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GENETIC DIVERSITY OF BEAN POD MOTTLE VIRUS (BPMV) AND DEVELOPMENT OF BPMV AS A VECTOR FOR GENE EXPRESSION IN SOYBEAN

Chunquan Zhang
University of Kentucky

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ABSTRACT OF DISSERTATION

Chunquan Zhang

The Graduate School

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ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for
the degree of Doctor of Philosophy in the College of Agriculture
at the University of Kentucky

By

Chunquan Zhang

Lexington, Kentucky

Director: Dr. Said A. Ghabrial, Professor of Plant Pathology

Lexington, Kentucky

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Bean pod mottle virus (BPMV), a member of the genus *Comovirus* in the family *Comoviridae*, is widespread in the major soybean-growing areas in the United States. The complete nucleotide sequences of the genomic RNAs of the naturally occurring partial diploid strain IL-Cb1 were determined. Intermolecular RNA1 recombinants were isolated from strain IL-Cb1 and characterized at the molecular level. Structurally similar recombinant RNA1 was also generated after four passages in soybean derived from plants previously inoculated with a mixture of infectious RNA1 transcripts from two distinct strains.

BPMV was developed as a plant viral vector that is appropriate for gene expression and virus-induced gene silencing (VIGS) in soybean. The foreign gene was inserted between the movement protein (MP) and the large coat protein (L-CP) coding regions. The recombinant BPMV constructs were stable following several serial passages in soybean and relatively high levels of protein expression were attained. Successful expression of several proteins with different biological activities was demonstrated from the BPMV vector.

Double infection of soybean by BPMV and SMV triggers a synergistic interaction leading to a serious disease. To investigate the underlying mechanism, helper component-protease (HC-Pro) genes from several SMV strains and TEV were expressed from BPMV vectors. The recombinant BPMV vectors carrying the HC-Pro genes from SMV strain G7 or TEV induced very severe symptoms on soybean whereas constructs containing the HC-Pro gene from SMV isolate P10, a mild strain with an apparent defect in synergism, induced only very mild symptoms. Transient agroinfiltration assays using GFP-transgenic *Nicotiana benthamiana* showed that HC-Pro from SMV isolate P10 was not a RNA silencing suppressor, whereas those of SMV strain G7 and TEV exhibited strong suppressor activities. Analysis of chimeric HC-Pro genes and point mutations indicated that a positively charged amino acid at position 144 is critical for the suppressor function of not only SMV HC-Pro but also other potyvirus HC-Pro proteins. Although amino acid substitution at position 144 resulted in changes in small RNA profile, it did not affect HC-Pro stability.

KEYWORDS: Bean pod mottle virus, soybean mosaic virus, diploid reassortments, recombination, viral vector, VIGS, synergism, RNA silencing suppression.

Chunquan Zhang

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Said A. Ghabrial
Director of Dissertation
Lisa J. Vaillancourt
Director of Graduate Studies
07 / 19 / 2005

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1. Overview of plant RNA virus recombination

1.1 Introduction

Viruses are simple and adaptable. Rapid evolution of RNA viruses can lead to the emergence of new species, more severe strains, break of host resistance, expanded host ranges or enhanced vector transmission. The major driving forces for the evolution of RNA viruses are: mutation, reassortment and RNA recombination (Roossinck, 1997).

Mutation: Since most plant viruses use RNA as their genome or genome replication intermediate (caulimoviruses), the error-prone nature of viral encoded RNA-dependent-RNA-polymerase (RdRp or reverse transcriptase in case of caulimoviruses) leads to high error rate. The average error rate of RdRp is 10^{-3}-10^{-4} per nucleotide while that of cellular DNA-dependent DNA polymerases is on the order of 10^{-11} (Hull, 2001). Although the mutation rate is high, the mutation frequency at the population level varies dramatically for different viruses. Host and vector can influence the virus variation (Fraile *et al.*, 1997). The recent finding of RNA editing-like base modification (deamination), reported in several animal viruses, can be another way of introducing mutations (Bass, 2002; Jayan & Casey, 2002). It remains to be determined if plant viruses undergo similar modifications since plant cells clearly utilize RNA editing mechanisms and contain the appropriate enzymes.

Reassortment: Viruses with multipartite genomes can exchange segments upon co-infection. The new reassortants may have new features or exhibit better fitness (Hou *et al.*, 1996; Fraile *et al.*, 1997; Hull, 2001).

Recombination: RNA Recombination is a process that joins two noncontiguous RNA fragments together. This could occur in the same viral RNA genome, among different viral RNAs or even between viral and host RNAs (Cheng & Nagy, 2003).

Viral genomic RNA recombination was first discovered in poliovirus (Hirst, 1962; Ledinko, 1963). The first case of RNA recombination in a plant RNA virus was found in *Brome mosaic virus* (BMV; Bujarski & Kaesberg, 1986). Based on nucleotide sequence comparisons, RNA recombination is now known to be widespread among animal, plant, and bacterial viruses (Lai, 1992; Chetverin, 1997). Other than recombination between viruses, RNA recombination is also suggested to occur between viral RNAs and host RNA (including transgenes). It was found that RNA recombination between *Plum Pox Virus* (PPV) containing a defective CP gene and transgenic *Nicotiana benthamiana* expressing the wild type CP gene can restore a wild type virus (Varrelmann *et al*., 2000). RNA recombination between plant viruses and transgenic plants thus pose a problem for agricultural biotechnology (Rubio *et al*., 1999). Based on sequence comparisons, host genes are also found in viruses. An exon of tobacco chloroplast DNA was found in *Potato leaf roll virus* (PLRV) genome (Mayo & Jolly, 1991; Meyers *et al*., 1991.)

Plant viral RNA recombination can occur for both segmented and nonsegmented viruses. Detection methods of RNA recombination include different types of PCR-based methods, cDNA cloning and sequencing and phylogenetic analysis (Worobey & Holmes, 1999). Mostly, the detection is a reflection of accumulative results of RNA recombination, which is subject to the recombinant's fitness including many factors like host range and vector transmission. Although RNA recombination is suggested in many plant RNA viruses by phylogenetic analysis, information on molecular characterization of the recombinant cDNA clones is only available for a limited number of plant viruses (Table 1.1).

Viral RNA recombination has two major effects: to purge accumulated deleterious changes of viral genomic RNA population and to create or spread beneficial combinations of mutations or genetic elements within species or between species (Worobey & Holmes, 1999). This may have major impact on the virus and its host. For example, the recent highly transmissible severe acute respiratory syndrome (SARS) coronavirus outbreak in Asia was predicted to be caused by host jumping due to interspecies RNA recombination (Stavrinides & Guttman, 2004).

1.2 Mechanisms of RNA recombination

The most acceptable RNA recombination classification system was proposed by Nagy and Simon in 1997. In this system, there are three different models proposed for the mechanism of RNA recombination, including RNA breakage-ligation, breakage-induced template switching and replicase-driven template switching model (Nagy & Simon, 1997). Sometimes, the first model is also called nonreplicase-driven RNA recombination as opposed to the second and third models of replicase-driven RNA recombination.

The breakage-ligation model is based on the well characterized DNA recombination model. In this model, RNAs can break and religate thus creating new RNA recombinants due to the ligation of two or more different RNA fragments. This mechanism was shown for RNA splicing-mediated by RNA site-specific ribozymes (Morl & Schmelzer, 1990). A similar mechanism termed as nonreplicative transesterification mechanism was proposed for recombination of Qβ phage-associated RNA. First line of evidence showed that altering the 3'-OH (required for ligation) of the acceptor RNAs influenced the formation of recombinants (Chetverin *et al.*, 1997). Second line of evidence came as viable recombinant viruses were recovered from nonreplicating and nontranslatable parts of a viral RNA genome, suggesting that no replicase was involved in RNA recombination for the early steps (Gmyl *et al*., 1999; Gmyl *et al*., 2003). Both precise and imprecise viral RNA recombinants were generated through this nonreplicative recombination process.

As a replicase-driven RNA recombination model, the breakage-induced template-switching model requires RdRp for template switch. This model requires the cleavage of the donor RNA to force the RdRp to pause. The pausing will promote RNA recombination. Basically, this model is a combination of the replicase-driven template-switching and breakage-ligation models (Nagy & Simon, 1997).

The replicase-driven template switch model has been extensively studied. It predicts that a sequential event is required for RNA recombination to take place. Firstly, the viral replicase falls off from the donor template during the nascent strand RNA synthesis. Then the replicase binds to a acceptor template (new RNA or another region of the donor RNA) and uses the nascent strand RNA (from donor RNA template) as a primer and continues RNA synthesis on the acceptor RNA. The newly synthesized RNA will contain a recombinant genome from both donor and acceptor RNA templates (Nagy & Simon, 1997). Replicase-driven RNA recombination mechanism is further classified into three different classes: similarity-essential (requires base-pairing), similarity-nonessential (secondary structures are involved) and similarity-assisted recombination (a combination of base-pairing and secondary structures is involved in recombination).

Many factors involved in the RNA replication process influence RNA recombination. In the replicase-driven RNA recombination model, RNA elements, viral encoded RdRp and host factors are the major components that influence the process.

The RNA elements in recombination include the primary sequences and secondary structures. Both the donor and acceptor RNA primary sequences (including the nucleotide composition, sequence length and position of the primary sequences) are important factors that are involved in RNA recombination. It has been reported that the length and position of AU- and GC-rich sequences could influence the frequency and precision of RNA recombination (Nagy *et al*., 1999). In some plant viruses, AU-rich sequences were found to increase the RNA recombination frequency for *Tomato bushy stunt virus* (TBSV) and *Citrus tristeza virus* (CTV) (Shapka & Nagy, 2004; Vives *et al*., 2005).

In the similarity-nonessential and similarity-assisted recombination, RNA secondary structures play an important role in template switching. Formation of a stable stem-loop structure between two viral RNAs enhances recombination for murine coronavirus mouse hepatitis virus (MHV) and BMV (Nagy & Bujarski, 1993; Rowe *et al*., 1997; Figlerowicz, 2000). In the case of *Turnip crinkle virus* (TCV) associated satC RNA recombination, it was found that both the sequences and the secondary structural hairpin are key for RNA recombination (Cascone *et al*., 1993; Nagy *et al*., 1998). In an *in vitro* study, Kim and Kao (2001) showed that both primary and secondary structures could affect the efficiency of template switching for BMV and *Bovine viral diarrhea virus*.

As a major component in the replicase-driven RNA recombination model, the replicase complex (with the viral-encoded RdRp as a key component) plays a significant role in RNA recombination. The replicase complex interacts with the RNA elements and hence has an extensive effect on RNA recombination. Mutations in the replicase components can alter the distribution of recombination hot spots (e.g. mutations in the helicase-like domain of BMV 1a protein, Nagy *et al*., 1995), inhibit non-homologous recombination (e.g. a single amino acid mutation in the polymerase domain of BMV 2a protein, Figlerowicz *et al*., 1997) or influence the generation of recombinants (mutations in the RNA binding motif of p33 or p92, Panaviene & Nagy, 2003). It has been reported that about 100 host genes can influence BMV viral RNA replication in yeast (Kushner *et al.*, 2003).

2. Overview of plant virus synergism

2.1 Introduction:

As plants are usually hosts to more than one virus, mixed infections with more than one virus are not uncommon. Interactions between plant viruses can take place in two ways: changes in transmission properties and enhancement of replication in the host plant.

Vector transmission change caused by transcapsidation is one important type of plant virus interaction (Taliansky & Robinson, 2003). Umbraviruses are mechanically transmissible under experimental conditions. But in nature, umbraviruses depend on an assistor virus belonging to the luteovirus family for aphid transmission between plants in a transcapsidation way. The transcapsidation happens in a mixed infection by an umbravirus and an assistor virus with the CP of the assistor virus forming aphid-transmissible hybrid virus particles that encapsidate umbraviral RNA. Other than dependence on assistor virus for vector transmission, umbraviruses can systemically infect plants and replicate independently (Taliansky & Robinson, 2003).

Mechanical transmission property can be changed too by plant viral interactions. *Potato leaf roll virus* (PLRV) is not transmitted mechanically in nature (transmitted by aphids). But coinoculation of tobacco with sap from PLRV and *Pea enation mosaic virus* -2 (PEMV-2, an umbravirus) single infected leaves showed that PEMV-2 can act as a ‘helper’ that enables PLRV to be transmitted mechanically (Taliansky & Robinson, 2003). The exact mechanism is still unknown.

An important plant viral interaction is synergism, which has been reported for many virus-host systems (Table 1.2). Synergism can induce enhancement in symptom severity that cannot be accounted for by just additive effects of single infections. The synergistic interaction of *Potato viruses X* (PVX) and *Potato virus Y* (PVY) was first reported in 1955 (Rochow & Ross, 1955); double infection with PVX and PVY causes more severe symptoms on potato than induced by single infections. Since this finding, PVX and PVY have been extensively used in studies on synergism induced by plant virus double infections. This synergism is not the result of increased level of doubly infected cells but rather by increased accumulation level of PVX genomic RNA and subgenomic RNAs in doubly infected leaf cells (Goodman & Ross, 1974; Vance, 1991).

2.2 RNA silencing as a plant defense response to viral infection

Unlike animals, which have an adaptive immune system, plants have no similar antigen recognition system for defense against specific intruding organisms. Instead, plants share another widely conserved RNA-based defense system, RNA silencing. RNA silencing [or PTGS (post-transcriptional gene silencing)] in plants, quelling in fungi and RNA interference in animals] is a sequence-specific RNA degradation process. After its discovery in 1998, RNA silencing is now known to occur widely in living organisms from fungi, nematodes, plants to mice. RNA silencing can target host genes, transgenes, viroids, viral genomic RNAs and parasitic genetic elements like transposons. RNA silencing can cause sequence specific RNA destruction, transcription block and translation inhibition (Novina & Sharp, 2004).

RNA silencing in plants can be triggered by double-stranded RNA, which is used by most plant viruses as an intermediate for either replication or sub-genomic RNA production. The dsRNAs can be cleaved by DCLs (plant Dicer-like homologues of *Drosophila*) to produce short interfering dsRNA (siRNA, 21 to 25 nucleotides in length). The siRNA can also be used by plant-encoded RdRp as templates for siRNA amplification. The siRNAs are incorporated into a multi-subunit endonuclease silencing complex called RNA-induced silencing complex (RISC). Within the activated RISC, single-stranded siRNAs act as guides to bring the complex into contact with complementary mRNAs and thereby cause sequence homology-dependent RNA degradation (Roth *et al*., 2004). Plant viruses when carrying endogenous or transgene homologous sequences can induce RNA silencing as well and is called virus induced gene silencing (VIGS). RNA silencing is triggered not only by plant viruses but also by viroids (Wang *et al*., 2004).Many host factors are involved in this process including host RdRp, translation initiation factors, helicase, RNase III-like proteins, transmembrane proteins and some functionally unknown proteins (Agrawal *et al*., 2003).

2.3 Plant viruses use diverse strategies against RNA silencing

RNA silencing is a complex plant defense process involving many components in its initiation, systemic establishment and maintenance. To establish a successful infection, plant viruses either encode proteins to suppress RNA silencing or escape this defense at different steps of this process. This feature of plant viral interaction is used for dissecting the mechanism of RNA silencing pathway and viral strategies against this process (Agrawal *et al*., 2003).

Some plant viruses encode proteins that suppress RNA silencing. Three major methods are used in identifying RNA silencing suppressors: (1) the transient suppressor expression assay, (2) the RNA silencing reversion assay, and (3) the stable suppressor expression assay. The transient expression assay method is to co-infiltrate a mixture of two bacterial strains (one for silencing induction and the other contains candidate suppressor gene) into a plant leaf (usually *Nicotiana benthamiana* transgenic for a reporter gene) and then examine the infiltrated patch over time for silencing of the reporter (usually GFP fluorescence or GUS staining). The silencing reversion assay method is to infect a silenced plant with the candidate virus and check whether the silenced phenotype can be reversed by viral infection. The stable expression assay method is to produce a stable transgenic line expressing a candidate suppressor of silencing and then cross this line with a series of well-characterized transgenic lines with silenced reporter genes which are checked for suppression function (Roth *et al*., 2004).

With the methods described above, two major classes of suppressors have been identified (Table 1.3). The first class of suppressors affects siRNA metabolism in plants. Usually, they affect the function of siRNA by either blocking the creation of siRNA by Dicer or blocking the function of siRNA through binding to them. One example is P19, which can sequester siRNA and thus suppress RNA silencing. The binding of siRNA by P19 prevents the incorporation of siRNA into the RISC complex as guide for specific cleavage (Silhavy *et al*., 2002).

Another class of suppressors affects systemic silencing. This class includes many suppressors (Table 1.3). A good example is CMV 2b, which primarily targets systemic silencing by blocking signal movement. The blocking can prevent the initiation of silencing but can not reverse already established silencing (Bucher *et al*., 2003; Guo &

Ding, 2002). The exact mechanism of blocking is unknown though it was shown that CMV 2b could localize to the nucleus (Lucy *et al*., 2000). PVX p25 (MP protein) also suppresses systemic silencing by blocking silencing signal production or transmission. And this suppression is correlated with the absence of a slightly-larger class of small RNAs (Voinnet *et al*. 2000; Hamilton *et al*., 2002).

HC-Pro is another type of suppressor that is highly effective against silencing. It can block systemic silencing and reverse an established silencing (Hamilton *et al*., 2002, Mallory *et al*., 2003). As different viruses suppress different pathways in the RNA silencing process or escape silencing effect, the strong silencing suppression function of HC-Pro can complement other viral counter defense strategies. In mixed infections involving potyviruses, this effect thus enhance the accumulation of a broad range of unrelated plant viruses. This RNA silencing and its suppression accounts for the large number of potyvirus-associated synergistic diseases in plants (Roth *et al*., 2004).

2.4 Structural features of the multifunctional protein HC-Pro

The potyvirus HC-Pro is a multifunctional protein with vector transmission, systemic movement, genome amplification, proteinase and counter-defense functions. It has three major regions, the N-terminal, central and C-terminal regions.

The N-terminal region of HC-Pro has a highly conserved tetrapeptide Lys–Ile/Leu–Thr /Ser–Cys [K(I/L)(T/S)C; designated KITC] within a Cys-rich motif. The N-terminal KITC motif and the C-terminal PTK (Pro–Thr–Lys) motif are involved in aphid transmission. The N-terminal KITC motif binds to the aphid vector's stylets while the C-terminal PTK motif mediates the binding of HC-Pro to the viral capsid protein's N-terminal DAG (Asp-Ala-Gly) motif (Plisson *et al*., 2003). It has been proposed that HC-Pro occurs as a dimer in infected plants, and that the N-terminal region was sufficient for dimerization (Urcuqui-Inchima *et al*., 2001).

Other than the PTK motif, the C-terminal region has cysteine protease-like proteinase function, which cleaves itself from the polyprotein chain upon translation. This region is also involved in cell-to-cell movement, as shown by microinjection assays (Rojas *et al*., 1997) that this region is important for increasing the size exclusion limit (SEL) of plasmodesmata.

The central region of HC-Pro has a diverse function in genome amplification, suppressor activity, and systemic movement within the host plant. This domain is responsible for non-specific binding to nucleic acids with a preference for single-stranded RNA (Plisson *et al*., 2003). Alanine-scanning mutational analysis showed that mutations in this region could reduce genome amplification in protoplasts, systemic movement in plants and abolish RNA silencing suppression function. The fact that these mutants could only be partially complemented by transfection of plants expressing the wild-type HC-Pro of TEV implied that suppression of RNA silencing is not the only the reason for the defect. Steps in systemic movement like entry into and exit from the vascular system may require this region. Alanine scanning has also shown that the central region is critical for suppression of RNA silencing (Kasschau *et al*., 1997). As mentioned before, this region is critical for suppressor function, which is the underlying mechanism of synergism, mutations within the central region of TEV HC-Pro abolished induction of synergism with PVX (Shi *et al*., 1997).

Table 1.1 A list of plant RNA viruses with documented reports of RNA recombination

Genus	Virus	Reference
Alfamovirus	*Alfalfa mosaic virus*	Van der Kuyl *et al*., 1991
Bromovirus	*Brome mosaic virus*	Bujarski & Kaesberg, 1986
	Cowpea Chlorotic mottle virus	Allison *et al*., 1990
Carmovirus	*Turnip crinkle virus*	Cascone *et al*., 1990
Cucumovirus	*Cucumber mosaic virus*	Chen *et al*., 2002
		Fernandez-Cuartero *et al*., 1994
		Garcia-Arenal, 1994
	Tomato aspermy virus	Aaziz & Tepfer, 1999
Luteoviridae	*Sugarcane yellow leaf virus*	Moonan *et al*., 2000
		Smith *et al*., 2000
Potyvirus	*Plum pox potyvirus*	Glasa *et al*., 2002
	Turnip mosaic virus	Ohshima *et al*., 2002
		Tomimura *et al*., 2003
	Yam mosaic virus	Bousalem *et al*., 2000
	Zucchini yellow mosaic virus	Gal-On *et al*., 1998
Tobamovirus	*Tobacco mosaic virus*	Beck & Dawson, 1990
		Gibbs, 1999
Tombusvirus	*Cucumber necrosis virus*	White & Morris, 1994
	Tomato bushy stunt virus.	

Table 1.2 Synergistic interactions among plant viruses

Host	Viruses	References
Tobacco	*Tobacco mosaic virus* (TMV; genus *Tobamovirus*) & *Cucumber mosaic virus* (CMV, *Cucumovirus*)	Garces-Orejuela & Pound, 1957
Barley	TMV & *Barley stripe mosaic virus* (BSMV; genus *Hordevirus*)	Dodds & Hamilton, 1972
Potato	*Potato virus X* (PVX; genus *Potexvirus*) & *Potato virus Y* (PVY; genus *Potyvirus*)	Rochow & Ross, 1955
Tobacco	PVX & PVY	Damirdagh & Ross, 1967
Cereals	*Barley yellow dwarf virus* (B)-PAV, BYDV-RPV (genus *Luteovirus*)	Miller and Rasochova, 1997
Corn	*Maize chlorotic mottle virus* (MCMV, genus *Machlomovirus*) & *Sugarcane mosaic virus* (SCMV, genus *Potyvirus*)	Goldberg & Brakke, 1987
Squash	CMV & *Zucchini yellow mosaic virus* (ZYMV; genus *Potyvirus*)	Wang *et al*., 2002
Soybean	*Soybean mosaic virus* (SMV, genus *Potyvirus*) & BPMV or CPMV (genus *Comovirus*)	Calvert & Ghabrial, 1983; Anjos *et al*., 1992
Cucumber	ZYMV & CMV	Poolpol & Inouye, 1986
Sweet potato	*Sweet potato feathery mottle virus* (SPFMV, genus *Potyvirus*) & *Sweet potato chlorotic stunt virus* (SPCSV, genus *Crinivirus*)	Karyeija *et al*, 2000
Corn	*Wheat streak mosaic virus* (WSMV, genus *Rymovirus*) & MCMV	Scheets, 1998

Table 1.3 A list of reported RNA silencing suppressors

Genus	Virus	Suppressor	Suppression activity			Reference
			Local silencing	Systemic silencing	Silencing reversed	
Carmovirus	TCV	CP	Yes	Yes	No	Qu *et al*., 2003; Thomas *et al*., 2003
Closterovirus	BYV	P21	Yes	-	-	Reed *et al*., 2003
Comovirus	CPMV	Small CP	Yes	-	-	Canizares *et al*., 2004
Cucumovirus	CMV,TAV	2b	Yes	Yes	No	Li *et al*., 2002
Furovirus	BNYVV	P14	Yes	-	Yes	Dunoyer *et al*., 2002
Geminivirus	ACMV	AC2	Yes	Yes	Yes	Dong *et al*., 2003; van Wezal *et al*., 2002
Hordeivirus	BSMV	γb	Yes	-	-	Yelina *et al*., 2002
Pecluvirus	PCV	P15	Yes	-	Yes	Dunoyer *et al*., 2002
Polerovirus	BWYV	P0	Yes	No	-	Pfeffer *et al*., 2002
Potexvirus	PVX	P25	No	Yes	No	Voinnet *et al*., 2000; Hamilton *et al*., 2002
Potyvirus	PVY, TEV	HC-Pro	Yes	Yes	Yes	Mallory *et al*., 2003; Hamilton *et al*., 2002
Sobemovirus	RYMV	P1	-	-	Yes	Voinnet *et al*., 1999
Tenuivirus	RHBV	NS3	Yes	-	Yes	Bucher *et al*., 2003
Tombusvirus	TBSV	P19	Yes	Yes	No	Voinnet *et al*., 2003; Qu & Morris, 2002
Tospovirus	TSWV	NSs	Yes	-	Yes	Bucher *et al*., 2003; Takeda *et al*., 2002

-, not tested.

Virus abbreviations: TCV (*Turnip crinkle virus*), BYV (*Beet yellows virus*), CPMV (*Cowpea mosaic virus*), BYSV (*Beet yellow stunt virus*), CMV (*Cucumber mosaic virus*), TAV (*Tomato aspermy virus*), BNYVV (*Beet necrotic yellow vein virus*), ACMV (*African cassava mosaic virus*), TYLCV-C (*Tomato yellow leaf curl virus*), BSMV (*Barley stripe mosaic virus*), PCV (*Peanut clump virus*), BWYV (*Beet western yellows virus*), PVX (*Potato virus X*), PVY (*Potato virus Y*), TEV (*Tobacco etch virus*), RYMV (*Rice yellow mottle virus*), RHBV (*Rice hoja blanca virus*), TBSV (*Tomato bushy stunt virus*), TSWV (*Tomato spotted wilt virus*).

3. Development of plant viral gene expression vector

Plants are now used as a general gene expression platform for protein production. One major plant-based protein expression method is nuclear transformation and the regeneration of transgenic plant lines. It is widely used but has some disadvantages as low transformation rates for some plant species, time consuming and the inability to transfer the transgene between plants. Another transformation-based method is the organelle (including chloroplast and plastid) transgenic systems (Maliga, 2002; Daniell *et al.*, 2002). Both chloroplast and plastid systems can achieve high yields. The chloroplast transgenic system is restricted to only several plant species. The transplastomic method has protein modification problems like glycosylation. As an alternative to stable transgenic lines, transient expression has been developed for gene expression. This includes two methods, infiltration with *Agrobacterium tumefaciens* and inoculation with plant viral gene expression vectors. Both of them are good for laboratory test of target proteins. The agroinfiltration process has scale problem and some host plants are recalcitrant to infiltration (Fischer *et al.*, 2004).

The use of plant viral vectors provides an alternative method of gene expression in plants with many advantages. It is rapid, flexible, high throughput, and can achieve high level of gene expression with reduced cost. Plant viral vectors have been tested in many applications such as vaccine studies with antigen and antibody production, functional studies of diverse genes such as pathogen avirulence genes and RNA silencing suppressors and large molecule biochemical engineering. Viral vectors also present excellent tools for studies on functional genomics. They can be used to complement other gene function discovery methods, including metabolic profiling, protein or RNA profiling, T-DNA modification, and other approaches. Driven by these interests, many plant viral vectors have been developed (Table 1.4). The plant viral gene expression system is an integrated gene expression system depending on the viral life cycle as well as on plant virus interactions. The plant viral vector usually is tested first using ‘full virus’ strategy. Then both the plant part and the viral vector are further modified using ‘the deconstructed virus’ vector strategy to enable efficient and controlled target gene expression in plants (Gleba *et al.*, 2004).

The viral vector constructed using the ‘full virus’ vector strategy is designed to behave as a wild type virus in plants. Additional to virally- encoded genes, a gene of interest is inserted into the viral genome for expression. Historically, this is the first approach developed for viral vector-mediated gene expression. These vectors were infectious systemically in plants (with exception for the simple gene replacement method) and (relatively) stable. Based on the genomic expression strategy of the virus involved, foreign genes are expressed using different approaches including gene replacement, subgenomic mRNA and fusion protein.

Gene replacement was the first attempted method for viral vector development (Takamatsu, *et al*., 1987). A viral gene, usually CP or MP gene, is replaced with a foreign gene of interest (Table 1.4, BMV and TMV). The disadvantage of this method is that loss of the viral gene function causes problems in the viral life cycle. For example, BMV CP-replacement vectors could not move from cell to cell in an infected leaf (French *et al*., 1986).Although TMV-CP replacement vectors could move from cell to cell, they could not move systemically in plants (Takamatsu *et al*., 1987). Nevertheless, this method is useful in protoplast systems to produce recombinant protein products for analysis.

For viruses using subgenomic mRNAs for gene expression, the element containing a foreign gene under the control of a viral subgenomic promoter is inserted into the viral genome in addition to all required viral genes. Thus, target genes were expressed via subgenomic mRNA (Table 1.4, AMV, BMV, BYV, PVX, TBSV, TRV and TMV). Using heterologous promoter from other viruses instead of the viral vector own homologous promoter can improve viral vector stability (Shivprasad *et al*., 1999). Another strategy is bicistronic subgenomic mRNA strategy tested with the PVX vector (Toth *et al*., 2001). A GFP gene was placed under the control of PVX CP subgenomic promoter and upstream of the PVX CP gene. Between GFP gene and PVX CP gene, an internal ribosome entry sequence (IRES) was inserted so that the downstream PVX CP gene can be translated from the bicistronic subgenomic mRNA. Vectors using both methods still have all viral encoded genes and are able to move systemically with the inserted foreign gene.

The foreign gene can be expressed as a fusion protein by inserting the foreign gene in frame with an existing virus open reading frame (ORF). The foreign gene is usually flanked with additional proteinase cleavage sites to facilitate the processing of the fusion protein for release of the foreign protein (Table 1.4, AMV, BSMV, BYV, CPMV, PPV, PVX, PVY, TBSV, TEV, TMV, WSMV and ZYMV). The flanking proteinase cleavage site can be peptide sequence recognized by viral vector-encoded proteinase or other peptide that has self cleaving activity. Foot and mouth disease virus 2A cleavage sequence was successfully used as the heterologous cleavage site (Santa, *et al*., 1996). Flanking foreign gene with non-viral vector encoded proteinase cleavage peptide has the advantage of avoiding the duplication of homologous nucleotide sequences and thus the viral vector is rendered more stable. Epitope display is a special type of peptide expression. By design, the epitope is displayed usually on the surface of virions so mostly epitope coding sequence is fused to the CP gene.

Under the 'deconstructed virus' vector strategy, some viral functions are eliminated or rebuilt. These functions are then either provided by hosts (these hosts are genetically modified to provide those functions) or replaced with analogous functions that are not derived from the virus vector. Such integrated systems have the advantages of more efficient and controlled gene expression, and have improved safety by preventing any escape of infectious viral particles outside the host plant. But it is designed for industrial protein production and not suitable for laboratory research as it deviates from the concept of transient expression system (Gleba, *et al*., 2004).

An alternative strategy of this method is host chromosome integration of a whole viral vector amplicon (partial viral vector sequence that can replicate itself in plant cells). The amplicon is usually under control of an inducible promoter. By activation, the amplicon (with foreign gene) theoretically is induced in the whole plant without the need for systemic movement **(**Mallory *et al*., 2002; Marillonnet *et al*., 2004). It is much safer than the full-virus strategy as no mature virions are produced and thus possible secondary infection is avoided.

Many problems had been realized and solved, at least partially, with many years of development of viral vectors. The first is foreign gene instability. This problem can be partially resolved by using heterologous sequences in either subgenomic strategy or fusion strategy (Shivprasad *et al*., 1999). Genetic drift is another concern for multiple passages as plant RNA viruses have high mutation rates that introduce mutations into the nonviral target gene sequence. However, it was shown that some selective advantage can be gained from single base mutations in nonviral sequences for several viral vectors (Pogue *et al*., 2002). Plant viral vector systems have other unique challenges, as gene expression requires both the viral part and the plant part. Other than environmental conditions, a viral vector system is also subject to host defense responses because essentially it is a systemic infection process. A careful selection and genetic modification of hosts (like expression of viral MP, CP genes or known RNA silencing suppressors) may enhance foreign gene expression (Voinnet *et al*., 1999; Pogue *et al*., 2002).

4. Plant VIGS vectors and their applications

Plant viral gene expression vectors may be used as virus induced gene silencing vectors (VIGS) as well. Kumagai (1995) first reported that the phytoene desaturase (PDS) gene in *N. benthamiana* was silenced by a TMV VIGS vector. Generally, a 300-500 nucleotides fragment of the target sequence in sense or antisense direction is inserted into the viral vector to induce posttranscriptional gene silencing. The fragment can be a conserved region of a gene family for targeting multiple genes, a specific sequence of a single gene within a family for single gene silencing or a combination of multiple gene specific sequences for simultaneous silencing of several distinct genes (Burch-Smith *et al*., 2004; Peele *et al*., 2001; Turnage *et al*., 2002). Plants infected with the virus vector containing the fragment will exhibit systemic suppression of the targeted gene expression leading to a loss-of-function phenotype. It has been successfully used for identifying genes in plant defense (Burch-Smith *et al*., 2004), genes in metabolic pathways (Darnet & Rahier, 2004) and genes involved in plant development (Liu *et al*., 2004).

Originally, infectious viral RNA or DNA with target gene sequence was inoculated onto plants for induction of gene silencing. The viral vector genome was further placed under the control of the CaMV 35S promoter in binary vectors for *Agrobacterium*-mediated expression in plant cells. This *Agrobacterium*-mediated introduction of viral genome into plants avoids the laborious process of producing viral transcripts *in vitro*. *Agrobacterium* containing viral genomes can be applied by stabbing leaves using a toothpick, infiltrating with a syringe, or just spraying onto leaves depending on the plant species and type of experiment.

VIGS has many advantages compared with traditional techniques for gene function studies. VIGS avoids plant transformation that is laborious and time consuming. It can test genes whose deletion is lethal in development. VIGS can target a gene in a family, the whole family or multiple genes simultaneously. The VIGS test can be done within a single generation with only a single plant and the result can be rapidly scaled up and repeated. Unlike nuclear transformants, VIGS allows rapid comparisons of gene function between species and different genetic backgrounds (Burch-Smith *et al.*, 2004). Since the development of TMV as a VIGS vector (Kumagai *et al.*, 1995), many viruses including both RNA viruses and DNA viruses were engineered for host gene function studies (Table 1.4). A VIGS based 'fast-forward' genetics method was tested in *N. benthamiana*. Nearly 5000 cDNAs were screened with the PVX vector and many genes involved in defense signaling were identified (Lu *et al.*, 2003).

Despite its advantages, certain limitations are inherent in VIGS as a technique for loss-of-function studies. The expression of a target gene is not completely shut down and low level transcript of some genes can produces enough functional protein for phenotype. The silencing of the target gene is not uniform and may complicate the analysis of results. The VIGS is a systemic infection of host plants and host response to pathogen infection may mask some phenotypes. The VIGS process is also subject to environmental effects, especially temperature. The possible suppression of non-target genes is also a concern that may complicate the results. Some of these questions can be solved with better knowledge of the genome sequencing data, a well- controlled environmental conditions and an appropriate host gene control (like PDS) for silencing effectiveness (Burch-Smith *et al.*, 2004).

At present, the most reliable and effective VIGS vectors have a limited host range including *Arabidopsis*, barley, *N. benthamiana*, tobacco and tomato (Table 1.4). Still, the power of systemic gene knockout and overexpression of plant viral vectors needs to be further explored. New plant viral vectors (as well as VIGS vector) developed for major crops like corn, rice and soybean will greatly facilitate studies on functional genomics.

Table 1.4 A list of reported plant virus gene expression vectors and VIGS vectors

Genus	Virus	Host	Expression method	VIGS vector	References
Alfamovirus	AMV	*N. benthamiana*	Free protein via subgenomic RNA; CP gene fusion protein	-	Sanchez-Navarro, *et al.*, 2001
Bromovirus	BMV CCMV	barley, cowpea	Free protein via subgenomic RNA; CP gene replacement	-	French, *et al.*, 1986; Allison, *et al.*, 1988
Caulimovirus	CaMV	turnip	ORF II replacement	-	Brisson, *et al.*, 1984
Begomovirus	CbLCV	*Arabidopsis*	CP gene replacement	Yes	Turnage, *et al.* 2002
Clostrovirus	BYV	tobacco	Free protein via proteolytic fusion, Free protein via subgenomic RNA	-	Hagiwara, *et al.*, 1999
Comovirus	CPMV	cowpea	Free protein via proteolytic processing; CP fusion protein,	-	Gopinath, *et al.*, 2000; Porta, *et al.*, 1994 and 1996
Geminivirus	TGMV	*N. benthamiana*	CP gene replacement	Yes	Kjemtrup, *et al.* 1998; Peele, *et al.* 2001
Hordivirus	BSMV	barley	Gene fusion	Yes	Haupt, *et al.*, 2001; Holzberg, *et al.*, 2002;
Potexvirus	PVX	*N. benthamiana*	Free protein via subgenomic RNA; CP gene fusion protein	Yes	Chapman, *et al.*, 1992; Lu, *et al.* 2003; Toth, *et al.*, 2001

Table 1.4 (continued) A list of reported plant virus gene expression vectors and VIGS vectors

Genus	Virus	Host	Expression method	VIGS vector	References
Potyvirus	TEV, PVY, ZYMV, PPV	*N. benthamiana, N. clevelandii,* potato, tobacco, squash, melon, and cucumber	Free protein via proteolytic processing; CP gene fusion protein	-	Arazi, *et al*., 2001; Dolja, *et al*., 1992; Fernandez-Fernandez, *et al*., 2001
Rymovirus	WSMV	wheat, barley, oat and maize	Free protein via proteolytic processing; CP gene fusion protein	-	Choi, *et al*., 2000
Tobamovirus	TMV, ToMV	*N. benthamiana*	Free protein via subgenomic RNA; CP gene replacement; CP gene fusion protein	Yes	Donson, *et al*., 1991; Fitzmaurice, *et al.,* 2002; Kumagai, *et al.,* 1995; Lacomme, *et al.,* 2003; Takamatsu, *et al*., 1987
	SVISS*	tobacco		Yes	Gossele, *et al.,* 2002
Tobravirus	TRV	*N. benthamiana* tomato	Free protein via subgenomic RNA	Yes	Liu, *et al.,* 2002; MacFarlane & Popovich, 2000; Ratcliff, *et al.,* 2001
Tombusvirus	TBSV	*N. benthamiana*	Free protein via subgenomic RNA; CP gene fusion protein	-	Joelson, *et al*., 1997

Virus abbreviations: AMV (*Alfalfa mosaic alfamovirus*), BMV (*Brome mosaic bromovirus*), BSMV (*Barley stripe mosaic hordeivirus*), BYV (*Beet yellows closterovirus*), CbLCV (*cabbage leaf curl virus*), CCMV (*Cowpea Chlorotic mosaic virus*), CPMV (*Cowpea mosaic comovirus*), PPV (*Plum pox potyvirus*), PVX (*Potato X potexvirus*), PVY (*Potato Y potyvirus*), TBSV (*Tomato bushy stunt tombusvirus*), TEV (*Tobacco etch potyvirus*), TGMV (*Tomato golden mosaic bigeminivirus*), TMV (*Tobacco mosaic virus*), ToMV (*Tomato mosaic tobamovirus*), TRV (*Tobacco rattle tobravirus*), WSMV (*Wheat streak mosaic rymovirus*) and ZYMV (*Zucchini yellow mosaic potyvirus*).

-, not tested *, SVISS is the abbreviation of a satellite virus-induced silencing system.

5. *Comoviruses*

Introduction

Viruses in the family *Comoviridae* have segmented positive-sense single stranded RNA genomes. The family includes three genera that differ in vector specificity. Viruses in the genus *Nepovirus* are transmitted by longidorid nematodes, whereas viruses in the genera *Fabavirus* and *Comovirus* are transmitted, respectively, by aphids (in a nonpersistent manner) and chrysomelid beetles (Hull, 2001).

