MOLECULAR STUDIES ON SOYBEAN MOSAIC VIRUS-SOYBEAN INTERACTIONS

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Molecular Studies on Soybean Mosaic Virus-Soybean Interactions

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(ABSTRACT)

In the U.S., soybean mosaic virus (SMV) is classified into seven strain groups, designated G1 to G7, based on their different responses on resistant soybean [*Glycine max* (L.) Merr.] cultivars. These responses are: symptomless or resistant (R), necrotic (N), and mosaic or susceptible (S). The gene-for-gene model has been proposed for SMV-soybean interactions. In the majority of cultivars, a single dominant gene, *Rsv1*, confers both the R and N responses. In the first part of this study, the coat protein (CP) genes of two SMV strains, G1 and G6 were isolated, cloned, and sequenced. Gene isolation was done by reverse transcription-polymerase chain reaction (RT-PCR) on partially purified virus preparation without prior RNA extraction. Amplified products were blunt-end ligated into pNoTA/T7 vector and transformed into competent cells. Sequencing was performed in both directions on heat-denatured double-stranded plasmids. The predicted 265 amino acid sequence of the CP of G1 and G6 strains were 98.9% identical, with only two amino acid differences. Correlating the CP sequences of G1, G2, G6, and G7, with their virulence on resistant soybean cultivars indicated that the CP is not likely to be the R- and/or N-determinant in the SMV-soybean system. The second part of the study involved studying the pathogenesis of G1, G6, and G7 strains on inoculated leaves of R, N, and S soybean cultivars by leaf imprint immunoassay. Results indicated four types of reactions: i) susceptible, showing unrestricted replication and spread; ii) immune, where no virus was detected; iii) systemic spread, showing unrestricted replication but limited spread along the veins; and iv) restricted replication and spread, where infection was restricted to few foci along the veins. Results of this study indicated that *Rsv1-*

mediated resistance is a multicomponent type of resistance that involves both inhibition of virus replication as well as cell-to-cell movement. The third part of the study aimed at investigating *Rsv1*-mediated resistance at the cellular level. For this purpose, an SMV-soybean protoplast system was developed. Protoplast isolation was based on a combined cellulase-pectolyase Y-23 digestion and metrizamide-sorbitol gradient purification protocol. Virus inoculation of protoplasts was facilitated by either polyethelene glycol (PEG) or poly-l-ornithine (PLO), and method of detection was by Western blotting using antiserum to whole virus. Inoculation by PEG was successful, but results were irreproducible because of the adverse effect of PEG on protoplast viability. Inoculation by PLO was inconclusive because of the high background from residual inoculum.   Additional research is needed before a protoplast system can be used to study the mechanism of *Rsv1* resistance to SMV at the cellular level.

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CHAPTER I

Literature Review

1. Characteristics of soybean mosaic virus (SMV)-soybean pathosystem

SMV is a disease that occurs worldwide wherever soybean is grown, causing severe yield reductions (Demski and Kuhn, 1989). SMV can also contribute to crop loss by causing seed coat mottling thus decreasing seed quality (Kennedy and Cooper, 1967). In the United States, it is believed that the virus was introduced in the beginning of this century with the first soybean plant introductions from the Orient (Piper and Morse, 1910). The mosaic symptoms characteristic of the disease were first described in 1915 (Clinton, 1916), but not until 1921 when the virus nature of the disease was first established (Gardner and Kendrick, 1921).

1.1. Physical and biological properties of SMV

SMV particles are flexuous rods about 746 nm in length (Bos, 1972). The thermal inactivation point (10 min) is 55-60 C and sometimes higher; the dilution end point is usually around 10^{-3}; and the longevity in sap is about 2-4 days at room temperature. The virus is most stable at pH 6.0 and loses infectivity at pH values below 4 and above 9 (Galvez, 1963).

The host range of SMV is restricted to *Leguminosae* with the exception of the two species: *Chenopodium album* L. and *C. quinoa* Willd. In nature, soybean, and less frequently bean (*Phaseolus vulgaris* L.), are the only natural hosts (Bos, 1972). SMV is readily transmitted by mechanical inoculation of plants with infected sap. It is also transmitted through seeds. Seed transmission comprises the most common source of virus for primary spread in nature because SMV basically has no other overwintering hosts (Hill et al., 1980). In addition, SMV is transmitted by a number of aphid species in the non-persistent manner. This mode of transmission constitutes the most common form of secondary spread in the field (Abney et al., 1979).

1.2. Genetics of interaction of SMV and soybean

Cho and Goodman (1979) classified SMV into seven strain groups, G1 to G7, by assigning numerous SMV isolates from commercial soybean fields and from USDA soybean germplasm collections into groups on the basis of their differential reactions on a set of resistant soybean cultivars (Table I.1). These reactions are: i) symptomless or resistant (R) in which the plant is indistinguishable from uninoculated plants; ii) necrotic (N) which is characterized by brown local and systemic spots and discoloration of leaves, leaf veins, petioles and stems, bud blight, and defoliation, often leading to the death of the plant; and iii) mosaic or susceptible (S) giving rise to chlorosis, puckering of leaves and stunting. The type strain was designated G1, the least virulent, infecting none of the resistant cultivars. Isolates that infected more cultivars were considered more virulent than G1, hence given higher numbers *i.e.*, G2-G7.

In 1986, Roane et al. proposed the gene-for-gene model for SMV-soybean resistance (R) interaction. In 1983, the same authors reported that a single dominant gene in cv. York gives resistance to G1, but the gene was given no symbol. Kiihl and Hartwig (1979) studied the inheritance of resistance to SMV in cvs. PI96983 and Ogden using G2 and G3. Resistance in PI 96983 to both strains was found to be controlled by a single dominant allele, designated *Rsv*. Ogden, resistant to G2 but producing necrosis to G3, had another allele at the same locus which

Table I.1. Reactions of soybean cultivars to SMV (Cho and Goodman, 1979; Chen et al., 1991; 1994).

Cultivar	R gene	Symptoms caused by SMV						
		G1	G2	G3	G4	G5	G6	G7
Essex	*rsv1*	S	S	S	S	S	S	S
York	*Rsv1^y*	R	R	R	N	S	S	S
Kwanggyo	*Rsv1^k*	R	R	R	R	N	N	N
Marshall	*Rsv1^m*	R	N	N	R	R	N	N
Ogden	*Rsv1^t*	R	R	N	R	R	R	N
PI96983	*Rsv1*	R	R	R	R	R	R	N

N, Necrotic; R, Resistant; S, Susceptible.

they designated *rsv^t*. The latter allele was demonstrated to be recessive to *Rsv* but dominant to susceptibility. Buzzell and Tu (1984) found a dominant gene in the cv. Raiden (PI 360844) for resistance to G7 and G7A to be at a different locus than the above and labeled it *Rsv2*. Lim (1985) reported that cvs. Suweon 97 (PI 483084) and PI 486335 had resistance to all seven strain groups controlled by a single dominant gene located at loci different from each other and from *Rsv*, but he did not test for allelism. Another inheritance study by Chen et al. (1993) revealed that resistance in cv. PI 486335 is controlled by two independent dominant genes, one of which is located at the *Rsv* locus. Buss et al. (1989) found single allelic dominant genes conferring resistance to G1 in cvs. Marshall and Kwangyo. In 1991, Chen et al. changed the symbol of cv. PI96983 allele *Rsv* to *Rsv1* and reported that cvs. PI96983, Ogden, York, Marshall, and Kwanggyo each have a single dominant gene conferring resistance to G1, and that these genes are alleles at the *Rsv1* locus. The gene symbols *Rsv1^y*, *Rsv1^m*, and *Rsv1^k* were assigned to the resistance alleles in York, Marshall, and Kwangyo, respectively. The symbol for Ogden gene *rsv1^t* was changed to *Rsv1^t* because of its dominance nature. In 1992, Bowers et al. found that cvs. Buffalo and HLS both have a single dominant resistance gene that were located at different loci. They did not test for allelic relationship to other reported genes and thus did not assign gene symbols. The chromosomal location of *Rsv1* has been identified (Yu et al., 1994). By the use of molecular markers *Rsv1* has been assigned to linkage group "F" in soybean (Yu et al., 1994).

The necrotic response in soybeans to infection with SMV was first reported by Kendrick and Gardner (1924) and confirmed by later reports (Bowers et al., 1992). The N response was often observed when less virulent strains were inoculated to cultivars that gave an S reaction to more virulent strains. This response has been considered a hypersensitive type of resistance (Buzzell and Tu, 1989; Paschal and Goodman, 1978; and Bowers et al. 1992). However, necrosis in this case is rarely restricted to the infection site. Instead, the virus, along with necrosis, spreads systemically to other parts of the plants leading to tip necrosis and eventual death of the plant. Therefore, although the N response is a manifestation of resistance, it is more deleterious to the plant than the disease or the S response (Chen et al. 1994).

Support for the fact that the N response is a hypersensitive resistance response comes from the work of Choi (1991). In one set of experiments, he demonstrated that the N response, but not the R or S, was accompanied by the activation of a cascade of defense responses. The appearance of necrosis paralleled the synthesis of fifteen acidic proteins which were similar in molecular weights as well as in enzymatic function to the well characterized pathogenesis related proteins of tobacco reacting hypersensitively to tobacco mosaic virus (Linthorst, 1991).   In another set of experiments, Choi et al. (1989) also demonstrated that during the N response an increase of gene expression of phenylalanine ammonia lyase, chalcone synthase, and peroxidase enzymes took place. These are key enzymes in several pathways leading to the production of secondary products such as the antimicrobial phytoalexins and the cell wall metabolites that are usually induced in response to pathogen attack.

The genetics of the N response is less certain than that of the R response. Kwon and Oh (1980) concluded that necrosis is controlled by a single dominant gene and that it is dominant to the S response. Buzzell and Tu (1989) also reported that stem tip necrosis in the cv. Columbia is a hypersensitive temperature dependent response and that it is dominant to mosaic and conditioned by a single dominant gene designated *Rsv3*. Other reports (Chen et al., 1994; Bowers et al., 1992) indicated that the resistance gene *Rsv1* conditions both the R and the N response. These reports demonstrated that the N response was associated with the heterozygous condition which may indicate incomplete dominance. F1 plants in resistant X susceptible crosses could either be symptomless or necrotic. The reason why not all heterozygous plants are necrotic is not known.

Kiihl and Hartwig (1979) proposed that necrosis is a response to a heavy inoculum. However, soybean plants can still become necrotic when inoculated with a given strain, no matter if the plants are mechanically inoculated with undiluted sap from infected plants or inoculated with the much smaller dose provided by aphids (Bowers et al., 1992). Therefore, these reports (Bowers et al., 1992; Chen et al., 1994) considered necrotic plants together with symptomless plants as the resistant class in the genetic analysis of resistant x susceptible crosses. In addition to the heterozygous condition, Chen et al. (1994) proposed that the N response is also controlled by the virus strain and the resistance allele, *i.e.* the N response is often observed when less virulent strains are inoculated to cultivars that gave a S reaction to a more virulent strain or when more virulent strains are inoculated on cultivars that are R to less virulent strains (Table 1). In addition, the temperature has been suggested to be a factor affecting the expression of this response. However, this factor has never been investigated experimentally. With respect to the R response, low temperature was suggested to break resistance and induce a systemic spread of SMV in resistant soybean lines (Mansky et al., 1991).

1.3. Classification and taxonomy of SMV

SMV belongs to family *Potyviridae* which contains 198 viruses (Barnett, 1991, 1992; Ward and Shukla, 1991). This family is characterized by having flexuous filaments 680-900 nm long and 11 nm wide, containing one type of coat protein and inducing host-cell associated "pinwheel" inclusion bodies. *Potyviridae* contains three recognized genera based on sequence diversity and vector transmission : 1) *Potyvirus* is the largest among plant virus groups (180 definite and possible members), contains aphid-borne viruses and is typified by potato virus Y (PVY). SMV belongs to this genus; 2) *Bymovirus* is composed of the fungal-transmitted viruses with barley yellow mosaic virus as the type species; and 3) *Rymovirus* contains mite-transmitted viruses with ryegrass mosaic virus as the type species. The family also contains two possible genera: *Ipomovirus* and *Macluravirus* which are transmitted by whiteflies and aphids, respectively.

2. Potyvirus molecular biology

The replication cycle of potyviruses involves: entry of the virus particle into the plant cell - uncoating of the virus - translation of the genome on host ribosomes -polyprotein processing to produce functional proteins - transcription of the negative sense copy of the parental positive sense strands - use of the negative sense strand as a template to produce a progeny of positive sense strands - assembly of the positive sense RNA strands and coat protein subunits to form virus particles - movement of the virus within the plant - vector transmission from plant to plant (Mathews, 1991; Shukla et al., 1994).

There has been a tremendous increase in the knowledge of potyvirus molecular biology in the past decade. This increase can be attributed to two breakthroughs which led to greater understanding of potyvirus genome structure and expression (Riechmann et al., 1992). The first breakthrough was achieved in 1986 when the complete nucleotide sequences of two members of this group, tobacco etch virus (TEV; Allison et al., 1986) and tobacco vein mottling virus (TVMV; Domier et al., 1986), were first reported. To date, the complete sequences of eleven other potyviruses have been determined. These are: bean yellow mosaic virus (BYMV; Guyatt et al., 1996), johnsongrass mosaic virus (JGMV; Gough and Shukla, 1993), papaya ringspot virus (PRSV; Yeh et al., 1992), pea seed-borne mosaic virus (PSbMV; Johansen et al., 1991), peanut stripe virus (PStV; Gunasinghe et al., 1994), pepper mottle virus (PepMoV; Bowman Vance et al., 1992), plum pox virus (PPV; Lain et al., 1989; Maiss et al., 1989), potato virus A (PVA; Puurand et

al., 1994), potato virus Y (PVY; Robaglia et al., 1989), soybean mosaic virus (Jayaram et al., 1992), turnip mosaic virus (TuMV; Nicolas and Laliberte, 1992), and yam mosaic virus (YMV; Aleman et al., 1996). Comparison of these potyviruses have revealed sequence similarities with other plus-sense RNA groups of plant viruses, such as como- and nepoviruses, as well as with animal viruses (Domier et al., 1987; Goldbach, 1987; Goldbach and Wellink, 1988). Such similarities have led to the establishment of the current picture of the potyvirus genetic map and to the proposed functions for gene products that have not been determined.

The second breakthrough came about with the isolation of full-length complementary DNA (cDNA) clones of several potyviruses from which infectious transcripts can be generated. This availability of functional cloned viruses has proven to be a powerful way to circumvent difficulties in the study of the molecular biology of RNA viruses caused by the lack of well-defined mutants and by the inability to engineer changes in their RNA genome (Riechmann et al., 1992). To date, the infectious clones of four potyviruses have been reported: PPV (Riechmann et al., 1990; Maiss et al., 1992), TVMV (Domier et al., 1989), and zucchini yellow mosaic virus (ZYMV; Gal-On et al., 1991).

Despite these discoveries, the complete picture of the functions of potyviral gene products is far from complete. Besides the coat protein, the activities of only three other proteins have been supported by experimental evidence.

The following literature review focuses on recent studies of the potyvirus molecular biology. Because most potyvirus research has been concentrated on TEV and TVMV, the focus of this review will mainly be on these two viruses.

2.1. Particle structure

The potyvirus virion is a flexuous rod, 720-850 nm long and 11-12 nm wide, made up of approximately 2000 coat protein (CP) subunits, 30-36 kD each, arranged in a helical manner around the viral RNA genome (Dougherty and Carrington, 1988; Riechmann et al., 1992; Shukla et al., 1994). This RNA genome is monopartite, plus-sense, and single stranded of about 10 kb. It has a poly (A) tail at the 3' end and a genomic-linked virion protein (VPg) covalently linked to the 5' end, the latter being the only protein besides the CP that is detected in the virus particle.

2.2. Genome structure and organization

The potyvirus RNA genome is plus-sense i.e. it functions as a messenger RNA (mRNA) that is translated into one polyprotein which is subsequently cleaved by virus-encoded proteinases into at least nine mature proteins. The generalized map of the potyvirus genome along with the positions of the various genes is presented in Fig. I.1. The genome also contains 5' and 3' nontranslated regions (NTRs) (Reichmann et al., 1992). The length of the 5' NTR is in the range of 131-205, whereas the 3' NTR of different potyviruses is quite heterogeneous in size and sequence.

Only two SMV strains, G2 and G7, have been sequenced to date (Jayaram et al., 1992). The RNA genome of both strains consists of 9588 nucleotides [excluding a poly (A)] and encodes a polyprotein of 3066 amino acids. As shown in Fig. I.1 and Table I.2, the order of genes in SMV genome is considered to be: 35 kDa P1 protein (amino acids 1-308), 51 kDa HC-Pro (309-765), 42 kDa P3 protein (766-1164), 71 kDa CI protein (1165-1798), 6k (1799-1852), 21

P1	HC-Pro	P3	CIP	6 K	NIa	NIb	CP

Fig. I.1. Generalized map of the potyvirus genome (Jayaram et al., 1992; Reichmann et al., 1992).
Abbreviations: P1, first protein; HC-Pro, helper component-proteinase; P3, third protein; CIP, cylindrical inclusion protein; 6k, 6 kDa protein; NIa, nuclear inclusion a; NIb, nuclear inclusion b; CP, coat protein.

Table I.2. Nucleotide and amino acid differences between SMV-G2 and -G7 (Jayaram et al., 1992).

Region	Nucleotides			Amino acid		
	Total	Dif.	% Dif.	Total	Dif.	% Dif.
5'NTR	131	13	10	NA	NA	NA
P1	924	52	6	308	20	6
HC-Pro	1371	54	4	457	11	2
P3	1197	85	7	399	22	6
CIP	1903	155	8	634	27	4
6K	161	9	6	54	0	0
NIa (VPg)	567	36	6	189	2	1
NIa (Pro)	729	45	6	243	2	1
NIb	1551	78	5	517	12	2
CP	798	31	4	265	3	1
3'NTR	259	18	7	NA	NA	NA

Abbreviations: 5'NTR, 5' nontranslated region; P1, first protein; HC-Pro helper component-proteinase; P3, third protein; CIP, cylindrical inclusion protein; 6k, 6 kDa protein; NIa (VPg), nuclear inclusion a (viral protein-genome linked); NIa (Pro), nuclear inclusion a (proteinase); NIb, nuclear inclusion b; CP, coat protein; 3'NTR, 3' non translated region.

kDa NIa proteinase (1853-2041), 27 kDa VPg (2042-2284), 58 kDa NIb (2285-2801), and the 30 kDa CP (2802-3066). The nucleotide and amino acid sequence identities between the two strains are 94% and 97%, with the greatest variability occurring in the 5' region specifically in the P1 and P3 proteins (Table 2). Amino acid comparison of SMV strain G7 with TEV, TVMV, PVY, PPV and SMV-G2 shows that the most conserved regions among the five potyviruses are CI, NIb and the coat protein. SMV CP shows greatest similarity to PVY and TEV CP but overall, SMV is more similar to PPV.

2.3. Genome expression and gene function

a. Translation

As mentioned earlier, the RNA genome of potyviruses functions as a mRNA and has a VPg instead of a conventional cap attached to its 5' end. Other than that, very little is known about the translation process of potyviruses and the few reports that exist in this area have concentrated on the role of the 5' NTR.

In case of TEV, the 5' NTR has a translation enhancement activity (Carrington & Freed, 1990). Fusion of the 144 nucleotide leader to the reporter gene encoding ß-glucuronidase (GUS) enhanced protein expression eight- to 21-fold when tested *in planta.* A similar result was obtained in a cell-free system where translation of GUS transcripts were found insensitive to cap analog m7GTP. This indicates that the translation enhancement ability of this region is cap-independent in that translational initiation bypasses the normal cap recognition step.

The 5' NTR of PSbMV (Nicolaisen et al.,1992), PVY (Levis et al., 1992), and TuMV (Basso et al., 1994) gave similar results. In the latter study, a stable hairpin loop was placed before or after the 5' NTR leader. When the viral leader was positioned after the hairpin loop, a significant level of GUS activity was measured. On the other hand, when the hairpin loop was positioned after the viral leader, no GUS activity was measured. From these results, the authors suggested that ribosomes bound to an internal site within the TuMV 5' NTR and then presumably scanned the sequence for the initiator AUG.

A study was conducted on an infectious clone of PPV to determine which AUG, the first at nucleotide position 36 or the second at 147, is the initiation codon (Reichmann et al., 1991). Deletion of a part of PPV 5' NTR from nucleotide 19 to 101, or introduction of a mutation into the first AUG, did not impair *in vitro* translation of the PPV transcripts indicating that the second AUG is employed *in vivo*. The hypothesis that this second AUG is recognized through a ribosomal internal entry mechanism was tested by placing PPV 5' NTR internally in a bicistronic recombinant clone. The second cistron driven by the 5' NTR was inefficiently translated indicating that PPV translation might proceed through a conventional leaky scanning mechanism.

b. Polyprotein processing

Three proteins, NIa, HC-Pro, and P1, are responsible for the proteolytic cleavages needed to process the potyviral polyprotein. The NIa is a proteinase that catalyzes most of the proteolytic events processing the C-terminal two-thirds of the polyprotein, while HC-Pro and P1 are responsible for their autocatalytic release from the N-terminus of the polyprotein.

The NIa proteinase is known to accumulate in the nucleus by virtue of its specific nuclear localization signals (Restrepo-Hartwig et al., 1990; Carrington et al., 1991). This protein is

multifunctional consisting of two domains: the 22 kDa Vpg domain at the N-terminus which is necessary for RNA replication, and the 27 kDa proteinase at the C-terminus (Dougherty and Parks, 1991; Parks et al., 1992). The proteinase domain of NIa is separated from the N-terminal part by post-translational proteolysis at an internal cleavage site in some but not all of the molecules. However, this cleavage event does not seem to be important for the NIa proteinase activity. The NIa proteinase has been considered to be structurally and functionally related to the cellular serine proteinases such as trypsin and chymotrypsin on the basis of sequence homology (Bazan and Fletterick, 1988; Dougherty and Selmer, 1993). The putative catalytic triad of histidine, aspartic acid, and cysteine (which replaces serine) has been proposed (Dougherty et al., 1989; Ghabrial et al., 1990). The NIa cleavage sites are defined by conserved heptapeptides (EXXYXQ_S\G), and seven of these sequences (named A to F and v) are found along the potyvirus polyprotein (Dougherty and Carrington, 1988; Reichmann et al., 1992; Dougherty and Selmer, 1993). These cleavage sites were identified by N-terminal sequencing of proteins purified from infected tissue and by analysis of the products of proteolytic processing from all seven sites both *in vitro* and from *Escherichia coli* Castellani and Chalmers. It is known that each site has different traits in respect to *cis* or *trans* processing, partial or complete cleavage and different reaction profiles (for review see Reichmann et al., 1992).

The P1 proteinase was characterized recently as a serine-type enzyme that catalyzes cleavage at a tyrosine-serine dipeptide between itself and HC-Pro (Verchot et al., 1991). The P1 proteinase cleaves preferentially by an autoproteolytic mechanism, and its activity *in vitro* requires a cellular factor present in extracts from plants (Verchot et al., 1992). The catalytic triad consists of histidine, aspartic acid and serine (Verchot et al., 1992). The C-terminal half of P1 (147 amino acids) contains the complete functional proteinase, whereas the N-terminal 157 amino acids is dispensable for proteinase activity. Among potyviruses, the P1 protein is the least conserved region of the polyprotein (Domier et al., 1987; Verchot and Carrington, 1995a), ranging in size from 30 kDa to 63 kDa. Actually, the N-terminal half of P1 is hypervariable both in length and in sequence.

c. Genome amplification

The process of potyvirus replication is poorly understood. Most of our current knowledge in this area centers on the functions of some of the gene products in this process. This knowledge has resulted from *in vitro* biochemical and mutational analysis of several potyviruses, as well as from amino acid comparisons with other more studied picornaviruses. These biochemical and mutational studies have indicated that most gene products function either directly or indirectly in genome amplification. Some of them, like CI, NIa and NIb, contribute essential enzymatic functions, whereas others provide only accessory replication-enhancing functions.

