Chapter Four

Characterization of the Resistance Conditioned by the *Rsv4* Gene for Resistance to SMV in Soybean

ABSTRACT

Resistance to *Soybean mosaic virus* (SMV; Genus *Potyvirus*; Family *Potyviridae*) in soybean (*Glycine max* [L.] Merr.) is controlled by single dominant genes at three distinct loci identified by genetic studies, molecular mapping, and responses to SMV pathotypes. The mechanism of resistance at the *Rsv4* locus was investigated by tracking virus accumulation and movement over time. Soybean lines Peking, PI 88788, V94-5152, and Essex-*Rsv4* isoline with the *Rsv4* gene derived from V94-5152 were inoculated with SMV strains G1, G6 and G7 to monitor events during the infection process. Leaves were sampled 2, 4, 9, 14, 19 and 26 days post inculation and tested for SMV presence by leaf immuno-printing. The number of infection sites, rate and extent of viral movement, both cell-to-cell and long-distance, were all reduced in comparison to that in the susceptible cv. Essex or the resistant PI 96983 (*Rsv1*). Virus accumulation was reduced in the inoculated leaves and not detected in the upper, non-inoculated trifoliolate leaves. Vascular movement of the virus was also delayed and restricted. PI 88788 and Peking were more resistant to SMV than were Essex-*Rsv4* and V94-5152, suggesting different alleles. It was concluded that *Rsv4* resistance is non-strain-specific, non-necrotic to any strain, and does not function through a hypersensitive or necrotic response. Its mechanism is to reduce infection and accumulation, and limit and delay local and systemic invasion of plants by restricting both cell-to-cell and long distance movement of the virus.

Keywords: *Glycine max*, Virus Resistance, Virus Movement, *Soybean mosaic virus*

INTRODUCTION

Soybean mosaic virus (SMV; Genus *Potyvirus*; Family *Potyviridae)*, the causal agent of soybean mosaic, is one of the most important viruses in soybean (*Glycine max* [L.] Merr.) and occurs worldwide (Brunt et al., 2003). If plants are infected at an early stage, the result is reduction of pod set, seed size and weight, increase in seed coat mottling and decrease in seed quality. Late infections with SMV, however, have a limited effect on yield and seed quality (Hill 1999; Hill et al., 1987). Cho and Goodman (1979, 1982) classified SMV into seven different strain groups based on the differential reactions of various SMV isolates on resistant soybean cultivars. The strain groups were named G1 to G7 with the low numbered groups being least virulent and the high numbered being more virulent on this set of soybean cultivars [Essex, Davis (York), Kwangyo, Marshall, Ogden, and Buffalo (PI 96983)].

Resistance to SMV in soybeans has been detected in different cultivars; these appear not resistant to all of the SMV strains (Ma et al., 1995). Resistance is regulated by a single dominant gene in most of the resistant soybeans, including those cultivars used by Cho and Goodman (Chen et al., 1991). *Rsv*, later named *Rsv1,* a single dominant gene, was identified from PI 96983. The *Rsv1* alleles are usually resistant to the lower numbered strains and display mosaic or necrotic reactions to the higher numbered strains. Six alleles of *Rsv1* have been identified (Buss et al., 1989, Chen et al., 1991) that react differently to different strains of SMV. The *Rsv1* locus conditions both the necrotic and the resistant reactions (Chen et al., 1994). Hajimorad and Hill (2001) reported that the *Rsv1*-mediated resistance to SMV-N is HR-independent and is similar to extreme resistance of *Rx* to *Potato virus X* (PVX; Genus; Family). Cultivars carrying the *Rsv1* locus, which are susceptible to lower virulence strains, show a necrotic reaction to more virulent strains of the virus. Similarly, cultivars showing a resistance reaction to lower virulence strains of SMV develop a necrotic reaction to more virulent SMV strains (Chen et al., 1994). Such reactions are consistent with a gene for gene model proposed by Roane et al. (1986). The *Rsv1* locus maps to linkage group F of the soybean genome and is linked to a cluster of resistance genes (Yu et al., 1994, 1996).

Molecular and genetic studies identified two other genes for resistance to SMV in soybeans mapping to regions distinct from the *Rsv1* locus; these have been named *Rsv3* and *Rsv4* (Chen et al., 1993, Gunduz et al., 2001, Gunduz et al., 2002). *Rsv3* maps to soybean linkage

group MLG B2 carrying a cluster of disease resistance genes such as soybean cyst nematodes, and an *Rps5* region conferring resistance to *Phytophthora sojae* (Jeong et al., 2002). The resistance conditioned by *Rsv3* alleles to SMV pathotypes is different from that of *Rsv1*. This locus is susceptible to low numbered strains (G1-G4) but resistant to high numbered strains (G5-G7).

This paper examines the mechanisms of resistance in plants carrying the *Rsv4* gene for resistance to SMV. Gunduz et al. (2004) reported that PI 88788 infected with SMV resulted in a late susceptible phenotype when *Rsv4* was heterozygous. In the homozygous state, no virus symptoms appeared and they suggested that *Rsv4* restricts vascular and cell-to-cell movement of the virus. Therefore, the objectives of this study were to determine differences in four different *Rsv4* carrying soybean cultivars with respect to: (i) the different reactions to three SMV pathotypes, (ii) virus accumulation, (iii) the extent of virus cell-to-cell movement, (iv) the extent of long distance movement, and (v) and the extent of vascular movement.

Two independent resistance genes have been identified in PI 486355 (Chen et al., 1993; Ma et al., 1995). One is at the *Rsv1* locus, the other is independent from *Rsv1* and *Rsv3* and termed *Rsv4*. *Rsv4* was separated by segregation into LR2, a selection of PI 486355 x Essex. V94-5152, a reselection from LR2 gave line, was registered as a germplasm (Buss et al., 1997). *Rsv4* has been mapped to linkage group MLG D1b (Hayes et al., 2000). This *Rsv4* gene is completely dominant. This gene is resistant to all strains of SMV with no necrosis involved. Soybeans heterozygous for *Rsv4* show a late susceptible phenotype with SMV (Ma et al., 1995; Gunduz et al., 2004).

PI 88788 soybean was introduced as germplasm from China for its resistance to soybean cyst nematode. In addition, PI 88788 was found to be resistant to SMV (Gunduz et al., 2004). Genetic studies showed that the PI 88788 resistance locus is independent of *Rsv1* and *Rsv3* and allelic to *Rsv4*. Peking, carrying the *Rsv4*, is another traditional Chinese cultivar used extensively in soybean breeding programs (Yang et al., 2000). Essex-*Rsv4* isoline, a backcross between V94-5152 and Essex (*rsv*; susceptible), was developed by G.R Buss (Dept. Crop and Soil environmental Sciences, Virginia Polytechnic Institute and State University, Blacksburg, VA.)

