농학박사학위논문

Capsicum annuum 에서의 *Pepper mottle virus* 저항성유전자의 동정 및 분자적 특성 구명

Isolation and Molecular Characterization of a *Pepper mottle virus* Resistance Gene from *Capsicum annuum*

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Phu Tri Tran

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Phu Tri Tran

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지도교수 김국형

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농생명공학부 식물미생물전공

Phu Tri Tran

Phu Tri Tran 의 박사학위논문을 인준함
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BY
PHU TRI TRAN

MAJOR IN PLANT MICROBIOLOGY
DEPARTMENT OF AGRICULTURAL BIOTECHNOLOGY

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Phu Tri Tran

ABSTRACT

In plants, gene-mediated resistance is one of defense mechanisms that prevents or reduces virus infection. Based on their mode of inheritance, plant resistance genes are classified as recessive or dominant (*R* genes). In this study, a method based on agroinfiltration and virus inoculation was developed for the simple and inexpensive screening of candidate *R* genes that confer a hypersensitive response (HR) to plant viruses. The well-characterized resistance genes *Rx* and *N*, which confer resistance to *Potato virus X* (PVX) and *Tobacco mosaic virus* (TMV), respectively, were used to optimize a transient expression assay for detection of HR in *Nicotiana benthamiana*. Infectious sap of PVX and TMV were used to induce HR in *Rx*- and *N*-infiltrated leaves, respectively. When this method was used to screen 99 candidate *R*

genes from pepper, an *R* gene (named *Pvr9*) that confers HR to the potyvirus *Pepper mottle virus* was identified. Molecular characterization of *Pvr9* revealed that this gene putatively encodes for a CC-NBS-LRR protein containing 1298 amino acids. *Pvr9* is predicted to be located on chromosome 6 where other *R* genes are clustered. NIb is the elicitor of the *Pvr9*-mediated hypersensitive response but no direct interaction between the *R* gene and the elicitor was detected in yeast two hybrid system or bimolecular fluorescence complementation assays. NIb from several potyviruses elicited the hypersensitive response, and the elicitation was determined by two internal regions of PepMoV NIb. Inoculation of pepper with PepMoV resulted in a minor increase in *Pvr9* transcription in the resistant cultivar *Capsicum annuum* 'CM334' and a slight down-regulation in the susceptible cultivar *C. annuum* 'Floral Gem'. The 5′ upstream region of *Pvr9* from cultivar CM334 had higher transcription activity than the region from cultivar Floral Gem. The cultivars CM334 and Floral Gem have non-functional *Pvr9* homologs with loss-of-function mutations in the CC, NBS, and LRR domains.

Key words: Hypersensitive response, *v*irus resistance, NBS-LRR genes, pepper, *Pepper mottle virus*, screening method, *Pvr9*, elicitor, NIb

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GENERAL INTRODUCTION

Plants have two major types of disease resistance, basal defense and resistance gene mediated defense. Basal defense provides first line of defense to the infection by a wide range of pathogens (Ausubel, 2005). However, adapted pathogenic microbes deploy a variety of effectors that are able to overcome the basal defense of host plants (Da Cunha et al., 2007; De Jonge et al., 2011; Kasschau and Carrington, 1998). In turn, plants have also evolved various types of resistance genes to avoid or combat to pathogen.

Genetically, plant disease resistance genes can be classified into two groups: dominant and recessive genes. Recessive resistance is controlled by a passive mechanism in which the pathogen is unable to utilize the host cell machinery for infection (Diaz-Pendon et al., 2004). Dominant resistance (*R*) genes confer an active resistance by encoding resistance proteins that recognize specific pathogen effectors or so-called avirulent (Avr) factors (Chisholm et al., 2006). In gene for gene concept, plant producing an *R* gene product are particularly resistant towards a pathogen that produces the corresponding avirulent (*Avr*) gene product (Flor, 1971).

The majority of plant *R* genes encode proteins with a nucleotide-binding site (NBS) and a leucine-rich repeat (LRR) domain

(Ellis et al., 2000). Based on the presence of different domains at the N-terminal portion of the NBS-LRR proteins, these NBS-LRR gene products were classified into two subgroups: the TIR-NBS-LRR proteins that contain a Toll-like domain, and the CC-NBS-LRR proteins that are characterized by a coiled-coil domain (Moffett, 2009; Van Ooijen et al., 2007). The NBS domain comprises a functional nucleotide binding pocket capable of binding and hydrolyzing ATP (Tameling et al., 2002), and responsible for resistance signaling (Rairdan and Moffett, 2006). The LRR domain is made up of individual repeat with the consensus leucine rich repeat motif (Jones and Jones, 1997) that is involved in Avr recognition (Botella et al., 1998; Ellis et al., 2007). A large number of NBS-LRR encoding sequences have been isolated from various plant species through map-based cloning, transposon tagging as well as sequence homology (Leister et al., 1996)

Most plant *R* genes control resistance through hypersensitive responses (HR) or extreme resistance (ER), in which the pathogen is localized to a few cells or in one cell, respectively (Carr et al., 2010). ER, termed “cellular resistance” or “immunity”, shows very little or no visible effect (Fraser, 1990; Fraser and Van Loon, 1986). In contrast to ER, HR includes the rapid death of cells at the infection site associated with defense gene expression. During HR, there are a number of

biochemical changes, for example, the presence of an oxidative burst, and production of phytoalexins, hydrolytic enzymes, pathogenesis related proteins, salicylate, proteinase inhibitors, and the deposition of lignin and callose (Heath, 2000; Richael and Gilchrist, 1999). For these distinct characteristics, HR has been widely used as a marker for high-throughput screening of resistance genes *in planta* (Bendahmane et al., 2000; Berberich et al., 2008; Ma et al., 2012)

Peppers (*Capsicum* ssp.), which are grown worldwide under various geographic conditions, are exposed to many pathogens (Green and Kim, 1991). Forty-nine virus species belonging to 15 different taxonomic groups have been shown to infect pepper (Hanssen et al., 2010). Among these viruses, members of the genus *Potyvirus* are the most numerous (Janzac et al., 2008; Moury et al., 2005). In this genus, *Pepper mottle virus* (PepMoV) infects mostly *Capsicum sp.* and is aphid-transmitted in a non-persistent manner in the field. The virus formed flexuous rod-shaped particle of 730 nm × 11 nm containing a single plus sense RNA genome of ~10 kb in length. The genome of PepMoV have a single open reading frame (ORF) that are translated into a large polyprotein, which is hydrolyzed and translated as a 350 kDa polyprotein precursor (Vance et al., 1992).

Pepper plants carry potyvirus resistance genes, most of which

are recessive. *pvr1* in *C. chinense* ‘P1152225’ and ‘PI1559236’ confers broad-spectrum recessive resistance to *Tobacco etch virus* (TEV), *Pepper mottle virus* (PepMoV), and *Potato virus Y* (PVY) (Greenleaf, 1956, 1986). The allele *pvr2*^{1} of *C. annuum* ‘Yolo RP10’ and ‘Yolo Y’ controls the recessive resistance to PVY pathotype 0 (Cook, 1960; Gebre Selassie et al., 1983). The allele *pvr2*^{2} from *C. annuum* ‘PI 264281’, ‘SC46252’, and ‘Florida VR2’ confers recessive resistance to PVY pathotype 1 and TEV (Cook and Anderson, 1959; Gebre Selassie et al., 1983). In addition, polygenic resistance to PVY consisting of a combination of quantitative trait loci and the locus *pvr2* was found in *C. annuum* ‘Perennial’ (Caranta et al., 1997). A third locus, *pvr3*, contains a monogenic recessive gene in *C. annuum* ‘Avelar’, which is resistant to PepMoV (Zitter and Cook, 1973). The recessive loci *pvr5 and pvr8* from *C. annuum* ‘Criollo de Morelos 334’ (CM334) provide resistance to PVY pathotype 0 and pathotype 1 isolate P-62-81, respectively (Andrés et al., 2004; Dogimont et al., 1996). A recessive gene *pvr6*, which is complementary with *pvr2*^{2} for resistance to *Pepper veinal mottle virus* (PVMV), was found in *C. annuum* ‘Perennial’ (Caranta et al., 1996).

The dominant gene *Pvr4* from *C. annuum* CM334 confers a broad spectrum and extreme resistance to potyviruses (Dogimont et al.,

1996). The *R* gene *Pvr7* from *C. chinense* ‘PI 159236’ is tightly linked to *Pvr4* on chromosome 10 and confers a hypersensitive response to PepMoV Florida (V1182) strain (Grube et al., 2000). Recently, a novel resistance gene of *C. annuum* ‘NW4’ that confers resistance to *Chilli veinal mottle virus* (potyvirus, ChiVMV) was mapped on chromosome 6 (Lee et al., 2013). However, none of these *R* genes has been cloned or characterized at the molecular level.

In this study, I developed a method for the screening of genes that confer resistance to viruses based on virus inoculation and transient over-expression of candidate *R* genes by agroinfiltration in *Nicotiana benthamiana*. By using this method, I determine an *R* gene, named *Pvr9*, conferring hypersensitive response to PepMoV in *N. benthamiana*. Further studies were carried out to determine the molecular characteristics of the *Pvr9* gene and its elicitor. I showed that this *R* gene putatively encodes for 1298 amino acids of a possible CC-NBS-LRR protein and is predicted to be located on pepper chromosome 6. We also showed that PepMoV NIb is the elicitor of the *Pvr9*-mediated hypersensitive response. Finally, I used mutational analyses to identify the amino acid residues that are important to the function of the *R* gene and the elicitor.

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CHAPTER I

A simple method for screening of plant NBS-LRR genes that confer a hypersensitive response to plant viruses and its application for screening candidate pepper genes against *Pepper mottle virus*

Phu-Tri Tran, Hoseong Choi, Saet-Byul Kim, Hyun-Ah Lee, Doil Choi and Kook-Hyung Kim
Department of Agricultural Biotechnology
and Plant Genomics and Breeding Institute, Seoul National University, Seoul 151-921, Korea

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ABSTRACT

Plant NBS-LRR genes are abundant and have been increasingly cloned from plant genomes. In this study, a method based on agroinfiltration and virus inoculation was developed for the simple and inexpensive screening of candidate *R* genes that confer a hypersensitive response (HR) to plant viruses. The well-characterized resistance genes *Rx* and *N*, which confer resistance to *Potato virus X* (PVX) and tobamovirus, respectively, were used to optimize a transient expression assay for detection of HR in *Nicotiana benthamiana*. Infectious sap of PVX and *Tobacco mosaic virus* were used to induce HR in *Rx*- and *N*-infiltrated leaves, respectively. The transient expression of the *N* gene induced local HR upon infection of another tobamovirus, *Pepper mild mottle virus*, through both sap and transcript inoculation. When this method was used to screen 99 candidate *R* genes from pepper, an *R* gene that confers HR to the potyvirus *Pepper mottle virus* was identified. The method will be useful for the identification of plant *R* genes that confer resistance to viruses.

INTRODUCTION

Most plant viruses have the ability to systemically infect their hosts. This process involves a series of interactions between the genome and proteins of the virus and proteins of the host (Schoelz, 2006). To systemically infect the host after initial invading a small number of its cells, viruses express their proteins, replicate their genomes, and move from invaded cells to other cells (Pallas and Garc á, 2011). In turn, plants have developed a variety of defense mechanisms to interrupt viral infection and prevent the disease development. RNA silencing, for example, degrades virus RNA and helps plants recover after virus infection (Ratcliff et al., 1997). Some viruses, however, have adapted to RNA silencing and are able to counter this defense by deploying suppressors of RNA silencing (Kasschau and Carrington, 1998).

To prevent such adapted viral pathogens, plants have evolved additional defense mechanisms that are mediated by resistance genes (Kang et al., 2005). Such genes are classified into two groups: i) recessive resistance genes provide a passive resistance in which the pathogen is unable to utilize the host cell machinery (Diaz-Pendon et al., 2004), and ii) dominant resistance genes (*R* genes) confer an active

resistance by encoding resistance proteins that recognize pathogen effectors or so-called avirulence (*Avr*) factors (Chisholm et al., 2006). Most *R* genes encode proteins with a nucleotide-binding site (NBS) and a leucine-rich repeat (LRR) region (Ellis et al., 2000). *R* genes can generate extreme resistance (ER), in which the pathogen is localized to a single cell, or a hypersensitive response (HR), in which the pathogen is localized to a few cells (Carr et al., 2010). HR is easier to recognize and quantify than ER because HR produces visible symptoms and distinctive histological and biochemical changes (Heath, 2000; Richael and Gilchrist, 1999).

Agrobacterium-mediated transient expression enables the rapid accumulation of a large amount of recombinant proteins in plants (Horsch and Klee, 1986). Using this approach and HR as an indicator, researchers have screened for plant *R* genes. The *Potato virus X* (PVX) resistance gene *Rx2* from *Solanum acaule*, for example, was isolated from among 200 *Rx1* homologues by agroinfiltration in transgenic *Nicotiana tabacum* leaves expressing PVX coat protein (Bendahmane et al., 2000). In another example, candidate resistance genes to *Phytophthora infectans* were screened by transient expression in *N. benthamiana* (Li et al., 2011). Transient expression is widely used to screen candidate genes because it is fast, inexpensive, and reproducible.

In this study, we developed a method for the screening of genes that confer resistance to viruses based on virus inoculation and transient over-expression of candidate *R* genes by agroinfiltration in *N. benthamiana.* The advantages of this protocol are: i) it does not require a transgenic host plant that expresses an elicitor, and ii) it can be carried out for viruses with unknown elicitors. With this method, we isolated an *R* gene that induces HR in response to *Pepper mottle virus* (PepMoV).

MATERIALS AND METHODS

I. Plant virus isolates and generation of PMMoV transcripts

The viruses used in this study include PVX USA strain (Park, 2006), *Tobacco mosaic virus* (TMV) U1 strain (V01408), *Pepper mild mottle virus* (PMMoV) Kr strain (AB216003), and PepMoV Kr strain isolate 134 (EU586123). Each virus isolate was obtained from a local lesion host at 7 to 14 days post-inoculation and was maintained in *N. benthamiana.*

To make PMMoV transcripts, total RNAs were extracted from PMMoV-infected *N. benthamiana* leaves using Isol-RNA lysis reagent (5Prime, Hamburg, Germany) according to the manufacturer's instructions. PMMoV cDNA was amplified by RT-PCR using GoScript Reverse Transcriptase (Promega, WI, USA) and LA *Tag* DNA polymerase (Takara, Tokyo, Japan). Sequences of the primers were designed to produce a T7 promoter and *Sma*I restriction enzyme (RE) site on the corresponding 5′ and 3′ end of the PCR products, respectively (Table 1). Amplified PMMoV dsDNA was then cloned into the pGEM®-T Easy Vector (Promega) and linearized by *Sma*I (Takara) for *in vitro* transcription. PMMoV transcripts were synthesized using T7 RNA polymerase (Takara) and G(5')ppp(5')G RNA cap structure

Table 1. Primers used for cloning of candidate *R* genes, PMMoV cDNA, the *Rx* gene and the *N* gene.

Primer name	Primer sequence (5′ → 3′)	Amplified sequence
Rpi-blb2 ortholog Fw	CCAATCCCTCTACGGATGGAAAAA CGAAAAGATAWTG	*Rpi-blb2* orthologs
Rpi-blb2 ortholog Rv	TATCCTCCTACGGTCCTACTTAAAT AAGGGGATATG	*Rpi-blb2* orthologs
T7 promoter-PMMoV Fw[a]	TAATACGACTCACTATAGTAAATTT TTCACAATTTAACA	PMMoV
PMMoV SmaI Rv[b]	CCCGGGTGGGCCGCTACCCGCGG TTC	PMMoV
Rx *Mlu*I Fw	CGACGCGTATGGCTTATGCTGCTG TTAC	*Rx*
Rx *Mlu*I Rv	CGACGCGTCTACTCGCTGCAGAA AAATA	*Rx*
N Fw	ATGGCATCTTCTTCTTCTTCTTCTA G	*N*
N Rv	TCACCCATTGATGAGCTCATAAAA GG	*N*
N Promoter *Sac*II-*Kpn*I Fw	GGCCGCGGTACCCAAGTTGACAG TTACCTCTTTCTCCTCC	*N* promoter
N 492 Rv [c]	GAGCCTTTGAGATTGGCCGCT	*N* promoter
N 6039 Fw	TGTCGCGCATGACCCAGAAACT	*N* terminator
N Terminator *Mlu*I-*Kpn*I Rv	CGACGCGTGGTACCTAGAATTTGT CAACAATGTTAGCTCA	*N* terminator
N 2512 Fw	ACGACATAAGGGAAGGGGGCA	*N* Intron 3
N 5634 Rv	GGTAGCTGTGTAAGCCTCTGGC	*N* Intron 3

[a]Fw stands for forward primer, and Rv stands for reverse primer.
[b]The fused enzyme sites are included in primer names, with the underlined sequences.
[c]The number indicates the start position of the primer on the genomic sequence of the *N* gene.

analog (NEB, Hertfordshire, England) as described previously (Ryu, 2005).

II. Construction of vectors for transient expression of the resistance genes *Rx* and *N*

For amplification of the *Rx* gene, chromosomal DNAs were extracted from leaves of the transgenic *N. benthamiana*, which contained the cDNA sequence of *Rx*, using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The *Rx* gene was amplified by PCR using EX*Tag* DNA polymerase (Takara). Sequences of the primers were designed so that the *Mlu*I RE site was incorporated into the two ends of the *Rx* gene (Table 1). The amplified *Rx* gene was digested by *Mlu*I and then cloned into the modified pPZP212 binary vector (Fig. 1A) (Park et al., 2009) using *T4* DNA ligase (Promega).

