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Genetic Analysis of Soybean Mosaic Virus (SMV) Resistance Genes
in Soybean [*Glycine max* (*L.*) *Merr*.]

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Crop, Soil, and Environmental Sciences

by

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ABSTRACT

Soybean mosaic virus (SMV) causes the most serious viral disease in soybean worldwide. Seven SMV strains, G1 - G7, and three independent multi-allelic loci for SMV resistance, *Rsv1*, *Rsv3*, and *Rsv4*, have been identified. In the initial study, 299 soybean germplasm lines were genotyped for *Rsv4* region, inoculated with SMV-G1 and G7 strains, and classified into several resistance groups. The *Glyma.02g121400* locus was sequenced from ten soybean accessions, and alignment of the sequences revealed three SNPs displaying 100% polymorphic consistency when a soybean genotype carrying the *Rsv4* gene was present. A cross between V94-5152 × Lee 68 was made to create linkage map revealing a distance of 3.6 cM between the *Rsv4* and the closest SNP. Five *Rsv4* candidate genes have been proposed in this region. In the second study, three SMV R-genes were pyramided by crossing J05 and V94-5152. The gene-pyramided line GP20, was crossed with Williams 82, F_2 plants were genotyped and collated with phenotypic data of $F_{2:3}$ lines inoculated with SMV-G1 and G7 strains. The results confirmed a successful incorporation of three genes into one soybean line. In the third study, soybean germplasm PI 438307 was crossed to Essex for the inheritance study, and to three differential parents for the allelism test. F_2 population and $F_{2:3}$ lines derived from all four cross combinations were screened with SMV-G7 strain. Additionally, F_2 generation of PI 438307 x Essex were genotyped with two SSRs. The results revealed that resistance to SMV in PI 438307 is controlled by a single dominant gene at the *Rsv4* locus. PI 438307 plants exhibited a unique symptoms; therefore, a new allele *Rsv4-v* was assigned to SMV resistance in PI 438307. In the final study, PI 96983 and York were crossed to evaluate allelomorphic relationship between *Rsv1* and *Rsv1-y*. To break possible linkage, 3000 F_2-plant population was phenotyped using the SMV-G1 strain. Occurrence of susceptible and segregating lines indicated tight linkage between two genes

positioned in a distance of 2.2 cM. The *Rsv2* symbol was proposed to be assigned instead of *Rsv1-y*. Results from this research may accelerate breeding efforts to develop multi-virus resistant crops.

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Thank you.

Mariola Klepadlo

Fayetteville, May 2016

DEDICATION

To my mother Józefa Klepadło...

...for life, love, and education. Mom, I love you.

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ABBREVIATIONS

6K1 - Viral Protein 6K1

6K2 - Viral Protein 6K2

BCIP - 5-bromo-4-chloro-3-indolyphosphate

CI - *Cylindrical Inclusion* Viral Protein

cM - CentiMorgan Distance Unit

CP - Coat Protein

CTAB - Cetyl Trimethyl Ammonium Bromide

df - Degrees of Freedom

DNA - Deoxyribonucleic acid

dNTP - Deoxynucleotide Mix

EST - Expressed Sequence Tag

G1-G7 - SMV Strain Nomenclature

GP - Gene Pyramiding

HC-Pro - Helper Component - Proteinase Viral Protein

HSP - Heat Shock Protein

IRES - Internal Rybosome Entry Site

EDTA - Ethylene Diamine Tetraacetic Acid

ER - Resistant Response at early stages

KASP - Kompetitive Allele Specific PCR

LOD - Logarithm of Odds Ratio

MAS - Marker-assisted Selection

MLG - Molecular Linkage Group

N - Necrotic Response

NBS-LLR - Nucleotide-binding Site and Leucine-rich Repeat

NBT - Nitro-blue Tetrazolium

NIa-VPg - Viral Protein NIa-VPg

NIa-Pro - Viral Protein NIa-Pro

NIb - Viral Protein Nib

ORF – Open Reading Frame

P1 - Viral Protein 1

P3 - Viral Protein 3

P3N-PIPO - Viral Protein P3N-PIPO

PBS - Phosphate-buffered Saline

PCR - Polymerase Chain Reaction

PVP - Polyvinylpyrrolidone

qRT-PCR - Quantitative Real-Time PCR

R - Resistant Response

R-Gene - Resistance Gene

RAPD - Random Amplified Polymorphic DNA

RdRp - RNA-dependent RNA Polymerase

RFLP - Restriction Fragment Length Polymorphism

RIL - Recombinant Inbreed Line

RNA - Ribonucleic Acid

rpm - Rotations Per Minute

Rsv - SMV Resistance Gene Nomenclature

RT - Room Temperature

S - Susceptible Response

SDS - Sodium Dodecyl Sulfate

SEL - Size Exclusion Limit

SMV - Soybean Mosaic Virus

SNP - Single Nucleotide Polymorphism

SSR - Simple Sequence Repeat

TAE - *Tris/Acetate/EDTA* Buffer

TBE - Tris/Borate/EDTA Buffer

TF - Transcription Factor

USDA-ARS - United States Department of Agriculture - Agricultural Research Services

UTR - Untranslated Region

VPg - Virus Genomic Protein Linked Molecule

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

SOYBEAN PRODUCTION

An estimated eight hundred million people around the world suffer from chronic food shortage, and millions more may go hungry due to current and future food crises. To meet this need, the United Nations has called for a 70% increase in food production by 2050 when a world population is expected to exceed 9 billion people (FAO, 2009). High-yielding crops can help feed a growing world population; therefore, improving seed quality, and developing tolerance/resistance to biotic and abiotic factors is a key to improving worldwide food production. From 2009 to 2014, the soybean yield increased from 44.0 to 47.8 bushels/acre in the U.S., reaching 50.0 bushels/acre in Arkansas (SoyStats, 2015).

In 2014, the United States was the leader in worldwide soybean production (34%) followed by Brazil (30%), Argentina (18%), and China (4%). In 2014, approximately 83.7 million acres (33.9 million hectares) in the U.S. were planted with a total production of approximately 4 billion bushels (108 million metric tons). Whereas soybean can be grown throughout the United States, the majority are planted in the Midwest, the Midsouth, the Southeast, and the Atlantic coast. Iowa, Illinois and Minnesota are the top producers, while Arkansas is on the 10th position (SoyStats, 2015).

Soybean [*Glycine max (L.) Merr*.] is the top provider crop of oil and proteins in the world, and due to these tremendous values it is referred as “the miracle crop”. Soybean quality is typically determined by the protein, oil, saccharides, and mineral content of the seed. About 90% of total soybean meal production is used to supply livestock fodder; a part of soybean production is processed for human consumption (e.g. soy milk or tofu), and for industrial use (e.g. biodiesel, inks, plastics, solvents and cosmetics) (Singh, 2010).

SOYBEAN MOSAIC VIRUS

Soybean mosaic virus (SMV) is a plant pathogenic virus (*Potyviridae* family, potyvirus genus). The genome is composed by a linear, positive sense, single-stranded RNA approximately 10 kb long (Tolin, 1999). The RNA, accounting for 5.3% of the virus particle, encodes two different polyproteins that are proteolytically cleaved by self-encoded proteases into 11 mono- or multi-functional proteins (P1, HC-Pro, P3, P3N-PIPO, 6K1, CI, 6K2, NIa-VPg, NIa-Pro, NIb, and CP) (Chowda-Reddy et al., 2011; Gagarinova et al., 2008; Jayaram et al., 1992; Wen and Hajimorad, 2010; Zhang et al., 2009). The inactivation of SMV is possible by raising the temperature to 55-60°C for 10 minutes, or apply pH < 4 or > 9 (Tolin, 1999).

Disease symptoms caused by SMV were first observed and documented in the U.S. by Clinton in 1915 (Clinton, 1916). Later, Gardner and Kendrick (1921) reported that SMV-infected plants had mosaic dark green areas on leaves and the leaflets were misshapen and stunted. Of the 100 viruses that can infect soybean (Singh, 2010; Tolin, 1999), SMV is the biggest threat for soybean industry (Mandhare and Gawade, 2010; Singh, 2010). SMV causes the most common and serious viral disease of soybeans and also for many other commercially important plants worldwide. SMV may cause significant yield losses and deterioration of seed quality via reduction of seedling viability and vigor, seed coat mottling, flower abortion, reduction of pod set, seed number and size (Buss et al., 1989; Gunduz et al., 2004; Hill et al., 1987; Mandhare and Gawade, 2010; Ren et al., 1997; Ross, 1983). Moreover, SMV infection may result in seed composition of higher protein and lower oil content (El-Amrety et al., 1987; Wang et al., 2001). Depending on soybean genotype and SMV strain, yield can be reduced by 25% (Ren et al., 1997), 60% (Cho et al., 1977) with some studies documenting a 90% reduction in yield (Wang et al., 2001).

SMV is transmitted vertically in about 30 plant species through seed by infected embryo and through pollen derived from an infected plant that can be transfer via wind and insects (Hill et al., 1987; Tolin, 1999). It may also be transmitted within a season by aphids (*Aphis glycines*) mouth parts in a non-persistent, non-circulative, stylet borne manner using a virus protein, the helper component protein (HC-Pro) which facilitates binding of virus particles to the aphid maxillary stylet (Ivanov et al., 2014). Aphids can acquire the virus after short probing, and usually retains the virus for a short period of time (minutes), they may carry SMV for a relatively short distances; however, strong winds may effectively spread SMV in a long distance. Due to the relatively easy transmission of the disease, it is difficult to control the virus and produce SMV-free seeds (Gardner and Kendrick, 1921; Balgude et al., 2012). For genetic studies and breeding purposes, SMV infection may be obtained by mechanical inoculation. Inoculum is composed of infected leaves smashed with mortar and pestle in a potassium phosphate buffer solution, and both unifolate leaves dusted with abrasive are rubbed by a pestle dipped into the inoculum (Buss et al., 1985; Chen et al., 1991).

Maintenance of SMV strains may be achieved in three major ways. For short-term usage, *in vivo* continuous periodic infections of susceptible cultivars can be performed (Chen et al., 1991). Chen et al. (1988) reported that SMV can also be maintained *in vitro* via virus infected callus culture (Mozzoni and Chen, 2010). For long-term maintenance, SMV may be stored *ex vivo* by freezing infected leaf tissues at -80°C (Ma et al., 1995).

Various classification systems of SMV strains have been established in different countries. Conover (1948) proposed that SMV strains could be identified based on the differential reactions of soybean genotypes. Currently, SMV is classified into strains based on virulence on differential soybean genotypes (Pu et al., 1982). In Japan, five strains (A-E) have

been identified (Takahashi et al., 1963; 1980). In South Korea, G1-G7, SMV-N, G5H, G7a and G7H have been named (Seo et al., 2009). In China, strains have been grouped according to geographical regions and soybean responses, in 21 groups (SC1-SC21) (Li et al., 2010). In the United States, SMV has been grouped into seven strains (G1 through G7) where G1 strain is the least and G7 strain is the most virulent on different soybean cultivars (Tables 1, 2) (Cho and Goodman, 1979).

SMV adapts and develops overtime, resulting in emergence of new strains that overcome SMV resistance in soybean. SMV-N, G5H, G7a and G7H have recently emerged in the Korean peninsula (Seo et al., 2009). In the early 1980s, SMV-G5 strain caused about 80% of yield losses, whereas in the late 1980s, SMV-G5H was the dominant strain, responsible for over 65% of observed lose (Cho et al., 1983; Kim, 2003). More recently, SMV-G7H became the most prevalent strain accounting for approximately 50% of the SMV incidence (Kim et al., 2003; Seo et al., 2009). Due to the genetic variability of SMV and strong selection pressure, resistance-breaking isolates evolve in time, including CN18 identified in soybean fields in South Korea (Choi et al., 2005). Also, recombinant soybean mosaic virus (SMV-R) was recently identified and classified as a novel strain in Chongqing, China, exhibiting different pathogenicity on soybeans compared with other SMV strains (Yang et al., 2014). For those reasons, there should be extra caution when controlling SMV in soybean to avoid the evolutionary race between the host and the virus.

SMV DISEASE SYMPTOMS

The symptoms induced by SMV depend on many factors including the host genotype, virus strain, plant age at infection, and environment (Buss et al., 1989; Chen et al., 1991). Development of soybean cultivars with genetic resistance to SMV seems to be the most efficient strategy to control the disease. The first and most important step in production of soybeans with SMV resistance is to identify germplasm with resistance and study the genetic mechanisms before an introduction into a breeding program (Foolad and Panthee, 2012; Song et al., 2010). Individual cultivar reactions to SMV strains are classified into three main responses; susceptible (mosaic), necrotic (systemic necrosis), or resistant (symptomless) (Fig. 1) (Chen et al., 1991; Cho and Goodman, 1979).

The susceptible response is characterized by vein clearing, curled leaves, puckering, downward cupping, and reduction of leaf blade size. Infected plants are often stunted due to shortening of steams and petioles (Fig.1). As the disease progresses, a noticeable reduction in pod set numbers and size occurs, decrease of seeds size with characteristic coat mottling, reduction of secondary roots and bacterial nodulation, problems with seed germination and seedling vigor are also significant (Balgude et al., 2012; Bos, 1972; Cho and Goodman, 1979; Gardner and Kendrick, 1921). Susceptible plants often survive and finish plant life cycle, however, SMV infections at reproducible stages of plant development can significantly reduce yield (Cho et al., 1977; Ren et al., 1997; Tolin, 1999; Wang et al., 2001). A host plant is considered fully susceptible when the virus can successfully complete its replication, cell-to-cell movement trough plasmodesmata, and long distance movement through vascular tissues (Carrington and Whitham, 1998; Soosaar et al., 2005). The delayed vascular movement of SMV results in symptoms referred to late susceptible (LS) or early resistant (ER). Late susceptible

plants express resistance to SMV about 20 days after inoculation, and then susceptibility as small chlorotic to bronze lesions on one or more leaflets (Gunduz et al., 2004).

The necrotic symptoms indicate extreme hypersensitive reaction of the host to SMV. In general, the necrotic reaction provides yellow and brown discoloration on upper leaves, stunting of the entire plant, browning the stems and petioles, defoliation, and ultimately plant death (Fig.1). The necrotic symptoms are a protective system which is activated in response to SMV in order to reduce spreading the disease within the crop (Li et al., 2009; Ma et al., 2003; Matthews, 1991). Some soybean lines, e.g. PI 507389 and PI 96983, develop necrotic symptoms in a short period after infection leading to plant death at the V1 developmental stage, whereas other lines, e.g. PI 547857, need more time to develop the necrotic symptoms (Ma et al., 2003). In necrotic plants, viral replication, cell-to-cell and long distance movement are reduced although the virus is still detectable by molecular and immunohistochemical methods (Matthews, 1991). Genetic studies suggested that necrotic plants should be classified as resistant when evaluating segregating populations as the necrotic reaction is associated with heterozygous stage of the *Rsv1* locus (Chen et al., 1989; 1994).

Resistant soybeans exhibit no disease symptoms and are indistinguishable from non-infected plants (Fig.1). A host plant is resistant if it can block viral replication, cell-to-cell or long distance movement; therefore, SMV is not detectable in these plants (Soosaar et al., 2005). Chemical and cultural control of SMV is neither economical nor environmentally friendly (Mattews, 1991; Singh, 2010). Deployment of genetic resistance is considered to be the most effective alternative to control the disease (Chen et al., 1991; Shi et al., 2009).

SMV GENETIC RESISTANCE

The inheritance of resistance to SMV has been extensively studied. Three independent dominant genes for SMV resistance (R-genes) have been discovered, and named as *Rsv1, Rsv3* and *Rsv4* (Buss et al. 1997; Buzzel and Tu 1989 Kiihl and Hartwing, 1979). *Rsv* nomenclature confers resistant reaction and dominant nature, and *rsv* susceptible reaction via carrying a recessive allele (Ma et al., 2004). The *Rsv2* locus was initially assigned as a resistance gene in OX670 soybean but later abandoned when confirmed to carry *Rsv1* and *Rsv3* (Gunduz et al., 2001).

The *Rsv1* locus was the first SMV resistance gene identified and it is the most common in soybean germplasm. *Rsv1* contains ten alleles *Rsv1, Rsv1-t, Rsv1-y, Rsv1-m, Rsv1-k, Rsv1-r, Rsv1-s, Rsv1-n*, *Rsv1-h,* and *Rsv1-c* identified in PI 96983, Ogden, York, Marshall, Kwanggyo, Raiden, LR1, PI 507389, Suweon 97, and Corsica, respectively (Buss et al., 1994; Chen et al., 1991, 2001, 2002; Kiihl and Hartwig, 1979; Ma et al., 1995; Roane et al., 1983; Shakiba et al., 2013). Most of these alleles exhibit partial dominance and confer resistance to less virulent strains from SMV-G1 through G3 and susceptibility or necrosis to the more virulent G5 - G7 strains (Table 1). The first allele, *Rsv1*, was found in PI 96983 and displays resistance to G1 through G6, and necrosis to G7 strain (Kiihl and Hartwing, 1979). Ogden cultivar carries *Rsv1-t* allele and shows necrotic response when inoculated with G3 and G7 strain (Chen et al., 1991; Gunduz et al., 2002; Li et al., 2010; Ma et al., 2002). York (*Rsv1-y* allele), Kwanggyo (*Rsv1-k* allele), and Raiden (*Rsv1-r* allele) are resistant to less virulent strains, and are susceptible or necrotic to more virulent strains (Chen et al., 1991, 2001; Roane et al., 1983). Marshal (*Rsv1-m* allele) expresses resistance to strains G1, G4 and G5, and necrosis to the rest of strains (Chen et al., 1991). PI507389 (*Rsv1-n* allele) does not show any resistance but necrosis when infected

with SMV-G1, G2, G5 and G6 (Ma et al., 2003). Suweon 97 carries the most valuable *Rsv1-h* allele that confers resistance to all SMV strains (Chen et al., 2002). Recently, a new allele *Rsv1-c* has been identified in Corsica that confers early resistance at the seedling stage (ER) to G2, G5, and G7 strains (Shakiba et al., 2013).

The *Rsv3* locus contains six alleles identified so far. These alleles exhibit complete dominance and confer resistance to more virulent strains G5 - G7, and susceptibility to the less virulent strains G1 - G4 (Table 1). *Rsv3* alleles were identified in OX686, L29, Harasoy, PI 61944, PI 61947, and PI 399091 (Buzzel and Tu, 1989; Buss et al., 1999; Cervantes, 2012; Gunduz et al., 2001; Shakiba et al., 2012b). L29 and Harasoy alleles display susceptibility to G1 through G4, and resistance to G5 through G7 (Buss et al., 1999; Gunduz et al., 2001). OX686 allele shows necrosis to G1 through G4, and resistance to G5 through G7 (Buzzel and Tu, 1989). PI 61944 (*Rsv3-n* allele) displays mix responses of necrosis and mosaic when infected by G1 or G2 strain, and confers resistance to G4, G5, G6, and G7 (Cervantes, 2012). PI 61947 (*Rsv3-h* allele) shows the same response as PI 61944 with the exception of mix reaction necrosis/mosaic to G3 (Shakiba et al., 2012b). PI 399091 (*Rsv3-c* allele) confers early resistance to G3 and G7, full resistance to G5, and susceptibility to G1, G2, and G6 (Shakiba et al., 2012b).

The *Rsv4* locus has three alleles identified in V94-5152, PI 88788, and Beeson (*Rsv4-b*) and confers resistance to all or most strains (Buss et al., 1997; Gunduz et al. 2004; Ma et al., 2002; Shakiba et al., 2011, 2013). This gene is dominant, non-necrotic and mostly non-strain specific (Table 1) (Saghai Maroof et al., 2010). The genotype V94-5152 carries *Rsv4* gene conferring resistance to all strains (Buss et al., 1997) and it is derived from the cultivar Columbia carrying both *Rsv3* and *Rsv4* genes (Ma et al., 2002). Due to top-level resistance, there is a high

interest of pyramiding the *Rsv4* locus with *Rsv1* and *Rsv3* loci as a defensive strategy for multiple SMV strains (Chen et al., 1994).

In most soybean cultivars, resistance is conferred by a single dominant gene that makes it an easy target for genetic manipulation. Resistance that is controlled by single gene occurs in 80% of all studied SMV resistant cultivars (Table 1) (Buss et al., 1989; Chen et al., 1991; Kang et al., 2005; Ma et al., 1995; Wang et al., 1998). Some resistant soybeans contain two complementary SMV resistance genes in various combinations (Table 2), reducing vulnerability of plant during virus infection (Chen et al., 1993; Liao et al., 2011; Shi et al., 2009; Zheng et al., 2006).

SMV resistance genes and host symptoms have been compared and summarized in Table 1 and 2 (Chen et al., 1991). The presence of each R-gene in soybean genotypes from Table 1 and 2 have been evaluated and confirmed by genetic and inheritance studies (Chen and Choi, 2008; Shi et al., 2008a, 2011); however, there is no information about those genes in most of the available germplasm collections. The germplasm collection was previously screened via SMV infections, and based on symptoms, R-genes have been proposed (Shakiba et al., 2012a; Shi et al., 2008b, 2012; Zheng et al., 2005).

MOLECULAR MECHANISMS OF SMV INFECTION

The molecular interactions between SMV and the host are complex and many mechanisms are still unknown. The virus is released directly into the host cell via mechanical damage of soybean tissue (Ivanov et al., 2014).

In susceptible plants, after entry into the cell the coat protein (CP) is removed first (virion encapsidation) and then the genetic information is translated. The genome is composed of

positive-sense, single stranded RNA with the virus genome-linked (VPg) protein at the 5'UTR (untranslated region), and a poly-A tail at the 3'UTR. The genome is a direct template for translation using the cap-independent internal ribosome entry site (IRES) for initiation of translation (Ivanov et al., 2014). Two products of translation are produced as precursors of functional proteins: (A) long polyprotein as a result of translation of the entire genome, (B) short polyprotein P3N-PIPO produced via ribosomal frameshift. After translation, polyproteins are subjected to proteolytic processing by three self-encoded proteases to yield mature proteins (Ivanov et al., 2014; Soosaar et al., 2005).

There are few main components of SMV infection: entry, uncoating, translation, replication, cell-to-cell and long-distance movement. Shortly after translation, the viral genome is replicated by its own replicase RdRp (RNA-dependent RNA polymerase) in association with cytoplasmic membranes that create a specific micro-environment to protect viral genome from silencing (Ivanov et al., 2014; Matthews et al., 1991; Soosaar et al., 2005). Some of the copied molecules are coated (virion assembly) while some copies remain uncoated and move into the neighbor cells through plasmodesmata as a nucleoprotein complex including viral movement proteins (CP, HC-Pro, CI, and p6K) that are capable of increasing a plasmodesmatal size exclusion limit (SEL) and mediate the passage of viral molecules between cells by interaction with the plant cytoskeleton. Long-distance movement occurs when the virus spreads through the vascular system and can infect cells located far from the initial infection point (systemic infection) (Rojas et al., 1997; Soosaar et al., 2005; Wei and Wang, 2008).

From the 11 viral proteins produced after translation, CP, VPg, HC-Pro, CI, and P3N-PIPO may play a role in viral transport through plasmodesmata (Dolja et al., 1994; Rojas et al., 1997; Wei et al., 2010; Wen et al., 2010). Long-distance movement via phloem is poorly

understood but four viral proteins, CP, HC-Pro, VPg, and 6K2 are associated with this phenomenon (Dolja et al., 1994). Babu et al. (2008) used microarray technology to detect expression changes of Williams 82 SMV-susceptible genome infected by SMV-G2 strain. Many genes of hormone metabolism, cell wall biogenesis, chloroplast functions and photosynthesis were significantly down-regulated at 14 days of post inoculation. The genes involved in defense were up-regulated at the late stages suggesting that the response to SMV was delayed and the plant could not combat the infection.

Molecular interactions between SMV and soybean R-genes have not been extensively studied. In a study of *Rsv1*-SMV interactions, Hajmorad et al. (2005, 2008) discovered that P3 is an elicitor of *Rsv1*-mediated necrosis; however, lack of P3 is not sufficient for G7 to gain virulence. Zhang et al. (2009) noticed that N- and C-terminal regions of the viral CI protein are required for *Rsv3*-mediated resistance. Based on the same strategy, Chowda-Reddy et al. (2011) described that the P3 of G2 strain is an avirulent elicitor for *Rsv4*.

MAPPING OF SMV RESISTANCE

The *Rsv1* gene was mapped on chromosome 13 (MLG F) by performing a cross PI 96983 (R) × Lee 68 (S), generating F_2 population and using two RFLP (pA186 and pK644a) and one SSR (SM176) markers linked to the *Rsv1* locus with distances of 1.5, 2.1, and 0.5 cM, respectively (Yu et al., 1994). One RAPD marker ($OPN11_{980/1070}$), and one SCAR marker ($SCN11_{980/1070}$) were also found linked to *Rsv1* with the same distance of 3.03 cM (Zheng et al., 2003). Gore et al. (2002) constructed a high resolution map with one RAPD, four SSRs, and 19 RFLPs, and concluded that the *Rsv1* gene is closely linked to the SSR marker Satt510 (<2.4 cM). In another study, a PCR-based primer Rsv1-f/r was developed based on *3gG2* gene with a

distance of 0 cM to the *Rsv1* (Shi et al., 2008b). Ma et al. (2011) mapped SC14Q resistance on chromosome 13 between Satt334 and MY750, with genetic distances of 0.6 and 0.5 cM, respectively, approximately corresponding to a physical distance of 1.18 Mb. Additionally, one SNP marker, MY525, was developed between Satt334 and MY750, and the interval was further narrowed to a 616 Kb region. Yang et al. (2013) mapped the resistance gene SC7 in PI 96983 to a 380 Kb region. In study by Zheng et al. (2014), a cross Qihuang 1 (R) × Nannong 1138-2 (S) was used to study inheritance and linkage mapping of the SC3 R-gene. The results indicated that a single dominant gene (R_{SC3Q}) located on chromosome 13 controls SMV resistance in Qihuang 1. Two SSR markers BARCSOYSSR_13_1114 and BARCSOYSSR_13_1136 were found flanking the two sides of the gene with the interval of 651 kb. In the same study, quantitative real-time PCR (qRT-PCR) analysis of the candidate genes showed that five genes *Glyma13g25730 (Glyma.13g187600), Glyma13g25750 (Glyma.13g187900), Glyma13g25950 (Glyma.13g190300), Glyma13g25970 (Glyma.13g190400), and Glyma13g26000 (Glyma.13g190800)*, were likely to be involved in soybean SMV resistance. Yan et al. (2015) performed linkage analysis using 184 RILs of a cross Kefeng No.1 (R) × Nannong 1138-2 (S), and association analysis using 191 soybean germplasm. The SC7 gene was positioned between BARCSOYSSR_13_1128 and BARCSOYSSR_13_1136 on chromosome 13.

The *Rsv3* gene was mapped on chromosome 14 (LG B2) by making two crosses L29 (R) × Lee 68 (S) and Tousan 140 (R) × Lee 68 (S), and using data collected from F_2 generations. The *Rsv3* gene was flanked by A519F/R at a distance of 0.9 cM and M3Satt at 0.8 cM (Jeong et al., 2002). Moreover, *Rsv3* was mapped in J05 cultivar using Sat_424 (1.5 cM) and Satt726 (2.0 cM) (Shi et al., 2008a). Recently, five nucleotide-binding leucine-rich repeat (NBS-LLR) genes *Gylma14g38500*, *Gylma14g38510*, *Gylma14g38540*, *Gylma14g38560*, and *Gylma14g38590*

were reported as *Rsv3* candidates (Suh et al. 2011). Wang et al. (2011b) crossed Dabaima (R) × Nannong1138-2 (S) and mapped the SC4 resistance on chromosome 14 (MLG B2) flanked by a 100 Kb interval between BARCSOYSSR_14_1413 and BARCSOYSSR_14_1416. Quantitative real-time PCR further identified *Glyma14g38510* (Wm82.a2.v1: *Glyma.14g204600*), *Glyma14g38560* (Glyma.14g205000) and *Glyma14g38580* (*Glyma.14g205200*) to be likely involved in this resistance.

