A DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Molecular Characterization of Nonhost Resistance of Pepper against *Phytophthora infestans* Based on Effector-induced Cell Death

감자역병균의 **Effector**가 유도하는 세포사멸 반응을 기반으로 한 고추의 비기주 저항성 기작

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ABSTRACT

Nonhost resistance (NHR) is the resistance of a plant species against the vast majority of potential pathogens. The mechanism of NHR remains poorly understood but it is considered that NHR shares many components with host resistance such as physical/chemical barriers, PAMPs-triggered immunity, and effector-triggered immunity (ETI). Based on the possibility of polygenic control in ETI of NHR, the main hypothesis of this thesis is that multiple interaction between resistance genes and effectors underpin NHR. Pepper is a nonhost plant against *Phytophthora infestans* which causes severe potato

late blight disease in the world. Hypersensitive response (HR) cell death was observed on epidermal cells of pepper infected with *P. infestans*, which suggests that ETI is a major factor of NHR of pepper against *P. infestans* because HR is a typical response of ETI induced by the interaction between resistance (R) genes and cognate effectors. To screen cell death induced by *P. infestans* effectors, 100 pepper accessions were inoculated with 54 RXLR effectors. The findings that multiple effectors induced cell death on pepper accessions imply that multiple putative R genes could be present in nonhost pepper. To investigate how many pepper genes are involved in cell death induced by an effector, inheritance study of effector-induced cell death in F2 population was performed. The segregation ratio of cell death induced by effectors was 15:1, 9:7, and 3:1, which means that multiple pepper genes control cell death by single effector. To identify nonhost R gene(s) which recognize multiple core effectors of *P. infestans* and confer durable resistance, a total of 445 NB-LRR genes have been cloned based on pepper genome information. Avrblb2, one of core effectors of *P. infestans*, is critical for virulence of *P. infestans*. It has seven paralogs in *P. infestans* genome and induced cell death on all pepper accessions tested. Using agro-coinfiltration assay, cell death induced by the interaction between pepper NB-LRRs and Avrblb2 effectors was screened and CaNBARC114 showed cell death when

co-expressed with Avrblb2. Transient expression of CaNBARC114 into *N. benthamiana* elevated resistance against *P. infestans* but transgenic potato carrying CaNBARC114 showed no resistance to *P. infestans*. Taken together, this study provides insight into durable NHR based on multiple interactions between RXLR effectors and nonhost factors.

Keywords: *Capsicum annuum*, *P. infestans*, Nonhost resistance, RXLR effectors, Nucleotide-binding site leucine-rich repeat (NB-LRR), Hypersensitive response (HR)

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LIST OF ABBREVIATIONS

NHR	Nonhost resistance
PAMP	Pathogen-associated molecular pattern
NB-LRR	Nucleotide binding site leucine rich repeat
PVX	*Potato Virus X*
Avr	Avirulence factor
GFP	Green fluorescent protein
HR	Hypersensitive response
ETI	Effector-triggered immunity
PTI	PAMP-triggered immunity
RT-PCR	Reverse transcriptase polymerase chain reaction
TOE	Transient overexpression

GENERAL INTRODUCTION

Plants are immobile and are therefore constantly exposed to numerous microbial pathogens. As a result, plants have evolved to defend themselves and have developed an immune system comprised of several components including physical barriers, antimicrobial compounds, pattern recognition receptors (PRRs), and resistance (R) genes (Jones and Dangl, 2006; Dodds and Rathjen, 2010). When plants are challenged with pathogens, PRRs on plasma membranes recognize pathogen-associated molecular patterns (PAMPs) and plants trigger a basal resistance response called PAMP-triggered immunity (PTI). To overcome the defense response induced by PTI, microbial pathogens secrete a set of effectors via the Type III secretion system (T3SS) for bacteria or haustoria for filamentous pathogens (Galán and Collmer, 1999; Kamoun, 2006). Effectors modulate plant physiology and modify host proteins to increase pathogen virulence (Chisholm *et al.*, 2006). Plants also have hundreds of R genes that mainly encode nucleotide-binding and leucine-rich repeat (NB-LRR) proteins (Meyers *et al.*, 1999; Hulbert *et al.*, 2001). The proteins encoded by R genes interact with avirulence (Avr) effector proteins to induce a rapid and strong resistance response called effector-triggered immunity (ETI). An ETI response is typically associated

with the hypersensitive response (HR), which is localized programmed cell death to restrict pathogen growth in plant cells (Jones and Dangl, 2006; Dangl *et al.*, 2013).

Most plant R genes belong to the NB-LRR superfamily. Depending on the N-terminal domain, plant NB-LRR genes can largely be classified into two groups: Toll/interleukin-1 receptor (TIR) – NB-LRRs (TNLs) and coiled-coil (CC)-NB-LRRs (CNLs) (McHale *et al.*, 2006; Lukasik and Takken, 2009). The N-terminal TIR and CC domains are involved in the formation of homo-dimers required to activate defense signaling (Burch-Smith and Dinesh-Kumar, 2007; Takken and Goverse, 2012). Some TIR domains are sufficient to induce cell death upon transient expression (Bernoux *et al.*, 2011). The central NB-ARC domain comprises of three subdomains: NB, ARC1, and ARC2. The ARC domain was named based on its presence in APAF-1 (apoptotic protease-activating factor-1), R proteins, and CED-4 (Caenorhabditis elegans death-4 protein) (van der Biezen and Jones, 1998; van Ooijen *et al.*, 2008). The NB-ARC domain acts as nucleotide-binding pocket and hydrolyzes ATP to induce conformational changes in R proteins (Takken *et al.*, 2006; Takken and Goverse, 2012). Conserved motifs, including the P-loop, RNBSA to D, and MHD (methionine-histidine-aspartate) in NB-ARC domains, play important roles in controlling R gene

activation (Meyers *et al.*, 1999; Bendahmane *et al.*, 2002; van Ooijen *et al.*, 2008). The C-terminal LRR domains function in protein-protein interaction with more variable sequences than N-terminal or NB-ARC domains (Padmanabhan *et al.*, 2009). The LRR domain forms horse-shoe shaped and interacts with NB-ARC domain to maintain the "OFF" state in the absence of pathogen effectors (van Ooijen *et al.*, 2007; Lukasik and Takken, 2009; Takken and Goverse, 2012). Upon pathogen attack, R proteins directly or indirectly interact with effectors and shift into the "ON" state to activate defense signaling.

NB-LRRs in host resistance

Flor hypothesized the mode of action of the R-effector interaction and termed it the "gene-for-gene hypothesis" (Flor, 1971). This hypothesis explains how resistance is triggered by interactions between host resistance gene and cognate avirulence gene of the microbial pathogen. Some evidence supports the hypothesis, including *Pi-ta* and *Rpiblb1* genes. *Pi-ta* is a rice resistance protein against *Magnaporthe oryzae* that directly interacts with AvrPita (Jia *et al.*, 2000). A single amino acid change determined susceptible allele of Pi-ta suggesting R-Avr specificity (Bryan *et al.*, 2000). Furthermore, it is known that *Rpiblb1*, a late blight resistance gene of *Solanum*

bulbocastanum, interacts with IPI-O (Avrblb1) based on yeast two-hybrid screens and co-Immunoprecipitation (co-IP) experiments (Chen *et al.*, 2012).

However, many R genes indirectly recognize effectors. This is called the "guard hypothesis" in which R genes guard host proteins ("guardee") modified by pathogen effectors and activate signal transduction pathways (Dangl and Jones, 2001). *Arabidopsis* RIN4 (RPM1-interacting protein 4) is a well-studied guardee protein targeted by multiple effectors. RPM1 and RPS2 sense RIPK-dependent phosphorylation and cleavage of RIN4 by AvrRpm1 or AvrRpt2 effectors of bacterial pathogen *Pseudomonas syringae*, respectively (Mackey *et al.*, 2002; Mackey *et al.*, 2003; Liu *et al.*, 2011). In addition to AvrRpm1, AvrRpt2, and AvrB, HopF2pto targets RIN4 to promote virulence activity (Wilton *et al.*, 2010). RIN4 negatively regulates PAMP-induced signaling and interacts with H+ATPase to resist pathogen invasion, which implicates RIN4 as a link between PTI and ETI (Kim *et al.*, 2005; Liu *et al.*, 2009). *BSL1*, which encodes putative plant phosphatase, has recently been characterized as a guardee protein against the oomycete pathogen *Phytophthora infestans* and NB-LRR type R2 guards BSL1 (Saunders *et al.*, 2012). R2 and Avr2 interacted with BSL1, and knock-down of BSL1 expression impaired the interaction between R2 and Avr2 effectors.

In addition, the decoy model has been proposed. Decoy means the proteins

specializes in perception of the effector by R protein without function in disease development or resistance (Van der Hoorn and Kamoun, 2008). Recently, the mechanism of several NB-LRR pairs has been studied and it proposes integrated decoy hypothesis. One NB-LRR of NB-LRR pairs detects the presence of specific effectors by direct binding via integrated domains in the NB-LRRs called integrated decoy or sensor domains (Maqbool *et al*., 2015; Cesari *et al*., 2013; César*i et al*., 2014; Williams *et al*., 2014.). PopP2, a cognate effector of *Arabidopsis* RRS1/RPS4 pair, possesses acetyltransferase function and targets host transcription factors such as WRKY to manipulate host defense response (Le Roux *et al*., 2015). Interestingly, WRKY domain integrated into C-terminal of RRS1 has been targeted by PopP2, which disrupts RRS1/RPS4 association and activates RPS4-dependent immunity (Williams *et al*., 2014.; Le Roux *et al*., 2015). The conserved mechanism of NB-LRR pairs across both monocot and dicot plants provides the insight into the nature of plant defense response.

Genome sequencing of *P. syringae* revealed that it has 298 putative virulence genes including approximately 50 T3SS effector genes. It is also known that the *P. infestans* genome has ~550 RXLR effector genes that could be regarded as putative Avr factors (Buell *et al.*, 2003; Haas *et al.*, 2009). It seems reasonable that plants have evolved surveillance systems to monitor

guardee(s) or decoy(s) targeted by multiple effectors rather than individual R-Avr interactions.

NB-LRRs involved in nonhost resistance

Identified host R genes have been deployed to improve plant resistance but the breakdown of resistance has often been observed in the field (McDonald and Linde, 2002). Conversely, nonhost resistance (NHR) has emerged as a source of durable resistance (Ayliffe *et al.*, 2008). NHR is the resistance of an entire plant species to an entire pathogen species (Heath, 2000; Lipka *et al.*, 2008). It is known that several components shared with host resistance are involved in NHR (Fan and Doerner, 2012). Among them, ETI is considered as one of the core components, and the evolutionary concept of NHR explains that the relative contribution of ETI to NHR increases with decreased the evolutionary distance between nonhost and host plants (Schulze-Lefert and Panstruga, 2011).

Until now, only a few NB-LRR type R genes have been identified as nonhost resistance genes. *Rxo1* was isolated from maize, which is a nonhost plant against *Xanthomonas oryzae* pv. *oryzicola* (Xoc) that causes bacterial leaf streak disease in rice (Zhao, BY *et al.*, 2004). Rxo1 recognizes AvrRxo1 of *Xoc* and confers resistance when it is transferred to susceptible rice (Zhao,

B *et al.*, 2004; Zhao *et al.*, 2005). Rxo1 also showed resistance against *Burkholderia andropogonis* which cause bacterial stripe on maize. This implies that a single R gene confers resistance to a nonhost pathogen and an unrelated host pathogen. In addition, White rust resistance 4 (*WRR4*) encodes TNL protein and has been identified from nonhost *Arabidopsis* ecotypes showing variable degrees of resistance using map-based cloning (Borhan *et al.*, 2008). *Brassica napus*, a host plant of white rust (*Albugo candida*) transformed with *WRR4*, and transgenic plants showed resistance against *A. candida*. However, nonhost plants carrying non-functional copy of those R genes still showed resistance against the pathogens (Zhao *et al.*, 2005; Borhan *et al.*, 2010). In the near future, the genome information of nonhost plants and pathogens could provide insight into the mechanisms of NHR.

NB-LRR functions beyond resistance

Several studies indicated that R genes also function as non-immune receptors during signal transduction for plant development. *Arabidopsis ADR1* encodes a CNL protein and conveys broad-spectrum disease resistance (Grant *et al.*, 2003). Enhanced expression of *ADR1* confers drought tolerance, suggesting that there is some overlap between disease resistance and abiotic stress signaling pathways (Chini *et al.*, 2004). *Arabidopsis chilling-sensitive*

2 (*chs2*) mutant exhibited temperature-sensitive growth defects similar to what is observed during defense responses when grown below 16℃ (Huang *et al.*, 2010). A gain-of-function of *RPP4* mutation has been identified using map-based cloning of *chs2* mutant which showed increase of pathogenesis-related (PR) genes expression and accumulation of hydrogen peroxide and salicylic acid (SA) when it was grown at 16℃, which result in chilling sensitivity (Huang *et al.*, 2010). Similarly, an *Arabidopsis chs3* mutant exhibited arrested growth and chlorosis at 16℃ by defense response activation (Yang *et al.*, 2010). *CHS3* was cloned using map-based cloning and was identified as a TNL-LIM type R gene that controls the freezing tolerance phenotype. The observed de-regulation of R genes in a temperature-dependent manner suggests that R genes mediate temperature sensitivity of plant growth.

R genes also control plant morphology. The mutant of *UNI* that encodes an NB-LRR type protein showed early termination of inflorescence stem growth with morphological changes and elevated expression of PR and cytokinin-responsive genes (Uchida *et al.*, 2011). AtTIP49a identified as a factor associated with TATA-binding protein complex is important for sporophyte and female gametophyte viability and serves as a negative regulator of RPP5- and RPP2-mediated resistance (Holt III *et al.*, 2002). Taken together, the

findings suggest a functional link between plant development and R gene-induced defense responses although the degree to which this is a side effect of autoimmunity remains to be fully investigated.

NB-LRR gene family in plant genomes

Over the past two decades, sequencing technologies have been rapidly developed and used to assess plant-microbe interactions. Their use enables genome-wide analyses of NB-LRR genes based on the NB-ARC domain (Meyers *et al.*, 2003; Meyers *et al.*, 2005; Guo *et al.*, 2011). The sequenced genomes of various plant species have revealed hundreds of NB-LRR genes (McHale *et al.*, 2006). A. thaliana, the first plant species sequenced, has 159 NB-LRR genes including 43 CNLs and 83 TNLs (Meyers *et al.*, 2003; Guo *et al.*, 2011). Solanaceae plants carry more than twice the number of NB-LRR genes than *Arabidopsis* and possess more CNLs than TNLs (Guo *et al.*, 2011; Consortium, 2012; Jupe *et al.*, 2012; Kim *et al.*, 2014). However, TNLs have not been identified in cereal crop species, which suggests that TNLs have evolved after the divergence of monocots and dicots (Goff *et al.*, 2002; Meyers *et al.*, 2002; Meyers *et al.*, 2003; McHale *et al.*, 2006). NB-LRR genes belong to a rapidly evolving gene family, and their compositions differ among plant species (Clark *et al.*, 2007; Rafiqi *et al.*, 2009). For instance, 185

NB-LRR genes (21 CNLs and 103 TNLs) were identified in *A. lyrata* and more differences in R gene composition have been observed at the genus level. Recently, R gene enrichment and sequencing (RenSeq) method has been established and enables re-annotation of NB-LRR family genes with identification of novel NB-LRR genes (Jupe *et al.*, 2013; Andolfo *et al.*, 2014). Also, partial type R proteins such as TIR-NB (TN) or TIR-unknown domain (TX) have been identified in plant genomes and the role of those proteins in basal defense has been characterized (Nandety *et al.*, 2013). *Arabidopsis* ecotype Col-0 has 30 TX genes and 21 TN genes and the overexpression of the genes showed various phenotypes associated with basal defense response such as elevated SA level (Nandety *et al.*, 2013). Genome-wide analysis of NB-LRR genes may broaden the scope to understand the mechanism of plant resistance.

Until now, numerous NB-LRR-type R genes have been cloned from a variety of plant species. Most of them have been isolated using map-based cloning approaches, and a few were cloned using transposon tagging or comparative genomics approaches. Corresponding avirulence factor and host interactor proteins have been characterized using genomic libraries and interactor screening such as yeast two hybrid screens or co-IP. With the sequencing technology development, comparative genome-wide analyses of

the NB-LRR family within and between species provide new insight into the evolution of the NB-LRR family and serve as a valuable and rapid means to identify R genes through a genome-assisted R gene identification approach. Combining conventional map-based cloning with genome information could shorten the development of trait-linked markers and identify candidate genes (Jander *et al.*, 2002). Similarly, core effectors that are found in several isolates could be easily predicted using comparative genomics, and high-throughput screening of effector activity could accelerate R gene identification (Cooke *et al.*, 2012). Merging biochemical approaches with genomic data could deepen our understanding of R gene regulatory mechanisms.

This study focused on the understanding of molecular basis of NHR based on ETI using pepper and *P. infestans*. The thesis work is based in two sections and addresses the following topics:

Chapter 1: Multiple Recognition of RXLR Effectors is Associated with Nonhost Resistance of Pepper against *Phytophthora infestans*

Chapter 2: Identification of a Nonhost Resistance Gene of Pepper Recognizing Multiple Core Effectors of *P. infestans* using Genome-based Approach

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CHAPTER 1

Multiple Recognition of RXLR Effectors is Associated with Nonhost Resistance of Pepper against *Phytophthora infestans*

The research described in this chapter has been published in *New Phytologist* DOI: 10.1111/nph.12861 and Figure 1-1b was added.

ABSTRACT

Nonhost resistance (NHR) is a plant immune response to resist most pathogens. The molecular basis of NHR is poorly understood, but recognition of pathogen effectors by immune receptors, a response known as effector-triggered immunity, has been proposed as a component of NHR. Pepper showed a localized cell death response upon inoculation with *P. infestans*, suggesting that recognition of effectors may contribute to NHR in this system. The 54 *P. infestans* RXLR effectors were transiently expressed in pepper accessions using optimized heterologous expression methods. Pepper accessions recognized as many as 36 effectors. Among the effectors, PexRD8 and Avrblb2- induced cell death in a broad range of pepper accessions. Segregation of effector-induced cell death in an F2 population derived from a cross between two pepper accessions fit 15:1, 9:7, or 3:1 ratios, depending on the effector. These genetic data suggest that a single or two independent/complementary dominant genes are involved in the recognition of RXLR effectors. Multiple loci recognizing a series of effectors may underpin NHR of pepper to *P. infestans* and confer resistance durability.

INTRODUCTION

Plants are challenged by numerous pathogens including fungi, bacteria and viruses. However, most plants are resistant to most pathogens and disease is the exception in nature (Huitema *et al*., 2003). This phenomenon is known as nonhost resistance (NHR), an immune response of plant species against all isolates of a microorganism that cause disease on other plant species (Sumit *et al*., 2012). NHR is the most durable and strong resistance form of plants (Heath, 2000; Gurr and Rushton, 2005), and may have the potential to improve crop resistance to pathogens (Fan and Doerner, 2012). However, the molecular mechanism underlying NHR remains to be elucidated, because classical genetic approaches are limited since most nonhost plants cannot be crossed with susceptible host plants (Niks and Marcel, 2009).