For amplification of the *N* gene and its regulatory sequences, total RNAs and chromosomal DNAs were extracted from *N. glutinosa* leaves using Isol-RNA Lysis Reagent and the DNeasy Plant Mini Kit, respectively. From the total RNA, full-length cDNA of the *N* gene (Whitham et al., 1994) was amplified by RT-PCR using GoScript reverse transcriptase and LA *Taq* DNA polymerase. The intron 3, promoter (AB453947), and terminator of the *N* gene were amplified by PCR from the chromosomal DNA using EX *Taq* DNA polymerase. Primers for the amplification of the cDNA and intron 3 were designed

based on the genomic sequence of the *N* gene (U15605). Primers for amplification of the promoter and terminator were designed based on previous studies (Baker and Dinesh-Kumar, 2002; Haque et al., 2008). RE sites *Sac*II-*Kpn*I and *Mlu*I-*Kpn*I were fused to the 5′ end of the promoter and the 3′ end of the terminator, respectively (Table 1). The cDNA was cloned into the pGEM®-T Easy Vector (Promega). The terminator, intron 3, and promoter were respectively inserted to downstream, between exon 3–4, and upstream of the cDNA via pairs of RE sites *Sac*I-*Mlu*I, *Sal*I-*Bgl*II, and *Sac*II-*Xba*I. The full-length *N* gene with the respective promoter, intron 3, and terminator was then cloned into the modified pCAMBIA 0380 (Fig. 1B), in which the pUC8 multiple cloning site (MCS) was replaced with pUC18 MCS, via the *Kpn*I RE site. The REs and *Taq* DNA polymerases were purchased from Takara.

III. Cloning candidate *R* genes from pepper

A total of 99 candidate sequences from different pepper cultivars (more than 20 varieties of *Capsicum* spp. from Korea, India, Japan, Mexico, Taiwan, Thailand, and USA) were amplified and cloned into the modified pMBP1 binary vector (Fig. 1C) by ligation-independent cloning (Oh et al., 2010). These isolated *R* candidates,

which contained the NBS-LRR domain, included 47 orthologs of *Rpi-blb2* (Vossen et al., 2005) (Late blight resistance gene from *Solanum bulbocastanum*, DQ122125), 22 orthologs of *R3a* (Huang et al., 2005) (Late blight resistance gene from *Solanum tuberosum*, AY849382), and 30 expressed sequence tags (EST) from the pepper database (http://passport.pepper.snu.ac.kr/). The *Rpi-blb2* orthologs were amplified with *Rpi-blb2* ortholog primers (Table 1). The other NBS-LRR sequences were amplified with different primer pairs based on predicted sequences from the database (data not shown). The NBS-LRR domain was predicted using gene-prediction algorithms including Fgenesh (Salamov and Solovyev, 2000) and Interproscan (Zdobnov and Apweiler, 2001).

IV. Inoculation of *N. benthamiana* plants with virus

Crude sap containing PVX, TMV, PMMoV, or PepMoV was prepared by grinding 100 mg of systemically infected leaves in 1 ml of phosphate buffer (0.05M, pH 7.5) as described previously (Grube et al., 2000a) with minor modifications. The *in vitro* transcription mixes of PMMoV, which contained approximately 200 ng/μl of RNA transcript (experimentally determined), were diluted twice with phosphate buffer (0.1 M, pH 7.5). Phosphate buffer (0.05 M, pH 7.5) was used in mock

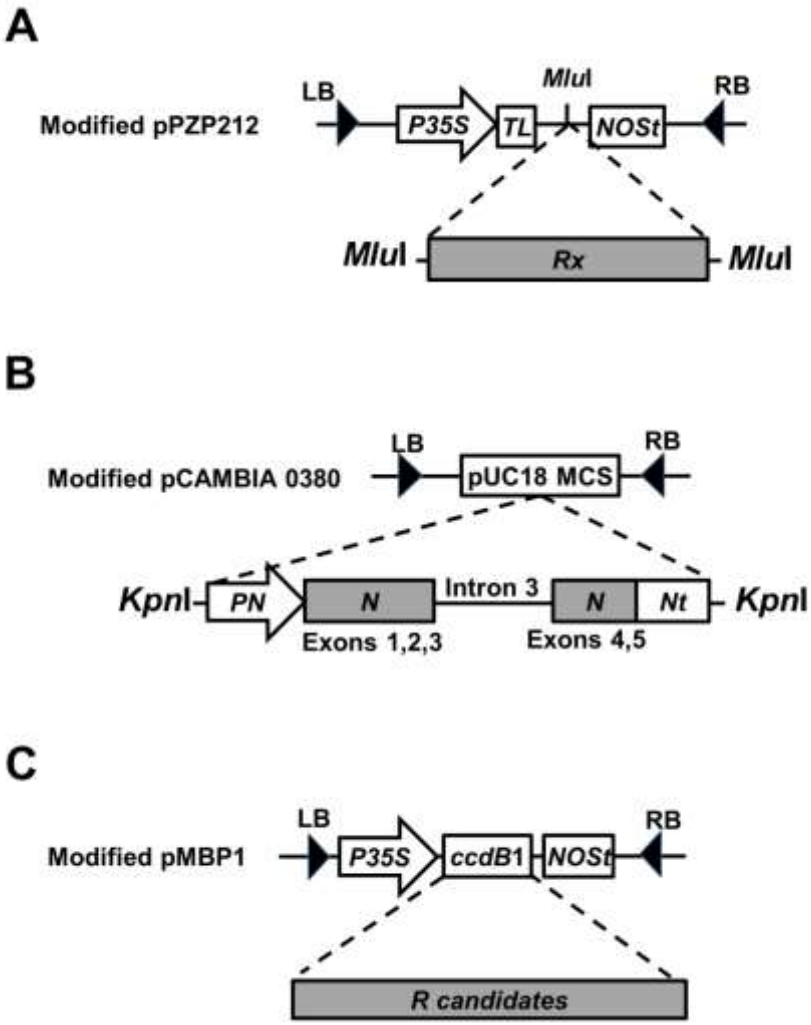

Fig. 1. Schematic representation of constructs used for *Agrobacterium*-based transient expression of *R* genes and candidates. (A) The *Rx* gene was cloned into modified pPZP212 under control of CaMV 35S promoter (*p35S), Tobacco etch virus* leader sequence (*TL*) and NOS terminator (*tNOS*). (B) The cassette of the *N* gene and its regulatory sequences, which were cloned in modified pCAMBIA 0380 binary vector, included *N* natural promoter (*pN*), *N* coding sequences (consisting of 2 parts: *5′-N*, exon 1–3; and *3′-N*, exon 4–5), *N* intron 3, and *N* natural terminator (*tN*). (C) *R* candidates were cloned in modified pMBP1 vector under control of CaMV 35S promoter and NOS terminator.

inoculation. Three of the top and fully expanded leaves of N. benthamiana were dusted with carborundum and rubbed gently with 50 µl of the crude sap or diluted transcripts. The inoculated leaves were then washed with sterile-distilled water to remove the remaining abrasive.

V. *Agrobacterium*-mediated transient expression

N. benthamiana seedlings were grown at 25°C with 60% humidity and a 16-h light and 8-h dark photoperiod in a growth chamber. Four weeks old *N. benthamiana* plants were used for the agroinfiltration. *Agrobacterium tumefaciens* strain GV3101 was used for all controls and *R* candidate clones. Kanamycin was used for selection of modified pCAMBIA 0380 and pMBP1 vector-based clones, and spectinomycin was used for selection of pPZP212 vector-based clones; both antibiotics were used at 50 µg/ml in YEP broth (1 % yeast extract, 2 % peptone). Agroinfiltration in *N. benthamiana* was conducted as described previously (Bendahmane et al., 2000; Ma et al., 2012; Wydro et al., 2006) with minor modifications. First, *Agrobacterium* clones were individually added to 1 ml of YEB broth containing 50 µg/ml of rifamycin and appropriate antibiotics for vector selection. After the broth cultures had grown for 24 h at 28℃ and 200 rpm, 0.1ml of each was transferred to 4.9 ml of YEP broth containing

the same antibiotics and 200 μM acetosyringone. The 5-ml cultures were grown under the same conditions for 16 h before the *Agrobacterium* cells were collected by centrifugation at room temperature and 2000×g for 10 min. The cells were suspended and diluted in MMA buffer (pH 5.7, 10 mM MES, 10 mM $MgCl_2$, and 200 μM acetosyringone). The optical density of each bacterial suspension at 600 nm wavelength (OD_{600}) was measured with an UV/visible spectrometer (Ultrospec 3100 pro, Biochrom, Cambridge, England). The suspensions were then diluted to the desired concentrations. Fully expanded leaves of *N. benthamiana* were gently infiltrated by syringe at two abaxial sides. The infiltrated leaves were photographed with a digital camera (NIKON 7200, Tokyo, Japan).

VI. 3,3'-Diaminobenzidine (DAB) staining

To visualize reactive oxygen species (ROS) generated by dead cells *in situ*, DAB staining was performed as described previously (Wong et al., 2007) with minor modifications. Briefly, agroinfiltrated leaves were detached and incubated in 1 mg/ml of DAB in PBS buffer (pH 7.2) for 3 h. Then the leaves were bleached in 95% ethanol at 65℃ for overnight. The DAB-stained leaves were photographed with a digital camera (NIKON 7200, Tokyo, Japan).

VII. Measurement of ion leakage

The degree of cell death was estimated by measuring ion leakage from leaf discs as described previously (Rizhsky et al., 2004) with minor modifications. Leaves discs (9 mm in diameter and prepared by punching between veins of leaves with a cork borer) were floated in an excess of distilled water for 5 minutes to remove leakage resulting from injury. For each measurement, five leaf discs were transferred to a 15-ml tube containing 10 ml of double-distilled water. After the tube was incubated for 3 h at room temperature, the conductivity of the washing fluid was measured with a conductivity meter (LaMotte, Maryland, USA), and this first conductivity reading is referred to as value A. The leaf discs were then returned to the washing fluid in the 15-ml tube, which was tightly capped and incubated at 65℃ overnight. After the tube cooled to room temperature, the conductivity of the washing fluid was measured again, and this second conductivity reading is referred to as value B. Ion leakage for each sample was expressed as a percentage, i.e., (value A of sample - value A of water) ÷ (value B of sample - value B of water) × 100.

RESULTS

I. Effect of *Agrobacterium* concentration on HR level

To determine the *Agrobacterium* concentration that results in the highest HR level without background cell death, the *Agrobacterium* clone harboring the *N* or *Rx* gene was suspended in MMA buffer at five densities (0.1, 0.3, 0.5, 0.7, and 0.9 in OD_{600}), and the five suspensions of each clone were infiltrated into the same leaf. At 2 days post-agroinfiltration (dpa), TMV was rub-inoculated into the *N*-infiltrated leaves, and PVX was rub-inoculated into the *Rx*-infiltrated leaves. Phosphate buffer (0.05 M, pH 7.5) was used for mock inoculation to check for self-HR of the infiltrated leaves. The infiltrated leaves were photographed at 3, 6, and 8 days post-inoculation (dpi).

Following mock inoculation, no HR was observed on leaves infiltrated with either gene (Fig. 2, A–C and G–I). This demonstrated that the agroinfiltration had no background effects. Following virus inoculation, the HR appeared clearly at 6 dpi (Fig. 2, E and K) without much difference among concentrations from 0.1 to 0.5 for *Rx* and from 0.3 to 0.9 for *N*. The onset of HR in this experiment was slightly delayed relative to that occurring after agroinfiltration of *R* candidates into transgenic tobacco plants expressing a viral elicitor (Bendahmane et al., 2000). At 8 dpi, HR symptoms were similar at all concentrations

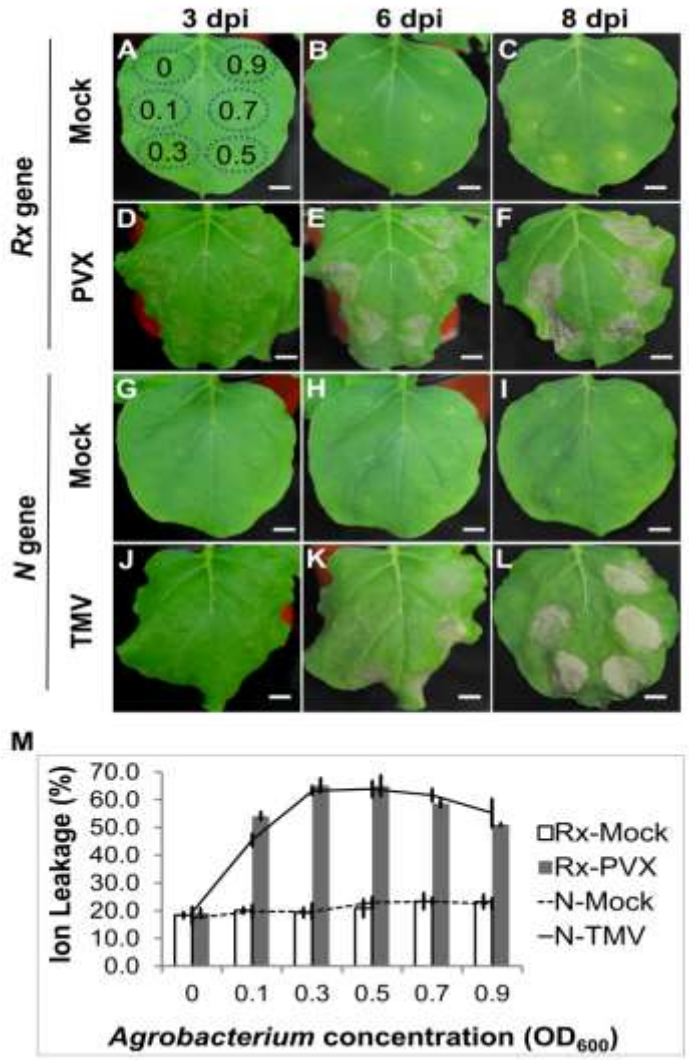

Fig.2. Optimization of *Agrobacterium* concentration. Leaves were inoculated 2 days post-agroinfiltration and were photographed at 3, 6, and 8 days after inoculation (dpi): (A–C) *Rx* agroinfiltration and mock inoculation, (D–F) *Rx* agroinfiltration and PVX inoculation, (G–H) *N* agroinfiltration and mock inoculation, and (J–L) *N* agroinfiltration and TMV inoculation. (M) HR levels of *N* gene- and *Rx* gene-infiltrated leaves as affected by *Agrobacterium* concentration were estimated by ion leakage (as indicated by electrical conductivity) at 6 dpi. Dotted circles in A indicate the areas infiltrated on all leaf samples; the numbers inside indicate the *Agrobacterium* OD_{600} concentrations. Scale bars in A–L=1 cm.

for both genes (Fig. 2, F and L), indicating the absence of significant differences in HR formation among all tested concentrations. The infiltrated–inoculated leaves at the onset of HR (6 dpi) were collected and used for measurement of ion leakage (Fig. 2M). For both genes, ion leakage was highest (i.e., HR level was highest) at bacterial OD_{600} concentrations between 0.3 and 0.5. A bacterial OD_{600} concentration of 0.4 was used for further optimization.

II. Effect of virus inoculation method on HR level

Viruses require an incubation period to replicate and move throughout the host plant (Carrington et al., 1996). Therefore, the time interval between *R* gene infiltration and virus inoculation may affect the HR level. To check this hypothesis, we conducted sap-inoculation at different time points relative to agroinfiltration.

In one experiment, the *Agrobacterium* suspension containing the virus resistance gene (*Rx* or *N*) was infiltrated, and then the corresponding virus sap was inoculated at 0, 1, or 2 dpa. The leaves were examined for visible HR and for hydrogen peroxide production, which is a hallmark of HR (Levine et al., 1994), at 3 dpi. No visible HR was evident in *Rx*-infiltrated leaves (Fig. 3, A–C) or in *N*-infiltrated leaves (Fig. 3, G–H) when the viruses were added 0, 1, or 2 dpa.

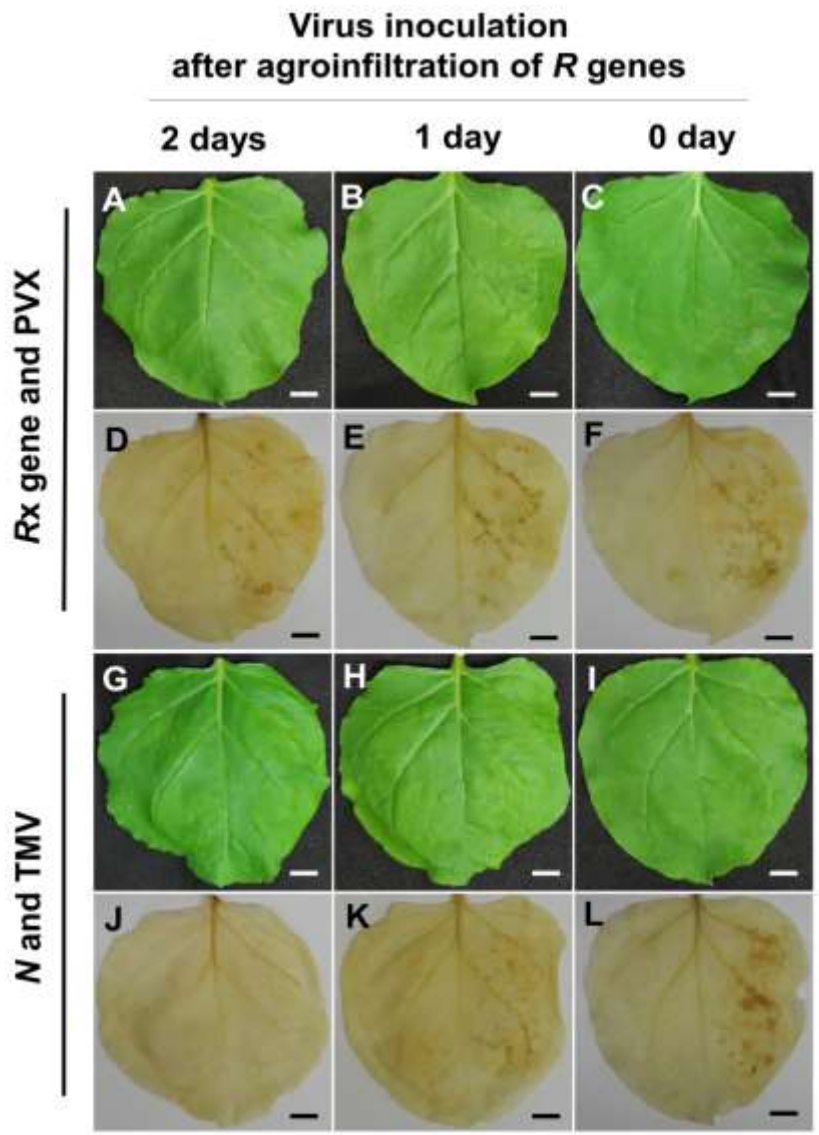

Fig.3. HR as affected by the time elapsed between *R* gene agroinfiltration and subsequent virus inoculation. On each leaf, the left side was infiltrated with empty vector and the right side was infiltrated with the resistance gene. PVX and TMV were inoculated 2 days (A, D and G, J), 1 day (B, E and H, K), or just a few minutes (C, F and I, L) after the agroinfiltration. At 3 days after virus inoculation, visible HR was recorded on intact leaves, and H_2O_2 was then detected by DAB staining in the same leaves. Scale bars in A–M=1 cm.

