GENETIC MAPPING OF GREENBUG RESISTANCE LOCI IN SORGHUM [*Sorghum bicolor* (L.) Moench] & EXPRESSION ANALYSIS OF CANDIDATE GENES IN RESPONSE TO GREENBUG INFESTATION

By

SOMASHEKHAR M. PUNNURI

Master of Science in Agriculture
University of Agricultural Sciences
Dharwad, Karnataka. India
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Dissertation Approved:

Dr. Yinghua Huang

Dissertation Adviser

Dr. Andrew Doust

Dr. William J. Henley

Dr. Yanqi Wu

Outside Committee Member

Dr. Mark E. Payton

Dean of the Graduate College

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DEDICATION

This dissertation is dedicated to my beloved grandma Gouramma, my sweet little princess Saanvi, my wife Pallavi and my parents.

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CHAPTER I

INTRODUCTION

Sorghum

Sorghum, *Sorghum bicolor* (L.) Moench, a self pollinated C_4 species with diploid chromosomes (2n = 20), has recently gained increasing popularity because of its drought tolerance and potential uses in biofuel production, and as a palatable diet among millions of people in Africa and Asia. In the United States, sorghum is more popular as feed stock for poultry and as fodder for dairy animals, but has recently received commercial interest due to increasing demand for biofuel production. In the United States, sorghum is ranked as the third most important cereal crop and claims second highest production worldwide. In the United States, in 2010, an area of 5.40 million acres was planted in sorghum producing 345.3 million bushels (www.usda.gov/nass 2010 reports). The United States is the number one sorghum exporter in the world market, with a share of 65-70% of world trade, the majority of which is exported to Mexico (www.grains.org/sorghum 2009-2010).

Greenbug

Greenbug, *Schizaphis graminum* (Rondani) (Homoptera: Aphididae), is a severe pest on sorghum in the states of the Great Plains. Greenbugs are diploid organisms (2n = 8) with a relatively small genome size of 387 Mb and has very little highly repetitive DNA (Sun and Robinson 1966; Maa et al. 1992; Finston et al. 1995). Greenbugs are small, pear shaped, yellowish-to bluish-green aphids with a dark green stripe along the midrib of their abdomen. Greenbugs are classified as piercing and sucking insects based on their feeding behavior, injecting toxins and sucking the phloem sap while transmitting viral diseases. Greenbugs are known to be the first introduced aphid with significant economic losses in the prominent winter areas of North America (Van-Emden and Harrington 2007). Greenbugs exist in both winged and wingless form, which have a high asexual reproductive capacity in high temperature growing regions of sorghum.

Review on greenbugs and their occurrence on sorghum

Although greenbugs were first introduced and observed in 1882, they were not considered detrimental to sorghum production until the first damaging biotype C was discovered in 1968 (Harvey and Hackerott 1969; Van-Emden and Harrington 2007). Sorghum breeding efforts started immediately to overcome this new pest of sorghum, but breeders sequentially witnessed the appearance of new greenbug biotypes over the previously developed resistant hybrids. There were four biotypes C, E, I and K that appeared one after the other from 1968 until 1997 (Harvey and Hackerott 1969; Porter et al. 1982; Harvey et al. 1991; Harvey et al. 1997). There existed a great biotypic variation among these greenbugs, which resulted in the identification of several biotypes, but of these, only three, C, E, and I, caused significant

losses to sorghum production above the economic threshold damage (Porter et al.1997; Shufran et al. 2000). Among these three, biotype I, first found in Stevens County, Kansas in 1990 (Harvey et al. 1991), is the most distinguishable and more commonly found in fields associated with heavy infliction on sorghum (Burd and Porter 2006). The annual loss due to greenbug on sorghum production is estimated to be $248 million to the sorghum growers in the United States (INTSORMIL 2006).

Greenbug-Sorghum Interaction

Greenbugs, which occupy the largest portion among the phloem feeding insects, use phloem sap as a source of nutrients, by using their specialized mouthparts, the stylet to drain photoassimilates from sieve elements of the phloem. These phloem-feeders penetrate their mouthparts through epidermal and mesophyll cells to reach to the sieve tubes of the phloem in the vascular bundle (Dixon 1998). Greenbugs also inject toxins into the plant, which basically destroy the photosynthetic machineries in the leaf tissue; this destruction is manifested in the form of red necrotic spots with surrounding pale-yellow discoloration on leaves of affected plants (Reese and Schmidt 1986; Miles 1989; Ryan et al. 1990; Girma et al. 1998; Maa et al. 1999). Saliva forms the first line of contact when these insects attack a plant and reach the phloem sap through the breakdown of the cell wall (Miles 1999; Goggin 2007). Greenbugs possess two kinds of saliva: gelling saliva or stylet sheath saliva and non-gelling saliva or watery saliva. These two kinds of saliva vary in their constituents and have different roles in invading the plant phloem sap (Miles 1999; Will et al. 2009). Aphids obtain a diet rich in carbohydrates but deficient in essential amino acids from the phloem and these amino acids are primarily supplied by the endosymbionts present in the aphid gut which have a role in provisioning the nutrients (Buchner 1965; Douglas 1989; Prosser et al. 1992;

Wilkinson and Douglas 1996; Douglas 1998). Therefore, saliva also acts as a medium of transmission for several microbes including viruses to the plant cells (Powell 2005).

Aphids that feed on phloem sap induce different responses from the host plants compared to other insects (Thompson and Goggin 2006). These phloem-feeding insects cause minimum mechanical damage triggering limited local wound responses. It is now clear that the disruption of cell wall and membrane integrity initiates the primary responses in plants and then the saliva which contains elicitors are recognized by the plant leading to further activation of an array of defensive response genes through signaling pathways (Kus´nierczyk et al. 2008; Morkunas et al. 2011). The response to these wounds is perceived as similar to pathogen invasion in plants (Walling 2000); therefore, signaling pathways in greenbug-induced plants may overlap with plant responses to pathogens (Huang 2007). The watery saliva, composed of several enzymes and elicitor molecules, induces plant defense responses through eliciting cascades of signals (Baumann and Baumann 1995; Miles 1999). Sorghum plants exhibit several responses through an orchestration of multiple signaling pathways in response to greenbug feeding; these pathways involve communication between messenger compounds through cross-talk, eventually manifested in the form of early signaling and differential gene expression (Zhu-Salzman et al. 2004; Park et al. 2006; Smith and Boyko 2007). These endogenous signaling molecules such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) induce transcriptional changes in plant defense genes upon aphid infestation (Dicke and Hilker 2003; Thompson and Goggin 2006).

Smith and Boyko (2007) have proposed two processes involving plant-aphid interaction. One of the processes, which highlights on gene-for-gene interaction, is specific for aphid-resistant plants only. The other process involves general or basal defense response which is expressed

in both aphid-resistant and aphid-susceptible plants. Plant resistance (R) genes and their homologues are thought to be involved in aphid resistance (Van der Biezen and Jones 1998; Smith and Boyko 2007). Plants recognize aphid effector proteins through the use of R gene products containing transmembrane pattern recognition receptors (PRRS) or nucleotide-binding leucine-rich-repeat (NB-LRR) protein sequences (Morkunas et al. 2011). To date, there are two dominant resistance genes, *Mi-1* and *Vat*, that have been isolated in plants, both of which share structural similarities and encode NBS-LRR proteins involved in specific recognition of aphids (Dogimont et al. 2010). Transcriptional studies in sorghum have shown the involvement of gene sequences with LRR motifs and *Xa1* in response to greenbug attack (Zhu-Salzman et al. 2004; Park et al. 2006). These studies support the involvement of R gene-mediated resistance in sorghum-greenbug interaction.

Role of molecular markers in greenbug resistance breeding

The sorghum genome, consisting of 730 Mb (Megabase pair of DNA), is exemplary as a grass model species. Its genome size is one third that of the maize genome and 75% larger than that of the rice genome (Paterson et al. 2009). The lower level of genome duplication and small genome size of sorghum has driven many researchers to harness genomic resources for structural and functional analyses (Paterson 2008).

Sorghum linkage maps have been developed for some time, due to the availability of abundant polymorphic DNA markers. Linkage mapping in sorghum is relatively easy because of the diploid nature of the sorghum genome (Paterson 2008). DNA markers are neutral to the environment, cost effective and time saving, which have accelerated the efforts to elucidate the entire architecture of the sorghum genome. Mapping in sorghum began in

1990, and there are at least 26 published papers as of 2005, that have been reviewed (Zhi-Ben et al. 2006). These maps have incorporated several different markers, such as the simple sequence repeat (SSR) markers, the restriction fragment length polymorphism (RFLP) markers, the amplified fragment length polymorphism (AFLP) markers and in few cases, the randomly amplified polymorphic DNA (RAPD) markers. There are several other works in the last five years contributing to the sorghum maps, prominent among them is the consensus map developed by Mace et al. (2009) using diversity array technology (DArT) markers. Consensus map helps the integration of the genetic map into physical maps for better understanding of the functions of genes. The completion of the sorghum genome sequence project has more recently opened new avenues of research for functional analyses especially in the development of gene-based markers. Sorghum molecular marker maps, saturated with several kinds of markers, will play a pivotal role in tagging loci that govern quantitative traits like greenbug resistance. These molecular markers will facilitate the dissecting the genetic mechanisms underlying insect resistance in sorghum (Yencho et al. 2000).

QTL mapping

In the last decade, research on greenbug resistance has taken advantage of different molecular markers in developing quantitative trait loci (QTL) maps. To date, there are five published QTL maps for greenbug resistance in sorghum (Agrama et al. 2002; Katsar et al. 2002; Nagaraj et al. 2005; Wu et al. 2007; Wu and Huang 2008). The RFLP map that was developed using four different F_3 populations showed nine different loci contributing to greenbug resistance against four different biotypes (C, E, I and K). These loci originated from different distinct resistant sources, highlighting the use of disparate resistant source possessing allelic variation at particular locus in breeding for greenbug resistance (Katsar et

al. 2002). Agrama et al. (2002) measured chlorophyll loss upon damage inflicted by two biotypes of greenbug (I and K) using the SPAD index, which revealed nine loci from seven linkage groups contributing to biotype-specific and biotype non-specific resistance and tolerance in sorghum. A similar study using chlorophyll loss as an indicator in a set of recombinant inbred lines showed three to five loci accounting for 9-19% tolerance to greenbug biotypes I and K (Nagaraj et al. 2005). In a more recent study, a large amount of phenotypic variation for greenbug resistance to two biotypes, I and K, was observed with a major locus located on chromosome 9 (Wu et al. 2007; Wu and Huang 2008). These findings further enhance our understanding about greenbug-sorghum interactions; that the inheritance of greenbug resistance is polygenic and involves distinct regions of the genome.

Rationale and Significance

Sorghum is an important cereal crop in tropical and subtropical regions of the world possessing a C_4 metabolism and capable of surviving high temperature (Paterson 2008). Sorghum has special importance in the United States as it generates major revenue through exports, as livestock feed and raw material for biofuel industry. Sorghum production in the United States is centered in five states of the central and southern Great Plains, including Kansas, Texas, Nebraska, Oklahoma and Missouri accounting for approximately 89 percent of total nationwide production. Greenbug, *Schizaphis graminum* (Rondani), is a severe pest on sorghum in the states of the southern and central Great Plains, causing huge losses to sorghum production. Greenbug not only causes losses by damage on the sorghum leaf, but also as a vector that transmits several viral diseases, including maize dwarf mosaic virus (MDMV) and sugarcane mosaic virus (SCMV). The best way to control such diseases is through the control of aphids that carry these viruses. The loss of sorghum crop incurred due

to greenbug damage ranks in the hundreds of millions of dollars to sorghum farmers. Systemic insecticides are known to control greenbug damage, but are often associated with high costs, development of insecticide resistance and environmentally unfriendly effects, especially apparent in drastic reduction of beneficial insects. The undesirable effects of systemic insecticides should lead to increased interest in host plant resistance against greenbug infestation as better alternative to chemical control measures and as an effective means of integrated pest management practices (Smith 2004). Breeders have developed several greenbug resistant cultivars over a period of time, but have often encountered the emergence of new greenbug biotypes. Hence there is a need to screen better resistant sources from a wide collection of germplasm, which offer a durable resistance. Better sorghum breeding strategies involving known sources of greenbug resistance would facilitate deploying of resistant cultivars into greenbug management programs. Molecular breeding takes advantage of DNA markers that are neutral to the environment and cost and time effective for development of resistant cultivars.

This project will enhance our understanding of plants' innate resistance by elucidating the functions of genes expressed during greenbug-sorghum interaction. The results from the expression studies will facilitate the identification of differentially-expressed genes between resistant and susceptible plants. This project will also identify molecular markers linked to greenbug resistance which can be further incorporated into marker-assisted selection and map-based cloning of greenbug resistant genes. The results of these efforts have a larger impact on sorghum economics and the agricultural environment. Sorghum growers will benefit by the decreased costs incurred on insecticides, as well as from increased yield.

Objectives and overview of the study

With the above background, I undertook two projects, with several objectives, which are explained in detail in the following two chapters. The first project is detailed in chapter II, with a broad objective of identifying quantitative trait loci (QTL) in an F_2 mapping population of sorghum developed using a greenbug-resistant source, PI 607900. We identified major loci for greenbug resistance on sorghum chromosome 9. These results were supported by previous studies which also identified major loci for greenbug resistance on chromosome 9. These loci accounted for large portions of phenotypic variation observed for greenbug resistance. This study developed several gene-based markers associated with insect resistance and R-gene mediated resistance. The identified greenbug resistance loci were in the vicinity of receptor-like kinase *Xa21*-binding protein 3, a gene known to increase *Xa21*-mediated resistance in rice. Therefore, we wanted to confirm the expression of *Xa21*-mediated resistance in sorghum against greenbug. We also wanted to indentify new loci for greenbug resistance in a population that has undergone genomic recombination. Our second project, detailed in chapter III, involved an intercross population derived from a previously used F_2 population of sorghum. This project had two basic objectives: the first objective was to compare and locate QTLs in an intercross population and identify candidate genes for greenbug resistance in the QTL region; the second objective was to elucidate the expression pattern of these selected candidate genes in two sorghum parents (one greenbug-resistant and one greenbug-susceptible) using real-time PCR analysis. We identified that the same region on chromosome 9 was conferring greenbug resistance with narrow confidence interval compared to previous results from F_2 QTL analysis. Relative quantification of gene expression for four selected candidate genes revealed two of these genes, receptor–like *Xa21*

binding protein 3-like and map kinase phosphatase were differentially expressed between two contrasting parents.