A mutational study was conducted on a full-length cDNA clone of TVMV to identify the gene products involved in viral replication (Gamble Klein et al., 1994). Mutants with linker insertions at known locations throughout the TVMV genome were constructed. None of the mutants with inserts in the P3, CI, 6K, NIa, or NIb was able to produce progeny viral RNA in protoplasts or plants indicating that the proteins of these genes are directly involved in replication. Two other mutants, one in P1 and one in HC-Pro were also replication defective suggesting that the effects of these proteins might be indirect by affecting polyprotein processing. The study, however, provided no insight on the mechanism of replication.

The CI protein is the principal component of distinctive cylindrical inclusions that accumulate in the cytoplasm of infected cells (Dougherty and Hiebert, 1980) and is postulated to perform a direct role in potyviral replication. Early on, amino acid sequence studies on this protein

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had indicated the presence of nucleotide-binding motif (NTBM) composed of cassettes of amino acids (GXXGXGKS) that are present in most positive strand RNA viruses (Domier et al., 1987; Gorbalenya and Koonin, 1989; Lain et al., 1989). Sequence comparisons with several viral and cellular proteins suggested that these NTBM-containing proteins possess a helicase activity indicating that RNA unwinding is probably a general requirement for genomic amplification.

The first *in vitro* study on this protein was done on PPV (Lain et al., 1990, 1991). The CI protein was purified and shown to be able to unwind RNA duplexes. This activity was dependent on the hydrolysis of NTP to NDP and inorganic phosphate and thus could be considered as an RNA helicase activity. In the *in vitro* study, the protein was only able to unwind double strand RNA substrates with 3' single strand overhangs indicating that the helicase activity functions in the 3' to 5' direction.

Sequence comparison studies among several potyviruses and other prokaryotic and eukaryotic NTBM-containing proteins indicate that the helicase activity is rather confined to the N-terminus of the CI protein. The involvement of the C-terminus in other functions in the life cycle of the virus such as the attachment of the replication complex to membranes in the host cell has been suggested (Domier et al., 1987).

The NIb protein is the most conserved region in the potyvirus polyprotein. It is believed to be the RNA-dependent RNA polymerase of potyviruses based on sequence homology with the RNA-dependent RNA polymerases of a variety of both animal and plant plus-strand RNA viruses (Domier et al., 1987; Kamer and Argos, 1984). The NIb protein of potyviruses contains the consensus motif GDD which is conserved. The study of these polymerases in the replication process has been greatly hampered by the lack of a pure and stable RNA polymerase. This is because the isolation and characterization of this protein from infected cells has been hindered by two factors: first, the presence of an inducible host RNA-dependent RNA polymerase activity; second, the low extractability of active RNA polymerases from cellular membranes and their tight association with endogenous RNA template (Matthews, 1991). The only report in this respect with a potyvirus comes from the work of Martin and Garcia (1991), who isolated a crude membrane extract with RNA polymerase activity from PPV-infected tobacco leaves that successfully catalyzed the *in vitro* synthesis of PPV RNA. Based on electrophoretic mobility and differing sensitivity to RNase, *in vitro* products probably corresponded to replicative form, replicative intermediate RNA, and to the single-stranded RNA.

Potyvirus genomic RNA has a VPg instead of a methylated cap covalently attached to its 5' end. The VPg protein was identified as a 22 to 24 kDa protein and mapped to the N-terminus of the NIa-proteinase (Siaw et al., 1985; Shahabuddin et al., 1988; Riechmann et al., 1989; Murphy et al., 1990; Laliberte et al., 1992). In a proportion of NIa molecules *in vivo*, a self-cleavage reaction occurs between the N-terminal VPg and the C-terminal proteinase domains at suboptimal cleavage site (Dougherty and Parks, 1991). The TVMV VPg is linked to TVMV RNA via a tyrosine residue (Murphy et al., 1991). Mutants of TVMV in which this tyrosine was changed did not replicate (Murphy et al., 1996). Sequence comparison with other picornaviruses have suggested that the VPg functions in RNA replication acting as a primer and/or by cleaving a replicative form of the RNA (Reichmann et al., 1992).

Between the CI and the NIa in the viral polyprotein is a small polypeptide called the 6-kDa protein. Little information concerning the function of this protein during virus replication is available. However, in transgenic plants expressing TEV 6-kDa/NIa polyprotein, the 6-kDa inhibited the nuclear translocation activity of NIa suggesting that the small polyprotein may play a regulatory role in subcellular transport of NIa (Restrepo-Hartwig and Carrington, 1992). In 1994,

Restrepo-Hartwig and Carrington introduced mutations into TEV-GUS constructs that were targeted to coding sequences of the 6-kDa protein and its N- and C-terminal cleavage sites to inhibit the cleavage of the protein from CI and NIa, respectively. These mutations resulted in viruses that were nonviable in tobacco protoplasts suggesting that the 6-kDa is essential for virus replication. The subcellular localization properties of the 6-kDa was also investigated. This was accomplished by using the 6-kDa/GUS fusion protein in transgenic plants. In fractionation studies the 6-kDa/GUS with the crude membrane fraction and immunogold labelling localized it to membranous proliferations associated with the periphery of the nucleus. These results plus the fact that the protein contains a hydrophobic domain led the authors to conclude that the 6 kDa functions as an anchor that binds the replication complexes to membranes.

The N-terminus of all potyviral HC-Pro contains a highly conserved arrangement of a histidine and several cysteine residues that are postulated to affect genome amplification by forming a "zinc finger" motif characteristic of nucleic acid-binding transcriptional factors. To investigate the ability of HC-Pro to bind nucleic acids, Maia and Bernardi (1996) cloned PVY HC-Pro in *E. coli* and purified it. Electrophoretic mobility shift assays demonstrated that HC-Pro acts as a sequence non-specific RNA-binding protein thus providing an experimental confirmation for the nucleic acid-binding activity of the HC-Pro.

Experimental evidence also exists for a possible role for the N terminal domain of HC-Pro in potyvirus replication (Atreya et al., 1992; Atreya and Pirone, 1993; Dolja et al., 1993). In the former study several mutants of a TVMV infectious clone were constructed. Individual substitutions in place of histidine and three cysteines in the putative zinc finger motif of the HC-Pro had profound effects on virulence *i.e.* the virus was either noninfectious or could not be detected by ELISA or PCR. Transcripts with insertion or deletion mutagenesis in this part of HC-Pro were also unable to infect plants suggesting that the N-terminal domain may be an essential feature for TVMV replication.   The potyviral CP was also found to have a stimulatory role in genome amplification. This role was identified through analysis of the effects of deletions and frameshift-stop codon mutations that were introduced in the TEV genome (Mahajan et al., 1996). The CP coding sequence appeared to stimulate genome amplification through two distinct mechanisms: i) the process of translation of at least the 5' half of the CP coding sequence but not the CP translation product, and ii) the CP sequence contains one or more *cis*-acting RNA control elements.   The authors argued that the stimulatory effect of the process of translation could be due to a secondary structure of RNA within the 5' half of the CP sequence that is altered by passage of the ribosomes, or that translation might be required for *cis*-preferential delivery of nascent replication proteins to a site near the genome 3' end. Alternatively, the association of the translational apparatus with that part of the genome might promote genome stability.

Using the two hybrid system, Hong et al. (1995) were able to demonstrate the interactions of TVMV NIb protein with the CP in yeast cells. Mutations in the conserved GDD domain of the NIb diminished this interaction. The authors suggested the possibility of involvement of the CP in the replication apparatus to regulate the levels of plus and minus strand RNAs in infected cells as proposed for alfalfa mosaic virus.

Recent evidence also implicates the P1 protein as an accessory factor in genome amplification (Verchot and Carrington, 1995a, 1995b). TEV mutants with deletion of the whole P1 coding region accumulated in protoplasts to approximately 2 to 3% the level of the wild virus. Also, these mutants were complemented by P1 expressed in transgenic hosts. These results indicate that P1 functions in *trans* to stimulate genome amplification. Support of these studies comes from the fact that P1 exhibits nonspecific single-stranded RNA binding activity (Brantley and Hunt, 1993; Soumounou and Laliberte, 1994).

d. Virion assembly

To date, very little is known about the mechanism of assembly of the CP monomers with the viral RNA to form potyvirus particles. *In vitro* studies have indicated that PVY particles can be dissociated into monomers by treatment with a high salt concentration or at a pH below 6 or above 9 (McDonald and Bancroft, 1977). They can then be reassociated to form long flexuous potyvirus-like virus particles in the presence or absence of RNA. It has been proposed that groups of seven to eight CP monomers polymerize to form stacked- rings which then assemble further into filamentous rods of 11 nm in diameter and of varying lengths up to several micrometers. Potyvirus particles mildly treated with trypsin, which removes the surface-exposed N- and C- termini, can also be dissociated and reassociated into potyvirus-like particles indicating that all of the necessary information required for polymerization of the CP is located within its core (Shukla et al., 1988; Jagadish et al., 1993).

Cloning and expression of the potyvirus JGMV CP in various host systems such as *E. coli* and yeast have been achieved (Gal-On et al., 1990; Jagadish et al., 1991, 1993). Results showed that the CP of JGMV expressed in *E. coli* and yeast could readily assemble in the absence of RNA to form JGMV particles with a stacked-ring appearance. Electron microscopy of sections of *E. coli* showed that these particles were formed inside the host cell and were arranged in vast arrays of parallel strands. Mutational changes of some of the residues within the core region disrupted the self assembly process (Jagadish et al., 1993). The highly conserved residues RQ (arginine and glutamine) at positions 194/195 in the core region, previously proposed to be involved as a pair in the construction of a salt bridge crucial for the assembly process (Dolja et al., 1991), were targeted for site-directed mutagenesis. The results suggested that these two residues were crucial for potyvirus structure and assembly, but they may not necessarily be involved as a pair in a common salt bridge.

e. Virus spread

i) Spread within plants

Spread of virus from the site of infection to other parts of the host plant includes two distinct processes: First, cell-to-cell movement, an active process mediated by virus-encoded movement proteins (MP), by which the virus moves through intercellular connections, the plasmodesmata (Hull, 1991; Maule, 1991; Deom et al., 1992; Carrington et al., 1996). Second, long-distance movement, which is a complex process by which the virus enters, circulates within, and exits the vascular tissues, mainly the phloem (Seron and Haenni, 1996; Carrington et al., 1996).

In potyviruses, cell-to-cell and long-distance movement are understood poorly and proteins with dedicated movement functions have not yet been identified. Initially, the P1 protein was proposed extensively in the literature to function as the potyviral MP (Domier et al., 1987; Riechmann et al., 1992). However, recent evidence indicates no role of P1 in virus movement (Verchot and Carrington, 1995a, 1995b). In the former study, mutations, insertions and deletions in the N-terminal non-proteolytic domain of TEV P1 coding sequence had no effect on virus movement and systemic infection. Mutations in the C-terminal proteolytic domain, on the other hand, rendered the virus non-viable.

In addition to MP, CP is also involved in the movement of several plant viruses. In case of

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potyviruses, the first direct evidence of the role of CP in cell-to-cell and long-distance transport came from the work of Dolja et al. (1994, 1995). In these studies, the infectious clone of TEV genome carrying a copy of GUS between the P1 proteinase and the HC-Pro (TEV-GUS) was used (Dolja et al., 1992). This allowed direct observation and quantitation of virus movement in plants by detecting GUS activity. Two types of mutations were constructed in the CP of TEV-GUS. Mutants with substitutions of conserved residues within the core domain were assembly-defective and unable to move from cell-to-cell. Deletion mutants lacking most of the N- or C-terminal domain which comprise regions exposed on the virion surface were assembly-competent and possessed a slow cell-to-cell movement phenotype. These mutants, however, were completely impaired in long-distance transport. Both cell-to-cell and systemic movement defects were complemented in transgenic plants expressing the wild type TEV CP. These data indicate that different domains within the CP have specific roles in cell-to-cell and long-distance transport. Furthermore, since N- and C-terminal truncated mutants were able to form virions and were only partially inhibited in cell-to-cell movement, evidence was, therefore, provided that virus assembly is not the only role of CP during transport (Dolja et al. 1994, 1995).

In addition to the N- and C-termini of the CP, recent evidence suggests that the HC-Pro functions in long-distance transport (Cronin et al., 1995). A clone of TEV-GUS with tripeptide substitution mutation in the conserved sequences within the central region of HC-Pro failed to show symptoms on the upper leaves. However, this mutant was able to amplify in protoplasts indicating that the movement and replication functions of HC-Pro were distinct. The histochemical analysis indicated that this mutant was able to reach as far as the phloem companion cells of inoculated leaves, indicating that the block might have been at the point of virus entry into the sieve elements. The authors (Cronin et al., 1995) proposed that probably HC-Pro and the CP (or virions) interact to form active complexes that facilitate both insect transmission and long-distance movement (see below).

ii) Spread from plant to plant/aphid transmission

Natural spread of potyviruses from plant to plant is accomplished by aphids in the non-persistent manner. Two virus-encoded proteins are needed to accomplish this process: the HC-Pro and the CP (Pirone, 1991).

Previously, it had been proposed that the HC-Pro functions by binding potyvirus particles to the food canal of aphid vectors (Govier and Kassanis, 1974; Berger and Pirone, 1986). Later, transmission electron microscopy-immunogold labeling and autoradiography studies provided direct evidence supporting this hypothesis (Ammar et al., 1994; Wang et al., 1996). In the latter study, mouth parts of aphids were sectioned after feeding through membranes on purified virions of TEV and TVMV in presence of a functional or defective HC-Pro. Of aphids fed on TVMV with a functional HC-Pro, 57% had virus particles associated with the epicuticle of their food canal. No virus particles were found in aphids fed on a non-transmissible combination. Autoradiography of intact stylets fed on ^{125}I-labeled TEV gave similar results. Of aphids that fed on TEV with a functional HC-Pro, 48% retained label in stylets, while label was barely detectable in stylets of aphids fed on TEV and a non-functional component. These results gave strong evidence that aphid transmissibility is dependent upon retention of virions in the mouth parts of aphids and that HC-Pro functions by facilitating such retention.

Comparisons of the derived amino acid sequences of HC-Pro of PVC, a non-transmissible strain of PVY, with that of PVY and three other potyviruses revealed two amino acid changes within

areas of conserved boxes in the N-terminus of the HC-Pro, one from lysine _ glutamine within lysine-isoleucine-threonine-cysteine (KITC) box and second from isoleucine _ valine within isoleucine-aspartic acid (ID) box (Thornbury et al., 1990). By site-directed mutagenesis, a lysine was introduced instead of a glutamic in the KITC box of TVMV and total loss of helper activity was recorded (in addition to reduction in virus accumulation and attenuation of symptoms) (Atreya et al., 1992, 1993). However, it is proposed that more than one region in the HC-Pro is functionally involved in aphid transmissibility. Recently, a threonine _ alanine substitution within the proline-threonine-lysine (PTK) box located in the C-terminal half of HC-Pro of a non-transmissible strain of zucchini yellow mosaic virus (ZYMV) was also observed (Huet et al., 1994). The KITC box, related to loss of helper activity in PVC and TVMV, was not changed in this strain.

The mode of action of these mutations at the molecular level in aphid transmission is not known. Two possible mechanisms have been suggested (Huet et al., 1994): (i) HC-Pro serves as a link between the virus and the stylet, thus one box (either KITC or PTK) is required to attach to the virus and the other (KITC or KTP) is required to attach to the aphid's stylet; or (ii) these boxes are co-involved in dimer formation of the biologically active helper, since some evidence has been provided that the HC-Pro is biologically active only in a dimer form (Thornbury et al., 1985).

Successful transmission of potyviruses by aphids also depends on the CP. Initially, after the sequence of the CP region of a number of potyviruses has been determined, it became apparent that the conserved amino acid motif aspartic acid-alanine-glycine (DAG) near the CP N-terminus in the aphid transmissible (AT) potyviruses is essential for aphid transmissibility (Lain et al., 1988; Maiss et al., 1989; Gal-On et al., 1990, 1992). In SMV-N, a non-aphid transmissible isolate of SMV-G2, the sequence DAD is present instead of the DAG box (Eggenberger et al., 1989).

The first direct evidence that an amino acid substitution in the third position of the DAG triplet can affect aphid transmissibility was provided by the work of Atreya et al. (1990). Comparison between TVMV-AT and TVMV-NAT (not aphid transmissible) revealed a single difference, glycine _ glutamic acid, in the DAG box. A cDNA fragment representing the CP gene of TVMV-NAT was substituted into a full-length cDNA clone of TVMV-AT. The resultant hybrid virus could not be transmitted by aphids.

To further define the nature of the amino acids in the N-terminal domain of TVMV CP that are involved in the aphid transmissibility of the virus, site-directed mutagenesis was used to alter the amino acid residues in the DAG triplet and other N-terminal residues (Atreya et al., 1991, 1995). In the first position of the triplet, an acidic or neutral amino acid, but not a basic one, imparted transmissibility. In the second position a small nonpolar residue was needed. In the third position, glycine seemed to be critical. The amino acid following the DAG does not appear to be conserved among potyviruses. However, the change from the wild-type lysine _ glutamine resulted in loss of aphid transmissibility of TVMV. Atreya et al (1991) suggested that the motif involved in aphid transmissibility should be expanded to DAGX.

The mechanism of action of the amino acids in DAG in regulating aphid transmissibility is unknown. It has been proposed that the DAG sequence near the N-terminus interacts, perhaps by binding, with the HC-Pro to allow aphid transmission to occur (Atreya et al., 1990). However, direct evidence for this is lacking.

3. Potyvirus coat protein

Of the potyviral genome, the CP has been the most extensively characterized. So far, the CP

sequences of 30 potyviruses have been determined (Shukla et al., 1994). This interest in the CP comes mainly from its usefulness in taxonomic and evolutionary studies, in diagnosis, and in CP-mediated resistance (Beachy et al., 1990). The importance of the CP in these studies stems from the following facts (Riechmann et al., 1992): i) the CP is a unique gene product that is characteristic of the group, ii) the CP is the only structural protein (besides the VPg) that is detected in the virus particle and accounts for 95% of the virion, iii) its involvement in virus-plant interaction, and finally iv) the CP is the most accessible gene to clone since it is the last gene located at the 3' end of the potyvirus genome.

The CP of potyviruses is involved in several biological functions with the primary one being to encapsidate and protect the viral RNA. As discussed in the preceding sections, other functions of the CP are aphid transmission, cell-to-cell and long distance movement, as well as in genome amplification. Another role of the CP of potyviruses may be in plant-virus interaction as in host recognition leading to host range specificity and symptom induction, as well as a determinant of host resistance/pathogenicity. More on the latter will be presented later in the "plant resistance to viruses" section.

3.1. Molecular weight (MW)

The MW of the potyvirus CPs are in the range of 28-40 kDa. Size estimates of this CP have been accomplished by two ways: by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and by sequence data (Shukla and Ward, 1989a). The MWs reported in the literature based on SDS-PAGE have varied considerably. This variability could be attributed to two causes (Shukla et al., 1994): first, to varied gel concentration and other differences in electrophoretic conditions, and second to differential cleavage at two different sites in the polyprotein junction between the polymerase protein NIb and the coat protein. In addition to this variation, there have been many reports indicating the heterogeneity of the CP bands i.e. the presence of two or more bands migrating in the gel. This has been attributed to the partial degradation of the CP, which initially involves removal of the surface exposed N- and C-termini by proteases of host or microbial origin (Shukla and Ward, 1989a; Shukla et al., 1994). A recent study by Hassan et al. (1994) has attributed the occurrence of multiple bands to the composition of the extraction buffer. This study indicated that extraction of PSbMV proteins from plant tissue with a buffer containing SDS and/or urea lead to mobility changes for the proteins in SDS-PAGE resulting in two bands: one fast with an apparent MW of 36 kDs, and the other slow with a MW of 39 kDa. However, no slow form was seen when denaturating agents were omitted from extraction buffer. Additionally, when infected leaves were submerged in boiling water for 15 sec before extraction no slow form was seen. *In vitro* transcription and translation of a cDNA containing the CP coding sequence preceded by an artificial start codon gave a protein with a size of 36 kDa indicating that the fast form of PSbMV CP was the authentic CP, while the slow form had been modified in some way. To ascertain whether the CP could be converted to the slow form *in vitro*, purified PSbMV particles were mixed with healthy plant sap with or without urea. Western blots revealed both the slow and fast form in samples incubated with urea, while the virus incubated without urea contained the fast form. The authors considered glycosilation to be unlikely since the reaction could take place after high speed centrifugation *i.e.* in absence of plant membranes, and suggested the possibility that denaturating conditions may expose regions of the CP to a soluble plant enzyme leading to this conversion.

3.2. CP structure

One major feature of the potyvirus CP subunit is that it is composed of three regions: the N-terminus, core protein, and the C-terminus. The N- and C-terminus are surface exposed and are liable to be removed upon mild trypsin digestion, while the protein core is trypsin resistant (Allison et al., 1985; Shukla et al., 1988). The core particle *i.e.* trypsin-treated particles without the N- and C-termini are still infectious and are indistinguishable structurally from untreated particles indicating that these termini are not required for particle integrity (Shukla et al., 1988; Dolja et al., 1994, 1995). Furthermore, treated particles can be disassembled and reassembled into potyvirus-like particles indicating that the N- and C-termini are not required for virus assembly and that all of the necessary information required for polymerization of the CP is located within its core region (Shukla et al., 1988; Jagadish et al., 1993).

As mentioned before, the CP from different potyviruses varies in size and ranges from 251 to 332 amino acids (Shukla and Ward, 1989a; Shukla et al.,1994). These size differences are largely due to variation at the N-terminus which ranges from 19 to 97 residues. Additionally, this region varies considerably in sequence among the different potyviruses. In contrast, the C-terminal region varies in length by only two residues (18-20) and show significant sequence identity. The core protein region also shares striking identity in both sequence (65%) and in length (214-217 residues).

The sequences of the CP of four strains of SMV have been determined. These are SMV-VA, -N, -G2, and -G7. SMV-N is considered to be an aphid non-transmissible version of SMV-G2 with only three nucleotide changes and one amino acid change (glycine _ aspartic acid) in the DAG box implicated in aphid transmissibility (Eggenberger et al., 1989, Jayaram et al., 1991). Based on biological properties, SMV-VA was identified to belong to G1 strain group (Hunst and Tolin, 1982). However, sequence data of this strain indicated that it was a distinct potyvirus more related to TEV (69% homology) than to SMV-N (58% homology) (Gunyuzlu et al., 1987; Shukla and Ward, 1988) (see next paragraph). Sequence analysis of both SMV-G2 and -G7 has indicated that the CP gene occupies the 3'-terminal 798 bases, followed by a NTR of 259 bases long [excluding a poly (A) tail] (Jayaram et al., 1991). The gene product consists of 265 amino acids starting with a serine at position 1. The surface-exposed N- and C-termini in both strains are 24 and 18 nucleotides respectively. SMV-G2 and -G7 are highly similar with 90% homology at the nucleotide level with 31 base differences and 98.8% amino acid sequence identity with three changes: one in the N-terminus at position 8, one substitution in the core at position 217, and one at 264 at the end of the C-terminus.

High-performance liquid chromatography (HPLC) peptide profiles of CP tryptic digest and amino acid composition data have been obtained for 14 strains of SMV (Jain et al., 1992). Results indicated that the 14 strains, including SMV-VA were highly similar to SMV-N. No evidence was found for the reported CP sequence of SMV-VA (Gunyuzlu, 1987), implying that the sequenced SMV-VA clone was rather a minor contaminant of the original SMV-VA isolate (Jain et al., 1992).

