농학박사학위논문

Pepper mottle virus의 증식 및 병징 발현 관련 바이러스인자의 분자생물학적 특성 구명

Molecular characterization of pepper mottle virus replication and symptom determinants

2023년 2월

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Molecular characterization of pepper mottle virus replication and symptom determinants

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by

MIAO FANG

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A THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Molecular characterization of pepper mottle virus replication and symptom determinants

UNDER THE DIRECTION OF DR. KOOK-HYUNG KIM

SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
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MAJOR IN PLANT MICROBIOLOGY
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Molecular characterization of pepper mottle virus replication and symptom determinants

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Major in Plant Microbiology

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ABSTRACT

Pepper mottle virus (PepMoV) is a destructive pathogen that infects various solanaceous plants, including pepper, bell pepper, potato, and tomato. In this study, I described the current understanding of the molecular characteristics of PepMoV and its interactions with host plants in Chapter I. In Chapter II, I characterized the virus multiplication and symptom determinants of PepMoV. There are 13 PepMoV isolates from nine regions of Korea, causing different symptoms on inoculated indicator host plants. To further identify the responsible symptom determinant(s) and explore viral protein functions of PepMoV, isolate 134 and 205136 were used in this study. Isolate 134 causes necrosis and yellowing, while 205136 causes severe mottle and yellowing symptoms on *Nicotiana benthamiana*. All chimeric and site-directed mutants contain the PepMoV 134 genome as a backbone with specific regions switched for those from

counterparts of PepMoV 205136. Results from my study provide direct evidence that the helper component-proteinase (HC-Pro) and the nuclear inclusion protein b (NIb)-coat protein (CP) regions are involved in virus accumulation and symptom determinants. In addition, I mapped to amino acid residues tyrosine, glycine, and leucine at positions 360, 385, and 527, respectively, in the HC-Pro region to participate in faster viral accumulation or movement in the plant. The residue valine at position 2773 of NIb plays an essential role in isolate 134 symptom development. As part of this study, I seek to gain insight into viral factors involved in the PepMoV infection cycle and a better understanding of plant-virus interactions. These findings complement the insufficiency of the gene function study of the PepMoV virus and provide a novel perspective for the protein function study of the Potyvirus.

Keywords: pepper mottle virus, HC-Pro, NIb, virus-host interaction, viral symptom determinant, *Nicotiana benthamiana*

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Chapter I

Pepper mottle virus and its host interactions:

Current state of knowledge

This chapter is a slightly modified version of the manuscript that previously has been published in Viruses

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ABSTRACT

Pepper mottle virus (PepMoV) is a destructive pathogen that infects various solanaceous plants, including pepper, bell pepper, potato, and tomato. In this study, I summarize what is known about the molecular characteristics of PepMoV and its interactions with host plants. Comparisons of symptom variations caused by PepMoV isolates in plant hosts indicates a possible relationship between symptom development and genetic variation. Researchers have investigated the PepMoV–plant pathosystem to identify effective and durable genes that confer resistance to the pathogen. As a result, several recessive *pvr* or dominant *Pvr* resistance genes that confer resistance to PepMoV in pepper have been characterized. On the other hand, the molecular mechanisms underlying the interaction between these resistance genes and PepMoV-encoded genes remain largely unknown. Our understanding of the molecular interactions between PepMoV and host plants should be increased by reverse genetic approaches and comprehensive transcriptomic analyses of both the virus and the host genes.

Keywords: pepper mottle virus; *Potyvirus*; pepper resistance gene; virus–host interaction

INTRODUCTION

Pepper mottle virus (PepMoV), which is in the genus *Potyvirus* and the family *Potyviridae* (Zitter, 1972; Valli et al., 2015), has been isolated from economically important solanaceous plants including pepper (*Capsicum* sp.), potato, and tomato in North America, India, and Asia (Zitter, 1972; Rodriguez-Alvarado et al., 2002; Verhoeven et al., 2002; Ogawa et al., 2003; Kim et al., 2009; Cheng et al., 2011; Melzer et al., 2012; Kaur et al., 2014). PepMoV is transmitted by several species of aphids but can also spread via mechanical inoculation, grafting, and infected seeds (Zitter, 1975; Sharma et al., 2018). PepMoV causes various symptoms in host plants, including severe or mild mottling mosaic, necrosis, vein clearing or necrosis, and leaf curling or yellowing (Han et al., 2006; Kim et al., 2008). When pepper plants are co-infected with PepMoV and cucumber mosaic virus, synergistic disease development is the result (Murphy and Kyle, 1995; Murphy and Bowen, 2006).

PepMoV has a single-stranded positive-sense RNA genome that is about 10 kb in length (Vance et al., 1992; Warren and Murphy, 2003; Kim et al., 2008). The PepMoV RNA is expressed as a large polyprotein, which is catalyzed and cleaved into smaller mature proteins in the host (Vance et al., 1992; Ala-Poikela et al., 2019). Variation in symptoms, pathogenicity, and molecular properties among PepMoV isolates in Korea suggested that certain virus-encoded proteins determine host specificity or pathogenicity

(Jonson et al., 2009; Kim et al., 2009; Shen et al., 2020). Recent studies of compatible/incompatible responses between PepMoV and host plants revealed dynamic interactions between PepMoV-encoded viral proteins and host proteins (Jiang et al., 2015; Kim et al., 2015; Tran et al., 2015). The development of full-length infectious clones of PepMoV has enabled researchers to investigate the interaction between PepMoV and hosts as well as to explore the feasibility of using PepMoV as a viral vector for the stable expression of heterologous genes in plants (Song and Ryu, 2017; Tran et al., 2019). Here, I review what is known about PepMoV with a focus on recent major findings concerning the interactions between PepMoV and its plant hosts.

DIVERSITY AND PATHOGENIVITY

PepMoV was reported first in Arizona and Florida in the early 1970′s and is considered as a species in the *Potyvirus* genus (lineage 5; (Wylie et al., 2017; Gibbs et al., 2020). Compared to potato virus Y (PVY), which is one of the major potyviruses infecting pepper, PepMoV forms relatively long and thin pinwheel inclusions in infected leaves and also differs in the symptoms it induces, its serological characteristics, the molecular weights of its proteins, and its nucleic acid contents (Purcifull et al., 1975; Hiebert and Purcifull, 1992; Han et al., 2006).

To date, PepMoV has been reported from many regions in North America, East Asia, and India, and full genome sequences of 23 isolates are available and sequences of 45 isolates are partially released at the NCBI database. A phylogenetic analysis based on the deduced amino acid sequences indicated that all 13 Korean isolates of PepMoV formed one cluster that was distinct from American isolates (Kim et al., 2009). A recent report indicated that full genome sequences of an isolate of PepMoV from Hunan China (PepMoV HN) are closely related to 18 PepMoV isolates from Korea that reported in previous studies (Kim et al., 2009; Lee et al., 2011; Zhang et al., 2019). However, when the authors of the latter study analyzed coat protein (*CP*) genes of nine PepMoV isolates from pepper in Southern China, they detected two distinct groups and subgroups.

Based on its genetic variation and on its symptom severity and pathogenicity on different host plants, researchers divided 13 Korean isolates of PepMoV into two groups

(Kim et al., 2008; Kim et al., 2009). Having acquired data on the highest ratio of synonymous (dS) to non-synonymous (dN) base substitutions for the P1 and 6K2 genes of PepMoV and on amino acid (aa) variation encoded by the 6K2 gene, Kim et al. suggested that the P1 and 6K2 genes might be involved in PepMoV host specificity and pathogenicity (Kim et al., 2009). As discussed later in this study, Kim et al. also described a system that could be used to identify viral-encoded proteins affecting pathogenicity and host specificity.

GENOME ORGANIZATION

Like all potyviruses, PepMoV forms a flexuous rod-shaped virion that consists of about 2000 copies of CP subunits with an Mr of 30.8 kDa [(Revers and García, 2015). The virion contains a positive-sense single-stranded viral genomic RNA with a genome-linked protein (VPg) at its 5′-terminal end and a poly(A) tail at its 3′-terminal end (Vance et al., 1992; Revers and García, 2015). The PepMoV genome is translated into a large polyprotein that is catalyzed by three potyvirus proteases (P1, HC-Pro, and NIa) and catalyzed into 10 mature viral proteins (P1, HC-Pro, P3, 6K1, CI, 6K2, VPg, NIa, NIb, and CP) (Vance et al., 1992; Warren and Murphy, 2003). The existence of a short open reading frame, termed P3N-PIPO, embedded within the P3-encoding region of the polyprotein is a universally conserved feature and has conserved coding capacity throughout the genus (Urcuqui-Inchima et al., 2001; Chung et al., 2008; Mäkinen and Hafrén, 2014) (Figure 1A).

Both of approaches described in the previous paragraph have been used to construct full-length cDNA clones of PepMoV. A Korean isolate of PepMoV (PepMoV-Vb1) was cloned downstream of a bacteriophage SP6 promoter and was tagged with green fluorescent protein (GFP), which was inserted between the coding regions for NIb and CP in the plasmid (Lee et al., 2011). In vitro RNA transcripts from this clone were infectious and stably expressed GFP in tobacco and pepper plants (Lee et al., 2011). Although infectious clones are useful and fundamental

tools in studying virus–host interactions, some challenges remain in constructing and stably delivering infectious clones into host plant using *E. coli*-based plasmids. For example, toxicity to *E. coli* has been reported for plasmids containing full-length clones of several viruses including citrus tristeza virus, tobacco etch virus (TEV), and influenza A virus; such toxicity makes it difficult or even impossible to use the clone for molecular manipulation (Satyanarayana et al., 2003; Bedoya and Daròs, 2010; Gao et al., 2012). During their modification and construction, full-length clones in *E. coli* have also been reported to be unstable for plant RNA viruses including potyviruses (Johansen, 1996; López-Moya and García, 2000; Olsen and Johansen, 2001; Bedoya and Daròs, 2010; Tuo et al., 2015) and tobraviruses (Ratcliff et al., 2001; Constantin et al., 2004). To reduce or minimize undesired toxic effects or instability in *E. coli*, insertion of introns into the viral genome has been extensively used (Johansen, 1996; López-Moya and García, 2000). For example, insertion of the potato ST-LS1 intron 2 sequence into the NIa coding region of PepMoV increased the stability of the infectious clone (Tran et al., 2019). Relative to traditional pPepMoV infectious clones, the modified infectious clone inserted plant intron into pPepMoV could restore infectivity and maintain plasmid stability. *Agrobacterium*-mediated inoculation of this modification clone showed faster symptom induction compared to non-modified clones when the same amount of *Agrobacterium* cell suspension was inoculated in plants (Tran et al., 2019). In addition, the resulting symptom intensity was similar to that following sap inoculation (Tran et al., 2019).

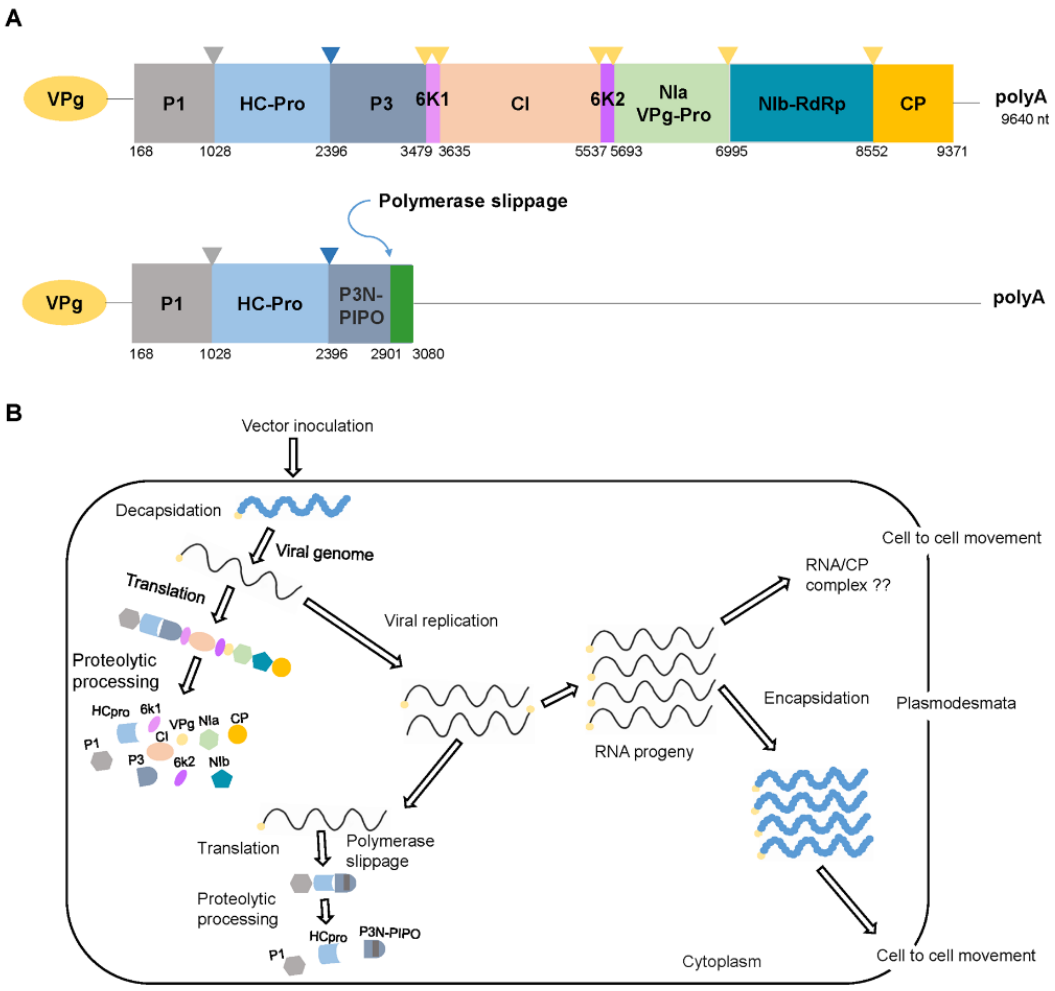

Figure 1 Genome organization and schematic representation of replication of pepper mottle virus (PepMoV). (**A**) The genomic maps of PepMoV. The genome is translated into a large polyprotein that is catalyzed by three potyviral proteases (P1, HC-Pro, and NIa) and cleaved into 10 mature viral proteins (marked in different colors). The next depicted represents a short open reading frame, termed P3N-PIPO, embedded within the P3-encoding region of the polyprotein. (**B**) Schematic representation of replication in a plant cell. The cycle begins (left upper corner) when the viral particle or RNA enters the cell from infected cells or initially

inoculated by its vector. The genomic RNA undergoes decapsidation, translation, and proteolytic processing to generate mature proteins. The replication complex uses the positive genomic RNA to generate a complementary negative genomic RNA, which functions as a template for the synthesis of numerous genomic RNAs. After replication, the progeny RNAs can be encapsidated and acquired by vectors to be transmitted again, or they can move to adjacent cells through plasmodesmata.

