QTL MAPPING OF PHYTOPHTHORA ROOT ROT RESISTANCE AND APHID RESISTANCE IN SOYBEAN

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ABSTRACT

QTL MAPPING OF PHYTOPHTHORA ROOT ROT RESISTANCE AND APHID RESISTANCE IN SOYBEAN

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Soybean [Glycine max (L.) Merr.] is one of the most important crops worldwide. As the largest soybean producer, the United States produces $40.2 billion US dollar worth soybeans in 2011. Biotic stresses such as soybean cyst nematode (SCN), Phytophthora root rot (PRR), sudden death syndromes (SDS) and soybean aphids are threatening soybean production and plant health. Three independent projects were performed to identify genetic resistance against PRR, caused by *Phytophthora sojae*; to identify genetic resistance against soybean aphid, *Aphis glycines* Matsumura; and to develop breeder-friendly simple sequence repeat (SSR) and single nucleotide polymorphism (SNP) markers to facilitate marker-assisted selection (MAS) in breeding for PRR resistance and aphid resistance.

In the first project, a major locus *Rps*1 was identified for resistance to PRR in Michigan elite soybean E00003 (*Glycine max*) through a genetic mapping approach. A bi-parental mapping population was evaluated in the greenhouse for PRR resistance against *P. sojae* races 1, 4, and 7, in 2009 and 2010 using a modified rice grain inoculation method. The heritability of the PRR resistance ranged from 83% to 94%. A major locus contributing 50% to 76% of the phenotypic variation was detected within a 3 cM interval in the *Rps*1 region. Based on the specific responses to the tested races, this locus was determined to be *Rps*1k. Two SSRs and three SNPs were found located within the predicted functional gene and are highly associated with PRR resistance in the mapping population.

In the second project, two major quantitative trait loci (QTLs) for soybean aphid resistance were detected and confirmed in E08934, derived from a wild type soybean, *Glycine soja*. Aphid resistance phenotyping was conducted one season in the greenhouse and three seasons in field cages. The broad-sense heritability of aphid resistance from field trials was 0.84. After genotyping with SNPs and SSRs, two major QTLs were detected ($P < 0.001$) in intervals of 13.5 cM between Sat_382 and Satt455 on Chr. 8, and 3.5 cM between Satt693 and Sat_370 on Chr. 16. The locus on Chr. 8 explained 40.8% of the phenotypic variation in the greenhouse trial, and 46.4, 19.5 and 39.1% of the 2009, 2010 and 2011 field trials, respectively. The locus on Chr. 16 explained 12.5 to 22.9% of the phenotypic variation. Further, these two loci were both confirmed in a validation population. The no-choice test indicated both loci confer antibiotic resistant to aphids. The novel locus on Chr. 8 (LG A2) is denoted as *Rag6,* since it showed significant partially dominant effect in the validation population ($P < 0.05$).

In the third project, nearly 4,000 F_2 plants from bi-parental populations were employed to fine map partially dominant locus *Rag*3 derived from PI 567543C. A major locus explaining 44.7% of the phenotype variance was detected in QTL analysis of 376 F_2 lines and confirmed as *Rag*3 between markers MSUSNP16-10 (Gm16_6262227) and MSUSNP16-12 (Gm16_6423098) on chr.16. After genotyping all 3802 F_2 progeny and 983 F_3 progeny of 102 F_2 recombinants, two F_3 recombinants with crossovers between 6.26 and 6.47 Mbp were identified and confirmed according to their F_4 progeny. *Rag*3 was fine mapped to 207 Kbp between MSUSNP16-10 (Gm16_6262227) and SNP at Gm16_6469551.

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CHAPTER 1

LITERATURE REVIEW

SOYBEAN PRODUCTION

Soybean (*Glycine max*) is a legume species native to East Asia (Hymowitz, 2008). Ever since the cultivation of soybean, its food products: bean curd (tofu), soybean milk, soy sauce and soybean paste have always been immensely popular among East Asian countries, such as China, Japan and Korea. As the origin of soybean cultivation, China was the largest soybean producer and exporter before mid-20th century (Qiu and Chang, 2010). In 1765, soybean was first introduced to the US by a sailor Samuel Bowen (Brachfeld and Mary, 2007). In the 1950s, the United States became and still is the largest soybean-producing country in the world, followed by Brazil, Argentina, China, India and other countries (FAO, 2008). In 2010, 258.9 million metric tons of soybeans were produced worldwide. Ninety-one million metric tons were produced in the US, which is 35% of the world soybean production, followed by Brazil 27%, Argentina 19% and China 6% (SoyStats, 2011).

Soybean has become one of the most valuable crops worldwide not only for human consumptions, but also for its protein and oil content for livestock feed and biofuel (Masuda and Goldsmith, 2009). The rapidly growing demand of animal-based diet in developing countries leads to increased demand of soybean production as livestock feed. As an oilseed, soybean can be used to extract methyl esters of fatty acids (FAMEs) to produce biodiesel with basic catalyst (Fabbri et al., 2007). Biodiesel is recommended to help reduce the emissions of greenhouse gases, and soybean has a smaller environmental impact for biofuel production than sunflower seed (Requena et al., 2011).

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BIOTIC STRESSES-DISEASES AND INSECTS

Like many other field crops, soybean production is always under the threats of biotic stresses, such as diseases and insects (Graham and Vance, 2003). Up to 30% of the world crop yield is reduced by these factors, and the number may be even larger in developing countries due to virus transmission by insect pests (Christou and Twyman, 2004). At least 10% of the global food production loss is caused by plant diseases (FAO, 2000). For example, common bean (*Phaseolus vulgaris*) losses to root rot are estimated as $4 million year^{-1} in the State of Minnesota alone (Graham and Vance, 2003). The yield loss in soybean production to diseases increased from 11% worldwide in 1994 to 23% in 2003 (Hartman et al., 1999; Oerke, 2006). Eleven percent of the loss was due to parasitic bacteria and fungi, 1% to viruses and 11% to animal pests including nematodes (Hartman and Hill, 2010).

According to Wrather and Koenning (2011), a total of 168 million t of soybean yield was lost due to diseases from 1996 to 2010. In the report, soybean cyst nematode (SCN, *Heterodera glycines* Ichinohe) ranks number one in most devastating diseases, causing more than 61.5 million t soybean yield loss from 1996 to 2010. Phytophthora root rot (PRR, *Phytophthora sojae*), seedling diseases and sudden death syndrome (SDS, *Fusarium solani* f. sp. *glycines*) ranked second to fourth in total yield reduction from 1996 to 2010. The yield loss caused by Phytophthora root rot was estimated as much as 18 million t. Seedling disease caused 14 million t of yield loss. Weather conditions played a critical role in seedling diseases reducing yield in Illinois, Kansas, Minnesota, North Dakota, and Ohio (Wrather and Koenning, 2006). SDS affected soybean production most in Arkansas, Iowa, Illinois, Indiana, Minnesota, and Tennessee,

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and was especially severe in Illinois and Indiana. The total yield loss caused by SDS was over 12 million t. Damage caused by Charcoal root rot (*Macrophomina phaseolina*) and Sclerotinia stem rot (*Sclerotinia sclerotiorum*) varied significantly between years, due to changes in weather and pathogens. Rust (*Phakopsora pachyrhizi*) was first confirmed on soybean in nine states in 2004: Alabama, Arkansas, Florida, Georgia, Louisiana, Missouri, Mississippi, Tennessee, and South Carolina (Wrather and Koenning, 2006).

Insect pests are another category threatening soybean production. The ecology and management of insect pests in soybean has been reviewed in literatures since 1970 (Altieri and Whitcomb, 1980). Based on their feeding guild, soybean insect pests are categorized into three classes: defoliators (Lepidoptera, Coleoptera, Orthoptera), phloem feeders (Hemiptera) and seed feeders (Coleoptera, Diptera) (O'Neal and Johnson, 2010). Defoliators have chewing mouthparts that can either remove leaf area or destroy the leaf surface, such as grasshoppers (Orthoptera), beetles (Coleoptera), larva or caterpillars of moths and butterflies (Lepidoptera) (Singh, 2010). In spite of yield reduction, insects can pass diseases to their plant host through their feeding habits, for example, grasshoppers is known to transmit *Tobacco ring-spot virus* into soybeans (Dunleavy, 1957).

The phloem feeders, such as the three-cornered alfalfa hopper (*Spissistilus festinus*), sweet-potato whitefly (*Bemisia tabaci*) and soybean aphid (*Aphis glycines*) from the order Hemiptera significantly impact on soybean production (O'Neal and Johnson, 2010). These pests have piercing-sucking mouthparts that enter vascular tissue, consume phloem and excrete sugar content. Besides, these phloem feeders can also transmit viruses to the plant, such as *Tobacco ring-spot virus*, *soybean mosaic virus* and *soybean dwarf virus* (Clark and Perry, 2002; Kloepper et al., 2004; Morales and Anderson, 2001; Pedersen et al., 2007).

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Other insect pests in the category of seed, pod, stem and root feeders, can cause significant yield loss as well. The pentatomid stinkbugs such as *Nezara viridula* also have piercing-sucking mouthparts that can cause seed decay and severe abortion of pods leading to severe late-growth stage damage and reduction in soybean yield (O'Neal and Johnson, 2010). Just like other insect pests, stinkbugs can transmit diseases like yeast spot disease (Kimura et al., 2008a; Kimura et al., 2008b).

MANAGEMENT OF PHYTOPHTHORA ROOT ROT AND SOYBEAN APHIDS

Phytophthora root rot caused by the oomycete *Phytophthora sojae* can occur at any soybean growth stage. It is the second most devastating disease in soybean production. Yield loss caused by this disease in the US was estimated at 18 million t from 1996 to 2010. Severe damage of soybean yield occurred mostly in the North Central states, such as Illinois, Indiana, Minnesota, Missouri and Ohio, and southern states like North Carolina, Louisiana, Kentucky and Oklahoma (Wrather and Koenning, 2006; Wrather and Koenning, 2011).

As a soil-borne pathogen, *Phytophthora sojae* infects host plants through the root system when the soil moisture favors the disease development (Ho, 1969). For disease management of the monocyclic soil-borne disease caused by *Phytophthora sojae,* avoiding or reducing the amount or efficacy of primary inoculum is of considerable importance (Appiah et al., 2004; Smilde et al., 1996). Knowing the history of the soil and planting with clean certificated seeds on well-drained or raised beds in the field with clean irrigation systems and sanitation equipment are good practices to reduce disease incidence (Cadle-Davidson and Gray, 2006). Using fungicides

at the first appearance of disease and rotating the types of fungicides used could be effective

controlling PRR but with increased input cost (Ando and Grumet, 2006; Barbetti et al., 2007;

Olanya et al., 2006). Crop rotation with non-host crops is also crucial for soil-borne disease

management (Ruppel et al., 1988). The management of PRR has been studied when the pathogen

is present (Dorrance et al., 2009; Rehm and Stienstra, 1993; Zhang and Xue, 2010). In Rehm and

Stienstra's study (1993), they investigated whether management practices such as variety

selection, fungicide use and potash fertilization could reduce damage in areas where PRR was

known to be a problem. In conclusion, they found that yield loss was most severe when a

susceptible soybean cultivar was grown, and alternatively using a combination of the chemical

Ridomil and a resistant soybean variety can reduce the PRR severity (Rehm and Stienstra, 1993).

Application of chemical fungicides can be quite expensive. The residuals of harmful chemicals

may pollute natural environments (Vanderborght et al., 2011) and kill beneficial insects (Meikle

et al., 2012) or animals (Engelhaupt, 2008). In addition, there is a risk of "super-pesticide-

resistant" pathogen development induced by high fungicide pressure (Cheatham et al., 2009;

Gressel, 2009). Selecting genetically resistant cultivars has always been suggested as the most

prominent strategy to manage PRR (Baker and Cook, 1974; Dorrance et al., 2009; Plank, 1963).

The soybean aphid (*Aphis glycines* Matsumura) is considered an invasive species into the

Americas from Asia (Kogan and Turnipseed, 1987). As mentioned earlier, *Aphis glycines*

Matsumura is classified in the order Hemiptera with typical piercing-sucking mouthparts that

feed on plant sap leading to severe water and nutrient deficiency (O'Neal and Johnson, 2010).

Since the first found in Wisconsin in July of 2000, soybean aphids have made a significant

impact on soybean production in the United States. Before the aphid was a problem, few

insecticides had been needed in the twelve north-central states (Fernandez-Cornejo and Jans,

1999; Ragsdale et al., 2011). With the presence and rapid outbreak of soybean aphid across these soybean growing states, the insecticide application has increased from 4 to 14 M ha year^{-1} (O'Neal and Johnson, 2010). It is challenging to control aphids with effective management practices and at the same time minimizing the risk of insecticide resistance (Lawrence, 2009; O'Neal and Johnson, 2010). As the first commercial insecticide, neonicotinoids have been widely used against phloem and leaf feeding insect pests, such as aphids, whiteflies and potato beetles (Jeschke and Nauen, 2008). The compounds can be used as seed treatments or a foliar formulation (Buchholz and Nauen, 2002; Elzen, 2001; Mukherjee and Gopal, 2000; Weichel and Nauen, 2004). However, a recent study on insecticidal seed treatments for soybean aphids suggested that insecticidal seed treatments hardly benefit soybean production of the Northern Great Plains (Seagraves and Lundgren, 2012). Even with multiple spray treatments, the insecticide application timing is crucial to control soybean aphids. When population density is high, it is most effective to apply at the R2 and R3 stages (Myers et al., 2005). In addition to limited improvement on aphid management and the massive cost of practical inputs, insecticides can leave traceable residues that pollute the environment and harm beneficial insects and natural enemies (Derksen et al., 2008; Ohnesorg et al., 2009; Southwick et al., 2004). Therefore, integrated pest management (IPM) has been suggested as the best solution to control soybean aphids (Hodgson et al., 2004; Irwin, 1999; Johnson et al., 2009; Ragsdale et al., 2011; Rosenheim et al., 2011; Song and Swinton, 2009; Wiarda et al., 2012). To developing robust and sustainable IPM practices, breeding and releasing host plant resistant cultivars can be a remarkably effective (Diaz-Silveira and Herrera, 1998; Kaster and Gray, 2005; Smith and Clement, 2012; Soleimannejad et al., 2010; Vieira et al., 2011; Wiarda et al., 2012).

QTL MAPPING, FINE MAPPING AND MARKER-ASSISTED SELECTION

Genomic regions that associate with quantitative traits are known as quantitative trait loci (QTLs). Crop yield, seed quality, oil or protein content and many disease resistance traits are superb examples of quantitative traits with continuous distribution of trait values. QTL mapping studies have made remarkable progress by taking advantage of molecular marker development since the 1980s. The marker types include: amplified fragment length polymorphism (AFLP) markers, simple sequence repeat (SSR) markers and single nucleotide polymorphism (SNP) markers and so on (Collard et al., 2005; Gupta et al., 2001; Hollis and Hindley, 1988; Lander and Botstein, 1989; Miles and Wayne, 2008; Paterson et al., 1988; St Clair, 2010; Williams et al., 1991; Young, 1996). There are two main strategies for QTL mapping, linkage-based mapping and association mapping (Donnelly and Wiuf, 1999; Lander and Botstein, 1989; Manly and Olson, 1999; March, 1999; Shifman and Darvasi, 2005; Sterken et al., 2012).

Linkage-based mapping utilizes a genetic linkage map as the backbone for QTL analysis (Gardiner et al., 1993). It estimates the map distance between marker and QTL by the recombination fraction (Lander and Botstein, 1989; Wu et al., 2002). In a typical linkage-based QTL mapping experiment, a mapping population developed by crossing two inbred lines is genotyped to construct a genetic linkage map. The position of quantitative trait loci can be located on the genetic linkage map (Lander and Botstein, 1989). Further, epistasis between QTLs and interactions between QTL and environmental factors can be addressed and better understood (Doerge, 2002; Mao and Da, 2004; Purcell and Sham, 2000). However, due to the limited

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recombination events in meiosis during population development, traditional linkage mapping has lower resolution of QTLs (McMullen et al., 2009).

Association mapping, commonly referred as "linkage disequilibrium mapping", does not require any linkage map. Instead, it takes advantage of historical recombination events between the marker and QTL since such nonrandom co-segregation (linkage disequilibrium) was ever introduced into a population. With more opportunities for recombination, association mapping provides higher resolution of QTLs (Rabinowitz, 1997; Risch and Merikangas, 1996; Wu et al., 2002; Xiong and Guo, 1997). However, association mapping has several bottleneck issues, such as high false discovery rate, population structure, rare variants with low allele frequency and difficulty in distinguishing strong disequilibrium and loose linkage from weak disequilibrium and tight linkage (Brachi et al., 2010; Donnelly and Wiuf, 1999; Hewitt, 2008; March, 1999; Mezmouk et al., 2011; Qin et al., 2010; Sorkheh et al., 2008; Sterken et al., 2012; Weir, 2008; Whittaker et al., 2000). Therefore, linkage-based mapping still plays a pivotal role in genetic mapping of high-heritability disease and insect resistance loci with higher power and low false discovery rate (Duan et al., 2007; Grisi et al., 2007; Sabatti et al., 2003; Stoeckli et al., 2009). Some particularly stimulating research has been proposed to combine the two methods. Wu et al. (2002) proposed a new mapping strategy combining the strength of both methods in the EM-implemented maximum-likelihood framework for an outcross population. More recently, Yu et al. (2008) developed a nested association mapping population in maize to utilize the advantages for both methods to decode the maize genome. The variety, B73 was crossed to 25 diverse founders to develop 5,000 immortal genotypes from genome reshuffling (Yu et al., 2008).

Prosperous progress in DNA-based molecular markers and QTL mapping is making marker-assisted selection (MAS) more and more popular and effective (Bernardo, 2008; Chaisan

et al., 2012; Collard et al., 2005). For example, MAS has been used for development of disease resistance in bean and cowpea (Kelly et al., 2003), resistance for soybean cyst nematode (Diers, 2005), and other disease resistance and agronomic traits (Ha et al., 2007; Kumar et al., 2011; Maroof et al., 2008; Sebastian et al., 2010; Zhu and Sun, 2006). However, these successful examples are far fewer than the number of QTLs that have been identified in the literature (Bernardo, 2008). A potential pitfall for MAS can be undesired recombination between markers and functional genes. Therefore, to better facilitate MAS, fine mapping of QTL is needed to provide precise location of QTL with high resolution, closely linked molecular markers, and even markers that locate exactly within functional genes. There are usually two approaches for fine mapping, traditional linkage mapping with a large set of progeny from bi-parental crosses, and association mapping.

RESISTANCE LOCI FOR PHYTOPHTHORA AND APHIDS AS WELL AS OBJECTIVES OF THE RESEARCH CONDUCTED IN THIS DISSERTATION

To date, nine loci with 15 alleles (*Rps*1a, b, c, d, k, *Rps*2, *Rps*3a, b, c, *Rps*4, *Rps*5, *Rps*6, *Rps*7, *Rps*8 and *Rps*Yu25) have been reported confer race-specific resistance to *Phytophthora sojae* (Anderson and Buzzell, 1992; Athow and Laviolette, 1982; Athow et al., 1980; Burnham et al., 2003b; Buzzell and Anderson, 1992; Demirbas et al., 2001; Diers et al., 1992; Gao and Bhattacharyya, 2008; Gordon et al., 2006; Kilen et al., 1974; Mueller et al., 1978; Sandhu et al., 2005; Sun et al., 2011; Weng et al., 2001). The locations of these loci were updated according to Cornelius et al. (2005) and summarized in Table 1.1. In Chapter 2, the research objectives were

to identify PRR resistance in an advanced breeding line E00003 from Michigan State University and to develop breeder-friendly molecular markers to facilitate MAS for PRR.

For soybean aphids, five quantitative resistance loci with seven alleles have been published (*Rag*1, *rag*1, *Rag*2, *Rag*3, *rag*3, *rag*4 and *Rag*5) underlying aphid resistance (Hill et al., 2006a; Hill et al., 2006b; Hill et al., 2009; Jun et al., 2012; Li et al., 2007; Mian et al., 2008a; Mian et al., 2008b; Zhang et al., 2009; Zhang et al., 2010). Their locations are summarized in Table 1.2. The objectives of the study in Chapter 3 were to identify aphid resistance loci of E08934 and develop molecular markers for MAS. In Chapter 4, the research aims were to fine map the partially dominant allele *Rag*3, seeking tightly linked SNP markers for use in MAS and for further genetic studies such as cloning and function studies of this locus.

Table 1.1. Quantitative resistance loci conferring race-specific resistance to Phytophthora root rot according to soybean consensus map (Song et al., 2004). Table contents were updated according to Cornelius et al. (2005).

