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Diversity, Identification, Mapping and Pyramiding of Genes for Resistance to Soybean Mosaic Virus [Glycine max (L.) Merr.]

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DIVERSITY, IDENTIFICATION, MAPPING AND PYRAMIDING OF GENES FOR RESISTANCE TO *SOYBEAN MOSAIC VIRUS* [*Glycine max* (L.) Merr.]

DIVERSITY, IDENIFICATION, MAPPING AND PYRAMIDING OF GENES FOR RESISTANCE TO *SOYBEAN MOSAIC VIRUS* [*Glycine max* (L.) Merr.]

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of the requirements for the degree of
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By

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ABSTRACT

Soybean Mosaic Virus (SMV) is a prevalent viral pathogen transmitted by aphids and via seed, causing significant yield loss and quality reduction. There are seven SMV strain groups (G1 to G7) and three resistance loci (*Rsv1*, *Rsv3*, and *Rsv4*) reported in soybean. New sources of SMV resistance would be valuable for breeding cultivars with durable resistance to multiple strains. The objectives of this research were to analyze genetic diversity of SMV-resistant soybean germplasm at the molecular level; to pyramid three genes from different sources for durable SMV resistance; and to identify and map new genes/alleles for differential reactions to SMV strains. One hundred and fourteen SMV-resistant germplasm genotypes collected worldwide were screened with 98 simple sequence repeat (SSR) markers. The 114 germplasm genotypes were grouped into four clusters with the largest group containing most of Asian genotypes. *Rsv1* alleles were found to be most common, while *Rsv3* and *Rsv4* were rare among all the SMV resistant germplasm tested. PI 96983 (*Rsv1*) was crossed with Columbia (*Rsv3*, *Rsv4*) to pyramid all three SMV resistance genes with the aid of SSR markers. The molecular screening of a F_2 plant population derived from PI 96983 x Columbia showed good fit to the expected genotypic ratios at each marker locus; one out of 70 plants was identified to contain three resistance genes in homozygous condition (*Rsv1+Rsv3+Rsv4*), while 26 additional plants also contained three genes but at the heterozygous condition. $F_{2:3}$ lines inoculated with G1 and G7 strains confirmed the presence of specific alleles and SMV resistance. A new SMV resistant parent PI 61944 with unknown gene(s) (*Rsv-?*) was crossed with the susceptible parent Essex (*rsv*), and resistant lines PI 96983 (*Rsv1*), L29 (*Rsv3*), and V94-5152 (*Rsv4*) to investigate the inheritance and allelism of SMV resistance. F_2 plants were screened with selected SSR markers, and the $F_{2:3}$ lines were screened with SMV G1 and G7. SMV screening and molecular analysis revealed that the PI 61944 carries a new allele at the *Rsv3* locus on linkage group (LG) B2. This research has

identified genetic diversity among SMV-resistant soybean germplasm, combined three genes for durable resistance in soybean, and discovered a new SMV resistance allele. These germplasm will be useful in soybean breeding programs where SMV resistance is an objective. This research also demonstrated that SSR technology is extremely useful in marker assisted selection (MAS).

This dissertation is approved for recommendation
to the Graduated Council.

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DEDICATION

To my mother because without her this would not have been possible, Gloria I love you.

To my sister Teresa and brother Cuahutemoc Cervantes who always care for me.

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CHAPTER I

Introduction and Literature Review

Introduction

Soybean [*Glycine max* (L.) Merr.], the leading oilseed crop produced and consumed in the world, is a major economic crop in North America, Europe, and South and Central America (Hymowitz, 2004). In 2010 the U.S. was the largest producer with 3.4 billion bushels, followed by Brazil (70 million bushels) and Argentina (49.5 million bushels) (SoyStats, 201). In North America soybean is grown in the eastern half of the continent, from coastal areas of the Gulf of Mexico north to southern Canada. Because these areas have adequate moisture to produce the crop successfully, soybean production extends west in the U.S. to the Dakotas, Nebraska, Oklahoma, and Texas (Wilcox, 2001). The state of Arkansas is the tenth largest producer of soybean annually (Arkansas Ag Statistics, 2011) with a planted area of 3,190 thousand acres and a total production of 110.2 million bushels (SoyStats, 2012).

Soybean has a variety of edible and industrial uses and can be consumed as edamame, milk, flours, and edible fats and oils. Soybean is also used for livestock feeding of poultry, swine, beef, and dairy; and among the industrial uses are oil production and biodiesel fuel; therefore, soybean is called the "miracle crop" (SoyStats, 2012). As any other profitable crop, soybean manifests adversities, including diseases, with Soybean Mosaic Virus (SMV) being one of the most. SMV can cause yield losses up to 90%, affecting seed size and oil content (Wang et al., 2001); and during 2003 the estimated reduction of soybean yield due to virus was 18, 663 bushel in the state of Arkansas (Wrather and Koenning, 2006). Much of the yield losses and quality reduction are due to abortion of flowers, reduced pod set, reduction in the number and weight of seeds, and seed discoloration (coat mottling) (Steinlage et al., 2002).

SMV is transmitted via infected seed from one generation to the next one; by aphids (*Aphis glycines*), by feeding directly on soybean or transmitting viruses; and mechanically

though sap (Garcia-Arenal and McDonald, 2003; Zhang et al., 2009). It is reported that when transmission of SMV by the *Aphis glycines* is low, the transmission is also poor through the seed. This tendency has been associated with amino acid sequence of the HC-Pro (helper component/protease) and CP (coat protein) coding regions changes, most often with mutations in the DAG motif (Domier et al.2007).

There are seven strain groups of SMV based on the classification of virulence (G1-G7), G1 being the least and G7 the most virulent strains (Cho and Goodman, 1979). Individual plant reactions to the different strains are classified as resistant (R, symptomless), necrotic (N, necrosis), or susceptible (S, mosaic). The SMV resistance is controlled by single dominant genes, and there are three independent loci reported *Rsv1*, *Rsv3*, and *Rsv4* (Buzzell and Tu, 1984; Gunduz et al., 2004; Hayes et al., 2000). These loci are at the linkage groups (LG) F, B, and D1b, respectively, in the soybean molecular map (Song et al., 2004).

To control viral diseases, genetic resistance is preferred because it is an effective and economical method for disease management in soybean (Zheng et al., 2005), however finding durable resistance to SMV by conventional breeding methods requires laborious screening and genetic testing. The use of molecular markers and plant breeding methodologies can be successful for finding a target disease resistance gene (Garcia-Arenal and McDonal, 2003; Yu et al., 1996).

The objectives of this research were to analyze genetic diversity of SMV resistant soybean germplasm at the molecular level, to pyramid three genes from different sources for durable SMV resistance, and to identify and map new genes/alleles for differential reactions to SMV strains.

Literature Review

The genus *Glycine* (Willd.) belongs to the family *Fabacea*, subfamily *Papilionoideae*, tribe *Phaseoleae*, subtribe *Glycininae* (Palmer and Hymowitz, 2004). There are two species usually recognized within the genus *Soja*, *Glycine max* and *Glycine soja*. *G. soja* is considered as the ancestor of *G. max*, and its semiwild relative should be regarded as taxonomically distinct from *G. soja* since both are domesticated. In addition, *G. gracilis* could be an intermediate species between *G. max* and *G. soja* (Chen and Nelson, 2004).

The soybean genome is of average size compared to many other plants. Schumutz et al. (2010) carried out exhaustive research to analize the soybean genome *Glycine max* var. Williams 82. The diploid chromosome number of 2n=40 compromise 1,115 megabases (Mb), and is organized in 20 linkage groups (LG) and/or in 20 chromosomes pairs, known as the genetic map of the soybean (Song et al., 2004; SoyBase, 2012). Based on this genetic map, 4,991 single nucleotide polymorphisms (SNPs) and 1,028 simple sequence repeats (SSRs) have been identified. These molecular markers are the tools to localize genes on the soybean molecular map, and detect linked molecular and/or phenotypical markers to facilitate breeding programs.

Improvement in seed yields of soybean by conventional breeding is considered efficient, but molecular techniques may provide faster genetic gains. Genes associated with the important agronomic traits such as seed yield, lodging, plant height, seed filling period, and plant maturity in soybean are identified through molecular techniques (Pathee et al., 2007). Molecular techniques have also been used in soybean to locate genetic alleles for seed composition traits, reproductive traits, time of flowering and maturity traits, and response to nutritional factors (Palmer and Hymowitz, 2004).

SMV Characteristics

Soybean mosaic virus (SMV) is a plant virus from the Potyviridae family, and is constituted by a single-strand RNA of approximately 10 kb. Longevity *in vitro* (LIV) is 2 to 5 days in infective sap and 7 days in dried leaves at 25 to 33°C, and it is quiet immunogenic (Sutic et al., 1999; Wang et al., 2001). Virons contain 5.3% nucleic acid, 94.7% protein, and 0% lipid, and can be found in roots, cortex, epidermis, phloem, testa, and embryos (Goodman, 1980).

Using 98 isolates of SMV from seeds of the USDA germplasm collection, Cho and Goodman (1979) classified SMV into seven strain groups, G1 (the least aggressive) through G7 (the most aggressive); they also reported that cultivars resistant to less virulent strains could be severe necrotic when inoculated with more virulent strains. Subsequently, the G7A and C14 strains were added to the classification (Lim, 1985). In most plant-virus systems analyzed, seed transmission is dependent upon both host and virus genotypes (Domier et al., 2011). SMV strains can be maintained *in vivo*, conducted by frequent inoculations in greenhouse-grown susceptible plants, or *ex vivo*, storage in refrigerator or direct freezing of plant tissue or in vitro as in callus culture (Mozzoni and Chen, 2010).

SMV Reactions

Host reactions to SMV include resistance (R, symptomless); necrosis (N, localized and/or systemic necrosis), and susceptibility (S, mosaic). SMV symptoms are influenced by temperature, therefore they persist or disappear (Goodman, 1980; Ross, 1969). Resistant plants are vigorous and undistinguishable from non-inoculated plants. The necrotic reaction gives rise to brown discoloration of leaf veins, yellowing of the leaves (systemic necrosis), stunting of the plant, browning of petioles and stems, defoliation, and eventually plant death. The susceptible

plants show leaves that gradually curl, the leaf margins twist downward with chlorosis developing between them, and leaf blades are smaller and somewhat brittle (Bowers et al., 1992; Sutic et al., 1999). Susceptible plants yield poorly when they get infected in an early stage but yield reduces significantly when infection occurs during reproductive stages (Cho and Goodman, 1982).

Necrosis is a protective system activated in response to infection with pathogens; this reaction is the result of infection of soybean possessing resistance to prevalent SMV strains by a more virulent strain of the same virus. In cultivars carrying hypersensitivity genes, control is achieved either by the virus being restricted to the initial sites of inoculation, or by rapid plant death resulting in reduced secondary spread within the crop (Cho and Goodman, 1982; Kyrichenko et al., 2007; Walkey, 1991). SMV reaction is controlled by the virus strain, host genotype, and the environment (Bowers et al., 1992).

Lethal necrosis (LN) particularly in the soybean line PI 507389 develops N symptoms in a very short time after inoculation lending to a severe stem-tip necrosis and plant death; PI 507389 carries the allele *Rsv1-n* at the *Rsv1* locus that confers N to G1 but no R to any other strains of SMV. The allele for LN response is recessive to the allele for R and co-dominant with the allele for S, and the S allele may be dominant over the LN allele at a later stage in response to more virulent SMV strains (Ma et al., 2003). Other studies also reported that necrotic symptoms appeared when soybean cultivars resistant to less virulent SMV strains were infected by more virulent strains (Li et al., 2006). Stem tip (STN) necrosis is a SMV strain-specific, thermo-sensitive response associated with SMV resistance gene (Zheng et al., 2005). However, only cultivars having *Rsv1* allele exhibit dosage and temperature effect on SMV expression, these cultivars tend to develop STN in homozygous or heterozygous state (Li et al., 2009).

SMV Genetics

It is reported that 80% of the resistance in SMV is monogenically controlled and only half of it show dominant inheritance (Kang et al., 2005). Symbols *Rsv*, and *rsv* were initially used to designate an allelomorphic series involved in SMV resistance (Cho and Goodman, 1982; Kiihl and Hatwing, 1979), with *Rsv* conferring resistance reaction and *rsv* conferring susceptible reaction. Resistance to different strains controlled at separate loci makes it possible to incorporate many genes into one cultivar for multiple resistances (Ma et al., 2004)

There are three independent loci for SMV resistance, *Rsv1*, *Rsv3*, and *Rsv4*. Cultivars Essex and Lee 68 are susceptible to all SMV strains and carry a recessive allele symbolized as *rsv*; they are used as recessive parents in most of the of the inheritance studies. The *Rsv2* locus was reported in cultivar Raiden (PI 360844) (Buzzell and Tu, 1984); however, subsequent studies confirmed that the gene in cultivar Raiden is allelic to the *Rsv1* locus and a new allele symbol was assigned, the *Rsv1-r* (Chen et al., 2001).

There are nine alleles reported at the *Rsv1* locus. Most of the *Rsv* alleles at the *Rsv1* locus confer resistance to the strains G1-G3 and susceptibility or necrosis to G5-G7. The resistance alleles may show systemic necrosis in the heterozygous state (Hayes et al., 2000). The first resistance allele found in the PI 96983 and *Rsv1* symbol was assigned. Cultivar Ogden produces necrosis after inoculation with G3 strain; it carries a single gene allelic to *Rsv1*, and named as *Rsv1-t*. York (*Rsv1-y*), Kwanggyo (*Rsv -k*), and Raiden (*Rsv1-r*) express resistance to lower-numbered strains but susceptibility or necrosis to the higher-numbered strains. Marshal (*Rsv-m*) is resistant to G1, G4, and G5, and necrotic to the rest four strains. Swedon 97 (*Rsv1-h*) shows resistance to all strains. PI 507389 (*Rsv1-n*) is the only genotype carrying an allele at the *Rsv1*

locus conditioning necrosis to G1 and no resistance to any other SMV strains (Buss et al., 1989; Chen et al., 1991; Chen et al., 2002; Ma et al., 2003; Roane et al., 1983).

Cultivars that carry alleles al the *Rsv3* locus show resistance to higher-numbered strains (G5-G7) but they are susceptible to lower-numbered strains (G1-G4). *Rsv3* was first found in a line derived from cv. Columbia for resistance to SMV strains G5-G7 (Buzzell and Tu, 1984). Subsequently, other *Rsv3* alleles were identified in cultivars L29, and OX 686, and OX 670; they confer necrotic or susceptible reaction to strains G1-G4 and resistance to strains G5-G7 (Buss et al., 1999; Ma et al., 2002).

Rsv4 confers resistance to all known strains in the genotype V94-5152 (Buss et al., 1997). Another allele of *Rsv4* was found in the PI 88788 which present late susceptibility (LS) reaction to all strains; this reaction is an early restriction to virus movement through the vascular system and later the virus invades newly developing leaves resulting in venial chlorosis and green islands in the LS genotype (Gunduz et al., 2004).

Having more than one dominant gene for SMV resistance reduces the vulnerability of cultivars to suffer devastating infestation. There are several soybean genotypes that contain multiple genes for SMV resistance. Among these genotypes is PI 486355 (*Rsv1Rsv4*) with two independent genes that exhibit incomplete dominance and give resistance to all strains (Chen et al., 1993). Columbia (*Rsv3Rsv4*) has two complementary resistance genes and confers resistance to all strains but necrosis to G4 (Ma et al., 2002). Cultivars Tousan 140 and Hourei (both with *Rsv1Rsv3*) carry two resistance genes *Rsv1* and *Rsv3*. The *Rsv1* allele confers resistance to strains G1-G3 but not strains G5-G7 and the *Rsv3* allele conditions resistance to G5-G7 and

susceptibility to G1-G3, therefore these two cultivars are resistant to all strains (Gunduz et al., 2002). Zao18 (*Rsv1Rsv3*) also shows resistance to strains G1-G7 (Liao et al., 2002).

Molecular Mapping of SMV Loci

The genes for resistance to SMV have been molecularly mapped. The chromosomal location of *Rsv1* was identified using RFLPs and SSRs markers in a cross between soybean line PI 96986 as the resistant parent and cultivar Lee 68 as the susceptible parent. *Rsv1* was mapped on the soybean linkage group (LG) F (chromosome 13) in a F_2 population constructed from a cross between PI 96983 x Lee 68 (Yu et al., 1994).

The gene *Rsv3* confers resistance to the more virulent strain groups G5 through G7, and conditions mosaic reaction to the less virulent group G1 through G4. *Rsv3* was mapped on LG B2 (chromosome 14) based on the data collected from two F_2 populations, L29 (*Rsv3*) x Lee68 (*rsv3*) and Tousan 140 (*Rsv3*) x Lee68 (*rsv3*) (Jeong et al., 2002).

Gene *Rsv4* confers resistance with complete dominance to all SMV strains G1 to G7 identified in the U. S. In contrast to cultivars carrying *Rsv1* alleles which show systemic necrosis in the heterozygous state, the *Rsv4* locus in PI 486355 shows resistance without necrosis in both the heterozygous and homozygous states, and it produces no necrotic or hypersensitive type reactions. A F_2 family derived from the cross between the resistant line LR2 (*Rsv4*) and the susceptible line Lee68 (*rsv4*) was evaluated for a mapping study to determine the *Rsv4* locus in soybean. Results showed that the *Rvs4* locus was at the LG D1b (chromosome 2) on the soybean map (Hayes et al., 2000). Because *Rsv4* confers resistance to all seven strains, there is an interest in pyramiding this gene with other resistance loci such as *Rsv1* and *Rsv3* to incorporate multiple genes in soybean for defense against multiple SMV strain infection. The ability to pyramid resistance genes into a single cultivar is greatly expedited by the use of closely linked molecular

markers. Since genes such as *Rsv4* can mask presence of other genes, selecting lines that contain multiple genes is not always possible by simple phenotypic methods (Chen et al., 1994).

Inheritance of Resistance to SMV

Most of the results on the inheritance of resistance to SMV strains showed that resistance was conferred with a single dominant gene. The first SMV resistance gene was found in the PI 96983 and symbolized as *Rsv* (Kiihl and Hartwing, 1979). Subsequently, nine alleles had been reported in York, Marshall, Raiden, Kwanggyo, Ogden, Suweon 97, LR-1, and PI 507389 carry the *Rsv1*, *Rsv1-y*, *Rsv1-m*, *Rsv1-r*, *Rsv1-k*, *Rsv1-t*, *Rsv1-h*, *Rsv1-s*, and *Rsv1-n* alleles, respectively (Chen and Choi, 2007). However, inheritance studies of resistance in cultivar Zao18 crossed with the SMV-susceptible cultivar Lee 86, and with resistant lines PI 96983, L29, and V94-5152 which carry *Rsv1*, *Rsv3*, and *Rsv4*, respectively, indicated that Zao18 possess two independent dominant genes for SMV resistance, one of which is allelic to the *Rsv3* locus; the other is allelic with *Rsv1*. The presence of both genes (*Rsv1* and *Rsv3*) in Zao18 confers resistance to SMV strains G1-G7 (Liao et al., 2002).

