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Inheritance of *Phytophthora infestans* Effector-Induced Hypersensitive Cell Death in Chili Pepper (*Capsicum* spp.)

감자역병균 effector 에 의해 유도된 고추에서의 과민성 세포 사멸의 유전

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UNDER THE DIRECTION OF DR. DOIL CHOI
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
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ABSTRACT

Non-host resistance is the common and durable resistance against most potential microbial pathogens in most of plant species. Non-host resistance consists of various levels of defense including preformed and induced resistance. To elucidate non-host resistance at molecular levels, cytological interaction between *Phytophthora infestans* and Solanaceae family plants was investigated. The non-host resistance was hypothesized to correlate with the presence of multiple genes that interact specifically with RXLR effector, thereby facilitating durable resistance in pepper. To test this, non-

host interaction of pepper plants against a potato blight pathogen (*Phytophthora infestans*) was used using recombinant PVX virions. Genetic analyses of hypersensitive cell death in pepper induced by RXLR effectors of *P. infestans* revealed that multiple factors both in the host and the pathogen are involved in effector-induced cell death. The results could be a starting point to elucidate the complicated mechanism of non-host responses in Solanaceae plants against pathogenic oomycetes, primarily *Phytophthora* spp.

Key words: *Capsicum annuum*, Non-host resistance, *Phytophthora infestans,* RXLR effector, Recombinant PVX virions

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INTRODUCTION

The agricultural industry is an undoubtedly important part of life for all human. In the past, the Irish famine caused by a potato blight, threatened human survival and brought about an emigration from their native land to a new settlement area. From late 19th century, the human population has rapidly increased, while food production has not been enough to sustain human population. Hence, agricultural crops are considered to be an instrument of national power and are beginning to be regarded as a political weapon. Plant diseases are one of major reasons for unstable crop-supply. In fact, late blight induced by *Phytophthora infestans* is still the cause of multibillion-dollar losses annually for the production of both potatoes and tomatoes (Kamoun, 2001).

Plants are exposed to a broad range of pathogens, such as bacteria, fungi, viruses, and nematodes in their whole life cycle. To survive against those pathogens, plants have multiple layered immune systems. The surfaces of plants are the front lines in their defense systems. For infections, pathogens must penetrate the surface of plants. When pathogens enter extracellular space through leaves, roots, stomata, or wounds, they encounter new barriers, such as cell walls and cellulose-based support. On the external face of the host cell, receptor proteins, known as pattern recognition receptors (PRRs), recognize conserved microbial elicitors, called pathogen associated molecular patterns (PAMPs), such as bacterial flagellin, oomycete glucans, and fungal chitin (Felix et al., 1999; Gust et al., 2007;

Boller & Felix, 2009). When PAMPs are recognized by PRRs, PAMP-triggered immunity (PTI) is activated and this helps prevent colonization by the pathogens. The second resistance class is induced when intercellular receptor recognize the pathogen virulence molecule called the effector. This interaction induces effector-triggered immunity (ETI), which is qualitatively stronger and faster and is accompanied by a hypersensitive response (HR) (Jones & Dangl, 2006; Schornack et al., 2009; Stassen et al., 2011). HR has been reported as a programmed cell death in the local region surrounding infection sites. In addition to the oxidative burst associated with HR, the production of antimicrobial compounds and the expression of defense-related genes are also related to resistance response (Baker et al., 1997). As repeating ETI and PTI, both plants and pathogens have co-evolved (Inuma et al., 2007). This concept is called the 'zigzag model' and it explains the mechanism of host resistance. Classical breeding uses these traits; breeders transfer resistance genes through conventional transfer methods (Dodds & Rathjen, 2010). However, host resistance is easily broken down and disease proliferates once more. Therefore, new methods or mechanisms to mediate resistance against pathogens are required. Non-host resistance (NHR) emerges as new way to control plant diseases.

NHR is known to have a durable and stable resistance response that prevents infection of most potential microbe pathogens (Heath, 2000; Nurberger & Brunner, 2002; Lipka et al., 2008). Beyond the range of host plants, pathogens are not able to cause an infection because of host specificity (Niks & Marcel, 2009). Therefore, it is possible to explain why resistance is the rule and disease is the exception

(Huitema et al., 2003). The type of NHR that has been studied is one that typically followed the host's pathosystem. The molecular basis of NHR remains poorly understood. Classical genetic approach to study NHR is limited due to sexual incompatibility and sterility (Jeuken et al., 2008). Subsequently, only a few inheritance studies have been conducted in the field of NHR. More studies are necessary to elucidate the NHR.

In this study, the molecular level mechanism of NHR was tested with non-host pepper and RXLR effectors of *P. infestans* causing potato late blight disease. To investigate non-host resistance, the phenotypes of host and non-host plants infected by *P. infestans* were compared. Resistance level was shown to be involved in establishing NHR between pepper and *P. infestans*. Using inoculation with recombinant PVX virions, four pepper germplasms were screened against 54 RXLR effectors originated from *P. infestans*. Among the pepper germplasms, two pepper accessions were chosen based on the screening results of cell death induced by effectors of *P. infestans*. To determine the inheritance of the *P. infestans* effector-induced hypersensitive cell death in pepper, F_2 population were derived from two accessions AC09-202 and AC09-226. The segregation ratios of the hypersensitive cell death suggested that multiple factors were mediated in effector-induced cell death. These results support the finding that polygenic host factor proteins interact with the RXLR effector at the ETI level. The results provide a new insight into understanding the molecular mechanism of non-host resistance between oomycetes pathogen and Solanaceae plants.

LITERATURE REVIEW

Non-host resistance

NHR is a phenomenon shown by most plant species that have resistance against microbes and virus, which have ability to infect other plants (Jones & Takemoto, 2004; Mysore & Ryu, 2004; Lipka et al., 2010). NHR suggests a new possibility of plant resistance due to its durability in nature and effectiveness against broad range of pathogens (Fan & Doerner, 2012). However, the molecular basis of NHR is still largely unknown, because genetic incompatibility between host and non-host plants has been obstacles for genetic approaches (Kamoun, 2001; Jeuken et al., 2008).