The *Rsv4* was mapped on chromosome 2 (LG D1b) by crossing V94-5152 (R) × Lee 68 (S) using data of the F_2 generation. *Rsv4* was flanked between Satt542 at 4.7 cM and Satt558 at 7.8 cM (Hayes et al., 2000). Later, two ESTs markers AI856415-g or AI856415-S and BF070293-S were mapped at 2.8 cM on one side of the gene, and two ESTs markers AW307114A (3.3cM) and AW471852A (2.4 cM) on the other side (Hwang et al., 2006). In addition, Fu et al. (2006) mapped the SC7 resistance in Kefeng No.1 to a 2.65 Mb region on chromosome 2. SSR markers Satt266, Satt634, Satt558, Satt157, and Satt698 were reported to be linked to the SC7 with distances of 43.7, 18.1, 26.6, 36.4 and 37.9 cM, respectively. Recently, several studies focused on fine mapping of the *Rsv4* locus have been reported. Saghai Maroof et al. (2010) utilized the whole genome shotgun sequence for fine mapping the *Rsv4* gene in two populations D26 (R) × Lee 68 (S) and V94-5152 (R) × Lee 68 (S). Six markers were used to localize the gene in 1.3-cM region in both mapping populations with a physical interval of less than 100 kb on chromosome 02. In this region, ten candidate genes *Gylma02g13360*, *Gylma02g13370*, *Gylma02g13380*, *Gylma02g13390*, *Gylma02g13400, Gylma02g13410, Gylma02g13420, Gylma02g13430, Gylma02g13440,* and *Gylma02g13450* were proposed. Wang et al. (2011a) analyzed populations derived from Kefeng No.1 (R) × Nannong 1138-2 (S) to map SC8 resistance gene. Two SSR markers BARCSOYSSR_02_0610 and

BARCSOYSSR_02_0616 were identified that flank both sides of the gene with a 200 kb interval between them on chromosome 02. Further, expression analysis determined five candidate genes, *Glyma02g13310* (Glyma.02g120700), *Glyma02g13320* (Glyma.02g120800), *Glyma02g13400* (Glyma.02g121500), *Glyma02g13460* (Glyma.02g121900), and *Glyma02g13470* (Glyma.02g122000). In a recent study by Li et al. (2015), based on a cross of Kefeng No.1 (R) × Nannong 1138-2 (S) and SSR markers, the *Rsc18A* locus was mapped on chromosome 2 within a 80 Kb region; 6 putative genes were predicted, and three, *Glyma02g127800*, *Glyma02g128000*, and *Glyma02g128200*, displayed differences at the amino acid level.

Yan et al. (2015) used a set of 191 soybean accessions for association mapping and 184 RILs derived from Kefeng No.1 (R) × Nannong 1138-2 (S) to identify and fine-map soybean genes associated with resistance to SMV strain SC7. Among 19 SNPs detected via association analysis, BARC-021625-04157 was located in the 2.65 Mb region, and fine-mapped to the *Rsv4* region of approximately 158 kb between BARCSOYSSR_02_0621 and BARCSOYSSR_02_0632 on chromosome 2. From the fifteen genes within this region, three SC7 candidate genes *Glyma09g34200* (Wm82.a2.v1: *Glyma.09g208900*, NBS-LRR type gene), *Glyma11g08480* (*Glyma.11g079900*, HSP40 gene), and *Glyma16g27560* (*Glyma.16g159700*, serine carboxypeptidase-type gene) have been proposed.

In addition, Yang and Gai (2011) crossed 'RN-9' (R) × '7605' (S) in order to study inheritance of resistance to SC15 Chinese SMV strain. Results indicated that a single dominant gene, designated as *RSC15*, conferred the SMV resistance. The genetic linkage analysis was used to map SC15 resistance between Sat_213 and Sat_286 with distances of 8.0 and 6.6 cM on chromosome 6 (MLG C2).

SMV RESISTANT ISOGENIC LINES

Isogenic lines are genetically identical pure-breeding group of individuals with a difference of a single gene (and its linkage drag) introduced into a susceptible cultivar by backcrossing technique. Two sets of isogenic lines for SMV resistance alleles have been released for breeding and genetic study purposes. These isogenic lines have been evaluated and their SMV resistance allele in each isogenic line was identified (Buss et al., 1997; Saghai Maroof et al., 2008; Wang et al., 2006).

Williams isogenic lines were developed by Dr. R.L. Bernard, Dep. of Crop Sciences, University of Illinois (Wang et al., 2006). Williams isogenic lines, designed as L-series, was derived by crossing Williams with 10 resistant lines: PI 96983, Buffalo (2×), Raiden, PI 486355, Suweon 97, Ogden, Marshall, Dorman, Hardee, and then backcrossing, resulting in isolines possessing different alleles of *Rsv1* and *Rsv3* loci.

Essex isogenic lines (V-series) were developed by Dr. G.R. Buss, Dep. of Crop and Soil Environmental Sciences, Virginia Polytechnic Institute and State University (Buss et al., 1997; Li et al., 2009). Four out of six isolines V94-3971, V262, V229, and V97-9003 were derived from backcrosses of Epps (*Rsv1*) × Essex (*rsv*), PI 507389 (*Rsv1-n*) × Essex (*rsv*), L29 (*Rsv3*) × Essex (*rsv*), and V94-5152 (*Rsv4*) × Essex (*rsv*), respectively. Essex isolines show resistant, necrotic, or susceptible reactions when infected by the same SMV strain. For example, infection by G1 strain provides resistance in V94-3971 and V97-9003, necrosis in V262, and susceptibility in V229. Induced symptoms do not depend on virus strain but do depend on a host genotype.

MARKER-ASSISTED SELECTION AND GENE PYRAMIDING

The most effective way of controlling SMV is incorporation of genetic resistance into the susceptible genotype either via classical breeding or genetic engineering (transgenesis and gene editing) (Liu et al., 2012; Soosaar et al., 2005). Gene pyramiding (GP) is an excellent tool combining multiple resistance genes by performing crosses or a series of backcrosses. The soybean-SMV interactions have been studied at the molecular level and gene pyramiding can be implemented applying marker-assisted selection (MAS). MAS has been widely used in disease resistance selection by implementation of molecular markers (especially SSR and SNP) in order to identify genes or combine genes into a single target genotype (Collard and Mackill, 2008; Fooland and Panthee, 2012). Some soybean lines with resistance to all SMV strains contain two complementary resistance genes in diverse combinations that cannot be distinguished by plant reactions to SMV strains. Pyramiding of all three genes (*Rsv1*, *Rsv3*, and *Rsv4*) can be performed through MAS using linked molecular markers in order to develop new soybean lines with multiple SMV resistance genes (Fooland and Panthee, 2012; Shi et al. 2012; Song et al., 2010; Suh et al., 2011). To find polymorphism between parents it is necessary to conduct an initial screening using molecular markers at the MLG B2, D1b, and F (Table 3). Molecular markers are the basis for an efficient MAS in scientific research and commercial soybean breeding. The availability of various molecular markers closely linked to each of the resistance genes makes the identification of these genes possible (Collard and Mackill, 2008).

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Table 1. Reactions of soybean genotypes carrying a single resistance gene to seven Soybean mosaic virus strains in the U.S.: R, resistant (symptomless), N, necrotic (systemic necrosis); S; susceptible (mosaic); ER, early resistant at seedling stage; N/S, mixture of necrotic and susceptible reactions; R/N, mixture of resistant and necrotic reactions.

NAME	ORIGIN	G1	G2	G3	G4	G5	G6	G7	GENE	REFERENCE
PI 96983	Korea	R	R	R	R	R	R	N	*Rsv1*	Kiihl and Hartwing, 1979
Suweon 97	Korea	R	R	R	R	R	R	R	*Rsv1-h*	Chen et al., 2002
York	USA	R	R	R	N	S	S	S	*Rsv1-y*	Chen et al., 1991
Raiden	Japan	R	R	R	R	N	N	R	*Rsv1-r*	Chen et al., 2001
Kwanggyo	Korea	R	R	R	R	N	N	N	*Rsv1-k*	Chen et al., 1991
Ogden	USA	R	R	N	R	R	R	N	*Rsv1-t*	Chen et al., 1991
Marshall	USA	R	N	N	R	R	N	N	*Rsv1-m*	Chen et al., 1991
PI 507389	USA	N	N	S	S	N	N	S	*Rsv1-n*	Ma et al., 2003
LR1	USA	R	R	R	R	N	N	R	*Rsv1-s*	Ma et al., 1995
Corsica	USA	S	ER	S	-	ER	S	ER	*Rsv1-c*	Shakiba et al., 2012
L29	USA	S	S	S	S	R	R	R	*Rsv3*	Buss et al., 1999
OX 686	Canada	N	N	N	N	R	R	R	*Rsv3*	Buzzel and Tu, 1989
Harosoy	Canada	S	S	S	S	R	R	R	*Rsv3*	Gunduz et al., 2001
PI 61944	China	N/S	N/S	R	-	R	R	R	*Rsv3-n*	Cervantes, 2012
PI 61947	China	N/S	N/S	R/N	-	R	R	R	*Rsv3-h*	Shakiba et al., 2012
PI 399091	Korea	S	S	ER	-	R	S	ER	*Rsv3-c*	Shakiba et al., 2012
V94-5152	USA	ER	ER	ER	ER	ER	ER	ER	*Rsv4*	Buss et al., 1997
PI 88788	China	ER	ER	ER	ER	ER	ER	ER	*Rsv4*	Gunduz et al., 2004
Beeson	USA	ER	ER	S	-	R	ER	R	*Rsv4-b*	Shakiba et al., 2012

Table 2. Reactions of soybean genotypes carrying none, two or three resistance genes to seven Soybean mosaic virus strains in the U.S.: R, resistant (symptomless); N, necrotic (systemic necrosis); S, susceptible (mosaic).

NAME	ORIGIN	G1	G2	G3	G4	G5	G6	G7	GENE	REFERENCE
Essex	USA	S	S	S	S	S	S	S	*rsv*	Chen et al., 1991
Lee 68	USA	S	S	S	S	S	S	S	*rsv*	Chen et al., 1991
Hourei	Japan	R	R	R	R	R	R	R	*Rsv1 Rsv3*	Gunduz et al., 2002
OX 670	Canada	R	R	R	R	R	R	R	*Rsv1 Rsv3*	Gunduz et al., 2001
Tousan 140	Japan	R	R	R	R	R	R	R	*Rsv1 Rsv3*	Gunduz et al., 2002
J05	China	R	R	R	R	R	R	R	*Rsv1 Rsv3*	Zheng et al., 2006
Zao18	China	R	R	R	R	R	R	R	*Rsv1 Rsv3*	Liao et al., 2002
Jindou 1	China	R	R	R	R	R	R	R	*Rsv1 Rsv3*	Shi et al., 2012
PI 486355	Korea	R	R	R	R	R	R	R	*Rsv1 Rsv4*	Chen et al., 1993; Ma et al., 1995
Columbia	Korea	R	R	R	R	R	R	R	*Rsv3 Rsv4*	Ma et al., 2002
8101	China	R	R	R	R	R	R	R	*Rsv1Rsv3 Rsv4*	Liao et al., 2011

Table 3. PCR-based markers and their positions in relation to three SMV resistance loci in soybean linkage map.

MARKER	MLG	Chr. No.	cM	LOCUS	REFERENCES
Sat_297	F	13	59.6	*Rsv1*	Cregan et al., 2003; Song et al., 2004
Sat_229	F	13	62.8	*Rsv1*	Cregan et al. 2003; Song et al., 2004
Satt114	F	13	63.7	*Rsv1*	Cregan et al., 2003; Song et al., 2004
Sat_234	F	13	66.6	*Rsv1*	Cregan et al., 2003; Song et al., 2004
SOYHSP176	F	13	68.4	*Rsv1*	Yu et al., 1996; Cregan et al., 2003
Sat_154	F	13	68.9	*Rsv1*	Cregan et al., 2003; Song et al., 2004
Rsv1-f/r	F	13	69.1	*Rsv1*	Shi et al., 2006; Shi et al., 2008
Satt510	F	13	71.4	*Rsv1*	Gore et al., 2002; Cregan et al., 2003
Sat_317	F	13	73	*Rsv1*	Cregan et al., 2003; Song et al., 2004
Sct_103	F	13	74.1	*Rsv1*	Song et al., 2004
Sat_120	F	13	76	*Rsv1*	Gore et al., 2002; Cregan et al., 2003
Satt334	F	13	78.1	*Rsv1*	Cregan et al., 2003; Song et al., 2004
Satt063	B2	14	93.5	*Rsv3*	Jeong et al., 2002; Cregan et al., 2003
A519	B2	14	96.7	*Rsv3*	Jeong et al., 2002
M3Satt	B2	14	97.5	*Rsv3*	Jeong et al., 2002
Satt560	B2	14	97.9	*Rsv3*	Cregan et al., 2003; Song et al., 2004
Sat_424	B2	14	100.1	*Rsv3*	Cregan et al., 2003; Song et al., 2004
Satt726	B2	14	100.6	*Rsv3*	Cregan et al., 2003; Song et al., 2004
Satt687	B2	14	113.6	*Rsv3*	Cregan et al., 2003; Song et al., 2004
Satt558	D1b	2	43.9	*Rsv4*	Hayes et al. 2000; Cregan et al., 2003
BF070293-S	D1b	2	46	*Rsv4*	Hwang et al., 2006
AI856415-g	D1b	2	46	*Rsv4*	Hwang et al., 2006
AI856415-S	D1b	2	46	*Rsv4*	Hwang et al., 2006
BI470504	D1b	2	46.5	*Rsv4*	Song et al., 2004; Hwang et al., 2006
Satt634	D1b	2	46.6	*Rsv4*	Cregan et al., 2003; Song et al., 2004
Sat_254	D1b	2	46.9	*Rsv4*	Cregan et al., 2003; Hwang et al., 2006
BF070293	D1b	2	47.3	*Rsv4*	Cregan et al., 2003; Song et al., 2004
A1856415	D1b	2	50.1	*Rsv4*	Cregan et al., 2003; Song et al., 2004
AW307114A	D1b	2	51.1	*Rsv4*	Hwang et al., 2006
AW471852R	D1b	2	51.2	*Rsv4*	Hwang et al., 2006
Satt296	D1b	2	52.6	*Rsv4*	Cregan et al., 2003; Song et al., 2004
Satt542	D1b	2	53	*Rsv4*	Hayes et al., 2000; Cregan et al., 2003

Table 4. Previously reported candidate genes for the *Rsv1*, *Rsv3*, and *Rsv4* SMV resistance genes.

Genome Assembly Wm82.a1.v1	Genome Assembly Wm82.a2.v1	Physical Position	Possible Function	R-Gene	References
Glyma13g25730	*Glyma.13g187600*	30134637..30143817	LRR Kinase	*Rsv1*	Yang et al., 2013
Glyma13g25750	*Glyma.13g187900*	30174410..30180072	LRR Kinase	*Rsv1*	Yang et al., 2013
Glyma13g25950	*Glyma.13g190300*	30388583..30392233	LRR Kinase	*Rsv1*	Yang et al., 2013
Glyma13g25970	*Glyma.13g190400*	30402029..30409606	LRR Kinase	*Rsv1*	Yang et al., 2013
*Glyma13g26000**	*Glyma.13g190800*	30423894..30430435	LRR Kinase	*Rsv1*	Hayes et al., 2004; Yang et al., 2013
Gylma14g38500	*Glyma.14g204500*	46946496..46957734	LRR Kinase	*Rsv3*	Suh et al., 2011
Gylma14g38510	*Glyma.14g204600*	46968705.46974585.	LRR Kinase	*Rsv3*	Suh et al., 2011; Wang et al., 2011b
Gylma14g38540	-	na		*Rsv3*	Suh et al., 2011
Gylma14g38560	*Glyma.14g205000*	47005574..47019661	LRR Kinase	*Rsv3*	Suh et al., 2011; Wang et al., 2011b
Glyma14g38580	*Glyma.14g205200*	47041931..47046048	Cytochrome P450	*Rsv3*	Wang et al., 2011b
Gylma14g38590	*Glyma.14g205300*	47046209..47056610	LRR Kinase	*Rsv3*	Suh et al., 2011
Glyma02g13310	*Glyma.02g120700*	11904074..11910578	Cytochrome P450	*Rsv4*	Wang et al., 2011a
Glyma02g13320	*Glyma.02g120800*	11926840..11931251	LRR Kinase	*Rsv4*	Wang et al., 2011a
Gylma02g13360	-	11983986..11999753	Unknown	*Rsv4*	Saghai Maroof et al., 2010
Gylma02g13370	-	12006720..12013300	Unknown	*Rsv4*	Saghai Maroof et al., 2010
Gylma02g13380	*Glyma.02g121400*	12028928..12030693	Unknown	*Rsv4*	Saghai Maroof et al., 2010
Gylma02g13390	-	na	na	*Rsv4*	Saghai Maroof et al., 2010
Gylma02g13400	*Glyma.02g121500*	12065640..12082937	MADS Box TF	*Rsv4*	Saghai Maroof et al., 2010; Wang et al., 2011a
Gylma02g13410	-	na	na	*Rsv4*	Saghai Maroof et al., 2010
Gylma02g13420	-	12084616..12089110	Unknown	*Rsv4*	Saghai Maroof et al., 2010
Gylma02g13430	-	na	na	*Rsv4*	Saghai Maroof et al., 2010
Gylma02g13440	-	na	na	*Rsv4*	Saghai Maroof et al., 2010
Gylma02g13450	-	12106072..12107969	Unknown	*Rsv4*	Saghai Maroof et al., 2010
Glyma02g13460	*Glyma.02g121900*	12112034..12115027	Unknown	*Rsv4*	Wang et al., 2011a
Glyma02g13470	*Glyma.02g122000*	12115284..12118493	Unknown	*Rsv4*	Wang et al., 2011a
Glyma02g14160	*Glyma02g127800*	13010651..13015848	LRR Kinase	*Rsv4*	Li et al., 2015
Glyma02g14190	*Glyma.02g128000*	13048160..13051248	Decarboxylase	*Rsv4*	Li et al., 2015
Glyma02g14200	*Glyma02g128200*	13093448..13095566	Methyltransferase	*Rsv4*	Li et al., 2015

-, no reported correspondence between genome assemblies; na, data not available; **3gG2* gene name; LRR, Leucine-Rich Repeat.

Figure 1. Foliar symptoms of soybean genotypes in response to Soybean mosaic virus G7 strain: resistant line L29 showing no symptoms of the disease (left); susceptible cultivar York displaying mosaic symptoms (middle); necrotic response of PI 96983 displaying a systemic necrosis and plant death (right).

CHAPTER TWO

GENETIC DIVERSITY AND SNP MARKERS FOR RESISTANCE
TO SOYBEAN MOSAIC VIRUS IN SOYBEAN

ABSTRACT

Soybean mosaic virus (SMV) causes a substantial decrease in soybean yield and reduction of seed quality. The most effective management strategy to control the virus is the deployment of host resistance. Seven SMV strains, and three independent multi-allelic loci for SMV resistance have been identified previously. The goal of this research was to detect single nucleotide polymorphisms (SNPs) associated with SMV resistance at the *Rsv4* locus. Ten soybean accessions, with confirmed resistance genes, were used for sequencing the candidate gene *Glyma.02g121400*. Alignment of these sequences revealed three SNPs displaying 100% consistency for genotypes carrying the *Rsv4* gene. These SNPs were applied for a rapid screen of diverse soybean germplasm using the Sequenom iPLEX Gold platform, phenotyped with SMV-G1 and G7 strains to determine phenotype and classified into several groups carrying the proposed R-gene. The population of V94-5152 (*Rsv4*) × Lee 68 (*rsv*) was screened using novel SNPs to create a genetic map with improved resolution to determine the location of the *Rsv4*. To observe the recombination frequencies within the population, three additional SNPs on both sides of the *Glyma.02g121400* gene were added. A linkage map revealed a distance of 3.6 cM between the *Rsv4* locus and the closest SNP, thus shifting the putative *Rsv4* region downstream on chromosome 2. With regard to this distance, five candidate genes have been proposed. The genomic position of the discovered SNPs, linked to the *Rsv4,* could increase screening precision and accelerate breeding efforts to develop multi-strain resistant crops.

INTRODUCTION

Soybean mosaic virus infects soybean [*Glycine max* (L.) Merr.], considerably reducing yield and seed quality (Ren et al. 1997; Tolin 1999). The virulence of SMV has diverged during the co-evolution of virus and host, leading to the emergence of strains that display different levels of virulence. In the United States, SMV isolates have been grouped into seven strains, G1-G7, where G1 is the least and G7 is the most virulent strain upon infecting differential soybean accessions (Cho and Goodman 1979).

Disease symptoms depend on host genotype, virus strain, time of infection, and environmental conditions (Chen et al. 1994; Li et al. 2009; Ren et al. 1997). Phenotypic reactions are classified into three major categories: resistant (R), susceptible (S), and necrotic (N) (Cho and Goodman 1979). Susceptible soybean genotypes typically display transient vein clearing followed by mosaic symptoms. As the disease progresses, leaf areas develop puckering or more general rugosity and leaf edges twist downward (Hill 1999; Tolin 1999). Necrotic symptoms are characterized by yellow discoloration of leaves, stunting, browning of steams and petioles, defoliation, and finally plant death (Buzzell and Tu 1989; Chen et al. 1994; Li et al. 2009; Ma et al. 2003).

Chemical and cultural control of SMV is neither economically nor environmentally-friendly, and deployment of genetic resistance is the most effective alternative to manage the disease (Carrington and Whitham 1998; Shakiba et al. 2012b). Three multiallelic resistance loci, *Rsv1*, *Rsv3*, and *Rsv4*, have been previously reported (Buss et al. 1997; Buzzel and Tu 1989; Kiihl and Hartwing 1979), and mapped on chromosome 13 (MLG F), 14 (MLG B2), and 02 (MLG D1b) respectively (Hayes et al. 2000; Jeong et al. 2002; Yu et al. 1994). Resistance to SMV is probably controlled by a single dominant gene (Buss et al. 1997; Chen et al. 1994;

Shakiba et al. 2012a); however, two or three complementary genes have also been identified (Liao et al. 2011; Ma et al. 2002; Shi et al. 2013). The *Rsv1* gene confers resistance to less virulent strains (G1-G7), whereas the *Rsv3* locus displays resistance to more virulent strains (G5-G7). Genotypes carrying the *Rsv4* locus display resistance to most or all strains identified in U.S. (G1-G7). Based on symptoms of genotypes, it is possible to predict classify them into groups of the resistance gene they carry; however, due to masking effect of each SMV gene, soybean lines resistant to all SMV strains cannot have their genes predicted (Chen et al. 1991; Zheng et al. 2005).

The *Rsv4* locus harbors at least three alleles identified in V94-5152, PI 88788, and Beeson (*Rsv4-b*) (Buss et al. 1997; Gunduz et al. 2004; Shakiba et al. 2012). The alleles exhibit complete dominance and confer resistance to all SMV strains (G1 - G7); however, they may express delayed and mild susceptibility exhibiting mosaic symptoms (ER) in some genotypes at a later stage (Buss et al. 1997; Gunduz et al. 2004) and delaying virus replication and movement (Ma et al. 1995). The *Rsv4* gene was found to function in a non-strain specific and non-necrotic manner (Buss et al. 1997; Gunduz et al. 2004).

Several research studies focused on mapping the *Rsv4* locus have been conducted. Microsatellite markers (SSRs) Satt634 (46.6 cM) and Satt542 (53 cM) were previously found to flank the *Rsv4* (Hayes et al. 2000). At a later date, two ESTs markers AI856415 (46 cM) and BF070293 (46 cM) were mapped at 2.8 cM on one side of the gene and two ESTs markers AW307114 (51.1 cM) and AW471852 (51.2 cM) were mapped on the other side (Hwang et al. 2006). Saghai Maroof et al. (2010) utilized the whole genome shotgun sequence to map the *Rsv4* in two populations D26 (*Rsv4*) × Lee 68 (*rsv*) and V94-5152 (*Rsv4*) × Lee 68 (*rsv*). Six new SSR markers were used to localize the gene in 1.3-cM region in both mapping populations with a physical interval of less than 100 kb on chromosome 02. In this region (Gm02:11,651,991-

11,771,944), ten candidate genes were proposed: *Gylma02g13360*, *Gylma02g13370*, *Gylma02g13380*, *Gylma02g13390*, *Gylma02g13400, Gylma02g13410, Gylma02g13420, Gylma02g13430, Gylma02g13440,* and *Gylma02g13450*. Wang et al. (2011) analyzed populations derived from Kefeng No.1 (R_{SC8}) × Nannong 1138-2 (*rsv*) to map a gene that causes resistance to the Chinese SMV strain SC8. Two SSR markers BARCSOYSSR_02_0610 and BARCSOYSSR_02_0616 were identified that flank both sides of the gene with 200 kb interval (Gm02:11,567,483-11,782,246). Expression analysis determined five candidate genes: *Glyma02g13310* (correspondence for Wm82.a2.v1: *Glyma.02g120700*), *Glyma02g13320* (*Glyma.02g120800*)*, Glyma02g13400* (*Glyma.02g121500*), *Glyma02g13460* (*Glyma.02g121900*), and *Glyma02g13470* (*Glyma.02g122000*). Ilut et al. (2015) used a population V94-5152 (*Rsv4*) × Sowon (*rsv*) BC_3F_2 to fine-map the *Rsv4* to a 94 kb interval (12,071,517-12,165,890). Eleven candidate genes were proposed in the 12,065,640-12,163,084 region: *Glyma.02g121500, Glyma.02g121600, Glyma.02g121700, Glyma.02g121800, Glyma.02g121900, Glyma.02g122000, Glyma.02g122100, Glyma.02g122200, Glyma.02g122300, Glyma.02g122400*, and *Glyma.02g122500*. Yan et al. (2015) used a set of 191 accessions for association mapping and 184 RILs derived from Kefeng No.1 (R_{SC7}) × Nannong 1138-2 (*rsv*) to fine-map soybean genes associated with resistance to SMV-SC7 strain. Among 19 SNPs, BARC-021625-04157 was located in the 2.65 Mb region between two closest SSR markers Satt266 and Satt634, and fine-mapped to a region of approximately 158 kb (11805400-11975404) on chromosome 2 containing fifteen genes. In research by Li et al. (2015), using a cross of Kefeng No.1 (R) × Nannong 1138-2 (S) and SSR markers, the *Rsc18A* locus was mapped on chromosome 2 within 80 Kb region (Gm02:13,010,651-13,095,566); six putative genes were predicted, and three of them, *Glyma02g127800*, *Glyma02g128000*, and *Glyma02g128200*, displayed differences at the amino acid level.

Molecular markers provide a powerful substitution for the labor intensive and slow process of phenotyping; however, the value of markers is limited because the exact positions of SMV R-genes in the soybean genome cannot be determined. Due to the limited number of simple sequence repeats (SSRs) in the genome, marker implementation has recently shifted to single nucleotide polymorphism (SNP) technologies, which allow for the saturation of a specific region with different marker densities (Shi et al. 2011). Now that the soybean genome has been sequenced (Schmutz et al. 2010), molecular markers can be connected to the specific positions of interest in the genome, thereby improving the information provided by SNPs. New markers can be detected from different cultivars by PCR-sequencing of short DNA fragments or large chromosomal regions using next generation sequencing (NGS).

The specific objectives of this study were to: (a) discover and validate SNP markers for marker-assisted selection (MAS); (b) assess genetic diversity of soybean germplasm towards SMV resistance; and (c) map the *Rsv4* locus and propose candidate gene(s). The goal of this research was to discover SNPs associated with SMV resistance at the *Rsv4* locus and thus allow for more effective ways to analyze and manage data, integrate phenotypic results, and apply new tools for breeding purposes.

MATERIALS AND METHODS

Plant material and growth conditions

A total of 299 soybean accessions, including 40 checks with known R-genes, were used to identify SMV resistance. An average of 12 seeds from each genotype were planted in three sets; one for iPLEX genotyping and two for phenotyping by SMV-G1 and G7 strains. The greenhouse was maintained at 25-28°C and 14 h photoperiod at the Harry R. Rosen Alternative Pest Control Center of University of Arkansas, Fayetteville, AR.

DNA extraction and iPLEX genotyping

A bulk of young trifoliate leaves was collected from each line for DNA extraction. Genomic DNA was extracted from young leaves using the modified CTAB (cetyltrimethylammonium bromide) method (Doyle and Doyle 1990). Frozen leaves were crushed to powder with metal beads using TissueLyser II (Qiagen), then 750 µl extraction buffer (2% CTAB, 100mM Tris-Cl, 20mM EDTA pH 8.0, 1.4M NaCl, and 1% of volume β-mercaptoethanol) was added to each tube and incubated at 60°C. After one hour, tubes were cooled down and 1 ml chloroform:isoamyl alcohol (24:1) was added to each tube. Samples were centrifuged at 12,851 g for 15 min. The supernatant was transferred to a new tube and incubated with RNase A for one hour at 37°C. For DNA precipitation, 1 ml 95% ethanol was added and tubes were gently inverted several times. Samples were centrifuged at 20,817 g for 5 min and DNA pellets were washed with 75% ethanol. DNA was dissolved in 200 µl sterilized distillated water, and total concentration was measured using a NanoDrop™ ND-2000 (Thermo Scientific).

For initial screening, a total of 11 SNP primers linked to the *Rsv4* were preselected for random testing for polymorphisms among the 40 checks, covering the region between 11,904,074-12,107,969 on chromosome 2 and containing previously reported candidate genes (Shaghai Maroof et al. 2010; Wang et al. 2011). Screening with the three discovered SNPs, ss244712651, ss244712651, and ss244712653 was performed by multiplex PCR. Genotyping was conducted at the University of Minnesota Genomics Center using the Sequenom iPLEX Gold genotyping platform, followed by mini-sequencing reactions in a single well. The size of reaction products was determined directly by MALDI-TOF mass spectrometry, yielding genotype information.

