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Pyramiding for Durable Insect Resistance: A Case Study of Developing Soybean Cultivars with Stable Aphid Resistance

By

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A creative component submitted to the graduate faculty

In partial fulfilment of the requirements for the degree of

Master of Science

Major: Plant Breeding

Program of Study Committee:
Major Professor Dr. Shui-zhang Fei, Dr. Thomas Lubberstedt

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Introduction

Many factors play a role in the evolution of agricultural crops and modern plant breeding. I can think of no greater force than that of resistance durability. Resistance durability may be described as the strength of resistance and duration of resistance displayed within a specific crop population against a specific or multiple biotic pest(s) or abiotic stresses.

What genes should be selected to establish sufficient pest resistance? There is no more important step in the process of combining genes than the initial step of choosing and crossing specific donor genotypes for the purpose of integration within a single line. Multiple resistance genes need to be available for this process, which is made possible when plant breeders have access to a wide range of genetic diversity that can be evaluated from large collections of accession data.

The soybean aphid epidemic which occurred in the United States during the early 2000's as well as the response to the issue as it relates to establishing resistance durability within soybean crops will be the focus of this Creative Component. Additionally, resistance breakthroughs in the battle against alternative soybean pests will be discussed. Parallels may easily be drawn between the aphid epidemic experienced in soybean and the progression of pest problems occurring in other major crops. The goal is to show how breeders utilized pest resistance genes in order to establish increased resistance durability in superior soybean lines by using gene pyramiding. Genotypes carrying single dominant genes have contributed to higher yields by establishing pest resistance, but have quickly become susceptible because of evolving pest biotypes. Gene stacking has diversified the concept of gene integration for crop resistance and also has exposed unexpected results which include continued usefulness of previously susceptible genotypes. Additionally, the use of biotechnology in the form of marker assisted selection (MAS) and genome wide association studies (GWAS) has enhanced the gene pyramiding process. Ultimately, the implementation of MAS, GWAS, and reconstituted use of susceptibility alleles at resistance loci establishes gene pyramiding as superior for establishing durable resistance compared to the use of single dominant genes.

Prevalence of soybean aphid in the United States

Soybean aphid has currently become a major pest for soybean crop in the northern United States and was first found during the year 2000 in the Midwest (Cooper et al., 2015). U.S. soybean farmers produced 2.8 billion bushels with a value of over $15 billion dollars between 2000 and 2002 (Kim et al., 2008). This production covered over 2.9 million ha. Losses of 5 to 19 bushels per acre by damages caused by soybean aphids was the normal scenario for U.S. farmers in the northern states (Kim et al., 2008). Yield losses have been reported by as much as 50% within the state of Minnesota during the year of 2001 (Ostlie, 2002). 80% of the U.S. soybean fields were infested by soybean aphid by 2004 (Venette and Ragsdale, 2004). Twenty-three soybean growing states reaching as far south as Mississippi and Georgia were affected by soybean aphid by 2005 (Li et al., 2007). Threshold-based application of broad spectrum foliar insecticides was the primary method of treatment during this time. Unfortunately, this method has its deficiencies. Many soybean fields in the north central soybean growing states crossed the economic threshold

of 250 aphids per plant, by which applying chemical pesticides would avoid economic damage (yield loss that exceeds the cost of chemical control) to the soybean crop (Rice, 2004).

Soybean aphids reduce yield by inflicting leaf damage that may include curling, wilting, yellowing, and premature dropping. Stunted plants may also occur. Additionally, seed quality is affected when a reduction in the number of pods and seeds take place. Seeds that exhibit reduced weight may appear discolored and deformed (Difonzo and Hines, 2002). Another potentially large threat posed by the aphid is its ability to transmit certain plant viruses such as alfalfa mosaic virus*,* soybean dwarf virus*,* and soybean mosaic virus *to* soybean (Hill et al., 2001).

Single dominant gene confers *Rag1* aphid resistance to soybean in 'Dowling'

Dowling was one of the first genotypes to be identified with carrying genes associated with aphid resistance in soybean since the arrival and establishment of soybean aphid as a major pest within the U.S. Soybean aphid was controlled by threshold-based application of broad spectrum insecticides prior to the discovery of a few aphid resistance genotypes which included 'Dowling'. Breeders needed to determine if this resistance was a heritable trait. The goal for researchers was to make crosses with 'Dowling' and self-pollinate to produce offspring which would then be tested for the inheritance of the potential trait (Hill and Hartman, 2006).

The resistant parent ('Dowling') was crossed with susceptible parents Loda and Williams 82 (Hill and Hartman, 2006). Subsequently, all crosses were self-pollinated to produce the $F_{2:3}$ generation. F_1 and F_2 plants were tested in greenhouse conditions. Crosses as well as individuals were self-pollinated in greenhouse conditions and were segregated as to not allow for foreign pollination. A scale of 0 to 4 was utilized for evaluation with 0 representing no aphid present to 4 representing dense colonies with plant damage. 0, 1, and 2 ratings were recognized as resistant (R) while 3 and 4 were designated as susceptible (S). F1s of Dowling X Loda, Dowling X Williams 82, and selfed progeny of Dowling were resistant. Selfed progeny of Loda and Williams 82 were susceptible. Final analysis revealed a single dominant gene, which was designated as *Rag1*, representing antibiosis resistance (Hill and Hartman, 2006). This analysis is verified when $F_{2:3}$ individuals display a 1:2:1 genotypic ratio, which is indicative for the presence of monogenic inheritance. Additionally, F_2 plants displayed a 3:1 phenotypic ratio.

