A DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Genome-based identification of *Pvr4* conferring resistance against potyvirus in *Capsicum annuum*

고추의 **Potyvirus** 저항성 유전자 ***Pvr4***의 분리

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Genome-based identification of *Pvr4* conferring resistance against potyvirus in *Capsicum annuum*

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ABSTRACT

Pvr4 is a resistance gene showing broad-spectrum resistance against multiple potyviruses, including *Pepper mottle virus* (PepMoV), *Pepper severe mosaic virus* (PepSMV), and *Potato virus Y* (PVY). *Capsicum annuum* landrace 'CM334' is known to have *Pvr4*, but it has not been cloned and the mechanism of resistance is unknown. To identify the avirulence factor in potyviruses corresponding to Pvr4, a total of eleven viral cistrons of PepMoV were expressed into potyvirus-resistant (*Pvr4*) and -susceptible (*pvr4*) pepper plants. Hypersensitive response (HR) was observed only when a RNA-dependent RNA polymerase (NIb) of PepMoV was expressed in *Pvr4*-haboring pepper leaves in a genotype-specific manner. In addition, the over-

expression of NIb proteins of other potyviruses including PepSMV, PVY also induced HR in *Pvr4*-harboring pepper plants. These results indicate that NIbs of PepMoV, PepSMV, and PVY may play important roles as avirulence factors for Pvr4 in pepper plants. To identify *Pvr4* resistance gene against potyvirus in pepper, genome-based cloning with two populations including BC1F3 and F2 populations was performed. High-density molecular markers including 32 co-dominant markers were developed in the TG420 marker region using tomato and pepper genome. Three SNP markers showed a co-segregation with *Pvr4* in two populations and *Pvr4* is located within 350 kb region containing sixteen annotated genes. Among them, eight genes were coiled-coil (CC) nucleotide-binding site leucine-rich repeat (NB-LRR) and they were clustered in this region. Transient over-expression of the eight NB-LRR type genes and NIb of PepMoV in susceptible pepper (*C. annuum* 'Jupiter') and *Nicotiana benthamiana* leaves revealed that only one gene (*CA10g21170*) induced HR at 2 dpi. The relative amounts of PepMoV RNA transcripts and protein were significantly suppressed in the leaves that transiently over-expressed of CA10g21170 after 3 dpi. Furthermore, CA10g21170 showed resistance against other potyviruses including PepSMV and PVY. Consequently, these results prove that CA10g21170 is indeed Pvr4 leading to recognize NIb and suppress PepMoV, PepSMV and PVY

replication. *Pvr4* consisted of seven exons and encodes a CC-NB-LRR type protein with 1746 amino acids. Genomic region of *Pvr4* including exon and intron is 13,870 bp. *In planta* assays using the TRV-based gene silencing revealed that silencing of *HSP90*, *SGT1* and *RAR1* in *N. benthamiana* suppressed HR induced by Pvr4 and NIb of PepMoV. To confirm the heterologous *in planta* expression of *Pvr4* in other plant, *Pvr4*-harboring transgenic potato were generated. Inoculation of virus validated that the replication of PVY-O was significantly suppressed in the transgenic upper leaves. Taken together, the cloned potyvirus resistance gene, *Pvr4* could provide information for the application of broad-spectrum potyvirus resistance in crop breeding, as well as for understanding potyvirus resistance mechanisms in plants.

Keywords: *Capsicum annuum*, Pvr4, resistance gene, potyvirus, *Pepper mottle virus* (PepMoV), *Peppper severe mosaic virus* (PepSMV), *Potato virus Y* (PVY), nucleotide-binding site leucine-rich repeat (NB-LRR), RNA-depenedent RNA polymerase (NIb), hypersensitive response (HR)

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LIST OF ABBREVIATIONS

Pvr4	Potyvirus resistance gene 4
Pvr7	Potyvirus resistance gene 7
NB-LRR	Nucleotide binding site leucine rich repeat
PepMoV	*Pepper mottle virus*
PepSMV	*Pepper severe mosaic virus*
PVY	*Potato virus Y*
TEV	*Tobacco etch virus*
Avr	Avirulence factor
RdRp	RNA-dependent RNA polymerase
GFP	Green fluorescent protein
ER	Extreme resistance
HR	Hypersensitive response
ETI	Effector-triggered immunity
PTI	PAMP-triggered immunity
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
TOE	Transient overexpression
TRV	*Tobacco rattle virus*
VIGS	Virus-induced gene silencing
BAC	Bacterial artificial chromosome

GENERAL INTRODUCTION

Plants have a complex immune system that includes several layers, which enables them to defend against pathogen from fungi, bacteria and viruses (Chisholm et al., 2006). One of the first layers, the recognition of these structures/proteins, named pathogen associated molecular patterns (PAMP), takes place by pattern recognition receptors (PRRs) on plant plasma membranes. These PRRs recognize conserved structures of pathogens, and induce PAMP triggered immunity (PTI) response (Dangl and Jones, 2001). Plant pathogens need to suppress the PTI in plants, and secrete effector protein to induce effector triggered susceptibility (ETS) into plant cell. To overcome ETS, dominant resistance gene (*R* gene) products directly or indirectly recognize the presence of a specific effector produced by pathogens, named avirulence factor (Avr), leading to effector-triggered immunity (ETI).

Most viral resistance is monogenically controlled by resistance gene in plants (Kang et al., 2005a). The first step for genetic studies of viral resistance is to verify whether the resistant response is inherited, if so, the number of resistance genes involved and their mode of inheritance. Two main types of plant resistance to viruses have been characterized according to the mode of action to restrict viral replication/movement (Kang et al., 2005a). Recessive

resistance results in a passive mechanism in which the lack of interaction between the plant host factor and the viral factor suppresses its replication. Eukaryotic translation initiation factors 4E (eIF4E) is one of the example of recessive resistances against potyviruses (Kang et al., 2005b; Robaglia and Caranta, 2006). The lack of elF4E and its elFiso4E blocks its physical interaction with the potyviral genome-linked protein (VPg) and results in plant resistance (Kang et al., 2005b). On the other hand, dominant genetic resistance in plant defense system is based on a gene-for-gene (Flor, 1971) or guard hypothesis (Van Der Biezen and Jones, 1998; Dangl and Jones, 2001). In this concepts, the disease resistance in plants requires an *Avr* gene in the pathogen and a corresponding *R* gene in the host. If either is inactive or absent, plants are susceptible to pathogen. The majority of cloned resistance genes encode proteins of a conserved nucleotide-binding site (NBS) in the center of the protein and a leucine-rich repeat (LRR) domain at the C-terminal end and a Coiled-coil (CC) or Toll and Interleukin-1 receptor (TIR) domain at the N-terminal end (Maule et al., 2007; Moffett, 2009).

Direct or indirect interaction between the dominant resistance protein of plant and the viral effector encoded by the virus avirulence gene triggers a hypersensitive reaction (HR) or an extreme resistance (ER) (Cooley et al., 2000; Vidal et al., 2002; Mestre and Baulcombe, 2006; Slootweg et al., 2010).

ERs were defined by the absence of any visible symptoms after inoculation suggesting that this mechanism occurs rapidly enough to restrict virus replication in the plant cell level (Bendahmane et al., 1999; Mestre et al., 2000; Hajimorad and Hill, 2001). For example, *Rx*-mediated extreme resistance against *Potato virus X* (PVX) in potato does not involve a necrotic cell death at the site of initial infection, but an interaction between Rx and coat protein of PVX showed a rapid cell death in *N. benthamiana* (Bendahmane et al., 1995; Bendahmane et al., 1999). However, HR is represented by visible necrotic local lesions initiated by programmed cell death (PCD) surrounding the infection site and a restriction of the cell-to-cell movement of the virus occurs within 2-3 days (Solomon-Blackburn and Barker, 2001; Kang et al., 2005a). The *N* gene-*Tobacco mosaic virus* (TMV) interaction has issued as a model system for the study of HR-mediated resistance in plants (Whitham et al., 1994).

Solanaceae is a large family which comprises of more than 3,000 species including important vegetable species, such as potato (*Solanum tuberosum*), tomato (*Solanum lycopersicum*), eggplant (*Solanum melongena*), and pepper (*Capsicum* spp.) (Peters et al., 2012). Most of plants belong to Solanaceae have the same number of chromosome (n = 12) but vary in genome size. Syntenic and co-linear relationships have been demonstrated among pepper,

tomato, and potato, indicating possibility of comparative genome researches and the applications among Solanaceous genomes (Lefebvre et al., 2002; Wu et al., 2009b; Wu et al., 2009a; Peters et al., 2012). Recent study revealed that the pepper (*Capsicum annuum*) genome shared highly conserved syntenic blocks with the tomato genome, its closest relative within the Solanaceae family (Kim et al., 2014).

Pepper (*Capsicum* spp.) is widely used as a vegetable, condiment and food additive. *Capsicum* genus comprises of about 30 species, and 5 species (*C. annuum*, *C. frutescens*, *C. baccatum*, *C. chinense*, and *C. pubescens*) are domesticated. Among them, *C. annuum* is the most widely cultivated species (Wang and Bosland, 2006). World-wide green pepper production in 2013 reached 2 million ha with more than 31 million metric tons harvested (FAOSTAT, 2013). As a result of its world-wide geographical distribution, peppers are exposed to many pathogens and particularly to more than 20 viruses that cause loss of production and the poor quality of fruit (Green and Kim, 1991). Since chemical controls are ineffective to control the enormous damage caused by viral infections and the application of plant genetic resistance is thought to be the only method to protect crops from the damage caused by viruses (Janzac et al., 2009). Among these viruses, members of the genus *Potyvirus* include some of the most destructive plant viruses such as

PepMoV, PepSMV, PVY and TEV in pepper as well as other Solanaceous plants.

Resistance against Potyvirus has been reported in *C. annuum* landrace Criollo de Morelose 334 (‘CM334’) (Dogimont et al., 1996; Caranta et al., 1999; Grube et al., 2000; Janzac et al., 2008; Janzac et al., 2009). The single dominant gene *Pvr4* in *C. annuum* ‘CM334’ confers extreme resistance against multiple potyviruses, including PepMoV, PepSMV, *Pepper yellow mosaic virus* (PepYMV), *Ecuadorian rocoto virus* (ERV), *Peru tomato virus* (PTV) and all isolates of PVY (Dogimont et al., 1996; Caranta et al., 1999; Grube et al., 2000; Lefebvre et al., 2002; Janzac et al., 2008; Janzac et al., 2009). The broad resistance controlled by *Pvr4* has remained durable over almost 20 years (Janzac et al., 2009). The *Pvr4* locus was mapped with linked marker (TG420) on pepper chromosome 10 sharing the order of the markers with tomato, potato and eggplant in the lineage (Tanksley et al., 1992; Wu et al., 2009b; Wu et al., 2009a; Kim et al., 2011). Though *Pvr4* is crucial genetic source in pepper breeding, it has not been cloned and the mechanism of resistance is still unknown.

To date, many resistance genes have been isolated by map-based cloning using molecular markers which is one of the most important genetic tools for plant genetics and breeding (Bendahmane et al., 1999; Kang et al., 2005a;

Tomita et al., 2011). Genome sequencing projects in crops including pepper and tomato contribute to molecular marker development as well as genome study (Schmutz et al., 2010; Consortium, 2012; Kim et al., 2014). From the genome sequence data of major crops, a large number of molecular markers linked with agronomic traits such as resistance to abiotic and biotic stresses have been discovered for marker assisted selection (MAS) and to map loci of interest (Varshney et al., 2005; Varshney et al., 2006). Based on this approach, candidate gene-based marker discovery provides valid information for gene cloning.

This study focused on the identification of an avirulence factor of potyvirus against Pvr4 and genome-based cloning of *Pvr4* conferring broad resistance to potyvirus in pepper. Moreover, Pvr4-mediated signaling components were characterized in *Nicotiana benthamiana* and *Pvr4*-haboring transgenic potato. Studies performed the following topics:

Chapter 1: Identification of RNA-dependent RNA polymerase (NIb) as the avirulence factor of potyvirus in *Pvr4*-harboring pepper plants

Chapter 2: Genome-based cloning of *Pvr4* conferring multiple potyvirus resistance from *Capsicum annuum* ‘CM334’

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CHAPTER 1

Identification of RNA-dependent RNA polymerase (NIb) as the avirulence factor of potyvirus in *Pvr4*-bearing pepper plants

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ABSTRACT

Potyviruses are one of the most destructive viral pathogens of Solanaceae plants. In *Capsicum annuum* landrace ‘CM334’, a broad-spectrum gene, *Pvr4* is known to be involved in resistance against multiple potyviruses, including *Pepper mottle virus* (PepMoV), *Pepper severe mosaic virus* (PepSMV), and *Potato virus Y* (PVY). However, a potyvirus avirulence factor against Pvr4 has not been identified. To identify the avirulence factor corresponding to Pvr4 in potyviruses, there were performed *Agrobacterium*-mediated transient expressions of potyvirus protein coding regions in potyvirus-resistant (*Pvr4*) and -susceptible (*pvr4*) pepper plants. Hypersensitive response (HR) was observed only when a RNA-dependent RNA polymerase (NIb) of PepMoV, PepSMV, or PVY was expressed in *Pvr4*-bearing pepper leaves in a genotype-specific manner. In contrast, HR was not observed when the NIb of *Tobacco etch virus* (TEV), a virulent potyvirus, was expressed in *Pvr4*-bearing pepper leaves. Our results clearly demonstrate that NIbs of PepMoV, PepSMV, and PVY serve as avirulence factors for Pvr4 in pepper plants.

INTRODUCTION

Potyviruses belong to the family *Potyviridae* which represents the largest plant viruses, and severely affect the production of economically important crops. Several members of the genus *Potyvirus* including *pepper mottle virus* (PepMoV), *pepper severe mosaic virus* (PepSMV), *potato virus Y* (PVY) and *tobacco etch virus* (TEV) have a wide range of hosts such as potato, pepper, and tomato in Solanaceae plants (Ivanov et al., 2014). The genome of potyviruses is composed of a single-stranded RNA with a length of ~9.7 kb, which covalently links with a viral-encoded protein (VPg) at its 5'-end and contains a 3'polyadenylated tail. All members of potyviruses encode two polyproteins, a larger polyprotein of about 3,000 amino acids and the shorter one translated from a 2+ frameshift in the P3 coding region (Quenouille et al., 2013). These polyproteins are cleaved by viral proteases subsequently generating eleven mature proteins (Chung et al., 2008).

To date, functions of PVY viral proteins are the most well studied among potyviruses in response mechanisms against plant host factors to trigger the plant immune system (Hong et al., 1995; Mestre et al., 2000; Janzac et al., 2010; Moury et al., 2011; Quenouille et al., 2013; Tian and Valkonen, 2013). For example, PVY VPg interacts with a recessive resistance protein, pvr2 in

pepper which is also known as a member of eukaryotic initiation factor 4E (elF4E) (Ayme et al., 2007). Another PVY viral protein, HC-Pro is known to function broadly in potato and tobacco by interacting with elF4E and its elFiso4E (Ala-Poikela et al., 2011), and is also involved in HR-like cell death in potato by responding to resistance genes called NC_{tbr}, NC_{spl} and Ny_{tbr} (Moury et al., 2011). A PVY protease, NIa protease (also called NIaPro) was found to be required for *Ry*-mediated resistance of potato against PVY (Mestre et al., 2000). While these PVY viral proteins have structural analogy with other potyvirus proteins, they do not always function similar. For instance, a PepMoV NIaPro which exhibits 63.5% identity in sequence with a PVY NIaPro showed HR in *Ry*-mediated resistance; whereas, a TEV NIaPro failed to induce HR although it shares 45.9% identity with the PVY NIaPro(Mestre et al., 2000).

PepMoV was first reported as an atypical pepper isolate of PVY (Zitter, 1972), is known to cause a serious disease in pepper (Abdalla et al., 1991). However, functions of PepMoV-encoded proteins mostly remain unknown.

The completion of the pepper genome sequencing project using *Capsicum annuum* landrace ‘CM334’ (hereafter ‘CM334’) provides a tremendous amount of information and facilitates characterization of multiple disease resistance genes in pepper (Kim et al., 2014). ‘CM334’ contains a

single dominant resistance gene, referred as *Pvr4*, which confers resistance against all strains of PepMoV, PepSMV, and PVY, but not to TEV (Caranta et al., 1999; Grube et al., 2000; Arnedo-Andrés et al., 2002; Janzac et al., 2009; Janzac et al., 2010; Kim et al., 2011). The *Pvr4*-mediated resistance in pepper plants exhibits extreme resistance or HR to multiple potyviruses which is not yet found in any other Solanaceae host plants such as tomato and potato (Janzac et al., 2010; Kim et al., 2011). Although *Pvr4* has been mapped to chromosome 10 of the pepper plant, it was not isolated, and subsequently the molecular mechanism of *Pvr4*-mediated resistance to PepMoV infection has not been elucidated (Kim et al., 2011). Only a mutation of a RNA-dependent RNA polymerase (RdRp, also called NIb, hereafter NIb) area in PVY genome has been reported to confer virulence against *Pvr4*-bearing pepper plants (Janzac et al., 2010). However, a corresponding viral component that plays a role as an avirulence factor against *Pvr4* in pepper plants remains to be identified.

In this study, there were screened all eleven proteins from PepMoV to identify the avirulence factor for the single dominant resistant gene, *Pvr4*, in ‘CM334’. Viral cistrons of PepMoV were cloned into an *in planta* expression vector for screening against *Pvr4*-segregating F2 populations derived from a cross between ‘CM334’ (*Pvr4*) and ‘Jupiter’ (*pvr4*) cultivar. It was revealed

that NIbs from multiple potyviruses function as avirulence factors for Pvr4 in ‘CM334’.

MATERIALS AND METHODS

Plant Materials

Six different *C. annuum* L. lines, including three resistance [‘CM334’ (*Pvr4/Pvr4*), an F1 hybrid (*Pvr4/pvr4*), and a resistant homozygotic F2 (*Pvr4/Pvr4*) from a cross between ‘CM334’ and ‘Jupiter’] and three susceptible lines [‘ECW’ (*pvr4/pvr4*), ‘Jupiter’ (*pvr4/pvr4*), and a susceptible homozygotic F2 (*pvr4/pvr4*) from a cross between ‘CM334’ and ‘Jupiter’] against PepMoV, were confirmed by viral inoculation and co-segregating DNA marker (Kim et al., 2011). Briefly, to confirm resistance in pepper plants, it was inoculated 4 to 6 weeks old leaves with PepMoV-GFP modified from PepMoV-Vb1 (Lee et al., 2011) and performed an enzyme-linked immunoassay (ELISA) to detect PepMoV according to the manufacturer’s protocol (Agdia, Elkhart, IN, USA). The genotypes of F1 and F2 lines were confirmed by *Pvr4*-linked co-segregating marker (PCAPS15) to distinguish the *Pvr4* and *pvr4* genes (Kim et al., 2011). Transient assays were performed with 4 to 6 week-old pepper plants. All pepper plants were grown in a growth chamber at 22–25℃ with 60% relative humidity and a 14:10-hour light-dark cycle.

Application of *Pvr4*-linked CAPS Marker for Identification of Pepper Genotype

For detection of *Pvr4*-linked markers, PCR products that were amplified with the marker primer were digested with XhoI. *Pvr4*-linked CAPS marker (PCAPS15) allows discernment of the *Pvr4* allele as *Pvr4*/*Pvr4*, *Pvr4*/*pvr4*, or *pvr4*/*pvr4* (Kim et al., 2011). As shown in Fig. 1, XhoI digestion of the PCR products generated 550- and 270-bp fragments for *Pvr4* and 470- and 350-bp fragments for *pvr4*.