In another study PI 88788 was crossed with susceptible cultivars Essex and Lee 68 and with the resistant lines PI 96983, L29, and V94-5152. The progeny was inoculated with SMV strains G1 and G7 and data analyses showed that resistance in PI 88788 to SMV-G1 was controlled by a single, partially dominant gene; however, to SMV-G7, the same gene was completely dominant. This gene was independent of the *Rsv1* and *Rsv3* loci, but allelic to *Rsv4* in V94-5152 (Gunduz et al., 2004). In addition, cultivar Suweon 97 was crossed to susceptible cultivars Essex and Lee 68, and with SMV resistant cultivars possessing the *Rsv1*, *Rsv3*, and *Rsv4* genes for inheritance and allelism tests. The segregation ratio of the resistance x susceptible crosses was 1 resistant: 2 segregating: 1 susceptible in the $F_{2:3}$ lines, so Suweon 97 has a single

dominant gene for SMV resistance. Presence of necrotic plants in the cross suggests the Suweon 97 gene for SMV resistance is incompletely dominant. No segregation for susceptibility to five SMV strains was found in the cross of Suweon 97 x PI 96983, suggesting that the Suweon 97 gene is an allele at the *Rsv1* locus. Allelism test indicated that this gene is independent of *Rsv3* and *Rsv4*. Suweon 97 is immune to all SMV strains in the U.S., thus a valuable source of genetic resistance (Chen et al., 2002).

Tousan 140 and Houri cultivars were crossed with SMV susceptible cultivar Lee 68, and with lines possessing *Rsv1*, *Rsv3*, and *Rsv4*. Inheritance and allelism tests studies indicated that Tousan 140 contained two SMV resistance genes; one of them genes was an allele of *Rsv1*, conferring resistance to SMV-G1 through G3 and susceptibility to SMV G5 through G7 while the other one, an allele of *Rsv3*, expresses the contrary reaction. Hurei is resistant to SMV-G1 and possesses two SMV resistance genes, which are also alleles of *Rsv1* and *Rsv3*. The *Rsv1* allele, expresses resistance to SMV-G1 and necrosis or susceptibility to G7, and the *Rvs3* allele may express resistance to SMV-G7 but susceptibility to G1. Presence of both genes increase resistance to more strains than what is provided by either of the single genes. This justifies the strategy of pyramiding of the multiple genes to provide more effective and durable resistance to SMV (Gunduz et al., 2002).

Likewise, inheritance of SMV resistance in J05, and determination of the allelic relationship of resistance genes in J05 with three resistance genes (*Rsv1*, *Rsv3*, and *Rsv4*) showed that J05 contained two independent dominant genes for SMV resistance, one at the *Rsv1* locus conferring resistance to G1 and necrosis G7 and the other at the *Rsv3* locus conditioning resistance to G7 but susceptibility to G1. The combination of both *Rsv1* and *Rsv3* in J05 provides resistance to all SMV strains (G1-G7). Soybean genotypes with resistance to all SMV strains are

rare, and most commercial soybean cultivars are susceptible to SMV; therefore, identification of new soybean genotypes such as J05 with resistance to all SMV strains are valuable in breeding for SMV resistance (Zheng et al., 2006). Since SMV resistance could be easily broken, there is necessity of find new sources of cultivars that carry novel genes for resistance.

Gene Pyramiding

Previous SMV inheritance studies are valuable to develop gene pyramiding for SMV resistance; those identified alleles can be combined with the MAS approach for breeding purposes. Different studies have demonstrated its success in diverse crops such as rice, in which the *Xa21* and cry1Ab/cry1Ac genes conferring resistance to bacterial blight and lepidopteran insects, respectively, were successfully pyramided into a derived line of 'Minghui 63' using MAS (Jiang et al., 2004). Double haploid lines and MAS strategies were utilized to pyramid the *rym4*, *rym5*, *rym 9* and *rym11* genes conferring resistance to Barley Yellow Mosaic Virus (BaYMV) in barley (Werner et al., 2005). Barloy et al. (2007) pyramid the *CreX* and *CreY* genes, involved in the resistance response to cereal cyst nematode (CCN), in wheat through MAS approach.

MAS methodology makes possible the phenotypic selection in virus resistance research where the lack of virus strains differentiation is the principal restriction. With MAS we can identify, at early generation, lines that possess one, two or more resistance genes and ptherwise cannot be recognized by mechanical inoculation. Then, pyramiding multiple resistance genes in a single genotype could be achieved, as in several studies. Shi et al. (2008) used PCR-based markers to link to the three SMV resistant genes, and identify advanced soybean lines derived from a cross between J05 (*Rsv1Rsv3*) and V94-5152 (*Rsv4*) that presumably carry the three resistance genes for SMV.

Soybean Genetic Diversity

Knowledge of the genetic diversity of the crop is necessary for parental selection that maximizes genetic improvement, consequently more accurate and complete descriptions of genotypes and patterns of genetic diversity could help to determin future breeding strategies and facilitate introgression of diverse germplasm into the current commercial soybean genetic base (Thompson et al., 1998). Many agronomic traits, pedigrees, geographic origins, and isozymes have been used for assessments of soybean genetic diversity. However these methods generate limited data that have influence of growing environment on agronomic trait evaluation, and there are possible errors of incomplete information in the documentation of pedigrees and origins of accession collections; therefore those methods have been replaced by DNA marker analysis (Burnham et al., 2002; Wang et al., 2006).

The primary gene pool of soybean, which consists of *G. max* cultivars, land races, and the wild annual *G. soja*, is useful to plant breeders for selecting germplasm. The major *Glycine* germplasm collections exist in Australia, Brazil, China, Germany, India, Indonesia, Japan, Russia, Republic of Korea, Taiwan, Ukraine, and the United States. In addition, within the genus *Glycine* subgenus *Glycine* there are 22 recognized wild perennial species that represent a reservoir of useful genes to improve the cultivated soybean (Palmer and Hymowitz, 2004).

There is a limited genetic base of North America soybean cultivars, which is due to the contribution of fewer than 20 plant introductions (PIs) to the primary gene pool and to the repeated use of related parents in breeding programs (Gizlice et al., 1994). However, nowadays it is possible to estimate the diversity of the PIs by DNA marker analysis and PIs carrying desirable alleles would serve as candidates for parents in breeding program; therefore the more genetically diverse the PIs are from the elite parents, the more likely they are to possess unique alleles for

traits of interest. Currently SSR markers have been shown to be highly polymorphic in soybean, and the SSR alleles typically show monogenic-codominant inheritance that enables classification of homozygotes and heterozygotes in a segregating population. The ability of SSRs to distinguish among elite soybean genotypes and PIs with agronomic merit may assist with the transfer of favorable alleles from PIs into elite soybean cultivars (Narvel, 2000).

Through SSRs utilization, Brown-Guedira et al. (2000) assessed the relationship of 18 major ancestors of North American soybean germplasm with 87 plant introductions (PIs) that are potential new sources of genetic variation for soybean breeding programs. The most stable grouping was among ancestors that corresponded with known relationship based on pedigree and maturity, and several groups of PIs are distinct from the majority of the ancestors.

The use of exotic germplasm in soybean cultivar development generally has been limited to a small number of introductions that have served as sources of genes for resistance to disease and insect pests and have contributed little to overall genetic diversity. As assumption, lines that have yields similar to commercial cultivars and have a parent that are genetically distinct from those same cultivars, are good candidates for inclusion in a breeding program to expand genetic diversity and increase yield. The most important possible sources of SMV resistance genes in U. S. commercial cultivars are genetically associated with the frequent utilization of ancestral cultivars of North America CNS and Ogden cultivars, indicating possible loss of resistance via genetic drift because lack of selection pressure for SMV resistance (Wang et al., 2005). There is a relatively high frequency of SMV resistance in major ancestral lines in U. S. cultivars; among them are CNS and Ogden cultivars which do not provide resistance to all SMV strains. The limited germplasm base of North America soybean cultivars increases the vulnerability of the

crop to changes in pathogen and pest populations and threatens the ability of breeders to sustain genetic improvement (Brown-Guedira et al., 2000).

Through time researchers have implemented the use of molecular markers for studying many aspects related with soybean diversity. Fei and Chen (1996) used RAPD markers for analysis of genetic diversity of the *Glycine* genus to classify 21 accessions from 10 different species. A genetic diversity study among 18 soybean genotypes selected for increasing the protein content of varieties adapted for central European growing conditions recommended combined use of a limited number of RAPD and SSR markers as means of evaluating genetic relationship of genotypes in absence of pedigree data (Doldi et al., 1997). Molecular analyses of soybean diversity in U.S. using RFLP and SSR show clear separation of northern and southern cultivars and the limited diversity in southern gene pool, and that the actual elite pool gene is less diverse than the ancestral pool which could limit breeding progress (Cornelious and Sneller, 2002).

Estimations of genetic variation in soybean within and among China, South Korea, and Japan accessions reveled by RAPD markers indicated that the mean genetic distance within China is more extensive than that within Japan or South Korea. These kinds of estimations are useful for establishing strategies for sampling and managing germplasm (Li and Nelson, 2001). A study of genetic diversity in annual *Glycine* showed that soybean has lost many rare sequence variants and has undergone numerous allele frequency changes throughout its history, and that the bottleneck with the most impact was domestication (Hyten et al., 2006).

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CHAPTER II

Genetic Diversity of Soybean Germplasm Resistant to Soybean Mosaic Virus

Abstract

Soybean Mosaic Virus (SMV) is a prevalent viral pathogen transmitted by aphids and via seed, causing significant yield loss and quality reduction. There are seven SMV strain groups (G1 to G7) and three resistance loci (*Rsv1*, *Rsv3*, and *Rsv4*) reported in soybean. New sources of SMV resistance would be valuable for breeding cultivars with durable resistance to multiple strains. The objective of this research was to analyze genetic diversity of SMV resistant soybean germplasm at the molecular level. One hundred and fourteen soybean germplasm collected worldwide were previously identified as SMV resistant. Eighty genotypes were proposed to carry alleles at the *Rsv1* locus for resistance to G1; the other 34 genotypes were categorized as resistant to SMV G1 and/or G7 and may carry alleles at *Rsv1*, *Rsv4*, or a combination of both. DNA genotypes were screened with 98 simple sequence repeat (SSR) markers, generating 298 fragments with an average of 2.6 bands per marker for each one of the genotypes. Marker diversity indexes ranged from 0.14 to 0.78 with an average of 0.51, and genetic similarity among all genotypes evaluated ranged from 0.37 to 0.95 with an average of 0.66. Similar results were observed for genotypes resistant to only G1 vs. genotypes with resistance to G1and/or G7 strains, that is genotypes resistant to G1 are more similar among them than those resistant to G1and/or G7 strains. Cluster analysis with UPGMA dendrogram grouped the 80 soybean genotypes resistant to G1, and the 34 genotypes resistant to G1 and/or G7 into four main clusters with a 0.58 coefficient of similarity, most of them Asian germplasm lines. The SMV resistant germplasm used in this study showed adequate genetic diversity in the genome other than the regions for SMV resistance. This information on SMV-resistant germplasm will be helpful to breeders in selecting parents for crossing in their breeding programs where both SMV resistance and using diverse gene pools are objectives.

Introduction

Soybean [*Glycine max* (L.) Merr.], one of the most important oilseed crops around the world, can be strongly affected by diverse pathogens, including Soybean Mosaic Virus (SMV), which affects seed quality, and decreases yield. SMV is transmitted in three modes, mechanical, by seed from one generation to the next one, and by aphids. There are seven strain groups based on the classification of SMV virulence (G1-G7), G1 the least and G7 the most virulent strains (Cho and Goodman, 1979). Individual plant reactions to the different strains are classified as resistant (R, symptomless), necrotic (N, systemic necrosis), or susceptible (S, mosaic). The SMV resistance is controlled by single dominant genes, and there are three independent loci reported *Rsv1*, *Rsv3*, and *Rsv4* (Buzzell and Tu, 1984; Gunduz et al., 2004; Hayes et al., 2000). These loci are at the linkage groups (LG) F, B, and D1b, respectively, in the soybean molecular map (Song et al., 2004). The *Rsv1* allele provides resistance to G1-G3 strains and susceptibility to G5-G7; and nine alleles are well dentified (Table 1). Genotypes reported having the *Rsv3* allele provides resistance to the highest strains (G4-G5) but susceptibility to lowest strains (G1-G4). The *Rsv4* gene confers resistance to the highest SMV strains, but only few genotypes have been identified.

Genetic resistance is preferred for the control of viral diseases because is practical, economic, and effective. This method often fails because resistance-breaking pathogen genotypes increase in frequency (Garcia-Arenal and McDonald, 2003), therefore it is important to continuously identify sources of resistance to the SMV virus. Zheng et al. (2005) studied 212 diverse soybean genotypes on the basis of their differential reactions to SMV strains, and identified 116 genotypes resistant to strains G1, G7, or to both strains; they determined that most of the genotypes carry the *Rsv1* allele, others the *Rsv3* or the *Rsv4*, and some a combination of two alleles. Research was conducted to screen 127 genotypes with six SMV strains, the results

showed the presence of alleles at *Rsv1* locus in 84 genotypes and new alleles at the three SMV loci were identify in 23 genotypes (Zheng et al., 2008). In another study, 253 soybean accession collected from 26 countries were screened with SMV strains G1-G7 but G4. Results showed that 75 accessions were resistant to some of the strains, and only 21 resistant to all strains utilized (Li et al., 2010).

Several researches have as principal purpose studied the soybean diversity at the molecular level to interpret soybean origin. Nevertheless there are not studies that analyze the diversity of soybean germplasm resistant to SMV. The objective of the present research is to assess at the molecular level genetic diversity of 114 soybean genotypes characterized in a previous study as resistant to SMV on basis of their differential reactions to SMV-G1 and SMV-G7 strains.

The primary gene pool of soybean, which consists of *G. max* cultivars, land races, and the wild annual *G. soja*, is useful to plant breeders for selecting germplasm to develop a breeding program. The genus *Glycine* subgenus *Glycine* recognizes 22 wild perennial species that represent a reservoir of useful genes to improve the cultivated soybean (Palmer and Hymowitz, 2004). Vavilob documented that the center of original domesticated is where the greatest diversity of types occur for a particular crop, and that soybeans belongs to the Chinese center of origin (Hymowitz, 2004). The Chinese national collection of soybean landraces includes more than 23,000 soybean germplasm lines collected and preserved in the National Gene Bank at the Institute of Crop Science, Chinese Academy of Agricultural Sciences (Li et al., 2008).

Defining a center of origin is important for researchers in order to find the soybean gene pool. However, several studies have shown that domestication and founding events create genetic bottlenecks that decrease the genetic diversity and how improving crops reduces the

diversity (Hyten, et al., 2006). The results of an analysis of the genome-wide variation in soybean using 17 wild and 14 domesticated soybean genomes demonstrated that the allelic diversity in wild soybeans was higher than in cultivated soybeans across the entire genome, which probes a negative effect caused by a genetic bottleneck and/or influence by human selection in cultivated soybeans (Lam et al., 2010). In the U.S. the combination of few introduced plants and intensive plant breeding has narrowed the genetic diversity among North America elite soybean cultivars (Min et al., 2010); this also increases the vulnerability of the crop to changes in pathogen and pest populations (Brown-Guedira et al., 2000; Gizlice et al., 1994). With the relative high frequency of the SMV resistance in major ancestral lines, resistance to SMV may be more common than expected (Wang et al., 2005). Even though, the potential of soybean breeding is enormous because a small fraction of the existing accessions in germplasm collections contribute to the genetic base of the present cultivars (Mulato et al., 2010).

Germplasm variability information is necessary to select the breeding strategies, to facilitate the introgression of genes of interest into commercial cultivars, to understand the evolutionary relations among accessions, to better sample germplasm diversity and to increase conservation efficiency; consequently, genetic improvement is maximized (Mulato et al., 2010; Thompson et al., 1998).

Agronomic traits, pedigrees, geographic origins, and isozymes have all been used for assessments of soybean genetic diversity. However these generate limited data that are influenced by growing environment on agronomic trait evaluation which can interfere with the results and generate incomplete information in the documentation of pedigrees and origins of accession collections. Currently, those methods have been replaced by DNA marker analysis; this is an alternative method of estimating the diversity of genotypes that would serve as

candidates for parents in breeding programs (Burnham et al., 2002; Wang et al., 2006). The ability of molecular markers, as simple sequence reapet (SSR), is to distinguish among soybean genotypes with agronomic merit than could be transfer of favorable alleles from soybean accessions into elite soybean cultivars (Narvel et al., 2000). Monitoring the genetic variability within the gene pool of elite breeding material could make crop improvement more efficient by the directed accumulation of favored alleles thus decreasing the amount to be screened (Singh et al., 2010).

According with An et al. (2009), an ideal molecular marker should be rich in polymorphism, easy to work with, robustness, and cost effectiveness, therefore researchers have prioritize the use of SSRs. SSR or microsatellite markers are tandem repeatet of a motif of two or three base of pairs that can be used to reveal a series of several alleles of each locus studied. The alleles detected are co-dominant, and then two homologous alleles can be observed in heterozygous individuals (Grivet and Noyer, 2003). Currently, an extensive development of SSR markers has been made over the set of 20 pairs of chromosome of the soybean genome which facilitates molecular screening (Song et al., 2004).

Through several studies it has been probe the efficiency of genetic differentiation among soybean accessions at the molecular level, Doldi et al. (1997) analyzed the genetic diversity of 18 early maturity soybean genotypes using random amplified polymorphic DNA (RAPD) and SSR markers, and concluded that SSRs present more heterogeneity and polymorphism and that data combined with RAPD give very good agreement information. Another study was conducted to identify genetic diversity of 1000 soybean accessions for photoperiod insensitivity using SSRs and amplified fragment length polymorphism (AFLPs) markers; and the authors determined that

identifying genetically diverse parents based for desirable traits based on molecular markers would be a good approach for the production of desirable progeny (Singh et al., 2010).
Since most of the SMV resistant ancestors of the North America public soybean cultivars are CNS, Ogden, Lincoln, Mandarin, and Peking (Wang et al., 2005) and reactions to SMV are not commonly reported at the cultivar registrations, knowing the genetic diversity of soybean resistant to SMV can be useful to breeders for helping to select parents with specific desirable traits and some level of resistance to SMV. This study will provide information the relatedness of the soybean germplasm resistant to SMV, and how these germplasm may be used.

Materials and Methods

Plant Materials

One hundred and fourteen soybean genotypes resistant to SMV were selected from a total of 212 soybean genotypes previously characterized for reactions to SMV G1 and G7 (Zheng et al., 2005). Among the 114 genotypes selected, 80 were proposed to carry alleles at the *Rsv1* locus for resistance to G1 (Table 3); the other 34 genotypes were resistant to strains G1 and G7, or only to G7, and presumably carrying *Rsv4*, *Rsv1-r*, or *Rsv1-h* and some a combination of the two resistant genes *Rsv1Rsv3*, *Rsv1Rsv4*, or *Rsv3Rsv4* (Table 4). The 114 soybean genotypes originated from different countries were provided by the USDA Soybean Germplasm Collection, USDA-ARS, at the University of Illinois, and from Virginia Polytechnic Institute and the State University.

