1990

Interactions of soybean mosaic virus with susceptible and resistant lines of soybean

Louis Michael Mansky
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Interactions of soybean mosaic virus with susceptible and resistant lines of soybean

Mansky, Louis Michael, Ph.D.
Iowa State University, 1990
Interactions of soybean mosaic virus with susceptible and resistant lines of soybean

by

Louis Michael Mansky

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

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Iowa State University
Ames, Iowa

1990
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GENERAL INTRODUCTION

Host Plant Resistance

Plant viruses cause losses in yield and quality of many economically important crops. Although such losses may be difficult to quantify, it has been estimated losses in most crops are between 1 and 10 %, with occasional, localized catastrophic losses (Carr, 1984).

Few methods exist to control plant virus disease. Chemotherapy has been used successfully for elimination of virus from propagation lines in tissue culture, but has not been extensively used in the field (Bailess et al., 1977). Disease avoidance has also been used. Strategies used for disease avoidance include the use of pathogen-free seed; import restrictions and quarantine regulations; soil sterilization and, when possible, direct control of vectors such as insects and nematodes by using chemical insecticides and nematocides (Fraser, 1985). These measures can be effective and have high selectivity and relatively low environmental impact. If they fail, an alternative method(s) for control is necessary.

Host plant resistance, conditioned by a single or a few genes, is perhaps the most important strategy for control of viral infection. The main advantages of host plant resistance include: (1) it is inherited; (2) it is generally environmentally sound; (3) it is highly selective; and (4) the cost
is relatively low, after the initial establishment of the breeding program. The main disadvantages are the relatively high selection pressure for mutants of pathogens to overcome resistance and that breeding for resistance may be at the expense of other desirable characters.

The study of the biology of host plant resistance to viruses is important from the practical economic standpoint of the development of new strategies for crop protection. Also, from the viewpoint of developing new fundamental principles, host plant resistance provides an interesting model for the study of host-parasite interactions. From this, the interaction of host and pathogen genomes and the nature of the recognition event between the host and pathogen can be studied. Plant viruses provide a good choice for a model to study the molecular aspects of recognition and interaction because of their relatively simple structure. The study of the molecular aspects of recognition between plant cells and viruses can also be used as a model to study eukaryotic developmental biology since, as different tissues respond differentially to transduced signals in response to viral challenge, similar types of molecular recognition and signalling events may be involved in development.

Resistance of plants to viruses, although difficult to categorize due to poorly understood mechanisms, has been classified. Resistance can be broadly categorized into host or non-host resistance. Although a matter of debate, a resistant plant has been defined most commonly as one that restricts virus multiplication, whereas a susceptible plant allows
multiplication and spread of virus, resulting in characteristic symptom development. Resistance, in the extreme case, can be termed immunity; therefore, in many cases, host and non-host resistance may also be referred to as host and non-host immunity. Host immunity or resistance occurs within a species that can be a host for a particular virus. Non-host immunity or resistance applies to a particular plant species where there are no detectable signs of virus multiplication or pathogenesis after inoculation. Another term, tolerance, is used to define a plant in which viral multiplication occurs with or without the expression of symptoms and economic loss. With respect to crop quality and yield, tolerance may be, in certain instances, the equivalent of resistance.

Three models have been proposed by Fraser (1985) to account for non-host resistance. The first (positive-type model) invokes an active defense response in all members of the non-host species to totally inhibit virus multiplication; this is essentially the equivalence of functional immunity. The second (negative-type model), acts by the lack of plant-specific components necessary for multiplication, e.g., receptor or replicase subunit. In this model, specific recognition events necessary for multiplication do not occur. In the third proposed model, physical and chemical barriers preclude multiplication and pathogenesis. Such barriers may include physical barriers which do not exist at the protoplast level, or chemical barriers which alter pH or ionic strength of cells within the plant. Non-host resistance is most likely involved in the determination
of host range control. Although common, non-host resistance is difficult to exploit in plant breeding due to the large number of genes involved.

Classical studies regarding the genetics of resistance have involved crossing resistant and susceptible parents followed by analysis of the F₁ and F₂ plants and those plants resulting from subsequent backcrossings. In the majority of reported examples, segregation ratios have identified Mendelian control by a single dominant allele (i.e., where the F₁ displays the same or a similar resistance reaction as the homozygous parent). Virus isolates exist that can overcome resistance genes for most of these dominant resistance alleles. Indeed, virus isolates that break resistance genes appear to be common in nature. Fraser (1985) estimates that virulent isolates have been reported for about 60% of all known resistance genes.

Two models were proposed by Fraser (1985) to explain the action of resistance genes: the positive action model proposes direct interference with viral replication and viral pathogenesis; the negative action model proposes that the resistance gene is a mutant allele of a wild type gene that confers susceptibility to viral replication and pathogenesis (e.g., polymerase subunit).

In the positive action model, an antiviral factor would specifically interfere with the viral multiplication cycle. Siegel (1979) proposed six potential targets in the virus life cycle: (1) entry into the host cell; (2) uncoating of the viral nucleic acid; (3) translation of viral proteins; (4)
replication of viral nucleic acid; (5) assembly of the progeny virions; (6) spread of virus to new cells and new hosts. In contrast, Fraser (1982) identified five potential targets based upon more functional criteria: (1) transmission from one host to another; (2) disease establishment; (3) systemic movement; (4) replication of the virus; and (5) tolerance. In theory, a resistance mechanism could be involved in the interruption of viral replication at any one or several of these points.

**Induced Host Plant Resistance**

Host plant resistance can be induced or constitutive. Induced host resistance is not normally expressed in the plant but is stimulated by the virus. This type of host resistance allows some viral multiplication to occur; therefore, in a strict sense, it is not true immunity. The most extensively studied type of induced resistance is that of the hypersensitive response. In this situation, the virus is localized in small necrotic areas and the plant remains essentially disease-free (Goodman et al., 1986). The model used for these studies was tobacco containing either the N or N' genes for resistance to TMV.

In tobacco, symptoms on some plants are known to be controlled by the genes, N and N' (Holmes, 1938; Matthews, 1981). The N gene, found naturally in *Nicotiana glutinosa*, confers hypersensitive resistance to all TMV strains tested. In these plants, virus is confined to areas near
the necrotic local lesions. The \( N' \) gene, originally from *Nicotiana sylvestris*, induces a hypersensitive response to some TMV strains. Other strains move systemically causing typical mosaic symptoms. Antiviral factor (AVF), a glycoprotein similar in structure to human interferon, has been suggested to be a regulatory element that influences expression of the \( N \) gene (Sela et al., 1987; Edelbaum et al., 1990).

Differences among TMV strains, that induce different symptoms in plants containing the \( N' \) gene have been extensively studied. Saito et al. (1987) studied the function of the resistance gene using two strains of TMV. The OM (common strain) strain of TMV induces systemic mosaic in plants expressing the \( N' \) gene, while the L (tomato strain) strain of TMV induces the hypersensitive response. By constructing recombinant viruses, Saito et al. (1987) found that the viral sequence that induces the necrotic response in \( N' \) gene-expressing plants is located in the coat protein gene of TMV-L.

Knorr and Dawson (1988) identified TMV sequences responsible for inducing local lesion formation in plants containing the \( N' \) gene by using cDNA clones to construct genomic recombinants between the common strain genome and a local-lesion-inducing mutant. Nucleotide sequences conferring the mutant phenotype were incorporated into infectious transcripts that were used to inoculate leaves of *N. sylvestris* and the formation of either local lesions or a systemic infection were observed. Analysis of the sequence from the mutant that converted the
hybrid genome to the mutant phenotype revealed a point mutation in the mutant that occurred in the coat protein gene, changing the codon from specifying serine to phenylalanine. This study demonstrated that the coat protein gene of TMV encodes both the virion structural protein and the function that mediates the outcome of infection in *N. sylvestris*.

Saito et al. (1989) constructed several mutants of the L strain of TMV that had insertions or deletions in the coat protein gene. Frame-shift mutants that caused premature termination of translation of the coat protein caused no necrotic local lesions on plants containing the *N′* gene. Mutants that resulted in the expression of coat protein derivatives with one amino acid inserted after residue 56, 101, or 152 caused necrotic local lesions on plants containing the *N′* gene. Deletion mutants lacking the coding region for fewer than the C-terminal 13 amino acid residues caused necrotic local lesions, whereas mutants lacking the coding region for the C-terminal 38 residues caused no necrotic local lesions. These data suggest that modifications of the coat protein gene affect its ability to induce the hypersensitive response on plants containing the *N′* gene.

The *N* gene has been extensively studied as a model to isolate and characterize the gene(s) and gene product(s) responsible for the hypersensitive response (Matthews, 1981; Dunigan et al., 1987). Smart et al. (1987) identified mRNAs and proteins specific to the hypersensitive response by utilizing the temperature-sensitive nature of the response. Four polypeptides specific to the response were identified by cell-free
translation of the identified mRNAs. Differential hybridization experiments (Dunigan et al., 1987), designed to identify the gene(s) unique to plants containing the N gene, permitted the screening of over 100,000 clones; however, none were specific to the hypersensitive response.

Another type of induced host resistance is referred to as systemic acquired resistance (SAR). If lower leaves of plants are inoculated with virus, followed by subsequent inoculation of upper leaves, the symptoms are less severe on the upper leaves. SAR can be induced by a number of different pathogens and with certain chemicals, including aspirin (van Loon, 1987). A family of basic proteins, known as pathogenesis-related proteins (PR proteins), have been associated with this response (van Loon, 1987). Activities associated with these proteins include beta-1,3 glucanases that may be active against fungi, and alpha-amylase and proteinase inhibitors that may be active against insect damage (Bol and van Kan, 1988). Antiviral functions have yet to be associated with the PR proteins.

One type of induced resistance, that has been used as a practical virus control measure, is cross protection. Classical cross protection describes resistance to infection of a severe strain of virus induced by prior inoculation of the same plant with a mild strain of the same virus. Transgenic tobacco plants that express the coat protein of a mild strain of TMV have been shown to mimic classical cross protection. Such plants are resistant to inoculation with a severe strain of the virus but not
resistant to viral RNA (Powell-Abel et al., 1986). This phenomenon has also been shown with alfalfa mosaic (AMV), tobacco streak (TSV), cucumber mosaic (CMV), and potato virus X (PVX) and Y (PVY) viruses (Loesch-Fries et al., 1987; Tumer et al., 1987; van Dun et al., 1987; 1988; Cuozzo et al., 1988; Hemenway et al., 1988; Hoekema et al., 1989; Lawson et al., 1990). To summarize, induced host resistance has been extensively studied in systems described by the \( N \) genes of tobacco, SAR, and viral cross protection.

**Constitutive Host Plant Resistance**

The second type of host resistance is constitutive resistance that is expressed in the plant, even in the absence of virus. Several model systems have been reported in the literature that have addressed the cellular and molecular basis of mechanisms involved in this type of resistance. The following examples represent some of the more extensively studied models.

The resistance genes \( Tm-1, \ Tm-2, \) and \( Tm-2^2 \) confer resistance in tomato to TMV, the type member of the tobamovirus group. Plants homozygous for the \( Tm-1 \) gene inhibit multiplication of TMV more effectively than heterozygous plants. In \( Tm-1 \) gene-conferred resistance, a delay of 8-40 days occurs between inoculation and virus multiplication.
Motoyoshi and Oshima, 1977). This observation suggests that the antiviral product was present at inoculation and did not need activation.

The effect of growth temperature on the Tm genes in tomato is complicated. The Tm-1 gene severely reduced virus multiplication and completely suppressed symptoms induced by TMV strain 0 when plants were grown at constant temperatures from 20 to 35°C (Fraser and Loughlin, 1982). In contrast, TMV strain 1 caused more severe symptoms at higher temperatures. This suggests that multiplication of strain 1 is inhibited at 25°C, but not at 33°C (Fraser and Loughlin, 1982). Tomato plants containing the Tm-2 or Tm-2 genes for TMV resistance are symptomless at normal temperatures after inoculation with strain 0; severe systemic necrosis occurs at elevated temperatures (Schroeder et al., 1967; Cirulli and Alexander, 1969; Pelham, 1972).

Protoplasts from plants containing the Tm-1 gene did not support multiplication of the TMV L strain when inoculated with either intact virions or with RNA (Motoyoshi and Oshima, 1979). Polymerase extracts from susceptible infected and resistant infected (with a resistance-breaking TMV strain) could synthesize the replicative form (RF) from TMV RNA in vitro. Extracts from susceptible uninfected and resistant plants could not synthesize RF.

Comparison of the genomic structure and function of virulent and avirulent strains of TMV has provided a better understanding of the mechanism of action of the Tm-1 gene. Watanabe et al. (1987) described...
the isolation of the resistance-breaking Lta 1 strain of TMV, that was isolated from a plant containing the Tm-1 gene that had been inoculated with the TMV L strain. Lta 1 spreads systemically in and causes mosaic symptoms on tomato plants homozygous for Tm-1. The L strain neither spreads nor produces symptoms on such plants. Comparison of the genomic sequences of strains Lta 1 and L revealed two base substitutions in the Lta strain that resulted in amino acid changes in the 130 and 180 K proteins (Meshi et al., 1988), that are putative viral-encoded replicase proteins. Infectious transcripts of the L strain having either one or both of the amino acid changes found in the Lta 1 strain were used to inoculate plants homozygous for the Tm-1 gene. An infectious transcript of the L mutant with both amino acid changes multiplied and produced symptoms in plants homozygous for Tm-1 similar to Lta 1; an infectious transcript with only a single amino acid change efficiently multiplied in protoplasts and tomato plants containing the Tm-1 gene. Virus recovered from only the plants containing the Tm-1 gene, which had been inoculated with transcripts containing only a single amino acid change, revealed a second amino acid change near the mutagenized residues. This data suggests viral RNA replication may be the antiviral target of disease resistance involving the Tm-1 gene.

Although resistance was operative at the protoplast level for Tm-1, the resistance genes Tm-2 and Tm-2² appear not to act at the protoplast level (Motoyoshi and Oshima, 1975; 1977). Therefore, it has been
speculated that resistance encoded by $Tm$-$2$ and $Tm$-$2^2$ may operate at the level of organized plant tissue (Motoyoshi and Oshima, 1975; 1977). Further evidence comes from mixed infection experiments. Taliansky et al. (1982a, b) found that the Ls 1 strain of TMV spread in plants containing the $Tm$-$2$ gene from the conducting tissue into the mesophyll only when plants were pre-infected with potato virus X (PVX). These experiments suggest that the $Tm$-$2$ resistance gene functions by inhibition of TMV movement from the conducting to the mesophyll tissues; the presence of PVX may have complemented a TMV mutant defective in its movement function. Recent studies with transgenic tobacco plants have shown that TMV encodes a gene for a 30 K protein that potentiates virus movement (Deom et al., 1987; 1990); the Ls 1 strain of TMV was shown to be specifically defective in potentiating virus movement (Deom et al., 1987; 1990). This implies that, in the mixed infection experiments of Taliansky et al. (1982a, b), complementation of the virus-encoded movement functions occurred. A resistance-breaking isolate of TMV derived from the L strain, Ltb 1, spreads systemically in tomato plants homozygous for the $Tm$-$2$ gene (Meshi et al., 1989). Nucleotide sequence analysis of Ltb 1 revealed two amino acid changes, as compared with the L isolate, in the 30 K movement protein (Meshi et al., 1989). These data suggest that virus movement is the antiviral target of the $Tm$-$2$ gene. Young et al. (1988) have used RFLP mapping to isolate
DNA clones linked to the *Tm-2* gene; this may allow future functional studies of the *Tm-2* gene product.

The interaction of cowpea mosaic virus (CPMV) with cowpea has provided another extensively studied model system for virus disease resistance in plants. A survey of over 1000 cowpea lines by Beier et al. (1977) found that 65 lines were operationally immune to CPMV. To be classified as immune, the seedlings were expected to remain free of symptoms and detectable virus after inoculation with a virus concentration 100 times greater than that that infected susceptible cowpea. However, the protoplasts from almost all of the immune lines supported the multiplication of CPMV. One cultivar, Arlington, did not support CPMV multiplication when compared with results from protoplasts isolated from the susceptible cultivar, Blackeye 5. Cowpea severe mosaic virus (CPSMV), another member of the comovirus group, infected virtually all cowpea lines and their protoplasts, including Arlington.