There are 15 approved members in the genus *Comovirus: Andean potato mottle virus* (APMoV), *Broad bean stain virus* (BBSV), *Bean rugose mosaic virus* (BRMV), *Broad bean true mosaic virus* (BBTMV), *Bean pod mottle virus*(BPMV), *Cowpea mosaic virus* (CPMV, type member), *Cowpea severe mosaic virus* (CPSMV), *Glycine mosaic virus* (GMV), *Pea green mottle virus* (PGMV), *Pea mild mosaic virus* (PMiMV), *Quail pea mosaic virus* (QPMV), *Radish mosaic virus* (RaMV), *Red clover mottle virus* (RCMV), *Squash mosaic virus* (SqMV) and *Ullucus virus C* (UVC) (Wellink *et al*., 2000). Comoviruses have a bipartite genome consisting of two positive-sense single-stranded RNA molecules that are separately encapsidated in icosahedral particles (T=1, pseudo T=3) with a diameter of 28 to 30 nm. Three sedimenting components, designated top (empty capsids), middle (containing RNA2 or M-RNA) and bottom (containing RNA1, B-RNA) components are resolved when purified virions are subjected to density gradient centrifugation (Wellink *et al*., 2000). The comovirus virions are composed of 60 copies of two coat proteins, large and small coat protein subunits both encoded by RNA2. The host range of each member is usually limited to one plant family. For example, the natural hosts of CPSMV and BPMV are limited to species in the family *Leguminosae* (*Fabaceae*; Valverde & Fulton, 1996).

5.1 Comovirus genome organization and expression

The complete nucleotide sequences of many comoviruses have been reported including BPMV (Di, *et al*., 1999; MacFarlane, *et al*., 1991; Gu *et al*., 2002), CPMV (Lomonossoff & Shanks, 1983; van Wezenbeek *et al*., 1983), CPSMV (Chen and Bruening, 1992a, b), RCMV (Shanks & Lomonossoff, 1992; Shanks *et al*., 1986), SqMV (Han *et al*., 2002). The sequences of RNA2 and the RdRp gene of APMoV have also been published (Shindo *et al*., 1993; Krengiel *et al*., 1993). The sizes of RNA1 of comoviruses range from 5.9 to 7.2 kb and those of RNA2 vary from 3.5 to 4.5 kb. Both RNA1 and RNA2 have a 5' end covalently genome-linked viral protein (VPg) and a 3' end poly (A) tail (Wellink *et al*., 2000). Comoviruses use polyprotein synthesis and cleavage strategy for gene expression. CPMV RNA1 contains a single large open reading frame (Lomonossoff & Shanks, 1983; van Wezenbeek *et al*., 1983) encoding a 200 kDa polyprotein precursor which is subsequently processed into 5 mature gene products designated from 5' to 3' as protease cofactor (Co-pro, 32K), helicase (58K), VPg, proteinase (Pro, 24K) and RNA dependent RNA polymerase (Pol or RdRp, 87K). The Co-pro functions as a cofactor for cleavage of the RNA2-encoded polyprotein precursor. It is also involved in the regulation of RNA1 polyprotein processing as well as targeting the replication complex to host membranes where viral RNA replication takes place (Peters *et al*., 1992; Carette *et al*., 2002a). It is a cytotoxic protein inducing necrotic lesions in *Nicotiana benthamiana* when expressed from the *Tobacco rattle virus* (TRV) expression vector (Carette *et al*., 2002b). The 58K helicase protein has a nucleotide-binding motif (NTBM), which is characteristic of viral RNA helicases (Gorbalenya *et al*., 1990). Mutation in the NTBM debilitated its binding capacity to ATP, and as a consequence viral RNAs were not able to replicate in cowpea protoplasts (Peters *et al*., 1994). The 60K protein (helicase+VPg) has been reported to interact in the yeast two hybridization system with host proteins including the translation elongation factor eEF-1β, which is a putative component of the replication complex of positive sense RNA viruses (Carette *et al*., 2002c). The 60K protein was also shown to induce the formation of small membranous structures in both plant and insect cells by using the TRV and baculovirus expression systems, respectively (Carette *et al*., 2002b; van Bokhoven *et al*., 1992). The VPg was proposed to prime viral RNA transcription (Lomonossoff *et al*., 1985; Pouwels *et al*., 2002a). The 24K protein is a member of the trypsin-like serine proteinase family and the major player in processing both RNA1 and RNA2 encoded polyproteins (Verver *et al*., 1987; Dessens & Lomonossoff, 1991). Although the C-terminal region of the 87K protein has an RNA-dependent RNA polymerase domain, it was proposed that the 110-kDa protein (87K+24K) is the polymerase because it is the only viral protein found in association with purified viral replication complex (Eggen *et al*., 1988).

CPMV RNA2 has two start codons and thus can be translated into either a 105K or 95K polyprotein (Rezelman *et al*., 1989). The 105 K polyprotein is processed to 58K CR (cofactor of RNA2 replication), large coat protein (LCP) and small coat protein (SCP) while the 95K polyprotein is processed to the 48K MP (movement protein), LCP and SCP (Pouwels *et al*., 2002a). The 58K CR protein shares its carboxy terminal region with the 48K MP protein. CR is required for RNA2 replication possibly by targeting RNA2 to the replication complex (van Bokhoven *et al*., 1993a). The MP is divided into two regions based on their functions. The N-terminal region is responsible for the induction of the tubular structures through which viral particles move from cell-to-cell (Wellink *et al*., 1993; Pouwels *et al*., 2002b). The C-terminal region has binding capacity of viral particles (specifically to the LCP), ssRNA and rGTP (Carvalho *et al*., 2003; Carvalho *et al*., 2004). The virus-encoded LCP and SCP can form virus-like particles when expressed separately in insect cells or protoplasts (Wellink *et al*., 1996, Shanks and Lomonossoff, 2000). CPMV SCP has been identified as a suppressor of RNA silencing. The C-terminal 16 amino acids, which are exposed on the surface of virions, are particularly important for suppressing RNA silencing and cannot be substituted by the equivalent counterpart of BPMV (Canizares *et al*., 2004).

5.2 Viral genome replication and virus movement

The RNA1 of CPMV codes for all the proteins required for replication and is capable of replication alone in cowpea protoplasts (Goldbach *et al*., 1980). In contrast, the replication of RNA2 is dependent on RNA1-encoded proteins (van Bokhoven *et al*., 1993b). The replication is associated with host membranes presumably small membranous vesicles derived from ER (De Zoeten *et al*., 1974; Eggen *et al*., 1988; Carette *et al*., 2000; Carette *et al*., 2002a). Proliferation of the ER membranes similar to that induced by CPMV infection can be induced by individually expressed Co-pro or 60K (58K+VPg) (Carette *et al*., 2002b). Following replication, viral RNAs are packaged into virus particles in the cytoplasm where replication and translation occur (Carette *et al*., 2002a). That capsid proteins can assemble into virus-like particles when expressed in insect and protoplast cells suggesting that no other viral proteins are required for capsid assembly (Wellink *et al*., 1996; Shanks & Lomonossoff, 2000). Then the virions travel intracellularly from the assembly site to the cell periphery through an unknown mechanism.

CPMV moves from cell-to-cell in the form of viral particles via tubular structures that are pierced through the cell wall (van Lent *et al*., 1990). Intact plasmodesmata are not essential for the formation of these tubular structures induced by CPMV infection (van Lent *et al*., 1991; Kasteel *et al*., 1996; Carvalho *et al*., 2003). CPMV MP is the only viral protein required for the formation of these structures and MP mutants defective in tubule induction are unable to move from cell-to-cell (Kasteel *et al*. 1993; Wellink *et al*., 1993; Kasteel *et al*., 1996; Bertens *et al*., 2000). A recent model about CPMV cell-to-cell movement is proposed as follows (Poulwels *et al*., 2002a; Pouwels *et al*., 2003): A proportion of MP binds virus particles at viral RNA replication/virion assembly sites and guides the MP-virion complex to plasma membranes without participation of cytoskeleton and secretary pathways (Carvalho *et al*., 2003). The majority of MP or GTP-binding MP diffuses to the cell periphery and is targeted to the plasma membranes (Pouwels *et al*., 2002b). In the plasma membranes, MP and MP-virion complex accumulate via interaction with membrane residing proteins. Tubules initiated in punctuate structure form within plasmodesmata through polymerization of MP and MP-virion complex, thereby encaging virus particles. The growing tubules eventually extend to the neighboring cells where the tubular structures disassemble. Then the virions are released into the neighboring cells that are thus infected (Pouwels *et al*., 2003).

CPMV moves systemically through the phloem. CPMV is able to replicate in all types of vascular cells except companion cells (CC) and sieve cells (SC). CPMV moves from phloem parenchyma cells to CC and from CC to SC in a way different from the tubule guided movement because tubule structures are absent between those cells (Silva *et al*., 2002). Though CPMV invades both major and minor veins of the inoculated leaves, it is only unloaded from major veins. Mostly, detail of CPMV systemic movement is still largely unknown.

5.3 Transmission of comoviruses

All comoviruses are mechanically transmissible. Seed transmission has been documented for 6 members in the genus *Comovirus* and the transmission frequencies range from 1% to 10% (Hull, 2001). The BPMV seed transmission frequency is as low as

0.1% or lower, suggesting that transmission might occur as a consequence of seed coat infection rather than embryo infection (Giesler *et al*., 2002). Embryo infection by comoviruses derives either from the pollen or the ovule. Seedling infections of SqMV and BBMTV from ovule have been reported. In addition, BBMTV was able to infect the seedling through either the virus-containing pollen or ovule (Gergerich and Scott, 1996). Most comoviruses are transmitted by leaf feeding beetles in the families *Chrysomelidea, Coccinellidae, Curculionidea or Meloidae*, except GMV, PMiMV and UVC whose beetle vectors have not been identified. The efficiency of comovirus transmission is highly dependent on the species of beetle vector and the species of host plant. For instance, BRMV is transmitted by the bean leaf beetle (*Cerotoma trifurcata*) at a frequency close to 80%. In contrast, the transmission frequencies by banded cucumber beetle (*Diabrotica balteata)* and *D. adelpha* are only 20% and 10%, respectively (Gergerich & Scott, 1996). Comoviruses do not replicate in their beetle vectors (Hull, 2001). Unlike aphid transmission, little is known about the viral encoded proteins that may be involved in beetle transmission.

5.4 Diversity of the genus *Comovirus*

The complete nucleotide sequences of six species in the genus *Comovirus* have been reported (Lomonossoff and Ghabrial, 2001). For BPMV, two distinct subgroups of strains have been reported based on nucleic acid hybridization and nucleotide sequence analyses (Gu *et al*., 2002; Gu & Ghabrial, 2005). By using experimental and diagnostic host reactions and/or serological assays, ten species in the genus *Comovirus* have been reported to contain more than one strain (Lomonossoff, 2001, Valverde & Fulton, 1996). Six biotypes of SqMV were reported based on host range as well as symptomatology. The biotypes are classified into two serological groups by agar double-diffusion serological tests (Nelson & Knuhtsen, 1973). Further nucleotide sequencing and nucleotide hybridization analysis showed that there are at least two subgroups for SqMV isolates collected in the United States (Haudenshield & Palukaitis, 1998). Similarly, two subgroups as well as reassortants have been shown to exist by nucleic acid hybridization analysis using probes prepared from cDNA clones of strains O and S of RCMV (Oxelfelt *et al*., 1992). Although distinct strain subgroups and reassortants were characterized, there is no reported experimentally tested RNA recombination between distinct RNAs in the genus *Comovirus*. As a driving force of RNA virus evolution, RNA recombination is not only important to extend our knowledge of virus evolution but also to understand basic biology like host range and symptomatology, which are important in practice. The existence of two subgroups as well as reassortants in BPMV provides a good opportunity to molecularly characterize RNA recombination of comoviruses.

6. Research objectives and outline

The major objectives of my research are to: (1) decipher the genetic diversity among natural isolates of *Bean pod mottle virus*; (2) characterize at the molecular level intermolecular RNA1 recombinants of BPMV; (3) develop BPMV as a gene expression and VIGS vector in soybean and (4) investigate the role of SMV HC-Pro in the synergism induced by double infection with BPMV and SMV. The information generated in this study should be useful to the understanding of BPMV-host interaction.

Chapter Two

Characterization of a partial diploid severe strain of *Bean pod mottle virus* (BPMV) and nucleotide sequence comparisons with other BPMV strains

Introduction

Viruses with mutltipartite RNA genomes are able to reassort their genome segments either in their hosts or insect vectors as a consequence of mixed infections. Viral genetic reassortment has been reported for viruses belonging to several families of mutltipartite RNA viruses including the families *Bunyaviridae*, *Bromoviridae*, *Arenaviridae*, *Potyviridae* (genus *Bymovirus*), *Reoviridae* and *Orthomyxoviridae* (Ushijima *et al*., 1981; Barry *et al*., 1985; Murphy and Webster, 1990; Hendenson *et al*., 1995; Kashiwazaki and Hibino, 1996; Fraile *et al*., 1997). Partial diploid reassortment, which arises as a consequence of dual infection, refers to the genotype of the virus progeny that is diploid for one or more genome segments and haploid for the other genome segments. This phenomenon has been observed with both plant and animal viruses in the laboratory under experimental conditions (Kashiwazaki and Hibino, 1996; Fraile at al, 1997; Rodriguez *et al*, 1998). However, it is not clear if genetic reassortment in natural populations also results in the emergence of partial diploid reassortants. Viruses with partial diploid genomes were shown to be transient and evolve into either the parental genotypes or genome reassortants (Kashiwazaki and Hibino, 1996; Rodriguez *et al*, 1998). The generation of diploid reassortment can potentially plays an important role in the evolution, pathogenesis and epidemiology of some of these mutltipartite viruses. Two types of approaches are used to study genetic reassortment. The first one is sequence-based phylogenetic analysis. Additional examples of genetic reassortment are reported as more viral genomic sequences become available (Hendenson *et al*.; 1995; Miranda *et al*., 2000; Lin *et al*., 2004). The second is experimental detection of reassortment using diverse methods including RNase protection assay (Fraile *et al*., 1997), RT-PCR

(Kashiwazaki and Hibino, 1996; Rodriguez *et al*., 1998) and Northern blot hybridization analysis (White *et al*., 1994).

BPMV is a member of the genus *Comovirus* in the family *Comoviridae* (Goldbach *et al*., 1995). Like other comoviruses, BPMV has a bipartite positive-strand RNA genome consisting of RNA1 (approximately 6.0 kb) and RNA2 (approximately 3.6 kb), which are separately encapsidated in isometric particles 28 nm in diameter. Sequences of several strains of BPMV have been reported (Di *et al*., 1999; Gu and Ghabrial, 2005; MacFarlane *et al*., 1991). In this chapter, I report the complete nucleotide sequences and deduced amino acid sequences of the genomic RNAs of a severe strain, IL-Cb1, which is a partial diploid reassortant. Interestingly, by using nucleic acid hybridization analysis and RT-PCR, another severe strain of BPMV, K-Ho1, was also shown to be a partial diploid reassortant. Both IL-Cb1 and K-Ho1 are naturally occurring partial diploid reassortant strains of BPMV, which induce strikingly severe symptoms.

Materials and Methods

BPMV viral isolates and plant growth conditions

The BPMV field isolates, IL-H14, K-G7, K-Ha1, K-Ho1 and IL-Cb1, were collected from different locations in Kentucky and Illinois (Gu *et al*., 2002). The virus isolates were maintained in the soybean cultivar Essex in a greenhouse with 16 h / 8 h. light / dark conditions.

RNA extraction and nucleic acid hybridization analysis

Viral RNAs were extracted from purified virions according to the procedure of Peden and Symons (1973). Procedures for slot blot hybridization analysis and for preparation of radiolabeled probes were previously described (Gu *et al*., 2002). Following hybridization, the slot blots were exposed to a phosphorimager screen and the images were visualized with a PhosphorImager 445 SI system and analyzed with the ImageQuant 4.1 program (Molecular Dynamics, Sunnyvale, CA).

cDNA synthesis and cloning

For IL-Cb1 cDNA cloning, RNA1 and RNA2 were purified from low-melting agarose following electrophoretic separation of the viral RNAs. cDNA synthesis was carried out using the SuperScript choice system (Invitrogen Corporation, Carlsbad, CA). First strand cDNA synthesis was primed with oligo(dT)$_{12-18}$ primers. Following addition of *Eco*RI adapters to the ends of the double stranded cDNA, it was ligated into *Eco*RI-linearized pGEM 3ZF(+) vector (Promega, Madison, WI). For the viral genomic RNA 5' end cloning, the 5' RACE system (Invitrogen Corp.) was used and the final PCR product was cloned into the pGEM-T easy vector (Promega).

Reverse transcription-polymerase chain reaction (RT-PCR) was used for cloning of type II RNA1 cDNA from BPMV K-Ho1. BPMV RNA1 specific primer R1-Rev1 was used for RNA1 first strand cDNA synthesis with Superscript II (Invitrogen). The BPMV type II RNA1 specific primer pair (Han-RNA1-For5th and Han-RNA1-Rev4th) was used for type II RNA1 sequence amplification. Following PCR, the product was cloned into pGEM-T easy vector.

Sequencing and sequence analysis

For IL-Cb1 cDNA sequencing, multiple independent cDNA clones containing large inserts were selected and used for sequencing. Universal M13 and gene-specific sequencing primers were used for sequencing. M13 forward and reverse primers were used to sequence the 5' and 3' terminal nucleotides of selected cDNA clones and primers based on the generated sequences were synthesized for subsequent sequencing (Tables 2.1). For sequencing of type II RNA1 cDNA from K-Ho1, the PCR product was used as template and corresponding primers are listed in Table 2.1.

CEQ 2000 Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter Inc, Fullerton, CA) and CEQ™ 8000 Genetic Analysis System were used for sequencing. Sequence analysis was performed using the DNA strider (CEA, France) and Vector NTI programs (Informaxinc, Frederick, Maryland).

Results and discussion

The naturally occurring severe strains IL-Cb1 and K-Ho1 are partial diploid reassortants

By cloning and sequencing of IL-Cb1, both type I and type II RNA1 cDNAs were cloned and sequenced (Figures 2.2, 2.3, and 2.4). IL-Cb1 was passaged twice on a local lesion host and the two types of RNA1 were separated in different local lesion isolates (Chapter Three). Slot blot hybridization analysis of the original field isolate (Figure 2.1) and its local lesion derivatives (Chapter Three) as well as RT-PCR cloning all showed that only type I RNA2 was present in BPMV IL-Cb1. So severe strain BPMV IL-Cb1 is diploid for RNA1 but haploid for RNA2. Another severe strain K-Ho1, from which only type I RNA1 was previously cloned, showed a weak hybridization signal to a type II RNA1 cDNA probe (Figure 2.1). To test if type II RNA1 is also present, specific PCR was performed and type II RNA1 was cloned (Figure 2.6). The cloned type II RNA1 from K-Ho1 is unique to type II RNA1 from both K-Ha1 and IL-Cb1 (Figure 2.6). This ruled out the possible contamination of the strains in handling. Therefore, the severe strain K-Ho1 is also a partial diploid reassortant (this study and Gu and Ghabrial 2005).

Emergence of new virus variants as a consequence of genomic reassortment has been observed in natural populations of animal and plant multipartite viruses (Hendenson *et al.*; 1995; Miranda *et al.*, 2000; Lin *et al.*, 2004). Detection of partial diploid reassortants is important in viral evolution and viral virulence monitoring. Both the virus host and the viral vector could be the site for the generation of diploid reassortants. Aphid transmission experiments indicated that a diploid reassortment of CMV was detected in one out of 64 infected tomato plants. However, it was not clear how this diploid reassortment of CMV emerged (Fraile *et al.*, 1997). The role of beetle transmission in the emergence and maintenance of BPMV partial diploid reassortants needs to be critically studied. BPMV accumulates in beetles feeding on infected plants, but it does not replicate in its vector (Ghabrial and Schultz, 1983). With large beetle populations and concomitant high incidence of BPMV, individual beetles may accumulate more than one strain and introduce the mixture into healthy plants. It is not likely to introduce different strains sequentially to the same plant via viruliferous beetles due to cross-protection. In any case, the beetle vectors are the key players in attaining mixed infections and subsequent generation of partial diploid reassortants and new strains that may cause more severe symptoms.

This study presents strong evidence for the presence of stable partial diploid reassortants among field isolates of BPMV. The BPMV partial diploid reassortants are stable at least under greenhouse conditions since both IL-Cb1 and K-Ho1 strains have been maintained in soybean by successive passages at monthly intervals for many years. Whether the diploid reassortants are stable under field conditions has not been experimentally determined. It will be interesting to determine whether haploid reassortants can be generated from the diploid reassortants by beetle transmission since the haploid descendents were isolated by local lesion isolation (Chapter Three). It is possible that the diploid reassortants may serve as a reservoir for the emergence of new strains of multipartite viruses.

Sequence analysis of BPMV strain IL-Cb1

The complete nucleotide sequences of K-G7 RNA1 and RNA2 have been reported (Di *et al.*, 1999; MacFarlane *et al.*, 1991). To decipher the diversity among isolates of BPMV, multiple cDNA clones representing the entire lengths of the genomic RNAs of the severe strain IL-Cb1, were sequenced (Figures 2.2-2.4). At least three clones were used to sequence each RNA. The lengths of the coding and noncoding regions of RNA2 and RNA1 were determined (Figures 2.2-2.4). The percentage nucleotide and deduced amino acid sequence identity of RNA1 and RNA2 among BPMV strains is shown in Figure 2.5.

The complete nucleotide sequences of RNA2 is shown in Figure 2.2. It has 3649 nucleotides, and it is 13 and 24 nucleotides shorter than those of K-G7 and K-Ha1 RNA2, respectively. It was proposed that the translation of CPMV produces two carboxy coterminal polyproteins depending on which start codon is used (van Wezenbeek *et al.*, 1983). The ORFs of IL-Cb1 RNA2 code for two large carboxy co-terminal polyproteins with molecular masses of 113,508 and 113,517 Da, respectively (Figure 2.2). The larger RNA2 ORF is predicted to initiate translation at the AUG at nucleotide positions 443 and to terminate at the UGA at nucleotide positions 3499. The smaller ORF is predicted to start at the AUG at nucleotide positions 749 and to terminate at the same UGA as the larger ORF. Nucleotide sequence alignment of RNA2 of BPMV strains showed that IL-Cb1 has the highest percentage sequence identity (96.6%) with K-G7 RNA2 (Figure 2.5). This result is in good agreement with those of slot blot hybridization analysis, which placed IL-Cb1 RNA2 in subgroup I with K-G7. Although IL-Cb1 has the highest deduced amino acid sequence identity with K-G7 (98%), the difference in identity is not as large as those based on nucleotide identities between different strains (Figure 2.5).

IL-Cb1 contains two types of RNA1. Type I RNA1 is 5986 nucleotides in length excluding the poly (A) tail (Figure 2.3). The complete nucleotide sequence of type II RNA1 is 5989 in length (Figure 2.4). Similar to BPMV strain K-G7 as well as other members of the genus *Comovirus*, sequence analysis of RNA1 of either type I or type II revealed the presence of a single open reading frame (ORF). The type I RNA1 ORF is predicted to initiate at the AUG at nucleotide position 369 and to terminate at the UAG at nucleotide position 5923. The type I RNA1 ORF encodes a polyprotein of 1851 amino acids with calculated molecular mass of 209,491 Da. It is one amino acid longer than that of K-G7 (Di, *et al*., 1999). The type II RNA1 ORF is predicted to initiate at the AUG at nucleotide position 370 and to terminate at the UAG at nucleotide position 5926. The RNA1 ORF codes for a polyprotein with calculated molecular mass of 209,309 Da. The sequence context (ACAAC**AUG**AA) surrounding the start codon in the RNA1 ORFs of type I and type II RNA1 are identical to those of K-G7. Sequence comparisons indicated that type I RNA1 shares very high nucleotide identities with K-G7 RNA1 and K-Ho1 (97.9% and 99.1%, respectively; Figure 2.5B). While the amino acid sequence identity between different RNA1 is not as large as that of nucleotide sequence identity. The percentage of nucleotide sequence identity scores between IL-Cb1 type I RNA1 and IL-Cb1 type II or K-Ha1 (type II) is 85% and 85.3% respectively. The percentage of nucleotide sequence identity scores between IL-Cb1 type II RNA1 and IL-Cb1 type I, K-G7 (type I) and K-Ho1 (type I) is 85%, 83.1% and 82.4% respectively. Relatively higher identity scores, however, were obtained for the deduced amino acid sequences (Figure 2.5). These results are consistent with those of slot blot hybridization, which placed IL-Cb1 type I RNA1 in subgroup I and IL-Cb1 type II RNA1 in subgroup II.

Table 2.1 Primers used in cloning and sequencing

Primer name	Sequence	Position
R1-Rev1	TTTATATTTAAACACACTCATTGCACATAG	5963-5992
	Primers for type I RNA1 cDNA sequencing	
BH-R1-For	TACTGAAGTCCTCGCTCGTTTG	475-496
2BM-R1-For	TGCATTTTGGCTAGCTCC	1034-1051
4HopR1-For	TGAAGGCTTACATGTCGC	1569-1586
5HopR1-For	AACATGGCTGGTTTGGAG	2021-2038
6HopR1-For	TGAACAAGGAAGGCGAGTG	2544-2526
BHR1-Rev	TCCAAGCAGTAGTAGGTAAAC	5672-5652
2BM-R1-Rev	TCACACTCTGACAACTTTC	5109-5091
4HopR1-Rev	ATTCCAACTTGGCAACTC	4554-4537
5HopR1-Rev	TATCCTTCATGCTCTGTGC	4008-3990
6HopR1-Rev	AGAAAAGGTCCCAAGCAG	3452-3435
	Primers for type II RNA1 cDNA sequencing	
Han-R1-For-2nd	TGTGGCTGTGAAGAGGATACTGAAG	461-485
Han-R1-For-3rd	TTGAAGAGGCTGCGAAGG	969-986
Han-R1-For-4th	AGGAAACTTTTGGGATTGG	1460-1478
Han-R1-For-5th	ATTTCAAGTGCTCCATACC	2000-2018
Han-R1-For-6th	TCTCTGCTGGAAGGAAGGAC	2490-2509
Han-R1-Rev-1st	TGCTTCTGAAGGTAATTGAC	5598-5616
Han-R1-Rev-2nd	AGAACCACTAAAGTAAGGC	4996-5104
Han-R1-Rev-3rd	TCCATAGGGAGAATAGTGAAGC	4439-4470
Han-R1-Rev-4th	TGTTCTGTGCCCACCAATC	3984-4002
Han-R1-Rev-5th	TCACTGTCCCAGCAAAAAAG	3439-3468
	Primers for IL-Cb1 RNA2 sequencing	
C1-R2-Rev1	TAGCCCATTCAGAACTCCAC	3304-3322
C1-R2-For1	TCAACTGTGGGGATTTAG	420-440
C1-R2-Rev2	CACTGGTATTGTGGACACTGAAC	2748-2800
C1-R2-For2	GAAAAACACTTGGGCATTGGT	498-515
C1-R2-Rev3	CTTTCCCATCCATCTATTCAACA	2355-2377
C1-R2-For3	GGTATCCCTGCTGATGTTCTT	770-790
C1-R2-Rev4	TCATTCCTTTGATACGGTGGG	1974-1994
C1-R2-For4	GGTGCTATGCTTCTGGTTGAT	1178-1198
C1-R2-Gsp1	ATCAGCAGGGATACCTTTGT	765-784
C1-R2-Gsp2	CAGTAGTGAGAAAGGACGGAAGAAT	608-632
C1-R2-Gsp3	GCACCAATGCCCAAGTGTTTTTCAA	416-440

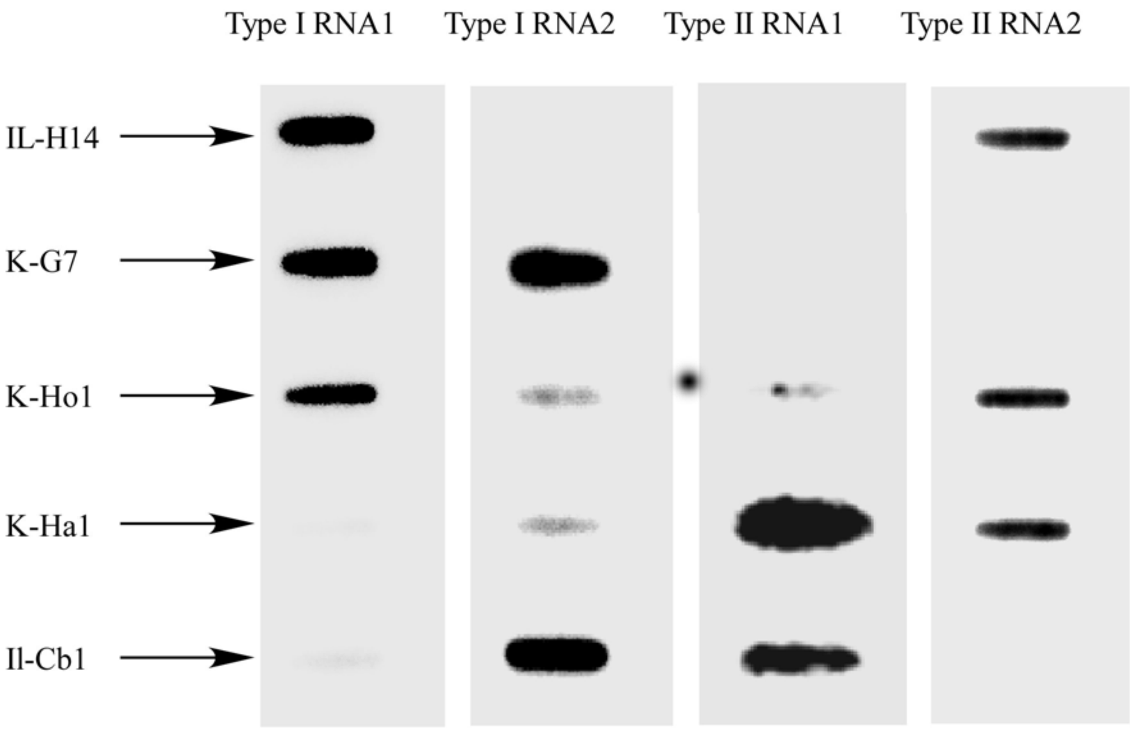

Figure 2.1. Slot blot hybridization analysis of RNA isolated from purified virions of five *Bean pod mottle virus* (BPMV) isolates. RNA samples were blotted (200 ng) onto a Hybond-N^+ (Amersham, Piscataway, NJ) membrane and hybridized with cloned cDNA probes designated as following: Type I RNA1 from K-G7 RNA1; Type I RNA2 from K-G7 RNA2; Type II RNA1 from K-Ha1 RNA1 and Type II RNA2 from K-G7 RNA2.

Figure 2.2. The complete nucleotide sequence of BPMV IL-Cb1 RNA2. The deduced amino acid sequence of the polyprotein encoded by RNA2 is indicated in the one-letter code below the nucleotide sequence.

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   1 TATTAAAATTTTCATAAGATTTGAAATTTTGATAAACCGCGATCATAGGTTGCCGCACCTTAAAACCGGAAACAAAAGCA 80

  81 ATCGTTACTTGATTTCAAAGACTTCTCAATTTCTCTCTACATTTCTTGTATACGGCTTTCAAAGTGAAAGAAAATCACTC 160

 161 TCTGTGCTGGTCACAGACTTCGTGAATCATTTTCTTTCCGCTCTCAGTTCATTTGCCAAACACTCTCCTATTTGACATAG 240

 241 GACTTCGTGTCAGGTTTGAACTTCTCTTATCTCTCTTTCTCGGTTCTTCATCATCTTCTGGGCTAGTGCTCTCACTCTCC 320

 321 TATCTGGTATAGGACTTCGTGAGTAGACTTTCCCATTTCTTCTCTTTTCTCCCACTTCTTCTTGTTCTACACACTGCTGT 400

 401 TCAAAGTGGTCTTATTTGAAAAACACTTGGGCATTGGTGCAA ATG TTT GCT TCA TTC ATC TTT TCT GGT   469
   1                                            M   F   A   S   F   I   F   S   G     9

 470 GAC AAT AAG CTT ACT GAG AAA ACA ATT TTC AAC TGT GGG GAT TTA GAT ATT TTG GTT GTT   529
  10 D   N   K   L   T   E   K   T   I   F   N   C   G   D   L   D   I   L   V   V     29

 530 TAT TAT ACA ATA GCC ACT CAG TTC AGG AAG TTT CTT CCT CAT TAT ATT AGG TGG CAT TTG   589
  30 Y   Y   T   I   A   T   Q   F   R   K   F   L   P   H   Y   I   R   W   H   L     49

 590 TAT ACG CTG TTG ATT TAT ATT CTT CCG TCC TTT CTC ACT ACT GAA ATC AAG TAC AAG CGA   649
  50 Y   T   L   L   I   Y   I   L   P   S   F   L   T   T   E   I   K   Y   K   R     69

 650 AAT TTG AGC AAC GTT CAT ATT TCT GGC TTG TTC TAC GAT AAT AGG TAT AAA TTC TGG ACT   709
  70 N   L   S   N   V   H   I   S   G   L   F   Y   D   N   R   Y   K   F   W   T     89

 710 AAG CAC GAC AAA AAT CTT GCC TTA ACA GAA GAG GAG AAG ATG GAA GTG ATT AGA AAC AAA   769
  90 K   H   D   K   N   L   A   L   T   E   E   E   K   M   E   V   I   R   N   K     109

 770 GGT ATC CCT GCT GAT GTT CTT GCA AAG CGC GCT CAT GAA TTT GAA AAA CAT GTC GCT CAT   829
 110 G   I   P   A   D   V   L   A   K   R   A   H   E   F   E   K   H   V   A   H     129

 830 GAA AGT CTC AAG GAT CAA ATT CCT GCT GTT GAT AAG TTG TAC TCT ACT AAG GTT AGT AAA   889
 130 E   S   L   K   D   Q   I   P   A   V   D   K   L   Y   S   T   K   V   S   K     149

 890 TTT GCA AAA ATT ATG AAT CTT AGA CAG AGT GTT GTT GGT GAT CTC AAA CTT CTT ACT GAT   949
 150 F   A   K   I   M   N   L   R   Q   S   V   V   G   D   L   K   L   L   T   D     169

 950 GGG AAG TTG TAT GAG GGT AAG CAC ATT CCT GTA TCT AAT ATT AGT GCG GGA GAA AAT CAT   1009
 170 G   K   L   Y   E   G   K   H   I   P   V   S   N   I   S   A   G   E   N   H     189

1010 GTG GTG CAG ATA CCC TTG ATG GCA CAG GAG GAA ATT CTG TCT TCT AGT GCA AGT GAT TTC   1069
 190 V   V   Q   I   P   L   M   A   Q   E   E   I   L   S   S   S   A   S   D   F     209

1070 AAG ACT GCT ATA GTA AGC AAA AGT AGC AAA CCT CAA GCT ACA GCA ATG CAT GTT GGG GCT   1129
 210 K   T   A   I   V   S   K   S   S   K   P   Q   A   T   A   M   H   V   G   A     229

1130 ATA GAA ATT ATC ATT GAT AGT TTC GCT AGC CCC GAT TGC AAC ATA GTT GGT GCT ATG CTT   1189
 230 I   E   I   I   I   D   S   F   A   S   P   D   C   N   I   V   G   A   M   L     249

1190 CTG GTT GAT ACA TAT CAC ACT AAT CCT GAA AAT GCA GTC CGT AGT ATT TTT GTT GCA CCC   1249
 250 L   V   D   T   Y   H   T   N   P   E   N   A   V   R   S   I   F   V   A   P     269

1250 TTT AGG GGT GGT AGA CCC ATT CGG GTT GTT ACT TTC CCA AAC ACC ATT GTG CAG ATT GAA   1309
 270 F   R   G   G   R   P   I   R   V   V   T   F   P   N   T   I   V   Q   I   E     289

1310 CCA GAT ATG AAC TCA AGG TTT CAA CTT TTG AGT ACA ACC ACC AAT GGT GAC TTT GTT CAA   1369
 290 P   D   M   N   S   R   F   Q   L   L   S   T   T   T   N   G   D   F   V   Q     309

1370 GGA AAA GAT CTT GCA ATG GTT AAG GTT AAT GTA GCA TGT GCT GCT GTA GGC TTA ACA TCA   1429
 310 G   K   D   L   A   M   V   K   V   N   V   A   C   A   A   V   G   L   T   S     329

1430 AGT TAC ACT CCA ACT CCA TTG TTA GAA TCT GGT CTG CAG AAA GAT AGG GGT CTT ATT GTT   1489
 330 S   Y   T   P   T   P   L   L   E   S   G   L   Q   K   D   R   G   L   I   V     349

1490 GAA TAT TTT GGA AGA ATG TCT TAT GTT GCT CAT AAC ATC AAT CAA CCT CAA GAG AAA GAT   1549
 350 E   Y   F   G   R   M   S   Y   V   A   H   N   I   N   Q   P   Q   E   K   D     369

1550 TTG TTG GAG GGA AAT TTT TCC TTC GAT ATT AAA TCT CGC TCC AGG TTA GAG AAA GTT TCT   1609
 370 L   L   E   G   N   F   S   F   D   I   K   S   R   S   R   L   E   K   V   S     389
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Figure 2.2. continued.

