AN ABSTRACT OF THE THESIS OF

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Richard O. Hampton

Pea seed-borne mosaic potyvirus pathotype 1 (PSbMV-P1) has the ability to infect most genotypes of *Pisum sativum*. The exception are those genotypes that are homozygous recessive for the *sbm-1* gene. The life cycle of PSbMV pathotype 4 (PSbMV-P4) is unaffected by the *sbm-1/sbm-1* genotype. Infectious clones of P1-P4 recombinants were used to define the genomic segment in P1 that is inhibited by the *sbm-1* gene.

Transcripts generated *in vitro* from these clones were initially tested for infectivity by mechanical inoculation onto the susceptible genotype Early Freezer 680 (EF680). Those recombinants that proved to be infectious were then tested for pathogenicity to PI269818, a *sbm-1/sbm-1* genotype. The P4 genomic substitution, which enabled P1 to infect PI269818, was made progressively smaller until one PSbMV-P1 coding region was established as the determinant for infectivity in PI269818. This study demonstrated that the VPg

coding region is responsible for the inability of PSbMV-P1 to infect PI269818. Whether P1 life cycle disruption occurred at the nucleotide or amino acid levels is unknown.

In conjunction with the definition of the P1 coding region inhibited by *sbm-1*, the point of virus life-cycle disruption was investigated. PSbMV-P1 resistant plants were inoculated with P1 purified virus or RNA. A time line of infection was established for inoculated and noninoculated leaves. Protoplasts generated from P1 susceptible- and resistant-plant leaves were transfected with P1 RNA, followed by ELISA testing for presence of P1 coat protein. Whereas P1 RNA was fully infectious to EF680 protoplasts, P1 coat protein was undetectable in PI269818 protoplasts transfected with P1 RNA. RT-PCR of RNA extracted from PI269818 P1 inoculated leaves revealed no P1 RNA amplification. When P1 inoculated PI269818 leaves were used to inoculate EF680, no viral infection was detected. These results suggest that a complete lack of P1 replication is occurring in *sbm-1/sbm-1* peas. The potyviral VPg protein has been implicated in viral replication. This study demonstrates that the VPg is the determinant of P1 pathogenicity in PI269818 and consistent with the proposed role of VPg in viral replication, PSbMV-P1 viral life-cycle disruption in the *sbm-1/sbm-1* genotype occurs at an early time in viral replication.

Interactions Between Pea Seed-borne Mosaic Virus Pathotype 1 and *Pisum sativum* Resistance Gene *sbm-1*

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Major Professor, representing Botany and Plant Pathology

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Chair of Department of Botany and Plant Pathology

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Dean of Graduate School

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Karen E. Keller, Author

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Chapter 1

Introduction

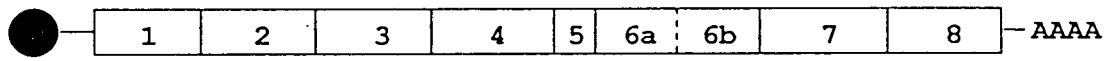
Luria and Darnell (1967) define viruses as "entities whose genome is an element of nucleic acid either DNA or RNA, which reproduce inside living cells and use their synthetic machinery to direct the synthesis of specialized particles, the virion, which contain the viral genome and transfer it to other cells". Thus defined, viruses are obligate, intracellular parasites at the genetic level. The genomes of plant viruses are small, coding for between three and sixteen proteins (Fraser, 1988). Due to the small number of viral proteins encoded and the complete reliance of plant viruses on their host for life cycle completion, it has long been speculated that host cells provide specific components required for virus synthesis. Viruses vary widely in their host ranges (Mathews, 1991). Some viruses are confined to a single host (i.e. barley stripe mosaic virus; Timian, 1974). Other viruses, such as cucumber mosaic and tomato spotted wilt, have large host ranges. In both examples, recognition events between a virus and host plant must be specific. If this were not the case, every virus would be able to infect every host. In addition, some host plants contain gene(s) conferring specific virus resistance. In a host species, this is known as cultivar resistance (Fraser, 1988). This type of resistance can be further broken down into induced (expressed

by the host plant after viral invasion) or constitutive resistance (expressed by the plant in the absence of virus).

Cultivar resistance has given plant virologists a model system to study the type(s) of virus-host interactions occurring in a given host. Tremendous advances in plant molecular virology have allowed virologists to manipulate the viral genome, while still maintaining an infectious viral entity. There are three common approaches used to undertake genetic studies of virus/host interactions (Mansky, 1993). The first is a direct comparison of amino acid or nucleotide sequences of closely related viruses, only one of which is able to infect a given plant genotype. A variation on this approach is genetic recombination of two closely related viruses. Genetic recombination has allowed virologists to apply classical genetic methods to define viral genomic sequences responsible for pathogenicity. A second, alternate approach involves isolation of protoplasts from resistant plants, followed by inoculation with nucleic acid or virions of the non-virulent virus. Determination of replication, or the lack of it, at a single cell level, has allowed scientists to develop hypotheses about the mode of viral life cycle inhibition. If replication occurs at the single cell level, inhibition of cell-to-cell movement is considered a possible mechanism of virus resistance. Lastly, mechanisms of cell-to-cell movement have been studied in an attempt to elucidate one of the most basic steps in viral pathogenesis (i.e., systemic

infection) and to investigate the mode of virus resistance that inhibits movement.

Pea seed-borne mosaic potyvirus (PSbMV) is a typical member of the potyvirus family (Johansen et al., 1991). The genome consist of a single positive-sense (+) strand of RNA. The genome can potentially code for nine proteins. The 5′ end of the genome is covalently linked to a small, virus encoded protein, the VPg. The genome sequence is terminated by a poly-adenylated sequence. Translation of the genome generates a polyprotein which is subsequently cleaved into the nine individual protein components by three viral encoded proteinases (Reichmann et al., 1992). Fig. 1 is a schematic of the potyvirus 5′ structure (VPg), untranslated regions (5′ and 3 UTRs), coding regions, and poly-adenylated 3′ terminus. Included in Fig. 1 is the proposed or known functions of the nine viral proteins. Fig. 2 demonstrates the polyprotein cleavage events performed by the three proteinases. The viral genome is encapsidated by a single species of coat protein, to form a filamentous particle (Shukla et al., 1994). Three pathotypes of the virus have been defined according to the *Pisum sativum* gene that confers resistance to the individual pathotype. Hagedorn and Gritton (1973, 1975) reported that PSbMV-P1 was unable to infect peas that were homozygous recessive for the *sbm-1* gene (located on pea chromosome 6). Genes *sbm-2* (chromosome 2) and *sbm-3* (chromosome 6) confer resistance to P2 (Provvidenti and Alconero, 1988a).



Potyvirus Proteins		Known/Proposed Protein Funtion(s)
1	P1	Proteolytic processing, cell-to-cell movement
2	HC-Pro	Proteolytic processing, insect transmission
3	P3	Unknown
4	CI	Replication (helicase, ATPase)
5	6K	Replication, membrane binding
6	NIa	
6a	VPg	Replication
6b	Pro	Polyprotein processing, replication
7	NIb	Replication, RNA-dependent RNA polymerase
8	CP	Encapsidation and insect tranmission

Figure 1. The potyvirus genome including, 5′ and 3′ untranslated regions, coding regions, and proposed/known functions of the virally encoded proteins (Reichmann et al., 1992).

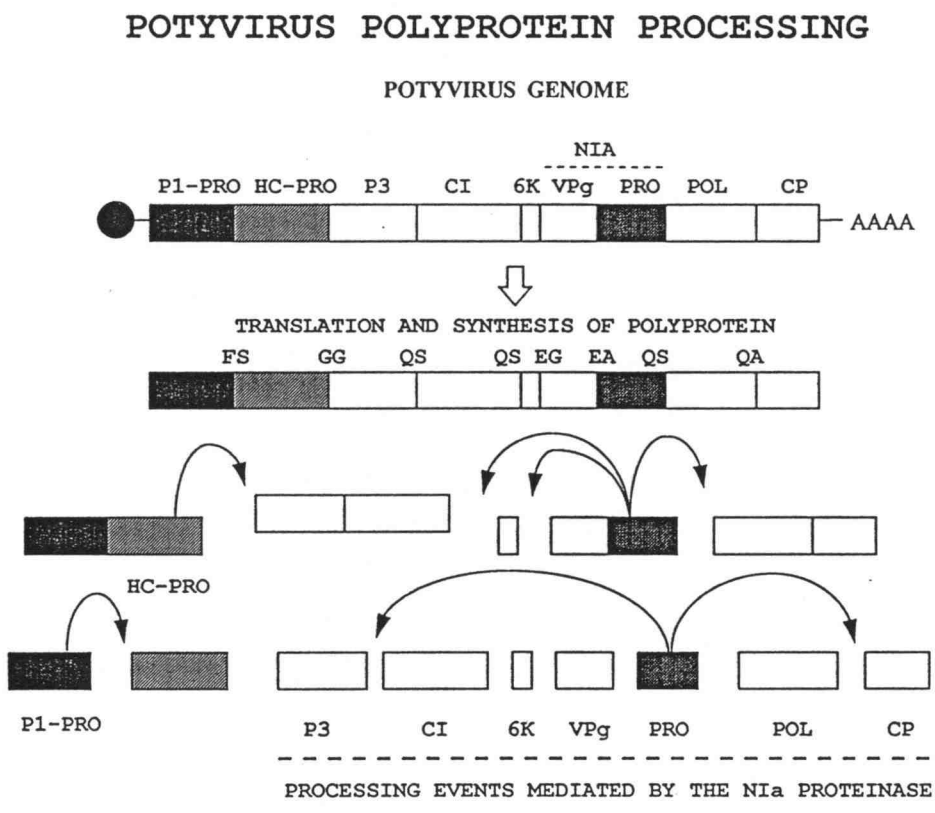
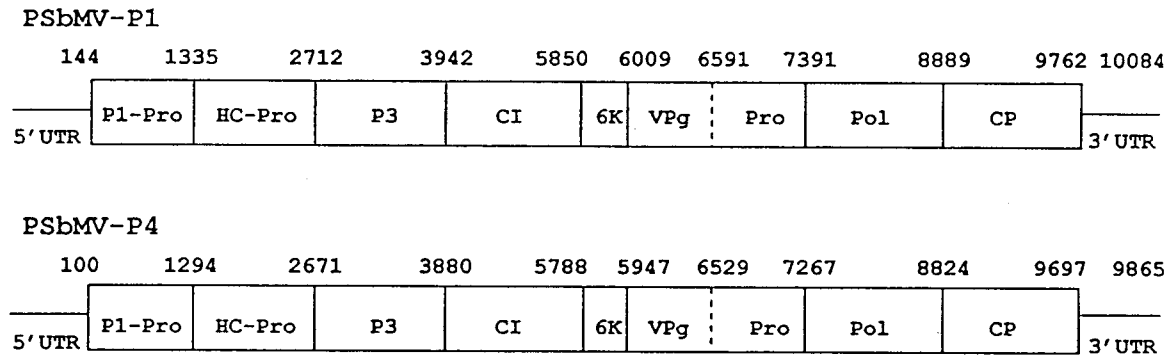


Figure 2. Schematic of the proposed potyvirus proteinase processing events (Reichmann et al., 1992).

Resistance to P4 is conferred by the *sbm-4* gene (chromosome 6; Provvidenti and Alconero, 1988b).