Discovery of soybean aphid biotypes

Breeders of 'Dowling' were excited about the establishment of aphid resistance as a heritable trait. *Rag1* was revealed but there was another issue. The probability of additional biotypes within *A. glycine* posed a problem for *Rag1* genotypes that could be susceptible. There were no known additional *A. glycine* biotypes upon the establishment of 'Dowling' (Bhusal et al., 2013). Researchers needed to determine if there were more *A. glycine* biotypes because they knew the broad use of cultivars with a single dominant gene could cause a breakage in resistance and widespread crop damage. Researchers devised a study to determine if there was *A. glycine* variation within the US by showing if aphid populations are able to colonize on *Rag1* genotypes (Kim et al., 2008).

The study began with two sets of aphids being collected from different locations. The first set was taken from crop fields in Illinois where resistance was consistently high. The other set was taken from Ohio crop fields where information regarding resistance breakage of *Rag1* was being reported. Eight soybean genotypes were used for the study with all showing resistance in Illinois and a few showing susceptibility in Ohio. They were 'Dowling', LD05-16611, PI 200538, 'Jackson', PI 567597C, and PI 567541B. Each of the eight were grown separately and treated with an Ohio and Illinois isolate individually in a choice and non-choice test (Kim et al., 2008).

Results of the tests show PI 200538 and PI 567597C were both resistant to both aphid biotypes, and PI567541C exhibits moderate resistance to both biotypes. The remaining genotypes were susceptible. This study indicates there is a separate and distinct *A. glycine* biotype within the state of Ohio that is capable of breaking resistance of the *Rag1* gene. Thus, a broader genetic background with more than a single dominant gene is required to establish aphid resistance in the future (Kim et al., 2008).

Single dominant gene confers *Rag2* aphid resistance in 'Wyandot'

'Wyandot' was released as a high yielding and late maturing food grade soybean cultivar from Ohio State University in 2006. The genotype was developed to be an elite cultivar with a genetically diverse background (St. Martin et al., 2006). Traits were outstanding in this cultivar except for a susceptibility to soybean aphids and powdery mildew (Mian et al., 2016).

The USDA-ARS and Ohio Agricultural Research and Development Center accepted the responsibility of trying to incorporate soybean aphid and powdery mildew resistance into the cultivar 'Wyandot' (Mian et al., 2016). Approximately 200 soybean genotypes were screened for resistance to soybean aphids in a greenhouse test using soybean aphid collected in Wooster, OH. PI 243540, PI 567301B, and PI 567324 were identified as highly resistant against the soybean aphids collected from soybean fields in Ohio *(Ohio biotype)*. Additionally, PI 243540 and PI 567301B were highly resistant to the soybean aphids collected from the fields in Illinois *(Illinois biotype)*. PI 567324 exhibited moderate resistance against the aphid from Illinois. The three additional plant introductions were confirmed to have a moderate level of resistance to the Ohio collection of soybean aphids (Mian et al., 2008). Ohio State University chose PI 243540 for their breeding project. The objective was to backcross the *Rag* 2 allele that transfers resistance to biotypes 1 and 2 of soybean aphid and the Rmd_PI243540 allele that transfers resistance against powdery mildew from PI 243540 into the cultivar 'Wyandot'. SSR markers were selected via bulk segregant analysis in order to identify the genomic location on the soybean map for the *Rag2* allele, which was discovered to be derived from a different location than *Rag1* (Mian et al., 2008b). PI 243540 is a cultivar from Japan that has desirable agronomic traits. These traits include resistance to lodging, superior germination rate, and vigorous early seedling growth. Seeds are large and round with clear hilum and shiny yellow seed coat. 'Wyandot' is a food grade cultivar with most seed traits similar to those of PI 243540 (Mian et al., 2016). 'Wyandot-14' was the result of a backcross breeding strategy that was initiated between PI 243540 and 'Wyandot'.

‘Wyandot-14’ would display excellent resistance against two biotypes of soybean aphid and also powdery mildew (Mian et al., 2016).

The USDA-ARS and Ohio State University crossed an additional Plant Introduction from Asia with ‘Wyandot’. PI 567301B from China was crossed with ‘Wyandot’ during the summer of 2006 to create recombinant inbred lines RILs (Lee et al., 2017). These lines provide soybean breeders with valuable germplasm for research purposes. PI 567301B contains one major gene *(RAG5)* for resistance against soybean aphids as well as an additional minor gene which were identified by fine mapping (Hesler et al., 2013). Also, PI 567301B demonstrates genetic resistance to several fungal pathogens and insect pests including *F. graminearum, A. glycines, and M. diffusa* (Lee et al., 2017). The aggregate gene resistance of PI 567301B has an advantage when compared to the single dominant gene of PI 243540. However, soybean aphid resistance genes located within PI 567301B display only antixenosis type resistance, while the single dominant gene within PI 243540 displays antibiosis type resistance. Antibiosis resistance is considered a stronger type of resistance due to the ability of the host plant to have injurious or damaging effects on the life of an insect pest, whereas antixenosis resistance refers to non-preference of the insect pest to the host plant in the form of reduced colonization. Additionally, PI 567301B contains undesirable agronomic traits which poses a problem with possible linkage drag during a backcross breeding strategy. This is perhaps the reason why PI 243540 was used to cross with ‘Wyandot’ to produce a high quality cultivar in ‘Wyandot-14’, while PI 567301B was crossed with ‘Wyandot’ to produce breeding lines for germplasm research purposes (Lee et al., 2017).