Materials and Methods

Genetic Material and Growth Conditions

Genetic lines used were 'PI 88788', 'Peking', 'V94-5152', and 'Essex-*Rsv4'* isoline (Essex-*Rsv4* BC4) of soybean each carrying the *Rsv4* gene for resistance to SMV, and the susceptible Essex (*rsv*). Test plants (six/pot) were grown from seed in 11x10 cm pots filled with MetroMix360® (Scotts-Sierra Horticultural Products Co., Marysville, OH 43401). Osmocote® 18%N, 6%P, 12%K, (Scotts-Sierra Horticultural Products Co., Marysville, OH) was used as a supplemental fertilizer. G. R Buss (Dept. Crop and Soil Environmental Sciences, Virginia Polytechnic Institute and State University, Blacksburg, VA) graciously supplied seeds of all cultivars.

Virus Inoculations

SMV strains G1 (Hunst and Tolin, 1982), G6, and G7 used in previous studies (Chen et al., 1991) were the virus inocula. Virus cultures were maintained in greenhouse-grown cvs. Essex or York and transferred to new seedlings every 2-4 wks; they were verified periodically for pathotype using a set of resistant soybean cultivars (Cho and Goodman 1979; Chen et al., 1991). Inoculum was extracted, using a chilled mortar and pestle, from young leaves of plants infected for 2-3 weeks with SMV G1, G6 or G7. Test plants (three pots; three plants/pot) were inoculated using a 1:10 (1 g leaf tissue /10 ml 0.01M neutral sodium phosphate buffer) inoculum and placed on a greenhouse bench in a completely randomized design (CRD). Whole, fully-expanded unifoliolate leaves, pre-dusted with carborundum abrasive, were inoculated by rubbing with a pestle moistened with inoculum, and rinsed with tap water immediately. A more concentrated (1 g: 5 ml) inoculum was also used to inoculate PI 88788 and Essex-*Rsv4* plants.

Leaf-Immunoprint Preparation and Development

Virus movement in plants (both cell-to-cell and long distance) was monitored by immunological detection of virus in imprints of individual leaves. Leaves were removed from plants and imprinted over a time course (2, 4, 9, 14, 19, 26 days post inoculation, dpi) onto 9 cm

disks of hardened filter paper (S&S 410 Filter Paper, Schleicher and Schuell, Keene, NH) (Gera, 1994). Following decoloration with 5% Triton X-100, the imprints were processed by a modified Western blot procedure described by Lin et al. (1990) using polyclonal antibodies (1:10,000) raised against SMV particles (Hunst and Tolin, 1982), followed by goat anti-rabbit IgG (whole molecule) conjugated with alkaline phosphatase (1:15,000) (Sigma-Immuno Chemicals, St Louis, MO 63178), and 5-bromo-4-chloro-3-indolyl phosphate and nitro tetrazolium salt (NBT/BCIP) substrate (Zymed Laboratories, Inc., San Francisco, CA). For each time point, one inoculated unifoliolate leaflet and one non-inoculated first trifoliolate leaflet were sampled from each of three plants. Sampling was also done on two other biological replicates of Essex (January 2001 and October 2001).

Data Collection from Leaf Prints

Total number of infection sites, average size of an infection site, and total leaf area infected from three leaves were recorded from digitized images of the leaf immunoprints using an Alpha Innotech Imager (Mod 4912-2010/0000) and the Alpha Innotech Analysis software (Alpha Innotech Corporation, San Leandro, CA 94577). Analysis of variance was performed using SAS statistical package (North Carolina State University, Raleigh, NC). Analysis was performed on the maximum number of infection sites and corresponding size for each cultivar-strain combination, which was not the same for all the combinations. The maximum leaf area invaded was recorded at 26 dpi.

Detection of Virus in Longitudinal and Cross Sections from Stems

Cross sections and longitudinal stem sections from Essex and Essex-*Rsv4*, inoculated with SMV G1, G5, G6, G7 and non-inoculated controls, were blotted onto 25 mm Whatman 0.2 μm cellulose nitrate discs (Whatman, Maidstone, England) and 0.45 μm NitroPure nitrocellulose membranes (Osmonics™ Westborough, MA 01581), respectively. Serological detection followed a protocol similar to that of the leaf prints. Digital scans of the developed blots were done using the Scan Jet ADF HP scanner.

Enzyme Linked Immuno Sorbent Assay (ELISA)

Test plants (three/pot) of Essex-*Rsv4*, V94-5152, PI 88788 and Essex were inoculated with SMV G1, G5, G6, and G7 and placed randomly on a greenhouse bench. Buffer inoculated plants were used as controls. One inoculated unifoliolate and one non-inoculated first trifoliolate leaf were sampled from two plants of the same cultivar. Extractions were done from fresh tissue (1:50) in extraction buffer [0.05 M sodium carbonate pH 9.6; 1% polyvinyl pyrrolidone (PVP)]. Samples were incubated in uncoated 96 well plates (Dynatech Immulon ™) overnight at 4°C. Wells were washed 4-5 times with PBS-Tween at room temperature, incubated at 37°C with virus particle (CP) polyclonal antibodies (1:10,000) raised against SMV particles (Hunst and Tolin, 1982) followed by another 4-5 washes with PBS-Tween at room temperature. Wells were then incubated with goat anti-rabbit IgG (whole molecule) conjugated with alkaline phosphatase (1:15,000) (Sigma-Immuno Chemicals, St Louis, MO 63178) at 37°C. After rinsing a final time with PBS-Tween, we added paranitrophenyl phosphate (pNPP) substrate and recorded absorbance at 405 nm was recorded using the Spectramax plate reader (Molecular Devices, Sunnyvale, CA) 45 min later.

RESULTS

Symptom Appearance

Symptoms on Essex (*rsv*) and the *Rsv4* carrying cultivars inoculated with SMV-G1, G6 and G7 were recorded peridocally. The susceptible cultivar Essex showed symptoms typical of SMV by 9 days post inoculation (dpi) with the three SMV strains G1, G6 and G7. Inoculated leaves had visible chlorotic lesions with rust-colored margins at the point of infection with chlorosis spreading to the veins. Non-inoculated first and second trifoliolate leaves displayed typical mosaic and chlorotic veins by 9 dpi (Fig. 4.1A). In contrast, all of the *Rsv4* carrying cultivars remained free of symptoms for 26 dpi, the duration of the experiment. Figure 4.1B-D shows non-symptomatic Essex-*Rsv4* plants inoculated with SMV strains G1, G6 and G7, respectively, at 14 dpi. Cvs. Peking, PI 88788, and V94-5152 responded similarly to inoculation with the three SMV strains (not shown).

