgia to plant soybean on Bowen's farm near Savannah, GA in 1765 (Hymowitz and Harlan, 1983). Bowen, a seaman employed by the East India Compnany, grew soybean on his plantation and made soy sauce and vermicelli from them for export to England. In 1851 John H. Lea was the first person to plant soybeans in the Midwest near Alton, Illinois, where they were disseminated throughout the rest of the region in the following years (Hymowitz, 1986).

In 2004, soybean was planted on nearly 30.5 million hectares in the USA. The 2004 soybean crop produced almost 3.1 billion bushels worth an estimated $18 billion. Approximately 35% of the soybeans produced in 2004 in the USA were exported, with most exports sent to the European Union, Japan, Mexico, China, and Taiwan (Economic Research Service, http://usda.mannlib.cornell.edu/usda/ers/OCS//2000s/2006/OCS-04-04-2006_Special_Report.pdf; verified 12 July 2007).

Soybean has a wide variety of uses. Soybeans are usually processed and separated into two components, meal and oil. Soybean meal is by far the most important livestock protein feed, making up nearly 65% of the world protein feed supply (Economic

Research Service, http://usda.mannlib.cornell.edu/usda/ers/OCS//2000s/2006/OCS-04-04-2006_Special_Report.pdf; verified 12 July 2007). Soybean oil is a major source of food-grade vegetable oil, and is also used to produce biodiesel and paint.

Throughout history, soybean growers have been forced to deal with yield losses due to disease. Soybean diseases are primarily caused by viruses, bacteria, fungi, and nematodes. Growers depend on plant breeders to provide them with cultivars that possess disease resistance, which is often the most cost effective and environmentally benign method of reducing crop damage caused by soybean diseases.

The objectives of this research are to enhance soybean resistance to southern soybean stem canker (caused by the fungus *Diaporthe phaseolorum* (Cooke & Ellis) Sacc. f. sp. *meridionalis* Morgan-Jones) and Southern root-knot nematode (*Meloidogyne incognita* (Kofoid and White) by (1) mapping the genomic location of three soybean resistance genes to southern stem canker using SSR markers, and (2) determining if a Southern root-knot nematode resistance QTL is linked to the *T*-locus conditioning pubescence color.

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CHAPTER 2

# REVIEW OF LITERATURE – SOUTHERN SOYBEAN STEM CANKER

## Research Impetus

Stem canker, caused by the fungus *Diaporthe phaseolorum* (Cooke & Ellis) Sacc. is an economically and culturally important disease of soybean (*Glycine max* (L.) Merr.). Major revenue losses in the form of reduced yield potential have been observed in the USA and most other major soybean producing countries in the world. In 1994, seven of the top ten soybean producing countries reported yield losses due to stem canker, including (in order of greatest to least estimated loss) Brazil, Paraguay, USA, Argentina, Bolivia, Italy, and Canada. Of the various diseases and pests of soybean, stem canker caused the second highest estimated yield loss at 1.9 million metric tons in these countries, with only soybean cyst nematode (*Heterodera glycines* Ichinohe) causing higher estimated losses (Wrather et al., 1997). Stem canker in North America has been found in the midwestern and southeastern regions of the USA and in Ontario, Canada. Stem canker has especially been a problem in the southern USA in recent years. In terms of percent of infected plants, infestation rates as high as 80% have been observed in some areas of the Southeast (Krausz and Fortnum, 1983). The economic impact of losses in the Southeast were estimated to cost $37 million in 1983 (Backman et al., 1985). More recently, yield losses due to stem canker in the USA were estimated to be valued at $67.1 million in 2003 (Wrather, 2004).

**Taxonomy**

It is generally accepted that stem canker is caused by a fungal member of the *Diaporthe*/*Phomopsis* complex. The telomorphic, or sexually reproductive stage of this complex is identified as *Diaporthe phaseolorum*. The anamorphic, or asexually reproductive stage is identified as *Phomopsis phaseoli* (Morgan-Jones, 1989). Further classification of the fungal organism causing stem canker has been under continuous debate since it was first connected to the disease. Welch and Gilman (1948) were the first to distinguish stem canker from pod and stem blight, a disease of soybean and other crops caused by *Diaporthe phaseolorum* var. *sojae* (Lehman) Wehm. However, they incorrectly suggested that a strain of *Diaporthe phaseolorum* var. *batatatis* (Harter & Field) Wehm. was the causal organism of stem canker. Athow and Caldwell (1954) were the first to correctly identify the fungus causing stem canker and gave it its own varietal name, *Diaporthe phaseolorum* (Cke. & Ell.) Sacc. var. c*aulivora* Athow & Caldwell. They differentiated *D. phaseolorum* var. *caulivora* from *D. phaseolorum* var. *sojae* by its lack of a conidial (anamorphic) stage, the shape and grouping of its perithecia, the shape and length of its perithecial beaks, and the smaller size of its asci and ascospores. However, in the late 1950's and 1960's evidence began accumulating that the causal organisms of stem canker and pod and stem blight were not in fact distinct varieties of *D. phaseolorum*. Both the similar responses of the two proposed varieties to radiation-induced mutation *in vitro* and the identification of a conidial stage of *D. phaseolorum* var. *caulivora* (Threinen et al., 1959) provided the first evidence of a possible misclassification. Whitehead (1966) reported finding a strain of var. *sojae* that caused symptoms on soybean similar to stem canker, further invalidating this separation.

However, strains associated with stem canker can consistently be distinguished from other strains of *D. phaseolorum*. As a result, Kulik (1984) suggested distinguishing strains causing stem canker as different *formae speciales* from those lacking the pathogenic capability to induce the stem canker disease. This division recognized that the difference between strains was mostly in the host-pathogen relationship, with few morphological and *in vitro* behavioral differences otherwise distinguishing the strains. Backman et al. (1985) observed differences between strains of fungi that caused the stem canker disease. Differences in strains that were isolated from plants with stem canker symptoms in the northern USA and strains that were isolated from plants with stem canker symptoms in the southeastern USA were great enough to justify partitioning northern and southern biotypes into separate *formae speciales*. In 1989, Morgan-Jones officially recognized three *formae speciales* of *D. phaseolorum* (Morgan-Jones, 1989): (1) the organism associated with pod and stem blight was recognized as *Diaporthe phaseolorum* (Cooke & Ellis) Sacc. f. sp. *sojae* Morgan-Jones; (2) the organism associated with stem canker in the northern USA and Canada was recognized as *Diaporthe phaseolorum* (Cooke & Ellis) Sacc. f. sp. *caulivora* Morgan-Jones; and (3) the organism associated with stem canker in the southeastern USA was recognized as *Diaporthe phaseolorum* (Cooke & Ellis) Sacc. f. sp. *meridionalis* Morgan-Jones. The latter was differentiated from f. sp. *caulivora* based on differences in colony appearance, stromatal size and occurrence, perithecium morphology production and overall arrangement, conidiomata formation, ascospore size and shape, growth response to different temperatures, and host symptom development. However, the classification of these fungi as *formae speciales* has not been universally accepted, and they are still often

referred to as varieties in the literature. For the purposes of this review, *D. phaseolorum* f. sp. *caulivora* will be referred to as DPC, and *D. phaseolorum* f. sp. *meridionalis* will be referred to as DPM. Based on differential host cultivar reactions to certain isolates, several races of DPC have been identified (Higley and Tachibana, 1987; Keeling, 1985). Presently, no specific races of DPM have been identified.

**Disease History**

Northern stem canker was first observed in the late 1940's. Although it probably existed before that time period, it was not differentiated from pod and stem blight. By the early 1950's stem canker incidence had increased dramatically due to the widespread use of two highly susceptible soybean cultivars, Blackhawk and Hawkeye. The repeated planting of these two cultivars allowed the DPC population to increase over time to levels high enough to greatly reduce soybean yield (Hildebrand, 1952). Moving away from the use of Blackhawk and Hawkeye to other cultivars eliminated stem canker as a disease concern by the late 1950's (Weaver et al., 1984).

In contrast, the history of southern stem canker is more complex. Significant damage due to southern stem canker was first reported in Mississippi in 1973, followed by Alabama in 1977, Tennessee in 1981, South Carolina and Georgia in 1982, Florida, Louisiana, and Arkansas in 1983, and Texas in 1984 (Backman et al., 1985). Unlike northern stem canker, outbreaks of southern stem canker could not be traced back to one or two highly susceptible cultivars because the cultivars grown in each state had a wide range of reactions to the pathogen. Presently, stem canker still causes yield losses in some areas of the USA, but the development of resistant cultivars and altered cultural

practices (both discussed in detail in later paragraphs) have dramatically reduced the number of outbreaks in the USA.

**Disease Symptoms**

Stem canker is named for the symptoms it causes in the plant. The first symptoms of both northern and southern stem canker appear during the plant's early reproductive stages as small reddish-brown superficial lesions on the stem, usually near a lower leaf node. The lesions expand longitudinally and form reddish-brown cankers that become slightly sunken as the growing season progresses. Older lesions may appear dark brown with a grayish-brown center and a reddish-brown margin (Fernandez et al., 1999). Tissue above and below the canker remains green (Grau et al., 2004). Lesions of northern stem canker range from 2 to 10 cm in length and usually girdle the stem. The dead stem tissue blocks the upward flow of water through the vascular tissue, and the seed-bearing portion of the plant becomes water-stressed and usually dies before the plant's full yield potential can be reached (Hildebrand, 1952). The leaves usually remain attached after plant death (Fernandez et al., 1999), but in some cases a top dieback can occur (Hobbs et al., 1981).

In southern stem canker the lesions expand along the length of the stem, but rarely girdle the stem (Fernandez et al., 1999). Leaves usually remain attached and show symptoms of interveinal chlorosis and necrosis. A phytotoxin plays a role in foliar symptoms and premature plant death (Lalitha et al., 1989). As in northern stem canker, plants with southern stem canker retain their leaves after death. Necrosis of the terminal meristem occasionally occurs, causing a characteristic shepherd's crook curling. Premature plant death causes a reduction in seed number and size (Fernandez et al.,

1999). The reduction in seed size caused by the disease contributes to even greater yield loss during harvest as small seeds are often lost during the harvesting process (Hildebrand, 1952). Since the cankered stem tissue also becomes dry and brittle any significant lateral force applied to the plant at this point can easily snap the stem, further contributing to yield loss (Hildebrand, 1956).

**Epidemiology**

Most epidemiological research involving stem canker has involved DPM rather than DPC due to the greater threat and economic impact of DPM on soybean production. Plants are infected with DPM early in the growing season during the early vegetative growth stages from V3 through V10 (Fehr et al., 1971). Infection occurs primarily by rain splashing inoculum from infected soybean residue from the previous growing season onto the new crop during heavy rains (Ploetz and Shokes, 1985). Both conidia and ascospores are capable of causing infection (Ploetz and Shokes, 1985). Plants are usually infected through scars on stems, petioles, or petiole bases (Ploetz and Shokes, 1987b). Infection can also occur in the leaf tissue, but much less frequently than in other plant parts. Leaf infection does not lead to stem canker symptoms as often as does infection of stems, petioles, or petiole bases (Ploetz and Shokes, 1987a). Once a plant becomes infected, there is a latent period where no disease symptoms can be observed. Symptoms then appear during the early reproductive growth stages, usually during early podfill, or the R3 stage of development (Fehr et al., 1971). After stem canker symptoms have been observed, perithecia and pycnidia are occasionally produced in the cankers before the end of the growing season on highly susceptible cultivars. These perithecia and pycnidia can

produce ascospores and conidia, respectively, which can serve as a secondary inoculum source during the growing season. The secondary inoculum may cause infection, but probably does not contribute to yield loss or disease development. Instead, it probably serves to increase the inoculum potential for the following growing season (Fernandez et al., 1999). In moderately susceptible cultivars, reproductive structures begin to develop after plant senescence in late winter. The inoculum then overwinters on colonized plant debris left in the field after harvest (Grau et al., 2004). Ascospores and conidia are released in late April through June and infect the following season's crop during the V3 through V10 development stages primarily by the previously mentioned mechanisms, at which point the cycle of disease is complete.

The pathogen is spread from one area to another by high winds during rainstorms, by the transfer of infected debris, and to a lesser extent by infected seed. Splashing, blowing rain, and high winds during an early season storm can spread spores up to 2 m from a point source (Damicone et al., 1990). The movement of infested soybean debris contributes to the spread of the disease over greater distances (Backman et al., 1985; Grau et al., 2004). There has been much debate about the spread of stem canker via infested seed. Research has shown that seed infested with DPC has a greatly reduced germination rate (Hildebrand, 1952). Further research indicated that planting DPC infested seed does not increase the incidence of stem canker nor does it decrease the yield of the resulting plants in the absence of any other inoculum source (Hildebrand, 1956). Ploetz and Shokes (1987b) also conclude that DPM-infested seed does not play a substantial role in the spread of southern stem canker from one field to another. They also point out that no research has shown that seed infested with DPM results in infected

plants when the seed is planted with no other exposure to the pathogen. Backman et al. (1985) however, argue that severe cases of stem canker have developed with seed as the only apparent inoculum source. Seed infestation rarely exceeds 1% in plants infected with DPM, but can range from 10% to 20% in plants infected with DPC (Fernandez et al., 1999). As shown by the various results in the literature, the exact role of infected seed in the spread of stem canker is unclear, and more research concerning the matter may prove beneficial.

Alternate hosts for DPC and DPM may play a role in the spread of stem canker. Alternate hosts for DPC include alfalfa (*Medicago sativa* L.), barley (*Hordeum vulgare* L.), flax (*Linum usitatissinum* L.), lambsquarters (*Chenopodium album* L.), snapbean (*Phaseolus vulgaris* L.), and to lesser extents red clover (*Trifolium pratense* L.), white sweetclover (*Melilotus alba annua* Desr.), corn (*Zea mays* L.), rye (*Secale cereale* L.), oats (*Avena sativa* L.), barnyard grass (*Echinochloa crus-galli*), rough pigweed (*Amaranthus retroflexus* L.), witch grass (*Panicum capillare* L.), wheat (*Triticum aestivum* L.), and green foxtail (*Setaria viridis* L. Beauv.) (Frosheiser, 1957). These alternate hosts were found *in vitro* by evaluating the reproductive ability of a fungal strain on a putative host grown in culture media. While most of the alternate hosts were asymptomatic, stem canker symptoms were observed on alfalfa, red clover, and snapbean. At least 16 alternate hosts have been found for DPM. DPM that was isolated from cotton (*Gossypium hirsutum* L.) in Mississippi has been found to cause stem canker symptoms on soybean (Roy and Miller, 1983). Black et al. (1996) evaluated 17 weed species common to Louisiana soybean fields for their potential as alternate hosts. Of the 17 species they evaluated, 14 were identified as potential alternate hosts. They include

black nightshade (*Solanum nigrum*), entireleaf morning glory (*Ipomoea hederacea* var. *integriuscula*), hairy indigo (*Indigofera hirsuta*), hemp sesbania (*Sesbania exaltata*), ivy-leaf morning glory (*Ipomoea hederacea*), northern joint-vetch (*Aeschynomene virginica*), pitted morning glory (*Ipomoea lacunosa*), prickly sida (*Sida spinosa*), redweed (*Melochia corchorifolia*), sicklepod (*Cassia obtusifolia*), smallflower morning glory (*Jacquemontia tamnifolia*), spiny amaranth (*Amaranthus spinosus*), tall morning glory (*Ipomoea purpurea*), and wild poinsettia (*Euphorbia heterophylla*). Only two of these species, hemp sesbania and hairy indigo, showed stem canker symptoms. After isolating a phytotoxin from DPM that plays a role in the foliar symptoms of southern stem canker, Lalitha et al. (1989) tested 12 plant species for reaction to both the isolated toxin and the fungal strains from which the toxin was isolated. Only soybean and lima bean (*Phaseolus lunatus*) expressed symptoms of stem canker in the presence of the phytotoxin and the fungal strains. As a result, lima bean is considered to be an additional alternate host for DPM.