REPLICATION AND MOVEMENT: FUNCTIONS OF VIRAL PROTEINS

In research on the role of virus-encoded proteins during the virus infection process, use of the infectious full-length cDNA clones has provided reliable information on viral RNA replication and movement (Dolja et al., 1992; Deng et al., 2015). Almost all of the potyviral proteins are involved in viral replication (Revers and García, 2015) (Figure 1B). For example, the potyviral proteins HC-Pro, CI, VPg, NIb, and CP have multiple functions during viral infection, and CI, CP, HC-Pro, VPg, and P3N-PIPO have been implicated in viral intercellular movement (Deng et al., 2015). The protein functions of PepMoV are largely unknown. However, the possible roles of PepMoV-encoded proteins could be expected from the reported functions of the other closely related potyviruses.

The P1 protein of potyviruses is a chymotrypsin-like serine proteinase that cleaves itself at C-terminus (Quenouille et al., 2013; Revers and García, 2015). P1 is the most divergent and variable protein among potyvirus- encoded proteins (Yang et al., 2021). The TEV P1 protein has been shown to function *in trans* to stimulate genome amplification (Verchot and Carrington, 1995). The function of clover yellow vein virus P1 has been reported for its involvement in eIF4E-mediated recessive resistance (Nakahara et al., 2010). The potato virus V (PVV) P1 does not have direct association with RNA silencing suppression, but self-cleavage activity of P1 affects RNA silencing suppression indirectly by modulating function of HC-

Pro (Pasin et al., 2014).

The HC-Pro is a cysteine protease and well-established multitasking protein that is involved in many potyviral infection processes such as aphid-mediated transmission, RNA silencing suppression, genome replication, symptom expression, and long-distance movement (Maia et al., 1996; Hasiów-Jaroszewska et al., 2014). HC-Pro could interact with several other potyviral proteins and many host factors (Yang et al., 2021). Two conserved motifs, i.e., the N-terminal 'KITC' and the C-terminal 'PTK' motifs, have been identified in HC-Pro (Blanc et al., 1998). Site-directed mutation replacing lysine to glutamic acid (K59E) within the KITC motif using several PVY isolates abolishes the interaction of HC-Pro with aphid stylets and aphid transmissibility of PVY (Blanc et al., 1998). It is reported that the PVY HC-Pro interacts with three *Arabidopsis* 20S proteasome subunits (PAA, PBB, and PBE), which is related to the antiviral response (Jin et al., 2007). In addition, HC-Pro from three potyviruses, including potato virus A (PVA), PVY, and TEV could interact with the eukaryotic translation initiation factors (eIF4E) and eIF(iso)4E of *Nicotiana tabacum* and eIF(iso)4E and eIF4E of potato suggesting possible new role(s) in potyvirus infection cycle (Ala-Poikela et al., 2011).

The protein P3 is also one of the well-characterized multifunctional potyviral proteins. Dual roles of TEV P3 in virus movement and replication have been reported (Langenberg and Zhang, 1997; Cui et al., 2010). A polymerase slippage mechanism on P3 cistron leads to the production of P3N-PIPO (Chung et al., 2008), which localizes at PD and involves in the viral cell-to-cell movement in conjunction with CI protein

(Wei et al., 2010). P3 plays crucial roles as virulence and symptom determinants (Jenner et al., 2003).

The 6K1 of plum pox virus (PPV) is required for viral replication and is a necessary viral element of the viral replication complexes (VRC) at the early infection stage (Cui and Wang, 2016).

The multifunctional CI protein, as part of the VRC, participates in viral genome replication. In addition to replication, it also functions in viral cell-to-cell and long-distance systemic movement, probably by interacting with the recently reported viral P3N-PIPO protein (Wei et al., 2010; Sorel et al., 2014a; Deng et al., 2015). There is genetic evidence suggested that CI protein of TEV interacts directly with plasmodesmata and CP-containing ribonucleoprotein complexes to facilitate intracellular movement (Carrington et al., 1998). The lettuce mosaic virus CI has been shown to interact with the viral VPg and with lettuce eIF4E (Tavert-Roudet et al., 2012) and involved in the eIF4E-mediated resistance-breaking (ABDUL-RAZZAK et al., 2009; Sorel et al., 2014b).

The potyviral 6K2 protein has been found to be involved in long-distance movement and symptom development (Spetz and Valkonen, 2004; Jiang et al., 2015; González et al., 2019). In addition, potyviral 6K2 exhibits critical components of the VRC with NIb, HC-Pro, P3, CI, and NIa (Shen et al., 2020). The potyviral 6K2 induces proliferation of ER membrane for construction of VRCs at ER exit sites in cellular coatomer protein I- and II-dependent manner (Wei and Wang, 2008; Jiang et al., 2015). The TEV 6-kDa protein is membrane associated and has been shown to

be necessary for virus replication (Restrepo-Hartwig and Carrington, 1994). It contains transmembrane (TM) domain at N-terminal region and putatively luminal domain at C-terminal region (Lerich et al., 2011; Lõhmus et al., 2016). The TM domain of potyviral 6K2 protein is typically required for targeting and anchoring to the ER membrane (Lõhmus et al., 2016). The N-terminal region of TEV 6K2 includes a D(X)E motif which is crucial for ER exit of the 6K2-induced replication vesicles (Lerich et al., 2011).

Potyvirus VPg contains two nuclear localization signals (NLSs) and nucleotide triphosphate binding motifs (Jiang and Laliberté, 2011). VPg is required for several viral processes, including translational initiation of viral RNA and replication (Jiang and Laliberté, 2011). Potyviral VPg can form an intrinsically disordered state of the protein and this structural flexibility provides accessible interaction complexes with different virus or host proteins to enable its diverse functions (Jiang and Laliberté, 2011; Yang et al., 2021). Including PVA VPg, it requires host eIF4Es to promote viral RNA replication as well as the viral translation products (Eskelin et al., 2011). In contrast, interaction between VPg and host eIF4E and eIF(iso)4E might be involved in translation inhibition in host cellular mRNAs (Eskelin et al., 2011; Coutinho de Oliveira et al., 2019). VPgs in PVY, TEV, and turnip mosaic virus (TuMV) can inhibit cellular cap-dependent translational initiation in vitro through binding with eIF4E or eIF(iso)4E (Grzela et al., 2006; Khan et al., 2008; Miyoshi et al., 2008). The interaction between VPg

and eIF4E is also related to recessive resistance response against several potyviruses (Kang et al., 2005a; Wang and Krishnaswamy, 2012). TuMV 6K2–VPg–NIa complex, membrane-associated precursor form, is found within vesicular structures derived from the ER where replication might occur (Dufresne et al., 2008). TuMV VPg also has RNA silencing suppressor activity by inducing degradation of suppressor of gene silencing 3 (SGS3), which is involved in RNA silencing pathway (Cheng and Wang, 2017).

Potyvirus NIa-Pro is a cysteine protease and generally functions proteolytic processing of the potyviral polyprotein (Yang et al., 2021). NIa-Pro accesses differential cleavage efficiency, and it affects host range and viability of potyviruses (Rodamilans et al., 2018). Previous studies have described how PVY and PepMoV NIa were able to elicit *Ry*-mediated HR in *Solanum stoloniferum* by sharing the same recognition/cleavage site for NIa (Mestre et al., 2000). Expression of NIa-Pro interferes ethylene signaling pathway and enhances aphid fecundity in TuMV-infected *Arabidopsis* (Casteel et al., 2015). NIa-Pro relocalization from cytoplasm and nucleus to the vacuole of the cell during TuMVand PVY infections when in the presence of the aphid vector has reported (Bak et al., 2017). This relocalization confers the ability to promote vector performance to potyvirus NIa-Pro (Bak et al., 2017). Recent study suggested that PepMoV NIa was involved in pathogenicity and suppression of host antiviral defense response (Gong et al., 2020).

NIb of potyviruses acts as an RNA-dependent RNA polymerase (RdRp) or RNA replicase, and is therefore required for potyviral genome replication (Shen et

al., 2020). Beyond its major role as an RdRp during viral infection, NIb also has additional functions such as a recruiter that interacts with many pro-viral host factors participated in the assembly and activation of the VRC, a suppressor of host defense response, a target of host antiviral defense, and an elicitor that activates effector-triggered immunity (ETI) (Shen et al., 2020). One of the characterized functions of PepMoV NIb was associated with *Pvr4*- or *Pvr9*-mediated hypersensitive response (HR) (Kim et al., 2015; Tran et al., 2015).

The potyviral CP has also been reported to participate in the regulation of viral RNA replication (Revers and García, 2015; Kežar et al., 2019). Potyvirus CP is indispensable for viral intra- and intercellular movement (Dolja et al., 1995; Martínez-Turiño and García, 2020). Including PVA CP, the CP-vRNA interaction regulates virion assembly/disassembly and coordinates switch between viral RNA translation and replication (Besong-Ndika et al., 2015; Yang et al., 2021). Both terminal regions of the PVY CP have a crucial role in PVY infectivity. However, only the N-terminal region of CP is essential for virus-like particle (VLP) formation (Kežar et al., 2019). Recent findings for TuMV CP also suggest functions of the N-terminal region of CP in virion maturation and/or termination of virion formation. However, several studies demonstrated that the involvement of the N-terminal of potyviral CP in cell-to-cell movement and systemic infection varies from virus-to-virus (Dai et al., 2020). N-terminal domains of TuMV and zucchini yellow mosaic virus CPs were dispensable for viral cell-to-cell and long-distance movements. However, the same regions in TEV and PVY are necessary for

establishing cell-to-cell movement and systemic infection (Dolja et al., 1994; Arazi et al., 2001; Kežar et al., 2019; Dai et al., 2020). In contrast, C-terminal regions of TuMV, PVY, and TEV CPs were shown to be associated with viral cell-to-cell and long-distance movement (Arazi et al., 2001; Besong-Ndika et al., 2015; Martínez-Turiño and García, 2020). The aromatic residue tryptophan at core domain (W^{122}) of CP in tobacco vein banding mosaic virus, which is highly conserved residue among potyviruses, plays a role in maintaining stability of CP during viral replication and this involvement in viral cell-to-cell movement was also observed with the same residue of watermelon mosaic virus and PVY (Yan et al., 2021). An additional role as the pathogenicity determinant for CP has been reported in PVY (Baebler et al., 2020). The highly conserved DAG motif in the N-terminal domain of CP is responsible for aphid transmission by mediating the interaction between CP and HC-Pro (Llave et al., 2002; SEO et al., 2010).

So far, extensive research has mainly focused on the defense response-related PepMoV-encoded proteins and their corresponding host genes. Although functions of HC-Pro remain to be established in PepMoV, the protein is likely to be involved in replication and systemic movement. Likewise, PepMoV NIb might have roles in symptom developmentand virus replication, which is supported by my recent study.

RESISTANCE GENES AGAINST PEPMOV

Plants have evolved multi-layered systems to defend against viral invasion, including RNA silencing, regulation of RNA stability, ubiquitination-mediated protein degradation, autophagy, HR, *R* gene-mediated resistance responses, and systemic acquired resistance (Mandadi and Scholthof, 2013; Li and Wang, 2019; Yang et al., 2021). Several studies have described incompatible interactions between PepMoV and pepper plants; the host symptoms associated with those resistance responses have been used to identify efficient resistance (*R*) genes for application in plant breeding (Tran et al., 2014; Kim et al., 2015). Characterized host genes are listed in Table 1.

Table 1.Reported resistance genes against PepMoV.

Resistance Genes	Resistance Type	Target Virus [a]	Source	References
pvr1	Recessive	PepMoV, TEV and PVY	*Capsicum chinense* PI159236 and PI152225	(Yoon et al., 2020) (Liu et al., 2016)
pvr3	Recessive	PepMoV and TEV	*C. annuum* 'Avelar'	(Murphy et al., 1998)
Pvr4	Dominant	PepMoV and PVY	*C. annuum* 'CM334'	(Janzac et al., 2009)
Pvr7	Dominant	PepMoV	*C. chinense* PI159236	(Liu et al., 2016)
Pvr9	Dominant	PepMoV	*C. annuum* 'CM334'	(Tran et al., 2015)

[a]PepMoV, pepper mottle virus; TEV, tobacco etch virus; PVY, potato virus Y.