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1610 TCT ACG AAG GCA CAA TTT GTC AGT GGA AAA ACT TTT AAA TAT GAT ATA ATT GGT GCT GGT  1669
 390 S   T   K   A   Q   F   V   S   G   K   T   F   K   Y   D   I   I   G   A   G    409

1670 TCA CAT TCT TCT GAG GAA GTT TCT GAG GAA AAG AAT CAG GGA AAG ACA AAG CAT GTT GAT  1729
 410 S   H   S   S   E   E   V   S   E   E   K   N   Q   G   K   T   K   H   V   D    429

1730 GCT AGG TTG AGG CAA AGA ATA GAT CCA CAA TAC AAT GAA GTT CAA GCT CAA ATG GAA ACA  1789
 430 A   R   L   R   Q   R   I   D   P   Q   Y   N   E   V   Q   A   Q   M   E   T    449

1790 AAT CTA TTC AAA TTG TCT CTT GAT GAT GTT GAG ACT CCA AAA GGT TCC ATG TTA GAC CTC  1849
 450 N   L   F   K   L   S   L   D   D   V   E   T   P   K   G   S   M   L   D   L    469

1850 AAG ATT TCC CAA TCT AAG ATT GCA CTT CCC AAA AAT ACA GTT GGA GGG ACC ATT TTG CGC  1909
 470 K   I   S   Q   S   K   I   A   L   P   K   N   T   V   G   G   T   I   L   R    489

1910 AGT GAT CTG TTG GCA AAT TTC TTG ACA GAA GGC AAT TTT AGA GCA AGT GTT GAT TTG CAA  1969
 490 S   D   L   L   A   N   F   L   T   E   G   N   F   R   A   S   V   D   L   Q    509

1970 CGT ACC CAC CGT ATC AAA GGA ATG ATT AAA ATG GTG GCT ACA GTT GGC ATT CCT GAA AAC  2029
 510 R   T   H   R   I   K   G   M   I   K   M   V   A   T   V   G   I   P   E   N    529

2030 ACA GGT ATA GCA CTG GCT TGT GCA ATG AAT AGT TCC ATT AGA GGG CGT GCC AGT TCT GAT  2089
 530 T   G   I   A   L   A   C   A   M   N   S   S   I   R   G   R   A   S   S   D    549

2090 ATC TAT ACC ATT TGT TCG CAA GAT TGT GAA CTA TGG AAT CCT GCT TGT ACA AAA GCA ATG  2149
 550 I   Y   T   I   C   S   Q   D   C   E   L   W   N   P   A   C   T   K   A   M    569

2150 ACC ATG TCA TTT AAT CCA AAC CCA TGT TCT GAT GCA TGG AGT TTG GAA TTT CTT AAA CGT  2209
 570 T   M   S   F   N   P   N   P   C   S   D   A   W   S   L   E   F   L   K   R    589

2210 ACT GGG TTC CAC TGT GAC ATT ATT TGC GTT ACT GGA TGG ACT GCA ACT CCA ATG CAA GAT  2269
 590 T   G   F   H   C   D   I   I   C   V   T   G   W   T   A   T   P   M   Q   D    609

2270 GTT CAA GTT ACA ATT GAT TGG TTC ATT TCC TCT CAG GAG TGC GTT CCC AGA ACC TAT TGT  2329
 610 V   Q   V   T   I   D   W   F   I   S   S   Q   E   C   V   P   R   T   Y   C    629

2330 GTT TTG AAT CCA CAA AAT CCT TTT GTG TTG AAT AGA TGG ATG GGA AAG CTG ACT TTT CCT  2389
 630 V   L   N   P   Q   N   P   F   V   L   N   R   W   M   G   K   L   T   F   P    649

2390 CAA GGC ACT TCT CGA AGT GTT AAA AGG ATG CCT CTT TCT ATA GGA GGA GGA GCT GGT GCT  2449
 650 Q   G   T   S   R   S   V   K   R   M   P   L   S   I   G   G   G   A   G   A    669

2450 AAA AAT GCC ATT CTC ATG AAT ATG CCA AAT GCA GTT CTT TCA ATG TGG AGG TAC TTT GTA  2509
 670 K   N   A   I   L   M   N   M   P   N   A   V   L   S   M   W   R   Y   F   V    689

2510 GGA GAT CTT GTT TTC GAA GTT TCA AAG ATG ACC TCT CCT TAC ATT AAA TGT ACA GTA TCT  2569
 690 G   D   L   V   F   E   V   S   K   M   T   S   P   Y   I   K   C   T   V   S    709

2570 TTT TTC ATA GCA TTT GGA AAT TTG GCT GAT GAT ACC ATC AAT TTT GAA GCT TTT CCT CAT  2629
 710 F   F   I   A   F   G   N   L   A   D   D   T   I   N   F   E   A   F   P   H    729

2630 AAA TTG GTG CAG TTT GGA GAA ATT CAA GAA AAA GTT GTG CTA AAA TTT TCA CAA GAG GAG  2689
 730 K   L   V   Q   F   G   E   I   Q   E   K   V   V   L   K   F   S   Q   E   E    749

2690 TTT CTC ACA GCA TGG TCC ACT CAG GTG CGT CCT ACA ACA ACC TTG TTG GCT GAT GGG TGC  2749
 750 F   L   T   A   W   S   T   Q   V   R   P   T   T   T   L   L   A   D   G   C    769

2750 CCA TAT TTG TAT GCT ATG GTG CAT GAT AGT TCA GTG TCC ACA ATA CCA GGT GAT TTT GTC  2809
 770 P   Y   L   Y   A   M   V   H   D   S   S   V   S   T   I   P   G   D   F   V    789

2810 ATT GGT GTC AAG TTG ACG ACC ATA GAA AAC ATG TGC GCA TAT GGA CTT AAT CCT GGT ATT  2869
 790 I   G   V   K   L   T   T   I   E   N   M   C   A   Y   G   L   N   P   G   I    809

2870 TCA GGT TCT CGT CTT CTT GGC ACC ATT CCT CAA TCT ATC TCT CAG CAG ACC GTT TGG AAT  2929
 810 S   G   S   R   L   L   G   T   I   P   Q   S   I   S   Q   Q   T   V   W   N    829

2930 CAA ATG GCA ACA GTG AGA ACA CCA TTG AAC TTT GAT TCA AGC AAA CAA AGC TTT TGC CAA  2989
 830 Q   M   A   T   V   R   T   P   L   N   F   D   S   S   K   Q   S   F   C   Q    849

2990 TTT TCT ATA GAT CTC CTT GGT GGA GGC ATC TTA GTA GAC AAA ACT GGA GAT TGG ATT ACA  3049
 850 F   S   I   D   L   L   G   G   G   I   L   V   D   K   T   G   D   W   I   T    869

3050 CTT GTG CAA AAT TCT CCA ATT AGT AAT CTA TTG AGA GTT GCT GCC TGG AAG AAG GGT TGT  3109
 870 L   V   Q   N   S   P   I   S   N   L   L   R   V   A   A   W   K   K   G   C    889
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Figure 2.2. continued.

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3110 CTG ATG GTT AAA ATT GTA ATG TCT GGA AAT GCA GCA GTT AAG AGG AGT GAT TGG GCA TCA  3169
 890 L   M   V   K   I   V   M   S   G   N   A   A   V   K   R   S   D   W   A   S    909

3170 TTA GTG CAA GTG TTC CTA ATA AAT AGT AAT AGT ACA GAG CAC TTT GAT GCA TGC AAG TGG  3229
 910 L   V   Q   V   F   L   I   N   S   N   S   T   E   H   F   D   A   C   K   W    929

3230 ACT AAA TCA GAA CCA CAT TCG TGG GAA TTG ATT TTT CCA ATA GAA GTG TGT GGT CCC AAT  3289
 930 T   K   S   E   P   H   S   W   E   L   I   F   P   I   E   V   C   G   P   N    949

3290 AAT GGT TTT GAA ATG TGG AGT TCT GAA TGG GCT AAT CAA ACT TCA TGG CAT CTA AGT TTC  3349
 950 N   G   F   E   M   W   S   S   E   W   A   N   Q   T   S   W   H   L   S   F    969

3350 CTT GTT GAT AAT CCC AAA CAA TCC ACG GTT TTT GAT GTT CTT TTA GGG ATT TCA CAA AAC  3409
 970 L   V   D   N   P   K   Q   S   T   V   F   D   V   L   L   G   I   S   Q   N    989

3410 TTT GAA ATT GCT GGA AAC ACT TTA ATG CCA GCT TTC TCT GTT CCA CAG GCC AAT GCC AGA  3469
 990 F   E   I   A   G   N   T   L   M   P   A   F   S   V   P   Q   A   N   A   R    1009

3470 TCT TCT GAA AAT GCA GAA TCT TCT GCA TGA TCTGGTAGTAGCGTTTTCTTTTTATTTGTTTTTGTTTCCA 3539
1010 S   S   E   N   A   E   S   S   A   *                                            1019

3540 ATCAAATAAAGGAAATTAGGC ATG ACC CTC GTC TGA GA ATG CTC TGC CTA TTT GAA AAT TTC CAC 3604


3605 ACC TCT TTT AAG TAT TGT AAT GGT ATG TGA AGTGTGTGTTATTTTAAAAAAAA                  3657
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Figure 2.3. The complete nucleotide sequence of type I RNA1 from BPMV IL-Cb1. The deduced amino acid sequence of the polyprotein encoded by RNA1 is indicated in the one-letter code below the nucleotide sequence.

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   1 TATTAAAATTTTCATAAGATTTGAAATTTTGATAAACCGCGATCATAGGTTGCCGCACCTTAAAACCGGAAACAAAAGCA 80

  81 ATCGTTACTTGATTTCAAAGACTTCTCAATTTCTCTCTACATTTCTTGTATACGGCTTTCAAAGTGAAAGAAAATCACTC 160

 161 TCTGTGCTGGTCACAGACTTCGTGAATCATTTTCTTTCTGCTCTCAGTTCATTTGCTGAACACTCTCCTATTTGATATAG 240

 241 GACTTCGTGTCAGATTTGAACTTCTCCTATCTCTCTTTCTCGGTTCTTCATTTGATTTCAAATTTCTCTGAAATTTAAAT 320

 321 TTCTTTTGACATTTTGAACTTTGTGTTGGCCTCATTTGAAAAACAAC ATG AAG TTC TAT CCT GGT CAA AAT  391
   1                                                 M   K   F   Y   P   G   Q   N    8

 392 ATT TCT GAA ATT GTT TAC CAC TTT CAG AGT AAT GAG ACA GCC AAT AGG TTA GAT GCA TAT  451
   9 I   S   E   I   V   Y   H   F   Q   S   N   E   T   A   N   R   L   D   A   Y    28

 452 TTT GCC TGT GGC TGT GAG GAG GAT ACT GAA GTC CTC GCT CGT TTG AAG CAG TGT AAC CCT  511
  29 F   A   C   G   C   E   E   D   T   E   V   L   A   R   L   K   Q   C   N   P    48

 512 CGT CTG CTT CAT TTG TCA TAT GCT GCC TTT TGT TTA GAA ATG GGC AGT CAT TCA ATA GAG  571
  49 R   L   L   H   L   S   Y   A   A   F   C   L   E   M   G   S   H   S   I   E    68

 572 GAA ATG GAA TAT GAT GAT GGG GAA TTA ATT TTT TCC TAT TTT CAA AAT TTT TTG CTT TCC  631
  69 E   M   E   Y   D   D   G   E   L   I   F   S   Y   F   Q   N   F   L   L   S    88

 632 ATC GTT TCC AAC TCT TCT AAA ACA ACC AAA TTG AGA GCA TAC ATT CGT TCA ACA TTT GCA  691
  89 I   V   S   N   S   S   K   T   T   K   L   R   A   Y   I   R   S   T   F   A    108

 692 TAT CAT TTT CAG CAT TTT GTT GAA TTT GAT CAA TAC ACA AAT GAT TCT CTC AAT ACT GTA  751
 109 Y   H   F   Q   H   F   V   E   F   D   Q   Y   T   N   D   S   L   N   T   V    128

 752 GAT ACA AGT GTA TCA GCC CAA GGG ATA GCA GAC TTG GCT CTC TCT ATG GTC AGA TGG ATA  811
 129 D   T   S   V   S   A   Q   G   I   A   D   L   A   L   S   M   V   R   W   I    148

 812 CCC ACT CAG ATT AAA AAA GTT GTT AAT TTT GGT GTG GGA TCT GTT ATA GAG TCT TTT TCA  871
 149 P   T   Q   I   K   K   V   V   N   F   G   V   G   S   V   I   E   S   F   S    168

 872 GAG CAT TTT AAT AAG CTC TTG ATG CAA TAT TGT CCA ATA GTT TTT CAA GCT TTC AGC TGG  931
 169 E   H   F   N   K   L   L   M   Q   Y   C   P   I   V   F   Q   A   F   S   W    188

 932 GTT AAC AAT ATT TGG ACA ATG GTC AAA GAA TGG ATA GAA GAA GCT GCG AAA GAA ATT TCA  991
 189 V   N   N   I   W   T   M   V   K   E   W   I   E   E   A   A   K   E   I   S    208

 992 TGG TTC TTG CAA GGA TGT AAA GAG CTG CTA GCC TGG GGA ATG TGC ATT TTG GCT AGC TCC  1051
 209 W   F   L   Q   G   C   K   E   L   L   A   W   G   M   C   I   L   A   S   S    228

1052 TGT GCT CTA GGA TTG GTT GAA AAA TGC CTT ATC TCT TTG GGC ATG ATT TCT GAA TCT TTT  1111
 229 C   A   L   G   L   V   E   K   C   L   I   S   L   G   M   I   S   E   S   F    248

1112 GAT TTG GTT GGT TTG TTT GTT CGA TCT GCC ATT GTG GGA GCT TTC TGT GTT TCC ATA AAA  1171
 249 D   L   V   G   L   F   V   R   S   A   I   V   G   A   F   C   V   S   I   K    268

1172 ACT GGT AAG TTC ATC ACA AAC AGT GAG TTG ATT ACT TGT GCT ACC ATT GCA GTT TCT ACA  1231
 269 T   G   K   F   I   T   N   S   E   L   I   T   C   A   T   I   A   V   S   T    288

1232 ATA GCA ACT GTA ATG TCT CAG GCT TTT AAG CCT TCT GAA GAG ATT AAG GGA CAG TTC CAA  1291
 289 I   A   T   V   M   S   Q   A   F   K   P   S   E   E   I   K   G   Q   F   Q    308

1292 GCC CTT TCA GTT CTA GAA GGG TTG GCA ACA CAG CTC ACT TCA TTT TGT GAC ACA TCT TTA  1351
 309 A   L   S   V   L   E   G   L   A   T   Q   L   T   S   F   C   D   T   S   L    328

1352 GTT GCT ATG GGA AAA ACC TGC ACA GCT TTT AAT CAA ATT TGC ACT GCT GGC AAA AAT GTT  1411
 329 V   A   M   G   K   T   C   T   A   F   N   Q   I   C   T   A   G   K   N   V    348

1412 AAG GTG ATT GCA GGT AGG TTA CTA GAA GTT GTT TCC AAT TTT GTC AGA AAA TTA TTA GGA  1471
 349 K   V   I   A   G   R   L   L   E   V   V   S   N   F   V   R   K   L   L   G    368

1472 TTG GAC AGT GTT TTT CTC AGA GAT GCT GCA CTC ATC TTT TCT CAA GAT GTG GAT GGG TGG  1531
 369 L   D   S   V   F   L   R   D   A   A   L   I   F   S   Q   D   V   D   G   W    388
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Figure 2.3. continued.

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1532 TTG CGT AAC ATC AGT TGG TGC CAA GAA CAG TTT TTG TTG AAA GCT TAC ATG TCA CAA GAT  1591
 389 L   R   N   I   S   W   C   Q   E   Q   F   L   L   K   A   Y   M   S   Q   D    408

1592 GAT CTT ATT GTC CTG CGC TCT TTA GTT GTC AAA GGT GAA AGA ATG AGG GAA CAG ATG CTT  1651
 409 D   L   I   V   L   R   S   L   V   V   K   G   E   R   M   R   E   Q   M   L    428

1652 GAA GGA GAA GTT AAG GTG TCT CCA AGT GTT TGC AAC CTT ATT GTC AAA GGC TGT GAA GAA  1711
 429 E   G   E   V   K   V   S   P   S   V   C   N   L   I   V   K   G   C   E   E    448

1712 GCA AAT AAA TTG ATG CGT GAG AGC GCA CTT CAT TGT TCA AAA ACA ATT AGG AAG ATT CCT  1771
 449 A   N   K   L   M   R   E   S   A   L   H   C   S   K   T   I   R   K   I   P    468

1772 TTT GTT ATT TTT GCT CAC GGT GAA TCC CGG GTT GGG AAA TCT CTG CTG GTT GAT AGG CTA  1831
 469 F   V   I   F   A   H   G   E   S   R   V   G   K   S   L   L   V   D   R   L    488

1832 ATC ACA GAT TTC TGT GAT CAT TTG GAA ATT GGA GAA GAT GCT GTG TAC TCA AGG AAT CCA  1891
 489 I   T   D   F   C   D   H   L   E   I   G   E   D   A   V   Y   S   R   N   P    508

1892 TCA GAT CCT TTC TGG AGT GGA TAT AGA AGG CAG CCA ATT GTT ACT ATT GAT GAT TTT GCT  1951
 509 S   D   P   F   W   S   G   Y   R   R   Q   P   I   V   T   I   D   D   F   A    528

1952 GCT GTT GTT TCG GAG CCA TCT GCT GAA GCT CAG TTA ATT CCA TTA GTT TCA AGT GCT CCT  2011
 529 A   V   V   S   E   P   S   A   E   A   Q   L   I   P   L   V   S   S   A   P    548

2012 TAT CCA TTA AAC ATG GCT GGT TTG GAG GAA AAG GGA ATG CAC TTT GAT TCC CAG ATC ATG  2071
 549 Y   P   L   N   M   A   G   L   E   E   K   G   M   H   F   D   S   Q   I   M    568

2072 ATG TGT TCT TCA AAT TTT TTA GAG CCG TCT CCT GAA GCT AAA ATT AGA GAT GAT ATG GCT  2131
 569 M   C   S   S   N   F   L   E   P   S   P   E   A   K   I   R   D   D   M   A    588

2132 TTT AGA AAT CGA AGA CAT GTG CTG ATC ACA GTT GAA CTC AAA CCT GGG GTT GAA TAT GAT  2191
 589 F   R   N   R   R   H   V   L   I   T   V   E   L   K   P   G   V   E   Y   D    608

2192 GAG AGT GAT TTT ACT AAA AAT CAG CGA TAT TTG CTG AAA ACT TGG TTT CAT GAT CAT TAT  2251
 609 E   S   D   F   T   K   N   Q   R   Y   L   L   K   T   W   F   H   D   H   Y    628

2252 GTT GTA GAC CAA ACT TTC GAG TCT TAT GCT GAT CTG CTG GCA CAT TGT TTT ACC AAG TGG  2311
 629 V   V   D   Q   T   F   E   S   Y   A   D   L   L   A   H   C   F   T   K   W    648

2312 GAG AGA CAT GTT AAG GAG CAA GAG TCA AAT CTG TCT CAA ATT AAG GGC AAG AAA AAT GAA  2371
 649 E   R   H   V   K   E   Q   E   S   N   L   S   Q   I   K   G   K   K   N   E    668

2372 AGT GGT CAT TTC AAT AAC TTT CAA CAA CTT ATG GAT TTG GCT GTT TCA TGG AAC CTT AGT  2431
 669 S   G   H   F   N   N   F   Q   Q   L   M   D   L   A   V   S   W   N   L   S    688

2432 GCA GAT ATC ATG AAA AAC AGG ATC AAG GCT GAG AGA AAT GAC ATG GTT TAT GTT TTT TCT  2491
 689 A   D   I   M   K   N   R   I   K   A   E   R   N   D   M   V   Y   V   F   S    708

2492 GCA GGG AGG AAG GAT AAA ATT TTT CAT TGT TTT CTG AAC AAG GAA GGC GAG TGC ACG GTT  2551
 709 A   G   R   K   D   K   I   F   H   C   F   L   N   K   E   G   E   C   T   V    728

2552 CGT CCT GAT TCA ATA GAT GAT CCT GAA GCG CAA GCT TTG CTC AAA GCT TCA GAG ACA ATG  2611
 729 R   P   D   S   I   D   D   P   E   A   Q   A   L   L   K   A   S   E   T   M    748

2612 CTC ATG AAA GCC TAT GCC TTC CTC AAA TAC AAT AAT GCA ACA AAT TTG ATT GTC AGA ACC  2671
 749 L   M   K   A   Y   A   F   L   K   Y   N   N   A   T   N   L   I   V   R   T    768

2672 CAT TTG GCA GAA CTG GTG AAT GAA GAT TTC TAT GAT GAG AAA TTC AAT TTC ATT GGA ACA  2731
 769 H   L   A   E   L   V   N   E   D   F   Y   D   E   K   F   N   F   I   G   T    788

2732 ATT GGA ACA CCG GCT TTT CAT CGC CAA ATA GCT GCA CAT TTG GAA AAG ATG CCA TTG TGG  2791
 789 I   G   T   P   A   F   H   R   Q   I   A   A   H   L   E   K   M   P   L   W    808

2792 CAA AAA GCA ATT TTG TGT GGA ATG GGA CAT TGT TTG TCT CGG AAA AGC AAA GAA ACC TGG  2851
 809 Q   K   A   I   L   C   G   M   G   H   C   L   S   R   K   S   K   E   T   W    828

2852 TAT ACT GGT ATG AAG GAG AAA TTT GTG CAG ATG ATG AAA AGC ATC TAT GAA ACT GAA GTC  2911
 829 Y   T   G   M   K   E   K   F   V   Q   M   M   K   S   I   Y   E   T   E   V    848

2912 ACA GAT TGG CCA GTG CCA TTG AAA ATC ATT TCT GGT ACT ATT CTA GCC ACC ATT TTG GGA  2971
 849 T   D   W   P   V   P   L   K   I   I   S   G   T   I   L   A   T   I   L   G    868

2972 ACA ACT TTT TGG AAG TTA TTT TCC TTT TTA AGG GAT GCT GGT AAT GGA GGT GTT TTT GTT  3031
 869 T   T   F   W   K   L   F   S   F   L   R   D   A   G   N   G   G   V   F   V    888

3032 GGT AAT GTT GCT TCA GCA TTT ACT ACA TCA AGT GTG CTC GAA GCG CAA AGC CGA AAA CCC  3091
 889 G   N   V   A   S   A   F   T   T   S   S   V   L   E   A   Q   S   R   K   P    908
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Figure 2.3. continued.

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3092 AAC AGA TAT GAG GTC TCT CAA TAT AGG TAT CGC AAT GTG CCA ATA AAG CGC AGA GCG TGG  3151
 909 N   R   Y   E   V   S   Q   Y   R   Y   R   N   V   P   I   K   R   R   A   W    928

3152 GTT GAG GGC CAA ATG TCT TTT GAT CAA TCA GTG GTA GCA ATT ATG TCA AAA TGT AAA GCC  3211
 929 V   E   G   Q   M   S   F   D   Q   S   V   V   A   I   M   S   K   C   K   A    948

3212 AGT ATG AGA ATG GGA AAC ACT GAT GCT CAA ATT TTG ATG GTT CCA GGG CGT AGA TTC ATT  3271
 949 S   M   R   M   G   N   T   D   A   Q   I   L   M   V   P   G   R   R   F   I    968

3272 GCA CAT GGT CAT TTT TTC AAG AAT CTC ACC CAA AAA GTT AGA GTC CAA ATT GTT ACT TCT  3331
 969 A   H   G   H   F   F   K   N   L   T   Q   K   V   R   V   Q   I   V   T   S    988

3332 GAG AAA ACC TAT TGG CAT GTG TAC GAT CCT GAT AAA TTT CAA ATG TTT GAT AAC AGT GAA  3391
 989 E   K   T   Y   W   H   V   Y   D   P   D   K   F   Q   M   F   D   N   S   E    1008

3392 ATC GGG TTG TAT ACA AAT CCA ACT TTG GAG GAC ATC CCA CAT TCT GCT TGG GAC CTT TTC  3451
1009 I   G   L   Y   T   N   P   T   L   E   D   I   P   H   S   A   W   D   L   F    1028

3452 TGC TGG GAC AGT GAG AAA ACT CTG CCA AAT AAT TTT TCT GCT GAA TTG CTT TCC TGT AAA  3511
1029 C   W   D   S   E   K   T   L   P   N   N   F   S   A   E   L   L   S   C   K    1048

3512 TTG GAC ACT GTT ACG GGA CAA TAT TAC CCA GAA TGG GCT CCA ATA AAT TGT CGA GTA CAT  3571
1049 L   D   T   V   T   G   Q   Y   Y   P   E   W   A   P   I   N   C   R   V   H    1068

3572 CGG CAA CCA ATT CAC ATA ACT GAA GGG AAT TAT GTT AGG AAA CAA GAT GTA AGC ATC GAA  3631
1069 R   Q   P   I   H   I   T   E   G   N   Y   V   R   K   Q   D   V   S   I   E    1088

3632 TAT GAT GCC TGC ACA ATT CCT AAT GAT TGT GGA TCT CTG GTG GTT GCT AAG GTC GGA AAT  3691
1089 Y   D   A   C   T   I   P   N   D   C   G   S   L   V   V   A   K   V   G   N    1108

3692 CAC AAG CAA GTT GTT GGT TTT CAT GTT GCT GGA AGC AAA GGA AGA TTG GGC TAT GCT TCA  3751
1109 H   K   Q   V   V   G   F   H   V   A   G   S   K   G   R   L   G   Y   A   S    1128

3752 TTA ATA CCA TAT GTT GAG CCT GTG GTA CAA GCC CAA AGT GCT GAA GTC TAT TTT GAC TTT  3811
1129 L   I   P   Y   V   E   P   V   V   Q   A   Q   S   A   E   V   Y   F   D   F    1148

3812 TTT CCT GTG GAA GTT GAT AGT CAA GAG GGA GTT GCT CAT ATT GGT GAA CTC AAA TCT GGA  3871
1149 F   P   V   E   V   D   S   Q   E   G   V   A   H   I   G   E   L   K   S   G    1168

3872 GTT TAT GTA CCA CTG CCC ACA AAA ACT AAT CTT GTG GAA ACT CCC AAA GAA TGG CAG TTG  3931
1169 V   Y   V   P   L   P   T   K   T   N   L   V   E   T   P   K   E   W   Q   L    1188

3932 GAT TTG CCT TGT GAT AAG ATT CCA AGT GTG TTA ACC ACT ACT GAT GAG AGA TTG GTT GGC  3991
1189 D   L   P   C   D   K   I   P   S   V   L   T   T   T   D   E   R   L   V   G    1208

3992 ACA GAG CAT GAA GGA TAT GAC CCA TTT CTT GGT GGT ATT CAA AAA TAT GCA ACT CCC ATG  4051
1209 T   E   H   E   G   Y   D   P   F   L   G   G   I   Q   K   Y   A   T   P   M    1228

4052 ATG CCT CTT GAT GAG GAG ATT CTT TCC AAA GTT GCA CAA GAC ATG GTT GAA GAA TGG TTT  4111
1229 M   P   L   D   E   E   I   L   S   K   V   A   Q   D   M   V   E   E   W   F    1248

4112 GAT TGT GTT GAT GAG GAG GAT ACA TTT GAA GAA GTT TCT TTG AGT GCT GCA CTC AAT GGT  4171
1249 D   C   V   D   E   E   D   T   F   E   E   V   S   L   S   A   A   L   N   G    1268

4172 GTT GAA GGT TTG GAT TAC ATG GAA CGC ATT CCT CTT GCC ACT TCA GAG GGT TTT CCT CAT  4231
1269 V   E   G   L   D   Y   M   E   R   I   P   L   A   T   S   E   G   F   P   H    1288

4232 GTT CTG TCC AGG AAA AAT GGT GAA AAA GGC AAG AGA AGA TTT GTC ACT GGA GAT GGT GAA  4291
1289 V   L   S   R   K   N   G   E   K   G   K   R   R   F   V   T   G   D   G   E    1308

4292 GAA ATG TCA CTA ATT CCT GGT ACC AGT GTT GAA GAA GCA TAC AAT AAA TTG ACT GTT GAA  4351
1309 E   M   S   L   I   P   G   T   S   V   E   E   A   Y   N   K   L   T   V   E    1328

4352 CTA GAA AAG TGT GTT CCA ACA TTG GTT GGC ATA GAA TGT CCC AAA GAT GAA AAA CTT CCC  4411
1329 L   E   K   C   V   P   T   L   V   G   I   E   C   P   K   D   E   K   L   P    1348

4412 CGT CGC AAA ATT TTT GAT AAA CCC AAG ACG CGC TGC TTC ACC ATA CTT TCT ATG GAA TTT  4471
1349 R   R   K   I   F   D   K   P   K   T   R   C   F   T   I   L   S   M   E   F    1368

4472 AAT CTA GTG GTG CGT CAA AAA TTC TTG AAT TTT GTG CGA TTC ATT ATG AAG AAA AGG GAC  4531
1369 N   L   V   V   R   Q   K   F   L   N   F   V   R   F   I   M   K   K   R   D    1388

4532 AAA TTG AGT TGC CAA GTT GGA ATC AAT CCA TAT TCT ATG GAG TGG ACT GGT TTG GCA AAT  4591
1389 K   L   S   C   Q   V   G   I   N   P   Y   S   M   E   W   T   G   L   A   N    1408

4592 AGA CTG TTG AGC AAG GGA AAT GAC ATT TTG TGT TGT GAC TAT GCT AGT TTT GAT GGT CTG  4651
1409 R   L   L   S   K   G   N   D   I   L   C   C   D   Y   A   S   F   D   G   L    1428
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Figure 2.3. continued.

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4652 ATA ACT AAG CAA GTT ATG AGC AAG ATG GCA GAA ATG ATA AAC AGT CTT TGT GGT GGA GAT  4711
1429 I   T   K   Q   V   M   S   K   M   A   E   M   I   N   S   L   C   G   G   D    1448

4712 GAG AAA CTG ATG CGT GAG AGA ACA CAT CTT CTG TTA GCT TGT TGC TCC AGG ATG GCA ATC  4771
1449 E   K   L   M   R   E   R   T   H   L   L   L   A   C   C   S   R   M   A   I    1468

4772 TGT AAA AAA AAT GTT TGG AGA GTT GAG TGT GGT ATC CCT TCT GGA TTT CCA CTC ACT GTT  4831
1469 C   K   K   N   V   W   R   V   E   C   G   I   P   S   G   F   P   L   T   V    1488

4832 ATC TGT AAT AGC ATT TTC AAT GAG ATG CTT ATC AGA TAT AGT TAT GAA AAG TTG TTG CGT  4891
1489 I   C   N   S   I   F   N   E   M   L   I   R   Y   S   Y   E   K   L   L   R    1508

4892 CAA GCT AAG GCT CCT AGT ATG TTT CTC CAG TCT TTT AAA AAT TTT ATT TCT TTG TGT GTT  4951
1509 Q   A   K   A   P   S   M   F   L   Q   S   F   K   N   F   I   S   L   C   V    1528

4952 TAT GGA GAT GAT AAT TTA ATT AGT GTT CAT GAG TAT GTT AAG CCA TAT TTT AGT GGT TCT  5011
1529 Y   G   D   D   N   L   I   S   V   H   E   Y   V   K   P   Y   F   S   G   S    1548

5012 AAA TTA AAA AGT TTC CTA GCT AGT CAT AAC ATC ACC ATT ACT GAT GGA ATT GAC AAA ACT  5071
1549 K   L   K   S   F   L   A   S   H   N   I   T   I   T   D   G   I   D   K   T    1568

5072 AGT GCG ACT TTA CAG TTT AGA AAG CTG TCA GAG TGT GAT TTT CTT AAA AGA AAT TTC AAG  5131
1569 S   A   T   L   Q   F   R   K   L   S   E   C   D   F   L   K   R   N   F   K    1588

5132 CAA ATG TCC AAT GTT TTG TGG GTA GCT CCT GAA GAC AAA GCT AGT TTG TGG TCA CAA TTA  5191
1589 Q   M   S   N   V   L   W   V   A   P   E   D   K   A   S   L   W   S   Q   L    1608

5192 CAC TAT GTT TCA TGT AAC AAT TTG GAA ATG CAA GAA GCT TAT CTT GTT AAC TTG GTT AAT  5251
1609 H   Y   V   S   C   N   N   L   E   M   Q   E   A   Y   L   V   N   L   V   N    1628

5252 GTG TTG CGT GAG TTG TAC CTG CAC AGT CCA GAA GAA GCT CGT CAG TTG AGA AGA AAG GCT  5311
1629 V   L   R   E   L   Y   L   H   S   P   E   E   A   R   Q   L   R   R   K   A    1648

5312 CTC TCT CGT ATT GAG TGG TTG CAA AAA GCT GAT GTG CCC ACC ATA GCA CAA ATT GAA GAA  5371
1649 L   S   R   I   E   W   L   Q   K   A   D   V   P   T   I   A   Q   I   E   E    1668

5372 TTT CAT TCA ATG CAG AGG ATT ATG AAT GCT CCT GAT TCA AAT GAT AAT ATT GAT CTC TTG  5431
1669 F   H   S   M   Q   R   I   M   N   A   P   D   S   N   D   N   I   D   L   L    1688

5432 TTG AGC ATC GAC TTG TTG GGT CTT CAG GGT GCA GGC AAG GCC TTC CCA AAT AAG ATT GTG  5491
1689 L   S   I   D   L   L   G   L   Q   G   A   G   K   A   F   P   N   K   I   V    1708

5492 TTT GAT GAT AAA TTG GTA TTG GCA AAT ACA CAA GAA TTT TTT GAT GGA AAT TTT CCA ACA  5551
1709 F   D   D   K   L   V   L   A   N   T   Q   E   F   F   D   G   N   F   P   T    1728

5552 GAT TCT TGG TTA CCA ATA TTT GTT AAT TGT CTT TAC CCT GTG AGT CAA TTG CCC GCA GAA  5611
1729 D   S   W   L   P   I   F   V   N   C   L   Y   P   V   S   Q   L   P   A   E    1748

5612 GCT GTC ATT GTT AAT GTT GTT TGT GGG AGT GGG CGC GGT GGT TTG CCT ACT ACT GCT TGG  5671
1749 A   V   I   V   N   V   V   C   G   S   G   R   G   G   L   P   T   T   A   W    1768

5672 ATT AGT TCT GCA GTT AAC AAT CGC TCC TCA GAT ATC AAT AAG AAA ATT CGG ACA GCG CTT  5731
1769 I   S   S   A   V   N   N   R   S   S   D   I   N   K   K   I   R   T   A   L    1788

5732 GGA AAA GGT AAG AAA ATT GTC TTT TTG ACT AGA GTT GAT CCT TTT CCT GTG GCC TTG TTA  5791
1789 G   K   G   K   K   I   V   F   L   T   R   V   D   P   F   P   V   A   L   L    1808

5792 GCT GTT CTT TTT GGT GTT AAG AAT GAA ATT CTG AGT TCT AAT GCC ACA AAT CCA ATG TTG  5851
1809 A   V   L   F   G   V   K   N   E   I   L   S   S   N   A   T   N   P   M   L    1828

5852 ACA AGG CTT CTT GAG AAC TGC AAG AGT CTT AAA TAT TTG GTT GAT GAG TGT CCT TTT GCA  5911
1829 T   R   L   L   E   N   C   K   S   L   K   Y   L   V   D   E   C   P   F   A    1848

5912 TTT GTT AAC TAG TTTGTAATATTTTGTTCACTTAAATAAAGCGCATTACT ATG TGC AAT GAG TGT GTT    5979
1849 F   V   N   *

5980 TAA ATATAAAAAAAAAAAA                                                             5998
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Figure 2.4. The complete nucleotide sequence of type II RNA1 from BPMV IL-Cb1. The deduced amino acid sequence of the polyprotein encoded by RNA1 is indicated in the one-letter code below the nucleotide sequence.

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   1 TATTAAAATTTTCATAAGATTTGAAATTTTGATAAACCGCGATCACAGGTTGCCGCACCTTAAAACCGGAAACAAAAGCA 80

  81 ATCGTTACTTGTTCTCAAGAATCTTCAAACTTTCTTCTGTTTTCTCTGCACTCGGTTCTTCAGGACAAGAAAATCACTCT 160

 161 CTGTATTGATTACAGACTTCGTGAATCATTTTCTTTTCAACTTTCAGTTCACTTGCTGAACACTCTCCTATAAATATATA 240

 241 GGACTTCGTGTCAGATTTGAACTTTTCCTGTTTCTTTCTCGGTTTTCTTTCTTATTCTCATCTTCTTTAAATTTTAAGGC 320

 321 TCGCATTTTGTTTCTTTAAACTTTCTGTTGTACTCATTTGAAATACAAC ATG AAG TTT TAT CCA GGA CAA   390
   1                                                   M   K   F   Y   P   G   Q     7

 391 AAT GTC TCT GAA ATT GTT TAC CAT TTT CAG AGT AAT GAG ACA GCT AAT AGG CTT GAT GCT  450
   8 N   V   S   E   I   V   Y   H   F   Q   S   N   E   T   A   N   R   L   D   A    27

 451 TAT TTT GCC TGT GGC TGT GAA GAG GAT ACT GAA GTC CTC GCT CGC TTG AAG CAA TGT AAC  510
  28 Y   F   A   C   G   C   E   E   D   T   E   V   L   A   R   L   K   Q   C   N    47

 511 CCT CGG CTA CTG CAT TTA TCT TAT GCT GCT TTC TGT TTG GAA ATG GGC AGT CAT TCT GTT  570
  48 P   R   L   L   H   L   S   Y   A   A   F   C   L   E   M   G   S   H   S   V    67

 571 GAA GAA ATA GAA TAT GAT GAC GGA GAG TTG GTT TTC TTG TAT TTC CAA AAT TTT CTA CTC  630
  68 E   E   I   E   Y   D   D   G   E   L   V   F   L   Y   F   Q   N   F   L   L    87

 631 TCC ATA GTG TCC AAT TCT TCC AAG ACA GCA AAT CTG AGA GCA TAC ATA CGT TCA GCT TTT  690
  88 S   I   V   S   N   S   S   K   T   A   N   L   R   A   Y   I   R   S   A   F    107

 691 GCA TAT CAT TTT CAG CAC TTT GTT GAA TTT GAT CAA TAT ACA AAT GAC TCT CTC AAT GTG  750
 108 A   Y   H   F   Q   H   F   V   E   F   D   Q   Y   T   N   D   S   L   N   V    127

 751 ATG GAT ACA AGC GTA TCT GCT CAA GGA ATT GCA GAT TTG GCT CTG TCC ATG GTC AGA TGG  810
 128 M   D   T   S   V   S   A   Q   G   I   A   D   L   A   L   S   M   V   R   W    147

 811 ATT CCT ACT CAA ATT AAA AAA GTT GTG AAT TTT GGG GTA GGA TCG GTC ATA GAA TCC TTT  870
 148 I   P   T   Q   I   K   K   V   V   N   F   G   V   G   S   V   I   E   S   F    167

 871 TCA GAG CAT TTC AAT AAG CTC ATA ATG CAA TAT TGT CCA ATA GTG TTC CAA GCT TTT AGC  930
 168 S   E   H   F   N   K   L   I   M   Q   Y   C   P   I   V   F   Q   A   F   S    187

 931 TGG GTT AAC AAT ATT TGG ACC ATG GTT AAA GAA TGG ATT GAA GAG GCT GCG AAG GAA ATT  990
 188 W   V   N   N   I   W   T   M   V   K   E   W   I   E   E   A   A   K   E   I    207

 991 TCT TGG TTC CTG CAA GGT TGT AAG GAA TTG TTA GCT TGG GGA ATG TGT ATT CTA GCT AGT  1050
 208 S   W   F   L   Q   G   C   K   E   L   L   A   W   G   M   C   I   L   A   S    227

1051 TCC TGT GCT TTG GGA TTG GTT GAA AAA TGT CTC ATT TCT CTA GGC ATG ATT TCT GAA TCT  1110
 228 S   C   A   L   G   L   V   E   K   C   L   I   S   L   G   M   I   S   E   S    247

1111 TTT GAT TTG GTT GGT TTG TTT GTT CGA TCA GCC ATT GTT GGA GCC TTC TGT GTT TCT ATC  1170
 248 F   D   L   V   G   L   F   V   R   S   A   I   V   G   A   F   C   V   S   I    267

1171 AAG ACG GGC AAG TTT GTT TCA AAT AGT GAG TTG ATC ACA TGT GCT ACC ATT GCA GTT TCT  1230
 268 K   T   G   K   F   V   S   N   S   E   L   I   T   C   A   T   I   A   V   S    287

1231 ACA ATT GCA ACT GTT ATG TCT CAA GCT TTC AAA CCT TCT GAA GAA ATT AAA GGG CAA TTC  1290
 288 T   I   A   T   V   M   S   Q   A   F   K   P   S   E   E   I   K   G   Q   F    307

1291 CAG GCT CTT TCT GTT TTA GAG GGA TTG GCA ACA CAA CTC ACT TCA TTT TGT GAC ACA TCT  1350
 308 Q   A   L   S   V   L   E   G   L   A   T   Q   L   T   S   F   C   D   T   S    327

1351 TTG ATT GCC ATG GGA AAA ACT TGC ACA GCA TTT AAT CAA ATT TGT ACT GCT GGA AAA AAT  1410
 328 L   I   A   M   G   K   T   C   T   A   F   N   Q   I   C   T   A   G   K   N    347

1411 GTT AAG GTG ATT GCT GGC AGA TTG TTG GAT GTA GTT TCC AAT TTT GTA AGG AAA CTT TTG  1470
 348 V   K   V   I   A   G   R   L   L   D   V   V   S   N   F   V   R   K   L   L    367

1471 GGA TTG GAT AGT GCT TTT CTT AGA GAT GCA GCG CTC ATC TTC TCT CAA GAT GTT GAT GGT  1530
 368 G   L   D   S   A   F   L   R   D   A   A   L   I   F   S   Q   D   V   D   G    387
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Figure 2.4. continued.