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CHAPTER II

GENETIC MAPPING OF GREENBUG, BIOTYPE I RESISTANCE LOCI IN SORGHUM [*Sorghum bicolor* (L.) Moench]

Abstract

Greenbug is a major damaging insect to sorghum production in the United States. Among various virulent greenbug biotypes, biotype I is the most predominant and severe for sorghum. This experiment was conducted to identify the genomic regions contributing resistance to greenbug biotype I in sorghum. An F_2 mapping population consisting of 371 individuals developed from a cross of BTx623 (susceptible, seed parent) by PI 607900 (resistant, pollen parent) were tested and scored in the greenhouse with two parallel experiments, each consisting of three replications. Significant differences in resistance were observed between the two parental lines and their F_2 progeny in response to greenbug feeding at 7, 10, 14 and 21 days after infestation. A linkage map spanning a total length of 729.5 cM across the genome was constructed with 102 polymorphic SSR markers (69 genomic and 33 EST SSRs). Single marker analysis revealed 29 markers to be significantly associated with the plant response to greenbug feeding damage.

The results from interval mapping, composite interval mapping and multiple interval mapping analyses identified three major QTLs for greenbug resistance on chromosome 9. These QTLs collectively accounted for 34 to 82 % of the phenotypic variance in greenbug resistance. Minor QTLs located on chromosome 3 explained 1 % of the phenotypic variance in greenbug resistance. The major allele for greenbug resistance was on chromosome 9 close to receptor-like kinase *Xa21*-binding protein 3. These markers are useful to screen resistant genotypes. Furthermore, the markers tagged to QTL regions can be used to enhance the sorghum breeding program for greenbug resistance through marker-assisted selection and map-based cloning.

Introduction

Sorghum is the fifth most important cereal crop worldwide with its multifaceted uses such as food, fiber and biofuel. The crop also has large commercial value in cattle, poultry and dairy industries as it is mainly used as feed for animals in the United States. Insect attack is one major factor limiting sorghum production. Sorghum is attacked by more than 150 insect pests, of which aphids are the most prominent (Young and Teetes 1977; Sharma 1993). Greenbug, *Schizaphis graminum* (Rondani), a sap-sucking aphid, is one of the most devastating to sorghum productivity (Teets 1980). Greenbugs feed on the leaves of grasses and cereals, absorbing nutrients present in the sieve elements while incorporating phytotoxins to produce visible symptoms in the plants, including chlorosis and red necrotic spots (Van-Emden and Harrington 2007).

Over the past four decades, sorghum growers have witnessed the emergence of several resistance-breaking biotypes of greenbug causing severe damage to the crop,

especially in the United States Great Plains. The identification of a serious greenbug problem began with the discovery of biotype C in 1968 (Harvey and Hackerott 1969), and later by the discovery of other biotypes E, I and K within the next three decades. These resistance-breaking biotypes could overcome the sorghum hybrids developed for previous biotypes (Porter et al. 1982; Harvey et al. 1991; Harvey et al. 1997). Among these four biotypes, biotype I, which was identified and designated in 1990, is of economic importance because it causes huge losses in sorghum yield (Harvey et al. 1991; Kofoid et al. 1991; Teets and Pendleton 2000).

Host plant resistance is perceived as an eco-friendly, socially acceptable and effective component of integrated pest management in deploying resistant cultivars against insect damage (Bramel-Cox et al. 1986; Andrews et al. 1993; Sharma 1993; Sharma and Ortiz 2002; Smith 2004). Therefore, host plant resistance is of prime importance in the arsenal of aphid-plant interaction. Multiple mechanisms are involved in plant defense response to aphid feeding through early signaling and differential expression of gene pathways. Several messenger compounds communicating through cross-talk in the multiple signaling pathways are induced when greenbugs attack (Smith and Boyko 2007). Transcriptional reprogramming induced by phloem-feeding insects within their host plants involves physiological and biochemical changes in phloem tissue (Thompson and Goggin 2006). Sorghum responds to greenbug feeding by activating an array of defense responsive genes through signaling pathways, which may overlap with responses to pathogens (Huang 2007). Different transcriptomic studies with microarray profiling have focused on the greenbug-sorghum interaction and emphasized the role of signaling compounds and defense-activated genes. Suppression subtractive hybridization

revealed the down-regulation of cysteine proteinase inhibitors and the up-regulation of genes such as *Xa1*, antimicrobial proteins and other signaling compounds in response to greenbug damage on sorghum plants (Park et al. 2006). Another transcriptomic study also identified the differential expression of 82 greenbug responsive genes, including a LRR-containing glycoprotein sequence and other defense related proteins in plants infested with greenbug (Zhu-Salzman et al. 2004). These studies have shown the prominent role of plant R genes in defense response to greenbug attack through signal transduction pathway.

Molecular markers have diverse utility in dissecting genes for greenbug resistance and in better understanding the genetic basis and mechanism of resistance (Yencho et al. 2000). Recent progress in sorghum genomics has availed the genome sequence to the public to aid in the development of several different types of molecular markers, including gene-based markers. Furthermore, post-genomic progress has accelerated linkage mapping experiments for all economically important traits including greenbug resistance (Paterson 2008; Paterson et al. 2009). Genic microsatellites provide an opportunity to tag traits such as insect resistance due to their inherent nature that are derived within the gene sequence and have several advantages over genomic SSRs (Varshney et al. 2005).

In the past decade, the use of molecular markers has helped to identify suitable greenbug resistant sorghum, which has fostered marker-assisted breeding programs for greenbug resistant crops. To date, five independent QTL mapping experiments have been conducted in sorghum to identify greenbug resistance to four different greenbug biotypes (Agrama et al. 2002; Katsar et al. 2002; Nagaraj et al. 2005; Wu et al. 2007; Wu and

Huang 2008). In these studies, seven disparate genetic sources of resistance were used, which revealed multiple genomic regions accounting for resistance to greenbug biotypes C, E, I, and K. Among these studies, Katsar et al. (2002) identified at least three loci present on chromosome SBI05, SBI06 and SBI07 conferring resistance to greenbug biotype I. Agrama et al. (2002) quantified chlorophyll loss caused by greenbug injury and detected nine genomic regions affecting both biotype-specific and biotype non-specific resistance and tolerance to Biotype I and K. Of the seven QTLs detected by Agrama and colleagues (2002) that were associated with biotype-specific resistance and tolerance to greenbug damage, three markers present on chromosomes SBI02, SBI05 and SBI09 were linked with biotype I-specific resistance and tolerance. A similar study by Nagaraj et al. (2005) using chlorophyll loss as an indicator to greenbug damage identified three QTLs present on the sorghum chromosome SBI01 and SBI04 for biotype I resistance and tolerance. The recent study conducted by Wu and Huang (2008) have shown a major QTL located on sorghum chromosome SBI09 conditioning resistance to biotype I. It is obvious from these studies that resistance and tolerance to greenbug damage originates from multiple regions of the genome depending on the resistance source contributed by various genotypes and that some of the alleles are biotype-specific, and others are biotype non-specific.

Despite the economic importance of continuous breeding efforts to develop resistant sorghum cultivars, progress has been slow in identification of greenbug biotype I resistance sources for incorporation into existing greenbug resistance management practices. The resistance to aphid attack is governed by very few resistance loci and alleles, considering the meager sources of resistance (Dogimont et al. 2010). Previous

work indicated that sorghum line PI 607900 contained strong resistance to biotype I and was genetically distinct from other known major resistant genptypes (Tuinstra et al. 2001; Wu and Huang 2006). Tuinstra et al. (2001) reported PI 607900 (KS 97) had superior general combining ability of greenbug biotype I resistance compared to PI 550610, which carried one major and one minor QTL on SBI09 resistant to greenbug biotype I (Wu and Huang 2008). Our objective in the present research was to identify genomic regions associated with greenbug biotype I resistance in sorghum accession, PI 607900 using SSR markers. Microsatellite markers, diagnostic to biotype I resistance, developed in this study will be a useful tool in identifying resistant genotypes from the sorghum germplasm pool. Additionally, with the accessibility of the sorghum genome sequence, the precise location of QTLs can also be inferred.

Materials and Methods

Selection of resistant source for the mapping population

The parental lines of sorghum for our QTL study were BTx623 (susceptible parent) and PI 607900 (resistant parent). BTx623 is the cultivar utilized in the sorghum genome sequencing project (Sorghum Genomics Planning Workshop Participants, 2005) and is susceptible to greenbug biotype I. PI 607900, also known as KS 97, is highly resistant to greenbug biotype I (Tuinstra et al. 2001). PI 607900 was developed by Dr. Gerald Wilde at Kansas State University using IS 2388 as a heterogenous seed source from South Africa (Wilde and Tuinstra 2000). In our preliminary screening for greenbug biotype I resistance, involving three major resistant sources (PI 550607, PI 550610, PI 607900) and two susceptible checks, we confirmed the PI 607900 accession as an outstanding

greenbug biotype I-resistant line among currently available sorghum lines (Wu and Huang 2006 unpublished data). We produced an F_2 population of 371 individuals by selfing F_1 plants of the two parents. The F_2 population and their $F_{2:3}$ families were utilized in respective genotyping and phenotyping experiments.

Marker analysis

All the markers utilized in this study were microsatellite markers. The information of nuclear SSR primers was obtained from publically available sorghum linkage maps (Brown et al. 1996; Taramino et al. 1997; Dean et al. 1999; Bhattramakki et al. 2000; Kong et al. 2000; Cordeiro et al. 2001; Schloss et al. 2002). In addition, we developed additional nuclear SSRs and genic SSRs in the present investigation. Genic microsatellites were developed either in-silico (gene based SSR) using the sequence data base from the Phytozome website (http://www.phytozome.net/sorghum) or using collective sequence information from various EST databases (EST SSR). SSRIT, a SSR identification tool (http://www.gramene.org/db/markers/ssrtool) (Temnykh et al. 2001) was used to search the presence of microsatellites among these sequences. The search criteria to mine the core repeat motif was set to identify the maximum repeat motif length group using decamer option with the five repeats as the minimum threshold number of repeats in the sequence. These sequences containing the SSR were further utilized in designing primers to amplify the repeat motifs with flanking sequences. Primer 3.0 software, v 0.4.0 was used with default parameters to obtain both forward and reverse flanking primer sequences (Rozen and Skaletsky 2000). The expected PCR product size was set to 100-300 bp, 40-60% GC content with optimum of 50%, and an annealing temperature of 55°C to 58°C. The forward primer was extended with the M13 primer

sequence (5'-CACGACGTTGTAAAACGACG-3') before the 5' end of the sequence. The standard naming system for the newly developed marker was followed as proposed by De Vicente et al. (2004).

DNA was extracted from 1.5-month-old seedlings of F_2 plants grown in the greenhouse using a modified CTAB (cetyl-trimethyl-ammonium bromide) procedure as described by Murray and Thompson (1980) except that we used a different method for grinding tissue samples (drill with a blunt 1 ml tip). The final concentration of DNA was diluted to10 ng/µl as a working stock of PCR DNA template. We first screened all 401 available SSR markers with DNA from the parents, which resulted in identification of 107 polymorphic markers for the genotyping experiment. The PCR reaction volume and amplification procedure were followed as described by Wu and Huang (2008). We conducted the PCR reactions for genotyping all 371 F_2 individuals along with the parental lines in a PTC-220 Dyad Thermal Cycler (MJ Research Inc, MA, USA) and 2720 thermal cycler (Applied Biosystems, CA, USA). The PCR reactions were performed with an initial denaturation step of 94°C for 5 min proceeded by 13 cycles of denaturation at 94°C for 20 sec, primer annealing at 58°C for 1 min, primer extension at 72°C for 30 sec, followed by 27 cycles of denaturation at 94°C for 20 sec, annealing at 55°C for 1 min, extension at 72°C for 30 sec, and a final primer extension at 72°C for 10 min. The PCR products were separated in 6.5 % polyacrylamide gels mounted using a LI-COR 4300 DNA Analyzer (LI-COR Biosciences, Lincoln, NE, USA). The electrophoretic conditions were as follows: 1500 V, 40 mA, 40 W, and 45°C for 2.00 hr. The two parents were added as controls either in the beginning of the 700 dye gel or at the end wells in the 800 dye gel. The DNA banding pattern in the gel was scored

manually. For each marker, we recorded whether the individual was homozygous for the BTx623 or PI 607900 allele, heterozygous (both BTx623 and PI 607900 allele present), or was missing the marker amplification.

Phenotyping and data analysis

Phenotyping for greenbug resistance was conducted with the F_2-derived F_3 sorghum families. Two phenotyping experiments were performed, each arranged in a randomized complete block design consisting of three blocks. Within a block, each of the 371 F_2-derived F_3 families ($F_{2\text{-}3}$) was represented with two seedlings along with two parental lines. Two $F_{2\text{-}3}$ seeds from a single genetic family were planted together in a cell present in a growing tray of 12″ X 20″ X 1.75″ size filled with Redi-Earth soil. The two experiments were conducted in different section of the greenhouse with a gap of three days in sowing time.

Greenbug biotype I cultures were reared on barley (cultivar 'Schuyler') seedlings in the greenhouse of USDA-ARS, Stillwater until ready for use. For infestation, barley seedlings co-cultivated with greenbugs were cut and placed immediately between the rows of 12-day-old sorghum seedlings, equally and effectively infesting all the sorghum seedlings. F_2-derived F_3 families ($F_{2\text{-}3}$) were phenotyped for greenbug feeding response at 7 d, 10 d, 14 d, and 21 d post-infestation. Evaluation of the response of sorghum seedlings to greenbug feeding was conducted using a visual estimation to class damage to seedling foliage with a discrete scale that ranged from 1 to 6, where 0 = no damage, 1 = < 20% damage, 2 = 20-40% damage, 3 = 40-60% damage, 4 = 60-80% damage, 5 = > 80% damage, and 6 = dead (Starks and Burton 1977). The greenhouse conditions were

maintained with constant temperature (28 +/- 2 °C) and constant photoperiod (14 L:10 D) throughout the experiment (Wu et al. 2007; Wu and Huang 2008).

SAS software version 9.2 (SAS Institute 2008) was used to estimate heritability and variance components. The means and standard errors for parental lines and F_2-derived F_3 families were calculated using PROC MEANS. For all analyses, we calculated the mean of greenbug response of the two seedlings within an F_2-derived F_3 family raised within a single growing cell. To determine whether the F_2-derived F_3 families differed in greenbug resistance, we performed analysis of variance (ANOVA) with family and block as random factors (PROC MIXED). Similarly, to determine whether the two parental lines differed in greenbug resistance at the 4 time points, we performed an ANOVA with family as a fixed factor (PROC GLM). REML estimates of variance components were obtained using SAS/MIXED. We calculated the heritability of greenbug resistance at 7, 10, 14, and 21 d post-infection on a plot (block) basis and family mean basis following the REML univariate mixed-model analysis described by Holland et al. (2003). The phenotypic correlations among greenbug resistance at the four time points were estimated using multivariate REML module.