The secondary and tertiary structure of the potyvirus CP has not yet been elucidated (Shukla and Ward, 1989a, Shukla et al., 1994). However, structural predictions for PVY-D suggested ten a-helices and four ß-sheets. Seven of the a-helices are either overlapping or separated by only one or two amino acids reducing the number to seven. These structural predictions for PVY-D suggest features similar to those in other rod-shaped plant viruses such as TMV and potato virus X (PVX) as depicted in Fig. I.2. TMV and PVX are also known to have the N- and C-termini exposed on the particle surface.   In addition, the well-studied TMV structure
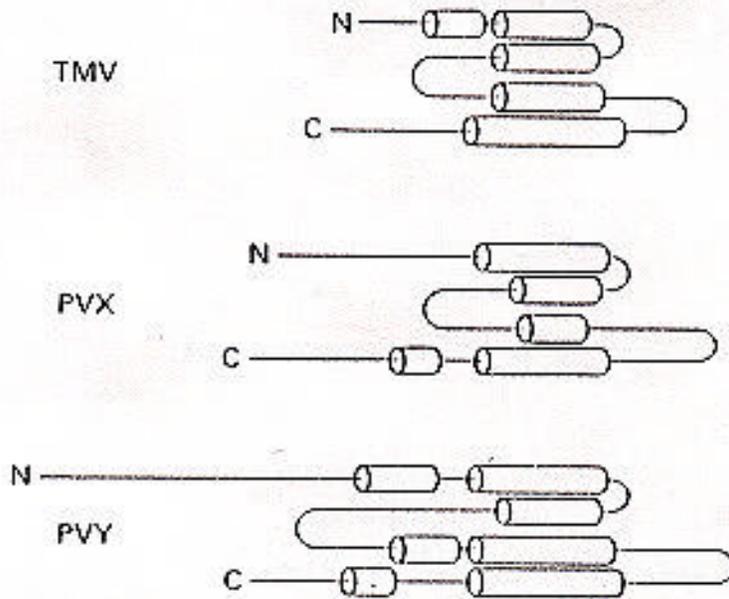
**Fig. I.2.** Schematic drawings of the coat protein subunit secondary structures of the tobamovirus tobacco mosaic virus (TMV), the potexvirus potato virus X (PVX) and the potyvirus potato virus Y (PVY). The predicted folding pattern and arrangement of a-helices (indicated by cylinders) of PVX and PVY are compared with a simplified cartoon of the known structure of TMV. Reproduced from Shukla et al., 1988.

shows that the central part of the molecule consists of a group of four a-helices which are arranged closely parallel or antiparallel to each other. These helices are held together by four short antiparallel ß-sheets and intra- and inter-subunit salt bridges.

As mentioned earlier, the potyvirus particle is made up of approximately 2000 CP subunits arranged in a helical manner around a single copy of viral RNA (Shukla et al., 1994). The pitch of the nucleocapsid is 3.3 nm with 7-8 CP subunits per ring. The estimated number of nucleotides associated with each CP subunits is six.

The dissociation and reassembly of PVY particles have been studied *in vitro* (McDonald et al., 1976; McDonald and Bancroft, 1977). These studies showed that the CP can readily dissociate into its constituent subunits in a high salt and/or at pH of 6 or 9. By adjusting these conditions, the CP subunits readily formed long flexuous particles in absence of RNA. These subunits first polymerized to form stacked-rings, each about 40 nm long then these further assembled into filamentous particles of 11 nm in diameter and of varying lengths up to several micrometers. The precise way in which the PVY particles assemble is not known, but it was suggested that the stacked-ring structure may be an intermediate in the virus assembly, the RNA being threaded into the small central cavity of the ring causing a conformational shift from the stacked-ring to the helical form (McDonald and Bancroft, 1977; Shukla et al., 1994). Alternatively, the virus may be directly formed from further monomers of small assembly aggregates, the stacked-ring particles being disassembled by RNA before reassembly around it.

3.3. CP importance in potyvirus taxonomy

By the late 1980s, the taxonomy of the potyvirus group was in a very unsatisfactory state because of its size, complexity and variability. It was suggested then that successful resolution of potyvirus detection and identification presents a major challenge for plant virologists (Shukla and Ward, 1988; Shukla and Ward, 1989b). This unsatisfactory state of potyvirus taxonomy was attributed partly to the large size of this group, since it is the most rapidly growing and is the largest among the 35 different plant virus groups (Matthews, 1991). The main cause, however, is the large variation among potyviruses and the inability to understand the taxonomic significance of this variation. It was believed, on the basis of biological properties and inconsistent serology that stains of potyviruses form a continuous array (continuum hypothesis) between two or more viruses in such a way that boundaries separating distinct potyviruses cannot be sharply defined (Hollings and Brunt, 1981). Recently, however, amino acid sequences of potyvirus CPs have been adopted as a basis for the identification and classification of potyviruses into distinct viruses and strains (Shukla and Ward, 1988; Shukla and Ward, 1989b). This criterion proved to be inconsistent with the "continuum" hypothesis and showed a clear demarcation between distinct viruses and between strains. Computer analysis of the available potyvirus CP amino acid sequence data revealed a bimodal distribution of sequence homologies. Sequence homology between distinct potyviruses ranged from 38 to 71% (average 54%) with major differences in the length and sequence of their N-termini and high homology in the C-terminal three-quarters of the CP. In contrast, strains of individual viruses exhibit homology of 90 to 99% (average 95%) and have very similar N-terminal sequences.

4. Plant resistance to viruses

Plant resistance to viruses is of two types (Fraser, 1987; Mansky and Hill, 1993): i) non-host resistance, in which all members of a species are completely resistant; this type is not

considered in this review, ii) host resistance which occurs when some members of a species are susceptible and others are resistant. The latter type of resistance is usually controlled by either a single dominant gene or a small number of genes, and thus it is mostly used by the plant breeder.

Available data indicates that host resistance to viruses can be one of two types. It can be direct constitutive resistance resulting from the direct inhibition of an essential virus function which is presumed to be conferred directly by the product of the resistance gene. Alternatively, host resistance to viruses can be induced. In this type of resistance, which is termed hypersensitive response (HR), the virus is restricted as a result of the elicitation in the plant of a pre-existing defense reaction. Such activation is postulated to occur following the plant recognition of the virus, its genome, or a nonstructural protein encoded by the virus. In other words, HR may be induced following specific interaction, which could be direct or indirect, between a host resistance gene (R) and a corresponding pathogen avirulence gene (Avr) (gene-for-gene model) (Flor, 1971; Keen, 1990). Despite the highly specific Avr-R interaction that initiates the process, the resulting induced resistance (referred to as acquired resistance) is non-specific and is effective against diverse pathogens. The visual manifestation of HR is usually, but not always, a necrotic lesion at the infection site.

The molecular mechanism underlying resistance is poorly understood. Generally, plant resistance to viruses occurs by interruption of the virus life cycle at one or more of the following stages (Matthews, 1991): 1) entry into the cell, 2) uncoating of the virus genome, 3) translation of the viral proteins, 4) replication of the viral genome, 5) assembly of progeny viruses, and 6) spread of the virus both to other cells and to new hosts. In the broadest sense, we can say that plant resistance to viruses can work on the replication cycle within the individual cell and in this case resistance is termed immunity. Alternatively, virus replication can occur in the inoculated cell(s) but does not proceed further due to an ineffective virus movement. A valuable experimental tool to characterize the mechanism of virus resistance is the use of plant protoplasts. Virus resistance can be expressed in protoplasts and in this case resistance is more likely to be caused by inhibition of virus replication. Alternatively, if the protoplasts from resistant plants support virus multiplication, then resistance operates by inhibiting cell-to-cell movement and/or subsequent systemic spread of the virus.

In viral systems, the starting point for the analysis of resistance, in general, has been the identification of viral genes associated with overcoming resistance. In most studies, such genes, usually termed resistance-breaking determinants, are often identified by sequence analysis; *i.e.*, to correlate sequence variability with virulence. Examples of such approach will follow. To precisely map resistance determinants, however, of great use has been the production of infectious clones which allows the manipulation of the viral genome by *in vitro* mutagenesis or by the making of hybrid strains. In cases of HR, transgenic plants were of use, where the putative Avr gene is introduced into the genome of the resistant cultivar thus resulting in overall necrosis or HR.

In the general sense, only few virus-host systems have been described in the literature. Three systems have been well characterized, however, and these are tobacco mosaic virus (TMV)-tobacco, TMV-tomato, and potato virus X (PVX)-potato. With respect to potyviruses, the literature is scarce and the mechanism of resistance/pathogenicity of only very few viruses of the 180 or so members of the group has been studied. As most of the research in this area has concentrated on the above three systems, the emphasis of this review will be on these three systems and on what is available on the potyvirus genus.

4.1. TMV-tobacco

The *N'* gene of *Nicotiana sylvestris* Speg. et Comes confers an HR against most strains of TMV but not others (U1 and OM) (Culver et al., 1991b). Saito et al. (1987) was the first to map the induction of HR in this system to the CP of TMV. This was demonstrated through the production of hybrids between TMV-L which produce HR in *N. sylvestris* and TMV-OM which causes a systemic infection. When TMV-L is replaced by the CP of TMV-OM, HR was not produced and systemic infection ensued. The cloning of nitrous acid mutants of TMV-U1 capable of inducing HR and the subsequent exchange of specific segments of these mutants into the wild-type TMV-U1 cDNA clone revealed that a point mutation in the CP is responsible for the induction of HR (Knorr and Dawson, 1988). This point mutation resulted in a single amino acid substitution of serine to phenylalanine at position 148 in the CP. To establish whether additional mutations in the CP could also induce HR, Culver and Dawson (1989a) made four individual nucleotide mutations in the CP of TMV-U1 strain resulting in four different amino acid substitutions. These four mutants induced HR in *N. sylvestris* indicating that changes throughout the CP gene could induce HR. The expression of the altered CP, and not the RNA sequence, during virus replication was found to be required for the induction of HR (Culver and Dawson, 1989b). An HR-inducing mutant with an alteration in the CP translational start codon, preventing the production of the CP but maintaining an intact CP ORF was produced. The resultant mutant failed to induce HR although it replicated efficiently. Transgenic *N. sylvestris* plants that expressed only the CP gene of an HR-inducing strain displayed an HR phenotype and resulted in necrosis and the collapse of the entire plant (Culver and Dawson, 1991b; Pfitzner and Pfitzner, 1992). This result indicated that the CP by itself is sufficient for eliciting HR. Recently, two studies (Culver et al., 1994; Taraporewala and Culver, 1996) provided evidence demonstrating a critical role for a specific CP structural site in eliciting HR. Specific substitutions were created within the CP of the elicitor strain P20L to identify structural areas essential for host recognition. All of the substitutions that led to a change in host response resided within the three-dimensional CP structure along the right face of the molecule's helical bundle that disrupt interface regions between adjacent CP subunits. The authors suggested that the aggregation state of the subunits may influence induction of the HR. Alternatively, the strong association between CP subunits in virulent strains may mask a universal elicitor domain from the putative *N'* receptor.

The *N* gene originally identified in *Nicotiana glutinosa* L. is a single dominant gene that confers an HR against all strains of TMV except tomato mosaic virus- Ob strain (ToMV-Ob) (Culver et al., 1991b). This resistance gene has been isolated recently, thus becoming the first plant gene for virus resistance gene to be cloned (Whitham et al., 1994). It has been shown that protoplasts isolated from leaves of *N* gene tobacco plants fully support virus replication without an HR (Otsuki et al., 1972). Thus, multicellular infection or the cell wall appears to be necessary for the induction of HR. Several lines of evidence suggest that the CP of TMV is not the elicitor of HR for the *N* gene. TMV mutants with a deletion of the entire CP ORF or with a replacement with the bacterial chloramphenicol acetyltransferase ORF were found to induce HR in *N* gene plants (Takamatsu et al., 1987). Transgenic Xanthi NN tobacco plants that expressed TMV MP did not exhibit necrosis in absence of infection (Deom et al., 1991). Additionally, a recombinant virus in which the MP of TMV was replaced with that of ToMV-Ob resulted in HR (Padgett and Beachy, 1993). Thus, the MP is not directly involved in the induction of HR. Chemical mutagenesis of the infectious TomMV-Ob cDNA clone with hydroxylamine was done and a mutant that induced HR was identified (Padgett and Beachy, 1993). Sequence analysis of this mutant revealed a single nucleotide change in the 126 kDa replicase gene leading to a proline _ leucine change. Re-creation of this mutation in the parental TomMV-Ob cDNA clone by site-directed mutagenesis also resulted in induction of HR.

4.2. TMV-tomato

Three genes, *Tm-1*, *Tm-2, Tm-2²*, have been recognized in tomatoes (*Lycopersicon esculentum* Mill.) as conferring resistance to TMV (Pelham, 1966; Culver et al., 1991b). The latter two are alleles and produce an HR type of resistance. The expression of these resistance factors can be displayed as either a necrotic local lesion resulting in resistance, or systemic necrosis resulting in severe disease. The type of necrosis displayed is dependent upon the type of strain, the genotype of the plant, and environmental conditions, especially temperature. *Tm-2* and *Tm-2²* do not affect virus replication in protoplasts (Motoyoshi and Oshima, 1977). *Tm-2* resistance is known to be broken by coinoculation with PVX, which belongs to another taxonomic group (Atabekov and Dorokhov, 1984). These observations thus suggested that the *Tm-2* and *Tm-2²* resistance mechanism affects the cell-to-cell movement of the virus. Comparisons of the nucleotide sequences of resistance-breaking virulent mutants to that of the wild-type virus revealed amino acid substitutions at different positions in the 30 kDa movement protein (Meshi et al., 1989; Calder and Palukaitis, 1992). All of these substitutions, however, involved change in the local charge of the 30 kDa movement protein. These results suggested that resistance breakage may involve electrostatic interactions between 30 kDa protein and some host factor encoded by *Tm-2/Tm-2²* locus. However, it is not known whether the wild-type 30 kDa protein acts as an elicitor of HR in tomato (Culver et al., 1991b).

Unlike the resistance conferred by the *Tm-2* and *Tm-2²* genes, *Tm-1* resistance is symptomless and also expressed in protoplasts (Motoyoshi and Oshima, 1977) indicating that it operates at the level of virus replication. The amino acid composition of the CP has been compared between virulent and avirulent strains but no strong correlation with the virulence was observed (Dawson et al., 1979). Comparison of the genomic sequences between virulent and avirulent strains, however, have revealed two base substitutions resulting in amino acid changes in the 126 and 180 kDa replicase proteins (Meshi et al., 1988). The two substitutions were introduced into the avirulent strain and the resultant mutant multiplied in *Tm-1* tomato. A strong correlation was found between the ability to overcome the resistance and a decrease in local net charge, suggesting the involvement of an electrostatic interaction between the viral 126 and 180 kDa proteins and a putative host resistance factor. However, replacement of one of these amino acids which is glutamine at position 979 by amino acids that does not involve change of charge of the protein like lysine also broke resistance (Hamamoto et al., 1997). This result indicates that the decrease in the net charge is not the major reason for overcoming *Tm-1* resistance.

4.3. PVX-potato

A number of genes have been identified in potato (*Solanum tuberosum* L.) that confer strain specific resistance to PVX. The genes *Nx* and *Nb* are single dominant genes that confer an HR response in incompatible interactions with PVX. Based on their response on cultivars carrying these genes, PVX is classified into four strain groups (Cockerham, 1970): i) group 1 strains induce a necrotic response on potato cultivars with either the *Nx* or *Nb* gene, ii) group 2 strains induce necrosis on *Nb* cultivars, iii) group 3 strains induce necrosis on *Nx* cultivars, and iv) group 4 strains overcome both resistance genes. The necrosis induced by PVX on plants with the *Nx* and *Nb* genes is expressed phenotypically either by the development of necrotic local lesions on the inoculated leaf or as a systemic necrosis. The systemic infection termed apical necrosis results in death of the apical tissue and eventually the death of the plant. The type of necrosis induced usually depends on both inoculum concentration and environmental conditions, especially temperature (Adams et al.,

1986a). Experiments on the effects of *Nx* in potato protoplasts have provided conflicting data. Adams et al. (1985) reported that *Nx* did not affect virus multiplication, whereas Foxe and Prakash (1986) reported the opposite result.

Another class of resistance genes in potato that is also inherited as a single dominant character are the two *Rx* genes: Rx_{adg} and Rx_{acl}. These two loci, which are unlinked, have been introgressed into various potato cultivars from wild potato relatives (Cockerham, 1970). Both genes confer in most instances an identical symptomless reaction with no visible signs of infection (Tozzini et al., 1991). *Rx*-mediated resistance is effective against all strains of PVX except the group 4 strain PVX_{HB} (Moreira et al., 1980). This reaction, which has been termed extreme resistance or immunity, is unusual in that it is expressed at the level of protoplasts and leads to complete suppression of virus multiplication (Adams et al., 1986b, Kohm et al., 1993). However, one allele of *Rx*, $Rx_{acl}{}^{n}$ is associated with HR rather than the extreme resistance phenotype (Cockerham, 1970). Additionally, there are reports that if potato carrying Rx is graft inoculated, there is low-level accumulation of PVX in the systemic tissue and some necrosis (Goulden and Baulcombe, 1993). Despite the complete suppression of virus multiplication in *Rx* plants, *Rx*-mediated resistance has been found to be an induced effect based on the observation that protoplasts of *Rx* potato become resistant to PVX_{HB}, cucumber mosaic virus (CMV), or TMV if co-inoculated with an avirulent strain of PVX (Kohm et al., 1993).

The genetics of the PVX-potato pathosystem is viewed from the standpoint of gene-for-gene model of plant-pathogen interaction. Hybrid viruses based on an avirulent strain (PVX_{UK3}) and the virulent PVX_{HB} strain have demonstrated that overcoming *Nx*-mediated resistance maps to the CP gene (Kavanagh et al., 1992). Sequence comparison between the avirulent strain PVX_{DX} and the virulent strain PVX_{DX4} has indicated a single amino acid change, glutamine _ proline (Santa Cruz and Baulcombe, 1993). Constructing hybrid viruses by replacing the CP gene of a full-length PVX clone with the corresponding sequences from PVX_{DX} and PVX_{DX4} has shown that a single nucleotide difference was sufficient to alter the outcome of *Nx*-resistance. The Avr determinant of PVX on *Rx* potato was also found to be the coat protein (Kavanagh et al., 1992; Goulden et al., 1993; Querci et al., 1993, 1995). Querci et al. (1993) compared the nucleotide and amino acid sequence of PVX_{HB} and avirulent strains and highlighted the presence of eight residues unique for the HB strain. Mutational analysis and secondary structure predictions revealed that only two of the eight residues were of importance for overcoming resistance. Goulden et al. (1993) analyzed a series of hybrid and mutant isolates of PVX and concluded that overcoming extreme resistance in *Rx* plants is affected by two amino acids in the CP with one at position 121 (threonine _ lysine) representing the major determinant.

4.4. Cowpea mosaic virus (CPMV)-cowpea

A well-characterized example for direct resistance in plant viruses is that of the cowpea [*Vigna unguiculata* (L.) Walp.] cv. Arlington against the comovirus cowpea mosaic virus (CPMV). Arlington-derived resistance is controlled by a single dominant locus in crosses with the susceptible Blackeye 5 cowpea (Ponz et al., 1988). Among the more 1000 lines tested, only Arlington protoplasts resisted infection by CPMV indicating that Arlington-derived resistance restrict replication of CPMV (Beier et al., 1977, 1979). Later the CPMV-resistance factor of Arlington was found to be a specific inhibitor of the virus protease that process the polyprotein 95 kDa into the coat proteins and the cell-to-cell movement protein (Ponz et al., 1988).

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4.5. Potyviruses

The Burley tobacco (*Nicotiana tabacum* L.) cv. Tennessee 86 (TN86) is resistant to most strains of tobacco vein mottling virus (TVMV), the virus being restricted to epidermal cells of inoculated leaves (Gibb et al., 1989). One strain, designated TVMV-S, overcomes this resistance and infects TN86 systemically. Resistance in TN86 is controlled by a single recessive gene, designated *va*, which in the homozygous condition provides resistance to TVMV, and medium tolerance to PVY and TEV. On the other hand, *N. tabacum* cv. Kentucky (KY 14) is susceptible to these three potyviruses. Protoplasts from both KY14 and TN86 supported accumulation of TVMV, although lower amounts were detected in TN86 protoplasts. These results suggest that resistance is primarily due to restricted systemic movement although a reduction in the rate of virus replication may also play a role (Gibb et al., 1989).

Site-directed mutagenesis introduced into the TVMV HC-Pro gene 5'-terminal region has provided evidence that this domain is an essential element for TVMV infectivity and virulence, in addition to aphid transmissibility (Atreya et al., 1992; Atreya and Pirone, 1993). Plants of the cv. KY 14 inoculated with TVMV mutants developed delayed symptoms which shortly disappeared. The virus and viral RNA followed a similar pattern, in that they were noticeably lower than the wild-type TVMV and decreased to undetectable levels over time. TVMV with deletions in the HC-Pro were unable to infect plants. Furthermore, these deletion mutants were not complemented in transgenic plants expressing the HC-Pro. The authors (Atreya and Pirone, 1993) suggested the possibility that the HC-Pro nucleotide sequence itself was involved in the interaction with the host.

Construction and analysis of infectious transcripts from TVMV-S strain infectious clone have been achieved recently (Nicolas et al., 1996). A determinant responsible for resistance breaking has been mapped to the VPg cistron. Variant TVMV-S transcripts containing changes within the VPg exhibited a 75% reduction in infectivity on cv. TN86 as measured from number of infected plants.

Comparison of two full-length TVMV cDNAs whose *in vitro* transcripts induce wild-type or attenuated symptoms in tobacco plants cv. KY14 has shown that the determinant of symptom severity is located in the noncoding 3'-NTR of the viral RNA (Rodriguez-Cerezo et al., 1991). Results of sequence analysis and genome exchange experiments indicated that a 58 nucleotide segment consisting of patterns of adenine and uracil residues in the 3'-NTR is responsible for the symptom attenuation phenotype. On the other hand, the amount of virus produced in the plants as determined by ELISA and Northern blot analysis was not altered. The mechanism by which the mutated transcripts affects symptom severity is not known, but it was suggested that alterations in the secondary structure formed by the 3'-NTR of the viral RNA may be involved.

Potato virus Y, the potyvirus type member, is an important pathogen of cultivated potato and other members of the *Solanaceae*. Resistance in potato against PVY has been introgressed from wild *Solanum* species (Barker and Harrison, 1984). This resistance ranges from a symptomless reaction (extreme resistance) to necrotic local lesion to systemic necrosis. Mesophyll protoplasts from several resistant potato clones reacting hypersensitively to PVY became infected when inoculated with PVY. In contrast, protoplasts from potato cultivars possessing the dominant gene R_y, which confers a symptomless reaction, were resistant to infection. This study suggests that R_y resistance works on virus replication rather than inhibition of cell-to-cell movement.

The nature of resistance of *Solanum brevidens* Phil. to PVY has been investigated (Valkonen et al., 1991). Leaf protoplasts of this plant were found susceptible to PVY. In addition, between 0.01 to 0.1% of protoplasts prepared from inoculated and systemic leaves of *S. brevidens* were found infected. These results indicated that resistance in *S. brevidens* to PVY could be associated with slow cell-to-cell spread rather than slow virus replication.

Resistance in pepper (*Capsicum annuum* L.) plants against PVY is conferred by a recessive allele y^a in the homozygous condition (Cook, 1961). Pepper-PVY isolates have been classified into pathotypes 0, 1, and 1^{-2} (Arroyo et al., 1996). PVY isolates against which y^a confers resistance belong to pathotype 0, whereas pathotype 1-PVY isolates are able to break resistance. Resistance mechanisms in cv. Yolo Y (y^a y^a) have been investigated (Arroyo et al., 1996). Results of fluorescence immunochemical analysis indicated that PVY-0 is able to multiply in protoplasts prepared from Yolo Y leaves mechanically inoculated with the virus indicating that resistance is due to impaired cell-to-cell movement.

5. Objectives of the study

This study, in general, is aimed at understanding some aspects of SMV-soybean interaction. The SMV-soybean system provides an excellent tool for studying the mechanism underlying virus-host interactions in general for the following reasons: i) SMV is a member of the largest plant virus group, constituting about 30% of all total known plant viruses (Barnett, 1991); ii) a number of naturally-occurring SMV strains that produce differential symptoms on soybean cultivars exist; and iii) soybean cultivars and lines of well-defined genotypes are available.

In the first part of this study, sequence analysis of the CP gene of two strains of SMV has been conducted. SMV-G1 is an isolate from Virginia and was originally named SMV-VA (Hunst and Tolin, 1982). Sequence data of the CP of this strain indicated that it was rather a distinct potyvirus (Gunyuzlu et al., 1987). This was in contrast to peptide profile analysis which indicated otherwise, suggesting that the sequenced strain was rather a contaminant of the original one (Jain et al., 1992). Therefore, sequencing of this strain becomes of importance in order to resolve this discrepancy, especially since SMV-G1 is used extensively in the genetic studies.