‘Jackson’ and ‘Dowling’ marked a beginning of using host-plant resistance to control aphids in soybeans, but soybean aphid evolution requires an expanded look at genetic resistance in soybean (Hill et al., 2004). The crosses made with ‘Wyandot’ marked an improvement in soybean aphid resistance by preventing the colonization of a second biotype. Nevertheless, the evolution of aphid resistance makes the prospect of further research into genetically resistant genes necessary for the security of the soybean crop species.

Individual genotypes show resistance against specific aphid biotypes

Soybean aphid colonization on *Rag1* and *Rag2* genotypes was similar to that of susceptible checks in greenhouse and field testing conducted at South Dakota State University in 2011 and 2012 (Bhusal et al., 2013). Over three hundred soybean accessions were involved in the testing which included resistant and susceptible genotypes. PI 603712 was the only genotype that consistently exhibited resistance to soybean aphid in all tests (Bhusal et al., 2013), which means that this genotype may possess additional unknown Rag genes. This information is an indication that the benefit of extended resistance durability is made possible when crosses are made between genotypes exhibiting multiple resistance genes. Alternatively, genotypes carrying single dominant genes seemingly become susceptible one by one as they are identified and integrated separately within germplasm. *Rag1* gene was the first to be identified in ‘Dowling’. Subsequently, additional aphid resistant genotypes were discovered. ‘Jackson’ is controlled by a single dominant gene that is allelic to *Rag1*. The *Rag2* gene was found in PI 243540 and PI 200538. *Rag4* and *rag1C* were both found in PI 567541B. *Rag3b* was found in PI 567543C and *rag1b* was found

in PI 567598B. Therefore, there is opportunity to combine various rag genes and incorporate them within germplasm to increase resistance durability (Bhusal et al., 2013).

Genome Wide Association Study (GWAS) reveals additional soybean aphid resistance genes in soybean crops

Strives were made in the early 2000's in the fight against soybean aphid with the introduction of a few host-plant resistant cultivars after the discovery of the pest in the U. S. Soybean researchers thought the newly found host-plant resistant cultivars were good for the soybean crop industry, but suspected that there were additional resistance genes available within the genome of the soybean species. Researchers analyzed a broader genetic scope of the soybean crop species in order to reveal genes that confer resistance to the soybean aphid pest. This objective was realized by initiating a Genome Wide Association Study (GWAS) to identify single nucleotide polymorphisms (SNPs) associated with resistance to soybean aphids (Hanson et al., 2018). Two sets of information are needed to complete a GWAS. The first set of information are phenotypic data compiled for this study from 2,366 soybean accessions, courtesy of USDA's soybean germplasm collection in Urbana, Illinois, that were made available from published studies on screenings of cultivated soybean *(Glycine max)* and wild soybean *(Glycine soja*) with aphids exhibiting Biotype 1, 2, or 3 characteristics (USDA-ARS, 2016) These studies were done separately and accumulated in a database over time. The second set of information was genotypic data compiled across the soybean genome of each accession within the USDA's soybean germplasm collection in Urbana, Illinois. The SoySNP50K high density genotyping array identified 42,509 common SNPs within each accession (Song et al., 2013, 2015). The SNPs were revealed from 18,480 cultivated soybean *(Glycine max)* accessions and 1,168 wild soybean *(Glycine soja)* accessions stored within the USDA's soybean germplasm collection (Song et al., 2015). Associations between genotypic data in the form of an aggregate number of SNPs acquired from soybean accessions in the USDA's germplasm collection and phenotypic data in the form of approximated aphid counts from previously published studies were analyzed with the programming language package *rrBLUP* for genome-wide association mapping. The *rrBLUP* package provided a *p*-value score (–log[*p*]) for each SNP that would identify the strength of association between each SNP and aphid occurrences within the accessions. Additionally the *p*-value was graphically displayed via a Manhattan plot (Hanson et al., 2018).

45 SNPs were significantly associated with changes in soybean aphid resistance, and located on 18 of the 20 soybean chromosomes (Hanson et al., 2018). A substantial number of SNPs were found on chromosomes 7, 8, 13, and 16, which are chromosomes presently recognized as carrying soybean aphid resistance genes. Additionally, GWAS produced surprising results of significant SNPs being found on chromosomes 1, 2, 4, 5, 6, 9, 10, 11, 12, 14, 17, 18, 19, and 20 where aphid resistance genes had not yet been mapped (Hanson et al., 2018). This implies that there are many soybean aphid resistance genes within the soybean genome that are yet to be discovered. SNPs may be traced back to accessions where these undiscovered resistance genes are located. Ultimately, these accessions will be used as germplasm sources for resistance and improved resistance durability (Hanson et al., 2018).