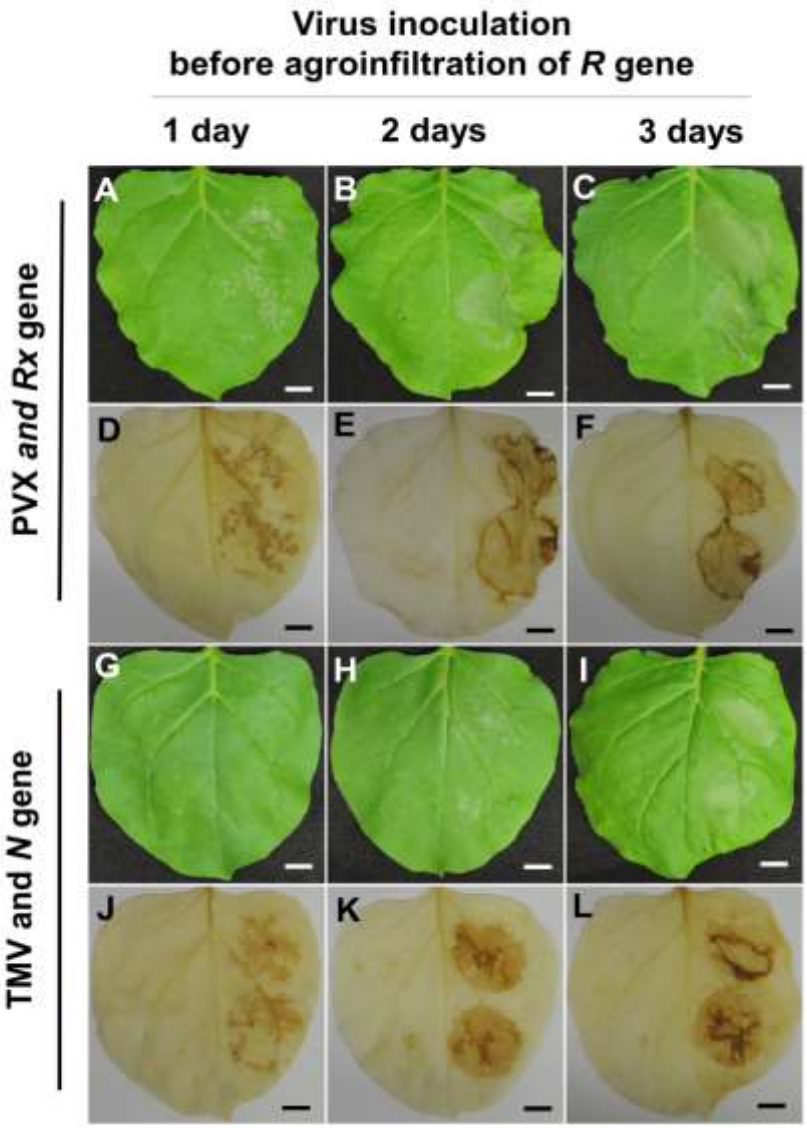

Fig. 4. HR as affected by the time elapsed between virus inoculation and subsequent *R* gene agroinfiltration. On each leaf, the left side was agroinfiltrated with empty vector and the right side was agroinfiltrated with the resistance gene. PVX and TMV were inoculated 1 day (A, D and G, J), 2 days (B, E and H, K), and 3 days (C, F and I, L) before the agroinfiltration. Visible HR was recorded on intact leaves, and H_2O_2 was then detected by DAB staining in the same leaves at 2 days after agroinfiltration. Scale bars in A–M=1 cm.

Hydrogen peroxide, however, was detected by DAB staining. The intensity of DAB staining decreased as the time between infiltration and inoculation increased for both *Rx*–PVX (Fig. 3, D–F) and *N*–TMV (Fig. 3, J–L).

A second experiment tested whether reversing the order of virus inoculation and agroinfiltration affected the HR level. Inoculation with the PVX or TMV was done first, and agroinfiltration was carried out 1, 2, or 3 dpi. The leaves were examined for visible HR and hydrogen peroxide production at 2 dpa. The level of visible HR increased as the time between inoculation and infiltration increased for both PVX–*Rx* (Fig. 4, A–C) and TMV–*N* (Fig. 4, G–I). The same pattern was evident with DAB staining (Fig. 4, D–F and J–L). For further screening, virus inoculation was done 2 days before agroinfiltration.

If the virus sap used for inoculation contains multiple viruses, and this could occur because mixed infections of plant viruses are common in nature (Syller, 2012), determining which virus was interacting with the host *R* gene would be difficult. This could be eliminated as a potential problem by the use of infectious viral transcripts in place of sap from an infected host plant. To test whether *in vitro*-transcribed viral RNA can induce HR in *R* gene-agroinfiltrated

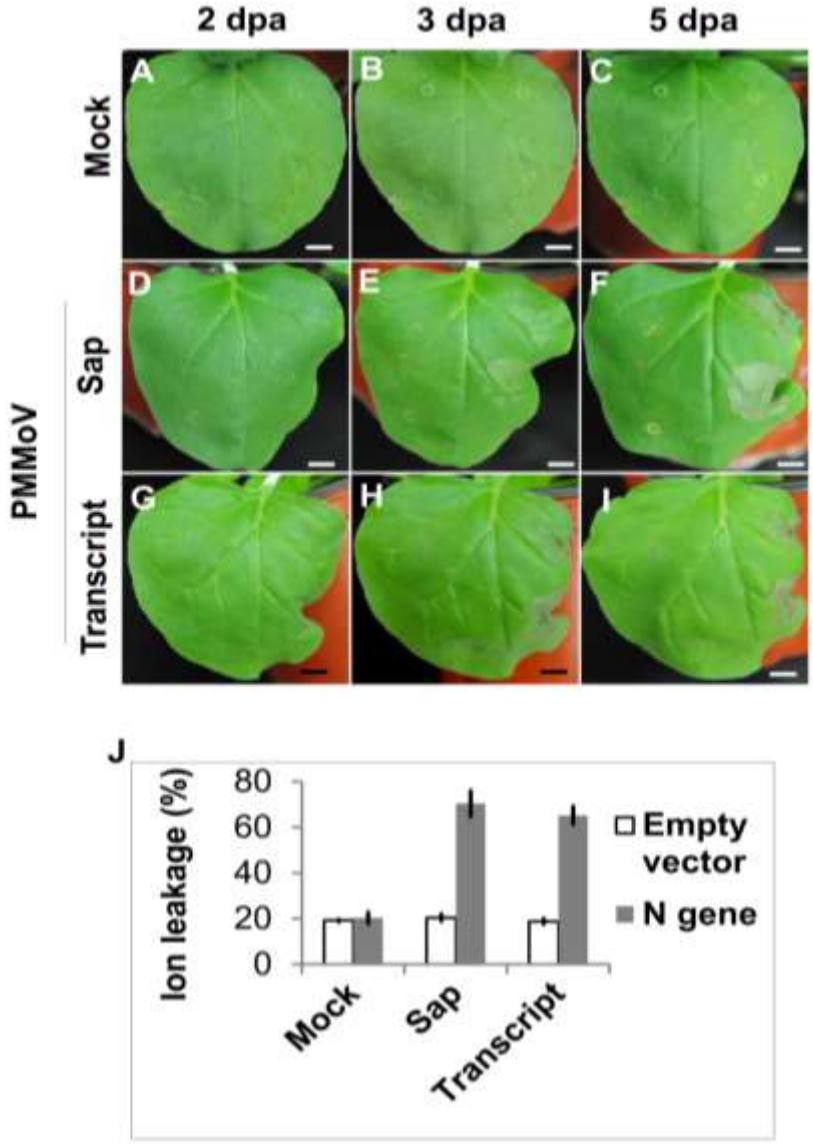

Fig.5. HR induced by *N* gene agroinfiltration of leaves previously inoculated with PMMoV sap or PMMoV transcripts. On each leaf, the left side was agroinfiltrated with empty vector and the right side was agroinfiltrated with the *N* gene 2 days after mock inoculation (A–C), PMMoV sap inoculation (D–E), and PMMoV transcript inoculation (G–I). Visible HR was recorded on intact leaves 2, 3, and 5 days after agroinfiltration. (J) HR levels were estimated based on ion leakage 3 days after agroinfiltration. Scale bars in A–I =1 cm.

leaves, we generated the viral transcripts using *in vitro* transcription driven by the T7 promoter. Given that the *N* gene reacts with all tobamoviruses except TMV Ob strain (Tobias et al., 1982), the tobamovirus PMMoV was used to induce the *N* gene-dependent HR. *N. benthamiana* leaves were inoculated with PMMoV sap and transcripts 2 days before the *N* gene agroinfiltration. The PMMoV transcripts triggered HR in the *N* gene-agroinfiltrated leaves, and HR first appeared 3 dpa (Fig. 5, E and H). HR levels (as indicated by ion leakage) were similar with sap and transcript inoculation (Fig. 5J). This result demonstrated that virus transcripts can be used as an alternative to virus sap for induction of *R* gene-mediated HR.

III. Application of the optimized screening method to identify candidate *R* genes in pepper that confer HR upon PepMoV inoculation

Based on the results presented here, the optimum screening procedure involved:

1) Inoculation of *N. benthamiana* leaves with infectious virus sap or transcripts.

2) Agroinfiltration of suspensions of *Agrobacterium* carrying the candidate *R* gene into the inoculated leaves 2 days after virus inoculation, at a bacterial OD_{600}concentration between 0.3 and 0.5.

3) Observation of HR on infiltrated leaves from 2 to 5 days after agroinfiltration.

PepMoV belongs to the genus potyvirus, and potyviruses are the most common viruses in peppers (Janzac et al., 2008; Moury et al., 2005). Several dominant resistance genes to potyviruses have been mapped (Grube et al., 2000b) but no NBS-LRR gene resistant to potyvirus has been isolated. To find pepper NBS-LRR genes conferring HR in response to PepMoV, we screened 99 candidate *R* genes from pepper using the optimized protocol. Among the candidates, an *Rpi-blb2* ortholog from *C. anuuum* cv. 'Floral Gem' (USA), named *RP28*, conferred HR in response to virus infection. As shown in Fig. 6, two *Rpi-blb2* orthologs, *RP1* and *RP28*, were agroinfiltrated on the *N. benthamiana* leaves after PepMoV sap inoculation. The absence of a response to mock inoculation demonstrated that the candidate did not generate a background HR (Fig. 6, A–D). Visible HR was evident at 3 dpa when leaves were inoculated with PepMoV sap and subsequently agroinfiltrated with *RP28* (Fig. 6, I–L); the same pattern was evident when DAB staining was used to detect hydrogen peroxide (Fig. 6, E–H and M–P). Based on the ion leakage assay, the HR levels were similar with the candidate *RP28* and the positive controls (Fig. 6Q). These results demonstrated that the optimized screening protocol can be used to identify candidate *R* genes in plants.

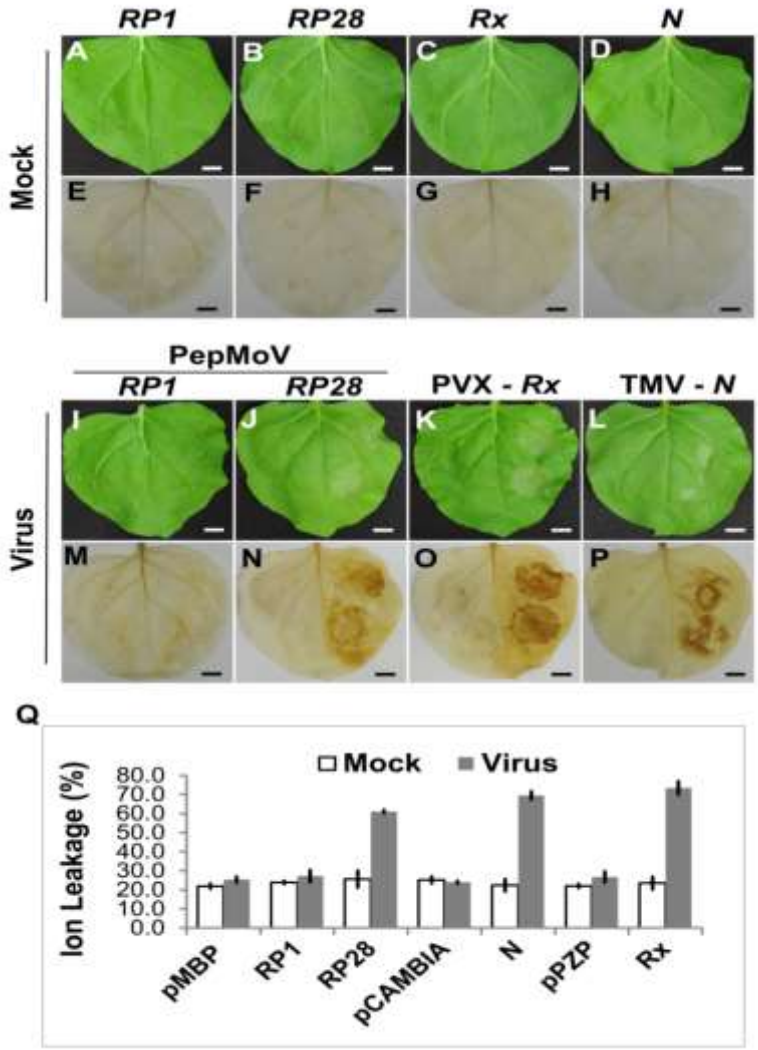

Fig. 6. Screening of candidate *R* genes for the ability to confer resistance to PepMoV. The leaves were first inoculated with virus and 2 days later were agroinfiltrated with candidate *R* genes. On each leaf, the left side was infiltrated with empty vector, and the right side was infiltrated with the candidate or positive control. HR was recorded on intact leaves, and H_2O_2 was detected by DAB staining in the same leaves 2 days after agroinfiltration. (A–C) and (E–H), responses of *RP1*, *RP28*, and two positive controls to mock inoculation; (I, M) responses of *RP1* to PepMoV; (J, N) responses of *RP28* to PepMoV; (K, O) responses of *Rx* to PVX; (L, P), responses of *N* to TMV; (Q) HR level was estimated by ion leakage for *RP1*, *RP28*, and the positive controls. Scale bars in A–P=1 cm.

DISCUSSION

An important factor influencing transient expression following agroinfiltration is *Agrobacterium* concentration. In previous studies, the tested concentrations had OD_{600} values from 0.1 to 1.5, and values between 0.5 and 1.0 resulted in the greatest expression (Amoah et al., 2001; Song and Sink, 2005; Wroblewski et al., 2005; Wydro et al., 2006). Results from the current study with *Rx* and *N* were consistent with these previous reports in that the bacterial OD_{600} concentrations that produced the highest HR levels were between 0.3 and 0.5.

In the protocol described here, the factor that most affected HR induction was virus inoculation method. In previous reports concerning the detection of resistant responses of *R* genes, pathogens were inoculated at the same time as or after agroinfiltration of the *R* genes (Li et al., 2011; Seo et al., 2006; Tomita et al., 2011). In this study, we found that HR was stronger when the virus inoculation preceded the agroinfiltration. Because protein expression resulting from agroinfiltration is detectable at 2 dpa, peaks at 3–4 dpa, and declines during 4–10 dpa (Horsch and Klee, 1986; Janssen and Gardner, 1990), this inoculation sequence may help synchronize the expression of *R* genes with that of plant virus genes. In other words, the interaction

between the *R* gene and viral elicitor may be stronger if a high level of elicitor is available in the plant at the time of infiltration. According to the results with PVX or TMV inoculation, virus infections might require at least 2 days to produce enough viral proteins to trigger a strong response from the infiltrated *R* genes.

Another important condition for enhancing transient expression is the suppression of RNA silencing, which significantly increases the protein level. Post-transcriptional gene silencing was proposed as a limiting factor for transient expression (Johansen and Carrington, 2001). The tombusvirus silencing suppressor p19 enhances the transient expression of proteins (Voinnet et al., 2003). In tobacco species, however, p19 is an *Avr* determinant and can induce HR in the infiltrated area at 3–4 dpa (Angel et al., 2011). Therefore, we did not use p19 in the development of a protocol to detect *R* genes.

R candidates can be obtained from plants in several ways. Genetic mapping, for example, can be used to isolate many open reading frames from a specific locus. Thus, three genes and seven pseudogenes responsible for tobamovirus resistance were isolated from the *L3* locus in pepper (Tomita et al., 2011). In another approach, primers designed from sequence motifs of conserved regions of *R* genes in one species are used to isolate *R* candidates from the same or

other species. For example, 200 *Rx1* homologues were amplified from *Solanum acaule* by PCR (Bendahmane et al., 2000). For all of these methods, transient expression is necessary to determine which genes are truly *R* genes.