Linkage and QTL mapping

The genetic map was constructed using MAPMAKER/EXP 3.0 (Lander et al. 1987) with a logarithm of odds score (LOD) of 3.0 and maximum linkage threshold of 40 cM. First, a few known markers were used as anchoring markers to ascertain the number of linkage groups that were formed in the present mapping population using the 'Group' command. Assignment of linkage group to a specific chromosome was defined based on the

previous map information and the current number of linkage groups obtained in this experiment. The linkage groups were assembled, ordered and named based on the nomenclature given by Kim et al. (2005). The best order among the markers was chosen after using the 'Compare' command. The rest of the markers were added using the 'Try' command. Finally, the 'Ripple' command was executed to confirm the best possible order for constructing the framework map with log-likelihood threshold value of 2.0. The relative map distances between the markers were estimated by translating the recombination fractions into genetic mapping distances using the Kosambi mapping function (Kosambi 1944).

The output files were fed into QTL Cartographer version 2.5 (Wang et al. 2010) for QTL analysis. The empirical LOD threshold significant values for declaring QTLs associated with each trait was determined by conducting a 1000 permutation test (Churchill and Doerge 1994). Single Marker Analysis, Simple Interval Mapping and Composite Interval Mapping were performed prior to Multiple Interval Mapping (MIM) analyses to have a glimpse of the significant associated markers and the variation explained by these markers. We selected a new model in the Multiple Interval Mapping module by selecting QTLs that had high LOD values in Composite Interval Mapping, Interval Mapping and Single Marker Analysis. The optimum position of QTLs and significant QTLs were tested in an iterative manner. The *P* value chosen for declaring a significant QTL was 0.05 with LOD values obtained from 1000 permutation tests.

Results

Resistance source and phenotypic analysis

Based on the phenotyping data, plant response to greenbug feeding varied significantly in resistance between the two parents, BTx623 and PI 607900 as well as among the F_2-derived F_3 families (Table 1). Heritability values were high, ranging from 71% - 83% for all four greenbug resistance traits which are derived on a family mean basis of plant responses. These values are reported along with various components of variation (Table 2). All four greenbug resistance traits were positively correlated, with coefficients from 0.89 to 0.97 (Table 3).

Mapping and QTL detection

Of the 401 SSR markers, 33 % of nuclear SSRs and 20-25 % of ESTs and gene based SSRs were polymorphic. We obtained 107 polymorphic markers for genotyping among F_2 individuals, which consisted of 73 genomic SSRs, 30 EST-based SSRs, and 4 gene-based SSRs. We developed and used 48 novel markers, including 34 genic markers and 14 nuclear SSRs (Table 4). There were 18 markers that deviated from the expected segregation ratio in this mapping experiment. Of these, 13 markers belong to chromosome 2, which deviated towards PI 607900. The linkage map included 13 groups with 102 markers, which spanned a total length of 729.5 cM (Fig. 1). The linkage map covered nine chromosomes, except chromosome SBI05. Five markers (sb6_036-SBI03, Xtxp303, Xtxp299-SBI05, Xtxp224-SBI07, and Starssbem94-SBI09) were unlinked and two of these markers were assigned to chromosome 5 in previous sorghum maps. The marker order and map distances were in consensus with previously published maps (Wu and Huang 2007; Mace et al. 2009).

Single marker analysis identified 29 markers linking to four greenbug resistance traits with R^2 (%) values explaining from 1-72 % of the phenotypic variation within these traits. All the markers present on chromosome 9 were significantly associated with the greenbug resistance traits measured. Interval Mapping and Composite Interval Mapping results were corroborative in identification of the major interval influencing greenbug resistance, Starssbnm 78 – Starssbnm 102.

Initial MIM results indicated a consistent QTL for the four different time points of the study in the intervals of Starssbnm 78 – Starssbnm 81, Starssbnm 81 – Starssbnm 102, and one minor QTL near Starssbnm 47 – Strassbnm 64. Additional microsatellite markers were developed within these regions for candidate genes of insect resistance using the information from the phytozome sorghum database. At least three markers were polymorphic for the homologue similar to the receptor-like kinase *Xa21*-binding protein 3 gene, but one of them was not consistent and failed to produce sufficient information, hence only two markers were used in the mapping experiment. In addition, we developed one marker each for the chitinase gene and the jasmonate precursor, OPDA (12-oxo-phytodienoic acid) gene.

The identified QTLs with their corresponding genetic effects are summarized in Table 5. A major QTL for greenbug resistance response from plants for all time points was evident between the interval Starssbnm 81 – Starssbnm 102 located on chromosome 9 after incorporating gene-based markers (Fig. 2). The locus associated with the interval Starssbnm 93-Starssbem 296 was consistent across all traits, but explained different phenotypic variation. Eight significant QTLs were detected for all traits together with LOD values from 2.5 to 138.3. Of the eight QTLs, two minor QTLs were located on

chromosome 3b, between Starssbem 162 and Starssbem 265, and between Xtxp16 and Starssbem 162, which explained 1.3 percent and 1.0 percent of greenbug resistance at day 14 and 21, respectively. These minor QTLs are reported here owing to the high LOD values associated with them. The intervals Starssbem 286 – Starssbnm 93 and Starssbem 298 – Starssbnm 102 together accounted for 74.5 percent of phenotypic variation for the trait on day 7, but the position of the QTL was closer to Starssbnm 93. The markers Starssbnm 93 and Starssbem 296 were tagged to greenbug resistance across all times post-infestation, and hence a major QTL resides at this region, which we designated Qstsgr-sbi09i (Q-QTL, st-Stillwater-ARS, sgr-*Schizaphis graminum* resistance, sbi09i-sorghum bicolor chromosome 9 first QTL). This region involving Starssbnm93 and Starssbem 296 was responsible for 82.4 percent of phenotypic variation at day 21 post infestation. The final model at day 14 post-infestation explained the highest phenotypic variation among all traits and this model consisted of alleles present on chromosomes 9 and 3b accounting for 85.3 percent phenotypic variation. Two more QTLs were designated Qstsgr-sbi09ii and Qstsgr-sbi09iii, identified in the intervals of Starssbem 298 – Starssbnm 102 and Starssbnm 78 – Starssbnm81, respectively. Although the QTL Qstsgr-sbi09iii is associated with LOD values less than 3.0, it is reported here as it was responsible for high phenotypic variation. Hence, the major allele responsible for greenbug resistance was closer to receptor-like kinase *Xa21*-binding protein 3. The minor QTL on chromosome SBI03 was designated Qstsgr-sbi03. Additive and partial dominance effects were associated with all of the QTLs identified in this study. The negative sign associated with additive effects indicated that increasing allelic effect was derived from the resistant source (PI 607900) for all the identified loci. However, we also

observed a few more putative QTLs on sorghum chromosome SBI 3b (Xtxp285-Xtxp34), SBI 6b (Xtxp57-Xcup37) and SBI 10 (Xtxp320-ESR78), which explained either less phenotypic variation (less than 1%) or were associated with low LOD values. QTLs with an increased effect from the susceptible parent (BTx623) were also observed to be associated with the chitinase gene, but were not reported here due to a lack of consistency and low LOD values. No significant QTLs were associated with the marker Starssbem 274 developed for the OPDA (12-oxo-phytodienoic acid) gene, a precursor for jasmonate synthesis.

Discussion

Since the outset of extensive sorghum greenbug attack in 1968, varieties resistant to various biotypes had been developed but were frequently overtaken by newly emerging greenbug biotypes. Screening efforts to identify new sources of resistance have been a vital component of sustainable practices for greenbug management. Genomics-assisted techniques have hastened sorghum breeding efforts by facilitating marker-assisted selection for developing greenbug resistant varieties. The availability of the sorghum genome sequence has aided in developing novel markers for use in the current study. To our understanding, this is the first published map to tag genic SSRs for greenbug resistance in sorghum, which has further relevance to sorghum gene expression involved in aphid response.

Resistance to greenbug biotype I was governed by a complimentary gene action between two major dominant genes (Tuinstra et al. 2001). PI 607900 is a genetically distinct source of resistance against greenbug biotype I (Wu et al. 2006). Resistance is manifested in the distinctive categories; antibiosis, antixenosis and tolerance (Painter

1951). The resistance categories for this source were classified as antibiosis and tolerance (Wilde and Tuinstra 2000). However, later studies have shown that resistance to greenbug biotype I is controlled by polygenes (Agrama et al. 2002; Katsar et al. 2002; Nagaraj et al. 2005; Wu and Huang 2008). In this study several genomic regions contribute to greenbug resistance in resistant sorghum line PI 607900. The identification of a major QTL for resistance to greenbug biotype I on chromosome 9 corroborates earlier mapping efforts for greenbug resistance (Wu and Huang 2008). Moreover, sorghum chromosome SBI09 also harbored genes for resistance to different greenbug biotypes, including C and E (Agrama et al. 2002; Katsar et al. 2002; Wu et al. 2007). Furthermore, the major QTL accounting for the highest phenotypic variation was consistently observed in the interval of Starssbnm 93 – Starssbem 296 or near Starssbnm 93 at all four post-infestation times. Equally important, all the major QTLs are located in the interval of Starssbnm 78 – Starssbnm 102. The high phenotypic variation can be attributed to a bigger mapping population size, more closely spaced markers and a clear phenotypic response. The selection of plants with a combination of the above flanking markers for Qstsgr-sbi09i would better assist in precise selection of a greenbug resistant variety compared to using a single marker alone. The region between Starssbnm 78 – Starssbnm 102, which roughly corresponds to 1.02 Mb of physical distance on sorghum chromosome SBI09, contained several potential putative candidate genes. Most genes prominent and relevant to disease and insect resistance were the homologues similar to receptor-like kinase *Xa21*-binding protein 3 (Song et al. 1995), the chitinase gene, cysteine protease and amino acid selective channel protein. The QTLs identified for correlated traits for greenbug resistance resided in the same region of the chromosome;

similar results were observed in the earlier mapping experiments due to the phenomenon of pleiotropy of a single gene or tight linkage of a few genes affecting the trait (Aastveit and Aastveit 1993; Agrama 1996; Agrama et al. 2002; Wu et al. 2007; Wu and Huang 2008; Satish et al. 2009).

Genetic and molecular basis of aphid resistance has been reviewed and deciphered more recently with an emphasis on the involvement of R gene products in aphid resistance among agriculturally important crops (Thompson and Goggin 2006; Smith and Boyko 2007; Howe and Jander 2008; Dogimont et al. 2010). The genetic diversity of the sorghum gene pool from various parts of the world was assessed to identify resistance genes associated with greenbug attack from different resistant donors (Radchenko and Zubov 2007). Resistance to greenbugs was often conferred by either a few genes on a gene-for-gene basis or controlled by polygenes (Puterka and Peters 1995; Tuinstra et al. 2001; Dogimont et al. 2010). Aphid resistant plants are characterized with specific responses involving a gene-for-gene interaction and resistance in such a case involves loci containing NBS-LRR sequences (Smith and Boyko 2007; Dogimont et al. 2010). In the present findings, alleles accounting for a major proportion of variation were more closely linked to a homologue similar to receptor-like kinase *Xa21*-binding protein 3 than to a chitinase gene found in the nearby interval. Detection of consistent alleles upstream of this gene indicates a regulatory role of the R gene involved in herbivore damage. Moreover, this project particularly suggests the involvement of *Xa21* gene in a defensive response mounted by the plant. The up-regulation of *Xa1* gene in greenbug-infested sorghum plants supports our finding that similar but slightly different genes are involved in greenbug response (Park et al. 2006). However, it remains to be determined

whether the *Xa21* plays a direct or indirect role in modifying response of the plant to greenbug damage.

In conclusion, this study identified major QTLs in the marker interval Starssbnm 78 – Starssbnm 102 on SBI-09 for greenbug resistance in sorghum. This project aided in the development of molecular markers and in the identification of the location of these markers on the chromosomes for future map-based cloning experiments. The efforts to improve sorghum breeding programs for greenbug resistance management can be accelerated by using these tagged molecular markers.

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Tables

Table 1 Means ± SE for four greenbug resistance traits for two parental lines and F_2-derived F_3 families. Resistance traits include greenbug damage response 7 d (GDR07), 10 d (GDR10), 14 d (GDR14), and 21 d (GDR21) post-infestation. The degrees of freedom for family effect in ANOVA testing for variation between parental families was 1, 49

Greenbug damage response trait	Differences between parental lines				Differences among F_2-derived F_3 families		
	Mean ± S.E BTx623	Mean ± S.E PI 607900	*F* ratio	*P* value	Mean ±S.E	*Z* value	*P* value
GDR07	5.23 ± 0 .30	1.55 ± 0.09	313.13	<0.0001	2.77 ± 0.02	11.04	<0.0001
GDR10	5.93 ± 0.06	2.11 ± 0.12	369.94	<0.0001	3.46 ± 0.03	12.00	<0.0001
GDR14	6.00 ± 0.00	2.19 ± 0.06	1332.48	<0.0001	3.99 ± 0.03	12.38	<0.0001
GDR21	6.00 ± 0.00	2.66 ± 0.09	480.39	<0.0001	4.48 ± 0.02	12.17	<0.0001

Table 2 Variance components and heritability associated with greenbug resistance trait among F_2:F_3 families

Greenbug damage response trait	σ^2_g	σ^2_{ge}	σ^2_e	h^2 ± SE (plot basis)	h^2± SE (family mean basis)
GDR07	33.42	7.57	56.91	0.34±0.02	0.71±0.02
GDR10	45.87	4.99	50.28	0.45±0.02	0.80±0.01
GDR14	47.71	4.13	44.46	0.49±0.02	0.83±0.01
GDR21	44.99	2.25	51.14	0.45±0.02	0.82±0.01

Variance components expressed in percentage

σ^2_g variance associated with genotypes

σ^2_{ge} variance associated with genotype X environment

σ^2_e Residual variance

h^2 on plot basis where one experimental unit is considered as plot

h^2 on family mean basis using means of F_2:F_3 families

Table 3 Phenotypic correlations (r) among four different greenbug resistance traits at $P < 0.0001$

Trait	GDR10	GDR14	GDR21
GDR07	0.93754	0.91663	0.89239
GDR10		0.96254	0.93588
GDR14			0.97254

Table 4 List of novel microsatellite markers used in construction of linkage map for BTx623 X PI 607900