The mechanism of NHR comprises of several components, including pre-formed and induced immunity (Thordal-Christensen, 2003). Pre-formed immunity includes physical barrier, for example, cuticular wax, and chemical, antimicrobial compounds. Induced immunity includes pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) (Thordal-Christensen, 2003; Zhao *et al*., 2005; Jones and Dangl, 2006). PAMPs are conserved components of pathogens that are

recognized by pattern recognition receptors at the surface of plant cells, which activate basal defense responses referred collectively as PTI. To suppress PTI, pathogens secrete effectors into plant cells that are specifically recognized by plant resistance (R) proteins leading to ETI (Jones and Dangl, 2006). Defense responses induced by PTI and ETI involve an oxidative burst and transcriptional reprogramming, mediated by signaling machinery (Tsuda and Katagiri, 2010). However, ETI can be distinguished from PTI by the induction of localized programmed cell death in the former, known as the hypersensitive response (HR) (Hammond-Kosack and Jones, 1997; Heath, 2000; Cunnac *et al*., 2009). The relative contribution of ETI and PTI to NHR may relate to the evolutionary divergence time between host and nonhost plants. It was suggested that the relative contribution of ETI increases with decreasing phylogenetic divergence time between two plant species (Schulze-Lefert and Panstruga, 2011).

There is some evidences to show the involvement of ETI in NHR. HopQ1-1, a type III effector of *Pseudomonas syringae* pv. *tomato* elicited HR in nonhost *Nicotiana benthamiana* and a HopQ1-1 deletion strain caused disease in the nonhost plants suggesting a role for ETI in NHR (Wei *et al*., 2007). Similarly, HopAS1 broadly present in *P. syringae* strains triggered NHR in Arabidopsis, whereas pathogenic strains had a truncated form of HopAS1 (Sohn *et al*.,

2012). Resistance genes may also play a role in NHR. *Rxo1* identified from maize encodes R protein that interacts with avrRxo1 and confer resistance against the nonhost pathogen *Xanthomonas oryzae* pv. *oryzicola* which causes bacterial leaf streak in rice (Zhao *et al*., 2005). When *Rxo1* was transferred to susceptible rice, multiplication of the bacteria was inhibited. Another R protein in NHR is WRR4, which was identified from *Arabidopsis* ecotype Col-0 infected with *Albugo candida* using a natural variation in immunity of *Arabidopsis* accessions (Borhan *et al*., 2008). Transfer of *WRR4* to susceptible *Brassica napus* rendered the plant resistant to *A. candida* (Borhan *et al*., 2010). However, nonhost plants carrying a non-functional copy of *WRR4* retained resistance to the pathogen, suggesting polygenic control of NHR (Borhan *et al*., 2008).

Phytophthora infestans, an oomycete pathogen, is one of the most devastating pathogens in the world. It causes potato late blight on Solanaceae crops such as potato and tomato, but not on pepper. Oomycete pathogens secrete effectors into host cells that suppress host defense responses and modify host cell processes (Dodds and Rathjen, 2010; Bozkurt *et al*., 2012). The effectors have conserved RXLR motifs in their N-terminal domains that play an important role in targeting the host cells, and they contain W, Y, and L motifs in their C-terminal domains (Whisson *et al*., 2007; Jiang *et al*., 2008).

The genomic sequence of *P. infestans* reveals approximately 550 genes encoding possible RXLR effectors. Most of these are found in the expanded repetitive DNA-rich regions, suggesting that they have rapidly evolved (Haas *et al*., 2009; Win *et al*., 2012). Using genomic information, a collection of non-redundant RXLR effectors of *P. infestans* has been cloned into *Potato virus X* (PVX)-based transient expression vectors (Torto *et al*., 2003; Vleeshouwers *et al*., 2008; Oh *et al*., 2009). High-throughput screening systems, such as PVX agro-infection, have been optimized in Solanaceae plants, allowing rapid identification of cognate R genes that interact with RXLR effectors (Takken *et al*., 2000; Torto *et al*., 2003; Vleeshouwers *et al*., 2006). Two of these genes are Rpi-blb1 which interacts with Avrblb1 (Vleeshouwers *et al*., 2008), and Rpi-blb2 which recognizes Avrblb2 (Oh *et al*., 2009). This genomic information and the development of experimental methods that enable effector-omics analyses of plant-microbe interactions, provide a promising approach for studying nonhost interaction.

Here, we hypothesized that multiple interactions between nonhost plant factors and pathogen effectors establish durable NHR. To test this hypothesis, we optimized *in planta* transient expression methods to screen interactions between pepper accessions and RXLR effectors of *P. infestans*. Using the results from the screening, we performed genetic analyses on an F2

population to investigate how plant factor(s) control effector-induced cell death. From the inheritance study, we conclude that multiple interaction between plant factor(s) and effectors of *P. infestans*, could be one of the determinants of the durable nature of NHR in pepper against *P. infestans*.

MATERIALS AND METHODS

Plant materials and growth conditions

To screen nonhost interactions between pepper and RXLR effectors of *P. infestans*, a total of 100 pepper accessions from various regions were kindly provided by Dr. Byoung-Cheorl Kang (Seoul National University, Korea). Seeds of the pepper accessions were sterilized with 0.1% sodium hypochlorite (NaOCl). Seven days after germination at 30°C, all the seedlings were transferred to controlled chambers with a 16 h light and 8 h dark period at room temperature until cotyledons were fully expanded. Four plants from each accession were transplanted into a 32-plug form tray filled with horticultural bed soil composed of cocopeat (65%-70%), peat moss (8%-12%), and vermiculite (10%-14%) according to the manufacturer's information (Baroker, Seoul Bio Co., Ltd., Seoul, Korea).

P. infestans spore infection

For *P. infestans* inoculation assays, 4-week-old *Capsicum annuum* cv. CM334 (nonhost), *Solanum lycopersicum* cv. Heinz (susceptible host) and *Solanum tuberosum* cv. Daeji (susceptible host) were used. The *P. infestans* isolates 88069, 40707, 40718, 43071, and 43072 provided by the RDA Gene

Bank of Korea were used. All isolates were grown on rye sucrose agar media as previously described at 17°C in the dark for 10 days (Kamoun *et al*., 1998). To release the zoospores from sporangia, the plates were flooded with autoclaved distilled cold water, gently rubbed with a sterile cell scraper, and incubated at 4°C for 1.5 h. The zoospores were counted under a hemocytometer, and the concentration was adjusted to 5×10^4 zoospores/ml. The detached leaves of pepper, potato and tomato were placed in the rectangle plate overlaid with a wet paper towel to maintain high humidity. The 10 μl droplets of zoospores were applied onto the detached leaves and incubated at 17°C. The inoculated leaves were sampled at 0, 24, 48 and 72 h post-inoculation (hpi).

Trypan blue staining

Upon inoculation of *P. infestans*, leaf discs containing zoospore droplets were excised at 5 days post inoculation (dpi) and stained with lactophenol-trypan blue staining (10 ml lactic acid, 10 ml glycerol, 9.3 ml phenol, 10 ml distilled water, and 20 mg trypan Blue) under vacuum as described (Yeom *et al*., 2012). The leaf discs were boiled in the lactophenol-trypan solution, incubated overnight in staining solution, and cleared with chloral hydrate (2.5 g/ml). The cleared leaf discs were mounted in 50% glycerol solution and

observed using stereo microscopy (Dimis-M, Siwon Optical Technology Co., Ltd., Korea) and differential interference contrast microscopy.

Reverse transcription-PCR

Total RNA from *P. infestans*-inoculated leaves was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA) and 3 μg of total RNA were reverse-transcribed using Superscript II (Invitrogen, Carlsbad, CA). To determine *in planta* effector expression, PCR was performed using gene-specific primers (10 pmol/μl, listed in Table 1-2). The following cycling conditions were used : 1 cycle of 94°C for 3 m; 30 cycles (or 35 cycle for pepper) of 94°C for 20 s, 58°C for 20 s and 72°C for 45 s. The actin gene (designated ActA) for the pathogen was used as control for monitoring transcript levels (Bos *et al*., 2010). The PCR product were detected by gel electrophoresis on 2% agarose gel.

PexRD effectors of *P. infestans*

All effectors were cloned into a binary Potato Virus X-based pGR106 vector in *A. tumefaciens* strain GV3101 (Holsters *et al*., 1980; Jones *et al*., 1999) for *in planta Agrobacterium*-mediated transient expression. Both

pGR106-dGFP and pGR106-PexRD2 were used as negative and positive controls, respectively (Table 1-1).

PVX agro-infection

Recombinant *Agrobacterium* carrying pGR106-PexRD effectors was incubated at 28°C on a YEP agar plate containing kanamycin (50 mg/l) and rifampicin (50 mg/l) for 2 days. Using toothpicks, the *Agrobacterium* was inoculated by piercing 5-week-old pepper leaves. Duplicate experiments were performed twice on different days. Cell death induced by effectors was monitored at 14 dpi.

***In planta* expression using recombinant PVX virions**

Agrobacterium carrying PexRD effector genes in binary vector pGR106 were cultured overnight at 28°C in YEP liquid media with antibiotics as described above and resuspended in infiltration buffer (10 mM MgCl2, 10 mM MES, and 150 μM acetosyringone). The culture was diluted to a final OD600 of 0.5 and incubated with gentle shaking at room temperature for 3 h, then infiltrated into the expanded leaves of *N. benthamiana* with a 1-ml disposable syringe without a needle. At 7 dpi, 1 g upper leaves that emerged after systemic infection of PVX were collected and ground in 5 ml of 0.05 M

potassium phosphate buffer (pH 7.4) using a pestle and mortar to make the sap inoculum carrying recombinant PVX virions. The inoculum was rubbed onto the leaves of 4-week-old pepper plants using carborundum 400 mesh. Each virion was applied to two leaves of each plant to prevent virions to be mixed. Then, distilled water was applied onto the inoculated leaves to wash out the carborundum. Cell death on the inoculated leaves was monitored up to 7 dpi, and necrotic cell death was observed by destaining chlorophyll with 100% ethanol.

Table 1-1. Description of the tested PexRD genes

Effector Names	PITG Gene ID	Number of Homologs Tested	RXLR	dEER	Average Number of Accessions With Cell Death
PexRD1	PITG_15287	1	RQLR	EDGEER	0
PexRD2	PITG_21422, PITG_11350, PITG_11383, PITG_11384, PITG_22935	1	RLLR	ENDDDSEAR	43
PexRD3	PITG_09160	1	RFLR	EGDNEER	13
PexRD4	NA	1	RFLR	DEER	2
PexRD6, ipiO, Avrblb1	PITG_21388	3	RSLR	DEER	5
PexRD7, Avr3a	PITG_14371	2	RLLR	EENEETSEER	7
Pex147-2, Avr3a paralog		1	RLLR	EESEETSEER	6
Pex147-3, Avr3a paralog		1	RFLR	EENEETSEER	3
PexRD8	PITG_14739, PITG	1	RLLR	DDDDEEER	22
PexRD10	PITG_23206	1	RKLR	EER	4
PexRD11	PITG_23206	2	RLLR	DEGELTEER	3
PexRD12	PITG_16233, PITG_16240	2	RSLR	DSDDGEER	7
PexRD13	PITG_08812	2	RQLR		7
PexRD14	PITG_14961, PITG_14962, PITG_14954, PITG_14959	2	RLLR	ETGNQEER	6
PexRD16	PITG_06087	2	RSLR	EER	4
PexRD17	PITG_08599	2	RVLR	EIEAETER	1
PexRD21	PITG_13452	1	RLLR	EREVQEER	2
PexRD22	PITG_13306	2	RFLR	EDASDEER	4
PexRD24	PITG_04314	2	RSLR	ETSEDEEER	8
PexRD26	PITG_07947	2	RVLR	DEER	1
PexRD27	PITG_13628	1	RLLR	DSEER	0
PexRD28	PITG_03192	1	RSLR	ETSEDEEER	1
PexRD31	PITG_16402	1	RSLR	EDQEGDEER	2
PexRD36	PITG_23132	2	RHLR	DDEER	3
PexRD39/40, Avrblb2	PITG_20300	3	RSLR		19
PexRD41	PITG_04089	3	RSLR		3
PexRD44	PITG_17063	1	RFLR	QEEGVFEER	2
PexRD45	PITG_09632	2	RSLR		2
PexRD46	PITG_18685	3	RSLR		3
PexRD49	PITG_05750	1	RLLR	EEER	3
PexRD50	PITG_06099	2	RLLR		2
PexRD51		2	RFLR	EER	0

*Modified from Oh *et al*., 2009.

RESULTS

Nonhost resistance of pepper against *P. infestans* is associated with hypersensitive cell death

To investigate how nonhost pepper plants respond to *P. infestans* and characterize its nonhost defense response, the detached leaves of pepper, tomato and potato plants were inoculated with a zoospore suspension of *P. infestans* isolate 88069 (5×10^4 spores/ml). The interaction with *P. infestans* was cytologically examined using UV light and trypan blue staining to visualize dead cells (Fig. 1-1a). HR cell death appeared at 6 hpi which suggest that *P. infestans* induce rapidly resistance response (Fig.1-1b). Five days after inoculation, dead cells at the infection site were readily shown by their brown color under white light and autofluoresced on a red background under UV light. The germinated zoospores of *P. infestans* were shown and germ tubes were elongated on pepper leaves (Fig. 1-1a, upper right). The HR on penetrated epidermal cells was apparent on pepper leaves and remained limited to those cells (Fig. 1-1a, upper right). In contrast to the responses in pepper, the biotrophic growth of *P. infestans* was apparent 5 days after inoculation in tomato and potato leaves and hyphae with sporangia, necrotic death of mesophyll cells spread throughout the leaves (Fig. 1-1a, lower right

panel).

NHR is defined as resistance displayed by all accessions of a plant species against all strains of a pathogen. To further examine the pepper - *P. infestans* interaction, we tested five accessions of pepper with four isolates of *P. infestans* (40707, 40718, 43071, and 43072) collected from different regions of Korea (Fig. 1-2). All accessions displayed localized cell death typical of the HR to all four isolates. The HR was limited to penetrated epidermal cells in all accessions inoculated with three of the isolates, whereas in pepper infected with isolate 40707, the induced HR was not completely confined to the infected cells and extended to adjacent cells. The finding that NHR of pepper to *P. infestans* is associated with the HR suggests the possible presence of pepper R genes that interact with *P. infestans* effectors.

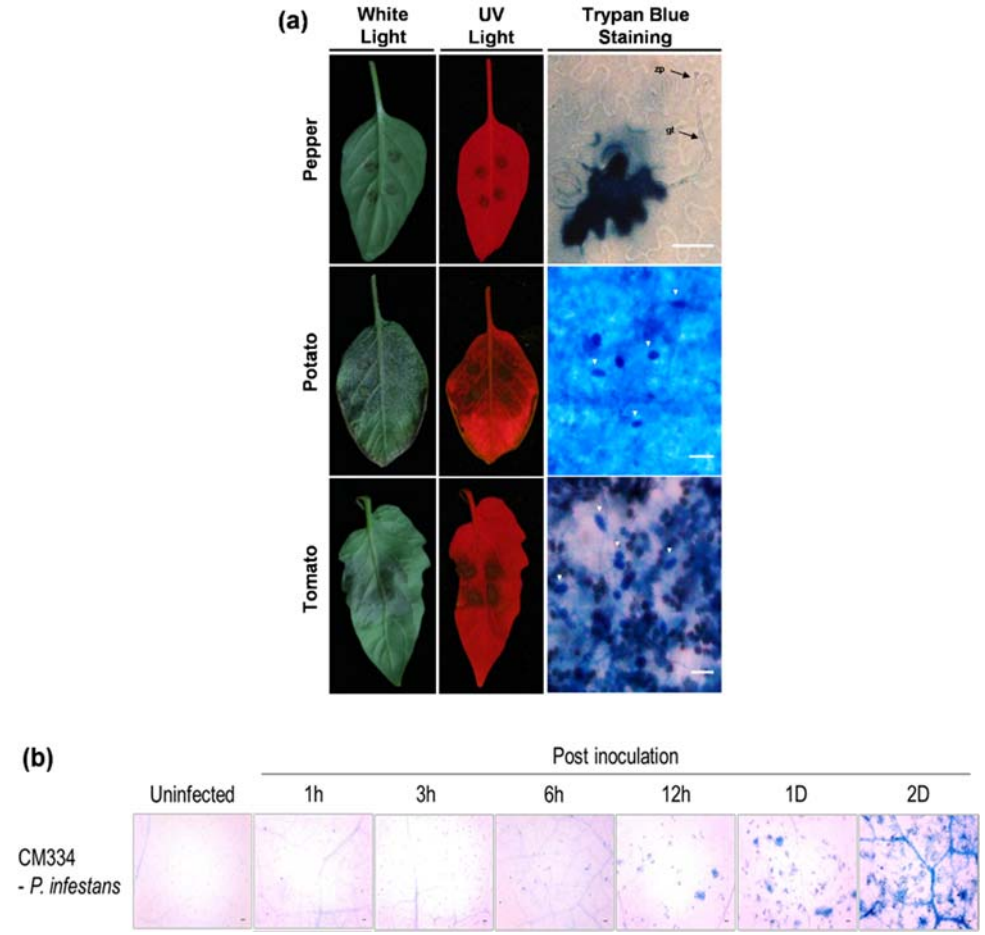


Figure 1-1. Nonhost response of pepper against *P. infestans* is associated with hypersensitive cell death.

(a) Detached leaves of nonhost pepper landrace CM334 and host plants (potato cv. Daeji and tomato cv. Heinz) were infected with a zoospore suspension of *P. infestans* 88069 (5×10^4 / ml). Leaf pieces containing zoospore droplet were excised at 5 dpi, examined for hypersensitive cell death and sporangia development under UV illumination, and visualized by trypan blue staining. White arrows indicate sporangia formation on potato and tomato leaves. zp : zoospore; gt germ tube.

(b) Leaf spots inoculated with *P. infestans* were harvested at 1, 3, 6, 12, 24 (1D), and 48 hpi (2D). Harvested leaf samples were stained by trypan blue and visible cell death response appeared at 6 hpi.

