QTL DISCOVERY FOR JAPANESE BEETLE RESISTANCE IN APHID-RESISTANT GERMPLASM, STACKING APHID-RESISTANT GENES, AND METABOLITE PROFILING STUDIES IN SOYBEAN

By

Desmi Indumali Chandrasena

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ABSTRACT

QTL DISCOVERY FOR JAPANESE BEETLE RESISTANCE IN APHID-RESISTANT GERMPLASM, STACKING APHID-RESISTANT GENES, AND METABOLITE PROFILING STUDIES IN SOYBEAN

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As public institutions and seed companies incorporate soybean aphid (*Aphis glycines* Matsumura) resistance genes into soybean [*Glycine max* (L.) Merr.] cultivars, it is important to retain resistance to insect defoliators. In order to improve MSU aphid-resistant germplasm for resistance to Japanese beetle (*Popillia japonica* Newman), a population was derived from a cross between E06906 (MSU) and another aphid-resistant source, LD05-16060 (Uni. of Illinois) that showed lower susceptibility to Japanese beetle. A QTL (Quantitative Trait Loci) mapping approach with a subset of 94 individuals on 15 chromosomes indicated the presence of five QTL (QTL-M, QTL-G, QTL-H, QTL-D1b and QTL-E) previously reported to confer resistance to several other soybean defoliators, and one published QTL to confer Japanese beetle-resistance. More importantly, three new QTL were found on LG-A1, LG-A2, and LG-C2; they were also detected with 234 individuals. Candidate gene analysis for resistant QTL found key enzymes involved in flavonoid biosynthesis pathway, thus a comprehensive flavonoid profiling study was conducted using High Performance Liquid Chromatography/tandem Mass Spectrometry (HPLC/MS/MS) on three susceptible and three resistant lines. Thirty two distinct peaks corresponding to glycosides or aglycones of Daidzein, Genistein, Glycetein, Kaempferol, Naringenin, and Quercetin were found in damaged and un-damaged leaflets. Higher abundances of flavonoids were found in damaged leaflets of LD05-16060. It appears that differential

susceptibility to Japanese beetle observed between LD05 and E06906, can be explained by differences in feeding-deterrent and phago-stimulant flavonoids. Furthermore, this mapping population gave an opportunity to stack *Rag1* and *rag3* aphid-resistant genes. Pyramiding multiple resistance genes, particularly with different modes of action, has great potential to provide durable resistance. This dissertation also reports MSU soybean breeding program's research on stacking *rag3,* rag*4*, *rag1b,* and *rag1c* aphid- resistant genes from a population of 727 F_2 individuals derived from two (Plant Introduction) PI s. Four trials in greenhouse and field were conducted for phenotypic evaluations. SSR and SNP markers linked to these genes were used for the genotypic selections. Repeatedly in all trials *rag3-rag1c* lines outperformed other lines showing great consistency in their resistance. Additionally, *rag3-rag4-rag1c* and *rag3-rag4-rag1b* stacks also provided significantly more resistance than other gene combinations.

DEDICATION

This dissertation is dedicated to my loving parents Mr. and Mrs. Chandrasena, my sister Madhumali, and to my dear husband Rasanga, for their love and un-conditional support.

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CHAPTER 1.0

Literature Review

Soybean: Origin, history, production, and uses

Soybean [*Glycine max* L. (Merrill)] is a cultivated crop, native to East Asia. It was grown in China for more than 5,000 years as a food and as a component of drugs (Wu et al. 2004). Linnaeus originally introduced the genus *Glycine* in 1737, in his first edition of *Genera Plantarum*. The cultivated soybean first appeared in the *Species Plantarum* by Linnaeus, under the name *Phaseolus max* L. The combination *Glycine max* was proposed by Merrill in 1917.

Soybeans spread to other Asian countries nearly 2,500 years ago (Wu et al. 2004). They were first brought to America in mid-1770s, by trading ships from Asia (Smith 1994). By 1898, the United States Department of Agriculture (USDA) began introducing new varieties of soybeans from Asia. Soybean became an important field crop beginning in the 1940s (Smith 1994). Currently, 31 states in the United States grow soybeans. The top three states with most acreage in 2011 were Illinois, Minnesota, and Iowa (NASS 2012). In 2012, 76.1 million acres of soybean were grown in the United States. In 2011, 3.06 billion bushels of soybeans were produced in the United States (NASS 2012).

Soybeans are high in nutrition and are an important source of vegetable protein; beans contain on average 38% protein. About 18% of the bean consists of oil (0.5% lecithin), which is rich in polyunsaturated fatty acids (54% linoleic acid, 22% oleic acid, and 7.5% linolenic acid) and

contains no cholesterol. The rest of the bean consists of moisture (14%), soluble carbohydrate (15% sucrose, stachyose, raffinose, others), and insoluble carbohydrate or dietary fiber (15%) (Singh et al. 2008).

Nearly all soybeans grown in the United States are used for producing oil and as feed for livestock (Smith 1994). Another important product of soybean oil is biodiesel. Biodiesel is a clean-burning, alternative fuel made from vegetable oils that can be used in compression-ignition (diesel) engines. Since soybean oil is the top oil produced in the United States, the development of biodiesel has mainly focused around soy oil. One bushel of soybean produces about 1.5 gallons of biodiesel (NBB 2009).

Soybean aphid biology and ecology

The soybean aphid, *Aphis glycines* Matsumura (Hemiptera: Aphididae), is a native of Asia, and is one of the most serious insect pests of soybean (Yu et al. 1989, Wang et al. 1996, Wu et al. 1999, Sun et al. 2000, Hill et al. 2004, Ragsdale et al. 2004). It has been a soybean pest for many years in China, Japan, South Korea, the Philippines, Indonesia, Malaysia, Thailand, Vietnam, and Russia (Wu. et al. 2004). Soybean aphid causes heavy yield loss; yield reduction up to 70% was reported in China when infestations occurred (He et al. 1995, Wang et al. 1996). In recent years, the soybean aphid was discovered in Australia (1999), Canada (2000), and the United States (2000) (Wu et al. 2004). By 2004, it was found in 24 states in the United States, including Michigan, and in three Canadian provinces (Ragsdale et al. 2004, Rutledge and O'Neil 2006).

The morphological characteristics of the soybean aphid were described in detail, by Chen and Yu (1988) and Takahashi et al. (1993). A combination of characters such as body color, black cornicles, and host range distinguish it from other *Aphis* species (Voegtlin et al. 2004.). Similar to other common *Aphis* species, soybean aphid nymphs have four instars; wing development occurs in the third and fourth instars (Zhang 1988). Adults may be winged (alate) or non-winged (apterous). Both of these forms can produce offspring. In general, nymphs range from 0.58 - 1.4 mm in length. Winged viviparous females generally have a long-ovoidal form, are 0.96 - 1.52 mm in length, have red-brown compound eyes and a black head. The wingless viviparous females have an ovoid form, and are 0.95 -1.29 mm in length. (Wu et al. 1999). Soybean aphid can reproduce parthenogenically or sexually depending on the time of the year to complete their life cycle.

Life cycle of soybean aphid

In China, soybean aphid alternates between its primary host buckthorn, *Rhamnus davurica* Pall., and secondary host(s) which are primarily cultivated soybean *G. max* and wild glycine species, *Glycine soja* Sieb.& Zucc. (Wang et al. 1962). There are two confirmed overwintering hosts in North America; *Rhamnus cathartica* L., common buckthorn, an invasive woody plant of European origin and *Rhamnus alnifolia* L'Héritier, the native alder leaf buckthorn (Voegtlin et al. 2004, Ragsdale et al. 2004). The secondary host of soybean aphid in North America is chiefly cultivated soybean, *G. max.*

The soybean aphid is heteroecious holocyclic, spending life on different hosts with sexual reproduction during a portion of its life cycle (Wang et al. 1962, Zhang and Zhong 1982, Ragsdale et al. 2004). The life cycle starts in spring, when eggs hatch and develop into wingless fundatrices. These fundatrices produce a second generation of wingless females. On the primary host *Rhamnus*, the third and subsequent generations of primarily winged morphs are generated, who emigrate in search of a secondary host in summer, typically cultivated soybean. Throughout the season, many overlapping generations of both wingless and winged morphs are produced on soybean. Later in autumn, under reduced photoperiod and temperature, winged gynoparae are produced on soybeans that move in search of *Rhamnus* again. On *Rhamnus*, they produce nymphs that develop into oviparae. The gynoparae also form males, on soybean, that later emigrate to *Rhamnus* in search of oviparae. Once the males and oviparae mate, their overwintering eggs are deposited on *Rhamnus (*Ragsdale et al. 2004).

Damage and economic impact

Although soybean aphid is reported as a significant pest in Asia (Wang et al. 1994), damage to soybean in the United States has been significantly greater over a short period of time, than in its native habitat (Ragsdale et al. 2004, Wu et al. 2004). Soybean aphid reduces yield directly via plant feeding, and indirectly through reduction in seed protein content (Wang et al. 1994). Plants with heavy infestations show wrinkled and distorted foliage, early defoliation, stem and leaf stunting, reduction in number of pods and seed weight, and even plant death (Wang et al. 1962, Wang et al.1996, Lin et al. 1992, 1994; He et al. 1995, Wu et al. 1999, DiFonzo and Hines 2002, Wu et al. 2004, Diaz-Montano et al. 2006, Ragsdale et al. 2011). Honeydew excreted by aphids

builds up on foliage, and supports the growth of sooty mold, affecting plant photosynthesis, yield, and seed quality (Chen and Yu 1988).

Significant yield loss can occur due to feeding damage by soybean aphid. In China, yield was reduced up to 52% when soybeans in the early vegetative stage (first node stage) were inoculated with 220 aphids per plant (Wang et al. 1994). Soybean aphid feeding results in reduction of seed yield, and also reduction in seed quality (e.g., discoloration, deformation) which could be a major concern for food-grade soybean growers and consumers (Mian et al. 2008a).

In addition to yield loss from the direct feeding, another threat posed by the aphid is its ability to transmit plant viruses to soybean (alfalfa mosaic virus, soybean dwarf virus, soybean mosaic virus) and other crops (Iwaki et al. 1980, Hill et al. 2001, DiFonzo 2006, DiFonzo and Agle 2008). In Michigan, soybean aphid outbreaks often coincide with high virus levels in cucumber (*Cucumis sativus* L.), squash (*Cucurbita* spp.), pumpkin (*Cucurbita* spp.), and dry beans (*Phaseolus* spp.) (DiFonzo 2006, DiFonzo and Agle 2008). Since soybean aphid is a relatively recent pest to colonize soybean in the United States, its full consequences as a virus transmitter to soybeans and other crops is still unknown (Mian et al. 2008a).

Management of soybean aphid

Several factors affect soybean aphid populations on soybean, including environmental factors (e.g., temperature, precipitation, and humidity), number of overwintering aphid eggs, cultural

practices (e.g. planting time and soybean variety), and natural enemies (Wu et al. 1999, Wu et al. 2004). Soybean aphid can be controlled by a number of distinct tactics including biological control, chemical control, and host plant resistance. These control options can be used individually or together.

In Asia, the complex of natural enemies attacking soybean aphid includes the predators *Propylaea japonica* (Thunberg), *Harmonia axyridis* (Pallas), and *Harmonia. arcuata* (Fabricius) (Coleoptera: Coccinellidae), and several species of syrphid and lacewing larvae (Van den Berg et al. 1997, Wu et al. 2004). In North America, the dominant soybean aphid natural enemies are mainly generalist predators, such as lady beetles (Coccinellidae), green lacewings (Chrysopidae) and, pirate bugs (*Orius* spp.*)*. *Orius insidiosus* Say is present in the field prior to the arrival of soybean aphid, due to its ability to feed on alternative small prey and on the soybean plant itself (Costamagna et al. 2008). Studies also show that lady beetles play an important role in suppressing soybean aphid population (Fox et al. 2004, Costamagna and Landis 2006, 2007; Costamagna et al. 2008).

During outbreak years, cultural practices and biological control are not sufficient to keep soybean aphids under control, thus growers currently rely on chemical control. Numerous pesticides have been tested and applied to manage soybean aphid in China (Chen and Yu 1988,, Wu et al. 1999, Sun et al. 2000). In North America, the most commonly used foliar insecticides are chlorpyrifos, acephate, esfenvalerate, permethrin, and λ-cyhalothrin (NASS 2006). Many of these insecticides are highly toxic and have a broad spectrum of activity. In 1999, prior to the

discovery of soybean aphid, less than 1% of the soybean acreage in Michigan was treated with insecticides (NASS 2000). In 2005, an outbreak year, 42% of Michigan soybean acres were treated, indicating the rapid increase in insecticide use since the discovery of this pest. Similar increases were observed in many north-central states (NASS 2000; 2006).

Significant insecticide costs have been inevitable with soybean aphid control since its introduction in the North central States. Song et al. (2006) estimated a total yield loss exceeding 350 million bushels in the north-central states, if soybeans were left untreated. In 2004, Michigan soybean growers have reported spending $8-12/acre for insecticide application (Song et al. 2006).

Ragsdale et al (2007) developed an economic threshold (ET) to reduce unnecessary insecticide applications against soybean aphid. The average ET over all control costs, market values, and yield was 273 ± 38 aphids per plant. This ET provided a 7-d lead-time before soybean aphid populations exceeded the economic injury level (EIL) of 674 ± 95 aphids per plant (Ragsdale et al. 2007). This ET currently does not take into consideration, factors that may influence soybean aphid populations such as, weather conditions, and natural enemy populations. To date, use of insecticides is the only cost effective method to manage soybean aphid outbreaks in field. However, chemical control of soybean aphid is not widely accepted by organic soybean growers and consumers (Mian et al. 2008a).

Types of host plant resistance

Developing host plant resistance to control soybean pests is a more environmental-friendly alternative to insecticides. Host plant resistance to insects could be classified as non-preference, antibiosis, or tolerance (Painter 1951). The term '*antixenosis*' was later used to replace non preference (Kogan and Ortman 1978). *Antibiosis* is a type of resistance that refers to a host plant that has a detrimental effect on the physiology and life history of an insect pest (lethal or sub lethal). Antixenosis resistance affects pest behavior by discouraging feeding and/or oviposition due to morphological (e.g., dense pubescence) or biochemical (presence of a deterrent compound or absence of an attractant) factors. *Antibiosis* type of resistance can pose lethal effects to the insect. This resistance can impair growth; affect pupal weights, fitness directly or indirectly affecting fecundity and maturity of the insect. The type (s) of resistance present in a host plant can be differentiated with choice and no-choice tests. A choice test provides clues on antixenosis (non-preference) where the insect is given a variety of choices to feed on. Similarly, antibiosis effects of a specific host plant can be identified using a no-choice test, where lethal effects will be shown if the insect feeds on the only food source available. Both these tests have been widely adapted in identifying soybean aphid resistance (Mensah et al. 2005, Hill et al. 2006a, Mian et al. 2008) and soybean defoliation-resistance (Yesudas et al. 2010).

Tolerance is a way of host plant adapting to withstand damage by the insect thus, pose no risk to insect while the plant merely increases the threshold. It is widely accepted that these mechanisms overlap for several insects. Thus several soybean aphid-resistant sources have been found to have both types of resistance. Also for defoliators such as Corn earworm (*Helicorverpa Zea* Say)

several resistant sources with combined effects of antibiosis and antixenosis were found (Rector et al. 1998, 2000). Because antibiosis brings lethal effects to the insect, it poses heavy selective pressure and biotypes can be produced. Antixenosis and tolerance, or finding sources with both antixenosis and antibiosis will provide more durable resistance against insects. Therefore more emphasis should be given on breeding for varieties with multiple resistance mechanisms.

History of host plant resistance and pyramiding genes for soybean aphid resistance

One way to reduce dependence on insecticides for soybean aphid is to grow cultivars with aphid resistance. In China, the native range of both soybean and soybean aphid, resistance was reported in both cultivated soybean, *G. max* (Fan, 1988; Sun et al., 1991), and in a wild relative, *G. soja* (Yu et al., 1988 and 1989). He et al. (1995) found that resistant Chinese cultivars had lower aphid populations and were less preferred for feeding than susceptible varieties. The first soybean aphid-resistant lines in the United States, Dowling and Jackson, were identified by Hill et al. (2004); antibiosis resistance in these cultivars is controlled by single dominant genes, *Rag* and *Rag1*, respectively (Hill et al., 2006a, 2006b). Mian et al. (2008a) identified a different resistant gene, *Rag2*, in PI 243540, and other breeding programs reported additional lines displaying antibiosis and antixenosis resistance (Diaz-Montano et al., 2006; Hesler et al., 2007; Hesler and Dashiell, 2008).

In Michigan, Mensah et al. (2005) screened 2147 soybean accessions, originating in northern China for aphid resistance. In greenhouse and field studies, they found two maturity group (MG) III accessions from Shandong Province exhibited antibiosis resistance (Mensah et al. 2005,

2008). These accessions, PI 567541B and PI 567598, each had two recessive genes that acted epistatically (Mensah et al., 2008). These genes were named *rag1c* and *rag4* for PI 567541B (Zhang et al., 2009), and *rag1b* and *rag3* for PI 567598B (D. Wang pers. comm.). Zhang et al. (2010) discovered that PI 567543C could be mainly controlled by a single dominant gene *Rag3*.

Most new aphid-resistant sources are identified by preliminary screening for aphid abundance in contained environments such as greenhouses or field cages with artificial infestation of soybean aphids (Hill et al. 2004, Mensah et al. 2005, Diaz-Montano et al. 2006, Mian et al. 2008a). Hill et al. (2004) tested three resistant sources (Dowling, Jackson, PI 71506) with five susceptible lines in field plots confined in a cage after artificial infestation and found three resistant sources had significantly lower aphid indices (0-9 scale) compared to most susceptible lines. They also studied per plant yield attributes such as height, dry mass, and number of pods, and 100-seed weight with and without imidacloprid treatment (Hill et al. 2004). Under heavy artificial infestation, Dowling had no significant differences in yield components between insecticide treated and untreated plants (p= 0.05) suggesting successful resistance, which was later, identified as antibiosis (Hill et al., 2006a).

However, as a further step, conducting field trials of these resistant lines under natural aphid pressure enable breeders and entomologists to discover vital information on the efficacy of resistance under field conditions, yield response, and need to integrate other management tools such as natural predators and insecticide treatments with aphid-resistant lines for more effective control.

Efficacy of *Rag1* aphid-resistance have been investigated by few groups to date (Krupke and Guo 2011, Hodgson and VanNostrand 2011). Krupke and Gou (2011) investigated the efficacy of *Rag1* alone (provides only moderate resistance) and when combined with a thiamethoxem seed treatment. They found that both the *Rag1* gene and the seed treatment affected soybean aphid growth rates when used alone. Additionally this combination improved the resistance to soybean aphid. Multi-year and multi-location field trials investigating the efficacy of *Rag* 1 has been conducted by Iowa State University. Hodgson and VanNostrand (2011) reported suppression of soybean aphid by *Rag1* alone and when combined with insecticidal seed treatments.

Identifying the importance of these field evaluations, a multi-state study evaluated many aphid-resistant breeding lines in replicated field plots including E06901, E06905, E06906, and aphid-susceptible lines. Following the same design, in 2007, a multivariate analysis across different locations in six north-central states with 18 soybean lines revealed three groups of breeding lines based on aphid infestation level (log CAD). E06901, E06905, and E06906 were termed 'group 1' with most resistance, where all other lines grouped into either 'group 2' or 'group 3' exhibiting lower or no resistance in a cluster analysis done with data collected from all participating states (IA, IL, MI, WI, SD, and MN) (Chiozza, 2009).

However, a possible negative impact to host plant resistance can be posed by the rise of new biotypes (Auclair, 1989, Smith 1989). If only a single gene is responsible for antibiosis resistance (such as *Rag1*) there is high probability for soybean aphids to overcome this resistance in a relatively short time. In a study that tested two soybean aphid biotypes from Illinois (Biotype

1) and Ohio (Biotype 2), *Rag1* resistance was not effective against the Ohio biotype, thus these soybean aphids were able to colonize breeding lines with *Rag1* (Kim et al. 2008). More recently another biotype namely 'Biotype 3' has been identified (Hill et al. 2010) which survived on both *Rag1* and *Rag2*.

Therefore pyramiding multiple resistance genes, particularly with different modes of action, in the same cultivar have great potential of providing a more durable resistance against soybean aphid (Mian et al. 2008a). This concept of 'gene pyramiding' could be a valuable addition to numerous efforts made by soybean breeders to develop aphid-resistant cultivars with long lasting resistance. Recently, a study reported efficacy of two stacked aphid-resistant genes on new soybean germplasm (Wiarda et al. 2012). Development of soybean aphid on lines with only *Rag1 or Rag2* alone and both genes combined or in the absence of both genes were tested after artificial infestations in cages. Additionally, the impact of gene stacking on yield was also reported (Wiarda et al. 2012). This study confirmed significant aphid suppression by stacked *Rag1-Rag2* genes than alone; less yield reduction was also reported when resistant sources were stacked. Chapter 3.0 in this dissertation , reports MSU soybean breeding program's research on stacking *rag3, rag1b* and *rag4, rag1c* aphid-resistant genes.

Importance of assessing defoliation resistance in soybean aphid-resistant lines

As previously stated, one of the major constrains to growing soybean in the United States is the susceptibility of many cultivars to soybean aphid, *Aphis glycines* Matsumura. To date, several aphid resistant genes have been found. Mensah et al. (2005) found four accessions from Shandong province (China), resistant to soybean aphid. Plant Introductions (PI) 567543C and PI

567597C exhibited antixenosis while PI 567541B and PI 567598B exhibited antibiosis (Mensah et al. 2005, 2008).

In 2007, a trial evaluating aphid resistance was conducted in Michigan as part of a wider multi-state project. Three sister lines (E06901, E06905, E06906), developed at MSU from PI567598B, showed excellent aphid resistance in this trial (Chiozza. 2009). However in laboratory and field assessments for Japanese beetle (*Popillia japonica* Newman) defoliation among *rag1b* and *rag3* aphid-resistant lines (E06901, E06905, and E06906) was higher when compared with a *Rag1* aphid-resistant line (LD05-16060), and aphid-susceptible lines (DKB27-53, SD01-76R, and Titan RR) (Chandrasena et al. 2012). Under natural insect pressure, the percentage of leaflets consumed by Japanese beetle was greater on *rag1b* and *rag3* lines (50-86%) than LD05-16060 (11%) and SD01-76R (5%). Defoliation on the three-most-damaged trifoliates was higher on *rag1b* and *rag3* lines (49-54%), and its aphid-susceptible parent, Titan RR (35%), than LD05-16060 (5%) and its aphid-susceptible parent, SD01-76R (1%). Similarly, in laboratory choice and no-choice tests, greater leaf area was removed from *rag1b* and *rag3* lines. There was more feeding on LD06-16060 under no-choice conditions than under choice conditions, suggesting LD05-16060 was more attractive to Japanese beetle in the absence of a preferred line (non-preference). This was surprising because most commercial soybean lines have some resistance to defoliation by Japanese beetle (Hammond, 1994). Although aphid resistance is the major priority in our breeding program, it is also very important to retain resistance to defoliators such as Japanese beetle while incorporating agronomically important traits.

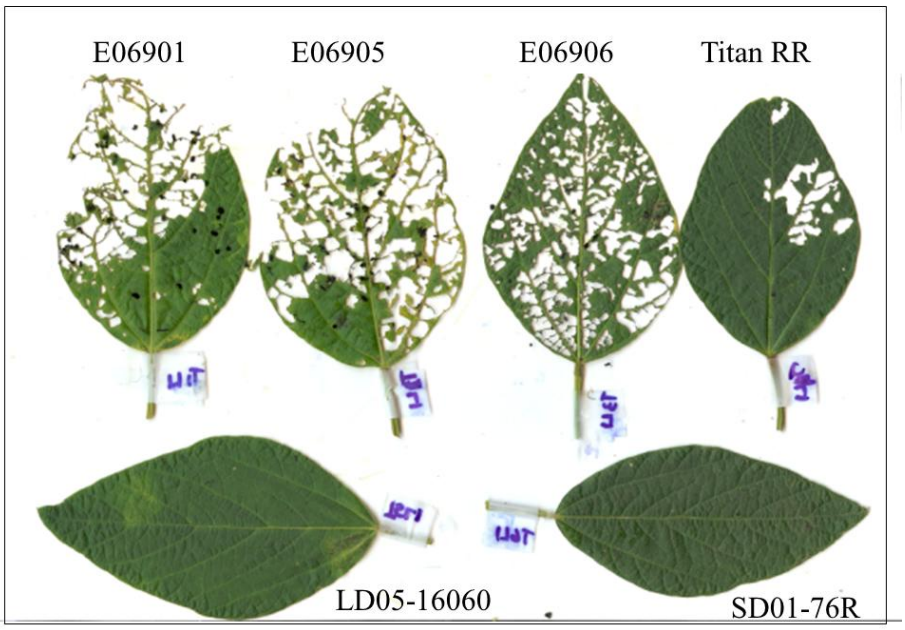

Figure 1.1. Image showing scanned leaflets of six soybean lines in a detached leaflet choice-test in 2008. E06901, E06905, E06906, and, Titan RR (top row, from left to right), LD05-16060 (bottom row left) and, SD01-76R (bottom row right) 48 h after exposure to Japanese beetles. "For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation."

Japanese beetle biology, impact, and management

The Japanese beetle, *Popillia japonica* Newman is an introduced pest from Japan, and was first discovered in North America in 1916 in a nursery near Riverton, New Jersey during a routine inspection (Fleming 1972, 1976). It is a common destructive pest of turf and landscape plants; adults feed on more than 300 species of wild and cultivated plants in 79 families (Fleming 1972, Ladd 1986; 1988). It damages fruit crops, field crops such as soybean and maize, and many garden crops (Potter and Held 2002). Some of the preferred plants are grape, early apples,

cherry, peach, plum, raspberry, rose, zinnia, soybean and corn. Adults mainly feed on fruits and leaves; larvae feed on roots. Due to its high success rate Japanese beetle is now reported in 28 states and Canada (NAPIS 2009).

The Japanese beetle is univoltine, completing its life cycle in one year throughout most of its range in the United States (Fleming 1972). However, in cooler climates, it can take up to two years to complete a single generation (Crocker et al. 1995). Adult Japanese beetles feed on tissue between the leaf veins, leaving a characteristic lace-like skeletonized structure. Feeding usually begins at the top of the plant, regardless of height, on the upper and outermost leaves (Potter and Held 2002, Cook and Gray 2004). Severely injured leaves turn brown, die, and drop off the plant. Damage caused by foliar feeding of adult Japanese beetles occurs in soybean during the middle of the growing season (July - August) when the plants are in the reproductive stages (Turnipseed and Kogan 1976, Cook and Gray 2004).

On soybean, leaf feeding occurs in July and August in the Midwestern United States, when plants are flowering and filling pods (Turnipseed and Kogan, 1976; Cook and Gray, 2004). Although Japanese beetle adults are frequently present in Michigan soybean fields, feeding by this species alone is rarely enough to merit treatment (DiFonzo and Warner, 2010). Instead, producers consider overall defoliation from multiple insect species to make a treatment decision. Action thresholds for soybean defoliation in the Great Lakes region generally range from 30% to 40% pre-bloom, decreasing to 15% between bloom and pod fill, and 25% thereafter (Eisley and Hammond, 2007). Several foliar insecticides are recommended for Japanese beetle in the event of an outbreak (DiFonzo and Warner, 2010).

Breeding and QTL discovery for defoliation-resistance in soybean

Development of soybean cultivars with insect resistance was a focus of U.S. soybean breeders for more than 30 years (All et al., 1989). Although Japanese beetle feeding on soybean is not a severe threat in most soybean-growing regions in the United States, soybean lines were screened for defoliation as early as the 1940s. Coon (1946) assessed Japanese beetle defoliation on 26 soybean genotypes using a numerical damage scale and concluded that all were susceptible. However, based on his ratings, he confirmed that four cultivars (Chief, Viking, Illini, Wilson) were less susceptible to Japanese beetle feeding than others. Furthermore, his studies confirmed that increased beetle feeding resulted in decreased yield. In the 1960s, the Japanese PI 229358 was one of the first found to be resistant to Mexican bean beetle (MBB), *Epilachna varivestis* Mulsant (Van Duyn et al., 1971, 1972). The PIs 229358, 171451, and 227687 showed greater resistance to this beetle in a choice-test when planted with other genotypes (cultivars and lines). Furthermore, in a laboratory forced-feeding test, these same PIs were the least-consumed among 29 genotypes, which Van Dyan et al. (1971) presumed to be either, due to absence of feeding stimulants or presence of feeding deterrents. Mexican bean beetle feeding on these three PIs also had reduced longevity and fecundity.

These three PIs were the main sources of defoliation-resistance to several insects in soybean (Lambert and Tyler 1999, Zhu et al., 2006) and served as donor parents to develop defoliation-resistant soybean in conventional breeding programs (Van Duyn et al., 1971, 1972). Breeding for defoliation-resistance became a major objective in several breeding programs during the 1970 after the identification of three germplasm accessions with resistance to the Mexican bean beetle (PI 171451, PI 227687 and PI 229358). The latter was found to be resistant to several major

lepidopteron soybean pests. However, there have been some difficulties to incorporate some of the resistant genes to elite cultivars due to yield drag. Also the progeny did not possess the same level of resistance as the parent PI, due to various combined effects of genes that were not present in progeny. Classical genetic studies on inheritance of defoliation-resistance on PI 171451, PI 227687, and PI 229358 have shown the presence of quantitative inheritance for Soybean Looper (SBL) and MBB (Sisson et al. 1976; Kenty et al. 1996). Other populations derived from PI 229358 showed inheritance of SBL resistance from few major genes (Kilen et al. 1977, Kenty et al. 1996). With the advent of DNA markers, QTL conferring resistance to several soybean defoliators were reported (Rector et al. 1998, 2000; Zhu et al. 2006, 2008). Restriction fragment length polymorphisms (RFLPs) were used to map QTL underlying antibiosis and antixenosis resistance to Corn Ear Worm (CEW) from PI 171451, PI 227687, and PI 229358 (Rector et al. 1998, 1999 and 2000). Later, Zhu et al. (2006) mapped three QTL from PI 229358 conferring either antibiotic or antixenosis resistance to three key soybean defoliators (MBB, CEW and SBL) namely QTL-G, QTL-H, and QTL-M. The QTL on linkage group M (QTL-M) is one of the most important major QTL conferring both antixenosis (37%) effect and antibiosis (22%) effect to soybean defoliators (Rector et al. 1998, 1999 and 2000; Komatsu et al. 2005, Zhu et al. 2006). QTL-G provides only antibiosis, while QTL-H has antixenosis effects (Parrott et al. 2008). However the most effect is reported from QTL-M and also when QTL-G and QTL-H are combined with QTL-M, thus breeding efforts has been focused on introgressing QTL-M into elite soybean varieties. Another limitation to introgression of this QTL is the possible linkage drag (Parrott et al. 2008). Thus Zhu et al. (2007) successfully fine mapped the QTL –M to 0.52 cM map interval with Williams 82 genomic sequence, further assisting in introgression of this QTL-M without unnecessary linkage drag.

Table 1.1. List of some known QTLs conferring resistance to insect defoliators on soybean

Resistance source	Linkage Group	Marker Interval	Description
PI 229358 (QTL M)	M	*satt220-satt626*	Japanese PI with defoliation resistance to **many defoliators (coleopteran and other)** Zhu et al. (2006), Zhu et al. (2008)
PI 229358 (QTL G)	G	*satt472-satt191*	**SIR** (Soybean Insect Resistant QTL) Zhu et al. (2006), Zhu et al. (2008)
PI 229358 (QTL H)	H	*satt122-satt541*	**SIR** (Soybean Insect Resistant QTL) Zhu et al. (2006), Zhu et al. (2008)
PI 229358	D1b	*Satt141-Satt290*	**Corn Ear Worm** resistance (Rector et al. 1998, 2000)
Forrest	B1	*Satt583-Satt415*	Cultivar Forrest has partial resistance to Japanese beetle (JB). QTL **specifically confer resistance to JB**. Yesudas et al. (2010)
Forrest	A2	*Satt632-A2D8*	
Forrest	N	*Satt009-Satt530*	
Forrest	A1	*Satt386*	
Forrest	I	*Satt440*	
Forrest	F	*Sat_039-Satt160*	
Forrest	D2	*Satt464-Satt488*	

Although defoliation by Japanese beetle is not yet reported to cause serious economic loss in many soybean cultivars, it is also very important to retain resistance to defoliators such as Japanese beetle while incorporating agronomically important traits in new breeding lines.

Chapter 2.0 in this dissertation, reports a study conducted to identify defoliation-resistant QTL in soybean aphid-resistant germplasm. With this project, we aimed to identify new soybean germplasm with both, durable aphid-resistance, and defoliation-resistance to Japanese beetle.

Plant-herbivore communications: an overview of signaling by plant metabolites

Chandrasena et al. (2012) reported elevated susceptibility in some aphid-resistant germplasm developed by Michigan State University. However, information about underlying biochemical factors leading to differential susceptibility in this germplasm has not been explored; hence sufficient biochemical analyses were important. Additionally, understanding the genes underlying JB-resistant QTL can potentially reveal very important information for novel resistant gene discovery. Chapter 4.0 in this dissertation describes a comprehensive biochemical study conducted to identify key soybean metabolites responsible for herbivory by Japanese beetle.

Secondary metabolites are a blend of complex molecules produced by the plant, with or without insect feeding (induced or non-induced). These compounds can be highly diverse among and within plant species, and may be produced by more than one biosynthetic pathway inside the plant (Figure 1.2). It is a general observation that these initial signals elicited by the plant will in turn trigger more aggregation of the same species or serve as chemical cues for predators to locate host insects. In this section more specific examples of Japanese beetle aggregation and feeding induced by several plant compounds on many host species will be discussed. These belong to a diverse array of chemicals, thus explanation of specific details on their chemical structure and biosynthesis is beyond the scope of this research. However it is important to

emphasize main types of compounds (volatiles and non-volatiles) and their common biosynthetic pathways.

Biosynthetic pathways of common plant secondary metabolites

There is intense research continuing in the area of signal transduction pathways within plants, which enable communication between sites of damage and sites of systemic release of secondary metabolites. The primary biosynthetic pathways responsible for production of major compounds have been thoroughly studied (Figure 1.2). Below is a very simple outline of primary and secondary metabolic pathways synthesizing a vast majority of volatiles and non-volatiles.

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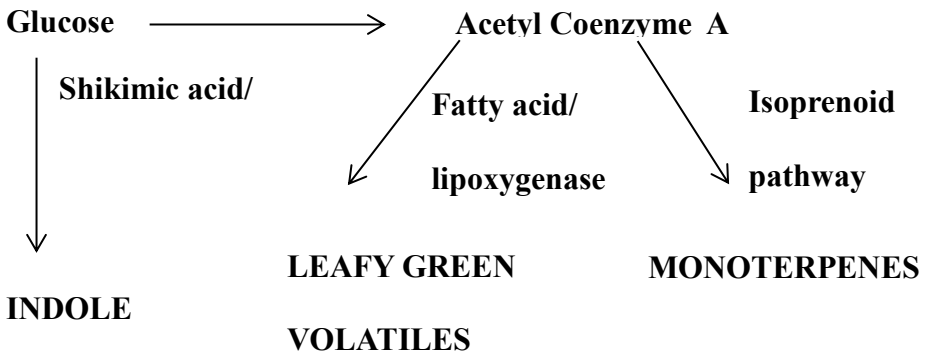

Figure 1. 2. Primary and secondary metabolic pathways leading to release of majority of secondary metabolites in herbivore damaged plants (reproduced from Pare' and Tumlinson, 1995)

At least four biosynthetic pathways are known to be responsible for production of volatiles and non-volatiles.

1) The isoprenoid pathway - produces monoterpenes and sesquiterpenes.
2) The fatty acid/lipoxygenase pathway - produces green leaf volatiles and jasmone

3) The shikimic acid/tryptophan pathway - results in several volatiles , including indole
4) Non volatiles such as sugars, isoflavonoids and flavones are produced as byproducts of glycolytic pathway

Host feeding by Japanese beetle

Feeding behavior of this generalist is initiated by the attempts to locate host plants in the vicinity by olfaction, followed by the determination/selection of hosts by olfaction and ingestion. It is believed that plant volatiles play a key role in host locating the host while many plant-derived non-volatiles act as phago-stimulants or antifeedents leading to final selection. (Ahmad 1982, Keathley al.1999, Teparkum 2000, Potter and Held 2002).

Host-locating strategies for Japanese beetle.

