A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Effector-omics approaches for identifying *Phytophthora capsici* effectors inducing hypersensitive cell death in *Nicotiana benthamiana*

Effector-omics 접근을 통한 *Nicotiana benthamiana*에서 과민성 세포 사멸을 유도하는 고추역병균 effector 동정

AUGUST 2020

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ABSTRACT

Over time, plants and pathogens are engaged in arms race for survival. Pathogen secretes arsenals of effector proteins into the plant cells to manipulate the host defense. As a counterdefense, plants recruit nucleotide binding leucine rich repeat (NLR) proteins to recognize those effectors. The perception of an effector by a NLRs induces effector-triggered immunity (ETI) which is often accompanied by hypersensitive localized cell death (HR). To identify NLRs involved in ETI as a source of resistance, effector-omics approaches are employed. Particularly, much attention has been made on a large effector class with conserved RXLR domain. In this study, novel *Phyophthora capsici* RXLR effector candidates inducing HR-

mimic cell death were investigated. Total 25 putative *P. capsici* RXLR effectors were predicted by bioinformatic tools and subjected to analyze cell death-inducing activity in *Nicotiana benthamiana.* As a result, 9 *P. capsici* RXLR effector candidates were selected and further examined to characterize the cell death in *N. benthamiana*. The selected *P. capsici* effector candidates could induce defense-related gene expression upon ectopic expression in *N. benthamiana* indicating that the effector-induced cell death is a mimic of HR. Moreover, the induction of cell death by selected effectors required SGT1, a chaperone component of NLR signaling. These results suggest that the identified *P. capsici* RXLR effector candidates may act as novel avirulent factors that induce cell death through recognizing specific NLRs in *N. benthamiana*. Taken together, the newly identified *P. capsici* effector candidates could contribute to further understanding of the mode of action in effector-triggered HR cell death in plants as well as identifying corresponding NLR genes in *N. benthamiana* for further application to develop disease resistant crops.

Keywords: Effector-triggered immunity (ETI), Nucleotide-binding leucine rich repeat (NLR), Hypersensitive response (HR), RXLR effector, *Phytophthora capsici*

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LIST OF ABBREVIATIONS

PAMP	Pathogen-associated molecular pattern
PRR	Pattern recognition receptors
PTI	PAMP-triggered immunity
R	Resistance
NLR	Nucleotide-binding leucine rich repeat
ETI	Effector-triggered immunity
NLR	Nucleotide-binding, leucine rich repeat
HR	Hypersensitive response
ROS	Reactive oxygen species
MAPK	Mitogen-activated protein kinase
ORF	Open reading frame
PVX	*Potato virus X*
LIC	Ligation-independent cloning

INTRODUCTION

In nature, plants are constantly exposed to various pathogens. For plants, the recognition of 'non-self' is a much bigger issue compared to animals because of their lack of adaptive immune system. Plants have evolved a versatile, multi-layered immune system to protect themselves against pathogens. Along with preformed barriers, such as cell wall, cuticle, and phytoanticipin, plants employ a two-tier defense mechanism induced by pathogen attacks (Jones and Dangl, 2006; Dodds and Rathjen, 2010). The first layer is the perception of pathogen-associated molecular patterns (PAMPs) which are typical patterns of pathogens, such as bacterial flagellin or fungal chitin. At the plant cell surface, pattern recognition receptors (PRRs) recognize PAMPs and turn on immune responses called PAMP-triggered immunity (PTI) (Zipfel, 2014). As a counterdefense, pathogens exploit a large array of effector proteins to manipulate the host defense. To ward off the pathogen effectors, plants have recruited the second immune layer called effector-triggered immunity (ETI) which is a result of dynamic coevolution of plant and pathogen. In plants, resistance (R) proteins recognize the effectors and activate defense responses. Most of the R proteins are nucleotide-binding leucine rich repeat (NLR) proteins which act as intracellular receptors (Jones and Dangl, 2006). The effectors recognized by R proteins and involved in ETI are usually regarded as avirulent factors. ETI is often accompanied by hypersensitive response (HR), a localized cell death at the infection site. HR appears as superficial macroscopic cell death resulting in suppression of

disease progress (Balint-Kurti, 2019).

NLRs recognize effectors directly or indirectly in plant cells and become activated by conformational changes (Cui *et al.*, 2015). NLR activation initiates immune signaling. Several components serve as nodes of NLR-mediated signal transduction pathways (Chiang and Coaker, 2015). A conserved chaperone complex plays an important role in resistance mediated by NLR proteins recognizing pathogen effectors. This molecular chaperone complex which is composed of Supressor of the G2 allele of *SKP1* (SGT1), Heat shock protein 90 (HSP90) and Required for MLA12 resistance 1 (RAR1) contributes to the stability of NLRs by facilitating the folding of the proteins (Shirasu, 2009). Non-race specific disease resistance1 (NDR1), a plasma membrane integrin like protein, is known to be required for the activation of multiple NLR-mediated defense responses (Chiang and Coaker, 2015). Some NLRs known as helper NLRs deliver the signals from other NLRs sensing effector proteins. Activated disease resistance 1 (ADR1) and NB-LRR required for hypersensitive response-associated cell death (NRC) are widely known helper NLR groups. These helper NLRs also contribute to various NLR-dependent ETI responses (Baggs *et al.*, 2017; Li *et al.*, 2015; Chiang and Coaker, 2015). The perception of effectors by NLRs also activates various defense responses which are associated with HR. Complex defense signaling network is involved in ETI, including production of reactive oxygen species (ROS), calcium flux, mitogen-activated protein kinases (MAPKs) cascade activation, controlling homeostasis of plant defense hormones, and transcriptional reprogramming of defense-related genes.