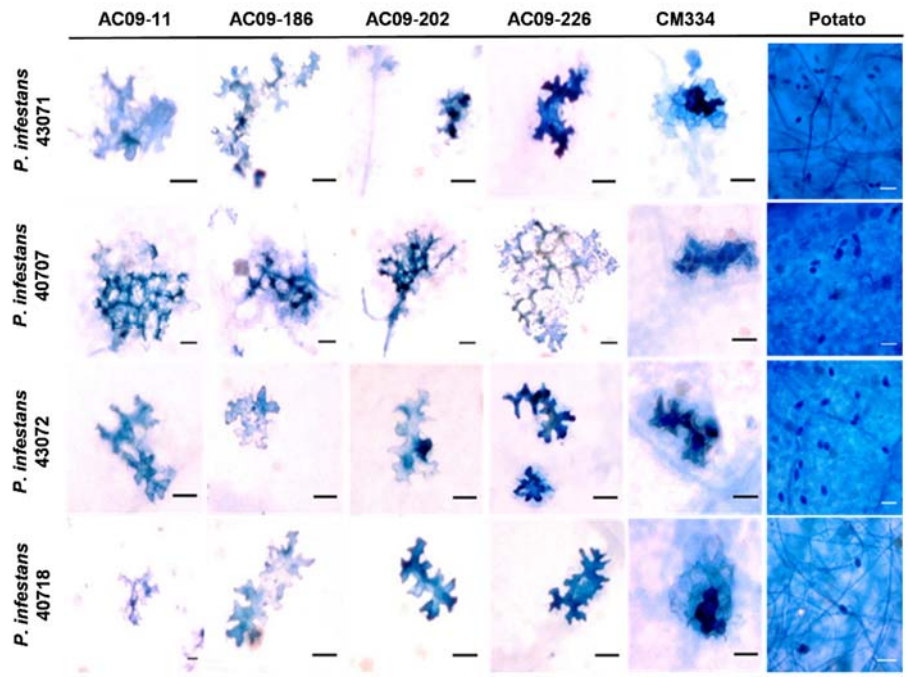


Figure 1-2. Hypersensitive cell death was confirmed by interactions between several pepper accessions and four isolates of *P. infestans* 40707, 40718, 43071, and 43072.

The detached leaves of five pepper accessions (AC09-11, AC09-186, AC09-202, AC09-226, and CM334) were inoculated with four isolates of *P. infestans* and stained with trypan blue. Compared to susceptible control potato, pepper accessions showed cell death response restricted to epidermal cells. Scale bar represents 50 μm.

Nonhost pepper shares expression profiles of the effector genes of *P. infestans* with host plants

To determine whether recognition of *P. infestans* effectors is relevant to nonhost resistance in pepper, we first examined whether effector genes are expressed during the interaction. Expression of the RXLR effectors used in this study was detected in pepper and potato plants infected with *P. infestans* using gene-specific primers (Fig. 1-3; Table 1-2). The Kazal-like serine protease inhibitor EPI1 is an *in planta*-induced gene that may be involved in suppression of PTI (Tian *et al*., 2005; Attard *et al*., 2008). EPI1 was expressed in *P. infestans*-infected pepper, and the expression of other RXLR effector genes in nonhost pepper was similar to that in host potato, which suggests that the expression of *P. infestans* effectors in nonhost pepper is induced with those in host plants in a similar pattern. PexRD2, PexRD8, and PexRD11 showed no detectable expression until 3 dpi. The expression of Avrblb2 was induced during early infection of pepper by *P. infestans* similar to the expression observed during potato infection.

In conclusion, the expression of several *P. infestans* effector genes during interactions with pepper indicates that these effectors could be targeted by the pepper immune response.

Table 1-2. The list of primers used in this study

Effector Name	Forward (5'->3')	Reverse (5'->3')	Ref.
PiActA	CATCAAGGAGAAG CTGACGTACA	GACGACTCGGCGG CAG	Bos *et al*., 2010. PNAS
PiEPi1	CATGCTCAAAGCC CGCAAGTCATCA	TTATCCCTCCTGCG GTGTCACCTT	AY586273
PexRD1	GCTACAGAAAATA CAGACCCTGTTA	GTATTTAATGTATT TCCCCCAATTC	GQ869413
PexRD2	AGCTGGGATGACT GTTGACGACT	TCAGCCACATAGTT GAGGTACCGC	GQ869414
PexRD3	AGTATTCAAGACG AAGTCTTTATCG	ATACTTGACAGTTT TCAACCTT	GQ869415
PexRD6	GTTTTCTATCTCAA ACTCTGTGGAA	TTATCGTACCAGTT TTTGAATCTGT	GQ869419
Avr3a	CTAAATGAGGAGA TGTTTAATGTGG	AGGTGCATCATGT AGCTATTGTAGA	GQ869420
PexRD8	GCGAATGCTGCCA ACCTCTTCAA	GTAGTACCAGCTG CGGTACAGCA	GQ869424
PexRD11	AGGCTTACTGGAT AAGAAATAGTGTG	CTATTTGTACCCCT GTCCCTTT	GQ869426
PexRD12	GACTGCTTAATGGT ATGACAGATTT	ACCACTTCTTCGAC TTCTTAATGTA	GQ869428
PexRD13	CTTCGAGAACCAG CATCAGT	GATCGCTCTAGCTT CTTTCAATG	GQ869430
PexRD14	CAACCAAGAAGAA AGGACAATTAAC	CTGTAAACAGAAA TTCATCGCTTT	GQ869432
PexRD16	GATGAACAACAAC CAGGAATTT	AGAAGGAGTAGTA GCCGAGGTATAG	GQ869434
PexRD17	ATACGTCAAGAAA CACTGCTCTATC	AAGTCTAGATAGA AGTGACCAACGA	GQ869436
PexRD21	ACCACTACCTAGC	TATCTCCAGCTTCT	GQ869438

	ATCGTCAATA	GCCTTTAAC	
PexRD22	AAACTCAGTCCAG	ATTTTAAGCTTGAA	GQ869439
	ACCAAGACAT	GATTTTCTCCT	
PexRD24	GTTATATTCGTGAA	TACGAGTTGGTTTT	GQ869441
	AAGCTCAAAAA	GTAGATACGAG	
PexRD26	GTACAGATGAATC	GGATACTTTTTCTG	GQ869443
	TTGACAACGTC	ATAGGCAGTAAC	
PexRD27	GCTACCAGATATA	AAGCCTTCTTCTTG	GQ869445
	GAAACGTTCAAG	CTTACATTATC	
PexRD31	AATATTATGTACCC	TGTTAAAAAGGTA	GQ869447
	AGTTCTCGATG	CTTGATCCAATC	
PexRD36	ACTGGTCTTAAAG	CTTTTGAGCATTTT	GQ869448
	GAACAAGAAGAT	GTTGATAGTTT	
Avrblb2	GGAAAAACGTAGT	GATACTTCGCTCAA	GQ869461
	CCAGAGTACTAAC	CCTTTTACAG	
PexRD41	GCTCTTATCAGACA	AATCAGTTTGCTCA	GQ869463
	CTTTTACCAAA	TGTCTTTTTC	
PexRD44	CTAAACATACCAA	ACTGTTCATCATTC	GQ869466
	GCGATTTCTG	TAACACGAAAG	
PexRD49	GAATGATCAAAAA	AGTCTTGATCTTGG	GQ869472
	GGGAATTTCT	ACTCGTAAAA	
PexRD50	GAAGACTCCAAGA	CGTAGTAGTCGAC	GQ869474
	ACGTGAAAC	ATAACCTGTGTA	
INF1	ACACGTCGTTTAAT	GTACGAGTACACG	U50844
	CAGTGCTC	TTGAGTACCAG	

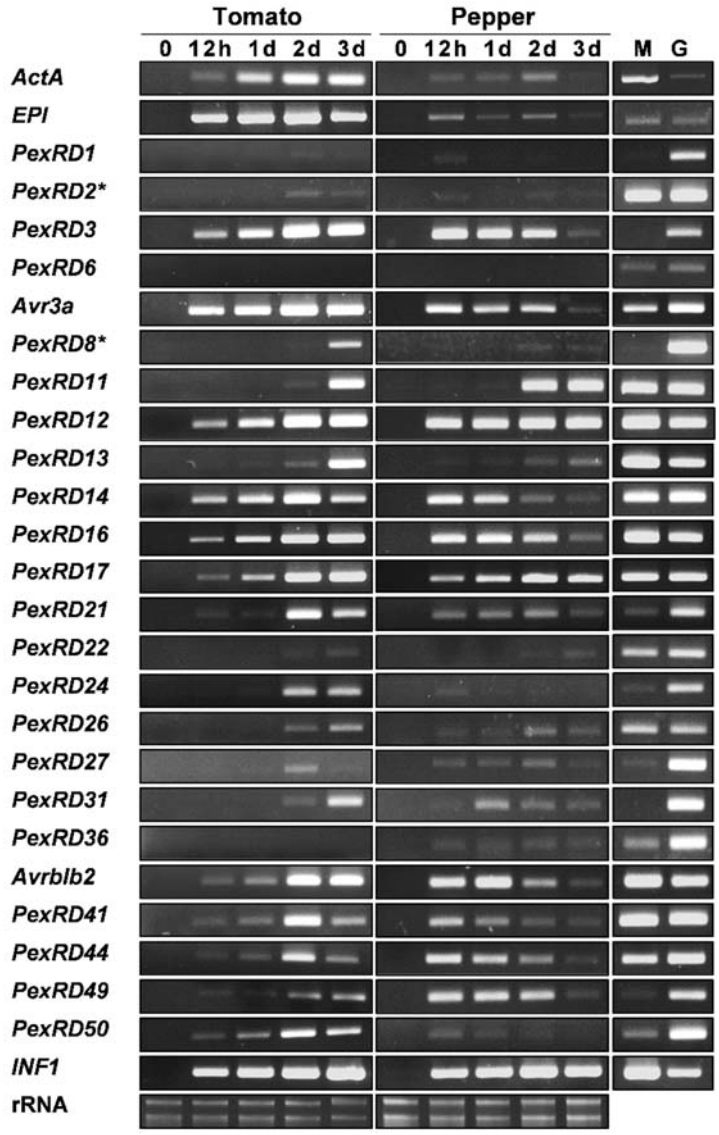


Figure 1-3. Nonhost pepper shares expression profiles of the effector genes of *P. infestans* with host plants.

Detached leaves of pepper and potato upon inoculation with *P. infestans* isolate 88069 were harvested at 0, 1, 2, and 3 dpi (t0, t1, t2, and t3,

respectively). Total RNA was extracted from the harvested samples. Reverse transcription-PCR was performed to observe *in planta* expression of PexRD effector genes. Constitutive *ActA* and *in planta*-induced *EPI* were used as controls. This experiment was repeated twice with similar results. M, *P. infestans* mycelium RNA; G, *P. infestans* genomic DNA; H2O, water.

Optimization of a heterologous effector expression system in pepper

Effector-omics or high-throughput approaches for the rapid assignment of activities to effector genes require effective heterologous expression systems. To establish such a system in pepper, we applied a PVX agro-infection method for the *in planta* expression of effectors in pepper, as depicted in Figure 1-4a. This method is well suited for high-throughput screening of effector activity in Solanaceae plants. To confirm the suitability of this approach in pepper, four elicitors known to induce the necrotic response in Solanaceae plants, specifically *INF1* (Kamoun *et al*., 1998), *PiNPP1* (Kanneganti *et al*., 2006), *CRN2* (Torto *et al*., 2003), and *PexRD2* (Oh *et al*., 2009), were wound-inoculated on leaves of 4-week-old pepper plants. *Agrobacterium* strain GV3101 carrying those elicitors and negative control dGFP were inoculated onto both sides of the mid-vein of fully expanded pepper leaves using toothpicks. Among the four different elicitors, only PexRD2 caused cell death on pepper leaves at 14 dpi (Fig. 1-4). Considering the induced cell death by PexRD2, the PVX agro-infection method is as applicable in pepper as it is in other Solanaceae plants.

Figure 1-4. Optimization of a heterologous effector expression system in pepper.

(a) The PVX agro-infection method is depicted. *Agrobacterium* carrying pGR106-RXLR effectors was wound-inoculated onto pepper leaves.

(b) PexRD2 and elicitins (INF1, NPP1, and CRN2) which are known to induce cell death were inoculated on both sides of mid veins in pepper leaves. PexRD2 but not elicitins induced cell death on inoculated pepper leaves at 14 dpi.

Pepper accessions respond to a diversity of *P. infestans* effectors

To perform a primary screen of the nonhost interaction, we inoculated the infection-ready collection of 54 *P. infestans* RXLR effectors onto the leaves of 100 pepper accessions using the PVX agro-infection method (Fig. 1-5). The effectors are putative secreted effectors from expressed-sequence tag data of *P. infestans* and have been cloned into the binary PVX vector pGR106 for high-throughput screening of effector activities (Randall *et al*., 2005; Oh *et al*., 2009). Among 54 effectors, nine effector families including PexRD6, PexRD7, PexRD16, PexRD24, Avrblb2, PexRD41, PexRD44, PexRD49, and PexRD50 were recently determined as core effectors using criteria that measure induction of expression during infection and presence in the genome of three *P. infestans* isolates (Cooke *et al*., 2012). The pepper accessions originating from various areas including the United States, China, and Japan were selected for screening of nonhost interactions. Most of them belong to *Capsicum annuum* L. with a few accessions of *C. chinense* (Table 1-3). After inoculation of RXLR effectors in duplicate, effector-induced cell death was monitored at 14 dpi. *Agrobacterium* carrying pGR106-dGFP and pGR106-PexRD2 were used as negative and positive controls, respectively. Considering the relatively low efficiency of *Agrobacterium*-mediated transient expression on pepper, we judged “cell death” when either one of the

duplicate inoculation sites showed cell death. Cell death confirmed twice was marked as "+" (Tables 1-3 and 1-4).

Among 100 pepper accessions, 55 showed no cell death response by inoculation of *Agrobacterium*-carrying the positive control (PexRD2), which may indicate incompatibility of these accessions with *Agrobacterium* and low growth of *Agrobacterium* resulting in insufficient expression of effectors. Three accessions (AC09-15, AC09-20, and R09-135) showed the necrotic cell-death response triggered by the negative control pGR106-dGFP, as evidenced by susceptible response to PVX developing systemic mosaic symptoms and leaf crinkling at 14 dpi (Table 1-3). Those three accessions were excluded for further screening. From the screening results of the other 42 accessions showing the HR against positive control PexRD2, each accession variously responded to at least one effector, showing a distributed cell death response among pepper accessions. The average number of effectors inducing cell death on each pepper accessions was seven and many accessions reacted to multiple effectors. Each pepper accession showed cell death by *in planta* expression of 1 to 36 effectors, respectively. These results support the hypothesis that there are multiple recognitions in nonhost interactions between pepper and effectors of *P. infestans* (Table 1-4).

The RXLR effectors that interacted with pepper accessions could be

divided into two groups: one, broadly recognized, and another, specifically recognized effectors. The effectors recognized by a broad variety of pepper accessions included PexRD8 and PexRD39/40, known as the Avrblb2 family (Table 1-1; Fig. 1-6). More than one-half of the tested accessions showed the HR to those effectors. The specifically recognized effectors showed various responses, depending on the pepper accession. Generally, homologous effectors induced cell death on the same pepper accessions. For example, PexRD12-1 and PexRD12-2 exhibited 99% nucleotide sequence similarity and both triggered cell death on pepper accessions AC09-12, R09-120, AC09-202, and AC09-52. Furthermore, PexRD24-1 and PexRD24-2, sharing 94% identity, elicited cell death on pepper accessions AC09-72, AC09-73, and AC09-10. Those accessions could be postulated to have cell death-inducing factors that interact with an effector family. Taken together, these results revealed that nonhost pepper plants show cell death by interactions with multiple RXLR effectors of *P. infestans*.

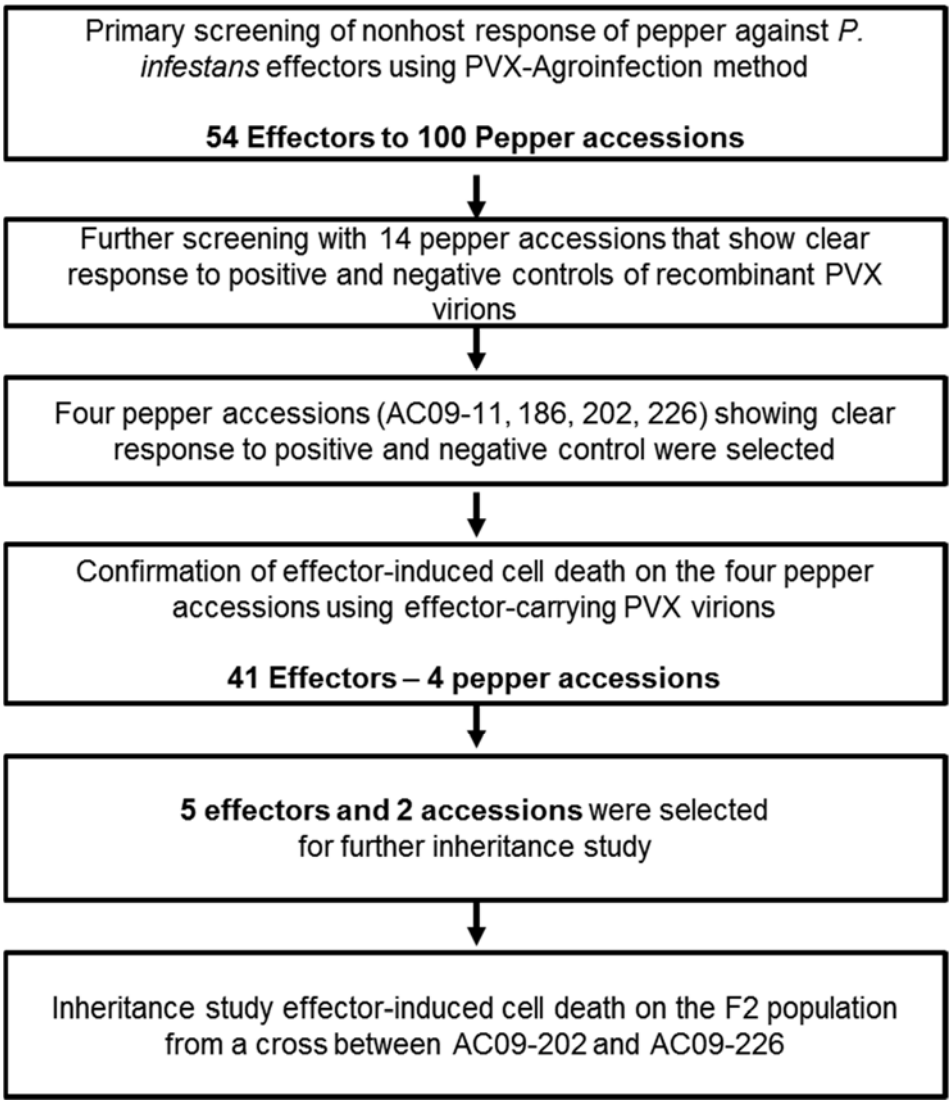


Figure 1-5. Work-flow of this study.

