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Characterization of the Tolerance Response in the Soybean KS4202 to *Aphis glycines* Matsumura

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to *Aphis glycines* Matsumura

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Since the introduction of the soybean aphid, *Aphis glycines* Matsumura, to the soybean growing regions of the United States, the soybean aphid has caused considerable economic damage and yield loss to soybean growers. The objectives of this research were to evaluate selected genotypes for resistance to the soybean aphid and characterize transcriptional changes in response to aphid feeding to better understand the underlying tolerant mechanism(s) in KS4202 and genes contributing to its tolerance response. A field study (2009) was conducted to evaluate selected soybean genotypes during their reproductive stages for resistance to *A. glycines*. The economic injury level (EIL) was reached in all genotypes during the 2009-growing season. Most of the genotypes showed no significant differences in yield or yield parameters with some minor exceptions for a few yield parameters. For KS4202, the average seed weight and the average number of seeds per pod for aphid infested treatments were significantly lower than their respective non-infested control plants. The mean number of aphids was significantly higher for KS4202 when compared to the other genotypes and the average

peak number of aphids for this genotype was almost 5 times the economic threshold. The second component of this research was to characterize transcriptional changes in response to aphid feeding to better understand the underlying tolerant mechanism(s) in KS4202 and genes contributing to the tolerance response. Comparing gene expression levels between infested and control plants for KS4202, over 550 genes had a higher expression level in response to aphid feeding, while, over 650 genes had a lower expression level in response to aphid feeding. For K03-4686 (susceptible), over 150 genes had a higher expression level in response to aphid feeding, whereas, over 750 genes had a lower expression level when comparing infested to control plants. This research will significantly add to the understanding of the mechanisms of soybean aphid tolerance in soybeans and allow for the continual development of improved soybeans varieties with soybean aphid resistance.

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Introduction and Thesis Objectives

The soybean aphid, *Aphis glycines* Matsumura, has become a serious pest of soybean, *Glycine max* Merr, since its introduction into the soybean-growing region of the United States in the early 2000s. Much research has focused on identifying resistant sources of soybean; however, the concentration on this research has been on antibiotic and antixenotic genotypes rather than on tolerant sources. Furthermore, most studies have been conducted on seedling soybeans, even though the soybean aphid does not typically arrive in Nebraska until soybean plants have reached the reproductive stages. Although many antibiotic and antixenotic sources have been identified, little is known about the mechanisms of resistance and how soybean feeding impacts the physiology and biochemistry of the plant. Therefore, the focus of this research was to evaluate selected genotypes for resistance to the soybean aphid, characterize the tolerance response of the soybean genotype KS4202, and investigate the underlying mechanisms and genes conferring tolerance.

Objectives:

1) Evaluate soybean genotypes during their reproductive stages for resistance to *Aphis glycines* under field conditions.
2) Characterize transcriptional changes in response to aphid feeding to better understand the underlying tolerant mechanism(s) and genes contributing to the tolerance response.

CHAPTER 1

Introduction and Literature Review

Soybeans.

Soybeans (*Glycine max* (L.) Merrill) are an important crop in the United States and throughout the world. Between 75.7 million and 77.4 million acres of soybeans were planted each year between the 2008 and 2010 growing seasons, producing 3.0 billion to 3.4 billion bushels (USDA 2011 (A); USDA 2011 (B); USDA 2011 (C)). In Nebraska, 4.7 million to 5.1 million acres were harvested each year producing 226 million to 268 million bushels (USDA 2011 (A); USDA 2011 (B); UNL Cropwatch 2008; UNL Connect 2010). Soybeans are grown all around the world and have a variety of uses, including for animal and human consumption, biofuels, and several other industrial uses such as hydraulic fluids, lubricants, and plastics.

Aphid Biology in North America.

The soybean aphid, *Aphis glycines* Matsumura, is a native crop pest of eastern Asia and was first confirmed in the North Central growing region of the United States during the 2000 growing season, though several reports indicate the arrival of the aphid in previous years (Dai and Fan 1991; Ragsdale et al. 2004). Since the arrival of the soybean aphid in North America, aphids have been found in 30 states as well as several south Canadian provinces causing considerable damage (NAPIS 2011; Ragesdale et al. 2011; Venette and Ragsdale 2004).

Soybean aphids exhibit a heteroecious and holocyclic lifecycle. This means that the aphids alternate hosts and produce sexual offspring during part of their lifecycle

(Ragsdale et al. 2004). The primary hosts of the soybean aphid in North America are the *Rhamnus* spp., usually that of the common buckthorn, *R. cathartica* L. Alder buckthorn, *R. alnifolia* L'Héritier and lanceleaf buckthorn, *R. lanceolata* Pursh, have also been shown to serve as possible hosts (Ragsdale et al. 2004; Voegtlin et al. 2004; Voegtlin et al. 2005).

Soybean aphids will overwinter as eggs on buckthorn, often surviving temperatures down to the eggs' supercooling point of -34°C, which may limit the potential locations for overwintering (Ragsdale et al. 2004; McCornack et al. 2005). The eggs hatch in the spring and develop into wingless fundatrices. These aphids will reproduce parthenogenetically, resulting in a second generation of apterous viviparous females. The third generation consists of winged viviparous females, which migrate to the secondary host, soybeans (Hill et al. 2004a; McCornack et al. 2004; Ragsdale et al. 2004). During the late spring and the early summer, overlapping generations can be found which may consist of both winged and wingless morphs of viviparous females. The rate of reproduction during this period is heavily dependent on temperature. Optimum temperatures are between 20-25°C for fecundity, generation time, and life expectancy, while temperatures above 30°C may significantly reduce aphid numbers and inhibit development (McCornack et al. 2004; Ragsdale et al. 2004). At a temperature of 27.8°C, soybean aphid numbers can double in a day and a half when no natural enemies are present (McCornack et al. 2004). A significant increase has been shown in the proportion of migratory forms (alatoid nymphs and adults) during the beginning of soybean seed set, which coincides with decreasing photoperiod (Hodgson

et al. 2005). Gynoparous females are produced on soybean in the fall and migrate to the primary host, *Rhamnus* spp., where they feed and produce pheromone-emitting wingless female offspring called oviparae (Ragsdale et al. 2004; Zhu et al. 2006). Males are also produced on soybean and migrate to the wintering host, where mating occurs and overwintering eggs are laid (Ragsdale et al. 2004). The soybean aphid does not generally appear on soybeans in Nebraska until late June to mid-July, whereas, other regions of the country tend to detect the soybean aphid on soybeans as early as the beginning of June. This could possibly be because of low numbers of *Rhamnus* spp. in Nebraska, requiring the soybean aphid to migrate from other states . Because of this, the soybean aphid is not normally seen in Nebraska until soybeans are in their reproductive stages (Brosius et al. 2007).

Impact of Soybean Aphid in North America.

Soybean aphids typically inhabit the undersides of soybean leaves beginning with their infestation on the younger trifoliate leaves. As the plant begins to mature and aphid numbers begin to climb, aphids begin infesting the lower canopy. As aphid numbers grow, aphids can be found throughout the plant on leaves, petioles, pods, and stems (Blackman and Eastop 2000; Ragsdale et al. 2004). Soybean aphids will pierce the stem of their hosts in order to withdraw phloem contents, which may lead to viral infection (e.g. soybean mosaic virus), stunted plants, poor canopy development, and a reduction in photosynthesis. Sooty mold buildup may also occur due to high levels of honeydew accumulation (Clark and Perry 2002; Ostlie 2002; Domier et al. 2003; Davis et al. 2005). High aphid numbers may have severe consequences on overall plant

performance (growth and yield). Some of these consequences include a reduction in the number of pods, the number of seed per pod, and individual seed weight (Myers et al 2005; Beckendorf et al. 2008). Another consequence of heavy soybean aphid feeding is the reduction of seed oil concentration. At levels that fall below 19% concentration, the marketability becomes less desirable (Beckendorf et al. 2008). Due to the potential yield loss as a result of aphid feeding, it is vital to develop management strategies to reduce the overall effects of the soybean aphid.

Economics of the Soybean Aphid.

Without proper management strategies for controlling the soybean aphid, the economic impact to growers can be severe. In 2003, several North Central states were impacted by soybean aphid injury. In Illinois, over 0.5 million hectares of soybeans were injured from aphid feeding resulting in a $45 million loss to farmers (Steffey 2004; Hill et al. 2010). In Minnesota, over 1.6 million hectares of soybeans were injured resulting in an $80 million loss to farmers (Associated Press 2003; Hill et al. 2010). A recent economic impact study on the soybean aphid predicts an annual $3.6-4.9 billion loss to the soybean industry without proper management tool availability (Kim et al. 2008). These numbers were predicted based on the insecticide application cost, the severity of the aphid outbreak, and the price elasticity of the soybean supply.

Methods for Managing the Soybean Aphid in North America.

Chemical Control.

There are several control methods that can be used to manage the soybean aphid. These control methods include chemical control, biological control, and cultural

control (including plant resistance). The primary control method for managing soybean aphid is chemical control. Upon arrival of the soybean aphid in the early 2000s, it was the sole method of aphid management (Hill et al. 2004b). The economic impact of the soybean aphid on yield is substantial, thus promoting growers to apply insecticides to prevent yield loss (Myers et al. 2005). Although insecticides quickly limit aphid injury, surviving soybean aphids can rapidly reproduce in the absence of natural enemies following an insecticide application (Myers et al. 2005). Timing is another difficulty that accompanies chemical control of the soybean aphid. If an application is made too early, aphid numbers are likely to recover or reinvest, which could lead to an impact on yield (Myers et al. 2005). Alternatively, waiting too long and allowing aphid densities to peak could mean that most of the feeding damage has already been done. In the perfect world, one insecticide application would be made right before aphid densities reach an economic injury level. Similar to the North Central United States, chemical control is widely used in China. Dai and Fan (1991) report as many as four insecticide applications may be used in a single growing season. Conflicting recommendations can be found in the literature on when to treat the aphid to get the greatest benefit. Wang et al. (1996) recommends a chemical application at the end of June, while Lin et al. (1994) recommends an application during the early reproductive stages of soybeans. Baute (2002) reported insecticide applications in Canada being applied during the R1 reproductive stage. Applications during this period in Canada appeared to give the most benefit in reducing numbers and protecting crop yields. For many parts of the North Central US, peak aphid densities occur during late July to early August (Ragsdale et al.

2004; Myers and Gratton 2006). Sampling studies have indicated that aphid populations are more likely to reach damaging levels in later-planted soybeans (June) over early-planted soybeans (May) (Myers et al. 2005). This observation may be related to more favorable conditions for aphids paired with younger soybean plant tissue for them to feed on. Myers et al. (2005) show that chemical treatments are best applied during the V1 vegetative stage, as well as the R3 reproductive stage. Results from Myers et al. (2005) indicate that treatment during R2 and R4 reproductive stages were consistently less effective in improving yield. Since soybean aphid populations are usually rare in the field during the V1 vegetative stage, it would seem unnecessary to apply insecticides for control, especially since a second treatment may be likely within a few weeks. For the R2 stage, treatments appear to be beneficial and are not significantly different from treatments in the R3 stage, although experimental results indicate a slight yield improvement for applications in the R3 stage (Myers et al. 2005). Once the soybean canopy has fully developed (around the R4 stage), it appears that insecticide applications are not as effective because aphids may be protected in the lower canopy, allowing for some populations to rebuild which could necessitate a second insecticide application (Myers et al. 2005).

Biological Control.