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1531 TGG CTG CGC AAT ATC AGC TGG TGT CAG GAA CAA TTC CTA CTG AAA GCA TAC ATG TCT CAA  1590
 388 W   L   R   N   I   S   W   C   Q   E   Q   F   L   L   K   A   Y   M   S   Q    407

1591 GAT GAT CTT ATT GTC TTG CGT TCC TTA GTT GTC AAA GGT GAA AGA ATG AGA GAG CAA ATG  1650
 408 D   D   L   I   V   L   R   S   L   V   V   K   G   E   R   M   R   E   Q   M    427

1651 TTG GAA GGA GAG GTT AAA GTG TCT CCC AGT GTT TGT AAT CTT ATT GTA AAA GGT TGT GAA  1710
 428 L   E   G   E   V   K   V   S   P   S   V   C   N   L   I   V   K   G   C   E    447

1711 GAA GCA AGT AAG TTG ATG AGA GAA AGT GTG CTA CAT TGT TCA AAG ACT GTA CGG AAA ATT  1770
 448 E   A   S   K   L   M   R   E   S   V   L   H   C   S   K   T   V   R   K   I    467

1771 CCA TTT GTC ATT TTT GCA CAC GGT GAT TCT CGT GTT GGA AAA TCT TTG CTA GTT GAT AGA  1830
 468 P   F   V   I   F   A   H   G   D   S   R   V   G   K   S   L   L   V   D   R    487

1831 CTT ATC ACA GAT TTT TGT GAT CAT CTG GAA ATT GGG GAG GAT GCT GTT TAT TCA AGG AAT  1890
 488 L   I   T   D   F   C   D   H   L   E   I   G   E   D   A   V   Y   S   R   N    507

1891 CCT TCG GAT CCT TTC TGG AGT GGG TAT AGG AGA CAA CCA ATC GTC ACT ATT GAT GAT TTT  1950
 508 P   S   D   P   F   W   S   G   Y   R   R   Q   P   I   V   T   I   D   D   F    527

1951 GCT GCT GTT GCA TCA GAG CCA TCT GCT GAG GCT CAA TTG ATT CCA TTA ATT TCA AGT GCT  2010
 528 A   A   V   A   S   E   P   S   A   E   A   Q   L   I   P   L   I   S   S   A    547

2011 CCG TAC CCA TTG AAC ATG GCA AGT TTA GAG GAA AAG GGA ATG CAC TTT GAT TCC CAG ATC  2070
 548 P   Y   P   L   N   M   A   S   L   E   E   K   G   M   H   F   D   S   Q   I    567

2071 ATG ATG TGC TCT TCA AAT TTT TTG GAA CCT TCT CCT GAA GCC AAA ATT AGA GAT GAC ATG  2130
 568 M   M   C   S   S   N   F   L   E   P   S   P   E   A   K   I   R   D   D   M    587

2131 GCT TTT AGA AAT AGA AGA CAT GTT TTG ATA ACA GTT GAA CTT AAA CCT GGA GTG GAG TAT  2190
 588 A   F   R   N   R   R   H   V   L   I   T   V   E   L   K   P   G   V   E   Y    607

2191 GAT GAG AGC GAT TTT ACC AAA AAT CAG CGA TAT TTA CTC AAA ACT TGG TTT CAT GAT CAT  2250
 608 D   E   S   D   F   T   K   N   Q   R   Y   L   L   K   T   W   F   H   D   H    627

2251 TAT GTT GTA GAC CAA ACT TTT GAA TCT TAT GCT GAT CTT TTG GCA TAT TGC TTC ACT AAA  2310
 628 Y   V   V   D   Q   T   F   E   S   Y   A   D   L   L   A   Y   C   F   T   K    647

2311 TGG GAG AGA CAT GTG AAG GAA CAA GAG TCT AAT TTG TCT CAA ATT AAA GGT AAG AAA AGT  2370
 648 W   E   R   H   V   K   E   Q   E   S   N   L   S   Q   I   K   G   K   K   S    667

2371 GAG AGT GGC CAT TTC AAT AAT TTT CAA CAA CTT ATG GAT TTG GCA GTT TCA TGG AAT CTC  2430
 668 E   S   G   H   F   N   N   F   Q   Q   L   M   D   L   A   V   S   W   N   L    687

2431 AGT GCG AAT ATC ATG AAG GAA CGA ATC AAA GCT GAT AAA AGT GAT ATG GTT TAT GTC TTC  2490
 688 S   A   N   I   M   K   E   R   I   K   A   D   K   S   D   M   V   Y   V   F    707

2491 TCT GCT GGA AGG AAG GAC AAA ATT GTA CAT TGC TTC TTG AAT AAA GAA GGT GAA TGT AGT  2550
 708 S   A   G   R   K   D   K   I   V   H   C   F   L   N   K   E   G   E   C   S    727

2551 ATA CGT CCT GAT TCA ATA GAA GAT CCA GAA GCA CAA CTT TTG CTC AAA GCT TCA GAA ACT  2610
 728 I   R   P   D   S   I   E   D   P   E   A   Q   L   L   L   K   A   S   E   T    747

2611 ATG CTC ATG AAA GCT TAT GCT TTT TTG AAG TAC AAC AAT GCG ACC AAT TTG ATT GTC AGG  2670
 748 M   L   M   K   A   Y   A   F   L   K   Y   N   N   A   T   N   L   I   V   R    767

2671 ACC CAT TTG GCA GAA TTG GTC AAT GAG GAC TTT TAT GAT GAA AAG TTT AAT TTC ATT GGC  2730
 768 T   H   L   A   E   L   V   N   E   D   F   Y   D   E   K   F   N   F   I   G    787

2731 ACA ATT GGG ACT CCA GCT TTT CAT CGG CAA ATT GCT GCA CAC TTG GAG AAA ATG CCA TTA  2790
 788 T   I   G   T   P   A   F   H   R   Q   I   A   A   H   L   E   K   M   P   L    807

2791 TGG CAA AAA GCA ATT TTG TGT GGA ATG GGA CAT TGT TTG TCT CGG AAA AGC AAA GAA ACA  2850
 808 W   Q   K   A   I   L   C   G   M   G   H   C   L   S   R   K   S   K   E   T    827

2851 TGG TAT TCT GGG ATG AAG GAA AAA TTT GTA CAA ATG ATG AAG AGC ATC TAT GAA ACA GAA  2910
 828 W   Y   S   G   M   K   E   K   F   V   Q   M   M   K   S   I   Y   E   T   E    847

2911 GTT ACA GAT TGG CCA GTA CCA CTG AAA ATT ATT TCT GGA ACC ATT CTT GCA ACA ATT TTG  2970
 848 V   T   D   W   P   V   P   L   K   I   I   S   G   T   I   L   A   T   I   L    867

2971 GGA ACA ACC TTC TGG AAA CTC TTT TCC TTT CTT AGA GAT GCT GGT AAT GGG GGA GTT TTT  3030
 868 G   T   T   F   W   K   L   F   S   F   L   R   D   A   G   N   G   G   V   F    887

3031 GTT GGT AAT GTT GCT TCA GCA TTC ACA ACC TCA AGT GTG CTC GAG GCC CAA AGT AGA AAA  3090
 888 V   G   N   V   A   S   A   F   T   T   S   S   V   L   E   A   Q   S   R   K    907
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Figure 2.4. continued.

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3091 CCC AAC AGA TAT GAA GTT TCC CAA TAC AGA TAT CGC AAT GTG CCA ATA AAG CGC AGA GCA  3150
 908 P   N   R   Y   E   V   S   Q   Y   R   Y   R   N   V   P   I   K   R   R   A    927

3151 TGG GTT GAA GGC CAG ATG TCT TTC GAT CAG TCT GTA GTA GCA ATT ATG TCT AAA TGC AAA  3210
 928 W   V   E   G   Q   M   S   F   D   Q   S   V   V   A   I   M   S   K   C   K    947

3211 GCC AGC ATG AGA ATG GGA AAC ACT GAT GCT CAA ATT TTG ATG GTT CCA GGG CGC AGA TTC  3270
 948 A   S   M   R   M   G   N   T   D   A   Q   I   L   M   V   P   G   R   R   F    967

3271 ATA GCT CAT GGA CAT TTC TTC AAA AAT CTG ACT CAG AAA GTG CGA GTC CAA ATT GTT ACA  3330
 968 I   A   H   G   H   F   F   K   N   L   T   Q   K   V   R   V   Q   I   V   T    987
3331 TCT GAA AAG AGT TAC TGG CAT GTT TAT GAT CCT GAC AAG TTT CAG ATG TTT GAC AAC AGT  3390
 988 S   E   K   S   Y   W   H   V   Y   D   P   D   K   F   Q   M   F   D   N   S   1007

3391 GAA ATA GGC CTT TAT TAT AAT CCC ACT TTG GAA GAT ATC CCA CAT TCA GCT TGG GAC CTT  3450
1008 E   I   G   L   Y   Y   N   P   T   L   E   D   I   P   H   S   A   W   D   L   1027

3451 TTT TGC TGG GAC AGT GAA AAA ACC TTG CCA AAT AAT TTT TCT GCA GAA TTG CTC TCT TGC  3510
1028 F   C   W   D   S   E   K   T   L   P   N   N   F   S   A   E   L   L   S   C   1047

3511 AAA TTG GAT ACT GTT ACT GGC CAA TAT TAT CCT GAG TGG GCT CCA ATC AAT TGT CGA GTT  3570
1048 K   L   D   T   V   T   G   Q   Y   Y   P   E   W   A   P   I   N   C   R   V   1067

3571 CAT CGA CAA CCA ATT CAC ATC ACT GAA GGA AAT TAT GTC AGA AAG CAA GAT GTC AGC ATT  3630
1068 H   R   Q   P   I   H   I   T   E   G   N   Y   V   R   K   Q   D   V   S   I   1087

3631 GAA TAT GAT GCA TGT ACA ATT CCA AAT GAT TGT GGT TCA TTG GTT GTT GCC AAG GTT GGA  3690
1088 E   Y   D   A   C   T   I   P   N   D   C   G   S   L   V   V   A   K   V   G   1107

3691 AAT CAC AAA CAA ATT GTT GGT TTC CAT GTT GCT GGA AGT AAA GGA AGA CTG GGA TAT GCT  3750
1108 N   H   K   Q   I   V   G   F   H   V   A   G   S   K   G   R   L   G   Y   A   1127

3751 TCA TTG ATA CCA TAT GTT GAG CCA GTT GTG CAA GCT CAA AGT GCT GAA GTT TAC TTT GAC  3810
1128 S   L   I   P   Y   V   E   P   V   V   Q   A   Q   S   A   E   V   Y   F   D   1147

3811 TTC TTC CCT GTG GAG GTT GAT AGT CAA GAA GGA GTT GCT CAC ATT GGT GAA TTG AAA TCT  3870
1148 F   F   P   V   E   V   D   S   Q   E   G   V   A   H   I   G   E   L   K   S   1167

3871 GGT GTC TAT GTT CCA CTG CCT ACA AAA ACC AAT TTA GTG GAA ACT CCC AAG GAA TGG CAA  3930
1168 G   V   Y   V   P   L   P   T   K   T   N   L   V   E   T   P   K   E   W   Q   1187

3931 CTG GAT CTA CCT TGT GAC AAA ATT CCT AGT GTC TTG ACT ACA ACT GAT GAG AGA TTG GTA  3990
1188 L   D   L   P   C   D   K   I   P   S   V   L   T   T   T   D   E   R   L   V   1207

3991 GGC ACA GAA CAT GAG GGA TAT GAT CCT TTT CTT GGT GGA ATT CAA AAA TAT GCC ACT CCC  4050
1208 G   T   E   H   E   G   Y   D   P   F   L   G   G   I   Q   K   Y   A   T   P   1227

4051 ATG ATG CCC CTA GAT GAA GAA ATT CTT TCT AAG GTT GCA CAA GAT ATG GTT GAG GAA TGG  4110
1228 M   M   P   L   D   E   E   I   L   S   K   V   A   Q   D   M   V   E   E   W   1247

4111 TTT GAT TGT GTT GAT GAG GAG GAT TCC TTT GAG GAA GTT TCT TTA AGT GCA GCA CTC AAT  4170
1248 F   D   C   V   D   E   E   D   S   F   E   E   V   S   L   S   A   A   L   N   1267

4171 GGT GTT GAG GGT TTG GAC TAT ATG GAA AGA ATT CCT CTT GCC ACA TCT GAG GGT TTT CCT  4230
1268 G   V   E   G   L   D   Y   M   E   R   I   P   L   A   T   S   E   G   F   P   1287

4231 CAT GTG CTT TCA CGT AAA AAT GGC GAA AAA GGC AAA AGG AGA TTT GTC TCT GGG GAT GGT  4290
1288 H   V   L   S   R   K   N   G   E   K   G   K   R   R   F   V   S   G   D   G   1307

4291 GAA GAG ATG ACA TTG ATT CCG GGA ACC AGT GTT GAA GAA GCT TAC AAC AAG CTA ATA GTT  4350
1308 E   E   M   T   L   I   P   G   T   S   V   E   E   A   Y   N   K   L   I   V   1327

4351 GAA CTT GAA AAA AGT GTT CCT ACA TTG GTT GGC ATT GAA TGT CCC AAG GAT GAG AAA CTT  4410
1328 E   L   E   K   S   V   P   T   L   V   G   I   E   C   P   K   D   E   K   L   1347

4411 CCT CGT CGC AAA ATT TTT GAC AAA CCT AAG ACG CGC TGC TTC ACT ATT CTC CCT ATG GAG  4470
1348 P   R   R   K   I   F   D   K   P   K   T   R   C   F   T   I   L   P   M   E   1367

4471 TTT AAT CTT GTG GTT CGT CAG AAG TTT TTA AAT TTT GTG AGG TTC ATT ATG AAG AAA AGG  4530
1368 F   N   L   V   V   R   Q   K   F   L   N   F   V   R   F   I   M   K   K   R   1387

4531 GAC AAG CTT AGT TGT CAA GTT GGA ATC AAC CCA TAC TCC ATG GAA TGG ACT GGT TTG GCC  4590
1388 D   K   L   S   C   Q   V   G   I   N   P   Y   S   M   E   W   T   G   L   A   1407

4591 AAT AGA TTA TTG AGT AAG GGC AAC GAT ATT CTG TGT TGC GAT TAT GCT AGT TTT GAT GGT  4650
1408 N   R   L   L   S   K   G   N   D   I   L   C   C   D   Y   A   S   F   D   G   1427
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Figure 2.4. continued.

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4651 TTG ATT ACC AAG CAA GTT ATG AGC AAG ATG GCA GAA ATG ATA AAC AGT CTT TGT GGT GGT  4710
1428 L   I   T   K   Q   V   M   S   K   M   A   E   M   I   N   S   L   C   G   G    1447

4711 GAT GAA AAA TTG ATG CGT GAA AGG ACA CAC CTG CTG TTG GCT TGT TGT TCA AGG ATG GCA  4770
1448 D   E   K   L   M   R   E   R   T   H   L   L   L   A   C   C   S   R   M   A    1467

4771 ATT TGT AAG AAA GAT GTT TGG AGA GTT GAA TGT GGA ATC CCT TCT GGA TTT CCG CTC ACA  4830
1468 I   C   K   K   D   V   W   R   V   E   C   G   I   P   S   G   F   P   L   T    1487

4831 GTT ATT TGC AAT AGC ATT TTT AAT GAG ATG CTC ATT AGA TAT AGC TAT GAA AAA TTA CTG  4890
1488 V   I   C   N   S   I   F   N   E   M   L   I   R   Y   S   Y   E   K   L   L    1507

4891 CGC CAG GCT AAA GCT CCA AGT ATG TTT TTA CAA TCC TTC AAA AAT TTT GTT TCT TTG TGT  4950
1508 R   Q   A   K   A   P   S   M   F   L   Q   S   F   K   N   F   V   S   L   C    1527

4951 GTG TAT GGT GAT GAT AAC TTT ATT AGT GTT CAT GAA TAT GTC AAG CCT TAC TTT AGT GGT  5010
1528 V   Y   G   D   D   N   F   I   S   V   H   E   Y   V   K   P   Y   F   S   G    1547

5011 TCT AAA TTG AAA AGC TTT CTA GCT GGT CAT AAT ATC ACC ATT ACT GAT GGT ATT GAC AAA  5070
1548 S   K   L   K   S   F   L   A   G   H   N   I   T   I   T   D   G   I   D   K    1567

5071 ACT AGT GCA ACT TTG CAA TTT AGA AAG TTA GCA GAT TGT GAT TTT CTC AAG AGA AAC TTT  5130
1568 T   S   A   T   L   Q   F   R   K   L   A   D   C   D   F   L   K   R   N   F    1587

5131 AAG CAG ATG TCT AAT GTT TTG TGG GTG GCT CCT GAG GAT AAG GCG AGT TTA TGG TCA CAA  5190
1588 K   Q   M   S   N   V   L   W   V   A   P   E   D   K   A   S   L   W   S   Q    1607

5191 CTT CAT TAT GTT TCG TGT AAT AAT CTG GAA ATG CAA GAA GCT TAT CTT GTC AAT CTC GTA  5250
1608 L   H   Y   V   S   C   N   N   L   E   M   Q   E   A   Y   L   V   N   L   V    1627

5251 AAT GTG TTA CGA GAA TTG TAT TTG CAC AGT CCA GAG GAA GCT CGC CAA TTG AGA AGG AAA  5310
1628 N   V   L   R   E   L   Y   L   H   S   P   E   E   A   R   Q   L   R   R   K    1647

5311 GCT CTC TCT CGT ATT GAA TGG CTG CAA AAA GCT GAT GTG CCT ACC ATA GCA CAG ATT GAA  5370
1648 A   L   S   R   I   E   W   L   Q   K   A   D   V   P   T   I   A   Q   I   E    1667

5371 GAG TTT CAT TCG ATG CAG AGG ATG ATG AAT GCT CCT GAT TCA AAT GAT AAT ATT GAT CTA  5430
1668 E   F   H   S   M   Q   R   M   M   N   A   P   D   S   N   D   N   I   D   L    1687

5431 CTG TTG AGC ATT GAT TTG TTG GGT TTA CAA GGA GCA GGT AAA GCT TTT CCA AAC AAG ATT  5490
1688 L   L   S   I   D   L   L   G   L   Q   G   A   G   K   A   F   P   N   K   I    1707

5491 GTT TTT GAT GAT AAG CTT GTG TTG GCT AAC ACA CAA GAA TTC TTT GAT GGA AAT TTT CCA  5550
1708 V   F   D   D   K   L   V   L   A   N   T   Q   E   F   F   D   G   N   F   P    1727

5551 GTA GAT TCT TGG TTA CCA ATT TTT GTG AAT TGT CTT TAT CCT GTA AGT CAA TTA CCT CCA  5610
1728 V   D   S   W   L   P   I   F   V   N   C   L   Y   P   V   S   Q   L   P   P    1747

5611 GAA GCT GTC GTT GTA AAT GTT ACA TGT GGT AGT GGA CGT GGT GGT TTA CCC ACC ACT GCT  5670
1748 E   A   V   V   V   N   V   T   C   G   S   G   R   G   G   L   P   T   T   A    1767

5671 TGG ATT AGT TCT GCA GTT AAC AAT CGC TCC TCA GAT ATC AAC AAA AAG ATT CGC ACA GCA  5730
1768 W   I   S   S   A   V   N   N   R   S   S   D   I   N   K   K   I   R   T   A    1787

5731 CTT GGG AAA GGT AAG AAA ATT GTT TTT CTT ACT AGA GTT GAT CCT TTT CCA GTA GCT TTA  5790
1788 L   G   K   G   K   K   I   V   F   L   T   R   V   D   P   F   P   V   A   L    1807

5791 CTG GCT GTT CTT TTT GGC GTT AAG AAT GAA ATT CTG AGT TCT AAC GCC ACC AAC CCT ATG  5850
1808 L   A   V   L   F   G   V   K   N   E   I   L   S   S   N   A   T   N   P   M    1827

5851 CTG ACG CGA CTT CTT GAG AAC TGT AAG AGT CTC AAA TAT CTG GTT GAT GAG TGT CCT TTT  5910
1828 L   T   R   L   L   E   N   C   K   S   L   K   Y   L   V   D   E   C   P   F    1847