These 114 genotypes were grown in the Rosen Alternative Pest Control Greenhouse at Fayetteville, AR greenhouse during spring 2007. Five to ten seeds of each genotype were planted in a 3.5 inch plastic pot containing Redi-earth commercial soil mix (Sun Gro Horticulture Canada, Ltd). Temperature of the greenhouse was maintained between 20-25°C with a 14 hr

photoperiod. At V3 stage (Fehr and Caviness, 1974), young trifoliolate leaves were collected from plants of each genotype for DNA extraction. Leaf samples were kept in labeled plastic bags and stored at -80°C. Subsequently, frozen leaves were ground with liquid nitrogen to powder, placed into 2.0 uL micro tubes, and kept at -80C until DNA extraction. The extractions were performed using the CTAB method (Kato and Palmer, 2004).

Molecular Analysis

To analyze the genetic diversity of 114 soybean genotypes resistant to SMV, 149 simple sequence repeat markers (SSR) were selected from the genetic linkage map of the soybean (Song et al., 2004). A criterion of inter-marker genetic distance of approximately 15 cM was considered to have an adequate coverage of each of the 20 soybean LGs (20 chromosomes) (Table 2). Ninety eight out 148 makers were informative and used in the diversity analysis.

PCR reaction of DNA from each genotype was performed for each of the polymorphic markers. The PCR reaction mixture had a volume of 15.0 µL consisting of 3.0 µL of 5x Green GoTaq flexi buffer (Promega Corporation), 0.9 µL of $MgCl_2$, 1.0 µL of dNTP, 7.6 µL autoclaved filtered water, 0.2 µL GoTaq DNA polymerase, 1.0 µL of primer (1 µM), and 1.3 µM of template DNA. Microplates of 96 samples containing the reaction mixture were vortexed for 3 s to mix well the reaction solution. The microplate was collocated to ICycler Thermal Cycler (Bio-Rad Laboratories Inc., CA) with a program of 94°C for 4 min for preheat, 33 cycles of 25 s at 94°C for denaturation, 25 s at 47°C for annealing, 25 s at 68°C for extensions, and final cycle of 5 min at 72°C.

The PCR products were separated on 2-5% agarose gels (Agarose 3:1, Amresco Inc., Solon, OH), containing 0.50 µg/mL ethidium bromide, in 1x TBE buffer (0.089M Tris base, 0.089M boric acid, and 0.002M EDTA). The percentage of agarose used for preparing the gels

varied and was adjusted o control band separation. The DNA banding patterns were photographed using UV light.

DNA amplifications for each informative marker were scored as 1 for presence and 0 for absence of the marker allele. For each SSR marker, number of alleles was quantified and Shannon diversity index was calculated (H). The H score of each marker was estimated based on $H = - \Sigma Pi2 \ln (Pi)$, where *Pi* was the frequency of the ith allele. Genetic similarity (S) between pairs of genotypes were calculated using Dice similarity coefficient, and S matrix were used for unweighthed pair-group method using arithmetic averages (UPGMA) to generate cluster tree using NTSYSpc 2.1 software (Rohlf, 1992). Genetic distance was calculated according to [GD = (1-Sij)1/2], where GD = genetic distance (Zhang et al., 2010).

Results and Discussion

Results from the molecular analysis showed that 98 out of 148 SSR markers detected DNA polymorphism among genotypes generating 298 fragments with an average of 2.6 bands per marker (ranging 2 to 4) for each one of the genotypes. The number allele per marker locus in this study was lower than what was observed in other studies (Shi et al., 2010; Zhang et al., 2010). This difference could be due to the fact that the germplasm used in the present study was selected strictly for SMV resistance and therefore they tend to have a narrow genetic base. These data could also imply that breeders might have shared and used similar parental materials in their breeding programs, resulting in lines with less genetic diversity.

When the 114 genotypes were pairwise compared for each marker allele, results showed that marker diversity indices (H) ranged from 0.14 to 0.78 with an average of 0.51 (Table 2). Again, the diversity was relatively lower in our study than that reported in other studies (Shi et al., 2010; Zhang et al., 2010). When genotypes were compared based on resistance to specific

strains, diversity indices ranged from 0.22 to 0.74 with an average of 0.42 for the group with resistance to G1 only, and from 0.18 to 0.72 with an average of 0.45 for group with resistance to G1 and/or G7 strain. This is expected because of genetic similarity within each group containing similar genes and/or of a small population in each group compared to the entire collection in this study. Likewise, when genetic similarity (S) was compared, the similarity coefficient ranged from 0.37 to 0.95 with an average of 0.66 for all the genotypes evaluated, from 0.37 to 0.94 with an average of 0.65 for the genotypes with resistance to G1 strain, and from 0.33 to 0.76 with an average of 0.54 for genotypes with resistance to G1 and/or G7.

Both diversity index analysis and genetic similarity showed similar results for genotypes resistant to only G1 vs. genotypes with resistance to G1and/or G7 strains, that is genotypes resistant to G1 are more similar among them than those resistant to G1and/or G7 strains. The resistance to G1 strain is mostly controlled by *Rsv1* alleles, which are abundant and common in the SMV resistant germplasm and most of them were originated in Asia (Table 3, Zheng et al., 2008). It is reasonable to assume that germplasm with *Rsv1* alleles were widely distributed and used as crossing parents in different breeding programs. It is also possible that the germplasm with *Rsv1* alleles share common ancestors, resulting in high genetic similarity. In contrast, genotypes carrying *Rsv3*, *Rsv4* alleles showed less genetic similarity, and most them were from the orient where soybean was originated and domesticated. The few lines with *Rsv3* or *Rsv4* found in the U.S. were all derived from the resistance source from Asia.

Cluster analysis with UPGMA dendrogram grouped the 80 soybean genotypes resistant to G1, and the 34 genotypes resistant to G1 and/or G7 into four main clusters with a 0.58 coefficient of similarity (Fig. 1a and b). Although germplasm appeared to distribute somewhat randomly across the four cluster groups according to their origins, germplasm with the same

origin in a cluster group tend to cluster together. This genetic similarity within a geographic region is expected because of breeding for local adaptations. For example, 58 genotypes from the U.S. were grouped in the cluster number III (Fig. 1b), most of which contain an *Rsv1* allele for resistance to the G1 strain. Another group showing high genetic similarity (0.90 coefficient of similarity) included L78-379, L81-4420, L84-2112, L93-3327, and L92-8580. In fact, these line are backcrossed isolines of Williams soybean with different *Rsv1* alleles (*Rsv1*, *Rsv1-y*, *Rsv1-t*, or *Rsv1Rsv4*) Zheng et al. (2008).

It has been reported that the Chinese cultivars CNS and Peking are the most common ancestors of the North America cultivars (Wang et al., 2005). In our analysis, CNS was included in cluster I with only five genotypes in the group. These genotypes are all old cultivars and all from Asia. Peking, the other common ancestral genotype, carrying *Rsv1Rsv4* alleles, was grouped in cluster II, has been widely used as a major source of resistance to soybean cyst nematode. The genotypes in cluster groups I and II appeared to be mostly old cultivars from Asia. They are still valuable germplasm in terms of providing genetic diversity and disease resistance. In contrast, some of the modern cultivars from Southern U.S. including Brim, Holladay, Young, Dillon, and Prolina, are grouped in the clusters III and IV. Based this cluster analysis, when genetic diversity is desired, a breeder should select parents from cluster I and II for crossing in their breeding program. However, some genotypes originated from Korea, Japan, China, and Russia were also present in all clusters and can be used as diverse parents with SMV resistance in the U.S. breeding programs.

Cluster analysis for genotypes resistant only to G1 strain is shown in Figure 2. Similarly, the most the ancient cultivars were in a distinct cluster from others, although most of them may contain the same/similar alleles for SMV resistance. Most of the North American cultivars group

together with a coefficient of 0.70, indicating their genetic similarities. It is worth noting that Brim soybean is grouped together with two Chinese cultivars Youbian 30 and Kefeng 1, and three were remotely distinctive from the old Asian cultivars Chang-uwal and Kyeong-du (Fig. 1 and 2). Genotypes with extreme diversity, particularly between old and new, would be valuable for genome sequence in gene discovery for genetic studies of domestication and evolution. The dendogram for genotypes resistant to G1 and/or G7 strains (Fig. 3) showed more diversity than genotypes with resistance to G1. Most of the genotypes contain *Rsv3*, or *Rsv4*, both, or eone combined with *Rsv1* allele. It is interesting to see Yuwoltae is most distinctive from PI 171434 (Fig. 3) as also shown in Fig.1, both could provide genetic diversity if desired. Overall, the SMV resistant germplasm used in this study showed adequate genetic diversity in the genome other the regions for SMV resistance, although the level of diversity was slightly lower than that observed in other studies. This research also showed the genetic similarities among all the germplasm via pairwise comparisons, which further grouped the genotypes studied into four clusters. Therefore, the genetic diversity information on SMV-resistant germplasm will be helpful to breeders in selecting parents for crossing in their breeding programs where both SMV resistance and using diverse gene pools are objectives.

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Table 1. Reaction of different soybean genotypes to seven Soybean Mosaic Virus (SMV) strains identified in U.S.

Genotype	Reaction to SMV strain†							Gene	References
	G1	G2	G3	G4	G5	G6	G7		
Essex/Lee68	S	S	S	S	S	S	S	*rsv*	Chen et al., 1991
PI 96983	R	R	R	R	R	R	N	*Rsv1*	Kiihl and Hartwing, 1979
Suweon 97	R	R	R	R	R	R	R	*Rsv1-h*	Chen et al., 2002
York	R	R	R	N	S	S	S	*Rsv1-y*	Roane et al., 1983; Chen et al., 1991
Raiden	R	R	R	R	N	N	R	*Rsv1-r*	Chen et al., 2001
Kwanggyo	R	R	R	R	N	N	N	*Rsv1-k*	Chen et al., 1991
Ogden	R	R	N	R	R	R	N	*Rsv1-t*	Chen et al., 1991
Marshall	R	N	N	R	R	N	N	*Rsv1-m*	Chen et al., 1991
PI 507389	N	N	S	S	N	N	S	*Rsv1-n*	Ma et al., 2003
LR1	R	R	R	R	N	N	R	*Rsv1-s*	Ma et al., 1995
L29	S	S	S	S	R	R	R	*Rsv3*	Buss et al., 1999
V94-5152	R	R	R	R	R	R	R	*Rsv4*	Buss et al., 1997
Hourei	R	R	R	R	R	R	R	*Rsv1Rsv3*	Gunduz et al., 2002
PI 486355	R	R	R	R	R	R	R	*Rsv1Rsv4*	Chen et al., 1993; Ma et al, 1995
Columbia	R	R	R	N	R	R	R	*Rsv3Rsv4*	Ma et al, 2002

† R, resistant (symptomless); N, necrotic (system necrosis); S, susceptible (mosaic).

Table 2. Ninety-eight simple sequence repeat (SSR) markers used for genetic diversity analysis of soybean resistant to SMV.

Marker	LG	Chromosome	Position (cM)	Allele No.	Marker Diversity Index (H)
Sat_356	A1	5	42.80	2	0.48
Satt385	A1	5	64.74	3	0.63
Satt390	A2	8	9.14	2	0.50
Satt424	A2	8	60.89	4	0.71
Satt599	A2	8	85.58	2	0.50
Satt437	A2	8	107.05	2	0.27
Satt409	A2	8	145.57	4	0.57
Stt378	A2	8	165.73	2	0.48
BE808308	B1	11	0.00	3	0.53
Sat_270	B1	11	21.99	3	0.78
Satt453	B1	11	123.96	3	0.65
Satt126	B2	14	27.63	3	0.14
Sct_034	B2	14	51.45	2	0.47
Satt272	B2	14	71.68	2	0.47
Satt063	B2	14	93.49	3	0.54
Satt687	B2	14	113.61	2	0.42
Sat_140	C1	4	41.43	3	0.64
Satt396	C1	4	24.11	3	0.50
Satt578	C1	4	65.08	2	0.25
Satt195	C1	4	84.81	3	0.61
Satt670	C1	4	85.37	2	0.27
Satt524	C1	4	120.12	2	0.47
Satt681	C2	6	3.15	2	0.50
Satt227	C2	6	26.65	3	0.51
Satt291	C2	6	45.76	2	0.49
Sat_153	C2	6	61.98	2	0.47
Satt286	C2	6	101.75	3	0.52
Satt307	C2	6	121.27	2	0.50
Satt371	C2	6	145.48	2	0.43
Sat_413	D1a	1	5.93	3	0.58
Sat_353	D1a	1	36.23	3	0.53
Satt179	D1a	1	56.20	4	0.69
Satt407	D1a	1	99.59	2	0.38
Sat_279	D1b	2	3.79	3	0.61
Satt634	D1b	2	46.62	3	0.63
Sat_069	D1b	2	102.60	3	0.56
Sataga002	D1b	2	126.45	2	0.29
Sctt008	D2	17	3.16	2	0.46

Continuation at next page

Table 2a. Continuation of Table 2

Marker	LG	Chromosome	Position (cM)	Allele No.	Marker Diversity Index (H)
Satt669	D2	17	67.71	2	0.48
Sat_338	D2	17	87.16	2	0.31
Satt186	D2	17	105.45	2	0.46
Sat_220	D2	17	128.73	3	0.52
Satt575	E	15	3.30	3	0.55
Satt720	E	15	20.80	2	0.50
Satt699	E	15	41.24	2	0.46
Satt685	E	15	56.70	3	0.42
Sat_390	F	17	1.79	3	0.60
Satt423	F	17	20.56	3	0.59
Sat_309	F	13	41.47	3	0.58
Sat_297	F	13	59.60	3	0.67
Sat_229	F	13	62.79	3	0.62
Satt114	F	13	63.69	3	0.63
Sat_234	F	13	66.55	3	0.64
Sat_317	F	13	72.97	2	0.48
Satt362	F	13	82.83	2	0.49
Satt522	F	13	119.19	2	0.50
Satt275	G	18	2.20	2	0.47
Satt610	G	18	10.92	3	0.62
Satt356	G	18	12.18	2	0.50
Satt235	G	18	21.89	2	0.36
Sat_308	G	18	43.09	2	0.33
Satt505	G	18	63.00	3	0.48
Satt612	G	18	80.38	2	0.50
Satt635	H	12	4.88	2	0.50
Satt568	H	12	27.64	2	0.46
Satt442	H	12	46.95	3	0.62
Satt142	H	12	86.49	2	0.49
Satt317	H	12	89.52	2	0.50
Satt434	H	12	105.74	2	0.49
Satt239	I	20	36.94	2	0.48
Sat_268	I	20	55.10	3	0.62
Sat_155	I	20	98.06	4	0.62
Satt249	J	16	11.74	2	0.41
Sat_366	J	16	52.84	2	0.50
Sat_393	J	16	90.33	3	0.62
Satt539	K	9	1.80	2	0.49
Satt273	K	9	56.62	2	0.50
Sat_293	K	9	99.10	2	0.49

Continuation next page

Table 2b. Continuation of Table 2a

Marker	LG	Chromosome	Position (cM)	Allele No.	Marker Diversity Index (H)
Satt588	K	9	117.02	3	0.48
Satt723	L	19	1.07	2	0.31
Sat_099	L	19	78.23	3	0.64
Satt229	L	19	93.89	3	0.59
Satt373	L	19	107.24	3	0.64
Sat_245	L	19	115.07	4	0.66
Sat_316	M	7	21.00	3	0.45
Satt435	M	7	38.94	3	0.63
Satt626	M	7	58.60	2	0.36
Satt680	M	7	77.19	3	0.59
Satt551	M	7	95.45	3	0.62
Satt346	M	7	112.79	2	0.47
Satt336	M	7	133.83	3	0.53
Sct_195	N	3	2.44	2	0.60
Satt152	N	3	22.67	2	0.33
Satt347	O	10	42.29	2	0.50
Sat_282	O	10	63.81	3	0.59
Satt477	O	10	82.09	3	0.62
Satt592	O	10	100.38	3	0.60
Sat_190	O	10	129.80	3	0.65
Mean				**2.5**	**0.51**

Table 3. Soybean genotypes with resistance phenotype to SMV G1 and postulated to carry alleles at *Rsv1* locus.

Possible resistant gene†	Source	Genotype		
Rsv1-y	Korea	Chang-uwal	KLS 743-1-2	PI 399022
		Chankon	Kyeong-du	PI 399091
		KAERI 540-4	Kyongsang Pukdo	PI 96257
		KAERI 543-3	ORD 8113	Yuwoltae B
		KAERI-GNT 361-2-2	PI 339999	Yuwoltae C
		KAERI-GNT 390-8	PI 398289	Yuwoltae D
		KAERI-GNT 681-1-9	PI 398877	Yuwoltae E
	Japan	Jitsuka	Nooki 1	Kosuzu
		Fukuyutaka	Shibahara Mame	
	China	PI 90401	Qi Huang 1 B	Xu dou 1
		Qi Huang 1 A	Qi Huang 1 C	
	Russia	Mocinave 7		
	U.S.	Brim	Dillon	Ripley
		Calhoun	Doles	Toano
		Clifford	Hatwing	York
		Cook	L85-2308	Young
		Corsica	Musen	
		Davis	Prolina	
Rsv1-n *Rsv1*	Japan	PI 507389		
Rsv1-t *Rsv1-m*	China	PI 61944		
Rsv1-k	Japan	Tousan 122	PI 181555	Tousan 26
		Ani 31	PI 181557	Tousan 58
		Hakuho 1	Sakyuu Ki Mam	Tousan 65
		Iwate No. 1	Shakkin-nashi	Xu dou 2
		Iwate wase kurome	Shimoda Shitachi	
		Nohrin No. 3	Shin 4	
		PI 181550	Tokishi	
	China	Ching Tao 21	Ke feng 1	
Rsv1 *Rsv1-t*	U.S.	L78-379	L78-379	Mercury
		Holladay	L81-4420	Pace
		Hood	L84-2112	Saturn
		Johnston	L93-3327	
Rsv1-k *Rsv1*	Korea	PI 96983	Kwangyo	

† Not specific alleles have been assigned.

Table 4. Soybean genotypes with resistance phenotype to SMV G1 and/or G7 and controlled by *Rsv1-r*, *Rsv1-h*, *Rsv3*, or *Rsv4* genes.

Possible resistant gene†	Source	Genotype		
Rsv1-r	Korea	Chuzu	KAERI-GNT390-4-3	Suweon 97
Rsv1-h		KAERI-GNT 220-7-5	PI 438307	Yuwoltae
Rsv4		Chuzu	KAERI-GNT390-4-3	Suweon 97
Rsv1Rsv3				
Rsv1Rsv4	Japan	PI 181554	Miyagi Shirome	Tohoku No. 1
		Azeminori	Okushireme	Tousan 140
		Enrei	Raiden	Tsuronoko
		Houjaku Kuwazu	Suzuyutaka	
		Hourei	Tanrei	
	China	PI 171434	Da bai ma	Peking
		Columbia	Jindou 1	
	U.S.	Beeson	L92-8580	Virginia
		L88-8431	V94-5152	
Rsv3	China	CNS		
	Canada	Harosoy		
	US	Bryan	Cornell	Hardee

† Not specific alleles have been assigned.