Because plant NBS-LRR genes are very abundant (Kohler et al., 2008; Wanderley-Nogueira et al., 2007; Zhou et al., 2004), screening based on transient expression of candidate genes might be cumbersome when the elicitor is unknown. The same problems could occur with co-agroinfiltration of candidate *R* genes and viral genes. Tobamoviruses, for example, have four open reading frames (Lewandowski, 2005), and at least four transgenic lines might be required if the screening were carried out in the above manner; four co-agroinfiltrations would be needed for each *R* candidate. The situation is more complex if the *R* gene has more than one elicitor. In soybean, for example, *Rsv-1* confers resistance to *Soybean mosaic virus* with corresponding elicitors HC-Pro and P3. This problem could be partially solved by co-agroinfiltration of virus infectious clones and *R* candidates. For example, the resistance gene *RT4-4* was co-agroinfiltrated with an infectious clone of *Bean dwarf mosaic virus* (Seo et al., 2006), and *L3* gene candidates were tested with an infectious clone of PMMoV by co-agroinfiltration (Tomita et al., 2011). However, the limited availability of

Agrobacterium-based infectious clones limits the use of this co-expression strategy.

In the protocol developed here, *N. benthamiana* was mechanically inoculated with virus infectious sap 2 days before agroinfiltration with candidate *R* genes. After infection, plant viruses translate their genes to proteins, replicate the genome in plant cells, and move systemically (Carrington et al., 1996). It follows that viral proteins may occur throughout the plant before agroinfiltration of the candidate *R* gene. In this respect, the infected plant is comparable to a transgenic plant expressing viral elicitor.

Virus sap obtained from natural sources often contains multiple viruses (Syller, 2012), and if the virus sap used in our protocol did contain multiple viruses, identifying the main virus interacting with the *R* gene would be difficult. The problem of mixed infection in virus sap can be solved by the use of infectious transcripts. The transcripts obtained by *in vitro* transcription of viral cDNA clones behave like normal viruses (Ahlquist et al., 1984). In this study, the HR levels obtained by agroinfiltration of the *N* gene were similar whether plants had been inoculated with PMMoV transcripts or sap containing PMMoV.

Although our protocol enables the detection of HR, it does not

enable the detection of ER. Our protocol would also be unsuitable for viruses that cause lethal systemic necrosis (Heaton et al., 1991; Ozeki et al., 2006) because death of the entire plant would prevent observation of HR on infiltrated leaves. In using this protocol, researchers must select susceptible host plants that are suitable for agroinfiltration and that do not exhibit systemic death in response to virus infection. In addition, the sap inoculation method used in this study could not be used for those viruses that are only transmitted by non-mechanical ways. In this case, an *Agrobacterium*-based infectious clone (agroinfection) could be used as an alternative (Grimsley et al., 1986). Because agroinfiltration requires time for the transcription of RNA from cDNA (the RNA is detectable at 18 h after agroinfiltration) (Narasimhulu et al., 1996), the time interval between agroinfection and agroinfiltration with the *R* candidate must also be considered.

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CHAPTER II

Molecular characterization of *Pvr9*, a pepper *Rpi-blb2* ortholog conferring a hypersensitive response to *Pepper mottle virus* (a potyvirus) in *Nicotiana benthamiana*

Phu-Tri Tran, Hoseong Choi, Doil Choi
and Kook-Hyung Kim
Department of Agricultural Biotechnology
and Plant Genomics and Breeding Institute, Seoul National University,
Seoul 151-921, Korea

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ABSTRACT

Pvr9, an *Rpi-blb2* ortholog from pepper, confers a hypersensitive response to *Pepper mottle virus* (PepMoV) in *Nicotiana benthamiana*. This gene putatively encodes for 1298 amino acids and is predicted to be located on chromosome 6. NIb is the elicitor of the *Pvr9*-mediated hypersensitive response but no direct interaction between the *R* gene and the elicitor was detected in Y2H or BiFC assays. NIb from several potyviruses elicited the hypersensitive response, and the elicitation was determined by two internal regions of PepMoV NIb. Inoculation of pepper with PepMoV resulted in a minor increase in *Pvr9* transcription in the resistant cultivar *C. annuum* 'CM334' and a slight down-regulation in the susceptible cultivar *C. annuum* 'Floral Gem'. The 5′ upstream region of *Pvr9* from cultivar CM334 had higher transcription activity than the region from cultivar Floral Gem. The cultivars CM334 and Floral Gem have non-functional *Pvr9* homologs with loss-of-function mutations in the CC, NBS, and LRR domains.

INTRODUCTION

In plants, gene-mediated resistance is one of defense mechanisms that prevents or reduces virus infection (Kang et al., 2005). Based on their mode of inheritance, plant resistance genes are classified as recessive or dominant. Recessive resistance genes provide a passive resistance in which virus multiplication is compromised by the incompatible interactions between the virus and host factors (Diaz-Pendon et al., 2004). Dominant resistance genes (*R* genes), in contrast, confer an active resistance by encoding resistance proteins (R proteins) that recognize pathogen effectors or so-called avirulence (*Avr*) factors (Chisholm et al., 2006).

In pepper, several resistance genes to potyvirus have been described (Grube et al., 2000c). Two dominant resistance genes resistant to potyvirus have been mapped in chromosome 10: *Pvr4 and Pvr7*. *Pvr4* originating from *Capsicum annuum* "Criollo de Morelos 334" has a broad spectrum and extreme resistance to potyviruses (Dogimont et al., 1996). *Pvr7* tightly linked to *Pvr4*, which is from *Capsicum chinense* "PI159236", confer HR to PepMoV Florida (V1182) strain (Grube et al., 2000a). Recently, a novel codominant molecular marker for an *R* gene that confers resistance to *Chilli veinal mottle*

virus (potyvirus, ChiVMV) was mapped on chromosome 6 of *C. annuum* ‘NW4’ (Lee et al., 2013). Till now, none of these genes are cloned and characterized at molecular level.

We previously screened for genes that confer resistance to viruses based on virus inoculation and transient over-expression of candidate *R* genes by agroinfiltration in *Nicotiana benthamiana*; using this method, we isolated an *Rpi-bl2* ortholog (named the *Pvr9* gene) that confers a hypersensitive response to PepMoV in *N. benthamiana* (Tran et al., 2014). Here, we determine the molecular characteristics of the *Pvr9* gene and its elicitor. We show that this *R* gene putatively encodes for 1298 amino acids of a possible CC-NBS-LRR protein and is predicted to be located on pepper chromosome 6. We also show that PepMoV NIb is the elicitor of the *Pvr9*-mediated hypersensitive response. Finally, we use mutational analyses to identify the amino acid residues that are important to the function of the *R* gene and the elicitor.

MATERIALS AND METHODS

I. Plant materials and virus inoculum

C. annuum ‘Floral Gem’ and *C. annuum* ‘CM334’ were used for observation of gene expression as well as for isolation of *Pvr9* homologs and *Pvr9* upstream sequences. *N. benthamiana* was used for *Agrobacterium*-based transient expression of *Pvr9* constructs, viral genes, and promoter constructs. PepMoV isolate 134 was maintained in *N. benthamiana*; extraction of viral sap and inoculation were described previously (Tran et al., 2014).

II. Cloning and mutagenesis

To identify the cDNA sequence of *Pvr9*, total RNAs were extracted with Isol-RNA lysis reagent (5Prime) from *Pvr9* agroinfiltrated leaves at 2 days after agroinfiltration. The total RNAs were then treated with RQ1 DNase I (Promega) to remove DNA contamination. Double-stranded cDNA of *Pvr9* was amplified by RT-PCR with primers 1 and 2 (Table 1) using GoScript reverse transcriptase (Promega) and EX *tag* DNA polymerase (Takara). The PCR product was ligated to the pGEM Teasy vector (Promega) by *T4* DNA ligase (Promega). The sequence of the *Pvr9* cDNA clone was

Table 1. Primers for cloning, mutagenesis, and detection

No.	Primer name	Primer sequence (5′ to 3′)
1	Pvr9 *Mlu*I[a] Fw[b]	CGACGCGTATGGAAAAACGAAAAGATA TTGAAGA
2	Pvr9 *Mlu*I Rv[b]	CGACGCGTCTACTTAAATAAGGGGATAT CCTCCT
3	Pvr9 1380[c] Rv	TGAGTCCGGGGCTTTCAGAT
4	Pvr9 1093 Fw	ACCGCAAGCACTTCAGGGGC
5	Pvr9 2339 Rv	GGGCAGCTCTTGTTTCCAAATGCC
6	Pvr9 2034 Fw	TCAAGTTACCGGCCCTGATTC
7	Pvr9 3272 Rv	TCAATGGACAGCACTCGCAACT
8	Pvr9 2941 Fw	AGGCGGTATGGTAAACACCTCT
9	Walker adaptor-1	GTGAGCGCGCGTAATACGACTCACTATA GGGNNNNATGC
10	Walker adaptor-2	GTGAGCGCGCGTAATACGACTCACTATA GGGNNNNGATC
11	Walker adaptor-3	GTGAGCGCGCGTAATACGACTCACTATA GGGNNNNTAGC
12	Walker adaptor-4	GTGAGCGCGCGTAATACGACTCACTATA GGGNNNNCTAG
13	Walker primer-1	GTGAGCGCGCGTAATACGA
14	Walker primer-2	GTAATACGACTCACTATAGGG
15	Pvr9 144 Rv	TCCTATCAAATTTTCAAAGCCACA
16	Pvr9 97 Rv	GGGATTTCTTCAAAATAGAATGCTG
17	Pvr9 3912 Fw	AGAGAGCCCTCATGTTGAAGA
18	Pvr9 3965 Fw	ATATGGGAGGGGACGAGCTT
19	Pvr9 homolog *Mlu*I Fw	CGACGCGTATGGAAAGAGTAAAAGAAA ATGAACTAGTGA
20	Pvr9 homolog *Mlu*I Rv	CGACGCGTCTACTTGAATAACGGGATATTA TGCCGG
21	Scaffold1553 217610 Fw	GATTGTGATCAAGTTCTCTGGCAC
22	Scaffold1553 224484 Rv	ACCAATCACCAAACCATGGGCTAACA
23	Scaffold1553 221126 Fw	TCCGGTCCACTTGGGGTAATTTTCAT
24	Scaffold1553 224827 Rv	CCCAACATGGTGCAGCTAGA
25	Scaffold1553 228180 SacI-SacII Rv	TAGGAGCTCCGCGGAACAAAAAGAGGGG CACCCA
26	Scaffold1553 224326 Fw	TCCTCACCTGAGTCTTGGCTCC
27	Scaffold1553 220269 Rv	GGTCAAAAGTGTTGCTCGTGG
28	Scaffold1553 220071 Fw	TTGCCGCTAGAAGCAGAACC

29	Scaffold1553 219063 Rv	TGTCATTTTTCACTTCACAAGACT
30	Scaffold1553 218922 Fw	GTTAGGGCCCTTCTCCAACC
31	Scaffold1553 223304 Fw	CAAGTAATGCCCTACCCCCAG
32	Scaffold1553 223646 Rv	TGTGTGGGCTTACTAGGGGT
33	Scaffold1553 222274 Fw	CAGATTGCATTGTTTTGTTCACGA
34	Scaffold1553 222496 Rv	CTAGTTCGCAACCCTCTCGT
35	Scaffold1553 225808 Rv	ACAACTTCTGGCTGTTGTCGAATAAC
36	Scaffold1553 225660 Fw	TGTTCCATCAACTTTTCCCAGTT
37	Scaffold1553 227186 Rv	CACTTCCAACCCGAAGGTCC
38	Scaffold1553 227133 Fw	ACCCCTCCCTAGCTCCCCT
39	P1 *Mlu*I Fw	CGACGCGTATGGCAACTAACGTTATTCAGTTTG
40	P1 *Mlu*I Rv	CGACGCGTATACTGTTCCATATGAAGTACAGTTG
41	HC *Mlu*I Fw	CGACGCGTATGTCAACACCTGAAGCATTTTGG
42	HC *Mlu*I Rv	CGACGCGTTTGGTGGATGACCTGTTTTTC
43	P3 *Mlu*I Fw	CGACGCGTATGGGAACTGTAGAGAATCATAAAGTGAAAATAG
44	P3 *Mlu*I Rv	CGACGCGTTTGGTGGATGACCTGTTTTTC
45	6K1 *Mlu*I Fw	CGACGCGTATGAGATCAACTGAAGATCTCAAG
46	6K1 *Mlu*I Rv	CGACGCGTCTGATGTCTAACTTCTGAACCTAGTG
47	CI *Mlu*I Fw	CGACGCGTATGTCTTTGGATGATTTTGTGAATAC
48	CI *Mlu*I Rv	CGACGCGTCTGGTGATGCACAAATTGTAAC
49	6K2 *Mlu*I Fw	CGACGCGTATGTCCAAGTCTTCTCTTGCGAAGG
50	6K2 *Mlu*I Rv	CGACGCGTCTGATGACTCACTTCAGACATCTTTC
51	NIa-VPg *Mlu*I Fw	CGACGCGTATGGGACGCTCTAAGACGAAAAG
52	NIa-VPg *Mlu*I Rv	CGACGCGTTTCGTGCTTCACAACTTCCTTTG
53	NIa-Pro *Mlu*I Rv	CGACGCGTGCGAAAACTTTAATGAGGGGCC
54	NIa-Pro *Mlu*I Rv	CGACGCGTTTGCTCCCTCACACTTTCAC
55	NIb *Mlu*I Fw	CGACGCGTATGGCACACACATCACCTTGGATGC

56	NIb *Mlu*I Rv	CGACGCGTCTGATGATGAACTTCATATGTACCAC
57	CP *Mlu*I Fw	CGACGCGTATGAGCAGCTCAAGATCAGATACATT
58	CP *Mlu*I Rv	CGACGCGTCATATTCCTGACCCCAAGCAG
59	P3N-PIPO overlap Fw	TGGCGTGGACCTTGGAGAAAAAATTATCAAGGC
60	P3N-PIPO overlap Rv	GCCTTGATAATTTTTTCTCCAAGGTCCACGCCA
61	P3N-PIPO *Mlu*I Rv	CGACGCGTTTAGTTCCTTGCTTGGTGCG
62	PVY-NIb *Mlu*I Fw	CGACGCGTATGGCTAAACATTCTGCGTGGATGTA
63	PVY-NIb *Mlu*I Rv	CGACGCGTTTGATGGTGCACTTCATAAGCATC
64	PVA-NIb *Mlu*I Fw	CGACGCGTATGGGATGTGATAGCAAGTGGCTATT
65	PVA-NIb *Mlu*I Rv	CGACGCGTTTGGAAATACACCATGTCATCTTCTT
66	TuMV-NIb *Mlu*I Fw	CGACGCGTATGACCCAGCAGAATCGGTGGAT
67	TuMV-NIb *Mlu*I Rv	CGACGCGTCTGGTGATAAACACAAGCCTCAACAC
68	ZYMV-NIb *Asc*I Fw	GGCGCGCCATGAGCAAGCGAGAAAGATGGGTTTA
69	ZYMV-NIb *Asc*I Rv	GGCGCGCCCTGGAGCATMACAGTGTCTCCTTG
70	SMV-NIb *Mlu*I Fw	CGACGCGTATGGGGAGAAAGGAAAGATGGGTTTT
71	SMV-NIb *Mlu*I Rv	CGACGCGTTTGTAAGGACACTGATTCACAGCAG
72	NIb 555 *Mlu*I Fw	CGACGCGTATGACACGGACTTTCACAGCAG
73	NIb 615 *Mlu*I Fw	CGACGCGTATGGATGATTTCAACAACCAGTTTTA
74	NIb 1082 *Mlu*I Rv	CGACGCGTCAGTGTGTCCAATAAGTTTTCACGTT
75	NIb 1335 *Mlu*I Rv	CGACGCGTTGGGTATCCCCATGATTCAA
76	NIbΔ(186-235)-a Rv	*GGCAAAGCTGTCTTATTAGCCTCTAT*CTTTTCCT^{D}
77	NIbΔ(186-235)-b Fw	*ATAGAGGCTAATAAGACAGCTTTGCC*TGATGGAT
78	NIbΔ(236-369)-c Rv	*AAAAATGACTTGACAAAAGCTTAT*TCCATCCACC