I. Recessive Resistance Genes

In incompatible interactions between a plant virus and host, resistance responses can be mediated by recessive or dominant host genes (Figure 2) (Kang et al., 2005a; Robaglia and Caranta, 2006; Wang and Krishnaswamy, 2012; Tran et al., 2015). Recessive resistance genes are produced by the loss or mutation of a host factor that has an important function in disease development (Murphy and Kyle, 1995; Kyle and Palloix, 1997; Mandadi and Scholthof, 2013). Recessive resistance genes are thought to be more durable and to provide more broad-spectrum resistance than dominant *R* genes (Pavan et al., 2010). Recessive resistance genes are more common than dominant resistance genes, especially against potyvirus infections, and function at single-cell level and thereby limit cell-to-cell movement (Kang et al., 2005a).

In *Capsicum* spp., *pvr1* and *pvr3* have been characterized as two unlinked recessive loci that confer distinct kinds of resistance to PepMoV (Murphy et al., 1998). The *pvr1* gene, which was identified in *C. chinense* PI159236 and PI152225, confers relatively broad resistance to PepMoV, TEV, and PVY (Kang et al., 2005a; Yoon et al., 2020). In contrast, the *pvr3* gene, which was identified in *C. annuum* 'Avelar', confers a different type of resistance to PepMoV than to TEV and PVY (Murphy et al., 1998). The mechanisms of resistance responses in *Capsicum* spp. against PepMoV differ depending on whether the response is *pvr1*- or *pvr3*-mediated (Murphy and Kyle, 1995; Murphy et al., 1998). *C. chinense* PI 152225 and PI 159236, which contain *pvr1,* do not support replication of PepMoV at the cellular level, whereas *C. annuum* 'Avelar', which contains *pvr3* allows for

PepMoV accumulation in inoculated leaves and its movement into the vascular system but not its spread into upper leaves (Murphy and Kyle, 1995; Murphy et al., 1998; Guerini and Murphy, 1999). However, this restriction of systemic movement was collapsed when PepMoV was co-infected with cucumber mosaic virus (Murphy and Kyle, 1995; Guerini and Murphy, 1999). Other recessive genes, i.e., *pvr2* and *pvr6*, that are located on the pepper chromosomes 4 and 3, respectively, confer digenic recessive resistance to another pepper potyvirus, pepper veinal mottle virus (Ruffel et al., 2006). However, the effect of *pvr2* and *pvr6* in response to the infection of PepMoV remains to be determined.

The eukaryotic translation initiation factors (eIF4Es) have been identified and cloned from diverse hosts as resistance genes that are natural, recessive, and inherited (Figure 2A) (Yeam et al., 2005; Wang and Krishnaswamy, 2012). The *pvr1* locus encodes an eIF4E homolog, and *pvr6* is expected to encode eIF(iso)4E (Kang et al., 2005a). Recessive resistance against several potyviruses in plant hosts is conditioned by mutations in eIF4E and its isoforms (Ruffel et al., 2005; Robaglia and Caranta, 2006; Wang and Krishnaswamy, 2012). Using transgenic tomato progeny with ectopic expression of the *pvr1*, researchers documented dominant resistance to several potyviruses, including PepMoV and TEV (Kang et al., 2007). Moreover, resistance induced by mutation of eIF4E1 in tomato, obtained by TILLING platform or by Clustered Regularly Interspaced Palindromic Repeats/CRISPR-associated protein 9 (CRISPR/Cas9)-mediated targeted mutagenesis, was enhanced against PepMoV but not against TEV (Gauffier et al., 2016; Yoon et al., 2020). These studies

indicated that *pvr1* or eIF4E greatly affects the resistance/susceptibility to plant viruses, especially to PepMoV, although the specific mechanism is unclear. In general, previous studies have suggested that eIF4E is also required for cell-to-cell movement and viral RNA replication during potyvirus infections (Kang et al., 2005a; Kang et al., 2005b; Wang and Krishnaswamy, 2012). The interaction between eIF4E and potyviruses will be discussed later in this study.

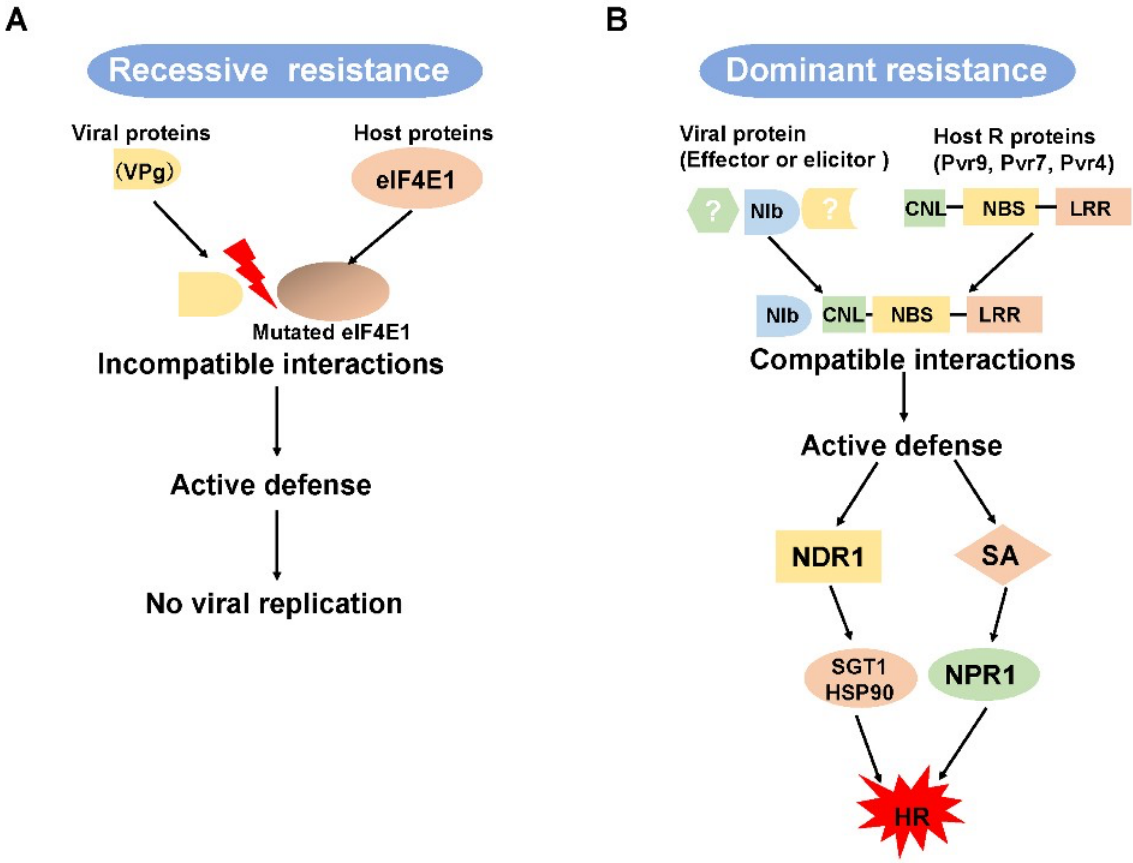

Figure 2. A scheme of host genes that may mediate recessive and dominant resistance and an explanation of the R protein-mediated signaling pathway. (**A**) Recessive resistance results from a host factor, the loss or mutation of which causes an incompatible interaction between a viral protein and a host protein. (**B**) Dominant resistance results from a compatible interaction between a viral effector and plant R proteins. Pvr9-mediated hypersensitive response requires several proteins, like NDR1 and the SGT1-HSP90 complex. Pvr9-mediated HR might also involve the SA pathway. NBS, nucleotide-binding site; LRR, leucine-rich repeat; CC, coiled-coil motif; HR, hypersensitive response; SA, salicylic acid.

II. Dominant R Genes

Dominant *R* genes, corresponding to pathogen effector-encoding or avirulence (*Avr*) genes, confer an active resistance resulting in the development of an HR that limits pathogen spread (de Ronde et al., 2014; Baebler et al., 2020) or that provides extreme resistance (ER) to a broad range of potyviruses (Collmer et al., 2000; Zhang et al., 2012; Baebler et al., 2020). The major class of *R* genes encode proteins consisting of a nucleotide-binding site (NBS), a leucine-rich repeat (LRR) region at the C-terminal, and Toll/Interleukin-1 receptor homology or a coiled-coil (CC)-domain at the N-terminal end (Figure 2B) (de Ronde et al., 2014).

Dominant resistance genes such as *Pvr4*, *Pvr7*, and *Pvr9* confer HR against potyviruses in pepper (Janzac et al., 2009; Tran et al., 2015; Liu et al., 2016). The *Pvr7* gene from *C. chinense* PI159236 and the *Pvr4* gene from *C. annuum* 'CM334' confer ER to PVY and PepMoV. *Pvr7* was tentatively re-designated as *Pvr4* in recent study (Venkatesh et al., 2018).

Pvr4 encodes a coiled-coil nucleotide-binding leucine-rich repeats (CNLs)-type protein, and ectopic expression of *Pvr4* in *N. benthamiana* confers resistance against PepMoV (Kim et al., 2017). Kim et al. found that *pvr4* in the susceptible allele from *C. annuum* 'ECW' had higher similarity with the coiled-coil nucleotide-binding domain than with the LRR domain, which might be involved in specific recognition of Avr factors (Mondragón-Palomino et al., 2002; Goritschnig et al.,

2016). Researchers have demonstrated that the NIb of several potyviruses including PepMoV serves as an avirulence factor for *Pvr4* in pepper. The NIb of PepMoV, pepper severe mosaic virus, and PVY induced an HR, but the NIb of TEV could not induce HR-like cell death in *Pvr4*-bearing pepper. Kim et al. suggested that the differences in resistant responses among four potyviruses might be related to the low sequence identity of NIb with TEV compared with that of other potyviruses (Kim et al., 2015).

Another *R* gene, *Pvr9*, is orthologous to *Rpi-blb2* of *Solanum bulbocastanum* and was isolated via screening of Agrobacterium-based transient expression of candidate *R* genes that were able to induce an HR upon PepMoV infection in *N. benthamiana* (Tran et al., 2014). *Pvr9* is expected to be located on pepper chromosome 6, and encodes 1298 amino acids that contain CNLs-type protein domains (Tran et al., 2015). PepMoV infection in pepper resulted in a minor increase in *Pvr9* gene expression in the resistant cultivar *C. annuum* 'CM334' but in a slightly reduced expression of the susceptible allele in the susceptible cultivars *C. annuum* 'FloralGem' (Tran et al., 2015). Tran et al. also demonstrated that PepMoV NIb elicits the *Pvr9*-mediated HR, which is similar to the *Pvr4*-mediated HR (Kim et al., 2015; Tran et al., 2015).

CHARACTERIZATION OF INTERACTING VIRUS AND HOST FACTORS

I. Host Responses upon PepMoV Infection

Previous research has demonstrated that virus infection of plants affects host gene expression and metabolism, which results in altered host development and growth defects (Whitham et al., 2006). The changes in host gene/protein expression depend on whether the interaction is compatible or incompatible but also varies with plant species (Whitham et al., 2006). In early interactions between potato and PVY, for example, comparative transcriptomic analysis showed that transcriptional changes in compatible and incompatible reactions in one host shared more overall similarities in the response to PVY inoculation than compatible reactions between two different hosts (Goyer et al., 2015). The latter study also showed that a different cascade of molecular changes was triggered by two different PVY strains (Goyer et al., 2015). Although these previous studies documented changes in global gene/protein expression and in pathways in diverse host species following infection by different viruses, little is known about host responses to PepMoV infection under different conditions at a genome-wide level.

To identify pathways related to the *Pvr9*-mediated HR against PepMoV infection, researchers silenced selected genes using tobacco rattle virus-based virus-induced gene silencing and thereby assessed their functions (Tran et al., 2016). The

results showed that *Pvr9*-mediated HR requires the host genes *HSP90*, *SGT1*, *NDR1*, and *NPR1* genes but not the *EDS1* gene (Tran et al., 2016). This indicated that *Pvr9*-mediated HR might involve the salicylic acid (SA) pathway but not the jasmonic acid (JA), ethylene (ET), reactive oxygen species (ROS), or nitric oxide (NO) pathways (Figure 1) (Tran et al., 2016). Further research is needed to clarify the role of the SA pathway in *Pvr9*-mediated HR and the contribution of the HSP90-SGT1 complex to plant immunity against PepMoV.

Recent research showed that potato virus X vector-mediated expression of PepMoV NIa, which is highly conserved among potyviruses, resulted in severe mosaic symptoms and triggered a HR. In the latter study, Gong et al. observed significantly increased expression levels of host genes including the ER-localized binding protein (Bip) and heat shock protein 90-2 (HSP90-2) in NIa-expressed plant, whereas the expression of the basic leucine zipper protein 60 (bZIP60) was not changed by NIa expression. Given that BIP and HSP90-2 are required for the stabilization of many proteins in response to endoplasmic reticulum (ER) stress, the authors suggested that NIa might induce ER stress (Gong et al., 2020).

II. PepMoV–Host Interaction: Avirulence and Virulence Genes

Although the exact mechanism by which eIF4E mutations control resistance remains to be elucidated, protein–protein interaction(s) between viral elicitor(s) and the host receptor(s) might contribute to the resistance responses. In this regard, it is noteworthy that the potyviral protein VPg, which is required for viral infection,

interacts with eIF4E to induce infection; mutations in eIF4E. However, prevent VPg binding and thus inhibit viral infection, resulting in a resistance response (Kang et al., 2005b; Wang and Krishnaswamy, 2012). At the same time, amino acid substitutions in VPg that restore its binding to the mutated eIF4E can break down the resistance (Ala-Poikela et al., 2019; Yang et al., 2021). Continuous co-evolution between viral effectors and their host counterparts has apparently resulted in the diversification of both genes.