Rps loci	Chr.[†]/Linkage Group	Flanking markers (cM)	Reference
*Rps*1	3/N	Satt159─Satt009 (27.1 ─ 28.5)	Weng et al. 2001
*Rps*2	16/J	A233_1─A724_1 (83.2 ─ 84.9)	Diers et al. 1992; Demirbas et al. 2001
*Rps*3	13/F	A757_1─R045_1 (63.1 ─ 70.1)	Diers et al. 1992; Demirbas et al. 2002
*Rps*4	18/G	A586_2 (111.2)	Diers et al. 1992; Demirbas et al. 2003
*Rps*5	18/G[††]	T005_2 (81.5)	Diers et al. 1992; Demirbas et al. 2004
*Rps*6	18/G	Not defined	Demirbas et al. 2001
*Rps*7	3/N	Satt009─Satt125 (28.5 ─ 40.6)	Weng et al. 2001
*Rps*8	8/A2	Sat_040─Satt228 (118.6 ─ 154.1)	Burnham et al. 2003b
*Rps*Yu25	3/N	Sat_186─Satt530 (30.1 ─ 32.8)	Sun et al. 2011

[†] Chromosome;

[††] Possibly (Diers et al. 1992; Demirbas et al. 2004).

Table 1.2. Quantitative resistance loci to soybean aphids based on soybean consensus map (Song et al., 2004).

Aphid resistance genes	Chr.[†]/Linkage Group	Flanking markers (cM)	Reference
*Rag*1/*rag*1	7/M	Satt540─Satt435 (35.9─38.9)	Hill et al., 2006a; Hill et al., 2006b; Li et al., 2007
*Rag*2	13/F	Satt114─Satt510 (63.7─71.4)	Mian et al., 2008b; Hill et al., 2009
*Rag*3/*rag*3	16/J	Sat_339─Satt414 (28.0─37.0)	Zhang et al., 2010; unpublished data
*rag*4	13/F	Satt649─Satt348 (5.4─15.3)	Zhang et al., 2009
*Rag*5	13/F	BARC-060107-16382[††]	Jun et al., 2012

[†] Chromosome;
[††] Single nucleotide polymorphism (SNP) marker.

CHAPTER 2

PHYTOPHTHORA ROOT ROT RESISTANCE IN SOYBEAN E00003

ABSTRACT

Phytophthora root rot (PRR), caused by the oomycete *Phytophthora sojae*, is a devastating disease in soybean production. Using resistant cultivars has been suggested as the best solution for disease management. Michigan elite soybean E00003 is resistant to *P. sojae* and has been used as a PRR resistance source in breeding of new cultivars. However, the genetic control of PRR resistance in E00003 is unknown. To facilitate marker-assisted selection (MAS), the PRR resistance loci in E00003 and their map locations need to be determined. In this study, a genetic mapping approach was employed to identify major PRR resistant loci in E00003. The mapping population consists of 240 F_4-derived lines developed by crossing E00003 with the *P. sojae* susceptible line PI 567543C. In 2009 and 2010, the mapping population was evaluated in the greenhouse for PRR resistance against *P. sojae* races 1, 4, and 7, using a modified rice grain inoculation method. The population was genotyped with single sequence repeat (SSR) and single nucleotide polymorphism (SNP) markers. The heritability of resistance in the population ranged from 83% to 94%. A major locus, contributing 50% to 76% of the phenotypic variation, was mapped within a 3 cM interval in the *Rps*1 region. Based on the specific responses to the tested races, this locus was determined to be *Rps*1k. To pinpoint the functional gene, the interval was further saturated with more BARCSOY SSRs and SNPs with TaqMan® assays. Two SSRs and three SNPs were found located within the predicted functional gene; these are highly associated with PRR resistance in the mapping population. These breeder-friendly molecular markers can be used to improve MAS for PRR resistance.

INTRODUCTION

Phytophthora root rot (PRR), caused by *Phytophthora sojae*, is the second most destructive disease of soybean [*Glycine max* (L.) Merr.] in the United States (Wrather and Koenning, 2006). Significant disease epidemics have occurred in the North Central states, such as Illinois, Indiana, Minnesota, Missouri and Ohio, and also in the southern states such as North Carolina, Louisiana, Kentucky and Oklahoma (Wrather and Koenning, 2006). The total estimated loss in U. S. soybean yield due to the disease was over 18 million t from 1996 to 2010 (Wrather and Koenning, 2011), equivalent to 7 to 8 billion U. S. dollars (O'Brien, 2010). Yield loss can vary with weather conditions. In Ohio, for instance, yield losses averaged 11% in years with rainy springs and 8% in years with normal spring precipitation (Dorrance and Mills, 2009).

Phytophthora sojae infects soybeans at any growth stage primarily via the root system. High soil moisture favors disease development since *P. sojae* zoospores are only produced in saturated soil (Ho, 1969). The disease can only be partially managed using methods such as cultural practices and seed treatments with fungicides (Dorrance and Dennis, 2009; Schmitthenner, 1985). Integrated management strategies that combine host resistance with seed treatments were studied by Dorrance et al. (2009), who concluded that selecting resistant cultivars of the greatest importance.

Based on the results of six decades of research, both partial and race-specific resistances to *P. sojae* have been identified (Burnham et al., 2003a; Dorrance et al., 2004). Nine loci, with a total of 15 alleles, have been reported responsible for race-specific resistance (Anderson and Buzzell, 1992; Athow and Laviolette, 1982; Athow et al., 1980; Burnham et al., 2003b; Buzzell and Anderson, 1992; Demirbas et al., 2001; Diers et al., 1992; Gao and Bhattacharyya, 2008;

Gordon et al., 2006; Kilen et al., 1974; Mueller et al., 1978; Sandhu et al., 2005; Sun et al., 2011; Weng et al., 2001).

A high yielding Michigan soybean line, E00003, was found resistant to *P. sojae* races 4 and 7 in the Uniform Soybean Tests in Northern States 2002 (Crochet, 2002). There is insufficient evidence to trace the ancestor that contributed the PRR resistance to this line, thus it is unknown whether it carries any new PRR resistance genes or some specific alleles at existing resistance loci. The objective of this study was to characterize PRR resistance in E00003, and to develop breeder-friendly markers to facilitate marker-assisted selection.

MATERIALS AND METHODS

Genetic mapping strategy was applied to identify PRR resistance loci in E00003 genome. Phenotypic data were obtained from greenhouse trials, and genotypic data were obtained with SSRs and SNPs. Resistance loci suggested in the literature were first tested with SSRs in those regions.

Mapping population

The genetic mapping population consists of 240 F_4-derived lines using the single seed descent method (Roy, 1973) from the cross E00003 $\times$ PI 567543C. E00003 is resistant to *P. sojae*, whereas PI 567543C (Mensah et al., 2005), from Shandong Province in China, and is susceptible to *P. sojae*.

Evaluation for *Phytophthora sojae* resistance

Three *P. sojae* races, 1, 4, and 7, were used to evaluate the mapping population and its two parents in the MSU Plant Science Greenhouses in a total of six trials in 2009 and 2010. In each pathogen-inoculated trial, 12 seeds from each inbred line as one replicate, plus 36 seeds from each parent as three replicates, were planted in Baccto$^{®}$ soil mix (Michigan Peat Company, Houston, Taxes). The pathogen isolates were kindly provided by Dr. Anna Dorrance from the Department of Plant Pathology at Ohio State University. The greenhouse was maintained at 26/15 ℃ day/night temperature, and sodium vapor lights were used to supplement light intensity during the day (14 h).

For a higher throughput evaluation, the rice grain inoculation method was adapted, originally developed for evaluation of tree species for phytophthora resistance (Holmes and Benson, 1994). As used by Holmes and Benson, the rice inoculum were mixed well with sand, incorporated into a peat-vermiculite medium and sprinkled on the surface of a tray containing tree seeds. The method was modified by burying three rice grains together with one soybean seed in soil, providing the most appropriate disease pressure to differentiate PRR resistance level in our preliminary tests. This method allows the pathogen to penetrate the plant root naturally, mimicking natural infection for this soil-borne disease. In a 250-mL flask, 25 g white long-grain rice and 20 mL distilled water were mixed thoroughly. The flask was covered with double-layered aluminum foil and autoclaved for 40 min twice in 24 h. After cooling, the rice grains in the flask were inoculated with three disks (5 mm diam.) of *P. sojae* mycelia obtained from the margin of an actively growing culture for each race. The flask was shaken daily to prevent the grains from compacting and ensure that all grains were uniformly colonized. After about 12 to 14 d of incubation, the rice grains were sampled and assayed for fungal colonization on V8 medium (Miller, 1955) 2 d prior to use as inoculum.

Each soybean seed was buried in the soil with three grains of inoculated rice at a depth of 2.5 cm in 12.5-cm-deep square pots (700051C, T.O. Plastics, Clearwater, Minnesota). The pots were watered every other day for 14 d, and the numbers of live seedlings were counted. For the six trials, survival index (SI) of line j for trial i was calculated as follows: $SI_{ij} = [(\text{the number of plants of line } j \text{ surviving in the trial } i) /12] \times 100$. SI values of the parents, E00003 and PI 567543C, were calculated as $SI = [(\text{the number of plants surviving in the trial } i) /36] \times 100$. The SI ranges from 0% for the most susceptible lines to 100% for the most resistant lines.

Genetic analysis

Genotyping of the mapping population with SSR markers was carried out as described by Zhang et al. (2010). All genomic regions that had been suggested in the literature were considered as potential PRR resistance loci (Table 2.1). Bulked segregant analysis (BSA), as proposed by Michelmore et al. (1991), was used to obtain possible genomic regions of major PRR resistance. For each inoculation trial, 15 resistant lines with the largest SI, and 15 susceptible lines with the smallest SI, were selected to form one resistant bulk pool and one susceptible bulk pool. A total of 132 SSR markers covering the genomic regions with reported PRR resistance loci were first selected to test the bulks. Genomic regions potentially associated with PRR resistance were saturated further with more SSR markers and additional BARCSOY SSR markers (Song et al., 2010). Then, a subpopulation of 94 individual lines, as well as the two parents, was genotyped with polymorphic and trait-associated markers identified by BSA. The remaining lines of the entire mapping population were then genotyped with the markers that showed association with PRR resistance in the first 94 lines.

Ten DNA samples including the susceptible and resistant parents, two segregating bulks, and six resistant inbred lines from the mapping population were genotyped with Illumina® BeadChip (Illumina, Inc., San Diego, California) containing over 52,000 soybean SNP markers (Song et al., 2011). Three polymorphic SNPs associated with PRR resistance among the 10 samples were selected as candidates for TaqMan® SNP allele-specific genotyping assays for the population. The sequences were subjected to the Customer Taqman® Assay Design Tools of Applied Biosystems (ABI, Foster City, California) to obtain allele-specific primers and probes, which were synthesized by ABI. The entire mapping population was genotyped with allele-

specific SNP Assays. Taqman[®] probe-based PCR reactions were carried out in 384-well plates

with a total volume of 3 uL/well on a LightCycler[®] 480 (Roche Applied Science, Indianapolis,

Indiana). The PCR reaction mixture for the Taqman[®] assay consisted of 20 ng of genomic DNA,

0.15 uL of 10X Taqman[®] Assay, and 1.5 uL of 2X ABI Genotyping Master mix containing a

modified Taq DNA polymerase, reaction buffer, $MgCl_2$, and dNTPs (ABI). After 10 min pre-

incubation at 95 ℃, 45 PCR cycles were conducted with 10 sec denaturation at 95 ℃, 30 sec

annealing at 60 ℃, and 10 sec extension at 72 ℃. A final melting cycle for nonspecific amplicon

screening was performed by raising the temperature to 95 ℃ for 10 sec, lowering the

temperature to 40 ℃ for 30 sec, then increasing the temperature to 83 ℃ with continuous

fluorescent acquisition followed by cooling to 40 ℃ on the LightCycler[®] 480. Data were

analyzed by the Roche Applied Science software version 1.5.0.

Data analysis

      SI from the six inoculated trials were calculated and analyzed by the statistical software R.

The broad-sense heritability (h^2) (Fehr, 1987) of PRR resistance to three races was calculated as

follows: $h^2 = \dfrac{\sigma_g^2}{\sigma_g^2 + \sigma_2^2 / r}$

, where σ_g is standard deviation of genotype factor, σ_e is standard deviation from error term and r

is the number of years. The two years were treated as two replications, with $r = 2$. Correlation

coefficients for SI of the same pathogen race in 2009 and 2010 were estimated. Tukey's

procedure was used for multiple line comparisons. A linkage map was constructed using

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JoinMap 3.0 with the Kosambi function and a LOD score of 3 (Van Ooijen and Voorrips, 2001).

Composite interval mapping (CIM) was performed using QTL Cartographer V2.5 (Wang et al.,

2008) to locate PRR resistance loci with LOD threshold determined by 1000 permutations. The

plots of LOD scores and the locus positions were generated using MapChart (Voorrips, 2002).

RESULTS AND DISCUSSION

Phenotype distribution

Phytophthora sojae races 1, 4 and 7 were used to screen the mapping population for

resistance to PRR. Race 25 was only tested with the two parents of the mapping population,

since both parents are partially resistant to race 25. SI of the susceptible parent PI 567543C

varied from 0.0 to 5.6%, while that of the resistant parent E00003 ranged from 41.7 to 91.7%

(Table 2.2). E00003 and the population showed lower SI means in trials inoculated with race 4 in

both years, which indicates that E00003 is less resistant to race 4. The SIs of three replicates of

each parent were very closely correlated ($P < 0.0001$), implying minor experimental error in the

trial. Therefore, one replication was sufficient for each trial. The broad sense heritability ranged

from 83 to 94%. The correlations of race-specific SI between the two years were all significant

for all three *P. sojae* races, 89.1, 77.2 and 78.3% respectively. Continuous phenotypic

distribution of SI with major peaks was observed for each trial, with SI ranging from 0 to 100%

(Fig. 2.1).

PRR resistance locus mapping

The resistant and susceptible bulks were first genotyped with previous markers from the regions listed in Table 2.1 to investigate whether the *Rps* loci in E00003 was among those reported in the literature. From the bulk segregant analysis (BSA), a cluster of SSR markers on Chromosome 3 (linkage group N) were polymorphic and associated with PRR phenotypic data in the two bulks. These polymorphic SSR markers from BSA were further tested with 94 lines from the mapping population. SSR markers Satt631, Satt675, Satt485, Satt584 and Satt624 appeared to be associated with the phenotypic data, and were confirmed by genotyping the rest of the population. The five SSR markers formed one linkage group in the linkage analysis with JoinMap (Van Ooijen and Voorrips, 2001), and a major QTL was located between Satt631 and Satt675 within a 20 cM interval when the data were analyzed using the CIM method in WinQTLCart 2.5 (Wang et al., 2008) (data not shown). The interval was associated with the *Rps*1 region (Gardner et al., 2001). Though *Rps*1 is close to *Rps*7, *Rps*7 was eliminated because it is susceptible to races 1, 4, 7 and 25 (Dorrance et al., 2004). According to criteria on responses to *P. sojae* races (Dorrance et al., 2004) , the PRR resistance allele in E00003 was determined to be *Rps*1k, as it showed partial resistance to race 25 (data not shown). Within the flanking region by Satt631 and Satt675, there is another recently identified *Rps* locus, *Rps*Yu25 (Sun et al., 2011). The authors claimed that *Rps*Yu25 is a novel locus based on different responses to a set of various pathogen isolates, and developed a flow chart as a dichotomous key to differentiate *Rps* loci. The set of pathogens used in that study was not comparable with pathogen isolates that we used; thus, it was not obvious to eliminate *Rps*Yu25as a candidate. However, we were able to exclude *Rps*Yu25 after we further pinpointed the gene with SNP markers (discussed in analysis with SNP markers below).

With the goal of developing breeder-friendly SSRs that locate within a functional region, a total of 220 BARCSOY SSRs (Song et al., 2010) between Satt631 and Satt675 were screened with the bulks, and polymorphic BARCSOY SSRs were further genotyped with the entire population. Two BARCSOY markers, BARCSOYSSR_03_0249 and BARCSOYSSR_03_0250, were determined to be strongly associated with *Rps*1k. These two markers are located within the candidate genes for resistance to *P. sojae* identified by Gao et al. (2005). To further identify the region containing the resistance locus in E00003, a new linkage map was constructed by adding the two BARCSOY SSRs to the five SSRs mapped earlier (data not shown). The marker order followed the same order as was observed in consensus map and a latest map integrated with BARCSOY SSRs (Song et al., 2004; Song et al., 2010) and was highly comparable to that region of soybean genomic map. The interval containing the resistant locus was less than 2 cM between marker BARCSOYSSR_03_250 and Satt675. The LOD score was estimated at 28.9 to 53.3. The R^2 ranged from 0.46 to 0.67 with additive effect from 18.8 to 33.4%. Since the R^2 only partially explained the total genetic variance as estimated by heritability, we investigated whether other PRR resistance loci existed. A total of 1328 SSRs evenly spread through the entire genome were screened with the DNA bulks, and only 15 SSRs were found to be polymorphic. However, none showed association with PRR resistance in the entire mapping population. Therefore, no other major PRR resistance loci were detected in E00003.

Analysis with SNP Markers

Of the 52,041 SNPs on the soybean BeadChip, 17,830 (34.3%) were polymorphic between the two parents. Among these SNPs, 200 (1.1%) showed consistent genotypes between resistant parent and resistant bulk, and susceptible parent and susceptible bulk. Among the

genotypes of the six resistant lines for these 200 SNPs, 76 SNPs were found to be associated with the PRR resistance. All these 76 SNPs are located on Chromosome 3 (linkage group N), confirming that no other major PRR resistance loci reside on other chromosomes. Of the 76 SNPs spreading from the physical position of 2,025,790 bp to 22,595,547 bp, 56 SNPs (73.7%) are clustered within the region 3,613,821bp to 5,948,099 bp, covering the *Rps*1k candidate gene region, (4,457,810 bp to 4,641,921 bp) (Gao and Bhattacharyya, 2008). This candidate gene family encoding coiled coil-nucleotide binding-leucine rich repeat (CC-NB-LRR)-type proteins could be involved in a hypersensitive response, producing pathogen recognition and defense response initiation (Gao and Bhattacharyya, 2008). Six SNP markers were designed for TaqMan[®] SNP allele-specific genotyping assays at the following positions, Gm03_4487138, Gm03_4563499, Gm03_4567214, Gm03_4576493, Gm03_4578272, Gm03_4610670 (The numeric string indicates SNP position). MSUSNP03-1 (Gm03_4487138), MSUSNP03-2 (Gm03_4563499), and MSUSNP03-3 (Gm03_4610670) were selected to genotype the entire mapping population using TaqMan[®] SNP allele-specific genotyping assays.

A new linkage map was constructed while adding the three SNPs (Fig. 2.2), and QTL analysis was performed using this new map. The QTL positions on the new map are shown in Fig. 2.2, and LOD scores and R^2 are given in Table 2.3. The LOD scores were estimated to be between 32.3 and 62.6. The R^2 ranged from 0.50 to 0.76, with additive effects from 19.2 to 33.0%. These results indicated that about 50 to 76% of the phenotypic variation can be explained by *Rps*1k. The remaining heritability was due to some other minor effect QTL or experimental errors.

The positions of LOD peaks in Table 2.3 were estimated at 24.4 to 26.3 cM based on the position of markers Satt631 and Satt675 from soybean consensus map (Song et al., 2004). Considering physical positions, this candidate gene region is located within interval 4,475,877 to 4,563,799 bp (Fig. 2.2), within the *Rps*1k candidate interval, 4,457,810 bp to 4,641,921 bp (Gao and Bhattacharyya, 2008). *Rps*Yu25 is located between 3,338,620 and 3,465,436 bp by converting the flanking markers Satt152 and Sat_186 from interval 30.1 to 32.8 cM (Song et al., 2010; Sun et al., 2011). Therefore, *Rps*1k is a different locus than *Rps*Yu25.

To validate the gene action of *Rps*1k, SI for each genotype of the three polymorphic allele-specific SNP markers was estimated using the combined SI of two years' data for the same race (Table 2.4). Strong evidence of additive gene action was detected, since the average SI for the heterozygous type was significantly smaller than that for the homozygous resistant type, significantly larger than for the homozygous susceptible type, and not significantly different from the average of the two homozygous types at size of 0.05.

In spite of advances in quantitative molecular genetics, due to genomics, computation, and statistics, the bottleneck in genetic analysis is now phenotyping, rather than genotyping (Walsh, 2009). The detection of *Rps*1k in the Michigan elite line E00003 has shown that the rice grain inoculation method is an effective high-throughput phenotyping approach to detect *Rps* loci with major effects. The method provides the opportunity for the soil-borne pathogen to interact with its host, mimicking natural infection of soybean, and avoid injury to epidermal tissues, which occurs in the hypocotyl splitting method. The rice grain method saves labor and time of splitting soybean seedlings. Within 14 days, thousands of rice grains can be prepared in a single patch as pathogen inoculum, and they are easier to handle. Compared with the vermiculite layer test (Thomison et al., 1991), growing the pathogen on one layer of agar in a petri dish for each

single pot is unnecessary. However, to detect partial resistance to PRR, lesion length

measurement using a slant board test (Burnham et al., 2003a) has proven effective (Tucker et al.,

2010). Although there might be some confounding effect with germination rate or soil factors,

the validity of our findings was not affected. A separate germination test with non-pathogen-

inoculated seeds was conducted in the greenhouse in 2010. In the entire mapping population, 90%

of the lines had a germination rate above 87.5%. The germination rate was calculated and

applied as a phenotypic trait in the QTL analysis, where no QTL with LOD more than 1.0 were

detected within the region of interest (data not shown). Further, we adjusted SI with germination

rate to ΔSI as follows: ΔSI$_{ij}$ = the number of surviving seedlings of line j in the non-pathogen

germination test minus the number of plants surviving of line j in the original trial. There was a

only slight improvement on heritability and R^2 in QTL analysis over the unadjusted SI (data not

shown). Thus, we used unadjusted SI for simplicity.