NHR is composed of multiple protective layers that include preformed and inducible defense system (Nurnberger & Lipka, 2005). Preformed defense such as the plant cytoskeleton, actin microfilaments and cuticle layers plays a role as the first obstacle for pathogen invading. Inducible defense mechanisms initially occur when highly conserved molecules of pathogen, known as PAMP, is recognized by plant receptor (Zipfel, 2008). The recognition leads to PTI which is accompanied by MAP kinase activation and production of reactive oxygen species. For suppression of PTI, pathogens secrete effectors. Avirulence effector-resistance gene interaction causes ETI involving HR.

Since the traits of durability resistance and the potential to transfer resistance gene to non-host plant, NHR is of highly interest to breeders. The first report about

transferring non-host resistance gene is the maize *Rxo1* gene which controls resistance to non-host pathogen *Xanthomonas oryzae* pv. *oryzicola* into rice (Zhao et al., 2005). In addition, *WRR4* confers broad-spectrum white rust resistance gene from *Arabidopsis thaliana* successfully transfer into *Brassica napus* (Borhan et al., 2010). These results provide evidences that NHR could be a novel source of disease control.

P. infestans and RXLR effectors

P. infestans is a hemibiotrophic pathogen. Plant infection has occurred by *P. infestans* followed a two-step that early biotrophic phase and second phase cause host tissue necrosis (Van Damme et al., 2012).

The biotrophic phase, up to 36 h post inoculation, pathogens penetrate the plant wall and parasitize host cells simultaneously suppressing defense responses (Schulze-lefert, 2004). The second phase, pathogens secrete hundreds of proteins, called as effectors, that act in the apoplast or are translocated into the cytoplasm (Schornack et al., 2009). Some oomycete effectors are recognized by intracellular host resistance proteins, triggering ETI as interaction between effectors and resistance proteins (Rafiqi et al., 2009). Two classes of effectors RXLR and crinkler including LFLAK motif facilitate delivering effectors into host cells (Birch et al., 2006; Stassen et al., 2011; Van Damme et al., 2012). The ability of RXLR motif is confirmed an experiment that native Avr3a from *P. infestans* induces HR on plant expressing R3a, while an avr3a mutant substituted RXLR and EER motif

by alanine residues has no HR (Whisson et al., 2007). The oomycete effectors, ATR1 and Avr3a, have revealed evidence that these prompt programmed cell death when effectors reach the cytoplasm and target intercellular proteins which can activate plant immunity in the meantime (Kale & Tyler, 2011).

To this day, oomycete genome sequences were determined with next-generation sequencing technologies, enabling prediction about hundreds of putative effectors. Based on bioinformatics data, the RXLR effectors only exist in Peronosporales clade, especially *Phytophthora* and downy mildews RXLR effector genes (Bozkurt et al., 2012). Genome sequencing technology revealed that *P. infestans* has 563 RXLR effector genes (Hass et al., 2009; Bos et al., 2010).

Resistance genes

Most plant resistance genes encode proteins that have a nucleotide-binding site (NB) and leucine-rich repeats (LRR) (Tameling & Takken, 2008). The function of resistance gene is known for recognition of pathogen-derived avirulence (AVR) effectors (Eitas & Dangl, 2010; Elmore et al., 2011). The resistance gene and AVR effector interaction initiate a series of signaling cascades leading to disease resistance (Hammond-Kosack et al., 1997). Recognition of AVR effector occur either direct interaction with matched pairs of plant resistance genes and pathogen AVR effectors, or indirect interaction, through the perception of modification in host target by AVR effectors (Chisholm et al., 2006; Van der Hoorn & Kamoun, 2008). One of examples for direct interaction is between

Hyaloperonospora arabidopsidis ATR1 and *A. thaliana* RPP1 (Rehmany et al., 2005). For indirect interaction, *Pseudomanas syringae* AvrB and AvrRpm1 are recognized *by A. thaliana* RPM1 activated by phosphorylation of RIN4 (Mackey et al., 2002; Caplan et al., 2008).

During resistance gene-AVR effector interaction, NB domains act a part to bind and hydrolyze ATP or GTP, whereas the LRR motif is generally involved in protein-protein interactions (Leister & Katagiri, 2000). The typical LRR domain has 21-25 amino acids per repeat and forms a large helix of multiple structures. Plant NB-LRR proteins can be subdivided into TIR and non-TIR classes based on a group of the sequences that precede the NB domain (Young, 2000; DeYoung & Innes, 2006). The TIR class of plant NB-LRR proteins contains an amino-terminal domain with homology to the Toll and interleukin 1 receptors. TIR-NB-LRR genes occur in dicots and are rare or absent in monocots (Bai et al., 2002). The non-TIR class mostly has a coiled-coil structure or leucine zipper structure (Lupas, 1996; Qureshi et al., 1999). Generally, resistance genes evolve at a faster rate than most genes. Rapid evolution of resistance genes is driven by an evolutionary arms race between pathogens and their hosts (Bent & Mackey, 2007; Ashfield et al., 2012).

MATERIALS AND METHODS

Plant materials and growth conditions

Four accessions of *Capsicum* spp. (AC09-11, AC09-186, AC09-202, and AC09-226) were obtained from Prof. Byeong-Cheorl Kang at Seoul National University, Seoul, Korea. All plants were grown in a walk-in chamber under 16 h photoperiod at 22°C.

Two pepper accessions, AC09-202 and AC09-226 (*C. annuum*), were crossed in a glasshouse. Parent plants, F_1, and F_2 were used for genetic analysis of inheritance of *P. infestans* effector-induced hypersensitive cell death. The plant materials used in this study are listed in Table 1.

