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Screening and identification of *Phytophthora infestans* core effectors that induced hypersensitive cell death on pepper (*Capsicum annuum* L.)

고추에 과민성 세포 사멸을 유도하는 감자역병균의 core effector 분리 및 동정

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SEUNGYEON SEO

MAJOR IN HORTICULTURAL BIOTECHNOLOGY
DEPARTMENT OF PLANT SCIENCE
THE GRADUATE SCHOOL OF
SEOUL NATIONAL UNIVERSITY

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BY
SEUNGYEON SEO

MAJOR IN HORTICULTURAL BIOTECHNOLOGY
DEPARTMENT OF PLANT SCIENCE
GRADUATE SCHOOL SEOUL NATIONAL UNIVERSITY

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APPROVED AS A QUALIFIED THESIS OF SEUNGYEON SEO
FOR THE DEGREE OF MASTER OF SCIENCE
BY THE COMMITTEE MEMEBERS

CHAIRMAN _____
Jin Hoe Huh, Ph.D.

VICE-CHAIRMAN _____
Doil Choi, Ph.D.

MEMBER _____
Byung-Cheorl Kang, Ph.D.

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ABSTRACT

Nonhost resistance (NHR) is a durable form of resistance in a plant species against all isolates of a pathogen species. Effector-triggered immunity (ETI) induced by the interaction between plant resistance genes and pathogen effectors is suggested as one of components to establish NHR. We inoculated 57 recombinant PVX virions harboring core effectors of *Phytophthora infestans* on pepper accessions (*Capsicum annuum* L.). Each pepper accession showed a diverse hypersensitive cell death. This result confirmed multiple interactions between pepper and *P. infestans* effectors. Among 57 core effectors, 7 effectors which induced the clear hypersensitive cell death in *C. annuum* cv. CM334 were selected for further study and

agro-infiltrated with resistance gene analogs (RGAs) to test the relationship to ETI, but not in pepper. Four effectors that showed defense response like cell death in only ECW-30R, not CM334, were chosen for an inheritance study for the genetic basis of the effector-induced cell death. Segregation ratios of F2 population from a cross between CM334 and ECW-30R were 3 : 1 and 9 : 7, indicating multiple host factors are underpin the HR response in pepper plants. This study could provide the starting points for isolation of the nonhost resistance gene of pepper against *P. infestans*.

Key words: *Capsicum annuum* L., Core effectors, Effector-triggered immunity, Hypersensitive response, Nonhost resistance, *Phytophthora infestans*

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LIST OF ABBREVIATIONS

Avr	Avirulence
CC	Coiled-coil
CE	Core effector
ETI	Effector-triggered immunity
HR	Hypersensitive response
NLPs	Nep1-like proteins
LIC	Ligation-independent cloning
LRR	Leucine-rich repeat
NBS	Nucleotide-binding site
NHR	Nonhost resistance
PAMPs	Pattern-associated molecular patterns
PTI	PAMP-triggered immunity
PRRs	Pattern recognition receptors
RXLR	Arg-X-Leu-Arg
PVX	*Potato virus X*
R	Resistance
RGAs	Resistance-gene analogs

TIR	Toll-interleukin-1 receptor

INTRODUCTION

Plant disease is the exception in nature. Nonhost resistance (NHR) is the immunity of a plant species against pathogen species including fungi, bacteria and viruses (Lee *et al*., 2014; Stam *et al*., 2014). NHR is the most durable form of resistance form of a plant species against all isolates of a pathogen species (Fan and Doerner, 2012). The mechanism of NHR involves several components, including pre-formed and induced immunity (Lee *et al*., 2014). Pre-formed immunity includes structural barriers and antimicrobial metabolites, but many pathogens succeed in breaking this barriers system (Pieterse *et al*., 2009). Plants perceive infecting pathogens and induce their innate immune system, such as pathogen-associated molecular patterns (PAMPs)-triggered immunity (PTI) and effector-triggered immunity (ETI) (Pieterse *et al*., 2009). PAMPs are conserved molecular structures in pathogens like flagellin, chitin and glycoproteins and are recognized by pattern recognition receptors (PRRs), which trigger the basal resistance that is called PTI (Pieterse *et al*., 2009). Pathogen effectors secreted into plant cells suppress PTI and promote virulence for pathogen growth and replication (Elmore *et al*., 2011). In turn, plants resistance (R) gene(s) recognize pathogen effector(s), which result in induction of a programmed cell death referred as hypersensitive response (HR) at an infection site (Pieterse *et al*., 2009).

There is an evolutionary model that ETI is a key element of NHR in nonhost species closely related with host species and some evidences support the model (Schulze-Lefert and Panstruga, 2011). Potato, tomato and pepper belong to Solanaceae family. The oomycete *Phytophthora infestans* causes potato late blight

disease in potato and tomato, but not in pepper. The effector activity of *P. infestans* 54 RXLR were screened in pepper accessions (*Capsicum* spp.) and several effectors induced HR on pepper which suggests that ETI is main factor of NHR of pepper against *P. infestans* (Lee *et al*., 2014). Nonhost R genes, WRR4 and RXO1, have been identified from *Arabidopsis* and maize, respectively. Host plants transformed with those nonhost R genes showed resistance against cognate pathogens (Borhan *et al*., 2008; Zhao *et al*., 2005). NHR induced by R genes may be conserved across plant lineages (Maekawa *et al*., 2012).

Most of plant R genes encode nucleotide-binding site (NBS) domain and leucine-rich repeat (LRR) domain (Elmore *et al*., 2011; Yue *et al*., 2012). NBS-LRR proteins can be divided two major groups according to N-terminal domains, Toll/interleukin-1 receptor (TIR) or coiled-coil (CC) domain (Heidrich *et al*., 2012; Michelmore *et al*., 2013). Genome-wide analysis revealed that hundreds of NBS-LRR genes exist in plant genomes, suggesting the importance of the NBS-LRR family (Yue *et al*., 2012). Especially, pepper has approximately 700 NBS-LRR genes with orthology to known R genes of Solanaceae (Kim *et al*., 2014 ; Yeom *et al*., unpublished).