**Factors Determining Disease Severity**

In order to provide protection for soybean against southern stem canker, a considerable amount of research has evaluated the factors that affect the severity of the disease. The growth stage at the time of infection, environmental conditions such as air temperature and moisture, cultural practices, and the presence of other pests have been shown to affect the extent to which soybean will react to DPM. The percent of infected soybean plants in a given field can increase from a negligible amount to epidemic levels in as little as one year. In one previously uninfested field, 1.6% of soybean plants had

symptoms of stem canker in the first season stem canker was found at that location in 1983. During the 1984 growing season, 72.4% of the plants had symptoms of stem canker at the same location (Rothrock et al., 1985). It is clear that a combination of many external factors played a role in such a dramatic and rapid increase in the effect of stem canker on the crop grown in this field.

The growth stage of the host at the time of introduction to DPM has been shown to play a significant role in how stem canker affects the performance of the plant. It was first observed in northern stem canker that if soybean was infected with DPC early in the growing season, both seed quality and quantity would be greatly reduced, but if the infection occurred later in the growing season, the effects were negligible (Hildebrand, 1952; Hildebrand, 1956). The findings that early infection appeared to be related to final disease severity were also confirmed for southern stem canker. Rothrock observed that in 1985, 34% of plants of the moderately susceptible cultivar Hutton were infected by 21 days after planting (DAP) and 37% of these sampled plants died prematurely. The following year, 4% of the Hutton plants sampled 24 DAP were infected, and 6% of the sampled plants died (Rothrock et al., 1988). DPM exposure at the V3 growth stage is associated with the highest disease severity. Severity is progressively reduced when exposure to the pathogen is delayed until stages V4 to V10 (Grau et al., 2004). In order to more fully understand the relationship, Smith and Backman (1989) conducted two different experiments to determine if planting date and infection at different soybean developmental stages influenced the occurrence or severity of the disease. In one experiment all plots were planted at the same time and inoculated at various growth stages. In the second experiment, the plots were planted at different times and all of the

plots were inoculated at the same time. Similar results were obtained from both experiments despite the different experimental designs, cultivars, and environmental conditions. The data from both experiments were fitted to Cauchy distribution models that predicted an exponential increase in disease (measured at R6) for plants exposed to inoculum at stages V1 to V3 (12 to 22 DAP). Their models predicted that disease incidence and severity would then progressively decrease when plants were exposed to inoculum at V5 through R2. Their experiments suggest that disease severity largely depends on time of infection relative to plant growth stage. Additional studies that used the development of foliar symptoms as a measurement of disease incidence and seed weight and number as measurements of disease severity determined that stem canker symptoms can occur regardless of growth stage at the time of infection (Rupe et al., 1999). However, foliar symptoms, which are usually observed after the presence of cankers, require a minimum incubation period of 34 to 41 days and a reproductive developmental stage. Foliar symptoms have never been observed earlier than R2 (the onset of flowering) even when inoculation occurs as early as V1. These results indicate that plants inoculated at V1 have the longest incubation period, while plants inoculated at R2 have the shortest incubation period for the disease.

Environmental conditions during vegetative growth play a role in the development of stem canker. Of these, air temperature has the greatest effect (Grau et al., 2004). A wide range of temperatures promote maximum recovery of DPM from artificially inoculated soybean plants, but upper and lower temperature limits do appear to exist. Growth chamber experiments have determined that temperatures of 28 to 34°C promote the highest levels of DPM recovery after artificial inoculation, with optimal

recovery at 28.5°C. Temperatures of 10 to 22°C are associated with less infection (Rupe et al., 1996). Infection at temperatures above 40°C is not known to occur (Ploetz and Shokes, 1987a). Moisture during vegetative growth of the plant also appears to play a role in stem canker infection. After spore deposition has occurred, either a prolonged and continuous wetting event such as rainfall or irrigation, or many discontinuous periods of wetting facilitate infection and stem canker development (Damicone et al., 1987). Stem canker epidemics are more likely to occur in an irrigated field than a nonirrigated field (Subbarao et al., 1992). Rupe et al. (1996) combined treatments of various temperatures (15 to 30°C) and moisture periods (8 to 96 h) on artificially inoculated soybean plants grown in the greenhouse and measured the recovery of DPM from surface-sterilized stem and petiole sections placed on potato dextrose agar (PDA) after the experiment was completed as a measure of infection. Maximum recovery of DPM was observed at wetness periods of 72 to 96 h and temperatures of 28 to 34°C. A regression model ($R^2$ = 0.847) fitted to the data predicted high fungal recovery over a broad range of near-optimum temperatures and longer wetness periods.

Certain cultural practices have been found to affect disease severity. Tilling under infected debris and delayed planting have been shown to reduce disease severity (Grau et al., 2004; Hilty, 1991; Rothrock et al., 1985; Rothrock et al., 1988). Doublecropping soybean with wheat has been shown to increase disease severity (Rothrock et al., 1985; Rothrock et al., 1988). Crop rotations in general have not been shown to reduce stem canker infestations (Grau et al., 2004). Tilling that buries infested soybean residue reduces disease development by lowering or eliminating the number of spores near aboveground plant parts that would otherwise be available to colonize crops

early in the growing season (Hilty, 1991; Rothrock et al., 1985; Rothrock et al., 1988). Delayed planting can reduce stem canker incidence and severity by avoiding the critical time period when spores are released and deposited onto plants (Grau et al., 2004). However, the reduced yield potential usually associated with delayed planting is often greater than the reduced yield potential of crops with stem canker. Doublecropping soybean after wheat has been shown to promote the onset of stem canker symptoms (Rothrock et al., 1985). Stem canker incidence and severity is significantly higher during doublecropping with wheat than in monocropping with soybean alone, but there is no increase in DPM infection. This indicates that the relationship between cropping system and stem canker is related to symptom expression rather than rates of infection (Rothrock et al., 1988).

The severity of stem canker symptoms can change when a host plant is parasitized by other organisms. Three-cornered alfalfa hopper (*Spissistilus festinus* Say; TCAH) feeding on soybean causes characteristic symptoms described as main stem feeding girdles. TCAH infestations reduce soybean yields by causing plant death, reducing seed weight, and causing seed loss during harvest due to stem lodging and breakage. Stem canker symptom severity, especially canker length, is usually increased in plants with main stem girdles induced by TCAH when compared to plants parasitized with stem canker alone (Padgett et al., 1994; Russin and Boethel, 1986). This relationship appears to be mostly additive. However, the authors qualify their findings by saying the relationship between these two pests could be due to a general reduction in plant vigor caused by TCAH rather than any actual physiological relationship within the plant (Russin and Boethel, 1986). Soybean looper (*Pseudoplusia includens* Walker), a

defoliating insect of soybean, and soybean cyst nematode (*Heterodera glycines* Ichinohe) a parasitic nematode of soybean, have been found to reduce the severity of stem canker symptoms when either soybean looper, soybean cyst nematode, or both pests are found on plants with stem canker (Padgett et al., 1994; Russin et al., 1989).

DPM produces a phytotoxin after it infects its host that leads to the foliar symptoms associated with southern stem canker. Different strains of DPM have been found to produce different amounts of toxin. Disease severity has been shown to be related to the amount of phytotoxin to which a plant has been exposed (Lalitha et al., 1989). Lalitha et al. (1989) were the first to isolate this phytotoxin from soybean plants with stem canker symptoms that were infected with single-spore isolates of DPM. They reintroduced the isolated toxin in serial dilutions to healthy soybean plants and found a positive linear relationship ($R^2 = 0.61$) between disease severity and toxin concentration. The single-spore isolates of DPM used in this experiment produced different amounts of toxin in culture. The amount of toxin produced in culture by a given DPM strain was associated with the length of cankers caused by inoculating plants with that DPM strain and with the length of cankers caused by exposing the plant to the isolated phytotoxin produced by the strain.

**Disease Control**

When, where, and even if stem canker outbreaks will occur in a given growing season is difficult to predict and depends on many external factors. Therefore, most control strategies focus on preventing the spread of the disease, reducing inoculum levels in the field, avoiding conditions that promote infection, and using resistant cultivars

rather than fungicides. To prevent the spread of the disease from infested fields to uninfested fields, Backman et al. (1985) recommended cleaning equipment before moving it from an infested field to a noninfested field. They also discouraged planting infested seed in a noninfested field even though the spread of the disease via seed is debatable. Infected soybean residue can also spread the disease if it is moved from one field to another (Grau et al., 2004). Inoculum levels in the field are reduced by tilling under infected soybean debris, as discussed in earlier paragraphs. To avoid conditions that promote infection, planting later in the season has also been recommended in some cases. Delaying planting until late June or July avoids the initial burst of inoculum released during May and early June. However, delayed planting is not guaranteed to prevent infection because spores may still be released in late June and July, and plants may still be infected if temperature and rainfall conditions are within the parameters for infection. Chemical control with fungicides is certainly possible, but is not always economically feasible. Fungicides can be an economically effective means of controlling stem canker if they are part of a management strategy that also employs the use of cultivars that are moderately resistant to the disease (Weaver et al., 1984). Control of stem canker cannot be achieved with fungicides paired with highly susceptible cultivars because production costs would quickly exceed the crop returns. Using fungicides with highly resistant cultivars provides little or no benefit in terms of profits (Backman et al., 1985). If foliar fungicides are utilized, they must be applied well before any evidence of disease. Backman et al. (1985) suggested that fungicides be applied during early vegetative growth periods when spores are actively being produced to prevent infection even though symptoms may not appear until much later in the growing season.

**Sources of Resistance**

In most cases, stem canker can be controlled by removing or deep-tilling infested soybean debris combined with planting resistant cultivars. Cultivar resistance to stem canker is conditioned by at least five different dominant resistance genes. One resistance allele at any of these loci will condition resistance to DPM. The exact functions of these genes are not known, but in each case the resistant reaction is activated after infection has occurred, and thus does not prevent infection itself (Ploetz and Shokes, 1987a). In the late 1970's and early 1980's, soybean cultivars and breeding lines were observed to have different reactions to DPM under natural infection in the field (Keeling, 1982; Weaver et al., 1984). In most cases, the cultivar Tracy-M had significantly lower disease levels than other cultivars, and was considered highly resistant. The suspected source of resistance in Tracy-M is the cultivar CNS, which is a common ancestor of several highly resistant cultivars including Tracy-M and 'Bay' (Keeling, 1982). To determine the mode of resistance in Tracy-M, it was crossed to the highly susceptible breeding line J77-339 (Kilen et al., 1985). The parents, $F_1$, $F_2$, and $F_3$ generations were artificially inoculated and evaluated for resistance. All members of the $F_1$ generation were resistant, indicating that the resistant allele(s) in Tracy-M was dominant. The $F_2$ generation segregated in an approximate 15:1 (resistant: susceptible) ratio. The $F_3$ generation segregated in an approximate 7:8:1 (resistant: segregating: susceptible) ratio. The segregation ratios in the $F_2$ and $F_3$ generations indicate the presence of two major dominant genes conditioning resistance to stem canker in Tracy-M. Kilen and Hartwig (1987) used $F_4$ lines of individual $F_3$ plants from an earlier study (Kilen et al., 1985) that were uniformly

resistant in order to separate the two resistance genes in Tracy-M into two separate lines. To do this, they chose two $F_4$ lines that were uniformly resistant, crossed them to each other and to J77-339 and evaluated them for resistance. The $F_2$ generation of the populations created from the $F_4$ lines crossed to J77-339 segregated in a 3:1 ratio (resistant: susceptible). When plants of the two $F_4$ lines were crossed to each other they segregated 15:1 (resistant: susceptible). Their results indicated that the two $F_4$ lines both had single dominant resistance genes at two different loci. They designated these two resistance genes *Rdc1* and *Rdc2*. In 1994, two germplasm lines, D85-10404 and D85-10412, were released with resistance to stem canker (Kilen and Hartwig, 1994). The source of resistance in D85-10404 was *Rdc1* and the source of resistance in D85-10412 was *Rdc2*, both from Tracy-M (Kilen et al., 1985). Additional resistance genes have been found in the cultivars Crockett and Dowling (Bowers et al., 1993). In that study, Crockett, Dowling, and Tracy-M were used as resistant parents and 'Coker 338' and 'Johnston' as susceptible parents. They crossed each resistant parent to each susceptible parent and to the other resistant cultivars. $F_1$ plants from each cross were backcrossed to the susceptible parents. $F_2$ plants from the original crosses were harvested individually and used to create $F_{2:3}$ lines. Members of all generations were then evaluated for resistance. Based on their segregation ratios, Crockett and Dowling were each found to contain single dominant genes conditioning resistance to stem canker. The resistance genes from Crockett and Dowling were at different loci from each other and also from *Rdc1* and *Rdc2* found in Tracy-M. They assigned the symbols *Rdc3* and *Rdc4* to the resistance genes found in Crockett and Dowling respectively. Additional sources of resistance to stem canker have been found in two soybean plant introductions (Tyler,

1995). PI 230976 and PI 398469 both have single dominant genes conditioning resistance to stem canker that are at different loci from *Rdc1-4*, but as of yet have not been assigned symbols. It is not known if the genes in these plant introductions are at different loci from each other.

Mapping the *Rdc* genes will allow soybean breeders to more efficiently select for stem canker resistance in their cultivars and breeding lines. Delineating the genomic regions where stem canker resistance is located to a smaller segment of a linkage group with the use of SSR markers also facilitates the development of even more "breeder friendly" markers such as single nucleotide polymorphisms (SNPs) that are more tightly linked to the resistance gene. These SNPs and SSRs may ultimately be used for marker assisted selection (MAS) in breeding programs in lieu of phenotyping for disease resistance using the more traditional greenhouse and field methods.