Pathogen preparation and inoculation

P. infestans isolates were grown in rye sucrose agar medium for 8 days at 15°C in darkness. The plate was flooded with autoclaved distilled water, rubbed with a sterile cell scrapper, and then incubated at 4°C for 1 h (Caten & Jinks, 1968). The zoospores released from the sporangia were counted by using a hemocytometer and the concentration was adjusted to 5×10^4 zoospores/ml. Detached leaves from 6-week-old Solanaceous plants (*C. annuum*, *Solanum tuberosum*, and *S. lycopersicum*) were seperately placed into square dishes (125 × 125 × 20 mm; SPL Life Science, Pocheon, Korea) with wet paper to maintain high humidity. The leaves were inoculated by pipetting 10 µl droplets on the abaxial side

Table 1. Pepper accessions used in this study.

Pepper accesion	Species	Cultivar name	Origin
AC09-11	*Capsicum annuum*	Numex Big Jim	USA
AC09-186	*C. chinense*	Bisbas Rotfruechtig	Yemen
AC09-202	*C. annuum*	Nitrianska Krajova	Russia
AC09-226	*C. annuum*	Serrano	USA
CM334	*C. annuum*	Criollo de Morelos 334	Mexico

(Vleeshouwers et al., 2000). Multiple *P. infestans* isolates used in this study are shown in Table 2.

Histological and cytological analysis

To investigate hyphal structures and stain dead cells, trypan blue staining was conducted (Koch & Slusarenko, 1990). The lactophnol-trypan blue solution was prepared by dissolving 5 mg trypan blue in a solution of 2.5 ml lactic acid, 2.5 ml glycerol, 2.325 ml phenol, and 5 ml distilled water. Leaf discs (1 cm diameter) were vacuum-infiltrated with the lactophenol-trypan blue working solution for 5 min two or three times. Whole leaves were boiled for 4 min in the water bath and incubated overnight at room temperature. The tissues were destained several times in chloral hydrate (2.5 g chloral hydrate dissolved in 1 ml distilled water) and then mounted in 70% glycerol and representative phenotypes were photographed under a light microscope.

RNA extraction and gene expression analysis

Total RNA was isolated from leaves of *Capsicum* spp., *S. tuberosum*, and *S. lycopersicum* to analyze the proliferation of *P. infestans* infection. Tissues were immediately frozen in liquid nitrogen and stored at -80^{o}C for later RNA extraction. RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The first-strand cDNA synthesis by using 5 μg total RNA with Oligo (dT) and SuperScript II reverse transciptase (Invitrogen) for reverse transcription mediated

Table 2. *P. infestans* isolates used in this study.

Isolate	Description	Origin
88069	Wild type	S. Kamoun[a]
208M2	Transformed with GFP expression vector p34GFN	A.S. Ammour[b]
40706	Wild type	RDA Gene Bank[c]
40707	Wild type	RDA Gene Bank
40718	Wild type	RDA Gene Bank
43701	Wild type	RDA Gene Bank
43072	Wild type	RDA Gene Bank

[a]Wageningen Agriculrutal University, Wageningen, Netherlands

[b]University of Fribourg, Fribourg, Switzerland

[c]Rural Development Administration (RDA) Gene Bank Center, Suwon, Korea

polymerase chain reaction (RT-PCR). Gene-specific oligonucleotides (Table 3) were based on The National Center for Biotechnology Information (NCBI) Reference Sequence database (http://www. ncbi. nlm. nih. gov/RefSeq/).

RXLR effectors of *P. infestans*

In planta expression clones of 54 RXLR effectors were gifts from Dr. Sophien Kamoun at The Sainsbury Laboratory, Norwich, UK. The cloning product was transformed into *Agrobacterium* strain GV3101 (Jones et al., 1999). pGR106:ΔGFP and pGR106:PexRD40 (170-1) were used as negative and positive controls, respectively (Kavanagh et al., 1992).

Inoculum with recombinant PVX virions

Nicotiana benthamiana plants used for transient overexpression of 54 RXLR effectors. *A. tumefaciens* GV3101 carrying pGR106-PexRD effectors was used for delivering T-DNA constructs into the four-leaf stage of *N. benthamiana. A. tumefaciens* cultures were incubated at 28^{o}C and harvested by centrifugation at 1,000 g for 15 min. The pellet was resuspended with 1 ml infiltration buffer (10 mM $MgCl_2$, 10 mM 2-(*N*-morpholino)ethanesulfonic acid, and 200 μM acetosyringone) to an OD_{600} = 0.5. The resuspension was incubated at room temperature for 3 h prior to syringe infiltration. At 7 days after inoculation, infected systemic leaves were ground in 50 mM potassium phosphate buffer

Table 3. Primers used for RT-PCR.

Primer	Sequence (5'→3')
Epi1_F[a]	CATGCTCAAAGCCCGCAAGTCATCA
Epi1_R	TTATCCCTCCTGCGGTGTCACCTT
Ef2_F	TGACGCTATCGCCAAGGAATCGAC
Ef2_R	TAACGCTGAGCCGTAATGGGGGA
CaActin_F	CCACCTCTTCACTCTCTGCTCT
CaActin_R	ACTAGGAAAAACAGCCCTTGGT
PotatoActin_F	GATGGCAGAAGGCGAAGATA
PotatoActin_R	GAGCTGGTCTTTGAAGTCTCG
TomatoActin_F	GGCGATGAAGCTCAATCCAAACG
TomatoActin_R	GGTCACGACCAGCAAGATCAAGACG

[a]F, R, forward and reverse orientation, respectively.

(pH 7.0) and immediately used for inoculum.

Inoculation of pepper with recombinant PVX virions

Leaves from 4-week-old pepper were inoculated with PVX sap while rubbing. The plants were predusted with carborundum (400 meshes) and then inoculum was applied gently to the upper surface. The inoculated leaves were rinsed with distilled water of 3 min after inoculation (Ritter et al., 1991).

RESULTS

P. infestans triggers HR in pepper

Potato and tomato have been reported to play a role as hosts against *P. infestans*. However, pepper is known to be non-host against *P. infestans* under field conditions.