Biological control is also being considered as an alternative to chemical control. Some difficulties of biological control are that programs do not occur in a vacuum and hold the potential for unknown environmental risks (Hokkanen and Lynch 1995; Follett and Duan 2000; Wajnberg et al. 2001). The potential risks of biological control should

be weighed against the risks of not beginning the control method. Before moving forward with biological control, two decisions should be made: (1) is there warrant for biological control importation and (2) which species should be introduced? Since the arrival of the soybean aphid in the early 2000s, several aphid predators and parasitoids have been identified to aid in its management. These include nine dipteran predators and six hymenopteran parasitoids (Kaiser et al. 2007). Exploration of natural enemies was conducted in China and Japan from 2001 to 2002 with the desire to introduce selected aphid parasitoids. Several parasitoid species were found in Southeastern Asia soybean fields including that of *Aphelinus albipodus* (Aphelinidae), *Lysiphlebus fabarum* (Marshall) and *Lipolexis gracilis* Förster (Braconidae: Aphidiinae) (Heimpel et al. 2004). These parasitoids were effective at low soybean aphid densities. Several strains of these parasitoids have been imported to Newark, DE for continued study. A non-Japanese strain of *A. albipodus* has already been released in parts of the Western United States in the early 1990s to help control the Russian wheat aphid (Hopper et al. 1998; Prokrym et al. 1998; Heimpel et al. 2004) and are now established in several US states including California, Idaho, Kansas, Nebraska, and Oklahoma (Prokrym et al 1998; Burd et al. 2001; Heimpel et al. 2004). During the summer of 2001, recoveries of these strains were found in soybean fields in Wyoming. In laboratory settings, individuals of this strain were confirmed as parasitizing soybean aphid. As a result, the USDA Animal and Plant Health Inspection Service (APHIS) had begun mass rearing of this strain (Heimpel et al. 2004). By the 2002 field season, three strains of parasitoids, 2 *A. albipodus* and 1

L. gracilis, had successfully passed through quarantine and provide a secondary option to combat the soybean aphid (Heimpel et al. 2004).

Just as parasitoids can be used to combat the soybean aphid, several arthropod predators of the soybean aphid exist as well. In parts of Asia, soybean aphids can be suppressed by more than 30 species of predators (Quimio and Calilung 1993; van den Berg et al. 1997; Chang et al. 1994; Wang and Ba 1998; Wu et al. 2004; Rutlegde et al. 2004) including the coccinellid beetle *Harmonica arcuata* (F.), and the staphylinid beetle *Paederus fuscipes* Curtis (van den Berg et al. 1997). According to Rutledge et al. (2004), approximately 30 species of ground dwelling Coleoptera from the family Carabidae were found to aid in suppression of the aphid in Indiana and Michigan soybean fields. Rutledge et al. (2004) also indicated the potential for 9 foliar-foraging Coleopteran species from the Cantharidae and Coccinellidae families, 4 heteropterans, 3 neuropterans, 2 dipterans, and a Lampyrid as potential predators. Predators that occur early and in high numbers (e.g. *Orius insidiosus* (Say)) appear to have a higher probability of preventing an outbreak than those that appear later in the season (*Harmonia axyridis* (Pallas)) (Rutledge et al. 2004). Because soybean aphids are typically found in the upper soybean canopy, one would expect to find more foliage dwelling predators aiding in aphid suppression, although the ground dwelling predators may have some suppression influences. Rutledge et al. (2004) found that the most common aphid predators in the field were the minute pirate bug and multicolored Asian lady beetle. In fact, more than 85% of all predators found in their Indiana field location were these two predators, feeding on aphids in both adult and immature stages. Fox (2002)

found that *A. glycines* survival was reduced in field trials where predators were present. These predators appear to be effective because they show a strong numerical response, especially in areas of high aphid densities (Rutledge et al. 2004).

Cultural Control.

Cultural control is another method that can be used to reduce soybean aphid population. Significant yield protection and effective control of aphids by their natural enemies was observed with the interplanting of maize and soybeans (Wang and Ba 1998; Wang et al. 2000; Wu et al. 2004). A similar effect was observed when soybean and maize seeds were sown in the same holes. In China, breeding programs for insect selection exist along with disease resistant varieties. These varieties may differ significantly between each other when selecting for soybean aphids (Wu et al. 2004). According to Hu et al. (1992, 1993), soybean varieties with higher lignin content inhibited soybean aphid infestation while varieties with higher nitrogen content appeared to be more susceptible to soybean aphid damage.

Host Plant Resistance.

According to Smith (2005), plant resistance to arthropods is "the sum of the constitutive genetically inherited qualities that result in a plant of one cultivar or species being less damaged than a susceptible plant lacking these qualities." Susceptibility is defined as "the inability of a plant to inherit qualities that express resistance to arthropods (Smith 2005)." The resistance of a plant is measured on a relative scale based on the degree of resistance in comparison to the susceptible control plant that is more severely damaged or killed under identical experimental conditions. The

measurement of resistance should also be based on a resistant control with a known, predetermined level of resistance. These relative measurements are a necessity as resistance is "influenced by environmental fluctuations occurring over both time and space (Smith 2005)."

Host plant resistance can be divided into three categories. These are: (1) antibiosis, (2) antixenosis, and (3) tolerance. The categories of resistance were originally described by Painter (1951) and more precisely defined by Horber (1980) as functional categories. Antibiosis occurs when "the negative effects of a resistant plant affect the biology of an arthropod attempting to use that plant as a host (Smith 2005)." The effects of an antibiotic plant can range from mild to lethal. This could be the result of either chemical or morphological plant defenses. Lethal, acute effects often affect the larvae and eggs while chronic effects can lead to mortality affecting older larvae and pre-pupae, which may fail to pupate (Smith 2005). Individuals that survive the effects of antibiosis will often see reduced body size and biomass, reduced fecundity, and prolonged period of development in the immature stages (Smith 2005). Antixenosis, originally described as 'non-preference' by Painter (1951), denotes "the presence of morphological or chemical plant factors that adversely alter arthropod behavior (Smith 2005)." As a result, the arthropod may seek out an alternative host plant. Some of the factors include thickened plant epidermal layers, waxy deposits on the leaves, or a change in trichome density on the leaf surface. Both of the above plant resistance categories, antibiosis and antixenosis, may impose selection pressure on arthropod pests. As a result, it is possible to see biotype development.

Biotypes can be defined as "populations within an arthropod species that differ in their ability to utilize a particular trait in a particular plant genotype (Gallun and Khusk 1980; Wilhoit 1992; Pedigo 1999; Smith 2005)." Although I have given a definition for biotype, there is no fully recognized definition in the scientific community. Until recently, soybean aphid biotypes had been relatively unknown in North America. Over the past few years, several soybean breeding lines have been in developed that express resistance. Some of these lines included those possessing the *Rag1* gene. In 2006, Kim et al. (2008) reported dense colonies of aphids surviving on plants containing the *Rag1* gene in research fields within the state of Ohio. According to Kim et al. (2008), aphid numbers were similar to that which could be found on the susceptible, Williams 82. Based on observations noted between isolates collected in Illinois and Ohio, the Ohio isolate was distinguishable from the isolate because large colonies could grow and survive while the Illinois isolate could not colonize plants containing *Rag1* (Hill et al. 2010). As a result of the soybean aphid biotype discovery, it became clear that the aphids could adapt to these genes showing that further biotype development is possible. Hill et al. (2010) found a third aphid isolate and possible biotype outside of Springfield, Indiana in 2007. This particular isolate drew attention as they had found populations building on a new breeding line containing the *Rag2* gene. After several years of testing, the Indiana isolate was found to readily colonize plants containing the *Rag2* breeding lines which distinguished itself from biotypes 1 (Illinois isolate) and 2 (Ohio isolate). As a result, isolate 3 was confirmed to be a new biotype in the United States. With that, Hill et al. (2010) confirmed the Indiana isolate as biotype 3. As new

breeding lines and new combinations of the *Rag* gene become available, it will be vital to continue searching for new soybean aphid biotypes in the future to prevent large scale outbreaks and protect farmer's soybean yields.

It is possible to find soybeans that are resistant to soybean aphid under the final category of host plant resistance, tolerance. Tolerance can be defined by the ability of the plant to withstand or recover from damage caused by arthropod populations (Smith 2005). Tolerant plants are known to produce a greater amount of biomass over non-tolerant, susceptible cultivars (Smith 2005). There are five primary factors that may result in a plant possessing tolerance. These factors include (1) increased net photosynthetic rate, (2) high relative growth rate, (3) increased branching/tillering after apical dominance release, (4) pre-existing high levels of carbon found in the root system, (5) the ability to transfer stored carbon from the roots to the shoots, and (6) increased oxidative enzyme activity (Gawronska and Kielkiewicz 1999; Strauss and Agrawal 1999; Smith 2005; Heng-Moss et al. 2004; Franzen et al. 2007). Unlike the other two forms of host plant resistance, tolerance is a plant response. As a result, tolerance imposes minimal if any selection pressure on the insect. The pest is more likely to remain avirulent to the plant (Smith 2005). Another benefit to tolerance is that the effects of beneficial arthropods will be enhanced because the symptoms of antibiosis and antixenosis will be next to nothing.

Over the past decade, several screening studies have been conducted to identify resistant soybean genotypes. Three of the first reported genotypes to show resistance to the soybean aphid include 'Dowling,' 'Jackson,' and PI-71506 (Hill et al. 2004b).

Dowling and Jackson, along with their ancestor 'Palmetto,' possess strong antibiosis while PI-71506 is antixenotic. For Jackson and Dowling, a single dominant gene appears to be responsible for the antibiotic resistance to the soybean aphid (Hill et al 2006a; Hill et al. 2006b). Screening studies performed by Diaz-Montano et al. (2006) also identified additional sources of resistance to the soybean aphid. In his study, resistance was indicated in varieties K1639 and Pioneer 95B97. In these varieties, characteristics of both antibiosis and antixenosis appeared to be present. His study also went on to suggest the presence of antixenosis in addition to the antibiosis found in Dowling, Jackson, and Palmetto (Diaz-Montano et al. 2006). Further, he reported reduced fecundity and longevity of the soybean aphid in genotypes Dowling and Jackson when compared to the susceptible, 'Pana.' Li et al. (2004) found a high percentage of mortality and no maturation of first instar soybean aphids on genotypes Dowling and PI-200538. This observation would suggest a higher level of antibiosis in these two genotypes when compared against Jackson. The resistance provided by the *Rag1* gene, found in Dowling, and the *Rag* gene, found in Jackson, has since broken down in the field leading to possible biotype development (Kim et al. 2008).

A study completed at Michigan State University focused on genotypes that are typically grown in parts of Northern China. These genotypes were chosen because of the similarity in climate between China and the North Central region of the United States. A total of 2147 soybean plant introductions (PI) from maturity groups 0 to III were evaluated (Mensah et al. 2005). From these maturity groups, 5 PIs from maturity group 0, 530 PIs from maturity group I, 979 PIs from maturity group II, and 633 PIs from

maturity group III were evaluated. Williams 82 was included in this study as a susceptible check while Dowling, Jackson, PI-71506, or some combination of the three, was included as the resistant check(s). The first evaluation was a choice test evaluating the preference of soybean aphid colonization and determining whether or not the PI was resistant. If the choice test indicated resistance, a second evaluation, a non-choice test, was conducted. The non-choice test would be used to determine if the genotype was antibiotic or antixenotic. Of the 2147 PIs chosen for evaluation, only six lines were rated as resistant during the 2002 and 2003 growing seasons in the choice tests (Mensah et al. 2005). The PIs rated as resistant were PI-567543C, PI-567597C, PI-567541B, PI-567598B, PI-603392, and PI-603418C with all lines belonging to maturity group III (Mensah et al. 2005). For the non-choice tests, PIs PI-567541B and PI-567598B had adverse effects on the soybean aphids and thus possessed antibiosis (Mensah et al. 2005). PI-567543C and PI-567597C did show resistance in the choice test, but failed to show that resistance again in the non-choice test (Mensah et al. 2005).