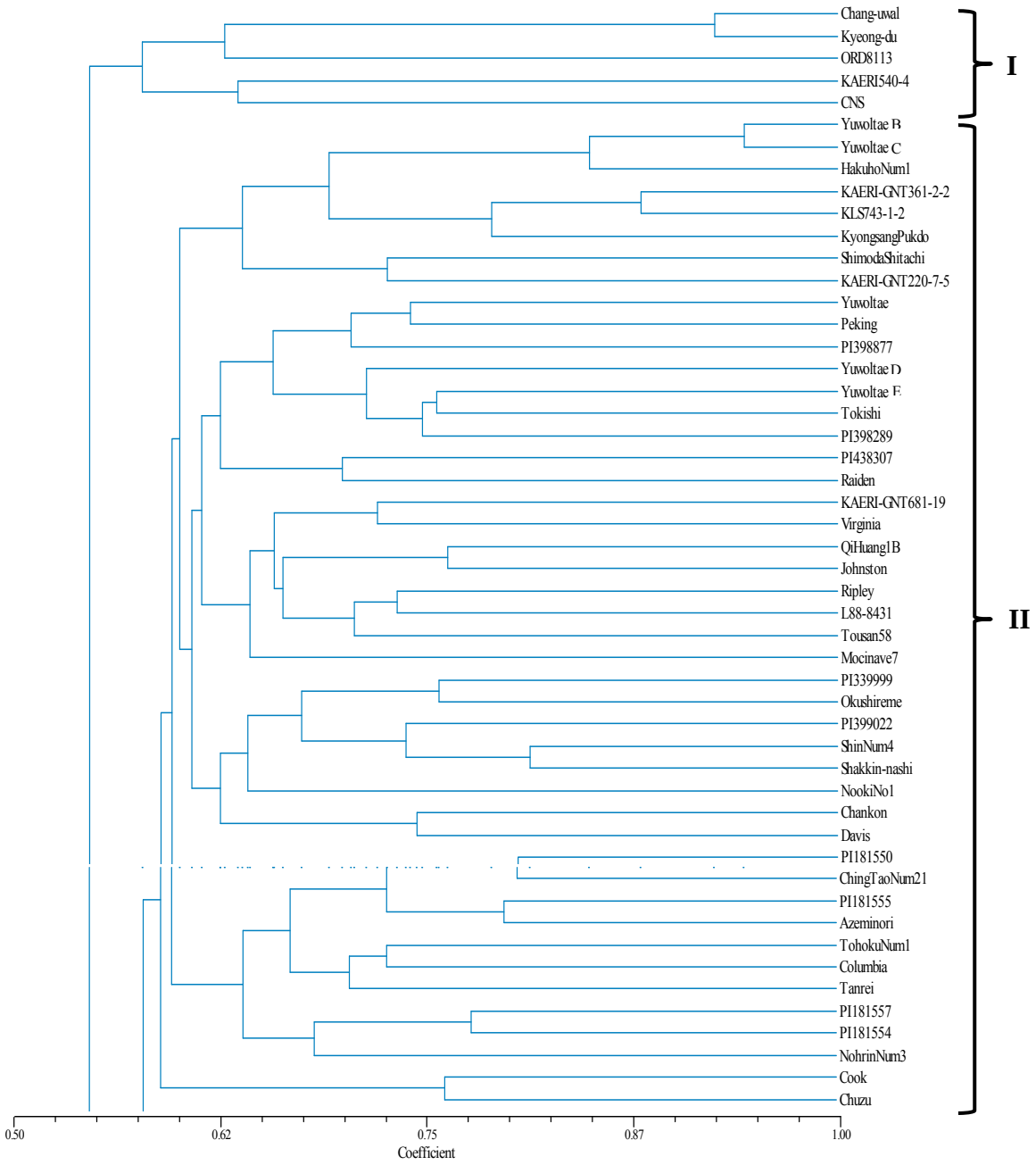

Figure 1a. Dendogram of 114 soybean genotypes resistant to SMV clusters I and II, by Unweighted Pair-Group Method with Arithmetic average (UPGMA).

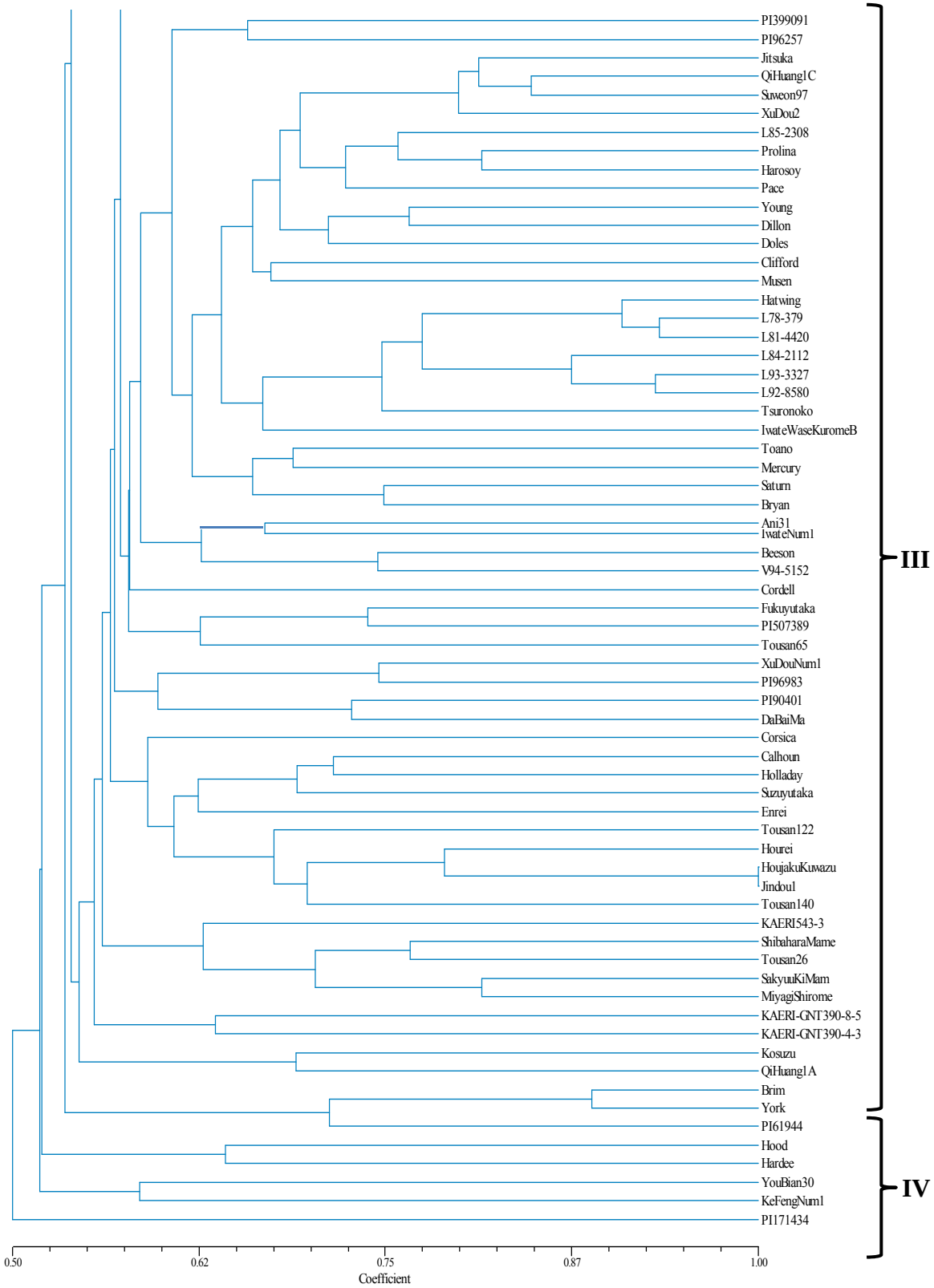

Figure 1b. Dendogram of 114 soybean genotypes resistant to SMV clusters III and IV, by Unweighted Pai-Group Method with Arithmetic average (UPGMA).

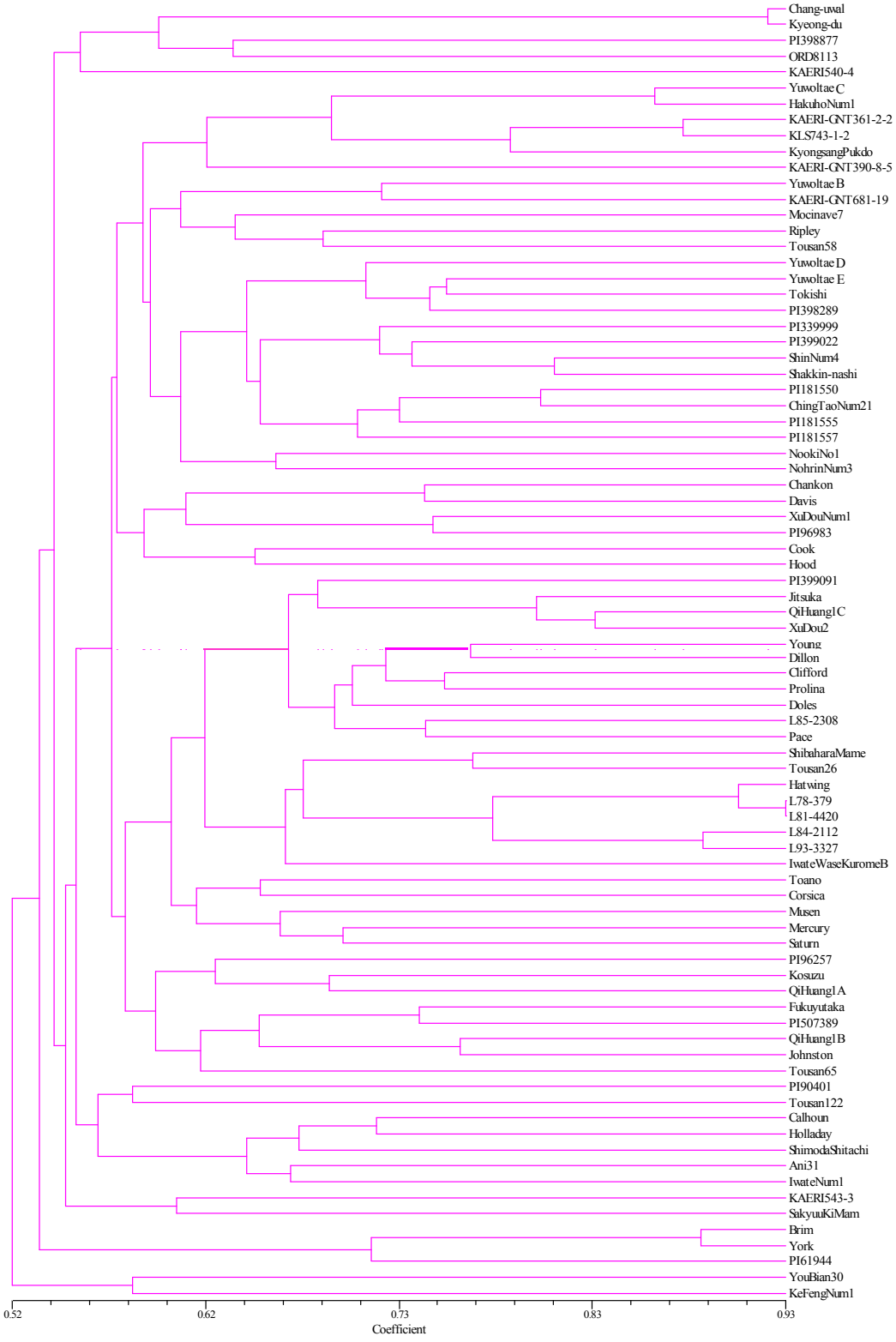

Figure 2. Dendogram of 80 soybean genotypes resistant or necrotic to SMV-G1, by Unweighted Pair-Group Method with Arithmetic average (UPGMA).

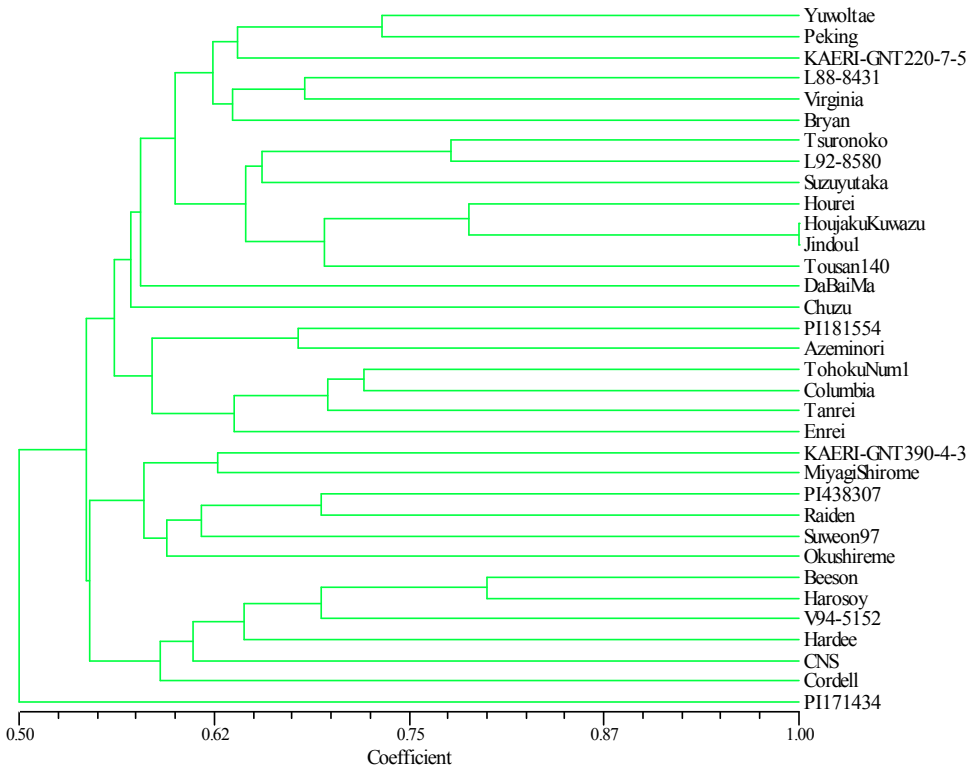

Figure 3. Dendogram of 34 soybean genotypes resistant or necrotic to SMV-G1 and/or G7 by Unweighted Pair-Group Method with Arithmetic average (UPGMA).

CHAPTER III

Pyramiding of Three Genes for SMV Resistance in Soybean Using Molecular Markers

Abstract

Soybean [*Glycine max* (L.)] is one of the leading oilseed crops worldwide and can be affected by different pathogens such as Soybean Mosaic Virus (SMV). SMV is transmitted by aphids and via seed, causing significant yield loss and quality reduction. There are seven SMV strain groups (G1 to G7) and three resistance loci (*Rsv1*, *Rsv3*, and *Rsv4*) reported in soybean. Soybean sources with one or two SMV resistant genes had been already reported, but there are not sources that may carry three genes of resistance for durable resistance to multiple strains. Breeding methodologies and marker assisted selection (MAS) facilitates pyramiding resistance genes to diseases as SMV. A cross between genotypes PI 96983 and Columbia carrying the SMV resistance genes *Rsv1* and *Rsv3Rsv4* was realized to develop a F_2 population (GP-134) and generation advance for $F_{2:3}$ lines for this gene-pyramiding research. SSR marker analysis data from the closest markers to each *Rsv* loci on each F_2 plant were used to predict the F_2 genotype according to a proposed genetic model based on segregation of three independent genes (*Rsv1*, *Rsv3*, and *Rsv4*). Chi-square test showed good fit overall based on 27 classes. Same result was observed when the 27 classes were pooled according to the number of resistance genes. Using this model, it was possible to detect lines with one, two, three or zero SMV resistance genes in the GP-134 F_2 population. The F_2 genotypes were verified by the phenotypes of $F_{2:3}$ lines when inoculated with G1 and G7. Using SSR markers closely linked to the three resistance loci make it possible to differentiate individual plants carrying specific genes. This research served as a proof-of-concept for marker assisted selection (MAS) in a practical breeding program in an attempt to select for specific genes and/or for gene pyramiding.

Introduction

Soybean [*Glycine max* (L.) Merr.], one of the most important oilseed crops around the world, can be strongly affected by diverse pathogens as Soybean Mosaic Virus (SMV) which decline seed quality, and decreases yield. SMV is transmitted in three modes, mechanical, by seed from one generation to the next one, and by aphids. The SMV resistance is controlled by single dominant genes at three independent loci reported *Rsv1*, *Rsv3*, and *Rsv4* (Buzzell and Tu, 1984; Gunduz et al., 2002). The *Rsv1* gene was identified on the soybean linkage group (LG) F (chromosome 13) in a F_2 population constructed from a cross between PI 96983 x Lee 68 (Yu et al., 1994). The gene *Rsv3* was mapped on LG B2 (chromosome 14) based on the data collected from two F_2 populations, L29 (*Rsv3*) x Lee 68 (*rsv3*) and Tousan 140 (*Rsv3*) x Lee68 (*rsv3*) (Jeong et al., 2002). Moreover, the *Rvs4* gene was localized on the LG D1b (chromosome 2) on a family derived from the cross between the resistant line LR2 (*Rsv4*) and the susceptible line Lee68 (*rsv4*) (Hayes et al., 2000).

There are seven strain groups based on the classification of SMV virulence (G1-G7), G1 the least and G7 the most virulent strains (Cho and Goodman, 1979). Different resistance genes often confer resistance to different SMV strains, the *Rsv1* allele provides resistance to G1-G3 strains and susceptibility to G5-G7; and nine alleles are well identified. Genotypes reported having the *Rsv3* allele provides resistance to the highest strains (G4-G5) but susceptibility to lowest strains (G1-G4). The *Rsv4* gene confers resistance to the highest SMV strains, but only few genotypes are identified. Rare are the genotypes having two alleles for SMV resistance, and there are not genotypes reported to carry the presence of three. Therefore, it is important to develop soybean lines carrying three SMV resistance alleles for having resistance to all strains of this disease; this resistance will be more durable and difficult to break.

SMV resistance in soybean is reported in the genotypes PI 96983, York, Marshall, Raiden, Kwanggyo, Ogden, Suweon 97, LR-1, and PI 507389 carry the alleles *Rsv1*, *Rsv1-y*, *Rsv1-m*, *Rsv1-r*, *Rsv1-k*, *Rsv1-t*, *Rsv1-h*, *Rsv1-s*, and *Rsv1-n*, respectively (Chen and Choi, 2007) (Table 1). Alleles at the *Rsv3* locus were identified in cultivars L29, OX 686, and OX 670 (Buss et al., 1999; Buzzell and Tu, 1984; Ma et al., 2002); and alleles at the *Rsv4* locus on genotypes V94-5152 and PI 88788 (Buss et al., 1997; Gunduz et al., 2004). Few genotypes are reported to carry two alleles at different locus PI 486355 (*Rsv1Rsv4*), Columbia (*Rsv3Rsv4*), Zao18 (*Rsv1Rsv3*), and Tousan 140 and Hourei (both with *Rsv1Rsv3*) (Chen et al., 1993; Gunduz et al., 2002; Liao et al., 2002; Ma et al., 2002); and there are not cultivars reported to have three alleles at the SMV resistance loci.

