CLONING AND USING A QTL FOR INSECT RESISTANCE IN SOYBEAN

By

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(Under the Direction of Wayne Parrott)

ABSTRACT

Soybean is one of the world's most important feed crops. Leaf damage caused by insects reduces yield and seed quality. Development of plants that resist insect damage is the most economical and environmentally sustainable way to control insects. Resistance to leaf-chewing insects is available in the soybean accessions PI 229358 and PI 227687; it is conferred by the quantitative trait loci QTL-M, QTL-H, and QTL-G in PI 229358, and QTL-E in PI 227687. Chapter two details the characterization of novel combinations of insect resistance QTLs, ME and MGHE, that provide high levels of resistance against leaf-chewing insects, and the combination of ME with Bt (*cry1Ac*) that enhances the effect of Bt. Chapter three describes the development of the elite germplasm lines Benning-ME and Benning-MGHE. This chapter provides the graphical genotypes used to determine the QTL introgressions in each NIL, and KASP markers for marker-assisted selection of QTLs. Chapter four covers the evaluation of insect-resistant soybean meal for broiler chickens. In a 21-day feeding trial, the performance broiler was equivalent for Benning, Benning^{M}, and Benning^{MGH} diets. There is no indication that meal produced from soybean seed carrying QTL-M, QTL-G, and QTL-H would not be as safe as the insect-susceptible Benning soybean cultivar when used for animal feed. Chapter five details the cloning and validation of *GmORUGA*, the gene for QTL-M, which is by far the major determinant of leaf-chewing

insect resistance in soybean. Resistance corresponds to a mutation of TG**G** (^{275}W) to TG**A** (stop), which leads to a truncated protein. Resistance is achieved by the loss-of-function of *GmORUGA*. Complementing a QTL-M resistant line with the susceptible *GmORUGA* allele restores susceptibility, and silencing *GmORUGA* in susceptible lines results in resistance. *GmORUGA* is a genistein 7-O-glucosyltransferase induced after caterpillar damage. The functional enzyme in susceptible soybean contributes to maintaining a constant concentration of genistin after insect attack. This enzyme is not active in QTL-M plants, and genistin is reduced and condensed tannins (CT) increases, presumably through redirection of metabolic flux to produce CTs in lieu of genistein. This is the first report of an insect resistance gene that operates via the isoflavone pathway.

INDEX WORDS: Insect Resistance, Soybean, Leaf-chewing Insects, Antixenosis, Antibiosis, Quantitative Trait Loci, QTL Pyramids, *cry1Ac*, Near Isogenic Lines, QTL-M, *GmORUGA*, genistein glucosyltransferase, condensed tannins

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INTRODUCTION AND LITERATURE REVIEW

Introduction

Soybean [*Glycine max* (L.) Merr.] production is limited by pests. Worldwide, 11% of the crop is lost to animal pests, including insects (*1*). In the USA, the leaf-chewing insects corn earworm [CEW, *Helicoverpa zea* (Boddie)], soybean looper [SBL, *Chrysodeixis includens* (Walker)], velvetbean caterpillar [VBC, *Anticarsia gemmatalis* (Hübner)] and bean leaf beetle, [*Cerotoma trifurcata* (Forster)] are among the most important insect pests of soybean (*2*). Leaf-chewing insects are capable of defoliating soybean plants entirely. Although soybean can withstand moderate levels of leaf damage, high levels of defoliation greatly reduce seed yield and quality (*3*). In 2013, the southern states of Alabama, Arkansas, Louisiana, Mississippi, North Carolina, Tennessee, and Virginia; harvested just 13.6% of the U.S. supply; yet farmers in these states spent $262 million on insect control, and despite the control efforts, yield losses to insects amounted to $234 million. Thus the combined costs of insect control and yield loss were equivalent to $500 million (*4*). The amount of insecticides applied to soybeans in the USA have quadrupled between 2002 and 2012, and as a result the soybean's impact to freshwater ecotoxicity has increased by 3-fold (*5*).

Developing insect-resistant cultivars is a cost-effective and environmentally friendly method to control insect pests. Soybean accessions resistant to leaf-chewing insects have been available since the late 1960s (*6*).

Abbreviations:

Bt: δ-endotoxin

CEW: corn earworm (*Helicoverpa zea*)

Cry: crystal protein from *Bacillus thuringiensis*

EIL: economic injury level

ET: economic threshold

HPR: host-plant resistance

IPM: integrated pest management

LG: linkage group

LOXs: lypoxigenases

MBB: Mexican bean beetle

NIL: near-isogenic line

OP: organophosphates

PIs: plant introductions

PPOs: polyphenol oxidases

QTL: Quantitative trait locus

RFLP: restriction fragment length polymorphism

SBL: soybean looper (*Chrysodeixis includens*)

SSR: simple sequence repeat

TBW: tobacco budworm

VOCs: volatile organic compounds

However, deployment of the resistance genes found in these accessions into commercial soybean cultivars has been hampered by a lack of understanding of the genetic basis of resistance to most insects, in addition to the difficulty of developing insect-resistant cultivars that yield equivalently to the existing cultivars (*7*).

Impact of insect pests in crop production

Plants and insects have coexisted for the last 350 million years, if the earliest form of both plants and insects are considered (*8*). Fossils of plant leaves from the Permian period showing insect-damage are evidence that plants and insects have been in competition for about 270 million years (*9*). According to the coevolutionary theory of Ehrlich and Raven (*10*), insect feeding on plants has been a major factor in increasing species diversity in both insect herbivores and their plant hosts.

Approximately two-thirds of all known plant-feeding insects are leaf-eating coleopteran or lepidopteran species that have evolved mouthparts for chewing, snipping or tearing tissue (*11*). Alternatively, piercing-sucking insects, such as thrips and true-bugs, use a tube-like mouthpart to syphon the liquid content of wounded cells. Leafminer insects develop in, and feed on soft tissue between epidermal cell layers. Aphids, whiteflies, and leafhoppers are able to insert a specialized stylet between cells, then establishing a feeding site in the phloem (*12*).

From the beginning of agriculture, pests have been a major problem for crop production. Crop plants have had to compete with animal pests, plant pathogens and weeds (*1*). Despite an approximate annual investment of $40 billion in the worldwide application of pesticides, and the use of biological and non-chemical control methods, pests cause pre-harvest loss of 35% to 42% of potential crop production. Insect pests are responsible for 14%

of this loss (*13*). In the USA, annual crop losses to pests are about 37%, of which 13% is due to insects. Unfortunately, in the last 50 years, despite a more than 10-fold increase in the amount and toxicity of synthetic insecticides, the percent of yield lost to insects has nearly doubled (*14*).

Mechanisms of host-plant resistance to insects

From an agricultural perspective, insect resistance refers to individual genotypes, which suffer less damage from certain insects than other genotypes of that crop. Insect resistance is an integral part of integrated pest management programs (IPM) (*15*). Three categories of host-plant resistance to insects (HPR) have been described; non-preference, antibiosis, and tolerance (*16*). Antibiosis is a type of resistance in which the plant has a detrimental effect on insect growth, development, and/or reproduction; this can be manifested as decreased growth rate, lower larval weights, decreased fitness, and other factors that limit the insect's survival. Non-preference was renamed as antixenosis by Kogan and Ortman (*17*), and is a type of resistance in which the plant affects insect behavior by discouraging oviposition, colonization, or feeding.

The underlying traits conferring antixenosis can be morphological (e.g. dense pubescence) or biochemical (presence of a deterrent, or absence of an attractant. Tolerance refers to the ability of a plant to grow, reproduce, and yield normally despite supporting a density of insects that would otherwise cause significant damage in a susceptible plant. Plant defenses can be also be indirect, in association with a third species, such as recruitment of natural enemy (i.e. parasitoid or predator) of insect pests by production of volatile organic compounds (VOCs) (*18*). Direct plant defenses can be classified as physical/morphological and biochemical traits. Physical/morphological defenses are usually the first feeding barrier

encountered by herbivores; this category includes structural traits such as leaf surface wax, thorns (spinescence), trichomes (pubescence), increased cell-wall thickness, lignification, suberization, divaricated branching, incorporation of granular minerals into plant tissue, and toughened leaves (sclerophylly). The latter trait plays an active role in reducing the palatability and digestibility of tissue, therefore reducing leaf damage (*19*). Pubescence plays an important role in plant defenses against many insects. Trichome density affects herbivory mechanically, by interfering with the movement of insects on the plant surface and reducing the access to the leaf epidermis for oviposition and feeding (*20*). Glandular trichomes act as combination of physical and chemical defenses, as they can secrete secondary metabolites, including flavonoids, terpenoids, and alkaloids, which can be poisonous, repellent, or act as a trap (*21*).

Biochemical defenses include compounds involved in resistance and susceptibility to insect pests. Defensive secondary metabolites are compounds that do not affect the normal growth and development of the plant, but can reduce the palatability of plant tissue (*12*). These compounds can be constitutively stored as inactive forms (phytoanticipins), or induced in response to insect and microbial attacks (phytoalexins). During herbivory, phytoanticipins are usually activated by β-glucosidase, which then mediates the release of biocidal aglycone metabolites (*22*). The classical example of phytoanticipins exists in crucifers, where glucosinolates are hydrolyzed by myrosinases during tissue disruption to produce isothiocyanates (*23*). Phytoalexins include isoflavonoids, terpenoids, and alkaloids that affect insect survival (*24*). The classical example of phytoalexins exists in maize, where high concentration of maysin, a C-glycosyl flavone, provides resistance (antibiosis) to corn earworm (*25*).

Secondary metabolites have been widely studied for their role in direct plant defenses; however most of the signaling pathways are yet to be identified. Study of secondary metabolite profiles coupled with gene-expression analysis could lead to the identification of novel signaling molecules involved in plant resistance against to herbivores (*26*). Among the secondary metabolites, flavonoids and tannins have been widely studied in plant-insect interactions.

Flavonoids protect plants against pests by influencing insects' behavior, growth, and development (*27*). More than 5000 flavonoids have been reported in plants; they are divided into anthocyanins, flavones, flavonols, flavanones, dihydroflavonols, chalcones, aurones, flavan, and proanthocyanin. From these, flavonols, flavones, proanthocyanidins, flavan-3-ols, flavonones, flavans, and isoflavonoids have been studied as feeding deterrents against many insect pests (*28*). Flavanones such as 5-hydroxyisoderricin,7- methoxy-8- (3-methylbutadienyl) and 5-methoxyisoronchocarpin isolated from *Tephrosia villosa* (L.), *T. purpurea* (L.), and *T. vogelii* Hook, respectively are feeding deterrents against *Spodoptera exempta* (Walk.) and *S. littoralis* Bios (*29*). In arabidopsis, overexpression of a transcription factor controlling flavonoid production has been reported to confer resistance against *S. frugiperda* (J.E. Smith) (*30*). The isolated isoflavonoids judaicin, judaicin-7-*O*-glucoside, 2-methoxyjudaicin, and maackiain, from wild relatives of chickpea, act as antifeedants against *Helicoverpa armigera* (Hubner), *S. littoralis,* and *S. frugiperda* (*31*).

The role of tannins in plant defense against various abiotic and biotic stresses, and their response to insect damage has been studied in many plants species (*32*). Tannins are astringent and bitter polyphenols that act as feeding deterrents to many insect pests, affecting insect growth and development by binding to proteins, reducing nutrient absorption efficiency, and causing midgut lesions (*21, 32*). Tannins precipitate proteins nonspecifically

(including digestive enzymes in herbivores). They also chelate the metal ions, reducing their bioavailability to herbivores. When insects ingest tannins, these reduce protein digestibility, and therefore nutritive value of plant tissue. Condensed tannins, also known as proanthocyanidins, have diverse structures and functions. In leaves of English oak [*Quercus robur* (L.)], (+) -catechin, (+) - gallocatechin, and vanillin inhibit the winter moth larvae [*Operophtera brumata* (L.)] (*33*). In groundnut [*Arachis villosulicarpa* (L.)], procyanidin polymers act as feeding deterrent to the cowpea aphid (*Aphis Craccivora* Koch) (*34*). Condensed tannins from Alaska paper birch reduce pupal weight and survival of the spear-marked black moth [*Rheumaptera hastata* (L.)] larvae (*35*). In trembling aspen (*Populus tremuloides* Michx.), the enzyme dihydroflavonol reductase involved in the tannin branch of the phenylpropanoid pathway, is induced by feeding by the forest tent caterpillar (*Malacosoma disstria* Hübner), and the satin moth [*Leucoma salicis* (L.)]; Tannins are systemically induced in neighboring leaves of a damaged plant (*36*). Insects can tolerate tannins by hydrolyzing them rapidly, or avoid any damaging effects by restricting the passage of tannins, by adsorbing them on the thick peritrophic membrane, and by inhibiting tannin-protein complex formation via surfactants in the midgut (*37*).

Plant defensive proteins, such as lectins, proteinase inhibitors (PIs), polyphenol oxidases (PPOs), and lipoxygenases (LOXs), are important components of plant resistance to insects, as they are capable of disrupting the insects' nutrition; these enzymes have been thoroughly studied in different plant-insect interactions, and are reviewed by War et al. (*26*).

Attempts to link biochemistry to genetics has proceeded slowly. Thirty quantitative trait loci (QTLs) or single genes for resistance to insects have been identified in major crop species (*38, 39*); however an understanding of the molecular basis for insect resistance is lagging, relative to that achieved for genes that confer resistance to plant pathogens. Of these

insect resistance genes, one of the best characterized is the *Mi* gene from tomato, which confers resistance to root-knot nematode (*Meloidogyne incognita*), potato aphid (*Macrosiphum euphorbiae* Thomas), and whiteflies (*Trialeurodes vaporariorum*)(*40*). This resistance (R) gene belongs to the nucleotide-binding site-leucine-rich repeat family of R genes, which includes many genes involved in the classic gene-for-gene interaction between plant and pathogens. Resistance conferred by R genes is usually highly specific, and the *Mi* gene provides greater resistance to European isolates of potato aphid than to North American isolates (*41*). In contrast, much less is known about genes that confer resistance to chewing insects, even though these tend to cause the greatest amount of crop loss.

Soybean

The genus *Glycine* Willd. is a member of the Fabaceae, subfamily Papilionodeae, and tribe Phaseoleae. *Glycine* is further divided into two subgenera *Glycine* (perennial) and *Soja* (annual). The subgenus *Soja* includes the cultivated soybean [*G. max* (L.) Merr.], and its wild relative *G. soja* Sieb. and Zucc. It is widely believed that cultivated soybean was domesticated from *G. soja* which is native to eastern Asia. *G. soja* has a viney growth habit, and produces small black seeds in pods that shatter, whereas *G. max* has an upright plant habit, and produces yellow or black seeds in pods that usually do not shatter. Both species produce small, cleistogamous flowers (*42*).

Production and uses

Soybean is the world's most widely used legume crop. In 2015, the world soybean production was estimated at 320.2 million metric tons (*43*). The USA is the largest producer, harvesting 34% of the world's production, with a value estimated at $40.3 billion. Forty-five

percent of the USA production is exported (*44*). The second largest soybean producer is Brazil (29%), followed by Argentina (19%), China (6%), India (4%), Paraguay (3%), Canada (2%), and other countries (4%) (*45*).

Soybean seeds are an important source of nutrition for humans and animals, as they are composed of approximately 40% protein, 21% oil, 34% carbohydrate, and 4% ash. About 6% of the soybean is used in traditional foods such as soy sauce, soymilk, soy paste, tempeh, miso, tofu, and natto. Approximately 85% of the world's soybean crop each year is processed into soybean meal and seed oil. Two percent of the soybean meal is used for human consumption, with the remaining 98% used in animal feed. The seed oil is primarily used to for human consumption. Fifty-five percent is used as cooking and salad oil, 24% as baking and frying fats and oils, 4% as ingredient in margarine, 7% for food and industrial uses, and 11% as substrate for biodiesel (*46*).

History of soybean in the USA

Soybean was introduced to North America in 1765 by Samuel Bowen. Bowen, a former sailor who had traveled to China, brought the Chinese vetches (soybean) seeds with him to the port of Savannah in colonial Georgia. Henry Yonge, the Surveyor General of Georgia planted the seeds on his plantation, per Bowen's request (*47*). Bowen started planting soybean on his property on Thunderbolt, GA since 1766, and in 1767 he received a Royal Patent to produce soy sauce from the soybeans grown in America. The word "soybean" was most probably coined by Dr. James Mease in 1804, to refer to the bean from which soy sauce was produced (*48*).

Throughout the late 1700s and 1800s, several soybean introductions to the USA occurred. In this period only eight soybean cultivars were grown in small trials across the country, and the occasional crop was grown for commercial-scale hay or forage (*49*), through the early 1900s. Due to cottonseed scarcity and high prices in 1915, the US-grown soybean seeds were crushed for oil and protein meal for the first time in Elizabeth City, North Carolina (*50*). In 1941 was the first year that the area of soybean harvested for seeds exceeded the land harvested for forage in the USA. By the late 1940s and early 1950s, the USA had become a world leader in soybean production (*51*).

Management of leaf-chewing insects pests

The primary method of controlling insect pests of crops worldwide since the 1940s has been through the use of synthetic chemical insecticides. However, the extensive use of chemicals insecticides in agriculture through the mid-1960s resulted in insecticide-resistance, resurgence of primary pests, upsurges of secondary pests, and environmental contamination (*52*). The overuse of pesticides was brought to public attention in 1962 by the controversial publication, *Silent Spring* (*53*), thus helping the promotion of the integrated control concept that has since then come to be known as integrated pest management (IPM). IPM is the integrated approach to manage pest populations below economic injury levels (EIL) by utilizing all suitable techniques and methods (*52*). IPM is not limited to insect pests; it also refers to the control of weeds, pathogens, and non- arthropod animals. However, the concept was founded in an entomological context and is used most often in reference to insect management.

IPM practices entail systematic scouting for crop growth, crop damage, pest development, and natural predators, as well as the utilization of EILs to determine proper

actions during pest outbreaks. The development of EILs led to the concept of economic thresholds (ETs) by Stern et al. (*54*) as a means to reduce the amount of applied insecticides and promote a framework for sustainable agriculture; ETs utilized in conjunction with population monitoring have made chemical insecticide application more effective, economical, and compatible with other management tactics.

Soybean grown in the southern USA is more likely to be damaged by insects, than soybean grown in the more temperate regions of the Midwest. This is due to longer growing seasons, which allow multiple insect generations per year; and higher winter temperatures, which allow insects to overwinter until the next growing season. According to the most recent update for soybean insect losses in the southern USA, the most important insect-pests of soybeans in 2013, in both in terms of control costs and yield losses were the CEW, SBL, and the stink-bug complex formed by the green [*Acrosternum hilare* (Say)], southern green [*Nezara viridula* (L.)], the brown [*Euschistus servus* (Say)], and the brown marmorated stink bug [*Halyomorpha halys* (Stål)] (*4*). Yield losses due to the most recently introduced kudzu bug [*Megacopta punctatissima* (Fabricius)] are estimated to be up to 60%, for heavily infested plants (*55*).

In the past, organophosphorous (OPs) and carbamate compounds were routinely utilized to control pest outbreaks in soybean. Although OPs are considered the most cost-effective compounds for producers, a shift to pyrethroids and other new chemistries led to the reduction of insecticides' environmental impact, since these new insecticides are effective at much lower doses (*2*). Resistance to pyrethroid insecticides has been documented in SBL in areas where soybean and cotton have been grown in close proximity (*2*). Unfortunately the amount of insecticides applied to soybeans in the USA has quadrupled between 2002 and 2012, and as a result soybean's impact to freshwater ecotoxicity has increased by 3-fold (*5*).

Planting insect-resistant cultivars is crucial to reduce production costs, and the frequency of insecticide applications (*7*).

Planting insect-resistant soybean can also be an integral part of an IPM program. IPM programs are used to control leaf-chewing insects in soybean. Although soybean plants can withstand moderate levels of leaf damage, a high level of defoliation greatly reduces seed yield and quality (*3*). The efficient use of insecticide applications depends on ETs, which are based on percent of defoliation and are used to monitor insect populations in the field to prevent them from reaching levels that may cause economic losses. The suggested ETs for leaf-chewing insects in soybean are 35% defoliation during the vegetative stages and 20% defoliation during the reproductive stages (*56*).

Insect-resistant soybean accessions

Discovery of soybean resistance to insects can be divided in two main events (*57*). The first event was 82 years ago, with the serendipitous discovery that plant pubescence conferred resistance to the potato leafhopper [*Empoasca fabae* (Harris)] (*58, 59*). Incorporating this trait into commercial soybean cultivars resulted in population suppression, which relegated the potato leafhopper insect to non-pest status. The second event occurred 46 years ago, when Van Duyn et al. (*6, 60*) screened a set of accessions from the soybean germplasm collection for resistance to Mexican bean beetle [MBB, *Epilachna varivestis* Mulsant]; at that time, MBB infestations were severely damaging the soybean production in South Carolina. In this screening, three plant introductions (PIs) from Japan, PI 229358 ("Sodendaizu"), PI 171451 ("Kosamame"), and PI 227687 ("Miyako White") (*61*) were found to be highly resistant to MBB. Resistance was measured as number of egg masses per plant, larval weight, adult number, and levels of damage (*6, 60*). These PIs became the soybean

models for research on insect resistance inheritance, resistance mechanisms, compatibility of plant resistance with IPM management, and genotype by environment interactions. Besides their resistance to MBB, these PIs also have been reported to be resistant to multiple coleopteran, lepidopteran, and hemipteran insects that are major economic pests of soybean worldwide (*62-75*).

Since the PIs' discovery, they were frequently used as donor parents in breeding programs in the USA and other countries to develop insect-resistant cultivars (*57*). By the mid 1980s, soybean-breeding programs in 10 USA states were using one or more of the PIs (*76*). Unfortunately, it was difficult to capture the PIs' levels of resistance in progenies derived from crosses with adapted high-yielding germplasm. For instance, Hatchett et al. (*77*) demonstrated that only two of five PI 229358-derived breeding lines, selected for resistance to MBB and SBL were as resistant as PI 229358 to CEW and tobacco budworm [TBW, *Heliothis virescens* (Fabricius)]. Similarly, Smith and Brim (1979 a,b) found that lines derived from either PI 229358 or PI 227687 and selected for resistance to MBB, were not very effective at deterring CEW (*78, 79*). Kilen and Lambert (*80*) found F_3 lines derived from crosses between the PIs that exhibited transgressive segregation for both susceptibility and resistance, which suggested that these three PIs have at least some different genes for insect-resistance.

It was also difficult to capture the yield potential and agronomic qualities of the elite parent in crosses between these PIs and the elite parents. Although numerous breeding lines with insect resistance derived from these PIs have been released from breeding programs as resistance sources, only three resistant cultivars have been released: 'Lamar', 'Crockett', and 'Lyon'. None of these cultivars could compete agronomically with the best contemporary cultivars (*7, 57, 81-83*).

Research on the inheritance of insect resistance from PI 171451, PI 227687, and PI 229358 indicated a quantitative inheritance, involving a few major genes (*84, 85*). The availability of molecular markers made it possible to identify the number of insect-resistance QTLs for each PI, their contribution to resistance, and their locations in the soybean genome.

Insect resistance in PI 229358

Rector et al. (*86-88*) initially used RFLPs to find a major QTL for resistance on Linkage Group (LG) M (now chromosome 7) of PI 229358 and PI 171451, and referred to it as QTL-M. In mapping populations derived from crosses between each of the PIs and the susceptible cultivar Cobb, QTL-M is associated with both antixenosis and antibiosis resistance to CEW, accounting for 37% of the observed variance for antixenosis, and for 22 to 28% of the variance for antibiosis. This same region of chromosome 7 has been associated with resistance to the common cutworm [CCW, *Spodoptera litura* (Fabricius)] in another genotype, PI 594177 (*67, 89-92*).

QTLs on other chromosomes also are associated with the resistance of PI 171451 and/or PI 229358 to CEW. QTL-H (now chromosome 12) conditions antixenosis in PI 229358 and PI 171451, with R^2 values ranging from 9 to 19% (*88*). QTL-G (now chromosome 18) conditions antibiosis (R^2 = 19%) in PI 229358 (*88*). However, QTLs G and H are only "active" if QTL-M is present in the genome. In a set of eight BC_5F_2- derived lines, Zhu et al. (*93*) determined that the main effect of QTL-H (antixenosis) and QTL-G (antibiosis) are not significant ($P > 0.05$) when the QTL-M locus allele corresponds to the susceptible background; lines carrying the PI229358 allele at QTL-M are more resistant if the PI 229358 alleles are present at QTL-H or QTL-G. The locations of QTL-H and QTL-

M were confirmed more precisely using simple sequence repeat (SSR) markers in combination with the earlier RFLP data (*93, 94*).

Insect resistance in PI 227687

Using SSR markers, Hulburt (*95*) identified a major insect-resistance QTL in a mapping population from a PI 227687 x 'Cobb' cross. This QTL (QTL-E) on LG E (now chromosome 15) of PI 227687 conveys both antibiosis and antixenosis. QTL-E co-maps with the *Pb* locus that controls sharp (*Pb_*) vs. blunt (*pbpb*) leaf pubescence in soybean (*96*). Although there are earlier reports on the effect of pubescence traits on soybean resistance to insects (*97-99*), this is the first report that a sharp-trichome locus co-localizes with an insect resistance QTL. Hulburt et al. (*100*) confirmed that sharp-trichome near-isogenic lines (NILs) from 'Clark' and 'Harosoy' are more resistant to lepidopterans as compared to the blunt-trichome cultivars. Nevertheless, given that Lambert and Kilen (*68*) showed that PI 227687's resistance is graft-transmissible, it remains possible that resistance is really due to an as of yet unidentified gene linked to *Pb*.

Pyramiding insect-resistance QTLs

Pyramiding is a strategy whereby multiple desirable genes for the same trait are combined into a single genetic background (*101*). This strategy can be used in the development of insect-resistant cultivars; it permits genes with different modes of action to be combined to obtain more durable resistance. To determine the effects of the insect-resistance QTLs from PI 229358 on the effectiveness of Bt. Jack^{cry1Ac} (soybean cultivar Jack engineered with Bt) (*102*) and PI229358 were crossed, followed by two backcrosses to Jack^{cry1Ac}. Jack^{cry1Ac} expresses a synthetic *cry1Ac* transgene and under field conditions is resistant to CEW and

VBC (*103*). SSR markers were used for marker-assisted backcrossing to obtain individuals carrying the QTL regions from PI229358; F_3-derived lines with QTL-M and/or *cry1Ac* were identified and evaluated for resistance (*104*). SBL larvae feeding on leaves from the line with both *cry1Ac* and QTL-M resistance genes had significantly lower weights than larvae feeding on plants with either resistance gene alone. This demonstrates that QTL-M enhances the effectiveness of Bt; the results are very relevant because SBL is not as sensitive to *cry1Ac* as CEW and VBC (*104*). Other BC_2F_3 lines with *cry1Ac*, QTL-H, and QTL-M in all possible combinations were developed in a similar fashion from the same cross, and were tested for 2 years in the field for resistance to CEW and SBL. These field trials confirmed the ability of QTL-M to enhance the effectiveness of *cry1Ac*. In addition, the plants were tested with the YHD2 strain of tobacco budworm (TBW), which was selected for its resistance to *cry1Ac* (*105*). As expected, YHD2 caterpillars were not affected by *cry1Ac* soybean, but the same strain feeding on *cry1Ac* soybean with QTL-M suffered detrimental effects, thus illustrating the importance of additional, unrelated genes to enhance the effectiveness of Bt in soybean (*106*). Overall, pyramiding Bt with genes with other modes of action is considered an effective strategy for durable resistance to insects (*105, 107, 108*).

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CHAPTER 2

PYRAMIDS OF QTLs ENHANCE HOST-PLANT RESISTANCE AND BT-MEDIATED RESISTANCE TO LEAF-CHEWING INSECTS IN SOYBEAN

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Abstract

Plant resistance to leaf-chewing insects minimizes the need for insecticide applications, reducing crop production costs and pesticide concerns. In soybean [*Glycine max* (L.) Merr.], resistance to a broad range of leaf-chewing insects is found in PI 229358 and PI 227687. PI 229358's resistance is conferred by three quantitative trait loci (QTLs): M, G, and H. PI 227687's resistance is conferred by QTL-E. The letters indicate the soybean Linkage groups (LGs) on which the QTLs are located. This study aimed to determine if pyramiding PI 229358 and PI 227687 QTLs would enhance soybean resistance to leaf-chewing insects, and if pyramiding these QTLs with Bt (*cry1A*c) enhances resistance against Bt-tolerant pests. The near-isogenic lines (NILs): Benning^{ME}, Benning^{MGHE} , and Benning$^{ME+cry1Ac}$ were developed. Benning^{ME} and Benning^{MGHE} were evaluated in detached-leaf and greenhouse assays with soybean looper [SBL, *Chrysodeixis includens* (Walker)], corn earworm [CEW, *Helicoverpa zea* (Boddie)], fall armyworm [FAW, *Spodoptera frugiperda* (J.E. Smith)], and velvetbean caterpillar [VBC, *Anticarsia gemmatalis* (Hübner)]; and in field-cage assays with SBL. Benning$^{ME+cry1Ac}$ was tested in detached-leaf assays against SBL, VBC, and Southern armyworm [SAW, *Spodoptera eridania* (Cramer)]. In the detached-leaf assay, Benning^{ME} showed the strongest antibiosis against CEW, FAW, and VBC. In field-cage conditions, Benning^{ME} and Benning^{MGHE} suffered 61% less defoliation than Benning. Benning$^{ME+cry1Ac}$ was more resistant than Benning^{ME} and Benning^{cry1Ac} against SBL and SAW. Agriculturally relevant levels of resistance in soybean can be achieved with just two loci, QTL-M and QTL-E. ME+*cry1Ac* could present an opportunity to protect the durability of Bt genes in elite soybean cultivars. These results should assist the development of effective pest management strategies, and sustainable deployment of Bt genes in soybean.

Abbreviations:

bp: base pair

Bt: *Bacillus thuringiensis*

Chr: chromosome

cM: centimorgans

CEW: corn earworm (*Helicoverpa zea*)

FAW: fall armyworm (*Spodoptera frugiperda*)

IPM: integrated pest management

LG: linkage group

PI: plant introduction

QTL: quantitative trait locus

SBL, soybean looper (*Chrysodeixis includens*)

SSR: simple sequence repeat

SNP: single nucleotide polymorphism

VBC: velvetbean caterpillar (*Anticarsia gemmatalis*)

SAW: southern armyworm (*Spodoptera eridania*)

Introduction

The production of soybean [*Glycine max* (L.) *Merr*], one of the world's primary sources of vegetable oil and protein (*1*), is often limited by pests. Worldwide, 11% of the crop is lost to animal pests, including insects (*2*). In the USA, the insect pests causing the most impact are: corn earworm [*Helicoverpa zea* (Boddie)], soybean looper [*Chrysodeixis includens* (Walker)], velvetbean caterpillar [*Anticarsia gemmatalis* (Hübner)], bean leaf beetle, [*Cerotoma trifurcata* (Forster)], green stink bug [*Chinavia hilaris* (Say)], and southern stink bug [*Nezara viridula* (L)] (*3*). The corn earworm (CEW), soybean looper (SBL), velvetbean caterpillar (VBC), and bean leaf beetle are chewing insects capable of defoliating plants entirely. Although soybean plants can withstand moderate levels of leaf damage, high levels of defoliation greatly reduces seed yield and quality (*4*). The efficient use of insecticide applications depends on economic thresholds (ETs), which are based on percent of defoliation and are used to monitor insect populations to prevent them from reaching levels that may cause economic losses. The suggested ETs for leaf-chewing insects in soybean are 35% defoliation during the vegetative stages and 20% defoliation during the reproductive stages (*5*).