Table 1-3. Cell death induced by RXLR effectors of *P. infestans* in pepper accessions

Origin	Species	Cultivar name	Accession ID	GFP	Pex RD2
No cell death by PexRD2					
USA	*C. annuum*	Filus blue	07A-003	-	-
Thailand	*C. annuum*	CHINDA2	07A-216	-	-
Italy	*C. annuum*	CO 0725	07A-204	-	-
Japan	*C. annuum*	Ohyatsubua	AC09-13	-	-
USA	*C. annuum*	Starburst	07A-014	-	-
Unknown	*C. annuum*	Paul smith & quot	07A-222	-	-
Unknown	*C. annuum*	PBC646/PBC378*5	07A-218	-	-
Ethiopia	*C. annuum*	Unknown	AC09-63	-	-
Guatemala	*C. annuum*	Unknown	AC09-54	-	-
Ethiopia	*C. annuum*	Unknown	AC09-62	-	-
Unknown	*C. annuum*	Ikom II	AC09-55	-	-
Ethiopia	*C. annuum*	Unknown	AC09-71	-	-
Yemen	*C. chinense*	BISBAS	AC09-183	-	-
Yemen	*C. chinense*	BISBAS	AC09-184	-	-
Yemen	*C. chinense*	BISBAS ROTFRUECHTIG	AC09-186	-	-
Yemen	*C. annuum*	BISBAS	AC09-187	-	-
USA	*C. annuum*	Numex Bailey Piquin	AC09-219	-	-
USA	*C. annuum*	Black cuban	AC09-228	-	-
USA	*C. chinense*	Numex Suave Red	AC09-230	-	-
USA	*C. annuum*	Numex Halloween	AC09-223	-	-
USA	*C. annuum*	Numex Thanksgiving	AC09-221	-	-
Mexico	*C. annuum*	CHILI SERRANO	07A-207	-	-
Russia	*C. annuum*	Karkovskij	07A-203	-	-
Japan	*C. annuum*	Nagayatsubusa	AC09-09	-	-
China	*C. annuum*	Unknown	AC09-21	-	-
China	*C. annuum*	Unknown	AC09-22	-	-
China	*C. annuum*	Unknown	AC09-32	-	-

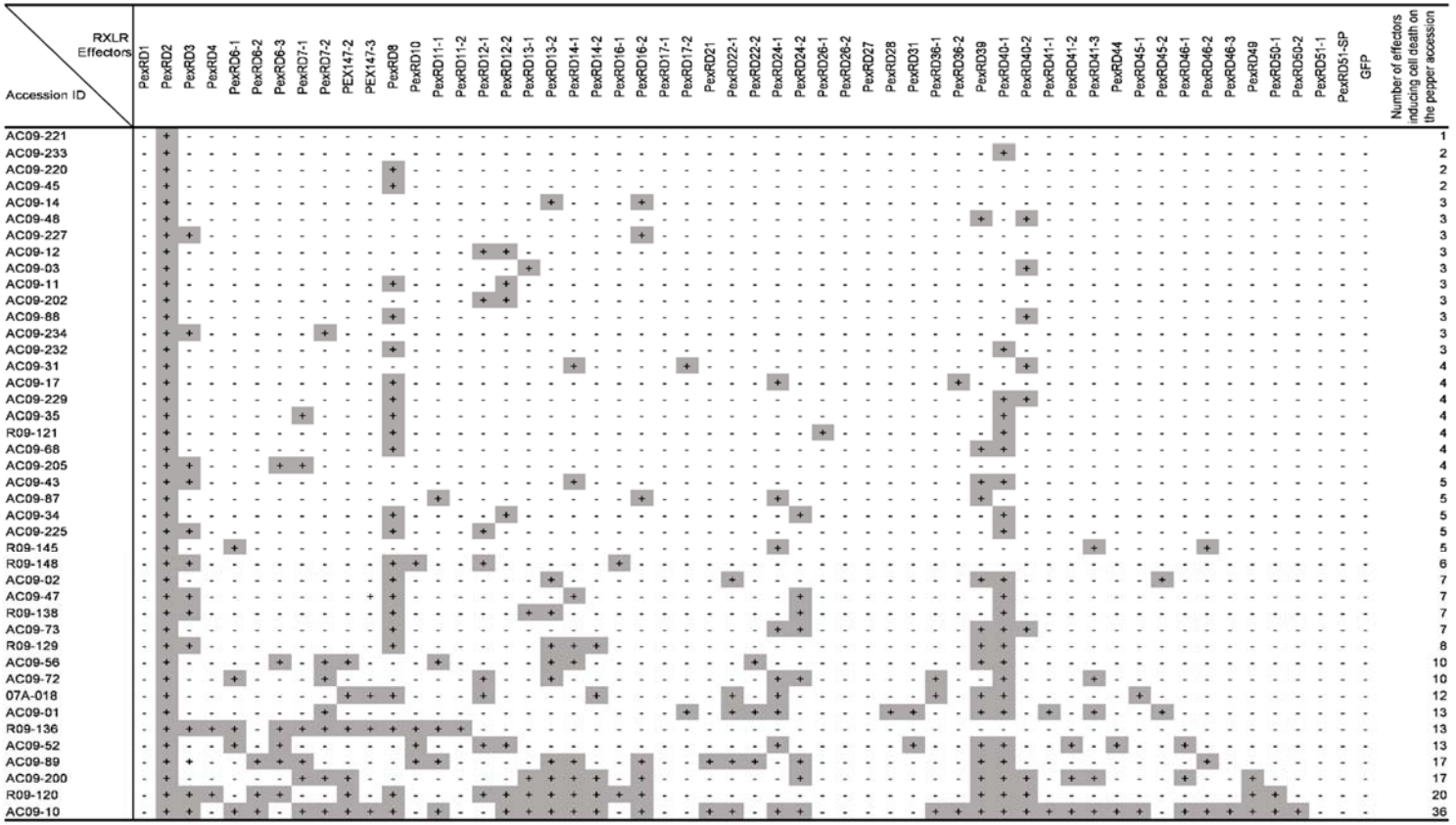
Honduras	*C. annuum*	Unknown	AC09-59	-	-
China	*C. annuum*	Five Colour Pepper	AC09-50	-	-
USA	*C. annuum*	Takanotsume	AC09-199	-	-
USA	*C. annuum*	Numex Conquistador	AC09-218	-	-
USA	*C. annuum*	Serrano	AC09-226	-	-
Unknown	*C. annuum*	98HES106 PBC30-4	R09-111	-	-
Unknown	*C. annuum*	98HES117 B4006-1	R09-112	-	-
Unknown	*C. annuum*	98 misang 8	R09-113	-	-
Unknown	*C. annuum*	Hot Pepper AL	R09-114	-	-
Unknown	*C. annuum*	Chilly Long	R09-116	-	-
Unknown	*C. annuum*	Hot Pepper Orissa local-3	R09-117	-	-
Unknown	*C. annuum*	SVS Banglaor	R09-118	-	-
Unknown	*C. annuum*	AnKur-228	R09-119	-	-
Unknown	*C. annuum*	ChiangRai VM	R09-123	-	-
Unknown	*C. annuum*	PBC59 Bhaskar	R09-124	-	-
Unknown	*C. annuum*	PBC134 LCA-305	R09-125	-	-
Unknown	*C. annuum*	PBC141 X-235	R09-126	-	-
Unknown	*C. annuum*	PBC157 HuaySithon	R09-127	-	-
Unknown	*C. annuum*	PBC455	R09-128	-	-
Unknown	*C. annuum*	PBC580 Chillies Giants	R09-131	-	-
Unknown	*C. annuum*	PBC585	R09-132	-	-
Unknown	*C. annuum*	PBC586	R09-133	-	-
Unknown	*C. annuum*	PBC636 Galkunda Miris 01146	R09-134	-	-
Unknown	*C. annuum*	TW99	R09-139	-	-
Unknown	*C. annuum*	G4	R09-140	-	-
Unknown	*C. annuum*	Unknown	R09-147	-	-
Unknown	*C. annuum*	Unknown	R09-143	-	-
Unknown	*C. annuum*	Unknown	R09-144	-	-
Cell death induced by negative control					
China	*C. annuum*	Unknown	AC09-20	+	+
Japan	*C. annuum*	Shishi	AC09-15	+	+
Unknown	*C. annuum*	99Gonmyung1	R09-135	+	+

Positive cell death response by PexRD2					
USA	*C. annuum*	5502	07A-018	-	+
China	*C. annuum*	Su-Tsu	AC09-01	-	+
Japan	*C. annuum*	Fushhimikara	AC09-02	-	+
Japan	*C. annuum*	Goshiki	AC09-03	-	+
Japan	*C. annuum*	Nikko	AC09-10	-	+
Slovakia	*C. annuum*	Nitrianska Krajova	AC09-11	-	+
Slovakia	*C. annuum*	Nitrianska Tenkosteena	AC09-12	-	+
Japan	*C. annuum*	Sapporofuto	AC09-14	-	+
Austria	*C. annuum*	Wiener Calvill	AC09-17	-	+
China	*C. annuum*	Da-Chun-Pao	AC09-31	-	+
Argentina	*C. annuum*	Unknown	AC09-34	-	+
Argentina	*C. annuum*	Unknown	AC09-35	-	+
USA	*C. annuum*	Unknown	AC09-43	-	+
Mexico	*C. annuum*	Unknown	AC09-45	-	+
USA	*C. annuum*	Keystone wonder Giant	AC09-47	-	+
USA	*C. annuum*	Keystone resistant	AC09-48	-	+
Unknown	*C. annuum*	Cecei	AC09-52	-	+
China	*C. annuum*	Siau-Fung-Tsong	AC09-56	-	+
Yugoslavia	*C. annuum*	HS - X - 3	AC09-68	-	+
Geogia	*C. annuum*	Kutansuri	AC09-72	-	+
Ukaraine	*C. annuum*	Krupnyj Zeltyj 903	AC09-73	-	+
North Korea	*C. annuum*	Sok Hu	AC09-87	-	+
North Korea	*C. annuum*	Sopul	AC09-88	-	+
North Korea	*C. annuum*	Nam Cju	AC09-89	-	+
USA	*C. annuum*	Santaka	AC09-200	-	+
USA	*C. annuum*	Numex Big Jim	AC09-202	-	+
USA	*C. annuum*	Numex 6-4	AC09-205	-	+
USA	*C. annuum*	Numex Christmas	AC09-220	-	+
USA	*C. annuum*	Numex Thanksgiving	AC09-221	-	+
USA	*C. annuum*	Numex Sunglo	AC09-225	-	+
USA	*C. annuum*	Numex Mirasol	AC09-227	-	+

USA	*C. annuum*	deArbol	AC09-229	-	+
USA	*C. annuum*	Floral Gem	AC09-232	-	+
USA	*C. annuum*	Spanish Piquillo	AC09-233	-	+
USA	*C. annuum*	Santa fe grande	AC09-234	-	+
Unknown	*C. annuum*	PX23595(No.8)	R09-120	-	+
Unknown	*C. annuum*	PX24195(No.10)	R09-121	-	+
Unknown	*C. annuum*	PBC479 ANK-72	R09-129	-	+
Unknown	*C. annuum*	99Gonmyung2	R09-136	-	+
Unknown	*C. annuum*	Tombak-2	R09-138	-	+
Unknown	*C. annuum*	95SLU DiRe-9	R09-145	-	+
Unknown	*C. annuum*	Unknown	R09-148	-	+

* - : No cell death; + : Cell death.

Table 1-4. Pepper accessions respond to a diversity of *P. infestans* effectors



* - : No cell death; + : Cell death.
* PexRD39/40 : Avrblb2 family effectors.

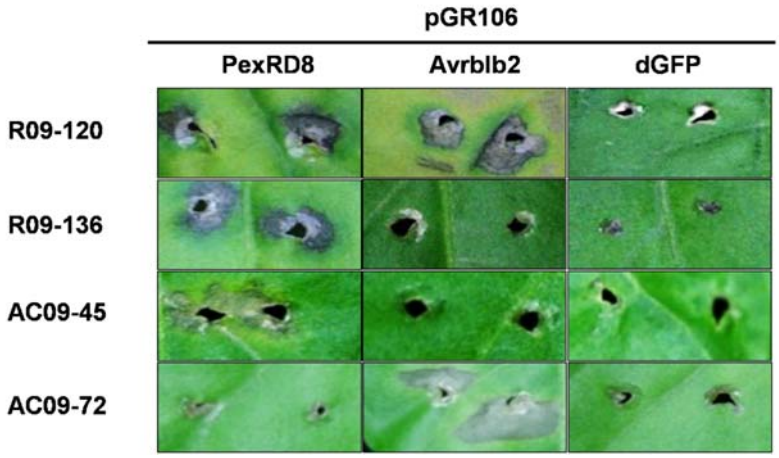


Figure 1-6. PexRD8 and Avrblb2 are recognized by a variety of pepper accessions. Hypersensitive cell death was observed in pepper accessions after wound inoculation with *Agrobacterium* carrying pGR106-PexRD8 and Avrblb2 at 14 dpi.

Validation of the pepper hypersensitive response to *P. infestans* effectors using recombinant PVX virions

Some of the pepper accessions that did not show any reaction with the PVX agro-infection assay may be recalcitrant to *A. tumefaciens*-mediated transient assays. To confirm the positive interactions of pepper to *P. infestans* effectors for a further inheritance study and exclude the effect of *Agrobacterium*, we developed a PVX-virion-mediated screening method. As depicted in Figure 1-7a, recombinant PVX virions carrying RXLR effectors propagated in *N. benthamiana* were harvested from upper leaves that showed PVX symptoms and were manually applied on carborundum-dusted leaves of the pepper accessions. HR cell death was solely developed by PVX-mediated *in planta* expression of RXLR effectors.

Susceptible responses of pepper against PVX, including severe necrosis, have been reported in 11 cultivars of *C. annuum* species (Shi *et al*., 2008). To select accessions for the application of the recombinant virion method, we screened 14 pepper accessions for their response to PVX following inoculation of the negative control PVX-dGFP and positive control PVX-PexRD2 (Fig. 1-8; Table 1-5). Twelve out of 14 pepper accessions were randomly selected from the pepper accessions showing cell death induced by PexRD2. The other two accessions (AC09-186 and AC09-226) were chosen

from the pepper accessions showing no effector-induced cell death. Upon inoculation of the recombinant virions, some accessions, including AC09-56 and AC09-200, showed cell death against PVX-dGFP, which may indicate that these accessions contain intrinsic R genes against PVX. For further study of inheritance of effector-induced cell death, we selected four pepper accessions, AC09-11, AC09-186, AC09-202 and AC09-226 showing no cell death response induced by PVX-dGFP and a clear response against PVX-PexRD2. All accessions showed cell death against PexRD2 but no cell death against PVX-dGFP (Fig. 1-7b).

Of 54 RXLR effectors, 13 effectors (PexRD6-3, PexRD12-1, PexRD12-2, PexRD24-2, PexRD26-1, PexRD26-2, PexRD31, PexRD39, Pex46-2, PexRD49, PexRD50-2, and PexRD51) were excluded, due to the difficulty of obtaining recombinant PVX virions. In total, recombinant PVX virions of 41 RXLR effectors were tested on four accessions (Table 1-6). Effector-induced cell death by PexRD8, PexRD13, Avrblb2, and PexRD50 are shown in Figure 1-7b. PexRD8 resulted in cell death on all accessions except AC09-202, confirming the results of the primary screening. Interestingly, the PexRD13-1 and Avrblb2 families (PexRD40-1 and PexRD40-2) were recognized by all four accessions. In potato, PexRD13-1 elicited cell death on *Solanum bulbocastanum* and *S. pinnatisectum* which are both resistant to *P. infestans*.

Avrblb2 families induced cell death in both resistant and susceptible Solanum species (Vleeshouwers *et al*., 2008). These similar patterns of cell death induced by the PexRD8 and Avrblb2 families suggest that pepper has common plant factors or cognate R genes interacting with those effectors. Some effectors elicited cell death on one or two accessions. For example, PexRD24 induced cell death on AC09-11 and AC09-226. PexRD50-induced cell death was only observed on AC09-202. Overall, we confirmed the multiple interactions between pepper and effectors of *P. infestans*.

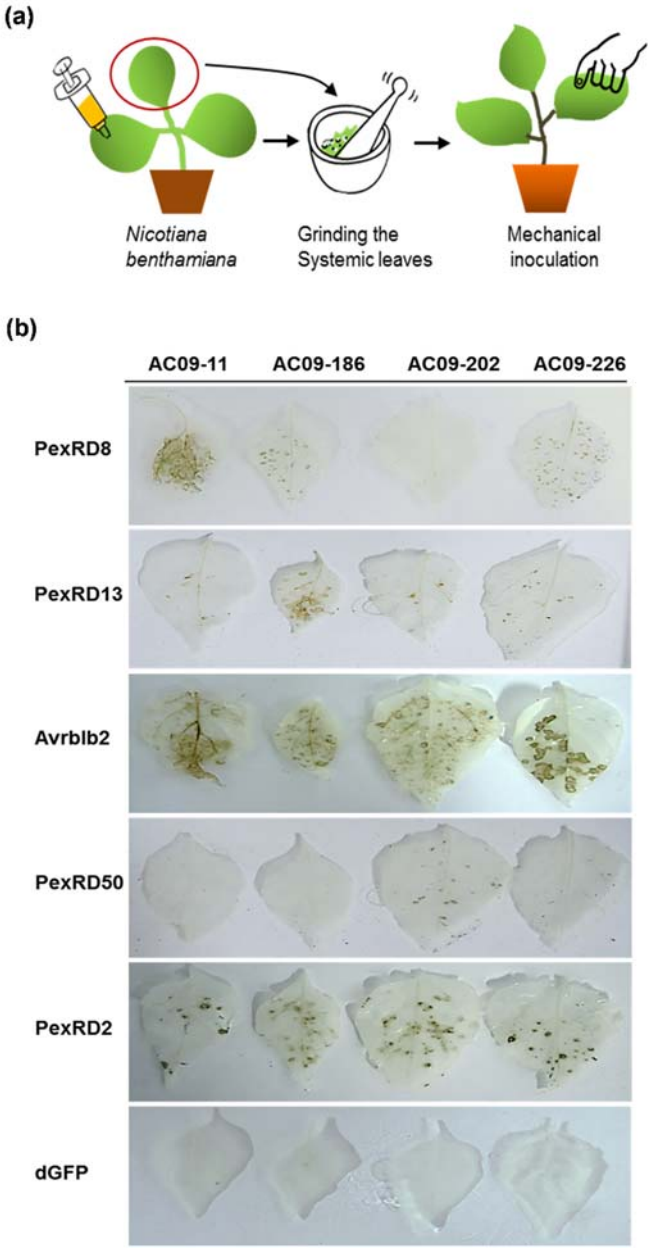


Figure 1-7. Validation of effector-induced cell death using recombinant PVX virions.

(a) Overview of the PVX-mediated transient expression method is shown.

Agrobacterium carrying RXLR effectors was inoculated into lower leaves of *N. benthamiana*. Upper leaves showing virus symptom were ground to produce a PVX-inoculum carrying RXLR effectors. The inoculum was mechanically inoculated on pepper leaves to validate the effector-induced cell death.

(b) Four pepper accessions (AC09-11, 186, 202, and 226) were wound-inoculated with recombinant PVX virions (PexRD8, PexRD13, Avrblb2, and PexRD50). PVX virions of PexRD2 and dGFP were used as positive control and negative control, respectively. Inoculated leaves were harvested at 5 dpi and cleared in 100% ethyl alcohol to remove chlorophyll to visualize the cell death.