79	NIbΔ(236-369)-d Fw	*ATAAGCTTTTGTCAAGTCATTTTT*CTGATTTAGG
80	NIbΔ(370-445)-e Rv	*TGAGTTAATTGCAGTGTGTCCA*ATAAGTTTTCAC
81	NIbΔ(370-445)-f Fw	*TGGACACACTGCAATTAACTCA*TGAGATTCGAAG
82	SMV-NIb 555 Rv	TTTGTTTGCTTGCACTTTTTCAATTG
83	SMV-PepMoV-NIb 556 Fw	*CAATTGAAAAAGTGCAAGCAAACAAA*ACACGGACTTTCACAGCAGC
84	SMV-NIb 706 Fw	AGAAGTTTACCCGATGGATGGG
85	PepMoV-SMV-NIb 705 Rv	*CCCATCCATCGGGTAAACTTCT*CAAAAGCTTATTCCATCCACCAT
86	SMV-NIb 1084 Rv	GAGAGTGTCATACAGCCATGTGTC
87	SMV-PepMoV-NIb 1085 Fw	*GACACATGGCTGTATGACACTCTC*TCAAGTCATTTTTCTGATTTAGGGC
88	SMV-NIb 1336 Fw	GAATTGCTGCAAGAGATCCGC
89	PepMoV-SMV-NIb 1335 Rv	*GCGGATCTCTTGCAGCAATTC*TGGGTATCCCCATGATTCAATC
90	Pvr9 2242 Fw	GGAAAGCGCAACACTGATCC
91	Pvr9 3167 Fw	CAAACCTTTGGAATCTAGAAACCCT
92	Pvr9 733 Fw	GGACACTTCCTTTGGGAGGA
93	Pvr9 1301 Fw	GTGCAACTCGAGATTTGCTGG
94	Pvr9 1886 Fw	CAATCACTGGTATGCCGGGT
95	Pvr9 1984 Rv	TGCACCATGCACAAATGTCG
96	Pvr9 D492 Fw	TGTGGCTTAT**GAC**GCTAAAGATGTCATT[e]
97	Pvr9 D492 Rv	CATCTTTAGC**GTC**ATAAGCCACATCTAA
98	Pvr9 Q492 Fw	TGTGGCTTAT**CAG**GCTAAAGATGTCATT
99	Pvr9 Q492 Rv	CATCTTTAGC**CTG**ATAAGCCACATCTAA
100	Pvr9 K492 Fw	TGTGGCTTAT**AAG**GCTAAAGATGTCATT
101	Pvr9 K492 Rv	CATCTTTAGC**CTT**ATAAGCCACATCTAA
102	Pvr9 P492 Fw	TGTGGCTTAT**CCG**GCTAAAGATGTCATT
103	Pvr9 P492 Rv	CATCTTTAGC**CGG**ATAAGCCACATCTAA
104	Pvr9 L652 Fw	TATTGATGTT**CTT**GATGAGCTAAGGAGA
105	Pvr9 L652 Rv	TTAGCTCATC**AAG**AACATCAATATTCTC
106	Pvr9 G652 Fw	TATTGATGTT**GGT**GATGAGCTAAGGAGA
107	Pvr9 G652 Rv	TTAGCTCATC**ACC**AACATCAATATTCTC
108	Pvr9 P652 Fw	TATTGATGTT**CCT**GATGAGCTAAGGAGA
109	Pvr9 P652 Rv	TTAGCTCATC**AGG**AACATCAATATTCTC
110	Pvr9 3327 Fw	CTCACAGTTAGAGAACTTGAGAGAATTAG
111	Pvr9 3383 Rv	TCTTTTGAATAGGAAAGCACGAGT

112	Pvr9 3403 Fw	AGGTTCCCCAATCTTCAAGAGC
113	Pvr9 3468 Rv	TCGCTCTGTTGAGTAATCCCA
114	Pvr9 3530 Fw	GTTCAAATTCCAATGACAGTGGGGC
115	Pvr9 3599 Rv	TTGGAAGGGAAGTGAAAATCCCA
116	Ca-GAPDH 300 Fw	TCTTCGTCCTTACAAAACTCTGGT
117	Ca-GAPDH 397 Rv	CCACCCGTCTGTGACTTTGG
118	Nb-F-Box Fw	GGCACTCACAAACGTCTATTTC
119	Nb-F-Box Rv	ACCTGGGAGGCATCCTGCTTAT
120	PepMoV-CP 598 Fw	GGTCTGGCTCGATACGCATT
121	PepMoV-CP 690 Rv	TGCTGCTGCTTTCATTTGGA
122	Pvr9 29 Fw	CAAACAACTCATTGGCGTCGT
123	Pvr9 128 Rv	ACAGCTTTTCGATCCTCTTCGT
124	GFP 334 Fw	GAGGTGAAGTTCGAGGGCGA
125	GFP 425 Rv	AGCTTGTGCCCCAGGATGTT

[a] The fused enzyme sites are included in the primer names in italics; the underlined sequences are recognition sites.

[b] Fw stands for forward primer, and Rv stands for reverse primer.

[c] The number indicates the start nucleotide position from the start codon.

[d] The overlapped sequences are italicized.

[e] The mutated codon is in bold.

checked by sequencing with M13 primers and *Pvr9*-specific primers (Table 1, primers 3–8). The *Pvr9* cDNA clone was then digested by *Mlu*I and ligated to the modified pPZP212 vector (Park et al., 2009).

The outermost 5′ and 3′ genomic sequences of *Pvr9* in pepper were identified by chromosome walking as described previously (Reddy et al., 2008). First, total genomic DNA was extracted from leaves of pepper cultivars CM334 and Floral Gem with the DNeasy Plant Mini Kit (Qiagen). Strand-displacement reactions were then conducted using *phi29* DNA polymerase (NEB) and walker adaptors (Table 1, no. 9–12). Finally, chromosome walking reactions were carried out by nested PCRs with walker primers and locus-specific primers (Table 1, primers 13–18). Sequences of the PCR products were checked by sequencing with locus-specific primers.

To isolate homologous genomic *Pvr9* sequences from various pepper genotypes, PCR amplifications were conducted with primers designed from the chromosome walking (Table 1, primers 19 and 20) using genomic total DNAs as templates. The PCR products were cloned to the pGEM-T Easy vector and confirmed by the same procedure used for *Pvr9*. The *Pvr9* homologs were then transferred from the pGEM-T easy vector to the modified pPZP212 via the *Mlu*I RE site.

To identify the upstream sequences of *Pvr9*, full *Pvr9* with identified outermost sequences was blasted against the pepper genome database (http://cab.pepper.snu.ac.kr/). A highly homologous sequence (> 99.7% identity to *Pvr9*) of 5574 base-pairs upstream of *Pvr9* was found in scaffold4966 (11,394 bps) of pepper scaffold database version 1.1. The 5574 bps were then found (100% identical) in counter-orientation from nucleotide 223206 to nucleotide 217,633 in scaffold1553 (total 558,846 base-pairs) of pepper scaffold database version 1.2. From this scaffold, three overlapped fragments upstream of *Pvr9* (3,872, 3,537, and 3,798 base pairs) were amplified with specific primers (Table 1, primers 21–26,) and stepwise cloned to the pGEM-T easy vector via TA cloning, *Nhe*I/*Sac*I, and *Sac*I RE sites. Plasmids of transformants were confirmed by specific primers (Table 1, primers 27–38). The united 10,348 base pair upstream sequences were then digested by *Sac*II, end-blunted by Klenow fragment (Takara), and ligated to the modified promoterless vector GFP-pCAMBIA0380 by blunt-end ligation using T4 ligase.

To identify and characterize the elicitor of *Pvr9*, nine genes of PepMoV isolate 134 (EU586123) were amplified by RT-PCR from total RNAs of PepMoV-infected tissue using the viral gene flanking primers (Table 1, primers 39–56) with fused start codons at the 5′ ends. *NIbs*

from PVY, PVA, TuMV, ZYMV, and SMV were amplified by RT-PCR with the flanking primers (Table 1, primers 61–70; the start codon was fused at the 5′ end of the primers) using total RNA from the virus-infected tissue. The truncated in-frame PepMoV *NIbs* were amplified from full-length PepMoV *NIb* with PepMoV *NIb o*utermost primers (Table 1, primers 53 and 54) and internal primers (Table 1, primers 71–74). To make internally deleted PepMoV *NIbs*, small DNA segments were made by PCRs with PepMoV outermost primers and overlapping primers (Table 1, primers 75–80); final PCR products of internally deleted *NIbs* were made by overlap extension PCRs (Ho et al., 1989) from two short segments (Fig 5B; segments a and b for NIbΔ(186-235), segments c and d for NIbΔ(236-369), segments e and f for NIbΔ(370-445)). To make SMV-PepMoV *NIb* hybrids, small PCR segments were amplified with SMV *NIb* outermost primers (Table 1, primers 69 and 70), SMV internal primers (Table 1; primers 85, 87, 89, and 91), and overlapping primers (Table 1; primers 86, 88, 90, and 92); overlapping PCRs were carried out from small segments (Fig 5C; segments 1, 2, and 3 for hybrid 1; segments 4, 5, and 6 for hybrid 2; segments 1, 2, 7, 5, and 6 for hybrid 3). The *Mlu*I-digested PCR products of the non-mutated and mutated *NIbs* were cloned to the modified pPZP212 via the *Mlu*I RE site.

To make swapping constructs of *Pvr9*, *Pvr9* and *homologs* were first cloned to the pGEM-Teasy vector; the swapping of the N terminal region, central region, and C terminal region between *Pvr9* and *homologs* was carried out by restriction enzyme digestion (ligation via *Sac*I–*Nhe*I, *Nhe*I–*Hpa*I, and *Hpa*I–*Apa*I) or by overlap extension PCR with some *Pvr9* outermost primers and internal primers (Table 1, primers 111 and 112). These swapped products were transferred from the pGEM-T easy vector to the modified pPZP212 via the *Mlu*I RE site.

To make point mutations for *Pvr9*, site-directed mutagenesis was carried out by overlap extension PCR (in Table 1, primers 93–110 were used for comparative analysis of *Pvr9* and *homolog 1*, and primers 112–118 were used for the analysis of *Pvr9* and *homolog 2*). The mutants were transferred from the pGEM-T easy vector to the modified pPZP212 via the *Mlu*I RE site.

The sequence data have been submitted to the NCBI database. The accession number of *Pvr9* with 10.3 kbps upstream sequence, *homolog 1,* and *homolog 2* with 10.3 kbps upstream sequence respectively are KM590984, KM590985, and KM590986.

III. Agroinfiltration and cell death detection

Agrobacterium tumefaciens GV3101 was used for all constructs in the modified pPZP212 vectors. Agroinfiltration was conducted as described previously (Tran et al., 2014). For detection of the hypersensitive response, infiltrated leaves were detached 2 days after agroinfiltration and were stained with 3,3'-diaminobenzidine (DAB) and trypan blue (TB) as described previously with minor modifications (Van Wees, 2008). The infiltrated leaves and DAB-stained leaves were imaged with a digital camera (Nikon 7200). *Differential interference contrast* (*DIC*) images of TB-stained tissues were obtained with an Axio Imager A1 Microscope *(*Carl Zeiss Ltd.) and a *20X-objective lens*.

IV. Real-time RT-PCR

For quantification of transcripts of PepMoV, plant genes, and transiently expressed genes, total RNAs were collected from pepper or tobacco leaves and treated with Dnase I (RQ1, Promega) to remove DNA contamination. Real-time PCR was then carried out with biological triplicates and technically replicates in a LightCycler 480 instrument (Roche) using a qRT-PCR kit (PrimeScript, Takara) as described by the manufacturer. Reference amplifications of pepper GAPDH (Wan et al., 2011) and *N. benthamiana* F-box (Liu et al., 2012)

were conducted with specific primers (Table 1, primers 119–122). Primers specific to PepMoV CP (Table 1, primers 123 and 124) were used for detection of PepMoV. Specific and exon-exon spanning primers were used for detection of *Pvr9* transcripts (Table 1, primers 125 and 126). Primers specific to GFP (Table 1, primers 127 and 128) were used to detect GFP transcripts. Ct values of target or reference genes were recorded with LightCycler 480 software (version 1.5) and were statistically calculated with Microsoft Excel 2010 software as previously described (Schmittgen, 2006).

V. Yeast two-hybrid and bimolecular fluorescent complementation assays

For yeast two-hybrid assays, double-stranded cDNAs of *Pvr9* and PepMoV *NIb* were re-amplified and cloned to vectors pGBKT7 and pGADT7 of the Matchmaker yeast two-hybrid system (Takara) for fusion with the DNA-binding domain (BD) or transcription-activation domain (AD), respectively. Yeast co-transformation and growth protocol were carried out with yeast strain AH109 as described by the manufacturer. Co-transformation of pGADT7::T (simian virus 40 large T–antigen) and pGBKT7::53 (murine p53) was used as a positive control. Co-transformation of pGADT7-T and pGBKT7-Lam (human

lamin C) was used as a negative control. Co-transformation of empty vectors only (pGADT7 and pGBKT7) was used to test the background growth of yeast. Serial 10-fold-diluted broths (10^{-1}, 10^{-2}, 10^{-3}) from each co-transformation were re-spotted on a low stringency medium (without leucine or tryptophane), a medium stringency medium (with 2.5 mM of 3-amino-1,2,4-triazole and without histidine, leucine, or tryptophane), and a high stringency medium (without adenine, histidine, leucine, or tryptophan). Growth on medium stringency or high stringency media is indicative of an interaction.

For bimolecular fluorescence complementation, double-stranded cDNA of *Pvr9* and PepMoV *NIb* was cloned to pPZP-nYFP and pPZP-cYFP vectors so that N-terminal (nYFP) and C-terminal (cYFP) fragments of YFP were fused in frame at the N terminus of target genes in the modified binary vector pPZP212. The nYFP-fused SMV-CP (nYFP::SMV-CP) and cYFP-fused SMV-CP (cYFP::SMV-CP) were used as positive controls (Kang et al., 2006). Co-agroinfiltration of the empty vectors pPZPnYFP and pPZPcYFP was used as a negative control to check the background GFP signal. The binary vectors were introduced into *Agrobacterium tumefaciens* GV3101. *Agrobacterium*-based transient co-expression in *N. benthamiana* leaves was carried out as described above. Protein–protein interactions in the living plant cells

were visualized based on YFP fluorescence at 36 h after co-agroinfiltration with an Axio Imager A1 Microscope a 20X objective lens, and a green fluorescent filter.

RESULTS

I. Characterization of *Pvr9*

According to the accepted proposed revision of nomenclature for potyvirus resistance genes in *Capsicum* (Kyle and Palloix, 1997), the pepper *Rpi-bl2* ortholog that confers a hypersensitive response to PepMoV in *N. benthamiana* was previously designated *Pvr9* (Tran et al., 2014). The response caused by *Pvr9* upon PepMoV infection was confirmed in the current study by agroinfiltration of *Pvr9* and PepMoV inoculation in *N. benthamiana* as described previously (Tran et al., 2014). Two days after agroinfiltration, a hypersensitive response on infiltrated leaves was detected by trypan blue staining combined with light microscopy (Fig. 1A, left panel). The accumulation of H_2O_2, the hallmark of the hypersensitive response (Levine et al., 1994), was detected by DAB staining (Fig. 1A, right panel).

The coding sequence of *Pvr9* was determined from the transiently expressed transcripts of *Pvr9* in *N. benthamiana* leaves. By RT-PCR, cloning, and sequencing, an open reading frame of 3897 nucleotides was identified. Sequence alignment of the mRNA and genomic DNA revealed an intron from nucleotide 43 to nucleotide 169 of the genomic sequence (Fig. 1B, upper panel). This open reading

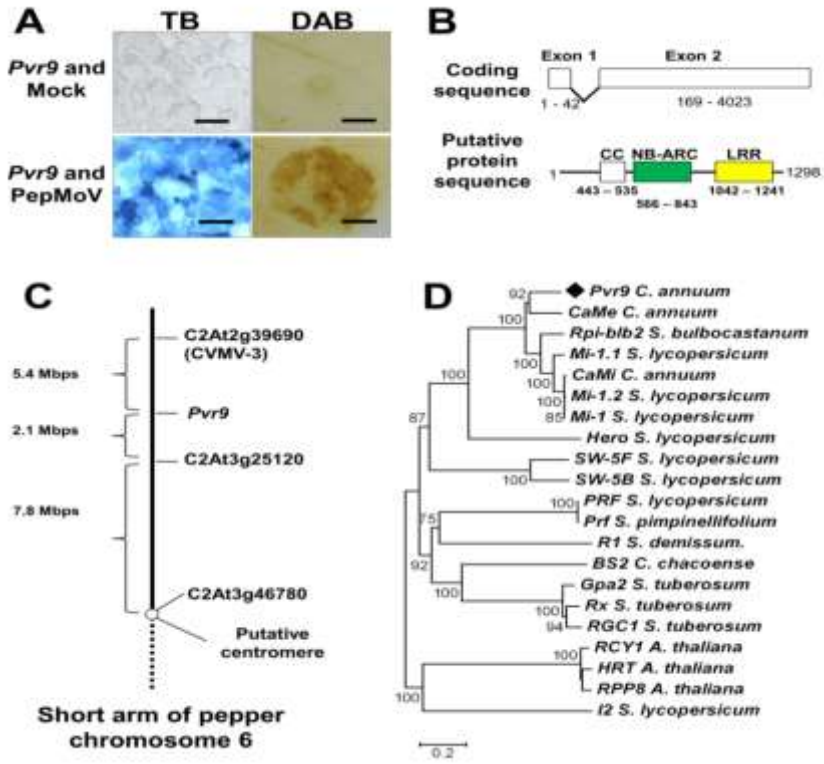

Fig. 1. Characterization of *Pvr9*. (A) Responses of *Pvr9* to mock inoculation (*Pvr9* and Mock) or PepMoV inoculation (*Pvr9* and PepMoV) in *N. benthamiana* as indicated by trypan blue staining (TB, left panel, scale bar = 50 μm) and DAB staining (DAB, right panel, scale bar = 5 mm). (B) The upper diagram indicates the representative structure of the *Pvr9* coding sequence, which includes two exons and one intron (numbers indicate nucleotide position from the start codon); the lower diagram indicates the representative structure of the Pvr9 putative protein, which includes a possible coiled-coil domain (CC), a nucleotide-binding domain (NB-ARC), and a leucine-rich repeat domain (LRR) (the number indicates the amino acid position from the N terminal). (C) Predicted relative position of *Pvr9* on the short arm of pepper chromosome 6 with COSII markers C2At2g39690 (CVMV-3), C2At3g25120, and C2At3g46780. (D) Phylogenetic tree of Pvr9 and other characterized R proteins; species names of the plants from which the *R* gene were isolated are indicated in italics; scale bar indicates 0.2 substitutions per amino acid position; the bootstrap values expressed as a percentage of 1000 replicates are indicated at each node.

frame putatively encodes 1298 amino acids (Fig. 1B, lower panel). Domain and motif searching (Jones et al., 2014) detected an NBS domain from amino acid residue 566 to residue 843 (green box in lower panel, Fig. 1B), which included motifs of kinase-1a (known as the P-loop or Walker A with motif GxxxxGKS/T, in which x indicates any residue), kinase-2 (known as the Walker B with motif hhhhDD/E, in which h is mostly a hydrophobic residue), kinase 3a (motif hhhhToR, in which o is an alcoholic residue), and a hydrophobic domain (GPLP motif). LRR motif scanning (Bej et al., 2014) revealed an LRR domain from residue 1005 to residue 1241 (yellow box in lower panel, Fig. 1B) with at least five leucine-rich repeat (motif LxxLxLxxNxL, in which L is Leu/Ile/Val/Phe and N is Asn/Thr/Ser/Cys). There is a possible CC domain from residue 443 to residue 535 (white box in lower panel, Fig. 1B) including a possible leucine zipper and a heptad repeat before the NBS domain.