Many plant-derived volatiles facilitate the location of hosts for Japanese beetle although there is insufficient evidence to conclude that these beetles exclusively seek volatiles induced only by their susceptible hosts. Loughrin et al. (1996a) tested volatile compounds emitted by leaves of four crabapple (*Malus spp.*) cultivars susceptible to Japanese beetle and four relatively resistant cultivars. A total of twelve compounds, mostly terpenes were identified from intact leaves. Four terpenes; (*E*)-β-ocimene, caryophylene, germacrene D, and (*E*,*E*)-α-farnesene levels were significantly high in susceptible cultivars, whereas resistant cultivars had elevated amounts of (*E*)-4,8-dimethyl; 1,3,7-nonatriene, and linalool. Although there were differences in quantities of individual volatiles, their results indicated that some resistant cultivars were as attractive as susceptible cultivars thus their variation in defoliation was not due to the variations in attractive compounds among resistant and susceptible plants rather to the variation in non-volatiles

components in susceptible and resistant cultivars. Evidently Japanese beetle does not seem to discriminate the complex blend of volatiles released by resistant and susceptible crab apple varieties. This is supportive of the postulate that they do not exclusively seek volatiles induced only by their susceptible hosts. In addition, this study also showed that this generalist is attracted to a range of plants that release volatiles regardless of their suitability (Loughrin et al. 1996a, 1996b; Potter and Held 2002).

Host plant selection

Host selections by insects have been studied for decades; several theories have been advanced. Fraenkel (1959) proposed the "token stimulus theory" postulating that host plant selection by insects is specifically determined by phytochemicals (glycosides, phenols, tannins, terpinoids, alkaloids, and saponins) (Teparkum 2000). Since Japanese beetles are attracted to a range of plants regardless of their suitability, is could be speculated that host-acceptance or host-rejection is determined at the leaf surface by olfaction and/or by taste (chemoreception) (Potter and Held 2002).

Olfaction

Many observers of Japanese beetle suggest that once landed on a plant, a beetle is faced with a decision to either accept or reject the plant. This decision may be supported by olfaction.
To demonstrate the role of olfaction (sense of smell) in host location, Ahmad (1982) conducted laboratory choice feeding assays with adult Japanese beetle. He demonstrated that intact beetles (with antennae) located highly preferred foliage more frequently over less preferred foliage while

beetles with their antennae removed could not locate the more preferred foliage as frequently as intact beetles.

Studies using a Y-tube olfactometer (a Y shaped glass tube that allows insects to make either one of two choices and proceed at the junction) where adult Japanese beetle were given a choice of a preferred apple leaf disc, and an odorless paper disc showed that 50% of the beetles were observed on the leaf after 1 minute; of those who first chose the leaf, 92% stayed on the leaf after 15 minutes. More (58% of the beetles) were observed than on the leaf disc after 15 minutes (Teparkum 2000). Results indicated that substantial number of Japanese beetles relied on smell even prior to contact with its preferred host.

Phago-stimulants

Several plant-derived sugars including sucrose, maltose, fructose, and glucose serve as strong phago-stimulants for Japanese beetle (Ladd 1986; 1988, Potter and Held 2002). Ladd (1986) showed that several natural sugars stimulated feeding in adult Japanese beetle. Feeding response for sixteen naturally occurring carbohydrates; pentoses (ribose, xylose, lyxose, and arabinose); hexoses (fructose, mannose, glucose and galactose); disaccharides (sucrose, melibiose, maltose, and trehalose); trischarides (melezitose and raffinose); a polyhydric alcohol (sorbitol), and sorbose in agar/cellulose media were evaluated for feeding response by field-collected adult Japanese beetle. Sucrose, maltose, fructose and glucose acted as strong phago-stimulants while arabinose, xylose and raffinose induced moderate feeding stimulance. Another interesting finding was the lack of response to sorbitol; a constituent widely spread in Rosaceae that stimulated feeding in many lepidopteron pests. Of all the families attacked by Japanese beetle,

Rosaceae includes the largest number of severely damaged plants (Ladd 1986). Ladd (1988) evaluated sucrose (0.01-1 M) and 13 other naturally occurring sugars (0.1 M) on acetate-cellulose membrane filter disks as feeding stimulants for Japanese beetle larvae and concluded that sucrose, maltose, fructose, glucose, and trehalose stimulated larval feeding. All of the above sugars except trehalose have also been reported as strong phago-stimulants to adult Japanese beetle (Ladd 1986).

Anti-feedents

Several plant-derived deterrents are known as anti-feedents to Japanese beetle. Eight foliar phenolics found in *Malus spp.* were incorporated into an artificial diet to test the response. Four of them; phloridzin, phloretin, naringenin, and catechin were anti-feedants, whereas quercetin and rutin were not deterrents, but phago-stimulants (Fulcher et al. 1998). A triterpene in cucurbits named cucurbitacin B is responsible for repelling Japanese beetle from them (Tallamy et al. 1997). This compound behaved as a potent deterrent to several other chrysomelids. Keathley et al. (1999) showed that Bradford pear, a plant that is normally rejected by Japanese beetle, gained palatability after freezing and thawing the leaves. They proposed that deterrents, possibly phenolics that cause feeding-resistance could be compartmentalized in vacuoles without release followed by enzymatic degradation . Upon damage these deterrents are freely released and oxidized by enzymatic reactions.

Some cyanogenic glycosides such as prunasin, herniarin and coumarin, present in resistant *Prunus spp*., were reported as potent anti-feedants for Japanese beetle (Potter and Held 2002).

It is evident that many plant volatiles and non-volatiles affect aggregation of adult Japanese beetle. Initial signals sent from feeding-induced plants attract more beetles and increase the members in an aggregation. Furthermore there is significant evidence to support that Japanese beetle relies heavily on chemoreception of these compounds for both host location and host plant selection. Final acceptance or rejection of a host is determined primarily upon contact of the host rather than from a distance where plant-derived compounds play a key role as odors, phago-stimulants and/or anti-feedents (Potter and Held 2002).

In soybean specifically, many secondary plant compounds serve as feeding deterrents to herbivorous insects, including isoflavonoids (Treutter, 2006, Chen et al. 2008). Several soybean flavonoids produced through phenylpropanoid pathway play a key role in plant defense against herbivory. The isoflavones afrormosin, coumestrol, and phaseollin are abundant in soybean leaves (Caballero et al. 1986, Dakora 1995). Thus, increased or decreased feeding of Japanese beetle on different varieties of the same host can be related to differences in phagostimulants or deterrent compounds.

Metabolite profiling methods for soybean leaves

Metabolic profiling techniques are a widely adopted approach to reveal and compare biochemical differences among plant tissue. With the advent of hybrid systems such as GC-MS (Gas Chromatography-Mass spectrometry) and LC-MS (Liquid Chromatography-Mass spectrometry), precision and accuracy of separation of compounds have become efficient. LC-MS is known to be an effective approach to analyze plant secondary compounds in a wide polarity range and is proven to be a better approach for larger molecules such as sugar

derivatives, lipids and flavonoids (Liu et al. 2001). High Performance Liquid Chromatography (HPLC) is a popularly used method for analyzing complex mixtures including flavonoids. Numerous research groups have analyzed compounds derived from soybeans by HPLC (Eldridge 1982; Hardin and Stutte 1980; Lookhart et al. 1978; Murphy and Stutte 1978, Cavaliere et al. 2007).

The basic structure of 'flavonoids' have a common C6-C3-C6 flavone skeleton with a three-carbon bridge between the phenyl groups (Cavaliere et al. 2007) (Fig. 1.3). The collective noun 'flavonoids' includes sub classes such as, isoflavones, flavonols, flavanones, anthocyanins, catechins, and chalcones. The groups differ according to the degree of unsaturation and degree of oxidation of the three-carbon segment. (Cavaliere et al. 2007).

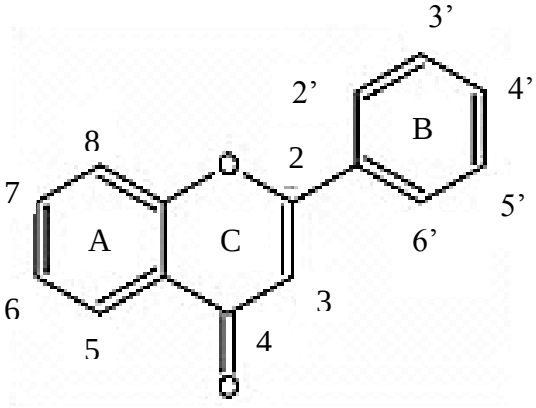

Figure 1.3. Base structure of flavonoids (Cavaliere et al. 2007)

Information about underlying genetic or biochemical factors that leads to differential Japanese beetle susceptibility on soybean germplasm has not been explored to date. Chapter 4.0 is describing an investigation carried out to uncover biochemical differences between selected

Japanese beetle-susceptible and resistant soybean germplasm, which is also harboring different aphid-resistant genes. It can be hypothesized that differences in plant metabolite (induced or constitutive) profiles lead to differential susceptibility to Japanese beetle on this aphid-resistant germplasm.

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CHAPTER 2.0

A whole-genome scan for QTL conferring resistance to Japanese beetle in aphid-resistant soybean germplasm.

Introduction

One of the major constrains for growing soybean in the United States is susceptibility of many cultivars to soybean aphid, *Aphis glycines* Matsumura, an invasive pest from China introduced to North America in 2000 (Ragsdale et al. 2004). Soybean aphid causes serious damage to soybean resulting in significant yield loss (Wu et al. 2004, DiFonzo and Hines 2002). To date, insecticides are the primary means to manage outbreaks in the field. Several aphid resistant sources have been found since the first discovery of soybean aphid in the United States . Mensah et al. (2005) found four accessions, resistant to soybean aphid. Plant Introductions (PI) 567543C and PI 567597C exhibited antixenosis while PI 567541B and PI 567598B exhibited antibiosis (Mensah et al. 2005, 2008).

In 2007, a trial evaluating aphid resistance was conducted in Michigan as part of a wider multi-state project, three sister lines (E06901, E06905, E06906), developed at MSU from PI 567598B, showed excellent aphid resistance in this trial (Chiozza 2009). However, elevated feeding by Japanese beetle (*Popillia japonica* Newman) was observed on these lines, compared to other breeding lines and commercial varieties in the trial (Table 2.1.). This was surprising because most commercial soybean lines have some resistance to defoliation by Japanese beetle (Hammond, 1994). Although aphid resistance is the major priority in breeding programs, it is

also very important to retain resistance to defoliators while incorporating agronomically important traits.

Japanese beetle is established in 28 U.S. states and Canada (NAPIS 2009). It is a common destructive pest of turf and landscape plants, feeding on more than 300 species of wild and cultivated plants in 79 families (Potter and Held, 2002). On soybean, leaf feeding occurs in July and August in the Midwestern United States, when plants are flowering and filling pods (Turnipseed and Kogan, 1976; Cook and Gray, 2004). Adults feed on tissue between leaf veins, usually of the upper and outermost leaves, leaving a characteristic lace-like skeletonizing (Hammond 1994; Cook and Gray, 2004). Although Japanese beetle adults are frequently present in Michigan soybean fields, feeding by this species alone is rarely enough to merit treatment (DiFonzo and Warner, 2010). Instead, producers consider overall defoliation from multiple insect species to make a treatment decision. Action thresholds for soybean defoliation in the Great Lakes region generally range from 30% to 40% pre-bloom, decreasing to 15% between bloom and pod fill, and 25% thereafter (Eisley and Hammond 2007).

In laboratory and field assessments for Japanese beetle (*Popillia japonica* Newman), defoliation among *rag1b* and *rag3* aphid-resistant lines (E06901, E06905, and E06906) was compared with a *Rag1* aphid-resistant line (LD05-16060), and aphid-susceptible lines (DKB27-53, SD01-76R, and Titan RR) (Chandrasena et al. 2012). Under natural insect pressure, the percentage of leaflets consumed by Japanese beetle was greater on *rag1b* and *rag3* lines (50-86%) than LD05-16060 (11%) and SD01-76R (5%). Defoliation on the three-most-damaged trifoliates was higher on *rag1b* and *rag3* lines (49-54%), and its aphid-susceptible parent Titan RR (35%), than LD05-

16060 (5%) and its aphid-susceptible parent, SD01-76R (1%). Similarly in laboratory choice and no-choice tests, greater leaf area was removed from *rag1b* and *rag3* lines. There was more feeding on LD06-16060 under no-choice conditions than under choice conditions, suggesting LD05-16060 was more attractive to Japanese beetle in the absence of a preferred line**.** These assessments confirmed that E06901, E06905, and E06906 were consistently more preferred by Japanese beetle among other aphid-resistant and aphid-susceptible lines. Hence in 2008 a breeding population was developed by crossing two parents both having aphid resistance but differential susceptibility to Japanese beetle (LD05-16060 -Japanese beetle resistant, E06906-Japanese beetle susceptible).

Table 2.1. Visual estimates of Japanese beetle feeding on soybean aphid-resistant and susceptible soybean lines in a field trial in East Lansing, MI (2007 and 2008).

Soybean line or cultivar	Aphid resistance gene(s)	% leaflets fed on in 2007	% defoliation on nine leaflets in 2008
E06906	*rag1b*, *rag3*	86.2 ± 3.7 a	53.6 ± 5.3 a
E06905	*rag1b*, *rag3*	58.6 ± 6.9 b	49.7 ± 6.7 a
E06901	*rag1b*, *rag3*	49.9 ± 7.2 c	49.4 ± 4.3 a
Titan RR	none	n/a	34.6 ± 4.5 b
DKB27-53	none	15.0 ± 6.1 d	n/a
LD05-16060	*Rag1*	11.1 ± 2.7 de	5.2 ± 1.8 c
SD01-76R	none	5.2 ± 1.2 e	1.2 ± 0.7 d

Within each year, means followed by different letters are significantly different (p<0.05).

Development of soybean cultivars with insect resistance was a focus of U.S. soybean breeders for more than 30 years (All et al. 1989). Although Japanese beetle feeding on soybean is not a severe threat in most soybean-growing regions in the United States, soybean lines were screened for defoliation as early as the 1940s. Coon (1946) assessed Japanese beetle defoliation on 26 soybean genotypes using a numerical damage scale and concluded that all were susceptible. However, based on his ratings, he confirmed that four cultivars (Chief, Viking, Illini, Wilson) were less susceptible to Japanese beetle feeding than others. Further, his studies confirmed that increased beetle feeding resulted in decreased yield. In the 1960s, the Japanese PI 229358 was one of the first found to be resistant to Mexican bean beetle (MBB), *Epilachna varivestis* Mulsant (Van Duyn et al., 1971, 1972). The PIs 229358, 171451, and 227687 showed greater resistance to this beetle in a choice-test when planted with other genotypes (cultivars and lines). Furthermore, in a laboratory forced-feeding test, these same PIs were the least-consumed among 29 genotypes, which Van Dyan et al. (1971) presumed to be either due to absence of feeding stimulants or presence of feeding deterrents. MBB feeding on these three PIs also had reduced longevity and fecundity.

These three PIs were the main sources of defoliation-resistance to several insects in soybean (Lambert and Tyler 1999, Zhu et al. 2006), and served as donor parents to develop defoliation-resistant soybean in conventional breeding programs (Van Duyn et al. 1971, 1972). Breeding for defoliation-resistance became a major objective in several breeding programs during the 1970 after the identification of three germplasm accessions with resistance to MBB (PI 171451, PI 227687 and PI 229358). The latter was found to be resistant to several major lepidopteron soybean pests. However, there have been some difficulties to incorporate some on the resistant

genes to elite cultivars due to yield drag. Also the progeny did not possess the same level of resistance as the parent PI due to lack of various combined effects of genes that were absent in progeny.

Classical genetic studies on inheritance of defoliation-resistance on PI 171451, PI 227687, and PI 229358 have shown in several locations the presence of quantitative inheritance for soybean Looper (SBL) and MBB (Sisson et al. 1976; Kenty et al. 1996). Other populations derived from PI 229358 showed inheritance of SBL resistance from few major genes (Parrott et al. 2008).

With the advent of DNA markers, QTL conferring resistance to several soybean defoliators were reported (Rector et al., 2000; Zhu et al., 2006, 2008). Restriction fragment length polymorphisms (RFLPs) were used to map QTL underlying antibiosis and antixenosis resistance to Corn Ear Worm (CEW) from PI 171451, PI 227687, and PI 229358 (Rector et al. (1998, 1999 and 2000) used. Later Zhu et al. (2006) mapped three QTL from PI 229358 conferring either antibiotic and/or antixenosis resistance to three key soybean defoliators (MBB, CEW and SBL) namely QTL-G, QTL-H and QTL-M. More recently, Yesudas et al. (2010) identified QTL from seven chromosomes conferring resistance specifically to Japanese beetle in a recombinant inbred population. A list of all known defoliation-resistant QTL was recently published by Parrott et al. (2008).

The advanced breeding line E06906 possess higher aphid-resistance, however many agree that durability of these aphid-resistance genes can be improved by pyramiding other resistance genes. Moreover, with the discovery of new aphid biotypes our breeding program has identified the

importance of stacking several sources of aphid resistance for durable aphid resistance in soybean. To address both these issues, E06906, a *rag3/rag1b* aphid-resistant line with higher susceptibility to Japanese beetle, was crossed with LD05-16060, a *Rag1* resistant line also showing less susceptibility to Japanese beetle. With this project, the main objective was to develop new soybean germplasm with both, durable aphid-resistance, and defoliation-resistance to Japanese beetle. Specifically this population possesses unique germplasm with stacked aphid-resistant genes from both LD05-16060 and E06906 thus harbor new sources of aphid resistance. More importantly, we anticipated identifying QTL conferring resistance to Japanese beetle defoliation in this aphid-resistant germplasm.

Although defoliation by Japanese beetle is not yet reported to cause serious economic loss in many soybean cultivars, it is also very important to retain resistance to defoliators such as Japanese beetle while incorporating agronomically important traits to MSU aphid-resistant germplasm. Soybean varieties with resistance to both insects will save Michigan soybean growers cost of insecticides and reduce pollution resulted from insecticide applications.

It was hypothesized that resistance to Japanese beetles and resistance to soybean aphids are controlled by separate genes thus two traits could be combined through marker assisted breeding. We have identified DNA markers linked to the multiple sources of aphid resistance present in this population, thus DNA markers can be used to distinguish *Rag1* resistance, '*rag3/rag1b'* resistance, and progeny with both forms. A progeny with multiple stacked genes may harbor durable resistance. E06901, E06905, and E06906 aphid resistant lines were more susceptible to Japanese beetle, suggesting the possibility of some association between the two

traits. With a whole-genome scan it was anticipated to identify DNA markers linked to defoliation-resistant QTL in this population, and to select progeny with resistance to both insects.

Objective 1: Marker assisted selection of soybean aphid-resistant QTL using already known DNA markers that are linked with both forms of soybean aphid resistance.

Objective 2: Identify and detect QTL conferring resistance to Japanese beetle by whole genome scanning with polymorphic SSR markers, identify tightly liked markers for new QTL.

Objective 3: Release novel germplasm with resistance to Japanese beetles and soybean aphids.

Materials and Methods

Plant material

An initial population of 235 F_2 plants was developed from a cross between two aphid-resistant lines (LD05-16060 and E06906) which differed in source of aphid-resistance and in susceptibility to Japanese beetle in a 2007 preliminary field study. The advanced breeding line E06906 was derived from a cross of the aphid resistant PI 567589B *(rag3* and *rag1b*) by the aphid-susceptible 'Titan RR' by the MSU soybean breeding program. The aphid-resistant line, LD05-16060 was derived from a cross between the aphid resistant 'Dowling' (*Rag1)* and the aphid- susceptible SD01-76R by the Soybean breeding program at Uni. Of Illinois. Therefore the population was expected to segregate for both aphid resistance and for resistance to Japanese beetles.

Phenotypic data collection for aphid resistance

The first evaluation for soybean aphid resistance in a field choice test was conducted in summer of 2009 at the Agronomy Farm of Michigan State University (MSU), East Lansing , MI. A choice test provides clues on antixenosis (non-preference) when the insect is given a variety of choices to feed on. Similarly antibiosis effects of a specific host plant can be identified using a no-choice test, where lethal effects will be shown if the insect fails to feed on the only food source its' left with. 235 $F_{2:3}$ families along with its parents were planted in a replicated randomized complete design in an aphid and predator-proof polypropylene cage with 0.49-mm size mesh (Redwood Empire Awning Co., Santa Rosa, CA, USA) (Figure 2.1). Each line consisted of 10-15 plants in a single 30 cm long plot with 60 cm row spacing. Each plant per line was individually rated for aphid damage using a standard scale developed by Mensah et al. (2005). Two wingless aphids were placed on the top-most unopened trifoliate at the V1 stage (Fehr and Caviness 1977). The sources of aphids were field-collected aphids from the same year. Visual ratings on aphid infestation were taken 3 weeks after infestation using a scale of 0–4 developed by Mensah et al. (2005, 2008), where 0 = no aphids; 0.5 = fewer than 10 aphids per plant, no colony formed; 1 = 11–100 aphids per plant, plants appear healthy; 1.5 = 101–150 aphids per plant, plants appear healthy; 2 = 151–300 aphids per plant, mostly on the young leaves or tender stems, plants appear healthy; 2.5 = 301–500 aphids per plant, plants appear healthy; 3 = 501–800 aphids per plant, young leaves and tender stems are covered with aphids, leaves appear slightly curly and shiny; 3.5 = more than 800 aphids per plant, plants appear stunted, leaves appear curled and slightly yellow, no sooty mold and few cast skins; 4 = more than 800 aphids per plant, plants appear stunted, leaves appear severely curled and yellow and are covered with sooty mold and cast skins.

Phenotypic data collections for Japanese beetle

Field choice-tests

The first field evaluation for Japanese beetle feeding was carried out in summer of 2009 at the MSU agronomy farm, East Lansing, MI. A family of 235 families was planted in a single 2 feet plot (20 seeds per plot) in south-north orientation with replications on either side of the cage. Each replication consisted of approximately 4700-4800 plants. Planting orientation was critical in 2008 (when the F_2 generation was planted) since it was observed that Japanese beetles moved to the west end in an east-west oriented row and fed mostly on the plants at west end. Approximately 10,000 Japanese beetles were collected using beetle traps with floral lures placed in surrounding fields. Those beetles were released inside the cage after the aphid rating was completed. To ensure equal infestation on all rows, beetles were evenly distributed and hand-released on to plants. Also floral lures were tied to poles to equally attract beetles to front, center and, back of cage.

In September, defoliation caused by beetles was assessed using a damage scale developed by DiFonzo and Chandrasena (Figure 2.2). A rating of 0-5 was given for each of three leaflets of the most-damaged trifoliate in each plant in first replication (A fully un-damaged leaflet was given a rating of 0, 1 means ≤10% defoliation on leaflet, 2 means ≤less than 20% defoliation, 5 means ≤ 50% defoliation). Due to high labor and time associated with this assessment we conducted this evaluation for only one replication. Similarly, each plant in every plot was given a percentage for the overall defoliation on the plant. This evaluation was conducted for both replications.

In 2010, due to lack of success of experiment with beetles confined inside the cage, we conducted a replicated field study in an open field in Entomology farm in close proximity to an asparagus patch which appeared to be a breeding site for Japanese beetles (Figure 2.3). These hill plots were organized in 2 replications. Both the first replication (which was the closest to the asparagus patch) and the adjoining second replication had randomized 235 $F_{2:4}$ lines. Four replicated plots of each E06906 and LD05-16060 were randomly planted throughout the study site for each replication. The experiment was set up this way to allow beetles to choose among all lines for feeding based on their preferences without leaving their natural habitat. Two rows of floral lures fixed to bamboo sticks were placed along the two edges of the site to maximize equal attraction of beetles to all plots. The poles with lures were approximately 1.5 m away for the edges.

Susceptibility to Japanese beetle of each plant in these 235 families were assessed using three indices; Pest severity (PS) was recorded as described by Yesudas et. al. (2010). In first week of September, when majority of lines reached R5-R6 (early pod fill) stages, pest severity was measured using newly developed rating scale from 0-9 that scored the plants based on the percentage of defoliation on the whole plant with increments of 0.5 (Figure 2.3). 0 = no defoliation on whole plant, 0.5 = not more than 5% defoliation on whole plant or only less than 5% of total leaf area from whole plant is removed by feeding, 1.0 = 5.1-10% defoliation on whole plant, 1.5= 11-15% defoliation on whole plant 2.0 = 16-20% defoliation on whole plant , 3.0= 21-30% defoliation on whole plant, 4.0= 31-40% defoliation on whole plant, 6.0= 51-60% defoliation on whole plant, 9.0 = 81-90% defoliation on whole plant.

Other indices used for assessing susceptibility are defined as following. Pest incidence (PI) was the percentage of damaged plants per plot. A new index, pest occurrence (PO) was defined as the percentage plants having Japanese beetle in each plot. In addition, maximum number of beetles on a plant within plots was also recorded when majority of plants were in blooming stage.

Forced-feeding no-choice tests

In 2011, a subset of 120 $F_{2:4}$ lines were selected to represent 40 most resistant, 40 moderately resistant, and 40 least resistant lines based on previously recorded PS data, to conduct a detached leaflet forced-feeding no-choice assay. This no-choice forced feeding assay was developed in 2008 and yielded results that led to measure significant differential feeding among six lines including LD05-16060 and E06906 (Chandrasena et al, 2012). Hence, this assay was conducted to collect feeding measurements under no-choice conditions for QTL detection.

In late July, an undamaged leaflet from the middle canopy was collected from a randomly selected field-grown plant (R5-6 stages) from each 120 lines. Each detached leaflet was placed individually in a 15 mm x 150 mm diam. Petri dish. A single leaflet was tested per line while four replications were included for E06906 and LD05-16060. Each Petri dish was labeled with a unique identifier. Next approximately 300 adult Japanese beetles collected from an asparagus field on the same day were held in a cooler for 5-6 h starvation period prior to placing two beetles in each Petri dish. After 48 h, the feeding damage on each leaflet was visually assessed using an available defoliation scale for soybean (Figure A1, appendix). Since this scale did not exceed 50% defoliation, a new scale was developed to rate plants to as high as 100% defoliation (Figure A2, appendix).

DNA extraction

The young fully unopened trifoliates were bulk harvested for each line ($F_{2:3}$) and from their parents, after rating for aphids and Japanese beetle feeding in 2009. CTAB (hexadecyltrimethyl ammonium bromide) method as described by Kisha et al. (1997) was used to extract genomic DNA from tissue samples. DNA concentration was measured using a ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, Delaware, USA).

Marker Assisted selection (MAS) for aphid-resistance

It was reported through mapping experiments (Hill et al. 2006a, 2006b) that *Rag1* locus mapped on chromosome 7 (formerly Linkage Group (LG) M) between SSR markers *satt5*40 and *satt*463. Furthermore, fine mapping of this region confirmed that *satt540* maps 7.3 cM away from *Rag1* locus (Kim et al. 2010). This marker was polymorphic between the two parents thus was used in scoring for *Rag1* resistance. It was reported through mapping experiments that *rag3* (on chromosome 16, formerly LG-J) mapped closest to SSR marker *satt414* (Guorong Zhang, pers.comm). This marker was polymorphic between the two parents, thus was used in scoring for *rag3* resistance. Initially a random subset of 94 was screened for both forms of aphid resistance. Later the remaining individuals were scored for aphid resistance after visualization on a polyacylamide gel. Whole population scoring for both markers was duplicated for accuracy.

In addition, the second recessive gene in PI567598B, *rag1b* is reported to be mapped on chromosome 7 (formerly LG-M) closest to SSR marker *satt435* (Guorong Zhang, pers.comm). *Satt435* is also mapped close to *Rag1*. Therefore this marker was not suitable to be used to

distinguish *Rag1* from *rag1b* in this population. However individuals with both *Rag1* and *rag3* genes may have the potential for more durable aphid resistance due to stacking of multiple aphid-resistant genes. Therefore we expected the lines that possess *rag* genes, and lines with stacked genes, to perform better than lines with only *Rag1*.

Genomic DNA with simple sequence repeat (SSR) markers was run on a MJ Tetrad^{TM} thermal cycler (MJ Research, Waltham, MA, USA) for PCR. After PCR, the amplified products were separated on 6% non-denaturing polyacrylamide gels using electrophoresis unit DASG-400-50 (C.B.S. Scientific Co., Del Mar, CA, USA) as described by Wang et al. (2003). After staining with ethidium bromide, the bands were visualized under UV light, and scored for polymorphism.

Linkage map construction and whole-genome mapping with SSR

Approximately 1016 SSR markers were mapped on soybean consensus map developed by Song et al. (2004). Two parents were first screened for polymorphism between these 1016 markers on a 6% non-denaturing polyacyramide gel electrophoresis unit. The polymorphic markers between the two parents were further selected to genotype a random subset of 94 individuals from the mapping population for markers distributed among 15 chromosomes (the remaining 5 chromosomes had few markers that were polymorphic between the two parents, thus were not considered for screening the population). Markers from every 10-15 cM in the consensus map were selected for this screening process. Markers associated with new QTL peaks in the 94 individuals were tested for the entire population of 235 lines to saturate the genomic regions with additional markers. Each linkage map for 15 chromosomes was constructed using JoinMap 4.0 with Kosambi mapping function. Each linkage group consisted of maximum number of markers

in fixed order as determined on the integrated map by Song et al. (2004) (Gm consensus 4.0). Gm composite 3.0 map (www.soybase.org) was used to determine the order when markers were not found on the latest 2004 map. A minimum LOD score of 1.0 or lower was used to map markers for each linkage group as they were created separately. This also allowed maximum markers to be placed in fixed order. The genetic linkage maps were drawn using MapChart function in JoinMap 4.0. Single Marker Analysis (SMA), Composite interval mapping (CIM), and Multiple Interval Mapping (MIM) methods were applied to detect QTL positions using QTL Cartographer V2.5 with the standard model Zmapqtl 6 (Wang et al. 2008).

Data analyses

Pearson correlations were conducted with the CORR procedure of SAS (Sas Institute 2010). Broad sense heritability estimates for PS trait was measured using Analysis of Variance (ANOVA) results from SAS statistical software. For CIM using QTL cartographer v.2.5, forward and backward regression method was used to select markers as cofactors. The walking speed chosen for CIM was 1 cM. A manual LOD threshold of 2.5 was used to detect QTL with 94 individuals on 15 linkage maps. The empirical LOD threshold at the 5% probability level was determined by a 1,000-permutation test (Churchill and Doerge 1994) for QTL reported with the whole population, and with forced-feeding assays. Multiple Interval Mapping (MIM) was applied to detect and further refine QTL positions and to obtain QTL x QTL interactions and their effects. MIM was conducted with model 6.0 (standard model) with 94 individuals. Forward and backward regression method was used. The final maps combined with LOD scores for QTL peaks were drawn using MapChart 2.2 (Voorrips 2002).

Results and Discussion

Phenotypic evaluations for aphid-resistance

In 2009, the 235 $F_{2:3}$ families were first evaluated for aphid resistance in a predator-proof large field cage. The standard aphid scoring method by Mensah et al (2005) was used to rate individual plants. The population derived from E06906 x LD05-16060 consisted of single dominant *Rag1* and recessive *rag3/ rag1b* alleles, thus was segregating for resistance genes from both sources. Consistent with the previous reports of breakdown of *Rag1* (Wiarda et al. 2012, Hill et al. 2011), LD05-16060 showed weaker resistance to soybean aphid than E06906 in both 2009 and 2011 evaluations (Table 2.2.). Results showed a 0.83 correlation between aphid-resistance data for the two replications in 2009. The correlation between 2009 and 2011 mean aphid scores for the entire population was 0.511($P<0.001$). Zhang et al. (2009) reported higher heritability of.0.95-0.96 for resistance derived from a population with similar PI (PI 567543C) carrying two recessive *rag* genes in field; this indicated that those may be the only major genes controlling aphid-resistance in that population. In contrast, this population harbored multiple resistant genes from both PI 567598B and Dowling, thus only the combined effect of the phenotype can be observed.

Table 2.2. Mean Aphid-resistance scores for two parent soybean lines from the two-year field evaluations

Year	E06906	LD05-16060
2009	0.51 ± 0.44	0.7 ± 0.22
2011	0.64 ± 0.24	2.25 ± 1.16

Figure 2.1. Large field cage used for evaluating 235 F_2-derived population (E06906 x LD05-16060) for aphid resistance

Phenotypic evaluations for Japanese beetle resistance

2009 aphid-cage: The first evaluation for Japanese beetle feeding was carried out in the same field cage as for evaluation of aphid resistance in 2009. However this design was not successful to collect accurate phenotypic data for QTL mapping. Despite the placement of floral lures, there was uneven distribution of Japanese beetles inside the cage. There was significant aggregation around the corners of the field cage. Only a weak positive correlation for whole-plant defoliation (r=0.22) was observed between the two replications. Based on the field data, almost zero correlation between aphid-resistance and Japanese beetle resistance was observed in both

replications (0.07 for rep 1, -0.02 for rep 2). The beetles moved to the west end because it was warmer there in the afternoon. Therefore it was finally decided that the data collected on defoliation was not reliable to use for mapping Japanese beetle-resistant QTL. The lower correlation between traits may have been caused due to inadequate reliability of 2009 data for feeding.

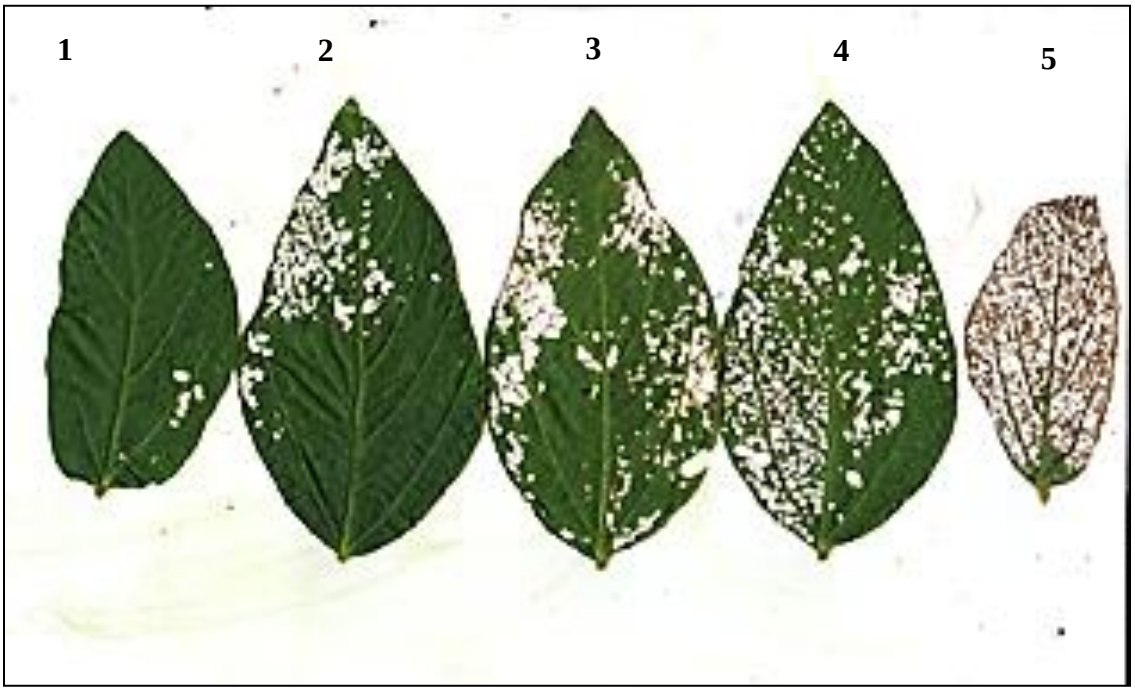

Figure 2.2. Rating scale for assessing Japanese beetle defoliation based on three most-damaged soybean trifoliates of 235 $F_{2:3}$ lines

Figure 2.3a-e. Pest Severity (PS) scale used for assessing defoliation by Japanese beetle in field choice tests on $F_{2:4}$ and $F_{2:5}$ mapping populations of 235 families

Figure 2.3 a-e (cont'd)

Figure 2.4.The study site used for evaluation of the soybean mapping population for feeding by Japanese beetle

Field choice-tests: Due to the problem with previous evaluations inside cages, there was a need to change the experimental design in order to get reliable phenotypic data for Japanese beetle feeding. In 2010, more reliable phenotypic data for Japanese beetle susceptibility were obtained from the field plots near the natural habitat of Japanese beetles. PI, PS and PO indices were used to assess lines for susceptibility for Japanese beetle. Mean PS, PI, and PO values for the two parents are presented in Table 2.3.