Sequence data of the CPs of SMV-G2 and -G7 are already available (Jayaram et al., 1991, 1992). Therefore, by sequencing the CP of SMV-G6 we will have sequences of representatives of the two most and two least virulent SMV strain groups. Correlation of sequence differences of these strains with their differential responses will indicate if the CP has any specific function as an R- or/and N-determinant.

The second part of the study is focused on studying the pathogenicity of SMV strains G1, G6, and G7 on soybean cultivars Essex, Marshall, York, and PI 96983. The objective is to determine if restricted virus accumulation in the R and N response is due to reduction of virus multiplication or restriction of local or systemic virus movement. This involves doing immunoprints of inoculated leaves at different time intervals to determine the general pattern of virus movement and location of viral antigens in susceptible, necrotic, and resistant soybean cultivars.

In the third part of the study, developing an SMV protoplast system was the goal. This system involves three methods: protoplast isolation and purification, virus inoculation of protoplasts and detection of virus replication in the inoculated protoplasts. The ultimate aim of developing such methods is to be able to conduct experiments in which susceptibility/immunity of protoplasts

isolated from the different cultivars to inoculation with various SMV strains can be assessed. Correlating such results with the responses at the intact plant level will give an indication whether *Rsv1* affects the virus replication or cell-to-cell movement.

The specific objectives of this research are the following:

1. To isolate, clone, and determine the sequence of the CP of SMV-G1 (VA strain) used in the genetic studies.

2. To isolate, clone and determine the sequence of SMV-G6 CP gene.

3. To correlate the CP sequence changes of SMV-G1 and -G6, as well as the previously published G2 and G7, with their differential phenotypic reactions on *Rsv1*-containing soybean cultivars as a possible indication of a specific function of the CP as an R and/or N-determinant.

4. To study the pathogenicity over time of virus strains G1, G6, and G7 on the susceptible (Essex) and resistant *Rsv1*-carrying soybean cultivars (Marshall, PI96983, and York).

5. To develop a protoplast experimental system in soybean including methods of protoplast isolation, inoculation with SMV, and detection of virus replication.

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CHAPTER II

The Nucleotide Sequences of the Coat Proteins of Soybean Mosaic Virus Strains do not Correlate with their Virulence on *Rsv1*-Containing Soybean Cultivars

ABSTRACT

The complete nucleotide sequences of the coat protein (CP) genes of the soybean mosaic virus (SMV) strains G1 and G6 were determined. Isolation of the genes was done by performing reverse transcription-polymerase chain reaction (RT-PCR) on partially purified virus preparation without prior RNA purification. Primers used for the PCR were designed based on conserved regions of the previously sequenced G2 and G7. Amplified products were blunt-end ligated into pNoTA/T7 vector and transformed into competent cells. Sequencing was performed manually on heat denatured double stranded plasmids in both directions. Two clones from each strain were sequenced. The two clones of G1 were almost identical. However, those of G6 were only 93.5% identical with one of the clones having a sequence identical to G1 indicating the possibility that the original culture was a mixture of strains. Therefore, G6 was purified by a single lesion transfer and tested on a differential host range to confirm its identity. Subsequently, it was cloned and sequenced again. The resulting sequences of the two clones of this purified strain were identical and different from all other sequences. Therefore, this sequence was considered as the true representative of G6. The 265 predicted amino acid sequences of G1 and G6 strains were 98.9% identical with only two amino acid differences in the N-terminus. Correlating the CP sequences of G1, G2, G6, and G7 strains with their virulence on resistant soybean cultivars indicated that the CP is not likely to be the resistance-breaking/pathogenicity determinant gene in the SMV-soybean system.

INTRODUCTION

Soybean mosaic virus (SMV) is one of the most prevalent diseases of soybean [Glycine max L. (Merr.)] in the world. It results in severe yield losses, as well as reduction of seed quality due to mottling of the seed coat (Demski and Kuhn, 1989). In the United States, SMV is classified into seven strain groups, G1 to G7, based upon their differential reactions on resistant soybean cultivars (see Table I. 1, Chapter I) (Cho and Goodman, 1979). These reactions are: susceptible or systemic mosaic (S), necrosis (N), and symptomless or resistance (R). G1 has been found to be the least virulent, causing no symptoms on any of the resistant cultivars. The other strains are more virulent and hence are given higher numbers; *i.e.* G2 to G7. Gene-for-gene model has been suggested for SMV- soybean interaction (Roane et al., 1986). On the part of the host, inheritance of resistance (R) in the majority of cultivars tested is governed by a single dominant gene, designated *Rsv1* (Kiihl and Hartwig, 1979; Chen et al., 1991). In addition to *Rsv1*, genetic studies have indicated the existence of at least two other genes that are not at the *Rsv1* locus (Buzzell and Tu, 1984; Lim, 1985; Chen et al., 1993). So far, up to six alleles of *Rsv1* have been identified (Buss et al., 1989; Chen et al., 1991). However, none of these alleles confers resistance to all SMV strains. The location of *Rsv1* has been mapped by the use of molecular markers to linkage group "F" (Yu et al., 1994). The N response in the SMV-soybean system is considered a type of hypersensitive response (HR). Support of this fact comes from the work of Choi (1991) who demonstrated that the N response is accompanied by the activation of several defense responses, like the synthesis of a set of proteins similar in enzymatic function and molecular weights to those of the well-studied pathogenesis-related (PR) proteins of tobacco (*Nicotiana tabacum* L.) reacting hypersensitively to tobacco mosaic virus (TMV) (Linthorst, 1991). The information on the genetics of the N reaction, however, is limited. Chen et al. (1994) have indicated that *Rsv1* conditions both the N and R reactions, and that two situations of SMV-strain x *Rsv1* allele interaction produce the N reaction: i) alleles are necrotic in the homozygous state when they are dominant to alleles which are resistance or susceptible to the same strain, ii) alleles which are resistant in the homozygous state to a certain strain often exhibit necrosis when they occur in a heterozygous condition with a susceptible locus.

SMV is classified in the *Potyvirus* genus, family *Potyviridae*, which is the largest among all plant viruses (Barnett, 1991, 1992; Shukla et al., 1994). The potyvirus virion is a flexuous rod, 680-900 nm long and 11-15 nm wide, made up of a monopartite single-stranded plus-sense RNA genome of about 19 kb which is encapsidated in about 2000 molecules of coat protein (CP) monomers, 30-36 kDa each, arranged in a helical configuration (Dougherty and Carrington, 1988; Reichmann et al., 1992; Shukla et al., 1994). The genomic RNA has a poly (A) tail at the 3' end and a genomic-linked virion protein (VPg) covalently linked to the 5' end. The genome functions as a mRNA that is translated into one polyprotein which is subsequently cleaved by virus-encoded proteinases into at least nine mature proteins (see Fig. I. 1, Chapter I). Only SMV strains G2 and G7 have been sequenced so far (Jayaram et al., 1992). The RNA genome of both strains consists of 9588 nucleotides [excluding a poly (A) tail] and encodes a polyprotein of 3066 amino acids. The nucleotide and amino acid identities between the two strains are 94 and 97%, respectively, with the greatest variability occurring in the 5' region, specifically in the P1 and P3 proteins.

The CP of potyviruses has been shown to be involved in several biological functions. Other than the primary function of encapsidating and protecting the viral RNA, the CP has been implicated in aphid transmission (Atreya et al., 1990, 1991, 1995; Gal-On et al., 1992). Within the N-terminus, there exists a conserved DAG triplet which has been shown to be essential for aphid transmission. The mechanism of action, however, of the amino acids in DAG in regulating aphid transmissibility is unknown. The CP is also involved in virus spread within the plant. Dolja et al.

(1994, 1995) indicated that different domains within the CP have specific roles in cell-to-cell and long-distance movement. The CP role in this respect is not clear, but seems to be other than virion assembly. The potyviral CP was also found to have a stimulatory role in genome amplification (Mahajan et al., 1996) by providing *cis*-acting elements during replication. Another role of the CP of potyviruses is virus-plant interaction such as in host recognition leading to host range specificity and symptom induction. A good example is the case of sugarcane mosaic virus (SCMV) in which sequence comparisons among the N-terminus of the CPs of several strains indicated a correlation with their host range (Xiao et al., 1993). Involvement of the CP in virus-host interactions as a determinant of resistance-breaking/pathogenicity is also well-documented. Thus far, there are two virus genes that have been identified with a typical avirulent (Avr) function; *i.e.* the product of *avr* gene is an elicitor of the hypersensitive response (HR). The first viral Avr gene to be identified was the CP of the common strain of TMV. A single amino acid change (serine _ phenylalanine) in this protein was responsible for the induction of HR on *Nicotiana sylvestris* Speg. et Comes carrying the *N'* gene (Knorr and Dawson, 1988; Culver and Dawson, 1989a, 1989b). Transgenic tobacco plants expressing only the CP gene of an HR-inducing strain exhibited an HR phenotype or total necrosis indicating that the CP by itself is sufficient for the elicitation of this response (Culver and Dawson, 1991a; Pfitzner and Pfitzner, 1992). The other example involves the CP of potato virus X which is the Avr-determinant on potato (*Solanum tuberosum* L.) carrying the *Nx* gene (Kavanagh et al., 1992). A single amino acid difference (glutamine _proline) in this protein was identified to be necessary for producing HR (Santa Cruz and Baulcombe, 1993).

The sequences of the coat protein of four strains of SMV have been determined so far. These are SMV-G2 and -G7 (Jayaram et al., 1991), SMV-N (Eggenberger et al., 1989), and SMV-VA (Gunyuzlu, 1987; Gunyuzlu et al., 1987). Sequence analysis of SMV-G2, -G7, and -N has indicated that the CP gene occupies the 3'-terminal 798 bases, followed by a non-translated region (NTR) of 259 bases excluding a poly (A) tail. The gene product consists of 265 amino acids starting with a serine. The surface-exposed N-and C-termini are 24 and 18 amino acids, respectively. SMV-N is considered to be an aphid non-transmissible version of SMV-G2 with only three nucleotide changes and one amino acid change (glycine _ aspartic acid) in the DAG box implicated in aphid transmissibility (Eggenberger et al., 1989). Based on biological properties, SMV-VA was identified to belong to G1 strain group (Hunst and Tolin, 1982). However, sequence data of this strain indicated that it was a distinct potyvirus more related to TEV (69% identity) than to SMV-N (58% identity) (Gunyuzlu, 1987; Shukla and Ward, 1988). High-performance liquid chromatography (HPLC) peptide profiles of CP tryptic digest and amino acid composition data have been obtained for 14 strains of SMV (Jain et al., 1992). Results indicated that the 14 strains, including SMV-VA, were highly similar to SMV-N. No evidence was found for the reported CP sequence of SMV-VA, implying that the sequenced SMV-VA clone was rather a minor contaminant of the original SMV-VA isolate. SMV-G2 and -G7 are highly similar with 90% homology at the nucleotide level with 31 base differences and 98.8% amino acid sequence identity with three changes: one in the N-terminus at position 8 (aspartic acid in G2 _ glutamic acid in G7), one substitution in the core at position 217 (methionine _ isoleucine), and one at 264 at the end of the C-terminus (proline _ glutamine).

The main objective of this study is to isolate and isolate the CP of SMV-G1 and -G6. SMV-G1 was originally named SMV-VA (Hunst and Tolin, 1982), and as mentioned earlier, previous sequence data (Gunyuzlu, 1987) indicated that it was not related to SMV-N in contrast to the biological properties as well as to peptide profile analysis (Jain et al., 1992) of the strain which indicated otherwise. Therefore, sequencing of this strain becomes of importance in order to resolve this discrepancy. Sequence data of the CPs of SMV-G2 and -G7 are already available (Jayaram et al., 1991). By sequencing the CP gene of G6 in addition to G1, we will have sequences of the two most and two least virulent SMV strains. Correlation of sequence differences of these strains with

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their virulence on various soybean cultivars will indicate if the CP has any specific function as an R- or N-determinant.

MATERIALS AND METHODS

Virus strains and plant material

The strain SMV-G1 used in this study was originally collected from soybean cv. Lee in Virginia and designated SMV-VA. Based on reactions on soybean differentials, it was classified to belong to SMV-G1 strain group of Cho and Goodman (1979) (Hunst and Tolin, 1982). SMV-G2 through G7 were originally obtained from Dr. R.M. Goodman (University of Illinois, Urbana, IL) and were described previously by Cho and Goodman (1979). Virus strains were maintained and propagated in their susceptible host cvs. Essex or Lee 68 (for G1 through G4) and York (for G6 and G7) by mechanical transmission.

Virus purification

Virus particles were isolated from SMV-infected second or third trifoliolate leaves 2-3 wk after inoculation. The purification scheme was essentially as that of Hunst and Tolin (1982) with some modifications as follows. Approximately 5-6 gm of SMV-infected leaves were homogenized in a mortar and pestle with extraction buffer consisting of 0.5 M sodium citrate (pH 7.0) plus 2% (v/v) 2-mercaptoethanol at a ratio of 4-5 ml for each gm of leaves. Clarification of the extract was done by adding 8% (v/v) chloroform/butanol (1:1) of total volume followed by low speed centrifugation at 13,000 g for 10 min. The virus was purified from the supernatant by the addition of polyethylene glycol (PEG) 6000 to 4% (w/v) along with NaCl to 0.3 M. After stirring and incubation in the cold for at least 1 hr, the extract was centrifuged at 13,000 g for 15 min. The pellet was resuspended in 5 mM Tris-HCl buffer (pH 8.0 at 25 C) and subjected to a second round of purification by high speed centrifugation at 171,000 g for 1.5 hr. The virus-containing pellet was resuspended by gentle shaking using the above mentioned buffer at a ratio of 10 μl for each gm of infected leaves used initially. All steps were performed in the cold at 4 C or on ice.

cDNA synthesis by RT-PCR

First strand cDNA synthesis was obtained by reverse transcription using Moloney murine leukemia virus RNase H⁻ reverse transcriptase (RT) Superscript II kit (BRL, Gaithersburg, MD). This was accomplished directly from the partially purified virus preparation described above without prior RNA purification as described by Wyatt et al. (1993) with modifications. To 12 μl of the virus suspension, the following were added to make up a total of 20 μl: 1 μl RT (200 U/μl); 2 μl of 10x buffer [1x buffer contains: 20 mM Tris-HCl (pH 8.4 at 22 C), 50 mM MgCl$_2$, and 0.1 μg bovine serum albumin (BSA)]; 2 μl oligo d(T)12-18 (0.5 μg/μl); 2 μl of 50 mM dithiothreitol (DTT); 1 μl of 10 mM deoxyribonucleoside triphosphates (dNTP) mixture; and 1 μl RNAase inhibitor (RNasin; 26 U/μl) (Promega, Madison, WI). The mixture was incubated at 42 C for 45 min, followed by four cycles of freeze (-70 C for 10 min) and thaw (42 C for 20 min). This was followed by heating at 70 C for 15 min to inactivate the enzyme. The mixture was then stored at -20 C until further use.

Second strand synthesis and the isolation of the CP gene was accomplished by PCR. The oligonucleotide primers (DNAgency, Malvern, PA) used were designed on the basis of conserved regions in SMV-G2 and -G7 sequences determined previously (Jayaram et al., 1992). These primers, designated P5 and P6, are designed to amplify a 903 bp fragment spanning the CP cistron

(Table II.1). The 5' primer (P6) begins 39 nucleotides upstream of the start codon of the CP gene, and of the 28 nucleotides of the primer, 21 are identical in both G2 and G7. The 3' primer (P5) begins 46 nucleotides downstream from the CP end. Twenty three of the 29 nucleotides are identical in the above strains. The remainder of the primers is composed of flanking *Xba* I site and additional nucleotides for cloning purposes.

DNA amplification was carried out in a 80 µl reaction using 1-3 µl of the cDNA preparation; 50 pmol of each of P5 and P6 primers; 1 µl of Thermalase *Tbr* polymerase (2U/µl) (Amresco, Solon, OH) (for SMV-G1), or cloned *Pfu* polymerase (2.5 U/µl) (Stratagene, La Jolla, CA) (for SMV-G6); 8 µl 10X buffer provided with each enzyme [for thermalase *Tbr*, 1x buffer contains: 10 mM Tris-HCl (pH 8.8 at 25 C), 1.5 mM $MgCl_2$, 50 mM KCl, and 0.1% Triton X-100; and for cloned *Pfu* enzyme 1x buffer contains: 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 200 mM Tris-HCl (pH 8.8) 20 mM $MgSO_4$, 1% Triton X-100 and 1000 µg/ml BSA]; and 1 µl of 10 mM dNTP mixture. The reaction mixture was overlayed with 50 µl of mineral oil and subjected to an initial 5 min incubation at 95 C, and then to a thermal cycling scheme of 35 cycles, including: 5 cycles of 1 min melting at 95 C, 1 min annealing at 40 C, and 2 min synthesis at 72 C; and 30 cycles of 1 min at 95 C, 1 min at 50 C, and 2 min at 72 ; followed by a final cycle of 72 C for 10 min.
To characterize the amplified fragments for each strain as the CP of the virus, 10 µl aliquot of the crude PCR reaction products were digested with *Hin*d III and analyzed on 1 % agarose gels (Maniatis et al., 1982).

cDNA cloning

PCR amplified fragments were cloned in the plasmid vector pNoTA/T7 using Prime PCR Cloner System kit from 5 prime _ 3 prime (Boulder, CO) according to the manufacturer's instructions. In summary, the PCR amplified fragments were first cleaned on columns, their ends modified, and then blunt-end ligated into the dephosphorylated vector. Ligated plasmid DNA was used to transform competent cells of *Escherichia coli* Castellani and Chalmers (cells provided in Prime PCR Cloner System kit) which were later plated on Luria broth plates containing 100 µg/ml ampicillin, isopropylthio-ß-D-galactoside (IPTG), and 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-gal). White colonies were selected and plasmid DNA was purified by the alkali lysis method from 3 ml of overnight culture (Maniatis et al., 1982). Positive clones containing the correct insert; *i.e.* 903 bp CP fragment, were determined by digesting purified plasmid with *Xba* I and *Hin*d III and electrophoric separation on 1% agarose gels.

Sequencing

Manual DNA sequencing was conducted by the dideoxynucleotide chain termination method using the modified T7 DNA polymerase (Sequenase version 2.0) sequencing kit (United States Biochemicals, Cleveland, OH) and 5% Long Ranger sequencing gels (FMC Biochemicals, Rockland, ME) following manufacturer's directions. Plasmid DNA from positive clones was purified by PEG as described (Kraft et al., 1988). Heat-denatured double-stranded plasmids were sequenced on both strands using vector-specific bordering oligonucleotide primers (M13 Universal forward and reverse primers, USB) as well as internal synthetic primers derived from SMV-N sequence (kindly provided by A. Eggenberger, Iowa State University, Ames, IA). These internal primers are shown in Table II.2. Sequence data was compiled by sequencing two independent clones. Nucleotides which were found to differ were confirmed by sequencing additional clones.

Table II.1. Synthetic oligonucleotide primers synthesized based on SMV-G2 and -G7 sequences (Jayaram et al., 1992) and used in PCR amplifications of the coat protein genes of SMV-G1 and -G6.

Primer	Position in genome	Nucleotide sequence (5' _ 3')	Length (base)
P5	9359-9379	**TGCTCTAG**ATAAAGCGACCCGAAATGAT	28
P6	8475-8494	**CGTTCT**AGATATCTTGAAGTGCTGGATTT	29

*: Bases in bold are *Xba* I site plus additional nucleotides and thus not from SMV-G2 and -G7 sequence.

Table II.2. Synthetic oligonucleotide primers based on SMV-N sequence (Eggenberger et al., 1989) used in sequencing the coat protein genes of SMV-G1 and -G6*.

Primer	Position in genome	Nucleotide sequence (5' _ 3')	Length (base)
Al3	9163-9144	TCTAAAACACCAAACAGGGC	20
Al7	9013-9030	TCTCAGATGCAGCAGAAG	18
Al8	8775-8792	CGAGCAACAAGAACACAG	18
Al10	8939-8922	GTGATGATGGATGGAGAG	18

* : In addition to M13 forward and reverse bordering primers.

Comparison of sequences

The nucleotides and the derived amino acid sequences were compared to SMV-G2 and -G7 as well as to other potyviruses from sequences stored in the Gene Bank. Pairwise alignment, multiple alignment and dendrograms were performed using the Lasergene computer program (version 1.61; DNASTAR Inc., Madison, WI) according to the Martinez/Needleman-Wunsch and to the Clustal methods.

RESULTS AND DISCUSSION

In this study, two SMV strains, G1 and G6, were isolated, cloned and sequenced. The starting material was a preparation of partially purified virus obtained from diseased cv. Essex or York soybean plants. First strand cDNA synthesis was achieved without prior RNA purification by reverse transcription using an oligo d(T) primer. PCR amplifications were carried out on 1-3 μl of cDNA preparation. The primers used for amplification were designed on the basis of SMV-G2 and -G7 sequences determined previously (Jayaram et al., 1992) and were chosen to yield PCR products spanning the CP cistron. The length of the amplified fragments (approximately 900 bp) obtained corresponded to those expected from position of the primers on SMV-G2 and -G7 sequences (Figs. II.1 and 2).

An *Xba* I flanking restriction sites were introduced into the amplification primers for cloning purposes. However, several attempts to clone using this approach had failed. Finally, the blunt end ligation approach was employed using the cloning kit from 5 prime _ 3 prime (Boulder, CO). Several full-length CP clones were obtained for each strain. The specificity of the clones was validated by digestion with *Xba* I and *Hin*d III enzymes which yielded 330 and 550 bp fragments expected from the CP gene of SMV (Fig. II.3).

The nucleotide sequence of the SMV-G1 and -G6 strains were determined from two full-length CP clones sequenced in both directions. These clones are designated as G1-42 and G1-44 for SMV-G1, and G6-1 and G6-6 for SMV-G6. No sequence heterogeneity was found between G1-42 and G1-44 clones except for two nucleotides. The identity of these bases was confirmed by checking the sequence of a third clone, G1-48. The limited heterogeneity between G1 clones is expected since the strain is most commonly used in the genetic studies and has been passed through single lesions on bean cv. Top Crop (*Phaseolus vulgaris* L.) several times in the past (S.A. Tolin, personal communication).

With respect to SMV-G6, the two clones sequenced, G6-1 and G6-6, were only 93.5% identical. In fact, one clone, G6-1, was identical to the G1 sequence. The other, G6-6 was different from all the sequenced strains (Fig. II. 4). It should be mentioned, however, that unlike SMV-G1 strain, SMV-G6 had not been single lesioned since its acquisition (S.A. Tolin, personal communication). Therefore, a single lesion transfer for this strain was carried out, using soybean cv. Kwangyo upon which SMV-G6 gives necrotic lesions. In an attempt to transfer four individual lesions, only one successfully replicated in cv. York (a suscept of SMV-G6). The virus from this single lesion was propagated on cv. York and then tested on the differential host range. The expected characteristic symptoms of SMV-G6 were observed. After virus purification and cloning, two clones, G6-4 and G6-20 were sequenced. These two clones had identical sequences and were different from both G6-1 and G6-6.