Manhattan plots for Biotype 1 aphids on *G. max* and *G. soja* accessions revealed SNPs, which were substantially associated with soybean aphid population counts on chromosomes 2, 7, and 13. Significant SNPs were also found on chromosomes 5, 9, 10, 11, 16, 17, 18, 19, and 20, where no aphid resistance genes had been documented (Hanson et al., 2018). Plots for Biotype 2 aphids on *G. max* and *G. soja* accessions showed that SNPs were substantially associated with soybean aphid population counts on chromosomes 1, 4, 6, 8, 10, 12, 13, and 14, while plots for Biotype 3 aphids were substantially associated with soybean aphid population counts on chromosomes 5, 8, 10, 13, and 19. (Hanson et al., 2018). SNPs were substantially associated with soybean aphid population counts in a combined analysis for all aphid biotypes on chromosomes 1, 5, 6, 18, and 19 where aphid resistant *(Rag)* genes have not been reported. A significant SNP was found on chromosome 13 within the range of *Rag2* and *Rag5* aphid resistance genes (Hanson et al., 2018).

GWAS enables soybean breeders to evaluate the genetic diversity of cultivated and wild soybean accessions via search of SNPs associated with soybean aphid resistance. Genome Wide Association Mapping can directly use available genotypic and phenotypic data, which saves valuable time and resources required for development of specialized linkage mapping populations. In other words, it is possible to further explore the genetic basis of soybean aphid resistance without the need to develop mapping populations for each resistant accession. Historical recombination events are analyzed via GWAS as opposed to the more time consuming research of analyzing recombination events within individual bi-parental populations (Hanson et al., 2018).

Additionally, GWAS serves as an example of how biotechnology has impacted the plant breeding industry for the better and has transitioned more breeding activity from the field to the office. Phenotypic and genotypic information from thousands of accessions can now be organized by GWAS into useful statistical data via R programming language for plant breeders, saving valuable time when compared to field experimentation. Also, accessions can be genotyped using the powerful SoySNP50k high throughput genotyping array to identify SNPs. These types of biotechnological tools will only become more advanced and expand throughout the plant breeding industry as they improve efficiency and speed.

The full scope of genetic diversity within the soybean genome has to be analyzed in order to effectively complete GWAS. Breeders needed to acquire 100% of soybean resistance SNPs within the soybean genome in order to accomplish this goal. Phenotypic data were compiled from 2,366 soybean accessions that were made available from a published study on the screening of cultivated soybean *(Glycine max)* and wild soybean *(Glycine soja*) with aphids exhibiting Biotype 1, 2, or 3 characteristics. Additionally, genotypic data in the form of 42,509 common SNPs were revealed from each of 18,480 cultivated soybean *(Glycine max)* accessions and 1168 wild soybean *(G. soja)* accessions stored within the USDA's soybean germplasm collection. This massive collection of phenotypic and genotypic accession data enhances GWAS and future gene pyramiding projects.

GWAS generates population structure for soybean accessions

An analysis of the population structure of the total USDA soybean collection was conducted for seed protein and oil content. Large accession numbers are a key element for improved GWAS analysis. This study included genotypic data in the form of 52,041 SNPs derived from 14,430 *G.max* and *G.soja* accessions using the Illumina infinium soySNP50k genotyping technology. 52,041 SNPs were reduced to 36,513 when markers associated with minor alleles were removed. Additionally, phenotypic data of existing oil and protein content were collected from field evaluations and comprised a total of 12,116 G. max accessions (Bandillo et al., 2015).

An admixture and principal component analysis was used to summarize the genetic structure and variation of the population and produced informative data with respect to population structure. Subpopulations were organized according to genetic similarity. Information was also organized according to region of origin and maturity group (Bandillo et al., 2015). Region of origin designations included China, Korea, Japan, Asia, America, Europe, and Russia. Maturity group designations included 000-0, I, II, III, IV, V, VI, and VII-X. Maturity group is characterized by temperature and photoperiod response to latitude.

Accessions from Japan and Korea were more closely related to each other than with accessions from China. Also, ancestors of American soybean mostly share ancestry with subpopulation 2 and subpopulation 5. This type of information is important as it may be useful when making crosses between donor lines and recombinant parents when backcross strategy is used to establish heterozygous lines for a gene stacking strategy. A similar background is useful to promote sufficient combining ability for hybrid vigor in offspring (Bandillo et al., 2015).

Optimization of pyramid strategy begins with crossing donor lines

Genes from multiple donor lines may be integrated into one superior line by gene pyramid breeding strategy. Heterozygosity for all desired traits must be established as the initial step. This is accomplished when a backcross strategy is conducted separately between each donor genotype and a superior recurrent genotype with respect to other traits. The sets of backcrossed plants should then be crossed (Ishii and Yonezawa, 2007). An example of this strategy is when four plants produced separately from the backcross method are then crossed evenly between each other to produce the heterozygous state of the genes within the donor lines. Four plants (1, 2, 3, and 4) are crossed (1 x 2) x (3 x 4). 1 x 2 should be similar to 3 x 4 (Ishii and Yonezawa, 2007). The product is a F_1 plant that is in a suitable heterozygous state (25%-50%-25%) for donor traits and also should exhibit the superior agronomic traits of the parents. The F_1 plant is then self-pollinated to produce plants that are homozygous for the trait or traits of interest (Ishii and Yonezawa, 2007).