Primer name	Forward sequence (5'-3')	Reverse sequence (5'-3')	SSR Motif	Tm value	Expected size
Starssbem279	CACCTTCCTTCCTTCCTTCC	ATGTCAGCTTCGAGCACCTT	(gga)6	60	180-200
Starssbem69	GGCAATTTGGCAAGCAAT	CTCTTCTCCTTTCCACGCTG	(cagg)5	64	145-175
Starssbem169	ATAACCAACCCCGGAAACTC	AATCTGAAGCGCACCAAAAC	(agat)7	64	200-250
Starssbem111	CGTCCTGGAGCAAAGGTTAC	TTTCCACTCGGCTCTTGTCT	(tg)11	63.9	250-300
Starssbem197	GTATCCATCCATCCCACCAC	AGCACCACGAAGGAAGTCAC	(gagc)7	64.3	250-300
Starssbem04	CAGCACCACAACTGATCCAT	TATTGACACGCAGGTAACGC	(ta)9	63.6	145-175
Starssbem99	TCGCTTTCTCCCCTCTACAA	GAAGTCGGCGTTCATCTCTC	(ga)9	63.9	175-200
Starssbem70	GACATCTACTTCTTCGCGCC	TGATGCGTCACAAACTCACA	(tgta)5	64.2	145-175
Starssbem126	CAGAGCATACCTCCCCTGAA	TTGAATCGGTTGCATGGATA	(agc)15	63.8	200-250
Starssbem208	ATAGGGACACGGCAGCACTA	ACCCAGGTGAAGATGATCCA	(ag)10	60.3	145-175
Starssbem16	TCACCTCCTTTTTCTCCCCT	AGAGCTCGTACGCCTTCTTG	(tg)13	63.5	250-300
Starssbem44	AGCTCTGCTGATCTGACGGT	AGCTTGCTCGTGTGTGATTG	(acc)7	64.1	145-175
Starssbem187	AATGCAGATCCGACTGGC	CAGATAAAAGCAGCGTGCAA	(gagg)5	63.9	200-220
Starssbem204	CATTTCAAATCGCCACTAGC	GTGTTGCGGTTTCCTTGTTT	(ga)9	60	100-145
Starssbem12	CGAGCTCAACATACAGGCAA	CCAAGGCTGAGGTCAAGAAG	(ac)9	63.8	220-250
Starssbem82	CCACAGGGCTTATCCAAGAA	TTTACATGTGCCAGAACACA	(ta)11	60.2	145-175
Starssbem215	TCTTCCTTCCTTTTTCGGGT	CATTGTCCCTCACTCCTGGT	(tc)25	60	200-220
Starssbem136	TGCTTCCCACTAGACCATCC	GAACGATGGAAGCCATGAAC	(cac)6	64.4	100-145
Starssbem23	CGGGTCTTCATCTCCCTCTC	GGTCAACACATTTTTGCCCT	(gcc)6	63.6	145-175
Starssbem162	ATTGGTTTTGTTCCCAATCG	GCAAGACCAATAACCCCATC	(atgt)6	63	100-145

Starssbem265	AATGCATCAGCA TCAACTCG	AGTGAGCAACACA CACGTCA	(cgta)5	58.9	100-120
Starssbem170	CGGAGAGCATGA GGATTGAT	CGCAGCTGATACT GATTGGA	(atgt)5	63.9	250-300
Starssbem280	ACCAACCTGCCTA CCATCAG	CCTCGGCCATTAC CTTACCT	(gctc)5	60.3	200-225
Starssbem266	CCGTGAGAAGGA AAGTTAAATCAG	AGGGAGGAGGAC CTTATGGA	(attc)5	59.9	100-145
Starssbem95	TTCTTAACCTCCA TGCCTGC	AATTGAACTCGAA TTGGGGG	(ct)10	64.7	100-145
Starssbem151	GAGAGCTACGGC TGGGAGAT	TCATCACATCCTC CTCCCTC	(gca)8	64.1	230-250
Starssbem18	CAGCTAACACCA CCCTCGTT	CCAGATCCAGCAG GAAGAAG	(tc)10	63.8	250-300
Starssbem78	CTCAGTTCAGCAG CAGCAGT	CCATCGATCGAGC TCTCTGT	(ag)11	64.5	100-120
Starssbem77	CACGAGGCAAAG ACACAGC	GCATCGCCATCTC TCTCTTC	(ga)14	64.0	100-120
Starssbem94	TCATATGGGGTGT GATGTGG	AAGGCAATGTCCA CAAAAGG	(gt)10	63.7	100-120
Starssbem274	TGAAACTCGGAC TGACGATG	GTTGCGGCAAGTA AAAGGAGG	(cgga)6	59.8	175-200
Starssbem286	GGTGGCCACTGTC TTCTTGT	CAGCAGCATCTGG TTGAAGA	(gcg)5	60.1	200-250
Starssbem296	GATGTTCGACTCC CTGCAC	CCGTTCTCCAGCA GCACCT	(gtc)3	61	220-250
Starssbem298	TGCCTCCTCTTCC TCTTCCT	AGGACCATGGACA GCACCT	(tctgc)8	57.8	145-175
Starssbnm07	GCAGCTTAAGGG CAAAAGAA	AATTGGTCGACAA TGGGAAG	(at)9	59.61	145-175
Starssbnm42	CGACGACGCTAC TACTGCTG	GCAAAGCAAATAA GGCAAGG	(gtc)6	59.8	175-200
Starssbnm60	CTCGTAAGGGGT CAGCAGAG	TAAATGGCCCACC TTCAGTC	(tgag)5	60	175-200
Starssbnm47	GGAGGCCAACAA CCAACTAA	TGGGTGGGAAAAA GAAAAGA	(aat)7	59.9	220-250
Starssbnm64	AAACAGCACAGG AGGGAGTG	GATGTCCGTCAGA GGAGGAG	(cagctc)5	59.7	175-200
Starssbnm73	TGGTGAGGTACTC CCTCCAG	CAAGATTTTGAGG CCAGCAT	(at)7	60.0	175-200
Starssbnm21	GGGAATGCAAAA AGGAGTGA	AGGAAGACGGAA GAGGAAGC	(ct)7	59.9	200-230
Starssbnm35	TTGTGCCCCATAC TCCTCTC	GGCCAACTAGACG CAAATGT	(ag)10	60	175-200
Starssbnm37	GTTGCACGCTATC ACTCTGC	GTGGTTCAGGAGC AATGGTT	(at)8	59.6	175-200

Starssbnm78	AGGTGATGACAG GGATGGAG	CGGGTATGTAGGC CAGAATC	(ttc)15	59.9	230-250
Starssbnm81	CCATGCTTGCTCA GTTCCTT	CGGCGACACAAAC TCTATGA	(aat)41	59.8	220-250
Starssbnm93	GATCGGCGTGAA AACAAAAT	TTTGGTGTCAATC CCAGTGA	(cag)8	59.9	175-200
Starssbnm102	TGCATTGCTGAAA GCCTAAA	CCTGTGCTGTGAC TGCATCT	(ccat)11	59.5	220-250
Starssbnm104	GGGAGGGAGAGA GGAGTGTC	AGCGTCGCTAAGG GTTCATA	(tc)7	59.8	200-230

Legend:

Starssbnm stands for Stillwater-ARS (Stars) Sorghum bicolor (sb) nuclear microsatellite (nm)

Starssbem stands for Stillwater-ARS (Stars) Sorghum bicolor (sb) est microsatellite (em)

Table 5 Estimates of greenbug resistance QTLs with their genetic effects

Traits	Chromosome/ marker interval/	QTL position	LOD	Genetic effects Additive	Dominance	R^2 value (% effect)
GDR07	SBI09 Starssbem286-Starssbnm93	22.57	3.9	-0.6180	-0.1746	39.8
	SBI09 Starssbem298-Starssbnm102	25.57	3.1	-0.5470	-0.2043	34.7
						74.5 R^2_F
GDR10	SBI09 Starssbnm93-Starssbem296	23.57	26.9	-1.2139	-0.2814	64.7
	SBI09 Starssbnm78-Starssbnm81	16.28	2.5	-0.3442	-0.2575	17.6
						82.3 R^2_F
GDR14	SBI09 Starssbnm93-Starssbem296	23.57	27.31	-1.3369	-0.2050	67.0
	SBI09 Starssbnm78-Starssbnm81	16.28	2.5	-0.3645	-0.1425	17.3
	SBI03B Xtxp16-Starssbem162	24.50	3.7	-0.1641	-0.1487	1.0
						85.3 R^2_F
GDR21	SBI09 Starssbnm93-Starssbem296	23.57	138.3	-1.3945	-0.1091	82.4
	SBI03B Starssbem162-Starssbem265	30.22	4.5	-0.1547	-0.1022	1.3
						83.7 R^2_F

Legend:

GDR07, GDR10, GDR14, and GDR21, represents greenbug damage response scored at 7, 10, 14, and 21 days after infestation

R^2_F Total phenotypic variation explained by final model

'-' sign associated with resistant parent PI 607900

Figures

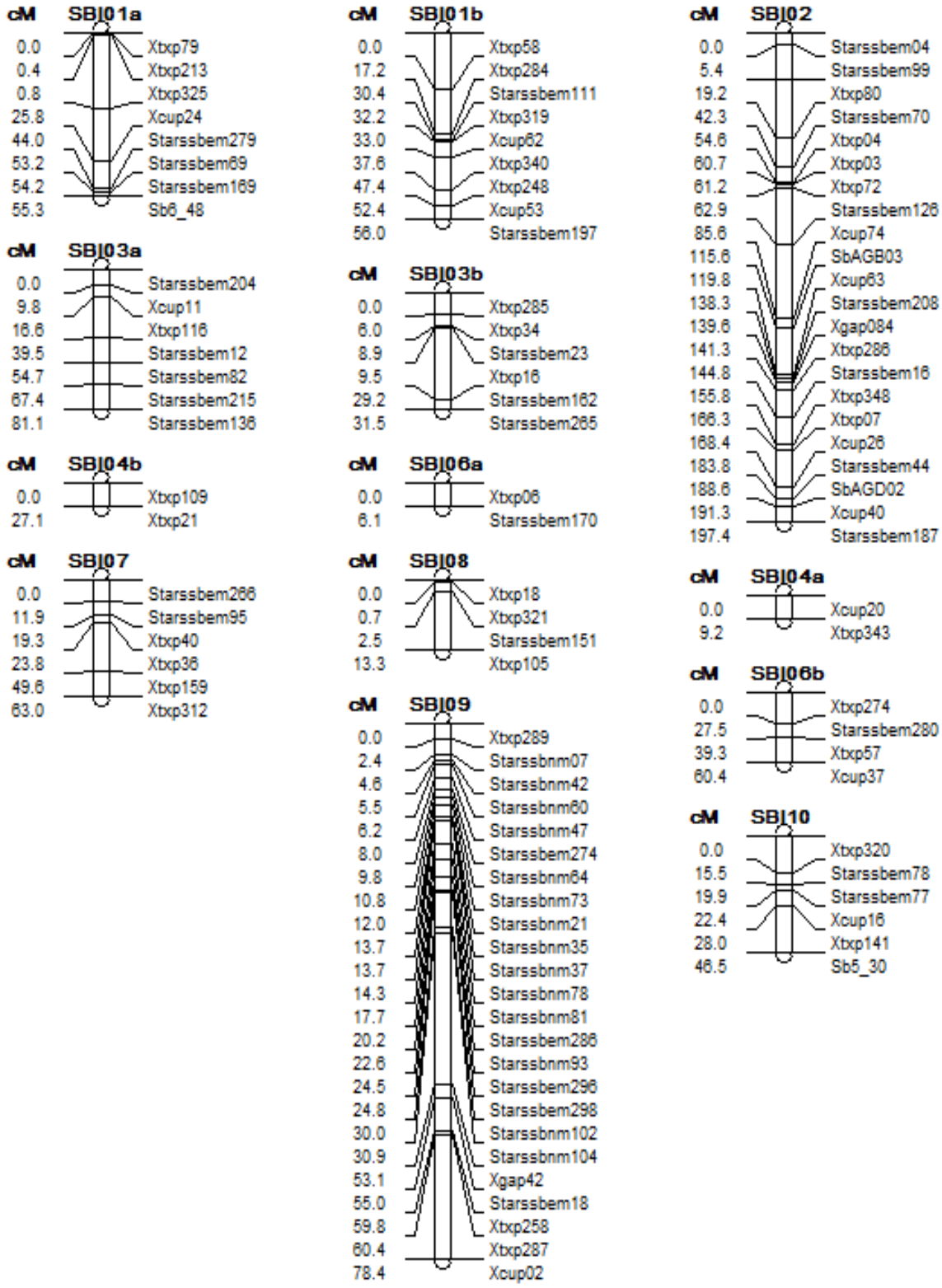

Fig. 1 Linkage map for BTx623 X PI 607900 cross in F_2 population

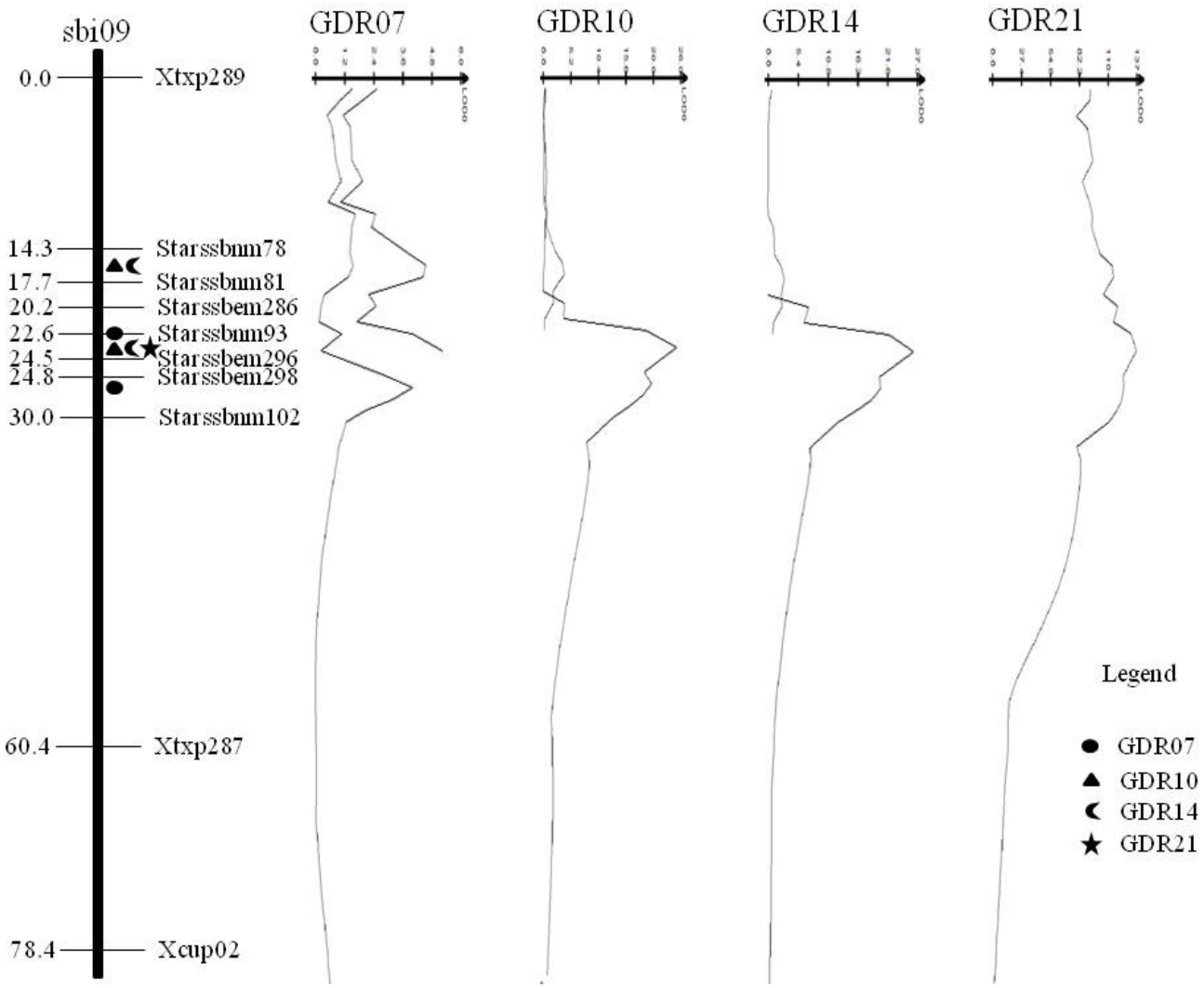

Fig. 2 The major QTLs identified in this study. The LOD score peak profile using MIM analysis for four greenbug resistance traits identified on chromosome 9. GDR07, GDR10, GDR14, and GDR21, represents greenbug damage response scored at 7, 10, 14, and 21 days after infestation. The identified QTLs for four traits are designated with symbols given in legend and distances between markers is in centimorgan (cM)