A third of the world's soybean crop was produced in the USA in 2013 (*6*). The southern states of Alabama, Arkansas, Louisiana, Mississippi, North Carolina, Tennessee, and Virginia; harvested just 13.6% of the U.S. supply; yet farmers in these states spent $262 million on insect control to produce a $5 billion crop. Despite the control efforts, yield losses to insects amounted to $234 million. Thus the combined costs of insect control and yield loss were equivalent to $500 million. CEW, SBL, and stink bugs were the most important species, both in terms of control costs and yield losses (*7*). The need to lower cost of production, along with increased concern over insecticide residues in the food chain and environment are incentives to develop insect-resistant cultivars to use in integrated pest

management (IPM) strategies. However, these efforts have been hampered by a lack of understanding of the genetic basis of resistance to most insects, in addition to the difficulty of developing insect-resistant cultivars that yield equivalently to the existing cultivars (*8*).

The Japanese soybean landraces 'Kosamame' (PI 171451), 'Miyako White' (PI 227687), and 'Sodendaizu' (PI 229358) are the most widely used sources of resistance to defoliating insects (*9*). They were initially discovered to be resistant to Mexican bean beetle [*Epilachna varivestis* (Mulsant)] by Van Duyn (*10, 11*). They also have been reported to be resistant to multiple coleopteran, lepidopteran, and hemipteran insects that are major economic pests of soybean worldwide (*12-25*).

Resistance to defoliating insects in PI 171451, PI 227687, and PI 229358 is conferred via both antibiosis and antixenosis (*26, 27*). Antibiosis is a type of resistance in which the plant has a detrimental effect on insect growth, development, and/or reproduction (*28*). Antixenosis or non-preference is a type of resistance in which the plant affects insect behavior, by discouraging oviposition, colonization, or feeding (*28, 29*). Initial attempts to transfers insect resistance from these plant introductions (PIs) to elite soybean lines were hindered by poor agronomic qualities of the PIs, and by quantitative inheritance of resistance (*30*). The advent of marker-assisted selection (MAS) has made possible reduce many of the issues caused by linkage drag (*31*).

To understand the genetic basis of resistance in these PIs, Rector et al. (*26, 27, 32*) identified a major QTL on Linkage Group (LG) M (now chromosome 7) of PI 171451 and PI 229358. This locus, named "QTL-M" accounts for 37% of antixenosis variance, and up to 28% of antibiosis variance. In addition there are two minor QTLs involved in resistance. QTL-H on chromosome (formerly LG H) conditions antixenosis in PI 229358 and PI 171451, and QTL-G on chromosome 18 (formerly LG G) conditions antibiosis in PI 229358.

Zhu et al. (*33*) demonstrated that QTL-H, and QTL-G only have a detectable effect if QTL-M is present in the genome. These minor QTLs have usually been missed by conventional breeding programs (*34*).

Hulburt (*35*) identified a major insect-resistance QTL in a mapping population from a PI 227687 x 'Cobb' cross. This QTL (QTL-E) on LG E (now chromosome 12) of PI 227687 conveys both antibiosis and antixenosis. QTL-E co-maps with the *Pb* locus that controls sharp (*Pb_*) vs. blunt (*pbpb*) leaf pubescence in soybean (*36*). Although there are earlier reports on the effect of pubescence traits on soybean resistance to insect (*37-39*), is the first report that a sharp-trichome locus co-localizes with an insect resistance QTL. Hulburt et al. (*40*) confirmed that sharp-trichome NILs from 'Clark' and 'Harosoy' are more resistant to lepidopterans, compared to the blunt-trichome cultivars. Nevertheless, given that Lambert and Kilen (*18*) showed that PI 227687's resistance is graft-transmissible, it remains possible that resistance is really due to an as of yet unidentified gene linked to *Pb*.

Pyramiding is used to combine multiple desirable genes for the same trait into a single genetic background (*41*). This strategy is advantageous for development of insect-resistant cultivars; it permits genes with different modes of action to be combined to obtain more durable resistance. Accordingly, Walker et al. (*42*) demonstrated that QTL-M enhances the effectiveness of Bt in soybean plants expressing the *cry1Ac* transgene, while Santos et al. (*43*) found that the use of cowpea trypsin inhibitor counteracted the effects of Cry1Ac in arabidopsis. In addition, Zhu et al. (*44*) analyzed sixteen NILs carrying all possible combinations of the insect-resistance QTLs from PI 229358 and the *cry1Ac* transgene in a 'Benning' background (*45*). CEW and SBL bioassays confirmed that Cry1Ac is more effective in the presence of insect-resistance QTLs from PI 229358.

The main goal of this research is to enhance soybean resistance to leaf-chewing insects by identifying the best combination of host-plant resistance QTLs. The objectives of this study were to: (i) develop NILs containing novel combinations of the insect resistance QTLs from PI 229358 and PI 227687; (ii) characterize the NILs for their resistance to defoliating insects, and (iii) evaluate the effect of the combination of QTL-M, QTL-E, and Bt for controlling Bt-tolerant pests.

Materials and methods

Characterization of Benning^{ME} and Benning^{MGHE}

Development of near-isogenic lines

The BC_6F_2-derived NILs, Benning^{ME} and Benning^{MGHE} [ie, Benning with QTLs M and E in the first case and M, G, H, and E in the second case, backcrossed into it], were developed using a marker-assisted backcross approach (**Fig 2.1**). Benning, a Maturity Group VII elite cultivar adapted to Georgia, was used as the recurrent parent. The NIL development took approximately 10 years, and started before SNPs were commonplace. Simple sequence repeat (SSR) markers linked to each QTL were used during backcross and selfing generations to select lines carrying a specific QTL combination. The flanking markers were: Sat_258 (5'-GCGCAATAGATAATCGAAAAACATACAAGA-3' and 5'-GCGGGGAAAGTGAAAACAAGATCAAATA-3') and Satt702 (5'-GCGGGGTTCTGTGGCTTCAAC-3' and 5'-GCGCATTGGAATAACGTCAAA -3') for QTL-M (*46*); Sct_199 (5'-GCGACAATGGCTATTAGTAACAATCA-3' and 5'-GCGATTTTCTATTTTCCTCACAGTG-3') and Satt191 (5'-CGCGATCATGTCTCTG -3' and 5'-GGGAGTTGGTGTTTTCTTGTG-3') for QTL-G (*44*); Sat_334 (5'-GCGTAACGTAGCAAATTGACTATAAGA-3' and 5'-

GCGTGTGCAAAGACAATTTCAATGA-3') and Sat_122 (5'-GTGACAAATGGATGGACAATAG-3' and 5'-AAGAAAAATAAAATAATGTAGAGTGGTGAT-3') for QTL-H (*44*); and Sat_112 (5'-TGTACAGTATACCGACATAATA-3' and 5'-CTACAAATAACATGAAATATAAGAAATA-3') and Satt411 (5'-TGGCCATGTCAAACCATAACAACA-3' and 5'-GCGTTGAAGCCGCCTACAAATATAAT-3') for QTL-E (*35*). Primer sequences for the SSR markers were obtained from SoyBase (http://www.soybase.org) (*47*). Genomic DNA isolation, PCR, and electrophoresis protocols for SSRs were performed as described by Zhu et al. (*44*). Single nucleotide polymorphism (SNP) markers (Ortega 2016) were used to genotype the plants used in the bioassays.

Defoliation

To estimate defoliation percentage, a soybean leaf defoliation chart (**Fig. 2.2**) was built from a collection of chewed leaves for which the percentage of consumed leaf area was calculated in ImageJ (*48*). A chart including 5% increments was the most useful to estimate the percent defoliation in NILs carrying the minor insect-resistance QTLs (QTL-G and QTL-H) in combination with the major QTLs (QTL-M and QTL-E).

Bioassays

SBL, CEW, fall armyworm [*Spodoptera frugiperda* (J.E. Smith)], and VBC caterpillars were used to evaluate the insect-resistant NILs performance in antibiosis, antixenosis, and field-cage assays. Eggs were obtained from Benzon Research Inc. (Carlisle, PA). Eggs were incubated for 72 hr at 25°C in a 600-ml (20 oz) clear polystyrene cup (Letica Corporation,

Rochester Hills, MI, USA) sealed with a dome lid (Letica Corporation); the cup contained 7 ml of plaster of Paris saturated with water to maintain 75% relative humidity. Neonate caterpillars were used to infest the bioassays.

Detached leaf experiments

Antibiosis (non-choice) assays were used to determine the effect of the ME and MGHE QTL combinations on caterpillar weight-gain. Benning (susceptible check), Benning^{M}, Benning^{E}, Benning^{H}, Benning^{G}, and Benning^{MGH} were included in each experiment. The NILs were tested for antibiosis to SBL, CEW, FAW, and VBC. Each species was evaluated independently using a randomized complete block design with 15 replications. Each replication included one plant from each genotype as the experimental unit. The experimental procedures included: i) One seed was planted in a 450-ml polystyrene foam cup filled with Fafard 2 mix (Conrad Fafard, Agawam, MA) with three holes were punched in the bottom to provide drainage. Plants were grown in an insecticide-free greenhouse under a photoperiod of 16 h. Sunlight was supplemented with 400 J s^{-1} Phillips ED-18 high-pressure sodium lamps (Phillips Inc., Andova, MA, USA) to keep the plants in a vegetative stage. The temperature was regulated to approximately 28°C during the day, and 20°C at night. Newly expanded trifoliolate leaves were collected once plants reached the V4 stage (*49*). One trifoliolate leaf was placed into a 600-ml (20-oz) clear polystyrene cup (Letica Corporation) sealed with a dome lid (Letica Corporation). Each cup contained 7 ml of plaster of Paris saturated with water, to maintain 75% relative humidity. Five SBL or FAW neonate caterpillars were placed in each cup, whereas only one CEW and VBC neonate was used per cup, with two cups per plant, to avoid cannibalism. Infested cups were placed in a growth chamber set at 27°C, and a 14-h light period was maintained with

fluorescent lights (T8 F032/730/Eco, Sylvania Octron, Danvers, MA, USA) providing ca. 40 µmol photons m^{-2} s^{-1} (*44*) (**Fig 2.3a**). Trifoliolate leaves were replaced with fresh leaves on the 4th day, and subsequently whenever 60% of the leaf area had been consumed. The average percentage of defoliation was estimated based on the appearance of the entire leaf.. The experiment was terminated after 7 days; caterpillars were immobilized by placing the cups at 4°C for 24 h. Caterpillars from each cup were weighed and their mean weights were used for analysis of variance.

Greenhouse experiments

Antixenosis (choice) assays were used to evaluate caterpillars' feeding preference when foliage of the null, M, E, H, G, ME, MGH, and MGHE NILs formed a canopy. The percentage of leaf area consumed by SBL, CEW, FAW, and VBC was determined for the each entire plant. Each insect species was tested independently using a randomized complete block design with 15 replications, with one plant from each NIL as the experimental unit. One seed was planted in a 450-ml polystyrene foam cup as described previously and grown in an insecticide-free greenhouse with the conditions as described above. Once plants reached the V4 stage, each block was transferred to a 24 x 24 x 36" polyester-mesh cage (BioQuip products, Rancho Dominguez, CA, USA) (**Fig 2.3b**). Each plant was infested with 10 neonate caterpillars. Since leaves of neighboring plants were in contact with each other, the caterpillars were able to move from plant to plant at will. Feeding was terminated when defoliation of Benning was higher than 50%, which took approximately 10 days. Percent defoliation of each entire plant was estimated by at least three researchers, and the mean of the estimates for each plant was used for an analysis of variance.

Field-cage experiments

This assay was designed to evaluate resistance to SBL under field conditions; resistance was scored as percent defoliation, which includes the effects of antibiosis and antixenosis. A field-cage containing the null, M, E, H, G, ME, MGH, and MGHE NILs was installed at the University of Georgia Plant Sciences Farm (**Fig 2.3c**). The experiment was planted on 1 July 2013 in a randomized complete block design with 15 replications. The experimental unit was a 6-plant hill plot (*50*); each block contained one plot per NIL. Hills were spaced 76.2 cm apart and were thinned to six plants after germination. A single border row of Benning hill-plots surrounded the experiment. After the plants reached the V2 stage, a cage covered with 0.9 x 0.9 mm Saran screen (Asahi Kasei, Tokyo, Japan) was placed over the experimental area. This confined the test insects and prevented immigration of parasitoids, predators, and other insect pests. The hill plots were infested when plants reached the V3 stage. Each hill plot was initially infested with 200 caterpillars. After that, 50 neonate caterpillars were added to the each hill-plot twice a week for 2 consecutive weeks. The percent defoliation for each hill of plants was estimated by four researchers at 5, 7, 11, and 14 days after the first infestation. A second field-cage containing the null, M, E, ME, MGH, and MGHE NILs was planted in 26 August 2013. This cage was infested and evaluated for defoliation as described for the first cage.

Characterization of Benning$^{ME+cry1Ac}$

Line development

The Benning$^{ME+cry1Ac}$ line was developed from a cross between Benning^{MGH} and Benning^{cry1Ac} (*44*); the breeding scheme is shown in **Fig 2.4**. The presence of QTL-M and QTL-E was confirmed by genotyping for Sat_258 and Satt702, and for Sat_112 and Satt411,

respectively. The presence of *cry1Ac* was confirmed by PCR, using the primers described by Stewart et al. (*51*).

Cry1Ac toxin in leaf tissue

The *cry1Ac* and ME+*cry1Ac* plants were tested for *cry1Ac* expression using the Cry1Ab/Cry1Ac ImmunoStrip test (Agdia Inc., Elkhart, IN, USA). Two leaf punches were collected per plant. Samples were ground in 300 µl of SEB4 extraction buffer (Agdia Inc.) using a GenoGrinder 210 (Spex SamplePrep, Metuchen, NJ, USA). Leaf extracts were processed according to the manufacturer's instructions.

Detached leaf experiments

SBL, VBC, and southern armyworm (SAW) [*Spodoptera eridania* (Cramer)] were used in non-choice assays to determine the effect of the ME+*cry1Ac* pyramid on caterpillar weight gain. These species were chosen because they vary in their sensitivity to Cry1Ac; SBL and VBC are susceptible, while SAW is resistant (*52*). Eggs were obtained from Benzon Research Inc. (Carlisle, PA). In each assay, Benning, Benning^{ME}, and Benning^{cry1Ac} were included as controls. The assays were set up and evaluated, as described in the previous section. Each assay consisted of a randomized complete block design with six replications. For the SAW assay, one cup containing five caterpillars was used to test each plant.

Data analyses

Data recorded from antibiosis, antixenosis, and field-cage assays were analyzed using JMP statistical software version 10.0 (SAS Institute, Inc., Cary, NC). Each dataset was tested for normality using the Shapiro-Wilk test ($P < 0.05$) (*53*). A one-way ANOVA test ($P < 0.01$)

was used to detect any difference among genotypes and experimental blocks, and a post-hoc Tukey-Kramer multiple comparison test ($P < 0.05$) (*54-56*) was used to determine significant differences between genotypes.

Results

Characterization of Benning^{ME} and Benning^{MGHE}

Detached leaf experiments

The results for the non-choice assays are shown in **Fig 2.5**. MGHE had the strongest antibiotic effect against SBL; SBL feeding on Benning^{MGHE} were 48% smaller than those feeding on Benning. However, ME had the strongest antibiotic effect against CEW, FAW, and VBC. CEW feeding on Benning^{ME} weighed 83% less than CEW feeding on Benning. FAW feeding on Benning^{ME} weighed 69% less than those feeding on Benning. Finally, VBC feeding on Benning^{ME} weighed 70% less than VBC feeding on Benning. Lines carrying QTL-H and QTL-G did not show antibiosis to any of the insect species.

Greenhouse experiments

Results for the SBL, CEW, FAW, and VBC choice assays are shown in **Fig. 2.6**. The pyramided NILs Benning^{ME}, Benning^{MGH}, and Benning^{MGHE} were the least defoliated across the four experiments. In the SBL and CEW bioassays, the combinations ME and MGHE were as resistant as MGH ($P > 0.05$). Benning^{ME} tended to have less SBL defoliation than Benning^{MGH} and Benning^{MGHE}; however, this difference was not significant. Similarly, Benning^{MGHE} tended to have less CEW defoliation than Benning^{ME} and Benning^{MGH} (13.3%). In the FAW and VBC bioassays, Benning^{ME} was more resistant than Benning^{MGH}, but not significantly different from Benning^{MGHE} ($P > 0.05$).

Benning and Benning^{G} were the most susceptible lines averaged across experiments. Benning^{H} showed resistance to CEW and VBC assays; however, QTL-H alone failed to protect the plants from SBL and FAW caterpillars. Benning^{M} and Benning^{E} were the most resistant single-QTL NILs. QTL-M and QTL-E provided similar levels of resistance against SBL, VBC, and FAW. Nonetheless, Benning^{M} was significantly more resistant against VBC than Benning^{E}.

Field-cage experiments

Defoliation progression in cage 1. The mean percentage of defoliation on each NIL at 5, 7, 11, and 14 days after infestation are shown in **Fig 2.7**. At 5 days, defoliation ranged between 12 and 18%, and no significant differences were observed between the NILs. At 7 days, Benning showed the most defoliation (32%) and Benning^{ME} was the least defoliated (14%). At this time-point, caterpillars were actively moving between hills, and towards the Benning hills used as borders. At 11 days, susceptible and resistant hills were easily distinguishable (**Fig 2.8**); Benning still showed the most defoliation (63%) and Benning^{ME} was the least defoliated (26%). At day 14, the rate of feeding was significantly slower; few caterpillars had migrated to the resistant NILs, but the majority of them were located on the cage's mesh.

Defoliation in cage 1. The data collected at 11 days after infestation were analyzed to determine differences in levels of resistance among NILs. This time-point was selected, because the plants were highly defoliated and the caterpillars were still highly active. Benning^{ME} (21%), Benning^{MGH} (25%), and Benning^{MGHE} (27%) were the most resistant lines in this cage, followed by Benning^{E} (52%) and Benning^{M} (38%), which were moderately

resistant. Benning (63%) Benning^{H} (62%), and Benning^{G} (61%) were the most susceptible (**Fig 2.9a**).

Defoliation in cage 2. Benning^{G} and Benning^{H} were excluded, because in the first cage they were not resistant to SBL. Benning^{MGHE} (27%) was the most resistant line in this cage, followed by Benning^{ME} (34%) and Benning^{MGH} (39%). Benning^{E} (65%) and Benning^{M} (45%) were more defoliated than Benning^{ME} and Benning^{MGH} in this cage; however Benning^{E} and Benning^{M} were less defoliated than Benning (75%) (**Fig 2.9b**).

Characterization of Benning$^{ME+cry1Ac}$

Detached leaf experiments

The results for the non-choice assays are shown in **Fig 2.10**. The pyramid of QTL-M, QTL-E, and *cry1Ac* showed enhanced antibiosis against SBL and SAW when compared to Benning^{ME} and Benning^{cry1Ac}. SBL fed on Benning^{ME} and Benning^{cry1Ac} weighed 61% and 43% less than SBL fed on Benning. However, the strongest antibiotic effect against SBL was observed in Benning$^{ME+cry1Ac}$; these caterpillars weighed 88% less than Benning-fed caterpillars. SAW fed on Benning^{ME} and Benning^{cry1Ac} weighed 68% and 59% less than SAW fed on Benning. The strongest antibiotic effect against SAW was observed on Benning$^{ME+cry1Ac}$; these caterpillars weighed 89% less than those fed on Benning. VBC fed on Benning^{ME} weighed 81% less than VBC fed on Benning. VBC fed on Benning^{cry1Ac} died at the first instar; their weight was 98% less than Benning-fed VBC. VBC fed on Benning$^{ME+cry1Ac}$ also died at the first instar; therefore the effect of QTL-M and QTL-E could not be measured for this species.

Discussion

PI 229358 and PI 227687 have been used in soybean breeding programs worldwide to introgress resistance to chewing-insects. This is the first time that the resistance of NILs carrying pyramids of insect resistance QTLs from PI 229358 and PI 227687 has been evaluated. The rationale was based on work by Lambert and Kilen (*57*), showing that F_1 progeny from PI 229358 x PI 227687 are more resistant than either parent. In this study it was demonstrated that the QTL combinations ME and MGHE are able to confer high levels of resistance against multiple insect species via antibiosis and antixenosis, in the cultivar, Benning. The ME and MGHE NILs exhibit similar levels of resistance in all but one of the bioassays. Therefore there is no indication that the addition of QTL-G and/or QTL-H, to the ME combination is required to reach agriculturally relevant levels of resistance. Although the results of are encouraging, a limitation of this study might be that ME and MGHE were characterized in a single genetic background (Benning), due to the time and resources needed to develop the NILs. Nevertheless, QTLs M (*34, 42, 58*) and E (*35, 40*) have been verified to work in different backgrounds when independently tested. From a breeding perspective, introgressing just QTL-M and QTL-E into an elite cultivar is simpler than introgressing all four QTLs. As the number of QTLs increases, pyramiding in an elite line, becomes increasingly difficult; especially when selection involves several traits at a time (*59*). Furthermore, QTL-G is associated with a yield penalty (*60*). Altogether, pyramiding the major insect-resistance QTLs from PI 229358 and PI 227687 presents an effective genetic combination to deploy host-plant resistance to insects in soybean.

In Brazil, the genetically modified MON 87701 x MON 89788 soybean, which expresses the Bt toxin Cry1Ac, is used for the integrated pest management of lepidopteran pests (*61*). This soybean is resistant to SBL, VBC (*62*), tobacco budworm [*Heliothis virescens*

(Fabricius)] (*63*), and the recently imported old world cotton bollworm [*Helicoverpa armigera* (Hübner)](*64*). However Cry1Ac is not sufficient to protect soybeans from FAW, SAW, and the velvet armyworm [*Spodoptera latifascia* (Walker)] (*52*). Frequent SAW outbreaks have been already reported in Brazil (*65, 66*); SAW's high defoliation capacity (*67*) and its large populations, make this species an important pest that can cause severe economic losses to Brazilian soybean production. A synergistic relationship between *cry1Ac* and the insect resistance QTLs from PI 229358 was previously reported. (*42, 44*). PI 227687 has shown resistance to SAW via antibiosis (*68*). There was interest in determining if the combination of QTL-M, QTL-E and *cry1Ac* would also provide enhanced resistance to lines with only the *cry1Ac* transgene or the QTLs by themselves. Benning$^{ME+cry1Ac}$ was developed and characterized in antibiosis assays. This line is more resistant than Benning^{ME} and Benning^{cry1Ac} against SBL and SAW. Although this combination would need to be thoroughly studied in antixenosis field cage assays and, if possible, in field tests with natural pest infestations; the results from the antibiosis assays indicates the potential of combining QTL-M, QTL-E and *cry1Ac* to improve soybean resistance to insects that are naturally tolerant to *cry1Ac*. The use of this pyramid as part of a resistance management strategy (*69*) could help preserve the effectiveness of Bt, which could lead to durable resistance to leaf-chewing insects in soybean.

Breeding high-yielding soybean cultivars with agriculturally relevant levels of insect-resistance has been a long-term goal. In the past, lines carrying only PI 229358 QTLs were either lower yielding (e.g., Benning^{MGH} (*31*), or not highly-resistant in the field (e.g., Benning^{MH} (*44*). With only two insect resistance QTLs, Benning^{ME} is at least as resistant to several important lepidopteran pests as Benning^{MGH}, without carrying QTL-G. Lines carrying QTLs from PI 229358 QTLs enhance the resistance provided by *cry1Ac* in lines like

Benning$^{MH+cry1Ac}$ (*44*). The combination of ME+*cry1Ac* described here could present an opportunity to effectively deploy Bt, in a pyramid with host-plant resistance genes.

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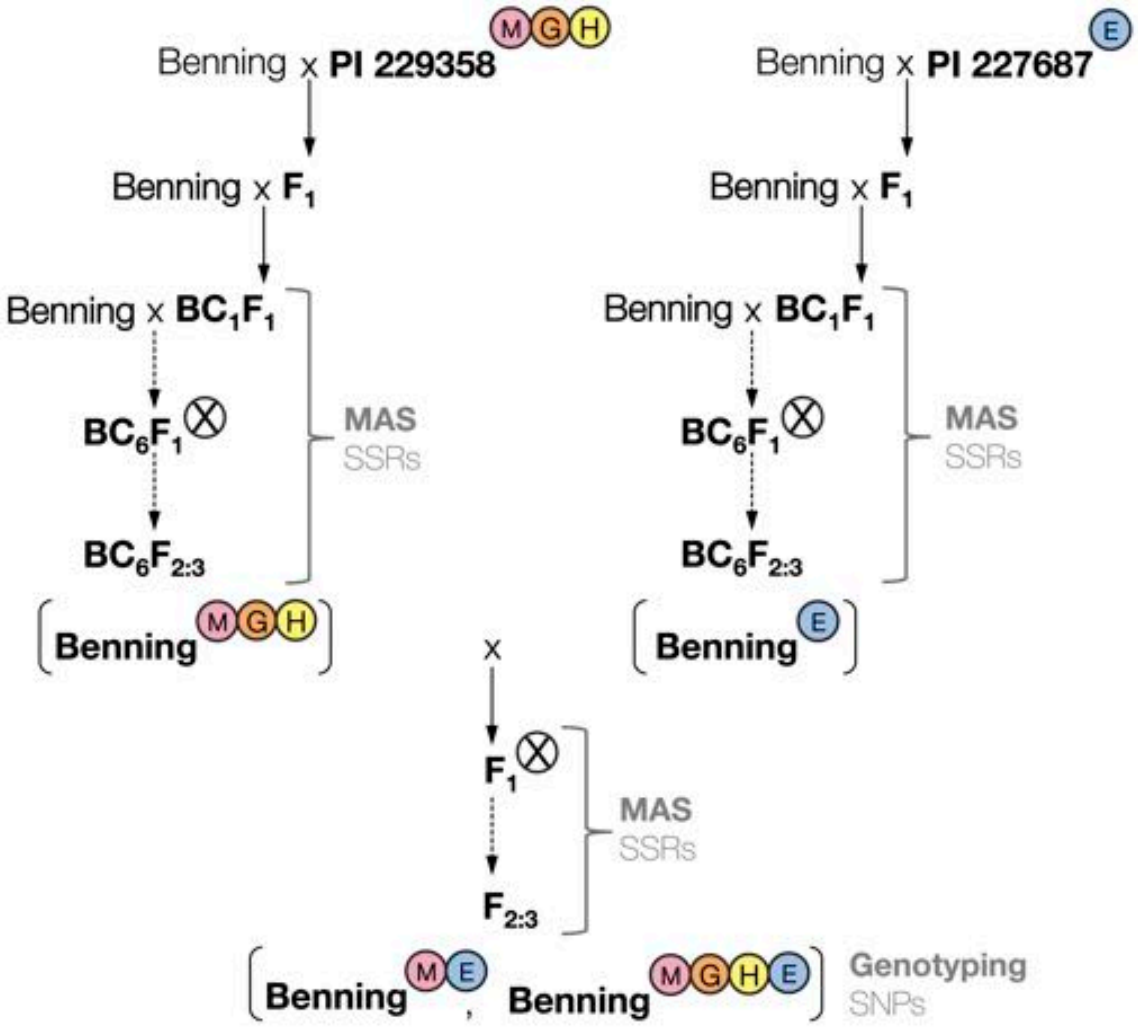

Figure 2.1. Breeding scheme for pyramiding insect-resistance QTLs in Benning. Benning^{MGH} (Zhu et al. 2007) and Benning^{E}, developed from a cross between Benning and PI 227687, were crossed; and the QTL combinations Benning^{ME} and Benning^{MGHE} were selected in the progeny. SSRs were used for marker-assisted selection (MAS) of QTL pyramids in each generation, and SNPs (Ortega 2016) were used to genotype the plants used in the bioassay

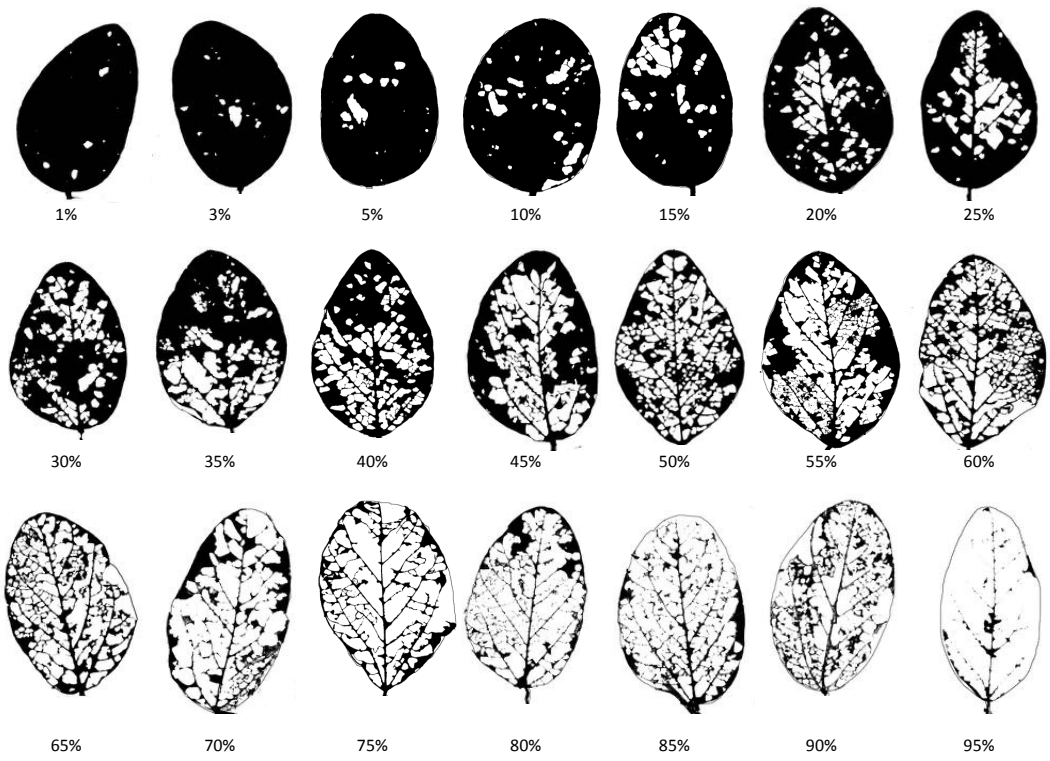

Figure 2.2. Soybean defoliation chart. Percentage of leaf area consumed by herbivores was calculated using Image J.

Figure 2.3. Insect bioassay settings. (a) Detached-leaf assay: caterpillars feeding on soybean leaves were contained in plastic cups. (b) Greenhouse assay: each cage contained caterpillars feeding on a block of test soybeans. (c) Cage built at the UGA Athens Plant Sciences farm to perform the field-cage assays.

