

THE EFFECTS OF TEMPERATURE ON THE DURABILITY OF
RESISTANCE OF SOYBEAN TO SOYBEAN MOSAIC VIRUS

by

Jonathan P. Flora

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Approved:

Dr. Sue A. Tolin, Chairperson

Dr. Glenn R. Buss

Dr. Herman Warren

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Dr. Sue A. Tolin, Chairperson

Department of Plant Pathology, Physiology and Weed Science,
Virginia Polytechnic Institute and State University

(ABSTRACT)

The objectives of this study were to determine the effects the temperature sensitivity of alleles of *Rsv₁* in soybean (*Glycine max* (L.) Merr.). Soybean cultivars carrying alleles of *Rsv₁* were exposed to several heat treatments designed to induce heat shock protein production prior to inoculation with soybean mosaic virus (SMV). The heat treatment methods were similar to those employed in the research with *N* gene-tobacco mosaic virus studies. The soybean cultivars used were Lee 69, York, Kwanggyo, Ogden and PI96983, carrying the *Rsv*, *RsvI-y*, *RsvI-k*, *RsvI-t*, and *RsvI* alleles of *Rsv₁*, respectively, and were selected to provide a range of reactions to selected SMV pathotype groups. For example *RsvI-y* and *RsvI-k* give a necrotic response to SMV G4 and SMV G6, respectively, while both are resistant to SMV G1. To determine the durability of resistance under heat shock conditions, the symptoms were observed for changes in the phenotype of the resistance response. Immunological techniques were employed to determine the vascular movement and localization of the viral antigen in the plant. Heat treatments used were found to induce HSP but to have no effect on the resistance phenotype. A detached leaf assay was used to test the same *Rsv₁* alleles at constant high temperatures. Primary trifoliolate leaflets were removed and inoculated, then placed into a continuously lighted incubator at 20 °C or 30 °C. Leaf immunoprint assays were used to determine the localization of the viral antigen. The visible symptoms for necrotic lesions and veins were observed for necrotic phenotype-pathotype combinations but mosaic symptoms were not observed on detached leaves, as expected for inoculated leaves. The detached leaf assay confirmed that no change from the expected resistance response of the *Rsv₁* alleles occurred at 30 °C. A breakdown of resistance to SMV at high temperature had been reported in soybean by Tu and Buzzell (1987). The resistance gene in which the high temperature breakdown occurred has been determined to be *Rsv₃*. Using cultivars and breeding lines carrying *Rsv₃* a similar experiment was attempted in growth chambers. Preliminary results suggest that *Rsv₃* is temperature sensitive.

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TABLE OF CONTENTS:

Title and Signature Page	i
Abstract	ii
Acknowledgments	iii
Table of Contents	iv
List of Tables	vi
List of Figures	vii

Chapter I

Introduction and Literature Review	1
Introduction	1
Soybean Mosaic Virus	2
Soybean resistance to SMV	3
Plant resistance to virus	4
Temperature sensitive resistance genes	4
Virus pathogenesis and temperature	6
Viral movement protein relationship to HSPs	7
Effects of low temperature on viral pathogenesis	7
Effect of heat shock on viral replication	7
Virus movement in plants	8
Short-distance movement	8
Long-distance movement	12
Methods for tracking virus movement	14
Heat Shock	15
Heat shock response	15
Heat shock induction	16

Chapter II

Effects of Heat Shock on Soybean <i>Rsv₁</i> Resistance to Soybean Mosaic Virus	17
Introduction	17
Materials and Methods	18
Plant material	18
Virus and inoculation methods	18
Detached leaves	19
Heat treatment	19
Immunological detection of SMV	20
Determination of HSP increase	21

Enzyme linked immunosorbant assay of HSP's	22
Results	22
Effect of heat treatment on symptom development	22
Evidence for heat shock protien activation	24
Comparative SMV disribution in inoculated leaves	26
Comparative vascular movement of SMV	28
Response at two temperatures of detached leaves to SMV	29
Discussion	31

Chapter III

Effect of temperature on <i>Rsv₃</i> Gene of Soybean for Resistance to Soybean Mosaic Virus	38
Introduction	38
Materials and Methods	38
Plant material	38
Virus and inoculation methods	38
Immunological detection of SMV	39
Results	40
Effect of temperature on symptom development	40
SMV replication and movement in inoculated leaves	40
Effect of temperature on vascular movement of virus	42
Discussion	45

Chapter IV

Final Discussion	50
Summary of Heat Shock Treatments on <i>Rsv₁</i>	50
Summary of Temperature on <i>Rsv₃</i>	50
Speculation on soybean resistance properties	50
Final Conclusions	51
Literature cited	52
Vita	58

LIST OF TABLES:

Table 2-1	Reaction of soybean cultivars to SMV pathotypes	18
Table 2-2	Days following heat treatment to the first appearance of visible symptoms	23
Table 2-3	Activation of HSP production in leaves and hypocotyles of soybean and detection by ELISA	24
Table 2-4	Effect of temperature on the localization SMV in detached leaves incubated at high and low temperatures	26
Table 2-5	Effect of HS on the vascular movement of SMV	29
Table 2-6	Effect of HS on the localization of SMV in the inoculated leaves	33
Table 3-1	Response of <i>Rsv₃</i> - containing soybean cultivars to SMV pathotypes	39
Table 3-2	Appearance of symptoms on <i>Rsv₃</i> - containing soybean cultivars after inoculation with SMV	40
Table 3-3	Extent of invasion of SMV in leaves of <i>Rsv₃</i> - containing soybean cultivars	43
Table 3-4	Detection of SMV in trifoliolate leaves of <i>Rsv₃</i> - containing soybean cultivars	44

LIST OF FIGURES:

Figure 2-1	Histogram of HSP activation	25
Figure 2-2	Comparison of localization of viral antigen in York	27
Figure 2-3	Comparison of effects of heat shock on localization of viral antigen in inoculated leaves	30
Figure 2-4	Symptom development on detached trifoliolate leaves incubated at 30 °C day 6 p.i.	32
Figure 2-5	Comparison of localization of viral antigen in detached leaves incubated at 30 °C	34
Figure 2-6	Comparison of localization of viral antigen in detached leaves incubated at 20 °C	35
Figure 3-1	Effect of temperature on the movement of SMV G1 antigen within the inoculated leaf for plants held at 30 °C	41
Figure 3-2	Effect of temperature on the movement of SMV G7 antigen within the inoculated leaf for plants held at 22 °C	42
Figure 3-3	Effect of temperature on the movement of SMV G1 antigen into trifoliolate leaflets for plants held at 30 °C	46
Figure 3-4	Effect of temperature on the movement of SMV G7 antigen into trifoliolate leaflets for plants held at 22 °C	47

CHAPTER I

Introduction and Literature Review

INTRODUCTION:

To further the understanding of interaction between soybean (*Glycine max* (L.) Merr.) and soybean mosaic virus (SMV), it is important to determine if resistance genes are temperature sensitive, since many resistance genes have been found to be temperature sensitive in other systems. Plant geneticists at Virginia Tech investigating the genetics of resistance have often noticed variability in symptoms at different times of the year, and attributed this variability to high temperature influences. The sensitivity of resistance genes to high temperature has been reported for many viral resistance genes, for example, in tobacco (*Nicotiana tabacum* L.) the *N* gene for resistance to tobacco mosaic virus (TMV) (Yarwood, 1958), the *I* (ts) gene in bean (*Phaseolus vulgaris* L.) for resistance to bean common mosaic virus (BCMV) (Fisher & Kyle, 1994), and in soybean the *Rps* resistance gene to *Phytophthora sojae* (Gijzen *et. al.*, 1996). In addition to the temperature sensitivity of resistance genes, the potential for viral movement from initially infected cells might be a factor of molecular chaperoning functions of heat shock proteins or of viral-encoded genes.

In viral temperature sensitive (TS) resistance systems, the breaking or change in the resistance phenotype is often accompanied by the lack of viral localization. The hypersensitive response or a subliminal infection that restricts viral movement at normal temperatures might be affected at high temperatures. In soybean it has been reported that a systemic necrosis changed to a systemic mosaic at temperatures above 28 °C (Tu and Buzzell, 1987). Studies on the effect of heat treatments, whose conditions are similar to those known to induce heat shock in plants, have been shown to increase the number of local lesions or to induce lesions in latent infections (Yarwood, 1958).

Many methods have been employed to test for the presence of the virus in tissue to determine if the virus moves. Grinding sections of host tissue and inoculating to an indicator host proves the presence of infectious virus in the tissue tested, however that method is laborious and time consuming. Nucleic acid and immunological techniques can be employed to determine the presence of viral nucleic acid or antigen, respectively. The detection of viral antigen by press blotting has been correlated with the presence of infectious virus in the leaf (Mansky *et al.*, 1990). By blotting whole leaves the localization of the viral antigen within the leaf can be determined.

This study seeks to determine if resistance genes in soybean are temperature sensitive. The approach includes observing changes in appearance of visible resistance phenotype reactions, or a change in the pattern of systemic movement and the localization of the viral antigen. A change in the movement observed from the expected phenotype reaction in the various heat treatments when compared to control plants is considered as possible breaking of resistance. Several methods of brief heat treatments will be applied to cultivars carrying alleles at the *Rsv₇* locus. In addition, preliminary

tests of several cultivars carrying an allele of *Rsv₃* will be conducted in a similar manner to that done by Tu and Buzzell (1987) to determine the TS of that resistance allele.

The specific objectives of my research are:

1. To determine if heat shock treatment changes the phenotype of the resistance response to selected *Rsv₁* alleles and selected SMV groups.
2. To determine if heat shock treatments and high temperature incubation of detached leaves changes the localization of SMV antigen in the leaf and plant tissue for selected *Rsv₁* alleles and selected SMV groups.
3. To determine if resistance responses of selected *Rsv₃* alleles to selected SMV groups change when plants are grown at high temperature.

SOYBEAN MOSAIC VIRUS:

Soybean mosaic virus (SMV) is a member of the *Potyviridae*, the largest and most economically important group of plant viruses (Matthews, 1991). In soybean it is the most important virus and is found in all soybean growing regions of the world (Bos, 1972). Transmission of SMV can occur by three methods: (i) it can be easily transmitted mechanically, (ii) it is transmitted in a non-persistent manner by aphids, and (iii) in nature the main source of spread is through seeds (Bos, 1972).

The SMV virion is a flexuous rod, about 750 nm in length by 15-18 nm wide (Bos, 1972). The virus consists of a monopartite plus-sense single-stranded RNA genome of about 10,000 bases. The genomic RNA is polyadenylated at the 3' end and has a genome-linked small viral protein (VpG) covalently linked at the 5' end (Ghabrial *et al.*, 1990). The virion is formed from multiple copies of a single protein, molecular weight 29,900 (Jain *et al.*, 1992). SMV proteins are produced from a single polyprotein.

SMV isolates are grouped based on the phenotypic response of selected resistant soybean cultivars. There are seven SMV groups (G1-G7) classified on the basis of phenotypic response on differential soybean lines by Cho and Goodman (1979). The SMV groups are not individual strains, but a population of isolates with similar reactions on the differential cultivars. Strains of SMV have also been distinguished by aphid transmission and antigenic properties, but for this study the reaction of the groups to the differential cultivars is considered (Jain *et al.*, 1992).

SMV symptomatology: The symptomatology of the phenotypic responses are systemic mosaic, systemic necrosis and resistant. Systemic mosaic, a permissive infection of a plant having no

resistance to the virus, does not restrict the virus from cell-to-cell or vascular movement. The systemic mosaic appears as a light and dark green mosaic pattern with leaf distortion depending on the cultivar. Necrosis is a hypersensitive response of a resistance gene and is characterized most often as the appearance of brown lesions, surrounded by a yellow halo, on the inoculated leaves. Necrosis is most often not confined to an inoculated leaf, but instead the virus spreads to subsequent leaves and stems where it induces necrosis, resulting in severely stunted plants that often develop tip necrosis (Chen *et. al.*, 1994). There are no symptoms apparent in resistant plants, and no virus can be recovered or detected.

SOYBEAN RESISTANCE TO SMV:

Soybean *Rsv₁* resistance to SMV: The resistant and necrotic reactions to SMV are conditioned by a single, dominant, nuclear-inherited gene (Chen *et. al.*, 1994). There appear to be several alleles at the *Rsv₁* locus for SMV resistance, which interact differentially with different SMV groups, four of which are listed in Table 2-1 (Chen *et. al.*, 1994). The necrotic and resistant reactions segregate together in a 3(R+N):1 ratio to susceptibility. When a resistant or necrotic plant, carrying an allele at the *Rsv₁* locus, is crossed with a susceptible plant, the resistant allele is dominant to susceptibility. Heterozygotes of crosses between *Rsv₁* and *rsv* plants often give a necrotic response; therefore providing evidence for necrosis being considered a resistance response (Chen *et. al.*, 1994). Chen *et. al.* (1994), also noted that the alleles that exhibit complete resistance to specific strains in the homozygous state often respond with a necrotic reaction to the same strain when they occur in a heterozygote with a susceptible allele.

The alleles at the *Rsv₁* locus are derived from several sources. The gene at the *Rsv₁* locus was determined to be a single dominant gene conferring resistance in York to SMV G1 (Roane *et al.*, 1983). Later it was determined that the single dominant resistance genes in York, Ogden, Kwanggyo and PI96983 were all alleles at the same *Rsv₁* locus (Chen *et al.*, 1991). The alleles were assigned gene symbols *Rsv₁-y*, *Rsv₁-t*, *Rsv₁-k* and *Rsv₁* for York, Ogden, Kwanggyo and PI96986, respectively (Chen *et al.*, 1991).

The *Rsv₁* gene has been mapped with microsatellite and restriction fragment length polymorphism (RFLP) markers to linkage group F in soybean Yu *et al.*, 1994; 1996). It was linked to low molecular weight (LMW) heat shock protein (HSP) gene (Yu *et al.*, 1994). A distance of 0.5 centiMorgans separated *Rsv₁* and the soybean microsatellite marker 176 constructed from sequences flanking a simple sequence repeat region of (AT)₁₅ in the soybean HSP176 a LMW HSP (Yu *et al.*, 1994; Nagao *et al.*, 1985).

Effects of temperature on SMV resistance have been examined in a few experiments but the work on the temperature sensitivity (TS) of resistance genes is not complete. Tu and Buzzell (1987) reported that systemic lethal necrosis that occurred at 20 °C and 24 °C due to SMV G1 infection changed to systemic mosaic at 28 °C and 32 °C. In soybean, 10 °C was found to break the resistance

in a soybean line PI96983 carrying *Rsv₁* to SMV G1 to which it is normally resistant (Mansky *et al.*, 1991). It was suggested that resistance may be repressed at low temperatures and that this observation may not be unique to the SMV-soybean system (Mansky *et al.*, 1991).

PLANT RESISTANCE TO VIRUS:

Host resistance to virus infection is mediated by resistance genes that a plant may carry. Resistance is different from immunity or non-host resistance in that an active factor is expressed by the genotype of the plant (Zaitlin and Hull, 1987). Susceptibility or resistance operates at four levels as identified by Zaitlin and Hull (1987), (i) for total immunity the virus does not replicate; (ii) in subliminal infections some virus replicates in the initially infected cells, but does not spread to adjacent cells; (iii) the hypersensitive reaction, the host restricts the virus to a few cells around the site of infection; and (iv) total susceptibility, most of the cells become infected. Resistance occurs when the virus infection and replication cycle, including movement from the site of infection, is interrupted.

Tobacco *N* gene resistance to TMV infection: The resistance gene for plant viral infections has been extensively studied in the tobacco *N* gene/TMV system. The *N* gene was introduced into tobacco from *Nicotiana glutinosa* L. In this system the hypersensitive reaction of the *N* gene mediates resistance. Infection of a tobacco plant carrying the *N* gene causes the formation of necrotic local lesions when plants are inoculated with TMV. The spread of all strains of TMV prevented temperatures below 30 °C, whereas above 30 °C extensive invasion of the plant occurs (Fraser, 1983). The highly localized infections of TMV in tobacco with *N* gene resistance restricts the infection to a few cells.

TEMPERATURE SENSITIVE RESISTANCE GENES:

Many plant resistance genes for various pathogens are affected by high and low temperatures. Resistance to fungal, bacterial and viral pathogens is effected by high temperature in many plants conditioned by many resistance genes. The mechanism for the breakdown in resistance by the temperature extremes is not well understood for most of the reactions but the fact that it occurs is well documented. One of the most studied resistance breakdown systems is the *N* resistance gene in tobacco. In soybean heat shock conditions, genes for resistance to *Phytophthora sojae* are known to be temperature sensitive. In bean, resistance to SMV and BCMV conferred by the *I* gene is temperature sensitive and applying high temperature is part of the method for detecting the presence of the gene in the breeding process. Resistance to SMV in soybean was reported to be temperature sensitive, however at the time of the studies the complete genetics of the resistance gene was not known (Tu and Buzzell, 1987; Mansky *et al.*, 1991).

The classic temperature sensitive resistance gene is the *N* gene in tobacco. The TS of the *N*

gene was noted in 1931 when it was observed that *N. glutinosa* inoculated with TMV exhibited 1-2 mm necrotic lesions at 21 °C, formed larger and more quickly spreading necrotic lesions at 28 °C, and with no necrosis, a mosaic pattern developed at 35 °C (Samuel, 1931). Beyond the early reports of the TS of this gene numerous studies have been done on this system. The hypersensitive response of the *N* gene is overcome at high temperatures and the virus spreads throughout the plant; however, extensive necrosis of the tissues invaded by the virus at high temperature results when the plants are transferred from 32 °C to 22 °C (Weststeijn, 1981). The hypersensitive response is not completely inactivated at high temperatures, but is unable to be expressed until temperatures are lowered. Tobacco plants (cv. Samsun NN) grown at 22 °C could be changed from reacting with a necrotic local lesion to systemic mosaic for up to 11 days following inoculation. Weststeijn (1981) suggested that the final stages of localization is an inhibition of the spread of the virus, which was broken down at high temperature. Also, the temperature sensitivity of tobacco and TMV mutants led to the discovery of viral movement proteins. At temperatures above 28 °C the restriction of TMV is broken down and at 32 °C there is no hypersensitive response at all. The necrotic reaction changes to a susceptible, permissive infection at high temperatures.