All these concerted processes drive resistance against pathogens (Cui *et al.*, 2015).

Since progress in 'effector'-triggered immunity has been made, much attention has been focused on effectors involved in ETI. Studies of effectors in all genus, including bacteria, fungi and oomycete, have been performed (Chisholm, 2006). Particularly oomycetes translocate a large class of effectors with conserved Arg-Xaa-Leu-Arg (RXLR) domain at N-termini into plant cells (Anderson *et al.*, 2015). It is known that the conserved RXLR domain followed by N-terminal signal peptide, is required for the proper translocation into the host cell (Whisson *et al.*, 2007). Variable C-terminal regions of RXLR effectors, so-called effector domains, exhibit the effector activity (Schornack *et al.*, 2009). Comparative analysis based on RXLR domain has revealed that a large number of RXLR effectors are conserved in *Phytophthora* and downy mildew lineage. Total 563 RXLR genes were predicted in *Phytophthora infestans*, 374 in *Phytophthora sojae*, and 134 in *Hyaloperonospora arabidopsidis (Hpa)*, respectively (Anderson *et al.*, 2015).

Many RXLR effectors have been identified as avirulent factors which activate ETI. Several RXLR effectors and their corresponding NLRs were elucidated (Anderson *et al.*, 2015). Avr1, Avr2, Avr3a, Avr4, Avrblb2, and IPI-O of *P. infestans*, Avr1a, Avr1b-1, Avr1c, Avr1d, Avr1k, Avr3b, and Avr3c of *P. sojae*, and ATR1, ATR5, ATR13, and ATR39 of *Hpa* were identified (Anderson *et al.*, 2015). For example, *P. infestans* RXLR effector Avrblb2, which has a canonical RXLR domain, induces HR with *Solanum bulbocastanum* NLR Rpiblb2. The perception of Avrblb2 by Rpiblb2

leads to resistance against *P. infestans* (Oh *et al.*, 2009). With the revolution of genomics, novel effectors were identified using bioinformatic analysis. ATR39-1, a *Hpa* RXLR effector, was identified based on the computational prediction. ATR39-1 exhibited avirulence activity in one ecotype of Arabidopsis and its cognate NLR, RPP39, was identified in turn (Goritschnig *et al.*, 2012).

Effector repertoires of oomycete pathogens, such as *P. infestans* and *P. sojae*, have been revealed substantially (Anderson *et al.*, 2015). However, despite the destructiveness of *P. capsici*, little is known about the molecular mechanisms under *P. capsici* effectors compared to other species. *P. capsici* causes blight and crown and fruit rot on broad range of vegetables including all cucurbits, pepper, tomato, eggplants, and even lima beans. Over 1 billion dollars of worldwide vegetable production is threatened by *P. capsici* each year (Lamour *et al.*, 2012a). After the first draft genome of *P. capsici* was completed, available sequenced genomes have been expanded (Lamour *et al.*, 2012b; Reyes-Tena *et al.*, 2019) and 357 candidate RXLR effectors were predicted from the reference genome by bioinformatic analysis. However, only one *P. capsici* RXLR effector, a homolog of *P. infestans* Avr3a, has been functionally characterized but the corresponding NLR gene is still undisclosed.

In this study, HR-like cell death inducing *P. capsici* RXLR effector candidates were investigated using effector-omics approaches. Total 25 putative *P. capsici* RXLR effectors were selected using a newly devised bioinformatic pipeline. Transient overexpression of selected *P. capsici* effector candidates triggered varying

degrees of cell death in *N. benthamiana*. To verify the activity of cell death-inducing *P. capsici* effector candidates, effector-triggered induction of defense-related gene expression were examined. In addition, silencing of downstream signaling components of NLR-mediated defense response was performed. The newly identified RXLR effector candidates may become a source of resistance against *P. capsici*.

MATERIALS AND METHODS

Plant materials and growth conditions

N. benthamiana seeds were sown in 200-plug form tray filled with horticultural bed soil (Farmhannong, Seoul, Korea) and grown in a growth chamber under conditions of 16-h/8-h light/dark photoperiod, temperature of 23-24℃, and relative humidity of 60%. After two weeks, seedlings were transplanted into pots.

Identification of *P. capsici* RXLR effector candidates

Phyca11 scaffolds and proteins were obtained from the *Phytophthora capsici* sequencing consortium website (https://mycocosm.jgi.doe.gov/Phyca11/Phyca11.home.html). Two separate approaches were used to identify *P. capsici* RXLR effector candidates.

All open reading frames (ORFs) were extracted from Phyca11 scaffolds (EMBOSS getORF; Rice *et al.*, 2000). effectR was used to predict candidate RXLR effectors from the extracted ORFs (Tabima and Grünwald, 2019). Predicted RXLR effectors without signal peptide were filtered out using signalP5.0 (Armenteros *et al.*, 2019).

Target Gene Family Finder (TGFam-Finder) was used to annotate *P. capsici* RXLR effectors (Kim *et al.*, 2020). InterProsScan5 was used for domain identification (Jones *et al.*, 2014). Target domain ID was set as PF16810 according to Pfam protein families database. Putative RXLR effectors of 20 oomycete species were used as resource proteins: including *Phytophthora infestans, P. parasitica, P. halstedii, P. capsici, P. ramorum, P. sojae, P. kernoviae, Hyaloperonospora arabidopsidis, Phytopythium vexans, Pythium ultimum var. ultimum, P. irregulare, P. iwayamai, P. arrhenomanes, P. aphanidermatum, Albugo laibachii, A. candida, Saprolegnia diclina, S. parasitica, Aphanomyces astaci,* and *A. invadans*. The 20 oomycete genome data was obtained from Oomycete Gene Order Browser (OGOB; McGowan *et al.*, 2019). RXLR effector candidates were extracted from genome data using effectR as previously described.

