NONHOST RESISTANCE TO BEAN POD MOTTLE VIRUS IN

NICOTIANA BENTHAMIANA

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Junyan Lin

Graduate Program in Plant Pathology

The Ohio State University

2013

Dissertation Committee:

Dr. Feng Qu, Advisor

Dr. Anne E. Dorrance

Dr. Margaret G. Redinbaugh

Dr. John Finer
ABSTRACT

Nonhost resistance refers to the microbial resistance mounted at the species level, namely none of the cultivars of the (plant) species is susceptible to a given pathogen. This form of disease resistance is considered to be the most robust and durable form of plant resistance. However, little is known about how nonhost plants engage viral pathogens. The goal of my thesis research was to elucidate the mechanisms of anti-viral nonhost resistance using Bean pod mottle virus (BPMV) and Nicotiana benthamiana (Nb) as experimental models. BPMV is a single-stranded, positive sense (+) RNA virus with two separately encapsidated genomic RNAs - RNA1 and RNA2. Nb – a common model plant – is a nonhost to BPMV. Soybean and lima bean, two host plants of BPMV, were also examined in parallel with Nb to uncover the differences in responses to BPMV infection that might contribute to nonhost resistance. I first examined the replication efficiency of BPMV RNAs in Nb cells. A GFP-tagged BPMV was introduced into Nb and its RNA accumulation level as well as its protein expression was evaluated. In the presence of P19, a heterologous virus suppressor of RNA silencing, BPMV RNA1 replication was readily detectable in the Nb nonhost cells, whereas RNA2 replication was detected only after a significant delay. These results suggested that Nb resists BPMV invasion by
selectively targeting the replication of BPMV RNA2. Furthermore, RNA silencing is an integral component of the anti-BPMV nonhost resistance, as neither BPMV RNA1 nor RNA2 was detectable in Nb cells in the absence of P19. Since RNA2 was selectively repressed by Nb, The next objective was to identify unique features encoded by BPMV RNA2 that could be targeted by the nonhost resistance using the lima bean and soybean host systems. BPMV RNA2 encodes two unique cis-acting elements: the first is a cis-acting RNA element located at 5’ untranslated region (UTR) that was determined to be critical for the synthesis of negative (-) sense of RNA2. This element, referred to as stem-loop C (SLC), consists of a big, 15 base end loop, and a stem of 16 base pairs interspersed with one single-base bulge and one two-base mismatch. The other cis-acting element is a 58 kilodalton (K) protein (P58) encoded by RNA2. Mutagenesis of P58 indicated that the N-terminal 14k domain of P58 is sufficient to support the accumulation of RNA2. N-terminal extension of P58 compromised BPMV infectivity. Furthermore, a series of complementation experiments were designed and confirmed that the P58 is a cis-acting protein that assisted only the RNA from which it is translated. Overall, the completion of my thesis project reveals for the first time that nonhost plants attacked by bipartite RNA viruses could mount effective defenses by selectively targeting one of the genome segments. I also uncovered critical differences in the replication of the genome segments of the bipartite BPMV. These discoveries will contribute to the exploitation of nonhost resistance resource to achieve effective control of BPMV infection in soybean plants.
To my husband Dr. Peng Zhao and my parents
ACKNOWLEDGMENTS

I would like to thank my adviser, Dr. Feng Qu. No word can appropriately express my sincere gratitude for all his support, endless encouragement and guidance throughout my PhD course. He helped me grow to be a better researcher and professional. His profound knowledge in critical thinking made me open eyes to a new way of understanding the plant-virus interactions.

I would also like to thank my candidacy and dissertation committee: Dr. Anne Dorrance, Dr. Peg Redinbaugh, and Dr. John Finer, who gave me invaluable advice and suggestions on my PhD research.

I would like to thank all the former and current members in Feng’s lab, Xiucun zhang, Ahmed Khamis Ali, Ping chen, Jasleen Singh, Junping Han, Godwill Chewachong, Xiaofeng Zhang, Ashlina Chin and Jiangbo Guo, for all their precious helps and friendships. Thank Dr. Lucy Stewart and other members of USDA, ARS Corn and Soybean Research Unit for sharing equipment. Thank Cheri Nemes for making transgenic soybean plants for my studies. Thank Carlos Hernandez-Garcia and Ning Zhang for assistance with particle bombardment in Dr. John Finer’s lab. Thank Dr. Said Ghabrial at University of Kentucky
for providing me with BPMV RNA1 infectious cDNA construct and Drs. Steve Whitham and John Hill at Iowa State University for providing me with the RNA2-GFP construct.

This project was supported in part by an OARDC Graduate Seed award, by North Central Soybean Research Program and by the Ohio Soybean Council foundation. I would also like to thank the Department of Plant Pathology at the Ohio State University for providing me the scholarship.

I would like to give a special thanks to my loving family: my husband, Dr. Peng Zhao, for all his invaluable love and support; and my parents, Mr. Shengjia Lin and Ms. Meixin Nong, for giving me endless love, care, support, inspiration and encouragement throughout my life.
VITA

August 1986............................................Born-Guangxi, China

July 2008..................................................B.S.in Biological Sciences, China Agricultural University

September 2008 to present .........................Graduate Research Associate, Department of Plant Pathology, The Ohio State University

PUBLICATIONS


FIELDS OF STUDY

Major Field: Plant Pathology
TABLE OF CONTENTS

Abstract ................................................................................................................................. ii

Acknowledgments ................................................................................................................ v

Vita ........................................................................................................................................ vii

Table of contents .................................................................................................................. ix

List of Figures ........................................................................................................................ xii

Chapter 1. Introduction ........................................................................................................ 1

Overview ............................................................................................................................... 1

BPMV is a serious soybean pathogen with no known conventional resistance
resources ............................................................................................................................. 3

N. benthamiana is an excellent model nonhost of BPMV .................................................... 6

Multi-partite (+) RNA viruses use contrasting strategies to access replication
factories ................................................................................................................................... 7
Nonhost plants use different mechanisms to combat virus infections .................. 10

Research Objectives ........................................................................................................... 15

Chapter 2. Nonhost resistance in *Nicotiana benthamiana* engages the bipartite *Bean pod mottle virus* by selectively targeting the replication of the viral RNA2 .................. 17

Abstract........................................................................................................................................ 17

Introduction ........................................................................................................................................ 19

Materials and Methods ............................................................................................................. 22

Results ........................................................................................................................................... 25

Discussion....................................................................................................................................... 35

Acknowledgements ....................................................................................................................... 37

Chapter 3. A stem-loop structure in the 5' untranslated region of *Bean pod mottle virus* RNA2 is specifically required for RNA2 accumulation .................................................. 42

Abstract........................................................................................................................................ 43

Introduction ........................................................................................................................................ 45

Materials and Methods ............................................................................................................. 49

Results ........................................................................................................................................... 52

Discussion....................................................................................................................................... 59

Acknowledgements ....................................................................................................................... 62
Chapter 4. The Bean pod mottle virus RNA2-encoded 58K protein is required in cis for RNA2 accumulation ................................................................. 67

Abstract ........................................................................................................ 68

Introduction ................................................................................................... 69

Materials and Methods ............................................................................... 73

Results ......................................................................................................... 76

Discussion ................................................................................................... 88

Acknowledgements ...................................................................................... 91

Chapter 5. RNA-silencing-mediated antiviral defense constitutes an important component of nonhost resistance against Bean pod mottle virus in Nicotiana benthamia ................................................................. 96

Abstract ........................................................................................................ 97

Introduction ................................................................................................... 98

Materials and Methods ............................................................................... 101

Results ......................................................................................................... 105

Discussion ................................................................................................... 111

Acknowledgements ...................................................................................... 113

Bibliography ................................................................................................ 117
LIST OF FIGURES

Figure 1.1 Genome organization of BPMV RNAs. The numbers denote the sizes of the two RNAs (5,986 and 3662 nucleotides), and their encoded open reading frames (ORFs). The shaded boxes represent mature functional proteins released from the single ORFs by the viral protease (Pro). Other proteins on RNA1 are, C-Pro: protease cofactor; HEL, putative helicase; VPg: genomic linked viral protein (also shown attached to the 5’ end of both RNAs). RdRP: viral RNA-dependent RNA polymerase. Proteins encoded by RNA2 are, P58: function unknown; MP: movement protein, overlaps with P58 but starts from the second AUG of RNA2; L-CP and S-CP, large small capsid protein subunits............ 16

Figure 2.1 Replication-independent expression of BPMV coded proteins in Nb leaves. Binary constructs designed to express individual BPMV-coded proteins were delivered into Nb leaves with agro-infiltration. Protein samples extracted from the infiltrated leaves at 5 dai were separated on 11% SDS-PAGE gels, transferred to nitrocellulose membranes, and subjected to Western Blot (WB) with anti-HA antibody to reveal the presence of each protein. (A) Protein expression levels in the absence of a viral silencing
suppressor (VSR, TCV CP in these experiments). (B) Protein levels in the presence of the TCV CP VSR. ................................. 38

**Figure 2.2** Selective replication of BPMV RNA1 in *Nb*. (A) Schematic representation of binary constructs containing full length cDNAs of BPMV genomic RNAs and their derivatives. (B) Semi-quantitative RT-PCR evaluation of the relative accumulation levels of BPMV RNA1 and RNA2 in *Nb* leaves agro-infiltrated with the constructs shown on the top of the panels. ................................. 39

**Figure 2.3** BPMV RNA2 replication failed to occur despite active RNA1 replication in the same *Nb* cells. (A) Constructs (RNA1-R2G and R1m-R2G) that ensure the delivery of both BPMV RNA1 and RNA2 into the same *Nb* cells. (B) Assessing the replication competence of RNA1-R2G and R1m-R2G in the host cells of BPMV. Top two panels: RNA1-R2G (left) and R1m-R2G (right) delivered to the cotyledons of lima bean with particle bombardment. Bottom two panels: Soybean plants infected with the saps of bombarded lima bean cotyledons. (C) Evaluation of the relative accumulation levels of BPMV RNA1 and 2 [both (+) and (-)] in agro-infiltrated *Nb* leaves with semi-quantitative RT-PCR. ................................. 40

**Figure 2.4** BPMV RNA2-coded polyprotein was processed by RNA1-coded protease in a replication-independent manner in *Nb*. (A) *Nb* leaves infiltrated with constructs shown above each leaf. (B) Western blot (WB) analysis of the protein samples extracted from
the leaves shown in A, showing the presence of processed GFP only in leaves that contained both RNA1 (R1m) and R2G.

**Figure 3.1** RNA2 5’ UTR contains key *cis*-acting elements required for RNA2 accumulation. (A) Schematic representation of BPMV RNA1 and RNA2 (R2G) constructs used in this study, together with RNA1m, a nonreplicating RNA1 mutant, and a number of R2G mutant with altered 5’ or 3’ UTRs. A construct that combines the changes in R2G-1U5 and -1U3 was also generated but not shown in the diagram. (B) Infectivity of mutant constructs assessed with particle-bombarded lima bean cotyledons and subsequently rub-inoculated soybean plants. Constructs tested are indicated above the respective panels. The images of GFP fluorescent cotyledons were taken 48 hour post bombardment (hpib), whereas those of systemically infected soybean leaves taken 2 week post inoculation (wpi). (C) Verification of active replication in lima bean cotyledons with (-) RNA2-specific RT-PCR. A RT-PCR product of a lima bean actin mRNA was used as controls. (D) Verification of active replication in systemically infected soybean leaves with (-) RNA2-specific RT-PCR. A RT-PCR product of a soybean actin mRNA was used as controls.

**Figure 3.2** Identification of SLC as a *cis*-acting element essential for RNA2 accumulation. (A) Schematic representation of R2G mutants that contained various forms of insertions/deletions within the second half of 5’ UTR of RNA2 (nt position 263-466). (B) RNA secondary structure of the nt 263-466 region predicted using the MFold
The three putative stem-loop structures are referred to as SLA, SLB, and SLC, respectively. (C) Infectivity of mutant constructs assessed with particle-bombarded lima bean cotyledons and subsequently rub-inoculated soybean plants. Mutants tested are indicated above the respective panels. Images were recorded in a manner similar to those in Fig. 3.1B. (D) and (E) Verification of active replication in lima bean cotyledons and systemically infected soybean leaves with (-) RNA2-specific RT-PCR.

**Figure 3.3** The functionality of SLC depends on the integrity of the central portion of the stem. (A) Predicted secondary structures of mutRR, mutLL, mutRL, and 460BamHI. The altered nts in each mutant are in lower case letters. Detailed description of the mutants is provided in the main text. (B) Infectivity of mutant constructs assessed with particle-bombarded lima bean cotyledons and subsequently rub-inoculated soybean plants. Mutants tested are indicated above the respective panels. (C) and (D) Verification of active replication in lima bean cotyledons and systemically infected soybean leaves with (-) RNA2-specific RT-PCR.

**Figure 3.4** Conservation of SLC structure in RNA2 of various BPMV isolates. The SLC element was identified from an Iowa (IA-Di1) isolate, and shared with 100% identify by five additional isolates. Limited nt variations found in two Kentucky (KY) isolates and one Ohio (OH) isolates, none of which caused significant changes in the overall structure.
Figure 4.1 P58 is required for RNA2 accumulation. (A) Schematic representations of BPMV RNA1, RNA1m (a nonreplicating RNA1 mutant), RNA2-GFP (R2G) and a series of R2G mutants used in this study, accompanied by images of lima bean cotyledons bombarded with these constructs. The mutation (GDD→AAH) contained in RNA1m is highlighted in the grey box. Details of RNA2 mutants are provided on their respective diagrams. The images of bombarded cotyledons were taken at 48 hour post bombardment (hpb), using a camera that is part of a dissecting microscope, illuminated with long wavelength UV light (left panels, size bar = 0.5mm). Some infection foci are highlighted with arrows. The close-up images of infection foci were taken with a fluorescence microscope (right panel, size bar = 10µm). (B) Replication of BPMV in bombarded cotyledons as measured by strand-specific, semi-quantitative RT-PCR that detects (-) RNA1 (top panel) and (-) RNA2 (middle panel). Total RNA was extracted at 48 hpb and treated with RNase-free DNase I. Same amounts of RNA were subjected to sqRT-PCR. A RT-PCR product of a lima bean actin mRNA was used as a control. 

Figure 4.2 Compensatory mutants isolated from lima bean cotyledons bombarded with RNA1 + R2G-mP58. (A) The nucleotide (nt) sequences and the deduced amino acid (aa) sequences of the compensatory mutants (R2G-N36-P58, R2G-N3-P58 and R2G-N3/N36-P58) were aligned with those of the original R2G-mP58 mutant to highlight the position and identity of the compensatory mutations. Only the section with mutations is shown. All except the revertant (R2G) retained that original ATG→gct change. Nontranslatable aas are shown in grey and translatable aas are shown in black. The compensatory
mutations at the nt level and their corresponding aa (M for methionine) are underlined. Numbers underneath the name of each mutant represent the number of clones obtained for the corresponding mutants. (B) Infectivity of the mutants as depicted by photographs of GFP fluorescent cotyledons (top panels; 48 hpb. Size bar = 0.5mm), as well as systemically infected soybean leaves [bottom panels; 2 week post inoculation (wpi)]. (C) Verification of active replication in lima bean cotyledons with (-) RNA2-specific, sqRT-PCR. 

**Figure 4.3** P58 is a *cis*-acting protein. (A) Complementation between RNA2 mutants with defects in P58 and MP. The diagrams of the mutants used, as well as those included in each of the complementation experiments, are shown on the left, with the outcome of infections/complementations shown on the right. (B) Complementation of P58 and MP defects with replication-independent expression of corresponding proteins. (C) & (D) Results of (-) RNA2-specific sqRT-PCR used to confirm the complementation results shown on (A) and (B), respectively. 

**Figure 4.4** Replication-related proteins coded by RNA1 are all *cis*-acting. (A) Schematic representation of the constructs used in this set of experiments (top), and their fate in singly or co-bombarded cotyledons (bottom). (B) Confirmation of results shown in (A) using (-) RNA1-specific, sqRT-PCR. (C) The defect of RNA1m cannot be complemented by functional RdRP provided by wild-type RNA1. Digestion with BsrBI, which cuts the PCR fragment of R1m mutant but not that of wild-type RNA1 (lane 1 and 2) was used to
detect potential R1m-specific PCR product amplified from RNA1 + R1m-2G co-bombarded cotyledons. (D) Schematic representation of five RNA1 mutants tested in this study. The names of the mutants, together with the specific aa changes introduced by these mutants, are highlighted in their corresponding boxes.

**Figure 5.1** Differential replication efficiency of BPMV RNAs in agro-infiltrated *Nb* leaves at 5, 10, 15 days post infiltration (dpi). (A) Schematic representation of four constructs used in this study: pAI101-RNA1 containing cDNA of BPMV RNA1 flanked by P35S and T35S; pAI101-RNA1m carrying a mutated RNA1 defective in RdRP; pAI101-RNA1-R2G containing both full length cDNA of BPMV RNA1 and RNA2:GFP (R2G); pAI101-R1m-R2G containing the mutated RNA1(R1m) and R2G. (B) Evaluation of the relative accumulation levels of BPMV (-) strand RNA1 and RNA2 in agro-infiltrated *Nb* leaves at 5, 10 and 15 dpi as measured by semi-quantitative RT-PCR with 24 cycles. *Nb* actin (NbACT) mRNA was also detected as control to ensure a similar account of RNA was used in all RT-PCR reactions. (C) *Nb* leaves were infiltrated with agrobacterium carrying RNA1-R2G or R1m-R2G constructs. Then one infiltrated leaf per treatment was collected at 5, 10, 15 dpi (a-f) and used to inoculate soybean plants. GFP fluorescence was observed on soybean local inoculated leaves at 5dpi (middle panel) and systemic leaves at 10dpi (bottom panel). (D) Western blot analysis of GFP protein extracted from leaves infiltrated with RNA1-R2G or R1m-R2G at 5, 10 and 15 dpi. ... 114
**Figure 5.2** Nb-P19 is at least 1000 fold more resistance to BPMV than soybean. (A) A diagram of a dilution series generated by making continuous 1:10 dilutions. Each dilution was divided into two equal halves and used to inoculate BPMV host soybean leaves and nonhost Nb-P19 leaves. (B) Comparison of the susceptibility of two types of plants to BPMV. The number underneath represents the total number of GFP foci observed on respective inoculated leaves. NC: not countable. (C) BPMV-GFP systemic infection on wildtype Nb or Nb-P19 leaves at three weeks post inoculation (wpi). The number represents the infection ratio of BPMV in Nb plants. 60 plants of each type of Nb were evaluated in this study.

**Figure 5.3** Evaluation of GFP expressions of pCAMBIA1300-sGFP-viral genes on Nb cells. (A) Schematic representation of pCAMBIA1300-based GFP expression constructs of BPMV proteins. Each viral gene was driven by 35S promoter while sgfp was driven by Gmubi promoter. (B) sGFP mRNA (top panel) and siRNA (middle panel) were analyzed by northern blot using five different sGFP oligos as specific radio labeled probe. Ethidium bromide staining of ribosomal RNA (bottom panel) provides a control for RNA loading.
CHAPTER 1. INTRODUCTION

OVERVIEW

In the field of plant-microbe interactions, the term nonhost is invoked to describe the plant species that is resistant to a given pathogen at the species level, meaning none of its varieties (cultivars, biotypes, ecotypes, etc) can be infected by this pathogen. It is obvious that most plants are nonhosts to most pathogens, as each plant species is typically susceptible to only a small subset of pathogenic microbes (Heath, 2000; Holub and Cooper, 2004; Mysore and Ryu, 2004; Schulze-Lefert and Panstruga, 2011). Nonhost plants defend themselves against most of the pathogenic microbes with diverse, highly sophisticated self-defense strategies, which are collectively referred to as nonhost defense (Heath, 2000; Mysore and Ryu, 2004; Uma, et al., 2011).

In contrast to the better characterized race-specific resistance mediated by Resistance (R) genes that are present in limited varieties of a plant species, nonhost resistance is shared by all varieties of the same species, and hence considered to be more
durable. The R-gene mediated defense establishes pathogen race- and plant cultivar-specific resistance, in which some varieties of a plant species are resistant and others susceptible to a given pathogen (Dangl and Jones, 2001; Schulze-Lefert and Bieri, 2005; Soosaar et al., 2005). This resistance is often governed by single R genes, the products of which can interact, directly or indirectly, with the specific elicitors produced by the so-called avirulence (avr) genes of pathogens (Flor, 1971; Hammond-Kosack and Jones, 1997; Kang, et al., 2005; Maule, et al., 2007). A number of antiviral R genes have been identified that confer antiviral resistance with the corresponding R proteins. For instance, the N protein of tobacco confers resistance to Tobacco mosaic virus (TMV), whereas the Rx protein of potato confers resistance to Potato virus X (PVX) (Whitham et al., 1996; Ishibashi et al., 2007; Tomita et al., 2011). These R genes have been used to protect crops from viruses, although resistance-breaking virus mutants often emerge after the introduction of these genes (Strasser and Pfitzner, 2007; Ishibashi et al., 2012). By contrast, nonhost resistance is rarely overcome, and hence considered to be more durable (Heath, 2000; Mysore and Ryu, 2004).