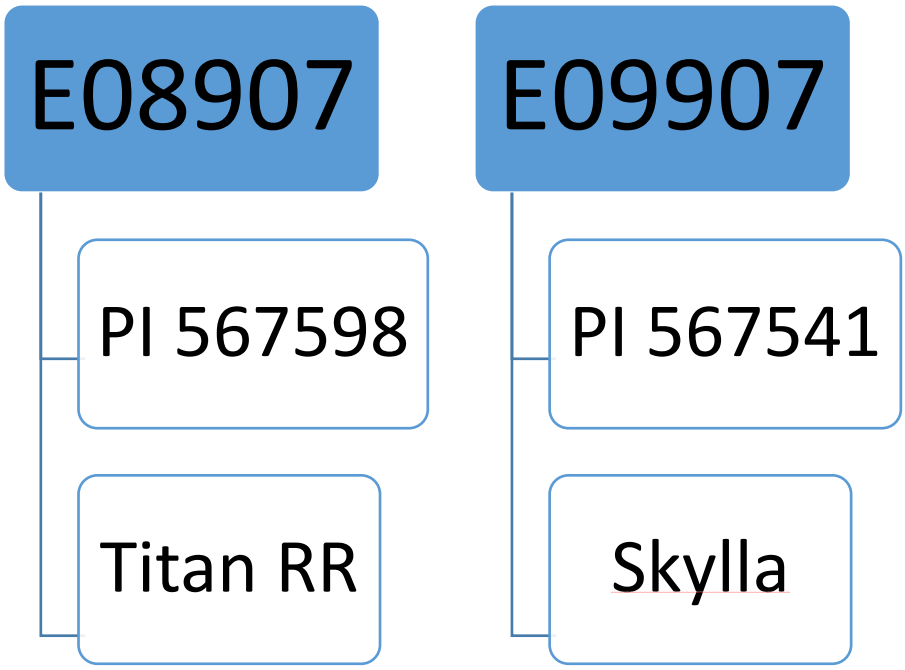
Alternatively, the strategy is different when a backcross strategy is not utilized. Tandem crosses must then occur with donors contributing fewer target markers happening first within the scheme followed by tandem crosses of donors with more numerous genetic markers to produce a heterozygous state within a single line. A single homozygous plant must then be selected via self-pollination strategy as the second major step in the process of producing superior crosses for an enhanced pyramiding process (Ishii and Yonezawa, 2007).

Pyramiding rag genes for aphid resistance in soybean

The state of Michigan offered clues regarding the status of soybean aphid biotypes in 2011 when soybean germplasm carrying the *Rag2* gene was consistently overcome in crop fields. 'Dowling' and 'Jackson' also showed extensive soybean aphid damage. This would suggest that an additional third biotype and possibly a fourth biotype existed within Michigan soybean fields (Chandrasena et al., 2015). The prevalence of new aphid biotypes poses problems for resistance in germplasm carrying a single major resistance gene. Another example of a compromise in resistance occurred in the state of Ohio. *Rag*1 resistance was not effective against the Ohio biotype when the Illinois *(Biotype 1)* and Ohio *(Biotype 2)* were evaluated across fields in the early 2000's (Kim et al., 2008). The breaking of resistance results in a chain-like pattern where the breeder must stay one step ahead with resistance breeding-strategy. Breaking the chain of resistance started when the Ohio biotype 2 aphids are able to colonize on germplasm with the single *rag1* gene (Kim et al., 2008). An additional biotype 3 was identified and was able to colonize on breeding lines with the *rag*2 gene (Hill et al., 2010). Later, biotype 4 was found to colonize lines with either *rag*1 or *rag*2 or a combination of the two (Alt and Ryan-Mahmutagic, 2013). Gene pyramiding may be the answer to the problem of pests quickly breaking resistance in crops. This approach of combining genes and incorporating them within germplasm has successfully produced longer and stronger resistance in crops with less yield reduction (McCarville et al., 2014). Soybean breeders learned from past experiences with soybean aphids and began to stack aphid resistance genes in an effort to increase resistance durability.

Researchers began the breeding study with the objective of evaluating soybean aphid damage on soybean lines containing individual or a combination of *rag*3, *rag*4, *rag*1b, and *rag*1c aphid resistance genes. A secondary objective of the study was to identify the genetic interactions of these genes in greenhouse and field conditions. The process began when breeders developed a population from a cross between advanced breeding lines E08907 and E09907. E08907 inherited the *rag3* and *rag1b* genes from PI 567598B, while E09907 inherited the *rag4* and *rag1c* genes from PI 567541B. Both PI 567598B and PI 567541B were found after screening 2,147 accessions indigenous to China. Subsequently, the resistant parent PI 567598B was crossed with the susceptible parent 'Titan RR' to produce the progeny E08907. The progeny E09907 was produced by a cross of resistant parent PI 567541B and susceptible parent 'Skylla' (Chandrasena et al., 2015).

Donor line crosses produce E08907 and E09907 advanced lines.



Advanced lines E09907 and E08907 were crossed to produce the F_1 plant which was bulk harvested during the summer of 2009. An interesting note is that parent lines PI 567598B and PI 567541B were not backcrossed into the susceptible parents to produce E09907 and E08907. A backcross strategy for each may have ensured superior agronomic traits are passed to each advanced line when compared to a single cross strategy unless both donor parents possess similar superior traits as the recombinant parents.