Cloning of Potyvirus Cistrons for *in planta* Expression

For cloning of PepMoV cistrons for *in planta* expression, specific primers to amplify each coding regions and the *NIb* from PepSMV (NC_008393) (Ahn et al., 2006), PVY (EF026074.1) (Baldauf et al., 2006) and TEV (M11458.1) (Janzac et al., 2009) were designed for use in the ligation-independent cloning (LIC) method by adding adapter sequences with: 5'-CGACGACAAGACCCT ATG (adaptor sequence) – viral coding region specific sequence - 3' and 5' - GAGGAGAAGAGCCCT TCA (adaptor sequence) - viral coding region specific sequence – 3' (Oh et al., 2010; Bae et al., 2013). *P3N-PIPO* cistron was generated by overlap PCR including a PIPO coding region in the GGAAAAAA motif to place the *PIPO* ORF in-frame

with the N-terminal half of the P3 coding region (Yu et al., 2004; Szewczyk et al., 2007; Chung et al., 2008; Vijayapalani et al., 2012). For cloning of PepMoV cistrons for western blot, specific primers added HA tag (TACCCATACGACGTCCCAGACTACGCT) to amplify *NIb*, *CP* and *HC-Pro* were designed for use in the ligation-independent cloning (LIC) method by adding adapter sequences with: 5'- GAGGAGAAGAGCCCT (adaptor sequence) TCA AGCGTAGTCTGGGACGTCGTATGGGTA– viral coding region specific sequence - 3' in C-terminal region (Table 1-1). As a control, Coat Protein (CP) coding regions from PepSMV and PVY-0 were designed for use in the ligation-independent cloning (LIC) method by adding adapter sequences. All amplified PCR products were cloned by LIC method into the pCAMBIA 2300-LIC vector containing the CaMV 35S promoter and the NOS terminator cassette (Oh et al., 2010; Bae et al., 2013). A total 15 fmol of purified PCR product was treated with T4 DNA polymerase (NEB) in reaction buffer containing 10 mM dATP at 22℃ for 30min and 70℃ for 20min for inactivation of T4 DNA polymerase. The pCAMBIA 2300-LIC vector was digested with *PstI* and treated with T4 DNA polymerase with 10 mM dTTP. T4 DNA polymerase-treated PCR products and pCAMBIA 2300-LIC vector were mixed and incubated at room temperature for 30 min (Oh et al., 2010). The mixture was transformed into *E. coli* DH10b competent cells.

The entire sequence of cloned cistrons was confirmed by DNA sequencing at the National Instrumentation Center for Environmental Management (NICEM, Seoul, Korea). Each cloned vector was transformed into *Agrobacterium tumefaciens* strain C58C1 for transient *in planta* expression assays (Wroblewski et al., 2005).

Table 1-1. Primer sequences used in this study

	Primer name	Sequence
1	PepMov-LIC-P1-F	CGACGACAAGACCCTATGGCAATTAACGTTATTCAG
2	PepMov-LIC-P1-R	GAGGAGAAGAGCCCT TCAATACTGTTCCATATGAAGTACAGTTGC
3	PepMov-LIC- HC-Pro-F	CGACGACAAGACCCT ATGTCAACAtCTGAAGCATTTTGGAG
4	PepMov-LIC- HC-Pro-R	GAGGAGAAGAGCCCT TCAACCAACTCTATAGTGCTTTATCTCAG
5	PepMov-LIC-P3-F	CGACGACAAGACCCT ATGGGAACTGTAGAGAATCATAAAGTG
6	PepMov-LIC-P3-R	GAGGAGAAGAGCCCT TCATTGGTGGATGACCTGTTTTTCATCATA
7	PepMoV-P3N-R	GTAAGAGGCCTTGATAATTTTTTTCCTAATTCTTTCCATTGGTCGTGTAAGAGGCC
8	PepMov-PIPO-F	GGAAAAAAATTATCAAGGCCTCTTAC
9	PepMov-P3N-PIPO-LIC-R	GAGGAGAAGAGCCCT TTA GTTCCTCGCTTGGTGCGCACTG
10	PepMov-LIC-6K1-F	CGACGACAAGACCCT ATGAGATCAACTGAAGATCTCAAG
11	PepMov-LIC-6K1-R	GAGGAGAAGAGCCCT TGACTGATGTCTAACTTCTGAACCTAG
12	PepMov-LIC-6K2-F	CGACGACAAGACCCT ATGTCCAAGTCTTCTCTTGCGAAG
13	PepMov-LIC-6K2-R	GAGGAGAAGAGCCCT TGACTGATGACTCACTTCAGACATCTTTC
14	PepMov-LIC-Cl-F	CGACGACAAGACCCT ATGTCTTTGGATGATTTTGTGAATAC
15	PepMov-LIC-CI-R	GAGGAGAAGAGCCCT TCACTGGTGATGCACAAATTGTAAC
16	PepMov-LIC-VPg-F	CGACGACAAGACCCT ATGGGACGCTCTAAGACGAAAAG
17	PepMov-LIC-VPg-R	GAGGAGAAGAGCCCT TCATTCGTGCTTCACAACTTCCTTTG
18	PepMov-LIC-NIa-F	CGACGACAAGACCCT ATGGCGAAAACTtTAATGAGGGGC
19	PepMov-LIC-NIa-R	GAGGAGAAGAGCCCT TCATTGCTCCCTCACACATTCACTC
20	PepMov-LIC-NIb-F	CGACGACAAGACCCT ATGGCACACACATCACCTTGGATG
21	PepMov-LIC-NIb-R	GAGGAGAAGAGCCCT TCACTGATGATGAACTTCATATGTACCAC
22	PepMov-LIC-CP-F	CGACGACAAGACCCT ATGAGCAGCTCAAGATCAGACAC
23	PepMov-LIC-CP-R	GAGGAGAAGAGCCCT TCACATATTTCTGACCCCAAGC
24	PeSMV-LIC-NIb-F	CGACGACAAGACCCT ATGGCTAGCCATTCAGCATGGATG
25	PeSMV-LIC-NIb-R	GAGGAGAAGAGCCCT TCATTGATGATAAACTTCATATGGACG
26	PVY-LIC-NIb-F	CGACGACAAGACCCT ATGGCTAAGCACTCTGCGTGGATG
27	PVY-LIC-NIb-R	GAGGAGAAGAGCCCT TCATTGATGGTGTACTTCATAAGAGTC
28	PeSMV-LIC-CP-F	CGACGACAAGACCCT ATGGCCGATACAACTGTTGATG
29	PeSMV-LIC-CP-R	GAGGAGAAGAGCCCT TCATGTGTTTCTAACCCCAAGC
30	PVY-LIC-CP-F	CGACGACAAGACCCT ATGGCAAATGACACAATCGATGC

31	PVY-LIC-CP-R	GAGGAGAAGAGCCCT TCACATGTTCTTGACTCCAAGTAG
32	PepMov-LIC-frame-shifted NIb-F	CGACGACAAGACCCT ATGCACACACATCACCTTGGATG
33	PepMov-HC-Pro-HA-LIC-R	GAGGAGAAGAGCCCT TCAAGCGTAGTCTGGGACGTCGTATGGGTAACCAACTCTATAGTGCTTTATCTCAG
34	PepMoV-NIb-HA-LIC-R	GAGGAGAAGAGCCCT TCAAGCGTAGTCTGGGACGTCGTATGGGTACTGATGATGAACTTCATATGTACCAC
35	PepMoV-CP-HA-LIC-R	GAGGAGAAGAGCCCT TCAAGCGTAGTCTGGGACGTCGTATGGGTACATATTTCTGACCCCAAGC

***In planta* Expression Assay in Pepper Plants**

After transformation, the cultured cells were centrifuged and re-suspended in induction buffer (10 mM $MgCl_2$, 10 mM MES pH 5.6, and 200 μM Acetosyringone), and cells were incubated at room temperature for 2 h before agro-infiltration. The concentration of *Agrobacterium* cells was adjusted to 0.5 at OD_{600}, and then the cells were subjected to pressure infiltration using needleless syringe (Oh et al., 2009). Empty vector and vector with necrosis-inducing protein (NIP) from *Phytophthora sojae* were infiltrated into one pepper leaf as a negative or positive control, respectively (Qutob et al., 2002). All experiments were performed as three biological replicates. Cell death on the leaves was observed at two or three days after *Agrobacterium* infiltration. Inoculated leaves were cleared in 100% ethanol to remove chlorophyll in order to visualize the cell death. Total RNA was extracted from pepper plant using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. First strand cDNA was synthesized using 3 μg total RNA with oligo (dT) and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) for RT-PCR. Oligonucleotides used in RT-PCR were described in Table 1-1.

Immunodetection of PepMoV-encoded proteins

To confirm the *in planta* expression of viral proteins, it was representatively decided to design three HA-tagging constructs out of eleven viral-encoded proteins. HA tag sequence was added at C-terminal of PepMoV NIb, CP and HC-Pro (See Material and methods, Cloning of Potyvirus Cistrons for *in planta* Expression). These constructs were transformed into *Agrobacterium* C58C1 and the cells were fully infiltrated into *N. benthamiana* leaves. Total protein was extracted from leaves of *N. benthamiana* with extraction buffer as described in Win *et al* (Win et al., 2011) at 1 day and 2 days after infiltration of each construct. Protein concentrations were measured by Bradford assay (Thermo Scientific, Waltham, Massachusetts, United States), and equal amounts were loaded onto polyacrylamide gels. After transfer, western blot analysis was accomplished to detect protein expression by using an anti-HA antibody (Abcam, Cambridge, UK) and an anti-rabbit horseradish peroxidase conjugate (Abcam, Cambridge, UK).

RESULTS

Genotypes and PepMoV Accumulation in Pepper Plants

To confirm Pvr4-mediated resistance in pepper plants, it was performed genotype screening by PCR with the PCAPS15 marker, and then utilized ELISA to detect PepMoV accumulation (Kim et al., 2011). When the marker was applied in pepper, *Pvr4*-harboring pepper genotypes showed 550- and 270-bp fragments, while *Pvr4*-lacking (*pvr4*-) plant genotype showed 470- and 350-bp fragments. In our results, ‘CM334’, F1 hybrid and the resistant homozygotic F2 (RR) lines contained band patterns of *Pvr4*-harboring genotype, whereas the other peppers had band patterns of *Pvr4*-lacking genotype (Fig. 1-1). Resistance against PepMoV could be confirmed by ELISA with a PepMoV antibody, which presents an accumulation of virus. Lower values (ELSIA value < 0.2) which were detected with ‘CM334’, F1 hybrid and the resistant homozygotic F2 lines represented that PepMoV replication was limited in those peppers. On the other hand, ‘ECW’, ‘Jupiter’ and the susceptible homozygotic F2 (SS) lines showed higher values (ELSIA value > 0.4) (Fig. 1-1). These results indicated that *Pvr4*-harboring plants successfully repressed the growth of PepMoV virus and that resistance phenotypes of pepper plants against PepMoV co-segregated with their

genotypes. From these conclusions, it was decided to use these pepper lines for screening the avirulence factor of potyviruses.

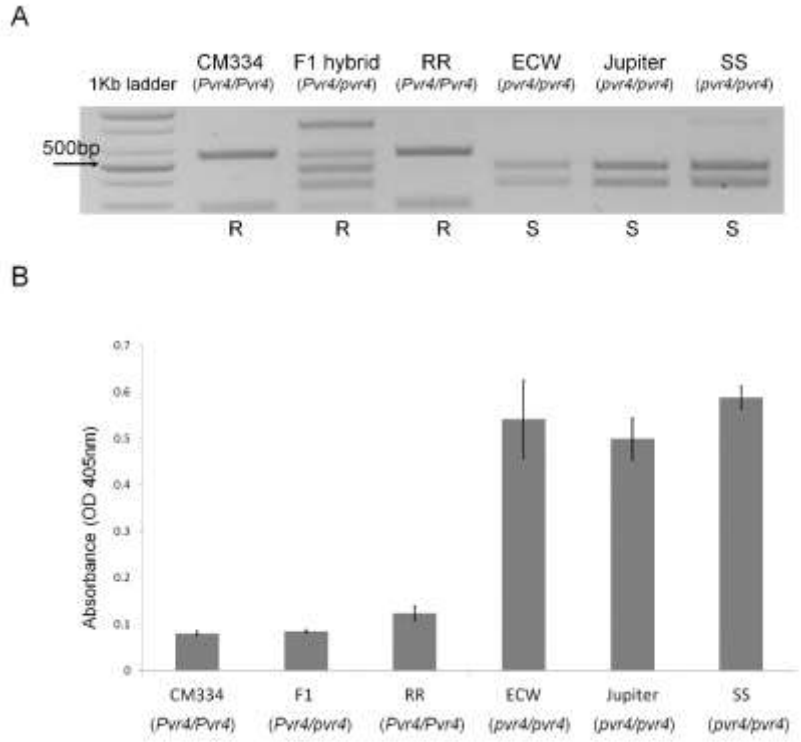

Figure 1-1. Genotypes and genotype-specific accumulation of PepMoV in pepper plants. (A) Identification of genotype in relation to *Pvr4* using the CAPS marker (PCAPS15). *Pvr4*-harboring pepper genotypes have 550- and 270-bp fragments, while *pvr4*-plants have 470- and 350-bp fragments. RR; a resistant homozygotic F2, SS; a susceptible homozygotic F2. Genotype of each plant is depicted under the cultivar name, and phenotypes of plants are also described under the images. R denotes resistant, and S denotes susceptible. (B) Detection of accumulated PepMoV by ELISA. Resistance against PepMoV was confirmed by ELISA with PepMoV antibody, which presents an accumulation of virus. Genotype of each plant is depicted under the cultivar name. Pepper leaves were sampled at 15 dpi. Error bars represent standard deviations. This result and subsequent figures show a representative experiment of three biological replicates.

Identification of NIb as the Avirulence Factor of PepMoV in *Pvr4*-bearing Pepper Plants

To identify the avirulence factor of PepMoV, it was performed *in planta* expression analyses with eleven viral proteins of PepMoV in pepper plants (Table 1-2). First, PepMoV coding regions were dissected and cloned into the pC2300-LIC binary vector with a 35S promoter (Quenouille et al., 2013; Ivanov et al., 2014). For *in planta* expression analyses, each clone was infiltrated in all six pepper cultivars, respectively. As results, HR-like cell death was observed only in the PepMoV NIb-expressing leaves in a genotype-specific manner. However, the HR-like cell death was absent when other viral cistrons were infiltrated (Fig. 1-2 and Fig. 1-3).

To test whether each clone from PepMoV interacts with Pvr4 at the protein level, there were picked three clones, NIb, HC-Pro, and CP from PepMoV and generated HA-tagged constructs (PepMoV NIb:HA, PepMoV HC-Pro:HA and PepMoV CP:HA). Each protein expression was detected by western blot experiments using anti-HA at 24 and 48 hours after infiltration in *N. benthamiana* (Fig. 1-2). To verify that these proteins still have their activity in *Pvr4*-mediated resistance, it was performed *in planta* expression of these HA-tagged proteins in ‘CM334’ and also observed HR-like cell death with PepMoV NIb:HA regardless of whether the HA tag was present or not.

Over-expression of other cistrons such as PepMoV HC-Pro and PepMoV CP did not induce HR-like cell death in ‘CM334’ (Fig. 1-2). This results suggested that the PepMoV NIb protein works as the avirulence factor in *Pvr4*- containing ‘CM334’.

Table 1-2. PepMoV cistrons used in this study

Name of cistron	Size (bp)	Function	References
P1	861	serine protease	(Verchot et al., 1992)
HC-Pro	1368	helper-component protease	(Ala-Poikela et al., 2011)
P3	1083	potyviral membrane protein	(Restrepo-Hartwig and Carrington, 1994; Chung et al., 2008)
6K1	156	unknown	-
CI	1902	cylindrical inclusion	(Wei et al., 2010)
6K2	156	potyviral membrane protein	(Restrepo-Hartwig and Carrington, 1994)
VPg	564	viral protein genome-linked	(Elena and Rodrigo, 2012)
NIa (Pro)	738	nuclear inclusion A	(Carrington and Dougherty, 1987)
NIb	1557	RNA dependent RNA polymerase	(Hong and Hunt, 1996; Janzac et al., 2010)
CP	819	coat protein	(Atreya et al., 1995)
P3N-PIPO	771	cell-to-cell movement	(Chung et al., 2008; Vijayapalani et al., 2012)

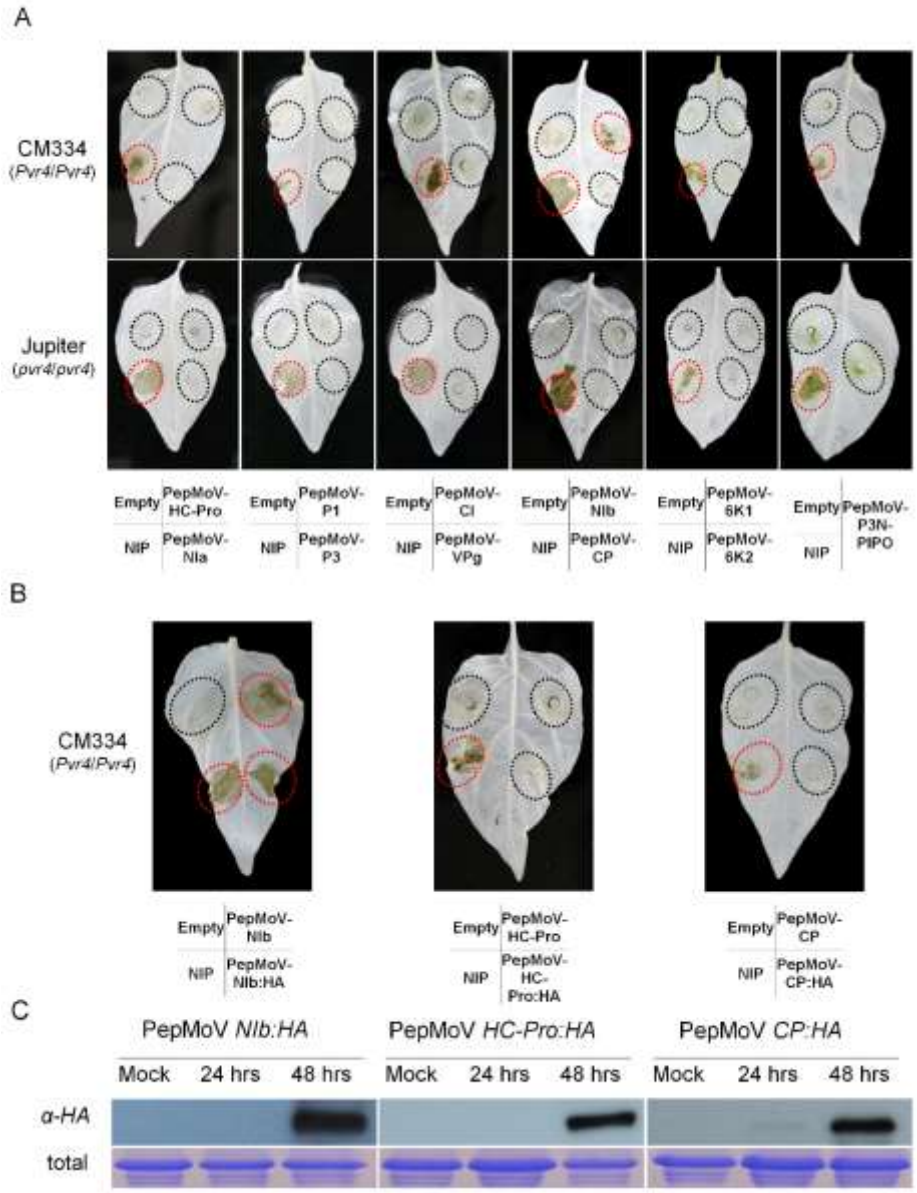

Figure 1-2. Identification of NIb as the HR-inducing avirulence factor against *Pvr4*-bearing pepper plants. (A) Transient expression of PepMoV viral proteins in 'CM334' and 'Jupiter'. Eleven cistrons from PepMoV were infiltrated into 'CM334' and 'Jupiter'. At 3 dpi, leaves were cleared with 100% ethanol to remove chlorophylls in

order to visualize the cell death. For this and subsequent experiments, Empty vector and vector with necrosis-inducing protein (NIP) from *P. sojae* were infiltrated as a negative or positive control, respectively. Regions of infiltration were marked with ovals and the area of cell death was marked as red. Inoculated viral cistrons were depicted under panels. (B) Transient expression of HC-Pro:HA, CP:HA and NIb:HA in 'CM334'. Plant responses with HA-tagged proteins were tested in *Pvr4*-harboring plants ('CM334'). Inoculated viral cistrons were depicted under panels. (C) Expression of PepMoV NIb:HA, CP:HA and HC-Pro:HA proteins in *N. benthamiana* leaves. 5-week-old tobacco leaves were collected at 24 hpi and 48 hpi. Untreated leaves were used as mock for negative controls. Each protein was immunodetected by using anti-HA antibody. Coomassie blue–stained total proteins were shown as loading controls.