A more recent study completed by Mian et al. (2008) focused in on the use of the two different soybean aphid biotypes: the Illinois isolate (biotype 1) and the Ohio isolate (biotype 2). These authors evaluated approximately 200 genotypes under both field and greenhouse conditions. The Ohio biotype has been shown to overcome the resistance found in Dowling and Jackson, while it appears that the Illinois biotype (original introduction) has remained suppressed by the resistance previously found (Kim et al. 2008; Mian et al. 2008). From their study, a total of nine genotypes were found to show resistance to the soybean aphid. Genotype PI-243540 appeared to show strong

antibiosis, which is being controlled by a single dominant gene (Kang et al. 2008). Genotypes PI-567301B appeared to show strong antixenosis. It is important to note that the above screening studies used to evaluate resistance to the soybean aphid were completed during the early seedling stages (Hill et al. 2004b, Diaz-Montano et al. 2006, Mian et al. 2008).

While many resistant sources have been identified, very few studies have focused on identifying tolerant soybeans and characterizing their mechanisms. Over the past decade, the main focus on tolerance in resistance studies has been directed toward the seedling stages. Over the next few years, it will likely become more important to expand the research to the later vegetative and reproductive stages of soybeans. There is also a need to identify the genes and mechanisms of this resistance.

Next Generation Sequencing - Illumina Genome Analyzer.

Since Sanger et al. (1977) first described dideoxynucleotide sequencing of DNA, technology has allowed the DNA sequencing process to grow into a powerful large-scale production enterprise that requires the use of devoted robotics, bioinformatics, large scale computer databases, and instrumentation (Mardis 2008). When analyzed with the appropriate computational algorithms, the ability to answer questions about the mutational spectrum of an organism, from a single base to large copy polymorphisms on a genome wide scale, will radically change our understanding of model organisms. Next generation sequencing will allow scientists to do more with less funding. Next generation sequencing methods will give scientists the ability to process millions of sequence reads in parallel rather than the traditional 96 reads at a time (Mardis 2008).

With these types of runs, conventional vector based cloning and *Escherichia coli* based amplification stages found in capillary sequencing are eliminated as next generation sequencing reads are built from fragment libraries (Mardis 2008). Sequence ready libraries can be prepared from DNA fragments that originate from a variety of front end processes and are prepared for sequencing by ligating specific adaptor oligonucleotides to both ends of each DNA fragment. As a result, little input DNA is needed to build the library.

With continual upgrades in technology, next generation DNA sequencers have changed the way researchers study genetics. The genome analyzer, also known as the Illumina sequencer, now gives researchers the ability to produce hundreds of mega-bases of sequence information from a single run (Quail et al. 2008). Since soybeans are of high agronomic value in several areas of the world, the detection of a dense and genome wide set of single nucleotide polymorphisms (SNPs) in relevant germplasm is an essential goal for trait discovery and for agronomic improvement (Rafalski 2002; Palaisa et al. 2003; Wilson et al. 2004; Yu and Buckler 2006; Eathington et al 2007; Deschamps et al. 2010). Reference assemblies provide an essential resource to rapidly position sequences and genetic variations onto a physical map and provide a detailed context when overlaid with associated genome annotations (Hillier et al. 2008).

As a result of a recent public initiative, a genome assembly has already been constructed from a shotgun sequence of soybean cultivar Williams 82. The annotation of this assembly remains an ongoing process (Deschamps et al. 2010). The current construct of the soybean cultivar Williams 82 genome is rather complex. The estimated

size of the genome is around 1.15 Gb with a repeat content believed to be between 60 and 70%. A high number of paralogous sequences are found within the transcribed regions of the construct (Arumuganathan and Earle 1991; Shoemaker et al. 1996; Foster-Harnett et al. 2002; Nelson and Shoemaker 2006; Deschamps et al. 2010). The repeated sequences found within the genome are generally comprised of autonomous and non-autonomous transposable elements, with this class making up a majority of the soybean genome (Mudge et al. 2004; IRGSP 2005; Schlueter et al. 2007; Deschamps et al. 2010).

An effort by Hyten et al. (2008), examined the success rate of converting verified SNPs (single nucleotide polymorphisms) into working assays. A custom 384 SNP GoldenGate (Illumina) assay was designed with SNPs that were discovered through the re-sequencing of five diverse accessions that are the parents of three recombinant inbred line mapping populations. The 384 SNPs used were predicted to segregate into one or more of the recombinant inbred line populations. Allelic data was successfully generated for 89% of the SNP loci (342 of 384) when used in the three recombinant inbred line mapping populations. These results would indicate that the complexity of the soybean genome had little to no impact on the conversion of discovered SNPs into assays. The high success rate of the GoldenGate (Illumina) assay validates the technique for creating high density genetic maps in species where SNP markers are available.

The onset of the Illumina technology has allowed for the rapid re-sequencing of genomes on a large scale for a fraction of the cost and time commitments in comparison to some of the traditional technology (Shendure and Ji 2008). The development of

reduced representation libraries (RRLs) or cDNA libraries are two effective ways to target coding regions of a genome to avoid sequencing repetitive data. As a result, analysis should be a bit less tedious. The Illumina sequencer allows one to focus in on the transcriptome, which will allow for a reduction in the complexity of the genome being sequenced. Overall, the Illumina sequencer is relatively new, but holds great potential in the world of genetics, especially to those in which agronomic practices can be positively impacted.

CHAPTER 2

Evaluation of reproductive stage soybeans for resistance to the soybean aphid, *Aphis glycines* Matsumura (Hemiptera: Aphididae) in the field.

*This chapter represents a compilation of work done by Lanae Pierson in 2007 and Travis Prochaska in 2009. Sections from Pierson's thesis have been incorporated in this chapter.

Introduction

Soybeans (*Glycine max* (L.) Merrill) are an important commodity in the United States and throughout the world. Since the introduction of the soybean aphid, *Aphis glycines* Matsumura, to the United States in the early 2000s, aphids have spread to 30 states and several Canadian provinces (Hartman et al. 2001; Alleman et al. 2002; Venette and Ragsdale 2004; Beckendorf et al. 2008; NAPIS 2011). The soybean aphid has caused considerable economic damage to soybean growers since its introduction.

Soybean aphids exhibit a heteroecious and holocyclic lifecycle (i.e. the aphid alternates hosts and produces sexual offspring during part of its lifecycle) (Ragsdale et al. 2004). The primary hosts of the soybean aphid in North America consist of *Rhamnus* spp., the most suitable being common buckthorn, *R. cathartica* L. Alder buckthorn, *R. alnifolia* L'Héritier, and lanceleaf buckthorn, *R. lanceolata* Pursh, have also been shown to serve as possible hosts (Ragsdale et al. 2004; Voegtlin et al. 2004; Voegtlin et al. 2005). The secondary host is soybean (Hill et al. 2004a; McCornack et al. 2004; Ragsdale et al. 2004). The soybean aphid does not generally appear on soybeans in Nebraska until late June to mid-July, whereas, other regions of the country they tend to detect the soybean aphid on soybeans as early as June. This could be because of the lack of significant populations of *Rhamnus* spp. in Nebraska. Because of this, the soybean aphid

is not usually reported in Nebraska until soybeans are in their reproductive stages (Brosius et al. 2007).

Initial infestations of soybean aphids in soybean are typically found on the undersides of young, tender leaves. As the plant matures and aphid numbers increase, the aphids can be detected throughout the soybean canopy on leaves, petioles, stems, and pods (Blackman and Eastop 2000; Ragsdale et al. 2004). Soybean aphid feeding can affect the plant in several ways, including the removal of photosynthates causing a reduction in photosynthesis (Ostlie 2002). Soybean aphids can also transmit viral diseases such as soybean mosaic virus and soybean stunt. Extreme honeydew accumulation may cause a buildup of sooty mold (Ostlie 2002; Clark and Perry 2002). Yield losses of up to 50% have been reported (Wang et al. 1994; DiFonzo and Hines 2002; Ragsdale et al. 2004; Mensah et al. 2005). Soybean aphids can reduce soybean yield by reducing the number of pods, number of seeds per pod, and individual seed weight (Myers et al. 2005; Beckendorf et al. 2008). Since the soybean aphid has the potential to have severe effects, several strategies have been developed to manage this pest including chemical, biological, and cultural control methods (Wang and Ba 1998; Wang et al. 2000; Ostlie 2002; Hill et al. 2004b, Wu et al. 2004, Rutledge and O'Neil 2005; Brosius et al. 2007).

Although continued progress has been made in developing effective management strategies for the soybean aphid, it remains essential to continue exploration of alternate aphid management options in order to reduce insecticide use.

The identification and deployment of aphid-resistant soybean cultivars remains an important management option.

Over the past decade, several screening studies have identified resistant soybean genotypes. Three of the first reported genotypes to show resistance to the soybean aphid included 'Dowling,' 'Jackson,' and PI-71506 (Hill et al. 2004b). Dowling and Jackson, along with their ancestor 'Palmetto,' exhibit strong antibiosis while PI-71506 exhibits antixenosis. A single dominant gene appears to be responsible for the antibiotic resistance observed in Jackson and Dowling (Hill et al 2006a; Hill et al. 2006b). Diaz-Montano et al. (2006) reported resistance in varieties K1639 and Pioneer 95B97. Both antibiosis and antixenosis appear to be present in these varieties. His study also suggested the presence of antixenosis in addition to the antibiosis found in Dowling, Jackson, and Palmetto (Diaz-Montano et al. 2006). He reported lower soybean aphid fecundity and longevity in genotypes Dowling and Jackson when compared to the susceptible, 'Pana.' Li et al. (2004) reported a high percentage of mortality and no maturation of first instar soybean aphids on genotypes Dowling and PI-200538. This observation would suggest a high level of antibiosis in these two genotypes when compared to Jackson. The *Rag1* gene, found in Dowling, and the *Rag* gene, found in Jackson, that confer resistance have since broken down in the field leading to possible soybean aphid biotypes (Kim et al. 2008).

A study completed at Michigan State University focused on evaluating genotypes typically grown in parts of Northern China for aphid resistance. These genotypes were chosen because of the similarity in climate between China and the North Central region

of the United States. A total of 2147 soybean plant introductions (PIs) from maturity groups 0 to III were evaluated (Mensah et al. 2005). From these maturity groups, five PIs from maturity group 0, 530 PIs from maturity group I, 979 PIs from maturity group II, and 633 PIs from maturity group III were evaluated. "Williams 82" was included in this study as a susceptible check while Dowling, Jackson, PI-71506, or some combination of the three was included as the resistant check. Choice and non-choice tests were used to determine antibiosis or antixenosis. Of the 2147 PIs chosen for evaluation, only six maturity group III lines were rated as resistant (Mensah et al. 2005). The PIs rated as resistant were PI-567543C, PI-567597C, PI-567541B, PI-567598B, PI-603392, and PI-603418C (Mensah et al. 2005). The lines PI-567541B and PI-567598B exhibited antibiosis (Mensah et al. 2005), while PI-567543C and PI-567597C exhibited antixenosis. The remaining two lines, PI-603392 and PI-603418C, appeared to show signs of tolerance, as they did not show signs of severe damage (Mensah et al. 2005). Although there appears to be signs of tolerance in these genotypes, the authors concluded that several more years of yield and dry matter studies should be completed before confirming the tolerance.

More recent studies have focused on evaluating soybean germplasm for resistance to the two different soybean aphid biotypes, the Illinois isolate (biotype 1) and the Ohio isolate (biotype 2). Mian et al. (2008) evaluated approximately 200 soybean genotypes in field and greenhouse studies. The Ohio biotype has overcome the resistance found in Dowling and Jackson, while the Illinois biotype remains susceptible (Kim et al. 2008; Mian et al. 2008). A total of nine soybean genotypes were found to

show resistance to the soybean aphid. Genotype PI-243540 showed strong antibiosis and genotypes PI-567301B showed strong antixenosis (Kang et al. 2008). It is important to note that the above screening studies were conducted during the early seedling stages (Hill et al. 2004b, Diaz-Montano et al. 2006, Mian et al. 2008).