PRR resistance locus *Rps*1k was detected and confirmed in E00003 successfully.

Additionally, two SSRs and three SNP allele-specific endpoint genotyping markers,

MSUSNP03-1 (Gm03_4487138), MSUSNP03-2 (Gm03_4563499), and MSUSNP03-3

(Gm03_4610670) were identified and developed within the candidate gene region for the

resistant line E00003. With these breeder-friendly SSR and SNP markers, marker-assisted

selection can be performed efficiently and effectively, by using E00003 or other soybeans

containing *Rps*1k as source for PRR resistance.

Table 2.1. *Rps* loci and QTLs underlying resistance to Phytophthora root rot and their locations on the soybean composite map. Linkage group names, marker names, and marker positions are updated as shown on soybean composite map (Song et al., 2004). Table contents updated according to Cornelius et al. (2005).

Rps loci or QTL	Linkage Group	Flanking markers (cM)	Reference
*Rps*1	N	Satt159─Satt009 (27.1 ─ 28.5)	Weng et al. 2001
*Rps*2	J	A233_1─A724_1 (83.2 ─ 84.9)	Diers et al. 1992; Demirbas et al. 2001
*Rps*3	F	A757_1─R045_1 (63.1 ─ 70.1)	Diers et al. 1992; Demirbas et al. 2002
*Rps*4	G	A586_2 (111.2)	Diers et al. 1992; Demirbas et al. 2003
*Rps*5	G[†]	T005_2 (81.5)	Diers et al. 1992; Demirbas et al. 2004
*Rps*6	G	Not defined	Demirbas et al. 2001
*Rps*7	N	Satt009─Satt125 (28.5 ─ 40.6)	Weng et al. 2001
*Rps*8	A2	Sat_040─Satt228 (118.6 ─ 154.1)	Burnham et al. 2003b
QTL	F	Satt252─Satt423 (16.1 ─ 20.6)	Burnham et al. 2003a
QTL	D1b	Satt266─Satt579 (59.6 ─ 75.9)	Burnham et al. 2003a
*Rps*Yu25	N	Sat_186─Satt530 (30.1 ─ 32.8)	Sun et al. 2011

[†] possibly (Diers et al. 1992; Demirbas et al. 2004).

Table 2.2. Survival index (mean $\pm SE$) of the mapping population with 240 F$_4$ derived lines and the parents E00003 and PI 567543C in the six *P. sojae* inoculated greenhouse trials conducted in 2009 and 2010. Survival index = the number of surviving plants / total number of seeds planted $\times 100\%$.

Trial	Year	*P. sojae* race	Survival index			Range
			Parents		F4-derived lines	
			PI 567543C	E00003	Population mean$\pm SE$[†]	
1	2009	1	0.0±0.0	91.7±0.0	48.7±36.2	0.0 – 100.0
2		4	0.0±0.1	44.4±25.5	26.8±27.0	0.0 – 100.0
3		7	5.6±4.8	69.4±29.3	46.7±34.2	0.0 – 100.0
4	2010	1	0.0±0.0	61.1±17.3	51.2±39.7	0.0 – 100.0
5		4	0.0±0.1	41.7±25.0	36.8±32.7	0.0 – 100.0
6		7	2.8±4.8	80.6±12.7	50.7±39.1	0.0 – 100.0

[†] *SE*, standard error.

Table 2.3. Phytophthora root rot (PRR) resistance locus detected in the mapping population E00003 ×PI 567543C using composite interval mapping (CIM) method. Chromosome/linkage group 03/N is based on Soybase (Grant et al., 2010).

Trial	Year	*P. sojae* race	Peak Pos. (cM) [†]	Marker near the peak	LOD (threshold) [††]	R^2	Additive effect
1		1	24.4	BARCSOYSSR_03_0250	62.6(3.7)	0.76	31.6
2	2009	4	26.3	MSUSNP03-1 (Gm03_4487138) [§]	32.3(2.5)	0.50	19.2
3		7	24.4	BARCSOYSSR_03_0250	32.1(2.4)	0.59	26.8
4		1	25.4	BARCSOYSSR_03_0250	56.01(2.8)	0.69	33.0
5	2010	4	24.4	BARCSOYSSR_03_0250	36.6(3.2)	0.56	24.8
6		7	26.2	MSUSNP03-1 (Gm03_4487138)	58.9(3.6)	0.70	32.9

[†] Peak position converted according to consensus map (Song et al., 2004);

[††] LOD score threshold for each trial determined by 1000 permutations in CIM by WinQTLCart (Wang et al., 2008);

[§] Single nucleotide polymorphism (SNP) markers from Song et al. (2011).

Table 2.4. Average survival index for different genotypes of the three single nucleotide polymorphism (SNP) markers in the mapping population E00003 ×PI 567543C. Numbers followed by different letters within the same row are significantly different at size of 0.05. The three SNP markers are MSUSNP03-1 (Gm03_4487138), MSUSNP03-2 (Gm03_4563499), and MSUSNP03-3 (Gm03_4610670).

SNP Marker	E00003 type	Heterozygous type	PI 567543C type	Average of PI 567543C and E00003 types
Race 1				
MSUSNP03-1[†]	82.8a	58.7b	13.6c	48.2b
MSUSNP03-2	82.6a	57.5b	14.2c	48.4b
MSUSNP03-3	82.1a	57.1b	12.7c	47.4b
Race 4				
MSUSNP03-1	53.9a	31.1b	5.9c	29.9b
MSUSNP03-2	53.4a	29.2b	6.5c	30.0b
MSUSNP03-3	54.0a	29.4b	7.2c	30.6b
Race 7				
MSUSNP03-1	79.6a	59.8b	14.6c	47.1b
MSUSNP03-2	79.1a	58.5b	15.7c	47.4b
MSUSNP03-3	79.1a	59.6b	15.5c	47.3b

[†] Allele-specific SNP markers designed from 52K Soybean Bead chip (Song et al., 2011).

Figure 2.1. Phenotypic distribution of survival index (SI) of the mapping population among 240
F_4 derived lines from PI 567543C $\times$ E00003. Parent SIs were indicated by arrows. SI was
calculated as: SI_{ij} = [(the number of plants surviving of line j in the inoculated trial i) /12] $\times$ 100.
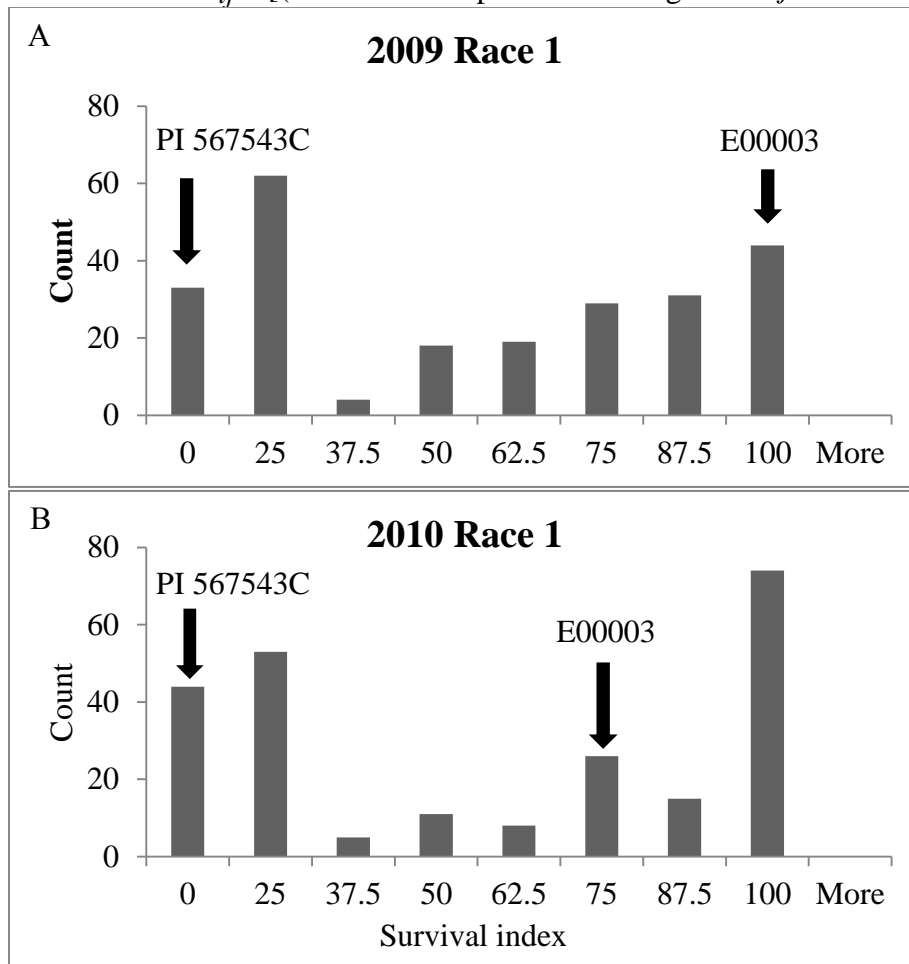
Figure 2.1. (cont'd)
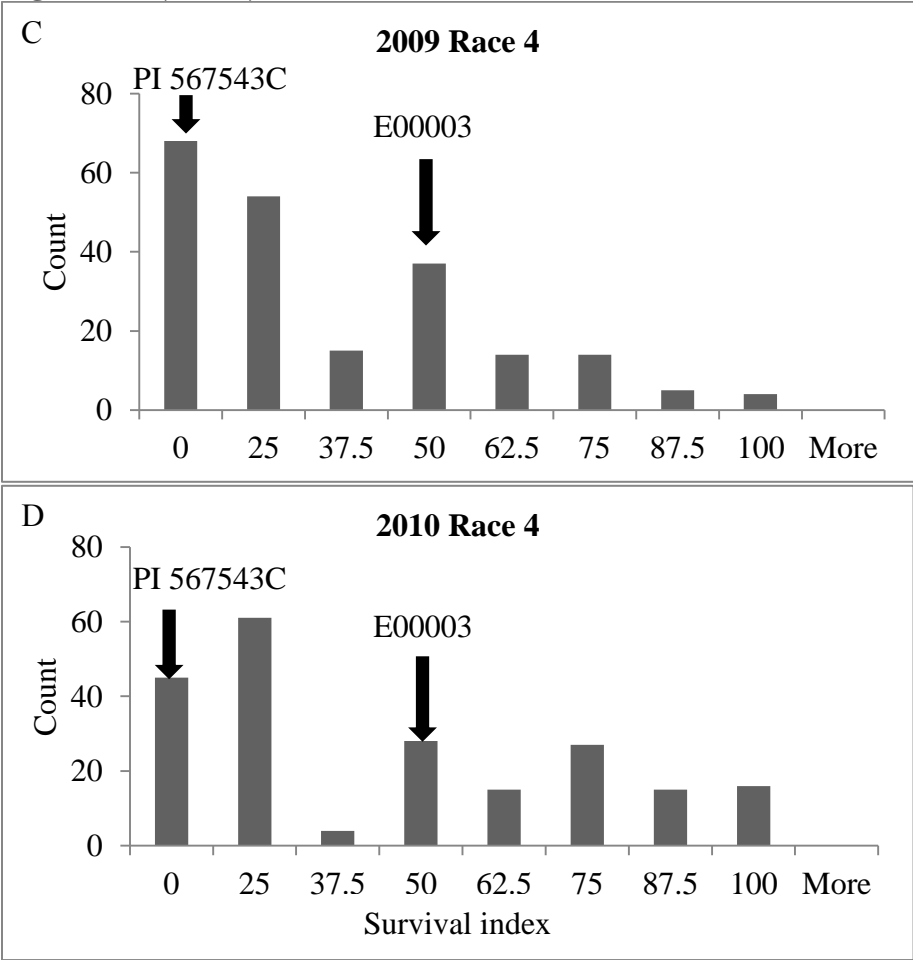
Figure 2.1. (cont'd)
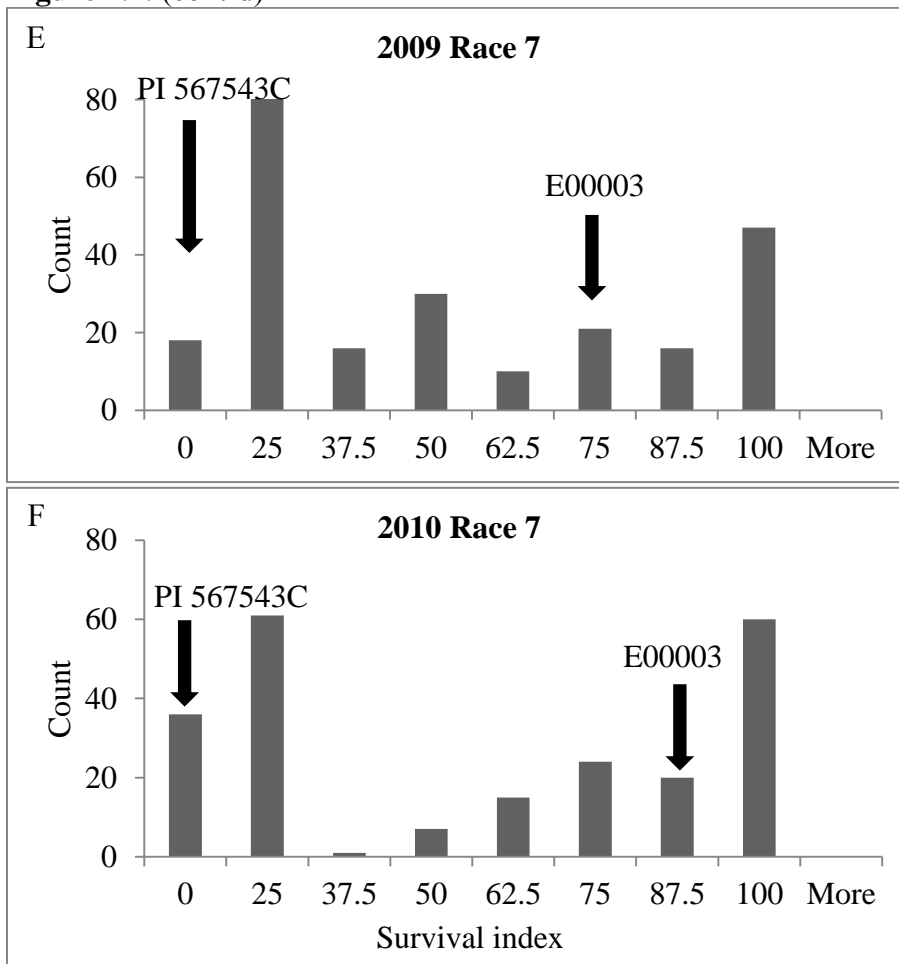
Figure 2.2. Location of Phytophthora root rot resistance locus in E00003 as mapped with the composite interval mapping method. Linkage map of the SSR markers and the SNP markers was constructed with the genotypic data from the mapping population. Bars show intervals of loci mapped with different trials.
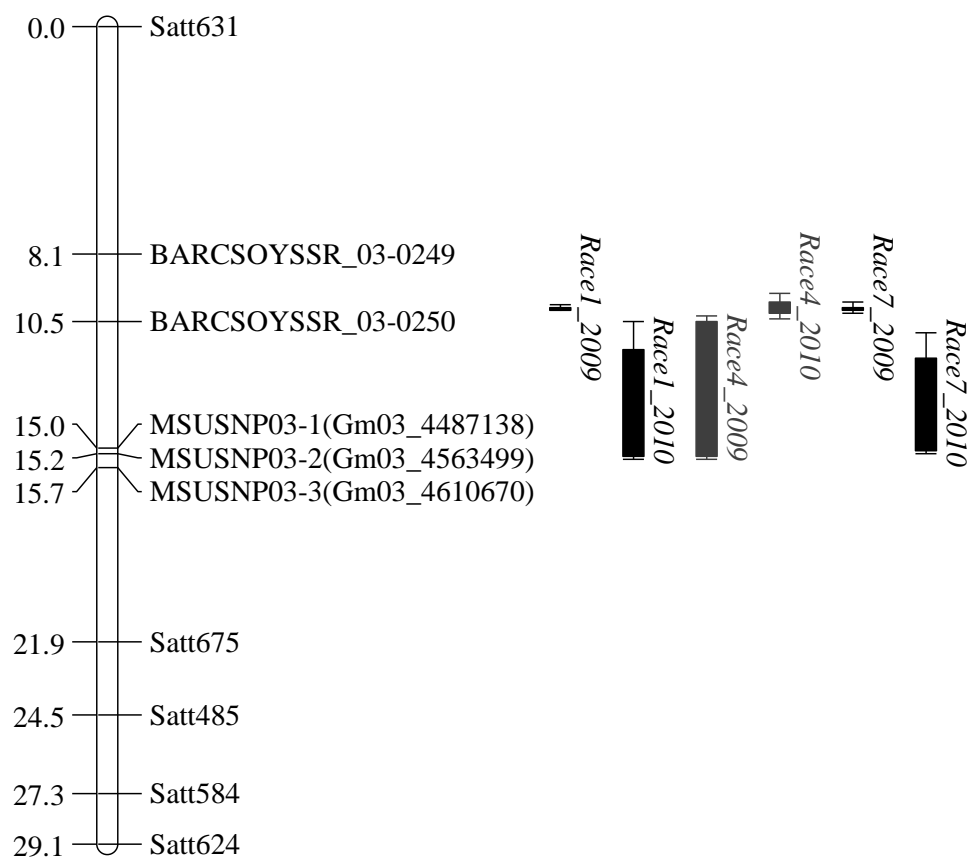
CHAPTER 3

APHID RESISTANCE IN WILD SOYBEAN *GLYCINE SOJA*

ABSTRACT

Soybean aphid can cause significant quality damage and yield loss. Host-resistance is cost-effective and environmentally friendly solution. E08934, a single plant selection from a wild type soybean, *Glycine soja* accession 85-32, is resistant to aphids. To map the resistance loci in E08934 conferred from *G. soja*, a mapping population consisting of 140 F_3-derived lines was developed by crossing E08934 with the aphid susceptible line E00003 (*Glycine max*). Aphid resistance phenotyping was conducted one season in the greenhouse and three seasons in the field cages. The broad-sense heritability for aphid resistance from field trials is 0.84. According to the results of genotyping eight resistant and eight susceptible lines on the 6K soybean SNP Beadchip, three genomic regions, 38.8 to 43.9 mega base pair (Mbp) on Chromosome (Chr.) 8 (linkage group (LG) A2), 4.8 to 11.3 Mbp and 24.6 to 28.5 Mbp on Chr. 16 (LG J) were detected with clusters of polymorphic SNPs consistent with the aphid damage index. After genotyping the entire population, using polymorphic SSRs from these three regions integrated with SNP markers in TaqMan$^{®}$ assays, two major QTLs were detected to be significant ($P < 0.001$) in intervals of 13.5 cM between Sat_382 and Satt455 on Chr. 8, and 3.5 cM between Satt693 and Sat_370 on Chr. 16, respectively. The locus on Chr. 8 explained 40.8% of the phenotypic variation in the greenhouse trial, and 46.4, 19.5 and 39.1% in the field trials in 2009, 2010 and 2011, respectively. The locus on Chr. 16 explained 12.5 to 22.9% of the phenotypic variation. Further, these two loci were both confirmed in a validation population consisting of 252 F_2-derived lines from cross E08934 with E08929, an accession from PI 567541B. The no-choice test indicated both loci provide antibiotic resistant to aphids. The novel locus between Sat_382

and Satt455 on Chr. 8 (LG A2) is denoted as *Rag6,* since it showed significant partially dominant effect in the validation population ($P < 0.05$). A new aphid resistance source is of foremost importance, considering the problem of evolving aphid biotypes that may defeat the existing resistance gene. With closely linked SNPs to these resistance loci, breeding for aphid resistance from wild type soybean can be performed more effectively.

INTRODUCTION

The soybean aphid (*Aphis glycines* Matsumura) was found in the Midwest region of the United States in 2000, since then it has dispersed to most soybean-growing states and become a serious destructive insect pest in the USA (Jun et al., 2012; Kim et al., 2008; Mian et al., 2008a; Ragsdale et al., 2004; Zhang et al., 2010). Categorized as phloem feeders, the aphids (Hemiptera) have typical piercing-sucking mouthparts that enter vascular tissue and feed on the plant by sucking phloem sap, causing curling, wilting, yellowing, stunting and plant death under heavy infestations (O'Neal and Johnson, 2010; Zhang et al., 2010). By jeopardizing water and nutrient absorption from leaves and stem, soybean aphids can significantly reduce biomass production and seed oil concentration (Beckendorf et al., 2008). Ostlie (2002) reported Minnesota soybean yield loss to aphids exceeded 50% in 2001. Maximum possible yield loss due to soybean aphid was estimated to be 48 to 75% for infestation stage starting at the V5 stage and the R2 stage (Fehr and Cavinese, 1977) based on study of caged soybean plants (Catangui et al., 2009; Catangui et al., 2010; O'Neal et al., 2010). Other severe threats of virus-transmission and seed quality losses were also reported due to soybean aphid infestation (Clark and Perry, 2002; Davis et al., 2005; Hartman et al., 2001; Hill et al., 2001; Iwaki et al., 1980; Mueller and Grau, 2007; Pedersen et al., 2007).