Dr. E. Johansen (1990) first cloned and sequenced PSbMV-P1. This work was then extended on PSbMV-P4 (Johansen et al., in press). There are minor differences between these two pathotypes, in coding region and untranslated region size (Fig. 3). P1-P4 amino acid homologies vary among the different coding regions (Fig. 4). Johansen (personal communication) created an infectious clone of PSbMV-P1 by inserting the full length P1 cDNA into a vector containing a bacteriophage T7 promoter (Petty, 1988). T7 polymerase was used to generate RNA runoff transcripts of the full lenth viral sequence. Using site directed mutagenesis, Johansen engineered common restriction sites into clones of both the P1 and P4 pathotypes. The new restriction sites allowed the two genomes to be divided into four segments (Fig. 5). Recombinants between the two pathotypes were then constructed. Johansen (personal communication) later constructed a full length infectious clone of PSbMV-P4 by inserting P4 cDNA downstream from the cauliflower mosaic virus (CaMV) 35S promoter. This enabled double-stranded DNA to be directly inoculated into peas.

The objective of the present study was delineation of the exact PSbMV-P1 coding region which prevents P1 from establishing an infection in peas containing *sbm-1/sbm-1*. Dr. R. Provvidenti provided seeds of *P. sativum* P.I. 269818, a



Genomic Region	Nucleotide Number P1	P4
5'UTR	143	99
P1-Pro	1191	1194
HC-Pro	1377	1377
P3	1230	1209
CI	1908	1908
6K	159	159
VPg	582	582
Pro	738	738
Pol	1560	1557
CP	873	873
3'UTR	322	168

Figure 3. Genomic size comparison of PSbMV-P1 and -P4 untranslated and coding regions.

PSbMV Pathoypes P1 and P4 Amino Acid Homology

(expressed as an percentage)

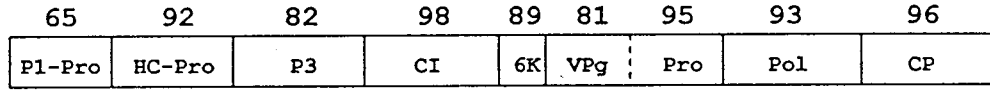


Figure 4. PSbMV-P1 and -P4 amino acid homologies (% similarity) among the different coding regions.

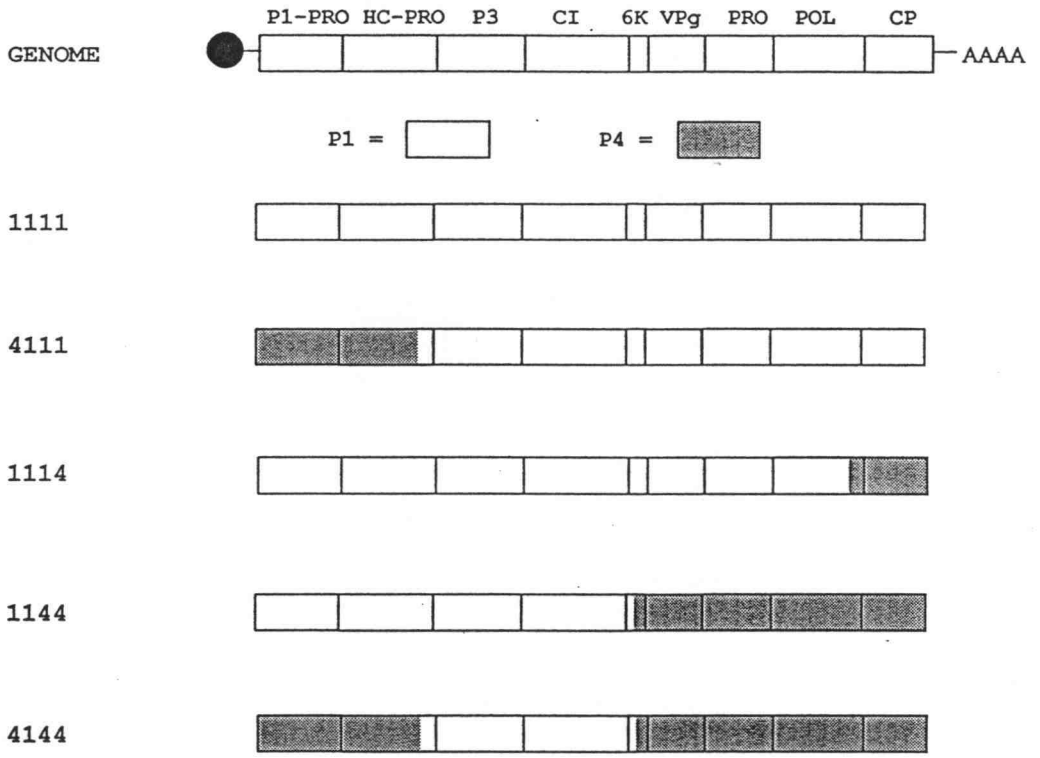


Figure 5. E. Johansen's PSbMV-P1 and -P4 recombinant clones used to initially screen pea genotypes.

genotype that is homozygous recessive for the *sbm-1* gene. This information also would define one of the determinants of P1 capacity to infect plants of the *Sbm-1/Sbm-1* or *Sbm-1/sbm-1* genotypes. These plants were inoculated with parental PSbMV-P1, -P4, or P1-P4 recombinant virus. Using anti-PSbMV antiserum, enzyme-linked immunosorbent assay (ELISA) was used to determined infectivity.

Investigation of PSbMV-P1 pathogenesis in the *sbm-1/sbm-1* genotype has been the secondary focus of this research. In an attempt to discern the point at which the virus life cycle was disrupted, PSbMV-P1 virus or RNA inoculated whole plants were ELISA tested, including inoculated and systemic (noninoculated) leaves. Protoplasts were isolated from resistant and susceptible pea genotypes. These protoplasts were then transfected with P1 viral RNA. Protoplasts were ELISA tested using anti-PSbMV polyclonal antiserum to determine whether replication occurred at the single cell level.

Chapter 2

Literature Review

Introduction

The association of a virus with its host is an intimate one. The intimacy of the relationship derives from the fact that viruses can only replicate inside living cells and, thus, are in all cases obligate parasites. Viruses are biologically inert outside their host and are completely reliant on their host, whether animal, fungal, bacterial, or plant, for virus synthesis. Siegel (1979) identified six steps in the virus life cycle which host resistance genes could potentially disrupt: 1) entry into the cell, 2) uncoating of the viral genome, 3) translation of proteins from viral RNA, 4) replication of the viral genome, 5) assembly of new virus particles, and 6) movement of the virus, both cell-to-cell and systemically through the host. In an attempt to investigate the individual steps that lead to successful pathogenesis, plant virologists have used plants whose genomes contain identified genes conferring virus resistance. These genes, whether dominant or recessive, must prevent one or more stages of the virus life cycle. Discerning the point at which the viral life cycle is disrupted could assist in elucidating the complex relationship between virus and plant host.

Tobacco Mosaic Tobamovirus

Many laboratories have investigated the point at which the viral life cycle is disrupted in plants containing genes conferring virus resistance. The predominant form of resistance to plant viruses, in fact, is conferred by a single, dominant gene (Fraser, 1986). One of the most thoroughly investigated virus/host resistance systems is that of tobacco mosaic tobamovirus (TMV) and tomato cultivars containing single, dominant genes for resistance to TMV pathotypes.

TMV is a rod-shaped, single-strand, positive-sense, RNA virus. The 5′ terminus of the genome is capped, whereas the 3′ terminus is composed of a tRNA like structure (Brunt, 1986). The genome encodes at least three non-structural proteins and the coat protein (Goelet et al., 1992; Ohno et al., 1984). Two TMV resistance-conferring genes in tomato (*Tm-1*, *Tm-2*, and *Tm-2*^{2}) have been identified, one of which is comprised of two identified alleles (Pelham, 1966). TMV pathotypes are defined by their ability to infect tomato plants whose genomes contain one or more of these resistance genes (Pelham, 1972). Group 0 isolates cannot infect genotypes which contain any of the resistance genes. Genes *Tm-1* and *Tm-2* can be overcome by group 1 and group 2 isolates, respectively, whereas group 1.2 isolates overcome both *Tm-1* and *Tm-2*.

The *Tm-1* gene appears to interfere with viral replication. This gene strongly inhibits synthesis of both viral RNA and proteins in both tomato plants and protoplasts (Motoyoshi and Oshima, 1977; Motoyoshi, 1982; Wantabe et al., 1987). Plants homozygous for *Tm-1* were more resistant to infection than heterozygotes (Fraser and Loughlin, 1980; Fraser et al., 1980; Motoyoshi, 1982; Watanabe et al., 1987) indicating that *Tm-1* is gene dosage dependent. When TMV RNA was used to inoculate the *Tm-1* tomato genotype, resistance to infection remained intact, successfully demonstrating that the source of resistance was not at the uncoating stage in the viral life cycle (Motoyoshi and Oshima, 1979). Meshi et al. (1988) sequenced a *Tm-1* resistance breaking strain of TMV-L (strain TMV-0), designated Ltal. This sequence was compared to the TMV-L sequence. It was found that the 130 and 180 K genes had undergone two base changes in the Ltal strain, resulting in two amino acid substitutions: Gln (at aa position 979) to Glu and His (at position 984) to Tyr. The translation products of these two genes are thought to be involved in viral replication (Ishikawa et al., 1986). When Yamafuji et al. (1991) engineered changes in the L strain sequence, in order to duplicate the Ltal strain sequence, RNA transcripts were infectious in the *Tm-1* genotype. This work demonstrated that these two amino acid substitutions were sufficient to overcome *Tm-1* resistance and supported earlier work suggesting virus replication was hindered in *Tm-1* plants. The amino acid

substitutions, in the resistance breaking strain, caused an overall change in electrostatic charge in the putative TMV polymerase protein. It was surmised that this charge difference could influence host protein-viral protein interaction(s). These results suggest that the product of the *Tm-1* gene was unable to inhibit the altered protein and that it was interacting directly with the native TMV polymerase protein or was influencing other host protein virus protein interactions.

Protoplasts generated from tomato plants containing *Tm-2* and *Tm-2²* (allelic) resistance-conferring genes supported replication of TMV-L, making the mechanism(s) of resistance different from that of *Tm-1* (Motoyoshi and Oshima, 1977; Nishiguishi and Motoyoshi, 1987). The *Tm-2* geneotype, previously infected with potato virus X (PVX), was not resistant to TMV-L, whereas the *Tm-1* genotype maintained resistance under identical conditions (Taliansky, et al., 1982). This suggests that PVX was able to provide TMV-L with a movement function in *Tm-2* genotypes. In these experiments, TMV-L was not able to replicate in a *Tm-1* genotype previously infected with PVX. It can be reasoned that either the *Tm-2* gene product either inhibits the TMV-L movement protein or that it is unable to recognize it. TMV-Ltb, a *Tm-2* resistance breaking strain of TMV-L (Meshi et al., 1987), provided evidence that two amino acid substitutions in the 30 kDa movement protein (Deom et al., 1990) allowed systemic

infection in resistant tomato plants (Meshi et al., 1989). It is possible that protein-protein interactions, between host and viral components, are disrupted by the subsequent change in overall charge caused by these substitutions (Meshi et al., 1988; Calder and Palukaitis, 1992).

Cowpea Mosaic Comovirus

Cowpea mosaic virus is a member of the comovirus group of plant viruses. Its genome is composed of two RNA molecules, designated RNA-B and RNA-M. The RNAs are packaged separately in isometric particles with 60 copies each of two coat proteins. The RNAs, like those of the animal picornaviruses, are polyadenylated and have a protein (VPg) attached to their 5′ termini (Goldbach and van Kammen, 1985; van Kammen and Eggen, 1986). RNA-B codes for a proteinase that is responsible for cleavage of RNA-M polyprotein translation product into the two CPMV capsid components (Pelham, 1979; Franssen et al., 1982; Wellink et al., 1986).