Among the putative RXLR effectors of two separate data sets, candidates which were differentially expressed during early infection stage and biotrophic phase in the microarray expression profiles of a previous study were selected (Jupe *et al.*, 2013).

Plasmid construction

Effector domain of 25 selected *P. capsici* RXLR effector candidates were synthesized artificially in pTwist cloning vectors (LNCbio, Seoul, Korea). Except

one failed sequence, a total of 24 effector fragments were amplified using gene-specific primers with adaptor sequences: 5' – CCAATCCCTCTACG – gene specific sequence – 3' and 5' – TATCCTCCTACG – gene specific sequence – 3'. Amplicons were cloned into potato virus X (PVX) based vector pICH31160 (pkw) by ligation-independent cloning (LIC) method (Oh *et al.*, 2010). Each cloned effector candidate was transferred to *Agrobacterium tumefaciens* strain GV3101 for *in planta* expression assay.

In planta expression assay in *N. benthamiana*

A. tumefaciens GV3101 carrying various constructs were grown overnight at 30°C in LB medium. The cells were centrifuged and resuspended in infiltration buffer [10 mM MgCl2, 10 mM MES (pH5.6), and 150 uM acetosyringone] to an optical density at 600 nm (OD_{600}) of 0.4 for *P. capsici* effector candudates. The suspension was pressure-infiltrated into expanded leaves of four-week-old *N. benthamiana* using needleless syringe. Inoculated leaves were harvested at 5-6 days post infiltration (dpi) and cell death response was monitored. Cell death phenotype was detected under white light using FOBI (Neoscience, Seoul, Korea) and cell death-associated fluorescence was detected using Azure c400 fluorescent imaging system (Azure biosystem, Dublin, CA, USA).

Gene expression assay

Total RNA was extracted using TRIZOL reagent (MRC, OH, USA), and cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen, CA, USA). Quantitative RT-PCR at 95°C for 5min, followed by 40 cycles of denaturation at 95°C for 15 sec and 55°C for 1 min with gene-specific primers was performed using CFX96 real-time PCR instrument (Bio-Rad, USA) and powerup SYBR green supermix (Applied Biosystems, MA, USA). *NbEF1a* was used to normalize gene transcripts level. Primers used in this study are listed in Table1.

Virus-induced gene silencing (VIGS) in *N. benthamiana*

Agrobacterium with pTRV1, pTRV2-GFP, pTRV2-SGT1, pTRV2-NDR1, pTRV2-ADR1, pTRV2-NRC2/3/4 were constructed as previously described. *Agrobacterium* carrying pTRV1 and pTRV2-target genes were prepared as previously described in *in planta* expression assay. Suspension of pTRV1 and pTRV2-target gene were mixed in a 1:1 ratio to final OD_{600} as 0.15 each and infiltrated into two leaves of two-week-old wild type *N. benthamiana* for VIGS. After 2-3 weeks of VIGS, upper leaves were used for subsequent inoculation of effector candidates-containing *Agrobacterium* and further experiments.

Table 1. Primers used for qRT-PCR in this study

Primer name	Sequence (5' - 3')
EF1a_F	AGCTTTACCTCCCAAGTCATC
EF1a_R	AGAACGCCTGTCAATCTTGG
Hin1_F	GCCATGCCGGAATCCAATTT
Hin1_R	TTGCAGAGGCAGCCAAAGAGA
PR1_F	AATAGGGTAGCGGCCTTTGC
PR1_R	CGGCGGCTAGGTTTTCG
RbohB_F	CAGTCTCTTCACCATGCCAAAAA
RbohB_R	CCCACAATAGAAGACCCCAACT
SGT1_F	TTTGTGGAATCTAATGGGAC
SGT1_R	CAAACAAAACAAACGTCAC
NDR1_F	CAAAGGTGTTCGTTACGATG
NDR1_R	CCTGAACCTGCAATTGTAAAAC
ADR1_F	GGAAAAGTTCTCAACTCTGGTC
ADR1_R	CTCTGTTCCATATTAAACCTAACG
NRC2_F	GCTAAGCTTCACAAGGACAAAG
NRC2_R	CAGATTGATCTTCATCTTGAAGG
NRC3_F	GACGATAAAAATAAATTTGCTCAGTGG
NRC3_R	CATCAGCAACCCCTTTTGCTC
NRC4_F	GAACAGTGGAAAGTGTTGGTGG
NRC4_R	GGTCATGAATTCCTTTGACCTC