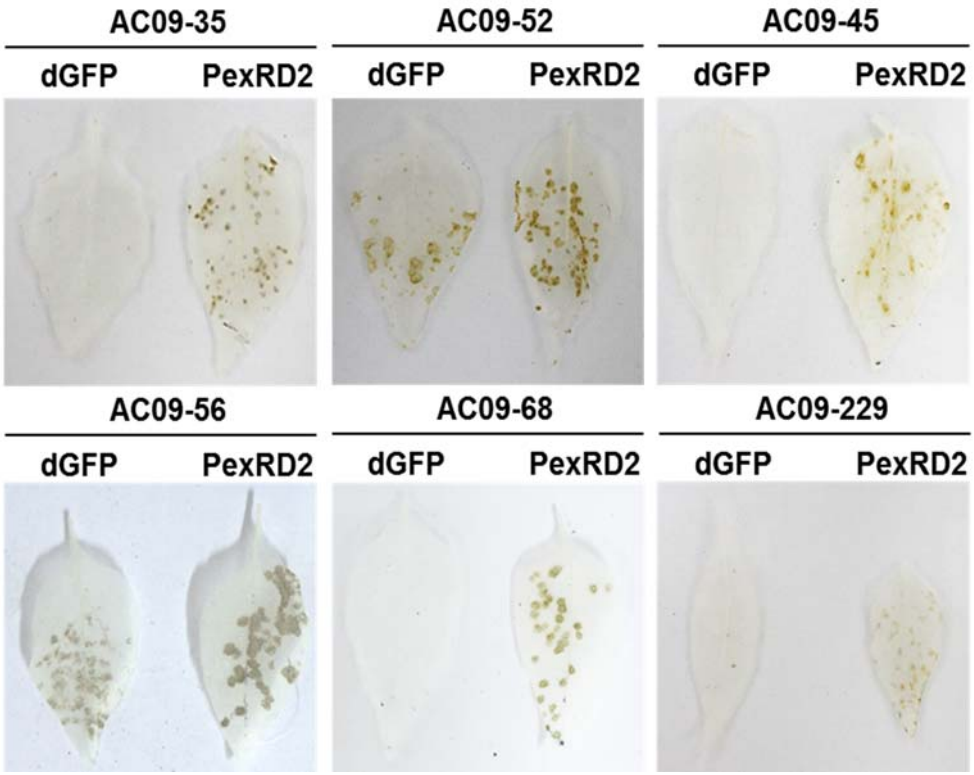


Figure 1-8. The response of pepper accessions against PVX upon inoculation of the negative control PVX-dGFP and positive control PVX-PexRD2.

Pepper accessions chosen were inoculated with recombinant PVX virions including negative control PVX-dGFP and positive control PVX-PexRD2. The response against recombinant PVX virions were monitored until 7 dpi. The inoculated leaves of pepper accessions were harvested and destained with 100% ethanol. The photographs were taken at 7 dpi.

Table 1-5. The response of pepper accessions upon inoculation of negative control PVX-dGFP

Species	Cultivar name	Accession ID	Response against PVX-dGFP
C. annuum	Nitrianska Krajova	AC09-11	No cell death
C. annuum	Unknown	AC09-35	No cell death
C. annuum	Unknown	AC09-45	No cell death
C. annuum	Cecei	AC09-52	Cell death
C. annuum	Siau-Fung-Tsong	AC09-56	Cell death
C. annuum	HS - X - 3	AC09-68	No cell death
C. annuum	Krupnyj Zeltyj 903	AC09-73	Cell death
C. chinense	BISBAS ROTFRUECHTIG	AC09-186	No cell death
C. annuum	Santaka	AC09-200	Cell death
C. annuum	Numex Big Jim	AC09-202	No cell death
C. annuum	Numex Sunglo	AC09-225	Cell death
C. annuum	Serrano	AC09-226	No cell death
C. annuum	deArbol	AC09-229	No cell death
C. annuum	Floral Gem	AC09-232	No cell death

Table 1-6. Hypersensitive cell death induced by 41 RXLR effectors in pepper accessions using inoculation with recombinant PVX virions

P.infestans RXLR effectors	Pepper accessions			
	AC09-11	AC09-186	AC09-202	AC09-226
PexRD1	-	-	-	-
PexRD2	+	+	+	+
PexRD3	-	-	-	-
PexRD4	-	-	-	-
PexRD6-1	-	-	-	-
PexRD6-2	-	-	-	-
PexRD7-1	-	-	-	-
PexRD7-2	+	-	+	-
Pex147-2	-	-	-	-
Pex147-3	-	-	nd	-
PexRD8	+	+	-	+
PexRD10	+	-	-	-
PexRD11-1	-	-	-	-
PexRD11-2	-	-	-	-
PexRD13-1	+	+	+	+
PexRD13-2	+	-	+	-
PexRD14-1	-	-	-	-
PexRD14-2	-	-	-	-
PexRD16-1	-	-	-	-
PexRD16-2	+	-	+	-
PexRD17-1	-	-	-	-
PexRD17-2	-	-	-	-
PexRD21	nd	nd	+	-
PexRD22-1	-	-	-	-
PexRD22-2	-	-	-	-
PexRD24-1	+	-	-	+
PexRD27	-	-	-	-
PexRD28	-	-	-	-
PexRD36-1	+	-	-	-
PexRD36-2	-	-	-	-
PexRD40-1	+	+	+	+
PexRD40-2	+	+	+	+
PexRD41-1	+	-	-	-
PexRD41-2	nd	-	-	-
PexRD41-3	-	nd	+	-

PexRD44	-	-	-	-
PexRD45-1	-	-	-	-
PexRD45-2	-	-	-	nd
PexRD46-1	+	-	+	-
PexRD46-3	-	-	+	-
PexRD50-1	-	-	+	-

* PexRD40-1/PexRD40-2 : Avrblb2 family effectors.

* nd : Not determined.

Multiple loci determine responses of pepper to *P. infestans* RXLR effectors

Based on the diversity of effector-induced cell death in pepper accessions, an inheritance study was performed to determine the genetic basis of effector-induced cell death in pepper. Two accessions, AC09-226 and AC09-202, were crossed to generate an F2 population. Among the effectors to which these two pepper accessions reacted differentially, PexRD8 and PexRD24 caused cell death on AC09-226, whereas PexRD41-3, PexRD46, and PexRD50 induced cell death on AC09-202.

PVX virions expressing the five effectors (PexRD8, PexRD24, PexRD41, PexRD46, and PexRD50) were manually inoculated onto two leaves per each plant of F1 and F2 population. Each F2 plant was treated with only one recombinant virion. Six days later, all inoculated leaves were scored for effector-induced cell death. To determine whether PVX affects the cell death induced by effectors on F2 plants, we applied the virion of negative control PVX-dGFP on 65 F2 plants, resulting in no cell death responses on any of the tested F2 plants. Observations of cell death induced by five effectors in F1 progeny indicated that plant factor(s) to determine the cell death phenotype were dominant alleles. The F2 distribution of PexRD8-induced cell death showed no significant deviation from the 15:1 ratio of cell death at the 0.05

probability level, suggesting that two unlinked dominant genes interact with the PexRD8 effector (Table 1-7). The F2 segregation of cell death induced by PexRD24, PexRD46 and PexRD50 fitted the expected 9:7 ratio, consistent with two independent gene loci that interact in complementary dominant epistasis to trigger effector-induced cell death. These results indicate that pepper has a set of genes that recognize one RXLR effector. The cell death phenotype induced by PexRD41-3 segregated in a 3:1 ratio, suggesting that cell death is conditioned by a single dominant gene. Taken together, our data support the idea that multiple interactions of plant factors with effectors underpin the NHR of pepper against *P. infestans*.

Table 1-7. Multiple dominant genes mediate effector-induced cell death in F2 population from the cross of AC09-226 to AC09-202

		F2				
	F1	Observed ratio		Expected ratio		
Effector	HR+/HR-	HR+	HR-	HR+: HR-	Chi square	*p*-value
PexRD8	8 : 0	141	14	15 : 1	2.047	0.152
PexRD24-1	15 : 0	101	81	9 : 7	0.042	0.837
PexRD41-3	15 : 0	101	31	3 : 1	0.162	0.687
PexRD46-1	15 : 0	83	57	9 : 7	0.524	0.469
PexRD50-1	15 : 0	27	17	9 : 7	0.468	0.494

DISCUSSION

NHR is a durable resistance that includes both preformed immunity and induced immunity. Here, we report on the interaction between pepper, a Solanaceae plant, and the destructive potato late blight pathogen, the oomycete *P. infestans*. We performed a cytological investigation to examine the cellular responses of this interaction, and concluded that a localized HR is a major response of NHR in pepper against *P. infestans*. This finding is consistent with a previous study, in which NHR to *P. infestans* in *Solanum* spp. and *Arabidopsis* was associated with the HR (Vleeshouwers *et al*., 2000; Huitema *et al*., 2003). It was recently reported that PAMPs such as flagellin and the glycoprotein CBEL have elicitor activity and induce HR-like cell death (Khatib *et al*., 2004). However, the HR, a form of programmed cell death, is more frequently induced by interactions between R genes and the cognate effectors (Khatib *et al*., 2004; Naito *et al*., 2008; Coll *et al*., 2011). Based on our finding, a set of effectors of *P. infestans* were expressed during interactions with both host potato and nonhost pepper. We suggest that multiple recognition of effectors by an arsenal of R genes in pepper is an important factor in durable NHR. Pepper, tomato, and potato are all in the Solanaceae family and have a relatively close evolutionary distance; thus, it

seems reasonable that pepper might carry R genes that detect RXLR effectors of *P. infestans* and that ETI would therefore contribute to NHR (Schulze-Lefert and Panstruga, 2011).

As an initial step in the characterization of the interaction between RXLR effectors of *P. infestans* and pepper, we inoculated 54 PexRD effectors into 100 pepper accessions using a PVX agro-infection method. This method has been used to study the response by effectors in several Solanum species (Vleeshouwers *et al*., 2006; Vleeshouwers *et al*., 2008) and is considered appropriate for the high-throughput screening of effector activity that induces cell death. However, pepper has relative low efficiency of *Agrobacterium*-mediated transformation, and gene transfer by *Agrobacterium* might not be easily performed (Lee *et al*., 2004). Functional studies in pepper mainly have been conducted by gene silencing using viruses rather than overexpression. In this study, we optimized the method and confirmed the response to effectors of pepper with recombinant PVX virions for *in planta* overexpression of effectors. This method made it possible to exclude *Agrobacterium* and achieved high efficiency in a transient expression assay. For instance, pepper accessions AC09-186 and AC09-226 used for the inheritance study did not show any cell death induced by effectors using the PVX agro-infection method but clear cell death induced by effectors was

observed after inoculation with recombinant PVX virions.

Several RXLR effectors of *P. infestans* are known to be recognized by R genes of Solanum species. R gene-avirulence effector pairs include R2-Avr2 (Lokossou *et al*., 2009), R3a-Avr3a (Armstrong *et al*., 2005), Rpi-blb1-Avrblb1 (Vleeshouwers *et al*., 2008), and Rpiblb2-Avrblb2 (Oh *et al*., 2009). Our finding that pepper recognized several RXLR effectors of *P. infestans* which is a nonhost pathogen suggests that pepper has R genes that interact with RXLR effectors from this pathogen. Based on comparative genomic analysis of *Arabidopsis* accessions, R genes were found to rapidly evolve, having higher polymorphism than plant gene families involved in basic biological processes such as ribosomal function and transcriptional regulation (Clark *et al*., 2007). Each pepper accession showed a cell death response to each RXLR effector, which could reflect a high number of polymorphisms of the R gene family among the pepper accessions. Each pepper accession exhibited cell-death responses to at least one effector, and 1 to 36 of the tested 54 RXLR effectors, indicating multiple interactions of the nonhost pepper with RXLR effectors. The 54 effectors used in this study account for approximately 10% of the effectors in the *P. infestans* genome, indicating that pepper could have more interactions with effectors of *P. infestans*. As a consequence, we cannot conclude that AC09-221, showing a cell death

response to only the PexRD2 effector, would be compatible to a *P. infestans* isolate not producing a PexRD2 effector. Furthermore, we identified some effectors including PexRD8 and PexRD39/40, termed Avrblb2, which trigger cell death on a broad range of pepper accessions. PexRD8 is known to suppress INF1-induced cell death and Avrblb2 is recognized by the late blight resistance protein Rpi-blb2 identified from *Solanum bulbocastanum* (van der Vossen *et al*., 2005; Oh *et al*., 2009). PexRD8 and Avrblb2 also induce cell death on most of *P. infestans*-resistant wild *Solanum* species (Vleeshouwers *et al*., 2008). However, *N. benthamiana*, a member of the Solanaceae family, did not respond to either PexRD8 or Avrblb2 (Oh *et al*., 2009). These results suggest that conserved genes exist in pepper and potato that can interact with PexRD8 and Avrblb2. We also tested interactions with Avrblb2 using recombinant PVX virion in 10 pepper accessions, which included the 9 of 14 pepper accessions used in the screening of the response against PVX in addition to the reference cultivar 'CM334' (Table 1-5). Avrblb2 which is conserved in *P. infestans* isolates induced cell death in the 10 pepper accessions tested, suggesting that pepper might have target(s) or R gene(s) with conserved functions that interact with Avrblb2. The putative R gene(s) that interact with Avrblb2 could play an important role in NHR and are likely to be distinct from Rpiblb2 as Rpiblb2 orthologous cloned from several

pepper accessions did not recognize Avrblb2 (Lee *et al*., unpublished data).

Our genetic data suggest that several major genes are associated with the interaction with *P. infestans* effectors. Those genes could be R gene(s). In a previous study, the genetic analysis of the response to *P. syringae* effectors in nonhost lettuce showed that a linkage group, containing chromosome regions including multiple R gene analogs, determine the cell death phenotype (Wroblewski *et al*., 2009). In this study, PexRD8-induced cell death in an F2 population from a cross between AC09-226 and AC09-202 showed a 15:1 phenotypic segregation ratio, indicating that two independent dominant alleles (e.g. A-B-, aaB-, and A-bb) are required to confer cell death. A dual R-gene system (RRS1-Ws and RPS4-Ws) showed resistance to *Colleotrichum higginisianum* (Narusaka *et al*., 2009). Arabidopsis RPP2A and RPP2B, members of TIR-NB-LRR, are required for resistance against *Peronospora parasitica* isolate Cala2 (Sinapidou *et al*., 2004). Furthermore, the segregation of effector-induced cell death by PexRD24, PexRD41-3, and PexRD46 in the F2 population was consistent with a 9:7 ratio, suggesting that two dominant complementary genes (e.g. A-B-) are associated with effector-induced cell death. The mode of action of two putative R genes in NHR could be supported by several studies in which two R genes act cooperatively in the resistance response. The tomato *NRC1* gene encodes a coiled-coil (CC)-

nucleotide binding (NB)-leucine-rich repeat (LRR) type resistance protein and involves an HR signaling pathway mediated by the *Cf-4* resistance gene (Gabriels *et al*., 2007). Similarly, *NRG1*, encoding CC-NB-LRR, mediates resistance together with the tobacco *N* gene against *Tobacco mosaic virus* (Peart *et al*., 2005). In our experimental system, the inverse model, the interaction of an R gene with multiple effectors, could not be demonstrated. Arabidopsis RPM1 induces the resistance response by the interaction with two unrelated effectors of *P. syringae*, AvrB and AvrRpm1. Similarly, a dual interaction of an R gene with different effectors is likely to occur in nonhost interactions. We cannot rule out the possibility that the combination of the interactions between multiple R genes and effectors establishes durable NHR.

Our finding that pepper has the ability to respond to *P. infestans* effectors raises some interesting questions. *P. infestans* is a specialized pathogen of *Solanum* spp. native to Central and South America but is known to infect neither pepper nor related *Capsicum* spp. Why then, would the nonhost pepper carry genes that detect effectors of *P. infestans*? There are several possibilities. It is possible that pepper was formerly a host of *P. infestans* during its evolutionary history. Another explanation is that pepper does not directly respond to *P. infestans* effectors but rather to the cellular perturbations that these effectors trigger. This would be consistent with

models of indirect recognition as suggested by the guard model (Innes, 2004). Recently, recognition of the *P. infestans* AVR2 effector by the *Solanum* R2 gene was shown to require an additional host protein, phosphatase BSL1 (Saunders *et al*., 2012). R2-mediated resistance to *P. infestans* was compromised specifically by the silencing of BSL1. The association of AVR2 with BSL1 and the requirement of AVR2 for interaction of R2 with BSL1 suggest the role of BSL1 as an intermediary host protein. In addition, Antonovics *et al*. (2013) hypothesized that the evolutionary mechanism for pathogen specialization by co-evolution with the host results in NHR that reduces the virulence on other potential hosts (Antonovics *et al*., 2013). These authors explained that nonhost plant species that are closely related to the host may possess the gene variants required for the resistance. However, those variants would be less exposed to selection pressure. As hypothesized, it is possible that pepper has R genes orthologous to *Solanum* species that recognize the effectors. On the basis of criteria such as sequence identity and chromosome location, 319 putative orthologous pairs of R gene families were identified from the comparative analysis between tomato and potato (Andolfo *et al*., 2013). Analysis of pepper genome revealed that 395 NB-LRRs are clustered with those of tomato and potato. Additionally, pepper has several NB-LRRs with orthology to known Solanaceae R genes (Kim *et al*., 2014).

Inversely, pepper is host to *Phytophthora capsici* which causes serious blight disease. This means that pepper R genes should have evolved to resist *P. capsici* in the evolutionary arms race. As a consequence of adaptation to *P. capsici*, pepper R genes could recognize *P. infestans* effectors that have homology with *P. capsici* effectors. Genome-wide analysis of the evolution of NB-LRRs and effectors could provide clues to elucidate the mechanism of NHR.

NHR is the most durable form of plant resistance and therefore practical and scientific interest on NHR has increased. However, it is poorly understood, partly due to interspecific genetic incompatibility. We performed functional profiling of the responses of nonhost pepper using effectors of *P. infestans* based on effector genomics. This experimental approach could possibly be exploited in other nonhost plant-pathogen interactions, which might lead to the identification of the corresponding active R genes and to the understanding of the molecular mechanism of NHR. The recently completed pepper genome sequence combined with our results could offer a new approach for identifying nonhost R genes against late blight (Kim *et al*., 2014). R genes interacting with multiple effectors may confer durable resistance through functional interfamily transfer and provide a powerful source to breed broad-spectrum disease resistance.

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CHAPTER 2

Identification of Nonhost Resistance Genes of Pepper Recognizing Multiple Core Effectors of *P. infestans* Using Genome-based Approach

ABSTRACT

Nonhost resistance (NHR) is the resistance of a plant species against vast majority of pathogens but the mechanism remains poorly understood. Plant resistance (R) genes recognize pathogen effectors and induce strong defense response called effector-triggered immunity (ETI) which is considered as a major factor of NHR. It is known that multiple recognition of *P. infestans* effectors is associated with NHR of pepper. Most of identified R genes belong to NB (Nucleotide binding site)-LRR (Leucine-rich repeat) family. To identify nonhost R genes, genome-based identification approaches have been developed. A total of 445 pepper NB-LRR genes predicted from genomic information were cloned into an *in planta* expression vector to identify nonhost R genes against *Phytophthora infestans* which is a nonhost pathogen of pepper. *In planta* co-expression screenings using pepper NB-LRRs and Avrblb2 core effectors which trigger cell death on all tested pepper accessions have been performed. CaNBARC114 induced cell death when co-expressed with Avrblb2 effectors. Overexpression of CaNBARC114 in *Nicotiana benthamiana* showed significant decrease in lesion size of *P. infestans* infection. However, CaNBARC114 in transgenic potatoes did not enhance resistance against *P. infestans*. This study proposes genome-based R gene

identification strategy and provides insights into contribution of integrated defense responses to durable NHR.