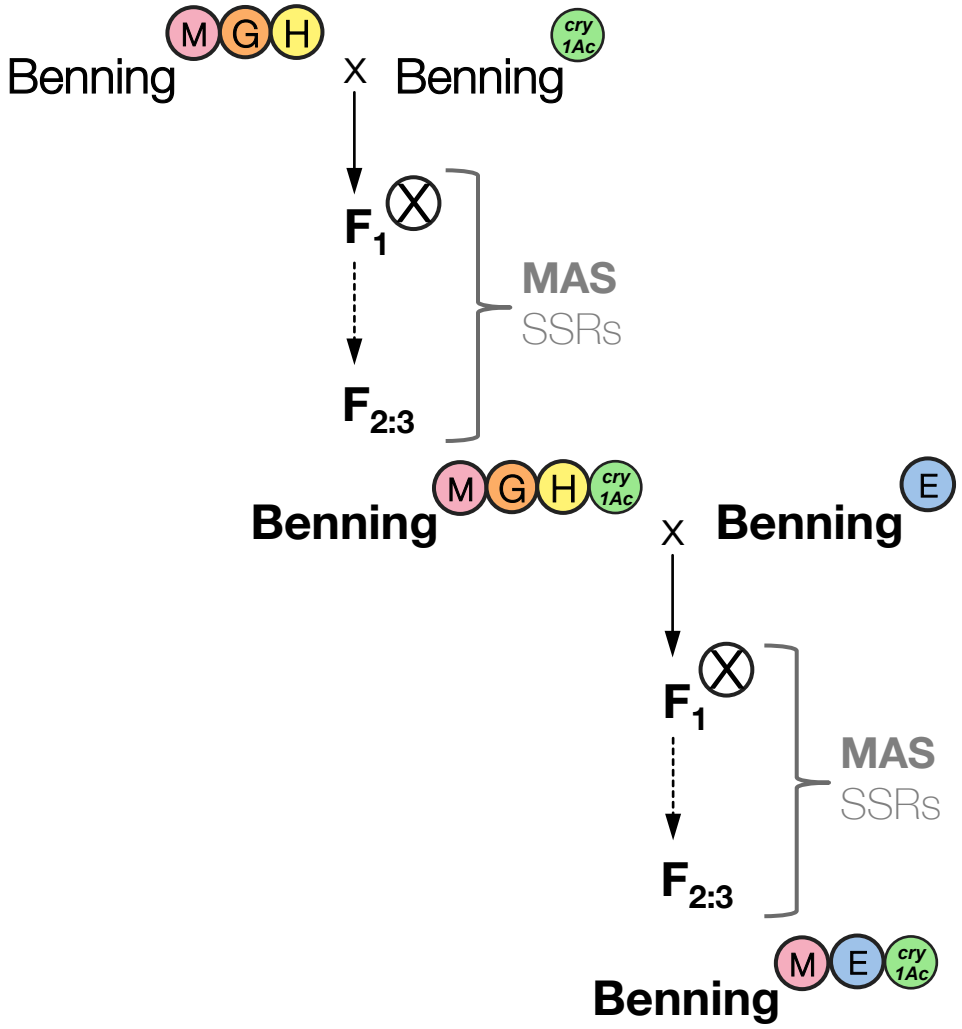

Figure 2.4. Breeding scheme for pyramiding insect-resistance QTLs and *cry1Ac* in Benning. SSRs were used for marker assisted selection (MAS) of QTLs in each generation. SNPs (Ortega 2016) were used to genotypethe plants used in the phenotyping assays.

Figure 2.5. Mean weight of SBL, CEW, FAW, and VBC caterpillars after feeding on insect-resistant NILs during detached-leaf (antibiosis) assays. Significant differences (Tukey-Kramer post-hoc test, $p < 0.05$) between NILs are indicated by letters.

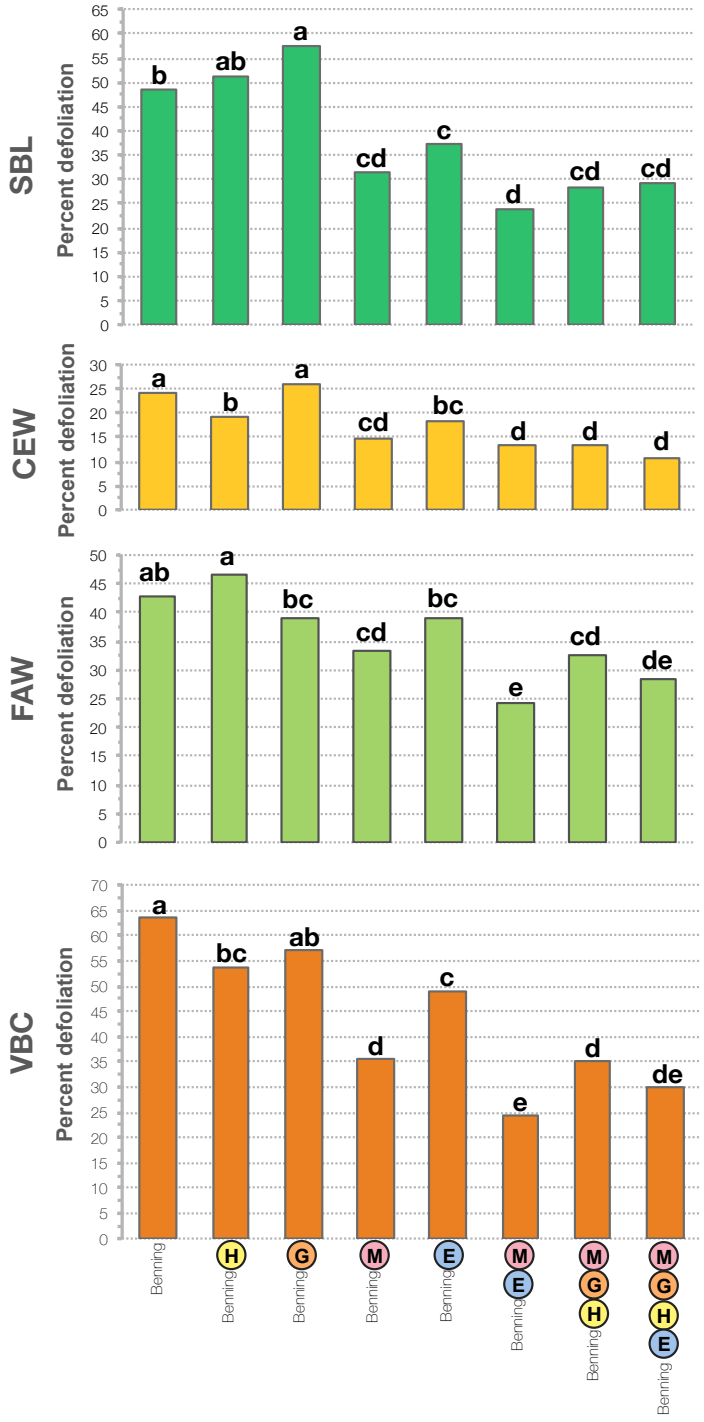

Figure 2.6. Mean defoliation by SBL, CEW, FAW, and VBC caterpillars on NILs during greenhouse (antixenosis) assays. Significant differences (Tukey-Kramer post-hoc test, $p < 0.05$) between NILs are indicated by letters.

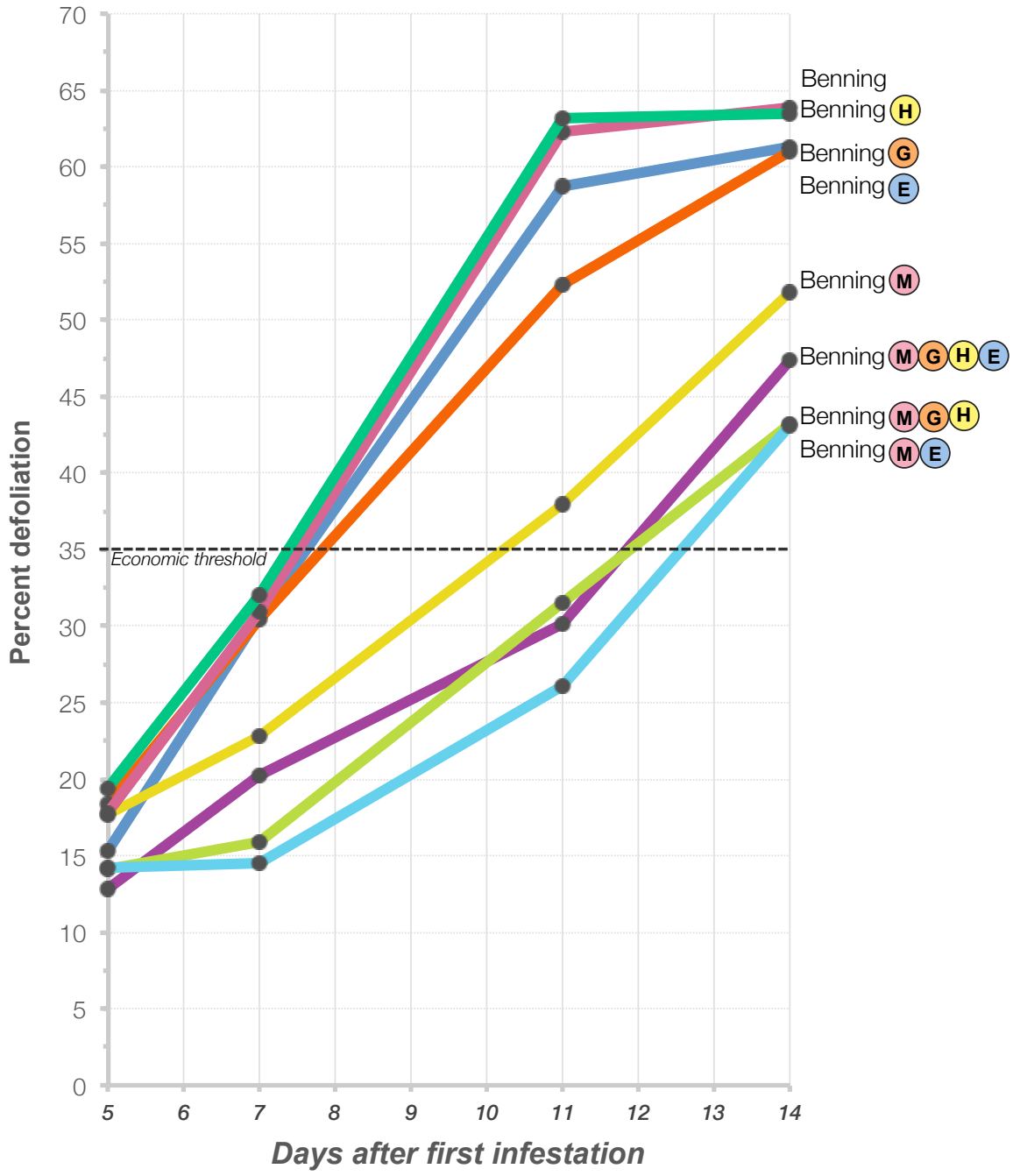

Figure 2.7. Feeding progression of SBL in the first field cage. Percentage of defoliation per hill was recorded at 5, 7, 11, and 14 days after the first infestation. Each time point shows the mean defoliation per NIL.

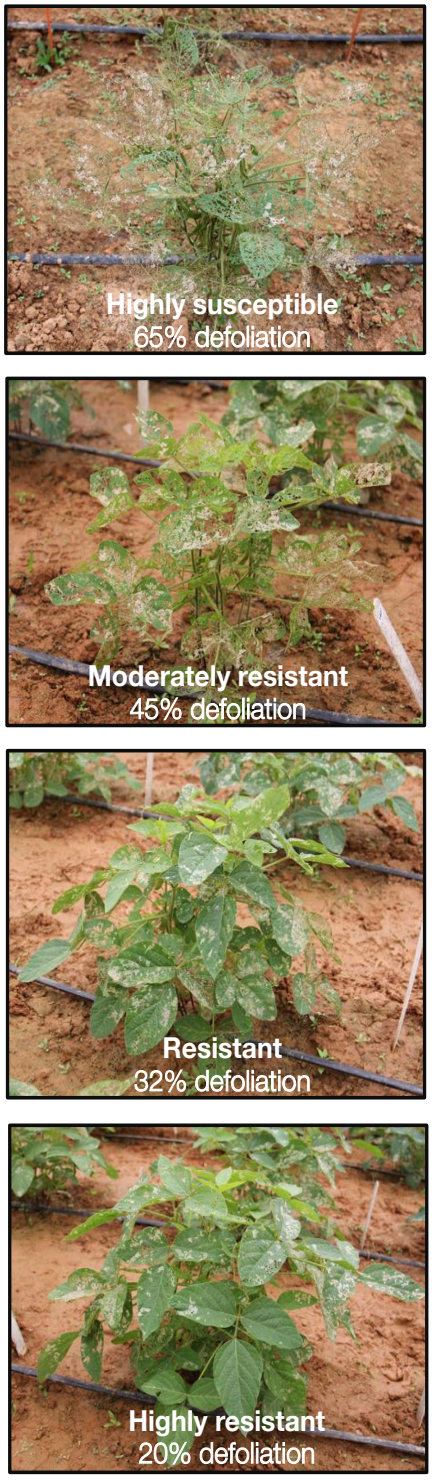

Figure 2.8. Leaf damage on NILs exposed to SBL feeding in the field cage, at 11 days after infestation.

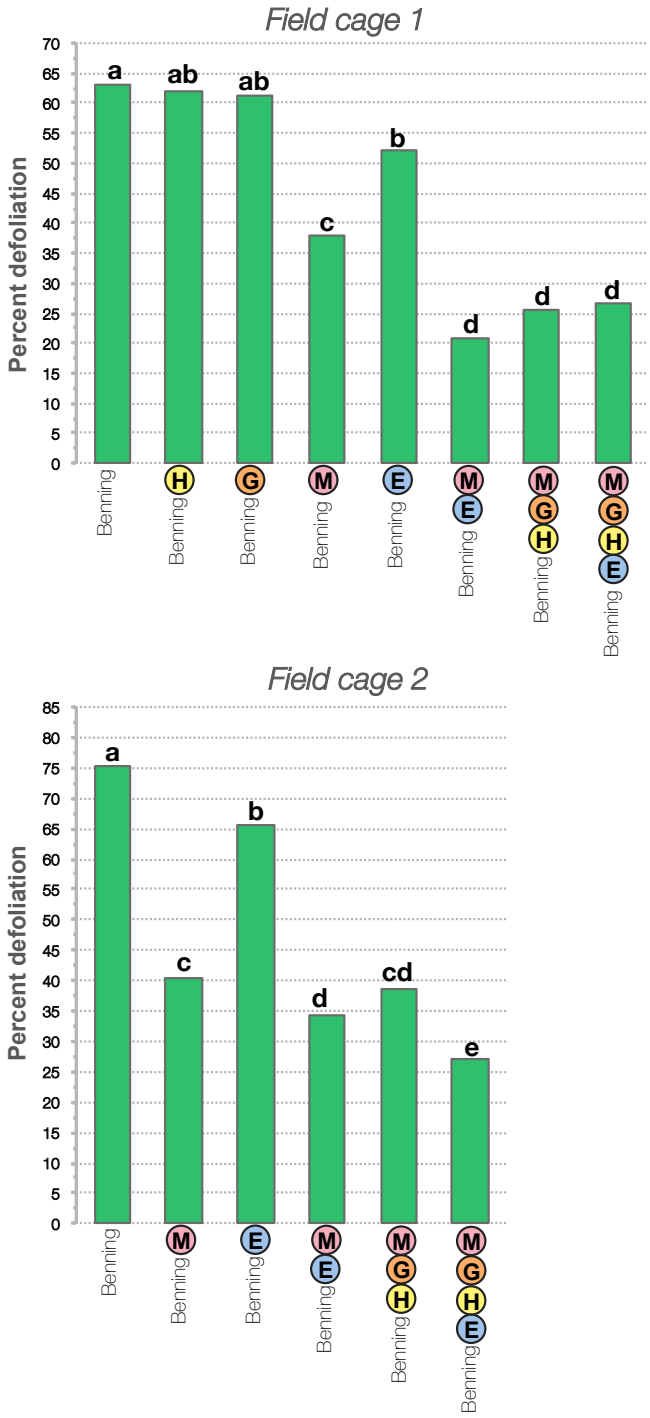

Figure 2.9. Mean defoliation by SBL at 11-days after infestation, in the first and second field cage. Significant differences (Tukey-Kramer post-hoc test, $p < 0.05$) between NILs are indicated by letters.

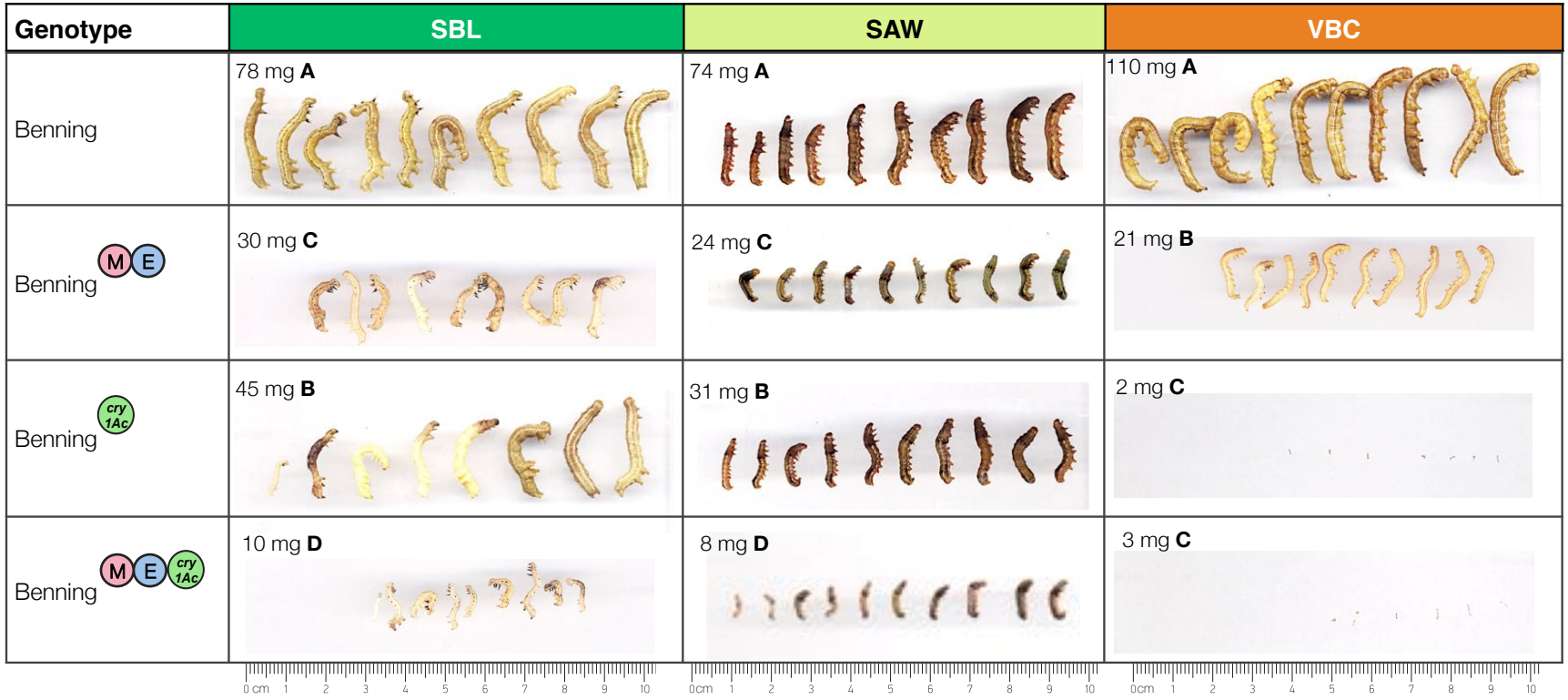

Figure 2.10. Mean weight of SBL, SAW, and VBC caterpillars after feeding on insect-resistant Benning$^{ME+cry1Ac}$ during detached-leaf (antibiosis) assays. Significant differences (Tukey-Kramer post-hoc test, $p < 0.05$) between NILs are indicated by letters.

CHAPTER 3

REGISTRATION OF TWO SOYBEAN GERMPLASM LINES CONTAINING LEAF-CHEWING INSECT RESISTANCE QTLs FROM PI 229358 AND PI 227687 INTROGRESSED INTO 'BENNING'

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Abstract

Two soybean [*Glycine max* (L.) Merr] germplasm lines, Benning-ME (Reg. No. GP-___, PI _____) and Benning-MGHE (Reg. No. GP-___, PI _____), were developed by the University of Georgia Agricultural Experiment Stations. Control of insect pests is crucial in soybean production; host-plant resistance reduces the need for insecticide applications, thus diminishing production costs and pesticide concerns. In soybean, resistance to a broad range of leaf-chewing insects is found in the Japanese plant introductions PI 229358 and PI 227687. PI 229358's resistance is conferred by QTL-M, QTL-H, and QTL-G. PI 227687's resistance is conferred by QTL-E. To enhance soybean resistance to leaf-chewing insects, PI 229358 and PI 227687's QTLs were pyramided in Benning-ME and Benning-MGHE, which are near-isogenic lines of 'Benning', obtained through marker-assisted backcrossing. Under field conditions Benning-ME and Benning-MGHE sustain 67% and 57% less defoliation than Benning, respectively. To determine the QTL introgressions in each line, high-density SNP genotypes were obtained using the SoySNP50K iSelect BeadChip (Illumina, San Diego, USA). To facilitate selection of lines carrying a specific QTL pyramid, KASP markers were developed for high-throughput genotyping. These lines are valuable genetic resources for breeding of host-plant resistance to insects in soybean. The combination of QTL-M and QTL-E provides agriculturally relevant levels of resistance, and with only two loci, the use of this pyramid is feasible in a breeding program.

Abbreviations:

bp: basepair

Chr: chromosome

CEW: corn earworm (*Helicoverpa zea*)

KASP: Kompetitive Allele Specific PCR

LG: linkage group

PI: plant introduction

QTL: quantitative trait locus

SBL, soybean looper (*Chrysodeixis includens*)

SNP: single nucleotide polymorphism

SSR: simple sequence repeat

VBC: velvetbean caterpillar (*Anticarsia gemmatalis*)

FAW: fall armyworm (*Spodoptera frugiperda*)

Introduction

Insect pests affect soybean production. Particularly, high-levels of leaf damage by chewing insects indirectly impact seed yield and quality (*1*). Corn earworm [CEW, *Helicoverpa zea* (Boddie)], soybean looper [SBL, *Chrysodeixis includens* (Walker)], velvetbean caterpillar [VBC, *Anticarsia gemmatalis* (Hübner)], and bean leaf beetle, [*Cerotoma trifurcata* (Forster)] are among the most economically important insects affecting U.S. production (*2, 3*). Plant resistance to these pests reduces the need for insecticide applications, thus diminishing production costs and pesticide concerns. The Japanese soybean landraces PI 229358 and PI 227687 have been widely used as source for resistance to leaf-chewing insects (*4*). Resistance in these PIs is conferred via antibiosis and antixenosis. Antibiosis encompasses the detrimental effects on insect physiology (*5*), and antixenosis refers to the discouragement of insect colonization or feeding (*6, 7*). PI 229358's resistance is conferred by three quantitative trait loci (QTLs). QTL-M confers antibiosis and antixenosis, QTL-G confers antibiosis, and QTL-H confers antixenosis (*8, 9*). QTL-G and QTL-H are minor QTLs that are only expressed if QTL-M is present (*10*). PI 227687's resistance is conferred by QTL-E, via antibiosis and antixenosis (*7*). Benning NILs carrying each and all of the PI 229358's QTLs were released by Zhu et al., (*11*). G05-Ben229IR-MGH is the most resistant of these lines (*12*). However, QTL-G is associated with yield-drag (*13*), which hinders the deployment of this pyramid.

The objective of this research is to enhance soybean resistance to leaf-chewing insects by combining the QTLs from PI 229358 and PI 227687. The new NILs, i) Benning-ME (QTL-M and QTL-E) and ii) Benning-MGHE (QTL-M, QTL-G, QTL-H, and QTL-E) are highly resistant to CEW, SBL, VBC and fall armyworm [FAW, *Spodoptera frugiperda* (J.E. Smith)] (*14*). Pyramiding two insect resistance loci, i.e. QTL-M and QTL-E, is feasible in a

breeding program. To characterize the QTL introgressions in Benning-ME and Benning-MGH, high-density SNP genotypes were obtained using the SoySNP50K iSelect BeadChip (Illumina, San Diego, USA). Kompetitive Allele Specific PCR (KASP) assays were designed to detect SNP alleles flanking each QTL; these assays facilitate high-throughput genotyping and selection of breeding lines carrying a specific QTL combination.

Methods

Development of the insect-resistant germplasm lines

The germplasm lines Benning-ME and Benning-MGHE are BC_6F_2-derived near-isogenic lines (NILs) developed from [Benning (7) x PI 229358] x [Benning (7) x PI 227687]. Benning is a Maturity Group (MG) VII cultivar derived from a F_4 plant descended from the cross 'Hutcheson' x 'Coker 6738' (*15*). The insect resistance sources PI 229358 (MG VII) and PI 227687 (MG VIII) are Japanese cultivars known as 'Soden-daizu' and 'Miyako White', respectively (*4*). Simple sequence repeat (SSR) marker were used during backcross and selfing generations to select lines carrying a particular QTL combination: Sat_258 and Satt702 for QTL-M (*16*); Sct_199 and Satt191, for QTL-G (*12*); Sat_334 and Sat_122, for QTL-H (*12*); and Sat_112 and Satt411, for QTL-E (*7*). Primer sequences for the SSR markers were obtained from SoyBase (http://www.soybase.org) (*17*). Genomic DNA isolation, PCR, and electrophoresis protocols for SSRs were performed as described by Zhu et al. (*12*). In brief, the breeding scheme was as follows: $Benning^{MGH}$, the $BC_6F_{2:3}$ Benning near-isogenic line (NIL) carrying QTL-M, QTL-G, and QTL-H was developed using a marker-assisted backcross approach from a cross between Benning and PI 229358; $Benning^{E}$, The $BC_6F_{2:3}$ Benning NIL carrying QTL-E, was developed in a marker-assisted backcross approach from a cross between Benning and PI 227687; $Benning^{MGH}$ was crossed to

Benning^{E}; Finally, the $F_{2:3}$ plants carrying the QTL pyramids QTL-M and QTL-E; and QTL-M, QTL-G, QTL-H, and QTL-E were identified through marker-assisted selection. Seed increased from the $F_{2:3}$ lines were used for the insect resistance bioassays (Ortega et al., 2016).

Graphical genotypes

The SoySNP50K iSelect BeadChip (Illumina, San Diego, USA) (*18*), which contains 52,041 SNPs distributed throughout the soybean genome, was used to genotype Benning, PI 229358, PI 227687, Benning^{MGH}, Benning^{E}, Benning^{ME} and Benning^{MGHE}. The SoySNP50K assays were performed at Michigan State University using an Illumina iScan platform (Illumina, San Diego, USA). SNP genotype calling was done in GenomeStudio v2011.1 software (Illumina, San Diego, USA).

Polymorphic SNPs between Benning and PI 229358, Benning and PI 227687, and Benning vs. PI 229358 and PI 227687 were identified using FlapJack (*19*). A graphical genotype (*20*) of Benning^{MGH}, Benning^{E}, Benning^{ME} and Benning^{MGHE} was created using Graphical Genotypes GGT 2.0 (*21*). The introgressions at QTL-M (chromosome 7), QTL-G (chromosome 18), QTL-H (chromosome 12), and QTL-E (chromosome 15) were estimated using the graphical genotypes.

Development of KASP genotyping for insect resistance QTLs

Genomic DNA isolated from seeds of Benning NILs was used for the SNP-genotyping assays. The DNA extraction protocol was modified from Kamiya and Kiguchi (*22*). Briefly, cotyledonary tissue was harvested with a scalpel and placed into a 2-ml tube, and 600 µl of digestion buffer [10 mM Tris-HCL (pH 7.8), 5mM EDTA, 0.5% SDS, 0.5% NP-40, 0.5%

Tween-20] and 2 µl of Proteinase K (20mg ml^{-1}) were added. The tube was vortexed for 10 min, incubated at 55°C for 45 min, and left at room temperature for 15 min. Six hundred µl of phenol/chloroform/isoamyl-alcohol (25:24:1) were added to the tube; the sample was mixed by inversion to form an emulsion and centrifuged at 13,200 rpm for 6 min. The supernatant was transferred to a 1.5-ml tube and mixed with 500 µl of chloroform/isoamyl-alcohol (24:1); the tube was centrifuged at 13,200 rpm for 6 min, and the supernatant was transferred to a 1.5- ml tube. The chloroform/isoamyl-alcohol extraction was repeated until a clear supernatant was obtained. DNA was precipitated by adding 1 vol of isopropanol; the tube was centrifuged at 13,200 rpm for 6 min and the supernatant was discarded. Finally, the DNA pellet was washed with 70% ethanol, dried, and resuspended in 50 µl of TE/RNase buffer (10 mM Tris-HCL [pH 8.0], 1 mM EDTA, 100 µg ml^{-1} RNAse A).

The KASP genotyping system (KBioscience Ltd., Hoddesdon, England) was used to develop a high-throughput genotyping assay of insect-resistant NILs. The KASP assay included QTL-flanking SNPs for QTLs G, H, and E; which were identified from the graphical genotypes of Benning^{ME} and Benning^{MGHE}. The assay also included the functional SNP for the insect-resistance gene in QTL-M (Ortega 2016). Each 4-µl KASP reaction consisted of 2 µl of 2X KASP mastermix, 2 µl of 10-20 ng μl^{-1} genomic DNA, and 0.106 µl of primer mix (allele-specific primers at 1.4 µM, and common primer at 3 µM). PCR reactions were performed in a T100™ Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA), using a *Taq* polymerase activation period (94°C for 15 min), followed by a touchdown amplification step consisting of 10 cycles of 94°C for 20 sec and 65°C for 1 min (decreasing 0.8°C per cycle), then 29 cycles of 94°C for 20 sec and 57°C for 1 min. The KASP assay was read in a LightCycler480 (Roche Diagnostics, Germany); a single fluorescence acquisition was recorded after incubating the samples at 37°C for 1 min.

Characteristics

Like Benning, Benning-ME and Benning-MGHE have determinate growth habit and belong to MG VII. Both lines have purple flowers, tawny sharp pubescence, tan pods, yellow seed coat, and sharp trichomes. Benning-ME has brown hilum, and Benning-MGHE has brown hilum of varying intensity. They are also similar to Benning in plant height, lodging score, and seed quality score.

Graphical Genotyping of Benning NILs

From the 52,041 SNPs in the SoySNP50K chip, 12,367 SNPs were polymorphic between Benning and PI 229358. In the Benning^{MGH} genome, 91.5% of the polymorphic SNP loci carried Benning alleles, while 8.5% carried PI 229358 alleles (**Fig 3.1a**). 14,587 SNPs were polymorphic between Benning and PI 227687. In Benning^{E} , 98.0% SNP loci carried Benning alleles and 2.0% carried PI 227687 alleles (**Fig 3.1b**). 6,645 SNPs were polymorphic between Benning and both plant introductions. In Benning^{ME}, 94.3% SNP loci carried the Benning allele and 5.7% carried either PI229358 or PI 227687 (**Fig 3.1c**); while in Benning^{MGHE}, 91.3% SNP loci carried the Benning allele and 8.7% carried either PI229358 or PI 227687 (**Fig 3.1d**). PI introgressions were also detected in other regions of the NILs' genome (**Table 3.1**).