The oomycete *P. infestans* secrets hundreds of effectors that carry a conserved amino-terminal motif Arg-X-Leu-Arg (RXLR, X means any amino acid.), required for translocation into plant cells (Cooke *et al*., 2012; Haas *et al*., 2009; Kamoun, 2006). The genome of *P. infestans* T30-4 has been sequenced and approximately 550 RXLR effectors were predicted (Haas *et al*., 2009). The genome of aggressive isolates including 13_A2, NL07434, and US22 revealed that there are

conserved effectors which could be sensed by resistance genes (Cooke *et al*., 2012). Among the conserved effectors, 57 effectors were chosen as core effectors of *P. infestans* with the induction of *in planta* expression (Witek *et al*., unpublished). The core effectors of *P. infestans* were cloned into binary *Potato virus X* (PVX)-based vector for screening of functional expression *in planta* (Torto *et al*., 2003). PVX-based transient expression provides an efficient approach for analyzing plant-microbe interaction between *P. infestans* and pepper accessions (Lee *et al*., 2014).

With developed sequencing technology, plant resistance genes and pathogen effectors could be predicted from genome sequences, which make it possible to overcome the difficulty of classical genetic approach to study NHR. In this study, we primarily screened cell death induced by core effectors of *P. infestans* on three pepper cultivars including CM334 to select candidate effectors which could interact with pepper resistance genes. With candidate effectors, we screened the interaction with pepper R gene analogs (RGAs) cloned from CM334 and performed inheritance study of effector-induced cell death. This study present evidence of multiple interaction in nonhost resistance between pepper and *P. infestans* and provide the basis to identify nonhost resistance genes.

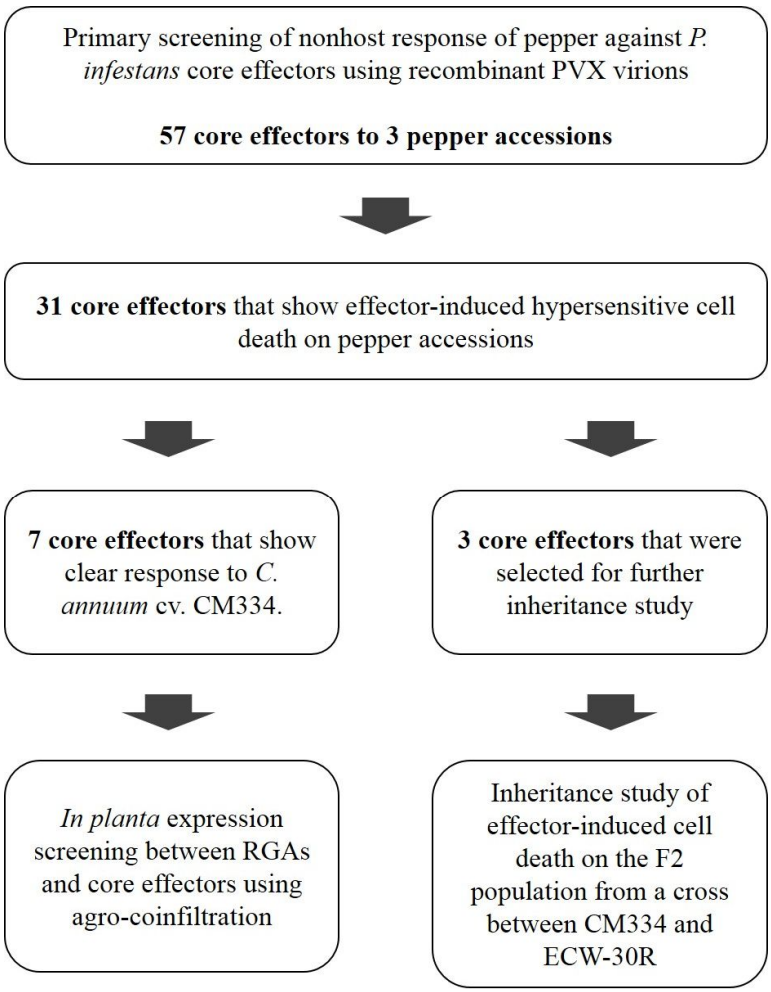

Figure 1. Work flow in this study.

LITERATURE REVIEW

Nonhost resistance

Disease is the exception in nature, as most plants have a resistance to a broad range of pathogens including fungi, bacteria and viruses (Stam *et al*., 2014). This resistance form is known as NHR, defined that a plant species has a durable resistance against all isolates of a pathogen species (Fan and Doerner, 2012). However, NHR is poorly understood.

The mechanism of NHR involves several components, such as the pre-formed and induced immunity. Pre-formed immunity includes structural barriers and antimicrobial metabolites (Pieterse *et al*., 2009). Plant surfaces are layered with the cuticle which acts first physical barriers against plant pathogens (Thordal-Christensen, 2003). For example, wax of the cuticle component is important to stop the development of an appressorium of fungi (Tsuba *et al*., 2002). Also, plants produce antimicrobial enzymes and secondary metabolites to inhibit the growth of pathogens, including saponins, phenolics, isoflavonoids and many others (Papadopoulou *et al*., 1999).

However, many pathogens succeed in breaking the previous immunity system and face another plant defense system, called induced immunity (Stam *et al*., 2014). Pathogens have common features like flagellin and chitin, referred to a PAMPs, which are highly conserved within a class of pathogens (Jones and Dangl, 2006). PRRs in the cell surface recognize PAMPs and activate innate immune responses preventing pathogens proliferation, so called PTI (Pieterse *et al*., 2009).

Pathogens could overcome and suppress PTI by secreted effectors and promote a virulence for their growth and replication (Elmore *et al*., 2011). In turn, plants R gene recognizes pathogen effectors, resulting that ETI induces a programmed cell death, referred as HR (Thomma *et al*., 2011). A series of induced immunity response in a plant-pathogen interaction is depicted by the zigzag model (Jones and Dangl, 2006).