Table 2.3. Mean PS, PI, and PO scores for Japanese beetle on parent soybean lines in 2010

Damage index	Replication	E06906	LD05-16060
		Mean ± SD	Mean ± SD
Whole plant defoliation (% PS)	1	22.1 ± 7.4	5.4 ± 1.9
	2	9.8 ± 4.4	4.1 ± 2.1
Damaged plants/total (% PI)	1	75.3 ± 28.0	26.7 ± 14.6
	2	37.8 ± 18.4	15.3 ± 14.6
Plants with Japanese beetle /total (% PO)	1	39.4 ± 19.1	9.5 ± 7.9
	2	16.2 ± 19.7	1.9 ± 3.8

In both replications, LD05-16060 showed lower scores for PS, PI and PO, yet again confirming its low susceptibility to Japanese beetle (partial resistance).The parent line E06906, had less scores for all 3 indices in second replication than in the first replication, suggesting may be the distance from the asparagus plot made Japanese beetles feed on the first replication more than on the distant second replication.

Correlation between three indices for Japanese beetle and aphid resistant scores were studied (mean pest severity vs. mean aphid resistance, pest incidence vs. mean aphid resistance, pest occurrence vs. mean aphid resistance). However, correlation for PS between the two replications was weak (0.24) and because the distance from the asparagus patch was different for the two replications, correlation analyses was done independently for the two replications (Table 2.4). There was weak positive correlation between pest severity (which appeared to be the best index for assessing herbivory on the plant) and aphid scores in the first replication (0.128) and almost

zero negative correlation between aphid resistance and PS in second replication (-0.064). In addition there were weak positive or zero correlations between PI and PO indices with aphid scores. Hence, based on these observations there is insufficient evidence to believe of any strong correlation between the two traits in this population.

Table 2.4: Trait correlations for $F_{2:4}$ 235 soybean lines in field choice tests, 2010

Index	Replication 1	Replication 2
Aphid resistance vs. PS	0.128	-0.064
Aphid resistance vs. PI	-0.056	-0.050
Aphid resistance vs. PO	0.175	-0.042

For field evaluations in 2011 and 2012, only the pest severity measurement was collected. Although there was even distribution of beetles throughout both replications in 2012, there was severe leafhopper (*Empoasca fabae* Harris) feeding in majority of plots planted in replication two. It was observed that plants fed by leafhoppers were less attractive to Japanese beetles, thus zero or very little feeding was observed on plants when severe leafhopper damage was present. The weak correlation (0.16) between the two replications in 2012 study site could have been caused by this unexpected negative impact. Hence, only pest severity data recorded from the first replication was used for QTL mapping.

Table 2.5. Percent mean pest severity (PS) scores for Japanese beetle between parent soybean lines in field choice tests 2010-2012.

Year/trait	E06906	LD05-16060
2010_PS	22.4 ± 7.4 %	5.4 ± 1.9%
2011_PS	27.8 ± 11%	10.4 ± 5.3%
2012_PS	37.5 ± 1.5%	7.3 ± 0.4%

Trait (PS) distributions for field-choice tests conducted through 2010-2012 are shown in Figure 2.5a- f). The distribution for PS_ 2010 appeared skewed, however the PS scores for parents were significantly apart (Figure 2.5a). In both 2011 and 2012, the PS distributions appeared to behave normal. The Pest Incidence (PI) was an estimate of percentage of plants fed within a line; however this appeared to be a less valuable measure due to failure of this estimate to measure and compare the severity of defoliation among lines. Broad sense heritability for defoliation-resistance was relatively low (0.44), with 0.18-0.62 90% Confidence Interval for PS_2010.

Source of variance	DF	Mean Square	Mean Square ID
Genotype	75	0.8519	M1
Error	74	0.4777	M2

Broad sense heritability was calculated using above ANOVA output and substituting mean square components in the equation below.

$$h^2 = \frac{M1 - M2}{M1}$$

Due to unreliable measurements collected from the second replication in 2012, heritability measurements were not calculated for that year. Based on these findings it appeared that aphid-resistance and Japanese beetle resistance are independently controlled in both MSU and non-MSU germplasm tested. Additionally, Chandrasena et al. (2012) reported that *rag3/rag1b* aphid-resistant lines were not different from the aphid-susceptible Titan RR when it came to susceptibility to Japanese beetle. Similarly both *Rag1* line (LD05-16060) and aphid-susceptible isoline SD01-76R, were not different for Japanese beetle susceptibility irrespective of the aphid resistance trait.

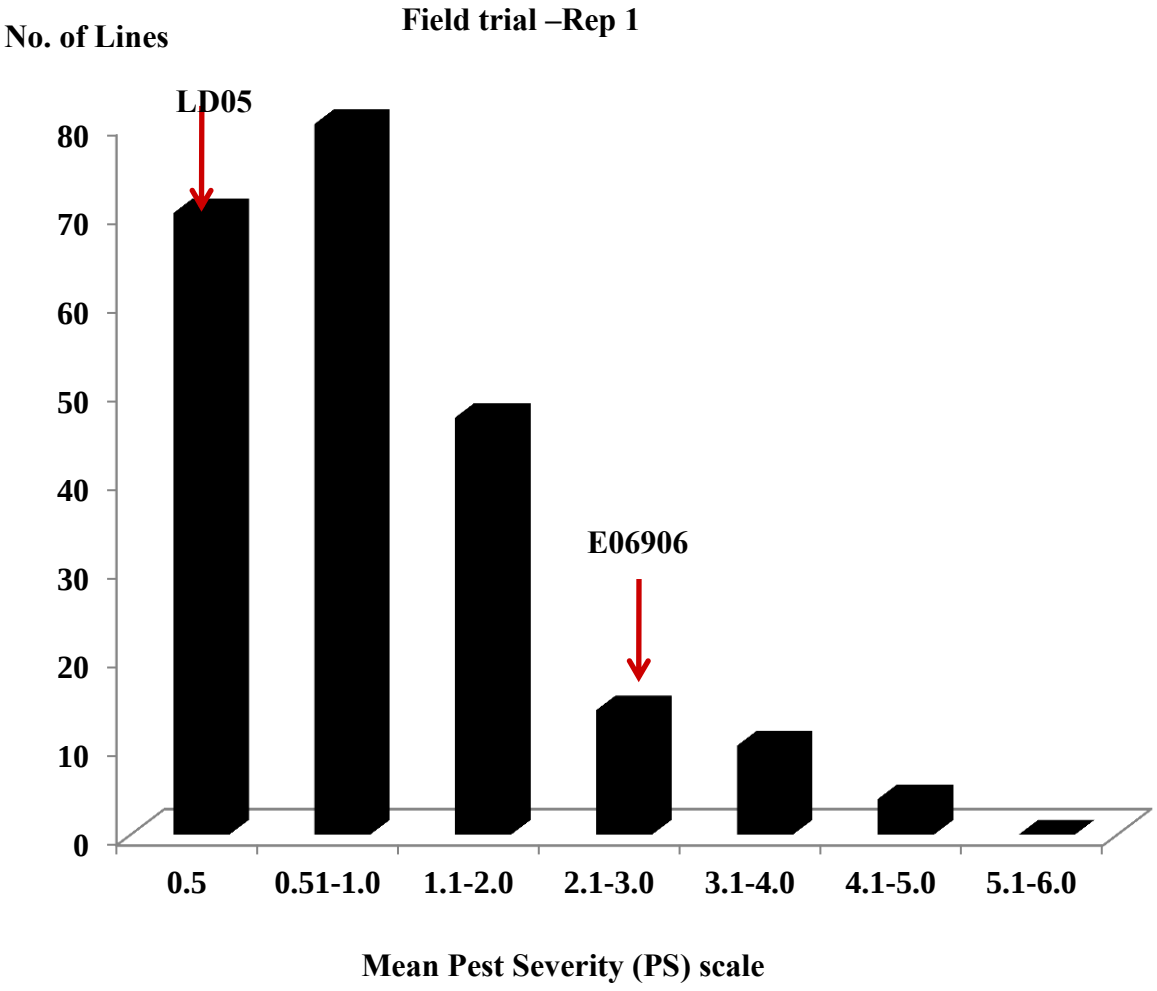

Figure 2.5a-f: Trait distributions for pest severity indices, 2010-2012

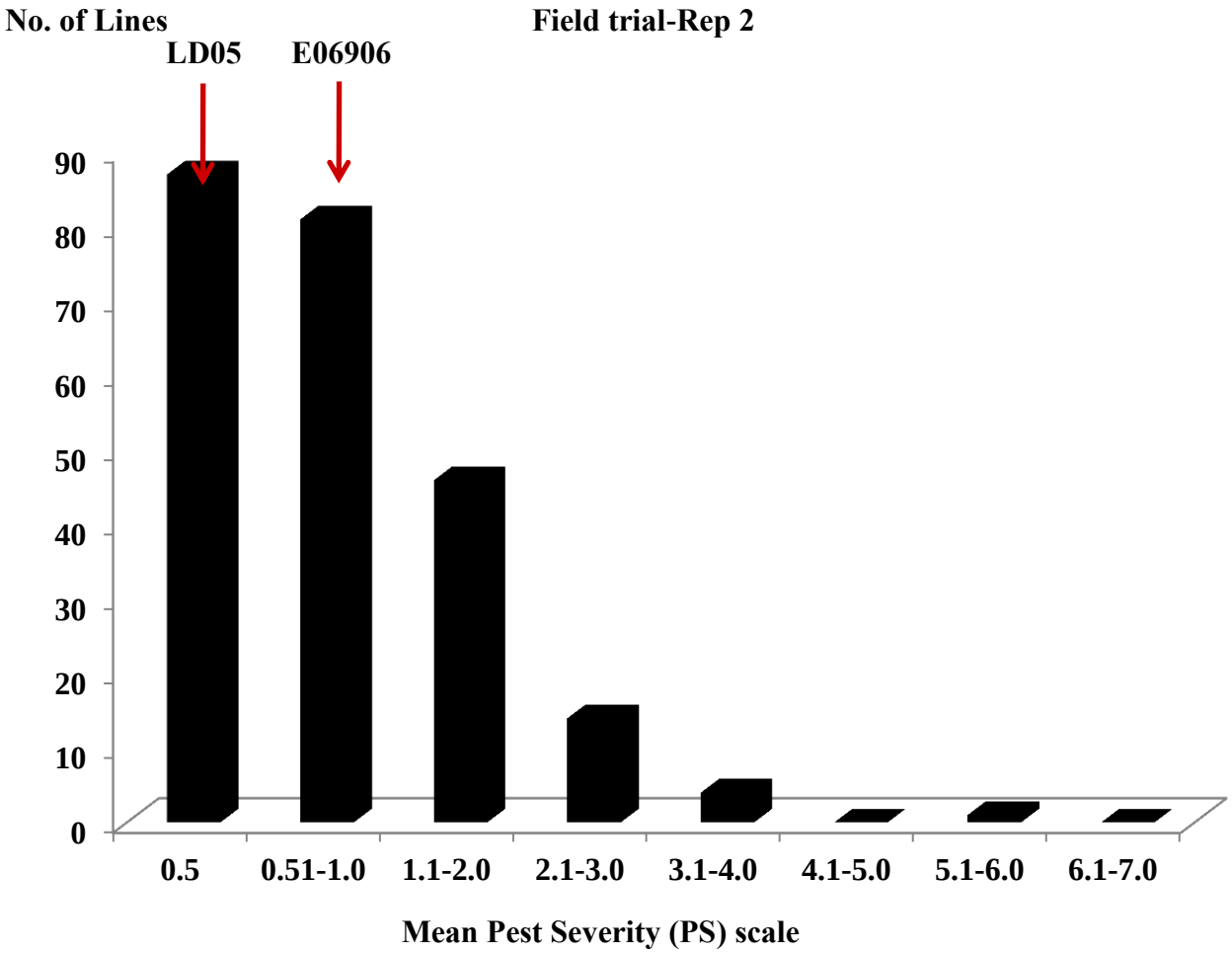

Figure 2.5a-f (cont'd)

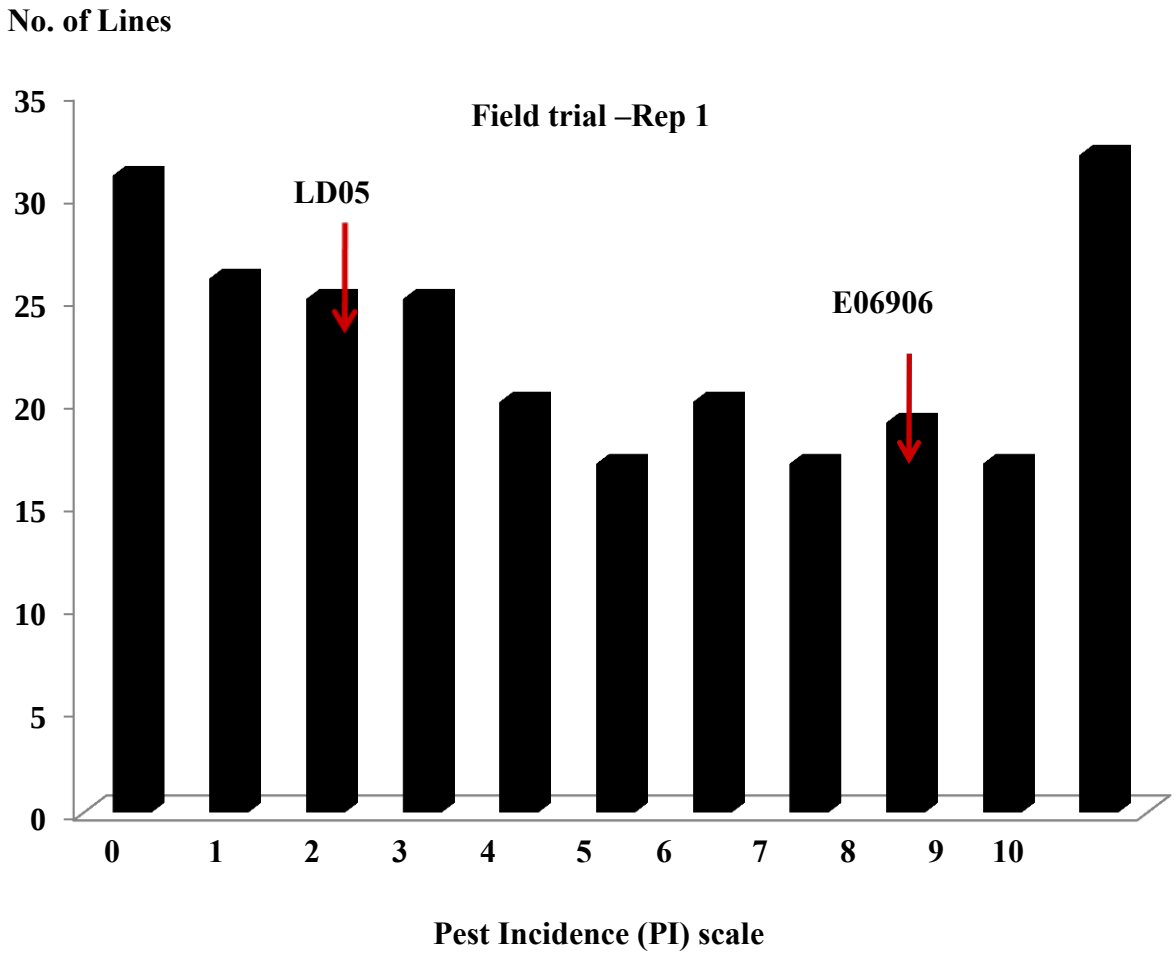

Figure 2.5a-f. (cont'd)

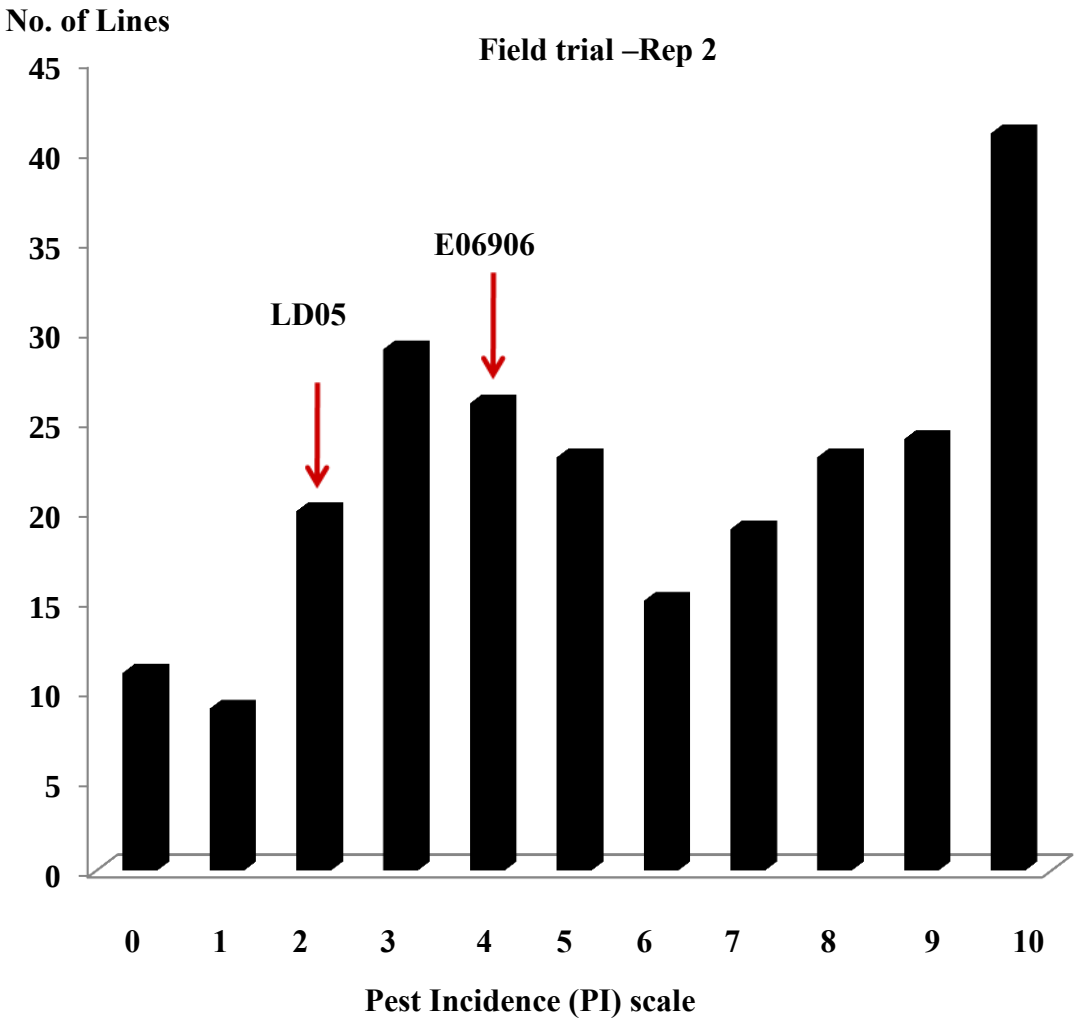

Figure 2.5a-f (cont'd)

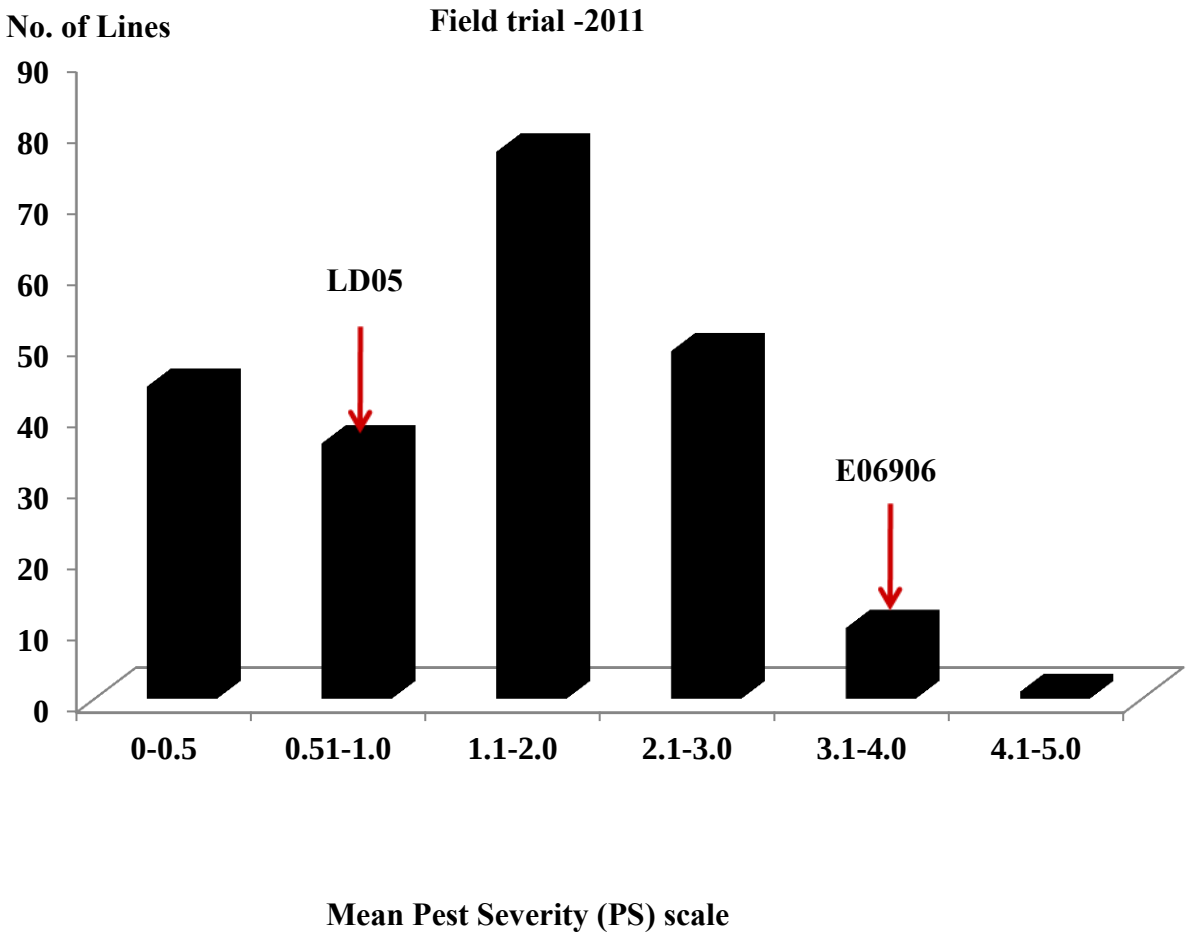

Figure 2.5a-f (cont'd)

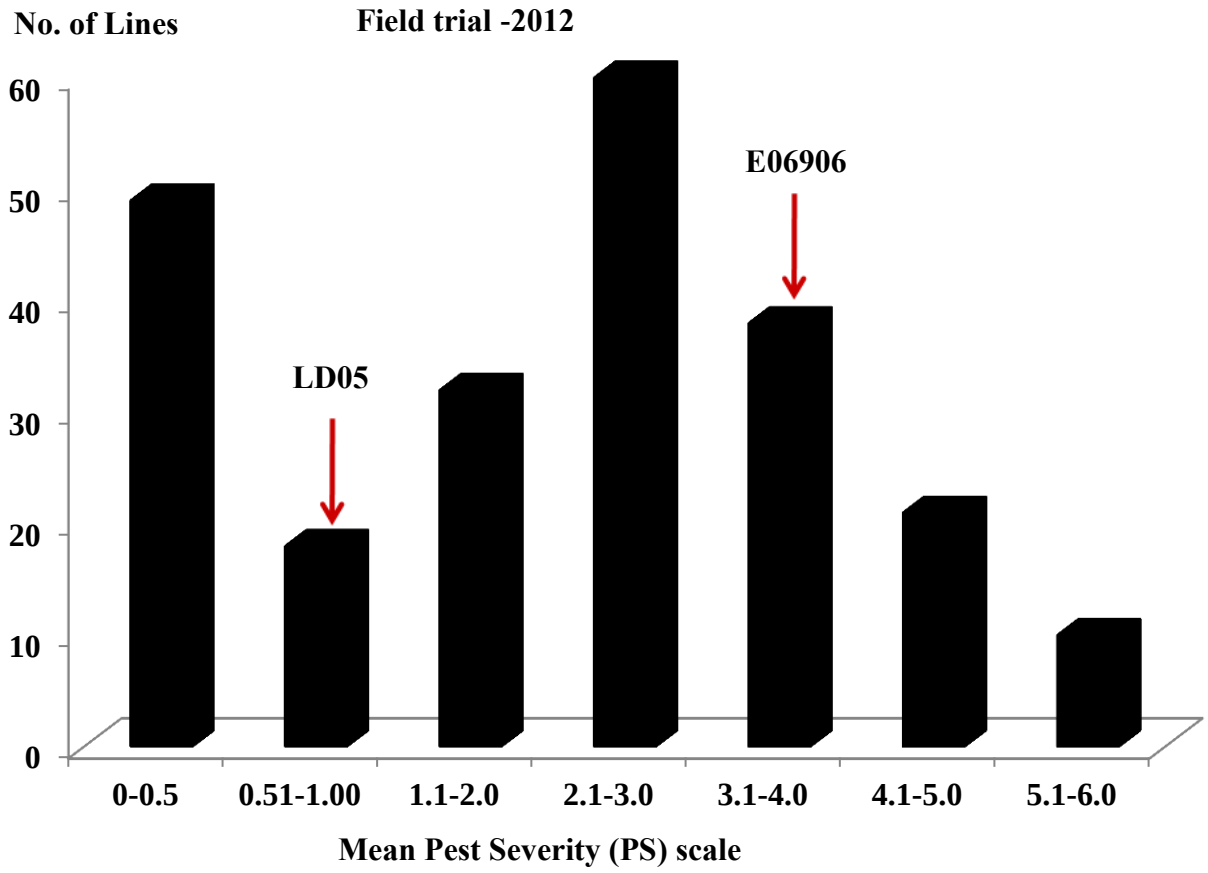

Figure 2.5a-f (cont'd)

No -choice feeding assays: Forced feeding by two beetles confined to a leaflet, belonging to randomly chosen 120 lines, (Resistant, Susceptible, and moderately resistant) among 235 lines were measured as another phenotype for verifying QTL already identified with field-choice tests. Percent defoliation on a leaflet after 48 h of feeding was recorded from 113 lines (seven lines were excluded due to un-healthy appearance of leaves). No-choice tests help to distinguish antibiosis and antixenosis effects of a QTL.. This phenotype was used to detect or further confirm QTL identified by PS trait. The trait distribution for 113 lines is shown in Figure 2.6. The mean defoliation on E06906 was heavy (88 ± 2.5%), while the mean defoliation on LD05-16060 was relatively low (34 ± 7.7%), again confirming that resistance conferred by LD05-

16060 was antixenosis (Figure 2.7) . Previous no-choice tests by Chandrasena et al. (2012) on LD05-16060, also confirmed antixenosis (non-preference) in LD05-16060.

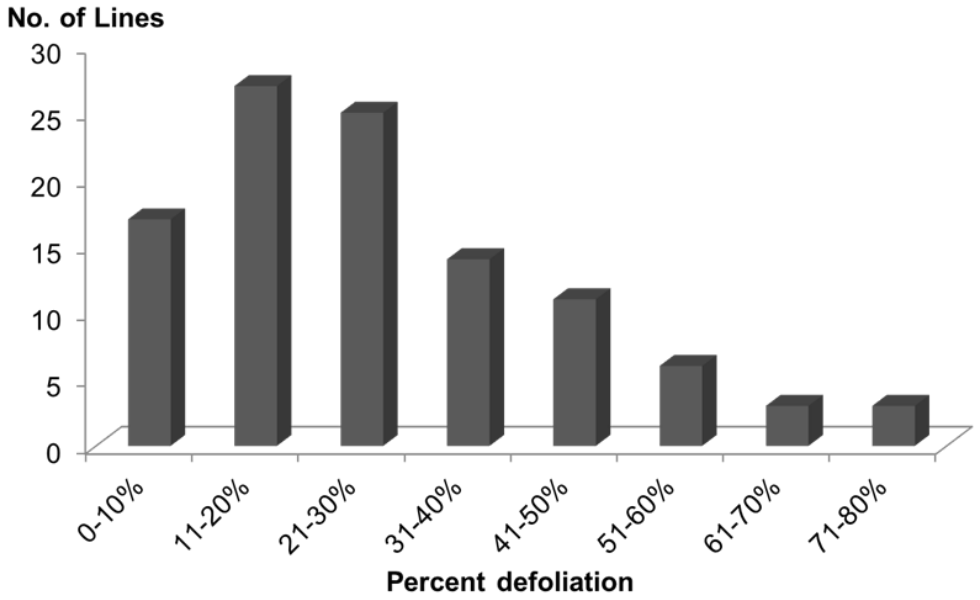

Figure 2.6. Distribution of forced-feeding defoliation trait for Japanese beetle among soybean leaflets from 113 $F_{2:5}$ soybean lines.

Figure 2.7. Four replicates of E06906 and LD05-16060 after 48h in no-choice feeding assay for Japanese beetle

MAS for aphid-resistance

DNA extracted using CTAB protocol yielded good quality DNA with concentrations ranging from 587 ng/µL -3801 ng/µL. The DNA quality (260/280) ratio ranged between 1.42 for low quality samples to 2.13.for best quality DNA. DNA from all individuals was used with PCR and gel electrophoresis for MAS.

Pedigree selection of best individuals conferring resistance to both insects

After phenotypic data collection for PS in 2010, the best $F_{2:4}$ families that show resistance to both insects with mean PS score not more than 0.5 and aphid index not more than 0.5 were selected, (see appendix from detailed protocol for selections). Seeds were individually harvested from the best individual within a line and these individuals were genotyped for the aphid – resistance source(s) (Table 2.6). Lines with *rag3*, and *rag3* stacked with *Rag1* were further advanced until F_6 generations eliminating the lines with susceptibility to both insects after phenotypic evaluations every year. The selected $F_{5:6}$ lines were planted in hill-plots to evaluate for agronomic traits such as yield, lodging, and maturity.

Table 2.6. Aphid resistance genes in best individuals within $F_{2:4}$ soybean lines conferring highest resistance to both insects in 2010.

Individual_ID	Aphid-resistance gene	
10	*Rag1*	
39	*Rag1*	
69	*Rag1*	
48	*Rag1*	
60	*Rag1*	*rag3*
61	*Rag1*	*rag3*
229	*Rag1*	*rag3*
70	*Rag1*	*rag3*
136	*Rag1*	*rag3*
137	*Rag1*	*rag3*
150	*Rag1*	*rag3*
160	*Rag1*	*rag3*
174	*Rag1*	*rag3*
189	*Rag1*	*rag3*
198	*Rag1*	*rag3*
205	*Rag1*	*rag3*
225	*Rag1*	
89	*rag3*	
101	*rag3*	
103	*rag3*	
107	*rag3*	
111	*rag3*	
115	*rag3*	
119	*rag3*	
121	*rag3*	

Linkage Map construction

From a total of 1016 SSR markers based on the consensus map (Song et al. 2004), nearly 300 markers from all 20 soybean linkage groups were polymorphic between LD05-16060 and E06906. From this, 130 markers were successfully genotyped on a subset of 94 lines along with parents (Table 2.7). An example of a gel photo is shown in Figure 2.8. 119 markers were mapped in fixed order to 15 linkage groups in final maps. JoinMap 4.0 was used to create genetic maps for every linkage group (Figure 2.9). 11 out of 130 markers were eliminated because they inflated the maps, thus was unable to be included in final fixed order. The total length of the genetic map spanning 15 chromosomes was 2168.2 cM. The average interval length between markers in fixed order was 18.2 cM. The longest chromosome was LG-D1b (209.2 cM) while the shortest was LG-E (61.3 cM). The minimum number of markers mapped in fixed order on a chromosome was 5 (in LG-B1) while the maximum number of markers mapped in fixed order on a chromosome was 12 (in LG-C2). Majority of the markers were successfully mapped in the same order as the latest reference map published on SoyBase (www.soybase.org), however some markers caused heavy inflations thus were removed. The highest deviation of marker order from consensus map was observed in LG-C2 fixed order. Next, genome scan on 15 chromosomes for QTL conferring resistance to Japanese beetle was conducted with a subset of 94 individuals.

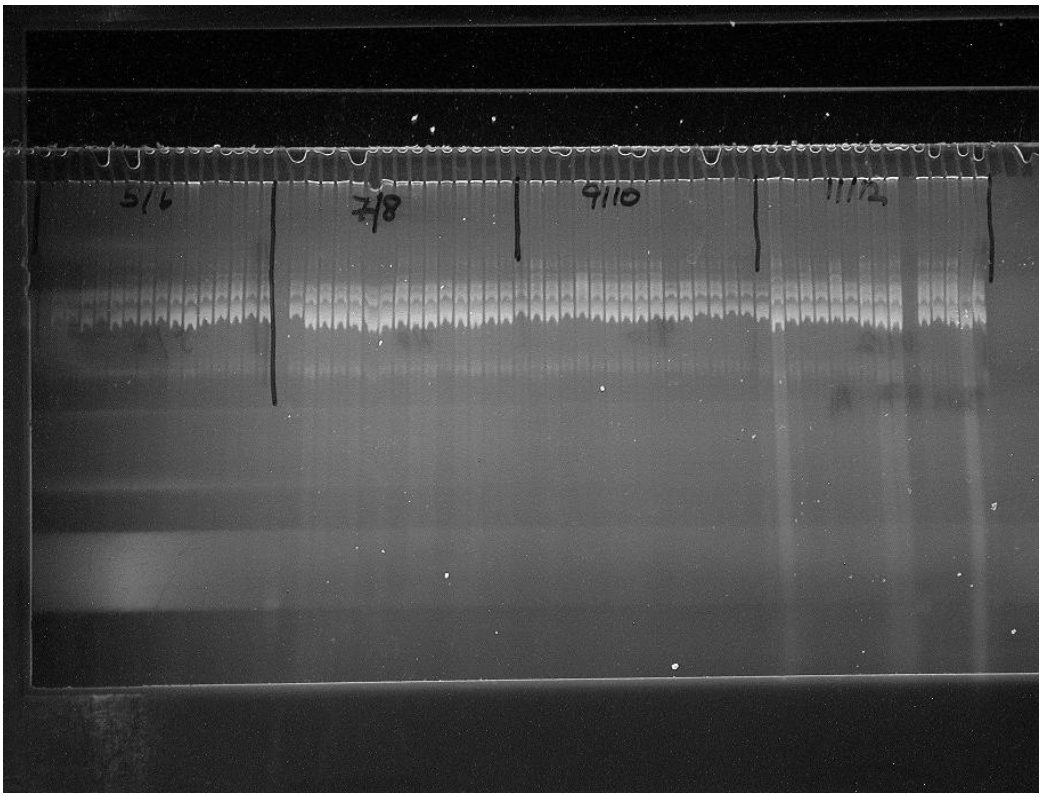

Figure 2.8. A subset of individuals ready to be genotyped for *Sat*_271 on LG-A1 (gel visualized after Ethidium bromide staining)

Table 2.7. SSR markers utilized in creating genetic linkage maps for 15 soybean chromosomes.