The *N* gene is affected also by heat treatment prior to inoculation with TMV. In addition to the changes exhibited when the plants are maintained at high temperature, high temperature treatment prior to inoculation can change the resistance reaction. The number of local lesions was increased about 7 times when leaves were dipped in water at 45 °C for 60 s prior to inoculation with TMV (Yarwood, 1952). It has also been reported that heating plants at 36 °C for two days prior to inoculation increased the number of lesions formed by TMV on tobacco plants (Kassanis, 1952).

There are heat shock plant reactions to viral infections reported. In addition to the brief heat treatments, Yarwood (1958) reported that “heating only the inoculated leaf, without heating the growing points or other parts of the plant was sufficient to induce systemic infection” in pinto bean inoculated with TMV, heated to 50 °C for 10-40 s. Foster and Ross (1975) show that symptomless infection in tobacco without the *N* gene can be activated to produce necrotic lesions by dipping a leaf in a 50 °C water bath.

In bean (*Phaseolus vulgaris* L.) a single dominant gene segregated as a unit gene with the *I* gene for resistance to several potyviruses (Fisher and Kyle, 1994). Plants containing this gene respond with lethal necrotic reactions when inoculated with SMV, but are symptomless following inoculation with bean common mosaic (BCMV) and some other potyviruses (Fisher and Kyle, 1994). At temperatures above 32 °C, resistance to BCMV breaks down and a necrotic phenotype develops that is similar to the lethal necrosis that is induced by SMV at any temperature (Kyle and Provvidenti, 1993). This is an example of a TS resistance gene for resistance to potyviruses.

An opposite reaction from the *I* gene in bean was reported to occur in SMV G1 inoculated soybean. High temperature changed systemic necrosis to systemic mosaic, similar to the reaction of the *N* gene at high temperature. Tu and Buzzell (1987) reported that systemic lethal necrosis occurred at 20 °C and 24 °C due to SMV G1 infection changed to systemic mosaic at 28 °C and 32 °C.

Many genes for fungal and bacterial pathogens are also TS as well. Several *Rps* genes and alleles for resistance to *Phytophthora sojae* in soybean are temperature sensitive. Low temperature post-inoculation susceptibility could be induced by transferring inoculated hypocotyls immediately to a 30 °C water bath after inoculation, but only for certain resistance genes. For example *Rps1-a* and *Rps6* are gene considered to be TS by post-inoculation incubation at 33 °C, but *Rps1-c* and *Rps5* are not TS (Gijzen *et al.*, 1996). However, with *Rps1-c* in cultivars Williams 79 and Harosoy BC4, the non-TS resistance was broken by pre-treating cotyledons in 44 °C water bath for 12 min (Gijzen *et al.*, 1996). It should be noted that the 44 °C water bath pre-treatments of hypocotyls is very similar to conditions used by Lin *et al.*, (1984) in induce heat shock protein production (reviewed on p.23). The results of Gijzen *et al.* (1996) suggested to them that susceptibility induced by short, high temperature pre-inoculation treatments is fundamentally different from susceptibility induced by post-inoculation incubation at moderately elevated temperatures. Their results also suggest that the “heat shock” treatment is a very stringent measure of the TS of a resistance gene, but they did not test genes generally considered to be TS to see if they were TS by this method.

In general many resistance genes of plant resistance responses have been found to be TS. Other TS resistance genes not discussed in detail include: *Tm-1* gene in tomato for resistance to TMV (Fraser and Loughlin, 1982), potato mop-top virus (PMTV) induction of ringspots in tobacco carrying the *N* gene following heat shock (Jones, 1973), and temperature having an effect on the development of necrosis in potato carrying the *Nx* gene to potato virus X (PVX) (Adams *et al.*, 1986). Tobacco inoculated with PMTV and held at 22 °C does not normally develop the necrotic ringspots that develop when plants are held at 15 °C; however, when dipped into 50 °C water for 40 s the necrotic ringspots develop within 3 day of treatment (Jones, 1973). Also, potato inoculated with PVX - DX developed necrotic lesions within 8 days post-inoculation when plants were held at 15 °C to 20 °C, but lesions failed to develop or were very slow to develop when plants were held at 10 °C or 25 °C (Adams *et al.*, 1986).

VIRUS PATHOGENESIS AND TEMPERATURE:

Heat shock is a universal response in cells of all types exposed to high temperature. Conservation of heat shock proteins has been studied extensively and it has been confirmed by both protein and nucleic acid sequences. The function of most heat shock proteins is to repair the cell physiology by chaperoning proteins and enzymes damaged by high temperature out of the cell. Sequence similarities have been found between heat shock proteins and virus movement proteins, CPs, and HSPs encoded in some viruses. Using the DOTHELIX/GENBEE program Agranosky *et al.* (1991) found a statistical significance of about 10 deviations above the random expectation for the 65 kDa protein of beet yellows virus to heat shock protein 70 (HSP70). It has been suggested that virus evolution has involved the acquisition of cellular genes for macromolecular trafficking as the progenitor to viral movement proteins (Agranosky *et al.* 1991).

Viral movement protein relationship to HSPs: Some viral movement proteins and the 90 kDa

HSP have conserved sequences (Koonin *et al.*, 1991). Relationships were found between a diverse group of plant virus movement proteins to the family of ubiquitous cellular 90 kDa heat shock proteins (Koonin *et al.*, 1991). This diverse group included: Tobamoviruses, Caulimoviruses, Comoviruses, Nepoviruses, and Closteroviruses with 'LPL', 'G', 'D' conserved domains with eukaryotic HSP90. However, the group of plant viral movement proteins compared did not include the potyviral movement protein since it is not known. Thus its relationship to HSP 90 is not known. Constitutive HSPs appear to have important roles in protein transport and are thought to act as molecular chaperones in post-heat stress conditions (Agranovsky *et al.*, 1991).

Effects of low temperature on viral pathogenesis: Low temperature generally slows resistance development and viral movement but the effects on viral replication was not determined (Kassanis, 1952). In soybean a temperature shift from 20 °C at the time of inoculation with SMV G2 to 10 °C, 15 °C, 20 °C, 25 °C, 30 °C, or 35 °C for 10 days post inoculation, then returned to 20 °C found that the 10 °C broke the resistance of the *Rsv₁*, for which it is normally resistant (Mansky *et al.*, 1991). It was suggested that resistance may be repressed at low temperatures and that this may not be unique to the SMV-soybean system (Mansky *et al.*, 1991).

Effects of HS on viral replication: Induction of host heat shock response does not affect the ability of TMV to replicate in heat treated plants. The HS response is characterized by a rapid induction of HS gene transcription coupled with a precipitous decline in the transcription of most other genes (Gurley and Key, 1991). With a precipitous decline in transcription and production of cellular proteins, HS potentially could limit the infection potential of virus if it limits the replication and production of proteins and nucleic acids are that required by the virus for a successful infection. In a TMV infection in tobacco (cv. Xanthi) where the plants were shifted from 25 °C to 37 - 40 °C, the synthesis of most host proteins was immediately inhibited, whereas the synthesis of TMV proteins was not inhibited (Dawson and Boyd, 1987). They did not report changes in plant resistance response or virus localization for this study. Dawson and Boyd (1987) suggest that there are some structural or spacial differences between the two classes of mRNAs. In the TMV system the induction of HS does not limit the ability of the virus to replicate itself or to produce viral proteins required for viral replication.

VIRUS MOVEMENT IN PLANTS:

Virus movement in plants is an area that has been the subject of many studies and recent reviews (Carrington *et al.*, 1996 ; Deom *et al.*, 1992; Hull, 1989; Mushegian and Koonin, 1993; (Séron and Haenni, 1996). The following is a summary of several review articles with a few specific examples added. The movement of viruses in plants is different from the movement of viruses in animal systems and is also different than most other plant pathogens. Plants cells are fundamentally different from animal cells in that they have cell walls through which the virus needs to move, as well as the cell membrane, in order to get from cell to cell. Virus invasion of the plant requires the movement of the viral genome from infected cells into neighboring healthy cells (Citovsky *et al.*,

1992). The virus enters the plant cell through mechanical damage inflicted by a biological vector or by mechanical abrasion (Hull, 1989). At the time of inoculation only a few cells of the plant become infected. As the virus replicates itself in the initial cell and produces more copies of the virus, some are encapsidated by the coat protein and others may be associated with virus movement protein (Matthews, 1991). If, after entering and replicating, the progeny virus genomes are not capable of moving into adjacent healthy cells, a subliminal infection is said to occur, and the host appears to be resistant (Deom *et al.*, 1992). During a successful whole plant infection there are two phases of virus movement: (i) short-distance, slow, cell-to-cell movement to neighboring cells; and (ii) rapid, long distance movement via the vascular tissues of plants, mainly the phloem (Deom *et al.*, 1992; Hull, 1989).

Short-distance movement: The first phase of virus movement is from the initially infected cell to adjacent healthy cells. In animal systems viruses move cell-to-cell by endocytosis but plants have cell walls which prevent endocytosis. It is generally accepted that in plants, cell-to-cell movement of the virus is through cytoplasmic intercellular connections, the plasmodesmata (Mushegian and Koonin, 1993). The plasmodesmata are plasma membrane-lined cylindrical pores that traverse the cell wall (Fujiwara *et al.*, 1993). The function of the plasmodesmata is to link the cytoplasm of the cells to the symplast to provide for the movement of water, nutrients, and some cell products between the cells (Hopkins, 1995). It is this connection through the cell walls which is thought to allow for the movement of the virus between cells.

The entire virus particle is too large to move through the plasmodesmata. The primary plasmodesmata is formed during cytokinesis of mesophyll cells, is approximately 50 nm in diameter, and is lined with plasma membrane that connects the two adjoining cells (Carrington *et al.*, 1996). Between the plasma membrane lining of the plasmodesmata there is an appressed endoplasmic reticulum termed a desmotubule, which links the endomembrane systems of the neighboring cells (Fujiwara *et al.*, 1993; Carrington *et al.*, 1996). The membrane lining and the desmotubule limit the size of macroparticles which can diffuse passively through the plasmodesmata. The size exclusion limit (SEL) for the passive diffusion of macromolecules through the plasmodesmata is approximately 800 to 1000 Dalton (Da) (Ding *et al.*, 1992). Embedded in both the plasma membrane and the desmotubule are proteinaceous particles, and the spaces between the particles form a series of microchannels or a cytoplasmic sleeve of approximately 1.5-3.0 nm in diameter within the plasmodesmata (Fujiwara *et al.*, 1993; Deom *et al.*, 1992; Hull, 1989). It is through the microchannels of the plasmodesmata that the virus moves between the cells (Carrington *et al.*, 1996; Fujiwara *et al.*, 1993). Plant virus particles easily exceed the molecular weight SEL and the physical size restrictions of the plasmodesmata as the smallest diameter of flexuous rods is 10 nm and 17 nm for rigid plant viruses (Walkey, 1991).

The entire virus particle is not needed for infection. The virus particle cannot move cell-to-cell as animal viruses do by endocytosis, the fusion with cell membranes, because the cell wall precludes that. The size of the plasmodesmata restricts movement of the virus particle through the

plasmodesmata to neighboring cells. “Virus infection of plants requires movement of the viral genome from infected into neighboring healthy cells” (Citovsky, *et al.* 1992). The only method for cell-to-cell movement is by genome movement. For successful infection all that is needed is the nucleic acid genome for most viruses to initiate the infection process (Citovsky *et al.*, 1992). The function of a virus is to replicate itself so that it can continue to perpetrate. However, the SEL of the plasmodesmata is smaller than the size of the free viral nucleic acid genome with an average diameter of approximately 10 nm (Deom, *et al.*, 1992). Free viral genomic nucleic acid is considered to be restricted due to its size from movement through the plasmodesmata to adjoining cells. The virus or its genome cannot move cell-to-cell by passive transport through the plasmodesmata because of the SEL (Hull, 1989).

The modification of the plasmodesmata or active transport of the viral genome is required for movement between cells. Many viruses are known to encode proteins which are specifically required for movement and are known as movement proteins, because they impart the property of cell-to-cell movement to the viral genome. The function of the movement protein of TMV strain Ls1 was determined in an experiment using a temperature-sensitive (TS) mutant. The TS mutant replicated but could not move out of initially infected cells at the restrictive temperature of 32 °C to 33 °C (Deom *et al.*, 1987). Since the mutation in the TS-TMV mapped to a 30-kDa protein, this protein of TMV became known as a movement protein (Deom *et al.*, 1987). Mushegian and Koonin stated that “frequently, a plant virus protein is referred to as movement protein if (i) it is not a capsid protein; and (ii) disruption of the coding sequence of this protein abolishes infection in whole plants but has no effect on replication in protoplasts” (Mushegian and Koonin, 1993). An early function discovered of movement proteins is that during co-infections of a virus for which the plant is permissive with one that is not, the MP will transport the genome of the non-permissive virus, allowing the second virus which could previously not invade the plant to now spread throughout the plant.

Once the function of the protein was determined the mechanism of how the protein facilitates the movement of the viral genome through the plasmodesmata has been sought. The movement protein and viral genome complex has been observed as thin linear structures by electron microscopy, and purified TMV 30-kD movement protein has been shown to bind ssDNAs and ssRNA nonspecifically biochemically (Citovsky *et al.*, 1992). One study found that a fluorescein isothiocyanate (FITC) labeled 35-kD movement protein of red clover necrotic mosaic virus (RCNMV) spread from cell-to-cell in three minutes when the purified FITC labeled protein was microinjected into the cell (Fujiwara *et al.*, 1993). Also, a mutant MP was tested by the same method and was found to be unable to move from the initially injected cell (Fujiwara *et al.*, 1993). Based on the data obtained from their experiments on the cell-to-cell trafficking of the RCNMV MP's the researchers concluded that the MP moved via the plasmodesmata. Electron microscopy of thin sectioned TMV-infected leaves with immunogold labeling of the TMV 30-kD MP found that protein was localized to the inside of the plasmodesmatal channels (Hull, 1989). Another immunogold labeling experiment for ultra thin sectioned electron microscopy found the 33.6 kD NS_M movement

protein of tomato spotted wilt virus (TSWV) in close association with plasmodesmata (Kormelink *et al.*, 1994). Further studies in the function of plant viral MP's suggests that rather than just mediating viral movement through the plasmodesmata that the MP are involved in the active transport of the viral genome from cell-to-cell.

Two types of cell-to-cell movement have been identified, but they may not be the only methods for cell-to-cell movement. The first type is characterized by the 30kDa MP of TMV, which interacts with the plasmodesmata to facilitate the movement of a non-virion form of the virus from cell-to-cell and was described above. The second type of cell-to-cell movement is one in which icosahedral virus particles appear to pass along or through tubular structures through the plasmodesmata and it is characterized by cowpea mosaic virus (CPMV) and cauliflower mosaic virus (CaMV) (Hull, 1989). Complete virions have been observed in the plasmodesmata by electron microscopy and it suggests that the tubules, made from MP and possibly cellular constituents, have an internal diameter roughly the width of the virions (Carrington *et al.*, 1996). One especially important feature of this system is that the MP have the ability to induce the tubules in insect cells and Kastel *et al.* 1996 suggests "...that these MPs interact with fundamentally important cellular components that are conserved across the boundaries of the animal and plant kingdoms." Hull (1989) suggests that the mechanisms for cell-to-cell movement through the plasmodesmata are truly different and whether they are the only methods will require further experimentation.

SMV produces pinwheel structures in infected cells. The localization of the pinwheel structure is similar to the tubules of CPMV and CaMV. In ultrastructural studies of SMV infected soybean, the pinwheels were observed in mesophyll and paraveinal mesophyll cells (Hunst, 1981; Hunst and Tolin, 1983). The pinwheel's location in the infected cell is similar to the tubules in that they are in association with the plasmodesmata and the cytoplasmic strands containing SMV particles (Hunst and Tolin, 1983). The paraveinal mesophyll of soybean are cells spread out at the level of the phloem where their function has been proposed to be involved in interveinal conductance of photosynthates (Fisher, 1967). The virus was detected in the paraveinal cells at day 9 post-inoculation (p.i.) (Hunst and Tolin, 1983).

In potyviruses, however, less is known about their cell-to-cell movement, and the movement functions of genome-encoded proteins have yet to be identified. As described for TMV, the MP interacts with the plasmodesmata and the non-assembled viral genome. Based on mutations in the tobacco etch virus (TEV) coat protein which debilitate virus movement, movement defects appear to be independent of virus assembly (Dolja *et al.*, 1994). Potyviral CP is encoded by the C-terminus of the polyprotein (Dolja *et al.*, 1994). Mutations in the N-terminal domain of the TEV CP results in an altered CP that retained the ability to assemble the virion, but cell-to-cell movement was slowed and virus was unable to move from the inoculated leaves (Dolja *et al.*, 1994, 1995). These results strongly suggest a direct function for the coat protein in movement. Two possibilities are proposed for mechanisms of potyviral cell-to-cell movement: (i) virions are assembled and transported as complete virions between cells; or (ii) a non-virion ribonucleoprotein is transported, similar to TMV

and others (Dolja *et al.*, 1994). More study is required before the cell-to-cell movement is understood for potyviruses, since possibly other viral encoded proteins are involved (Dolja *et al.*, 1994). Despite the repeated observation of pinwheels associated with plasmodesmata, none of the recent reviews on virus movement have suggested a role for them in potyvirus cell-to-cell movement.

While it has now been generally established that plant viral MP's facilitate the movement of the viral genome through the plasmodesmata by increasing the SEL it has been proposed in recent review articles that in fact the MP's mediate active transport between cells. It has been suggested "...that the trafficking of macromolecules is a normal function of plasmodesmata, which was presumably usurped during convolution by plant viruses to facilitate cell-to-cell spread of the viral genetic material" (Fujiwara *et al.*, 1993). The modification of the protein channels in the plasmodesmata modify the gating properties of the plasmodesmata, but the fact that the MP's themselves move through the plasmodesmata suggest that they function as molecular chaperones for the viral genome. Studies have suggested that there is intracellular targeting of the replicated viral positive-stranded RNA genome to movement protein localized in the endomembrane surfaces, so that the replicated viral genome is bound to MP (Carrington *et al.*, 1996). The genome replication is targeted to endomembrane bound MP and the endomembrane systems of the plants are linked by the desmotubule through the plasmodesmata. The MP's bind to single-stranded copies of their own genome to form extended protein-nucleic acid structures that represent the transport complexes (Deom, *et al.*, 1992). The protein-nucleic acid transport complexes are targeted for intracellular production or transport to the endomembrane system. In this form, the transport complex of MP and viral genome is in position to move through the plasmodesmata.