In pursuit of chemical control against phloem feeders such as aphids and white flies, neonicotinoids were the first commercial insecticide used as seed treatments or foliar formulations (Buchholz and Nauen, 2002; Elzen, 2001; Jeschke and Nauen, 2008; Mukherjee and Gopal, 2000; Weichel and Nauen, 2004). However, insecticides significantly increase

agriculture input cost and may leave residuals in the environment, harming the natural enemies of pests and beneficial insects (Derksen et al., 2008; Ohnesorg et al., 2009; Southwick et al., 2004). Many studies have suggested that host-resistance is a more environmental friendly solution for control of soybean aphid in comparison with chemical control (DiFonzo, 2009; Hill et al., 2004; Jun et al., 2012; Mensah et al., 2005; Mensah et al., 2008; Mian et al., 2008a; O'Neal and Johnson, 2010; Song and Swinton, 2009; Wiarda et al., 2012; Zhang et al., 2009; Zhang et al., 2010). Plant host resistance can be categorized as antibiosis and antixenosis (Painter, 1951). Antibiosis involves toxic secondary metabolites in host plant that disturb insect biology and reproduction, while antixenosis resistance repels insects with non-preference for specific plants. Endeavors have been made in identifying host resistance sources within these two categories. These include PI 567541B, PI 567598B and PI 230977 with antibiosis, and PI 567543C, PI 567597C, PI 595099, PI 567301B with antixenosis resistant to aphids (Hesler et al., 2007; Jun et al., 2012; Mensah et al., 2005).

Genetic studies are of crucial importance to better employ the resistance genes. To date, seven major resistance alleles *Rag*1, *rag*1, *Rag*2, *Rag*3, *rag*3, *rag*4 and *Rag*5 have been reported for aphid resistance from cultivars and plant introductions. Dominant allele *Rag*1 (Hill et al., 2006a; Hill et al., 2006b; Li et al., 2007) and recessive gene *rag*1 (Zhang et al., 2009) were mapped to Chr. 7 (LG M) between marker Satt435 and Satt540. Dominant allele *Rag*2 (Mian et al., 2008b), *Rag*5 (Jun et al., 2012) and recessive *Rag*4 (Zhang et al., 2009) were mapped to Chr. 13 (LG F). Partially dominant allele *Rag*3 (Zhang et al., 2010) was mapped on Chr. 16 (LG J) between marker Sat_339 and Satt414. Further, commercial aphid resistant soybean germplasm has been released, such as "*Sparta*" from Michigan State University and aphid tolerant varieties with *Rag*1 gene from Monsanto. However, it is remaining challenging that aphid resistant genes

can be overcome by new aphid biotypes (Hill et al., 2010). Therefore, identifying new sources

with new aphid resistance loci is of considerable importance and urgency.

Wild type soybean, *Glycine soja* (*G. soja*), has been proven resistant to a broad range of

disease and insects, such as *mosaic virus*, soybean cyst nematode and soybean aphid (Diers et al.,

2005; Hill et al., 2004; Kabelka et al., 2005; Kabelka et al., 2006; Li and Cao, 2011; Pazdernik et

al., 1997; Ram et al., 1984; Wang et al., 2001; Winter et al., 2007). Yang et al. (2004) identified

three *G. soja* accessions, 85-32, 85-39 and 85-1, with aphid resistance. To adapt the aphid

resistance genes from *G. soja* to cultivated background, 85-32 was further crossed with Jiyu 71,

an aphid susceptible variety. E08934 is a single F_4 plant selection that is resistant to soybean

aphids, derived from the cross Jiyu 71 $\times$ 85-32. The objective of this study was to identify aphid

resistant loci in E08934, which was derived from *G. soja* accession 85-32 using linkage-based

quantitative trait locus (QTL) mapping with SSR and SNP markers, confirm resistance in a

different genetic background and determine resistance type.

MATERIALS AND METHODS

Linkage-based QTL mapping was employed with bi-parental populations to locate and validate aphid resistance loci. A mapping population consisting of 140 F_3-derived lines was used for initial mapping, and a confirmation population of 240 F_2-derived lines was used to validate the aphid resistance loci.

Aphid resistance loci mapping

Population development and phenotype evaluation

The mapping population was developed by crossing aphid-resistant line E08934, derived from a *G. soja* accession, with an aphid-susceptible, advanced breeding line E00003 (*Glycine max*), and followed with single seed descent. The population was evaluated in choice-test for aphid resistance in the greenhouse 2010, and in the field cages, in 2009, 2010 and 2011. Only the parents were replicated three times in each trial, rather than the entire mapping population, because aphid resistance has been considered as a high heritability trait (about 0.90) according to previous study (Zhang et al., 2009). In the spring of 2010, one greenhouse trial was conducted in the Plant Science Greenhouse at Michigan State University (MSU) in East Lansing, Michigan. In the summers of 2009, 2010 and 2011, field trials were performed independently on the Agronomy Farm of MSU in East Lansing. The greenhouse maintenance, planting strategy in the field cage, aphid infestation, evaluation scale (Mensah et al., 2005; Mensah et al., 2008) were adopted as described by Zhang et al. (2010). The phenotypic data was scored when the susceptible parent E00003 reached the maximum of the rating scale, with more than 800 aphids

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per plant. An aphid damage index (DI) defined as DI = ∑ (scale value × no. of plants in the

category) / (4 × total no. of plants) × 100, ranging from 0 being no infestation to 100 being the

most severe damage (Mensah et al., 2005; Mensah et al., 2008) was used as phenotypic indicator

in quantitative trait analysis.

DNA preparation and marker genotyping

Plant tissues of the mapping population and the two parents were collected during the

field cage trial in 2009. CTAB (hexadecyltrimethyl ammonium bromide) DNA extraction

protocol was adopted from Zhang et al. (2010). Two segregating bulks were formed with 15

most resistant and 15 most susceptible lines for the purpose of a bulk segregant analysis (BSA),

proposed by (Michelmore et al., 1991). Firstly, these two bulks, together with the two parents,

were genotyped on 52K soybean SNP Beadchip, covering the entire soybean genome with more

than 52,000 SNPs (Song et al., 2011). Then, eight lines selected from the resistant pool and eight

lines from the susceptible pool were individually genotyped on newly developed 6K soybean

SNP Beadchip (Illumina, CA), covering the most gene-abundant genomic regions throughout the

soybean genome (Qijian Song et al. unpublished data).

After the selective genotyping above, simple sequence repeat (SSR) markers from

candidate genomic regions were used to genotype the two segregating pools and the two parents.

In association analysis, SSRs correlated with traits were used to genotype the remaining lines of

the entire mapping population.

To achieve better resolution, SNP markers for TaqMan[®] SNP allele-specific genotyping

assays were designed with the information from SNPs located within candidate region on 52K

and 6K chips. These SNPs were further genotyped with the entire mapping population. Taqman[®]

probe-based PCR protocol using LightCycler$^®$ 480 was performed as described by Zhang et al.

(2012 *submitted to Crop Sci.*).

Statistical analysis and mapping analysis

The statistical correlation analysis on aphid damage index data of the four trials was

carried out with R software. The broad-sense heritability of DI from the three-year field trial was

estimated according to Fehr (1987) as follows: $Y_{ij} = \mu + Genotype_i + \varepsilon$. *i=1,2,...140; j=2009,*

2010 and 2011. Linkage maps were constructed with JoinMap 4.0 using Kosambi's and LOD of

3.0 in regression method (Van Ooijen, 2006). Composite interval mapping (CIM) in QTL

Cartographer Version 2.5 (Wang et al., 2008) with 1000 permutations described by (Zhang et al.,

2010) was conducted to determine the locations of the aphid resistance loci from *G.soja.* The

maps with locus positions were visualized by MapChart (Voorrips, 2002). No-choice test data

were also analyzed with one-way analysis of variance in R software, at significance level of 0.05.

Validation of aphid resistance loci

To confirm the location and determine the gene action of the aphid resistance loci

identified from E08934, a validation population was developed by crossing E08934 with E08292,

an aphid-resistant accession from PI 456741B with recessive alleles *rag*1 and *rag*4 (Zhang et al.,

2009). In both summer of 2010 and 2011, the entire validation population together with the two

parents were evaluated for aphid resistance in field-cage trials similar to the mapping population.

DNA extraction was performed using the CTAB method. Polymorphic and associated SSRs and

SNPs from the regions identified in the mapping population, along with markers in region *rag*1 and *rag*4 were genotyped in the validation population. Linkage and mapping analysis were carried out in the same manner as above in the mapping population. Data of gene action and gene combination in the validation population were analyzed in one-way analysis of variance with Bonferroni correction in R software, at a significance level of 0.05.

No-choice test

In the spring of 2012, three replications of aphid no-choice test were performed with E00003 (susceptible control), PI 567598B (antibiotic resistant control), line 24 (possessing *Rag*3(?) on Chr. 16), line 131 (possessing *Rag*6 on Chr. 8), and line 19 (possessing both *Rag*3(?) and *Rag*6) from the mapping population, in the Plant Science Greenhouse at MSU. In each replication, eight seeds from each line were planted in a 105-mm-diameter and 125-mm-deep plastic pot, maintained at 26/15 ℃ day/night temperature with sodium vapor lights as supplement. Each plant was infested with two healthy wingless aphids at the V1 stage (Fehr and Cavinese, 1977) and immediately covered with a mesh-cage from bottom to top (Mensah et al., 2008). Aphid resistance was rated, and the aphid damage index was calculated, as mentioned in the mapping population phenotype evaluation, when the susceptible control E00003 reached the maximum of the rating scale, the most severe damage stage. Data of average damage index were analyzed using one-way analysis of variance in R software.

RESULTS

Phenotype distribution of the mapping population

The aphid damage index (DI) of the two parents, E08934 and E00003, together with the 140 F_3-derived lines from the mapping population is summarized in Table 3.1. In all four trials, resistant parent E08934, derived from *G. soja* accession had significantly lower DI than susceptible parent E00003 ($P < 0.001$). DI of the F_3-derived lines from the mapping population varied significantly ($P < 0.001$) from 6.3 to 100.0 with standard errors of 19.6 to 27.4. The correlations between the three field trials are 0.71, 0.65 and 0.63 ($P < 0.05$). The broad-sense heritability for DI of the field trials was estimated as 0.84 ($P < 0.001$). The distribution of DI from the mapping population in the greenhouse trial and the three year field trials are shown in Fig. 3.1 A-D. The field trials in 2009 and 2010 showed similar patterns that are bimodal with a ratio of 1:1. However, no pattern was observed in the 2009 greenhouse trial and the 2011caged field trial (Fig. 3.1).

Aphid resistance locus mapping

Overall, 95% of the SNPs from the 52K SNP Beadchip called the genotype successfully for the two parents and the two segregating bulks from the mapping population, with 37.6% polymorphic SNPs between the two parents and 26.4% between the two bulks. Polymorphic SNPs with genotypes consistent with sample phenotypes were distributed throughout all linkage

groups. Interestingly, polymorphic SNPs from LG A2 and J clustered more intensively than other groups (data not shown).

From the genotypic data of 6K SNP Beadchip with eight resistant and eight susceptible lines selected from the two bulks, three regions were detected with clustered SNPs that significantly correlated with aphid resistance for all four trials ($P < 0.001$, data not shown). They are 38.8 to 43.9 mega base pair (Mbp) on LG A2, 4.8 to 11.3 Mbp and 24.6 to 28.5 Mbp on LG J. 104 SSRs were tested on LG A2 and J on consensus map (Song et al., 2004). Satt209 and Satt455 on LG A2, Satt693 and Sat_370 on LG J showed highly consistent genotypes with the aphid resistance for the two parents and bulks, and further approved correlations with the entire mapping population. Therefore, to cover ±20 cM region nearby, a total of six SSRs from LG A2, eight SSRs from LG J, were genotyped with the remaining lines of the entire mapping population. Eight TaqMan$^{®}$ SNP allele-specific genotyping markers were developed, including MSUSNP08-1 (Gm08_23293155), MSUSNP08-2 (Gm08_40320904), MSUSNP08-3 (Gm08_41114696) and MSUSNP08-4 (Gm08_45189358) derived from 38.8 to 45.2 Mbp of LG A2, MSUSNP16-10 (Gm16_6262227), MSUSNP16-11 (Gm16_6413214), MSUSNP16-12 (Gm16_6423098) and MSUSNP16-15 (Gm16_8051585) derived from 4.8 to 11.3 Mbp region of LG J. With the genotypic data of SSRs and SNPs, nine markers formed one linkage group with 57.7 cM lone (Fig. 3.2A), denoted as Chr. 8/LG A2 and 12 markers formed another linkage group with 36.8 cM in length (Fig. 3.2D) Chr.16/LG J, and are highly comparable with consensus map (Song et al., 2004) (Fig. 3.2-B and E). The linkage maps were used with CIM method in QTL analysis. On LG A2 (Chr. 8), one QTL was consistently detected between SNP MSUSNP08-2 (Gm08_40320904) and Satt209 for all four trials (Table 3.2; Fig. 3.2A), explaining 19.5 to 46.4% of the phenotypic variation. On LG J (Chr. 16), a QTL located within

the interval between Satt693 and Sat_370 was identified for 2010 greenhouse trial, 2009 and

2010 field trials, explaining 12.5 to 22.9% of the phenotypic variation. As expected, the

resistance allele comes from E08934. However, no significant interaction was detected between

these two loci with multiple-interval mapping method in QTL Cartographer (data not shown).

Aphid resistance loci validation

The validation population contained 252 F_2-derived lines from the cross of E08929 $\times$

E08934. The aphid damage index of the population was continuously distributed, but skewed to

the resistant category in both field trials 2010 and 2011. The correlation between the two trials

was 69.7% ($P < 0.05$). After the entire validation population was genotyped, seven SSRs with

three SNPs from LG A2 (Chr. 8) (Song et al., 2010) formed one linkage group (Fig. 3.2C). Ten

SSRs with two BARCSOYSSR markers formed LG J (Chr. 16) and six SSRs formed LG M (Chr.

7) in Fig. 3.2F-G. The marker order is highly comparable with consensus map but with inflation

(Fig. 3.2B-H). Marker data with phenotypic traits were analyzed with QTL Cartographer in CIM

method. On Chr. 8, one QTL was detected MSUSNP08-1 (Gm08_23293155) and Satt455 for

both trials (Fig. 3.2C). That interval is equivalent to 13.5 cM onto consensus map between

marker Sat_382 and Satt455 (Fig. 3.2B). This confirms the location of the resistance locus

identified on Chr. 8 in the mapping population. This locus was denoted as *Rag*6 for convenience

and will be discussed later.

For LG J (Chr. 16), one QTL was detected within the same interval as in the mapping

population between Satt693 and Sat_370 in the field trial 2011. The interval detected by field

trial 2010 between Satt693 and Satt456 is larger, but covering the region identified in the

mapping population (Fig. 3.2F). This confirmed the finding of the locus on LG J in the mapping population.

As one of the parents of the validation population, E08929 is an accession from PI 567541B, therefore, it theoretically carries aphid resistance loci *rag*1 and *rag*4. The QTL detected from LG M (Chr. 7) in both field trials confirms the location of *rag*1 between Satt540 and Satt435 (Fig. 3.2G-H). These three loci detected in the validation population explained 4.1 to 13.9% of the phenotypic variation individually (Table 3.2). Though they explained just a small proportion of the phenotypic variation, all three loci detected from the two years field trials are significant by 1000 permutations ($P < 0.01$). The resistance allele of the locus on Chr. 7 comes from the parent E08929, and the other two resistance alleles on Chr.8 and Chr. 16 respectively come from parent E08934. The recessive allele *rag*4 was not detected in the validation population.

Gene action in validation population

To determine the gene action of the loci in the validation population, the 252 F_2-derived lines were divided into three genotypic groups for the markers that are closest to the peak LOD score, Satt540 for *rag*1, Satt209 for the locus on LG A2, and BARCSOYSSR_16_0371 for *Rag*3(?) (Fig. 3.3). Average DI for each genotypic group was calculated and compared in Table 3.3. We first tested whether the average DI significantly differs among the three genotypic groups for each marker by one-way analysis of variance (ANOVA). The results showed the association was significant even after adjusting for multiple testing (Bonferroni correction) ($P < 0.0001$, Table 3.3). The pairwise differences were calculated among three groups for each

marker to investigate gene actions. For marker Satt540, the closest marker to *rag*1, the average DI of the heterozygous group was significantly ($P < 0.05$) lower than that of the homozygous group of allele from E08934, but not significantly different from that of the homozygous group of allele from E08929 in both 2010 and 2011 field data (Fig. 3.3A). This result suggests that *rag*1 from parent E08929 may have a partially dominant effect instead of a recessive effect. For marker Satt209, the closest marker to the locus on LG A2, the average DI of the heterozygous group was significantly ($P < 0.05$) lower than that of the homozygous group of allele from E08929, but not significantly different from that of the homozygous group of allele from E08934 in both years (Fig. 3.3B), suggesting partially dominant effect of this locus. The location of this novel locus from *G. soja* is different from that of the locus identified by Jun et al. (2012) on LG A2. Therefore, we denote it as *Rag*6 using the conventions of the Soybean Genetics Committee.

For marker BARCSOYSSR_16_0371 on LG J (Chr. 16), located within the interval of *Rag*3(?), the average DI of the heterozygous group was neither significantly different from that of the homozygous group of allele from E08929, or from that of the homozygous group of allele from E08934 in both years (Fig. 3.3C) at size of 0.05. However, the average DI of the homozygous group of allele from E08929 was significantly different from that of the homozygous group of allele from E08934 ($P < 0.05$). Therefore, further study is required to determine the gene action of *Rag*3(?) on LG J from *G. soja*.

Combination of aphid resistance loci in validation population

Progeny was separated that possess different aphid resistance genes or without any resistance gene from the validation population. The average DI of different combination of

*Rag*3(?)*, Rag*6 and *rag*1 from the 2010 field trial was estimated (data not shown) and plotted in

Fig 3.4. Average DI was calculated for progeny that have no resistance genes, one resistance

gene, two resistance genes combined and all three genes combination of *Rag*3(?), *Rag*6 and *rag*1

(Fig. 3.4). A clear trend of decreasing DI can be observed as more resistance genes are combined.

However, the difference of average DI observed was not significant due to the small number of

individuals in each combination. This trend may become significant and more meaningful if

more progeny were included.

No-choice test in the mapping population

The DI of E00003, PI 567598B, line 24 (*Rag*3(?)), line 131(*Rag*6), and line 19 (*Rag*3(?)

*and Rag*6) from the mapping population were analyzed and summarized in Table 3.4. Fig. 3.5

shows the bar graph of the DI with 95% confidence interval derived from one-way analysis of

variance conducted at size of 0.05. Though the average DI of PI 5767598B is significantly lower

than that of line 24 (*Rag*3(?)), are higher than that of line 131(*Rag*6), and line 19 (*Rag*3(?) *and*

*Rag*6), the average DI of all these lines are significantly lower than that of the susceptible check

E00003. The aphid resistance loci in PI 567898B were proven to be antibiosis (Mensah et al.,

2005); therefore, we conclude that the locus *Rag*3(?) and the new locus *Rag*6 both possess

antibiosis aphid resistance.

DISCUSSION

A novel aphid resistance gene *Rag*6 from wild type soybean *Glycine soja* was mapped in an interval between marker Sat_382 and Satt455 on Chromosome 8 in the F_3-derived mapping population, equivalent to 13.5 cM on soybean consensus map (Song et al., 2004). It was further confirmed to the same interval in a validation population. To date, *Rag*5 is the only gene reported, which has been mapped to Chr. 8 and located between Satt437 and Satt327 interval (Jun et al., 2012). The location of *Rag*6 is different from that of *Rag*5, because the marker Satt209 closest to the peak of LOD score is 18.6 cM distance away from Satt327, based on soybean consensus map (Song et al., 2004). Therefore, we denote it as *Rag*6 based on the conventions of the Soybean Genetics Committee.

Meng et al. (2011) reported one QTL *qRa_1* located near marker Satt470 on Chr. 8, conferring aphid resistance in a high isoflavone content variety "Zhongdou27". Compared to consensus map (Song et al., 2004), *Rag*6 is 6.2 cM away from marker Satt470. Due to the lack of information of the exact location of *qRa_1*, we cannot conclude whether *Rag*6 is different from *qRa_1*.

In the validation population, the distribution of phenotypic traits and the aphid damage index were skewed to the resistant category, because both parents E08934 and E08929 possess resistance loci. Based on the results in Fig. 3.4, the more resistance genes a progeny has, the more resistant it shows. Therefore, the validation population skewed to the resistant category. Theoretically, E08929 as an accession from PI 567541B, should carry both *rag*1 and *rag*4 genes (Zhang et al., 2009). However, *rag*4 was not detected in the validation population. According to

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SNP genotypic data from SNP Beadchip, E08929 was heterozygous in the *rag*4 region based on *rag*4 fine mapping results (Jiazheng Yuan unpublished data). With *Rag*6, *Rag*3(?) and *rag*1 present, the effect of the *rag*4 allele from the heterozygous parent E08929 was masked in the validation population. Another possibility is that E08929 did not inherit *rag*4 from the original plant introduction. More detailed information of sequencing might be helpful in a future study to verify if *rag*4 is present in E08929.