INTRODUCTION

Plants are constantly exposed to numerous microbial pathogens and have developed immune systems comprised of several components including physical/chemical barriers, PAMPs-triggered immunity (PTI), and effector-triggered immunity (ETI) (Allen *et al.*, 2004; Jones and Dangl, 2006). When plants are challenged with pathogens, pattern recognition receptors (PRRs) recognize pathogen-associated molecular patterns (PAMPs) which are conserved microbial structures that are shared by different pathogens (Nürnberger and Brunner, 2002). The interaction between PRRs and PAMPs triggers a basal resistance response called PTI (Zipfel, 2009). To overcome the defense response induced by PTI and to manipulate host physiology, pathogens secrete a set of effectors into plant cells (Chisholm *et al.*, 2006). Plant resistance (R) genes which encode nucleotide-binding leucine-rich repeat (NB-LRR) proteins interact with effector proteins directly or indirectly to induce a rapid and strong resistance response called ETI. Localized programmed cell death named hypersensitive response (HR) is a typical response of ETI (Dodds and Rathjen, 2010).

Identified R genes from resistant cultivars have been deployed to improve plant resistance but the breakdown of resistance has often been observed in

the field due to rapid evolution of pathogens (McDonald and Linde, 2002). Nonhost resistance (NHR), which is the resistance of an entire plant species against an entire pathogen species, is considered as a potential source of durable resistance (Heath, 2000; Rodrigues *et al.*, 2004). R gene-mediated resistance is one of the core components of NHR but only a few nonhost R genes such as *RXO1* and *WRR4* have been identified (Zhao *et al.*, 2005; Borhan *et al.*, 2008). However, nonhost plants carrying non-functional copy of these R genes still showed resistance against the pathogens pointing to the multigenic nature of NHR (Zhao *et al.*, 2005; Borhan *et al.*, 2010). Furthermore, high-throughput screenings of cell death induced by *P. infestans* effectors on nonhost pepper plants revealed that multiple genes are associated with effector-induced cell death (Lee *et al.*, 2014). Taken together, we can postulate that multiple R genes or host proteins, rather than a single R gene, recognize multiple effectors, leading to durable NHR.

Genetic approaches to identify nonhost R genes have been hampered due to interspecific hybrid sterility (Niks and Marcel, 2009). This difficulty could be overcome by genomics approaches and experimental tools. Over the past two decades, sequencing technologies have been rapidly developed and used to assess plant-microbe interactions (Raffaele and Kamoun, 2012; Boyd *et al.*, 2013). Genome-wide analyses of the NB-LRR gene family within and

between plant species provide insight into the evolutionary history and help us identify nonhost R genes (Jupe *et al.*, 2013; Yu *et al.*, 2014). Similarly, core effectors of pathogens that are commonly found in several isolates could be easily predicted using comparative genomics, and effector-omics could accelerate R gene identification (Vleeshouwers *et al.*, 2008; Cooke *et al.*, 2012).

Phytophthora infestans is one of the most devastating pathogen in the world and causes severe potato late blight on potato and tomato but not on pepper. The genome of *P. infestans* has revealed that it has approximately 550 RXLR effector genes, potentially regarded as putative avirulence (Avr) factors (Haas *et al.*, 2009). Cooke *et al*., performed comparative genome analyses of several aggressive isolates of *P. infestans* including 06_3928A, NL07434, and reference isolate T30-4 (Cooke *et al.*, 2012). It was revealed that 45 RXLR effectors are consistently induced *in planta* in the three isolates and are named as core effectors. Those effectors include known Avr factors including Avr2, Avrblb1, and Avrblb2. Among them, Avrblb2 is a cognate effector of Rpi-blb2 identified from wild potato *Solanum bulbocastanum* and *P. infestans* genome carries highly homologous paralogs of Avrblb2 (Vossen *et al.*, 2005; Oh *et al.*, 2009; Oliva *et al.*, 2015). Also, Avrblb2 enhances *P. infestans* virulence and accumulates around haustoria during the infection,

which suggests that it plays an important role in pathogenesis of *P. infestans* (Bozkurt et al., 2011).

In this study, we hypothesized that nonhost R genes of pepper which recognize multiple Avrblb2 effectors of *P. infestans* could be a great source for durable resistance. To identify nonhost R genes, 479 NB-LRR genes of pepper have been predicted using genome information and cloned into *in planta* expression vectors. *In planta* co-expression screenings using NB-LRRs and Avrblb2 effectors have been performed and one NB-LRR gene was found to induce cell death when it was co-expressed with Avrblb2 effectors. Resistance against *P. infestans* was tested in transgenic potatoes carrying the NB-LRR gene. This study could propose genome-based approaches to identify NHR genes to engineer durable resistance and underpin NHR mechanism by ETI.

MATERIALS AND METHODS

Plant materials and growth conditions

Capsicum annuum ‘CM334’, of which whole genomic sequences has been completely defined, was used to screen cell death induced by core effectors. The seeds were sterilized with 0.1% sodium hypochlorite (NaOCl). When radicle appeared, all of the seedlings were transferred to 200-plug form tray filled with horticultural bed soil (Baroker, Seoul Bio Co,. Ltd, Seoul, Korea) and grown under a 16h : 8h, light : dark period at 23°C in controlled chambers. When cotyledons were fully expanded, the seedlings were transferred to 50-plug form tray and grown under same conditions mentioned above. *Nicotiana benthamiana* seeds were sown in 200-plug form tray and grown in pots and maintained under the same conditions.

NB-LRR gene cloning

Based on genomic prediction of NB-LRR gene family (Seo *et al*., unpublished), 479 NB-LRR genes carrying motifs which are important for R gene function have been cloned into *in planta* expression vector pCAMBIA2300-LIC using ligation-independent cloning (LIC) method (Aslanidis and de Jong, 1990; Oh *et al.*, 2010). The vector was modified to

contain the *ccdb* gene for LIC. BlastX and FgeneSH pipeline (Salamov and Solovyev, 2000) were used to predict the gene barriers of the NB-LRR genes. Gene specific primers which anneal outside of the gene barriers were designed and LIC adaptor sequence (Forward 5'-CGACGACAAGACCCT, Reverse 5'-GAGGAGAAGAGCCCT) was added onto the 5' terminals of each forward and reverse primers, respectively. PCR amplification was performed with the primers using genomic DNA of CM334 using PrimeSTAR GXL DNA polymerase (Takara Bio, Shiga, Japan) to minimize PCR errors. The PCR regimen for 32 cycles was 98°C for 10s, 60°C for 15s, 72°C for 2 min. The amplicons purified with AMPure XP beads (Agencourt bioscience, Beverley, MA, USA) and *Pst*I-digested linear vector were treated with the T4 DNA polymerase (NEB, Beverley, MA, USA) to generate complementary overhang. The treated amplicons and vector were combined to be annealed each other and the mixture was transformed into *E. coli*. All the cloned NB-LRR genes were sequenced to confirm the predicted genomic sequence and the constructs carrying the gene was transformed *into Agrobacterium tumefaciens* GV3101 for further *in planta* screening

P. infestans **core effectors**

The 57 core effectors of *P. infestans* which are different to 45 core

effectors predicted by Cooke *et al*. were kindly provided by Dr. Kamoun and Dr. Jones (Sainsbury laboratory, England). The core effectors were predicted based on comparative genomic analysis of four isolates (T30-4, NL07434, P17777, and 06_3928A). Those effectors are commonly present in all four isolates and highly expressed at 2 or 3 dpi following *P. infestans* infection on potato. The effectors were chemically synthesized not amplified by PCR and cloned into PVX vector pICH31160. The constructs were transformed into *Agrobacterium* GV3101.

P. infestans zoospore infection

P. infestans T30-4 was grown on rye sucrose agar media as previously described (Lee *et al.*, 2014). To release the zoospores, the plates were flooded with distilled water, gently rubbed with a sterile cell scraper, and incubated at 4°C for 1h. The detached leaves were inoculated by pipetting 10 μl zoospore droplets on the abaxial side in the rectangular plates overlaid with a wet paper. The disease symptom was observed under UV light at 7 dpi.

PVX-mediated transient expression

To screen cell death induced by *P. infestans* core effectors on pepper, PVX-mediated transient expression method was performed as previously

described (Lee *et al.*, 2014). Briefly, the PVX inoculum carrying each effector gene was prepared from *N. benthamiana* and it was mechanically inoculated on pepper leaves. At 7 dpi, the cell death response was monitored after removal of chlorophyll with 100% ethanol.

Agro-coinfiltration assays in *N. benthamiana*

Agrobacterium GV3101 carrying the constructs of NB-LRR gene and core effectors were incubated overnight in YEP medium containing antibiotics (rifampicin (50 μg/ml) and kanamycin (50 μg/ml)) at 28°C with vigorous shaking. The overnight cultures were harvested by centrifugation for 10 min at 3000 rpm and re-suspended in infiltration medium (10 mM MgCl2, 10 mM MES (pH 5.6), and 150 μM acetosyringone). The OD_{600} of the *Agrobacterium* was adjusted to 0.75 for NB-LRR and 0.4 for core effectors. *Agrobacterium* carrying core effectors were mixed with *Agrobacterium* carrying pepper NB-LRR genes in a 1:1 ratio and the mixture was infiltrated into *N. benthamiana* leaves using syringe. Cell death response was monitored from 3 to 7 dpi.

Reverse transcription-PCR (RT-PCR)

Six leaf disks were harvested using a cork borer from the pepper leaves

inoculated with *P. infestans*. Total RNAs were extracted using Trizol reagent (Invitrogen, Carlsbad, CA) and reverse-transcribed using Superscript II (Invitrogen, Carlsbad, CA). To determine *in planta* effector expression, PCR was performed using gene-specific primers (Table 2-1).

Table 2-1. The list of primer sequences used in this study

Effectors	Forward (5'->3')	Reverse (5'->3')	PITG ID[1)]
Avrblb2B	ATCAAGTTTTTCAACT TCTTCACCTTTTTTGC	TATATTCTACGTTGCTC TTGCCTTTGC	PITG 04097
Avr12	TCC GAG GTT TCT CGA CTG GAT CAT CTA	CGA CCT CAG TTC TTG CTG AAG GC	PITG 20857
Avr10	TTA CGA AGA TGT TTT CTT ACG GCT AAT CAA GT	TCT CCT GAC AAA AGA TTC CTT CGT ATC GC	PITG 04194
Avr15_16	GAA TCC CGA CGA AAC TCG GCT CTT A	TCG CTG CCT TCT TAC CGA CGA ATG	PITG 18670/18685
Avr9	TCT ACG CTG TAC TTG CCA TTG CG	GGC TAA GGA TTT CCA GAT GCC ACC AT	PITG 22727
Avr6	TGCGTCTCAGTCTCAG TT	TCAGCTTCTCTGCCTTC T	PITG 23193
Avrblb2	GGAAAAACGTAGTCC AGAGTACTAAC	GATACTTCGCTCAACC TTTTACAG	PITG20300/ 04090/ 20303/18683 /04085

1) *P. infestans* T30-4 genome locus tag.

RESULTS

Genomic prediction and cloning of pepper NB-LRR family

Plant NB-LRR proteins have the central NB-ARC (nucleotide-binding adaptor shared by APAF-1, R proteins, and CED-4) domains which act as nucleotide-binding pocket and hydrolyzes ATP to induce conformational changes in R proteins (Takken *et al.*, 2006; Takken and Goverse, 2012). Conserved motifs, including P-loop, RNBSA to D, and MHD in NB-ARC domains, play important roles in controlling R gene activation. Based on the presence of NB-ARC domains, 755 NB-LRR genes from pepper genome (v.1.5) were predicted using in-house developed bioinformatics pipelines (Kim *et al.*, 2014). The predicted NB-LRR genes were divided into 13 groups of CNLs (CC-NB-LRRs) and 1 group of TNLs (TIR-NB-LRRs) depending on phylogenetic analysis and OrthoMCL clusters (Seo *et al*., unpublished) (Table 2-2). Among them, 479 NB-LRR genes carrying conserved motifs in NB-ARC domains were filtered as potentially full-type NB-LRR genes, which were subjected to be cloned into the binary vector pCAMBIA2300 and fused behind CAMV 35S promoter.

To predict the sites of translational start and termination, the genomic sequences including 5kb upstream and downstream all of the NB-LRR genes

were analyzed using Blast X and FgeneSH program. All of the genes were amplified from genomic DNA of CM334 to consider splicing variants and cloned using LIC method. Out of 479 NB-LRR genes, 34 genes were not amplified by PCR due to misprediction of genome sequence because NB-LRR genes are multi-copy genes so that reference assembly could contain underrepresented or mis-assembly of multi-copy genes (Church *et al.*, 2011). All cloned 445 NB-LRR genes have been sequenced to confirm genomic DNA sequence and 60% of the genes were perfectly matched or showing SNPs with predicted sequences. The other NB-LRR genes containing mismatched sequences resulted from genomic mis-assembly because genome sequence of the genes have insertion or deletion, or gaps. Then, the constructs of NB-LRR genes have been transformed into *Agrobacterium* GV3101 for further *in planta* expression.

Table 2-2. Summary of cloned NB-LRR genes of pepper

	Number of Predicted NB-LRRs	Number of Cloned NB-LRRs	Number of Not Amplified or Partial NB-LRRs	Known NB-LRRs	Remark
TNL	54	53	1	*N, Bs4, Gro1-4, RY-1*	
CNL-G1	75	66	9	*CaMi, Rpi-blb2, Hero, Mi-1.2*	Pepper-expanded
CNL-G2	91	85	6	*Bs2*	Pepper-expanded
CNL-G3	23	22	1	*R1, Prf*	
CNL-G4	33	32	1	*L1, RPS1-K, I2, R3a*	
CNL-G5	11	11	0	*R2, Rpi-blb3, Rpi-abpt*	
CNL-G6	29	25	4	*Sw-5*	
CNL-G7	19	16	3	*Rpi-blb1*	
CNL-G8	14	14	0	*NRC1*	
CNL-G9	51	48	3	-	
CNL-G10	38	33	5	*RGC2, RPS2, RPS5*	
CNL-G11	7	7	0	*Tm-2, Tm-2a*	
CNL-G12	9	9	0	*Rx, Rx2, Gpa2*	
CNL-G13	1	1	0	*R-FOM-2*	Pepper-contracted
None -grouping	24	23	1	*HRT, RCY1, RPP8, NRG1, RPM1*	
Total	479	445	34		

Avrblb2 core effectors of *P. infestans* induced cell death on CM334

The Avrblb2 effector is thought to be important for *P. infestans* virulence and suppresses PTI response (Oh *et al.*, 2009; Bozkurt *et al.*, 2011; Zheng *et al.*, 2014). Avrblb2 is also recognized by the NB-LRR protein Rpi-blb2 (Oh *et al.*, 2009). Structure-function analyses revealed that amino acid 69 of Avrblb2 which is positively selected residue is critical for activation of Rpi-blb2 (Oh *et al.*, 2009; Oliva *et al.*, 2015). Avrblb2 has seven paralogs in the genome of *P. infestans* followed by a recent expansion from an ancestral single Avrblb2 gene and the effector was identified only in the sister species which are in the same clade with *P. infestans,* whereas not in *P. capsici*, the causal organism of severe blight on pepper (Oliva *et al.*, 2015). The expression of Avrblb2 was induced during *P. infestans*-infected pepper and pepper accessions tested showed cell death when Avrblb2 was ectopically expressed (Lee *et al.*, 2014). Based on the previous study, it appears that Avrblb2 is an essential effector of *P. infestans*.

Using the genome of four isolates of *P. infestans*, a total of 57 core effectors were predicted based on two criteria : 1) commonly present in the genome of four isolates and 2) highly induced during potato infection by *P. infestans*. The core effectors includes Avrblb2 and four paralogs (PITG04085 (Core17), PITG04090 (Core19), PITG18683 (Core73), PITG20300

(Avrblb2-Core74), and PITG20301 (Core75)). These five effectors called Avrblb2 core effectors below. PVX carrying Avrblb2 core effectors induced cell death on the leaves of *C. annuum* CM334 (Fig. 2-1a). As mentioned above, amino acid at position 69 of Avrblb2 affect the recognition by Rpi-blb2. Mutation of amino acid 69 to phenylalanine (Phe-69) failed to induce Rpi-blb2-mediated cell death even after 10 dpi (Oliva *et al*., 2015). However, substitution of the amino acid 69 to proline (Pro-69) instead of phenylalanine did not induce cell death on all the pepper accessions tested suggesting putative pepper R gene(s) recognizing Avrblb2 core effectors could have no homology with Rpi-blb2. Furthermore, protein sequence alignment of Avrblb2 core effectors revealed the polymorphism in C-terminal of the effectors but it did not affect the cell death on pepper (Fig. 2-1b).

In addition to Avrblb2 core effectors, Belhaj *et al*., (personal communication) performed genome-wide analysis of Avrblb2 family effectors using *P. infestans* T30-4 genome based on sequence similarity. A total of 19 effectors were identified as Avrblb2 family and 3 effectors (PITG20301, PITG04086, and PITG22604) were excluded because these effectors were duplicated. Using 16 Avrblb2 family effectors, phylogenetic analysis was performed. Avrblb2 family effectors were divided into three clusters and Avrblb2 core effectors belong to cluster I (Fig. 2-2). To

investigate the cell death induced by the effectors, the leaves of CM334 were inoculated with PVX carrying Avrblb2 family effectors. Out of 16 effectors, 12 effectors including Avrblb2 core effectors triggered cell death on pepper leaves (Fig. 2-3). However, only Avrblb2 core effectors were highly expressed during pepper infection by *P. infestans* (Fig. 2-4). Except for weak expression of PITG18670 and PITG18685, the other effectors were rarely expressed, which means Avrblb2 core effectors plays important roles in *P. infestans* infection in nature.

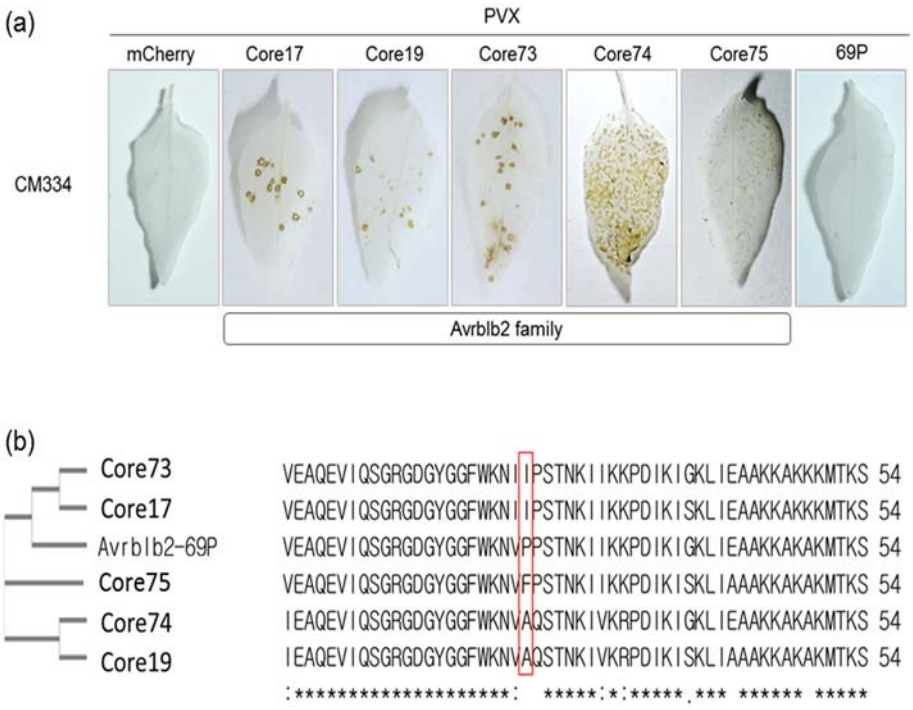

Figure 2-1. Avrblb2 core effectors of *P. infestans* induced cell death on CM334.