Genetic crosses of Arlington and Blackeye 5 cowpeas indicated that resistance was inherited as a simple dominant trait (Kiefer et al., 1984). Protoplasts from these progeny are also resistant, indicating that operational immunity observed in Arlington seedlings and resistance in protoplasts are both reflections of resistance acting within the cell.

To test the hypothesis that resistance was acting at the level of proteolytic processing of CPMV polyproteins, Sanderson et al. (1985) developed an assay that detected proteolytic activity in extracts of
Arlington cowpea protoplasts that inhibited the formation of the 48 and 60 K proteins encoded by CPMV, that normally are produced from a 95 K polyprotein by a CPMV-encoded protease (Goldbach and Krijt, 1982). Extracts from Blackeye 5 cowpea protoplasts had a negligible amount of this activity.

To extrapolate these observations to the whole plant, Ponz et al. (1987) examined extracts from Arlington cowpea leaves. The inhibitor of proteolytic processing of the CPMV-encoded 95 K polyprotein precursor was present along with proteinases that degraded CPMV proteins; in addition, inhibitors of translation of CPMV RNAs were observed. These three activities were at higher concentrations in leaf extracts of Arlington than in extracts of Blackeye 5 cowpeas. The proteinases degraded CPMV and CPSMV proteins equally well; the activities were not coinherited with immunity against CPMV in progeny of cowpea crosses. The CPMV polyprotein processing inhibitor was inherited and virus-specific, implying activity in providing immunity to CPMV. Ponz et al. could not definitively determine whether the inheritance of the translation inhibitor activity was coinherited with immunity, but speculated that this activity may contribute to immunity against CPMV.

Another model system for studying virus disease resistance in plants containing a single dominant allele is that described for CMV and cucumber. Resistance to CMV in cucumber has been studied in two cultivars, Chinese Long (Barbara and Wood, 1972, 1974; Coutts and
Wood, 1977) and Kyoto (Maule et al., 1980); this has led to some confusion in the literature because observations made with one resistant cultivar have been assumed to be true for the other cultivar without experimental proof. However, the data from each suggest that disease resistance in each has a similar mechanism (Barbara and Wood, 1972; 1974; Coutts and Wood, 1977; Maule et al., 1980).

Symptom formation in Chinese Long was suppressed and virus multiplication was reduced to about one-tenth of that in a susceptible cultivar, but without elimination of systemic spread (Amemiya and Misawa, 1977). Virus accumulation was observed in Chinese Long up to 36 h after inoculation, when inhibition was detected (Amemiya and Misawa, 1977). This suggested that disease resistance was activated during one of the early stages in the virus multiplication cycle. Further support of this hypothesis, and indication that activation specifically requires the transcription of DNA, have come from studies showing that the development of resistance is inhibited by treatment with actinomycin D (Barbara and Wood, 1974; Amemiya and Misawa, 1977), alpha-amanitin (Amemiya and Misawa, 1977) or ultraviolet light (Levy et al., 1974). These agents were effective only on plants treated more than 12 to 24 h after inoculation. A short temperature treatment of resistant plants at 35°C for 6 h after virus inoculation also prevented the development of resistance, suggesting the possible involvement of a thermolabile protein
(Ameniya and Misawa, 1977). These data suggest that resistance is inducible and not constitutively expressed.

Inoculation of protoplasts from Kyoto cucumber plants resulted in lower levels of virus and a lower proportion of protoplasts in that virus antigen could be detected than in protoplasts from susceptible cucumber plants (Maule et al., 1980). This evidence suggests resistance to CMV may operate at a level in the virus multiplication cycle that occurs within the cell and not at cell-to-cell spread. When CMV RNA was used to inoculate protoplasts, resistance was maintained, suggesting that attachment or uncoating of the virus may not be the target of resistance. When viral RNA synthesis was assayed, resistance could also be detected. This further indicated that resistance may act at the transcriptional or translational levels. Boulton et al. (1985) reported, in contrast to data from intact plants of Chinese Long, that treatment of Kyoto protoplasts with actinomycin-D and ultraviolet light did not prevent inhibition of virus multiplication. These authors proposed that resistance was composed of two components. One requires activation after infection and is associated with cell-to-cell movement (this has only been observed with Chinese Long and not with Kyoto). The second acts at the level of multiplication within the cell (this has only been observed with Kyoto and not with Chinese Long). In protoplasts, the suggestion is that only the second mechanism would function.
Resistance at the cellular level has also been studied in the cowpea chlorotic mottle virus (CCMV)-cowpea system. In a classic study by Wyatt and Kuhn (1979), CCMV multiplied in the inoculated leaves of the resistant cowpea line PI 186465, but the virus did not spread systemically. The rate of virus multiplication in inoculated, resistant leaves was only 5 to 10% of that in susceptible leaves of California Blackeye. When the virus produced in resistant plants was analyzed, RNA 3 was present in very small amounts. Susceptible plants inoculated with virus showed the normal level of RNA 3. RNA 4, a monocistronic mRNA for viral coat protein, was found in normal amounts in resistant plants. RNA 3 encodes the coat protein and a putative movement protein. A hypothesis to explain resistance in PI 186465 suggests that the antiviral target is directed at lowering the amount of mRNA that encodes for a putative movement protein. Therefore, the level of this protein may determine susceptibility or resistance. Kuhn et al. (1981) reported, through inheritance studies, that virus spread in the resistant PI 186465 was controlled by a single dominant gene.

Potato virus X (PVX), the type member of the potexvirus group, and potato lines resistant to PVX have been used to study disease resistance. Resistance to PVX in potato lines is conditioned by the dominant genes \(N_x\) and/or \(N_b\) (Cockerham, 1943). PVX strains have been differentiated into four groups based upon their reaction to the two genes. Inoculation of plants with group 1 strains does not result in symptoms on lines with
either or both genes. Group 2 strains can infect potato lines containing the \( \mathcal{A} \) gene. Group 3 strains are the most common in nature and infect cultivars with the \( \mathcal{N} \) gene. Group 4 strains can systemically infect cultivars with either or with both resistance alleles. Foxe and Prakash (1986) reported that protoplasts from the King Edward cultivar containing the \( \mathcal{N} \) gene, supported a low level of PVX group 3 strain multiplication, compared to virus multiplication in protoplasts from a susceptible cultivar using either intact virus or PVX RNA. This suggests that resistance may involve inhibition of virus multiplication within the cell.

All these examples of constitutively expressed disease resistance are controlled by a single dominant allele. In the bean common mosaic virus (BCMV)-\textit{Phaseolus} bean model, resistance is controlled by a recessive gene system (Drijfhout, 1978). Resistance to BCMV requires the cooperative action of the \( \mathcal{B} \) gene with one or more genes at the \( \mathcal{B}-1, \mathcal{B}-2, \) or the \( \mathcal{B}-3 \) (collectively termed the \( \mathcal{B}-x \) ) loci. Drijfhout (1978) reported that virus isolates lacking specific virulence genes generally did not spread systemically. Systemic symptoms were induced only in lines homozygous for \( \mathcal{B}-u \) and one of the \( \mathcal{B}-x \) genes. From these studies, BCMV was differentiated into 10 pathotype groups on the basis of phenotypic response on nine differential bean cultivars. Fraser (1985) has suggested that \( \mathcal{B}-u \) is effective only in combination with a \( \mathcal{B}-x \) gene to prevent systemic spread, but \( \mathcal{B}-1 \) alone may function against symptom
formation. Plants heterozygous for both \textit{bc-u} and \textit{bc-1} showed no suppression of systemic mosaic, indicating that the genes were recessive.

Resistance in plants homozygous for \textit{bc-u} and \textit{bc-x} was effective in all leaves of bean plants except in the inoculated leaves. These data indicate that a very long induction period was necessary, or that resistance did not completely inhibit multiplication within cells or cell-to-cell virus movement within the inoculated leaves. BCMV is known to be translocated in the phloem (Ekpo and Saettler, 1975), so resistance may involve inhibition of phloem loading of BCMV and the blocking of systemic spread.

Constitutive Host Plant Resistance to Potyviruses

Four model systems can be cited from the largest and most economically important group of plant viruses, the potyvirus group. Teleologically, one may expect the greatest diversity of resistance mechanisms evolved from the largest group of plant viruses. Therefore, studies of resistance with potyviruses may increase the probability of discovering new fundamental principles regarding resistance mechanisms in plants to viruses that apply to all model systems.

Potato and potato virus Y (PVY), the type member of the potyvirus group, have been described primarily with reference to breeding programs for virus-resistant potatoes. Resistance in potato to PVY has
been classified by symptomatology ranging from systemic necrosis to no detectable symptoms (Barker and Harrison, 1984). Multiplication of PVY was detected by immunofluorescence in protoplasts made from a large number of the resistant potato cultivars thought to contain a single dominant allele conditioning resistance to PVY. However, protoplasts from the cultivars Corine and Pirola, which are cultivars known to contain the \( Ry \) resistance gene to PVY from *Solanum stoloniferum* and that are symptomless when inoculated with PVY, were found to be resistant to infection by both intact virus and PVY RNA. These studies seem to suggest that resistance in Corine and Pirola may involve inhibition of virus multiplication within the cell and not cell-to-cell spread; the other lines appear to possess resistance to PVY that may inhibit cell-to-cell spread.

Numerous studies regarding the genetics of resistance in maize to maize dwarf mosaic virus (MDMV) have been reported (Johnson, 1971; Josephson and Naidu, 1971; Scott and Rosenkranz, 1975; Naidu and Josephson, 1976; Roane et al., 1983; Mikel et al., 1984). These studies suggested that resistance to MDMV is most likely conditioned by relatively few genes. The maize inbred Pa 405, one of the most-studied resistant inbreds to MDMV, is symptomless when inoculated with MDMV strains A and B (Lei and Agrios, 1986; Louie, 1986). MDMV-B replicated to high titers and spread locally in inoculated leaves, but did not spread systemically. Infectivity of virus recovered from resistant plants was comparable to that from susceptible plants, which Lei and Agrios (1986)
suggested was an indication of resistance acting at some other level than virus inactivation. Immunofluorescent staining of inoculated resistant leaves suggested that the number of infection loci were low as compared to leaves from susceptible plants. Lei and Agrios (1986) speculated that virus spread through the leaf vascular system may be inhibited in resistant plants. Although not specifically stated by the authors, cell-to-cell spread may be affected in Pa 405.

Several linkage studies relating gene(s) conditioning MDMV resistance to other markers have located resistance gene(s) on chromosome 6 (Scott and Nelson, 1971; Scott and Rosenkranz, 1975; McMullen and Louie, 1989; Scott, 1989). Roane et al. (1989a) have shown that several inbred maize lines have single dominant genes conditioning resistance to MDMV A. Scott (1989) found that linkage exists between the MDMV-A resistance gene and the endosperm color gene \((Y_f)\) in several maize inbreds, not including Pa 405. The data revealed that most of the resistant lines have a single gene linked to the endosperm color gene, but that other gene(s) for resistance to MDMV also exist. The gene linked to \(Y_f\) is most likely dominant for all but one of the lines studied. McMullen and Louie (1989) used RFLP analysis to identify a gene controlling symptom response to MDMV on chromosome 6 in the maize inbred Pa 405. This gene, \(Mdm\,1\), was essential for resistance, since plants lacking this gene developed generalized mosaic symptoms. Roane et al. (1989b) named this gene \(Rmd\,1\), and found the gene was
located on the centromere side of $Y_1$ in Pa 405 and two other lines; the
gene was also found to be linked to the gene conditioning endosperm
type, $su2$.

Tobacco vein mottling virus (TVMV) and tobacco have been
recently studied as a model for potyvirus disease resistance in plants.
Gibb et al. (1989) compared virus multiplication in resistant (Tn 86) and
susceptible (Ky 14) cultivars of tobacco. TVMV did not spread to
uninoculated leaves, but was recovered from inoculated leaves.
Immunostaining of epidermal leaf strips revealed that viral coat and
cylindrical inclusion proteins were detected in Ky 14 as early as 5 days
post inoculation. In contrast, detection occurred 15 days after inoculation
in the resistant Tn 86. Similar results were obtained by immunostaining
mesophyll cells from each of the cultivars. The spread and distribution in
Tn 86 of TVMV-S, a TVMV isolate that systemically infects Tn 86, were
similar to that of TVMV in Ky 14. Electroporation of protoplasts from both
resistant and susceptible cultivars supported TVMV multiplication,
although lower amounts were detected in protoplasts from resistant
plants. Gibb et al. (1989) concluded that resistance in Tn 86 was due
primarily to restriction of virus movement, but conceded that a reduction in
the number of initial infection sites and the rate of virus accumulation may
also play a role.

Soybean and soybean mosaic virus (SMV) represent a potentially
informative system for the study of potyvirus disease resistance. Strains
of SMV have been placed into groups (G1-G7, G7a and C14) based upon their pathogenicity to differential soybean lines (Cho and Goodman, 1979; 1982; Buzzell and Tu, 1984; Lim, 1985; Chen et al., 1988a). In addition, several soybean lines contain a single dominant allele that confers resistance to most SMV strains (Kiihl and Hartwig, 1979; Roane et al., 1986a, b; Buss et al., 1987; 1988). The line PI 96983 contains the single dominant allele *Rsv*, that confers resistance to all virulence groups except G7 and G7a (Buss et al., 1988; Chen et al., 1988a). L78-379, a resistant line derived from the susceptible line Williams Union with *Rsv* from PI 96983 (R. Bernard, Univ. of Illinois, Urbana, personal communication), is also resistant to all virulence groups except G7 and G7a (Chen et al., 1988a). The soybean cultivar Davis is susceptible to SMV strains G4-G7 and G7a, as are the cultivars York, Dorman, and Ware (Roane et al., 1986b; Chen et al., 1988a); these cultivars are thought to possess a single dominant resistance gene that is allelic to *Rsv* from PI 96983 (Roane et al., 1986b). Table 1 summarizes the discussed examples of constitutive host plant resistance to viruses.

**Use of Protoplasts for Analysis of Disease Resistance**

These virus-host combinations represent most of the well-characterized systems for the study of disease resistance to viruses in plants. One of the principle experimental approaches used in these
Table 1. Examples of constitutive host plant resistance to viruses

<table>
<thead>
<tr>
<th>Plant host</th>
<th>Resistance gene(s)</th>
<th>Virus</th>
<th>ts / tr&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Infection of protoplasts</th>
<th>Virulent isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tomato</td>
<td>Tm-1</td>
<td>TMV</td>
<td>ts</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Tomato</td>
<td>Tm-2</td>
<td>TMV</td>
<td>ts</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Tomato</td>
<td>Tm-2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>TMV</td>
<td>ts</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Cowpea cv. Arlington</td>
<td>nd&lt;sup&gt;d&lt;/sup&gt;</td>
<td>CPMV</td>
<td>nd</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Cucumber</td>
<td>nd</td>
<td>CMV</td>
<td>nd</td>
<td>No</td>
<td>nd</td>
</tr>
<tr>
<td>Cowpea</td>
<td>nd</td>
<td>CCMV</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Potato</td>
<td>Nx, Nb</td>
<td>PVX</td>
<td>nd</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Bean</td>
<td>bc-u, bc-x&lt;sup&gt;c&lt;/sup&gt;</td>
<td>BCMV</td>
<td>nd</td>
<td>nd</td>
<td>Yes</td>
</tr>
<tr>
<td>Potato</td>
<td>Ry</td>
<td>PVY</td>
<td>nd</td>
<td>No</td>
<td>nd</td>
</tr>
<tr>
<td>Maize</td>
<td>Mdm 1</td>
<td>MDMV</td>
<td>nd</td>
<td>nd</td>
<td>Yes</td>
</tr>
<tr>
<td>Tobacco</td>
<td>nd</td>
<td>TVMV</td>
<td>nd</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Soybean</td>
<td>Rsv</td>
<td>SMV</td>
<td>nd</td>
<td>nd</td>
<td>Yes</td>
</tr>
</tbody>
</table>

<sup>a</sup>(tr) = temperature resistant; (ts) = temperature sensitive.
<sup>b</sup>(nd) = not determined or not reported.
<sup>c</sup>Recessive alleles.
systems is the use of protoplasts for analysis of the effect of resistance on the viral multiplication cycle. Two types of conclusions have been drawn. In the situation that occurs less frequently, protoplasts from resistant plants do not support virus multiplication. Examples of this type of resistance mechanism include: the $Tm\text{-}1$ gene of tomato, the Arlington cultivars of cowpea, Kyoto cucumber, the $Nx$ gene in potato, and the $Ry$ gene of potato model system. Resistance in these cases is more likely caused by inhibition of virus multiplication within the individual cell than by inhibition of cell-to-cell spread. The more common observation is that protoplasts from plants resistant to systemic viral infection support viral multiplication, suggesting that resistance is caused by inhibition of cell-to-cell spread and subsequent long distance movement of virus. Examples include the $Tm\text{-}2$ gene in tomato plants resistant to TMV, most of the cowpea lines surveyed for resistance to CPMV, and most potato cultivars resistant to PVY. The models for disease resistance that have been studied in greatest detail have been those in which resistance acts at the cellular level and is observed in protoplasts. This may be of coincidence and due to the interests of the investigators. If any general conclusion can be drawn about natural selection of disease resistance, it appears that resistance which does not function at the protoplast level is more prevalent in nature and it may be the most successful way for a host plant to resist viral infection. If this is the case, the fundamental study of virus
movement in plants at the molecular level could provide information that would be of practical use in understanding disease resistance.