5911 GCA TTT GTG AAC TAG T ATG TAA TATTTTATTCACTTAAATAAAGCGCATTACT ATG TGC AAT GAG   5975
1848 A   F   V   N   *

5976 TGT GTT TAA ATATAAAAAAAAAAAA                                                     6000
```

A

	K-G7	IL-Cb1*	K-Ho1	K-Ha1
K-G7		96.6	85.7	84.4
IL-Cb1	98.0		84.7	83.5
K-Ho1	96.4	96.8		98.8
K-Ha1	96.3	96.7	99.4	

B

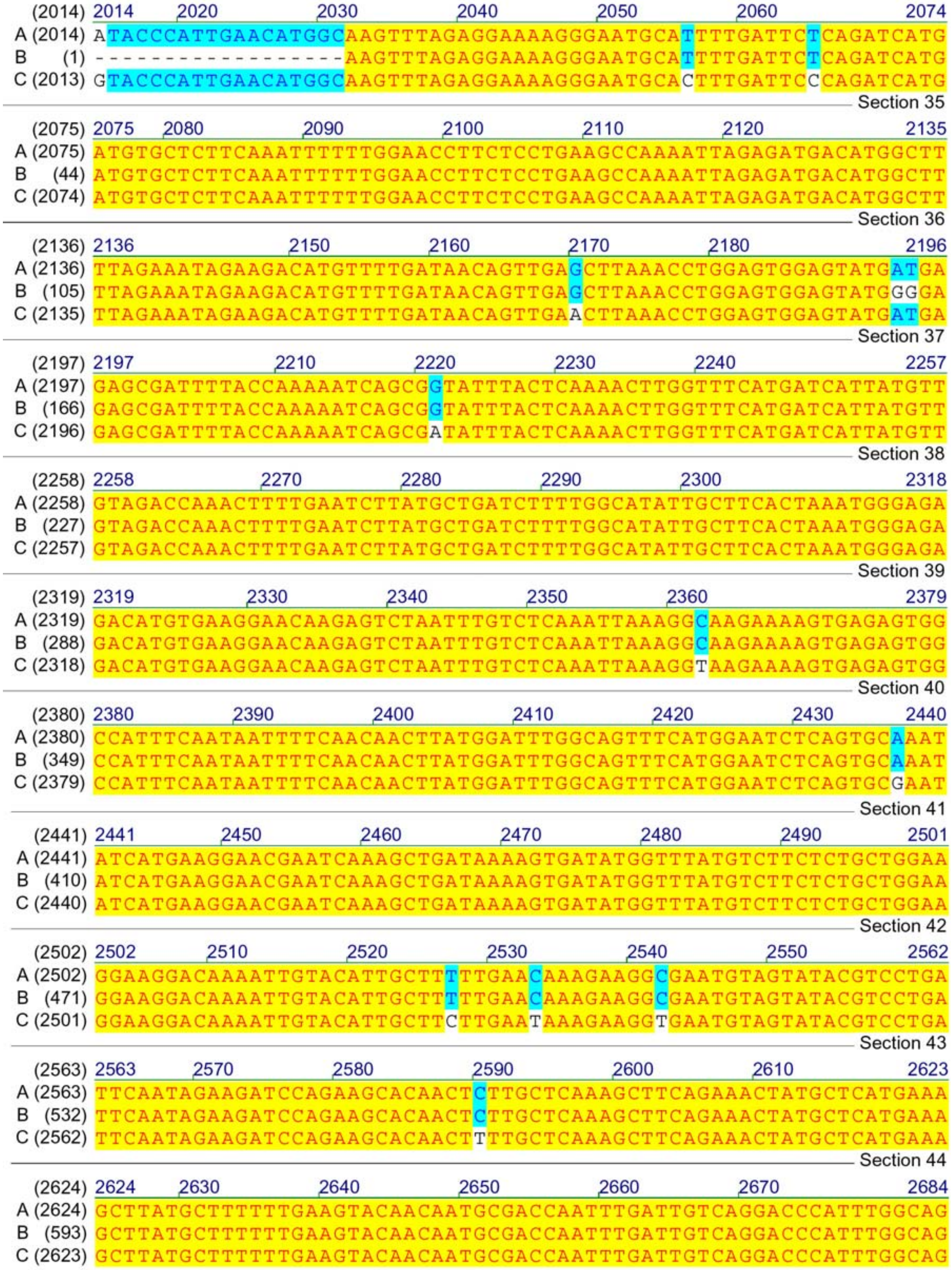
	K-G7	IL-Cb1 * (type I)	IL-Cb1 * (type II)	K-Ho1	K-Ha1
K-G7		97.9	83.1	97.9	83.6
IL-Cb1 (type I)	97.9		85	99.1	85.3
IL-Cb1 (type II)	95.7	97.0		82.4	98.5
K-Ho1	98.0	99.6	97.2		82.8
K-Ha1	95.9	97.1	99.8	97.3	

Figure 2.5. Percentage nucleotide and deduced amino acid identity of RNA2 and RNA1 between BPMV strains.

(A) Full-length RNA2 nucleotide sequence identity, above diagonal, and deduced amino acid sequence identity, below diagonal. (B) Full-length RNA1 nucleotide sequence identity, above diagonal, and deduced amino acid sequence identity, below diagonal. Values are the identity scores generated by the GAP program in the UWGCG package.

* : Gene Bank accession numbers for IL-Cb1 RNA2, RNA1 (type I) and RNA1 (type II) are AY744933, AY744931 and AY744932, respectively.

Figure 2.6. Sequence alignment of type II RNA1 from three strains of BPMV. A, K-Ha1; B, K-Ho1; C, IL-Cb1.



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(2014) 2014  2020        2030       2040       2050       2060        2074
A (2014) ATACCCATTGAACATGGCAAGTTTAGAGGAAAAGGGAATGCATTTTGATTCTCAGATCATG
B    (1) ------------------AAGTTTAGAGGAAAAGGGAATGCATTTTGATTCTCAGATCATG
C (2013) GTACCCATTGAACATGGCAAGTTTAGAGGAAAAGGGAATGCACTTTGATTCCCAGATCATG
                                                               Section 35
(2075) 2075 2080       2090       2100       2110       2120          2135
A (2075) ATGTGCTCTTCAAATTTTTTGGAACCTTCTCCTGAAGCCAAAATTAGAGATGACATGGCTT
B   (44) ATGTGCTCTTCAAATTTTTTGGAACCTTCTCCTGAAGCCAAAATTAGAGATGACATGGCTT
C (2074) ATGTGCTCTTCAAATTTTTTGGAACCTTCTCCTGAAGCCAAAATTAGAGATGACATGGCTT
                                                               Section 36
(2136) 2136          2150       2160       2170       2180             2196
A (2136) TTAGAAATAGAAGACATGTTTTGATAACAGTTGAGCTTAAACCTGGAGTGGAGTATGATGA
B  (105) TTAGAAATAGAAGACATGTTTTGATAACAGTTGAGCTTAAACCTGGAGTGGAGTATGGGGA
C (2135) TTAGAAATAGAAGACATGTTTTGATAACAGTTGAACTTAAACCTGGAGTGGAGTATGATGA
                                                               Section 37
(2197) 2197         2210       2220       2230       2240             2257
A (2197) GAGCGATTTTACCAAAAATCAGCGGTATTTACTCAAAACTTGGTTTCATGATCATTATGTT
B  (166) GAGCGATTTTACCAAAAATCAGCGGTATTTACTCAAAACTTGGTTTCATGATCATTATGTT
C (2196) GAGCGATTTTACCAAAAATCAGCGATATTTACTCAAAACTTGGTTTCATGATCATTATGTT
                                                               Section 38
(2258) 2258         2270       2280       2290       2300             2318
A (2258) GTAGACCAAACTTTTGAATCTTATGCTGATCTTTTGGCATATTGCTTCACTAAATGGGAGA
B  (227) GTAGACCAAACTTTTGAATCTTATGCTGATCTTTTGGCATATTGCTTCACTAAATGGGAGA
C (2257) GTAGACCAAACTTTTGAATCTTATGCTGATCTTTTGGCATATTGCTTCACTAAATGGGAGA
                                                               Section 39
(2319) 2319         2330       2340       2350       2360             2379
A (2319) GACATGTGAAGGAACAAGAGTCTAATTTGTCTCAAATTAAAGGCAAGAAAAGTGAGAGTGG
B  (288) GACATGTGAAGGAACAAGAGTCTAATTTGTCTCAAATTAAAGGCAAGAAAAGTGAGAGTGG
C (2318) GACATGTGAAGGAACAAGAGTCTAATTTGTCTCAAATTAAAGGTAAGAAAAGTGAGAGTGG
                                                               Section 40
(2380) 2380       2390       2400       2410       2420       2430     2440
A (2380) CCATTTCAATAATTTTCAACAACTTATGGATTTGGCAGTTTCATGGAATCTCAGTGCAAAT
B  (349) CCATTTCAATAATTTTCAACAACTTATGGATTTGGCAGTTTCATGGAATCTCAGTGCAAAT
C (2379) CCATTTCAATAATTTTCAACAACTTATGGATTTGGCAGTTTCATGGAATCTCAGTGCGAAT
                                                               Section 41
(2441) 2441        2450       2460       2470       2480       2490      2501
A (2441) ATCATGAAGGAACGAATCAAAGCTGATAAAAGTGATATGGTTTATGTCTTCTCTGCTGGAA
B  (410) ATCATGAAGGAACGAATCAAAGCTGATAAAAGTGATATGGTTTATGTCTTCTCTGCTGGAA
C (2440) ATCATGAAGGAACGAATCAAAGCTGATAAAAGTGATATGGTTTATGTCTTCTCTGCTGGAA
                                                               Section 42
(2502) 2502       2510       2520       2530       2540       2550      2562
A (2502) GGAAGGACAAAATTGTACATTGCTTTTTGAACAAAGAAGGCGAATGTAGTATACGTCCTGA
B  (471) GGAAGGACAAAATTGTACATTGCTTTTTGAACAAAGAAGGCGAATGTAGTATACGTCCTGA
C (2501) GGAAGGACAAAATTGTACATTGCTTCTTGAATAAAGAAGGTGAATGTAGTATACGTCCTGA
                                                               Section 43
(2563) 2563      2570       2580       2590       2600       2610       2623
A (2563) TTCAATAGAAGATCCAGAAGCACAACTCTTGCTCAAAGCTTCAGAAACTATGCTCATGAAA
B  (532) TTCAATAGAAGATCCAGAAGCACAACTCTTGCTCAAAGCTTCAGAAACTATGCTCATGAAA
C (2562) TTCAATAGAAGATCCAGAAGCACAACTTTTGCTCAAAGCTTCAGAAACTATGCTCATGAAA
                                                               Section 44
(2624) 2624   2630       2640       2650       2660       2670          2684
A (2624) GCTTATGCTTTTTTGAAGTACAACAATGCGACCAATTTGATTGTCAGGACCCATTTGGCAG
B  (593) GCTTATGCTTTTTTGAAGTACAACAATGCGACCAATTTGATTGTCAGGACCCATTTGGCAG
C (2623) GCTTATGCTTTTTTGAAGTACAACAATGCGACCAATTTGATTGTCAGGACCCATTTGGCAG
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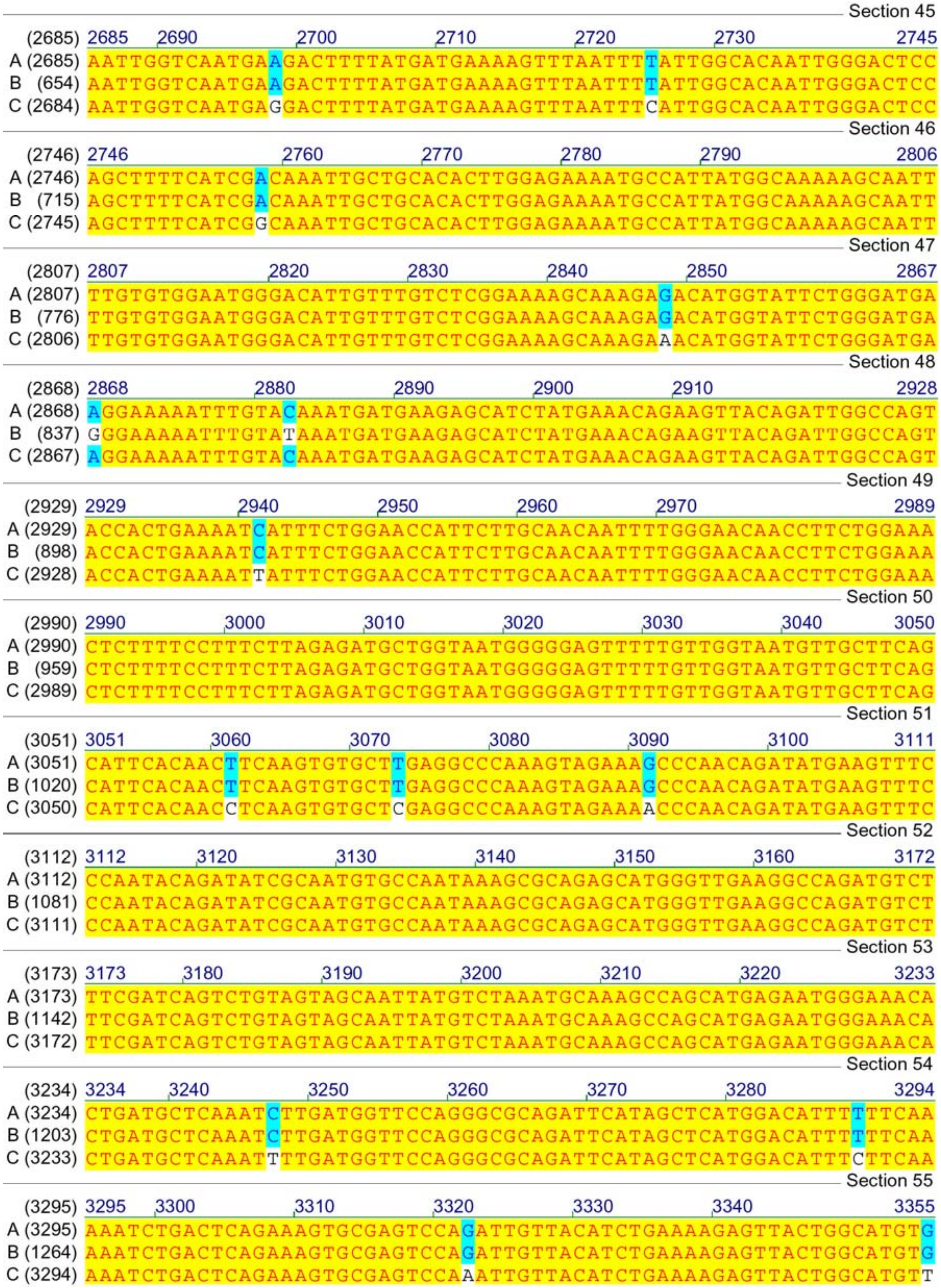
Figure 2.6. continued.



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                                                                  Section 45
    (2685) 2685    2690           2700           2710           2720           2730             2745
  A (2685) AATTGGTCAATGAAGACTTTTATGATGAAAAGTTTAATTTTATTGGCACAATTGGGACTCC
  B  (654) AATTGGTCAATGAAGACTTTTATGATGAAAAGTTTAATTTTATTGGCACAATTGGGACTCC
  C (2684) AATTGGTCAATGAGGACTTTTATGATGAAAAGTTTAATTTCATTGGCACAATTGGGACTCC
                                                                  Section 46
    (2746) 2746                 2760           2770           2780           2790             2806
  A (2746) AGCTTTTCATCGACAAATTGCTGCACACTTGGAGAAAATGCCATTATGGCAAAAAGCAATT
  B  (715) AGCTTTTCATCGACAAATTGCTGCACACTTGGAGAAAATGCCATTATGGCAAAAAGCAATT
  C (2745) AGCTTTTCATCGGCAAATTGCTGCACACTTGGAGAAAATGCCATTATGGCAAAAAGCAATT
                                                                  Section 47
    (2807) 2807                 2820           2830           2840           2850             2867
  A (2807) TTGTGTGGAATGGGACATTGTTTGTCTCGGAAAAGCAAAGAGACATGGTATTCTGGGATGA
  B  (776) TTGTGTGGAATGGGACATTGTTTGTCTCGGAAAAGCAAAGAGACATGGTATTCTGGGATGA
  C (2806) TTGTGTGGAATGGGACATTGTTTGTCTCGGAAAAGCAAAGAAACATGGTATTCTGGGATGA
                                                                  Section 48
    (2868) 2868                2880           2890           2900           2910             2928
  A (2868) AGGAAAAATTTGTACAAATGATGAAGAGCATCTATGAAACAGAAGTTACAGATTGGCCAGT
  B  (837) GGGAAAAATTTGTATAAATGATGAAGAGCATCTATGAAACAGAAGTTACAGATTGGCCAGT
  C (2867) AGGAAAAATTTGTACAAATGATGAAGAGCATCTATGAAACAGAAGTTACAGATTGGCCAGT
                                                                  Section 49
    (2929) 2929                2940           2950           2960           2970             2989
  A (2929) ACCACTGAAAATCATTTCTGGAACCATTCTTGCAACAATTTTGGGAACAACCTTCTGGAAA
  B  (898) ACCACTGAAAATCATTTCTGGAACCATTCTTGCAACAATTTTGGGAACAACCTTCTGGAAA
  C (2928) ACCACTGAAAATTATTTCTGGAACCATTCTTGCAACAATTTTGGGAACAACCTTCTGGAAA
                                                                  Section 50
    (2990) 2990          3000           3010           3020           3030           3040       3050
  A (2990) CTCTTTTCCTTTCTTAGAGATGCTGGTAATGGGGGAGTTTTTGTTGGTAATGTTGCTTCAG
  B  (959) CTCTTTTCCTTTCTTAGAGATGCTGGTAATGGGGGAGTTTTTGTTGGTAATGTTGCTTCAG
  C (2989) CTCTTTTCCTTTCTTAGAGATGCTGGTAATGGGGGAGTTTTTGTTGGTAATGTTGCTTCAG
                                                                  Section 51
    (3051) 3051         3060           3070           3080           3090           3100       3111
  A (3051) CATTCACAACTTCAAGTGTGCTTGAGGCCCAAAGTAGAAAGCCCAACAGATATGAAGTTTC
  B (1020) CATTCACAACTTCAAGTGTGCTTGAGGCCCAAAGTAGAAAGCCCAACAGATATGAAGTTTC
  C (3050) CATTCACAACCTCAAGTGTGCTCGAGGCCCAAAGTAGAAAACCCAACAGATATGAAGTTTC
                                                                  Section 52
    (3112) 3112        3120           3130           3140           3150           3160        3172
  A (3112) CCAATACAGATATCGCAATGTGCCAATAAAGCGCAGAGCATGGGTTGAAGGCCAGATGTCT
  B (1081) CCAATACAGATATCGCAATGTGCCAATAAAGCGCAGAGCATGGGTTGAAGGCCAGATGTCT
  C (3111) CCAATACAGATATCGCAATGTGCCAATAAAGCGCAGAGCATGGGTTGAAGGCCAGATGTCT
                                                                  Section 53
    (3173) 3173      3180           3190           3200           3210           3220          3233
  A (3173) TTCGATCAGTCTGTAGTAGCAATTATGTCTAAATGCAAAGCCAGCATGAGAATGGGAAACA
  B (1142) TTCGATCAGTCTGTAGTAGCAATTATGTCTAAATGCAAAGCCAGCATGAGAATGGGAAACA
  C (3172) TTCGATCAGTCTGTAGTAGCAATTATGTCTAAATGCAAAGCCAGCATGAGAATGGGAAACA
                                                                  Section 54
    (3234) 3234     3240           3250           3260           3270           3280           3294
  A (3234) CTGATGCTCAAATCTTGATGGTTCCAGGGCGCAGATTCATAGCTCATGGACATTTTTTCAA
  B (1203) CTGATGCTCAAATCTTGATGGTTCCAGGGCGCAGATTCATAGCTCATGGACATTTTTTCAA
  C (3233) CTGATGCTCAAATTTTGATGGTTCCAGGGCGCAGATTCATAGCTCATGGACATTTCTTCAA
                                                                  Section 55
    (3295) 3295    3300           3310           3320           3330           3340            3355
  A (3295) AAATCTGACTCAGAAAGTGCGAGTCCAGATTGTTACATCTGAAAAGAGTTACTGGCATGTG
  B (1264) AAATCTGACTCAGAAAGTGCGAGTCCAGATTGTTACATCTGAAAAGAGTTACTGGCATGTG
  C (3294) AAATCTGACTCAGAAAGTGCGAGTCCAAATTGTTACATCTGAAAAGAGTTACTGGCATGTT
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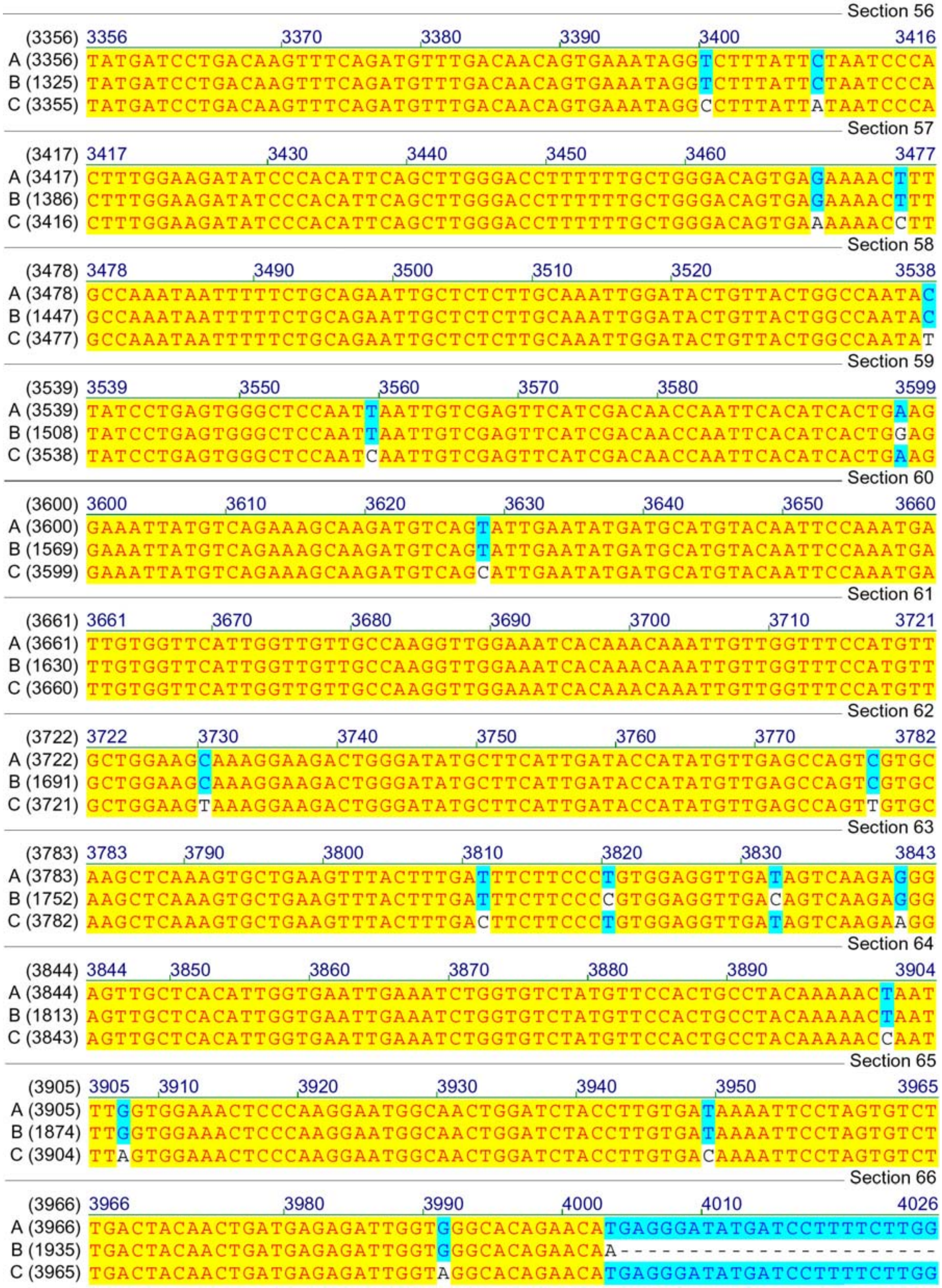
Figure 2.6. continued.



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                                                                  Section 56
(3356) 3356         3370      3380      3390      3400            3416
A (3356) TATGATCCTGACAAGTTTCAGATGTTTGACAACAGTGAAATAGGTCTTTATTCTAATCCCA
B (1325) TATGATCCTGACAAGTTTCAGATGTTTGACAACAGTGAAATAGGTCTTTATTCTAATCCCA
C (3355) TATGATCCTGACAAGTTTCAGATGTTTGACAACAGTGAAATAGGCCTTTATTATAATCCCA
                                                                  Section 57
(3417) 3417         3430      3440      3450      3460            3477
A (3417) CTTTGGAAGATATCCCACATTCAGCTTGGGACCTTTTTTGCTGGGACAGTGAGAAAACTTT
B (1386) CTTTGGAAGATATCCCACATTCAGCTTGGGACCTTTTTTGCTGGGACAGTGAGAAAACTTT
C (3416) CTTTGGAAGATATCCCACATTCAGCTTGGGACCTTTTTTGCTGGGACAGTGAAAAAACCTT
                                                                  Section 58
(3478) 3478        3490      3500      3510      3520             3538
A (3478) GCCAAATAATTTTTCTGCAGAATTGCTCTCTTGCAAATTGGATACTGTTACTGGCCAATAC
B (1447) GCCAAATAATTTTTCTGCAGAATTGCTCTCTTGCAAATTGGATACTGTTACTGGCCAATAC
C (3477) GCCAAATAATTTTTCTGCAGAATTGCTCTCTTGCAAATTGGATACTGTTACTGGCCAATAT
                                                                  Section 59
(3539) 3539        3550      3560      3570      3580             3599
A (3539) TATCCTGAGTGGGCTCCAATTAATTGTCGAGTTCATCGACAACCAATTCACATCACTGAAG
B (1508) TATCCTGAGTGGGCTCCAATTAATTGTCGAGTTCATCGACAACCAATTCACATCACTGGAG
C (3538) TATCCTGAGTGGGCTCCAATCAATTGTCGAGTTCATCGACAACCAATTCACATCACTGAAG
                                                                  Section 60
(3600) 3600       3610      3620      3630      3640      3650    3660
A (3600) GAAATTATGTCAGAAAGCAAGATGTCAGTATTGAATATGATGCATGTACAATTCCAAATGA
B (1569) GAAATTATGTCAGAAAGCAAGATGTCAGTATTGAATATGATGCATGTACAATTCCAAATGA
C (3599) GAAATTATGTCAGAAAGCAAGATGTCAGCATTGAATATGATGCATGTACAATTCCAAATGA
                                                                  Section 61
(3661) 3661      3670      3680      3690      3700      3710     3721
A (3661) TTGTGGTTCATTGGTTGTTGCCAAGGTTGGAAATCACAAACAAATTGTTGGTTTCCATGTT
B (1630) TTGTGGTTCATTGGTTGTTGCCAAGGTTGGAAATCACAAACAAATTGTTGGTTTCCATGTT
C (3660) TTGTGGTTCATTGGTTGTTGCCAAGGTTGGAAATCACAAACAAATTGTTGGTTTCCATGTT
                                                                  Section 62
(3722) 3722    3730      3740      3750      3760      3770       3782
A (3722) GCTGGAAGCAAAGGAAGACTGGGATATGCTTCATTGATACCATATGTTGAGCCAGTCGTGC
B (1691) GCTGGAAGCAAAGGAAGACTGGGATATGCTTCATTGATACCATATGTTGAGCCAGTCGTGC
C (3721) GCTGGAAGTAAAGGAAGACTGGGATATGCTTCATTGATACCATATGTTGAGCCAGTTGTGC
                                                                  Section 63
(3783) 3783    3790      3800      3810      3820      3830       3843
A (3783) AAGCTCAAAGTGCTGAAGTTTACTTTGATTTCTTCCCTGTGGAGGTTGATAGTCAAGAGGG
B (1752) AAGCTCAAAGTGCTGAAGTTTACTTTGATTTCTTCCCCGTGGAGGTTGACAGTCAAGAGGG
C (3782) AAGCTCAAAGTGCTGAAGTTTACTTTGACTTCTTCCCTGTGGAGGTTGATAGTCAAGAAGG
                                                                  Section 64
(3844) 3844   3850      3860      3870      3880      3890        3904
A (3844) AGTTGCTCACATTGGTGAATTGAAATCTGGTGTCTATGTTCCACTGCCTACAAAAACTAAT
B (1813) AGTTGCTCACATTGGTGAATTGAAATCTGGTGTCTATGTTCCACTGCCTACAAAAACTAAT
C (3843) AGTTGCTCACATTGGTGAATTGAAATCTGGTGTCTATGTTCCACTGCCTACAAAAACCAAT
                                                                  Section 65
(3905) 3905 3910      3920      3930      3940      3950          3965
A (3905) TTGGTGGAAACTCCCAAGGAATGGCAACTGGATCTACCTTGTGATAAAATTCCTAGTGTCT
B (1874) TTGGTGGAAACTCCCAAGGAATGGCAACTGGATCTACCTTGTGATAAAATTCCTAGTGTCT
C (3904) TTAGTGGAAACTCCCAAGGAATGGCAACTGGATCTACCTTGTGACAAAATTCCTAGTGTCT
                                                                  Section 66
(3966) 3966           3980      3990      4000      4010            4026
A (3966) TGACTACAACTGATGAGAGATTGGTGGGCACAGAACATGAGGGATATGATCCTTTTCTTGG
B (1935) TGACTACAACTGATGAGAGATTGGTGGGCACAGAACAA-----------------------
C (3965) TGACTACAACTGATGAGAGATTGGTAGGCACAGAACATGAGGGATATGATCCTTTTCTTGG
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Chapter Three

Characterization of RNA1 recombinants from a partial diploid reassortant isolate of *Bean pod mottle virus* (BPMV).

Introduction

Viral RNA recombination is a process that links together two noncontiguous RNA regions to produce a new RNA containing genetic information from more than one source (Cheng and Nagy, 2003). It is considered a major driving force for the evolution of viruses (Roossinck 1997). Since the first report of RNA recombination in a plant RNA virus, *Brome mosaic virus* (BMV; Bujarski and Kaesberg, 1986), evidence has been accumulating for RNA recombinational events in a number of other plant RNA viruses. The availability and abundance of viral genomic sequences made it possible to predict viral RNA recombination based on phylogenetic analysis. Another method is based on experimental recovery of recombinants from known parental populations. The establishment of an *in vitro* recombination system (Cheng and Nagy, 2003) allows detailed examination of the recombination factors, namely donor and acceptor RNA sequences. But the unavailability of such systems in other host-virus systems undermines its usefulness for RNA recombination under natural selection. Although many viral RNA recombinational events were implied by phylogenetic analysis, examples of experimentally confirmed plant viral RNA recombination were limited to a few viral genera (Allison *et al*., 1990; Bousalem *et al*., 2000; Cascone *et al*., 1990; Chen *et al*., 2002; Gibbs, 1999; Glasa *et al*., 2002; Moonan *et al*., 2000; Tomimura *et al*., 2003; Van der Kuyl *et al*., 1991; White and Morris, 1994).

Bean pod mottle virus (BPMV), a major soybean viral pathogen, is a member of the genus *Comovirus* in the family *Comoviridae* (Goldbach *et. al.,* 1995). BPMV has a bipartite positive-strand RNA genome consisting of RNA1 (approximately 6.0 kb) and RNA2 (approximately 3.6 kb) that are separately encapsidated in isometric particles 28 nm in diameter. The genomic RNAs have a small basic protein (viral genome-linked protein, VPg) covalently linked to their 5′ termini and are polyadenylated at the 3’ end.

The BPMV genome is expressed via the synthesis and proteolytic processing of polyprotein precursor strategy. BPMV RNA1 codes for five mature proteins required for replication (from 5′ to 3′, a protease cofactor [Co-pro], a putative helicase [Hel], a VPg, a protease [Pro], and a putative RNA-dependent RNA polymerase [RdRp]), whereas RNA2 codes for a putative cell-to-cell movement protein and the two coat proteins. (Goldbach *et. al.,* 1995, Lomonossoff and Ghabrial 2001).

Based on their genomic sequences, BPMV isolates have been classified into two subgroups, I and II (Gu *et al*., 2002). Naturally occurring reassortants between the two subgroups have also been described (Gu *et al*., 2002 and unpublished). The complete nucleotide sequences of the two genomic RNAs of BPMV strains K-G7, K-Ho1 and K-Ha1 have been determined (Di and Ghabrial 1999, Gu *et. al.,*2002, Gu and Ghabrial 2005, MacFarlane *et. al*., 1991). In this study, RNA recombinants were isolated from the BPMV natural reassortant IL-Cb1, and characterized at the molecular level. Similar recombinants were also recovered from an artificial viral population composed of subgroups I and II strains. To our knowledge, this is the first report of RNA recombination in both a natural isolate and an artificial pseudorecombinant in a member of the genus *Comovirus.*

Materials and methods

Virus isolation and propagation

The field isolate IL-Cb1 was collected from Carbondale, Illinois and was maintained in the greenhouse in the soybean cultivar ‘Essex’. Virion purification from infected leaf tissues was carried out as previously described (Ghabrial *et al*., 1977). Strains K-G7, K-Ha1, and K-Ho1 were previously described (Gu *et al*., 2002). The complete nucleotide sequences of the genomic RNAs from isolates IL-Cb1 have been determined and the sequences have been deposited in the GenBank under accession numbers AY744933, AY744931 and AY744932 for RNA2, RNA1 (type I) and RNA1 (type II) respectively.

Local lesion isolation

BPMV isolate IL-Cb1 was passed through two consecutive local lesion transfers on *Phaseolus vulgaris* cv Pinto. Dilute inoculum was prepared from IL-Cb1-systemically infected soybean leaves and used to inoculate 16 Pinto bean leaves by rub-inoculation. A total of 16 single lesions were selected, one from each of the 16 leaves, and used to inoculate a new set of 16 Pinto bean leaves. One lesion from each of the second set 16 Pinto bean leaves was then used to inoculate soybean seedlings.

RNA extraction and Northern blot hybridization

Viral RNAs were extracted from purified virions by the SDS-phenol method (Peden and Symons 1973). Northern blot hybridization analysis was performed using cloned cDNA probes to full- length RNA1 from isolate K-Ho1 (type I) and K-Ha1 (type II) as well as to PCR fragments corresponding to different regions of RNA1 or RNA2 from isolates IL-Cb1 and K-Ho1. Purified virion RNAs were separated by electrophoresis on 0.8% glyoxal gel (Sambrook and Russell, 2001), and transferred onto Hybond-N$^+$ membranes (Amersham biotech, Piscataway, NJ), according to the manufacturer's instructions. The membranes were then prehybridized, hybridized and air-dried, as described by Gu *et al.* (2002). The probes were prepared by the Rediprime^{TM} II random prime labeling system (Amersham Pharmacia biotech). The membranes were exposed to a phosphorimager screen (Molecular Dynamics, Sunnyvale, CA) for 12-24 hours. The images were visualized by a PhosphorImager 445 SI system and analyzed with the ImageQuant 4.1 program (Molecular Dynamics).

cDNA cloning

Viral RNA was extracted from purified virions of strain IL-Cb1, and the viral RNA1 and RNA2 were purified from low-melting agarose following electrophoretic separation of the viral RNAs. cDNA synthesis was carried out using the SuperScript choice system (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. First strand cDNA synthesis was primed with oligo $(dT)_{12-18}$ primers. Following addition of *Eco*RI adapters to the ends of the double stranded cDNA, it was ligated into *Eco*RI-linearized pGEM 3ZF(+) vector (Promega, Madison, WI). For the 5' end of RNA2 cloning, 5' RACE system (Invitrogen Corp.) was used and the final PCR product was cloned into the pGEM-T easy vector (Promega, Madison, WI).

Reverse transcription-polymerase chain reaction (RT-PCR)

The primers used for sequencing and RT-PCR are listed in Table 3.1. RNA1 3' end specific primer, R1-Rev1 (Table 3.1), was used for RNA1 first strand cDNA synthesis with Superscript II^{RT} (Invitrogen). Briefly, 4 µg viral RNA and 20 pM primer were mixed together in a total 11 µl final volume. The mixture was first incubated at 70 °C for 10 minutes and at 37 °C for 20 minutes. After incubation, 4 µl 5X first strand buffer, 2µl 0.1M DTT, 1 µl dNTP and 2 µl Superscript II ^{RT} enzymye were added. The reaction was incubate at 37 °C for 90 minutes and the product was subjected to PCR.

The cDNA was subjected to PCR using different combinations of primers shown in Table 3.1. HiFi Taq DNA Polymerase (Invitrogen) was used for PCR. Thermal cycling conditions for 5' end RT-PCR were: 94°C for 2 min, 3 cycles at 94° C for 30 sec, 47° C for 30 sec and 68° C for 3 min, 30 cycles of 94° C for 30 sec, 58° C for 30 sec and 68° C for 3 min, followed by 15 min at 68° C. Thermal cycling condition for 3' end and other specific RT-PCR were: 94°C for 2 min, 32 cycles of 94° C for 30 sec, 58° C for 30 sec and 68° C for 3 min, followed by 15 min at 68° C. The PCR products were cloned into the pGEM-T easy vector (Promega) and sequenced by cycle sequencing using M13 universal primers and the sequence-specific primers listed in Table 3.1. CEQ 2000 Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter Inc, Fullerton, CA) and CEQ™ 8000 Genetic Analysis System were used for sequencing. Sequence analysis was performed using the DNA strider (CEA, France) and Vector NTI programs (Informaxinc, Frederick, Maryland).

Construction of full length infectious cDNA clone

IL-Cb1 RNA2 full length cloning: Two IL-Cb1 RNA2 cDNA clones, IL-Cb1-609 & IL-Cb1-128 (both cloned in pGEM 3zf (+) from the Superchoice system) were selected and digested with *Eco*RV. The 5' end of clone IL-Cb1-609 (5 kbp containing the vector backbone) and the 3' end of clone IL-Cb1-128 (1.6 kbp) were gel purified and ligated together using the standard T4 ligase protocol. The ligation product was used as a template for PCR with primers F1 and R1. The PCR product was cloned into the pGEM-T easy vector and used for *in vitro* transcription and inoculation.

RNA1 full length cloning: Either the mosaic or the chimeric RNA1 full length infectious cDNA clones were constructed using the same strategy as that used for RNA2. RT-PCR clones for both the 5' and 3' ends with correct orientations were chosen for construction of full length cloning. Clone Ha10H46, which contains the 3' end type II

RNA1 from IL-Cb1 was used to construct the artificial recombinant HoHa8 (which contains the 5'end of type I RNA1 and the 3' end type II RNA1). Clone HoHa8 was digested with *Sal*I and self- ligated to remove the 3' end *Sac*II site (thus produce clone HoHa82). Clone HoHa82 was selected and digested with *Sac*II and *Xho*I to remove the 5' end type I RNA1 and the 6 kbp fragment was gel purified. LL3B15 and 201-14 were sequentially digested with *Sca*I, *Sac*II and *Xho*I and the 3 kbp fragments were gel purified and ligated to the 6 kbp fragment from HoHa82. Two clones, LL3-F1 and 201-F1, from these two ligations were infectious.

In vitro **transcription and inoculation**

Full length BPMV RNA1 and RNA2 cDNA clones were linearized with *Sa*lI. A 100 µl reaction mixture containing 40 mM Tris-HCl pH7.5, 6 mM MgCl, 2 mM spermidine, 10 mM DTT, 50 units RNasin (Promega), 0.1 mM GTP, 0.5 mM each of ATP, CTP and UTP, 0.5 mM RNA Cap structure analogue [m^{7}G(5')ppp(5']G (New England Biolabs), 50 units T7 RNA polymerase (Stratagene) and 4 µg linearized DNA was incubated at 37 °C for 2 hours. After incubation, samples (5 µl) of transcription reaction mixture were analyzed by electrophoresis on 1% agarose gel to assess yield and quality. The RNA transcripts were used to inoculate fully expanded primary leaves of one-week old soybean seedlings by rub inoculation.

Results

Both mild and severe isolates can be derived from strain IL-Cb1 by local lesion isolation

Previous studies on cDNA cloning and sequencing of the genomic RNAs of BPMV strain IL-Cb1, which induces very severe symptoms on soybean, indicated that it contained at least two genetically distinct types of RNA1 (types I and II) but only one type of RNA2 (type I; Chapter Two). Following passage through two consecutive single lesion transfers on *Phaseolus vulgaris* cv 'Pinto', a total of 16 local lesion isolates were selected for further studies. Among the 16 local lesion isolates, 13 induced very severe symptoms comparable to the parental field isolate IL-Cb1. Of the remaining three, one induced moderate symptoms (local lesion isolate number 9 or LL-9) and two were mild isolates (LL-1 and LL-10). Two severe isolates (LL-3 and LL-16) in addition to the moderate and mild isolates LL-9, LL-1 and LL-10 were selected for further analysis (Figure 3.1).

Both the parental strain IL-Cb1 and its local lesion isolates contain only one type of RNA2

Northern hybridization analysis using cloned cDNA probes to RNA2 (both types I and II) showed that IL-Cb1 and its five local lesion isolates contained only type I RNA2 (Figure 3.2, panels B and C). Genomic RNAs extracted from purified virions of the parental strain and its 5 local lesion isolate derivatives were used as templates for RT-PCR with the primer pair F1 and R1 (Table 3.1). This primer pair anneals to strictly conserved terminal sequences at the 5' and 3' ends of BPMV genomic RNAs. The PCR products were cloned and more than 10 clones for each of these isolates were sequenced. The RNA2 sequences for all five local lesion isolates were indistinguishable from that of the parental isolate IL-Cb1. The complete nucleotide sequence of strain IL-Cb1 RNA2 has been deposited in the GenBank under accession number AY744933.

The BPMV strain IL-Cb1 contains at least two types of RNA1

cDNA cloning and sequencing of IL-Cb1 RNA1 indicated the presence of both type I and type II RNA1 as well as chimeric (clone IL-Cb1-210) and mosaic RNA1 (clone IL-Cb1-201) containing regions derived from both types (Figure 3.3). Northern hybridization analysis showed that strain IL-Cb1 RNA1 hybridized to both type I- and type II-specific probes (Figure 3.2, D and E). Whereas a strong hybridization signal was observed with the type II-specific probe, a weaker signal was obtained with the type I probe.

RNA1 from the mild local lesion isolates LL-1 and LL-10 hybridized only to type II-specific probes. RT-PCR cloning and sequencing confirmed that only type II RNA1 is present in these two mild isolates. The moderate isolate LL-9, on the other hand, contained only type I RNA1 (Figure 3.2 D and E). This was also confirmed by RT-PCR cloning and sequencing using the BPMV RNA1 specific primer pair F1 and R1-Rev1.

Although the two severe isolates, LL-3 and LL-16, showed similar hybridization profiles to that of the moderate isolate LL-9 with hybridization signals to only type I RNA1 (Figure 3.2, D and E), RT-PCR cloning and sequencing indicated that isolate LL-16 contains both types of RNA1. Isolate LL-3, on the other hand, contained type I RNA1, but not type II, and intermolecular recombinants between the two types (clones LL3-B15 and LL3-119; Figure 3.3). The existence of the recombinant LL3-B15 in the original field isolate, IL-Cb1, was further verified by RT-PCR cloning and sequencing using several pairs of primers targeting different regions in the recombinant RNA1 (Figure 3.3). Initially, two overlapping cDNA fragments were amplified from IL-Cb1 RNA using two pairs of primers corresponding to the 5'-end (primers F1 and R1-Rev-XhoI; Table 1) and the 3' end (primers Han-R1-For6 and R1-Rev1; Table 3.1 and Figure 3.3) of the recombinant RNA1. Both the 5' end (3.0 kbp) and 3' end (3.5 kbp) fragments were cloned and sequenced (Figure 3.3). Sequence analysis confirmed that recombinant LL3-B15 does indeed occur in the natural isolate IL-Cb1. Furthermore, the existence of this recombinant in the local lesion isolate LL-3 was established by RT-PCR cloning and sequencing. In addition to the overlapping 3.0 and 3.5 kbp cDNA fragments described above, a 3.3 kbp fragment that includes the recombination site and flanking sequences was also cloned and sequenced from LL-3 RNA using primers HopSpeI-For and HanRev3400 (Figure 3.3). Moreover, a 5.7 kbp near full length RT-PCR product corresponding to the recombinant RNA1 was amplified from LL-3 RNA using primers HopSpeI-For and HanSpeI-Rev. Cloning and sequencing of this RT-PCR product provided strong evidence that the full-length recombinant RNA1 exists in isolate LL3 even though at a low level not detectable by northern blot analysis of purified virion RNA (Figure 3.2). As a control for RT-PCR, RNA extracted from LL-9 (type I) and LL-10 (type II) virions were mixed together and used as templates for RT-PCR with primers HopSpeI-For and Han-Rev3400, specific for type I and type II RNA1, respectively. No PCR products were generated in this control experiment (data not shown) indicating that the recombinants were derived from the viral RNA population and are not RT-PCR artifacts.

Chimeric and mosaic clones from IL-CB1 and its local lesion isolates induced mild symptoms

Both type I and type II RNA1 were cloned from the natural isolate IL-Cb1 and its local lesion derivatives. RNA from purified virions of Isolates LL-9 (Type I) and LL-10 (type II) was used as templates for RT-PCR along with primers F1 and R1-Rev1 to generate full-length RNA1 cDNA clones. Sequencing analysis of these full-length clones showed that LL-10 and LL-9 each contains only one type of RNA1, type II and type I respectively. The RNA1 sequences of LL-10 and LL-9 were identical to the those of RNA1 sequences from their original IL-Cb1 isolate (Gene Bank accession numbers: IL-Cb1 type I RNA1, AY744931; IL-Cb1 type II RNA1, AY744932). A mosaic RNA1 (IL-Cb1-201), resulting from double recombination events, was identified from the natural IL-Cb1 isolate by RT-PCR using primers F1 and R1-Rev-XhoI (Figure 3.3). A full-length cDNA clone of this mosaic RNA1 was infectious and induced mild symptoms on soybean (Figure 3.1). The RNA1 deletion mutant LL3-119 was amplified from LL-3 using primers F1 and R1-Rev-XhoI. Since it lacks part of the helicase gene, no attempt was made to produce a full length cDNA clone. The recombinant IL-Cb1-210 (or LL3-B15, primers F1 & R1-Rev-XhoI, Figure 3.3) was infectious and caused only mild symptoms on soybean. When transcripts from full-length infectious cDNA clones of the mosaic and chimeric recombinant RNA1 (clones IL-Cb1-201 and IL-Cb1-210, respectively) were used to inoculate soybean seedlings, only mild symptoms were produced.

The RNA1 recombinants are less competent in accumulation in mixed infection

The recombinant RNA1, which was purified from soybean plants previously inoculated with transcripts from LL3-B15 RNA1 and IL-Cb1 RNA2 cDNAs, showed strong hybridization signals to both types of RNA1-specific probes whereas the field isolate IL-Cb1 showed weaker signals to type I RNA1 (Figure 3.2). Four cDNA fragments representing the 5’ and 3’ regions of both types of RNA1 were generated by PCR using full-length cDNA clones of the two RNA1 types as templates. Schematic representation of the two types of RNA1 and the four cDNA fragments, fragment b and d (type I), c and e (type II), are shown in Figure 3.4, panel F). Although fragment-based probes were similar to the probes prepared from full-length RNA1 cDNA in their reaction to all isolates, the recombinant LL3-B15 showed unique profile. It hybridized to the 5’ end type I RNA1 probe (derived from fragment b) and the 3’ end type II RNA1 probe (derived from fragment e). However, it did not hybridize to the 5’ end type II RNA1 probe (fragment c) nor to the 3’ end type I RNA1 probe (fragment d). The finding that LL3-B15 RNA1 exhibited a strong hybridization signal to the e fragment probe (3’ end region of type II RNA1) whereas no signals were evident with LL3 RNA1 using the same probe, indicates that the accumulation level of the recombinant LL3-B15 is lower than can be detected in the mixed infections with other RNA1 types present in isolate LL3 (Figure 3.4 E).

Recovery of recombinant RNA1 from soybeans plants inoculated with transcripts derived from two genetically-distinct cloned RNA1 cDNAs

The finding that the intermolecular recombinant RNA1 LL3-B15 occurs in the field isolate IL-Cb1 and its single lesion derivative (LL3) prompted the question of whether a comparable recombinant RNA1 can be generated from mixed infections with transcripts from full-length cDNA clones of both types of RNA1. To address this question, soybean plants were inoculated with transcripts derived from cloned K-Ho1 RNA1 (type I) and K-Ha1 RNA1 (type II) cDNAs along with K-Ho1 RNA2 (type II) transcript. Progeny virus from transcript-inoculated systemically infected leaves was subjected to four serial passages in soybean. Purified virions were prepared from the 4th passage plants and virion RNA was used as a template for RT-PCR cloning and sequencing. Using primers HopSpeI-For and HanRev3400, a single PCR product, corresponding to the 3.3 kbp fragment (Figure 3.3), was generated, gel purified and sequenced. Sequence analysis showed that it was similar to LL3-B15 and resulted from a recombinational event between K-Ho1 RNA1 and K-Ha1 RNA1 (Figure 3.5, HoHa recombinant). Sequence alignment indicated that the recombination region is AU-rich (81.8%) and that it is flanked downstream by another AU-rich region (75%). The immediate upstream region of the recombination site is 60% AU-rich.

Discussion

The identification of natural recombination events between virus strains is important in considerations of virus evolution. Plant RNA virus recombination can occur between different virus species and the role of recombination in evolution has been confirmed experimentally for luteoviruses, nepoviruses, bromoviruses, potyviruses and cucumoviruses (Miller *et al.* 1995, Smith *et al.* 2000, Le Gall *et al.*, 1995, Van der Kuyl *et al.* 1991, Varrelmann *et al.* 2000, Fernandez-Cuartero *et al.* 1994). The present study provides the first report on intermolecular recombination in a comovirus.

Previous studies on genetic diversity of BPMV documented the occurrence in nature of two subgroups of BPMV strains (subgroups I and II) as well as reassortants between these two subgroups (Gu *et al.*, 2002; Gu and Ghabrial, 2005). Furthermore, evidence was recently presented that some of the naturally occurring strains that induce very severe symptoms in soybean are partial diploid reassortants, i.e., they are diploid for RNA1 and haploid for RNA2 (Gu and Ghabrial, 2005; this study). The occurrence of such partial diploids at high frequency suggests that mixed infections with two distinct strains are also common. The role of the beetle vectors in the increased incidence of mixed infections was discussed in Chapter Two. The isolation and characterization of intermolecular BPMV RNA1 recombinants (this study) provides undisputed evidence that two distinct BPMV strains may replicate in the same cells. The occurrence of recombinants and reassortants in the natural populations of BPMV and their roles in the emergence of new strains that induce different symptoms are of considerable significance to studies on virus evolution and BPMV epidemiology.

The finding that the recombinant RNA1 generated by infection with transcripts comprising a partial diploid pseudorecombinant is structurally similar to the naturally occurring recombinants suggests that the site of recombination is a hot spot for recombination. Furthermore, the fact that three types of recombinants (mosaic, chimeric and deletion recombinants) that were characterized in this study share similar recombination sites further supports the presence of a recombination hot spot. Moreover, sequence analysis showed that the recombination region has AU rich sequences characteristic of recombination hot spots (Figure 3.5). It is also interesting that the recombination hot spot is located in the helicase gene, which was previously shown to be a symptom severity determinant (Gu and Ghabrial, 2005).

Our results on the molecular characterization of BPMV recombinants, particularly the discovery of deletion recombinants, favor the replicase-driven template switching mechanism (copy choice model) as the underlying mechanism for the generation of BPMV recombinants. It is of interest that all natural recombinants characterized so far have their 5'-end regions derived from type I RNA1 and their 3'-end regions derived from type II RNA1 suggesting that this genome arrangement is more fit for survival than the reverse arrangement. In this regard, the finding that the natural recombinants retain the C-terminal half of the helicase coding region is of considerable interest in view of the fact that the type I-derived C-terminal half of helicase is a major contributor to enhanced symptom severity (Gu and Ghabrial, 2005). Type II BPMV strains, which induce only mild symptoms on soybean, are the most widely occurring BPMV strains (Gu *et al.*, 2002), and thus are the most adapted to their hosts. Although the natural recombinants, which were derived from the severe partial diploid strain IL-Cb1 via local lesion isolation, induced mild symptoms, they were apparently less competent for replication and accumulation than wild type RNA1 in mixed infections.

Table 3.1 Primers used for PCR and sequencing

Primer name	Sequence	Position
R1-Rev1	TTTATATTTAAACACACTCATTGCACATAG	5963-5992
F1	taatacgactcactatagTATTAAAATTTTCATAAGATTT GAAATTTTGATAAACCG	1-39
R1	TTCCGCGGCCGCTATGGCCGACGTCGACTTTTT TTTTTTTTTTTTT	Poly(A)$_{18}$-SalI
HanRev3400	TGGGATTATAATAAAGGCCT	3397-3416
R1-For-XhoI	TCAAGTGTGCTCGAGGCCCAA	3062-3082
R1-Rev-XhoI	TTGGGCCTCGAGCACACTTGA	3082-3062
R1-Rev690	CAAAATGCTGAAAATGATATGC	692-713
Ho3-Rev	TGTTGAAAATTATTGAAATGACCACTTTCAT	2368-2398
HopSpeI-For	TTGTATACAGCTTTCAAAGTGAA	126-148
HanSpeI-Rev	TCGCGTCAGCATAGGGTTG	5860-5840
	Primers for type I RNA1 cDNA sequencing	
BH-R1-For	TACTGAAGTCCTCGCTCGTTTG	475-496
2BM-R1-For	TGCATTTTGGCTAGCTCC	1034-1051
4HopR1-For	TGAAGGCTTACATGTCGC	1569-1586
5HopR1-For	AACATGGCTGGTTTGGAG	2021-2038
6HopR1-For	TGAACAAGGAAGGCGAGTG	2544-2526
BHR1-Rev	TCCAAGCAGTAGTAGGTAAAC	5672-5652
2BM-R1-Rev	TCACACTCTGACAACTTTC	5109-5091
4HopR1-Rev	ATTCCAACTTGGCAACTC	4554-4537
5HopR1-Rev	TATCCTTCATGCTCTGTGC	4008-3990
6HopR1-Rev	AGAAAAGGTCCCAAGCAG	3452-3435

Primers used in this chapter are arranged in four major categories. Primers used for two types of RNA1 sequencing, RNA2 sequencing and those used for RT-PCR.

Table 3.1 continued.

Primer name	Sequence	Position
	Primers for type II RNA1 cDNA sequencing	
Han-R1-For-2nd	TGTGGCTGTGAAGAGGATACTGAAG	461-485
Han-R1-For-3rd	TTGAAGAGGCTGCGAAGG	969-986
Han-R1-For-4th	AGGAAACTTTTGGGATTGG	1460-1478
Han-R1-For-5th	ATTTCAAGTGCTCCATACC	2000-2018
Han-R1-For-6th	TCTCTGCTGGAAGGAAGGAC	2490-2509
Han-R1-Rev-1st	TGCTTCTGAAGGTAATTGAC	5598-5616
Han-R1-Rev-2nd	AGAACCACTAAAGTAAGGC	4996-5104
Han-R1-Rev-3rd	TCCATAGGGAGAATAGTGAAGC	4439-4470
Han-R1-Rev-4th	TGTTCTGTGCCCACCAATC	3984-4002
Han-R1-Rev-5th	TCACTGTCCCAGCAAAAAAG	3439-3468
	Primers for IL-Cb1 RNA2 sequencing	
Cb1-R2-Rev1	TAGCCCATTCAGAACTCCAC	3304-3322
Cb1-R2-For1	TCAACTGTGGGGATTTAG	420-440
Cb1-R2-Rev2	CACTGGTATTGTGGACACTGAAC	2748-2800
Cb1-R2-For2	GAAAAACACTTGGGCATTGGT	498-515
Cb1-R2-Rev3	CTTTCCCATCCATCTATTCAACA	2355-2377
Cb1-R2-For3	GGTATCCCTGCTGATGTTCTT	770-790
Cb1-R2-Rev4	TCATTCCTTTGATACGGTGGG	1974-1994
Cb1-R2-For4	GGTGCTATGCTTCTGGTTGAT	1178-1198
Cb1-R2-Gsp1	ATCAGCAGGGATACCTTTGT	765-784
Cb1-R2-Gsp2	CAGTAGTGAGAAAGGACGGAAGAAT	608-632
Cb1-R2-Gsp3	GCACCAATGCCCAAGTGTTTTTCAA	416-440

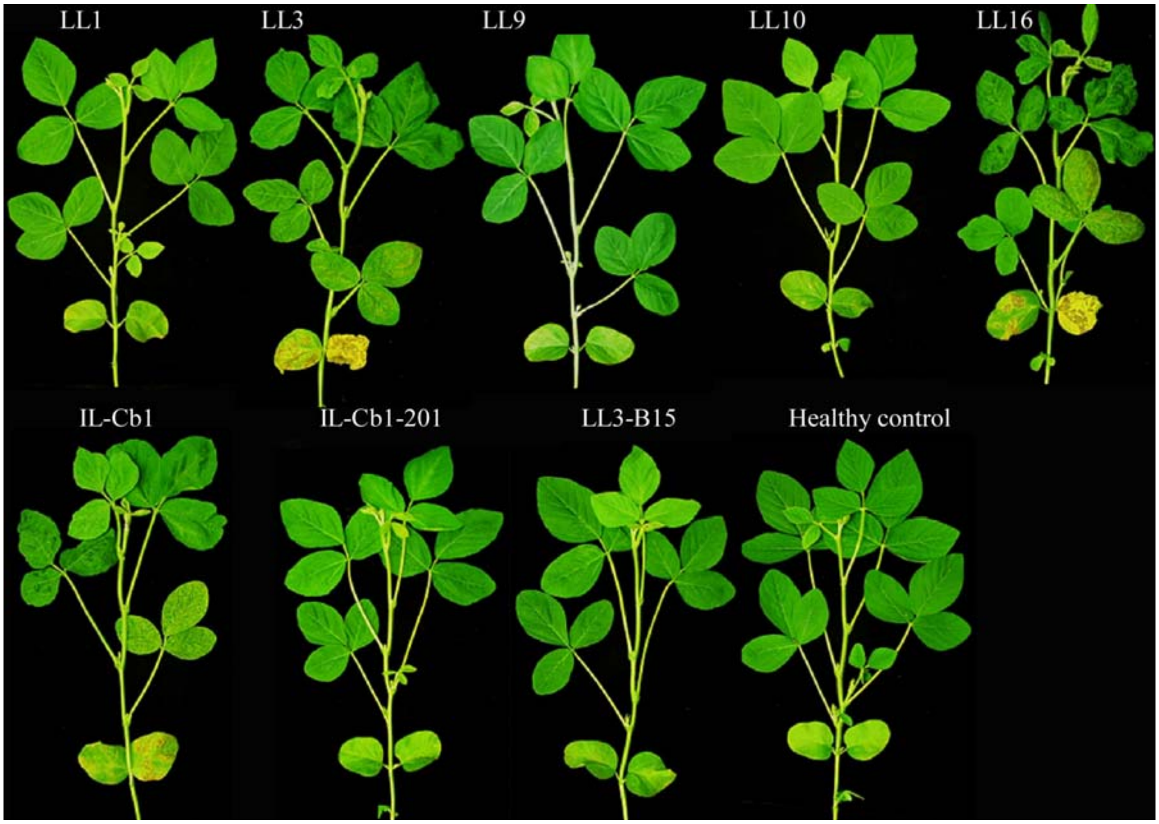


Figure 3.1. Symptoms induced by wild type and local lesion isolates of BPMV.

The designations of the various isolates are indicated at the top of the individual photographs. The local lesion (LL) isolates derived from the field isolate IL-Cb1, were designated LL1to LL16. Photographs of the symptoms induced by LL1, LL3, LL9, LL10 and LL16. are shown on the top row. Symptoms induced by the parental isolate IL-Cb1, the recombinants IL-Cb1-201and and LL3-B15 and the healthy control are shown on the bottom row. Leaf extracts prepared from systemically infected leaves were used as inocula in all cases except for the recombinants. In the latter case, transcripts derived from full-length recombinant RNA1 (IL-Cb1-201 or LL3-B15) plus IL-Cb1 RNA2 cDNA constructs were used to inoculate soybeans. Photographs were taken 21 dpi.

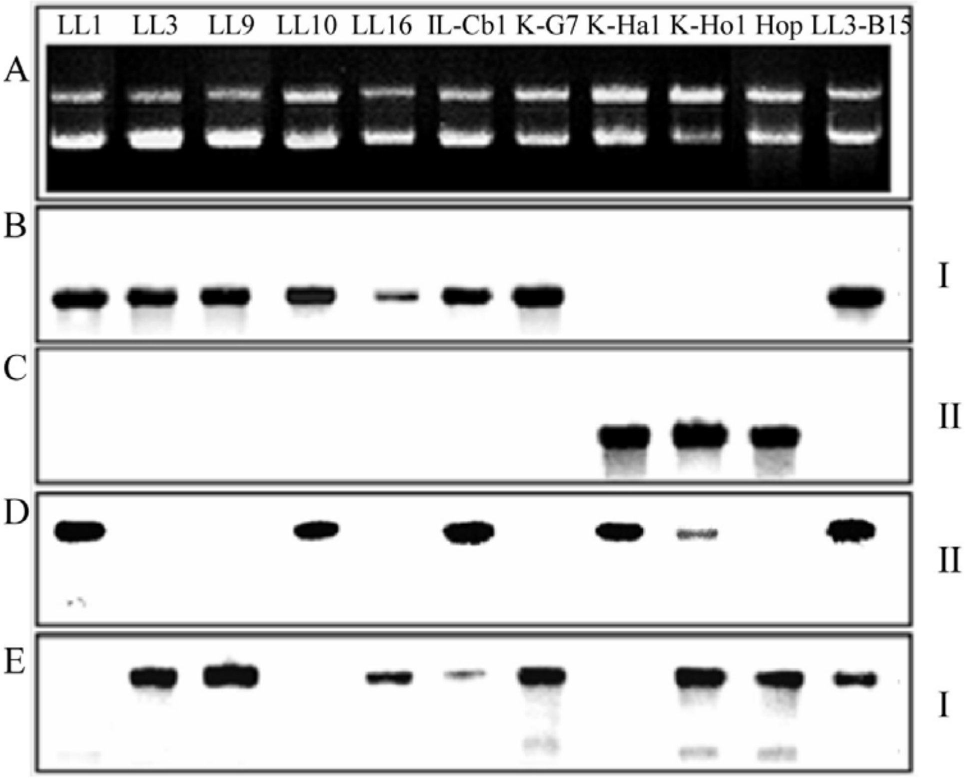


Figure 3.2. Northern blot hybridization analysis of genomic RNA isolated from purified virions of various wild type and local lesion isolates of BPMV.

The BPMV isolate designations are indicated at the top of the figure. The local lesion (LL) isolates LL1, LL3, LL9, LL1o and LL16 were derived from the field isolate IL-Cb1. K-G7, K-Ha1 and K-Ho1 are field isolates that belong to subgroup I, subgroup II and a partial diploid reassortant between the two subgroups, respectively. Hop, virions purified from soybean plants previously inoculated with transcripts derived from K-Ko1 RNAI (I) and RNA2 cDNA constructs. LL3-15, virions purified from soybean plants previously inoculated with transcripts derived from full-length recombinant RNA1 (LL3-B15) plus IL-Cb1 RNA2 cDNA constructs.

A, levels of RNA loading were assessed by ethidium bromide staining.

B, probe was prepared using full-length IL-Cb1RNA2 cDNA as a template (Type I).

C, probe was prepared using full-length K-Ha1 RNA2 cDNA as a template (Type II).

D: probe was prepared using full-length K-Ha1 RNA1 cDNA as a template (Type II)

E: probe was prepared using full-length K-Ho1 RNA1 (I) cDNA as a template (Type I)

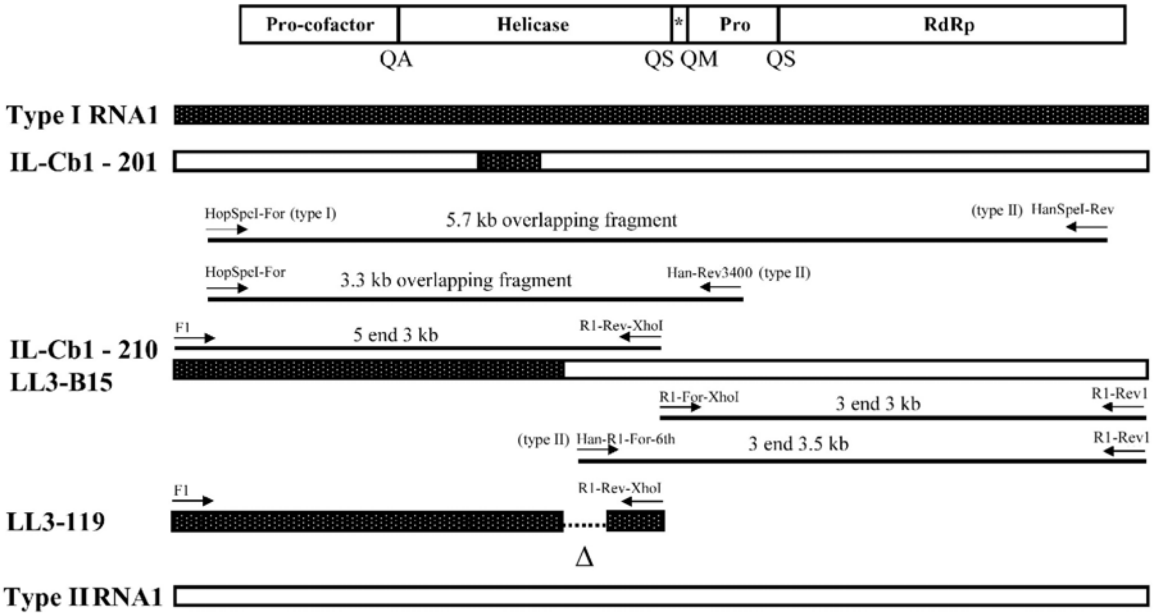


Figure 3.3. Schematic representation of the RNA1 cDNA clones derived from the field isolate IL-Cb1 and its local lesion isolate LL3.

A diagram of BPMV RNA1 genome organization is shown at the top.

Type I RNA1 (black rectangular) and type II RNA1 (open rectangular) cDNA constructs are shown at the top and bottom of the diagram, respectively. .

IL-Cb1-201 is a mosaic RNA1 cDNA clone derived from the natural isolate IL-Cb1.

IL-Cb1-210 is a chimeric RNA1 cDNA clone derived from the natural isolate IL-Cb1.

LL3-15 is a chimeric RNA1 cDNA clone derived from the local lesion isolate LL3.

LL3-119 is a deletion recombinant RNA1 cDNA clone derived from the local lesion isolate LL3.

The line drawings above the IL-Cb1-210 and LL3-15 constructs represent the types of cDNA clones that were amplified by RT-PCR from these recombinant RNA1s.

The lines drawings below the IL-Cb1-210 and LL3-15 constructs represent the types of cDNA clones that were amplified by RT-PCR from th 3’ end of these recombinant RNA1s.

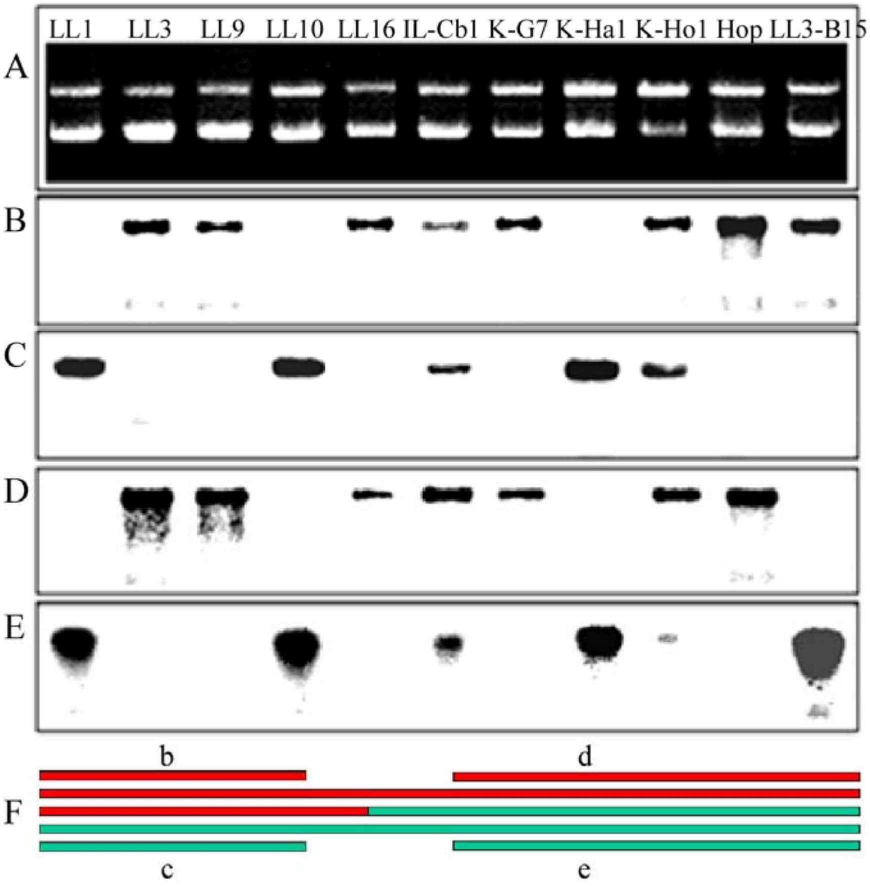
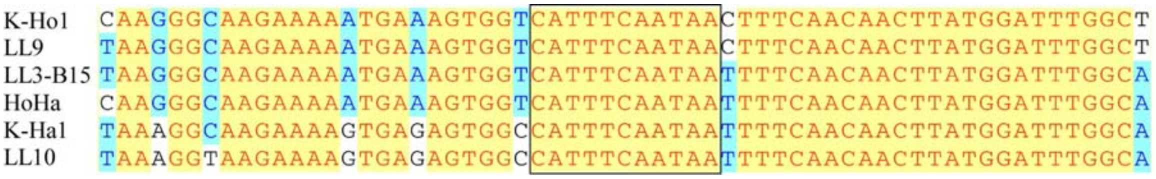


Figure 3.4. Northern blot hybridization analysis of genomic RNA isolated from purified virions of various wild type and local lesion isolates of BPMV.

See legend to Figure 2 for explanations of the virus isolate designation.

A, levels of RNA loading were assessed by ethidium bromide staining; B: probe was prepared from a 2 kbp PCR product (b) derived from the 5' end of K-Ho1 RNA1 (I) cDNA; C, probe was prepared from a 2 kbp PCR product (c) derived from the 5' end of K-Ha1 RNA1 (II) cDNA; D, probe was prepared from a 3 kbp PCR product (d) derived from of the 3' end of K-Ho1 RNA1 (I) cDNA; E: probe was prepared from a 3 kbp PCR product (e) derived from of the 3' end of K-Ha1 RNA1 (II) cDNA; F, schematic representation of the probes used in this figure. Dark gray rectangular represents type I RNA1 and light gray rectangular represents type II RNA1. A diagram of the recombinant LL3-15 is drawn in the center between the diagrams of type I and II RNA1.



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K-Ho1    CAAGGGCAAGAAAAATGAAAGTGGTCATTTCAATAACTTTCAACAACTTATGGATTTGGCT
LL9      TAAGGGCAAGAAAAATGAAAGTGGTCATTTCAATAACTTTCAACAACTTATGGATTTGGCT
LL3-B15  TAAGGGCAAGAAAAATGAAAGTGGTCATTTCAATAATTTTCAACAACTTATGGATTTGGCA
HoHa     CAAGGGCAAGAAAAATGAAAGTGGTCATTTCAATAATTTTCAACAACTTATGGATTTGGCA
K-Ha1    TAAAGGCAAGAAAAGTGAGAGTGGCCATTTCAATAATTTTCAACAACTTATGGATTTGGCA
LL10     TAAAGGTAAGAAAAGTGAGAGTGGCCATTTCAATAATTTTCAACAACTTATGGATTTGGCA
```