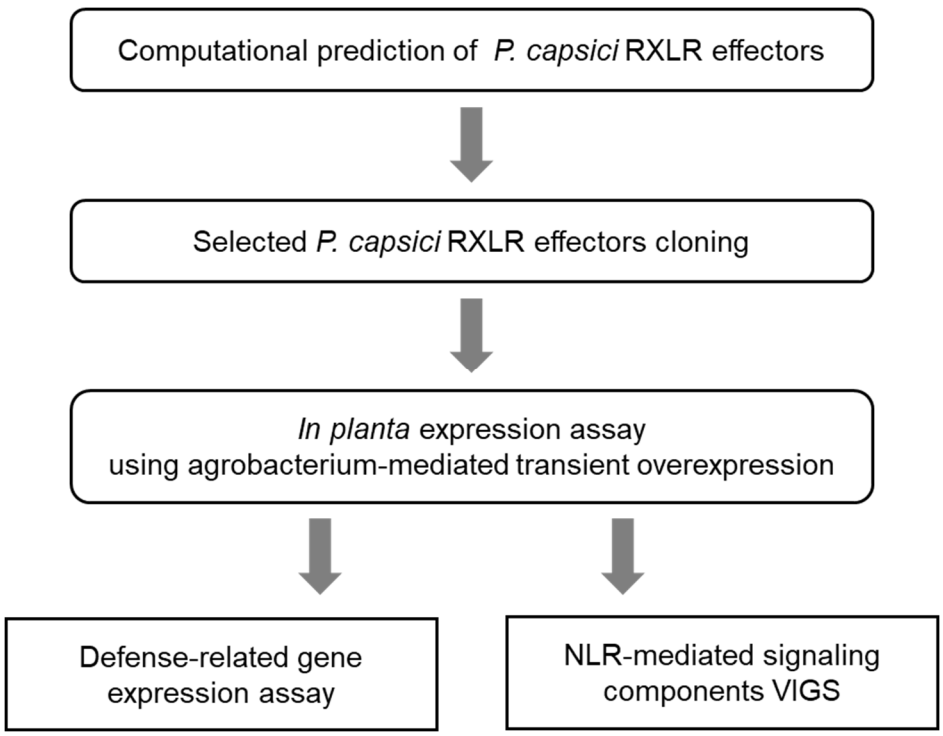


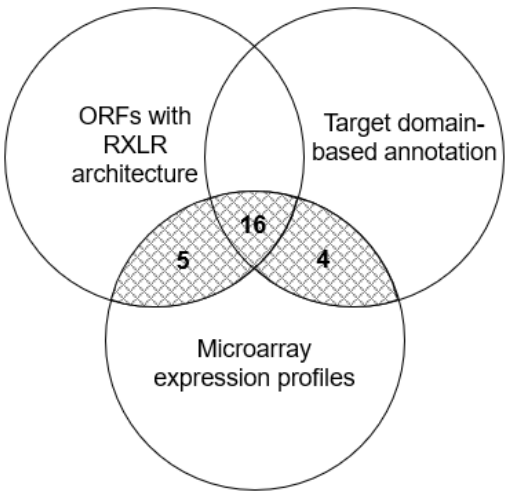
Figure 1. Workflow of this study

RESULTS

P. capsici effector candidates were selected using bioinformatic tools

To identify *P. capsici* RXLR effector candidates, a new pipeline employing three data sets, including ORFs extraction, target domain-based annotation, and microarray expression profiles of a previous study, was devised (Figure 2A). In this pipeline, two separate bioinformatic approaches were used (Figure 2B). In first approach, 268 ORFs with RXLR effector architecture were extracted. In second approach, 217 putative *P. capsici* RXLR effectors were predicted using RXLR domain-based annotation. The 20 species of oomycete RXLR effector proteins were used as references. These two data sets were merged with the previous microarray expression data set. Total 25 *P. capsici* effector candidates were selected for further studies.

(A)



(B)

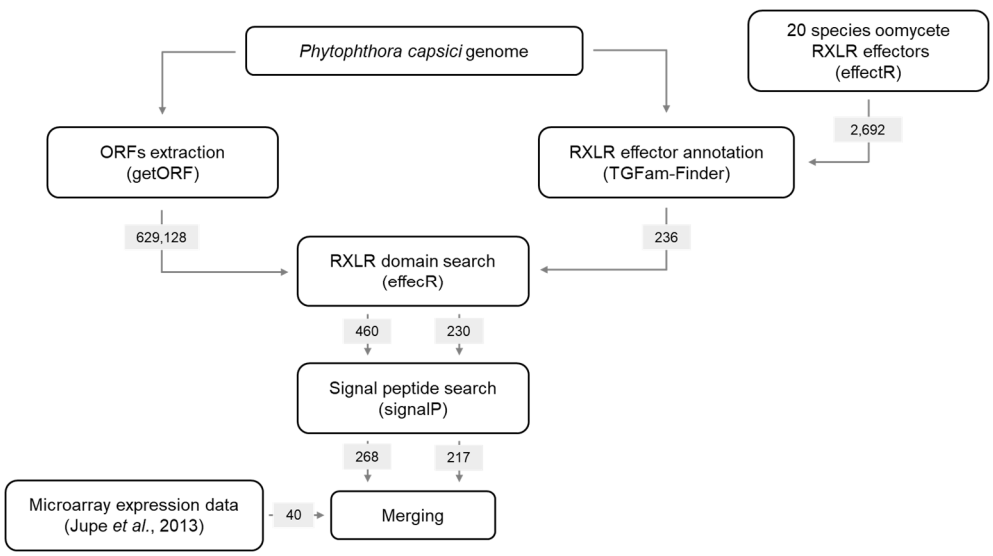


Figure 2. Putative *P. capsici* RXLR effectors are predicted by serial bioinformatic analysis.

(A) Three separate data sets used in RXLR effector identification pipeline. The number of selected effector genes are indicated in the stripped part of the venn

diagram. (B) Overall scheme of *P. capsici* effector candidate selection processes. Specific numbers of the genes in each step are shown in the gray boxes. Programs used in each step are given in parenthesis.