The physical position of *Pvr9* in the pepper genome was predicted by nucleotide blasting against chromosome data sets of the pepper genome database (cab.pepper.snu.ac.kr). The highest homologous sequences (> 99%) of 4041 base pairs belonged to chromosome 6 in pepper *C. annuum* Zunla-1 and *C. annuum* var. *grabriusculum*. They were determined to be located from nucleotide

213,329,659 to nucleotide 213,326,619 of *C. annuum* Zunla-1 chromosome 6 and from nucleotide 195,062,197 to nucleotide 195,058,157 of *C. annuum* var. *grabriusculum* chromosome 6. In Fig. 1(B), the position of *Pvr9* on pepper chromosome 6 is schematically represented relative to the previously published markers C2At3g25120, C2At2g39690, C2At3g46780, and CVMV-3 (Bombarely et al., 2011; Lee et al., 2013; Wu et al., 2009). *Pvr9* is located between C2At2g39690 and C2At3g25120, and its distance to the closest marker (C2At3g25120) is about 2.1 mega base pairs.

To determine the relationship of Pvr9 with the other characterized plant resistance proteins, we conducted NCBI protein blasting using deduced Pvr9 amino acid sequences and found 20 homologous accessions with identities from 27 to 73%. These homologous sequences belong to the CC-NBS-LRR class and originate from peppers (*Capsicum annuum* and *C. chacoense*), tomatoes (*Solanum lycopersicum* and *S. pimpinellifolium*), potatoes (*S. bulbocastanum, S. desmissum,* and *S. tuberosum)*, and *Arabidopsis thaliana*. The genes encoding the homologous sequences include *Rpi-bl2* (nucleotide accession no. DQ122125), *Mi-1* (AF091048), *Mi-1.1* (NM_001247693), *Mi-1.2* (NM_001247134), *CaMi* (DQ465824), *CaMe* (FJ231739), *Hero* (AJ457051), *SW-5F* (JX026925), *SW-5B*

(AY007366), *PRF* (U65391), *Prf* (AF220602), *R1* (AAL39063), *BS2* (AF202179), *Gpa2* (AJ249449), *Rx* (AJ011801), RGC1 (AF266747), *RCY1* (AB087829), *HRT* (AF234174), *RPP8* (AF089710), and *I2* (AF118127). By using the neighbor joining method in MEGA6 software (Tamura et al., 2013), we constructed an un-rooted and scaled phylogenetic tree from putative amino acid sequences of Pvr9 and the other functional R proteins (Fig. 1D). The analysis showed that Pvr9, Rp-blb2, and nematode resistance proteins (CaMe, Mi-1, Mi-1.1, Mi-1.2, and CaMi) formed a monophyletic clade; Pvr9 was closest to CaMe, the nematode resistance protein in pepper.

II. Elicitor identification and characterization

In gene-for-gene models, a hypersensitive response usually results from the recognition of a pathogen avirulence determinant (an elicitor) by a plant R protein (Rouxel and Balesdent, 2010). To determine which PepMoV gene triggers the hypersensitive response mediated by *Pvr9*, we cloned the viral genes from PepMoV isolate 134 (Fig. 2A) (Kim et al., 2009) in the modified vector pPZP212 and transformed the cloned genes into *Agrobacterium*. The transformants were then co-infiltrated into *N. benthamiana*. As shown in Fig. 2(B), co-agroinfiltration of transformants expressing *NIb* and *Pvr9* triggered

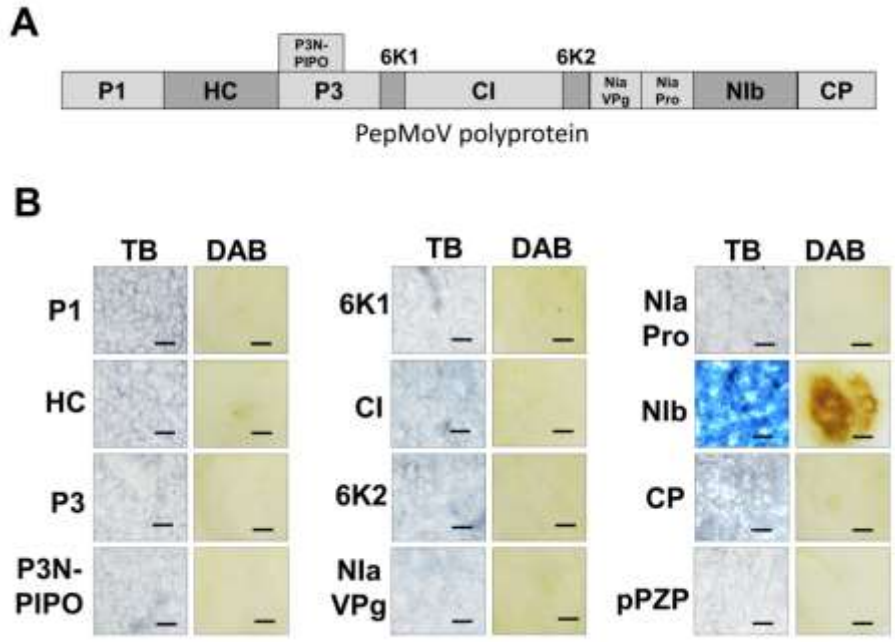

Fig 2. Identification of the elicitor of *Pvr9*. (A) Representative map depicts PepMoV polyprotein with different premature products (P1, HC, P3, 6K1, CI, 6K2, NIa, NIb, and CP). (B) Responses of *Pvr9* to different PepMoV genes at 2 days after co-agroinfiltration; the empty modified pPZP212 vector was used as a control; the hypersensitive response and H_2O_2 were indicated by trypan blue staining (TB, left panel, scale bar = 50 μm) and DAB staining (DAB, right panel, scale bar = 5 mm), respectively.

a hypersensitive response but co-infiltration of the other transformants did not. This demonstrated that PepMoV NIb is the elicitor of the hypersensitive response.

The interaction between Pvr9 protein and PepMoV NIb protein was checked by yeast two-hybrid (Y2H) and bimolecular fluorescent complementation (BiFC) assays. In the first Y2H assay, double-stranded cDNA of *Pvr9* or PepMoV *NIb* was cloned to prey and bait vectors. The yeasts were co-transformed with transcription activation domain (AD)-fused NIb (pGADT7::NIb) and DNA-binding domain (BD)-fused Pvr9 (pGABKT7::Pvr9) or were co-transformed with AD-fused Pvr9 (pGADT7::Pvr9) and BD-fused NIb (pGABKT7::NIb). Following the yeast co-transformations, none of yeast cultures could grow in the low, medium, or high stringency media, indicating the absence of interaction between the proteins (Fig. 3A). In the second BiFC assay, N-terminal and C-terminal fragments of YFP in the modified binary vector pPZP212 were fused in frame to the N terminus of Pvr9 (pPZPnYFP::Pvr9 and pPZPcYFP::Pvr9) or NIb (pPZPnYFP::NIb and pPZPcYFP::NIb); co-agroinfiltration of pPZPnRFP::Pvr9 and pPZPcRFP-NIb or co-agroinfiltration of pPZPnRFP-NIb and pPZPcRFP::Pvr9 did not generate any fluorescent signal (Fig. 3B). Thus, neither the Y2H assay nor the BiFC assay

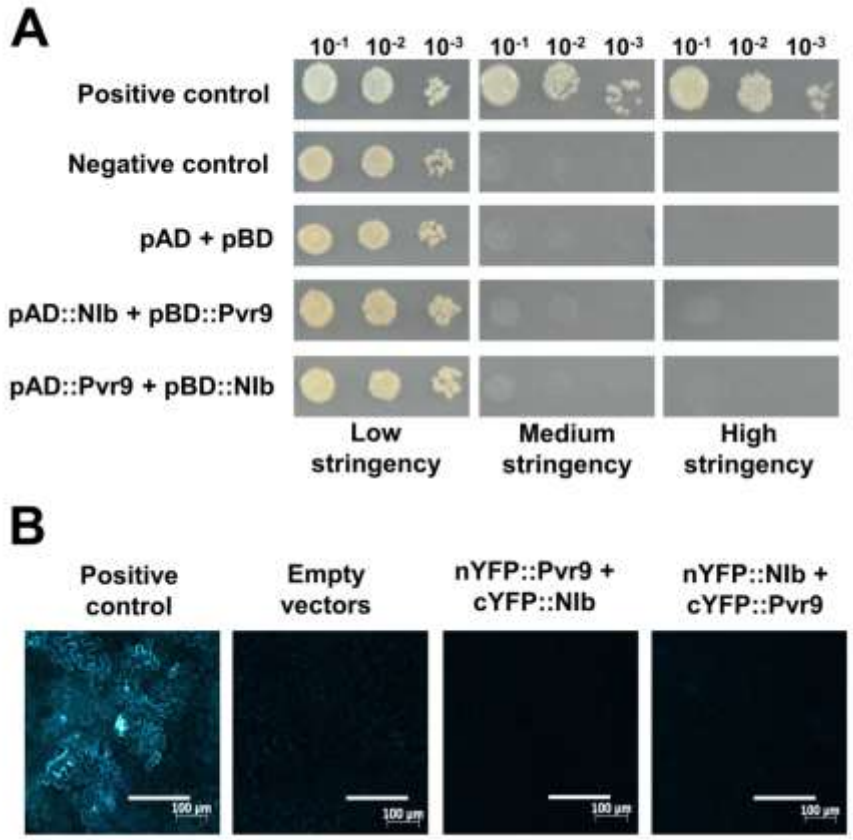

Fig. 3. Interaction of Pvr9 with PepMoV NIb. (A) Yeast two-hybrid assay for the interaction between Pvr9 and PepMoV-NIb. Pvr9 and PepMoV NIb were fused to the transcription-activation domain (AD) and the DNA-binding domain (BD). The yeast transformations of positive control plasmids, negative control plasmids, empty vectors, AD-fused NIb and BD-fused Pvr9 plasmids, and AD-fused Pvr9 and BD-fused NIb plasmids are indicated as PC, NC, pAD + pBD, pAD::NIb + pBD::Pvr9, and pAD::Pvr9 + pBD::NIb, respectively. Serial 10-fold diluted broths (10^{-1}, 10^{-2}, 10^{-3}) from each co-transformation were re-spotted on low stringency, medium stringency, and high stringency media. (B) Bimolecular fluorescence complementation assay of the interaction between Pvr9 and PepMoV-NIb. The co-agroinfiltration of controls, nYFP-fused Pvr9 and cYFP-fused NIb, and nYFP-fused NIb and cYFP-fused Pvr9 are indicated as positive control, negative control, nYFP::Pvr9 + cYFP::NIb, and nYFP::NIb + cYFP::Pvr9, respectively. Scale bar = 200 μm..

revealed a direct interaction between Pvr9 and PepMoV NIb.

To determine whether other potyvirus NIbs elicit a response from Pvr9, the *NIb* gene from PVY, *Potato virus A* (PVA), *Turnip mosaic virus* (TuMV), *Soybean mosaic virus* (SMV), and *Zucchini yellow mosaic virus* (ZYMV) were cloned to the modified pPZP212. Co-agroinfiltration of these transformants expressing other potyvirus NIbs with Pvr9 showed that the *NIbs* from PVY, PVA, and TuMV triggered the hypersensitive response. However, the ZYMV *NIb* triggered only a weak hypersensitive response, and the SMV *NIb* failed to trigger a hypersensitive response (Fig. 4A). To explore the phylogenetic relationships among these NIb proteins, we generated an un-rooted and scaled phylogenetic tree from the NIb amino acid sequences of the 37 non-gramineae infecting potyviruses. As shown in Fig. 4(B), the NIbs of PepMoV, PVY, and PVA belonged to a monophyletic group, and the NIbs of ZYMV and SMV belonged to another clade. Branching of the phylogenetic tree which is usually interpreted as a specification event in phylogenetics, especially at the early nodes (Fig. 4B), revealed that the NIb of TuMV shared a common ancestor with the NIb of PepMoV rather than with the NIbs of ZYMV and SMV.

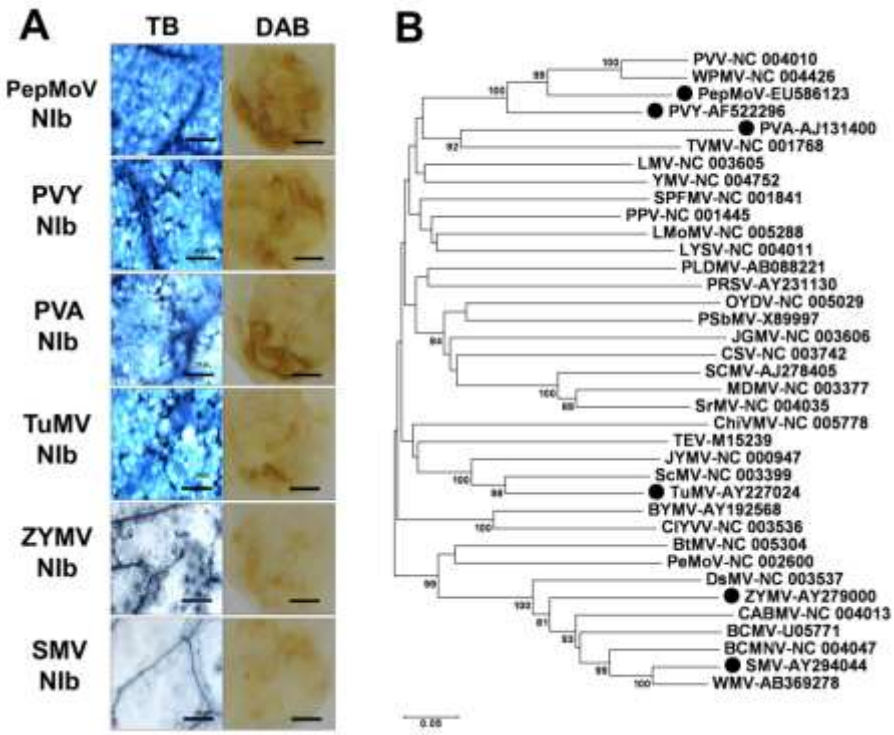

Fig. 4. Pvr9 response to several potyvirus NIbs and phylogenetic tree of potyvirus NIbs. (A) Responses of Pvr9 to PepMoV-NIb, PVY-NIb, PVA-NIb, TuMV-NIb, ZYMV-NIb, and SMV-NIb as indicated by trypan blue staining (TB, left panel, scale bar = 200 μm) and DAB staining (DAB, right panel, scale bar = 1 cm). (B) Phylogenetic tree of NIbs from non-Gramineae-infecting potyviruses; an un-rooted and scaled phylogenetic tree was constructed from putative amino acid sequences of NIbs from 37 non-Gramineae infecting potyviruses; black dots in the phylogenetic tree indicate positions of the tested NIbs; scale bar indicates 0.05 substitutions per amino acid position; the bootstrap values expressed as percentage of 1000 replicates are indicated at each node (70% cut off).

III. Mapping of the elicitor interaction domain

To find those portions of the NIb protein that are required to elicit the Pvr9 response, several truncated mutants were generated by deletions from the two ends of PepMoV NIb. Truncated mutants that lacked amino acid residues 1 to 185 (NIbΔ(1-185)) and residues 445 to 517 (NIbΔ(445-517)) still triggered the Pvr9 response. However, the truncated mutants lacking amino acid residues 1 to 235 and residues 370 to 517 failed to trigger a response (Fig. 5A, NIbΔ(1-235) and NIbΔ(370-519), respectively). These results suggested that the residues 186 to 235 and residues 370 to 445 are important for NIb elicitor activity. In support of this inference, internal deletion mutants lacking the residues 186-235 and 370-445 elicited no Pvr9 response or a greatly attenuated response while the mutant lacking 236 to 396 still triggered the Pvr9 response (Fig. 5B; NIbΔ(186–235), NIbΔ(370–445), and NIbΔ(236–369)). To confirm the role of these regions in eliciting the Pvr9 response, we constructed chimeras by replacing the residues 186 to 235, residues 370 to 445, or both of these regions of SMV *NIb* with the corresponding PepMoV regions (Fig. 5C). As shown in Fig. 5(D), the double replacement (SMV-NIb hybrid 3) caused SMV NIb to trigger the Pvr9 response while the single replacements (SMV-NIb hybrid 1 and hybrid 2) did not.