The transport complex of the viral genome and the MP are able to take advantage of the existing cellular transport system of the cell. Based on the understanding of current research there are three general steps proposed for the passage of the MP-genome transport complex through the plasmodesmata: (i) binding of the MP-genome transport complex at, and internalization into, the channel, (ii) transport through the channel, and (iii) release into the adjacent cell (Carrington *et al.*, 1996). It has been proposed that the MP-genome transport complex is actively transported by the escort proteins, chaperones, and/or molecular motors of the plasmodesmatal trafficking apparatus. This hypothesis of active transport is based on research into cell-to-cell trafficking of macromolecules and all of the studies into the function of viral MP's (Lucas *et al.*, 1993).

Long-distance movement: Plant viruses move relatively short distances by moving from cell-to-cell, whereas long distance movement is rapid movement via the vascular tissues of plants, mainly the phloem. Less is known about the specific methods that viruses use for long-distance movement. Long-distance movement, also known as systemic movement, is really vascular movement because the virus is moving through the vascular tissue of the plant (Séron and Haenni, 1996). There are three steps to long-distance movement for the plant virus: (i) entry into the sieve elements through plasmodesmata of surrounding vascular parenchyma or companion cells and, (ii) translocation through the vascular systems by bulk flow to tissues that are sinks for photoassimilate, and (iii) exit

from the vascular system into uninfected host tissue (Séron and Haenni, 1996; and Carrington *et al.*, 1996). The specifics of the actual movement events has yet to be determined by researchers, but some of the properties of plant viruses that effect long-distance movement have been suggested.

Long-distance movement can be very rapid once the virus enters the vascular system. The limiting factor is the entry into the vascular system. Carrington *et al.* (1996) suggests that entry into the vascular system may involve a novel set of viral and host factors because of companion cell/sieve element plasmodesmata have different gating capacities than do mesophyll cells. The methods of entry into and exit from the vascular systems has been proposed to take place through the plasmodesmata on the sides of vascular bundles or through the ends of minor veins (Séron and Haenni, 1996). Modification of the secondary plasmodesmata by transgenically expressed TMV 30-kD MP in tobacco occurs between mesophyll cells, but it does not change the SEL of plasmodesmata linking bundle-sheath and phloem-parenchyma cells (Ding *et al.*, 1992). Microinjection experiments have established that low molecular weight fluorescent dyes can move from companion cells to the sieve element but that the upper limit of the SEL of plasmodesmata connection has not been established (Lucas and Wolf, 1993). Lucas and Wolf (1993) suggest that, based on the SEL for the plasmodesmata connecting the companion cells to the sieve elements, it is unlikely that proteins can move by simple diffusion and most likely interact with plasmodesmatal substructural components. The movement form of the virus maybe different for long-distance than for cell-to-cell movement. Entry into the vascular system from the mesophyll cells is through the plasmodesmata but some other factors are involved for entry into the vascular system.

The viral encoded MP's might have some role in vascular movement, but evidence suggests a strong role of the coat protein and entire virion. The specific functions of the viral encoded MP for long distance movement in the vascular system is difficult to distinguish from its obligatory role in cell-to-cell movement (Séron and Haenni, 1996). Studies on the behavior of coat protein-defective strains of TMV and of TMV RNA suggest that coat protein sequences might be essential at some stage of long-distance spread but not necessarily in the form of virus coat protein (Hull, 1989). The function of the coat protein (CP) in the modification of SEL at the bundle-sheath/phloem-parenchyma has been proposed because specific mutations in the TMV CP either disable or greatly impair vascular movement while not affecting cell-to-cell movement (Deom *et al.*, 1992). The requirements and mechanisms for cell-to-cell movement have been well described but more work is needed on the entry to the vascular system to understand the requirements for viral long-distance movement.

Long-distance movement of potyviruses is known to affected by the CP. The long-distance movement of TEV can be knocked out by a mutation in the N-terminal domain of the TEV CP (Dolja, *et al.*, 1994). Surface-oriented amino acids and carboxyl regions of the CP are not required for cell-to-cell movement but are required for long-distance movement (Dolja, *et al.*, 1994). Also, the central region of the helper-component-proteinase (HC-Pro) is also required for long-distance movement (Schaad and Carrington, 1996). Schaad and Carrington (1996) also report that mutations in the C- as well as the N-terminal region are long-distance movement defective and that the domains

contribute little to virion assembly or stability. There are several requirements of potyviruses for long-distance movement and more work needs to be completed before the specific requirements are determined.

Translocation and movement of the virus or infective elements through the vascular system has been well studied. While the mechanism of movement into and out of the vascular systems may not be known, long-distance movement has been long recognized. In a classic experiment with TMV in tomato, Samuel in 1934 used an infectivity assay to determine the systemic movement of the virus with time (Matthews, 1991). The virion or infective element is translocated passively over long-distances in the plant by the bulk flow of the photoassimilates from source to sink leaves in the phloem (Séron and Haenni, 1996). Once the virus enters the phloem, movement may be very rapid, with values of 1.5 cm/hr and 8 cm/hr reported for TMV in tobacco (Matthews, 1991). It is generally accepted that long-distance movement of the virus or infective element is transported via phloem for most plant viruses, with only a few viruses that are beetle-transmitted capable of being transported long-distance through the xylem (Hull, 1989).

The developmental conditions of the host plant has a strong impact on the long-distance movement of the virus. The virion or infective element is passively transported through the sieve elements downward to the roots and then upward to upper leaves (Atabekov & Dorokhov, 1984). The many studies of long-distance movement have found that the virus is transported to the roots and young developing leaves first and that the infection of mature leaves is slower. The evidence for movement from mature leaves to developing upper leaves strongly suggests that the infective element is transported with the photoassimilates from mature or source leaves to young or photoassimilate sink cells. The pressure-flow hypothesis for translocation in the symplast presents a model where the mass transfer of photoassimilate solute from the source cells to the sink cells along a hydrostatic pressure gradient and the virus is then translocated passively by the mass flow (Hopkins, 1995). Typically symptoms in mature plants are not typically as severe as infections in young plants. This can be explained because young plants have strong sinks in the developing leaves and the virus is transported to the young leaves by the mass flow. Infection of mature plant is slower as expected because mature leaves are source leaves and transport is out of them, rather than into them.

Exit from the vascular system is the final event required for effective long-distance movement and systemic infection of the plant. Again, little is known about the specific requirements for exit from the vascular system. The lack of protein synthesis in the vascular system implies that viral factors needed for translocation and exit must follow the virus or infective elements into the vascular system (Séron and Haenni, 1996). The function of the plasmodesmata as organelles for informational trafficking of macromolecules involved in the regulation of gene expression has been proposed (Lucas & Van Der Schoot, 1993). Receptors for macromolecules and potentially for viral movement complexes have yet to be identified but may contribute to host range specificity and may explain how the virus moves from the vascular system into uninfected areas of the plant (Lucas *et al.*, 1993; Carrington *et al.*, 1996). The method of viral exit from the vascular system is not yet known but

many researchers propose that host elements are involved, possibly an active transport function out of the vascular system.

Methods for tracking virus movement: Various methods have been used to track virus movement in plants. Immunological, biological, nucleic acid, and electron microscopy methods have been used to detect viral movement in plants. Matthews (1991) described the classic long-distance movement experiments of TMV in tomato done by Samuel (1931):

“He inoculated the terminal leaflet and then followed the spread of the virus with time by cutting up sets of plants into many pieces at various time. He incubated the pieces to allow any amounts of virus to increase and then tested for the presence of the virus by infectivity” (Matthews, 1991).

The classic work of Samuel on the movement of the virus used a biological assay for the detection of the virus. By applying immunological technology a rapid method can be applied for detection of viruses in plant tissue and can be used to track movement. A potential problem with using immunological methods for detection is that the antibodies are made to the viral antigen. The antigen is usually the coat protein of the virus and the virus may not always move as an intact virion. By using nucleic acid probes the nucleic acid of the virus would be detected.

By using a method developed by Mansky *et al.* (1990) and refined by Gera (1994) the rate and type of systemic or local movement can be determined by the localization of the virus in the inoculated leaf. Immunoprint detection of SMV in inoculated leaves allows for the interpretation of virus movement from the site of initial infection. Mansky *et al.* (1990) used a hydraulic press to blot leaves to nitrocellulose membrane (0.45-um, Micron Separations Inc.), whereas Gera (1994) used a hammer and #8 Ruled or #410 filter paper (Schleicher & Schuell).

HEAT SHOCK:

Heat shock proteins production is universal and highly conserved in all types of cells. The HS phenomenon has been extensively described for *Drosophila*, as well as for other insect, avian and mammalian cells (Barnett *et al.*, 1980). Soybean is the model system for heat shock study in plants. Heat shock is defined as the response to rapid high temperature increase usually 8-12 °C above normal. The response is very well conserved in all types of cells, bacteria, single celled eukaryotes, animal and plants. When heat shock occurs, the production of most cellular proteins is drastically reduced and the production of heat shock proteins increases rapidly (Nagao *et al.*, 1985).

Heat shock response: The heat shock (HS) response is very highly conserved and has been studied extensively in plant and animal cells. HS is an induced response after an 8-12 °C shift up from the normal growing temperature and is characterized by a rapid induction of HS gene transcription coupled with a precipitous decline in the transcription of most other genes (Gurley and Key, 1991).

The HS response is very rapid and heat shock genes are activated and start producing mRNA easily detectable on Northern blots within 3 to 10 min of exposure to HS temperatures (Key *et al.*, 1985). The function of heat shock proteins (HSP) has been strongly correlated with the development of thermal tolerance (Kimpel *et al.*, 1990). In plants there are the high molecular weight (HMW) HSPs which are highly conserved and at least three groups of low molecular weight (LMW) HSPs (Key *et al.*, 1985, DeRocher *et al.*, 1991). Plants differ from animal cells in that the low molecular weight (LMW) HSPs are diverse in size and amino acid sequence but the HMW HSPs are highly conserved across a broad spectrum of organisms (Nagao *et al.*, 1985).

The induction of HS is the plant's response to high temperature stress. The function of HS is generally considered to provide protection or thermotolerance for the organism to otherwise lethal temperatures (Lin *et al.*, 1984). The HS function for thermotolerance is very important for plants since they have only limited cooling capacities, through evapotranspiration, highly dependant on the availability of moisture in the soil. During the heat of the day when light and air temperatures can heat the plant tissues beyond its normal temperature, the HS function is to provide thermotolerance to prevent the death of plant tissues. In the field, mature soybeans produced mRNAs for LMW HSPs on days when the field temperatures reached above 38 °C; however the level was lower in irrigated fields than non-irrigated fields (Kimple and Key, 1985). Plants can cool themselves to below the ambient air temperatures by evapotranspiration but the maximum difference between irrigated and non-irrigated soybeans is approximately 5.5 °C (Kimple and Key, 1985). The ability for plants to cool themselves is often not enough to maintain tissues in the normal temperatures range so the HS system allows plants to acquire thermotolerance to survive the extreme temperatures.

Heat shock induction: Heat shock is induced by a shift up 8-12 °C in the normal temperature at which time HSP are induced. The induction of HSP gives the plant protection so that it can withstand high temperature which otherwise may be lethal. This is referred to as thermotolerance. The acquisition of thermotolerance can occur by several methods; a previous moderate HS, a gradual temperature increase, a severe HS with recovery period (Lin, *et al.*, 1984, DeRocher *et al.*, 1991). Thermotolerance requires time for the HS response to occur and for the synthesis and accumulation HSPs before exposure to the lethal temperature (Lin, *et al.*, 1984). Once thermotolerance has been acquired the plant can survive high temperatures which otherwise would be lethal.

LMW HSPs in plants are numerous and produced at high levels during heat stress. Plants have many LMW HSPs, in contrast to most other eukaryotes which only have a few (Vierling, 1991). The LMW HSPs are not expressed at detectable levels in leaves under normal growing temperatures but they are the most abundant proteins induced by heat stress (Vierling, 1991). In soybean LMW HSPs are sized from 15 to 27 kDa and 20,000 mRNA copies of each LMW HSPs can accumulate in 2 hours at 40 °C (Nagao *et al.*, 1985). The multiple LMW HSPs in soybean are members of a super family of LMW HSPs. There are four gene families of LMW HSPs in plants, two classes encoding cytoplasmic proteins, one class encoding chloroplast-localized protein and the final class encoding endomembrane proteins (Vierling, 1991). In soybean seedlings at least 27 different LMW

HSPs were detected by two-dimensional IEF/SDS-PAGE incubated for 3 hours at 40 °C (Mansfield and Key, 1987). The 15 to 18 kDa class of LMW HSPs reached a maximum level (1.54 mg/100 mg protein) by 4 h when incubated at 40 °C and remains at fairly constant level of production when incubated for 24 h as determined by immunological protein assay (Hsieh *et al.*, 1992). A brief HS exposure at 45 °C for 10 min followed by incubation at 28 °C accumulated 15 to 18 kDa LMW HSPs to the level of 0.76 to 0.98 ug/ 100 ug in 2 day-old hypocotyl tissue (Hsieh *et al.*, 1992).

Chapter II

Effects of Heat Shock on Soybean *Rsv₁* Resistance to Soybean Mosaic Virus

INTRODUCTION:

To further the understanding of interaction between soybean (*Glycine max* (L.) Merr.) and soybean mosaic virus (SMV), it is important to determine if *Rsv₁* and its alleles are temperature sensitive, since many resistance genes have been found to be temperature sensitive. Plant geneticists at Virginia Tech investigating the genetics of the resistance have often noticed variability in symptoms at different times of the year, and attributed this to high temperature influences. The sensitivity of resistance genes to high temperature has been reported for many viral resistance genes. Tu and Buzzell (1987) reported that higher temperature changed stem tip necrosis to mosaic in SMV G1 inoculated soybean. Examples include the tobacco *N* gene for resistance to tobacco mosaic virus (TMV) (Yarwood, 1958), the *I* (ts) gene in bean for resistance to bean common mosaic virus (BCMV), blackeye cowpea and cowpea aphid-borne mosaic virus (BICMV and CAMV) (Fisher & Kyle, 1994), and in soybean the *Rps* resistance gene to *Phytophthora sojae* (Gijzen *et. al.*, 1996).

Soybean is one of the model systems for the study of heat shock proteins (HSP) in plants. HSPs production is universal and highly conserved in all types of cells. Heat shock (HS) is induced after an 8-12 °C shift up from the normal growing temperature and is characterized by a rapid induction of HS gene transcription coupled with a precipitous decline in the transcription of most other genes (Gurley, 1991). Heat shock genes are activated and start producing mRNA easily detectable on Northern blots within 3 to 10 min of exposure to HS temperatures (Key *et al.*, 1985). Low molecular weight (LMW) HSPs are not expressed at detectable levels in leaves under normal growing temperatures but they are the most abundant proteins induced by heat stress (Vierling, 1991). *Rsv₁* is closely linked to a microsatellite marker for a low molecular weight HSP (Yu *et. al.*, 1994). In soybean LMW HSPs are sized from 15 to 27 kDa and 20,000 mRNA copies of each LMW HSPs can accumulate in 2 hr at 40 °C (Nagao *et al.*, 1985).

The resistant and necrotic reactions to SMV are conditioned by a single, dominant, nuclear-inherited gene (Chen *et. al.*, 1994). There are to be several alleles at the *Rsv₁* locus for SMV resistance, which interact differentially with different SMV strain/pathotypes, four of which are listed in Table 2-1 (Chen *et. al.*, 1994). There are seven SMV pathotype groups (G1-G7) classified on the basis of phenotypic response on differential soybean lines by Cho and Goodman (1979). The symptomology of one of the phenotypes is systemic mosaic, a permissive infection of the plant not having resistance to the pathotype, with no restriction of movement. Necrosis is a hypersensitive resistance response of the *Rsv₁* allele and brown lesions, surrounded by a yellow halo, appear on the inoculated leaves. Necrosis is not confined to an inoculated leaf, but instead the virus spreads to subsequent leaves and stems necrosis is induced, resulting in severely stunted plants that often

develop tip necrosis (Chen *et al.*, 1994). There are no symptoms apparent in resistance plants, and no virus can be recovered or detected.

The purpose of this study was to investigate the potential of high temperature to affect the response to SMV of soybean plants carrying alleles of the *Rsv₁* resistance gene. Extreme high temperature treatments were used to induce heat shock protein production. Observations were made to note changes in expected phenotype expressed by the *Rsv₁* allele to the SMV pathotype. The localization and movement of the virus was determined by immunological methods.

Table 2-1: Interaction of selected soybean cultivars (Chen *et al.*, 1994) containing *Rsv₁* and SMV pathotypes (Cho and Goodman, 1979) used for temperature sensitivity tests.

Cultivar	R gene	Reaction of cultivars to SMV pathotypes			
		G1	G4	G6	G7
Lee 68	<i>rsv</i>	S ^a	S	S	S
York	<i>Rsv₁-y</i>	R ^c	N ^b	S	S
Kwanggyo	<i>Rsv₁-k</i>	R	R	N	N
Ogden	<i>Rsv₁-t</i>	R	N	R	N
PI96983	<i>Rsv₁</i>	R	R	R	N

^aS = Susceptible - Characterized by systemic mosaic, a mild mosaic pattern on trifoliate leaves.

^bN = Necrotic - Local necrosis characterized by circular dark brown or reddish lesions, surrounded by a yellow halo of variable size on the inoculated leaf leading to systemic necrosis. Systemic necrosis is characterized by necrotic lesions developing on upper leaves and then often spreading to the stem tip and sometimes leading to the death of the plant.

^cR = Resistant - No symptoms, no virus recoverable.

MATERIALS AND METHODS:

Plant materials: Five cultivars of soybean were used, each carrying a different *Rsv₁* allele. These were Lee 68, York, Kwanggyo, Ogden and PI96983, carrying the *rsv*, *Rsv₁-y*, *Rsv₁-k*, *Rsv₁-t*, and *Rsv₁* alleles of the *Rsv₁* SMV-resistance gene, respectively. The plants were grown under greenhouse conditions in 10 cm plastic pots with a soil-less potting medium (Scotts Metro-Mix 360), six plants per pot. Seed were supplied by G. R. Buss.