**Virus Movement in Plants**

The importance of virus movement in plants has been recently recognized (Baulcombe and Hull, 1989); virus movement in relation to disease resistance, host range, and host specificity has been reviewed (Atabekov and Dorokhov, 1984; Hull, 1989). Virus movement in plants consists of short-distance cell-to-cell movement via plasmodesmata and long distance spread through conductive tissues. Two models have been proposed to explain how viruses move cell-to-cell. The first model implies a specific interaction between the virus and the plant leading to translocation of virus. The second model involves a non-specific, 'gated' intercellular connection that allows abnormally large molecules, including viruses, to pass through. A recent report used a novel method to deliver non-plasmalemma-permeable fluorescent probes to the cytosol of spongy mesophyll cells of tobacco leaves to study the plasmodesmatal size exclusion limits in transgenic plants that express the TMV movement protein gene (Wolf et al., 1989). This study was consistent the second model. Although it is unknown how the 30 K protein of TMV potentiates virus movement cell-to-cell (Deom et al., 1987; 1990), the 30 K protein has been identified as a RNA and single-stranded DNA binding protein
(Citovsky et al., 1990). Saito et al. (1990), using mutants of the L strain of TMV, have suggested that both the coat protein and the assembly of origin of TMV are involved in long-distance movement, and that virus particles may play a major role in the movement process.

Early evidence implicating a viral-encoded movement function came from two types of experiments. The first was from studies revealing subliminal viral infection of plants and infection of isolated protoplasts. Examples include TMV in cowpea and cotton (Sulzinski and Zaitlin, 1982) and cauliflower mosaic virus (CaMV) in cotton (Hussain et al., 1987), as well as TMV in tomato containing the $7m-2$ gene, CPMV in most resistant cowpea lines, and PVY in most resistant lines of potato.

The second line of evidence came from complementation experiments where infection of a plant with one virus (called the "helper virus") allowed systemic infection of a second virus. Complementation in the virus transport function has been implied from mixed infection experiments between related and unrelated viruses (e.g., brome mosaic virus helped by TMV to spread in tomato and bean, TMV helped by PVX to spread in plants containing the $7m-2$ gene, TMV helped by barley stripe mosaic virus to spread in wheat, and potato leaf roll virus helped by PVY to spread in potato) (Taliansky et al., 1982a, b; Carr and Kim, 1983; Barker, 1987; 1989). Such studies have been interpreted to imply that the movement protein of a helper virus is likely to have some general effect.
on cellular physiology that ultimately results in cell-to-cell movement of virus.

A recent report by Malyshenko et al. (1989) showed complementation between various tobamoviruses. Malyshenko et al. concluded from this study that complementation of virus transport may be non-specific since complementation occurred between viruses with putative transport proteins that differed extensively in amino acid sequence (Saito et al., 1988; Melcher, 1990). These studies imply not only a viral-encoded movement protein, but the apparent non-specific nature of complementation in virus movement (Saito et al., 1988; Melcher, 1990). In summary, virus movement, though not completely understood, is an important aspect in the study of disease resistance in plants.

Use of Single Resistance Genes for Studying Disease Resistance

The second principle experimental approach (along with the use of protoplasts for analysis of the effect of resistance on the viral multiplication cycle) used in these systems has involved classical genetic studies that have suggested virus disease resistance often involves a single dominant resistance allele. This suggests a possible gene-for-gene relationship between the resistance gene and the virulence gene. If this is the case, then most of the known virus-host resistance systems should
be fairly amenable to genetic and molecular analyses. Those systems that have a large number of virus pathotypes could make the identification of virulence gene(s) relatively easier; such systems include TMV-tomato, TMV-tobacco, BCMV-bean, PVX-potato, SMV-soybean. In addition, systems with well characterized single resistance genes in characterized genetic backgrounds will make isolation and characterization of the resistance gene feasible. The *Tm*-2 gene, conferring resistance to TMV, and *Mdm* 1 (*Rdm* 1) gene, conferring resistance to MDMV, are examples of disease resistance where attempts have been made to isolate and characterize the resistance gene responsible.

**Research Presented in This Dissertation**

Soybean mosaic virus (SMV) and *Glycine max* (L.) Merr. (soybean) were used as a model system to study virus disease resistance in plants. Aspects of the model investigated were: (1) characterization of factors influencing disease resistance; (2) identification of virus-encoded functions altered in resistant plants; (3) effects of leaf tissue extracts from resistant soybean on the cell-free translation of SMV RNA.

Observations of the interaction of the known strains of SMV with soybean lines of varying susceptibility to infection, led to the following hypotheses: (1) Environmental conditions (e.g., temperature) may influence disease resistance in lines of soybean resistant to infection by
SMV; (2) Co-inoculation of soybean plants with virulent and avirulent strains of SMV may allow functional complementation that could lead to systemic movement of the avirulent virus strain in the resistant lines of soybean; and (3) Leaf extracts from resistant soybean may affect the cell-free translation of SMV RNA.

Part I describes the development of a technique to detect virus location in infected leaf tissue. In Part II, this technique is used, as a test of the first hypothesis, to study the effects of temperature on the maintenance of resistance in soybean to SMV. Part III presents a series of complementation experiments with virulent strains of SMV as helper viruses, as a test of the second hypothesis. In Part IV, as a test of the third hypothesis, the effects of fractionated leaf extracts from either the susceptible soybean cultivar Williams '82 or the resistant cultivar Davis, on the translation of SMV RNA in rabbit reticulocyte lysates were investigated. Part V presents the cell-free translation profiles of representatives from strain groups of SMV, in addition to other unclassified SMV isolates, using both wheat germ extracts and rabbit reticulocyte lysates.

**Explanation of Dissertation Format**

This dissertation is written in the Alternate format. Each major division (Parts I-V) is a complete manuscript modified to conform with the
specifications of the Iowa State University Thesis Office. Each part has its own abstract, introduction, materials and methods, results, discussion, and literature cited. Following these five parts is a general discussion of the entire dissertation. Part I was published in Plant Molecular Biology Reporter 8:13-17 (1990). Part II has been submitted for publication to Phytopathology.
PART I: PLANT VIRUS LOCATION IN LEAF TISSUE BY PRESS BLOTTING
PART I: PLANT VIRUS LOCATION IN LEAF TISSUE BY PRESS BLOTTING

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Iowa State University
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ABSTRACT

A method, termed press blotting, is described which allows localization of plant virus in infected leaf tissue. Press blotting should have broad applicability to identifying the distribution and location of proteins and nucleic acids in plants.
INTRODUCTION

The recent development of the tissue printing technique has been useful for determining the location and distribution of specific proteins and nucleic acids (Cassab and Varner, 1987; McClure and Guilfoyle, 1989). Although several tissue types have been successfully printed onto nitrocellulose paper (e.g., seed, cotyledon, root, hypocotyl), leaf tissue, for technical reasons, has not been readily amendable to tissue printing. Navot et al. (1989) recently described a method (squash blot) whereby tomato leaves, roots, stems, flowers, and fruits were squashed onto a nylon membrane by using a glass rod or pen. Although tomato leaf tissue can be easily squashed onto membranes, leaf tissues from other less succulent plants are not as amenable. In attempts to localize the distribution of a plant virus in infected leaf tissue, we investigated various methods for blotting leaf tissue onto membranes. The method developed (press blot) uses a hydraulic press for tissue blotting. The press blot is a logical extension of tissue printing and should be of use in studying the distribution, during development, of plant proteins and mRNAs. The press blot should be applicable to a wider range of plant leaf tissues than the squash blot.
MATERIALS AND METHODS

Special Solutions and Equipment Required

The hydraulic press was a Carver laboratory press (model B) from Fred S. Carver Inc., Summit NJ. Nitrocellulose (cat. no. 162-0114), gelatin (cat. no. 170-6537), protein A-gold (cat. no. 170-6524), and the gold enhancement kit (cat. no. 170-6538) were from Bio-Rad (Richmond, CA). Monoclonal antibody S-10 used in the experiments has been described (Hill et al., 1989). Solutions of TBS (130 mM NaCl, 15 mM Tris-HCl, pH 7.2) and blocking buffer (1.5% gelatin in TBS) were prepared and used.

Preparation of Plant Material

Soybean plants (Glycine max cv. Williams '82) were used as the source of plant material. Soybean leaf tissue represents plant leaf tissue that is not very amenable to squash blottting. Plants were maintained in a growth chamber with an 18 h day length at 22°C. Primary leaves were inoculated (before development of trifoliate leaves) with an isolate of soybean mosaic virus (SMV). Soybean trifoliate leaves were sampled 10 days postinoculation. As a control, soybean trifoliate leaves from uninoculated plants were sampled. Samples were either immediately
used for the press blot or were treated at -70° C for 10 min and returned to room temperature before press blotting.

**Press Blot**

Two pieces of Whatman no. 1 filter paper were placed below a piece of dry nitrocellulose (the filter paper acts as an absorbent for excess sap that is forced through the nitrocellulose). Leaf samples were placed onto dry nitrocellulose with the undersurface of the leaf in direct contact with the membrane. This sandwich was wrapped in Saran Wrap and placed in the hydraulic press, applying 10,000 psi for 1 min. The sandwich was disassembled, and the nitrocellulose was allowed to air dry.

**Detection of Virus**

For detection of virus by antibody, press blots were analyzed as follows:

1) Press blots were blocked to eliminate nonspecific binding of detecting antibodies by incubating in blocking buffer for 30 min at 37° C.

2) The blots were incubated for 15 h at 37° C in blocking buffer containing a 1:2000 dilution (from ascites fluid) of monoclonal antibody to SMV coat protein.

3) Blots were washed in TBS for 30 min.
4) Blots were placed in an undiluted protein A-colloidal gold solution and incubated until color development.

5) Color was enhanced by using a colloidal gold enhancement kit.
RESULTS

Press blotting of virus-infected leaf tissue is a simple and efficient method for determination of virus location in leaf tissue. The blotting parameters of 10,000 psi for 1 min yielded optimal extrusion of plant sap from leaf tissue. When tissue was subjected to a freeze/thaw cycle of 10 min at -70°C before blotting, a very distinct press blot of leaf tissue is obtained. For example, in Figure 1, a detailed image of the blotted leaves is observed. When blots were probed with antibody to SMV coat protein, only virus-infected soybean leaves reacted with the antibody. Lower background was observed with nitrocellulose matrices than with nylon-based membranes.

The best leaf image was obtained when leaves were frozen before blotting. For applications where location of enzymatic activity is desired, leaf tissue can be blotted without prior treatment, but we found less detail of soybean leaf tissue when it was immediately blotted. Blotting by electrophoretic transfer was also tried as an alternative to using high pressure as a method for blotting leaf material onto nitrocellulose paper. Image resolution was lower as compared with the press blot (data not shown). Infected and uninfected tissue could be readily differentiated, although virus was detected only near leaf veins. We therefore found press blotting the superior method.
DISCUSSION

We present here a method for the location of proteins that should be of general use to plant molecular biologists. The method should also be applicable for the location of nucleic acids. The ability to locate proteins and nucleic acids in plants by press blotting should have several applications. One is the study of location and expression of plant viral pathogens. The ability to determine virus location in entire leaves should allow analysis of disease symptomatology in relation to sites of viral location. We have shown that the method has immediate application to the simple and efficient distribution and location of virus in leaf tissue. Similarly, press blotting should also be of utility to plant developmental biologists who wish to locate the distribution of particular plant proteins or mRNAs. Thus, this method extends the tissue printing technique to leaf tissue. The simplicity of the procedure should make its use routine in the plant molecular biology laboratory.
LITERATURE CITED


Figure 1. Press blot of soybean leaf tissue. Trifoliolate leaves from soybean plants were kept at -70°C for 10 min and returned to room temperature before press blotting. Pressure at 10,000 psi was applied for 1 min by using a hydraulic press. Blots were incubated with a monoclonal antibody to soybean mosaic virus coat protein. Detection of antibody binding was done by using protein A-coupled colloidal gold particles followed by silver-stain enhancement. The light-colored leaf press blot (right side) represents uninoculated leaf tissue; the dark-stained leaf press blot (left side) represents virus-infected leaf tissue
PART II: EFFECTS OF TEMPERATURE ON THE MAINTENANCE OF RESISTANCE IN SOYBEAN TO SOYBEAN MOSAIC VIRUS
PART II: EFFECTS OF TEMPERATURE ON THE MAINTENANCE OF RESISTANCE IN SOYBEAN TO SOYBEAN MOSAIC VIRUS

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ABSTRACT

The effects of temperature upon naturally occurring disease resistance to soybean mosaic virus (SMV), strain G2, were studied by using resistant soybean lines PI 96983, L78-379, and Davis. When plants were shifted from 20° C to 10° C after inoculation for 10 days, viral coat protein of SMV-G2 accumulated in trifoliate leaves of resistant plants inoculated with SMV-G2, but not when shifted to the higher temperatures tested. Infectious SMV was recovered from these leaves by local lesion assay. Temperature had no apparent affect on the accumulation of coat protein of SMV-G2 in trifoliate leaves of inoculated plants of the susceptible cultivar Williams '82. Moreover, temperature did not influence accumulation of coat protein in susceptible and resistant lines inoculated with a resistance-breaking strain, SMV-G7.
INTRODUCTION

Temperature is a significant factor that can influence the disease process of viruses in plants (Matthews, 1981). However, studies specifically addressing the effects of temperature on virus disease resistance known to be conditioned by one or a few genes are limited. The most notable examples are illustrated by the $Tm$ genes in tomato. The $Tm$-1 gene completely suppressed symptoms induced by tobacco mosaic virus (TMV), strain 0, when plants were grown at constant temperatures from 20 to 35° C; high temperatures severely reduced virus multiplication (Fraser and Loughlin, 1982). In contrast, TMV strain 1 caused more severe symptoms at higher temperatures. Strain 1 multiplication was inhibited at 25° C, but not at 33° C (Fraser and Loughlin, 1982). Tomato plants containing the $Tm$-2 or $Tm$-2$^2$ genes for resistance to TMV are symptomless at normal temperatures after inoculation with strain 0 but severe systemic necrosis occurs at elevated temperatures (Cirulli and Alexander, 1969; Pelham, 1972; Schroeder et al., 1967). Similar effects of high temperature on resistance to TMV have been reported with the $N$ and $N'$ genes in tobacco (Fraser, 1983; van Loon, 1975) and with transgenic tobacco expressing the coat protein of TMV (Nejidat and Beachy, 1989).