LG-A1	**LG-A2**	**LG-B1**	**LG-B2**	**LG-C1**	**LG-C2**	**LG-D1a**
Sat_271	*Satt*429	*Satt*484	*Satt*601	*Satt*294	*Satt*277	*Satt*267
Sat_217	*Sat_138*	*sdtt444*	*Satt*560	*Satt338*	*Satt371*	*Satt198*
*Satt*200	*Sat_259*	*Sat_270*	*Satt168*	*Satt399*	*Sat_263*	*Satt203*
Satt211	*Satt377*	*Sat_272*	*Sat_177*	*Satt565*	*Satt316*	*Satt283*
Satt042	*Satt228*	*Sat_128*	*Satt577*	*Satt195*	*Satt433*	*Satt032*
Sat_137	*sct_067*		*Satt556*	*Satt646*	*Satt307*	*Sat_159*
Satt155	*Satt455*		*Satt065*	*Satt524*	*Satt640*	*Satt320*
Satt648	*Satt589*		*Satt020*	*Satt180*	*Satt289*	*Sat_201*
Satt385	*Satt390*		*Sat_358*		*Sat_286*	
Satt599	*Satt187*				*Satt202*	
Satt684	*Satt209*				*Satt336*	
	Sat_319				*Satt489*	
	Sat_377					

LG-D1b	**LG-D2**	**LG-M**	**LG-E**	**LG-G**	**LG-H**	**LG-J**	**LG-L**
Sat_096	*Satt328*	*Satt702*	*Satt268*	*Sat_117*	*Sat_180*	*Sat_412*	*Satt481*
Sat_202	*Satt486*	*Satt323*	*Satt685*	*Sat_185*	*Sat_410*	*Sat_350*	*Sat_182*
Sat_173	*Satt461*	*Satt220*	*Satt575*	*Sat_203*	*Satt192*	*Satt244*	*Satt156*
Satt266	*Satt002*	*Satt626*	*Satt212*	*Satt115*	*Sat_214*	*Sat_151*	*Satt143*
Satt459	*Satt615*	*Satt245*	*Satt606*	*Satt191*	*Sat_142*	*Sat_224*	*Satt229*
Satt290	*Satt447*	*Sat_003*	*Satt598*	*Satt427*	*Satt302*		*Satt373*
Satt558	*Satt226*	*Satt250*	*Satt204*	*Sat_088*	*Satt353*		*Satt006*
Sat_211	*Satt301*	*Sat_258*	*Satt452*	*Satt275*			*Sat_448*
Satt634		*Satt636*					
Satt579		*Satt567*					
Sat_351		*Sat_258*					

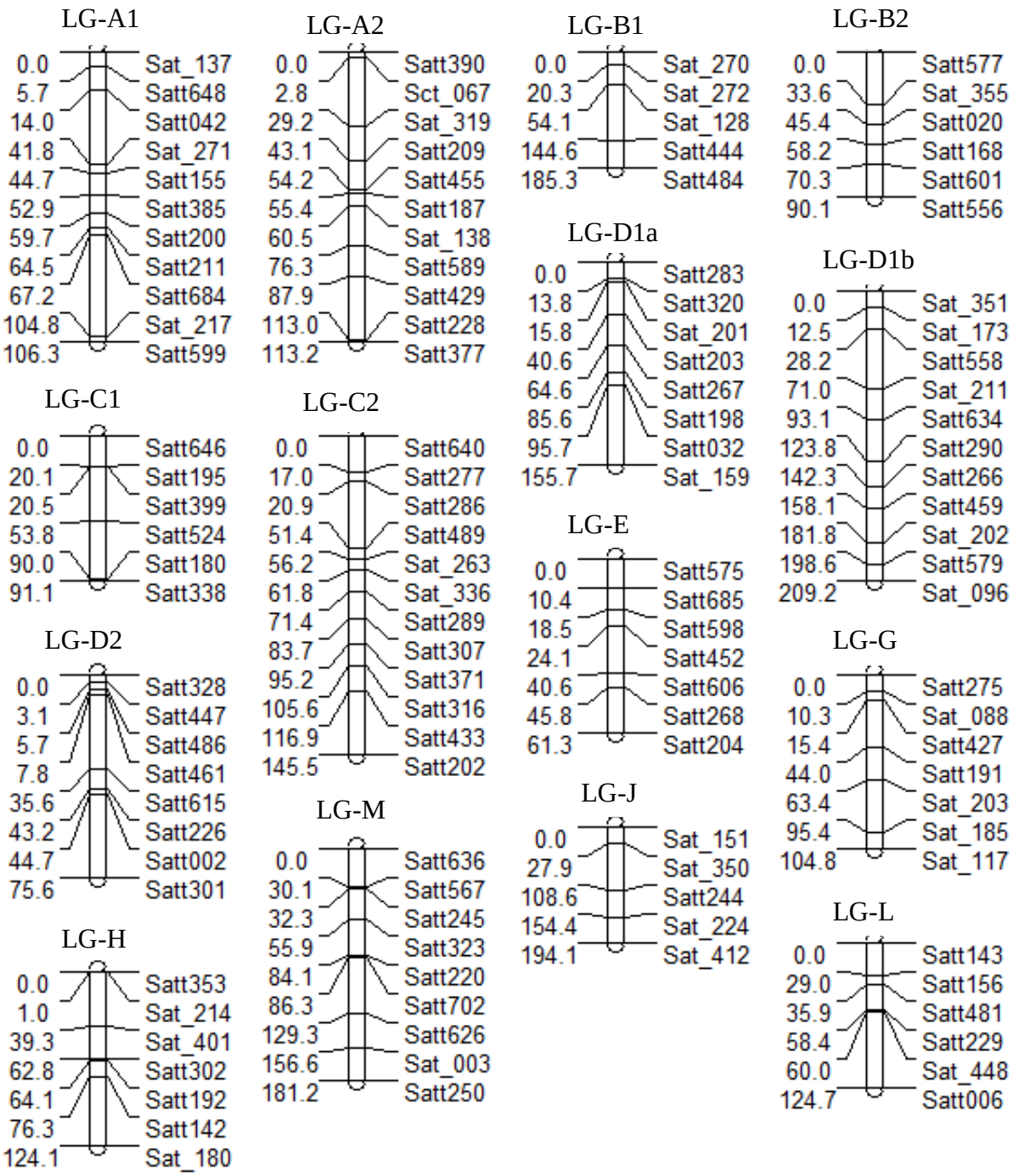

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Figure 2.9. Linkage maps of 15 soybean chromosomes with 119 SSR in fixed order created with JoinMap 4.0.

QTL mapping

Single marker analysis: Single marker associations with pest severity traits PS_2010, PS_2011, and PS_2012 were discovered on several linkage groups. Significant single marker-trait associations at 5% probability level are listed in Table 2.8. Graphs corresponding to some significant QTL are shown in appendix. Some QTL were detected with more than one trait thus provided a better possibility to be a true QTL linked to that marker. *Sct_067* on LG-A2 appeared to be significantly associated with both PS-2010 and PS_2012. Another position on LG-A2 was detected by PS_2011 data only. Similarly a significant marker-trait association was observed for *Sat_271* on LG-A1. Another important finding from this initial QTL analysis for Japanese beetle was that these results provided strong evidence of presence of several known major-defoliation resistant QTL in this population. The results show the potential of these known QTL to confer resistance to Japanese beetle defoliation as well. A major QTL reported to confer resistance to several insect defoliators, namely QTL-M, was detected with significant associations to *Satt626* with PS-2010 and PS_2012. *Satt626* is a tightly linked marker to QTL-M, which was reported by several groups investigating defoliation-resistant QTL in soybean (Rector et al. 1998, 2000; Zhu et al. 2006, Zhu et al. 2008). *Satt*353, a possible marker linked with another known defoliation resistant QTL, QTL-G was detected with PS-2010 and PS_2011 traits. QTL-G has shown to provide resistance to Corn Ear Worm (Rector et al. 1998, 2000) which is flanked by *Satt*472-*Satt*191 (89-103 cM, Gm consensus 4.0) on LG-G. In this population, two significant associations were detected with *Sat*_117 (76.cM, Gm consensus 4.0) on LG-G and *Satt*185 (100.3cM, Gm consensus 4.0) on LG-G. Therefore these significant-marker trait associations may correspond to the known QTL-G. Another soybean defoliation-resistant QTL on LG-H has been identified by Rector et al. (1998 , 2000) for CEW resistance, which mapped between

*Satt*541-*Satt*122 (63-72cM, Gm consensus 4.0). However in LG-H, a different marker *Satt*353 (13.8 cM, Gm consensus 4.0) was associated with PS_2010 and PS_2011. This marker was further away from the known QTL position thus single marker analysis did not confirm presence of QTL-H. Two markers were associated with PS_2010 in D1b. Another known QTL in LG-D1b for CEW has been reported (Rector 1998, 2000). QTL-D1b mapped between *Satt*141–*Satt*290 . These two markers were not identified as significant ones in SMA, however, the significant marker *Satt*266 mapped in close proximity to *Satt29*0 (18cM apart) in D1b linkage map. This may be a good indication of presence of another known QTL, QTL-D1b in the population. Other marker-trait associations were consistently found on LG-L, LG-B2 and LG-E. All these marker-trait associations were further investigated with CIM to discover their link to a true, known or new QTL.

Table 2.8. Single marker associations with pest severity (PS) traits for Japanese beetle defoliation with a subset of 94 soybean lines derived from E06906 x LD05-16060.

Trait	LG (Chr.)	Significant marker	F (P<0.05)
PS_2010	A2 (05)	*Sct_067*	4.72 (0.032)
	D1b (02)	*Satt558*	4.67 (0.033)
	D1b (02)	*Satt266*	3.99 (0.049)
	M (07)	*Satt626*	6.48(0.03)
	E (15)	*Satt452*	6.76(0.011)
	E (15)	*Satt606*	6.59(0.012)
	G (18)	*Sat_117*	6.46 (0.013)
	H (12)	*Satt353*	4.31(0.041)
	L (19)	*Satt143*	5.822(0.018)
	L (19)	*Satt006*	6.795(0.011)
PS_2011	A1(08)	*Sat_217*	7.228 (0.009)
	A2 (05)	*Satt209*	4.11(0.045)
	H (12)	*Satt353*	7.27(0.008)
	L (19)	*Satt448*	4.733(0.032)
PS_2012	A2 (05)	*Sct_067*	4.43(0.038)
	B1 (11)	*Satt168*	7.169(0.009)
	B2 (14)	*Satt020*	5.723(0.019)
	D2 (17)	*Satt461*	5.476(0.021)
	M (07)	*Satt626*	5.72(0.019)
	G (18)	*Satt275*	9.78(0.002)

Composite Interval Mapping (CIM) with a subset of 94 individuals

Composite Interval Mapping (CIM) with 94 individuals derived from E06906 x LD05-16060 consistently detected several QTL with one or more traits (Table 2.9). A highly significant QTL peak (LOD 2.66) was detected on LG-A1 with PS_2010 that showed 11% effect. The same QTL was detected with PS_2012 with a lower LOD score with 10% effect. Despite the relatively low

LOD score, the marker interval overlapped with previous years' interval. Moreover, three flanking markers for this QTL, *Satt200*, *Satt211*, and *Sat*_217mapped within 15cM in Gm consensus 4.0. Due to consistent detection of this significant peak with SMA and CIM, and with high QTL effect, there is evidence to believe the presence of a new QTL conferring resistance to Japanese beetle in this population (Figure 2.10). In addition to this, another new significant QTL was detected on LG-C2 with PS_2010 and PS_2012 with LOD scores 5.7 and 6.3 respectively. However, this QTL was only responsible for 3-5% of phenotypic variation with PS traits (Figure 2.14).

Presence of three known QTL for insect defoliation resistance (QTL-M, QTL-G, and QTL-H) were again detected. A significant QTL (LOD >2.5) was consistently detected between *Satt323*-*Satt702*- *Satt 626* interval. This peak explained 31% of total phenotypic variation of the PS_2010 trait. The same flanking markers has been identified for QTL-M after fine mapping experiments (Zhu et al. 2008), thus there was reliable evidence of presence of QTL-M in this population. Similarly QTL-G also showed major effect for conferring Japanese beetle resistance in this population. QTL-G was mapped on *satt472-satt191* interval (Zhu et al 2006, 2008). In this population a highly significant QTL was repeatedly mapped in the same interval (*Sat_185 – Sat_117*) on LG-G explaining 47% of phenotypic variation in 2010 (Figure 2.13). Additionally as mentioned before, these two markers were also detected by SMA.

Flanking markers *Satt472-Sat191,* were mapped approximately 89-103cM (Gm consensus 4.0) on LG-G. While two significant peaks were detected in this population, with *Sat_117* (76.cM Gm consensus 4.0) on LG-G and *Satt185* (100.3cM Gm consensus4.0) on LG-G, it can be

assumed that QTL-G was present in this population. Similarly, a significant QTL corresponding to QTL-H was also detected in this population (Figure 2.15). QTL-M, G, and H have been detected together in accessions derived from Japanese PI 229358. This discovery strongly indicates that LDO5-16060 might have been derived from this PI. A peak corresponding to QTL-D1b was also detected in all three years; PS_2010 peak was highly significant explaining 22% of the variation. It was detected in the same marker interval *Satt634-Satt290* for all three traits, while the reported position was slightly shifted further away between *Satt191-Satt290*. Due to significance of association to *Satt290*, and close proximity to the published QTL it could be concluded that the QTL found with this study in LG-D1b appear to be the same as previously reported QTL linked to CEW resistance.

Two potential peaks were observed on LG-E and LG-A2. Although the LOD did not reach the 2.5 threshold, due to detection of a peak (LOD =1.65) between *Sct_067- Sat_319* interval, and because SMA also detected significant marker-trait associations to *Sct_067*, there is solid evidence to support the presence of a new QTL in A2 associated with *Sct*_067. Also this QTL explained 18% of phenotypic variation in 2010. The same peak was detected in other two years only with a slight shift, now to be flanked by *Sat 319_Satt185* (Figure 2.17). Despite the LOD <2.5, QTL present on LG-E was both detected with CIM and SMA (Figure 2.16). A QTL on LG-E is reported to be associated with CEW resistant from PI 227687 (Hurburt et al. 2001). This region was later reported to confer insect-resistance through dense pubescence (pb). It is possible that the same QTL was detected in our population. All significant QTL detected with CIM on 94 individuals are shown on MapChart graphs in the following pages.

Table 2.9. Composite Interval Mapping (CIM) with pest severity (PS) traits for Japanese beetle defoliation with a subset of 94 soybean lines derived from E06906 x LD05-16060.

LG (Ch.)	Trait	Marker Interval	LOD	R^2
A1 (08)	field choice_PS2010	*Satt200-Satt211*	2.66	11%
	field choice_PS2012	*Satt200-Sat_217*	1.5	10%
A2 (05)	field choice_PS2010	*Sct_067-Sat_319*	1.65	18%
	field choice_PS2011	*Sat_319-Satt187*	1.1	8%
	field choice_PS2012	*Sat_319-Satt187*	0.91	6%
C2 (06)	field choice_PS2010	*Satt286-Satt489*	5.7	3%
	field choice_PS2012	*Satt286-Satt489*	6.3	5%
D1b (02)	field choice_PS2010	*Satt634-Satt290*	3.1	22%
	field choice_PS2011	*Satt634-Satt290*	1.6	ns
	field choice_PS2012	*Satt634-Satt290*	2.2	ns
M (07)	field choice_PS2010	*Satt702_Satt323*	2.9	31%
	field choice_PS2011	*Satt323-Satt702*	2.1	7%
	field choice_PS2012	*Satt702-Satt626*	2.7	9%
G (18)	field choice_PS2010	*Sat_185-Sat_117*	3.9	47%
	field choice_PS2012	*Sat_185-Sat_117*	2	7%
E (15)	field choice_PS2010	*Satt452-Satt606*	2.3	17%
H (12)	field choice_PS2012	*Satt142-Sat_180*	4.6	10%

LG- Linkage Group
ns - not significant

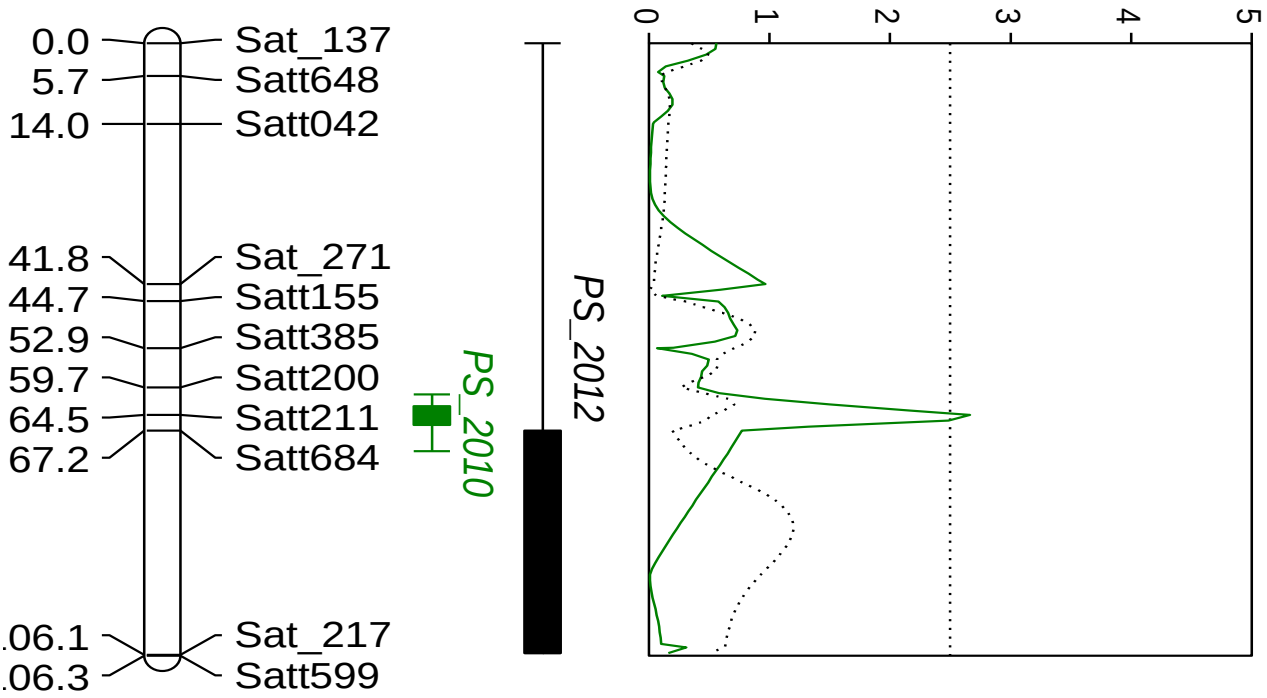

Figure 2.10. Composite Interval Mapping (CIM) in LG-A1 with pest severity (PS) traits for Japanese beetle defoliation on a subset of 94 soybean lines derived from E06906 x LD05-16060 .

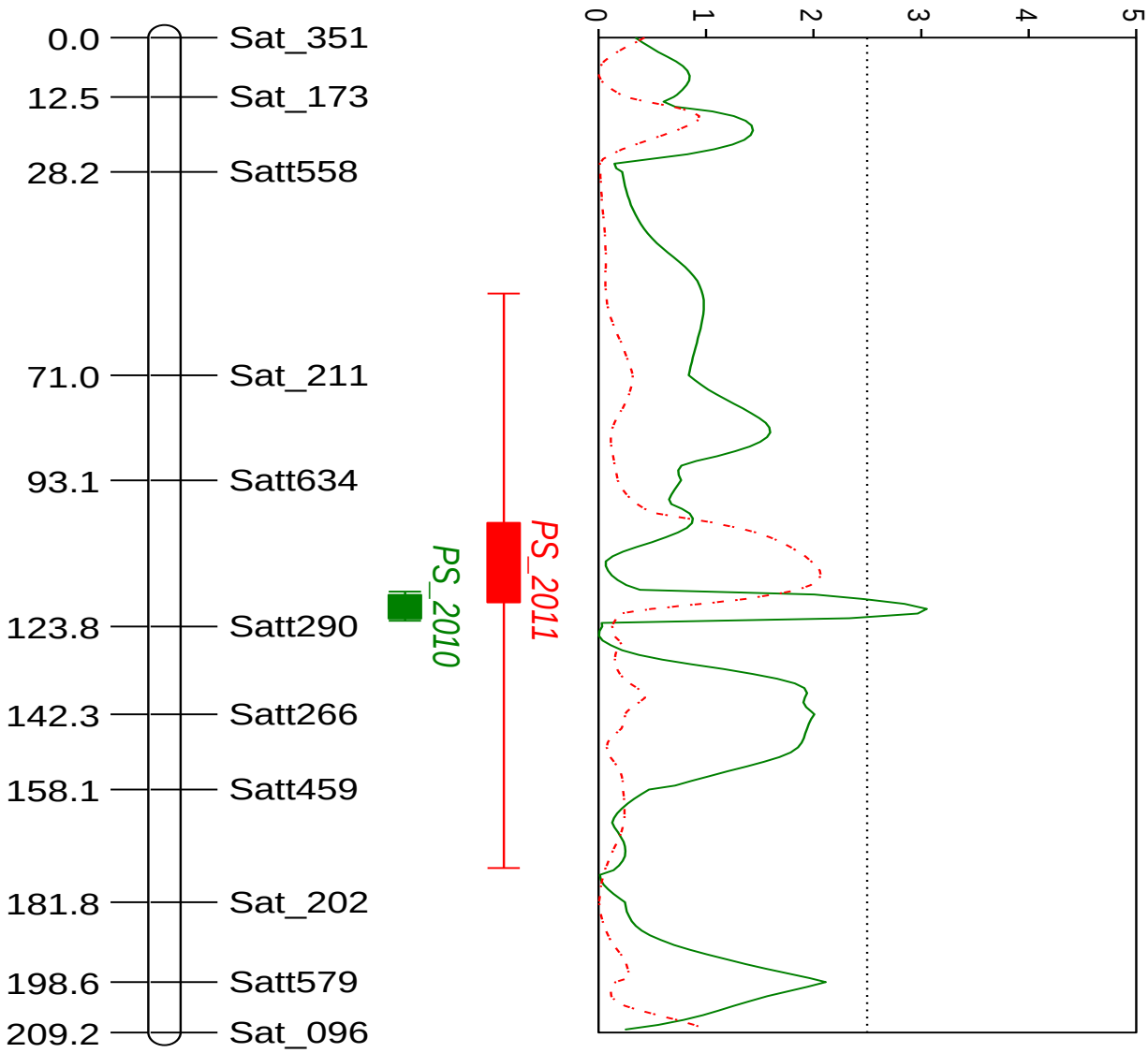

Figure 2.11. Composite Interval Mapping (CIM) in LG-D1b with pest severity (PS) traits for Japanese beetle defoliation on a subset of 94 soybean lines derived from E06906 x LD05-16060

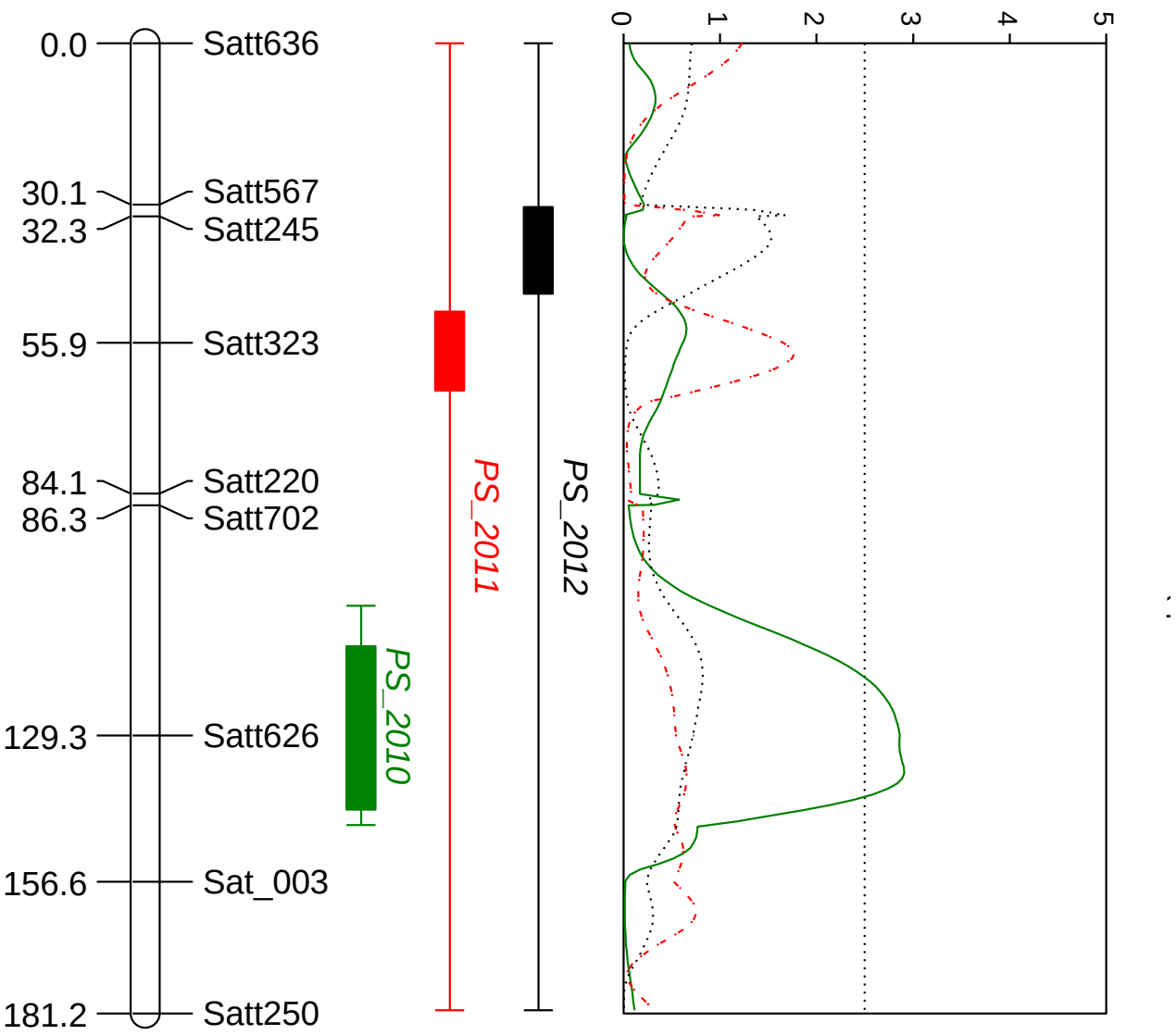

Figure 2.12 . Composite Interval Mapping (CIM) in LG-M with pest severity (PS) traits for Japanese beetle defoliation on a subset of 94 soybean lines derived from E06906 x LD05-16060

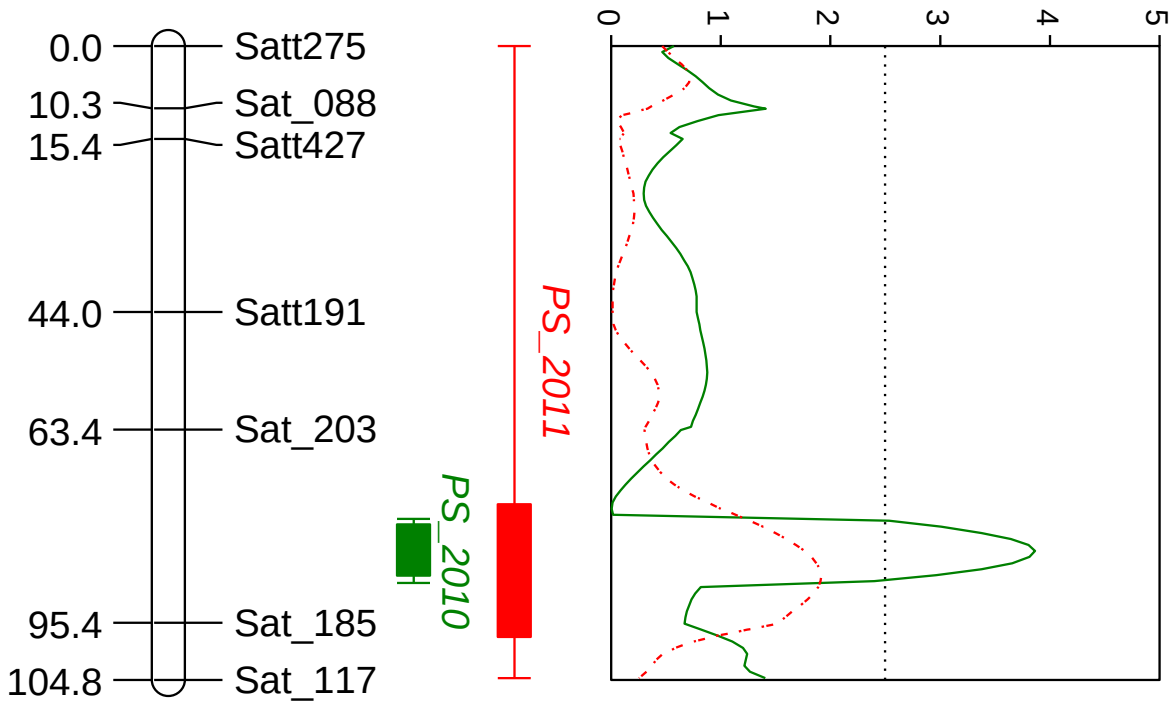

Figure 2.13. Composite Interval Mapping (CIM) in LG-G with pest severity (PS) traits for Japanese beetle defoliation on a subset of 94 soybean lines derived from E06906 x LD05-16060

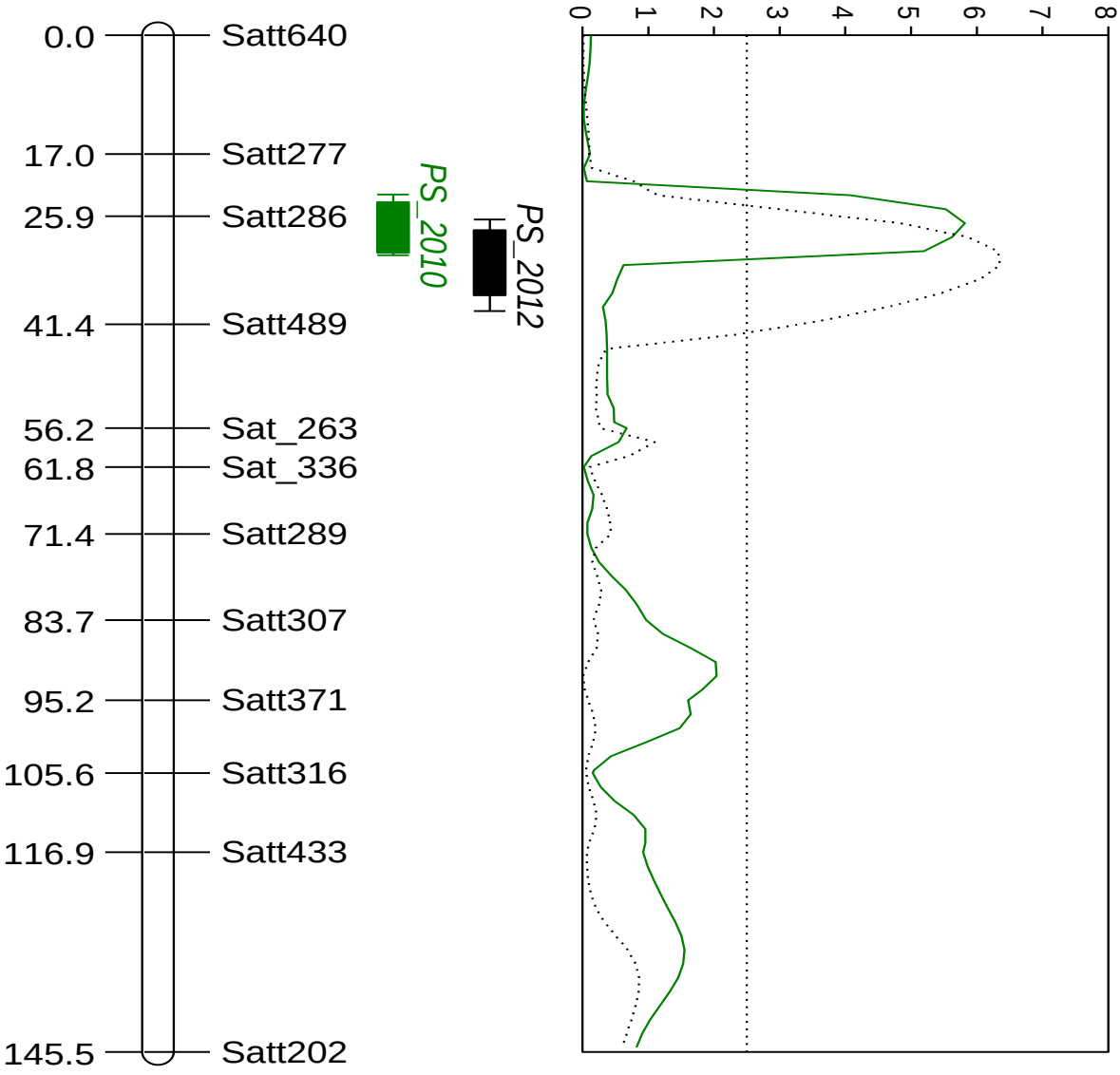

Figure 2.14. Composite Interval Mapping (CIM) in LG-C2 with pest severity (PS) traits for Japanese beetle defoliation on a subset of 94 soybean lines derived from E06906 x LD05-16060

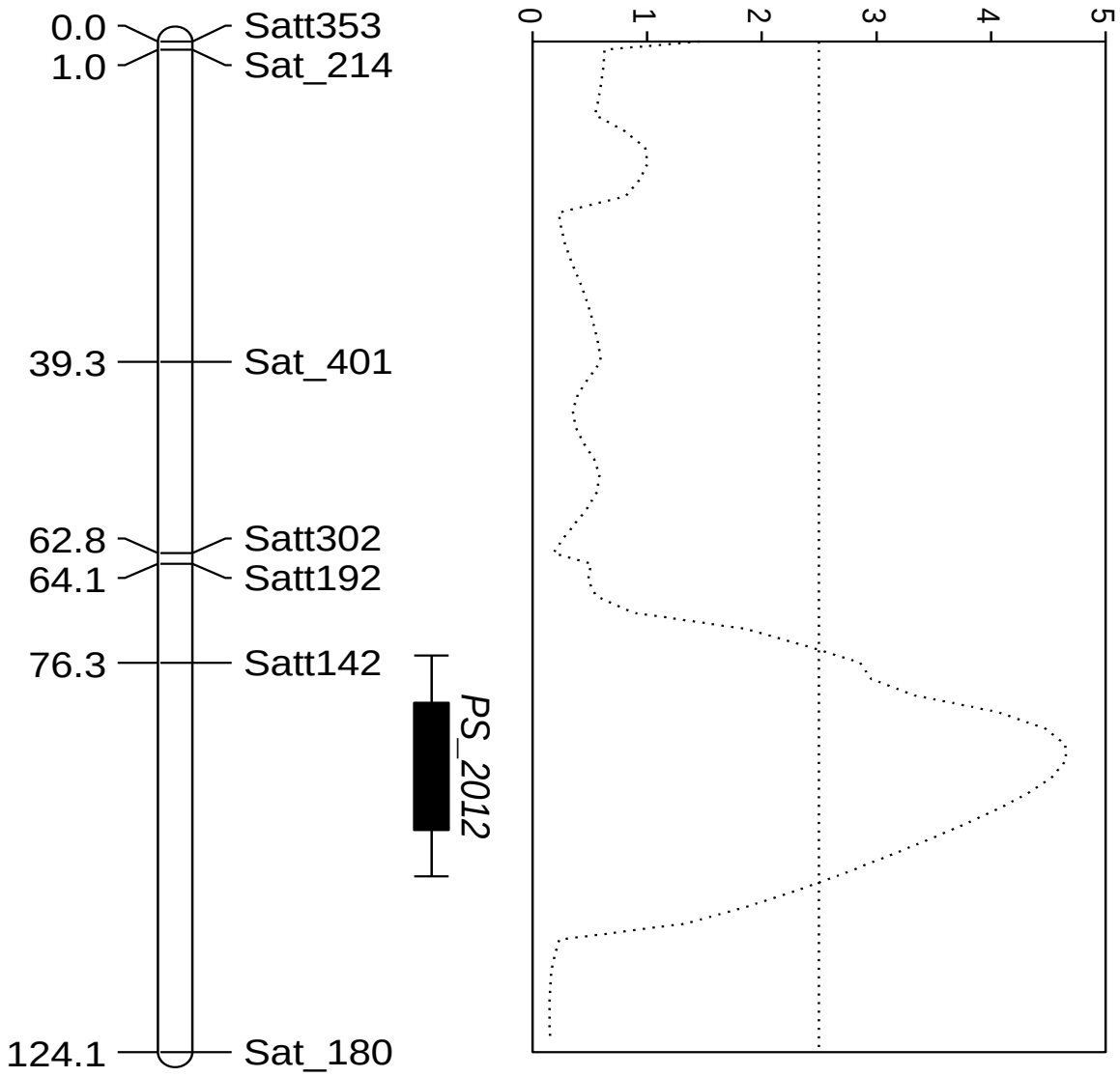

Figure 2.15. Composite Interval Mapping (CIM) in LG-H with pest severity (PS) traits for Japanese beetle defoliation on a subset of 94 soybean lines derived from E06906 x LD05-16060