SNP for marker assisted selection of Insect-resistance QTLs

The introgression of PI-derived DNA for each QTL, SSR markers used for selection of the NILs, and new SNP markers are shown in **Fig 3.2**. Five SNP loci from the SoySNP50K chip were converted to KASP markers for QTL-H; five and four SNPs were also converted to KASP markers for QTL-G and QTL-E respectively. Primer sequences for

the KASP markers are listed in **Table 3.2**. Each KASP marker effectively distinguished the Benning allele from the plant-introduction allele when they were validated using the insect-resistant NILs genotyped with the SoySNP50K chip (**Fig 3.3**). These KASP markers still need to be validated for MAS in an insect-susceptible genetic background other than Benning. Nonetheless, the Glyma07g14530 marker in QTL-M is the functional SNP; hence the PI 229358 allele is unique to insect-resistant lines carrying QTL-M.

Availability

Seeds of Benning-ME and Benning-MGHE will be maintained by the Georgia Agricultural Experiment Stations at the University of Georgia, Athens GA 30602. A small sample of seeds may be requested from the corresponding author for research purposes. Seeds of Benning-ME and Benning-MGHE have been deposited in the National Germplasm System (Urbana, IL), where they will be available for distribution after submission.

Conclusions

Breeding high-yielding soybean cultivars with agriculturally relevant levels of resistance to leaf-chewing insects has been a long-term goal. This is the first time that QTLs from PI 229358 and PI 227687 have been pyramided to enhance soybean resistance to insects. The germplasm lines Benning-ME and Benning-MGHE would be useful to soybean breeders for simultaneous selection of QTL-M and QTL-E; and QTL-M, QTL-G, QTL-H, and QTL-E respectively. Both NILs exhibit similar levels of resistance; therefore Benning-ME is very useful if breeders prefer to introgress only QTL-M and QTL-E, and/or exclude QTL-G because of the yield penalty. In addition, the KASP genotyping assays would assist the selection of lines carrying a specific QTL combination. Furthermore, the graphical-

genotypes for the QTL-introgression provide a reference for fine mapping and cloning of candidate genes responsible for insect-resistance.

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Table 3.1. Distribution of SoySNP50K SNPs used to draw the graphical genotypes. Benning and PI alleles are listed for each chromosome.

Chr	LG	Benning[MGH]			Benning[C]			Benning[MC]			Benning[MGHC]		
		Total	Benning	PI 229358	Total	Benning	PI 227687	Total	Benning	PI 229358 PI 227687	Total	Benning	PI 229358 PI 227687
1	D1a	522	517	5	445	443	2	173	171	2	173	171	2
2	D1b	830	577	253	963	924	39	453	450	3	453	441	12
3	N	505	504	1	617	597	20	260	233	27	260	208	52
4	C1	660	655	5	775	772	3	441	439	2	441	439	2
5	A1	732	731	1	842	839	3	468	467	1	468	437	31
6	C2	504	502	2	697	696	1	249	249	0	249	249	0
7	M	620	410	210	851	843	8	366	194	172	366	196	170
8	A2	662	657	5	952	947	5	377	376	1	377	369	8
9	K	509	502	7	482	475	7	198	195	3	198	195	3
10	O	547	545	2	637	631	6	330	327	3	330	327	3
11	B1	523	468	55	516	481	35	253	249	4	253	210	43
12	H	523	327	196	583	580	3	350	350	0	350	265	85
13	F	670	636	34	817	813	4	386	384	2	386	363	23
14	B2	699	694	5	723	712	11	340	339	1	340	339	1
15	E	913	854	59	660	641	19	388	331	57	388	375	13
16	J	529	523	6	695	684	11	312	296	16	312	307	5
17	D2	607	572	35	681	678	3	368	367	1	368	367	1
18	G	1024	873	151	1300	1268	32	431	391	40	431	347	84
19	L	402	401	1	931	928	3	257	257	0	257	257	0
20	I	386	371	15	420	336	84	245	203	42	245	202	43
Total		12367	11319	1048	14587	14288	299	6645	6268	377	6645	6064	581

Table 3.2. KASP markers for selection of insect-resistance QTLs.

SNP ssID #	Chr.	bp	FAM primer	VIC primer	Common Primer	Benning Allele	PI 229358 Allele
Glyma07g14530	*Gm07*	11,281,192	GAAGGTGACCAAGTTCATGCTTGGGTGTGAATGTTTATTGTGA	GAAGGTCGGAGTCAACGGATTTGGGTGTGAATGTTTATTGTGG	CTGCTCTTGGCAGAGTGTGCCACC	A	G
ss715613669	*Gm12*	9,189,112	GAAGGTGACCAAGTTCATGCTCAACACCTAGTTTTTTACCACAACA	GAAGGTCGGAGTCAACGGATTAACACCTAGTTTTTTACCACAACG	TCTGTTTAAAAGGTCAACCTCTCC	A	G
ss715613710	*Gm12*	9,972,984	GAAGGTGACCAAGTTCATGCTAACCTCATGTAAATGTTGTCA	GAAGGTCGGAGTCAACGGATTAACCTCATGTAAATGTTGTCG	GACGATTGACGACCCTTGTT	T	C
ss715611351	*Gm12*	10,445,762	GAAGGTGACCAAGTTCATGCTTAAGCCTCTCCTCGCTTTTGCT	GAAGGTCGGAGTCAACGGATTAGCCTCTCCTCGCTTTTGCC	ATGCAATGATTGGGTGCTAAG	T	C
ss715611380	*Gm12*	11,157,215	GAAGGTGACCAAGTTCATGCTGTGGTGAAGATGGTGGGCA	GAAGGTCGGAGTCAACGGATTGTGGTGAAGATGGTGGGCG	CCAAGCGACATCGTTTCTTT	A	G
ss715611524	*Gm12*	14,384,675	GAAGGTGACCAAGTTCATGCTATGACACCTAGATCTGGTGCA	GAAGGTCGGAGTCAACGGATTATGACACCTAGATCTGGTGCG	AGAGCGTGAGCAGGATTCTG	C	T
ss715631954	*Gm18*	53,905,333	GAAGGTGACCAAGTTCATGCTGAGGATGCAACGGCTGTGGTA	GAAGGTCGGAGTCAACGGATTGAGGATGCAACGGCTGTGGTG	CCACGGTCTACGCCTCACCC	G	A
ss715631979	*Gm18*	54,061,528	GAAGGTGACCAAGTTCATGCTCAGGCAAGGGCTAAGATGC	GAAGGTCGGAGTCAACGGATTTCAGGCAAGGGCTAAGATGT	TTTCAAAAGTATCCATTTGTTGC	G	A
ss715631994	*Gm18*	54,137,764	GAAGGTGACCAAGTTCATGCTCTAGCTCCTGTTCATCAGAAATCTG	GAAGGTCGGAGTCAACGGATTCTAGCTCCTGTTCATCAGAAATCTT	AAAATTTCCTGGCTGGGTTT	T	G
ss715632003	*Gm18*	54,191,166	GAAGGTGACCAAGTTCATGCTGAAGGATTAAATAAAAAACACTCACA	GAAGGTCGGAGTCAACGGATTGAAGGATTAAATAAAAAACACTCACC	CCAGAAGTTCACCATCACCA	C	A
ss715632041	*Gm18*	54,391,672	GAAGGTGACCAAGTTCATGCTCAATTCGATTTTTGGATAATGC	GAAGGTCGGAGTCAACGGATTCCAATTCGATTTTTGGATAATGT	TCCACTTGGCAATTTACGTG	C	T
SNP ssID #	**Chr.**	**bp**	**FAM primer**	**VIC primer**	**Common Primer**	**Benning Allele**	**PI 227687 Allele**
ss715623001	*Gm15*	714,829	GAAGGTGACCAAGTTCATGCTTCTGTTCAAACTCATGCAGAAGA	GAAGGTCGGAGTCAACGGATTCTGTTCAAACTCATGCAGAAGG	CAAATTCCGCGAGGTAAGTC	A	G
ss715623005	*Gm15*	718,311	GAAGGTGACCAAGTTCATGCTTCGCGTCTCTTGGTGTCAG	GAAGGTCGGAGTCAACGGATTCGCGTCTCTTGGTGTCAA	CTAAAGGCACAGGCCTCCAT	T	C
ss715621274	*Gm15*	2,152,272	GAAGGTGACCAAGTTCATGCTTGGAGGGTGGTTATAGGTCTTGT	GAAGGTCGGAGTCAACGGATTGGAGGGTGGTTATAGGTCTTGC	GTAAAAATCAACCACAGATGAGC	G	A
ss715621275	*Gm15*	2,154,788	GAAGGTGACCAAGTTCATGCTGACACCCGATCAAGATTCAAG	GAAGGTCGGAGTCAACGGATTGACACCCGATCAAGATTCAAA	CGAGGTCCTTGTATGGGTTG	T	C

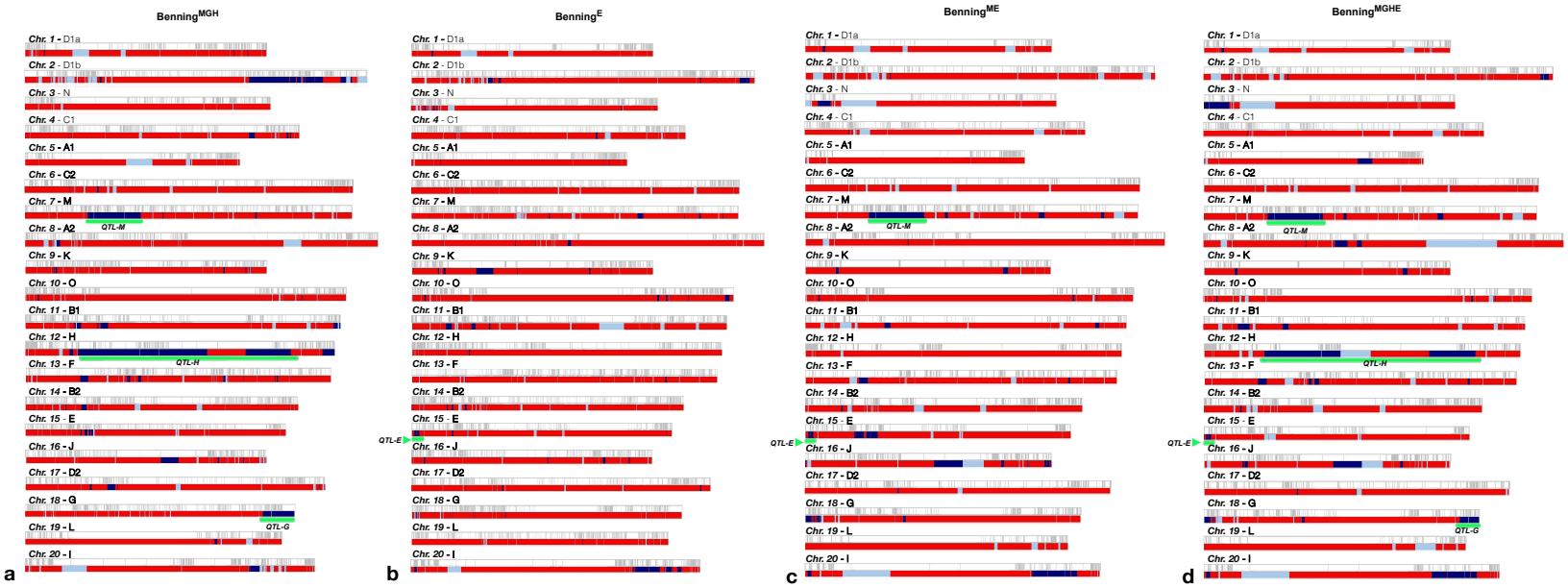
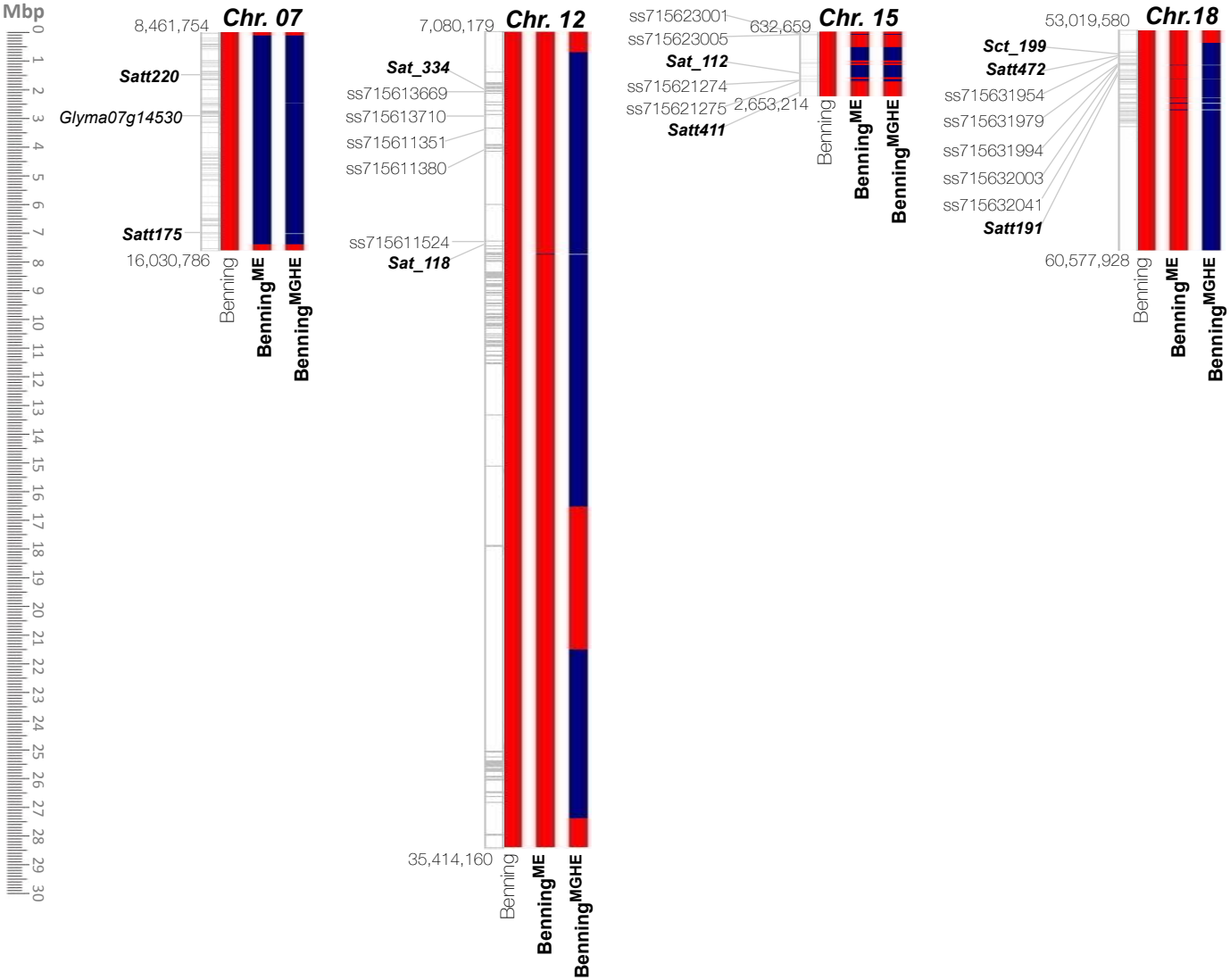


Figure 3.1. Graphical genotypes. (a) Benning^{MGH}, built with polymorphic markers between Benning and PI 229358; (b) Benning^{E}, built with polymorphic SNPs between Benning and PI 227687; (c) Benning^{ME} and Benning^{MGHE}, built with polymorphic SNPs between Benning, and PI 229358 and PI 227687. Red indicates Benning alleles, dark blue indicates PI alleles, and light blue indicates heterozygous loci.


Mbp
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8,461,754
Chr. 07
Satt220
Glyma07g14530
Satt175
16,030,786
Benning
BenningME
BenningMGHE
7,080,179
Chr. 12
Sat_334
ss715613669
ss715613710
ss715611351
ss715611380
ss715611524
Sat_118
35,414,160
Benning
BenningME
BenningMGHE
ss715623001
ss715623005
632,659
Chr. 15
Sat_112
ss715621274
ss715621275
Satt411
2,653,214
Benning
BenningME
BenningMGHE
53,019,580
Chr.18
Sct_199
Satt472
ss715631954
ss715631979
ss715631994
ss715632003
ss715632041
Satt191
60,577,928
Benning
BenningME
BenningMGHE

Figure 3.2. Insect resistance QTLs showing PI introgressions in Benning^{ME} and Benning^{MGHE}. Red indicates Benning alleles, dark blue indicates PI alleles, and light blue indicates heterozygous loci. SSR markers used for line development, and new KASP marker are indicated by the arrows.

Figure 3.3. Example of SNP assays using the KASP genotyping system for detection of insect-resistance QTLs. FAM (blue, X-axis) and VIC (green, Y-axis) were used as fluorescent labels to detect each allele.

CHAPTER 4

THE SUITABILITY OF SOYBEAN MEAL FROM INSECT-RESISTANT SOYBEANS FOR BROILER CHICKENS

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[1]María A. Ortega, Adam J. Davis, H. Roger Boerma, and Wayne A. Parrott. 2016. *Journal of Agricultural and Food Chemistry*. 64(11):2209-2213.

Abstract

Benning^{M} and Benning^{MGH} are near-isogenic lines (NILs) of the soybean cultivar Benning, which contain insect-resistance quantitative trait loci (QTLs) from the soybean accession PI 229358. Benning^{M} contains QTL-M, which confers antibiosis and antixenosis. In addition to QTL-M, Benning^{MGH} contains QTL-G, which confers antibiosis; and QTL-H, which confers antixenosis. Soybean meal was produced from Benning and the NILs. Nutritional composition, digestible amino acid content, and nitrogen-corrected true metabolizable energy (TME_N) were equivalent among soybean meals. A 21-day broiler feeding trial was carried out to determine if the QTLs affect soybean meal quality. Weight gain and fed-to-gain ratio were evaluated. No biologically significant differences were detected for broilers fed on Benning, Benning^{M}, and Benning^{MGH}. This demonstrates that soybean meal produced from the insect resistant NILs is equivalent to soybean meal produced from their non-insect resistant parent cultivar, for broiler weight gain.

Abbreviations:

NIL: near isogenic line

PI: plant introduction

QTL: quantitative trait loci

TME_N: nitrogen-corrected true metabolizable energy

Introduction

Broiler feeding trials have become a standard test to assess the nutritional suitability of genetically modified crops (*1*). Some jurisdictions even consider them as providing a screen to guard against the unintentional presence of harmful side effects from the modification (*2, 3*). In contrast, similar traits obtained via conventional breeding are seldom tested for safety (*4*). Insect resistance in soybean [*Glycine max* (L.) Merr] is an example of a trait that can be obtained either transgenically (*5*) or conventionally (*6*).

Soybean seeds are a major protein source for animal feed (*7*). Worldwide, 11% of the crop is lost to animal pests, including insects (*8*), of which leaf-chewing insects are economically important in the southern USA (*9*). Although soybean can withstand moderate leaf damage, high levels of defoliation greatly reduce seed yield and quality (*10*). Therefore, plant resistance to leaf-chewing insects is essential for preventive pest management; it promotes efficient use of insecticides, diminishing crop production and environmental concerns. In soybean, non-transgenic resistance to a broad range of leaf-chewing insects (*11-26*) is found in the Japanese soybean landrace 'Sodendaizu' PI 229358 (*27*), from where it has been bred into several modern cultivars.

PI 229358's resistance is conferred via antibiosis and antixenosis (*28, 29*). In antibiosis, the plant has detrimental effects on insect growth, development, and/or reproduction (*30*). In antixenosis, the plant affects insect behavior by discouraging oviposition, colonization, and/or feeding (*30, 31*). Three quantitative trait loci (QTLs) confer PI 229358's resistance. QTL-M, on chromosome 7, provides both antibiosis and antixenosis. QTL-H, on chromosome 12, conditions antixenosis; while QTL-G, on chromosome 18, conditions antibiosis (*28, 29*). QTL-M is required for the expression of QTL-H and QTL-G

(*32*). The chemical nature of the resistance conferred by these QTLs remains largely unknown.

Inasmuch as the products from PI 229358 QTLs are detrimental to insect growth and behavior, there is a concern that meal derived from such insect resistant soybean seed could also have detrimental effects on animals when used for feed. Although rare, a few past efforts to develop disease-resistant cultivars through conventional breeding led to unacceptable levels of undesirable metabolites. The potato cultivar Lenape accumulated high levels of glycoalkaloids (*33*), and disease-resistant celery containing high levels of furanocoumarins was associated with dermatitis among grocery store personnel (*34, 35*). Therefore, it is prudent to ensure that soybean meal produced from plants carrying QTL-M, QTL-G, and QTL-H is as safe and wholesome as soybean meal produced from seed without these QTLs.

To determine if the addition of insect-resistance QTLs has negative effects on the feed quality of soybean meal, soybean meals were produced from soybean NILs containing the QTLs described earlier. Digestible amino acid content and nitrogen corrected true metabolizable energy (TME_N) were measured for each soybean meal, and diets containing each soybean meal were evaluated in a 21-day broiler feeding trial.

Materials and methods

Soybean meal production

Benning (*36*) and its insect-resistant NILs (*6*) were used in this study. Benning^{M} contains QTL-M, while Benning^{MGH} contains QTL-M, QTL-G, and QTL-H. **Fig. 4.1** shows Benning, Benning^{M}, and Benning^{MGH} plants in the field, exposed to soybean looper caterpillars. Benning is highly defoliated, whereas Benning^{M} is moderately defoliated, and Benning^{MGH} is the least defoliated. The NILs are similar to Benning for most agronomic

characteristics, including seed quality score, and protein, and oil content (*6*). Benning, Benning^{M}, and Benning^{MGH} were grown in 2011 at the University of Georgia Plant Sciences Farm. To avoid agronomic differences due to environment and ensure that differences among soybean meals were due to genotype, the lines were planted on the same date and in the same field prepared the same way. A total of 250 kg of seeds was harvested from each line. The soybean seeds were processed into meal at the Food Protein R&D Center, Texas A&M using industry-standard procedures (*37*).

Soybean meal composition

Proximate composition and amino acid content were determined for each soybean meal. Amino acid, dry mater, and crude protein analysis were performed according to AOAC methods 994.12, 930.15, and 990.03, respectively (*38*). Digestible amino acid content and nitrogen-corrected true metabolizable energy (TME_N) were determined according to Sibbald (*39*), and Dale and Fuller (*40*). To determine the digestible amino acid content of each soybean meal, the digestive tracts of 8 cecectomized 60-week old White Leghorn roosters (*Gallus gallus domesticus* L.) were cleared by a 30-hour feed withdrawal. Each rooster was precision-fed 35 g of soybean meal; 8 unfed roosters served as controls. Roosters were distributed to each treatment in a completely randomized design. For each individual rooster, excreta were collected for 48 hours after feeding; the samples were dried and analyzed for amino acid content (*38*). The amino acid digestibility protocol was modified to determine TME_N, in that non-cecectomized roosters were used in the assay. Excreta were analyzed as previously described for corrected true metabolizable energy (TME_N) (*39, 40*). Animal

procedures were approved by the Institutional Animal Care and Use Committee of the University of Georgia.

Broiler assay

The procedure was adapted from Davis (*41*). One hundred and eighty 1-day-old Cobb x Cobb male broiler chicks were selected from a larger population for uniform body weights. The chicks were maintained in electrically heated brooder batteries (24 pens per battery); each pen housed 5 chicks. The chicks were given constant illumination and free access to water. Diets were formulated on a digestible amino acid basis (**Table 4.1**). The dietary treatments, Benning, Benning^{M}, and Benning^{MGH}, were assigned in a completely randomize design to each pen. The experiment included 12 replicate pens per treatment. The experimental diets were fed until the chickens were 21 days of age. Weight gain and feed consumption were recorded for each pen at 7, 14, and 21 days of age. Animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Georgia.

Statistical analysis

Data were analyzed using JMP statistical software version 10.0 (SAS Institute, Inc., Cary, NC). Each dataset first was tested for normality using the Shapiro-Wilk test ($P < 0.05$) (*42*). A one-way ANOVA test ($P < 0.01$) was used to detect differences among soybean genotypes; and a post-hoc Tukey-Kramer multiple comparison test ($P < 0.05$) (*43-45*) was used to determine significant differences between soybean genotypes.

Results and discussion

Soybean meal composition

The overall nutrient profiles of soybean meal from Benning, Benning^{M}, and Benning^{MGH} were comparable (**Table 4.2**), despite minor differences that were detected on amino acid digestibility among soybean meals (**Table 4.2**). These variations could be the result of slight differences in seed composition, or differences in the small-batch processing of the meals. The TME_N values were similar for the three soybean meals, 2560, 2569, and 2544 kcal kg^{-1} for Benning, Benning^{M}, and Benning^{MGH} respectively (**Table 4.3**). The protein content in the prepared diets was not affected, as each diet was supplemented to adjust for the differences in meal composition (**Table 4.4**).

Broiler assay

The performance of the Cobb x Cobb male broilers was equivalent for Benning, Benning^{M}, and Benning^{MGH} diets, when measured at 7, 14, and 21 days of age (**Fig 4.2**). No statistically significant differences were found among diets for weight per chick, weight-gain per chick, and feed to gain ratio at 7, 14, and 21 days of age (**Table 4.4**). The Cobb-Vantress guideline for ideal broiler weight at 7, 14, and 21 days of age is 170, 449, and 885 grams respectively (*46*). The mean weight of Benning-, Benning^{M}-, and Benning^{MGH}-fed broilers was very close to the ideal weight.

Animal feeding trials are routinely conducted to determine the nutritive value of transgenic crops. In feeding assays with broiler chicks, Kan and Hartnell (*47*) demonstrated that insect-resistant soybean meal is nutritionally equivalent to non-transgenic cultivars, and McNaughton et al. (*48*) determined that high-oleic soybean seeds were comparable to non-transgenic controls. Although the insect-resistant lines Benning^{M} and Benning^{MGH} were

developed through conventional breeding and their agronomic characteristics are similar to those of Benning, the rationale for this study was to ensure that the insect-resistance QTLs derived from PI 229358 do not alter the nutritional value and safety of soybean meal. Since PI 229358 has been used in soybean breeding programs worldwide as a source of genetic resistance to leaf-chewing insects, the results of this study are highly relevant. Overall, the nutritional composition of Benning^{M} and Benning^{MGH} soybean meals is equivalent to Benning soybean meal. No biologically significant differences were detected among broiler chicks fed on Benning, Benning^{M}, and Benning^{MGH} for weight, weight gain, and fed-to-gain ratio; therefore there is no indication that meal produced from soybean seed carrying QTL-M, QTL-G, and QTL-H would not be as safe as the insect-susceptible Benning soybean cultivar when used for animal feed.

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Table 4.1. Nutritional composition of experimental diets containing soybean meal from Benning, and its insect-resistant isolines, Benning^{M} and Benning^{MGH}.

	Diet[1]		
	Benning	**Benning^{M}**	**Benning^{MGH}**
Ingredient %			
Corn	52.980	52.174	50.644
Soybean meal	40.387	41.156	42.289
Soybean oil[2]	2.697	2.742	3.142
Limestone	1.297	1.298	1.299
Dicalcium Phosphate	1.189	1.182	1.174
Salt	0.266	0.270	0.266
Sodium Carbonate	0.245	0.243	0.248
L-Lysine, HCl 78.8%	0.151	0.136	0.155
DL- Methionine 99%	0.378	0.380	0.370
L-Threonine, 98%	0.068	0.075	0.071
Choline Chloride 60%	0.020	0.020	0.020
Quantum Phytase XT 2,500	0.020	0.020	0.020
Vitamin mix[2]	0.227	0.227	0.227
Mineral mix[3]	0.075	0.075	0.075
Calculated analysis			
AME (kcal/kg)	3031	3031	3031
Crude protein (%)	23.467	22.836	22.927
Calcium (%)	0.950	0.950	0.950
Available phosphorus (%)	0.475	0.475	0.475
Digestible total sulfur (%)	0.950	0.950	0.950
Digestible lysine (%)	1.250	1.250	1.250
Digestible threonine (%)	0.812	0.812	0.812

[1]Starter diet was fed from day 1 to 21 days of age.
[2]Restaurant's Pride Advantage Soybean Oil (F.A.B, Inc., Alpharetta, GA, USA).
[3]Vitamin mix (DSM Nutritional Products Ltd., Pendergrass, GA, USA) provided the following per 100 g of diet: vitamin A, 551 IU; vitamin D_3, 110 IU; vitamin E, 1.1 IU; vitamin B_{12}, 0.001mg; riboflavin, 0.44 mg; niacin, 4.41 mg; d-pantothenic acid, 1.12 mg; choline, 19.13 mg; menadione sodium bisulfate, 0.33 mg; folic acid, 0.55 mg; pyridoxine HCl, 0.47 mg; thiamin, 0.22 mg; d-biotin, 0.011 mg; and ethoxyquin, 12.5 mg.
[4]Mineral mix (Southeastern Minerals Inc., Bainbridge, GA, USA) provided the following in mg per 100 g of diet: Mn, 6.0; Zn, 5.0; Fe, 3.0; I, 0.15; Cu, 0.05; and Se, 0.05.

Table 4.2. Crude protein, amino acid content, and amino acid digestibility of Benning, Benning^{M}, and Benning^{MGH} soybean meals

	Benning	**Benning^{M}**	**Benning^{MGH}**
Total content		**%**	
Dry matter	89.44	92.10	90.27
Amino Acid			
Alanine	2.11	2.00	2.01
Arginine	3.37	3.21	3.11
Aspartic acid	5.21	4.94	4.89
Cysteine	0.71	0.67	0.70
Glutamic acid	8.11	7.70	7.45
Glycine	2.01	1.91	1.90
Histidine	1.25	1.19	1.18
Isoleucine	2.11	2.01	1.98
Leucine	3.48	3.32	3.25
Lysine	2.91	2.78	2.74
Methionine	0.59	0.56	0.56
Phenylalanine	2.46	2.38	2.31
Proline	2.26	2.28	2.23
Serine	2.41	2.29	2.19
Threonine	1.90	1.81	1.80
Tryptophan	0.62	0.61	0.59
Tyrosine	1.20	1.12	1.08
Valine	2.27	2.17	2.14
Digestibility			
Alanine	83.10 ± 0.27^{b}	84.95 ± 0.14^{a}	85.22 ± 0.21^{a}
Arginine	84.25 ± 0.13^{c}	91.53 ± 010^{a}	88.06 ± 0.09^{b}
Aspartic acid	85.17 ± 0.14^{c}	87.75 ± 0.16^{a}	86.26 ± 0.01^{b}
Cysteine	72.02 ± 0.05^{b}	76.06 ± 0.15^{a}	74.46 ± 1.14^{ab}
Glutamic acid	86.96 ± 0.18^{b}	89.54 ± 0.15^{a}	89.08 ± 0.05^{a}
Glycine	71.00 ± 0.17	71.00 ± 0.00	70.35 ± 0.43
Histidine	85.88 ± 0.09^{c}	88.97 ± 0.03^{a}	86.61 ± 0.11^{b}
Isoleucine	86.97 ± 0.05^{c}	89.71 ± 0.05^{a}	88.66 ± 0.07^{b}
Leucine	87.86 ± 0.02^{c}	90.66 ± 0.07^{a}	89.83 ± 0.12^{b}
Lysine	87.30 ± 0.04^{c}	90.81 ± 0.15^{a}	88.67 ± 0.07^{b}
Methionine	90.57 ± 0.00^{c}	92.32 ± 0.06^{a}	91.43 ± 0.06^{b}
Phenylalanine	89.22 ± 0.16^{c}	91.61 ± 0.09^{a}	90.82 ± 0.09^{b}
Proline	84.23 ± 0.05^{c}	88.16 ± 0.09^{a}	87.11 ± 0.03^{b}
Serine	86.31 ± 0.23^{b}	88.57 ± 0.12^{a}	86.81 ± 0.18^{b}
Threonine	82.09 ± 0.17^{b}	83.86 ± 0.19^{a}	83.05 ± 0.17^{ab}
Tryptophan	89.20 ± 0.11	89.22 ± 0.06	89.50 ± 0.28
Tyrosine	91.24 ± 0.06^{c}	93.44 ± 0.09^{a}	92.38 ± 0.09^{b}
Valine	86.28 ± 0.02^{b}	88.84 ± 0.19^{a}	88.18± 0.09^{a}
Total AA	38.53	37.96	36.72
Total EAA	18.15	18.01	17.38
Total NEAA	20.38	19.95	19.34

[1] Amino acid and dry mater analysis were performed according to AOAC methods(*38*)

994.12 and 930.15, respectively.
[2] Amino acid digestibility was determined according to Sibbald(*39*).
[3] Values are mean ± SEM. Means within a row with a different letter subscripts differ, $P < 0.05$.
[4]AA: amino acid; EAA: essential amino acid; NEAA: non-essential amino acid.
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Table 4.3. Chemical composition and nitrogen corrected true metabolizable energy (TME_N) content of Benning, Benning^M, and Benning^{MGH} soybean meals.