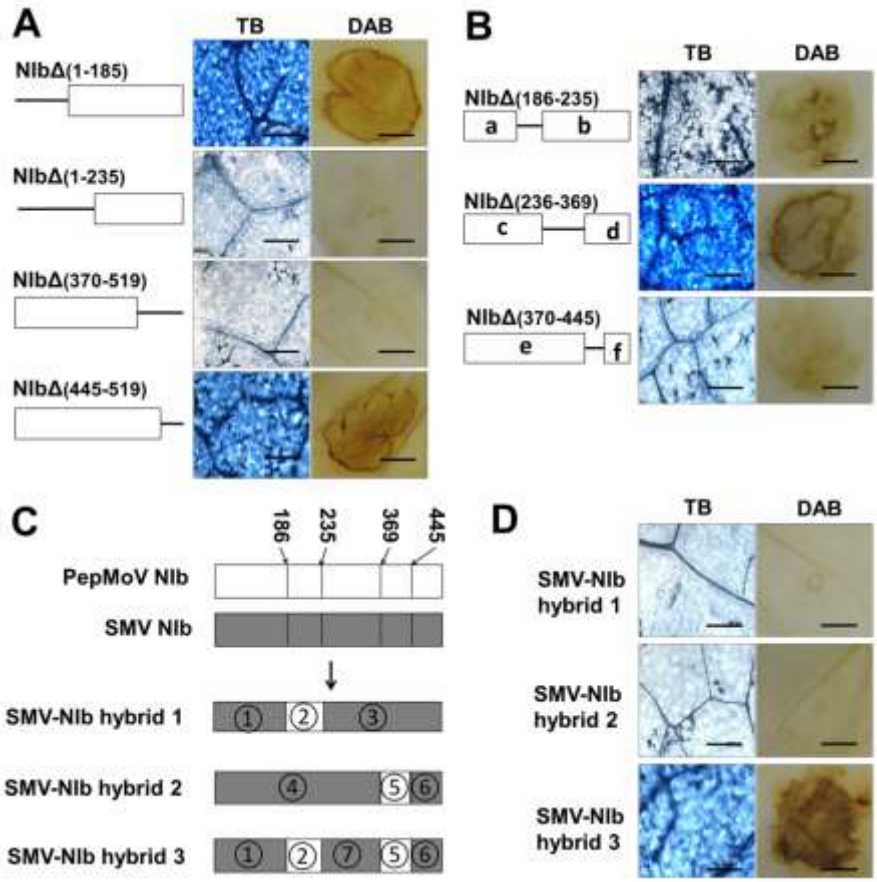

Fig. 5. Characterization of the NIb elicitor. (A) Responses of *Pvr9* to different *NIb* fragments, which were truncated from the N terminal (NIbΔ(1-185), NIbΔ(1-235)) or from the C terminal ((NIbΔ(370-519) and NIbΔ(445-519)); the numbers in parentheses indicate the amino acid positions of the deleted residues. (B) Responses of *Pvr9* to different internally deleted NIbs (NIbΔ(186-235), NIbΔ(236-369), and NIbΔ(370-445)). (C) Representative structures of SMV-NIb hybrids that were made from SMV-NIb background and residues 186-235 and 370-445 from PepMoV-NIb; the numbers indicate the amino acid position in the PepMoV NIb sequence. (D) Response of *Pvr9* to different SMV-NIb hybrids. The responses (in a, b and d) were detected at 2 days after co-agroinfiltration by trypan Blue (TB, left panel, scale bar = 200 μm) and DAB staining (DAB, right panel, scale bar = 1 cm).

IV. Expression of *Pvr9* in susceptible and resistant pepper cultivars in response to PepMoV infection

Transcription patterns of *Pvr9* were monitored in pepper cultivars *C. annuum* 'Floral Gem' and *C. annuum* 'CM334', which are respectively susceptible and resistant to PepMoV (Dogimont et al., 1996). First, tissues from the inoculated plants were collected, and total RNAs were extracted before inoculation (day 0) and after inoculation (from day 1 to day 15). Infectivity of PepMoV was then checked by symptom observation and real-time RT-PCR (qRT-PCR) with PepMoV CP-specific primers. The transcription level of *Pvr9* was assessed by qRT-PCR with the reference gene *GAPDH*.

Following inoculation, leaf stunting was evident at 7 dpi (days after inoculation) and yellowing was evident at 15 dpi in Floral Gem but not in CM334 (Fig. 6A). The qRT-PCR signals representing PepMoV CP transcripts increased in Floral Gem but not in CM334 (Fig. 6B). In Floral Gem, the signal logarithmically increased in inoculated (local) leaves and drastically increased in upper leaves (systemic) after 5 dpi . However, the signal decreased in both local and systemic leaves of CM334 (except for a slight increase in local leaves by 3 dpi). *Pvr9* transcript levels were generally stable in the upper leaves of both pepper cultivars (line graphs, Fig. 6C, D). In virus-inoculated leaves of CM334, the *Pvr9* transcript level increased slightly from 2 to 10 dpi in

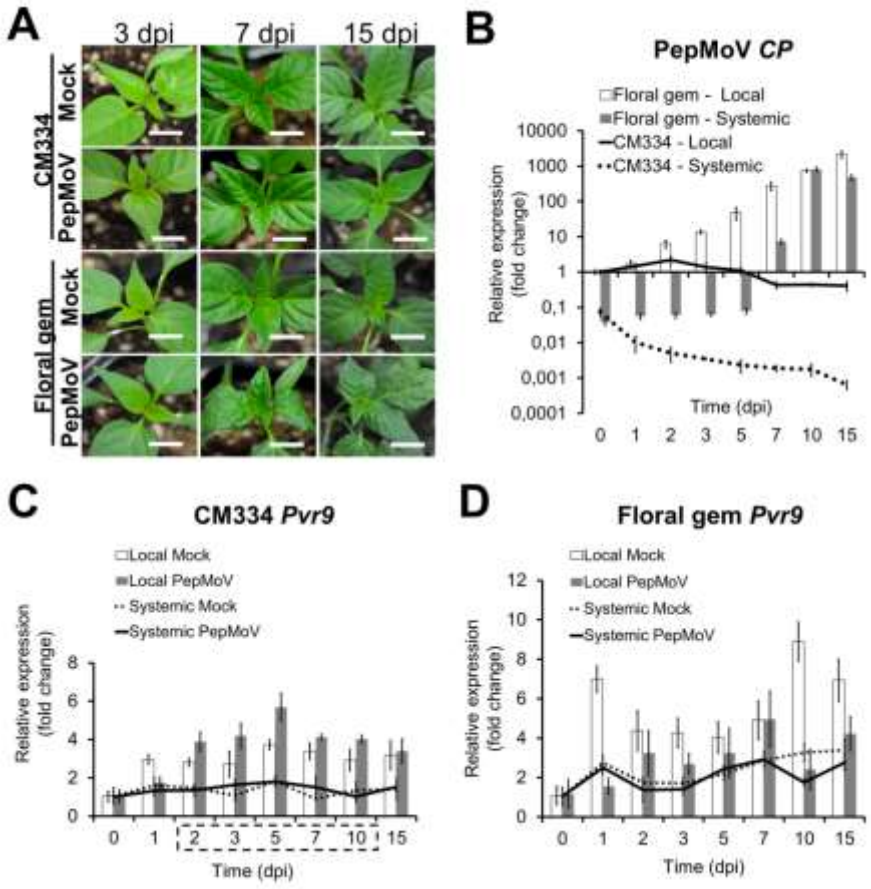

Fig. 6. PepMoV infectivity and Pvr9 expression profiles in peppers. (A) Symptoms of pepper cultivars CM334 and Floral Gem at 3, 7, and 15 days after inoculation (dpi) with PepMoV (PepMoV) or mock (Mock); scale bars = 2 cm. (B) Replication of PepMoV in the inoculated leaves (Local) and upper leaves (Systemic) of pepper cultivars CM334 (line graphs) and Floral Gem (column graphs) was expressed as relative expression (fold change) of PepMoV CP in comparison to samples collected before inoculation. (C,D) Relative expression of *Pvr9* in inoculated leaves (Local, columns graphs) and upper leaves (Systemic, line graphs) of pepper cultivars CM334 and Floral Gem following mock and PepMoV inoculation and in comparison to the sample collected before inoculation (at 0 dpi). The error bars (b–d) indicate ±SD (standard deviation) of biological triplicates.

comparison to mock inoculation (white and grey bars, Fig. 6C). In Floral Gem, however, *Pvr9* transcription was not induced by PepMoV inoculation; at some time points (1, 3, 10, and 15 dpi), the transcript levels were lower in PepMoV-inoculated leaves than in mock-inoculated leaves (white and grey bars, Fig. 6D).

Transcription of a gene is usually affected by upstream regulatory sequences. This could contribute to the susceptibility and differential expression patterns of *Pvr9* among the tested pepper cultivars. To find the promoter sequence of *Pvr9*, 10.3-kb upstream sequences of *Pvr9* were isolated by chromosome walking and PCRs from Floral Gem and CM334 pepper. These sequences were fused to GFP and cloned into modified promoterless GFP-pCAMBIA 0380 (Fig. 7a). *Agrobacterium*-mediated transient expression showed that the sequence from CM334 induced expression of GFP at 2 days after agroinfiltration (Fig. 7b). The sequence from pepper Floral Gem induced a weak expression of GFP that was detectable by qRT-PCR. The level of GFP transcription driven by the *Pvr9* upstream sequence was about 5-fold higher for CM334 than for Floral Gem (Fig. 7c).

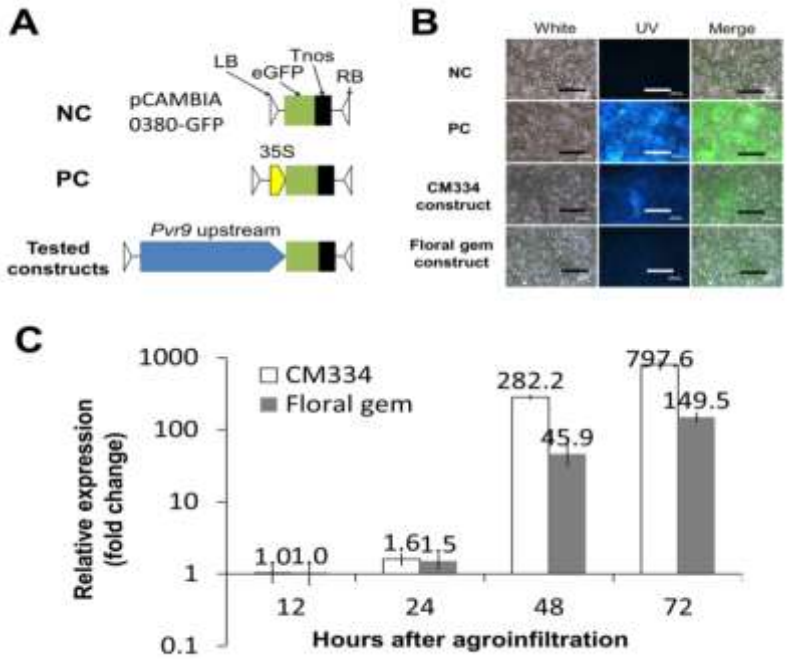

Fig. 7. Promoter activity of upstream sequences of *Pvr9*. (A) Representative structures of transient expression vectors for promoter activity assay. The clones of negative control, positive control and *Pvr9* upstream sequences were indicated as NC, PC and tested constructs For the construction of a negative control clone (NC), eGFP was fused to the upstream of the Tnos terminator (Tnos) and between the left border (LB) and right border (RB) of the binary promoterless vector pCAMBIA0380. For the construction of a positive control clone (PC), the 35S promoter was cloned to the 5′ end of eGFP of the NC clone. For determination of promoter activity of upstream sequences, the 10.3-kb upstream sequences of *Pvr9* were constructed in the same way as the PC clone. (B) GFP signal of the negative controls, positive controls, and *Pvr9* upstream sequences from pepper cultivars CM334 (CM334 construct) and Floral Gem (Floral Gem construct) as indicated by fluorescent microscopy; scale bars = 200 μm. (C) GFP transcription levels driven by *Pvr9* upstream sequences as determined by real-time RT-PCR. The samples were collected at 12, 24, 48, and 72 h after agroinfiltration. The number above each column indicates the relative expression of GFP (fold change) in comparison to the samples collected at 12 h after agroinfiltration. The error bars indicate ±SD of biological triplicates.

V. Important amino acid residues for Pvr9 function

Pvr9 was isolated from cultivar ‘Floral Gem’ (Tran et al., 2014). As described above, however, this cultivar is susceptible to PepMoV. To determine whether the *Pvr9* gene from this cultivar is still functional, the genomic sequence of *Pvr9* was re-isolated from Floral Gem and was named *homolog 1*. As indicated by the amino acid alignment in Fig. 8, homolog 1 had an insertion of six amino acids (NELVRK) between residue 6 and 7, and 16 substitutions (K3R, R4V, D6E, L14F, D44G, D74G, Q210R, S287P, K384E, E492G, A652T, V840A, S862G, Y874H, R1238W, I1288V, Q1291R, E1292H, and D1293N; the first letter, the number, and the second letter stand for the substituted residue, the position of the corresponding residue in Pvr9, and the substituting residue, respectively). Co-agroinfiltration of *homolog 1* and PepMoV *NIb* in *N. benthamiana* did not elicit any response (clones H1, Fig. 9A). To find regions responsible for the loss-of-function, hybrids of *Pvr9* and *homolog 1* were generated. Via the restriction enzyme (RE) sites *Nhe*I and *Hpa*I in the coding sequence, Pvr9 protein was divided into three segments: an N terminal segment from amino acid residue 1 to 261; a central segment from residue 262 to 680; and a C terminal segment from residue 681 to 1298. Each segment was mutually exchanged between *Pvr9* and *homolog 1*, and

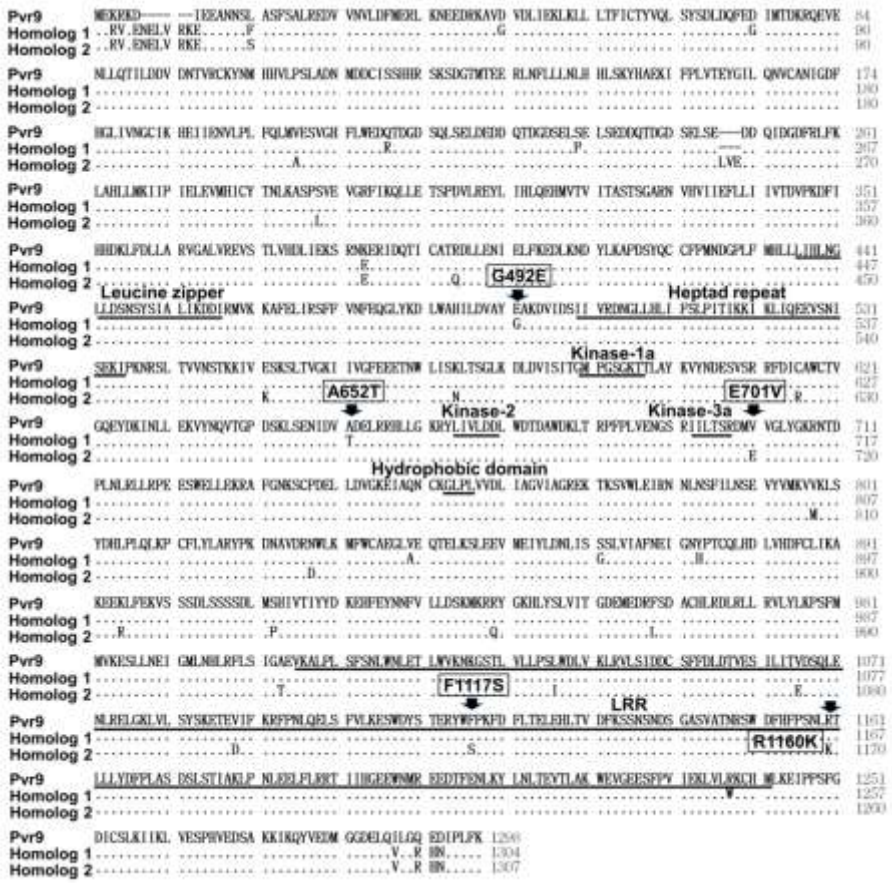

Fig. 8. Amino acid alignment of Pvr9 and two homologs from pepper cultivars CM334 and Floral Gem. The alignment was carried out using the ClustalW method with MegAlign software (DNAstar, Madison WI, USA). (.) indicates the identity of amino acids, and (-) indicates deletions. The underlines show predicted domains and motifs, with the names indicated. The boxed text with arrows indicates amino acid substitutions that affect the function of Pvr9.