From this study, novel aphid resistance allele and locus with molecular markers were identified from exotic soybean source, *Glycine soja*. It is of great importance and urgency to identify new aphid resistance genes in new resistant sources. With the detailed information on SSR and SNP markers that are closely linked with the new aphid resistance genes, breeders can effectively introgress the resistance genes from *G. soja* into aphid susceptible cultivars.

Table 3.1. Aphid damage index (DI) for two parents of the mapping population, E08934 and E00003, and 140 F_3-derived lines, in the greenhouse and field trials.

Trials	Parents		F_3-derived lines		
	E08934	E00003	Mean	Range	$SE^{\dagger\dagger}$
Greenhouse					
2010	$26.6a^{\dagger}$	84.4b	47.3	14.1-85.9	27.4
Field cage					
2009	12.5a	87.5b	46.4	12.5-87.5	19.6
2010	12.5a	87.5b	52.3	12.5-93.8	26.1
2011	20.8a	100.0b	57.7	6.3-100.0	23.1

DI = $\sum$ (scale value × no. of plants in the category) / (4 × total no. of plants) × 100, ranged from 0 being no infestation and 100 being the heaviest infestation (Mensah et al., 2005);

[†] Means followed by different letters in the parent column within the same row are significantly different at significant level of 0.0001;

[††] SE standard error.

Table 3.2. Aphid resistance loci identified in the mapping population E00003 ×E08934 and validation population E08929 ×E08934 with composite interval mapping method with 1000 permutations. MSUSNP08-1 (Gm08_23293155), and MSUSNP08-2 (Gm08_40320904) are single nucleotide polymorphism (SNP) markers derived from 6K SNP chip; MSUSNP16-11 (Gm16_6413214) and MSUSNP16-12 (Gm08_6423098) are derived from 52K SNP chip.

Population	Trials	Chr/LG[†]	Peak Pos.[††]	Flanking markers	LOD	R^{2}[§]	a[#]
E00003 × E08934	Greenhouse						
	2010	8/A2	18.8	MSUSNP08-2-Satt455	19.5	40.8	12.7
		16/J	25.2	MSUSNP16-11-MSUSNP16-12	10.6	22.9	9.7
	Field cage						
	2009	8/A2	18.8	MSUSNP08-2-Satt455	22.9	46.4	19.0
		16/J	25.2	Satt693-Sat_370	10.3	16.7	11.9
	2010	8/A2	18.8	Satt455-Satt209	9.1	19.5	11.7
		16/J	25.2	Satt693-Sat_370	7.0	12.5	10.6
	2011	8/A2	20.8	MSUSNP08-2-Satt455	16.0	39.1	14.7
E08929 × E08934	Field cage						
	2010	7/M	11.1	Satt540-Satt435	7.3	8.4	8.5
		8/A2	79.8	MSUSNP08-1-Satt455	4.6	6.4	-7.1
		16/J	16.5	BARCSOYSSR_16_0383-Satt456	6.4	6.9	-6.1
	2011	7/M	11.1	Satt540-Satt435	10.8	13.9	10.7
		8/A2	81.8	MSUSNP08-1-Satt455	3.4	4.1	-4.9
		16/J	15.5	BARCSOYSSR_16_0383-Sat_370	10.2	12.5	-7.8

[†] Chromosome/linkage group. The chromosome number and linkage group name are according to the SoyBase (Grant et al., 2010);

[††] Peak position is presented in centi Morgan (cM);

[§] R^{2}, percentage of phenotypic variation that can be explained by the locus;

[#] Additive effect.

Table 3.3. Summary of average damage index for genotypic groups of markers near LOD peak from validation population E08929 × E08934.

Trials	E08934 type	Heterozygous type	E08929 type	*P* values (F-test)
		Satt540-Chr. 7 (LG. M)		
2010 field	45.8a[†]	31.8b	24.4b	8.598E-07
2011 field	55.0a	36.0b	29.4b	2.226E-10
		Satt209-Chr. 8 (LG. A2)		
2010 field	19.8a	27.0a	48.3b	2.2E-16
2011 field	29.8a	33.6a	48.8b	5.191E-09
		BARCSOYSSR_16_0371-Chr. 16 (LG. J)		
2010 field	25.2ab	30.4abc	39.5bc	0.00002309
2011 field	27.5ab	37.2abc	46.6bc	3.209E-09

[†] Means followed by different letters within the same row are significantly different at significant level of 0.001.

Table 3.4. Summary of aphid damage index (DI) from no-choice test with selected F_3-derived lines with resistance genes from mapping population.

Test lines	Average DI	SE[†]
E00003	86.98	1.17
PI 567598B	11.46	1.17
Line 24 (*Rag*3(?))	19.27	1.17
Line 131 (*Rag*6)	5.73	1.17
Line 19 (*Rag*3(?), *Rag*6)	4.46	1.53

[†] *SE* standard error.

Figure 3.1. Phenotypic distribution of soybean aphid damage index of mapping population with140 F$_3$-derived lines by E00003 ×E08934 (A-D), and of validation population with 252 F$_2$-derived lines from E08929 ×E08934 (E-F). Parents are indicated by *arrows. y-axis* is the count of plants that fall into each category. A 2010 greenhouse trial; B-D 2009, 2010 and 2011 caged field trials; E-F validation population in 2010 and 2011 field trials.
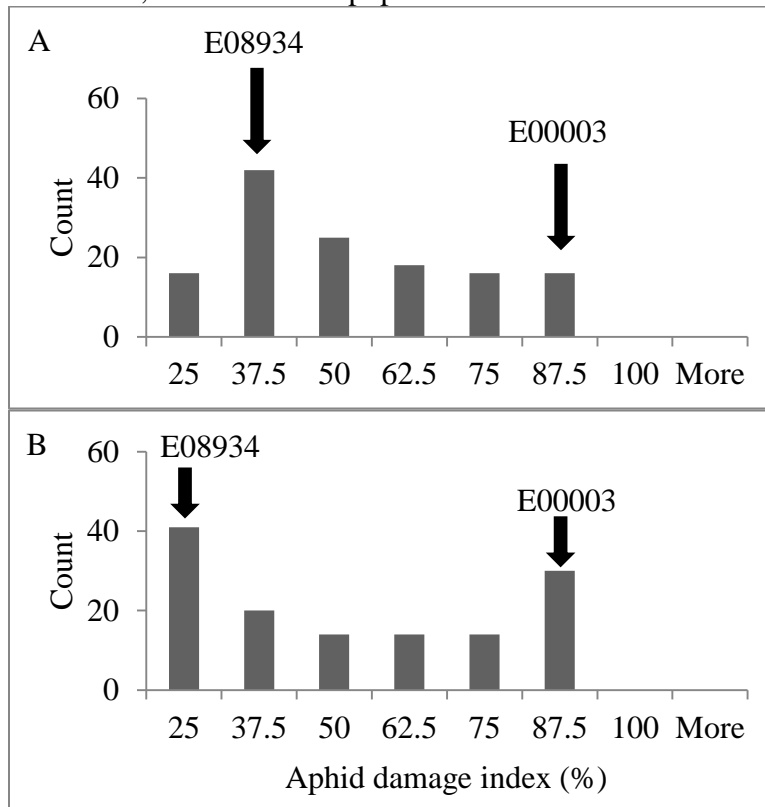
Figure 3.1. (cont'd)
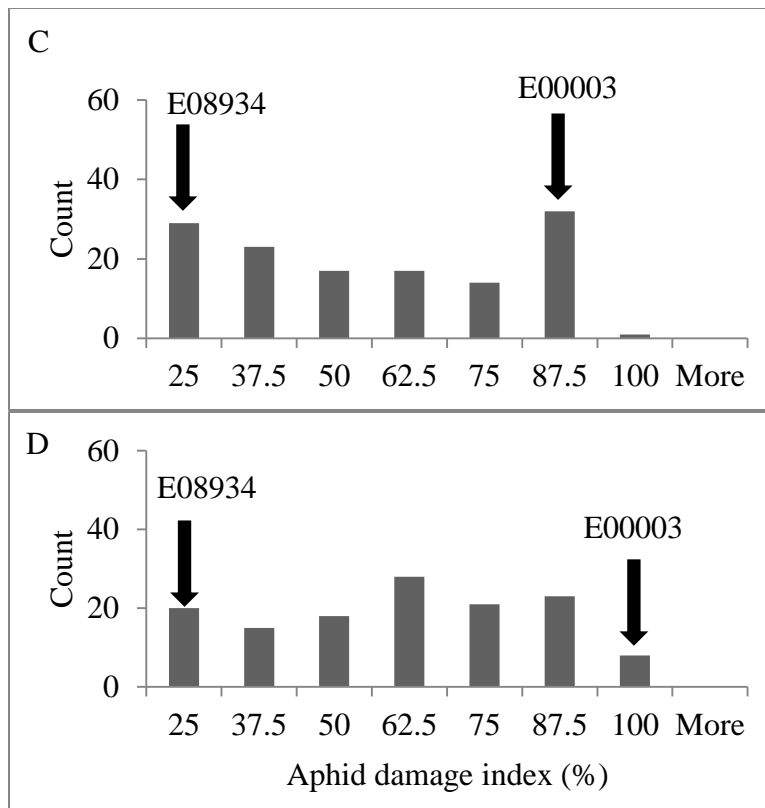
Figure 3.1. (cont'd)
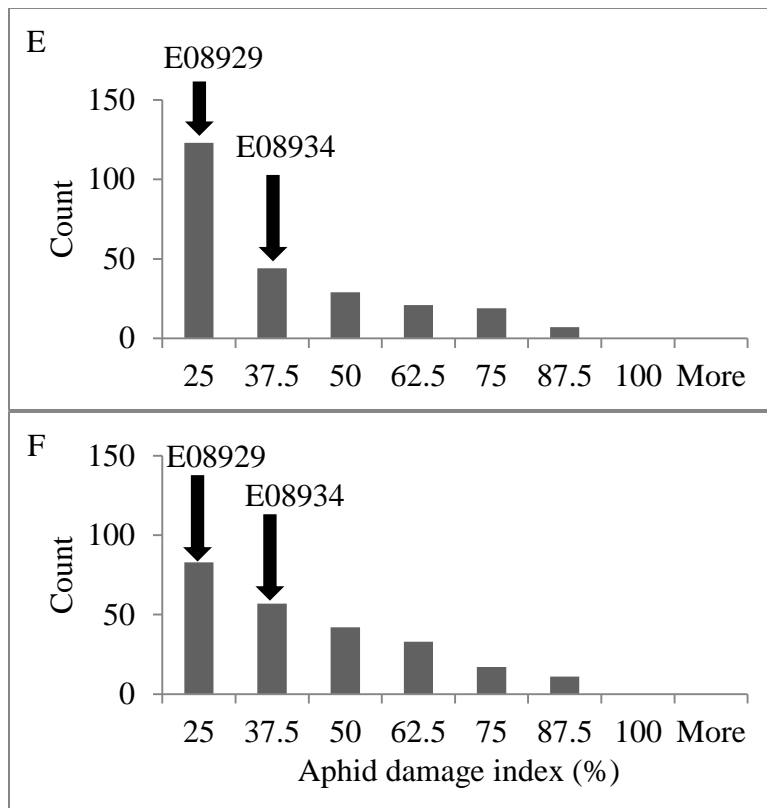
Figure 3.2. Location of soybean aphid-resistant loci detected in the mapping population E00003 ×E08934 and validation population E08929 ×E08934 with the composite interval mapping method with 1000 permutations. 1-LOD and 2-LOD intervals of each locus are indicated by *thick* and *thin bars*. Threshold line was drawn with $P = 0.01$ from 1000 permutations. A) Map of linkage group A2 (Chromosome 8) with the aphid resistance locus on the *right* from four trials in the mapping population, with LOD score plot on the *right*; B) The soybean consensus map of linkage group A2 (Chromosome 8) (Song et al., 2004); C) Map of linkage group A2 (Chromosome 8) with the aphid resistance locus on the *right* from two field trials in the validation population, with LOD score plot on the *right*; D) Map of linkage group J (Chromosome 16) with the aphid resistance locus on the *right* from four trials in the mapping population, with LOD score plot on the *right*; E) The soybean consensus map of linkage group J (Chromosome 16) (Song et al., 2004); F) Map of linkage group J (Chromosome 16) with the aphid resistance locus on the *right* from two field trials in the validation population, with LOD score plot on the *right*; G) Map of linkage group M (Chromosome 7) with the aphid resistance locus on the *right* from two field trials in the validation population, with LOD score plot on the *right*; H soybean consensus map of linkage group M (Chromosome 7) (Song et al., 2004) MSUSNP08-1 (Gm08_23293155), MSUSNP08-2 (Gm08_40320904), MSUSNP08-3 (Gm08_41114696) and MSUSNP08-4 (Gm08_45189358) derived from 38.8 to 45.2 Mbp of LG A2 on 6K SNP Beadchip, MSUSNP16-10 (Gm16_6262227), MSUSNP16-11 (Gm16_6413214), MSUSNP16-12 (Gm16_6423098) and MSUSNP16-15 (Gm16_8051585) derived from 4.8 to 11.3 Mbp region of LG J on 52K SNP Beadchip.

Figure 3.2. (cont'd)

A

Chr.8

Figure 3.2. (cont'd)

B

Chr.8

106.3 — Sat_392
111.5 — Sat_310
115.9 — Satt421
116.4 — Sat_382
116.6 — Sat_377
116.7 — Satt470
122.9 — MSUSNP08-2
123.3 — Sat_138
128.4 — Satt209
128.8 — MSUSNP08-3
129.9 — Satt455

154.0 — MSUSNP08-4
154.1 — Satt228

159.6 — Satt538
162.0 — Satt429
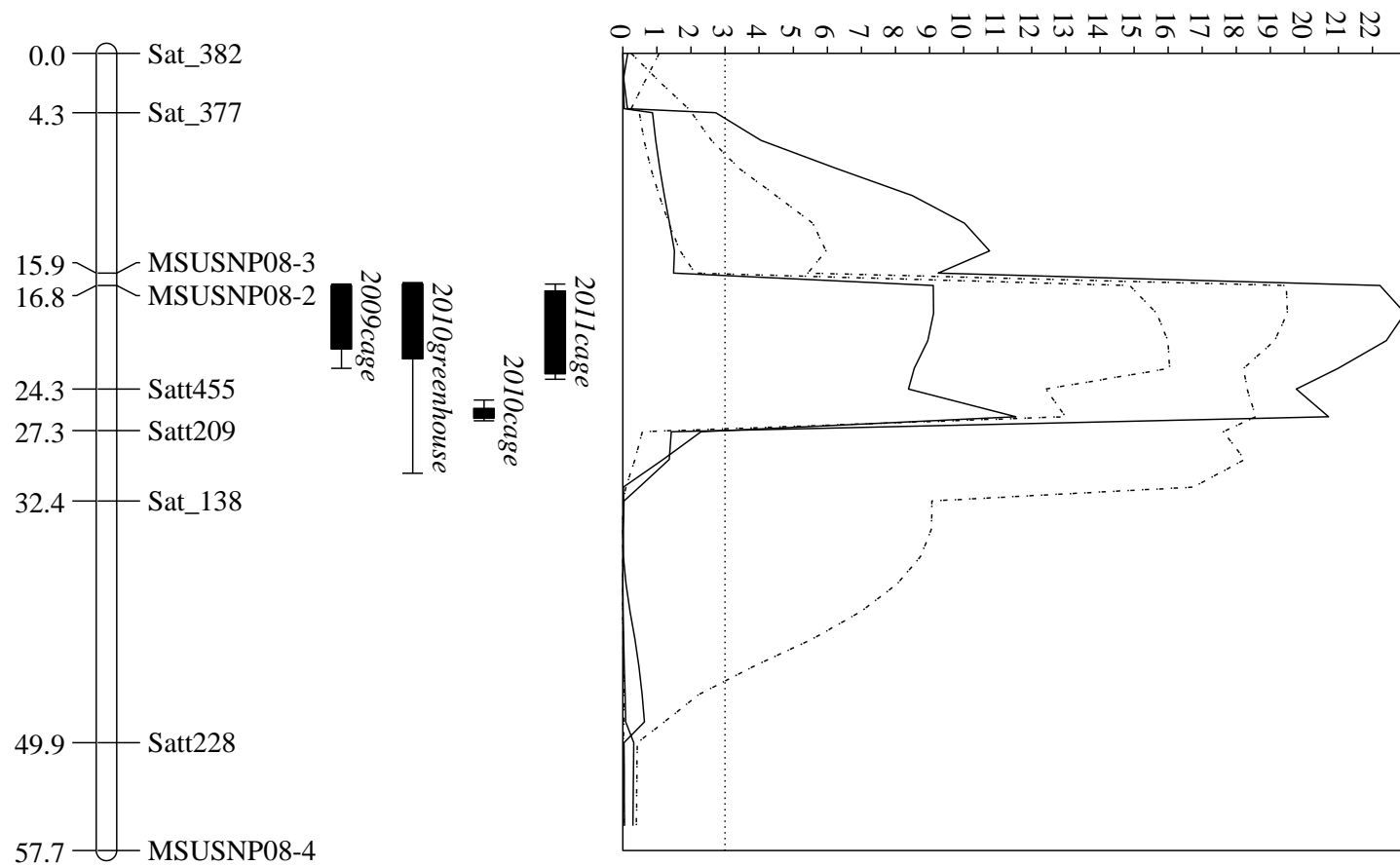
165.7 — Satt378
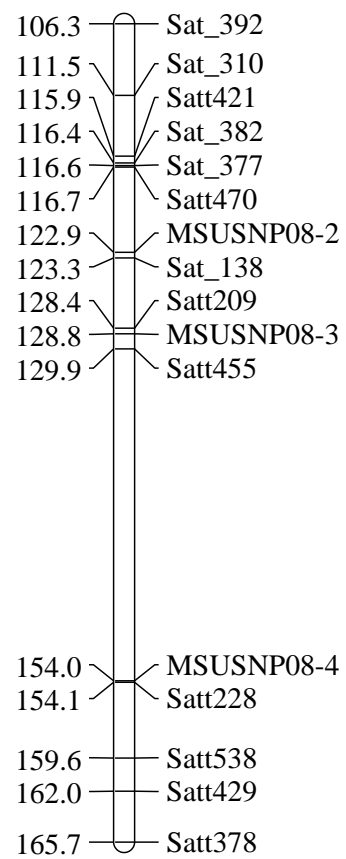
Figure 3.2. (cont'd)

C

Chr.8
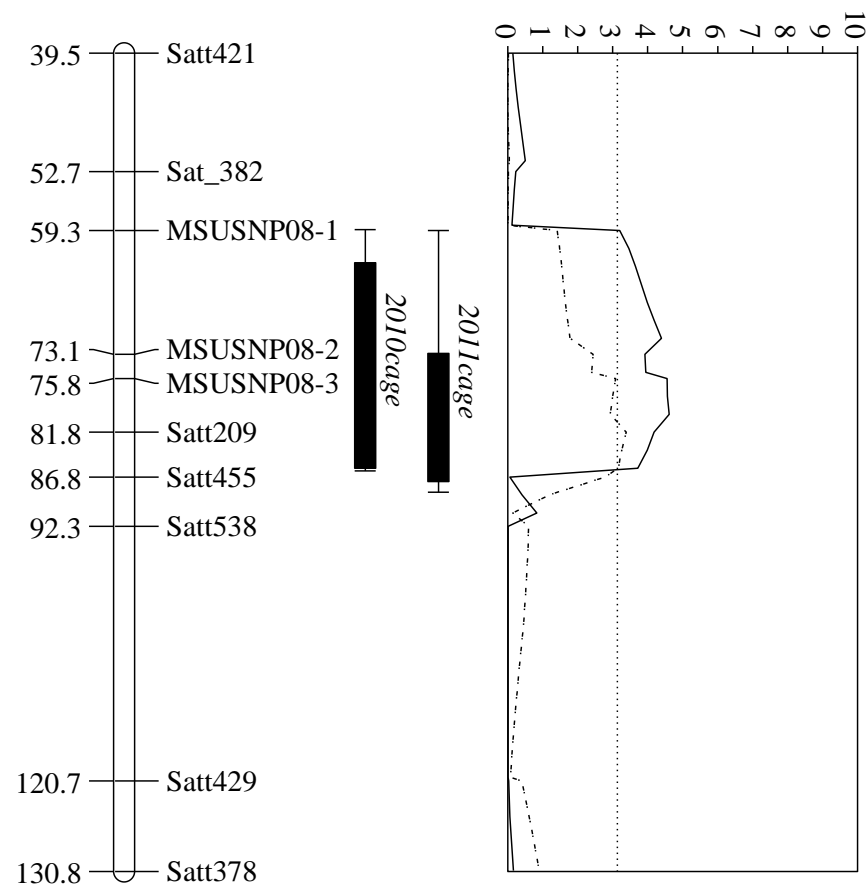
Figure 3.2. (cont'd)