Sequencing

Ten genotypes with known SMV reactions and resistance loci were used for sequencing the *Glyma.02g121400* locus and its flanking regions: PI 96983 (*Rsv1*), V94-3971 (*Rsv1*), L29 (*Rsv3*), V229 (*Rsv3*), Harosoy (*Rsv3*), V94-5152 (*Rsv4*), V97-9003 (*Rsv4*), PI 88788 (*Rsv4*), Essex (*rsv*), and Williams 82 (*rsv*). Three pairs of gene-specific primers were designed via BatchPrimer3 software to amplify overlapping fragments of approximately 600 bp long covering a chromosomal region of 1,539 bp. Each polymerase chain reaction (PCR) mixture consisted of 15×Green GoTaq Flexi Buffer (Promega), 45mM $MgCl_2$, 2.5mM dNTPs, 5mM primer mix, 1U Taq (Promega), and 80ng DNA. PCR products were amplified with a program of 94°C for 5 min initial denaturation; 30 cycles of 45 s at 94°C denaturation, 45 s at 47°C primers annealing, 45 s at 68°C extension, and 5 min at 72°C final extension after the last cycle. After PCR, amplified products were separated on 1.2% high-melting agarose (Amresco) gels containing GelRed, in 1×TAE buffer. Amplified DNA fragments were visualized under UV light, extracted from the gel, and purified by Zymoclean™ Gel DNA Recovery Kit.

Sequencing of both DNA strands of the products was performed using ABI 3130xe Genetic Analyzer (Applied Biosystems) for capillary electrophoresis at the DNA Resource Center**,** Center of Excellence for Poultry Science, University of Arkansas, Fayetteville, AR. Sequences of both DNA strains were aligned to the Williams 82 reference genome (Grant et al. 2010) and data were analyzed using BioEdit (Clustal W function).

Population development and KASP genotyping

In order to map and validate discovered SNPs linked to the *Rsv4* locus, a population V94-5152 (*Rsv4*) × Lee 68 (*rsv*) was developed at the Arkansas Agricultural Research and

Extension Center, University of Arkansas, Fayetteville, AR. Pubescence color and SSR marker Sat_254 were used to confirm true F_1 hybrids.

Leaf tissues were collected from 766 F_2 plants for DNA extraction. Three additional putative SNPs on each side of the *Glyma.02g121400* gene were designed to observe recombination frequencies within a population. Genotyping was conducted using KASP™ assay (LGC Genomics, Beverly, MA) according to Semagn et al. (2014). A Chi-square test (χ^2) was used to determine the goodness of fit of observed recombination fraction from the F_2 population to the expected genetic ratios. In addition, corresponding $F_{2:3}$ lines were used for phenotypic screening with SMV-G7 strain inoculation in the greenhouse.

For linkage and genetic map construction, F_2 genotypic data and $F_{2:3}$ phenotypic results were collated by JoinMap version 3.0 at a logarithm of odds ratio (LOD) of 3.0 to indicate linkage. Recombination values were converted to genetic distances using LOD value for a single linkage group. Whole genome information available at Phytozome 10.3 (www.phytozome.net/soybean) and SoyBase were used to define the soybean candidate genes.

SMV inoculation

Two SMV strains, G1 and G7, were used to screen the germplasm collection and G7 strain was used to phenotype $F_{2:3}$ lines of V94-5152 × Lee 68. Strain identities were confirmed by their foliar reactions on sets of differentials including: PI 96983 (*Rsv1*), York (*Rsv1-y*), V262 (*Rsv1-n*), L29 (*Rsv3*), V229 (*Rsv3*), V94-5152 (*Rsv4*), V97-9003 (*Rsv4*), and Lee 68 (*rsv*). SMV was introduced into each plant by mechanical inoculation of at least 15 individuals/genotype according to Chen et al. (1991). Briefly, the inoculum was prepared by grinding infected leaves in ice-cold 0.01M potassium phosphate buffer (pH=7.2) at an approximate dilution 1:10 (w/v). Both unifolate leaves of each plant (before V1 stage) were pre-dusted with 600-mesh

carborundum, and rubbed with a pestle dipped in the inoculum. Inoculations using two strains were performed in separate greenhouses to prevent cross contamination. The greenhouse conditions were maintained at 28°C with a 14 h photoperiod at the Harry R. Rosen Alternative Pest Control Center, University of Arkansas in Fayetteville, AR.

Foliar reactions to each SMV strain were monitored each week, compared with set of checks 2-4 weeks after inoculation, and classified foliar reactions into three major phenotypes as resistant, susceptible, and necrotic. Based on specific reaction of symptoms obtained from G1 and G7 infection, R-genes were proposed. Phenotyping was further compared and collated with SNP genetic marker results for validation of SNPs accuracy in marker-assisted selection (MAS).

RESULTS

Initial genotyping

Eleven putative SNPs linked to the *Rsv4* were preselected (Soybase SNP list) to randomly test for polymorphisms among 40 soybean checks with known SMV resistance (Table 1) using the Sequenom iPLEX Gold genotyping platform covering the region 11,904,074-12,107,969 on chromosome 2 and containing previously proposed candidate genes (Shaghai Maroof et al. 2010). In this run, a single SNP (ss244712651) displayed polymorphism and this became a deciding factor to sequence the region where the SNP was located. The location of this SNP was found by pair-wise comparisons of the SNP-flanking sequence with the reference genome of Williams 82 (Grant et al. 2010; Schmutz et al. 2010). The SNP was identified in the coding sequence of the *Glyma.02g121400* locus (Gm02:11,692,905-11,694,242), which was previously reported as a candidate SMV resistance gene (Shaghai Maroof et al. 2010).

Sequencing

Ten soybean accessions with known SMV resistance alleles were used as checks for the *Glyma.02g121400* locus sequencing, including two without R-loci, two carrying the *Rsv1* locus, three with the *Rsv3*, and three with the *Rsv4* (Table 2). Final sequences of 1,539 bp of each check genotype were aligned to their complementary strands to assure quality of the sequencing procedure. Clustal W analysis revealed three polymorphic SNPs: ss244712651 (Gm02:11,693,196), ss244712652 (Gm02:11,693,604), and ss244712653 (Gm02:11,693,900). These SNPs displayed perfect polymorphic consistency when a soybean genotype carrying the *Rsv4* gene was present (Table 2). The results were confirmed by direct comparisons of the sequences with the reference genome of Williams 82 (Wm82.a2) at SoyBase.

Germplasm classification

Identified SNPs, ss244712651, ss244712651, and ss244712653, were used for large scale testing of 299 soybean accessions by the Sequenom iPLEX Gold genotyping platform. The results were compared and combined with phenotypic data obtained via inoculations with SMV-G1 and G7 strains. The reactions of 40 soybean checks displayed the expected foliar symptoms as reported in previous studies (Table 1) thus confirming the integrity of the SMV strains used in this study. Phenotypic and genotypic results of the soybean checks revealed perfect consistency indicating 100% accuracy between SNP markers, response to the virus, and the *Rsv4* locus (Table 5). The soybean checks carrying *Rsv4* displayed a characteristic nucleotide pattern of A-G-G obtained from ss244712651, ss244712652, and ss244712653 respectively. Checks without the *Rsv4* locus exhibited the T-C-A nucleotide pattern (Tables 3, 4).

Soybean germplasm collection was separately phenotyped with SMV-G1 and G7 strains to observe differences in reaction of symptoms, and lines with unknown SMV resistance genes

were classified into several groups based on symptoms pattern of the 40 checks; however, genotypes displaying resistance to both strains could not be differentiated based on phenotype due to the masking effect of multiple resistance genes that can be present as single genes or in combination within a single soybean genotype. This unclassified group could potentially carry some alleles at the *Rsv1* (*Rsv1-r, Rsv1-h*) and *Rsv4* locus, or a combination of two (*Rsv1+Rsv3, Rsv1+Rsv4, and Rsv3+Rsv4*), and three (*Rsv1+Rsv3+Rsv4*) R-genes. These accessions were further differentiated by the *Rsv4* locus presence/absence based on a specific SNP marker pattern (A-G-G vs. T-C-A) obtained from genotyping by three identified SNPs (Tables 3, 4, 5).

A total of 299 accessions were divided into two sub-groups as carrying the SNP pattern of A-G-G (potentially carrying the *Rsv4*) or T-C-A (without the *Rsv4* locus). The grouping was accomplished using the phenotypic results obtained from inoculation by SMV strains. There were 62 accessions classified into the first sub-group (A-G-G), whereas 29 of them displayed resistant reactions to both, G1 and G7 strains (Table 5). The second sub-group (T-C-A) of 237 accessions was further sorted into fractions of genotypes with absence of any SMV R-gene (*rsv*) (Table 3) or carrying alleles at the *Rsv1* and *Rsv3* loci (Table 4). Among this sub-group, 70 accessions were susceptible to both strains and classified as *rsv*; 56 lines displayed resistance to G1 strain and systemic necrosis to G7; and therefore they potentially carry *Rsv1, Rsv1-k, Rsv1-t,* or *Rsv1-m* alleles of the *Rsv1* locus; 57 showed resistance to G1 and susceptibility to G7, probably carrying *Rsv1-y* allele at the *Rsv1* locus; 21 lines were resistant to both strains presumably carrying *Rsv1-h, Rsv1-r, Rsv1-s,* or a combination of two loci *Rsv1+3*; only 3 genotypes potentially carrying *Rsv1-n* allele; and 20 with alleles of the *Rsv3* locus. In addition, 10 lines displayed unique reaction pattern and were categorized as sources of possible new alleles of the *Rsv1* or *Rsv3* loci (Table 4).

Rsv4 linkage and mapping

SNPs validation was accomplished using V94-5152 (*Rsv4*) × Lee 68 (*rsv*), and 766 F_2 plants were screened with the three SNPs linked to the *Rsv4* locus. For mapping purposes, four additional SNPs on each side of the target SNP markers at the *Glyma.02g121400* gene were added to observe recombination frequency in the region of interest. Six SNPs were polymorphic, including three SNPs previously discovered by sequencing displaying 1A:2H:1B segregation for a single dominant gene within the mapping population (Table 6). Also, phenotyping results of inoculated $F_{2:3}$ lines by SMV-G7 strain fitted into the 1R:2H:1S ratio of single dominant gene segregation (data not shown).

Those results were used to assess linkage between the *Rsv4* resistance gene and SNPs, based on 766 individuals derived from the population. All markers were mapped on one side of the *Rsv4* with the closest marker, ss244712671, located in genetic distance of 3.58 cM upstream the DNA sequence (Table 6). Other SNPs, ss244712652, ss244712653, ss244712651, ss244712591, and ss244712184, were positioned at 3.62, 3.65, 3.72, 3.8, and 4.12 cM to the locus respectively (Table 6). The total genetic distance of 3.58 cM was translated into physical distance of 700 kb (Schmutz et al. 2010), and with regard to this interval, the physical region of 12,100,000 - 12,600,000 bp on chromosome 2 was closely analyzed. Williams 82 sequence annotation database (www.phytozome.net/soybean) retrieved 43 putative genes in the target region. Among them, there were only three genes with kinase functions and two transcription factors (Table 7).

DISCUSSION

The reference genome of Williams 82 (Grant et al. 2010; Schmutz et al. 2010) does not carry genetic resistance to SMV at the *Rsv4* locus. For this reason, researchers need to rely on mapping and sequencing of short chromosomal fragments derived from soybean accessions resistant to the virus. In this study, a non-standard method of SNP identification was applied by choosing DNA regions through proposed R-genes in previous studies and testing them with putative SNPs. After sequencing of the *Glyma.02g121400* locus, three SNPs were found and used for screening of a germplasm collection to assess genetic diversity and to validate marker accuracy in tagging the *Rsv4* gene for SMV resistance.

Based on distinct reaction pattern of each differential genotype to SMV strains, it was possible to divide most of other soybean accessions into groups with predicted SMV R-gene. Moreover, in some cases, differentiation of specific alleles was possible; however, phenotyping using only two SMV strains could not distinguish genotypes into all alleles. Also, soybean accessions susceptible to both SMV strains were classified as the ones that did not carry any SMV resistance gene. Our phenotypic results were consistent with previously published studies where phenotypic screening was performed on soybean germplasm collection (Li et al. 2010; Shakiba et al. 2012a, 2012b; Zheng et al. 2005).

Although this classification system seem to be efficient, it becomes a restraint when trying to separate soybean accessions resistant to all strains. If a given accession shows resistance to both G1 and G7, it may be due to several gene/allele combinations: *Rsv1-h, Rsv4*, *Rsv1Rsv3*, *Rsv1Rsv4*, *Rsv3Rsv4*, or *Rsv1Rsv3Rsv4*. In such situations, allelism/inheritance studies or use of molecular markers are necessary for efficient assessing the genetics of SMV resistance.

The SNPs identified in this study were useful for differentiating soybean germplasm for specific R-genes; however, it did not provide information on how many genes each accession carries.

The consistent results of genotyping and phenotyping allowed for the identification of lines carrying resistance alleles at the *Rsv4* locus. Among these, the majority come from Asia (China, Korea, Japan, and Russia), Africa (Zimbabwe, Algeria, and South Africa), North America (USA), and Europe (France and Bulgaria) (data not shown). China is the origin of soybean and it was not surprising that most of *Rsv4*-resistant genotypes come from Asia, whereas significant part of susceptible genotypes were from non-Asian countries.

In this study, 766 individuals derived from V94-5152 (*Rsv4*) × Lee 68 (*rsv*) assisted in the development of a linkage map for the *Rsv4* region with six SNP markers where ss244712671 was the closest one linked to the *Rsv4* locus with genetic distance of 3.6 cM. Because this marker is located at 11,697,977 position on chromosome 02 (MLG D1b), the *Rsv4* gene is located downstream of the DNA sequence at the physical chromosomal position of about 12,400,000 bp; however, this region may be much expanded due to presence of heterochromatin condensed structure.

In previous studies, the *Rsv4* region was fine-mapped within a small size physical interval; however, the gene is still elusive (Ilut et al. 2015; Li et al. 2015; Saghai Maroof et al. 2010; Wang et al. 2011; Yan et al. 2015). Our results were based on a large population size that indicated the analyzed *Rsv4* region should shift downstream in chromosome 2. According to this distance, we marked three candidate genes with the kinase function and two potential transcription factors (Suh et al. 2011). *Glyma.02g121900* and *Glyma.02g122000* encode leucine-rich repeat receptor-like protein kinases that may function in cellular signal transduction pathways as a part of the two-component system responsible for rapid cascade of reactions upon SMV infection (SoyBase 2016). Similarly, *Glyma.02g123700* encodes a highly conserved

hosphatidylinositol kinase-like protein, an enzyme responsible for signaling pathways that regulate functions of cell metabolism, survival, and vesicle trafficking (Engelman et al. 2006; SoyBase 2016). *Glyma.02g122900* and *Glyma.02g124300* encode a BSD domain-containing protein and Myb-like protein, respectively, that could work as transcription factors (TF), and could have a role in DNA binding and regulating gene expression during SMV infection (Doerks et al. 2002, SoyBase 2016).

Our prediction of *Rsv4* gene candidates was in agreement with the conclusions of Wang et al. (2011), Ilut et al. (2015), and Li et al. (2015). Molecular mechanisms of disease resistance are very complex that may be controlled by a network of genes (Marone et al. 2013; Suh et al. 2011). The candidate genes must be further investigated by designing gene specific SNPs based on full genome sequencing, expression analysis, and eventually transforming them into a susceptible soybean cultivar. It is also possible that the *Rsv4* gene may belong to a different family than the genes with NBS-LRR domain displaying an unknown functionality and therefore other genes present in this region may be considered.

Traditional ways of breeding for resistance require germplasm screening to identify sources of resistance, studying the mode of inheritance, introgression of the resistance in elite cultivars, and testing their performance under pathogen infection in the field. Identification of SNPs for MAS or genomic selection shorten the duration of a breeding program, increase the selection efficiency, and substitute for phenotypic screening. Our studies have provided information on the approximate location of the *Rsv4* gene. Finding the exact location of SMV R-genes will facilitate cloning and incorporation of them into susceptible cultivars. Development of a new approach to combat the disease caused by SMV is going to be more feasible when we know where the genes are located and what molecular functions they have. The SNPs discovered

in this study will enable a more effective way to analyze and manage genotyping results, integrating phenotypic data, and applying new tools to breeding programs.

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Table 1. Differential reactions to *Soybean mosaic virus* of soybean checks.

Check	Reactions to SMV[a]							R-Gene
	G1	G2	G3	G4	G5	G6	G7	
Essex	S	S	S	S	S	S	S	*rsv*
Lee 68	S	S	S	S	S	S	S	*rsv*
Williams	S	S	S	S	S	S	S	*rsv*
Williams 82	S	S	S	S	S	S	S	*rsv*
PI 96983	R	R	R	R	R	R	N	*Rsv1*
V94-3971	R	R	R	R	R	R	N	*Rsv1*
L78-379	R	R	R	R	R	R	N	*Rsv1*
Suweon 97	R	R	R	R	R	R	R	*Rsv1-h*
L92-8580	R	R	R	R	R	R	R	*Rsv1-h*
York	R	R	R	N	S	S	S	*Rsv1-y*
Davis	R	R	R	N	S	S	S	*Rsv1-y*
L85-2308	R	R	R	N	S	S	S	*Rsv1-y*
Raiden	R	R	R	R	N	N	R	*Rsv1-r*
L88-8431	R	R	R	R	N	N	R	*Rsv1-r*
L88-8440	R	R	R	R	N	N	R	*Rsv1-r*
Kwanggyo	R	R	R	R	N	N	N	*Rsv1-k*
Ogden	R	R	N	R	R	R	N	*Rsv1-t*
L93-3327	R	R	N	R	R	R	N	*Rsv1-t*
Marshall	R	N	N	R	R	N	N	*Rsv1-m*
L84-2112	R	N	N	R	R	N	N	*Rsv1-m*
PI 507389	N	N	S	S	N	N	S	*Rsv1-n*
V262	N	N	S	S	N	N	S	*Rsv1-n*
Corsica	S	ER	S	-	ER	S	ER	*Rsv1-c*
L29	S	S	S	S	R	R	R	*Rsv3*
V229	S	S	S	S	R	R	R	*Rsv3*
Harosoy	S	S	S	S	R	R	R	*Rsv3*
PI 61944	N/S	N/S	R	-	R	R	R	*Rsv3-n*
PI 61947	N/S	N/S	R/N	-	R	R	R	*Rsv3-h*
V94-5152	ER	ER	ER	ER	ER	ER	ER	*Rsv4*
Peking	ER	ER	ER	ER	ER	ER	ER	*Rsv4*
Virginia	ER	ER	ER	ER	ER	ER	ER	*Rsv4*
V97-9003	ER	ER	ER	ER	ER	ER	ER	*Rsv4*
PI 88788	ER	ER	ER	ER	ER	ER	ER	*Rsv4*
PI 438307	R	R	R	R	R	R	ER	*Rsv4-v*
Beeson	ER	ER	S	-	R	ER	R	*Rsv4-b*
Zhao shu 18	R	R	R	R	R	R	R	*Rsv1+3*
Hourei	R	R	R	R	R	R	R	*Rsv1+3*
Tousan 140	R	R	R	R	R	R	R	*Rsv1+3*
PI 486355	R	R	R	R	R	R	R	*Rsv1+4*
Columbia	R	R	R	R	R	R	R	*Rsv3+4*
N8101	R	R	R	R	R	R	R	*Rsv1+3+4*

[a] Foliar symptoms under SMV infection with different strains (G1-G7) isolated in U.S.: R, resistant (symptomless); N, necrotic (systemic necrosis); S, susceptible (mosaic); ER, early resistant at seedling stage; N/S, mixture of symptoms: necrotic and susceptible; R/N, mixture of symptoms: resistant and necrotic; -, missing data.

Table 2. Phenotypic characterization of ten checks to *Soybean mosaic virus* infection used for sequencing of the *Glyma.02g121400* locus.

Genotype	Reactions to SMV[a]		R-Gene	*Rsv4* SNP markers[b]		
	G1	G7		ss244712651	ss244712652	ss244712653
Williams 82	S	S	*rsv*	T	C	A
Essex	S	S	*rsv*	T	C	A
PI 96983	R	N	*Rsv1*	T	C	A
V94-3971	R	N	*Rsv1*	T	C	A
L29	S	R	*Rsv3*	T	C	A
V229	S	R	*Rsv3*	T	C	A
Harosoy	S	R	*Rsv3*	T	C	A
V94-5152	R	R	*Rsv4*	A	G	G
V97-9003	R	R	*Rsv4*	A	G	G
PI 88788	R	R	*Rsv4*	A	G	G

[a] Foliar symptoms under infection with SMV-G1 and G7 strains: R, resistant (symptomless); N, necrotic (systemic necrosis); S, susceptible (mosaic).

[b] SNPs located at the *Glyma.02g121400* locus, displaying the T-C-A and A-G-G patterns for different genotypes.

Table 3. Soybean lines without identified *Soybean mosaic virus* (SMV) resistance, displaying the T-C-A SNP pattern (checks underlined).

Soybean Accession	SMV Reactions[a]		R-Gene
	G1	G7	
1133 (PI 96984); 5693 (PI 88306); 7413 (PI 90479-1); 8085 (PI 70242); Accomac (PI 597388); Akanedzumime (PI 243516); Aobishi (PI 243519); Avery (PI 518663); BARC-19 (PI 652935); Bedford (PI 548974); Boggs (PI 602597); Braxton (PI 548659); Bryan (PI 542712); Camp (PI 553044); Chamberlain (PI 548635); Charleston (PI 567902); Chesapeake (PI 583366); Cisne (PI 593256); Darby (PI 614154); Dare (PI 548987); Daruma niju (PI 80834-1); Edison (PI 542711); Egyptian (PI 506417); Essex (PI 548667); Fayette (PI 518674); Gail (PI 548978); Gordon (PI 553047); Harper 87 (PI 518667); Hartwig (PI 543795); Haskell (PI 572238); Iroquois (PI 593259); KAS 200-23-1 (PI 398371); KAS 353-8 (PI 509080); KAS 540-27 (PI 458184); KLS 906 (PI 399045); Kurakake Daizu (PI 506949); Lamar (PI 533604); Lee 68 (PI 559369); Lyon (PI 576857); Macon (PI 593258); Manokin (PI 559932); Maverick (PI 598124); Mitchell (PI 548679); Murasaki No Mi (PI 417169); Mustang (PI 595363); No. 50 (PI 54610); Pearl (PI 583367); Pharaoh (PI 548645); Pickett 71 (PI 548982); Pyramid (PI 512039); Roanoke (PI 548485); Scott (PI 548613); Semmes (PI 548661); Sherman (PI 548614); Shiro Aki Daizu (PI 417310); Shironomai (PI 538409); Spry (PI 553051); Stafford (PI 508269); Stonewall (PI 53 1068); Stressland (PI 593654); Suzuhime (PI 494182); Tanba Kuro (PI 507336); Thorne (PI 564718); Union (PI 548622); Usuda Zairai (PI 507504); Vinton (PI 548618); Vinton 81 (PI 548625); Williams (PI 548631); Williams 82 (PI 518671); Woods Yellow (PI 548496).	S	S	*rsv*

[a] Foliar symptoms under infection with SMV-G1 and G7 strains: S, susceptible (mosaic).

Table 4. Soybean lines potentially carrying *Rsv1* and *Rsv3* showing the T-C-A SNP pattern.

Soybean Accession	SMV Reactions[a]		R-Gene
	G1	G7	
6053 (PI 89061); 552 (PI 96257); 1248 (PI 82210); 1384 (PI 97235); 7381 (PI 90401); Aze daizu (PI 416807); Brim (PI 548986); Calhoun (PI 576440); Casa Grande (PI 159923 A); Chang-uwal (PI 157410); Chankon (PI 84949); Cook (PI 553045); Davis (PI 553039); Dillon (PI 592756); Doles (PI 576154); Dorman (PI 548653); Fukuyutaka (PI 506675); GL 2678B/96 (PI 603167); H-060013 (PI 417582); Jitsuka (PI 494181); KAERI 540-4 (PI 407975 B); KAERI 543-3 (PI 407994); KAS 530-5 (A) (PI 407907 A); KAS 530-5 (B) (PI 407907 B); KAS 643-8 (A) (PI 424159 A); KAS 643-8 (B) (PI 424159 B); KAS 643-8 (C) (PI 424159 C); KAS172-9-2 (PI 398289); KLS 121 (PI 398877); KLS 743-1 (PI 399012); Kosuzu (PI 594208); Kyeong-du (PI 157447); Kyongsang pukdo (PI 399107); L85-2308 (PI 547873); Mejiro (PI 507033); Musen (PI 599333); No. 23 (PI 339999); Okute mame (PI 19986); ORD 8113 (PI 407788 A); Ping ding huang (PI 567577); Prolina (PI 597389); Qi Huang No.1 (B) (PI 468408 B); Qi Huang No.1 (C) (PI 468408 C); Ripley (PI 536636); Rokugastu daizu (PI 507189 A); Shibahara mame (PI 417288); Suzumaru (PI 593972); Toano (PI 508268); Tockikubo (PI 417387); Xu dou No.1 (PI 556950); Xu dou No.2 (PI 495020); York (PI 553038); You bian 30 (PI 518716); Young (PI 508266); Yuwoltae (C) (PI 339868 C); Yuwoltae (D) (PI 339868 D); Yuwoltae (E) (PI 339868 E); Yuwoltae (F) (PI 339868 F).	R	S	*Rsv1-y*
197 (PI 471938); 1132 (PI 96983); 19-1 (PI 235339); 30-1 (PI 235344); Choutan shirome (PI 416841); Chuzu (PI 86740); Clifford (PI 596414); Epps (PI 548977); F.A.V. 24-3 (PI 264555); Fengsan Iu tsao shen (PI 504481); Hakuho No.1 (PI 248511); Holladay (PI 572239); Hood (PI 548980); Iwate wase kurome (PI 506809); Johnston (PI 508267); Kantou 63 (PI 417005); Kawanagare (Iwate) (PI 417015); Kou kei 74 (PI 417071); Kwang kyo (PI 406710); L78-379 (PI 547844); L80-5227 (PI 547851); L81-4420 (PI 547857); L84-2112 (PI 591513); L93-3327 (PI 591515); Lu tsao shen (PI 504488); Mao 205 (PI 518287); Marshall (PI 548693); Mocinave 7 (PI 507690); Mukden (PI 548391); No. 31 (PI 181550); No. 38 (PI 181555); No. 40 (PI 181557); Ogden (PI 548477); Okushirome (PI 423888); Pace (PI 602496); Pulaska zolta wczesna (PI 417559); Saturn (PI 583837); Seneca (Cornell) (PI 235340); Shakkin-nashi (PI 229352); Shimoda Shitachi (PI 246367); Shin No.4 (PI 219789); Shiro higo (PI 594268A); Sundar No.1 (PI 504504); Suzuyataka (PI 561395); Tachiyutaka (PI 594289); Tanrei (PI 594295); Tohoku No.1 (PI 229359); Tousan 101 (PI 507439); Tousan 122 (PI 561397); Tousan 26 (PI 417412); Tousan 58 (PI 507396); Tousan 65 (PI 507403); Tousan kei B62 (PI 417423); V94-3971; Yao tou (PI 504487); Yatsufusa (PI 507548).	R	N	*Rsv1* *Rsv1-k* *Rsv1-t* *Rsv1-m*
37-2 (PI 407765); Bukalasa 2 (PI 381659); Sakyuu ki mame (PI 417263); Ching tao No.21 (PI 200460); Enrei (PI 385942); Hingukongu (PI 87013); Hourei (PI 561394); Ito san (PI 438494); Jiunong 21 (PI 612735); L88-8431 (PI 547885); L88-8440 (PI 547886); L92-8580 (PI 591516); Miyagi shirome (PI 417159); Okatsu mame (PI 507127); Raiden (PI 360844); Suweon 97 (PI 483084); Tousan 140 (PI 561398); Tsuronoko (PI 561392); Zao shu 18 (PI 603290); Zhao shu 18 (PI 612732).	R	R	*Rsv1-h* *Rsv1-r* *Rsv1-s* *Rsv1+3*
He feng 25 (PI 518703); Tousan 50 (PI 507389); V262.	N	S	*Rsv1-n*
Corsica (PI 559931); Cordell (PI 533605); Enoki (PI 59849); Freedom (PI 636463); Graine jaune unie (PI 189891); H 67-6 (PI 323555); H 67-7 (PI 323556); Harosoy (PI 548573); Hutcheson (PI 518664); L29; OCB 81 (PI 504510); Paoting (PI 179825); PLSO-63 (PI 346307); PLSO-70 (PI 346308); V229; VIR 5532 (PI 438427).	S	R	*Rsv3* *Rsv1-c*
7385 (PI 90402); Kakira 13 (PI 381668).	N	R	*Rsv3*
568 (PI 61944); 586 (PI 61947).	N/S	R	*Rsv3*
E dou No.2 (PI 436563); Krasnoarmejskaja (PI 404167); Shang tsai (PI 103079); Sherwood (PI 417578); Tailungyuan (PI 62 199); Tun czou (PI 404164).	R/N	R	*Rsv1-?* *Rsv3-?*
CNS-65F (PI 283332); Kolhida 4 (PI 404159); Tekkyou seitou (PI 417380).	R/N	S	*Rsv1-?*
7618 (PI 91346).	S	N	*Rsv1-?*

[a] Foliar symptoms under infection with SMV-G1 and G7 strains: R, resistant (symptomless); N, necrotic (systemic necrosis); S, susceptible (mosaic); R/N and N/S, mixed symptoms.