Mutation of eIF4E affects the infectivity of PepMoV in tomato (Yoon et al., 2020), but there is no biological evidence for a correlation among mutated eIF4E and viral proteins of PepMoV. In the case of PVA, VPg and HC-Pro interact with each other and with eIF4E and eIF(iso)4E proteins (Ala-Poikela et al., 2011; Ala-Poikela et al., 2019). A recent study revealed that HC-Pro and VPg can both interact through the eIF4E-binding motif YXXXXLΦ, which is similar to the motif in eIF4G (Ala-Poikela et al., 2019). In the latter study, Ala-Poikela et al. analyzed and compared the central region of VPg that contains a putative 4E-binding motif among 40 potyviruses (Ala-Poikela et al., 2019); they found a putative eIF4E-binding motif in the VPg of PepMoV (YADIVDV), but that motif is slightly different from that of PVA (YTDIRLI), which is similar to the eIF4E-binding motif in the VPg of PVY (YADIRDI) (Cheng et al., 2011).

Several potyvirus proteins have also been identified as elicitors of resistance or determinants of avirulence, and these correspond to dominant resistance proteins in plants (Huang, 2021). As previously noted, the NIb of PepMoV serves as an avirulence factor for *Pvr4* in pepper plants (Kim et al., 2015). However, an interaction between

Pvr9 and its elicitor or so-called avirulence factor NIb of PepMoV was not detected in the model plant *N. benthamiana* (Tran et al., 2015). The authors of the latter study suggested that the interaction depended on a third unknown factor that was present in *N. benthamiana* but not in pepper.

An HR was also triggered when *Pvr9* was co-expressed with NIbs from PepMoV, PVY, PVA, and turnip mosaic virus, but not with NIbs from zucchini yellow mosaic virus or soybean mosaic virus (Tran et al., 2015). Although evidence was lacking for the direct binding between *Pvr9* and NIb in yeast or in plants, the mutational analyses suggested their possible relationship between *Pvr9* and NIb (Tran et al., 2015). The amino acid substitutions E492G, V701E, F1117S, and R1160K in *Pvr9* failed to trigger an NIb-elicited HR in plants, while internal regions of NIb (the residues 186–235 and 370–445) are essential for NIb elicitor activity (Tran et al., 2015).

III. PepMoV–Host Interaction: Viral RNA Silencing Suppressors

In many potyviruses, VPg and especially HC-Pro help block or interfere with RNA silencing (Yang et al., 2021). A recent study showed that treatment with dsRNA targeting HC-Pro or NIb inhibited PepMoV accumulation in *N. benthamiana* (Yoon et al., 2021). However, it was not clear whether this inhibitory effect was caused by reducing expression of these target genes.

As noted earlier, the use of a potato virus X-based NIa expressing vector

indicated that PepMoV NIa might be responsible for symptom development in *N. benthamiana* (Gong et al., 2020). In addition, Gong et al. found that PepMoV NIa functions as a potent suppressor of host transcriptional gene silencing by negatively affecting the DNA methylation pathway in plant hosts (Gong et al., 2020). These results suggested that PepMoV NIa might inhibit global DNA methylation by regulating expression of essential genes involved in RNA-directed DNA methylation including *NbAGO4*, *NbMET1*, *NbDRM2*, and NbCMT3 (Gong et al., 2020).

GENOME-WIDE APPROACHES FOR IDENTIFYING ADDITIONAL HOST FACTORS

In the last decade, many researchers studied plant–virus interactions by focusing on genome-wide expression patterns of host and virus genes (Zanardo et al., 2019). The genome-wide analyses, especially of transcriptomic data, have allowed researchers to predict some of the major biological processes that are affected by virus infection and to detect genes that are differentially expressed under specific conditions or during different stages of virus– plant interactions (Zanardo et al., 2019). In *C. annuum* 'Zunla-1' pepper plants, for example, transcript profiles of CMV-Fny infected leaves showed different expression patterns at different time points (Zhu et al., 2018). Kim et al. reported the comprehensive transcriptomic profiling obtained from *C. annuum* at different time points after infection by *Phytophthora infestans*, PepMoV, or tobacco mosaic virus (Kim et al., 2018). Further detailed analysis based on global transcriptomic data will be useful for identifying host factors involved in infection or resistance to infection and for elucidating host molecular networks that respond to virus infection.

CONCLUDING REMARKS AND FUTURE PROSPECTS

PepMoV is one of the most important pathogens of solanaceous vegetables worldwide. Although the roles of each encoded PepMoV gene can be inferred by comparison with analogous genes in other potyviruses, the role(s) or function(s) of each PepMoV protein remain poorly characterized. Use of a PepMoV infectious clone will help researchers to identify the determinants of PepMoV pathogenicity/virulence and to understand the replication and movement of the virus in infected host plants.

In this study, I disclosure that the HC-Pro and the NIb regions are involved in PepMoV accumulation and symptom determinants. In addition, I characterized that amino acid residues tyrosine, glycine, and leucine at position 360, 385, and 527, respectively, in the HC-Pro region participate in faster viral accumulation or movement in the plant. The residue valine at position 2773 of NIb plays a crucial role in isolate 134 symptom development. As a part of my study, these results may provide new insight into viral vector involved in the PepMoV infection cycle and a better understanding of plant-virus interactions.

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Chapter II

Identification of viral genes involved in pepper mottle virus replication and symptom development in *Nicotiana benthamiana*

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ABSTRACT

Pepper mottle virus (PepMoV) infects primarily *Capsicum* species, including pepper and bell pepper which are important vegetable and spice crops in Korea. We have previously collected 13 PepMoV isolates from nine regions comprising five provinces, causing different symptoms on inoculated indicator host plants in Korea. To further identify the responsible symptom determinant(s) and explore viral protein functions of PepMoV, two out of 13 isolates, including 134 and 205136, were used in this study. Isolate 134 causes necrosis and yellowing, while 205136 causes severe mottle and yellowing symptoms on *N. benthamiana*. All chimeric and site-directed mutants contain the PepMoV 134 genome as a backbone with specific regions switched for those from counterparts of PepMoV 205136. Effects of all mutants compared with 134 after inoculation onto *N. benthamiana* by agroinfiltration. Results from my study provide direct evidence that the HC-Pro and the NIb-CP regions are involved in virus accumulation and symptom determinants. In addition, I mapped to amino acid residues tyrosine, glycine, and leucine at position 360, 385, and 527, respectively, in the HC-Pro region participate in faster viral accumulation or movement in the plant. The residue valine at position 2773 of NIb plays an essential role in isolate 134 symptom development. As part of this study, I seek to gain insight into viral factors involved in the PepMoV infection cycle and a better understanding of plant-virus interactions. These findings complement the insufficiency of the gene function study of the PepMoV virus and provide a novel perspective for the protein

function study of the *Potyvirus*.

Keywords: pepper mottle virus, HC-Pro, virus-host interaction, viral symptom determinant, *Nicotiana benthamiana*

INTRODUCTION

Pepper mottle virus (PepMoV), a member of the *Potyvirus*, the largest genus of plant RNA virus, causes significant losses of economically important crops (Rojas et al., 1997; Kim et al., 2008). PepMoV forms a flexuous rod-shaped virion with a length of 730 nm and a diameter of 12 nm containing a single-stranded plus sense RNA genome of 9.7 kb in length (Warren and Murphy, 2003; Kim et al., 2008). Viral genomic RNA, covalently linked to a viral-encoded protein (VPg) at the 5' end and containing a polyadenylated tail at the 3' end, encodes a large polyprotein cleaved by three virus-specific proteases to yield 11 mature proteins (Chung et al., 2008). As in all potyviruses, PepMoV encodes two polyproteins, a large polyprotein of approximately 3,068 amino acid residues and the shorter one translated from a 2+ frameshifting of the P3 cistron, as a fusion to the amino (N)-terminal part of P3 (P3N-PIPO) (Quenouille et al., 2013).

PepMoV infects most Capsicum sp. and is transmitted by aphids in a non-persistent manner in fields (Han et al., 2006). Our laboratory has previously isolated 13 PepMoV isolates, which were collected from nine regions comprising five provinces in Korea. These 13 PepMoV isolates caused different symptoms on indicator host plants, i.e., *Nicotiana tabacum* cv. Xanthi-nc and *N. benthamiana* (Kim et al., 2009). Among them, isolate 134 caused necrosis and yellowing symptoms in *N. benthamiana,* while isolate 205136 caused mild mottle symptoms in *N. tabacum* and severe mottling and yellowing symptoms in *N. benthamiana*.

Among research on identifying viral pathogenicity determinants, PVY is one of the intensively studied viral pathogens (Scholthof et al., 2011; Quenouille et al., 2013). The HC-Pro is the critical protein encoded by potyviruses, characterized in detail. The HC-Pro intervenes in several steps of the virus replication cycle: 1) genome multiplication and replication at the single-cell level; 2) cell-to-cell and systemic movements (Kasschau et al., 1997; Rojas et al., 1997; Sáenz et al., 2002); and 3) symptom intensity (Shiboleth et al., 2007; Torres-Barceló et al., 2008; Yambao et al., 2008). HC-Pro can be schematically divided into three regions: an N-terminal region essential for the aphid transmission process, a C-terminal region harboring the proteinase activity and the suppression of plant defenses based on the RNA silencing machinery by binding small interfering RNAs (siRNAs), and a central region implicated in all other functions (Plisson et al., 2003; Lakatos et al., 2006; Shiboleth et al., 2007; Varrelmann et al., 2007). These various properties could account not only for the general involvement of the potyvirus HC-Pro in symptomatology and synergy with co-infecting viruses for symptom severity but also for its roles in virus multiplication and systemic movement.

The NIa protease of potyviruses has been identified as a multiprotein that plays various roles in virus infection (Xiao et al., 2022). The tobacco etch virus (TEV) NIa has RNA binding activity; it can interact with NIb protein and contributes to viral accumulation (Schaad et al., 1996; Daròs and Carrington, 1997). NIa of papaya ringspot virus (PRSV) is involved in host specificity (Chen et al., 2008). The NIa of PepMoV has been reported as a pathogenicity determinant and contributed to releasing DNA

methylation of *N. benthamiana* (Gong et al., 2020). The NIb protein has been shown to act as the RNA-dependent RNA polymerase (RdRp) involved in the replication of viral RNA (Hong and Hunt, 1996). The NIb protein of TEV possesses several functions, including RdRp and nuclear translocation activities (Li et al., 1997). In addition, PepMoV NIb functions as an elicitor for the potyvirus-resistant 4 (Pvr4) and Pvr9-mediated hypersensitive responses (Kim et al., 2015; Tran et al., 2015). NIb from several other closely related potyviruses also elicited a similar hypersensitive response (Tran et al., 2015). The P3 protein of TuMV, which is an influential pathogen of *Brassica* species and other crops worldwide (Walsh et al., 2002), has been identified as a symptom and avirulence determinant in Brassicas. The viral RdRp or RNA replicase is expected to contribute to indirectly defining pathogenesis by affecting virus replication and thus virus accumulation (García and Pallas, 2015). Based on what has been discussed above with potyviruses, including PVY, TuMV, and TEV, the functions of their proteins might differ depending on the virus species; up to now, the protein functions of PepMoV remain largely unknown.

In terms of plant RNA viruses, *Potyvirus* is the largest genus; they have been well documented. However, those species' viral protein functions differ from virus to virus. Research on PepMoV and its protein functions are largely unknown. In this study, I revealed the crucial region(s) of PepMoV for symptom development and systemic infection using a full-length infectious clone of PepMoV 134 expressing green fluorescent protein (GFP; pPepMoV-134:GFP) (Tran et al., 2019). my results suggested

that the HC-Pro is involved in PepMoV genome replication and systemic movement, the C-terminal region of the NIb plays a vital role in developing viral symptoms and multiplication. In addition, amino acid substitution mutations revealed that the valine residue at position 2773 in the C-terminal region of the NIb protein is critical in causing different symptoms without noticeably affecting the systemic movement of the virus. Taken together, my results adequately fill in the large gaps in PepMoV viral protein functions. Additionally, my findings provide insight into viral proteins implicated in the PepMoV infection cycle and a novel perspective for the biology study of potyvirus.

MATERIALS AND METHODS

I. Plant and Virus Sources

N. benthamiana was used as an indicator host plant in this study. *N. benthamiana* seedlings were selected for inoculation when plants were three weeks old. *N. benthamiana* plants were grown in a growth chamber at 25°C under a 16/8 h (light/dark) photoperiod. The PepMoV 134 and 2051356 isolates have been described and characterized previously (Kim et al., 2009). Infectious full-length cDNA clones of PepMoV 134 (pPepMoV: GFP-134) were previously constructed (Tran et al., 2019). Isolate 205136 was propagated in *N. benthamiana* plants with infected tissue and collected virus-infected systemic leaves after symptom observation (Figure 1A).

II. Generation of Chimeric and Site-Directed Mutants

The complete genome sequence of isolate 205136 was divided into 6 fragments and exchanged individually with the corresponding pPepMoV:GFP-134 plasmid region. The main 11-kb section was divided using conserved restriction enzyme sites to make reciprocal exchanges between pPepMoV:GFP-134 and counterparts DNA fragments of isolate 205136 of the 1.8-kb *Bsp*120I- *Acc*65I, 4.2-kb *Acc*65I-*Pac*I, 2kb *Pac*I-*Sac*I, 2.8-kb *Sac*I-*Mss*I and 0.52-kb *Sac*I- *Mss*I and 2.5-kb *Mss*I-*Mlu*I fragments. Chimeras were constructed by exchanging counterparts between pPepMoV:GFP-134 and DNA fragment of isolate 205136 using available restriction enzyme sites common to the plasmid of

pPepMoV:GFP-134 and DNA fragments of isolate. The used restriction enzyme sites in the PepMoV genome are shown in Figure 1B.