**Phenotypic Evaluation**

It would seem logical that the best method for evaluating plants for resistance to stem canker is the observation of plants in the field under natural conditions. However, occurrence of the disease in the field is sporadic and unpredictable from year to year. To ensure continuous progress in research combating stem canker, a repeatable and reliable method of artificial inoculation quickly became necessary. There are two widely used methods of artificial inoculation. They are the toothpick method and the spore suspension method. Keeling (1982) developed a method for inoculating seedlings with a toothpick 10 DAP in a greenhouse, or 30 DAP in the field. This method is a modified version of an earlier protocol developed by Crall (1952). In Keeling's study, seedlings

were evaluated in a greenhouse for resistance 10 days after inoculation. Plants were considered susceptible if they were dead after 10 days, moderately resistant if they were still alive but had symptoms of stem canker, and resistant if they showed no symptoms of stem canker. Plants inoculated in the field were observed 60, 90, and 100 days after inoculation and rated for resistance based on the length of the canker extending from the point of toothpick insertion. Data generated by both the field and greenhouse inoculation methods were correlated with the results for natural field infestation. Toothpick inoculation has since been confirmed by other researchers to be a reliable alternative to evaluation under natural field conditions (Weaver et al., 1988). Another widely used method of artificial inoculation involves spraying plants with spore suspensions during their vegetative growth stages (Frosheiser, 1957). With this method, inoculum density was shown to be linearly related to incidence of infection for densities of $1 \times 10^3$ to $1 \times 10^6$ spores/mL $H_20$ (Ploetz and Shokes, 1987a). However, further research is needed to simulate naturally occurring disease development in the field as closely as possible. Evaluating plant resistance using the spore suspension method has been compared to the toothpick inoculation method and infection under natural field conditions and has been shown to be as reliable as the toothpick method (Keeling, 1988).

**Genotypic Evaluation**

SSR markers are polymerase chain reaction (PCR)-based markers that target specific nucleotide repeats in the soybean genome and provide a highly polymorphic marker system in soybean. Most soybean SSR primers are developed from non-coding DNA located in flanking regions of $(AT)_n$ or $(ATT)_n$ repeats (Akkaya et al., 1992). The

number of repeats at a given SSR locus can vary in different genotypes. This genotypic variation can be utilized as a tool for gene mapping (Diwan and Cregan, 1997). SSR markers are codominant and evenly spaced throughout the soybean genetic map (Cregan et al., 1999). At least 1000 SSR primer pairs have been developed in soybean. These SSR markers have been mapped to the USDA consensus genetic map of soybean that includes marker positions and genetic distances based on multiple soybean mapping populations (http://soybase.ncgr.org).

Bulked segregant analysis (BSA) is a powerful method of rapidly identifying molecular markers linked to a specific gene (Michelmore et al., 1991). In BSA, DNA samples from a segregating population from a single cross are pooled together based on their expression of a particular trait (in this case, response to stem canker) and analyzed using molecular markers. This method relies on the fact that the two bulks will be genetically similar to the respective parents in areas of the genome associated with the trait used to differentiate them, but essentially heterogeneous for all genomic regions not associated with that trait. BSA has been used extensively for successfully locating disease resistance genes in soybean (Demirbas et al., 2001; Jeong et al., 2002; Mian et al., 1999).

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# CHAPTER 3

# REVIEW OF LITERATURE – SOUTHERN ROOT-KNOT NEMATODE

## Research Impetus

Plant parasitic nematodes, microscopic roundworms, are rarely seen by the human eye but are almost ubiquitously distributed throughout the world. Plant-parasitic nematodes live on or in plant roots and are a detriment to their host. Several plant-parasitic nematodes are capable of causing significant economic losses in soybean (*Glycine max* L. Merr). Of these, the southern root-knot nematode (*Meloidogyne incognita* (Kofoid and White) Chitwood; RKI) is one of the most important and most devastating plant-parasitic nematodes in Georgia and the southeastern USA. One estimate attributed a $38.76 million loss from reduced soybean yields caused by *Meloidogyne* spp. in 2003 (Wrather, 2004).

## Symptoms

Plants that have been severely parasitized by RKI typically exhibit both above ground and below ground symptoms. Above ground symptoms are similar to other abnormalities that cause nutrient and water deficiencies, such as stunted growth, chlorotic leaves, increased wilting during periods of mild water stress, and reduced yield. Below ground symptoms are more diagnostic and include swollen, knotted roots and in some cases under developed root systems. Knots, also called galls, are areas where the entire

circumference of the root is swollen, and are usually associated with the site of RKI parasitism (Niblack et al., 2004)

**Life Cycle**

*Meloidogyne incognita* belongs to a specific class of nematodes known as the sedentary endoparasites. Endoparasitic nematodes completely enter and migrate within plant roots. At 27°C, RKI takes 25 days to complete its life cycle (Agrios, 1997). RKI is mitotically parthenogenic. A juvenile RKI possesses the ability to develop into a male or a female. Females usually produce eggs through mitotic cell division, thus not requiring male fertilization. Males are generally uncommon except in stressful environmental conditions. After going through its first molt and hatching, the second-stage juvenile (J2) RKI penetrates and enters the root just behind the root cap, and migrates through the root intercellularly in the root cortex until it reaches the protoxylem pole (Hussey, 1985). Once the nematode reaches this preferential location of the plant root, it establishes multiple feeding sites called giant-cells and becomes sedentary in response. Giant-cells are formed in response to parasitism proteins expressed in RKI esophageal gland cells and secreted into the host root cells. One secretory peptide functions as a ligand for host transcription factors that regulate normal root development. As a result, host root cells are modified into large multinucleate cells with thickened walls, extensive ingrowths and dense cytoplasm through the process of repeated karyokinesis (nuclear division) without cytokinesis (cellular division) (Huang et al., 2006). Giant-cells serve as supermetabolic protein factories that supply the nematode with the nutrition it needs to complete its life cycle, but are a detriment its host (Huang, 1985). After 10 to 12 days of feeding, the J2

RKI stops feeding and undergoes three consecutive molts over a time period of 2 days to become an adult female. To complete its life cycle, the adult deposits eggs in a gelatinous egg sac on the root's surface. Egg production usually coincides with a period of increased feeding site growth in the plant and increased nutrient demand for the parasitizing RKI (Hussey, 1985). Although giant-cells are beneficial to RKI they are a liability for the plant, resulting in nutrient and water deficiencies. After egg production, death completes the adult female's life cycle and giant-cells degenerate.

**Disease Control**

RKI can be effectively controlled using several nematicides, but these are expensive and hazardous to the health of humans and many other animals. Many of the most effective nematicides have been banned from use because of their harmful effects. Nematicides lower profit margins when used on low-value crops such as soybean (Niblack et al., 2004). Other methods of control include rotation with nonhost crops and the use of resistant cultivars (Weaver et al., 1988b). RKI has a wide host range, so an economically acceptable nonhost may not be available in some soybean production areas. Growing resistant cultivars is the most profitable and desirable method a grower can use to reduce crop damage due to RKI. Therefore, it is important for soybean breeders to incorporate RKI resistance into the cultivars they develop if they are to be grown in areas where RKI is present.

**Disease Resistance**

In most soybean diseases such as those caused by fungi, bacteria, and viruses, host resistance is measured by the ability, or the lack of ability of a pathogen to cause disease within the host. However, host resistance to parasitic nematodes is more accurately described as the ability, or lack of ability of the parasite to develop and reproduce on the host plant (Triantaphyllou, 1987). Methods for measuring nematode reproduction on soybean include counting the number of root galls on the roots of each infected plant, and counting the number of eggs produced on each plant by adult female RKI. These methods are discussed in detail in later paragraphs of this paper.

Despite the presence of reliable methods to measure resistance to RKI, few studies have characterized the genetic variation for RKI resistance in soybean, which is useful in determining the most efficient strategy for improving quantitative traits in crops (Weaver, 1989). However, RKI-resistant cultivars were successfully developed prior to knowing the genetic control of resistance (Hartwig and Epps, 1977). This could be due to the fact that RKI resistance is controlled by relatively few genes compared to most quantitative traits. Boquet et al. (1976) conducted one of the first studies to determine the inheritance of resistance to RKI. They screened $F_1$, $F_2$, and $F_3$ plants of a cross of D69-6344 (resistant) by D69-8178 (susceptible) for variation in egg production and determined that resistance to the Wartelle race of RKI was conditioned by at least one major, partially dominant gene with several gene modifiers. The major gene was determined to come from the cultivar Laredo. In a similar manner, analysis of $F_1$, $F_2$, and $F_3$ populations of crosses between 'Bossier' (susceptible) and 'Forrest' (resistant) screened for RKI gall formation determined that RKI resistance in Forrest is conditioned

by a single additive gene designated as *Rmi1* (Luzzi et al., 1994a). Luzzi et al (1994b) used greenhouse screens to asses galling in $F_1$, $F_2$, and $F_3$ populations of resistant x susceptible and resistant x resistant crosses in order to draw conclusions about the inheritance of RKI resistance in two soybean plant introductions. The overall mean number of galls for the $F_1$ and $F_2$ populations of PI96354 (resistant) x Bossier (susceptible) was more similar to the midparent value than to either of the parental means. This indicated that inheritance of RKI resistance from PI96354 was largely additive and neither resistance nor susceptibility was dominant. Data from their $F_2$ and $F_3$ populations indicated that resistance was quantitatively inherited. Luzzi et al (1994b) evaluated three different sources of resistance, Forrest, PI96354, and PI417444. Based on their screens of crosses of these three sources in different combinations, the source of resistance in Forrest, *Rmi1*, was determined to be different from the source of resistance in either of the two plant introductions. This same study also showed that PI96354 and PI417444 did not contain unique resistance genes for RKI gall. In previous studies PI96354 was found to have a greater ability to suppress nematode reproduction than PI417444 (Luzzi et al., 1987). PI96354 appears to have resistance to both gall formation and RKI reproduction that are conditioned by different genes (Luzzi et al., 1994b).

There are varying accounts of the importance of genotype x environment (G x E) interaction in resistance to RKI. The fact that greenhouse screens (Harris et al., 2003; Hussey et al., 1991; Riggs et al., 1988) and field screens (Birchfield and Harville, 1984; Kinloch et al., 1985) in single environments are used to select for resistance in genotypes intended to be grown in broad base populations of environments is an indication that G x E interaction during the selection process may not be as important for RKI resistance as it

is for other quantitative traits. Regardless of how few environments are used to evaluate RKI resistance, significant variation in environmental conditions exist even within the same greenhouse (Boerma and Hussey, 1992). Before resistant cultivars are released, they should be screened in nematode-infested fields in several environments (Boerma and Hussey, 1992). Another issue involved with the G x E interaction in RKI resistance is the fact that different races of RKI have been reported. If resistance to different RKI races is conditioned by different soybean resistance genes (race-specific resistance), then some soybean cultivars will perform better in the presence of certain RKI populations than others. Swanson and Van Gundy (1984) found that RKI races 1,3,and 4 produced 5,000 to 15,000 eggs per root system in greenhouse tests on the cultivar Pickett 71, and only 300 to 600 on the cultivar Centennial. On the other hand, RKI race 2 produced 1,200 eggs on Pickett 71 and 8,000 eggs on Centennial in the same experiment. This cultivar x race interaction was confirmed in a second greenhouse experiment in the same publication. However, Windham and Barker (1986) refuted these results when they found egg production of RKI races 1 and 3 to be higher on Centennial than that of race 2. They concluded that RKI resistance was population specific but not race specific.

**Heritability Estimates of RKI Resistance**

Estimates of heritability are important for determining the relative ease in which a certain trait can be selected under a given testing regime and for prediction of the amount of progress that can be made during selection for the trait (Burton, 1987). Luzzi et al (1994b) calculated both variance component and parent-progeny correlation estimates for the heritability of RKI resistance. They evaluated the crosses of the susceptible cultivar

Bossier and two resistant plant introductions, PI96354 and PI417444. To calculate variance component heritability estimates they used three replications of 40 $F_3$ families from each cross at one greenhouse location. Each plot consisted of seven plants of a single $F_3$ family. They calculated heritability based on $F_3$ family means using the formula $h^2 = \sigma^2_{F3}/(\sigma^2_{F3} + \sigma^2_e/r)$, and based on single $F_3$ plants using the formula $h^2 = \sigma^2_{F3}/(\sigma^2_{F3} + \sigma^2_e)$, where $\sigma^2_{F3}$ = variation among $F_3$ families, $\sigma^2_e$ = error variance, and r = number of replications. For the RKI resistance in PI96354, heritability was 0.91 and 0.78 based on family means and single plants, respectively and 0.92 and 0.79 for the PI417444 source of resistance. Luzzi et al. (1994b) calculated the standard-unit heritability for the RKI resistance in PI96354 using parent-progeny correlations (Frey and Horner, 1957) using the same number of replications, families, locations, and plot size as previously mentioned, and the formula $h^2 = \sigma_{F2F3}/(\sigma^2_{F2} \times \sigma^2_{F3})^{1/2}$ where $\sigma_{F2F3}$ = covariance between $F_2$ plants and their $F_3$ progeny. They found this value to be 0.73. Tamulonis et al (1997) calculated a variance component heritability estimate of 0.69 based on 101 $F_{2:3}$ families of a cross of PI96354 with Bossier. Their selection unit consisted of two replications, one location, and seven plants per family. These moderate to high heritability estimates indicate that resistance to RKI found in these soybean plant introductions is controlled by relatively few genes and that incorporation of this resistance into previously susceptible genotypes can be done relatively quickly.