Table 5. Soybean lines potentially carrying the *Rsv4* locus showing the A-G-G SNP pattern (checks underlined).

Soybean Accession	SMV Reactions[a]		R-Gene
	G1	G7	
11/45S95 (PI 170896); 5913 (PI 88788); Beeson (PI 548510); Bergerac (PI 153309); Columbia (PI 548317); Da Bai Ma (PI 556948); Dun cuan (PI 404165); Fen dou 31 (PI 574477); Il-soy (PI 157435); Jin dou No.1 (A) (PI 578494 A); Ju xuan 23 (A) (PI 578498 A); Ju xuan 23 (B) (PI 578498 B); Ke feng No.1 (PI 556949); N8101 (PI 654355); No. 36 (PI 181554); No. 42A (PI 171434); Pekin kuro diazu (PI 417243); Peking (PI 548402); Rhosa (PI 324924); S-17 (PI 84594); SAO 196-C (PI 438335); SS74185 (PI 486355); V94-5152 (PI 596752); V97-9003; VIR 2980 (PI 438307); VIR 964 (PI 437482); Virginia (PI 548422); VU-5817 (PI 438357 A); Yuwoltae (B) (PI 339868 B).	R	R	*Rsv4* *Rsv1+4* *Rsv3+4* *Rsv1+3+4*
PI 339870; PI 399091; A.K. (Harrow) (PI 548298); CNS (PI 548445); Hardee (PI 548666); Hubert 33 (PI 229738); Kaigen's Kingenzu (PI 88486); KAS 301-14 (PI 458120); Kuro masshokutou (Kou 205) (PI 417094); Shin 2 (PI 507239); Wilson (PI548427).	S	R	*Rsv3* *Rsv1-c*
Akita ani (PI 506516); Ani 31 (PI 229314); Iwate No.1 (PI 229325); Kantou 9 (PI 506840 A); Mercury (PI 583835); Nohrin No.3 (PI 224271); Nooki No.1 (PI 229341); Shou outou (PI 417345 A); Tokishi (PI 229361); Tousan 52 (PI 507391).	R	N	*Rsv1* *Rsv1-k* *Rsv1-t* *Rsv1-m*
Azeminori (PI 219782); KLS 806-1(PI 399022); Qi Huang No.1 (PI 561375).	R	S	*Rsv1-y*
Tej sen da baj pi (PI 404172).	S	N	*Rsv1-?*
Jin dou No.1 (B) (PI 578494 B); KAS 390-4 (PI 398593).	N/S	R	*Rsv3*
Dyn haj hun mao czy (PI 404185); Gun li huang (PI 567541 A); Moshito (PI 81786); N230A (PI 79727); ORD 8113 (PI 407788C); Tun san si he czao (PI 404170).	S	S	*rsv*

[a] Foliar symptoms under infection with SMV-G1 and G7 strains: R, resistant (symptomless); N, necrotic (systemic necrosis); S, susceptible (mosaic); N/S, mixed symptoms.

Table 6. SNP markers used for competitive allele specific PCR (KASP) genotyping the *Rsv4* region and their genetic distances in a F_2 population V94-3132 × Lee 68.

SNP[a]	Position	Type	V94-5152	Lee 68	χ^2	*p*-value	Distance[b]
ss244712184	11613852	[T/C] = Y	C	T	0.49	0.7816	- 4.12
ss244712591	11685678	[T/A] = W	A	T	0.49	0.7816	- 3.80
ss244712651	11693196	[T/A] = W	A	T	0.72	0.6966	- 3.72
ss244712652	11693604	[C/G] = S	G	C	0.63	0.7292	- 3.62
ss244712653	11693900	[A/G] = R	G	A	0.61	0.7384	- 3.35
ss244712671	11697977	[T/G] = K	G	T	0.41	0.8145	- 3.58

[a] SNPs located in the coding sequence of the *Glyma.02g121400* gene were underlined.

[b] Genetic distances between SNPs and the *Rsv4* locus (in cM).

Table 7. Gene annotations of *Rsv4* candidate genes identified in relevance of a distance between analyzed SNPs and the *Rsv4* gene.

Locus	Position[a]	Protein/Family[b]	Function
Glyma.02g121900	12,112,034-12,115,054	Leucine-rich repeat receptor-like kinase	Signal transduction
Glyma.02g122000	12,115,287-12,118,397	Leucine-rich repeat receptor-like kinase	Signal transduction
Glyma.02g122900	12,259,463-12,264,960	BSD domain-containing protein	Transcription factor
Glyma.02g123700	12,351,993-12,355,050	Phosphatidylinositol 4-kinase	Signal transduction
Glyma.02g124300	12,425,993-12,427,856	Myb domain-containing protein	Transcription factor

[a] Physical position on chromosome 2 (in bp) of the *Rsv4* candidate genes retrieved from the reference genome Wm82.a2.v1.
[b] Possible protein identified based on presence of specific domains (SoyBase).

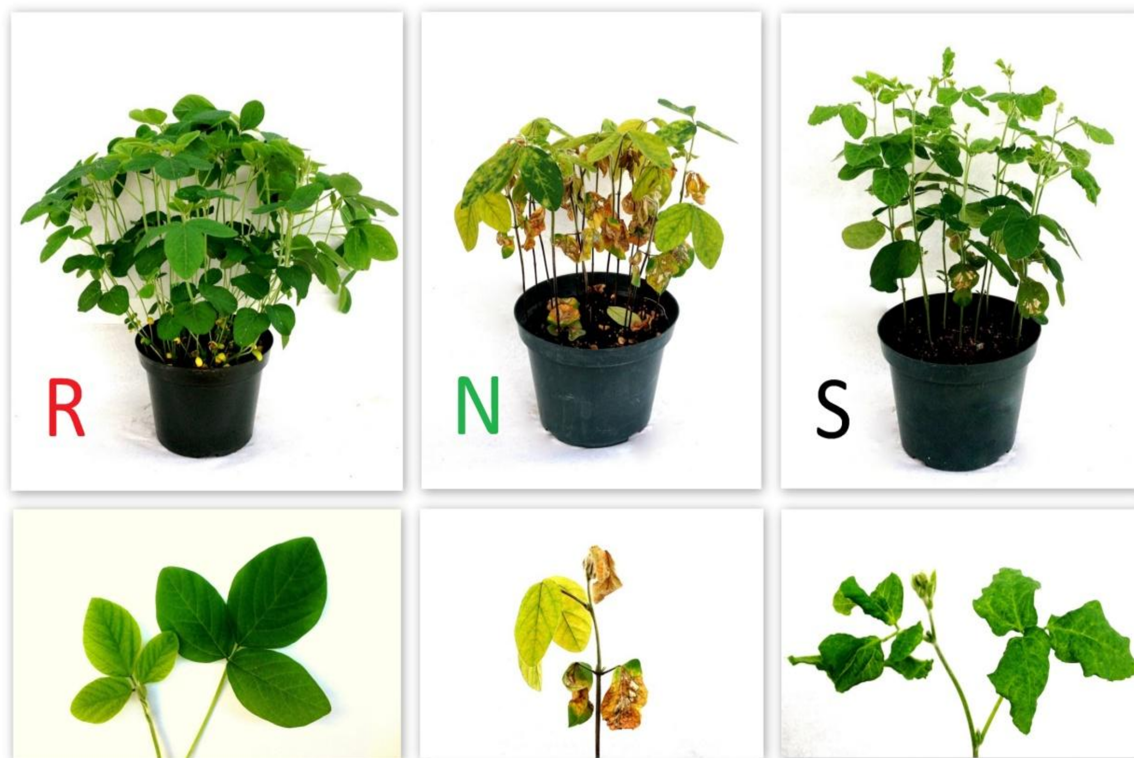

Figure 1. Foliar symptoms of soybean genotypes in reaction to soybean mosaic virus (SMV): R, resistant, showing no symptoms of the disease; N, necrotic with systemic tip necrosis; S, susceptible line displaying typical mosaic symptoms of the SMV disease. Upper part presents entire plants, and lower part exhibits a detailed view.

position 1473 [A/T] = W, in Williams 82 at 11693196bp = ss244712651

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Query: 1321     aagaacttcaatgaggttgttgttgatgatggtgcagaaagtgattcaagttctgatctg 1380
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 11693348 aagaacttcaatgaggttgttgttgatgatggtgcagaaagtgattcaagttctgatctg 11693289

Query: 1381     tttgaattgcaaaactatgacttgagatactattcaagtggcctacctgtctatgaaact 1440
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 11693288 tttgaattgcaaaactatgacttgagatactattcaagtggcctacctgtctatgaaact 11693229

Query: 1441     accaacatggatagcatcaagagaggagcaccwatttccaatggccctctgtgatgtttg 1500
                |||||||||||||||||||||||||||||||| |||||||||||||||||||||||||||
Sbjct: 11693228 accaacatggatagcatcaagagaggagcaccaatttccaatggccctctgtgatgtttg 11693169

Query: 1501     gtgtacaatatttttcttccttctttaattggttaaggtttaatatttagcatgttagaa 1560
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 11693168 gtgtacaatatttttcttccttctttaattggttaaggtttaatatttagcatgttagaa 11693109

Query: 1561     gctatgaaaaaaggaaaatctattagattttgcttgtttcccccagggtttcatgatttc 1620
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 11693108 gctatgaaaaaaggaaaatctattagattttgcttgtttcccccagggtttcatgatttc 11693049
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position 1065 [G/C] = S in willams82 at 11693604bp = ss244712652

```
Query: 901      agctcaagcactgcagattcaaagtccttgtactcctccttgagttcagggtttagaact 960
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 11693768 agctcaagcactgcagattcaaagtccttgtactcctccttgagttcagggtttagaact 11693709

Query: 961      cctccttatgtacaaacaccaacaaagagctgcaaggaattcagaaccttctcttcagaa 1020
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 11693708 cctccttatgtacaaacaccaacaaagagctgcaaggaattcagaaccttctcttcagaa 11693649

Query: 1021     aacaagcatgcactgtccttttcagcaaagtacaacaataacaasaacaacaatggacaa 1080
                |||||||||||||||||||||||||||||||||||||||||||| |||||||||||||||
Sbjct: 11693648 aacaagcatgcactgtccttttcagcaaagtacaacaataacaagaacaacaatggacaa 11693589

Query: 1081     catgtaagatcatcaacagcaaccaccactttgcaaaatgagtttttgtgggatgagaag 1140
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 11693588 catgtaagatcatcaacagcaaccaccactttgcaaaatgagtttttgtgggatgagaag 11693529

Query: 1141     aaaaagagggaaccaacaacaacaacaaccttgttggatgataatagcaaccacaaacac 1200
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 11693528 aaaaagagggaaccaacaacaacaacaaccttgttggatgataatagcaaccacaaacac 11693469
```

position 769 [T/C] = Y in Willams82 at 11,693,900bp = ss244712653

```
Query: 601      aggcaccatcatcatcatcatcatggacatagagctgccagaatcagcttagacatgcca 660
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 11694068 aggcaccatcatcatcatcatcatggacatagagctgccagaatcagcttagacatgcca 11694009

Query: 661      atgagaagcttgctcccacagcaattccatggcatggagaagcaaatcatcatgaaggag 720
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 11694008 atgagaagcttgctcccacagcaattccatggcatggagaagcaaatcatcatgaaggag 11693949

Query: 721      aagaagcacaagcagcctagctctcctggtggaaggcttgcaagcttcytgaactctctc 780
                |||||||||||||||||||||||||||||||||||||||||||||||| |||||||||||
Sbjct: 11693948 aagaagcacaagcagcctagctctcctggtggaaggcttgcaagcttcttgaactctctc 11693889

Query: 781      ttcagccaatcagcatcaaagaagaagaagtcaaataagtcaagctcacagtccatgaaa 840
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 11693888 ttcagccaatcagcatcaaagaagaagaagtcaaataagtcaagctcacagtccatgaaa 11693829

Query: 841      gatgaagatgagagccctggtggaaggaggagaagaaggagcagcattagccatttcaga 900
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 11693828 gatgaagatgagagccctggtggaaggaggagaagaaggagcagcattagccatttcaga 11693769
```

Figure 2. Blast output of sequenced V94-5152 (*Rsv4*) soybean accession against SoyBase database (http://soybase.org) of Williams 82 (*rsv*) reference sequence. Discovered SNPs located in the *Glyma.02g121400* gene were marked in black box.

```
                                         1470        770             1060
Glyma02g13380                            CACCAATTTC  GCTTCTTGAACTCT  ATAACAAGAAC
Sample 1 - PI96983 (Rsv1)                ..........  ..............  ...........
Sample 31 - PI96983 (Rsv1)               ..........  ..............  ...........
Sample 2 - V94-3971 (Rsv1)               ..........  ..............  ...........
Sample 32 - V94-3971 (Rsv1)              ..........  ..............  ...........
Sample 3 - L29 (Rsv3)                    ..........  ..............  ...........
Sample 33 - L29 (Rsv3)                   ..........  ..............  ...........
Sample 4 - V229 (Rsv3)                   ..........  ..............  ...........
Sample 34 - V229 (Rsv3)                  ..........  ..............  ...........
Sample 5 - Harosoy (Rsv3)                ..........  ..............  ...........
Sample 35 - Harosoy (Rsv3)               ..........  ..............  ...........
Sample 6 - V94-5152 (Rsv4)               ....T.....  .....C........  .......C...
Sample 36 - V94-5152 (Rsv4)              ....T.....  .....C........  .......C...
Sample 7 - V97-9003 (Rsv4)               ....T.....  .....C........  .......C...
Sample 37 - V97-9003 (Rsv4)              ....T.....  .....C........  .......C...
Sample 8 - PI 88788 (Rsv4)               ....T.....  .....C........  .......C...
Sample 38 - PI 88788 (Rsv4)              ....T.....  .....C........  .......C...
Sample 9 - Essex (rsv)                   ..........  ..............  ...........
Sample 39 - Essex (rsv)                  ..........  ..............  ........N..
Sample 10 - Williams 82 (rsv)            ..........  ..............  ...........
Sample 40 - Williams 82 (rsv)            ..........  ..............  ...........
```

Figure 3. Clustal W (BioEdit) output of ten soybean checks for the target *Glyma.02g121400* (*Glyma02g13380*) sequences aligned to Williams 82 sequence (Wm82.a2.v1) (SoyBase). Positive and negative strand of each DNA was sequenced and aligned. Letters designate changes in nucleotides (SNPs), dots indicate no change while blasting with the Williams 82 (*rsv*), the "N" letter indicates a possibility of presence of any base.

>Glyma02g13380.1 class=Sequence position=Gm02:11692903..11694668 (- strand)

```
ACAACCACGT ACTTAACACC AAACTCCCAC TCAAACCCAT AGAAGCATAT AGAATCCAAG GAAACACAGC TCTTTGCTCC
CCACAATCCC TAACAAAATC CTCTGGTTTT TAACAGCATG AGGAGGATAA AAAGCACTGT TCCCCACTTA TATCTTTATT
GCTAATGCAC ATATTCACAT CATGTGTGGA CCTCACACAA AAACCTCCAA AACTCCCTCA TCATATAAAA TTATGCCTTG
AGTTATTGAT TAACCTAAAC TGACCCTCCC CACTACTCTC CATCATTCGA AACCCAGTAT CCCCCCCCCC TTCTTGTTAC
ATATTGCTAT ATCCATAATT CCACACACCA TTTCATTCAT TCATTCACCT CTTGTTACAT ATATAACTTG TTAATACAAG
TCCTAAACTC AAACTTGCAC CACACTATGT CCATAGCAGG CCTTATAGAC CCAGAAATGA ATCACAACAA GTCCTTCCAC
CGGCGAAATA ACTCCGGCGA GCTCGATGTG TTTGAGGCAG CAAGGTACTT CTCAGGATAC AGTGAAGTTC TTGGCTCCAC
CACCACCACC TACACTCAGA AGATCAATAT GAGAGAAGAA AGGCACCATC ATCATCATCA TCATGGACAT AGAGCTGCCA
GAATCAGCTT AGACATGCCA ATGAGAAGCT TGCTCCCACA GCAATTCCAT GGCATGGAGA AGCAAATCAT CATGAAGGAG
AAGAAGCACA AGCAGCCTAG CTCTCCTGGT GGAAGGCTTG CAAGCTTCYT GAACTCTCTC TTCAGCCAAT CAGCATCAAA
GAAGAAGAAG TCAAATAAGT CAAGCTCACA GTCCATGAAA GATGAAGATG AGAGCCCTGG TGGAAGGAGG AGAAGAAGGA
GCAGCATTAG CCATTTCAGA AGCTCAAGCA CTGCAGATTC AAAGTCCTTG TACTCCTCCT TGAGTTCAGG GTTTAGAACT
CCTCCTTATG TACAAACACC AACAAAGAGC TGCAAGGAAT TCAGAACCTT CTCTTCAGAA AACAAGCATG CACTGTCCTT
TTCAGCAAAG TACAACAATA ACAASAACAA CAATGGACAA CATGTAAGAT CATCAACAGC AACCACCACT TTGCAAAATG
AGTTTTTGTG GGATGAGAAG AAAAAGAGGG AACCAACAAC AACAACAACC TTGTTGGATG ATAATAGCAA CCACAAACAC
TTATCAGAGA AACAAAAGAA CAACAACAAC AAGGGAAGTC ATGAGTTATT ACTTGAGAAA GATAGGATGT TAGTGGACAA
CAAGTACTCA TCAGAAGAGA AGGAAACCAC CACTCAATTC AAGAACTTCA ATGAGGTTGT TGTTGATGAT GGTGCAGAAA
GTGATTCAAG TTCTGATCTG TTTGAATTGC AAAACTATGA CTTGAGATAC TATTCAAGTG GCCTACCTGT CTATGAAACT
ACCAACATGG ATAGCATCAA GAGAGGAGCA CCWATTTCCA ATGGCCCTCT GTGATGTTTG GTGTACAATA TTTTTCTTCC
TTCTTTAATT GGTTAAGGTT TAATATTTAG CATGTTAGAA GCTATGAAAA AAGGAAAATC TATTAGATTT TGCTTGTTTC
CCCCAGGGTT TCATGATTTC AACTGATCCT TTCAATACTT TTTTTTTTCT GTGTACATAT TGGAATGTTG GCTTGTCTTA
TCTAATTTCA TGATCTAATG TCCTTTGCTT TTGGACCTTT GTTTTTAGAG TGCAAAAACA AAAACAAAAC AAAAGTTAAT
GCCCAC
```

Figure 4. Physical positions of three SNPs identified (marked in black box) at the sequenced *Glyma.02g121400* (*Glyma02g13380*) coding sequence (marked in light grey) and its flanking sequences (marked in dark grey). Three SNPs: ss244712653 [T/C]=Y (position 769; in Williams 82 at 11,693,900bp), ss244712652 [G/C]=S (position 1065; in Williams 82 at 11693604bp), and ss244712651 [A/T]=W (position 1473; in Williams 82 at 11693196bp) were identified by Blast function available at SoyBase website.

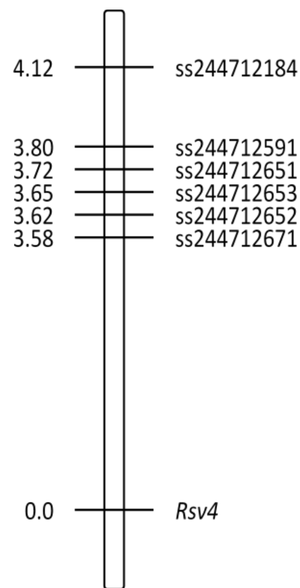

Figure 5. Genetic linkage map of partial chromosome 2 (MLG D1b) created with JoinMap using data from the $F_{2:3}$ population derived from V94-5152 (*Rsv4*) × Lee 68 (*rsv*). Genetic distances (in cM) between discovered SNPs and the *Rsv4* gene were indicated on the left side.

CHAPTER THREE

VALIDATION OF MARKER-ASSISTED GENE PYRAMIDING FOR SOYBEAN MOSAIC VIRUS RESISTANCE

ABSTRACT

Soybean can be infected and severely damaged by *Soybean mosaic virus* (SMV) causing a significant decrease in soybean yield. To prevent or reduce this destruction, pyramiding of SMV resistance genes (R-genes) is of vital importance leading to durable crop protection against multiple strains of the pathogen. Three SMV resistance genes *Rsv1*, *Rsv3*, and *Rsv4* have been pyramided by crossing two SMV resistant accessions J05 (*Rsv1+Rsv3*) and V94-5152 (*Rsv4*) using marker-assisted selection (MAS). In this study, we tested ten $F_{4:7}$ lines for a presence of all three R-genes at the homozygous stage using simple sequence repeat (SSR) and single nucleotide polymorphism (SNP) markers. For inheritance study, we crossed the GP20 (*Rsv1+3+4*) line with homozygous recessive parent Williams 82 (*rsv*) and 155 F_2 plants were genotyped by three SSR markers linked to the *Rsv1*, *Rsv3*, and *Rsv4* loci, and $F_{2:3}$ lines were separately inoculated with SMV-G1 and SMV-G7 strains to determine plants foliar symptoms. The results confirmed a successful integration of three SMV R-genes into one soybean background displaying segregation of three independent genes in the progeny. The gene pyramiding line GP20 provides durable resistance to all SMV strains, thus helping the host in an evolutionary race with the virus. We propose the GP20 line for future release as a source of SMV resistance in soybean breeding programs worldwide.

INTRODUCTION

SMV causes the most devastating viral disease in soybean-growing areas around the world and results in deterioration of seed quality and significant yield losses up to 90% in severely infected fields (Ren et al. 1997; Wang et al. 2001). In the United States, SMV isolates have been classified into seven strains (G1 - G7) based on a set of differential cultivars (Cho and Goodman 1979). Individual soybean reactions to these strains are classified into three main responses as resistant (R, symptomless), susceptible (S, mosaic) or necrotic (N) (Cho and Goodman 1979; Chen et al. 1991).

To date, three multiallelic SMV R-genes have been identified: *Rsv1*, *Rsv3*, and *Rsv4* (Buss et al. 1997; Buzzel and Tu 1989; Kiihl and Hartwig 1979). These genes follow a Mendelian mode of major gene inheritance and each expresses a distinct pattern of reaction to the seven SMV strains. The *Rsv1* locus confers resistance to less virulent strains (G1 - G4), and susceptibility or necrosis to more virulent strains (G5 - G7). In contrast, the *Rsv3* harbors resistance to more virulent strains (G5 - G7), and susceptibility to less virulent strains (G1 - G4) (Chen et al. 1991). The *Rsv4* provides resistance to G1 - G7, but may express early resistance (ER) at the seedling stage and mild susceptibility at later developmental stages (Buss et al. 1997). The *Rsv1*, *Rsv3*, and *Rsv4* R-genes have been physically mapped on chromosome 13 (MLG F) (Yu et al. 1994), 14 (MLG B2) (Hayes et al. 2000), and 2 (MLG D1b) (Jeong et al. 2002) respectively.

Limited number of SMV resistant resources is available in soybean breeding programs (Shakiba et al. 2012a). Most of resistant soybean accessions carry a single dominant gene, and only a few contain two R-genes in various combinations (*Rsv1+3*, *Rsv1+4*, or *Rsv3+4*) (Chen et al. 1993; Gunduz et al. 2002; Liao et al. 2002; Shakiba et al. 2012b). Recently, all three genes

were identified in the Korean landrace '8101' (Liao et al. 2011). Combination of two or three genes for SMV resistance diminishes vulnerability of the plant by conferring complementary resistance to multiple viral strains (Chen et al. 1993; Shi et al. 2009).

SMV adapts and develops overtime, resulting in emergence of new strains that overcome resistance in soybean (Ivanov et al. 2014). Due to the genetic variability of SMV and strong selection pressure, resistance-breaking isolates SMV-N, G5H, CN18, G7a and G7H have recently emerged in the Korean peninsula (Ahangaran et al. 2013; Choi et al. 2005; Kim et al. 2003; Seo et al. 2009). Also, recombinant soybean mosaic virus (SMV-R) was recently identified and classified as a novel strain in Chongqing, China, exhibiting different pathogenicity on soybeans compared with other SMV strains (Yang et al. 2014).

Qualitative resistance is often less durable because of rapid changes of virulence caused by counter-evolution of a host and its pathogen (Ivanov et al. 2014). This gene-specific resistance is usually considered as a gene-for-gene type of response, and is relatively easy to manipulate in both genetic research and breeding programs; however, their use is often limited to a specific race or strain of a pathogen (Ivanov et al. 2014; Rubiales et al. 2015). The main objective of gene pyramiding (GP) is to obtain an ideal genotype with all genes of desirable traits. Pyramiding of multiple SMV R-genes in a single soybean genotype is needed to provide more durable and non-race-specific resistance for soybean improvement (Shi et al. 2009).

Due to dominance and epistasis of genes governing disease resistance, pyramiding is difficult using conventional breeding methods; however, it is often performed using marker-assisted selection (MAS), also called as marker-assisted pyramiding. MAS is a method of selecting desirable individuals in a breeding scheme to improve or develop new cultivars based on indirect selection on traits of interest by molecular markers that assist phenotypic selections

for crop improvement (Collard and Mackill 2008). The general principle of MAS is existence of polymorphisms, natural variations in DNA sequence that have no adverse effect on the individuals, and if the location of a polymorphism is known, it can serve as a landmark for locating specific genes (Jeong et al. 2002; Yu et al. 1994). Since these markers and genes are linked to each other on the same chromosome, they tend to be inherited together by the standard laws of inheritance from one generation to the next (Collard and Mackill 2008).

Up to now, two attempts have been made to pyramid SMV resistance genes in soybeans. Saghai Maroof et al. (2008) pyramided SMV resistance genes *Rsv1*, *Rsv3* and *Rsv4* using three Essex isogenic lines V94-3972 (*Rsv1*), V229 (*Rsv3*) and V97-9003 (*Rsv4*), resulting in lines with two- and three-gene combinations. In their study, F_2 plants were screened by two flanking SSR markers per locus. Two gene and three gene isogenic lines of *Rsv1+Rsv3*, *Rsv1+Rsv4* and *Rsv1+Rsv3+Rsv4* acted in a complementary manner conferring resistance against six SMV strains; whereas isogenic lines of *Rsv3Rsv4* displayed a late susceptible reaction to the selected SMV strains. Subsequently, Shi et al. (2009) pyramided three SMV resistance genes from a cross between J05 (*Rsv1+Rsv3*) and V94-5152 (*Rsv4*) using eight PCR-based markers. Two SSR markers (Sat_154 and Satt510) and one gene-specific marker (Rsv1-f/r) were used for selecting plants containing *Rsv1*, Satt560 and Satt063 for *Rsv3*, and Satt266, AI856415, and AI856415-g for *Rsv4*. Five $F_{4:5}$ lines were identified to be homozygous for all eight marker alleles and presumably carry all three SMV resistance genes that would potentially provide multiple and durable resistance to SMV.

In the present study, we validated the pyramided lines created by Shi et al. (2009), and confirmed a successful transfer of SMV resistance alleles at each of these three loci into a single soybean background using classical breeding and molecular marker approach.

MATERIALS AND METHODS

Plant material and initial screening

We planted 11 $F_{4:7}$ pyramided (GP) lines, derived from the cross J05 (Rsv1+3) × V94-5152 (Rsv4) developed by Shi et al. (2009), in the field at Arkansas Agricultural Research and Extension Center, Fayetteville, AR. Young trifoliate leaves were collected and genomic DNA was extracted from fresh leaves using the CTAB method (Doyle and Doyle 1990) with minor modifications. For initial screening, we picked twenty plants from each line for genotyping using SNP and SSR markers screening (Table 1). We used three polymorphic SNPs, ss244712651 (Gm02:11,693,196), ss244712652 (Gm02:11,693,604), and ss244712653 (Gm02:11,693,900), to confirm the presence of the Rsv4 locus and genotyped the lines at the Genomics Center, University of Minnesota, Minneapolis, MN, using Sequenom iPLEX platform. We also employed five SSR markers Sat_317 (Gm13: 30,984,436-30,984,483) and Sat_154 (Gm13:27,312,436-27,312,485) for presence of Rsv1 locus, Sat_424 (Gm14:46,983,684-46,983,731) and Satt560 (Gm14:47,849,680-47,849,691) for Rsv3 locus, and Satt634 (Gm02:11,441,849-11,441,887) for Rsv4 locus. Moreover, we phenotyped minimum 50 plants per GP line by mechanical inoculations with SMV-G1 and G7 strains in a greenhouse.

Population development and genotyping

To study inheritance of SMV resistance, we crossed the GP20 line with homozygous recessive cultivar Williams 82 and monitored F_2 plants for hypocotyl and flower color segregation at the Arkansas Agricultural Research and Extension Center of University of Arkansas, Fayetteville, AR. We collected leaf tissue for DNA extraction and used three selected polymorphic SSR markers Sat_317 for Rsv1, Sat_424 for Rsv3 and Satt634 for Rsv4 locus for

molecular screening of each F_2 plant derived from the validation population GP20 × Williams 82.