Amplification of DNA fragments to produce chimeras and amino acid substitution mutants was carried out by PCR with virus-specific primer pairs. To introduce site-directed mutagenesis (SDM) in pPepMoV:GFP-134, DNA fragments of isolate 205136 were amplified for 40 cycles using PfuUltra II Fusion HS DNA polymerase (Agilent Technologies, U.S.A.) and appropriate primer pairs: mutant4 I2374V Fw and mutant4 I2374V Rv primers for the mutant4 I2374V, mutant4 V2773D Fw and mutant4 V2773D Rv primers for the mutant4 V2773D, mutant4 T2789A Fw and mutant4 T2789A Rv primers for the mutant4 T2789A, mutant4 T2805A Fw and mutant4 T2805A Rv primers for the mutant4 T2805A. The PCR products were purified through a DNA purification kit (NucleoSpin Gel and PCR clean-up Kit, MACHEREY-NAGEL, Germany). The mutagenized PCR fragments, amplified to contain the region from MssI to MluI sites, were digested with MssI and MluI and inserted into pPepMoV:GFP-134, which was digested with MssI and MluI. The sequences of all constructed chimeras and mutants were validated by DNA sequencing. All primers for constructing chimeras and amino acid substitution mutants are listed in Table 1.

III. Plant inoculation and virus assessment

To evaluate the effects of all modified pPepMoV:GFP-134 on *N. benthamiana*, all expression clones were prepared using a plasmid purification kit (MACHEREY-

NAGEL, Germany), including pPepMoV:GFP-134, then transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation device (ECM 830, BTX, USA). The presence of transformants in the *Agrobacterium* was detected by PCR with specific primers. The agroinfiltration was conducted as described previously (Yoon et al., 2021). The optical density of each bacterial suspension was measured at OD_{600} with a UV/visible spectrometer (Ultrospec 3100 Pro, Biochrom, Cambridge, England). Then the suspensions were diluted to the OD_{600} to 0.5. Fully expanded leaves of three-week-old plants were infiltrated by syringes to their backsides. The treated leaves were photographed with a digital camera (Nikon7200, Tokyo, Japan). Necrosis symptom differentiation was assessed visually at 12 dpi. PepMoV local and systemic movements on inoculated plants were verified by observing GFP expression under UV light at 5 and 8 dpi.

IV. Protoplast isolations

For protoplast isolation, inoculated with wild-type 134 and chimeric virus suspensions (OD_{600}=0.5) onto plants by agroinfiltration and then incubated for 2 hours in a growth chamber at 25°C. Protoplasts were isolated from infected sliced leaves (remove the petiole and midrib) and digested with 10ml enzyme solution (cellulose enzyme solution including 1% Cellulase R10 (Yakult), 0.5% Macerozyme R10 (Yakult), 0.45 M Mannitol and 20 mM MES (pH 5.7) for 14 to16 hours at 25°C, 10 rpm shaking for 10 mins before collection. And then, dilute the enzyme/protoplast solution with an equal

volume of W5 washing solution. Protoplasts were collected using filtration followed by centrifugation at 750 rpm 4°C for 3 mins in a swinging-bucket rotor. Rewash protoplast with 2 ml of W5 washing buffer, centrifuge at 750 rpm 4°C for 3 min, and then resuspend in 1 ml of WI incubation solution.

V. RNA Extraction and cDNA synthesis

Total RNAs were isolated from healthy and virus-infected *N. benthamiana* leaves using RNAiSO Plus reagent (TaKaRa) according to the manufacturer's instructions. The viral RNA extraction protocol of protoplast is referred from a previous study (Fabian and Andrew White, 2007).

Equal amounts of total RNAs (1 µg) were used for cDNA synthesis using M-MLV reverse transcriptase (Promega, USA) and oligo (dT_{15}) primer (Bioneer). cDNAs were used for virus detection and quantification of viral RNA accumulation for further experiments. PepMoV was detected by PCR using virus-specific primer pair (Table 1).

VI. RT-qPCR

RT-qPCR reactions were conducted using IQ™ SYBR Green Supermix (Bio-Rad, USA) based on manual instruction in a CFX 384 Real-Time PCR detection system (Bio-Rad, USA). The RT-qPCR analysis was conducted as previously reported (Widyasari et al., 2022). The endogenous gene actin was used as a reference gene to normalize the qPCR results. Melting-curve analysis was carried out using the Bio-Rad CFX manager

v.1.6.541.1028 software. Experiments were repeated three times with at least three replicate plants in three independent experiments. The primer sets used for qPCR are shown in Table 1.

Table 1 Primers and restriction enzymes used in this study.

Primer name	Sequence 5'-3'	Restriction enzymes	Purpose
pCAMBIA 6601 Fw	GCTGGCTGGTGGCAGGATA	Bsp120I Acc65I	Construction of 134_{P1}
P1 KpnI Rv	GATGAACGTTAACGGTACCCGAATACTGTTCCATATGAAGTACAGTTGC		
PepMoV 948 Fw	GTTCGCGGAAAATCGGATGG	Acc65I PacI	Construction of $134_{HC\text{-}Pro}$
PepMoV 2511 Rv	ACATGACACTTGGCCTGTAGATCCC		
PepMoV 2222 Fw	TGACGCAGAGCTGCCTCGTAT	PacI SacI	Construction of $134_{P3\text{-}CI}$
PepMoV 4727 Rv	TGCGGTGCCCTTCTGTATGCG		
PepMov 3940 Fw	ACGGCAGGGTTTTGCTGATA	SacI MssI	Construction of $134_{CI\text{-}NIa}$
PepMoV NIa-Intron2 ST-LS1 Fw	AACAGACAGGTTTGTTTCTGCTTCTACCTTTGAT		
Intron2 ST-LS1-PepMoV NIa Rv	GAGACACACCCTAAACATCACCATGTTTTGGTCA		Construction of 134_{NIa}
PepMoV 7262 Rv	TGCGTCGCAATCGACTACTCCT		
PepMoV 6988 Fw	GGGAGCAAGCACACACATCACCT	MssI MluI	Construction of $134_{NIb\text{-}CP}$
pSNU1 241 Rv	TCGCAAGACCGGCAACAGGA		

mutant4 I2374V Fw	**TATCATTAGAGTTGTTGTATACATGCAGA**	**MssI MluI**	**Construction of 134_{I2374V}**
mutant4 I2374V Rv	**TCTGCATGTATACAACAACTCTAATGATA**		
mutant4 V2773D Fw	**GAGCTTCAGGACTACCTCAGA**	**MssI MluI**	**Construction of 134_{V2773D}**
mutant4 V2773D Rv	**TCTGAGGTAGTCCTGAAGCTC**		
mutant4 T2789A Fw	**TTGAATGTGGTGCATATGAAGTTCA**	**MssI MluI**	**Construction of 134_{T2789A}**
mutant 4 T2789A Rv	**TGAACTTCATATGCACCACATTCAA**		
mutant4 T2805A Fw	**GATACATTGGATGCTGGAGAGGAGA**	**MssI MluI**	**Construction of 134_{T2805A}**
mutant4 T2805A Rv	**TCTCCTCTCCAGCATCCAATGTATC**		
PepMoV diag Fw	**ATGAGCAGCTCAAGATCAGATACATTG**		**PepMoV detection primer**
PepMoV diag Rv	**CATATTCCTGACCCCAAGCA**		**PepMoV detection primer**
PepMoV 5836 Fw	**GCACAACCGTTGGCATGGGC**		**PepMoV quantification in RT-qPCR**
PepMoV 5950 Rv	**TCCATTTGTGCACCTGTGAGTGG**		**PepMoV quantification in RT-qPCR**
Nb Actin real time Fw	**CCAGGTATTGCTGATAGAATGAG**		**Internal control for RT-qPCR**
Nb Actin real time Rv	**CTGAGGGAAGCCAAGATAGAG**		**Internal control for RT-qPCR**

VII. Statistical analysis

Experiments were conducted at least three times with three individual plants (biological replicates) in each experiment. Statistical analysis was performed using IBM SPSS statistics 26 for Windows software. The data were subjected to a one-way analysis of variance (ANOVA). The means of values were compared using Duncan's least significant range test ($p<0.05$). Graphs were generated using GraphPad Prism.

RESULTS

I. Symptom observations of chimeric mutants of PepMoV isolate 134 and 205136

The reported 13 PepMoV isolates caused different symptoms in *N. benthamiana,* respectively. In this work, I selected two isolates, 134 and 205136, which displayed visible and significantly different symptoms (Figure 1A). Based on a previous study (Tran et al., 2019), I used a full-length infectious clone of isolate 134 (pPepMoV:GFP-134) to investigate the differences in symptoms development caused by 134 and 205136 in detail; I constructed six PepMoV:GFP-134 chimeric clones whose specific viral region was precisely replaced with corresponding regions of PepMoV 205136 isolate. I designed chimeric regions based on available restriction enzyme sites of PepMoV-134 and 205136 viral cDNA for exchanging and generating chimeric clones from pPepMoV:GFP-134 as a backbone vector (Figure 1B). In addition, all chimeric mutants contained GFP downstream of the P1 region to evaluate GFP-expressing PepMoV in plants. A scale card was developed to classify mutants' differences in GFP fluorescence expression and symptom development. GFP fluorescence expression and symptom development for all mutants are summarized in Figure 1C.

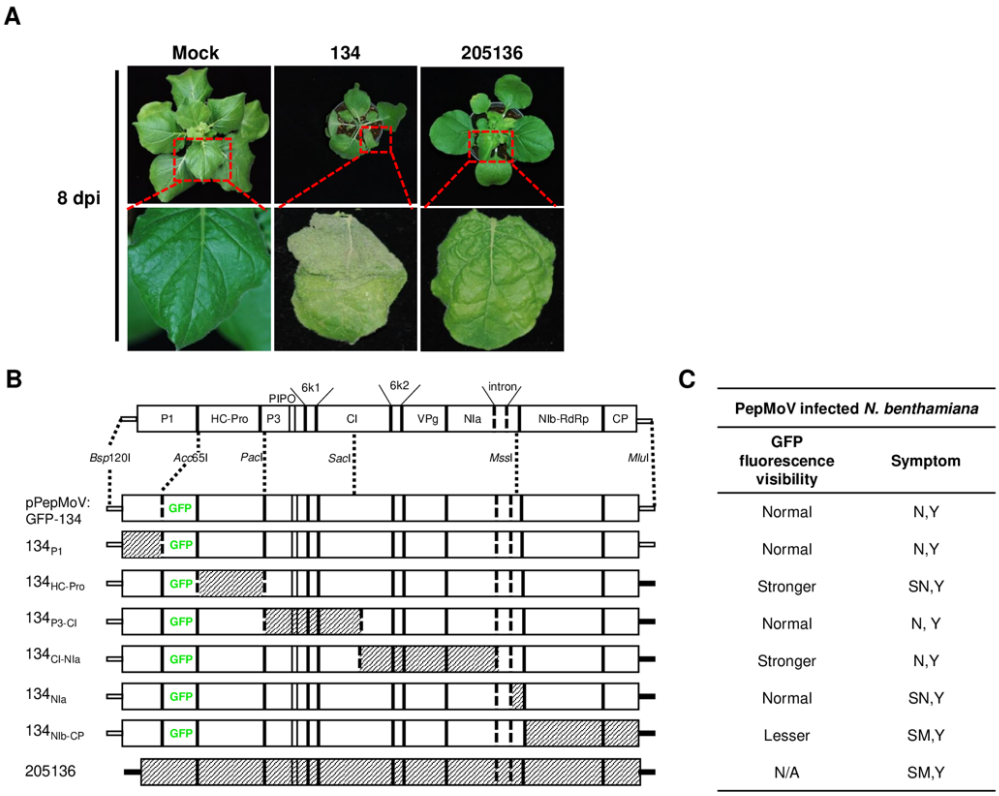

Figure 1. (A) Symptoms on *N. benthamiana* leaves infected with pepper mottle virus (PepMoV) isolates 134 and 205136. Results from upper systemic (non-inoculated) leave at 8 days post-inoculation (dpi) are shown. (B) Schematic representation of chimeric mutants of PepMoV isolates 134 and 205136. Chimeric mutants were constructed by substituting counterparts between 134 and 205136. pPepMoV:GFP-134 and 205136-derived regions are indicated by blank and hatched boxes, respectively. (C) Summary of 134 mutants shown in Figures 2 and 3. GFP fluorescence representing virus infection and symptoms of the other six chimeric viruses were compared with PepMoV isolate134. Viral accumulation was observed at 5 and 8 dpi, and symptom development was detected at 12 dpi. N, necrosis; Y, yellowing; SN, severe necrosis; SM, severe mottle; N/A, not available.

Symptom development of pPepMoV:GFP-134 and the chimeric mutants were monitored at 12 dpi. In the previous study, I confirmed consistent results in pPepMoV:GFP-134 infected *N. benthamiana* plants. pPepMoV:GFP-134 caused necrotic and yellowing symptoms on systemic leaves. Most symptoms appeared at 12 dpi in all tested plants except $134_{NIb\text{-}CP}$ infected plants. $134_{NIb\text{-}CP}$ was unable to display necrosis symptoms (Figure 2A). $134_{NIb\text{-}CP}$ infected plants did not show necrosis symptoms at 16 dpi. Similar to isolate 134 (pPepMoV:GFP-134), 134_{P1}, $134_{P3\text{-}CI}$, and $134_{CI\text{-}Nia}$ induced necrosis symptoms on local and systemic leaves. In contrast, $134_{HC\text{-}Pro}$ and 134_{NIa} caused more severe necrotic symptoms when compared with 134. These results indicated that NIb and/or CP proteins of PepMoV might be responsible for causing different symptoms of PepMoV. Additionally, the HC-Pro and C-terminal of NIa may participate in causing necrosis symptoms of PepMoV.