In 1977, Beier et al. screened 1,031 cowpea (*Vigna sinensis*) lines for resistance to cowpea mosaic virus isolate SB (CPMV-SB). Sixty-five of these lines were shown to be immune to mechanical inoculation of CPMV-SB. Protoplasts, isolated from fifty-five of these lines, were inoculated with CPMV-SB purified virus. Protoplasts from 64 of these 65 lines were susceptible to infection. Protoplasts from variety 293453 (*cv.* Arlington) proved to be immune to CPMV-SB

infection, but were found to be susceptible to CPMV-DG (Beier, 1979). Arlington cowpeas were also susceptible to cowpea severe mosaic comovirus (CSMV; Goldbach and Krijt, 1982). Arlington cowpeas exhibit a very specific form of virus resistance to CPMV-SB. A single dominant gene was responsible for this resistance (Bier, 1979). Keifer et al. (1984) demonstrated that CPMV-SB capsid protein and negative strand RNA accumulated to a much reduced level in Arlington protoplasts than in protoplasts from susceptible *cv.* California Blackeye 5. The authors concluded that Arlington protoplasts specifically restricts the replication of CPMV, but not CSMV, by reducing or preventing the production of CPMV proteins, including capsid proteins and RNA-dependent RNA-polymerase.

Ponz et al. (1988) demonstrated that cleavage of the two CPMV capsid proteins occurred at extremely reduced rates in Arlington cowpea, when compared to cleavage events occurring in a susceptible cultivar. He postulated that a host inhibitor of the viral proteinase prevented proper cleavage of the polyprotein. *In vitro* translation of CPMV RNA, followed by the addition of partially fractionated Arlington leaf extracts, revealed inhibitors of CPMV polyprotein processing. CPMV RNA was not degraded to a greater degree in the Arlington extract assay than in the susceptible cowpea extract assay. CSMV RNA translation products were processed correctly in Arlington extracts, indicating specificity of polyprotein

processing inhibition. The observed characteristics of the inhibitor of polyprotein processing in Arlington cowpea extracts were consistent with the postulated role of a factor that mediates the immunity of Arlington cowpea to CPMV-SB infection.

Potato Potexvirus X

The genome of potato virus X (PVX) is composed of a single stranded RNA molecule. The 5′ terminus is capped and the 3′ segment terminates in a poly(A) tract. The particles are semi-flexous rods. The coat protein is translated from a subgenomic RNA (Harbison et al., 1988; Forester et al., 1988).

Complete absence of PVX replication occurs in potato genotypes possessing the dominant *Rx* resistance-conferring gene (Cockerman, 1970). Two PVX strains were used to construct a viral recombinant, producing a *Rx* resistance breaking virus. The genomic sequence responsible for this resistance breakdown occurred in the coat protein coding region (Kavanaugh et al., 1992). The means by which coat protein or coding region sequence could interfere with virus replication was unknown. However, Culver et al. (1991) demonstrated that the coat protein of TMV is an elicitor of the hypersensitive response in *N′* genotype tobacco plants. It is conceivable that the PVX coat protein also initiates a hypersensitive response in potato plants of the *Rx/Rx* genotype.

Cowpea Chlorotic Mottle Bromovirus

Cowpea chlorotic mottle bromovirus (CCMV) comprises a tripartite genome and isometric virions containing a single strand, positive-sense, RNA (Kuhn, 1964). RNA 1 and RNA 2 encode two replicase proteins (Kroner et al., 1989; Traynor et al., 1991). RNA 3 encodes the movement protein and coat protein, both dispensable for viral replication (Allison et al., 1990; Pacha et al., 1990).

CCMV is unable to move systemically in resistant cowpea plants. Rogers et al. (1973) determined that this resistance is conferred by one recessive gene pair. Kuhn et al. (1981) proposed that the recessive gene be designated *mv*. Although CCMV replicated in inoculated leaves of resistant cowpeas (Kuhn, et al., 1981; Wyatt and Kuhn, 1979), virus multiplication was greatly reduced in the inoculated leaves of a resistant *vs* a susceptible cultivar. Quantitative comparison of viral RNAs recovered from inoculated leaves of resistant *vs* susceptible plants revealed that RNA 3 was significantly limited in the resistant line, whereas the levels of RNA 1 and RNA 2 were the same in susceptible and resistant lines. Inhibition of RNA 3 replication would reduce the amount of movement protein available, thus decreasing cell-to-cell spread of CCMV. While this inhibition of RNA 3 replication might be a factor in CCMV cowpea resistance, the authors state that restricted virus movement is the probable mechanism of resistance (Wyatt and Kuhn, 1979) and mention the

difficultly of distinguishing between uniformly reduced virus replication in all infected cells *vs* restricted virus movement from a limited number of cells competent for high virus replication.

Southern Bean Mosaic Sobemovirus

Southern bean mosaic sobemovirus (SBMV) is an icosohedral, single-stranded, positive-sense RNA virus. The genome is covalently linked to a virally encoded protein at the 5′ end and terminates at the 3′ end with a hydroxyl group. The coat protein is translated from a subgenomic RNA (Hull, 1988).

The cowpea (SBMV-C) and bean (SBMV-B) strains of SBMV are serologically related and share many of the same properties (Ghabrial et al., 1967). They differ in their ability to infect cowpea and bean cultivars (Tremaine and Hamilton, 1983). SBMV-C is unable to accumulate in bean (*Phaseolus vulgaris* L.) whereas SBMV-B is unable to infect cowpea (*Vigna unguiculata*). Molefe et al.(1983) demonstrated that bean plants which were inoculated with SBMV-C sustained a low level of virus replication in inoculated leaves. Inoculation of isolated bean protoplasts with SBMV-C, SBMV-B or SBMV-C+SBMV-B showed that SBMV-C could replicate as well as SBMV-B (Fuentes and Hamilton, 1991). These researchers facilitated the cell-to-cell spread of SBMV-C by coinfecting cv. Bountiful bean with sunhemp mosaic tobamovirus (SHMV). However, only SHMV

was able to move systemically long distance through conductive tissues. Fuentes and Hamilton (1993) investigated the localization of SBMV-C in bean using thick and thin layer sections of bean co-infected with SHMV. They discovered SBMV-C capsid protein in the epidermal and mesophyll cells, but not in conductive tissues. No assembled SBMV virions were found in the mesophyll cells. This lack of assembly was not correlated to coat protein degradation. Coat protein isolated from bean and from SBMV-C inoculated cowpea migrated at the same rate on SDS-polyacrylamide gels. Assembled virions were apparently not essential for cell-to-cell spread of the virus, but may be required for long distance transport of the virus.

Potyviruses: General

Potyviruses are positive stranded, RNA viruses. The genome, consisting of approximately 10,000 nucleotides, is covalently linked to a virus-encoded protein (Vpg) at the 5′ end and is polyadenylated at the 3′ terminus (Riechmann et al., 1992). The genome structure consists of a single open reading frame, coding for one large polypeptide. At least nine gene products are produced from this polypeptide by the action of three virally encoded proteinases (Dougherty and Carrington, 1988; see Fig. 1). While the functions of the P1-Pro and P3 proteins are unknown, P1-Pro has been shown to posses nucleic acid binding properties, preferentially binding ssRNA (Brantly and Hunt, 1993). Helper component (HC-Pro) is

essential for aphid transmissibility and has a cis-acting proteinase activity (Pirone and Thornbury, 1983; Carrington et., 1989). P3 protein has been observed in infected cells (Rodriguez-Cerezo and Shaw, 1991), but its function remains unknown. The CI protein has *in vitro* RNA-dependent ATPase and helicase activities (Lian et al., 1990, 1991). The NIa functions as a serine-type proteinase (Pro) and is responsible for six of the polyprotein cleavages (Carrington and Dougherty, 1987; Carrington et al., 1988). The NIa protein has two domains; the VPg protein comprises the N′ terminus the NIa, whereas the Pro protein makes up the C′ terminal domain. A *cis*-cleavage event, by the NIa Pro, produces the Vpg protein, in addition to the Pro protein. The VPg is found covalently linked to the 5′ end of the viral RNA (Shahabuddin et al., 1988; Murphy et al., 1990). The NIb has been inferred, by amino acid sequence comparisons with other viral proteins, to be the RNA-dependent RNA-polymerase (Koonin, 1991). CP is the single-species coat protein subunit. By analogy to the animal picornaviruses, to which the plant potyviruses are closely related (Goldbach, 1987), potyviruses are likely to be replicated in the cytoplasm, in membrane bound complexes. Sequence homology or protein biochemical similarities between picornaviruses and potyviruses has lead to the proposal that the CI, NIa, and NIb proteins of potyviruses are involved in replication (Goldbach, 1990; Kuhn and Wimmer, 1987).

Soybean Mosaic Potyvirus

Soybean lines that contain *Rsv*, *Rsv*$_2$, or *Rsv*$_3$ dominant alleles are resistant to different strains of soybean mosaic virus (SMV; Buss et al., 1987; Kihl and Hartwig, 1979). Resistance conferred by each gene can be overcome by distinct SMV strains. Several soybean lines lose resistance at low temperatures (Mansky et al., 1991). Cell free translation of a SMV strain inhibited by gene *Rsv*, demonstrated no inhibition of viral polyprotein processing (Mansky et al., 1992). A SMV strain capable of establishing infection in *Rsv/Rsv* genotypes, was able to compliment a nonvirulent strain, allowing the latter strain to move systemically (Mansky, 1990). Nucleotide sequence comparison between two SMV strains differing in their ability to infect the *Rsv/Rsv* genotypes, revealed the greatest variation in the P3 (42 kDa) protein, followed by the P1-Pro (35 kDa) protein, CI, and HC-Pro proteins (Jayaram et al., 1992). An amino acid comparison of the two viruses revealed areas of local net charge differences in the P1-Pro and P3 proteins. Although the function of these proteins is not known, local net charge differences have been proposed to affect electrostatic interactions between a host factor and non-structural viral proteins involved in *Tm-1*-conferred resistance to TMV in tomato (Meshi et al., 1988).

Tobacco Vein Mottling Potyvirus

Tobacco vein mottling virus (TVMV) is able to infect the inoculated leaves of resistant tobacco cultivar TN 86 in an inoculum concentration dependent manner (Gibb et al., 1989). Viral coat protein and cylindrical inclusion proteins were detected in susceptible tobacco cultivar, KY 14, 5 days post-inoculation. TN 86 produced these proteins 15 days post-inoculation, and only in epidermal cells. No virus was detected in TN 86 mesophyll cells. Protoplasts from susceptible and resistance cultivars supported TVMV replication; however virus accumulation was restricted in TN 86 protoplasts. The ability of TN 86 protoplasts to support replication of TVMV-S suggests interference with cell-to-cell movement of the virus; however, the delay and reduction in replication could also play a role in Tn 86 resistance.

Potato Potyvirus Y

Several forms of resistance are found in potato cultivars possessing the *Ry* dominant gene (Barker and Harrison, 1984). Protoplasts of most potato cultivars support the synthesis of PVY. However, potato cultivars Pirola and Corine both contain the *Ry* gene and protoplasts from these cultivars do not support replication of PVY. These results suggest that cell-to-cell movement of PVY is inhibited by the *Ry* gene or the *Ry* gene product in these cultivars.

Maize Dwarf Mosaic Potyvirus

The *Mdm-1* (*Rdm-1*) locus, in maize, is responsible for resistance to maize dwarf mosaic virus (MDMV; Johnson, 1971). Maize line Pa405 develops no symptoms when inoculated with MDMV pathotypes -A or -B (Louie, 1986), although a high titer of virus is reached in Pa405 inoculated leaves (Lei, 1986). Cell-to-cell spread of the virus occurs in the inoculated leaves; however, the virus is unable to move systemically in infected plants (Lie, 1986). Initial infection foci were smaller in leaves of resistant *vs* susceptible cultivars (Lei, 1986). These findings suggest that interference with virus movement, in resistant maize plants, prevents MDMV from establishing a systemic infection.