Restriction of Virus Accumulation and Movement in the Inoculated Leaves

The relationship between symptom appearance and viral antigen accumulation was confirmed by immunoprint analysis of the leaves of Essex and the resistant cvs. Essex-*Rsv4*, V94-5152, PI 88788 and Peking. Figure 4.2A shows an SMV-G1 inoculated leaf from a susceptible Essex plant and its corresponding immunoprint (Fig 4.2B). The dark purple color resulting from enzyme/substrate action corresponds to the sites of virus accumulation in the leaf. By 9 days, the virus moved from the initial infection sites to the vasculature of the leaf. The sites of virus accumulation match those of the chlorotic lesions and veins on leaves of Essex. Figure 4.2B also shows virus accumulation in the leaf petiole. Leaves from a time course experiment sampled at 4, 9, 19 and 26 dpi from Essex inoculated with SMV-G1 are shown in Figure 4.3A-D.

SMV rapidly invaded the inoculated leaves of *Essex* (Figure 4.3A-D). In contrast, SMV movement was delayed and restricted in all the resistant lines carrying the *Rsv4* gene. Leaf prints from non-symptomatic SMV-G1 inoculated unifoliolate leaves of *Rsv4* carrying cultivars sampled at 2, 4, 9, 14, 19 and 26 dpi from the same time course experiment showed a delay in detection of virus particles by about 10 days compared to the susceptible cv. Essex. Leaf prints taken at 2, 4 and 9 dpi showed no virus accumulation (Figure 4.3E-H). Virus presence was first detected at 14 dpi (Figure 4.3I-L). From 14 dpi to 26 dpi, the end of the time course experiment, the virus movement was restricted to the initial sites of infection in the inoculated leaves. The virus did not invade the leaf vasculature (Figure 4.3M-P) even when the virus moved across leaf veins. A similar delay in detection of virus accumulation in the *Rsv4* cultivars was found for SMV-G6 and G7 (Figure 4.4A-F).

The number of infection sites from three leaves of Essex and the *Rsv4* carrying cultivars inoculated with SMV strains G1, G6 and G7 is presented in Table 4.1. This number is the maximum number of sites counted before the virus moved into the leaf vasculature and the sites coalesced. For Essex, this number was derived from leaves sampled 4 dpi for the three strains of SMV. However, for the *Rsv4* carrying lines it was derived from leaves sampled at 19 dpi. No virus was detected in the non-inoculated controls (all values were zero for number of sites, size of infection sites and percent leaf area infected; data not shown). Numbers were higher in Essex versus all the *Rsv4* carrying cultivars for the three strains. SMV-G1, G6 and G7 resulted in similar number of infection sites in Essex-*Rsv4* and its parent V94-5152. PI 88788 and Peking

had considerably lower numbers of infection sites for the three strains compared to the two other resistant cultivars. The differences among the three strains were not statistically significant, however.

Following G1 inoculations, infection sites were larger in diameter in the *Rsv4* carrying cultivars 19 dpi (Table 4.2) versus the Essex at 4 dpi diameters (data not shown). Infection sites were similar between Essex-*Rsv4* and V94-5152 and smaller than those in PI 88788 and Peking. Inoculating V94-5152, Peking and PI 88788 with SMV-G6 and G7 gave larger sites of infection than the same cultivars inoculated with SMV-G1. These results showed that the *Rsv4* cultivars do not give the same reaction in terms of limiting the spread of virus from the initial infection foci and that the three strains of SMV tested differ in the rate of movement and accumulation away from the initial sites of infection.

The most informative data were the percentage of leaf area invasion at 26 dpi (Table 4.3). The percentage leaf area infected was significantly higher for the susceptible Essex than the resistant cultivars. By 26 dpi, the virus invaded approximately the whole leaf area in Essex. These data show a similar pattern to the number and size of infection sites in separating the different cultivars and the strains. For each of the three strains taken separately, Essex-*Rsv4*, and its parent V94-5152 showed similar percentages of leaf invasion. PI 88788 and Peking were similar for all of the SMV strains tested. SMV-G1 resulted in a similar extent of invasion with all the *Rsv4* cultivars. Percentage of leaf area invaded by both SMV-G6 and SMV-G7 was higher in Essex-*Rsv4* and V94-5152 versus PI 88788 and Peking. The responses of PI 88788 and Peking were similar for all three strains tested.

Systemic Movement Iinto Trifoliolate Leaves

Non-inoculated upper trifoliolate leaves were sampled over time and tested serologically for SMV by immunoassay. SMV was detected in non-inoculated first and second trifoliolate leaves from susceptible Essex plants inoculated with SMV-G1 at the unifoliolate stage, by 9 dpi (Figure 4.5A-B). Virus particles were present in the petioles, mid-veins, and the leaf tissue as indicated by immunoprints. In contrast, no virus accumulation was detected in the non-inoculated first trifoliolates of resistant cultivars until 19 dpi. SMV accumulated around the mid-vein in the center of the leaf in PI 88788 and Peking inoculated with SMV-G7 (Figure 4.6A and 4.6B respectively). In another first trifoliolate from Peking inoculated with SMV-G7 at 43 dpi

(Figure 4.6C), SMV was present as a distinct patch along the leaf mid vein in an interveinal area. Figure 4.6D shows SMV in the mid vein and secondary veins of SMV-G6 inoculated V94-5152 at 26 dpi. SMV was detected in only a few of the trifoliolate leaves of the *Rsv4* carrying cultivars of over 200 leaves tested (data not shown). Preliminary experiments from Essex-*Rsv4*, PI 88788 and V94-5152 plants inoculated with the higher inoculum (1:5) of SMV-G1, G6 and G7 showed no difference from the 1:10 (data not included).

Vascular Movement

The SMV movement was restricted in the vascular tissue of the resistant *Rsv4* carrying cultivars compared to the susceptible Essex (Figure 4.7). Longitudinal stem sections from SMV-G1 inoculated Essex, sampled 7 dpi, (Fig. 4.7A) show virus accumulation and movement in the stem both above and below the unifoliolate leaf petioles and above the internode to the first trifoliolates. No SMV accumulation was detected in any of the longitudinal stem section from Essex-*Rsv4* inoculated with any of the six SMV strains tested at 35 dpi (data not included). Cross sections of stems and petioles also showed high accumulation of SMV in Essex (Figure 4.7B-C) but not in Essex-*Rsv4* (Figure 4.7D). Virus accumulation was detected in one section only, from Essex-*Rsv4* inoculated with SMV-G2 at 35 dpi (Figure 4.7E). The intensity of the substrate was low, indicating a relatively low accumulation of SMV in the vascular tissue from the resistant soybean cultivar.

Virus Accumulation in Essex and Essex-Rsv4

Indirect ELISA was used to assess the relative SMV titer. Absorbance values at 405 nm (corresponding to the wavelength at which maximum absorbance of substrate occurs) from inoculated unifoliolates and non-inoculated trifoliolates of Essex, Essex-*Rsv4*, V94-5152 and PI 88788. Essex showed higher SMV titer after inoculation with any of the four strains of SMV compared to those resistant cultivars (Table 4.4). Samples were considered positive for SMV if their absorbance values were higher than that of the healthy plus 3x the standard deviation of the healthy. Samples from the inoculated unifoliolate leaves were ELISA positive, indicating virus replication and accumulation occurred. *Rsv4* trifoliolates were ELISA negative for all SMV

strains tested, supporting the lack of virus detection in immunoprints from non-inoculated trifoliolates.