Serial passaging experiments were performed by sap inoculation to determine the stability of the altered symptomatology for six mutants. Inoculation of sap extracts from 134_{P1}, $134_{HC\text{-}Pro}$, $134_{P3\text{-}CI}$, $134_{CI\text{-}NIa}$, and 134_{NIa} induced consistent necrotic symptoms. However, $134_{NIb\text{-}CP}$ caused only yellowing and mottle symptoms compared to 134. As a result, most chimeric mutants sustained stable viral infectivity in the plant host (Figure 2B).

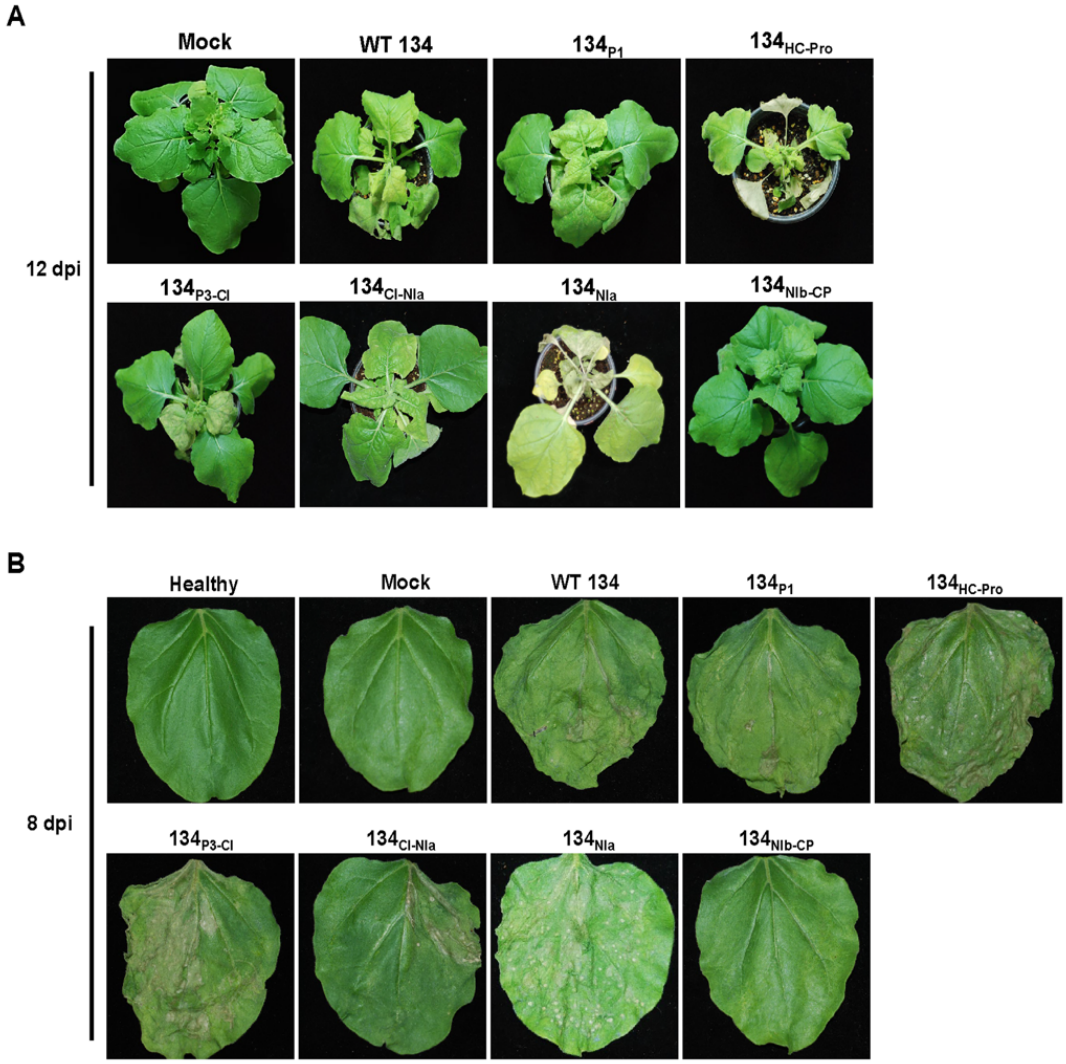

Figure 2. Symptom development of systemic and infected leaves on *N. benthamiana* plants caused by 134 and the other mutants. (A) Symptoms of 134 and chimeric mutants at 12 dpi. (B) Passage experiments with sap extracts of 134 and six chimeric mutant viruses inoculated onto *N. benthamiana.*

II. GFP fluorescence representing chimeric virus infections in *N. benthamiana*

The GFP-expressing virus accumulation of chimeric mutant viruses was monitored under UV light (Figure 3A). At 5 dpi, inoculated leaves of the $134_{P1,}$ $134_{P3\text{-}CI}$, and 134_{NIa} displayed no noticeable difference in GFP intensity compared with 134. The $134_{HC\text{-}Pro}$ and $134_{CI\text{-}NIa}$ produced more vigorous GFP intensity on the local leaves and faster systemic movement to upper leaves, indicating that PepMoV accumulation in those mutants was higher than that of 134. On the contrary, the $134_{NIb\text{-}CP}$ induced distinct lesser viral accumulation on inoculated leaves than 134. At 8 dpi, GFP expression of 134- and chimeric viruses-inoculated plants moved to the upper non-inoculated leaves (Figure 3B). The $134_{HC\text{-}Pro}$ and $134_{CI\text{-}NIa}$ infected plants showed vigorous GFP intensity, and $134_{NIb\text{-}CP}$ infected plants showed weak GFP expression in systemic leaves.

To determine whether the symptoms caused by chimeric mutants correlated with viral RNA accumulation, I conducted quantitative real-time PCR (RT-qPCR). After the 134 and four selected chimeric mutants were inoculated onto *N. benthamiana*, total RNAs were extracted from local inoculated leaves at 1 to 3 dpi. I also included another mutant ($134_{\Delta 6k2\text{-}CP}$) of pPepMoV:GFP-134 as a negative control, which did not contain the 6K2 to CP region in the 134 viral genome and lacks replication activity since it does not have RdRp. At the early viral replication stage, no significant difference was observed among 134 and chimeric mutants at 1 dpi based on RT-qPCR analysis (Figure 3C). Although the mutant $134_{HC\text{-}Pro}$ showed increased viral RNA accumulation (185%) and the $134_{NIb\text{-}CP}$ showed reduced viral RNA accumulation (23%) at 3 dpi compared to 134 these are

consistent results with their phenotype on plants, these changes were insignificant due to high experiment deviations (Figure 3, panels A and B). In the case of 134_{NIa} and 134_{P1}, viral RNA accumulation in those mutants showed similar accumulation levels compared to 134. This result suggests that the NIb-CP region of 205136 would adversely impact RNA accumulation, cell-to-cell and systemic movement of isolate 134. In contrast, the HC-Pro region might play an imperative role in virus replication and systemic movement (Figures 3B and C).

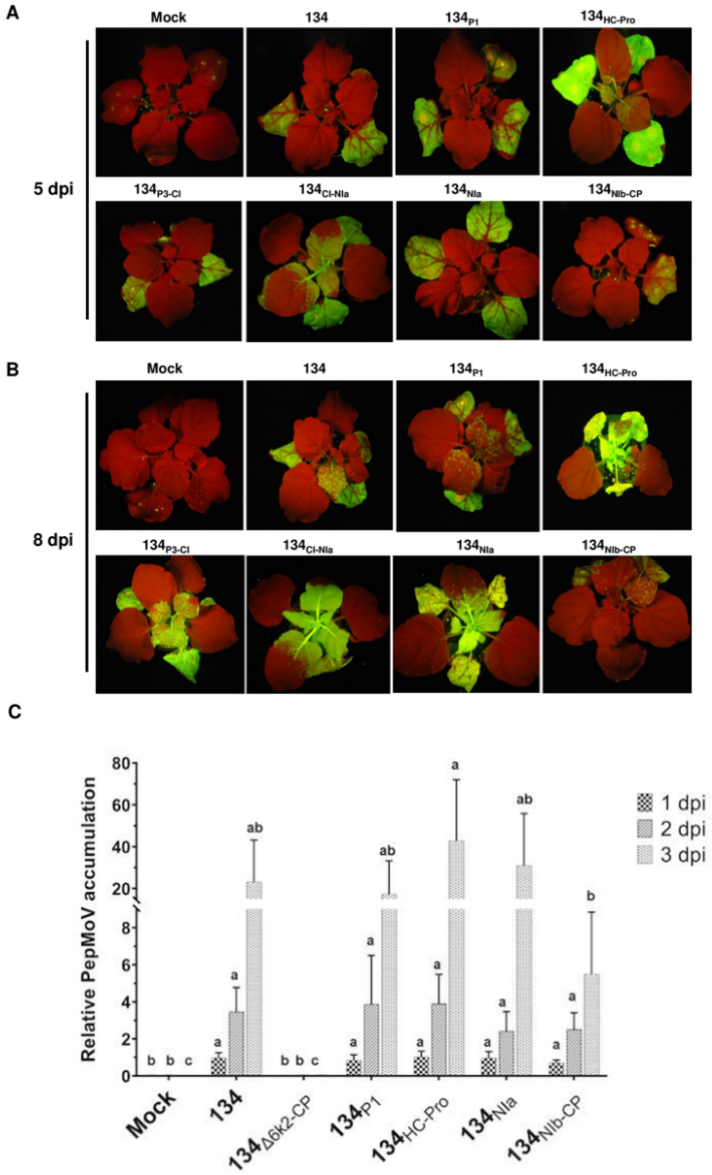

Figure 3. GFP fluorescence representing chimeric viruses infection and symptom development of parental and mutant viruses on local and systemic leaves. PepMoV-134 and chimeric mutants were inoculated onto *N. benthamiana* plants by agro-infiltration. The plant age used for inoculation is around 3 weeks old. Differences in virus

replication among 134 and mutant viruses were monitored from 5 to 12 days post-inoculation. (A) Results of inoculated leaves at 5 days post-inoculated (dpi). (B) Results of systemic leaves at 8 dpi. (C) Virus accumulation at 1 dpi, 2 dpi, and 3 dpi, respectively. Mean values with different letters above the bars indicate significant differences at $P < 0.05$ according to a one-way ANOVA and Duncan's least significant range test.

III. Evaluation of PepMoV replication on chimeric mutants at a single cell

Since the RT-qPCR results indicated no significant differences between 134 and chimeric viruses at 1dpi (Figure 3C), I isolated protoplasts from those mutant-infected plants to identify the conditions of viral accumulation differentiation. As a result, on a single cellular level, viral RNA accumulation of all mutants was significantly lower than 134 (Figure 4). When the viruses were present in the cells that were initially infected, there were no distinguishing characteristics among the mutants; however, as the viruses moved from cell to cell, the impacts of chimeric mutants became apparent.

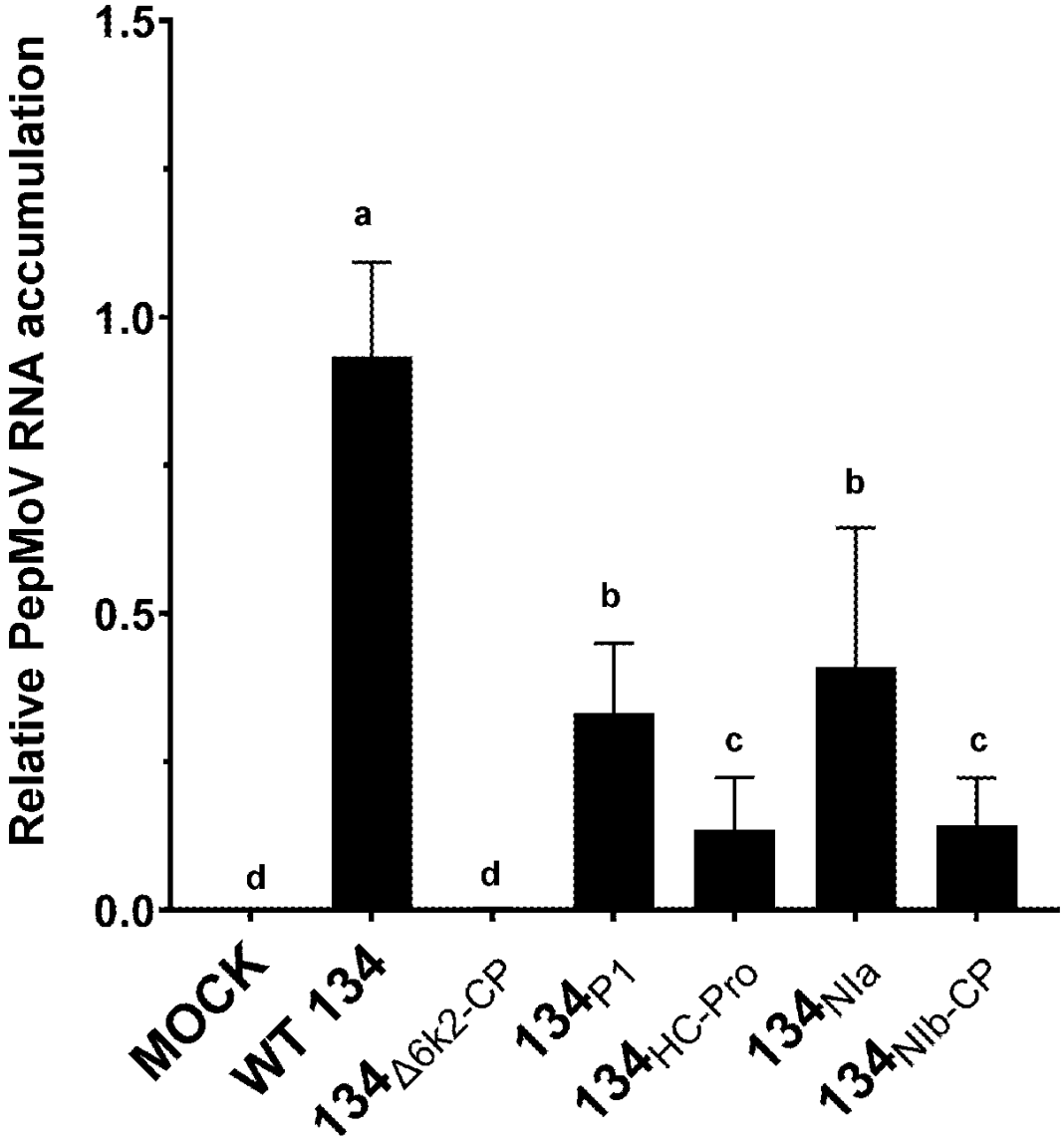

Figure 4. Quantification of protoplast viral accumulation at 1 dpi. Mean values with different letters indicate significant differences ($P < 0.05$) between 134 and chimeric mutants according to a one-way ANOVA and Duncan's least significant range test ($P < 0.05$).