**RKI Resistance QTL**

Restriction fragment length polymorphism (RFLP) markers were first linked to RKI resistance QTL when Tamulonis et al. (1997) found a major RKI resistance QTL that had additive gene action on linkage group (LG) O, and was linked to the RFLP marker G248A-1, and a minor resistance QTL with dominant gene action on LG-G that was linked to the RFLP markers K493H-1 and Cs008D-1. To find these marker-trait associations, they initially evaluated 101 $F_{2:3}$ lines from a cross of PI96354 with Bossier for galling in a greenhouse screen. Seven plants from each line were screened in two replications with an initial inoculum of 3,000 eggs per plant. To collect genotypic information, they digested DNA from the 101 $F_2$ plants that gave rise to the $F_{2:3}$ families with the restriction enzymes *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, and *Taq*I. The digested DNA was then electrophoresced in an agarose gel. DNA probes were hybridized to the digested $F_2$ DNA to detect polymorphic fragments. A total of 127 RFLP markers were used to derive the genotype of each $F_2$ plant at each marker. They used linear regression to identify markers that were associated with RKI resistance by using $F_2$ genotype ($A_1A_1$, $A_1A_2$, $A_2A_2$) as the predictor variable and average $F_{2:3}$ family gall number as the response variable. Interval mapping was also used to determine the position and LOD value for each QTL. Using regression analysis, they determined that four RFLP markers were significantly associated with RKI gall number. They were A882D-1 and G248A-1 on LG-O and A890V-1 and K493H-1 on LG-G. The highest LOD score (LOD = 6.9) from interval mapping indicated that a major RKI resistance QTL was located near RFLP marker G248A-1 that explained 31% of the phenotypic variation in gall number. They could not determine if the LOD score at this marker was the true maximum because no

markers distal to G248A-1 were available. However, they used simulated markers to determine that the most likely position of the QTL and maximum LOD score, 16.2, was 10 to 15 centiMorgans (cM) distal to G248A-1. A second minor QTL was detected in the interval from K493H-1 to Cs008D-1 on LG-G that explained 14% of the variation in gall number. The peak LOD score of 2.4 was found 5.4 cM from K493H-1. When simple sequence repeat (SSR) markers in soybean became available, Li et al (2001) combined the data from Tamulonis et al (1997) with data for SSR markers available in the previously identified regions for the QTL on LG-O and LG-G. Li et al (2001) used the same mapping population, phenotypic data, and RFLP genotypic data from Tamulonis et al (1997), but added genotypic information from 20 SSR markers. In addition to regression analysis and interval mapping, Li et al (2001) used composite interval mapping to determine the most likely interval for the QTL while controlling for the effects of QTL in other regions. The major QTL on LG-O mapped between SSR markers Satt492 and Satt358, had additive gene action, and explained 55.8% of the variation in gall number. The maximum LOD score of 16.3 was found 3.1 cM from Satt492. This location is within the region 10 to 15 cM from G248A-1 identified by Tamulonis et al (1997) as the region most highly associated with RKI resistance using simulated markers. Li et al (2001) believe that this major QTL most likely corresponds to the RKI resistance gene *Rmi1*. On LG-G, Li et al (2001) found two peaks for potential QTL that expressed dominance using interval mapping. However, composite interval mapping indicated that a single minor QTL on LG-G mapped between SSR markers Satt012 and Satt505, and explained 18% of the phenotypic variation in gall number. The highest LOD score of 3.6 was found 4 cM from Satt012. Interestingly, the peak LOD score in this study was 29.9

cM away from the peak LOD score found in the previous study, which composite interval mapping had determined to be a ghost peak. Li et al (2001) provide a logical explanation for this, pointing out that Tamulonis et al (1997) found two peaks in LOD score on LG-G. They found one peak between K493H-1 and Cs008D-1 that they reported to be the location of the minor QTL, and an additional peak between L156V-1 and A890V-1 that was not statistically significant. Both of the RFLP markers used to define the latter peak in Tamulonis et al (1997) are flanking markers of the region Li et al (2001) found to be the true QTL using SSR markers Satt012 and Satt505. Li et al (2001) concluded that by increasing the marker density in the LG-G region that they achieved a better resolution of the QTL's true location.

Both the major QTL on LG-O and the minor QTL on LG-G have been confirmed as QTL in independent experiments (Ha et al., 2004; Li et al., 2001). Ha et al (2004) used six SSR markers flanking the QTL on LG-O to track the inheritance of this QTL in six cycles of breeding in 48 different soybean lines. They found strong evidence of cosegregation of RKI resistance and a 200-bp band at Satt358. They also identified the cultivar Palmetto as the most likely ancestral contributor of *Rmi1* in most of the RKI-resistant elite cultivars in maturity groups V, VI, VII, and VIII. Li et al (2001) confirmed the minor QTL on LG-G by accounting for the variation associated with this QTL in a different set of meiotic events. They used a $BC_2F_2$ population of Prichard x G93-9009, where Prichard was the recurrent parent. G93-9009 has resistance alleles at both the major QTL on LG-O and the minor QTL on LG-G. Prichard only has the resistance allele at the QTL on LG-O. They phenotyped $BC_2F_2$ plants for gall number and tested three SSR markers on LG-O near the QTL. All of the plants that were homozygous for

the G93-9009 alleles at the three SSR markers had significantly fewer galls than the plants that were homozygous for the Prichard alleles at the same SSR markers. Because PI96354 is an ancestor of G93-9009, their results indicated that marker-assisted selection for the minor QTL on LG-G in a population that was already fixed for resistance at the major QTL on LG-O allowed them to recover the high level of RKI resistance present in PI96354.

The two QTL that condition resistance to RKI explain a large proportion of the genetic variation for gall number. The heritability estimate ($h^2$) of 69% used in Tamulonis et al (1997) and Li et al (2001) can be considered as the upper limit for the amount of genetic variation that can be explained by QTL (Knapp et al., 1990). The coefficient of determination ($R^2$) obtained using regression analysis represents the amount of phenotypic variation explained by a given QTL or multiple QTL depending on the regression model used. The proportion of the total genetic variation explained by a given QTL or multiple QTL can be expressed as the $R^2/h^2$. Tamulonis et al (1997) used multiple regression for the two QTL model to get a $R^2$ value of 39%. The two QTL they found explained 57% (39/69 = 0.57) of the total genetic variation for gall number. By increasing the marker density on LG-O and LG-G, Li et al (2001) were able to increase the $R^2$ value from multiple regression for the two QTL to 57%. As a result they were able to explain 83% (57/69 = 0.83) of the total genetic variation. The remaining 17% of the total genetic variation left unexplained by these two QTL could eventually be accounted for with a tighter marker-QTL linkage (possibly using single nucleotide polymorphism (SNP) markers) or by finding additional QTL conditioning RKI resistance.

As evident by this literature review, much is known about RKI and the QTL in soybean associated with resistance to this parasite. However, based on the amount of variation in gall or egg number explained by previously found QTL, it is evident that resistance QTL may exist in addition to those on LG-O and LG-G. Learning more about the nature and location of these QTL and introgressing them into improved cultivars would better equip soybean growers in their efforts to manage this parasite.

**Resistance Screens**

Resistance to nematodes in soybean has been measured in a variety of different ways. Some studies measure resistance in terms of the nematode's ability to reproduce on the plant. These experiments involve growing plants in soil with a uniform initial number of RKI eggs or juveniles in greenhouse pots and counting the number of eggs or egg masses produced on each plant after a certain period of time. Results are usually reported as the total number of eggs per plant, the total number of egg masses per plant, the number of eggs per unit of total root weight, or the number of egg masses as a percentage of the total root surface. Luzzi et al (1987) used both the total number of eggs per plant and the number of eggs per gram of root tissue as measures of resistance for evaluation of genotypes included in the USDA Southern Soybean Germplasm Collection. Egg mass indices are often used. Indices vary from study to study with some based on predetermined classes, and others established experimentally based on the performance of resistant and susceptible check cultivars included in the test. Hussey and Boerma (1981) suggested standardizing indices and allowing selection for greater resistance by using resistant and susceptible checks as an internal standard within each study. For

example, for an index with six discrete classes (0 to 5), the mean of the resistant check or parent should serve as the midpoint value for class 2 and the mean of the susceptible check or parent should serve as the starting value for class 5. Hussey and Boerma (1981) used an index for the total number of RKI egg masses per root system where root systems with no egg masses were given a score of 0, root systems with 1 to 2 egg masses were given a score of 1, root systems with 3 to 10 egg masses were given a score of 2, root systems with 11 to 30 egg masses were given a score of 3, root systems with 31 to 100 egg masses were given a score of 4, and root systems with more than 100 egg masses were given a score of 5.

Other studies measure the plant's ability to prevent tissue damage caused by the nematode. These experiments also involve growing plants in soil with a uniform initial number of RKI eggs or juveniles in greenhouse pots, but root galls are counted rather than eggs or egg masses. Gall indices are often used to measure nematode damage similarly to how egg mass indices are used to estimate nematode reproduction. Luzzi et al (1987) used several different gall indices for their experiments, all of which were based on the performance of resistant and susceptible check cultivars. Hussey and Boerma (1981) found that the gall index they used was positively correlated with the egg mass index, the total number of eggs per plant, and the number of eggs per gram of root. A significant negative correlation between gall number and yield has also been reported (Kinloch et al., 1985) and the ultimate goal of breeding for resistance to RKI is to maintain a high yield in the presence of nematodes. However, one must use caution when evaluating resistance based on gall numbers. Gall formation and nematode

reproduction can be controlled by different genes (Luzzi et al., 1987) that may be mutually exclusive of each other (Hussey and Boerma, 1981).

Some studies have even used yield and plant vigor based on above ground symptoms as indirect measures of resistance (Williams et al., 1973). These measures are almost unavoidably confounded with other genetic and environmental factors. The advantage of using yield or plant vigor ratings is that they allow screening for tolerance to the disease. This cannot be done in a greenhouse screen where only galling or reproduction is measured. A tolerant genotype is one that sustains nematode feeding and reproduction similar to that of a susceptible genotype but has higher yield and agronomic quality characteristics under nematode pressure than a susceptible intolerant genotype (Weaver, 1989).

Evaluation and selection for RKI resistance is typically done using greenhouse screens rather than field studies. Screening genotypes in the field requires natural infestation or artificially inoculated field microplots. In such situations, nematode density can vary greatly and more than one nematode species is often present. Greenhouse screening allows genotypes to be evaluated continuously throughout the year in an environment where the nematode species and inoculum concentration can be controlled, and soil contamination with other pathogens of soybeans or nematodes can be minimized by using sterilized soil (Hussey and Boerma, 1981). A standard greenhouse procedure for selecting soybean genotypes for RKI resistance was described by Hussey and Boerma (1981). A summary of their method is as follows: RKI populations are maintained on a susceptible variety of tomato. Galled roots from infected tomato plants are cleaned using water and then shaken for 4 minutes in a 0.5% NaOCl solution to

extract the nematode eggs from the roots (Hussey and Barker, 1973). The solution, including eggs, is passed through a series of sieves, the smallest of which is used to collect the eggs and wash away the NaOCl solution. Inoculum (egg) concentration is determined and adjusted to the desired level using a stereoscopic microscope. Three seeds of each test genotype are planted in a container filled with methyl bromide-fumigated sandy loam soil. Seven to ten-day-old seedlings are thinned to one per container and inoculated with the egg suspension. Metal halide lamps, automatic drip irrigation, and 20-20-20 (N-P-K) fertilizer are used to enhance nematode infection. Once it has been determined that symptoms (galling or egg number) are sufficient on a susceptible check cultivar, all plants are topped and roots are washed prior to examination. Roots of the test genotypes are then either examined for galls, egg masses, or total egg number. The most current modifications in this procedure are presented in Ha et al (2004).

Unless it is determined that egg production and gall formation are not related, selection is usually based on evaluating plant roots for gall number rather than egg production. Gall number is used more often simply because counting galls is less expensive and time-consuming than isolating and counting eggs from each entry in the experiment. When evaluating genotypes for RKI resistance, it is also important to consider initial inoculum concentration. If the concentration is too high it may be difficult to distinguish resistant from susceptible plants (Luzzi, 1986). Typically, testing genotypes from crosses where both parents were resistant requires a high initial inoculation density of nematode eggs to completely distinguish between resistant and highly resistant genotypes. Testing genotypes from crosses where only one of the parents

is resistant requires a lower initial inoculum density (Luzzi et al., 1987). To normalize variations in greenhouse environments, resistant and susceptible checks should be included in each experiment as internal standards within each experiment.

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# CHAPTER 4

# SSR MAPPING OF THREE SOYBEAN RESISTANCE GENES TO SOUTHERN STEM CANKER[1]

[1]Shearin, Z.P., Narvel, J.M., Yates, J.L. Bowers, G.R., and H.R. Boerma. To be submitted to *Crop Science*.

**Abstract**

Southern stem canker, caused by the fungus *Diaporthe phaseolorum* (Cooke & Ellis) Sacc., is a very devastating disease of soybean worldwide, and particularly in the southern USA. The most successful method for controlling stem canker involves the use of resistant soybean cultivars. The resistance genes *Rdc1*, *Rdc3*, and *Rdc?(PI398469)* have been identified using traditional phenotypic segregation ratios. The objective of this study was to locate *Rdc1*, *Rdc3*, and the *Rdc?(PI398469)* genes on the USDA consensus soybean genetic linkage map. Our approach to mapping the *Rdc* loci involved crossing a source of a single *Rdc* gene with the highly susceptible soybean breeding line J77-339 to create $F_2$ or $F_{2:3}$ soybean mapping populations. Greenhouse and field screens for stem canker reaction (resistant vs. susceptible) produced the expected 3:1 (resistant: susceptible) segregation ratio for all three mapping populations. Simple sequence repeat (SSR) markers were tested using bulk segregant analysis on $F_2$ populations of 'Crockett' x J77-339 and PI 398469 x J77-339, while single nucleotide polymorphism (SNP) markers were tested on an $F_{2:3}$ population of D85-10404 to determine the linkage group on which each gene resides. Additional polymorphic SSR markers were tested in the putative genomic regions of each stem canker resistance gene. The *Rdc3* gene from Crockett and the *Rdc?(PI398469)* gene from PI 398469 both mapped to the top of LG-B2 within approximately 6 cM of each other. The *Rdc1* gene from D85-10404 mapped to LG-D1b within 10 cM of Satt428.

## Introduction

Southern stem canker, caused by the fungus *Diaporthe phaseolorum* (Cooke & Ellis) Sacc., is an economically important disease of soybean, *Glycine max* (L.) Merr.. In North America, stem canker has been found in the midwestern and southeastern regions of the USA and in Ontario, Canada. Yield losses due to stem canker in the USA were estimated to cost growers $67.1 million in 2003 (Wrather, 2004). The disease has especially been a problem in the southern USA in recent years. After previously being considered as separate varieties, the northern and southern biotypes of stem canker-causing fungi have been classified as separate *formae speciales*, with the organism associated with stem canker in the northern USA and Canada classified as *D. phaseolorum* (Cooke & Ellis) Sacc. f. sp. *caulivora* Morgan-Jones (DPC), and the organism associated with stem canker in the southern USA classified as *D. phaseolorum* (Cooke & Ellis) Sacc. f. sp. *meridionalis* Morgan-Jones (DPM; (Morgan-Jones, 1989). However, the classification of these fungi as *formae speciales* has not been universally accepted, and they are still often referred to as varieties in the literature.