Through years, breeders have been trying to develop single soybean lines for improving cultivars with durable resistance to multiple strains of SMV which could be possible with techniques as gene pyramiding (Kumar and Nayak, 2010). Phenotyping soybean lines resistant to all SMV strains is challenging because genetic actions as epistasis; however, genotyping at the molecular level makes possible to pyramid the SMV alleles in a soybean line and to identify individuals that may carry one, two, or three alleles is feasible. According to Ye and Smith (2010) the objectives of gene pyramiding include: 1) enhancing trait performance by combining two or more complementary genes; 2) remedying deficits by introgressing genes from others sources; 3) increasing the durability of disease tolerance and/or disease resistance; and 4) broadening the genetic basis of released cultivars. The goal of gene pyramiding is to obtain near-homozygous breeding lines that are fully homozygous for the desirable alleles of the target genes using the minimum number of generations of selections and the lowest genotyping and phenotyping cost (Ye and Smith, 2008). Conventional gene pyramiding may encounter

difficulties as screening protocols that require extensive artificial disease inoculation with several races of the pathogen due to race specificity of many of these genes after each cycle of crossing, an effective selection method of differentiating races, it could be expensive, and it also may have epistatic effects. Therefore, the availability of PCR-based and tightly linked molecular markers has facilitated pyramiding through MAS (Saghai Maroof et al. 2008). Identification by host reaction to SMV strains G1-G7 is no distinguished if there is a combination of two or three SMV genes in a genotype, therefore the MAS approach is useful to develop soybean lines with high levels of resistance.

MAS approach use molecular markers as a primordial tool, and due to their usefulness in characterizing and manipulating genetic loci responsible for monogenic and polygenic traits, these are valuable tools for crop improvement. Markers linked to resistance genes can obviate the need of resistance testing to identify resistant individuals in early generations of breeding populations reducing the number of progeny maintained (Torres, 2010). Among different kind of markers, Simple Sequence Repeated (SSRs) or microsatellites are the most common markers used on gene pyramiding projects, specifically in soybean. These markers are tandem repeated of a motif of two or three base of pairs that can be used to reveal a series of several alleles of each locus studied; the alleles detected are codominant, and then two homologous alleles can be observed in heterozygous individuals (Grivet and Noyer, 2003). Their high level of allelic diversity increase the possibility of detecting polymorphism between parents of populations derived from the hybridization of adopted soybean genotypes (Iqbal and Lightfoot, 2004).

The gene pyramiding strategy has been developed in different species, and mostly used to improve disease and insect resistance. The genes *Bt* and *Xa21*, conferring resistance to lepidoptera insects and bacteria blight respectively, were pyramided into a cytoplasmic male

sterile *indica* line 'Minghuig 3' and results showed that pyramiding gene have a yield-stabilizing effect on the recipient line and its hybrids reflected satisfactory yield and resistance against insect damage and disease (Jiang et al., 2004). In the same species, genes *xa5*, *xa13* and *Xa21* conferring resistance to bacteria blight (BB) were pyramided trough MAS in the high-yielding but BB-susceptible rice cultivar PR106; results demonstrated increased and wide-spectrum resistance of populations in PR106 lines having pyramided genes to the six predominant races of *Xanthomonas orizae* pv. *orizae* (*Xoo*) pathogen isolates (Singh et al., 2001). The genes for greenbug resistance in *Triticum aestivum Gb2*, *Gb3*, and *Gb6* and their pyramided forms *Gb2*/*Gb3*, *Gb2*/*Gb6*, and *Gb3*/*Gb6* were tested for effectiveness against different biotypes; results reveals no additional resistance protection in plants with the pyramided resistance genes (Porter et al., 2000).

In soybean, Shi et al. (2008) developed soybean lines resistant to SMV derived from the cross between parents J05 x V94-5152, these $F_{2:3}$ lines were identified by MAS to carry the three resistance SMV alleles and showed resistance to G1 and G7 strains. In another study, Saghai Maroof et al. (2008) created an isogenic line of the susceptible cultivar Essex containing one, two, or three *Rsv* loci, and results showed isolines containing *Rsv1Rsv3*, *Rsv1Rsv4* and *Rsv1Rsv3Rsv4* confer resistance against all SMV strains but isolines of *Rsv3Rsv4* displayed a late susceptible reaction to the SMV strains. These studies prove the efficiency of the MAS for developing soybean lines trough gene pyramiding.

Inheritance and molecular documentation of SMV resistance in soybean facilitates the development of soybean germplasm with durable resistance to SMV. Therefore accompaniment of conventional breeding and MAS approaches can be used to find soybean lines carrying he three resistance genes to SMV. In addition, molecular markers help to select desirable traits in

base to marker linked to the gene of interest, making possible the genotyping in early generations as F_2.

The soybean PI 96983 was the first genotype reported to carry the R*sv1* allele, conferring resistance to strains G1-G6 and necrotic to strain G7 (Kiihl and Hartwing, 1979). Soybean cultivar Columbia was reported to carry *Rsv3Rsv4* alleles and confers resistance to G7 and late susceptibility to G1 (Ma et al., 2002). Through this research a cross between parents PI 96983 (*Rsv1*) and Columbia (*Rsv3Rsv4*), carry out different alleles for resistance to SMV, was made with the objective of incorporate the *Rsv1, Rsv3,* and *Rsv4* alleles having as possible result a genotype in homozygous condition to the three genes conferring resistance to the seven SMV strains.

Materials and Methods

Plant Materials

In order to develop a population for this gene-pyramiding research, a cross between genotypes PI 96983 and Columbia carrying the SMV resistance genes *Rsv1* and *Rsv3Rsv4*, respectively, was made during winter 2006-spring 2007 at the winter nursery in Costa Rica. Because there was a low percentage of success to obtaining hybrid F_1 seed, the same cross was made at the Arkansas Agricultural Experimental Station at Fayetteville, AR in summer 2008. The F_1 plants were grown and individually harvested in Fayetteville, AR in 2009.

During summer 2010, F_2 seeds were space-planted in a single row to develop a F_2 population designated as GP-134 (Gene Pyramiding *Rsv 1*, *3*, and *4*). At the V3 stage (Fehr and Caviness, 1979), plants were individually tagged and samples of the youngest trifoliate were taken for DNA extraction. The leaf samples were placed into individually labeled plastic bags, and freeze-dried for 48 to 72 h. Dry leaves were grounded with liquid nitrogen to powder,

placed into 2.0 uL micro tubes, and kept at -80°C until DNA extraction. Plants also were individually harvested at maturity, and the derived $F_{2:3}$ lines were used for SMV inoculation in the greenhouse.

DNA Extraction

The DNA extraction was done using the CTAB method (Kato and Palmer, 2004). Briefly, the extraction buffer [10% CTAB (hexadecyltrimethyl ammonium bromide), 1% 1-10 phenanthroline, 1*M* Tris-HCL, 05*M* EDTA pH 8.0 and 5*M* NaCl] plus 1000:1 of *β*-mercaptoethanol was preheated for 10 min at 62-65 °C, then 750 µL of this extraction buffer was added to each 2.0 µL sample tube. Sample tubes were mixed until a homogeneous solution was achieved, and incubated at 62-65 °C in a water bath, with shaking the samples every 15 min. After 1 h, tubes were left at room temperature for 10 min.

Subsequently, 750 µL chloroform:isoamyl alcohol (24:1) was added to each sample, and gently mixed. Sample tubes were placed in an orbital shaker for about 15 min then centrifuged at 4-10°C at 13,000 rpm for 15 min. Then, the upper layer was transferred to a new 1.5 µL tube.

To precipitate the DNA, 750 µL of 95% cold (-20°C) ethanol /15m*M* NH_4Ac was added, tubes were then gently inverted and incubated at room temperature for 20 min to wash the DNA for 30 min. The DNA was centrifuged, ethanol poured out, and the tube placed under the hood to dry for 1 hr.

The last step was to dissolve the DNA in 200 µL of 0.1x TE buffer [Tris (hydroxymethyl aminomethanol ethylenediamine tetracetic acid)] (pH 8.0). The samples were stored at -20°C until use, at which time DNA concentration of each sample was tested using a spectrophotometer.

SSR Analysis

In order to find polymorphism between parents, a total of 28 SSR markers were selected for the initial screen: nine on LG F (chromosome 13) for *Rsv1*, eight on B2 (chromosome 14) for *Rsv3* and eight on D1b (chromosome 2) for *Rsv4*. These markers covered 21.6, 21.8 and 15.0 cM of LG F, B2, and D1b, respectively (Table 2). Polymorphic markers between parents were used to screen the F_2 population from PI 96983 x Columbia.

PCR Procedure

Each PCR reaction mixture had a total volume of 15.0 µL consisting of 3.0 µL of 5x Green GoTaq flexi buffer (Promega Corporation), 0.9 µL of $MgCl_2$, 1.0 µL of dNTP, 7.6 µL autoclaved filtered water, 0.2 µL GoTaq DNA polymerase, 1.0 µL of primer (1 µM), and 1.3 µM of template DNA. Microplates of 96 samples containing the reaction mixture were vortexed for 3 s to mix well the reaction solution. The microplate should be collocated to ICycler Thermal Cycler (Bio-Rad Laboratories Inc., CA) with a program of 94°C for 4 min for preheat, 33 cycles of 25 s at 94°C for denaturation, 25 s at 47°C for annealing, 25 s at 68°C for extension, and a final extension 5 min at 72°C for.

Electrophoresis

After PCR, samples were loaded in 3 % agarose gels containing 1x TBE buffer [10 x TBE buffer (108 g Tris, 55 g boric acid, and 40 mL EDTA in 1L deionized distilled water)]. Sampler were run at 120 V/10 cm for 2 h, and visualized under UV light.

SMV Screening

Seventy $F_{2:3}$ lines from PI 96983 x Columbia were grown at the Rosen Alternative Pest Control Greenhouse at Fayetteville, AR during summer 2011 for reaction to SMV. Plants were in

8 inch plastic pots containing Redi-earth commercial soil mix (Sun Gro Horticulture Canada, Ltd). Temperature of the greenhouse was maintained between 20-25°C with a 14 hr photoperiod.

For each $F_{2:3}$ line, 12 to 20 plants were mechanically inoculated with G1 or G7, and classified into the three different reactions: resistant (R, symptomless), systemic necrotic (N, necrotic lesions and spots on both inoculated and non-inoculated leaflets, petioles, and stems), and susceptible (S, mosaic) (Ma, et al., 2003). Inoculations of G1 and G7 were conducted in separate greenhouses to avoid strain contamination. SMV strains were maintained in susceptible plants grown in each greenhouse.

Systemically infected leaves of soybean cultivars Essex (for G1) and/or Lee68 (for G7) were grounded in 0.05 M potassium phosphate buffer (pH 7.2) with mortar and pestle (1 g leaf tissue :10 ml buffer). Inoculum was applied with cheesecloth pads to both unifoliolate leaves (V1 stage) of each plant that had been previously dusted with 600-mesh carborundum. Inoculated soybean plants were monitored for the symptom expression on a regular basis for 3-4 weeks.

Data Analysis

For the SSR analysis a score designation A was given if the plant is homozygous dominant, B if it is homozygous recessive, and H if it is heterozygous for the locus of interest. A genetic model based on three *Rsv* independent genes segregation was proposed to detect the number of genes in GP-134 lines. For each marker locus, a Chi-square test was conducted to evaluate the goodness of fit to the expected 1:2:1 marker segregation ratio. In the overall analysis, each plant was assigned a combined numerical genotypic designation representing the presence of each gene. For example, a genotypic designation 1+0+4 is given to an individual if markers alleles for both *Rsv1* and *Rsv4* were detected. Then, all 27 genotypic classes based on the marker analysis were tested for goodness of fit to the segregation ratio expected from the

three independent genes (Table 4). The 27 genotypic classes were then grouped in to eight classes on the basis of the presence of number of resistance alleles regardless of their dominance conditions (Table 5) and Chi-square test was performed accordingly.

To confirm the predicted F_2 genotypes of GP-123 population by molecular markers, $F_{2:3}$ lines were inoculated with SMV G1 and G7. The $F_{2:3}$ lines were classified according to the reaction of individual plants into R, S, or segregating for R/S, and R/N/S. A marker genotype of an F_2 plant was confirmed if the corresponding $F_{2:3}$ line exhibited the SMV reaction expected for the specific locus (Table 6) among all 27 possible genotypic classes. Then the overall confirmation was done for the pooled data of eight genotypic classes based on number of resistance genes.

Results and Discussion

The GP-134 population consisting of 70 individuals derived from the cross between PI 96983 and Columbia was used to conduct molecular analysis for each of the three SMV resistance loci , *Rsv1* on LG F (chromosome 13), *Rsv3* on B2 (chromosome 14), and *Rsv4* on D1b (chromosome 2). For detecting the presence of *Rsv1*, *Rsv3*, and *Rsv4* in the GP-134 lines, nine SSR markers on LG F, eight on B2, and eight on D1b were selected to screen the parents and polymorphic markers were used to screen the pyramiding population (Table 2). Among the nine markers covering 21.6 cM region at *Rsv1*, five were polymorphic (Sat_234, Satt510, BACSOY_13_1133, Satt335, and Sat_375) and segregated with a good fit to the expected 1:2:1 ratio (Table 3). In the screening for *Rsv3*, five (Satt534, Sct_064, Sat_424, Satt726, and Sat_009) out of eight markers covering a 21.8 cM region at *Rsv3* on LG B2 were polymorphic between parents (Table 2); all five but one showed good fit to the 1:2:1 segregation ratio in the pyramiding population (Table 3). Five of eight SSR markers close to the *Rsv4* region were

polymorphic (Sat_211, Satt701, Sat_254, Satt296, and Satt542) (Table 2) and showed a good fit to the expected 1:2:1 ratio in the pyramiding population (Table 3).

Due to the inability of distinguishing progeny lines carrying more than one of three resistance genes based on SMV phenotypes, SSR marker data on each F_2 plant were used to predict the F_2 genotype according to a proposed genetic model based on segregation of three independent genes (*Rsv1*, *Rsv3*, and *Rsv4*) in the pyramiding population (Table 4). Using this model, it was possible to detect lines with one, two, three or zero SMV resistance genes in the GP-134 population. In this model, numerical genotype was assigned to lines with and without any of the three SMV resistance genes in either homozygous or heterozygous condition. Number one was assigned for the presence of *Rsv1*, three for *Rsv3*, four for *Rsv4*, and cero for non-presence of any of the three alleles (Table 4). For example, if the closest marker (Satt510) for *Rsv1* and Sct_064 for *Rsv3* amplified in an individual, but not Satt296 for *Rsv4*, this individual would be assigned a genotype of 1+3+0. With all the three maker data sets compiled, the genotypes of the GP-134 population were tested for good of fit to the three gene segregation ratio, and the Chi-square test showed a good fit overall ($X^2 = 21.771$ $p = 0.701$) (Table 4). This Chi-square test was conducted based on 27 possible genotype classes predicted from the three gene model. If these 27 genotypic classes were re-grouped into 8 pooled classes according to the number of resistance genes (*Rsv1+Rsv3+Rsv4*, *Rsv1+Rsv3*, *Rsv1+Rsv4, Rsv3+Rsv4*, *Rsv1*, *Rsv3*, *Rsv4*, *rsv1+rsv3+rsv4*) with each locus either in homozygous or heterozygous conditions, an excellent fit ($X^2 = 3.685$; $p = 0.8153$) was also obtained to a ratio of 27(all 3 genes) : 9 each of the two gene combinations : 3 each of the one gene type : 1 with all susceptible alleles (Table 5). These data further confirmed the frequency of allele combinations at the molecular level and the observed phenotypic ratio expected from the segregation of three genes in the pyramiding

population. No genotype of *rsv1rsv3rsv4* was detected using SSR markers and no progeny showed homogeneous susceptible reaction to SMV in this population. This could be attributed to the small population size used in this study. However, other seven classes with one, two, or three genes exhibited good fits to the expected ratios. In most cases, homozygous alleles were confirmed in genotypes with one, two, or three resistance genes (Fig. 1-3).

The F_2 genotypes predicted by the molecular analysis were verified by the phenotypes of $F_{2:3}$ lines when inoculated with SMV G1 and G7 (Table 6). Most of the F_2 genotypes (49 out 70) based on the maker allele classification were consistent with the $F_{2:3}$ phenotypes. There were some missing data points due to poor germination of some of the $F_{2:3}$ lines. However, the majority of the F_2 genotypic classes were consistent with their corresponding F_3 phenotypes, except for the group without any resistance genes and those only with the *Rsv4* gene (Table 6). Comparing the two sets of data from G1 and G7, we found more consistency between F_2 genotypic and F_3 phenotypic data with G7 inoculation (59 out of 63 cases) than with G1 inoculation (41 out of 55 cases) excluding the missing data (Tables 6 and 7). This is likely because resistance to G7 is conferred by two genes (*Rsv3* and *Rsv4*), and therefore more maker data were available to confirm the F_2 genotypes, given a relatively small population. Nevertheless, the occasional missing data point in each of the genotype classes did not appear to affect the marker/phenotype confirmation because other plants/lines in each genotypic category were used to draw the conclusion.

With a small population of each $F_{2:3}$ line screened, it would be difficult to distinguish between lines heterozygous and lines homozygous for a specific locus. It was anticipated that finding individuals with homozygous alleles for all markers at each locus would be extremely

difficult, if not possible, with a rather small population. Therefore, if the majority of the markers at a resistance locus were present in an individual or the closest marker to that locus present, the individual was assigned to the marker genotype with the specific resistance gene. Then, if most of the $F_{2:3}$ lines in each genotypic class exhibited the expected phenotype, we conclude that the F_2 phenotype was confirmed with the molecular data. Furthermore, when all 27 classes of possible genotypes were pooled on the basis of the number of genes present, either in the homozygous or heterozygous condition, six out of eight genotypic classes were confirmed by the SMV phenotypes. Evidently, the two or three gene combinations were more frequently confirmed than the single genes. Again, this is because the more marker data points were available to confirm the genotypes with multiple genes than the single genes. Overall, 27 out of 70 lines were identified to contain three gene combinations, although most of them were in the heterozygous state. These lines would be very valuable for selecting homozygous lines with all three resistance genes.

The single dominant gene in PI 96983 (*Rsv1*) confers resistance to G1 and necrosis in response to G7 (Kiihl and Hartwing, 1979), and Columbia contains two independent genes, *Rsv3* and *Rsv4*, conferring resistance to G1 and G7 strains (Ma et al., 2002). Therefore, it was expected that some of the progeny would carry all three genes, although in a very low frequency. However, it is not possible to identify such progenies using phenotypic reaction to SMV inoculation. Using SSR makers closely linked to the three resistance loci made it possible to differentiate individual plants carrying specific genes, even at a very early stage, such as F_2 generation in this study, thereby improving the efficiency of the breeding process. This research served as a proof-of-concept for marker assisted selection (MAS) in a practical breeding program in an attempt to select for specific genes and/or for gene pyramiding. The implication of this

research includes progeny lines with all three SMV resistance genes and genetic potential for durable resistance to multiple SMV strains.