However, molecular mechanisms underpinning the nonhost resistance remain relatively unexplored compared with the well-studied R gene-mediated resistance. Although recent studies suggest that nonhost resistance to bacterial and fungal pathogens enlist multi-layered defense mechanisms that encompass Pathogen associated molecular pattern (PAMP)-triggered immunity (PTI) as well as R-gene mediated resistance (Mysore and Ryu, 2004; Nurnberger and Lipka, 2005; Jones and Dangl, 2006; Boller and Felix, 2009; Uma, et al., 2011), the nature of antiviral nonhost defense remains poorly
understood. This knowledge gap has prevented the exploitation of nonhost resistance resources to achieve effective control of virus diseases in crop plants.

**BPMV IS A SERIOUS SOYBEAN PATHOGEN WITH NO KNOWN CONVENTIONAL RESISTANCE RESOURCES**

*Bean pod mottle virus* (BPMV) was first discovered on *Phaseolus vulgaris* cv. Tendergreen in South Carolina in 1945, and was first reported on soybean fields in Arkansas and North Carolina in 1948 (Giesler et al., 2002). Although initially found in the southern and southeastern parts of United States, it has been steadily spreading to northern parts of U.S., becoming a nationwide threat to soybean production (Giesler et al., 2002; Ziems et al., 2007). BPMV infection induces various symptoms on soybean plants. They range from a mild chlorotic mottling of foliage to severe mosaic symptoms on younger leaves, stunting, and delayed maturity (Giesler et al., 2002), which lead to yield losses of up to 50% in some areas or years (Giesler et al., 2002; Redinbaugh et al., 2010). The plant maturity, weather condition and the time of virus infection, determine the impact of BPMV infection on yield: symptom severity may lessen with mature plant during hot weather; earlier infection results in more severe losses (Giesler et al., 2002). BPMV infection also delays pod formation and reduces seed size, weight and number (Giesler et al., 2002). In addition to yield reduction, mild or even symptomless infections of BPMV may lead to discoloration of the seed coat with black or brown pigmentation,
compromising the commercial value of the crop (Giesler et al., 2002; Redinbaugh et al., 2010).

BPMV is transmitted by several leaf-feeding beetles (Coleoptera) in the families *Chrysomelidae, Coccinellidae* and *Meloidae* (Giesler et al., 2002). Bean leaf beetle (*Cerotoma trifurcate*) acquires BPMV after a single bite of an infected soybean plant, and remains transmission-competent for at least 72 hours, although BPMV probably does not replicate in the beetle since viral titer decreases gradually after feeding on healthy plants (Pitre and Patel, 1975; Giesler et al., 2002). The efficiency of virus transmission is highly related with species of beetle vector. For example, the transmission frequency of *C. trifurcate* is close to 80%, while the transmission frequency of *Diabrotica adelpha* is only 10% (Gergerich and Scott, 1996; Giesler et al., 2002).

BPMV is a member of the comovirus genus in the *Comovirinae* subfamily of *Secoviridae* (Sanfacon et al., 2009). Virus particles in the *Comovirinae* are icosahedral in symmetry, non-enveloped, and are approximately 28 to 30 nm in diameter (Wellink et al, 2000). BPMV is a single stranded, positive (+) sense RNA virus with two separately encapsidated genomic RNAs (gRNAs) of RNA1 and RNA2 (Giesler et al., 2002). Virions can be separated by density gradient centrifugation into three components: top, middle and bottom (Giesler et al., 2002). The middle and bottom components contains single molecules of RNA1 (ca. 6,000) and RNA2 (ca. 3,700) (Giesler et al., 2002; Gu and Ghabrial, 2005; Fig. 1.1), respectively, whereas the top component only contains empty particles (Giesler et al., 2002). Both gRNAs are polyadenylated at the 3’ termini and are
covalently bound by a small viral genome-linked protein (VPg) at the 5’ termini (Giesler et al., 2002; Gu and Ghabrial, 2005).

Each BPMV gRNA encodes one single open reading frame (ORF), whose translated polyprotein is then proteolytically processed into mature functional proteins (MacFarlane et al., 1991). RNA1 encodes proteins involved in the replication of viral RNAs and polyprotein processing. The processing of RNA1-encoded polyprotein gives rise to a 32 kilodalton (K) protease cofactor (C-Pro), a 58K putative helicase (HEL), a VPg, a 24K protease (Pro), and a 87K putative viral RNA-dependent RNA polymerase (RdRP) (MacFarlane et al., 1991; Di et al., 1999; Fig. 1.1). Proteinase and putative helicase have been reported to be determinants of BPMV symptom development (Gu and Ghabrial, 2005). Notably, BPMV RNA2 encodes two overlapping polyproteins, as its translation can initiate from two separate, in-frame AUG codons [at nucleotides (nt) positions 467 and 773 of RNA2; Fig. 1.1]. Both polyproteins are processed twice at the same sites to produce four different mature proteins: the larger N-terminal protein of 58K with unknown function(s); the smaller N-terminal protein which is the movement protein (MP); and two capsid protein subunits (L-CP and S-CP; Lomonossoff, et al., 2001; Fig. 1.1). Since RNA2 does not encode any replication-related functions, its replication relies on RNA1-encoded replication proteins. How RNA2 recruits the replication complex induced by RNA1 encoded protein(s) is still unknown. This unsolved question will be addressed in Chapter 3 and Chapter 4.

Despite of many years of efforts by soybean breeding community, no soybean variety has been found to be resistant to BPMV (Hill et al., 2007; Ziems et al., 2007).
Alternative management measures, including planting varieties that are relatively tolerant to BPMV and using insecticide to control BPMV-transmitting bean leaf beetle, have had limited success (Hill et al., 2007). Recently, it was found that planting soybean varieties resistant to insect feeding was unable to prevent the spread of BPMV (Redinbaugh et al., 2010). Therefore, there is an urgent need for identifying a durable resistance solution to BPMV-caused soybean diseases by examining nonhost resistance response to BPMV.

**N. BENTHAMIANA IS AN EXCELLENT MODEL NONHOST OF BPMV**

The host range of BPMV is restricted to legumes (Giesler et al., 2002). In a study of BPMV experimental host range, 25 species including 20 genera of plants were evaluated for their susceptibility to BPMV. Several varieties of snap bean and dry bean, as well as soybean (*Glycine max*) and lima bean (*Phaseolus lunatus*), were determined to be susceptible to BPMV (Giesler et al., 2002).

However, the model plant *N. benthamiana* (*Nb*) does not support detectable reproduction of BPMV, thus is considered to be a nonhost of BPMV (Zhang and Ghabrial, 2006). *Nb* has long been an experimental model for the plant virology community. It is also well suited for large-scale biochemical studies because it is relatively easy to rear, fast growing, and matures to a reasonably large size. Importantly, it is particularly amenable to agro-infiltration, a highly efficient *Agrobacterium*-mediated transient expression procedure. Transient expression through agroinfiltration is simple and effective, involving the injection of *A. tumefaciens* into leaves and then monitoring
the expression of transient transgene in the infiltrated tissue during the next few days (Sparkes, et al., 2006). This procedure is being increasingly used by virologists and plant biologists alike to elucidate biochemical properties of viral as well as plant proteins without having to rely on the lengthy stable transformation approach (Goodin et al., 2008). In summary, *Nb* is an ideal model plant for the investigation of plant nonhost resistance to BPMV.

**MULTI-PARTITE (+) RNA VIRUSES USE CONTRASTING STRATEGIES TO ACCESS REPPLICATION FACTORIES**

Despite differences in viral genome organization and coding strategies, (+) RNA viruses share basically similar strategies for genome replication. The genomic RNA(s) serve first as the mRNA to translate the replication proteins, and then the replication proteins bind to the 3’ end of (+) genomic RNA(s) and convert it into negative sense (-) replication intermediates. The (-) RNAs in turn serve as templates for synthesis of progeny (+) RNAs (Nagy and Pogany, 2010; den Boon and Ahlquist, 2010). To facilitate the viral replication process, most (+) RNA plant viruses were found to encode at least two proteins required for genome replication. One protein is named as auxiliary protein (AP), which accumulates to very high concentrations in the infected cells but lacks the conserved RdRP motif, whereas the other is the RdRP which is the core protein that catalyzes the nucleotide polymerization step, yet it accumulates to only 3-5% of AP levels (den Boon and Ahlquist, 2010).
AP plays a critical role in the early steps of viral RNA replication. The replication of (+) RNA virus genomes is thought to take place in partially enclosed spherules, vesicles or multi-vesicular bodies, termed virus replication complexes (VRCs). The VRCs are formed by conformational changes of membranes of cellular organelles, most frequently endoplasmic reticulum (ERs). The conformational change of organellar membranes is induced by viral AP, which in turn occupies the interior surface of VRCs at a ratio of up to 100 AP copies per VRC. In contrast, only one or a few copies of RdRP and viral RNA are present in every VRC, both recruited by AP. VRCs have been observed in many (+) RNA viruses-infected plant cells, including those infected by comoviruses (den Boon and Ahlquist, 2010; Carette et al, 2000). These membrane compartments provide protective enclosed structures that concentrate and sequester viral replication elements, coordinate replication steps and shield RNA replication intermediates from cellular antiviral responses (den Boon and Ahlquist, 2010; Laliberte and Sanfacon, 2010).

Notably, different genomic RNA segments of multi-partite (+) RNA viruses were found to be recruited into VRCs using different mechanisms. Many viral genomic RNAs are also mRNAs for the translation of AP, hence may be brought to VRCs by APs in a translation-coupled manner (Yi and Kao, 2008; Wang et al., 2009). Specifically, AP-encoding viral RNA, as exemplified by RNA1 of *Brome mosaic virus* (BMV) and *Red clover necrotic mosaic virus* (RCNMV), is not recruited unless the same viral RNA copy serves as a competent template for AP translation (Yi and Kao, 2008; Iwakawa et al., 2011). This recruitment mechanism is hence referred to as translation-coupled.
On the other hand, viral RNA(s) encoding non-AP proteins must possess additional specificity determinants in the form of specific secondary structures or stem-loops (SLs) that are recognized by AP or RdRP, which in turn carries the corresponding viral RNAs to VRCs (Baumstark and Ahlquist, 2001; Iwakawa et al., 2011). For example, BMV consists of three genomic RNAs, RNA1, RNA2 and RNA3. RNA1 and RNA2 encode 1a and 2a, two proteins required for genome replication (serving AP and RdRP functions, respectively). The smaller RNA3 encodes the cell-to-cell movement and capsid proteins (MP and CP). It has been long established that BMV RNA3 encodes a unique, cis-acting RNA structure that specifically interacts with 1a to dramatically enhance the replication of RNA3 (Baumstark and Ahlquist, 2001; and refs therein). Similarly, RCNMV RNA1 encodes N-terminally overlapping replication proteins, P27 and P88. P88 carrying RdRP motif was found to function in cis for the replication of RNA1 (Okamoto et al, 2008), whereas P27 interacts directly with a Y-shaped RNA structure in 3’ untranslated region (UTR) of RNA2 then brings RNA2 into VRCs (Iwakawa et al., 2011; and refs therein). A somewhat unique example is RNA3 of Alfalfa mosaic virus (AMV), which does not encode AP or RdRP. The replication of AMV RNA3 was dependent on the coat protein (CP), which binds to the 3’-terminus of RNA3 to induce RNA conformational changes, thus positioning the RdRP at the initiation site for (-) strand synthesis (Neeleman et al, 2004; Reichert et al, 2007).

Unlike BMV, RCNMV or AMV, which translate the final protein products directly from genomic or subgenomic mRNAs, viruses like BPMV translate one polyprotein per genomic RNA segment, which is then proteolytically processed into functional proteins.
Nevertheless, available data suggest that these two types of viruses replicate using similar strategies. Studies by Carette and colleagues (2000) showed that *Cowpea mosaic virus* (CPMV) induced a massive proliferation of ER membranes, which aggregate into electron-dense structures near nuclei of infected cells. They further demonstrated that these morphological changes could be induced by C-Pro or HEL-VPg homologs of CPMV. They further observed that CPMV RdRP as well as viral RNA co-localized with these ER-based structures (Carette et al., 2002a; b). These data suggest that CPMV RNAs replicate in membrane-based VRCs as well. These striking similarities among multi-partite (+) RNA plant viruses further imply that the two RNA segments of BPMV might also use different mechanisms to access VRCs.

**NONHOST PLANTS USE DIFFERENT MECHANISMS TO COMBAT VIRUS INFECTIONS**

As obligate intracellular parasites, plant viruses are unique in that they use the host cellular protein synthesis system and other cellular components to synthesize pools of new proteins and nucleic acids that participate in the assembly of new virions. These characteristics are conducive to the evolution of certain unique plant antiviral defense strategies that target virus reproduction. These may include preventing viral protein translation by making the cellular translational machinery incompatible with viral RNA, engaging viral replication-related proteins through protein-protein interactions, clearing viral RNA with RNA silencing, or capturing viral Avr proteins with R proteins (Ishibashi
et al., 2007; Ishibashi et al., 2009; Robaglia and caranta, 2006; German-Retana et al., 2008; Nieto et al., 2011; Moffett, 2009). Hence, the nonhost incompatibility between a virus and a plant can be conditioned by multiple layers of antiviral defense that either actively suppresses viral pathogenesis, or passively evades viral multiplication (Pallas and Garcia, 2011). Below, I will review our current understanding of nonhost plant antiviral defenses.

Some nonhosts subdue virus attacks by targeting the replication of virus genome. Ishibashi and colleagues (2009) examined the molecular basis of the nonhost resistance to *Tobacco mild green mosaic virus* (TMGMV) and *Pepper mild mottle virus* (PMMoV) in tomato plants. Both TMGMV and PMMoV infect tobacco systemically but neither infects tomato. They found that transgenic expression of one tomato protein, tm-1, was enough to disrupt the multiplication of TMGMV and PMMoV in tobacco. They further demonstrated that the resistance conferred by tm-1 was due to the physical binding of tm-1 protein with one of the viral replication proteins, thus preventing viral RNA replication. Was tm-1 the only factor that prevents the TMGMV infection? If yes, mutants of TMGMV that escape tm-1-mediated resistance might be expected to appear. Indeed such mutants were isolated from tm-1 expressing tobacco plants. However, these mutants showed limited virus replication and systemic necrosis in nonhost tomato, suggesting that additional inhibitory factors may be present in tomato. In other words, tomato employs multilayered defense to fully combat the invasion of TMGMV (Ishibashi et al., 2009).

By contrast, Nieto and colleagues (2011) took a different approach to identify the nonhost resistance factor conferring resistance to *Melon necrotic spot virus* (MNSV) in
They had established earlier that \textit{Nb} was resistant to most strains of MNSV except for MNSV-264. Notably, the MNSV-264 strain also breaks race-specific resistance in MNSV-resistant melon varieties. The race-specific resistance to MNSV in melons is conferred by a specific isoform of the eukaryotic translational initiation factor 4E (eIF4E). Recessive resistance mediated by eIF4E is due to the absence of an eIF4E isoform compatible with the RNA of the corresponding virus in the cells of resistant plants, thus the inability to translate viral proteins required for RNA replication. The authors hypothesized that a similar \textit{Nb} eIF4E isoform might also be responsible for the nonhost resistance to MNSV strains other than MNSV-264. To test this hypothesis, they expressed the eIF4E isoform from MNSV-susceptible melons in \textit{Nb} leaves. This alone enabled the replication of a MNSV strain otherwise incapable of replicating in ordinary \textit{Nb} leaves (Nieto et al., 2011). This study suggests that a \textit{Nb} eIF4E isoform confers nonhost resistance to MNSV by thwarting the translation of viral proteins, especially the viral RdRP required for viral RNA replication.

Both of the above mentioned anti-viral nonhost determinants were discovered based on the involvement of their homologs in race-specific resistance. Specifically, tm-1 was initially identified as the resistance protein to \textit{Tomato mosaic virus} (ToMV) in a few accessions of tomato (Ishibashi et al., 2007), whereas isoforms of eIF4E have been known to exert race-specific resistance to many different viruses (Truniger and Arenda, 2009). Thus, nonhost resistance may in some cases simply reflect the functionality of other forms of microbe resistance at the species level.
RNA silencing, also known as RNA interference, is an essential antiviral defense that recognizes and processes double-stranded RNA (dsRNA) or partially double-stranded hairpin-loop RNA (hpRNA) formed during virus replication (Ding and Voinnet, 2007). The dsRNAs serve as the replication intermediate of RNA viruses, whereas hpRNAs are frequently formed inside viral RNA genomes to serve critical regulatory functions (Miller and White, 2006; Wu et al., 2010). The processing of these ds/hpRNAs leads to accumulation of small interfering RNA (siRNA) with different sizes (21, 22, or 24 nucleotides). These siRNAs in turn target viral RNAs in a sequence-specific manner for degradation, achieving viral resistance through the destruction of viral genetic materials.

RNA silencing plays a primary role in limiting the invasion of viruses in nonhost plants. Antiviral RNA silencing operates in all plants, yet in susceptible hosts it is rigorously counteracted by virus-encoded silencing suppressors (VSRs) that target one or more components of the RNA silencing pathway (Ding and Voinnet, 2007). During the host plants-virus interactions, the adapted viruses encode VSRs that co-evolve with their plant targets. This may in turn make VSRs less capable of engaging their corresponding targets in nonhost plants, especially when the nonhosts are evolutionarily distant from virus-susceptible plants (Schulze-Lefert and Panstruga; 2011). This selection process could account for some of the nonhost antiviral resistance, as elegantly demonstrated by Jaubert and colleagues (2011). These authors showed that nonhost resistance to Potato virus X (PVX) in Arabidopsis is primarily due to the inability of PVX to overcome RNA
silencing-mediated defense, thus revealing a distinct role of RNA silencing in nonhost resistance.

Conversely, although \( R \) genes are commonly identified in a subset of varieties of a given plant species, they could function as nonhost resistance determinants if they are present in all varieties of a given plant species. In fact, the tobacco \( N \) gene, which confers resistance to TMV in tobacco (\( N. \) \textit{tabacum}), was originally identified from \( N. \) \textit{glutinosa}, a different plant species resistant to TMV at the species level (Marathe et al., 2002). A similar case can be made for the \textit{Arabidopsis} gene \textit{Constitutive PR expression 5} (\textit{CPR5}), which confers nonhost resistance to \textit{Brome mosaic virus} (BMV), but otherwise is also involved in \( R \) gene-mediated resistance to bacterial and fungal pathogens (Fujisaki et al., 2009).