Soybean line	**TME_N**		**Protein**	**Fat**	**Moisture**	**Ash**
	As is	Dry				
	kcal/kg	kcal/kg	----------%----------			
Benning	2560±21	2789±23	49.30	0.87	8.20	6.09
Benning^M	2569±23	2880±26	46.39	0.89	10.80	5.90
Benning^{MGH}	2544±23	2832±26	46.15	1.17	10.14	6.03

[1]TME_N was determined using the methodology described by Dale and Fuller(*40*).

Table 4.4. Growth performance of Cobb x Cobb male broilers fed Benning, Benning^{M}, and Benning^{MGH} diets from 1 to 21 days of age.

	Body weight gain (g/bird)	Feed to gain
1 to 7 days of age		
Benning	126 ± 3	1.11 ± 0.01
Benning^{M}	129 ± 2	1.14 ± 0.01
Benning^{MGH}	127 ± 4	1.11 ± 0.02
1 to 14 days of age		
Benning	395 ± 10	1.20 ± 0.02
Benning^{M}	396 ± 7	1.22 ± 0.02
Benning^{MGH}	389 ± 8	1.19 ± 0.01
1 to 21 days of age		
Benning	848 ± 20	1.35 ± 0.03
Benning^{M}	853 ± 13	1.38 ± 0.02
Benning^{MGH}	826 ± 11	1.36 ± 0.02

[1]The values are means ± SEM.

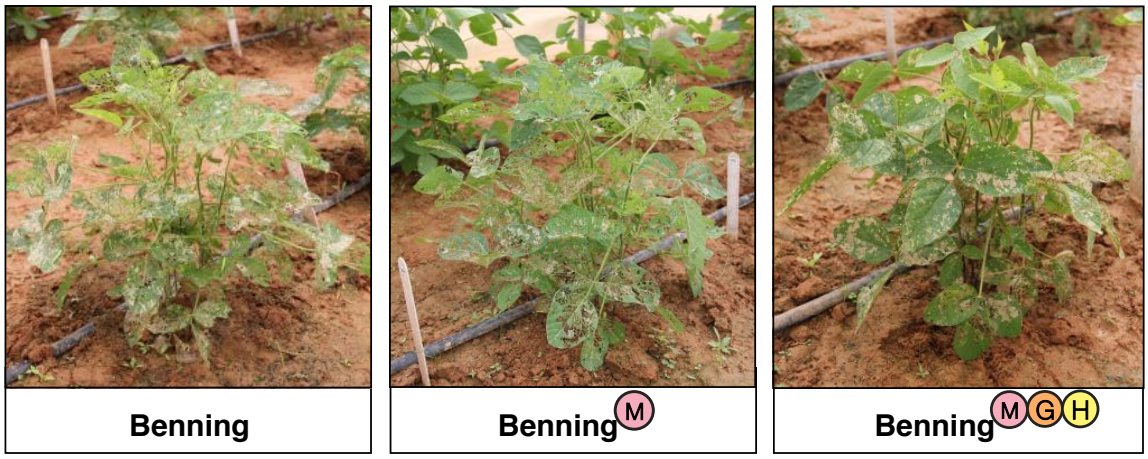

Figure 4.1. Benning, Benning^{M}, and Benning^{MGH} soybeans exposed to soybean looper caterpillars in a field-cage assay.

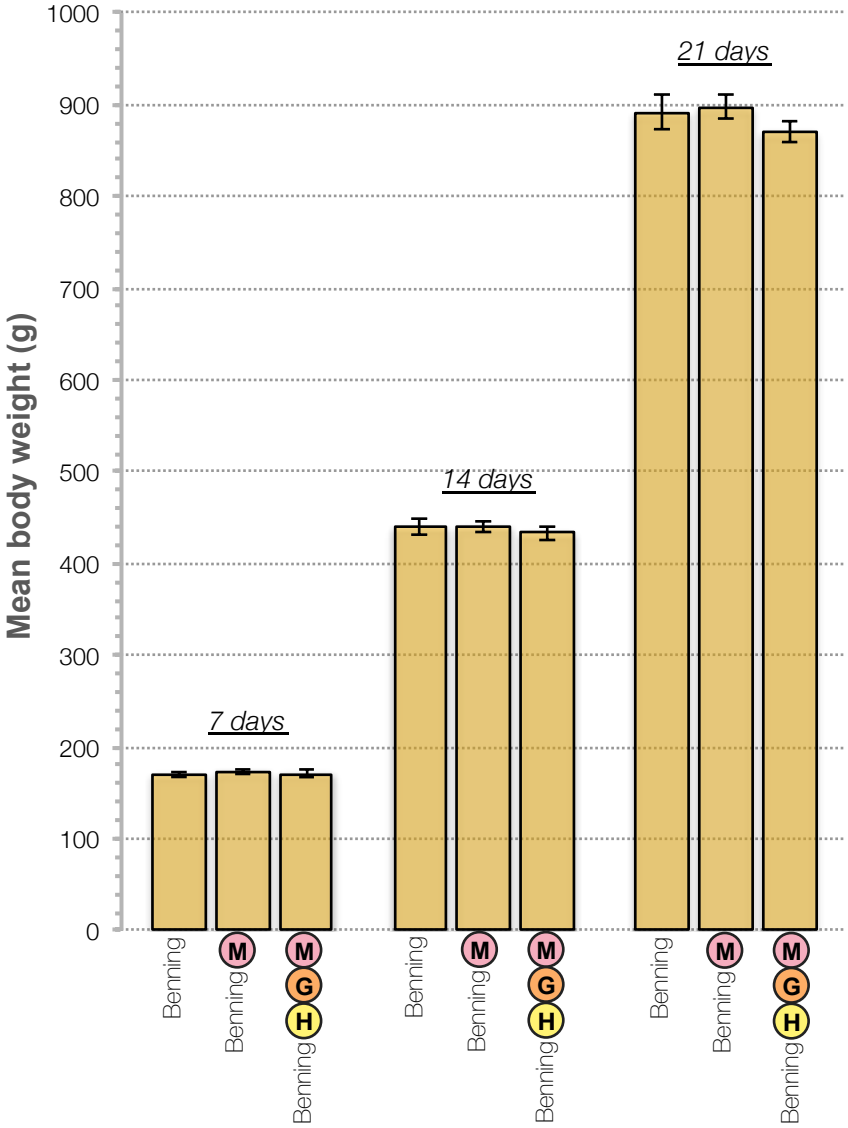

Figure 4.2. Weight of Cobb x Cobb male broiler chickens feeding on Benning, Benning^{M}, and Benning^{MGH} soybean meals at 7, 14, and 21 days of age.

CHAPTER 5

GMORUGA CONTROLS SOYBEAN RESISTANCE TO LEAF-CHEWING CATERPILLARS

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Abstract

Caterpillars and beetles are among the most economically damaging defoliating insects. The quantitative trait locus QTL-M is a major determinant for resistance to leaf-chewing insects in soybean. *GmORUGA*, the gene underlying QTL-M, was cloned and found to encode a wound-inducible genistein glucosyltransferase. Susceptibility is prevalent in the U.S. soybean germplasm, and resistance is caused by a mutation that results in a truncated protein. Expressing the susceptible allele in resistant plants restores susceptibility, and silencing the susceptible allele results in resistance. After insect attack, leaves from resistant plants contain less genistin, an isoflavone glycoside, and accumulate condensed tannins. *GmORUGA* is the first leaf-chewing insect resistance gene cloned from soybean; its discovery clarifies the role of isoflavones and condensed tannins in soybean resistance to leaf-chewing insects, which will lead to developing insect-resistant cultivars that ultimately can be planted to lower production costs and reduce insecticide applications.

Abbreviations:

BAC: bacterial artificial chromosome

bp: base pair

Chr: chromosome

CT: condensed tannins

GmUbiP: *Glycine max* ubiquitin promoter

HPLC: high performance liquid chromatography

INDEL: insertion-deletion

Kb: kilobase pair

LG: linkage group

MAS: marker-assisted selection

miRNA: micro RNA

MIGS: miRNA induced gene silencing

NIL: near-isogenic line

PI: plant introduction

PVDF: polyvinylidene Difluoride

PDA: photodiode array

qRT-PCR: real-time quantitative reverse transcription PCR

QTL: Quantitative trait locus

RACE: rapid amplification of cDNA ends

rbcsT: *Pisum sativum* rubisco terminator

RSL: recombinant substitution line

SBL: soybean looper (*Chrysodeixis includens*)

SHaM: Soybean histodifferentiation and maturation

SNP: single nucleotide polymorphism

SSR: simple sequences repeat

StUbiP: *Solanum tuberosum* ubiquitin promoter

StUbiT: *Solanum tuberosum* ubiquitin terminator

UGT: UDP-glucosyltransferase

Introduction

Soybean [*Glycine max* (L.) *Merr*] is one of the world's primary sources of vegetable oil and protein (*1*). However, each year 11% of the worldwide crop is lost to animal pests including insects (*2*). A third of the world's soybean is produced in the USA (*3*). In 2014, in the Southern USA alone, $500 million of the soybean production was lost to insects, including control costs and damages (*4*). Unfortunately the amount of insecticides applied to soybeans in the USA has quadrupled between 2002 and 2012, and as a result, the soybean's impact to freshwater ecotoxicity has increased three-fold (*5*). The need to lower cost of production, along with increased concern over insecticide residues in the food chain and environment, are incentives to develop insect-resistant soybean cultivars.

1. The leaf-chewing insects corn earworm [*Helicoverpa zea* (Boddie)], soybean looper [SBL, *Chrysodeixis includens* (Walker)], velvetbean caterpillar [*Anticarsia gemmatalis* (Hübner)], and bean leaf beetle, [*Cerotoma trifurcata* (Forster)] are among the most economically important pests of soybeans in the USA (*4, 6*). Although soybean plants can withstand moderate levels of leaf damage, high levels of defoliation greatly reduce seed yield and quality (*7*). The Japanese soybean landrace Sodendaizu (PI 229358) (*8*) is one of the most widely used sources of host-plant resistance to insects in soybean breeding programs. Since its discovery in 1971, PI 229358 has been reported to be resistant to multiple coleopteran, lepidopteran, and hemipteran insects (*9-24*). PI 229358's resistance is conferred by three quantitative trait loci (QTLs) named M, G, and H, that function via both antibiosis and antixenosis (*25, 26*). Antibiosis is a type of resistance in which the plant has a detrimental effect on insect growth, development, and/or reproduction (*27*). Antixenosis or non-preference is a type of resistance in which the plant affects insect behavior, by discouraging oviposition, colonization, or feeding (*27, 28*). QTL-M confers both

types of resistance (*25, 26*), accounting for 37% of antixenosis variance, and up to 28% of antibiosis variance (**Fig 5.1**). QTL-H and QTL-G only have a significant effect if QTL-M allele from PI 229358 is present in the genome (*29*). Introgressions of QTL-M have been verified in thirteen insect-resistant soybean lines developed in several soybean breeding programs through phenotypic selection, before molecular markers were available (*30*), thus establishing that QTL-M is effective in different genetic backgrounds. Furthermore, QTL-M enhances insect resistance when pyramided with host-plant resistance and Bt-mediated resistance in soybean (*31-33*).

Although QTL-M is by far the major determinant of leaf-chewing insect resistance discovered in soybean to date, the gene controlling QTL-M and the mechanisms by which insect-resistance is achieved are still unknown. For that matter, the basis for insect resistance remains poorly understood in most other crops (*34*). The objectives of this study are: (i) to identify and validate the gene underlying QTL-M, and (ii) to study secondary metabolite responses that may be involved in soybean's response to attack by chewing insects, and are associated with resistance. The ultimate goal of this work is to gain a better understanding of the basis for resistance to chewing insects in soybean, which will lead to the design of sustainable insect resistance management strategies in the longer term.

Results and Discussion

Genetic mapping placed QTL-M in a 0.52-cM interval (1.7 Mb) on its namesake Linkage Group M, now soybean chromosome 7 (Chr 7) (*29*). To fine-map QTL-M to an interval suitable for cloning, a set of recombinant substitution lines (RSLs) containing different PI 229358 introgressions in QTL-M, in a susceptible 'Benning' (*35*) background (**Fig 5.2A**) was developed. Insect resistance (**Fig 5.2B**) co-segregated with a 182-kb segment (**Fig 5.2C**). In Williams 82

(*36*), this 182-kb segment contains 11 annotated gene models (*37*), none of which resemble any canonical gene involved in plant resistance to insects (*38*). To obtain the corresponding sequence in PI 229358, clones from the PI 229358 BAC library (*39*) were identified by probe hybridization (**Fig 5.2C**), and a contig was assembled using BAC-end sequences. Two overlapping clones were sequenced (**Fig 5.2D**), and their assembled sequence was aligned to that of Williams 82 (**Fig 5.2E**). The 182-kb segment in Williams 82 corresponds to a 187-kb segment in PI 229358. The differences between PI 229358 and Williams 82 consist of 216 SNPs, 68 INDELs, including a 5.4-kb insertion in PI 229358, and 588-bp and 599-bp deletions in PI 229358. Polymorphisms were found in the coding sequence of seven genes, therefore narrowing down the possible candidate genes to these seven (**Table 5.1**). The three large INDELs in PI 229358 were excluded as candidate sequences for insect resistance because the same alleles are also found in the susceptible Benning.

To narrow the number of candidate genes for QTL-M resistance, the sequences for the seven polymorphic genes between PI 229358 and Williams 82 were also obtained from a panel of 34 of insect-susceptible soybean accessions, including the 32 accessions that form most of the USA soybean ancestral germplasm pool (*40*), and Benning and Jack (*41*) (**Fig 5.3A**). In this approach, all genes for which a SNP had the same allele in PI 229358 and any of the susceptible accessions were excluded as candidate genes. Only two genes, Glyma0714470, a predicted Ploop-NTPase, and Glyma07g14530, a predicted isoflavone glucosyltransferase, have SNP alleles that are unique to PI 229358 (**Fig 5.3B**). RT-PCR indicated that both of these genes are expressed in soybean leaves before insect attack, and Glyma07g14530 was upregulated after insect attack in both the susceptible Benning and the resistant Benning^{M} (*42*) (**Fig 5.4A**).

Since full-length complementary DNAs (cDNAs) are essential for the functional analysis of genes and their products, attempts were made to clone full-length cDNAs of Glyma07g14470 and Glyma07g14530 from Benning and Benning^{M}. The Glyma07g14470 full-length cDNA could not be recovered; its sequence is a chimera between Glyma01g45590 and Glyma03g35470, and the amplified products corresponded to either of these genes. To overcome this, a genomic clone of Glyma07g14470 first was expressed in arabidopsis. The full-length cDNA isolated from arabidopsis showed that contrary to the original annotation, its SNP is part of an intron (**Fig 5.4B**). Although a RT-PCR product (170 bp) matching Glyma07g14470's sequence was obtained from soybean leaves as shown in **Fig 5.4A**, it is likely that this product was amplified from another soybean-gene transcript with sequence similarity to the annotated Glyma07g14470. This gene was eliminated in most recent update for the soybean gene models (Glyma 2.0) (http://www.soybase.org). For Glyma07g14530, the full-length cDNA isolated from Benning and Benning^{M} leaves consists of a single 1476-bp intronless reading frame; thus the original gene model (Glyma 1.0) was also mis-annotated. In the corrected annotation, the unique SNP is no longer in an intron.

To further confirm if Glyma07g14530 is the QTL-M gene, both Glyma07g14470 and Glyma07g14530 were sequenced from a panel of 17 cultivated and wild soybean (*Glycine soja* Sieb. & Zucc.) accessions that have been reported to be insect-resistant, though such resistance has no always been verified (*43, 44*). PI 227687, which has resistance that does not map to QTL-M (*25, 26, 45*), was included (**Fig 5.3C**). The PI 229358 allele for Glyma07g14470's SNP was shared with six resistant accessions, including PI 227687; this further eliminates Glyma07g14470 as the candidate gene, as the resistance of PI 227687 maps to a different chromosome altogether. In contrast, the PI 229358 allele for Glyma07g14530 is shared with four other resistant

accessions, but not with PI 227687. Collectively, these results strongly suggest that Glyma07g14530 is the QTL-M gene (**Fig 5.3D**).

Based on its full-length cDNA, the Glyma0714530 protein in the susceptible soybeans is 491 amino acids long. The resistant SNP allele (cDNA position 825) is a mutation of TG**G** (^{275}W) to TG**A** (stop), which leads to a truncated protein of 274 amino acids. The Glyma07g14530 promoter contains four W-box motifs (TTGAC), which are predicted binding sites for WRKY transcription factors involved in regulating plant immune responses to biotic and abiotic stresses (*46*). A time-course assay was used to determine if Glyma07g14530 expression in leaves is induced by chewing-insect feeding. The gene is induced in whole plants as early as 24 hr after infestation; in the 72-hr samples, Glyma07g14530 transcripts were up to 40-fold higher than those in non-infested leaves. Even in detached leaves, a 27-fold higher induction took place. No significant differences in induction level were found between resistant and susceptible soybeans, or between Jack and Benning, thus indicating that resistance is not caused by differences in transcription levels (**Fig 5.4C**). Therefore the hypothesis was that resistance is achieved by the loss-of-function of Glyma07g14530, whereas susceptibility is conferred by the functional gene. This hypothesis is supported by evidence that QTL-M resistance is inherited as a partially recessive locus. Plants heterozygous for QTL-M have an intermediate level of resistance (*25*).

Transgenic soybean lines were used to test the loss-of-function hypothesis. In complementation lines, the susceptible allele Glyma530-S was expressed in Jack^{M} plants to restore susceptibility. Jack expressing Glyma530-R was used as a control. To test the effect of silencing Glyma530-S in Jack, a miRNA-induced gene-silencing construct, carrying miR1510 (*47*) fused to 100-bp of the Glyma07g14530 gene was expressed in Jack. Jack^{M} plants carrying

the transgenic construct were the controls. The complementation lines in which both transgene and native Glyma530 gene were expressed were evaluated for insect resistance in bioassays with SBL caterpillars (**Fig 5.5**). The same was done for the silencing lines in which the transgene was expressed but the native Glyma530 gene was silenced. In the T_2 generations, in a greenhouse (choice) assay to measure resistance as percent defoliation, $Jack^M$ plants expressing Glyma530-S were more defoliated than those of $Jack^M$ and less defoliated than Jack (**Fig 5.6A**). In contrast, silencing lines of Jack expressing 1510:530 were less defoliated than plants the Jack controls (**Fig 5.6B**). Similarly, in the T_3 generations, in a growth chamber (non-choice) assay in which resistance is measured as caterpillar weight, caterpillars feeding on $Jack^M$ plants expressing Glyma530-S were larger than caterpillars feeding on $Jack^M$ itself; two of the lines produced caterpillars that were as big as those fed on Jack (**Fig 5.6C**), while those feeding on silencing lines of Jack were smaller (**Fig 5.6D**). Altogether, these experiments confirmed that complementing a QTL-M resistant line with the susceptible Glyma530-S allele restores susceptibility, and that silencing Glyma530-S in susceptible lines results in resistance (**Fig .5.7**). Therefore the Glyma07g14530 gene present in most of the soybean germplasm is an allele for susceptibility, and the mutation found in PI 229358 and the other resistant accessions is responsible for the insect resistance associated with QTL-M. Because of its role in resistance to leaf-chewing insects, the gene has been named *GmORUGA*, derived from the Spanish word for caterpillar.

GmORUGA is a predicted glycosyltransferase (GT). GT enzymes catalyze the transfer of a sugar group from a donor to a target molecule; the target molecule can be a lipid, a protein, or an oligosaccharide (*48*). GTs are classified into 98 superfamilies ((*49*), http://www.cazy.org). The superfamily 1 (GT1) contains the UDP-dependent glycosyltransferases (UGTs), which use UDP-activated sugars (e.g., UDP-glucose, UDP-galactose, and UDP-rhamnose) as sugar donors

(*50*). GT1 contains all the plant secondary products glycosyltransferases (PSPGs), which modify all major secondary metabolites, including phenolics, terpenoids, cyanohydrins, thiohydroximates, and alkaloids (*51*). All PSPGs contain a sequence of 44 amino acid residues known as the PSPG box that contains the amino acid residues that interact with the UDP sugars (*51*). The PSPG box is used to classify each GT1 in subgroups. Based on the sequence of the PSPG motif (**Fig 5.8B**), *GmORUGA* can be classified as a group E enzyme (*52*). In *GmORUGA,* the PSPG motif starts at amino acid residue 336. As a result, the truncated protein associated with *GmORUGA*-R lacks the PSPG motif, which further indicates that resistance is due to loss of function.

In arabidopsis, group E consists of 21 genes classified in the UGT families 71 and 72, and the gene UGT88A1 (*53*). *GmORUGA* is classified in the family 72, subfamily B (www.phytozome.jgi.doe.gov), and shows the highest homology with UGT72B2, which glycosylates hydroxycoumarins in vitro (*54*). The soybean genome contains 182 putative GT1-like genes; 36 of these genes are classified as group E enzymes. During the evolution of higher plants, the phylogenetic groups A, D, E, G, and L have expanded more than the other groups. To identify the putative function of *GmORUGA,* the protein sequence was blasted (BLASTP) to identify homologous, putative and characterized, genes in other plant species. The most similar proteins were all found in legumes. The phylogenetic tree containing *GmORUGA* and the related PSPGs identified by BLASTP is shown in **Fig 5.8A**.

Of the legume UGTs with characterized functions, the kudzu isoflavone-7-glucosyltransferase GT07O02; which is also a group E, family 72, subfamily B enzyme; is the protein with a match to *GmORUGA,* suggesting that soybean's *GmORUGA* could be an isoflavone-7-glucosyltransferase. Isoflavones are a type of flavonoids almost exclusively produced in

legumes. Group-E UGTs seem to be enriched in legumes. In kudzu, from the 15 UGT enzymes cloned by He et al. (*55*), six UGTs including GT07O02 were classified in group E. Caputi et al (52) performed a phylogenetic analysis of group-E UGTs in higher plants. In this study, the family 72, subfamily B clade was formed by two genes from saffron [*Crocus sativus* L.], three genes from arabidopsis, three genes from grape [*Vitis vinifera* L.], four genes from poplar [*Populus trichocarpa* Torr. and Gray], four genes from yellow monkeyflower [*Mimulus guttatus* DC.], seven genes from sorghum [*Sorghum bicolor* (L.) Moench], eight genes from rice [*Oryza sativa* L.], eleven genes from apple [*Malus domestica* Borkh.], and twenty-four genes from soybean. This further shows that the family 72, subfamily B clade of UGTs has expanded in legumes.

In soybean, the isoflavone aglycones are daidzein, genistein, and glycitein, which are converted by UGTs into daidzin, genistin, and glycitin respectively (*56*). Two isoflavone-7-glucosyltransferases, *GmIF7GT* and *GmUGT4*, have been characterized in soybean (*57, 58*). However, the soybean genome encodes a large number of UGT homologs that are highly transcribed and whose function is still unknown (*59*). The more than 7000 flavonoids identified in different plant species originate from combinatorial modifications to a common aromatic structure, and glycosylation is one of their most predominant modifications (*60*). Insects landing or walking on a leaf encounter flavonoid aglycones that can accumulate on the leaf surface, before encountering flavonoid glycosides that are stored in vacuoles within the leaf (*61*). It is generally accepted that flavonoids, along with other plant polyphenols, can play a role in plant interaction with insect herbivores (*62*); however the mechanisms by which these compounds regulate the insect behavior remains unknown.

To determine if *GmORUGA* plays a role in isoflavone glycosylation, isoflavone profiles of Benning, Benning^{M}, Jack, and Jack^{M} leaves were compared before (0 hr) and post-infestation (72

hr). The leaf samples were analyzed by HPLC to determine the concentration of the flavanol aglycones kaempferol and quercetin, and the flavanol glycoside rutin (quercetin-3-O-rutinoside); the isoflavone aglycones glycitein, genistein, daidzein, and formononetin; and the isoflavone glycosides glycitin (glycitein-7-O-glucoside), genistin (genistein-7-O-glucoside), and daidzin (daidzein-7-O-glucoside) in the leaf samples.

Genotype- specific differences were found between Benning and Jack. The major compound in Benning is daidzin (1281 ± 216 μg g^{-1} leaf tissue), and no differences were observed between Benning and Benning^{M}, before or after infestation. The major compound in Jack is rutin (1909 ± 38 μg g^{-1} leaf tissue). Glycitin was not detected in Benning, but it is present in Jack. The only compound that is different between susceptible and QTL-M plants, independent of genotype, is genistin. The measured concentrations of genistin were the same between Benning and Benning^{M}, and Jack and Jack^{M} before insect attack. However, after insect attack, genistin concentration was significantly reduced in both Benning^{M}, and Jack^{M} (**Fig 5.9**). These results suggest that *GmORUGA* is a genistein 7-O-glucosyltransferase that is induced in leaf tissue after caterpillar damage to produce genistin. In agreement with the loss-of-function model, the functional enzyme in the susceptible soybean contributes to maintaining a constant concentration of genistin after insect attack, whereas this enzyme is not active in the QTL-M plants, and genistin levels are significantly reduced.

Variation in genistin levels could be associated with insect resistance, if metabolic flux within the phenylpropanoid pathway gets re-routed in QTL-M plants. The rationale for this is that levels of isoflavone aglycones do not increase in QTL-M plants (**Fig 5.9**), and naringenin, which is also used to synthesize anthocyanins and condensed tannins (CTs), is immediately upstream of genistin (**Fig 5.10A**). Red/brown spots indicating CT accumulation are observed in

QTL-M plants after infestation (**Fig 5.10C**). Therefore, the levels of CTs were compared between susceptible and QTL-M plants. Tannins have been studied in the last 50 years for their role on plant resistance to insects, albeit with inconclusive results. For instance, CT induction in leaf tissue of *Populus* species is associated with increased resistance to herbivores (*63*). However transgenic hybrid aspen [*Populus tremula* L. *x* . *tremuloides* Michx.] plants containing high CT levels, as a result of the overexpression of the transcription factor *PtMYB134*, were preferred by the forest tent caterpillar [*Malacosoma disstria* Hübner] and the gypsy moth caterpillar [*Lymantria dispar* L.], over the non-transgenic control (*64*). The network of regulatory proteins and transcription factors controlling the CT pathway and its response to environmental stimuli, such as caterpillar feeding, remain largely unknown (*65*). CT levels, estimated in a colorimetric assay, increase in both Benning^{M} and Jack^{M} after infestation, while they remain constant in Benning and Jack (**Fig 5.10D**), presumably through a redirection of metabolic flux to produce CTs in lieu of genistein.

Genes for resistance to leaf-chewing insects have been described in plants, but none provide any insight into insect resistance for soybean. What these genes have in common is the accumulation of a metabolite that is detrimental to insects. For instance, in arabidopsis, a loss-of-function version of an epithiospecifier gene (*ESP*) deters cabbage looper (*Trichoplusia ni* Hübner) by promoting the formation of isothiocyanate instead of nitriles (*66*). In maize, a recessive allele of a QTL for resistance to CEW increases the concentration of maysin, a C-glycosyl flavone with antibiotic effects to these caterpillars (*67*). However, in their natural habitats plants encounter a variety of insect pests, thus resulting in complex plant-insect interactions. Therefore, in the same plant, the induction of a gene that provides resistance against a particular insect pest, may lead to susceptibility to a different insect pest. This phenomenon has been described in maize by Tzin et al. 2015 (*68*). In maize cultivars carrying QTLs for resistance to the beet armyworm [BAW,

Spodoptera exigua Hübner], feeding by BAW caterpillars induces methylation of DIMBOA-Glc, resulting in the production of MBOA, which is a caterpillar deterrent. Alternatively, upon feeding by the corn leaf aphid [*Rhopalosiphum maidis* Fitch], DIMBOA-Glc is converted to DIMBOA, which is an aphid deterrent. Tzin et al. found that beet armyworm-induced DIMBOA-Glc methylation promotes progeny production in corn leaf aphids, when aphids fed on leaves already exposed to beet armyworms. A similar phenomenon could explain why *GmORUGA* is functional and upregulated by leaf damage, in leaf-chewing-insects susceptible soybeans, which are the majority of the soybean germplasm; and how the loss-of-function of *GmORUGA* gave rise to resistance. However, the pest that was or is still controlled by the functional gene has not been found yet. This is the first report of a gene for resistance to leaf-chewing insects that operates via the isoflavone pathway.

Materials and Methods

Plant materials

The Japanese soybean landrace 'Sodendaizu' (PI 229358) is the source of the resistant allele of QTL-M (*8*). Benning is an elite soybean variety adapted to Georgia that is susceptible to leaf-chewing insects (*35*), as are almost all other soybean varieties. Five soybean Recombinant Substitution Lines (RSLs) representing different recombination events in the QTL-M region were used to fine map the insect resistance locus. RSL 42, 47, 48, and 50 are resistant to leaf-chewing insects, and RSL 54 is susceptible. To generate the RSLs, Benning^{M} (i.e., Benning with the resistant allele of QTL-M introgressed into it) which was developed from Benning x PI 229358 through Marker-Assisted Selection (MAS) was crossed to Benning, to obtain a population

of 1,991 $BC_7F_{1:2}$ plants. This population was screened through MAS, as described by Zhu (*29*), to identify heterozygous recombinant lines, which were self-pollinated to obtain homozygous RSLs (**Fig 5.2A**). The cultivar Jack (*41*) and the BC_3F_2-derived $Jack^M$ (*69*) were used for biolistic transformation. Jack, $Jack^M$, Benning, and the $BC_6F_{2:3}$-derived $Benning^M$ (*42*) were used for the gene expression, isoflavone profiling, and condensed tannins assays.

Identification of Ch.07 segment required for QTL-M resistance

To determine if the RSLs were susceptible or resistant to leaf-chewing insects, they were evaluated in antixenosis assays with SBL, as described by Zhu et al. (*29*). To identify the PI 229358 introgression in the RSLs, these were genotyped for SNP and SSR loci within the QTL-M region (*29*), between Sat_258 and Satt702 (*37*).