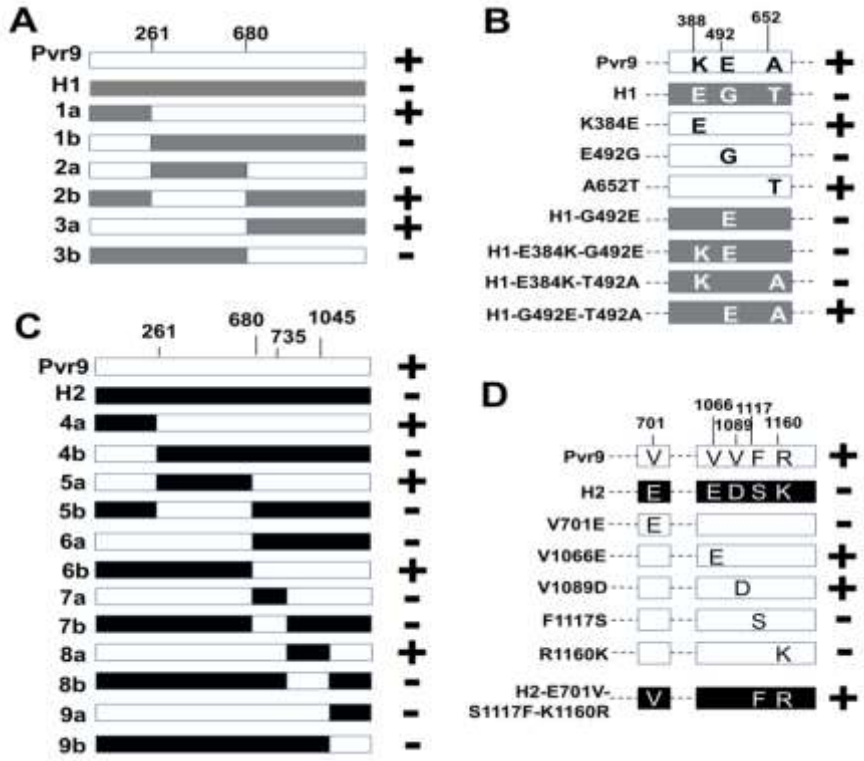

Fig. 9. Mutational analyses of Pvr9 and homologs. Responses of *Pvr9*, the homologs, and mutants were determined by co-agroinfiltration with PepMoV *NIbs*. The presence or absence of a hypersensitive response in each of the constructs is indicated by plus (+) or minus (-) signs. (A) Swapped constructs were made from Pvr9 and homolog 1 (H1) by exchange of the N terminal fragment (1a and 1b), the central fragment (2a and 2b), and the C terminal fragment (3a and 3b) of the proteins. (B) Point mutations at the central region of Pvr9 include K384E, E492G, and A652T. The C terminal point mutants derived from homolog 1 include H1-G492E, H1-E384K-G492E, H1-E384K-T492A, and H1-G492E-T652A. (C) Swapped constructs were made from *Pvr9* and *homolog 2* by exchange of the N terminal fragment (4a and 4b), the central fragment (5a and 5b), and the C terminal fragment (6a and 6b) of the proteins. The C terminals of Pvr9 and homolog 2 were then analyzed by additional swapped constructs (7a and 7b, 8a and 8b, 9a and 9b). (D) Point mutations at the C terminal region of Pvr9 include V701E, V1066E, V1089D, F1117S, and R1160K. The triple mutant of homolog 2 simultaneously contains the mutations E701V, S1117F, and K1160R (as H2-E701V-S1117F-K1160R).

the constructs were co-agroinfiltrated with PepMoV *NIb*. As shown in Fig. 9(A), the replacement of segment 262–680 of Pvr9 with the corresponding segment of homolog 1 resulted in loss of Pvr9 function (clone 2a); in a mutual relationship, homolog 1 gained the function when it contained segment 262–680 from Pvr9 (clone 2b); the other exchanges did not cause loss-of-function in Pvr9 (clone 1a and 3a) or gain-of-function in homolog 1 (clone 1b and 3b). The amino acid alignment between Pvr9 and homolog 1 revealed three amino acid changes in segment 262–680 of homolog 1: K384E, E492G, and A652T. To determine which change is responsible for the loss-of-function, we used PCR mutagenesis to generate single mutants at residue 384 from K to E, at residue 492 from E to G, or at residue 652 from A to T; only the mutation at residue 492 caused the loss-of-function (Fig. 9B, clone E492G). To investigate the function of residue 492, substitutions E492D, E492K, E492Q, and E492P were made; however, all of these mutants lost the function (data not shown). Non-reciprocally, the substitution of the corresponding G492 to E in homolog 1 did not recover the function (clone H1-G492E); however, double mutagenesis revealed that the function of the mutant with G492E and T492A was completely recovered (Fig. 9B, clone H1 G492E-T492A). To investigate the possible function of residue 652 in Pvr9, A652 was

replaced by L, G, or P; these mutations did not prevent Pvr9 from responding to *NIb* but did reduce the response (i.e., it reduced the number of hypersensitive spots per total infiltrated spots) (data not shown).

To determine whether the *Pvr9* from the PepMoV resistant cultivar CM334 is functional, a genomic homologous sequence of *Pvr9* was isolated and named *homolog 2*. The putative amino acid alignment (Fig. 8) showed that homolog 2 protein has an insertion of six amino acids (NELVRK) between residue 6 and 7, an insertion of three amino acids (LVE) between residue 243 and 244, and 25 substitutions (K3R, R4V, D6E, L14S, V199A, S287L, K384E, R495Q, E552K, K575N, C616R, V701E, V798M, N828D, K895R, S913P, R940Q, F959L, A1004T, L1037I, V1066E, V1089D, F1117S, R1160K, I1288V, Q1291R, E1292H, and D1293N). As was the case with *homolog 1*, co-agroinfiltration of *homolog 2* and PepMoV *NIb* in *N. benthamiana* did not elicit any response (Fig. 9C, clone H2). To investigate the responsible region for the loss-of-function, we generated several swapped clones via the RE sites *Nhe*I and *Hpa*I or via overlap extension PCR. In Fig. 9(C), only the exchange of segment 681–1298 caused loss-of-function in Pvr9 and gain-of-function in homolog *2* (clones 6a and 6b); the other exchanges did not alter the function of

Pvr9 (clones 4a and 5a) and homolog *2* (clones 4b and 5b). To narrow the searching region, we generated additional swapped clones by overlap extension PCR; the exchanges of segment 681–735 and segment 1045–1298 caused loss of Pvr9 function (clones 7a and 9a) while the exchange of segment 735–1045 did not (clone 8a); however, none of these segment replacements resulted in gain-of-function in homolog 2. These results suggested that two or more amino acid substitutions independently cause the loss-of-function in homolog *2*. Amino acid alignment between Pvr9 and homolog 2 revealed one amino acid substitution in segment 681–735 (V701E) and eight substitutions in segment 1045–1298 (V1066E, V1089D, F1117S, R1160K, I1288V, Q1291R, E1292H, and D1293N). Among them, substitutions I1288V, Q1291R, E1292H, and D1293N are also present in the C terminus of homolog 1, which functioned normally in several chimera clones (Fig. 9A, clones 2b and 3a). We therefore made and investigated only five single-point mutants from Pvr9 including V701E, V1066E, V1089D, F1117S, and R1160K. As shown in Fig. 9(D), the point mutants V701E, F1117S, and R1160K led to the loss of Pvr9 function while the others did not; the function of homolog 2 was completely recovered in the triple mutant with E701V, S1117F, and K1160R (clone H2 E701V-S1117F-K1160R).

DISCUSSION

The domain predictions revealed the presence of NBS and LRR domains in the putative protein sequence of *Pvr9*. However, the CC or TIR domain of NB-LRR proteins could not be detected in *Pvr9* by prediction software. The upstream sequence of the NBS domain implicates the presence of a CC domain, which usually contains a heptad repeat (Mason and Arndt, 2004). In addition, the phylogenetic analysis showed that *Pvr9* is in same group with CC-NBS-LRR genes. Thus, these results suggest that *Pvr9* belongs to the CC-NBS-LRR class.

Resistance genes in plants are usually present in clusters of tightly linked genes (Hulbert et al., 2001). In CM334 pepper, two dominant potyvirus resistance genes, *Pvr4* and *Pvr7*, are tightly linked and located on chromosome 10 (Grube et al., 2000b). The *Pvr9* gene characterized in this study is not related to those *R* genes but might be linked to another dominant potyvirus *R* gene, *CVMV*, which was recently mapped on chromosome 6 of *C. annuum* 'NW4' (Lee et al., 2013). That both *Pvr9* and *CVMV* were found between the markers C2At2g39690 and C2At3g25120 in the short arm of chromosome 6 also suggests that the genes may be linked. Because the distance between the two markers was large (around 7.5 mega base pairs),

however, additional research is needed to elucidate the relationship between *Pvr9* and *CVMV*.

Functional *R* genes can be isolated by mapping with molecular markers associated with the resistance traits (Bendahmane et al., 1997) or by transposon tagging from resistance plants (Whitham et al., 1994). However, *Pvr9* was previously cloned from a genomic DNA pool of various pepper cultivars based on sequence homology (Tran et al., 2014). Genome-wide analyses have revealed that *R* genes are very diverse and abundant in plant genomes (Kohler et al., 2008; Meyers et al., 2003; Wanderley-Nogueira et al., 2007; Zhou et al., 2004). Therefore, this *R* gene isolation strategy could detect *R* genes whose functions are not expressed in the original host but can be expressed in other plants. This is the case with *Pvr9*, which was isolated from a PepMoV-susceptible pepper but which conferred resistance to PepMoV in *N. benthamiana*.

Pvr9 does not respond to PepMoV response in pepper probably because an unknown factor that mediates the recognition of PepMoV NIb by Pvr9 protein is lacking in pepper. The failure to detect a direct interaction between the *R* gene and the elicitor (Fig. 4) suggests that a third factor is present in *N. benthamiana* but not in pepper. Since neither homolog 1 from Floral Gem nor homolog 2 from CM334 is

capable of inducing hypersensitivity, it is also worth noting that the homolog 1 is probably an allelic variant of Pvr9 in the pepper Floral Gem population. How the Pvr9 homologs present or evolve in the pepper Floral Gem as well as in the other cultivars remained to be clarified. As complete genome sequencing often identifies numerous allelic variants (sometimes hundreds) for a given gene, further study will determine significance of the genotype and corresponding phenotype using a sufficiently large population.

The fact that the homolog 2 from the CM334 cultivar was not functional indicated that Pvr9 is not necessary to the Pvr4 harboring pepper. However, the Pvr9 transcription patterns seemed to link with susceptibility to the virus. The promoter activity driven by the Pvr9 upstream sequence may contribute to the differences of Pvr9 transcription in both cultivars. In that case, *Pvr9* could be one auxiliary component that is up-regulated when resistance blocks virus infection and down-regulated by the successful virus infection. The presence of additional partners that interact with *Pvr9* and possible regulatory mechanisms of *Pvr9* expression remain to be clarified.

Because it carries the GDD motif of the polymerase and has RNA-dependent RNA polymerase activity, NIb is the potyviral replicase (Hong and Hunt, 1996). NIb is also an elicitor of the

resistance response; for instance, NIb of PVY strain M^SN^R is the elicitor of the hypersensitive response in root-knot nematode-resistant tobacco (Fellers et al., 2002). In our study, the potyvirus NIb also elicited the hypersensitive response when *Pvr9* was transiently expressed in *N. benthamiana.*

Avirulence factors of viral plant pathogens usually act as elicitors of resistance conferred by *R* genes but can also act as virulence factors. For example, the helicase domain triggers the *N* gene-mediated hypersensitive response to the TMV U1 strain but also enables the TMV Ob strain to overcome the resistance (Abbink et al., 2001). The CP elicits the hypersensitive response of *Rx*-transgenic tobacco to PVX but also carries the resistance-breaking determinant in PVX strain HB (Querci et al., 1995). Several potyvirus proteins have been proven to be elicitors of resistance or determinants of avirulence with respect to dominant resistance genes in plants. For example, TuMV CI is an avirulence factor of oilseed rape *R* genes *TufB01* and *TurB05* (Jenner et al., 2000; Jenner et al., 2002); TuMV P3 is an avirulence factor of oilseed rape *R* genes *TurB03* and *TurB04* (Jenner et al., 2002; Jenner et al., 2003); SMV P3 and HC are elicitors of the soybean *R* gene *Rsv-1* (Eggenberger et al., 2008); and PVY NIa is the elicitor of potato *R* gene *Ry* (Mestre et al., 2000). As reported by Janzac and colleagues, a

mutation in the NIb of PVY confers virulence toward the *Pvr4*-based resistance of pepper and a high competitiveness cost in the susceptible cultivar (Janzac et al., 2010). In terms of a gene-for-gene relationship, this NIb could be an avirulence factor that is recognized by genotypes of the host plants that harbor the matching resistance gene. The elicitor characteristic of NIb in our study supported the likelihood that NIb is a novel avirulence determinant of potyvirus resistance.

The interaction between *Pvr9* and *NIb* seems relatively conserved among the potyviruses. The intensity of the responses of Pvr9 to different NIbs (in terms of TB and DAB staining) was positively correlated with the similarity of the potyvirus NIb to PepMoV NIb. This suggests that the weak or non-elicitation ability of ZYMV NIb and SMV NIb is due to a weak phylogenetic relationship between these NIbs and PepMoV NIb. Responses of *Pvr9* to truncated and internally deleted PepMoV *NIb* constructs demonstrate that at least two internal sequences are responsible for the elicitor activity of the NIb protein. This activity was recovered in SMV NIb by replacement of the corresponding regions from PepMoV NIb. Given that NIb is an avirulence factor, these regions could be important to viral pathogenicity. This hypothesis should be tested in the future.

From the CM334 and Floral Gem pepper cultivars, we

identified two *Pvr9* homologs that are no longer capable of a NIb-elicited hypersensitive response. The amino acid substitutions E492G, V701E, F1117S, and R1160K were determined to cause the loss of Pvr9 function. The substitution E492G in the possible CC domain of homolog 1 lies between a possible leucine zipper and a heptad repeat (Fig. 8). In the Rx protein, the CC domain interacts with NBS-LRR moieties in pathogen recognition and signaling (Rairdan et al., 2008). The CC domain is also important in pathogen recognition as in "guard" model. For example, the CC domains of NBS-LRR proteins RPM1 and RPS2 interact with *Arabidopsis* RIN4 protein during recognition of the *Pseudomonas syringae* effectors AvrRpm1, AvrB, and AvrRpt2 (Axtell and Staskawicz, 2003; Mackey et al., 2003; Mackey et al., 2002); AvrRpt2 interacts with *Arabidopsis* PBS1 kinase, which binds to the CC domain of NBS-LRR protein RPS5 (Shao et al., 2003). Any point mutation at residue 492 cause loss of Pvr9 function indicates that this residue is critical. These substitutions could disrupt the interaction between domains of Pvr9 or/and compromise the downstream signaling. In addition to residue E492, homolog 1 carries an important substitution A652T at corresponding residue 652 of Pvr9 (Fig. 8); the substitution from T to A is necessary for the functional recovery of this homolog. However, the mutation A652T did not prevent *Pvr9* from

conferring the hypersensitive response to PepMoV *NIb*. As described above, the comparison between Pvr9 and homolog 1 (Fig. 8) revealed one insertion and 16 substitutions. This suggests that another residue(s) might functionally compensate for the role of residue 652 in the Pvr9 background. This hypothesis will need to be tested in further mutagenesis analyses.

The loss-of-function substitution V701E is adjacent to a putative kinase-3a motif (V701E, Fig. 8; clone V701E, Fig 9D). Previous mutation analyses of *R* genes identified many amino acid substitutions in the NBS-ARC domain that compromise ATP/GFP binding/hydrolysis activity and lead to the loss-of-function (Takken et al., 2006). The hhhhToR signature (h is a mostly hydrophobic residue, and o is an alcoholic residue) in kinase-3a corresponds to the sensor I motif in AAA+ ATPases and functions in γ-phosphate sensing (Iyer et al., 2004; Ogura and Wilkinson, 2001). Further research is required to determine whether the V701E substitution affects this sensing activity.

The LRR domain contains the protein recognition motifs LxxLxLxxNxL which appear to provide a versatile structural framework for the formation of protein–protein interactions (Kobe and Kajava, 2001). LRR domain of *R* genes interacts directly (Deslandes et al., 2003; Dodds et al., 2006; Krasileva et al., 2010) or indirectly

(Axtell and Staskawicz, 2003; Mackey et al., 2003; Mackey et al., 2002) with pathogen effectors. In many plant R proteins, this domain is also thought to have co-evolved with pathogen effectors (Dodds et al., 2006; Ellis et al., 2000). The loss-of-function substitutions F1117S and R1160K in the LRR domain could interfere with interactions between this domain and the NIb target. The mechanism of the proposed indirect interaction between Pvr9 and NIb is unclear.

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Capsicum annuum 에서의 *Pepper mottle virus* 저항성유전자의 동정 및 분자적 특성 구명

Phu Tri Tran

ABSTRACT

식물에서 유전자에 의해 매개되는 저항성 반응은 바이러스 감염을 차단하거나 감소시키는 방어 기작 중 하나이다. 이러한 식물의 저항성 유전자들은 이들이 어떻게 자손에게 전달되는지에 따라 우성 및 열성 유전자로 분류된다. 이 연구에서는 Agroinfiltration과 바이러스 접종을 기반으로 바이러스에 대한 식물체의 과민성반응을 유도함으로써 보다 간단하고 저 비용으로 저항성 유전자들을 선별할 수 있는 방법이 개발되었다. *Potato virus X*(PVX) 및 *Tobacco mosaic virus*(TMV)에 대한 저항성에 기여하며 그 특성이 잘 규명된 유전자인 *Rx*와 *N* 유전자가 *Nicotiana benthamiana*에서 과민성반응을 검출하기 위한 transient expression assay를 최적화하는데 이용되었으며

이러한 *Rx* gene 및 *N* gene이 처리된 잎에 HR 반응을 유도하기 위하여 PVX와 TMV에 감염된 조직의 즙액접종이 각각 사용되었다. 이러한 선별 방법을 이용하여 고추에서 99개의 저항성 유전자 후보군으로부터 Potyvirus에 속하는 *Pepper mottle virus*의 감염에 대하여 과민성반응을 일으키는 저항성 유전자(*Pvr9*)이 동정되었으며 이러한 *Pvr9* 유전자의 분자적 특성 규명을 통하여 이 유전자가 1298개의 아미노산으로 이루어진 CC-NBS-LRR 으로 추정되는 단백질을 암호화 하고 있다는 것이 밝혀졌다. *Pvr9*은 다른 저항성 유전자들이 모여 있는 6번 염색체에 위치하고 있는 것으로 예측된다. 바이러스의 유전자 산물 중 하나인 NIb는 *Pvr9*에 의해 매개되는 과민성반응의 elicitor로써 작용하며 Y2H 와 BiFC 방법을 이용하였을 때 *R* gene 산물과 elicitor간의 직접적인 상호작용은 나타나지 않았다. PepMoV의 NIb와 근연관계가 가까운 몇몇 Potyvirus의 NIb들도 과민성반응을 유도하였으며 이러한 과민성반응의 유도는 PepMoV NIb의 두 internal region에 의해 결정된다. PepMoV를 고추에 접종한 결과 *Pvr9*의 전사가 저항성 품종인 *Capsicum annuum* ‘CM334’ 에서는 소폭 증가하였으나 감수성 품종인 *C. annuum* ‘Floral Gem’ 에서는 하향조

절 되었다. 이는 CM334 품종에서 분리된 *Pvr9*의 5’ 상위 지역에서 Floral Gem에서 분리된 *Pvr9*에 비해 높은 transcription activity가 나타났기 때문인 것으로 여겨진다. 또한 CM334와 Floral Gem 품종에는 non-functional *Pvr9* homolog가 존재하고 이는 CC, NBS 그리고 LRR domain의 아미노산 변이에 의하여 그 기능을 상실한 것으로 나타났다.