Multiple *P. capsici* RXLR effector candidates induce cell death in *N. benthamiana*

To investigate the response of *N. benthamiana* to *P. capsici* RXLR effector candidates, 24 effector candidates were transiently overexpressed in *N. benthamiana*. Recombinant agrobacteria carrying the effector candidates cloned into PVX-based vector pICH31160 were infiltrated into four-week-old *N. benthamiana*. BCL2 associated X (BAX) and mCherry were used as a positive and a negative control respectively. As a result, *P. capsici* effector candidates induced varying degrees of cell death in *N. benthamiana* (Figure 3A). The intensity of cell death was estimated upon the arbitrary scale from 0 to 4 (Figure 3B and 3C). In total, 9 out of 24 *P. capsici* RXLR effector candidates triggered cell death. Pathogen effectors which act as avirulent factors usually interact with plant NLR proteins to induce HR cell death. These results suggest that the cell death inducing *P. capsici* RXLR effector candidates could be recognized by endogenous NLR of *N. benthamiana* and elicit HR phenotype.

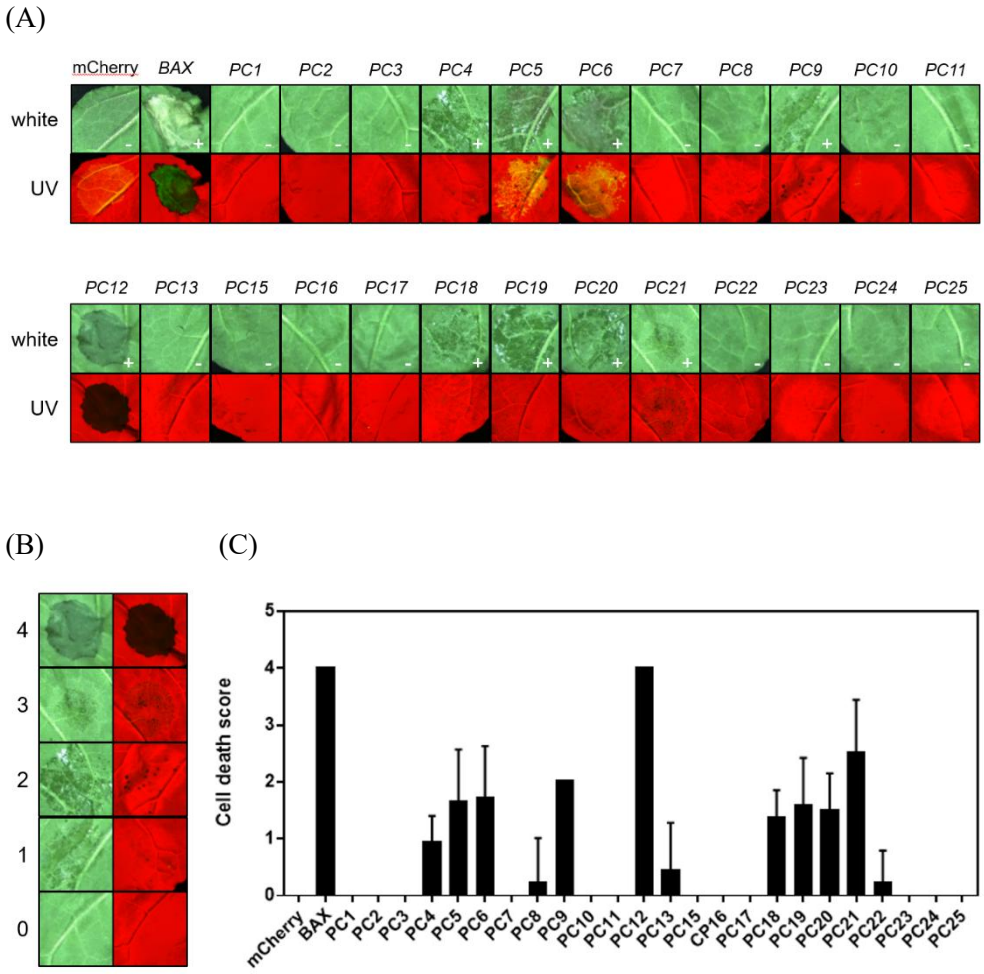


Figure 3. Multiple *P. capsici* RXLR effector candidates induce cell death in *N. benthamiana*.

(A) Cell death responses triggered by *P. capsici* RXLR effector candidates. Cell death phenotype was visualized under white and UV light. Pictures were taken at 6 dpi. mCherry and BAX were used as a negative and positive control, respectively. The plus symbol (+) indicates that cell death was observed, and the minus symbol (-) indicates that cell death was not. (B) Cell death index. Intensity of cell death was estimated according to an arbitrary scale from 0 to 4. Index 0 indicates no cell death.

Index 1 indicates only weak glittered cell death observed at abaxial side. Index 2 indicates definite glittered cell death without observed signal under UV condition. Index 3 indicates necrosis-like cell death with weak detection under UV condition. Index 4 indicates a strong cell death similar to BAX. (C) Intensity of cell death induced by *P. capsici* RXLR effector candidates according to the cell death index. Bars represent mean ± SD of 14 infiltration sites.

Cell death induced by *P. capsici* effector candidates mimics hypersensitive cell death

To verify whether cell death induced by the effectors is related to NLR–mediated defense responses, the transcript accumulation of known defense-related genes, including *Harpin-induced 1* (*Hin1), Pathogenesis-related gene 1 (PR1)*, and *Respiratory burst oxidase homolog B (RbohB)*, was monitored by quantitative RT-PCR. *Hin1* is a common gene used as a cell death marker. It is induced in response to HR-inducing elicitors (Gopalan *et al.*, 1996). *PR1* is a plant-inducible defense gene upon the activation of salicylic acid-dependent signaling (Glazebrook, 2005). *RbohB* is responsible for ROS production which plays a key role in resistance against plant pathogens (Adachi *et al.*, 2015). Randomly selected 5 *P. capsici* effector candidates were examined. mCherry and a known avr gene, *Avrblb2*, and its corresponding R gene, *Rpiblb2*, were used as negative and positive control, respectively. *P. capsici* effector candidates, mCherry, and Avrblb2-Rpiblb2 were expressed using agrobacterium-mediated transient overexpression in *N. benthamiana* leaves. Total RNA was extracted from *N. benthamiana* leaves at 24, 36, and 48 hours after infiltration. Overexpression of several effectors resulted in higher transcription level compared to the negative control (Figure 4). *Hin1* and *RbohB* were upregulated by all 5 effectors. In the case of PC12, it did not induce the accumulation of *PR1* transcript. These results indicate that cell death induced by *P. capsici* effector candidates mimics HR cell death except PC12.