Disease resistance of soybean to soybean mosaic virus (SMV) provides an appropriate model to study disease resistance. First, there
are a large number of known virus strains that differ in pathogenicity. Second, several lines of soybean have been identified with known single, dominant genes conferring resistance to SMV. Third, SMV is a member of the largest group of plant viral pathogens (the potyvirus group) of economic importance.

Isolates of SMV have been placed into virulence groups (G1-G7, G7a and C14) based on the phenotypic response of differential soybean lines to virus inoculation (Buzzell and Tu, 1984; Cho and Goodman, 1979, 1982; Lim, 1985; Roane et al., 1986b). In addition, several soybean lines contain a single dominant allele conferring resistance to strains of SMV (Kiihl and Hartwig, 1979; Buss et al., 1987, 1988; Roane et al., 1986a, b). The line PI 96983 contains the single dominant allele, Rsv, that confers resistance to all strains except G7 and G7a (Buss et al., 1988; Chen et al., 1988). L78-379, a resistant line derived from a cross of the susceptible soybean cultivar Williams Union with PI 96983 containing Rsv (R. Bernard, Univ. of Illinois, Urbana; personal communication), is also resistant to all virulence groups except G7 and G7a (Chen et al., 1988; Roane et al., 1986b). The soybean cultivars Davis, Dorman, Ware, and York are susceptible to strains SMV-G4, G5, and G6, as well as G7 and G7a (Chen et al., 1988; Roane et al., 1986b). These lines are thought to possess a single, dominant resistance gene from PI 96983 that is allelic to Rsv (Roane et al., 1986b).
The objective of this research was to develop experimental parameters to manipulate disease resistance by testing the effects of temperature on resistance. Low temperatures allowed virus to overcome disease resistance and induce systemic spread of SMV in resistant soybean lines.
MATERIALS AND METHODS

Plants and Viruses

The soybean lines and virus strains used in this study are described in Table 1. Plants were maintained in growth chambers operating at constant temperatures between 10 and 35° C with a daylength of 18 h and an irradiance of 50 W/m². The two fully expanded primary leaves of soybean seedlings 5-10 cm tall, grown at 20° C, were mechanically inoculated by using infectious sap prepared in 50 mM sodium phosphate buffer, pH 7.0. Leaves were rinsed with running water immediately after inoculation.

Temperature-shift of Inoculated Plants

Seedlings grown in pots at 20° C (approx. 5-8 plants per pot) were inoculated with either SMV-G2, SMV-G7, or were mock-inoculated (50 mM sodium phosphate buffer, pH 7.0) and placed at a constant 10, 15, 20, 25, 30, or 35° C for 10 days, and then returned to 20° C for an additional 10 days. Plant height was measured at inoculation and at 10 and 20 days postinoculation.
Press Blotting

On the tenth day after returning to 20° C, secondary or tertiary trifoliate leaves from plants were sampled for presence of virus antigen by press blotting as previously described (Mansky et al., 1990), using monoclonal antibody S10 to the coat protein of SMV (Hill et al., 1989).

Local Lesion Assay

Recovery of infectious SMV from trifoliate leaves of soybean lines was tested by local-lesion assay. Primary, secondary, or tertiary trifoliate leaves (approximately 0.25 g of leaf tissue) collected 10 days after plants were returned to 20° C were ground in 1 ml of 50 mM sodium phosphate buffer, pH 7.0. Assays were done in triplicate for three plants of each inoculated soybean line grown at defined temperatures. The expressed sap was used to inoculate detached leaves of Phaseolus vulgaris cv. Top Crop as described (Milbrath and Soong, 1976).
RESULTS

Plant Growth Measurements

Growth of virus-inoculated soybean lines, as measured by plant height, was generally directly correlated with temperature (Fig. 1). Growth of "mock-inoculated" soybean lines was similar to that of virus-inoculated plants (data not shown). Because trifoliate leaves did not develop on plants grown at 10°C, plants were transferred to the original growth temperature (i.e., 20°C) for an additional 10 days. To compare results obtained in this experiment with those from plants grown at other temperatures, all plants were returned to the original growth temperature at the same time.

Press Blotting Analyses of Virus Location in Upper Leaves

Press blotting of trifoliate leaves maintained at 10°C after inoculation showed that SMV coat protein accumulated in all virus-inoculated plants. The presence of coat protein was not evident in mock-inoculated plants (Fig. 2). SMV strain G7 coat protein appeared at all temperatures. Only in Williams '82 did SMV-G2 coat protein accumulate at all temperatures. In resistant lines, viral coat protein was found with plants shifted to 10°C for 10 days, but not with plants shifted to 15, 20, 25,
30, or 35°C for 10 days. Presence of SMV coat protein in trifoliate leaves was also confirmed (data not shown) by double-sandwich enzyme-linked immunosorbent assay (Chen et al., 1982).

**Virus Infectivity in Trifoliate Leaves**

To correlate the presence of viral coat protein in trifoliate leaves, as determined by press blotting, with viral infectivity, other leaf samples from the same plants were used for local-lesion assay (Table 2). Samples which contained infectious virus, as indicated by local lesion formation, correlated with the detection of viral coat protein in other leaf samples from the same plants (Fig. 2). Variation in the presence of local lesions occurred in different samples from the same plant; however, at least two of the three samplings always contained infectious virus.
DISCUSSION

The detection of SMV coat protein in trifoliate leaves of plants inoculated with SMV was used as a criterion for susceptibility or resistance; these data were confirmed by local lesion assays (Table 2) and ELISA (data not shown). Since a minimum concentration of 1 mg virus per ml is needed to produce local lesions with SMV (Milbrath and Soong, 1976), local lesion formation suggested that a relatively high concentration of virus was present in the trifoliate leaves sampled.

Breaking of disease resistance in plants by low temperature has not been previously reported. Most studies addressing the effects of temperature on resistance to viruses have examined only elevated temperatures (Fraser, 1983; Fraser and Loughlin, 1982; van Loon, 1975) and not low temperatures. However, the breaking of disease resistance reported here may not be unique to the SMV-soybean system and could exist in other systems.

Resistance in the line L78-379 was derived from soybean line PI 96983; thus, the response of L78-379 was expected to be similar to that of PI 96983 (Roane et al., 1986a, b). In contrast, resistance in the cultivar Davis is derived from an allelic gene (Cho and Goodman, 1979; Roane et al., 1986b); in addition, the reaction of Davis to SMV strains G4, G5, and G6 is also different from PI 96983 and L78-379 (Chen et al., 1988; Cho and Goodman, 1982; Roane et al., 1986b). However, resistance in Davis
was also altered by low temperature; this may suggest a similar resistance mechanism to the G2 strain in these lines.

It may be important to follow and quantitate viral multiplication during and after the low temperature shift. Trifoliate leaves on soybeans do not develop at 10°C during a 10-day period; therefore, only primary leaves could be used for analysis at 10°C. If primary leaves are inoculated on only one-half of the leaf with virus, the other side of the leaf could be used to determine virus spread. Analysis of the effect of low temperature on viral multiplication in protoplasts may also yield significant results.

The mechanism responsible for disease resistance is unknown. The results of this study suggest that disease resistance may be repressed at low temperatures, even after plants are returned to higher temperatures. Alternatively, resistance may be overcome at low temperatures, thus allowing for viral multiplication; but resistance may eventually be restored to inhibit viral multiplication when plants are returned to a higher temperature.

In our initial studies, we have manipulated temperature to induce susceptibility of several soybean lines resistant to a strain of SMV. The development of an experimental procedure to alter resistance may be a useful tool to study disease resistance.
LITERATURE CITED


Table 1. Symptoms induced in soybean lines inoculated with two strains of soybean mosaic virus

<table>
<thead>
<tr>
<th>Soybean lines</th>
<th>Resistance genes</th>
<th>Phenotypic response of virus-inoculated plants&lt;sup&gt;a&lt;/sup&gt;</th>
<th>G2&lt;sup&gt;b&lt;/sup&gt;</th>
<th>G7</th>
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<tr>
<td>Williams '82</td>
<td>-</td>
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<td>S</td>
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<td>PI 96983</td>
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<td>Rsv</td>
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<td>N&lt;sup&gt;c&lt;/sup&gt;</td>
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</tr>
<tr>
<td>Davis</td>
<td>Rsv?</td>
<td>0</td>
<td>S</td>
<td></td>
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<sup>a</sup> 0= no reaction; N= systemic necrosis; S= systemic mottling; data from Roane et al. (1986b).

<sup>b</sup> Isolate la 75-16-1 (Hill and Benner, 1980a,b).

<sup>c</sup> Determined in this study.
Figure 1. Height of SMV-inoculated soybean lines at various times during temperature treatments. Height of soybean lines inoculated with SMV-G2 or SMV-G7 was recorded at inoculation (day 0), at temperature shift (day 10), and when leaves were sampled for analysis by press blot and local lesion assay (day 20). Data are the mean and standard deviation (vertical bars) for the height of all virus-inoculated plants of each soybean line transferred to 10, 15, 20, 25, 30, or 35°C.
Figure 2. Press blotting of trifoliolate leaves from soybean lines grown at various temperatures after “mock” or mechanical inoculation with either SMV strains G2 or G7. Soybean lines “mock-inoculated” or inoculated with SMV-G2 or SMV-G7 were shifted to 10, 15, 20, 25, 30, or 35°C. Plants were then returned to 20°C for 10 days. Secondary or tertiary trifoliolate leaves were collected and analyzed by press blot. Comparison of results from leaves within individual panels is appropriate; however, because of the experimental protocol, direct comparison of leaves in different panels is inappropriate. Dark-stained press blots represent virus-infected leaf tissue; light-colored press blots represent uninfected leaf tissue.
<table>
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<th>Temp. (°C)</th>
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<th>PI 96983</th>
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<td>Mock</td>
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Figure 2. cont.
<table>
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<th>Temp. (°C)</th>
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Inoculum: Mock SMV-G2 SMV-G7

Temp. (°C): 10, 15, 20, 25, 30, 35
Table 2. Results of local-lesion assays of soybean lines inoculated with SMV strains G2 or G7 or "mock-inoculated" with phosphate buffer

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<th>SMV-G7</th>
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<sup>a</sup>Three samples of trifoliate leaves were assayed from three different plants of soybean lines inoculated on primary leaves and subjected to temperature treatment. + = presence of local lesions; - = absence of local lesions.
PART III: COMPLEMENTATION OF PLANT POTYVIRUS PATHOGENIC STRAINS IN MIXED INFECTION
PART III: COMPLEMENTATION OF PLANT POTYVIRUS PATHOGENIC STRAINS IN MIXED INFECTION

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Iowa State University
Ames, Iowa
ABSTRACT

Co-inoculation of soybean lines resistant to SMV strain G2 with either SMV-G7 or SMV-G7a and SMV-G2 resulted in systemic spread of the avirulent SMV-G2 as well as the virulent isolates. Systemic movement of SMV-G2 was demonstrated by re-isolation of virus from local lesions and identification of viral coat protein present in infected tissue. Thus, SMV-G7 and -G7a complement a G2 function which is inactive in resistant plants inoculated with G2 alone.
INTRODUCTION

Soybean mosaic virus (SMV) is a member of the large and economically important potyvirus group of plant viruses (Bos, 1972). Potyviruses are characterized by a single-stranded, positive-sense RNA genome of approximately 10 kb, a single type of coat protein, and a flexuous rod morphology; considerable information is available regarding genomic structure and expression (reviewed by Dougherty and Carrington, 1988). SMV and its natural host, soybean (Glycine max L. Merr), represent a potentially useful system to study plant-virus interactions.

SMV isolates have been grouped (G1-G7, G7a and C14) based upon pathogenicity on a set of differential soybean lines (Cho and Goodman, 1979; 1982; Buzzell and Tu, 1984; Lim, 1985; Roane et al., 1986b; Chen et al., 1988). Several soybean lines, including PI 96983 and L78-379, contain a single dominant allele, Rsv, which confers resistance to all SMV strains except G7 and G7a (Kiihl and Hartwig, 1979; Roane et al., 1986a, b; Buss et al., 1987; 1988; Chen et al., 1988). The soybean cultivar Davis, containing a gene that is allelic to Rsv from PI 96983 (Roane et al., 1986a), is susceptible to SMV strains G4-G7 and G7a, as are the cultivars York, Dorman, and Ware (Roane et al., 1986b; Chen et al., 1988). In contrast, the cultivar Williams '82 is susceptible to
isolates representing strains G1-G7 (Cho and Goodman, 1979; 1982), G7a, and C14.

It has recently been shown that low temperature induces susceptibility to the G2 strain of SMV in the lines PI 96983, L78-379, and Davis (Mansky et al., unpublished). Extracts of leaf tissue from the soybean cultivar Davis have no discernible effects on the cell-free translation of SMV-G2 RNA that are associated with disease resistance (Mansky et al., unpublished), suggesting that the resistance mechanism does not alter viral translation or proteolytic processing.

Little is known concerning how the Rsv gene functions or of the genetic differences between the known virulence groups of SMV. Complementation between plant viruses in mixed infection of plant resistant to one of the viruses have suggested resistance acting at the level of virus movement (Taliansky et al., 1982a, b; Atabekov and Dorokhov, 1984). To test whether complementation between viruses could lead to systemic spread of SMV-G2 in soybean lines resistant to SMV-G2, mixed inoculation experiments were performed. In this study, we demonstrate that infection of soybean with virulent and avirulent strains of SMV allows systemic spread of the avirulent strain, SMV-G2.
MATERIALS AND METHODS

Viruses and Plants

The SMV strains G2 (isolate la 75-16-1), G7, and G7a, as well as the soybean lines used in this study have been described previously (Cho and Goodman, 1979; 1982; Hill and Benner, 1980a, b; Buzzell and Tu, 1984; Lim, 1985; Roane et al., 1986b; Buss et al., 1987; 1988; Chen et al., 1988). Primary leaves of soybean seedlings were mechanically inoculated with infectious sap prepared by grinding 2 g of virus-infected leaf tissue in 20 ml of inoculation buffer (IB; 50 mM sodium phosphate, pH 7.0). Leaves were rinsed with water immediately after inoculation and plants were maintained in growth chambers at 20° C, with a daylength of 18 h and irradiance of 50 W/m².

Isolation of SMV Strains from Mixed Infection

Symptomatic trifoliate leaf samples from plants which had been inoculated on primary leaves with two SMV isolates were ground in IB and then sap was used to mechanically inoculate leaves of the local lesion host *Phaeseolus vulgaris* cv. Top Crop as described by Milbrath and Soong (1976). Individual local lesions were excised from leaves with a cork borer, ground in 100 ul of IB, and the homogenate was used to
inoculate the susceptible cultivar Williams '82. Infected trifoliate leaves from these plants were used as a source for a second cycle of virus strain isolation by local lesion assay followed by inoculation to Williams '82.

Analysis of Isolated SMV Strains by Reaction on Differential Soybean Lines

Sap from infected trifoliate leaves of Williams '82 soybean inoculated with individual local lesions was used to inoculate primary leaves of the soybean lines Williams '82, PI 96983, L78-379, and Davis. Plants were observed for symptom development for 4 weeks.

Identification of SMV Isolates by Signature Analysis

Wells in Dynatech Immulon I removewell strips (Dynatech Labs. Inc., Chantilly, VA) were coated with monoclonal antibody S-7 (Hill et al., 1989) (12 ug protein/ml, 50 ul/well) in carbonate coating buffer (0.05 M NaCO₃, pH 9.6). After incubation for 2 h at 20° C, wells were washed four times with BLOTTO (Johnson et al., 1984), prepared in 0.02 M sodium phosphate, pH 7.4, containing 0.85 % NaCl (PBS), and then submerged overnight in BLOTTO at 20° C. Uninoculated trifoliate leaves of soybean (1g) were ground in 2 ml of PBS, squeezed thru a double layer of cheesecloth, and centrifuged at 12,000 x g for 3 min. The
supernatant was diluted (1:10) in PBS and used as a diluent to prepare two-fold serial dilutions (1:1) of sap extracts from infected trifoliate leaf tissue that was diluted (1:10) in PBS prior to serial dilutions. After overnight incubation at 20°C, plates were washed four times with BLOTTO and antigen (100 ul) was added to plates. Plates were incubated at 20°C for 2-3 h, antigen was aspirated, and plates were washed four times with BLOTTO.