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Table 1. Reaction of different soybean genotypes to seven Soybean Mosaic Virus (SMV) strains identified in U.S.

Genotype	Reaction to SMV strain†							Gene	References
	G1	G2	G3	G4	G5	G6	G7		
Essex/Lee68	S	S	S	S	S	S	S	*Rsv*	Chen et al., 1991
PI 96983‡	**R**	**R**	**R**	**R**	**R**	**R**	**N**	***Rsv1***	**Kiihl and Hartwing, 1979**
Suweon 97	R	R	R	R	R	R	R	*Rsv1-h*	Chen et al., 2002
York	R	R	R	N	S	S	S	*Rsv1-y*	Roane et al., 1983; Chen et al., 1991
Raiden	R	R	R	R	N	N	R	*Rsv1-r*	Chen et al., 2001
Kwanggyo	R	R	R	R	N	N	N	*Rsv1-k*	Chen et al., 1991
Ogden	R	R	N	R	R	R	N	*Rsv1-t*	Chen et al., 1991
Marshall	R	N	N	R	R	N	N	*Rsv1-m*	Chen et al., 1991
PI 507389	N	N	S	S	N	N	S	*Rsv1-n*	Ma et al., 2003
LR1	R	R	R	R	N	N	R	*Rsv1-s*	Ma et al., 1995
L29	S	S	S	S	R	R	R	*Rsv3*	Buss et al., 1999
V94-5152	R	R	R	R	R	R	R	*Rsv4*	Buss et al., 1997
Hourei	R	R	R	R	R	R	R	*Rsv1Rsv3*	Gunduz et al., 2002
PI 486355	R	R	R	R	R	R	R	*Rsv1Rsv4*	Chen et al., 1993; Ma et al, 1995
Columbia‡	**R**	**R**	**R**	**N**	**R**	**R**	**R**	***Rsv3Rsv4***	**Ma et al, 2002**

† R, resistant (symptomless); N, necrotic (system necrosis); S, susceptible (mosaic).
‡ Genotypes used for gene pyramiding analysis.

Table 2. Simple sequence repeat (SSR) markers used for gene-pyramiding of SMV resistance genes from PI 96983 (*Rsv1*) and Columbia (*Rsv3Rsv4*) soybean.

SSR Marker	Linkage Group (LG)	Position (cM)	Polymorphic†	SMV resistance locus	Reference‡
Sat_234	F	66.5	*	*Rsv1*	Song et al., 2004
Sat_154	F	68.9		*Rsv1*	Song et al., 2004
Satt510	F	71.4	*	*Rsv1*	Cregan et al., 1999
BACSOY_13_1133	F	72.0	*	*Rsv1*	N/A
Sct_033	F	74.1		*Rsv1*	Cregan et al., 1999
Satt335	F	77.7	*	*Rsv1*	Cregan et al., 1999
Sat334	F	78.1		*Rsv1*	Cregan et al., 1999
Sct_188	F	85.3		*Rsv1*	Cregan et al., 1999
Sat_375	F	88.1	*	*Rsv1*	Song et al., 2004
Sat_009	B2	78.7	*	*Rsv3*	N/A
Satt534	B2	87.6	*	*Rsv3*	Cregan et al., 1999
Sct_064	B2	89.3	*	*Rsv3*	Cregan et al., 1999
AW620774	B2	90.3		*Rsv3*	Song et al., 2004
Satt063	B2	93.5		*Rsv3*	Cregan et al., 1999
Satt560	B2	97.9		*Rsv3*	Cregan et al., 1999
Sat_424	B2	100.1	*	*Rsv3*	Song et al., 2004
Satt726	B2	100.5	*	*Rsv3*	Song et al., 2004
Sat_211	D1b	38.0	*	*Rsv4*	Song et al., 2004
Satt698	D1b	38.0		*Rsv4*	Song et al., 2004
Sat_173	D1b	38.6		*Rsv4*	Song et al., 2004
Satt701	D1b	40.0	*	*Rsv4*	N/A
Sat_254	D1b	46.9	*	*Rsv4*	Song et al., 2004
AI856415	D1b	50.1		*Rsv4*	Song et al., 2004
Satt296	D1b	52.6	*	*Rsv4*	Cregan et al., 1999
Satt542	D1b	53.0	*	*Rsv4*	Cregan et al., 1999

† * polymorphic between PI 96983 (*Rsv1*) and Columbia (*Rsv3Rsv4*), others were monomorphic.
‡ N/A, no available in published reference, but available in SoyBase (http://soybase.org/).

Table 3. Co-segregation of the *Rsv1*, *Rsv3*, and *Rsv4* locus and linked SSR markers on the LG F, B2, and D1b in the F_2 population from PI 96983 x Columbia.

Markers	Locus	Segregation of SSR marker alleles†				$X^2_{(1:2:1)}$	*P*
		A	H	B	Total		
Sat_234	*Rsv1*	24	29	19	72	3.42	0.181
Satt510	*Rsv1*	22	31	18	72	1.59	0.451
BC_13_1133	*Rsv1*	19	33	20	72	0.53	0.768
Satt335	*Rsv1*	22	31	19	72	1.64	0.441
Sat_375	*Rsv1*	13	37	16	66	1.24	0.537
Sat_009	*Rsv3*	16	46	10	72	6.56	0.038
Satt534	*Rsv3*	15	42	14	71	2.41	0.299
Sct_064	*Rsv3*	15	43	13	71	3.28	0.194
Sat_424	*Rsv3*	16	29	27	72	0.08	0.048
Satt726	*Rsv3*	18	32	22	72	1.33	0.513
Sat_211	*Rsv4*	12	34	17	63	1.19	0.551
Satt701	*Rsv4*	12	31	19	62	1.58	0.452
Sat_254	*Rsv4*	17	33	17	67	0.02	0.993
Satt296	*Rsv4*	20	36	15	71	0.72	0.698
Satt542	*Rsv4*	15	37	18	70	0.49	0.784

† No. of F_2 plants genotyped with SSR markers.

Table 4. Proposed genetic model for pyramiding three independent SMV resistance genes using SSR markers in the population GP-134 from PI 96983 x Columbia.

Possible Genotype†	SSR marker allele‡			Numerical Genotype	Frequency	
	Satt510 (*Rsv1*)	Sct_064 (*Rsv3*)	Satt296 (*Rsv4*)		Expected	Observed
R1R1 R3R3 R4R4	1	3	4	1+3+4	1.09 (1/64)	1
R1R1 R3R3 ***R4r4***	1	3	4	1+3+4	2.19 (2/64)	3
R1R1 R3R3 ***r4r4***	1	3	0	1+3+0	1.09 (1/64)	1
R1R1 ***R3r3 R4R4***	1	3	4	1+3+4	2.19 (2/64)	4
R1R1 R3r3 ***R4r4***	1	3	4	1+3+4	4.38 (4/64)	6
R1R1 R3r3 ***r4r4***	1	3	0	1+3+0	2.19 (2/64)	3
R1R1 ***r3r3 R4R4***	1	0	4	1+0+4	1.09 (1/64)	0
R1R1 r3r3 ***R4r4***	1	0	4	1+0+4	2.19 (2/64)	3
R1R1 r3r3 ***r4r4***	1	0	0	1+0+0	1.09 (1/64)	2
R1r1 R3R3 R4R4	1	3	4	1+3+4	2.19 (2/64)	1
R1r1 R3R3 ***R4r4***	1	3	4	1+3+4	4.38 (4/64)	2
R1r1 R3R3 ***r4r4***	1	3	0	1+3+0	2.19 (2/64)	1
R1r1 ***R3r3 R4R4***	1	3	4	1+3+4	4.38 (4/64)	3
R1r1 R3r3 ***R4r4***	1	3	4	1+3+4	8.75 (8/64)	7
R1r1 R3r3 ***r4r4***	1	3	0	1+3+0	4.38 (4/64)	7
R1r1 ***r3r3 R4R4***	1	0	4	1+0+4	2.19 (2/64)	2
R1r1 r3r3 ***R4r4***	1	0	4	1+0+4	4.38 (4/64)	7
R1r1 r3r3 ***r4r4***	1	0	0	1+0+0	2.19 (2/64)	0
r1r1 R3R3 R4R4	0	3	4	0+3+4	1.09 (1/64)	0
r1r1 R3R3 ***R4r4***	0	3	4	0+3+4	2.19 (2/64)	2
r1r1 R3R3 ***r4r4***	0	3	0	0+3+0	1.09 (1/64)	3
r1r1 ***R3r3 R4R4***	0	3	4	0+3+4	2.19 (2/64)	2
r1rsv1 R3r3 ***R4r4***	0	3	4	0+3+4	4.38 (4/64)	6
r1r1 R3r3 ***r4r4***	0	3	0	0+3+0	2.19 (2/64)	3
r1r1 ***r3r3 R4R4***	0	0	4	0+0+4	1.09 (1/64)	0
r1r1 r3r3 ***R4r4***	0	0	4	0+0+4	2.19 (2/64)	1
r1r1 r3r3 ***r4rsv4***	0	0	0	0+0+0	1.09 (1/64)	0
	$X^2 = 21.771$ df = 26 $p = 0.7011$					70

† *R1 = Rsv1*, *r1 = rsv1*, *R3 = Rsv3*, *r3 = rsv3*, *R4 = Rsv4*, *r4 = rsv4*; Non-bold symbols signify the same genotype as above. ‡ 1, presence of *Rsv1*; 3, presence of *Rsv3*; 4, presence of *Rsv4*.

Table 5. Pooled classification of F_2 plants based on number of SMV resistance genes segregating in the population GP-134 from PI 96983 x Columbia.

Classified Genotype	**Genes**	**Expt. Freq.**	**Obs. Freq.**
1+3+4	*Rsv1+Rsv3+Rsv4*	27	35
1+3+0	*Rsv1+Rsv3*	9	9
1+0+4	*Rsv1+Rsv4*	9	7
0+3+4	*Rsv3+Rsv4*	9	10
1+0+0	*Rsv1*	3	3
0+3+0	*Rsv3*	3	4
0+0+4	*Rsv4*	3	2
0+0+0	*rsv1+rsv3+rsv4*	1	0
		64	70

$X^2 = 3.685$ df = 7 $p = 0.8153$

Table 6. Comparison of F_2 marker genotype and corresponding $F_{2:3}$ line phenotype in response to SMV G1 and G7 of the gene pyramiding population from PI 96983 x Columbia.

Possible genotype†	No. F_2 plants‡	SMV Phenotype of Corresponding $F_{2:3}$ lines§															
		G1							G7								
		Expt.	Obs.						Expt.	Obs.							
R1R1 R3R3 R4R4	1	R	-						R	+							
R1R1 R3R3 ***R4r4***	3	R	**na**	**na**	+				R	+	**na**	+					
R1R1 R3R3 ***r4r4***	1	R	+						R	+							
R1R1 ***R3r3 R4R4***	4	R	-	**na**	+	-			R	+	+	+	+				
R1R1 R3r3 ***R4r4***	6	R	+	-	-	-	**na**	+	R/S	+	+	+	+	**na**	+		
R1R1 R3r3 ***r4r4***	3	R	**na**	-	+				R/S	**na**	+	+					
R1R1 ***r3r3 R4R4***	0	R	**na**						R	**na**							
R1R1 r3r3 ***R4r4***	3	R	**na**	+	+				R/S	+	+	+					
R1R1 r3r3 ***r4r4***	2	R	-	+					N	-	-						
R1r1 R3R3 R4R4	1	R	-						R	-							
R1r1 R3R3 ***R4r4***	2	R/N/S	+	+					R	+	+						
R1r1 R3R3 ***r4r4***	1	R/N/S	+						R	+							
R1r1 ***R3r3 R4R4***	3	R	+	**na**	**na**				R	+	-	+					
R1r1 R3r3 ***R4r4***	7	R/N/S	+	+	+	+	+	+	+	R/N/S	+	+	+	+	+	+	+
R1r1 R3r3 ***r4r4***	7	R/N/S	+	+	+	+	+	+	+	R/N/S	+	+	+	+	+	+	+
R1r1 ***r3r3 R4R4***	2	R	-	-					R	-	+						
R1r1 r3r3 ***R4r4***	7	R/N/S	+	+	+	+	+	+		R/N/S	+	+	+	+	+	+	+
R1r1 r3r3 ***r4r4***	0	R/N/S	**na**						R/N/S	**na**							
r1r1 R3R3 R4R4	0	R	**na**						R	**na**							
r1r1 R3R3 ***R4r4***	2	R/S	+	+					R	+	+						
r1r1 R3R3 ***r4r4***	3	S	-	-	**na**				R	+	+	+					
r1r1 ***R3r3 R4R4***	2	R	-	-					R	**na**	+						
r1rsv1 R3r3 ***R4r4***	6	R/S	+	+	+	**na**	**na**		R/S	+	+	+	+	+			
r1r1 R3r3 ***r4r4***	3	S	+	**na**	-				R/S	+	**na**	+					
r1r1 ***r3r3 R4R4***	0	R	**na**						R	**na**							
r1r1 r3r3 ***R4r4***	1	R/S	**na**						R/S	**na**							
r1r1 r3r3 ***r4rsv4***	0	S	**na**						S	**na**							

† *R1 = Rsv1*, *r1 = rsv1*, *R3 = Rsv3*, *r3 = rsv3*, *R4 = Rsv4*, *r4 = rsv4*; Non-bold symbols signify the same genotype as above.

‡ No. F_2 plants with SMV resistance alleles detected by SSR markers

§ Expt. = expected phenotype of $F_{2:3}$ lines in response to inoculation with SMV G1 or G7; Obs. = observed phenotype of $F_{2:3}$ lines in response to inoculation with SMV G1 or G7: +, lines consistent with expected phenotype and F_2 marker data; -, lines inconsistent with expected phenotype and F_2 marker data; na, data not available.

Table 7. Phenotypic confirmation of $F_{2:3}$ lines in response to SMV G1 and G7 in pyramiding of three resistance genes in a soybean population from PI 96983 x Columbia.

Classified Genotype	Genes	No. of $F_{2:3}$ lines†	No. of $F_{2:3}$ lines confirmed by inoculation with	
			G1	G7
1+3+4	*Rsv1+Rsv3+Rsv4*	27	15	24
1+3+0	*Rsv1+Rsv3*	12	11	12
1+0+4	*Rsv1+Rsv4*	12	8	10
0+3+4	*Rsv3+Rsv4*	10	5	8
1+0+0	*Rsv1*	2	1	0
0+3+0	*Rsv3*	6	1	5
0+0+4	*Rsv4*	1	0	0
0+0+0	*rsv1+rsv3+rsv4*	0	0	0

† Lines combined on the basis of genotypic category with regard to the number of genes present regardless of their homozygous or heterozygous state.

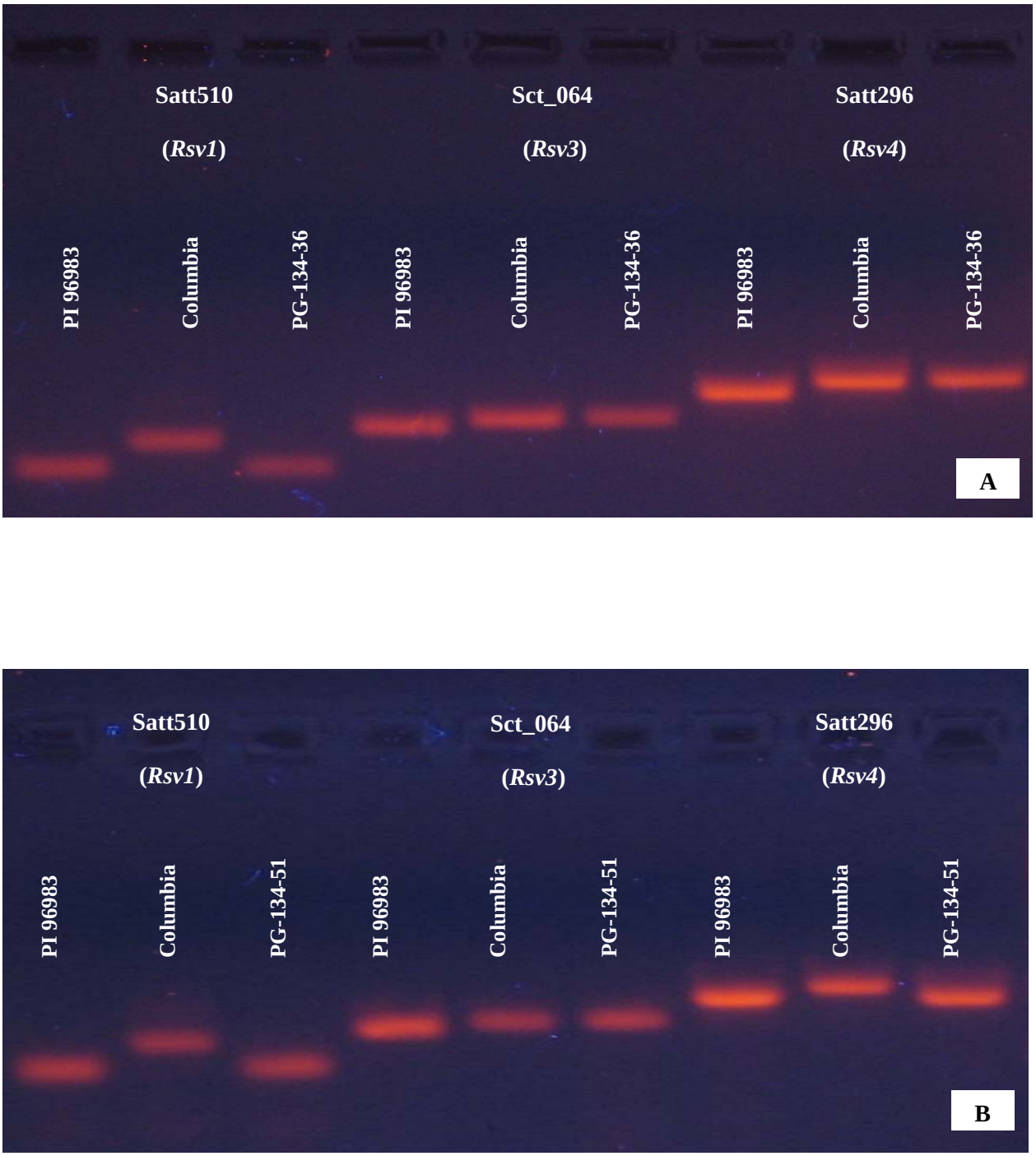

Figure 1. Comparison of DNA amplification patterns of parents and F_2 plants derived from PI 96983 x Columbia using specific SSR markers closely linked to SMV resistance loci *Rsv1*, *Rsv3*, and *Rsv4*. A, Plant GP-134-36 with genotype 1+3+4. B, Plant GP-136-51with genotype 1+3+0.