IV. Generation of amino acid substitution clones of $134_{HC\text{-}Pro}$

In Figure 2, $134_{HC\text{-}Pro}$ and 134_{NIa} infected plants showed more severe necrotic symptoms when compared with 134. $134_{HC\text{-}Pro}$-infected plants showed more significant viral accumulation and faster movement at the early infection stage. An amino acid sequence alignment was conducted to determine the crucial domain of HC-Pro in PepMoV. Sequence comparison indicated that HC-Pro contains only three different amino acids (Figure 5A). I generated single and double amino acids substitution mutants to identify the crucial amino acid in $134_{HC\text{-}Pro,}$ which is responsible for faster virus accumulation and movement. The six $134_{HC\text{-}Pro}$-derived mutants were generated by adjusting the amino acid positions 360, 385, and 527 in PepMoV amino acid sequences and named $134_{HC\text{-}Pro/Y360H}$, $134_{HC\text{-}Pro/G385S}$, $134_{HC\text{-}Pro/L527P}$, $134_{HC\text{-}Pro/Y360H\&G385S}$, $134_{HC\text{-}Pro/Y360H\&L527P}$, and $134_{HC\text{-}Pro/G385S\&L527P}$ (Figure 5B). I inoculated the plant by agro-infiltration and observed the GFP expression of all site-directed mutants. All plants exhibited similar GFP intensity at 2 and 3 dpi compared to 134. Therefore, I conducted RT-qPCR to quantify and compare viral RNA accumulation among these six mutants (Figure 5C). The results showed that all three single amino acid substitution mutants showed reduced accumulation levels compared to $134_{HC\text{-}Pro}$ but maintained significantly increased viral RNA accumulation compared to the 134. In addition, $134_{HC\text{-}Pro/Y360H\&G385S}$, $134_{HC\text{-}Pro/Y360H\&L527P}$, and $134_{HC\text{-}Pro/G385S\&L527P}$, which are only one amino acid changed from 134, displayed higher RNA accumulation than 134 in Figure 5C, suggesting that

each amino acid substitution (H360Y, S385G, and P527L) in 134 can increase the viral RNA accumulation.

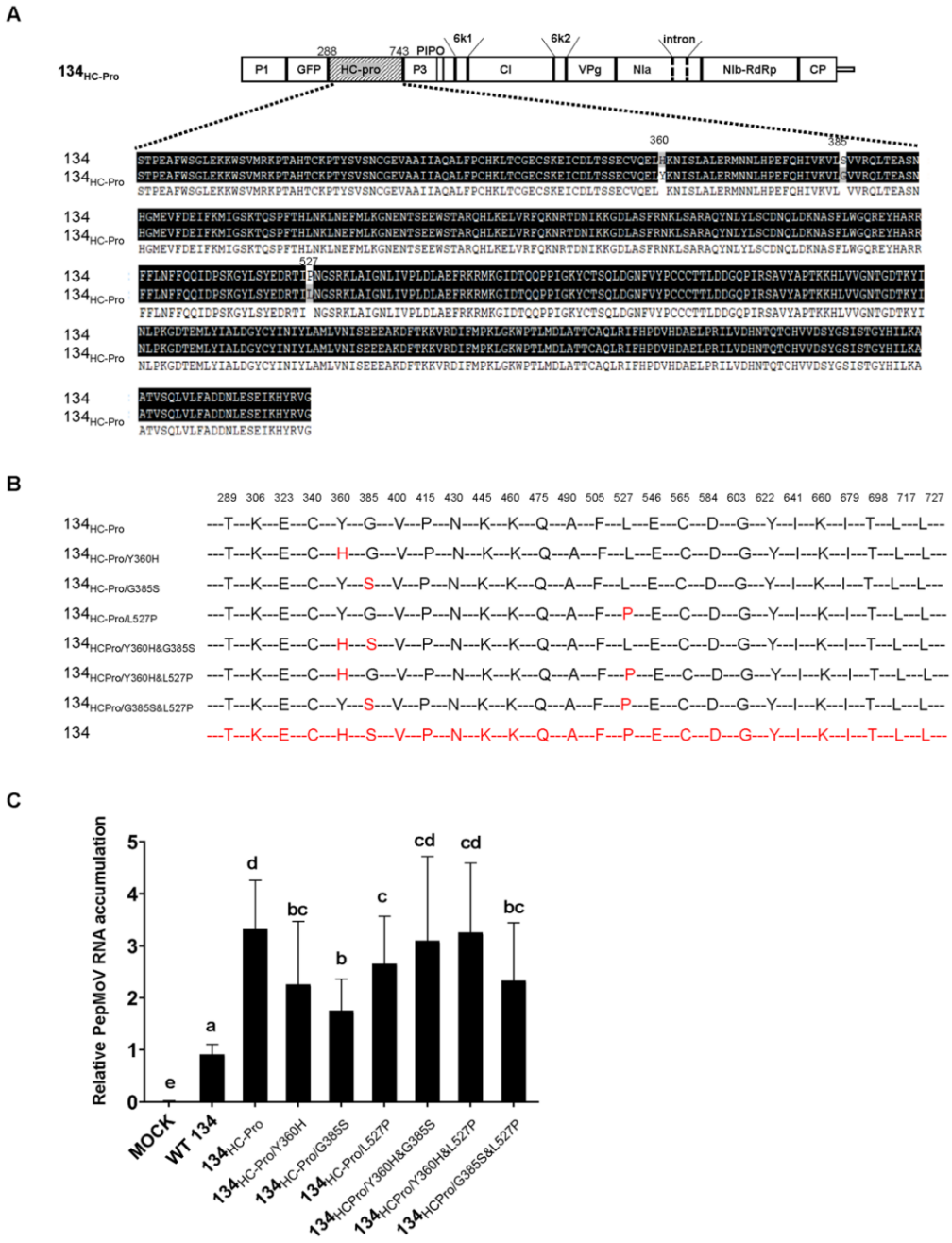

Figure 5. (A) Amino acid alignment sequence of the HC-Pro of PepMoV isolates 134 and $134_{HC\text{-}Pro}$. Dark highlighted amino acids are identical. Numbers are amino acid positions on the poly-protein precursor. (B) Schematic of PepMoV isolates $134_{HC\text{-}Pro}$-

derived amino acid substitution mutants. Single or double amino acid substitution mutations (red) were introduced in $134_{HC\text{-}Pro}$. The positions of substituted amino acids on the polyprotein precursor are indicated at the top of the amino acid sequence. (C) Quantification of $134_{HC\text{-}Pro}$-derived mutants accumulation at 2 dpi. Plant tissues were collected from inoculated leaves at 2 dpi. Letters indicate a significant difference among 134, $134_{HC\text{-}Pro}$ and derived mutants according to a one-way ANOVA and Duncan's least significant range test ($P < 0.05$)

V. Amino acid substitution alters symptom development of PepMoV-134$_{NIb-CP}$

I showed that 134$_{NIb-CP}$ induced weak viral symptoms and lower viral RNA accumulation than 134 (Figure 2). I assumed that the switched NIb and CP region changed severe symptoms caused by isolate 134 to mild symptoms caused by isolate 205136. To confirm whether the symptom differences between isolate 134 and 205136 might relate to this NIb-RdRp and CP region, I compared the amino acid sequence of the corresponding regions of isolate 134 and 205136 (Figure 6A). The amino acid differences are located at positions 2,374, 2,773, 2,789, and 2,805, three amino acids located in the NIb protein region, and the other is in the N-terminal of CP (Figure 6A). To identify crucial amino acid(s) among these four amino acids in the NIb and CP for symptom development, I constructed four 134$_{NIb-CP}$-derived mutants which contained a single amino acid mutation (Figure 6B). The amino acid substitution mutants were named 134$_{I2374V}$, 134$_{V2773D}$, 134$_{T2789A}$, and 134$_{T2805A,}$ respectively.

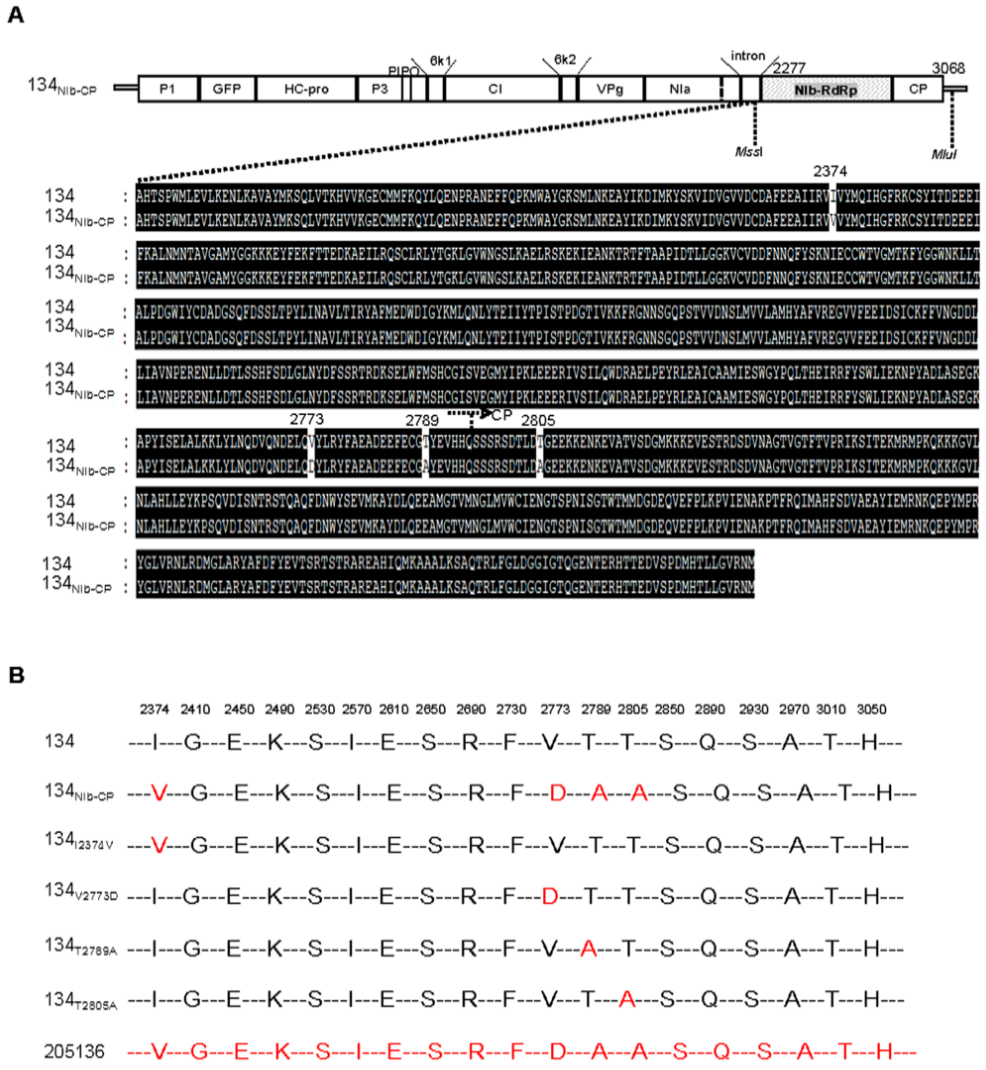

Figure 6. Amino acid sequence alignment of NIb-CP region between wild type 134 and 134$_{NIb\text{-}CP}$. (A) Alignment of the deduced amino acid sequence of the NIb and CP of PepMoV isolates 134 and 205136. Dark highlighted amino acids are identical. Numbers are amino acid positions on the poly-protein precursor. (B) Schematic of PepMoV isolates 134-derived amino acid substitution mutants. The positions of substituted amino acids on the polyprotein precursor are indicated at the top of the amino acid sequence.