All seeds were planted and grown out to produce a population of 727 F_2 plants. The 727 F_2 lines, the aphid-resistant checks PI 567541B and PI 567598B and a susceptible parent check 'Skylla' were all planted in the plant sciences greenhouse at Michigan State University in the fall of 2010. Eight seeds were planted per pot with sodium vapor lights being used for supplemental lighting. Two wingless aphids collected from Michigan soybean fields were placed at the top upper and unopened trifoliate leaf of each soybean plant at the v1 stage of development. A single seed was harvested from a select number of the 727 F_2 lines. Evaluations for selection was based upon an aphid visual rating scale from 0 for no aphids to 4 for more than 800 aphids per plant. Each line was also evaluated according to an assigned damage index (DI). The DI ranges between 0% for no aphid infestation to 100% for the most severe aphid damage (Chandrasena et al., 2015).

A single seed was planted from each F_2 line during the spring of 2011. Unfortunately, not all F_2 lines were able to produce viable seed because of either germination issues or aphid damage. Subsequently, 633 $F_{2:3}$ plants were evaluated with the same checks as described in the prior evaluation and again exposed to aphids from Michigan soybean fields. Checks were replicated twice. Multiple seeds were harvested from each plant in the $F_{2:3}$ line (Chandrasena et al., 2015).

A field test study was conducted during the summer of 2011 at Michigan State University which focused upon a choice test that measured level of resistance of both antibiosis and antixenosis types. Susceptible parent 'Skylla' and resistant parents PI 567541B and PI 567598B were included as checks. Dowling, Jackson, LD05-16060 *(Rag1)*, LD08-12430a, and PI 567301B (Hesler et al., 2013) were also included as resistant checks. 633 $F_{3:4}$ lines, resistant checks, and susceptible checks were planted in a randomized complete block design. A single replication of 10 to 15 plants per line were planted in each row and infected with two aphids each. Aphids were collected from Michigan soybean fields. 190 plants were selected individually and based on molecular markers in such a way that all available combinations of *rag* genes from the population were selected (Chandrasena et al., 2015).

Seeds harvested from each of the 190 plants represented $F_{3:5}$ seeds. $F_{3:5}$ seeds were planted, grown out, evaluated in a replicated field choice test during the summer of 2013. The 190 $F_{3:5}$ lines, parents, and same checks as used with the previous evaluation were evaluated in a randomized complete block design with three replications in the field. Plants in each row were individually infested with aphids and rated for aphid damage using the 0 to 4 scale and also the damage index (Chandrasena et al., 2015).

Table 1. Genotypic groups of 190 F_3–derived lines from the mapping population E08907 × E09907 with different combinations of rag genes.

rag genotype	*n*
*rag*1b	5
*rag*1c	7
*rag*1b/c	12
*rag*3+*rag*1b	87
*rag*3+*rag*1c	7
*rag*3+*rag*1b/c	15
*rag*4+*rag*1b	3
*rag*4+*rag*1c	8
*rag*4+*rag*1b/c	17
*rag*3+*rag*4+*rag*1b	7
*rag*3+*rag*4+*rag*1c	10
*rag*3+*rag*4+*rag*1b/c	12
Total lines selected	190

The above chart was originally developed in (Chandrasena et al., 2015)

Twelve different resistance gene combinations in individual plants were categorized by marker genotyping (Table 1).

KASP SNP markers of LGC Genomics in Beverly, MA. were previously identified from fine mapping studies (Bales et al., 2013b; Yuan et al., 2014a; Yuan et al., 2014b) as tightly linked to SSR markers

and *rag* loci. These custom-designed KASP markers were used to determine the genotypes at the four aphid-resistant loci within the population. Both resistant parents PI 567598B and PI 567541B each contained two loci to genotype for resistance. PI 567598B contained the following SNPs: Gm16_5773005_A_G and Gm16_7072168_A_C are closely linked to *rag3* on chromosome 16, while SNP07-4 is closely linked to *rag1b* on chromosome 7 (Song et al., 2013). PI 567541B contains the following SNPS: SNP07-4, SNP07-5rhL2, and SNP07-7rhR2 are all closely linked to *rag*1c also on chromosome 7, while *Satt348* and *Satt649* are closely linked to *rag*4 on chromosome 13 (Zhang et al., 2009).

Table 2. Mean soybean aphid damage index (%) of F_3–derived lines from E08907 × E09907 population with different rag gene combinations across different trials. Standard error of the means was for each genotype group. The aphid DI resistance threshold is at 30%.