While many resistant sources have been identified, very few studies have focused on identifying tolerant soybeans and characterizing their resistance mechanisms. Over the past decade, most resistance screening studies have been conducted on the seedling stages. It is important to expand the research and evaluations to later vegetative and reproductive stages of soybeans. This may help researchers to better understand the impact of soybean aphid injury on soybean physiology and how the soybean plant defends itself against soybean aphid feeding. The objective of this research was to evaluate selected genotypes for resistance to the soybean aphid in the later vegetative and reproductive stages.

Methods and Materials

2007 Field Study.

Six soybean genotypes were evaluated for resistance to soybean aphid in a field study at the University of Nebraska Northeast Research and Extension Center Haskell Agricultural Laboratory (HAL), Concord, NE. The genotypes selected for evaluation were 'Dowling' (reported to have resistance in the seedling stage), 'Jackson' (reported to have resistance in the seedling stage), K-1621 (reported to have resistance in the seedling stage), K-1639-2 (reported to have resistance in the seedling stage), KS4202 (reported to be susceptible in the seedling stage), and Asgrow 2703 (unknown resistance) (Hill et al.

2004b, Diaz-Montano et al. 2006). The soybean variety Asgrow 2703 is a commercially available variety commonly grown in northeastern Nebraska (T. Hunt, personal communication). Genotypes were planted with each replication containing an aphid infested and an aphid non-infested treatment.

Standard agronomic practices for northeastern Nebraska were used to maintain experimental plots. Fields were disked twice in the spring prior to planting. Soybeans were planted under the traditional corn-soybean rotation in an Alcester-silt loam soil. Soybeans were irrigated six times by an overhead lateral irrigation system during the growing season (2.5 cm of water each time). Pursuit (DG)® and Cobra® herbicides were used to control weeds.

Experimental design was a randomized complete block with six replications. Plots were three rows wide and 1.5 meters long. Because of limited seed quantity, the center of the center row was planted with nine seeds of the designated genotype in the middle of 0.46 meters. The two outer rows, as well as the outer portions of the center row, were planted with Asgrow 2703 to serve as a buffer. Soybeans were planted on 6 June 2007.

Because natural soybean aphid colonization was very light and sporadic, plots were artificially infested with 10 aphids per plant on 4 August 2007 from leaflets containing 10-50 aphids that were obtained from buffer rows. An infested leaflet was placed on the upper node of one soybean plant in the middle of the row and in each end of the experimental rows. Warrior® was sprayed on the non-infested plots (the control plots) on 16 July 2007, to prevent aphid infestation.

Three plants were chosen at random on a weekly basis from July 12 to September 6, 2007 for non-destructive evaluation and data collection. Aphids were counted and plants were assigned a damage rating. Damage ratings were based on a 1 to 5 damage scale where 1 - ≤10% yellowing discoloration; 2 – 11-30% yellowing discoloration; 3 – 31-50% yellowing discoloration; 4 – 51-75% yellowing discoloration; and 5 - ≥76% of leaf area with yellowing discoloration or dead tissue (Heng-Moss et al. 2002; Heng-Moss et al. 2004; Hill et al. 2004b; Pierson 2010b). Plant height and the growth stage (vegetative or reproductive) were also recorded (Fehr et al. 1971). Towards the end of the experiment, data collection was taken from four of the six replications. This was done to alleviate time constraints.

A more informative measure of aphid pressure than peak aphid number is accumulated aphid days, which is a measure of aphid pressure over time. Aphid days = $((N_1+N_2)/2)*T$, where N_1 is the number of aphids per plant on the previous sampling date, N_2 is the numbers of aphids per plant on the following sampling date, and T is the number of days in between the two sampling dates (Hanafi et al. 1989). In order to gain a better understanding of the total aphid pressure over the growing season, accumulated aphid days were calculated.

Soybean harvest occurred on 25 October 2007. All plants from each treatment (4 to 10 plants per plot) from the four replications sampled throughout the study were cut at the soil line and wrapped in brown wrapping paper for later processing. Yield components were then evaluated to determine the effect of soybean aphid injury to yield: number of pods per plant, number of seeds per pod, average dry seed weight,

average dry pod weight, dry weight of stem, and total plant biomass (Hill et al. 2004b; Svehla 2007; Beckendorf et al. 2008).

2009 Field Study.

The 2009 field study was similar to that of the 2007 field study with a few minor exceptions. Only four of the six genotypes were evaluated in the 2009 study: K-1621, K-1639-2, KS4202, and Asgrow 2703. Genotypes 'Dowling' and 'Jackson' are from higher soybean maturity groups which need a longer growing season for complete maturity and are usually grown south of Nebraska, as a result, these two genotypes were removed from the study as full yield potential was rarely met. Once again, two plots per genotype were planted in each replication, one infested and the other non-infested. Planting occurred on 28 May 2009.

Standard agronomic practices for northeastern parts of Nebraska were used to plant and maintain the experimental plots. As with 2007, fields were disked twice in the spring shortly before planting. Soybeans were planted in a corn-soybean rotation in an Alcester-silt loam soil. Unlike 2007, experimental plots were not irrigated in 2009 because the irrigation system was inoperative. Dual® II Magnum® and Resource® herbicides were used to control weeds.

Experimental design was a randomized complete block with six replications. Plots were four rows wide and three meters long. The two center rows were planted with approximately 100 seeds per row of the designated genotype. The outer two rows were planted with Asgrow DKB 27-52 to serve as a buffer (seed supply was limited).

The level of infestation in the field was again inadequate, so plots were artificially infested on the 15 July 2009 using the technique described for 2007. Warrior® was sprayed on the non-infested control plots on 10 August 2009 to prevent aphid infestation. Unlike 2007, four plants were randomly selected from each plot for aphid and injury evaluation on a weekly basis from 1 July 2009 through 24 September 2009. Following each evaluation, accumulated aphid days were calculated. Each plant was assigned a damage rating using the previously described 1-5 scale. Plant height, vegetative and reproductive stage was recorded each week.

Harvest was completed on 5 November 2009. Ten plants were randomly selected from each plot. Each soybean sample was wrapped in brown wrapping paper and stored for later processing.

Statistical Analysis.

Damage ratings, aphid numbers, accumulated aphid days, and yield components were analyzed using mixed model analyses (PROC MIXED, SAS Institute 2002). When there was a significant treatment effect ($P \leq 0.05$) means were separated using Fisher's least significant differences (LSD) procedures (PROC MIXED, SAS Institute 2002).

Results and Discussion

Aphid numbers.

Overall aphid pressure was significantly higher in 2009 than in 2007, so data for each year were analyzed separately. The current economic threshold for the soybean aphid on soybeans is 250 aphids per plant with populations increasing (Ragsdale et al. 2004). In 2007, most genotypes did not reach the economic threshold, let alone yield

damaging levels. Genotype KS4202 was the only genotype to exceed the economic threshold and had over twice the number of aphids recorded on Asgrow 2703 on 15 August 2007, the day of peak aphid population (Table 1, Figure 1). In 2009, all genotypes exceeded economic thresholds, and KS4202 again had approximately twice as many aphids as the other three genotypes (Table 2, Figure 3).

In 2007, KS4202 accumulated just under 12,000 aphid days, while Asgrow2703 accumulated just under 4,500 aphid days (Figure 2). Genotype K-1621 accumulated just over 2,000 aphid days, while the remaining genotypes did not even reach 1,000 aphid days. Genotypes Jackson, Dowling, and K-1639-2 had fewer aphids over the entire growing season than KS4202 did on peak aphid day. Genotypes Jackson, Dowling, and K-1639-2 accumulated an average of 527.2 aphids per plant during the growing season while KS4202 accumulated an average of 578.2 aphids per plant on the peak aphid day of 15 August 2007 (Table 1, Figure 2).

In 2009, all genotypes exceeded 15,000 accumulated aphid days (Figure 4). KS4202 accumulated over 28,000 aphid days during 2009, which was nearly double that of 2007 (Figures 2 and 4), and nearly double that of Asgrow 2703, K-1621, and K-1639-2 in 2009 (Figure 4).

When comparing mean aphid numbers amongst the genotypes, mean aphid numbers were significantly different at the statistical level of $P \leq 0.05$ (Tables 1 and 2). Damage ratings for the week after peak aphid week are presented because the effects of severe aphid feeding are not always immediately visible (Tables 1 and 2).

Damage ratings.

In 2007, damage ratings were fairly consistent from one week to the next. This is not surprising since aphid numbers were very low for most of the genotypes tested. KS4202 was the only genotype in 2007 to exceed the economic threshold and reach population levels where significant injury would be expected. Even though KS4202 had relatively high aphid numbers, it maintained the lowest damage rating throughout the growing season (Table 1).

In 2009, damage ratings were higher for all infested genotypes when compared to 2007. Asgrow 2703, KS4202, and K-1621 soybean damage ratings remained fairly consistent or reduced from the week of peak aphid number to the following week (Table 2). Only genotype K-1639-2 saw an increase in damage from the peak aphid week to the following week (Table 2). This observation is interesting, since the mean aphid numbers was lower when compared to the other three genotypes which had lower damage ratings (Table 2).

Plant stage.

In 2007, aphids initially were observed in mid-July when the soybeans were in vegetative stages V5-V9. Aphid populations reached their peak in mid-August. For Dowling and Jackson, aphid peak occurred at stages V11-V17. The remaining genotypes all peaked in the reproductive stages with K-1639-2 peaking in reproductive stage R1, K-1621 peaking in R2, KS4202 peaking in R2-R3, and Asgrow 2703 peaking in R4-R5. Peak aphid populations occurred on 27 August 2009 with plant stages at R4-R6 for Asgrow 2703 and KS4202, R2-R4 for K-1621, R1-R2 and V9-V15 for genotype K-1639-2.

In 2007, aphids had little to no effect on plant development. Infested soybeans were generally in the same growing stages as their non-infested controls. In 2009, plant stages varied by as much as one reproductive stage.

Yield.

In 2007, there were no significant differences between aphid infested and non-infested control treatments for each genotype for any of the yield parameters tested: total biomass, average seed weight, and total seed weight, number of seeds per plant, number of pods per plant, or number of seeds per pod (Table 3). This is not surprising because the genotypes did not exceed the economic threshold with the exception of KS4202. Genotype KS4202 did surpass the economic threshold and reached aphid levels where yield loss would be expected, but there were no significant differences in yield or yield parameters between the aphid infested and the non-infested control treatments (Table 3).

Although aphid pressure was high enough to effect yield in 2009 (Figure 3 and 4), results were similar to 2007. Most of the genotypes showed no significant differences in yield or yield parameters with some minor exceptions for a few yield parameters (Table 4). For KS4202, the average seed weight (*P*=0.0179) and the average number of seeds per pod (*P*=0.0332) for aphid infested treatments were significantly lower than their respective non-infested controls (Table 4). For K-1639-2, the number of pods per plant (P-value=0.0459) and average number of seeds per pod (P-value=0.0453) for aphid infested treatments were significantly lower than their respective non-infested controls (Table 4).

For KS4202, two of the six yield components were significantly different in 2009 (average seed weight and average number of seeds per pod) while in 2007, no significant differences were indicated. This could be due to the difference in aphid numbers observed between the two years. In 2007, the average number of aphids for KS4202 peaked at 578.6 aphids (Table 1) and accumulated nearly 12,000 aphid days (Figure 2), which is at the lower range of where yield damage would be expected. In 2009, the average peak number of aphids for KS4202 was nearly double that in 2007, averaging around 1058.47, (Table 2), and KS4202 accumulated nearly 28,000 aphid days (Figure 4), which should easily result in significant yield loss. Similar patterns were also observed for the other genotypes (Tables 1 and 2). In 2009, the mean aphid numbers per plant were much higher for KS4202 when compared to the other genotypes. In fact, KS4202 had nearly twice as many aphids per plant than Asgrow 2703 (Figure 3) and the average peak number of aphids was almost 5 times the economic threshold.