To summarize, these four published examples illustrate that nonhost resistance to plant viruses that have been identified to date employ extremely diverse molecular mechanisms, ranging from RNA silencing, \( R \) gene-mediated resistance, eIF4E-mediated resistance, to other plant proteins with as-yet-unknown functions. It is noteworthy that the above mentioned modes of plant nonhost antiviral defense are also involved in plant resistance to the invasion of host-adapted viruses, therefore, the molecular mechanisms of nonhost resistance to viral pathogens may overlap with non-specific host resistance mechanisms and include both active and passive (lack of susceptibility factors) defense mechanisms.
RESEARCH OBJECTIVES

Based on the comprehensive review of current understanding of nonhost antiviral defense, I designed my thesis research with the goal of unraveling the molecular mechanism of the nonhost resistance encountered by BPMV in Nb. As I described earlier in this chapter, I chose to use BPMV as the virus model because it is a major pathogen of the economically important soybean crop. Conversely, I chose Nb as the model nonhost as it does not support the infection of BPMV. In addition, I also examined two BPMV host plants, namely soybean and lima bean, in parallel with Nb to uncover differences in responses to BPMV infections that could contribute to nonhost resistance. In order to understand how Nb prevents BPMV infection, my first objective was to develop a robust system for investigating the replication efficiency of BPMV genomic RNAs in Nb cells. The completion of the first objective suggested that among the two BPMV genomic RNAs, RNA2 replication was drastically delayed in Nb cells. That Nb selectively represses RNA2 replication implies that it might have exploited an inherent difference between the replication processes of RNA1 and RNA2. Therefore, my second objective was to identify the RNA2 specific features that facilitate RNA2 replication in BPMV hosts. In the final objective, I examined the role of RNA silencing mediated antiviral defense against BPMV in Nb. The outcomes of my study are expected to contribute to the understanding of molecular mechanism of anti-BPMV nonhost resistance, and accelerate the development of novel experimental procedures applicable to studies on other virus-nonhost interactions.
Figure 1.1 Genome organization of BPMV RNAs. The numbers denote the sizes of the two RNAs (5,986 and 3662 nucleotides), and their encoded open reading frames (ORFs). The shaded boxes represent mature functional proteins released from the single ORFs by the viral protease (Pro). Other proteins on RNA1 are, C-Pro: protease cofactor; HEL, putative helicase; VPg: genomic linked viral protein (also shown attached to the 5’ end of both RNAs). RdRP: viral RNA-dependent RNA polymerase. Proteins encoded by RNA2 are, P58: function unknown; MP: movement protein, overlaps with P58 but starts from the second AUG of RNA2; L-CP and S-CP, large small capsid protein subunits.
CHAPTER 2. NONHOST RESISTANCE IN NICOTIANA BENTHAMIANA ENGAGES THE BIPARTITE BEAN POD MOTTLE VIRUS BY SELECTIVELY TARGETING THE REPLICATION OF THE VIRAL RNA2

ABSTRACT

Nonhost resistance to plant viruses is highly prevalent in nature but its mechanisms are poorly understood. The current study sought to understand how Nicotiana benthamiana (Nb), a nonhost of Bean pod mottle virus (BPMV), respond to BPMV invasion. BPMV is a bipartite, positive sense (+) RNA virus with both genomic RNAs (RNA1 and RNA2) encoding single polyprotein precursors, which are subsequently processed into functional viral proteins. I first established that all of the functional proteins, as well as both polyprotein precursors, could accumulate to detectable levels in Nb leaves without triggering visible defense responses. I then rigorously examined
whether BPMV viral RNA replication could occur in individual Nb cells, and
demonstrated that BPMV RNA1 was capable of initiating active replication on its own at
5 days post infiltration (dpi) in the infiltrated Nb leaves. By contrast, RNA2 replication
was not detectable at 5 dpi despite the availability of replication proteins provided by
RNA1. Furthermore, I revealed that the activity of the protease, which is encoded on
RNA1, is independent of active RNA1 replication, and it readily processes RNA2-coded
polyprotein in trans in Nb. Together, our results uncovered a novel nonhost defense
mechanism that selectively targets the replication of BPMV RNA2. This finding
illustrates that the distinct replication requirements of different genomic RNAs of
multipartite viruses can be exploited by nonhost plants to mount effective defenses. Our
observation reinforces the mechanistic diversity of nonhost antiviral defenses.
INTRODUCTION

Most plants are hosts to a small subset of plant viruses, thus are nonhosts to most viruses. While some nonhosts could have escaped virus attack through certain passive mechanisms, such as a lack of plant factors essential for virus propagation, an increasing number of virus-nonhost interactions are being revealed to involve active defense mounted in the nonhost plants (Fujisaki et al., 2009; Ishibashi et al., 2009; Jaubert et al., 2011; Nieto et al., 2011). Nonhost resistance resources are far more abundant that the traditional resistance (R) gene-mediated, race-specific resistance as they are operative in a greater number of mostly unrelated plant species. Furthermore, the mechanisms of nonhost resistance are expected to be substantially more diverse as different plants likely have evolved different strategies to counter virus invasions. Therefore, elucidation of the antiviral resistance mechanisms of nonhost plants promises to provide novel, effective means for controlling virus diseases of host plants.

Nevertheless, our understanding of nonhost resistance to plant viruses remains limited, primarily because of the difficulty to track this type of resistance with traditional genetic approaches. In fact, mechanistic underpinnings are only starting to emerge for a few examples of plant virus-nonhost interactions. Notably, Ishibashi and colleagues (2009) discovered that tomato proteins tm-1, confers nonhost resistance to Tobacco mild green mosaic virus (TMGMV) and Pepper mild mottle virus (PMMoV) through physical binding with one of the viral replication proteins thus preventing viral RNA replication. Separately, Nieto and colleagues (2011) observed that nonhost resistance to Melon necrotic spot virus (MNSV) in Nicotiana benthamiana (Nb) is likely dependent on the
eukaryotic translational initiation factor 4E (eIF4E) in this plant. Interestingly, eIF4E has also been implicated in race-specific antiviral resistance. Therefore, these two examples suggest that at least some instances of nonhost resistance share similar mechanisms with race-specific resistance.

Conversely, nonhost resistance to *Brome mosaic virus* (BMV) in *Arabidopsis* was shown to require Constitutive PR expression 5 (CPR5), a protein known to repress *R* gene-mediated resistance to bacterial and fungal pathogens (Fujisaki et al., 2009). Separately, nonhost resistance to *Potato virus X* (PVX) in *Arabidopsis* has been attributed to the functionality of RNA silencing-mediated defense (Jaubert et al., 2011). The general conclusion drawn from the few reported examples appears to be that nonhost resistance to plant viruses employs extremely diverse molecular mechanisms, which in turn highlight the urgent need for additional research.

In an effort to develop a robust experimental system for examining nonhost antiviral defense, I have been using the highly efficient, *Agrobacterium*-mediated transient expression procedure (agro-infiltration) to probe the interactions between *Bean pod mottle virus* (BPMV) and its nonhost *Nb*. BPMV is a bipartite, positive sense (+) RNA virus of the *Secoviridae* family. The two BPMV genomic (g) RNAs, 6.0 and 3.6 kilobases (kb) in size, both encode single large open reading frames (ORFs), whose translated polyproteins are then processed into functional protein products by the viral protease (Pro) encoded on the larger gRNA (RNA1). All proteins required for viral RNA replication are encoded on RNA1, which include, from 5’ to 3’, a putative Protease cofactor (C-Pro), an RNA helicase (Hel), a small viral protein covalently linked to the 5’
end of gRNAs (VPg), Pro, and a viral RNA-dependent RNA polymerase (RdRP; Fig. 2.2A). RNA2 relies entirely on RNA1 for its replication but encodes the proteins required for the assembly of virus particles, as well as the viral spread from the initial invasion sites to whole infected plants. These include a 5’ proximal movement protein (MP), followed by two capsid protein subunits (L-CP and S-CP; Fig. 2.2A).

*Nb* is a widely used model plant for studying plant virus replication, cell-to-cell and systemic movement, as well as antiviral defense responses. Although it is known to be susceptible to a great number of common viruses, it is notably a nonhost of BPMV. I began my investigation of the BPMV-*Nb* interaction by examining whether *R* gene-mediated hypersensitive response (HR) plays a part in the nohost resistance response. I then asked whether the replication of BPMV genomic RNAs takes place in single *Nb* cells. I found that while RNA1 of BPMV underwent active replication in *Nb* cells, RNA2 delivered into the same cells was not replicated by the replication proteins provided by RNA1. Our results reveal a novel nonhost resistance mechanism which preferentially target one of the gRNAs of a bipartite virus.
MATERIALS AND METHODS

Assembly of binary constructs containing full length BPMV cDNAs

The BPMV cDNAs used in this study were acquired from several different sources. The RNA1 cDNA was derived from a BPMV strain (K-Ho1) isolated in Kentucky, whose sequence has been reported (Gu and Ghabrial, 2005). A replication-defective mutant of RNA1 cDNA (RNA1m) was created by changing the highly conserved GDD motif to AAH (the sequence of the DNA oligo is available upon request). This change also simultaneously created a new BsrB site to permit fast identification of the mutant in mixed infections. The RNA2 cDNA was synthesized in the current study from an Ohio isolate (OH) of BPMV. The RNA2:GFP cDNA has been reported previously (Zhang et al., 2010). All full length BPMV cDNAs were first cloned into the plasmid pRTL2 to acquire the 35S promoter (P35S) and terminator (T35S) of CaMV. The resulting expression cassettes, with BPMV cDNAs flanked by P35S and T35S, were then excised from pRTL2 and inserted into the binary vector pPZP212 (Qu et al., 2003) to produce pPZP-RNA1, -RNA1m, -RNA2, and -R2G.

To make the RNA1-R2G and RNA1m-R2G constructs, the binary vector pCAMBIA1300 was first modified to create pAI101. This was done by deleting the expression cassette for the HPT II gene, which confers resistance to hygromycin in transgenic plants but is unnecessary for transient expression mediated by agro-infiltration. R2G expression cassette was then inserted into pAI101 to create pAI-R2G. RNA1 and RNA1m cassettes were subsequently inserted into pAI-RNA2G, upstream of
R2G cassette, to create RNA1-R2G and R1m-R2G. The tandem direction of the inserted cassettes (Fig. 2.3A) was confirmed by digestions with appropriate restriction enzymes.

**Constructs for transient expression of individual BPMV proteins**

The plasmid pRTL2 was modified to create pRTL4i, which contains an Apal site, a NotI site, and the cDNA of a duplicated HA epitope tag immediately downstream. The cDNAs of individual BPMV proteins were then amplified with PCR, incorporating Apal and NotI sites to their 5’ and 3’ ends, respectively. In addition, the 5’ PCR oligos also contained nine extra nucleotides (AACAACATG) between the Apal site and BPMV sequence to equip every fragment with a translational start codon (ATG), and to place the start codon within the sequence context needed for optimal translational initiation. The 3’ ends of all fragments were designed with care to ensure their translation products include the 3’ dHA fusion. The sequences of all constructs were verified before they were mobilized, together with P35S and T35S, into the binary vector pPZP212.

**Transient expression mediated by Agrobacterium (agro-infiltration)**

The assembled binary constructs were transformed into the C58C1 strain of *A. tumefaciens* by electroporation (Qu et al., 2003). Single *Agrobacterium* colony was used to inoculate a 3 ml overnight culture. The 3 ml culture was then used, at 1:100 dilution, to start another fresh overnight culture to ensure all suspensions have similar amounts of living cells. The second overnight cultures were harvested and resuspended as described
previously (Qu et al., 2003). *Nb* leaves of three – four weeks old plants were infiltrated with a needle-less syringe.

**Particle bombardment of lima bean cotyledons**

Lima beans of the Anderson Bush variety was purchased from Earl May Seed and Nursery (Shenandoah, IA). The bombardment experiments were carried out following the procedure described by Hernandez-Garcia et al. (2010).

**Semi-quantitative RT-PCR**

Total RNAs were extracted from infiltrated *Nb* leaves with the TRIsure reagent (Bioline, Taunton, MA) at 5dpi. After verifying the quality of RNAs with gel electrophoresis, approximately 5 μg RNA per sample was treated with TURBO DNA-free DNase according to Manufacturer’s instruction (Ambion, Austin, TX). Strand-specific reverse transcription was carried out with appropriate primers, and reverse transcriptase purchased from Clontech (Palo Alto, CA). PCR was carried out using the EconoGreen PCR Master Mix (Lucigen, Middleton, WI).

**Protein extraction and Western blot**

Proteins were extracted from infiltrated *Nb* leaves as described previously (Cao et al., 2010) and subjected to Western blot analyses with monoclonal anti-HA antibody (Sigma, St. Louis, MO)
RESULTS

All BPMV-coded proteins accumulate to detectable levels without eliciting HR in Nb

A previous study by Zhang and Ghabrial (2006) found that BPMV strains isolated from Kentucky did not infect Nb systemically. To confirm that Nb is indeed a nonhost of BPMV, I inoculated Nb with saps prepared from soybean leaves infected with two additional strains of BPMV (isolated from Ohio and Iowa, respectively). I observed that, while the same inoculum caused leaf mottling and stunting in soybean, the simultaneously inoculated Nb plant remained as healthy as mock-inoculated controls. Furthermore, BPMV RNAs were undetectable in the inoculated or upper uninoculated leaves with a reverse-transcription coupled with polymerase chain reaction (RT-PCR) procedure (data not shown). These unpublished experiments corroborated the independent observation made by Zhang and Ghabrial (2006), establishing Nb as a nonhost of BPMV.

Previous studies suggest that nonhosts frequently engage non-adapted viruses with factors that confer race-specific resistance to other related viruses (Ishibashi et al., 2009; Nieto et al., 2011). I hence asked whether the nonhost resistance to BPMV in Nb was also conditioned by certain race-specific resistance factors. Since R gene-mediated resistance is the most common form of race-specific resistance, I first evaluated whether any of the BPMV-coded proteins is targeted by R gene-mediated resistance, using R gene-triggered hypersensitive response (HR) as the visible indicator. To accomplish this, I transiently expressed the individual BPMV proteins in Nb leaves. Seven of the eight BPMV-coded proteins, C-Pro, Hel, Pro, RdRP, MP, L-CP, and S-CP (see Fig. 2.2A for a detailed
genome organization map), were expressed as individual proteins, whereas VPg was expressed as a fusion with Hel due to its small size. A fused CP (F-CP) was also included that combined both L-CP and S-CP. All nine cDNAs were placed between the strong 35S promoter (P35S) and the corresponding transcriptional terminator (T35S) of *Cauliflower mosaic virus* (CaMV), cloned into the binary vector pPZP212 (Qu et al., 2003), and delivered into *Nb* leaves by agro-infiltration. To facilitate their detection, they were all tagged with a double HA (dHA) epitope tag at their C-termini.

I observed no visible HR in any of the infiltrated leaves (data not shown). To determine whether the individual BPMV proteins could be translated from the introduced constructs, I harvested the infiltrated leaves at five days post infiltration (5 dpi) and analyzed their protein extracts with Western blot to detect HA-tagged BPMV proteins. As shown on the top panel of Fig. 2.1, F-CP, but neither L-CP nor S-CP alone, was readily detected (lanes 8-10). In addition, BPMV RdRP was faintly detectable (lane 6).

The failure to detect other proteins could be due to clearance of their respective transcripts by RNA silencing. To assess this possibility, I re-delivered the BPMV-derived constructs together with a construct that expresses a viral suppressor of RNA silencing (VSR), namely the coat protein (CP) of *Turnip crinkle virus* (TCV; Qu et al., 2003). The bottom panel of Fig. 2.1 illustrated that suppression of RNA silencing permitted the detection of all nine BPMV proteins, although their levels of accumulation varied considerably. Nevertheless, HR was still absent from the infiltrated leaves (data not shown). These results demonstrated that BPMV-encoded proteins can be stably expressed in *Nb* leaves without triggering HR-mediated defense.
BPMV RNA1 and RNA2 each encode polyprotein precursors that are subsequently processed proteolytically into mature proteins. Therefore, although the individual BPMV proteins failed to elicit HR in Nb leaves, it remains possible that some of the processing intermediates could be targeted by an HR-mediated defense. To test this possibility, I then made binary constructs that harbor full length cDNAs of both BPMV RNAs and delivered these constructs into Nb leaves together with the TCV CP VSR using agro-infiltration. Both BPMV cDNAs were flanked by P35S and T35S, cloned into the binary vector pPZP212 (Fig. 2.2A, RNA1 and RNA2 diagrams). P35S in these constructs is expected to be recognized by the Nb DNA-dependent RNA polymerase II (PolII) to produce the primary BPMV transcripts, which are then translated into BPMV polyproteins. HR-like responses were never observed on leaves infiltrated with constructs that express BPMV RNA1, RNA2, or both (data not shown). Together, these results suggest that R gene-mediated defense likely does not play a major role in nonhost resistance against BPMV in Nb.

**RNA1, but not RNA2, replicates to detectable levels in the infiltrated Nb leaves at 5dpi**

I next tried to determine whether the nonhost resistance in Nb targets the replication of BPMV RNAs at the single cell level. To do this, I took advantage of the RNA1 and RNA2 constructs assembled earlier (see above). Since these constructs supported the transcription of primary BPMV transcripts by PolII independent of active viral RNA replication, I sought to distinguish the replication-dependent RNA1 by creating a
replication-defective mutant of RNA1. This mutant, referred to as RNA1m (Fig. 2.2A, second diagram), was produced by changing the highly conserved GDD (glycine-aspartic acid-aspartic acid) motif in viral RdRP to AAH (alanine-alanine-histidine). The GDD motif has been found previously to be essential for the RdRP function of other viruses (Koonin, 1991). In addition, in infiltrations without the TCV CP VSR, an empty vector (EV) was included to keep the number of constructs constant. I then assessed the replication of BPMV RNA1 and RNA2 using semi-quantitative RT-PCR (sqRT-PCR) at 5 dpi.

Results presented in Fig. 2.2B illustrate the contrasting replication competence of BPMV RNA1 and RNA2 in Nb cells. In the bottom panel, RT-PCR products of Nb actin (NbACT) mRNA were used as controls to ensure an equivalent amount of RNA was used in every sqRT-PCR reaction. Also, no PCR product was detected when the reverse transcriptase was omitted, indicating that all products were RNA-dependent (data not shown). Interestingly, wildtype RNA1-derived products were always more abundant than those derived from the non-replicating RNA1m mutant, regardless of the presence of VSR (Fig. 2.2B, top panel, compare lanes 1 with 2, 3 with 4). Nevertheless, VSR did cause an increase of about three fold for both RNA1 and RNA1m (Fig. 2.2B, top panel, compare lane 1 with 3, 2 with 4, and data not shown). These results strongly suggest that Nb cells permitted the replication of BPMV RNA1. Furthermore, as shown in lanes 5 and 6 (top panel), RNA1 replication occurred in the absence of RNA2, thus was independent of RNA2.
By contrast, RNA2 did not replicate concomitant with RNA1 because RNA2-specific products were at similar levels regardless of the functionality of viral RdRP (Fig. 2.2B, middle panel, compare lane 1, in which RNA1 provided the functional RdRP, with lane 2, in which RNA1m provided nonfunctional RdRP). This conclusion is further supported by the fact that, in the presence of a VSR, RNA2 again accumulated to similar levels in co-infiltrations that included RNA1, RNA1m, or even the empty vector (Fig. 2.2B, middle panel, lanes 3, 4, and 7). Together, these results suggested BPMV RNA2 replication was either arrested, or significantly delayed. Thus, the selective depression of RNA2 replication could be the primary reason for the nonhost resistance to BPMV in Nb.

Selective interference with BPMV RNA2 replication is not caused by the lack of RNA1-coded replication proteins in the same Nb cells

In the experiments described above, the RNA1 and RNA2 constructs were delivered into the Nb cells through co-infiltration of two Agrobacterium strains, each permitting the expression of one of the two BPMV RNAs. Since all viral proteins needed for RNA replication are encoded by RNA1, RNA2 replication could occur only in cells that received both RNA1 and RNA2 constructs. Consequently, the failure to detect active RNA2 replication could have resulted from inefficient entry of both RNA1 and RNA2 constructs into the same cells. To resolve this concern, I modified the binary vector pCAMBIA1300 to create pAI101, which allowed for the insertion of both BPMV RNA1 and RNA2 cassettes into the same construct (please see Materials and Methods for construct details). In addition, to facilitate convenient visual detection, I replaced RNA2
with R2G, an RNA2-derivative that contained GFP coding sequence between MP and L-CP coding regions (Fig. 2.2A, Zhang et al., 2010). The R2G cassette was inserted into pAI101 in tandem with RNA1 or R1m cassettes to create RNA1-R2G and R1m-R2G, respectively (Fig. 2.3A).

I first tested both RNA1-R2G and R1m-R2G for their competence in launching viral infections in the host plants of BPMV by delivering the constructs into cotyledons of lima beans with particle bombardment (Hernandez-Garcia et al., 2010). As shown in Fig. 2.3B, bright green fluorescent foci became visible on cotyledons bombarded with RNA1-R2G as early as 24 hours after bombardment. These foci continued to expand in size and brightness throughout our observation period (100 hour after bombardment). Under an epifluorescence microscope, these foci consisted of multiple cells with similar fluorescence intensity, reflecting active movement of the GFP-producing entity, rather than passive diffusion of GFP (Fig. 2.3B, inset in top left panel). By contrast, no GFP foci were seen on cotyledons bombarded with R1m-R2G, confirming the inability of RNA1m to initiate BPMV replication in host cells (Fig. 2.3B, top right panel). Together these data illustrated that RNA1-R2G is fully capable of initiating BPMV replication and cell-to-cell movement in lima bean cotyledons.

Since the particular lima bean variety I used for particle bombardment (Henderson Bush) did not support systemic infection of BPMV, I sought to confirm the competence of RNA1-R2G-generated viruses to systemically infect soybean plants. To do this, I ground lima bean cotyledons bombarded with both the RNA1-R2G and R1m-R2G constructs and used the saps from these cotyledons to inoculate soybean plants. As shown
in the bottom two panels of Fig. 2.3B, the viruses produced by the RNA1-R2G construct in lima bean cotyledons were able to infect soybean plants systemically, resulting in bright GFP fluorescence in upper un-inoculated leaves of soybean plants at two weeks after inoculation. GFP fluorescence was not detected in control plants inoculated with sap of R1m-R2G-bombarded cotyledons, further demonstrating that the fluorescence is dependent on active virus replication and systemic spread. The RNA1-R2G-infected plants also developed typical BPMV symptoms and caused stunting in plants (data not shown). Furthermore, viral RNA was readily detected in RNA1-R2G-infected plants but not in R1m-R2G-infected plants (data not shown). These experiments confirmed that the RNA1-R2G construct was fully competent at initiating BPMV infection in its native hosts. Since the R2G cassette in R1m-R2G is identical to that in RNA1-R2G construct, I consider the R2G of both constructs as being capable of facilitating successful BPMV infection in host systems.