Virus and inoculation methods: Four strain groups of SMV based on the virulence studies of Cho and Goodman (1979) were used in this study. SMV strain groups G1, G4, G6 and G7 were chosen to provide a range of resistance reactions on the *Rsv₁* alleles used in this study (Table 2-1) and

were the same as used in previous studies (Chen *et al.*, 1994). Virus inoculum was maintained in soybean in the greenhouse as previously described (Chen *et al.*, 1994; Jain *et al.*, 1992). Plants were inoculated mechanically with a 1:10 dilution of infected tissue in 0.01 M sodium phosphate buffer, pH 7.0, on fully expanded unifoliolate leaves lightly dusted with 600 mesh carborundum, and rinsed with tap water immediately. The plants were maintained in greenhouse conditions unless otherwise stated and observed daily for 21 days post inoculation (p.i.).

Detached leaves: The detached leaf assay employed the same alleles and cultivars and SMV pathotypes G4, G6, and G7. First, fully expanded trifoliolate leaves were removed from the plant by the petiole at the stem. The entire upper leaf surfaces were uniformly mechanically inoculated as described above. Two trifoliolates per SMV pathotype were then placed into a 10 cm Petri dish lined with a moist paper towel, and the dish was placed in a lighted incubator. For the 30 °C experiments the leaves were inoculated and then incubated for six days prior to immunoprinting. The experiment this was repeated four times. The low temperature detached leaf assay was conducted only twice and the leaves were maintained at 20 °C for eight days prior to immunoprinting. Observations for visible symptom development were made every second day.

Heat Treatment: Two methods were used to induce a heat shock (HS) response in the soybean plants. The first method was designed to induce HS throughout the entire plant by having the HS induction signal translocated from a single site of heat treatment. Treatments by dipping a single leaf in hot water had been used by Yarwood (1961) for experiments with TMV. The second method exposed the entire plant to temperatures which are known to induce heat shock (Kimple and Key, 1985; Key *et al.*, 1981) and was used to assure that HSP's were induced. Prior to all heat treatments, the plants were removed from the greenhouse or treated before the sun heated the greenhouse above ambient early morning temperatures. Control plants were maintained for comparison of symptom development.

In the single leaf HS induction treatment, two methods were used. The first method for single leaf treatment was accomplished by dipping a single unifoliolate leaf of each plant into a 50 °C water bath for 30 s. In the second method a single unifoliolate of each plant was placed for 45 s between two 5 cm² metal plates welded to Vice-Grips[®], previously heated to 50 °C in a water bath, to act as heat sinks. This method was designed to simulate the same induction of heat shock in a more convenient manner. The entire plant heat treatment was accomplished by placing pots into air heated to 50 °C for 5 min, in the dark. After all heat treatments the plants were returned to greenhouse conditions for 60 min prior to inoculation with the virus.

The unheated control, and whole plant hot air treatments were replicated 6 times, while the single leaf unifoliolate dip and heat sink were only replicated three times each. There were 6 plants per pot and one pot of plants per heat treatment and SMV phenotype combination. This yielded 45 pots and approximately (some seeds did not germinate or the plants were too small for inoculation) 270 plants per replication. This design always included unheated controls for each pathotype/allele

combination for direct comparison of symptom development because of possible environmental variations between each replication. Symptom observations were recorded when the majority of plants in the pot showed the same symptom.

Immunological detection of SMV: To detect the movement of the virus within the plant immunological detection methods were employed. Systemic movement, indicated by presence of viral antigen, was detected by blotting leaf tissue from the most recent fully expanded unifoliolates to nitrocellulose membrane (NitroPure, 0.45- μ m pore size; Micron Separation Inc., Westboro, MA). Movement of the virus within and out of the inoculated leaves was detected by blotting an entire inoculated unifoliolate leaf to #8 Ruled or #410 filter paper (Schleicher & Schuell, Keene, HN). This method is a modification of the press blotting method used by Mansky *et al.* (1990), as modified by Gera (1994). The viral antigen was detected by immunoassays using rabbit polyclonal antiserum against SMV-VA/G1, (Hunst and Tolin, 1982), alkaline phosphatase-labeled, Anti-Rabbit IgG (whole molecule) secondary antibody (Sigma Bio Sciences [A-3687], St. Louis, MO), and NBT/BCIP (nitro blue tetrazolium salt/5-bromo 4-chloro-3 indolyl phosphate) (Zymed Laboratories, Inc., San Francisco, CA) as substrate.

For the immunoblot detection of the vascular and extensive cell-to-cell movement SMV tissue blots were made at approximately day 14 p.i. A leaflet from the most recently fully expanded unifoliolate leaf was removed rolled up, torn and blotted by pressing edges to the nitrocellulose membrane. A plastic template containing 96 holes with the spacing of an ELISA microtiter plate was used to maintain equal spacing between the blots. The blots were allowed to dry and then developed as described below. Once developed the blots were rated relative to the positive and negative control for the intensity of the uniform dark blue color.

For the immunoprints, entire leaves (usually an inoculated leaf) with a 1 - 2 cm attached segment of leaf petiole were placed with the upper leaf surface facing up on a sheet of #8 ruled filter paper (#410 unruled was also used, but yielded a higher background) and sandwiched between sheets of copy paper. Then the sandwich was placed on to a flat hard surface (a level section of floor) and covered with clear acrylic sheet (5-7 mm thick). The leaves were pounded with light blows until a uniform green imprint could be seen on the single layer of white paper. The filter paper was carefully removed from the sandwich, the leaf skeleton removed and then the filter paper was allowed to dry. The filter paper was labeled with a sharp graphite pencil to record the information about the sample.

The following method was used to develop the immunoblots and immunoprints. The virus was detected from the clear imprint of the soybean leaf by a following the method of Lin *et al.* (1990) modified by Gera (1994). The filter paper (or nitrocellulose) was placed into 5% Triton X-100 (Sigma Bio Sciences). It was gently agitated for 10 - 20 min to decolorize the blots and leaf prints. The filter paper was rinsed in 1X KPS-Tween buffer (1X KPS: 0.10 M potassium phosphate, 0.75 M sodium chloride, pH 7.4) 1X KPS-Tween: 1X KPS + 0.05% Tween-20) for at least 3 min. The filter paper was placed into a blocking solution (1X KPS with 5% milk [Carnation® non-fat dry milk]

and 0.5% bovine serum albumin [BSA - Sigma Bio Sciences]) for 30 min with occasional shuffling of sheets to provide good exposure to all surfaces for more effective blocking. The filter paper was transferred directly to primary antibody against SMV diluted in 1X KPS to 1:15,000 for 60 min, again the sheets were repositioned to provide even exposure to the antibody. Following the primary antibody the filter paper was transferred to KPS-Tween buffer for three changes of rinse buffer of 15, 7, and 7 min each. The filter paper was next transferred to the secondary antibody (goat anti-rabbit whole fragment conjugated to alkaline phosphatase [Sigma Bio Sciences]) in a 1:10,000 to 1:25,000 dilution for 60 min. The filter paper was removed from the secondary antibody and rinsed three times as described above. The substrate was prepared by adding 10 ml of NBT to 80 ml of distilled water, swirled to mix, and then 10 ml of BCIP was added according to manufactures instructions. The filter paper was transferred to the substrate and a purple/blue color developed in about 5 min, but was left in the substrate for approximately 12 min to get a darker color. The filter paper was placed in distilled water for 10 min and then air dried and stored in the dark.

Determination of HSP increase: ELISA methods were employed to determine if HSP levels had increased by the conditions used for heat treatments in this study. The antibody was to the 15-18 kDa HSP of soybean (Hsieh *et al.*, 1992), and was obtained from Dr. Chu-Yung Lin, Taipei, Taiwan. It had been prepared from two major spots cut from the gel of LMW HSPs separated using two-dimensional IEF/SDS-PAGE, visualized by Coomassie blue staining, and injected into New Zealand White rabbit (Heish *et al.*, 1992). Using reported conditions 2-day-old soybean (cv. York) hypocotyls with the cotyledons removed were treated for 4 h at 40 °C and 25 °C, to serve as control to determine if this antibody could be used to detect an increase in HSPs by ELISA.

Test samples for plate trapped antigen ELISA to test for increase in LMW HSPs were prepared as follows. Two and a half grams of 2-day-old hypocotyls were incubated in 5 ml of incubation buffer (1% sucrose, 1 mM potassium phosphate, pH 6.0) for 4 h at 40 °C to induce HSPs and at 25 °C as a control. The tissue was homogenized using a Tekmar Tissumizer, equipped with a 080EN (8mm) probe, in 25 ml of HSP grinding buffer (500 mM sucrose, 200 mM Tris-HCL, 30 mM magnesium chloride, 100 mM potassium phosphate, 1mM dithiothriitol [DTT], 1mM phenylmethylsulfonyl fluoride [PMSF], pH 8.8) and filtered through a layer of Miracloth. The filtered homogenate was centrifuged for 15 min at 12,800 x g and the supernatant used for ammonium sulfate fractionation. A saturated solution of ammonium sulfate (AS) was added to the homogenate to 60% concentration by the following formula; where S_1 and S_2 are the initial and final concentration, and V_1 and V_2 are the volume of homogenate and volume of saturated AS to be added to the extract. $V_2 = V_1 (S_1 - S_2) / (S_2 - S_1)$ (Cooper, 1977). For example, to get a final solution of 60% AS in a 5 ml solution with 0% AS, 5.08 ml of saturated AS would be added. The solution was stirred for 1 h at 4 °C, then centrifuged for 30 min at 1200 x g and the supernatant saved. Saturated AS was added to the supernatant to a concentration of 100% as determined by the formula above and stirred at 4 °C overnight. The remaining precipitated proteins were collected by centrifugation for 10 min at 12,800 x g and resuspended overnight in HSP resuspension buffer (50 mM Tris-HCL, pH 8.8, 1 mM EDTA, 0.1% 2-mercaptoethanol).

To determine the induction of HSP in leaf tissue, plants were heat treated as described above and an unheated control was also maintained. At 1 and 4 h post heat treatment 1 g of leaves was harvested from all plants. The plants used for the determination had fully expanded primary unifoliolate leaves, however for the water dip and heat sink treatments only a single unifoliolate leaf was treated per plant and the opposite unifoliolate leaf harvested to determine if the HS response was translocated throughout the entire plant. The leaves were homogenized as above in 10 ml of HSP grinding buffer, AS fractionated, and clarified and fractionated as described above, then resuspended in 1 ml of HSP resuspension buffer. The fractionated protein at 1 g of leaf ml⁻¹ was used in the ELISA to determine in HSP levels.

Enzyme linked immunosorbant assay of HSP's: Once the samples were prepared by procedure described above, 200 µl were placed into a well according to a plan that allowed for multiple replications and for combinations of tested and control samples and the antibodies controls. The filled plate was covered and incubated for 1 hr at 37 °C. The samples were shaken out, and the plate was rinsed initially by filling wells with a fine stream of tap water. The plate was then shaken out immediately and blotted on paper towel. To rinse plate thoroughly, the wells were filled with rinse buffer (0.138 M sodium chloride, 1.5 mM potassium phosphate [monobasic and dibasic], 0.015 M sodium phosphate [monobasic and dibasic], 2.7 mM potassium chloride, pH 7.4, 0.05% Tween-20) from a rinse bottle. The plate was allowed to stand filled for 3 min between each rinse cycle for 3 rinse cycles. The contents of the plate were shaken out and blotted between each cycle.

The primary LMW HSP antibody was diluted 1:500 in PBS (0.138 M sodium chloride, 1.5 mM potassium phosphate [monobasic and dibasic], 0.015 M sodium phosphate [monobasic and dibasic], 2.7 mM potassium chloride, pH 7.4) and 100 µl placed in each well. The plate was covered and incubated at 4 °C overnight. After incubation the plate was rinsed and blotted 3 times as before. Following plate rinsing, 100 µl of the secondary antibody was placed in each well according to the plate plan in a 1:15,000 dilution of whole fragment GAR enzyme-conjugate. The plate was covered and the secondary antibody incubated at 37 °C for 4-6 hrs. After the incubation with secondary antibody enzyme-conjugate the plate was rinsed and blotted as before 3 times and then 200 µl of the substrate was added (FAST p-nitrophenyl phosphate tablet sets [N-2770] Sigma Bio Sciences). The optical density (O.D.) was read at 405 nm on an ELISA plate reader (Anthos Labtec Instruments Reader 2001) at 30 and 60 min following the addition of the substrate.

RESULTS:

Effect of heat treatment on symptom development: The effect of short duration pre-treatment at an excessively high temperature on the development of symptoms induced by SMV inoculation is summarized in Table 2-2. The times and temperatures reported for the three methods of heat treatment were the maximum exposure that did not kill leaf tissue immediately. Leaves treated by dipping in 50°C water for 30 sec, or by holding between two metal plates at 50 °C for 45 sec showed areas of collapsed cells by day 2 post-treatment, and abscised by day 5. Whole plants heated at 50°C

for 5 min showed reversible wilting but no tissue collapse. Heating leaves or plants at longer times or at higher temperatures resulted in immediate irreversible tissue collapse and/or plant death.

Table 2-2: Rate of symptom development in five cultivars of soybeans treated to induce heat shock proteins and then inoculated at the unifoliolate stage with three soybean mosaic virus (SMV) pathotypes inducing three symptom types.

Cultivar/SMV Pathotype	Days from inoculation to symptom appearances			
	NONE	HOT AIR	HOT WATER	HEAT SINK
Systemic Mosaic^a				
Lee 68/G1	7	7	7	7
Lee 68/G6	7	6	6	6
York/G6	8	7	8	7
Lee 68/G7	7	7	7	7
York/G7	8	7	7	7
Local Necrosis^a				
Kwanggyo/G6	6	7	7	6
Ogden/G7	11	11	11	11
Kwanggyo/G7	7	8	7	8
PI96983/G7	7	6	7	6
Systemic Necrosis^a				
Kwanggyo/G6	16	16	16	16
Ogden/G7	17	17	17	16
Kwanggyo/G7	15	15	15	15
PI96983/G7	14	14	14	14
Replications^d	6	6	3	3

^aSymptoms as described in Table 2-1.

^dThe number of replicates performed for each type of heat treatment, where each replicate represents one pot containing 6 plants.

No change in the type of symptom or in the rate of symptom development was observed for the average day the first symptoms were visible. There was no change in the type of symptom observed between the control non-heat treated plants and the various heat treated plants. Plants that developed a systemic mosaic to SMV pathotypes continued to develop the same symptoms. The local and systemic necrotic response of all of the heat treated plants of the *Rsv₁-k* allele (cultivar Kwanggyo) inoculated with SMV pathotype G6 was the same as the unheated control. The local and systemic necrotic response of all of the heat treated plants of the *Rsv₁-t*, *Rsv₁-k* and *Rsv₁* alleles

(cultivars Ogden, Kwanggyo, and PI96983, respectively) inoculated with SMV pathotype G7 was the same as the unheated controls. The observable resistance response of all of the alleles to SMV pathotypes G1, G6, and G7 was unchanged by the heat treatments. Plants which carry an *Rsv₁* resistance allele and are resistant to a particular SMV pathotype were observed to show no symptoms of viral infection and thus were not included.

Evidence for heat shock protein activation: The short duration, extreme heat treatments used in this study increased the relative level of HSP in soybean leaves. Table 2-3 presents the results of ELISA to measure the O.D. at 405 nm in fractionated samples adjusted to represent 1 g tissue ml⁻¹. Figure 2-1 presents the data in histogram form. The results with hypocotyl tissue heat treated by conditions reported to increase HSPs showed a greater than 50% increase in the absorbance at 405 nm over the room temperature control. This demonstrates for the first time an ELISA method for quantifying an increase of LMW HSP's. The antiserum had been previously reported as used in Western blotting methods (Hsieh *et al.*, 1992). At 1 hr post treatment of leaves, HSP level increased for the water bath, heat sink and hot air heat treatments relative to the untreated plant. This sampling time corresponded to the time the leaves were inoculated with virus, and demonstrated HSP levels were elevated during the initial stages of virus infection. At 4 hrs, only the hot water leaf treatment had an ELISA value greater than the untreated control.

Table 2-3. Activation of heat shock protein (HSP) production in leaves and hypocotyls of soybean and detection by plate-trapped antigen ELISA.

Heat Treatment	Optical Density at 405 nm			
	1 h ^a		4 h ^a	
	MEAN ^b	STD	MEAN ^b	STD
Untreated	0.250	0.001	0.251	0.105
Water Bath	0.313	0.029	0.348	0.004
Heat Sink	0.520	0.020	0.269	0.000
Hot Air	0.366	0.153	0.242	0.109
Hypocotyl 25 °C ^c	-	-	0.499	0.053
Hypocotyl 40 °C ^c	-	-	1.209	0.108

^a Time following heat treatment leaves were harvested for protein fractionation.

^b Mean of replicate wells.

^c Hypocotyl incubated in incubation solution at temperature for 4 h to test the effectiveness of LMW HSP antibody for use in ELISA.

HSP Activation

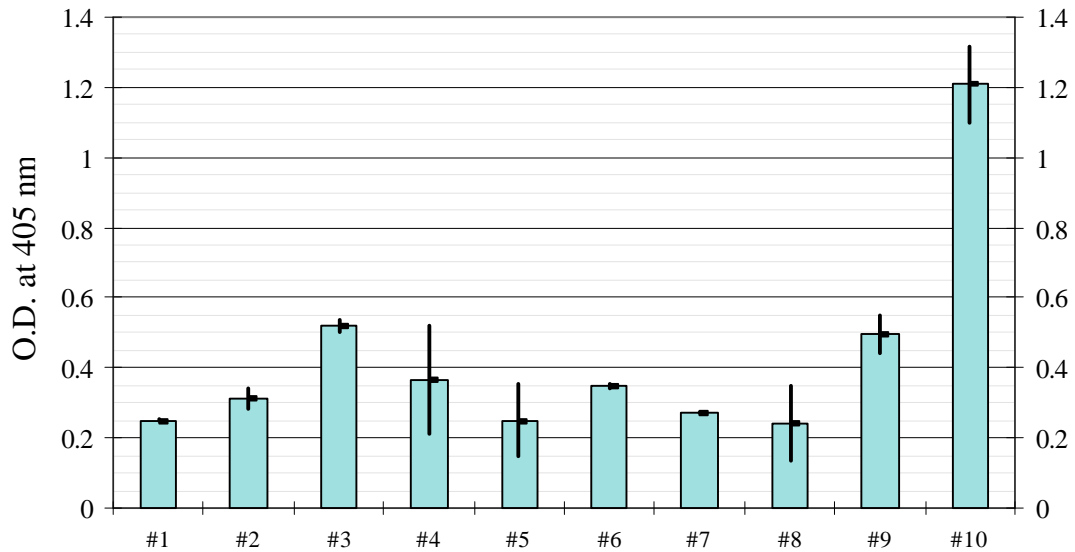


Figure 2-1. Activation of heat shock protein (HSP) production in leaves and hypocotyls of soybean and detection by plate-trapped antigen ELISA. (1) untreated soybean at 1 h. (2) Unifoliolate leaf dip in water bath, trifoliolate leaves harvested at 1 h. (3) Unifoliolate heat sink treatment, trifoliolate leaves harvested at 1 h. (4) Whole plant hot air treatment at 1 h. (5) untreated soybean at 4 h. (6) Unifoliolate leaf dip in water bath, trifoliolate leaves harvested at 4 h. (7) Unifoliolate heat sink treatment, trifoliolate leaves harvested at 4 h. (8) Whole plant hot air treatment at 4 h. (9) Hypocotyl incubated in incubation buffer for 4 hrs at 25 °C. (10) Hypocotyl incubated in incubation buffer for 4 hrs at 40 °C.