In field studies conducted by Beckendorf et al. (2008), yield components were evaluated using Pioneer 91B91. This soybean variety produced significantly fewer pods, fewer seeds per pod, and lower seed weights when compared to the non-infested plants of the same variety. All of these differences resulted in a lower overall yield (Beckendorf et al. 2008). In this study, not one of the tested genotypes had a significant reduction in all of the yield components (i.e. seeds per pod, number of pods, seed weight) as was reported in Beckendorf et al. (2008). The genotypes in this study had 0-2 significant reductions in yield components, which may not have been enough to observe the overall yield loss observed in the Beckendorf et al. (2008) study. However, it is

important to note that peak aphid numbers and accumulated aphid days were much higher in the Beckendorf study.

Implications.

Based on our results, genotypes may compensate for aphid feeding in different ways. When aphid numbers are high (5 times the economic threshold), KS4202 appears to tolerate severe aphid feeding without significant impact on yield. Further studies are necessary to fully describe the plant compensation for aphid feeding in KS4202. Asgrow 2703 appears to produce a similar number of seeds as its non-infested counterpart, although the seeds produced are slightly smaller. Genotype K-1621 tends to keep aphid numbers at moderate levels without allowing the aphid feeding to significantly reduce yield. Genotypes K-1639-2, Dowling, and Jackson appear to hinder aphid numbers by keeping them low, however, whether these genotypes are using antibiosis, antixenosis, or both to hold aphid populations down remains unclear. K-1639-2 may show some level of resistance, but that did not protect yield. The average number of pods per plant and the average number of seeds per pod were significantly lower when compared to the control (Table 4).

It is clear from the two field seasons that KS4202 is compensating for aphid feeding. Similar mechanisms of compensation are not only found in soybeans, but are common in other plant-insects systems as well. Resource reallocation is common in plants with insect herbivory. Some of the common methods to reallocation resources include mechanisms like tiller production, an increase or decrease in seed production, increased branching, smaller seed development, increased flowering, larger leaves,

delayed senescence, and many others. Many of the mechanisms are often dependent on stress factors such as plant competition, water stress, interactions of nutrients, root damage, air pollution and timing of defoliation (Morton and Watson 1948; Dixon 1971; Dyer and Bokhari 1976; Satoh et al. 1977; Inouye 1982; Kolodny-Hirsch and Harrison 1982; Lechowicz 1987; Benner 1988; Hendrix and Trapp 1989; Wisdom et al. 1989; Deregibus and Trlica 1990; Doak 1991; Reichman and Smith 1991; Swank and Oechel 1991; Trumble et al. 1993).

The results of this study support the findings by Pierson (2010b) and add evidence that KS4202 has some level of tolerance to soybean aphid feeding. The results from 2007 and 2009 indicate that KS4202 can support aphid populations without significant yield loss at levels where significant yield loss would be expected (Ragsdale et al. 2004). The common Nebraska variety, Asgrow 2703, appears to show signs of tolerance as well. None of the yield parameters were significantly different between the aphid infested and non-infested treatments. Although not significantly different, seeds that were produced appeared slightly smaller, even in the 2009 field study where aphid numbers were high. Future studies should continue to focus on gaining a better understanding of the compensation mechanism exhibited by KS4202 and Asgrow 2703 in response to aphid feeding.

Table 1: Mean damage ratings and mean number of soybean aphids per plant for field experiments in 2007.

Genotype	Mean Damage Rating (August 15)[1]	Mean Damage Rating (August 24)[1]	Mean Number of Aphids (August 15)[2]	Mean Number of Aphids (August 24)[2]
Asgrow 2703	1.6	1.7	254.9 b	156.0 a
K-1639-2	1.4	1.3	25.5 c	25.9 b
Dowling	1.3	1.3	17.2 c	40.2 b
Jackson	1.3	1.6	18.3 c	30.4 b
K-1621	1.2	1.5	106.3 bc	48.8 b
KS4202	1.1	1.3	578.6 a	251.2 a

Means within columns followed by the same letter are not significantly different (P>0.05), LSD test.
[1] Genotype*date interaction effect: F=0.5, df=5, 36, P=0.8; Genotype main effect: F=1.4, df=5, 36, P=0.24; Date main effect: F=1.9, df=1, 36, P=0.18; the standard error calculated by Proc Mixed was 0.2.
[2] Genotype*date interaction effect: F=2.9, df=5, 36, P=0.03; the standard error calculated by Proc Mixed was 55.4.

Table 2: Mean damage ratings and mean number of soybean aphids per plant for field experiments in 2009.

Genotype	Mean Damage Rating (August 27)[1]	Mean Damage Rating (September 3)[1]	Mean Number of Aphids (August 27)[2]	Mean Number of Aphids (September 3)[2]
Asgrow 2703	2.1	2.0	621.52 a	342.22 ab
K-1639-2	2.9	3.5	617.47 a	175.33 a
K-1621	2.4	2.0	556.15 a	204.37 ab
KS4202	2.3	2.4	1058.47 b	488.62 ab

Means within columns followed by the same letter are not significantly different (P>0.05), LSD test.

[1] No significant date x genotype interaction (P=0.4524); Main effect of genotype: F=5.6, df=3, 40, P=0.003; Main effect of date: F=0.14, df=1, 40, P=0.7; Genotype standard error is 0.3 (calculated by Proc Mixed); Date standard error is 0.2 (calculated by Proc Mixed).

[2] No significant date x genotype interaction (P=0.6618); Main effect of genotype: F=4.5, df=3, 40, P=0.008; Main effect of date: F=23.0, df=1, 40, P=<0.0001; Standard error was calculated by Proc Mixed as 121.21.

Table 3: Yield parameters for soybeans grown in 2007.

	Total Plant Biomass (g)			Average Seed Weight (g)		
Genotype	Aphid	No Aphid	p-value[1]	Aphid	No Aphid	p-value[1]
Asgrow	25.76±2.67	28.39±2.67	0.5128	0.118±0.006	0.129±0.006	0.2499
Dowling	18.21±1.92	19.98±1.92	0.5378	NA	0.210±0	NA
Jackson	16.79±2.66	16.92±2.66	0.9737	0.043±0.003	0.039±0.006	0.5438
K-1621	32.68±4.29	36.86±4.29	0.5170	0.091±0.003	0.091±0.003	0.9681
K-1639-2	27.58±6.22	35.49±6.22	0.4028	0.073±0.006	0.077±0.006	0.6706
KS4202	30.90±6.01	46.96±6.01	0.1079	0.133±0.006	0.143±0.006	0.2981

	Number of Seeds/Plant			Number of Pods/Plant		
Genotype	Aphid	No Aphid	p-value[1]	Aphid	No Aphid	p-value[1]
Asgrow	128.01±12.13	130.53±12.13	0.8879	53.91±4.54	54.25±4.54	0.9595
Dowling	0±0.35	0.50±0.35	0.3559	1.16±1.44	4.13±1.44	0.1960
Jackson	0.26±0.19	0.25±0.19	0.9765	4.35±2.02	4.13±2.02	0.9398
K-1621	152.32±24.61	176.87±24.61	0.5070	79.87±10.58	89.41±10.58	0.5469
K-1639-2	71.04±21.99	119.02±21.99	0.1738	46.33±12.77	73.73±12.77	0.1801
KS4202	120.48±25.40	173.50±25.40	0.1904	57.68±11.65	79.93±11.65	0.2256

	Total Seed Weight/Plant (g)			Average Number of Seeds/Pod		
Genotype	Aphid	No Aphid	p-value[1]	Aphid	No Aphid	p-value[1]
Asgrow	14.08±1.57	15.68±1.57	0.4992	2.369±0.04	2.392±0.04	0.6662
Dowling	NA	0.21±0	NA	0	0.030±0.02	0.3559
Jackson	0.73±0.03	0.75±0.02	0.9666	0.047±0.02	0.021±0.02	0.4054
K-1621	12.81±2.18	15.91±2.18	0.3550	1.879±0.08	1.958±0.08	0.5063
K-1639-2	5.493±2.15	9.347±2.15	0.2527	1.540±0.12	1.603±0.12	0.7182
KS4202	15.68±3.08	24.94±3.08	0.0777	2.078±0.05	2.089±0.05	0.8893

[1]Significantly different at P≤0.05 by least significant difference.

Table 4: Yield parameters for soybeans grown in 2009.

	Total Plant Biomass (g)			Average Seed Weight (g)		
Genotype	**Aphid**	**No Aphid**	**P-value**	**Aphid**	**No Aphid**	**P-value[1]**
Asgrow	13.7033±1.32	15.5250±1.32	0.3536	0.1531±0.00	0.1598±0.00	0.1874
KS4202	18.4550±2.53	22.4083 ± 2.53	0.2948	0.1252±0.01	0.1424±0.01	0.0179
K-1621	23.5933±3.31	23.4391±3.31	0.9744	0.06385±0.00	0.06436±0.00	0.7984
K-1639-2	39.2924±8.39	55.8730±8.39	0.1924	0.02320±0.01	0.03713±0.02	0.2881

	Number of Seeds / Plant			Number of Pods / Plant		
Genotype	**Aphid**	**No Aphid**	**P-value**	**Aphid**	**No Aphid**	**P-value[1]**
Asgrow	54.8333±4.44	58.7833±4.44	0.5438	25.2500±1.86	26.1167±1.86	0.7483
KS4202	72.8333±10.50	81.8667±10.50	0.5566	35.5833±4.62	37.9000±4.62	0.7304
K-1621	128.82±20.66	123.62±20.66	0.8623	67.2542±9.31	67.4061±9.31	0.991
K-1639-2	28.1528±33.04	116.69±33.04	0.0874	77.1144±16.91	131.60±16.91	0.0459

	Total Seed Weight / Plant (g)			Average Number of Seeds / Pod		
Genotype	**Aphid**	**No Aphid**	**P-value**	**Aphid**	**No Aphid**	**P-value[1]**
Asgrow	8.4167±0.86	9.4767±0.86	0.4039	2.1768±0.05	2.2501±0.05	0.3499
KS4202	8.9300±1.29	11.5240±1.29	0.184	1.9997±0.04	2.1547±0.04	0.0332
K-1621	8.2942±1.40	8.2683±1.40	0.9898	1.8098±0.10	1.7801±0.10	0.8323
K-1639-2	2.7813±3.11	9.0507±3.11	0.1844	0.1459±0.13	0.5568±0.13	0.0453

[1]Significantly different at P≤0.05 by least significant difference.