Resistance gene

According to the gene-for-gene hypothesis for plant-pathogen interactions, R gene(s) recognizes effector(s) which is secreted by pathogen and encodes a central NBS domain and a C-terminal LRR domain (Michelmore *et al*., 2013). NBS-LRR proteins are a large and variable gene families in plants (Yue *et al*., 2012). NBS domain consists of 3 subdomains such as the NB, ARC1 and ARC2 (which is shared by Apaf1, certain R genes and CED4) with conserved motifs including the P loop, MHD, GLPL and others (Meyers *et al*., 1999; Takken and Goverse, 2012). Subdomains of NBS domain form a STAND super family, then plant immune system could be progressed (Takken and Goverse, 2012). In other case of LRR domain, a LRR is consist of 20 to 29 amino acids having a conserved, 11-residue LxxLxLxxN/CxL motif. LRR domain constitutes protein-protein and protein-ligand interactions and has an important role for specific recognition of pathogen effectors (Gururani *et al*., 2012; Michelmore *et al*., 2013). NBS-LRR proteins can be divided into several groups, based on the amino acid motif organization and there are two major groups according to N-terminal domains, TIR

or CC domain (Gururani *et al*., 2012; Heidrich *et al*., 2012). NBS-LRR proteins directly and indirectly recognize specific pathogen effectors and trigger strong plant defense system, resulting in localized programmed cell death called HR (Michelmore *et al*., 2013). Therefore knowing R genes is important to establish a durable resistance of diverse plants.

P. infestans core effectors

The oomycete *P. infestans* is the destructive plant pathogen and infects important staple crops like potato and tomato worldwide (Cooke *et al*., 2012). Most importantly, *P. infestans* causes potato late blight, known as a cause of the Irish potato famine during the 19th century. During infection, oomycete secretes hundreds of effectors into the plant cell for manipulating plant physiology to promote host infection and colonization and effectors can be recognized by plant R genes (Haas *et al*., 2009; Jones and Dangl, 2006). Although the mechanism of oomycete secretion system remains poorly understood, oomycete effectors carry a conserved amino-terminal motif, RXLR, following a signal peptide of short, N-termianl, amino-acid sequences (Kamoun, 2006). RXLR motif is required for translocation into the cytoplasm of plant cells (Cooke *et al*., 2012; Haas *et al*., 2009; Kamoun, 2006). After translocation into plant cell, effector domain following RXLR motif encodes the protein for the biochemical activity (Win *et al*., 2012).

The genome of *P. infestans* has been sequenced including the reference genome of T30-4 and aggressive isolates, 13_A2, NL07434, and US2. Based on the genomes, the genes encoding hundreds of RXLR effectors were predicted and

conserved RXLR effectors in all four isolates were selected as core effectors (Cooke *et al*., 2012). Also, all selected core effectors showed the expression in host plants during 2 to 3 days after infection.

MATERIALS AND METHODS

Plant materials and growth conditions

C. annuum cv. AC09-226 were provided from Dr. Byoung-Cheorl Kang (Seoul National University, Seoul, Korea). Two pepper accessions of *C. annuum* cv. CM334 and ECW-30R were crossed for inheritance study in controlled glasshouse under similar conditions to walk-in chamber. Seeds of peppers were sterilized by dipping in 0.1% sodium hypochlorite (NaOCl) for 1 min and washed away with tap water. Then, seeds were germinated at 30℃. After radicle has emerged, seedlings were planted in 200-plug trays with horticultural bed soil (Baroker, Seoul Bio co., Ltd, Seoul, Korea) and transferred to a walk-in chamber under 16 h photoperiod at 22 to 24℃ with 55% humidity. As cotyledons were fully expanded, seedlings were transplanted into 50-plug trays for screening. Seeds of *N. benthamiana* were directly sown in pots filled with soil and grown at the same conditions as described above.

Core effectors of *P. infestans*

A. tumerfaciens Agl1 carrying *P. infestans* 57 core effectors were provided from Dr. Sophien Kamoun (The Sainsbury Laboratory, Norwich, UK) (Table 2). All core effectors were cloned into a binary *Potato Virus X* (PVX)-based vector pICH31160. For *in planta* expression, core effectors were transferred to *A. tumefaciens* strain GV3101. Both pICH31160-mCherry and pICH31106-NPP1 or

Avrblb2 family effectors were used as negative and positive controls, respectively.

Table 1. Pepper accessions used in this study.

Pepper accessions	Species	Cultivar name	Origin
CM334	*C. annuum*	Criollo de Morelos 334	Mexico
ECW-30R	*C. annuum*	Early Cal wonder	USA
AC09-226	*C. annuum*	Serrano	USA

Core effector name	Gene_ID	Annotation	Secretion
CE14	PITG_00582		YES
CE16	PITG_02860		YES
CE17	PITG_04085	AVRblb2 family	YES
CE18	PITG_04089	PexRD41	YES
CE19	PITG_04090	AVRblb2 family	YES
CE21	PITG_04266		YES
CE23	PITG_04269		NO
CE24	PITG_04276		NO
CE25	PITG_04314	PexRD24	YES
CE26	PITG_06087	PexRD16	YES
CE27	PITG_06099	PexRD50	YES
CE29	PITG_06478	PexRD18	YES
CE31	PITG_07550		YES
CE32	PITG_07630		YES
CE33	PITG_09160	PexRD3	NO
CE34	PITG_09216		YES
CE35	PITG_09218	PexRD52	NO
CE36	PITG_09732		YES
CE38	PITG_10232		YES
CE39	PITG_10540	PexRD5	YES
CE40	PITG_10654		YES
CE41	PITG_11947	PexRD33	YES
CE45	PITG_12057		NO
CE46	PITG_12737		YES
CE47	PITG_13044		NO
CE49	PITG_13048		NO
CE50	PITG_13093		YES
CE51	PITG_14371	AVR3a, PexRD7	YES
CE52	PITG_14884		NO
CE54	PITG_15039		YES
CE55	PITG_15110		YES
CE56	PITG_15114		NO
CE58	PITG_15123		NO
CE60	PITG_15125		NO
CE62	PITG_15127		NO
CE64	PITG_15278		YES
CE65	PITG_15753		YES
CE66	PITG_15930		YES
CE67	PITG_16275		NO
CE68	PITG_16294	AVRvnt	YES
CE69	PITG_16705		YES
CE70	PITG_17063	PexRD44	YES
CE71	PITG_17309		YES
CE72	PITG_17316		YES
CE73	PITG_18683	AVRblb2 family	YES
CE74	PITG_20300	AVRblb2 family, PexRD39	YES
CE75	PITG_20301	AVRblb2 family	YES
CE76	PITG_20922		NO
CE77	PITG_21362		YES
CE78	PITG_21388	AVRblb1, PexRD6	YES
CE79	PITG_21740		YES
CE80	PITG_22547		YES
CE81	PITG_22648		YES
CE82	PITG_22804		YES
CE83	PITG_22870	AVR2	YES
CE85	PITG_22922		YES
CE86	PITG_23226		YES