Figure 3.5. Nucleotide sequence alignment of the probable recombination region in six different BPMV RNA1s.

HoHa is a recombinant isolated from soybean plants infected a the partial diploid pseudorecombinant comprised of K-Ho1 RNA1 (I), K-Ha1 RNA1 (II) and K-Ho1 RNA2. See the legend to Figure 3.1 for explanations of the designations of the other five isolates. The potential recombination site is boxed.

Chapter Four

Bean pod mottle virus-based vectors for stable protein expression and sequence-specific virus-induced gene silencing in soybean

Introduction

Plant virus-based vectors for expressing heterologous proteins in plants present promising biotechnological tools to supplement conventional breeding and transgenic technology. Considering the speed with which a virus infection becomes established throughout the plant and the high yield of viral-encoded proteins that accumulate in plants, the use of viral vectors provides an attractive and cost effective means for the overproduction of valuable proteins in plants and for rapid evaluation of new traits.

Several different types of positive sense RNA plant viruses have been developed as vectors for production of recombinant proteins and peptides (Pogue *et al.*, 2002; Scholthof *et al.*, 1996). Depending on the structure of the viruses involved and their genome replication and expression strategies, a number of approaches including gene replacement, gene insertion, epitope presentation and complementation, have been utilized. Plant viral vectors are presently available for recombinant protein expression in a wide range of host plants including *Nicotiana benthamiana*, tobacco, squash, cucumber, wheat, barley, cowpea, *Nicotiana clevelandii*, *Chenopodium quinoa*, and *Arabidopsis* (Allison, *et al.*, 1988; Brisson, *et al.*, 1984; Choi, *et al.*, 2000; Constantin *et al.*, 2004; Dolja, *et al.*, 1992; Fernandez-Fernandez, *et al.*, 2001; French, *et al.*, 1986; Gopinath, *et al.*, 2000; Hagiwara, *et al.*, 1999; Haupt, *et al.*, 2001; Lacomme, *et al.*, 2003; Turnage, *et al.* 2002). Even with these advances, there are only a limited number of plant viral vectors that are suitable for systemic expression of foreign proteins in major crops like soybean. Soybean is a main source of oil and high-quality protein worldwide, and there is critical need for tools that allow for rapid evaluation of new traits involving expression of valuable proteins that confer disease/pest resistance and/or those that enhance the nutritional and commercial value of soybean. Here, we describe the development of *Bean* *pod mottle virus* (BPMV) as a gene vector capable of systemic expression of foreign genes in soybean.

Another important application of plant viral vector systems is in studies on host gene function. With more plant genomic information available, a high throughput tool is required. Virus-induced gene silencing (VIGS) is an exceptional reverse genetics tool that can be employed to generate mutant phenotypes for conveying function to unknown genes. VIGS has many advantages over other methods; it is quick and does not require plant transformation. In VIGS systems, viruses are designed to carry partial sequence of known or candidate genes in order to link their function to the mutant phenotype. The targets of VIGS can be a single gene, several members of a gene family or several distinct genes (Lu *et al.*, 2003; Peele *et al.*, 2001; Turnage *et al.*, 2002). Currently, many model host plants including *Nicotiana benthamiana*, tomato, tobacco, *Arabidopsis* and Cassava have been explored (Burch-Smith *et al*., 2004). With the current abundance of genomic information on soybean and model legume species (Stacey *et al*., 2004), it is timely to apply VIGS to soybean to enhance our knowledge of gene function in such a major legume crop. Here we also successfully show that BPMV can be used as a VIGS vector for gene function studies in soybean.

BPMV is a member of the genus *Comovirus* in the family *Comoviridae* (Lomonossoff and Ghabrial *et al.,* 2001). BPMV has a bipartite positive-strand RNA genome consisting of RNA1 (approximately 6.0 kb) and RNA2 (approximately 3.6 kb) that are separately encapsidated in isometric particles 28 nm in diameter. Two distinct subgroups of BPMV strains, designated subgroups I and II, have been previously isolated and extensively characterized (Gu *et al*., 2002; Gu and Ghabrial, 2005). The BPMV genome is expressed via the synthesis and subsequent proteolytic processing of polyprotein precursors. BPMV RNA1 codes for five mature proteins required for replication whereas RNA2 codes for a putative cell-to-cell movement protein (MP) and the two coat proteins (L-CP and S-CP). In this report, we demonstrate that BPMV-based vectors can be generated by inserting the gene of interest into the RNA2-encoded polyprotein open reading frame, between the MP and L-CP coding regions, and constructing additional proteinase cleavage sites to flank the foreign protein.

Materials and methods

Virus strains

BPMV strains K-Ho1, K-Ha1 and K-G7 have been previously described and their complete nucleotides sequences have been reported (Gu *et al.*, 2002; Gu and Ghabrial, 2005). The BPMV strains were propagated in the soybean cultivar ‘Essex’, and infected tissues were used for virion purification as previously described (Ghabrial *et al.*, 1977). *Soybean mosaic virus* (SMV) strains G6 and G7 were used for amplification of the HC-Pro coding regions. SMV strain designation was based on the differential reactions of soybean cultivars carrying resistance genes to SMV (Cho and Goodman, 1979; Gunduz *et al.*, 2004).

RNA extraction and northern hybridization analysis

Viral RNA was isolated from purified virions by the SDS-phenol method (Peden and Symons, 1973). Total RNA was extracted from plant tissue using a hot phenol method (Verwoerd *et al.*, 1989). For northern blot hybridization analysis, the RNA samples were denatured in the presence of glyoxal and dimethyl sulfoxide and separated by electrophoresis on a 0.8% agarose gel in 10 mM sodium phosphate buffer, pH 6.3 (Sambrook and Russell, 2001). RNA was transferred onto Hybond-N$^+$ membranes (Amersham, Piscataway, NJ), according to the manufacturer’s instructions. The membranes were then prehybridized, hybridized and air-dried as previously described (Gu *et al*., 2002). Full-length RNA1 and RNA2 cDNA clones of strain K-G7 (strain subgroup I) or K-Ha1 (strain subgroup II) were used as templates for probe preparation by the Rediprime^{TM} II random prime labeling system (Amersham, Piscataway, NJ) according to the manufacture’s instructions. The northern blots were exposed to a phosphorimager screen and the images were visualized with a PhosphorImager 445 SI system and analyzed with the ImageQuant 4.1 software program (Amersham).

Construction of BPMV RNA2 vectors

Full-length infectious BPMV RNA2 cDNA clones (pGG7R2 and pGHoR2), derived from subgroup I and II strains, respectively, were used for construction of the BPMV RNA2 vectors. Transcripts derived from plasmid pGHoR1 containing a full-length infectious RNA1 cDNA (type I, RNA1), were used along with transcripts from recombinant plasmids pGG7R2 or pGHoR2 in all inoculations. Plasmids pGHoR1, pGG7R2 and pGHoR2 were described previously (Gu *et al.*, 2005).

GFP constructs

The 5'-half of BPMV RNA2 cDNA in plasmids pGHoR2 or pGG7R2 (1830 bp) was amplified by PCR using the primer pair F1 and SwaI-Rev-R2 (Table 4.1) and the PCR products were cloned into the pGEM-T easy vector (Promega, Madison, WI). The resultant clones were digested with *Swa*I and *Nco*I and two clones, pGG7R2-1 and pGHoR2-1, were selected following verification by restriction enzyme digestion and nucleotide sequencing. Clones pGG7R2-1 and pGHoR2-1 were digested with *Aat*II, blunt-ended and self-ligated to remove the *Aat*II restriction site in the vector and to create the new constructs pGG7R2-2 and pGHoR2-2. The *GFP5* gene was amplified using plasmid pZGFP (Soldevila and Ghabrial, 2000) as a template and the primer pair GFP-For and GFP-Rev (Table 4.1). The PCR product was cloned into the pGEM-T easy vector and the resultant clone (pGGFP-1) was verified by sequencing. The pGG7R2-2 and pGHoR2-2 constructs were digested with *Swa*I and *Sal*I and ligated into similarly digested pGGFP-1 to generate constructs pGG7R2-3 and pGHoR2-3, respectively. The 3'-half of BPMV RNA2 cDNA in plasmids pGHoR2 or pGG7R2 (1841 bp) was amplified by PCR using the primer pair AatII-For-R2 and R1 (Table 4.1) and the PCR products were cloned into the pGEM-T easy vector (Promega) to generate clones pGG7R2-4 and pGHoR2-4, which were verified by sequencing. Clones pGG7R2-4 and pGHoR2-4 were digested with *Sac*I and *Pst*I, blunt-ended and self ligated to remove the vector *Sal*I site and to generate clones pGG7R2-5 and pGHoR2-5, respectively. Finally, clones pGG7R2-5 and pGHoR2-5 were digested with *Aat*II and *Sal*I and ligated into plasmids pGG7R2-3 and pGHoR2-3, previously digested with the same two enzymes, to produce the infectious constructs pBPG7R2-GFP and pBPHoR2-GFP.

DsRed constructs

The dsRed gene was amplified by PCR using plasmid pDsRed2-C1 (Clontech, Palo Alto. CA) as a template and the primer pair RFP-For and RFP-Rev (Table 4.1). The PCR product was cloned into the pGEM-T easy vector to generate clone pGdsRed-1, which was confirmed by sequencing. The dsRed gene was released from pGdsRed-1 by digestion with *Swa*I and *Aat*II and the resultant fragment was used ligated into plasmids pBPG7R2-GFP and pGHoR2-GFP, which were *Swa*I and *Aat*II-digested, to replace the *GFP* gene and generate the infectious constructs pBPG7R2-dsRed and pBPHoR2-dsRed, respectively.

Vector modification

To generate a suitable BPMV-RNA2 vector for cloning and expression of foreign genes, the GFP construct, pBPG7R2-GFP (Figure 4.1), was modified to remove most of the GFP sequences and to insert two new restriction sites. To introduce a *Bam*H1 restriction site into the BPMV RNA2 vector, primers VecModi-For1 and VecModi-Rev1, which partially anneal to each other, were subjected for PCR and the product was cloned into the pGEM-T easy vector and confirmed by sequencing (pVecModi-1). A similar approach was used to introduce a second *Msc*I restriction site into the BPMV RNA2 vector; primers VecModi-For2 and VecModi-Rev2 (Table 4.1), which partially anneal to each other, were subjected to PCR and the product was cloned into the pGEM-T easy vector and confirmed by sequencing (pVecModi-2). Plasmid pBPG7R2-GFP was digested with *Swa*I and *Msc*I and ligated into similarly digested pVecModi-2 to generate plasmid pGG7R2-6. The latter was then digested with *Cla*I and *Aat*II and ligated into similarly digested pVecModi-1 to generate the BPMV-RNA2 vector, designated pBPG7R2-V (Figure 4.1)

Bar constructs

The *bar* gene was released from plasmid pBG-GD (Straubinger *et al.*, 1992) by digestion with *Bgl*II, blunt-ended with Klenow large fragment DNA polymerase (Invitrogen, Carlsbad, CA, USA) and then digested with *Bam*HI. The DNA fragment containing the *bar* gene sequence was gel purified and ligated into pBPG7R2-V, previously digested *Msc*I and *Bam*HI, to produce pBPG7R2-Bar.

Constructs of RNA silencing suppressors

Tomato bushy stunt virus (TBSV) P19 gene was amplified from plasmid PZP-TBSVp19 (Qu, *et al.*, 2003) using the primer pair TBSV-P19-For and TBSV-P19-Rev (Table 4.1) and the resulting PCR product was cloned into pGEM-T easy vector. Clones in the correct orientation were selected and digested with *BamH*I and *Msc*I and the released P19 gene was cloned into *BamH*I-*Msc*I-digested pBPG7R2-V to produce pBPG7R2-P19. *Turnip crinckle virus* (TCV) coat protein (CP) gene was amplified from plasmid PZP-TCVCP (Qu, *et al*., 2003) using primers TCV-CP-For and TCV-CP-Rev (Table 4.1) and the resultant PCR product was cloned into pGEM-T easy vector. Clones in the correct orientation were selected and digested with *BamH*I and *Eco*RV and the released CP gene was cloned into *BamH*I-*Msc*I-digested pBPG7R2-V to produce pBPG7R2-TCP. The coding region of *Tobacco etch virus* (TEV) HC-Pro was amplified by PCR using plasmid pTEV7D, which contains a full-length cDNA of TEV-RNA (Dolja *et al*., 1992), as a template along with primers TEV-P2-For and TEV-P2-Rev (Table 4.1). The resultant PCR product was cloned into pGEM-T easy vector and clones in the correct orientation were digested with *BamH*I and *Eco*RV. The released HC-Pro gene was then cloned into *BamH*I/*Msc*I-digested pBPG7R2-V to produce pBPG7R2-HCPro(T). A RT-PCR approach was used to clone SMV HC-Pro coding region. A reverse primer (PBr; Table 4.1) was used for first strand cDNA synthesis with total RNA from soybean leaves infected with SMV strains G6 or G7 and a Superscript II reverse transcriptase kit (Invitrogen). To eliminate a *Bam*HI site in the SMV-HC-Pro coding region without changing the amino acid sequence, a two-step PCR method was used. In the first step two overlapping cDNA fragments containing the entire HC-Pro sequence (fragments A and B covering the 5' and 3' halves , respectively) were PCR amplified in separate reactions using first strand cDNA as a template and two pairs of primers (PAr and Paf and PBr and PBf). The reverse primer of fragment A (PAr; 23 nucleotides in length) is complementary to the forward primer of fragment B (PBf). An equimolar amount of each fragment was in the presence of primers PBr and Paf and used for the second step PCR. The final PCR product was cloned into pGEM-T easy vector and clones in the correct orientations were confirmed by sequencing. The inserted HC-Pro genes from strains G6 and G7 were digested with *Bam*HI and *Eco*RV and ligated into *BamH*I/*Msc*I-digested pBPG7R2-V to produce pBPG7R2-HCPro(S6) and pBPG7R2-HCPro(S7), respectively.

PDS constructs

Soybean genomic DNA was extracted from leaves of the soybean cultivar Essex using the method described before (Srinivasa *et al*., 2001). A 319 bp *PDS* fragment was PCR-amplified using the primer pair PDS-sen5-For and PDS-sen5-Rev (Table 4.1). The PCR product was digested with *BamH*I and *Eco*RV and ligated into *Bam*HI/*Msc*I-digested pBPG7R2-V to generate construct pBPG7R2-PDS.

Nucleotide sequencing

All sequencing was done using Big Dye Terminator DNA Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the ABI Prism 310 genetic analyzer. Sequence analysis was performed using the DNA strider (CEA, France) and Vector NTI programs (Informax Inc., Frederick, MD, USA).

In vitro transcription and inoculation

Plasmids pGHoR1 (containing cDNA to type I RNA1, from strain K-Ho1) and pCRHaR1 (containing cDNA to type II RNA1, from strain K-Ha1) were used as templates for *in vitro* transcription as previously described (Gu and Ghabrial, 2005). After transcription, 5µl samples of the reaction mixture were analyzed on a 1% agarose to assess yield and quality of the transcripts. RNA transcripts (a mixture of RNA1 and RNA2 transcripts) were used to inoculate fully expanded leaves of soybean by rub inoculation.

Protein expression and western blot analysis

Total protein extraction from soybean leaves was performed as described by Osherov and May (1998). Protein concentration was estimated by the Bio-Rad protein assay method (Bio-Rad Laboratories, Hercules, CA, USA). Known amounts of bacterially-expressed GFP was used as a standard in assays to assess expression level. For this purpose, the Wild type GFP gene was released from plasmid pIVEX2.3 (Roche Applied Science, Indianapolis, IN, USA) by digestion with *Xba*I and *Bam*HI and cloned into pET21d vector (EMD Biosciences, San Diego, CA, USA). The resulting clone was transformed into *E. coli* strain BL21 (DE3) and GFP expression was induced and purified according to manufacturer's instructions (EMD Biosciences). Western blot analysis was carried out as previously described (Srinivasa *et al.*, 2001) using antisera to BPMV CP and GFP (Chemicon international Inc., Temecula, CA, USA). GFP expression level was assessed using ImageQuant v5.2 (Amersham).

Fluorescence detection

Whole leaf green fluorescence images were acquired using BioChemi-V cooler camera mounted on Epi Chemi II Darkroom (UVP company, Upland, CA, USA). The settings were overhead excitation light 365 nm and filter set as SYBR Green (Hoechst Blue). The

Labworks Ver 4.0.0.8. software was used for acquiring images, which were exported as TIFF files.

Herbicide treatment

One-week old soybean seedlings were inoculated with the recombinant BPMV-bar construct. Two weeks later, the infected soybean plants were sprayed with the herbicide Liberty, which contains glufosinate-ammonium (GA) as the active ingredient (Aventis CropScience, Research Triangle PK, NC, USA), at a concentration of 0.1% GA (w/v) in deionized water. The soybean plants were photographed 3 weeks after herbicide treatment.

Results

Construction of BPMV RNA2 vectors

For development of BPMV as a viral vector for expression of heterologous proteins in soybean, the gene of interest was inserted into the RNA2-encoded polyprotein ORF between the movement protein (MP) and the large coat protein (LCP) coding regions. Additional proteinase cleavage sites were created to flank the foreign protein by duplicating the MP-LCP cleavage site (as exemplified by the GFP gene in Figure 4.1a). The coding sequences for the 8 C-terminal amino acids of the MP and the 19 N-terminal amino acids of the LCP were included for efficient processing. To minimize the chances of homologous recombination, thus instability, we took advantage of codon degeneracy by changing the third nucleotide in each codon (in accordance with BPMV codon usage) so that the encoded amino acid residues remain unchanged (Figure 4.1, diagram a). Initially, BPMV recombinant vectors expressing GFP or DsRed were constructed and shown to be infectious and stable. Under greenhouse conditions, the GFP construct was passed 4 times without any apparent reduction in fluorescence intensity (Figure 4.2).

The BPMV vector was further modified to include additional cloning sites (Figure 4.1, diagram b); foreign genes can be cloned by digesting the vector pBPG7R2-V with *Bam*HI and *Msc*I (for directional cloning) or by digestion with *Msc*I (for blunt end cloning). Two sets of BPMV RNA2 vectors corresponding to BPMV RNA2 strain subgroups I and II were constructed.

Several different genes that varied in size and biological activity were cloned into the BPMV RNA2 vectors utilizing the *Bam*HI and *Msc*I restriction sites in the modified vector (Figure 4.1b). In all cases, the foreign protein was placed between two artificial cleavage sites with duplication of 27 virus-derived amino acids, for efficient processing, as described for the GFP constructs. These genes ranged in size from 520 bp to 1400 bp (Figure 4.1c) and included the herbicide resistance *bar* gene (phosphinothricin acetyltransferase) and several viral-encoded suppressors of host-mediated RNA silencing (TBSV P19, TCV CP, SMV HC-Pro and TEV HC-Pro).

Expression levels of foreign genes from BPMV vectors

The GFP constructs were used to evaluate foreign gene expression levels in soybean. The primary leaves of 7-10 day old soybean seedlings were inoculated with the BPMV-GFP constructs derived from subgroup I or subgroup II BPMV RNA2. Three weeks postinoculation, total soluble proteins were extracted from first and second trifoliolate leaves and subjected to western blot analysis (Figure 4.3). Affinity-purified His-tagged GFP, which was expressed in *E. coli*, was used as a control (Figure 4.3, lane 7). Interestingly, the expression level provided by subgroup I RNA2 vectors was higher than that obtained with subgroup II RNA2 vectors in both the first and second trifoliolate leaves (compare lanes 3 and 5, Figure 4.3). To assess the GFP expression level, the western blot was scanned and the generated images of band intensity were analyzed by the ImageQuant v5.2 program (Amersham). The results indicated that GFP expression level accounted for 1% of total proteins in soybean.

Stability of the foreign gene expressed from BPMV RNA2 vectors

To assess the stability of inserted foreign genes during serial plant passages, virions were purified from soybean plants previously infected with the BPMV-GFP or BPMV-DsRed constructs. Following three passages of the recombinant BPMV vector, viral RNA was isolated from purified virions and subjected to northern hybridization analysis (Figure 4.4). Only a single band of the predicted size of the recombinant RNA2 containing the coding sequences for GFP or DsRed was resolved. No wild type RNA2 was detected even following extended overexposure of the blots. Furthermore, fluorescence due to expression of GFP or dsRed was readily detected in the seed coats from immature seeds (data now shown) suggesting that the foreign genes were stably expressed at a later developmental stage during pod formation.

Biological activity of gene products expressed from BPMV RNA2 vectors

Plants infected with the BPMV-bar construct were resistant to ammonium glufosinate when applied as a 0.1% solution (w/v) in deionized water (Figure 4.5a). In contrast, the noninoculated control, BPMV K-G7-infected plants and plants infected with the BPMV-GFP construct were killed within 3 weeks after herbicide treatment (Figure 4.5). Furthermore, plants infected with BPMV-bar construct were found to withstand ammonium glufosinate treatment at a concentration of 1% (w/v) in deionized water with little or no damage (data not shown).

It is known that certain RNA silencing suppressors encoded by plant viruses may enhance symptom severity induced by heterologous viruses (Pruss *et al.*, 1997; Yang and ravelonandro, 2002). Three different viral RNA silencing suppressors with apparently dissimilar underlying mechanisms (Roth *et al.*, 2004) were cloned into the BPMV vector and tested for their activity in soybean. The resulting constructs were infectious and the infected plants showed very severe symptoms including extensive stunting, leaf deformation, blistering and veinal necrosis compared with the mild mottling symptoms induced by infections involving wild type BPMV RNA2 (Figure 4.6). The severe symptoms induced by these constructs are reminiscent of the top necrosis syndrome induced by double infection of soybean plants with BPMV and SMV (Anjos *et al.*, 1992).

Silencing of phytoene desaturase in *Glycine max*

Virus-induced gene silencing (VIGS) is an attractive tool for studies of gene function. To determine whether a VIGS vector based on BPMV could be useful in silencing of endogenous soybean genes, a 318 bp fragment of the *phytoene desaturase* (*PDS*) gene, which is necessary for production of carotenoid pigment production, was inserted into the BPMV vector, and the resulting construct was used to infect soybean. Soybean plants inoculated with the recombinant BPMV-PDS developed typical photo-bleached leaves 2 weeks postinoculation indicating that that *PDS* gene had been silenced (Figure 4.7a). The

BPMV-PDS vector was stable when inoculum was prepared from the upper leaves and used to inoculate healthy soybean seedlings. Representative upper leaves harvested at 21 dpi from the second passage plants are shown in Figure 4.7. VIGS of the *PDS* gene was clearly evident regardless of the soybean cultivar used, Essex, Clark, Williams or York.

Discussion

This study represents the first report to demonstrate that BPMV-based vectors are suitable for efficient expression of heterologous proteins in soybean. The BPMV-RNA2 vector is indeed the only available plant-virus-based vector that is appropriate for expression of foreign proteins in soybean. Although the CPMV-RNA2 vector (Gopinath *et al*., 2000) could potentially be used as an expression vector in soybean, it is unstable and induces severe symptoms on soybean (Anjos *et al*., 1992). Furthermore, soybean is not a natural host for CPMV and the virus is not believed to be present in the United States (Lomonossoff and Shanks, 1999). Thus, CPMV-based vectors cannot be released in the field for practical applications. The instability of the CPMV-RNA2 vector appears to be related to homologous recombination, which may occur as a consequence of duplication of the cleavage sites that border the inserted foreign protein. In engineering the BPMV-RNA2 vector, we took advantage of the degeneracy of the genetic code and altered the nucleotide sequence of the duplicated regions without affecting amino acid sequence in order to minimize the chances of homologous recombination.

We demonstrated the BPMV-GFP vector was stable after four serial passages in soybean, and no traces of wild type virus were detected by northern hybridization analysis (Figure 4.4). The finding that the bright green fluorescence was maintained throughout the soybean plant including the seed coats of immature seeds provides further evidence for the endured stability of the GFP construct. At present, there are no commercially available soybean cultivars with resistance to BPMV. Because of the recent BPMV epidemic (Giesler *et al*., 2002), our laboratory as well as others are actively engaged in large-scale screening trials for BPMV resistance in various soybean germplasm accessions and transgenic lines. The BPMV vectors containing reporter genes such as GFP will provide a very useful tool for rapid screening of resistant plants.

The level of foreign gene expression, as exemplified by the BPMV-GFP vector, was estimated to account for 1% of total soluble proteins. This level is comparable to that reported for the PVX-based vectors (Culver, 1996). Expression of RNA silencing suppressors from recombinant BPMV vector showed significant enhancement in symptom severity (Figure 4.6) and the accumulation of the viral coat proteins, as assessed by immunoblot analysis and ELISA using an antiserum to BPMV CP (data not shown). Expression of RNA silencing suppressors in combination with recombinant BPMV vectors may be useful for enhanced levels of protein expression (Mallory *et al*., 2002; Voinnet *et al*., 2003). Although expression of more than one product (suppressor plus the proteins of interest) from the same BPMV vector is theoretically feasible (upper limit for insert size is 2.4 kbp), stability might be a concern because of the additional duplicated cleavage sites. Thus, expression of RNA silencing suppressors from co-infecting recombinant BPMV vector is probably a better approach.

Soybean is the top oilseed crop in the world and provides multi-billion dollar source of high quality protein. Some of the major goals of the soybean industry are to increase the level of plant resistance to environmental stress, targeted pests, and diseases in commercial varieties and to accelerate the process of developing and commercializing new traits into elite germplasm. The availability of the BPMV expression vector will allow rapid evaluation of candidate proteins with antifungal or insecticidal activities as well as other valuable proteins that may enhance the nutritional and commercial value of soybean. The potential advantages that make BPMV an attractive vector system are that the virus (including mild strains) multiplies to high levels in soybean (20-50 mg virus from 100 g leaf tissue; Ghabrial, unpublished) and that it is stable and easily purified. For inoculation purposes under greenhouse conditions, we successfully used purified recombinant BPMV virions or extracts from fresh or dried leaves from plants previously infected with the recombinant vector. For field applications, the use of a high pressure spray device would be more practical to apply the mixture of recombinant virions (or extracts from infected plants) and abrasive to production plants.

We have also demonstrated that the BPMV-based vector is suitable for use as a VIGS vector to study gene function in soybean. The bleached silencing phenotype of soybean plants inoculated with BPMV vector carrying a fragment of the soybean *PDS* gene was stable overtime as it continued to develop throughout the duration of the experiment (35 dpi). VIGS has proved to provide an impressive means to study gene function and has also demonstrated to be particularly useful in plants with genetic redundancy like soybean (Lawrence and Pikaard, 2003). The most widely used vectors are based on PVX or TRV (Liu *et al*., 2002; Lu *et al*., 2003) and their applications have been mainly studied in *N. benthamiana* where VIGS response is generally stronger and more enduring than in other plants (Lu *et al*., 2003). Recently, efficient VIGS systems have also been developed for a few additional host plants including barley, tomato and *Pisum sativum* (Constantin *et al*., 2004; Holzberg, *et al*., 2002; Liu, *et al*., 2002). There is currently an urgent need for a VIGS vector suitable for use in soybean considering the substantial wealth of available information on soybean genomics. None of the currently established VIGS vectors is appropriate for use in soybean. Although full-length cDNA infectious clones are available for the potyviruses SMV and *Clover yellow vein virus* (Hajimorad *et al*., 2003; Masuta *et al*., 1998), neither is suitable as a VIGS vector because they encode potent suppressors of RNA silencing. There are presently available more than 300,000 expressed sequence tags (ESTs) that are derived from over 80 different cDNA libraries representing a wide range of organs, developmental stages, genotypes and environmental conditions (Stacey *et al*., 2004). This soybean EST collection provides a large resource of publicly available genes and gene sequences that can potentially provide valuable insight into structure and function of this model crop legume. VIGS would present an ideal tool for large-scale functional genomics to convert the soybean sequence information into functional information. We demonstrated that BPMV-based vector is suited for this purpose.

A possible disadvantage of VIGS is that symptoms induced by virus infection may obscure the phenotype associated with silencing of the gene of interest. This should not be a problem with the BPMV-soybean system based on current knowledge of symptom severity determinants in BPMV. We have recently mapped BPMV-induced symptom severity to RNA1, and more specifically to the coding regions of the protease co-factor and the C-terminal half of putative helicase. Furthermore, we identified the amino acid positions that are responsible for differences in symptom severity between mild and severe strains (Gu and Ghabrial, 2005). Since BPMV RNA2 does not play a direct role in symptom severity and since it is the genomic segment that carries the foreign gene of interest, it is then a simple matter to avoid interference from virus symptoms by using RNA1 derived from a mild strain or from a strain engineered to cause only attenuated symptoms and enhanced production of the recombinant RNA2.