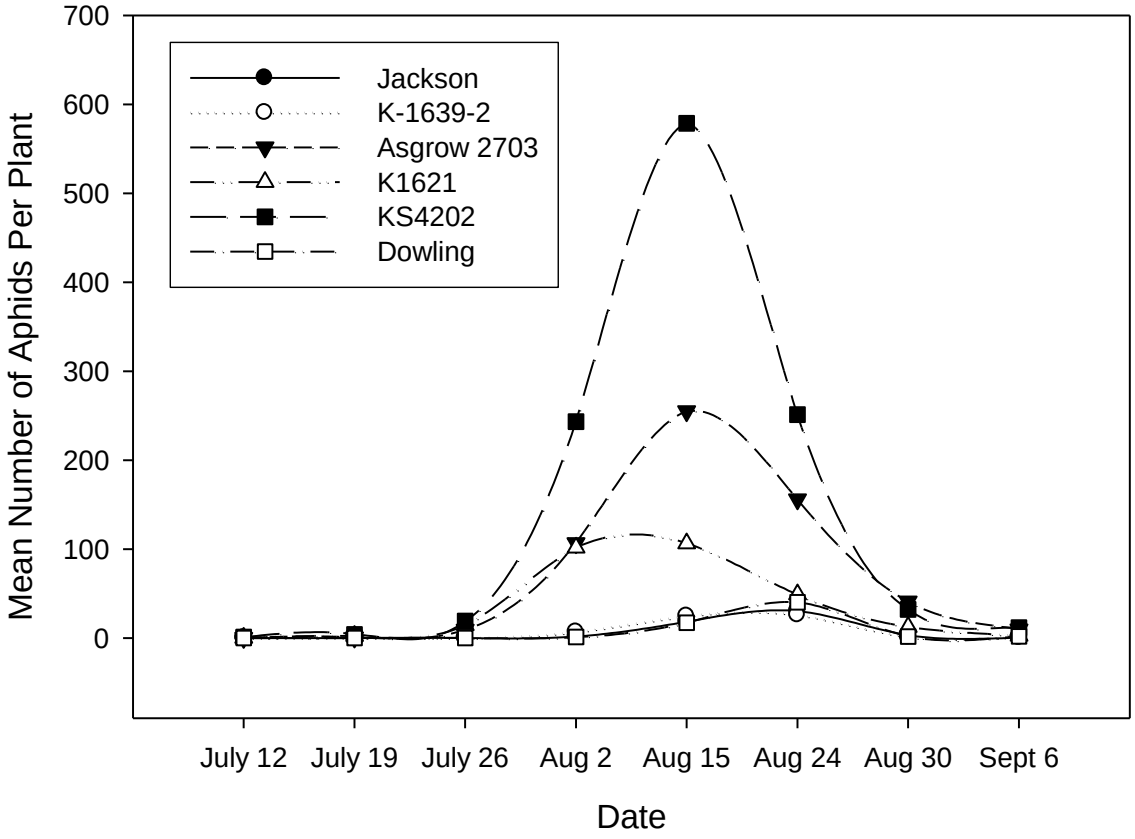

Figure 1. Mean aphid numbers for each genotype during weekly counts in 2007 growing season.

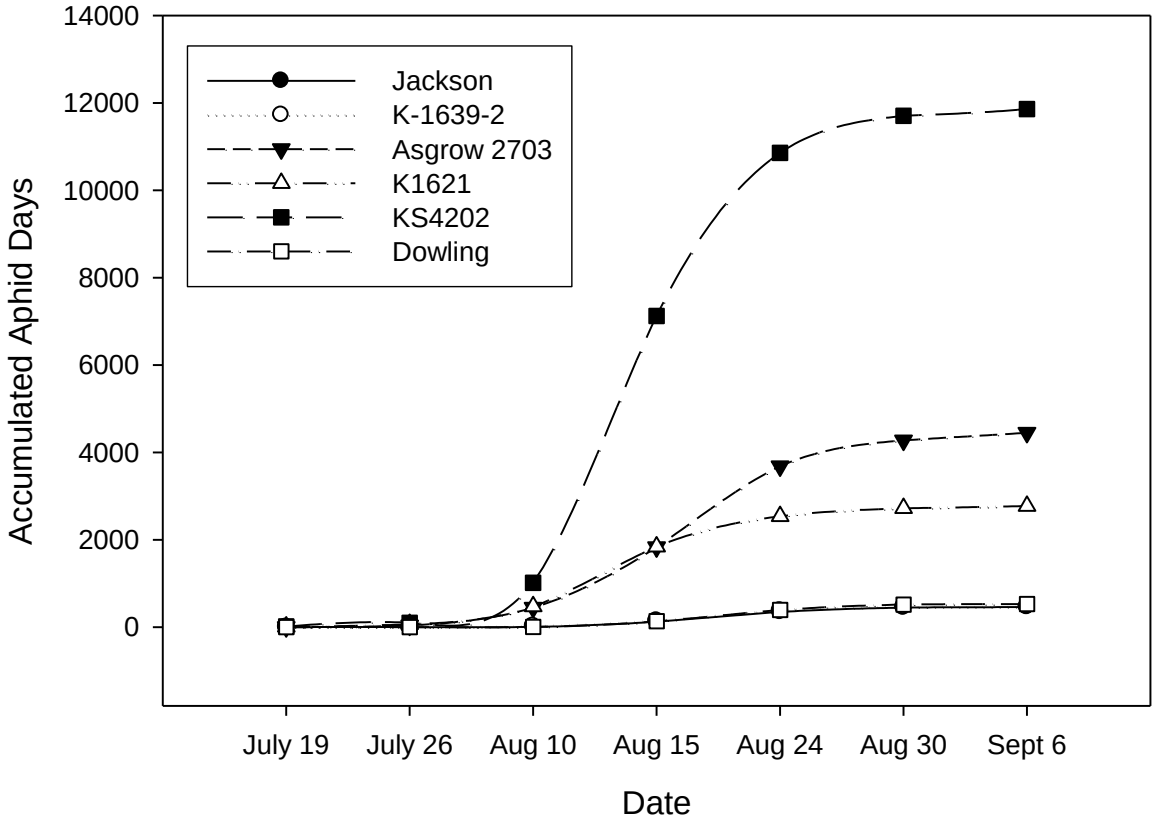

Figure 2. Accumulated aphid-days for each genotype in 2007 growing season.

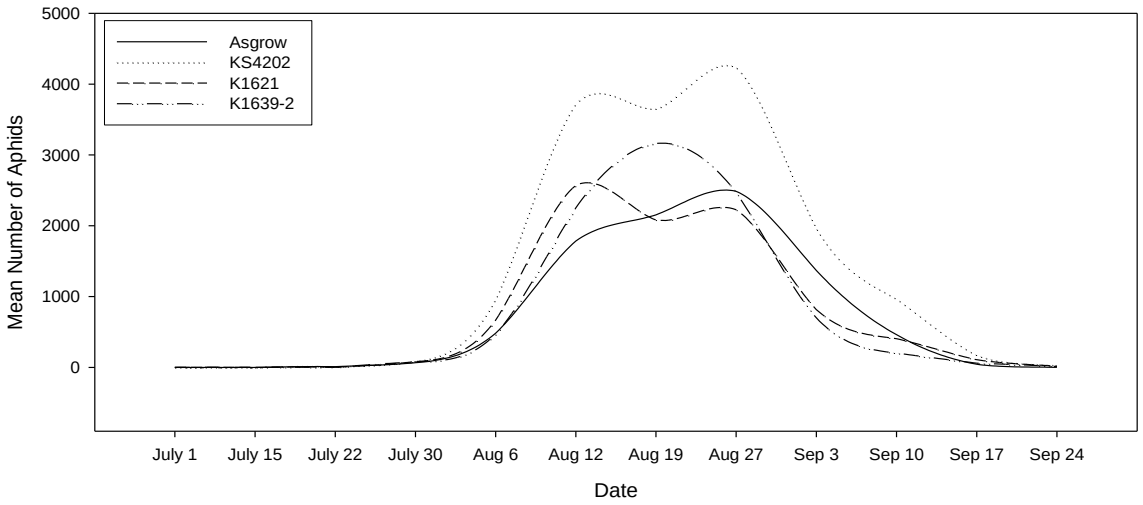

Figure 3. Mean aphid numbers for each genotype during weekly counts in 2009 growing season

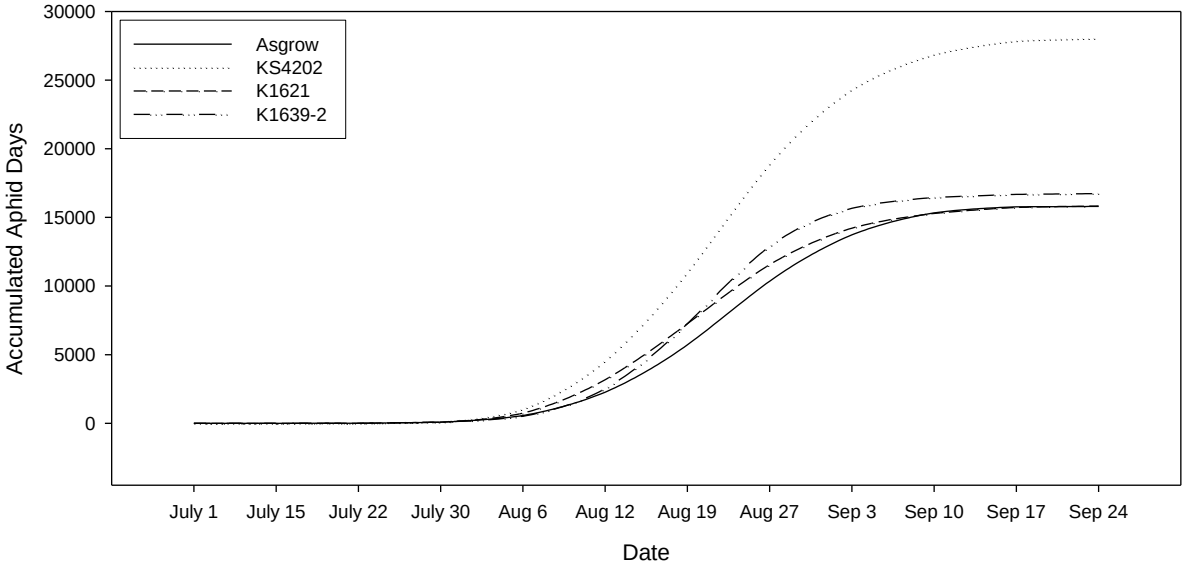

Figure 4. Accumulated aphid-days for each genotype in 2009 growing season.

Chapter 3

Illumina sequencing and transcriptional analysis of soybean genotypes KS4202 and K03-4686.

Introduction

Soybeans (*Glycine max* (L.) Merrill) are an important commodity in the United States and throughout the world. Since the introduction of the soybean aphid, *Aphis glycines* Matsumura, to the soybean growing regions of the United States in the early 2000s, aphids have spread to 30 states and several south Canadian provinces (Hartman et al. 2001; Alleman et al. 2002; Venette and Ragsdale 2004; Beckendorf et al. 2008; NAPIS 2011). Since its introduction, the soybean aphid has caused considerable economic damage and yield loss to soybean growers.

Several strategies have been developed to manage this pest including chemical, biological, and cultural control methods (Wang and Ba 1998; Wang et al. 2000; Ostlie 2002; Hill et al. 2004b, Wu et al. 2004, Rutledge and O'Neil 2006; Brosius et al. 2007). Recently, host plant resistance has gained attention as a viable management option. Soybeans that are antibiotic, antixenotic and tolerant have been identified (Hill et al. 2004b, 2006a, 2006b; Mensah et al. 2005; Diaz-Montano et al. 2006; Kang et al. 2008; Mian et al. 2008; Pierson 2010b). Although resistant (specifically tolerant) sources have been identified, limited information is available on how soybean aphid feeding impacts the physiology and biochemistry of the plant and the genes conferring tolerance. Illumina sequencing technology provides a powerful tool for identifying specific genes and their roles in regulating resistance in soybean.

The objective of this study was to characterize transcriptional changes in response to aphid feeding to better understand the underlying tolerant mechanism(s) and genes contributing to the tolerance response.

Materials and Methods

Two soybean genotypes were selected for Illumina sequencing to gain a better understanding of the tolerant response to soybean aphid feeding. The genotypes selected for sequencing included the tolerant genotype KS4202 and the susceptible genotype K03-4686 (Pierson 2009; Chandran 2011). Four seeds of each genotype were planted in potting media (34% peat, 31% perlite, 31% vermiculite, and 4 % soil mix) in 15 cm diameter round plastic pots (Hummert International, Earth City, MO). Plants were thinned to one plant per pot once seedlings emerged from the soil. Soybeans were grown to the V5 vegetative stage under 400-watt high intensity lamps with a 16:8 (L:D) hour photoperiod at a temperature of 23 ± 2˚C.