D

Chr.16
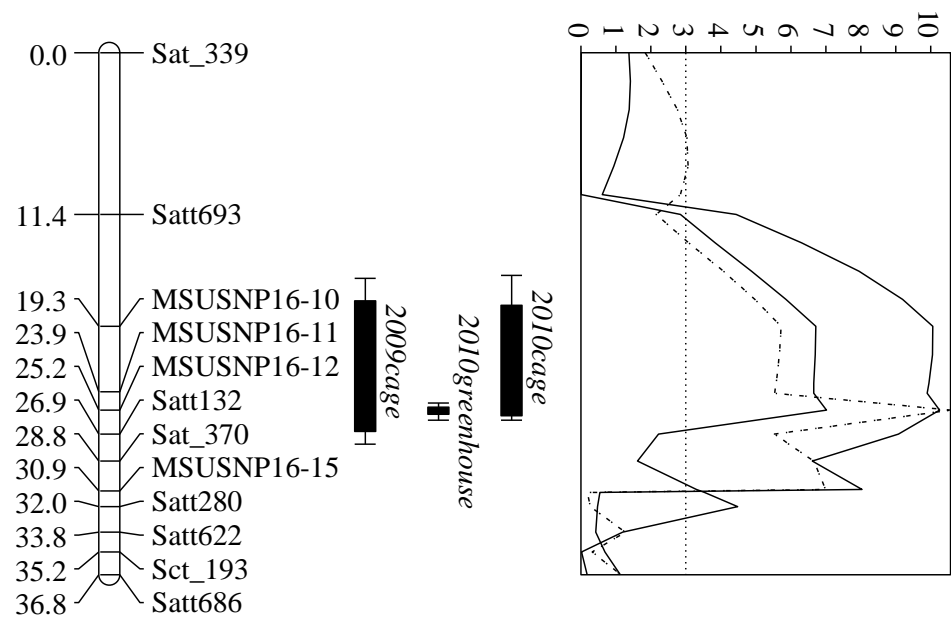
Figure 3.2. (cont'd)

E

Chr.16
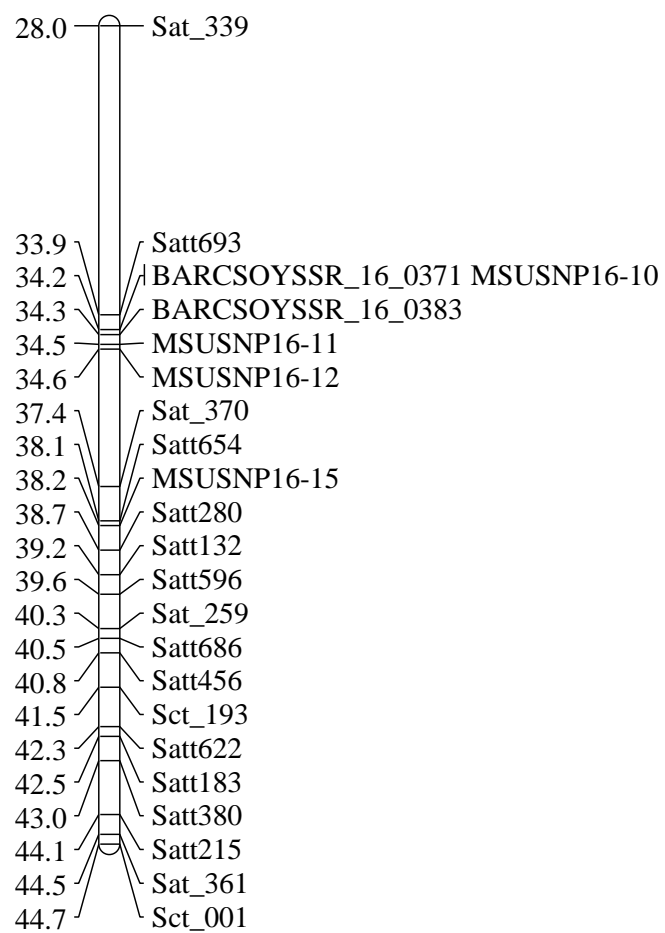
Figure 3.2. (cont'd)

F

Chr.16
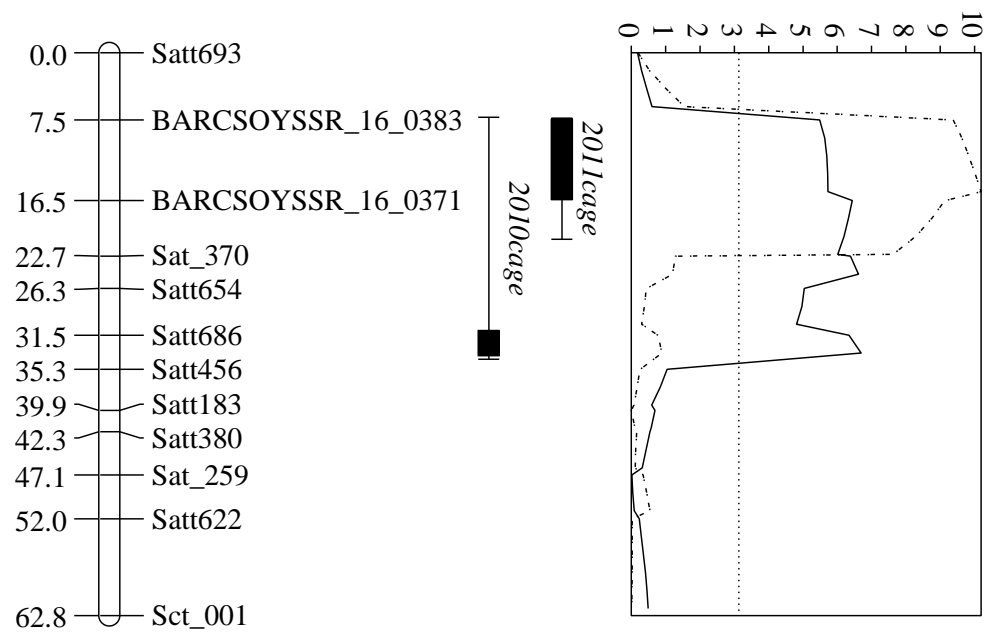
Figure 3.2. (cont'd)

G

Chr.7
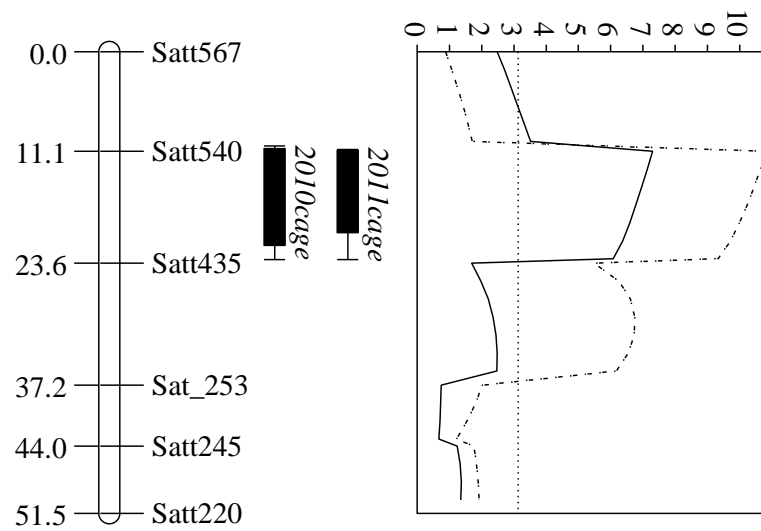
Figure 3.2. (cont'd)

H

Chr.7
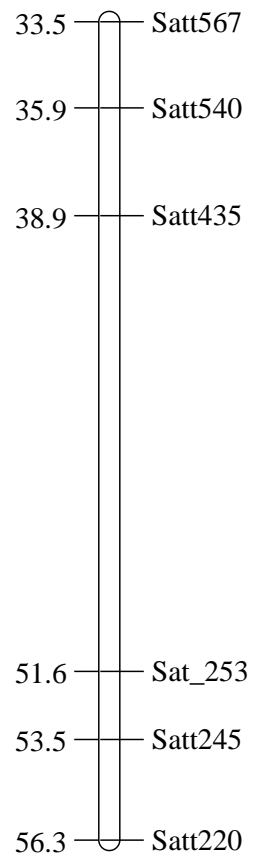
Figure 3.3. Average aphid damage index (DI) of progeny in three genotypic groups for each marker close to the aphid resistance locus detected in the validation population E08929 × E08934, in 2010 and 2011 field trials. The homozygous group with allele derived from parent E08934 was denoted as *AA*. The homozygous group with allele came from parent E08929 was denoted as *BB*. The heterozygous group with both alleles was denoted as *AB*. one-way analysis of variance was conducted at a significance level of 0.05. *Error bars* indicate 95% confidence interval of average DI. A) The average DI of genotypic group *AA*, *AB* and *BB* of marker Satt540 (close to *rag*1), three bars on the *left* are from field trial 2010, and bars on the *right* are from field trial 2011; B) The average DI of genotypic group *AA*, *AB* and *BB* of marker Satt209 (close to *Rag*6), three bars on the *left* are from field trial 2010, and bars on the *right* are from field trial 2011; C) The average DI of genotypic group *AA*, *AB* and *BB* of marker BARCSOYSSR_16_0371 (close to *Rag*3(?)), three bars on the *left* are from field trial 2010, and bars on the *right* are from field trial 2011.

Figure 3.3. (cont'd)

A
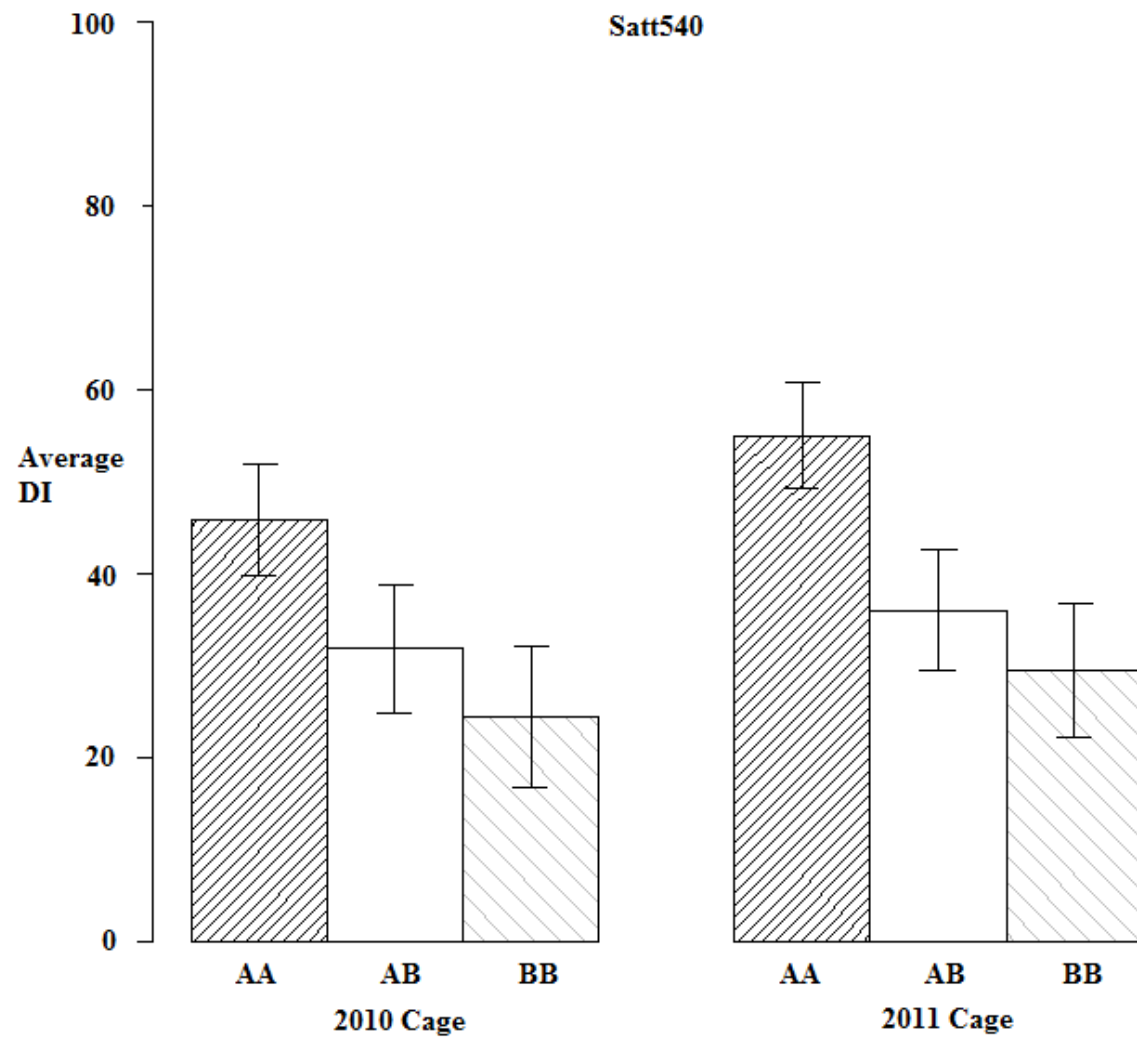
Figure 3.3. (cont'd)

B
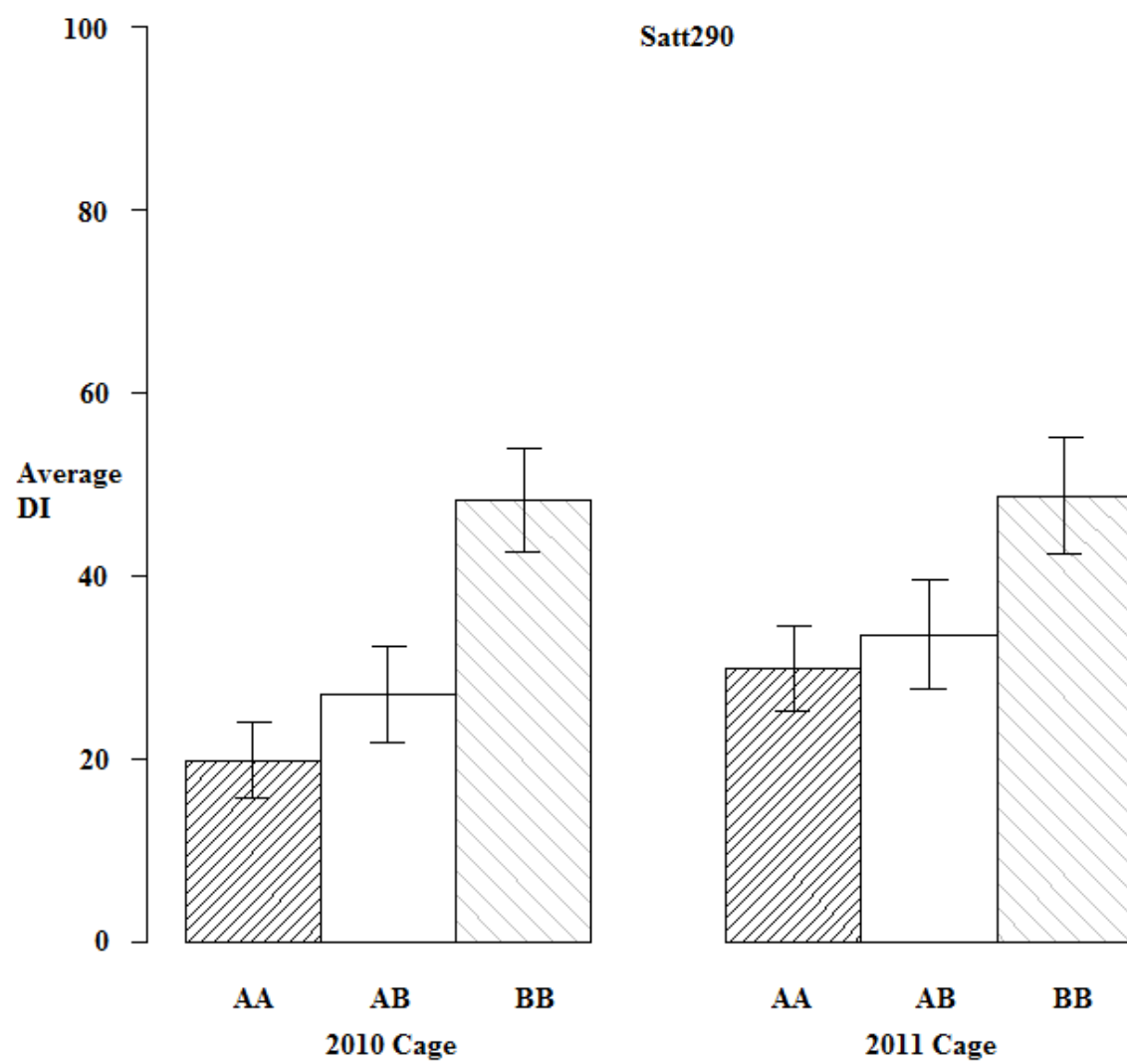
Figure 3.3. (cont'd)

C
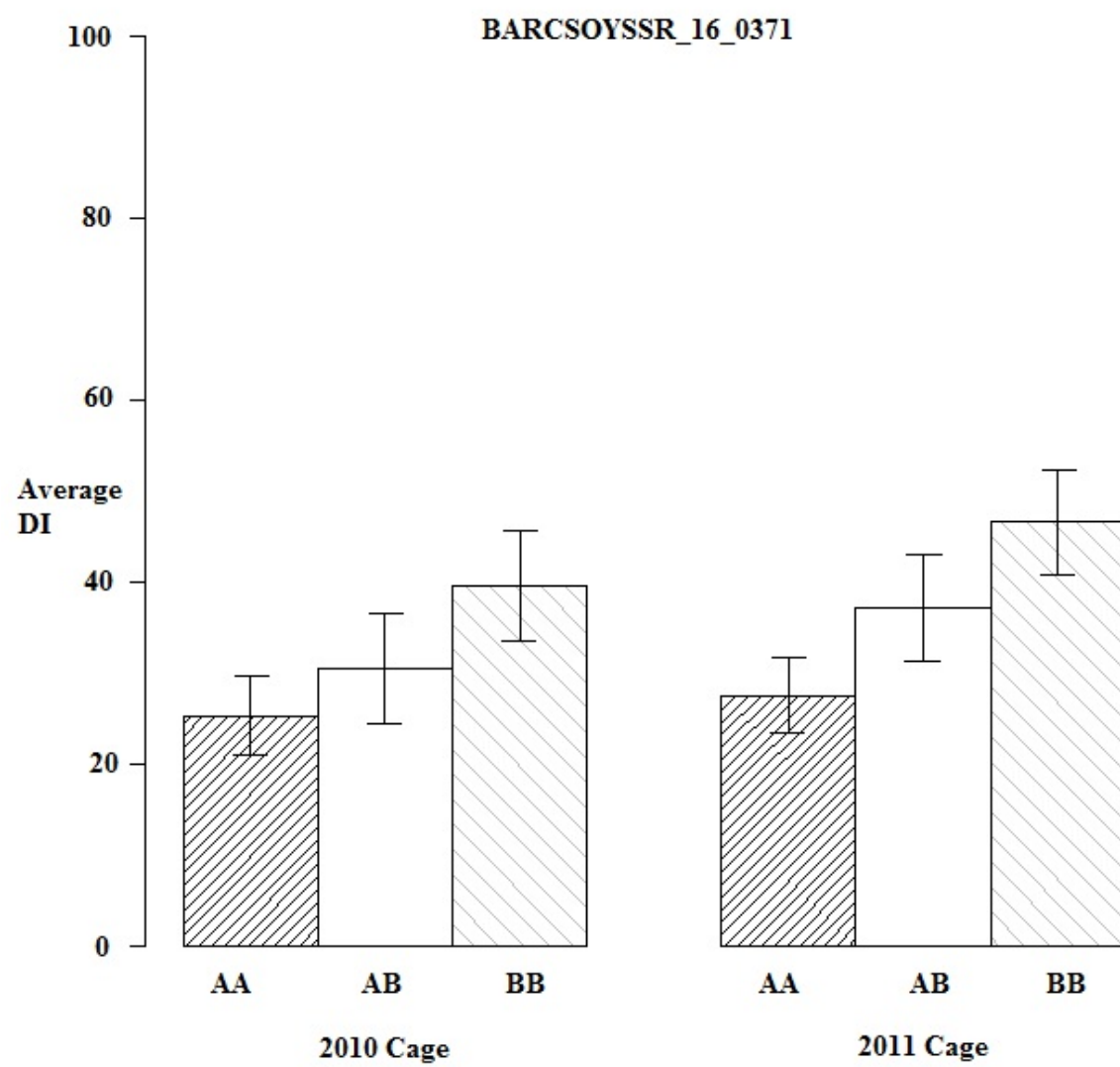
Figure 3.4. Average aphid damage index (DI) for progeny that have no resistance genes, one locus, two loci and all three loci from the validation population E08929 ×E08934 in 2010 field trial.
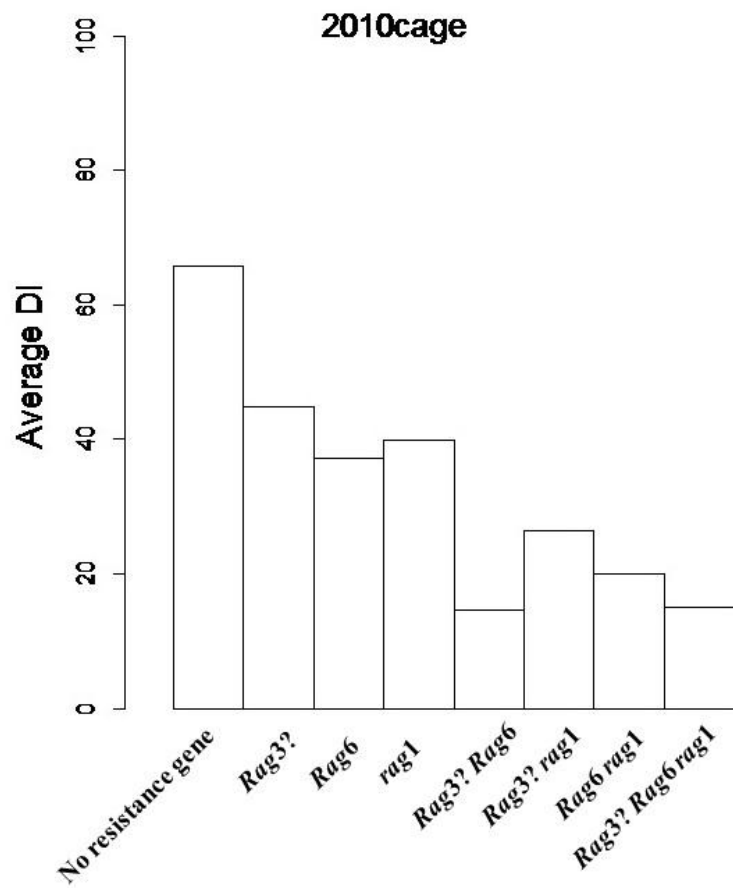
Figure 3.5. Average damage index (DI) from aphid no-choice test of selected lines from the mapping population E00003 ×E08934, line 24 with *Rag*3(?), line 131 with *Rag*6 and line 19 with both loci. E00003 was used as susceptible check, and PI 567598B was antibiosis resistance check. One-way analysis of variance was conducted at a significance level of 0.05. *Error bars* indicate 95% confidence interval of average DI.
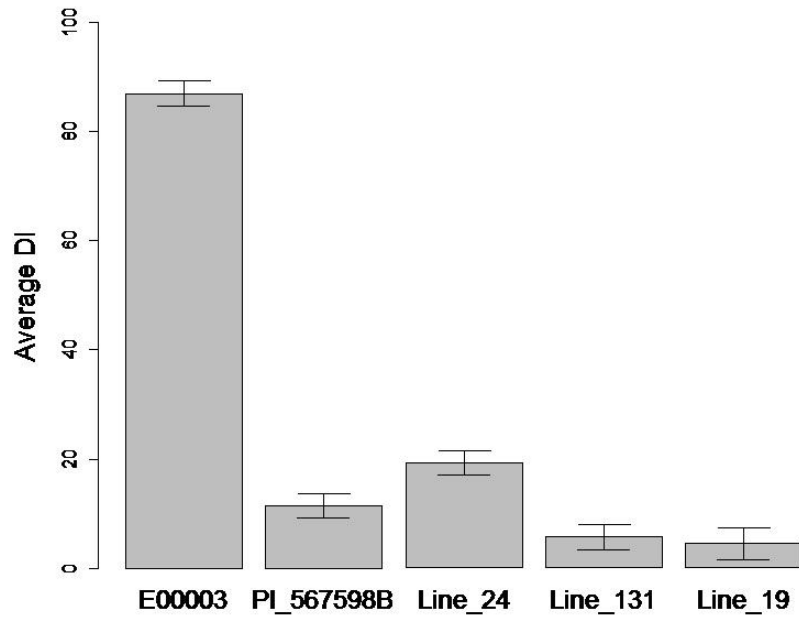
CHAPTER 4