Polymorphic SNPs between Benning and PI 229358 within the QTL-M region, were discovered using the soybean genome assembly version 4.0 (*36*). Briefly, the 13,800-14,100 Mb segment from Chr.7 was used as template to design 18 primer pairs that amplified 400-600-bp fragments every 50-100 kb (**Table 5.2**). DNA samples from PI 229358 and Benning were amplified with the 18 primer pairs in a 20-µl PCR reaction. Each reaction consisted of 2 µL of 40 ng μl^{-1} genomic DNA, 1X PCR reaction buffer, 2.5 mM $MgCl_2$, 100 µM of each dNTP, 0.2 µM of each primer, and 0.5 U of GoTaq Flexi DNA polymerase (Promega, Madison, WI). PCR reactions were performed in a T100™ Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA). The PCR program consisted of an initial denaturation (94°C for 5 min), followed by 35 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 40 sec, and a final step of 72°C for 5 min. The products were analyzed on a 1.5% agarose, 1X TBE gel to verify amplification of a single product. Each product was sequenced using the BigDye Terminator v3.1 Cycle

Sequencing Kit (PE ABI, Foster City, CA). The amplicons were analyzed on an ABI 3730 automated sequencer (PE-ABI, Foster City, CA). For each fragment, the PI 229358 and Benning sequences were aligned to identify SNPs. Each RSL was then genotyped by amplifying and sequencing the SNP-containing fragments.

Sequences for the SSRs markers, Sat_425 and Satt729 (**Table 5.2**) were obtained from SoyBase (http://www.soybase.org) (*37*) and were used to genotype the RSLs. For SSR genotyping, the PCR reactions were prepared using the protocol described by Li et al. (*70*). The PCR products were analyzed on an ABI 3730 automated sequencer, and the data was processed with GeneScan v. 2.1 and Genotyper v. 2.5 software (PE ABI, Foster City, CA) using the procedures described by Li et al.(*70*). The SNP and SSR marker data was used to build a graphical genotype of the QTL-M region for each RSL. The graphical genotypes were compared to the insect-resistance phenotypes, to identify the PI 229358 introgression that is shared among all the resistant RSLs, and therefore contains QTL-M.

PI 229358 BAC library screening, and BAC-clone sequencing

To assist the cloning of the insect-resistance gene, the PI 229358 BAC library reported by Zhu et al. (*39*) was screened to identify BAC clones in the QTL-M region. High-density BAC filters were prepared as described by Zhu et al. (*39*), to be used in a hybridization-based screening of the library. Three DNA probes: two flanking, and one within QTL-M (**Fig 5.2C**), were used to screen the BAC filters. Primers designed from the Williams 82 reference genome (*36*) (**Table 5.2**), were used amplify the radiolabeled (^{32}P) probes from PI 229358 genomic DNA, following standard techniques (*71*). BAC-end sequences were obtained to determine the clone's order in the QTL-M region contig, based on the Williams 82 reference genome. Briefly, each clone was

inoculated in 5 ml of LB broth supplemented with 12.5 µg ml^{-1} chloramphenicol, and shaken at 280 rpm for 16 h at 37°C. BAC DNA was isolated from the liquid cultures, using a standard alkaline-lysis method (*71*). The BAC-DNA was used as template for sequencing with the universal primers T7 and M13 reverse, using BigDye Terminator v3.1 Cycle Sequencing Kit (PE ABI). Lastly, two overlapping clones covering QTL-M (**Fig 5.2D**) were fully sequenced at the Clemson University Genomics Institute (Clemson, SC).

Annotation of BAC sequences

The sequences from the BAC clones 134P08 and 118D14 were assembled and annotated in Geneious version 8 (http://www.geneious.com) (*72*). The primers S72_3610K-F and SNP13885-R, (**Table 5.2**), flanking the PI 229358 introgression required for QTL-M resistance (**Fig 5.2C**), were mapped to the BAC-clone sequence assembly. The PI 229358 sequence between S72_3610K-F and SNP13885-R was aligned to the corresponding sequence in Williams 82 (Glyma.Wm82.a1, Gmax1.01), which was obtained from SoyBase, using the mauveAligner algorithm (*73*). The soybean gene models (Wm82.a1.v1) obtained from SoyBase were used to identify differences (SNPs and INDELs) in the gene-coding sequences that could be associated with insect resistance.

Identification of candidate genes for QTL-M resistance

A panel of 34 insect-susceptible soybean accessions, including the 32 accessions that form most of the USA soybean ancestral germplasm pool (*40*), and Benning and Jack, was used to narrow the number of candidate genes for QTL-M resistance (**Fig 5.3A**). Briefly, primers (**Table 5.2**) were designed to amplify the 7 genes containing SNPs polymorphic between the

susceptible Williams 82 and the resistant PI 229358 (**Table 5.1**). PCR products were amplified with KAPA HiFi polymerase (Kapa BioSystems, Boston, MA), using genomic DNA from each of the 32 insect-susceptible lines as template. PCR products were visualized in a 1X TAE + cytidine gel. Single products were extracted from the gel using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Orange, CA). The purified PCR products were sequenced from both ends, using the gene-specific PCR primers. Sequencing reactions were carried out using the BigDye Terminator v3.1 Cycle Sequencing Kit (PE ABI), and analyzed on an ABI 3730 automated sequencer (PE-ABI). The sequences were quality-trimmed, and mapped to each corresponding reference gene model in Geneious. Each sequence alignment was analyzed to determine the allele for each insect-susceptible accession at each SNP. All genes for which a SNP had the same allele in PI 229358 and any of the insect-susceptible accessions were excluded as candidates for insect-resistance.

A panel of 18 insect-resistant soybean accessions (*43, 44*), including cultivated and wild soybean, was used to further identify the candidate gene for QTL-M resistance (**Fig 5.3B**). Two genes, Glyma07g14470 and Glyma07g14530, which had SNP alleles unique to PI 229358 in the screening of the 32 insect-susceptible accessions, were sequenced from the insect-resistant accessions. The PCR, sequencing, and sequence analysis procedures were as described previously. Any gene sharing a SNP allele in common between PI 229358 and PI 227687was further excluded as a candidate for insect-resistance, because PI 227687's resistance does not map to QTL-M.

DNA and RNA isolation

Genomic DNA was isolated from unexpanded trifoliolate leaves using the modified CTAB procedure described by Zhu et al. (*29*). DNA samples were resuspended in 50 µl of TE/RNase buffer (10 mM Tris-HCL [pH 8.0], 1 mM EDTA, 100 µg mL^{-1} RNAse A). Leaf tissue harvested for RNA isolation was immediately frozen in liquid nitrogen. Total leaf RNA was isolated from 100 mg of tissue; the tissue was homogenized in Tri-Reagent Buffer (Sigma, St. Louis, MO), in a 2010 GenoGrinder (Spex SamplePrep, Metuchen, NJ). RNA samples were processed according to the manufacturer's instructions. RNA samples were resuspended in 50 µL of RNAse-free water (Ambion, Austin, TX), and stored at -20°C.

Expression of Glyma07g14470 and Glyma07g14530 in leaf tissue

RNA isolated from Benning and Benning^{M} plants was used for RT-PCR to determine if the candidate genes are expressed in leaf-tissue. Briefly, five Benning^{M} and Benning plants were planted in 450-ml polystyrene foam cups filled with Fafard 2 mix (Conrad Fafard, Agawam, MA) with three holes were punched in the bottom to provide drainage. Plants were grown in an insecticide-free greenhouse under a photoperiod of 16 h. Sunlight was supplemented with 400 J s^{-1} Phillips ED-18 high-pressure sodium lamps (Phillips Inc., Andova, MA, USA) to keep the plants in a vegetative stage. The temperature was regulated to approximately 28°C during the day, and 20°C at night. Expanded trifoliolate leaves were collected for RNA isolation once the plants reached the V4 stage (*74*), and the plants were immediately infested with 30 neonate SBL caterpillars. Expanded insect-chewed trifoliolate leaves were collected for RNA isolation, 72 h after infestation. RNA was isolated as described previously, and the RNA extracts were treated with Turbo DNAse (Ambion) to remove contaminating DNA. RT-PCR primers for

Glyma07g14470 and Glyma07g14530, and the rest of the genes in QTL-M were designed in Geneious (**Table 5.2**). The metalloprotease gene, Glyma03g29351, was used as a constitutive gene control; RT-PCR primers are described by Libault (*75*). RT-PCR primer sequences to amplify the wound-inducible pathogenesis-related 10 (PR10) gene were obtained from Graham (*76*). First-strand cDNAs were synthesized in a 20-µl Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA), containing 1 µg of RNA and 1 µl of 500 µg ml^{-1} $Oligo(dT)_{12-18}$. First-strand cDNAs were used as templates in gene-specific PCR reactions. Each reaction consisted of 2 µL of first-strand cDNA, 1X PCR reaction buffer, 2.5 mM $MgCl_2$, 100 µM of each dNTP, 0.2 µM of each primer, and 0.5 U of GoTaq Flexi DNA polymerase (Promega, Madison, WI). PCR reactions were performed in a T100™ Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA), using a standard PCR program modified for the annealing temperature of each primer pair. PCR products were visualized in 1.5% TBE gels.

Cloning of Full-length cDNAs from Glyma07g14470 and Glyma07g14530

To confirm the annotation of the Glyma07g14470 and Glyma07g14530 transcripts, rapid cDNA ends (RACE) PCR was performed using leaf RNA isolated from Benning and Benning^{M} as templates. The cDNAs were amplified using the SMARTer RACE cDNA kit (ClonTech, Mountain View, CA) according to the manufacturer's instructions. The gene-specific primers described in **Table 5.2** were used for amplification and sequencing of the RACE products. Full-length transcripts corresponding to Glyma07g14470 could not be amplified from leaf RNA samples, due to the gene's chimeric nature and homology with other soybean genes. As an alternative, Glyma07g14470 was expressed in arabidopsis. Briefly, the coding sequences of Glyma07g14470 were amplified with KAPA HiFi polymerase (KAPA BioSystems, using the

primers described in **Table 5.2**, from Benning and Benning^{M}. The purified PCR products were cloned into the pDONR/Zeo plasmid vector (Invitrogen), by recombination between the *att*L1 and *att*L2 sites. The coding sequences were then transferred into the pEarleyGate202 expression vector (*77*) by recombination between *att*B1 and *att*B2 sites. The arabidopsis ecotype Landsberg erecta was transformed by the floral dip method (*78*) using *Agrobacterium tumefaciens* strain GV3101 carrying the pEarleyGate202 expression vectors. T_1 transformants were selected with 50 mg L^{-1} BASTA (glufosinate ammonium). Leaf RNA was isolated from transformants as described previously. The expression of the transgenes was confirmed by RT-PCR as previously described. Rapid cDNA ends (RACE) PCR reactions were performed to clone the full-length transcripts. Finally, the 5'-RACE and 3'-RACE products obtained from Glyma07g14470 in arabidopsis, and Glyma07g14530 in soybean, were assembled and compared to the corresponding annotated gene models in Geneious.

qRT-PCR assays for Glyma07g14530

To determine if Glyma07g14530 is induced by caterpillar damage, time-course qRT-PCR experiments were set up to measure Glyma07g14530 transcript levels at 0, 24, 48, and 72 h after infestation. The first experiment was used to test whole-plants. Briefly, four sets of five plants from each Benning, Benning^{M}, Jack, and Jack^{M} were grown in the greenhouse as described previously. After the plants reached the V4 stage of development, 0-h leaf samples were collected for RNA isolation from the first set of plants, from each genotype. Immediately after, the rest of the plants were infested with thirty SBL caterpillars each. Samples from sets 2, 3, and 4 of plants, were collected at 24, 48, and 72 h after infestation, respectively. The second experiment was used to test detached leaves. Briefly, four sets of five plants from each Benning,

Benning^{M}, Jack, and Jack^{M} were grown in the greenhouse until they reached the V4 stage of development. Then, 0-h leaf samples were collected for RNA isolation from the first set of plants. Immediately after for the rest of the plants, a trifoliolate leaf was collected from each plant and placed a 600-ml (20 oz) clear polystyrene cup (Letica Corporation, Rochester Hills, MI, USA) sealed with a dome lid (Letica Corporation); the cup contained 7 ml of plaster of Paris saturated with water to maintain 75% relative humidity. Three neonate SBL caterpillars were added to each cup. Infested cups were placed in a growth chamber set at 27°C, and a 14-h light period was maintained with fluorescent lights (T8 F032/730/Eco, Sylvania Octron, Danvers, MA, USA) providing ca. 40 µmol photons m^{-2} s^{-1} (*32*). Samples from sets 2, 3, and 4 of detached leaves, were collected at 24, 48, and 72 h after infestation, respectively.

Leaf RNA extractions were carried out as described previously. The RNA extracts were treated with Turbo DNAse (Ambion) to remove contaminating DNA. Fifty ng of treated RNA were used as template for KAPA SYBR FAST One-Step qRT-PCR Kit (Kapa Biosystems). Briefly, 10-µl qRT-PCR reactions were performed in duplicated in a LightCycler 480II (Roche Diagnostics, Indianapolis, IN). The qRT-PCR program was 42°C for 5 min and 95°C for 3 min, for reverse transcription; and 45 amplification cycles of 95°C for 10 sec, 65°C for 20 sec, and 72°C for 1 sec. The melting curve analysis, used to confirm the specificity of the qRT-PCR reactions, was set up from 60°C to 95°C, at a ramp rate of 0.11°C $second^{-1}$. The primer set 530qRT-F6 and 530qRT-R6 (**Table 5.2**) was used to amplify Glyma07g14530. The metalloprotease gene was used as reference gene to normalize the expression of Glyma07g14530. The primers used to amplify the metalloprotease gene are the same as described previously. To calculate the relative transcript abundance of Glyma07g14530. The LightCycler 480II Software (Roche Diagnostics) was used to perform the following analyses. PCR efficiencies of each primer

pair were determined using PCR reactions containing serial dilutions of each PCR product as template. Ct values for each qRT-PCR reaction were calculated using the Absolute Quantification analysis program. The ratio of target gene in comparison to the reference gene was calculated using the formula described by Pfaffl (*79*).

Vector construction and soybean transformation

Three plasmid vectors were used for complementation and silencing of Glyma07g14530 in transgenic soybean lines. Two overexpression vectors used in the complementation assays were built as follow. The coding sequences for Glyma530-S and Glyma530-R were amplified from leaf DNA isolated from Benning and Benning^{M} respectively, with the primers *Asc*I-530-OE-F and *Avr*II-530-OE-R (**Table 5.2**) using the Phusion HF polymerase (New England Biolabs, Ipswich, MA). The plasmid pGmURNAi2 contains a gene-of-interest (GOI) expression cassette driven by the *Glycine max* ubiquitin (GmUbi) promoter (*80*) and the *Pisum sativum* rubisco (rbcS) terminator (*81*). The GOIs were cloned between GmUbi and rbcS using the *Asc*I and *Avr*II restriction sites. The PCR products and pGmURNAi2 were digested with *Asc*I/*Avr*II. After ligation, the plasmids were used to transform 10-beta Competent *E. coli* cells (New England Biolabs); colonies were selected in solid LB medium supplemented with 50 µg ml^{-1} ampicillin. Positive colonies were confirmed by PCR and grown in liquid LB medium supplemented with 50 µg ml^{-1} ampicillin. Plasmids were purified with the GenElute Plasmid Miniprep (Sigma-Aldrich). The GmUbiP:Glyma530-R:rbcST, and GmUbiP:Glyma530-S:rbcST cassettes were verified by Sanger sequencing. A miRNA-induced gene silencing (MIGS) vector was built to silence Glyma07g14530. A 100-bp target fragment was amplified from Glyma07g14530's coding sequence using the Phusion HF polymerase, with the primers *Asc*I-1510-530targ-F and *Avr*II-

530targ-R (**Table 5.2**). The miR1510 target identified by Jacobs et al. (*47*) was fused to the Glyma07g14530 sequence, in *Asc*I1510-530targ-F. The PCR product and the plasmid pGmURNAi2 were both digested with *Asc*I/*Avr*II. After ligation, the plasmids were used to transform 10-beta Competent *E. coli* cells. Colony screening, miniprep, and sequencing reactions, to check the GmUbiP:1510:530:rbcST cassette were performed as described previously. For biolistic transformation of soybean, the expression and silencing cassettes were cloned into the pSPH1 plasmid as I-*Ppo*I fragments. The Plasmid PSPH1 is derived from the pSMART HC Kan (Lucigen Corporation, Middleton, WI, AF532107), which was modified to contain the *hygromycin phosphotransferase* gene under the control of the *Solanum tuberosum* ubiquitin (StUbi3) promoter and terminator (StUbi3P:*hpt*:StUbi3T) (*82*), and the meganuclease I-*Ppo*I site.

To generate the transgenic soybean lines, somatic embryos from Jack and Jack^{M} were prepared and transformed using the procedure described by Trick et al. (*83*), with the following modifications. Somatic embryos were induced from immature cotyledons in Murashige and Skoog (*84*) basal medium containing 40 mg l^{-1} 2,4-D. The resulting somatic embryos were transferred to half the concentration of 2,4-D for maintenance. Jack embryos were transformed with GmUbiP:Glyma530-R:rbcST and GmUbiP:1510:530:rbcST pSPH1 plasmids in separate events, to generate OE:530-R complementation lines and 1510:530 silencing lines respectively. Jack^{M} embryos were transformed with GmUbiP:Glyma530-S:rbcST and GmUbiP:1510:530:rbcST pSPH1 plasmids in separate events, to generate OE:530-S complementation lines and 1510:530 silencing lines respectively.

For transformation, embryos were subjected to microprojectile bombardment, using 0.6-µm gold particles, with 10 ng of vector DNA per batch, shooting at the 7.58 MPa (1100 psi) setting. One week after bombardment, embryos were transferred to FNL medium (*85*)

containing 20 mg l^{-1} hygromycin, for selection of transgenic tissue. Transgenic embryos were selected at 6 to 8 weeks after bombardment. The selected embryos were propagated in FNL medium. Once enough tissue was available, they were transferred to SHaM (soybean histodifferentiation and maturation) medium (*86*). Transgenic soybean plants (T_0) were analyzed using standard molecular techniques to ensure the transgenes were present. For each transgenic line, the T_0 plants were self-pollinated, to obtain T_1 seed. To determine the zygosity of T_1 plants, the Invader assay (Hologic Inc., Madison, WI) (*87, 88*), was run on a Synergy 2 plate reader (BioTek Instruments Inc., Burlington, VA) according to manufacturer instructions. The assay contains a probe that produces fluorescence when bound to the *hpt* gene; this is used to quantify the relative abundance of *hpt* in each genomic DNA sample. Homozygous T_1 were self-pollinated to obtain T_2 seeds. Selected T_2 plants were self-pollinated to obtain T_3 seeds.

Characterization of transgenic soybean lines

Three independent lines from each Jack OE-530R and Jack 1510:530, and $Jack^M$ OE-530S and $Jack^M$ 1510:530 were characterized in bioassays against SBL caterpillars. The T_2 generations were evaluated in greenhouse (choice, antixenosis) assays, which are used to determine the caterpillars' feeding preference. The complementation lines Jack OE-530R and $Jack^M$ OE-530S were evaluated in the first bioassay. Briefly, ten T_2 plants from each line were grown as described previously. Leaf DNA was isolated from each plant, which was used as template for a PCR reaction with the primers Gmubi842-F and RbcSt110-R (**Table 5.2**), to confirm the presence of the transgenic cassette. Leaf RNA isolated from each PCR positive plant was used in qRT-PCR reactions to measure the relative expression of the native Glyma07g14530 and the transgenic construct OE:530S or OE:530R. qRT-PCR reactions were set up as

previously described. The primer set 530qRT-F6 and 530qRT-R6 was used to amplify the native Glyma07g14530, and the primer set qRbcSt-F and qRbcSt-R (**Table 5.2**) was used to amplify OE:530S or OE:530R constructs. Metalloprotease was used as the reference gene for relative quantification. Five T_2 plants, expressing both native gene and transgene, were used in the bioassay. The experimental design was a randomized complete block design with five replications. Each block contained one plant as the experimental unit. One plant from each complementation line, and one plant from each Jack and Jack^M were included in each block. The greenhouse bioassay was set up as described by Ortega et al. (*33*). Briefly, once the plants reached V4, each block was transferred to a 61 x 61 x 91 cm polyester-mesh cage (BioQuip products, Rancho Dominguez, CA, USA). Each plant was infested with 10 neonate SBL caterpillars. Since leaves of neighboring plants were in contact with each other, the caterpillars were able to move from plant to plant at will. Feeding was terminated when defoliation of Jack was higher than 50%, which took 10 days. Percent defoliation of each plant was estimated by at least three researchers, and the mean of the estimates for each plant was used for an analysis of variance. The silencing lines Jack 1510:530 and Jack^M 1510:530 were evaluated in the second bioassay. Fifteen T_2 plants from each line were grown and tested by PCR as described previously. qRT-PCR reactions, set up as previously described, were used to measure the relative expression of the native Glyma07g14530 and the transgenic construct 1510:530. To determine if the native gene was silenced in the transgenic lines expressing 1510:530, the expression level of Glyma07g14530 on each line was compared to the average relative Glyma07g14530 expression from five Jack or Jack^M plants. Five T_2 plants in which the native Glyma07g14530 was silenced were used in the bioassay, which was set up as described above for the complementation lines.

The T_3 generations were evaluated in growth chamber (non-choice, antibiosis) assays, which are used to determine caterpillar weight gain. The complementation lines Jack OE-530R and Jack^M OE-530S were evaluated in the first bioassay. Ten T_3 plants from each line were planted; the transgene's presence and expression was determined by PCR and qRT-PCR respectively as described previously, and five plants per line were selected for the bioassay. The experimental design was a randomized complete block design with 5 replications. Each block contained one cup as the experimental unit. One cup from each complementation line, and one cup from each Jack and Jack^M were included in each block. The growth chamber bioassay was set up as described by Ortega et al. (*33*), once the plants reached V4 stage. Briefly, for each plant, a trifoliolate leaf was placed into a 600-mL (20-oz) clear polystyrene cup (Letica Corporation) sealed with a dome lid (Letica Corporation). Each cup contained 7 mL of plaster of Paris saturated with water, to maintain 75% relative humidity. Five neonate SBL caterpillars were placed in each cup. The infested cups were placed in a growth chamber set at 27°C, and a 14-h light period was maintained with fluorescent lights (T8 F032/730/Eco, Sylvania Octron, Danvers, MA, USA) providing ca. 40 µmol photons m^{-2} s^{-1} (*32*). Trifoliolate leaves were replaced with fresh leaves on the 4th day, and subsequently whenever 60% of the leaf area had been consumed. The average percentage of defoliation was estimated based on the appearance of the entire leaf. The experiment was terminated after 7 days; caterpillars were immobilized by placing the cups at 4°C for 24 h. Caterpillars from each cup were weighed and their mean weights were used for analysis of variance.

Preparation of insects for bioassays

SBL eggs were obtained from Benzon Research Inc. (Carlisle, PA). The eggs were incubated for 72 hr at 25°C in a 600-ml (20 oz) clear polystyrene cup (Letica Corporation, Rochester Hills, MI, USA) sealed with a dome lid (Letica Corporation); the cup contained plaster of Paris as described. Neonate caterpillars were used to infest the bioassays.

Measurement of leaf isoflavones and isoflavone glycosides

Leaf samples from Bennning, Benning^{M}, Jack, and Jack^{M} were used for HPLC analyses to determine the effect of QTL-M on levels of leaf isoflavone and isoflavone glycosides. Briefly, two sets of five plants from each Benning, Benning^{M}, Jack, and Jack^{M} were grown in the greenhouse as described previously. After the plants reached the V4 stage of development, 0-h entire trifoliolate leaf samples were collected from the first-set plants. Immediately after, the plants in the second set were infested with thirty SBL caterpillars each. After 72 h, entire trifoliolate leaf samples were collected from the second-set plants. From each trifoliolate leaf sample, a sub-sample of 100-mg of leaf tissue was used for RNA isolation. The RNA extractions were used for qRT-PCR reactions to determine the relative expression of Glyma07g14530; qRT-PCR procedures were as previously described. For the HPLC analysis, the rest of each trifoliolate leaf was freeze-dried for in a FreezeMobile 25EL lyophilizer (Virtis, Gardiner, NY), and ground to a fine powder using a 2010 GenoGrinder (Spex SamplePrep). The powder was extracted on a platform shaker at 150 rpm for 16 h with 80% EtOH (25 µl mg^{-1}) and 20 µM of epigallocatechin gallate was used as an external standard. The extract was filtered through 0.2um PVDF, and 15ul aliquots were injected onto a Symmetry C18 column (4.6 × 75 mm, 100Å, 3.5 µm) held at 30 °C, and analyzed at 250 nm using a Waters Alliance 2695 HPLC equipped with PDA. The

mobile phase flow rate was 1 mL min^{-1} and consisted of buffers A [0.1 % (v/v) formic acid in water] and B [0.1 % (v/v) formic acid in acetonitrile], with the following gradient (0 min 95% A, 3 min 85% A, 4 min 85% A, 21 min 50% A, 21.1 min 20% A, 24 min 20 % A, 24.1 min 95 % A, 28 min 95 % A) using a linear gradient between time points. Compounds were quantified by comparison to authentic standards for the flavanol aglycones kaempferol and quercetin, and flavanol glycoside rutin (quercetin-3-O-rutinoside); and the isoflavone aglycones glycitein, genistein, daidzein, and formononetin, and the isoflavone glycosides glycitin (glycitein-7-O-glucoside), genistin (genistein-7-O-glucoside), and daidzin (daidzein-7-O-glucoside). Unknown compounds were expressed as genistin equivalents. All chemical standards were purchased from Cayman Chemical (Ann Arbor, MI), with the exception of formononetin that was from Indofine Inc, and rutin, that was from our in-house chemical library.

Colorimetric assay for condensed tannins

To determine if QTL-M affects the levels of condensed tannins in soybean leaves, the Benning, Benning^{M}, Jack, and Jack^{M} plants from the isoflavone assay were analyzed for condensed tannins using the butanol-HCl colorimetric assay described by Porter et al. (*89*). Briefly, 10 mg of ground freeze-dried tissue were transferred to a 2-mL Safe-Lock tube (Eppendorf, Hamburg, Germany). Next, 500 µL of methanol were added to each sample, and the samples were sonicated for 30 min in a FS-30 water-bath sonicator (Fisher Scientific, Pittsburg, PA). The tubes were spinned in a tabletop centrifuge for 30 sec, and 400 µL of supernatant were transferred to a new tube. Six hundred µL of chloroform and 800 µL of water were added to each tube, in this order. The tubes were vortexed, and centrifuged for 1 min at 15000xg. The supernatant, containing the water-soluble condensed tannins, was transferred to a

new tube. The samples were dehydrated in a Centrivap concentrator (Labconco Co., Kansas City, MO). Five hundred µL of butanol-HCl reagent (5% HCl in butanol) and 10 µL of 2% ferric reagent (2% ferric ammonium sulfate in 2M HCl) were added to each tube. The tubes were vortexed, and incubated at 95 °C for 20 min, and then cooled to room temperature. Each sample was read in duplicate wells, loading 100 µL per well, at 550 nm on a on a Synergy 2 plate reader (BioTek Instruments Inc).

Statistical analyses

2. Data recorded from qRT-PCRs, bioassays, HPLC analysis, and the condensed tannin assay were analyzed using JMP statistical software version 10.0 (SAS Institute, Inc., Cary, NC). Each dataset was tested for normality using the Shapiro-Wilk test ($P < 0.05$) (*90*). A one-way ANOVA test ($P < 0.05$) was used to detect any difference among treatments, and a post-hoc Tukey-Kramer multiple comparison test ($P < 0.01$) (*91-93*) was used to determine significant differences between treatments.

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Table 5.1. Polymorphisms between Williams 82 and PI 229358 for the 11 models contained in the QTL-M region.

Gene ID	Predicted annotation[1]	Size	Gene model[2]	Williams 82/PI 229358 polymorphisms[3]
Glyma07g14440	Unknown function	1,220 bp		^{832}T/C (^{278}stop/R)
Glyma07g14450	Unknown function	1,424 bp		Same sequence
Glyma07g14460	Cytochrome P450	2,610 bp		T/A, T/-, ^{726}A/C (^{242}silent)
Glyma07g14470	Ploop-NTPase	1,903 bp		^{500}G/A (^{167}P/L)
Glyma07g14480	MYB-related	2,014 bp		^{78}G/C (^{26}K/N), G/A, A/T, A/C, ^{639}T/A (^{213}silent)
Glyma07g14490	Phosphoglycerate mutase	3,128 bp		C/A, A/T, G/A
Glyma07g14500	**Pseudogene**, Helix-loop-helix	378 bp		Same sequence
Glyma07g14510	Glucosyl/Glucuronosyl transferase	1,705 bp		Same sequence
Glyma07g14520	Unknown function	1,891 bp		A/G
Glyma07g14530	Glucosyl/Glucuronosyl transferase	1,401 bp		^{747}G/A (^{249}W/stop)
Glyma07g14540	DNAJ/HSP40	3,703 bp		Same sequence

[1] Annotations based on the Wm82.a1.v1 gene models (www.soybase.org).
[2]Blue arrows represent exons, and blue lines indicate introns. Polymorphisms are indicated by orange triangles; details for each polymorphism are shown in the next column.
[3]For SNPs and/or INDELs in introns, the Williams 82 allele is shown first, followed by the PI 229358 allele. For SNPs in exons, the SNP position (bp) in the coding sequence is shown first, followed by the Williams 82 and PI 229358 allele; the effect of each polymorphism in the amino acid sequence is shown in parenthesis.