To identify the cytological basis of the non-host mechanism, three representative Solanaceae plants (*C. annum*, *S. tuberosum*, and *S. lycopersicum*) were inoculated with zoospore of *P. infestans* isolate 88069. On the leaves of the potato and tomato host plants, disease symptoms and visible hyphae caused by *P. infestans* were observed at 72 h after inoculation (hai) (Figs. 1D, 1E, 1G, 1H). However, HR cell death was observed in non-host pepper plants (Figs. 1A, 1B). In addition, plant cell death was examined under a light microscope following the lactophenol-trypan blue staining. Numerous dead cells were stained and sporangia were formed in the host plant (Figs. 1F, 1I), while only localized cell death appeared in the leaves of the pepper (Fig. 1C). Localized cell death suppressed the propagation of hyphae and sporangia.

To confirm the response between *P. infestans* and pepper, four isolates of *P. infestans* were inoculated to leaves of four pepper germplasms and potato. All types of *P. infestans* caused the localized cell death on the leaves of various germplasms (Fig. 2). The localized cell death did not spread all over the pepper leaves. This result implies that the localized cell death response on pepper is

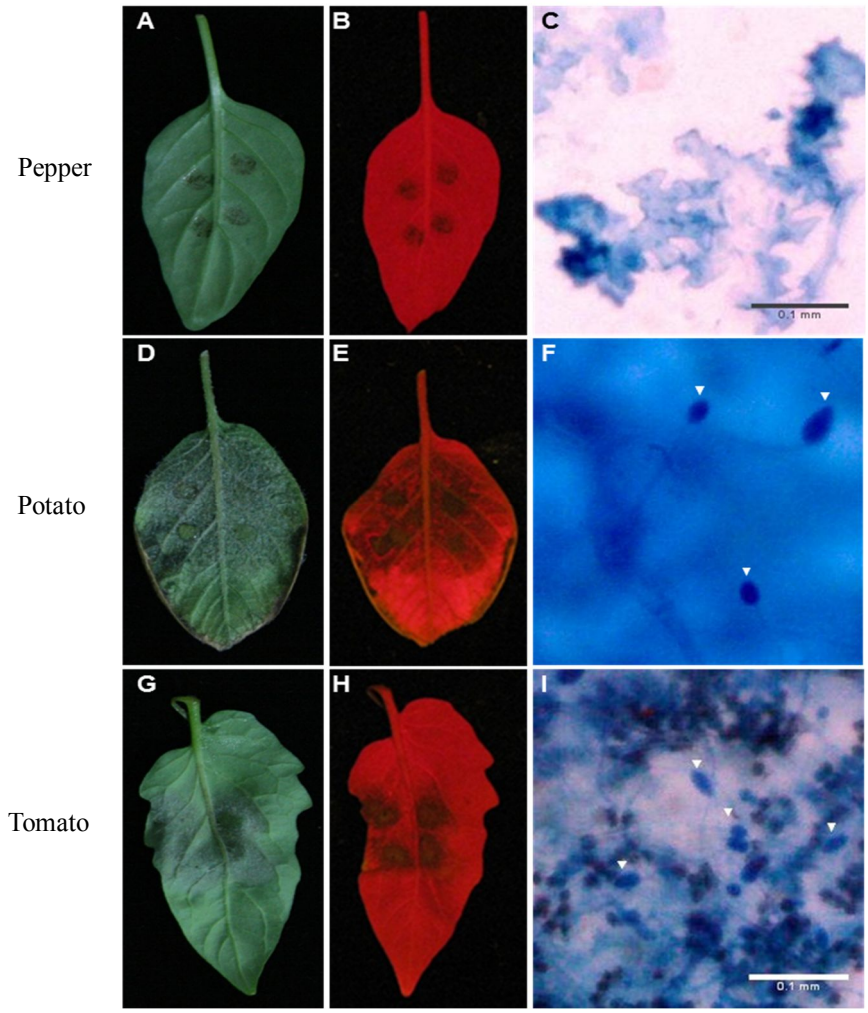

Fig. 1. Phenotypes of non-host and host plants following infection with *P. infestans.*

Detached leaves from non-host pepper (A-C) and host plants (potato (D-F) and tomato (G-I)) were infected by zoospore suspension of *P. infestans* 88069 (5×10^4/ml). Leaf pieces containing zoospore droplet were excised at 72 hai and examined for HR and sporangia development after lactophenol-trypan blue staining. Microscopic cell death on pepper epidermal cell was observed (C). White arrows indicate sporangia formation on potato and tomato leaves (F, I).