Bean Common Mosaic Potyvirus

Virus-host interactions in plants possessing recessive resistance-conferring genes have not been investigated as thoroughly as the dominant-gene systems. Drijfhout (1978), working with diallel crosses in bean (*Phaseolus vulgaris*), defined a number of recessive genes conferring resistance to bean common mosaic potyvirus (BCMV). The *bc-u* gene is not strain-specific, but does complement strain specific, recessive genes. Strain-specific genotypes *bc-1/bc-1*^{2} and *bc-2/bc-2*^{2} are either allelic or the respective genes are tightly linked. A third gene, *bc-3*, also was defined. Resistance is

effective when bc-u occurs in conjunction with at least one of the strain specific genes. More recent work, on BCMV pathogenesis in bean containing BCMV resistance-conferring genes (Day, 1984), indicated that unlimited BCMV multiplication occurs in inoculated leaves. In some heterozygous combinations (i.e. plants possessing both dominant and recessive alleles), virus replication was reduced. Unfortunately, BCMV isolates are not yet completely cloned and sequenced, precluding attempts to investigate relationships between respective BCMV pathotype genomes and resistance-conferring *P. vulgaris* genes.

Papaya Ringspot Potyvirus

Gibb et al. (1994) investigated the interactions of papaya ringspot virus type W (PRSV-W), formerly called watermelon mosaic virus 1, with resistant *Cucumis melo* L. (muskmelon) cv Cinco. Two growing temperatures were utilized for the experiments: 25 C and 40 C. In plants grown at 25 C, only PRSV inoculated cotyledons showed mild chlorotic flecking whereas inoculated true leaves remained symptomless. However, no virus was recovered from these symptomatic cotyledons. F1 hybrids generated by a cross between Cinco and a susceptible melon was named Cinbo. Cinbo differed from Cinco in that PRSV could be recovered from inoculated cotyledons, regardless of the growing temperature. Virus was recovered from inoculated true leaves of Cinbo as well, if plants were grown at 25 C.

Perhaps the most important finding in this research was the detection of virus in both cultivars, in 3-7% of leaf mesophyll cells, in inoculated and non-inoculated leaves. This suggests that limited long distance movement was occurring. Whether this was due to reduced replication of the virus or inhibition of long distance movement is unknown. The authors speculated that a slight delay in expression of host resistance response could allow limited virus replication and/or spread.

Pea Seed-borne Mosaic Potyvirus

Three pathotypes of pea seed-borne mosaic virus (PSbMV) have been defined according to the *Pisum sativum* (pea) genes that confer resistance to each pathotype (Hagedorn and Gritton, 1973; Gritton and Hagedorn, 1975; Provvidenti and Alconero, 1988a; Provvidenti and Alconero, 1988b). Genes *sbm-1*, *sbm-3*, and *sbm-4* are all located on pea chromosome six and confer resistance to PSbMV-P1, -P2, and -P4 respectively. Gene *sbm-2*, located on chromosome 2, also confers resistance to P2. PSbMV-P1 strain DPD1 was cloned and sequenced (Johansen et al., 1991). E. Johansen followed this prior work with the cloning and sequencing of PSbMV-P4 (Johansen et al., in press). Infectious transcripts were derived from the full length clones of P1, P4, and recombinants of these two pathotypes (Johansen, personal communication). These clones

and clones constructed specifically for this study were used to investigate the interactions between PSbMV-P1 and *sbm-1*.

Chapter 3

Materials and Methods

Virus Isolates

PSbMV-P1 isolate DPD1 was kindly provided by E. Johansen (The Danish Institute of Plant and Soil Science, Biotechnology Group, Lyngby, Denmark). DPD1 was originally obtained from a *Pisum* seed sample by the Danish Plant Directorate in Lyngby, Denmark. Johansen et al. (1991) subsequently cloned and sequenced this pathotype.

PSbMV-P4 isolate NY was provided by R. Provvidenti (Cornell Univ., Geneva, New York). P4 was characterized by Paul Kohnen et al. (1995). Johansen et al. (in press) cloned and sequenced PSbMV-P4.

PSbMV-P1, -4, and P1-P4 Recombinate Clones

Full length cDNA clones of PSbMV-P1 and P1-P4 recombinants initially used in this study were provided by E. Johansen. Additional P1-P4 recombinants were provided by Johansen or were constructed by the author following site directed mutagenesis (see Kunkel mutagenesis below) for the construction of common restriction sites in the P1 and P4 genomes. The shared restriction sites allowed cDNA genomic exchanges between clones of P1 and P4 using standard cloning

procedures (Sambrook et al., 1989). All clones used, except P4 (P1 6K) and P4 (P1 VPg), were cloned downstream from a T7 promoter (Petty, 1988). T7 polymerase was then used to generate full length infectious transcripts of those clones. Johansen (personal communication) cloned full lenth P4 cDNA downstream from the cauliflower mosaic (CaMV) 35S promoter. Johansen was then able to directly inoculate susceptible peas with this clone. Using P1 and P4 mutagenized clones provided by the author, she subsequently constructed P4 (P1 6K) and P4 (P1 VPg) downstream from CaMV 35S promoter. Fig. 6 illustrates the clones used in this study.

Virus Purification

PSbMV was recovered from peas harvested 2 wk post-inoculation using the method of Alconero (1986). A total of 120 g chilled tissue was homogenized in 120 ml cold extraction buffer (0.5 M potassium phosphate, 10 mM EDTA, and 0.1% Na_2SO_4; pH 7.5), 60 ml chloroform, and 60 ml carbon tetrachloride. Supernate was clarified by spinning 10,000 g for 15 min. Supernate was then filtered through glasswool. PEG 8000 4% was dissolved in the supernate by stirring for 1 hour at 4 C. Virus was pelleted by spinning at 10,000 g for 10 min. The pellet was resuspended in 40 ml 0.02 M potassium phosphate buffer (pH 8.2) containing 0.1% 2-mercaptoethanol. CsCl 38% (wt/vol) was added to the virus suspension. The CsCl mixture was spun at 79,000 g for 20 h at 6 C. After drawing virus

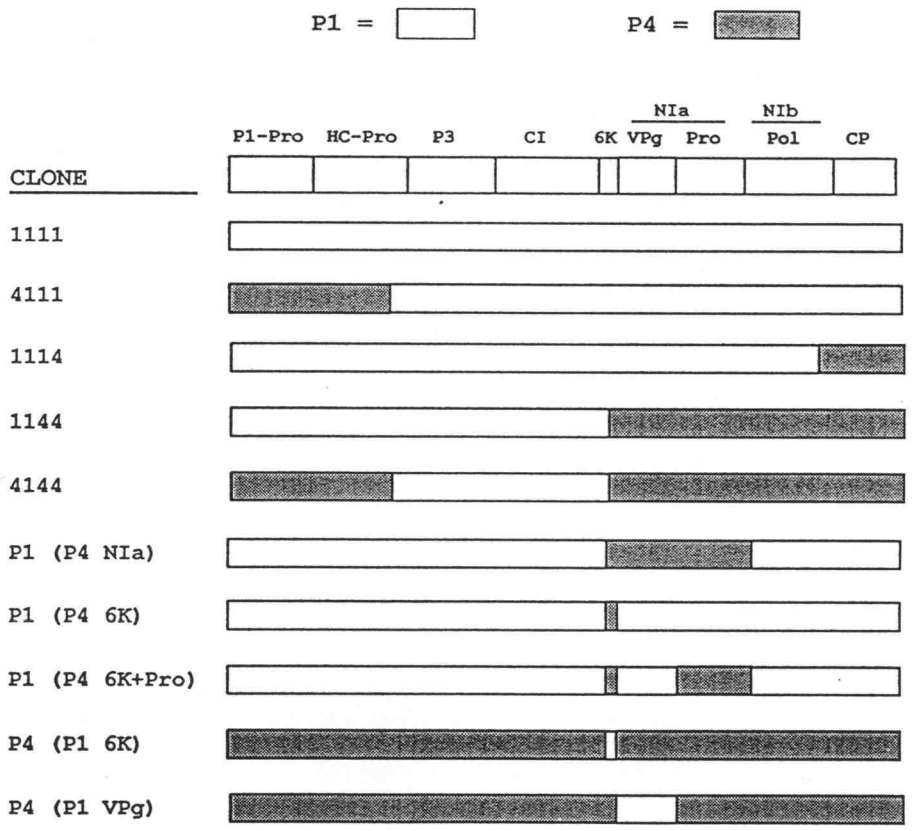


Figure 6. PSbMV P1-P4 recombinant clones used in this study to investigate P1 pathogenesis in the *sbm-1/sbm-1* PI269818 pea line.

bands from the CsCl gradients, the virus was precipitated by diluting the sample 1:1 with resuspension buffer (0.02 M potassium phosphate; pH 8.2), 0.1% 2-mercaptoethanol, and 0.6 M NaCl). PEG 8000 5% (wt/vol) was again dissolved for 1 h at 4 C. Virus was pelleted by spinning 10,000 g for 10 min. The pellet was resuspended in 0.5-1.0 ml buffer (0.02 M potassium phosphate and 0.1% 2-mercaptoethanol; pH 8.2). An extinction coefficient of 2.4 was used to calculate virus yield.

Serological Detection of PSbMV

Immuno-gamma globulin (IgG) was purified (see Appendix 1) from PSbMV polyclonal antiserum AS 4395 (provided by R.O. Hampton). IgG was conjugated to alkaline phosphatase (Appendix 1). DAS-ELISA was used to detect PSbMV-P1, P4, or P1-P4 recombinants following the method of Kohnen et al. (1995). IgG was diluted 1:1000 in coating buffer (1.59 g/L sodium carbonate, 2.93 g sodium bicarbonate, and 0.2/L g sodium azide; pH 9.6). A volume of 200 ul of IgG was added each wells of a Nunc Immunosorb ELISA plate. The plates were either incubated for 4 h at 37 C or overnight at 4 C. Plates were washed three times with wash buffer (8 g/L sodium chloride, 1.15 g/L dibasic sodium phosphate, 0.1 g/L monobasic potassium phosphate, 0.1 g/L potassium chloride, 0.1 g/L sodium azide, and 1 ml/L Tween 20; pH 7.4). Plant tissue was ground 1:10 (wt/vol) in virus buffer (20 g/L

polyvinylpyrrolidone MW=10,000 (Sigma), 2 g/L chicken egg ovalbumin (Sigma), 1 ml/L Tween 20 (Sigma), 8 g/L sodium chloride, 1.15 g/L dibasic sodium phosphate, 0.1 g/L monobasic potassium phosphate, 0.1 g/L potassium chloride, and 0.1 g/L sodium azide; pH 7.4). Plant sap was diluted 1:100 with virus buffer, before adding to the ELISA plate (200 ul/well). Plates were incubated and washed as before. PSbMV conjugate was diluted 1:5000 in virus buffer and added to the plates (200 ul/well). Incubation was performed as before. ELISA plates were again washed before the addition of 33 ug/ml *p*-nitrophenyl phosphate, disodium substrate (Sigma) in substrate buffer (97 ml diethanolamine/L distilled water (Sigma); pH 9.8) was added. Plates were allowed to develop for 1-24 h before an A_{405} value was recorded (BioTek EL309 Microplate Autoreader).