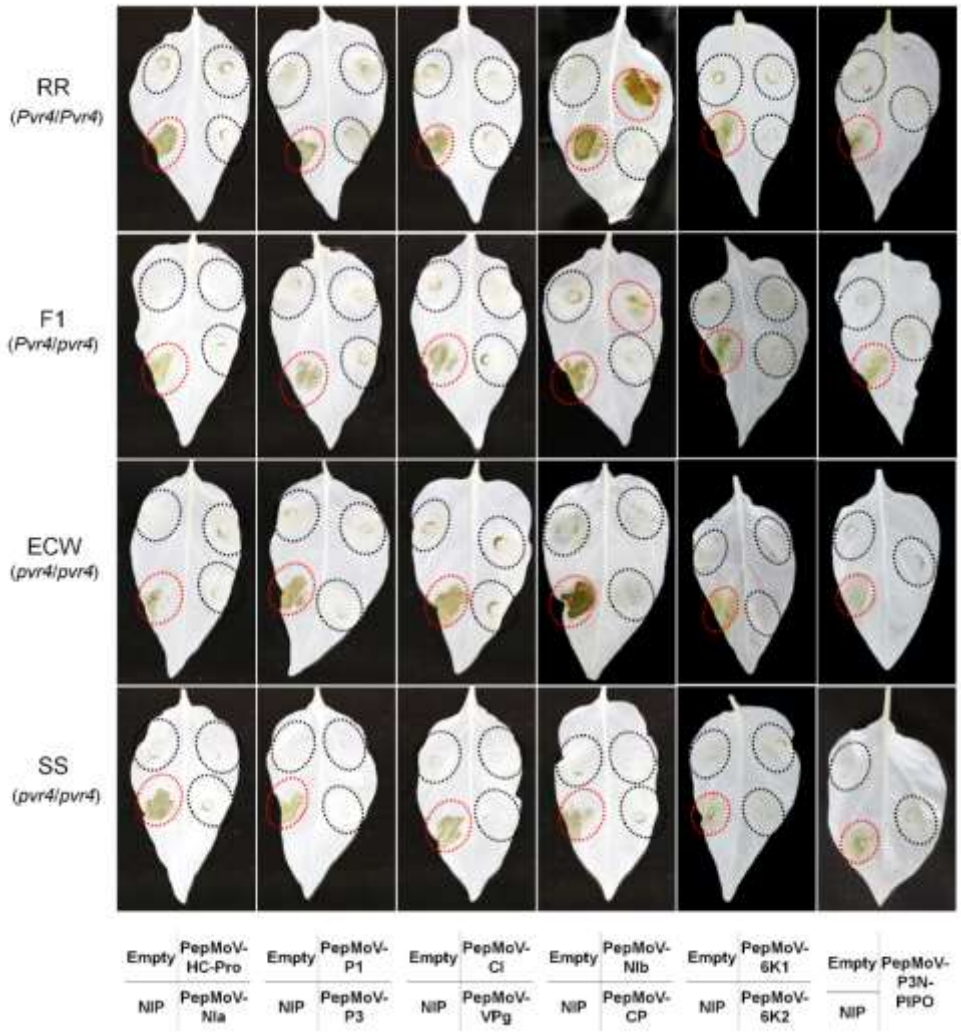

Figure 1-3. Identification of NIb as the HR-inducing avirulence factor against *Pvr4*-bearing pepper plants. Transient expression of PepMoV viral proteins in the resistant homozygotic F2 (RR), F1 hybrid, 'ECW' and the susceptible homozygotic F2 (SS). Eleven cistrons from PepMoV were infiltrated into four pepper cultivars.

To investigate the correlation of NIb-induced cell death with *Pvr4* in pepper, it was also examined the phenotypes of the F2 population derived from ‘CM334’ and ‘Jupiter’ by transient expression of *PepMoV NIb*. The genotypes of the F2 segregating progenies of the cross between ‘CM334’ and ‘Jupiter’ were clarified by the PCAPS15 marker analysis (Fig. 1-4). All *Pvr4*-bearing plants showed HR cell death while none of *pvr4*-plants show HR cell death (Fig. 1-4). This results implied that HR-like cell death phenotype induced by PepMoV NIb is related to *Pvr4*.

To confirm the *NIb* RNA itself does not cause HR-like cell death, the frame-shifted mutant of *NIb* (PepMoV-ΔNIb) was generated and transiently expressed in the F2 populations derived from ‘Jupiter’ and ‘CM334’. Expression of *PepMoV NIb* and *PepMoV-ΔNIb* were confirmed in pepper leaves tested by RT-PCR (Fig. 1-5). The NIb mutant did not induce HR-like cell death phenotype in any tested pepper plants while the in-frame NIb construct showed HR cell death (Fig. 1-5).

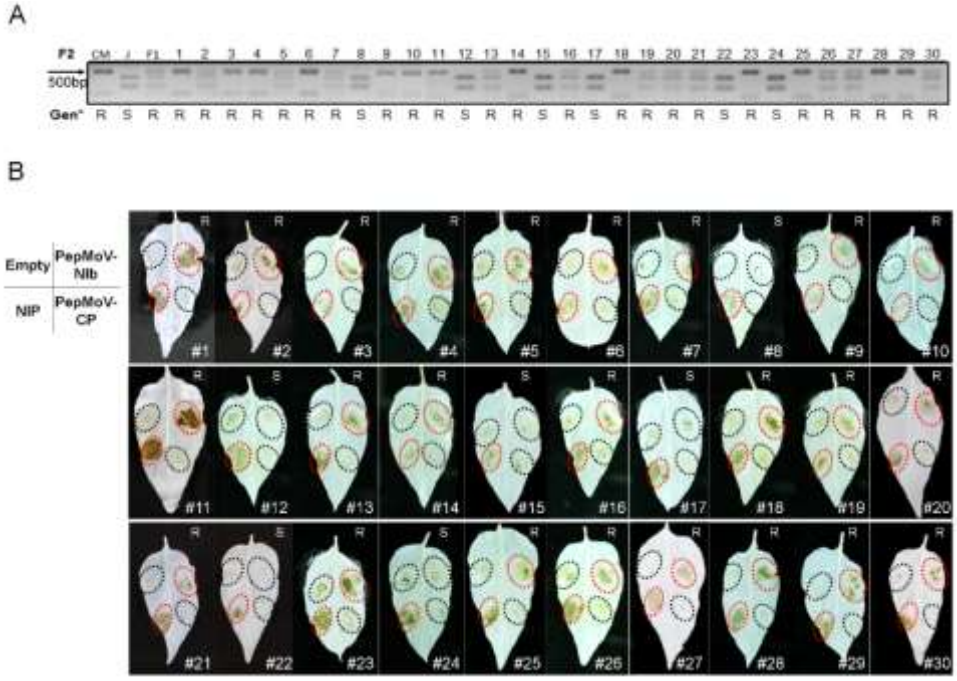

Figure 1-4. Correlation of genotypes and cell death phenotype of *Pvr4* against NIb in the F2 population. (A) Identification of genotype in relation to *Pvr4* using the CAPS marker (PCAPS15). Thirty plants of the F2 generation were tested to identify their genotypes. Genotypes of plants (Gen*) are described under the images as R (resistant) or S (susceptible). (B) Response of the F2 population plants derived from 'Jupiter' and 'CM334' to PepMoV proteins, NIb and CP. Thirty progenies of the F2 generation were tested to verify whether *Pvr4*-harboring plants show HR in response to PepMoV NIb. The F2 lines which showed HR cell death as well as *Pvr4* genotypes were marked as R. S represents the F2 lines which did not show HR cell death and were confirmed as *pvr4*-plants. Inoculated viral cistrons were depicted at the left of panel.

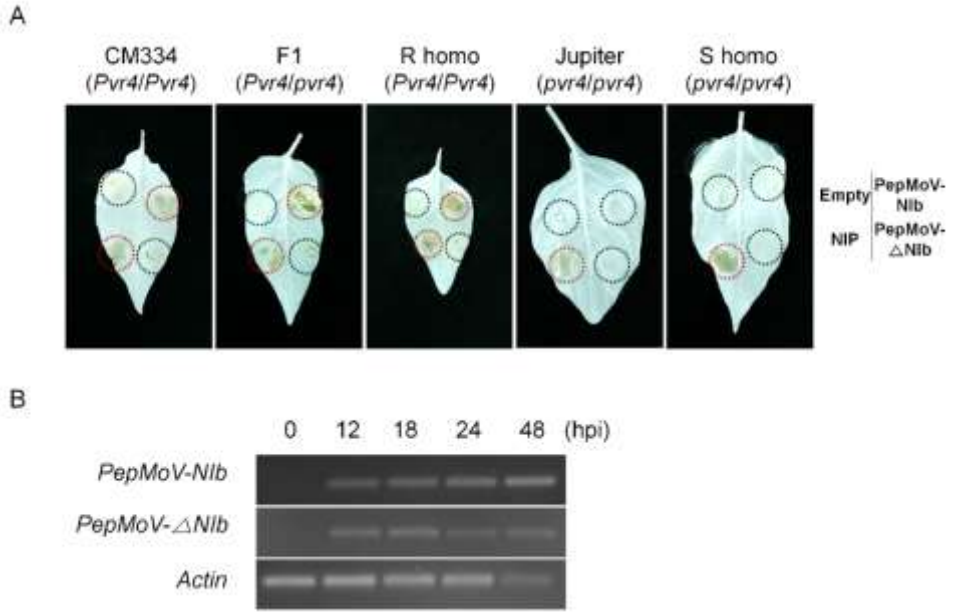

Figure 1-5. Verification of NIb-encoded protein as the avirulence factor against *Pvr4*-bearing pepper plants. (A) Response of five pepper cultivars after *in planta* expression of *NIb* or frame-shifted *NIb* mutant clone of PepMoV at 2-3 dpi. (B) RT-PCR of transient overexpressed PepMoV *NIb* and -*⊿NIb*. Pepper leaves were sampled at 0, 12, 18, 24 and 48 hours after transient overexpression. As a control, *actin* was used.

NIb proteins of other Potyviruses as Avirulence Factors in Pvr4-mediated Resistance

To test whether NIb proteins from other potyviruses function as avirulence factors, there were cloned NIb coding regions from potyviruses PepSMV and PVY into the pCAMBIA 2300-LIC vector and examined *in planta* expression assays with pepper plants. When each *NIb* cistron was transiently expressed in each pepper plants, HR-like cell death was observed only in *Pvr4*-containing plants (‘CM334’, the F1 hybrid, and the resistant homozygotic F2) (Fig. 1-6 and Fig. 1-7). These results indicate that NIbs of PepSMV and PVY also function as Pvr4 effectors in the plant immune system.

Since TEV is a virulent potyvirus to *Pvr4*-bearing pepper plants, it was tested whether TEV NIb interacts with Pvr4 and subsequently causes cell death. Thus, TEV NIb coding region was cloned into pC2300-LIC vector and *in planta* expressed in leaves of ‘CM334’ and ‘Jupiter’. However, HR-like cell death was not observed in any pepper leaves when the clone was infiltrated (Fig. 1-6). Taken together, although TEV has NIb like other potyviruses, TEV NIb could not induce HR-like cell death and additionally TEV shows virulence in *Pvr4*-bearing pepper plants (Table 1-3).

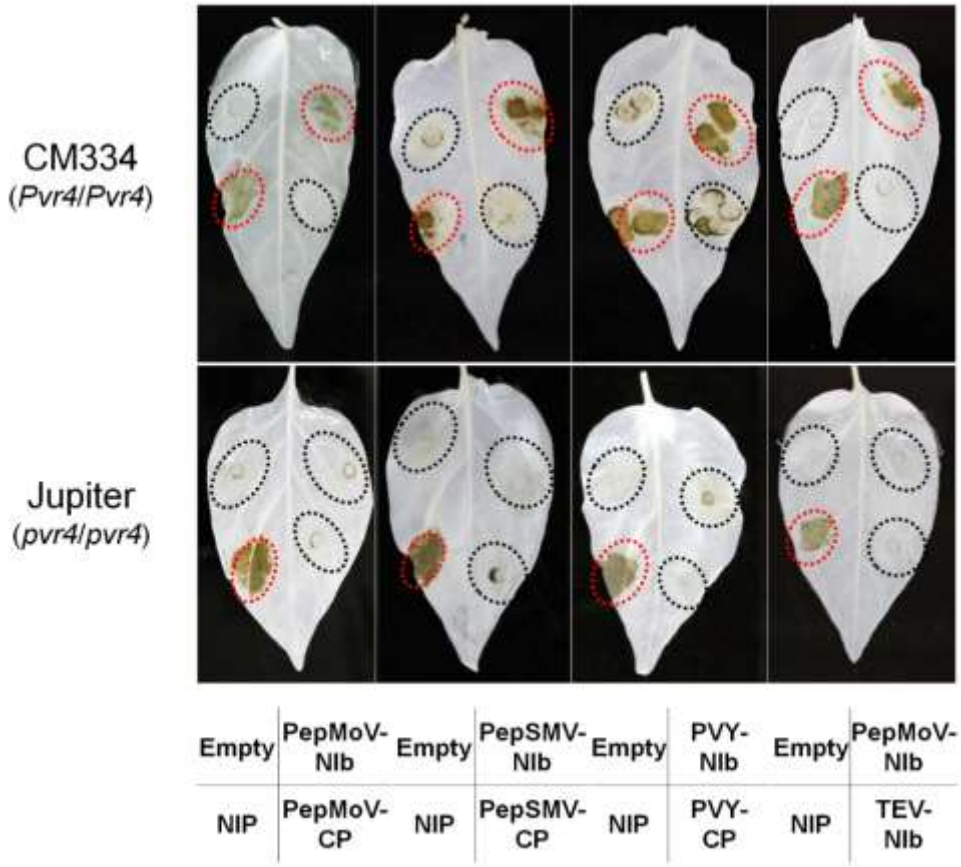

Figure 1-6. Confirmation of NIb as the HR-inducing avirulence factor against *Pvr4*-bearing pepper plants. *In planta* expressions of NIbs from four potyviruses were performed in 'CM334' and 'Jupiter', respectively.

Table 1-3. Resistance and HR induced NIb of potyviruses in *Pvr4*-harboring pepper plants

Potyvirus species	**NIb-induced HR**	**Virus resistance**	
		Phenotype	**References**
PepMoV (DQ631638)	+	R	In this study, (Janzac et al., 2009; Lee et al., 2011)
PepSMV (NC_008393)	+	R	(Ahn et al., 2006; Janzac et al., 2009)
PVY (EF026074)	+	R	(Valkonen et al., 1996; Janzac et al., 2009)
TEV (M11458)	-	S	(Valkonen et al., 1996; Janzac et al., 2009)

HR, hypersensitive response. R, resistant; S, susceptible.

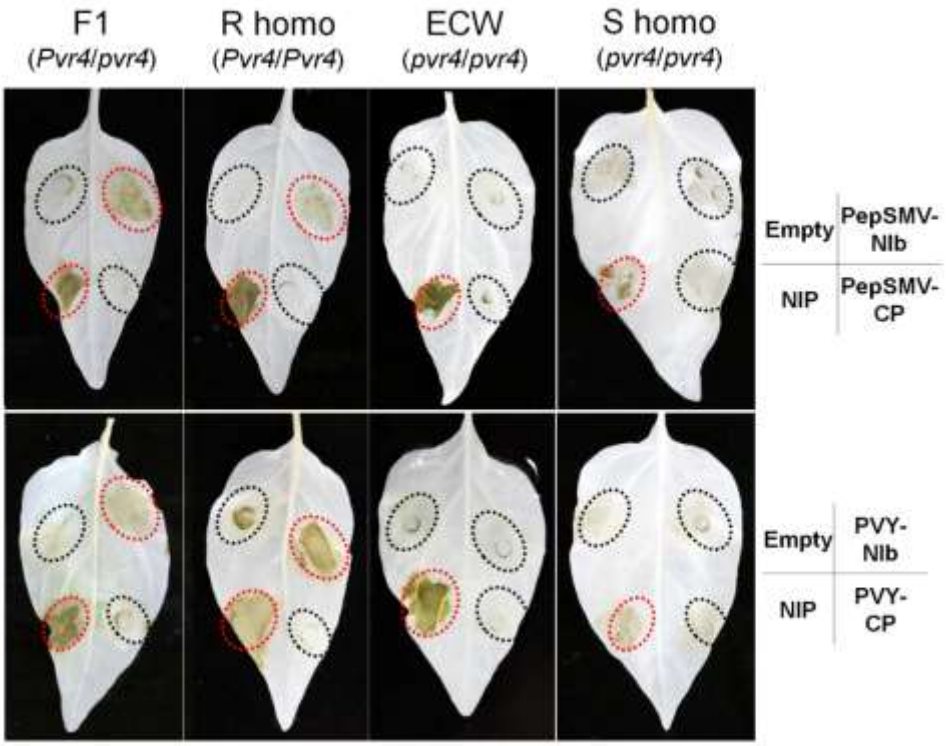

Figure 1-7. Confirmation of NIb as the HR-inducing avirulence factor against *Pvr4*-bearing pepper plants. *In planta* expressions of NIbs from four potyviruses were performed in four cultivars, respectively.

DISCUSSION

In this study, to identify an avirulence factor of PepMoV against Pvr4-based resistance, transient over-expression of a total of eleven viral cistrons of PepMoV into *C. annuum* 'CM334' (potyvirus-resistant, *Pvr4*) and *C. annuum* 'Jupiter' (potyvirus-susceptible, *pvr4*) were tested (Table 1-2) (Fig. 1-2). Eleven viral proteins of PepMov were dissected and each clones was transient over-expressed into pepper leaves including resistant pepper ('CM334', F1 hybrid crossed by 'CM334' and 'Jupiter', RR homozygotic line of self-pollinated BC1F2) and susceptible pepper ('ECW', 'Jupiter' and SS homozygotic line of self-pollinated BC1F2). Only NIb protein of PepMoV induced HR-like cell death in resistant pepper, and other viral cistrons did not show any cell death symptoms in all tested pepper leaves (Fig. 1-3). These results clearly demonstrate that the PepMoV NIb protein is the avirulence factor for *Pvr4* in pepper plants. This *in planta* expression assay had been used for identifying nonstructural protein (NSs) of *tomato spotted wilt virus* (TSWV) which was identified as avirulence (Avr) factor for *Tsw* resistance gene in pepper (de Ronde et al., 2013). Furthermore, it has been reported that NIb of PVY-O would be an avirulence factor against Pvr4 (Janzac et al., 2009). They analyzed nucleotide changes between the genome of the cDNA clone of

the avirulent SON41p isolate and its virulent variants. Mutant clone of SON41p was observed A8424G substitution in the NIb cistron of virulent variants of SON41p. This result indicated that one single nucleotide substitution conferred the virulence against *Pvr4*. Although direct evidence lacked, it was more likely that the avirulence factor corresponding to Pvr4 is the PVY NIb protein.

Moreover, to determine the correlation of NIb-induced cell death with *Pvr4* in pepper, thirty F2 individuals were screened by *Pvr4*-linked marker (PCAPS15) and *PepMoV NIb* was over-expressed into each pepper leaves. The result showed that PepMoV NIb induced HR-like cell death only in *Pvr4*-haboring pepper in genotype-specific manner (Fig. 1-4). PCAPS15 was *Pvr4*-linked marker developed in Kim et al. (2011). This result implied that HR-like cell death showed by PepMoV NIb is genetically related to Pvr4.

In a previous study, it was described that an untranslatable RNA sequence of the *Cymbidium Ringspot Virus* (CymRSV) CP might be a HR inducing elicitor in *Datura stramonium* (Szittya and Burgyán, 2001). To confirm the NIb RNA itself cause HR-like cell death, it was infiltrated the frame-shifted mutant of NIb (PepMoV-ΔNIb) in the *Pvr4*- or *pvr4*-harboring peppers. As expected, PepMoV-ΔNIb did not induce HR-like cell death in any tested pepper leaves (Fig. 1-5). This result indicated that HR-like cell death was not

induced by *NIb* RNA in resistant pepper plants, but by NIb protein.