Fungal infection by DPM occurs primarily by rain splashing inoculum from infected soybean residue from the previous growing season onto the new crop during heavy rains (Ploetz and Shokes, 1985). Plants are usually infected through scars on stems, petioles, or petiole bases (Ploetz and Shokes, 1987). Once a plant becomes infected, there is a latent period where no disease symptoms can be observed. The first symptoms of southern stem canker appear during the plant's early reproductive stages as small reddish-brown superficial lesions on the stem, usually near a lower leaf node. The lesions expand longitudinally and form reddish-brown cankers that become slightly

sunken as the growing season progresses. Older lesions may appear dark brown with a grayish-brown center and a reddish-brown margin, rarely girdling the stem (Fernandez et al., 1999). Tissue above and below the canker remains green (Grau et al., 2004). The dead stem tissue blocks the upward flow of water through the vascular tissue, and the seed-bearing portion of the plant becomes water-stressed and usually dies before the plant's full yield potential is reached (Hildebrand, 1952). Leaves usually remain attached to the plant and show symptoms of interveinal chlorosis and necrosis. A phytotoxin plays a role in foliar symptoms and premature plant death (Lalitha et al., 1989). Premature plant death causes a reduction in seed number and size (Fernandez et al., 1999).

The stem canker disease can usually be managed by removing or deep-tilling infected soybean debris combined with planting resistant cultivars. Removing or deep-tilling infected soybean debris is not possible for growers that use no-till management practices. Cultivar resistance to stem canker is conditioned by at least five different dominant resistance genes. In 1994, two germplasm lines, D85-10404 and D85-10412, were released with resistance to stem canker (Kilen and Hartwig, 1994). 'Tracy M' was the source of resistance for D85-10404 (*Rdc1)* and D85-10412 (*Rdc2)*. Both germplasm lines were developed from the cross of Tracy-M x J77-339 (Kilen et al., (1985). Additional resistance genes have been found in the cultivars Crockett (*Rdc3*) and Dowling (*Rdc4*) (Bowers et al., 1993). Both PI 230976 and PI 398469 have single dominant genes conditioning resistance to stem canker that are at different loci than *Rdc1, Rdc2, Rdc3, and Rdc4*, but as of yet have not been assigned gene symbols because it is not known if the genes in these plant introductions are unique (Tyler, 1995).

The objective of this study was to locate *Rdc1*, *Rdc3*, and the *Rdc?(PI398469)* genes on the USDA consensus soybean genetic linkage map (Song et al., 2004). This will allow soybean breeders to more efficiently select for stem canker resistance in their cultivar development programs.

**Materials and Methods**

In the summer of 2004, the stem canker resistant D85-10404, Crockett, and PI 398469 were each crossed to the highly susceptible breeding line J77-339 at the Univ. of Georgia Plant Sciences Farm near Watkinsville, GA. The $F_1$ generation of each cross was grown in a greenhouse on the Univ. of Georgia campus in Athens, GA during the winter of 2005. Seed of the $F_{1:2}$ generation was harvested in bulk from the Crockett x J77-339 and PI 398469 x J77-339 populations and gave rise to the $F_2$ plants used for mapping. Three $F_{1:2}$ rows from the cross D85-10404 x J77-339 were grown in the summer of 2005 at the Univ. of Georgia Plant Sciences Farm near Watkinsville, GA. Individual plants from each of the $F_{1:2}$ rows were harvested and single-plant threshed to create $F_{2:3}$ lines.

Screening for stem canker reaction was done in the greenhouse (D85-10404 x J77-339 population) and in the field (Crockett x J77-339 and PI 398469 x J77-339 populations) using methods similar to those presented by Keeling (1982). The DPM inoculum was isolated in August of 2006 from the stems of infected soybean plants grown in a field in Mississippi. The DPM isolate was subcultured in a potato dextrose agar (PDA) solution with toothpicks. Infested toothpicks were inserted into the hypocotyls of individually-labeled $F_2$ soybean seedlings from the Crockett x J77-339 and

PI 398469 x J77-339 populations planted in the field and 13 to 16 $F_3$ soybean seedlings of the D85-10404 x J77-339 population labeled according to $F_{2:3}$ family planted in clay pots. Plants were considered susceptible if they were dead after 10 d, moderately resistant if they were still alive but had stem lesions, and resistant if they showed no symptoms of stem canker.

For the Crockett x J77-339 and PI 398469 x J77-339 $F_2$ mapping populations, each $F_2$ plant was labeled and leaves were harvested from each plant for genomic DNA extraction prior to DPM inoculation. For the D85-10404 x J77-339 $F_{2:3}$ mapping population, leaves from each $F_3$ plant were harvested and combined with leaves of other plants from the same $F_2$ family prior to DNA extraction. The collected leaf tissue was lyophilized and ground to a powder. DNA was extracted using the CTAB method (Keim et al., 1988) and resuspended in TE buffer.

The PCR conditions for each SSR marker were similar to those used by Li et al (2001). Each reaction had a total volume of 10 µL and consisted of 2 µL of 50 ng/µL template DNA, 1.0X PCR buffer, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 0.2 µM each of fluorescently-labeled forward and reverse SSR primers, and 5 U/µl of Promega Taq DNA polymerase. PCR products were combined with a mixture of 2 µL formamide, 0.75 µL loading buffer, and 0.2 µL ROX-500 size standard in a 2:3 ratio (PCR product: loading buffer). The mixture was denatured at 95°C for 5 minutes and loaded into a 96-lane, 12-cm 19:1 (acrylamide: bisacrylamide) gel and electrophoresced at 750 V for 1.5 - 2 hours using an ABI Prism 377 DNA sequencer [Applied Biosystems Incorporated (ABI), Foster City, CA] in order to assign a parental genotype to each PCR product. The computer

program GeneScan Version 3.0 [Applied Biosystems Incorporated (ABI), Foster City, CA] was used to collect marker data.

SSR marker data on the $F_2$ plants was collected by using bulk segregant analysis (BSA), with one bulk consisting of $F_2$ plants with the resistant phenotype, and one bulk consisting of $F_2$ plants with the susceptible phenotype. Each bulk was made up of no more than 12 plants of a mapping population with each plant contributing approximately equal DNA to the bulk. Polymorphic SSR markers spaced approximately every 20 cM along the soybean genetic map on each of the 20 linkage groups were tested on the bulks and the parents of each cross. Using BSA, a putative location of the resistance locus was considered for further analysis when the band for the susceptible bulk matched the susceptible parent and bands for the resistant bulk matched both the resistant and the susceptible parents. Once a putative map location was found using BSA, these marker(s) were tested on the individual members of each bulk to verify the initial BSA results. After the confirmation of these markers for each cross, additional polymorphic SSR markers available in the region were tested on each member of the mapping population in order to resolve the most probable location of the resistance gene.

The genotypic and phenotypic data were analyzed using MAPMAKER/EXP V. 3.0 (Lander et al., 1987) to construct genetic linkage maps using the Kosambi mapping function (Kosambi, 1944). The stem canker rating (resistant or susceptible) was treated as a qualitative trait and mapped by treating the resistant rating as a dominant marker.

**Results and Discussion**

Plants from the two $F_2$ mapping populations, Crockett x J77-339 for mapping *Rdc3* and PI 398469 x J77-339 for mapping *Rdc?(PI398469)*, segregated 3:1 (resistant : susceptible) for stem canker reaction (Table 4.1). Given the difficulty of determining stem canker reaction on single plants of this population, the phenotypic reaction of the $F_2$ generation of D85-10404 x J77-339 segregating for *Rdc1* was determined by the phenotypes of their $F_{2:3}$ lines. Plants of the $F_2$ generation that produced all susceptible $F_3$ plants were classified as *rdc1 rdc1*, and all other $F_2$ plants were classified as *Rdc1* __. Based on their $F_{2:3}$ lines phenotype, the $F_2$ plants exhibited the expected 3:1 segregation ratio (Table 4.1).

When Satt577 located on LG-B2 was tested using BSA of plants from the PI 398469 x J77-339 $F_2$ mapping population, bulks of stem canker resistant plants had bands of similar size to bands from both the resistant parent and the susceptible parent, and bulks of stem canker susceptible plants had a single band matching the size of the susceptible parent (Figure 4.1). Similar results were found when Sat_264, also on LG-B2, was tested using BSA of plants from the Crockett x J77-339 $F_2$ mapping population (Figure 4.2). All of the other 130 SSR markers tested on the PI 398469 x J77-339 population and the 102 SSR markers tested on the Crockett x J77-339 population had both parental bands in the resistant and susceptible bulks. The putative identification of the linkage of *Rdc?(PI398469) and* Satt577 using BSA was verified by testing each plant used to make the bulks in individual PCR reactions with Satt577. Every plant in the resistant bulk had either a band of similar size to the resistant parent or both parental bands while every plant in the susceptible bulk had only a band of similar length to the

susceptible parent (Figure 4.3). Similar results were found when the putative linkage of *Rdc3* and Sat_264 was tested in individual PCR reactions with DNA from each plant used during BSA of the Crockett x J77-339 population (Figure 4.4). The BSA provided strong evidence that both *Rdc?(PI398469)* and *Rdc3* are located on LG-B2.

Based on results from BSA, additional SSR markers from LG-B2 were tested on the 157 $F_2$ plants of the PI 398469 x J77-339 population and on the 164 $F_2$ plants of the Crockett x J77-339 population. In addition, SSR markers from LG-D1b were tested on 94 $F_{2:3}$ lines from the D85-10404 population. The *Rdc?(PI398469)* gene from PI 398469 mapped to the segment of LG-B2 between Sat_177 and Sat_342, 3.9 cM from Sat_177 (Figure 4.5). Satt577, the marker identified as being associated with *Rdc?(PI398469)* using BSA, was only 5.5 cM from *Rdc?(PI398469)*. The *Rdc3* gene from Crockett mapped to the segment of LG-B2 between Sat_264 and Satt416, 7.8 cM from Sat_264 (Figure 4.6). Sat_264 was also the marker identified as being associated with *Rdc3* using BSA.

To map *Rdc1*, 41 single nucleotide polymorphism (SNP) markers were tested on the $F_{2:3}$ population of D85-10404 x J77-339. The Monsanto Company used SNP markers to putatively locate *Rdc1* on LG-D1b near SSR marker Satt579. In addition, SSR markers from LG-D1b were tested on 94 F2:3 lines from the D85-10404 x J77-339 population. The *Rdc1* gene from D85-10404 mapped to the segment of LG-D1b between Satt428 and Sat_415, 9.7 cM from Satt428 (Figure 4.7). The *Rdc1* gene was not linked to Sat_415, but was the nearest polymorphic marker on that side of the linkage group. Using forced linkage in the MAPMAKER/EXP program, Sat_415 was 62.7 cM from *Rdc1*. Linkage analysis of the SSR markers used to map *Rdc1*, *Rdc3*, and

*Rdc?(PI398469)* are in reasonable agreement with the USDA consensus soybean genetic linkage map (Song et al., 2004).

It is interesting that both *Rdc3* and *Rdc?(PI398469)* mapped to LG-B2 and were found to be tightly linked to each other (*Rdc?(PI398469)* mapped 3.9 cM from Sat_177 while *Rdc3* mapped 9.9 cM from Sat-177*)*. Tyler (1995) reported that a Crockett x PI 398469 $F_2$ population segregated approximately 15:1 (119 resistant plants: 5 susceptible plants) indicating that the *Rdc3* and *Rdc?(PI398469)* resistance genes were located at independently segregating loci. However, there was no $F_3$ progeny confirmation of the $F_2$ resistant or susceptible plants. The general agreement of the genetic distance between the SSR markers on the USDA consensus soybean map and our linkage maps of LG-B2 for PI 398469 x J77-339 and Crockett x J77-339 and the lack of map expansion after including the *Rdc?(PI398469)* and *Rdc3* genes provides evidence of the validity of our data. Given the precision of our mapping data, it is possible that *Rdc3* and *Rdc?(PI398469)* are located at the same locus. Although our data cannot resolve this issue, it is clear that the stem canker resistance gene(s) in Crockett and PI398469 are not at independent loci.

None of the *Rdc* genes presented here have mapped to known soybean resistance gene clusters (Soybase, http://soybeanbreederstoolbox.org). However, *Rdc1* on LG-D1b has mapped to within 30 cM of the *Rsv4* gene conferring soybean mosaic virus resistance, a QTL for *Sclerotinia* stem rot resistance, and at nearly the same location as the resistance gene analog RGA1f (Arahana et al., 2001; Hayes et al., 2000; Kanazin et al., 1996). Perhaps further research on mapping soybean disease resistance genes will reveal a new cluster of resistance genes in this region.

Marker assisted selection for these three stem canker resistance genes will require tighter marker-resistance gene linkages. Delineating the genomic regions where stem canker resistance is located to a smaller segment of a linkage group with the use of simple sequence repeat (SSR) markers facilitates the development of breeder friendly markers such as single nucleotide polymorphisms (SNPs) that could be more tightly linked to the resistance gene. The development of SNP markers may be especially helpful in achieving tighter marker linkages with *Rdc1* and *Rdc3*(Zhu et al., 2003). These SNPs and SSRs may ultimately be used for marker assisted selection in breeding programs in lieu of phenotyping for disease resistance using the more traditional greenhouse and field methods which require maintenance and culturing of the stem canker-causing fungus. Development of additional markers in the genomic regions of these stem canker resistance genes will empower breeders to select homozygous resistant plants and lines in early breeding generations and practice selection for other traits such as seed yield among stem canker resistant lines.