Detection of PSbMV by Reverse Transcription / Polymerase Chain Reaction (RT/PCR)

Total nucleic acid was extracted from less than 1 g of pea tissue. Tissue was frozen in liquid nitrogen, followed by grinding using a RNase free mortar and pestle. One milliliter of extraction buffer (2.0 mM Tris-HCl (pH 8.2), 2.0 mM EDTA, 2% SDS) was added to the powdered tissue along with 0.1 mg Proteinase K (Sigma). This solution was incubated at 37 C for 45 min. The solution was then extracted with an equal volume of Tris-equilibrated phenol, and the aqueous phase was

removed. This phase was combined with 0.4 volumes of 5 M ammonium acetate and 2.0 volumes of 95% ethanol. The sample was stored overnight at -20 C to precipitate the nucleic acid. The nucleic acid was centrifuged at 10,000 g for 15 min. The pellet was washed with 70% ethanol, dried under vacuum and resuspended in sterile double distilled water. The concentration of nucleic acid was quantitated spectrophotometrically at A_{260} (1 OD = 40 ug/ml).

Reverse transcription (RT) of viral RNA was performed using $\leq$ 1.0 ug total nucleic acid. The 20 ul RT reaction was performed in 50 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 1 mM each dNTP, 20 U RNasin (Promega), 50 U M-MLV reverse transcriptase (Gibco) and 100 pmoles of "downstream primer". The reaction was overlaid with 50 ul mineral oil and incubated at 42 C for 1 h. The reaction was then heated to 99 C for 5 min, followed by chilling on ice for 5 min.

The polymerase chain reaction (PCR) was carried out in the same tube. The PCR reaction (80 ul) was assembled and added to the 20 ul RT reaction, under the oil layer. The resulting 100 ul reaction consisted of 50 mM KCl, 2 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 0.2 mM dNTP, 100 pmoles of the upstream primer, and 2.5 U Taq DNA polymerase (Promega). Thirty-five cycles of amplification were performed at 94 C for 1 min., 37 C for 1 min., and 72 C for 2 min, in an Ericomp thermocycler.

PCR amplification products were resolved electorphoretically on 1% agarose gels, stained with ethidium bromide, and photographed.

Site Directed Mutagenesis Of PSbMV-P1 And -P4 Clones

Dr. V. Dolja provided the methodology and bacterial cell line RZ1032 used in the site directed mutagenesis procedure. Dolja's procedure is a modification of the Kunkel mutagenesis method (Kunkel, 1985; Kunkel et al., 1987). In addition, this study further modified the mutagenesis method. Primers containing the desired nucleotide changes were made by the Oregon State University Central Service Laboratory using an ABI Model 380A DNA synthesizer. An 165 ug/ml oligo stock solution was prepared in sterile, distilled water. The oligo was kinased [21.0 ul water, 2.5 ul stock oligo, 3.0 ul 10X polynucleotide kinase (New England Biolabs), 3.0 ul 10 mM ATP (Gibco), and 0.4 ul T4 polynucleotide kinase (New England Biolabs)] for 15 min at 37 C, followed by enzyme inactivation for 10 min at 70 C. The plasmid vector, containing the viral cDNA sequence to be mutagenized, was used to transform (Appendix 2) competent RZ1032 bacterial cells (Appendix 3). Single stranded DNA (ssDNA) complementary to the mutant oligo was prepared from this cell line (Appendix 4). The single-strand DNA concentration was adjusted to 0.5 ug/ul. The mutant oligo was annealed to ssDNA by mixing 4.0 ul ssDNA, 2.2 ul phosphorylated oligo, 1.8 ul annealing buffer (20 ul 5 M

$NaCl_2$, 10 ul 1 M Tris; pH 7.8, 4 ul 0.25 M EDTA, and 96 ul H_2O), and 4.0 ul ssDNA (0.5 ug/ul). The mixture was heated to 70 C for 3 min. The mixture was then incubated at 37 C for 30 min and finally kept on ice until the extension reaction was assembled. The extension reaction involved mixing 2.5 ul 10X T4 DNA polymerase (New England Biolabs), 9.5 ul 4 mM dNTPs (Pharmacia), 1.4 ul 10X BSA (New England Biolabs), 1.6 ul water, and 2.75 ul 10 mM ATP (Gibco), 0.75 ul T4 DNA polymerase (New England Biolabs), and 0.75 ul T4 DNA ligase (Promega). This mixture was held on ice for 5 min, then brought to room temperature for 5 min, incubated at 37 C for 2 h, followed by incubation overnight at 16 C. The extension reaction was used to transform competent TG-1 cells (cell line was obtained from E. Johansen). Transformed cells were CTAB mini-prepped (Appendix 5). Mini-prep DNA was digested with the appropriate restriction endonuclease enzyme to verify insertion of new restriction site. Restriction mapping was used to verify correct viral cDNA size and identity.

Preparation, Transfection, and Analysis of Protoplasts

Pea protoplasts were prepared using a modification of the methods of Demler, et al. (1993), Loesch-Fries and Hall (1980) and de Faria and de Zoeten (1986). Modifications were proposed and tested by Dr. Paul Kohnen. Six-to-8 day-old-pea seedlings were dark-conditioned for 24 h. Harvested leaves

were surface sterilized for 1 min in 70% ethanol, containing 150 ppm Tween 20. Leaves were then soaked for 6 minutes in 10% bleach, containing 450 ppm Tween 20. Leaves were rinsed five times in sterile distilled water. Leaves were cut into 2-3 mm strips and digested in 50 ml enzymatic mixture [20 mg/ml Macerase (Calbiochem), 1 mg/ml Cellulase R-10 (Yakult Honsha Co.), and 1 mg/ml BSA (Sigma) in 10% mannitol]. Digestion mixture was agitated 120 rotations/minute, at 28 C, for 1/4 h. Protoplasts were strained through two layers of sterile cheese cloth followed by 25 g centrifugation for 3 min. Protoplasts were resuspended in 8 ml 10 % mannitol. This rinsing procedure (centrifugation and resuspension of protoplasts in mannitol) was repeated two more times. After spinning protoplasts for a fourth time, the pellet was resuspended in 300 ul of 10% mannitol. Viral RNA (70 ug) was added to the protoplast mixture. A 40% PEG (Boehringer Mannheim) solution containing 3 mM $CaCl_2$ was added to the protoplast suspension. After mixing the solution for 10 sec, 10 ml 10% mannitol were added. The mixture was held at 4 C for 20 min. Protoplasts were rinsed twice in 8 ml 10% mannitol and resuspended in 12 ml incubation medium (10% mannitol, 0.2 mM KH_2PO_4, 1.0 mM KNO_3, 1.0 mM $MgSO_4$, 1.0 uM KI, 0.1 mM $CuSO_4$, 10 mM $CaCl_2$, 10 ug/ml gentamicin sulfate (Sigma), pH 6.5). Protoplasts were allowed to incubate for 3-4 days at approximately 22-28 C. Cells were pelleted as before. The protoplast pellet was resuspended in 800 ul of ELISA antigen

buffer (0.5 M KH_2PO_4/Na_2HPO_4, 150 mM $NaCl_2$, and 1.0 mM sodium diethyl-dithiocarbamate (NaDIECA; Sigma), pH 7.2). The resuspended cells were macerated by mortar and pestle. Macerate was added to a Nunc-Immuno Maxisorb ELISA plate and allowed to incubate overnight at 4 C. The plate was rinsed three times with wash buffer (8 g/L sodium chloride, 1.15 g/L dibasic sodium phosphate, 0.1 g/L monobasic potassium phosphate, 0.1 g/L potassium chloride, 0.1 g/L sodium azide, and 1 ml/L Tween 20; pH 7.4). PSbMV IgG derived from PSbMV AS 4395 (Hampton, personal communication) diluted 1:1000 in virus buffer (20 g/L polyvinylpyrrolidone MW=10,000 (Sigma), 2 g/L chicken egg ovalbumin (Sigma), 1 ml/L Tween 20 (Sigma), 8 g/L sodium chloride, 1.15 g/L dibasic sodium phosphate, 0.1 g/L monobasic potassium phosphate, 0.1 g/L potassium chloride, and 0.1 g/L sodium azide; pH 7.4) containing 1:50 wt/vol healthy plants sap, was added to the plate. The plate was incubated at 37 C for 2 h. After washing the plate as before, goat antirabbit alkaline phosphatase conjugate (Sigma) was added at a concentration of 1:5000 in virus buffer and allowed to incubate for 2 h at 37 C. Following a final plate wash, 33 ug/ml *p*-nitrophenyl phosphate, disodium substrate (Sigma) in substrate buffer (97 ml diethanolamine/L distilled water (Sigma); pH 9.8) were added. The plate was allowed to develop for 8 h before an A_{405} value was taken (BioTek EL309 Microplate Autoreader).

In vitro Transcription of Full Length PSbMV Clones and Inoculation of EF 680 Pea Plants

Transcripts were generated using Ambion's mMessage mMachine *in vitro* transcription kit. Template DNA was cut with Xba I, a unique restriction site in each clone, located at the 3′ terminus of the genome. Template DNA was treated with ProK (100-200 ug/ml; Ambion) and SDS (0.5%) for 30-60 min at 50 C, followed by phenol:chloroform extraction. A 1/10 volume of 0.25 M EDTA, 1/10 volume of 5 M ammonium acetate and two volumes of EtOH were added to the template mixture and chilled -20 C for 15 min. The DNA template was pelleted and resuspended in dH2O at a concentration of 0.5 mg/ml. The 20 ul transcription reaction was assembled at room temperature. The reaction included 4 ul RNAse free water, 2.0 ul 10X transcription buffer, 10.0 ul 2X ribonucleotide mix, 2.0 ul template DNA, and 2 ul of enzyme solution. The reaction was incubated at 37 C for 1-2 h. The reaction efficiency and transcript integrity was visualized by electrophoresing 1 ul of the 20 ul reaction through 1% agarose gel, staining with ethidium bromide and visualizing the gel under a UV light source. The reaction was stopped by the addition of 80 ul RNase free water and 100 ul 6 mM potassium phosphate buffer (pH 8.2).

Six-to-8-day-old-EF680 pea plants, greenhouse grown at 78/72 C daytime/nighttime temperatures under 12 h/day light conditions, were lightly dusted with carborundum. Each leaf

set was inoculated with RNA transcripts by gently abrading the leaf surface with 20 ul of the transcription reaction. Pipet tips and gloves were changed after each leaf was inoculated to prevent possible RNase degradation of transcripts. Plants were ELISA tested 6-14 days after inoculation for infectivity of transcripts.

Chapter 4

Results

Screening Parental PSbMV-P1, -P4, and P1-P4 Recombinant (1111, 1114, 1144, 4144, and 4111) Viruses for Ability to Infect PI269818, PI193586, cv. Bonneville, and cv. EF680 Pea Lines

After establishing infectious cDNA derived transcripts, cloned and parental viruses were maintained in cv. Early Freezer 680 (EF680) pea as inoculum sources for pea genotype resistance screening.

The two lowest leaf sets of Bonneville (*Sbm-1/Sbm-1)*, PI269818 *(sbm-1/sbm-1)*, and PI193586 (*sbm-1/sbm-1, sbm-4/sbm-4)* were inoculated with tissue extracts from infected EF680 peas. The highest inoculated leaf set and the upper adjacent noninoculated leaf set were sampled for ELISA tests. Virus inoculated and noninoculated leaves of all genotypes were sampled 14 days post-inoculation. ELISA results of the initial screening of all three genotypes are presented in Table 1. Absorbance (A_{405}) values were recorded 1 h after the addition of substrate. Neither recombinant nor parental viruses were able to infect PI193586 whereas all viruses were infectious in Bonneville, verifying trueness-to-type for both parent viruses (P1 and P4) and the P1-P4 recombinants. Only recombinants 1144 and 4144, in addition to P4, had the ability to infect PI269818, implicating the 3rd segment of P4 as the

Table 1. Parental and recombinant virus screening of susceptible and resistant *Pisum sativum* lines.