DISCUSSION

Immunological detection of virus in leaves was used to assess the resistance in *Rsv4*-carrying soybean cultivars by monitoring (tracking) the infection process in comparison to the susceptible Essex. This method also showed differences between *Rsv4*-carrying cultivars, indicating possible allelic differences. Virus accumulation and movement were tracked in susceptible (Essex) and resistant (*Rsv4*-carrying) soybean cultivars in a time course study. Inoculated unifoliolate and upper non-inoculated trifoliolate leaves were sampled and the number and diameter of infection sites and percentage leaf area infected were measured.

Virus Cell-to-Cell Movement

A delay in time to first detection and a reduction in the initial number of infection sites was observed for *Rsv4* cultivars. Data from immunoprints of inoculated unifoliolate leaves of Essex-*Rsv4*, V94-5152, PI 88788 and Peking clearly show virus accumulation to a detectable level is delayed by 10 days compared to the fully susceptible Essex for all three strains tested (SMV-G1, G6 and G7). Data also show a reduction by 50% or more in number of infection sites in the *Rsv4* cultivars versus Essex, and a dramatic reduction in percentage of leaf area invaded (up to 95%). Size of individual infection sites was greater in the *Rsv4* cultivars at 19 dpi versus Essex at 4 dpi simply because the virus had more time to move away from the center of infection points. However by the same sampling time (i.e., 19 dpi), SMV moved to the vasculature and invaded almost the entire leaf in the susceptible cultivar. Even though virus movement was restricted between 14 dpi (the initial detection of virus in *Rsv4* inoculated leaves) and 26 dpi, the initial infection sites formed patches of virus accumulation in the inoculated leaves that spread across the leaf veins. However, the virus did not appear to enter the veins and invade the vascular system. This suggested that the mechanism of resistance of the *Rsv4* involves not only limiting cell-to-cell movement away from the initial infection sites, but also restricting the virus entry into the vascular tissue.

These data support the hypothesis by Gunduz et al. (2004) that SMV-G1 and G7 completely invade inoculated leaves of Essex and that the *Rsv4* gene decreases the number of foci of infection and the leaf area invaded. However, our data contradicts the hypothesis that the size of infection foci and extent of virus invasion do not increase in PI 88788 inoculated with SMV-G1 beyond 23 dpi (Gunduz et al., 2004). Both values increased about 3-fold from 19 to 26 dpi. However, initial observations from leaf prints sampled at 32 dpi suggest no increase in the extent of virus invasion beyond 26 dpi (data not shown).

Restriction of Long Distance and Vascular Movement

In addition to restricting cell-to-cell movement, the *Rsv4* gene limited long distance movement of SMV. Leaf immunoprints from non-inoculated first trifoliolate leaflets of *Rsv4* cultivars showed no detectable virus. Fewer than one out of ten leaflet prints (first trifoliolates) were positive for SMV particles by immuno-detection at 19 or 26 dpi; no virus was detected in leaf prints prior to 19 dpi. In addition to limiting the long distance movement, *Rsv4* restricts the unloading of SMV from the vascular tissue to the adjacent cells and subsequent cell-to-cell movement. One example of resistance genes that limit long distance movement of virus include the *Arabidopsis RTM1* and *RTM2* genes that restrict movement of *Tobacco etch virus* (TEV; Genus *Potyvirus*; Family *Potyviridae*) (Chisholm et al., 2001). Another is *Arabidposis* resistant to *Lettuce mosaic virus* (LMV; Genus; Family) (Revers et al., 2003). Lack of virus in stem sections of Essex-*Rsv4* supports our hypothesis that this gene restricts both movement and accumulation of SMV in the vascular tissue, therefore limiting the long distance movement and the access to the vasculature.

Allele Specificity

Our data show that different *Rsv4*-carrying cultivars react differently to SMV infection. The resistance conditioned by *Rsv4* has been recently described from PI 88788 (Gunduz et al., 2004). No previous work has differentiated among the allele(s) of the *Rsv4* locus. Low numbers of infection sites and correspondingly larger diameters of infection sites were present in PI 88788 and Peking. In contrast, more but smaller infection sites were detected in Essex-*Rsv4* and V94-5152. Similarly, percentages of leaf area invaded by SMV were lower in Essex-*Rsv4* and V94-

5152 compared to the two other cultivars. The similarity in the extent of virus invasion between Essex-*Rsv4* and V94-5152 is not surprising since the former is derived from V94-5152. Although in the same group, the Essex-*Rsv4* isoline is more permissive to SMV than its parent V94-5152 since the *Rsv4* gene has been introduced into the susceptible Essex background. The difference between the two groups was also significant; PI 88788 and Peking were more resistant to SMV than the other two cultivars. This clearly supports our hypothesis that host background plays an important role in shaping the resistance response to disease. More significantly, this study showed that the *Rsv4* alleles are different between Essex-*Rsv4* and V94-5152, and PI 88788 and Peking. PI 88788 and Peking reacted similarly to all SMV strains tested and therefore do not appear to have distinct alleles of the *Rsv4*.

Differential Reactions of Rsv4 to SMV Pathotypes

Although *Rsv4*-based resistance is not strain specific, the resistance response is, i.e., the number and size of infection sites, and the extent of virus invasion are different for each pathotype with all the cultivars tested. SMV-G6, e.g., resulted in about two and six-fold increase in the percentage of leaf area invaded by SMV-G7 and SMV-G1, respectively. Similarly, V94-5152 was more resistant to SMV-G1 or G7. SMV-G7 was more severe on Peking than were strains G1 or G6, but this difference was not seen on PI 88788. The different *Rsv4* carrying cultivars reacted differently to the SMV pathotypes (Tables 4.1-3).

Mechanisms for virus resistance include the hypersensitive response (HR), extreme resistance (ER), gene silencing and resistance to movement. A normal viral infection cycle includes entry into the cell, replication of the viral genome, movement from cell-to-cell, entry into the vascular system for long distance movement, exit and subsequent cell-to-cell movement. Breaking one or more steps of this cycle can lead to resistance. An HR reaction involves activation of a cascade of defense responses. This includes localized cell death i.e., necrosis, activation of systemic acquired resistance (SAR), and accumulation of salicylic acid (SA) and pathogenesis related (PR) proteins. Tobacco *N* gene resistance to *Tobacco mosaic virus* falls in this category (Dinesh Kumar et al., 2000). Other HR responses involve activation of defense cascades conditioned by jasmonic acid (JA) and ethylene (ET) (McDowell and Dangl, 2000). ER is not associated with necrosis and results in no visible lesions on plants. Virus replication is

inhibited. Potato *Rx* gene for resistance to *potato virus X* (PVX) is an example of extreme resistance (Bendahmane et al., 1999).