Table 2. Description of the tested core effector of RXLR type.

Cloning of resistance-gene analogs

RGAs of *C. annuum* cv. CM334 were predicted and classified by Dr. Seon-in Yeom (Gyeongsang National University, Jinju, Korea). Gene-specific primers for RGAs were designed using primer design tool based on pepper genome v.1.5. Primers were designed to have melting temperature ranging from 56 to 60℃. For ligation independent cloning (LIC) method, all inserts had complementary overhangs with LIC vectors using the following primers: Forward- 5′ CGACGACAAGACCCT and reverse- 5′ GAGGAGAAGAGCCCT (Oh *et al*., 2010). Inserts were amplified by PCR with PrimeSTAR GXL DNA polymerase (TAKARA Bio inc., Shiga, Japan) using templates of genomic DNA of *C. annuum* cv. CM334 and purified by gel elution.

pCAMBIA2300-LIC vectors were generated by modification of pCAMBIA2300 vector. For LIC method, pCAMBIA2300-LIC vectors were digested by *Pst*I (New England Biolabs, MA, USA) for 1 h at 37℃. The linearized vectors were purified by gel elution.

Compatible overhangs were generated by treating T4 DNA polymerase (New England Biolabs) with 100 μM deoxyadenosine triphosphate (New England Biolabs) for inserts and 100 μM deoxythymidine triphosphate (New England Biolabs) for pCAMBIA2300-LIC vectors for 30 min at 22℃. For inactivating polymerase activity, both inserts and LIC vectors were incubated for 20 min at 75°C. The T4 DNA polymerase treated LIC vectors were mixed with each inserts and incubated for 20 min at room temperature and transformed into competent

Escherichia coli DH5α cell.

Inoculation of pepper leaves with recombinant PVX virions

A. tumefaciens carrying core effector genes in binary vector pICH31160 were cultured at 30℃ in YEP medium supplemented with kanamycin at 50 mg/L and rifampicin at 50 mg/L for 1 d, spun at 3000 rpm and the pellet was resuspended in infiltration buffer (10 mM $MgCl_2$, 10 mM MES and 150 μM acetosyringone). *A. tumefaciens* cultures were diluted to a final OD_{600} = 0.3 to 0.4 and incubated with gentle shaking at room temperature for 3 h. *A. tumefaciens* cultures were infiltrated into the expanded leaves of 2 week-old *N. benthamiana* with a 1 ml syringe without a needle. For sap transmission, 1 g of upper leaves that showed systemic symptoms of PVX were picked and grounded in 5 ml of 50 mM potassium phosphate buffer (pH 7.4) using a pestle and mortar at 7 days post inoculation (dpi). The leaves of four week-old pepper were dusted with 400-mesh carborundum and gently rubbed after dropping 30 μl inoculum. Cell death symptoms on the inoculated leaves were monitored from 5 to 7 dpi. The chlorophyll of the leaves showing cell death were destaining with 100% ethanol for overnight.

Agro-coinfiltration using RGAs and candidate effectors

A. tumefaciens cultures were incubated at the same conditions described previously. *A. tumefaciens* cultures were diluted to the OD_{600} = 0.4 for those

carrying core effector genes and the OD_{600} = 0.75 for those carrying RGAs genes. Each genes was incubated with gentle shaking at room temperature for 3 h. For the transient co-expression assays, cultures were mixed at 1:1 ratio and infiltrated into the expanded leaves of 4 to 6 week-old *N. benthamiana* with a 1 ml syringe without a needle. Cell-death symptoms at the infiltrated sites were monitored from 5 to 7 d.

RESULTS

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

Core effectors are conserved in four aggressive *P. infestans* isolates (Witek *et al*., unpublished) and show induced expression at biotroph stage followed by inoculation of host potato with *P. infestans* (Cooke *et al*., 2012). To identify whether *P. infestans* core effector induced cell death in pepper, we inoculated 57 core effectors in three pepper accessions including CM334, ECW-30R and AC09-226. In the previous study, 54 RXLR effectors of *P. infestans* were expressed on pepper accessions using the recombinant PVX virions to validate the effector-induced cell death (Lee *et al*., 2014). For high efficiency, core effectors were also cloned into PVX-based transient expression vector, pICH31160 (Scholthof *et al*., 1996). To develop recombinant PVX virions by *P. infestans* core effectors on peppers, *A. tumerfaciens* carrying core effector genes were infiltrated into *N. benthamiana,* then, upper leaves showing PVX symptoms were harvested. Each pepper accessions was inoculated with recombinant PVX virions carrying mCherry for negative control. Core effectors including CE17, CE19, CE73, CE74, and CE75 belonging to Avrblb2 family were used for positive control according to previous study (Lee *et al*., 2014).