	ELISA VALUES[a]					
	Noninoculated Leaves			Inoculated Leaves		
Inoculum[b]	P1269818[c]	PI193586[d]	Bonneville[e]	PI269818[c]	PI193586[d]	Bonneville[e]
P1	-0.002	0.001	0.323	0.013	0.004	0.377
1111	-0.003	-0.004	0.376	0.001	0.016	0.494
1114	0.004	-0.004	0.090	-0.003	-0.004	0.320
1144	0.429	0.000	0.430	0.061	0.000	0.449
4144	0.738	0.000	0.870	0.471	0.014	0.511
4111	-0.004	0.000	0.730	0.005	0.007	0.451
P4	0.378	0.000	0.261	0.006	0.002	0.446
Mock Inoculated	0.002	-0.004	-0.002	-0.004	-0.006	-0.002

[a] Absorbance (A_{405}) value average of two replicate ELISA wells. ELISA A_{405} values were recorded 1 hour after the addition of substrate.

[b] Macerated leaves from EF680 plants infected with either parental or recombinant virus served as the inoculum source.

[c] PI269818 is homozygous recessive for the *sbm-1* gene.

[d] PI193586 is homozygous recessive for *sbm-1* and *sbm-4* genes.

[e] Bonneville contains dominant genes for *Sbm-1* and *Sbm-4*.

determinant of P4 pathogenicity in the P1-immune genotype PI269818. P4, 1144, and 4144 inoculated leaves had low A_{405} values relative to noninoculated leaves.

A subsequent experiment was confined to PI269818 and a susceptible control, EF680. This experiment was designed to investigate restricted movement or reduced replication in inoculated leaves. Inoculated and non-inoculated leaves of PI269818 and EF680 were compared (Table 2) using the same procedure described above. Both 1 and 2 h absorbance values were recorded. Unexplainably, inoculated leaves of PI269818 had a higher absorbance value in the 21-h recording then inoculated leaves of the EF680 susceptible control. In an attempt to understand the significance these results, additional experiments were undertaken.

Differentiating Constitutive *vs* Delayed/Induced *sbm-1/sbm-1* Resistance and Investigation of Viral Uncoating Inhibition in P1 Virus- and RNA-Inoculated PI269818 Leaves

A time line of the infection process was established for P1-inoculated PI269818 and EF680 plants. The second fully expanded leaf set on 6-day-old PI269818 and EF680 plants were inoculated with extracts from P1-infected EF680 or with purified P1 RNA. P1 RNA concentration was 2 ug RNA/20 ul 3 mM sodium phosphate buffer (pH 9.0). Each leaf set was inoculated with 20 ul RNA. An inoculated leaf set was sampled from two individual plants and combined to form one ELISA

Table 2. ELISA data of PI269818 and EF680 PSbMV-P1,-P4, or P1-P4 recombinant virus inoculated and noninoculated *Pisum sativum* leaves.

	ELISA VALUES[a]							
	1 HOUR READING				21 HOUR READING			
	Non-Inoculated Leaves		Inoculated Leaves		Non-inoculated Leaves		Inoculated Leaves	
Inoculum[b]	PI269818[c]	EF680[d]	PI269818[c]	EF680[d]	PI269818[c]	EF680[d]	PI269818[c]	EF680[d]
P1	-0.005	0.454	0.002	0.003	0.027	>2.500[e]	0.221	0.045
1111	0.001	0.518	0.011	-0.007	0.046	>2.500[e]	0.241	0.032
1114	0.000	0.102.	0.005	0.002	0.052	2.313	0.172	0.016
1144	0.528	0.568	0.514	-0.003	>2.500[e]	>2.500[e]	>2.500[d]	-0.003
4144	0.782	0.468	0.410	-0.002	>2.500[e]	>2.500[e]	>2.500[d]	-0.007
4111	-0.006	0.827	-0.002	-0.008	0.046	>2.500[e]	0.191	0.098
P4	0.257	0.482	0.229	-0.002	>2.500[e]	2.413	>2.500	0.038
Mock Inoculated	-0.002	0.003	0.004	-0.002	0.011	0.000	0.034	-0.007

[a] The A_{405} values were recorded 1 and 21 h after the addition of substrate. Values reflect averaged A_{405} values recorded for five plants and two ELISA well repetitions per plant. The EF680 values are two averaged well repetitions from one sampled plant.

[b] Inoculum was parental or recombinant virus from EF680 leaf extracts.

[c] PI269818 is homozygous, recessive for *sbm-1*.

[d] EF680 is dominant for *Sbm-1*.

[e] >2.500 indicates that the absorbance (A_{405}) values exceeded the plate reader upper recording limit, A_{405}=2.5.

sample. Sampling began 24 h post-inoculation and continued for an additional eight sampling periods (Table 3). The first evidence of PSbMV-P1 in virus-inoculated EF680 leaves occurred at 24 to 48 h post-inoculation. Regardless of sampling time, no virus was detected in P1 virus inoculated PI269818 leaves. Lack of detectible virus in PI269818 inoculated leaves demonstrated that the *sbm-1/sbm-1* resistance is likely to be constitutively expressed in the PI269818 genotype. P1 RNA-inoculated EF680 leaves did not demonstrate detectable infection until 6 days post-inoculation. The delayed response to RNA inoculation could be related to experimental inoculum dosage. Virus inoculum, derived from P1-infected EF680 extracts, was not quantitated but probably had a higher initial concentration of P1 nucleic acid relative to the RNA inoculum.

Non-recovery of PSbMV-P1 From Inoculated PI269818 Leaves

P1-infected EF680 leaves were macerated and used to inoculate PI269818 plants. Six days post-inoculation, PI269818 inoculated leaves were macerated and used to back inoculate healthy EF680 plants. DAS-ELISA was used to screen the bioassay EF680 plants for P1 infection 12 days post-inoculation. No virus was detected in any of the EF680 bioassay plants, confirming evidence from prior experiments that neither localized nor systemic infection by PSbMV-P1 occurred in PI269818.

Table 3. ELISA results demonstrating constitutive *vs* delayed/induced *sbm-1/sbm-1* resistance and investigation of uncoating inhibition in P1 virus- and RNA-inoculated PI269818 peas.

	ELISA VALUES[a]			
	RNA Inoculum[c]		Virus Inoculum[d]	
Sampling time[b]	PI269818[e]	EF680[f]	PI269818[e]	EF680[f]
24	0.000	0.003	0.022	0.046
48	-0.005	0.002	0.009	0.170
72	0.003	0.000	0.008	0.468
120	0.000	0.008	-0.004	1.908
144	-0.006	0.136	0.002	1.909
216	0.000	0.366	-0.006	2.417
264	-0.006	0.688	0.000	2.413
288	-0.005	1.106	0.000	2.366
360	0.002	1.719	0.003	2.482

[a] A_{405} values were recorded 27 h after the addition of substrate. Duplicate wells were averaged. values.
[b] Hours post-inoculation.
[c] PSbMV-P1 RNA (2 ug/20 ul).
[d] Extract from PSbMV-P1 infected EF680 tissues.
[e] PI269818 pea line is homozygous recessive for *sbm-1*.
[f] EF680 pea line is dominant for *Sbm-1*.

Grafts of Healthy PI269818 Scions and PSbMV-P1, -P4, or P1-P4 Recombinant Virus Infected EF680 Stocks

PI269818 scions were grafted onto EF680 stocks. EF680 leaves were inoculated with parental or recombinant viruses at the time of grafting. DAS-ELISA was performed on the stock leaves to assure a valid inoculum source for graft transmission and appropriately test PI269818 susceptibility to P1 by this inoculation mode (Table 4). ELISA results confirmed that all EF680 stocks were infected with parental or recombinant viruses. Previous tests indicated that mechanical inoculation of PI269818 with PSbMV-P4, 1144, and 4144 led to a systemic infection, and for this reason these viruses were included as experimental controls of the graft-transmission experiment. These viruses were unable to produce systemic infection in the PI269818 scion.

RT-PCR Detection of PSbMV-P1 or -P4 Nucleic Acid in EF680, PI193586, and PI269818 Inoculated Leaves

PSbMV-P1 or -P4 infected EF680 tissue extract was used to inoculate PI269818, PI193586, and EF680 pea genotypes. Inoculated leaves were sampled 7 days post inoculation. P1- or P4- specific primers (Kohnen et al., 1992; 1995) were used according to the kind of inoculum applied. P1 and P4 infected EF680 leaves served as positive controls and noninoculated EF680 leaves provided the negative control. P1 and P4 inoculated EF680 plants generated amplification products of

Table 4. Graft transmission of pea seed-borne mosaic virus pathotype P1, P4, or P1-P4 recombinants from infected EF680 stocks to *Pisum sativum* PI269818 scions.

	ELISA RESULTS[a]	
Inoculum[b]	PI269818 Scion[c] (# infected scions/ # established grafts)	EF680 Stock[d] (# infected stocks/ # established grafts)
P1	0 / 3	3 / 3
1111	0 / 1	1 / 1
4111	0 / 1	1 / 1
1114	0 / 3	3 / 3
1144	0 / 3	3 / 3
4144	0 / 3	3 / 3
P4	0 / 3	3 / 3

[a] DAS-ELISA was used to verify the presence or absence of virus in both scions and stocks. Results are given as ratios; the number of infected scions or stocks to the number of established grafts.
[b] Extract from infected EF680 peas served as inoculum.
[c] PI269818 pea line is homozygous recessive for the *sbm-1* gene.
[d] EF680 pea line is dominant for the *Sbm-1* gene.

the predicted size, thus validating RT-PCR test accuracy (Table 5). Non-inoculated EF680 produced no product(s) from either primer pair, confirming the specificity of the primers for recognition of P1 or P4 RNA, respectively. P4-inoculated PI269818 plants produced a product identical in size to the product generated from P4 inoculated EF680. No product was detected from P1 inoculated PI269818. Neither P1 nor P4 inoculated PI193586 generated amplification products, demonstrating that no specific RNA was detectable in inoculated leaves and that this genotype is immune to both viruses. DAS-ELISA had been used in previous experiments for detection of viral coat protein. RT-PCR has been demonstrated to detect PSbMV concentrations in pea at 2.5- to 10-times lower concentrations than DAS-ELISA (Kohnen et al., 1992). The inability to detect P1 nucleic acid in the *sbm-1/sbm-1* genotype using this highly sensitive assay provides stronger evidence that complete inhibition of PSbMV-P1 replication is imposed by this genotype. Likewise, the lack of detectable viral nucleic acid in P1 or P4 inoculated PI193586 leaves further supports this conclusion.

PSbMV Infectivity Complementation of PSbMV-P1 by -P4 in PI269818 Peas

PI269818 plants were inoculated with PSbMV-P4. Seven days later the same leaves were inoculated with PSbMV-P1. Alternatively, PI269818 leaves were inoculated with P1 and P4

Table 5. Detection of pea seed-borne mosaic virus pathotype P1 or P4 in EF680, PI193586 and PI269818 inoculated leaves by RT-PCR amplification.

	Inoculum[a]	
Pea Genotype[b]	PSbMV-P1	PSbMV-P4
EF680	+	+
PI269818	-	+
PI193586	-	-

[a] Extract from PSbMV-P1 or -4 infected EF680 plants served as inoculum.