Table 5.2. List of primer sets used in this study

PCR products to identify SNPs in the QTL-M region	Primer name	Sequence (5'->3')
S72_3420K	S72_3420K-F	CCTAACTCTCTTTAACCTCGTC
	S72_3420K-R	CTTGGTTGGGAAGTTCTG
S72_3430K	S72_3430K-F	AGGCTATAGATTAAGAATGCTAAGTC
	S72_3430K-R	CTTCACATTTCCTCTTATTAACAACC
S72_3450K	S72_3450K-F	TGATGTGATGTGATGTGACG
	S72_3450K-R	CCTCTATGTATTTCAGAAATGTCTC
S72_3490K	S72_3490K-F	CAAATGTCAATGCAATAATAGCGT
	S72_3490K-R	GAAGGTTGATAGTAATGACTGGAG
S72_3510K	S72_3510K-F	AAGGTGTATCCAAGATTAGCC
	S72_3510K-R	TCTGATTAGAAAGTCAATGATTCCC
S72_3530K	S72_3530K-F	GATTGTCCAACGTTCAGG
	S72_3530K-R	ACTTGTCTAACACATTGATGCTA
S72_3550K	S72_3550K-F	TTTGGTTATGTGTGGCTGG
	S72_3550K-R	CTTCTAGTAGTTAAGGTCTTCCC
S72_3570K	S72_3570K-F	GCTTCAACTTCTCTTCCTATCC
	S72_3570K-R	GTAGGGCATAGAGACGCA
S72_3600K	S72_3600K-F	ACACCAGCACAAAGTCC
	S72_3600K-R	GTCTCATTCAGTTCTCGCA
S72_3410K	S72_3410K-F	TTGTGCAGATATGAAGCTCTTTAG
	S72_3410K-R	CCACCTTCACAATCACG
S72_3422K	S72_3422K-F	CAGGTGCTCTGTGTTGT
	S72_3422K-R	CTGGTTCACTGACCTTCG
S72_3430K	S72_3430K-F	CCAAGTCAGGTATGAGAATGC
	S72_3430K-R	TCCACATCCAACACAATCG
S72_3443K	S72_3443K-F	CTCCATAGTTCAAGTACAACCC
	S72_3443K-R	ACCATCCATCTTCATGTACG
S72_3452K	S72_3452K-F	TTTGCTACATGGCAGCTT
	S72_3452K-R	GACACTGAAACACAATGCC
S72_3589K	S72_3589K-F	CTGGATGATTGTGAGGAGAC
	S72_3589K-R	AGAACTCGTTGAATCGCT AT
S72_3600K	S72_3600K-F	ACAGAACATTGCATTCCCTA
	S72_3600K-R	GCTGATATGTTCACAAAGCCA
S72_3610K	S72_3610K-F	CAGGGACATGATATGATTAGCAA
	S72_3610K-R	GGTCACACCTTCCATATCTC
S72_3616K	S72_3616K-F	CCATACAATGTTGCACACG
	S72_3616K-R	CATATTAGTATACCAGGTGACGG
SNP003	SNP003-F	CATACTGATGATGGGCCG
	SNP003-F	GCCACTTCTCACACGTTT
Satt729	Satt729-F	AAGTGCTAGCCACAAGGTTGA
	Satt729-R	GATTTTGGTTTGCCCTTAGC
SNP119	SNP119-F	ATACAGCTCAATAGGTAGTACCAAT
	SNP119-R	TGGGAAAGGGTGCTTGATTCTA
SNP13870	SNP13870-F	TGCCGATAGTGTCAATGAGT
	SNP13870-R	ATTAATGTGATTGTTTGGACCTG
SNP13885	SNP13885-F	GCTACTTGAACACAATCGATG
	SNP13885-R	ACCTAACTTATTAGAATTTGCGTGA
SNP13892	SNP13892-F	TAACTACTTGTATCACACTAGCATCT

	SNP13892-R	ACTAAGTGTCTTGGATCGGA
SNP116	SNP116-F	CAGACATAAGTCCTCGATCAC
	SNP116-R	CCTAACGACAAAGGCCC
Sat_425	Sat_425-F	CCTTTGAGACGCAACTGAAAAT
	Sat_425-R	TGATGGTGGTGTTGGTGTAA
Sequencing primers for candidate SNPs		
Glyma07g14460	P450-SEQ-F1	AGCCATATCCATCCAAAGAC
	P450-SEQ-R1	TGGACCTTTGAGGAAACG
	P450-SEQ-F2	AGCTCATCTCAGCCTTCATA
	P450-SEQ-R2	GGAAGGACAACACCACTAA
	P450-SEQ-F3	GTGGTGGAATCTCACGTT
	P450-SEQ-R3	TTGGATGAGGCTGCTTAGT
	P450-SEQ-F4	TCAATTCAATGCTTTGGTGGAT
	P450-SEQ-R4	CATAAAGAAACAATGAACTGCCG
	P450-SEQ-F5	TCATCTTAGTGTAGTGTGATTCTTAGT
	P450-SEQ-R5	TGCAGCAATGAGAAGCC
	P450-SEQ-F6	TGAAATCTTTGCAAGCATCATAAC
	P450-SEQ-R6	CAATCGATCTCTGGAAAAGG
	P450-SEQ-F7	AATATGGACTCATCTGCTTAGG
	P450-SEQ-R7	AACTCTTGGAAAGGTGCTAAA
Glyma07g14470	PLNTP-SEQ-F1	ACACACATTCTAAGA CGGTTAC
	PLNTP-SEQ-R1	GTCGTTGGAGGAGAGGT
	PLNTP-SEQ-F2	GGATCAGTTACCACACCG
	PLNTP-SEQ-R2	ACAATGAGCACCTGCAAATA
	PLNTP-SEQ-F3	CCATTCTTCTACGGATTAAATAGTGT
	PLNTP-SEQ-R3	ATGCACCTGTTTCTTGAGAT
	PLNTP-SEQ-F4	TGGACTTCAGGGAGTTAGGA
	PLNTP-SEQ-R4	TCAAGTAGTGTTAATTAGTTGTTGGG
	PLNTP-SEQ-F5	TGGACTTCAGGGAGTTAGGA
	PLNTP-SEQ-R5	TCAAGTAGTGTTAATTAGTTGTTGGG
	PLNTP-SEQ-F6	CAGAAGTATTGCTAGTTGTGG
	PLNTP-SEQ-R6	CACAACACATCACAGAAAGAC
Glyma07g14480	MYB-SEQ-F1	GGGTAATTTCCACATTATTAATGGTT
	MYB-SEQ-R1	AACGAAGACGACACGAC
	MYB-SEQ-F2	AGAAGAAGATGAGATGCTACTGA
	MYB-SEQ-R2	ACACAAATGCAAGTAACAACCATAAA
	MYB-SEQ-F3	TGCTTCACATTGATCCTGG
	MYB-SEQ-R3	CAAATTGTGCCTGCAACTC
	MYB-SEQ-F4	CTAACACTGCAAGGGACG
	MYB-SEQ-R4	GCTATGTCTTGTTTAAGGAAATTTGTA
	MYB-SEQ-F5	CAGCAACTCCTAAATCGC
	MYB-SEQ-R5	CCTCAGAATTATCAATGTAGGGC
	MYB-SEQ-F6	CCCAGAGAATGTAGTAGTAGAC
	MYB-SEQ-R6	CCACAGGGAAATCATCGAA
	MYB-SEQ-F7	TGCATTTCCTCAGATTCCAG
	MYB-SEQ-R7	GAATATATCATTGCAGAATGCGT
	MYB-SEQ-F7-1	TTGCCCACAATTGATGAGC
	MYB-SEQ-R7-1	TTGCAGAATGCGTTGATACC
	MYB-UP-SEQ-F1	CTCTTTCTCCAACCCACG
	MYB-UP-SEQ-R1	CAAGTCCTCCTTCCTCCCTAA

	MYB-UP-SEQ-F2	GTTTGGAGGAAGAGTTGGT
	MYB-UP-SEQ-R2	ACATATAAATAGTAAATAAGTCTCCCAGG
	MYB-UP-SEQ-F3	TGATGTTGTCTTTTGTATTTGTCTTC
	MYB-UP-SEQ-R3	TTATTCACTTGCATTCTCCACC
	MYB-UP-SEQ-F4	GCCATGTTACCTGTACACT
	MYB-UP-SEQ-R4	TTTACCACCCAACTTCATTATACT
	MYB-UP-SEQ-F5	AGGGATTCCACAAAATATCTATACAAA
	MYB-UP-SEQ-R5	ACGGTTATTCATGGCAGT
Glyma07g14500	HLH-SEQ-F1	GTCACAACATCTTTAATTGATTAGGT
	HLH-SEQ-R1	AAAGATCAATAACATCAAGAGTGC
	HLH-SEQ-F2	CTACAGTTCATCAATCTGCGA
	HLH-SEQ-R2	GCATATTGCACAGGTAGAGT
	HLH-SEQ-F3	CATGGGATAAGAGGAACGC
	HLH-SEQ-R3	CTAAATAGATGTACTGCTCGCT
	HLH-SEQ-F4	AGAAAGGCACTCCATTTACT
	HLH-SEQ-R4	CTATCTCCAGCCATTGTTATCG
	HLH-SEQ-F5	AGTTTGAGTGAGTGAGGG
	HLH-SEQ-R5	TAAGCATCTCAGCCACG
	HLH-SEQ-F6	TGTGAGAAGAAAGGTTGCG
	HLH-SEQ-R6	TGGATGATTAAGCGAAACCGA
Glyma07g14510	UDP1-SEQ-F1	ATGAATTTCCACAGACCGA
	UDP1-SEQ-R1	GGATTAGTGGTAGAGAACGAG
	UDP1-SEQ-F2	CAATTCTTGAGTTCTCTAAGCG
	UDP1-SEQ-R2	CTTGTATGCTACACCTGATCG
	UDP1-SEQ-F3	ATATTTCCCATCCACGGC
	UDP1-SEQ-R3	GCATATACAGAAGGGATTCCTC
	UDP1-SEQ-F4	AATCTTTGGGTCCACTACG
	UDP1-SEQ-R4	TGAGCAAGGATTTGAACCTG
	UDP1-SEQ-F5	AGATTCTTGTGGGTGTTAAGAC
	UDP1-SEQ-R5	TCCACTTTAATGCCAACTGT
	UDP1-SEQ-F6	TGATGGTTTGAAAGTGGCTC
	UDP1-SEQ-R6	ACAACAGACATATTATGCAAGTG
Glyma07g14530	UDP2-SEQ-F1	ATGCAGCAGTACCTTTGAC
	UDP2-SEQ-R1	ACAAGGGAAGTAGATGTAGGATA
	UDP2-SEQ-F2	CTAAACAACAATGGAGTCTCTG
	UDP2-SEQ-R2	CCAAATGACACATAAAGAACTGA
	UDP2-SEQ-F3	AGCTACCCTCCTGTGTAT
	UDP2-SEQ-R3	CTAGTGTCAACGTTTGGTCT
	UDP2-SEQ-F4	CTGAACAAAGAACCAATGCG
	UDP2-SEQ-R4	ATACACAATAGTTGACTTTATCTCATACA
Endpoint RT-PCR primers		
Glyma07g14440	Glyma07g14440-F1	TTTCGTTTCTTTTGGAATCGCC
	Glyma07g14440-R1	GTTGCAAATGTAAGGCTCCTC
Glyma07g14450	Glyma07g14450-F1	CAGGGCCATAAAAAGATATTTCATGGGGTC
	Glyma07g14450-R1	GCTTCCAACAGCCCAAAAGACGCATAAAC
Glyma07g14460	Glyma07g14460-F1	CTGATTTGCTTGCACTTTTGCG
	Glyma07g14460-R1	CTTAGGGCCAACAAGCTCAAGGG
Glyma07g14470	pLoop-NTP-F2	CCCAGCAACTAGCATGCAACTTTTTCC
	pLoop-NTP-R2	TATTGGCTGGACTTCAGGGAGTT
Glyma07g14480	Glyma07g14480-F3	TCTCTTGCCCACAATTGATGAGC

	Glyma07g14480-R3	CTGAAAGAAAGGGAACCCAATGG
Glyma07g14490	Glyma07g14490-F1	TCAAAGCCCCAATTTCCATCTT
	Glyma07g14490-R1	GAGGTGGTTCTTGCAGTGT
Glyma07g14500	Glyma07g14500-F4	AAGCATTCCAACTTGGGCTTGAAGGAA
	Glyma07g14500-R4	GGTGGACCAAAGATGAACGTGGCTGAGATG
Glyma07g14510	Glyma07g14510-F3	TTCTATAAACCATGTCCACATTATGTGATC
	Glyma07g14510-R3	CCCCCAAGCTTAAGTTTTTCCAC
Glyma07g14520	Glyma07g14520-F2	GGCTGATGAAAAGGTGCATCAAT
	Glyma07g14520-R2	TCTTTGCCTTCTCCCTCCAC
Glyma07g14530	Glyma07g14530-F3	GTGAGTATAGAGATCACCCAAAC
	Glyma07g14530-R3	CTTCCAATTCCATGAAGCTATTG
Glyma07g14540	Glyma07g14540-F1	GCATGTTGAGAAGGGTATGC
	Glyma07g14540-R1	GATGGCTCATCATCATCTTCG
Full-length cDNA cloning		
Glyma07g14470	Glyma07g14470-R3	AAGTTGCATGCTAGTTGCTGGG
	Glyma07g14470-R5	CTGTCTTGTCAATTACAATCCA
	Glyma07g14470-F10	GCAAACAAACCTCTTTGACACTC
	Glyma07g14470-F11	CACATATGAGAAGGTCACATG
	Glyma07g14470-R13	GACCGTCGTGGGTTGGAGAAAGAGTTCCTC
Glyma07g14530	530-5-RACE	GGAGACGTTTTGCGAACTCGAGGATTG
	530-3-RACE	GCTGTGAGACCAAACGTTGACACTAGTG
	Glyma07g14530-F2	CCCTTGTTTCTATCCCAGCTTTC
	Glyma07g14530-R2	GTCCTCGAAGTACACTGAGGG
	Glyma07g14530-R3	CTTCCAATTCCATGAAGCTATTG
Glyma07g14470 primers for cloning in arabidopsis		
	Gm470-Phy-F Gm470-R	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGTTGTGACCGTCGTGGGTT GGGGACCACTTTGTACAAGAAAGCTGGGTGAACAAATTGCAGGGTTGCAGAAATG
qRT-PCR primers		
Glyma07g14530 (PCR efficiency = 1.93)	530qRT-F6 530qRT-R6	CGTTGCCAAAGATACCGTAGTGC GGATACACAGGAGGGTAGCTAC
Metalloprotease (PCR efficiency = 1.76)	Metalloprotease-F Metalloprotease-R	ATGAATGACGGTTCCCATGTA GGCATTAAGGCAGCTCACTCT
Transgene terminator (PCR efficiency = 1.71)	qRbcSt-F	GTTCGAGTATTATGGCATTGGGAAAACTG
	qRbcSt-R	CACAGTTCGATAGCGAAAACCGAAT
PR10 (PCR efficiency = 1.86)	PR10-F PR10-R	AGTTACAGATGCCGACAACG CCTCAATGGCCTTGAAGAGA
Cloning primers		
GmUbiP:Glyma530-R:rbcST and GmUbiP:Glyma530-F:rbcST	*Asc*I-530-OE-F *Avr*II 530-OE-R	ATTA**GGCGCGCC**ATGGAATCAGCGGCAAGAACA CACG**CCTAGG**GTCAGCAAGTAGGACGCAAAG
GmUbiP:1510-530:rbcST	*Asc*I-1510-530targ-F *Avr*II-530targ-R	ATATA**GGCGCGCC***AGGTGGAATAGGAAAAACAACT*GTGTACTTCGAGGACCTAAAC TATAT**CCTAGG**ATCTTAGGGTTTCCCTAACGG
Primers for confirmation of transgenic events		
StUbi3P:*hpt*:StUbi3T	Hygro117-F Hygro504-R	CGATGTAGGAGGGCGTGGATA GTCGTCCATCACAGTTTGCCA
GmUbiP:Glyma530-R:rbcST and GmUbiP:Glyma530-F:rbcST and GmUbiP:1510-530:rbcST	Gmubi842-F RbcSt110-R	CGAGATTGCTTCAGATCCGTA CCATTTCCATTTCACAGTTCG

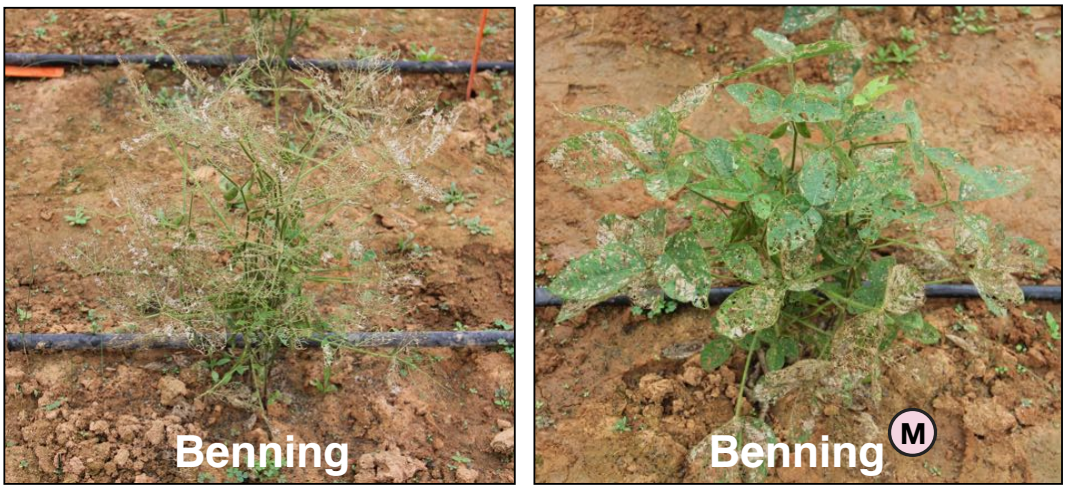
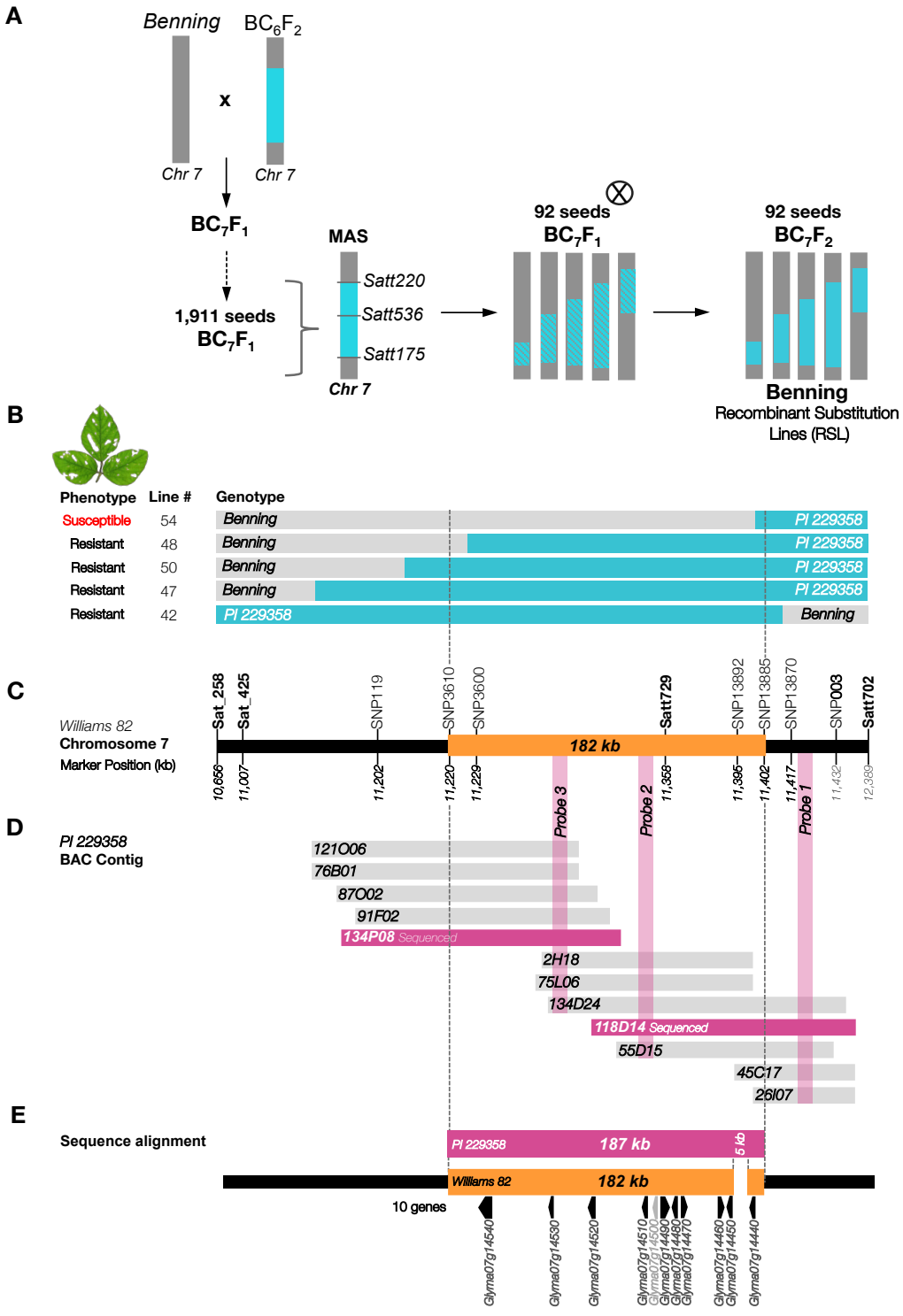


Figure 5.1. QTL-M confers resistance to leaf-chewing insects in soybean. The susceptible cultivar 'Benning' and its insect-resistant near isoline Benning^{M} exposed to SBL caterpillars in a field-cage assay.


A
Benning
BC6F2
x
Chr 7
Chr 7
BC7F1
1,911 seeds
BC7F1
MAS
Satt220
Satt536
Satt175
Chr 7
92 seeds
BC7F1
92 seeds
BC7F2
Benning
Recombinant Substitution
Lines (RSL)
B
Phenotype
Line #
Genotype
Susceptible 54 Benning PI 229358
Resistant 48 Benning PI 229358
Resistant 50 Benning PI 229358
Resistant 47 Benning PI 229358
Resistant 42 PI 229358 Benning
C
Williams 82
Chromosome 7
Marker Position (kb)
Sat_258
Sat_425
SNP119
SNP3610
SNP3600
Satt729
SNP13892
SNP13885
SNP13870
SNP003
Satt702
182 kb
10,656
11,007
11,202
11,220
11,229
11,358
11,395
11,402
11,417
11,432
12,389
Probe 3
Probe 2
Probe 1
D
PI 229358
BAC Contig
121O06
76B01
87O02
91F02
134P08 Sequenced
2H18
75L06
134D24
118D14 Sequenced
55D15
45C17
26I07
E
Sequence alignment
PI 229358
187 kb
5 kb
Williams 82
182 kb
10 genes
Glyma07g14540
Glyma07g14530
Glyma07g14520
Glyma07g14510
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Glyma07g14490
Glyma07g14480
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Glyma07g14440

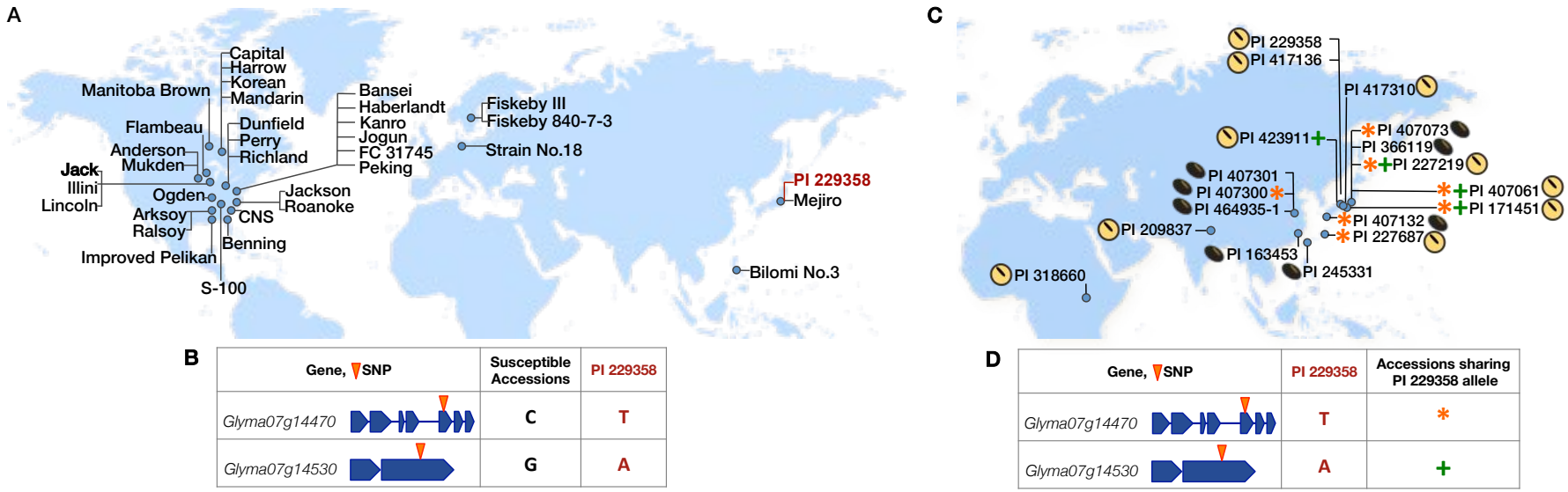

Figure 5.3. Identification of candidate genes for QTL-M insect resistance. (**A**) Panel of insect-susceptible soybeans. Polymorphic genes between PI 229358 and Williams 82 were sequenced in each accession to identify SNP loci carrying alleles unique to PI 229358. (**B**) Two gene models were identified that contained containing SNP alleles unique to PI 229358. (**C**) Panel of insect-resistant soybeans. Yellow seeds and brown seeds indicate cultivated and *G. soja* accessions, respectively. (**D**) Accessions carrying the PI 229358 allele for Glyma07g14470 and Glyma07g14530 SNPs are indicated by an asterisk and a cross, respectively. These two SNPs are also found in other insect-resistant genotypes.

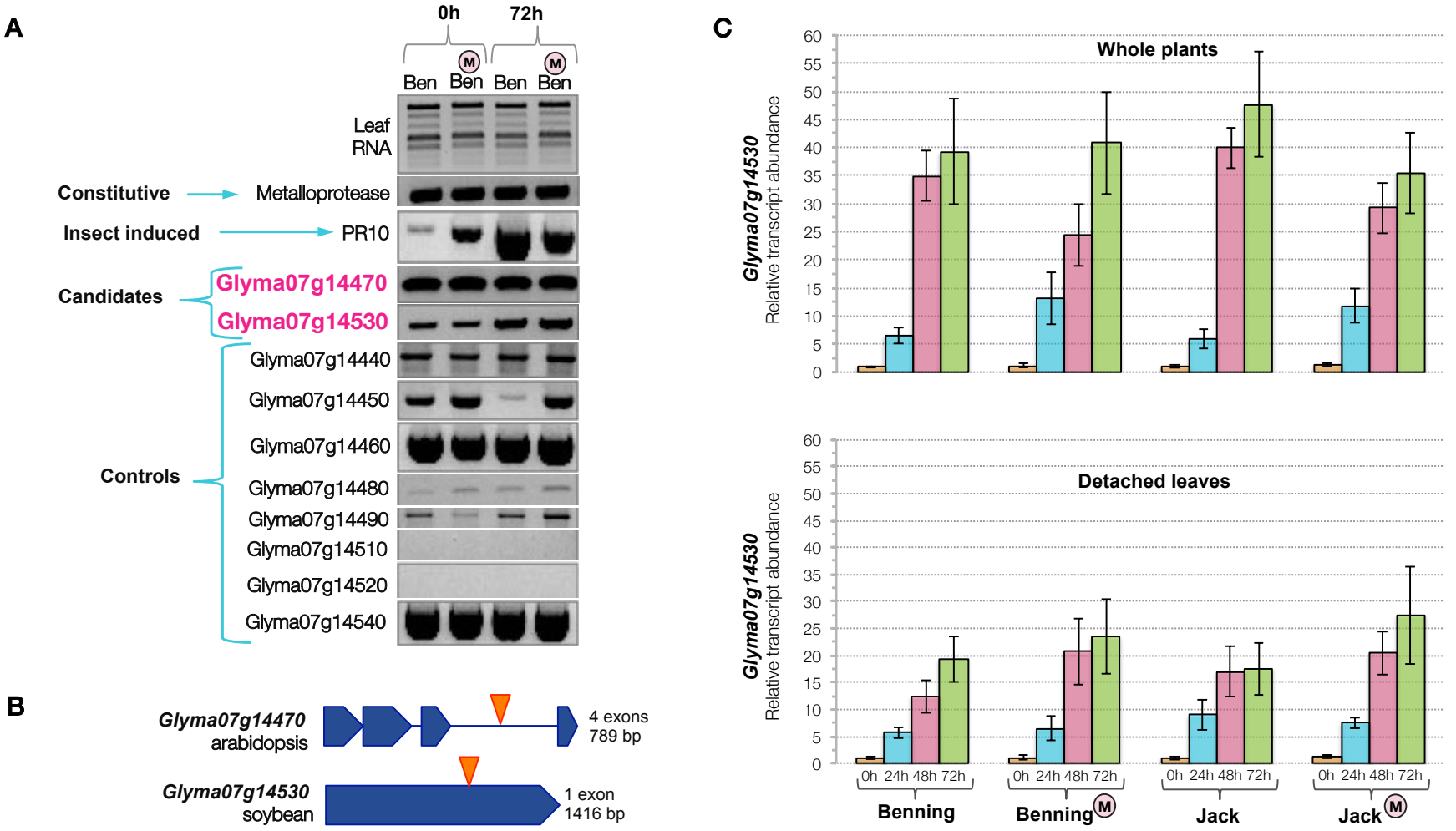

Figure 5.4. Molecular characterization of QTL-M candidate genes. (**A**) Expression of the candidate genes from the QTL-M region in leaf tissue. RNA isolated from Benning and Benning^{M} plants, at 0 hr and 72 hr after infestation with SBL caterpillars, was used as template for RT-PCR reactions. Metalloprotease, PR10, and neighboring genes in the QTL-M region were used as

controls. (**B**) Full-length Glyma07g14470 and Glyma07g14530 transcripts. Sequences were assembled from 5'-RACE and 3'-RACE products. (**C**) Relative expression of Glyma07g14530. Results from infested whole plants and detached leaves are shown in the top and bottom panels, respectively. RNA isolated from Benning, Benning^{M}, Jack, and Jack^{M} at 0, 24, 48, and 72 hr after infestation was used as template for qRT-PCR. Expression of Glyma07g14530 was normalized using metalloprotease as reference gene. Bars represent means ± SEM from five biological replicates. This gene is similarly upregulated in response to caterpillar infestation in both resistant and susceptible lines.

Figure 5.2. QTL-M region in soybean chromosome 7. (**A**) Development of RSLs derived from Benning x PI 229358, for positional cloning of QTL-M. (**B**) Graphical genotypes of five RSLs. Grey and blue indicate loci carrying Benning and PI 229358 alleles respectively. (**C**) Chr7 segment containing QTL-M. The bar shows the position of the molecular markers in Williams 82. All resistant lines contain the PI 229358 introgression between SNP3610 and SNP13885. (**D**) PI 229358 BAC-clone contig. Twelve clones were identified by hybridization with DNA probes; the contig was assembled using BAC-end sequences. Full-length sequences were obtained from two overlapping clones. (**E**) PI 229358 sequence aligned to Williams 82. Arrows indicate ten genes and a pseudogene annotated in this region.