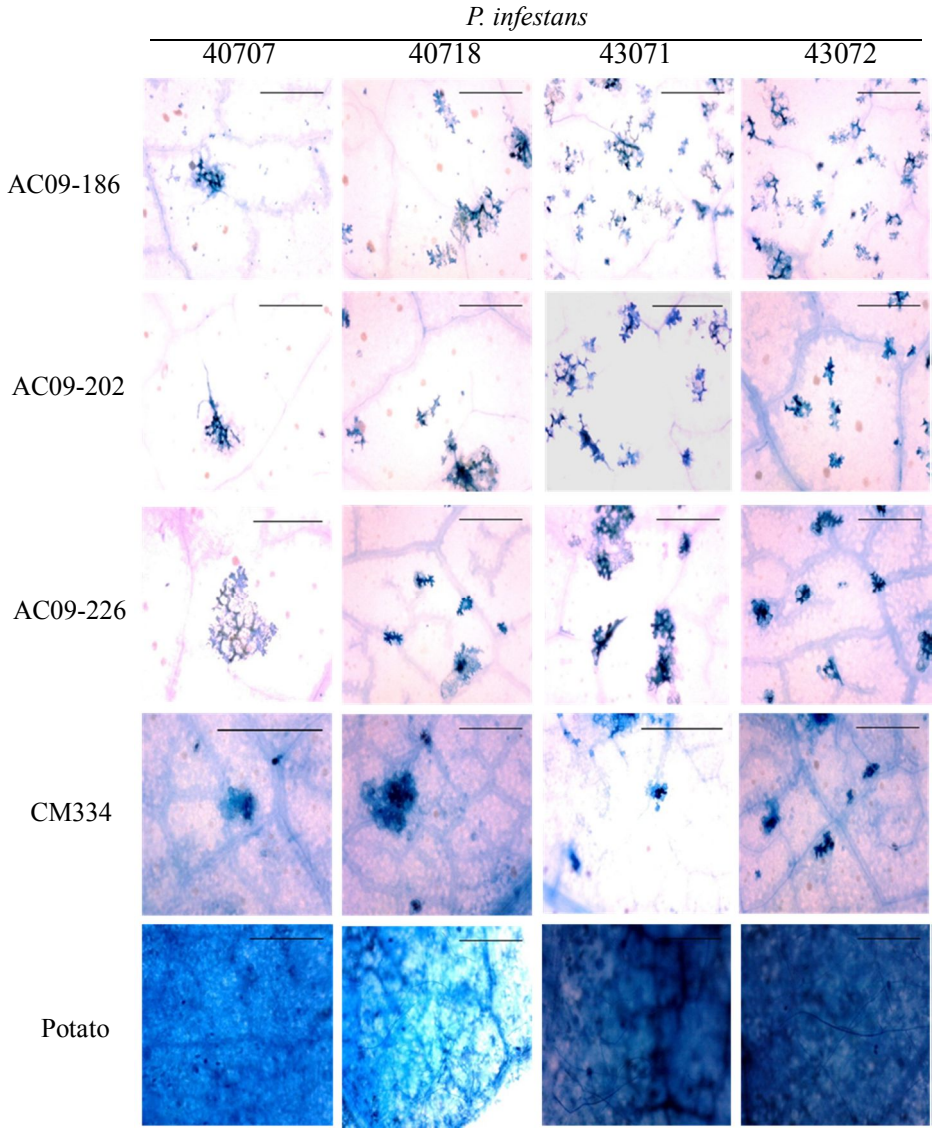

Fig. 2. Interaction between multiple isolates of *P. infestans* and various pepper accessions.

Detached leaves of potato and various pepper accessions (CM334, AC09-226, AC09-202, and AC09-186) were infected with zoospores of four *P. infestans* isolates at 2×10^4/ml. At 72 hai, the leaves were observed after lactophenol-trypan blue staining to visualize dead plant cells. Bars = 100 μm.

neither cultivar-specific nor isolate-specific.

The precise time of penetration in pepper by *P. infestans*, RT-PCR assay was conducted to confirm gene expression patterns with infected pepper leaves from various time points. *Epi1* was known as the *in planta*-induced Kazal-like protease inhibitor gene (Tian et al., 2004; Oh et al., 2009). *Elongation factor 2 (Ef2)* of *P. infestans* which has been reported as a constitutive gene (Torto et al., 2002; Kanneganti et al., 2006) was used for RT-PCR (Fig. 3). The RT-PCR results exhibit that *Ef2* and *Epi1* genes were expressed 24 hai. These results support the hypothesis that *P. infestans* penetrates non-host pepper plants and secreted proteins could act as avirulence factors. The putative resistance genes in pepper would interact with the RXLR effectors of *P. infestans*.

Effector-induced cell death using recombinant PVX virions as inoculumn

In a previous study, 106 pepper accessions of *C. annuum* were screened against 54 RXLR effectors using binary PVX-agroinfection. However, the PVX-agroinfection method was not reproducible, so an alternative screening method is required for further study (Lee, unpublished data). To investigate the interaction response in the non-host pepper plant, selected four pepper germplasms (AC09-11, AC09-186, AC09-202, and AC09-226) were inoculated with recombinant PVX virions. To express recombinant PVX virions *in planta*, *N. benthamiana* was used. Infected systemic leaves were ground and leaves of the peppers were mechanically

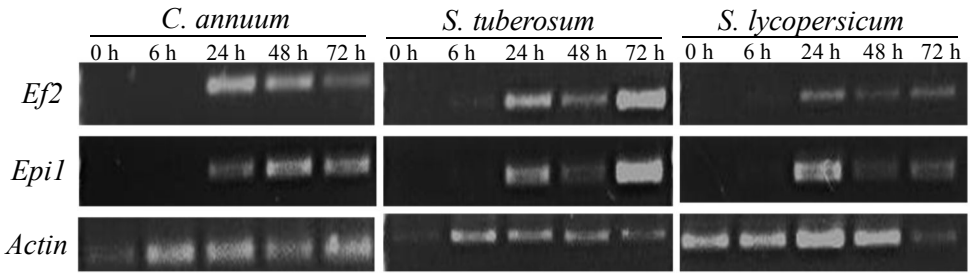

Fig. 3. Expression of *Ef2* and *Epi1* genes during *P. infestans* colonization in *Solanum* spp.

RT-PCR assay was conducted to confirm gene expression patterns with specific primers for *Ef2* and *Epi1*. Total RNA was isolated from infected leaves of tomato, potato, and pepper at 0, 6, 24, 48, and 72 hai.

inoculated. Effector-induced cell death in the inoculated leaves was monitored up to 6 day post inoculation (dpi). A profiling of the interaction response between four pepper accessions and 54 RXLR effectors is shown in Table 4.

From the screening data, a number of PexRD39/40 clones (the *Avrblb2* homologs) showed an interaction response to various pepper accessions. *Avrblb2* is known for being recognized by *Rpi-blb2* (Oh et al., 2009). The significant hypersensitive cell death phenotype of the pepper accessions to PexRD8, PexRD24 (113-1), PexRD41 (91-10), and PexRD50 (191-1) is shown in Fig. 4. In two accessions of *C. annuum* (AC09-202 and AC09-226), those effectors induced distinct differences in the types of effector-induced cell death.

Multiple dominant genes mediate effector-induced cell death in pepper

For the inheritance study, F_2 population was derived from two representative accessions with AC09-202 and AC09-226. Based on parent screening data, F_1 populations were inoculated with candidate effectors (PexRD8, PexRD24 (113-1), PexRD41 (91-10) PexRD46 (92-4), and PexRD50 (191-1)) and different degrees of effector-induced cell death between AC09-202 and AC09-226 were observed. HR-like cell death was monitored in the F_1 populations against five candidate effectors and all the inoculated leaves showed effector-induced cell death. To characterize the effector-induced response in the F_2 population, each F_2 plant was tagged and the number of plants that showed HR-like symptoms was scored at 7 dpi. The scheme

Table 4. Hypersensitive cell death induced by 54 RXLR effectors in pepper accessions using inoculation with recombinant PVX virions.