	Mean soybean aphid damage index (%) ± SE			
rag genes	Greenhouse 2010	Greenhouse 2011	Field 2011	Field 2013
*rag*1b	90.00 ± 2.50	85.00 ± 6.12	85.00± 7.24	62.94± 11.19
*rag*1c	53.57 ± 8.93	32.14 ± 8.99	71.97± 8.41	45.22± 8.94
*rag*1b/c	44.79 ± 8.35	26.04 ± 4.2	65.67± 6.67	52.01± 5.94
*rag*3+*rag*1b	14.22 ± 0.68	13.07 ± 0.35	32.47± 1.26	14.52± 0.38
*rag*3+*rag*1c	16.07 ± 3.57	12.50 ± 0.00	23.28± 1.94	13.20± 0.35
*rag*3+*rag*1b/c	12.50 ± 0.00	12.50 ± 0.00	30.32± 4.06	24.44± 4.87
*rag*4+*rag*1b	70.83 ± 29.17	79.17 ± 15.02	95.83± 4.17	69.07± 16.4
*rag*4+*rag*1c	18.75 ± 3.34	17.19 ± 4.69	31.13± 2.29	18.54± 2.31
*rag*4+*rag*1b/c	21.32 ± 2.57	19.85 ± 4.02	46.60± 6.29	38.90± 6.47
*rag*3+*rag*4+*rag*1b	12.50 ± 0.00	14.29 ± 1.79	40.38± 7.41	14.27± 0.85
*rag*3+*rag*4+*rag*1c	12.50 ± 0.00	12.50 ± 0.00	16.25± 2.67	13.49± 0.83
*rag*3+*rag*4+*rag*1b/c	13.54 ± 1.04	12.50 ± 0.00	24.13± 4.42	13.57± 0.63
Dowling	–	–	62.50± 0.00	16.67± 10.03

Jackson	–	–	71.67± 4.64	16.25± 11.11
LD05-16060 (*Rag*1)	–	–	83.93± 4.49	39.86± 6.46
LD08-12430a (*Rag*2)	–	–	72.81± 12.3	64.38± 9.26
PI 567301B	–	–	23.88± 4.22	47.33± 6.85
PI 567541B	12.50 ± 0.00	17.86 ± 2.53	47.92± 5.51	17.50± 10.90
PI 567598B	15.18 ± 1.49	12.50 ± 1.68	20.56± 2.42	8.33 ± 2.64
Skylla	85.42 ± 2.08	93.22 ± 3.93	96.88± 1.80	90.63± 2.55

The above chart was originally developed in (Chandrasena et al., 2015)

The data presented in Table 2 will assist plant breeders in determining the most effective resistance genes and gene combinations to integrate into soybean germplasm. The *rag*3*+rag*4*+rag*1c, *rag*3*+rag*1c, and *rag*3*+rag*1b gene combinations were the most effective and consistent in yielding resistance across the board for all trials. The most interesting finding showed that any gene that was in combination with *rag3* or *rag1c* exhibited good to excellent resistance to soybean aphid. An example of this is when *rag4* did not show acceptable aphid resistance individually, but exhibited excellent resistance when it was combined with *rag1c*. Also, *rag1c* was not as effective when evaluated individually, showing moderate resistance. This finding would suggest that *rag1c* possibly activates the *rag4* gene for superior resistance resulting in a type of epistatic affect or the rag1c gene suppresses the resistance susceptibility of *rag4. Rag1b* was the least resistance-mediating rag gene, while *rag4 + rag1b* provided the least resistance as a gene combination. Additionally, the *rag*4 and *rag*1b genes generally contributed less to soybean aphid resistance when compared to the much more resistant *rag1c* and *rag3* genes (Chandrasena et al., 2015).

Rag gene combinations outperformed individual rag genes consistently throughout the trials. Additionally, the broad genetic diversity of the soybean genome makes it possible for plant breeders to locate these resistant rag genes which prove vital in stabilizing soybean crop markets. Pyramiding or gene stacking increases resistance durability and is a more effective breeding strategy when compared to breeding for individual gene resistance (Chandrasena et al., 2015).

This gene pyramiding breeding strategy began with the selection of two resistant accessions (PI 567598B and PI 567541B) that were chosen from a group of 2147 accessions via fine mapping. Unfortunately, the GWAS study to identify aphid resistance genes occurred after the gene pyramiding study. Therefore, information obtained from GWAS was not available to assist during the selection of resistant parents for the gene stacking project. This circumstance is a testament

to breeders working with the best information available at the time. A different set of resistant parents may have been chosen by gene pyramid breeders had the GWAS data been available prior to the gene pyramiding study.

Pyramiding for soybean mosaic virus (SMV) resistance produces additional genotypes

SMV is a common soybean disease which affects most if not all soybean growing states within the U.S. Seed coat mottling is a common symptom which reduces overall seed quality. Leaves may also be affected as curling, necrosis, chlorosis, and rugosity are common (Shakiba et al., 2012). Infected plants carrying SMV may spread the disease to other plants by means of infected seeds, aphids, or mechanically (Maroof et al., 2008) (Shakiba et al., 2012). Soybean breeders desired to improve SMV resistance when a study was conducted to determine the effects of Rsv gene combinations on mosaic virus resistance. Specifically Rsv1, Rsv3, Rsv4 lines were first developed when a genotype that carried a single dominant SMV resistance gene was each backcrossed into a susceptible parent known as 'Essex' (Maroof et al., 2008). Backcrossing was carried to the BC_6 stage. V94-3972 carried the single dominant gene Rsv1, V229 carried Rsv3, and V97-9003 carried Rsv4. The three lines as well as sister lines were used in the study. Additionally, parental lines carrying the single Rsv genes were included with susceptible cultivar checks. Two gene combination lines were then produced when pairwise crosses were made (Rsv1+Rsv3, Rsv1+Rsv4, and Rsv3+Rsv4). The F_1 crosses were screened with molecular markers linked to the Rsv genes to ensure their crosses were accurate for the existence of Rsv genes (Maroof et al., 2008). They were then self-pollinated to obtain the F_2 generation. SMV isolates were introduced into testing and included Smv-G1, Smv-G2, Smv-G3, Smv-G5, and Smv-G7. Plants were inoculated with isolates and susceptible plants were discarded. Molecular markers linked to each resistance gene were used to screen and select individual plants at the F_2 generation for segregating Rsv genes. Susceptible plants were discarded while resistant plants were tested with PCR based RFLP molecular markers. Selected plants showed a homozygous dominant status for Rsv1+Rsv3, Rsv3+Rsv4 and Rsv1+Rsv4. The individual F_2 plants were crossed to produce lines with three gene combinations (Rsv1+Rsv3+Rsv4) (Maroof et al., 2008).