I then transformed both RNA1-R2G and R1m-R2G constructs into *Agrobacterium tumefaciens* (strain C58C1, Qu et al., 2003) and delivered the two constructs into *Nb* leaves with agro-infiltration. For infiltrations that included a VSR, I used p19 encoded by *Tomato bushy stunt virus* (TBSV) instead of TCV CP to rule out any specific effects attributable to TCV CP. Finally, in order to determine the replication of BPMV RNAs unambiguously, I used strand-specific RT-PCR to examine the accumulation levels of both (+) and (-) BPMV RNAs. As shown on Fig. 2.3C, top panel, while in these experiments the level of BPMV RNA1 originated from RNA1-R2G was only slightly higher than that from R1m-R2G in the absence of a VSR (lanes 1 and 2), it was greatly
elevated when the p19 VSR was present (compare lanes 3 and 4), again indicating that RNA1 replicated inside *Nb* cells. Likewise, the replication of RNA1 was not dependent on RNA2 as RNA1 alone resulted in a substantially higher levels of RNA1 accumulation than RNA1m (lanes 5 and 6) as long as the p19 VSR was present. The same RNA1 accumulation profile was detected for (-) RNA1 (Fig. 2.3C, fourth panel), further confirming the replication of BPMV RNA1 in *Nb* cells.

Most importantly, I corroborated the earlier finding concerning the replication of BPMV RNA2 in *Nb* cells. As I showed in Fig. 2.3B, the RNA2:GFP genome contained in RNA1-R2G and R1m-R2G constructs are fully capable of being replicated by RNA1-coded replication proteins in host plants of BPMV. Furthermore, the single construct format ensured that both RNA1 and RNA2 co-existed in the same cells. Nevertheless, same levels of RNA2 accumulated in cells containing RNA1-R2G and R1m-R2G, regardless of the presence of a VSR (Fig. 2.3C, second panel, compare lanes 1 and 2, 3 and 4). These data indicated that wildtype RdRP was just as powerless as the mutant RdRP at replicating RNA2 templates that are present in the same *Nb* cells. Therefore, the replication of BPMV RNA2 was selectively targeted in the nonhost *Nb*. The fact that (-) RNA2 showed the same accumulation pattern as (+) RNA2 suggests that this nonhost resistance targets RNA2 replication at an early step of viral RNA replication. Together these results establish a novel antiviral defense mechanism that selectively targets the replication of one of the genomic RNAs of a bipartite RNA virus.
The RNA2-coded polyprotein is properly processed in Nb cells

The discovery of selective inhibition of BPMV RNA2 replication by Nb prompted us to investigate which of the RNA1-coded proteins was prevented from interacting with RNA2 or RNA2-coded proteins. I first examined whether BPMV Pro could function in trans to process RNA2-coded polyprotein because the availability of the RNA2:GFP (R2G in both RNA1-R2G and R1m-R2G) expression cassette. As I showed in Fig. 2.3B, bright GFP fluorescence was detected in BPMV host plants, indicating the functionality of GFP. However, GFP needs to be properly processed from the RNA2-coded polyprotein in order to emit green fluorescence (Zhang and Ghabrial, 2006). Therefore, detection of GFP fluorescence signals proper processing of the RNA2-coded polyprotein by BPMV Pro in trans. I thus inspected the Nb leaves infiltrated with RNA1-R2G and R1m-R2G for GFP fluorescence. As shown on Fig. 2.4A, panels 1 and 2, no GFP fluorescence was detectable in the absence of the p19 VSR, consistent with the low levels of viral RNAs detected (Fig. 2.3C). However, faint GFP fluorescence was detected when either RNA1-R2G or R1m-R2G was co-infiltrated with the p19 VSR (Fig. 2.4A, panels 4 and 5). The intensity of GFP fluorescence was similar in both infiltrations, suggesting that: (i) Functional BPMV Pro could be produced from the non-replicating mutant RNA1; and (ii) the same Pro functioned in trans to process RNA2-coded polyprotein. Importantly, no GFP fluorescence was detected when RNA2:GFP was co-expressed with the VSR in the absence of RNA1 (Fig. 2.4A, panel 3), indicating that unprocessed RNA2:GFP polyprotein was unable to fluoresce. Consistent with these results, the processed GFP protein was detected in leaves 4 and 5 but not in leaves 1, 2, or 3 (Fig.
2.4B). I conclude from these findings that inability of RNA1-coded replication proteins to replicate RNA2 is not caused by the failure of Pro to process RNA2 polyprotein.
DISCUSSION

Nonhost resistance to plant viruses is an important yet less studied form of plant defense that could have very broad ramifications in the improvement of crop productivity. In the current study, I have utilized the BPMV-Nb nonhost system as a model to examine the mechanism of nonhost antiviral defense in plants and revealed that the replication of RNA2 of this bipartite RNA virus was selectively inhibited by Nb plants to thwart the reproduction process of BPMV in this plant. Unlike RNA1 of BPMV which encodes all proteins needed for viral RNA replication, RNA2 does not code for any replication-related protein and thus relies on RNA1-coded proteins for its replication. Our observation that RNA2 replication was selectively targeted in Nb cells suggests that these two genomic RNAs of BPMV are replicated through different mechanisms in permissive cells of host plants, and that this difference is seized upon by Nb cells to specifically prevent the replication of RNA2. Our investigation thus revealed an entirely novel antiviral defense mechanism by a nonhost plant.

While selective targeting of one of the genomic RNAs of a multi-partite virus by plant defense systems has not been reported before, it has been known that different genomic RNAs of multi-partite viruses access viral replication complexes (VRCs) via different molecular processes. For example, the RNA1 of Brome mosaic virus (BMV) and Red clover necrotic mosaic virus (RCNMV), have been proposed to enter VRCs through a translation-coupled mechanism because translation-defective BMV or RCNMV RNA1 failed to replicate (Yi and Kao, 2008; Iwakawa et al., 2011). On the other hand, other viral RNA(s) of these two viruses must possess additional specificity determinants.
in order to be replicated (Baumstark and Ahlquist, 2001; Chen et al., 2001; Iwakawa et al., 2011). Intriguingly, Yeh et al. (2000) observed that replication of RNA2 of the bipartite *Lettuce infectious yellow virus* (LIYV) was significantly delayed compared with its replicase-coding RNA1. Together these reports suggest that it may be a common feature of multi-partite RNA viruses to replicate different genomic RNA segments using different strategies. I reason that the replication of two genomic RNAs of BPMV likely also follows different paths. Our future directions would be to elucidate the replication strategies of BPMV RNA1 and RNA2 in its host plants, and to characterize the molecular events in *Nb* that subvert the replication of BPMV RNA2.
ACKNOWLEDGEMENT

I thank members of Feng Qu lab for frequent discussions and technical assistance, Dr. Lucy Stewart and other members of USDA Corn and Soybean Research Group for equipment sharing.
Figure 2.1 Replication-independent expression of BPMV coded proteins in Nb leaves. Binary constructs designed to express individual BPMV-coded proteins were delivered into Nb leaves with agro-infiltration. Protein samples extracted from the infiltrated leaves at 5 dai were separated on 11% SDS-PAGE gels, transferred to nitrocellulose membranes, and subjected to Western Blot (WB) with anti-HA antibody to reveal the presence of each protein. (A) Protein expression levels in the absence of a viral silencing suppressor (VSR, TCV CP in these experiments). (B) Protein levels in the presence of the TCV CP VSR.
Figure 2.2 Selective replication of BPMV RNA1 in Nb. (A) Schematic representation of binary constructs containing full length cDNAs of BPMV genomic RNAs and their derivatives. (B) Semi-quantitative RT-PCR evaluation of the relative accumulation levels of BPMV RNA1 and RNA2 in Nb leaves agro-infiltrated with the constructs shown on the top of the panels.
Figure 2.3 BPMV RNA2 replication failed to occur despite active RNA1 replication in the same Nb cells. (A) Constructs (RNA1-R2G and R1m-R2G) that ensure the delivery of both BPMV RNA1 and RNA2 into the same Nb cells. (B) Assessing the replication competence of RNA1-R2G and R1m-R2G in the host cells of BPMV. Top two panels: RNA1-R2G (left) and R1m-R2G (right) delivered to the cotyledons of lima bean with particle bombardment. Bottom two panels: Soybean plants infected with the saps of bombarded lima bean cotyledons. (C) Evaluation of the relative accumulation levels of BPMV RNA1 and 2 [both (+) and (-)] in agro-infiltrated Nb leaves with semi-quantitative RT-PCR.
Figure 2.4 BPMV RNA2-coded polyprotein was processed by RNA1-coded protease in a replication-independent manner in Nb. (A) Nb leaves infiltrated with constructs shown above each leaf. (B) Western blot (WB) analysis of the protein samples extracted from the leaves shown in A, showing the presence of processed GFP only in leaves that contained both RNA1 (R1m) and R2G.
CHAPTER 3. A STEM-LOOP STRUCTURE IN THE 5' UNTRANSLATED REGION OF BEAN POD MOTTLE VIRUS RNA2 IS SPECIFICALLY REQUIRED FOR RNA2 ACCUMULATION

This chapter is based on the following publication:

A stem-loop structure in the 5' untranslated region of Bean pod mottle virus RNA2 is specifically required for RNA2 accumulation. J Gen Virol. 94, 1415-1420.
ABSTRACT

*Bean pod mottle virus* (BPMV) is a bipartite, positive sense (+) RNA plant virus in the *Secoviridae* family. The RNA1 of this virus encodes all proteins needed for productive genome replication, hence is capable of replicate itself independent of RNA2. RNA2 on the other hand only encodes proteins needed for viral particle assembly and cell-to-cell movement, thus has to rely on RNA1 for replication. Exactly how RNA2 enlists RNA1-coded proteins for its replication is so far unknown. Here I report the identification of a stem-loop structure in RNA2 that is needed for RNA2 accumulation in host cells. To first determine if RNA2 replication relies on any *cis*-acting RNA element, I replaced the 5’ and 3’ untranslated regions (UTRs) of RNA2, separately as well as in combination, with their RNA1 counterparts, and evaluated the replication competence of the resulting mutants in both lima bean cotyledons and soybean plants. I observed that RNA2 replication was completely abolished by replacing its 5’ UTR with that of RNA1, revealing key *cis*-acting element(s) within RNA2 5’ UTR. I further mapped this *cis*-acting element to a 66 nt region immediately prior to the start AUG of RNA2 polyprotein. This element, referred to as stem-loop C (SLC), consists of a big, 15 base end loop, and a stem of 16 base pairs interspersed with one single-base bulge and one two-base mismatch. Additional dissection of SLC suggested that its functionality requires at least the middle portion of the stem to be base-paired. In addition, the two-base mismatch separating the middle and lower stem portions also appeared to be critical. In summary, I have identified the first *cis*-acting replication element in a secoviral RNA that
relies on a different genomic RNA segment for replication, and thus are well positioned to unveil additional molecular details of BPMV-plant interactions.
INTRODUCTION

The replication of viral genomes is a key step in the viral life cycle that depends on both virus-coded functions and a multitude of host elements (den Boon and Ahlquist, 2010; Nagy and Pogany, 2010). For positive sense (+) RNA viruses, this step is carried out primarily by RNA-dependent RNA polymerases (RdRPs) translated directly from viral genomic RNAs, via the synthesis of negative sense (-) replication intermediates. In addition, most (+) RNA viruses also encode a second protein, designated auxiliary protein (AP), that induces the proliferation and conformational changes of specific organellar membranes, forming protective enclosures that shield the replication process from the cellular environment (Schwartz et al., 2002). The complete membrane-based enclosure, consisting of viral RNA, RdRP, AP, as well as various host proteins recruited to assist in this process, is often referred to as the viral replication complex (VRC).

The question of how viral RNAs access VRCs has been extensively studied for a number of viruses. Many viral genomic RNAs are also mRNAs for the translation of AP, hence may be brought to VRCs by APs in a translation-coupled manner (Yi and Kao, 2008; Wang et al., 2009). Other viral RNAs have been found to encode specific secondary structures or stem-loops (SLs) that are recognized by AP or RdRP, which in turn carries the corresponding viral RNAs to VRCs. The latter mechanism is commonly used by viruses with multipartite genomes, as some of their genome segments encode functions unrelated to genome replication, thus have to rely on AP and RdRP encoded by other segments for their replication. This situation is best exemplified by Brome mosaic
virus (BMV) and Red clover necrotic mosaic virus (RCNMV). The genetic information of BMV is partitioned in three RNA segments, with RNA1 and 2 encoding 1a and 2a, two proteins required for genome replication (serving AP and RdRP functions, respectively). The smaller RNA3 encodes the cell-to-cell movement and capsid proteins (MP and CP). Accordingly, it has been long established that BMV RNA3 encodes a unique, cis-acting RNA structure that specifically interacts with 1a to dramatically enhance the replication of RNA3 (Baumstark and Ahlquist, 2001; and refs therein). Similarly, RCNMV RNA2, which encodes solely the viral MP, is recruited to VRCs through the specific interaction between a Y-shaped RNA structure in its 3’ untranslated region (UTR) and the RNA1-encoded p27, the RCNMV AP (Iwakawa et al., 2011).

Bean pod mottle virus (BPMV) is similar to RCNMV in that all viral proteins required for genome replication are encoded on RNA1 of its bipartite (+) RNA genome. In fact, the RNA1 of BPMV is known to replicate itself in single cells even in the absence of RNA2 (Gu and Ghabrial, 2005; Lin et al., unpublished). However, unlike RCNMV, each of the two BPMV genomic RNAs encodes one single polyprotein, from which multiple mature viral proteins are derived through post-translational processing by the virus-coded protease (Pro) (Gu and Ghabrial, 2005). Specifically, the RNA1-coded polyprotein is the precursor for five proteins: a putative protease co-factor (C-Pro), a putative RNA helicase (HEL) and possibly an AP, a small protein that covalently binds to the 5’ end of the viral genomic RNAs (viral protein genome-linked or VPg), Pro, and the viral RdRP (Fig. 3.1A). Notably, translation of BPMV RNA2 can initiate at two different start codons, leading to the production of two polyproteins that differ only at their N-
termi (MacFarlane et al., 1991; Fig. 3.1A). The larger polyprotein is thought to be processed into three mature proteins: an N-terminal 58K protein (p58K) with unknown functions, and two CP subunits (L-CP and S-CP. Fig. 3.1A), whereas the smaller one produces a smaller N-terminal mature protein that serves as the viral MP (Fig. 3.1A).

BPMV is a member of the comovirus genus in the *Comovirinae* subfamily of *Secoviridae*. *Secoviridae* is a plant virus family that contains a number of economically important viruses, including Rice tungro spherical virus, Maize chlorotic dwarf virus, and Cowpea mosaic virus (CPMV) (Sanfacon et al., 2009). Although viruses of *Secoviridae* and *Potyviridae* share similar coding strategies, they differ from each other in that secoviruses have icosahedral particle structures, two or more different capsid protein subunits, and an unusually small VPg. These and other attributes align secoviruses more closely with the animal-infecting viruses of *Picornavidae*, making them promising models for the more experimentally restrictive picornaviruses (Le Gall et al., 2008). However, despite their significance in agriculture and their potential in aiding human health research, our knowledge of the replication mechanisms of secoviruses remains rudimentary.

I initially became interested in BPMV because it is among the most important viral pathogens of soybean in the United States. To begin to understand the replication mechanisms of BPMV, I chose to first focus on the replication requirements of BPMV RNA2, and attempted to identify *cis*-acting RNA sequences or structures within RNA2 that are specifically needed for its own accumulation. Our results showed that while the 3’UTR of BPMV RNA2 can be replaced by its RNA1 counterpart without compromising
the viral infectivity, its 5’ UTR contains an important *cis*-acting element. I further
delineated this structural element to a 50 nucleotide (nt) stem-loop designated as SLC.
Importantly, our preliminary examination with site-directed mutagenesis suggests that the
maintenance of at least the middle portion of stem, but not the actual nt sequence of the
two stem strands, is critical for the function of SLC. Our result uncovers a *cis*-acting
RNA element in BPMV RNA2 that is needed for its own replication, and hence the
productive infection of BPMV. I speculate that this RNA structure plays an essential role
in shepherding RNA2 into VRCs programmed by RNA1-coded proteins.
MATERIALS AND METHODS

Constructs

The infectious cDNA of BPMV RNA1 was derived from a Kentucky isolate (K-Ho1) (Gu and Ghabrial, 2005). The replication-defective mutant of RNA1 cDNA (RNA1m) was created by changing the highly conserved GDD motif to AAH. A BsrBI site was introduced along with this change, allowing the identification of the mutant in mixed infections. A modified version of BPMV RNA2, which was derived from the IA-Di1 isolate and contained a GFP cDNA insert between MP and L-CP, was kindly provided to us by Dr. Steve Whitham (Zhang et al., 2010). This modified RNA2 cDNA was renamed R2G in the current study. The RNA1, RNA1m, and R2G full-length cDNAs were cloned into a modified version of pRTL2, with the BPMV inserts flanked by the 35S promoter (P35S) and terminator (T35S) of CaMV.

For the construction of R2G-1U5, RNA1 5’UTR was first PCR amplified from RNA1 cDNA with primers BPMV-R1/R2-1F (Xhol) (5’GAAGAACTCGAGTATTTAAATTTT-CATAAGATTTGAAATTTTGA3’) and BPMV-1U5-2gC-R (5’GAGAAAAATTAACGAAGAAACATGTTGTTTTCAAATGGAA 3’), which attaches the first 21 nt of the R2G coding sequence to the 3’ end of RNA1 5’ UTR. Simultaneously, the first 430 bp of R2G coding sequence was PCR amplified with primers BPMV-1U5-2gC-F (5’TGAAAAACACATGTTT-GCTTCGTTAATTTTCTCTG3’) and R2G-904R (5’GAAGAAGTCGACGAATACCAACTTG-TCAACAGCAGGAA3’). These two PCR fragments, with a 35 nt overlap, were then mixed and served as the template for a
bridging PCR with priemrs RNA1-1F(XhoI) and R2G-904R, leading to a chimeric fragment which combined RNA1 5’UTR and the first 430bp of R2G coding sequences. This fragment was then digested with XhoI and SacI (a unique restriction site at nt 822-827 of R2G), and used to replace the corresponding fragment in R2G XhoI and SacI. The R2G-1U3, R2G-1U5/2U5 were produced using essentially the same strategy (the sequences of DNA oligos are available upon request). The deletion and site-directed mutagenesis were likewise carried out using similar approaches. All mutant constructs were sequenced to confirm their identity before being used in experiments.

**Particle bombardment of lima bean cotyledons**

Lima beans of the Anderson Bush variety were purchased from Earl May Seed and Nursery (Shenandoah, IA). The bombardment experiments were carried out following the procedure described by Hernandez-Garcia et al. (2010).