P. infestans RXLR effector	Pepper accession			
	AC09-11	AC09-186	AC09-202	AC-09-226
PexRD1	–	–	–	–
PexRD2	–	–	+	+
PexRD3	–	–	–	–
PexRD4	–	–	–	–
PexRD6 (CU3)	–	–	–	–
PexRD6 (ipiO1)	–	–	–	–
PexRD7 (Avr3aKI)	–	–	–	–
PexRD7 (Avr3aEM)	+	–	+	–
Pex147-2	–	–	–	–
Pex147-3	–	–	nd	–
PexRD8	+	+	–	+
PexRD10	+	–	–	–
PexRD11 (21-1)	–	–	–	–
PexRD11 (43-1)	–	–	–	–
PexRD13 (98-3)	+	+	+	+
PexRD13 (98-4)	+	–	+	+
PexRD14 (99-4)	–	–	–	–
PexRD14 (99-5)	–	–	–	–
PexRD14 (56-2)	–	–	–	–
PexRD17 (MK88-4)	+	–	–	–
PexRD17 (MK90-1)	–	–	–	–
PexRD21	nd	nd	+	–
PexRD22 (68-2)	–	–	–	–
PexRD22 (66-1)	–	–	–	–
PexRD24 (113-1)	+	–	–	+
PexRD36 (45-1)	+	–	–	–
PexRD36 (45-10)	–	–	–	–
PexRD40 (170-1)	+	+	+	+
PexRD40 (170-7)	+	+	+	+
PexRD39/40-1	+	–	+	nd
PexRD39/40-2	+	–	+	+
PexRD39/40-4	+	+	+	+
PexRD39/40-6	nd	+	+	+
PexRD39/40-7	+	–	+	–
PexRD39/40-8	+	+	+	+
PexRD39/40-9	+	+	+	+
PexRD39/40-10	+	–	+	nd
PexRD41 (91-3)	+	–	–	–
PexRD41 (91-5)	nd	–	–	–
PexRD41 (91-10)	–	nd	+	–
PexRD44	–	–	–	–
PexRD45 (184-2)	–	–	–	–
PexRD45 (215-3)	–	–	–	nd
PexRD46 (92-4)	+	–	+	–
PexRD46 (92-12)	–	–	+	–
PexRD50 (191-1)	+	–	+	–

* +, effector-induced cell death; –, no cell death; nd, not determined.

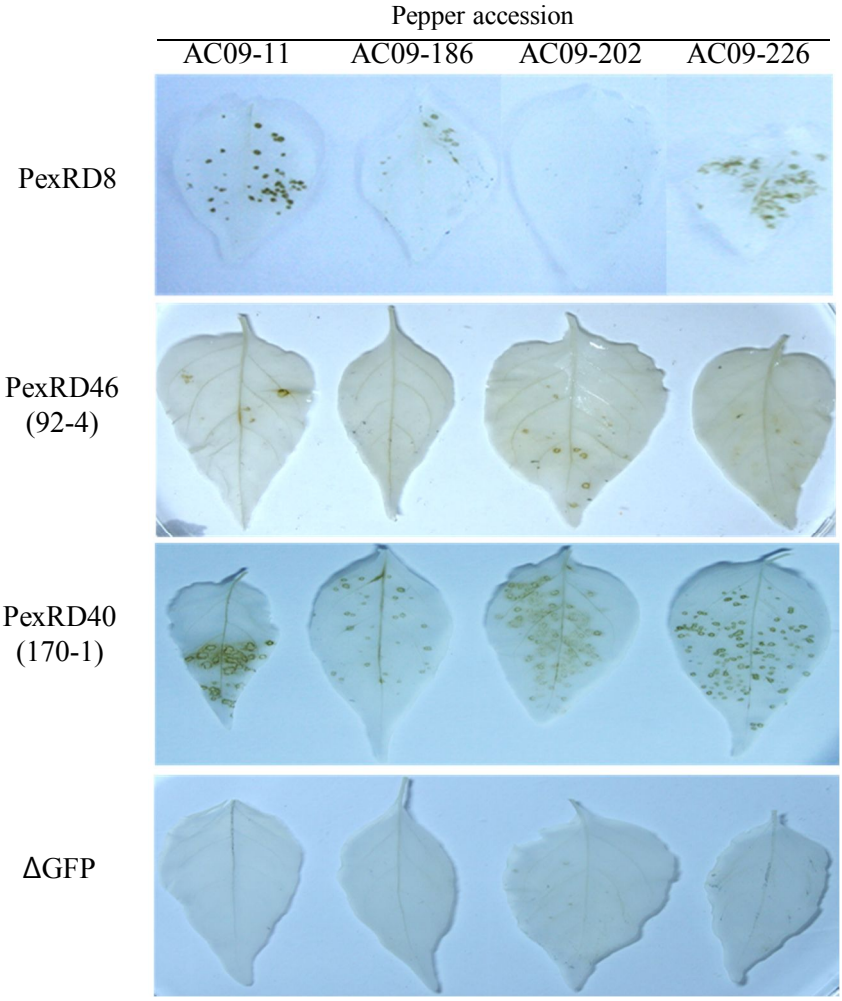

Fig. 4. Phenotypes of effector-induced cell death.

PVX carrying four effectors (PexRD8, PexRd46 (92-4), PexRD40 (170-1), and ΔGFP) were multiplicated using *N. benthamiana* plants. Four pepper accessions (AC09-11, AC09-186, AC09-202, and AC09-226) were wound-inoculated with PVX carrying effectors. The negative control was PVX-ΔGFP and positive control was PVX-RD40 (170-1). Inoculated leaves were harvested at 6 dpi and destained in 100% ethanol to remove chlorophyll.

of effector-induced cell death in the parent, F_1, and F_2 populations of *C. annuum* is shown in Fig. 5.