Pvr4 mediate resistance against PepMoV, PVY and PepSMV, but not TEV (Janzac et al., 2009). It was supposed that NIb of PVY and PepSMV would act commonly an avirulence factor corresponding to Pvr4. Over-expressed NIbs of PVY and PepSMV induced HR-like cell death in *Pvr4*-carrying pepper leaves with the same result of PepMoV NIb (Fig. 1-6, Fig. 1-7). In contrast to avirulent viruses against Pvr4, NIb of TEV did not induce HR-like cell death in any tested pepper leaves (Fig. 1-6). The reason why TEV NIb does not cause HR-like cell death is likely that it has a difference in structure compared to other three potyviruses NIbs. In previous study, TEV diverged from other three potyviruses in phylogenetic tree when parts of these nucleotide sequences were compared (Janzac et al., 2009). Furthermore, when it was compared the identity of NIb proteins among four potyviruses, TEV NIb had 61% identity compared with PepMoV, PepSMV and PVY, while three potyviruses have at least 76% identity. This result infers that TEV NIb, which has lower identity to other potyviruses NIbs, may not be recognized by Pvr4. In sum, these data strongly suggest that the high similarity of NIb protein sequences in avirulent potyviruses might be important for these proteins to function as avirulence factors. Subsequently, this would mediate a broad-spectrum stable resistance for *Pvr4*-bearing

pepper plants.

In this study, it was demonstrated that NIb proteins of three potyviruses are common avirulence factors for *Pvr4*-mediated resistance in pepper plants. These results may provide an efficient tool for the isolation of the broad-spectrum potyvirus resistance gene *Pvr4* from pepper, as well as for studying potyvirus resistance mechanisms in plants

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CHAPTER 2

Genome-based cloning of *Pvr4* conferring multiple potyvirus resistance from *Capsicum annuum* 'CM334'

ABSTRACT

Pvr4 is a resistance gene conferring broad dominant resistance against *Pepper mottle virus* (PepMoV), *Pepper severe mosaic virus* (PepSMV) and all isolates of *Potato virus Y* (PVY). The *Pvr4* locus of a landrace *Capsicum annuum* 'CM334' was mapped on south arm of pepper chromosome 10 linked with TG420. Based on synteny between tomato and pepper genomes, a high-density genetic map including thirty-two co-dominant markers was developed in flanking region of TG420. To perform genome-based cloning of *Pvr4*, three CAPS markers co-segregated with *Pvr4* in two populations derived from crosses between *C. annuum* 'CM334' and 'ECW' or 'Jupiter', were developed. Flanking region of *Pvr4* was delimited as 175kb containing eight *Pvr4* candidates encoding putative coiled-coil (CC) nucleotide-binding leucine rich repeat (NB-LRR). To identify *Pvr4*, *in planta* co-expression of the eight *Pvr4* candidates and *PepMoV-NIb* were transiently co-expressed in susceptible pepper (*C. annuum* 'Jupiter') and *Nicotiana benthamiana* leaves. One of these candidates, designated the *Pvr4* gene, induced hypersensitive response (HR) by co-expression with *PepMoV-NIb*. Furthermore, over-expression of *Pvr4* significantly suppressed replication of PepMoV, PepSMV and PVY in *N. benthamiana*. *Pvr4* consists of seven exons and encodes a

putative CC-NB-LRR type protein with 1746 amino acids. The heterologous expression of *Pvr4* in potato showed resistance against the PVY-O isolate. In *Pvr4*-mediated HR, Pvr4 activation by PepMoV-NIb is dependent on the co-chaperons SGT1, HSP90 and RAR1 in *N. benthamiana*. The identified *Pvr4* gene may provide a novel source of resistance for breeding potyvirus resistant crops including Solanaceae plants.

INTRODUCTION

Plant viruses cause significant losses of the most economically important crops in the fields and the loss attributable to virus disease for six major U.S crops (Corn, wheat, soybeans, cotton, rice and hay) was estimated to be about 1-2% of total yield (James, 1981). Among plant viruses, potyviruses are the most destructive plant viruses which result in considerable losses in a wide range of Solanaceae crops (Ivanov et al., 2014). Members of the genus *Potyvirus* including *Pepper mottle virus* (PepMoV), *Potato virus Y* (PVY), *Pepper severe mosaic virus* (PepSMV) and *Tobacco etch virus* (TEV) infect pepper as well as other Solanaceous plants (Adams et al., 2005). Since agricultural chemicals are ineffective to control the enormous damage caused by viral infections, the application of plant genetic resistance is considered as an alternative method to protect crops from the damage caused by viruses (Janzac et al., 2009). In accordance with development of advanced molecular biology techniques and genomics, to decrease the losses of crops by viruses via the application of plant defense mechanism is a shift away from chemical controls (Kenyon et al., 2014).

In plants, the most effective specific resistance to pathogens such as viruses is conferred by resistance (R) proteins. R proteins in host plants

recognize directly or indirectly the corresponding avirulence factor (Avr) in pathogens in a highly specific manner and trigger defense responses (Flor, 1971; Dangl and Jones, 2001). Most known *R* genes encode proteins containing NB (nucleotide binding) - ARC (Apaf-1, R gene, CED-4) followed by a LRR (leucine rich repeat) domain. NB-LRRs often have an additional domain in the N-terminal region such as putative leucine-zipper (LZ), coiled-coil (CC) or the Toll and Interleukin 1 receptor (TIR) (van Ooijen et al., 2007). Several studies have been reported the resistance mechanisms triggered by the interaction between plant R protein and an Avr factor of viruses (Kang et al., 2005a; Moffett, 2009). To date, nine dominant plant *R* genes to viruses have been identified including *L*, *Rx*, *Sw5*, *Tm-2*, *N* (from Solanaceous crops), *HRT*, *RCY1*, *RTM* (from *Arabidopsis*) and *RSV* (from soybean).

Two monogenic dominant genes have been known to confer resistance against potyvirus in *Capsicum* Spp. (Caranta et al., 1999; Grube et al., 2000). In *C. annuum* 'CM334', *Pvr4* confers dominant resistance against six potyviruses including PepMoV, PepSMV, *Pepper yellow mosaic virus* (PepYMV), E*cuadorian rocoto virus* (ERV), *Peru tomato virus* (PTV) and all PVY isolates (Dogimont et al., 1996; Janzac et al., 2009). Among these viruses, RNA-dependent RNA polymerases (NIbs) of PepMoV, PepSMV and PVY were defined as Avr factors against Pvr4 in pepper (Janzac et al., 2010;

Kim et al., 2015). *In planta* expression of *NIb*s of avirulent potyviruses into *Pvr4*-carrying pepper conferred a localized cell death (hypersensitive response, HR), while over-expression of *NIb* of virulent TEV did not induce HR (Kim et al., 2015). *Pvr7* in *C. chinense* 'PI159236-9093' was also reported as a dominant resistance gene to PepMoV and PVY, and was tightly linked to *Pvr4* (Grube et al., 2000; Arnedo-Andrés et al., 2002). *Pvr4 and Pvr7* are mapped on pepper chromosome 10, and closely linked to *Tsw* conferring dominant resistance against *Tomato spotted wilt virus* (TSWV) (Grube et al., 2000; Jahn et al., 2000). Although the genetic position of *Pvr4* is known to be closely linked to a tomato RFLP TG420 marker, the identity of *Pvr4* has been still unknown (Lefebvre et al., 2002; Kim et al., 2011).

In plant defense signaling, following pathogen recognition, the R protein activates a signaling cascade that coordinates plant defense responses to block pathogen spread by HR (Shirasu and Schulze-Lefert, 2000; Dangl and Jones, 2001; Kang et al., 2005b). Several genes have been identified as HR-related signaling components such as *ICSI* (*ISOCHORISMATE SYNTHASE 1*), *EIN2* (*ETHYLENE INSENSITIVE 2*) and *COII* (*CORONATINE INSENSITIVE 1*) which are also known to be involved in hormone synthesis and *RAR1* (*REQUIRED FOR MLA12 RESISTANCE 1*), *HSP90* (*HEAT SHOCK PROTEIN 90*) and *SGT1* (SUPPRESSOR OF THE G2 ALLELE OF SKP1)

acting as co-chaperones (Wildermuth et al., 2001; van Loon et al., 2006; van Ooijen et al., 2007; Shirasu, 2009) . In the case of *Pvr4*, despite of broad and important roles in resistance, its functions in the defense signaling pathway have not been elucidated.

With completion of the whole genome sequencing in several crop plants and pathogens, characterization of functional genes as well as marker assist selection are possible in a shorter time (Huang et al., 2005; Varshney, 2009; Consortium, 2011; Bombarely et al., 2012; Consortium, 2012; Hirakawa et al., 2014; Kim et al., 2014). In this study, by using pepper genome information, the genome-based cloning of *Pvr4*, a CC-NB-LRR type plant R gene derived *C. annuum* 'CM334', is reported. Co-expression of *Pvr4* and *NIb* of potyvirus triggered HR and over-expression of *Pvr4* conferred full resistance against a range of potyviruses in tobacco and potato. In addition, signaling components related to *Pvr4*-mediated HR were identified. Consequently, the transgenic application of *Pvr4* might be a novel approach for developing potyvirus resistant crops including Solanaceous plants.

MATERIALS AND METHODS

Plant materials

C. annuum ‘CM334’, ‘Jupiter’ and ‘ECW123R’ were used in this experiments. *C. annuum* ‘CM334’ (*Pvr4/Pvr4*) was used as a resistant line, and two varieties of peppers, *C. annuum* ‘Jupiter’ (*pvr4/pvr4*) and *C. annuum* ‘ECW123R’ (*pvr4/pvr4*) were used as susceptible lines. To perform genome-based cloning *Pvr4*, two mapping populations were generated by a cross between *C. annuum* ‘Jupiter’ and *C. annuum* ‘CM334’ and a backcross population from a cross between *C. annuum* ‘CM334’ and *C. annuum* ‘ECW123R’. An F2 population of 405 individuals was obtained from the *C. annuum* ‘Jupiter’ and *C. annuum* ‘CM334’ cross. The backcrossed population named BC1F3 of 1063 individuals was also obtained from the cross between *C. annuum* ‘CM334’ and *C. annuum* ‘ECW123R’. Total DNA was extracted according to the CTAB method (Hwang et al., 2009). *Nicotiana benthamiana* seeds were sown and grown in pots and maintained under a 16-h photoperiod at 24℃ for 4–5 weeks in chamber.

Phenotypic evaluation against potyviruses

PepMoV tagged with GFP (DQ631638.1), PepSMV (NC_008393), PVY-

O (EF026074.1) and TEV (M11458.1) were used for *Pvr4*-mediated resistance by propagating in *N. benthamiana*. One gram of infected tobacco leaves was macerated in 3 ml of 0.1 M phosphate buffer (pH 7.0) with a mortar. The 4-6 week old pepper leaves were dusted thoroughly with caborundum (600 mesh), gently rubbed with the inoculum, and washed immediately with tap water. Negative control plants were mock-inoculated with 0.1 M phosphate buffer (pH 7.0). After inoculation, the plants were kept in a growth chamber at 25℃. Potyviruses symptoms were first observed 2 weeks after inoculation, and the development of symptoms was monitored continuously until the experiment was completed. Double Antibody Sandwich (DAS)-ELISA (Agdia, Elkhart, IN, USA) were performed for monitoring of potyviruses accumulation at 14 dpi. Samples were considered positive for the presence of potyviruses when the absorbance value (405 nm) of each sample was greater than that of a negative control plant. Furthermore, to observe GFP expression, GFP-tagged PepMoV was rubbed in *Nicotiana tabaccum Xanti NC*.

Development of Cleaved Amplified Polymorphic Sequences (CAPS) markers

To develop *Pvr4*-linked markers based on the synteny between pepper

and tomato genome, flanking sequence of TG420 marker in chromosome 10 of pepper genome was analyzed. The gene coding regions of the tomato scaffold were predicted by FGENESH (http://linux1.softberry.com). The predicted amino acid sequences were used to search for the annotated genes using the BLASTP program (http://www.ncbi.nlm.nih.gov). The sequences of gene coding regions in tomato scaffold sequence were utilized to search for the homologous pepper sequences from pepper database (http://cab.pepper.snu.ac.kr). The primers were manually designed based on the intergenic regions of pepper sequences. The designed primers were used for parental screening using PCR method. For PCR analysis, 20-μl reaction mixtures were prepared, containing 100 ng of DNA, 1uM of each primer, 2.5 mM of each dNTP, 2.5 units *Taq* polymerase (TaKaRa Ex Taq™), 20 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 100 mM KCl, 1mM DTT, 0.5% Tween20, 0.5% NP-40 and 50% Glycerol. The PCR were performed for 40 cycles, using the following profile: 30 s DNA denaturation at 95℃, 30 s annealing at melting temperature, and 1 min elongation at 72℃. As a first step in PCR amplification, DNA was denatured for 5 min at 95℃ and finalized by an extra 5 min elongation step at 72℃. The amplification reactions were performed in a TaKaRa thermal cycler (TAKARA BIO INC, Kyoto, Japan). The PCR amplicons were purified with a Zymoclean PCR Purification Kit following

the manufacturer's protocol (Zymo Research, Irvine, CA, USA). Purified PCR products were sequenced at the National Instrumentation Center for Environmental Management (NICEM), Seoul National University, Seoul, Korea. Depending on the marker, the PCR product was digested with an appropriate restriction enzyme. Subsequently, the digested PCR products were analyzed by electrophoresis in agarose gels. Developed 32 markers were used for genotyping the BC1F3 and F2 populations. Genetic mapping of markers in the all populations were done using CarthaGene software.

BAC library screening and sequence analysis

A bacterial artificial chromosome (BAC) library consisting of 235,000 clones covering 12 X pepper genome (99%) constructed from *C. annuum* 'CM334' (Yoo et al., 2003) was used to develop 2D BAC pools. BAC pools were screened using the flanking markers 20172, 17918, 044832 and 1983 as the probes. Four BAC clones, those are covered in target region, were sequenced using PacBio and Roche 454 FLX Titanium at NICEM (Seoul National University, Seoul, Korea).

Cloning of *Pvr4* candidates by ligation-independent cloning

The region including *Pvr4*-linked markers on chromosome 10 were

selected from pepper database (http://cab.pepper.snu.ac.kr/). Based on sequences of NB-ARC genes cluster, primer sets were designed for cloning of eight NB-LRR genes predicted in this region. For cloning of *Pvr4* for *in planta* expression, specific primers to amplify each candidates were designed for use in the ligation-independent cloning (LIC) method by adding adapter sequences with: 5'- CGACGACAAGACCCT (adaptor sequence) – gene specific sequence - 3' and 5' – GAGGAGAAGAGCCCT (adaptor sequence) – gene specific sequence – 3'. To clone NB-LRR genes originated from gDNA, genomic DNA were extracted from *C. annuum* 'CM334' and *C. annuum* 'ECW' using CTAB method (Hwang et al., 2009). To construct expressing cDNA clones, RNA was extracted from *C. annuum* 'CM334' by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol. cDNA was synthesized from total RNA template (5 μg) using superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). All amplified PCR products were cloned by LIC method into the pCAMBIA 2300-LIC vector containing the CaMV (*Cauliflower mosaic virus*) 35S promoter and the NOS (nopaline synthase) terminator cassette. A total 15 fmol of purified PCR product was treated with T4 DNA polymerase (NEB, Ipswich, MA, USA) in 10X reaction buffer containing 10 mM dATP at 22℃

for 30 min and followed by 70℃ for 20min for inactivation of T4 DNA polymerase. The pCAMBIA 2300-LIC vector was digested with *PstI* and treated with T4 DNA polymerase with 10 mM dTTP. T4 DNA polymerase-treated PCR products and pCAMBIA 2300-LIC vector were mixed and incubated at room temperature for 30 min. The mixture was transformed into *E. coli* DH10β competent cells. The entire sequence of cloned candidates was confirmed by DNA sequencing at the NICEM (Seoul National University, Seoul, Korea). Each cloned vector was transformed into *Agrobacterium tumefaciens* strain C58C1 for transient *in planta* expression assays. After transformation, the cultured cells were centrifuged and re-suspended in induction buffer (10 mM MgCl2, 10 mM MES pH 5.6, and 200 μM Acetosyringone) and incubated at room temperature for 2 h before agro-infiltration.

Transient over-expression of *Pvr4* candidates and NIbs of potyviruses

For transient expression of candidates, pepper leaves were agro-infiltrated (absorbance at 600 nm [A 600] = 1.0 to 0.5 for *Pvr4* and NIb constructs), then harvested at 2-3 days post-infiltration. Empty vector and vector with necrosis-inducing protein (NIP) from *Phytophthora sojae* were infiltrated at A600 = 0.3 into one pepper leaf as a negative or positive control,

respectively. As a resistance gene and effector positive control, co-expression of *StR3a* and *Avr3a* was used in *N. benthamiana* leaves. All experiments were performed with 3 biological replicates. Inoculated pepper leaves were cleared in 100% ethanol to remove chlorophyll in order to visualize the cell death. Leaves of *N. benthamiana* were harvested and taken pictures under UV light from 3 to 7 dpi. Infiltrated pepper leaves were collected and de-stained in ethanol from 3 to 5 dpi.

Phenotypic evaluation of *Pvr4* in *N. benthamiana*

To measure PepMoV accumulation, *Pvr4*-bearing *Agrobacterium* was infiltrated on *N. benthamiana* leaves, and PepMoV was rubbed on leaves at 1 dpi. Empty vector (pCAMBIA 2300) or the *pvr4*-bearing *Agrobacterium* (isolated from *C. annuum* 'ECW') were used as a negative control. Infiltrated leaves were sampled at 1, 2, 3, 4 and 5 dpi. To validate PepMoV accumulation in transcript level, total RNA was extracted from pepper plant using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. First strand cDNA was synthesized using 3 μg total RNA with oligo (dT) and Superscript II reverse transcriptase for real-time PCR. Real-time PCR was performed using primer could detect VPg of PepMoV. Quantitative RT-PCR was performed using a Rotor-Gene 6000 apparatus (Qiagen, Hilden, North

Rhine-Westphalia, Germany) using SYBR Green (Invitrogen, Carlsbad, CA, USA). All calculations and statistical analyses were performed as described by the manufacturer. To normalize the expression level, actin transcript was used as an endogenous control for *N. benthamiana* and pepper plants.

To validate PepMoV accumulation in protein level, Double Antibody Sandwich (DAS)-ELISA (Agdia, Elkhart, IN, USA) was performed for monitoring virion accumulation at 14 dpi. Samples were considered positive for the presence of PepMoV when the absorbance value (405 nm) of each sample was greater than that of a negative control plant. *Pvr4*-mediated resistance against PepSMV and PVY were also tested in the same way with PepMoV.

Construction of the TRV-*Pvr4* vectors and VIGS in pepper

TRV2::*Pvr4*-N and TRV2::*Pvr4*-C containing the N-terminus and C-terminus of *Pvr4* cDNA were cloned into a TRV-based gene silencing vector (pTRV2) via LIC method (Dong et al., 2007). TRV2::*Pvr4*-N and TRV2::*Pvr4*-C were transformed into *Agrobacterium* strain GV3101 by the freeze-thaw method and the TRV-based VIGS on pepper was performed as described (Chung et al., 2004). GFP-tagged PepMoV was inoculated at 14 dpi on peppers.

Construction of the signaling components for VIGS assays in *N. benthamiana*

TRV2::RAR1, TRV2::HSP90, TRV2::SGT1 and TRV2::EDS1 were provided from Dinesh-Kumar's Lab (Liu et al., 2002a; Liu et al., 2002b; Liu et al., 2004). For TRV2::CRT1, PCR product was cloned into TRV2 vector using LIC method based on *CRT1* sequence of *N. benthamiana* (GQ855284.1) (Kang et al., 2010). For TRV2::NDR1, PCR product was cloned into TRV2 vector using LIC method based on *NDR1* sequence (AY438029.1) of *N. benthamiana* (Schornack et al., 2004). For TRV2::MEK2, PCR product was cloned into TRV2 vector using LIC method based on *MAPKK* sequence (AB360636.1) of *N. benthamiana* (Asai et al., 2008). For TRV2::COI1, PCR product was cloned into TRV2 vector using LIC method based on *COI1* sequence (AY428737.1) of *N. tabaccum* (Ekengren et al., 2003). For TRV2::EIN2, PCR product was cloned into TRV2 vector using LIC method based on *EIN2* sequence (EU998970.1) of *N. tabaccum*. TRV2::ICS1 was provided from Yoon et al., 2009 (Yoon et al., 2009).