Monoclonal antibodies specific to the SMV coat protein (Hill et al., 1989) were radioiodinated by using a reaction involving the oxidation of radiiodide by chloramine-T (Hill, 1990). Specific activities ranged from 0.79 to 3.37 x 10^6 cpm/ug. ^125^I-labeled antibodies, diluted in PBS-1% bovine serum albumin, were added at 30 ng protein/well and plates were incubated at 20°C for 2 h. Labeled antibodies were removed by aspiration and plates were washed five times with BLOTTO. Individual wells were removed, placed in glass tubes, and radioactivity was measured by using a gamma counter. Data were corrected for specific activity and radioactive decay of the ^125^I-labeled antibodies. An iterative least squares technique was used to estimate the multiple response functions between the mean of ln cpm and antigen dilution. Statistical methods used in the iterative alignment and curve fitting have been described (Wands et al., 1984; Ben-Porath et al., 1985; Monath et al., 1986). Computer-graphic plots of the aligned binding curves were generated. Antigenic identity (quantitatively and qualitatively identical
epitopes) was determined when aligned antigen binding curves of two virus antigen samples were superimposable with the 8 monoclonal antibodies used in the signature panel.
RESULTS

Pathogenicity of Virus Isolates from Mixed Infections

Inoculation of resistant soybean lines with avirulent SMV-G2 and virulent SMV-G7 resulted in the systemic spread of both virus strains in Davis, but only G7 was found to spread in PI 96983 and L78-379 as determined by re-isolation of virus strains by using local lesion assays and subsequent inoculation of resistant lines (i.e., PI 96983, L78-378, and Davis) (Table 1). The virulent strain G7 was recovered with higher frequency than SMV-G2 in Davis and Williams '82. Inoculation with G2 and G7a led to systemic spread of both virus strains in all soybean lines at comparable frequencies (Table 1). SMV-G7 was recovered from all lines inoculated only with G7, as was SMV-G7a (Table 1); SMV-G2 was never recovered from PI 96983, L78-379, or Davis when only G2 was used as the inoculum (Table 1).

Identification of SMV Isolates by Signature Analysis

Signature analysis of virus strains G2 and G7 from infected trifoliate leaves of Williams '82 revealed antigenic differences between the two viruses when the monoclonal antibodies S-9 and S-10 were used (Figs. 1, 2). Thus, the two virus strains could be distinguished antigenically by
using leaf tissue infected with either strain. Signature analysis using trifoliate leaves from the soybean lines PI 96983 and Davis, which were co-inoculated with G2 and G7 (Figs. 3, 4), were antigenically distinct from either SMV-G2 or G7 (Figs. 1, 2). Binding curves generated by using trifoliate samples from Williams '82 inoculated with SMV-G2 and G7 (Fig. 5) or from a 1:1 mixture of infectious sap extracts of Williams '82 infected with either G2 or G7 (Fig. 6) were found to be antigenically distinct from binding curves of either strain alone from Williams '82 (Figs. 1, 2). Table 2 summarizes the antigenic similarities and differences of the virus antigens analyzed.
DISCUSSION

The data show that soybean lines (i.e., PI 96983, L78-379, and Davis) inoculated with virulent and avirulent strains of SMV are susceptible to the avirulent strain. Systemic spread in resistant plants of SMV was indicated by using local lesion assay; strain identification was determined by virus re-isolation and the testing of pathogenic characteristics on differential soybean lines. These data show that the G7 strain induced spread of G2, in mixed infection, only in the resistant line Davis and not in PI 96983 or L78-379, while the G7a strain induced spread of the avirulent SMV-G2 in all resistant lines inoculated. In mixed infections, SMV strains G7 and G7a were isolated at a higher frequency relative to SMV-G2 in Williams '82, and in the resistant lines PI 96983, L78-379, and Davis, indicating that G7 and G7a may replicate more efficiently in these lines than the G2 strain. In addition, the presence of both SMV-G2 and G7 or G7a in Williams '82 confirmed that both strains could systemically infect the same plant without cross-protection. Systemic spread of the G2 strain in resistant lines inoculated with a helper virus strain suggests complementation of a G2 function by strains G7 and G7a.

To confirm these data, an alternative approach, strain-specific identification of virus antigen, was used to detect two virus strains in some infected soybean trifoliate leaf tissue. Comparison of Figs. 2 and 3
suggest the presence of both SMV strains G2 and G7 in trifoliate leaf tissue of the soybean lines PI 96983 and Davis. Non-alignment of antigen-antibody binding curves from leaf samples of soybean plants inoculated with G2 and G7, as compared to binding curves from G2 or G7-infected leaf tissue, was consistent with the presence of two virus antigens. The presence of the G2 strain in trifoliate tissue from PI 96983 inoculated with G2 and G7 does not agree with data in Table 1. However, this is probably an indication of too limited a number of local lesions analyzed, rather than the complete absence of the G2 strain in trifoliate tissue from PI 96983. Also, the radioimmunoassay is more sensitive than the local lesion assay for detection of virus. Thus, the data suggest the presence of the G2 strain in PI 96983 inoculated with both G2 and G7. Although the samples analyzed were restricted to inoculations with strains G2 and/or G7, these data suggest that the presence of G2 and G7a could also be detected from infected leaf tissue.

Mixed infection experiments have been commonly used for genetic analysis of vertebrate viruses and RNA phages. These often include tests for complementation or recombination by using two temperature sensitive mutants which are defective in different functions (Fields, 1985). Complementation, often one of the first genetic tests performed, can rapidly divide mutants into functional groups for further study. Such analyses have been useful for virus groups in which recombination analyses are not possible due to the low level or absence of
recombination (e.g., togaviruses, rhabdoviruses, and paramyxoviruses) (Pringle, 1975; 1977; Bratt and Hightower, 1977; Pfefferkorn, 1977). The most common type of complementation, nonallelic (intergenic), occurs when two mutants defective in different functions assist each other in the virus multiplication cycle by complementing the defective function. Allelic complementation has also been observed, although less frequently. It occurs when the gene product of both viruses is a multimeric protein and each virus is defective in different domains of the functional viral protein (Fincham, 1966). Complementation in mixed infections of plant viruses has been suggested (Dodds and Hamilton, 1976; Taliansky et al., 1982b; Atabekov and Dorokhov, 1984; Mushegian et al., 1989), but has not been extensively used with most RNA plant viruses for genetic analysis.

RNA recombination has been reported with plant viruses (Bujarski and Kaesberg, 1986; Robinson et al., 1987), and the mechanism of recombination has been investigated with poliovirus (Kirkegaard and Baltimore, 1986). Two lines of evidence suggest that the observations we report can be explained by complementation of SMV strains and not recombination. First, virus strains that were re-isolated possessed the same pathogenicity as strains G2, G7, or G7a. Second, signature analysis of virus antigen from soybean lines inoculated with G2 and G7 indicated differences (Table 2) compared to analysis of virus antigen from lines inoculated with either SMV-G2 or -G7; these differences suggest the
presence of two virus antigens. However, the presence of two virus antigens in itself does not preclude the possibility of recombination.

Although complementation apparently occurred between virulent and virulent strains of SMV, other plant viruses that systemically infect these lines could not complement the G2 strain. When the resistant soybean lines PI 96983, L78-379, and York were inoculated with SMV-G2 and either cowpea chlorotic mottle, tobacco ringspot, cowpea severe mosaic, or alfalfa mosaic viruses, no systemic spread of SMV was observed (unpublished data). This observation may be important by comparison with other reports of virus complementation.

Complementation in the virus transport function has been implied from mixed infection experiments by related and unrelated plant viruses (Taliansky et al., 1982a, b; Carr and Kim, 1983; Barker, 1987; 1989; Malyshenko et al., 1989). It has been suggested that the putative movement protein of a helper virus may have some general effect on cellular physiology, mediating cell-to-cell movement of both viruses (Taliansky et al., 1982a, b; Saito et al., 1988; Hull, 1989). Our observation of no complementation between SMV-G2 and unrelated viruses may be an indication that complementation at the level of virus transport does not affect disease resistance in PI 96983, L78-379, and Davis to SMV, or that the putative movement protein of SMV is more specific.

Extensive complementation analyses of all combinations of virulence strain groups of SMV could allow the construction of functional
groupings of SMV strains. This would be useful in future genetic studies by providing information regarding functional differences between strains. Functional groupings should also be useful for mapping the virulence gene(s). This knowledge may help to understand the mechanism of disease resistance.
LITERATURE CITED


Table 1. Identification of soybean mosaic virus (SMV) strains recovered from soybean lines inoculated with strains G2 and G7 or G7a or with G2, G7, or G7a

<table>
<thead>
<tr>
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<th>Soybean linea</th>
<th>No. of local lesionsb</th>
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<th>G2</th>
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<td>Helper virus (SMV-G7 or -G7a) was used to inoculate primary soybean leaves 2 days before inoculation with SMV-G2. Trifoliate leaves from these plants were used for subsequent analyses.</td>
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Figure 1. Signature analysis of SMV strains G2 from infected trifoliate leaves of Williams '82 (SBLINE=1). Monoclonal antibodies S-1, S-2, S-3, S-4, S-9, S-10, and S-12 were designated 1, 2, 3, 4, 9, 10, and 12, respectively; monoclonal antibody A1-2 was designated 11.
Figure 2. Signature analysis of SMV strains G7 from infected trifoliolate leaves of Williams '82 (SBLINE=1). Monoclonal antibodies S-1, S-2, S-3, S-4, S-9, S-10, and S-12 were designated 1, 2, 3, 4, 9, 10, and 12, respectively; monoclonal antibody A1-2 was designated 11.
Figure 3. Signature analysis results from mixed infection of SMV strains G2 and G7 from infected trifoliate leaves of PI 96983 (SBLINE=2). Designations of monoclonal antibodies were as described in Fig. 1
Figure 4. Signature analysis results from mixed infection of SMV strains G2 and G7 from infected trifoliate leaves of Davis (SBLINE=3). Designations of monoclonal antibodies were as described in Fig. 1.
Figure 5. Signature analysis results from mixed infection of SMV strains G2 and G7 from infected trifoliate leaves of Williams '82 (SBLINE=1). Designations of monoclonal antibodies were as described in Fig. 1.
Figure 6. Signature analysis results from a 1:1 mixture of infectious sap extracts from trifoliolate leaves of Williams '82 (SBLINE=1) inoculated with either SMV-G2 or -G7. Designations of monoclonal antibodies were as described in Fig. 1.
Table 2. Comparison of soybean mosaic virus (SMV) strains by signature analysis, indicating aligned or non-aligned antigen binding curves of two virus antigen samples with eight monoclonal antibodies

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*a(+) = aligned (superimposed) antigen binding curves, (-) = non-aligned (non-superimposed) antigen binding curves.

b(G2/G7) = 1:1 mixture of infectious sap from G2 or G7-infected trifoliate leaves of Williams '82.
PART IV: CELL-FREE TRANSLATION OF POTYVIRAL RNA INCLUDING EXTRACTS FROM DISEASE RESISTANT SOYBEAN
PART IV: CELL-FREE TRANSLATION OF POTYVIRAL RNA INCLUDING EXTRACTS FROM DISEASE RESISTANT SOYBEAN

Louis M. Mansky¹, Donald P. Durand¹, and John H. Hill²
From the Departments of ¹Microbiology and ²Plant Pathology
Iowa State University
Ames, Iowa
ABSTRACT

Extracts from disease-resistant soybean were added to cell-free translation reactions of soybean mosaic virus (SMV) RNA to investigate effects on translation and proteolytic processing (in vitro) that could be correlated with disease resistance. Extracts of leaf tissue from Davis (resistant to strain G2) and susceptible Williams '82 were fractionated into three broad peaks. Apparent proteinase activity was detected in peak II of both soybean cultivars in the cell-free translation of SMV-G2 or G7 RNAs. Inhibition of translation occurred in the presence of peak extracts from either soybean cultivar at concentrations of 500 ug/ml and/or 1000 ug/ml of protein. No inhibition of proteolytic processing was observed by extracts from either cultivar. Cell-free translation of G2 or G7 RNAs in the presence of fractionated leaf extracts from the cultivars Davis or Williams '82 revealed no differences that were correlated with disease resistance of the cultivars to the virus strains.
INTRODUCTION

Studies regarding the molecular basis of constitutive disease resistance to viruses in plants are limited. However, in one intriguing example of resistance, to cowpea mosaic virus (CPMV) in the cowpea cultivar Arlington, is associated with inhibition of a CPMV-encoded protease (Beier et al., 1979; Goldbach and Krijt, 1982; Sanderson et al., 1985). In uninoculated leaves of Arlington, the protease is both specific and inherited (Ponz et al., 1987). No single dominant allele has been identified as being responsible for this phenotype.

The molecular basis of constitutive disease resistance has been studied in tomato (Watanabe et al., 1987; Meshi et al., 1989). The $Tm$-1 gene in tomato confers resistance to tobacco mosaic virus (TMV). No detectable viral replication occurs in protoplasts when inoculated with either intact virus or viral RNA (Motoyoshi and Oshima, 1977; 1979). Watanabe et al. (1987) described the isolation of the resistance-breaking Lta 1 isolate of TMV, which spreads systemically in tomato plants homozygous for the $Tm$-1 gene; the parent L isolate neither spreads nor produces symptoms on such plants. Comparison of the genomic sequences of Lta 1 and L revealed two base substitutions in the Lta isolate that resulted in amino acid changes in the 130 and 180 K proteins (Meshi et al., 1988) of the Lta 1 isolate, which are thought to be the viral-encoded proteins of the viral replicase. Thus, viral RNA replication
appears to be the antiviral target of disease resistance induced by the $7m$-1 gene.

Another resistance-breaking isolate of TMV derived from the L strain, Ltbi, spread systemically in tomato plants homozygous for $7m$-2, a gene that also confers resistance to TMV. Nucleotide sequence analysis of Ltbi revealed two amino acid changes, as compared with the L isolate, in the 30 K movement protein (Meshi et al., 1989). The $7m$-2 gene does not confer resistance at the protoplast level (Motoyoshi and Oshima, 1975; 1977), suggesting that virus movement is the antiviral target of the $7m$-2 gene.

Wyatt and Kuhn (1979) have suggested that the molecular basis for disease resistance in the cowpea line PI 186465 to cowpea chlorotic mottle virus (CCMV) may be related to the low amount of RNA 3 produced in that line. Therefore, low levels of the putative movement protein, thought to be encoded by RNA 3 of members from the bromovirus group (Hull, 1989; Melcher, 1990), may limit virus spread. Inheritance studies have shown that virus spread is controlled by a single dominant gene (Kuhn et al., 1981).

Although there is considerable information regarding the genetics of reaction to SMV in soybean, little is known regarding the molecular basis for disease resistance. Known isolates of SMV have been grouped into strains (G1-G7, G7a and C14) based upon pathogenicity to differential soybean lines (Cho and Goodman, 1979; 1982; Buzzell and
Tu, 1984; Lim, 1985; Roane et al., 1986b; Chen et al., 1988). In addition, several soybean lines contain a single dominant allele, Rsv, which confers resistance to most strains of SMV (Kiihl and Hartwig, 1979; Roane et al., 1986a, b; Buss et al., 1987; 1988). The soybean cultivar Davis is susceptible to strains G4-G7 and G7a, as are the cultivars York, Dorman, and Ware (Roane et al., 1986b; Chen et al., 1988); these cultivars possess a resistance gene that is allelic to Rsv from PI 96983, which is susceptible to G7 and G7a (Roane et al., 1986a, b).