The *Rsv4* gene appears to employ multiple mechanisms for resistance to SMV including restricting virus accumulation and movement. Examples of virus resistance involving restriction to movement were reported for viruses with known movement proteins (MPs). MPs are involved with endogenous plant proteins facilitating movement of virus through plasmodesmata (Lucas 1999). Kasschau and Carrington (1998) reported that post transcriptional gene silencing (PTGS) restricts TEV movement by silencing viral movement components. No MPs have been identified in potyviruses, however. The cylindrical inclusion proteins of potyviruses are localized into pinwheel structures that align with plasmodesmata in the cell and interact with CP (Carrington et al., 1998). Hunst and Tolin (1983) showed the presence of pinwheel inclusions and cytoplasmic strand formation with SMV infection in soybean. We postulate that the *Rsv4* disrupts these formations and thereby disrupts the virus cell-to-cell movement.

In conclusion, the *Rsv4* is not strain specific (Ma et al., 1995, Hayes et al., 2000 and Gunduz et al., 2004); its phenotype is non necrotic with a late susceptibility developing in the heterozygous state only (Gunduz et al., 2004). We showed that *Rsv4* alleles are different among the various *Rsv4*-carrying cultivars. We propose the following allele designation: *Rsv4* for PI 88788 and Peking, $Rsv4^{v}$ for V94-5152 and Essex-*Rsv4*. We conclude that the mechanisms of *Rsv4* resistance to SMV include restricting viral cell-to-cell and long distance movement, restricting virus movement through the vascular tissue, and reducing accumulation of SMV in the vascular and leaf tissue.

A **B**

Figure 4.1. Symptom development in 'Essex' and *Rsv4*-containing cultivars inoculated with SMV. Susceptible Essex plant inoculated with G1 at 9 dpi (A), and resistant 'Essex-*Rsv4'*, inoculated with SMV-G1 (B), SMV-G6 (C), and SMV-G7 (D) at 14 dpi.

Figure 4.1 Continued

C

D

A **B**

Figure 4.2. Leaf immunoprints showing virus accumulation and detection in susceptible cv. 'Essex'. Corresponding leaf (A) and immunoprint (B) of 'Essex' unifoliolate inoculated with SMV G1 9 dpi. Sites of local and systemic lesions (A) correspond to those of virus accumulation (B).

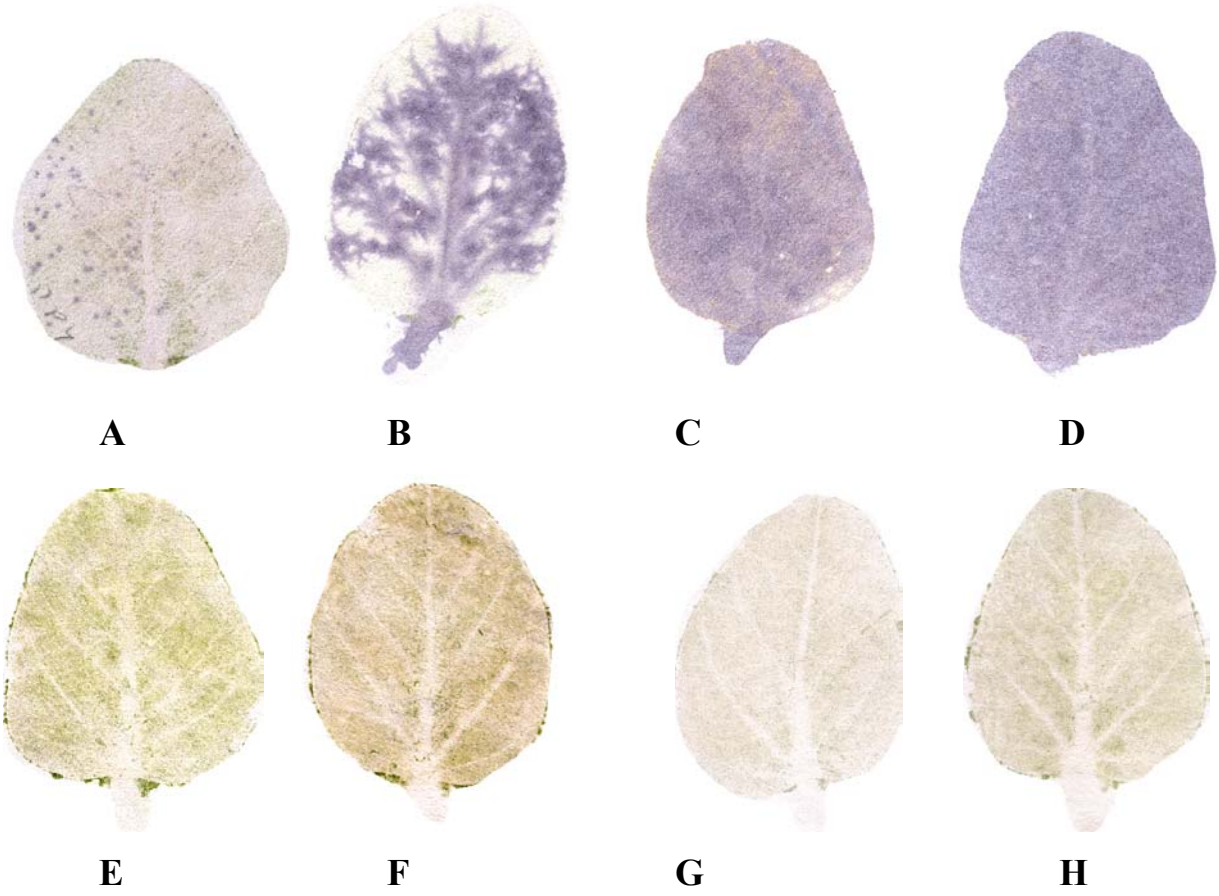
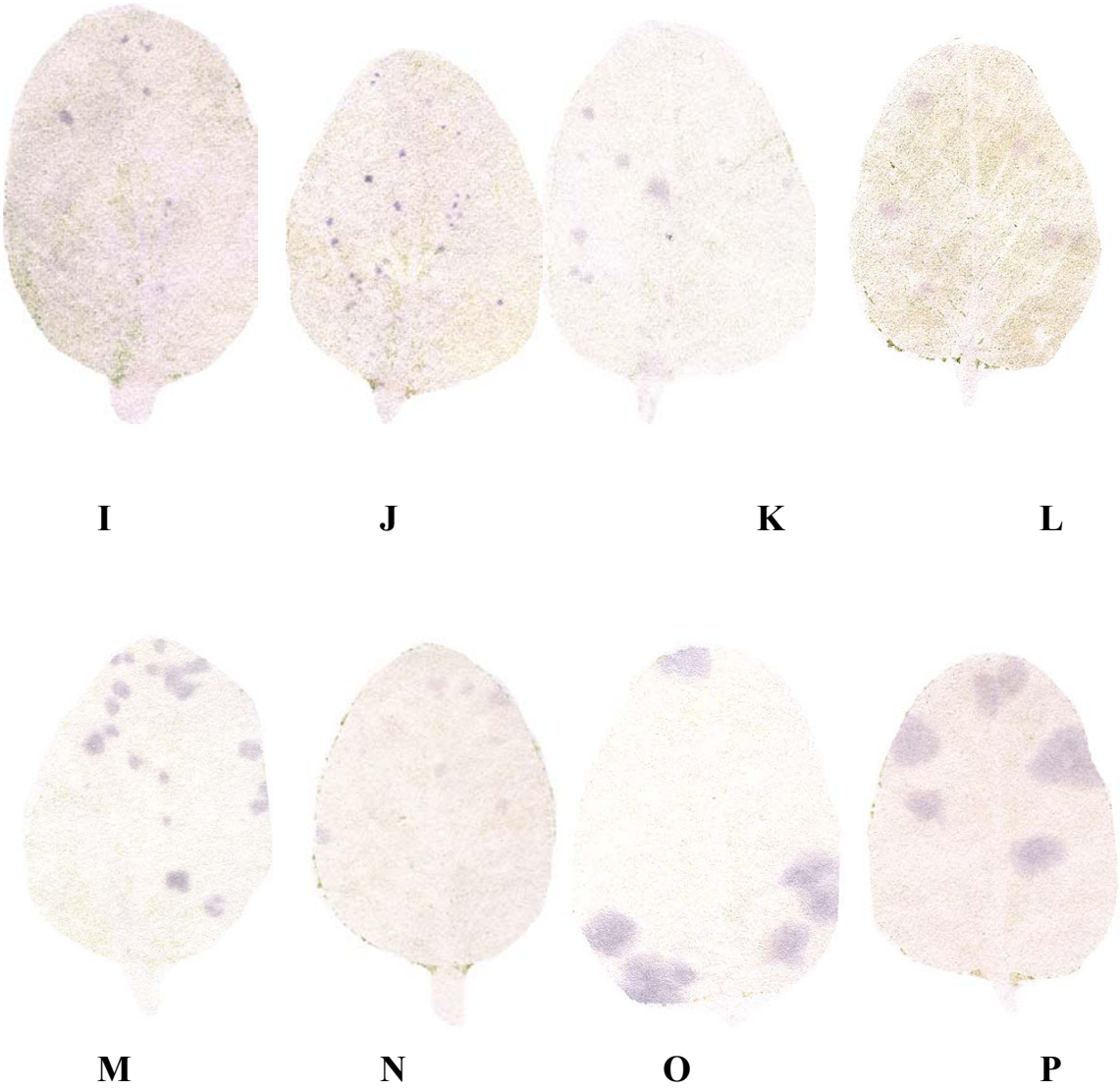