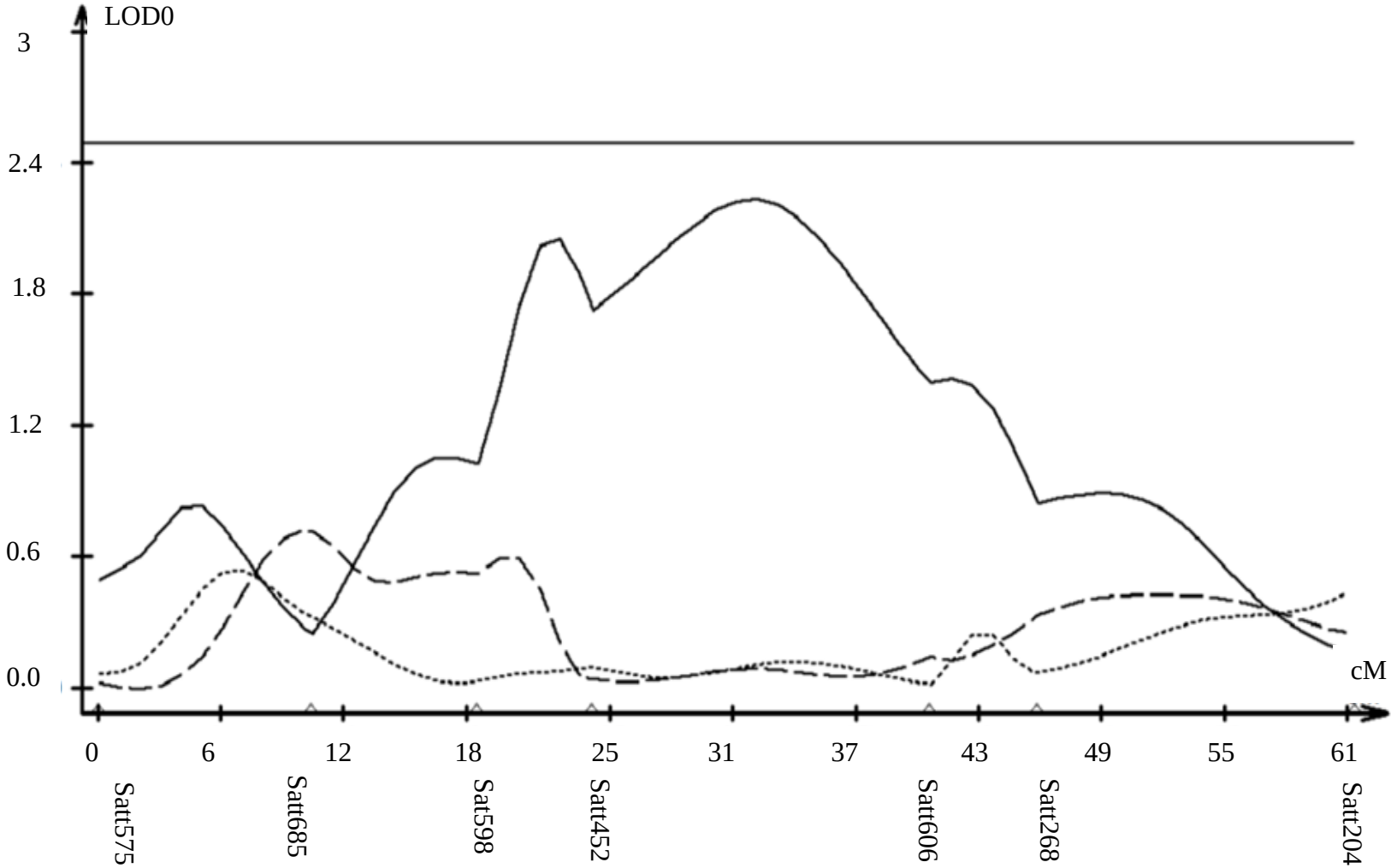

Figure 2.16. Composite Interval Mapping (CIM) in LG-E with pest severity (PS) traits for Japanese beetle defoliation on a subset of 94 soybean lines derived from E06906 x LD05-16060

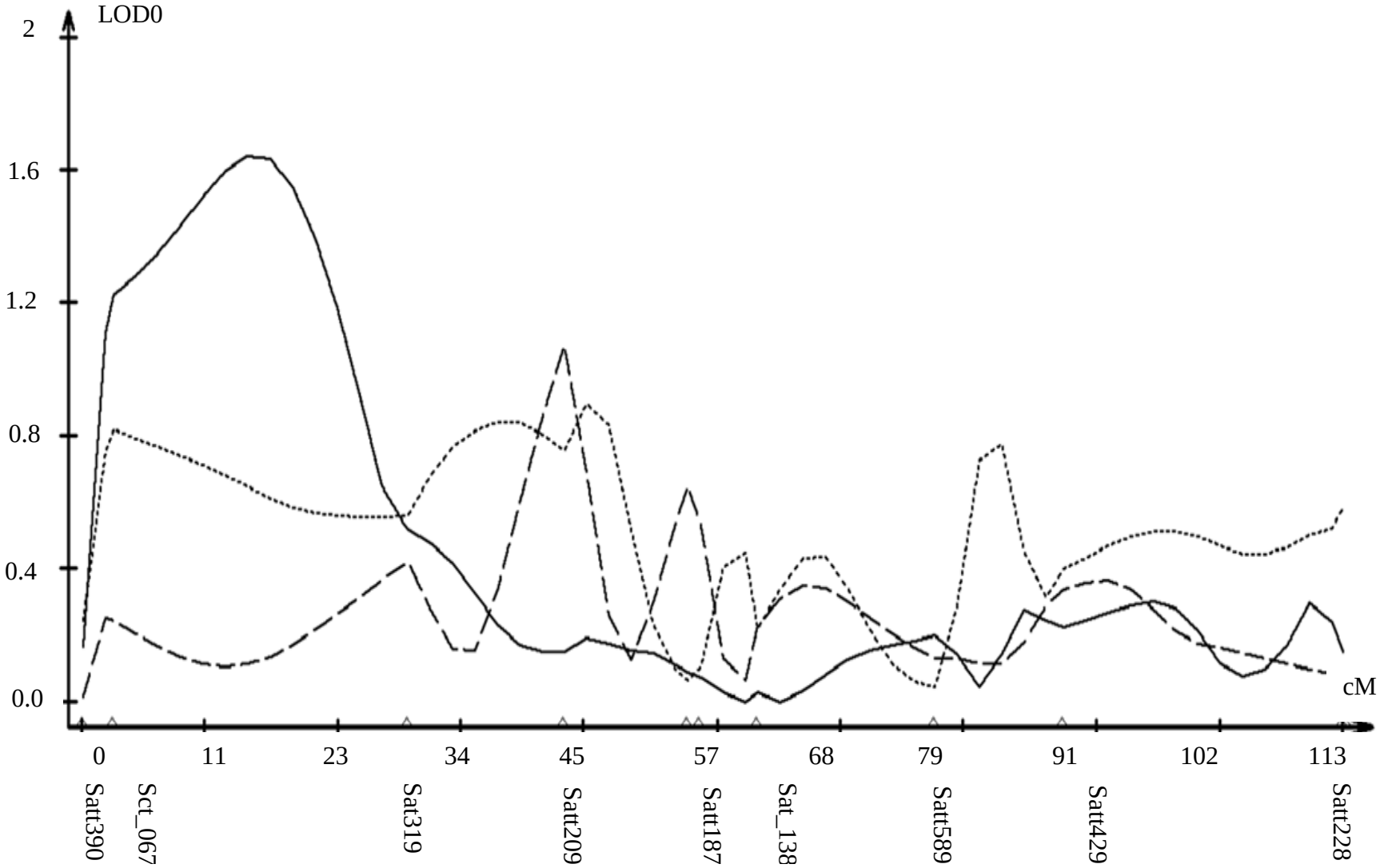

Figure 2.17. Composite Interval Mapping (CIM) in LG-A2 with pest severity (PS) traits for Japanese beetle defoliation on a subset of 94 soybean lines derived from E06906 x LD05-16060

There was sufficient evidence to confirm presence of known soybean defoliation-resistant QTL (M, G, H and D1b) in the population after genotyping with a subset of 94 individuals. However to further confirm the existence of the three new QTL detected with this population, QTL mapping with 234 individuals was carried out. Additionally, no-choice feeding data from 113 individuals was also used to further confirm these QTL (Table 2.10). 1000 permutations at 5% level detected a relatively low threshold of 1.4 for the entire population. This was used as the cut-off to further verify the new QTL with 234 individuals.

QTL on LG-A1 was detected after genotyping all individuals, for SMA and consistently with CIM for PS traits in the same genomic region. The highest variation explained by this QTL was 13.5% (Figure 2.18). Interestingly, the no-choice feeding tests also detected this QTL (Figure 2.21) indicating that this new QTL may possess antixenosis resistance in addition to antibiosis since it was detected with both choice and no-choice tests. The *Sct_067* marker from LG-A2 was also detected consistently with SMA. This marker was also identified with no-choice feeding assays. However the QTL position seemed to shift upstream from *Sct_067* after genotyping with 234 individuals with markers covering the first 50 cM region of this chromosome. No-choice feeding assays detected a peak (LOD = 1.3) between *Sat_117* and *Satt285* on LG-A2 (Figure 2.22). This inconsistency of the position may be caused by the presence of two QTL instead of one. Yesudas et al. (2010) detected a QTL for Japanese beetle defoliation close to *Satt632* (peak position 44 cM). It may be possible than this QTL was also detected in our population in addition to a new QTL corresponding to *Sct_067-Sat_319* interval.

Table 2.10. Single marker analysis and Composite Interval Mapping (CIM) for traits associated with Japanese beetle defoliation on 234 soybean lines derived from E06906 x LD05-16060.

		Single Marker Analysis		Composite Interval Mapping			
LG	**Trait**	**Marker**	**F (P<0.05)**	**Marker interval**	**LOD**	**Peak position**	**R^2**
A1	PS_2010	ns	ns	*Satt211-Sat_200*	3.3	83.4	13.5%
	PS_2011	*Sat_211*	4.644 (0.032)	*Satt211-Sat_217*	1.8	91.4	3%
	No-choice	*Sat_211*	4.766(0.031)	*Satt211-Sat_217*	2	89.1	13%
A2	PS_2010	*Satt187*	6.1 (0.014)	*Satt589_Satt177*	1.3	69.8	3%
	PS_2011	ns	ns	*Sat_319_Satt187*	1.3	44.4	1%
	PS_2011			*Satt177-Sat_215*	1.5	95.4	3%
	PS_2012	*Sct_067*	7.22(0.008)	*Satt187_Satt177*	2.55	69.9	6%
	No-choice	*Sct_067*	3.685(0.05)	*Satt177-Sat_215*	1.3	102.8	8%
	No-choice	*Sat_215*	6.181(0.014	*Sct_067_Sat_319*	1.0	34.6	2%
C2	PS_2010	*Satt371*	5.89 (0.016)	*Satt371-Satt202*	2.7	35.8	5%
	PS_2010	*Satt202*	8.923 (0.03)	ns			
	PS_2011	ns	ns	ns			
	PS_2012	ns	ns	*Satt286-satt489*	1.2	22.4	2%

LG = Linkage group
ns - QTL not significant in the population of 234 individuals
R^2 = Percentage of phenotypic variation explained by a QTL

Furthermore, a new QTL on LG- C2 was detected with slight shift of marker interval (Figure 2.20). Previously this QTL was detected between *Satt286- Satt489* (103-123 cM, in Gm consensus 4.0) with PS_2010 and PS_2012. However this time the QTL was mapped between *Satt371* and *Satt202* (127-114 cM, Gm consensus 4.0) when saturated with 5 makers from the same genomic region. Nevertheless this new QTL was repeatedly detected with SMA, and CIM with 94 and 234 individuals. Despite the differences in marker order on our linkage map, based on the mapping results, this QTL is linked to *Satt*286-*Satt*489 and *Satt*202-*Satt*371 overlapping

marker intervals within 20 cM distance on the Gm consensus 4.0 map. Final graphs for New QTL identified for Japanese beetle resistance with 234 individuals and forced feeding no-choice tests are shown below.

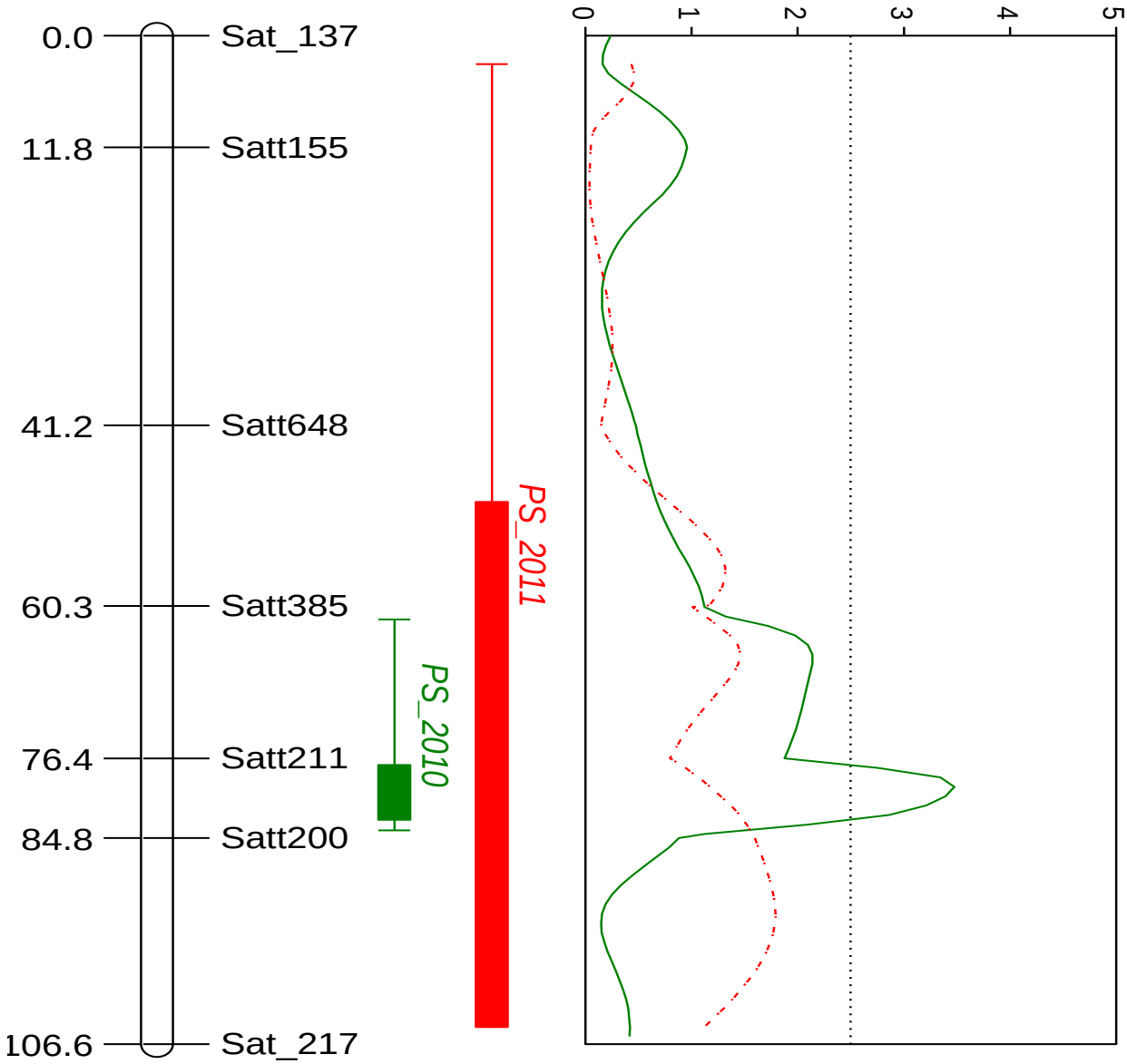

Figure 2.18. Composite Interval Mapping (CIM) in LG-A1 with pest severity (PS) traits for Japanese beetle defoliation on 234 soybean lines derived from E06906 x LD05-16060

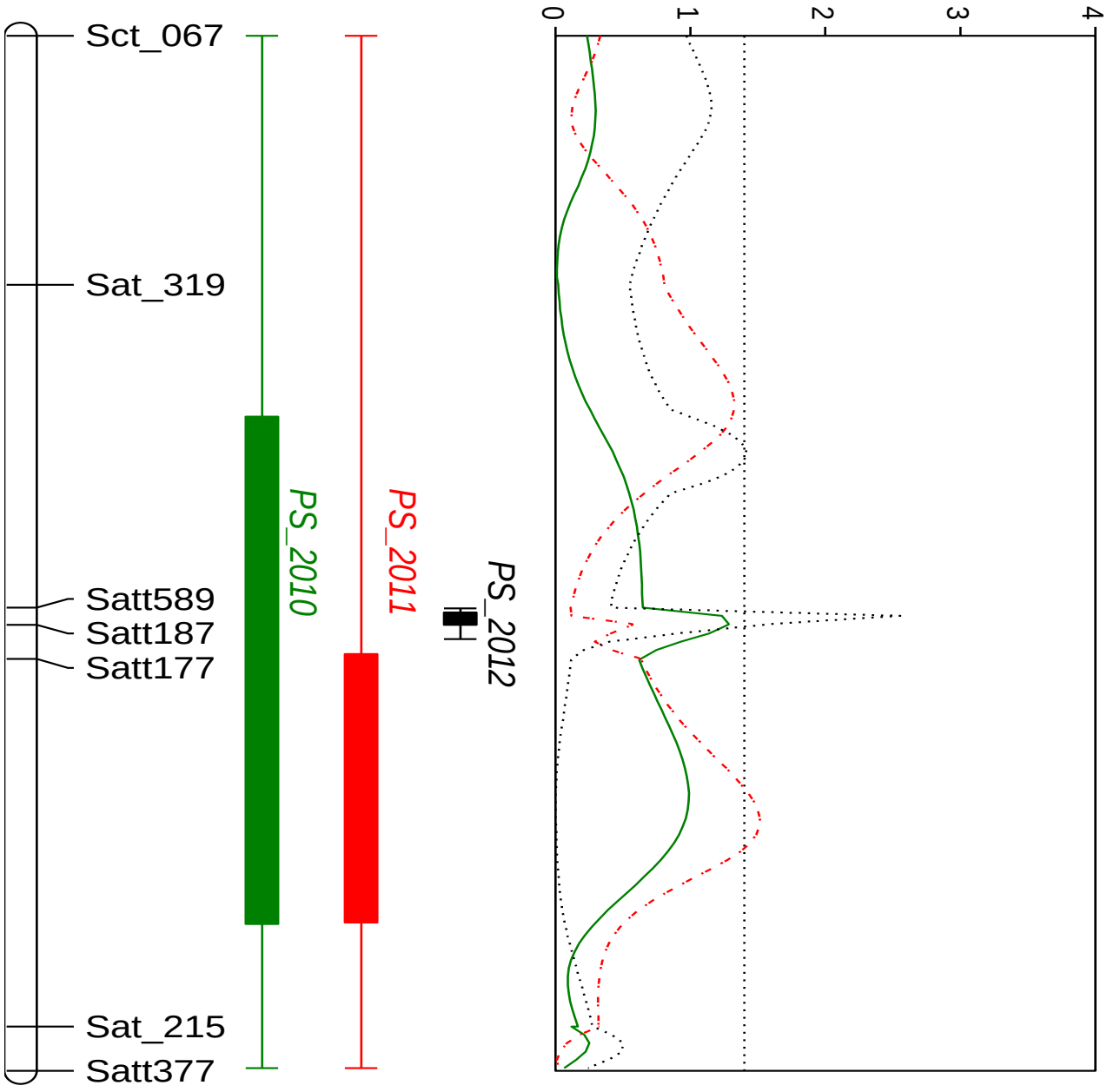

Figure 2.19. Composite Interval Mapping (CIM) in LG-A2 with pest severity (PS) traits for Japanese beetle defoliation on 234 soybean lines derived from E06906 x LD05-16060

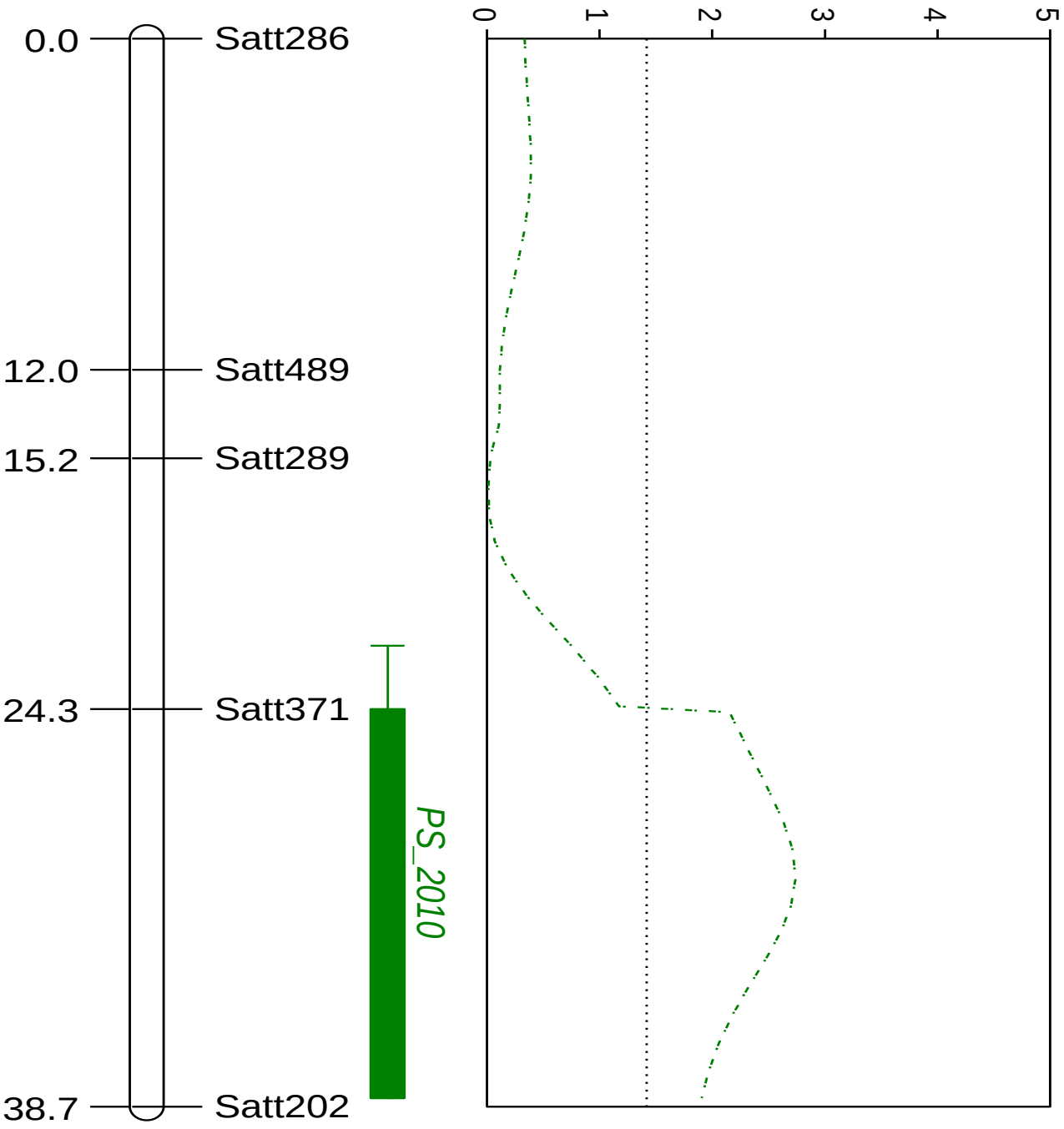

Figure 2.20. Composite Interval Mapping (CIM) in LG-C2 with pest severity (PS) traits for Japanese beetle defoliation on 234 soybean lines derived from E06906 x LD05-16060

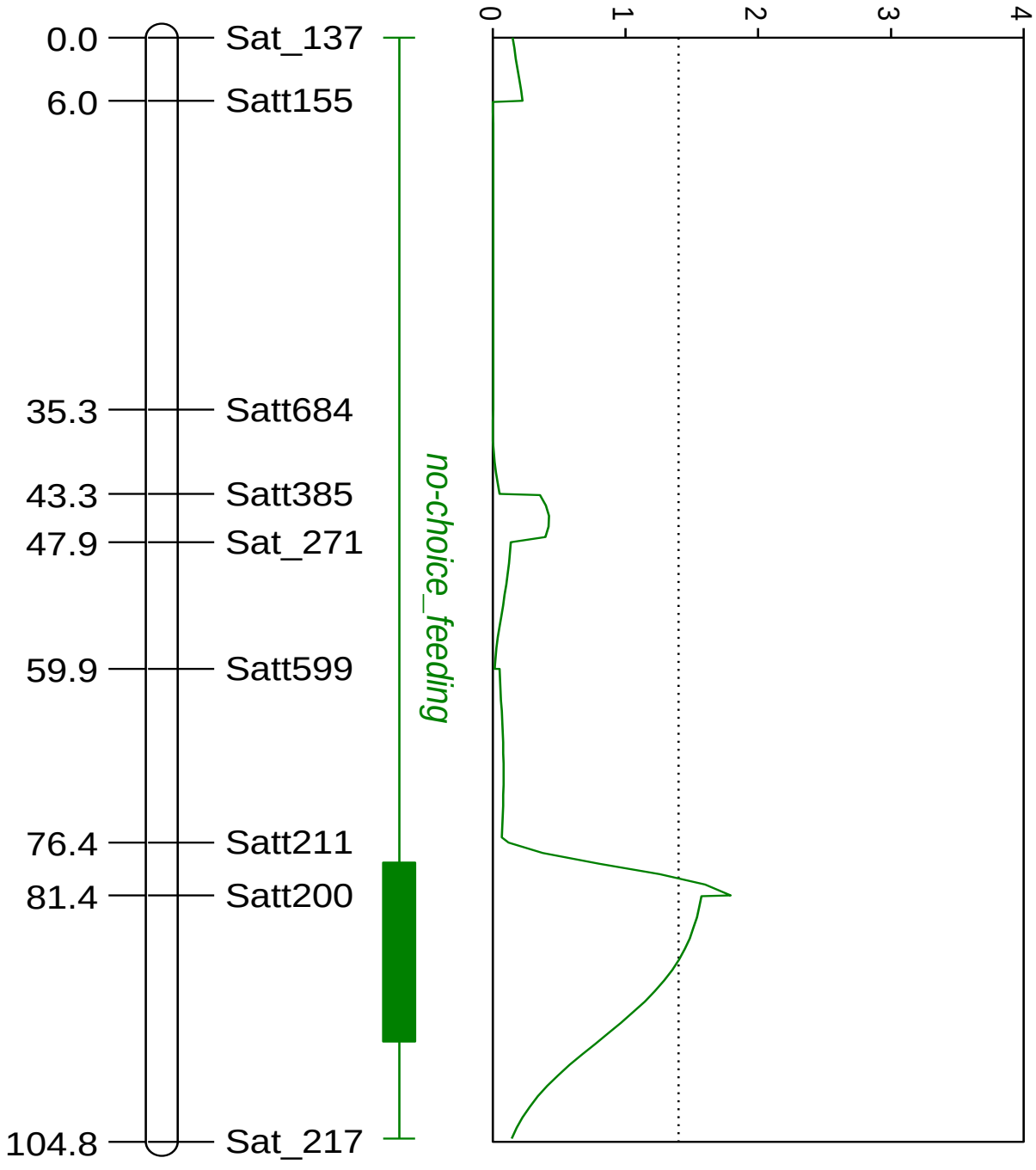

Figure 2.21: Composite Interval Mapping (CIM) in LG-A1 with no-choice feeding for Japanese beetle on 113soybean lines derived from E06906 x LD05-16060

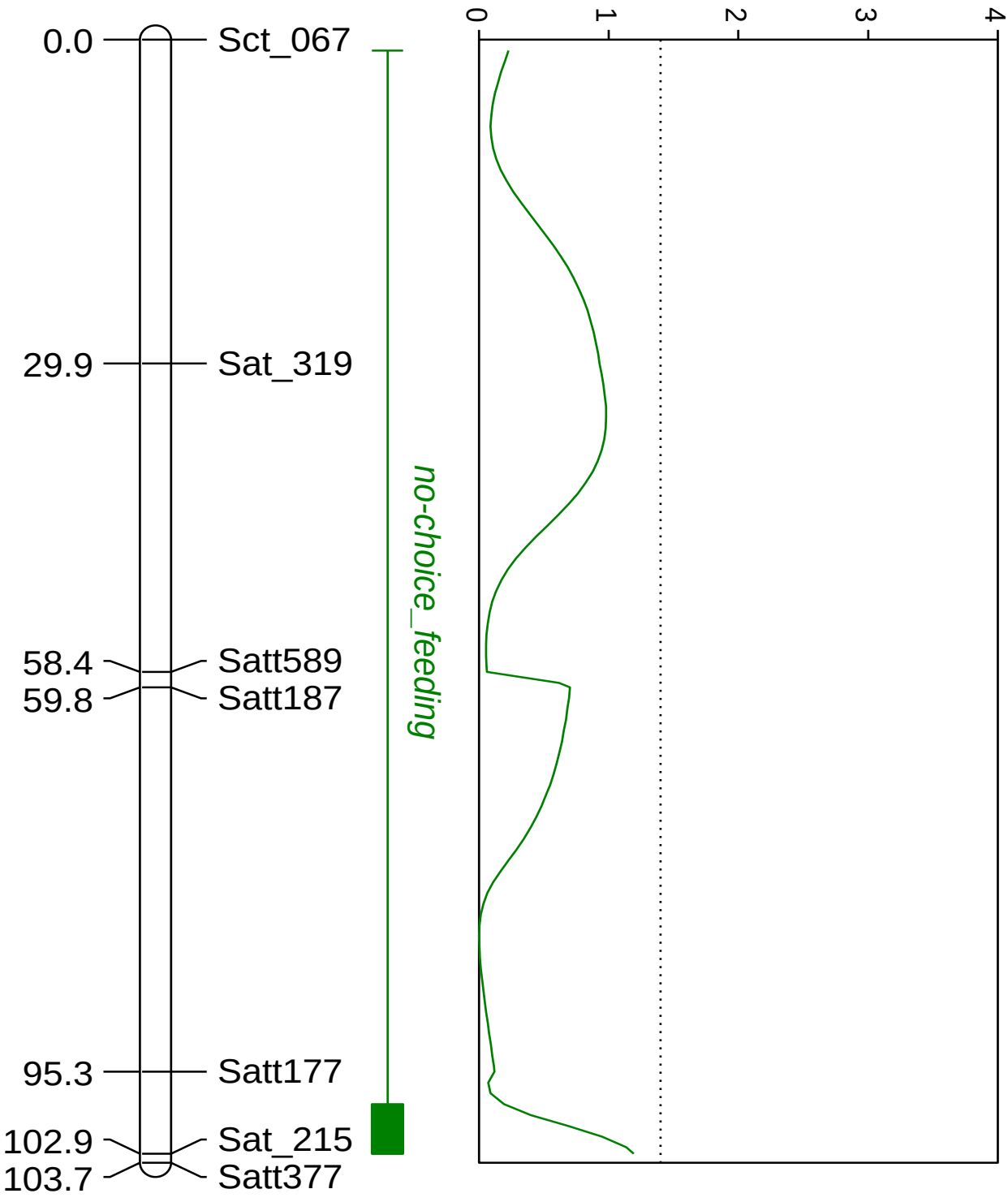

Figure 2.22: Composite Interval Mapping (CIM) in LG-A2 with no-choice feeding for Japanese beetle on 113soybean lines derived from E06906 x LD05-16060

Multiple Interval Mapping (MIM)

Multiple Interval Mapping (MIM) detected several QTL previously found through all of the above mapping methods (Table 2.11). The new QTL identified on LG-A1 was detected for all three traits, showing several significant interactions with other QTL. A1 QTL showed large dominant effect (0.73) with PS_2010 however it's interaction with QTL-A2 (another new QTL detected in this population) explained only 4.1% of phenotypic variation. In addition, the QTL-G and QTL-H appear to play a key role in explaining the phenotypic variation in this population. CIM identified that QTL-G alone explained 47% of phenotypic variation in with PS_2010 and 7% with PS-2011. The interaction between QTL-G and QTL-A2 identified 33.8% of phenotypic variation in 2010, thus QTL-G and QTL-H appear to have significant interactions with A1 and A2 QTL. Singer Marker analysis and CIM on LG-A2 with PS traits, forced feeding traits on 94 individuals, and whole population detected two peaks that led to strongly believe the presence of two QTL on A2. Interestingly MIM mapping also identified both these peaks. There is solid evidence to believe the presence of already published QTL (Yesudas et al. 2010) close to *Satt632*, and a new QTL detected conferring resistance to Japanese beetle downstream between *Sct*_067-*Sat*_319 region. Thus based on the trait used, either one or both of these QTL were detected in population derived from E06906 x LD05-16060. A potential peak identified before with CIM with 94 individuals on LG-E was again detected with MIM. A QTL on LG-B2 was identified with MIM and SMA. However no significant interaction between QTL-M and other QTL were detected with MIM.

Table 2.11. Multiple Interval Mapping (MIM) with pest severity (PS) traits for Japanese beetle defoliation on 234 soybean lines derived from E06906 x LD05-16060.

Trait	LG	Peak Position	Additive effect	Dominant effect	QTL interactions			
					QTLxQTL	Type	LOD	Effect %
PS_2010	A1	64.5	-0.0071	0.7332	A1xH	DxD	8.7885	4.10%
	A2	16.8	0.2604	-0.2196	A2xH	AxA	7.9856	0.40%
	G	84.4	0.4284	-1.1737	A2xG	DxA	5.1776	33.80%
	H	50.3	0.4863	-0.9449				
PS_2011	A1	55.5	-0.1597	0.0628	A1xA2	DxA	2.0615	10.40%
	A2	113.2	-0.1485	-0.2169	A1xA2	AxA	1.2998	10.40%
	E	24.2	0.0473	0.1179	A2xE	AxA	1.7903	10.20%
	G	64.2	-0.0246	0.3692	GxH	DxD	1.4626	9.20%
	H	87.4	-0.1292	0.5051				
PS_2012	A1	49.8	0.03	-2.3472	A1xD1b	DxA	10.134	1.00%
	B2	36.6	-0.9272	0.4672	B2xD1b	DxA	12.825	15.50%
	D1b	47.7	0.05	0.8911				

This study confirms the presence of significant interaction between several major and minor QTL. QTL already reported for conferring resistance to several other insect defoliators: QTL-M, QTL-G and QTL-H were consistently detected in this population with large effects (31% for M, 47% for G,). Also these QTL showed significant interactions among QTL-G and QTL-H. Effects and interactions of these three QTL have been studied extensive by many groups with regards to defoliation by several insects. However, to date, these QTL have not been studied or detected for conferring resistance to Japanese beetle. The QTL on linkage group M (QTL-M) is one of the most important major QTL conferring both antixenosis (37%) effect and antibiosis (22%) effect to soybean defoliators (Rector et al. 1998, 1999 and 2000; Komatsu et al. 2005,

Zhu et al. 2006). It was first mapped to approximately 30-cM interval, having a peak position to RFLP marker A584_4 on LG M conferring resistance to CEW. Its effects have been improved with the addition of cry1Ac transgene (Walker et al. 2004, Narvel et al. 2001). Later this QTL was mapped between markers *Satt*220 and *Satt*175 (Zhu et al. 2006). Fine mapping experiments confined the QTL to *Satt323-Satt702*. In this population, QTL-M was consistently detected in the same marker intervals.

It was reported that QTL-G provided only antibiosis, while QTL-H had antixenosis effects (Parrot et al. 2008). However the most effect was reported from QTL-M and also when QTL-G and QTL-H were combined with QTL-M, thus breeding efforts has been focused on introgressing QTL-M into elite soybean varieties. Another limitation to introgression of this QTL is the possible linkage drag. Zhu et al. (2007) successfully fine mapped the QTL –M to 0.52 cM map interval with Williams 82 genomic sequence further assisting in introgression of this QTL without unnecessary linkage drag. The study reported in this Chapter, confirms that these major QTL possess the ability to confer resistance to Japanese beetle defoliation in soybean.

More importantly, at least three new QTL were consistently detected with this study. QTL on LG-A1 was mapped to the same map interval by all methods studied. Since it was detected with both choice and no-choice assays it may possess both antixenosis and antibiosis resistance for Japanese beetle. A defoliation-resistant QTL for LG-A1 was published close to *Satt*382 (29.5 cM, Gm consensus 4.0) (Hurburt, 2001). However, the new QTL on LG- A1 was consistently detected in a new position further away from the reported position. Furthermore, results suggest

two new QTL detected on LG-A2 and LG-C2 with 234 individuals. However these new QTL may have only minor effects compared to QTL-M and QTL-G in this population.