The result of screening cell death on pepper triggered by core effectors in pepper accessions is given in Table 3. Among 57 core effectors, 31 effectors induced cell death response in pepper accessions. Twenty-three and 24 core effectors induced clear cell death response in CM334 and ECW-30R, but only 10

core effectors in AC09-226, respectively (Figure 2a). Eight core effectors of CE17, CE19, CE58, CE66, CE73, CE74, CE75 and CE86 presented cell death on all pepper accessions. Avrblb2 family effectors which showed 99% amino acid identity were broadly recognized by all pepper accessions similar with previous study (Lee *et al*., 2014). Of the eight core effectors, CE58, CE66 and CE86 had no significant homology to cloned RXLR effectors of *P. infestans* and each effectors did not share any similarity with other effectors. Interestingly, there was no core effector which caused cell death in only AC09-226, suggesting that conserved genes which recognize those *P. infestans* effectors may exist in pepper. Of fifty-seven core effectors, four core effectors are already known as Avr genes, CE51 in Avr3a, CE68 in Avrvnt, CE78 in Avrblb1 and CE83 in Avr2. In other words, those effectors have corresponding R genes and avirulence in host plants potato. None of them induced cell death in pepper accessions. This screening confirmed multiple interactions between *P. infestans* core effectors and pepper accessions, suggesting that resistance by R gene(s) confer NHR of pepper.

Table 3. Hypersensitive response induced by 57 core effectors in pepper accessions and *N. benthamiana* using recombinant PVX virions

Core effector	Pepper accesions			*N. benthamiana*
	CM334	ECW-30R	226	
CE14	-	+	-	+
CE16	-	+	-	-
CE17	+	+	+	-
CE18	-	-	-	-
CE19	+	+	+	-
CE21	-	+	-	-
CE23	-	-	-	-
CE24	-	-	-	-
CE25	-	-	-	+
CE26	-	-	-	+
CE27	-	-	-	-
CE29	-	-	-	+
CE31	+	+	-	-
CE32	-	-	-	-
CE33	-	-	-	+
CE34	+	-	-	+
CE35	+	-	-	+
CE36	+	-	+	+
CE38	-	-	-	+
CE39	+	+	-	+
CE40	+	+	-	+
CE41	+	-	-	-
CE45	-	-	-	-
CE46	-	-	-	-
CE47	-	+	-	+
CE49	+	+	-	+
CE50	-	-	-	-
CE51	-	-	-	-
CE52	-	-	-	-
CE54	-	-	-	+
CE55	+	-	-	-
CE56	-	+	-	+
CE58	+	+	+	-
CE60	-	-	-	-
CE62	-	-	-	-
CE64	-	+	-	-
CE65	+	+	-	+
CE66	+	+	+	-
CE67	-	+	-	-
CE68	-	-	-	+
CE69	-	-	-	+
CE70	+	+	-	+
CE71	-	-	-	+
CE72	-	-	-	+
CE73	+	+	+	-
CE74	+	+	+	-
CE75	+	+	+	-
CE76	-	-	-	-
CE77	+	+	-	+
CE78	-	-	-	+
CE79	+	-	+	-
CE80	-	+	-	-
CE81	-	-	-	-
CE82	+	+	-	+
CE83	+	-	-	-
CE85	-	-	-	-
CE86	+	+	+	+
Total HR	23	24	10	25

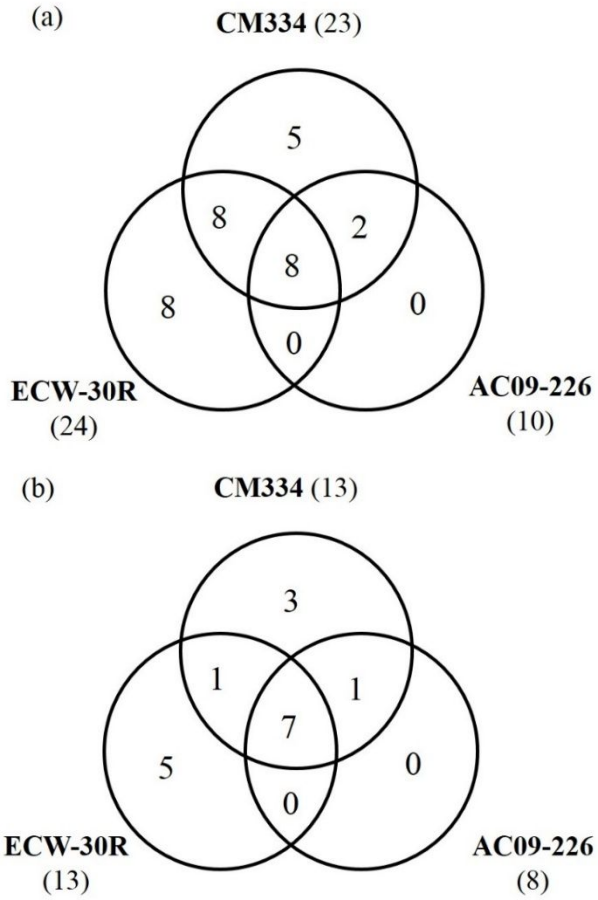

Figure 2. Venn diagram of cell death response against *P. infestans* core effectors in pepper accessions.

(a) Number of effector showed HR in each pepper accessions. Of 57 core effectors, 31 core effectors induced cell death in pepper. Eight effectors including Avrblb2 family effectors, CE58, CE66 and CE86 commonly caused cell death in all three pepper accessions. (b) Number of effector showed no cell death in *N. benthamiana* but showed cell death in pepper accessions. Thirteen core effectors showed cell death in CM334 and was selected for further study.

Screening for the interaction of RGAs of *C. annuum* cv. CM334 with *P. infestans* candidate core effectors.