V5 stage soybean plants were infested with 20 aphids per plant. Soybean aphids were obtained from a laboratory maintained colony (Biotype 1, Illinois Biotype). The treatment design was a 2x2x2 factorial design with 2 soybean genotypes (KS4202 and K03-4686), 2 infestation treatments (control and 20 aphids per plant), and 2 harvest dates (5 and 15 days). The experimental design was a completely randomized design with six replications.

Before destructively harvesting the plants for Illumina sequencing, damage ratings were performed using a 1-5 scale, where 1 = ≤ 10% yellowing discoloration; 2 = 11-30% yellowing discoloration; 3 = 31-50% yellowing discoloration; 4 = 51-75%

yellowing discoloration; and 5 = ≥ 76% of leaf area with yellowing discoloration or dead tissue (Hill et al. 2004b, Pierson et al. 2010a). Aphid number and plant stage were also recorded. The top two tri-foliates (youngest plant tissue) were harvested, flash frozen in liquid nitrogen, and stored at -80˚C until submission for Illumina sequencing.

Three biological replicates of each treatment were submitted to the University of Nebraska – Lincoln Biotechnology Center for Illumina Solexa sequencing. Samples were analyzed using the Illumina Genome Analyzer. Total RNA was isolated from the soybean samples and then complementary DNA was prepared from the total RNA. Purified mRNA was fragmented, annealed to high concentrations of random hexamers, and reverse transcribed. Oligonucleotide adapters complementary to sequencing primers were ligated to cDNA fragment ends and the resultant cDNA libraries were sized on an agarose gel. Two hundred bp fragments were excised and amplified by 15 cycles of polymerase chain reactions. Flowcell was used to perform 56 cycle sequencing by synthesis chemistry in the Genome Analyzer (www.illumina.com). Sequence reads were aligned with the soybean genome – *G. max* 109 (www.phytozome.org) using the Bowtie mapping program. Total mapping reads, average total alignment, average total alignment (%), and average total multi-mapping (suppression %) were compared between aphid-infested and control plants of KS4202 and K03-4686 at days 5 and 15. Only significant hits at the false discovery rate of less than 0.10 are reported. The cutoff for average fold change between the aphid-infested and control samples was 2.0. Protein homologues were identified using Blast2GO to annotate protein sequences with Gene Ontology terms.

Results and Discussion

Damage Ratings.

No evidence of visible plant damage was observed between infested KS4202 and K03-4686 plants at 5 (KS4202: 1.1 ± 0.09 and K03-4686: 1.3 ± 0.10) and 15 (KS4202: 1.1 ± 0.45 and K03-4686: 1.3 ± 0.11) days after aphid introduction.

Mapping Statistics.

KS4202 infested plants at days 5 and 15 had approximately 47.0% and 54.8% average total alignment, whereas, control plants had 50.5% and 55.95 average total alignment to the soybean genome. K03-4686 infested plants at days 5 and 15 had approximately 54.4% and 54.5% average total alignment while K03-4686 control plants had approximately 53.3% and 55.4% average total alignment to the soybean genome. The aphid-infested KS4202 treatment at day 5 had a total read number of 24,970,678 while infested KS4202 at day 15 had a total read number of 35,949,838. The aphid-infested K03-4686 treatment at day 5 had a total read number of 23,868,164, whereas, infested K03-4686 at day 15 had a total read number of 27,879,312. A detailed summary of the mapping statistics is provided in Table 1.

Comparing gene expression levels between infested and control plants for KS4202, 123 genes had a higher expression level in response to aphid feeding at day 5, while, 51 genes had a lower expression level in response to aphid feeding. By day 15, 467 genes had a higher expression level in infested plants when compared to control plants and 634 genes had a lower expression level between KS4202 infested and control plants (Table 1). For K03-4686, 86 genes had a higher expression level in response to

aphid feeding at day 5, whereas, 56 genes had a lower expression level when comparing infested to control plants. At day 15, 194 genes had a higher expression level in response to aphid feeding and 701 genes had a lower expression level in infested plants compared to control plants (Table 1).

Comparison Among Functional Processes.

KS4202 Response to Aphid Feeding.

A total of 16 functional processes exhibited a difference in gene expression level in response to soybean aphid feeding at day 5. Three functional processes showed a high level of differential gene expression in response to aphid feeding. The genes differentially expressed were grouped into the following functional processes: response to stimulus (21 genes), cellular process (44 genes), and metabolic process (59 genes) (Figure 1).

As seen for day 5, a total of 16 functional processes were again differentially expressed in response to soybean aphid feeding at day 15. The following four functional processes showed a high level of differential gene expression: response to stimulus (37 genes), cellular process (62 genes), the metabolic process (70 genes), and biological regulation (25 genes) (Figure 2).

K03-4686 Response to Aphid Feeding.

A total of 16 functional processes exhibited a difference in gene expression level in response to soybean aphid feeding at day 5. Two functional processes showed a high level of differential gene expression in response to aphid feeding. The genes differentially expressed were grouped into the following functional processes: response

to cellular process (33 genes) and metabolic process (29 genes) (Figure 3). The number of genes differentially expressed in K03-4686 was lower for these two categories than the number of genes differential expressed for KS4202.

As seen for day 5, a total of 16 functional processes were again differentially expressed in response to soybean aphid feeding at day 15 (Figure 4). The following four functional processes showed a high level of differential gene expression: response to stimulus (47 genes), cellular process (60 genes), the metabolic process (66 genes), and biological regulation (32 genes). The number of genes differentially expressed at 15 days after aphid introduction was similar between K03-4686 and KS4202.

Genes of Interest.

From the Blast2Go annotation sequence results, 20 genes of interest were selected from the list of genes with increased gene expression in the tolerant KS4202 plants (Table 2). Of specific interest are two peroxidase genes (Glyma04g39860 and Glyma06g15030) that had higher expression levels in the infested KS4202 plants when compared to KS4202 control plants at day 15 (Table 2). Pierson et al. (2010) also reported increased peroxidase activity in the tolerant KS4202 soybean in response to aphid feeding. Based on these findings, our proposed hypothesis is that tolerant soybean plants have the ability to elevate their level of reactive oxygen species (ROS)-scavenging enzymes, such as peroxidases, which enable them to efficiently remove ROS that accumulate in response to aphid feeding.

Table 2 reports the fold change between KS4202 control and infested plants, the Log_2 fold change, p-value, adjusted p-value, and the best match description using the

genome of *Arabidopsis* for the 20 genes of interest. Two WRKY genes expressed higher transcript abundance in the tolerant KS4202 plants in response to aphid feeding. WRKY genes have been reported to be involved in plant defense in other systems, such as wheat (Lapitan et al. 2008; Eck et al. 2010; Botha et al. 2010). Three genes encoding cytochrome P450s were also differentially expressed in the tolerant soybean. In plants, cytochrome P450s, which are involved in JA-mediated defense responses (Park et al. 2002), have been induced in aphid-resistant wheat and sorghum in response to *D. noxia* and *S. graminum*, respectively (Park et al. 2005; Boyko et al. 2006).

The first four genes listed in Table 2 were found to be associated with signal transduction in the soybean plant system. The differential expression of these four genes could be an important factor in the defense response of KS4202 to the soybean aphid. Future research on these genes could expand our understanding of the role of signal transduction in the defense response of tolerant plants and identify resistance mechanisms.

The Illumina sequence data generated from this project provides a comprehensive data set that will allow us to characterize transcriptional changes in response to aphid feeding to better understand the underlying tolerant mechanism(s) and genes contributing to the tolerance response. Further detailed analysis of this Illumina data set is required to fully understand the tolerance response of KS4202 to soybean aphids and identify specific genes responding to aphid feeding.

Table 1. Mapping statistics generated from the Bowtie program alignment.

Mapping Statistics				
	Total Reads	**Average Total Alignment**	**Average Total Alignment (%)**	**Average Total Multi-Mapping**
KS4202 Control (Day 5)	26,282,160	13,280,657	50.54%	35.90%
KS4202 Infested (Day 5)	24,970,678	11,733,023	46.99%	39.22%
KS4202 Control (Day 15)	39,291,337	21,963,116	55.91%	33.70%
KS4202 Infested (Day 15)	35,949,838	19,634,539	54.76%	34.26%
K03-4686 Control (Day 5)	22,705,911	12,098,790	53.30%	35.78%
K03-4686 Infested (Day 5)	23,868,164	12,980,612	54.41%	34.90%
K03-4686 Control (Day 15)	30,542,262	16,911,325	55.36%	33.92%
K03-4686 Infested (Day 15)	27,879,312	15,180,053	54.46%	34.24%

Table 2. Gene ID, fold change, Log_2 fold change, p-value, adjusted p-value, and best hit description of gene ID compared against the *Arabidopsis* genome. Genotype KS4202 at day 15.

	Gene ID	Fold Change	Log2 Fold Change	P-Value	Adjusted P-Value	Best-Hit Description (Arabidopsis)
1	Glyma06g41060	Inf	Inf	2.30E-08	5.81E-06	S-locus lectin protein kinase family protein
2	Glyma10g01140	Inf	Inf	5.11E-05	4.73E-03	AT-hook motif nuclear-localized protein 20
3	Glyma02g00840	Inf	Inf	1.58E-04	1.17E-02	phosphate transporter 1;7
4	Glyma08g45900	Inf	Inf	2.57E-03	9.52E-02	receptor-like protein kinase-related family protein
5	Glyma09g41530	17.45	4.13	7.42E-07	1.27E-04	HEAT repeat ;WD domain, G-beta repeat protein protein
6	Glyma0041s00240	16.02	4	7.27E-04	3.70E-02	hydroxyproline-rich glycoprotein family protein
7	Glyma05g03750	15.84	3.99	6.81E-06	8.94E-04	Subtilase family protein
8	Glyma04g39860	13.41	3.74	1.80E-03	7.17E-02	Peroxidase superfamily protein; Oxidative stress resp.
9	Glyma06g12620	12.39	3.63	7.15E-39	5.74E-35	Protein kinase superfamily protein
10	Glyma06g15030	11.71	3.55	6.41E-05	5.73E-03	Peroxidase superfamily protein; Oxidative stress resp.
11	Glyma05g22960	11.43	3.51	2.07E-08	5.28E-06	NAD(P)-binding Rossmann-fold superfamily protein
12	Glyma04g08380	11.36	3.51	1.43E-07	3.03E-05	hemoglobin 3
13	Glyma13g11820	11.13	3.48	1.38E-04	1.06E-02	Unclassified; Functional Annotations: neuropeptide signaling pathway, Copper binding octapeptide repeat, Bombesin-like peptide
14	Glyma12g31780	9.81	3.29	2.11E-22	5.29E-19	cellulose synthase-like B4
15	Glyma13g27470	8.33	3.06	2.41E-06	3.60E-04	Protein of unknown function, DUF584
16	Glyma17g13420	5.45	2.45	4.75E-04	2.66E-02	cytochrome P450, family 71, subfamily B, polypeptide 37
17	Glyma09g28970	3.71	1.89	1.79E-03	7.15E-02	Cytochrome P450 superfamily protein
18	Glyma08g10010	3.01	1.59	1.40E-05	1.64E-03	cytochrome P450, family 77, subfamily A, polypeptide 5 pseudogene
19	Glyma17g34210	2.49	1.32	2.13E-04	1.48E-02	WRKY DNA-binding protein 50; sequence-specific DNA binding
20	Glyma05g31800	2.29	1.19	1.57E-05	1.79E-03	WRKY DNA-binding protein 51; JA mediated signaling pathway