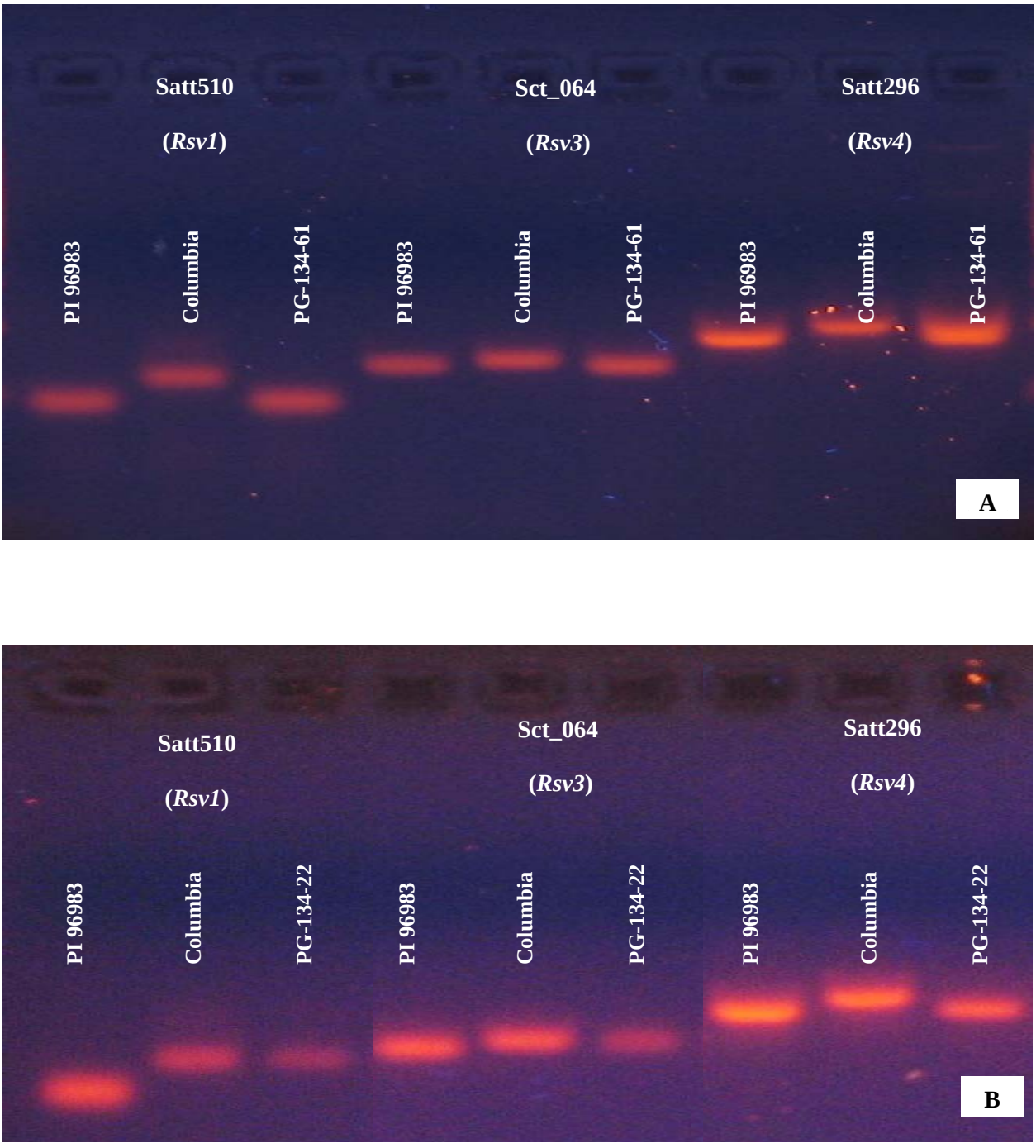

Figure 2. Comparison of DNA amplification patterns of parents and F_2 plants derived from PI 96983 x Columbia using specific SSR markers closely linked to SMV resistance loci *Rsv1*, *Rsv3*, and *Rsv4*. A, Plant GP-134-61 with genotype 1+0+4. B, Plant GP-136-22 with genotype 0+3+0.

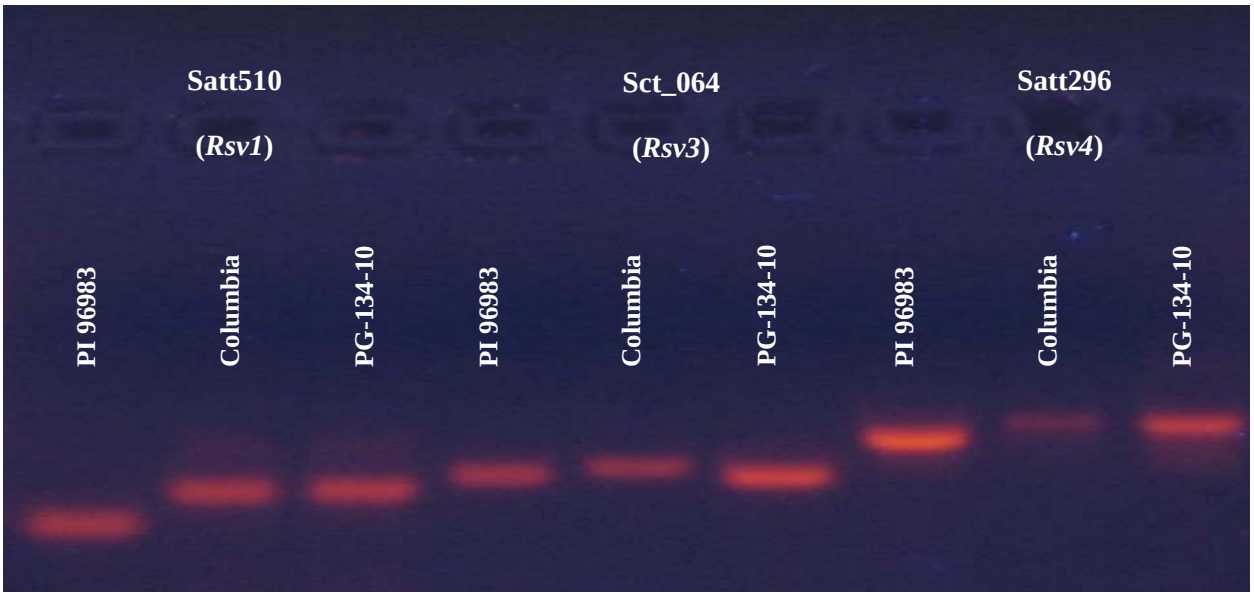

Figure 3. Comparison of DNA amplification patterns of parents and F_2 plants derived from PI 96983 x Columbia using specific SSR markers closely linked to SMV resistance loci *Rsv1*, *Rsv3*, and *Rsv4*. Plant GP-136-10 with genotype 0+3+4.

CHAPTER IV

Genetic Analysis of Resistance to Soybean Mosaic Virus in PI 61944 Soybean

Abstract

Soybean mosaic virus (SMV) is one of the most prevalent pathogen in soybean which is transmitted by aphis or infected seed. The most common method to control SMV is through development of resistant cultivars; however, they have been limited to single gene resistance which is ephemeral and highly vulnerable to dynamic and diverse plant pathogen populations. SMV resistance genes have been found into several soybean germplasm, however most of them do not provide resistance to all or at least to the most virulent strains. The objective of the current research is to identify new genes or alleles for differential reactions to SMV strains in the soybean genotype PI 61944. In order to develop F_2 populations and $F_{2:3}$ lines for SMV screening with G1 and G7 strains and mapping analysis, different crosses were made. The previously identify soybean resistant parent PI 61944 (*Rsv-?*) was crossed to the susceptible parent Essex (*rsv1*), and to the differential parents PI 96983 (*Rsv1*), L29 (*Rsv3*), V94-5152 (*Rsv4*), and PI 507389 (*Rsv1-n*) for allelism test. Results reveled that resistance to SMV in PI 61944 is controlled by a single dominant gene, and that this gene is not allelic to *Rsv1* locus as it was previously reported. Allelism test showed that PI 61944 is an allele at the *Rsv3* locus. This information was supported by molecular analysis which showed that PI 61944 is located at linkage group (LG) B2 (chromosome 14). PI 61944 exhibits a unique and different pattern from the reaction pattern of the reported *Rsv3* allele in L29. PI 61944 was N to G1 and R to G7 in this study, and N or S to G1 and G2; R or N to G3; and R to G5 to G7 in previous studies. Therefore, we propose that a new allele be assigned to the SMV resistance gene in PI 61944. Soybean sources carrying the *Rsv3* allele are rare to find into the soybean germplasm and confer resistance to most aggressive SMV strains; therefore, we expected PI 61944 may be useful choice in breeding for resistance.

Introduction

Soybean [*Glycine max*, (L.) Merr.], the most important oilseed crop worldwide, can be severely damage from different pathogens as Soybean Mosaic Virus (SMV). SMV has the facility to infect soybean and reduce soybean yield and seed quality. The primary inoculum of SMV is infected soybean seedlings derived from infected seeds; and secondary spread occur by activity of several aphids that transmit the virus (Hill et al., 1987). According to Cui et al. (2011) the current measures to control damages caused by SMV are (1) the development and use of soybean cultivars carrying at least one resistance gene, (2) the use of SMV-free seeds, (3) the selection of proper planting time, and (4) the control of aphid with pesticides. Complicating issues in breeding for soybean virus resistance is the emergence of *Aphis glycines*, an aphid that colonizes soybean by feeding directly on soybean or transmitting viruses; this aphid is reported to transmit at least 10 viruses including SMV (Saghai Maroof et al., 2008b). The most common method to control SMV in soybeans is through development of resistant cultivars, and it has been reported that the use of SMV resistant varieties prevented/reduced SMV and *Phomopsis* spp. seed infection; however conventional breeding programs have been limited to single gene resistance which is ephemeral and highly vulnerable to dynamic and diverse plant pathogen populations (Koning et al., 2002; Saghai Maroof et al., 2008a).

Several sources of SMV resistance have been found in the soybean germplasm but most of them do not provide resistance to all or at least to the most virulent strains. Identification of new sources of resistance to SMV that provides resistance to a broad and ever-changing range of SMV isolates will avoid genetic uniformity and potential vulnerability (Zheng et al., 2005). Therefore, the objective of the present research is to identify new genes or alleles for differential reactions to SMV strains in the soybean genotype PI 61944.

Soybean Mosaic Virus cause stunned plants and reduce flower and pod development decreasing seed yield and quality. This virus is transmitted seed-borne and by aphids, therefore genetic resistance is an alternative method of control. SMV was classified by Cho and Goodman (1979) based on the differential symptoms of cultivars Clark, Rampage, Davis, York, Kwanggyo, Marshall, Ogden, and Buffalo and categorized in seven strain groups of virulence (G1-G7) (Table 1); G1 is the least virulent strain which does not infect any of the resistant cultivars, and G7 the most virulent strain that infects all cultivars and causes necrosis and mosaic symptoms. The intermediated strains express noticeable symptoms, G2 show necrosis on Marshall; G3 also shows necrosis on Marshall and Ogden; G4 is necrotic in York and Davis; G5 mosaic in York and Davis and necrosis in Kwanggyo; and G6 cause the same as reaction than G5 and necrosis en Marshall (Li et al., 2010).

Individual plant reactions to the different strains are classified as resistant (R, symptomless) which produces no disease symptoms on plants, with no detectable and recoverable virus, and plants have vigorous appearance (Figure 1). Necrotic (N, stem-tip necrosis) develop very low level of virus replication and movement and almost non-detectable level of virus, include local necrosis, systemic necrosis, bud light, stunning of the plant and defoliation, and eventually plant death. Stem-tip necrosis is a hypersensitive reaction result of an incompatible reaction between the soybean and the SMV strain; it should not be classified as susceptible reaction (Buzzel and Tu, 1989). Local necrosis is hypersensitive reaction and necrotic lesions are restricted to the initial infection site on inoculated leaves; and systemic necrosis exhibit necrotic spots on upper leaves, brown discoloration of leaf veins, and browning of petioles and stems. Susceptible (S, mosaic) allows ample virus replication and systemic viral movement in plants, show leaves that gradually curl, leaf margins twist downward and chlorosis

developing between them; therefore virus and mosaic symptoms move and spread to new leaves as they develop . Expression and severity of SMV symptoms are temperature sensitive, genotype and SMV strain dependent (Bowers et al., 1992; Chen and Choi, 2007; Ma et al., 2002; Sutic et al., 1999).

SMV resistance is controlled by single dominant genes, and there are three independent loci, *Rsv1*, *Rsv3*, and *Rsv4*. The *Rsv1* was identified in the soybean line PI 96986 and provides resistance to the lowest and susceptibility to the highest strains, and nine resistance alleles *Rsv1-t*, *Rsv1-y*, *Rsv1-m*, *Rsv1-k*, *Rsv1-r*, *Rsv1-h*, *Rsv1-s* and *Rsv1-n* have been identified (Table 1) (Chen et al., 2001; Li et al., 2010). Consequently, the *Rsv1* locus was reported on the soybean molecular map linkage group (LG) F (chromosome 13) (Yu et al., 1994). The gene *Rsv3* was found in cultivar Columbia and L29, and confers resistance to the more virulent strain groups G5 through G7, and conditions mosaic reaction to the less virulent group G1 through G4 (Buzzell and Tu, 1984; Gunduz, et al., 2002). *Rsv3* locus was identified at the LG B2 (chromosome 14) based on the data collected from two F_2 different populations, L29 (*Rsv3*) x Lee68 (*rsv3*) and Tousan 140 (*Rsv3*) x Lee68 (*rsv3*) (Jeong et al., 2002). The *Rsv4* gene was reported in PI 486355 and confers resistance to all SMV strains G1 to G7, and show systemic necrosis in the heterozygous state. V94-5152 also carries *Rsv4* but confers early resistance to G1-G7 strains. The *Rvs4* locus was reported at the LG D1b (chromosome 2) on the soybean map in a population derived from the cross between lines LR2 (*Rsv4*) and Lee68 (*rsv4*) (Chen et al., 1994; Hayes et al., 2000).

Soybean genotypes carrying more than two genes for soybean resistance are not reported, however inheritance studies demonstrated that cultivars Zao18, J05, OX670, Tousan 140 and Hourei carry *Rsv1* and *Rsv3* genes, and confers resistance to SMV strains G1-G7 (Guduz et al.,

2004; Liao et al., 2002). In addition, Columbia carries *Rsv3* and *Rsv4* for resistance to all strains but G4 (Ma et al., 2002).

Most of the SMV resistant genes discovered are alleles at the *Rsv1* locus; however they provide resistance only to the lower strains. Therefore it is important to identify new sources of resistance to the higher and/or to all SMV strains. Through a diversity study of 212 soybean genotypes, Zheng et al. (2005) demonstrated that 116 genotypes were resistant to strains G1, G7, or to both SMV strains, and determined that most of the genotypes carry the *Rsv1* allele, others the *Rsv3* or the *Rsv4*, and some a combination of two alleles. Among these resistant genotypes, PI 61944 showed stem-tip necrosis after inoculation with G1 and mosaic symptom with G7; then virus was detected in both necrotic and mosaic plants. This reaction was similar to genotype PI 507389; therefore PI 61944 was postulated to be allelic to *Rsv1-n*. On another study 127 genotypes, including PI 61944, was conducted to screen them with six SMV strains; results showed that PI 61944 was susceptible (necrosis with mosaic symptoms) to the less virulent strains (G1 and G2), resistance to the more virulent strains (G5-G7), and necrotic or resistant reaction to G3 (Zheng et al., 2008). Simultaneously, PI 61944 showed amplification when analyzed with a polymerase chain reaction (PCR)-based marker for detection of 3gG2 at the *Rsv1* locus (Shi et al., 2008), which support the idea of PI 61944 may have a new allele at *Rsv1*. However, in a study of interaction between two strains of SMV, Shi et al. (2008) demonstrated that PI 61944 was necrotic to G1 and resistant to G7 strain through a single inoculation. This PI also showed necrotic reaction when inoculated with mixed strains G1+G7. Identifying new allele(s)/gene(s) with different strain SMV patter will provide a source of resistance to use in breeding programs.

Materials and Methods

Plant Materials

To study the inheritance of SMV resistance in PI 61944, a series of cross combinations was made. PI 61944 was crossed with the susceptible cultivar Essex to determine the number of genes and dominance status of the genes. PI 61944 was also crossed with genotypes with known SMV resistance genes, including PI 96983, L29 and V94-5152 carrying *Rsv1, Rsv3*, and *Rsv4*, respectively, to determine their allelic relationships. For each combination, several attempts were made at the Arkansas Agricultural Experimental Station at Fayetteville, AR in summer 2007. F_1 seeds were space-planted in summer 2008, and individually harvested. Part of the F_2 seed from each cross were kept for greenhouse inoculation and the other part were used for generation advancement. During summer 2009, two F_2 populations from each cross combination were spaced-planted in the field for DNA samples and for developing $F_{2:3}$ lines for SMV inoculation. Each F_2 plant was labeled, harvested, and threshed individually.

SMV Screening

To examine the reaction to SMV, approximately 80 seeds of F_2 population and 40 to 70 $F_{2:3}$ lines each with 30-40 plants from each of the five crosses combinations were grown at the Rosen Alternative Pest Control Greenhouse at Fayetteville, AR. Inoculations were conducted in two different greenhouses to avoid strain contamination. Plants were grown in 8 inch plastic pots containing Redi-earth commercial soil mix (Sun Gro Horticulture Canada, Ltd). Temperature of the greenhouse was maintained between 20-25°C with a 14 hr photoperiod.

Systemically infected leaves of soybean cultivars Essex (for G1) or Lee68 (for G7) were grounded in 0.05 M potassium phosphate buffer (pH 7.2) with mortar and pestle (1 g leaf tissue:10 ml buffer). Inoculum was applied with cheesecloth pads to both unifoliolate leaves (V1

stage) of each plant that had been previously dusted with 600-mesh carborundum. Inoculated plants were monitored for the symptom expression on regular basis at 8, 16, 24, and 32 days after inoculation, and classified into the three different reactions: resistant (R, symptomless), stem-tip necrosis (N, necrotic lesions and spots on both inoculated and non-inoculated leaflets, petioles, and stems), and susceptible (S, mosaic) (Ma, et al., 2004). F2:3 lines were classified based on the individual plant reactions into all R, all S, or segregating for R, N, and S.

In order to verify the virus symptoms, two representative leaves samples from each of the reaction class of the $F_{2:3}$ populations were assayed for SMV infection 32 days after inoculation by serological tissue blotting immunosorbent assay (Lin et al., 1990; Albrechtsen, 2005).

Molecular Analysis

Molecular analysis of a F_2 population derived from PI 61944 x Essex was grown and developed in the field in order to verify the PI 61944 locus. An F_2 individual plants were tagged when third trifoliate was developed, V3 stage (Fehr and Caviness, 1979). Samples of the youngest trifoliate were taken for DNA extraction, placed into individually labeled plastic bags, and freeze-dried for 48 to 72 h. Dry leaves were grounded with liquid nitrogen to powder, placed into 2.0 uL micro tubes, and kept at -80°C until DNA extraction.

The DNA extraction was done using the CTAB method (Kato and Palmer, 2004). Briefly, the extraction buffer [10% CTAB (hexadecyltrimethyl ammonium bromide), 1% 1-10 phenanthroline, 1*M* Tris-HCL, 05*M* EDTA pH 8.0 and 5*M* NaCl] plus 1000:1 of *β*-mercaptoethanol was preheated for 10 min at 62-65 °C, then 750 µL of this extraction buffer was added to each 2.0 µL sample tube. Sample tubes were mixed until a homogeneous solution was achieved, and incubated at 62-65 °C in a water bath, with shaking the samples every 15 min. After 1 h, tubes were left at room temperature for 10 min to cool down.