[b] EF680 pea line is dominant for both *Sbm-1* and *Sbm-4* genes. PI269818 pea line is homozygous recessive for the *sbm-1* gene. PI193586 is homozygous recessive for both *sbm-1* and *sbm-4* genes.

simultaneously. Each treatment included three plants. In all cases, P1- or P4-infected EF680 tissues served as inoculum sources. Both inoculated and noninoculated leaves were tested for the presence of P1 by RT-PCR 2 wk post-inoculation, using P1 specific primers (Kohnen et al., 1992). P1 was not detectable by RT-PCR in either inoculated or noninoculated leaves, whether inoculated after P4 or simultaneously with P4 (Table 6). P1-infected EF680 served as the positive control for the RT-PCR test, amplifying the expected P1 specific fragment size. The RT-PCR negative control, healthy EF680, was free of amplification product.

Analysis of EF680 and PI269818 Protoplasts Transfected with PSbMV-P1 RNA

Protoplasts isolated from EF680 and PI269818 were transfected with 70 ug PSbMV-P1 RNA. An equivalent number of non-transfected protoplast served as negative controls. The first protoplast isolation yielded 116 K cells of EF680 and 52 K cells of PI269818 for each treatment. The second isolation resulted in 56 K cells of EF680 and 42 K cells of PI269818 per treatment. Cells from the first isolation were allowed to incubate for 3 days post-transfection. Cells from the second isolation were incubated for 4 days before cells were harvested and assayed. Indirect ELISA results from the two individual protoplast isolations and transfections demonstrated that P1 was detectable in EF680 protoplasts in

Table 6. PSbMV-P1 RT-PCR amplification of P1-P4 inoculated PI269818 leaves.

	PI269818 Leaves	
	Inoculated[a,b]	Noninoculated[c]
PSbMV-P1 staggered inoculation with -P4[a]	-	-
PSbMV-P1 simultaneous inoculation with -P4[b]	-	-
P1 infected EF680[c]	+	+
Healthy EF680[c]	-	-

[a] PI269818 pea leaves were inoculated with extract from EF680 PSbMV-P4 infected peas, followed by inoculation with PSbMV-P1 infected EF680 extract six days later.

[b] Both PSbMV-P1 and -P4 infected EF680 pea extracts were used to simultaneously inoculate PI269818 peas.

[c] PSbMV-P1 infected EF680 peas served as the positive control for the assay, whereas noninoculated PI269818 and EF680 peas were used to verify lack of nonviral amplification.

Table 7. Indirect ELISA results obtained from PSbMV-P1 RNA transfected or non-transfected EF680 and PI269818 protoplasts.

ELISA RESULTS OF PROTOPLAST TRANSFECTION[a]

Protoplast Isolation 1[b]			
PI269818[c]/+RNA[g] (52,000)[d]	PI269818[c]/-RNA (52,000)[d]	EF680[f]/+RNA[g] (116,000)[d]	EF680[f]/RNA (116,000)[d]
-0.008	-0.001	0.401	0.015

Protoplast Isolation 2[c]			
PI269818[c]/+RNA[g] (42,000)[d]	PI269818[c]/-RNA (46,000)[d]	EF680[f]/+RNA[g] (56,000)[d]	EF680[f]/-RNA (56,000)[d]
0.008	0.003	0.432	0.024

[a] A_{405} values were recorded 8 h after the addition of substrate.
[b] Protoplast isolation 1 incubated 3 days post-transfection.
[c] Protoplast isolation 2 incubated 4 days post-transfection.
[d] The number of cells transfected in each treatment. The number of cells per ELISA plate well are one half this number, due to replication of ELISA samples. A_{405} values reflect an average of these 2 replications.
[e] PI269818 pea line is homozygous recessive for the *sbm-1* gene.
[f] EF680 pea line is dominant for the *Sbm-1* gene.
[g] PSbMV-P1 RNA (70 ug/transfection) was used for transfection of protoplasts.

both trials (Table 7), whereas PI269818 protoplasts contained no detectable virus in either trial (3- or 4-day incubation interval). Viral RNA (50 ug/ml) included in the ELISA test demonstrated that the inoculum contained no detectable coat protein contamination. Previous experiments have shown that PI269818 leaves are unable to support replication of PSbMV-P1.

Identification of the PSbMV-P1 Genomic Region Responsible for Loss of Pathogenicity in the PI269818 *Pisum sativum sbm-1/sbm-1* Genotype

Parental and recombinant viruses were screened for the ability to infect EF680, PI269818, and PI193586 (Fig. 7). All three genotypes were screened for virus presence by DAS-ELISA. There were no cases of P1, P4 or recombinant virus infection in PI193586. Inoculated EF680 contained ELISA detectable virus in all cases. Only recombinant viruses containing P4 NIa [1144, 4144, P1 (P4 NIa), and P4 (P1 6K)] were infectious to PI269818. Substitution of P1 VPg with P4 VPg resulted in a recombinant virus [P4 (P1 VPg)] that was unable to infect PI269818 while maintaining the ability to infect EF680.

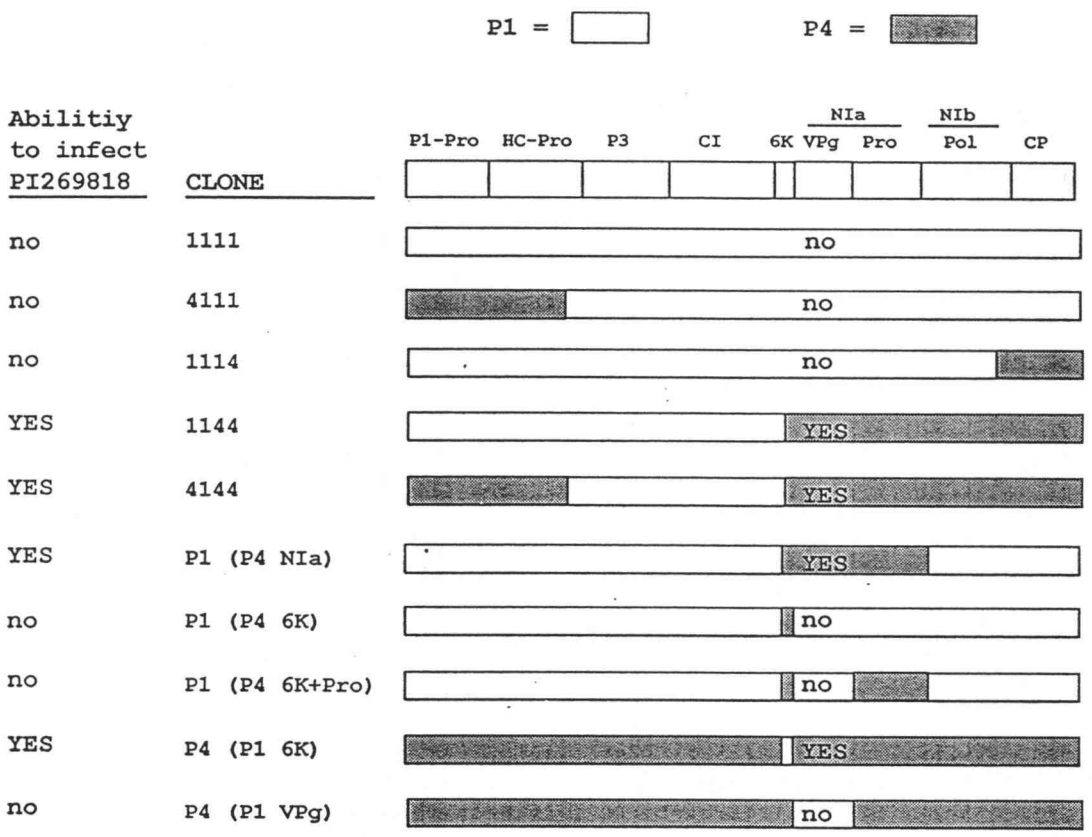


Figure 7. Diagrammatic representation of the pea seed-borne mosaic virus recombinant clones used in this study. DAS-ELISA was used to identify those clones that had the ability to infect PI269818. The P4 VPg confers the capability to infect this *sbm-1/sbm-1* genotype.

Chapter 5

Discussion

Replication of RNA viruses within a host involves four steps that may overlap chronologically; decapsidation, translation of the viral RNA, replication of the viral genome, and encapsidation of progeny RNA strands. Because PSbMV-P1 RNA was unable to establish either a local or systemic infection in the *sbm-1/sbm-1* genotype, it is our hypothesis that decapsidation is not the mechanism of immunity to P1 in this genotype.

Although PSbMV-P1 inoculated PI269818 leaves consistently had higher A_{405} ELISA values compared to EF680 P1 inoculated leaves, this might be explained by senesence of EF680 inoculated leaves, a typical response to inoculation. Neither viral RNA nor coat protein was detected in PSbMV-P1 inoculated or noninoculated leaves of PI269818 (*sbm-1/sbm-1*). Non-detection of virus in P1-inoculated PI269818 leaves could be the result of inhibited cell-to-cell movement from initially infected cells. Our attempt to separate long-distance viral movement from cell-to-cell movement by grafting healthy PI269818 scions with parental and recombinant PSbMV infected EF680 stocks was unsuccessful. It is not known how actively growing PI269818 scions, sharing vascular continuity with virus infected EF680 stocks, could exclude pathogenically competent viruses. However, the inablility of P1, 1111, 4111,

and 1114 viruses to systemically infect PI269818 scions is presumably unrelated to the action of *sbm-1* gene. Because protoplasts isolated from PI269818 do not support replication of P1, demonstrating that *sbm-1/sbm-1* resistance is effective against PSbMV-P1 not only on a whole plant basis but at the single cell level as well, this study surmises that inhibition of movement is not the cause of immunity to PSbMV-P1 infection in the *sbm-1/sbm-1* genotype. The success of P1 replication in EF680 protoplasts and a complete absence of replication in PI269818 protoplasts suggest that an early event (i.e. translation and replication) in PSbMV-P1 virus life cycle is disrupted by this genotype.

This study defined the VPg coding region as the determinant of pathogenicity in PI269818; infectious clones containing P1 VPg [1111, 4111, 1114, P1 (P4 6K), and P1 (P4 6K+Pro), and P4 (P1 VPg)] caused loss of infectivity whereas clones containing the P4 VPg [1144, 4144, P1 (P4 NIa), and P4 (P1 6K)] conferred pathogenicity in this genotype. In addition, the P4 VPg must be supplied in *cis*, demonstrated by the inability of PSbMV-P4 to provide P1 the capacity to infect the *sbm-1/sbm-1* genotype in a dual inoculation.

Murphy (1990) demonstrated that the TEV VPg was encoded for by the amino-terminal half of the 49-kDa protease coding region. Murphy et al. (1991) defined Tyr-1860 of the TVMV polyprotein as the residue covalently linked with the 5′ terminus of viral RNA. It is unknown whether the VPg

precursor (49-kDa protease) can attach to viral RNA. Although the specific form of this linkage is unknown, in the closely related picornaviruses the linkage is an O^4-(5'uridyl)tyrosine monophosphate linkage (Rothberg et al., 1978). The linking tyrosine is found in a conserved motif of NMYG (Murphy et al., 1991). PSbMV contains a similar sequence, NVYG, which is conserved between both P1 and P4 pathotypes, and thus is probably not a differential factor in P4 VPg conferred pathogenicity.