The error frequency of the enzyme *Pfu* polymerase which was used in PCR amplification of this strain is 1.3×10^{-6}. Therefore, the heterogeneity observed among the G6 clones cannot be attributed solely to misreadings caused by this enzyme. This heterogeneity may instead be due to the existence of different sequences in the natural population of that strain. This could result from the high error rate of the RNA-dependent RNA polymerase (10^{-3}-10^{-5}) which is thought to be due to the lack of any error-correcting mechanism for these polymerases (Matthews, 1991). This fact, coupled with the very high rate of virus replication, could have potentially led to rapid changes in the virus populations. Alternatively, SMV-G6 may well have been contaminated by other strains. One of the two single lesion transfers conducted in our laboratory (M. Baldwin, personal

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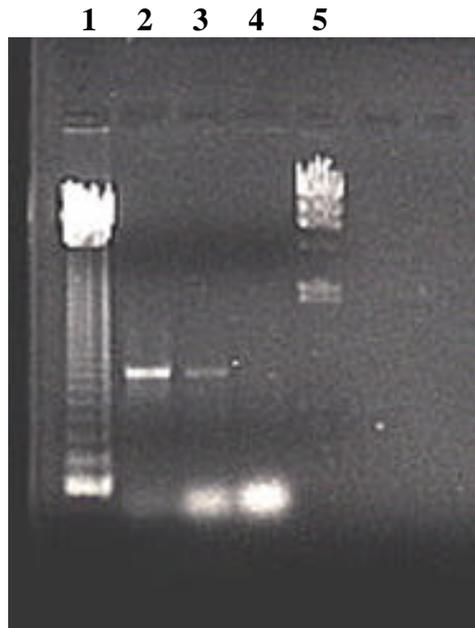
Fig II.1. Agarose gel stained with ethidium bromide showing the RT-PCR products for SMV-G1 coat protein. Lane 1, 123 bp ladder (Gibco BRL, Gaithersburg, MD); Lane 2, 10 μl amplified product from 1 μl cDNA; lane 3, from 2 μl cDNA; lane 4, from 3 μl cDNA; lane 5, *Hin*d III-digested phage _ DNA marker (Promega, Madison, WI).
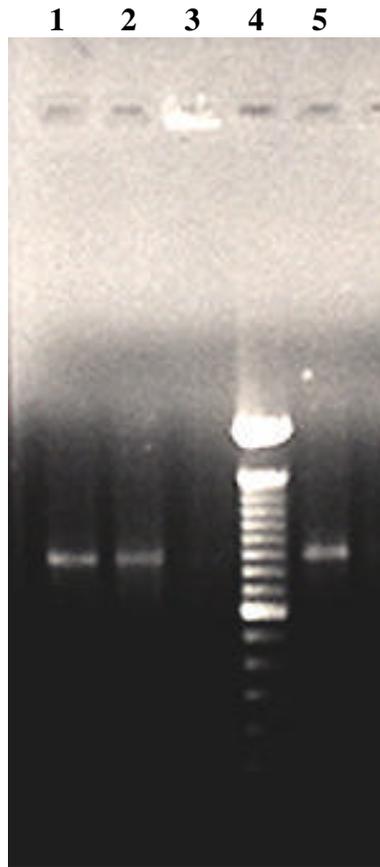
Fig. II.2. Agarose gel stained with ethidium bromide showing the RT-PCR products for SMV-G6 coat protein. Lane 1 and 5, 10 μl amplified product from 1 μl cDNA; lane 2, from 2 μl cDNA; lane 3, from 3 μl cDNA; lane 4, 100 bp ladder (Gibco BRL, Gaithersburg, MD).
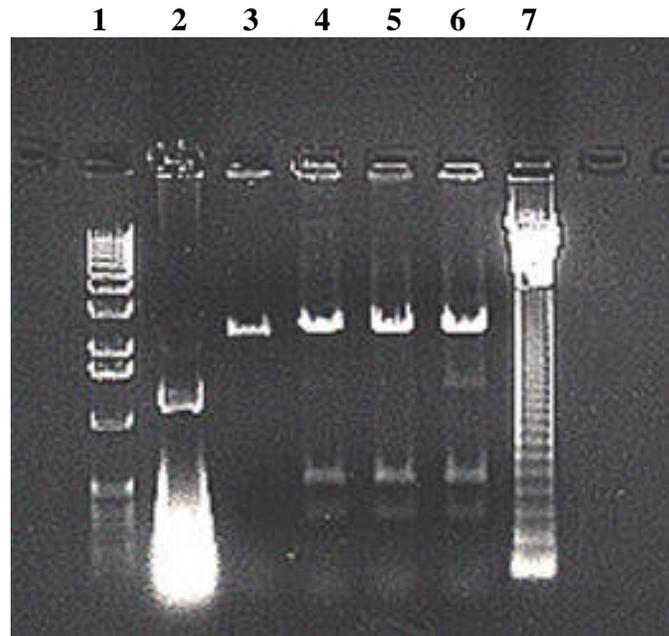
Fig. II.3. pNoTA/T7 plasmids containing RT-PCR products of the coat protein of SMV-G6. Plasmid DNA was purified from putative recombinant colonies and then digested with *Xba* I and *Hin*d III. Plasmids that released 330 and 550 bp fragments (lanes 4-6) were considered positive clones, plasmids with other size fragments were disregarded; lane 1 contains 1 kb molecular marker and lane 7 contains 123 bp ladder (both from Gibco BRL, Gaithersburg, MD); lane 2, uncut plasmid without insert; lane 3, cut plasmid without insert.

communication) gave SMV-G6 characteristic symptoms while the other gave an N response on PI96983 which is characteristic of SMV-G7. Additionally, one of the clones (G6-1) had a sequence identical to the G1 clones. These data support the suggestion that the starting viral population represented a mixture of strains. However, reversion to the putative parental strain (SMV-G1) can not be ruled out. Interestingly, the G6 strain from which the cDNA clones G6-1 and G6-6 were obtained was cultured and isolated from systemic infected leaves of cv. York plants, a cultivar that is resistant to G1. The systemic spread of G1 on this cultivar may be due to *trans*-complementation provided by the presence of a more virulent strain, like SMV-G6.

The clones G6-4 and G6-20, which resulted from a single lesion, gave characteristic host reactions for SMV-G6 and had identical sequences. Therefore, subsequent discussion will be restricted to this sequence, which will hereafter be considered as SMV-G6.

The complete nucleotide sequences of the CP genes and flanking sequences of SMV-G1 and -G6 are presented in Fig. II.4. The CP gene of both these strains are 798 nucleotides long, consistent with the sequences of G2 and G7. The nucleotide sequence identity between SMV-G1 and -G6 strains is 93.9% (Table II.3). Alignment and comparison of these sequences with the published CP sequences of SMV-G2 and -G7 shows more than 90% nucleotide sequence identity, the highest (98.2%) being between strains SMV-G6 and -G7. These data are in agreement with the 90 to 99% sequence identities observed amongst different strains of the same virus (Shukla and Ward, 1988). A noteworthy feature of the CP sequences of SMV-G1 and -G6 is that they have a deletion of one of the two stop codons that are present at the end of the CP gene of G2 and G7.

The SMV-G1 strain sequenced in this study is an isolate from Virginia and originally was named SMV-VA (Hunst and Tolin, 1982) or SMV-V (Shukla and Ward, 1988). Sequence data of the CP of this isolate previously indicated that it was rather a distinct potyvirus with only 58% sequence identity to SMV-N (Gunyuzlu, 1987; Shukla and Ward, 1988). However, comparisons of SMV-VA and SMV-N in soybean cultivars showed symptom differences typical of those generally found between strains of one virus (Shukla and Ward, 1988). Additionally, peptide profile analysis indicated high level of identity of SMV-VA (G1) to other SMV strains including SMV-N (Jain et al., 1992). Based on this information, it was suggested that the reported CP sequence of SMV-VA must have come from a contaminant of the original SMV-VA (Jain et al., 1992). SMV-G1 sequence reported in the present study supports this view. It is far from identical to the reported SMV-VA sequence and it does show high sequence identity to other SMV strains including SMV-G2 (an aphid transmissible version of SMV-N).

Transmission of potyviruses by aphids is dependent on the CP and a biologically active helper component. A tripeptide DAG near the N-terminus of the potyvirus CP has been predicted to play a key role in aphid transmission because it is conserved in aphid transmissible potyviruses and is altered in potyvirus strains that have lost aphid transmissibility (Atreya et al., 1990, 1991, 1995; Gal-On, 1992). Our SMV strains have been maintained over the years by repeated mechanical inoculations on susceptible soybean cultivars. Non-aphid transmissible strains of potyviruses are known to be selected for as a result of repeated serial transmissions by mechanical inoculation. Despite this fact, sequence data of SMV strains indicate the presence of this DAG box at position 10-12 in the N-terminus. Therefore, if these strains have lost their aphid transmissibility as would be expected, it is likely to be due to changes in the helper component rather than the CP gene.

Comparisons of the deduced amino acid sequences of the CP genesSMV-G1 and -G6, with those of the previously sequenced G2 and G7 (Jayaram et al., 1991) are shown in Fig. II.5. Similar to G2 and G7, the CP of G1 and G6 is 265 amino acid long. Amino acid sequence

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SMV-G1    AGATATCTTG AAGTGCTGGA TTTCACTCAT GNTGATGACT GTTGTGAATC    50
G1-44                           T A        T
SMV-G6                                     C
G6-6                           AC        C            C


SMV-G1    AGTGTCTCTA CAATCAGGCA AGGAGAAGGA AGGAGACATG GATGCAGGTA   100
G1-44
SMV-G6         CT                                  A
G6-6          CT              A A


SMV-G1    AGGATCCAAA GAAGAGCACC AGTAGTAGCA AGGGAGCTGA TACAAGCAGC   150
G1-44
SMV-G6                        C          A        G
G6-6               A         C          A        G


SMV-G1    AAAGATGTAA ATGTTGGATC AAAGGGGAAG GTGGTGCCGC GTTTGCAGAA   200
G1-44                                     A
SMV-G6                A A          T
G6-6                 A A          T


SMV-G1    GATTACAAGA AAGATGAATC TTCCAATGGT TGAAGGGAAG ATCATTCTCA   250
G1-44
SMV-G6                                                T
G6-6                                         A   T   T


SMV-G1    GTTTGGACCA CTTGCTTGAG TATAAACCTA ATCAGGTTGA TTTATTCAAT   300
G1-44
SMV-G6    C                    C      C                    C
G6-6      C A                  C                          C
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SMV-G1    ACTCGAGCAA CAAGAACACA ATTCGAAGCG TGGTACAATG CAGTTAAAGA   350
G1-44
SMV-G6                          G  T              T
G6-6                            G  T     A                    G


SMV-G1    TGAATATGAG CTTGACGATG AACAGATGGG CGTGGTTATG AATGGTTTCA   400
G1-44
SMV-G6                    T       G  A     T       C
G6-6                      T          A     T       C          T


SMV-G1    TGGTATGGTG CATCGACAAT GGCACATCTC CAGATGCCAA TGGCGTGTGG   450
G1-44
SMV-G6        T        T  T
G6-6          T        T  T


SMV-G1    GTGATGATGG ATGGAGAAGA ACAGATTGAA TATCCGCTGA AACCCATTGT   500
G1-44
SMV-G6                   G        A                  G
G6-6                     G                           G


SMV-G1    CGAGAATGCA AAACCAACTC TGAGGCAAAT TATGCACCAC TTCTCAGATG   550
G1-44
SMV-G6        A         G        T      A       C      T  T
G6-6      T  A                   T      A       C      T  T     T


SMV-G1    CAGCAGAAGC TTACATTGAG ATGAGAAATT CTGAAAGTCC GTATATGCCT   600
G1-44
SMV-G6                   T      A
G6-6
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SMV-G1     AGATATGGAC TACTGAGGAA TTTGAGAGAT AGAGAGCTAG CCCGCTATGC   650
G1-44                                                      A
SMV-G6               G  A  A            G            T
G6-6                                       C            T  G


SMV-G1     TTTTGATTTC TATGAGGTTA CTTCCAAAAC ACCAAACAGG GCAAGAGAAG   700
G1-44
SMV-G6         C              C        T                    G
G6-6                          C                            G


SMV-G1     CAATAGCGCA GATGAAGGCT GCAGCTCTTT CGGGAGTTAA CAACAAGTTG   750
G1-44
SMV-G6              A                 C
G6-6              A                 C                     C


SMV-G1     TTTGGACTTG ATGGGAACAT CTCAACTAAC TCCGAAAATA CTGAAAGGCA   800
G1-44
SMV-G6                 A  T          C
G6-6          G              C           G  C


SMV-G1     CACTGCAAGG GATGTGAATC AAAACATGCA CACTCTTTTG GGCATGGGCC   850
G1-44
SMV-G6                                          C
G6-6             A              G


SMV-G1     CACAGCAGTA AAGGCTAAGT AAATTGGTCA CAGTTATCAT TTCGGGTCGC   900
G1-44
SMV-G6                            C
G6-6                   G        C
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SMV-G1   .TTTAT                                                        905
G1-44
SMV-G6
G6-6
```

Fig. II.4. The nucleotide sequences of the coat protein (CP) genes and flanking sequences of SMV-G1 and -G6. SMV-G1 represents sequence of cDNA clone G1-42. G1-44 is another clone of SMV-G1 showing variations in two positions in the CP gene. SMV-G6 represents sequences of two cDNA clones (G6-4 and G6-20) obtained after purification of SMV-G6 culture. G6-6 is the sequence of a clone obtained before purification of SMV-G6 culture (see text). Bases below the SMV-G1 sequence are indicated only when they differ from SMV-G1. The start and end of the CP gene are marked by a vertical line. Primers used for CP cloning by RT-PCR are underlined.

Table II.3. Amino acid similarity (using Clustal method with PAM250 residue weight table) and nucleotide identity of the coat protein genes of SMV strains G1, G2, G6, and G7. G2 and G7 sequences are from Jayaram et al. (1991).

	Percent amino acid similarity			
	SMV-G1	SMV-G2	SMV-G6	SMV-G7
SMV-G1		98.8	98.9	98.5
SMV-G2	96.4		98.9	98.5
SMV-G6	93.9	94.9		99.2
SMV-G7	94.6	96.4	98.2	
	Percent nucleotide identity			

```
SMV-G1    SGKEKEGDMDAGKDPKKSTSSSKGADTSSKDVNVGSKGKV            40
SMV-G2              D         S       G
SMV-G6              E         S       G
SMV-G7              E         S       G
G6-6               D         N       G


SMV-G1    VPRLQKITRKMNLPMVEGKIILSLDHLLEYKPNQVDLFNTRA          80
SMV-G2
SMV-G6
SMV-G7
G6-6


SMV-G1    RATRTQFEAWYNAVKDEYELDDEQMGVVMNGFMVWCIDNG           120
SMV-G2
SMV-G6
SMV-G7
G6-6


SMV-G1    TSPDANGVWVMMDGEEQIEYPLKPIVENAKPTLRQIMHHF           160
SMV-G2
SMV-G6
SMV-G7
G6-6


SMV-G1    SDAAEAYIEMRNSESPYMPRYGLLRNLRDRELARYAFDFY           200
SMV-G2
SMV-G6
SMV-G7
G6-6


SMV-G1    EVTSKTPNRAREAIAQMKAAALSGVNNKLFGLDGNISTNS           240
SMV-G2                        M
SMV-G6                        M
SMV-G7                        I
G6-6                         M
```