Each polymerase chain reaction (PCR) mixture consisted of 15×Green GoTaq Flexi Buffer (Promega), 45mM $MgCl_2$, 2.5mM dNTPs, 5mM primer mix, 1U Taq (Promega), and 80ng DNA. We amplified PCR products with a program of 94°C for 5 min initial denaturation; 35 cycles of 25 s at 94°C denaturation, 25 s at 61ºC for Sat_317, 50ºC for Sat_424, and 48ºC for Satt634 primers annealing, 25 s at 72°C extension, and 5 min at 72°C final extension after the last cycle. We separated the PCR products in 6% non-denaturing polyacrylamide gel in 0.6 TBE and visualized by staining with ethidium bromide. To analyze the results from 155 F_2 samples, we used a scoring system "A" (GP20 parental allele), B (Williams 82 parental allele), and AB (presence of both parental alleles).

SMV inoculation

We used two SMV strains, G1 and G7, to screen the validation population (VP) of GP20 × Williams 82. To confirm strains identities, we observed foliar symptoms on sets of differentials including: PI 96983 (Rsv1), York (Rsv1-y), V262 (Rsv1-n), L29 (Rsv3), V229 (Rsv3), V94-5152 (Rsv4), V97-9003 (Rsv4), and Lee 68 (rsv). We introduced SMV into each plant by mechanical inoculation of at least 20 individuals per $F_{2:3}$ VP line according to Chen et al. (1991). We prepared the inoculum by grinding infected leaves in ice-cold 0.01M potassium phosphate buffer (pH 7.2) at an approximate dilution 1:10 (w/v). We pre-dusted both unifolate leaves before V1 stage with 600-mesh carborundum, and rubbed with a pestle dipped in the inoculum. To prevent cross contamination of SMV strains, we performed inoculations in separate greenhouses. The greenhouse conditions were maintained at 28°C with a 14 h

photoperiod at the Harry R. Rosen Alternative Pest Control Center, University of Arkansas in Fayetteville, AR. We monitored foliar reactions to each SMV strain each week, compared with set of checks 2-4 weeks after inoculation, and classified foliar reactions into four groups as all resistant (R), all susceptible (S), all necrotic (N) and segregating (R+S or R+N+S) phenotypes.

Data analysis

A Chi-square test (χ^2) was used to determine the goodness of fit of observed segregation ratios of three independent genes assortment based on the proposed genetic model (Table 2). This test was used for genotyping of F_2 plants (pooled classification) and phenotyping of $F_{2:3}$ lines separately. Pooled classification of F_2 plants was made based on a presence or absence of a particular SMV gene that was observed after genotyping, ignoring their homozygous or heterozygous stage. Also Chi-square was performed when the marker data was collated with phenotypic data of SMV-G1, SMV-G7, and SMV-G1 and G7 together.

RESULTS

Evaluation of pyramided lines

In a previous study, three SMV resistance genes, *Rsv1*, *Rsv3* and *Rsv4* were pyramided by crossing two resistant soybean accessions J05 (*Rsv1*+*Rsv3*) and V94-5152 (*Rsv4*) using marker-assisted breeding approach (Shi et al. 2009). In this research, we tested the homozygosity status of the $F_{4:7}$ GP lines using two SSR markers, Sat_317 and Sat_154, linked to the *Rsv1* locus; two SSR markers, Sat_424 and Satt560, linked to the *Rsv3* locus; one SSR marker, Satt634, and three SNP markers, ss244712651, ss244712652 and ss244712653, linked to the *Rsv4* locus (Table 1). Based on the SNPs, the "A-G-G" pattern was expected in genotypes

carrying the *Rsv4* gene, whereas the "T-C-A" pattern indicated an absence of this locus. In accordance to genotyping, we inoculated about 50 plants from each GP line with SMV-G1 and G7 strains to verify their resistance under greenhouse conditions (Table 1). Based on the marker and SMV inoculation results, we identified ten GP lines potentially carrying three SMV R-genes at homozygous stages. In order to confirm the presence of these three genes in the GP population, we selected the homozygous line GP20 (*Rsv1+3+4*) to perform further inheritance studies.

Validation population analysis

To create the validation population, we crossed SMV resistant $F_{4:8}$ GP20 line with the homozygous recessive at three analyzed loci Williams 82 (*rsv*) soybean cultivar susceptible to both SMV-G1 and G7 strains (Table 1). We employed three polymorphic SSR markers, Sat_317, Sat_424 and Satt634 to detect *Rsv1*, *Rsv3* and *Rsv4* genes respectively and assess a number of observed genotypes in 155 F_2 plants (Table 2). Sat_317 (position 72 cM on MLG F, approximate distance of 4 cM to the *Rsv1*) marker analysis revealed the genetic ratio of 39A:80H:36B. Similarly, Sat_424 marker (position 101.1 cM on MLG B2, approximate distance of 3 cM to the *Rsv3*) was scored as 46A:67H:42B, and Satt634 (position 46.4 cM on MLG D1b, approximate distance of 2 cM to the *Rsv4*) marker scored as 37A:74H:44B (Figure 2). Based on the Chi-square test, all marker results fit to a 1:2:1 genetic ratio (data not showed).

From the total of 155 F_2 samples, 62 displayed presence of all three R-genes with 3 plants being homozygous at all three loci (*R1R1R3R3R4R4*) (Table 3); 62 plants had alleles of two R-genes in various combinations (22 *Rsv1+3*, 16 *Rsv1+4*, and 24 *Rsv3+4* samples); 25 plants contained one single R-gene (6 with *Rsv1*, 13 with *Rsv3*, and 9 with *Rsv4*); and 3 plants were

homozygous recessive at all three loci (*r1r1r3r3r4r4*). These observed genotyping results (Table 3, Figure 2) were compared with expected genetic ratio of three independent genes assortment (Table 2) using a Chi-square (χ^2) goodness of fit test. The results showed a good fit to segregation of three independent genes in the VP population with a score of 7.2 and two-tailed p-value of 0.4 (Table 3).

We also analyzed the $F_{2:3}$ VP lines under greenhouse conditions to confirm the phenotypic reaction to inoculation about 25 plants per line with SMV-G1 and G7 strains separately (Table 3). We classified foliar symptoms of the VP lines infected by the G1 strain as 53 resistant lines (R), 27 susceptible lines (S) and 75 lines segregating (R+S). No necrotic symptoms occurred with G1 infection (Table 3, Figure 3). Infection by the G7 strain revealed 93 resistant lines (R), 3 necrotic lines (N), 3 susceptible lines (S), 27 segregating lines with two classes of reaction (R+S) and 29 segregating lines with three classes of reaction (R+N+S) (Table 3, Figure 3).

Genetic segregation analysis

We collated the observed $F_{2:3}$ phenotypic data of SMV-G1 only, SMV-G7 only, and SMV-G1 and G7 together with F_2 genotyping results (Table 3), and tested for the expected genetic ratio of three independent gene assortment (Table 2) using a two-tailed Chi-square (χ^2) goodness of fit test. Chi-square testing using molecular data and phenotypic results of SMV-G1 (with 81% accuracy) fit into the segregation of three independent genes with a χ^2 value of 10.19 and a p-value of 0.1781. The same results were obtained by testing molecular data and phenotypic results of SMV-G7 (85% accuracy) getting a χ^2 value of 9.77, and a p-value of 0.202. However, in testing for SMV-G1 and G7 together, from a total of 155 samples, 115 exhibited

consistency between F_2 molecular data and expected phenotype with SMV-G1 and G7 infection resulted in 74% accuracy. The results showed a χ^2 value of 17.23 and a p-value of 0.016. According to the criteria, this difference was considered to be statistically significant (with 99% confidence), and thus, the null hypothesis (H_0) of three independent genes segregation ratio was rejected due to small population size, marker distances to a specific R-gene, and experimental errors in phenotyping.

DISCUSSION

The purpose of gene pyramiding is to incorporate multiple genes from different parents into a single genotype to enhance trait performance (Collard and Mackill 2008). Improving qualitative traits, such as SMV resistance, is relatively easy because the presence of particular gene must have an effect on phenotypic performance of the plant (Saghai Maroof et al. 2008; Shi et al. 2009). Resistance breeding has been very successful in the past and provided various resistant crop varieties highly adapted to adverse growing conditions (Collard and Mackill. 2008; Saghai Maroof et al. 2008). For example, marker-assisted gene pyramiding has been used to pyramid major genes for resistance to blight (Huang et al. 1997) and blast (Fukuoka et al. 2015) in rice. In wheat, it was used for pyramiding *Pm2+Pm4a*, *Pm2+Pm21*, *Pm4a+Pm21* for powdery mildew (Wang et al. 2001) and the *Lr41*, *Lr42*, and *Lr43* genes for leaf rust resistance (Cox et al. 1994). In soybeans, multiple *Rpp* genes of Asian soybean rust (Yamanaka et al. 2015); and *rag*3, *rag*1b, *rag*4, and *rag*1c aphid-resistant genes were pyramided with help of MAS (Chandrasena et al. 2015).

In the study by Shi et al. (2009), $F_{4:5}$ lines have been identified as presumably carrying all three SMV resistance genes using the cross J05 (*Rsv1+3*) × V94-5152 (*Rsv4*). The soybean

accessions, used as the parents for gene pyramiding, were both resistant to SMV-G1 and G7 strains (Table 1). The $F_{4:5}$ GP lines were used to advance to the $F_{4:7}$ generation to reduce their heterozygosity levels. The GP lines displayed the same resistance as their parents, and for this reason, genotyping with available molecular markers was necessary for choosing the right GP parent for a validation population.

To verify the presence of all three SMV resistance genes in one soybean genotype our goal was to make a cross between the chosen inbred $F_{4:7}$ GP line, GP20 (*Rsv1+3+4*) and Williams 82 (*rsv*) to examine genetic segregation for SMV reaction and linked SSR markers (Figure 1). Observed genetic segregation of F_2 plants and phenotypic relationship of $F_{2:3}$ lines inoculated with SMV-G1 and G7 strains indicated the presence of three genes for SMV resistance at the homozygous state in the GP20 line (Table 3).

This study demonstrated three independent resistant genes segregating according to Mendelian laws that made it simple to predict 64 individuals as a minimal population size. One SSR marker per each SMV locus was used for tracing the presence or absence of the target genes, and their efficiency was good enough to fit into three independent genes segregation ratio (Table 3, Figure 2). However, these markers displayed 74% consistency when compared with $F_{2:3}$ phenotypic results of infection and both SMV strains, and the results did not fit into the three genes ratio. This could be due to possible inconsistency between genotyping and phenotyping data that was caused by using SSR markers that were not perfectly linked with three SMV loci. There is still a possibility for recombination between the gene and the marker located far from each other thus causing deviations in the results. For validation purposes, using one marker per locus was effective; however, it is advised to use at least two markers per locus while tracking SMV R-genes in a breeding program.

The successful effort on SMV resistance gene pyramiding using MAS was performed in previous studies using similar breeding strategies (Saghai Maroof et al. 2008; Shi et al. 2009); however, no validation cross was performed to confirm the number of pyramided genes in selected lines. The validation is often skipped as it needs several more years to confirm the results; therefore, the pyramided lines cannot be released to be used in breeding programs, and rather they are used in genetic studies. Our research provided the first evidence of successful incorporation of three dominant SMV resistance genes into the soybean GP20 line by performing a validation cross with the susceptible recessive line Williams 82. The confirmed GP20 line provides durable resistance to all SMV strains identified in the United States, thus protecting soybeans against an evolutionary race between host and pathogen.

This GP method was based on a cross between two distinct soybean germplasm lines, and selected progeny was a result of random gene shuffling that could potentially have an effect on expression of other important traits because gene pyramiding was not performed by backcrossing where crossing with the recurrent parent eliminate the linkage drag. Using the GP20 line as a donor parent for backcrossing with elite lines would be of higher importance in breeding programs in the future, and final progenies could be confirmed by background analysis using genome-wide molecular markers; therefore, it can be directly developed as a commercial variety. Molecular markers used for genotyping in this study could facilitate the backcrossing process by reducing the number of generations that breeders must evaluate to ensure the presence of desired SMV R-gene combination

The impact of molecular breeding is increasingly being appreciated by researchers as a method for improving the lower efficiency of traditional breeding methods. The strategy of introgression and screening multiple R-genes by molecular markers is a powerful method that

reduces the cost and the time required for the isolation of desirable recombinants with target resistance genes. It is important to test reliability of markers to predict the phenotype. To improve the effectiveness of MAS, it is necessary to identify markers as close as possible to the target gene to reduce the recombination frequency between the target gene and the marker. By providing broader and durable resistance against all existing SMV isolates, our inbreed GP20 line has been proposed as a potential future release, that is practical for breeders and will have a high impact on the yield stability and sustainability of soybean production when combined with backcrossing strategies.

Although SMV resistance loci have been reported in many soybean genotypes, most of the modern commercial cultivars are susceptible to SMV, particularly to more virulent strains (Zheng et al. 2005; Shakiba et al. 2012a). New resistance-breaking SMV strains cause a real danger, and for these reasons, gene pyramiding is crucial for breeding and production purposes and will contribute to provide effective resistance to a broad and ever-changing range of SMV pathotypes.

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Table 1. Genotypic and phenotypic characterization of soybean accessions used to create gene pyramiding and validation populations.

Soybean Accession	R-Gene	SSR[a]					SNP[b]			SMV[c]	
		Sat_317 (*Rsv1*)	**Sat_154** (*Rsv1*)	**Sat_424** (*Rsv3*)	**Satt560** (*Rsv3*)	**Satt634** (*Rsv4*)	**ss244712651** (*Rsv4*)	**ss244712652** (*Rsv4*)	**ss244712653** (*Rsv4*)	G1	G7
J05	*Rsv1+3*	+	+	+	+	-	T	C	A	R	R
V94-5152	*Rsv4*	-	-	-	-	+	A	G	G	R	R
GP20	*Rsv1+3+4*	+	+	+	+	+	A	G	G	R	R
Williams 82	*rsv*	-	-	-	-	-	T	C	A	S	S

[a] SSR genotyping: +, presence of SMV resistance locus; -, absence of SMV resistance locus.
[b] SNP genotyping: A, T, C, G correspond to DNA nucleotide changes.
[c] Symptoms upon infection by SMV-G1 and G7 strains: R, resistant (symptomless); S, susceptible (mosaic).

Table 2. Genetic model for segregation of three independent SMV resistance genes and $F_{2:3}$ phenotypic reactions in the validation population GP20 (*Rsv1+3+4*) × Williams 82 (*rsv*).

Genotype[a]	Sat_317 *Rsv1*	Sat_424 *Rsv3*	Satt634 *Rsv4*	Code[b]	Expected	Observed	SMV-G1[c]	SMV-G7[c]
R1R1 R3R3 R4R4	+	+	+	1+3+4	2.42 (1/64)	4	R	R
R1R1 R3R3 ***R4r4***	+	+	+	1+3+4	4.84 (2/64)	6	R	R
R1R1 R3R3 ***r4r4***	+	+	-	1+3+0	2.42 (1/64)	6	R	R
R1R1 ***R3r3 R4R4***	+	+	+	1+3+4	4.84 (2/64)	5	R	R
R1R1 R3r3 ***R4r4***	+	+	+	1+3+4	9.68 (4/64)	7	R	R+S
R1R1 R3r3 ***r4r4***	+	+	-	1+3+0	4.84 (2/64)	3	R	R+S
R1R1 ***r3r3 R4R4***	+	-	+	1+0+4	2.42 (1/64)	4	R	R
R1R1 r3r3 ***R4r4***	+	-	+	1+0+4	4.84 (2/64)	4	R	R+S
R1R1 r3r3 ***r4r4***	+	-	-	1+0+0	2.42 (1/64)	3	R	S
R1r1 R3R3 R4R4	+	+	+	1+3+4	4.84 (2/64)	5	R	R
R1r1 R3R3 ***R4r4***	+	+	+	1+3+4	9.68 (4/64)	8	R+N+S	R
R1r1 R3R3 ***r4r4***	+	+	-	1+3+0	4.84 (2/64)	4	R+N+S	R
R1r1 ***R3r3 R4R4***	+	+	+	1+3+4	9.68 (4/64)	10	R	R
R1r1 R3r3 ***R4r4***	+	+	+	1+3+4	19.36 (8/64)	17	R+N+S	R+N+S
R1r1 R3r3 ***r4r4***	+	+	-	1+3+0	9.68 (4/64)	9	R+N+S	R+N+S
R1r1 ***r3r3 R4R4***	+	-	+	1+0+4	4.84 (2/64)	4	R	R
R1r1 r3r3 ***R4r4***	+	-	+	1+0+4	9.68 (4/64)	4	R+N+S	R+N+S
R1r1 r3r3 ***r4r4***	+	-	-	1+0+0	4.84 (2/64)	3	R+N+S	R+N+S
r1r1 R3R3 R4R4	-	+	+	0+3+4	2.42 (1/64)	3	R	R
r1r1 R3R3 ***R4r4***	-	+	+	0+3+4	4.84 (2/64)	8	R+S	R
r1r1 R3R3 ***r4r4***	-	+	-	0+3+0	2.42 (1/64)	6	S	R
r1r1 ***R3r3 R4R4***	-	+	+	0+3+4	4.84 (2/64)	4	R	R
r1r1 R3r3 ***R4r4***	-	+	+	0+3+4	9.68 (4/64)	9	R+S	R+S
r1r1 R3r3 ***r4r4***	-	+	-	0+3+0	4.84 (2/64)	7	S	R+S
r1r1 r3r3 R4R4	-	-	+	0+0+4	2.42 (1/64)	3	R	R
r1r1 r3r3 ***R4r4***	-	-	+	0+0+4	4.84 (2/64)	6	R+S	R+S
r1r1 r3r3 ***r4r4***	-	-	-	0+0+0	2.42 (1/64)	3	S	S

[a] SMV allele symbols: *R1=Rsv1; r1=rsv1; R3=Rsv3; r3=rsv3; R4=Rsv4; r4=rsv4*; non-bold symbols signify the same genotype as above.

[b] Simplified genetic coding system for scoring R-genes: 1= *Rsv1*, 3= *Rsv3*, 4=*Rsv4*, 0=*rsv*.

[c] Phenotypic symptoms of $F_{2:3}$ lines upon SMV-G1 and G7 strains infection: R, resistant (symptomless); N, systemic necrosis; S, susceptible (mosaic).

Table 3. Collated classification of observed genotypic and phenotypic segregation in a population GP20 (*Rsv1+Rsv3+Rsv4*) × Williams 82 (*rsv*) to the expected genetic model.

Code[a]	R-gene(s)	No. F_2 lines genotyped[b]		No. $F_{2:3}$ lines phenotyped[c]		
		Expected	Observed	G1	G7	G1+G7
1+3+4	*Rsv1+3+4*	65.34 (27/64)	62	48	51	40
1+3+0	*Rsv1+3*	21.78 (9/64)	22	20	18	18
1+0+4	*Rsv1+4*	21.78 (9/64)	16	15	15	15
0+3+4	*Rsv3+4*	21.78 (9/64)	24	17	20	15
1+0+0	*Rsv1*	7.26 (3/64)	6	6	6	6
0+3+0	*Rsv3*	7.26 (3/64)	13	11	12	11
0+0+4	*Rsv4*	7.26 (3/64)	9	7	8	7
0+0+0	*rsv*	2.42 (1/64)	3	3	3	3
		155 (100%)	155 (100%)	127 (81%)	133 (85%)	115 (74%)
			$\chi^2 = 7.2$ $p = 0.4$	$\chi^2 = 10.19$ $p = 0.1781$	$\chi^2 = 9.77$ $p = 0.202$	$\chi^2 = 17.23$ $p = 0.016$**

[a] Simplified genetic coding system for scoring R-genes: 1= *Rsv1*, 3= *Rsv3*, 4=*Rsv4*, 0=*rsv*.
[b] Pooled classification of observed F_2 plants in comparison with the expected genetic model.
[c] Pooled classification of $F_{2:3}$ lines displaying consistent data between genotypic SSR marker results and phenotypic reactions to SMV-G1 and G7 strains; Chi-square scores were obtained by observed phenotypic and genotypic data; **, significance level of $p \leq 0.01$.

Table 4. Summary of F_2 genotypes and their corresponding $F_{2:3}$ phenotypes in response to soybean mosaic virus G1 and G7 strains of the gene pyramiding validation population from GP20 (*Rsv1+3+4*) × Williams 82 (*rsv*).

Possible Genotype†	No. F_2 Plants ‡	F2:3 Phenotypes for SMV-G1 §		F2:3 Phenotypes for SMV-G7 §	
		Expected	Observed	Expected	Observed
R1R1 R3R3 R4R4	4	R	- + + +	R	+ + + +
R1R1 R3R3 ***R4r4***	6	R	+ + + + + +	R	+ + + + + +
R1R1 R3R3 ***r4r4***	6	R	+ + + + + +	R	+ + + + + +
R1R1 ***R3r3 R4R4***	5	R	+ + + - +	R	+ + + + +
R1R1 R3r3 ***R4r4***	7	R	+ + + + + + +	R+S	- - + + - + -
R1R1 R3r3 ***r4r4***	3	R	- + +	R+S	- + -
R1R1 ***r3r3 R4R4***	4	R	+ + + +	R	+ + + +
R1R1 r3r3 ***R4r4***	4	R	- + + +	R+S	- + + +
R1R1 r3r3 ***r4r4***	3	R	+ + +	N+S	+ + +
R1r1 R3R3 R4R4	5	R	- - - - -	R	- + + + +
R1r1 R3R3 ***R4r4***	8	R+N+S	+ + + + + + + +	R	+ + + + + + + +
R1r1 R3R3 ***r4r4***	4	R+N+S	+ + + +	R	+ + + +
R1r1 ***R3r3 R4R4***	10	R	+ - - + + + + - + +	R	+ + + + + + + + + +
R1r1 R3r3 ***R4r4***	17	R+N+S	+ + + + + + + - + + + + + - - - +	R+N+S	+ + + + + - + + + + - + - - - + -
R1r1 R3r3 ***r4r4***	9	R+N+S	+ + + + + - + + +	R+N+S	+ + + + + - + + -
R1r1 ***r3r3 R4R4***	4	R	+ + + +	R	+ + + +
R1r1 r3r3 ***R4r4***	4	R+N+S	+ + + +	R+N+S	+ + + +
R1r1 r3r3 ***r4r4***	3	R+N+S	+ + +	R+N+S	+ + +
r1r1 R3R3 R4R4	3	R	- - -	R	+ + +
r1r1 R3R3 ***R4r4***	8	R+S	+ + + + + - + +	R	+ + + + + + + +
r1r1 R3R3 ***r4r4***	6	S	+ + - + + +	R	+ + + + + +
r1r1 ***R3r3 R4R4***	4	R	- + - +	R	+ + - +
r1r1 R3r3 ***R4r4***	9	R+S	+ + + - + + + + +	R+S	+ - + - + - + + +
r1r1 R3r3 ***r4r4***	7	S	+ + - + + + +	R+S	+ + - + + + +
r1r1 ***r3r3 R4R4***	3	R	- + +	R	+ + +
r1r1 r3r3 ***R4r4***	6	R+S	+ + + - + +	R+S	+ + + - + +
r1r1 r3r3 ***r4r4***	3	S	+ + +	S	+ + +

† SMV allele: *R1, Rsv1; r1, rsv1; R3, Rsv3; r3, rsv3; R4, Rsv4; r4, rsv4*; Non-bold symbols signify the same genotype as above.
‡ Frequency of F_2 plants with specific SMV resistance alleles detected by three SSR markers.
§ Expected and observed phenotypes of $F_{2:3}$ lines in response to SMV-G1 and G7 strains; +, lines consistent with expected phenotype and F_2 molecular data; -, lines inconsistent with expected phenotype and F_2 molecular data.

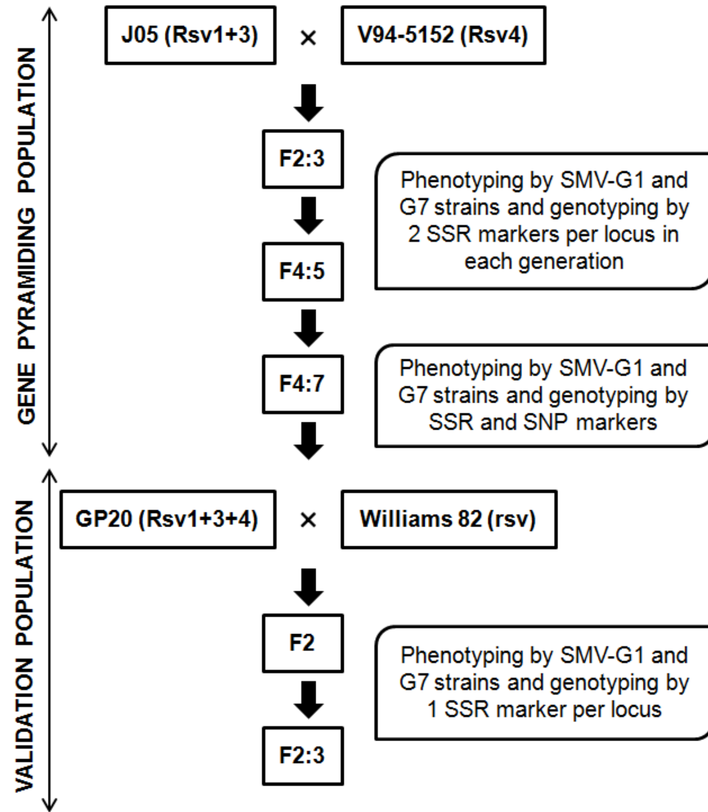

Figure 1. Schematic representation of pyramiding *Rsv1*, *Rsv3*, and *Rsv4* genes for SMV resistance using J05 (*Rsv1+3*) × V94-5152 (*Rsv4*) and validation cross of GP20 (*Rsv1+3+4*) × Williams 82 (*rsv*).

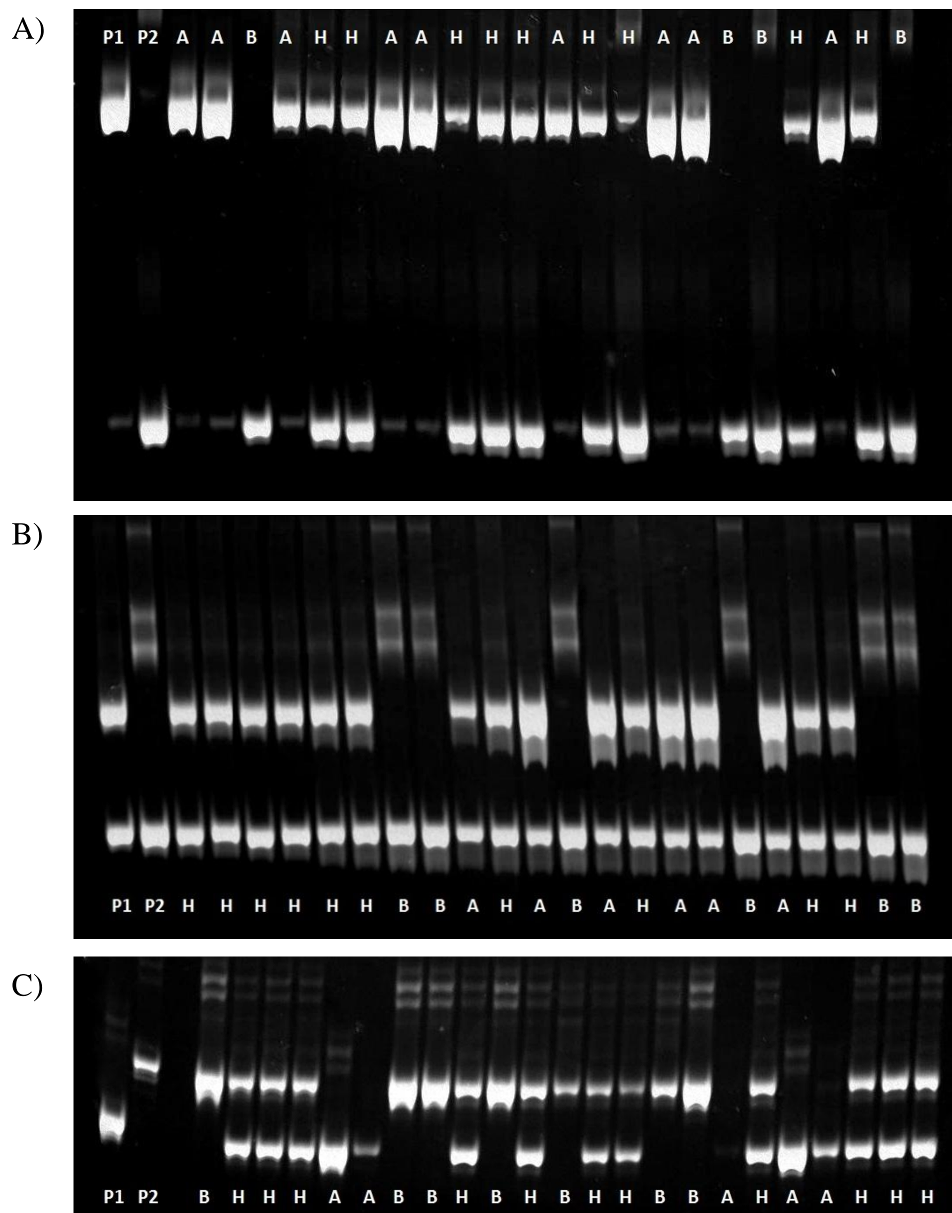

Figure 2. PCR amplification patterns of validation parents and F_2 population derived from a cross GP20 × Williams 82 using SSR markers linked to SMV resistance loci: A) Sat_317 (annealing temp. 61°C) linked to *Rsv1;* B) Sat_424 (annealing temp. 50°C) linked to *Rsv3*; C) Satt634 (annealing temp. 48°C) linked to *Rsv4.* P1, parent GP20; P2, parent Williams 82; A, resistance allele derived from GP20; B, susceptible allele derived from Williams 82; H, both alleles derived from both parents.