To compare infectivity and symptom development among the amino acid substitution mutants and 134, I inoculated the amino acid substitution mutants onto *N. benthamiana* plants by agro-infiltration. All four pPepMoV:GFP-134-derived single amino acid substitution mutants, 134_{I2374V}, 134_{V2773D}, 134_{T2789A}, and 134_{T2805A}, GFP-expressing virus existed on systemic leaves. Among them, the GFP intensity of the 134_{T2805A}-infected plant was stronger than $134_{NIb\text{-}CP}$ and the other site-directed mutants (Figure 7A). 134_{I2374V}, 134_{T2789A}, and 134_{T2805A} displayed necrotic symptoms on systemic leaves at 12 dpi (Figure 7, panels B and C). The result indicated that the amino acid substitution of valine to aspartic acid at position 2773 is sufficient for PepMoV 134 to alter symptom development. When I incubated infected plants longer, 134_{I2374V}, 134_{T2789A}, and 134_{T2805A} showed more severe necrosis symptoms. However, 134_{V2773D} and $134_{NIb\text{-}CP}$ did not appear with severe necrotic symptoms at 17dpi (Figure 7D). Taken together, the crucial residue valine at position 2773 of NIb plays an essential role in PepMoV-derived symptom development.

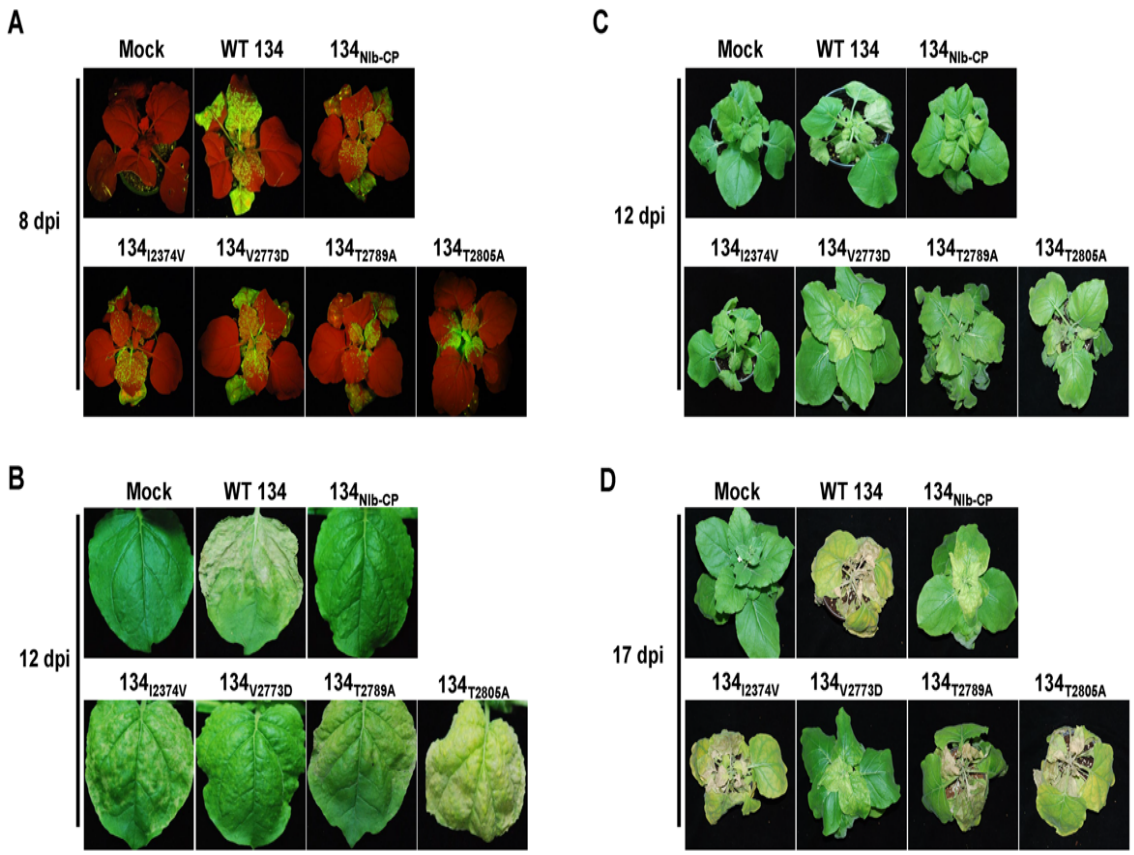

Figure 7. Virus replication and symptom development of 134 and substituted mutants on local and systemic leaves. PepMoV-134 and substituted mutants were inoculated onto *N. benthamiana* plants by agro-infiltration. The plants age used for inoculation is around 3 weeks old. The viral RNA accumulation difference among 134 and mutant viruses was monitored obviously for 5 days post-inoculation. (A) Observation of GFP expression on inoculated leaves at 8 dpi. (B) Results from systemic leaves at 12 dpi. Symptoms development of 134 and substituted mutants at 12 dpi (C) and 17 dpi (D).

DISCUSSION

The previous study described that PepMoV isolate 134 causes necrosis and yellowing, while 205136 causes severe mottle and yellowing symptoms in *N. benthamiana* plants (Kim et al., 2009). Here, I demonstrated that the $134_{NIb\text{-}CP}$ caused a more significant reduction in virus accumulation and mild symptoms than 134, suggesting that the C-terminal part of NIb is involved in PepMoV symptomatology and multiplication. In contrast, the $134_{HC\text{-}Pro}$ showed faster virus movement and increased severity indicating that HC-Pro plays an essential role in PepMoV symptom severity and systemic movement (Figure 3). To determine the crucial domain, I compared HC-Pro and NIb-CP amino acid sequences of 134 with counterparts of $134_{HC\text{-}Pro}$ and $134_{NIb\text{-}CP}$, respectively, and constructed single/double amino acid substitution mutants (Figures 5 and 6). As a result, I showed that the 385 positions of glycine of HC-Pro and 2773 positions of NIb might be involved in virus systemic movement and symptom development, respectively.

Because the HC-Pro protein of $134_{HC\text{-}Pro}$ was replaced from mild isolate 205136, theoretically, it could induce weaker symptoms and fewer virus accumulations. However, $134_{HC\text{-}Pro}$ caused more vigorous virus multiplication and faster long-distance movement than 134. It has been reported that the HC-Pro in the sugarcane mosaic virus and TuMV act as a viral RNA silencing suppressor (Han et al., 2016; Chen et al., 2017). The C-terminal HC-Pro protein in PVY also suppresses RNA

silencing-based plant defenses by binding to small interfering RNAs. I was curious if HC-Pro of PepMoV 134 has a strong VSR activity than 205136. However, I did not compare the VSR activities of HC-Pro from PepMoV isolate 134 and 205136 in this study and needs additional works. In addition, one of the previous studies said that substituting Ile for Arg at position 180 in the conserved motif Phe-Arg-Asn-Lys (FRNK) of potyviruses contributed to symptom expression (Gal-On, 2000). The sequence FRNK is a conserved motif in 18 potyviruses and many different isolates of those viruses (Gal-On, 2000). The mutation of the FRNK box to FINK (R180I) causes a drastic reduction in symptom severity of the leaves of various cucurbit species without noticeably affecting virus accumulation or infectivity. This mutation has been exploited for use in cross-protection (Shiboleth et al., 2007). PepMoV isolates134 and 205136 have conserved FRNK motifs and have identical amino acid sequences. Therefore, the Arg of the FRNK motif is not the primary reason for the symptomatic difference between PepMoV isolate 134 and 205136. In this study, I found another essential amino acid position in HC-Pro of PepMoV, which affected viral accumulation, systemic movement, and symptom determinants in PepMoV isolate 134.

I showed that the $134_{NIb\text{-}CP}$ and 134_{V2773D} could not reproduce the necrotic symptom induced by the 134, although titer in systemic upper leaves was reduced relative to 134. My results suggest that the C-terminal part of NIb is involved in PepMoV virus symptomatology and multiplication. Like the NIb protein of PepMoV, the *Oilseed rape mosaic virus* belongs to the genus *Tobamovirus*, in which RNA

replicase functions in virus accumulation and disease symptoms determination. This appears to be the case of determinants of systemic necrotic spots in tobacco (Mansilla et al., 2009). Virus' viral accumulation and symptomatology determinants are variable, depending on the specific virus family or strain. The symptoms and pathogenicity of different PepMoV isolates infect various plant hosts (Kim et al., 2009). Further work is required to identify crucial genes or motifs in PepMoV that might be involved in their symptoms and pathogenicity variation among the different plant hosts and virus isolates.

The $134_{HC\text{-}Pro}$ and $134_{CI\text{-}NIa}$, which substituted the HC-Pro region and half of the CI:NIa region replaced by counterpart regions of 205136, respectively, induced more robust virus distribution on the local leaves and faster long-distance movement on the systemic leaves. On the other hand, $134_{HC\text{-}Pro}$ showed distinctly more severe symptoms than 134, indicating that the HC-Pro protein of PepMoV roles in pathogenicity, virus accumulation, and systemic movement. Since I designed a chimeric construct based on the available restriction enzyme sites on pPepMoV:GFP-134 and 205136 isolate cDNA sequences, switched region in $134_{CI\text{-}NIa}$ construct included half of CI, 6K2, VPg, and N-terminal of NIa. Like $134_{HC\text{-}Pro}$, the $134_{CI\text{-}NIa}$ performed faster and stronger virus accumulation at 5 and 8 dpi (Figure 3, panels A and B); however, it did not display severe necrosis symptoms earlier than 134 (Figure 2A). Based on the virus development process in plants, the RT-qPCR (Figure 3C) showed quantitative viral accumulation differences in the initial stage, GFP observation

(Figures 3A and B) monitored virus cell-to-cell and systemic movement differences showed at the middle development stage, symptom observation (Figure 2A) displayed symptom differentiation at the later stage. In Figure 2A, the images of 134_{NIa} were taken at 12 dpi; it is the late stage of the PepMoV infection, and 134_{NIa} caused much more severe and accelerated necrotic symptoms than 134. However, in Figure 3 A and B, images of 134_{NIa} were taken at 5 and 8 dpi, respectively, at the early infection stage (5 dpi), GFP fluorescence expression did not show a clear difference compared to 134. However, 134_{NIa} developed its symptom much faster than 134 after 5 dpi; they already started to show necrosis symptoms at 8 dpi (data not shown). Additionally, in Figures 2A and 2B, because the objectives of the two experiments were different, I experimented using two different inoculation methods, i.e., agro-infiltration and sap inoculation (saps were collected from infected leaves), respectively. Meanwhile, the symptom images in Figures 2A and 2B were taken at 12 dpi and 8 dpi, respectively. As a result, the degree of $134_{HC\text{-}Pro}$ and 134_{NIa}-induced necrosis in Figures 2A and 2B was slightly different. The NIa from PepMoV has been demonstrated to determine pathogenicity (Gong et al., 2020). The result of this study said that 134NIa seems to have reached a similar conclusion with them.

Further, 134_{NIa}, generated by modifying the counterpart of NIa C-terminal between isolates 134 and 205136, contains 177 amino acids, and only two are different. This suggests that one of these amino acids may play an essential role in the pathogenicity factor (data unavailable). Even though I could not determine the specific proteins or

motifs involved in more robust virus accumulation and faster virus systemic movement in $134_{CI\text{-}NIa}$, further detailed experiments may clarify the specific roles of PepMoV viral proteins, whose functions remain unclear. This study may help to understand pathogenic determinant(s) in the PepMoV-*N. benthamiana* pathosystem and increase the understanding of the different host responses to PepMoV isolates.

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Pepper mottle virus 의 증식 및 병징 발현 관련 바이러스인자의 분자생물학적 특성 구명

팡미아오

ABSTRACT

Pepper mottle virus(PepMoV)는 고추, 피망, 감자, 토마토를 포함한 다양한 가지과 식물을 감염하는 파괴적인 병원체이다. 이 학위 논문에서는 PepMoV 의 분자적 특성에 대한 현재의 이해와 숙주 식물과의 상호작용에 대해 제 1 장에서 설명했다. 제 2 장에서는 PepMoV 의 바이러스 증식과 병징발현과 관련된 결정 인자를 확인하는 연구를 수행하였다. 국내 9 개 지역에서 13 개의 PepMoV 분리주가 수집되었는데 각 분리주들은 접종된 지표 기주 식물에 각기 다른 증상을 일으킨다. 본 연구에서는 PepMoV 의 감염 및 병징발현 관련 결정 인자를 확인하고 바이러스 단백질 기능을 탐색하기 위해 분리주 134 와 205136 을 사용하였다. PepMoV 분리주 134 는 괴사와 황화를 유발하며, PepMoV 분리주 205136 은 *Nicotiana benthamiana* 에 황화 증상을 유발한다. PepMoV 분리주 134 의 감염성 전장클론을 이용하여 PepMoV 분리주 205136 의 특정 영역의 대응 유전자로 치환된 돌연변이체들을 제작하여 연구에 사용하였다. 연구의 결과는 PepMoV 의 helper component-proteinase(HC-

Pro)와 nuclear inclusion protein b(NIb) 및 capsid protein(CP) 단백질 영역이 바이러스 증식 및 병징 결정 요인에 관여한다는 직접적인 증거를 제공하였다. 또한 HC-Pro 영역의 360, 385, 527 아미노산 위치에 존재하는 tyrosine, glycine 및 leucine 이 바이러스의 식물 내 증식 또는 이동 속도를 향상에 중요하게 관여하는 것을 확인하였다. NIb 의 경우 2773 번 위치에 있는 아미노산 valine 이 PepMoV 분리주 134 의 특징적인 병징 발현에 필수적인 역할을 한다는 것도 확인하였다. 이 연구의 결과들은 PepMoV 감염 및 병징 발현과 관련된 바이러스 요인에 대한 추가적인 정보를 제공하였다. 이러한 결과들은 식물-바이러스 상호 작용의 이해를 제고하는데 기여할 것이고, PepMoV 바이러스의 유전자 기능 연구의 부족함을 보완하고 potyvirus 들의 바이러스 단백질 기능 연구에 대한 새로운 관점을 제공할 것이다.

키워드: pepper mottle virus, HC-Pro, NIb, 바이러스-기주 상호 작용, 바이러스 병징 결정 인자, *Nicotiana benthamaiana*