Analysis of transcript levels by reverse transcription polymerase chain reaction (RT-PCR)

For semi-quantitative RT-PCR, total RNA was extracted using TRIzol

(Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For *N. benthamiana* genes, first-strand cDNA was synthesized using an oligo (dT) primer and 3 μg of total RNA with Superscript II reverse transcriptase, followed by RT-PCR with gene-specific primers. The PCR products were electrophoresed in agarose gels to quantify the amplified DNA.

Transformation of potato plants and selection of transgenic potato plants

Potatoes (*Solanum tuberosum* L. cv. Daeji) were cultivated in plate on Murashige and Skoog medium (pH5.8) containing MS salt, 30 g/l sucrose, Staba vitamin, 100 mg/l inositol and 8 g/l agar, which were kept at 24 ± 2℃ under light for 16 hr and dark for 8 hr. The Agrobacteria were cultured in 30ml liquid YEP medium containing 50mg/l kanamycin at 28℃ at 180 rpm in the dark (until OD600 reached 0.6). Leaves from 3 to 4 week old shoots were cut into 3 to 10 mm segments, immersed on the activated *Agrobacterium* suspension for 20 min, blotted dry on sterile filter paper, and co-cultured for 2 days on the co-culture medium at dark conditions. After 2days, the leaves were placed upside down on plant regeneration medium containing 0.01 mg/l NAA, 0.1mg/l GA3, 2 mg/l Zeatin, 100 mg/l kanamycin, 500 mg/l carbenicillin for the selection of stable transformants. Every 2 weeks, leaves with callus were transferred to new medium. After 7 to 8 weeks, the

regenerated shoots that grew to 1.0 cm in height were transferred to a MS medium (containing 100 mg/l carbenicillin and 50 mg/l kanamycin) to induce root.

Phenotypic evaluation of *Pvr4* in transgenic potato plants

To measure PVY accumulation against *Pvr4* in transgenic potato plants, PVY was inoculated on transgenic potato leaves at 14 days after acclimation. Upper leaves were sampled at 40 dpi. To validate PVY accumulation in transcript level, total RNA was extracted from transgenic potato plants using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. First strand cDNA was synthesized using 3 µg total RNA with oligo (dT) and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) for RT-PCR. RT-PCR was performed using primer could detect VPg of PVY. To validate PVY accumulation in protein level, Double Antibody Sandwich (DAS)-ELISA (Agdia, Elkhart, IN, USA) were performed for monitoring of potyviruses accumulation at 40 dpi. Samples were considered positive for the presence of PVY when the absorbance value (405 nm) of each sample was greater than that of a negative control plant.

RESULTS

Genetic analysis of *Pvr4* resistance to PepMoV

For efficient phenotyping, GFP-tagged PepMoV (PepMoV-GFP) was provided by Dr. Ki Hyun Ryu (Seoul Women's University, Seoul, Korea) and propagated in *Nicotiana.* To test *Pvr4*-mediated resistance, pepper and tobacco plants were screened with PepMoV-GFP. *C. annuum* 'CM334' showed no symptoms to PepMoV in inoculated leaves and GFP expression was not observed either under UV light (Figure 2-1 and 2-2). However, *C. annuum* 'ECW', 'Jupiter' and *N. tabaccum* showed severe mottling symptom in non-inoculated leaves, and GFP expressions were observed (Figure 2-1 and 2-2).

To perform fine mapping of *Pvr4*, two independent populations were generated. A backcross population (or BC1F3) was derived from a backcross between *C. annuum* 'CM334' (*Pvr4*/*Pvr4*) and *C. annuum* 'ECW123R' (*pvr4*/*pvr4*), and F2 population was derived from *C. annuum* 'Jupiter' (*pvr4*/*pvr4*) and *C. annuum* 'CM334' (*Pvr4*/*Pvr4*). All of the backcross population and F2 population showed a 3 R: 1 S segregation to PepMoV (Table 2-1). These data support that *Pvr4* is inherited as a single dominant resistance gene in pepper.

Table 2-1. Genetic analysis of PepMoV resistance using *C. annuum* 'CM334', *C. annuum* 'Jupiter' or *C. annuum* 'ECW123R', two populations

Plant material	Expected ratio (R:S)	Observed frequency		X^2	P
		R	S		
'CM334' (CM)	1:0	40	-	-	-
'Jupiter' (J)	0:1	-	29	-	-
'ECW123R' (E)	0:1		41	-	-
BC1F3	3:1	812	251	1.091	0.296
(J x CM) F1	1:0	19	-	-	-
(J x CM) F2	3:1	309	96	0.362	0.546

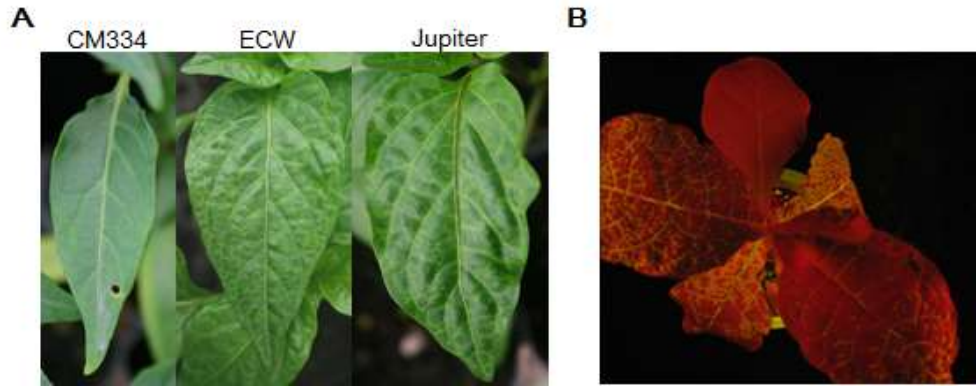

Figure 2-1. Disease symptoms of pepper plants infected with PepMoV-GFP. Pepper and tobacco plants were infected with PepMoV tagged with GFP and photographs were taken at 14 dpi. A. *C. annuum* 'CM334' showed resistance phenotype against PepMoV whereas *C. annuum* 'ECW' and *C. annuum* 'Jupiter' showed susceptible phenotypes. B. Virus infection was shown by UV treatment in *N. tabaccum* 'Xanti NC'.

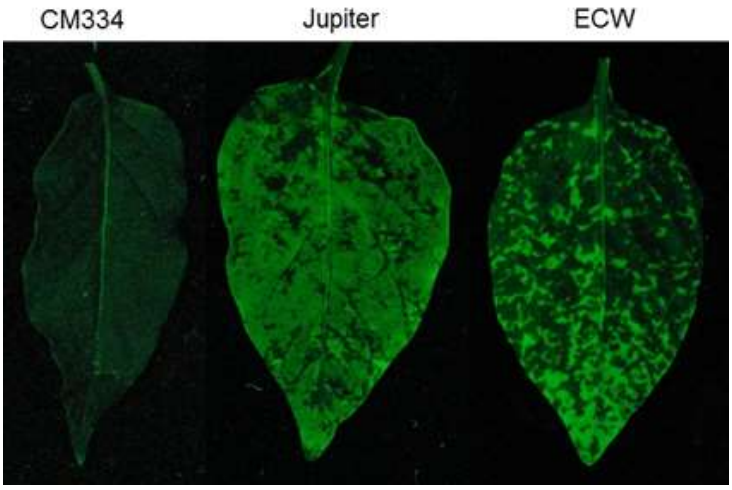

Figure 2-2. Green fluorescence in the systemic leaves of pepper *C. annuum* 'CM334', 'Jupiter' and 'ECW' inoculated with PepMoV-GFP. The photographs were taken at 20 dpi in UV light.

Comparative mapping of the *Pvr4* locus in tomato and pepper

In the previous study, it was reported that *Pvr4* is linked to TG420 in pepper chromosome 10 (Kim et al., 2011) sharing the same marker order with tomato, potato and eggplant in the lineage (Tanksley et al., 1992; Wu et al., 2009b; Wu et al., 2009a). Based on the syntenic relationship between the pepper and tomato genomes (Figure 2-3), about 3 Mb sequence including TG420 marker in tomato genome (Consortium, T.G., 2012) was isolated and used to obtain corresponding sequences of pepper in *C. annuum* 'CM334' genome (Livingstone et al., 1999; Kim et al., 2014).

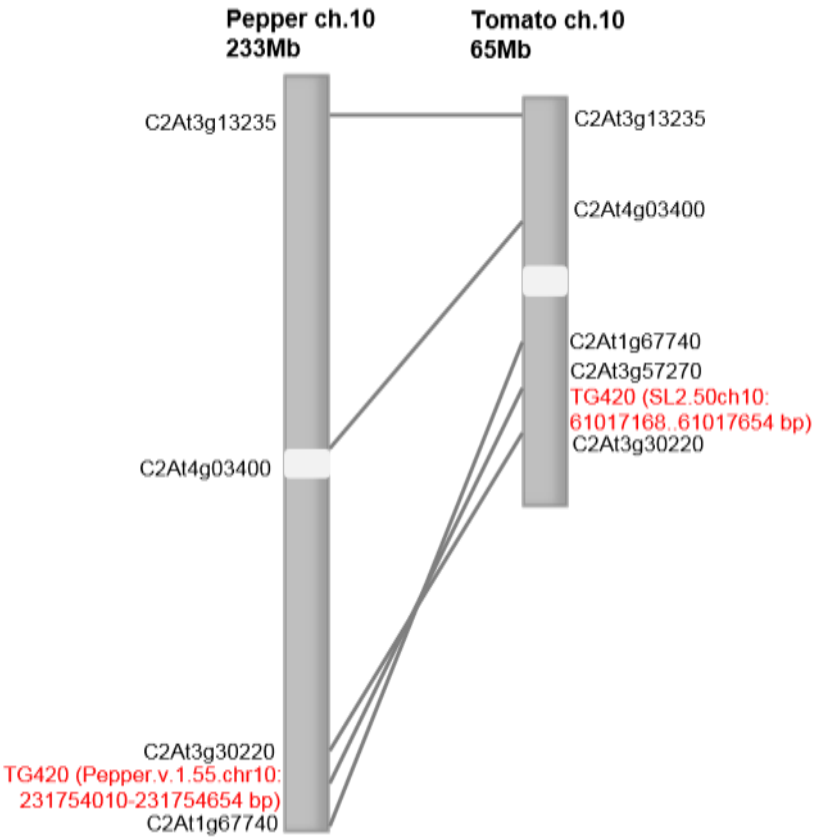

Figure 2-3. Comparative genetic map of pepper and tomato in the *Pvr4* region including TG420 marker.

Development of *Pvr4*-linked markers and identification of the *Pvr4* gene

To perform fine mapping of *Pvr4* using the comparative map information, the annotated genes in the TG420 region of pepper were analyzed and molecular markers were developed. Based on the fine map, an flanked region by the markers 20172 (0.3 cM) and 1072 (0.1 cM) (Figure 2-4), were delimited and this was confirmed that additional two markers of 61786 and 17918 were perfectly co-segregated with *Pvr4* in a total 1468 individuals of BC1F3 and F2 populations (Figure 2-4). To obtain accurate genome sequences of the *Pvr4* locus, 20172, 17918, 044832 and 1983 markers were used to screen bacterial artificial chromosome (BAC) library of *C. annuum* 'CM334' (Figure 2-5). Four BAC clones (575C14RO, 464C8RF, 229C11RA and 613C10RO), spanning the *Pvr4* locus were selected and were sequenced using PacBio and the Roche 454 FLX Titanium. The linear BAC sequences were aligned to the genome region between 20172 marker and 1983 marker. The delimited region contained a 295 kb flanking by 20172 and 1072 markers in the genetic map (Figure 2-4 and 2-6). The 295 kb sequences of *C. annuum* 'CM334' were analyzed by gene annotation.

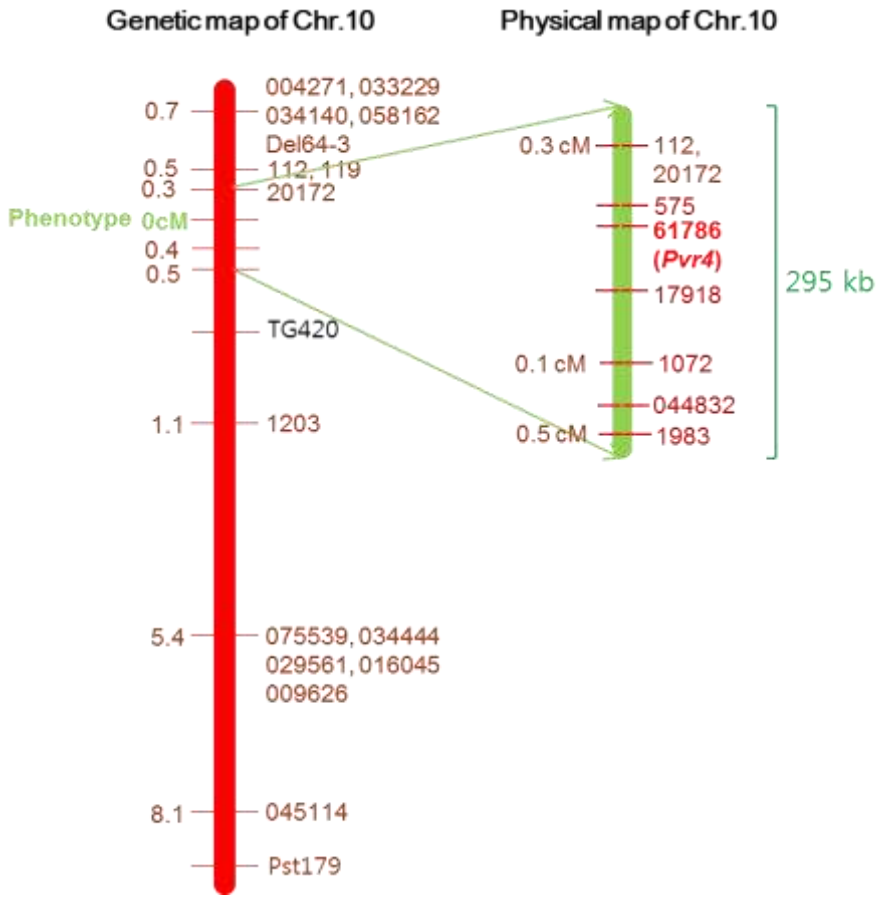

Figure 2-4. Genetic and physical map of the *Pvr4* locus in pepper. Thirty-two co-dominant markers were developed in this study and only twenty markers were showed in this figure. The *Pvr4* locus was delimited to 295 kb on physical map.

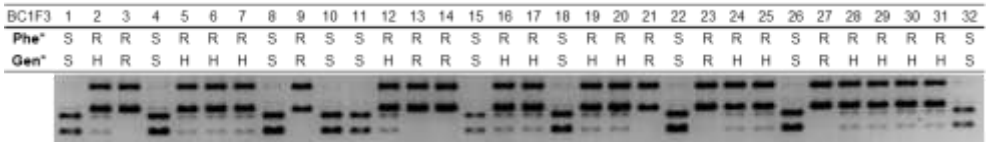

Figure 2-5. The 61786 marker (*Pvr4*) test result in BC1F3 population. Developed 61786 marker is co-segregated with the resistance phenotype against PepMoV in backcross population. * Phe means phenotyping and Gen means genotyping. In phenotyping, R means resistance phenotype and S means susceptible phenotype. In genotyping, R means resistance genotype, H means hetero genotype and S means susceptible genotype.

Candidate gene annotation in the *Pvr4* locus

In the delimited block of 295 kb, 16 annotated genes by gene annotation were obtained (Kim et al., 2014). Among them, eight genes were coiled-coil (CC) nucleotide-binding site leucine-rich repeat (NB-LRR) and they were clustered in this region (Figure 2-6): *CA10g21190*, *CA10g21180*, *CA10g21170*, *CA10g21150*, *CA10g21125*, *CA10g21120*, *CA10g21090* and *CA10g21040*. Only four NB-LRR genes were observed as intact CC-NB-LRR (*CA10g21190, CA10g21170, CA10g21150, and CA10g21120*) in this region, whereas the other 4 genes were partial. Furthermore, three SNP markers of 575, 61786 and 17918, which showed co-segregation with *Pvr4* in two populations, had been developed based on *NB-LRR* genes (Figure 2-5 and 2-6). Four *NB-LRR* genes (*CA10g21190, CA10g21170, CA10g21150, and CA10g21120*) were presumed to be *Pvr4* candidates.

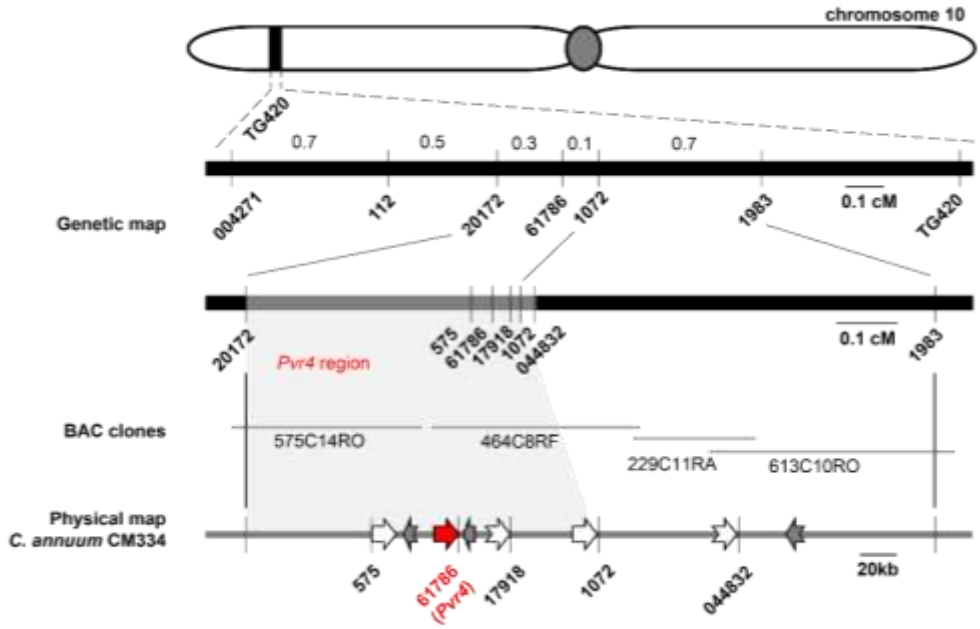

Figure 2-6. Genetic and physical maps of the *Pvr4* region. In the genetic map, the vertical lines represent the sites of *Pvr4*-linked molecular markers, and the above Arabic numeral of the vertical lines represent a genetic distance. The predicted genes *CA10g21190*, *CA10g21180*, *CA10g21170*, *CA10g21150*, *CA10g21125*, *CA10g21120*, *CA10g21090* and *CA10g21040* represent under gray vertical line in physical map. *NB-LRR* genes including full domain depicted by arrows and pseudo genes depicted by broken arrows. The 575C14RO, 464C8RF, 229C11RA and 613C10RO BAC clones are represented by overlapping gray lines.

Identification of *Pvr4* function by transient co-expression assay

To isolate *Pvr4* conferring resistance to PepMoV, *in planta* expression analysis was performed to test interactions between plant resistance protein and PepMoV-NIb. Based on gene annotation of *C. annuum* 'CM334' sequence, the gDNA and cDNA of four candidates in the physical block were identified and cloned into pCAMBIA 2300 vector under the CaMV 35S promoter by ligation-independent cloning (LIC) for further studies. Using the transient over-expression of *Pvr4* candidates together with *PepMoV-NIb*, HR induced by *Pvr4* were tested. A total four *NB-LRR* genes were tested by co-infiltration of *PepMoV-NIb* in *N. benthamiana* leaves (Figure 2-7). Among *NB-LRR* genes, only *CA10g21170* induced HR at 2-3 dpi (Figure 2-7). This result demonstrated that *CA10g21170* could be *Pvr4* which mediate the recognition of PepMoV-NIb. To confirm HR is triggered only by the resistance allele (*CA10g21170 - Pvr4*), co-expression assay was also tested using the susceptible allele (*CA10g21170 - pvr4*) in *N. benthamiana*. Transient expression of *pvr4* with *PepMoV-NIb* into *N. benthamiana* failed to induce HR (Figure 2-8).