CHAPTER III

QTL MAPPING FOR GREENBUG RESISTANCE IN SORGHUM AND GENE EXPRESSION STUDIES OF CANDIDATE GENES IN RESPONSE TO GREENBUG FEEDING

Abstract

Greenbug infestations to sorghum can cause severe and above economic threshold damage in the Great Plains of the United States. This study was conducted to identify quantitative trait loci and potential candidate genes within the QTL region responsible for greenbug resistance in an intercross population. In this study, we mapped quantitative trait loci (QTLs) responsible for greenbug resistance in sorghum using an intercross population derived from two parents, BTx623 (Greenbug-Susceptible line) and PI 607900 (Greenbug-Resistant line). Molecular markers for 101 loci were used to construct a linkage map which eventually facilitated tagging portions of the sorghum genome regions responsible for greenbug resistance. The loci for greenbug resistance were mapped to the region flanked by markers Starssbnm 93-Starssbnm 102 on chromosome 9. The locations of these loci were compared with our previous study on QTL analysis using a F_2 mapping population. The results were in concurrence with our findings in the F_2 QTL analysis. Further, this region contained several candidate genes for insect resistance including receptor–like *Xa21*-binding protein 3-like, map kinase phosphatase, a putative uncharacterized protein and inorganic pyrophosphatase.

These four candidate genes were subjected to differential gene expression analysis using real-time PCR. Relative quantification of gene expression in two parental lines was performed to assess the mean fold change upon greenbug feeding. The results from real-time PCR analysis revealed that receptor–like *Xa21*-binding protein 3-like and map kinase phosphatase were differentially expressed between the two contrasting parents. The markers/QTLs identified from this study can be effectively utilized in marker-assisted selection and map-based cloning experiments.

Introduction

Sorghum, *Sorghum bicolor* (L.) Moench, the fifth most important cereal crop, offers various utilities and advantages over other crops throughout the world and in the United States. This crop is particularly sought after for feed stock in the Great Plains, owing to its high performance in this climatic region. However in this region, sorghum yield is often reduced by insect pests, specifically the greenbug [*Schizaphis graminum* (Rondani)]. Since its discovery in 1990, greenbug biotype I, the most distinguishable biotype, has established sorghum as a predominant host crop (Harvey et al. 1991; Kofoid et al. 1991; Burd and Porter 2006). Greenbug damage to sorghum is detrimental in almost all stages of crop growth with observable symptoms such as red necrotic spots and chlorosis. The annual loss of sorghum due to greenbug damage is estimated around $248 million to US sorghum producers (INTSORMIL, 2006).

DNA markers have become increasingly popular genomic resources and have spurred the efforts towards the development of sorghum linkage maps. The linkage maps represent genome architecture by using DNA markers in linear order. Linkage maps have diverse utility because they form the basic framework for studying simple and complex

genetic traits (Tanksley 1993; Cone and Coe 2009). Genetic maps serve as a valuable source for comparative analysis among different crops (Mullet et al. 2001; Mace et al. 2009). Furthermore, linkage maps allow researchers to identify and tag significant genomic regions affecting trait such as insect resistance (Mohan et al. 1997; Yencho et al. 2000). Several linkage maps developed in sorghum have facilitated tagging of greenbug resistance (Agrama et al. 2002; Katsar et al. 2002; Nagaraj et al. 2005; Wu et al. 2007; Wu and Huang 2008). The alleles accounting for sorghum biotype I resistance have been documented through QTL studies, which support the involvement of multiple regions of the sorghum genome in greenbug resistance (Katsar et al. 2002; Agrama et al. 2002; Nagaraj et al. 2005; Wu et al. 2008).

The reliability of QTL mapping experiments depends on the type and size of the mapping population used, marker system employed and the insect/disease resistance scoring method involved in the bioassay. The type of mapping population which is primarily developed based on the objective of the study plays a crucial role in developing linkage maps for QTL studies, because the mapping population reflects the diversity of genetic information it can provide to the researcher (Young 2000). Although F_2 mapping populations are quick, easy to develop, transient and best suited for initial mapping, they are seldom used for fine mapping. For this reason, F_2-derived populations such as immortalized populations, advanced intercross lines, and intermated populations, have an advantage over F_2 mapping populations with respect to mapping resolution and accuracy. For example, the heterozygosity of parent (F_2) alleles are fixed and maintained in the pooled seeds of immortalized individuals (Gardiner et al. 1993; Hua et al. 2003). Advanced intercross lines and randomly crossed intermated populations, which

accumulate recombination events in short chromosome segments, increase the accuracy of fine mapping (Darvasi and Soller 1995; Liu et al. 1996; Lee et al. 2002). These recombinations between two loci, accumulated over generations by intermating within a population derived from $F_{2,}$ increase the ability to precisely identify a QTL map location and its effects (Liu et al. 1996; Darvasi 1998).

The results of mapping experiments can be further enhanced by using functional genomics (White 2001). Identification of a candidate gene is more efficient when QTL studies involving saturated maps are merged with gene expression studies (Nguyen 1999; Pflieger et al. 2001). A QTL region embeds a large number of candidate genes which are involved in functional polymorphism of the trait. Candidate genes are causative genes underlying or influencing known phenotypic trait variation (Rothschild and Soller 1997). After an initial QTL identification with a large QTL region which embeds several candidate genes influencing functional polymorphism for a given trait, candidate gene analysis could prove the necessary pathway for positional cloning of these loci (Zhu and Zhao 2007). A combination of approaches involving the positional candidate genes located in the vicinity of QTLs obtained from linkage studies, combined with functional candidate genes based on functional variation in a trait, would facilitate a better understanding of genes involved in the resistance trait like greenbug resistance (Byrne and McMullen 1996; Pflieger et al. 2001).

Sorghum, like any other plant, has innate mechanisms to tolerate and respond to aphid feeding through defensive responses which parallel pathogen-induced responses (Huang 2007). The recent understanding and unraveling of genetic and molecular interactions involved in several crops' defense response to aphid feeding, including

sorghum, have supported the role of plant R genes (resistant genes) (Smith and Boyko 2007; Dogimont et al. 2010). The up-regulation of glycoprotein sequences with LRR motif and *Xa1* in response to greenbug attack has clearly depicted the involvement of plant R genes in sorghum (Zhu-Salzman et al. 2004; Park et al. 2006). These transcriptomic studies have also shown that defense response of sorghum to greenbug attack is augmented through expression of numerous other genes and messenger molecules.

Real-time PCR is the most accurate detection system for measuring the initial amount of template used for amplification (Higuchi et al. 1992; Higuchi et al. 1993; Ginzinger 2002). Real-time PCR involves automated detection of fluorescence, which is directly proportional to the amount of product amplified. The fluorescence level detected, before the reaction enters into the plateau phase of amplification, is expressed as the C_T value or threshold cycle. The threshold cycle is the point at which the number of PCR cycles crosses the baseline of fluorescence to reveal the initial quantity of template. The expression analysis done through RT-PCR experiments can quantify the relative abundance of transcripts involved in resistance and the defense mechanism of plants. The relative quantification of gene expression studies using a house keeping gene is the most desired approach to quantify differences in expression level of target genes elicited in response to greenbug attack in two contrasting lines.

Our earlier efforts on QTL mapping for greenbug resistance in an F_2 mapping population showed promising results with the identification of resistance loci on sorghum chromosome SBI09. Utilizing this information, we aim to confirm and identify any new loci for greenbug resistance using an intercross population derived from an earlier F_2

population used in the previous project. Therefore, we had two objectives; (1) to identify QTLs in an intercross population and locate potential insect resistant candidate genes residing in the QTL region; (2) to elucidate the expression pattern of these genes in two parents using real-time PCR. The use of an advanced population would confirm and increase the accuracy of QTL map location with reduced confidence interval for the loci and their genetic effects. The relative gene expression of candidate genes from the QTL region would further enhance our outcome from the QTL studies.

Materials and Methods

QTL mapping in an intercross population

Objective: This experiment was performed to confirm the QTL positions identified from earlier studies and identify potential candidate genes in the QTL region.

Development of an Intercross Population

The mapping population used in this study was an intercross population, developed from an F_2 population of the cross between BTx623 and PI 607900. The procedure for developing an intermated population was initiated in F_3 lines. We chose 400 F_3 plants which were divided into two halves with 200 plants each. One half was used as the female group and plants were emasculated based on the pollen availability from the other group. One to one crossing was done with 200 paired plants. Upon successful crossing, we obtained 158 crossed seeds which were selfed to produce the first filial generation of the intercross of which 143 plants were used for genotyping. The second filial generation was used in the greenhouse trials for phenotyping of greenbug resistance. There was one

round of random intermating and one round of selfing prior to the use of material for linkage and QTL analysis respectively.

Genotyping

The genotyping procedure involved initial DNA extraction of 143 intercrossed plants using a modified CTAB procedure (Murray and Thompson 1980). Primers that were developed earlier and used in the F_2 mapping population were used for genotyping (chapter2). We proceeded with marker genotyping, as our parental polymorphic screening was accomplished in the first project using SSR markers (refer to chapter 2). We obtained 107 polymorphic SSR markers which were genotyped across 143 individuals and scored as A and B (allele from two parental lines) and H (heterozygote with alleles from both parents). Missing marker information was scored as "_". The PCR amplification protocol using fluorescence dye labeled (IR 700 and IR 800) primers was similar to that followed in chapter 2. The products obtained from two dyes were mixed and loaded on to LI-COR 4300 DNA analyzer (LI-COR Inc, NE, USA). Allele sizing was done by comparing to parental alleles which were used as controls in each gel.

Linkage map

Linkage analysis was performed utilizing the genotypic data obtained from 107 polymorphic markers, using MAPMAKER/EXP 3.0 (Lander et al. 1987). The markers were linked and ordered on linkage groups using minimum logarithm of odds (LOD) of 3.0 and a maximum linkage distance of 40 cM. The association of these markers was already known from previous linkage analysis, hence we performed other commands such as 'Compare' and 'Ripple' to determine the best order and final framework on the

chromosome. Kosambi mapping function was executed to calculate the relative mapping distances in cM (Kosambi 1944). The sorghum chromosomes were named in accordance with the internationally accepted names given by Kim et al. (1999).

QTL analysis

The identification of quantitative trait loci was carried through QTL cartographer version 2.5 by using suitable .map and .raw files from mapmaker. The best possible way to avoid association of false QTLs is to determine the significance level of the LOD threshold value for each trait through a 1000 permutation test given by Churchill and Deorge (1994). We performed 1000 permutation tests to identify the critical LOD values associated with four different greenbug resistant traits. Identification of significant markers, marker intervals with flanking markers from single marker analysis, interval mapping, and composite interval mapping, were used to include in the initial model for Multiple Interval Mapping (MIM) method of QTL analysis. The MIM analysis for each trait was initiated with the *p*-values set to 0.05 and LOD score obtained from the 1000 permutation tests. The MIM module in QTL cartographer includes step-wise and iterative mode of search for new QTLs along with optimizing position and effects of QTLs (Kao et al. 1999; Zeng et al. 1999). The initial model was tested in repeated mode to identify significant QTLs based on the Bayes Information Criterion values. Initially identified QTL positions and effects were stabilized and included in the final model.

Phenotyping and Statistical analysis

The phenotyping for greenbug resistance was carried out in a population which was selfed once after one generation of intercrossing. The phenotypic evaluations were done

as in our F_2 analysis (chapter 2). Greenbug damage scores were recorded at 7d, 10d, 14d and 21d post-infestation. We used the 0 to 6 scale (Starks and Burton 1977), where 0 indicated no damage and 6 indicates a completely dead plant, with intermediate scores of 1-to-5 indicating a 20% increase in damage for each respective increase in the scale. Phenotypic data were analyzed using SAS 9.2 (SAS Institute 2008). Parental scorings for greenbug resistance were subjected to ANOVA using the PROC GLM procedure. Scores from progeny lines were used in PROC MIXED and PROC CORR to estimate significant response and extent of correlation among the four different traits. Variance components and heritability values were determined using SAS code provided by Holland et al. (2003).

Quantification of gene expression using RT-PCR

Objective: We aimed to determine the expression pattern for a few candidate genes found in the identified QTL region.

Plant material

The two parental lines BTx623 (susceptible to greenbug) and PI 607900 (resistant to greenbug) were sown in pots in the greenhouse. The two lines were infested with greenbugs when seedlings were three weeks old. The greenbugs, which were initially reared on barley in ample amounts, were infested equally on two sorghum lines. The plant samples from leaf tissue and stem were collected at 1 day, 3 days, and 5 days after infestation. We also collected samples from two parental lines at 0 day before infesting greenbug which served as controls (calibrator sample) for the real-time PCR experiments. Two to three plant samples from each replication were combined and weighed to make

2.0 g of tissue for subsequent RNA extractions. There were eight samples per replication which were collected in liquid nitrogen and stored at -80°C. All samples were collected twice from different pots which represented two biological replications.

RNA extraction and cDNA synthesis

Total RNA was extracted for eight different samples using TRIzol reagent (Invitrogen, CA). RNA samples were quantified using a NanoDrop spectrophotometer and diluted to an equal concentration of 1 µg of RNA in all eight samples. The cDNA was synthesized by reverse transcribing mRNA with reverse transcriptase using the QuantiTect Reverse Transcription Qiagen Kit according to the manufacturer's instruction (Qiagen, CA). The kit also contains a prior step of removing genomic DNA contamination by treating with DNAase, therefore the cDNA synthesized was free from genomic DNA. The cDNA's and primers were checked using regular PCR.