Inheritance studies of effector-induced cell death with the two pepper accessions showed obvious segregation ratios that have genetic meaning. The segregation ratio for PexRD8 was observed to relate to a 15:1 ratio ($\chi^2 = 0.110$; $P < 0.735$), implying the presence of two independently inherited dominant genes (Table 5). In the case of the PexRD41 (91-10)-induced cell death, the segregation of the F_2 population fits to a 3:1 ratio ($\chi^2 = 0.667$; $P < 0.414$), indicating the presence of one independently inherited dominant resistance gene (Table 6). For PexRD24 (113-1), PexRD46 (92-4), and PexRD50 (191-1), the segregation ratios gave a near perfect fit to a 9:7 ratio (Tables 7, 8, 9). The result for the all candidate effectors are shown in Table 10. These results suggest that non-host resistance correlate with the presence of multiple host genes interacting with a specific RXLR effector.

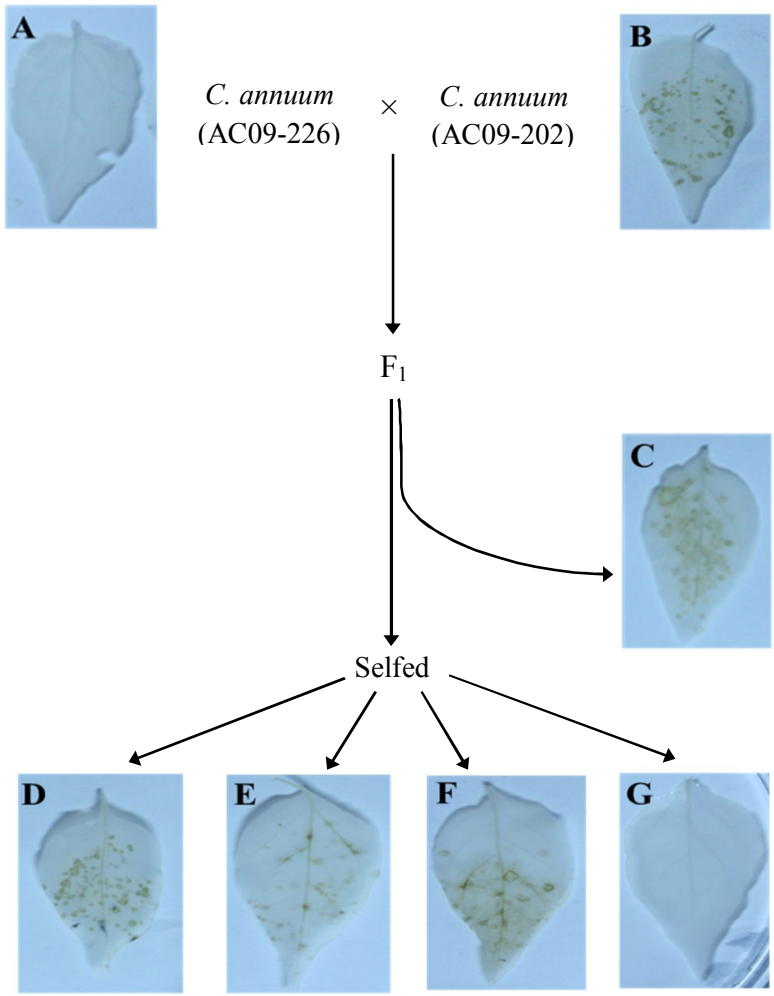

Fig. 5. Scheme of PexRD41 (91-10)-induced cell death in parent, F_1, and F_2 population of pepper.

Leaf of *C. annuum* AC09-226 was inoculated with PexRD41. Effector-induced cell death was not shown at 7 dpi (A). Leaf of *C. annuum* AC09-202 was inoculated with PexRD41. Effector-induced cell death was dramatically shown at 7 dpi (B). Leaf of F_1 derived from a cross between AC09-202 and AC09-226 was inoculated with PexRD41 (C). Leaf of self-pollinated F_2 was inoculated with PexRD41 (91-10). Segregation of effector-induced cell death was shown in F_2 population (D-G).

Table 5. Inheritance of PexRD8-induced cell death.

Plant material	Observed		Expected ratio		
	HR+	HR–	HR+ : HR–	χ^2	*P*-value
AC09-226	20	0	1:0	-	-
AC09-202	0	20	0:1	-	-
F_1 (226 × 202)	8	0	1:0	-	-
F_2 (226 × 202)	55	3	15:1	0.110	0.735

Table 6. Inheritance of PexRD41 (91-10) -induced cell death.

Plant material	Observed		Expected ratio		
	HR+	HR–	HR+ : HR–	χ^2	*P*-value
AC09-226	0	15	0:1	-	-
AC09-202	15	0	1:0	-	-
F_1 (226 × 202)	10	0	1:0	-	-
F_2 (226 × 202)	40	10	3:1	0.667	0.414

Table 7. Inheritance of PexRD24 (113-1)-induced cell death.

Plant material	Observed		Expected ratio		
	HR+	HR–	HR+ : HR–	χ^2	*P*-value
AC09-226	15	0	1:0	-	-
AC09-202	0	15	0:1	-	-
F_1 (226 × 202)	10	0	1:0	-	-
F_2 (226 × 202)	56	40	9:7	0.169	0.681

Table 8. Inheritance of PexRD46 (92-4) -induced cell death.

Plant material	Observed		Expected ratio		
	HR+	HR–	HR+ : HR–	χ^2	*P*-value
AC09-226	0	15	0:1	-	-
AC09-202	15	0	1:0	-	-
F_1 (226 × 202)	10	0	1:0	-	-
F_2 (226 × 202)	29	18	9:7	0.568	0.451

Table 9. Inheritance of PexRD50 (191-1)-induced cell death.

Plant material	Observed		Expected ratio		
	HR+	HR–	HR+ : HR–	χ^2	*P*-value
AC09-226	0	15	0:1	-	-
AC09-202	15	0	1:0	-	-
F_1 (226 × 202)	10	0	1:0	-	-
F_2 (226 × 202)	27	17	9:7	0.468	0.494

Table 10. Multiple dominant genes mediate effector-induced cell death in pepper.