Specific interaction of Pvr4 and PepMoV-NIb

To determine whether Pvr4 interacts with other potyvirus effectors,

PepMoV-NIb, PepSMV-NIb and *PVY-NIb* were co-expressed with *Pvr4* in *N. benthamiana*, and resulting in HR at 3 dpi, whereas co-expression with *TEV-NIb* did not induce HR (Figure 2-9). Furthermore, while *Pvr4* mediate HR by recognition *PepMoV-NIb*, but co-expression with *TSWV-NS*s, an effector of TSWV, did not show HR-like cell death into *N. benthamiana*. These results indicate that HR is induced by the specific interaction between Pvr4 and potyvirus NIb proteins (Figure 2-8). *In planta* analysis was also performed using PepMoV susceptible pepper (*C. annuum* 'Jupiter'). The HR was observed only the infiltration spot of *Pvr4* with *PepMoV-NIb* in 'Jupiter' leaves, but not in plants having *pvr4* (Figure 2-8). The HR caused by the interaction between *Pvr4* and *PepMoV-NIb* were same in pepper and tobacco leaves. This result indicated that NIb plays a role as an Avr factor specifically against Pvr4 (Kim et al., 2015).

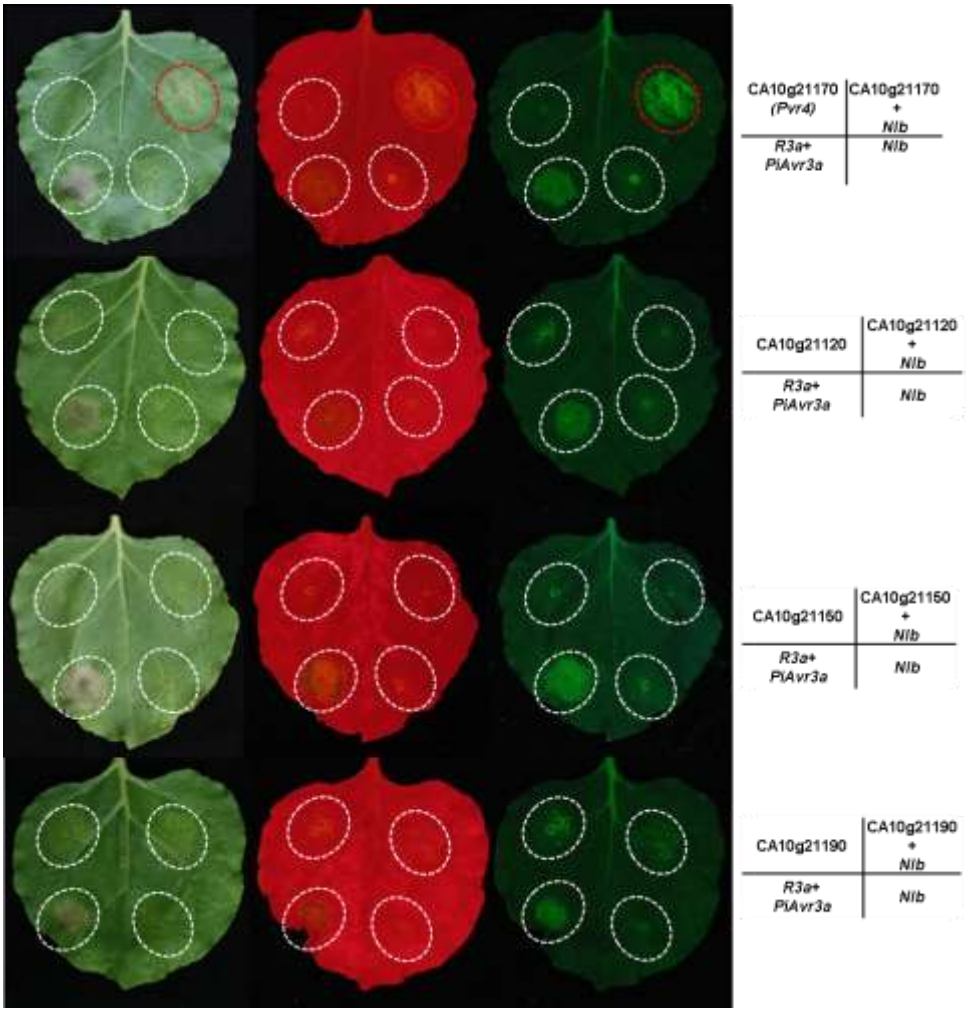

Figure 2-7. Transient co-expression assay of *Pvr4* candidate genes derived from *C. annuum* 'CM334' and *PepMoV-NIb* in *N. benthamiana*. Combination of *R3a* and *Avr3a* was used as a positive control. Inoculated genes were depicted right panels. Five days post infiltration, *N. benthamiana* leaves were harvested and visualized under UV light. *R3a*, *Phytophthora infestans* resistance gene derived from potato; *Avr3a*, *R3a* effector of *Phytophthora infestans*; NIb, PepMoV-NIb; CA10g21170, CA10g21120, CA10g21150 and CA10g21190; the *Pvr4* candidate genes derived from *C. annuum* 'CM334'.

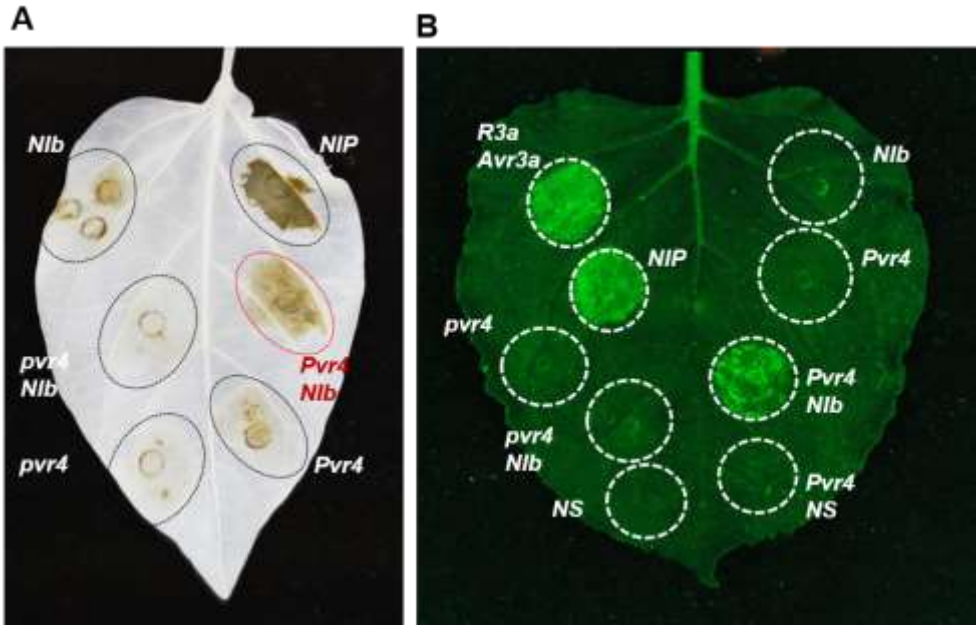

Figure 2-8. Transient co-expression assay in *C. annuum* 'Jupiter' (A) and *N. benthamiana* (B) leaves agro-infiltrated with *Pvr4* and *pvr4* together with effectors from viruses. (A) *Pvr4* and *pvr4* together with *PepMoV-NIb* by agro co-infiltration expressed in *C. annuum* 'Jupiter'. 'Jupiter' leaves were harvested and destained in ethanol to visualize at 3 dpi. (B) *Pvr4* and *pvr4* together with effectors from viruses by agro co-infiltration transiently expressed in *N. benthamiana* leaves. Combination of *R3a* and *Avr3a* was used as a positive control. Seven days post infiltration, *N. benthamiana* leaves were harvested and visualized under UV light. *R3a*, *Phytophthora infestans* resistance gene derived from potato; *Avr3a*, *R3a* effector of *Phytophthora infestans*; NIP, necrosis induced proteins derived from *Phytophthora sojae*; NS, NS cistron of TSWV; NIb, NIb cistron of PepMoV; *Pvr4*, *Pvr4* derived from *C. annuum* 'CM334'; *pvr4*, susceptible homolog derived from *C. annuum* 'ECW'.

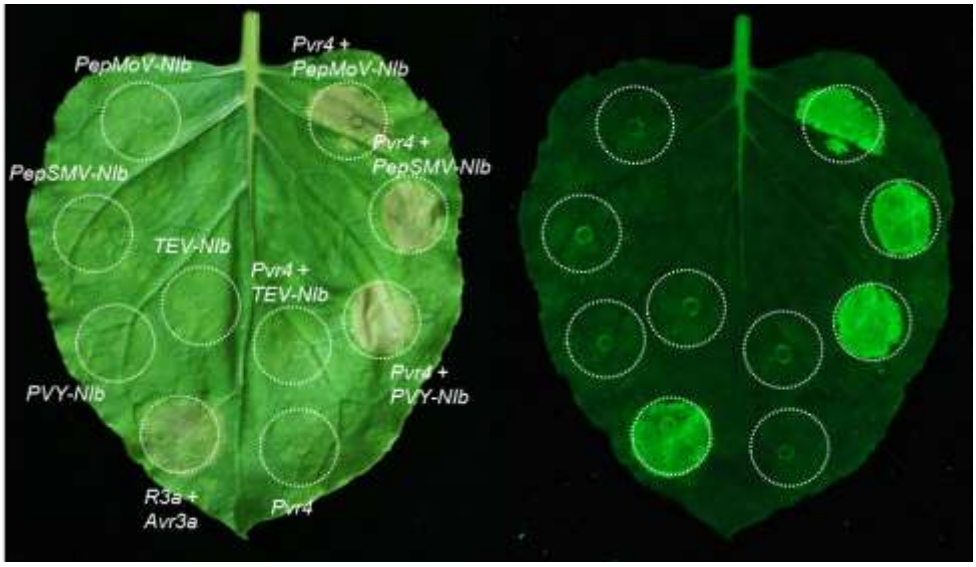

Figure 2-9. *N. benthamiana* leaves agro infiltrated with *Pvr4* derived from *C. annuum* 'CM334' and effectors from potyviruses. Combination of *R3a* and *Avr3a* was used as a positive control. Seven days post infiltration, *N. benthamiana* leaves were harvested and visualized under UV light. *R3a*, *Phytophthora infestans* resistance gene derived from potato; *Avr3a*, *R3a* effector of *Phytophthora infestans*; PepMoV-NIb, NIb cistron of PepMoV; PepSMV-NIb, NIb cistron of PepSMV; PVY-NIb, NIb cistron of PVY-O; TEV-NIb, NIb cistron of TEV; *Pvr4*, *Pvr4* derived from *C. annuum* 'CM334'.

Sequence analysis of the *Pvr4* and *pvr4* genes

To compare the intron/exon structures in the *Pvr4* and *pvr4* genes, genomic DNA regions were sequenced and analyzed by FGENESH (http://www.softberry.com/). *Pvr4* is consisted with seven exons and encoded a CC-NB-LRR type protein with 1746 amino acids. Genome region of *Pvr4* including exon and intron is 13,870 bp (Figure 2-10). Interestingly, five exons (from exon 2 to exon 6) in the LRR domain of *Pvr4* showed high nucleotide sequence identity from 89 to 99%. The intron regions (from intron 2 to intron 5) also showed high degree of sequence identity (99%). The susceptible allele, *pvr4*, isolated from *C. annuum* 'ECW' also encoded CC-NB-LRR type protein with 1,601 amino acids (Figure 2-10). The *pvr4* is consisted with 6 exons lacking one exon in the LRR domain compare to *Pvr4*. Moreover, the Pvr4 protein showed high degree of identity (98%) to the CC-NB domain of pvr4 but has lower identity (87%) in LRR domain (Figure 2-10).

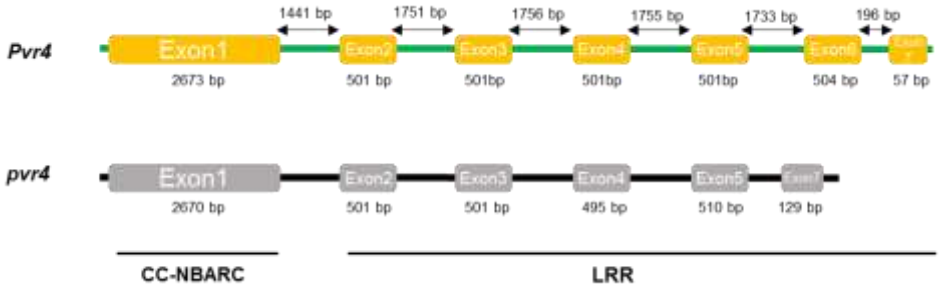

Figure 2-10. Gene structure of *Pvr4* (resistant allele) encodes seven exons and *pvr4* (susceptible allele) encodes six exons.

Resistance of *Pvr4* against potyviruses in *N. benthamiana*

When PepMoV was inoculated in *C. annuum* 'CM334', it appeared as extreme resistance (ER) with restricted virus replication and no apparent phenotype (Figure 2-1 and 2-2). However, the resistance response in *Pvr4*-carrying pepper has been observed ER or HR depending on virus isolates and inoculated organs such as cotyledon and leaves (Janzac et al., 2009). To investigate whether *Pvr4* also induce ER against avirulent potyviruses in *N. benthamiana* leaves, several potyviruses including PepMoV, PepSMV and PVY were first rubbed on half leaf of *N. benthamiana* and *Pvr4* was transiently over-expressed in the other half of the same leaves at 2 dai. In contrast to pepper, where potyvirus resistance induce ER, all avirulent potyviruses induced HR in the *Pvr4*-expressed region (Figure 2-11).

If the cloned *Pvr4* is a genuine resistance gene, it should suppress replication of PepMoV as well as induce HR. To investigate whether *Pvr4* confers resistance against PepMoV, the *Pvr4* and *pvr4* genes were overexpressed and followed by inoculation of PepMoV in *N. benthamiana* at 1 dai. Then, amounts of PepMoV RNA transcripts and protein levels were quantified by quantitative RT-PCR (qRT-PCR) and ELISA, respectively. In inoculated leaves that were overexpressed *Pvr4*, the relative transcripts of PepMoV was significantly suppressed after 3 dpi whereas in *pvr4*-expressed

leaves, the transcript levels were increased as high as those in empty vector (Figure 2-12B). In ELISA test, PepMoV accumulation in *Pvr4*-expressing *N. benthamiana* was significantly decreased than in empty vector-infiltrated or *pvr4*-expressed *N. benthamiana* (Figure 2-12A). Consequently, these results prove that CA10g21170 identified in this study encodes indeed *Pvr4* resistance protein recognizing NIb and suppressing PepMoV replication. Similarly, suppression of PepSMV and PVY replication was also tested as the same manner. Pvr4 also inhibited PepSMV and PVY accumulation in *N. benthamiana* (Figure 2-13). Taken together, these results suggest that the cloned *Pvr4* gene clearly confers resistance against broad spectrum of potyviruses.

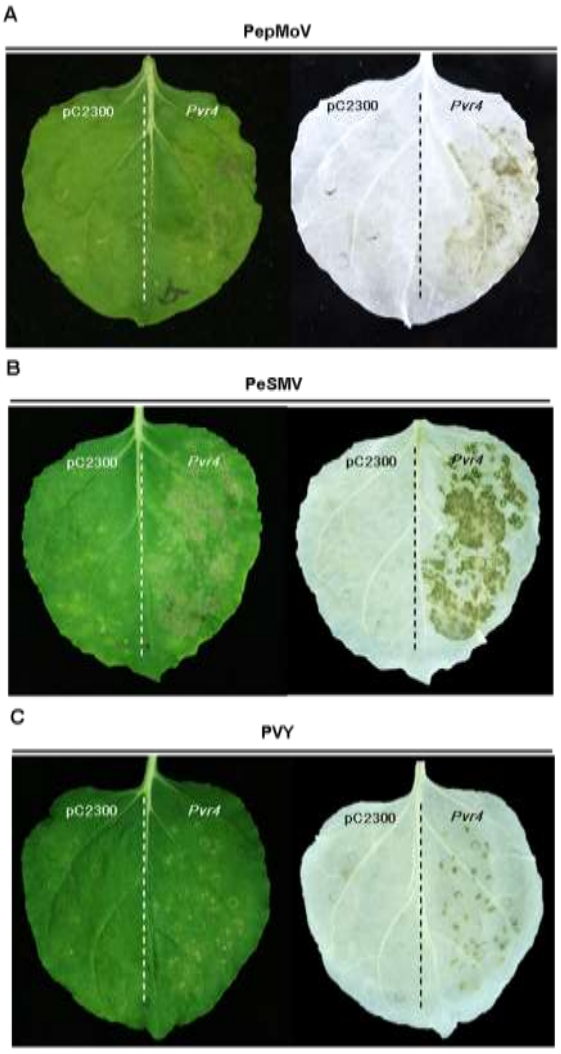

Figure 2-11. Resistance of *Pvr4* against potyviruses in *N. benthamiana*. HR was observed in *Pvr4* over-expressed right sides. PepMoV (A), PepSMV (B) and PVY (C) rubbed on *N. benthamiana* and pC2300 (empty vector) in left side and *Pvr4* in right side were inoculated at 2 dai.

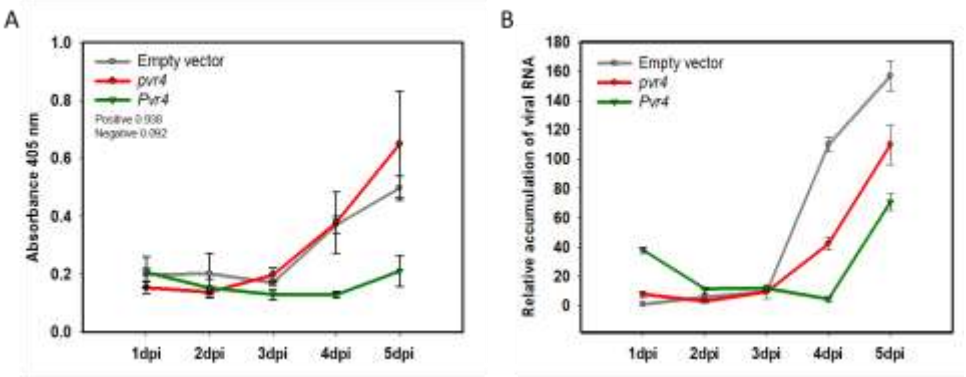

Figure 2-12. PepMoV accumulation in *N. benthamiana* overexpressing *Pvr4*. (A) PepMoV accumulation in *N. benthamiana* leaves transiently expressing *Pvr4* and *pvr4* by ELISA. (B) PepMoV accumulation in *N .benthamiana* leaves transiently expressing *Pvr4* and *pvr4* by quantitative real time RT-PCR. *Agrobacterium* harboring *Pvr4* or *pvr4* was inoculated in *N. benthamiana*, and PepMoV was rubbed at 1 dai. After rubbing virus, tobacco leaf discs were randomly sampled 5 leaf discs 5 plants at 1, 2, 3, 4 and 5 dpi. These samples were analyzed by ELISA and qRT-PCR. As a control, the leaves were inoculated with *Agrobacterium* harboring the empty vector pCAMBIA 2300. Quantitative real-time PCR was performed using the primers detect VPg of PepMoV. It was performed in five times. *Pvr4*, *Pvr4* derived from *C. annuum'* CM334'; *pvr4*, susceptible allele of *Pvr4*.