Comparative SMV distribution in inoculated leaves: Immunoprint detection of SMV in inoculated leaves permitted the analysis of virus replication at the site of initial infection, and an estimation of the rate and extent of cell-to cell and vascular movement by revealing localization of viral antigen. Results of one experiment are summarized in Table 2-4. Time course immunoprints showed that viral antigen, presumably in sites of infection, could be detected by as little as 48 to 72 h.p.i. in susceptible and some necrotic responses. In susceptible responses, the virus was able to spread over time into most of the leaf tissue of the inoculated leaf with no restriction, even though no symptoms were visible. The immunoprint assay consistently detected viral antigen in leaves showing necrotic lesions, but over time antigen concentrated in and near veins but was restricted from much of the leaf blade and . No antigen was detected in leaves of resistant cultivars, which also showed no symptoms. The rate of increase in viral antigen was accelerated at 30C, but the final response was unchanged (Table 2-4).

Table 2-4: Effect of temperature on localization of three pathotypes of soybean mosaic virus (SMV) in detached trifoliolate leaflets of five soybean cultivars inoculated and held at either 20C or 30C, compared to expected response. Leaf prints for immunological detection of viral antigen were made at 6 days post-inoculation (d.p.i.) for the high temperature and at 8 d.p.i. for the low temperature.

	Lee	York	Kwanggyo	Ogden	PI96983
SMV Pathotype	<i>rsv</i>	<i>Rsv₁-y</i>	<i>Rsv₁-k</i>	<i>Rsv₁-t</i>	<i>Rsv₁</i>
G4 @ 30° C	E ^a / S ^e	L ^b / N ^d	- ^d	L / N	-
G4 @ 20° C	E / S	L / N	-	L / N	-
G6 @ 30° C	-	-	L / N	0 ^c / R ^d	0 / R
G6 @ 20° C	-	-	L / N	0 / R	0 / R
G7 @ 30° C	-	-	L / N	L / N	L / N
G7 @ 20° C	-	-	L / N	L / N	L / N

^aE = extensive: virus detected in all veins and inter-veinal tissue.

^b0 = no virus detected in inoculated leaf.

^cL = localized: virus restricted to cells in and surrounding foci of infection and to leaf veins cells near infection foci.

^dS, R, N = expected response of susceptible, resistant, or necrotic phenotype as in Table 2-1.

Figure 2-2 shows the immunoprint results for mock, resistant, necrotic and susceptible reactions at day 9 p.i. No virus is detected in the mock or resistant plants in the inoculated leaves. Necrotic reactions are characterized by the restriction of the virus to areas near the presumed site of entry. A permissive infection is characterized by the lack of localization of the viral antigen at the site of entry, and extensive cell-to-cell and movement out of the leaf via the vascular system. In necrotic infections the viral antigen showed limited expansion into interveinal leaf tissue but it moved out of

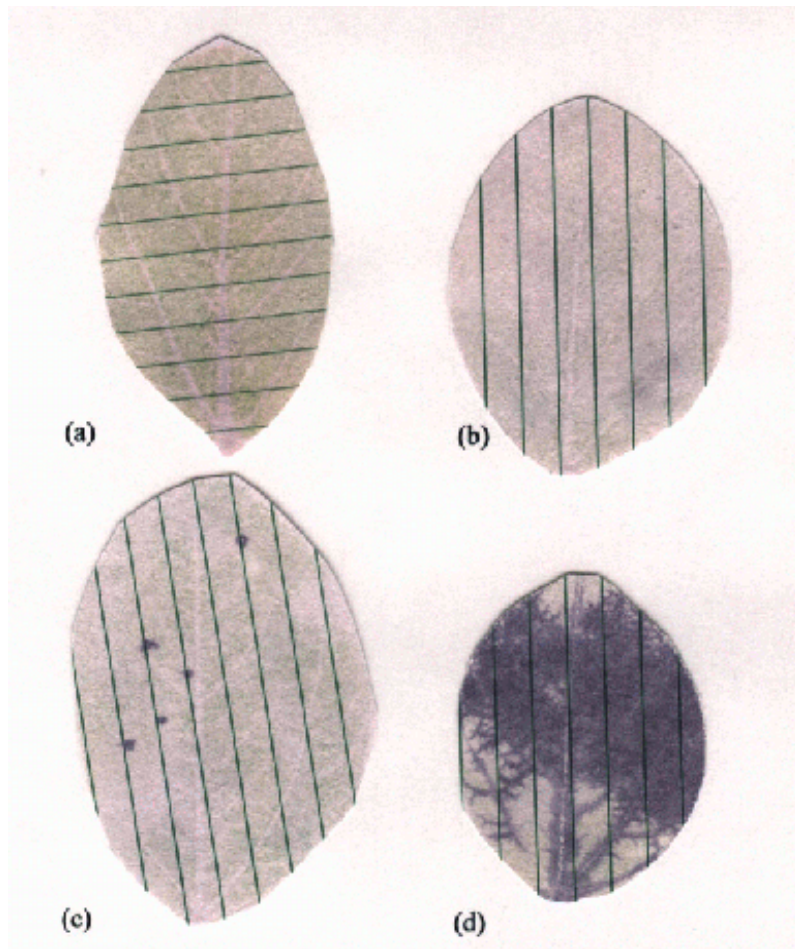


Figure 2-2. Comparison of localization of viral antigen with response of the soybean cultivar York to three soybean mosaic virus (SMV) pathotypes. Inoculated unifoliolate leaves were printed on day 7 post inoculation and reacted with antibody to SMV. **A:** Buffer (mock). **B:** SMV G1, resistant. **C:** SMV G4, necrotic **D:**SMV G7, susceptible. The dark blue color is the precipitate formed by the NBT/BCIP substrate and represents the presence of SMV antigen in the leaf tissue. The parallel green lines are artifacts of the filter paper used for blotting.

the inoculated leaf via the vascular system.

The immunoprints of control and heat treated plants were analyzed to determine if there was any change in the location of the virus in the inoculated leaves. SMV pathotype/*Rsv₁* combinations that give a necrotic response remained necrotic in heat treated plants. Also, tissue blot testing of SMV pathotype/*Rsv₁* combinations that give a susceptible response showed heat treated plants remained susceptible. Figure 2-2 shows several leaf immunoprints demonstrating that there was no change in the localization of the virus in the inoculated leaf of plants heat shocked or not heated prior to inoculation.

Comparative vascular movement of SMV: The presence of SMV antigen in the most recently fully expanded unifoliolate leaves was tested by tissue blotting. At day 10 to 17 p.i. tissue blots of the most recent fully expanded leaves were made. Results of a typical experiment in which leaves were sampled at day 14 p.i. are shown in Table 2-5. The developed tissue blots were rated relative to the negative and positive controls. In all susceptible plants, a uniform dark blue color was present, indicating a high concentration of viral antigen in all cells pressed to the membrane in the area of the template where the leaf tissue was blotted. In all resistant and mock inoculated plants, any blue color evident on the spot of the tissue blot was no greater than the background negative control, which had no viral antigen present and a negative rating. A uniform faint blue color or dark blue color over only a portion of the spot where the leaf tissue was blotted was interpreted to indicate either a low concentration of antigen or an irregular distribution of viral antigen in the cells pressed to the membrane. Spots giving a faint uniform blue or partial dark blue color always correlated with necrotic reactions. Thus, the virus antigen could not be reproducibly detected by tissue blotting in plants exhibiting systemic necrotic reactions due to restricted virus replication or localization in vascular tissue. This is attributed to the fact that a random tear across the leaf did not always contain cells with virus. However, since a partial reaction was never obtained with a susceptible or resistant response, it was thus an excellent indicator of a necrotic response.

Determination of the presence of SMV in upper leaves of plants with the five cultivar/pathotype combinations giving a susceptible reaction was consistently strong (Table 2-5). The whole plant hot air, leaf dip into hot water, or the heat sink treatments had no effect on the systemic movement of SMV pathotypes in plants which were susceptible to the pathotype inoculated. The heat treatments also had no effect on the systemic movement of the virus in plants of the cultivars that carried a resistance gene to the pathotype G1 or G6 inoculated, confirming that the gene conditioning resistance to that SMV pathotype was not temperature sensitive. Additionally, viral antigen was not detected in the upper leaves of any resistant plants with or without prior heat treatment. In upper leaves of plants of the four cultivar/pathotype combinations giving a necrotic reaction, detection of the viral antigen by the tissue blot method was difficult because of the restricted localization of the necrotic response. However, systemic movement of the virus was easily deduced by the symptoms produced by the necrotic reaction and

appeared to be unaffected by heat treatment.

Response at two temperatures of detached leaves to SMV: Detached trifoliolate leaves survived and maintained turgidity and green color for 18-21 days at 20 °C and for 10-14 days at

Table 2-5. Effect of heat shock induced by three heat treatment methods on the vascular movement and antigen distribution, compared to expected response, for three pathotypes of soybean mosaic virus (SMV) inoculated to five soybean cultivars at the unifoliolate stage within 1 hr of heat treatment. Torn edges of rolled trifoliolate leaflets were pressed to nitrocellulose membranes at 14 days post inoculation, and developed for immunological detection of viral antigen.

SMV pathotype - heat treatment	Lee <i>rsv</i>	York <i>Rsv_J-y</i>	Kwanggyo <i>Rsv_J-k</i>	Ogden <i>Rsv_J-t</i>	PI96983 <i>Rsv_J</i>
G1 - Untreated	+ ^a / S ^d	0 ^b / R ^d	0 / R	0 / R	0 / R
G1 - Hot Air	+ / S	0 / R	0 / R	0 / R	0 / R
G1 - Leaf Dip	+ / S	0 / R	0 / R	0 / R	0 / R
G1 - Heat Sink	+ / S	0 / R	0 / R	0 / R	0 / R
G6 - Untreated	+ / S	+ / S	~ ^c / N ^d	0 / R	0 / R
G6 - Hot Air	+ / S	+ / S	~ / N	0 / R	0 / R
G6 - Leaf Dip	+ / S	+ / S	~ / N	0 / R	0 / R
G6 - Heat Sink	+ / S	+ / S	~ / N	0 / R	0 / R
G7 - Untreated	+ / S	+ / S	~ / N	~ / N	~ / N
G7 - Hot Air	+ / S	+ / S	~ / N	~ / N	~ / N
G7 - Leaf Dip	+ / S	+ / S	~ / N	~ / N	~ / N
G7 - Heat Sink	+ / S	+ / S	~ / N	~ / N	~ / N

^a + = uniform dark blue color indicating high concentration of viral antigen in all cells pressed to the membrane.

^b 0 = no blue color, indicating no viral antigen present.

^c ~ = uniform faint blue color, or dark blue color over only a portion of the spot, indicating a low concentration or an irregular distribution of viral antigen in cells pressed to the membrane.

^d S, R, N = expected response of susceptible, resistant, or necrotic phenotype.



Figure 2-3. Comparisons of effects of heat shock on localization of viral antigen in three soybean cultivars inoculated at the unifoliolate leaf stage with soybean mosaic virus (SMV) pathotype G7 within 1 hr after treatment. Inoculated leaves were printed on day 9 post inoculation and reacted with antibody to SMV. **A, B, C, and D:** plants treated at 50 °C for 5 min. **E, F, G, and H:** plants held at 25 °C for 5 min. **A, E:** Ogden, necrotic; **B, F:** Kwanggyo, necrotic. **C, G:** York, susceptible; **D, H:** Lee 68, susceptible.

30 °C. Virus replication, movement, and symptom development progressed similarly to that in inoculated unifoliolate leaves on plants, and was as expected for the specific SMV pathotype/*Rsv₁* combinations. No symptoms were observed on the susceptible combination, G4 on *rsv*-Lee (Fig. 2-4, **D**), which looked the same as did resistant combinations of G6 on *Rsv*-PI96983 and *Rsv₁*-*t*-Ogden (Figs. 2-4, **B**, **E**). In necrotic responses, some local lesions were observed by day 8 p.i at 20 °C and by day 6 p.i. at 30 °C, as seen in Fig. 2-4. All of the necrotic combinations, namely G4 on *RsvI*-*t*-Ogden and *RsvI*-*y*-York, G6 on *Rsv₁*-*k*-Kwanggyo, and G7 on Ogden, Kwanggyo and PI96983, developed necrotic lesions of varying intensity and number but consistent with the expected response (Table 2.1).

The presence of viral antigen in the detached leaves was determined by leaf immunoprinting at 8 or 6 d.p.i. for leaves incubated at the low and high temperature, respectively. Incubation at the higher temperature accelerated the rate of development of both symptoms (results not shown) and extent of spread of viral antigen from an initial infection site. Results of nine *Rsv₁*/SMV pathotype interactions are summarized in Table 2-6 and shown in Figs. 2-5 and 2-6 as developed immunoprints of leaves held at 30 °C and at 20 °C, respectively. Necrotic and susceptible reactions were distinguished by more extensive spread of viral antigen in susceptible leaves. Extensive invasion of all leaf tissue and veins was observed in Lee/G4 cultivars/pathotype at 30°C (Fig. 2-5, **A**), but spread was much more limited at 20°C (Fig. 2-6, **A**). The necrotic response was induced in plants carrying any *Rsv₁* allele in leaves incubated at 20 °C as well as at 30°C. Further, the extent of localization of viral antigen in necrotic responses could be distinguished into two classes that corresponded to the cultivar and pathotype, rather than incubation temperature. In the following combinations, antigen was localized to infection foci and, to a limited extent, major veins: **B**: York/G4, **C**: Ogden/G4, **H**: Ogden/G7. Cultivars/pathotypes showing antigen localized to infection foci and spreading extensively along major and secondary veins included Kwanggyo/G6, Kwanggyo/G7, and PI96983/G7 (Fig 2-5 and 2-6, **D**, **F**, and **G**). No viral antigen was detected at either temperature in leaves with *Rsv₁*-*t* or *Rsv₁* (print not shown) resistance to the SMV pathotype G6 (Fig 2-5 and 2-6, **E**). Thus localization of the virus in the inoculated leaves was unchanged at two incubation temperatures.

DISCUSSION:

This series of experiments tested the potential change in the resistance response of alleles of the soybean *Rsv₁* gene for resistance to SMV following exposure to conditions inducing heat shock. No change was found in the phenotype of the resistance response of the selected *Rsv₁* alleles to SMV pathotypes under the conditions tested, in which the amount of heat shock proteins in heat treated plants was elevated at the time of inoculation. The phenotype of the resistance response was either a resistant plant where no infection occurred or a necrotic response where the virus is localized and the plant responded in a hypersensitive manner to the infection. A susceptible response occurred when the *Rsv₁* allele is not present, as with Lee 68 *rsv*, or when the allele can not confer resistance to the particular SMV pathotype. Plants that were resistant to

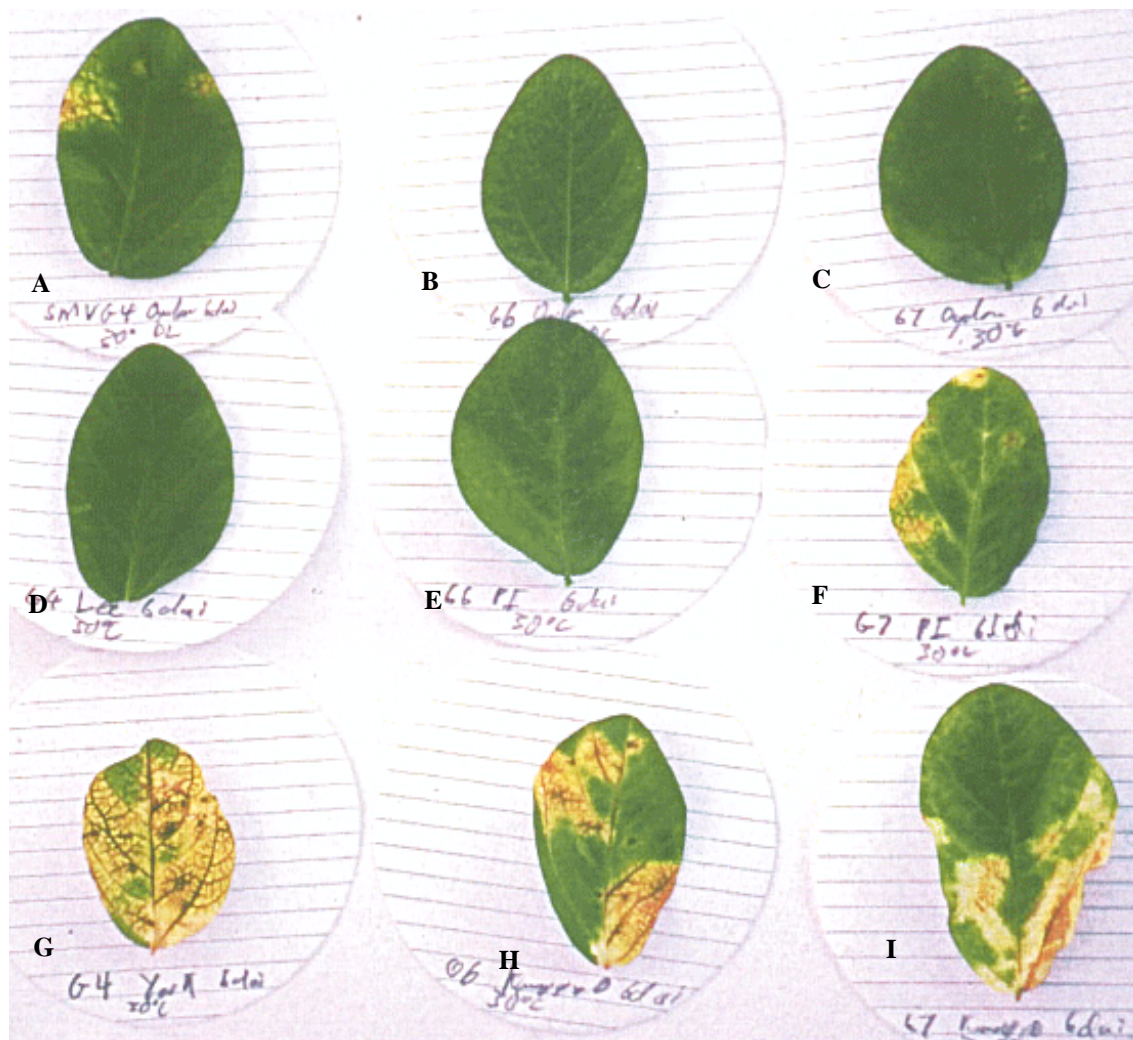


Fig. 2-4: Symptom development on detached trifoliolate leaves of five soybean cultivars at 6 days post inoculation with one of three pathotypes of soybean mosaic virus (SMV) and incubated at 30 °C. **A, D, and G:** SMV G4. **B, E and H:** SMV G6. **C, F and I:** SMV G7. **A, B, and C:** Ogden. **D:** Lee 68. **E, F:** PI96983. **G:** York. **H, I:** Kwanggyo.