Primer design for gene-specific primers

Given limited resources and time, we designed gene specific primers for four candidate genes in the putative QTL region using Primer 3.0 (Rozen and Skaletsky 2000). The cDNA sequences for four genes from the QTL region on chromosome 9 i.e. a putative uncharacterized protein (Sb09g001360), a hypothetical protein sequence similar to receptor-like Xa21-binding protein 3 (Sb09g001370), sequences similar to inorganic pyrophosphatase (Sb09g001530) and map kinase phosphatase (Sb09g001660), were retrieved from phytozome (a database for sorghum, http://www.phytozome.net/sorghum). The forward and reverse sequences for these genes were selected upon and synthesized (Bioneer Inc, Alameda, CA, USA) using Primer 3.0 (http://www-genome.wi.mit.edu/

genome_ software/other/primer3.html) with the following criteria: length of the primer - 18-23 nt, optimum = 20 nt; expected product size = 150- 300 bp; annealing temp = 58-60°C and GC = 45-60 % with an optimum of 50 %. These primers were checked at the sequence level to avoid primer dimers. The gene specific primers were designated GSP13, GSP15, GSP17 and GSP21. The list of primers developed for these four genes along with sequences are given in Table 1. The sequences of actin primers, F-5'-TAGTCCAGGGCAATGTAGGC 3', R-5' CCCAGATCATGTTCGAGACC 3', were taken from sorghum ESTs available in the public domain.

Real time PCR expression data analysis

The expression levels of four different candidate genes were analyzed using real-time PCR experiments. Real-time PCR experiment was performed with MyiQ™ Single-Color Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA). The common housekeeping gene, actin, was co-amplified along with 8 samples, which was further used in normalization during gene expression analysis. Initially, a standard curve was made for every primer along with the actin primer, for eight data points using 10-fold cDNA dilutions. The 15 µl final volume of PCR mix was made using 3µl of cDNA template dilution obtained from the standard curve, 12 µl mix of primer of 1µM and SYBR *Premix Ex Taq* ™green I dye fromTaKaRa mix (TAKARA BIO INC, CA). The amplification protocol was composed of initial denaturation at 95°C for 10 min, followed by (95°C for 10 sec, 58°C for 20 sec, 72°C for 20 sec) for 40 cycles, 95°C for 1 min, 55°C for 1 min, (55°C for 10 sec) for 81 cycles and finally hold at 4°C. The melt curve was started at 55°C with increase in 0.5°C until 95°C. The C_T values were obtained from MyiQ software. The level of expression of actin was constant in two parent lines at four

different time points. The formula for $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) for relative gene quantification was used as specified in Bio-Rad Real-Time PCR applications guide using delta delta C_T values (Bio-RAD Laboratories, Inc. Hercules, CA). The relative fold change is presented in Fig. 4. For each gene, a two-way fixed factor statistical model was used to assess the differences in expression using delta C_T values between families (two parents), time points, and their interaction. The statistical analyses were calculated using the GLIMMIX procedure in SAS version 9.2. Least-squares means (lsmeans) and differences of lsmeans were calculated using the LSMEANS command in SAS and the type I error rate was controlled by Tukey's adjustment. We had two biological replications, each consisting of eight samples including controls. PCR experiments were repeated twice (two technical replications) for each biological replication. In each technical replication, each sample was assayed in triplicate and the values averaged as one data point.

Results

Phenotypic analyses

The responses of the two parents were similar to our analyses in the first project. PI 607900 had very high resistance response to greenbug damage compared to BTx623. PI 607900 performed better when exposed to greenbugs, with mean damaging scores ranging from 1.15 ± 0.08 to 3.06 ± 0.09, while BTx623 was highly susceptible to greenbug damage, with mean damage scores from 5.35 ± 0.13 to 6.00 ± 0.00 across sampling times (Table 2). Progenies of the intercross population also showed significant difference in greenbug damage with mean values from 2.31 ± 0.06 to 5.09 ± 0.03 (p <0.001). The resistance to greenbugs was a highly heritable trait among progenies:

heritability values based on family mean basis ranged from 68% to 82% (Table 2). The associated variance components and phenotypic correlations for four traits are reported in Table 3 and Table 4, respectively. Trait 1 (7 days) and trait 2 (10 days) were highly correlated, followed by trait 2 (10 days) and trait 3 (14 days).

Linkage mapping in the intercross population

We used the same set of markers as in our first project to make a linkage map for the intercross population. However, we could map only 100 markers out of 107 (Fig. 1). The seven markers that were unlinked are as follows: Xcup24 on sbi01, Xtxp80 on sbi02, Xtxp299 and Xtxp303 on sbi05, Xtxp274 on sbi06, ESR 94 on sbi09 and Xtxp224 on sbi07. Overall, the order of markers on the map remained the same and was compared to established consensus maps (Mace et al. 2009). It is expected that because of random intercrossing in the mapping population, segregation distortion increases among alleles; we found segregation distortion for 53 markers, most of which were deviated towards PI 607900. The total length of the linkage map covered 858.3 cM of the total genome, encompassing nine sorghum chromosomes split into 15 linkage groups. However, we did not have enough markers on chromosome 5. The present map (858.3 cM) was expanded by 17.65% compared to our map from the F_2 population (729.5 cM). Some of the markers came very close, while others expanded drastically on the linkage map. As we cannot compare each of the markers, case by case, they are delineated here within each chromosome wise. Of the nine chromosomes represented, chromosomes SBI04 and SBI07 were reduced in length compared to the F_2 population map. The rest of the chromosomes showed an increase in genetic length. It is obvious that recombination between closely linked loci increased the genetic distance in this case. Moreover, the map

was expanded in the identified QTL region. We were particularly interested to see the region between Starssbnm 78 and Starssbnm 102 on chromosome 9, where we previously observed the QTLs for greenbug resistance in the F_2 mapping study. In the current study, this region was covered by 32.8 cM distance, an increase of two fold over 15.7 cM observed in our F_2 study. This would reduce the confidence interval associated with the QTL region.

QTL analysis

The results from single marker analysis explained all markers present on chromosome 9 as significant markers for four traits. The results of Multiple Interval Mapping (MIM) revealed two regions between Starssbnm 93 and Starssbem 296 and between Starssbem 298 and Starssbnm 102 as QTL harboring regions (Table 5). The two regions identified on chromosome 9 were supported by similar results in our F_2 QTL analysis (Fig. 2). The region between Starssbem 298-Starssbnm 102, accounted for 72.9 to 79.7 percent of variation for greenbug resistance observed at day 7 and day 10 with associated LOD values ranging from 47.98 to 54.99. The highest phenotypic variation of 73.3 to 80.9% for greenbug resistance was identified from the region of Starssbnm 93 and Starssbem 296 with associated LOD values of 41.05 to 50.36. These two regions were designated Qstsgrip09i (Starssbem 298-Starssbnm 102) and Qstsgrip09ii (Starssbnm 93-Starssbem 296), which showed an increased allelic effect for greenbug resistance originating from PI 607900 in the range of 1.00 to 1.95 units. The QTL identified for trait 3 (day 14) and trait 4 (day 21), Qstsgrip09ii, flanked by Starssbnm 93 and Starssbem 296 was 1 cM away from Starssbnm 93 and was also found consistently in our F_2 analysis. The QTL, Qstsgrip09i, was flanked by Starssbem 298-Starssbnm 102 for trait 1 (day 07) and trait 2

(day 10) and was also located at 1 cM away from Starssbem 298. The QTL location was always either upstream or downstream of the markers, Starssbem 296 and Starssbem 298, developed for a gene sequence similar to receptor-like *Xa21*-binding protein 3.

Gene expression analysis

After greenbug infestation, the two parents were visually observed for symptoms. BTx623 was highly susceptible with red necrotic spots spread all over the leaf while PI 607900 rarely developed any symptoms of infestation over three different time points, 1day, 3days and 5days (Fig. 3). The change in expression pattern of four genes, SP13, SP15, SP17 and SP21 upon greenbug feeding on susceptible plant BTx623 and resistant plant PI 607900 were quantified through real-time PCR. The transcript levels induced upon greenbug feeding in each of these plants were determined from threshold cycle (C_T) values. The C_T values were normalized by deducting actin C_T values to derive delta C_T value. The delta delta C_T values were then determined by subtracting their respective control C_T values from delta C_T values. The mean fold change in gene expression was calculated using delta delta C_T values at four different time points (0 day, 1 day, 3 days and 5 days) for each gene and is presented in graphs (Fig. 4). These results of mean fold changes were supported with statistical analysis where significant and non significant differences due to the delta (C_T) response values for the main effects and interactions in each gene, are presented in Table 6. The coefficient of variation for both biological replications, across two families (parents) of sorghum and across various time points within each gene was calculated using the mean delta C_T values (Table 7) obtained after greenbug infestation (Table 8). In general, three genes SP15, SP17 and Sp21, showed considerable variability between the two biological replicates. We found that gene

specific primer SP15, developed for a sequence similar to receptor-like *Xa21*-binding protein 3 was highly expressed in greenbug-susceptible parent, BTx623 compared to the greenbug-resistant parent, PI 607900 at all time points, with peak expression at 3 days; expression was further reduced at 5 days. The expression of this gene (SP15) went on increasing from day 1 to day 5 in PI 607900. The mean fold difference for SP15 and SP17 between two families was further corroborated by statistical analyses and was significant in gene SP15 at $p < 0.05$ and SP21 at $p < 0.07$. Among all the genes, SP17, developed for sequence similar to a gene coding for inorganic pyrophosphatase, showed the highest mean fold change at day 3 in BTx623, which was reversed with higher mean fold change in PI 607900 at day 5. However, this difference observed for SP17 was not significant ($p< 0.1$) between the two families. The pattern of SP21, a primer developed for a map kinase phosphatase gene, was similar to SP15. The gene specific primer, SP13, developed for a putative uncharacterized protein, did not exhibit differential expression and was down regulated at all time points. Hence, two of the genes, SP15 and SP21, showed statistical significance for differential expression due to family effect, while two other genes, SP13 and SP17, proved to be statistically non-significant for the observed family difference at $p< 0.1$. However, all four genes had a significant effect ($p<0.001$) due to time and gene expression for each gene varied with change in time.

Discussion

Over time, strategic deployment of greenbug resistant sorghum cultivars has helped to overcome the newly emerged biotypes. The breeding efforts have yielded new sources of resistance to biotype I (Andrews et al. 1993). Use of resistant cultivars as a core component of integrated pest management has shown promising, environmentally safe

and amiable results (Sharma and Ortiz 2002; Rooney 2004; Smith 2004). However, a vast number of potentially resistant lines need to be screened to be adopted into greenbug management programs which can be efficiently managed through marker-assisted selection (Huang 2011).

The classical experiments on PI 607900 showed this accession to be a promising resistant line with a possible role of two dominant genes acting through complimentary gene action (Wilde and Tuinstra 2000; Tuinstra et al. 2001). A worldwide collection of 40,000 sorghum germplasm accessions was evaluated for greenbug resistance, which resulted in identifying 21 resistant sources. Among these 21 lines, PI 607900 outperformed other lines with a damage rating of 1.1 (Huang 2011). Another study using AFLP diversity analysis, PI 607900 was genetically distant from other sources of resistance (Wu et al. 2006). Those preliminary works impelled us to use PI 607900 as a resistant source to identify the potential alleles conferring resistance to greenbug biotype I, because of its superior genetic and phenotypic performance compared to other sources.

Our mapping results using a F_2 population proved promising, since it harbored several candidate genes including a sequence similar to receptor-like *Xa21*-binding protein 3. We have avoided using terms like Immortalized F_2 population (IF_2) (Gardiner et al. 1993; Hua et al. 2003) or Advanced Intercrossed Lines (Darvasi and Soller 1995), as our procedure for developing the mapping population differed slightly. We employed a comparative QTL mapping approach to identify and locate QTLs for greenbug resistance between a F_2 population and a F_3 intercross population using genomic resources from Phytozome, a sorghum database. The present investigation involved an intercross mapping population derived from an earlier used F_2 mapping population. As our

investigation from F_2 study identified very closely linked markers to major loci for greenbug resistance on chromosome nine, which had a high density of markers, we were interested to see if the same region was confirming greenbug resistant alleles. Hence our interests were basically focused on detection of QTLs and their effects.

The concept of increasing recombination events by creating multiple meiosis in populations after F_2 by intercrossing for many generations was realized and utilized for QTL mapping in the last two decades (Darvasi and Soller 1995; Liu et al. 1996; Hua et al. 2003; Winkler et al. 2003). The idea of increasing recombination events was implemented in developing an intercross population using several random matings in F_3 lines. The increase in genetic length was not much of enhancement over our previous study, but we did see marginal enhancement on chromosome nine within the QTL region. We believe this is due to the limited number of individuals taken for intercrosses and one generation of random mating. Moreover, this confirms that the population size sampled for estimated gene effects has more impact than the number of markers and spacing between markers for increasing the resolving power of marker-QTL in a saturated map (Dravasi et al.1993; Darvasi and Soller1994). Furthermore, crossing was taken in F_3 lines which have reduced heterozygosity compared to the previous generation. The study by Falconer et al. 2006 showed that intermated populations do not always exhibit an expansion in map length. Our results include both expansion and shrinkage of distances between markers. We did not identify minor QTLs, which were otherwise detected in our earlier analysis. We found that major QTLs for greenbug resistance were consistent with respect to their map location on chromosome 9 which were attributable for a high phenotypic variation. This region flanked by Starssbnm 93-Starssbem 296, probably

harbors a candidate gene for greenbug resistance. Identification of two loci on chromosome 9 for four different traits was also reflected by their phenotypic correlations. We included only those significant QTLs in our final model which explained a high phenotypic variation and found that the QTL region was mapped to the same location as our previous study with less confidence interval for all traits measured. Moreover in this investigation, quantitative trait loci for all four traits for insect/greenbug resistance were found in the same cluster which is corroborated by other studies on insect resistance loci, shedding light on the concept of tight linkage and pleiotropy between correlated traits (Cai and Morishima 2002; Wu et al. 2007; Wu and Huang 2008; Satish et al. 2009). The recent availability of the complete sorghum genome sequence has made our efforts easy in harnessing likely candidate genes in the QTL regions (Paterson et al. 2009).