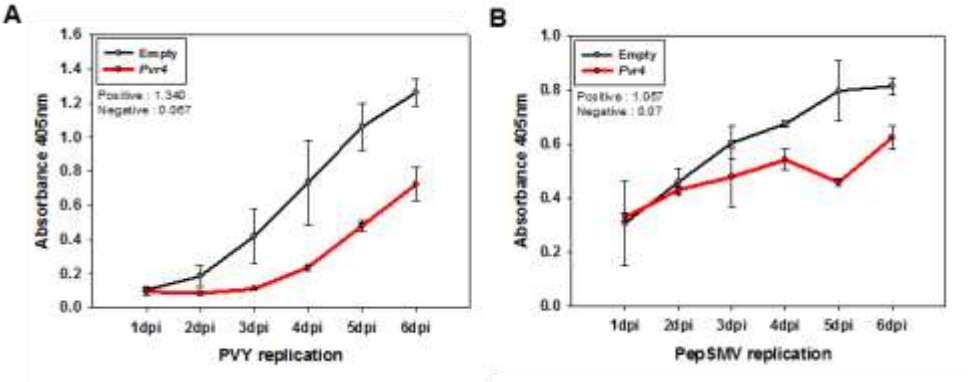

Figure 2-13. Accumulation of PVY-0 (A) and PepSMV (B) in *N. benthamiana* overexpressing *Pvr4*. (A) PVY accumulation in *N. benthamiana* leaves transiently expressing *Pvr4* detected by ELISA. (B) PepSMV accumulation in *N. benthamiana* leaves transiently expressing *Pvr4* detected by ELISA. *Agrobacterium* harboring *Pvr4* was inoculated in *N. benthamiana*, and PVY-0 and PepSMV were rubbed at 1 dai. After rubbing viruses, tobacco leaf discs were randomly sampled 5 leaf discs 5 plants at 1, 2, 3, 4, 5 and 6 dpi. As a control, the leaves were inoculated with *Agrobacterium* harboring the empty vector pCAMBIA 2300. These samples were analyzed by ELISA. It was performed in triplicate. *Pvr4, Pvr4* derived from *C. annuum* 'CM334'.

The nature of the *Pvr7* gene present in *C. chinense* 'PI159236'

There are two dominant resistance sources to PepMoV including *Pvr4* from *C. annuum* 'CM334' and *Pvr7* from *C. chinense* 'PI159236-9093', which was derived from backcrossing of *C. chinense* 'PI159236' and a *C. annuum* recurrent parent (Grube et al., 2000). These two genes were tightly linked each other (Grube et al., 2000). Since *Pvr7* is inherited dominantly, F1 hybrid derived from *C. chinense* 'PI159236' harboring *Pvr7* and a susceptible cultivar should show resistance response to PepMoV. To confirm *C. chinense* 'PI159236' has a dominant PepMoV resistance, F1 hybrid derived a cross between *C. chinense* 'PI159236' (resistant) and *C. annuum* 'ECW' (susceptible) was generated. For genetic analysis, PepMoV resistance and *Pvr4*-linked markers were tested in parents and F1 plants derived from the cross. Surprisingly, *C. annuum* 'ECW' and F1 plants were susceptible, while *C. chinense* 'PI159236' was resistant demonstrating that *C. chinense* 'PI159236' contains a recessive resistance gene (Figure 2-14). F1 plants showed the heterozygous genotypes for 119 and 575 markers (Figure 2-15). Assuming that *Pvr7* is tightly linked to *Pvr4*, I performed *Pvr4*-linked marker tests to examine the *Pvr7* locus in *C. chinense* 'PI159236-9093'. Molecular marker analysis revealed that the flanking region of the *Pvr7* locus of *C. chinense* 'PI159236-9093' is exactly the same as that of *C. annuum* 'CM334'

(Figure 2-15). Moreover, 61786, a marker derived from *Pvr4* was not present in the genomes of *C. chinense* 'PI159236', F1 hybrid and *C. annuum* 'ECW'.

To confirm whether *Pvr7*-harboring pepper also induce HR together with PepMoV-NIb, eight cistrons of PepMoV, PepSMV-NIb and PVY-NIb were transiently over-expressed in *Pvr7*-harboring pepper. When only NIb cistrons were overexpressed, HR was induced in *Pvr7*-harboring peppers (Figure 2-16). This results indicate that Avr factors of potyvirus against Pvr7 and Pvr4 are the same as NIb in peppers. Consequently, the genetic analysis results suggest that the *Pvr7* locus of *C. chinense* 'PI159236-9093' might be originated from *C. annuum* 'CM334'.

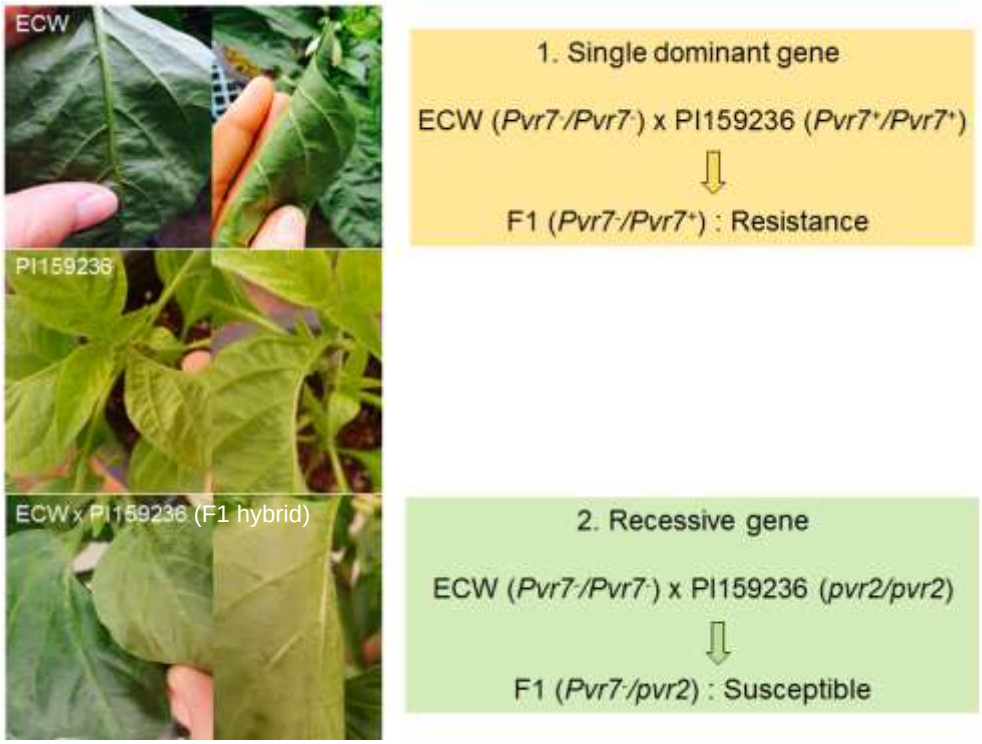

Figure 2-14. Symptoms of PepMoV infected peppers and a hypothesis demonstrating inheritance modes. *C. annuum* 'ECW' and F1 hybrid showed typical PepMoV symptoms whereas *C. chinense* 'PI159236' was resistant. PepMoV was inoculated on two youngest fully expanded leaves of *C. annuum* 'ECW' (susceptible), *C. chinense* 'PI159236' (resistant) and F1 hybrid derived from a cross between *C. annuum* 'ECW' and *C. chinense* 'PI159236'.

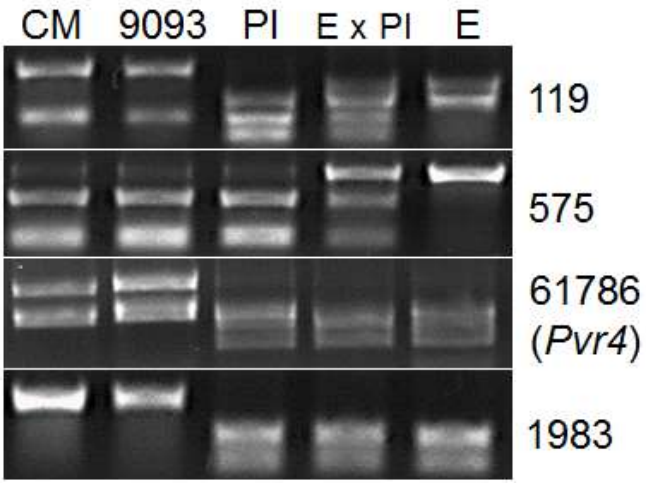

Figure 2-15. Genotype analysis of *Pvr4*-linked markers in different *Capsicum* species to reveal the genetic background flanking region of the *Pvr4* locus. 'CM' is *C. annuum* 'CM334' carrying *Pvr4*, '9093' is *C. chinense* 'PI159236-9093' carrying *Pvr7*, 'PI' is *C. chinense* 'PI159236', 'E' is *C. annuum* 'ECW' and 'E x PI' is F1 hybrid derived from a cross between *C. annuum* 'ECW' and *C. chinense* 'PI159236'. Marker patterns of *C. annuum* 'CM334' and *C. chinense* 'PI159236-9093' were all the same.

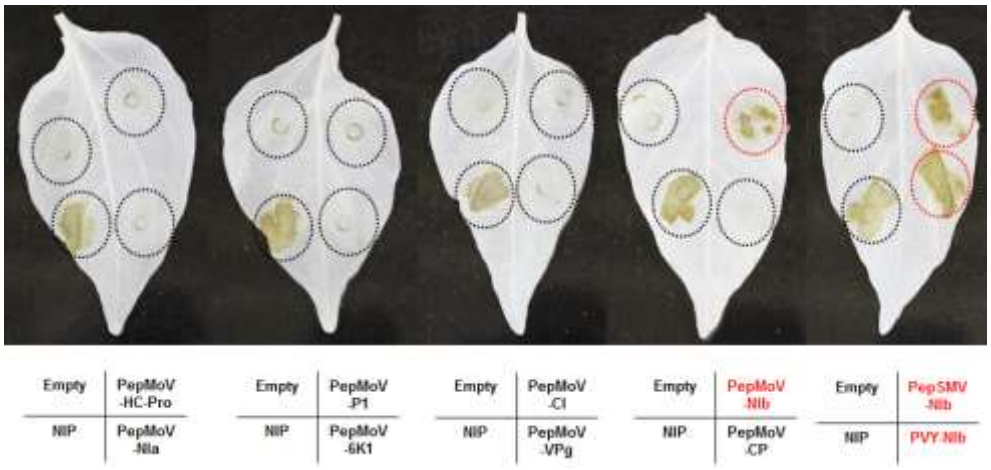

Figure 2-16. HR cell death symptoms in 9093 F2 individuals harboring *Pvr7*. Negative and positive control were used *Agrobacterium* C58C1 harboring pCAMBIA 2300 and NIP (necrosis induced proteins derived from *Phytophthora sojae*), respectively. 'Jupiter' leaves were harvested and destained in ethanol to visualize at 3 dpi.

Virus-induced gene silencing (VIGS) of *Pvr4*

To characterize *Pvr4*-mediated resistance, the loss of function phenotype of *Pvr4* was investigated by virus-induced gene silencing (VIGS) technique using *Tobacco rattle virus* (TRV)-based vector. For this approach, VIGS was performed in *Pvr4*-harboring peppers using TRV2::*Pvr4*-N and TRV2::*Pvr4*-C containing the N-terminus and C-terminus of *Pvr4* cDNA, respectively. *Pvr4*-silenced pepper plants showed a normal phenotype as that of control plants. To observe the replication of PepMoV in *Pvr4*-silenced plants, GFP tagged PepMoV was inoculated. As results, GFP signals were detected under UV light in both *Pvr4*-silenced as well as control plants (*Pvr4*-bearing peppers) indicating that VIGS method using TRV system was not suitable for PepMoV accumulation in pepper plants (Figure 2-17).

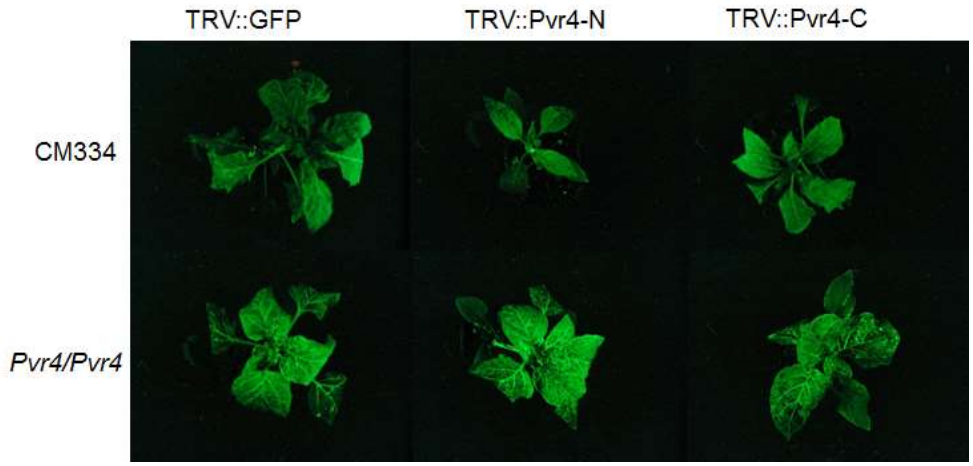

Figure 2-17. Virus-induced gene silencing of *Pvr4* in *C. annuum* 'CM334' and homozygote an F2 plant (*Pvr4/Pvr4*). Pepper plants were silenced by TRV2::*Pvr4*-N or TRV2::*Pvr4*-C, and PepMoV-GFP was inoculated at 20 dpi. TRV-ΔGFP was used as a negative control.

Signaling pathway of *Pvr4*-mediated HR

In *N. benthamiana, Pvr4* confers HR against PepMoV-NIb and also activate the defense-related signaling pathway. MEK2, HSP90, SGT1, RAR, COI1 and EIN2 are known to be involved in the signaling pathway leading to plant HR. To understand the role of these known signaling components in *Pvr4*-mediated resistance, VIGS experiments were performed in *N. benthamiana*. When *HSP90, RAR1* and *SGT1* were silenced in *N. benthamiana, Pvr4*-mediated HR were totally disappeared (Figure 2-18). This results implied that *Pvr4*-mediated resistance requires RAR1-SGT1-HS90 chaperone complex. However, silencing of MEK2, COI1 and EIN2 do not affect Pvr4/PepMoV-NIb-mediated HR cell death in *N. benthamiana*. This may indicate *Pvr4*-mediated HR cell death shares upstream signaling components with other R-gene but may have distinct downstream signaling pathways (Figure 2-19).

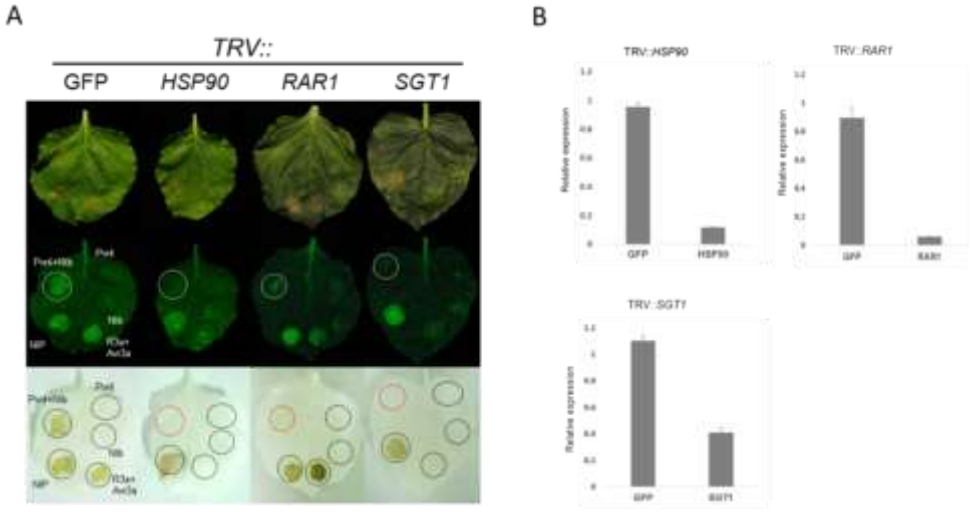

Figure 2-18. *Pvr4*-mediated HR requires the plant signaling components SGT1, HSP90 and RAR1. A. *HSP90, RAR1* and *SGT1* is silenced in *N. benthamiana*, respectively and HR was monitored by transient co-expression of *Pvr4* and *PepMoV-NIb*. As a negative control, TRV::ΔGFP is used. B. Relative expression of silenced gene in *N. benthamiana*. It was repeated in five times.

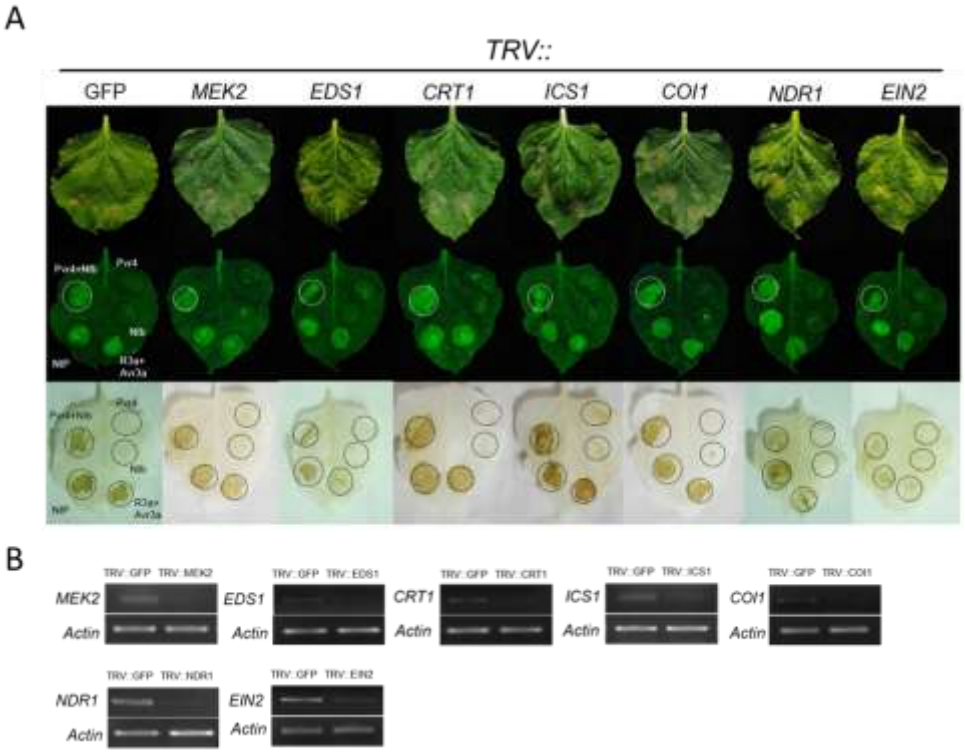

Figure 2-19. *Pvr4*-mediated HR is not mediated by MEK2, EDS1, ICS1, COII, NDR1 and EIN2 in *N. benthamiana*. A. *MEK2, EDS1, ICS1, COII, NDR1* and *EIN2* was silenced in *N. benthamiana*, respectively and HR was monitored. As a negative control, TRV::ΔGFP is used. B. Relative expression of silenced gene in *N. benthamiana*. *Actin* was used as a control. It was repeated in five times.

Resistance of transgenic potato harboring *Pvr4*

To test whether *Pvr4* confers resistance against potyvirus in potato, *Pvr4* was introduced into a susceptible potato ‘Daeji’. *Pvr4* cDNA was cloned into pCAMBIA 2300 binary vector by LIC method and named as pC2300-*Pvr4* (Figure 2-20). pC2300-*Pvr4* was transformed into a susceptible potato ‘Daeji’ by *Agrobacterium*-mediated transformation. As controls, pCAMBIA 2300 of empty vector and *NBARC575* were also introduced into potato. *NBARC575* is a NB-LRR gene, which is closely located near *Pvr4*. Transformation of potatoes was validated by RT-PCR using expressed *Pvr4* or *NBARC575* transcripts. A transgenic potato line highly expressing *Pvr4* were selected, and PVY-O isolates were infected together with control potatoes. Only *Pvr4*-harboring transgenic potato showed reduced PVY replication (Figure 2-21). This result suggest that horizontal transfer of *Pvr4* from pepper to potato could confer virus resistance and indicating presence of common downstream signaling component in potato and pepper.