Figure 3. Foliar symptoms of SMV infection on $F_{2:3}$ population derived from GP20 (R) × Williams 82 (S) cross: Resistant plants inoculated with SMV-G1 strain (upper left); Susceptible (mosaic) plants inoculated with SMV-G1 strain (upper right); Early systemic necrosis symptoms with SMV-G7 infection (lower left); Segregating line expressing resistant and susceptible (R+S) reactions (lower right).

CHAPTER FOUR

A NOVEL ALLELE AT THE Rsv4 LOCUS

FOR RESISTANCE TO SOYBEAN MOSAIC VIRUS

ABSTRACT

Soybean mosaic virus (SMV) is the most prevalent viral pathogen and economic threat to soybean production worldwide. Three independent genes harboring SMV resistance have been identified: *Rsv1*, *Rsv3*, and *Rsv4*. Although the resistance genes (R-genes) have been found in some germplasm, usually they provide protection to some, but not all, viral strains. The objective of this research was to identify a new source of SMV resistance in Korean soybean accession PI 438307. The soybean genotype PI 438307 was crossed with susceptible parent Essex (*rsv*), and differential parents PI 96983 (*Rsv1*), L29 (*Rsv3*), and V94-5152 (*Rsv4*). F_2 plants and $F_{2:3}$ lines derived from all four cross combinations were screened with SMV-G7 strain. Additionally, F_2 plants obtained from PI 438307 (R) x Essex (S) were genotyped with two simple sequence repeat (SSR) markers on chromosome 2 (MLG D1b). Inheritance and allelic studies revealed that resistance to SMV in PI 438307 is controlled by a single dominant gene allelic to the *Rsv4* locus. PI 438307 exhibited unique symptomology when compared to reported *Rsv4* alleles in V94-5152, PI 88788 and Beeson. PI 438307 was resistant to SMV-G1 through G6 and resistant at seedling stages to SMV-G7. Therefore, it was proposed that the new allele *Rsv4-v* should be assigned to the SMV resistance in this soybean accession. Soybean sources carrying *Rsv4* alleles are rare among the soybean germplasm and confer resistance to all or most SMV strains; therefore, this allele may be a good choice for breeding programs in the future.

INTRODUCTION

Among over one hundred viruses that are known to infect soybeans, soybean mosaic virus is the most common and detrimental pathogen causing substantial yield reduction and significant seed quality deterioration (Ren et al., 1997). Not only does it cause the mosaic disease in soybeans, but it also infects many other commercially important plants worldwide (Balgude et al., 2012). Susceptible soybean genotypes develop characteristic stunted growth and crinkled leaves, display reduction in seedling viability and vigor, and produce fewer, smaller, and often mottled seeds (Ross, 1983; Buss et al., 1989; Hill et al., 1987).

Host resistance is the preferred means of managing pathogens and preventing yield loses in economically important crops (Kang et al., 2005). Three independent multiallelic loci, *Rsv1, Rsv3* and *Rsv4* have been reported in soybean (Buss et al. 1997; Buzzel and Tu 1989 Kiihl and Hartwig, 1979), and mapped on chromosome 13 (MLG F), 14 (MLG B2), and 2 (MLG D1b), respectively (Hayes et al., 2000; Jeong et al., 2002; Yu et al., 1994). The *Rsv1* locus includes at least ten alleles (Chen et al., 1991, 2001, 2002; Kiihl and Hartwig, 1979; Roane et al., 1983; Shakiba et al., 2013) and generally confers resistance to less virulent strains (G1 - G4) and susceptibility or necrosis to more virulent strains (G5 - G7) (Table 1) (Chen et al., 1991; Gunduz et al., 2002; Ma et al., 2002; Li et al., 2010). The *Rsv3* locus contains at least six alleles (Buzzel and Tu, 1989; Gunduz et al., 2001; Cervantes-Bousher et al., 2015; Shakiba et al., 2012) and confers resistance to more virulent strains (G5 - G7) and susceptibility to less virulent strains (G1 - G4) (Table 1). The *Rsv4* locus has at least three alleles conferring resistance to all or most strains (G1 - G7) (Buss et al., 1997; Ma et al., 2002; Gunduz et al., 2004; Shakiba et al., 2013); however, often shows resistance at early vegetative stage and delayed mild susceptibility at a later stage (Table 1) (Buss et al., 1997; Gunduz et al. 2004)

To understand the principles of SMV infection and identify genetic sources of resistance,

extensive genetic studies need to be conducted. In 80% of all reported resistant cultivars, it was conferred by a single R-gene (Buss et al., 1989; Chen et al., 1991; Shakiba et al., 2012, 2013; Wang et al., 1998), only some soybean accessions contain two R-genes in diverse combinations (*Rsv1+3*, *Rsv1+4*, and *Rsv3+4*) (Chen et al., 1993; Gunduz et al., 2001; Liao et al., 2002; Ma et al., 1995; Shi et al., 2012; Zheng et al., 2006), and three R-genes have been identified in the Korean landrace '8101' (Liao et al., 2011). Presence of two or three genes for SMV resistance diminishes vulnerability of the plant by working in a complementary fashion to protect the host against multiple and ever-changing viral strains (Chen et al. 1993; Liao et al., 2011; Shi et al. 2012).

The old Korean plant introduction PI 438307 displayed resistance to SMV-G1 and SMV-G7, and therefore, it was assumed to carry either *Rsv1-r*, *Rsv1-h*, *Rsv4*, *Rsv1Rsv3*, *Rsv1Rsv4*, *Rsv3Rsv4*, or a new allele for SMV resistance (Zheng et al., 2005). Shi et al. (2008) observed the same reactions and postulated that PI 438307 carries *Rsv1-yRsv3* or *Rsv1-yRsv4* gene combinations because PCR-based marker Rsv1-f/r did not amplify a fragment of a 3gG2 gene, a candidate for *Rsv1*, suggesting that the R-gene in PI 438307 was not at the *Rsv1* locus, and leaving a possibility that this accession carries an allele at the *Rsv3* or *Rsv4*. In another study by Zheng et al. (2008), PI 438307 exhibited resistance to SMV-G1 through G6, and early resistance (ER) to G7 SMV strains (Table 1) indicating presence of new SMV resistance allele.

The objective of this study was to investigate a source of SMV resistance in PI 438307 soybean accession by performing genetic studies, and determine plant reaction symptoms of to all SMV strains identified in the United States. Identifying new allele(s)/gene(s) with specific symptom patterns under different SMV strain inoculations will provide new knowledge of resistance to utilize in breeding programs (Kang et al., 2005).

MATERIALS AND METHODS

Population development

PI 438307 (VIR2980), a Korean plant introduction, was provided from the Soybean Germplasm Collection, USDA-ARS. In this study, PI 438307 (*Rsv-?*) was crossed with a susceptible cultivar Essex (*rsv*) to study the inheritance of SMV resistance. To determine allelomorphic relationships with the reported resistance loci, PI 438307 was crossed with a set of resistant differential parents PI 96983, L29 and V94-5152 carrying *Rsv1*, *Rsv3* and *Rsv4*, respectively. All cross combinations were conducted in the field at the Arkansas Agricultural Research and Extension Center of University of Arkansas, Fayetteville. The F_1 hybrids were grown at 28°C and 14 h photoperiod in the Altheimer greenhouse of University of Arkansas, Fayetteville. Flower and/or pubescence color were used as morphological markers and Satt634 SSR marker was used to confirm true hybrids from each cross combination. One portion of the F_2 seeds of each cross was used for greenhouse inoculation and the second portion was planted in the field to advance $F_{2:3}$ lines.

SMV inoculations

The F_2 population and $F_{2:3}$ lines were used for inoculation using SMV-G7 strain kindly provided by Dr. Sue Tolin, Virginia Polytechnic Institute and State University. The strain identity and purity was confirmed on a set of differential soybean genotypes, including PI 96983 (*Rsv1*), York (*Rsv1-y*), V262 (*Rsv1-n*), L29 (*Rsv3*), V229 (*Rsv3*), V94-5152 (*Rsv4*) and Essex (*rsv*). The virus was introduced into at least 100 F_2 plants and 50 $F_{2:3}$ lines by mechanical inoculation according to Chen et al. (1991). The inoculum was prepared by grinding infected leaves in ice-cold 0.05M potassium phosphate buffer (pH=7.2) at an approximate rate of 1 g

tissue per 10 ml buffer. Both unifolate leaves of each plant, before V1 stage, were pre-dusted with 600-mesh carborundum and rubbed with a pestle dipped in the inoculum. The greenhouse conditions were maintained at 28°C and 14 h photoperiod at the Harry R. Rosen Alternative Pest Control Center, University of Arkansas, Fayetteville. Individual plant reactions to SMV-G7 strain were monitored each week and compared with set of differentials 4-6 weeks after inoculation. F_2 plants were classified into three distinct phenotypes as resistant (R), susceptible (S), or necrotic (N) whereas $F_{2:3}$ lines were grouped as all R, all S, or segregating (H) based on individual plant reaction. Additionally, PI 438307 was inoculated with seven U.S. SMV strains, G1 through G7, to establish symptoms of reaction, and the results were compared with known reactions of soybean genotypes carrying SMV resistance at all three loci (Table 1).

SMV detection

A dot blot serological procedure was performed to detect the presence of SMV in the F_2 plants derived from each cross and the corresponding parents three weeks after inoculation. Leaf samples were randomly picked from plants displaying resistant, susceptible, and necrotic symptoms. SMV-infected plant stock was used as a SMV-positive control, and SMV-free tissue was applied as a negative control. The procedure was performed as described by Tzanetakis et al. (2004). SMV-specific antibodies were provided by Dr. Ioannis Tzanetakis, University of Arkansas, Fayetteville. In short, leaf tissue was ground in 1 ml of water and 10 µl of each sap sample was blotted onto nitrocellulose membranes, washed twice with PBS and blocked by soaking in blocking buffer (PBS + 5% nonfat milk powder) for 1 h. After a washing with PBS-Tween, the membranes were transferred to SMV antiserum solution (1:1,000 to 1:25,000 diluted in PBS) and incubated at RT for 1 h. The membranes were rinsed three times with PBS-Tween solution for 5 min each, transferred to goat anti-rabbit alkaline phosphatase

conjugate (1:2,000 to 1:5,000 diluted in PBS containing 2% PVP-10,000 and 0.2% nonfat milk powder) and incubated at RT for additional 1 h. After triple washing with PBS-Tween solution for 5 min each, the filters were placed in substrate buffer (0.1 M Tris pH = 9.5; 0.1 M NaCl; 5 mM $MgCl_2$) containing precipitating substrate NBT/BCIP). Reactions were terminated by transferring the membranes to deionized water. The samples were considered as infected by SMV when the tissue dot changed to the brown/purple color after incubation with alkaline phosphatase.

DNA extraction and genotyping

Genomic DNA was extracted from F_2 plants of PI 438307 (R) × Essex (S) cross using the CTAB method (Doyle and Doyle, 1990) with minor modifications. In this procedure, frozen leaves were crushed to powder with metal beads using Qiagen Retsch TissueLyser Mm301 Mixer Mill Grinder. 750 μL of extraction buffer (2% CTAB, 100 mM Tris-Cl, 20 mM EDTA pH 8.0, 1.4 M NaCl and 1% volume β-mercaptoethanol) was added to each tube and incubated at 65°C in a water bath. After 1 hour of incubation, 1 ml chloroform:isoamyl alcohol (24:1) was added and samples were centrifuged at 12,000 rpm for 15 min at RT. To precipitate DNA, the upper layer was transferred to a new tube containing 1 ml ice-cold 95% ethanol. Pellets were washed in 1 ml 75% ethanol, dried for 2 hours, and dissolved in 200 μl nuclease-free water. DNA concentrations were measured using the NanoDrop ND-2000 1-Position spectrophotometer (Thermo Scientific).

Two SSR markers, Satt634 (Gm02: 11,441,849-11,441,887) and Satt296 (Gm02: 12,975,935- 12,975,997) linked to the *Rsv4* locus, were used for genotyping the F_2 plants PI 438307 (*Rsv-?*) × Essex (*rsv*). Each polymerase chain reaction (PCR) was consisted of 10×Green GoTaq Flexi Buffer (Promega), 45mM $MgCl_2$, 2.5mM dNTPs, 5mM primer mix, 1U Taq

(Promega), and 60ng DNA. PCR products were amplified with a program of 95°C for 10 min initial denaturation; 35 cycles of 25 s at 95°C denaturation, 25 s at 50°C for both primers annealing, 25 s at 72°C extension, and 5 min at 72°C final extension after the last cycle. PCR products were run in 6% non-denaturing polyacrylamide gel in 0.6 TBE and visualized by staining with ethidium bromide. To analyze the results, a scoring system of "A" for presence of PI 438307 allele, "B" for presence of Essex allele, or "H" for presence of both alleles was utilized.

In addition, PI 438307 was genotyped using three single nucleotide polymorphism (SNP) markers ss244712651 (Gm02: 11,693,196), ss244712652 (Gm02: 11,693,604), and ss244712653 (Gm02: 11,693,900), to confirm the presence of the *Rsv4* locus. Sequenom iPLEX genotyping was performed at the Genomics Center, University of Minnesota, Minneapolis. Based on these SNPs, the "A-G-G" pattern was expected in genotypes carrying the *Rsv4* gene, whereas the "T-C-A" pattern indicated absence of this locus.

Data analysis

Segregation ratios for SMV symptoms showed in F2 plants and F2:3 lines derived from all cross combinations were tested to fit expected genetic ratios of one, two and three genes segregations using a chi-square (χ^2) goodness-of-fit test. The necrotic plants were classified as resistant when evaluating segregating populations (Chen et al., 1994). A chi-square goodness of fit test was also used to compare molecular marker data to the expected genetic 1A:2H:1B ratio of a single dominant gene segregation.

RESULTS

Inheritance of SMV resistance in PI 438307

Identification of resistance genes in plant genome is usually based on genetic and phenotypic analysis of segregating populations to establish their inheritance and allelism tests (Buss et al., 1989; Chen et al., 1991; Gunduz et al., 2004; Liao et al., 2011). To determine inheritance of SMV resistance in PI 438307 soybean accession, a cross was performed between a resistant genotype in question, PI 438307 (*Rsv-?*), and susceptible cultivar Essex (*rsv*). Greenhouse SMV-G7 strain inoculations were used to evaluate observed segregation ratios of F_2 plants and $F_{2:3}$ lines with expected genetic ratios. Phenotypic results of F_2 population indicated a monogenic segregation pattern of 3R:1S (109R:31S) with $\chi^2 = 0.6$ and $p = 0.43$ (Table 2). The $F_{2:3}$ population from the same cross displayed a good fit to 1R:2H(R+S):1S ratio (23R:48H:18S) with $\chi^2 = 1.135$ and $p = 0.56$ (Table 3). In addition, dot blot results performed on F_2 population detected 22 samples with high concentration of SMV and 49 samples without the virus (Table 5), confirming a segregating population for SMV infection and reaction. These results indicated that PI 438307 carries a single dominant gene for SMV resistance.

Furthermore, the F_2 population was genotyped by two SSR markers and the results exhibited a good fit to the 1A:2H:1B ratio (Table 4, Figure 2). Satt634 revealed the 57A:132H:54B ratio with $\chi^2 = 1.75$ and $p = 0.416$ whereas Satt296 displayed 58A:129H:56B ratio with $\chi^2 = 0.86$ and $p = 0.65$. The molecular marker screening indicated that PI 438307 could carry an allele at the *Rsv4* locus for SMV resistance.

Allelic relationship of SMV resistance in PI 438307

The allelism test was performed by crossing PI 438307 (*Rsv-?*) with a set of differential resistant genotypes PI 96983 (*Rsv1*), L29 (*Rsv3*) and V94-5152 (*Rsv4*). To determine whether PI 438307 carries resistance at the *Rsv1* locus, a cross PI 438307 (*Rsv-?*, R) × PI 96983 (*Rsv1*, N) was performed. Analyzed 214 F_2 plants exhibited a digenic segregation ratio of 15(R+N):1S (148R+50N:19S) with $\chi^2 = 2.39$ and $p = 0.12$ (Table 2), whereas 56 $F_{2:3}$ lines showed a 7R:8H(R+N+S):1S (24R:29H:3S) segregation ratio with $\chi^2 = 0.116$ and $p = 0.94$ (Table 3). This segregation ratios indicated the presence of two dominant resistance genes thus confirming that PI 438307 does not carry the *Rsv1* gene for SMV resistance, and the resistance gene in this accession is located at a different locus. Dot blot results performed on F_2 population derived from this cross detected 22 samples with presence of SMV and 25 samples without the virus (Table 5), clearly showing genetic segregation for SMV infection and reaction within this the population.

To examine whether PI 438307 carries a resistance allele at the *Rsv3* locus, a cross PI 438307 (*Rsv-?*, R) × L29 (*Rsv3*, R) was performed. Upon infection by SMV-G7, investigated 145 F_2 plants showed a digenic ratio of 15R:1S (137R:8S) with $\chi^2 = 0.11$ and $p = 0.73$ (Table 2), and 73 $F_{2:3}$ lines derived from the same cross showed a good fit to the 7R:8H(R+S):1S segregation ratio (29R:39H:5S) with $\chi^2 = 0.58$ and $p = 0.74$ (Table 3). These results indicated that SMV resistance in PI 438307 is not harbored by the *Rsv3* locus. Dot blot results performed on F_2 population detected 20 samples with SMV and 34 samples without the virus (Table 5), confirming the digenic segregation for SMV infection.

To determine whether PI 438307 carries resistance at the *Rsv4* locus, a cross PI 438307 (*Rsv-?*, R) × V94-5152 (*Rsv4*, R) was performed. There was no phenotypic segregation observed

within 112 F_2 plants when inoculated with SMV-G7 (Table 2). Also, all 71 $F_{2:3}$ lines showed a complete resistance to the virus (Table 3). Lack of segregation in the progenies indicated that both parents PI 438307 and V94-5152 carry resistance alleles at the same locus. Dot blot results confirmed these results as the virus was not detected in any of analyzed 68 samples (Table 5).

Reactions of PI 438307 to various SMV strains

Visual symptoms upon SMV-G7 infection were detected in the susceptible parent Essex with typical venial clearing and mosaics in the first trifoliate leaves approximately one week after inoculation. PI 96983 plants initially developed venial clearing symptoms during the first few days after inoculation and then became necrotic approximately seven days later. In contrast, L29 did not exhibit symptoms of disease on trifoliate leaves at any time during the experiment, whereas V94-5152 displayed resistance with mild mosaics at late developmental stages. These observations ratified the identity and purity of the SMV-G7 strain, and confirmed the reactions of all parents (Table 1, Figure 1).

The inheritance and allelism studies indicated that the SMV resistance gene in PI 438307 was allelic to the *Rsv4* locus. To determine whether resistance in PI 438307 is due to a new allele at the *Rsv4* locus, it was necessary to compare SMV reaction pattern of this soybean accession with *Rsv4* alleles previously reported: V94-5152, PI 88788, and Beeson (Buss et al., 1997; Gunduz et al., 2004; Shakiba et al., 2013) (Table 1). The inoculation with seven SMV strains (G1 - G7) showed that PI 438307 exhibited different response pattern to SMV strains than genotypes with known *Rsv4* alleles. In this study, PI 438307 conferred full resistance to SMV-G1 through G6 strains, and resistance at seedling stage to SMV-G7 strain (Table1).

DISCUSSION

The greenhouse results confirmed genetic segregations in F_2 plants and $F_{2:3}$ lines advanced from crosses PI 438307 × Essex (*rsv*), PI 438307 × PI 96983 (*Rsv1*), and PI 438307 × L29 (*Rsv3*); but not from PI 438307 × V94-5152 (*Rsv4*) (Table 2, 3). This outcome provided evidence that PI 438307 carries a single dominant gene that is allelic to *Rs4* locus and is independent of *Rsv1* and *Rsv3* loci. Dot blot immunoassay verified and confirmed segregations in populations PI 438307 × Essex (*rsv*), PI 438307 × PI 96983 (*Rsv1*), and PI 438307 × L29 (*Rsv3*); however, in population derived from PI 438307 × V94-5152 (*Rsv4*), no virus was detected, indicating the resistant response. These results confirmed that the phenotypic classification used in this study was reliable for detecting genetic segregation and testing goodness-of-fit to the expected ratios.

Presence of the *Rsv4* allele in PI 438307 was validated using two polymorphic SSR markers flanking the *Rsv4* locus and covering 6 cM interval on chromosome 2 (MLG D1b). In addition, PI 438307 was genotyped by three SNP markers linked to the *Rsv4* locus displaying the "A-G-G" nucleotide pattern. Marker data supported the conclusion that the SMV resistance gene in the PI 438307 resides on chromosome 2 (MLG D1b) where the *Rsv4* locus was previously mapped (Hayes et al., 2000). Moreover, three SSR markers linked to the *Rsv1* on chomosome 13 (MLG F) and three SSR markers near the *Rsv3* on chromosome 14 (MLG B2) were used to screen the population PI 438307 × Essex (*rsv*) but no association was identified (data not showed), confirming that the R-gene in PI 438307 was allelic neither to *Rsv1* nor *Rsv3*.

The results from the inheritance and allelism studies, serological tests, and molecular marker analysis consistently proved that PI 438307 soybean accession carries a single dominant R-gene at the *Rsv4* locus.

Upon SMV infections using different strains identified in the U.S., PI 438307 was resistant to

six of them (G1 - G6), and resistant at early developmental stages to G7 strain. Our results were in agreement with previous phenotyping of this accession with G1 and G7 strains (Zheng et al., 2005; Shi et al., 2008), and G1 through G7 strains (Zheng et al., 2008). This reaction pattern was unique and different from resistance caused by other *Rsv4* alleles previously reported in V94-5152, PI 88788 and Beeson; therefore, we proposed that a novel allele *Rsv4-v* should be assigned to the SMV resistance in PI 438307.

Soybean genotypes carrying the *Rsv4* gene are rare in nature and only a few have been previously reported, including V94-5152, PI 88788, Beeson, PI 486355, Columbia, and 8101 (Buss et al., 1997; Chen et al., 1993; Gunduz et al., 2004; Liao et al., 2010; Ma et al., 1995, 2002; Shakiba et al., 2013). PI 438307 is an old plant introduction collected from North Korea and donated by Russian Federation in 1979 (http://www.ars-grin.gov/). There is no information available about its pedigree; therefore, it was not possible to analyze the *Rsv4* gene sources in the ancestors.

The novel *Rsv4-v* allele offers significant potential values for SMV genetic studies and breeding purposes. First of all, this allele contributes to genetic diversity as an option for plant breeders to improve soybean yield and seed quality, and therefore, save farmers' income. Second, it provides a mechanism of extra protection to variations in SMV pathogenicity. The *Rsv4-v* allele may provide additional blockade against dynamic nature of SMV virulence driven by natural selection and fitness that cause diversification of new strains defeating SMV R-genes (Kang et al., 2005). Third, the new allele may serve as a differential parent for identification and characterization of SMV strains, particularly G7. Fourth, PI 438307 carrying the *Rsv4*-v provides additional option to study molecular mechanisms of SMV-soybean interactions.

The *Rsv4-v* confers the strongest resistance to all U.S. SMV strains among known *Rsv4* alleles, and belongs to one of the most significant alleles among all R-genes followed by the *Rsv1-*

h allele of the *Rsv1* locus (Table 1), and therefore, PI 438307 becomes an excellent choice for breeding SMV resistance. Deployment of genetic resistance is considered to be the most economical and powerful method to control SMV infections, and a single dominant gene could be easily incorporated into elite breeding lines using backcrossing and marker-assisted selection.

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Table 1. Reactions of soybean genotypes possessing a single resistance gene to seven soybean mosaic virus strains.

NAME	ORIGIN	SMV REACTIONS †							GENE	REFERENCE
		G1	G2	G3	G4	G5	G6	G7		
PI 96983	Korea	R	R	R	R	R	R	N	*Rsv1*	Kiihl and Hartwing, 1979
Suweon 97	Korea	R	R	R	R	R	R	R	*Rsv1-h*	Chen et al., 2002
York	USA	R	R	R	N	S	S	S	*Rsv1-y*	Chen et al., 1991
Raiden	Japan	R	R	R	R	N	N	R	*Rsv1-r*	Chen et al., 2001
Kwanggyo	Korea	R	R	R	R	N	N	N	*Rsv1-k*	Chen et al., 1991
Ogden	USA	R	R	N	R	R	R	N	*Rsv1-t*	Chen et al., 1991
Marshall	USA	R	N	N	R	R	N	N	*Rsv1-m*	Chen et al., 1991
PI 507389	USA	N	N	S	S	N	N	S	*Rsv1-n*	Ma et al., 2003
LR1	USA	R	R	R	R	N	N	R	*Rsv1-s*	Ma et al., 1995
Corsica	USA	S	ER	S	-	ER	S	ER	*Rsv1-c*	Shakiba et al., 2012
L29	USA	S	S	S	S	R	R	R	*Rsv3*	Buss et al., 1999
OX 686	Canada	N	N	N	N	R	R	R	*Rsv3*	Buzzel and Tu, 1989
Harosoy	Canada	S						R	*Rsv3*	Shi et al., 2008
PI 61944	China	N/S	N/S	R	-	R	R	R	*Rsv3-n*	Cervantes, 2012
PI 61947	China	N/S	N/S	R/N	-	R	R	R	*Rsv3-h*	Shakiba et al., 2012
PI 399091	Korea	S	S	ER	-	R	S	ER	*Rsv3-c*	Shakiba et al., 2012
V94-5152	USA	ER	ER	ER	ER	ER	ER	ER	*Rsv4*	Buss et al., 1997
PI 88788	China	ER	ER	ER	ER	ER	ER	ER	*Rsv4*	Gunduz et al., 2004
Beeson	USA	ER	ER	S	-	R	ER	R	*Rsv4-b*	Shakiba et al., 2012
PI 438307	Korea	R	R	R	-	R	R	ER	*Rsv?*	Zheng et al., 2008

† G1 - G7, SMV strains; R, resistant (symptomless); N, necrotic (systemic necrosis); S, susceptible (mosaic); ER, early resistant at seedling stage; N/S, mixture of necrotic and susceptible; R/N, mixture of resistant and necrotic.

Table 2. Reactions of parents and F_2 populations from PI 438307 × Essex, and crosses of PI 438307 with allele differential genotypes (PI 96983, L29, and V94-5152) inoculated with soybean mosaic virus G7 strain.

Cross/Parent	Number of Plants Observed†						
	R	N	S	Total	Expected Ratio	χ^2	*p-value*
PI 438307 × Essex	109	0	31	140	3R:1S	0.6	0.4386
PI 438307 (ER)	14	0	0	14			
Essex (S)	0	0	17	17			
PI 438307 × PI 96983	148	50	19	214	15(R+N):1S	2.39	0.1217
PI 438307 (ER)	15	0	0	15			
PI 96983 (N)	0	19	0	19			
PI 438307 × L29	137	0	8	145	15R:1S	0.11	0.7344
PI 438307 (ER)	18	0	0	18			
L29 (R)	10	0	0	10			
PI 438307 × V94-5152	112	0	0	112	No segregation		
PI 438307 (ER)	15	0	0	15			
V94-5152 (ER)	12	0	0	12			

† R, resistant (symptomless); N, necrotic (systemic necrosis); S, susceptible (mosaic); ER, early resistance at seedling stage; the ER responses were categorized as R due to resistance during scoring of plants symptoms.

Table 3. Reactions of parents and $F_{2:3}$ lines from PI 438307 × Essex, and crosses of PI 438307 with allele differential genotypes (PI 96983, L29, and V94-5152) inoculated with soybean mosaic virus G7 strain.

Cross/Parent	Number of Plants Observed†						
	R	H	S	Total	Expected Ratio	χ^2	*p-value*
PI 438307 × Essex	23	48	18	89	1R:2H(R+S):1S	1.135	0.5669
PI 438307 (ER)	20	0	0	20			
Essex (S)	0	0	15	15			
PI 438307 × PI 96983	24	29	3	56	7R:8H(R+N+S):1S	0.116	0.9435
PI 438307 (ER)	9	0	0	9			
PI 96983 (N)	0	14	0	14			
PI 438307 × L29	29	39	5	73	7R:8H(R+S):1S	0.586	0.7458
PI 438307 (ER)	17	0	0	17			
L29 (R)	10	0	0	10			
PI 438307 × V94-5152	71	0	0	71	No segregation		
PI 438307 (ER)	15	0	0	15			
V94-5152 (ER)	14	0	0	14			

† R, resistant (symptomless); H, segregating (R+N+S); S, susceptible (mosaic); ER, early resistance at seedling stage; the ER responses were categorized as R due to resistance during scoring of plants symptoms.