Recurrent parent 'Essex' was used to produce multiple backcrossed offspring in the development of an F_1 population. The fact that the recurrent parent 'Essex' was the same in each backcross for the development of the population should confirm superior agronomic traits within the offspring. The GWAS which identified population structure of accessions previously discussed would be a useful tool to identify similar maturity group accessions for selection in developing superior crosses for pyramiding studies in soybean.

The results after testing concluded Rsv1+Rsv3 and Rsv1+Rsv4 to have complementary effect with superior resistance to all SMV isolates. Rsv1+Rsv3+Rsv4 lines were also resistant to all isolates. Alternatively, the Rsv3+Rsv4 combination resulted in what is described as late susceptibility or delay. This occurrence can be seen as a type of tolerance resistance (Maroof et al., 2008).

Gene combinations create new genotypes that exhibit additional sources of resistance. Rsv1 works well in combination with other Rsv genes but the Rsv3+Rsv4 gene combination that did

not include Rsv1 was not effective. Thus Rsv1 seems to activate other less resistance-mediating Rsv genes to create separate and distinct resistance genotypes.

Soybean reactions create opportunities in establishing resistance

Studies that involve reactions to pest exposure within crop accessions often give plant breeders important information with regard to resistance. Type of resistance is often associated with specific loci. These phenotypic data often reveal new information for plant breeders to evaluate as was the case for a study of soybean cultivar resistance to soybean mosaic virus that was conducted in greenhouse environments at the University of Arkansas. Researchers wanted to observe the reactions of a variety of commercial soybean cultivars to SMV (Shakiba et al., 2012). The testing began with collecting 303 soybean cultivars. The collection of cultivars would then be screened with six SMV strains which included G1, G2, G3, G5, G6 and G7 (Shakiba et al., 2012). Inoculation of all cultivars was completed exactly ten days after planting. Symptoms for SMV development was tracked and given a score on a weekly interval for a five week period. Each cultivar was then given a designation of either resistant, susceptible, necrotic, or mixed results which represented phenotypic data collected in the form of symptoms expressed within each of the plants. Reaction patterns of each tested soybean cultivar were compared to previous reaction results to SMV strains of specific cultivars that carry known genes or alleles. Tested cultivars that expressed identical reactions as previously tested cultivars were determined to possess the same genes or allele. Conversely, cultivars that expressed dissimilar reaction behavior as previously tested cultivars were determined to possibly possess a novel allele for SMV resistance (Shakiba et al., 2012)

Results indicated sixty-six cultivars to be resistant to strains G1 through G3 but susceptible to G5 through G7. Four cultivars were resistant to G5 through G7 but susceptible to G1 through G3. One cultivar was resistant to G1 through G3 but necrotic to G5 through G7. Most importantly, two cultivars exhibited resistance to all six SMV strains and thirty-four cultivars had reaction patterns that were uniquely different from existing cultivars with known genes. This would suggest there are possibly additional novel SMV resistance genes or alleles to identify and incorporate into germplasm for the establishment of more SMV resistant genotypes within crop production (Shakiba et al., 2012).

A possible testing scenario would be to use the phenotypic data from the two cultivars that exhibited total resistance to all six SMV strains and the thirty-three cultivars that exhibited unique reaction patterns into a GWAS. This phenotypic data could then be combined with genotypic data in the form of common SNPs identified by the SoySNP50K genotyping array within the thirty-five soybean cultivars. Useful SMV resistance genes will then be identified for gene pyramiding. Gene stacking opportunities expand the breadth of resistance breeding in crop production.

Conclusion

Plant breeders are at the forefront of efforts to improve crops in the wake of increasing threats from biotic and abiotic stresses that reduce yield. Soybean aphid is an example of an agricultural pest that reduces yields and profits for soybean farmers across the U.S. Soybean breeders were

eventually able to introduce aphid resistant cultivars to growers as breeding strategies became more advanced with the emergence of new aphid biotypes. Subsequently, farmers were able to depend less upon pesticides and more upon host-plant resistance. Single dominant genes were incorporated into commercial soybean genotypes to produce pest resistance and were effective for a while. Eventually this type of resistance becomes compromised to susceptibility. Breeders developed the strategy of pyramiding genes in order to accumulate resistance genes and to stay ahead of pest biotype evolution. Biotechnology advanced gene stacking strategies with the use of marker assisted selection and GWAS. Opportunities were also revealed such as the organization of population structure within accession data for future selection of donor line crosses. These donor line crosses will enable breeders to mate similar accessions together with superior agronomic traits. It is only through the continued use of biotechnology and gene pyramiding that resistance durability may be accomplished within the crop industry. Breeders must work in collaboration and leave knowledge for the next breeder in line in order to construct a successful continuum of pest resistance for the future of crop production.

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