After primary screening, we selected candidate core effectors of *P. infestans* for co-expression screening with *C. annuum* cv. CM334 RGAs in *N. benthamiana* using agro-coinfitration to test the NHR interaction between effector and the R gene (Figure 3). Agroinfiltraion has many advantages such as stable, speedy and inexpensive transformation procedure, compared to other transient expression system (Ma *et al*., 2012). The main benefit of agroinfiltration using syringe is that both core effector genes and RGAs can be expressed together in plants. Candidate effectors can be identified through hypersensitive cell death in infiltrated site when RGAs are corresponding R genes (Wroblewski *et al*., 2009). Besides, *N. benthamiana* was chosen for interaction screening because it has no cell death response against most *Agrobacterim* strains (Goodin *et al*., 2008). Upon agro-coinfiltration with RGAs and effectors, hypersensitive cell death response has been monitored as a result of the interaction. All core effector were infiltrated in *N. benthamiana* to confirm the necrotic response on their own because it interrupts the interaction screening. Thirty-two core effectors did not induce cell death in *N. benthamiana*, shown in Table 3. Of 32 core effectors, 7 effectors including CE31, CE41, CE55, CE58, CE66, CE79 and CE 83 surely induced cell death in *C. annuum* cv. CM334 (Figure 2b) and were finally selected for candidate core effectors for further study of interaction with RGAs (Figure 3).

In the pepper genome, 755 NBS-LRR family genes were predicted and clustered into thirteen CNL groups and one TNL group (Yeom *et al*., unpublished

data). NBS domain has conserved motifs which are important for proper function of R genes. Among 755 genes, 289 genes were excluded for cloning due to the gene structure of partial or pseudo genes. A total of 466 genes were selected as candidates for *in planta* interaction study and we have cloned the NBS-LRRs using LIC method (Yeom *et al*., unpublished). Comparative genome analysis in Solanaceae revealed that pepper has 158 RGAs classified with cloned functional R genes in *Solanum* spp. such as R1, R2, R3a, Rpiblb1, Rpiblb2, Rpiblb3 and Rpiabpt (Table 4). Sixty-one RGAs were cloned into pCAMBIA2300-LIC vector and transformed into *Agrobacterium* strain GV3101 for *in planta* co-expression with seven core effectors. Rpiblb2 and Avrblb2 effector were used as positive control. As a result, no combination of RGAs and core effectors induced hypersensitive cell death in *N. benthamiana*. This result indicated that pepper R genes which belong to other could interact with the effectors of *P. infestans*.

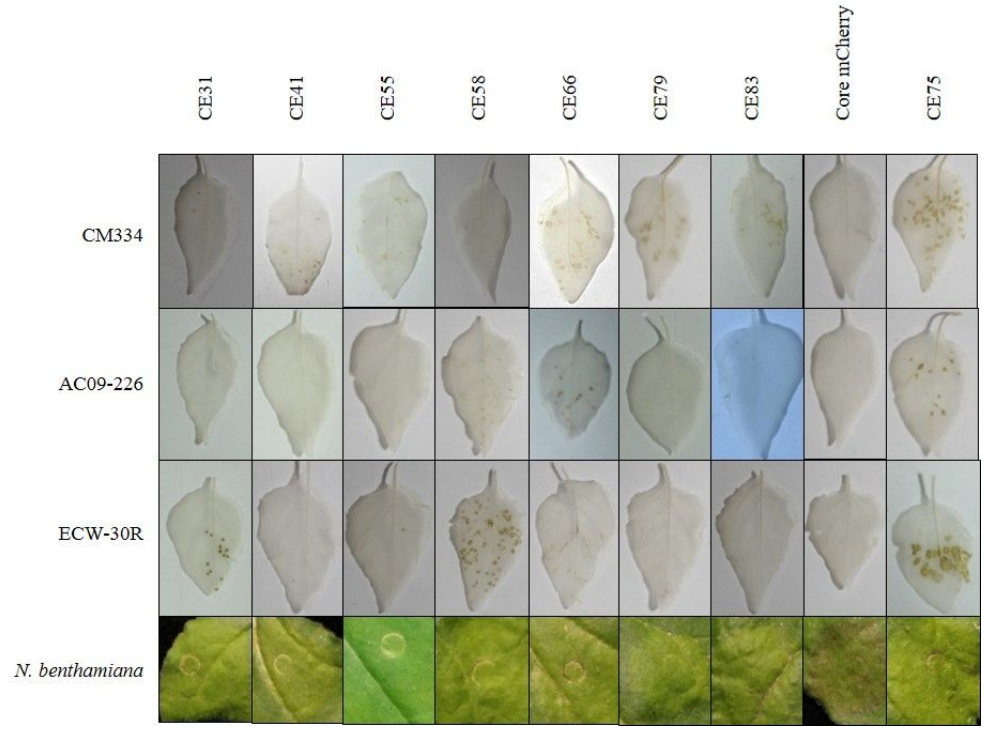

Figure 3. Effector-induced cell death by candidate core effectors on three pepper accessions and *N. benthamiana*

Seven core effectors showing no hypersensitive response in *N. benthamiana*, surely, induced cell death in *C. annuum* cv. CM334. Candidate core effectors were selected for co-expression with RGAs in *N. benthamiana* using agro-coinfitration.

Table 4. Interaction screening between *C. annuum* cv. CM334 RGAs with candidates core effectors.

Sub-group	Cloned R genes in *Solanum* spp.	No. of pepper RGAs	No. of cloned RGAs	No. of agro-coinfiltrated RGAs	No. of hypersensitive cell death
CNL-G1	Rpiblb2	75	54	34	-
CNL-G3	R1	23	7	2	-
CNL-G4	R3a	32	15	-	-
CNL-G5	R2, Rpiblb3	10	10	10	-
CNL-G7	Rpiblb1	18	15	15	-
Total number		158	101	61	-

Multiple loci determine response of pepper to *P. infestans* core effectors.