The goal of this study was to determine whether disease resistance to SMV in soybean acts upon the translational or polyprotein processing stage(s) of the SMV multiplication cycle. No correlation was found between the effect of leaf tissue extracts from resistant or susceptible soybean cultivars on the in vitro translation of SMV RNA or on proteolytic processing of translation products.
MATERIALS AND METHODS

Virus Strains, Soybean Cultivars, and Viral RNA Purification

The Davis cultivar is resistant to infection by SMV strains G2 (isolate 75-16-1) and susceptible to G7 (Buzzell and Tu, 1984; Roane et al., 1986b; Chen et al., 1988), while the soybean cultivar Williams '82 is susceptible to SMV-G2 and -G7 (Cho and Goodman, 1979; Hill and Benner, 1980 a, b; Wilcox, 1984). Soybeans were maintained in growth chambers at a temperature of 20°C and a daylength of 18 h with an irradiance of 50 W/m². Primary leaves of soybean seedlings were mechanically inoculated with infectious sap prepared in 50 mM sodium phosphate buffer, pH 7.0. Plants were rinsed with water immediately after inoculation.

SMV RNA was purified by using a procedure modified from Vance and Beachy (1984a). Sodium dodecyl sulfate (SDS) and ethylenediaminetetraacetic acid (EDTA) were added to purified SMV (Hill and Benner, 1980a) to concentrations of 1 % and 20 mM, respectively. This mixture was incubated at 65°C for 5 min. Protease (type XIV, Sigma) prepared in 200 mM sodium phosphate, pH 7.5, was added to 1 mg/ml, and the solution was incubated for 10 min at 37°C. The mixture was made 1.5 % with SDS and incubated at 55°C for 10 min. The RNA was extracted with phenol/chloroform and precipitated with 95 % ethanol.
SMV RNAs were analyzed for integrity by electrophoresis after denaturation with glyoxal (Maniatis et al., 1982).

Extract Preparation and Fractionation

Soybean leaf tissue extracts were prepared using a modified procedure developed for cowpea by Ponz et al. (1987). Leaf tissue from three-week-old uninoculated soybeans was harvested and used immediately or frozen at -20°C for up to 1 month before fractionation. Leaf tissue (50 g) was homogenized in 200 ml of ice-cold extraction buffer (EB; 80 mM Na2B3O7, 480 mM H3BO3, 290 mM NaCl, and 5 mM sodium ascorbate containing phenylmethysulfonyl flouride (PMSF) freshly dissolved to 1 mM).

The homogenate was filtered through four layers of cheesecloth and the filtrate was centrifuged for 10 min at 194,000 x g. The supernatant was loaded immediately onto a Sephadex G-25 column (42 cm X 5.2 cm, V0 = 110 ml) equilibrated with EB. The green colored void volume (50 ml) was precipitated with 1.2 volumes of saturated ammonium sulfate solution (4°C, pH 7.0) for 24 h at 4°C. The product was centrifuged at 194,000 x g for 15 min and the pellet resuspended in 20 ml of TDTT buffer (20 mM Tris-HCl, pH 6.8, 1 mM dithiothreitol) at 20°C. After clarification by centrifugation at 145,000 x g for 5 min, 5 ml of the supernatant was loaded onto a Sepharose Cl-6B column (60 cm X 1.5
cm, \( V_0 = 102 \text{ ml} \) equilibrated with TDTT. Fractions (2.0 ml) were collected at a flow rate of 10 ml/h. Peak fractions, eluted with TDTT and having an A280 greater than 0.10, were combined and precipitated with 1.2 volumes of saturated ammonium sulfate (4°C, pH 7.0) for 24 h at 4°C. Products were centrifuged at 194,000 x g for 15 min, resuspended in 2 ml of TDTT, and dialyzed for 48 h against TDTT. The solutions were then stored at -20°C until assayed. Protein concentrations were determined by using a Protein assay kit (Bio-Rad, Richmond, CA) according to the manufacturer’s instructions using bovine serum albumin as a protein standard.

**Cell-free Translation of Viral RNA**

SMV RNA was translated using micrococcal nuclease-treated rabbit reticulocyte lysates minus methionine (cat. no. L4210, Promega Corp., Madison, WI). Reaction mixtures (50 ul in final volume) included 10 mM creatine phosphate, 2.5 ug creatine phosphokinase, 2mM dithiothreitol, 2.5 ug calf liver tRNA, 79 mM potassium acetate, 500 uM magnesium acetate, 20 uM hemin, 50 units of RNasin (Promega), 40 uCi of \(^{35}\text{S}\)-methionine (New England Nuclear, > 800 Ci/mmol), a 20 uM amino acid mixture excluding methionine, and 1.0 ug of SMV RNA. Five microliters of fractionated leaf extracts from soybean tissue (at 5, 50, 500, or 1000 ug/ml of protein) or TDTT buffer were added to reactions. All
reactions were incubated at 30°C for 1 h. Reactions were analyzed directly or were stored at -20°C.

Electrophoresis of Translation Products

Five ul samples of translation products were diluted with 5 ul of sample buffer (75 mM Tris-HCl, pH 6.8, 2 % SDS, 10 % glycerol, 5 % 2-mercaptoethanol, 10 ug/ml bromophenol blue), heated to 100°C for 3 min, cooled, and fractionated on Laemmli SDS-polyacrylamide gels (Laemmli,1970) with a 4 % stacking and a 12.5 % resolving gel at a constant 200 V for 40 min. The gels were then soaked in fixing solution (50 % methanol, 10 % acetic acid), dried, and exposed to Kodak X-OMAT film at -70°C for 48 h with an intensifying screen.
RESULTS

Fractionation of Extracts from Soybean Leaves

Gel-exclusion chromatography on Sepharose CI-6B resolved soybean leaf tissue extracts from the SMV-G2 resistant cultivar Davis and the susceptible cultivar Williams '82 into three broad peaks of fractionated soybean extracts (Fig. 1). All three peaks eluted in the void volume, suggesting a macromolecular composition.

Cell-free Translation in Presence of Soybean Leaf Extracts

To test for potential ribonuclease activity in the peak extracts, 1 ug of a series of six synthetic poly (A)-tailed RNAs (0.24-9.5 kb RNA ladder, BRL, Gaithersburg, MD) was incubated at 30 C for 1 h in translation reactions containing peak extracts I, II, or III at protein concentrations of 1 mg/ml from either Davis or Williams '82; no substantial level of ribonuclease activity was observed that would suggest degradation of RNA (data not shown).

Translation of a homogeneous (data not shown) SMV-G2 RNA in the presence of soybean extract peak fractions from Davis leaves reproducibly revealed no accumulation of products at extract concentrations containing 500 ug/ml of protein or higher for peaks I, II,
and III (Fig. 2A, lanes 5, 6, 11, 12, 17, and 18). Addition of peak fractions I and II of Williams '82 to G2 RNA-directed translations resulted in no accumulation of translation products at protein concentrations of 500 ug/ml or higher (Fig. 2B, lanes 5, 6, 11, 12), but all concentrations of peak III added allowed accumulation of products (Fig. 2B, lanes 13-18). Comparison of the profile of translation products produced in the presence of peak extracts at lower concentrations from either cultivar with translation reactions with no soybean extracts added (Fig. 2A, B, lanes 1, 2, 7, 8, 13, and 14) suggested that no major inhibition of proteolytic processing occurred.

Translation of homogeneous (data not shown) SMV-G7 RNA in the presence of extract peak fractions from Davis soybean leaves reproducibly yielded no accumulation of translation products at protein concentrations of 500 ug/ml or higher (Fig. 3A, lanes 5, 6, 11, 12, 17, and 18). Peak fractions I and II from Williams '82 at protein concentrations of 500 and 1000 ug/ml allowed no accumulation of products (Fig. 3B, lanes 5, 6, 11, 12). Addition of peak III fractions at protein concentrations of 1 mg/ml resulted in no accumulation of translation products (Fig. 3B, lane 18). No inhibition of proteolytic processing was observed with peak extracts from either cultivar with the translation of G7 RNA, as compared to translations of G7 RNA with no soybean extract peak fractions added (Fig. 3A, B, lanes 1, 2, 7, 8, 13, and 14). Cell-free translation of brome mosaic virus RNA in the presence of extracts from either Davis or Williams
'82 soybean leaves revealed no accumulation of products at protein concentrations of 500 ug/ml or higher for peaks I, II, and III (data not shown).

When the green colored void volume from Davis leaf extracts was precipitated with higher concentrations of saturated ammonium sulfate (i.e., 2.0 volumes), there was no influence on the peak extracts obtained or their effects on the cell-free translation of SMV G2-RNA (data not shown).

Addition of Soybean Leaf Extracts After Translation

Soybean leaf peak fractions added to translation products of SMV-G2 RNA revealed no extensive proteolysis during a 30 C incubation for 1 h with peaks I and III (protein concentrations of 500 and 1000 ug/ml, respectively) of either Williams '82 (Fig. 4, lanes 2, 4) or Davis leaves (Fig. 4, lanes 5, 7); a low amount of reproducible proteolysis was observed with peak fraction II at protein concentrations of 500 ug/ml from Williams '82 (Fig. 4, lane 3) or Davis leaves (Fig. 4, lane 6). When peak fractions were boiled for 1 min prior to addition of translation products, no effect was observed with samples containing peaks I and III from both Williams '82 (Fig. 4, lanes 8, 10) and Davis leaves (Fig. 4, lanes 11, 13), but proteolysis was eliminated from samples containing protein peak II from both Williams '82 (Fig. 4, lane 9) and Davis leaves (Fig. 4, lane 12). Although
not quantitated, the concentration of putative proteolytic activity observed during incubation with peak fraction II from Williams '82 and Davis (Fig. 4, lanes 9 and 12, respectively) was similar.
DISCUSSION

Evidence is presented that suggests leaf extracts from SMV-resistant Davis or -susceptible Williams '82 cultivars do not exert differential effects on SMV RNA-directed cell-free translations that could be correlated to disease resistance. In general, addition of extracts at high protein concentrations (i.e., 500-1000 ug/ml) inhibited accumulation of SMV RNA translation products. This may be caused by nonspecific degradation of viral RNA by ribonucleases, degradation of proteins by proteinases, and/or inhibitors of translation.

The ribonuclease and putative proteinase activities observed in this study suggest that the absence of products in the presence of extract peaks I, II, and III from Davis (Fig. 2A, 3A, lanes 5-6, 11-12, and 17-18) and from Williams '82 (Fig. 2B, 3B, lanes 5-6 and 11-12; Fig. 3B, lane 18) was also due to other activities (e.g., inhibitors of translation). Therefore, since ribonuclease and proteinase activities apparently do not completely account for the absence of accumulated translation products, inhibitors of translation may be involved. The absence of accumulation of translation products occurred at the same protein concentrations (i.e., 500 ug/ml) for SMV-G2 and G7 RNAs translated in the presence of soybean leaf extract peaks I and II from both G2-resistant Davis and susceptible Williams '82. In the presence of soybean extract peak III from Davis, SMV-G2 and G7 RNA translation products did not accumulate at protein concentrations of
500 ug/ml or higher. For leaf extract peak III from the G2 and G7-susceptible cultivar Williams '82, G2 RNA translation products did not accumulate at protein concentrations of 1000 ug/ml from Williams '82 leaf extracts, while G7 RNA translation products accumulated at all protein concentrations of Williams '82 leaf extracts; however, these differences cannot be correlated with disease resistance. Therefore, these data suggest that inhibition of total protein accumulation was not correlated with disease resistance.

SMV, like other potyviruses, is thought to produce a polyprotein which is proteolytically cleaved to form mature viral proteins (Vance and Beachy, 1984a, b; Dougherty and Carrington, 1988). No evidence for inhibition of proper proteolytic processing was observed with soybean leaf extracts from Davis or Williams '82 when compared to translations in the absence of leaf extracts (Fig. 2A, 2B, 3A, 3B, lanes 1, 2, 7, 8, 13, and 14), and by comparison of SMV-G2 RNA translations with G2 RNA translation products produced during time course studies between 0 and 60 min (P. J. Berger and R. E. Andrews, Dept. Microbiol., Iowa State Univ., unpublished results). Translation profiles of SMV-G2 and G7 RNAs, in the absence of soybean leaf extracts, were distinctly different (Figs. 2, 3). This has been observed previously with other isolates from SMV strains and with strains of another potyvirus, maize dwarf mosaic virus (Berger et al., 1989; Mansky et al., unpublished). It has been suggested that these differences correlate with virulence (Mansky et al., unpublished).
The data presented in this report contrast with several observations made with CPMV and the cowpea line Arlington (Ponz et al., 1987). First, there was no evidence for inhibition of polyprotein processing with soybean extract peak fractions from either Davis or Williams '82 leaves as reported with Arlington. Second, proteinase activities from peak II fractions of susceptible Williams '82 or resistant Davis were present at similar levels. In contrast, peak II fractions from cowpea contained proteinase activity, which was less in susceptible cowpea leaves than in resistant leaves. Our data suggest that putative proteinases from peak II fractions of Williams '82 and Davis leaves may not contribute to disease resistance in soybean to SMV, in contrast to the suggestion of Ponz et al. (1987) that proteinases may be involved in immunity of Arlington cowpea to CPMV. Third, inhibitors of translation were found in: 1) all three soybean extract peaks of Davis leaves with translation of both SMV-G2 and G7 RNAs; 2) all three peaks of Williams '82 in translations of G7 RNA; and 3) peaks I and II of Williams '82 in translations of G2 RNA. In cowpea, inhibitors were found only in peak IV.

Disease resistance in soybean to SMV may involve antiviral activity at one or several stages in the viral multiplication cycle. The in vitro results presented here suggest that some stage(s) of the viral multiplication cycle other than translation and proteolytic processing (e.g., uncoating, RNA replication, assembly, virus movement) may be the target(s) of antiviral activity.
LITERATURE CITED


Meshi, T., Motoyoshi, F., Atsuko, A., Watanabe, Y., Nobuhiko, T., Okada, Y. 1988. Two concomitant base substitutions in the putative replicase genes of tobacco mosaic virus confer the ability to overcome the effects of a tomato resistance gene, Tm-1. EMBO J. 7:1575-1581.


Figure 1. Gel exclusion column chromatography of extracts from Davis (○-○) and Williams '82 (■-■) soybean leaves. Five ml of an ammonium sulfate precipitate dissolved in 20 mM Tris-HCl, 1 mM dithiothreitol, pH 6.8 (TDTT), was applied to a Sepharose CL-6B column, equilibrated, and eluted with the same buffer at a rate of 10 ml/h. The eluant, monitored at 280 nm, separated into three broad zones designated I-III.
Figure 2. Cell-free translation of SMV-G2 RNA in the presence of soybean leaf extracts from Davis (A) or Williams '82 (B) fractionated peaks. Added to translation mixtures containing rabbit reticulocyte lysate (35 ul), 50 units of RNasin, 40 uCi of $^{35}$S-methionine, and 1 ug of SMV-G2 RNA was 5 ul of either RNase-free water (lanes 1,7, and 13), TDTT (lanes 2, 8, and 14), or a dilution of peaks I (lanes 3-6), II (lanes 9-12), or III (lanes 15-18) from Davis or Williams '82 leaf tissue (protein concentrations of 5 ug/ml, lanes 3,9, and 15; 50 ug/ml, lanes 4, 10, and 16; 500 ug/ml, lanes 5, 11, and 17; 1000 ug/ml, lanes 6, 12, and 18). Solutions were incubated at 30° C for 1 h and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. Comparison of proteins from samples in lanes 1-6, 7-12, and 13-18 of each panel is appropriate; however, because each set (i.e., 1-6, 7-12, and 13-18) was analyzed by SDS-PAGE separately, direct comparison of molecular weights of proteins in different sets is inappropriate.
Figure 3. Cell-free translation of SMV-G7 RNA in the presence of soybean leaf extracts of Davis (A) or Williams '82 (B) fractionated peaks. Translations were identical to those performed in Fig. 2 except that translations were directed by 1 ug of SMV-G7 RNA. Solutions were incubated at 30 C for 1 h and analyzed by SDS-PAGE and autoradiography. Comparison of proteins from samples in lanes 1-6, 7-12, and 13-18 of each panel is appropriate; however, because each set (i.e., 1-6, 7-12, and 13-18) was analyzed by SDS-PAGE separately, direct comparison of molecular weights of proteins in different sets is inappropriate.
Figure 4. Proteinase activities of fractionated soybean leaf extracts. 