Figure 4.3. Tracking virus accumulation and movement form inoculated unifoliolate leaves. Leaf immunoprints from *Soybean mosaic virus* strain G1 (SMV-G1)-inoculated leaves of susceptible 'Essex' soybean (*rsv*) (A-D) sampled at 4, 9, 19, and 26 dpi, respectively, and resistant cvs. 'Essex-*Rsv4'* (E, I and M*)*, 'V94-5152' (F, J and N), 'PI 88788' (G, K and O) and Peking (H, L and P) sampled at 4, 14 and 26 dpi, respectively. Leaves were printed onto filter paper and developed by western analysis using whole virus particle antiserum to SMV, alkaline phosphatase conjugated goat anti-rabbit secondary antibodies and NBT/BCIP substrate. Purple color corresponds to sites of virus accumulation.

Figure 4.3 Continued



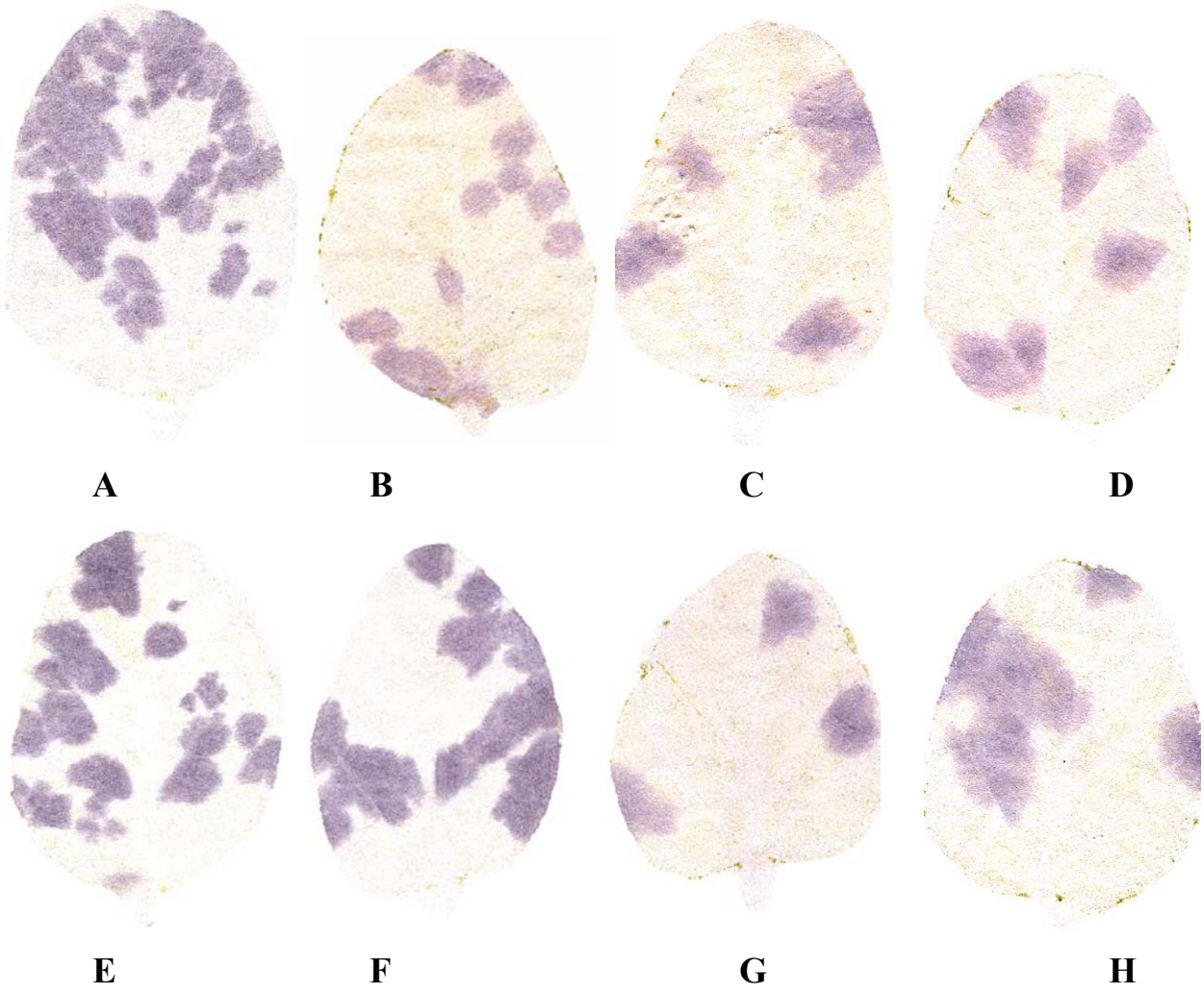


Figure 4.4. Tracking virus accumulation and movement from inoculated unifoliolate leaves. Leaf immunoprints of 'Essex-*Rsv4'* (A and E), 'V94-5152' (B and F), 'PI 88788' (C and G) and 'Peking' (D and H) inoculated with *Soybean mosaic virus* strains G6 and G7 at 26 dpi.

Figure 4.5. Detection of long distance movement of *Soybean mosaic virus*. Leaf immunoprints from non-inoculated (A) first trifoliate leaflet and (B) second trifoliolate leaf of 'Essex' inoculated with SMV-G1 at 9 days post inoculation. Virus inoculations were applied on the unifoliolate leaves (not shown).

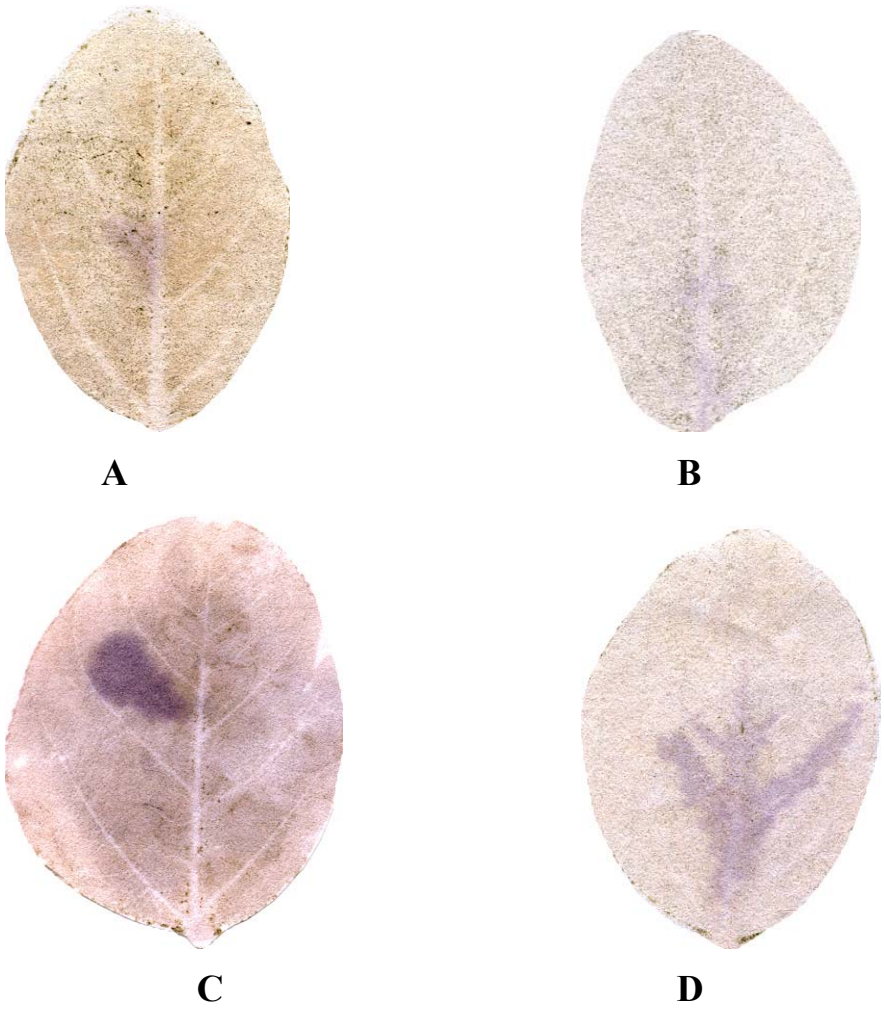


Fig.4.6. Detection of virus accumulation and movement from upper non-inoculated trifoliolate leaflets. Leaf immunoprints from non-inoculated trifoliolate leaflets of resistant soybeans carrying the *Rsv4* gene. (A) PI 88788 inoculated with SMV-G7 at 19 dpi; (B) Peking inoculated with SMV-G7 at 19 dpi, (C) Peking inoculated with SMV-G1 at 43 dpi and (D) V94-5152 inoculated with SMV- G6 at 26 dpi.