Due to limited resources and time, we chose to use four candidate genes for gene expression analysis. Our attempt was to find whether the chosen candidate genes showed a differential induction in response to greenbug attack. We could not make any conclusion from gene SP13, coding for a putative uncharacterized protein. We were more interested in knowing the expression pattern of SP15, a gene-specific primer for the sequence similar to a receptor-like Xa21-binding protein 3 (abbreviated as XB3), with an E3 ubiquitin ligase containing ankyrin repeat domain, that was shown to be required for the abundance of the Xa21 protein and for *Xa21*-mediated resistance (Wang et al. 2006). It was demonstrated that the XB3 protein interacts with Xa 21 by acting as its substrate and is required for its stability. *Xa 21* gene, which encodes a receptor-like kinase protein with a LRR motif in rice, is known to be involved in R gene-mediated resistance against races of *Xanthomonas oryzae* pv *oryzae* (Song et al. 1995). In our analysis the expression

of SP15 varied between the two parents, the expression was higher in the susceptible plants after infestation with greenbug. The expression level in the resistant plants increased gradually. This is in contrast to differential induction of the *XB3* gene, grouped one among the signal transduction genes, in resistant and susceptible melon plants upon aphid feeding (Samuel 2008). In our study, the observed expression pattern could be due to several factors involving sorghum-aphid interaction with high variation from environment, sample collection, aphid populations and nature of plant resistance. The mechanism of resistance expressed by PI 607900 toward greenbug attack are mainly antibiosis and tolerance (Wilde and Tuinstra 2000) which might be playing a critical role in manifestation of damage symptoms correlated with defense events. One more reason could be due to an interaction at the protein level, which might be different than at the transcript level. From our analysis, this gene might be grouped among those involved at the basal level of defense response in sorghum. Among the other three genes, SP21 followed a similar pattern of expression to SP15 and this might explain the reason map kinase phosphatases are involved in signal transduction events of sorghum defense response to greenbug attack along with receptor-like kinases. The two genes SP13 and SP17 were not significant in expression, with SP17 coding for inorganic pyrophosphatase that is likely to be involved in energy requirements (ATP synthesis) of cells coping with various events.

In conclusion, we identified a consistent region of the sorghum genome in two mapping studies. This region was located on chromosome 9 flanked by markers Starssbnm93-Starssbnm102. This region contained several candidate genes including receptor-like Xa21-binding protein 3 (XB3), which is known to increase R gene-mediated

disease resistance. The expression analysis revealed that these candidate genes are differentially regulated in response to greenbug attack on sorghum plants. However, further genetic analyses are required to confirm the precise role of these genes for greenbug resistance response in sorghum. The markers/QTLs identified in the study will have applications in MAS and map-based cloning experiments for the improvement of greenbug resistance in sorghum.

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Tables

Table 1 List of sequences developed for gene specific primers used in real-time PCR. The gene sequences were taken from Phytozome database for sorghum

Primer name	Forward sequence (5'-3')	Reverse sequence (5'-3')	Gene name/locus name	Tm	Expected size (bp)
SP13	TATCTACCT CGATGCCAA CC	GTAGCACCT CCCAAATCT CA	Putative uncharacterized protein. Sb09g001360 (Ref. Paterson et al. 2009)	51	176
SP15	AAGTTCATC AGCGAGCT AGACC	CTGACGACA CATCATCAA TGG	Sequence similar to Receptor-like Xa21-binding protein 3-like Sb09g001370 (Ref. Paterson et al. 2009)	54	178
SP17	ATTGATCAA GGAGAGGC AGAC	CAGAAAGT CGTTCACAG CAAC	Sequence similar to Putative inorganic pyrophosphatase Sb09g001530 (Ref. Paterson et al. 2009)	52	242
SP21	ACCGTCTAG TATTCGCAG GAC	GAAAACCTT GACAGGAA GAGC	Sequence similar to Map kinase phosphatase. Sb09g001660 (Ref. Paterson et al. 2009)	52	233

Table 2 Resistance response to greenbug damage among two parents and progenies from intercross population was measured at four different time points, 7 days (GDR07), 10 days (GDR10), 14 days (GDR14), and 21 days (GDR21) post-infestation using a 1 to 6 scale to estimate damage

Greenbug damage response	Parents		Progeny				
	Mean ± S.E BTx623	Mean ± S.E PI 607900	Difference	Mean ±S.E	Range		h^2 ± SE
GDR07	5.35 ± 0.13	1.15 ± 0.08	4.20***	2.31 ± 0.06	0.0	6.0	0.78 ± 0.02
GDR10	5.78 ± 0.08	1.65 ± 0.06	4.13***	3.42 ± 0.06	0.5	6.0	0.82 ± 0.01
GDR14	6.00 ± 0.00	2.06 ± 0.10	3.94***	4.28 ± 0.05	1.5	6.0	0.81 ± 0.02
GDR21	6.00 ± 0.00	3.06 ± 0.09	2.94***	5.09 ± 0.03	2.0	6.0	0.68 ± 0.03

*** significantly different at $p < 0.001$

h^2 -heritability on family mean basis derived from means of progenies of the intercross population

Table 3 The variance components of greenbug resistance measured for sorghum intercross progenies at four different time points, 7 days (GDR07), 10 days (GDR10), 14 days (GDR14), and 21 days (GDR21) post-infestation. Variance components expressed in percentage

Greenbug damage response	σ^2_g	σ^2_{ge}	σ^2_e
GDR07	53.89	22.12	21.65
GDR10	62.86	12.68	23.24
GDR14	62.21	10.95	25.49
GDR21	43.86	20.48	33.94

σ^2_g Variance associated with genotypes

σ^2_{ge} Variance associated with genotype X environment

σ^2_e Residual variance

Table 4 Phenotypic correlation coefficients observed for greenbug resistance measured at four different time points, $p < 0.0001$

Greenbug damage response	GDR07	GDR10	GDR14	GDR21
GDR07	1			
GDR10	0.93947	1		
GDR14	0.86013	0.92778	1	
GDR21	0.73276	0.79260	0.89664	1

Table 5 Quantitative trait loci (QTLs) for greenbug resistance identified in the intercross population along with their genetic effects and phenotypic variation (R^2)

Traits	Chromosome	Flanking marker	QTL/Position(cM)	LOD	Genetic effects Additive Dominance	R^2 (% effect)
GDR07	SBI09	Starsbem298-Starsbnm102	42.22 Qstsgrip09i	47.98	A -1.75 D -1.02	72.9
GDR10	SBI09	Starsbem298-Starsbnm102	42.22 Qstsgrip09i	54.99	A -1.90 D -0.93	79.7
GDR14	SBI09	Starsbnm93-Starsbem296	38.68 Qstsgrip09ii	50.36	A -1.65 D -0.17	80.9
GDR21	SBI09	Starsbnm93-Starsbem296	38.68 Qstsgrip09ii	41.05	A -1.00 D 0.23	73.3

Table 6 Two-way fixed factor F-statistics for each gene used in real-time PCR, showing effects due to family, time and family X time interactions

Type III Tests of Fixed Effects										
				SP13		SP15		SP17		SP21
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**	**F Value**	**Pr > F**	**F Value**	**Pr > F**	**F Value**	**Pr > F**
Family	1	8	3.02	0.1206	6.62**	0.0330	0.53	0.4867	4.32*	0.0713
Time	3	8	8.15	0.0081	57.17***	<.0001	47.58***	<.0001	27.14***	0.0002
Family X Time	3	8	0.86	0.5018	10.19**	0.0042	3.83*	0.0571	3.94*	0.0538

***** Significant at *P*< 0.001, ** Significant at *P*< 0.05, * Significant at *P*< 0.1**

Table 7 The raw mean delta Ct (Mean Dct) values for four candidate genes in two families (parents) of sorghum along with Standard Deviation (SD) and Standard Error (SE) at four different time points, 0 Day, 1 day, 3 days, and 5 days

Gene	Family	Time	Mean Dct	SD	SE
SP13	ODBTx623	0D	7.0050	0.31	0.22
SP13	1DBTx623	1D	8.0200	0.71	0.50
SP13	3DBTx623	3D	8.4025	1.13	0.80
SP13	5DBTx623	5D	8.9300	0.67	0.48
SP13	0DPI 607900	0D	7.0525	0.68	0.48
SP13	1DPI 607900	1D	8.9175	0.30	0.21
SP13	3DPI 607900	3D	9.6500	0.62	0.44
SP13	5DPI 607900	5D	9.0000	0.27	0.19
SP15	ODBTx623	0D	1.7300	0.42	0.30
SP15	1DBTx623	1D	2.0725	0.32	0.23
SP15	3DBTx623	3D	-0.1175	0.05	0.03
SP15	5DBTx623	5D	0.5250	0.09	0.07
SP15	0DPI 607900	0D	1.0150	0.24	0.17
SP15	1DPI 607900	1D	2.9925	0.03	0.02
SP15	3DPI 607900	3D	0.9325	0.30	0.21
SP15	5DPI 607900	5D	0.5950	0.27	0.19
SP17	ODBTx623	0D	-3.2000	0.55	0.39
SP17	1DBTx623	1D	-1.7875	0.69	0.49
SP17	3DBTx623	3D	-5.9875	0.12	0.08
SP17	5DBTx623	5D	-4.3600	0.98	0.70
SP17	0DPI 607900	0D	-3.1650	0.11	0.08
SP17	1DPI 607900	1D	-0.7375	0.51	0.36
SP17	3DPI 607900	3D	-4.9850	0.44	0.31
SP17	5DPI 607900	5D	-5.6350	0.46	0.33
SP21	ODBTx623	0D	-0.0125	0.67	0.47
SP21	1DBTx623	1D	1.8925	0.38	0.27
SP21	3DBTx623	3D	-1.9850	0.18	0.13
SP21	5DBTx623	5D	-1.3575	0.27	0.19
SP21	0DPI 607900	0D	-0.3750	0.12	0.09
SP21	1DPI 607900	1D	1.7250	0.42	0.30
SP21	3DPI 607900	3D	-0.1450	0.14	0.10
SP21	5DPI 607900	5D	-0.5450	1.07	0.76

Table 8 The overall mean delta Ct values for four candidate genes with associated Standard Deviation (SD) and Coefficient of Variation (CV)

Gene	FREQ	Mean_DCT	SD	CV
SP13	16	8.3721	1.03	12.38
SP15	16	1.2181	0.97	80.32
SP17	16	-3.7321	1.83	-49.17
SP21	16	-0.1003	1.35	-1350.35

Figures

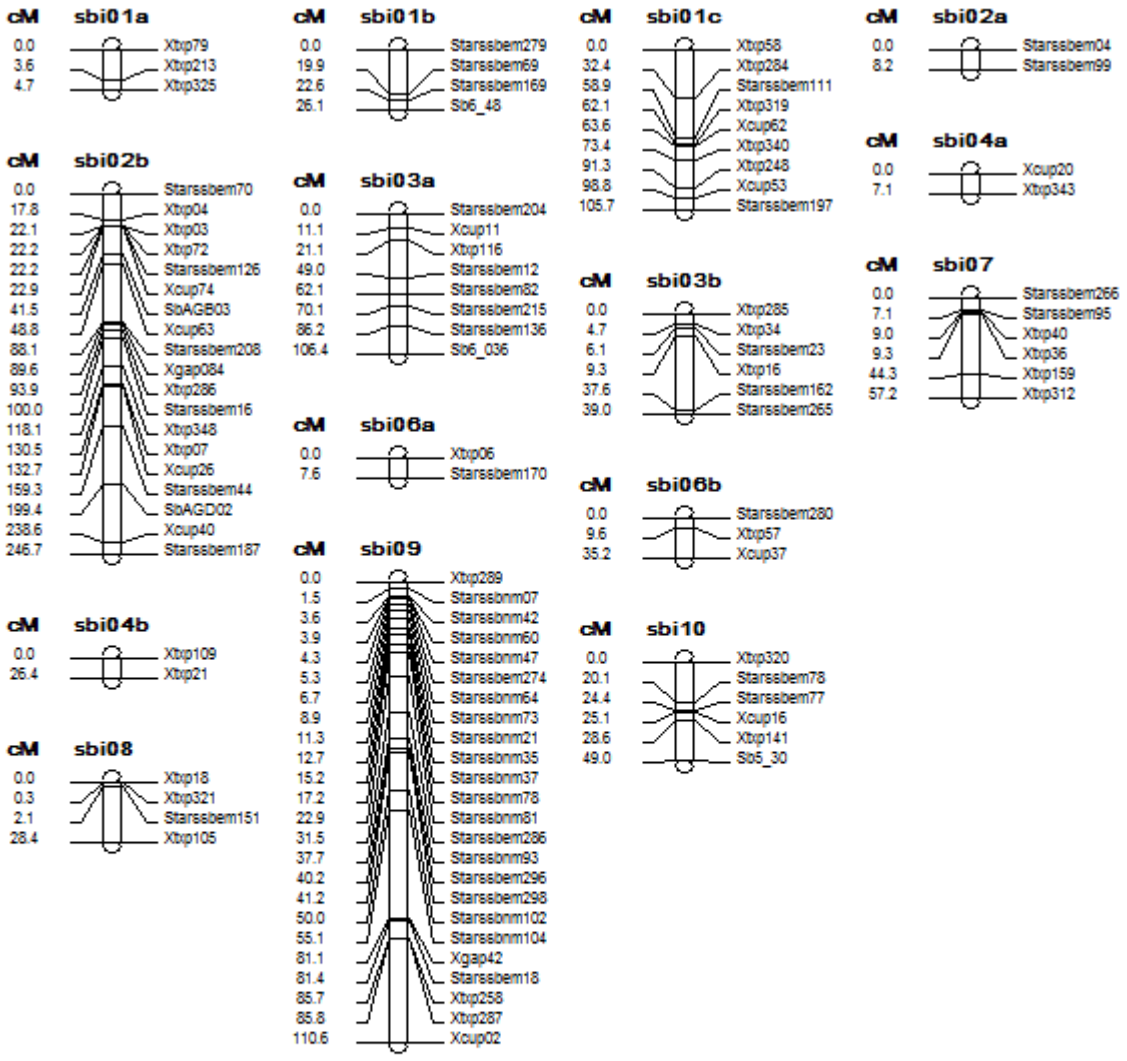


Fig.1 Linkage map constructed in intercross population of sorghum developed using F_3 progenies of BTx623 X PI 607900