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Table 4.1. Stem canker reaction in $F_2$ and $F_{2:3}$ populations of D85-10404 x J77-339, Crockett x J77-339, and PI 398469 x J77-339.

| Cross | Population | Resistance gene | Resistant | Susceptible | Total | $X^{2\dagger}$ |
|---|---|---|---|---|---|---|
| | | | no. | no. | no. | |
| D85-10404 x J77-339 | $F_{2:3}$ | *Rdc1* | 69 | 27 | 94 | 0.5 |
| Crockett x J77-339 | $F_2$ | *Rdc3* | 125 | 39 | 164 | 0.1 |
| PI 398469 x J77-339 | $F_2$ | *Rdc?(PI398469)* | 114 | 43 | 157 | 0.5 |

† Chi square values are for the expected 3:1 (resistant: susceptible) segregation ratios. $X^2_{(0.10)} = 2.71$ $X^2_{(0.05)} = 3.84$

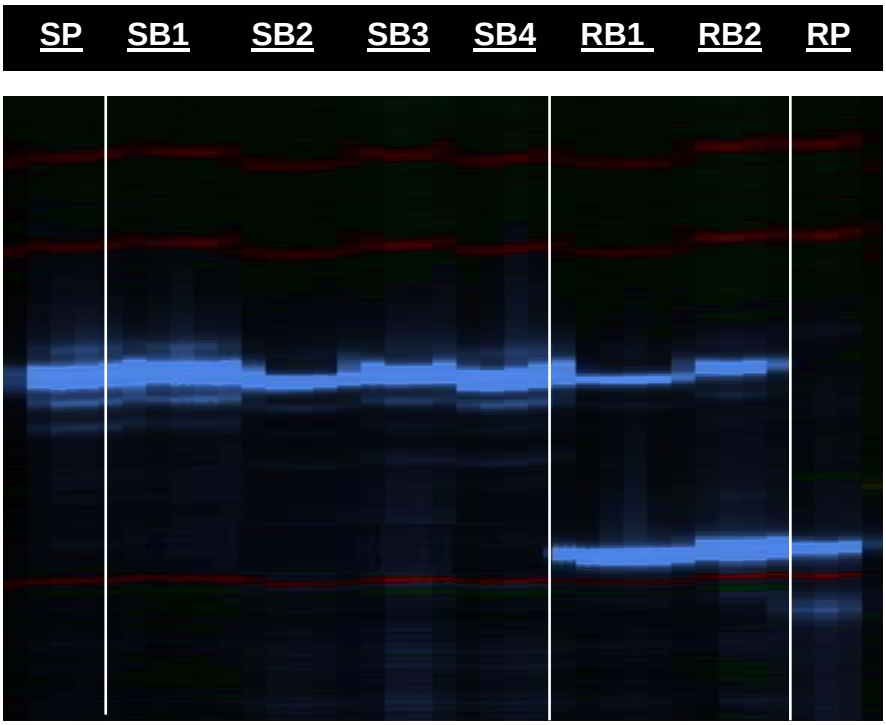


Figure 4.1. Bulk segregant analysis for SSR marker Satt577 on the PI 398469 x J77-339 $F_2$ mapping population (*Rdc?(PI398469)*). SP = susceptible parent; SB = susceptible bulk; RB = resistant bulk; RP = resistant parent

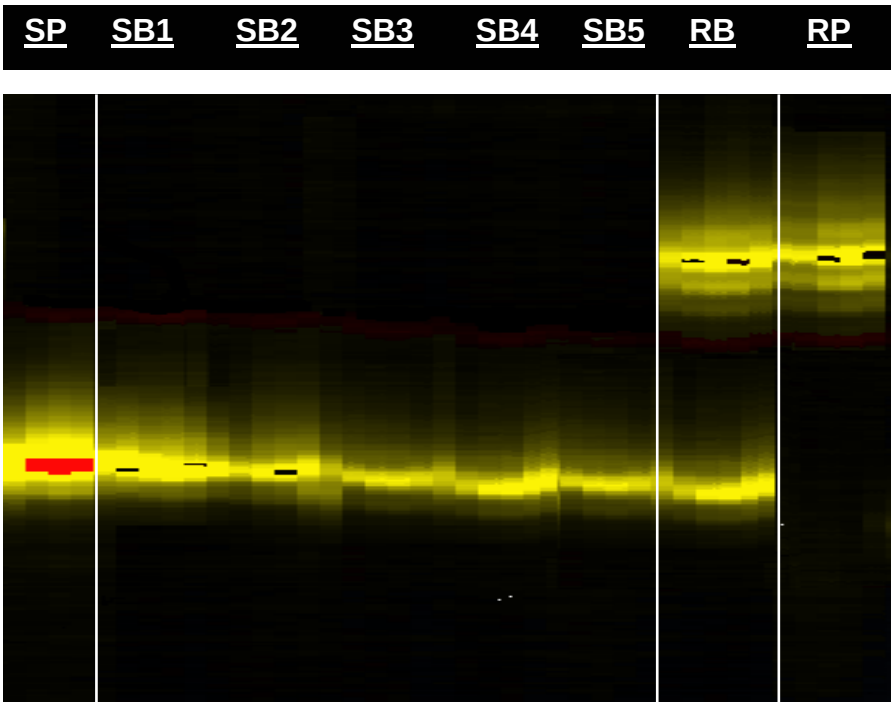


Figure 4.2. Bulk segregant analysis for SSR marker Sat_264 on the Crockett x J77-339 $F_2$ mapping population (*Rdc3*). SP = susceptible parent; SB = susceptible bulk; RB = resistant bulk; RP = resistant parent

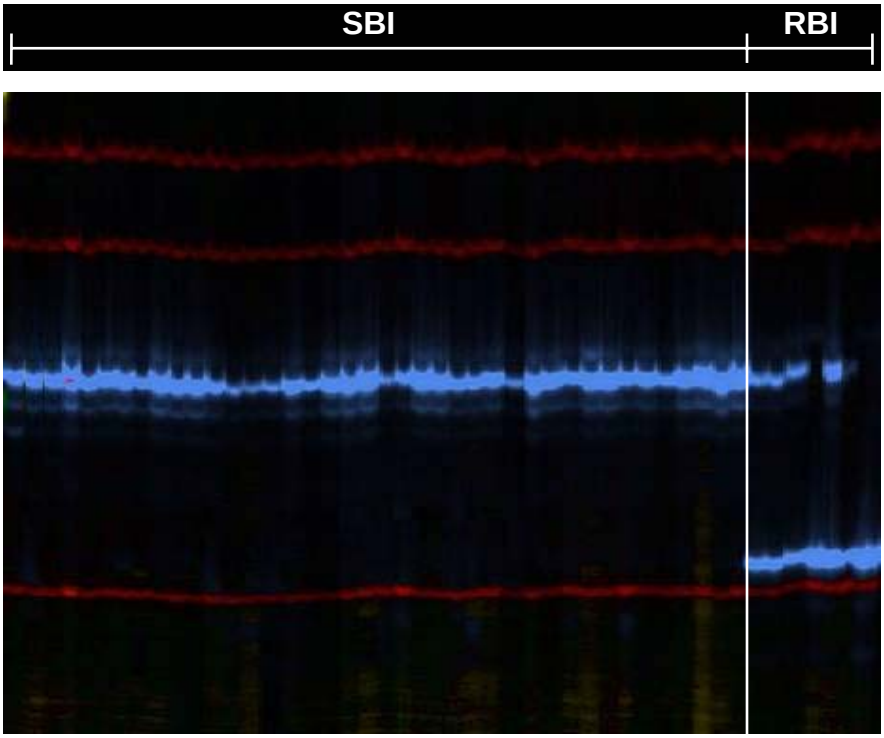


Figure 4.3. Amplification size of Satt577 in individual susceptible and resistant $F_2$ plants of PI 398468 x J77-339. SBI = 40 individual susceptible $F_2$ plants in 40 individual lanes; RBI = 8 individual resistant $F_2$ plants in 8 individual lanes

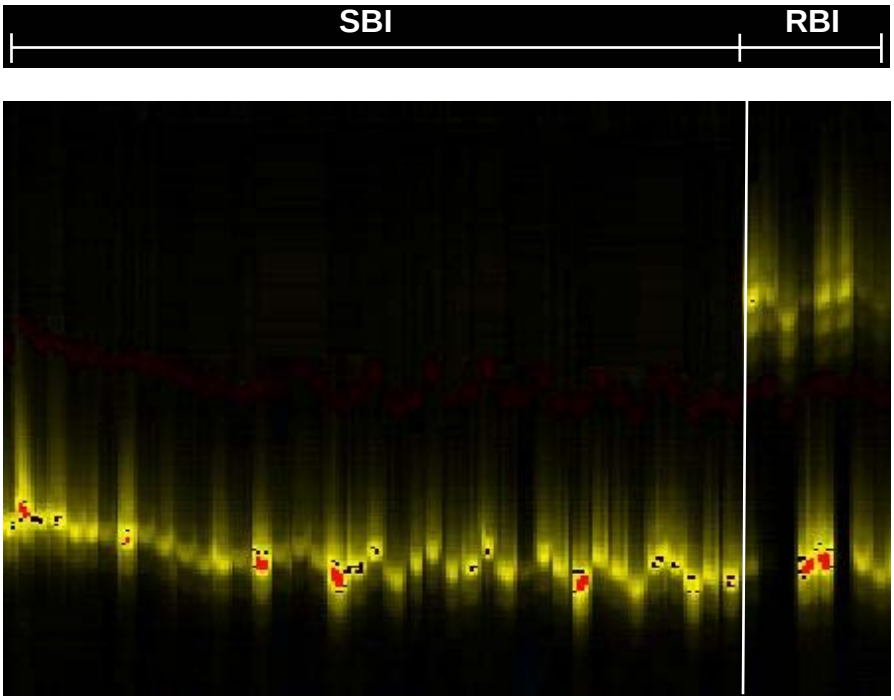


Figure 4.4. Amplification size of Sat_264 in individual susceptible and resistant $F_2$ plants of Crockett x J77-339. SBI = 40 individual susceptible $F_2$ plants in 40 individual lanes; RBI = 8 individual resistant $F_2$ plants in 8 individual lanes

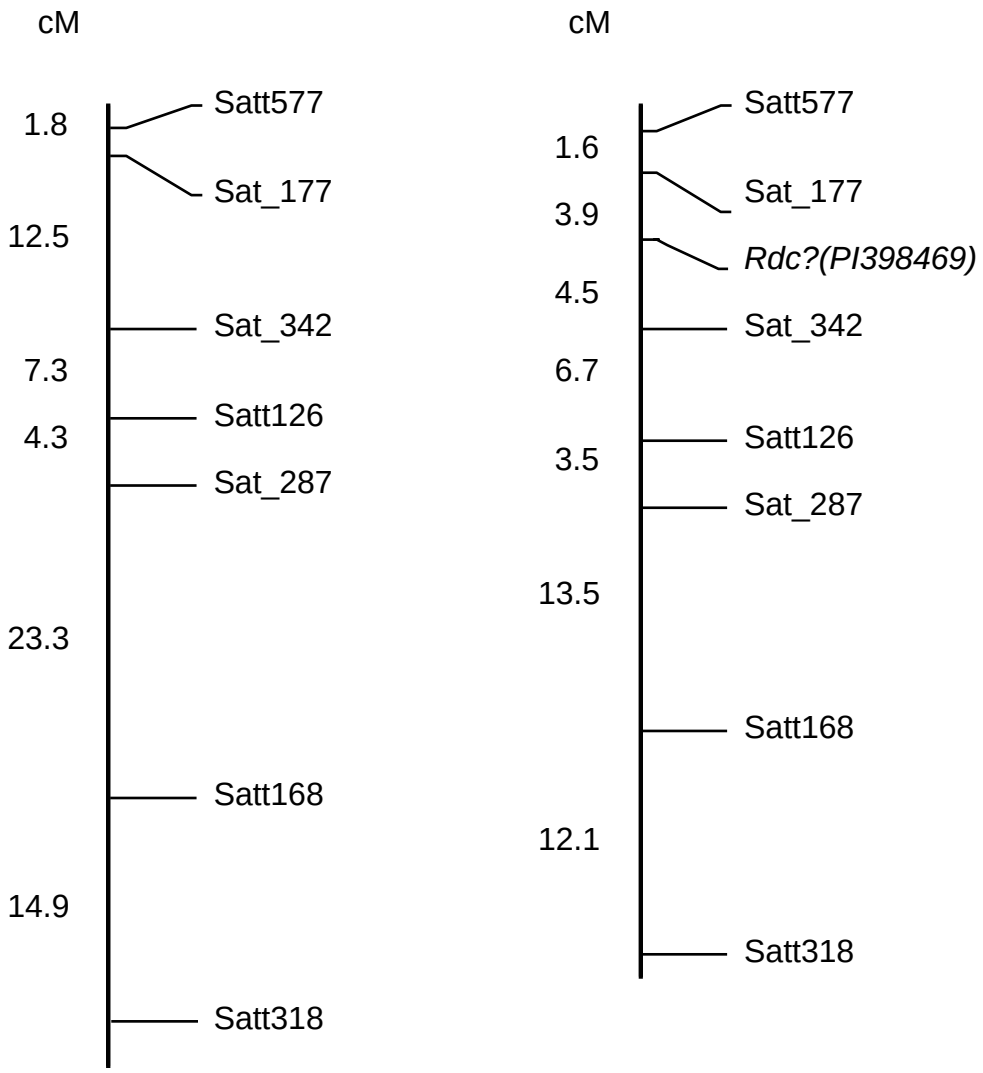


Figure 4.5. Linkage map of LG-B2 based on the USDA consensus soybean genetic linkage map (left) and a linkage map of LG-B2 including *Rdc?(PI398469)* based on 157 $F_2$ plants of PI 398469 x J77-339 (right).

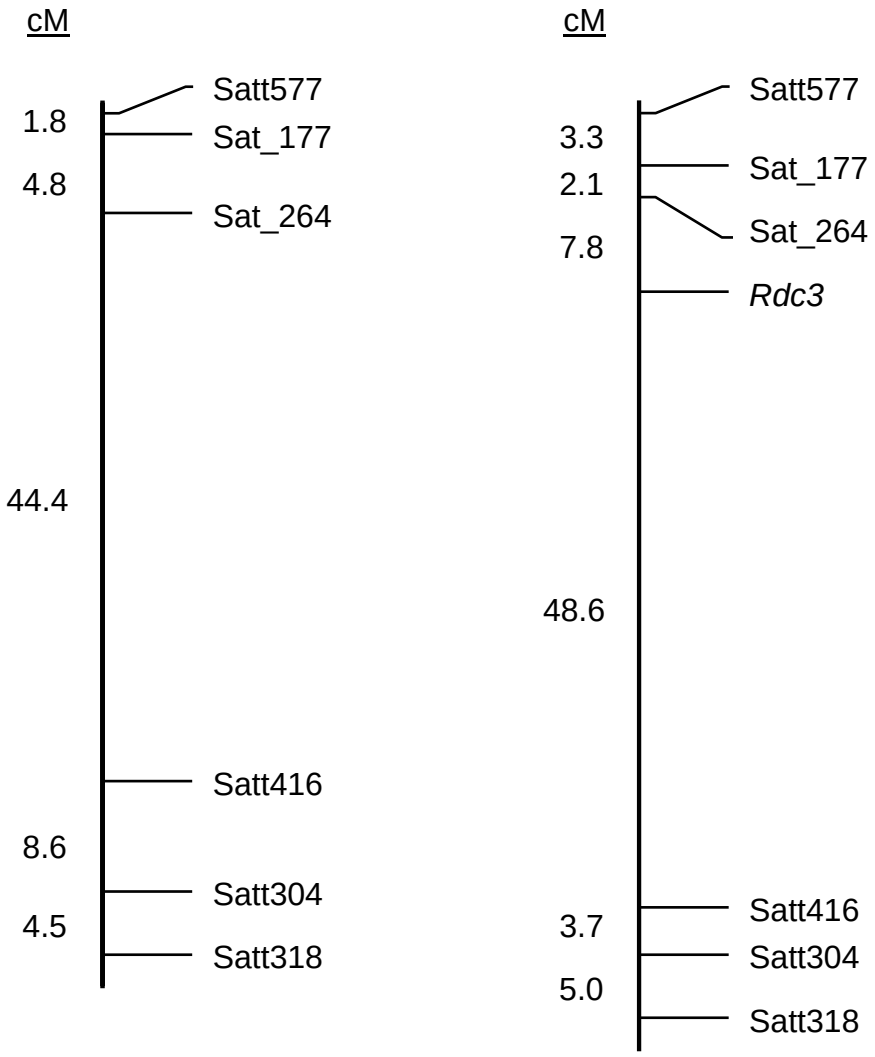


Figure 4.6. Linkage map of LG-B2 based on the USDA consensus soybean genetic linkage map (left) and a linkage map of LG-B2 including *Rdc3* based on 164 $F_2$ plants of Crockett x J77-339 (right).