```
SMV-G1      ENTERHTARDVNQNMHTLLGMGPQQ.                                    266
SMV-G2                               P
SMV-G6                               Q
SMV-G7                               Q
G6-6                                 Q
```

Fig. II.5. Alignment of the predicted amino acid sequences of the coat proteins of SMV strains G1, G2, G6, and G7. The derived amino acid sequence of cDNA clone G6-6, derived from SMV-G6 culture before purification, is also included (see text). Bases below SMV-G1 are indicated only where they differ SMV-G1. The G2 and G7 sequences are from Jayaram et al. (1991).

homology between the four strains is between 98-99%. There are two amino acid changes between G1 and G6. Both of these changes are present in the N-terminal region with the core protein with the C-terminal regions being identical. The first substitution is aspartic acid (in G1) _ glutamic acid (in G6) at position 8, and the second is aspartic acid _ glycine at position 26. The first substitution (aspartic acid _ glutamic acid) also occurs in G7 but not G2, while the aspartic acid at position 26 is characteristic of G1 only.

The phylogenetic tree of SMV strains with other selected potyviruses is illustrated in Fig. II.6. The four SMV strains G1, G2, G6, and G7 are closest to each other with 98-99% homology. Homology of these strains with SMV-China is lower being around 96% with most heterogeneity in the N-terminus. SMV is closest to WMV-2 (85%) and BCMV and PStV (76%) but has less similarity to JGMV, PPV, PVY, TEV, and TuMV (55-65%) (for abbreviations see legend of Fig II. 6).

The CP of potyviruses is the only structural protein (other than the VPg) detected in the virus and accounts for about 95% of the virus particle (Riechmann et al., 1992). The CP is also the first part of the virus to be detected by the host plant. Outer proteins of animal viruses are the primary determinants of pathogenicity. Additionally, in two virus-host systems (TMV-tobacco and PVX-potato), the viral genes eliciting HR reactions have been determined. In both cases, the viral gene has been found to be the CP where a single amino acid change has been found to change the host response (Knorr and Dawson, 1988; Santa Cruz and Baulcombe, 1993). How a single amino acid change in the CP alters the host response is not known. Possibly, this causes a change in the electrostatic interaction between a host component and the CP, or it might lead to changes in the three dimensional configuration of the CP enabling it to escape host recognition (Culver and Dawson, 1991b). In view of these facts, we speculated that the CP might also be the N- or R-determinant in SMV-soybean system. The very conservation of the CP sequences of the four SMV strains is remarkable in view of their different biological properties. Biologically, G1 is different from G2 in that the latter can produce an N reaction on cv. Marshall (see Table 1, Chapter I, p. 3). Amino acid sequence data, however, indicate that G1 has an aspartic acid instead of glycine in G2 at position 26 in the N-terminus (Fig.5). This change is not conservative and has the potential to change the host response since it involves changes in structure as well as in total charge. Aspartic acid is a polar residue with an acidic charge while glycine is nonpolar and is the smallest residue which allows it to fit into tight spaces. It is worthy to note that G2 shares this change with G6 and G7 and that all three strains produce an N response on Marshall. If this change is responsible for changing the response on Marshall, then we would expect strains G4 and G5, but not G3, to be similar to G1 and have an aspartic acid instead of a glycine in that position since they produce an R reaction on this host.

The other three amino acid changes present between the strains G2, G6 and G7 are all conservative. One change is aspartic acid (in G1 and G2) _ glutamic acid (in G6 and G7) at position 8. This substitution correlates with G6 and G7 being able to infect cv. York, but it does not involve change either in charge or in structure. Another change, methionine (in G1, G2 and G6) _ isoleucine (in G7) at position 217, is also a conservative change and it does not correlate with virulence. The third change, which is glutamine (in G1, G6, and G7) _ proline (in G2) at position 264, does involve a structural change of the C-terminus. However, this change of G2 does not have a specific host reaction that differentiates it from the other three strains.

In view of these findings, one can conclude that the CP sequence data of the SMV strains do not show strong correlation with virulence and thus is not likely to be the gene responsible for resistance-breaking in the soybean host. Future experiments involving the introduction of defined CP sequence differences in the full-length infectious clone of SMV will provide a definite answer.
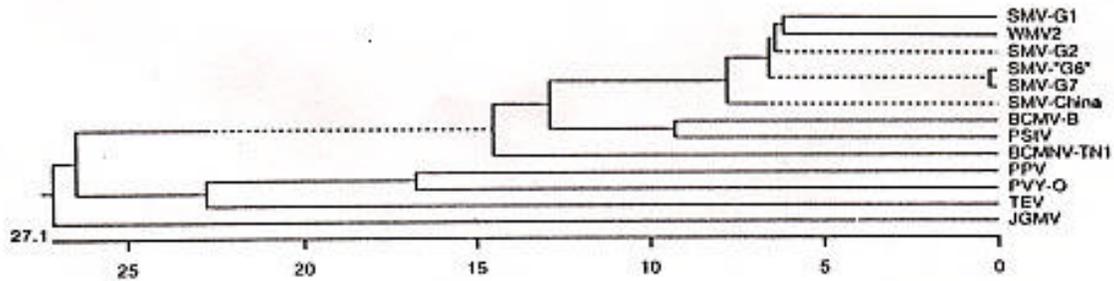
Fig. II.6. Phylogenetic tree obtained form the alignment of amino acid sequences of the coat proteins of selected potyviruses, using Clustal method with PAM250 residue weight table.
Abbreviations: BCMV-B, bean common mosaic virus strain B (Z15057); BCMNV-TN1, bean common mosaic necrosis virus strain TN1 (U19287); JGMV, Johsongrass mosaic virus (Z26920); PPV, plum pox virus (D13751); PStV, peanut stripe virus (X63559); PVY-O, potato virus Y strain O (X68226); SMV, soybean mosaic virus strains China (X63771), G1 (this study), G2 (S42280), G6 (this study), and G7 (S42280); TEV, tobacco etch virus M15239); WMV-2, watermelon mosaic virus 2 (D13913).

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CHAPTER III

Pathogenesis of Soybean Mosaic Virus to *Rsv1*-Containing
Soybean Cultivars

ABSTRACT

The pathogenesis of soybean mosaic virus (SMV) strains G1, G6, and G7 on the susceptible cultivar Essex, and on the resistant cultivars York, Marshall, and PI96983 has been investigated. Leaf immunoprints of inoculated leaves of the above combinations indicated four types of reactions: i) for G1-Essex, G6-Essex, G6-York, G7-Essex and G7-York, infection in these combinations started as pin-point foci that expanded over time until it covered the entire leaf; ii) for G1-York, G1-Marshall, G1-PI96983, and G6-PI96983, no virus was detected; iii) for G6-Marshall, detection of the virus was delayed and was sporadic, being restricted to few foci along the major veins; and iv) for G7-Marshall and G7-PI96983, detection of the virus was also delayed and starts as pinpoint foci with spread being eventually along the veins. Results of this study suggest that *Rsv1*-mediated resistance is a multicomponent type of resistance affecting both virus replication and cell-to-cell movement but not vascular systemic movement. A model to explain this resistance is proposed.

INTRODUCTION

Systemic infection of a host plant by a virus requires virus replication and movement from initially infected cells to other parts of the plant. To achieve this, the virus must first enter the host cell through a wound and then direct the synthesis of its own proteins and replicate its genome (Matthews, 1991). The virus particle or the viral genome then moves to adjacent epidermal or mesophyll cells through intercellular connections, the plasmodesmata. This cell-to-cell movement is an active process mediated by the interplay of virus-encoded movement proteins (MPs) and cellular factors (Hull, 1991; Maule, 1991; Deom et al., 1992; Carrington et al., 1996). The functional nature of MPs of different virus groups are distinct. The MPs of some rod-shaped viruses, namely tobamoviruses, bind directly to the viral genome and facilitate passage through plasmodesmata (Citovsky, et al., 1990, 1992). In other viruses, such as the isometric comoviruses, the MPs, in coordination with the coat protein (CP), form intercellular tubules through which intact viruses pass (Kasteel et al., 1993; Van Lent et al., 1990). Movement of the virus to distal parts of the plant, a process called long-distance movement, requires translocation through bundle sheath, vascular parenchyma and companion cells, followed by entry into the sieve elements through which the virus moves rapidly by bulk flow to tissues that are sinks for photoassimmilate (Carrington et al., 1996; Seron and Haenni, 1996). Invasion of cells in systemic tissues at a distance away from the initial site of infection requires exit into companion cells from sieve elements, then cell-to-cell movement into bundle sheath, mesophyll, and epidermal cells. In case of tobamoviruses, the viral functions for long distance transport differ from those required for cell-to-cell movement. For example, the CP is required for long-distance, but not cell-to-cell, movement of tobacco mosaic virus (TMV) (Dawson et al., 1988; Saito et al., 1990).

For potyviruses, long-distance and cell-to-cell movements have been studied little and mostly with tobacco etch virus (TEV). Potyviral proteins with dedicated movement functions have not been identified. In the past, P1 protein has been proposed to function as the potyviral MP. However, deletions and modifications of the P1 coding sequence of TEV had no effect on movement (Verchot and Carrington 1995a, 1995b). On the other hand, cell-to-cell movement functions have been mapped to the CP. Mutational analysis indicated that the core domain which is required for virus assembly is essential for cell-to-cell movement (Dolja et al., 1994, 1995; Jagadish et al., 1993). In contrast, the N-terminal (29 amino acids) and C-terminal (18 amino acids) regions, which comprise surface-exposed domains, are dispensable for cell-to-cell movement but necessary for long-distance transport. The central region of the helper component-proteinase (HC-Pro) is also required for long-distance movement. Sequence changes introduced into these regions result in mutants that are capable of assembly and cell-to-cell movement but not long-distance movement (Cronin et al., 1995).

Different approaches can be used to monitor the movement of the virus in tissues and through the vasculature (Seron and Haenni, 1996). One method involves scoring symptoms observed on the inoculated leaf and on the whole plant to establish if vascular movement has occurred. Other assays include the detection of viral proteins and/or nucleic acids in distinct parts of the plant by Northern blots, Western, tissue prints, immunocytochemistry and *in situ* hybridization. The generation of infectious clones of the TEV genome carrying an inserted copy of the reporter gene ß-glucuronidase (GUS) has been achieved (Dolja et al., 1992). This system provided a sensitive probe to analyze cell-to-cell and long-distance movement of TEV in plants. On primary inoculated leaves of tobacco and by 24 hr post inoculation (hpi), infection foci extending to about 10 cells in diameter were visible. These foci continued to expand radially over time and eventually fused. Virus movement was evident along the minor and major veins and into mesophyll cells adjacent to vascular tissue. TEV-GUS was also used to measure cell-to-cell movement and

long-distance movement in a resistant tobacco mutant line (V20) and a susceptible line (Havana 425) (Schaad and Carrington, 1996). The rates of cell-to-cell movement of virus in inoculated leaves were identical in the resistant and susceptible lines between 48 and 72 hr post inoculation. In contrast, long-distance movement was markedly restricted in the resistant line relative to susceptible line. The distribution of SMV in infected soybean leaf tissue by blotting the leaf onto membranes has been followed (Mansky, et al., 1990). In this study, leaves were blotted onto nitrocellulose membranes using a hydraulic press and the method of detection involved monoclonal antibody to SMV coat protein and protein A-colloidal gold solution. This technique had been used to study the effects of temperature on maintenance of resistance to SMV-G2 in soybean (Mansky et al., 1991). When resistant plants of soybean lines PI96983, L78-379, and Davis inoculated with SMV-G2 were shifted from 20 C to 10 C for 10 days, the coat protein and the infectious virus accumulated in trifoliolate leaves, unlike plants shifted to higher temperatures indicating that low temperature breaks resistance in soybean against SMV. It should be mentioned that, in this study, press blots detection of the virus coat protein had indicated a correlation between virus antigen and infectivity (Masnsky et al., 1991). In this study, detection pf the virus coat protein antigen was shown to be directly correlated with the presence the virus.

Three types of reactions are evident upon inoculation of soybean mosaic virus (SMV) strains into a set of resistant cultivars. These reactions are: susceptible or systemic mosaic (S), necrosis (N), and symptomless or resistant (R) (see Table I. 1, Chapter I). Accordingly, SMV is classified into seven strain groups, G1 to G7 (Cho and Goodman, 1979). G1 is the least virulent infecting none of the resistant cultivars. Higher numbers were assigned to other strains with increasing virulence from G1. The SMV-soybean system is governed by the gene-for-gene model (Roane et al., 1986). The N response is considered a hypersensitive response (HR) type of resistance (Buzzell and Tu, 1989). Additionally, the R and the N reactions are believed to be resistance responses governed in most cultivars by a single dominant gene, $Rsv1$ (Kiihl and Hartwig, 1979; Chen et al., 1991; Chen et al., 1994). So far, up to six alleles of $Rsv1$ have been identified (Kiihl and Hartwig, 1979; Buss et al., 1989; Chen et al., 1991). On the part of SMV, the gene(s) interacting with $Rsv1$ and mechanism of resistance is not known.

The objective of this research is to study the pathogenesis of SMV on soybean by comparing the extent of replication and the general pattern of virus movement in susceptible, necrotic, and resistant soybean cultivars. Three SMV strains, G1, G6, and G7, are used for this study. The reactions of these strains are assessed on the resistant soybean cultivars, Marshall, PI96983, and York (with the resistance alleles $Rsv1^m$, $Rsv1$, and $Rsv1^y$, respectively), as well as the susceptible Essex (with $rsv1$), thus having all the three responses (S, N, and R) in virus-host combinations (Table I. 1, Chapter I).

MATERIALS AND METHODS

Virus source and plant material

SMV was maintained in the greenhouse by periodically transferring the virus to young susceptible soybean cultivars (Essex for G1 and York for G6 and G7). The strain G1 used in this study was originally collected from Lee soybean in Virginia and named as SMV-VA. Based on reactions on soybean differentials, it was classified to belong to SMV-G1 strain group of Cho and Goodman (1979) (Hunst and Tolin, 1982). SMV-G6 and G7 were originally obtained from Dr. R.M. Goodman (University of Illinois, Urbana, IL) and were described previously by Cho and Goodman (1979). Cultures of SMV-G6 and -G7 were each passed through a necrotic single lesion on the resistant cultivar Kwanggyo to York (susceptible for G6 and G7). The cultivars used were Marshall, PI96983, and York (with the resistance alleles $Rsv1^m$, $Rsv1$, and $Rsv1^y$, respectively) as well as the susceptible Essex (with $rsv1$). The three varieties, Marshall, PI96983, and York, are all R to G1; Marshall is N to G6 and G7; and PI96983 is R to G6 and N to G7. Seeds were thankfully provided by G.R. Buss (Virginia Polytechnic Institute and State University, Blacksburg, VA).

Plant inoculation

The inoculum consisted of infected second or third trifoliolate leaves taken 2-3 wks after inoculation. These leaves were homogenized with a mortar and pestle in cold 0.01 M sodium phosphate buffer (pH 7.0) at a ratio of 1 gm leaves to 10 ml buffer. The sap was rubbed with a pestle onto unifoliolate leaves previously dusted with carborundum. Mock-inoculated controls were treated identically except buffer alone was used. Leaves were rinsed with tap water after inoculation.

Experimental design

Experiments were done under greenhouse conditions during the months of February and March. Virus strains were each inoculated on four cultivars: Essex, Marshall, PI96983, and York, making a total of 12 combinations. Seeds were planted in 15 cm dia. pots in the potting medium Metro-Mix 360 (Scotts-Sierra Horticultural Products Company, Marysville, OH). Three to four pots (5-6 plants per pot) per combination were used. Plants of one pot of each variety was mock-inoculated. Leaves were assayed for the virus at 4, 6, 9, and 12 days post inoculation (dpi). Except for G7-Marshall, the resistant and necrotic combinations were also assayed at 16 dpi. Two leaves of two plants in two different pots were assayed per combination per date. The experiment was conducted twice.

Leaf imprinting and immunodetection of the virus

Leaf imprint was performed as described (Gera, 1994) on S & S filter paper (#410; Schleicher and Schuell, Keene, NH). The leaf was placed with the lower surface on the filter paper and sandwiched between sheets of white copy paper. The sandwich was placed on a flat hard surface or on the floor and covered with a transparent acrylic sheet (1/4- 3/8" thick). With a 350 gm metal hammer, the acrylic sheet was hit with light blows until a uniform green imprint could be

seen on the upper white paper. The sandwich was disassembled and the filter paper with the green imprint of the leaf was allowed to dry.

Virus immunodetection on the leaf imprint was done as described by Lin et al. (1990) with some modifications. The filter was decolorized in 2% Triton X-100 for about 5 min with gentle shaking. The filter was then washed once for 3 min in KPS-T buffer (0.10 M K2HPO4, 0.75 M NaCl, pH 7.4 + 0.05% Tween-20) and placed into blocking solution [1X KPS-T buffer with 4% milk (Carnation non-fat dry milk) and 0.5% BSA]. After 30 min, the filter was transferred to a solution of primary antibody directed against purified SMV particles (Hunst and Tolin, 1982) diluted in KPS (1:10,000) for 1 hr on a shaker. After three rinses in KPS-T buffer, the filter was incubated in secondary antibody [alkaline phosphatase conjugated goat anti-rabbit IgG F(ab^{+2}) fragment or alkaline phosphatase conjugated goat anti-rabbit IgG (whole molecule), Sigma Chemical Co., St. Louis, MO] diluted in KPS (1:10,000) for 1 hr. The filter was transferred after three rinses in KPS-T to the substrate 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT; Zymed Laboratories Inc., San Francisco, CA) and developed for color for 15 min. This was followed by several rinses in distilled water to stop the reaction. The filter was stored in the dark to avoid fading.

The primary antibody used in this study was made in rabbits against SMV-G1 purified particles as described by Hunst and Tolin (1982). This antibody reacts equally to all SMV strains. It also detects as little as 100 ng of purified virus particles in a single spot on S & S filter paper with the intensity of blue color being proportional to the concentration of virus (Gera, 1994). Becauese of these results, and since Mansky et al. (1991) showed an exact correlation between virus antigen and the infectious virus, the assumption is made in this study that the detection of the virus antigen equates with the presence of the virus.

RESULTS

Time course of pathogenesis for soybean inoculated with SMV-G1

SMV-G1 produces systemic mosaic symptoms in Essex. The symptoms on inoculated leaves consist of chlorotic spots that are evident 6-7 dpi. Using leaf immunoprints, the virus was first detected 4 dpi (Fig. III.1, A). At this time, the infection is detected as pinpoint foci of 0.5-1 mm in diameter that are scattered over the surface of the leaf. By 6 dpi (Fig. III.1, B), these foci expanded radially to 1-2 mm diameter spots providing a potential for contact with the leaf veins. The virus antigen becomes detectable in the petiole indicating that the virus moves out of the inoculated leaf at this time. At 9 dpi (Fig. III.1, C), these spots have expanded further and start to coalesce and over the major veins. By day 12 (Fig. III.1, D), the virus has invaded almost all parts of the leaf. No virus was detected in mock-inoculated leaves (Fig. III.1, H).

Following SMV-G1 inoculation on the resistant cultivars York, Marshall, and PI96983, which constitute a symptomless reaction (R phenotype), the virus was not detected at 4, 6, 9, 12, and 16 dpi. Fig. III.1 (E-G) shows immunoprints of inoculated leaves of these cultivars at 16 dpi only.

Time course of pathogenesis for soybean inoculated with SMV-G6

Infection of SMV-G6 on cultivars Essex and York, which constitute a susceptible combination (S phenotype) proceeded in a manner similar to SMV-G1 on Essex. As shown in Fig.III. 2, A-D, The virus was detected on 4 dpi as 0.5-1 mm foci of infection that expanded until it invaded the major veins and almost the entire leaf. The virus was absent in mock-inoculated leaves (data not shown).

SMV-G6 usually produces local necrosis on inoculated leaves of Marshall (N phenotype) evident 8-10 dpi. By immunoprints, the virus was detected at 9 dpi (Fig. III.2, E-G) and only on some of the blotted leaves. The virus spread started as few restricted foci of infection along the veins. By 16 dpi, the virus had not progressed very far from the initial site of infection. No virus was detected on SMV-G6 inoculated leaves of PI96983 (R phenotype) even by 16 dpi (Fig.III. 2, H).

Time course of pathogenesis for soybean inoculated with SMV-G7

The response of SMV-G7 on inoculated leaves of Essex and York (S phenotype) was not different from other susceptible combinations (data not shown). Size, number and progression of infection foci were similar to those that appeared on other susceptible phenotypes. On Marshall and PI96983, which constitute a systemic necrosis phenotype, the virus was first detected on 6 dpi (Fig. III.3, B and F). The infection foci at this stage seemed to be similar to that on the susceptible Essex and York at 4 dpi. By day 9 pi, the virus had made contact with the veins and moved out of the inoculated leaf (Fig.III. 3, C and G). As evidenced by a more intense blue color, the antigen seemed to become more concentrated at the major and minor veins rather than in the leaf blade tissue. However, this virus strain seemed more invasive in Marshall than in PI96983. The extent of invasion increased at 12 and 16 dpi being again more greater at the veins (Fig. III.3, D and H). By 15 dpi, the inoculated leaves of Marshall had collapsed.