The role of VPg in translation and replication of the potyvirus genome is not well understood, but based on close similarities between potyvirus genome structure and translational strategy with other closely related viruses (i.e. picorna- and comoviruses) we can derive clues about the function of potyviral VPg. VPg covalent attachment to the viral RNA is not necessary for poliovirus infectivity (Rueckert, 1985). The effect of the 5' VPg on potyvirus infectivity is uncertain. Hari (1981) demonstrated reduced infectivity of tobacco etch potyvirus (TEV) RNA that was treated with protease: however, Riechmann et al. (1989) showed that plum pox potyvirus (PPY) was unaffected by the same treatment. This study and others (Riechmann, 1990) demonstrated that addition of an eukaryotic cap structure to *in vitro* derived transcripts was sufficient for infectivity, indicating that the VPg structure itself is dispensable for translation. Riechmann (1991) proposed a leaky scanning model

for PPV translational initiation. In this model ribosomes bind the 5′ UTR of viral RNA, scanning the RNA until the AUG initiation codon is reached, whereupon translation of the polyprotein begins. Carrington and Freed (1990) demonstrated that sequences within the 5′UTR served as cap-independent enhancers of TEV translation, providing further evidence that the potyviral VPg is dispensable for translation. This study does not disprove a role for P1 VPg in translational interference in PI269818 immunity to P1, however it seems unlikely, given our current knowledge of potyvirus translation.

Analogous to (+) ssRNA bacteriophages, (+) ssRNA viruses must replicate their genomes by first synthesizing (-) RNA on the genomic (+) ssRNA template. This (-) strand then serves as the template for progeny (+) genomic RNA. Many RNA viruses, in addition to potyviruses, contain a protein (VPg) covalently linked to the 5′terminus of the genomic RNA (Vartapetian and Bogdanov, 1987). VPg may serve as a primer for viral RNA replication (Shahabuddin et al., 1988) as in the proposed function of poliovirus VPg (Takeda et al., 1986). However, Takeda (1986) was unable to detect free poliovirus VPg-p-U (the proposed primer for replication) *in vivo*, nor has uridylation of VPg or its 60-kDa precursor been observed. Takeda (1986) did achieve *in vitro* uridylation of poliovirus VPg to VPg-pUpU, which in turn could be extended to longer RNA molecules. This work is the foundation for the model of VPg

priming of (+) and (-) strand RNA. Tobin et al. (1989) proposed that the VPg of poliovirus may be involved in cleavage of viral RNA replicative form, leading to self-catalyzed linkage of VPg to poliovirus RNA. Both (+) and (-) strand RNA of CPMV are covalently linked to VPg (Lomonossoff et al., 1985). This has led to the hypothesis that VPg is involved in an early stage of comovirus replication. CPMV replication has been proposed to occur in tight linkage to polyprotein processing (Eggen, and Van Kammen, 1988). In this model, the proteinase-polymerase and membrane binding protein-VPg or mem-VPg-Pro-pol proteins are tightly bound to cytoplasmic membranes in conjunction with a host factor. This structure would bind viral template RNA and initiate RNA replication. The proteinase would cleave the VPg and polymerase from the polyprotein. Viral RNA-dependent RNA-polymerase would then begin replication, using bound viral RNA as a template. The 5′ and 3′ termini of CPMV are capable of forming a hairpin loop upon addition of nucleotides to the 3′ terminus of the template. The VPg is proposed to perform a nucleophilic attack at this hairpin junction, simultaneously cleaving the hairpin and undergoing covalent attachment to the 5′ terminus of the new RNA strand. Many potyviruses contain a high content of aromatic amino acids (Tyr and Phe), Gly, and basic amino acids at the N-terminus of the 49 kDa protein (Lain, 1990). Query et al. (1989) illustrated this type of consensus to be typical of proteins that interact with RNA.

It is interesting to note that a ribonucleoprotein complex, composed of a cellular protein and two poliovirus proteins ($3C^{pro}$ and $3D^{pol}$) equivalent to potyviral 49 kDa (uncleaved Vpg and Pro) and polymerase, forms around the 5′ terminus of poliovirus RNA and is required for positive-strand RNA replication but is dispensable for negative-strand synthesis (Andino et al., 1990).

Dougherty and Parks (1991) identified the 49 kDa protease cleavage site between the VPg and Pro domains. The site is a sub-optimal cleavage site (Dougherty and Parks, 1989) and its cleavage probably represents a step in the viral RNA replication process. Potyviruses, unable to regulate their gene expression differentially at the levels of transcription and translation, may have evolved a method by which expression of gene products is controlled by sequential proteolytic events (Dougherty et al., 1989a). PSbMV-P1 and P-4 differ slightly at this cleavage site, however the amino acid differences are at non-essential sites (Dougherty et al., 1989a), leading us to conclude that these amino acid differences are probably unrelated to VPg pathogenicity in the *sbm-1/sbm-1* genotype.

This study does not define the region within the P1 VPg region that influences P1 pathogenicity in the *sbm-1/sbm-1* genotype. While there are conserved regions in the VPg coding region, between the two pathotypes, two areas differing in hydrophobicity occur. These areas are located between amino

acid residues 100-120 and again at the C terminus of the two isolates. What effect, if any, these differing regions have on P1-P4 VPg pathogenicity in the *sbm-1/sbm-1* genotype is unknown.

A minority of plant species derive resistance to viruses from homozygous recessive genes. This form of resistance might be explained by the lack of a recognizable host component, which is necessary for successful viral pathogenesis. Lack of a necessary host component would be consistent with our findings in this study; no detectable PSbMV-P1 RNA or coat protein in the P1 inoculated *sbm-1/sbm-1* genotype. Future work to define the exact region of P1 VPg influencing pathogenicity in this genotype would be of interest. More exciting work will involve discerning the exact point in replication in which the *sbm-1/sbm-1* gene interacts directly or indirectly with P1 VPg, resulting in blocked replication and total immunity to PSbMV-P1 infectivity. And most important perhaps, developing a better understanding of mechanisms by which viruses invade and replicate in their specific host.

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Appendices

Appendix 1

Preparation of IgG and Conjugate for ELISA

Protocol from Laboratory of R.O. Hampton

Preparation of IgG

Combine 1.2 ml whole antiserum with 1.2 ml water and add 2.4 ml 36% sodium sulfate. Vortex and let stand 10 min at room temperature. Centrifuge 12,000Xg for 15 min at 22 C. Add 10 ml 18% sodum sulfate to pellet, vortex and centrifuge as before. Resuspend pellet in 1 ml PBS and dialyze 24 hr at 4 C periodically changing dialysis buffer. Spectorphotometrically estimate IgG concentration (A_{280}=1.4) and adjust to 1 mg/ml. Determine appropriate dilution (usually 1000 fold) by ELISA evaluation.

Preparation of Alkaline Phosphatase Conjugated IgG

Add 1000 units lyophilized calf intestine alkaline phosphatase (Sigma) to 0.5 ml IgG (2 mg/ml) and vortex. Dialyze against PBS overnight at 4 C, changing buffer periodically. Measure final volume and, in a glass tube, add glutaraldehyde to a concentration of 0.2%. Vortex and dialyze as before. Determine optimal dilution (generally 1000-5000 fold) by ELISA evaluation.

Appendix 2

Preparation of Competent Cells for Long Term Storage

Protocol from Elisabeth Johansen

Streak cells on LB plate (or proper selection media if screening for F') and grow overnight 37 C. With a toothpick, select a couple of colonies to inoculate 5 mls of LB. Grow overnight 37 C, 250 rpm. Inoculate 100 mls of SOB broth (2% trypton, .5% yeast extract, 10 mM $NaCl_2$, 2.5 mM KCl; autoclave the above then add the following sterile filtered reagents to the following concentrations of 10 mM $MgCl_2$ and 10 mM $MgSO_4$.) with 500 ul overnight culture and grow for 4 h, 37 C, 250 rpm. Pour cells into a GSA bottle and spin 2500 rpm, 15 min., 4 C. Remove all liquid. Gently resuspend cells in 30 ml cold RF1 buffer [100 mM RbCl, 50 mM $MnCl_2(4H_2O)$, 10 mM $CaCl_2(2H_2O)$, 35 mM potasium acetate (from 1M pH 7.5), 15% glycerol (wt/vol). Adjust pH to 5.8 with acetic acid. Sterile filter. Store 4 C]. Leave on ice 1 hour. Pellet cells again, 2500 rpm, 15 min., 4 C. Remove all liquid. Gently resuspend cells in 8 mls RF2 buffer [10 mM RbCl, 75 mM $CaCl_2$, 10 mM MOPS (from .5M, pH 6.8), 15% glycerol (wt/vol). Adjust pH to 6.8 with NaOH. Sterile filter and store 4 C]. Leave on ice 15 min. Aliquot 50, 100 or 200 uls into labeled, chilled 1.5 ml eppendorf tubes. Freeze tubes immediately in liquid nitrogen. Store

tubes -70 C. Check for contaminants on antibiotic plates (use whatever your plasmid codes for.)

Appendix 3

Transformation of Bacterial Cells

Use 1-5 ul of plasmid DNA to inoculate competent cells. Leave on ice 30 min (not longer than 4 h). Heat shock 42 C, 45 sec. Leave on ice 2 min. Add SOC 4:1 (ie. 400 ul SOC to 100 ul cells). Shake slowly for 1 hour, 37 C. Plate on LB containing the appropriate antibiotics. Grow overnight at 37 C.

Appendix 4

Single-strand DNA Preparation

Protocol from T.Dawn Parks

With a toothpick, select several transformed colonies to inoculate a 5 ml LB + 5 ul Amp (50 mg/ml Amp stock; or appropriate antibiotic) culture. Let grow 16 h, 37 C, 300 rpm. Use 500 ul of overnight culture to inoculate 8 mls LB + 8 ul Amp (or other antibiotic). Grow 1 1/2 h, 37 C, 300 rpm. Add 50-100 ul helper phage. Shake 100 rpm 1-2 h. Inoculate 9 mls (X 2 if you need a lot of ssDNA) of LB containing 9 ul Amp (or other) and 10 ul Kanamycin (25 mg/ml Kan stock). Grow 37 C, 300 rpm, overnight. Transfer to 30 ml Corex tube, spin 10K 15 min. Pour supernant into clean 30 ml Corex tube containing 2.5 mls 2.5M $NaCl_2$/20% PEG 8000. Mix, then let stand RT for 30 min. Spin 10K, 10 min. Resuspend pellet in 380 ul TE + 1 ul 10 mg/ml RNase. Let stand RT for 30 min. Add 40 ul 10X ProK (10X ProK = 500 ug/ml proK, 100 mM Tris pH 7.8, 2% sarkosyl, 10 mM Edta pH 8.0). Heat virus solution to 55 C for 30 min, vortexing occasionally. Add 31.2 ul 5M $NaCl_2$. Use 400 ul phenol:$CHCl_3$ to extract 3 times. Add 900 ul of 95% EtOH. Place at -70 C for 30 min. Spin 15K 10 min. Wash pellet with 70% EtOH. Spin 15K for 3 min. Dry pellet. Resuspend in 25-50 ul water.

Appendix 5

CTAB Mini-Preps Suitable for Use in Cloning

Grow 5 ml overnight culture in LB. Pour 1 1/2 mls into a microfuge tube and spin 7K for 4 min. Remove supernant and resuspend pellet in 1 ml .1% STET. Add 15 ul of 50 mg/ml lysozyme and let stand 5 min at room temperature. Boil prep for 2 min. Spin 14K for 20 min. Remove pellet with a toothpick. Add 4 ul 10 mg/ml RNase and put at 37 C for 30 min. Add 50 ul of 5% CTAB (preheat CTAB at 55 C before using). Let stand at room temperature for 10 min. Spin 14K 10 min. Remove supernant. Resuspend pellet in 300 ul of 1.2 M $NaCl_2$. Phenol:CHCl extract twice. Precipitate DNA with 750 ul of 100% EtOH at -70 C for 30 min. Spin 14k for 15 min. Wash pellet with 70% EtOH and spin 14K for 3 min. Resuspend in 50 ul of water.