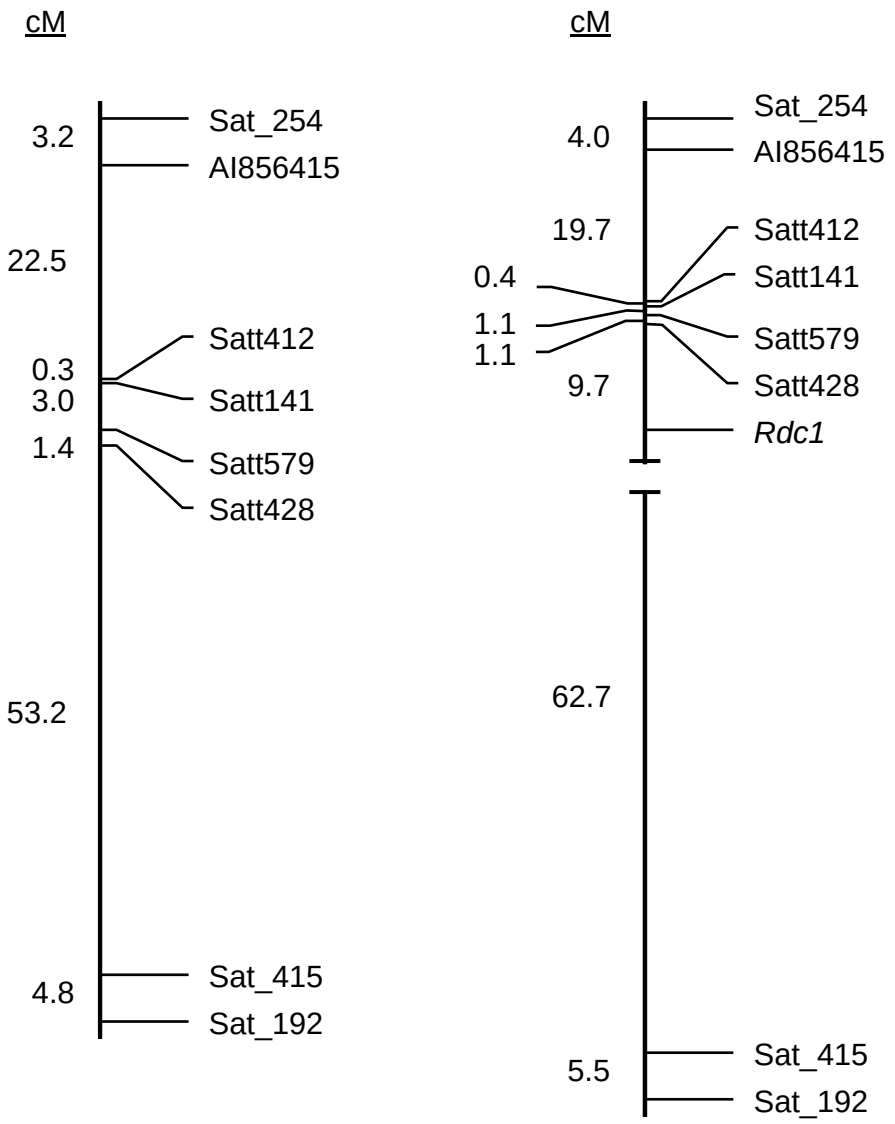


Figure 4.7. Linkage map of LG-D1b based on the USDA consensus soybean genetic linkage map (left) and a linkage map of LG-D1b including *Rdc1* based on 94 $F_{2:3}$ plants of D85-10404 x J77-339 (right). Sat_415 was the closest polymorphic SSR marker to *Rdc1* on that side, but was unlinked.

# CHAPTER 5

# A SOUTHERN ROOT-KNOT NEMATODE RESISTANCE QTL LINKED TO THE *T*-LOCUS IN SOYBEAN

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**Abstract**

The southern root-knot nematode (*Meloidogyne incognita* (Kofoid and White) Chitwood) (RKI) is one of the most devastating plant-parasitic nematodes in the southeastern United States. Growing soybean, *Glycine max* (L.) Merr., cultivars with RKI resistance is the most sustainable and environmentally benign method for growers to reduce crop damage due to RKI. G93-9009 is a soybean germplasm line that derived its RKI resistance from the highly resistant PI 96354. Two quantitative trait loci (QTL) for RKI resistance in PI 96354 have been mapped and confirmed, a major resistance QTL on LG-O near Satt358, and a minor QTL on LG-G near Satt012. However, not all the genetic variation for RKI resistance in a Bossier x PI 96354 mapping population was explained by these two QTL. One objective of this study was to determine if additional RKI resistance is associated with a QTL linked to the *T*-locus conditioning pubescence. Both the BoggsRR and BenningRR recurrent parents have tawny pubescence (*TT*) while the G93-9009 donor parent has gray (*tt*) pubescence. The T-locus has been mapped to LG-C2 of the USDA consensus soybean genetic map. This hypothesis was developed based on the observation that some advanced backcross families of both BoggsRR(6) x G93-9009 and BenningRR(5) x G93-9009 continued to segregate for pubescence color after phenotypic selection for RKI resistance of individual $F_2$ plants following each cycle of backcrossing. A second objective was to determine if there is a yield or seed quality reduction associated with the introgression of G93-9009 QTL for RKI resistance into BenningRR and BoggsRR using replicated field experiments. The gray lines from both advanced backcross populations averaged fewer RKI eggs on their roots than the tawny lines. These results suggest there is a previously undetected RKI resistance QTL on LG-

C2 near the *T*-locus and the RKI resistance allele was contributed by the gray pubescent parent G93-9009. There was no significant difference in seed yield between the highly RKI resistant gray lines and the somewhat less resistant tawny lines in either population.

## Introduction

Plant parasitic nematodes, which are microscopic roundworms, are rarely seen by the human eye but are almost ubiquitously distributed throughout the world. Most plant-parasitic nematodes live on or in plant roots and are a detriment to their hosts. Several plant-parasitic nematodes are capable of causing significant economic losses in soybean. Of these, the southern root-knot nematode, *Meloidogyne incognita* (Kofoid and White) Chitwood, (RKI) is one of the most widespread and most devastating plant-parasitic nematodes in the southeastern USA. Parasitism by RKI typically manifests in soybean as stunted growth, chlorotic leaves, increased wilting during periods of mild water stress, swollen and knotted roots, and reduced yield. Knots on the roots, also called galls, are areas where the entire circumference of the root is swollen, and are usually associated with the site of RKI parasitism (Niblack et al., 2004). The parasitic relationship between soybean and RKI is initiated when a female second-stage juvenile (J2) RKI penetrates and enters the root just behind the root cap, and migrates through the root intercellularly in the root cortex until it reaches the protoxylem pole (Hussey, 1985). Once the nematode reaches this preferential location of the plant root, it becomes sedentary and establishes multiple feeding sites called giant-cells. Giant-cells are formed in response to RKI esophageal gland cells expressing parasitism genes that encode proteins that are secreted into the host root cells. One secretory peptide functions as a ligand for plant

transcription factors that regulate normal root development. As a result, host root cells are modified into large multinucleate cells with thickened walls with extensive ingrowths, and dense cytoplasm through the process of repeated karyokinesis (nuclear division) without cytokinesis (cellular division) (Huang et al., 2006). Giant-cells serve as supermetabolic protein factories that supply the nematode with the nutrition needed to complete its life cycle, but are a detriment the host plant (Huang, 1985).

Growing soybean cultivars with RKI resistance is the most cost effective and environmentally benign method for growers to reduce crop damage due to RKI. G93-9009 is a soybean germplasm line that has the same high level of RKI resistance as its highly resistant parent PI 96354 (Li et al., 2001; Luzzi et al., 1996). The RKI resistance in PI 96354 is controlled by relatively few genes with moderate to high heritability (Luzzi et al., 1994; Tamulonis et al., 1997). Two quantitative trait loci (QTL) for RKI resistance in PI 96354 have been mapped and confirmed; a major resistance QTL on LG-O near Satt358 (Ha et al., 2004; Tamulonis et al., 1997), and a minor QTL on LG-G near Satt012 (Li et al., 2001). However, not all the genetic variation for RKI resistance in a 'Bossier' x PI 96354 mapping population was explained by these two QTL. The heritability estimate ($h^2$) for RKI gall number of 69% reported in Tamulonis et al (1997) and Li et al (2001) can be considered as the upper limit for the amount of genetic variation that can be explained by QTL (Knapp et al., 1990). The proportion of the total genetic variation explained by a given QTL or multiple QTL can be expressed as the $R^2/h^2$, where $R^2$ is the coefficient of determination from a multiple regression model that includes the identified QTL for the trait. Tamulonis et al (1997) used a multiple regression equation that included the LG-O and LG-G RKI QTL and obtained a $R^2$ value of 39% which results in

explaining 57% (39/69 = 0.57) of the genetic variation for gall number. By increasing the marker density surrounding the RKI QTL on LG-O and LG-G, Li et al (2001) were able to explain 57% of the phenotypic variation and 83% of the total genetic variation in gall number. The remaining 17% of unexplained genetic variation could potentially be accounted for by finding a marker more closely linked to the QTL or by finding previously undetected QTL.

Populations were developed at the Univ. of Georgia using backcrossing and RKI phenotypic selection in the greenhouse to introgress RKI resistance from G93-9009 into two high-yielding, glyphosate-tolerant breeding lines, G99-G725 and G99-G3438. These glyphosate-tolerant lines were backcross-derived lines of 'Boggs' (G99-G725; (Boerma et al., 2000) and 'Benning' (G99-G3438; (Boerma et al., 1997) and will hereafter be referred to as BoggsRR (G99-G725) and BenningRR (G99-G3438). Both BoggsRR and BenningRR possess tawny pubescence while G93-9009 has gray pubescence (Luzzi et al., 1996). Tawny pubescence color is produced by both the *TT* and *Tt* genotypes and while gray pubescence color is produced by the *tt* genotype (Woodworth, 1921). The *T*-locus is located on linkage group C2 of the USDA consensus soybean genetic map (http://soybeanbreederstoolbox.org/; verified 9 July 2007).

An evaluation of the $F_2$ plants from the fourth backcross to BenningRR and the fifth backcross to BoggsRR identified a number of gray pubescent plants. Given the LG-C2 location of the *T*-locus, the presence of these gray pubescent plants was unexpected based on both the number of backcrosses to BoggsRR and BenningRR and the phenotypic selection for highly RKI resistant $F_2$ plants as pollen donors for each backcross cycle (expected to maintain segments of the G93-9009 genome surrounding

the LG-G and LG-O RKI QTL). The first objective of this study was to determine if an additional RKI resistance QTL is linked to the *T*-locus conditioning pubescence color. A second objective was to determine if there was a yield reduction or affect on other agronomic traits associated with the introgression of G93-9009 QTL for RKI resistance into the BenningRR and BoggsRR genetic backgrounds.

**Materials and Methods**

In the summer of 2003, seed of four $BC_4F_{1:2}$ families from the cross BenningRR(5) x G93-9009 and 11 $BC_5F_{1:2}$ families from the cross BoggsRR(6) x G93-9009 were grown in the field at the Univ. of Georgia Plant Sciences Farm near Watkinsville, GA. After four cycles of backcrossing to BenningRR and five to BoggsRR and selecting the least galled $F_2$ plants from each backcross generation by artificial inoculation in the greenhouse to provide pollen for the next backcross cycle, three of the four BenningRR $BC_4F_{1:2}$ families and five of the 11 BoggsRR $BC_5F_{1:2}$ families were segregating for pubescence color. At maturity, 265 plants were selected from the BenningRR population along with 327 plants from BoggsRR population. These plants were selected based on their similarity to their recurrent parent except that an attempt was made to select both tawny and gray plants from each population. The plants were single-plant threshed to create $BC_5F_{2:3}$ lines of BoggRR and $BC_4F_{2:3}$ lines of BenningRR. During the winter of 2004 each line was screened in the greenhouse for resistance to RKI as described by Ha et al. (2004), and the presence of RKI resistance QTL from G93-9009 on LG-O and LG-G was confirmed using SSR markers. During the summer of 2004, 68 RKI resistant BenningRR $BC_4F_{2:3}$ lines and 60 BoggsRR $BC_5F_{2:3}$ lines were grown as

progeny rows the Univ. of Georgia Plant Sciences Farm. At maturity, six gray and eight tawny BenningRR $BC_4F_{2:4}$ lines and five gray and five tawny BoggsRR $BC_5F_{2:4}$ lines were selected and harvested in bulk.

*Field evaluation of pubescence color near isolines*

The lines from the BoggsRR and the BenningRR backcross populations were tested in separate experiments. For the BenningRR population a total of 20 entries (six gray $BC_4F_2$-derived lines, eight tawny $BC_4F_2$-derived lines, and six entries of BenningRR) were evaluated, while 16 entries were included for BoggsRR backcross experiment (five gray $BC_5F_2$-derived lines, five tawny $BC_5F_2$-derived lines, and six entries of BoggsRR). In 2005 both experiments were grown at the Plant Sciences Farm in a randomized complete block experimental design with four replications. In 2006, both experiments were grown in three environments (Univ. of Georgia Southwest Research and Education Center near Plains GA, an early planting (22 May 2006) at the Plant Sciences Farm, and a late planting (22 June 2006) at the Plant Sciences Farm. In each environment, the experiments were arranged in randomized complete block designs with three replications. The soil type was cecil coarse sandy loam (clayey, kaolinitic thermic, Typic Hapludults) at the Plant Sciences Farm, and faceville sandy loam (clayey, kaolinitic thermic, Typic Paleudults) at the Southwest Research and Education Center. Prior to planting, the experimental areas were fertilized with 131 kg $P_2O_5$ -261 kg $K_2O$ $ha^{-1}$, chisel-plowed, and fumigated with 99 kg $ha^{-1}$1,3-dichloropropene. The experiments were planted in two-row plots with a seed density of 27 seeds $m^{-1}$row and a row spacing of 76 cm. Each row was approximately 6.1 m in length at the time of planting and was

trimmed to a uniform length of 4.88 m within 3 weeks after emergence. After planting, weeds were controlled using pre-emergents 0.04 kg $ha^{-1}$ cloransulam-methyl (Benzoic acid, 3-chloro-2-{{(5-ethoxy-7-fluoro{1,2,4}triazolo{1,5-c}pyrimidin-2-yl)sulfonyl}amino}-, methyl ester), 0.89 kg $ha^{-1}$ s-metolachlor (2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl)-(S)-acetamide), and 0.01 kg $ha^{-1}$ Chlorimuron ethyl (ethyl 2-[[[[(4-chloro-6-methoxypyrimidin-2-yl)amino]carbonyl]amino]sulfonyl]benzoate). Throughout the growing season, the experiments were provided supplemental irrigation. All plots were mechanically end trimmed to a length of 3.66 m at the R6 stage of development (Fehr et al., 1971).