Table 2-6: Effect of heat shock induced by hot air on extent of invasion of three pathotypes of soybean mosaic virus (SMV) in five soybean cultivars inoculated at the unifoliolate leaf stage, compared to expected response. Leaf prints for immunological detection of viral antigen were made of inoculated leaves at 9 days post-inoculation (d.p.i.).

SMV pathotype - treatment	Lee	York	Kwanggyo	Ogden	PI96983
	<i>rsv</i>	<i>Rsv_i-y</i>	<i>Rsv_i-k</i>	<i>Rsv_i-t</i>	<i>Rsv_i</i>
G1 - Untreated	E ^a / S ^d	0 ^b / R ^d	0 / R	0 / R	0 / R
G1 - Heat Treated	E / S	0 / R	0 / R	0 / R	0 / R
G6 - Untreated	E / S	E / S	L ^c / N ^d	0 / R	0 / R
G6 - Heat Treated	E / S	E / S	L / N	0 / R	0 / R
G7 - Untreated	E / S	E / S	L / N	L / N	L / N
G7 - Heat Treated	E / S	E / S	L / N	L / N	L / N

^aE = extensive: virus detected in all veins and inter-veinal tissue.

^bO = no virus detected in inoculated leaf.

^cL = localized: virus restricted to cells in and surrounding foci of infection and to leaf veins cells near infection foci.

^dS, R, N = expected response of susceptible, resistant, or necrotic phenotype as in Table 2-1.

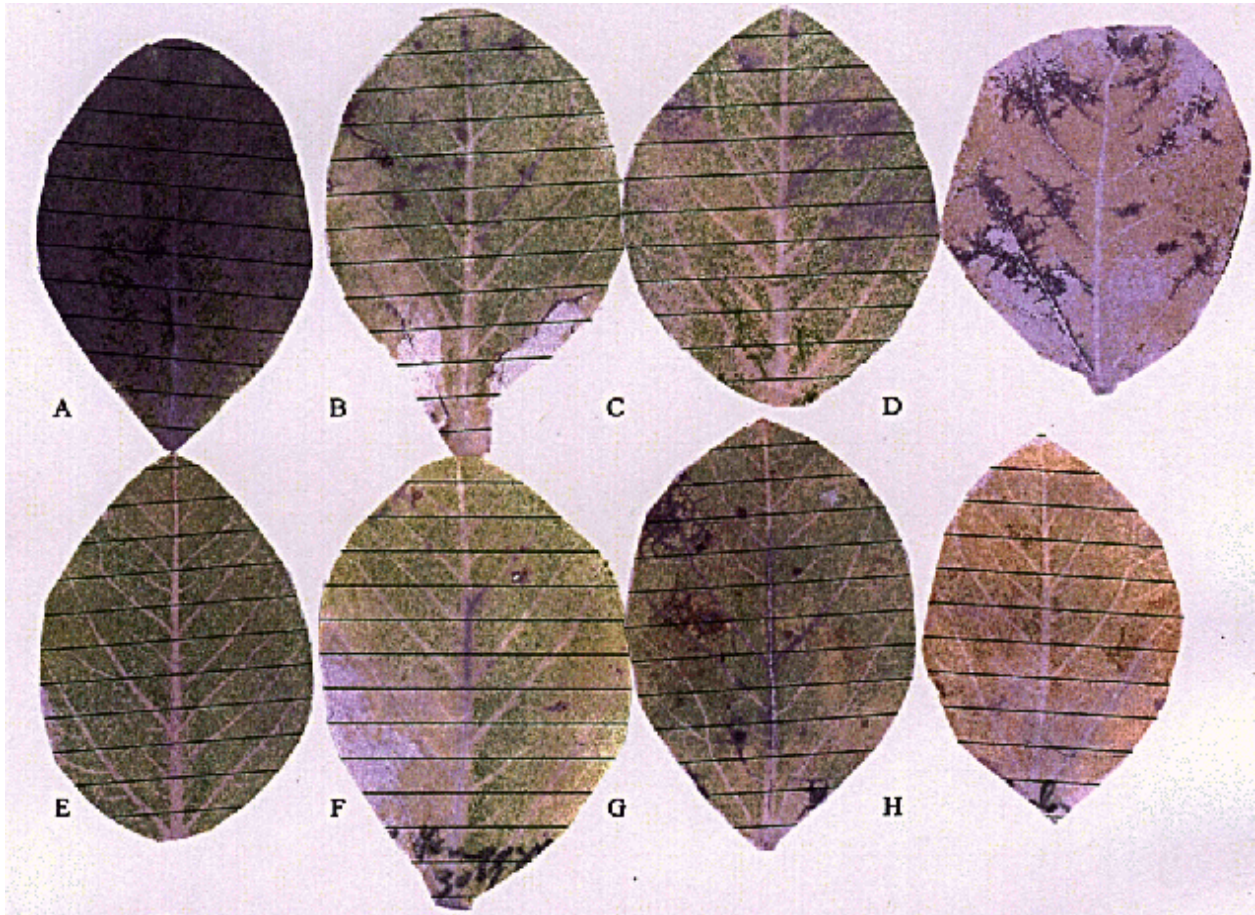


Figure 2-5. Comparison of localization of viral antigen in trifoliolate leaflets of three soybean cultivars inoculated with one of three soybean mosaic virus (SMV) pathotypes and held at 30 °C in continuous light. Leaves were printed on day 6 post inoculation and reacted with antibody to SMV. **A,B,C:** G4; **D,E:** G6; **F,G,H:** G7. **A:** Lee 68; **B:** York; **C, E, H:** Ogden; **D, F:** Kwanggyo; **G:** PI96983.

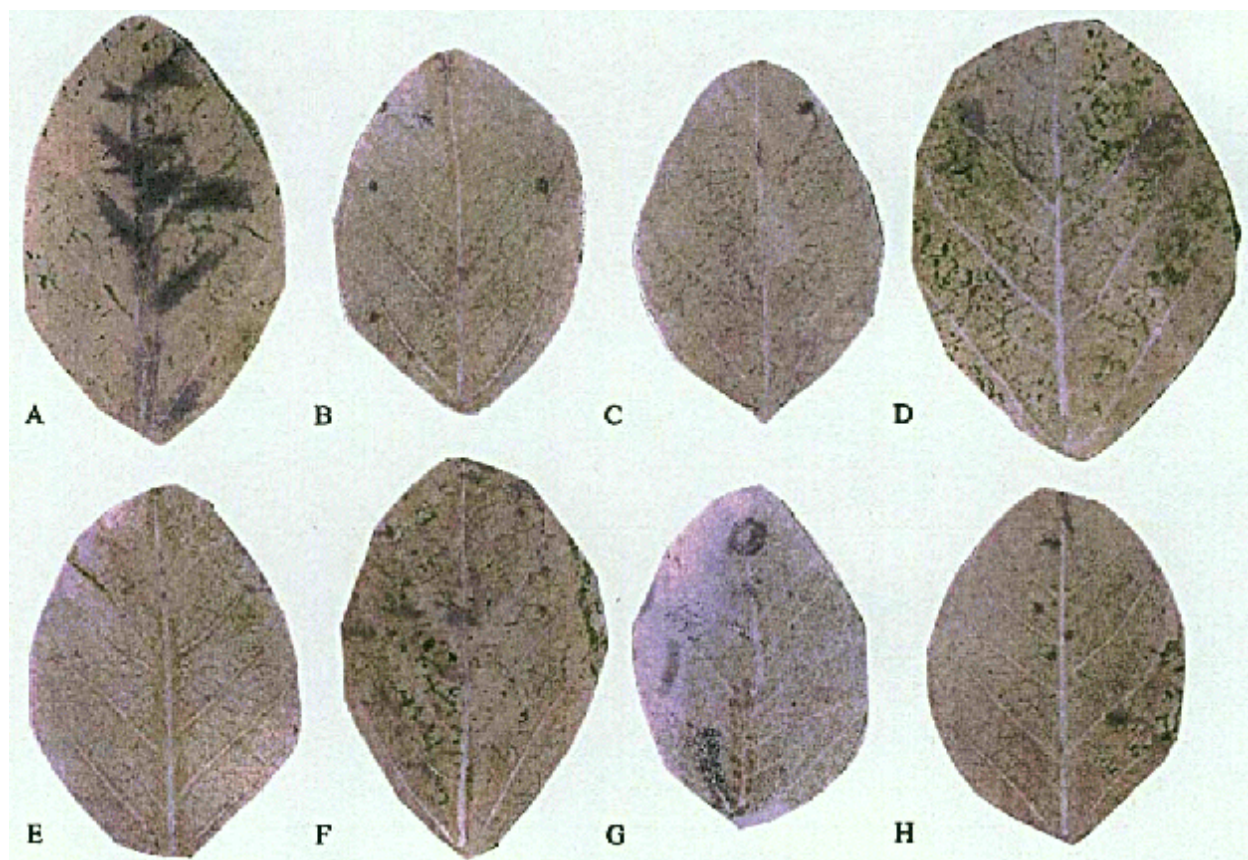


Figure 2-6. Comparison of localization of viral antigen in trifoliolate leaflets of three soybean cultivars inoculated with one of three soybean mosaic virus (SMV) pathotypes and held at 20 °C in continuous light. Leaves were printed on day 8 post inoculation and reacted with antibody to SMV. **A,B,C:** G4; **D,E:** G6; **F,G,H:** G7. **A:** Lee 68; **B:** York; **C, E, H:** Ogden; **D, F:** Kwanggyo; **G:** PI96983.

infection by a particular SMV pathotype remained resistant. Plants that produced the necrotic phenotypic resistance response remained necrotic following heat shock. The susceptible phenotypes continued to give systemic mosaic.

The rate of infection and systemic movement and the development of symptoms was also found to remain the same in the heat shocked plants as in untreated plants over the course of the infection. The heat treated plants showed similar extent and severity of symptoms as the control plants, regardless of response.

No change in the systemic movement of the virus was found between heat treated and control plants. The tissue blots of trifoliolate leaves showed that there was no change in the extent of systemic movement of virus. This method would have detected extensive or unrestricted systemic movement of the virus, a possible symptomless, latent infection of resistant plants, or a change from necrotic to susceptible, indicating a sensitivity of the resistance gene to heat pre-treatment.

Localization of the virus in inoculated tissue by the immunoprint method was consistent the phenotype determined by symptoms. No virus was detected in resistant reactions in either control or heat treated plants, which might be due to a restriction of replication of the virus or a very rapid hypersensitive cell response that does not allow time for the virus to move out of the initially infected cell. In necrotic responses viral antigen was limited to initial infection foci. A breakdown of the resistance gene at high temperature would be expected to lead to the lack of localization of the virus in the inoculated leaf, which was not detected in heat pre-treated plants.

The detached leaf method was a successful method for maintaining the leaf tissue and testing for the phenotype of resistance. It can potentially be used to test the resistance reaction of plants to numerous pathotypes while not actually infecting the whole plant. The results of the detached leaf assay testing the same alleles at a constant 30 °C temperature found no change in the localization of the virus in the inoculated leaf. The rate of detectable virus was slowed at 20 °C but that is attributed to the slowing of the plant's physiology at the cooler temperatures. By allowing more time before making the immunoprints for sufficient visible symptom development, the extent of viral localization could be compared with the high temperature detached leaves. In this experiment SMV pathotype G4 was added and G1 dropped to increase the number of necrotic reactions which were thought most likely to be effected by high temperature. The *Rsv₁* alleles did not change from their expected resistance response to the SMV pathotypes in the detached leaves incubated at the constant temperature of 30 °C.

The significance of the high temperature detached leaf assay is that in tobacco with the *N* gene inoculated with TMV infection the necrotic response is limited at 28 °C and above and the virus is not localized and spreads through the plant. At 32 °C no necrotic lesions develop at all and the virus spreads through the plant (Weststeijn, 1981). Tobacco detached leaf assays have been widely used to study the change in the resistance reaction. In soybean with the *Rsv₁* alleles tested, no change was

seen in the development of necrosis in genotype/SMV pathotype combinations that yield necrotic reactions. Using the detached leaf assay no change from necrosis to mosaic was observed by the various methods employed to determine if the reaction or localization to the virus changed. There was no change such as occurs in bean with the *I* gene for resistance to BCMV and several other potyviruses where resistance breaks down when plants are held above 32 °C and a necrotic phenotype develops (Kyle and Provvidenti, 1993). The two genotype/pathotype combinations used in the detached leaf assay that are resistant to SMV remained resistant. Finally, Tu and Buzzell (1987) reported that a SMV resistance gene in a line derived from Columbia was heat sensitive at 28 °C and 32 °C, changing from a necrotic expressed at 20 - 24 °C to mosaic infection. It has now been determined that the gene reported by them to be temperature sensitive is at the *Rsv₃* locus, which will be discussed in Chapter III. For temperatures up to 30 °C the, *Rsv₁* alleles tested expressed stable resistance responses.

A key difference between soybean virus resistance and *N* resistance is that the hypersensitive response of soybean does not localize the virus nor prevent extensive invasion of plant tissue. In soybean the necrotic reaction is typically a systemic lethal stem tip necrosis. Soybean *Rsv₁* resistance to SMV is also different from the *N* gene resistance reaction to TMV at high temperature. With the *N* gene the necrotic response restricts the virus to the inoculated leaf, but in soybean the necrotic response does not restrict the virus to the inoculated leaf, and the resulting systemic necrosis results in the eventual plant death. In *N* genes hypersensitive resistance response is overcome at 32 °C and the virus spreads throughout the plant, but when returned to below 28 °C the necrotic reaction occurs in all the cells that the virus had spread to, resulting in the death of those cells or possibly the plant (Weststeijn, 1981). The reaction of soybean virus resistance gene hypersensitive response is thus different than the *N* gene or *I* gene system.

The fact that the necrotic response remained similar to that of control plants likely means that the increase in HSP production does not effect the resistance response of the *Rsv₁* alleles. An increase in the severity of the necrotic response could have been interpreted as the result of a hypersensitive response when the virus was able to spread to a greater number of cells prior to induction of the hypersensitive response because the protein synthesis was potentially blocked by the HS response; however, the accumulation of viral protein was not tested. This was considered a possibility because of the linkage of *Rsv₁* to a microsatellite marker for a low molecular weight HSP (Yu *et. al.*, 1994). Also, if resistance had broken down it could have been attributed to the rapid induction of HS gene transcription coupled with a decline in the transcription of plant factors required for a resistance response. However, because no change was found in the development of the visible symptoms, no changes could be attributed to physiological changes caused by HS.

Chapter III

Effect of temperature on the *Rsv₃* Gene of Soybean for Resistance to Soybean Mosaic Virus

INTRODUCTION:

To further the understanding of interaction between soybean (*Glycine max* (L.) Merr.) and soybean mosaic virus (SMV), it is important to determine if *Rsv₃* is temperature sensitive, since many resistance genes have been found to be temperature sensitive. Plant geneticists at Virginia Tech investigating the genetics of the resistance have often noticed variability in symptoms at different times of the year, and attributed this to temperature influences. Tu and Buzzell (1987) reported that higher temperature changes stem tip necrosis to mosaic in SMV G1 infected soybean. The sensitivity of resistance genes to high temperature has been reported for many viral resistance genes. For example the tobacco *N* gene for resistance to tobacco mosaic virus (TMV) (Yarwood, 1958), the *I* (ts) gene in bean for resistance to bean common mosaic virus (BCMV), blackeye cowpea and cowpea mosaic virus (BICMV and CAMV) (Fisher & Kyle, 1994), and in soybean the *Rps* resistance gene to *Phytophthora sojae* (Gijzen *et. al.*, 1996).

The purpose of this study was to investigate the potential of high temperature to affect the movement of SMV in soybean plants carrying a copy of the *Rsv₃* resistance gene. Plants were inoculated with SMV and held in growth chambers to maintain constant temperature following inoculation. Observations were made to note changes in expected phenotype expressed in the plants inoculated with SMV. The localization and movement of the virus was determined by immunological methods.

MATERIALS AND METHODS:

Plant materials: Four cultivars of soybean were used, each carrying a resistance gene independent of *Rsv₁*. Seeds were provided by G.R. Buss, Blacksburg, VA. These included LR3 (derived from Columbia, by G. Ma and G.R. Buss), L29 (derived from Hardee, by R.L. Bernard, University of Illinois), Columbia, and Harosoy carrying *Rsv₃*, *Rsv₃ -h*, *Rsv₃ + Rsv₄*, and *Rsv₃?* SMV-resistance genes, respectively (Ma, 1995). The plants were grown under greenhouse conditions in 10 cm plastic pots with a soil-less potting medium (Scotts Metro-Mix 360), six plants per pot until the unifoliate leaves were nearly fully expanded.

Virus and inoculation methods: Two pathotype groups of SMV based on the virulence studies of Cho and Goodman (1979) were used in this study. SMV pathotype group G1 and G7 were chosen to provide a different extremes of reactions to the *Rsv₃* alleles used in this study, as shown in Table 3-1. The specific isolate of SMV G1 and SMV G7 were the same as used previously (Chen *et al.*, 1994; Ma, 1995). Plants were inoculated mechanically on fully expanded unifoliate leaves

lightly dusted with 600 mesh carborundum, and rinsed with tap water immediately. Inoculum was prepared from leaves 2-3 weeks post-inoculation (p.i.) ground in a mortar and pestle at a 1:10 dilution of tissue in 0.01 M sodium phosphate buffer, pH 7.0. The plants were maintained in greenhouse conditions for 4 h following inoculation, then transferred to the growth chamber and observed daily for 14 days p.i.