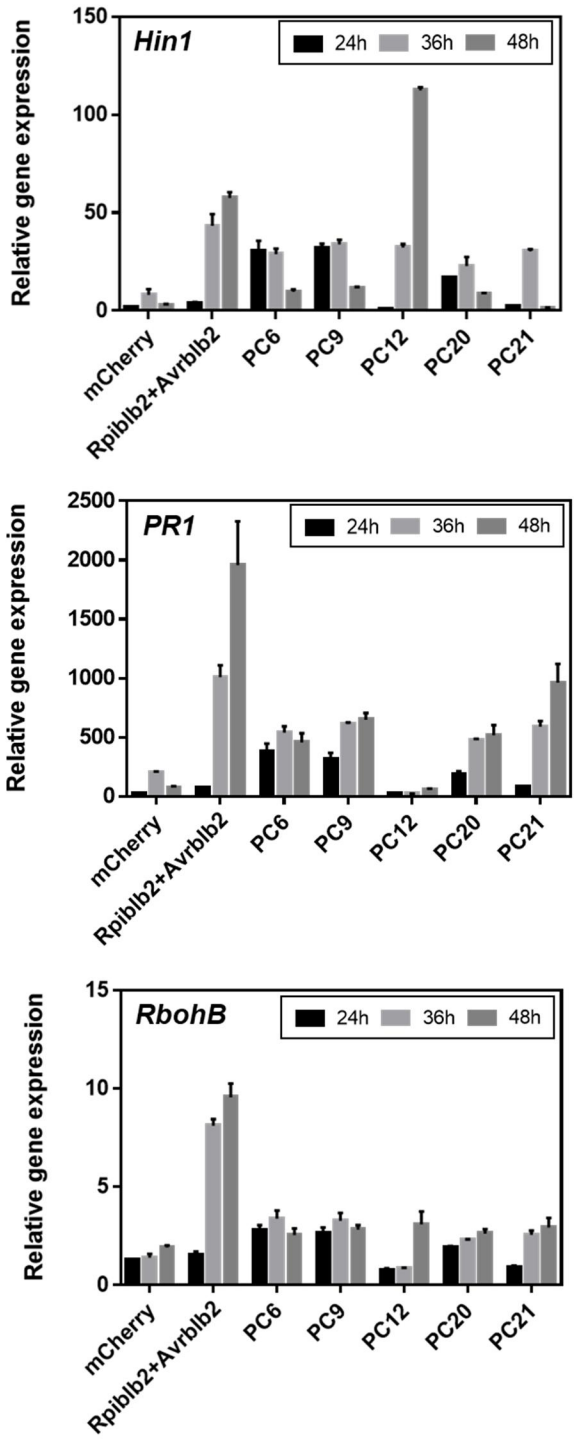

Hin1
24h
36h
48h
Relative gene expression
150
100
50
0
mCherry
Rpiblb2+Avrblb2
PC6
PC9
PC12
PC20
PC21
PR1
2500
2000
1500
1000
500
0
RbohB
15
10
5

Figure 4. Defense-related genes are upregulated by several *P. capsici* RXLR effector candidates.

Relative expressions of defense-related genes, *Hin1*, *PR1*, and *RbohB*, were examined by quantitative RT-PCR. mCherry and Rpiblb2-Avrblb2 pair were used as a negative and positive control, respectively. Randomly selected 5 *P. capsici* RXLR effector candidates were infiltrated into four-week-old *N. benthamiana* and collected at 24, 36, and 48 hours post infiltration (hpi). Transcript levels of the genes were normalized to that of *N. benthamiana EF1α*. Bars represent mean ± SD (n = 3).

Cell death mediated by *P. capsici* RXLR effector candidates are SGT1-depedent

Recent studies show several signaling components are required for NLR-mediated responses. Chaperone, NLRs, and various other components have been identified as signaling nodes of NLR-mediated responses. Therefore, whether cell death induced by *P. capsici* RXLR effector candidates was dependent on these signaling components or not was tested. VIGS was performed using TRV system to silence *NbSGT1, NbNDR1, NbADR1*, and *NbNRC2/3/4*. Cell death-inducing *P. capsici* effector candidates were agroinfiltrated in silenced *N. benthamiana*. In *SGT1*-silenced *N. benthamiana*, cell death induced by *P. capsici* effector candidates except PC12 was compromised while in *NDR1, ADR1*, and *NRC2/3/4*-silenced plants was not (Figure 5A). SGT1, NDR1, ADR1, and NRC2/3/4 transcripts were estimated by qRT-PCR 3 weeks after VIGS (Figure 5B). These results suggest that *P. capsici* RXLR effector candidates-mediated cell death is associated with SGT1-mediated signaling pathway. In addition, these results present the possibility that HR-mimic cell death induced by *P. capsici* effector candidates are NLR-mediated defense responses.