Data were recorded for maturity (date at which 95% of pods had reached their mature color), lodging (score 1 = all plants upright to 5 = all plants prostrate), and plant height (average distance from soil surface to apical meristem of three plants per plot). Once the plants in all plots had reached maturity, the plots were harvested using an ALMACO plot combine (ALMACO, Nevada, IA, USA). To determine protein and oil content, a 50 g seed sample from each plot was sent to the USDA-ARS National Center for Agricultural Utilization Research in Peoria, IL, where an 18 to 20 g seed sample was evaluated for protein and oil composition with a model 1255 Infratec NIR Food and Feed Grain Analyzer (Ultra Tec Manufacturing, Inc., Santa Ana, CA). Seed quality scores were based on visual observations (score: 1 = very good to 5 = very poor). Seed weight was measured from a 100-seed sample from each plot.

The data were analyzed (assuming blocks and environments as random effects and lines as fixed effects) by ANOVA using Agrobase software (Agronomix Software Inc., Winnipeg, Canada). T-tests were used to compare the mean performance of all

experimental lines from the same backcross population with gray pubescence to the mean of all experimental lines from the same backcross population with tawny pubescence.

*Greenhouse evaluation for RKI resistance*

The lines from both backcross populations were evaluated for RKI resistance in separate experiments using the greenhouse method developed by Hussey and Boerma (1981). For the BenningRR population the same 14 $BC_4F_2$-derived lines (six gray and eight tawny lines) as tested in the field experiment along with six check lines were planted in 10 replications of a randomized complete block experimental design. For the BoggsRR population the same 10 $BC_4F_2$-derived lines as tested in the field experiment along with four check lines were grown in 10 replications of a randomized complete block design.

Three seeds of each test genotype were planted in 10-cm clay pots filled with methyl bromide-fumigated sandy loam soil. Seven to ten-day-old seedlings were thinned to one per pot and inoculated with 5 mL of the egg suspension. The *M. incognita* population used as inoculum was maintained on the susceptible Black Beauty cultivar of eggplant (*Solanum melongena* L.) and eggs were collected with NaOCl (5.25%) as described by Hussey and Barker (1973). Inoculum (egg) concentration was determined and adjusted to approximately 1000 eggs $mL^{-1}$ using a stereoscopic microscope.

Metal halide lamps and automatic drip irrigation were used to enhance nematode infection, gall development, and nematode egg production. Once it was determined that the susceptible check cultivar Bossier was sufficiently symptomatic, the tops of all plants were removed and roots washed prior to examination. Eggs were collected from the

roots of each line using the 10.5% NaOCl method and the total number of eggs plant$^{-1}$ was determined by dilution and counting using a stereoscopic microscope.

Total egg number plant$^{-1}$ was used as a measure of the RKI resistance of each line and analyzed by ANOVA using Agrobase software (Agronomix Software Inc., Winnipeg, Canada). To ensure similar variances among resistant and susceptible lines, nematode egg data (X) were transformed to $\log_{10}(X + 1)$ values for the purposes of statistical analysis, but are reported as antilogs. For each backcross population a T-test was used to compare the mean number of eggs plant$^{-1}$ of lines with gray pubescence to the mean number of eggs plant$^{-1}$ of lines with tawny pubescence.

**Results and discussion**

Of the 128 backcross-derived $F_{2:3}$ lines identified as RKI resistant in the greenhouse and grown as progeny rows during the summer of 2004, 57 had tawny pubescence color, 21 had gray pubescence color, and 50 segregated for pubescence color. Based on their pedigrees, one would expect 96.9% BenningRR genome in the lines derived from the BenningRR $BC_4$ population and 98.4% BoggsRR genome in lines derived from BoggsRR $BC_5$ population outside of the regions harboring RKI resistance genes (Stam and Zeven, 1981). The high number of families with gray pubescence or segregating for pubescence color suggested that the region of the soybean genome surrounding the *T*-locus for pubescence color may be under selection pressure by the artificial selection for RKI resistance. Of the 14 BenningRR $BC_4F_{2:3}$ lines and the 10 BoggsRR $BC_5F_{2:3}$ lines, all were homozygous for the RKI resistance alleles at the RKI resistance QTL on LG-O and LG-G.

In both backcross populations the average number of RKI eggs plant$^{-1}$ in lines with gray pubescence was lower than lines with tawny pubescence (Table 5.1). In the BenningRR population, an average of 42 eggs plant$^{-1}$ were extracted from the roots of gray lines, which was significantly ($P < 0.05$) fewer eggs plant$^{-1}$ than the average of 170 eggs plant$^{-1}$ extracted from tawny lines. The recurrent parent BenningRR averaged 225 eggs plant$^{-1}$. In the BoggsRR population, an average of 6 eggs plant$^{-1}$ were produced on gray lines, which was significantly less ($P < 0.01$) than the 63 eggs plant$^{-1}$ averaged by the tawny lines. The tawny pubescent recurrent parent BoggsRR averaged 30 eggs plant$^{-1}$.

The overlapping range in the number of eggs plant$^{-1}$ for lines with tawny and gray pubescences in both backcross populations suggests that it is possible to select a tawny pubescent line with the same level of resistance as the highly RKI resistant, gray pubescent germlasm line G93-9009. This highly resistant, tawny line would be the result of a crossover between the RKI resistance QTL and the *T*-locus.

Based on the nematode egg data in Table 5.1, the RKI resistance appears to be greater in the lines from the BoggsRR backcross population than in the lines from the BenningRR backcross population. These data are likely an artifact of the two backcross populations being evaluated in different experiments. The continued segregation of the alleles at the *T*-locus in two independent advanced backcross populations derived from tawny (*TT*) recurrent parents and a gray (*tt*) resistant donor parent and the fact that the mean number of eggs collected from the advanced backcross-derived lines with gray pubescence was less than one fourth the mean number of eggs plant$^{-1}$ collected from lines with tawny pubescence in both populations is strong evidence of a previously undetected

RKI resistance QTL. The resistance allele at this QTL was inherited from the highly resistant, gray pubescence germlasm G93-9009.

There was no significant difference (P = 0.05) in the average yield seed between tawny and gray lines of either backcross population (Table 5.2). In the BenningRR backcross population the gray lines averaged 3302 kg $ha^{-1}$ compared to 3128 kg $ha^{-1}$ for the tawny lines. For the BoggsRR backcross population, the tawny lines averaged 87 kg $ha^{-1}$ less in seed yield than the average yield of 3107 kg $ha^{-1}$ for the gray lines. These results indicate that the RKI resistance QTL from G93-9009, including the resistance QTL associated with the *T*-locus, were introgressed into BoggsRR and BenningRR genetic backgrounds without any yield reduction.

In the BenningRR backcross population, no significant differences (P = 0.05) between tawny and gray backcross lines were observed for maturity, plant height, lodging, oil content, protein content, or seed quality (Table 5.2). However, seeds of the gray lines averaged 4 mg $seed^{-1}$ less weight than seeds of tawny lines. In the BoggsRR backcross population, no significant differences (P = 0.05) between tawny and gray backcross lines were observed in maturity, lodging, seed weight, or seed quality. The gray BoggsRR backcross lines averaged 3.6 cm shorter in plant height and produced seed with 7 g $kg^{-1}$ less oil and 10 g $kg^{-1}$ more protein than tawny lines.

As advanced backcross populations, no significant trait differences were expected among the lines except for trait(s) that have been intentionally transferred from the donor parent. However, in the genomic region of the *T*-locus on LG-C2 several QTL conditioning agronomic traits including yield, maturity, plant height, protein content, lodging, and seed weight have been identified (Chase et al., 1996; Cregan et al., 1999;

Csanadi et al., 2001; Diers et al., 1990; Mansur et al., 1993; Mian et al., 1998; Orf et al., 1999; Specht et al., 2001). Given this QTL-rich region of LG-C2 for these agronomic traits, it is interesting that only seed weight showed a significant difference between the tawny and gray lines of the BenningRR backcross population. These differences may be a result of different alleles in BenningRR and G93-9009 at the seed weight QTL near the *T*-locus. Additionally, G93-9009 and BenningRR could contain the same alleles for the QTL near the *T*-locus that condition the other agronomic traits where no significant differences were found. The significant differences between tawny and gray lines in the BoggsRR backcross population may also be the result of the agronomic QTL in the region near the *T*-locus. As mentioned above, QTL conditioning plant height and protein and oil content are located in this region of LG-C2. Fine mapping of the newly discovered RKI resistance QTL in the region near the *T*-locus may provide some insight on these issues.

This study has clearly established the presence of a previously undetected RKI resistance QTL near the T-locus on LG-C2. In addition, our data indicates it is possible to introgress the RKI resistance from G93-9009, including the newly reported RKI resistance QTL on LG-C2, into other genetic backgrounds without loss in seed yield or major modifications in other important agronomic traits.

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Table 5.1. Mean and range RKI eggs plant$^{-1}$ extracted from gray and tawny backcross-derived lines in greenhouse experiments.

| Lines | Entries | Mean | Range |
|---|---|---|---|
| | no. | eggs plant$^{-1}$ | no. plant$^{-1}$ |
| Bossier | 1 | 24920 | n/a |
| G93-9009 | 1 | 95 | n/a |
| BenningRR | 6 | 225 | 85 - 393 |
| BenningRR(5) x G93-9009 | | | |
| Tawny lines | 8 | 170a[†] | 53 - 353 |
| Gray lines | 6 | 42b | 0 - 85 |
| | | | |
| Bossier | 1 | 8080 | n/a |
| G93-9009 | 1 | 83 | n/a |
| BoggsRR | 1 | 30 | n/a |
| BoggsRR(6) x G93-9009 | | | |
| Tawny lines | 5 | 63a[‡] | 6 - 111 |
| Gray lines | 5 | 6b | 0 - 12 |

[†]Means with the same letter are not significantly different based on $LSD_{(0.05)}$

[‡]Means with the same letter are not significantly different based on $LSD_{(0.01)}$

Table 5.2. Mean agronomic performance of tawny and gray backcross lines of BenningRR(5) x G93-9009 and BoggsRR(6) x G93-9009.

| Population/line | Entries | Seed yield | Maturity | Plant height | Lodging | Oil content | Protein content | Seed weight | Seed quality |
|---|---|---|---|---|---|---|---|---|---|
| | no. | kg ha$^{-1}$ | d | cm | score[†] | g kg$^{-1}$ | g kg$^{-1}$ | mg seed$^{-1}$ | score[†] |
| BenningRR | 6 | 3498 | 49.7 | 101 | 1.8 | 205 | 406 | 138 | 1.3 |
| BenningRR(5) x G93-9009 | | | | | | | | | |
| Tawny lines | 8 | 3128a[*] | 51.2a | 96a | 2.1a | 198a | 405a | 137a | 1.6a |
| Gray lines | 6 | 3302a | 51.2a | 99a | 1.9a | 193a | 405a | 133b | 1.4a |
| BoggsRR | 6 | 3101 | 46.7 | 89 | 1.8 | 201 | 422 | 119 | 1.3 |
| BoggsRR(6) x G93-9009 | | | | | | | | | |
| Tawny lines | 5 | 3020a | 45.4a | 87a | 1.8a | 203a | 423a | 116a | 1.2a |
| Gray lines | 5 | 3107a | 45.0a | 83b | 1.6a | 196b | 433b | 115a | 1.1a |

[†]Score = a scale where 1 = all plants upright to 5 = all plants prostrate based on visual observations
[‡]Score = a scale from 1 = very good to 5 = very poor based on visual observations
[*]Means with the same letter are not significantly different based on $LSD_{(0.05)}$.

# CHAPTER 6

# CONCULSIONS

The research presented in the previous chapters has the objectives of enhancing soybean resistance to southern stem canker and southern root-knot nematode, two economically important diseases in the southern USA. In Chapter 4, two southern stem canker resistance genes, *Rdc?(PI 398469)*, and *Rdc3* Crockett have been mapped to LG-B2. The *Rdc?(PI398469)* gene from PI 398469 mapped to the segment of LG-B2 between Sat_177 and Sat_342, 3.9 cM from Sat_177. The *Rdc3* gene from Crockett mapped to the segment of LG-B2 between Sat_264 and Satt416, 7.8 cM from Sat_264. The *Rdc1* gene from D85-10404 mapped to the segment of LG-D1b between Satt428 and Sat_415, 9.7 cM from Satt428. Linkage analysis of the SSR markers used to map *Rdc1*, *Rdc3*, and *Rdc?(PI398469)* are in reasonable agreement with the USDA consensus soybean genetic linkage map. Mapping stem canker resistance genes allows soybean breeders to more efficiently select for stem canker resistance in their cultivars and breeding lines. Delineating the genomic regions where stem canker resistance is located to a smaller segment of a linkage group (LG) with simple sequence repeat (SSR) markers also facilitates the development of high throughput, breeder friendly markers such as single nucleotide polymorphisms (SNPs) that could be more tightly linked to the resistance gene. These SNP and SSR markers may ultimately be used for marker assisted selection in breeding programs in lieu of phenotyping for disease resistance using the more traditional greenhouse and field methods which require maintenance and culturing

of the stem canker-causing fungus. Marker assisted selection for these three stem canker resistance genes will require tighter marker-resistance gene linkages. The development of SNP markers may be especially helpful in achieving tighter marker linkages with *Rdc1* and *Rdc3*. Development of additional markers in the genomic regions of these stem canker resistance genes will empower breeders to select homozygous resistant plants and lines in early breeding generations and practice selection for other traits such as seed yield among stem canker resistant lines.

Identifying a Southern root-knot nematode (RKI) resistance quantitative trait locus (QTL) linked to the *T*-locus will allow breeders to introgress a higher level of RKI resistance from G93-9009 into their breeding material. Chapter 5 presents strong evidence for the existence of this QTL based on: 1) the *T*-locus was segregating in two independent advanced backcross populations, both of which were under selection for RKI resistance during each backcross generation; and 2) the mean number of eggs extracted from lines with grey pubescence was less than one fourth the mean number of eggs extracted from lines with tawny pubescence in both backcross populations. Replicated field experiments indicate that RKI resistance associated with the T-locus in G93-9009 was introgressed into two different genetic backgrounds without yield reduction through linkage drag.