Table 4.1 primers used in this study

Name	Sequence
F1	TAATACGACTCACTATAGTATTAAAATTTTCATAAGATTTGAAATTTTGATAAACCG
R1	TTCCGCGGCCGCTATGGCCGACGTCGACTTTTTTTTTTTTTTTTTT
Aat II-For-R2	GGACGTCGAGACTCCAAAAGGTTCCAT
Swa I-Rev-R2	AATTTAAATAGATTTGTTTCCATTTGAGC
GFP-For	AATTTAAATTGTCTCTTGATGATGTTGAAACACCCAAAGGATCAATGAGTAAAGGAGAAGAACTTTTCACT
GFP-Rev	GGACGTCGTCCAATGAAAGCTTAAACAAGTTAGTCTCCATTTGTGCTTGCACCTCGTTATATTGTTTGTATAGTTCATCCATGCCATGTG
RFP-For	ATTTAAATTGTCTCTTGATGATGTTGAAACACCCAAAGGATCAATGGCATCCTCTGAAGATGTTATCAAG
RFP-Rev	GACGTCGTCCAATGAAAGCTTAAACAAGTTAGTCTCCATTTGTGCTTGCACCTCGTTATATTGGGCGCCGGTGGAGTGG
VecModi-For1	AATTTAAATTGTCTCTTGATGATGTTGAAACACCC
VecModi-Rev1	TTGGCCAGGATCCTTTGGGTGTTTCAACATCATC
VecModi-For2	ATCGATGGCCACAATATAACGAGGTGCAAGCCCAAATGGAGACC
VecModi-Rev2	GACGTCGTCCAATGAAAGCTTAAACAAGTTGGTCTCCATTTGGG
SMV-P2-PAf	GGATCCTCCCAAAATCCTGAAGCTCAGTT
SMV-P2-PAr	ACTGTCAAAGATCCAAAAGAGTC
SMV-P2-PBf	GACTCTTTTGGATCTTTGACAGT
SMV-P2-PBr	TCATCCTCTGTTGCACGATATCACCAACTCT
TEV-P2-For	GGATCCAGCGACAAATCAATCTCTGAGGCA
TEV-P2-rev	GATATCTCCAACATTGTAAGTTTTCATTTCGGA
TBSV-P19-For	CGCGGATCCATGGAACGAGCTATACAAGGA
TBSV-P19-Rev	TGTGTTGGCCACTCGCTTTCTTTTTCGAAGGT
TCV-CP-For	CGCGGATCCATGGAAAATGATCCTAGAGTC
TCV-CP-Rev	ATTGGATATCAATCCTGAGTGCTTGCCATTTTCC
PDS-For	CCGCGGATCCGCCGCTTGTGGCTATATATCT
PDS-Rev	CACAGATATCTCCTGCACCGGCAATAACGAT

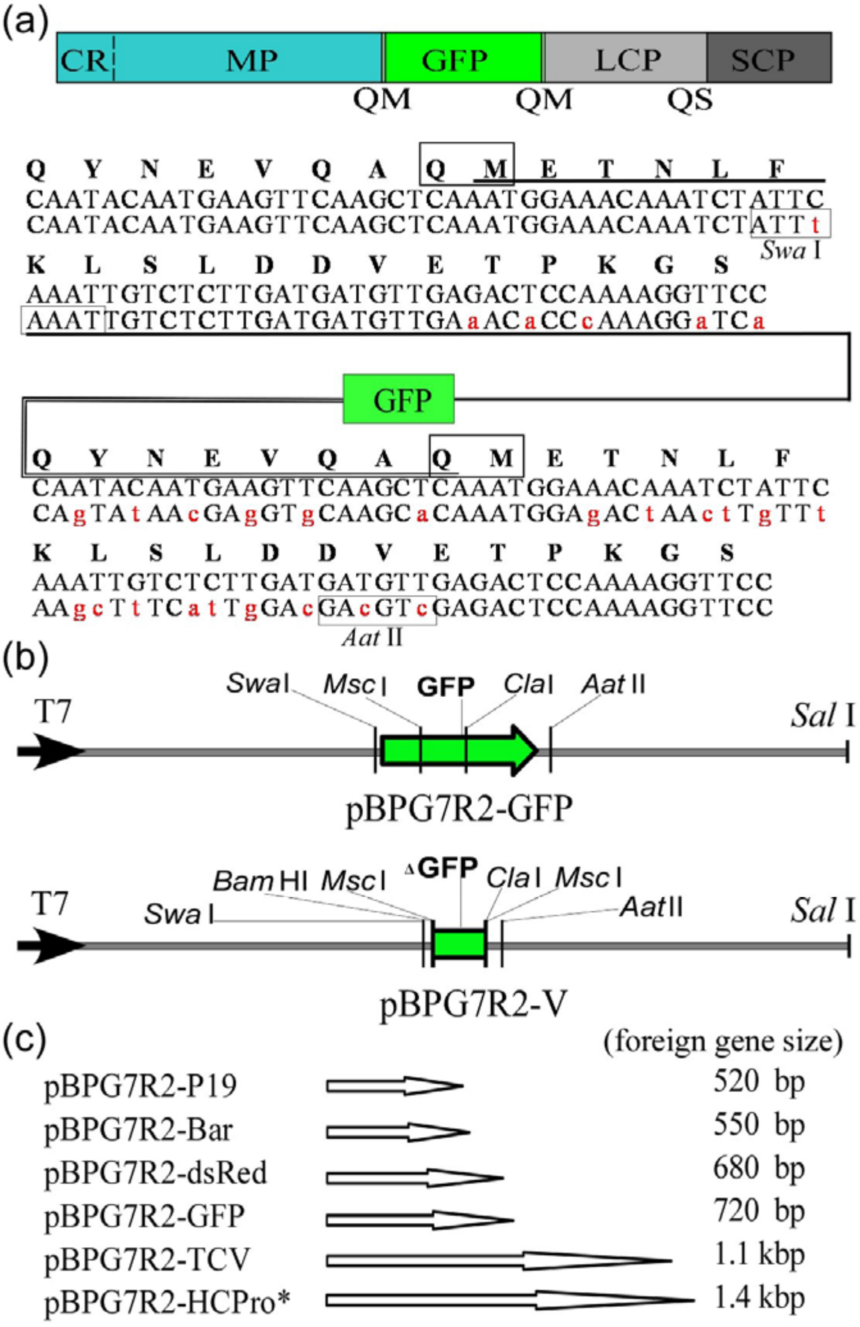

Figure 4.1. Schematic representation of BPMV RNA2 vector constructs.

(a) Genome organization of BPMV RNA2 and vector construction strategy. RNA2 is translated into two overlapping carboxy coterminal polyproteins. CR, RNA2 replication cofactor; MP, movement protein; L-CP, large coat protein; S-CP, small coat protein.

A foreign gene (*GFP*) is inserted between MP and L-CP coding sequences. The cleavage site (QM; boxed) is duplicated with the 8 C-terminal amino acids of the MP and the 19 N-terminal amino acids of the L-CP included for efficient processing. Amino acids, in the one-letter code, are indicated above the nucleotide sequences. Altered nucleotides are printed in red in lowercase. The *GFP* gene is shown as a green box. The introduced restriction sites, *Swa*I and *Aat*II, are boxed.

(b) Schematic presentation of BPMV RNA2 vector constructs. (Upper) construct pGG7R2-GFP with GFP inserted between two artificial proteolytic cleavage sites; the designation G7R2 indicates that RNA2 was derived from BPMV strain G7. (Lower) construct pGG7R2-V, which is a modified version of construct pGG7R2-GFP, contains additional restriction sites for cloning of foreign genes. A foreign gene can be cloned as a *Bam*HI-*Msc*I fragment in the pGG7R2-V vector after the vector is digested with same two enzymes. Alternatively, the foreign gene can be blunt-end ligated into *Msc*I-digested pGG7R2-V vector.

(c) A diagrammatic representation of the proteins expressed from the BPMV RNA2 vector listed in increasing order of their sizes: P19, *Tomato bushy stunt virus* P19 protein; Bar, phosphinothricin acetyltransferase; DsRed, DsRed red fluorescent protein; GFP, green fluorescent protein; TCVCP, *Turnip crinkle virus* coat protein; HCPro, potyvirus helper component-protease protein.

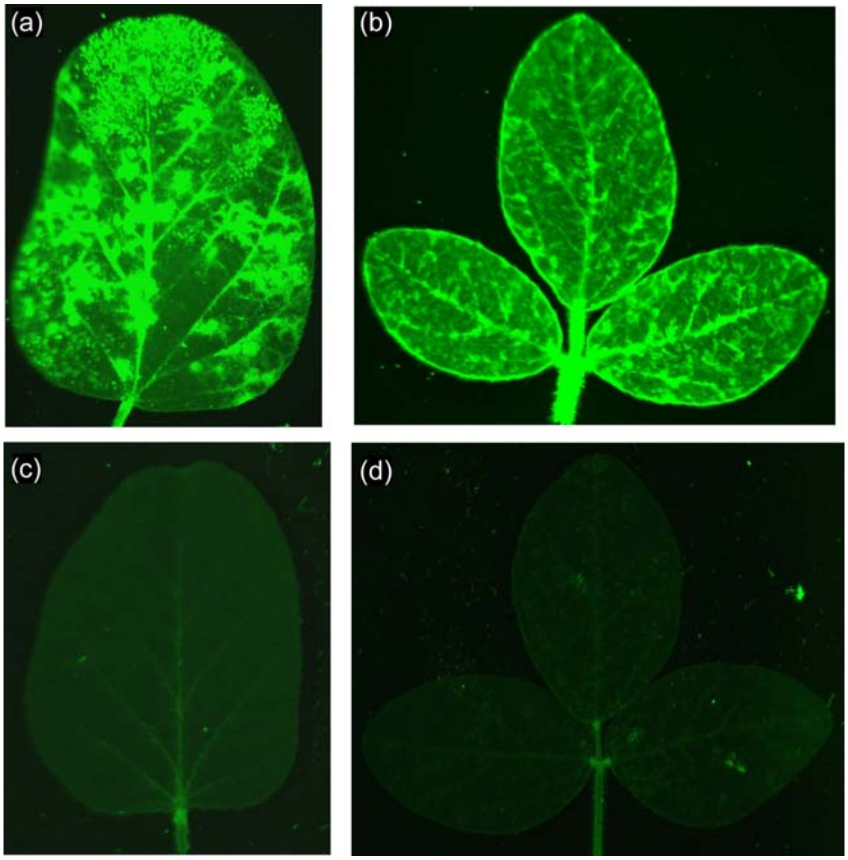

Figure 4.2. Green fluorescence on inoculated and systemic leaves of soybean plants. Soybean seedlings were inoculated on their primary leaves with leaf extracts prepared from plants infected with the BPMV-GFP construct after four serial passages in soybean. Alternatively, the primary leaves were inoculated with the wild-type K-Ho1 isolate or mock inoculated with buffer only.

(a) The primary leaf and (b) second trifoliolate leaf from a soybean plant, previously inoculated with the BPMV-GFP construct, showed intense green fluorescence under UV light. No fluorescence was detected on the mock-inoculated primary leaf (c) or on the second trifoliolate of K-Ho1-infected plants (d). Leaves in (a), (b) and (d) showed symptoms typical of isolate K-Ho1; mosaic and necrosis on inoculated leaves and mottling on systemic leaves. All leaves were photographed under UV light 11 days post-inoculation..

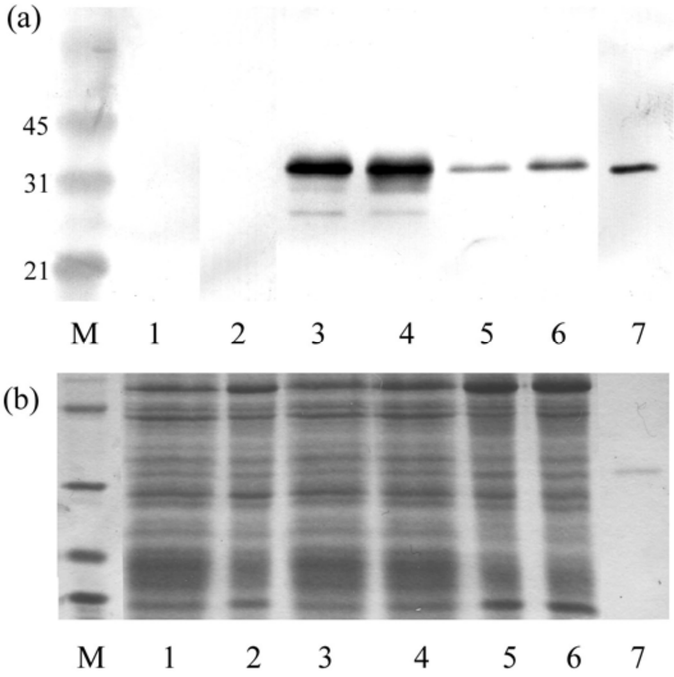

Figure 4.3. Immunoblot analysis of total proteins from soybean plants infected with GFP constructs

(a) Western blot analysis using an anti-GFP antiserum. Samples of total proteins (15 μg) extracted from soybean plants subjected to the following treatments were used: mock-inoculated (1st trifoliolate; lane 1), wild-type BPMV K-G7-infected (1st trifoliolate; lane 2), pGG7R2-GFP-infected (1st and 2nd trifoliolate leaves; lanes 3 and 4, respectively), and pGHoR2-GFP-infected (1st and 2nd trifoliolate leaves; lanes 5 and 6, respectively). Purified His$_6$-tagged GFP protein (50 ng) was included in lane 7. Lane M contains low molecular weight protein markers.

(b) Levels of protein loading were assessed by SDS-PAGE analysis and Coomassie blue staining of the proteins tested in (a).

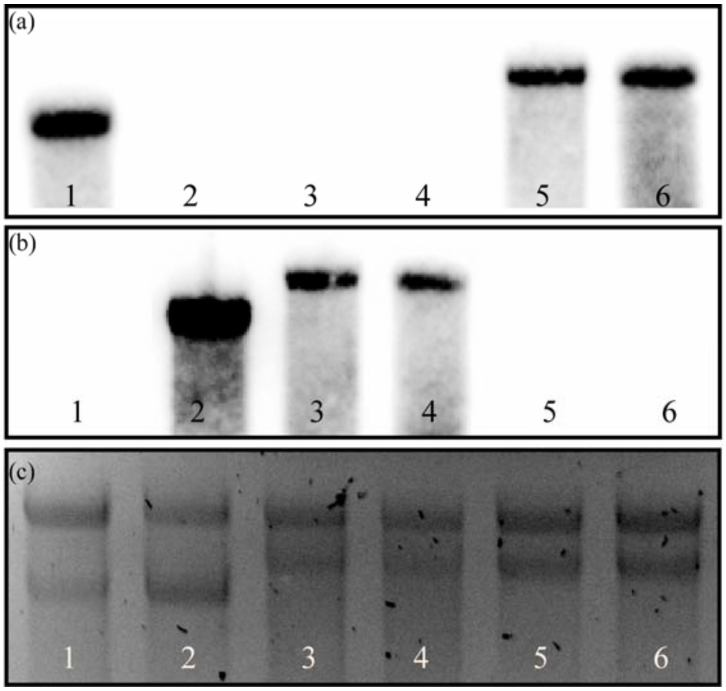

Figure 4.4. Stability of the *GFP* and *DsRed* genes expressed from the BPMV vectors.

(a and b) Northern blot hybridization analysis to assess the stability of foreign gene inserts. RNA extracted from purified virions from soybean plants previously inoculated with the following virus isolates or transcripts were used: 1, wild type strain K-Ho1; 2, wild type strain K-G7; 3, pGHoR1+pGG7R2-GFP transcripts; 4, pGHoR1+pGG7R2-DsRed transcripts; 5, pGHoR1+pGHoR2-GFP transcripts; and 6, pGHoR1+pGHoR2-DsRed transcripts. In panel (a) a probe specific for K-Ho1 RNA2 (type II) was used. In panel (b) a probe specific for K-G7 RNA2 (type I) was used. Note that the recombinant RNA2 constructs containing GFP or Ds-Red (lanes 3-6) are larger in size than those of the wild type RNA2 (lanes 1 and 2).

(c) Levels of RNA loading were assessed by ethidium bromide staining of viral RNA.

Figure 4.5. Herbicide resistance in soybean conferred by infection with the BPMV vector expressing the *bar* gene.

Soybean seedlings were inoculated onto the primary leaves with either wild type virus, transcripts from the BPMV-bar construct, transcripts from the BPMV-GFP construct or mock-inoculated with buffer alone. The herbicide treatment (0.1% amino glufosinate in deionized water) was applied to all plants when the second trifoliolate leaves were fully expanded. Photographs were taken 20 days after the herbicide treatment.

Soybean plants infected with: BPMV-bar construct (a); mock-inoculated control (b); wild type BPMV strain K-G7 (c); and BPMV-GFP construct (d) are shown.

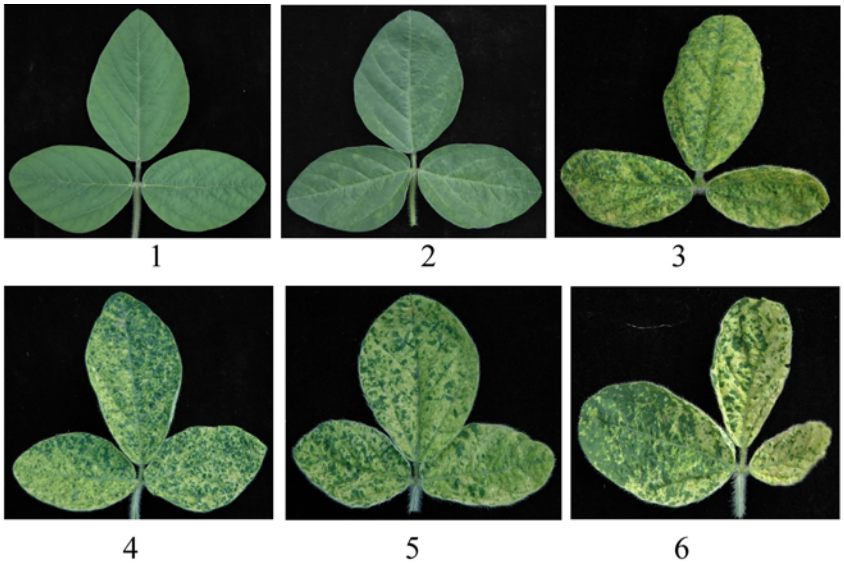

Figure 4.6. Enhancement of symptom severity in soybean plants infected with the BPMV vector carrying known viral suppressors of RNA silencing.

Photographs of first trifoliolate leaves from soybean plants inoculated with leaf extracts from plants infected with transcripts from pGHoR1 plus transcripts from: pGG7R2 (panel 2); pGG7R2-P19 (panel 3); pGG7R2-TCVCP (panel 4); pGG7R2-HCPro(S7) (panel 5); or pGG7R2-HCPro(T) (panel 6) are shown. A mock-inoculated control plant is shown in panel 1. Note enhanced symptom severity including necrosis on soybean plants infected with BPMV constructs carrying suppressors of RNA silencing (panels 3-6). The photographs were taken 2 weeks post inoculation.

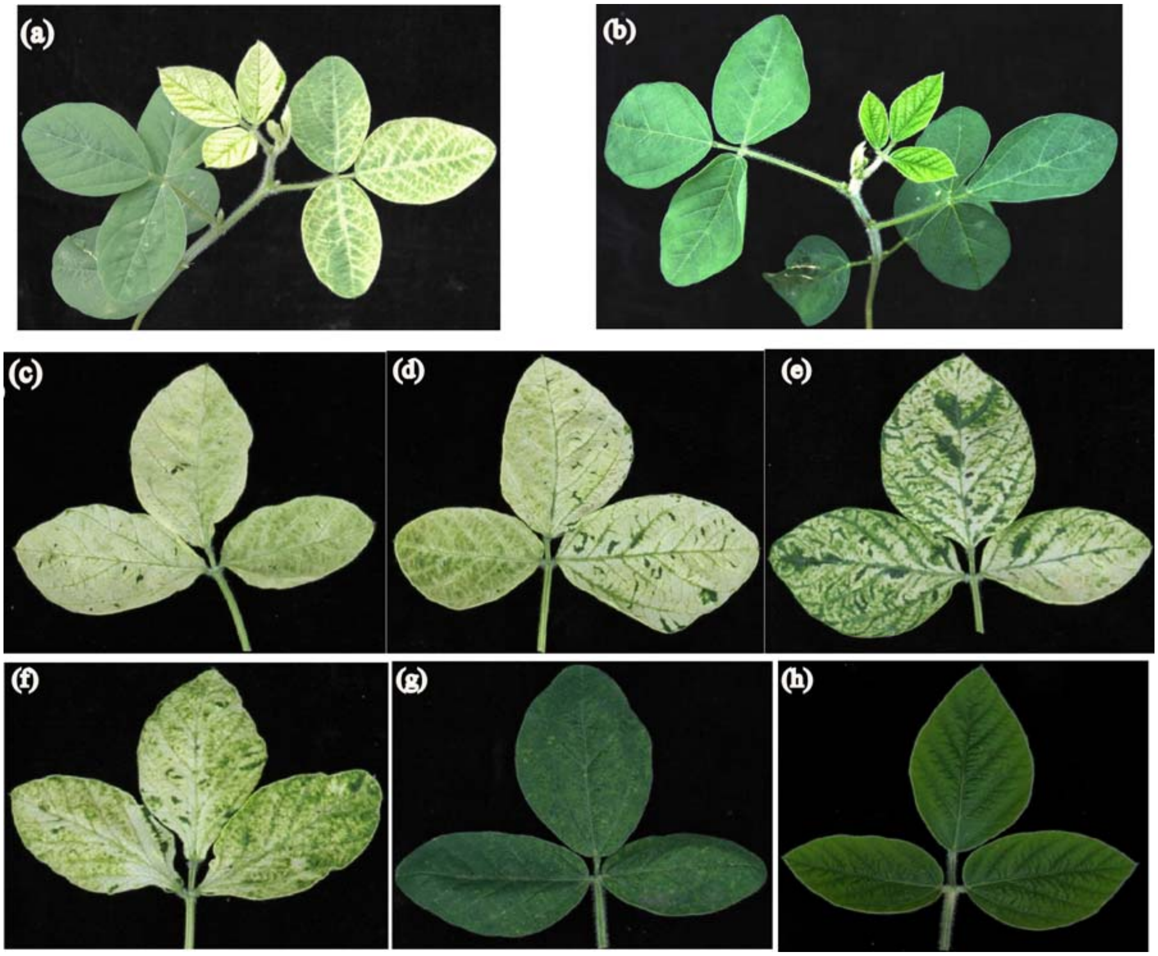

Figure 4.7. Virus-induced gene silencing (VIGS) of the soybean *PDS* gene.

(a, b) Phenotypes of soybean plants 21 days post-inoculation with the BPMV vector carrying a fragment of the soybean *PDS* gene (pGG7R2-PDS) and empty vector control (pGG7R2), respectively.

(c-f) Representative 3rd trifoliolate leaves from soybean plants previously inoculated with the pGG7R2-PDS vector showing different degrees of photobleaching are shown.

(g) A soybean plant previously inoculated with the vector control pGG7R2 showing typical mottling symptoms and no bleaching.

(h) A mock-inoculated soybean plant.

The photographs were taken 21days post-inoculation.

Chapter Five

HC-Pro suppressor function is required for synergistic interaction between *Soybean mosaic virus* and *Bean pod mottle virus*

Introduction

As plants are usually hosts to more than one virus, mixed infections are not uncommon. Some mixed infections can cause synergistic interactions with enhanced symptom severity that cannot be accounted for by merely the additive effects of single infections. Interestingly, potyviruses are often involved in such synergistic interactions. For example, co-infection of corn with the potyvirus *Maize dwarf mosaic virus* (MDMV) and *Maize chlorotic mottle virus* (MCMV, a *machlomovirus*) causes lethal necrosis (Goldberg and Brakke, 1987). Likewise, co-infection of soybean with the potyvirus *Soybean mosaic virus* (SMV) and *Bean pod mottle virus* (BPMV, a comovirus) causes top necrosis (Anjos *et al*., 1992; Calvert and Ghabrial, 1983). Furthermore, co-infection of *Nicotiana clevelandii* with the potyvirus *Potato virus Y* (PVY) and *Potato leaf roll virus* (PLRV, a polerovirus) enables PLRV to invade non-phloem tissues (Barker, 1987). Additionally co-infection of muskmelon with the potyvirus *Zucchini yellow mosaic virus* (ZYMV) and *Cucurbit aphid-borne yellow virus* (CABYV, a luteovirus) causes very severe symptoms (Bourdin and Lecoq, 1994). Severe symptoms in tobacco can be induced by co-infection with *Potato virus X* (PVX) and a variety of potyviruses including PVY, *Pepper mottle virus* (PepMoV), *Tobacco vein mottling virus* (TVMV) and *Tobacco etch virus* (TEV) (Vance, 1991; Vance *et al*., 1995). In each of these cases of potyvirus-associated synergism, an increase in the accumulation level of the non-potyvirus is correlated with increased symptom severity.

RNA silencing, a natural antiviral defense system, is used by plants against both viral and viroid infections. To establish a successful infection, plant viruses either encode proteins to suppress RNA silencing or escape this plant-mediated defense (Agrawal *et al*., 2003). Two major classes of suppressors have been identified. The first class of suppressors affects the small interfering RNAs (siRNAs) metabolism in plants by either blocking the creation of siRNA or blocking the function of siRNAs through binding to them. One example is the *Tomato bushy stunt virus* P19 protein, which binds to the siRNAs and thus prevents the formation of the RISC complex for specific cleavage (Silhavy *et al*., 2002). Another class of suppressors affects systemic silencing. A good example is the CMV 2b protein which primarily targets systemic silencing by blocking signal movement (Bucher *et al*., 2003; Guo & Ding, 2002). Potyvirus HC-Pro is a special type of suppressor that is highly effective against RNA silencing. It can block systemic silencing and reverse an established silencing (Hamilton *et al*., 2002, Mallory *et al*., 2003). As different viruses suppress different pathways in the RNA silencing process or escape silencing effect, the strong silencing suppression function of HC-Pro can complement other viral counter defense strategies. It was reported that the expression of either the 5′-proximal one-third of the potyviral genome in transgenic plants (Vance, 1991) or the P1/HC-Pro gene of TEV from a PVX-based vector (Pruss *et al*, 1997) can cause severe symptoms comparable to those induced by mixed infection. Furthermore, mutations in the central coding region of the HC-Pro gene abolished the PVX–potyvirus interactions, indicating direct involvement of the potyviral HC-Pro in the synergistic response (Shi *et al*., 1997).

In some cases, however, mixed infections involving potyviruses, may not lead to synergistic interactions , as in the cases of dual infections with *Peanut mottle virus* and either *Tomato spotted wilt virus* (Hoffmann *et al*., 1998) or *Bean pod mottle virus* (BPMV) (Anjos *et al*., 1992).

In this study, we used the BPMV- SMV dual infection system to investigate the underlying mechanism of synergism, specifically the role of HC-Pro in synergism. BPMV is a member of the genus *Comovirus* in the family *Comoviridae* (Goldbach *et al*., 1995). Like other comoviruses, BPMV has a bipartite positive-strand RNA genome consisting of RNA1 and RNA2, which are separately encapsidated in isometric particles 28 nm in diameter. Symptom severity due to BPMV infection was recently mapped to RNA1 (Gu and Ghabrial, 2005). BPMV has two subgroups of strains with distinct sequences (Gu *et al*., 2002). It was recently reported that the small coat protein of CPMV may function as a weak suppressor of RNA silencing (Canizares, *et al*., 2004). SMV has many strains and isolates with different level of interaction with BPMV. We have determined that a positively charged basic amino acid is required at position 144 for a diverse HC-Pro to function as suppressor. A variety of HC-Pro genes including chimeric HC-Pro between functional and nonfunctional suppressors were expressed in soybean from a BPMV vector. We found that synergism can be induced by the expression of a functional suppressor HC-Pro gene, which is tested by agro-infiltration in transgenic *Nicotiana benthamiana*.

Materials and methods

Virus strains and clones

BPMV strains K-Ho1, K-Ha1 and K-G7 have been previously described (Gu *et al*., 2002). Production of infectious cDNA clones of the genomic RNAs from these 3 strains have recently been reported (Gu and Ghabrial, 2005). The BPMV strains were propagated in the soybean cultivar 'Essex', and infected tissues were used for virion purification as previously described (Ghabrial *et al*., 1977). *Soybean mosaic virus* (SMV) strains G6, G7 and P10 were used for amplification of HC-Pro coding regions, as described below. SMV strain designation (G6 and G7) was based on the differential reactions of soybean cultivars carrying resistance genes to SMV (Cho and Goodman, 1979; Gunduz *et al*., 2004). SMV isolate P10 was obtained from a field grown soybean in Princeton, Ky. The binary vector containing TCV coat protein (TCV-CP) was provided by Dr. Jack Morris (Qu *et al*., 2003)

Plant growth conditions

Soybean and *Nicotiana benthamiana* plants were kept in the greenhouse or a growth chamber maintained at 22 °C with 16 h/8 h light/dark conditions. Disease symptoms were photographed with a digital camera (Nikon D70). Green fluorescence was photographed using the digital camera with a UV filter under excitation by a hand held UV lamp (Black-Ray long wave ultraviolet lamp model B 100 AP, UVP corp, Upland, CA, USA).

RNA extraction and gel blot analysis

Total RNA extraction and gel blot analysis were performed as described by Gu and Ghabrial (2005). Small RNA was extracted following the Qiagen method described by Llave *et al*. (2000). Small RNAs were separated by electrophoresis on 17%

polyacrylamide gels containing 7 M urea in 45 mM Tris-borate, pH 8.0/1 mM EDTA. After electrophoresis, small RNA was electroblotted in 90 mM Tris-borate, pH 8.0/2 mM EDTA to Hybond-N^+ membranes (Amersham Pharmacia) for 1 h at $3mA/cm^2$ gel, and UV crosslinked (1200 µJ, Stratalinker; Stratagene). The membrane was prehybridized in 50% (vol/vol) formamide, 5× Denhardt's solution, 0.1 mg/ml sheared salmon sperm DNA, 7% SDS, 0.3 M NaCl, and 50 mM phosphate buffer, pH 7.0, at 40°C for at least 1 h. Hydrolyzed GFP probe was directly added to a fresh hybridization solution. The hybridization was performed at 40°C overnight. The membrane was subsequently washed twice at 50 °C in 2X SSC & 0.2% SDS, once at 50 °C in 1X SSC & 0.1% SDS and once at 50 °C in 0.5X SSC & 0.1% SDS (1X SSC :0.15 M NaCl and 0.015 M sodium citrate). Prior to transfer, the gels were stained in ethidium bromide to confirm that equal amounts of RNA samples were loaded.

A plasmid containing full length mGFP5 sequence (Chapter Four) was digested with *Apa*I and *Cla*I, blunt-ended and self ligated to put the 3' end of mGFP gene (about 400 bp fragment) in sense direction between the T7 promoter and *Hind*III restriction site. After linearization with *Hind*III, *in vitro* transcription was performed as previously described (Gu and Ghabrial, 2005) except that α-^{32}p-dCTP was added and the cap analog was replaced with GTP. The GFP transcripts (20 µl) were hydrolyzed by adding 300 µl of 200 mM carbonate buffer (80 mM $NaHCO_3$ and 120 mM Na_2CO_3) and incubated at 60°C for 2 h. After hydrolyzation, 20 µl 3 M NaOAc /pH 5.0 was added to the 320 µl hydrolyzed probe and the 340 µl mixture was added directly to hybridization solution.

Infectious clones and *Agrobacterium* infiltration constructs

Production of infectious recombinant BPMV vector constructs containing SMV HC-Pro: SMV P10 and G7 wild type HC-Pro was previously described (Chapter Four). The two HC-Pro point mutations, P10-144-K and G7-144-I, were inserted into the BPMV vector following the same method.

Agrobacterium infiltration plasmids were all based on pRTL2 (Kasschau and Carrington, 2001) and pZP212 (Choi *et al*., 2004).

SMV HC-Pro: The HC-Pro genes for SMV strain G7 and isolate P10 were amplified from the previously described infectious clones (Chapter Four) with primer pair (SMV-P2-NcoI & SMV-P2-XbaI for wild type) and (SMV-P2-NcoI-His6 & SMV-P2-XbaI for

N-terminal His_6 tag). The PCR products were cloned into the pGEM-T easy vector (Promega, Madison, WI) and confirmed by sequencing (pTeasy-P10, pTeasy-P10-His, pTeasy-G7 and pTeasy-G7-His). HC-Pro genes were released from pTeasy-P10-His and pTeasy-G7-His by digestion with *Nco*I and *Xba*I and inserted into *Nco*I- *Xba*I-digested pRTL2 vector. The HC-Pro genes in the recombinant pRTL2 were under the control of TEV 35S promoter and terminator. These pRTL2-HC-Pro constructs were then digested with *Sph*I, blunt-ended with T4 DNA polymerase and ligated into *Sma*I-digested pZP212. Clones in the correct orientation (the 35S promoter, enhancer and terminator were arranged sequentially) were confirmed by *Xba*I digestion. The selected plasmids were then transformed into *Agrobacterium tumefaciens* strain C58C1. Plasmids pTeasy-P10-His and pTeasy-G7-His were used to generate chimeric HC-Pro clones between P10 HC-Pro gene and G7-HC-Pro gene. These two plasmids were digested with *Nde*I and *Bss*HII to produce 3.8 kbp and 0.6 kbp fragments, which were dephosphorylated and gel purified. The 0.6 kbp fragments were digested with *Sca*I to produce a 150 bp *Nde*I -*Sca*I fragment) and a 450 bp *Sca*I -*Bss*HII fragment. Different combinations of the P10 and G7 fragments (150 bp and 450 bp) were ligated and the ligation products were treated with T4 polynucleotide kinase. These phosphorylated fragments were then ligated to the 3.8 kbp fragment to produce the chimeric clones. These clones were then processed in the same manner as those of the wild type HC-Pro gene for insertion into the pZP212 vector for agroinfiltration (HC-Pro-C1 to HC-Pro-C4).

Plasmids pTeasy-P10-His and pTeasy-G7-His were used as templates with primer pair (SMV-ApaI-For & SMV-P2-XbaI) to introduce *Apa* I restriction site downstream from the coding region of the amino acid at position 144 (clones pTeasy-P10-His-ApaI and pTeasy-G7-His-ApaI). Four PCR reactions were performed: 1, template pTeasy-G7-His with primer pair SMV-P2-NcoI-His6 & G7-ApaI-I-Rev; 2, template pTeasy-P10-His with primer pair SMV-P2-NcoI-His6 & P10-ApaI-K-Rev; 3, template pTeasy-P10-His with primer pair SMV-P2-NcoI-His6 & P10-ApaI-H-Rev; 4, template pTeasy-P10-His with primer pair SMV-P2-NcoI-His6 & P10-ApaI-R-Rev. The PCR products were cloned into pGEM-T easy vector and the recombinant plasmids were digested with *Nco*I and *Apa*I and the released fragment was ligated into *Nco*I-*Apa*I-digested digested pTeasy-G7-

His-ApaI or pTeasy-P10-His-ApaI. The resulting clones were processed as described earlier for insertion into the pZP212 vector for infiltration.

TEV HC-Pro: full length *Tobacco etch virus* (TEV) RNA cDNA clone (Dolja *et al*., 1992) was used as a template with the primer pair (TEV-NcoI-for & TEV-BamHI-Rev) for PCR. The PCR product was cloned into pGEM-T easy vector. Sequencing confirmed clone was digested with *Nco* I & *BamH* I and the released HC-Pro gene was inserted into *Nco*I-*BamH*I- digested pRTL2. Then TEV *HC-Pro* gene was put into pZP212 vector for infiltration using the same method for SMV HC-Pro agroinfiltration constructs. Point mutations of TEV-HC-Pro were generated by PCR mutagenesis. With wild type TEV *HC-Pro* gene as a template, 4 PCR reactions were set up with 4 different primer pairs (TEV-NcoI-for & TEV-P2-Rev-I, TEV-P2-For-I & TEV-BamHI-Rev, TEV-NcoI-for & TEV-P2-Rev-R and TEV-P2-For-R & TEV-BamHI-Rev). The PCR products from the first two reactions were mixed in equimolar amounts as templates and overlapping PCR was performed using primer pair TEV-NcoI-for & TEV-BamHI-Rev, and the PCR product was cloned into pGEM-T easy. Similarly, the PCR products from the last two reactions were mixed in equal molar amounts and used as templates and overlapping PCR was performed using with primer pair TEV-NcoI-for & TEV-BamHI-Rev ,and the product was cloned into pGEM-T easy. The mutated TEV HC-Pro genes were then inserted into pZP212 for infiltration.

TVMV HC-Pro: A *Tobacco vein mottling virus* (TVMV) wild type HC-Pro was amplified from an infectious cDNA clone (Nicolas *et al*. 1996) with primers TVMV-P2-Nco-I and TVMV-P2-Rev-XbaI. The gene was then inserted into the pZP212, as described for other HC-Pro constructs. For point mutation analysis, overlapping PCR was performed. With TVMV wild type HC-Pro as a template, two primer pairs (TVMV-P2-Nco-I & TVMV-P2-K-Rev and TVMV-P2-K-For & TVMV-P2-Rev-XbaI) were used for amplification. The PCR products were mixed in equal molar amounts and amplified with the primer pair TVMV-P2-Nco-I & TVMV-P2-Rev-XbaI. The overlapping PCR product was cloned into pGEM-T easy vector and was processed for insertion into the pZP212 vector for use in the infiltration assay.

Agrobacterium growth condition and infiltration

The constructs expressing HC-Pro genes were transformed into *Agrobacterium tumefaciens* strain C58C1 and grown in LB medium containing rifampicin (100 µg/ml), spectinomycin (100 µg/ml) and tetracycline (5µg/ml) (An *et al*., 1988). The growth conditions of the transformed *A. tumefaciens* were as previously described (Goodin *et al*., 2002). The *Agrobacterium* infiltration experiments were performed as described by Qu *et al*. (2003). Transgenic *Nicotiana benthamiana* plants expressing GFP (line 16c) were obtained from Dr. David C. Baulcombe (The Sainsbury Laboratory, John Innes Center). Infiltrated plants showing fluorescence were photographed with a N90-S AF digital camera (Nikon, Tokyo).

In vitro transcription and inoculation

Plasmid pCRHaR1 (Gu and Ghabrial, 2005) containing a full-length RNA1 cDNA from BPMV strain K-Ha1, was linearized with *Sal* I and used as a template for *in vitro* transcription, as described by Gu and Ghabrial (2005). After transcription, 5µl samples of the reaction mixture were analyzed on a 1% DEPC agarose gel to assess the yield and integrity of the transcripts. 100 µl RNA1 transcripts and 100 µl RNA2 transcripts (different constructs) were mixed together and 15 µl were rub-inoculated onto soybean primary leaves.

Protein expression and western blot analysis

Total protein extraction from soybean or *Nicotiana benthamiana* leaves was performed as described by Osherov and May (1998). Protein concentration was estimated by the Bio-Rad protein assay method (Bio-Rad Laboratories, Hercules, CA, USA). Western blot analysis was carried out as previously described (Srinivasa *et al*., 2001) using antisera to BPMV CP or to the His-tag (Chemicon international Inc., Temecula, CA, USA). Protein accumulation level was assessed using ImageQuant v5.2 (Amersham).

Results

1. BPMV and SMV interact synergistically in dually infected soybean plants

Coinfection of soybean with SMV and BPMV is known to induce a serious disease “top necrosis”. To further study this phenomenon, the interactions of a very mild strain of

BPMV, K-Ha1, with mild (SMV isolate-P10) and severe SMV strains (strains G6 and G7) were examined. As previously reported, coinfection of soybean by SMV G6 or G7 and BPMV K-Ha1 caused very severe symptoms (Figure 5.1). Interestingly, no synergism was induced by coinfection of soybean with SMV P10 and BPMV K-Ha1 (Figure 5.1). Western blot analysis of total proteins extracted from soybean plants previously subjected to single or double infection with BPMV and SMV showed that enhanced symptom severity correlated well with virion accumulation levels (Figure 5.2).