The aphid-resistant gene *Rag*1 maps more than 10 cM apart from the current map location of QTL-M. However, no association between major QTL-M and *Rag1* has been found to date (Wayne Parott pers. comm). Due to presence of several major QTL reported from original PI source (PI 229358), there is strong evidence to believe that the advanced breeding line LD05-16060, inherited defoliation-resistance from this accession.

In 2011, a candidate gene analysis on a A1 QTL on soybean genomic browser (www. soybase.org) led to identification of a gene product, that may play a key role in conferring resistance to Japanese beetle (Chandrasena et al. 2012). This gene is a key enzyme, namely Acetyl –co-A-carboxylase (acc-C2) that plays a major role in catalyzing the formation of malonyl CoA from acetyl CoA, an early precursor in a pathway leading to both fatty acid and flavonoid biosynthesis in soybean (Reverdatto et al. 1999). Another isoform of Acetyl –co-A-carboxylase (acc-C3) was found underlying the newly identified QTL linked to *Sct_067* (see appendix for images from SoyBase genome browser).

More recently, candidate genes underlying major QTL-M has been studied (Ortega and Parrott 2012). High resolution mapping and cloning on this QTL has led to identification of another key enzyme, Flavonoid Glycosyl transferase, involved in the flavonoid pathway. This provides solid evidence that flavonoids may serve as feeding deterrents in certain soybean lines leading to resistance to defoliatiors. Additionally, this may explain the differential defoliation-resistance

observed between aphid-resistant LD05-16060 and E06906. Finally, the study resulted in advanced soybean lines with stacked aphid–resistant genes (*Rag1* with *rag3* and *rag1b*) combined with resistance to Japanese beetle, thereby conferring high levels of resistance to both insects.

Acknowledgements

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CHAPTER 3.0

Stacking *rag3, rag1b, rag4,* and *rag1c* aphid-resistant genes in soybean germplasm

Introduction

The soybean aphid, *Aphis glycines* Matsumura (Hemiptera: Aphididae), is an invasive pest introduced from Asia, that poses a significant threat to soybean [*Glycine max* (L.) Merr.] production in the United States (Yu et al. 1989, Wang et al. 1996, Wu et al. 1999, Sun et al. 2000, Hill et al. 2004, Ragsdale et al. 2004, Ragsdale et al. 2011). Since its discovery in the United States in 2000, soybean aphid was widely distributed in many regions of the Mid-western United States (Ragsdale et al. 2004). It is now reported in 24 US states including Michigan and in three Canadian provinces (Ragsdale et al. 2004, 2011; Rutledge and O'Neil 2006).

Although soybean aphid is reported as a significant pest in Asia (Wang et al. 1994), Its' damage to soybean in the United States has been significantly greater over a short period of time, than in its native habitat (Liu et al. 2004, Ragsdale et al. 2004, Wu et al. 2004). Direct feeding on plant sap is the most prominent damage that causes reduction of seed protein content (Wang et al. 1994). When heavily infested, plants show wrinkled and distorted foliage, early defoliation, stem and leaf stunting, reduction in number of pods and seed weight, eventually leading to premature plant death (Wang et al. 1962, Wang et al.1996, Lin et al. 1992, 1994; Wu et al. 1999, Wu et al, 2004, DiFonzo and Hines 2002, Diaz-Montano et al. 2006). Excess sugar secretions (honey dew) causes the growth of sooty mold on leaves impairing plant photosynthesis, thereby reducing yield, and seed quality (Chen and Yu 1988). Another threat posed by soybean aphid is the ability

to transmit plant viruses to soybean and several other crops (Iwaki et al. 1980, Hartman et al. 2001, Hill et al. 2001).

Several factors affect soybean aphid populations, including environmental factors (i.e., temperature, precipitation, and humidity), number of overwintering aphid eggs, cultural practices (i.e., planting time and soybean variety), and natural enemies (Wu et al. 1999, Wu et al. 2004). Soybean aphid can be controlled by a number of distinct tactics including biological control, chemical control, and host plant resistance. These control options can be used individually or together. Lady beetles (*Coccinellid spp*), green lace wings (*chrysopidae)* and minute pirate bugs (*Orius spp)* are among the few predators that feed on soybean aphid in the United States (Fox et al. 2004, Rutledge et al. 2004). However, biological control is not always sufficient to keep soybean aphid populations below damaging levels.

At present, insecticide treatments are widely used to effectively control soybean aphid. It is believed that yields can be protected, when timely and informed decisions are made by growers based on Economic Threshold and Economic Injury Levels (Johnson et al. 2009). However, heavy use of broad-spectrum insecticides can increase the possibility of developing resistance to commonly used insecticides for soybean aphid (Gao et al. 1993, Wu et al. 2004, Chandrasena et al. 2011).

Moreover, growing cultivars with aphid-resistance can be an environmentally safe alternative to use of insecticides. Host plant resistance to insects could be classified as non-preference, antibiosis, or tolerance (Painter 1951). The term '*antixenosis*' was later used to replace non -

preference (Kogan and Ortman 1978). *Antibiosis* is a type of resistance that refers to a host plant that has detrimental effects on the physiology and life history of an insect pest (lethal or sub lethal). Tolerance is a way of host plant adapting to withstand damage by the insect thus, pose no risk to insect while the plant merely increases the threshold.

Most new aphid-resistant sources are identified by preliminary screening for aphid abundance in contained environments such as greenhouses or field cages, with artificial infestation of soybean aphids (Hill et al. 2004, Mensah et al. 2005, Diaz-Montano et al. 2006, Mian et al. 2008a, Zhang et al. 2010). Hill et al. (2004) identified the first soybean aphid-resistant germplasm in the United States, namely 'Dowling' and 'Jackson'. In Michigan, Mensah et al. (2005) discovered four Maturity Group (MG) III accessions after screening 2147 soybean accessions originally from northern China. These four MG III accessions (PI 567543C, PI 567597C, PI 567541B, and PI 567597C) showed resistance to soybean aphid; PI 567543C and PI 567597C possessed antixenosis resistance while PI 567541B and PI 567597C possessed antibiosis (Mensah et al. 2005).

Single dominant genes control antibiosis resistance in Jackson *(Rag)* and Dowling (*Rag1*) (Hill et al. 2006a; 2006b). Both *Rag* and *Rag1* mapped to the same region on chromosome 07 (LG-M) (Li et al. 2007). Mian et al. (2008a) identified and mapped *Rag2*, in PI 243540 to a different chromosome (LG-F). In contrast to *Rag*, *Rag1* and *Rag2* resistance, controlled by single genes, PI 567541B and PI 567598B had two recessive genes that acted epistatically (Mensah et al. 2008). These genes were named *rag1c* (on linkage group M) and *rag4* (on linkage group F) for PI 567541B (Zhang et al. 2009). Names were designated as *rag1b*_provisional (on linkage group

M) and *rag3*_provisional (linkage group J) for resistant genes discovered from PI 567598B (Soybean Genetics Committee 2009). Recently, the QTL positions for PI 567541B were validated with custom designed TaqMan® and KASPar® SNP markers based on the genomic positions of a 52, 0000 Beadchip with Infinium assay (Illumina Inc. San Diego, CA) (Yuan et al. 2012).

A possible negative impact to host plant resistance can be posed by the rise of new biotypes (Auclair 1989, Smith 1989). If only a single gene is responsible for antibiosis resistance (such as *Rag1*) there is high probability for soybean aphids to overcome this resistance in a relatively short time. In a study where two soybean aphid biotypes from Illinois (Biotype 1) and Ohio (Biotype 2) were evalauated, *Rag1* resistance was not effective against the Ohio biotype, thus these soybean aphids were able to colonize breeding lines with *Rag1* (Kim et al. 2008). More recently another biotype namely 'Biotype 3' has been identified (Hill et al. 2010) which survived on both *Rag1* and *Rag2*.Therefore pyramiding multiple resistance genes, particularly with different modes of action, in the same cultivar has great potential of providing a higher and more durable resistance against soybean aphid (Mian et al. 2008a, Hesler et al. 2012).

This study reports MSU soybean breeding program's research on stacking *rag3,* rag*4, rag1b,* and *rag1c* aphid- resistant genes. A breeding population of 727 F_2 individuals was developed by combining multiple aphid resistant sources derived from two PIs. A cross between advanced breeding lines E08907 (harboring *rag3* and *rag1b* from PI 567589B) and E09907 (harboring *rag4* and *rag1c* from PI 567541B) produced this breeding population which was evaluated for

aphid resistance in four trials conducted in field and greenhouse, and was genotyped using molecular markers for corresponding resistant genes.

Specific objectives of this project were to assess and compare the impact of pyramiding multiple aphid resistant genes: *rag3*, *rag4*, *rag1b* and *rag1c* on conferring resistance to soybean aphid in both field and greenhouse trials, and to use SNP and SSR markers to genotype and select the best stacks for commercial release.

Materials and Methods

Plant material and phenotypic evaluations for soybean aphid resistance

An initial population of 727 F_2 plants were developed from a cross between advanced breeding line E08907 (*rag3* and *rag1b* from PI 567589B crossed with susceptible 'Titan RR') and E09907 (*rag4* and *rag1c* from PI 567541B crossed with susceptible 'Skylla') in 2009. As mentioned above, both parent lines were derived from two original PIs discovered by Mensah et al. (2005). Later, these advanced breeding lines were developed at Michigan State University (MSU).

The first evaluation on 727 F_2 plants was conducted in the Plant Sciences Greenhouse at MSU, East Lansing, Michigan in fall 2010. These 727 F_2 plants were planted as eight seeds per pot in 125 mm deep plastic pots with 105 mm in diameter. 26/15ºC day/night temperature was constantly maintained in the greenhouses with 14:10 L:D conditions. Supplemental light intensity was provided by sodium vapor lights during the day. In spring of 2011, single seed per each F_2 plant was planted in the greenhouse as described above. Each $F_{2:3}$ plant was individually

rated for aphid damage using a standard scale developed by Mensah et al. (2005) which is described later in this section.

The third evaluation for soybean aphid resistance, in a field choice test was conducted in summer of 2011 at the Agronomy Farm of MSU, East Lansing, MI. The population ($F_{3:4}$) along with its parents were planted in a randomized complete design, in an aphid and predator-proof polypropylene cage with 0.49-mm mesh size (Redwood Empire Awning Co., Santa Rosa, CA, USA). Based on previous experiments, aphid resistance in soybean is associated with very high heritability (Zhang et al. 2010), thus only a single replication was planted. Each line consisted of 10 -15 plants in a single 30 cm long plot with 60 cm row spacing. Each plant per line was individually rated for aphid damage using a standard scale developed by Mensah et al. (2005).

For both greenhouse and field trials the following method was used to infest and rate plants for soybean aphid resistance. Two wingless aphids were placed on the top-most unopened trifoliate at the V1 stage (Fehr and Caviness 1977). The sources of aphids were field-collected aphids from the same year and/or field-collected aphids from the previous summer for greenhouse evaluations in Spring. Visual ratings on aphid infestation were taken 3 weeks after infestation using a scale of 0–4 developed by Mensah et al. (2005, 2008), where 0 = no aphids; 0.5 = fewer than 10 aphids per plant, no colony formed; 1 = 11–100 aphids per plant, plants appear healthy; 1.5 = 101–150 aphids per plant, plants appear healthy; 2 = 151–300 aphids per plant, mostly on the young leaves or tender stems, plants appear healthy; 2.5 = 301–500 aphids per plant, plants appear healthy; 3 = 501–800 aphids per plant, young leaves and tender stems are covered with aphids, leaves appear slightly curly and shiny; 3.5 = more than 800 aphids per plant, plants

appear stunted, leaves appear curled and slightly yellow, no sooty mold and few cast skins; 4 = more than 800 aphids per plant, plants appear stunted, leaves appear severely curled and yellow and are covered with sooty mold and cast skins. A damage index (DI) for each line was calculated by the following formula: DI = ∑ (scale value x no. of plants in the category)/(4 x total no. of plants) x 100. The DI ranges between 0 for no infestation and 100 for the most severe damage (Mensah et al. 2005). This DI has been successfully used in previous experiments (Mensah et al. 2008, Zhang et al. 2010) as a good indicator of aphid resistance thus was used for current analysis.

After marker assisted selection, a final confirmation of aphid resistance on 120 best lines selected from the above 727 lines with 8 combinations of gene (s) (Table 3.1) were planted in the greenhouses in spring of 2012, following standard methods for planting, and was rated three and four weeks after infestation as described above.

Marker assisted selection for resistant genes

DNA extraction and marker analyses

The young fully unopened trifoliates were bulk harvested for each line ($F_{2:3}$) and from their parents, after rating for aphids in 2010. CTAB (hexadecyltrimethyl ammonium bromide) method was used to extract genomic DNA from tissue samples as described by Kisha et al. (1997). DNA concentration was measured using a ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, Delaware, USA).

Tightly linked SSR markers for the four mentioned aphid-resistant genes have been published to date. These markers were utilized for germplasm screening. Zhang et al (2009) reported that SSR marker *satt366* = BARCSOYSSR_16_036 mapped closely to *rag3* on chromosome 16 (formerly linkage group J). *Satt245* mapped closely to *rag1b* on Chromosome 7 (Formerly Linkage group M). *rag1c* was positioned in the intervals of *Satt299* and *Sat_244* on chrom 7. Genomic DNA with simple sequence repeat (SSR) markers was run on a MJ Tetrad™ thermal cycler (MJ Research, Waltham, MA, USA) for PCR. The SSR primer sequences were obtained from the SoyBase database (http:www.soybase.org). The SSR oligonucleotide primers were synthesized by Sigma Aldrich (St. Louis, MO). After PCR, the amplified products were separated on 6% non-denaturing polyacrylamide gels using electrophoresis unit DASG-400-50 (C.B.S. Scientific Co., Del Mar, CA, USA) as described by Wang et al. (2003). After staining with ethidium bromide, the bands were visualized under UV light, and scored for polymorphism.

A custom designed Taqman® SNP marker MSUSNP13-4 was used to screen for *rag4* allele in this population. Previous QTL mapping attempts confined *rag4* between the SSR markers *Satt348* and *Satt649* on soybean chromosome 13, formerly linkage group F (Zhang et al. 2009). Later a fine mapping experiment resulted in identifying a tightly linked SNP for *rag4* (Yuan et al. 2012).Thus custom made SNP marker MSUSNP 13-4 was used for this screening. The target SNP was validated by resequencing the flanking regions of the SNP based on reference genome, Williams 82 sequence (Yuan et al. 2012).

Custom Taqman® Assay Design Tools of Applied Biosystems (ABI, Foster City, CA, USA) was used to obtain the allele specific primers and probes. Taqman® probe based PCR reactions were

performed on a 384-well plate with a total volume of 3 uL/well on the LightCycler-480 instrument (Roche Applied Science, Indianapolis, IN, USA). The PCR reaction mixture for the assay consisted of 1-20 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, Foster City, CA, USA). After 10 min pre-incubation at 95 ºC, 45 PCR cycles were conducted with 10 s of denaturation at 95 ºC, 30 s of annealing at 60 ºC, and 10 s extension at 72 ºC. A final melting cycle for nonspecific amplicon screening was carried out by raising the temperature to 95 ºC for 10 s, lowering the temperature to 40 ºC for 30 s, and increasing the temperature to 83 ºC with continuous fluorescent acquisition followed by a cool down to 40 ºC on the LightCycler-480. Data were analyzed by the Roche Applied Science software version 1.5.0.

Statistical analyses

The DI for greenhouse and field trials were separately analyzed as the experimental designs and the source of infested aphids differed based on the year. Analysis of variance (ANOVA) was performed using the PROC GLM procedure in SAS (SAS Institute 2010). Mean comparisons were conducted using Tukey's Highest Significant Difference (HSD) at 5% significance level. Pearson correlation between DI, for each combination of different aphid resistance genes for 2011-field and 2012-greenhouse trials were conducted with the CORR procedure in SAS Institute (2010). When only a single plant was rated per line (first two greenhouse trials), Pearson correlation between aphid indices for each combination of different aphid resistance gene(s) were conducted with the CORR procedure of SAS (SAS Institute 2010).

Results and Discussion

The first evaluation of soybean aphid resistance on all 727 F2 plants derived from E08907 x E09907 was conducted in the greenhouses in 2010. The broad sense heritability of aphid resistance derived from PI 567541B (*Rag4* and *rag1c*) has been reported as 0.89-0.90 from greenhouse studies conducted by Zhang et al. (2009). An aphid rating of 1.5 or less has been classified as 'resistant' (Mensah et al. 2008). In the greenhouse, PI 567598B had an aphid rating of 1.0 at week-3 rating. The resistant PI 567541B had an aphid rating of 1.5 at week-3 rating. As described by Mensah et al. (2005) aphid indices for both sources could be categorized as resistant (1.5 or less). In the greenhouse the susceptible parent of E08907, Titan RR showed heavy infestation with an aphid index of 3.5. Similarly Skylla, the susceptible parent of E09907 had an aphid index of 3.5.

Based on marker screening, individuals belonging to eight different resistant gene combinations were categorized. Mean aphid index for each gene or combination is listed in Table 3.1. Marker assisted scoring with SSR and allele specific SNP marker helped to eliminate the heterozygous individuals for these genes, thus the selected individuals exhibited recessive homozygous state for each gene (*rag3, rag4, rag1b* and *rag1c*).

Due to the limitation of space for this large population in the greenhouses, only a single plant per line was planted for $F_{2:3}$ generation in 2011-greenhouse study. Similar to the previous study, the aphid index for each individual was recorded and later homozygous individuals among $F_{2:3}$ population having each different gene combination was selected as described above. The mean aphid indices are listed in Table 3.1. Based on the first evaluation, the strongest resistance was

shown both from the three gene stack *rag3-rag4-rag1c* and *rag1c* lines (0.50±0.0). The lowest resistance was shown from *rag4-rag1c* lines (1.5±1.68). However all combinations had an aphid index not more than 1.5 suggesting that presence of any one of these genes can be sufficient to confer significant level of resistance.

Similarly, in the second greenhouse evaluation in 2011, both *rag3-rag1c* and *rag1c* only lines showed the lowest aphid index (0.5±0.0). The highest aphid index was again obtained from *rag4-rag1c* lines (1.37±1.75). This was consistent with our observations from the first evaluation in 2010 (Table 3.1). Moreover, there was a strong positive correlation for aphid indices among individuals expressing same gene(s) between the first two greenhouse studies (Table 3.2) except for *rag3-rag4-rag1b* and *rag4-rag1b* lines. Correlations for other combinations were highly significant at P=0.05.

Damage Index (DI) has been used as a better estimate for comparing aphid susceptibility, since the formula is developed to give a single value based on overall aphid ratings given to all plants belonging to a particular line. This estimate was applied for field study in 2011 and the final greenhouse study in 2012, as each line consisted of several plants. Additionally, because of the percent estimate of damage, this estimate can be easily compared across any rating scale used for evaluating soybean aphid resistance. Mensah (2005) first developed and used DI to successfully screen and identify soybean aphid resistant germplasm from a pool of >2147 accessions. A DI ≤30%) was considered 'resistant' while DI>30% was considered 'susceptible'. Later, Mensah et al (2008), Zhang et al (2009, 2010), and Liu (2010) used this DI formula to successfully phenotype mapping populations for QTL discovery.

Mean value for damage index for each gene combination or gene for 2011 field study and 2012 greenhouse study is listed in Table 3.1. In the field, E09907 and its susceptible parent Skylla, had DI of 34.16 and 56.25 respectively, clearly indicating heavy aphid infestation on the susceptible line. However, both resistant E08907 and its susceptible parent, Titan RR showed higher DIs of 57.14 and 62.5 respectively. Similarly, PI 567541B (resistant source of E09907) had a higher damage index of 56.25 while the damage index of PI 567598B (resistant source of E08907) was much lower (DI= 16.7)

Significant differences appeared among 8 gene combinations or gene (s) or lines based on 2011 field evaluation (F= 3.49, P= 0.0019). The effect of *rag3* or *rag4* alone (with other genes absent) could not be detected as every genotype derived from the above cross possessed either *rag1b* or *rag1c* genes. Mean comparisons among DI with Tukey's separation are shown in Figure 3.1. Consistent with previous evaluations, statistically Highest DI (lowest resistance) was reported from *rag4-rag1c* lines (DI =59.3±8.8). Lines with gene stacks *rag3-rag4-rag1c* (DI = 31.0±4.0), *rag3-rag4-rag1b* (DI= 31.5±5.1) and *rag3-rag1c* (DI= 22.6± 5.1) showed statistically stronger aphid resistance than *rag4-rag1c* lines.

There was significant correlation between 3-week and 4-week rating for the 2012-greenhouse study conducted with the finally selected lines (Table 3.2). Strong positive correlations were observed for all combinations (P=0.05) except *rag4-rag1c* lines (r = 0.26, P = 0.5674). Failure to show correlation was consistently observed for *rag4-rag1c* lines in the previous greenhouse studies conducted in 2010 and 2011. A possible reason for this could be the weak nature of this combination and inconsistency in resistance, thus making this combination a less resistant

source. This study served as a final confirmation of results obtained from previous trials. The study also served the purpose of determining what stacks can be released commercially. In this study, susceptible 'Titan RR' and 'Skylla' showed heavy infestations with mean damage indices of 63% and 59 % respectively. In contrast to the earlier observations on E09907, both E08907 and E09907 both had low DI of 12.5%.

DI means were compared using Tukey's HSD for the final greenhouse study. These comparisons are shown in Figure 3.2. Consistent with all previous evaluations statistically highest DI (lowest resistance) was reported from *rag4-rag1c* lines (DI =29.4±6.9) again. Lines with rag*3 -rag1c* had the strongest resistance (DI= 9.1±4.4) and was significantly better than *rag4-rag1c* lines (Figure 3.2). However, statistical tests failed to differentiate any other genes or gene combinations from rag*4-rag1c* lines based on this trial. Lower DI values for the greenhouse trial in 2012 could have led to weaker separation among stacks.

Generally a higher DI was observed in the field than in the greenhouse for all resistant sources. It is possible than field conditions favor rapid aphid growth and reproduction than controlled environments. Also the differences in sources of aphids used for these studies might also have an impact. Despite the original DI threshold (30% DI) by Mensah (2004) given to classify soybean aphid resistance, several other greenhouse and field trials often recorded DI values slightly higher than 30%, even for resistant accessions. Zhang et al (2009) reported much higher DI of 60-75% for PI567541B in the field while DI was 25-29% for the same accession in the greenhouse. Zhang et al. (2010) reported DI of 35-44% for PI567543C for both greenhouse and field experiments.

The four trials with these different gene combinations provided some interesting results. Aphid ratings for single plants of F_2 and $F_{2:3}$ lines for 2010 and 2011 greenhouse trials gave aphid ratings, not more than 1.3 for all stacks except *rag4-rag1c* lines. Later, the field trial in 2011 with better estimate of DI, statistically separated both the three gene stacks *rag3-rag4-rag1c*, *rag3-rag4-rag1b a*nd *rag3-rag1c* as more resistant sources than *rag4-rag1c*. Poor performance of *rag4-rag1c* lines were again evident with the final greenhouse study in 2012, where it again clearly separated as the least resistant line. In support of this finding, higher damage indices were also observed for the original parent accession PI 567541B, and E09907 harboring *rag4* and *rag1c* genes. In conclusion, repeatedly in all trials *rag3-rag1c* lines outperformed the other lines showing greater consistency in their resistance. Moreover, there were sufficient evidence to believe than *rag3-rag4-1c* and *rag3-rag4-rag1b* stacks also possess strong resistance since they showed significantly low DI along with the *rag3-rag1c* lines in the 2011 field study.

Additionally, three combinations listed above also had very low aphid ratings in 2010 and 2011 greenhouse studies. Although there is good evidence, these observations are insufficient to confirm that lines with more gene stacks always outperform resistant lines with fewer aphid-resistant genes. Rather than a generalization, it should be understood that the durability and strength of a resistant line relies on many dynamics, such as the nature of the resistant gene (single dominant, partially dominant, or recessive), biotypes used, environmental, and physiological conditions impacting aphid growth and reproduction.

The concept of ‘gene pyramiding’ could be a valuable addition to numerous efforts made by soybean breeders to develop aphid-resistant cultivars with durable resistance. Recently, a study

reported efficacy of two stacked aphid-resistant genes in new soybean germplasm (Wiarda et al. 2012). Development of soybean aphids on lines with only *Rag1,* or *Rag2* alone, and both genes combined, or in the absence of both genes were tested after artificial infestations in cages. Additionally, the impact of gene stacking on yield was also reported (Wiarda et al. 2012). This study confirmed significant aphid suppression by stacked *Rag1/Rag2* than alone; less yield reduction was also reported when resistant sources were stacked. A recent study conducted by Hesler et al. (2012) on *Rag* (Jackson), *Rag1* (Dowling), and *Rag2* (Sugao Zarai, Sennari) lines reported inefficient performance of *Rag1* toward biotype 3, in agreement with several previous reports, it was concluded that soybean lines with a single aphid-resistance gene provided only limited resistance. Therefore, breeding strategies should be directed towards pyramiding resistant genes for more durable resistance leading to efficient management of soybean aphid.

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Table 3.1. Mean aphid ratings or mean Damage Index (DI) vs. resistant gene(s) for soybean lines derived from E08907x E09907.

Resistance gene (s)	**2010 greenhouse Mean aphid index ±SE**	**2011 greenhouse Mean aphid index ±SE**	**2011 field Mean DI ±SE**	**2012 greenhouse Mean D1 ±SE**
rag3, rag4, rag1b	0.72±0.61	0.68±0.46	31.03±4.03	18.21±4.23
rag3, rag4, rag1c	0.50±0	0.68±0.67	31.48±5.08	13.80 ±3.86
rag3, rag1b	0.68±0.51	0.61±0.43	41.80±2.65	11.87 ±2.30
rag3, rag1c	0.61±0.29	0.50±0	22.62±5.08	9.08±4.46
rag4, rag1b	0.72±0.44	1.17±1.09	47.27±5.86	16.17±4.73
rag4, rag1c	1.50±1.68	1.37±1.75	59.37 ±8.80	29.40± 6.69
rag1b only	0.81±0.86	0.71±0.74	39.55 ±3.67	11.08±2.57
rag1c only	0.5± 0	0.5±0	37.90±7.86	11.27±5.46

DI = ∑ (scale value x no. of plants in the category) /(4 x total no. of plants) x 100, ranging between 0 for no infestation and 100 for the most severe damage (Mensah et al. 2005)
SE= standard error

Table 3.2. Correlation for aphid index or Damage Index (DI) between trials for soybean lines derived from E08907x E09907 with different gene combinations.

Resistance gene(s)	**Aphid index Greenhouse 2010 vs. Greenhouse 2011**			**DI Greenhouse 2012 week 3 vs. week 4**		
	Correlation	**P value**	**N**	**Correlation**	**P value**	**N**
rag3, rag4, rag1c	1.0000	<0.0001*	11	0.97234	<0.0001*	11
rag3, rag4, rag1b	-0.167	0.6340	11	0.97323	<0.0001*	11
rag3, rag1b	0.59732	<0.0001*	44	0.97736	<0.0001*	34
rag3, rag1c	1.0000	<0.0001*	13	0.76930	0.0432*	7
rag4, rag1b	0.99011	0.0991*	5	1.0000	<0.0001*	5
rag4, rag1c	-0.346	0.3605	9	0.26392	0.5674	7
rag1b	0.83977	<0.0001*	27	0.89616	<0.001*	27
rag1c	1.0000	<0.0001*	6	0.94082	<0.0051*	6

* Values for correlation within trials are significant at P<0.05

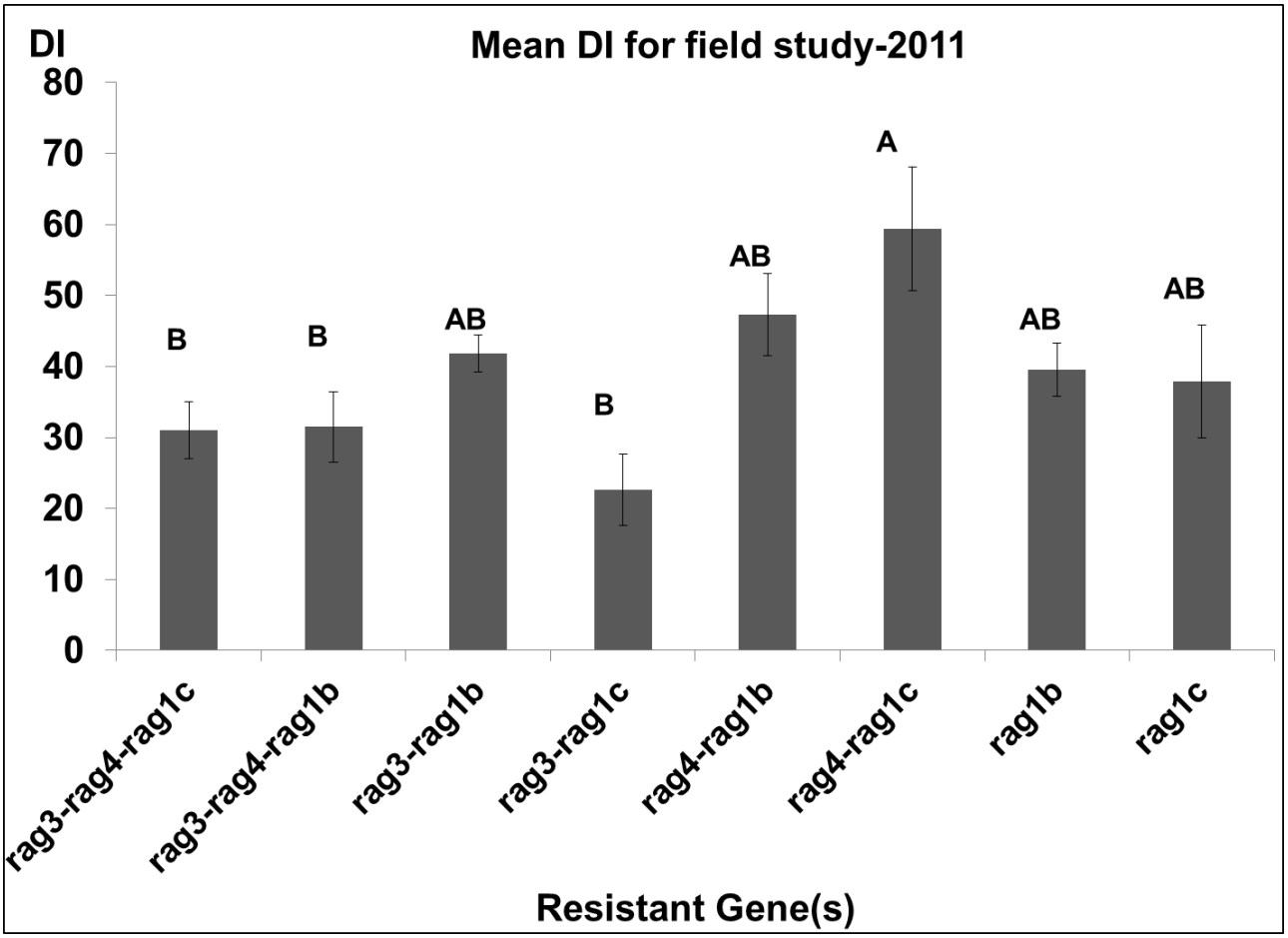

Figure 3.1. Mean Damage Index (DI) vs. resistant gene(s) for 727 $F_{3:4}$ soybean lines derived from E08907x E09907 in the 2011field trial.

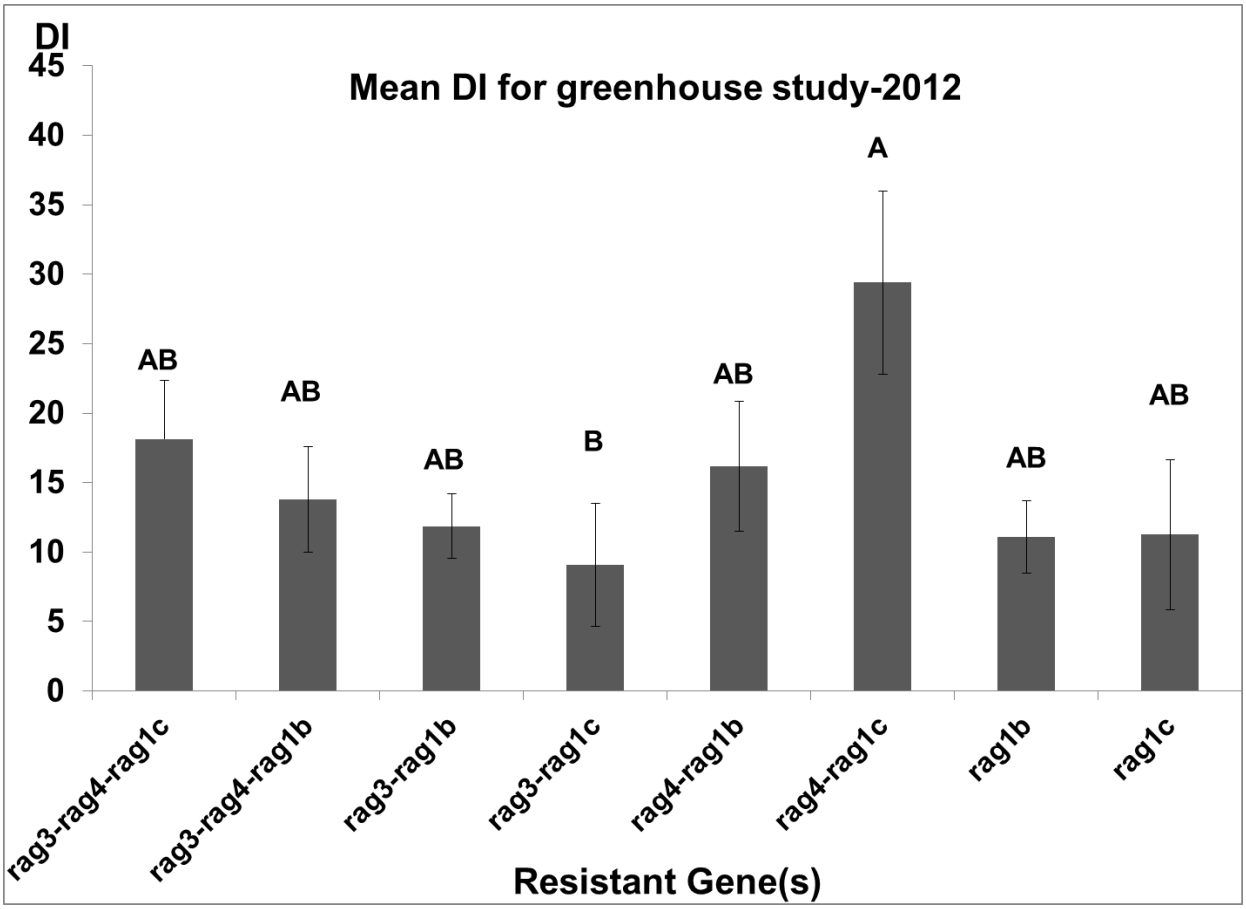

Figure 3.2. Mean Damage Index (DI) vs. resistant gene (s) for 120 $F_{4:5}$ soybean lines derived from E08907 x E09907 in the 2012 greenhouse trial.

Literature Cited

Literature Cited

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CHAPTER 4.0

A biochemical investigation to identify secondary metabolites conferring resistance to Japanese beetle in aphid-resistant soybean germplasm

Introduction

Soybean aphid is a major destructive pest in the United States. 50-70% yield loss can occur if a field is left untreated (He et al. 1995). Several aphid-resistant breeding lines have been developed by many breeding programs, but only very few lines have been successfully released due to breakdown of resistance. Thus far, aphid-resistant lines developed by MSU soybean breeding program continue to show reliable resistance since 2005. However, substantial evidence was collected from a multi-year field and laboratory investigation by Chandrasena et al. (2012) that confirmed increased susceptibility (>50% defoliation in field) to Japanese beetle on MSU aphid-resistant germplasm. Insect defoliators are important pests that pose a significant threat to soybean varieties (Hammond 2001). Action thresholds for soybean defoliation in the Great Lakes region generally range from 30% to 40% pre-bloom, decreasing to 15% between bloom and pod fill, and 25% thereafter (Eisley and Hammond 2007). Although most U.S. soybean cultivars show resistance to defoliation, it is important to monitor feeding by common defoliators such as Japanese beetle (*Popillia japonica* Newman) on new breeding lines.