(a) Avrblb2 family effectors (Core17, Core19, Core73, Core74, and Core75) and Avrblb2-69P mutant were inoculated on pepper leaves using PVX-mediated transient expression method. Photographs were taken at 5 dpi.

(b) Protein sequence analysis revealed that C-terminal region of the effectors has polymorphism. Red rectangular represents amino acid 69 position.

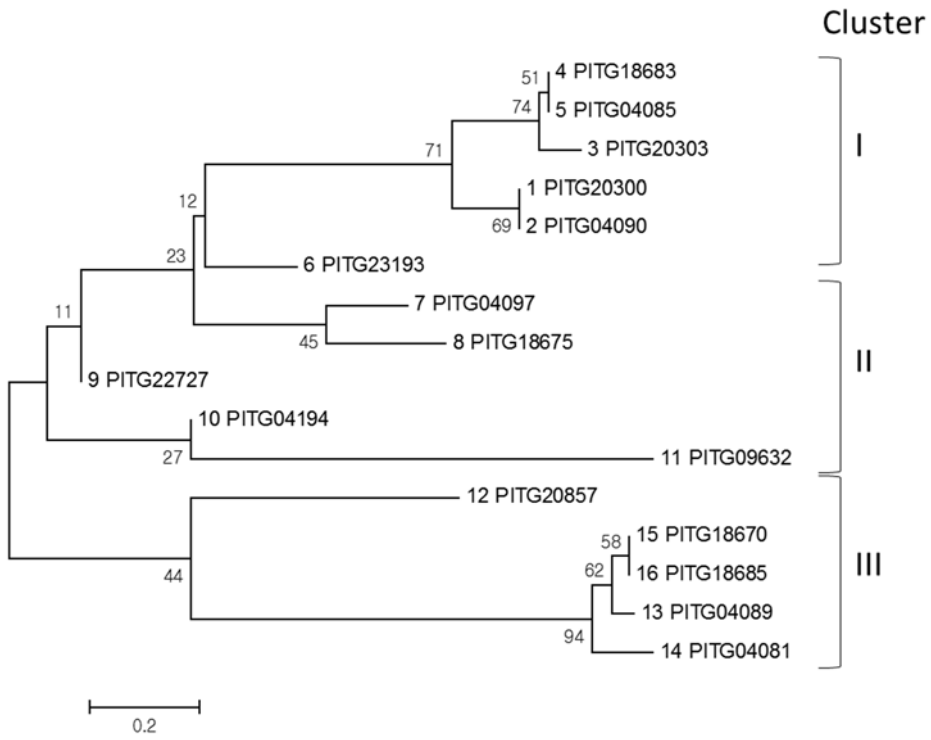

Figure 2-2. Phylogenetic analysis of Avrblb2 family effectors of *P. infestans* T30-4 genome.

A total of 16 effectors was used for constructing phylogenetic tree based on the effector domain using maximum likelihood. Cluster I included all the core effectors of Avrblb2.

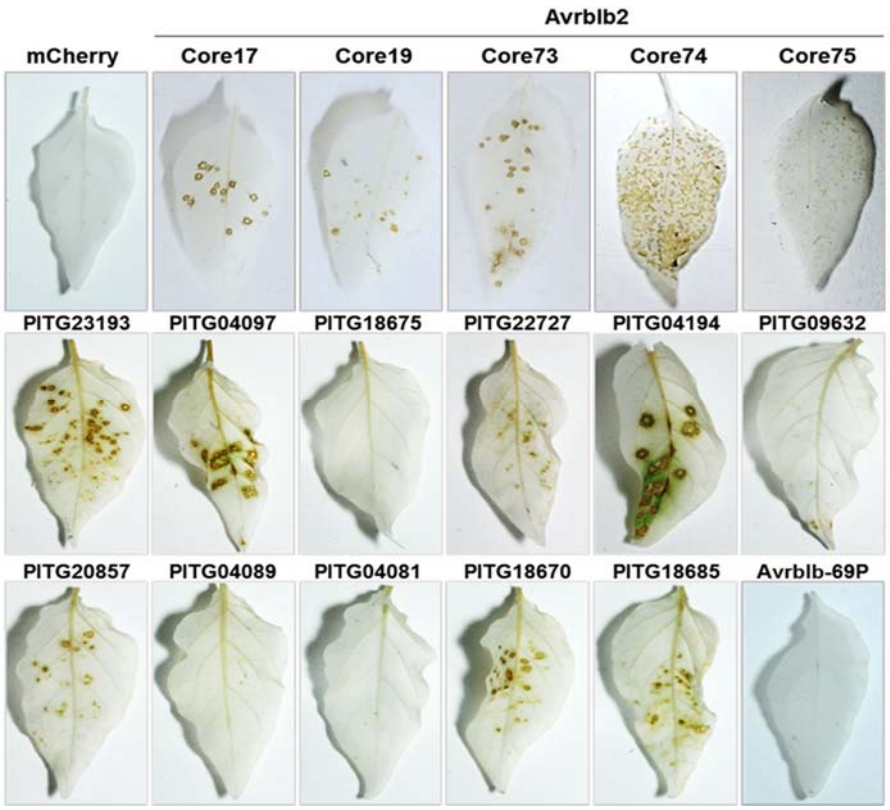

Figure 2-3. Twelve effectors of Avrblb2 family induced cell death on CM334 leaves.

PVX carrying all of 16 effector genes were mechanically inoculated on pepper leaves. The chlorophyll was removed with 100% ethanol and photographs were taken at 5 dpi.

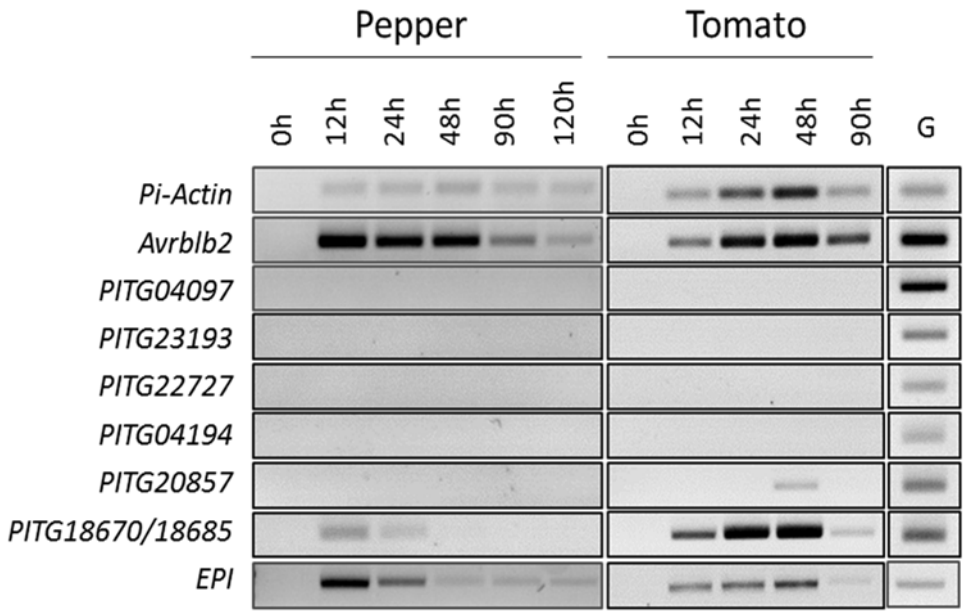

Figure 2-4. *in planta* expression of Avrblb2 core effectors was highly induced. Gene expression of Avrblb2 family effectors were confirmed using total RNAs extracted from *P. infestans*-infected pepper and tomato leaves at different time points. The expression of Avrblb2 core effectors and PITG18670/18685 was induced but the other effectors were rarely expressed. G, *Phytophthora infestans* T30-4 genomic DNA.

CaNBARC114 induced cell death when co-expressed with Avrblb2 core effectors in *N. benthamiana*

Because Avrblb2 core effectors induced cell death on all pepper accessions tested, forward genetic approach was impossible to isolate nonhost resistance gene(s) recognizing Avrblb2 core effectors. As an alternative strategy, genome-based identification approach was developed (Fig. 2-5). To explain the approach, NB-LRR genes as candidate resistance genes and core effectors were predicted from genomic information and cloned for *in planta* co-expression from pepper and *P. infestans*, respectively. Leaves of reference cultivar *C. annuum* CM334 were inoculated with PVX carrying core effectors and candidate core effectors were selected based on cell death. Then, *in planta* co-expression screenings between NB-LRR genes and candidate effectors were performed using agro-coinfiltration assay in *N. benthamiana*. The method is a versatile tool for screening the cell death response induced by the interaction between R genes and cognate effectors (Goodin *et al.*, 2008). Through the screening, candidate nonhost R genes interacting with Avrblb2 core effectors were selected based on induction of the cell death which is considered as the results of the interaction between R genes and effectors. Subsequently, resistance against *P. infestans* are tested in transgenic host potato expressing candidate R genes to identify nonhost R genes of pepper

against *P. infestans.*

To identify candidate nonhost R genes, agro-coinfiltration assay was performed in *N. benthamiana* using 445 pepper NB-LRR genes and Avrblb2 core effectors. The mCherry gene and Rpiblb2/Avrblb2 were used as a negative and positive control, respectively. Among 445 NB-LRR genes, 19 genes showed autoactivity (constitutive expression of defense response) when transiently overexpressed in *N. benthamiana* (Table 2-3). This result indicates that R gene regulators could be different between pepper and *N. benthamiana*.

As a result of agro-coinfiltration assay, CaNBARC114 (CA05g06820) triggered cell death with Avrblb2 core effectors (Fig. 2-6). However, CaNBARC114 had autoactivity in *N. benthamiana*, whereas not in pepper and potato. Autoactivity could mask the cell death induced by the interaction between CaNBARC114 and Avrblb2 effectors. To weaken its autoactivity, CaNBARC114 containing 2 kb of upstream sequence was cloned into a modified pCAMBIA2300 vector in which 35S promoter was removed to allow the gene to be expressed by native promoter. Autoactivity of CaNBARC114 under the control of native promoter was significantly weakened and prominent cell death was shown when co-expressed with Avrblb2 effectors (Fig. 2-6). To test whether CaNBARC114 recognizes other

core effectors, it was co-expressed with the 23 core effectors which did not induce cell death on *N. benthamiana*. In addition to Avrblb2 effectors, Core46 (PITG12737) and Core83 (PITG22870-Avr2) triggered cell death with CaNBARC114, which supports the multiple interaction hypothesis that single R gene recognizes multiple effectors of *P. infestans* leading to durable NHR.

CaNBARC114, a single-exon gene, is located on chromosome 5 and 2,585 bp long. It belongs to CNL-group5 having homology with R2, but not with Rpi-blb2. To look at synteny relationship of CaNBARC114 with tomato and *C. baccatum* in detail, SynOrth algorithm was used (Dong *et al*., 2009). As the result was shown in Figure 2-7, *Capsicum* species and tomato shared synteny in the region between CA05g06790 and CA05g06840. However, CANBARC114 has synteny only to the gene (Scaffold520.130) of *C. baccaum* but tomato did not have ortholog of CaNBARC114. In other words, CaNBARC114 is pepper-specific NB-LRR genes. This suggests that the gene may have appeared after speciation and conserved among *Capsicum* genus.

To investigate CaNBARC114-mediated resistance against *P. infestans*, CaNBARC114 and an empty vector control were transiently expressed in each half of the same leaf of *N. benthamiana* (Total 10 leaves). At 1 dpi, the leaves were detached and inoculated with zoospores of *P. infestans* T30-4. The lesion size by *P. infestans* was significantly reduced in the regions

expressing CaNBARC114 compared with empty vectors (Fig. 2-8).

For stable expression of CaNBARC114 and resistance test to *P. infestans*, 17 transgenic potato lines carrying CaNBARC114 under CaMV-35S promoter were generated. Expressions of CaNBARC114 in 17 transgenic lines carrying its genomic DNA were confirmed by RT-PCR (Fig. 2-9a). The ranges of the expression levels were different among the transgenic lines. Then, detached leaves of all transgenic potato lines were inoculated with *P. infestans* 43072 which is a virulent isolate to confirm the resistance. At 6 dpi, disease symptom was observed in all the transgenic lines expressing CaNBARC114, which suggests that the other factors of pepper could be required for CaNBARC114-mediated resistance in potato (Fig. 2-9b).

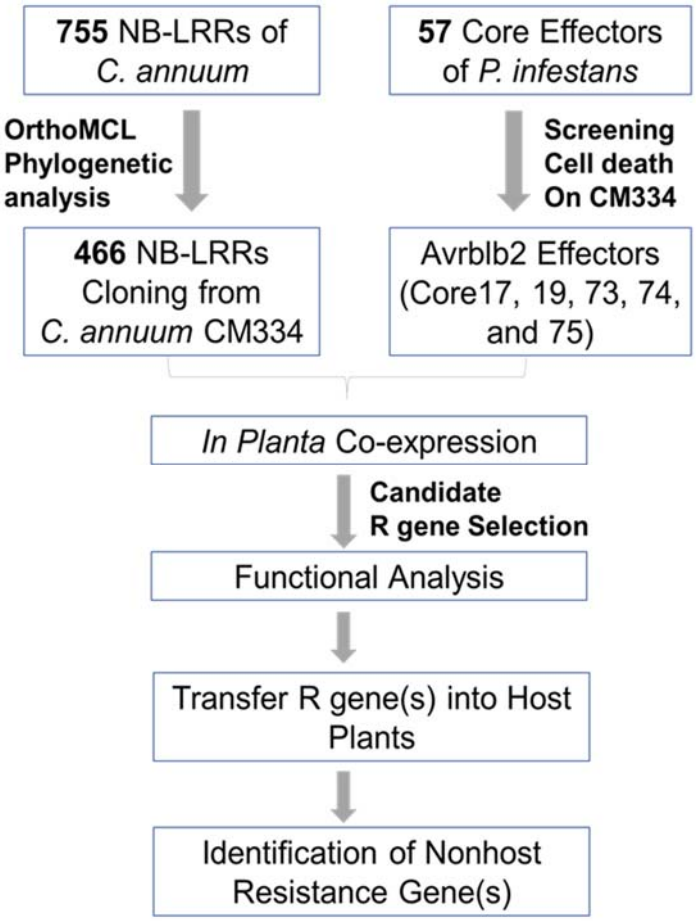

Figure 2-5. Strategy of genome-based identification of NHR genes.

The interaction between NB-LRRs and Avrblb2 effectors predicted from genome-based analysis are screened by Agro-coinfiltration assay. Resistance mediated by candidate R genes against *P. infestans* are tested in transgenic host plants.

Table 2-3. Heterologous expression of 19 pepper NB-LRRs showed autoactivity in *N. benthamiana*.

Groups	Known NB-LRR Genes	Number of Screened NB-LRRs	Number of Genes Showing Autoactivity
TNL	*N, Bs4, Gro1-4, RY-1*	51	5
CNL-G1	*CaMi, Rpi-blb2, Hero, Mi-1.2*	67	1
CNL-G2	*Bs2*	85	
CNL-G3	*R1, Prf*	23	
CNL-G4	*L1, RPS1-K, I2, R3a*	31	
CNL-G5	*R2, Rpi-blb3, Rpi-abpt*	11	2
CNL-G6	*Sw-5*	29	
CNL-G7	*Rpi-blb1*	19	1
CNL-G8	*NRC1*	14	
CNL-G9	-	48	1
CNL-G10	*RGC2, RPS2, RPS5*	33	3
CNL-G11	*Tm-2, Tm-2a*	7	1
CNL-G12	*Rx, Rx2, Gpa2*	9	
CNL-G13	*R-FOM-2*	1	
NG	*HRT, RCY1, RPP8, NRG1, RPM1*	23	5
Total		445	19

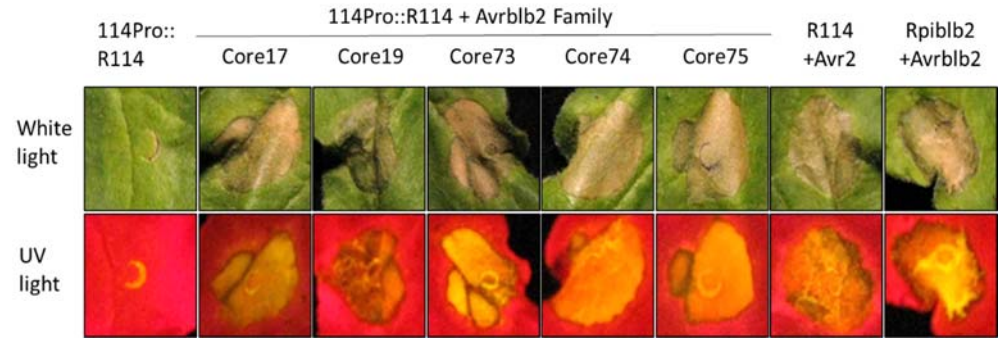

Figure 2-6. CaNBARC114 induced cell death when co-expressed with Avrblb2 core effectors and Avr2.

CaNBARC114 (R114) was transiently expressed under the control of the native promoter in *N. benthamiana*. When it was co-expressed with Avrblb2 and Avr2 (Core83) effectors, cell death was shown at 5 dpi. UV light showed autoflorescence by chlorophyll. Photographs were taken at 7 dpi.

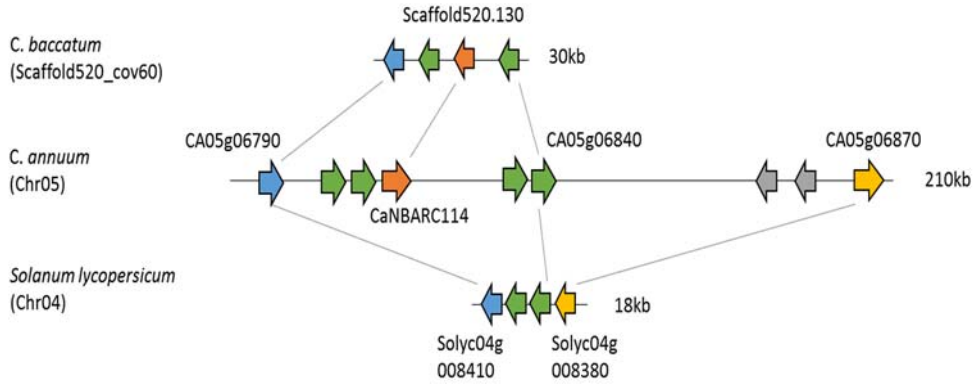

Figure 2-7. CaNBARC114 is a *Capsicum*-specific NB-LRR gene.
Synteny relationship was analyzed using SynOrth. CA05g06790 (Blue arrow) encodes pentatricopeptide repeat containing proteins and has synteny to *C. baccatum* and tomato (Solyc04g008410). Also, CA05g06840 encoding G-type lectin S-receptor-like Serine/threonine protein kinase showed conserved synteny between pepper and tomato. However, CaNBARC114 (Orange arrow) is located between CA05g06790 and CA05g06840 and has synteny only with *C. baccatum* Scaffold520.130 but not with tomato.