$[^{35}S]$methionine-labeled translation products from 1 h translations of SMV-G2 RNA in rabbit reticulocyte lysate were incubated at 30°C for 1 h in the absence (lane 1) or presence of extract peaks I, II, and III (protein concentrations of 500 μg/ml, 500 μg/ml, and 1000 μg/ml, respectively) from Williams '82 (peak I, lanes 2, 8; peak II, lanes 3, 9; peak III, lanes 4, 10) or Davis (peak I, lanes 5, 11; peak II, lanes 6, 12; peak III, lanes 7, 13) leaves which were added directly (lanes 2-7) or heated to 100°C for 1 min, cooled, and added to reaction mixtures (lanes 8-13). Reactions were analyzed by SDS-PAGE and autoradiography.
PART V: COMPARATIVE ANALYSIS OF TRANSLATION PRODUCTS FROM PATHOGENIC STRAINS OF SOYBEAN MOSAIC VIRUS
PART V: COMPARATIVE ANALYSIS OF TRANSLATION PRODUCTS
FROM PATHOGENIC STRAINS OF SOYBEAN MOSAIC VIRUS

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ABSTRACT

Cell-free translation products from isolates representing soybean mosaic virus (SMV) strains G1-G7 and G7a, along with several other SMV isolates, were analyzed. SMV RNAs were translated in both rabbit reticulocyte lysates and wheat germ extracts, yielding approximately twenty translation products for each strain from each translation system. Comparison of translation profiles by the presence or absence of proteins allowed for the formation of distinctive groups from each cell-free translation system. Groupings formed by analysis of products from rabbit reticulocyte lysates correlated with pathogenicity; groupings formed by analysis of products from wheat germ extracts did not correlate with any other biological properties of the isolates.
INTRODUCTION

Soybean mosaic virus (SMV) is a member of the largest group of plant viruses, the potyvirus group (Bos, 1972). Potyviruses are characterized by long flexuous rod morphology and by the ability to induce the formation of cytoplasmic pinwheel inclusion bodies in infected cells (Francki et al., 1985). The potyviral genome is a single-stranded, positive sense RNA of 10 kb with a 3' poly (A) tail and a 5' covalently attached VPg (Francki et al., 1985; Dougherty and Carrington, 1988). The genomic expression strategy involves the production of a single polyprotein which is subsequently cleaved into functional viral proteins; no subgenomic RNAs are produced (Vance and Beachy, 1984b; Valverde et al., 1986). Eight proteins are thought to be encoded by the potyviral genome, including a coat protein, putative replicase, two proteases, an aphid transmission factor, and a putative movement protein (Dougherty and Carrington, 1988).

Virulence groups (G1-G7, G7a and C14) of SMV isolates have been designated based upon pathogenicity to differential soybean lines (Cho and Goodman, 1979; 1982; Buzzell and Tu, 1984; Lim, 1985; Roane et al., 1986a, b; Chen et al., 1988). Little molecular information exists regarding biological differences among these groups. Analysis of SMV coat protein by Cleveland mapping and immunoblotting has allowed differentiation and grouping of SMV isolates (Hill et al., 1989).
To provide further information about SMV, we report the cell-free translation of RNAs from several isolates of SMV, and the comparison of their translation products.
MATERIALS AND METHODS

Viruses and Viral RNA

The SMV isolates used in this study have been previously described (Cho and Goodman, 1979; 1982; Hill and Benner, 1980; Lucas and Hill, 1980; Chen et al., 1982; Hunst and Tolin, 1982; Buzzell and Tu, 1984; Vance and Beachy, 1984a, b; Lim, 1985; Roane et al., 1986a, b; Chen et al., 1988; Hill et al., 1989) and, for purposes of this study, are designated as in Fig. 1. Virus isolates were purified as previously described (Hill and Benner, 1980). Viral RNA was isolated from purified virus by using a modification of the method described by Vance and Beachy (1984a). Sodium dodecyl sulfate (SDS) and ethylenediaminetetraacetic acid (EDTA) were added to purified SMV to concentrations of 1 % and 20 mM, respectively. This mixture was incubated at 65° C for 5 min. Protease (type XIV, Sigma) was added to 1 mg/ml, from a stock solution of 100 mg/ml in 200 mM sodium phosphate, pH 7.5, and the mixture was incubated for 10 min at 37° C. After addition of SDS to 1.5 %, the mixture was incubated at 55° C for 10 min. Protein was extracted with phenol/chloroform and viral RNA was precipitated with 95 % ethanol (Maniatis et al., 1982). Integrity of SMV RNAs was determined by electrophoresis after denaturation with glyoxal (Maniatis et al., 1982).
In vitro Translation of Viral RNA

SMV RNA was translated in micrococcal nuclease-treated rabbit reticulocyte lysate minus methionine (cat. no. L4210, Promega Corp., Madison, WI) or in wheat germ extracts minus methionine (cat. no. L4380, Promega Corp., Madison, WI). Reactions with rabbit reticulocyte lysates (50 ul in final volume) included 10 mM creatine phosphate, 2.5 ug creatine phosphokinase, 2mM dithiothreitol, 2.5 ug calf liver tRNA, 79 mM potassium acetate, 500 uM magnesium acetate, 20 uM hemin, 50 units of RNasin (Promega), 40 uCi of \(^{35}\text{S}\)-methionine (New England Nuclear, > 800 Ci/mmol), a 20 uM amino acid mixture excluding methionine, and 1.0 ug of SMV RNA. Reactions with wheat germ extracts (50 ul in final volume) included 10 mM creatine phosphate, 2.5 ug creatine phosphokinase, 5 mM dithiothreitol, 2.5 ug calf liver tRNA, 1.0 mM magnesium acetate, 100 mM potassium acetate, 0.5 mM spermidine, 1.2 mM ATP, 0.1 mM GTP, 12 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 50 units of RNasin ribonuclease inhibitor (Promega), 25 uCi of \(^{35}\text{S}\)-methionine (New England Nuclear, > 800 Ci/mmol), a 20 uM amino acid mixture excluding methionine, and 1.0 ug of SMV RNA. In vitro translation reactions in rabbit reticulocyte lysates were incubated at 30° C for 1 h, and translation reactions in wheat germ
extracts were incubated at 25° C for 1 h. Reactions were analyzed directly or were stored at -20° C.

Analysis of Translation Products

Five ul samples of translation products were diluted with 5 ul of sample buffer (75 mM Tris-HCl, pH 6.8, 2 % SDS, 10 % glycerol, 5 % 2-mercaptoethanol, 10 ug/ml bromophenol blue), heated to 100° C for 3 min, cooled, and fractionated in Laemmli SDS-polyacrylamide gels (Laemmli,1970) with a 4 % stacking and a 12.5 % resolving gel at a constant 200 V for 40 min. The gels were soaked in fixing solution (50 % methanol, 10 % acetic acid), dried, and exposed at -70° C to Kodak X-OMAT film with an intensifying screen.

Principal Component Analysis

Photographic negatives of autoradiograms were scanned at 900 nm by using a linear transport coupled to a monochrometer. The molecular weight of all polypeptides was calculated by regression analysis with reference to molecular weight standards run in each gel. A value of 1 was assigned to polypeptides present at a unique molecular weight, and a value of 0 was assigned to polypeptides not detected from one isolate, but present with another. The resulting data matrix was
analyzed by principal component analysis (Morrison, 1976). Principal component analysis was used to reduce the dimensionality of the data set, which contained a large number of interrelated variables, while retaining as much as possible of the variation present in the data set. This reduction was done by transforming to a new set of variables, the principal components, which were uncorrelated and ordered so that the first few retained most of the variation present in all of the original variables. Computation of the principal components was reduced to the solution of an eigenvalue-eigenvector problem for a positive-semidefinite symmetric matrix and summarized in two dimensions to reveal any significant groupings among SMV isolates.
RESULTS

Analysis of Translation Products from Rabbit Reticulocyte Lysates

SMV RNA-directed translations in rabbit reticulocyte lysates produced approximately 20 products for each isolate analyzed (Fig. 1). Two groups of virus isolates produced the same set of protein products (G1, G2, and la; G4, G5, and G6). Seven sets of isolates produced an identical set of proteins except for one protein: 1) G7 and G7a; 2) O and the G1, G2, and la group; 3) O and G3; 4) O and VA; 5) G3 and VA; 6) N and the G4, G5, and G6 group; and 7) G7 and VA. Thirteen proteins were produced by all isolates. Three proteins were produced by most virus isolates: namely, 65.9 K by all except G7 and G7a; 64 K by all except N and Br; and 28.7 K by all except G3. Five relatively infrequently produced proteins were identified as: 76.7 K by G7a; 53.9 K by Br; 51.3 K by G4, G5, G6, N, and Br; 41.5 K by G1, G2, and la; and 27.3 K by G1, G2, G3, 0, and la.

Analysis of Translation Products from Wheat Germ Extracts

SMV RNA-directed translation in wheat germ extracts also generated approximately 20 products from each isolate tested (Fig. 2).
Three groups of isolates produced the same proteins (G7 and G7a; G4, G5, Br, and la; G1 and VA). Four isolates identical except for 1 protein or polyprotein were: 1) G5 and the G1, VA group; 2) G2 and the G1, VA group; 3) G6 and the G4, G5, Br, and la group; and 4) O and the G4, G5, Br and la group. Nineteen proteins were made by all isolates. A 58.8 K protein was made by all isolates except G7, G7a and N. Five proteins were found to be produced by one or a few isolates: namely, 81.7 K by G3, G7, and G7a; 73.5 K by G2 and G6; 50.2 K by G1, G2, and VA; 43.2 K by O; and 36.9 K by N.

SMV Strain Groupings

Principal component analysis of translation profiles allowed grouping of SMV isolates from both rabbit reticulocyte lysates and wheat germ extracts (Fig. 3). Two groupings were found with profiles from rabbit reticulocyte lysates (G1, G2, la, and G3; G4, G5, and G6). The O isolate was most closely associated with the G1, G2, la group; N, Br, and VA were most closely associated with the G4, G5, and G6 group; and G7 and G7a were not closely associated with either grouping.

Analysis of profiles from wheat germ extracts revealed that the isolates formed the three groups G1, G6 and VA; G4, G5, la, and Br; and G7 and G7a. G2 was most closely associated with the G1, G6, and VA group; G3 was most closely associated with the G4, G5, la, and Br group;
N was most closely associated with G7 and G7a; and the O isolate was not found to be closely associated with either grouping.
DISCUSSION

The data reveal differences in translation product profiles of SMV isolates from both rabbit reticulocyte lysates and wheat germ extracts. To make comparisons between isolates, the presence or absence of proteins and not the amount of accumulated proteins was considered. Principal component analysis, which reduced the dimensionality of the data matrix while retaining as much of the variation as possible in the data set, suggested different groupings for the SMV isolates based upon the translation profiles and the two translation systems. Comparison of these groupings with known biological criteria, which differentiate isolates based upon pathogenicity (Cho and Goodman, 1979; 1982; Buzzell and Tu, 1984; Lim, 1985; Roane et al., 1986b; Chen et al., 1988), suggested the association of pathogenicity and translation profiles using rabbit reticulocyte lysates. The grouping of G1, G2, and G3 closely correlates with the observation that isolates characteristic of these strains are relatively less virulent (G1 being the least virulent), and isolates of the G4, G5, and G6 group correspond to moderately virulent strains (G4 being the least virulent of this group) (Cho and Goodman, 1979; 1982). The lack of close association with any group of G7 and G7a may correspond to the highly virulent characteristic of these strains (Cho and Goodman, 1979; 1982; Buzzell and Tu, 1984). The correlation between translation profile and pathogenicity allows for the prediction that the 1a and O isolates are
less virulent, and the N and Br isolates are moderately virulent; the SMV isolate la is a member of the G2 strain group (J. H. Hill, Dept. of Plant Pathology, Iowa State Univ., unpublished results). The SMV isolate VA has been placed in the G1 group (Gunyuzlu et al., 1987), but these data suggest differences between the representative G1 strain and the VA isolate. Analysis of groupings of SMV isolates from translation products produced in wheat germ extracts were not associated with any known biological characteristics. Therefore, these observations suggest that translation products of SMV RNAs produced in rabbit reticulocyte lysates are of biological significance, and may represent translation products produced in vivo.

The data presented in this study, which reveal differences in translation profiles among different strains of a potyvirus, agree with the observations of Berger et al. (1989) demonstrating different translation profiles of maize dwarf mosaic virus, strains A and B, RNAs in rabbit reticulocyte lysates. These differences may exist for strains of numerous potyviruses.

At present, it is unclear if this experimental approach will be useful for classification and identification of potyvirus strains. Potyvirus taxonomy is presently complex due to the large size of the group, the apparent variation that exists among members, and the lack of satisfactory taxonomic parameters that readily distinguish distinct viruses from strains (Shukia and Ward, 1989). At the present time, symptomatology and host
range, morphology, vector, and the production and morphology of cytoplasmic inclusions have been the principle criteria used for identification and classification of potyviruses (Edwardson, 1974a, b). Several approaches based upon viral molecular structure and including nucleic acid sequence homology and hybridization, coat protein structure, and serology have been proposed (reviewed by Shukla and Ward, 1989). It will presumably take several years before the utility of such approaches has been assessed.

Future comparative studies of translation profiles should include some system for ranking accumulation of individual proteins and polyproteins, because most strains which produced a protein of similar size do so in different amounts. It may also be useful to compare profiles of immunoprecipitation products from translations by using antibodies to SMV proteins to help identify specific polyproteins. For example, proteins (e.g., 41.5 K and 51.3 K) produced from translations in rabbit reticulocyte lysates were responsible for placing several SMV isolates into unique groupings (G1, G2, and Ia, and the G4, G5, G6, N, and Br groups, respectively); identification of these proteins may aid in understanding the correlation between translation profile and pathogenicity. Future studies should also include analysis of several isolates from the same virulence group. Preliminary data with G1 and the VA isolates suggested that differences in translation profiles exist when translated in rabbit reticulocyte lysates. Therefore, it is unclear whether the different profiles
correlate with only structural changes (e.g., RNA secondary structure) of
the viral genome or functional changes of mature viral proteins (e.g.,
proteases) that could lead to functional differences between isolates of
the same strain. Differences in secondary structure could partially
account for the variation in efficiency of translation observed (Figs. 1, 2).

These studies may allow for specific isolate identification, which
could be of use for studies of molecular epidemiology. Further biological
characterization of the SMV virulence groups (e.g., aphid vector
transmission and specificity; Lucas and Hill, 1980) may be useful to
provide further understanding of correlations between biological and
molecular biological criteria.
LITERATURE CITED


Figure 1. SMV RNA-directed translation in rabbit reticulocyte lysates. Translation mixtures contained rabbit reticulocyte lysate (35 ul), 50 units of RNasin, 40 uCi of $^{35}$S-methionine, and 1 ug of SMV RNA. Solutions were incubated at 30$^\circ$ C for 1 h and then analyzed by PAGE and autoradiography. Virus isolates are designated G1-G7, G7a, N, VA, Br (Brazil), 0, and la (la 75-16-1, Hill and Benner, 1980)
Figure 2. SMV RNA-directed translation in wheat germ extracts. Translation mixtures contained wheat germ extracts (25 ul), 50 units of RNasin, 25 uCi of $^{35}$S-methionine, and 1 ug of SMV RNA. Solutions were incubated at 25° C for 1 h, and analyzed by PAGE and autoradiography. Virus isolates are designated as in Fig. 1.
Figure 3. Plot of the first (PRIN 1) and second (PRIN 2) principal components revealing grouping of 13 SMV isolates on the basis of translation products from rabbit reticulocyte lysates (A) and wheat germ extracts (B). Virus isolates are designated as in Fig. 1.
GENERAL DISCUSSION

Several soybean lines have been shown to condition resistance to the potyvirus, soybean mosaic virus (SMV). Resistance is inherited as a single dominant allele, *Rsv*, providing functional immunity to most strains of SMV. The first part describes a method for virus location in leaf tissue that is used in the second part to study the influence of temperature upon the maintenance of resistance in several soybean lines to a virus isolate characteristic of SMV strain G2. When resistant soybean lines grown at 20°C were shifted, after inoculation to 10°C for 10 days, and then returned to 20°C for an additional 10 days, virus spread to trifoliate leaves. Temperature shifts to 15, 20, 25, 30, and 35°C did not influence the maintenance of resistance. Low temperature sensitivity of virus resistance has not been previously reported with other virus-host interactions, yet may exist. Temperature-sensitive resistance should be useful in the determination of resistance function, just as temperature sensitive virus mutants are useful for determining viral gene action. It is tempting to speculate that temperature has a direct effect on *Rsv* expression and function; however, the influence(s) of temperature maybe more complex than this. Because low temperature affects plant height, the general physiology of the soybean plant is significantly altered. This suggests that determining the mechanism by which low temperature treatment allows systemic spread of SMV may be difficult. The
observation of this phenomenon at the protoplast level may be useful for determining the point(s) at which the viral multiplication cycle is affected.