To test a genetic basis of effector-induced cell death in primary tested pepper accessions, CM334 and ECW-30R were crossed to develop an F2 population. Based on the previous screening, two pepper accessions were showed clear cell death response against *P. infestans* core effectors. Three core effectors of CE64, CE67 and CE80 were selected for inheritance study to the genetic basis of effector-induced cell death in pepper (Figure 4). Those effectors did not induce any cell death response in CM334 but induced cell death in ECW-30R. Recombinant PVX virions of three core effector were inoculated in hundreds of F1 and F2 plants. Dozens of F1 and F2 plants were also inoculated with recombinant PVX virions carrying mCherry as a negative control and showed no cell death suggesting that PVX did not affect the cell death phenotype on F1 and F2 plants. After seven days post inoculation, all inoculated plants were evaluated determining the cell death.

The cell death response in all F1 plants inoculated with candidate effectors suggested that the cell death phenotype is controlled by dominant genes. The segregation ratios of effector-induced cell death in F2 populations are shown in Table 5. The cell death phenotype induced by CE64 and CE67 segregated in a 3 : 1 ratio, indicating a single dominant gene is involved in the interaction with the effectors. The F2 segregation of cell death by CE80 was 9 : 7 ratio, suggesting two complementary dominant gene loci in epistasis. These results suggest multiple interactions between *P. infestans* core effector and pepper genes.

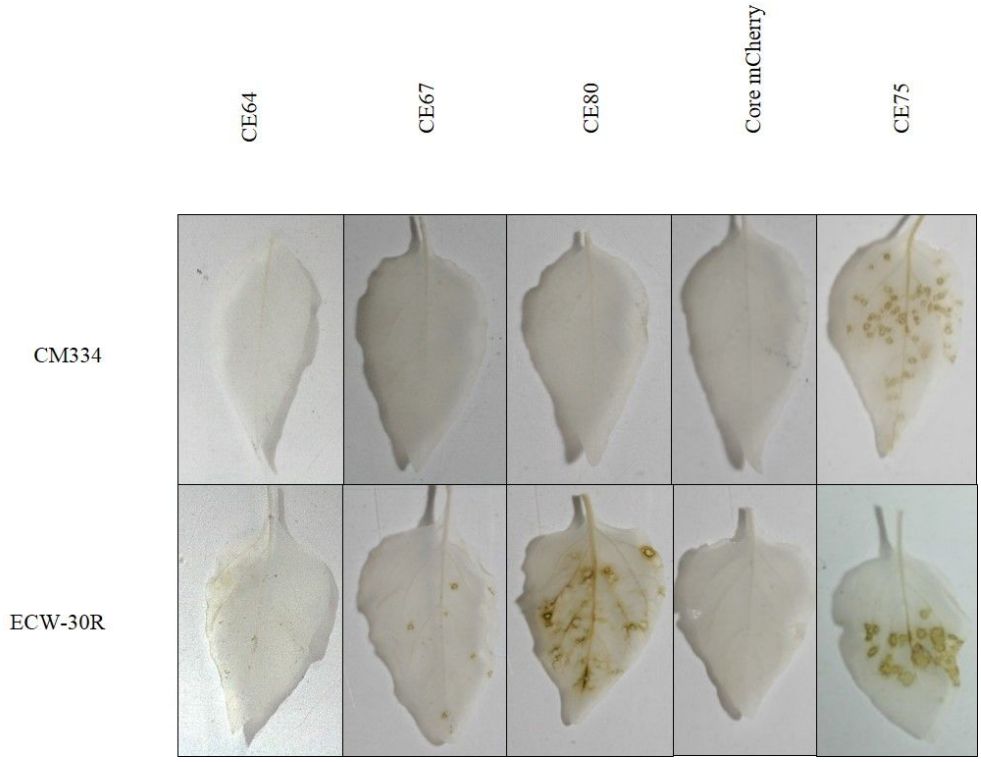

Figure 4. Hypersensitive cell death response of both CM334 and ECW-30R by candidate core effectors.

CE64, CE67 and CE80 did not show any cell death in CM334, but induced the clear cell death in ECW-30R. These effectors were used for the inheritance study to the genetic basis of effector-induced cell death in F1 and F2 plants obtained from a cross between CM334 and ECW-30R.

Table 5. Segregation of effector-induced cell death in the F1 and F2 population derived from a cross between CM334 and ECW-30R.

	F1	F2 (pepper accessions of *C. annuum cv.* CM334 x ECW-30R)				
		Observed ratio		Expected ratio		
Core effectors	HR+/HR-	HR+	HR-	HR+/HR-	Chi-square	P-value
CE64	14 : 0	89	26	3 : 1	0.350	0.553
CE67	16 : 0	114	33	3 : 1	0.510	0.475
CE80	19 : 0	85	63	9 : 7	0.084	0.771

DISCUSSION

The high-quality reference genome of various organisms gives an understanding of plant-pathogen interactions and could be a key tool to improve a plant resistance (Schneider and Collmer, 2010). The genome-wide analysis of resistance-gene analogs (RGAs) from *C. annuum* cv. CM334 and core effectors of *P. infestans* are powerful and effective tools to identify an R gene with a corresponding *Avr* gene. Previously, we investigated a NHR interaction related to ETI between three pepper accessions and core effectors of *P. infestans* and monitored HR responses as a result of interaction. The oomycete *P. infestans* causes a severe disease in the potato and tomato, not pepper, in the Solanaceae family. Pepper which is a nonhost plant having close evolutionary distance with host plants might have the R gene(s) which recognizes *P. infestans* RXLR effector, based on ETI (Schulze-Lefert and Panstruga, 2011). Lee *et al*., 2014 demonstrated that multiple recognitions of 54 RXLR effectors of *P. infestans* are associated with nonhost resistance of pepper and suggested that multiple pepper R gene(s) could interact with the effectors of *P. infestans*. However, 54 effectors used in Lee *et al*., 2014 are approximately 10% of *P. infestans* effectors therefore, these effectors do not represent other *P. infestans* effectors. To overcome this limitation, we used core effectors identified from four genomes of aggressive *P. infestans* isolates. According to our results, pepper accessions showed diverse numbers of the cell death against core effectors, suggesting that there are several possibilities why each pepper cultivars differently recognizes diverse effectors of *P. infestans*. First of all, among 57 core effectors, all tested pepper accessions