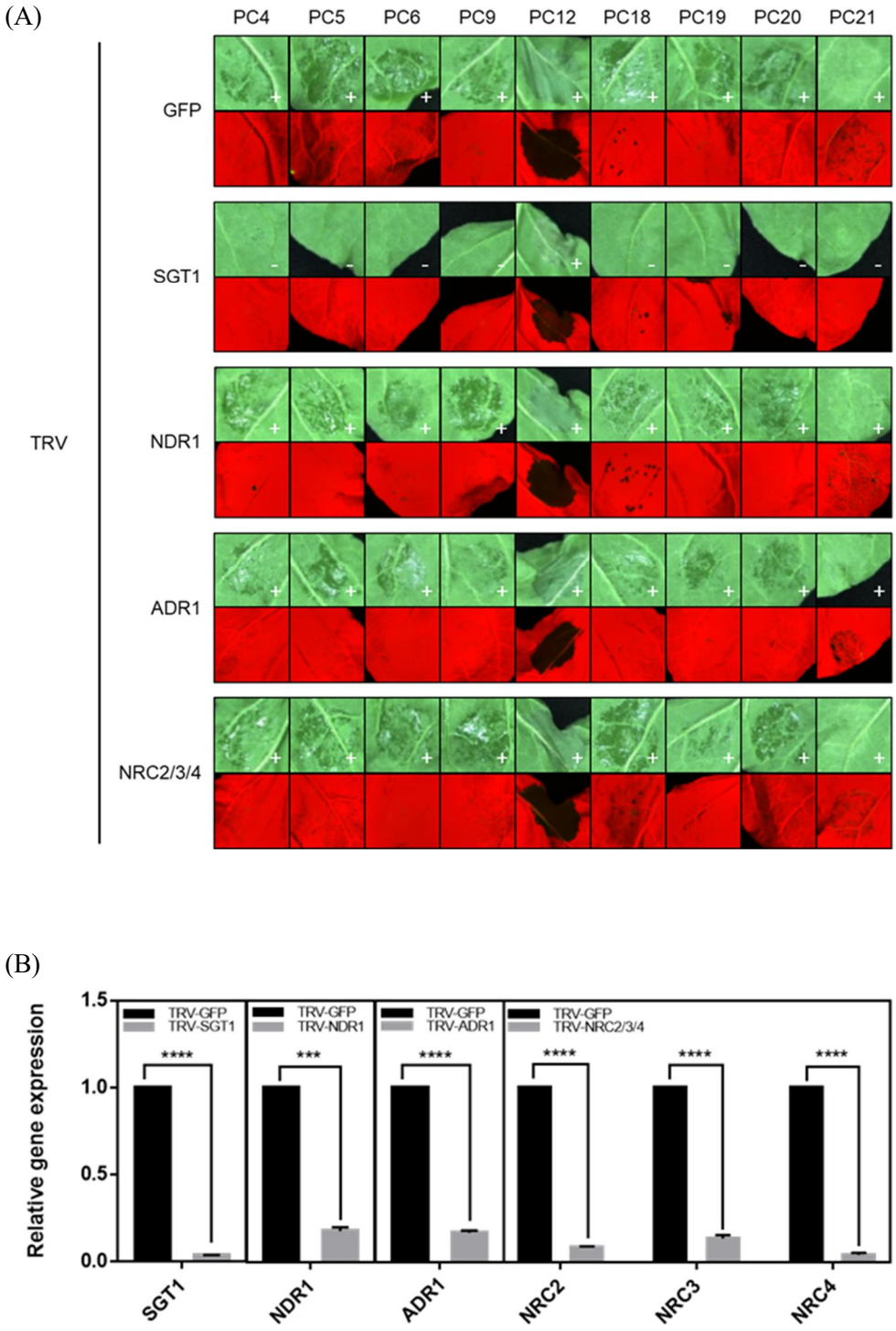

(A)
PC4
PC5
PC6
PC9
PC12
PC18
PC19
PC20
PC21
TRV
GFP
SGT1
NDR1
ADR1
NRC2/3/4
(B)
Relative gene expression
1.5
1.0
0.5
0.0
TRV-GFP
TRV-SGT1
TRV-NDR1
TRV-ADR1
TRV-NRC2/3/4
****
***
SGT1
NDR1
ADR1
NRC2
NRC3
NRC4

Figure 5. SGT1 is required for cell death triggered by *P. capsici* RXLR effector candidates

(A) Cell death phenotype in signaling components silenced *N. benthamiana*. Components of NLR-mediated pathway, including *SGT1, NDR1, ADR1*, and *NRC2/3/4* were silenced. Agrobacteria carrying cell death inducing *P. capsici* RXLR effector candidates were infiltrated into silenced *N. benthamiana* 2-3 weeks after VIGS. Pictures were taken at 6 dpi. The plus symbol (+) indicates that cell death was observed, and the minus symbol (-) indicates that cell death was not. (B) Relative transcript abundance of each gene in silenced *N. benthamiana*. Relative expression was measured by quantitative RT-PCR. Transcript levels of each gene were normalized to that of *N. benthamiana EF1α*. Transcript levels of each gene in *GFP*-silenced *N. benthamiana* was set as 1. Error bars represent mean ± SD (n = 3). Asterisks denote significant differences: ***P=0.0002, ****P<0.0001.

DISCUSSION

Globally plant diseases cause 220 billion dollars of crop yield losses annually (Chakraborty and Newton, 2011). Nowadays as genetic engineering has emerged as an effective strategy, efforts have been made to unveil the defense mechanisms of plants. Particularly, ETI accompanying HR, the robust defense response of plants, is activated by the perception of effectors by NLRs. Therefore, many attempts have been made to characterize avirulent effectors involved in ETI.