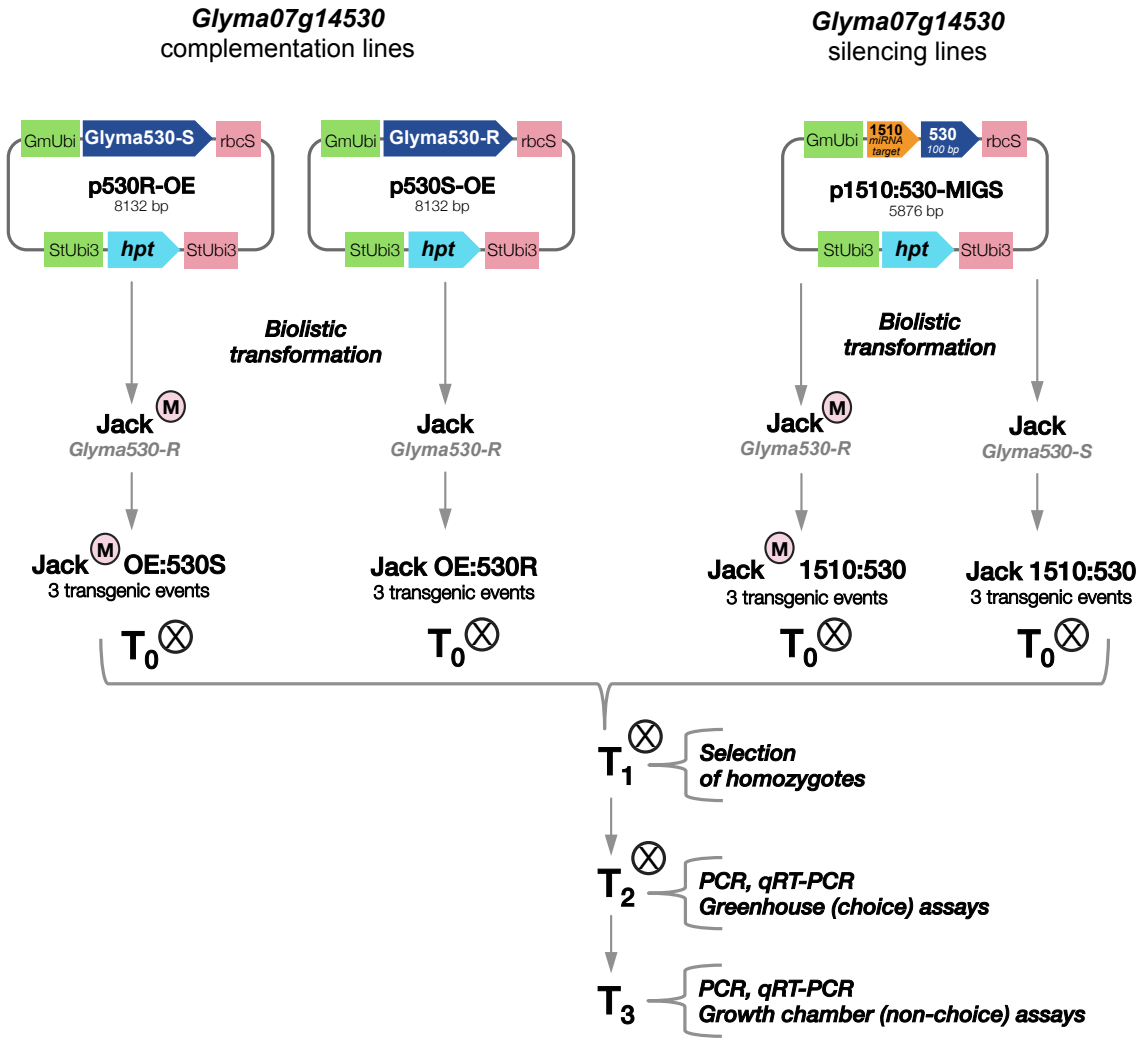

Figure 5.5. Development of transgenic soybean lines. The over-expression vectors p530S-OE and p530R-OE were used to generate complementation lines for Jack^{M} and Jack, respectively. The silencing vector p1510:530-MIGS was used to generate silencing lines for both Jack^{M} and Jack. T_0 and selected T_1 plants were self-pollinated. T_2 and T_3 plants were characterized for resistance against SBL caterpillars.

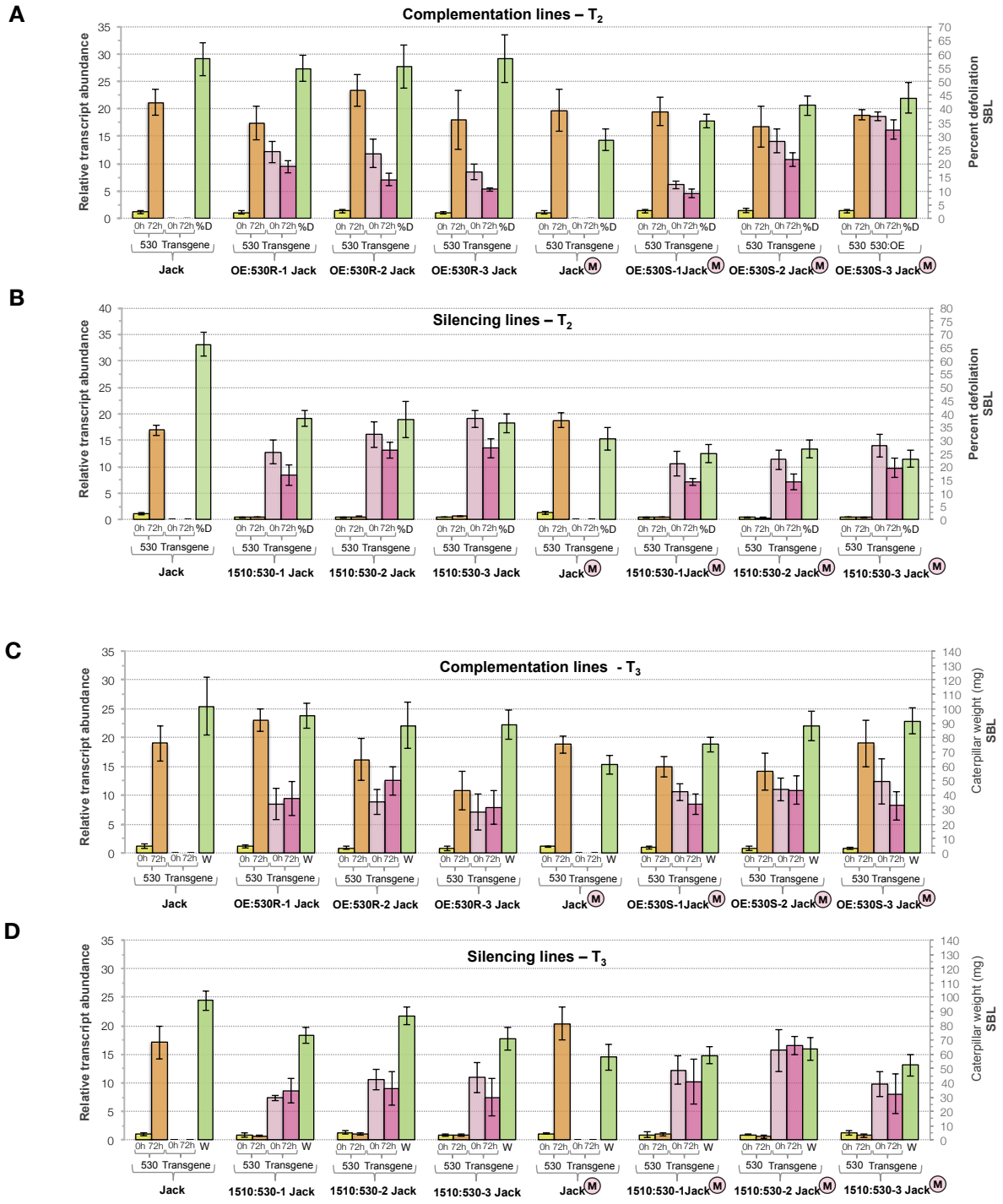

Figure 5.6. Characterization of T_2 and T_3 soybean lines for Glyma07g14530 relative transcript abundance 0 and 72 h post-infestation, % Defoliation (%D), and weight of caterpillars (W) feeding on the resulting lines. (**A**) Evaluation of Jack and Jack^M T_2 complementation lines in the greenhouse (choice) assay. (**B**) Evaluation of Jack and Jack^M T_2 silencing lines in the greeenhouse assay. (**C**). Evaluation of Jack and Jack^M T_3 complementation lines in the growth chamber (non-choice) assay. (**D**) Evaluation of Jack and Jack^M T_3 silencing lines in the growth chamber assay. Resistance was measured as percent

defoliation and caterpillar weight, in the greenhouse and growth chamber assays, respectively. SBL caterpillars were used in all the bioassays. Defoliation was estimated by three researchers, and the average was used for statistical analysis. Bars represent means ± SEM from five biological replicates. The average SBL weight per cup was used for statistical analysis. Bars represent means ± SEM from five biological replicates. For all assays, expression levels for Glyma07g14530 and transgene were determined by qRT-PCR, using metalloprotease as reference gene. Bars represent means ± SEM from five biological replicates.

Figure 5.7. Selected transgenic lines. (**A**) Defoliation on Jack, 530S:OE-3 Jack^{M}, and Jack^{M} T_2 lines, 10 days after SBL infestation. (**B**) SBL caterpillars are significantly larger after 7 days of feeding on 530S:OE-3 Jack^{M} compared to Jack^{M} (top row). SBL caterpillars are significantly smaller after feeding on 1510:530-3 Jack for 7 days compared to Jack (middle row). There is no difference in size of SBL feeding on Jack^{M} and 150:530-3 Jack^{M} T_3 lines (bottom row).

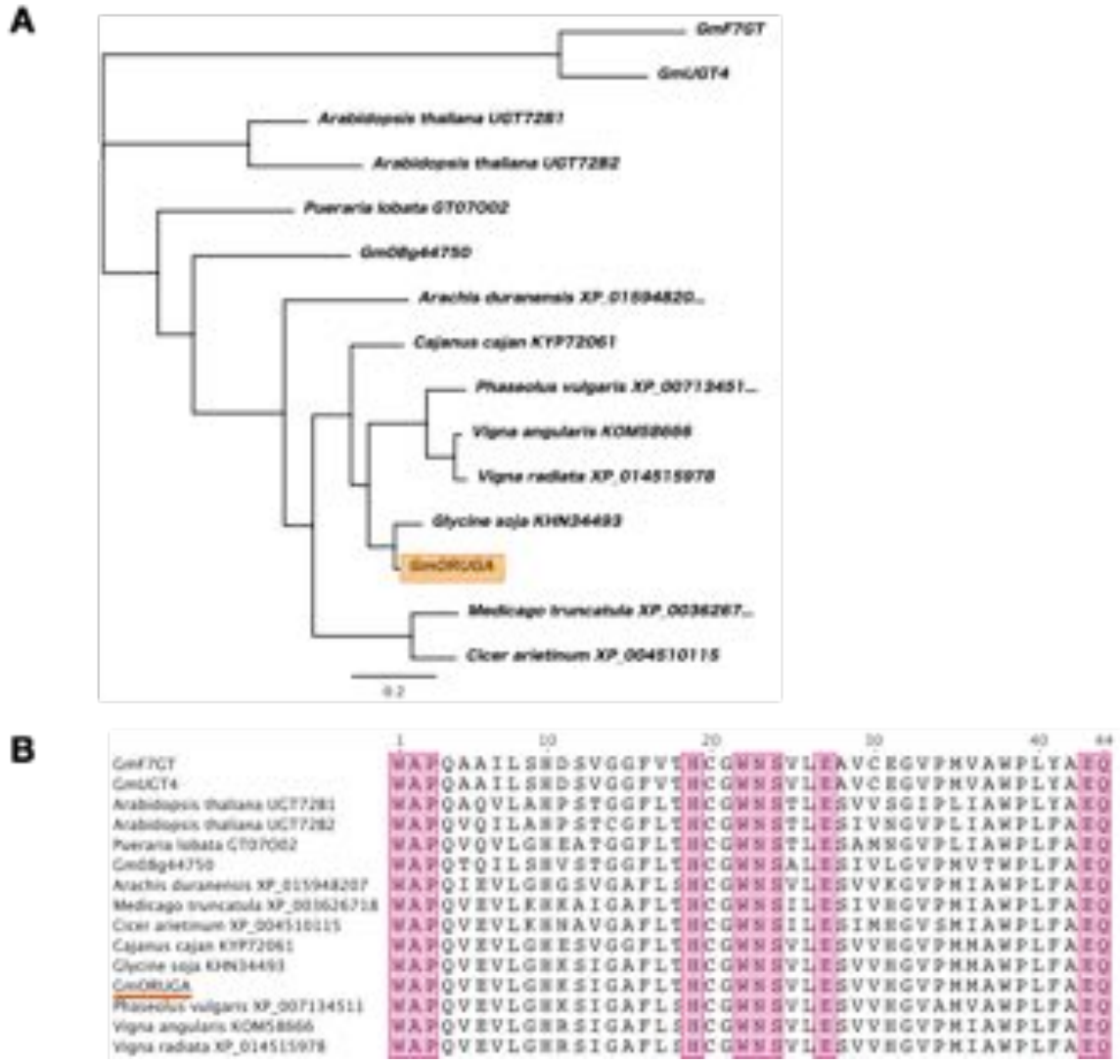

Figure 5.8. *GmORUGA* and related PSPGs. (**A**) Unrooted phylogenetic tree. The plant protein that are most similar to GmORUGA are predicted hydroquinone glucosyltransferases from pigeonpea [*Cajanus cajan* (L.) Millspaugh] (KYP72061), wild soybean (KHN34493), mung bean [*Vigna radiata* (L) Wilczek] (XP_014515978), chickpea [*Cicer aretinum* L.] (XP_004510115) and peanut [*Arachis duranensis* Krapov. and W.C Gregory] (XP_015948207); and the hypothetical protein LR48 from azuki bean [*Vigna angularis* (Willd.) Ohwi and Ohashi] (KOM58666), a hypothetical protein from common bean [*Phaseolus vulgaris* L.] (XP_007134511), an UDP-glucosyltransferase family protein from barrel medic [*Medicago truncatula* Gaertn.] (XP_003626718), and the isoflavone-7-glucosyltransferase GT07O02 from kudzu [*Pueraria lobata* (Willd.)] (ADV71365). The tree was constructed using the CLUSTALW plug-in in Geneious. Bar = 0.2 amino acid substitutions/site. UGT72B1 and UGT72B2 were included, as they are the arabidopsis group-E UGTs that are most similar to *GmORUGA*. GmF7GT and GmUGT4 are included as an outgroup, as they are not part of group E. (**B**) Conserved 44-aa PSPG motif. Residues highlighted in pink interact directly with the UDP-sugar, based on the available crystal structure of UGT72B1 from arabidopsis.

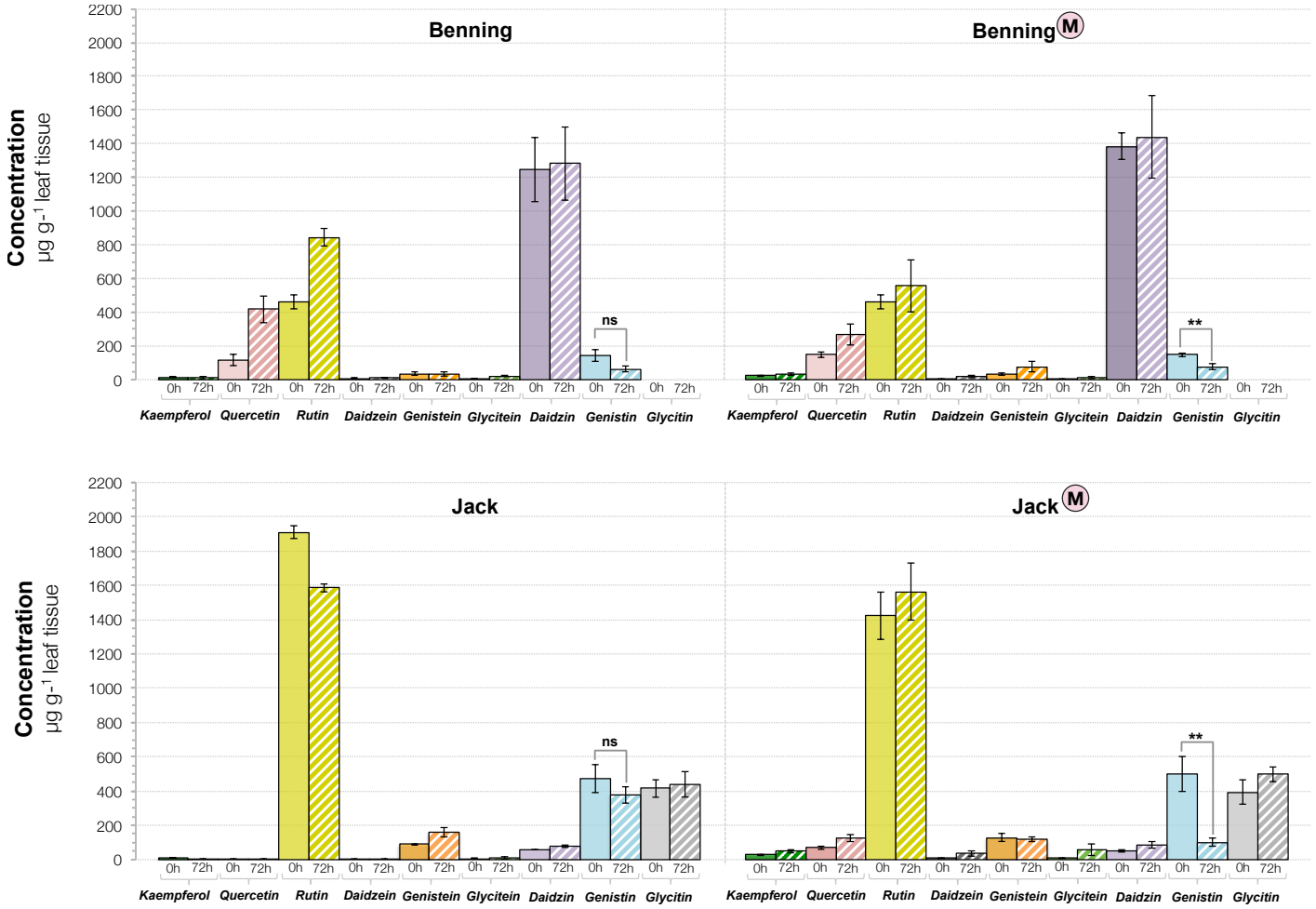

Figure 5.9. Isoflavone profiles of Benning, Benning^{M}, Jack, and Jack^{M}, at 0 and 72 hr after SBL infestation. For genistin: ** Highly significant differences (P<0.01); ns, not significant.

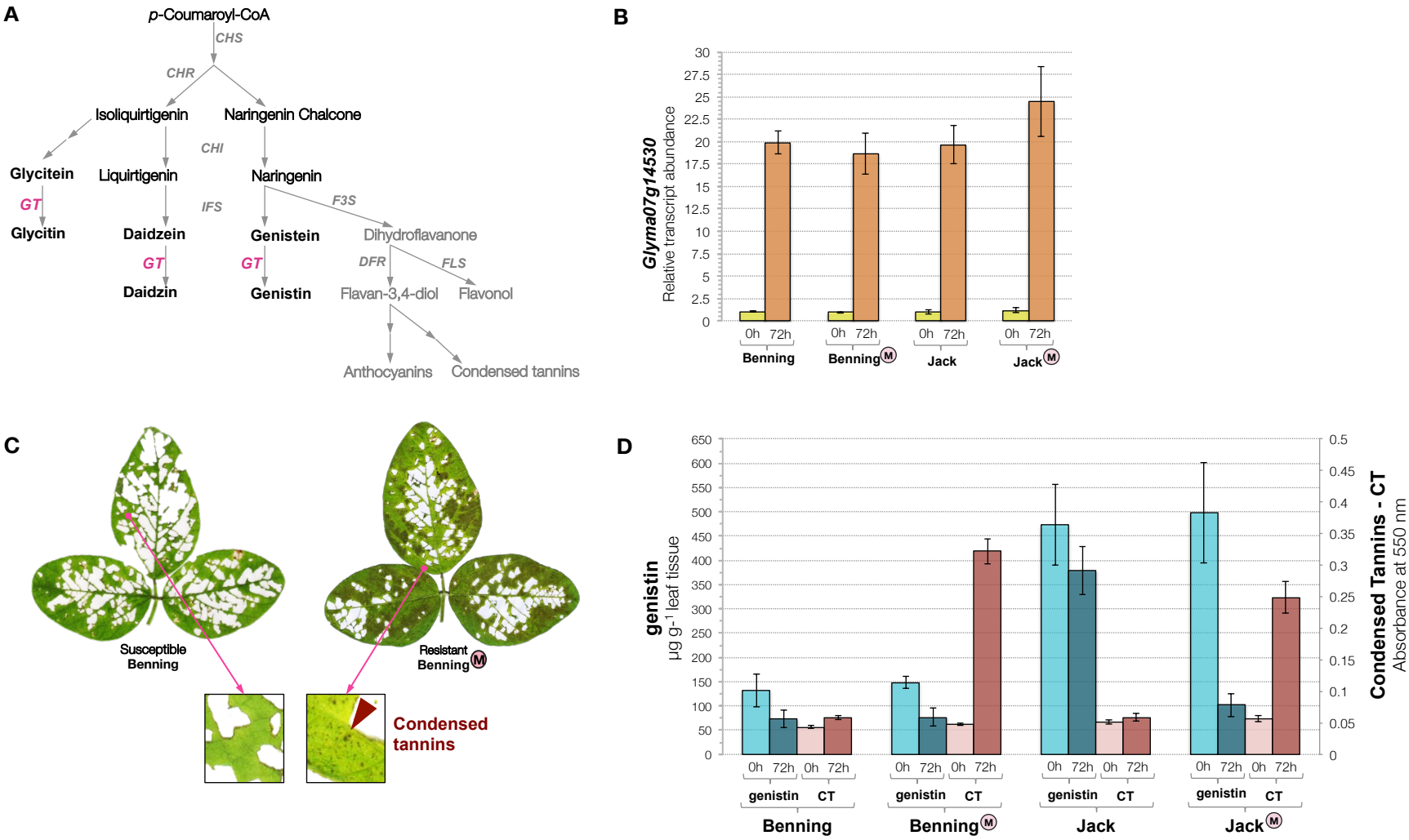

Figure 5.10. Genistin and condensed tannins (CT) in Benning, Benning^{M}, Jack, and Jack^{M}, at 0 and 72 hr after SBL infestation. (**A**) Illustration of the isoflavone and alternative anthocyanin/condensed tannins pathway in soybean. (**B**) For samples in panel D, in which Glyma07g14530 expression was measured by qRT-PCR using metalloprotease as reference gene. Bars represent means ± SEM from five biological replicates. (**C**) Leaves from Benning and Benning^{M} collected 10 days after SBL infestation. (**D**) Genistin and CT in leaf tissue. Note the increase in CT in the resistant lines. Bars represent means ± SEM from five biological

replicates. The resistant lines are accumulating CT as predicted. (The resistant allele of Glyma07g14530 affects genistin levels, indicating it is a genistein glucosyltransferase. The resistant allele prevents accumulation of genistin, leading to an accumulation of CT, resulting in resistance.

CHAPTER 6

CONCLUSIONS

Planting insect-resistant cultivars, as part of an IPM program to control insect pests, minimizes the frequency of insecticide applications, thus reducing crop production costs and concerns over pesticide residues in the environment. Leaf-chewing insects negatively impact soybean production, as high levels of defoliation reduce seed yield and quality. Soybean resistance to leaf-chewing insects is available in the Japanese landraces PI 229358 and PI 227687. Resistance is conferred by the quantitative trait loci (QTL) QTL-M, QTL-H, and QTL-G in PI 229358, and by QTL-E in PI 227687. This dissertation describes the characterization of novel combinations of insect resistance QTLs from PI 229358 and PI 227687, development of SNP markers for markers-assisted selection of insect-resistance QTLs, the evaluation of soybean meal from insect-resistant soybean for chicken feed, and the cloning of the gene responsible for QTL-M.

In near-isogenic lines (NILs) of the cultivar Benning, the QTL combinations ME and MGHE confer high levels of resistance against soybean looper [SBL, *Chrysodeixis includens* (Walker)], corn earworm [CEW, *Helicoverpa zea* (Boddie)], velvetbean caterpillar [VBC, *Anticarsia gemmatalis* (Hübner)], and fall armyworm [FAW, *Spodoptera frugiperda* (J.E. Smith)] caterpillars via antibiosis and antixenosis. The ME and MGHE NILs exhibit similar levels of resistance in all but one of the bioassays. Therefore there is no indication that the addition of QTL-G and/or QTL-H to the ME combination is required to reach agriculturally relevant levels of resistance. From a breeding perspective, introgressing just QTL-M and QTL-E into an elite cultivar is simpler than introgressing all four QTLs. As the number of QTLs

increases, pyramiding in an elite line, becomes increasingly difficult; especially when selection involves several traits at a time. Furthermore, QTL-G is currently associated with a yield penalty, although attempts are currently under way to remove that linkage drag. Altogether, pyramiding the major insect-resistance QTLs from PI 229358 and PI 227687 presents an effective genetic combination to deploy host-plant resistance to insects in soybean.

To determine if the combination of ME and *cry1Ac* (Bt) would also provide enhanced resistance in comparison to lines with only the *cry1Ac* transgene or the QTLs by themselves, Benning$^{ME+cry1Ac}$ was characterized in antibiosis assays. This line is more resistant than Benning^{ME} and Benning^{cry1Ac} against SBL and southern armyworm [SAW, Spodoptera eridania (Stoll)]. Although this combination would need to be thoroughly studied in antixenosis field cage assays and, if possible, in field tests with natural pest infestations; the results from the antibiosis assays indicates the potential of combining ME and *cry1Ac* to improve soybean resistance to insects that are naturally tolerant to *cry1Ac*. The use of this pyramid as part of a resistance management strategy could help preserve the effectiveness of *cry1Ac* and potentially other Bt genes, which could lead to durable resistance to leaf-chewing insects in soybean.

The elite lines Benning-ME and Benning-MGHE were developed for germplasm release; they would be useful to soybean breeders for simultaneous selection of QTL-M and QTL-E; and QTL-M, QTL-G, QTL-H, and QTL-E respectively. Benning-ME is very useful if breeders prefer to introgress only QTL-M and QTL-E, and/or exclude QTL-G because of the current yield penalty. Like the insect-susceptible Benning, Benning-ME and Benning-MGHE have determinate growth habit and belong to MG VII. Both lines have purple flowers, tawny sharp pubescence, tan pods, yellow seed coat, and sharp trichomes. Benning-ME has brown hilum, and Benning-MGHE has brown hilum of varying intensity.

They are also similar to Benning in plant height, lodging score, and seed quality score. The QTL introgressions in Benning-ME and Benning-MGHE were estimated in high-density SNP genotypes obtained using the SoySNP50K iSelect BeadChip. Kompetitive Allele Specific PCR (KASP) assays were designed to detect SNP alleles flanking each QTL; these assays facilitate high-throughput genotyping and selection of breeding lines carrying a specific QTL combination. Furthermore, the graphical-genotypes for the QTL-introgression provide a reference for fine mapping and cloning of candidate genes responsible for insect resistance in QTL-G, QTL-H, and QTL-E.

To determine if the addition of insect-resistance QTLs has negative effects on the feed quality of soybean meal, the suitability of soybean meal from the insect-resistant NILs Benning^{M} and Benning^{MGH} for broiler chickens was evaluated. Digestible amino acid content and nitrogen-corrected true metabolizable energy (TME_N) were measured for each soybean meal, and diets containing each soybean meal were tested in a 21-day broiler feeding trial. The overall nutrient profiles of soybean meal from Benning, Benning^{M}, and Benning^{MGH} were comparable, despite minor differences that were detected on amino acid digestibility among soybean meals. These variations could be the result of slight differences in seed composition, or differences in the small-batch processing of the meals. The TME_N values were similar for the three soybean meals, 2560, 2569, and 2544 kcal kg^{-1} for Benning, Benning^{M}, and Benning^{MGH} respectively. In the 21-day feeding trial, the performance of Cobb x Cobb male broilers was equivalent for Benning, Benning^{M}, and Benning^{MGH} diets. No statistically significant differences were found among diets for weight per chick, weight-gain per chick, and feed to gain ratio at 7, 14, and 21 days of age. Therefore there is no indication that meal produced from soybean seed carrying QTL-M, QTL-G, and QTL-H would not be as safe as the insect-susceptible Benning soybean cultivar when used for animal

feed. Since PI 229358 has been used in soybean breeding programs worldwide as a source of genetic resistance to leaf-chewing insects, the results of this study are highly relevant.

The gene controlling QTL-M, which is by far the major determinant of leaf-chewing insect resistance discovered in soybean, was still unknown. As major component of this dissertation, the insect resistance gene was identified in a fine-mapping approach, and its function was validated using transgenic soybean lines and NILs. The insect resistance gene corresponds to the soybean gene model Glyma07g14530. Because of its role in resistance to leaf-chewing insects, the gene has been named *GmORUGA*, derived from the Spanish word for caterpillar. At the same time OR reflects the surname of the person who cloned it, and UGA the location where the work took place. The resistant allele corresponds to a mutation of TG**G** (^{275}W) to TG**A** (stop), which leads to a truncated protein of 274 amino acids. *GmORUGA* is induced in whole plants as early as 24 hr after infestation. No significant differences in induction level were found between resistant and susceptible soybeans, thus indicating that resistance is not caused by differences in transcription levels. Therefore it was hypothesized that resistance is achieved by the loss-of-function of Glyma07g14530, whereas susceptibility is conferred by the functional gene. Transgenic soybean lines were used to test the loss-of-function hypothesis. Altogether, these experiments confirmed that complementing a QTL-M resistant line with the susceptible *GmORUGA* allele restores susceptibility, and that silencing *GmORUGA* in susceptible lines results in resistance. Therefore the Glyma07g14530 gene present in most of the soybean germplasm is an allele for susceptibility, and the mutation found in PI 229358 and the other resistant accessions is responsible for the insect resistance associated with QTL-M.

GmORUGA is a predicted UDP-glucosyltransferase (UGT). The isoflavone glucosyltransferase GT07O02 from kudzu [*Pueraria lobata* (Willd.)] is the closest protein match

to *GmORUGA*. Isoflavones are a type of secondary metabolites that are almost exclusively produced in legumes. To determine if *GmORUGA* plays a role in isoflavone glycosylation in soybean, isoflavone profiles of Benning, Benning^{M}, Jack, and Jack^{M} leaves obtained by HPLC were compared before and post-infestation. Genetic-background specific differences were found between Benning and Jack. The major compound in Benning was daidzin, and no differences were observed between Benning and Benning^{M}, before or after infestation. The major compound in Jack was rutin. Glycitin was not detected in Benning, but it is present in Jack. The only compound that is different between susceptible and QTL-M plants, independent of genetic background, is genistin. Concentrations of genistin were the same between Benning and Benning^{M}, and Jack and Jack^{M} before insect attack. However, after insect attack, genistin concentration is significantly reduced in both Benning^{M}, and Jack^{M}.

Therefore *GmORUGA* is a genistein 7-O-glucosyltransferase that is induced in leaf tissue after caterpillar damage to produce genistin. The functional enzyme in the susceptible soybean contributes to maintaining a constant concentration of genistin after insect attack, whereas this enzyme is not active in the QTL-M plants, and genistin levels are significantly reduced. To understand how variation in genistin levels could be associated with insect-resistance, the levels of condensed tannins (CT) were compared between susceptible and QTL-M plants. CTs are derived from the genistein precursor in a parallel pathway derived from the genistein precursor. Tannins have been studied in the last 50 years for their role on plant resistance to insects, albeit with inconclusive results. CT levels remain constant in Benning and Jack, while they increase in both Benning^{M} and Jack^{M} after infestation, presumably through a redirection of metabolic flux to produce CTs instead of genistein.

Genes for resistance to leaf-chewing insects have been described in plants, but none provide any insight into insect resistance for soybean. What these genes have in common is the accumulation of a metabolite that is detrimental to insects. This is the first report of a gene for resistance to leaf-chewing insects that operates via the isoflavone pathway. Discovering *GmORUGA*, and its role in isoflavone and tannin metabolism is a important contribution to understanding how plants evolved to defend from pests. The availability of this gene should assist the identification resistance genes in other crop plants, which will make it possible to develop insect-resistant cultivars, and design strategies to improve the insect-resistance genes' function and durability. Such cultivars are an important component of sustainable agricultural systems.