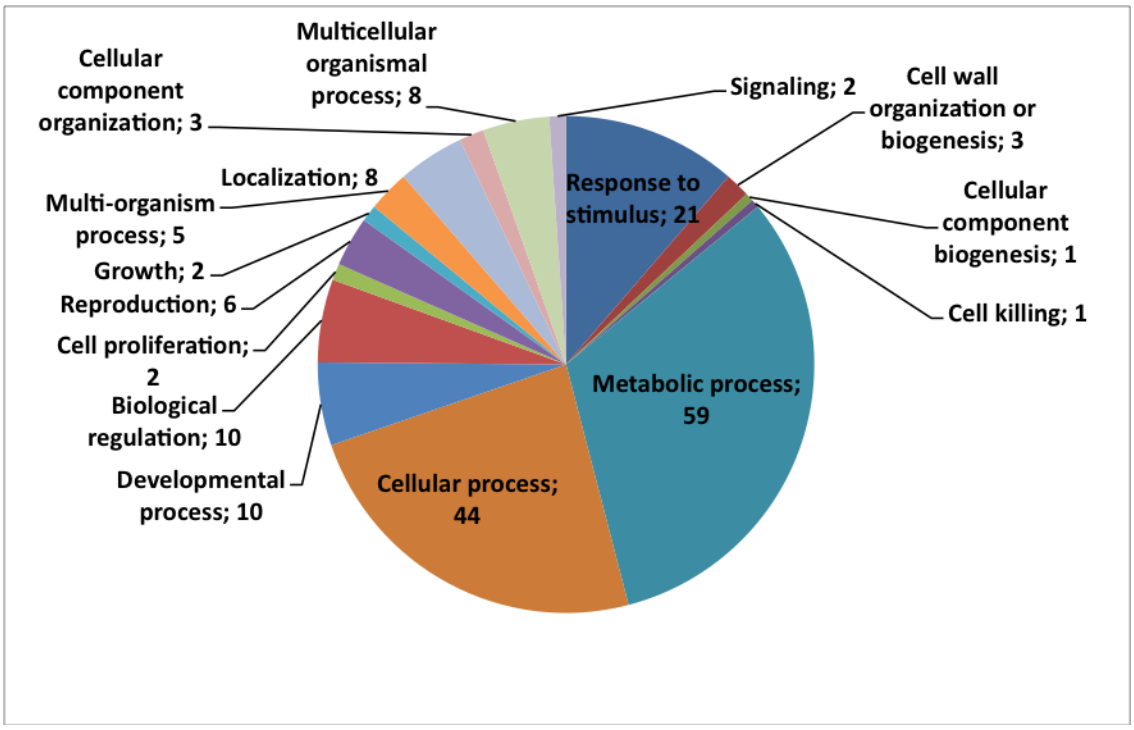

Figure 1. Functional process categories of the soybean genes responsive to soybean aphid feeding in KS4202 (tolerant) at day 5. Number indicates total genes differentially expressed in each category.

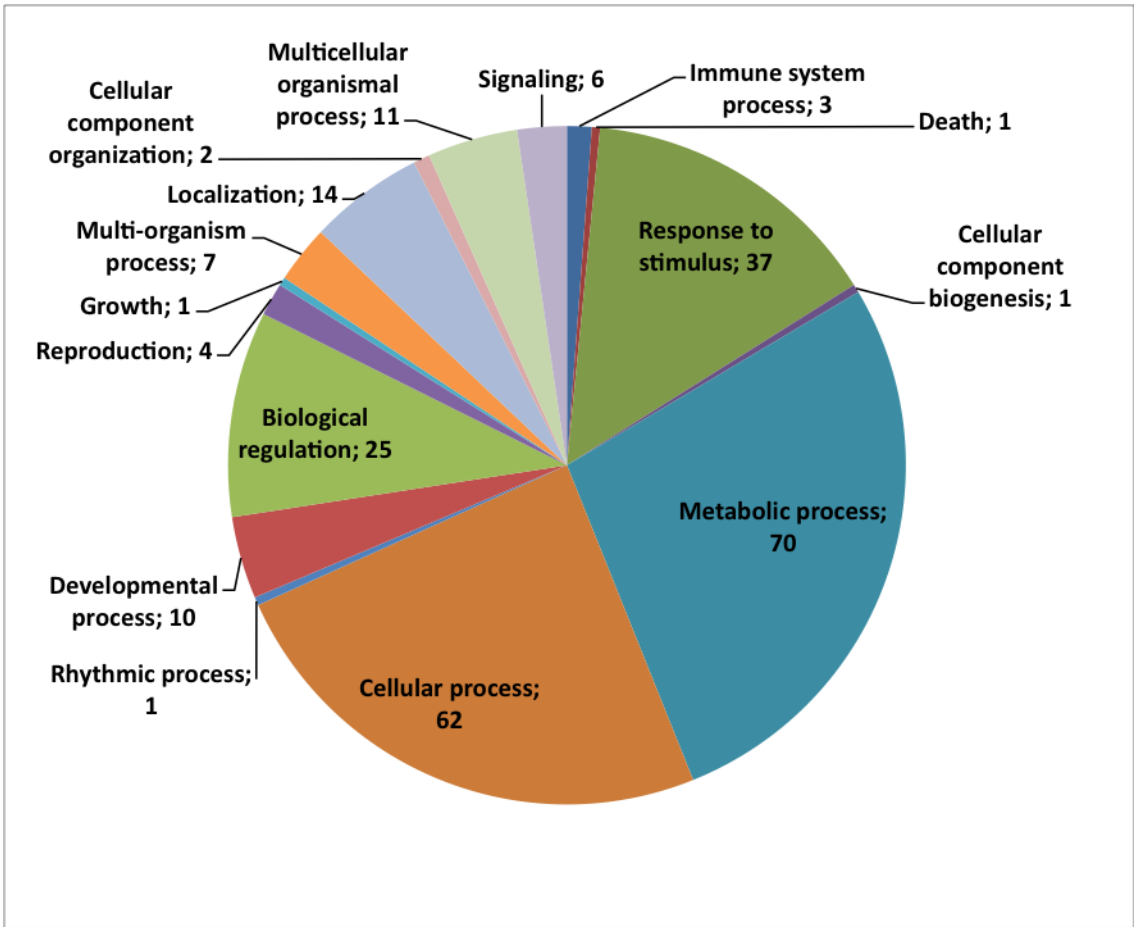

Figure 2. Functional process categories of the soybean genes responsive to soybean aphid feeding in KS4202 (tolerant) at day 15. Number indicates total genes differentially expressed in each category.

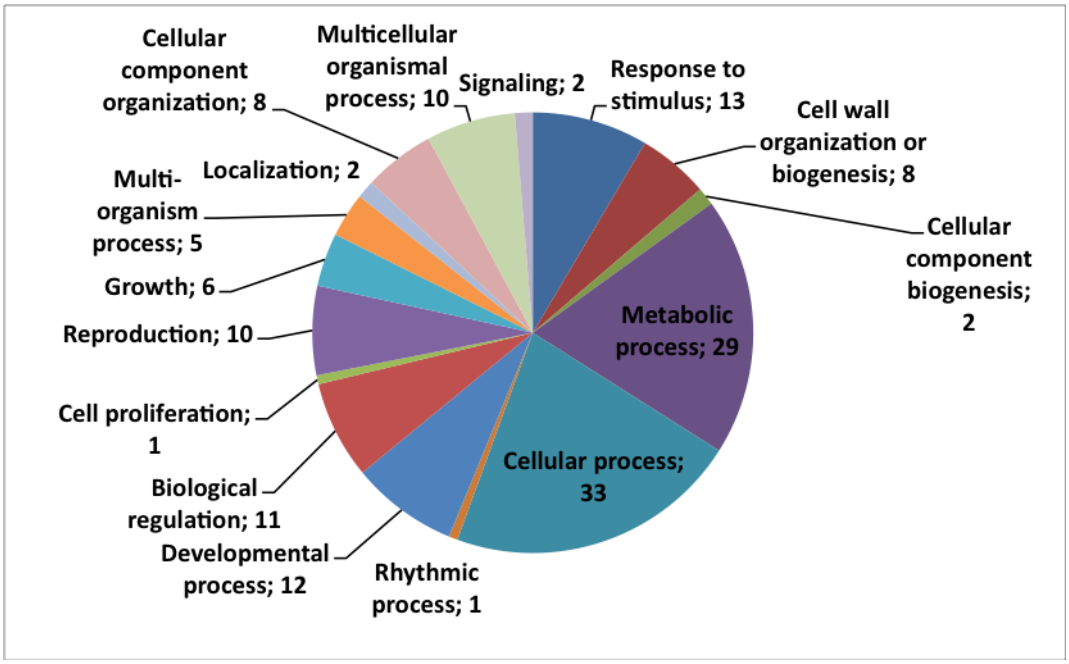

Figure 3. Functional process categories of the soybean genes responsive to soybean aphid feeding in K03-4686 (susceptible) at day 5. Number indicates total genes differentially expressed in each category.

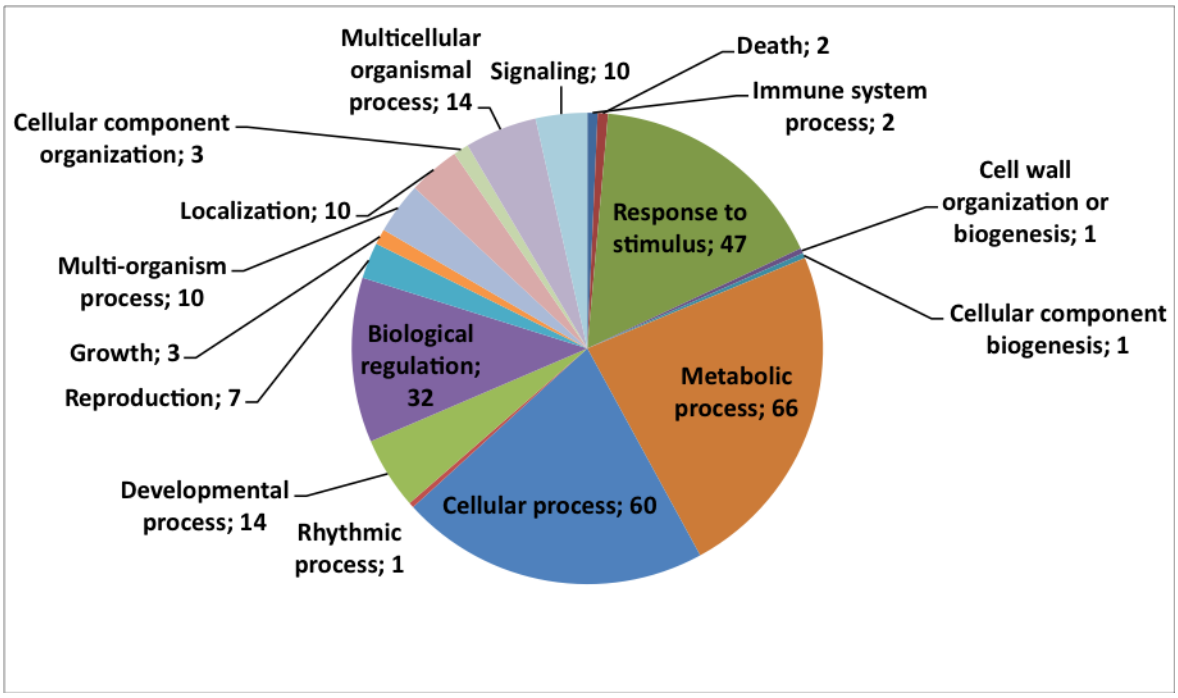

Figure 4. Functional process categories of the soybean genes responsive to soybean aphid feeding in K03-4686 (susceptible) at day 15. Number indicates total genes differentially expressed in each category.

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