**FINE MAPPING OF APHID RESISTANCE GENE *RAG*3 IN SOYBEAN PI 567543C
WITH BI-PARENTAL F$_2$ POPULATION**

ABSTRACT

Soybean aphid can cause total yield loss in severe infestation and seed quality loss due to virus-transmission. Chemical controls are not cost-effective and may harm beneficial insects in the environment. Since the first reported finding in Wisconsin, United States, many efforts have been made to identify host plant resistance to aphids. Partially dominant locus *Rag*3 was identified from PI 567543C and mapped to a 10 cM interval between simple sequence repeat (SSR) markers Sat_339 and Satt414 on chromosome 16 (linkage group J) (Zhang et al., 2010). The objectives of this study were to fine map *Rag*3 in bi-parental F_2 populations to identify tightly linked SNP markers to functional genes for marker-assisted selection. Advanced breeding lines E10902 and E10905 are derived from PI 567543C which is resistant to soybean aphid, while E07048 is susceptible to aphid. 1,889 F_2 lines were developed from a cross between E07048 $\times$ E10902 and 1,913 F_2 lines were also obtained from a cross between E10905 $\times$ E07048. All 1,889 F_2 lines from E07048 $\times$ E10902 were phenotyped for aphid resistance in the greenhouse, and a subset of 376 F_2 lines were genotyped with TaqMan$^{®}$ SNP markers at 6.16, 6.26, 6.41, 6.42 and 8.05 Mbp on chr.16. A major locus explaining 44.7% of the phenotypic variation was detected between markers MSUSNP16-10 (Gm16_6262227) and MSUSNP16-12 (Gm16_6423098) on chr.16 with QTL analysis of these 376 lines. All 3802 F_2 were genotyped with SNPs at 6.16 and 8.05 Mbp to select recombinants. 983 F_3 progeny of 102 F_2 recombinants were planted for aphid resistance evaluation in the greenhouse and genotyped with SNPs at 6.16, 6.26, 6.41, 6.423 and 6.424 Mbp on chr.16. Sixteen susceptible, heterozygous and recombinant

F$_3$ lines were selected and applied onto Infinium$^®$ SNP assays. Two F$_3$ recombinants with crossovers between 6.26 and 6.47 Mbp were identified and confirmed according to their F$_4$ progeny phenotypes and genotypes from the SNP Beadchip. In conclusion, *Rag3* is fine mapped to 207 Kbp between TaqMan$^®$ SNPs MSUSNP16-10 (Gm16_6262227) and Gm16_6469551_A_C with serine-threonine protein kinase coding genes as candidate functional genes. TaqMan$^®$ SNPs MSUSNP16-10 (Gm16_6262227), MSUSNP16-11 (Gm16_6413214) and MSUSNP16-12 (Gm16_6423098) can be used in MAS for aphid resistance of *Rag3* gene. Further genetics studies can be performed based on the candidate information provided in this study for gene cloning and functional analysis.

INTRODUCTION

Soybean aphid has become the most destructive insect pest in US soybean production since its first reported finding in Wisconsin in 2000 (Ragsdale et al., 2004). Yield losses to soybean aphids were estimated over 50% in Minnesota in 2001 (Ostlie, 2002). Insecticide application among the 12 north-central soybean growing states increased from 4 to 14 M ha per year due to the rapid outbreak of soybean aphids (O'Neal and Johnson, 2010). Additionally, since soybean aphid is a phloem feeder, it can transmit plant virus, such as *soybean mosaic virus*, *Tobacco ring-spot virus* to soybean, and *Potato virus Y* to potato (Kim et al., 2008; Mian et al., 2008a; Pedersen et al., 2007).

Aphid resistance has been considered a quantitative trait with a continuous phenotype distribution of damage index (Michel et al., 2009). Since chemical insecticides add more cost and may harm beneficial insects and the environment, breeding host resistant soybean has been proposed in the literature as a key component in effective integrated pest management (IPM) against soybean aphids (O'Neal and Johnson, 2010). Five loci, with seven alleles, *Rag*1*, rag*1*, Rag*2*, Rag*3*, rag*3*, rag*4 and *Rag*5 have been reported for aphid resistance conducting either antixenosis or antibiosis forms of resistance (Brachfeld and Mary, 2007; Jun et al., 2012; Kang et al., 2008; Li et al., 2007; Mian et al., 2008b; Zhang et al., 2009; Zhang et al., 2010). Among these resistance alleles, partially dominant *Rag*3 was mapped to a 10 cM interval between SSR markers Sat_339 and Satt414 on chromosome 16 (linkage group J) by Zhang et al. (2010). With the high density of SNP markers, pinpointing genes becomes possible to better facilitate marker-assisted selection (MAS) (Bernardo, 2008; Chaisan et al., 2012; Collard et al., 2005). However, to achieve higher mapping resolution, the occurrence of recombination is equivalently important

as high density SNPs. In this study, two large F_2 populations were developed with nearly 4,000 plants to increase the number of recombinants within the region of interest. The objectives of this study were to fine map partially dominant *Rag*3 with closely linked SNP markers for MAS, for gene cloning and further genetic studies.

MATERIALS AND METHODS

Plant materials

Two bi-parental F_2 populations were developed with 1,889 F_2 lines from the cross of E07048 $\times$ E10902 and 1,913 F_2 lines from E10905 $\times$ E07048. E10902 and E10905 are derived from PI 567543C which is resistant to soybean aphid, while E07048 is susceptible to aphid.

Aphid resistance phenotype evaluation

In total, 3802 F_2 plants, together with the parents, E07048, E10902 and E10905, were planted and inoculated with aphids in the spring of 2011, in the Plant Science Greenhouses at Michigan State University (MSU) in East Lansing, Michigan. Eight F_2 plants were grown in a 105-mm-diameter and 125-mm-deep plastic pots, maintained at 26/15°C day/night temperature with sodium vapor lights as supplement (Zhang et al., 2010). The parents were replicated three times to serve as susceptible and resistant checks. Two wingless aphids were transferred onto the un-expanded trifoliate at the V1 stage (Fehr and Caviness, 1977). After the susceptible parent E07048 reached severe infestation stage, with more than 600 aphids per plant, all 1,889 F_2 plants from cross E07048 $\times$ E10902 were rated for phenotype, using a simplified scale modified from Mensah et al. (2005; 2008), with $1 = 0 - 200$ aphids/plant^{-1} and healthy appearance; $2 = 201 - 600$ aphids/plant^{-1} and healthy appearance with slight curling; $3 = $ more than 600 aphids/plant^{-1} and stunting appearance with cast skins.

After associated the phenotypic data with the genotypic data of flanking SNPs

MSUSNP16-14 (Gm16_6164774) and MSUSNP16-15 (Gm16_8051585), F_3 progeny of F_2

recombinants were planted in the same greenhouse in the fall of 2011. The F_3 plants were

maintained and evaluated for aphid resistance in the same manner as the F_2 plants. Further, F_4

progeny of F_3 recombinants were phenotyped in the greenhouse in the spring of 2012 to confirm

the crossovers within the region of interest in the selected F_3 recombinants.

DNA preparation and marker genotyping

A quick DNA extraction method was adapted to obtain the genomic DNA from all F_2

plants. One young non-expanded trifoliate of each plant was collected into 96-deep-well PCR

plates and incubated in 94 ℃ oven with 100ul TE buffer (10mM Tris-HCl and 0.5M EDTA, pH

7.5) for 30 min. DNA was further diluted 10 times with $0.1 \times$ TE for TaqMan$^{®}$ assay in

LightCycler$^{®}$ 480 (Roche Applied Science, Indianapolis, IN). A TaqMan$^{®}$ assay protocol

described by Zhang et al. (unpublished data) was used for SNP allele-specific genotyping

analysis. In total, six TaqMan$^{®}$ SNPs were designed: MSUSNP16-14 (Gm16_6164774),

MSUSNP16-10 (Gm16_6262227), MSUSNP16-11 (Gm16_6413214), MSUSNP16-12

(Gm16_6423098), MSUSNP16-13 (Gm16_6424067) and MSUSNP16-15 (Gm16_8051585) (the

numeric string is the physical position of the SNP). A subset of 376 F_2 plants from the cross

E07048 $\times$ E10902 was genotyped with five SNPs, MSUSNP16-14 (Gm16_6164774),

MSUSNP16-10 (Gm16_6262227), MSUSNP16-11 (Gm16_6413214), MSUSNP16-12 (Gm16_6423098) and MSUSNP16-15 (Gm16_8051585). After QTL analysis, SNPs at 6.16 and 8.05 Mbp were used to determine recombinants for all F_2 lines. Genomic DNA F_3 and F_4 recombinants were quickly extracted and genotyped with SNP TaqMan[®] assay in the same manner as F_2 plants. All F_3 progeny of the F_2 recombinants were genotyped with SNPs MSUSNP16-14 (Gm16_6164774), MSUSNP16-10 (Gm16_6262227), MSUSNP16-11 (Gm16_6413214), MSUSNP16-12 (Gm16_6423098) and MSUSNP16-13 (Gm16_6424067). The selected 16 F_3 lines including susceptible lines, heterozygous lines and recombinants were applied onto Infinium[®] SNP assays with high resolution customer designed soybean Beadchip containing more than 52,000 SNPs (Song et al., 2011).

Data analysis

The histograms of phenotypic data were generated in Excel[®] 2010 for Windows[®]. The association between markers and phenotypes were performed in R, at significance level of 0.05. Linkage analysis was performed with the subset of 376 F_2 plants from the cross E07048 × E10902. A genetic linkage map was constructed in JoinMap 3.0 with Kosambi's and LOD = 3.0 (Van Ooijen, 2006) with the five SNPs between 6.16 and 8.05 Mbp. QTL analysis was conducted in QTL Cartographer Version 2.5 (Wang et al., 2008) with composite interval mapping (CIM) and multiple interval mapping (MIM) methods. MapChart (Voorrips, 2002) was used to visualize the position of the QTL.

RESULTS AND DISCUSSION

Phenotype of soybean aphid resistance

The phenotypes of all 1889 F_2 plants and its subset of 376 plants from cross E07048 $\times$

E10902 were plotted as histograms (Fig. 4.1). The ratio for the three scores among all F_2 plants

was 5.06: 8.48: 4.72, which was not significantly different from 1:2:1 in Pearson Chi-square test

(data not shown). The ratio of the F_2 plant subset was 1.24: 1.64: 0.88. F_2 plants from cross

E10905 $\times$E07048 were not rated due to technical difficulties.

Validation of *Rag*3 in F_2 population

With the genotypic data of 376 F_2 plants from the cross E07048 $\times$E10902, a 18.7 cM

long linkage map was constructed with five SNPs, MSUSNP16-14 (Gm16_6164774),

MSUSNP16-10 (Gm16_6262227), MSUSNP16-11 (Gm16_6413214), MSUSNP16-12

(Gm16_6423098)and MSUSNP16-15 (Gm16_8051585) (Fig. 4.2). The marker order is

consistent with the reported physical positions (Song et al., 2011). The slight inflation of the

linkage map was caused by genotyping errors from SNP TaqMan$^{\circledR}$ assay. One QTL was

detected for both CIM and MIM methods between SNPs MSUSNP16-10 (Gm16_6262227) and

MSUSNP16-12 (Gm16_6423098) (Fig. 4.2). The resistant parent E10902 was derived from PI

567543C with no other aphid resistance source integrated; therefore, we conclude that this QTL

is indeed *Rag*3. The LOD scores are 38.7 for CIM and 44.2 for MIM. R^2 was estimated

approximately as 0.447 in both CIM and MIM, suggesting that *Rag*3 can explain about 44.7% of

the phenotype variance among these 376 F_2 plants (data not shown).

Fine mapping *Rag*3 with recombinant lines

With the validation of *Rag*3 between 6.1 and 8.0 Mbp, SNPs MSUSNP16-14

(Gm16_6164774) and MSUSNP16-15 (Gm16_8051585) were used to select recombinants

within 6.1 and 8.0 Mbp conservatively. A total of 102 F_2 plants were identified as recombinants

within this region. After genotyping all 983 F_3 progeny with SNPs, MSUSNP16-14

(Gm16_6164774) and MSUSNP16-15 (Gm16_8051585), 16 F_3 lines were selected for further

genotyping with 52K SNP Beadchip. The genotypes of these 16 F_3 plants with 10 polymorphic

SNPs between 6.185 to 6.522 Mbp on Chr. 16 are shown in Table 4.1. First three lines with all

markers genotypes homozygous susceptible allele showed susceptible phenotype in the

greenhouse aphid resistance test. Nine segregating lines of these SNPs showed intermediate

resistance and resistance phenotypes, and the following two homozygous lines of the resistance

allele showed resistance phenotype. This evidence is consistent with the result from the QTL

analysis above, suggesting that *Rag*3 locates at least within 6.1.85 and 6.522 Mbp on Chr. 16.

Most interestingly, two aphid resistant F_3 lines, 2-499-2 and 2-277-6 were observed with

crossovers within this region (Table 4.1). For line 2-499-2, all SNPs within 6.185 and 6.262 Mbp

are homozygous susceptible type and the remaining SNPs to the right are alleles from the

resistant parent, indicating the genotype on the left of 6.262 Mbp has no effect on its phenotype.

Similarly for line 2-277-6, SNPs on the right of 6.470 Mbp do not affect the resistance phenotype. These two recombinants within 6.262 and 6.470 Mbp region narrowed the *Rag*3 region down to a 207 Kbp length on Chr. 16. To further confirm this finding, two F_4 progeny of 2-499-2 and 14 F_4 progeny of 2-277-6 were phenotyped and genotyped with 52K SNP Beadchip. As shown in Table 4.1, the phenotypes and genotypes of all the 16 F_4 progeny are highly consistent as their F_3 parents, confirming the 207 Kbp region of *Rag*3. The distance between SNPs of the two crossovers is actually 17 Kbp. To be conservative, one SNP more on each side with a total distance of 207 Kbp is considered to make sure *Rag*3 gene is covered. The limitation to further narrow this region down is the lack of recombination events and polymorphic SNP markers within this interval. Therefore, Illunima$^{®}$ Hi-Seq sequencing is ongoing to develop more SNP markers in the region of 6.2 to 6.4 Mbp. Progeny of segregating F_3 plants will continue to be planted for phenotyping and genotyping.

Association of SNPs MSUSNP16-10 (Gm16_6262227), MSUSNP16-11 (Gm16_6413214) and MSUSNP16-12 (Gm16_6423098) between genotypes and the phenotypes

Correlations of SNPs MSUSNP16-10 (Gm16_6262227), MSUSNP16-11 (Gm16_6413214) and MSUSNP16-12 (Gm16_6423098) genotypes and aphid resistance phenotypes in 376 F_2 subset of E07048 by E10902 and all F_3 progeny of 102 F_2 recombinants are summarized in Table 4.2. Among the subset of 376 F_2 lines, the correlation coefficients of SNP genotype and aphid resistance phenotype ranged from 0.52 to 0.68 with $P < 0.0001$. These

three SNPs explain 27 to 47% of the phenotypic variation. Among all 983 F$_3$ progeny, the correlation coefficients ranged from 0.60 to 0.66 with $P < 0.0001$, explaining 36 to 43% of the phenotypic variation. Therefore, these three TaqMan$^{\circledR}$ SNPs, MSUSNP16-10 (Gm16_6262227), MSUSNP16-11 (Gm16_6413214) and MSUSNP16-12 (Gm16_6423098) can be used as genotypic indicator for aphid resistance in MAS.

Table 4.1. Single nucleotide polymorphism (SNP) genotypes and phenotypes of selected F$_3$ and F$_4$ lines on 52K SNP Beadchip.

Physical Position (Mbp)		6.185	6.262	6.413	6.423	6.424	6.431	6.47	6.484	6.497	6.522
F$_3$ plants											
Line	Phenotype										
2-257-2	S[†]	S	S	S	S	S	S	S	S	S	S
3-386-9	S	S	S	S	S	S	S	S	S	S	S
4-58-40	S	S	S	S	S	S	S	S	S	S	S
rem18-5	Seg.[††]	H[§]	H	H	H	H	H	H	H	H	H
rem19-6	Seg	H	H	H	H	H	H	H	H	H	H
rem19-12	Seg	H	H	H	H	H	H	H	H	H	H
3-262-8	Seg	H	H	H	H	H	H	H	H	H	H
3-84-5	R[#]	H	H	H	H	H	H	H	H	H	H
2-291-1	R	H	H	H	H	H	H	H	H	H	H
4-31-4	R	H	H	H	H	H	H	H	H	H	H
3-386-2	R	H	H	H	H	H	H	H	H	H	H
4-248-7	R	R	H	H	H	H	H	H	H	H	H
2-381-30	R	R	R	R	R	R	R	R	R	R	R
3-38-2	R	R	R	R	R	R	R	R	R	R	R
2-499-2	R	S	**S**	**R**	R	R	R	R	R	R	R
2-277-6	R	R	R	R	R	R	**R**	S	S	S	S
F$_4$ plants											
2-499-2-1	R	S	**S**	**R**	R	R	R	R	R	R	R
2-499-2-2	R	S	**S**	**R**	R	R	R	R	R	R	R
2-277-6-1	R	R	R	R	R	R	**R**	S	S	S	S
2-277-6-2	R	R	R	R	R	R	**R**	S	S	S	S
2-277-6-3	R	R	R	R	R	R	**R**	S	S	S	S
2-277-6-4	R	R	R	R	R	R	**R**	S	S	S	S

Table 4.1. (cont'd)

Physical Position (Mbp)		6.185	6.262	6.413	6.423	6.424	6.431	6.47	6.484	6.497	6.522
	F4 plants										
Line	Phenotype										
2-277-6-5	R	R	R	R	R	R	**R**	**S**	S	S	S
2-277-6-6	R	R	R	R	R	R	**R**	**S**	S	S	S
2-277-6-7	R	R	R	R	R	R	**R**	**S**	S	S	S
2-277-6-8	R	R	R	R	R	R	**R**	**S**	S	S	S
2-277-6-9	R	R	R	R	R	R	**R**	**S**	S	S	S
2-277-6-10	R	R	R	R	R	R	**R**	**S**	S	S	S
2-277-6-11	R	R	R	R	R	R	**R**	**S**	S	S	S
2-277-6-12	R	R	R	R	R	R	**R**	**S**	S	S	S
2-277-6-13	R	R	R	R	R	R	**R**	**S**	S	S	S
2-277-6-14	R	R	R	R	R	R	**R**	**S**	S	S	S

[†] Susceptible;

[††] Segregating;

[§] Heterozygous;

[#] Resistant.