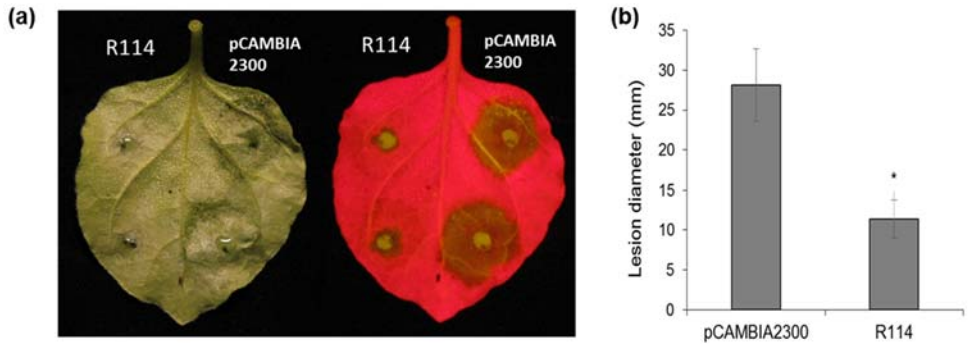

Figure 2-8. Overexpression of CaNBARC114 decreased lesion size by *P. infestans* in *N. benthamiana*.

(a) CaNBARC114 (R114) and pCAMBIA2300 as a control were transiently overexpressed in each half of the same leaf, followed by inoculation with *P. infestans*. At 5 dpi, overexpression of CaNBARC114 decreased lesion size by *P. infestans* (b) The lesion size by *P. infestans* showed a significant difference determined by a one-way ANOVA test ($p<0.05$). Error bars represent ± SE.

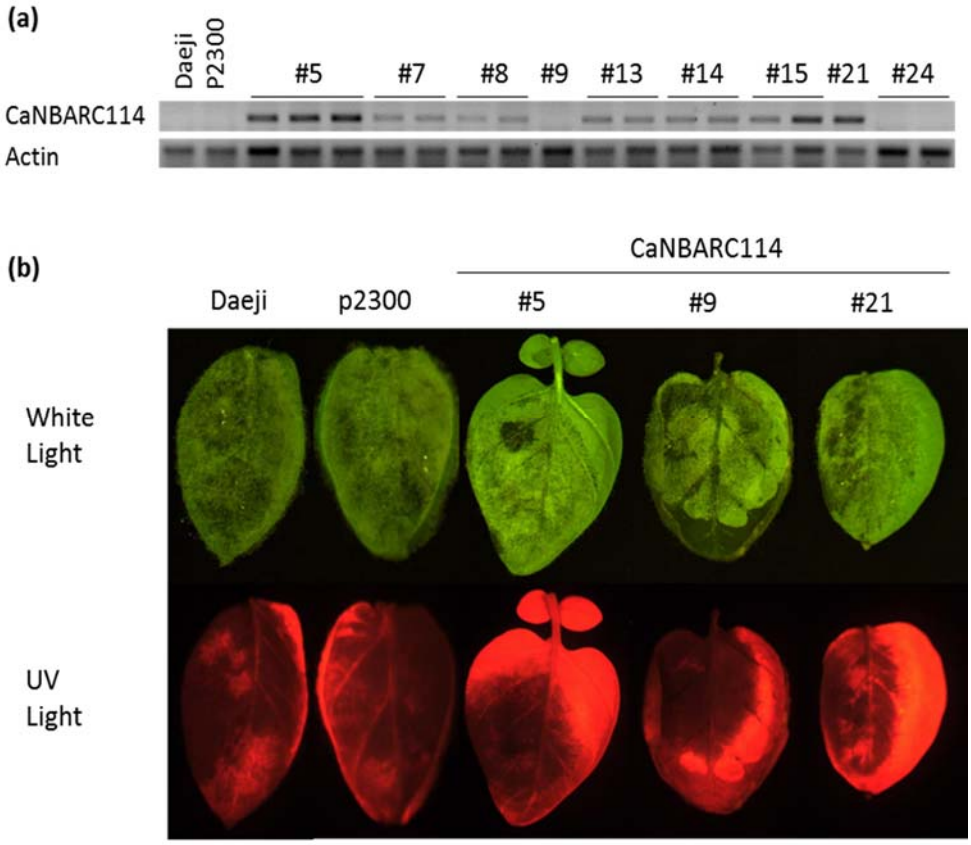

Figure 2-9. Transgenic potato expressing CaNBARC114 showed disease symptom upon inoculation with *P. infestans*.

(a) CaNBARC114 expression was confirmed using RT-PCR in nine transgenic lines.

(b) Nine transgenic lines of potato were inoculated with *P. infestans* 43072. All the transgenic lines expressing CaNBARC114 showed late blight disease symptom. UV light showed chlorophyll autoflorescence to visualize lesion area of late blight. Daeji was a cultivar used for generation of transgenic lines. p2300 represents transgenic carrying empty vector pCAMBIA2300.

DISCUSSION

ETI is considered as a major factor of NHR but few R genes have been identified as nonhost R genes due to interspecific hybrid sterility (Zhao *et al.*, 2005; Borhan *et al.*, 2008). Here, we hypothesized that NHR genes recognizing multiple effectors could confer durable resistance when transferred to a host crop plant. Genome-based identification approaches have been developed to identify nonhost R gene(s) of pepper against *P. infestans*. A total of 445 NB-LRR genes were cloned into an *in planta* expression vectors and the interaction between the genes and Avrblb2 core effectors has been screened by agro-coinfiltration assay. Among them, CaNBARC114 induced cell death when co-expressed with Avrblb2 effectors in *N. benthamiana*. Interestingly, CaNBARC114 which belongs to the group 5 having homology with R2 is not an ortholog of potato R protein Rpi-blb2 which recognizes Avrblb2 (Oh *et al*., 2009). A total of 75 pepper NB-LRR genes in the group 1 showed homology with Rpi-blb2 but none of them induced cell death when co-expressed with Avrblb2 effectors. Transient expression of CaNBARC114 in *N. benthamiana* showed significant reduction of lesion area caused by *P. infestans*. However, stable transgenic potato expressing CaNBARC114 showed no resistance against *P. infestans*. These

results raise some questions about the approach and the NHR mechanism as will be discussed below.

NB-LRR-type genes have been cloned for identification of nonhost R genes. There are considerable drawbacks in this approach. NB-LRR type genes are multi-copy gene family and associated with high levels of polymorphism (Clark *et al.*, 2007). Although the reference pepper genome is high quality, potential genomic mis-assembly or the gene missing could happen due to the repetitive and complex nature of NB-LRR gene family. Approximately 30 genes (6.3%) could not be amplified by PCR possibly as a result of mis-assembly. Also, some NB-LRR genes could be missed in the annotation, or be difficult to be predicted using the present version of pepper genome. To deal with this difficulty, Ren-seq (R gene enrichment sequencing) has been developed to discover more NB-LRR genes in tomato and potato (Jupe *et al.*, 2013). However, nonhost R genes could have relatively simple intron/exon structures because those genes might have been conserved within species and may be stably inherited such as *Rxo1* which is a nonhost R gene containing a single exon (Zhao *et al.*, 2005). In the predicted NB-LRR genes, genes having a simple structure are unlikely to be excluded from the gene annotation, which suggests that the NB-LRR constructs used in this study include potential nonhost R genes. In addition, start and termination sites of

the genes were re-analyzed using gene prediction program FgeneSH and Blast X to minimize annotation errors. Then, gene-specific primers and *Pfu* DNA polymerase were used to reduce errors in the cloning process. With several tactics, NB-LRR genes of pepper have been cloned for identification of nonhost resistance genes.

Why does the CaNBARC114 inducing cell death with *P. infestans* core effectors show no resistance against *P. infestans* in transgenic potato plants? First, other pepper proteins may be required for activation of CaNBARC114-mediated resistance. Recently, it was reported that hetero-dimer of two different R genes such as RGA4/RGA5 and RRS1/RPS4 mediate the resistance response (Cesari *et al.*, 2013; Césari *et al.*, 2014; Williams *et al.*, 2014). Similarly, other R gene(s) may be required for the activation of CaNBARC114-mediated resistance in potato. In addition, regulators for R gene activation and downstream signaling could have evolved in a specific manner in pepper. Ectopic expression of CaNBARC114 in *N. benthamiana* showed autoactivity. However, CaNBARC114 did not trigger autoactivity when it was transiently overexpressed and protein expression was confirmed in pepper, which suggests that regulators of CaNBARC114 autoactivity could be present in pepper (data not shown). In addition, it could be a suppressor or guard proteins. *Arabidopsis* mutants of SRFR1 (Suppressor of rps4-RLD)

showed autoactivity with accumulation of R proteins including SNC1, RPS2, and RPS4 (Li *et al.*, 2010). Also, RPS2 expression in *N. benthamiana* induced cell death by itself, but no cell death was observed when it was co-expressed with guard protein RIN4 (Day *et al.*, 2005). Based on the previous study, pepper-specific guard proteins could interact with CaNBARC114 and suppress its autoactivity but the potato orthologs of the guard proteins could interact with CaNBARC114 but not activate CaNBARC114-mediated resistance upon *P. infestans* inoculation. Most of identified R genes indirectly recognize effectors to guard host proteins modified by effectors and activate signal transduction pathway (Dangl and Jones, 2001). Recently, BSL1 have been identified as a guard protein to interact with Avr2 and associate with wild potato R2 protein to mediate disease resistance to *P. infestans* (Saunders *et al.*, 2012). Pepper ortholog of BSL1 (CaBSL1) was cloned because CaNBARC114 has homology with R2. CaBSL1 shared 93% amino acid identity with potato BSL1. Both CaBSL1 and CaNBARC114 were overexpressed in *N. benthamiana*, followed by *P. infestans* inoculation. However, it did not affect autoactivity of CaNBARC114 in *N. benthamiana* (data not shown).

Second, the cell death induced by Avrblb2 core effectors on pepper was not the result of R-Avr interaction. All Avrblb2 core effectors induced cell death

on all pepper accessions tested and Pro-69 mutant did not induce cell death. Based on the result, it was hypothesized that conserved putative R genes of pepper having no homology with Rpi-blb2 could recognize Avrblb2 core effectors. However, transient expression of Avrblb2 in potato triggered cell death on both resistant and susceptible *Solanum* species, suggesting that Avrblb2 effectors might perturb cell death signaling (Vleeshouwers *et al.*, 2008). Autoactivity of CaNBARC114 in *N. benthamiana* could be accelerated by Avrblb2 function. The cell death response by enhanced autoactivity of CaNBARC114 in *N. benthamiana* could not be a result of the R-Avr interaction, which could explain why CaNBARC114 showed no resistance in transgenic potato.

The hypothesis of this study that ETI is a major factor of NHR of pepper was based on rapid HR of pepper against *P. infestans* and cell death induced by multiple effectors on several pepper accessions (Lee *et al*., 2014). However, the results could not rule out the possibility that the component of NHR of pepper against *P. infestans* could be the other factors beyond ETI. Complex mechanisms such as physical barrier, antimicrobial compounds, PTI, and ETI establish NHR (Ayliffe *et al.*, 2008; Fan and Doerner, 2012). Recently, it was reported that a few PAMPs induced an HR-like cell death and PTI share defense signaling with ETI such as induction of a range of antimicrobial

compounds (Ingle *et al.*, 2006; Feechan *et al.*, 2015). Transcriptome of *P. infestans*-infected peppers gives us hints. Capsidiol synthesis-related genes were induced more than 100-fold at 6 hpi in *P. infestans*-infected peppers (unpublished data). Capsidiol, one of the well-studied phytoalexin, was isolated from pepper in 1972 and has antifungal activity (Stoessl *et al.*, 1972). FPP (Farnesyl pyrophosphate) derived from mevalonate pathway is converted to 5-epi-aristrochene by 5-epi-aristrochene synthase (EAS) and then 5-epi-aristolochene hydroxylase (EAH) converts 5-epi-aristolochene to capsidiol (Whitehead *et al.*, 1989; Facchini and Chappell, 1992; Ralston *et al.*, 2001). It has been reported that *P. infestans* is much more sensitive to capsidiol than *P. capsici* (Jones *et al.*, 1975; Giannakopoulou *et al.*, 2014). No growth of *P. infestans* was observed at 120 μM of capsidiol but *P. capsici* growth was affected by 1.5 mM or higher. *Nicotiana tabacum* which is nonhost of *P. infestans* also produces capsidiol, which suggests that capsidiol could be a main determinant of NHR (Park *et al.*, 2014; Li *et al.*, 2015). Preliminary findings that *EAS*-silenced pepper showed apparent spreading of *P. infestans* compared to negative controls indicate that capsidiol restricts *P. infestans* mainly on epidermal cells of infected tissues of pepper. Capsidiol is known to be regulated by MAPK (Mitogen-activated protein kinase) cascade which plays an important role in defense signaling of both PTI and ETI. Furthermore,

INF1 which is a major secreted elicitin of *P. infestans* induced capsidiol production in *N. benthamiana* (Yamamizo *et al.*, 2006; Matsukawa *et al.*, 2013). Identification of factors of pepper which initiate capsidiol synthesis remains challenging.

Using pepper and *P. infestans* as an experimental model, molecular basis of NHR of pepper was examined based on ETI. In this study, genome-based identification of NHR genes has been proposed but main factors of NHR of pepper against *P. infestans* still need to be characterized. Pepper and *P. infestans* have originated from different places and adapted to different environments (Aguilar-Meléndez *et al.*, 2009; Dorland & Went, 1947; Mizubuti and Fry, 1998). Favorable growth conditions of *P. infestans* is similar to growth conditions of host plants, which suggests that pathogens might evolve several weapons through co-evolution with hosts but they might have no chance to be ready for battles with nonhost plants (Antonovics *et al.*, 2013). The relationship of pepper and *P. infestans* can provide insight into the link between co-evolutionary arms race and NHR mechanism.

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ABSTRACT IN KOREAN

비기주 저항성은 한 종의 식물이 한 종의 병원균에 대해 보이는 절대적인 저항성으로 강력한 저항성 품종 육성을 위한 재료로써 주목을 받고 있지만, 그 기작은 아직 밝혀진 것이 많지 않다. 현재까지 밝혀진 기주 저항성의 기작을 바탕으로, 비기주 저항성에는 물리적/화학적 장벽을 포함하여, PTI와 ETI가 중요하게 작용할 것으로 추측하고 있다. 감자역병균은 전세계적으로 역병을 일으키고 있는 난균류의 병원균인데, 고추는 자연상태에서 감자역병균에 의해 병이 걸리지 않는 비기주 식물이다. 감자역병균을 고추에 접종하였을 때, 표피세포 수준에서 과민성 세포사멸이 일어나면서 저항성이 형성되는 것을 관찰하였고, 이것을 통하여 저항성 유전자와 병원균의 분비단백질인 effector 사이의 상호작용에 의해 나타나는 ETI가 중요한 비기주 저항성 요소일 것이라 추측하였다. 고추에서의 감자역병균 effector에 대한 반응을 살펴보기 위하여, 100종류의 고추 유전자원에 54개의 감자역병균 effector를 접종하여 나타나는 세포사멸 반응을 살펴보았을 때, 여러 개의 effector가 하나의 고추 유전자원에서 세포사멸 반응을 나타내었다. 세포사멸 반응이 저항성 유전자와 effector 사이의 상호작용에 의한 결과라는 것에 기반하여, 고추에는 여러 개의 effector와 상호작용할 수 있는 여러 개의 저항성 유전자가 존재할 것이라고 추측하였다. 그렇다면 하나의 effector에 의해 고추에서 나타나는 세포사멸 반응에 몇 개의 고추 유전자가 관여하는지 살펴보기 위하여, F2 집단에서의 Effector에 의한 세포사멸 반응의 분리비를 살펴보았다. 그 결과, 15:1, 9:7, 3:1의 분리비를 관찰할 수 있었고, 여러 개의 고추 유전자가 하나의 effector에 의한 세포사멸 반응을 조절한다는 것을 알 수 있었다. 이 결과는 기존 연구에서 비기주 저항성에 여러 개의

유전자가 관여할 것이라는 추측을 실험적으로 증명한 것이다.

다음으로, 비기주 저항성 유전자를 동정하기 위하여 유전체 정보를 활용한 동정방법을 고안하였고, 기존 저항성 유전자가 대부분이 NB-LRR의 구조를 갖는 것을 바탕으로 고추 전체 유전체에서 NB-LRR 유전자를 예측하였다. 그 중, 저항성 유전자로 기능하기 위한 중요 모티프를 갖는 445개의 유전자를 추려내어 모두 식물체 내에 발현이 가능한 벡터에 클로닝 하였다. 감자역병균의 effector 역시 비교유전체 분석을 통하여 감자역병균 내에 공통적으로 존재하며 식물체 내에서 발현이 증가하는 57개 core effector가 선발되었다. 그 중 Avrblb2 effector는 감자역병균이 병을 내는 데에 중요한 기능을 한다고 알려져 있으며, 지금까지 접종한 모든 고추 품종에 세포 사멸반응을 일으켜 비기주 저항성에 중요한 effector로 추측하였다. 클로닝 한 고추의 NB-LRR 유전자를 이용하여 Avrblb2 상호작용하는 고추의 저항성 유전자를 동정하고자 하였다. 담배를 이용하여 고추의 저항성 유전자와 Avrblb2 effector의 상호작용에 의해 나타나는 세포사멸 반응을 스크리닝 하였고, 그 결과 CaNBARC114 유전자가 Avrblb2와 함께 발현되었을 때, 세포사멸 반응이 나타남을 알 수 있었다. 저항성에 미치는 영향을 알기 위하여, CaNBARC114 유전자를 담배에 과발현 시킨 후, 감자역병균을 접종하였을 때 감자역병균의 병반이 대조군에 비해 유의하게 줄어든 것을 살펴보았다. 하지만, CaNBARC114가 형질전환된 감자에서는 감자역병균에 대한 저항성이 나타나지 않았다.

위의 결과들을 통하여 고추의 감자역병균에 대한 비기주 저항성에는 여러 개의 effector를 인지함으로써 성립되는 것을 알 수 있었고, 비기주 저항성 연구의 한계를 극복할 수 있는 유전체 정보를 이용한 저항성 유전자 동정 방법을 제시하였으며, 감자역병균의 effector와 함께 발현되었을 때 세포사멸 반응이 나타나는 고추 저항성 유전자를 찾을 수 있

었다. 더 나아가, 고추와 감자역병균, 고추와 고추역병균을 모델로 하여 공진화를 바탕으로 한 다양한 저항성 요소에 대한 연구가 비기주 저항성의 기작을 밝히는데 중요할 것으로 생각된다.