2. Expression of functional HC-Pro RNA silencing suppressors from a BPMV vector induces severe symptoms

RNA silencing and its suppression are known to play important roles in virus-host interactions. To determine whether suppression of RNA silencing is the underlying mechanism for SMV and BPMV synergistic interaction, the effect of inoculating soybean plants with recombinant BPMV vectors expressing SMV HC-Pro was studied. When SMV G7 (or G6) HC-Pro was expressed from the BPMV vector in soybean, the infected plants showed very severe symptoms similar to those induced by dual infection with the wild-type viruses (Figure 5.1). The BPMV multiplication level of the recombinant viruses was increased to a similarly high levels (Figure 5.2). Interestingly, expression of TEV-HC-Pro from the BPMV vector also induced severe symptoms in soybean, even though soybean is not a host for TEV. The inability of SMV P10 to interact synergistically with BPMV strain K-Ha1 was also examined by expressing SMV P10 HC-Pro from the BPMV vector. The recombinant vector induced mild symptoms comparable with those produced by dual infection with BPMV strain K-Ha1 and SMV isolate P10 (Figure 5.1).

To determine whether the differences between SMV strains G7 and P10 in their interactions with BPMV are due to differences in their RNA silencing suppression activities, an *Agrobacterium*-mediated transient expression system was used. The SMV G7 and SMV P10 HC-Pro genes were inserted into the pZP212 binary vector and the recombinant vectors were agro-infiltrated into *N. benthamiana* (transgenic for GFP) along with the recombinant GFP vector. No fluorescence was evident 5 days post infiltration with the recombinant pZP-HC-Pro (P10) whereas co-expression of GFP and

HC-Pro from SMV strain G7 induced intense fluorescence. Thus the results from the GFP transient agroinfiltration assays suggest that the SMV G7 HC-Pro is a functional RNA silencing suppressor whereas the SMV P10 HC-Pro is defective in its suppressor function (Figure 5.3). Amino acid sequence comparison of the HC-Pro of SMV strains P10 and G7 identified 16 amino acid positions that are different between these two strains. To pinpoint the amino acids that may be responsible for the observed differences in suppressor activity, several chimeric constructs between SMV G7 HC-Pro and SMV P10 HC-Pro were generated. GFP agroinfiltration assays with the chimeric constructs identified three amino acid positions in the central region of HC-Pro that may be responsible for the difference in suppressor activity (Figure 5.4). Whereas the difference at amino acid position 144 involves amino acids with dissimilar side chains, those at positions 229 and 260 involve similar non-polar amino acids. The functional SMV G7 HC-Pro has a positively charged basic amino acid (lysine) at amino acid position 144 whereas the non-functional P10 HC-Pro has an amino acid with a non-polar side group (isoleucine). Database searches with the BLAST program indicated that a lysine residue at position 144 in HC-Pro is highly conserved among potyvirus (Figure 5.4). Interestingly, *Tobacco vein mottling virus* (TVMV) HC-Pro contains leucine (non-polar) at this position. Point mutations were made at amino acid position 144 to determine whether a positively charged basic amino acid at this position is critical for potyvirus HC-Pro suppressor function. All possible positively charged basic amino acids (arginine, histidine and lysine) were used to replace isoleucine of SMV P10 HC-Pro at position 144. The results indicated that positively charged basic amino acid replacement restored the RNA silencing suppressor activity of P10 HC-Pros (Figure 5.4). Furthermore, substitution of lysine with isoleucine rendered SMV G7 HC-Pro nonfunctional as a silencing suppressor (Figure 5.4). Similar mutational analysis was performed with HC-Pro from TEV and TVMV. Constructs coding for a positively charged basic amino acid at position 144 were shown to function as suppressors of RNA silencing, whereas constructs coding for a non-polar amino acid at this position, including the wild type TVMV HC-Pro, did not function as suppressors in the transient agroinfiltration assay (Figure 5.4). These data strongly suggest that a positively charged basic amino acid at this position is conserved and critical for potyvirus HC-Pro to function as RNA silencing suppressor. This position was reported before to be involved in nonspecific RNA binding (Urcuqui-Inchima *et al.*, 2000).

Results from northern hybridization analysis suggest that the intensity of the green fluorescence, exhibited by the infiltrated leaf patches, correlates well with the accumulation of GFP mRNA (Figure 5.5a). No detectable GFP mRNA band was observed with the non-functional suppressors. To rule out the possibility that HC-Pro gene was knocked out during infiltration test, a PCR test was used to check the existence of HC-Pro gene before infiltration. Plasmid DNA was extracted from cultured *Agrobacterium* clones and used as template for PCR. The PCR results showed that the *HC-Pro* gene existed during the infiltration and negative result was not due to loss of *HC-Pro* gene during experimental handling (data not shown). Several SMV constructs were tested further for the existence of small RNA (siRNA) after transient assay (Figure 5.5b). The result showed that no detectable level of siRNA for functional suppressors (including the point mutations). In contrast, non-functional suppressors (including point mutations) readily produce similarly detectable level of siRNA. Taken together, the results of fluorescence, GFP mRNA level and siRNA accumulation, our data strongly suggest that the presence of a positively charged basic amino acid at position 144 is critical for potyvirus HC-Pro to function as a silencing suppressor.

3. Functional suppressor HC-Pro enhances symptom severity induced by infectious BPMV recombinants

In additional studies, the two HC-Pro point mutations were inserted into the BPMV vector to test their function in synergism. The functional suppressor mutant SMV P10 HC-Pro (isoleucine → lysine), when expressed in soybean by the BPMV vector caused enhanced symptoms in contrast with the very mild symptoms induced by wild type P10 HC-Pro construct (Figure 5.1). The reverse non-functional mutation for SMV G7 HC-Pro point mutant (lysine to isoleucine) abolished its function in inducing severe symptoms (Figure 5.1). The SMV wild type HC-Pro constructs and the point mutation constructs showed that a functional silencing suppressor HC-Pro is required for SMV and BPMV synergism.

4. Enhanced symptoms induced by G7 HC-Pro point mutations

As the experiments described so far were all done using RNA1 from the mild strain BPMV strains K-Ha1, it was of interest to determine whether the use of RNA1 derived from a severe strain would enhance the synergistic interaction. Furthermore, it was desirable to explain why the severe symptoms induced by the functional suppressor point mutation of SMV P10 (isoleucine to lysine) HC-Pro were not as severe as those induced by the wild type G7 HC-Pro (Figure 5.1). It is known that RNA1 from severe BPMV strain K-Ho1 can enhance BPMV replication thus increase the translation level of both RNA1 and RNA2 (Gu and Ghabrial, 2005). It was thus of interest to determine whether an increase in the accumulation levels of HC-Pro, which is expressed from BPMV RNA2, may influence symptom severity. All constructs used in the previous experiments were used together with BPMV K-Ho1 RNA1 to inoculate soybean and the resultant symptoms were denoted. Consistent with our previous results, the non-functional suppressor SMV P10 HC-Pro construct induced mild symptoms. The wild type functional suppressor SMV G7 HC-Pro and point mutation P10 HC-Pro (isoleucine to lysine) constructs induced very severe symptoms (Figure 5.6).

5. Protein stability is not a factor in the changes in suppressor function

The SMV HC-Pro proteins were labeled with His6 tag. I have determined that tagging with His6 does not influence HC-Pro function as a suppressor (data not shown). Hence, all SMV HC-Pro constructs used in agroinfiltration assays in *N. benthamiana* were done with labeled HC-Pro. Protein samples were extracted from infiltrated leaf area and subjected to western blot analysis using an antiserum to His6 tag. A prominent band corresponding to HC-Pro can be readily detected for all functional suppressor HC-Pro constructs, but not for the non-functional suppressor HC-Pro constructs. To test if the difference between SMV G7 and P10 HC-Pro is due to difference in protein stability, non-functional HC-Pro constructs were co-infiltrated with the functional suppressor TCV-CP construct, which is not labeled with the His6 tag, together with the GFP construct used for silencing induction. A band corresponding to HC-Pro could be seen when the functional suppressor TCV-CP was co-infiltrated (Figure 5.7). Compared with the functional SMV HC-Pro, the non-functional HC-Pro protein can sustain to a detectable level, which suggests that non-functional HC-Pro protein is stable enough as that of functional HC-Pro to be detected.

Discussion

BPMV was used as a gene expression vector to investigate the role of HC-Pro in the synergistic interaction between the comovirus BPMV and the potyvirus SMV. The results from these experiments combined with those from mutational analysis and transient agroinfiltration assays indicated that RNA silencing suppression is the most likely underlying mechanism for this synergistic interaction.

The results described in this chapter showed that a functional HC-Pro RNA silencing suppressor was required for the induction of severe symptoms when HC-Pro was expressed from the BPMV vector. Similar results were reported when the PVX vector was used to express the TEV HC-Pro (Kasschau and Carrington 2001; Pruss *et al*., 1997). As shown in Chapter Four, other functional suppressors like TCV-CP and TBSV-P19 that target different steps in the silencing process, when expressed in soybean from the BPMV vector, induced comparable severe symptoms . Evidence was recently presented that the small coat protein of CPMV, the type species of the genus Comovirus, functions as a weak suppressor of RNA silencing (Canizares *et al*., 2004). The finding that several mechanistically different suppressors, when expressed in soybean from the BPMV vector, could cause synergism suggest that comoviruses use a different strategy to counter RNA silencing-mediated host defense.

The availability of the mild isolate SMV P10, with apparent defects in its synergistic interactions with BPMV, allowed us to examine the functions of a potential natural non-suppressor HC-Pro protein in synergism. Transient agroinfiltration assays in *N. benthamiana* showed that P10 HC-Pro is not a silencing suppressor. This suggest that potyviruses with non-functional silencing suppressors could exist in nature. Some strains of two other potyviruses, *Peanut mottle virus* (PMV) and *Bean yellow mosaic virus* (BYMV), have also been reported not to interact synergistically with BPMV in mixed infections (Anjos *et al*., 1992). Based on the results of the present study, it is suggested that RNA silencing suppressors encoded by these two viruses might be defective. Based on the results from mutational analysis and production of chimeric constructs between the SMV *HC-Pro* from the severe strain G7 and the mild isolate P10, it was possible to identify the amino acid position responsible for the defective function in silencing suppression. TEV HC-Pro was subjected to extensive mutational analysis in order to map its functions in systemic movement, replication and silencing suppression (Kasschau *et al*., 1997; Kasschau and Carrington 2001). In the TEV study, mutants with RNA silencing suppression defects were identified and these mutants also showed defects in systemic movement and replication. Since RNA silencing-mediated antivirus plant defense is a conserved mechanism , silencing suppression is essential for successful viral establishment in plants. This feature makes it difficult to distinguish among HC-Pro functions in viral systemic movement, replication in host and silencing suppression. By using suppression defective P10 HC-Pro and silencing suppression defective point mutations from SMV G7 HC-Pro, it was possible to show that at least in SMV and BPMV synergism, silencing suppression is essential for a wild type synergism. However, other HC-Pro functions appear to be required for a strong synergism. In experiments where P10 HC-Pro suppression function was restored by substitution of isoleucine with a lysine at amino acid position 144, this point mutation induced severe symptoms ,but not as severe as that of the wild type SMV G7 HC-Pro (Figure 5.1). When the SMV G7 HC-Pro point mutation (Lys to Ile) was inoculated onto soybean along with RNA1 from the severe strain K-Ho1, it induced somewhat severe symptoms. One explanation is that in BPMV and SMV synergism, silencing suppression is required but not enough. Another possible reason is host factors may make difference in silencing process, a functional or non-functional suppressor in *N. benthamiana* might change its function in soybean. However, this scenario is unlikely as discussed before due to the mild symptoms induced by SMV P10.

Mutational analysis of several potyvirus HC-Pro proteins showed that a conserved positively charged basic amino acid at amino acid position 144 is critical for potyvirus HC-Pro to function as a suppressor (Figure 5.4). Carrington and co-workers have previously reported that charged amino acids are important to HC-Pro functions in silencing suppression, systemic movement and viral replication (Kasschau *et al*., 1997; Kasschau and Carrington 2001). The results of the extensive mutational analysis presented in this study established that at a conserved positively charged basic amino acid at amino acid position 144 is required for suppressor function of potyvirus HC-Pro. The region encompassing amino acid position 144 was previously reported to be involved in nonspecific RNA binding (Urcuqui-Inchima *et al*., 2000). Recently, the crystal structure of the potent silencing suppressor TBSV P19 was resolved (Vargason *et al*., 2003). Suppressor P19 functions as a homodimer form with the N termini from the homodimer in contact with the siRNA. Both of the N terminal α1 and α2 helixes are responsible for contacting with siRNA phosphates and sugar 2′ hydroxyls. There are several conserved positively charged amino acids in both of these two helixes (Vargason *et al*., 2003). It was also proposed that HC-Pro proteins function as oligomers, dimers, tetramers or hexamers (Plisson *et al*., 2003; Ruiz-Ferrer *et al*., 2004). The finding of a positively charged basic amino acid at position 144 implies that other positively charged amino acids in the central domain of HC-Pro might also be important for its suppressor function through interaction with, possibly, siRNAs or RNA containing complexes.

To address the question of whether substitution of the conserved Lys at position 144 may adversely influence the stability of HC-Pro protein, the TCV-CP construct was co-infiltrated with the non-functional HC-Pro constructs in the GFP agroinfiltration assay. Although the suppressor defective HC-Pro proteins were not detected by western blot analysis when their corresponding constructs were infiltrated alone, they were readily detected when coinfiltrated with a construct of the functional suppressor TCV-CP (Figure 5.7). In previous studies with TEV HC-Pro, it was shown that there were no differences in transcription level between functional and non-functional suppressor TEV HC-Pro genes (Kasschau *et al*., 1997; Kasschau and Carrington 2001). In the present study, it was shown that protein stability is not the reason for the lower accumulation level of non-functional HC-Pro protein in *N. benthamiana*. These data suggest that the low protein level of non-functional suppressor HC-Pro is due to their defects in silencing suppression.

Table 5.1 Primers used in this study

Name	Sequence
SMV-P2-BamHI	GGATCCTCCCAAAATCCTGAAGCTCAGTT
SMV-P2-EcoRV	TCATCCTCTGTTGCACGATATCACCAACTCT
SMV-P2-NcoI	ACCATGCCATGGCGTCCCAAAATCCTGAAGCTC
SMV-P2-NcoI-His6	CATGCCATGGCGCATCATCATCATCATCATTCCCAAAATCCTGAAG
SMV-P2-XbaI	CTAGTCTAGACTATTAACCAACTCTATAAAATTTCATC
SMV-ApaI-For	AAGGGCCCTCGGTGACACAAAGTGAGCT
P10-ApaI-K-Rev	AGGGCCCTTTCATTAGAGCCTTATTAATATCCT
G 7-ApaI-I-Rev	AGGGCCCTATCATCAGGGCCTTATTAATATCCT
P10-ApaI-H-Rev	GTGTCACTGAGGGCCCATGCATCAGGGCCTT
P10-ApaI-R-Rev	GTGTCACTGAGGGCCCACGCATCAGGGCCTT
TEV-P2-For-I	CAGCGAAATTCTGTTTATTGGCAATAAACTA
TEV-P2-Rev-I	AGTTTATTGCCAATAAACAGAATTTCGCTGA
TEV-P2-For-R	CAGCGAAATTCTGTTTCGTGGCAATAAACTA
TEV-P2-Rev-R	AGTTTATTGCCACGAAACAGAATTTCGCTGA
TEV-NcoI-for	CATCATCATCATCATAGCGACAAATCAATCTCTGA
TEV-NcoI-His-Nco	CCATGCCATGGCGCATCATCATCATCATCATAGCGA
TEV-BamHI	CGCGGATCCCTATTATCCAACATTGTAAGTTTTCATTT
TVMV-P2-Nco-I	CCATGCCATGGCGTCAACTGGTGATATATTTTGGAA
TVMV-P2-Rev-XbaI	CGCTCTAGACTATTAACCCACTTTATATTGTGCCATT
TVMV-P2-K-For	TCTTTTAAAGGGTTCAAAAGCCA
TVMV-P2-K-Rev	TGGCTTTTGAACCCTTTAAAAGA

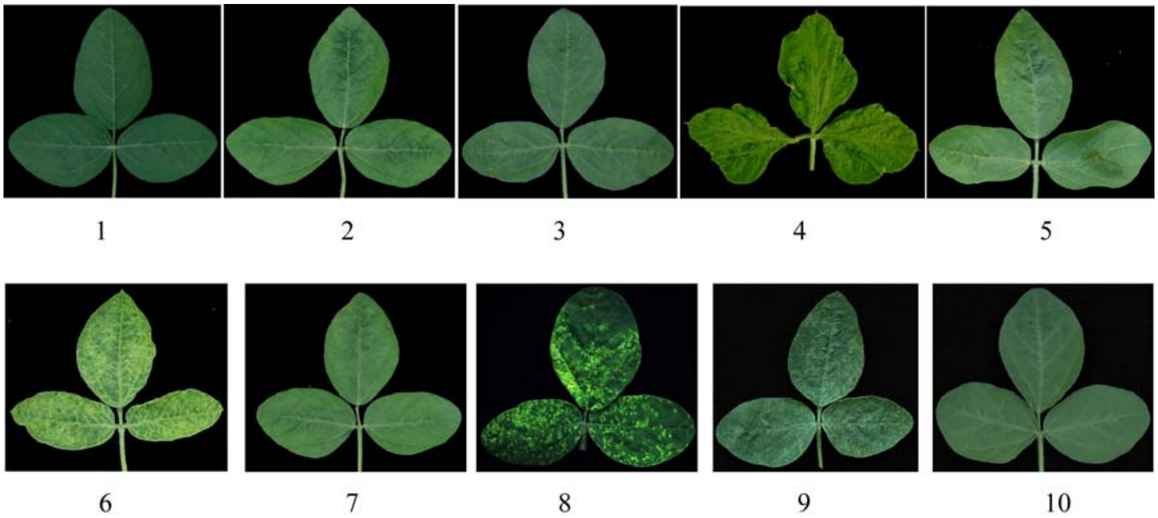

Figure 5.1. Symptoms induced in soybean by single or dual infections with field isolates of *Bean pod mottle virus* (BPMV) and *Soybean mosaic virus* (SMV) and with recombinant BPMV constructs.

(1), Healthy control; (2), BPMV strain K-Ha1; (3), SMV isolate P10; (4), SMV strain G7; (5), BPMV strain K-Ha1 plus SMV isolate P10; (6), BPMV strain K-Ha1 plus SMV strain G7; (7), transcripts from BPMV K-Ha1 RNA1 cDNA plus transcripts from BPMV-SMV-P10 HC-Pro construct; (8), transcripts from BPMV K-Ha1 RNA1 cDNA plus transcripts from BPMV-SMV-G7 HC-Pro construct; (9), transcripts from BPMV K-Ha1 RNA1 cDNA plus transcripts from BPMV- SMV-P10-K144 HC-Pro construct and (10), transcripts from BPMV K-Ha1 RNA1cDNA plus transcripts from BPMV- SMV-G7-I 144 HC-Pro construct.

The 2nd trifoliolate leaves were photographed 16 days postinoculation (dpi).

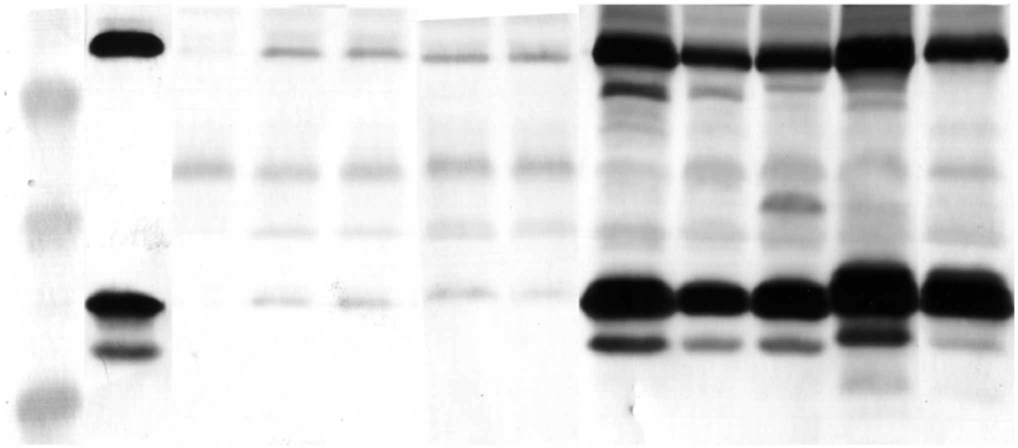

Figure 5.2. Western blot analysis using an antiserum to BPMV virions.

Total soluble protein was extracted from soybean plants previously inoculated with transcripts from different recombinant BPMV constructs and subjected to western blot analysis.

M, low molecular weight proteinstandards; 1, purified BPMV virions (2 μg); 2, healthy control; 3- 11, total protein extracted from 2nd trifoloiolate leaves of soybean plants (16 dpi) previously inoculated with BPMV field isolate or with transcripts from various recombinant BPMV constructs as follows. 3, BPMV wild type K-Ha1; 4, BPMV K-Ha1 RNA1 + BPMV K-G7 RNA2 (transcripts inoculated); 5, BPMV K-Ha1 RNA1 + BPMV K-G7 RNA2-SMV-P10 HC-Pro construct; 6, BPMV K-Ha1 RNA1 + BPMV K-G7 RNA2-SMV-G7 HC-Pro-I (K→I at amino acid position 144) construct; 7, BPMV K-Ha1 RNA1 + BPMV K-G7 RNA2-SMV-G7 HC-Pro; 8, BPMV K-Ha1 RNA1 + BPMV K-G7 RNA2-P10-HC-Pro-K (I→K at amino acid position 144); 9, BPMV K-Ha1 + BPMV K-G7 RNA2-TEV-HC-Pro; 10, BPMV K-Ha1 + SMV G7; 11, BPMV-K-Ha1 + SMV G6

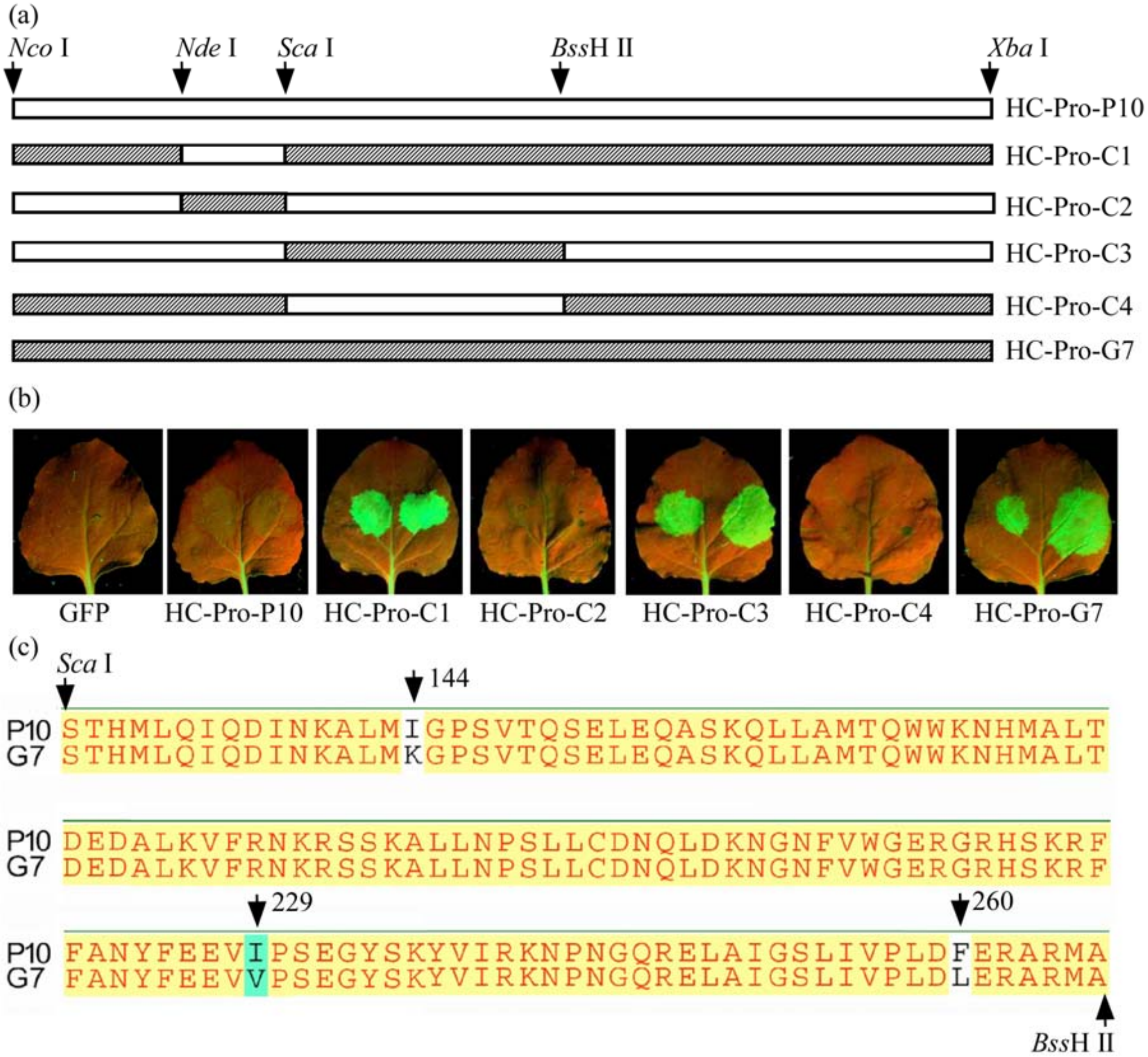

Figure 5.3. Mapping RNA silencing suppression function of SMV HC-Pro.

(a): Schematic representation of SMV HC-Pro constructs derived from SMV isolate P-10 (non-functional suppressor; open rectangular), strain G7 (functional suppressor; hatched rectangular, and chimeric constructs between the HC-Pro from the two strains. Restriction endonuclease sites used for generating the chimeric constructs are indicated with arrows. The construct designations are indicated to the right .

(b): Transient agroinfiltration assay for RNA silencing suppression function of SMV HC-Pro constructs. *Nicotiana benthamiana* plants were infiltrated with mixtures of *Agrobacterium tumefaciens* cells transformed with the GFP construct alone only (GFP) or mixed with cells transformed with the SMV HC-Pro constructs schematically represented in (a).

(c): Amino acid sequence alignment of SMV P10 and SMV G7 HC-Pro proteins for the region between the *Sca*I and *BssH*II restriction sites. The three amino acid positions that differ between the two strains are indicated with arrows.

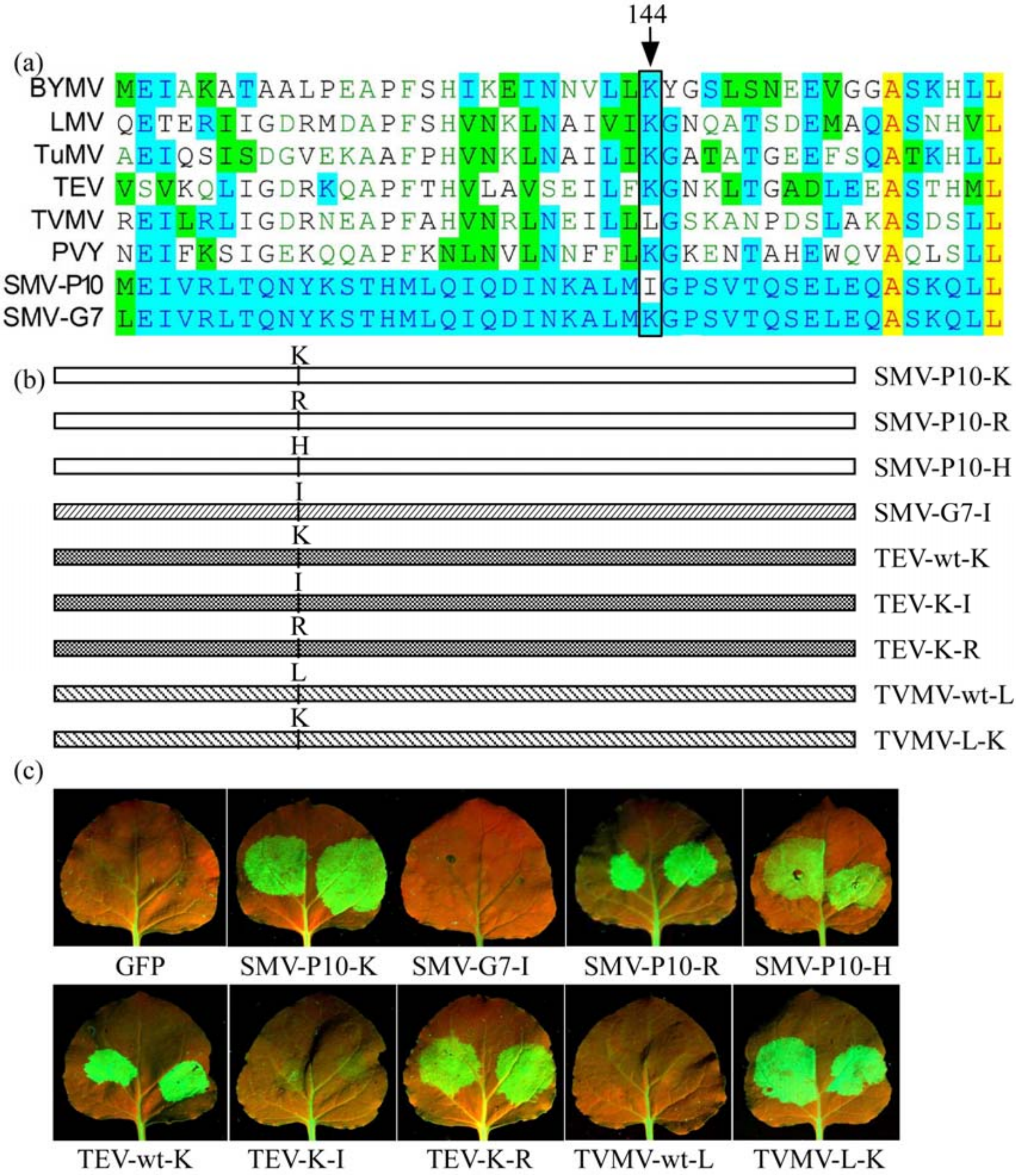

Figure 5.4. Mutational analysis of SMV HC-Pro.

(a): Amino acid sequence alignment of several potyviruses. HC-Pro proteins covering amino acid positions 118-161 (amino acid position 144 is boxed).

(b): Schematic representation of potyvirus HC-Pro amino acid point mutations at position 144. The substitute amino acid at position 144 is indicated above the position. Construct designations are indicated to the right.

(c): Transient agroinfiltration assay for RNA silencing suppression function of SMV HC-Pro point mutation constructs. GFP, plants were infiltrated with the GFP construct alone or mixed with the different SMV HC-Pro constructs schematically represented in (b).

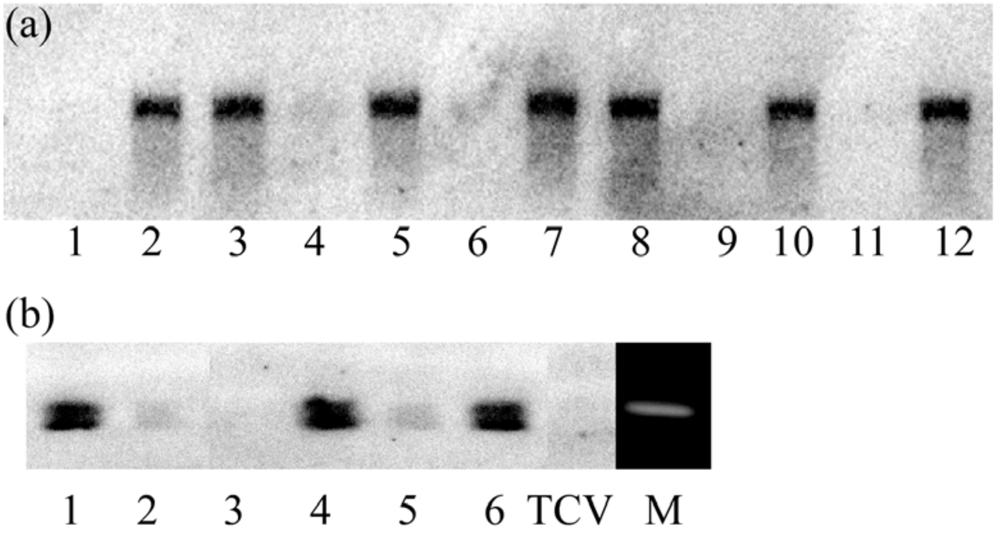

Figure 5.5. Nucleic acid hybridization analysis for assessing the accumulation of GFP mRNA and small RNAs in *Nicotiana benthamiana* leaves infiltrated with various HC-Pro constructs.

(a): Northern blot hybridization analysis of GFP mRNA accumulation level. Total RNA samples were extracted from *Nicotiana benthamiana* leaves infiltrated with the following constructs. 1, GFP alone ; GFP plus 2, TEV-K (wild type TEV HC-Pro has K at amino acid position 144); 3, SMV-G7; 4, SMV-P10; 5, SMV-P10-K (I→K at amino acid position 144); 6, SMV-G7-I (K→I at amino acid position 144); 7, SMV-P10-H (K→H at amino acid position 144); 8, SMV-P10-R (K→R at amino acid position 144); 9, TEV-I (K→I at amino acid position 144); 10, TEV-R (K→R at amino acid position 144); 11, TVMV-L (wild type TVMV HC-Pro has L at amino acid position 144); 12, TVMV-K (L→K at amino acid position 144). The probe was prepared using plasmid pGGFP-1 (see Chapter Four) containing full length GFP gene.

(b): Small RNA gel blot analysis. M, 20 bp dsDNA marker; Lanes 1 to 6 received the same samples as in (a). Lane 7, small RNA extracted from leaves infiltrated with TCV and GFP construct.

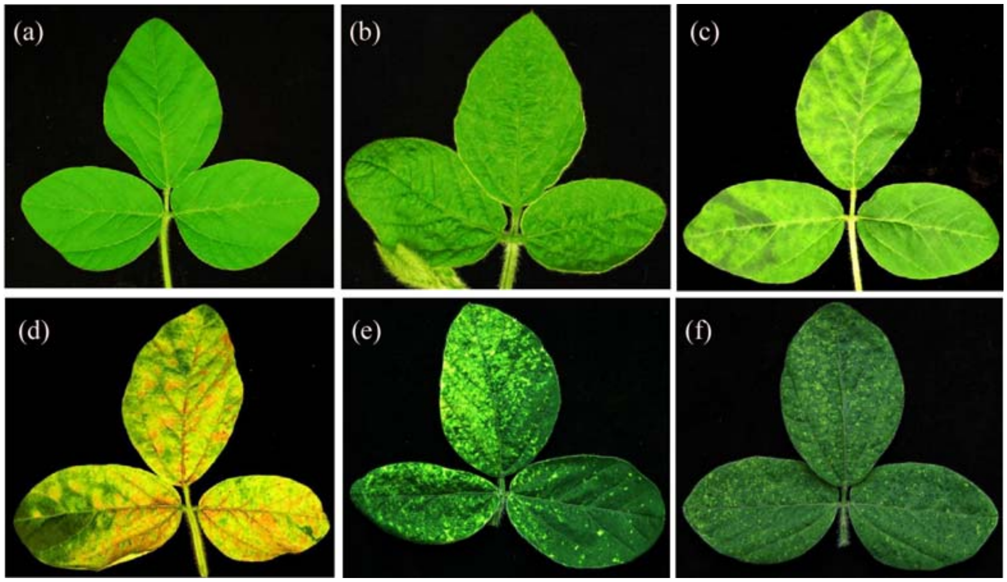

Figure 5.6. Symptoms induced in soybean by infection with recombinant BPMV constructs expressing SMV HC-Pro.

(a) Healthy control.

(b) BPMV K-Ho1 RNA1 cDNA and K-G7 RNA2 cDNA.

(c) BPMV K-Ho1 RNA1 cDNA and BPMV-SMV-P10 HC-Pro construct.

(d) BPMV K-Ho1 RNA1 cDNA and BPMV-SMV-G7 HC-Pro construct.

(e) BPMV K-Ho1 RNA1 cDNA and BPMV-SMV-P10 HC-Pro-K (I→K point mutation at amino acid position 144) construct.

(f) BPMV K-Ho1 RNA1 cDNA and BPMV-SMV-G7 HC-Pro-I (K→I point mutation at amino acid position 144) construct.

Pictures from b to f are from plants infected with transcripts derived from cDNAs.

The 2nd trifoliolate leaves were photographed 16 dpi.

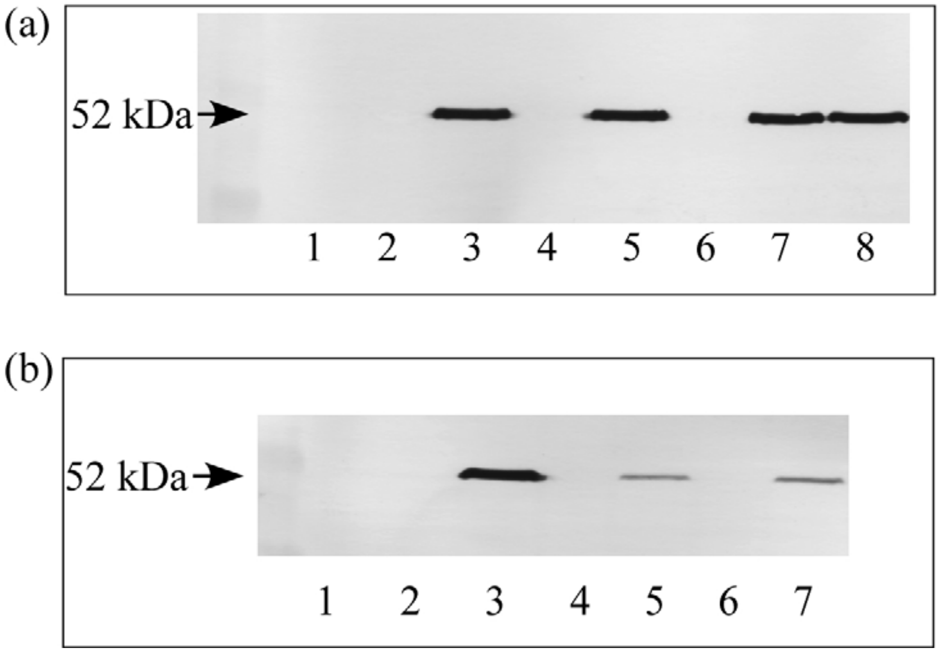

Figure 5.7. Immunoblot analysis of HC-Pro test in agroinfiltrated *N. benthamiana* tissue.

(a): Total protein samples (20 µg) extracted from leaves infiltrated with the following constructs were subjected to western blot analysis: 1, GFP alone or mixed with: 2, TEV-K; 3, SMV-G7; 4, SMV-P10; 5, SMV-P10-K; 6, SMV-G7-I; 7, SMV-P10-H and 8, SMV-P10-R. The antiserum used was a mouse anti-His tag antibody from Amersham Pharmacia Biotech Inc.

(b): Western blot analysis of HC-Pro proteins with non-functional suppressors. 1, GFP alone; or mixed with 2, TCV; 3, SMV-G7; 4, SMV-P10; 5, SMV-P10 + TCV; 6, SMV-G7-I; 7, SMV-G7-I +TCV.

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VITA

Name: Chunquan Zhang

Date/Place of Birth: June 28, 1971

Heilongjiang, P. R. China

Education

1993-1996: M.S, Northeast Agricultural University (China).

1989-1993: B.S. Northeast Agricultural University (China).

Professional Experience

1996-2000: Lecturer, Northeast Agricultural University (China).

1995-1996: Research Assistant, Chinese Academy of Sciences (China).

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Minghou Zhang, Peiwen Wei and **Chunquan Zhang** 1998 Comparison of virulence of SMV strains from districts of northeast Asian countries, Soybean Science Vol. 17, No.2, 101-107 (in Chinese).

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Membership in Professional Societies

The American Society for Virology.

The American Phytopathological Society.