The next two parts of this work deal with the influence of resistance on viral multiplication. The general questions addressed in these parts were: 1) what is the basis for viral pathogenicity?, and 2) what may be the antiviral target(s) of disease resistance? Part Three describes the functional complementation of SMV strains in mixed infections of resistant soybean lines. Combinations of an isolate characteristic of the avirulent G2 strain with isolates characteristic of the virulent strains G7 or G7a allowed systemic spread of SMV-G2. These experiments suggest that there is at least one gene required for infection that is encoded by G7 and G7a, whose gene product complements multiplication or spread of the G2 strain to induce susceptibility. A suggestion that could be made from these experiments is that complementation occurs at the level of viral proteins, implying that the antiviral target of resistance may involve the inhibition of viral protein function. An approach to finding the antiviral target would be the development of a genetically engineered complementation system. In this system, for example, various cloned portions of the G7 viral genome could be introduced into resistant soybean callus via Agrobacterium-mediated transformation using appropriate expression vectors to allow production of viral protein(s). Subsequent inoculation with SMV-G2 (or the G1 strain, which is avirulent on all soybean lines conferring resistance to SMV) will show whether the
expressed SMV-G7 viral proteins allow infection and spread of the avirulent strain. Soybean callus can be inoculated with SMV (Chen et al., 1988b) and portions of cloned potyviral genomic sequences have been expressed in transgenic tobacco plants (and not soybean) (Berger et al., 1989) indicating the feasibility of such an experiment. It will be important to observe susceptibility in callus from plants that are susceptible to SMV infection. L78-379 should be used for future resistance studies, because L78-379 is genetically identical to Williams except at the Rsv locus.

In the fourth part, the effects of protein extracts from the resistant soybean line Davis on the cell-free translation of SMV-G2 RNA was studied. Inhibitors of proteolytic processing have been implicated in another virus-host system, but this was not evident in this system at protein concentrations ranging from 5 ug/ml to 1000 ug/ml. No specific effects on proteolytic processing were observed. However, apparent proteinases and inhibitors of translation were observed. Proteinase activity was strongest in peak II fractions from both Davis and susceptible Williams '82, suggesting that this activity is not correlated with resistance. Inhibitors of translation associated with peak III fractions from Davis leaf tissue were stronger than those from Williams '82, but had the same effects on the cell-free translation of both SMV-G2 and -G7 RNAs. These data suggest that antiviral activity in the resistant line Davis is not associated with the effects observed from the addition of leaf extracts from resistant lines on the cell-free translation and proteolytic processing of
SMV. Future work should concentrate on other steps of viral multiplication (i.e., entry and uncoating, RNA replication, assembly, virus movement) as potential targets for antiviral activity. Because no *in vitro* assays currently exist for potyviral uncoating, RNA replication, or assembly, it would presently be very difficult to experimentally show antiviral activity directed at these stages of viral multiplication *in vitro*. An indication of antiviral activity on virus movement could be shown by the successful infection of protoplasts derived from resistant soybean lines; these experiments should be one of the short term goals of resistance research, mainly to allow further comparisons of this model to other systems.

In the fifth part, an investigation of the translation profiles in wheat germ extracts and rabbit reticulocyte lysates with isolates characteristic of known virulence strains of SMV was done, along with several unclassified isolates. Variation in translation profiles was found and analysis of these profiles suggested groupings. Groupings of translation profiles from rabbit reticulocyte lysates could be correlated with pathogenicity; however, groupings of translation profiles from wheat germ extracts could not be associated with other biological characteristics.
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My appreciation is expressed to my fellow graduate students, past and present, for their support and friendship. I would like to thank my family for their support and encouragement throughout my education. I would like to dedicate this dissertation to the memory of my parents, who were unable to see its completion.
APPENDIX A.

SUPPLEMENT TO PART I
Figure A1. Electroblot of soybean leaf tissue. Trifoliate leaves from soybean plants (cv. Williams '82) inoculated with SMV-G2 (isolate la 75-16-1) and collected 10 days post-inoculation were electroblotted to nitrocellulose, pre-soaked in electrophoresis buffer (25 mM Tris, pH 8.3, 192 mM glycine, 2 % v/v methanol) for 3 h at 60 V (0.17 A). Blots were incubated with a monoclonal antibody (S-10) to the SMV coat protein. Detection of antibody binding was by using protein A-coupled colloidal gold particles followed by silver-stain enhancement. Left: electroblot of uninfected tissue; Right: electroblot of SMV-infected leaf tissue.
APPENDIX B.

SUPPLEMENT TO PART II
Table B1. Results of ELISA of trifoliate leaves from soybean lines inoculated with isolates characteristic of soybean mosaic virus (SMV) strains G2 or G7 or "mock-inoculated" with phosphate buffer.

<table>
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<th>Shift Temperature</th>
<th>&quot;Mock&quot;</th>
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<th>SMV-G7</th>
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*Samples assayed were trifoliate leaves from plants inoculated on primary leaves. Ten days following inoculation, trifoliate leaves were sampled and tested for presence of viral coat protein antigen by using ELISA. + = presence of SMV coat protein; - = absence of SMV coat protein.*
APPENDIX C.

SUPPLEMENT TO PART III
Table C1. Results of co-inoculation of the soybean cultivar Williams '82 with soybean mosaic virus (SMV) strain G2 (isolate 75-16-1) and helper viruses a

<table>
<thead>
<tr>
<th>Helper virus</th>
<th>Days prior SMV inoculation</th>
<th>ELISA b</th>
<th>Helper virus</th>
<th>SMV-G2</th>
<th>Local lesion assay c</th>
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</table>
Soybean plants (i.e., Williams '82) were grown, under greenhouse conditions, until full extension of primary leaves. Plants, approx. 5-7 per pot, were inoculated with helper virus (i.e., 10, 8, 6, 4, 2, 0 days prior to SMV-G2 inoculation), and then with SMV-G2 on primary leaves by mechanical inoculation using phosphate buffer, pH 7.0.

Ten days following inoculation with SMV-G2, trifoliolate leaves were sampled and were tested for presence of viral coat protein antigen by using ELISA, and for viral infectivity by using local lesion assay on Phaseolus vulgaris cv. Top Crop.

For TRSV (tobacco ringspot virus), AMV (alfalfa mosaic virus), and CPMV (cowpea mosaic virus), Glycine max var. Pinto was used as the local lesion host; Glycine max cv. Williams was used as the local lesion host for CCMV (cowpea chlorotic mottle virus).
Table C2. Results of co-inoculation of soybean mosaic virus (SMV) strain G2 (isolate 75-16-1)-resistant soybean plants with SMV-G2 and helper viruses$^a$

<table>
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<th>Helper virus</th>
<th>Days prior SMV inoculation</th>
<th>ELISA$^b$ Helper virus</th>
<th>SMV-G2</th>
<th>Local lesion assay$^c$ Helper virus</th>
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</table>
Soybean plants (i.e., PI 96983, L78-379, and York) were grown, under greenhouse conditions, until full extension of primary leaves. Plants, approx. 5-7 per pot, were inoculated with helper virus (i.e., 10, 8, 6, 4, 2, 0 days prior to SMV-G2 inoculation), and then with SMV-G2 on primary leaves by mechanical inoculation using phosphate buffer, pH 7.0.

Ten days following inoculation with SMV-G2, trifoliolate leaves were sampled and tested for presence of viral coat protein antigen by using ELISA, and for viral infectivity by using local lesion assay on Phaseolus vulgaris cv. Top Crop.

For TRSV (tobacco ringspot virus), AMV (alfalfa mosaic virus), and CPMV (cowpea mosaic virus), Phaseolus vulgaris var. Pinto was used as the local lesion host; Glycine max cv. Williams was used as the local lesion host for CCMV (cowpea chlorotic mottle virus).

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</table>

* aSoybean plants (i.e., PI 96983, L78-379, and York) were grown, under greenhouse conditions, until full extension of primary leaves. Plants, approx. 5-7 per pot, were inoculated with helper virus (i.e., 10, 8, 6, 4, 2, 0 days prior to SMV-G2 inoculation), and then with SMV-G2 on primary leaves by mechanical inoculation using phosphate buffer, pH 7.0.

b Ten days following inoculation with SMV-G2, trifoliolate leaves were sampled and tested for presence of viral coat protein antigen by using ELISA, and for viral infectivity by using local lesion assay on Phaseolus vulgaris cv. Top Crop.

For TRSV (tobacco ringspot virus), AMV (alfalfa mosaic virus), and CPMV (cowpea mosaic virus), Phaseolus vulgaris var. Pinto was used as the local lesion host; Glycine max cv. Williams was used as the local lesion host for CCMV (cowpea chlorotic mottle virus).
Figure C1. Signature analysis of virus antigen 21 from infected trifoliate leaves of Williams '82 (SBLINE=1). Virus antigen 21 was isolated by using local lesion assay from trifoliate leaves of the soybean cultivar Davis inoculated on primary leaves with soybean mosaic virus (SMV) strains G2 (isolate la 75-16-1) and G7. This isolate was characterized as an isolate representative of the G2 strain group by inoculation the soybean lines Williams '82, PI 96983, L78-379, and Davis. Monoclonal antibodies S-1, S-2, S-3, S-4, S-9, S-10, and S-12 were designated 1, 2, 3, 4, 9, 10, and 12, respectively; monoclonal antibody A1-2 was designated 11.
Figure C2. Signature analysis of virus antigen 55 (local lesion isolate no. 5 SMV strains G2 (A, antigen 2) and G7 (B, antigen 7) from infected trifoliate leaves of Williams '82 (SBLINE=1). Virus antigen 55 was isolated by using local lesion assay from trifoliate leaves of the soybean cultivar Davis inoculated on primary leaves with soybean mosaic virus (SMV) strains G2 (isolate la 75-16-1) and G7. This isolate was characterized as an isolate representative of the G7 strain group by inoculation the soybean lines Williams '82, PI 96983, L78-379, and Davis. Monoclonal antibodies were designated as in Fig. C1.
### Table C3. Local lesion assay of resistant and susceptible soybean lines for selected strains of SMV

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<th>Virus strain</th>
<th>Soybean line or cultivar</th>
<th># of local lesions$^a$</th>
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<td>PI 96983</td>
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<td>Davis</td>
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</table>

$^a$Total number of local lesions from three replicate experiments with three assays per replicate.
Table C4. Local lesion assay of resistant and susceptible soybean lines for selected co-inoculated strains of SMV

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Soybean line or cultivar</th>
<th>#of local lesions from trifoliates leaves(^a)</th>
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<td>G1 + G2</td>
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<td>PI 96983</td>
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<td>Davis</td>
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<td>G3 + G2</td>
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<td></td>
<td>PI 96983</td>
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<tr>
<td></td>
<td>Davis</td>
<td>54</td>
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\(^a\)Total number of local lesions from three replicate experiments with three assays per replicate.
Figure D1. Gel exclusion column chromatography of extracts from Davis soybean leaves. Davis leaf extracts were loaded onto a Sephadex G-25 column (42 cm X 5.2 cm, Vo = 125 ml) equilibrated with EB, and the green colored void volume (56 ml) was precipitated with 2.0 volumes of saturated ammonium sulfate solution (4°C, pH 7.0) for 24 h at 4°C. The product was centrifuged at 194,000 x g for 15 min and the pellet resuspended in 20 ml of TDTT buffer (20 mM Tris-HCl, pH 6.8, 1 mM dithiothreitol) at 20°C. After clarification by centrifugation at 145,000 x g for 5 min, 5 ml of the supernatant was applied to a Sepharose CL-6B column (60 cm X 1.5 cm, Vo = 102 ml), equilibrated, and eluted with the same buffer at a rate of 10 ml/hr. The eluant, monitored at 280 nm, separated into three broad zones designated I-III.
Figure D2. Cell-free translation of SMV-G2 RNA in the presence of fractionated soybean leaf extracts, precipitated with 2.0 volumes of saturated ammonium sulfate, from the soybean cultivar Davis. Added to translation mixtures containing rabbit reticulocyte lysate (35 ul), 50 units of RNasin, 40 uCi of 35S-methionine, and 1 ug of SMV-G2 RNA was 5 ul of either RNase-free water (lanes 1, 7, and 13), TDTT (lanes 2, 8, and 14), or a dilution of peaks I (lanes 3-6), II (lanes 9-12), or III (lanes 15-18) from Davis leaf tissue extracts (protein concentrations of 5 ug/ml, lanes 3, 9, and 15; 50 ug/ml, lanes 4, 10, and 16; 500 ug/ml, lanes 5, 11, and 17; 1000 ug/ml, lanes 6, 12, and 18). Solutions were incubated at 30° C for 1 h and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. Comparison of proteins from samples in lanes 1-6, 7-12, and 13-18 of each panel is appropriate; however, because each set (i.e., 1-6, 7-12, and 13-18) was analyzed by SDS-PAGE separately, direct comparison of molecular weights of proteins in different sets is inappropriate.
APPENDIX E.

SUPPLEMENT TO PART V
Table E1. Translation patterns of soybean mosaic virus strains in rabbit reticulocyte lysates$^a$

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$^a$ (+) = presence of protein.
Table E2. Translation patterns of soybean mosaic virus strains in wheat germ extracts

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a (+) = presence of protein.
Figure E1. Plot of the first (PRIN 1) and second (PRIN 2) principal components revealing grouping of isolates characteristic of soybean mosaic virus strains G1-G7 on the basis of translation products from rabbit reticulocyte lysates.
Figure E2. Plot of the first (PRIN 1) and second (PRIN 2) principal components revealing grouping of isolates characteristic of soybean mosaic virus strains G1-G7 on the basis of translation products from wheat germ extracts.
Figure E3. Plot of the first (PRIN 1) and second (PRIN 2) principal components revealing grouping of isolates characteristic of soybean mosaic virus strains G1-G7 on the basis of translation products from rabbit reticulocyte lysates and wheat germ extracts.
Figure E4. Plot of the first (PRIN 1) and second (PRIN 2) principal components revealing grouping of isolates characteristic of soybean mosaic virus strains G1-G7 and isolates la (la 75-16-1) and VA on the basis of translation products from rabbit reticulocyte lysates.
Figure E5. Plot of the first (PRIN 1) and second (PRIN 2) principal components revealing grouping of isolates characteristic of soybean mosaic virus strains G1-G7 and isolates la (la 75-16-1) and VA on the basis of translation products from wheat germ extracts.
Figure E6. Plot of the first (PRIN 1) and second (PRIN 2) principal components revealing grouping of isolates characteristic of soybean mosaic virus strains G1-G7 and isolates la (la 75-16-1) and VA on the basis of translation products from rabbit reticulocyte lysates and wheat germ extracts.
Figure E7. Plot of the first (PRIN 1) and second (PRIN 2) principal components revealing grouping of 13 SMV isolates on the basis of translation products from rabbit reticulocyte lysates and wheat germ extracts. Virus isolates are designated as G1-G7, G7a, N, VA, Br (Brazil), 0, and la (la 75-16-1)
Figure E8. Plot of the first (PRIN 1) and second (PRIN 2) principal components revealing grouping of accumulated proteins from the translation products of 13 SMV isolates in rabbit reticulocyte lysates. Virus isolates are designated as G1-G7, G7a, N, VA, Br (Brazil), 0, and la (la 75-16-1)