Subsequently, 750 µL chloroform:isoamyl alcohol (24:1) was added to each sample in the flow-hood, and gently mixed. Sample tubes were placed in an orbital shaker for about 15 min then centrifuged at 4-10°C at 13,000 rpm for 15 min. Then, the upper layer was transferred to a new 1.5 µL tube.

To precipitate the DNA, 750 µL of 95% cold (-20°C) ethanol /15m*M* NH_4Ac was added, tubes were then gently inverted and incubated at room temperature for 20 min to wash the DNA for 30 min. The DNA was then spined down, ethanol poured out, and the tube placed under the hood to dry for 1 hr.

The last step was to dissolve the DNA in 200 µL of 0.1x TE buffer [Tris (hydroxymethyl aminomethanol ethylenediamin tetracetic acid)] (pH 8.0). The samples were stored at -20°C until use, at which time DNA concentration of each sample will be tested using a spectrophotometer.

SSR Analysis

In order to verify the allele and locus carried by genotype PI 61944, ten SSR markers from the LG B2 (chromosome 13) were selected with criteria of proximity to the *Rsv3* locus to test polymorphism between parents. These markers enclosed 25.2 cM of chromosomal area. Polymorphic markers were used to screen the F_2 populations for genotyping records.

Each PCR reaction mixture had a total volume of 15.0 µL consisting of 3.0 µL of of 5x Green GoTaq flexi buffer (Promega Corporation), 0.9 µL of $MgCl_2$, 1.0 µL of dNTP, 7.6 µL autoclaved filtered water, 0.2 µL GoTaq DNA polymerase, 1.0 µL of primer (1 µM), and 1.3 µM of template DNA. Microplates of 96 samples containing the reaction mixture were vortexed for 3 s to mix well the reaction solution. The microplate should be collocated to ICycler Thermal Cycler (Bio-Rad Laboratories Inc., CA) with a program of 94°C for 4 min for preheat, 33 cycles

of 25 s at 94°C for denaturation, 25 s at 47°C for annealing, 25 s at 68°Cfor extensions, and 5 min at 72°C for final extension.

After PCR, samples were loaded in 3 % agarose gels containing 1x TBE buffer [10 x TBE buffer (108 g Tris, 55 g boric acid, and 40 mL EDTA in 1L deionized distilled water)]. Sampler were run at 120 V/10 cm for 2 h, and visualized under UV light.

Data Analysis

The SMV phenotypic data from F_2 populations and $F_{2:3}$ lines were tested for goodness of fit to expected genetic segregation ratios by Chi-squared analysis. A single dominant gene is claimed if a 3R:1S in F_2 and 1R: 2 segregating:1S in $F_{2:3}$ were observed. If a 15R:1S in F_2 and 7R:8Segregating:1S in $F_{2:3}$ were observed, then two dominant genes were confirmed. Allelism is claimed if no segregation was observed. For the molecular screening, a score designation of A was given if the plant carries the dominant allele from PI 61944, B if it carried the recessive allele from Essex, and H if it carries both alleles from the parents. Segregation at each marker allele was also tested for goodness of fit to the expected 1:2:1 ratio by Chi-squared analysis.

Results and Discussion

Visual symptoms after inoculation with SMV G7 were detected in the susceptible parent Essex with typical venial clearing and mosaic in the first trifoliate leaves proximately eight days after inoculation. The mosaic symptoms sustained for 3-4 weeks after inoculation. In contrast, PI 61944, L29, and V94-5152 did not manifest symptoms of infection on trifoliate leaves at any time during the experiment. PI 96983 plants initially developed venial clearing symptoms during first few days after inoculation and then turned to necrosis approx.10 days after inoculation. These observations ratified the purity and identity of the G7 strain, and confirmed the reactions of the parents.

The results of F_2 population derived from PI 61944 (R) x Essex (S) showed a good of fit to the monogenic ratio of 3R:1S when inoculated with G7 (Table 2). The $F_{2:3}$ lines derived from same cross inoculated with G7 also showed a good fit to a ratio of 1all R:2 segregating (R+S):1all S (Table 3). These results indicated that resistance to G7 in PI 61944 is controlled by a single dominant gene.

The F_2 population from PI 61944 (R) x PI 96983 (N) appeared to be segregating for two genes when inoculated with G7 (Table 2). Although the segregation ratio is not a statistically acceptable fit to the expected ratio of 15(R+N):1S, the observed ratio is more deviated from the monogenic ratio of 3:1 than the digenic ratio. The $F_{2:3}$ lines derived from the same cross showed a good fit to the 7R(R+N):8 Segregating (R+N+S):1all S ratio when inoculated with G7 strain (Table 3). These results indicated that resistance to G7 in PI 61944 is not at the *Rsv1* locus.

Although there were ten susceptible plants observed in the F_2 populations derived from PI 61944 (R) x L29 (R) (Table 2), the $F_{2:3}$ lines derived from the same cross did not show segregation at all and all lines expressed resistance to G7 (Table 3). The absence of necrotic or susceptible plants in all 35 $F_{2:3}$ lines provided strong evidence that the gene for G7 resistance in PI 61944 is an allele at *Rsv3* locus.

The F_2 population from PI 61944 (R) x V94-5152 (R) exhibited a digenic segregation when inoculated with G7 strain (Table 2). The observed ratio was a poor fit to the digenic ratio, but was a better fit than the monogenic ratio. The $F_{2:3}$ lines from same cross showed a good fit to the 7 all R: 8 segregating (R+S):1all S ratio (Table 3). These results indicated that the resistance gene in PI 61944 is not at the *Rsv4* locus.

Virus detection was realized by serological test of each cross and the corresponding parents. Results confirmed genetic segregation in $F_{2:3}$ lines from PI 61944 x Essex, PI 61944 x PI96983, and PI 61944 x V94-5152; but not in the $F_{2:3}$ lines derived from PI 61944 x L29 (Table 4). These results support the conclusion that PI 61944 carries a single dominant gene that is allelic to *Rsv3* but independent of *Rsv1* and *Rsv4*.

In order to confirm the presence of the *Rsv3* allele in PI 61944, ten SSR markers near the locus on LG B2 were selected from the area covering 25.2 cM. Markers Satt534, Sct_064, Satt726, and Sat_424 showed polymorphism and were used to screen a F_2 population from PI 61944 x Essex. Results showed that all four marker alleles segregated in a monogenic fashion with a fit to the expected ratio of 1:2:1 (Table 5). These maker data further support the conclusion that the SMV resistance gene in PI 61944 resides at the *Rsv3* locus.

The results from the inheritance study, serologic tests, and molecular marker analysis consistently showed that PI 61944 carries a single dominant gene for resistance to SMV G7. This gene is at the *Rsv3* locus, but not at the *Rsv1* or *Rsv4* locus. PI 61944 was previously assumed to carry an allele at *Rsv1* locus based on inoculation results with two SMV strains (Zheng et al., 2005) where PI 61944 exhibited N to G1 and R to G7. This assumption was further adopted by another study where that PI 61944 was shown to be N or S to G1 and R to G7 (Zheng et al., 2008), which is similar to the pattern of SMV reaction in PI 507389 carrying an *Rsv1* allele (*Rsv1-n*) (Table 1, Ma et al, 2002). In this study, PI 61944 was N to G1 and R to G7. PI 61944 was showed to be N or S to G1 and G2; R or N to G3; and R to G5 to G7. This reaction pattern is unique and different from the reaction pattern of the reported *Rsv3* allele in L29 (Table 1). Therefore, we propose that a new allele be assigned to the SMV resistance gene in PI 61944.

In the molecular analysis, we used all the available SSR markers in the chromosome region where *Rsv3* is located. These markers covered an area of 19.2 cM in our linkage analysis which is 6.3 cM bigger than the corresponding fragment at the genetic map of soybean (Song et al., 2004; SoyBase, 2012). This discrepancy could be attributed to a different cross and a few markers used in this study. Our population was derived from a cross between two *G. max* lines, whereas the public map was constructed using a cross between *G.max* and *G. soja*. Nevertheless, all four polymorphic markers were co-segregating with the resistance gene in the population. It is worth noting that this chromosomal region contains a cluster of genes for disease resistance, such as resistance to two races of soybean cyst nematode, *Rps5* for resistance to *Phythophtora sojae* (Jeong et al., 2002). In addition, markers closed linked to the *Rsv1* locus on LG F and *Rsv4* on LG D1b were also used to screen the population from PI 61944 x Essex, but no association was found (data not showed), confirming that the gene in PI 61944 was not allelic to *Rsv1* and *Rsv4*.

Soybean genotypes carrying the *Rsv3* gene are rare and only few have been reported including cultivars OX 686, Harosoy, and L29 (Buss et al., 1999; Buzzell and Tu, 1989; Gunduz et al., 2001). PI 61944 is an old germplasm accession collected from China. It contains a new allele at the *Rsv3* locus and may also contribute to genetic diversity for crop improvement. Single dominant gene is feasible to use for genetic resistance and can be easily incorporated in breeding lines. *Rsv3* alleles confer resistance to most aggressive SMV strains and therefore the best choice in breeding for resistance.

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Table1. Reactions of differential soybean genotypes to seven Soybean Mosaic Virus (SMV) strains identified in the United States.

Genotype	Reaction to SMV strain†							Gene	References
	G1	G2	G3	G4	G5	G6	G7		
Essex, Lee68	S	S	S	S	S	S	S	*rsv*	Chen et al., 1991
PI 96983	R	R	R	R	R	R	N	*Rsv1*	Kiihil and Hartwing, 1979
Suweon 97	R	R	R	R	R	R	R	*Rsv1-h*	Chen et al., 2002
York	R	R	R	N	S	S	S	*Rsv1-y*	Roane et al., 1983; Chen et al., 1991
Raiden	R	R	R	R	N	N	R	*Rsv1-r*	Chen et al., 2001
Kwanggyo	R	R	R	R	N	N	N	*Rsv1-k*	Chen et al., 1991
Ogden	R	R	N	R	R	R	N	*Rsv1-t*	Chen et al., 1991
Marshall	R	N	N	R	R	N	N	*Rsv1-m*	Chen et al., 1991
PI 507389	N	N	S	S	N	N	S	*Rsv1-n*	Ma et al., 2003
L29	S	S	S	S	R	R	R	*Rsv3*	Buss et al., 1999
OX 686	N	N	N	N	R	R	R	*Rsv3*	Buzzel and Tu, 1989
OX 670	S	S	S	S	PR	PR	PR	*Rsv3*	Gunduz et al., 2001
PI 61944	N/S	N/S	R	-	R	R	R	*Rsv3-?*	Zheng et al., 2005
V94-5152	R	R	R	R	R	R	R	*Rsv4*	Buss et al., 1997

†R, resistant (symptomless); N, necrotic (systemic necrosis); S, susceptible (mosaic); PR, partial resistance.

Table 2. Reaction of parents and F_2 populations from crosses between PI 61944 and allele differential genotypes when inoculated with SMV G7.

Cross and parents	No. plants observed †						
	R	N	S	Total	Expected ratio	X^2	*p*
PI 61944 x Essex	48	0	21	69	3R:1S	0.87	0.30
PI 61944 (R)	9	0	0				
Essex (S)	0	0	10				
PI 61944 x PI 96983	45	0	7	52	15R(R+N):1S	4.62	0.03
PI 61944 (R)	7	0	0				
PI 96983 (N)	0	7	0				
PI 61944 x L29	67	0	10	77	No segregation		
PI 61944 (R)	8	0	0				
L29 (R)	6	0	0				
PI 61944 x V94-5152	65	0	10	75	15R:1S	6.42	0.011*
PI 61944 (R)	9	0	0				
V94-5152 (R)	9	0	0				

†R, resistant (symptomless); N, necrotic (systemic necrosis); S, susceptible (mosaic).

Table 3. Reaction of $F_{2:3}$ lines from crosses between PI 61944 and allele differential genotypes when inoculated with SMV G7.

Cross and parental genotypes	Observed Plants†				Expected Plants		
	R	H	S	Total	Ratio	X^2	*p*
PI 61944 x Essex	11	24	9	44	1R:2H(R+S):1S	0.54	0.76
PI 61944 (R)	7	0	0				
Essex (S)	0	0	10				
PI 61944 x PI 96983	22	45	2	69	7R(R+N):8N(R+N+S):1	1.33	0.25
PI 61944 (R)	6	0	0				
PI 96983 (N)	0	6	0				
PI 61944 x L29	35	0	0	35	No Segregation		
PI 61944 (R)	9	0	0				
L29 (R)	12	0	0				
PI 61944 x V94-5152	40	7	1	48	7R:8H(R+S):1S	1.42	0.23
PI 61944 (R)	6	0	0				
V94-5152 (R)	7	0	0				

†R, resistant (symptomless); N, necrotic (systemic necrosis); S, susceptible (mosaic).

Table 4. Tissue blotting of F3 populations from crosses between PI 61944 and allele differential genotypes and parents when inoculated with SMV G7.

Cross and parents	No. F_3 plants†			Phenotypic Classification
	+	-	Total	
PI 61944 x Essex	31	88	119	Segregating
PI 61944 (R)	0	2	2	Resistant
Essex (S)	2	0	2	Susceptible
PI 61944 x PI 96983	11	111	122	Segregating
PI 61944 (R)	0	2	2	Resistant
PI 96983 (N)	0	2	2	Necrotic
PI 61944 x L29	0	68	68	Resistant
PI 61944 (R)	0	2	2	Resistant
L29 (R)	0	2	2	Resistant
PI 61944 x V94-5152	5	32	37	Segregating
PI 61944 (R)	0	2	2	Resistant
V94-5152 (R)	0	2	2	Resistant

†+, Presence of virus; -, Absence of virus.

Table 5. Genotypic segregations in the F_2 population from PI 61944 x Essex, using selected SSR markers on LG B2.

Locus	Genetic distance (cM)	Genetic segregation†				$X^2_{(1:2:1)}$	*p*
		A	H	B	Total		
Satt534	0.0	11	31	19	61	2.11	0.35
Sct_064	3.9	9	27	21	57	5.21	0.07
Satt726	18.0	11	21	18	50	3.24	0.19
Sat_424	19.2	14	23	21	58	4.17	4.17

†A, allele amplified from resistant parent; B, allele amplified from susceptible parent; H, both resistant and susceptible parent alleles amplified.

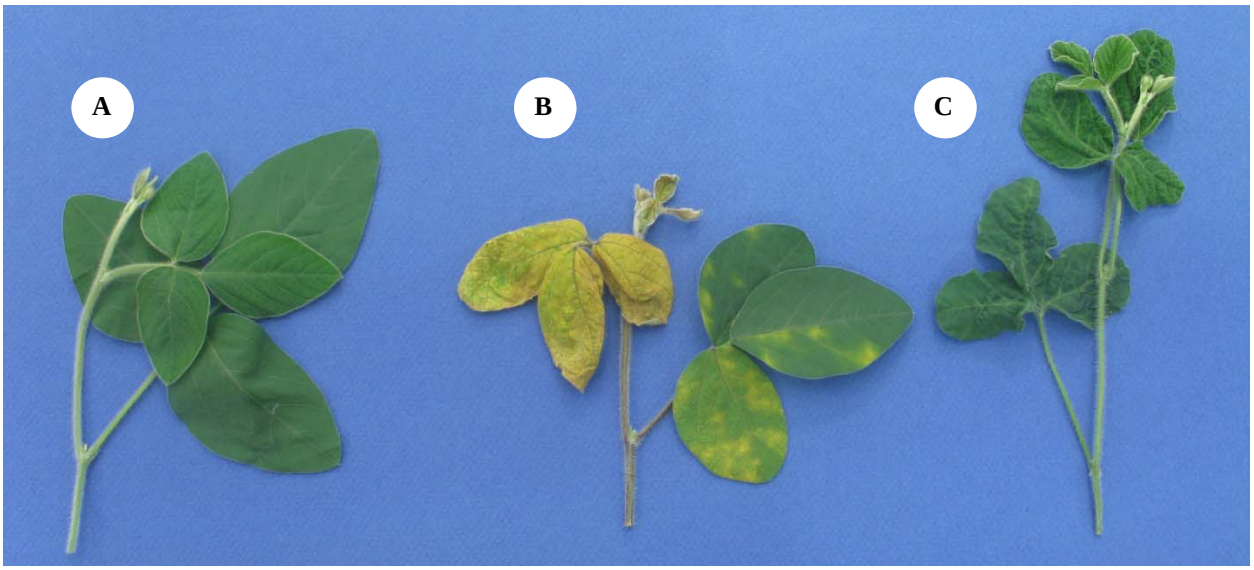

Figure 1. Soybean Mosaic Virus symptoms in soybean after inoculation with G7 strain. A, resistant (R). B, stem-tip necrosis (N). C, susceptible (S).

CONCLUSIONS

Through this research we analyze the genetic diversity at the molecular level of 114 soybean genotypes. This germplasm showed adequate genetic diversity in the genome other the regions for SMV resistance, although the level of diversity was slightly lower than that observed in other studies. This research also showed the genetic similarities among all the germplasm via pairwise comparisons, which indicated that the resistance to G1 strain is mostly controlled by *Rsv1* alleles, which are abundant and common in the SMV resistant germplasm and most of them were originated in Asia. Genotypes carrying *Rsv3*, *Rsv4* alleles showed less genetic similarity, and most of them were from the orient where soybean was originated and domesticated. Therefore, the genetic diversity information on SMV-resistant germplasm will be helpful to breeders in selecting parents for crossing in their breeding programs where both SMV resistance and using diverse gene pools are objectives.

Because of the inability of distinguishing progeny lines carrying more than one of three resistance genes based on SMV phenotypes, SSR marker data on each F_2 plant were used to predict the F_2 genotype according to a proposed genetic model based on segregation of three independent genes (*Rsv1*, *Rsv3*, and *Rsv4*) in the pyramiding population GP-134. Then, if most of the $F_{2:3}$ lines in each genotypic class exhibited the expected phenotype, we conclude that the F_2 phenotype was confirmed with the molecular data. Overall, 27 out of 70 lines were identified to contain three gene combinations, although most of them were in the heterozygous state. These lines would be very valuable for selecting homozygous lines with all three resistance genes.

A new allele for differential reactions to SMV strains was identified in the soybean genotype PI 61944. Results from inheritance study and allelism test revealed that resistance to SMV in PI 61944 is controlled by a single dominant gene, and that is allelic to *Rsv3* locus. This information was supported by molecular analysis which showed that PI 61944 is located at

linkage group (LG) B2 (chromosome 14). PI 61944 exhibits a unique and different pattern from the reaction pattern of the reported *Rsv3* allele in L29. PI 61944 was N to G1 and R to G7 in this study, and N or S to G1 and G2; R or N to G3; and R to G5 to G7 in previous studies. Therefore, we propose that a new allele be assigned to the SMV resistance gene in PI 61944. Soybean sources carrying the *Rsv3* allele are rare to find into the soybean germplasm and confer resistance to most aggressive SMV strains; therefore, we expected PI 61944 may be useful choice in breeding for resistance.