Table 4. Genetic segregation of selected polymorphic SSR molecular markers Satt634 and Satt296 (MLG D1b) in F_2 population derived from PI 438307 × Essex.

SSR†	Cross/Parent	Marker Segregation Observed‡						
		A	H	B	Total	Expected Ratio	χ^2	*p-value*
Satt634	**PI 438307 × Essex**	57	132	54	243	1A:2H:1B	1.75	0.416
	PI 438307 (R)	8	0	0	8			
	Essex (S)	0	0	8	8			
Satt296	**PI 438307 × Essex**	58	129	56	243	1A:2H:1B	0.86	0.65
	PI 438307 (R)	6	0	0	6			
	Essex (S)	0	0	7	7			

† SSR markers located close to the *Rsv4* locus.
‡ A, presence of resistance allele from PI 438307; B, presence of susceptible allele from Essex; H, presence of both alleles from the two parents.

Table 5. Tissue blotting of parents and F_2 populations from testcross PI 438307 × Essex, and crosses of PI 438307 with allele differential genotypes (PI 96983, L29, and V94-5152) inoculated with soybean mosaic virus G7 strain.

Cross/Parent †	Number of Plants ‡			Phenotype
	+	-	Total	
PI 438307 × Essex	22	49	71	Segregation
PI 438307 (ER)	0	5	5	Resistant
Essex (S)	5	0	5	Susceptible
PI 438307 × PI 96983	22	25	47	Segregation
PI 438307 (ER)	0	5	5	Resistant
PI 96983 (N)	0	5	5	Necrotic
PI 438307 × L29	20	34	54	Segregation
PI 438307 (ER)	0	5	5	Resistant
L29 (R)	0	3	3	Resistant
PI 438307 × V94-5152	0	68	68	Resistant
PI 438307 (ER)	0	5	5	Resistant
V94-5152 (ER)	0	4	4	Resistant

† R, resistant (symptomless); N, necrotic; S, susceptible (mosaic); ER, early resistant.
‡ +, presence of SMV; -, absence of SMV.

Figure 1. Major symptoms of soybean plants under SMV infection: resistant (R), necrotic (N), and susceptible-mosaic (S)

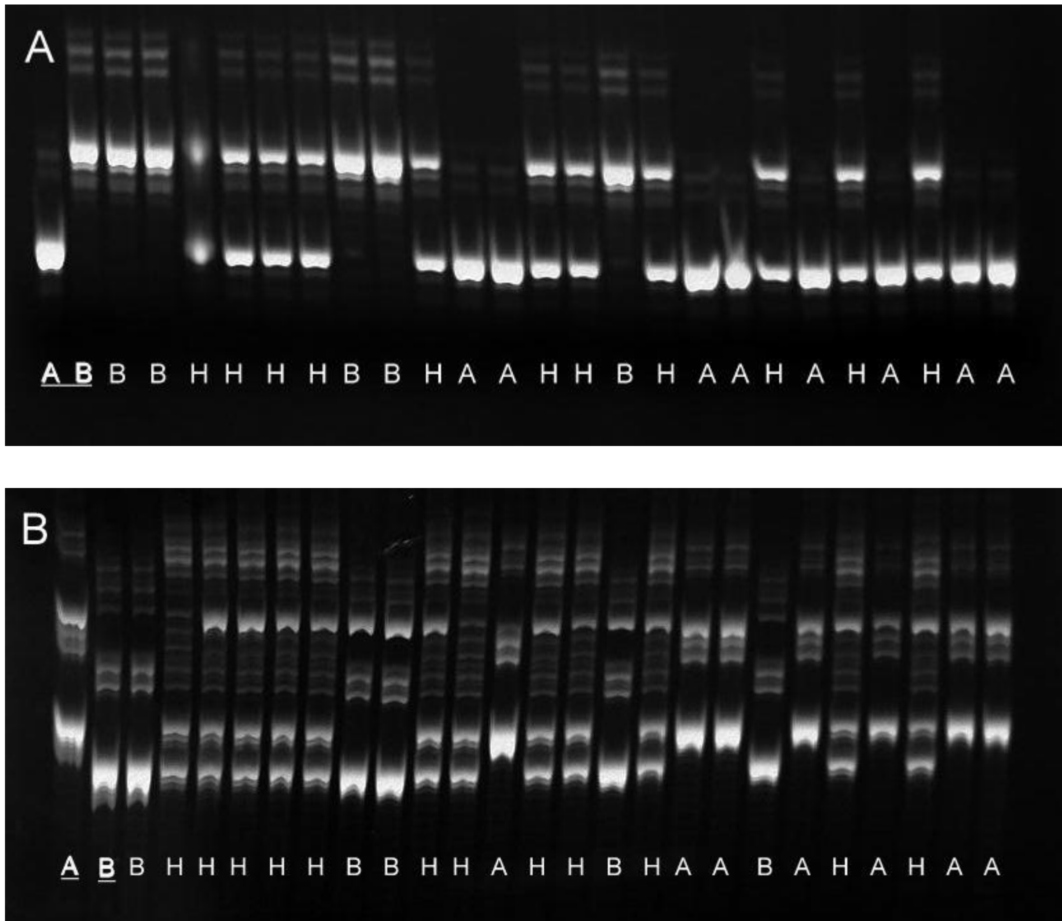

Figure 2. Amplification of SSR markers (A) Satt634 and (B) Satt296 in F_2 population from PI 438307 × Essex: A, resistance dominant allele from PI 438307; B, susceptible recessive allele from Essex; H, both alleles from PI 438307 and Essex; underlined samples correspond to the parents.

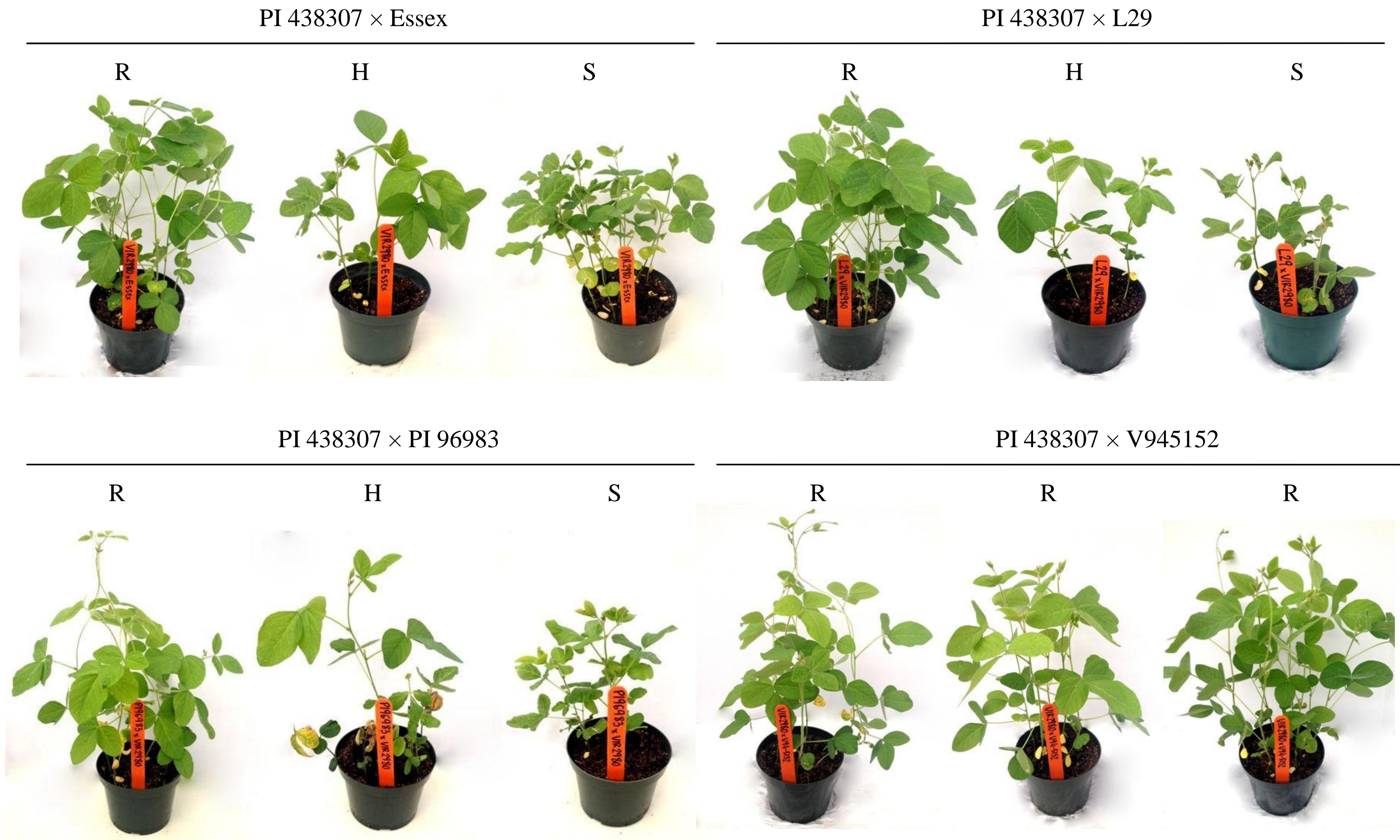

Figure 3. Symptoms of $F_{2:3}$ lines inoculated with SMV-G7: R, resistant; S, susceptible; H, heterozygous segregating (R+N+S).

CHAPTER FIVE

TWO TIGHTLY LINKED GENES OF SOYBEAN MOSAIC VIRUS RESISTANCE IN SOYBEAN

ABSTRACT

Soybean mosaic virus (SMV), a member of the *Potyviridae* family, is the most common virus negatively affecting yield and seed quality in soybean. Seven SMV strains, G1 through G7, and three independent SMV resistance genes (R-genes), *Rsv1, Rsv3* and *Rsv4*, have been previously identified. The *Rsv1* locus contains at least ten alleles displaying differential plant reactions to SMV strains, and it was mapped at very complex resistance-gene-rich region. In this study, two alleles of the *Rsv1* locus were analyzed crossing PI 96983 and York soybean accessions to evaluate whether *Rsv1* and *Rsv1-y* belong to the same or different but closely linked loci. To break possible linkage, 3,000 $F_{2:3}$ lines were developed and investigated using infections of the SMV-G1 strain in a greenhouse. The occurrence of segregating and susceptible lines indicated tight linkage between two genes. The recombination frequency (RF) was estimated using the maximum likelihood formula concluding that *Rsv1* and *Rsv1-y* are two distinct tightly linked loci located apart with genetic distance of 2.2 cM. We proposed a symbol of the *Rsv2* to be assigned for a new gene instead of *Rsv1-y*. This research provided the first evidence of two R-genes existence on chromosome 13, conferring resistance to different SMV strains. Both loci, *Rsv1* and *Rsv2*, can be easily transferred into susceptible cultivars in a breeding program to provide broad and durable protection against SMV strains with lower virulence.

INTRODUCTION

Soybean mosaic virus induces various disease symptoms in infected soybean plants including mild to severe mosaic symptoms or systemic necrosis (Chen et al., 1991; Gunduz et al., 2002; Kiihl and Hartwig, 1979). In the United States, SMV was classified into strains, G1 through G7, based on differences in the pathogenic variability, where G1 strain is the least and G7 strain is the most virulent upon infection of soybean cultivars (Cho and Goodman, 1979). Three independent SMV resistance loci, *Rsv1*, *Rsv3*, and *Rsv4*, have been identified and mapped on chromosome 13, 14, and 2, respectively (Hayes et al., 2000; Jeong et al., 2002; Yu et al., 1994).

The *Rsv1* is the most common SMV R-gene present among soybean germplasm (Chen et al., 1991; Kiihl and Hartwig, 1979; Yu et al., 1994), and contains at least ten alleles: *Rsv1* (PI 96983), *Rsv1-t* (Ogden), *Rsv1-y* (York), *Rsv1-m* (Marshall), *Rsv1-k* (Kwanggyo), *Rsv1-r* (Raiden), *Rsv1-s* (LR1), *Rsv1-n* (PI507389), *Rsv1-h* (Suweon 97), and *Rsv1-c* (Corsica) (Chen et al., 1991; 2001; 2002; Ma et al., 2003; Roane et al., 1983; Shakiba et al., 2013). All alleles at the *Rsv1* locus, except for Rsv1-h, confer resistance only to some, mostly less virulent SMV strains, and may be associated with necrosis (Table 1). The *Rsv1* allele, named the same as the locus, was discovered by performing a cross PI 96983 (*Rsv1*) × Lee 68 (*rsv*) what resulted in identification of SM176 marker 0.5 cM distant to *Rsv1* on chromosome 13 (MLG F) (Yu et al., 1994). The *Rsv1* allele is dominant and confers resistance to SMV-G1 though G6, and systemic necrosis to G7 strain (Table 1). The *Rsv1-y* allele was identified in York and it was confirmed that SMV resistance is triggered by a single dominant gene (Chen et al., 1991; Roane et al., 1983). York displays resistance to less virulent strains G1 - G3, necrosis to G4 and susceptibility to more virulent strains G5 - G7 (Table 1) (Cho and Goodman, 1979).

Rsv1-y and *Rsv1* have been recognized and classified as alleles of the *Rsv1* locus; however, several phenomena raised a question whether the *Rsv1-y* allele belongs to the *Rsv1* or it is just a distinct but tightly linked locus. According to the study by Shi et al. (2008), a PCR-based marker Rsv1-f/r for detection of the *Rsv1* candidate gene *3gG2* (Wm82.a2.v1: *Glyma.13g190400*), completely linked to *Rsv1,* could amplify a specific sequence from 55 soybean accessions carrying all *Rsv1* alleles except *Rsv1-y* present in York and 16 other genotypes. Recently, Yang et al. (2013) concluded that there might be one or two dominant R-genes tightly flanking the *Rsv1* locus by performing a cross of PI 96983 (R) × Nannong 1138-2 (S) and screening their recombinant inbreed lines (RILs) with molecular markers. The potential *Rsc-pm* gene confers resistance to the Chinese strains SMV-SC3, SC6, and SC17, was positioned between BARCSOYSSR_13_1128 and BARCSOYSSR_13_1136. The other gene *Rsc-ps* brings resistance to SMV-SC7, and was spotted between BARCSOYSSR_13_1140 and BARCSOYSSR_13_1155.

The *Rsv1* locus is located at resistance-gene-rich region on the long arm of chromosome 13 (MGL F) (Hayes et al., 2004; Yu et al., 1994) and is tightly linked to a cluster of genes containing N-terminal nucleotide binding site domain and C-terminal leucine-rich repeat domain (NBS-LRR) (www.soybase.org). This area of the chromosome is extremely complicated and besides resistance to SMV, it also locates R-genes to the soybean aphids (Kim et al., 2010) and other plant pathogens e.g. *Phytophthora* (Gunadi, 2012) and *Fusarium* (Ellis et al., 2012). The *Rsv1* locus on this chromosome seems to be complex itself with possibility having a variety of at least ten different copies of the same gene. Because of many tightly linked genes that confer resistance to other diseases are localized on this chromosome, mapping individual gene members

requires developing advanced techniques to detect single genes and then mapping these genes to independent loci (Hayes et al. 2004; Yang et al., 2013; Yu et al. 1994).

According to classical genetics, closely linked genes tend to be inherited together, and they don't segregate independently as they don't obey Mendel's Second Law of Independent Assortment (Xu, 2010). Genetic distance between two genes can be calculated based on recombination frequency occurring in bi-parental population. To measure this linkage, there must be linkage disequilibrium (LD) in the studied population to assess the allele independence at two or more loci. If two alleles from two different loci are found together more often than would be expected based on Mendelian segregation, it can be inferred that such alleles are in LD (Table 2). The stronger the linkage between two loci, the more difficult it is to observe recombination between them and the higher population size is required for detection (Flint-Garcia et al., 2003; Xu, 2010).

The goal of this study was to evaluate whether the two alleles *Rsv1* and *Rsv1-y* belong to the same or different but closely linked loci. To break the linkage between two closely linked genes, high population size was developed in order to increase a chance of crossing-over occurrence during meiosis. Based on Mendelian genetics, if *Rsv1* (R) and *Rsv1-y* (R) are different genes, then segregating and homozygous susceptible lines should appear in $F_{2:3}$ generation. The homozygous susceptible lines (*rsv1rsv1-y*) could bring evidence that recombination occurred between two closely linked genes and *Rsv1* and *Rsv1-y* belong to different loci.

MATERIALS AND METHODS

Population development and progeny test

Two soybean accessions, PI 96983 and York, were used in this research to determine allelic relationship between *Rsv1* and *Rsv1-y*. PI 96983 (*Rsv1*), a plant introduction from Korea, was crossed with York (PI 553038), a soybean cultivar developed in Virginia, USA; in the field at the Arkansas Agricultural Research and Extension Center of University of Arkansas, Fayetteville. The F_1 seeds were planted in the Altheimer greenhouse of the University of Arkansas, and true hybrids were indicated using purple flower color as a morphological marker. F_2 seeds were planted in the field lines, monitored for hypocotyl and flower color segregation, and tagged individually to advance 3,000 $F_{2:3}$ lines.

Progeny testing was performed in seventeen $F_{2:3}$ lines classified as resistant (R), segregating (R+N, R+S, or R+N+S) or susceptible (S). These lines were transferred into the field at the Arkansas Agricultural Research and Extension Center of University of Arkansas, Fayetteville, to obtain $F_{3:4}$ seeds. $F_{3:4}$ progeny lines were re-inoculated with SMV-G1 strain to observe symptoms.

SMV inoculation

The SMV-G1 strain has been chosen for this experiment due to resistant symptoms of both analyzed soybean accessions, PI 96983 and York, under infection. The SMV-G1 was kindly provided by Dr. Sue Tolin, Virginia Polytechnic Institute and State University, Blacksburg. The strain identity and purity was confirmed on a set of differential soybean genotypes, including PI 96983 (*Rsv1*), York (*Rsv1-y*), V262 (*Rsv1-n*), Corsica (*Rsv1-c*), L29 (*Rsv3*), V229 (*Rsv3*), V94-5152 (*Rsv4*) and Essex (*rsv*), and maintained by periodical passage to susceptible genotype Essex

(*rsv*). The virus was introduced into $F_{2:3}$ lines by mechanical inoculation of about 20 plants per genotype according to Chen et al. (1991) in the Altheimer greenhouse of University of Arkansas, Fayetteville, in batches of about 200 lines per day. Briefly, the inoculum was prepared by systematically grinding the infected Essex leaves in 0.05M potassium phosphate buffer (pH=7.2) at an approximate rate of 1 g tissue per 10 ml buffer. Both unifolate leaves pre-dusted with 600-mesh carborundum were gently rubbed with a pestle dipped in the inoculum. The greenhouse conditions were maintained at 28°C with a 14 h photoperiod. Foliar reactions to each SMV strain were monitored each week, compared with set of checks 2-4 weeks after inoculation, and classified into three distinct phenotypes as resistant (R), susceptible (S), and segregating (R+N+S, R+N or R+S). Every $F_{2:3}$ line containing less than ten inoculated plants was not included into final counting, unless all plants displayed mosaic symptoms.

SMV detection

Polymerase chain reaction (PCR) based detection of the SMV virus was performed in eight plants of the susceptible $F_{2:3}$ line. The Zymo Research ZR Plant RNA MiniPrep™ was used for extraction of total RNA, followed by cDNA synthesis by Reverse Transcription System (Promega) according to the manuals. SMV specific primers were used to detect its coat protein (CP) via SMV-CP130F: CCGCGTTTGCAGAAGATTAC and SMV-CP645R: AGCCTTCATCTGCGCTATT. SMV-infected soybean plants displaying resistant and susceptible symptoms were included as positive and negative control. Each PCR reaction mixture of a volume of 25 µl consisted of 2.5 µl of 15 µl of sterile water, 10x Taq buffer (GenScript), 4 µl of 2.5 mM dNTPs, 1.0 µl of 20 µM primers, 0.1 µl 5U/µl Green Taq DNA polymerase (GenScript), and 2.5 µl of cDNA template. The bands were amplified with a

program of 94°C for 2 min of initial denaturation, and 40 cycles of 30 seconds at 94°C of denaturation, 15 seconds at 58°C of primers annealing, 35 seconds at 72°C of extension; and 10 min at 72°C of final extension after the last cycle. Amplified products were separated on 6% polyacrylamide gels containing 0.5 μg/ml ethidium bromide in 0.5X TBE buffer. Sampler was run at 350 V for 2 hours and the bands were visualized under UV light.

DNA extraction and genotyping

Genomic DNA was extracted from tagged F_2 plants based on the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1990) with minor modifications. In this procedure, frozen leaves were crushed to powder with metal beads using Qiagen Retsch TissueLyser Mm301 Mixer Mill Grinder. 750 μL of extraction buffer (2% CTAB, 100 mM Tris-Cl, 20 mM EDTA pH 8.0, 1.4 M NaCl and 1% volume β-mercaptoethanol) was added to each tube and incubated at 65°C in a water bath. After 1 hour of incubation, 1 ml chloroform:isoamyl alcohol (24:1) was added and samples were centrifuged at 12,000 rpm for 15 min at RT. To precipitate DNA, the upper layer was transferred to a new tube containing 1 ml ice-cold 95% ethanol. Pellets were washed in 1 ml 75% ethanol, dried for 2 hours, and dissolved in 200 μl nuclease-free water. DNA concentrations were measured using the NanoDrop ND-2000 1-Position spectrophotometer (Thermo Scientific).

Molecular markers linked to the *Rsv1* locus, SOYBAR_SSR_1133-31, SOYBAR_SSR_1133-33, SOYBAR_SSR_1133-34, SOYBAR_SSR_1133-35, Sat_154, Sat_234, Sat_297, Sat_317, Satt114, Satt334, Satt510, and one gene specific primer Rsv1f/r, covering a chromosomal region of 13.37 cM, were tested for polymorphisms between parents, and Satt114 marker (Gm13: 27718778 - 27718828) was chosen as a background marker to test

$F_{2:3}$ and $F_{3:4}$ lines, and soybean differential checks. PCR was consisted of 4.3 µl autoclaved distillated water, 3 µl 5X Green GoTaq Flexi Buffer (Promega), 0.9 µl 25mM $MgCl_2$, 1.0 µl 2.5mM dNTPs, 0.2 µl 5 u/µl GoTaq Flexi DNA Polymerase (Promega), 1.0 µl 5µM Satt114 primers, and 3 µl 20 ng/µl DNA template. The products were amplified with a program of 95°C for 10 min of initial denaturation, 35 cycles of 25 seconds at 95°C of denaturation, 25 seconds at 48°C of primers annealing, 25 seconds at 72°C of extension; and 5 min at 72°C of final extension. After PCR, amplified products were separated on 6% polyacrylamide gels containing 0.5 µg/ml ethidium bromide in 0.5X TBE buffer. Sampler was run at 350 V for 2 hours and the bands were visualized under UV light.

Data analysis

Recombination frequency (RF) was calculated using maximum likelihood considering segregating (H) and susceptible (S) $F_{2:3}$ lines. In this method, the recombination fraction was computed using the Maximum likelihood (ML) estimator and calculated using a VB programming language. The RF between loci was transformed according to the Kosambi function using the formula for recombination fraction of two dominant genes segregation:

$$ML = n_1 \, log(2\text{-}2r^2) + \; n_2 \, log(2) + \; n_3 \, log(r^2)$$

Where: *ML* is the maximum likelihood, r is the estimated recombination fraction, n_1 is a number of resistant $F_{2:3}$ lines, n_2 is a number of segregating $F_{2:3}$ lines, and n_3 is a number of susceptible $F_{2:3}$ lines (Liu, 1997). Soybean pedigrees were extracted from the uniform soybean tests parentage information available on SoyBase (www.soybase.org).

RESULTS

Three thousands F_2 plants derived from the cross PI 96983 (*Rsv1*) × York (*Rsv1-y*) were individually threshed, hand-planted, and $F_{2:3}$ lines inoculated with the SMV-G1 strain. Results from 295 $F_{2:3}$ lines were discarded due to low number of seeds, low germination rate or missing genotyping data. From remaining 2,705 lines, 2,026 lines were classified as all resistant (R) (74.89 %), 516 lines segregating for resistance and necrosis (R+N) (19.07 %), 71 lines as segregating for resistance and susceptibility (R+S) (2.62 %), 91 lines as segregating for resistance, necrosis, and susceptibility (R+N+S) (3.36 %), and 1 susceptible line (S) (0.03 %) (Table 3, Figure 1). The sergeants classified into R+N, R+S, and R+N+S and were grouped together as the segregating $F_{2:3}$ population of 678 lines in total (25.06 %). From total of 2,705 $F_{2:3}$ lines investigated, only one line displayed susceptible symptoms on all eight infected plants, possibly leading to the *rsv1rsv1-y* homozygous genotype (*aabb*) (Table 3).

Seventeen $F_{2:3}$ lines displaying various symptoms were proceeded to develop $F_{3:4}$ lines for progeny testing to observe further segregations in next generations (Table 4). It was possible to test progenies from most of the resistant and susceptible plants; however, many progenies of necrotic F_3 plants did not produce seeds. Resistant and segregating progeny lines displayed expected results when infected with SMV-G1. Eight plants of the susceptible $F_{2:3}$ line displayed intense and unambiguous symptoms of SMV infection during entire life cycle starting with vein clearing, development of mosaics with strong puckering, and twisting leaf edges downward at late stage of infection (Figure 2). Moreover, all infected susceptible plants were stunted due to shortening steams and petioles. These plants displayed flower abortion and single or no pods were produced with a characteristic coat mottling, and re-inoculation of $F_{3:4}$ lines with SMV-G1 was not necessary as the virus was transferred to the next generation via infected embryos. In

addition, all plants from seventeen $F_{3:4}$ generation, used for the progeny test, were analyzed for virus detection by PCR (Table 4). The virus detection confirmed that phenotypic characterization and classification used in this study was in agreement with SMV infection. These results indicated that all eight plants of the susceptible $F_{2:3}$ line were infected with SMV displaying a band of ~500 bp.

The F_2 population and eight susceptible $F_{2:3}$ plants were tested with the background SSR marker, Satt114, to validate genetic segregation within the cross PI 96983 (*Rsv1*) × York (*Rsv1-y*) (Table3, Figure 3). The genotyping F_2 results (679A:1,349H:677B) fitted perfectly into the 1A:2H:1B genetic segregation ratio of a single dominant gene, with $\chi^2 = 0.021$, and $p = 0.98$. The results of this research displayed a characteristic pattern as most phenotypically segregating $F_{2:3}$ lines contained only the York allele (315 lines), and there was a significantly less segregating lines with the PI 96983 allele (117 lines). The parents, PI 96983 and York, displayed single polymorphic bands ("A" and "B"), whereas the F_2 susceptible sample amplified two bands that corresponded to both parents ("H"). Moreover, eight $F_{2:3}$ plants revealed three plants with both bands ("H") and five plants with a single PI 96983 band ("A") (Figure 3).

Recombination fraction (RF) was calculated using the maximum likelihood formula. The results revealed that the RF equals to 0.022 (2.2%). The genetic and physical distance between the *Rsv1* and *Rsv1-y* was calculated as the percentage of recombination between those genes. One centiMorgan (cM), a unit of recombinant frequency which is used to measure genetic distance, is equal to 1% RT (Griffiths et al., 2015). Based on this general rule, 2.2% recombination was estimated be equivalent to 2.2 cM. As the genetic distance of 1 cM in soybean equals to 200 Kb in euchromatine (Schmutz et al., 2010), the physical distance between the *Rsv1* and *Rsv1-y* corresponded to 440 Kb. It is important to point out that the linkage map

units like centiMorgans do not correspond to any fixed length of chromosome and can depend on many factors, e.g. frequency of crossover can be affected by location on chromosome (distance to the centromere) and proximity to another crossover.

York cultivar, resistant to G1 and susceptible to G7, was developed from the cross of Dorman (resistant to G1 and susceptible to G7) × Hood (resistant to G1 and necrotic to G7) (Figure 4). Dorman was developed from the cross of Arksoy 2913 (resistant to G1 and susceptible to G7) × Dunfield; whereas Hood was derived from N45-745 (resistant to G1 and necrotic to G7) × Roanoke (susceptible to G1 and G7). Ogden (resistant to G1 and necrotic to G7) and C.N.S. (susceptible to G1 and resistant to G7) were ancestors of N45-745.

DISCUSSION

This study was performed to evaluate allelomorphic relationship between *Rsv1* in PI 96983 and *Rsv1-y* in York using phenotypic response of soybean population developed from the cross PI 96983 (*Rsv1*) × York (*Rsv1-y*) that utilized artificial inoculations under controlled conditions in the greenhouse, which were ideal for development of SMV symptoms, and eliminated potentially ambiguous effects of the natural environment or mixed infections. Our hypothesis was that *Rsv1* and *Rsv1-y* are two distinct loci tightly linked that are inherited together in a very high frequency rate, and due to this reason, the *Rsv1-y* was incorrectly designated to belong to the *Rsv1* locus as one of its alleles. To break possible linkage between two closely located genes, high population size was necessary to be developed in order to increase the chance of the crossing-over occurrence. This experiment was proceeded with extreme carefulness as any source of contamination would affect the results. As the distance between *Rsv1* and *Rsv1-y* was unknown, a population size of 3,000 $F_{2:3}$ lines were developed

from the cross between PI 96983 and York. If the *Rsv1* (R) and *Rsv1-y* (R) are two different but linked loci, after crossing there should be some segregating and susceptible $F_{2:3}$ lines occurring at low frequency (Xu, 2010), thus the recombination between these genes could be indicated by a presence of fully susceptible lines to the SMV-G1 strain.