In an application of methods employed in the TMV/*N* gene research, at day 14 p.i. plants held at 30 °C were shifted to 22 °C. Effects on the symptoms and especially any change in the development of the hypersensitive response, were noted.

Table 3-1: The response of *Rsv3* - containing soybean cultivars to selected soybean mosaic virus (SMV) pathotypes.

Cultivar	R gene	G1	G7
Harosoy	<i>Rsv₃</i> [?]	S ^a	R ^b
LR3	<i>Rsv₃</i>	N ^c	R/n ^d
L29	<i>Rsv₃-h</i>	S	R
Columbia	<i>Rsv₃</i> & <i>Rsv₄-c</i>	LS ^e /n	R

^aS = susceptible, systemic mosaic symptoms.

^bR = resistant, no symptoms and no virus recovery.

^cN = necrotic, local necrotic lesions and systemic necrosis.

^dRn = resistant, with local necrotic lesions developing late.

^eLS = resistant initially, late-developing systemic symptoms.

Immunological detection of SMV: The material and methods used for the immunological detection of SMV were the same as described in Chapter II. Briefly, membranes or imprinted papers were reacted with antibody to SMV (Hunst and Tolin, 1982), and then with goat anti-rabbit whole fragment conjugated to alkaline phosphatase. A blue precipitate formed after incubation with NBT/BCIP substrate (Lin *et al.*, 1984). For tissue blot detection of systemic SMV, samples were taken at approximately day 4, 7, and 14 p.i. At day 4 p.i. a leaflet from the first trifoliolate leaf was removed, rolled up, torn in half, and blotted to a nitrocellulose membrane. At day 7 a leaflet from the first trifoliolate and one from the most recently fully expanded trifoliolate were blotted. Only the most recently expanded trifoliolate leaf was blotted at day 14 p.i. The most recent fully expanded leaflet was the same for each pathotype/temperature group, but was different between the pathotype/temperature groups. For example leaflets from the fourth trifoliolate was blotted for plants inoculated with SMV G7 at day 14 p.i. Once developed, the individual spots were rated relative to the positive and negative control for the intensity of the uniform dark blue color.

RESULTS:

Effect of temperature on symptom development: Systemic mosaic symptoms developed in Harosoy and L29 inoculated with SMV G1 in plants held at both 22 °C and 30 °C (Table 3-2). At 30 °C in LR3 inoculated with SMV G1, necrotic lesions developed on the inoculated leaf and in the trifoliolate leaves chlorotic spots developed with occasional necrotic flecks in the center of the chlorotic spot. In LR3 inoculated with SMV G1 and held at 22 °C, necrotic lesions developed on inoculated leaves, followed by systemic necrosis that appeared to develop into stem-tip-necrosis. Columbia inoculated with SMV G1 showed no symptoms at the lower temperature. At the higher temperature the inoculated leaf collapsed, remaining green, then dropped. No symptoms were observed in any of the cultivars inoculated with SMV G7 and held at either 22 °C or 30 °C.

The high to low temperature shift of plants at day 14 p.i. did not result in a rapid induction of necrosis (Data not included).

Table 3-2: Appearance of symptoms on four soybean cultivars when held at two temperatures after inoculation with two soybean mosaic virus (SMV) pathotypes.

SMV pathotype - treatment	Harosoy <i>Rsv₃?</i>	L29 <i>Rsv₃-h</i>	LR3 <i>Rsv₃</i>	Columbia <i>Rsv₃ & Rsv₄</i>
G1 - 22 °C	SM	SM	NL, STN ^e	NS
G1 - 30 °C	SM ^a	SM	NL ^b , CS ^c	NS ^d
G7 - 22 °C	NS	NS	NS	NS
G7 - 30 °C	NS	NS	NS	NS

^aSM = systemic mosaic.

^bNL = necrotic lesion on inoculated leaf.

^cCS = systemic chlorotic spots.

^dNS = no symptoms.

^eSTN = stem tip necrosis.

SMV replication and movement in inoculated leaves: In leaves inoculated with SMV G1, virus antigen was detected in Harosoy and L29 maintained at 30 °C and 22 °C, but there was a considerable difference in extent of invasion (Fig. 3-1). In LR3 inoculated with SMV G1 a high amount of viral antigen was detected in a few isolated spots in inoculated leaves of plants maintained at 30 °C, and either no virus or much smaller spots were detected at 22 °C (Fig. 3-1, **C, D**). No virus antigen was detected at either temperature in the inoculated leaves of Columbia inoculated with G1 (Fig. 3-1, **D, H**) or any of the cultivars inoculated with SMV G7 (Fig. 3-2).

Table 3-3 summarizes the detection of the virus in inoculated leaves as in Figures 3-1 and 3-2. Effects of temperature were seen in all types of infection.

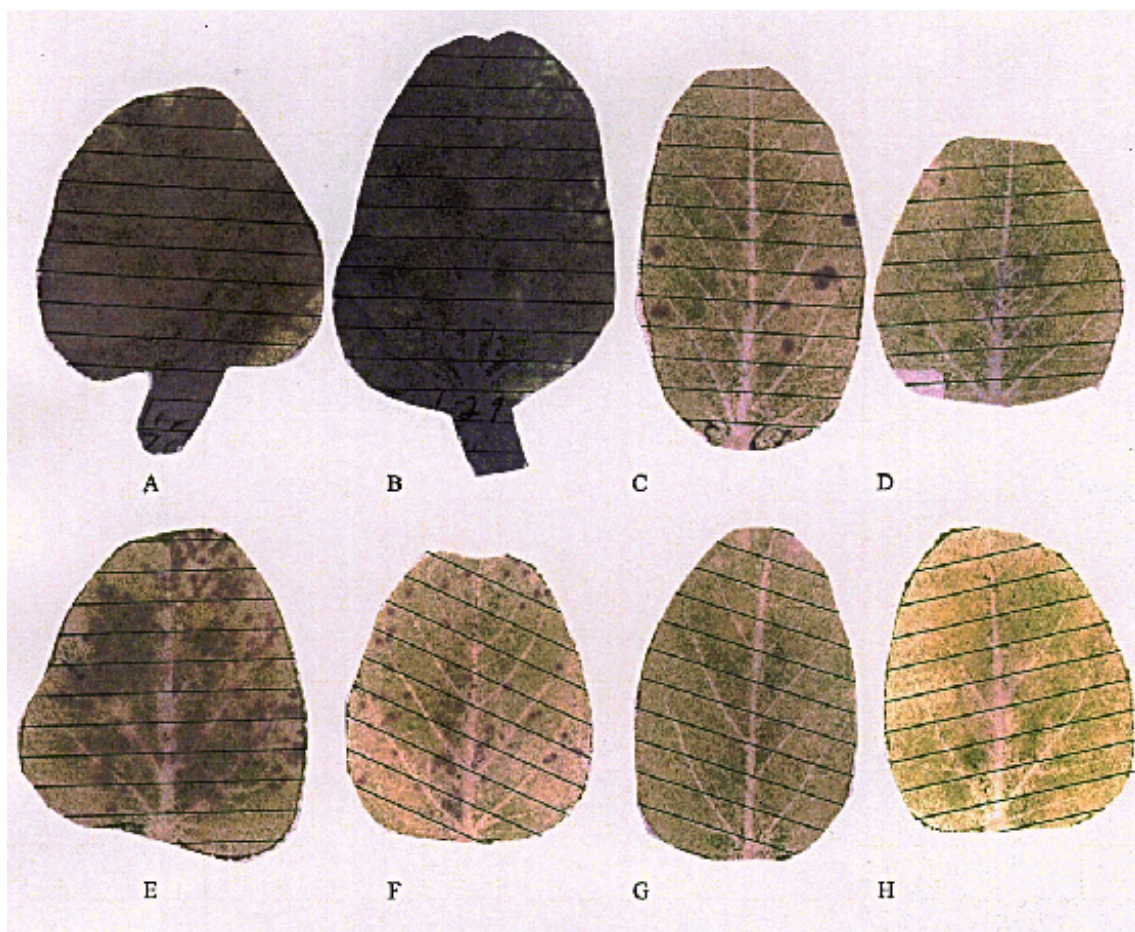


Figure 3 - 1. Effect of temperature on the movement of viral antigen within an inoculated unifoliolate leaf of four soybean cultivars after inoculation with soybean mosaic virus pathotype G1. Inoculated leaves were printed at day 7 after inoculation and reacted with antibody to SMV. **A, B, C,** and **D** are immunoprints from plants held at 30°C. **E, F, G,** and **H** are from plants held at 22°C. **A, E:** Harosoy. **B, F:** L29. **C, G:** LR3. **D, H:** Columbia.

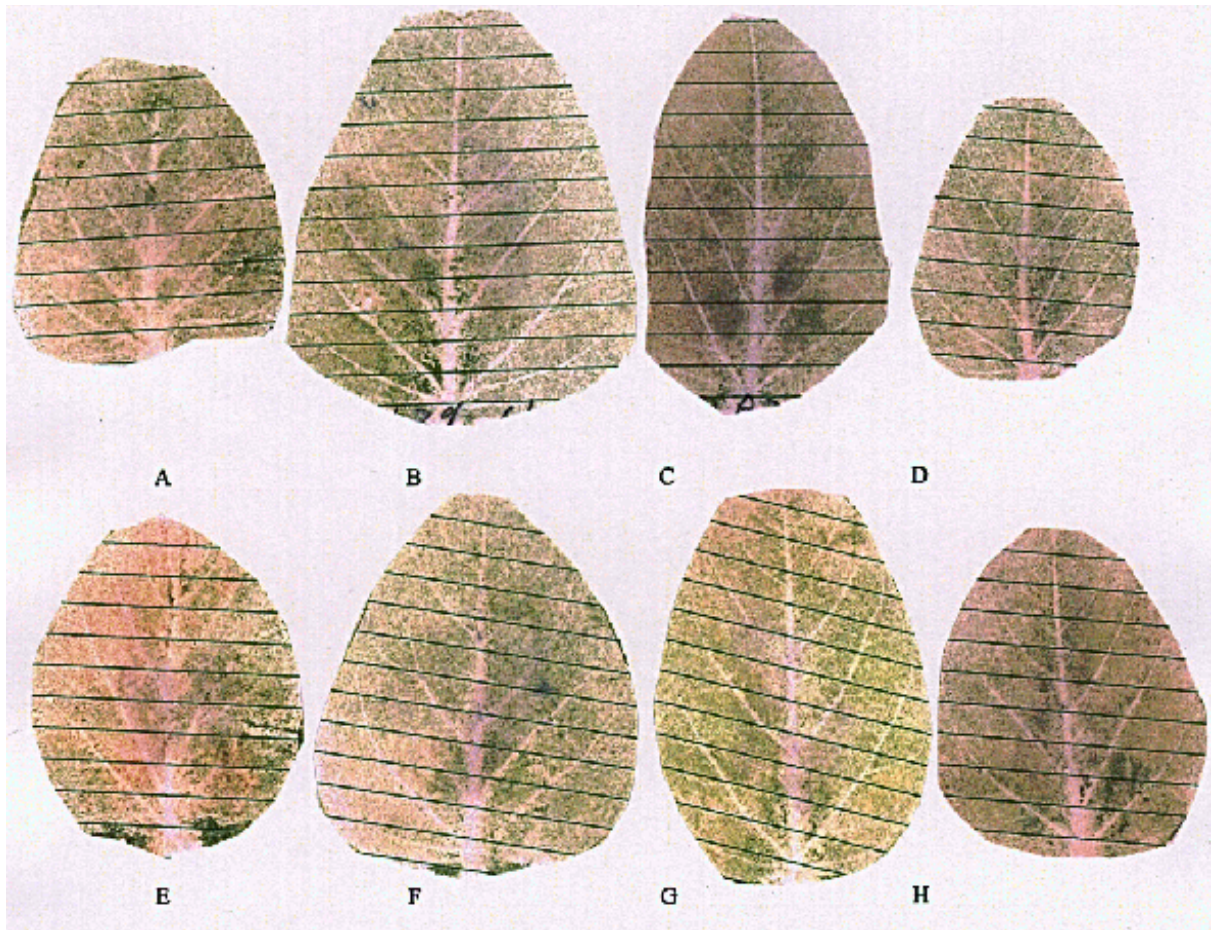


Figure 3 - 2. Effect of temperature on the movement of viral antigen within an inoculated unifoliolate leaf of four soybean cultivars after inoculation with soybean mosaic virus pathotype G7. Inoculated leaves were printed at day 7 after inoculation and reacted with antibody to SMV. **A, B, C, and D** are immunoprints from plants held at 30 °C. **E, F, G, and H** are from plants held at 22 °C. **A, E:** Harosoy. **B, F:** L29. **C, G:** LR3. **D, H:** Columbia. No antigen was detected in any leaves.

Table 3-3: Extent of invasion of two pathotypes of soybean mosaic virus (SMV) in four soybean cultivars inoculated at the unifoliolate leaf stage and held at two temperatures, compared to expected resistance response. Leaf prints for immunological detection of viral antigen were made of unifoliolate leaves at 7 days post-inoculation (d.p.i.) and trifoliolate leaflets at 14 d.p.i.

SMV pathotype - treatment	Harosoy <i>Rsv₃?</i>	L29 <i>Rsv₃-h</i>	LR3 <i>Rsv₃</i>	Columbia <i>Rsv₃ & Rsv₄</i>
G1 - 7 d.p.i.				
22 °C	L ^c / S ^d	L / S	0 / N ^d	0 ^b / LS ^d
30 °C	E ^a / S	E / S	L / N	0 / LS
G1 - 14 d.p.i.				
22 °C	E / S	0 / S	L / N	0 / LS
30 °C	E / S	E / S	L / N	0 / LS
G7 - 7 d.p.i.				
22 °C	0 / R	0 / R	0 / Rn	0 / R
30 °C	0 / R	0 / R	0 / Rn	0 / R
G7 - 14 d.p.i.				
22 °C	0 / R	0 / R	0 / Rn	-
30 °C	0 / R	0 / R	0 / Rn	-

^aE = extensive; virus detected in all veins and inter-veinal tissue of all leaves.

^b0 = no virus detected.

^cL = localized; virus restricted to cells surrounding site of infection and to veins of upper leaflet.

^dS, R, N, LS, Rn = expected reactions, as described in Table 3-1.

Effect of temperature on vascular movement of virus: The vascular movement of SMV into trifoliolate leaflets was confirmed by detection of viral antigen (Table 3-4). A uniform dark blue spot confirmed uniform presence of virus in leaves showing the systemic mosaic symptom in plants that were susceptible to the SMV pathotype. A uniform dark blue color indicated high concentration of viral antigen in all cells pressed to the membrane in the area of the template where the leaf tissue was blotted, as in the positive control. Spots with uniform intensity equal to the positive control were given a value of 2. A uniform faint blue color or dark blue color over only a portion of the area of the spot where the leaf tissue was blotted indicates a low concentration or an irregular distribution of viral antigen in cells pressed to the membrane and was given a value of 1. No blue color on the area of the tissue blot greater than the background of the negative control, indicating no viral antigen present, was rated as a negative and given a value of 0.

In the most recent fully expanded leaves at day 14 p.i. there was extensive invasion of

Table 3-4: Detection of viral antigen in trifoliolate leaflets, T_x, of soybean plants inoculated at the unifoliolate leaf stage with a soybean mosaic virus (SMV) pathotype and held at 22°C or 30°C. Leaflets from three individual plants were torn and blotted at various times post inoculation (p.i.) and rated for relative to the intensity of immunological reaction.

		Reaction Intesity Rating ^a			
Symptom ^b		Day 4 - T ₁	Day 7 - T ₁	Day 7 - T ₂	Day 14 - T _{MR}
SMV G1					
Harosoy 22 °C	SM	2, 2, 1 ^c	2, 2, 2 2, 2, 2	2, 2, 2 2, 2, 2	0, 0, 0
Harosoy 30 °C	SM	2, 2, 2	2, 2, 2 2, 2, 2	2, 2, 2 2, 2, 2	2, 2, 2
L29 22 °C	SM	0, 0, 0	2, 2, 0 2, 2, 2	0, 0, 0 0, 0, 0	0, 0, 0
L29 30 °C	SM	2, 2, 2	2, 2, 2 2, 2, 2	2, 2, 2 2, 2, 2	2, 2, 2
LR3 22 °C	STN	0, 0, 0	0, 0, 0 0, 0, 0	0, 0, 0 0, 0, 0	0, 0, 0
LR3 30 °C	CS	0, 0, 0	2, 1, 1 2, 1, 1	0, 0, 0 0, 0, 0	1, 1, 1
Columbia 22 °C	NS	0, 0, 0	0, 0, 0 0, 0, 0	0, 0, 0 0, 0, 0	0, 0, 0
Columbia 30 °C	NS	0, 0, 0	0, 0, 0 0, 0, 0	1, 1, 1 1, 1, 1	0, 0, 0
SMV G7					
Harosoy 22 °C	NS	0, 0, 0	0, 0, 0 0, 0, 0	0, 0, 0 0, 0, 0	0, 0, 0
Harosoy 30 °C	NS	0, 0, 0	0, 0, 0 0, 0, 0	0, 0, 0 0, 0, 0	0, 0, 0
L29 22 °C	NS	0, 0, 0	0, 0, 0 0, 0, 0	0, 0, 0 0, 0, 0	0, 0, 0
L29 30 °C	NS	0, 0, 0	0, 0, 0 0, 0, 0	0, 0, 0 0, 0, 0	0, 0, 1
LR3 22 °C	NS	0, 0, 0	0, 0, 0 0, 0, 0	0, 0, 0 0, 0, 0	0, 0, 0
LR3 30 °C	NS	0, 0, 0	0, 0, 0 0, 0, 0	0, 0, 0 0, 0, 0	0, 0, 0
Columbia 22 °C	NS	0, 0, 0	0, 0, 0 0, 0, 0	0, 0, 0 0, 0, 0	0, 0, 0
Columbia 30 °C	NS	0, 0, 0	0, 0, 0 0, 0, 0	0, 0, 0 0, 0, 0	0, 0, 0

^aSymptoms as in Table 3-2.