Although Japanese beetle is not reported to cause serious economic loss in most soybean cultivars, attempts to identify soybean QTL with defoliator-resistance indicate the pivotal importance of assessing defoliation by Japanese beetle in soybean breeding programs. Moreover, breeding for defoliation-resistance has been a priority of soybean breeders for more than three

decades (All et al. 1989). QTL conferring resistance to several insect defoliators, including Japanese beetle, were discovered in soybean germplasm (Coon 1946, Rector et al. 1998, 2000; Zhu et al. 2006, Zhu et al. 2008). Yesudas et al. (2010) identified QTL from seven chromosomes conferring resistance to Japanese beetle in a recombinant inbred population. Research reported in Chapter 2.0 on QTL discovery for Japanese beetle resistance in a population derived from E06906 x LD05-16060-16060 suggests that some new and known resistant QTL underlie key candidate genes involved in the flavonoid biosynthetic pathway (Chandrasena et al. 2012, Ortega and Parrott 2012). Thus, it was interesting to investigate the differences in metabolite profiles of these two soybean lines and some resistant and susceptible lines derived from this cross.

Thus far, information about underlying genetic or biochemical factors leading to differential susceptibility in this germplasm has not been explored; hence sufficient biochemical analyses are important. Additionally, understanding the genes underlying JB-resistant QTL can potentially reveal very important information for novel resistant gene discovery. Furthermore, attempts to identify soybean lines with defoliator-resistance indicate importance of uncovering the biochemical mechanisms leading to Japanese beetle resistance as well as for preventing future attacks by Japanese beetle.

Japanese beetle feeding behavior is a function of both host plant location and host plant selection (Potter and Held 2002). Plant volatiles may play a key role in locating suitable host plants from a distance. There is insufficient evidence to conclude that Japanese beetles seek volatiles induced only by preferred hosts, since they are attracted to a wide range of plant species regardless of

host suitability (Potter and Held 2002). The most common volatiles induced by Japanese beetle feeding are euginols, geraniols, jasmines, and phenyl-acetonitriles (Loughrin et al. 1996a, 1996b). Japanese beetles locate hosts primarily by olfaction, thus it is also possible that there are differences in the mixtures of volatiles released from susceptible and less preferred genotypes. In such case, Japanese beetle may be attracted from a distance to the susceptible lines in greater numbers due to more or different ratios of attractive volatiles released after initial damage by other beetles.

Once a Japanese beetle finds a host, host selection occurs by olfaction and/or by chemoreception (taste) where non-volatile compounds play a key role (Potter and Held 2002). For example, in *Malus* spp. flavonoid compounds, Quercetin and rutin were beetle phago-stimulants, phloridzin, phloretin, naringenin, and catechin were anti-feedants (Fulcher et al. 1998. More generally, several plant-derived sugars, including fructose, glucose, maltose, and sucrose are strong phago-stimulants for Japanese beetle (Ladd 1987, 1988; Potter and Held 2002) as well as the cyanogenic glycosides prunasin, herniarin, and coumarin which are present in resistant *Prunus* spp. (Potter and Held 2002). A triterpene, cucurbitacin B, repels beetles from cucurbits (Tallamy et al. 1997).

In soybean specifically, many secondary plant compounds serve as feeding deterrents to herbivorous insects, including flavonoids and isoflavonoids (Treutter 2006, Chen et al. 2008). Several soybean flavonoids produced through phenylpropanoid pathway play a key role in plant defense against herbivory. The isoflavones afromosin, coumestrol, and phaseollin are abundant with soybean looper, *Pseudoplusia includes (Walker)* damage (Caballero et al. 1986, Dakora

1995). Increased levels of phytoalexins are associated with a significant reduction in Mexican bean beetle feeding on soybean (Hart et al. 1983). Some soybean flavonoids, such as quercetin, have recently been tested for their effectiveness as feeding deterrents to Japanese beetle (Soybean Checkoff Research Database 2011). Thus increased or decreased feeding of Japanese beetle on different lines of the same host can be related to differences in phago-stimulants and/or deterrent compounds.

This particular study was aimed at investigating the biochemical differences (volatiles and non-volatiles) between the JB (Japanese beetle) susceptible and JB-resistant soybean germplasm. Due to previous findings of QTL underlying key enzymes in the flavonoid biosynthesis pathway, it was hypothesized that differences in flavonoids (induced or constitutive) lead to differential susceptibility to JB on different aphid-resistant germplasm. Thus a comprehensive biochemical study including a qualitative and a quantitative analysis was conducted on seven commonly reported soybean flavonoids, their abundant sugar conjugates (glysosides), and aglycones associated with herbivory in soybean (Cavaliere et al. 2007, O'Neill et al. 2010).

Materials and Methods

Plant material

As mentioned above, source material was obtained from an existing breeding population derived from a cross between LD05-16060, a *Rag1* aphid-resistant line that has shown less susceptibility to Japanese beetle, and *rag3*/*rag1b* aphid-resistant line E06906, which shows elevated susceptibility to Japanese beetle. This population has been scored for Japanese beetle-resistance

using a standard scale for two years (see Chapter 2.0). Therefore based on pest severity data from two years, two lines showing elevated susceptibility and two lines showing resistance were selected from $F_{4:5}$ generation, along with their parent lines for tissue extraction.

Tissue extraction (for volatile and non-volatile analysis)

Six soybean lines (E06906, LD05-16060, Res._1, Res._2, Susc._1 and Susc._2) were planted in the field at MSU entomology farm in summer (10-15 plants per line). Res._1, Res._2, Susc._1 and Susc._2 were $F_{4:5}$ lines derived from E06906 x LD05-16060. This particular site was repeatedly damaged by Japanese beetle, thus provided an ideal location for growing the plants. Typically, plant metabolite profiles differ vastly among different lines, hence all changes found between JB-resistant and susceptible lines cannot be attributed to herbivory. Therefore to find differences in metabolite profiles that may be closely associated with Japanese beetle herbivory, two measures were taken; flavonoid compounds that have been reported to play key roles in herbivory for soybean were investigated. Then, samples from damaged and un-damaged leaflets of each line were taken to see how those levels differed when exposed to feeding by Japanese beetle; samples taken from un-damaged plants represented the non-induced compounds, and a different leaflet from the same plant within the same line with damage represented the induced state. Each sample was replicated three times (each plant within line = biological replicate for a given sample).

Collected tissue was transferred to a 50 ml plastic vial (non polystyrene) at the study site, and appropriately labeled. Non volatile compounds were extracted using a solvent mixture: acetonytryl: 2-propanol: MQ water in 3:3:2 ratios. The solvent for volatile compound extraction

was MTEBE (Methyl Tertiary Ethyl Butyl Ether). Before extraction, the weight the damaged and un-damaged leaflets were recorded. 10 ml of solvent mixture was added to 50 ml vial, shaken vigorously for 2 minutes. Next, the tubes with solvent mixtures were refrigerated overnight. The next day, 1ml of solvent from each tube was transferred to 2ml auto-sampler vial, appropriately labeled and frozen in -80^0C until ready to be run on Gas Chromatography Mass Spectrometry equipment (GC/MS) and High Performance Liquid Chromatography/tandem Mass Spectrometry (HPLC/MS/MS) equipment. A total of 72 samples were extracted for this study; 36 each for the non-volatile and volatile analysis (6 lines x 3 replicates per line x damaged and undamaged state).

Metabolite profiling

First a baseline analysis on abundance of volatile compounds were conducted using (Gas Chromatography Mass Spectrometry equipment (GC/MS) at Mass Spectrometry facility at MSU, on a subset of all lines with or without damage (12/36 samples). A more comprehensive analysis on flavonoids was carried out using High Performance Liquid Chromatography/tandem Mass Spectrometry (HPLC/MS/MS) techniques at Mass Spectrometry facility for 36 samples.

A method for peak detection and analysis was developed with kind assistance from Ramin Vismeh at MSU mass spectrometry facility. An Electrospray Ionization (ESI) in positive ion mode was used to ionize analytes and collision induced dissociation (CID) with nitrogen gas was applied for fragmentation of selected flavonoid ions. Information dependent acquisition (IDA) was performed for initial screening of possible flavonoid ions (Ramin Vismeh, pers. comm). LC/MS/MS used in this work consisted of binary LC-20AD pumps (Shimadzu), a SIL-HTc

autosampler, and column oven coupled to a QTRAP 3200 mass spectrometer (AB/Sciex). All mass spectrometric analyses, including data processing, were performed using Analyst v. 1.4.2 software (AB/Sciex). Before mass spectrometry analysis, analytes were separated using an Ascentis Express C18 column (5 cm×2.1 mm; 2.7 μm particles) using a reversed phase binary gradient. Solvent A was 0.15% aqueous formic acid and solvent B was acetonitrile. Total solvent flow was maintained at 0.4 mL/min, and gradient elution was performed using the following solvent compositions: initial, 5 % B, held for 1 min; linear gradient to 28 % B at 9 min and then to 90 % B at 10 min with a hold of 2 min; sudden decrease to 5 % B at 12.01 min till 15 min for equilibration. Injection volume and column temperature were 5 μL and 45 °C, respectively.

MRM tandem mass spectrometry was performed on 32 selected ion transitions in positive ion mode and peak areas were automatically integrated using Analyst software (Ramin Vismeh , pers. comm). Fragment ions for MRM transitions were selected based on IDA data and published reference by Cavaliere et al. (2007).

Statistical analysis

Peak areas for several isomers (with same ionic mass) of the same flavonoid conjugate were averaged for 18 distinct compounds. Analysis of variance (ANOVA) was performed using the PROC GLM procedure of SAS (SAS Institute, 2010). Mean comparisons were conducted at 5% significance level with t-tests (P=0.05).

Results and Discussion

The analysis for volatile compounds detected only very weak signals for all samples of the subset (data not shown). This could be due to lack of volatiles in the extraction, or the method

used was not sufficiently sensitive. Since the most interest was on investigating the flavonoids, a more sensitive assay was conducted on non-volatiles. The mean weight of damaged and un-damaged leaflets for each line is listed in Table 4.1. Since quantitative measurements of each compound was compared among the lines, it was important to keep the size of leaflets as uniform as possible. Mean weight of damaged leaflets ranged between 0.57-0.87g, while mean weight of un-damaged leaflets ranged between 0.70-1.03g. The weights of leaflets did not vary drastically within damaged and un-damaged groups.

Table 4.1. Mean weights of damaged and un-damaged soybean leaflets from six soybean lines

Line	**Weight of the leaflet (g)**	
	Damaged (Mean ± SD)	**Un-damaged (Mean ± SD)**
E06906	0.77±0.11	1.03±0.06
LD05-16060	0.57±0.15	0.8±0
Sus._1	0.7±0.1	0.9±0.26
Sus._2	0.77±0.37	0.7±0.2
Res._1	0.77±0.30	0.83±0.11
Res._2	0.87±0.23	1.03±0.05

Based on generated enhanced product ion spectra (EPI), 32 ions (many of which had multiple isomers) were identified as flavonoids (Table 4.2), and were later monitored in all samples using Multiple Reaction Monitoring (MRM) to improve selectivity and reduce interference from other ions present. Manually selected EPI scans (MS/MS) were also performed in some cases to confirm and compare the fragment ions with published references (Cavaliere et al. 2007). A threshold of Signal/Noise (S/N=5) was used to identify the significant peaks.

Table 4.2. Profiled ion masses ($M+H^+$) of seven flavonoid compounds using Multiple Reaction Monitoring in six soybean lines

Quercetin	Daidzein	Genistein	Glycitein	Naringenin	Kaempferol or Luteolin
303	255	271	285	273	287
465	417	433	447	435	449
449	549	519	533	521	535
551	579	565	609		741
611	665	595	695		757
627	725	741			
773					

Analysis of damaged-leaflets

Compounds were not detected in their molecular form except for Genistein. However, 18 total compounds as sugar conjugates, or aglycones derived from six key soybean flavonoids (Kaempferol, Genistein, Glycitein, Daidzein, Naringenin, and Quercetin) were detected on leaflets damaged by Japanese beetle for six soybean lines (Table A2, appendix). The roman numerals after the name corresponds to the number of isomers of conjugate having the same ion mass. However, this method was sensitive to detect these forms separately. For the simplicity of analysis, areas of several isomers belonging to same sugar conjugate were averaged. The area under the peak corresponded to the relative abundance of each compound.

MRM is a widely adopted, and an accurate method for quantifying flavonoid compounds in plants without having to use expensive standards for each compound.

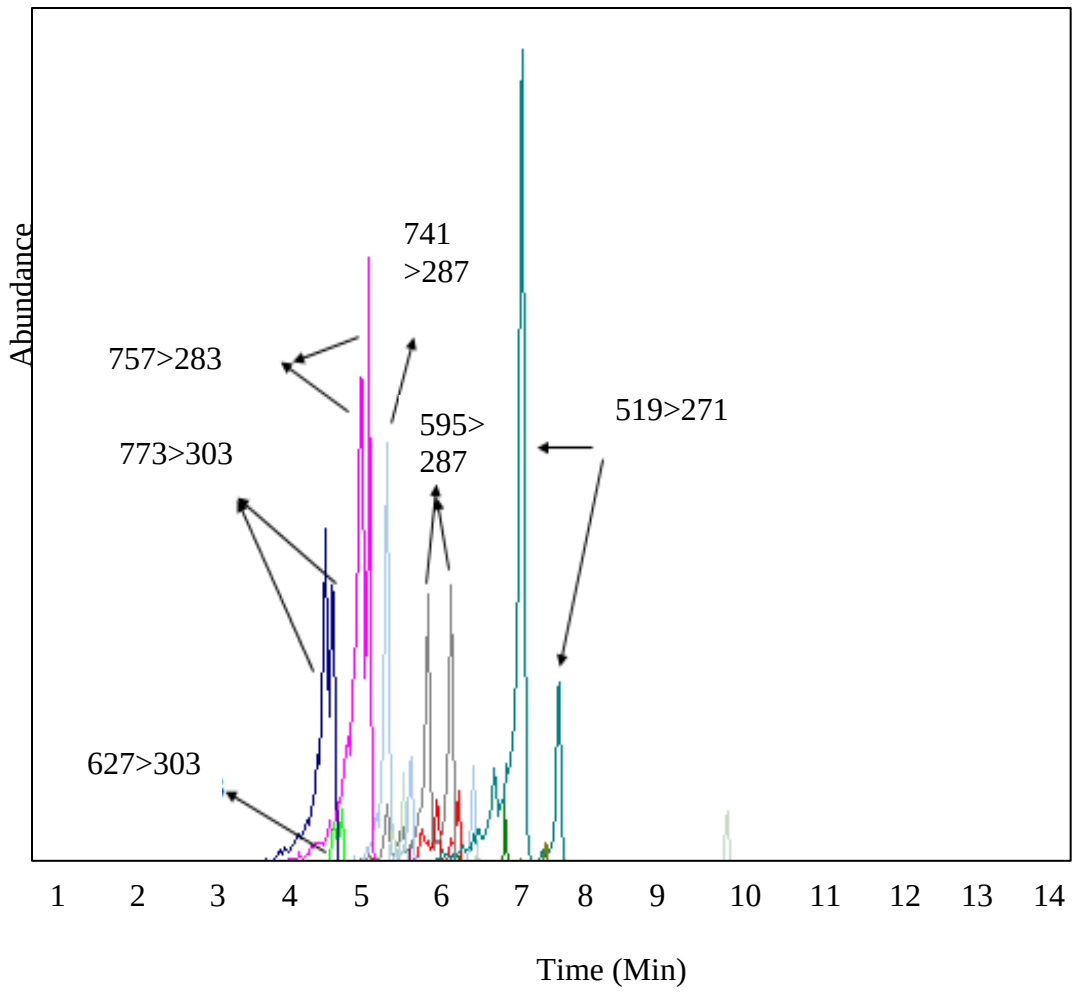


Figure 4.1. Selected ion chromatograms of 32 ion transitions from one of the soybean extract sample. Each color represents one specific transition.

*Some of the transitions for relatively abundant flavonoids are highlighted. (Ramin Vismeh, pers. comm).

Namely, mean areas for Daidzein O-hexoside (I,II) , Glycitein O-hexoside malonylated (I,II,III,IV), Genistein O-hexoside malonylated (I,II), Genistein O-hexosyl-pentoside (I,II), Kaempferol O-hexoside malonylated , Daidzein aglycone did not significantly differ among the six lines, therefore this study failed to find any direct association of above compounds to

herbivory. However based on this study only, it appears that these compounds are less likely to play any role for differential susceptibility to Japanese beetle, among the six soybean lines.

Highest peak area for several compounds was observed in damaged leaflets of LD05-16060. Abundances of 9 compounds were significantly higher in LD05-16060 than E06906 (Figure 4.2). Kaempferol aglycone levels were higher in E06906 than LD05-16060. The observed general trend was that there were more flavonoid compounds (high in abundance and in number) in damaged leaflets of LD05-16060, compared to E06906. Despite the evidence obtained from QTL mapping and candidate gene analysis for the presence of known major defoliating resistance QTL, such as QTL-M in this germplasm, it is difficult to infer that all differences observed between LD05-16060 and E06906 are related to Japanese beetle herbivory. However, if similar trends were detected between defoliation-resistant and susceptible lines derived from this cross, that could be direct evidence that these compounds are associated with herbivory by Japanese beetle. Such clear separation between all three resistant and three susceptible lines appeared for three compounds. Area corresponding to Genistein O-dihexoside was approximately 11 times more in LD05-16060 than E0906 (Table 4.3). Similarly, area for Genistein O-dihexoside was significantly more for both Res_1 and Res_2 than the two susceptible lines (3-14 fold increase than susceptible lines).

Abundance of Kaempferol O-rhamnoside O-hexosyl-rhamnoside was higher by 6-fold in LD05-16060 when compared to E06906 (Table 4.3). Resistant lines had significantly more Kaempferol O-rhamnoside O-hexosyl-rhamnoside (3-6 times more) than susceptible lines.

Similarly, there was significantly more Kaempferol O-hexosyl-rhamnoside (I,II) (10 fold increase) in LD05-16060 than E06906 (Table 4.3). The same trend was observed between resistant and susceptible lines (4-9 fold increase in abundance in resistant lines). Thus, there is good possibility for these three compounds to be directly related to differential susceptibility seen among these specific lines. Since dramatically high levels of these compounds were present in both LD05-16060 and two resistant lines, it could be speculated that elevated levels of above three flavonoid sugar conjugates can act as feeding deterrents to Japanese beetle. Although dramatically high levels of Glycetein aglycones were abundant in LD05-16060 (8-fold increase), the same trend was not obvious between the two resistant and susceptible lines, hence there was no direct evidence to identify this compound as a key deterrent leading to resistance.

Furthermore, Quercetin O-hexoside-malonylated (I,II) levels and Kaempferol aglycone levels were the same among two resistant lines (Res._1 and Res._2) and LD05-16060. But they did not significantly differ from all three susceptible lines; hence a direct relationship between these compounds and Japanese beetle herbivory cannot be confirmed.

Table 4.3. Abundance (area measured) of flavonoids that were significantly higher in damaged and un-damaged leaflets of LD05-16060

Compound	E06906	LD05-16060	Fold increase in LD05-16060
Damaged leaflets			
Naringenin O-hexoside	10,493	27,633	x3
Naringenin O-hexoside malonylated	21,167	67,667	x3
Genistein	1,450	4,237	x3
Genistein O-hexoside	80,997	214,217	x3
Genistein O-dihexoside	57,000	617,667	x11
Kaempferol O-hexoside (I,II,III)	25,353	71,656	x3
Kaempferol O-rhamnoside O-hexosyl-rhamnoside	375,000	2,310,000	x6
Kaempferol O-hexosyl-rhamnoside (I,II)	139,900	1,418,333	x10
Glycitein aglycone	79,866	601,666	x8
Un-damaged leaflets			
Genistein O-hexoside	34,957	110,235	x3
Genistein O-dihexoside	48,533	287,667	x6
Kaempferol O-rhamnoside O-hexosyl-rhamnoside	176,500.00	932,167.00	x5
Kaempferol O-hexosyl-rhamnoside (I,II)	342,000	1,323,000	x4
Glycitein aglycone	69,300	226,133	x3

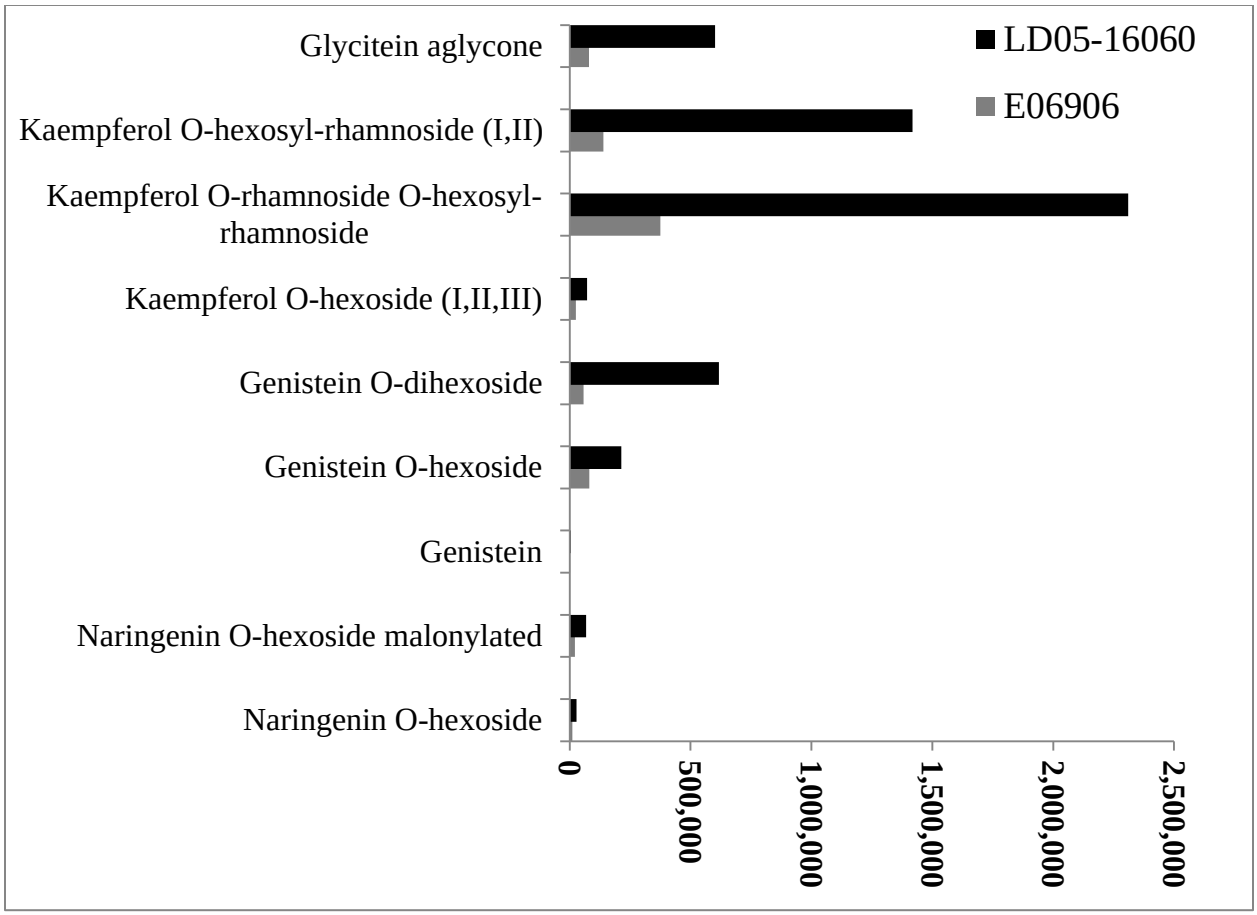


Figure 4.2: Abundance (Peak area measured) of nine flavonoids that were higher in damaged leaflets of LD05-16060 ($P<0.05$).

Analysis of un-damaged leaflets

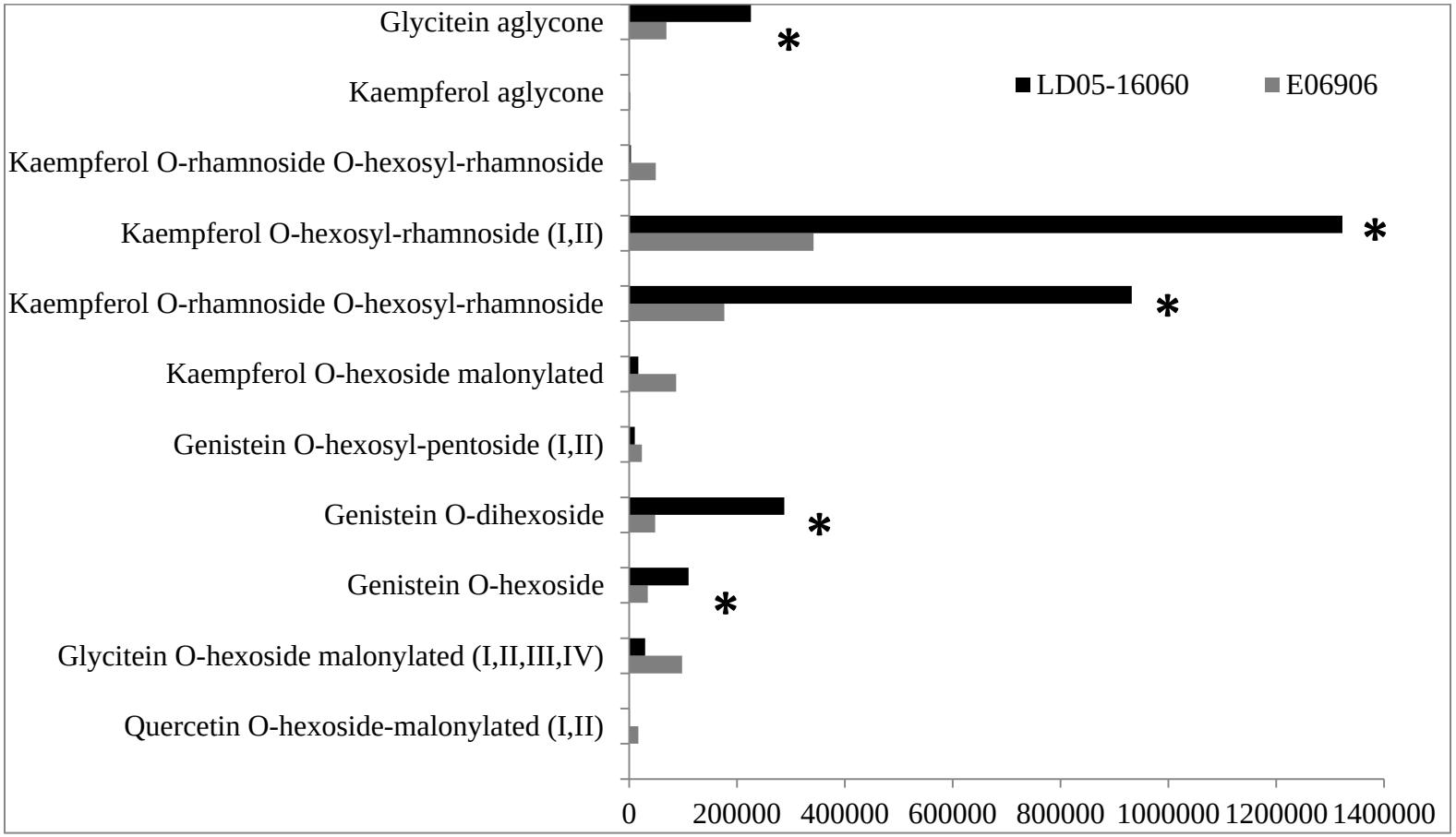
Levels of the same 18 compounds tested for damaged leaflets (Table 2, appendix) were analyzed from un-damaged leaflets collected from all six lines (Table A3, appendix). This analysis helped to compare how levels of the same compound differed with or without damage for a given soybean line.

Interestingly, abundances of six flavonoid compounds were significantly higher in E06906 than LD05-16060 for un-damaged leaflets (Figure 4.3). Those were Quercetin O-hexoside-malonylated (I,II), Glycitein O-hexoside malonylated (I,II,III,IV), Genistein O-hexosyl-pentoside (I,II), Kaempferol O-hexoside malonylated, Kaempferol O-rhamnoside O-hexosyl-rhamnoside, Kaempferol O-hexosyl-rhamnoside (I,II) and Kaempferol aglycone (Figure 4.3). However the general abundances of these compounds were very low. Similarly, the levels of compounds did not fluctuate drastically between damaged and un-damaged leaflets from E06906.

LD05-16060 showed significantly higher abundance for five compounds than E06906 (Quercetin O-hexoside-malonylated (I,II), Glycitein O-hexoside malonylated (I,II,III,IV), Genistein O-hexosyl-pentoside (I,II), Kaempferol O-hexoside malonylated, and Kaempferol O-rhamnoside O-hexosyl-rhamnoside) in un-damaged leaflets (Figure 4.3). Additionally, these abundances levels were dramatically high in LD05-16060. While the levels remained unchanged for E06906, when levels of the same flavonoid was compared on damaged and un-damaged leaflets within a line, it appeared that some dramatic fluctuations of flavonoid compounds were present between damaged and un-damaged LD05-16060 leaflets. Abundance of Kaempferol O-hexosyl-rhamnoside (I,II) changed from a 4-fold increase to a 10-fold increase than E06906 when undamaged and damaged LD05-16060 leaflets were compared (Table 4.3). Another example is Glycetein aglycone, where abundance (area) changed from 226,133 for un-damaged LD05-16060 leaflets to 601, 667 for damaged LD05-16060 leaflets (changed from 3-fold to 8-fold increase than E06906). Similar increases between damaged and un-damaged LD05-16060 leaflets were apparent for Genistein-O-dihexoside. Such distinct patterns did not appear for many

compounds in E06906 leaflets. This again indicates that increase of some feeding-deterrent flavonoids upon damage, can explain increased resistance in LD05-16060. It is possible that initial damage induce deterrent flavonoids in LD05-16060 as a defensive mechanism. Regardless of the state (damaged or un-damaged), relative abundance of several Genistein-derived, and Kaempferol-derived compounds were generally high in all lines. Following similar patterns observed between LD05-16060 and E06906, both resistant lines had significantly more Kaempferol O-hexosyl-rhamnoside (I,II) and Genistein-O-dihexoside than two susceptible lines. As observed with damaged leaflets, this consistently indicated the direct relationship of these compounds to herbivory.

To find if these compounds played any role in herbivory, Japanese beetle- resistant and Japanese beetle-susceptible lines were included from a population derived from the above cross. The differences observed between LD05-16060 and E06906 could perhaps be responsible for differential feeding. However, to confirm this, it was important to investigate other resistant and susceptible lines, and how levels within the same line fluctuated with or without exposure to Japanese beetle. Although several significant differences in compounds were observed between the two parent lines, only few compounds were distinguishably different between other resistant and susceptible lines. Two possibilities could be put forward to explain this phenomena; either those compounds were not directly responsible for herbivory, thus same pattern was not observed; or the phenotyping was not accurate for the resistant selections (susceptible lines may have appeared resistant, due to other conditions). However the two resistant lines were selected based on mean pest severity scores of two years, and they appeared resistant in both years.



Compounds followed by * were higher in LD05-16060 (P<0.05). Compounds without * were higher in E06906 (P<0.05).

Figure 4.3. Abundance (Peak area measured) of 11 flavonoid compounds showing significant differences between un-damaged leaflets of LD05 and E06906 (P<0.05).

Another interesting observation made from this analysis was the absence of four Quercetin-derived flavonoids in both LD05-16060 and resistant lines (Table 4.5). In both damaged and un-damaged leaflets of E06906 and susceptible lines, Quercetin O-hexosyl-rhamnoside (I,II), Quercetin O-di-hexoside(I,II), Quercetin O-rhamnoside O-hexosyl-rhamnoside (I,II), Quercetin O-hexoside levels were abundant. Except for Quercetin O-rhamnoside O-hexosyl-rhamnoside (I,II), all other compounds were below the detection threshold (S/N < 5) in both damaged and un-damaged leaflets of LD05-16060 and two resistant lines. Moreover, Fulcher (1998) reported Quercetin as a phago-stimulant for Japanese beetle in *Malus* spp. Due to supporting literature and since Quercetin-derived compounds were detected in higher abundances in all susceptible lines, it can be concluded that these compounds act as phago-stimulants, leading to the elevated herbivory in E06906 and susceptible lines.

Table 4.4. Abundance of four Quercetin-derived compounds in damaged and un-damaged leaflets of six soybean lines.

Compound	**E06906**	**Susc._1**	**Susc._2**	**LD05-16060**	**Res._1**	**Res._2**
Damaged						
Quercetin O-hexosyl-rhamnoside (I,II)	33533	25417	27433	*ND*	*ND*	*ND*
Quercetin O-di-hexoside(I,II)	25373	14100	24933	*ND*	*ND*	*ND*
Quercetin O-rhamnoside O-hexosyl-rhamnoside (I,II)	131983	118300	13266	*ND*	677	*ND*
Quercetin O-hexoside	30367	17233	23600	*ND*	*ND*	*ND*
Un-damaged						
Quercetin O-hexosyl-rhamnoside (I,II)	49550	2808	16783	*ND*	*ND*	*ND*
Quercetin O-di-hexoside(I,II)	44767	2293	15800	*ND*	*ND*	*ND*
Quercetin O-rhamnoside O-hexosyl-rhamnoside (I,II)	206333	23450	96167	*ND*	650	*ND*
Quercetin O-hexoside	47233	1840	15300	*ND*	*ND*	*ND*

ND = Abundances were below detection threshold (s/n<5)

Due to this germplasm's association to different aphid-resistant genes, some differences observed between E06906 and LD05-16060 leaflets could be explaining the differences in susceptibility to soybean aphid. In contrast to Japanese beetle, who is a defoliator, soybean aphid is a phloem feeder. Thus, the metabolite differences detected in phloem content rather than on leaflets may be useful to make any inferences. A mapping study conducted in china to identify QTL conferring aphid-resistance found that an aphid-resistant accession 'Zhongdou 27' (PI 567598B, the original resistant sources of E06906) possess QTL underlying genes responsible for high isoflavone content. They observed increased levels of Genistein, Daidzein and Glycetein from leaf tissue extracts of aphid-damaged and non-damaged plants of 'Zhongdou 27', thus concluded that elevated levels of Genistein, Daidzein and Glycetein may defend plants against soybean aphid (Meng et al. 2011).

Many compounds investigated in our study are associated to Japanese beetle herbivory in several host species (Potter and Held 2002). Recently, O'Neill et al. (2010) investigated the fluctuations of several flavonoids and their derivatives (Quercetin, Daidzein, Genistein, Kaempferol, Naringenin and Luteolin) in soybean foliage when affected by Japanese beetle damage, soybean aphid, and elevated CO_2 levels. A conclusion was made that majority of flavonoids were induced in response to damage by Japanese beetle while the levels remained unchanged in response to phloem-feeding. It was stated that these soybean flavonoids might have a large effect on leaf palatability.

In conclusion, it is clear that significant differences in several flavonoids appear between E06906 and LD05-16060; however not all differences could be attributed to herbivory. Regardless of

damage, several flavonoids were present in larger amounts (11- 10 fold increase than E06906) in LD05-16060. Additionally, higher levels of more flavonoid compounds (nine) were present in damaged leaflets of LD05-16060, where abundances also dramatically increased when compared to un-damaged leaflets. It appears that at least three compounds can be directly attributed to conferring resistance in LD05-16060. Dramatically higher levels of Kaempferol O-rhamnoside O-hexosyl-rhamnoside, Kaempferol O-hexosyl-rhamnoside (I,II), and Genistein-O-dihexoside were detected in all three resistant lines. Additionally, four Quercetin-derived compounds (that may act as phago-stimulants) were detected in E06906 and susceptible lines while those levels were below threshold in LD05-16060 and two resistant lines. Based on o these observations from the biochemical study, and strong evidence found on presence of candidate genes underlying new and known resistant QTL in this germplasm, it can be concluded that flavonoids play a large role in explaining differential Japanese beetle susceptibility between LD05-16060 and E06906.