responded against eight core effectors, meaning that pepper accessions have conserved R genes or guard proteins responding eight core effectors. Second, there are no effectors inducing the cell death only in AC09-226. We supposed that AC09-226 is a germ plasm collected from USA and considered as a domesticated pepper. The domesticated plants were optimized for a human preference like taste, color and size, leading to a reduction in a genetic diversity in the plant resistance (Aguilar-Melendez *et al*., 2009). We suggest the reason that AC09-226 showed the lower number of cell death response than other accessions, is a lack of resistance sources due to the domestication. Third, effectors, five in CM334 and eight in ECW-30R, induced cell death in only one pepper accessions. There are several explanations. The reason of the cultivar-specific phenotype is that CM334 and ECW-30R have biologically lots of differences. CM334 is a landrace cultivar from Mexican, Criollo de Morelos 334 and has displayed a high level of resistance to various pathogens like *P. capsici* and potyviruses (Pegard *et al*., 2005). ECW-30R is an isogenic line of Early Calwonder (ECW), obtained originally from USA. ECW-30R has a thick wall, called bell pepper, but no pungency. During domestication, ECW-30R was optimized for other characters more than the sources of resistance against pathogens. As well as, R genes are one of the reasons for the cultivar-specific cell death. Most R genes encode proteins with NBS and LRR domains. Because each domain has exerted different evolutionary rates, NBS domain is usually conserved and LRR domain is diverse (Zhou *et al*., 2004). Consequently, R gene are highly polymorphic depending on the cultivar (Yue *et al*., 2012). R genes of both CM334 and ECW-30R could show high polymorphism and then two pepper accession showed cultivar-specific cell death.

The necrotic response in *N. benthamiana* is important for the interaction screening using agro-coinfiltration. However, 25 core effectors caused the cell death response in the infiltration site on *N. benthamiana*. In spite of core effectors predicted to RXLR effectors, they act like Nep1-like proteins (NLPs) which induced the hypersensitive-like cell death in variety of plants (Pemberton and Salmond, 2004). The hemibiotrophic oomycete *P. infestans* penetrates a living plants cell like biotrophic pathogens, after then makes extensive necrosis for colonization and sporulation by necrosis-inducing proteins like NLPs (Kanneganti *et al*., 2006; Pemberton and Salmond, 2004). Of 25 core effectors, 11 effectors did not induced cell death on all pepper accessions, suggesting that the hypersensitive-like cell death by NLPs depends on the plant species in Solanaceae crop plants. We expressed core effectors in *N. benthamiana* using *Agrobacterium*-mediated method and in pepper accessions using PVX-mediated method. We hypothesized the infiltration and inoculation of effectors resulted in effectors inside of each plant cell. However those mechanisms are not completely benign. Accordingly, *N. benthamiana* and pepper accessions responded differently against same core effectors.

After we excluded core effectors showing cell death in *N. benthamiana*, candidate core effectors were co-expressed with RGAs of *C. annuum* cv. CM334 to identify R gene(s) which recognize effectors of *P. infestans*. We observed no cell death between RGAs and core effectors. There could be several reasons of no cell death. First of all, of 466 RGAs of CM334, two-third RGAs were cloned and only one-third RGAs were co-expressed with candidate core effectors. Therefore, for further study of co-expression between RGAs and core effectors, we could have a

chance to find the corresponding cognate R gene and effectors. Second, the putative R gene indirectly interacts with effectors, mediated by an accessory protein according to a guard (Dodds and Rathjen, 2010). The *Solanum demissum* R2 gene needs a phosphatase BSL1, an additional host protein for recognition of the *P. infestans* Avr2 effector (Saunders *et al*., 2012). Third, more than one NBS-LRR gene require the function in the disease resistance, such as both *RPP2A* and *RPP2B* of *Arabidopsis thaliana* NBS-LRR genes against *Peronospora parasitica* isolate Cala2 (Sinapidou *et al*., 2004). Similarly to our genetic data, a segregation ratio of induced cell death by CE80 showed 9 : 7 in an F2 population from a cross between CM334 and ECW-30R, suggesting that two independent dominant gene loci interact in complementary epistasis for effector-induced cell death.

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초 록

비기주 저항성은 한 식물종이 한 종의 병원체에 대해 갖는 저항성의 형태로, 대부분의 식물종이 갖는 지속적인 저항성 형태이다. 비기주 저항성이 성립되는 요소는 다양하지만, 특히 병원체가 분비하는 단백질을 식물의 저항성 유전자가 인지하여 나타나는 식물의 면역반응이 대두되고 있다. 이 관계를 설명하기 위해, 3개 고추 유전자원과 57개 감자역병균 분비단백질을 재료로 사용하였다. 재조합된 PVX 비리온을 이용하여 분비단백질을 고추 유전자원에 발현시킨 결과, 각 고추 유전자원에서 다양하게 과민성 세포 사멸이 나타나는 것을 관찰하였다. 이 결과를 토대로 고추 유전자원과 감자역병균의 분비 단백질 사이에서 다양한 상호작용이 있다는 것을 확인할 수 있었다. 이를 바탕으로, 고추 유전자원 중 CM334 품종에서 유전체 분석을 통해 예측된 저항성 유전자와 후보 effector를 함께 발현하여 과민성 세포 사멸을 관찰하였다. 또한 과민성 세포 사멸을 보이는 고추 유전자원과 보이지 않는 유전자원을 교배를 통해 F2 집단을 만들어, 후보 분비 단백질을 발현시켰다. 그 결과 후보 단백질에 따라 다양한 분리비를 보였다. 이는 고추 유전자원에서 나타나는 과민성 세포 사멸이 1개 이상의 요소에 의해 조절되는 것이라 생각된다. 본 연구는 고추 유전자원의 저항성 유전자와 감자역병균 effector 간의 상호작용이 비기주 저항성에 영향을 미친다는 결과를 본 것에 의의를 둔다.