Table 4.2. Correlations of single nucleotide polymorphism (SNP) with phenotypes in 376 F_2 lines from cross E07048 $\times$ E10902 and all 983 F_3 progeny of 102 F_2 recombinants. MSUSNP16-10 (Gm16_6262227), MSUSNP16-11 (Gm16_6413214) and MSUSNP16-12 (Gm16_6423098) were developed from 52K SNP Beadchip (Song et al., 2011).

SNP markers	376 F2 lines			983 F3 lines		
	Cor. Coe. [†]	*r* square	*p* value	*Cor. Coe.*	*r* square	*p* value
MSUSNP16-10	0.52	0.27	1.24E-17	0.60	0.36	1.24E-70
MSUSNP16-11	0.68	0.47	3.50E-45	0.66	0.43	7.96E-96
MSUSNP16-12	0.58	0.33	1.49E-30	0.61	0.38	6.52E-81

[†] Correlation coefficient.

Figure 4.1. Phenotype histogram of soybean aphid rating score of F_2 plants from bi-parental cross E07048 $\times$E10902, where E07048 is susceptible to aphid and E10902 is resistant. A. phenotype distribution of all 1889 F_2 plants with *y-axis* being the percentage of total plants for each score; B. phenotypes of a subset of 376 F_2 plants from the same population with *y-axis* being the number of F_2 plants for each score.
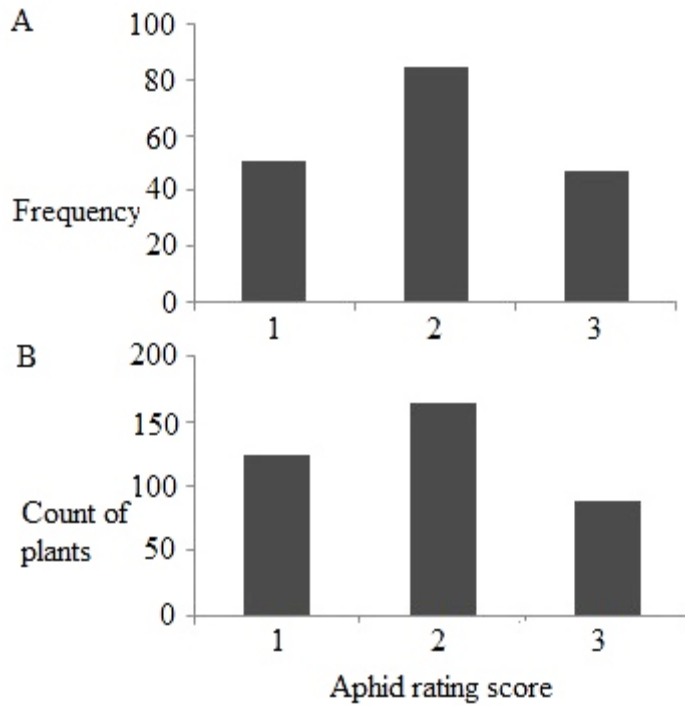
Figure 4.2. Validation *Rag*3 location on Chromosome 16 using a subset of 376 F₂ plants from cross E07048 ×E10902, with E07048 and E10902 being susceptible and resistant parent. QTL analysis was conducted with both composite interval mapping and multiple interval mapping methods with $P < 0.0001$. MSUSNP16-14 (Gm16_6164774), MSUSNP16-10 (Gm16_6262227), MSUSNP16-11 (Gm16_6413214), MSUSNP16-12 (Gm16_6423098) and MSUSNP16-15 (Gm16_8051585) are SNP markers with physical position indicated in the middle of the marker names. The black bar next to the linkage map indicates the location of *Rag*3 with composite interval mapping method; the grey bar on the *right* shows that with multiple-interval mapping method.

Chr.16
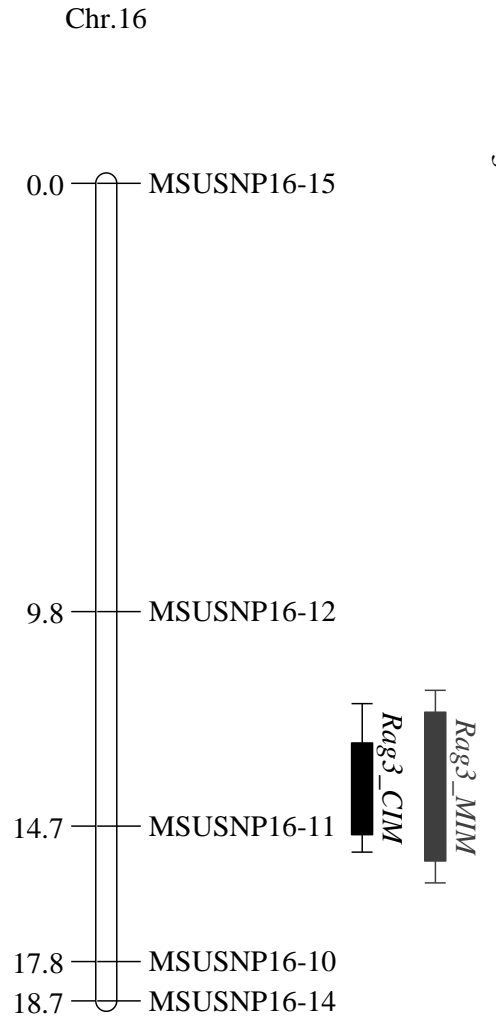
APPENDIX

GENOTYPING AND GENETIC ANALYSIS OF SUDDEN DEATH SYNDROMES IN SOYBEAN

Plant Materials and summary of phenotypes

The objective of this project was to identify new loci in GD2422 for resistance to sudden death syndromes (SDS). A total of 243 lines from two F_4-derived mapping populations GD2422 $\times$ LD01-5907 and GD2422 $\times$ Skylla were developed and phenotyped for SDS resistance with four replications at Decatur, Michigan in summer of 2011. Line 1- 129 derived from GD2422 $\times$ LD01-5907, and line 130- 243 are from GD2422 $\times$ Skylla. GD2422 and LD01-5907 are both resistant to SDS while Skylla is susceptible.

SDS phenotypic data were collected on three different dates, August 2, 15 and 22 of 2011. On the first two visits, a disease index (DX) described by Cathy Schmidt (Southern Illinois University, personal communications) was employed as a phenotypic indicator. DX was defined with two components: combing disease incidence (DI) and disease severity (DS) as followed: $DX = DI \times DS/9$, where DI is the percentage of plants with leaf symptoms, and DS is the most severe damage on each line, ranging from 1 (1-10% of leaf chlorotic) to 9 (premature death). On August 22, DX and average disease index (AvDX) was also collected and estimated. AvDX is modified as follows: $AvDX = DI \times \overline{DS}/9$. Instead of the worst symptoms of a line, $\overline{DS}$ is recorded as the average disease severity in the area with disease symptoms. Both DX and AvDX range from 0 to 100 for the most resistant type to the most susceptible type. DS, DX and AvDX of the first mapping population are plotted in Fig. A.1, and those of the second population are

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plotted in Fig. A.2. The heritability with 90% confidence interval of these traits is summarized in

Table A.1. August 15 disease index (Aug15DX) and August 22 average disease index

(Aug22AvDX) fit normal distribution and the correlation coefficient is 0.957 (data not shown).

These were selected as phenotypic indicators in QTL analysis. In the first mapping population,

the estimated heritability was all above 0.80. There are fewer lines in the second mapping

population with lower heritability ranging from 0.11 to 0.62. However, the heritability of the

traits used, Aug15DX and Aug22AvDX, are 0.43 and 0.58, respectively.

Selective genotyping

A subset of 93 lines was selected according to Table A.2 for genotyping on 6K SNP chip.

Aug22AvDX was divided into eight categories as the first column of Table A.2 and 93 lines

were selected from cross GD2422 $\times$ LD01-5907 to form a subset with a normal distribution for

initial QTL analysis. The phenotypic traits of the subset were plotted in Fig. A.3. Both Aug15DX

and Aug22AvDX showed normal distribution in the selected subset.

Genetic analysis

A genetic map was constructed with JoinMap 4.0 with the maximum likelihood method.

Polymorphic SNP markers on each chromosome are summarized in Table A.3. The genetic map

of the 20 chromosomes constructed with the selected subset was visualized in MapChart (data

not shown). Single marker analysis (SMA) was performed in Windows QTL Cartographer 2.5.

In the QTL analysis, there were 27 linkage fragments. The chromosomal labels in QTL

95

Cartographer and their corresponding chromosomes are listed in Table A.4. The result of SMA is

not shown here. As preliminary results show, two significant QTLs were detected on Chr. 18

(linkage group G) and Chr. 19 (linkage group L) with trait August 22AvDX. Several loci have

been previously identified as resistant to SDS on these two chromosomes (Grant et al., 2010).

Further analysis with SNP genotyping assays within potential regions found in SMA and more

phenotypic data are required to confirm if they are novel loci for SDS resistance.

Table A.1. Broad sense heritability of SDS phenotypic with 90% confidence intervals, August 2 disease severity (Aug2DS), August 2 disease index (Aug2DX), August 15 disease severity (Aug15DS), August 15 disease index (Aug15DX), August 22 disease index (Aug22DX) and August 22 average disease index (Aug22AvDX) of the two mapping populations from cross GD2422 $\times$ LD01-5907 and GD2422 $\times$ Skylla.

Trait	Heritability	90% CI upper limit	90% CI lower limit
GD2422 $\times$ LD01-5907			
Aug2DS	0.88	0.91	0.85
Aug2DX	0.84	0.87	0.79
Aug15DS	0.82	0.86	0.76
Aug15DX	0.9	0.92	0.87
Aug22DX	0.9	0.92	0.88
Aug22AvDX	0.88	0.91	0.85
GD2422 $\times$ Skylla			
Aug2DS	0.65	0.73	0.55
Aug2DX	0.58	0.67	0.46
Aug15DS	0.11	0.3	-0.14
Aug15DX	0.62	0.7	0.51
Aug22DX	0.43	0.55	0.26
Aug22AvDX	0.58	0.67	0.46

Table A.2. Normal distribution table for selective genotyping in mapping population GD2422 ×
LD01-5907.

Range of AvDX	Expected Count	Original Count	New Count
0 to 10	0.2	21	1
11 to 20	2.0	5	2
21 to 30	12.6	11	13
31 to 40	31.6	21	26
41 to 50	31.6	19	27
51 to 60	12.6	9	16
61 to 70	2.0	4	5
71 to 80	0.2	3	3

Table A.3. Summary of polymorphic single nucleotide polymorphism (SNP) markers on each chromosome between the two parents of mapping population GD2422 ×LD01-5907.

Linkage Group	# of polymorphic SNPs
LG01	48
LG02	114
LG03	49
LG04	62
LG05	69
LG06	216
LG07	91
LG08	83
LG09	208
LG10	60
LG11	59
LG12	22
LG13	184
LG14	22
LG15	87
LG16	152
LG17	110
LG18	162
LG19	182
LG20	22
Total Poly.	2002
Total SNPs	5361

Table A.4. Chromosome labels in QTL Cartographer 2.5 software and the corresponding chromosomes according to soybean consensus map (Qijian Song personal communication).

QTL Cartographer	Actual Chromosome
Ch-1, Ch-2	Chr.1-part1, 2
Ch-3, Ch-4	Chr.2-part1,2
Ch-5, Ch-6	Chr.3-part1, 2
Ch-7, Ch-8	Chr.4-part1,2
Ch-9	Chr.5
Ch-10	Chr.6
Ch-11	Chr.7
Ch-12	Chr.8
Ch-13	Chr.9
Ch-14	Chr.10
Ch-15, Ch-16	Chr.11-part1, 2
Ch-17	Chr.12
Ch-18	Chr.13
Ch-19	Chr.14
Ch-20, Ch-21	Chr.15-part1, 2
Ch-22	Chr.16
Ch-23, Ch-24	Chr.17-part1, 2
Ch-25	Chr.18
Ch-26	Chr.19
Ch-27	Chr.20

Figure A.1. Phenotype distributions of soybean line 1-129 from cross GD2422 ×LD01-5907. August 2 disease severity (Aug2DS), August 2 disease index (Aug2DX), August 15 disease severity (Aug15DS), August 15 disease index (Aug15DX), August 22 disease index (Aug22DX) and August 22 average disease index (Aug22AvDX). *Y-axis* is the count of number of plants that fall into each category.

Figure A.2. Phenotype distributions of line 130-243 of GD2422 × Skylla. August 2 disease severity (Aug2DS), August 2 disease index (Aug2DX), August 15 disease severity (Aug15DS), August 15 disease index (Aug15DX), August 22 disease index (Aug22DX) and August 22 average disease index (Aug22AvDX). *Y-axis* is the count of number of plants that fall into each category.
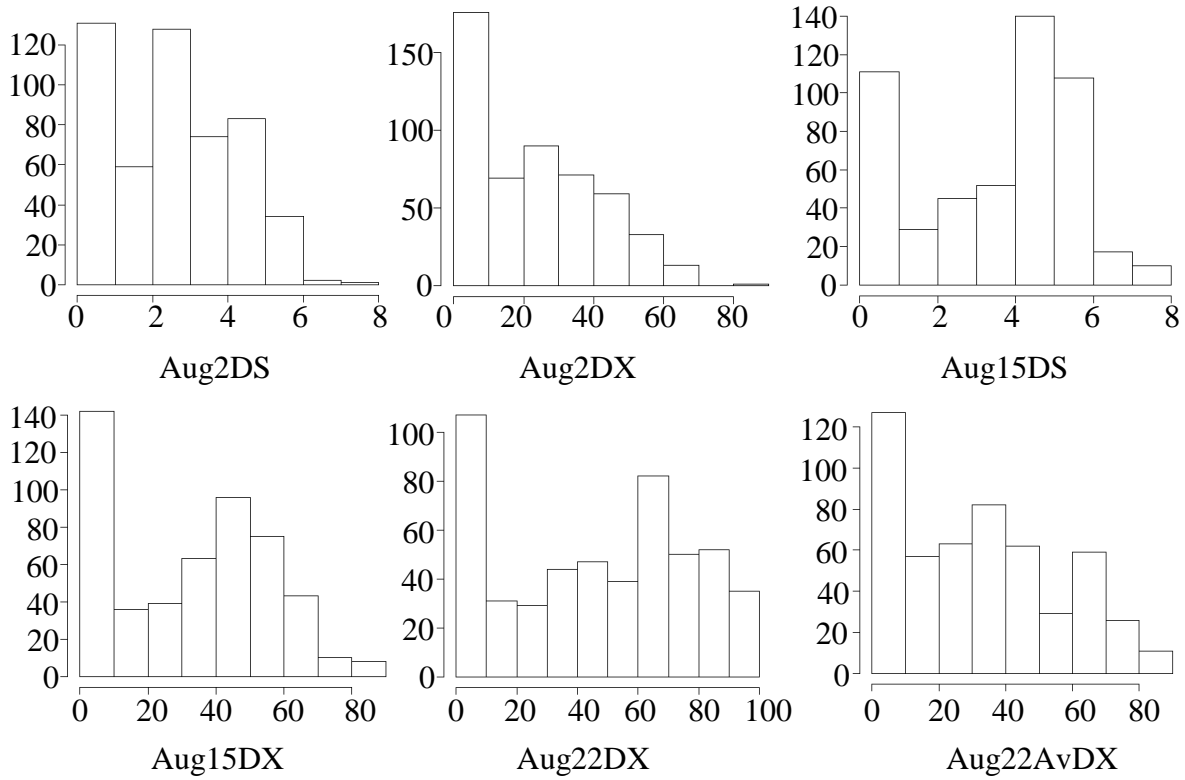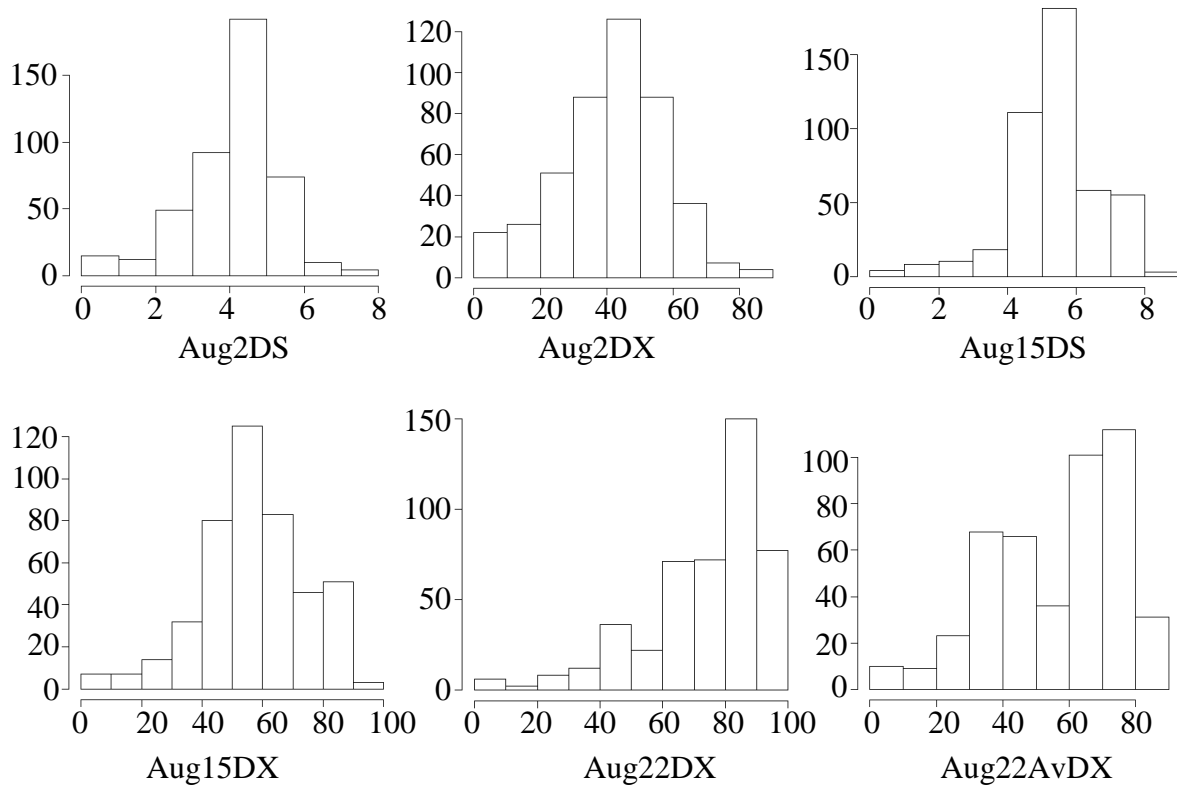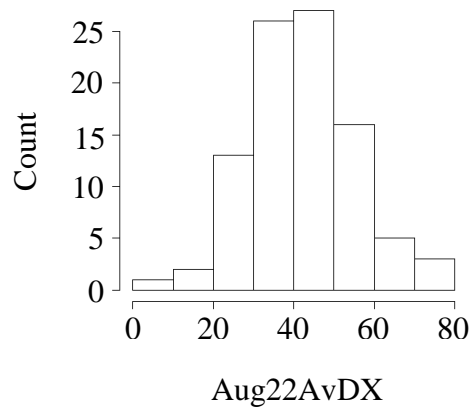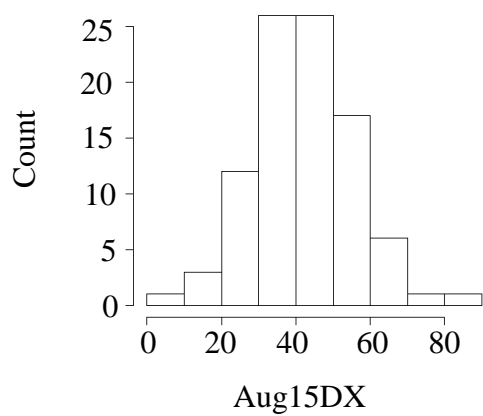
Figure A.3. Phenotypic distribution of the selected subset of population GD2422 ×LD01-5907. August 15 disease index (Aug15DX) and August 22 average disease index (Aug22AvDX).

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