**Inoculation of soybean seedlings**

The bombarded lima bean cotyledon was grinded in 1 ml inoculation buffer (0.01M sodium phosphate, pH7.0, 1% celite). The resultant extract was rub-inoculated on the first foliage leaves of one-week old soybean plants. Soybean plants were maintained under greenhouse conditions after inoculation.
Strand-specific RT-PCR

Total RNA were extracted from lima bean cotyledons 48 hours post bombardment using the protocol described by Louime and colleagues (2008), and from soybean leaves with the TRIsure reagent (Bioline, Taunton, MA). The quality and concentration of the isolated RNA was verified using NanoDrop ND-1000 (Thermo Fisher Scientific Inc.) and gel electrophoresis. Approximately 2 µg RNA per sample was treated with TURBO DNA-free DNase (Ambion, Austin, TX) according to manufacturer’s instruction. (-) strand-specific reverse transcription was carried out by using a forward primer [BPMV-R1/R2-1F (XhoI)], and reverse transcriptase purchased from Clontech (Palo Alto, CA), with 1 µg RNA per sample as the template. Subsequently, 1 µl cDNA per sample was used for PCR. PCR was carried out using the EconoGreen PCR Master Mix (Lucigen, Middleton, WI) with appropriate primers (the sequences of DNA oligos provided upon request). As controls, the actin mRNA of lima bean (PlACT) and soybean (GmACT) was also reverse-transcribed and PCR amplified in parallel with BPMV RNA2, with the following primers: PlACT-F (5’ GTTTCCAGCATTGTAGGTCGCTCT 3’), PlACT-R (5’ CTGACACCATCACCAGATCCAACA 3’), GmACT-F (5’GTAGTTGTTATGGGCCAGAAAG3’), and GmACT-R (5’CACCATCCCCAGAATCCAACACA3’).
RESULTS

A powerful experimental system for examining the replication and spread of BPMV

In order to identify cis-acting RNA elements functioning in the replication and spread of BPMV, I needed a reliable experimental system that allows us to monitor the accumulation of viral RNAs at both the single cell and the intact plant levels. The initial difficulty of developing a protoplast system amenable to BPMV infections prompted us to adopt an alternative approach to assess BPMV infectivity. In this new approach, I placed the cDNAs of BPMV RNA1 and RNA2 between the 35S promoter and terminator (P35S and T35S; Fig. 3.1A) of *Cauliflower mosaic virus*. Consequently, upon delivery of the resulting constructs into BPMV host cells, viral infection can be launched with RNAs transcribed intra-cellularly from the cDNAs, thus bypassing the need for producing *in vitro* transcripts. Next, to facilitate sensitive and convenient tracking of the infection process, I used as the surrogate for RNA2 an RNA2 derivative that contained a GFP insert between MP and L-CP (RNA2-GFP or R2G; Fig. 3.1A), which was shown previously to replicate to similar levels as wild-type RNA2 (Zhang et al., 2010). As a negative control, I generated a defective RNA1 cDNA, referred to as RNA1m (Fig. 3.1A), which encodes a nonfunctional RdRP due to the replacement of the highly conserved glycine-aspartic acid-aspartic acid (GDD) motif with alanine-alanine-histidine (AAH; Fig. 3.1A). Finally, I used particle bombardment to deliver these constructs into lima bean cotyledons, which were shown to be well suited for particle bombardment delivery of plasmid DNA (Hernandez-Garcia et al., 2012).
As shown in Fig. 3.1B, bombardment of lima bean cotyledons with RNA1 and R2G, but not with RNA1m and R2G, resulted in rigorous BPMV replication and cell-to-cell movement as indicated by brightly green fluorescent infection foci (Fig. 3.1B, top row, second and third panels). These infection foci cover multiple cells, as revealed by further inspection with epifluorescence microscope (data not shown). Additionally, extracts of the BPMV-positive cotyledons were highly infectious to soybean plants, leading to severe BPMV symptoms and bright GFP fluorescence in the systemically infected leaves (Fig. 3.1B, second row, second and third panels; and data not shown). The identity of R2G in both lima bean cotyledons and systemically infected soybean leaves were further confirmed with strand-specific reverse transcription-polymerase chain reaction (RT-PCR; Fig. 3.1C and 3.1D, lanes 2 and 3). In summary, these results demonstrated that our new approach permitted the observation of BPMV infection from single cell levels to whole plants, hence is suited for our study.

5’ UTR of BPMV RNA2 contains cis-acting RNA elements essential for its replication

Having established a robust system for the assessment of BPMV infectivity, I next examined the role of both 5’ and 3’ UTRs of BPMV RNA2 in RNA2 replication. To this end, I generated three R2G mutants in which the 5’ UTR, 3’ UTR, or both were replaced by their counterparts in RNA1, resulting in constructs R2G-1U5, -1U3, and -1U5/1U3, respectively (Fig. 3.1A). These constructs were then bombarded into lima bean cotyledons together with RNA1, and the bombarded cotyledons were observed on a daily
basis using a fluorescent dissecting microscope. As shown in Fig. 3.1B, while replacing the 3’ UTR of RNA2 with that of RNA1 exerted no detectable impact on the infectivity of the virus in both bombarded lima bean cotyledons and systemically infected soybeans (Fig. 3.1B, third row, first panel), doing the same with 5’ UTRs led to a complete loss of BPMV infectivity in both types of plants, as GFP fluorescence was undetectable in R2G-1U5-treated lima bean cotyledons even in single cells (Fig. 3.1B, first row, last panel; data not shown). Accordingly, replacing both UTRs also caused the loss of viral infectivity (Fig. 3.1B, third row, second panel). Since wild-type RNA1, which is known to replicate independently in single cells, was used to assist the replication of R2G mutants, the failure of R2G-1U5 to accumulate suggests that the 5’ UTR of RNA2 contained critical cis-acting element(s) essential for RNA2 replication.

To further delineate the RNA2-specific cis-acting RNA elements, I sought to make additional modifications within RNA2 5’ UTR. Pairwise comparison revealed that the first 262 nt of RNA1 and RNA2 5’ UTRs share a very high level of sequence identity (at least 91%), whereas the sequences after nt position 263 are much more divergent (Lin et al., data not shown). I first determined whether the few differences within the first 262 nt could affect the replication of RNA2 by substituting this portion of RNA2 5’ UTR for its RNA1 counterpart. The resulting construct, referred to as R2G-1U5 (262), was similarly competent as the wild-type R2G in both lima bean cotyledons and soybean plants (Fig. 3.1B, third panel in third and fourth rows; Fig. 3.1 C and D, lane 7). This result demonstrated that the first 262 nt of RNA1 and RNA2 5’ UTRs are interchangeable and thus do not contain cis-acting elements unique for RNA2. Nevertheless, this section of 5’
UTR contains essential \textit{cis}-elements shared by RNA1 and RNA2, as its deletion from R2G led to a complete loss of BPMV infectivity (Fig. 3.1B, third row, last panel). Together these results indicated that RNA2-specific \textit{cis}-acting structure does not reside in the first 262 nt of RNA2 5’ UTR.

A 66 nt region in the BPMV RNA2 5’ UTR contains an RNA2-specific \textit{cis}-acting element required for RNA2 accumulation

Having determined that the first 262 nt of the RNA2 5’ UTR was not uniquely required for RNA2 accumulation in host cells, I then moved to interrogate the rest of the 5’ UTR sequence (nt no. 263-466) for a potential role in RNA2 replication. Since most known \textit{cis}-acting RNA elements involve stretches of RNA sequences that fold into various forms of secondary structures, I first needed to determine the boundaries of these elements. To do that, I inserted a 36 nt non-BPMV sequence (termed “ha” as the majority of its sequence was derived the HA epitope tag cDNA; see Materials and Methods for details) between nt positions 466 and 467, and 263 and 264, to create constructs R2G-466ha and R2G-263ha, respectively (Fig. 3.2A). When delivered to lima bean cotyledons and subsequently soybean plants to examine their infectivity, both of them resulted in productive infections indistinguishable from wild-type R2G (Fig. 3.2C, top row, last panel; and third row, first panel). I conclude from these results that the potential RNA2-specific, \textit{cis}-acting element(s) must reside within the region from nt 263 to 466, and that the element(s) do not span across the boundaries at either ends.
I then further delimited the putative *cis*-acting element by creating deletion mutations within this region. To help guide the deletion mutagenesis, I used the Mfold algorithm to predict the RNA secondary structure of this region. As illustrated in Fig. 3.2B, this region could potentially fold into three stem-loops (SLs), referred to as SLA, SLB, and SLC, separated by unstructured sections of varying lengths. I thus generated three different deletion mutants of R2G, namely ΔSLA, ΔSLB and ΔSLC, each removing one of the SLs together with a few flanking nts (Fig. 3.2A). Upon delivery into lima bean cotyledons together with RNA1 via particle bombardment and subsequent passage into soybean seedlings, R2G-ΔSLA and R2G-ΔSLB caused rigorous BPMV infections essentially identical to wild-type R2G (Fig. 3.2C, third and fourth rows, second and third panels). By contrast, R2G-ΔSLC was unable to cause any detectable infection even at the single cell level (Fig. 3.2C, third row, last panel; and data not shown). These results were further confirmed with strand-specific RT-PCR detection of fragments of expected size (Fig. 3.2D and 3.2E). Collectively they strongly suggest that SLC constitutes a key *cis*-acting element for RNA2 replication, while the region covered by SLA and SLB are dispensable for productive BPMV infections.

**Maintaining the integrity of the stem of SLC is critical for its function**

With the identification of SLC as a key *cis*-acting structure of BPMV RNA2, I initiated a preliminary study to begin to understand the structural requirements of SLC. I chose the middle section of the stem for this investigation because this section contains the longest double-stranded stretch of SLC, with seven undisrupted base pairs (Fig.
3.3A). I first tried to disrupt the base pairs by changing four nts on the left arm of the stem to their complements on the right (UUCA to gggu), creating mutRR (Fig. 3.3A). A reciprocal mutLL mutant was similarly created by replacing UGGG on the right with acuu (Fig. 3.3A). MFold prediction suggested that the original SLC structure was severely disrupted in both mutants (Fig. 3.3A). In agreement with a critical importance of maintaining the stem, both mutants completely lost the ability to infect lima bean cotyledons (Fig. 3.3B, top row, last panel, and third row, first panel; and 3.3C, lanes 4 and 5). However, when the two mutations were combined to create mutRL in which the stem structure, but not its original sequence, was restored, wild-type infectivity was observed (Fig. 3.3B, third and fourth rows, middle panels; and Fig. 3.3C and 3.3D, lanes 6). These results indicated that the integrity of the middle section of the stem, and possibly the stem-loop structure as presented in Fig. 3.3A, is critical for the function of SLC.

I then examined the importance of the bottom part of the SLC stem consisting of one C-U mismatch and six base pairs (Fig. 3.3A). I disrupted this portion of structure by inserting four nts (aucc) after nt no. 461, hence creating a BamHI site in the corresponding cDNA. This mutant, referred to as R2G-460BamHI, completely abolished the BPMV infectivity (Fig. 3.3B, third row, last panel; and Fig. 3.3C, lane 7). It should be noted that the upper two thirds of SLC remained essentially undisturbed in this mutant. In addition, the six base pairs at the bottom of the stem also remained intact despite some changes in nt identity (Fig. 3.3A). Therefore, either the two base (C-U) mismatch needs to be faithfully preserved, or the identity of certain nts within this section of SLC is
critically important. In summary, our results identified SLC as a unique \textit{cis}-acting RNA structure required for RNA2 replication in the host cells of BPMV.

**All viable R2G mutants were genetically stable for at least three weeks**

To confirm the genetic stability of various viable R2G mutants in lima bean cotyledons as well as soybean plants, and to rule out the possibility of contamination by wild-type R2G, total RNA samples were extracted from soybean leaves systemically infected with R2G-1U3, -1U5 (262), -466ha, -263ha, -ΔSLA, -ΔSLB and -mutRL at three weeks after inoculation, and subjected to RT-PCR with primers that flank the respective mutations (data not shown). The amplified PCR products were then sequenced. I found no additional nucleotide changes other than the intended mutations in any of the mutants (data not shown). Therefore, mutations engineered in all of the viable R2G mutants appear to be genetically stable for the duration of our experiments, and do not appreciably affect the infectivity of BPMV.
DISCUSSION

BPMV is a member of the newly established virus family *Secoviridae* (Sanfacon et al., 2009). Despite of the economical importance of a number of viruses belonging to this family, as well as their potential to serve as model systems for human pathogenic picornaviruses, the molecular mechanisms that govern the replication of secoviral genomes have not been thoroughly investigated. In this report, I used a novel integrated approach to follow the replication and spread of BPMV from individual infected cells to systemically infected plants. Combined with carefully designed mutagenesis, this new approach allowed us to identify a stem-loop structure (SLC) within the 5’ UTR of BPMV RNA2 that is needed for the accumulation of RNA2 in infected cells. I further established that the maintenance of the middle portion of the stem, but not the identity of nts involved, is essential for the function of SLC. Additionally, the preservation of the two -base mismatch immediately below the middle portion of the stem is likely also critical.

BPMV is a bipartite (+) RNA virus that encodes all replication-related proteins on RNA1. Thus, SLC of BPMV RNA2 represents a new cis-acting element in a viral RNA segment that relies on a different genome segment for its replication (Baumstark and Ahlquist, 2001; Iwakawa et al., 2011).

Our study reveals the first RNA2-specific cis-acting element in a *Secoviridae* member. As discussed in Introduction, similar elements have been identified in BMV and RCNMV, two well studied multi-partite (+) RNA viruses that adopt a coding strategy different from BPMV and other *Secoviridae* members (Baumstark and Ahlquist, 2001; Iwakawa et al., 2011). To the best of our knowledge, such elements have not been
identified in other *Secoviridae* members. Although CPMV, another *Secoviridae* member with a similar genome organization as BPMV, has been subjected to exchanges of UTRs in a manner similar to our current study, no RNA2-specific *cis*-acting element was identified as both 5’ and 3’ UTRs of CPMV RNA2 could be replaced by their RNA1 counterparts without affecting the replication of RNA2 (van Bokhoven et al., 1993; Rohll et al., 1993). I speculate that CPMV RNA2 could possess a *cis*-acting element within its polyprotein-coding sequence. Alternatively, CPMV RNA2 could be brought to VRCs using a mechanism that does not rely on a *cis*-acting RNA element. It is worth noting that while BPMV RNA2 has a substantially longer 5’ UTR than RNA1, and shares significant sequence homology with RNA1 for the first 260 nts, CPMV RNA2 actually has a shorter 5’ UTR than RNA1, and does not share significant homology with RNA1 within this region (Lin et al., data not shown). Our data suggest that although BPMV and CPMV share similar genome organizations, they may have evolved different mechanisms for RNA2 replication.

Importantly, the conservation of SLC structure was also confirmed with phylogenetic analysis. As shown in Fig. 3.4, among the nine BPMV isolates for which RNA2 sequences are available through GenBank, six of them contained the exact same sequence as the isolate I examined (IA-Di1). The other three isolates (two KY, one OH), while containing a few nt variations, maintained the same SL structure (Fig. 3.4). I also note that the BPMV SLC is structurally similar to many of the *cis*-replication elements (CREs) present in *Picornavidae* viruses (Steil and Barton, 2009). While it remains to be determined how SLC ensures the accumulation of BPMV RNA2, the fact that SLC
mutants were unable to accumulate even in single cells implies that SLC might help deliver RNA2 to RNA1-programmed VRCs through direct or indirect interactions with AP(s) encoded by RNA1. However, other possibilities, such as enhancing the translation or stability of RNA2, cannot be completely ruled out. Our next goal is to test this model by evaluating the potential interactions between SLC and RNA1-coded proteins, and elucidating the intra-cellular process through which RNA2 associates with VRCs.
ACKNOWLEDGEMENTS

I greatly appreciate the generosity of Drs. Steve Whitham and John Hill at Iowa State University in providing us with the RNA2-GFP construct. I thank members of the Qu and Finer labs for stimulating discussions and technical assistances, Dr. Lucy Stewart and other members of USDA, ARS Corn and Soybean Research Unit for sharing equipment. This study was supported in part by an OARDC Graduate Seed award to me. Soybean virus-related research in Qu lab has been supported in part by grants from North Central Soybean Research Program and Ohio Soybean Council. I gratefully acknowledge Dr. Andy White for his critical reading of the manuscript, and numerous suggestions.
Figure 3.1 RNA2 5’ UTR contains key cis-acting elements required for RNA2 accumulation. (A) Schematic representation of BPMV RNA1 and RNA2 (R2G) constructs used in this study, together with RNA1m, a nonreplicating RNA1 mutant, and a number of R2G mutant with altered 5’ or 3’ UTRs. A construct that combines the changes in R2G-1U5 and -1U3 was also generated by not shown in the diagram. (B) Infectivity of mutant constructs assessed with particle-bombarded lima bean cotyledons and subsequently rub-inoculated soybean plants. Constructs tested are indicated above the respective panels. The images of GFP fluorescent cotyledons were taken 48 hour post bombardment (hpb), whereas those of systemically infected soybean leaves taken 2 week post inoculation (wpi). (C) Verification of active replication in lima bean cotyledons with (-) RNA2-specific RT-PCR. A RT-PCR product of a lima bean actin mRNA was used as controls. (D) Verification of active replication in systemically infected soybean leaves with (-) RNA2-specific RT-PCR. A RT-PCR product of a soybean actin mRNA was used as controls.
Figure 3.2 Identification of SLC as a cis-acting element essential for RNA2 accumulation. (A) Schematic representation of R2G mutants that contained various forms of insertions/deletions within the second half of 5’ UTR of RNA2 (nt position 263-466). (B) RNA secondary structure of the nt 263-466 region predicted using the MFold algorithm. The three putative stem-loop structures are referred to as SLA, SLB, and SLC, respectively. (C) Infectivity of mutant constructs assessed with particle-bombarded lima bean cotyledons and subsequently rub-inoculated soybean plants. Mutants tested are indicated above the respective panels. Images were recorded in a manner similar to those in Fig. 3.1B. (D) and (E) Verification of active replication in lima bean cotyledons and systemically infected soybean leaves with (-) RNA2-specific RT-PCR.
Figure 3.3 The functionality of SLC depends on the integrity of the central portion of the stem. (A) Predicted secondary structures of mutRR, mutLL, mutRL, and 460BamHI. The altered nts in each mutant are in lower case letters. Detailed description of the mutants is provided in the main text. (B) Infectivity of mutant constructs assessed with particle-bombarded lima bean cotyledons and subsequently rub-inoculated soybean plants. Mutants tested are indicated above the respective panels. (C) and (D) Verification of active replication in lima bean cotyledons and systemically infected soybean leaves with (-) RNA2-specific RT-PCR.
Figure 3.4 Conservation of SLC structure in RNA2 of various BPMV isolates. The SLC element was identified from an Iowa (IA-Di1) isolate, and shared with 100% identity by five additional isolates. Limited nt variations found in two Kentucky (KY) isolates and one Ohio (OH) isolate, none of which caused significant changes in the overall structure.
CHAPTER 4. THE BEAN POD MOTTLE VIRUS RNA2-ENCODED 58K PROTEIN IS REQUIRED IN CIS FOR RNA2 ACCUMULATION

This chapter has been submitted to Journal of Virology for publication:
ABSTRACT

_Bean pod mottle virus_ (BPMV) is a bipartite, positive sense (+) RNA plant virus in the _Secoviridae_ family. Its RNA1 encodes proteins required for genome replication, whereas RNA2 primarily encodes proteins needed for virion assembly and cell-to-cell movement. Although RNA2 has to rely on RNA1 for replication, it is not known whether RNA2 encodes any proteins that interact with RNA1-encoded proteins to facilitate its RNA2 reproduction. Incidentally, the function of a 58 kilo-dalton protein (P58) encoded by RNA2 has yet to be resolved. P58 and the movement protein (MP) of BPMV are two largely identical proteins resulting from translational initiations at two different in-frame start codons on RNA2, with P58 being 102 amino acids longer than MP at the N-terminus. In this report, I revealed a unique role for P58 by showing that RNA2 accumulation in infected cells was abolished when the start codon of P58 was mutated. This role of P58 is independent of MP as RNA2 was abundantly detectable in single cells when most of the region shared by P58 and MP was removed. Importantly, the function of P58 required the P58 protein, rather than its coding RNA, as compensatory mutations could be isolated that restored RNA2 accumulation by acquiring new start codons upstream of the original one. Most significantly, loss of P58 function could not be complemented by P58 provided _in trans_, thus suggesting that P58 functioned _in cis_ to facilitate the accumulation of the same RNA2 that served as its mRNA. Finally, I illustrated that all RNA1-encoded proteins are _cis_-acting relative to RNA1. Together, the results suggest that P58 functions by recruiting RNA1-encoded polyprotein to RNA2 to enable RNA2 reproduction.
INTRODUCTION

The replication process of positive sense (+) RNA viruses involves both virus-coded functions and host factors (den Boon and Ahlquist, 2010; Nagy and Pogany, 2010; Nagy et al., 2012). Most (+) RNA plant viruses encode at least two proteins that together with viral RNA form the core of viral replication complex (VRC). Typically multiple copies of a more abundantly expressed viral protein, commonly referred to as auxiliary protein (AP), interact with a certain type of membrane-based organelle to induce the formation of partially enclosed spherules that house viral genomic RNA (gRNA), another virus-encoded protein with RNA-dependent RNA polymerase activity (RdRP), as well as various host proteins (Schwartz et al., 2002; Nagy and Pogany, 2010). The replication of viral genome then takes place inside the protective spherules, or VRCs.