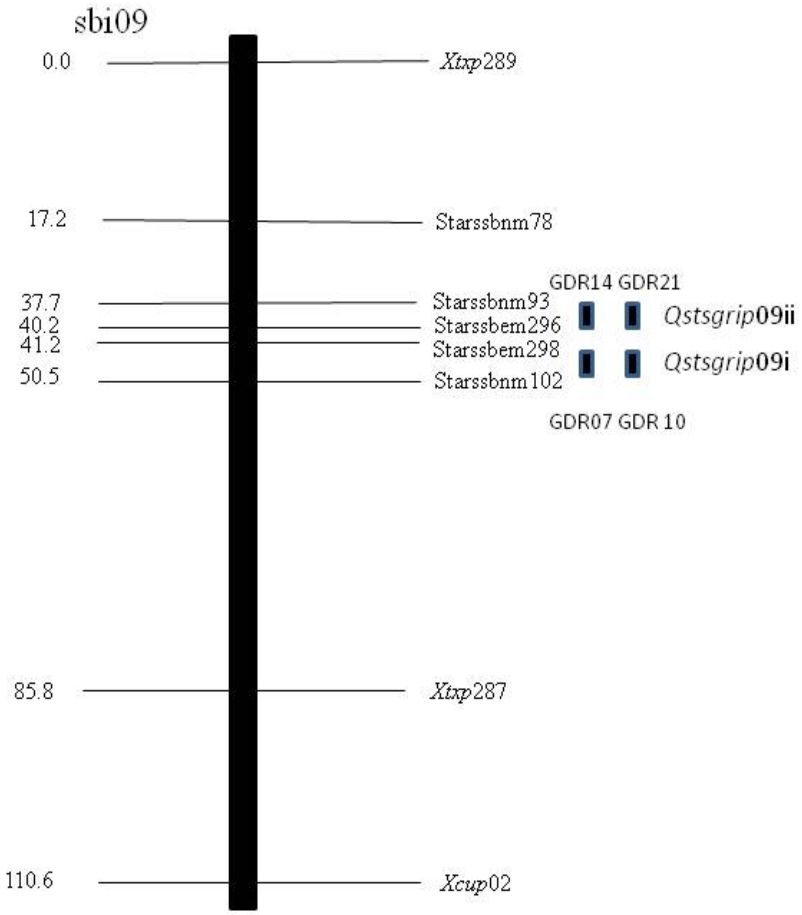
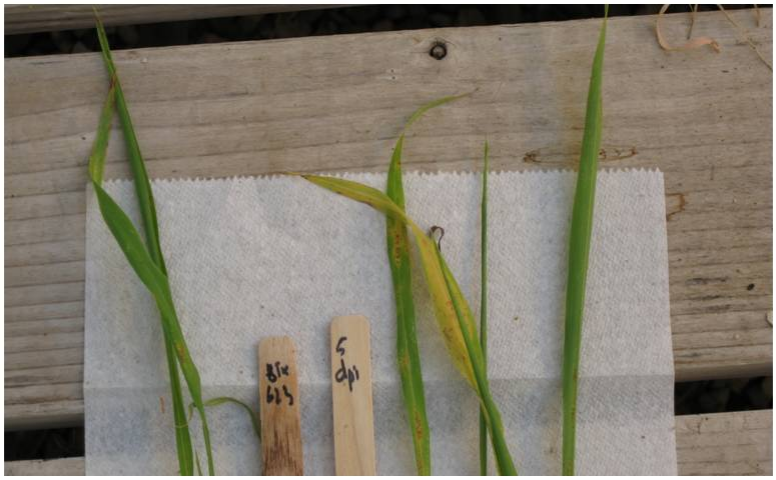


Fig. 2 Quantitative trait loci identified on sorghum chromosome 9 for four greenbug resistance traits measured at day 07 (GDR07), day 10 (GDR10), day 14 (GDR14) and day 21 (GDR21) in an intercross mapping population

a) BTx 623, susceptible parent collected at 5 days



b) PI 607900, resistant parent collected at 5 days

Fig. 3 Two families of sorghum showing expression symptoms of greenbug damage at 5 days post-infestation, a) Greenbug susceptible, BTx623 b) Greenbug resistant, PI 607900

Fig. 4a
Mean fold changes observed for the SP13 gene in two families of sorghum across four time points, 0day, 1 day, 3 days and 5 days

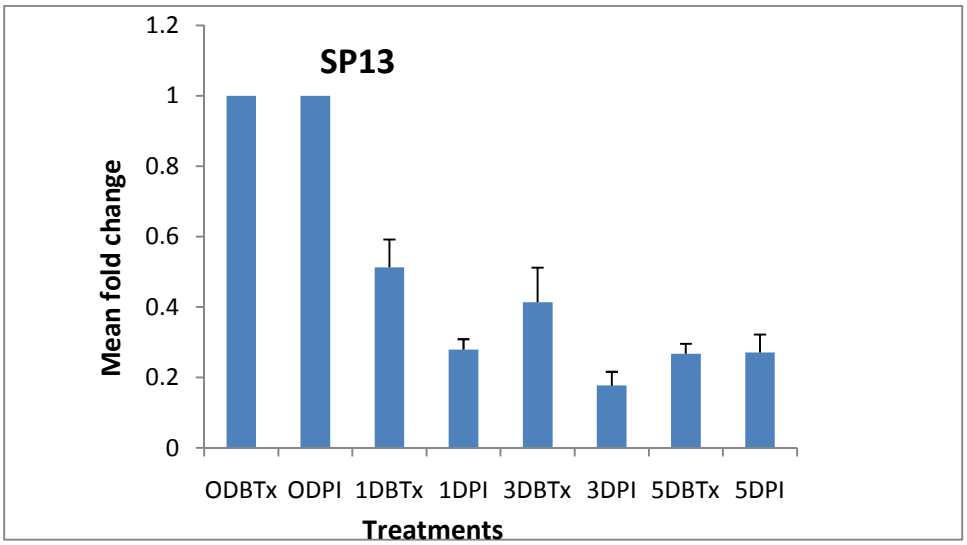


Fig. 4b
Mean fold changes observed for the SP15 gene in two families of sorghum across four time points, 0day, 1 day, 3 days and 5 days

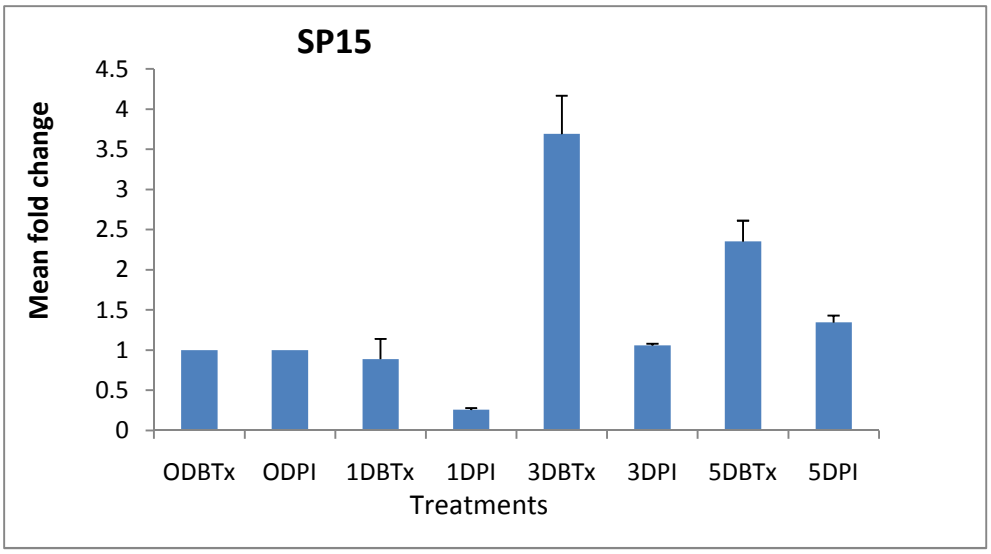


Fig. 4c
Mean fold changes observed for the SP17 gene in two families of sorghum across four time points, 0day, 1 day, 3 days and 5 days

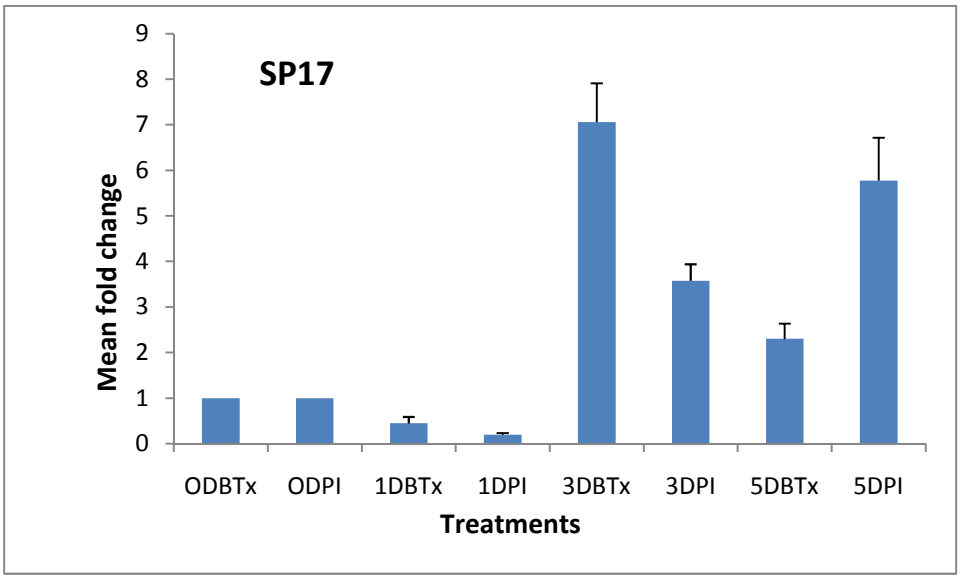


Fig. 4d
Mean fold changes observed for the SP21 gene in two families of sorghum across four time points, 0 day, 1 day, 3 days and 5 days

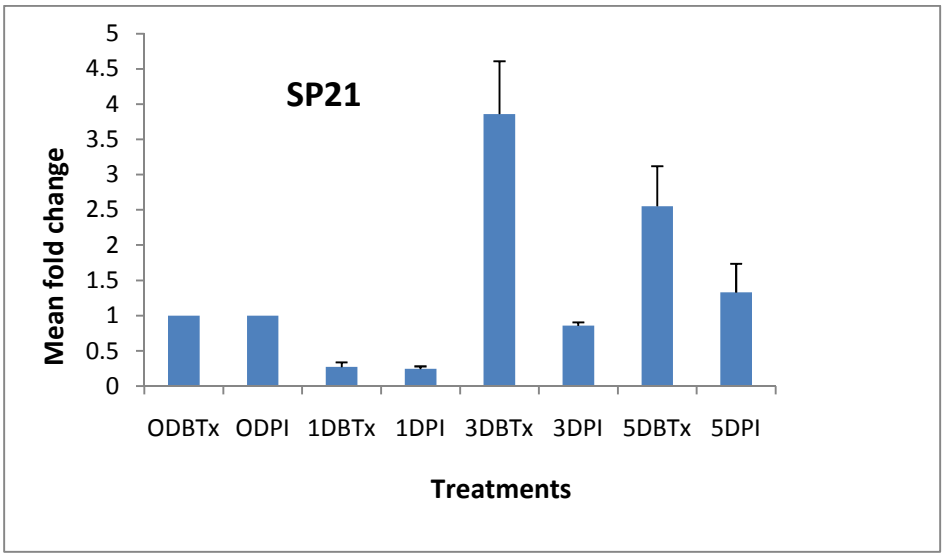


Fig. 4 Mean fold changes of each gene expression in two families of sorghum with their respective controls at four different time points: 0 day, 1 day, 3 days and 5 days. The data were normalized to actin and expressed as fold change. Mean fold changes represented are averages taken from values of two biological replications
a) SP13 b) SP15 c) SP17 d) SP21

VITA

Somashekhar M. Punnuri

Candidate for the Degree of

Doctor of Philosophy

Thesis: GENETIC MAPPING OF GREENBUG RESISTANCE LOCI IN SORGHUM [*Sorghum bicolor* (L.) Moench] & EXPRESSION ANALYSIS OF CANDIDATE GENES IN RESPONSE TO GREENBUG INFESTATION

Major Field: Plant Science

Biographical:

Education:

Completed the requirements for the Doctor of Philosophy in Plant Science at Oklahoma State University, Stillwater, Oklahoma in July, 2011.

Received Master of Science in Agriculture, Plant Biotechnology specialization at Institute of Agri-Biotechnology, University of Agricultural Sciences, Dharwad, Karnataka/India in 2004.

Received Bachelor of Science in Agriculture at ACD, University of Agricultural Sciences, Dharwad, Karnataka/India in 2001.

Experience: Teaching Assistant, Dept of Botany ,Oklahoma State University from Jan 2011 to May 2011. Research Assistant in Dr. Yinghua Huang's lab, USDA, ARS, Stillwater from Sept 2007 to Dec 2010, May 2011 to July 2011. Research Associate at Genetics and Plant breeding dept, UAS, Dharwad from Oct 2006 to July 2007. Agricultural Field Officer in Karnataka Bank Ltd. from June 2005 to March 2006. Junior Research fellow at Directorate of Rice Research Institute at Rajendra nagar, Hyderabad from Aug 2004 to May 2005.

Name: Somashekhar M. Punnuri Date of Degree: July, 2011

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: GENETIC MAPPING OF GREENBUG RESISTANCE LOCI IN SORGHUM [Sorghum bicolor (L.) Moench] & EXPRESSION ANALYSIS OF CANDIDATE GENES IN RESPONSE TO GREENBUG INFESTATION

Pages in Study: 94 Candidate for the Doctor of Philosophy

Major Field: Plant Science

Scope and Method of Study: Greenbug is a damaging pest of sorghum in the Great Plains of the United States. This study was taken with the objective to identify the genomic regions contributing resistance to greenbug biotype I using sorghum accession, PI 607900 as the resistance source. To accomplish this objective I undertook two projects involving three independent studies. These three studies involved linkage-based mapping and gene expression studies. The first study was conducted in an F_2 mapping population consisting of 371 individuals developed from a cross of BTx623 (susceptible to greenbug) by PI 607900 (resistant to greenbug) to identify QTLs contributing greenbug resistance in sorghum. The second study was performed to identify and confirm QTLs for greenbug resistance in an intercross population developed from a previously used F_2 mapping population. The third study was carried out to examine the differential expression of candidate genes induced by greenbug using real-time PCR experiments in two contrasting parental lines.

Findings and Conclusions: I found that two major loci for greenbug resistance were on sorghum chromosome 9 from two independent QTL mapping studies. The second QTL mapping project with an intercross population revealed potential candidate genes in a narrowed confidence interval compared to first project's QTL analysis with the F_2 population. These loci were mapped to a region on chromosome 9 flanked by markers Starssbnm 93-Starssbnm 102. The QTL mapping studies identified two novel loci for greenbug resistance using 48 newly developed nuclear and genic SSRs. The identified greenbug resistance loci were linked to a receptor-like kinase *Xa21*-binding protein 3, a gene known to increase *Xa21*-mediated resistance in rice. Relative quantification of gene expression in the two parental lines indicated that receptor–like kinase *Xa21*-binding protein 3 and map kinase phosphatase were differentially expressed upon greenbug infestation. The markers/QTLs identified in the study will have applications in MAS and map-based cloning experiments for the improvement of greenbug resistance in sorghum.

ADVISER'S APPROVAL: Dr. Yinghua Huang