	Observed		Expected ratio		
Effector	HR+	HR−	HR+ : HR−	χ^2	*P*-value
PexRD8	55	3	15:1	0.110	0.735
PexRD24	56	40	9:7	0.169	0.681
PexRD41	40	10	3:1	0.667	0.414
PexRD46	29	18	9:7	0.568	0.451
PexRD50	27	17	9:7	0.468	0.494

DISCUSSION

To elucidate NHR mechanisms, both histological observation and genetic studies were performed. PTI reactions, in general, include defense responses such as the production of reactive oxygen species and the accumulation of callose to enhance the cell wall (Asai et al., 2002; Yao et al., 2012). PTI has been reported that it is possible to suppress the penetration of pathogens without cell death (Nurnberger et al., 2004). As shown in Fig. 1, the phenotypes of leaves following infection with *P. infestans* showed that disease symptom was spread out at the infection site in the host. However, non-host pepper has localized cell death which would prevent plant from propagation of pathogen. This result implies that NHR against *P. infestans* is relevant not to PTI but to ETI level. *P. infestans* penetrates pepper cell wall and its effectors would be secreted into the cytoplasm. The effectors encounters protein encoded resistance genes. Of AVR-R interaction, NHR between pepper and *P. infestans* is presumed to be comprised.

In this study, 54 RXLR effector-induced hypersensitive cell death in chili pepper using inoculation with recombinant PVX virion were screened. The observations enable to the validation of previous work by Lee (2011, unpublished data), which showed the interaction between effectors of *P. infestans* and 106 pepper accessions using PVX-agroinfection. The previous method is appropriate for a large scale screening and facilitates the selection of pepper accessions contingent on occurrence of HR. However, PVX-agroinfection has been limited by a lack of reproducibility in pepper. Inoculation with recombinant PVX overcame

previous methodological problems. Among the 106 pepper accessions, four accessions that were used in this research were selected, allowing for different degrees of HR.

In screening data, homologues of PexRD39/40, which belongs to the Avrblb2 family, showed effector-induced HR in many accessions (Table 4). The Avrblb2 family is recognized inside plant cells by the *Rpi-blb2* resistance gene of the wild potato *Solanum bulbocastanum* (Oh et al., 2009). This result has opened the possibilities that *Capsicum* spp. has resistance gene which plays a role as *Rpi-blb2*.

There has been a strong relation between HR cell death and gene-for-gene resistance (Grant et al., 2000; Dodds et al., 2006). However, this study implies that the polygenic host factor protein interacts with the RXLR effector(s). The segregation ratio for PexRD8 fitted a 15:1 ratio, suggesting the presence of two independently inherited dominant genes (Table 5). The segregation ratio for PexRD24 (113-1), PexRD46 (92-4), and PexRD50 (191-1) was related to a 9:7, respectively (Table 10). The result supports two dependently inherited dominant genes. Of the PexRD41 (91-10), the segregation ratio fit the ritio 3:1 (Table 7). It indicates the presence of one independently inherited dominant resistance gene. These results prove that one effector can interact with two or more genes of non-host plants and open up additional possibilities that NHR resistance is established by gene(s)-for-gene(s) interaction.

The observation that pepper accessions have the ability to respond to most effectors of *P. infestans* raises some questions. *P. infestans* is a specialized

pathogen in Solanaceous plants that occurs in central and south america, but is not known to infect pepper nor related *Capsicum* species. If so, why would non-host pepper carries genes which perceive the effectors of *P. infestans*? There are several speculations. It is possible that pepper used to be a host of *P. infestans* during its evolutionary history. The Solanaceae genomes that have been studied that show a small number of chromosomal rearrangement in family (Livingstone et al., 1999; Wang et al., 2008; Park et al., 2011). Another explanation is that pepper does not directly respond to *P. infestans* effectors, but rather to the cellular perturbations these effectors trigger. This would be consistent with models of indirect recognition as postulated by the guard model (Innes, 2004). Indeed, recent recognition of the *P. infestans* AVR2 effector by the Solanum *R2* gene was shown to require an additional host protein, the phosphatase BSL1 (Saunders et al., 2012).

Combing these results with comparative genomics would be a powerful tools to rapidly identify the counterpart genes in pepper against *P. insfestans* effectors. This study demonstrates inheritance of *P. infestans* effector-induced cell death in pepper and may provide insight into the unknown non-host mechanism. Furthermore, the concept of multiple interactions between effector(s) from pathogen and resistance gene(s) in plants is to lay the foundation for unveiling non-host resistance.

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초록

비기주 저항성은 식물이 갖는 가장 일반적인 저항성으로 강력하고 지속적인 것으로 알려져 있다. 비기주 저항성은 식물이 가지고 있는 형태적인 특성 혹은 병원균에 의해 유도된 저항성 반응에 의해 일어난다. 비기주 저항성의 분자 수준의 메커니즘을 규명하기 위하여 가지과 식물에 감자역병균을 접종하여 세포학적인 상호작용을 확인하였다. 또한 비기주 저항성이 다중 유전자에 의해 조절됨을 입증하기 위하여 비기주 관계에 있는 고추와 감자역병균의 RXLR effector 사이에 상호작용을 재조합 PVX 비리온을 이용하여 실험하였다. 유전학적 연구를 위하여 고추에서 감자역병균의 RXLR effector에 의해 유도되는 과민성 세포 사멸을 1차 스크리닝하였고, 또한 F_2 교배 집단에서에서 후보 effector를 이용하여 상호작용 반응을 스크리닝하였다. 그 결과 과민성 세포 사멸이 기주와 병원균 간의 다중 요소에 의하여 일어나는 반응임을 밝혀내었고, 비기주 저항성은 이러한 다중 상호작용을 통하여 성립되는 것임을 실험적으로 증명하였다. 본 연구는 *Phytophthora*속의 병원균에 대한 가지과 식물의 비기주 저항성 기작을 밝혀내는 데 초석이 되는 의의를 갖는다.