(+) RNA plant viruses with multipartite genomes have proven to be intriguing models for examining replication mechanisms of (+) RNA viruses (Mine and Okuno, 2012). Indeed, two of the best studied (+) RNA viruses, *Brome mosaic virus* (BMV) and *Red clover necrotic mosaic virus* (RCNMV), partition their genetic information in multiple genomic RNA segments. This partition poses interesting challenges for the gRNA segments than encode no or only part of the VRC constituents. For example, both RNA3 of BMV and RNA2 of RCNMV encode neither AP nor RdRP, thus must encode the capacity to recruit these proteins for their own replication. In both of these cases, a specific RNA structural element has been identified that interacts with the AP of corresponding viruses to facilitate the entry of VRCs by these RNAs (Baumstark and Ahlquist, 2001; Iwakawa et al., 2011).
It should be noted that for both BMV and RCNMV, functional viral proteins are translated from viral gRNAs or subgenomic RNAs (sgRNAs) as final translation products. An alternative gene expression strategy used by many other viruses, including Bean pod mottle virus (BPMV) of genus Comovirus, family Secoviridae, is to pack all coding capacities into one large polyprotein per genome segment. These polyproteins are subsequently proteolytically processed to release functional proteins. Similar to RCNMV, BPMV is a bipartite (+) RNA virus, with its RNA1 encoding proteins required for genome replication, and RNA2 encoding proteins required for virion assembly and cell-to-cell movement (Gu and Ghabrial, 2005; Fig. 4.1). However, unlike RCNMV, functional BPMV proteins are produced through the processing of polyproteins translated from the two gRNAs by the viral protease (Pro) (MacFarlane et al., 1991). Given the significant difference in the gene expression strategy, an as-yet unresolved question is how RNA2 of BPMV and other comoviruses gain access to RNA1-encoded replication proteins.

The current study set out to resolve this question using BPMV as a model. Like all comoviruses, BPMV RNA1 encodes one polyprotein which is thought to be processed into at least five proteins: a putative protease co-factor (C-Pro), a putative RNA helicase (Hel), a small virus-coded protein (VPg) that binds to both genomic RNA segments at the 5’ termini, Pro, and the viral RdRP (Fig. 4.1A, Gu and Ghabrial, 2005). Strikingly, BPMV RNA2 actually encodes two, albeit largely overlapping, polyproteins, as its translation can initiate from two separate, in-frame AUG codons [at nucleotides (nt) positions 467 and 773 of RNA2; Fig. 4.1A]. These two polyproteins are hence mostly
identical, differing only at their N-termini (Fig. 4.1A). Both polyproteins are processed twice at the same sites to produce four different mature proteins: the larger N-terminal protein of 58 kilodalton (kDa), designated P58, with unknown function(s); the smaller N-terminal protein which is the movement protein (MP); and two capsid protein subunits (L-CP and S-CP; Fig. 4.1A). Since BPMV RNA2 does not encode any known replication-related protein, it primarily depends on RNA1-encoded proteins for its own replication.

In a previous study, I have succeeded in identifying a cis-acting RNA element in BPMV RNA2 using a particle bombardment-based experimental system (Lin et al., 2013). In the current study, I decided to take advantage of this system to resolve whether RNA2 encodes any proteins critical to RNA2 replication. I focused our primary attention on P58 because it was the only RNA2-encoded proteins with undefined function(s). Furthermore, there is some preliminary evidence that suggests the P58 counterpart of Cowpea mosaic virus (CPMV), a BPMV relative, might be necessary for CPMV RNA2 replication (van Bokhoven et al., 1993). Separately, RNA2 of Grapevine fanleaf nepovirus (GFLV), a virus more distantly related to BPMV, encodes an N-terminally located protein (2A) that was implicated in RNA2 replication (Gaire et al., 1999). Our results showed that eliminating the P58 coding capacity rendered RNA2 undetectable in BPMV host cells, and that the N-terminal 14 kDa portion of P58 was sufficient to preserve this activity. I further inferred that the translation of P58, rather than the RNA sequence coding for P58, is necessary for its function as compensatory mutations that restored BPMV infectivity always introduced alternative start codons for P58 at various
upstream positions. Mostly importantly, I determined that P58 functioned in a \textit{cis}-acting manner relative to its coding RNA, as P58 provided in \textit{trans} were unable to complement its defect. Finally, I also established that all RNA1-coded proteins were \textit{cis}-acting relative to RNA1. Together, these results strongly suggest that P58 functions to recruit RNA1-encoded polyprotein to RNA2 to facilitate RNA2 replication.
MATERIALS AND METHODS

Constructs

The infectious cDNA of BPMV RNA1 used in this study was derived from a Kentucky isolate (K-Ho1) of BPMV, and kindly provided to us by Dr. Said Ghabrial (Gu and Ghabrial, 2005). The RNA2 cDNA was originally derived from an Iowa isolate (IA-Di1), and was subsequently modified by Zhang and colleagues (2010) to include a GFP cDNA insert between MP and L-CP. The resulting construct, designated RNA2-GFP (R2G), was a kind gift from Dr. Steve Whitham. RNA1 and R2G cDNAs were subcloned into pRTL2 to acquire the 35S promoter and terminator (P35S and T35S) of Cauliflower mosaic virus (CaMV) using previously described procedures (Qu et al., 2003; Lin et al., 2013). Constructs containing both RNA1 and (RNA1m) and R2G cassettes were also produced for some experiments. All mutants were derivatives of RNA1 or R2G constructs. Sequences of primers used to produce the mutants are available upon request.

All mutants were generated by first producing mutation-containing PCR fragments with two-step PCR, and subsequently inserting the PCR fragments into the respective viral cDNA using appropriate restriction enzyme sites. For example, to produce R2G-mMP (Fig. 4.1A), two overlapping PCR fragments were first synthesized. The first fragment, encompassing nucleotide #1-800 of RNA2, incorporated the desired ATG→CCT mutation at nt #773-775 at its 3’ end. Conversely, the second fragment, covering nt #750-904 of RNA2, incorporated the same mutation at its 5’ end. These two fragments were then used as the template to produce a mutation-containing fragment spanning nt #1-904, which also contained an XhoI site prior to nt #1, and a unique SacI
site inherent to RNA2 cDNA at nt #822-827. This fragment was then digested with XhoI and SacI, and used to replace its counterpart in wild-type R2G cDNA. Wherever possible, a new restriction enzyme site was created at or near the mutation site to facilitate convenient screening of candidate clones. The identity of all mutants was all confirmed by sequencing before being used in our experiments. A slight variation of this strategy was used to produce R2G-CPMV14K, in that three instead of two fragments were first produced and served as the template for the secondary PCR, with the middle fragment substituting CPMV 14K for BPMV 12K.

For all RNA1 mutants, the conserved amino acid (aa) residues in each of the proteins were first identified through multiple alignments of at least six different comoviruses and nepoviruses. These conserved aas, shown on Fig. 4.4A, were then mutated using procedures as described above. In addition, two constructs that permit replication-independent expression of P58 and MP were produced by inserting their respective coding sequences into a modified version of pRTL2 (designated pRTL4i) that attaches a double HA tag to the C-termini of these proteins. The expression of both proteins was first confirmed in Nicotiana benthamiana leaves before being used in particle bombardment experiments (data not shown).

**Particle bombardment of lima bean cotyledons**

Lima beans of the Henderson Bush variety were purchased from Earl May Seed and Nursery (Shenandoah, IA). The bombardment experiments were carried out following the procedure described by Hernandez-Garcia et al. (2010).
Inoculation of soybean seedlings

The bombarded lima bean cotyledon was ground in 1 ml inoculation buffer (0.01M sodium phosphate, pH7.0, 1% celite). The resultant extract was rub-inoculated on the first foliage leaves of one-week old soybean plants. Soybean plants were maintained under greenhouse conditions after inoculation.

Negative (-) strand specific, RT-PCR

Total RNA were extracted from lima bean cotyledons 48 hours post bombardment using the protocol described by Louime and colleagues (2008). The quality and concentration of the isolated RNA was verified using NanoDrop ND-1000 (Thermo Fisher Scientific Inc.) and gel electrophoresis. Approximately 2 µg RNA per sample was treated with TURBO DNA-free DNase (Ambion, Austin, TX) according to manufacturer’s instruction. (-) strand-specific reverse transcription was carried out by using a primer with its sequence identical to nt #1-30 of RNA2 cDNA (sequence available upon request), and reverse transcriptase purchased from Clontech (Palo Alto, CA), with 1 µg RNA per sample as the template. Subsequently, 1 µl cDNA per sample was used for semi-quantitative PCR. PCR was carried out using the EconoGreen PCR Master Mix (Lucigen, Middleton, WI) with appropriate primers for 27 cycles of denaturation at 95 °C for 30 seconds, annealing at 60 °C for 30 seconds and extension at 72 °C for 1 minute. A cDNA fragment of the lima bean actin mRNA (PIACT1) was amplified in parallel to serve as the control (sequence of primers available upon request).
RESULTS

Tracking the replication and cell-to-cell movement of BPMV in detached lima bean cotyledons bombarded with BPMV cDNA constructs

In a previous report, I demonstrated that lima bean cotyledon cells supported robust BPMV multiplication after inoculation of BPMV cDNAs by particle bombardment (Lin et al., 2013). I further used this experimental system to characterize the stem-loop C (SLC), a cis-acting RNA element located in 5’ untranslated region (UTR) of BPMV RNA2 that is essential for RNA2 accumulation in these cells (Lin et al., 2013). Briefly, this experimental system utilizes cDNAs of BPMV RNA1 and RNA2 flanked by the 35S promoter and terminator (P35S and T35S; Fig. 4.1A) of CaMV to test the replication competency of each RNAs. As a result, these constructs would permit replication-independent transcription of BPMV RNAs by DNA-dependent RNA polymerase II (PolII) of the host plant once inside host cells. These primary BPMV RNAs would then initiate autonomous BPMV replication to produce infectious viruses. To distinguish between the PolII-driven primary transcripts and autonomously replicated BPMV RNA, I created R1m (also referred to as mRdRP in Fig. 4.4D), a replication-defective mutant of RNA1, by replacing the highly conserved glycine-aspartic acid-aspartic acid (GDD) motif of viral RdRP with alanine-alanine-histidine (AAH) (Fig. 4.1A, Fig. 4.4D).

Additionally, to visualize the progression of BPMV infection process, I adopted RNA2-GFP (R2G), a modified RNA2 containing a GFP insert between MP and L-CP (Fig. 4.1A; Zhang et al., 2010). Co-bombardment of RNA1 and R2G constructs into lima bean cotyledons resulted in rigorous BPMV replication and cell-to-cell movement as indicated...
by brightly green fluorescent infection foci encompassing multiple cells (Fig. 4.1A, e and e’). In addition, extracts of BPMV-positive cotyledons could infect soybean plants systemically as indicated by bright GFP fluorescence in upper un-inoculated leaves (Fig. 4.2B, a’). The presence of infectious BPMV in both lima bean cotyledons and systemically infected soybean leaves was further confirmed by the detection of RNA1 and R2G-specific, negative sense (-) RNA using strand-specific reverse transcription-polymerase chain reaction (RT-PCR) (Fig. 4.1B, top two panels, lane 3; and data not shown). As expected, the negative control, RNA1m+R2G, failed to produce any green fluorescent infection focus in either bombarded lima bean cotyledons or soybean leaves (Fig. 4.1A, f and f’; Fig. 4.1B, lane 2 of top two panels; and data not shown). Together, these experiments convinced us that this system is suitable for examining the function of individual BPMV-encoded proteins as well.

**P58 is required for RNA2 accumulation in host cells of BPMV**

As described earlier, P58 is the N-terminal utmost protein encoded by BPMV RNA2, and it differs from MP by harboring a 102 aa N-terminal extension. In order to determine its role in BPMV infections, I first determined if it shared any functional overlap with MP. To do this, I created a series of mutants shown on Fig. 4.1A that disrupted the expression of P58 and/or MP to varying degrees. These mutant constructs were then bombarded into cells of lima bean cotyledons together with a wild-type RNA1 construct, and observed 48 hours post bombardment with a fluorescence dissecting scope and a fluorescence microscope. As depicted on Fig. 4.1A, the R2G-mMP mutant lost the
AUG initiation codon of MP due to an AUG→CCU mutation (Fig. 4.1A). It is thus unable to produce an authentic MP, but should produce a near wild-type form of P58 [except for the methionine (AUG) to proline (CCU) change]. As shown in Fig. 4.1A, panel a, cotyledons treated with (RNA1 + R2G-mMP) developed numerous small, but bright GFP foci under the dissecting scope. When compared with cotyledons treated with positive control constructs (Fig. 4.1A, panel e), the dramatically reduced sizes of GFP foci strongly suggest that the cell-to-cell movement of the R2G-mMP mutant was severely debilitated. Further inspection with fluorescence microscopy showed that these foci commonly occupied 2 – 3 cells. Thus, the P58 protein translated from this mutant probably acted as a weak MP to enable very limited cell-to-cell movement of the mutant virus. These results illustrated that a functional BPMVMP requires its translation to initiate from the second AUG codon of RNA2, and that inclusion of the N-terminal 102 aa extension compromises its function in cell-to-cell movement.

However, the replication of either RNA1 or RNA2 (R2G) in these few infected cells was not appreciably affected, as the GFP foci were sufficiently intense to permit their detection even without any magnification (Fig. 4.1A, panel a; and data not shown). This conclusion was further corroborated by the R2G-ΔMP mutant, in which most of the MP coding sequence was eliminated with an in-frame deletion (Fig. 4.1A). GFP foci were again detected at large numbers with relative ease on cotyledons treated with (RNA1 + R2G-ΔMP) (Fig. 4.1A, panel b), although these foci were now limited in single cells, indicating a complete loss of cell-to-cell movement (Fig. 4.1A, panel b’). Nevertheless, the high intensity of the fluorescence was suggestive of robust replication
of BPMV RNAs in these individual cells. Collectively the results with the first two mutants suggest that BPMV MP does not play a significant role in RNA2 replication.

The next mutant I tested was R2G-mP58, in which the P58 AUG was mutated to GCU, yet the MP AUG remained intact. Surprisingly, most of the cotyledons treated with (RNA1 + R2G-mP58) failed to develop any GFP foci even when examined with fluorescence microscope (Fig. 4.1A, c and c’). This result is in stark contrast with earlier results showing that both MP mutants still replicated robustly in the cells they invaded. It hence indicates that P58 plays a distinct, non-MP role to facilitate the accumulation of BPMV RNA2. Furthermore, since the majority of the sequence shared by P58 and MP could be deleted without affecting RNA2 levels (the R2G-∆MP mutant; Fig. 4.1A, b and b’), this unique function of P58 is likely afforded by the first 120 aa of the protein, including the 102 aa (12 kDa) region not shared by MP (Fig. 4.1A).

Interestingly, an earlier study found that the P58 counterpart of CPMV, a close relative of BPMV, was also critical for the accumulation of RNA2 of that virus in protoplasts, and that the N-terminal 14 kDa region unique to CPMV P58 was sufficient for this role (Van Bokhoven et al., 1993). Therefore, even though the 14 kDa region of CPMV P58 and the 12 kDa region of BPMV P58 share little sequence similarity, it is still possible that they could complement each other. To test this possibility, I replaced the first 102aa region of BPMV P58 with its counterpart from CPMV. The resulting construct, R2G-CPMV14K, was then examined in lima bean cotyledons. As shown in Fig. 4.1A, panels d and d’, no GFP foci indicative of active replication were detected on
the treated cotyledons. This result suggests that this particular region of comoviral P58 is unique and irreplaceable for each virus.

Finally, I evaluated the ability of the four mutants to replicate by detecting (-) BPMV RNA in the treated cotyledons with a strand-specific RT-PCR procedure. By focusing solely on (-) RNA, I would capture only the replicating mutants that produce (-) RNA replication intermediates, while avoiding the interference by replication-independent transcription of (+) RNA driven by P35S (see Fig. 4.1A for construct details). As shown on Fig. 4.1B, top panel, (-) RNA1 was abundantly detected in the positive control (RNA1 + R2G) cotyledons, but absent in the negative control (RNA1m + R2G), as expected. Surprisingly, low level of (-) RNA1 could be detected in all four infections in which RNA1 was mixed with various RNA2 mutants, suggesting that our approach was sufficiently sensitive to detect infections that were limited to individual cells (lane 5), and that RNA2 accumulation is not needed for RNA1 replication (lanes 6 and 7). Similarly, low levels of (-) RNA2 were also detected for both MP mutants (middle panel, lanes 4 and 5). Nevertheless, (-) RNA2 was not detected in cotyledons containing either R2G-m58K or R2G-CPMV14K (lanes 6 and 7). These results are highly consistent with observed differences in the number, size and intensity of GFP foci, thus further confirming a critical role of P58 in BPMV RNA2 accumulation.

I hasten to note that, since the start codon of BPMV P58 lies only a few nts downstream of the cis-acting RNA stem-loop C (SLC) characterized in our previous study (Lin et al., 2013), I needed to rule out the possibility that altering the P58 start codon might have inadvertently disrupted the integrity of SLC, thus abrogating RNA2
replication. However, as I will show in the next section, this possibility is discounted by the isolation of several compensatory mutants that preserved the original mutation at the P58 start site but restored the translatability of P58 by introducing new start codons upstream.

**P58 functionality is restored by spontaneous second site mutations that extend the N-terminus of P58 by various lengths**

I described earlier that most of lima bean cotyledons bombarded with (RNA1 + R2G-mP58) were devoid of GFP foci, thus suggesting a lack of RNA2 replication. However, upon prolonged incubation (up to seven days after bombardment), a few bombarded cotyledons developed one or two isolated GFP foci that slowly expanded. To determine the underlying reason for the delayed development of GFP foci, I physically separated the focus-containing areas from the rest of the cotyledons and pooled them for RNA extraction. cDNA corresponding to the first 900 nt of BPMV RNA2 was then obtained by subjecting the extracted RNA to RT-PCR, and subsequently cloned into a plasmid vector. The sequences of the inserts were then determined for 16 recombinant plasmids. As summarized in Fig. 4.2A, none of the clones contained solely the original R2G-mP58 mutation (ATG→gct at the cDNA level). Similarly noteworthy is that only one of the 16 clones completely reverted to wild-type sequence (R2G). Rather, 15 out of 16 clones retained the ATG→gct mutation but also possessed additional novel changes upstream. Among them, ten clones, designated R2G-N3-P58, contained a T→A change nine nt upstream of the original mutation, thus creating a new ATG codon in-frame with
P58 and adding three additional aa to its N-terminus. Similarly, the R2G-N36-P58 variant, accounting for four of the 16 clones, recreated a new start codon for P58 at 36 aa upstream of the original mutation. Finally, one of the 16 clones (R2G-N36/3-P58) contained both of the new start codons.

To demonstrate the viability of these spontaneous mutants, I recreated full length cDNA constructs of R2G-N3-P58 and R2G-N36-P58, and tested their infectivity (together with RNA1) in lima bean cotyledons and subsequently soybean plants. As shown in Fig. 4.2B, panels c and c’, the R2G-N3-P58 mutant caused robust infections in both lima bean cotyledons and soybean plants that were indistinguishable from the wild-type R2G (Fig. 4.2B, a and a’). By contrast, the R2G-N36-P58 with a 36 aa N-terminal extension induced much smaller infection foci in bombarded lima bean cotyledons (Fig. 4.2B, b), yet was still able to infect soybean systemically (Fig. 4.2B, b’). Finally, the results of infection assays were confirmed with strand-specific RT-PCR that detected (-) RNA2 (Fig. 4.2C). Together these data demonstrated that it is the P58 protein, not the RNA sequence/structure coding for P58, which is needed for BPMV RNA2 accumulation.

**P58 is a cis-acting protein of BPMV RNA2**

To further elucidate the functional mechanism of P58, I sought to complement the loss of P58 function in the R2G-mP58 mutant with intact P58 expressed from both a different RNA2 construct, and a non-replicating, P58-expressing RNA. For this purpose, I created versions of R2G-mP58 and R2G-mMP that were no longer capable of
producing GFP fluorescence due to an in-frame deletion in their GFP coding sequence. These new mutants, designated R2G-mP58/ΔG and R2G-mMP/ΔG (Fig. 4.3A), would be expected to produce functional MP and P58, respectively, and complement the GFP-expressing R2G-mMP and R2G-mP58. I first successfully complemented the movement defect of R2G-mMP with R2G-mP58/ΔG. Despite its inability to synthesize GFP by itself, R2G-mP58/ΔG was able to cause the expansion of R2G-mMP and R2G-ΔMP-induced GFP foci to more than 10 cells (Fig. 4.3A, compare d/d’, e/e’ with a/a’, b/b’, respectively), hence demonstrating that functional MP provided by this construct could complement the loss of cell-to-cell movement in trans.

Interestingly, as shown in Fig. 4.3B, panels g/g’ and h/h’, MP provided by a non-replicating, P35S-driven construct complemented the movement defect of R2G-mMP and R2G-ΔMP to similar levels as R2G-mP58/ΔG, suggesting that the latter provided MP without replicating itself. This in turn raises the intriguing question of whether the R2G-mP58/ΔG mutant, which lacked P58 function and thus was unable to propagate RNA2 by itself, could utilize P58 (or its functional variant in R2G-ΔMP) provided by its complementation partners.