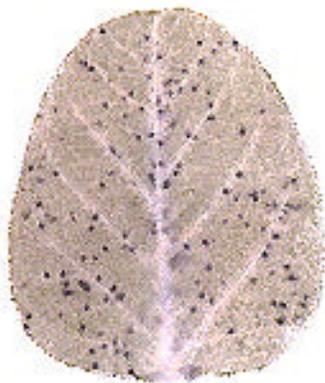
A   B
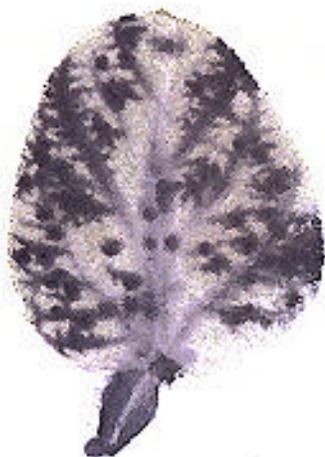
C   D
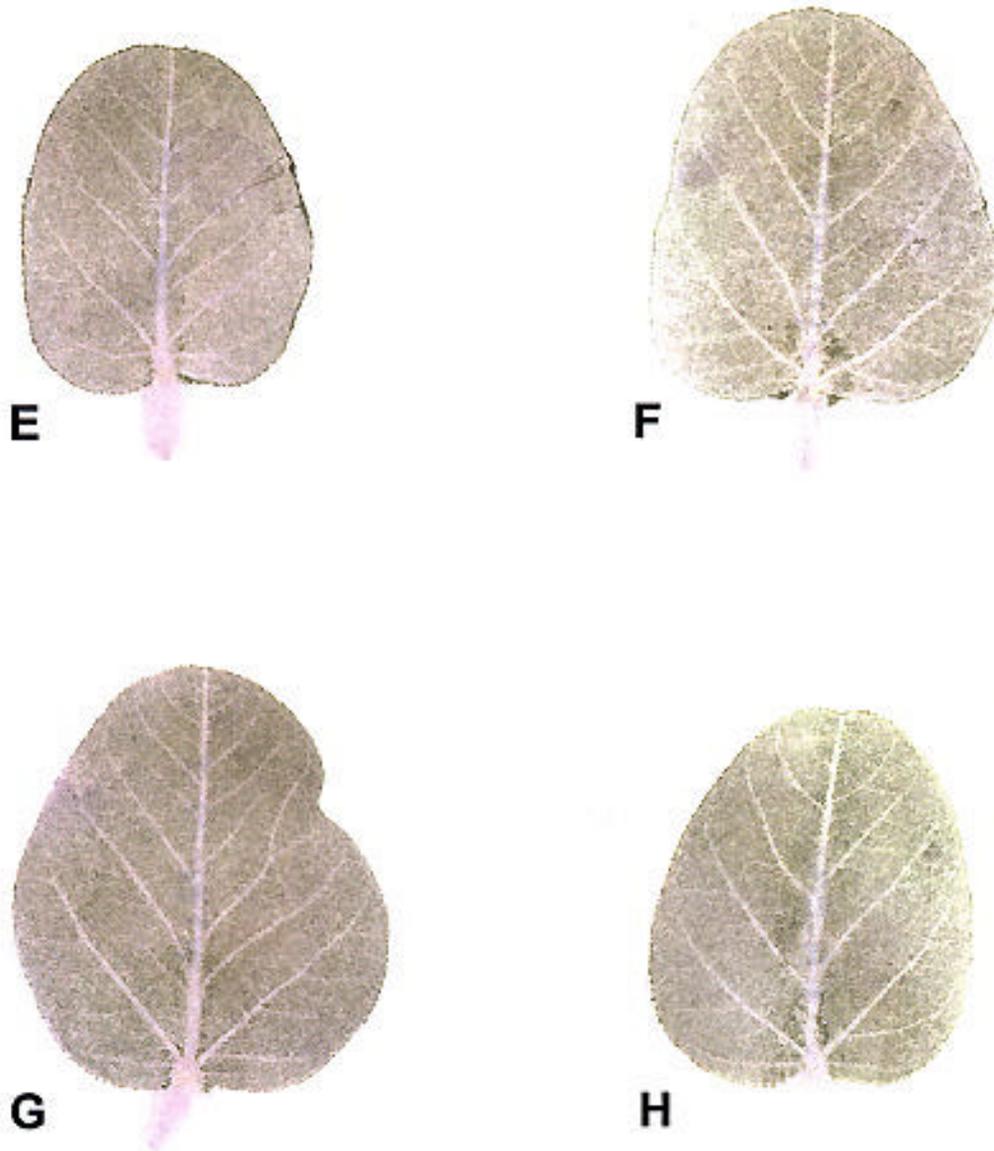
Fig. III.1. Immunoprints of unifoliolate leaves of soybean inoculated with SMV-G1. A-D, Cultivar Essex 4, 6, 9, and 12 dpi, respectively; E, York 16 dpi; F, Marshall 16 dpi; G, PI96983 16 dpi; H, mock-inoculated Essex.
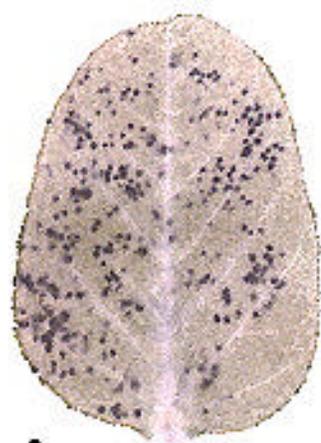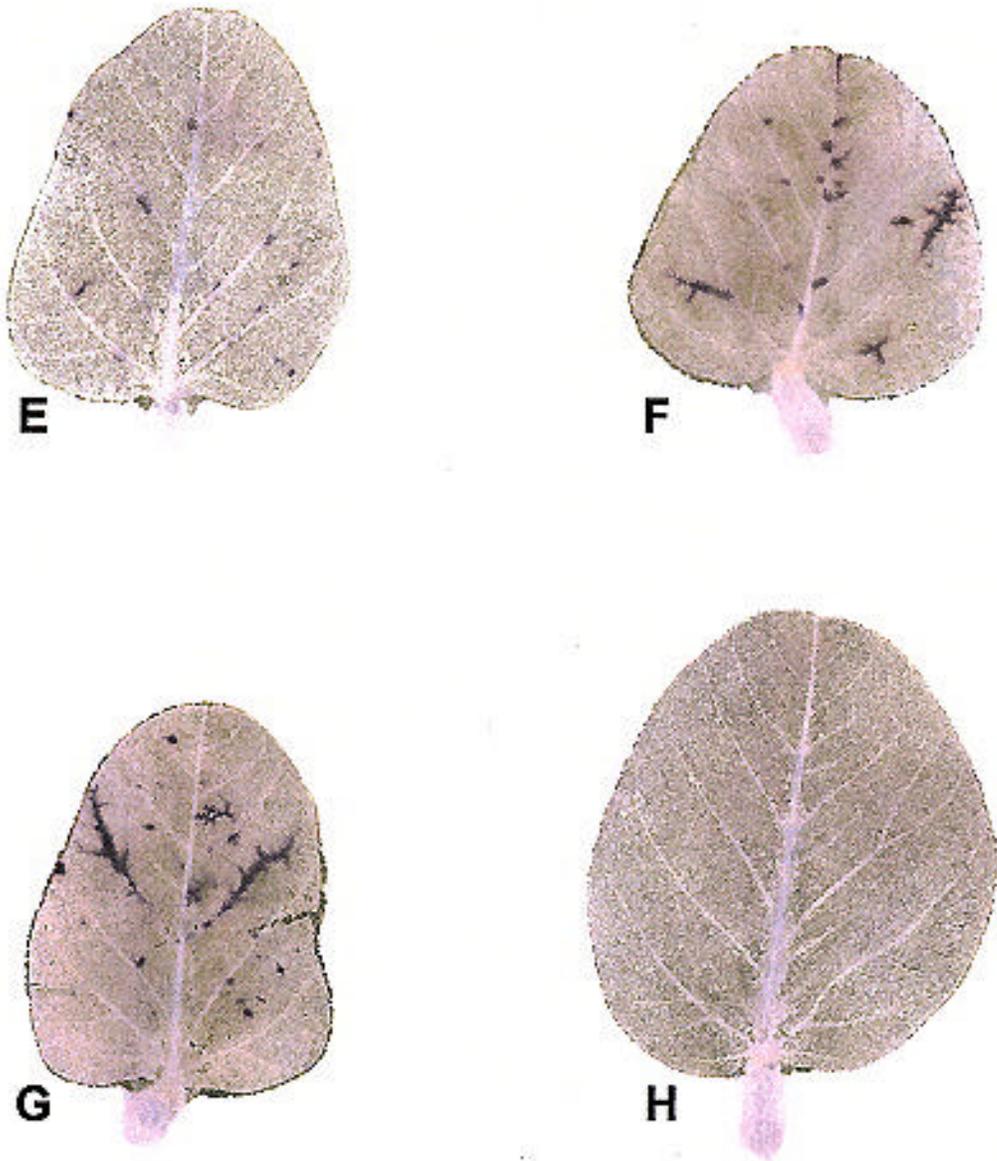
Fig. III.2. Immunoprints of unifoliolate leaves of soybean inoculated with SMV-G6. A-D, Cultivar York 4, 6, 9, and 12 dpi, respectively; E-G, Marshall 9, 12, and 16 dpi, respectively; H, PI96983 16 dpi.
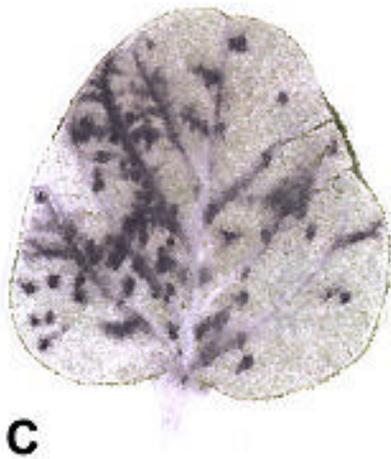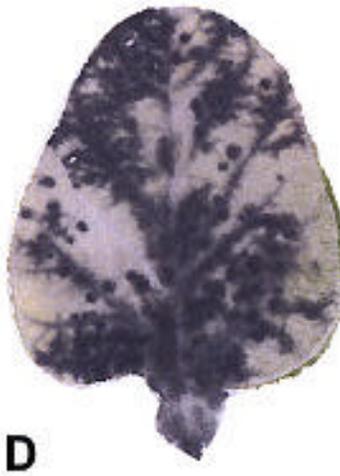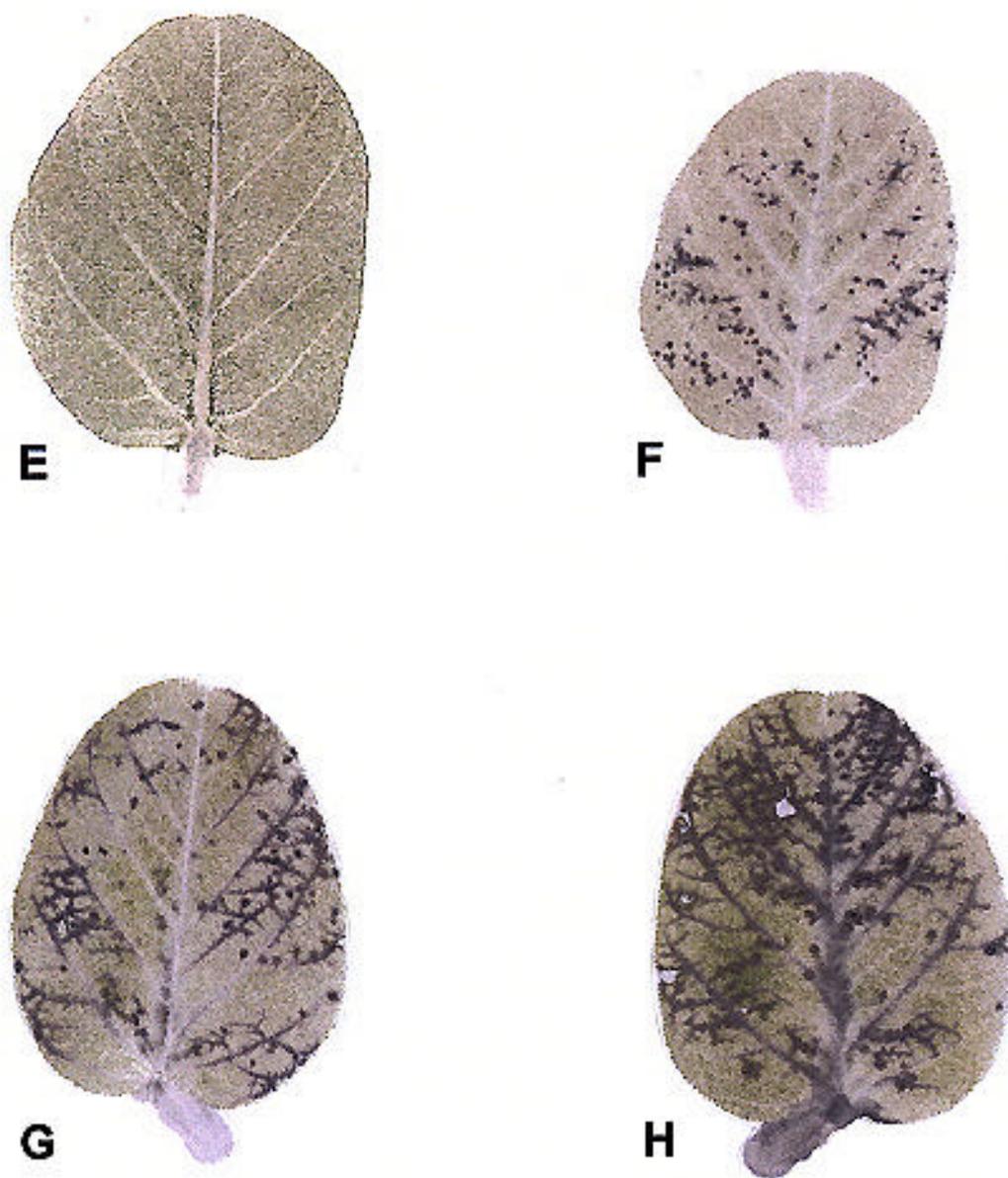
Fig. III.3. Immunoprints of unifoliolate leaves of soybean inoculated with SMV-G7. A-D, Cultivar Marshall 4, 6, 9, and 12 dpi, respectively; E-H, PI96983 4, 6, 9, and 12 dpi, respectively.

Delineation of four pathogenesis patterns

Four types of reactions were identified based on observations from the immunoprints of inoculated leaves of the four cultivars (Fig. III.4, A-D). The first type is manifested by the combinations G1-Essex, G6-Essex, G6-York, G7-Essex, and G7-York (Fig. III.4, A). These combinations display the susceptible mosaic phenotype at the whole plant level. By immunoprints, the virus was first detected as foci that expanded radially providing a potential for contact with the veins. Eventually, the virus invaded almost all the tissues of the leaf. These immunoprints indicate that the virus is unrestricted either in replication or in movement in the susceptible phenotype. The second type of reaction was observed on the immunoprints of G1-York, G1-Marshall, G1-PI96983 and G6-PI96983 (Fig. III.4, B). These combinations constitute the symptomless resistance phenotype. On immunoprints of such combinations, the virus was not detected indicating suppression of virus replication below detectable level on these cultivars. The third type of reaction was that of G7-Marshall and G7-PI96983 representing the systemic necrotic phenotype (Fig. III.4, C). The virus on these combinations was detectable later than in the susceptible combinations appearing at 6 rather than 4 dpi. Pathogenesis in these leaves started as in the susceptible combinations but as time progressed the virus seemed to be confined to the veins, with Marshall showing more virus spread in the leaf blade tissue than PI96983. The reaction of G6-Marshall constituted the fourth phenotype which is localized necrosis (Fig. III.4, D). The behavior of the virus in such combination was distinct from that of SMV-G7 on Marshall. The reaction was sporadic and when present it was delayed further and seemed to be restricted to only a few areas of the veins.
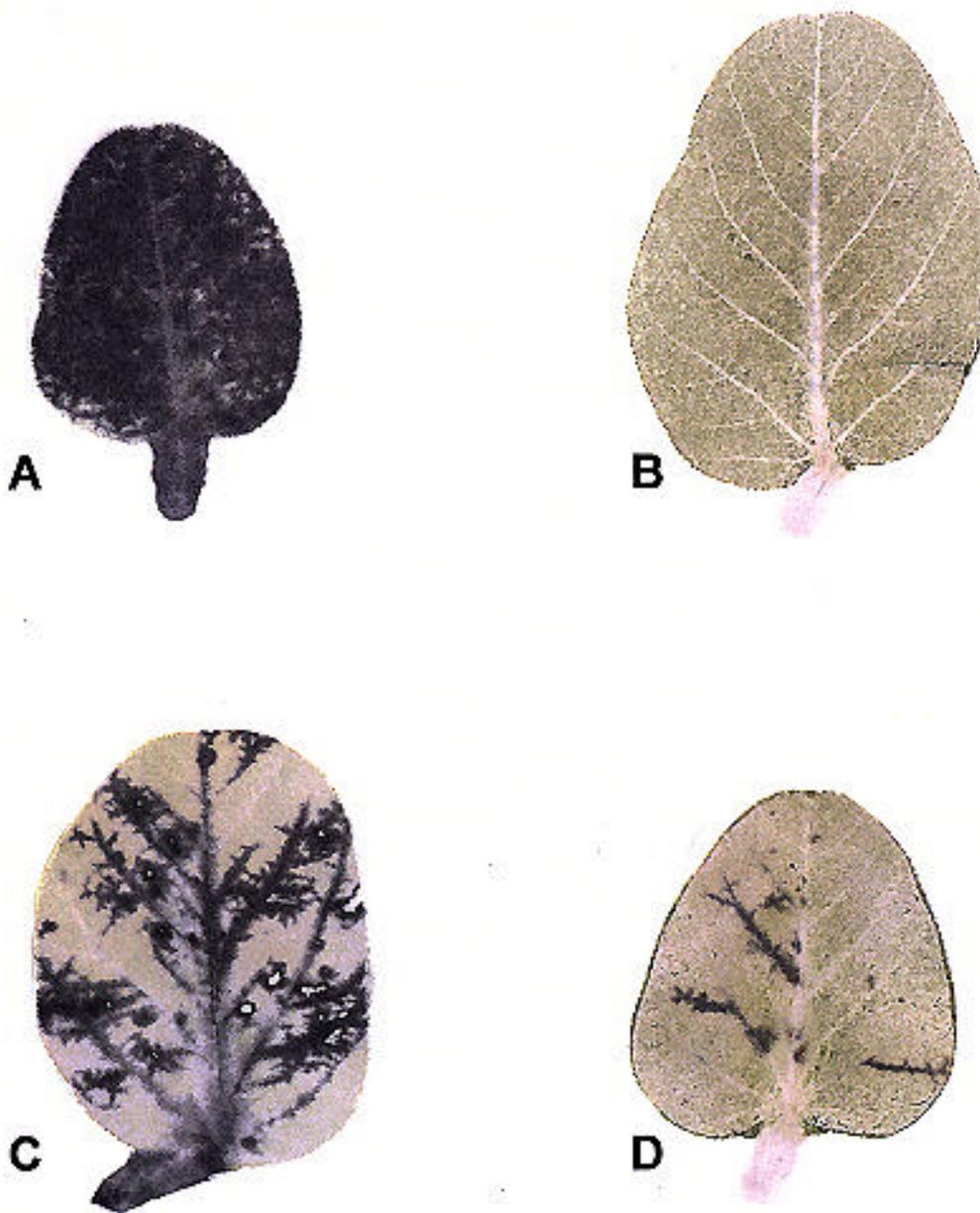
Fig. III.4. Four types of immunoprints for SMV-inoculated unifoliolate leaves of soybean. A, Cultivar York inoculated with SMV-G7 12 dpi; B, PI96983 inoculated with SMV-G6 16 dpi; C, PI96983 inoculated with SMV-G7 16 dpi; D, Marshall inoculated with SMV-G6 16 dpi.

DISCUSSION

In this study, a leaf immunoprint assay, which involves blotting the whole leaf onto filter paper, was carried out for SMV-inoculated soybean leaves. The assay included leaves of soybean cultivars Essex, York, Marshall, and PI96983 inoculated with SMV strains G1, G6, and G7 at specific time intervals after inoculation. Two leaves of different plants were assayed per combination per date. The whole experiment was conducted twice. Results were consistent from plant to plant and from one experiment to another. Thus, this method seems to provide a reliable, as well as a quick and economical way to study virus behavior on infected leaves.

Results of the present study strongly suggest that necrosis is also a manifestation of resistance as previously indicated by the genetic studies (Chen et al., 1994). They further demonstrate that *Rsv1* resistance involves both suppression of virus replication as well as inhibition of cell-to-cell movement. In the R response, resistance is complete and there is no detectable virus. In the case of G6-Marshall (local necrosis), the virus is occasionally detectable and, when present, it is only visible in some veins. This observation suggests that sometimes the virus is, in some way, able to overcome the suppression of replication that is exerted by *Rsv1^m*. The fact that the virus appeared only along the veins suggests that inhibition of cell-to-cell movement is also operable and effective in this case. In the cases of G7-Marshall and G7-PI96983 (systemic necrosis), the virus eventually overcomes the inhibition of replication and behaves as in the susceptible combination. However, and as time progresses, inhibition of cell-to-cell movement seems to build up as the virus appear to be mostly confined to the veins. This inhibition of cell-to-cell movement, however, seems to be less effective in the case of G7-Marshall than in G7-PI96963, for the virus showed more spread in the former. It is worth mentioning that although the behavior of the two alleles of PI96983 and Marshall, *Rsv1* and *Rsv1^m* respectively, with G7 was similar, *Rsv1^m* seemed less restrictive to the virus indicating that inhibition of cell-to-cell movement is less effective in this case. Such an observation also holds true at the gross plant level where necrosis is more invasive in the case of G7-Marshall. This observation further demonstrates that the type of reaction produced is determined by the resistance allele as well as by the virus strain. Alternatively, one can argue that the observed differences in the behavior of *Rsv1* and *Rsv1^m* alleles may be due to the effect of different genetic backgrounds. Availability of near isogenic lines for each of these two resistance alleles using the same recurrent parent may provide an opportunity for further analysis of these alleles.

Based on the observations from this study, one can conclude that *Rsv1* resistance is a multicomponent type of resistance that includes both inhibition of virus replication and cell-to-cell movement, but not systemic vascular movement, and that it is activated differentially depending on the virus strain and the specific resistance allele. Such a situation has been proposed for other virus pathosystems like potato virus X - potato (Rx) pathosystem (Goulden and Baulcombe, 1993). *Rx*, which is a single dominant gene in potato, confers a symptomless reaction, termed extreme resistance, with no visible signs of infection (Cockerham, 1970; Tozzini et al., 1991) similar to the R reaction in the SMV - soybean (*Rsv1*) system. *Rx*-mediated resistance is an induced type of resistance and is expressed at the level of protoplasts leading to complete suppression of virus multiplication (Adams et al., 1986; Kohm et al., 1993). However, several lines of evidence suggest that *Rx*-mediated resistance can have distinct components. One allele, *Rx^n*, is associated with a localized HR, with evidence of accumulation of virus in the inoculated cell and surrounding area, rather than extreme resistance (Cockerham, 1970). A mutant form of PVX overcomes Rx-mediated resistance in protoplasts but is still avirulent when inoculated to plants (Goulden and Baulcombe, 1993). Additionally, if potato carrying *Rx* is graft inoculated, there is a low-level accumulation of

PVX in the systemic tissue and some necrosis (Bagnall, 1961).

Results from this study provide evidence for the following model of *Rsv1*-mediated resistance. According to this model, *Rsv1* resistance controls both the N and the R responses. This argument can be further substantiated by the fact that, on rare occasions, the G6 strain was observed to produce a restricted veinal necrotic lesion on inoculated leaves of PI96983 (R combination) indicating that the boundaries separating the R and the N response are not distinct. By immunoprints, the virus was indeed detected on such leaves (data not shown). The fact that the N response is an HR type of reaction suggests that both of the N and R responses could be induced type of resistance (HR) despite the fact that the R is a symptomless reaction. In nonviral pathosystems, the induction of defense responses without being accompanied by the HR lesion phenotype has been reported (Jakobek and Lindgren, 1993). Transcripts for phenylalanine ammonia-lyase, chalcone synthase, and chitinase accumulated in common beans after infiltration with a mutant of *Pseudomonas syringae* pv. *tabaci* (Wolf and Foster) Young et al., even though an HR did not occur.

The overall picture of the proposed model describes *Rsv1* resistance as a two phase-process: a recognition phase followed by the response phase (Fig. III.5). In the recognition stage, the *Rsv1* recognizes the avirulence gene (Avr) of SMV, which is so far unknown. This event sets the stage for the response phase. The type of response that is activated depends on the nature of the components in the recognition reaction (the *Rsv1* allele and the Avr gene) and possibly the environmental conditions. In the case of the R response, defense responses that are triggered in the plant due to the interaction between the Avr and the *Rsv1* gene is successful in combating the virus, and thus the virus is not detectable. Changes in the Avr or the *Rsv1* genes, that lead to changes in their structure or surface charge, might affect their binding affinity to each other. Subsequently, an attenuated or a delayed defense response is produced allowing a reduced level of virus replication and cell-to-cell spread, but eventually succeeding in restricting the virus as in the localized necrosis phenotype. Alternatively, the binding affinity between the Avr and *Rsv1* might be altered to such a degree that the induced defense response is greatly attenuated or delayed, thus failing to restrict the virus, as in the systemic necrosis response.

An ultimate evidence of the above described model can be obtained by studying the mechanism of *Rsv1* resistance at the cellular protoplast level. According to this model, we will have to expect complete suppression of virus replication in the R response. In the local necrosis response, on the other hand, a lower branch level of resistance is expected with level of virus replication lower than in the susceptible phenotype. In the systemic necrosis a level of replication similar to that in the susceptible reaction might occur.
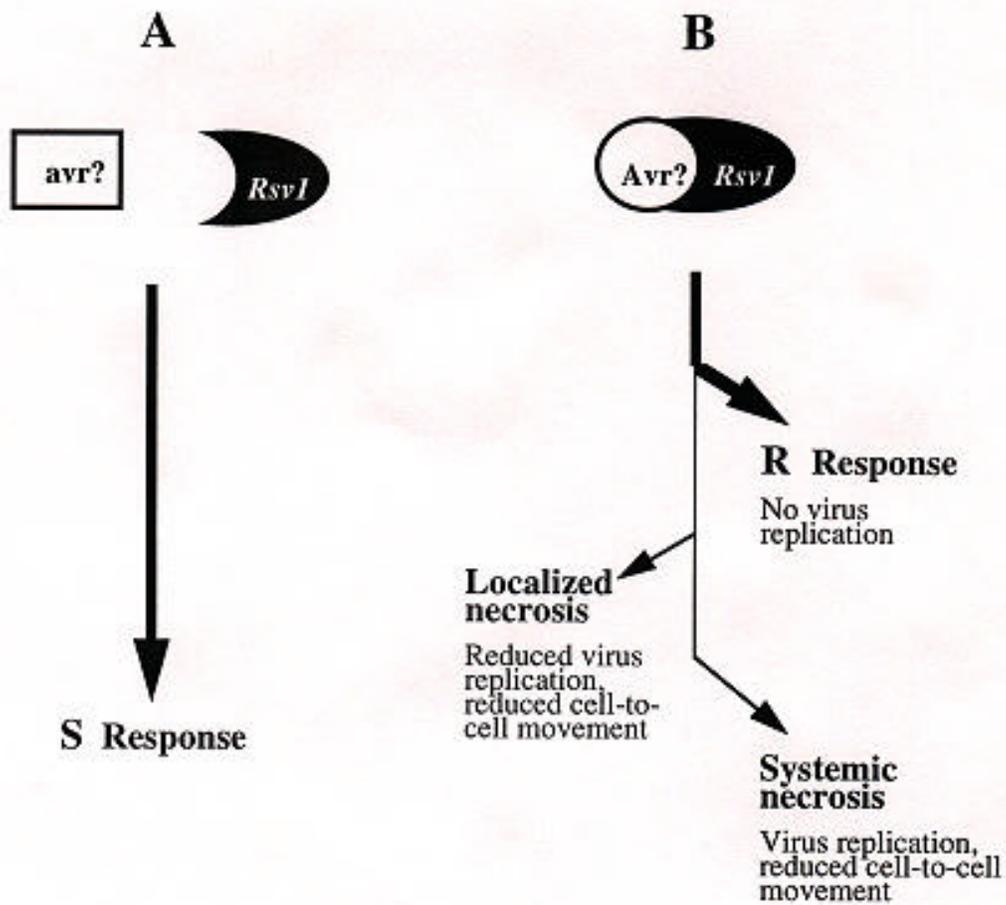
Fig. III.5. A model of the mechanism of *Rsv1*-mediated resistance.
A, The susceptible response (S): the recognition of SMV fails because *Rsv1* does not recognize the avr gene of SMV thus no resistance response; B, the R and N response: *Rsv1* recognizes the Avr gene of SMV, thus activating an N or R response depending on the virus strain, the resistance allele, and possibly the environmental conditions.

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CHAPTER V

Preparation of Soybean [*Glycine max* (L.) Merr.] Mesophyll Protoplasts and Transfection with Soybean Mosaic Virus

ABSTRACT

For the purpose of studying the nature of resistance in soybean [*Glycine max* (L.) Merr.] to soybean mosaic virus (SMV) at the cellular level, a soybean protoplast experimental system has been developed. This system includes: protoplast isolation and purification, virus inoculation and methods of detecting virus replication. Protoplast isolation was based on a combined cellulase-pectolyase Y-23 digestion and metrizamide-sorbitol gradient purification protocol. Virus inoculation was tried using either polyethylene glycol (PEG) or poly-L-ornithine (PLO), and detection was by Western blotting using antiserum to whole virus. Virus inoculation using PEG was successful but results were irreproducable because of the adverse effect of PEG on protoplasts viability. Inoculation using PLO was inconclusive because of the background from residual inoculum. Additional research is needed before soybean protoplasts can be used to investigate the mechanisms of resistance to SMV.

INTRODUCTION

Plant protoplasts provide a unique system to study the interaction between a certain virus and its host at the individual cell level. Materials such as whole plants, organs, or tissue are inadequate for this purpose because the number of cells initially infected by inoculation is extremely small and the stage of infection in individual cell becomes random as infection spreads (Takebe, 1975). The first report of protoplast inoculation with a virus was by Cocking (1966) for tobacco mosaic virus (TMV) in tomato (*Lycopersicon esculentum* Mill.) protoplasts. Since that time, protoplast technology has become a fundamental technique to study virus replication cycle occurring at the cellular level as well as for investigating mechanism of virus resistance and infection.

The molecular mechanism underlying host resistance is poorly understood. Generally, plant resistance to viruses occurs by interruption of the virus life cycle at one of two stages (Matthews, 1991): i) the replication cycle inside the cell (entry, uncoating, translation, replication, or assembly), or ii) spread of virus to other cells and parts of the plant. A valuable experimental tool to characterize the mechanism of virus resistance has been the use of plant protoplasts. Virus resistance can be expressed in protoplasts and in this case resistance is more likely to be caused by inhibition of virus replication. Alternatively, if the protoplasts from resistant plants support virus replication, then resistance operates by inhibiting cell-to-cell movement and/or subsequent systemic spread of the virus. Extreme resistance in potato (*Solanum tuberosum* L.) against the potyvirus type member, potato virus Y (PVY), is conferred by a single dominant gene designated R_y, which has been introgressed from a wild potato relative. Mesophyll protoplasts from resistant potato clones carrying R_y were found resistant to infection when inoculated with PVY (Barker and Harrison, 1984). This study suggests that R_y resistance works on the virus replication rather than inhibition of cell-to-cell movement. In contrast, resistance against PVY in the wild relative *Solanum brevidens* Phil. was found to be operable at the cell-to-cell movement step (Valkonen et al., 1991). Low numbers of protoplasts isolated from mechanically inoculated intact leaves of *S. brevidens* were infected, whereas high proportions of protoplasts became infected with PVY following protoplast inoculation. By comparison, resistance to tobacco mosaic virus (TMV), which induces a well-characterized HR-type defense response in tobacco (*Nicotiana glutinosa* L.), is not manifested at the protoplast level (Otsuki et al., 1972).

Three types of reactions are evident upon inoculation of soybean mosaic virus (SMV) strains into a set of resistant soybean [*Glycine max* (L.) Merr.] cultivars. These reactions are: susceptible or systemic mosaic (S), necrosis (N), and symptomless or resistant (R). Accordingly, SMV is classified into seven strain groups, G1 to G7 (Cho and Goodman, 1979). G1 is the least virulent infecting none of the resistant cultivars. Higher numbers were assigned to other strains with increasing virulence from G1. SMV-soybean system is governed by the gene-for-gene model (Roane et al., 1986). The R and the N reactions are believed to be resistance responses governed in most cultivars by a single dominant gene, *Rsv1* (Kiihl and Hartwig, 1979; Chen et al., 1991; Chen et al., 1994). The N response is considered a hypersensitive type of response (Paschal and Goodman, 1978; Buzzell and Tu, 1989) and is induced (Choi, 1991). On the part of SMV, the gene(s) interacting with *Rsv1* and mechanism of resistance are not known.

Plasmolysis of many higher plants is difficult to achieve. Soybean mesophyll cells plasmolyze with difficulty and give relatively low yields (Lee-Stadelmann et al., 1985). This is attributed to the tight adherence of the plasmalemma to the cell wall and/or to mechanical hinderance of protoplast contraction by starch filled chloroplasts in the case of older material. Most reports in

the literature describe isolation of soybean protoplasts from cell suspension culture or from immature cotyledons (Gamborg et al., 1983; Saleem and Cutler, 1986; Weber et al., 1986; Dhir et al., 1991; Wei and Xu, 1991; Honeycutt et al., 1992). However, such sources are not readily available and do not produce large numbers of protoplasts. The first report for isolation of mesophyll protoplasts was in 1981 by Schwenk et al. In this procedure, an enzyme mixture of 0.1% pectolyase Y-23 and 0.2% cellulase was utilized. However, protoplasts using this isolation method were more readily released from isolated cells than from sliced leaves. The only other report available for isolating soybean mesophyll protoplasts is that of Lin et al. (1983). His procedure was based on a combined cellulase (2%) - pectolyase Y-23 (0.1%) digestion and metrazamide-sorbitol gradient purification protocol. Based on this procedure, a 10 to 15% protoplast yield was obtained from fully expanded mature leaves and 20 to 30% yield from young expanding leaves, within 3 hrs.

Inoculation of plant protoplasts by viruses can be achieved by chemical, physical, and electrical methods. The use of poly-L-ornithine (PLO), a positively charged poly amino acid has been used successfully for the inoculation of protoplasts from a wide range of plant species with many viruses (Takebe, 1975; Gera, 1994). The mechanism of PLO stimulation of virus inoculation is not known. One possible function of PLO is to neutralize or reverse the surface charge of virus particles. This would facilitate adsorption of viruses onto the protoplast surfaces and may stimulate virus entry into the cell. Soybean protoplasts, isolated from liquid suspension culture, were successfully inoculated with cowpea mosaic virus (CPMV) and with southern bean mosaic virus (SBMV) by the use of PLO (Jarvis and Murakishi, 1980). As detected by fluorescent antibody, 70 to 90% and 30 to 35% of protoplasts were infected by CPMV and SBMV, respectively. Another method for protoplast inoculation is the use of polyethylene glycol (PEG). This method has been successfully used for inoculation of protoplasts with different viruses and viral RNA (Sander and Mertes, 1984). The mechanism of inoculation by PEG is not understood. An alternative method uses liposomes which encapsidate the viruses or viral RNAs and deliver them into the protoplasts (Fukunaga et al., 1981). The liposomes become attached to the protoplast membrane by using procedures developed for cell fusion. Additionally, electroporation of plant protoplasts with virus particles, but more commonly with virus RNA, has become a common method of inoculating protoplasts with viruses (Watts et al., 1987; Hibi, 1989).

As mentioned earlier, plant protoplasts have become important tools in plant virus research. However, drawing general conclusions concerning the relation between *in planta* and protoplast resistance may lead to erroneous conclusion (Mansky and Hill, 1993). A protoplast is an artificial system far removed from its environment by traumatic enzymatic treatment and may not function normally. Additionally, a number of actual or potential limitations must be kept in mind when working with protoplasts (Gera, 1994): i) protoplasts are very fragile; ii) protoplasts survive only 3 or 4 days and then decline and die ; iii) there are the quantitative variation in behavior among batches of protoplasts; iv) antibiotics added to the incubation medium may have adverse effects on virus replication; and v) experience is needed to obtain consistently good results.

The objective of this study is to develop a protoplast experimental system in soybean. This will include developing a method for isolating and purification of soybean protoplasts, an SMV inoculation method of soybean protoplasts, as well as developing a method of detecting virus replication in inoculated protoplasts. The ultimate aim of establishing such a system is to study SMV resistance at the cellular level. This can be accomplished be assessing susceptibility/immunity of protoplasts to various SMV strains. Correlating such results with the responses at the intact plant level of the various cultivars will give an indication whether *Rsv1* affects the virus replication or cell-to-cell movement, and whether the mechanism of resistance in the R response differs from that operating in the N response.

MATERIALS AND METHODS

Materials

All inorganic chemicals used in this study were purchased from Fisher Scientific (Pittsburg, PA). Biochemicals used in protoplast preparation and culture were obtained from Sigma (St. Louis, MO), unless otherwise stated. Soybean seeds were thankfully supplied by Dr. Glenn Buss (Virginia Polytechnic Institute and State University, Blacksburg, VA) and included the varieties: Essex, York and PI96983.

The strain SMV-G1 used in this study was originally collected from cv. Lee soybean in Virginia and named SMV-VA. Based on reactions on soybean differentials, it was classified to belong to SMV-G1 strain group of Cho and Goodman (1979) (Hunst and Tolin, 1982). The virus was maintained and propagated in its susceptible cv. Essex by mechanical transmission.

Plant growth conditions

Soybean plants were raised in 15 cm dia. pot (six plants per pot) with Metro-Mix 360 (Scotts-Sierra Horticultural Products Company, Marysville, OH) in a growth chamber and were irrigated once a week with Peters Professional water-soluble fertilizer solution (Scotts-Sierra Horticultural Products Company, Marysville, OH). Plants were grown under the following conditions: 1.8 uE $m^{-2}s^{-1}$, 12 hr photoperiod (25 C, light/20 C, dark) and 70% RH. Plants were kept in the dark for 24 hr prior to protoplast isolation. Only unifoliolate leaves or youngest trifoliolate leaves taken from plants less than 21 days old were used as a source for protoplast isolation.