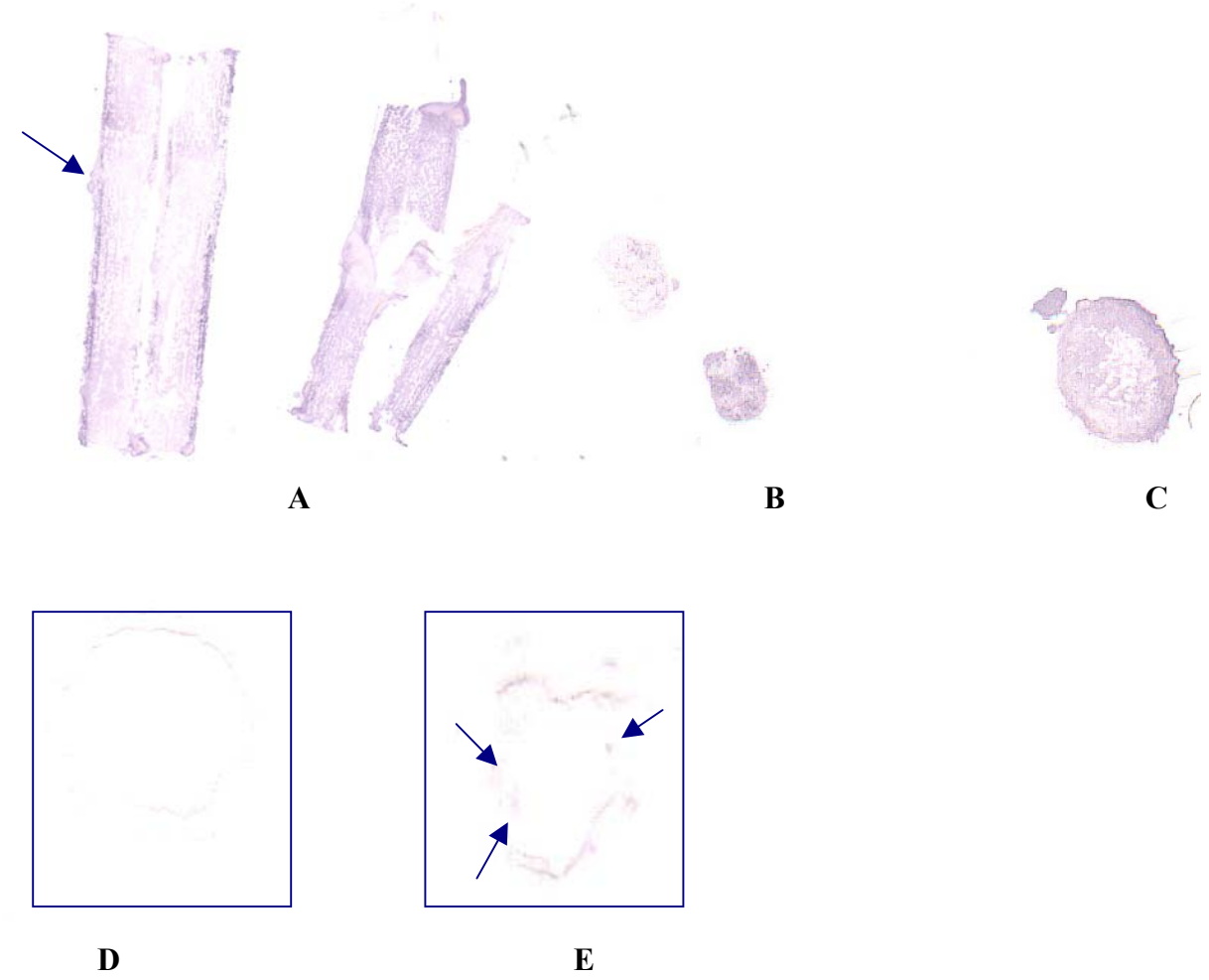


Figure 4.7. Detection of *Soybean mosaic virus* in the vascular tissue. Stem sections of 'Essex' 7 dpi and Essex-*Rsv4* inoculated with SMV 35 dpi. (A) longitudinal sections from SMV-G1 inoculated 'Essex' (arrow pointing to unifoliolate petiole attachment area, left; node leading to first trifoliolates shown on the right). B and C and show petiole cross sections from SMV-G1 inoculated 'Essex' unifoliolates and first trifoliolates; D and e show first trifoliolate petiole cross sections from 'Essex-*Rsv4'* inoculated with SMV-G6 and G2, respectively (arrows in E point to virus sites).

Table 4.1. Mean number of infection sites from susceptible and resistant soybean cultivars inoculated with *Soybean mosaic virus*. Maximum mean number of initial infection sites (before the virus moved into the vasculature of the leaf) from three inoculated leaves sampled from three different plants following inoculation with the SMV strains G1, G6 and G7.

Cultivar	**G1**	**G6**	**G7**	**Mean**
Essex	42.3	59.7	54.0	**52.0 A**
Essex-*Rsv4*	16.0	34.3	26.7	**25.7 B**
V94-5152	15.7	34.3	13.7	**21.2 B**
PI 88788	8.0	4.0	6.0	**6.0 C**
Peking	6.7	3.0	4.3	**3.6 C**

Data shown correspond to average numbers from three leaf blots from susceptible cultivar 'Essex' (*rsv*) sampled 4 days post inoculation (dpi), and resistant *Rsv4* cultivars 19 dpi.
Means followed by the same letter are not significantly different based on ANOVA and LSD (14.37) mean separation at $P \leq 0.05$.

Table 4.2. Mean diameter of infection sites (mm) from susceptible and resistant soybean cultivars inoculated with *Soybean mosaic virus.* Size of infection sites (before the virus moved into the vasculature of the leaf) from three inoculated leaves sampled from three different plants.

Cultivar	G1	G6	G7	LSD
Essex-*Rsv4*	1.7 **B** **(b)**	2.7 **B** **(ab)**	3.6 **B** **(a)**	1.62
V94-5152	1.4 **B** **(b)**	2.7 **B** **(a)**	2.8 **B** **(a)**	0.89
PI 88788	3.5 **A** **(b)**	5.7 **A** **(a)**	6.4 **A** **(a)**	1.99
Peking	4.3 **A**	6.1 **A**	5.3 **A**	NS
LSD	1.59	1.41	1.38	

Data shown correspond to average size of infection sites from three leaf blots from resistant cultivars 'Essex-*Rsv4'*, 'V94-5152', 'PI 88788' and 'Peking' sampled at 19 dpi.

Data analyzed by ANOVA at $P \leq 0.05$ for mean separation by LSD for each strain across cultivars (Columns; A-B) and for each virus strain in each cultivar (rows; a-b)

NS= not statistically different

Table 4.3. Mean percentage of leaf area invaded from susceptible and resistant soybean cultivars inoculated with three strains of *Soybean mosaic virus.* Percent leaf area infected 26 dpi with SMV strains G1, G6 and G7.

Cultivar	**G1**	**G6**	**G7**
Essex	95.6 **A**	100.0 **A**	98.9 **A**
Essex-*Rsv4*	7.4 **EF**	41.3 **B**	22.9 **CD**
V94-5152	4.3 **F**	28.9 **BC**	21.7 **CDE**
PI 88788	5.2 **F**	9.7 **DEF**	5.8 **F**
Peking	6.4 **F**	5.2 **F**	13.8 **DEF**

Data shown correspond to average percent leaf area invaded from three leaf blots from susceptible cultivar 'Essex' (*rsv*) and *Rsv4*-carrying resistant cultivars.

Means followed by the same letter are not significantly different based on ANOVA and LSD (12.45) mean separation at $P \leq 0.05$

Table 4.4. Relative virus titer from susceptible and resistant soybean cultivars inoculated with four strains of *Soybean mosaic virus*. Absorbance values at 405 nm from Essex, Essex-*Rsv4*, V94-5152 and PI 88788 inoculated with SMV G1, G5, G6 and G7 sampled 14 dpi.

		SMV strain			
Cultivar	**Leaf Position**	**G1**	**G5**	**G6**	**G7**
Essex	Unifoliolate	2.816	2.789	2.900	2.838
	Trifoliolate	2.372	2.494	2.712	2.777
Essex-*Rsv4*	Unifoliolate	0.164	0.147	0.149	0.138
	Trifoliolate	0.086	0.085	0.087	0.083
V94-5152	Unifoliolate	0.148	0.217	0.172	2.058
	Trifoliolate	0.063	0.068	0.054	0.073
PI 88788	Unifoliolate	0.230	0.518	0.308	0.985
	Trifoliolate	0.071	0.075	0.071	0.079

Mean absorbance from ELISA values of replicated duplicate wells from one inoculated unifoliolate and one non-inoculated first trifoliolate from each of two plants of each cultivar-strain combination sampled 14 dpi.

Values higher than 0.11 (mean absorbance of healthy plus 3 x standard deviation of healthy) are positive for SMV.

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