To resolve this question, I carried out the reciprocal complementation experiments using R2G-mMP/ΔG to provide P58 in trans to complement R2G-mP58 (Fig. 4.3A, bottom two diagrams). I reasoned that if the P58 provided by the former construct could be utilized by the latter, I would be able to detect GFP foci produced by the R2G-mP58 construct. However, as shown in Fig. 4.3A, panels f and f’, no GFP foci was detected even at the single cell level, indicating that P58 provided in trans was unable to
complement its loss in R2G-mP58. This result was similarly illustrated by the fact that P58 provided by a P35S-driven construct was likewise unable to complement R2G-mP58 (Fig. 4.3B, i/i'). Together these results strongly suggest that P58 is a cis-acting protein that functions only with the RNA2 molecules from which it is translated.

I also verified our findings using strand-specific RT-PCR. Note that one of the primers used for RT-PCR anneals to a region within the GFP coding sequence that was deleted in the ΔG constructs, thus the RT-PCR assay would only detect the replicating RNA2 mutants that encode full length GFP. As shown in Fig. 4.3C and D, I consistently detected more (-) RNA2-specific products in all cases where cell-to-cell movement was complemented (Fig. 4.3C and D, compare lanes 5 with 4, 7 with 6), thus corroborating the observed expansion of GFP foci. Conversely, no RNA2-specific product was detected in attempted P58 complementations, consistent with the absence of GFP focus on the respective cotyledons. In summary, the data so far implicates P58 as a cis-acting protein that facilitates RNA2 accumulation in BPMV-infected cells.

**Replication-related proteins encoded by BPMV RNA1 are all cis-acting relative to RNA1**

Having established that P58 is required in cis for RNA2 accumulation, I wondered if the cis-acting mode is unique to P58, or shared by other BPMV proteins functioning in the genome replication process. Since all other replication-related proteins are encoded by RNA1 but needed for RNA2 replication as well, it seemed counterintuitive to expect these proteins to be cis-acting. Nevertheless, I reasoned that
some of the RNA1-encoded proteins could still be cis-acting relative to RNA1 and hence must be recruited to RNA2 through RNA2-specific mechanisms, as previously established for other multi-partite viruses (Baumstark and Ahlquist, 2001; Yi and Kao, 2008; Iwakawa et al., 2011). To test this possibility, I first focused on BPMV RdRP as I had available an RNA1 mutant in which the highly conserved GDD motif of RdRP was changed to AAH, rendering RdRP nonfunctional. This mutant, designated RNA1m or mRdRP (Fig. 4.1A and 4.4A), was used in many of our previous experiments as a negative control.

To determine if the loss of RdRP function in this mutant could be complemented by functional RdRP provided in trans, I first created R1m-R2G, a new construct that combined the expression cassettes of both R1m and R2G into one single plasmid to ensure the maximal coexistence of both RNA segments in the same cells (Fig. 4.4A). A similarly configured positive control construct, RNA1-R2G, was also created (Fig. 4.4A). As shown in Fig. 4.4A, panel c, when bombarded into lima bean cotyledons alone, R1m-R2G failed to give rise to any GFP focus, thus verifying that the mutation in R1m abolished the replication of RNA1. When R1m-R2G was co-bombarded to cotyledons with a wild-type RNA1 construct, the replication of RNA2 was restored to levels similar to RNA1-R2G (Fig. 4.4A, compare lanes b with d), suggesting that wild-type RNA1 provided through a different construct readily supported RNA2 replication. Indeed, this was also confirmed by the detection of a (-) RNA1-specific RT-PCR fragment in these cotyledons (Fig. 4.4B, lane 4).
However, this complementation assay did not allow us to assess whether the defect in R1m was complemented by RdRP provided by wild-type RNA1 in trans. To do this, I took advantage of a BsrBI restriction enzyme site introduced into R1m at the time of mutant creation. As shown on Fig. 4.4C, lanes 1 and 2, digestion of control PCR products derived from RNA1 and R1m-containing plasmids failed to fragmentize the wild-type RNA1 product, but cut R1m product into two smaller fragments, confirming the presence of the BsrBI site, as well as the effectiveness of this enzyme. However, the equivalent, (-) RNA1-specific PCR products derived from cotyledons infected with RNA1-R2G or (RNA1 + R1m-R2G) were both resistant to BsrBI digestion (lanes 3 and 4), indicating that, despite its presence in the same cells with R2G, the inability of R1m to replicate was not restored by wild-type RNA1 that facilitated R2G replication. These results revealed two important insights: (i) the mutated RdRP of R1m, if produced, did not repress wild-type RdRP trans-dominantly; (ii) conversely, wild-type RdRP provided in trans was also incapable of complement the R1m defect. In conclusion, RdRP of BPMV is cis-acting relative to RNA1.

I went on to make four additional RNA1 mutants, mC-Pro, mHel, mVPg, and mPro, by replacing 2-3 conserved amino acids within each of the four corresponding RNA1 proteins. The detailed changes are summarized in Fig. 4D. These mutants were then analyzed with procedures similar to those used to examine R1m. None of these mutants could infect host cells when co-introduced into lima bean cotyledons together with R2G (data not shown). Furthermore, despite of repeated attempts, I was unable to complement
the defects in any of these mutants with wild-type RNA1. Thus we conclude that all RNA1 encoded proteins are *cis*-acting proteins relative to RNA1.
DISCUSSION

BPMV is a bipartite (+) RNA virus of the genus *Comovirus*, family *Secoviridae* (Sanfacon et al., 2009). Secoviruses share extensive similarities with animal viruses of *Picornaviridae* in terms of genome organization, mode of gene expression, as well as virion symmetry (Le Gall et al., 2008). One notable difference between these two virus families is that while picornaviruses typically have monopartite genomes, many secoviruses, including all comoviruses, have bipartite genomes. Like other comoviruses, the larger RNA1 segment of BPMV encodes all viral proteins required for its own replication, and replicates in single cells in the absence of RNA2 (Lin and Qu, unpublished). By contrast, RNA2 encodes mostly functions needed for virion assembly and cell-to-cell trafficking, hence has to rely on RNA1-encoded proteins for replication. Exactly how RNA2 recruits RNA1-encoded proteins to replicate itself is not well understood for BPMV or comoviruses in general. Earlier investigations based on CPMV, another comovirus, showed that mutations disrupting the coding region of the N-terminally located P58 ORF abolished RNA2 accumulation in protoplast cells, although it was not further examined as to whether the P58 protein itself or its coding RNA played a more critical role, and how this genome region facilitated RNA2 accumulation (van Bokhoven et al., 1993; Carette et al., 2002a; Sainbury et al., 2010). It was also found that the RNA2 of GFLV, a virus of the genus *Nepovirus of Secoviridae*, encodes a 5’ proximal 2A protein that is needed for RNA2 accumulation and co-localize with some of the RNA1-encoded proteins (Gaire et al., 1999). These earlier studies suggested that the
recruitment of RNA1-encoded proteins by RNA2 of bipartite secoviruses might invoke a shared mechanism that requires a RNA2-encoded protein.

In the current study, I have carried out an extensive investigation of P58 encoded by BPMV RNA2 with a newly developed system that allowed us to monitor BPMV RNA accumulation as well as viral cell-to-cell movement. With this new system, I was able to show that the P58 protein, rather than the sequence/structure of its coding RNA, is needed to ensure the accumulation of BPMV RNA2. I also determined that the function of P58 does not overlap that of MP, as most of the MP region could be deleted without compromising RNA2 replication in single cells. Most importantly, I found that P58 acts in cis, or at least cis-preferentially, to enable the accumulation of the same RNA2 molecule from which it is translated. Our results significantly deepened our current understanding of the 5’ proximally encoded P58 protein of BPMV, and lay the foundation for further mechanistic examination into the replication mechanism of BPMV RNA2.

How does P58 ensure the accumulation of RNA2 specifically? While this was not thoroughly investigated in the current study, several lines of evidence led us to speculate that P58 functions to recruit RNA1-encoded polyprotein to RNA2 for the purpose of replicating RNA2. First, in cotyledons treated with wild-type RNA1 and R2G-mP58 constructs, (-) RNA2 was never detected, despite the abundant availability of P35S-driven (+) RNA2 in these cells, and the fact that (-) RNA1 was readily detectable. Secondly, the cis-acting mode of P58 function is commonly associated with viral replication proteins (White et al., 1995; Yi and Kao, 2008; Wang et al., 2009; Iwakawa et
al., 2011). Indeed, most of the replication-related proteins encoded by poliovirus, a member of Picornaviridae, were found to be cis-acting (Novak and Kirkegaard, 1994). Our result showing that all of BPMV RNA1-encoded proteins are cis-acting further extends the list of cis-acting replication proteins, and conversely hints at a replication role for P58. Finally, the reported intracellular co-localization of GFLV 2A protein with some of the RNA1-encoded protein is also consistent with the involvement of one of RNA2-encoded proteins in the replication of RNA2 itself (Gaire et al., 1999). In addition, structural modeling suggests that the region of BPMV P58 not shared by MP contains a very strong transmembrane domain which could conceivably allow it to associate with the membrane-based VRCs (data not shown).

I have recently reported the identification of SLC, an RNA stem-loop structure within the 5’ UTR of BPMV RNA2 essential for RNA2 accumulation in infected cells (Lin et al., 2013). The characterization of P58 through the current study adds to the list of cis-acting elements needed to ensure efficient reproduction of BPMV RNA2. Our next goals are to understand how these cis-acting elements function to ensure the RNA2 accumulation in the infected cells, and whether they coordinate with each other to achieve the optimal outcome for the virus.
ACKNOWLEDGEMENTS

I greatly appreciate the generosity of Drs. Steve Whitham and John Hill at Iowa State University in providing us with the RNA2-GFP construct. I thank members of the Qu and Finer labs for stimulating discussions and technical assistances, Dr. Lucy Stewart and other members of USDA, ARS Corn and Soybean Research Unit for sharing equipment. This study was supported in part by an OARDC Graduate Seed award to me. Soybean virus-related research in Qu lab has been supported in part by grants from North Central Soybean Research Program and Ohio Soybean Council.
Figure 4.1 P58 is required for RNA2 accumulation. (A) Schematic representations of BPMV RNA1, RNA1m (a nonreplicating RNA1 mutant), RNA2-GFP (R2G) and a series of R2G mutants used in this study, accompanied by images of lima bean cotyledons bombarded with these constructs. The mutation (GDD→AAH) contained in RNA1m is highlighted in the grey box. Details of RNA2 mutants are provided on their respective diagrams. The images of bombarded cotyledons were taken at 48 hour post bombardment (hpb), using a camera that is part of a dissecting microscope, illuminated with long wavelength UV light (left panels, size bar = 0.5mm). Some infection foci are highlighted with arrows. The close-up images of infection foci were taken with a fluorescence microscope (right panel, size bar = 10µm). (B) Replication of BPMV in bombarded cotyledons as measured by strand-specific, semi-quantitative RT-PCR that detects (-) RNA1 (top panel) and (-) RNA2 (middle panel). Total RNA was extracted at 48 hpb and treated with RNase-free DNase I. Same amounts of RNA were subjected to sqRT-PCR. A RT-PCR product of a lima bean actin mRNA was used as a control.
Figure 4.2 Compensatory mutants isolated from lima bean cotyledons bombarded with RNA1 + R2G-mP58. (A) The nucleotide (nt) sequences and the deduced amino acid (aa) sequences of the compensatory mutants (R2G-N36-P58, R2G-N3-P58 and R2G-N3/N36-P58) were aligned with those of the original R2G-mP58 mutant to highlight the position and identity of the compensatory mutations. Only the section with mutations is shown. All except the revertant (R2G) retained the original ATG→gct change. Nontranslatable aas are shown in grey and translatable aas are shown in black. The compensatory mutations at the nt level and their corresponding aa (M for methionine) are underlined. Numbers underneath the name of each mutant represent the number of clones obtained for the corresponding mutants. (B) Infectivity of the mutants as depicted by photographs of GFP fluorescent cotyledons (top panels; 48 hpb. Size bar = 0.5mm), as well as systemically infected soybean leaves [bottom panels; 2 week post inoculation (wpi)]. (C) Verification of active replication in lima bean cotyledons with (-) RNA2-specific, sqRT-PCR.
Figure 4.3 P58 is a cis-acting protein. (A) Complementation between RNA2 mutants with defects in P58 and MP. The diagrams of the mutants used, as well as those included in each of the complementation experiments, are shown on the left, with the outcome of infections/complementations shown on the right. (B) Complementation of P58 and MP defects with replication-independent expression of corresponding proteins. (C) & (D) Results of (-) RNA2-specific sqRT-PCR used to confirm the complementation results shown on (A) and (B), respectively.
Figure 4.4 Replication-related proteins coded by RNA1 are all cis-acting. (A) Schematic representation of the constructs used in this set of experiments (top), and their fate in singly or co-bombarded cotyledons (bottom). (B) Confirmation of results shown in (A) using (-) RNA1-specific, sqRT-PCR. (C) The defect of RNA1m cannot be complemented by functional RdRP provided by wild-type RNA1. Digestion with BsrBI, which cuts the PCR fragment of R1m mutant but not that of wild-type RNA1 (lane 1 and 2) was used to detect potential R1m-specific PCR product amplified from RNA1 + R1m-2G co-bombarded cotyledons. (D) Schematic representation of five RNA1 mutants tested in this study. The names of the mutants, together with the specific aa changes introduced by these mutants, are highlighted in their corresponding boxes.
CHAPTER 5. RNA-SILENCING-MEDIATED ANTIVIRAL DEFENSE
CONSTITUTES AN IMPORTANT COMPONENT OF NONHOST
RESISTANCE AGAINST BEAN POD MOTTEL VIRUS IN
NICOTIANA BENTHAMIANA

A modification of this chapter will be submitted to Molecular Plant-Microbe Interactions
for publication.
ABSTRACT

RNA silencing is a highly conserved mechanism of eukaryotes that regulates the expression of endogenous as well as exogenous nucleic acids through sequence-specific targeting of RNA transcripts. Among many roles of RNA silencing is to defend cellular organisms against invasion of viruses. However, the contribution of RNA silencing to nonhost antiviral defense has not yet been well studied. I previously established that one of the defense strategies encountered by Bean pod mottle virus (BPMV) in the nonhost Nicotiana benthamiana (Nb) is to selectively repress the propagation of RNA2, one of the two genome segments of this bipartite virus. Here I show that, in the presence of a heterologous suppressor of RNA silencing (P19 of Tomato bushy stunt virus), BPMV RNA2 overcame the initial repression and regained the ability to replicate in Nb cells. Furthermore, transgenic Nb plants expressing a P19 transgene permitted delayed and inefficient systemic spread of BPMV. Consistent with the suggestion that BPMV infection is effectively restricted by RNA silencing in the nonhost Nb, none of the BPMV encoded proteins could detectably suppress RNA silencing (VSR) in Nb plants. Collectively our data indicate that the anti-BPMV defense in the nonhost Nb relies on at least two forms of active defense: selective repression of RNA2 replication and RNA silencing.
INTRODUCTION

As obligate intracellular parasites, plant viruses rely on multiple cellular processes of their hosts to synthesize pools of new proteins and nucleic acids that participate in the assembly of new virions. While host plants can achieve antiviral immunity through adaptive evolution at many steps, viruses could overcome individual defense barriers by quickly evolving various forms of counter-defense measures. Excellent examples of such counter-defense arsenal include suppressors of RNA silencing encoded by many plant viruses (Qu and Morris, 2005). Hence, the more durable forms of antiviral resistance represented by nonhost defense are expected to involve multiple layers of defense that coordinately fend off the attacks of the corresponding viruses (Pallas and Garcia, 2011).

RNA silencing is among the best understood antiviral defense mechanism of plants and invertebrate animals (Ding, 2010). RNA silencing exerts its antiviral defense role through recognizing and processing of double-stranded RNA (dsRNA) or partially double-stranded hairpin-loop RNA (hpRNA) formed by viral RNA in infected cells (Ding and Voinnet, 2007; Miller and White, 2006). The processing of these ds/hp RNAs by Dicer or its orthologs in plants [Dicer-like (DCL)] leads to accumulation of small interfering RNAs (siRNAs) of discrete sizes (21-25 nucleotides). These siRNAs are then incorporated into RNA-induced silencing complexes (RISCs), which in turn target viral RNAs in a sequence-specific manner for degradation, thus achieving viral resistance through the destruction of viral genetic materials (Ding, 2010).

Antiviral RNA silencing operates in all plants, yet in susceptible hosts it is rigorously counteracted by virus-encoded silencing suppressors that target one or more
components of the RNA silencing pathway (Qu and Morris, 2005; Ding and Voinnet, 2007; Alvarado and Scholthof, 2009). For example, P19 of *Tomato bushy stunt virus* (TBSV) binds to viral siRNAs, thereby preventing siRNAs from entering the RISC (Scholthof, 2006); whereas 2b of *Cucumber mosaic virus* (CMV) targets AGO1, the catalytic subunit of RISC (Zhang et al., 2006). Interestingly, virus-encoded silencing suppressors have been shown to enhance the infections and symptoms of other viruses, accounting for the synergistic effects of mixed infections by two or more different viruses (Voinnet, 2005; Latham and Wilson, 2008; Wu et al., 2010).

Although being suppressed by viral silencing suppressors in compatible virus-plant interactions, RNA silencing could conceivably play important antiviral roles in nonhosts, where the viral suppressors might not be able to engage their targets in RNA silencing pathway effectively. Indeed, Jaubert and colleagues (2011) observed that *Potato virus X* (PVX) gained the ability to infect Arabidopsis, which is normally a nonhost for PVX, when co-inoculated into the plants with a second virus, *Pepper ringspot virus* (PepRSV). They further observed that PVX was able to infect mutant Arabidopsis plants defective in RNA silencing-mediated defense, suggesting that RNA silencing plays a critical role in restricting PVX infections in the nonhost Arabidopsis.

*Bean pod mottle virus* (BPMV) is a bipartite, positive sense (+) RNA plant virus in the *Secoviridae* family (Sanfacon et al., 2009). It infects a number of legume species (Giesler et al., 2002), but not the experimental plant *Nicotiana benthamiana* (*Nb*), which was used as a model nonhost for BPMV in our work. In our previous study, I have examined the replication efficiencies of BPMV RNA1 and RNA2 in *Nb* leaves using
Agrobacterium-mediated infiltration assay. I concluded that the replication of BPMV RNA2 was selectively repressed in Nb, leading to the failure of BPMV RNA1 to exit individually infected cells. This conclusion was based on the observation that the replication of RNA1, but not RNA2, was detectable at five days post infiltration (dpi) in Nb leaves. Here I report that, when the observation time was extended to 10 dpi, I observed signs of RNA2 replication as well. Importantly, inclusion of the heterologous silencing suppressor P19 was necessary for the replication of both BPMV RNAs, thus suggesting a role of RNA silencing in the Nb nonhost resistance to BPMV. Consistent with suggestion, I found that transgenic Nb plants expressing a P19 transgene (Nb-P19) supported weak systemic infection of BPMV. Together, this report for the first time demonstrated that two different layers of defense, RNA silencing and selectively targeting of one of the bipartite genome RNAs, function coordinately to defeat BPMV invasion in the nonhost Nb.
MATERIALS AND METHODS

Constructs

The infectious cDNA of BPMV RNA1 used in this study was derived from a Kentucky isolate (K-Ho1) of BPMV, and kindly provided to us by Dr. Said Ghabrial (Gu and Ghabrial, 2005). A replication-defective mutant of RNA1 cDNA (RNA1m) was created by changing the highly conserved GDD motif to AAH (the sequence of the DNA oligo is available upon request). The RNA2 cDNA was originally derived from an Iowa isolate (IA-Di1), and was subsequently modified by Zhang and colleagues (2010) to include a GFP cDNA insert between MP and L-CP. The resulting construct, designated RNA2-GFP (R2G), was a kind gift from Dr. Steve Whitham. All full length BPMV cDNAs were first cloned into the plasmid pRTL2 to acquire the 35S promoter (P35S) and the corresponding transcriptional terminator (T35S) of Cauliflower mosaic virus (CaMV). The cDNAs of BPMV RNA1 or RNA1m flanked by P35S and T35S were then excised from pRTL2 and inserted into the binary vector pPZP212 (Qu et al., 2003) to produce pPZP-RNA1, -RNA1m. To created RNA1-R2G and R1m-R2G, RNA1/ RNA1m and R2G cassettes were excised from pRTL2 and cloned into a modified binary vector pAI101 to produce pAI- RNA1-R2G and -R1m-R2G.