PI 96983 was the first soybean accession where resistance to SMV was identified (Kiihl and Goodman, 1979), reassigned as dominant *Rsv1* locus (Chen et al., 1991), and mapped on chromosome 13 (MLG F) (Yu et all., 1994). Later, York was confirmed to be controlled by a single dominant gene (Roane et al., 1983). Both parents used for this research were previously analyzed by performing inheritance and *Rsv1* allelism tests (Chen et al., 1991; Kiihl and Hartwig, 1979; Roane et al., 1983). Based on the results of Chen et al. (1991), when York was crossed to a susceptible genotype Lee 68, nearly a one fourth of the plants observed in the F_2 population were necrotic (100R:45N:43S). When PI 96983 was crossed to a susceptible genotype Lee 68, only few necrotic plants were noticed (158R:5N:49S). In Chen et al. (1991) study, both populations fitted into a genetic ratio of a single dominant gene (3R:1S) when R and N were counted as resistant plants, what was in agreement with the previous reports of SMV resistance in York (Roane et al., 1983) and PI 96983 (Kiihl and Hartwig, 1979). Chen et al. (1991) found that the necrotic F_2 plants were indicated to be heterozygous for the resistance gene what was confirmed in F_3 population where the majority of the necrotic plants occurred in segregating rows, while homozygous rows were completely resistant. In this research, the same assumption was implemented that systemic necrosis is highly associated with plants at the heterozygous stage for the resistance allele *Rsv1*, but may be influenced by environment and genetic background.

The F_2 susceptible plant, possibly explaining the *rsv1rsv1-y* homozygous genotype (*aabb*), displayed two amplified bands, and its F_3 plants were scored as either A or H. This indicated that this $F_{2:3}$ line was derived from the original cross and it was not a source of contamination. For future analysis, whole genome genotyping (e.g. 50K SNP chip) of the susceptible line and its parents will be necessary to perform in order to identify if the susceptible line is a true progeny of the PI 96983 × York cross. The Satt114 marker could not be used to differentiate two potential genes as the amplified bands from each parents gave the resistant reaction. However, SMV-G7 strain could be used as an indicator because PI 96983 and York display different reaction patterns: necrosis and susceptibility, respectively (Table 1).

In this preliminary study, 2,705 $F_{2:3}$ lines were inoculated with the SMV-G1 strain, and one fully susceptible line was observed making an assumption that approximate number of 3,000 $F_{2:3}$ lines is the minimal population size to detect recombination between the two investigated genes. In general, if two soybean accessions, carrying resistance at the same locus, were crossed to each other, the following generations could display full resistance. However, in this study, the presence of one susceptible and 678 segregating $F_{2:3}$ lines provided an evidence that the *Rsv1* in PI 96983 and the *Rsv1-y* reside at two loci. Evidently, the frequency of susceptible lines was much lower (1 out of 2'705) than expected segregation of two independent genes (166 out of 2'705), therefore, the two genes seem to be closely linked. The results were additionally validated by performing the progeny test and observing further segregations of 17 $F_{2:3}$ lines including the susceptible line No. 3423 in reaction to SMV-G1. Interestingly, all plants derived from the susceptible line displayed mosaics in next generations and SMV was detected by PCR.

The presence of the susceptible line, as well as a big number of segregating lines indicated that two SMV resistance genes are located on chromosome 13. Based on the

segregating population, the recombination frequency was calculated and it was concluded that the R-genes are linked at genetic distance of 2.2 cM what corresponds to 440 Kb in the soybean genome. This distances need to be closer analyzed in the future, as only one susceptible line was observed. Also, SMV infection of about 20 plants per line could not identify all segregants. We propose to assign a symbol *Rsv2* for a new gene instead of the *Rsv1-y* nomenclature which was assigned by Chen et al. (1991) for SMV resistance present in York soybean accession.

Chen et al. (1991) performed an allelism test by crossing PI 96983 (R) × York (R), and analyzing 122 F_2 plants and 80 $F_{2:3}$ lines. A low level (about 0.6-1.3%) of necrotic plants and no susceptible lines were detected in F_2 (118R:4N:0S), and F_3 (79R:1H:0S) populations. The lack of segregation for susceptibility in both generations indicated a high probability that the resistance genes in these cultivars are alleles at a common locus; and therefore, the resistance in York was classified as an allele *Rsv1-y* of the *Rsv1* locus. Certainly, a tight linkage between *Rsv1-y* and *Rsv1* loci could not be detected by the population size used by Chen et al. (1991), as no segregation was observed in the progeny as a result of low recombination frequency between these two loci.

According to study by Shi et al. (2008), Rsv1-f/r PCR-based marker amplified the *3gG2* gene (Hayes et al., 2004), a strong candidate for *Rsv1*, from all soybean accessions carrying different *Rsv1* alleles except *Rsv1-y* present in York and 16 other genotypes. Unluckily, this marker could not be used in this experiment because the *Rsv1-y* allele cannot be detected at all, and the amplified *Rsv1* allele cannot be differentiated between homozygous and heterozygous state. Moreover, Yang et al. (2013) concluded that there might be an extra dominant R-gene tightly flanking the *Rsv1* locus conferring resistance to different SMV Chinese strains. The potential *Rsc-pm* R-gene (probably the *Rsv1*) was positioned between BARCSOYSSR_13_1128

(Gm13: 28,919,973- 28,920,014) and BARCSOYSSR_13_1136 (Gm13: 29,264,742-29,264,795), whereas *Rsc-ps* gene was positioned between BARCSOYSSR_13_1140 (Gm13: 29,301,702-29,301,734) and BARCSOYSSR_13_1155 (Gm13: 29,682,501- 29,682,520). The marker results proposed by Yang et al. (2013) suggested that the minimal distance between *Rsc-pm* and *Rsc-ps* was ~345 Kb. In the recent study, we detected a linkage distance of ~440 Kb between *Rsv1* and *Rsv2* loci. The molecular research made by Yang et al. (2013) was in agreement with our study, and we could suggest that the Chinese *Rsc-pm* and *Rsc-ps* R-genes might be equivalent to the American *Rsv1* and *Rsv2* loci. Gore et al. (2002) concluded that there is a possibility that PI 96983 may carry two linked genes controlling SMV infection, *Rsv1* and *Rvp1*. We also do not reject the possibility that PI 96983 could possess both R-genes, *Rsv1* and *Rsv2*, because only one susceptible line was observed in our experiment; nevertheless, a large number of segregating lines gave us the first evidence for existence of the *Rsv2* locus. If PI 96983 harbors SMV resistance at *Rsv1* and *Rsv2*, it could be possible that other soybean accessions with *Rsv1*-assigned alleles could carry an extra *Rsv2* locus as these linked genes tend to be inherited together, giving an additional protection against SMV. For example, PI 96983 could carry the same allele as Kwanggyo (*Rsv1-k*) or Ogden (*Rsv1-t*) and additional *Rsv2* locus which would contribute to additional resistance to wider range of SMV strains (Table 1). If this is true, the entire classification of ten identified alleles at the *Rsv1* locus need to be investigated and re-classified in the future.

The soybean genome is complex due to the presence of duplicate copies of genes that account for up to 80% of the total gene number. These copies are scattered throughout the genome and so are difficult to locate. In addition, the soybean genome contains large numbers of transposable elements which are mobile DNA pieces that may impact gene expression (Schmutz

et al., 2010). Therefore, it is highly possible that two SMV R-genes might be located in a close proximity. The *Rsv2* resistance gene in York seems to be derived from Arksoy through Dorman cultivars, even though Ogden occurred in its ancestors. The analysis of soybean genome shows that duplication and diversification of individual genes (paralogs) seems to be one of several forces to drive evolution of eukaryotic genomes via producing copies of a gene with similar but slightly different functions in the process pushed by natural selection (Lynch and Conery, 2000).

Viruses, such as SMV, have a high rate of mutations during their replication leading to the co-evolution of plant defenses in response to viral infections (Fraile and Garcia-Arenal, 2010). Among the seven U.S. strains of SMV, the G1 is the least, and G7 is the most virulent strain. SMV-G1 is also the most prevalent and predominant in nature (Cho and Goodman, 1979). The *Rsv1* is the most common in SMV resistant soybean germplasm, and most diverse multiallelic locus with ten indentified alleles (Li et al., 2010; Shakiba et al., 2012; Zheng et al., 2005). The SMV-G1 strain and the *Rsv1* gene must have gone through a long course of co-evolution in nature, which let to emerge new more aggressive strains and other resistance genes. The *Rsv2* (*Rsv1-y*) is also the most common R-gene in soybean germplasm collection (Li et al., 2010; Shakiba et al., 2012; Zheng et al., 2005); therefore, there is possibility that the *Rsv1* and *Rsv2* are the earliest resistance genes that confer resistance to the less aggressive SMV strains (Table1). The *Rsv1* locus appears to be very complex with abundant genetic diversity, and the *Rsv2* gene (*Rsv1-y*) is linked to the *Rsv1* locus. The region of a long arm of chromosome 13 contains the most complex sequences and it is known to contain a cluster of genes related to defense mechanisms. Since the *Rsv1* and *Rsv2* loci are located nearby, they most likely act as one genetic unit and can be transferred to the progeny feasibly in natural conditions as well as in breeding programs. The greatest advantage of having two commercially important genes linked

to each other is enhancement of protection against constantly evolving SMV strains, and reduction of genetic vulnerability to mutations. The two tightly linked genes identified in this study provide additional sources of genetic diversity and would be helpful in cloning of SMV R-genes and classical breeding of multiple resistances through marker-assisted selection (MAS).

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Table 1. Reactions of soybean genotypes carrying different alleles at the *Rsv1* locus to seven soybean mosaic virus strains.

Name	Reactions to SMV †							Allele	Reference
	G1	**G2**	**G3**	**G4**	**G5**	**G6**	**G7**		
PI 96983	R	R	R	R	R	R	N	*Rsv1*	Kiihl and Hartwig, 1979
York	R	R	R	N	S	S	S	*Rsv1-y*	Chen et al., 1991
Suweon 97	R	R	R	R	R	R	R	*Rsv1-h*	Chen et al., 2002
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
Corsica	S	ER	S	-	ER	S	ER	*Rsv1-c*	Shakiba et al., 2013

† G1 - G7, SMV strains; plant symptoms: R, resistant (symptomless); N, necrotic (systemic necrosis); S, susceptible (mosaic); ER, early resistance at seedling stage.

Table 2. Summary of genotypic and phenotypic frequencies in the F_2 and $F_{2:3}$ populations segregating for two genes based on independent assortment and complete linkage.

		Reaction to SMV-G1‡		SSR Amplification§		Genotypic frequency	
SMV Genotype	F_2 Genotype†	F_2	$F_{2:3}$	PI 96983 allele	York allele	Independent Assortment (50 cM)	Complete Linkage (0.0 cM)
Rsv1Rsv1-y	*AABB*	R	R	+	+	6.25	0
Rsv1Rsv1-y	*AABb*	R	R	+	+	12.5	0
Rsv1rsv1-y	*AAbb*	R	R	+	-	6.25	25
Rsv1Rsv1-y	*AaBB*	R	R	+	+	12.5	0
Rsv1Rsv1-y	*AaBb*	R	15(R+N):1S	+	+	25	50
Rsv1rsv1-y	*Aabb*	R+N	3(R+N):1S	+	-	12.5	0
rsv1Rsv1-y	*aaBB*	R	R	-	+	6.25	25
rsv1Rsv1-y	*aaBb*	R	3R:1S	-	+	12.5	0
rsv1rsv1-y	*aabb*	S	S	-	-	6.25	0

† A, presence of the *Rsv1* allele from PI 96983; B, presence of the *Rsv1-y* allele from York; a, presence of the *rsv1* allele from PI 96983; b, presence of the *rsv1-y* allele from York.
‡ R, resistant; R+N, segregation of R and N; R+N+S, segregation of R and N and S; R+S, segregation of R and S; S, susceptible.
§ +, presence of a specific allele; -, absence of a specific allele.

Table 3. Summary of the molecular data of F_2 population screened with SSR marker Satt114 closely linked to the *Rsv1* locus, and the phenotypic reactions of corresponded $F_{2:3}$ lines derived from PI 96983 (R) × York (R) to SMV-G1 strain.

F_2 Genotype†	$F_{2:3}$ Phenotypic Reaction to SMV-G1‡					Total
	R	R+N	R+S	R+N+S	S	
A	562	67	26	24	0	679
B	431	194	22	30	0	677
H	1033	255	23	37	1	1349
Total	2026	516	71	91	1	**2705**

† A, presence of the *Rsv1* allele (*AAbb* or *Aabb*); B, presence of the *Rsv1-y* allele (*aaBB* or *aaBb*); H, presence of both alleles *Rsv1* and *Rsv1-y* (*AABB*, *AABb*, *AaBB*, or *AaBb*).

‡ R, resistant; N, necrotic; S, susceptible; R+N, R+S and R+N+S, segregating line.

Table 4. Reactions of $F_{2:3}$ lines and soybean mosaic virus detection, and progeny test of $F_{2:4}$ lines infected with G1strain.

$F_{2:3}$ Line No.	$F_{2:3}$ Phenotype†	$F_{2:3}$ SMV Detection	$F_{3:4}$ Phenotype†
3018	14R+2S	- - - - - - - - - - - - - - + +	R R R R R S S R R R S R S S S S
3073	16R+2N+1S	- - - - - - - - - - - - - - - - + + +	R R R R R N R S N R R R R N N R na S
3093	10R+2N+1S	- - - - - - - - - - + + +	R R N R R R N S R R na na na
3203	9R+2S	- - - - - - - - - + +	S R R R R R R S R S S
3229	13R+1S	- - - - - - - - - - - - - +	R R S R R R R R R R S R S
3261	7R+3N+1S	- - - - - - - + + + +	N R N R S S R R N N S
3423	8S	+ + + + + + + +	S na na S na na na na
3645	9R+2N+2S	- - - - - - - - - + + + +	R R N S N R R R N R N S S
3785	21R	- -	R R R R N R R R R N R R R R R R R S R R R
3945	10R+1N+1S	- - - - - - - - - - + +	R R R R N R R R R N R S
4053	2R+4N+4S	- - + + + + + + + +	R R N R R R S na na S
4099	4R+7N+3S	- - - - + + + + + + + +	R R R R R na R na N na na S S S
4316	20R	- - - - - - - - - - - - - - - - - - - -	R R R R R R R R R R R R R R R N R R R R
4980	11R+2N	- - - - - - - - - - - + +	R R R R R R R N N R R na na
5743	9R+1N+1S	- - - - - - - - - + +	R N N R S R R R R R S
5865	14R	- - - - - - - - - - - - - -	R R R R R R R R R R R R R R
5920	11R+2N+2S	- - - - - - - - - - - + + + +	S R R R N N R R R R R R na S S

† Reaction of plants inoculated with SMV-G: R, resistance; N, necrosis; S, susceptibility (mosaics).

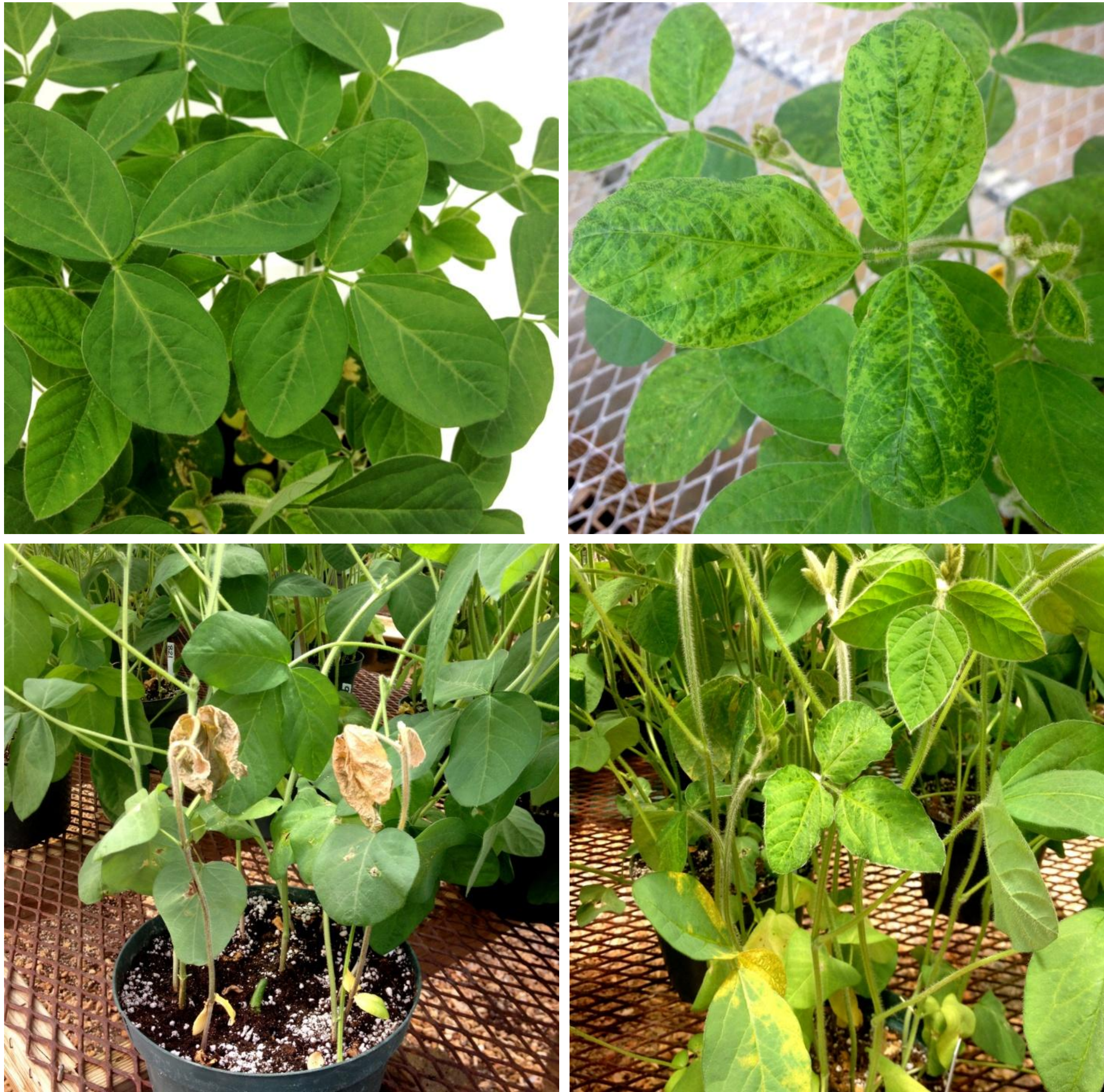

Figure 1. Symptoms representation of $F_{2:3}$ lines derived from the cross PI 96983 (R) × York (R) inoculated with SMV-G1 strain: resistant line (upper left); segregating line R+S displaying one susceptible plant (upper right); segregating line R+N displaying two necrotic plants (lower left), and segregating line R+N+S displaying resistant, susceptible and necrotic plants (lower right).

Figure 2. Foliar symptoms of the susceptible $F_{2:3}$ soybean line.

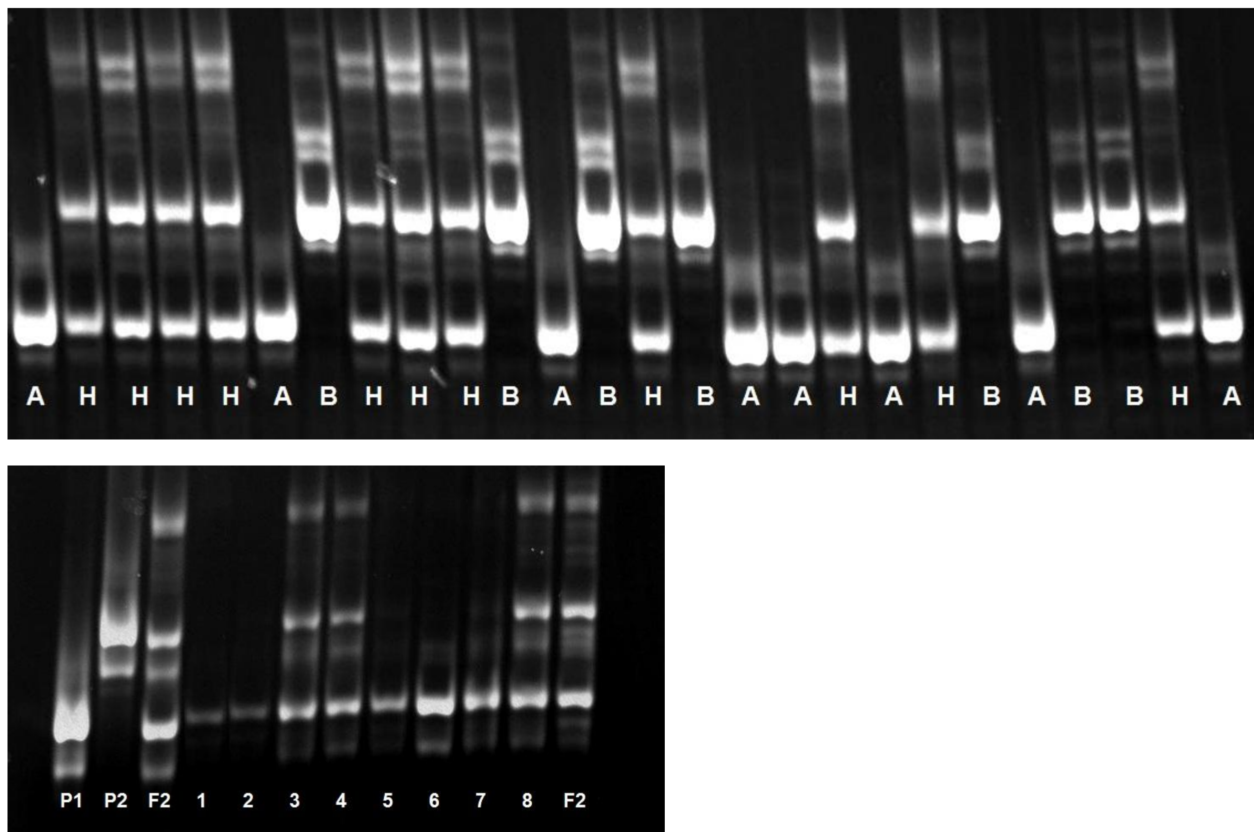

Figure 3. Amplification of SSR marker Satt114 in population derived from PI 96983 × York. Above: segregation of F_2 population: A, presence of the *Rsv1* allele from PI 96983; B, presence of the *Rsv1-y* allele from York; H, presence of both alleles from PI 96983 and York. Below: analysis of the $F_{2:3}$ susceptible line: P1, presence of *Rsv1* allele from PI 96983; P2, presence of *Rsv1-y* allele from York; F2, presence of both bands in the F_2 susceptible line; 1-8, $F_{2:3}$ progenies of the susceptible line.

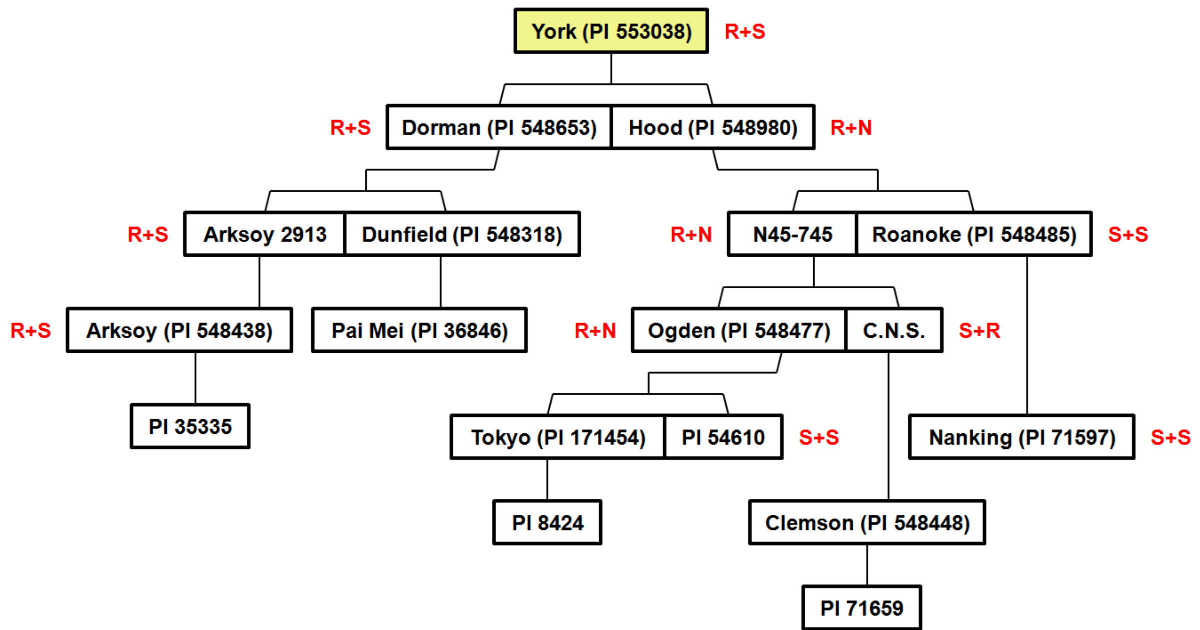

Figure 4. Analysis of York pedigree for SMV resistance: host reaction to SMV (G1+G7) strains; R, resistance; N, necrosis; S, susceptibility (mosaics).

<u>CONCLUSIONS</u>

Through this research we discovered six SNP markers for detection of the *Rsv4* SMV resistance locus in soybean. These markers were used to analyze the genetic diversity of 299 soybean accessions, and together with phenotyping, allowed for the classification into groups of potential SMV resistance genes. The markers were validated by segregating population and distances between the *Rsv4* and SNPs were calculated, what was the base of proposing *Rsv4* candidate genes.

In order to confirm a presence of three SMV resistance genes into one soybean line by gene pyramiding, an inheritance study was performed by crossing it with a homozygous recessive parent. The progenies were analyzed phenotypically and genotypically, confirming a successful incorporation of three SMV R-genes into one soybean line with three independent genes. This line will be proposed for future release as a source of SMV resistance for soybean breeding programs worldwide.

A new allele for differential reactions to SMV strains was identified in the soybean genotype PI 438307. Results from inheritance study and allelism test revealed that resistance to SMV in PI 438307 is controlled by a single dominant gene, allelic to the *Rsv4* locus. This information was supported by molecular analysis which showed that this gene is located on chromosome 2. PI 438307 exhibited a unique reaction pattern than other reported Rsv4 alleles; therefore we proposed that a new allele *Rsv4-v* be assigned to the SMV resistance in this soybean accession. This allele may provide additional protection against SMV virulence change over time driven by natural selection and fitness causing diversification of new strains that defeat host's R-genes.

No allelic relationship was found between the *Rsv1* in PI 96983 and *Rsv1-y* in York. This study demonstrated that *Rsv1* and *Rsv1-y* are two tightly linked genes. We proposed a symbol Rsv2 to be assigned to the SMV resistance in York. Since the Rsv1 and Rsv2 genes are linked on

chromosome 13, they can be easily transferred as one genetic unit in progeny in a breeding program, and it is possible that some soybean genotypes with identified *Rsv1* alleles may also possess the *Rsv2* locus, giving an additional protection against SMV.

The findings reported in this dissertation may assist researchers in future studies on SMV resistance, and may be helpful for breeders in selecting crossing parents for SMV resistance and accelerating breeding efforts to develop multi-virus resistant crops avoiding escapes due to pathogen evolution to overcome resistance.

VITAE

Mariola Klepadlo was born on September 4, 1985 in Krosno Odrzanskie, Poland. After graduating from the Broniewski High School in Krosno Odrzanskie, she was admitted to University of Szczecin, Department of Natural Sciences in 2004. Ms. Klepadlo received a B.S. and M.S. degrees with a major in Biotechnology in 2007 and 2009, respectively. During her M.S. program, she completed one-year Erasmus student exchange program sponsored by the European Union at the Mediterranean Agronomic Institute of Chania (MAICh), Greece. In 2009, she was admitted to the second M.S. program in Horticultural Genetics and Biotechnology at MAICh. During that time, she joined the Laboratory of Molecular Biology and Biotechnology (GMO Section) as assistant research scientist. In 2012, she was admitted to the Soybean Breeding and Genetics program in the Department of Crop, Soil, and Environmental Sciences, University of Arkansas to pursue her Ph.D. She worked as senior research assistant in soybean breeding development pipeline. During summer 2014, she joined the Key laboratory of biology and a genetic improvement of soybean, Ministry of Agriculture, Nanjing Agricultural University, China. She is a member of the American Society of Agronomy (ASA), National Association of Plant Breeders (NAPB), American Phytopathological Society (APS), and Gamma Sigma Delta Society (GSD).