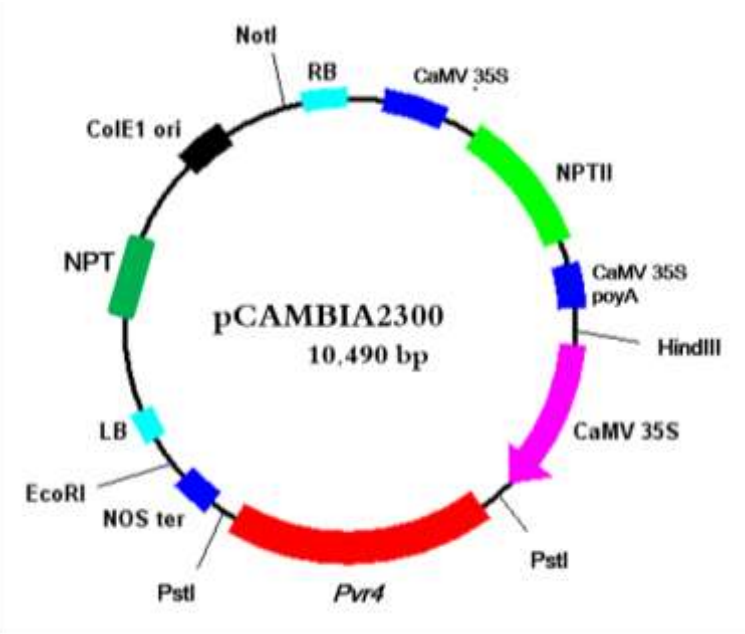

Figure 2-20. Diagram of pCAMBIA 2300-*Pvr4*. For *Pvr4*-harboring transgenic potato, *Pvr4* cDNA was cloned pCAMBIA 2300 vector by ligation-independent cloning (LIC).

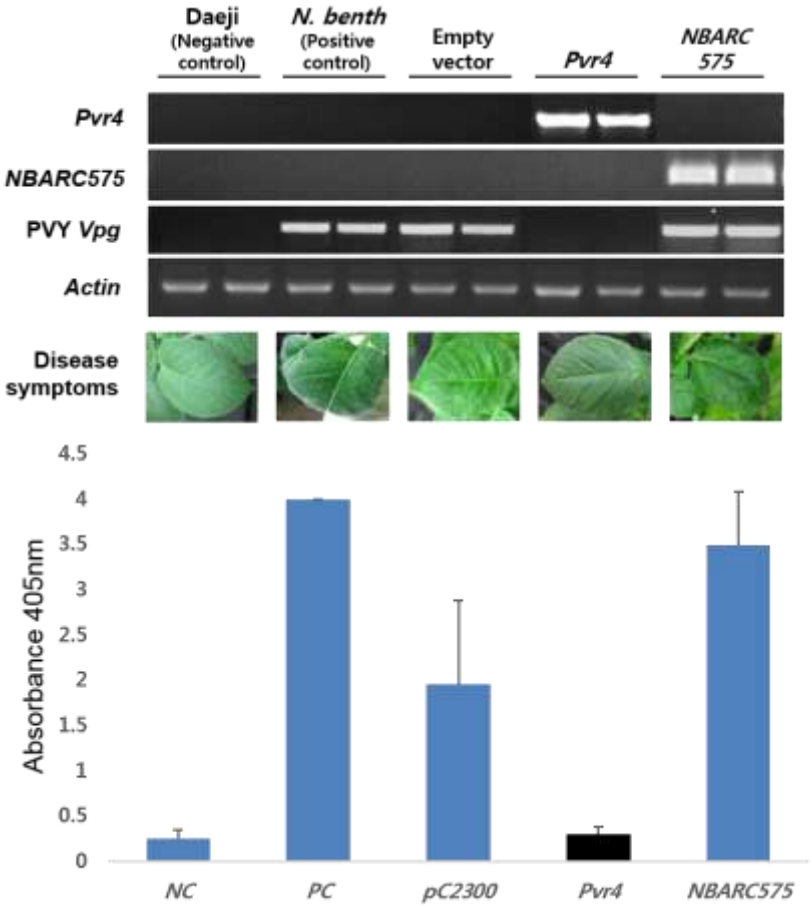

Figure 2-21. Resistance of *Pvr4*-harboring transgenic potato against PVY-0. (A) Expression of *Pvr4 or NBARC575* in transgenic potato plants transformed with the cDNA clone of *Pvr4 or NBARC575* driven by the CaMV 35S promoter. Expression of *VPg* of PVY-0 in transgenic potato plants after PVY-0 inoculation. Primers can detect were used to observe PVY replication. RT-PCR was carried out on total RNA from transgenic lines using the sequence-characterized gene specific primers. 'Daeji' not infected to PVY is used a negative control. The transgenic potato transformed with pC2300 into the susceptible 'Daeji' used for transformations were included as a positive control. All potato samples were sampled at 38 dpi.

DISCUSSION

Genome-based mapping of the *Pvr4* gene

In this study, we compared tomato and pepper genome information to identify the *Pvr4* resistance gene against various potyviruses. The flanking region of TG420 marker in pepper chromosome 10 is syntenic with that of tomato even though this region is inverted in pepper (Lefebvre et al., 2002; Wu et al., 2009b). This syntenic block is known to be resistance hot spot which contains several dominant virus-resistance genes such as *Pvr4*, *Pvr7* (resistant to Potyvirus) and *Tsw* (resistant to TSWV) (Grube et al., 2000; Jahn et al., 2000; Peters et al., 2012). Comparative genome research using syntenic and co-linear relationships have been demonstrated among pepper, tomato, and potato, indicating possibility of comparative genome researches and the applications among Solanaceous genomes (Lefebvre et al., 2002; Wu et al., 2009b; Wu et al., 2009a; Peters et al., 2012). For example, *R3a* resistance gene against *Phytophthora infestans* was identified from potato (*Solanum tuberosum*) by comparative genomics of tomato and potato (Huang et al., 2004). *R3a* is located in the R gene cluster of chromosome 11, where the tomato *I2* gene that confers resistance to *Fusarium oxysporum* f. sp. *Lycopersici* and the pepper *L* gene that confers resistance to *Tobacco mosaic*

virus (TMV) are located (Simons et al., 1998; Tomita et al., 2011). These relationships allow us to identify resistance genes and elucidate their functions.

Most disease resistance genes belong to NB-LRR family and more than half of them are clustered on the genomic region (Andolfo et al., 2013). For example, three copies of the *R3a* gene are duplicated in potato and seven copies of the *Mi-1* gene are tandem duplicated in tomato genome (Tameling et al., 2002; Huang et al., 2004; Andolfo et al., 2013). In this study, sixteen genes including NB-LRR, cupin and retrotransposon were annotated in the *Pvr4* locus. Among them, eight genes were CC-NB LRR, and they were also clustered in this region, which is similar to a number of NB-LRR genes clustered in plant genome (Xiao et al., 2001; Huang et al., 2005; Tomita et al., 2011). The eight NB-LRR genes were divided into two groups in *Pvr4* locus (Seo et al., unpublished). In one group, three NB-LRR genes consist of one exon and only one gene is a full type which has major motif such as P-loop, kinase2, GLPL and MHDV of major motif in NB-ARC domain (Yeom et al., unpublished). In contrast, another group including five NB-LRR genes have multiple exons and three of them are full type. The *Pvr4* locus was composed of duplication block of NB-LRR genes, which is similar to a high number of duplication events (CC-NB-LRR, NB-LRR, CC-NB, and NB type) in tomato

chromosome 4 (Andolfo et al., 2013). However, the region including TG420 in tomato does not contain NB-LRR gene cluster and unknown genes are located (Andolfo et al., 2013). Therefore, the *Pvr4* locus might be emerged by NB-LRR gene expansion in pepper after speciation between tomato and pepper.

Identification of the *Pvr4* gene function by transient co-expression assay

HR caused by interaction between a NB-LRR protein and corresponding Avr effector is the typical resistance response in plants (Flor, 1971; Dangl and Jones, 2001). Among eight NB-LRR genes above, *Pvr4* only induced HR with *PepMoV-NIb* (Avr factor of Pvr4) and each avirulent viruses-*NIbs* in pepper and tobacco leaves (Figure 2-7 and 2-8 and 2-9). However, *Pvr4* could not induce HR with *TSWV-NSs* (Avr factor of Tsw) or *TEV-NIb* of virulent potyvirus. These results support that the *Pvr4* gene of NB-LRR type confers HR by recognition of certain potyviruses-NIb specifically. TEV-NIb has lower identity to other potyviruses NIbs. This sequence diversity might be important for interaction with Pvr4. We expect to find the domain of NIb interacts with Pvr4 protein by comparative study among potyviruses-NIb. Furthermore, we did not investigate yet whether the interaction of Pvr4 and NIb is direct or indirect. In a previous study, *Arabidopsis* RPM1 induced HR

by recognition AvrRpm1 or AvrB secreted from *Pseudomonas syringae* which interact with and induce phosphorylation of RIN4 (Mackey et al., 2002). This indirect interaction is represented to 'guard hypothesis', which NB-LRR R proteins recognize the status of plant proteins targeted by pathogen effectors (Van Der Biezen and Jones, 1998; Dangl and Jones, 2001; McHale et al., 2006). Such indirect detection of pathogens allows the limited number of NB-LRR proteins to detect the multiple pathogen effectors (McHale et al., 2006). We speculate that Pvr4 detects the modification of the host factor conserved in pepper and tobacco by NIbs of multiple potyviruses or forms the complex of the host factor and NIbs, and elicits HR. Further in-depth interaction studies will need to be conducted.

Sequence analysis of the *Pvr4* and *pvr4* genes

In pepper and tobacco, Pvr4 induced HR with NIbs of multiple potyviruses, but pvr4 failed to function potyviruses NIb –dependent resistance response as well as HR (Figure 2-12 and 2-13). *Pvr4* consists of 7 exons showing high similarity between LRR domains while *pvr4* have 6 exons showing low similarity between LRR domains. CC-NB domains of Pvr4 and pvr4 shows 98 % similarity, but similarity of LRR domains is low compared to CC-NB domain. The LRR domain is made up of leucine repeats,

and the number of individual repeats vary greatly (Jones and Jones, 1997). This domain is known to be involved in determining recognition specificity of Avr factor (Mondragón-Palomino et al., 2002). Consistent with this idea, chimera analysis between highly similar NB-LRR proteins with different recognition specificities have shown that recognition specificity maps to the LRR domain (Mondragón-Palomino et al., 2002; Rairdan and Moffett, 2006; Tomita et al., 2011). The LRR domain of the pepper L proteins have been reported to determine the resistance spectrum, which is elucidated by domain swapping and mutational study (Tomita et al., 2011). The barley powdery mildew resistance proteins Mla1 and Mla6 recognize unrelated Avr effectors and the LRR and C-terminal non-LRR domains determine the specificity (Shen et al., 2003). Although we have not tested the chimera analysis study using the domains of Pvr4 and pvr4, we suggest that the LRR domains is important to recognize NIb.

Resistance of the *Pvr4* gene against potyviruses in *N. benthamiana*

Pvr4-mediated resistance confers ER in pepper (Figure 2-1) (Janzac et al., 2009). However, co-expression of *Pvr4* and avirulent viruses in *N. benthamiana* showed HR (Figure 2-11). Why Pvr4 confers ER in pepper and HR in *N. benthamiana*? In a previous study, Rx showed ER against PVX in

potato, but expression of PVX-CP (Avr of Rx) with 35S promoter in leaf cells showed HR cell death in the *Rx*-harboring plants (Bendahmane et al., 1999). This data may imply that an increased amount or a sustained expression of the CP elicitor can force the ER into HR-type (Shirasu and Schulze-Lefert, 2000). Even though Rx can suppress virus replication in potato protoplast, excessed CP expression might trigger HR by cell-to-cell communication over protoplast (Kohm et al., 1993). Likewise, our data suggest that the relatively limited *Pvr4* expression in the most tobacco leaf cells infected by virus may lead to HR cell death unlike ER of pepper system.

Signaling pathway of *Pvr4*-mediated HR

Silencing of genes related to signaling pathway suggests that *Pvr4*-mediated HR requires several defense signaling components. Plant NB-LRR proteins recognize viral proteins and induce defense response (Shirasu and Schulze-Lefert, 2000). Several components involved in defense signaling pathway associate with a member of the RAR1-SGT1-HSP90 chaperon complex, MAPK, and defense hormone synthesis (Pedley and Martin, 2005; van Loon et al., 2006; Shirasu, 2009). In *N. benthamiana*, RAR1 is required for N protein (Liu et al., 2002a), HSP90 mediate the resistance by N, Prf and R3a (Lu et al., 2003; Liu et al., 2004; Bos et al., 2006). The requirement of

SGT1 for plant immunity is shown by transient silencing of N, Bs2, Rx, Prf and R3a (Liu et al., 2002b; Leister et al., 2005; Bos et al., 2006; Mucyn et al., 2006). Consistent with this results in silencing experiments, RAR1, SGT1, and HSP90 are key regulators of *Pvr4*-mediated resistance in *N. benthamiana* (Figure 2-19 and 2-20). Taken together, these results imply that a number of NB-LRR proteins share signal molecules in defense signaling pathway.

The nature of the *Pvr7* gene present in *C. chinense* 'PI159236'

Pvr7 from *C. chinense* 'PI159236-9093' had been reported as another potyvirus resistance gene related to *Pvr4* (Grube et al., 2000). To identify the *Pvr7* gene, F1 hybrid obtained from a cross between *C. annuum* 'ECW' (PepMoV susceptible) and *C. chinense* 'PI159236' (PepMoV resistant) showed disease symptom by PepMoV-GFP (Figure 2-14). Based on previous study, *Pvr4*-linked marker test showed that *Pvr7* in *C. chinense* 'PI159236-9093' was mapped to the same locus with *Pvr4* in *C. annuum* 'CM334' (Figure 2-15)(Grube et al., 2000). In the genome of *C. chinense* 'PI159236', the *pvr1* gene that confers recessive resistance is known (Kang et al., 2005b). These results may indicate that *Pvr7* in *C. chinense* 'PI159236' may be originated from *C. annuum* 'CM334' by accidental introgression and indicating that *Pvr7* in the genome of *C. chinense* 'PI159236' is *Pvr4* from *C.*

annuum ‘CM334’.

Resistance of transgenic potato harboring *Pvr4*

We demonstrated that *Pvr4* was identified as evidence of HR induced by transient co-expression with NIb and resistance test against potyviruses. Therefore, we examined whether heterologous expression of *Pvr4*-harboring transgenic potato also showed resistance to PVY (Figure es2-21). In previous studies, *Prf*-harboring transgenic tobacco confers resistance to *P. syringae* and *R3a*-harboring transgenic potato or tomato confers resistance to *P. infestans* (Huang et al., 2005; Mucyn et al., 2006; Jia et al., 2010). However, only one *Pvr4*-harboring transgenic potato is verified in this study. A more search for more transgenic potato lines should be required.

In summary, we identified the *Pvr4* resistance gene conferring broad spectrum resistance against potyviruses using pepper genome information (Kim et al., 2014) and proved that *Pvr4*-harboring transgenic potato showed resistance to PVY. This would help us understand the overall mechanism underlying the recognition of potyvirus NIb by Pvr4. Furthermore, the cloned *Pvr4* may provide a novel resistance source against potyviruses in Solanaceous crop plants.

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ABSTRACT IN KOREAN

Pvr4 는 *Pepper mottle virus* (PepMoV), *Pepper severe mosaic virus* (PepSMV) 와 *Potato virus Y* (PVY)가 포함되어 있는 포티바이러스 (Potyvirus)에 대해서 광범위한 저항성을 나타내는 고추의 단일우성저항성유전자이다. *Pvr4* 를 가지고 있는 고추는 CM334 (*Capsicum annuum* landrace 'CM334')가 유일하게 알려져 있다. *Pvr4*는 강력한 저항성 유전자임에도 불구하고 아직 동정되지 않았으며 그에 대한 저항성 기작도 거의 연구되지 않고 있다. 본 연구는 *Pvr4*의 포티바이러스에 대한 저항성 기작을 밝히기 위한 방법으로써 *Pvr4*의 비병원성 단백질(avirulence factor, Avr)를 포티바이러스에서 분리하고 이를 이용하여 *Pvr4* 유전자를 동정함으로써 포티바이러스의 저항성 품종을 육종 및 개발하기 위하여 수행되었다. PepMoV가 생성하는 11개의 바이러스 단백질(PepMoV cistrons)을 분리하고 동정하였다. 분리한 바이러스 단백질 유전자들을 Pvr4(저항성단백질)를 가지고 있는 고추와 pvr4(이병성단백질)를 가지고 있는 고추에서 아그로박테리움(*Agrobacterium*)을 이용하여 과 발현시켰다. 마커 검정을 통해 *Pvr4*를 가지고 있음이 확인된 고추에서 유일하게 RNA-dependent RNA polymerase (NIb)가 과민감성 반응 (hypersensitive response, HR)을 유도하였다. 또한, *Pvr4*가 저항성을 나타내는 포티바이러스인 PepSMV와 PVY의 NIb 가 *Pvr4*를 가지고 있는 고추에서만 과민감성 반응을 나타내었다. 이를 통해 *Pvr4*가 나타내는 광범위한 저항성이 포티바이러스의 NIb를 비병원성 단백질로 인지하여 유도되는 것이라는 것을 확인하였다. 포티바이러스에 대해 단일우성저항성을 나타내는 *Pvr4* 유전자를 동정하기 위하여 'CM334'를 부로 하는 두 개의 서로 다른 집단인 BC1F3와 F2를 재료로 사용하였으며 고추의

게놈정보에 기반한 유전형 분석을 확인하였다. *Pvr4* 유전자가 토마토와 고추에서 잘 보존되어 있는 분자 마커인 TG420 과 연관되어 있다는 보고에 근거하여, TG420 마커를 포함하는 염기서열을 토마토를 기준으로 하여 고추에서 분리하였다. 확보된 고추시퀀스에서 *Pvr4*와 연관된 32개의 공우성 마커(co-dominant marker)를 개발하였으며 이 가운데 3개의 마커가 *Pvr4*와 공분리 되는 것을 두 개의 집단을 이용하여 확인하였다. 유전자 지도에서 확인된 *Pvr4* 유전자가 존재하는 영역은 물리적으로 350kb이며 16개의 유전자가 존재하는 것으로 예측되었다. 이 가운데 8개의 유전자는 coiled-coil nucleotide binding site leucine-rich repeat (CC-NB-LRR) 타입으로 크로모좀에서 일직선으로 나열하여 존재하는 것이 확인되었다. 8개의 *Pvr4* 후보 유전자들은 포티바이러스에 대해 이병성을 나타내는 고추 품종인 쥬피터(*C. annuum* 'Jupiter') 와 담배(*Nicotiana benthamiana*) 잎에서 PepMoV-NIb 와 함께 동시에 과발현되었으며, 한 개의 후보 유전자(CA10g21170)가 접종 후 2일차에 유일하게 과민감성 반응을 유도하는 것을 확인하였다. 또한, CA10g21170 유전자가 과발현된 담배잎에 PepMoV, PepSMV와 PVY를 접종하였을 때, 이들 바이러스의 증식이 현저히 억제되는 것이 관찰되었다. 이러한 실험결과를 통해 동정된 CA10g21170 유전자가 포티바이러스의 NIb를 비병원성 단백질로 인지함으로써 포티바이러스의 증식을 억제하는 *Pvr4* 임을 증명하였다. Pvr4는 1746개의 아미노산으로 이루어진 CC-NB-LRR구조의 단백질이며, BAC 분석을 통해 *Pvr4* 유전자 영역은 7개의 엑손(exon)과 6개의 인트론(intron)을 포함하는 13,870 bp으로 확인되었다. 담배에서 저항성 신호전달에 관여한다고 알려진 유전자들을 바이러스를 이용한 유전자 침묵현상(virus-induced gene silencing)을 이용하여 knock-down한 결과, HSP90, SGT1, RAR1 유전자의 발현이 감소하였을 때, Pvr4와 PepMoV-NIb의 상호작용에 의해 유도되는 과민감성 반응이

사라졌다. 이를 통해 Pvr4의 저항성 반응은 HSP90, SGT1 과 RAR1과 같은 샤페론(chaperon)단백질에 의해 매개된다는 결론을 도출하였다. 광범위한 저항성을 나타내는 *Pvr4* 유전자를 이종작물인 '대지'감자에 형질전환한 경우에도 PVY의 증식이 현저히 억제되었다. 이러한 실험결과들을 통해, 광범위한 포티바이러스에 대해 저항성을 나타내는 유전자인 *Pvr4*의 동정은 저항성 메커니즘에 대한 연구와 이종작물의 포티바이러스 저항성 품종 개발에 기여하는 중요한 발견이라고 할 수 있다.