To create constructs for the identification of VSR activity among BPMV-encoded proteins, seven of the eight BPMV-coded proteins, C-Pro, HEL, Pro, RdRP, MP, L-CP, and S-CP were expressed as individual proteins, whereas VPg was expressed as a fusion with HEL due to its small size. A fused CP (F-CP) was also included that combined both L-CP and S-CP. All nine cDNAs were placed between the P35S and T35S. At the same
time, a GFP expression cassette containing sGFP with Gmubi promoter (Hernandez-Garcia et al., 2010) and nos terminator was cloned into pRTL2. Finally, both individual viral protein expression cassette and the Gmubi-sGFP were excised from pRTL2 and inserted into the binary vector pCAMBIA1300. Sequences of primers used to produce these constructs are available upon request.

**Transient expression mediated by Agrobacterium (agro-infiltration)**

The assembled binary constructs were transformed into the C58C1 strain of *A. tumefaciens* by electroporation (Qu et al., 2003). Single *Agrobacterium* colony was used to inoculate a 3 ml overnight culture. The 3 ml culture was then used, at 1:100 dilution, to start another fresh overnight culture to ensure all suspensions have similar amounts of living cells. The second overnight cultures were harvested and resuspended the pellet in inoculation buffer (10mM MgCl₂, 10mM MES, 10μM Acetosyringone). The inoculants were adjusted to OD₆₀₀ equal to 1.0. *Nb* leaves of three – four weeks old plants were infiltrated with a needle-less syringe. The infiltrated leaves were kept in growth chamber with 12 hr daylight at 24 °C.

**Particle bombardment of lima bean cotyledons and image analysis**

Lima beans of the Anderson Bush variety was purchased from Earl May Seed and Nursery (Shenandoah, IA). Lima bean seeds of uniform size and shape were selected and surface sterilized in a 5% (v/v) bleach solution with slow agitation for 20 minutes, rinsed 4-7 times with autoclaved water. Five to six seeds were germinated in a Magenta box and
covered with moistened sterile paper towels for 4 days at 25°C. The bombardment experiments were carried out following the procedure described by Hernandez-Garcia et al. (2010), briefly, prior to bombardment, the seed coat was removed and each cotyledon was excised into two pieces. DNA plasmids were precipitated onto tungsten particles and delivered into the surface of cotyledons using Particle Inflow Gun. After bombardment, cotyledons were placed in sterile petri dishes containing 25 ml of OMS culture medium, containing MS salts, B5 vitamins, 3% (w/v) sucrose and 0.2% (w/v) Gelrite (pH 5.7).

The image of lima bean cotyledons were collected at 2 days post bombardment using image collection system, including a MZFLIII dissecting microscope (Leica, Heerbrugg, Switzerland) equipped with a "GFP-2" filter set (Excitation 480 ± 40 nm, Emission 510 nm), a Spot-RT CCD digital camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA). Lima bean cotyledons showing positive GFP expression were also observed using a Leica TCS SP5 confocal laser microscope (Molecular Cellular and Imaging Center, Wooster, OH).

**Semi-quantitative RT-PCR**

Total RNAs were extracted from infiltrated *Nb* leaves with the TRIsure reagent (Bioline, Taunton, MA) at 5 dpi. After verifying the quality of RNAs with gel electrophoresis, approximately 5 µg RNA per sample was treated with TURBO DNA-free DNase according to Manufacturer’s instruction (Ambion, Austin, TX). Strand-specific reverse transcription was carried out with appropriate primers, and reverse transcriptase purchased from Clontech (Palo Alto, CA). PCR was carried out using the
EconoGreen PCR Master Mix (Lucigen, Middleton, WI) with appropriate primers (the sequences of DNA oligos provided upon request) for 24 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 1 minute. As a control, the actin mRNA of *Nb* (NbACT) was also reverse-transcribed and PCR amplified in parallel with BPMV RNAs (Sequences of primers are available upon request).

**Northern blot**

Total RNAs were extracted from infiltrated *Nb* leaves at 5 dpi and subjected to RNA blot analysis to detect GFP mRNA and siRNA using previously described procedures (Qu et al., 2003). Different amounts of *Nb* RNA were used to detect mRNA (1 μg) and siRNAs (5 μg). The probes were a mixture of five different GFP specific oligonucleotides end labeled with ³²P (Sequences of primers are available upon request).

**Protein extraction and Western blot**

Proteins were extracted from infiltrated *Nb* leaves at 5dpi using protein extraction buffer (25mM Tris, pH=8.0; 1mM EDTA; 5mM DTT; 1ml extraction buffer per 0.5g plant tissue). Protein samples (2μg each) were separated on 12.5% polyacrylamide-0.1% SDS gels and subsequently transferred to polyvinylidene difluoride membranes for the detection of GFP with monoclonal anti-GFP antibody (Sigma, St. Louis, MO).
RESULTS

Replication of BPMV RNA1 was readily detectable whereas RNA2 replication was significantly delayed in the agro-infiltrated Nb leaves.

In a previous study, I examined BPMV replication in the nonhost Nb by infiltrating Nb leaves with Agrobacterium suspensions harboring a construct termed RNA1-R2G, which contains cDNAs of both BPMV RNA1 and RNA2-GFP (an RNA2 derivative that contains a GFP reporter), each sandwiched between the promoter and the terminator of the 35S RNA (P35S and T35S) (Fig. 5.1A). R1m-R2G, a replication-defective construct, in which the BPMV RNA1 cDNA was mutated at a conserved site to abrogate the function of viral RNA-dependent RNA polymerase (RdRP), was included as a negative control. I then analyzed the accumulation levels of (-) RNA replication intermediates of both RNA1 and RNA2 in the infiltrated Nb leaves at 5 days post infiltration (dpi). Those experiments initially led us to conclude that the replication of BPMV RNA2 was selectively repressed in the nonhost Nb.

To gain a more accurate comparison on the replication efficiency of RNA1 and RNA2 in Nb cells, we decided to more carefully evaluate the RNA1 and RNA2-specific products by including additional time points in our analyses, namely at 5, 10 and 15 dpi. To prevent the destruction of BPMV RNAs by RNA silencing, I also co-infiltrated a construct that expresses P19, the strong silencing suppressor encoded by TBSV (Scholthof, 2005; Ding and Voinnet, 2007). The infiltrated leaves harvested at these time points were subjected to strand-specific semi-quantitative RT-PCR (sqRT-PCR) to detect (-) viral RNAs. Results presented in Fig. 5.1B, illustrate the contrasting replication
competence of BPMV RNA1 and RNA2 in Nb cells. In the bottom panel, RT-PCR products of Nb actin (NbACT) mRNA were used as controls to ensure an equivalent amount of RNA was used in every sqRT-PCR reaction. Consistent with previous observation, wildtype RNA1-derived products were detectable by sqRT-PCR at all three time points (Fig. 5.1B, top panel, lanes 2, 4, 7, 9, 12 and 14). These results confirmed that Nb cells support the replication of BPMV RNA1 when P19 was present. In addition, the replication of RNA1 did not require RNA2 encoded products as RNA1-specific product was at similar levels in both RNA1-R2G and RNA1 alone samples (Fig. 5.1B, top panel, compare lanes 2 with 4, 7 with 9, 12 with 14).

By contrast, RNA2 replication was selectively delayed in the Nb nonhost cells, as RNA2 specific (-) RNA was undetectable in 5 dpi samples regardless of the functionality of viral RdRP (Fig. 5.1B, middle panel, compare lane 4, in which RNA1 provided the functional RdRP, with lane 5, in which R1m provided nonfunctional RdRP). This observation was further supported by the fact that the extracts of these 5 dpi infiltrated leaves of RNA1-R2G or R1m-R2G were unable to cause any local or systemic infection foci in soybeans (Fig. 5.1C, middle row, left two panels). These findings suggest that BPMV RNA2 replication was at least significantly delayed in Nb cells, making it impossible to detect its accumulation with either molecular (RT-PCR) or biological (soybean inoculation) means.

Interestingly, by 10 dpi, I began to observe multiple bright green spots on Nb leaves infiltrated with RNA1-R2G, but not on those with R1m-R2G (Fig. 5.1C, top row, e-f panels), the intensity of bright GFP fluorescence increased by 15 dpi, suggesting that they
were sites of active RNA2 replication. To confirm that the GFP fluorescence was due to the accumulation of infectious BPMV in Nb cells, I performed two sets of experiments. As shown in Fig. 5.1B, middle panel, RNA2-specific (-) RNA was detected from RNA1-R2G, but not R1m-R2G-treated leaves at 10 and 15 dpi, with a slight increase from 10 to 15 dpi (Fig. 5.1B, middle panel, compare lanes 9 with 10, lanes 14 with 15). These results were also consistent with the western blot data showing that GFP specific protein products accumulated to higher levels in leaves treated with RNA1-R2G than those with R1m-R2G at 10 and 15 dpi samples (Fig. 5.1D, top panel, compare lanes 4 with 5, lanes 6 with 7). Although GFP protein was also detected in R1m-R2G treatments, its levels were low and declined over time (Fig. 5.1D, compare lanes 3, 5, and 7), reflecting P35S-driven, replication-independent transcription of RNA2, which in turn supported low levels of translation of GFP-containing polyprotein. Furthermore, the extracts of 10 and 15 dpi Nb leaves, but not the 5 dpi ones, caused both local and systemic infections in soybean, again with a slight increase in focus numbers from 10-15 dpi (Fig. 5.1C, middle panel, compare i with j, k with l; bottom panel, compare i’ with j’, k’ with l’).

These new results demonstrated that, when RNA silencing-mediated antiviral defense was suppressed by a heterologous silencing suppressor (P19), RNA2 replication was drastically delayed, but not completely abolished. Notably, there was also a significant increase in RNA1 levels from 5 to 10 dpi, and this increase was largely independent of RNA2 (Fig. 5.1B, top panel, compare lanes 2 and 7, 4 and 9). Therefore, although RNA2 appeared to be more prone to repression in Nb cells, it is likely that both
RNA segments of BPMV replicated at suboptimal levels in *Nb* cells, even when RNA silencing was suppressed by P19.

While these results showed both BPMV RNAs could replicate in *Nb* cells inefficiently, it is important to point out that no replication could be detected for either RNA in the absence of TBSV P19. This in turn suggests that both BPMV RNAs are under active surveillance by RNA silencing-mediated antiviral defense. As I will show more definitively in the next section, RNA silencing is one of the key defenses against BPMV in *Nb*.

**RNA silencing constitutes an integral component of anti-BPMV defense in the nonhost *Nb***

I next tested whether suppression of RNA silencing in the entire *Nb* plant with transgenically expressed P19 could facilitate the systemic movement of BPMV. P19-transgenic *Nb* plants (*Nb*-P19), together with wild-type *Nb* as control, were rub-inoculated with extracts of soybean leaves containing rigorously replicating BPMV-GFP (RNA1-R2G). Surprisingly, starting from 7 dpi, a few fluorescent dots could be seen on the inoculated leaves of *Nb*-P19, but not wild-type *Nb* plants (Fig. 5.2B, f; data not shown). Furthermore, varying degrees of systemic movement of RNA1-R2G were also observed in 47 out of 60 *Nb*-P19 plants, with fluorescence mostly reaching a few upper uninoculated leaves or branches (Fig. 5.2C, panel d), whereas green fluorescence was never observed in systemic leaves of wildtype *Nb* plants inoculated with RNA1-R2G (Fig.5. 2C, panel c). This indicated that suppression of RNA silencing by P19 permitted
systemic movement of BPMV in *Nb*. Thus, RNA silencing is another crucial layer of the anti-BPMV nonhost resistance in *Nb*. The fact that once BPMV replication is initiated in inoculated leaves, the virus spread normally to neighboring cells and move systemically, suggested that there is no additional defense barrier in *Nb* at the cell-to-cell or systemic movement steps.

**The combined action of two defense layers caused *Nb* to be at least 1,000 fold more resistant to BPMV than soybean**

To obtain a quantitative estimation of the contributions of the two defense layers, I compared soybean and *Nb*-P19 side-by-side for their susceptibility to RNA1-R2G. The inoculums for this experiment were derived from one systemically infected soybean leaf. As shown in Fig. 5.2A, a dilution series was generated by making serial 1:10 dilutions. Each dilution was then divided into two equal haves, and used to infect 24 fresh soybean and 18 *Nb*-P19 plants, respectively. The numbers of GFP fluorescent foci were then counted for both types of plants. As shown in Fig. 5.2B, *Nb*-P19 leaves inoculated with the undiluted inoculum produced similar numbers of foci as the soybean leaves infected with 1: 1,000 diluted inoculum (Fig. 5.2B, compare d with f and e with g; NC: not countable). These quantitative results demonstrated that *Nb*-P19 is about 1,000 fold less susceptible to BPMV than soybean. Since *Nb*-P19 is defective in RNA silencing-mediated defense, I conclude that wild-type *Nb* is at least 1,000 times more resistant to BPMV.
None of BPMV proteins functions as an efficient VSR in nonhost Nb cells

Having established that a heterologous VSR P19 is needed to support the multiplication of BPMV in Nb plants, I hypothesize that BPMV-encoded silencing suppressor(s) are unable to function properly in Nb to engage the RNA silencing machinery of Nb, even though they are likely to be highly effective in BPMV hosts. To test this hypothesis, I performed the standard agroinfiltration assay in which a transiently expressed GFP gene was used as a visual indicator of RNA silencing as well as suppression of RNA silencing by various BPMV proteins (Voinnet et al., 2003). For these experiments, I used Gmubi3 (for Glycine max ubiquitin 3), a strong promoter from soybean characterized by Hernandez-Garcia et al. (2010), to driven transient expression of GFP, and P35S to drive the expression of all predicted BPMV proteins, as well as P19 serving as a positive control (Fig. 5.3A). The accumulation of GFP mRNA and siRNA was evaluated by Northern blot hybridizations at 5dpi. While co-expression of P19 and GFP resulted in higher levels of GFP mRNA accumulation (Fig. 5.3B, top panel, compare lane 10 with the other lanes) accompanied by lower levels of siRNAs (Fig. 5.3B, middle panel, compare lane 10 with the other lanes) in infiltrated Nb leaves, none of the mature proteins encoded by BPMV RNA1 or RNA2 could suppress GFP silencing reproducibly in Nb cells (Fig. 5.3B, compare lanes 1-9 with 10). These findings are consistent with those of Gu and Ghabrial (2005) and suggest that VSR(s) encoded by BPMV are ineffective in Nb cells.
DISCUSSION

In the current study, I have developed a model system consisting GFP-tagged BPMV and Nb nonhost to examine the mechanisms of nonhost antiviral defense in plants. I revealed that in the presence of a heterologous suppressor of RNA silencing (TBSV P19), BPMV RNA1 replication was readily detectable in the Nb nonhost cells at 5 dpi, whereas RNA2 replication was only detected after a significant delay. These results suggest that selective repression of RNA2 replication is one of the defenses operating in the Nb nonhost. Furthermore, the need for P19 in these experiments identifies RNA silencing as another important component of the anti-BPMV nonhost resistance. The combined action of these two types of antiviral defense enables Nb to be at least 1000 fold more resistant to BPMV than soybean. Therefore, enlisting multiple seemingly imperfect defenses may be a common defense strategy of nonhosts.

Importantly, I demonstrated that none of the BPMV-encoded proteins could serve as a silencing suppressor in Nb. This result confirms previous observations by others, and suggests that BPMV-encoded silencing suppressor(s) are unable to engage their respective targets in Nb (Gu and Ghabrial, 2005). It should be noted that a highly successful virus like BPMV is expected to effectively counteract the RNA silencing-based defense in its host plants. Thus BPMV-encoded silencing suppressor(s) likely function in a host–specific manner. This would be expected as viral silencing suppressors were evolved to neutralize the function of various silencing pathway components of the virus hosts (Ding, 2010). Conversely, in nonhost plant species that are evolutionarily distant from the virus hosts, these components may escape suppressor-caused
interference through the accumulation of polymorphisms (Schluze-Lefert and Panstruga, 2011).

How does Nb prevent the BPMV infection? In support the idea that Nb arrests the replication of BPMV RNA2 by exploiting the difference in replication of the two BPMV genomic RNAs, I have previously identified two RNA2 specific cis-acting elements, a 5’ UTR stem-loop structure (Lin et al., 2013) and an N-terminal 58K protein (unpublished), that could potentially facilitate RNA2 replication but are not needed for RNA1 replication in host cells. These two elements could potentially be targeted in nonhost cells. The selective suppression of BPMV RNA2 serves as the first layer of anti-BPMV defense in nonhost Nb, which allows the second layer of defense--RNA silencing, to destroy any BPMV RNAs that escaped the first defense layer.

The ability of BPMV to infect Nb-P19 suggests that once the two layers of defense are overcome, BPMV could move systemically in Nb plants, thus ruling out a significant role for other defense pathways. Therefore, the complete anti-BPMV defense in nonhost Nb consists of two layers of active defense: interference with RNA2 replication and RNA silencing.
ACKNOWLEDGEMENT

I thank members of Feng Qu lab for frequent discussions and technical assistance, Dr. Lucy Stewart and other members of USDA Corn and Soybean Research Group for equipment sharing. I greatly appreciate the generosity of Drs. Steve Whitham and John Hill at Iowa State University in providing us with the RNA2-GFP construct. This study was supported in part by an OARDC Graduate Seed award to J. L. Soybean virus-related research in Qu lab has been supported in part by grants from North Central Soybean Research Program and Ohio Soybean Council.
**Figure 5.1** Differential replication efficiency of BPMV RNAs in agro-infiltrated *Nb* leaves at 5, 10, 15 days post infiltration (dpi). (A) Schematic representation of four constructs used in this study: pAI101-RNA1 containing cDNA of BPMV RNA1 flanked by P35S and T35S; pAI101-RNA1m carrying a mutated RNA1 defective in RdRP; pAI101-RNA1-R2G containing both full length cDNA of BPMV RNA1 and RNA2:GFP (R2G); pAI101-R1m-R2G containing the mutated RNA1(R1m) and R2G. (B) Evaluation of the relative accumulation levels of BPMV (-) strand RNA1 and RNA2 in agro-infiltrated *Nb* leaves at 5, 10 and 15 dpi as measured by semi-quantitative RT-PCR with 24 cycles. *Nb* actin (NbACT) mRNA was also detected as control to ensure a similar account of RNA was used in all RT-PCR reactions. (C) *Nb* leaves were infiltrated with agrobacterium carrying RNA1-R2G or R1m-R2G constructs. Then one infiltrated leaf per treatment was collected at 5, 10, 15 dpi (a-f) and used to inoculate soybean plants. GFP fluorescence was observed on soybean local inoculated leaves at 5dpi (middle panel) and systemic leaves at 10dpi (bottom panel). (D) Western blot analysis of GFP protein extracted from leaves infiltrated with RNA1-R2G or R1m-R2G at 5, 10 and 15 dpi.
Figure 5.2 Nb-P19 is at least 1000 fold more resistance to BPMV than soybean. (A) A diagram of a dilution series generated by making continuous 1:10 dilutions. Each dilution was divided into two equal halves and used to inoculate BPMV host soybean leaves and nonhost Nb-P19 leaves. (B) Comparison of the susceptibility of two types of plants to BPMV. The number underneath represents the total number of GFP foci observed on respective inoculated leaves. NC: not countable. ILs: Local inoculated leaves. (C) BPMV-GFP systemic infection on wildtype Nb or Nb-P19 leaves at three weeks post inoculation (wpi). SL: systemic leave. The number represents the infection ratio of BPMV in Nb plants. 60 plants of each type of Nb were evaluated in this study.
**Figure 5.3** Evaluation of GFP expressions of pCAMBIA1300-sGFP-viral genes on Nb cells. (A) Schematic representation of pCAMBIA1300-based GFP expression constructs of BPMV proteins. Each viral gene was driven by 35S promoter while sgfp was driven by Gmubi promoter. (B) sGFP mRNA (top panel) and siRNA (middle panel) were analyzed by northern blot using five different sGFP oligos as specific radio labeled probe. Ethidium bromide staining of ribosomal RNA (bottom panel) provides a control for RNA loading.
BIBLIOGRAPHY


Carette J.E., Guhl K., Wellink J., Van Kammen A. (2002a) Coalescence of the sites of 

mosaic virus* 32- and 60-kilodalton replication proteins target and change the 

Chen J., Noueiry A., Ahlquist P. (2001) *Brome mosaic virus* Protein 1a recruits viral 

Dangl J.L., Jones J.D. (2001) Plant pathogens and integrated defence responses to 


Di R., Hu C.C., Ghabrial S.A. (1999) Complete nucleotide sequence of *Bean pod mottle
virus* RNA1: sequence comparisons and evolutionary relationships to other 


ARGONAUTE2 Mediates RNA-Silencing Antiviral Defenses against Potato virus X in Arabidopsis. Plant Physiology 156:1556-1564.


Sanfacon H., Wellink J., Le Gall O., Karasev A., van der Vlugt R., Wetzel T. (2009) Secoviridae: a proposed family of plant viruses within the order Picornavirales that combines the families Sequiviridae and Comoviridae, the unassigned genera