^b2 = equal to positive control; 1 < positive control, > negative control; 0 = negative control.

^cEach number within a column represents a leaflet from a single plant. Plants sampled at day 4 or day 14 were not always the same as those sampled at day 7. T₁ and T₂ at day 7 were from the same plant and are listed in corresponding order.

virus antigen in Harosoy inoculated with SMV G1 at both high and low temperatures (Fig. 3-3 A, E). In the upper leaves of L29 extensive invasion of SMV G1 antigen was observed at high temperature, but no antigen was detected in plants at 22 °C (Fig. 3-3 B, F). In LR3, the localized invasion of SMV G1 in trifoliolate leaves was similarly detected at high and low temperatures at day 14 p.i. (Fig. 3-3 C, G). In the upper leaves of plants inoculated with SMV G7 no antigen was detected in any of the cultivars at either temperature (Fig. 3-4).

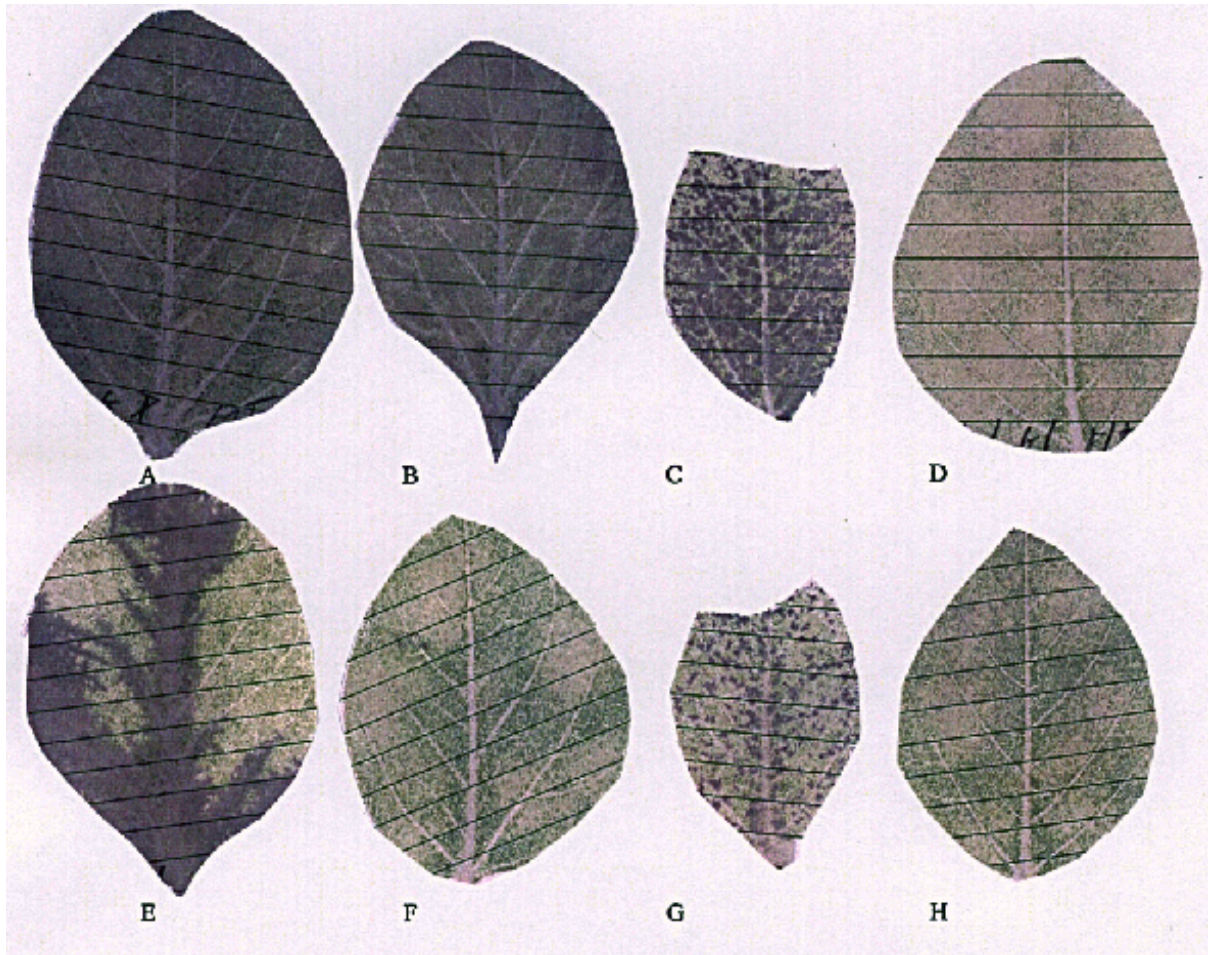
Table 3-3 summarizes the detection of viral antigen in upper trifoliolate leaves. Harosoy was susceptible to SMV G1, which was detected in upper leaves of plants maintained at both high and low temperatures. In L29 SMV G1 was detected in the upper leaves of plants maintained at 30 °C, but only detected in 2 of 3 blots of first trifoliolate leaves in plants maintained at 22 °C. In LR3 there was limited detection of systemic movement in the first trifoliolate at day 7 and in the fourth trifoliolate at day 14 for plants incubated at 30 °C (Table 3-3) and no detection at 20 °C. The virus antigen was not detected in the upper leaves of plants inoculated with SMV G7 with one exception, the most recent fully expanded trifoliolate leaflet at day 14 p.i. in L29 maintained at 30 °C.

DISCUSSION:

This series of experiment set out to test the potential change of the resistance response of the soybean *Rsv₃* resistance gene by high temperature. Based on the classic work with the TMV and tobacco *N* gene and reports of changes of the resistance response in soybean to SMV infection, a change in the resistance response was expected. The gene symbol *Rsv₃* was assigned by Tu and Buzzell in 1989. Recent genetics studies have determined that this is also present in cultivar Columbia is *Rsv₃* (Ma, 1995).

In this experiment the plants maintained after inoculation at low temperatures did not clearly develop the stem tip necrosis described by Tu and Buzzell (1987) when inoculated with SMV G1. This may be expected since the line used by them, OX686, was not available and the virus strain may be somewhat different.

In soybean with the *Rsv₃* alleles tested in this study, changes were observed in the development of necrosis in cultivar/SMV pathotype combinations that yield necrotic reactions at different temperatures. Tu and Buzzell (1987) reported that a SMV resistance gene in a line derived from Columbia was temperature sensitive at high temperatures of 28 °C and 32 °C, changing from a necrotic to mosaic infection. The results here are not complete enough to make a definitive statement about the temperature sensitivity of *Rsv₃* alleles. However, from the data it suggests the possibility that the allele carried by LR3, *Rsv₃* from Columbia, is temperature sensitive.



The detection of systemic movement of virus by the tissue blot method supports other

Figure 3 - 3. Effect of temperature on movement of viral antigen into trifoliolate leaflets of four soybean cultivars after inoculation of unifoliolate leaves with soybean mosaic virus pathotype G1. Leaves were printed at day 14 after inoculation and reacted with antibody to SMV. **A, B, C,** and **D** are immunoprints of a third trifoliolate from plants held at 30°C. **E, F G,** and **H** are of a second trifoliolate from plants held at 22°C. **A, E:** Harosoy. **B, F:** L29. **C, G:** LR3. **D, H:** Columbia.

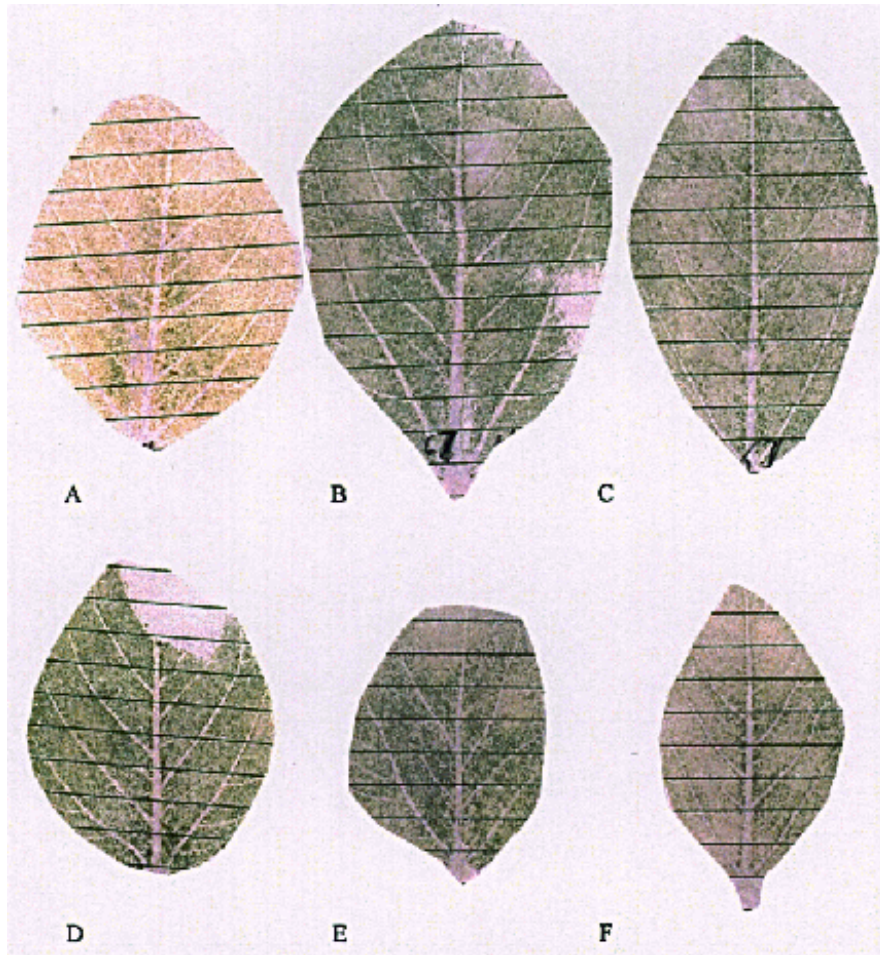


Figure 3 - 4. Effect of temperature on movement of viral antigen into trifoliolate leaflets of four soybean cultivars after inoculation of unifoliolate leaves with soybean mosaic virus pathotype G7. Leaves were printed at day 14 after inoculation and reacted with antibody to SMV. **A, B** and **C** are immunoprints of a third trifoliolate from plants held at 30°C. **E, F** and **G** are of a second trifoliolate from plants held at 22°C. **A, D:** Harosoy. **B, E:** L29. **C, G:** LR3. No antigen was detected in any leaves.

observations. The immunoprints of upper leaves of LR3, (Fig. 3-3 C) where the virus antigen was determined to be localized in the upper leaves of plants inoculated with SMV G1 and held at 30 °C for 14 day p.i., a consistent weak positive was found by the tissue blot method. The localized invasion of the virus in the upper leaf of LR3 explains why a viral concentration less than that in positive control can be obtained. Figure 3-3 (C, G) shows that virus was also detected in the upper leaves of LR3 held at 22 °C, but at a lesser intensity than in the plants held at 30 °C. The tissue blots did not detect virus in LR3 held at 22 °C for 14 day p.i. This difference is potentially due to a lower viral titer in the upper leaves of plants maintained at low temperature, which did not result in a spot of sufficient intensity by the tissue blot method to be considered a weak positive. The SMV G1/L29 the tissue blot test also supports the immunoprint data for the non-detection of virus in the upper leaves of L29 inoculated with SMV G1 held at 22 °C for 14 day p.i. (Fig. 3-3 F; Table 3-4). The potential for viral movement may be slowed at low temperature in the susceptible reactions of Harosoy and L29. The tissue blot results support the other observations made as to changes in the movement of the virus.

It should be noted that the different temperatures at which the plants were maintained in the growth chambers changed the rate of plant growth. Plant growth was more rapid at the higher temperature and at day 14 all the plants were larger than those at low temperature. By performing a time course study, the extent of viral localization could be compared between plants maintained at high and low temperature.

The apparent symptoms suggest that the gene in LR3 is temperature sensitive. At day 14 p.i. the LR3 plants inoculated with SMV G1 kept at 22 °C showed signs of stem-tip-necrosis. This necrosis, however, is not as pronounced as seen in *Rsv₁* cultivars, e.g. Kwanggyo/G6, in greenhouse conditions. When removed from low temperature after day 14 p.i. the growing point died, but the already expanded plant leaves did not. This type of stem-tip-necrosis was not observed in plants kept at 30 °C, but a slight fleck necrosis in the center of the chlorotic spots was evident. Shifting these LR3 plants at day 14 p.i. from 30 °C to 22 °C did not result in a rapid induction of necrosis or plant death, in contrast to the temperature sensitive *N* gene system (data not included).

No change in the resistance response of plants to SMV G7 was observed at the high or low temperatures used. In addition to specific examples mentioned above, the tissue blot tests also support the immunoprint and symptom data for the cultivar/SMV G7 combinations tested; i.e., there was no detection of virus in all plants inoculated with SMV G7, supporting the immunoprint data (Fig. 3-4, 3-5) and the symptom data (Table 3-2). The detection of viral antigen in a L29 plant inoculated with SMV G7, by the tissue blot at day 14 p.i., was possibly the result of contamination with SMV G1 likely to have occurred during the sampling process, but this was not determined.

The change observed for *Rsv₃* in soybean was different than the change in the resistance

response that occurs in bean with the *I* gene for resistance to BCMV and several other potyviruses where resistance breaks down above 32 °C and a necrotic phenotype develops (Kyle and Provvidenti, 1993). Also, SMV/*Rsv*₃ interaction is different from the *N* gene reaction with TMV at high temperature. With the *N* gene, the necrotic response restricts the virus to the inoculated leaf. In soybean the hypersensitive necrotic response virus is not restricted to the inoculated leaf, and the resulting systemic necrosis results in the eventual plant death. With the *N* gene, hypersensitive resistance response is overcome at 32 °C and the viruses spread throughout the plant, but when returned to below 28 °C the necrotic reaction occurs in all the cells into which the virus had spread, resulting in the death of those cells or possibly the plant (Weststeijn, 1981).

The effect that the high temperature has on the action of the resistance gene is not known. However, the study of temperature sensitive resistance genes could provide insight into the action of resistance genes or be useful in studies to map and clone these genes.

CHAPTER IV

Final Discussion

SUMMARY OF HEAT SHOCK TREATMENTS ON *Rsv₁*:

In this experiment heat shock was induced in the plants and heat shock proteins were present at the time of inoculation with SMV pathotypes. No change was observed in the resistance response of the *Rsv₁* alleles to the SMV pathotypes tested. In the following discussion of the conclusion of the *Rsv₃* high temperature results, the possibility of the hypersensitive response being unable to restrict the vascular movement of virus due to paraveinal mesophyll cells is presented. If it is likely that the virus can move before the hypersensitive response can restrict the virus and the resistance is fundamentally different from the *N* gene reaction, then heat shock treatments might not have effect on the *Rsv₁* mediated resistance.

SUMMARY OF HIGH TEMPERATURES ON *Rsv₃*:

The limited results suggest that the gene carried by LR3, *Rsv₃* from Columbia, is temperature sensitive. More work is required to fully characterize this reaction before a final determination can be made. The potential change observed for *Rsv₃* was different than the change in the resistance response that occurs in bean with the *I* gene for resistance to BCMV and several other potyviruses where resistance breaks down above 32 °C and a necrotic phenotype develops.

SPECULATION ON SOYBEAN RESISTANCE PROPERTIES:

It is possible that this difference might be due to movement of virus through the paraveinal mesophyll cells that are in soybean and not in tobacco. The paraveinal mesophyll are large cells, spreading at the level of the phloem where their function has been proposed to be involved in interveinal conductance of photosynthates (Fisher, 1967). These cells, which are proposed to be involved in phloem loading, are spread out and in contact with many different mesophyll cells. The spread of the virus in the interveinal tissue between the minor veins is extensive in early invasions characterized with susceptible responses, and is extensive late in the inoculated leaves of necrotic responses. The spread of the virus may be facilitated by these cells before the hypersensitive response can restrict the virus from moving to the vascular system. Once the vascular movement occurs at normal temperature and the virus spreads to developing tissue, the induction of the hypersensitive response in the developing invaded tissue results in stem tip necrosis. Then the stem tip and upper leaf necrosis observed is similar to that observed in tobacco with the *N* gene when it is returned to low temperatures after virus spread is allowed at high temperatures. One reason for the movement out of paraveinal mesophyll cells is that their plasmodesmata differ from those in the mesophyll. Also, they contact many different mesophyll cells allowing for a greater chance for the virus to enter the a phloem cell. Upon entry the virus

will have access to the vascular system which could account for its ability to spread prior to the hypersensitive localization. The paraveinal cells in soybean might be one of the reasons that the virus can move prior to the hypersensitive response which is able to restrict the virus as in the *N* gene system.

FINAL CONCLUSIONS:

More work is required to determine if soybean resistance genes are temperature sensitive; however, it seems likely that some may be. Many plant resistance genes are known to be temperature sensitive so it is not unlikely that soybean genes for resistance to viruses are as well. Based on the extensive research of TMV and *N* gene temperature sensitivity, which has been applied as a model system for many plant viruses, and the research into the movement of potyviruses, it seems that the system is different for potyviruses than for TMV. For SMV infection and soybean resistance to viruses, I think that it is the cell-to-cell movement restriction that is likely a major function of the resistance gene and that paraveinal mesophyll cells allow for easy access to the vascular system before the hypersensitive response can restrict cell-to-cell movement. The movement protein and likely resistance mechanisms appear in soybean appear to differ from the *N* gene, but more work is required to determine the specifics of how they differ.

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VITA:

Jonathan Flora was born on October 13, 1971. He graduated in 1990 from Montgomery High School, Montgomery Township, New Jersey. In the fall of 1990 he entered Bridgewater College in Bridgewater, Virginia, where he earned Bachelors of Science degree in Biology in May 1994. He entered the plant pathology graduate program at Virginia Polytechnic Institute and State University in the fall of 1994 under the supervision of Dr. Sue A. Tolin. In 1996 at the Potomac Division meeting of the American Phytopathological Society he received a Graduate Student Research Award, second place for "Flora, J. and Tolin, S.A. 1996. Heat shock does not alter resistance response of the soybean *Rsv₁* gene to soybean mosaic virus. *Phytopathology* 86 (11, Supplement): S121." Upon completion of his graduate work he will begin employment with Mycogen Seeds, St. Paul, Minnesota, at their Champaign Illinois, corn breeding station working in the Pathology Division.