Inoculum preparation

Virus particles used for protoplast inoculation were prepared by a purification scheme adopted from Hunst and Tolin (1982) with modifications. Approximately 5-6 gm of SMV-infected second or third trifoliolate leaves 2-3 wk after inoculation were homogenized in a mortar and pestle with extraction buffer consisting of 0.5 M sodium citrate buffer (pH 7.0) plus 2% (v/v) mercaptoethanol at a ratio of 4-5 ml for each gm of leaves. Clarification of the extract was done by adding 8% (v/v) chloroform/butanol (1:1) of total volume followed by low speed centrifugation at 13,000 g for 10 min. The virus was concentrated from the supernatant by the addition of PEG 6000 to 4% (w/v) along with NaCl to 0.3 M. After stirring and incubation in the cold for at least 1.5 hr, the extract was centrifuged at 13,000 g for 15 min at 4 C. The pellet was resuspended in 10 mM sodium phosphate buffer, pH 7.0 (for PEG-mediated inoculation) or 10 mM potassium phosphate, pH 7.0 (for inoculation by PLO) and subjected to a cycle of high speed centrifugation at 171,000 g for 1.5 hr. The virus-containing pellet was resuspended by gentle shaking in the above phosphate buffer at a ratio of 50 μl for each gm of infected leaves. The mixture was transferred to a 1.5 microfuge tube and centrifuged for 3 min. The optical density of the supernatant at 260 and 280 was measured spectrophotometrically to estimate virus concentration. An extension coefficient of 2.4 mg virus/ml was used for this purpose (Purcifull, 1966). All steps were performed in the cold at 4 C or on ice. The virus inoculum was used for protoplast inoculation on the same day it was prepared.

Protoplast isolation and purification

Protoplasts were isolated from young or mature mesophyll leaf tissue including the unifoliolate or first trifoliolates. Protoplast isolation and purification were performed using the procedure outlined by Lin (1983) with modifications. The lower epidermis of detached leaves was abraded using a cotton swab. Leaves were then floated in a Petri dish with the abraded side down on around 30 ml of holding solution [0.65 M sorbitol, 10 mM $CaCl_2.2H_2O$, 25 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES), 0.5 mM dithiothreitol (DTT), and 10 U/ml nystatin, pH 5.8). After 30 min, the holding solution was removed and replaced with 15 ml of cell wall-digesting enzyme mixture [2% (w/v) cellulase and 0.2% (w/v) pectolyase Y-23 (both enzymes were purchased from Karlan Chemicals, Santa Rosa, CA), 0.75 M mannitol, 10 mM $CaCl_2.2H_2O$, o.1% (w/v) bovine serum albumin (BSA), and 0.5 mM DTT, pH 5.5] in a shaker water bath at 50 rpm for 4-6 hr at 25 C in the dark. After incubation, the resulting mixture was filtered through stainless-steel filter mesh of 50 μm. Crude protoplasts were collected by centrifugation at 100 g for 4 min and then layered on top of a metrizamide-sorbitol gradient for purification by floatation. The gradient consisted of three layers of 5 ml each -- bottom layer of 0.2 M metrazamide and 0.39 M sorbitol, middle layer of 0.1 M metrizamide and 0.52 M sorbitol, and upper layer of 0.05 M metrizamide and 0.59 M sorbitol -- all in 10 mM $CaCl_2.2H_2O$ and 25 mM Hepes, pH 6.0. The gradient was centrifuged at 100 g for 5 min. Purified protoplasts were collected at the bottom interface for the mature leaves and next to the bottom interface for young leaves. Purified protoplasts were pelleted by adding 20 ml holding solution and centrifuging at 100 g for 4 min. Protoplasts were washed once with holding solution and resuspended in 5 ml of holding solution and kept at 4 C until further use. A sample of protoplasts was counted using a hemocytometer. The viability of protoplasts was determined by staining with 0.01% Evans Blue exclusion dye. The protoplasts were inoculated with the virus on the same day of isolation.

Inoculation of soybean protoplasts with SMV

Virus inoculation of soybean protoplasts was performed using PEG 3350 or 6000 and PLO (hydrobromide, 100,000-200,000). Inoculation in the presence of PEG was performed essentially by the method of Cassells (1980) for TMV inoculation of tobacco (*Nicotiana tabacum* L.) protoplasts. About 10^6 protoplasts were pelleted and resuspended in 0.5 ml of 0.6 M mannitol adjusted to pH 6.3. About 100-200 μg of virus was resuspended in 0.4 ml of phosphate buffered mannitol (PBM; 0.6 M mannitol in 10 mM sodium phosphate buffer, pH 6.3) and added to the protoplast suspension. To this, 0.1 ml of 28% PEG in 0.6 M mannitol (pH 6.3) was added. The solutions were gently swirled after each addition to ensure even mixing. The mixture was incubated at 4 C. After about 20 min, 0.3 ml of 28% PEG in 0.6 M mannitol plus 0.7 ml of 5m M sodium phosphate buffer, pH 6.3, containing 11 mM $CaCl_2.2H_2O$ in 0.6 M mannitol were added and the mixture was incubated for 1 hr at room temperature. The protoplasts were allowed to settle under gravity and the protoplast pellet was washed two times with 10 ml of 50 mM $CaCl_2.2H_2O$ in 0.6 M mannitol (pH 10.5) and one time with 10 ml protoplast culture medium [modified from Jarvis and Murakishi, 1980; 1X Murashige and Skoog basal salt mixture (MS), 1X Gamborg B5 vitamin mix (per liter: 10 mg thiamine HCl, nicotinic acid, 1 mg, pyrodixine 1 mg and myoinositol 100 mg), casein hydrolysate 1.5 gm/liter, 6 mM $CaCl_2.2H_2O$, 1 mM $CaH_4(PO_4)_2.H_2O$, 0.15 M glucose, 0.3 M sorbitol, pH 6.0]. After precipitation under gravity the protoplast pellet was resuspended in 20 ml culture medium, transferred to a 50 ml conical flask and incubated at room temperature in

diffuse room light for 48 hr or longer. A mock-inoculated treatment was included in the experiment. Protoplast viability was determined every 12-24 hr.

Another method of inoculation of protoplasts using PEG was also performed. The method is that of Dawson et al. (1978) and it involves resuspending freshly pelleted protoplasts (10^6) in 2 ml 40% PEG 3350 in 3 mM $CaCl_2.2H_2O$ containing 50 µg virus. Immediately after, 20 ml 0.7 M mannitol was gradually added and mixed well.   The protoplasts were left 30 min at room temperature and then washed twice with 0.7 M mannitol and cultured as before.

Virus inoculation of soybean protoplasts using PLO was accomplished by the following modifications of the procedure of Jarvis and Murakishi (1980).   The inoculation medium; consisting of: 25 µg virus/ml in 0.4 M sorbitol, 3 µg/ml PLO, 20 mM potassium phosphate, pH 6.3, and 5 mM $CaCl_2.2H_2O$; was prepared and allowed to pre-incubate for 15 min at room temperature. About 2-4 x 10^6 protoplasts were pelleted and resuspended in 5 ml inoculation medium and immediately diluted with 5 ml of 0.4 M sorbitol. The mixture was swirled gently and allowed to incubate at room temperature for 30 min. The protoplasts were then washed three times with 0.4 M sorbitol plus 10 mM $CaCl_2.2H_2O$ by centrifugation at 100 g for 1 min each time. The last resuspension was made in culture medium (as described above) and the protoplasts were incubated in 50 ml conical flasks, 20 ml/flask, at room temperature.

Western blot analysis of inoculated protoplasts

 Protoplasts were collected 48 hr or later after inoculation, washed once with 0.4 M sorbitol plus 10 mM $CaCl_2.2H_2O$, and sedimented in a microfuge tube. The protoplast pellet was stored at -70 C until assayed. Total proteins were extracted by vortexing or homogenizing the protoplast pellet in 100 µl of extraction buffer (0.05% Triton X-100 in 0.05 M K_2HPO_4/KH_2PO_4 buffer (pH 7.0) plus 5% 2-mercaptoethanol) and centrifugation at 12,000 g for 20 min at 4 C. About 25 µl of supernatant was mixed with an equal volume double concentrated Laemmli loading buffer, boiled for 5 min and separated on an sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). The proteins in the gel were electrotransferred to a nitrocellulose membrane (Nitropure; MSI, Westboro, MA) using the semi-dry Milliblot-SDE Electroblotting System (Millipore, Bedford, MA).

Following transfer, the membrane was incubated at room temperature for 2 hrs (or at 4 C overnight) in PBS (137 mM NaCl, 2.68 mM KCl, 8 mM Na_2HPO_4, 1.46 mM KH_2PO_4) containing 2% Carnation non-fat dry milk. The membrane was then placed in a 1:10,000 dilution in PBS of rabbit antibody to SMV (Hunst and Tolin, 1982). After three washes with PBS, the membrane was incubated for 1 hr with alkaline phosphatase-conjugated goat anti-rabbit IgG at 1:10,000 dilution in PBS. Following three washes with PBS, phosphatase activity on the membrane was revealed using 5-bromo-4-chloro-3-indolylphosphate (BCIP) as a substrate and nitroblue tetrazolium chloride (NBT) as an enhancer of the colorimetric reaction according to the manufacturers instructions (Zymed Laboratories Inc, San Francisco, CA).

RESULTS

Protoplast isolation and purification

Soybean protoplasts were successfully isolated by the procedure outlined giving a satisfactory yield of about 8-10 x 10^6 protoplast per gm of leaves. Source of protoplasts were young fully expanded unifoliolate or first trifoliate leaves. Age of plants seemed critical, for the yield obtained decreased proportionally with increasing plant age. These leaves were abraded on the underside and preplasmolized in a holding solution containing 0.6 M sorbitol as an osmoticum. Lower yields were obtained when this step was omitted. Different combinations and concentrations of enzymes; like cellulase and macerozyme R-10, cellulase and macerase, driselase, driselase plus macerozyme R10; or driselase and macerase; were tested and the only successful combination was 2% cellulase plus 0.2% pectolyase. Mannitol was used as an osmoticum in the digestion medium and it seemed to work better than sorbitol in this step. Initially, purification of protoplasts by floating on various concentrations of sucrose (10-40%) was attempted. However, the protoplasts seemed to sink to the bottom along with the plant debris whatever the concentration of sucrose used. Purification was successful using metrazamide column as outlined in the procedure of Lin (1983) and protoplasts usually floated into a broad band near the bottom interface.

Inoculum preparation

The method used for virus purification was a modification of that of Hunst and Tolin (1982). Initially, adjusting the pH of the extraction buffer (0.5 M sodium citrate) was of vital importance. Performing this step with unbuffer sodium citrate resulted in no virus pellet following PEG precipitation. Clarification of extract was performed with 8% (v/v) chloroform/butanol (1:1) of total volume instead of butanol alone. Including chloroform in this step gave a clearer extract than using butanol alone. The concentration and purification of the virus was carried out in two steps. First, the virus was precipitated using PEG plus salt. The pellet was resuspended in phosphate buffer and the virus resuspension was subjected to another high speed centrifugation. These two rounds of virus purification were found sufficient for the inoculation of protoplasts. Purification was not carried further by sucrose density gradient centrifugation and no second cycle of high speed centrifugation was performed because a significant decrease in virus yield resulted when the latter was done. On the average, 100-250 µg of virus/gm of SMV-infected leaves was obtained when the outlined procedure was performed.

Inoculation of soybean protoplasts with SMV

Two procedures of virus inoculation of protoplasts were attempted. The first involved the use of PEG. Soybean protoplasts seemed to be very sensitive to this agent and death of protoplasts in less than 24 hr after addition was almost always the outcome. Both PEG 3350 and 6000 were tried with similar results. Additionally, several percentages (5 to 30%) of PEG were tried and with three varieties of soybean; Essex, York, and PI96983. However, results were inconsistent. In one trial, 15% initial concentration of PEG seemed to be tolerated by the protoplasts. However, infection of protoplasts was not determined in that test. In few instances, inoculation using PEG at 28% was successful and a high percentage of protoplasts remained viable for about 72 (see results below). All attempts to reproduce the same results failed.

The other method of PEG-mediated inoculation, which involved resuspension of protoplasts in 40% PEG solution was not successful. In that case, death of protoplasts resulted shortly after PEG addition. This could be due to the high initial concentration of PEG.

The second procedure attempted for protoplast inoculation with the virus involved the use of PLO. Infection using this agent seemed to be successful and the viability of protoplasts was in general about 50% 48 hr following treatment.   However, the residual virus inoculum posed a problem in that the adsorbed virus particles that persisted on the surface of protoplasts could not be washed off and thus could not be differentiated from the virus that presumably had replicated inside the protoplasts (see below).

Detection of SMV replication in soybean protoplasts

Fig. IV.1 shows a Western blot illustrating SMV-G1 replication in soybean protoplasts (Essex) inoculated with SMV-G1 by the use of 28% PEG. About 10^6 protoplasts were inoculated with around 100-200 µg of SMV-G1, and cultured and sampled at times varying from 0 to 72 hr after inoculation. The virus was detected 36 hr after inoculation and its quantity increased until 72 hr. The virus was not detected at 0 hr indicating that the residual inoculum posed no problem when using this method (lane 6). The positive control on the blot consisted of SMV-G1 purified virions as well as sap from SMV-infected leaves. The latter control shows two bands. This observation has been reported many times for the potyviral coat protein (CP) and was attributed to the partial degradation of the CP by proteases of host or microbial origin (Shukla et al., 1994). A recent study (Hassan et al., 1994), however, has attributed the occurrence of multiple bands to the presence of denaturing agents in the extraction buffer like SDS or urea which might expose regions of the CP to a plant enzyme that modifies the size of the CP. Treatment of the infected leaves by submerging in boiling water prior to protein extraction, thus inactivating the enzyme, resulted in only one band (data not shown). No band was detected for the mock-inoculated protoplasts (lane 7) indicating no non-specific binding of SMV antiserum.

The results of soybean protoplast inoculation with SMV-G1 using PLO is shown in Fig. IV.2. About 2-4 x 10^6 of protoplasts were resuspended in an inoculation medium including 50 µg/ml of virus plus 3 µg/ml PLO. The protoplasts were cultured for 48 hrs before sampling. The virus was detected in both of the 0 hr (residual inoculum) and 48 hr samples. Thus, it was not possible to get definitive evidence for virus replication in the protoplasts. The residual inoculum of the virus could not be removed prior to protoplast culturing despite extensive washing. I could not explain the fact that two bands of different migration distances are seen in both the 0 and 48 hr samples. It is possible that the virus in the 48 hr treatment had indeed resulted from replication inside the protoplasts and thus was subjected to a plant enzyme that modified the virus CP to a heavier form. The fast form in the 0 hr treatment lane might have resulted from protein degradation of the virus inoculum during storage of the treatment until assayed, along with the other treatment, 48 hr later. As with protoplasts inoculated using PEG, mock-inoculated protoplasts also did not react with SMV antiserum.
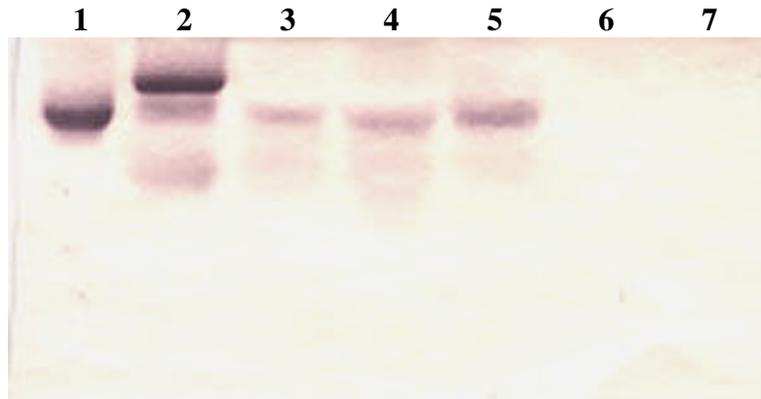
Fig. IV.1. Western blot analysis of SMV viral coat protein in soybean (cv. Essex) protoplasts using PEG-mediated inoculation. Protoplasts were either mock-inoculated or inoculated with purified SMV-G1 virions and total protein were electrophoresed in SDS-PAGE followed by electrophoretic transfer to nitrocellulose membranes. Protein blots were treated with antisera directed against SMV particles. Lane 1, 2 μl of purified SMV coat protein; lane 2, 10 μl of SMV-G1 infected sap; lane 3, 25 μl of proteins extracted from SMV-G1 inoculated protoplasts sampled 36 hr post inoculation (hpi); lane 4, from protoplasts sampled 56 hpi; lane 5, protoplasts sampled 72 hpi; lane 6, protoplasts sampled 0 hpi; lane 7, mock-inoculated protoplasts sampled 72 hpi.
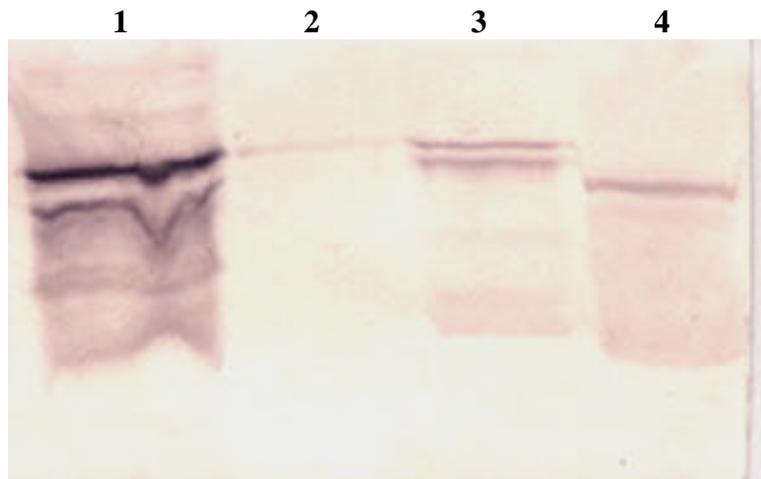
Fig. IV.2. Western blot analysis of SMV viral coat protein in soybean (cv. Essex) protoplasts using PLO-mediated inoculation. Protoplasts were either mock-inoculated or inoculated with purified SMV-G1 virions. Total proteins were extracted from protoplasts and electrophoresed in SDS-PAGE followed by electrophoretic transfer to nitrocellulose membrane. Blots were treated with antisera against SMV particles. Lane 1, 15 μl SMV-G1 infected sap; lane 2, 25 μl of total proteins extracted from mock-inoculated protoplasts sampled 48 hr post inoculation (hpi); lane 3, from SMV-G1 inoculated protoplasts sampled 48 hpi; lane 4, from protoplasts sampled 0 hpi.

DISCUSSION

Extensive studies have been conducted on the genetics of the SMV resistance in soybeans. However, nothing is known about the mechanism of interaction between this virus and its host at the molecular level. It is not known whether the resistance is a constitutive or an induced type of response. Furthermore, it is not known whether resistance is due to inhibition of virus multiplication inside the cell or it is due to restriction of cell-to-cell movement.

An experimental system; including: protoplast isolation and purification, virus inoculation, and a method for detecting virus replication in protoplasts is lacking for soybean. In this study, an attempt to develop such a system was initiated. The isolation and purification part of the system has been successfully achieved according to the method of Lin (1983) with some modifications. This is the only report (besides that of Schwenk, 1981) that exists in the literature for isolating soybean mesophyll protoplasts. Soybean and related species of the *Glycine* genus have been amongst the most difficult plant tissue from which to obtain protoplasts. Mature leaves have presented problems to many investigators because of the difficulty in obtaining viable protoplasts by enzyme digestion procedures. Many investigators have reverted to isolate protoplasts from cell suspension culture or from immature pods or cotyledons. Because of such factors as unavailability and ploidy level, leaves should be a superior source of protoplasts. In addition, protoplasts for the study of virus replication and attempts to see variation between resistance and susceptibility have generally been done with mesophyll protoplasts. Initially, different combinations of different enzymes were tried. It is only when the enzyme pectolyase Y-23 was included in the digestion medium, along with cellulase, that isolation of protoplasts was possible. For the purification part, a sucrose solution of 15 to 40% sucrose was tried repeatedly as recommended in many references. None of the attempts was successful and finally a metrazamide-sorbitol gradient was used successfully.

Two methods of virus inoculation were attempted. One involved the use of PEG, a method adopted from the TMV-tobacco system (Cassells, 1980). Based on a Western blot from a single trial, virus inoculation seemed to be successful. However, results were largely irreproducible. Although there exists a report in the literature describing the use of PEG to permeablize soybean protoplasts to macromolecules such as the fluorescent dye calcein (Cutler and Saleem, 1987), attempts to use this compound without affecting the viability of protoplasts have almost always failed. Soybean protoplasts seemed to be very sensitive to PEG and it was not possible to get the protoplasts to live more than few hours after its addition. Sometimes even immediate death of protoplasts occurred.

The second method for virus inoculation involved the use of the polycation, PLO. Soybean protoplasts seem to be able to tolerate this agent better than they tolerated PEG. In addition, a band on the blot, indicating the occurrence of virus multiplication in protoplasts, was sometimes seen. However, caution should be exercised regarding these results because a band of similar intensity was consistently obtained for the protoplasts that were harvested immediately after inoculation. This observation indicates that the virus residual inoculum persisted on these protoplasts and that may have given false positives. This problem of residual inoculum could have been avoided if method of detection involved using a probe other than the viral antigen, such as antibody to the cytoplasmic inclusion protein (CIP) (provided by J. Hill, Iowa State University, Ames, IA). However, these would be present at much lower amounts *in vivo* than the coat protein and thus could be undetectable in protoplasts. A tissue blot of an SMV-infected leaf, which presumably contain a high amount of virus, gave a very faint reaction using this antiserum.

Additional effort, which was not within the time limit of this project, is needed to answer the

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above questions. A reproducible system for achieving virus infection of protoplasts is needed. Additionally, an analysis that would permit detection of different levels of replication, as well as providing a clear-cut distinction between inoculated cells over time, is required. A follow up research may involve RNA extraction of the viral genome and inoculation of protoplasts via electroporation. Whether soybean protoplasts tolerate electroporation remains to be seen.

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