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APPENDIX

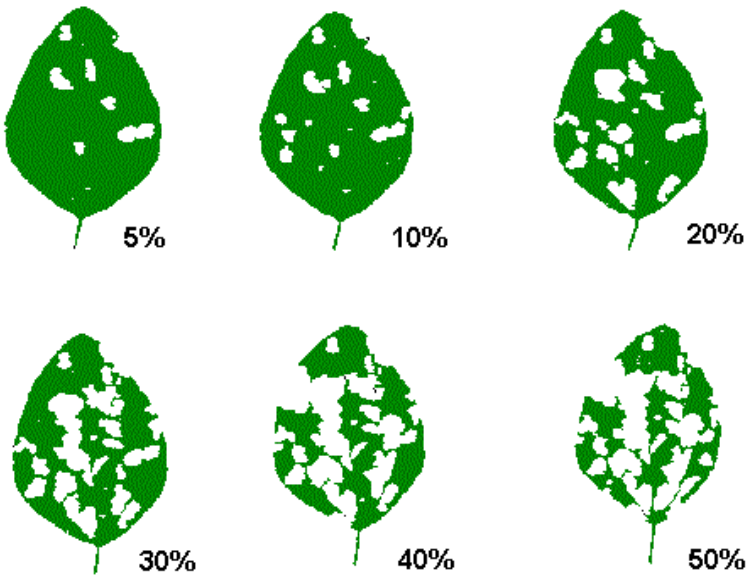

Figure A1. Scale used to rate defoliation (up to 50% defoliation) in no-choice forced- feeding assays in Chapter 2.0.

(source: http://extension.psu.edu/field-crop-news/news/2008/august-5)

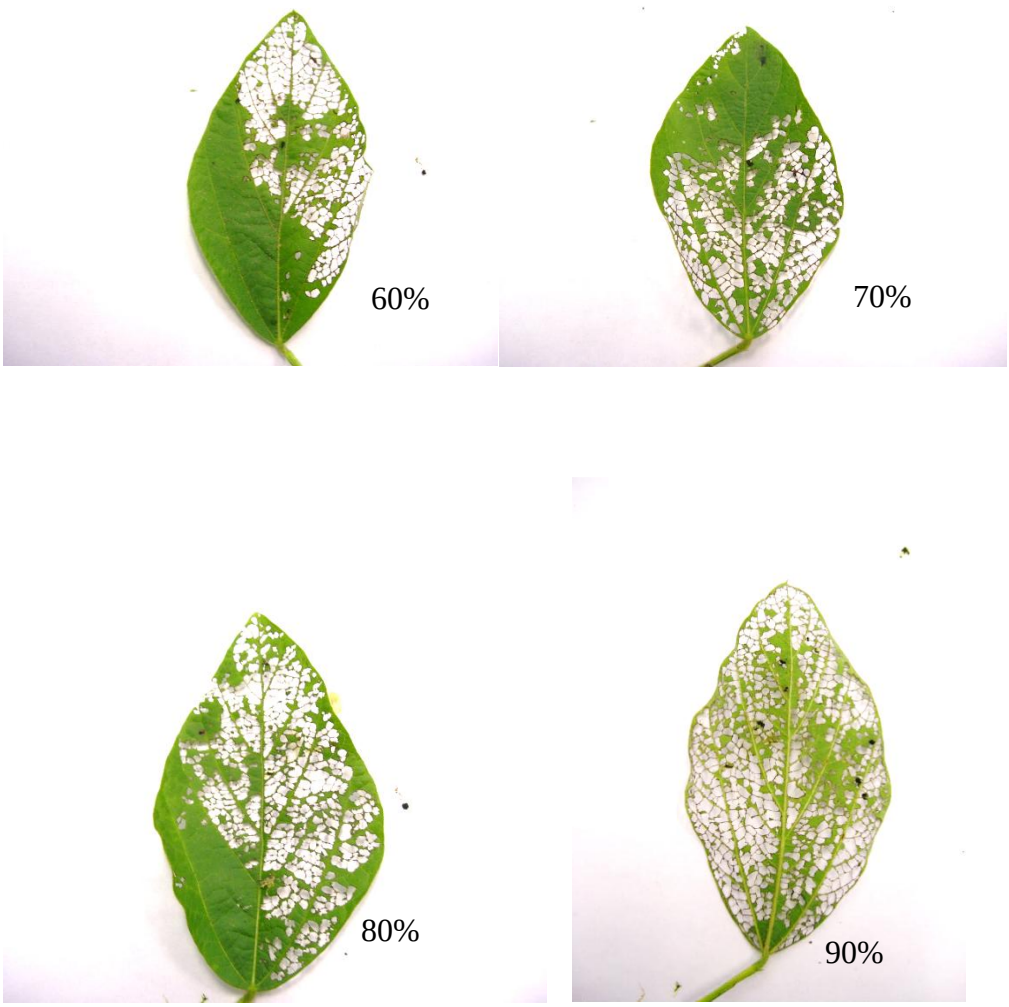

Figure A2. Scale developed to rate>50% defoliation in no-choice feeding assays in Chapter 2.0

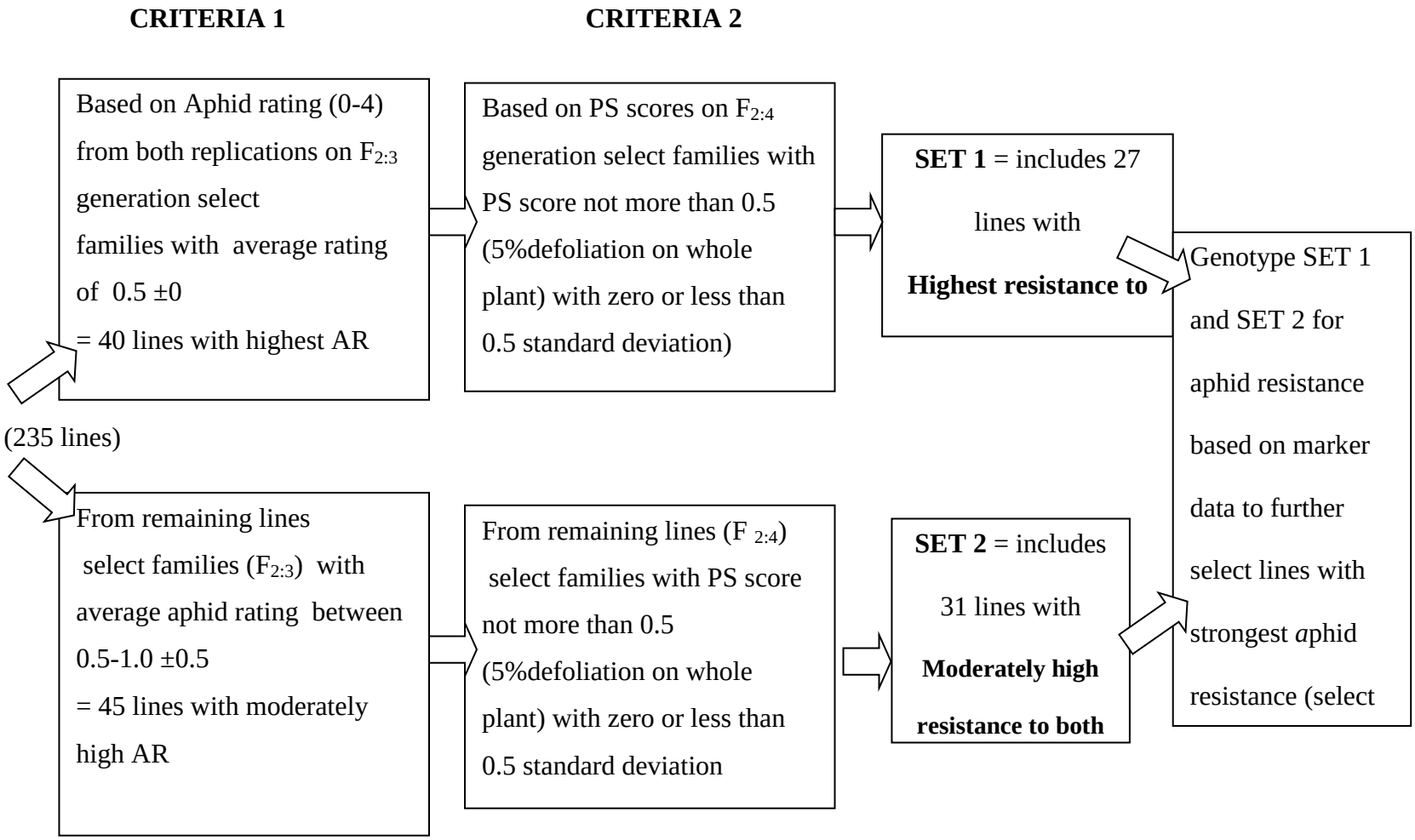

Figure A3. Criteria for selection of best individuals in 2010 (Chapter 2.0)

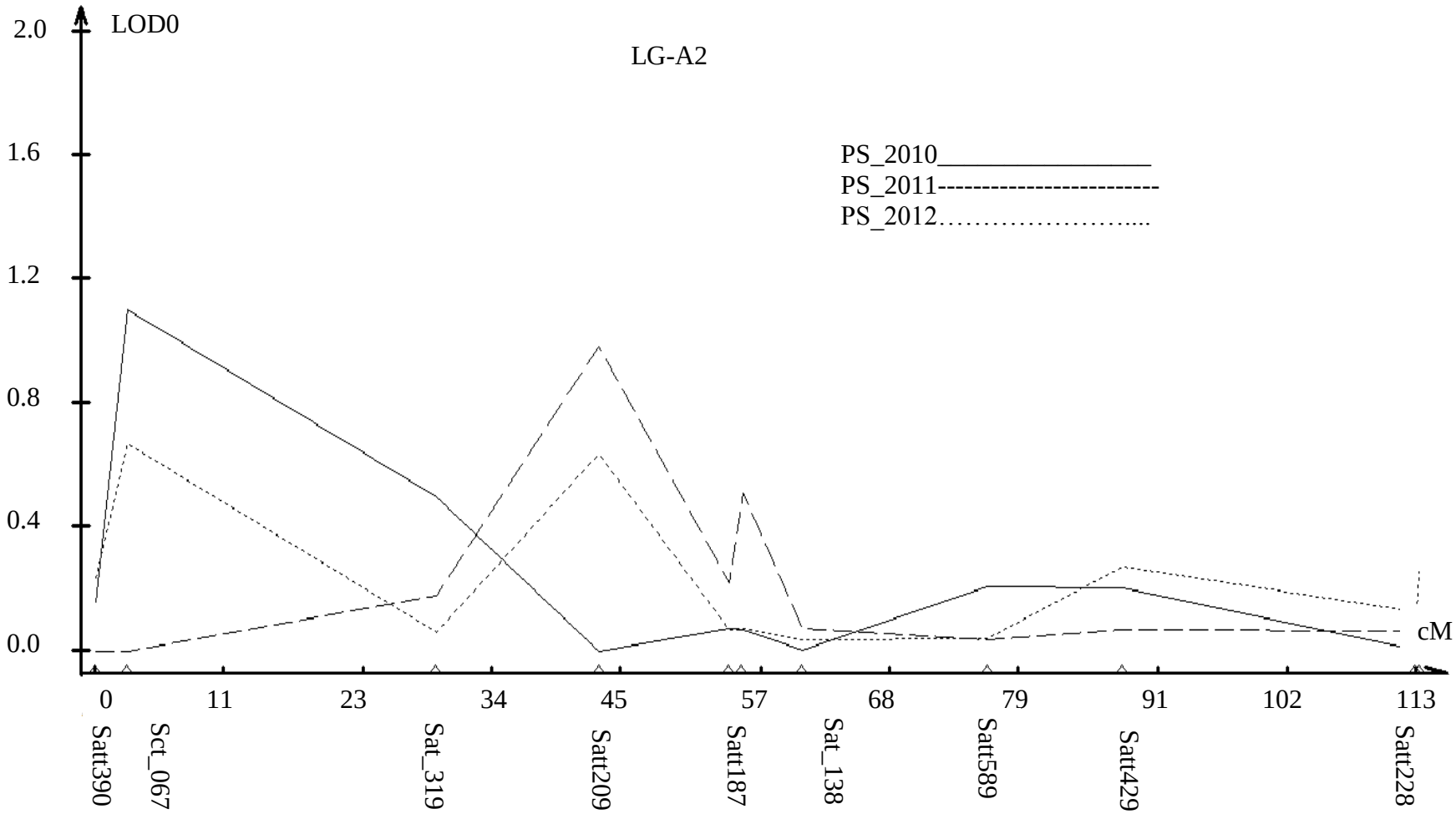

Figure A4. QTL detected on LG-A2 with single marker analysis with 94 individuals (Chapter 2.0).

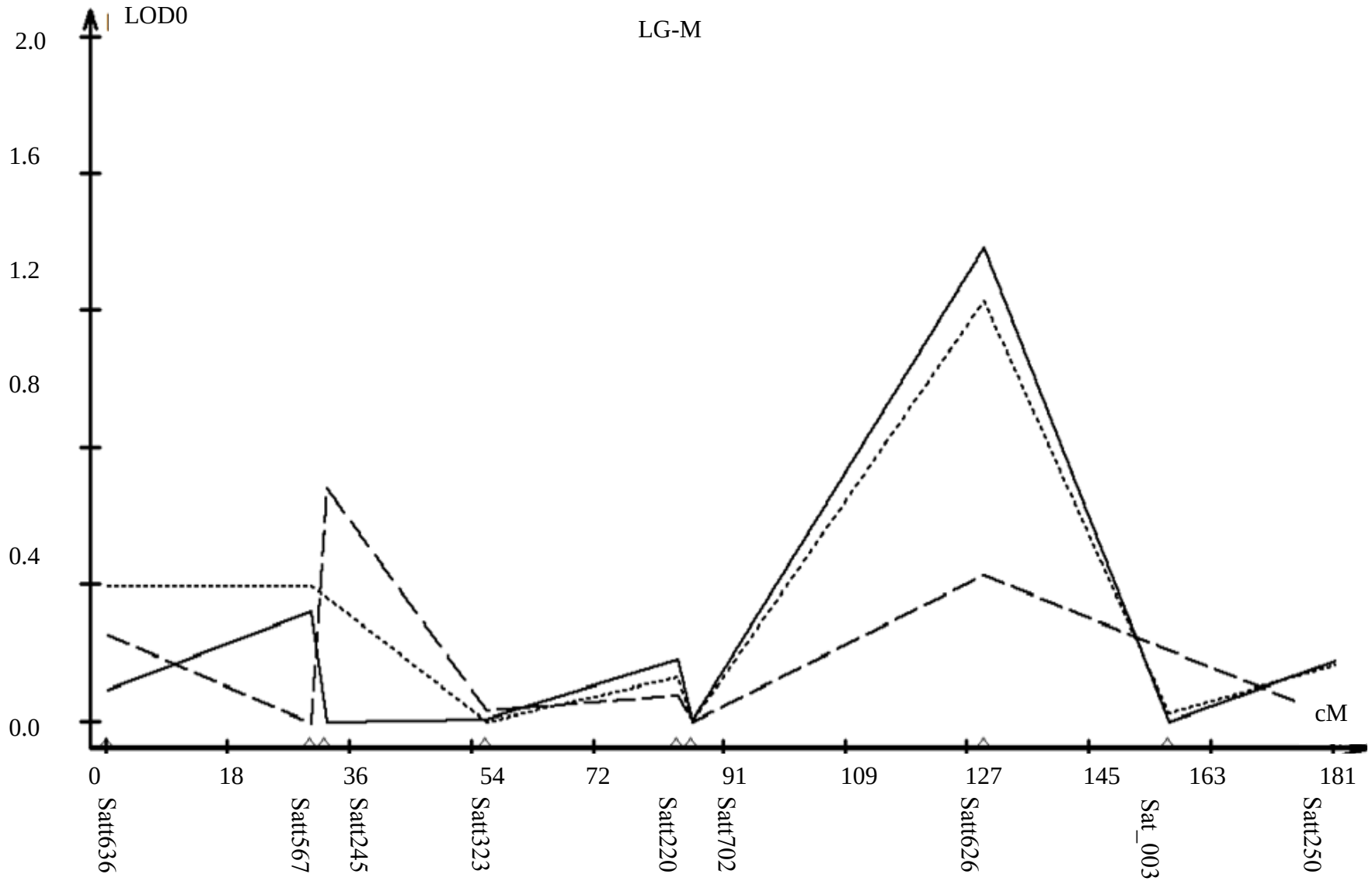

Figure A5. QTL detected on LG- M with single marker analysis with 94 individuals (Chapter 2.0).

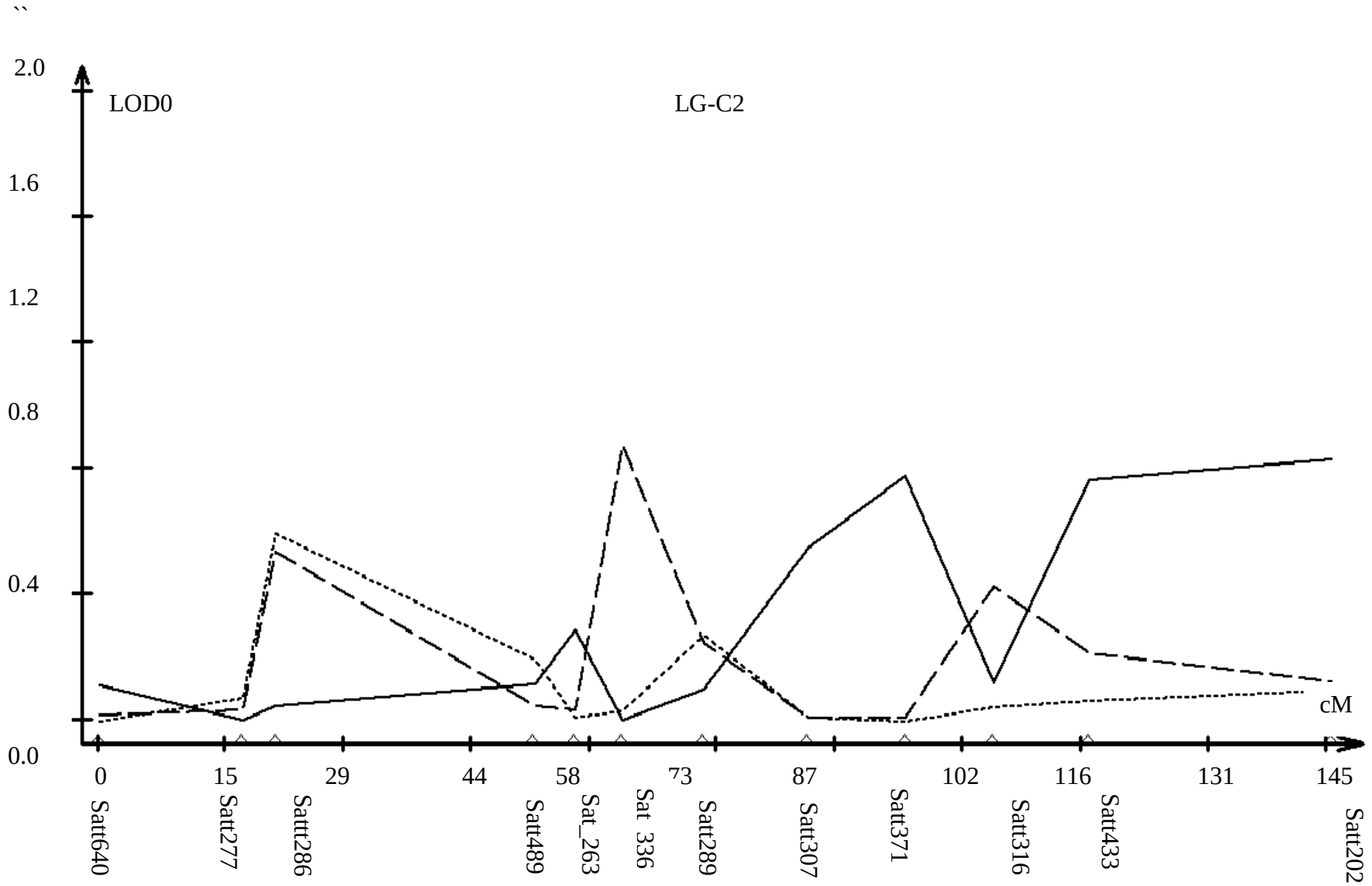

Figure A6. QTL detected on LG- C2 with single marker analysis with 94 individuals (Chapter 2.0).

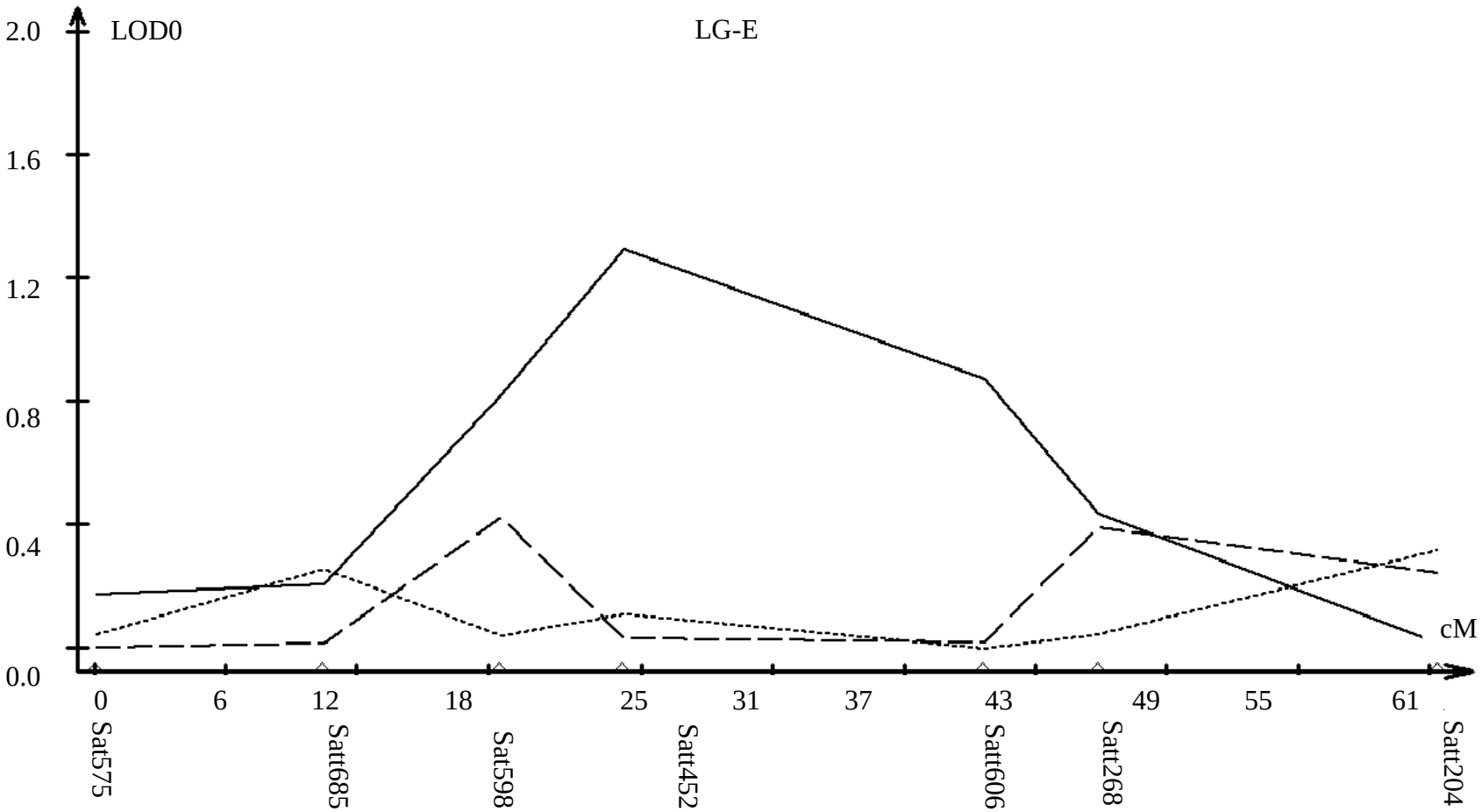

Figure A7. QTL detected on LG-E with single marker analysis with 94 individuals (Chapter 2.0).

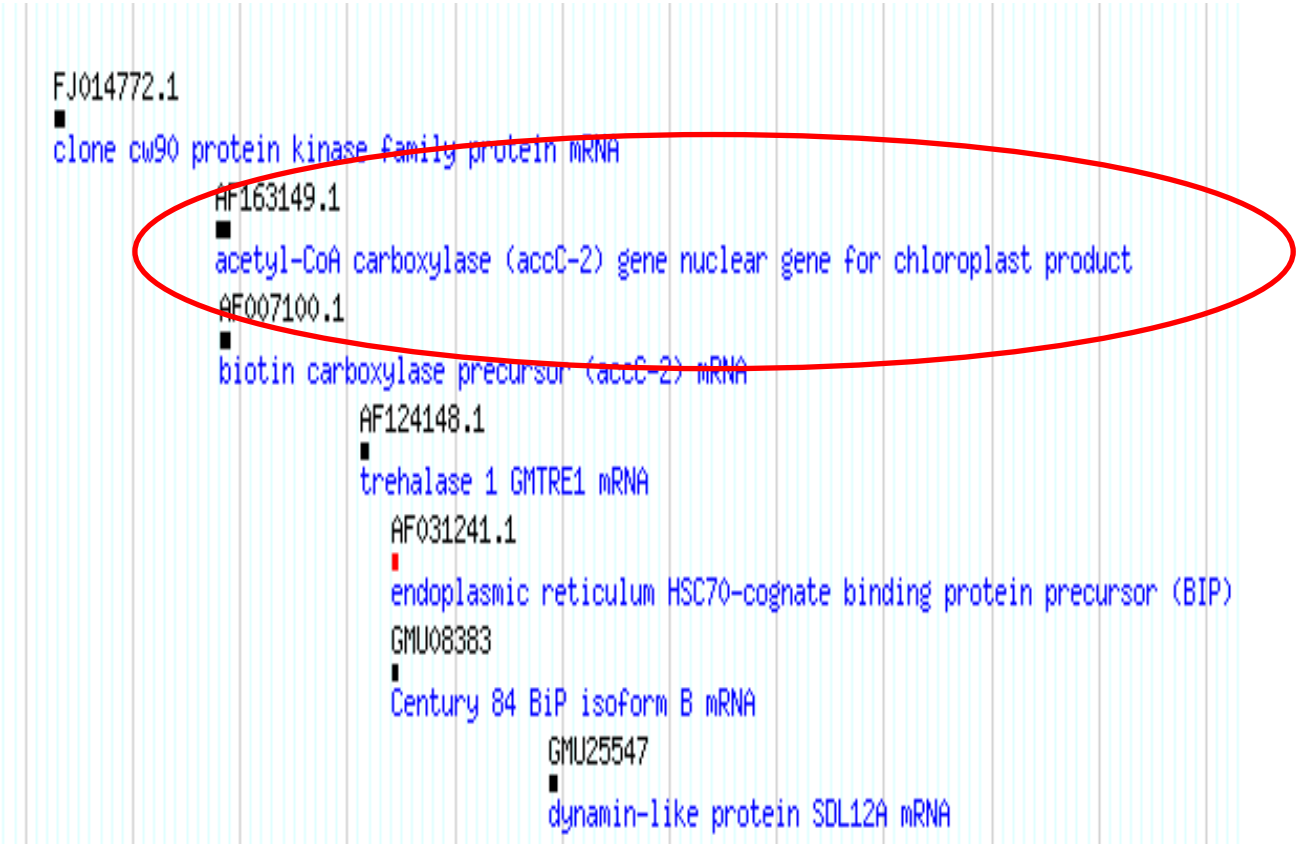

Figure A8. Candidate Gene analysis for A1 QTL on Soy Genome Browser (www. Soybase.org) showing acetyl-CoA carboxylase (accC-2) gene, nuclear gene for chloroplast product (Chapter 2.0)

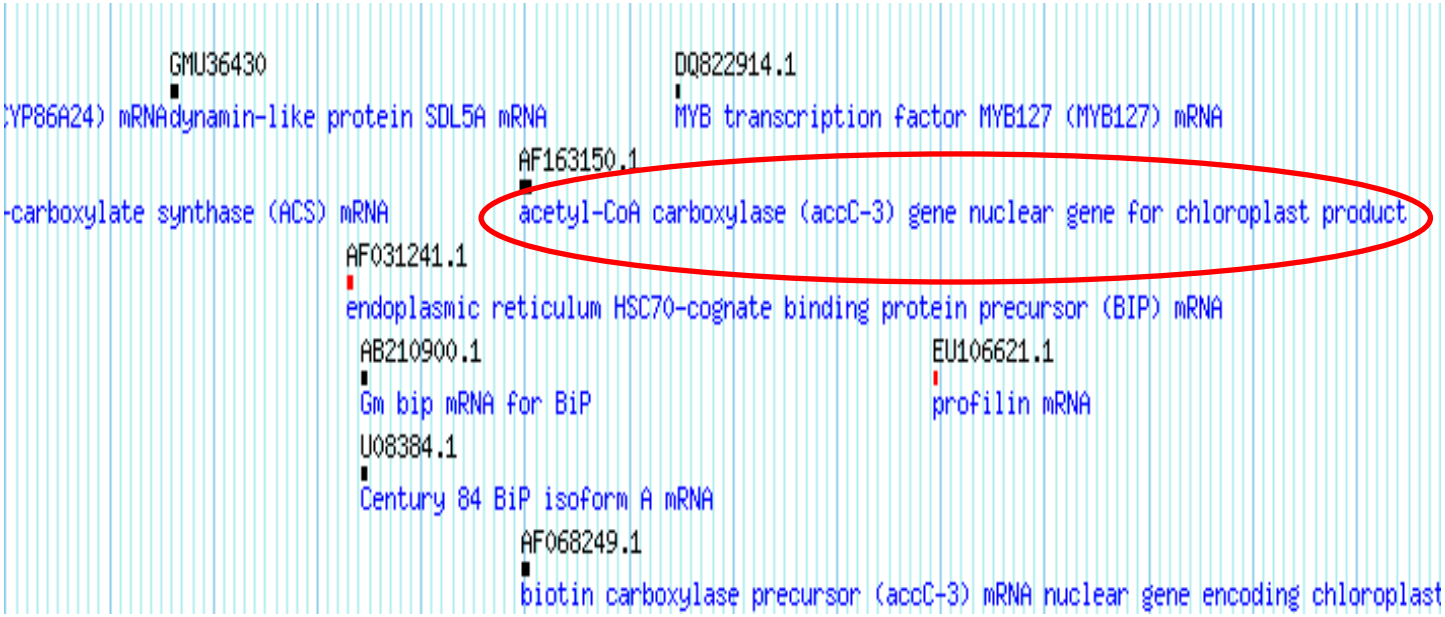

Figure A9. Candidate Gene analysis for A2 QTL on Soy Genome Browser (www. Soybase.org) showing acetyl-CoA carboxylase (accC-3) gene, nuclear gene for chloroplast product (Chapter 2.0)

Table A1. Weight of leaflets used for flavonoid analysis (Chapter 4.0)

ID	Sample #	Leaflet+ tube (g)	leaflet only (g)
906-D-1	3	14.7	0.9
906-D-2	7	14.5	0.7
906-D-3	11	14.5	0.7
LD05-D-1	15	14.2	0.4
LD05-D-2	19	14.4	0.6
LD05-D-3	23	14.5	0.7
S1-D-1	25	14.6	0.8
S1-D-2	30	14.5	0.7
S1-D-3	33	14.4	0.6
S2-D-1	39	14.3	0.5
S2-D-2	43	14.4	0.6
S2-D-3	47	15	1.2
R1-D-1	49	14.9	1.1
R1-D-2	53	14.5	0.7
R1-D-3	57	14.3	0.5
R2-D-1	61	14.8	1
R2-D-2	65	14.8	1
R2-D-3	69	14.4	0.6
906-ND-1	1	14.8	1
906-ND-2	5	14.8	1
906-ND-3	9	14.9	1.1
LD05-ND-1	13	14.6	0.8
LD05-ND-2	17	14.6	0.8
LD05-ND-3	21	14.6	0.8
S1-ND-1	26	14.6	0.8
S1-ND-2	29	14.5	0.7
S1-ND-3	35	15	1.2
S2-ND-1	37	14.5	0.7
S2-ND-2	41	14.3	0.5
S2-ND-3	45	14.7	0.9
R1-ND-1	51	14.7	0.9
R1-ND-2	55	14.7	0.9
R1-ND-3	59	14.5	0.7
R2-ND-1	63	14.8	1
R2-ND-2	67	14.8	1
R2-ND-3	71	14.9	1.1

Table A2. Abundance (Peak area measured) of six flavonoids, their sugar conjugates, and aglycones on damaged leaflets of six soybean lines

Compound	E06906	LD05-16060	Res._1	Res._2	Sus._1	Sus._2
Quercetin O-hexoside-malonylated (I,II)	8580 ab	1119b	1726b	1139b	11198a	8978ab
Daidzein O-hexoside (I,II)	3590a	5460a	8982a	2917a	5585a	7810a
Glycitein O-hexoside malonylated (I,II,III,IV)	17623a	9905a	49258a	8623a	20416a	53673a
Naringenin O-hexoside	10493bc	27633a	6933c	17367ac	21733ab	14950bc
Naringenin O-hexoside malonylated	21167c	67667a	54200ab	50500ab	44533abc	31433bc
Genistein	1450c	42377a	3663abc	2910abc	15967c	4133ab
Genistein O-hexoside	80997b	214217a	96083b	126233b	56723b	90088b
Genistein O-hexoside malonylated (I,II)	242,817a	470,500a	582,667a	320,900a	338,083a	413,817a
Genistein O-dihexoside	57000b	617,667a	201,333a	655,667a	67967b	45300b
Genistein O-hexosyl-pentoside (I,II)	12473a	15903a	8940a	13137a	13450a	13736a

Table A2(cont'd)

Kaempferol O-hexoside (I,II,III)	25353c	71655ab	55233bc	106711a	19098c	34914bc
Kaempferol O-hexoside malonylated	19003a	10810a	27877a	9701a	32613a	54003a
Kaempferol O-rhamnoside O-hexosyl-rhamnoside	375,000b	2,310,000a	1,193,6667a	2,583,333a	408,000b	408,333b
Kaempferol O-hexosyl-rhamnoside (I,II)	139,900b	1,418,333a	720,666a	1,460,000a	158,116b	169,633b
Kaempferol O-rhamnoside O-hexosyl-rhamnoside	31533ab	4123b	61063a	4067b	54800ab	46217ab
Kaempferol aglycone	6667a	1700b	567b	433b	2400b	2900b
Glycitein aglycone	79867b	601667a	365667ab	282667ab	59300b	415600a
Diadzein aglycone	7203a	13813a	12587a	3950a	4917a	12543a

*Means followed by the same letter are not significantly different within a compound in rows.

Table A3. Abundance (Peak area measured) of six flavonoids, their sugar conjugates, and aglycones on un-damaged leaflets of six soybean lines

Compound	E06906	LD05-16060	Res._1	Res._2	Sus._1	Sus._2
Quercetin O-hexoside-malonylated (I,II)	17350a	1232b	1214b	1192b	2316b	3107b
Daidzein O-hexoside (I,II)	8718a	10556a	6359a	2300a	2150a	1877a
Glycitein O-hexoside malonylated (I,II,III,IV)	98558a	29759b	24558b	5520b	5777b	3615b
Naringenin O-hexoside	7193b	9730b	8700b	28187a	12430b	13600b
Naringenin O-hexoside malonylated	22067b	44713b	43800b	53200a	23543b	18567b
Genistein	2357a	3137a	2169a	3667a	3179a	1807a
Genistein O-hexoside	34957b	110,235a	105,783a	127,450a	53100b	98115a
Genistein O-hexoside malonylated (I,II)	517,533a	348,333ab	359,483ab	227,517ab	117,133b	103,150b
Genistein O-dihexoside	48533b	287667a	295567a	603667a	38203b	96133b
Genistein O-hexosyl-pentoside (I,II)	23733a	10571b	10278b	11238b	7648b	13737b

Table A3 (cont'd)

Kaempferol O-hexoside (I,II,III)	28,367b	43,841b	49,333b	103,644a	20,374b	21,510b
Kaempferol O-hexoside malonylated	87617a	17430b	12257b	5440b	4765b	2372b
Kaempferol O-rhamnoside O-hexosyl-rhamnoside	176,500a	932,167b	722,050b	1,361,667b	119,233b	139,550b
Kaempferol O-hexosyl-rhamnoside (I,II)	342,000b	1,323,000a	1,123,333a	2,470,000a	287,500b	618,333b
Kaempferol O-rhamnoside O-hexosyl-rhamnoside	49550a	3496b	16625ab	3757b	41050a	33067ab
Kaempferol aglycone	2333a	233b	467b	267b	1767ab	5767a
Glycetin aglycone	69300b	226,133a	297,667a	294,333a	215,233ab	151,000ab
Diadzein aglycone	12667a	4620ab	9193ab	3379ab	5033ab	1770b

*Means followed by the same letter are not significantly different within a compound in rows.