Traditionally, effectors were identified using biochemical and genetic strategies. In recent years, with the advent of genomics, effectors have been identified based on sequence analysis followed by functional studies (Kamoun, 2006). Putative *P. capsici* RXLR effector candidates were predicted using a newly devised effector identification pipeline (Figure 2). Among putative effectors with signal peptide and RXLR domain, 25 effector candidates which are differentially expressed during early infection stage and biotrophic phase were selected for further studies. The sequence alignment of the selected 25 *P. capsici* RXLR effector candidates showed low similarities with previous databases in most of the cases. Only two *P. capsici* RXLR effector candidates, PC21 and PC23, showed high similarities with a known avirulent effector, Avr3a. In addition, 20 candidates out of 25 are present in at least 4 isolates of *P. capsici* genomes (Reyes-Tena *et al.*, 2019). This implies the potential of the identified *P. capsici* RXRL effector candidates to be

novel avirulent effectors.

In planta expression assay was performed to investigate whether *P. capsici* effector candidates induce cell death or not in *N. benthamiana*. As a result, 9 effector candidates triggered varying degrees of cell death in *N. benthamiana* (Figure 3). PC12 exhibited as strong cell death as BAX while other 8 effector candidates showed relatively weak cell death. In general, avirulent effectors are recognized by NLRs in plant cells and induce HR cell death in turn (Jones and Dangl, 2006). This suggests that the cell death-inducing *P. capsici* effector candidates might be recognized by endogenous *N. benthamiana* NLRs resulting in HR cell death at macroscopic level.

During ETI, defense-related genes are activated besides cell death. The expression of three defense-related marker genes, including *Hin1*, *PR1*, and *RbohB*, which are involved in three different defense-related pathways were examined (Figure 4). Except PC12, all three defense-related genes were upregulated by 4 *P. capsici* RXLR effector candidates among 5 randomly selected effector candidates. The relatively low expression levels of the 4 effector candidates might be correlated with the cell death intensities. PC6, PC9, PC20, and PC21 exhibited relatively weak cell death compared to Avrblb2-Rpiblb2-mediated cell death. Previous studies show NLR-mediated activation of defense-related genes is associated with HR (Cui *et al.*, 2015). This indicates cell death induced by several *P. capsici* effector candidates might mimic HR cell death. In the case of PC12, *PR1* was not upregulated and *RbohB* was induced at late time point despite it triggered strong cell death. This suggests

that PC12 might not be involved in defense responses and kill the cell via another mechanism.

Furthermore, HR cell death is the consequence of the series of signaling from the perception of effectors by NLRs. Various components are known to be involved in NLR-mediated signaling. Some NLRs do not perceive effectors directly but act as signaling nodes in downstream of other sensor NLRs (Baggs *et al.*, 2017; Li *et al.*, 2015; Chiang and Coaker, 2015). ADR1 and NRC2/3/4, well known helper NLRs, are required for HR or resistance signaling network (Jubic *et al.*, 2019). There are also non-NLR components, such as NDR1 and SGT1, which are essential for NLR-mediated responses. (Shirasu, 2009; Chiang and Coaker, 2015). Silencing of these certain genes known as signaling components of NLR-mediated cell death was followed. In *SGT1*-silenced *N. benthamiana*, cell death induced by 8 out of 9 *P. capsici* RXLR effector candidates were compromised. SGT1, a molecular chaperone, forms a complex with HSP90 and RAR1 to stabilize NLR folding (Sun *et al.*, 2020). VIGS of *RAR1* and *HSP90* could be done to understand more about the molecular mechanisms under the cell death. This provides the possibility that the perception of *P. capsici* RXLR effector candidates by endogenous *N. benthamiana* NLRs induced HR-mimic cell death. PC12 exhibited as strong cell death in *SGT1*-silenced *N. benthamiana* as in *GFP*-silenced *N. benthamiana*. This is in keeping with the precedent results. PC12 might not trigger NLR-mediated HR cell death but act as another factor, such as phytotoxin, which induces death in the plant cell.

Overall, in this study novel *P. capsici* RXLR effector candidates which could act as avirulent factors were identified. The *P. capsici* effector candidates contribute to the expansion of *P. capsici* RXLR effector repertoires and the understanding of the mode of action under HR. Identification of corresponding NLRs could be used as sources of the resistance against *P. capsici*.

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ABSTRACT IN KOREAN

오랜 시간동안, 식물과 병원균은 생존을 위해 끊임없이 싸워왔다. 병원균은 식물의 방어 기작을 조절하기 위해 effector 라는 단백질을 식물 세포 안으로 분비한다. 식물체 안에서는 이에 대한 방어 기작으로 nucleotide binding leucine rich repeat (NLR) 단백질이 effector를 인지하여 effector-triggered immunity (ETI)라는 면역 반응을 유도한다. ETI는 과민성 세포 사멸 반응을 수반하는데, 이는 국소적인 세포 사멸의 형태로 나타난다. 저항성 자원으로서 면역 반응에 관여하는 NLR을 찾아내기 위해 effector를 재료로 한 많은 연구들이 진행되었다. 특히 난균류에 보존되어 있는 RXLR 도메인을 바탕으로 effector-omics 접근이 이루어지고 있다. 본 연구에서는, 과민성 세포 사멸 반응을 보이는 새로운 고추역병균의 RXLR effector후보군을 동정했다. 생물정보학적 분석을 통해 예측된 25개의 고추역병균 effector 후보군들 중 9개가 *Nicotiana benthamiana*에 세포 사멸 반응을 유도하였다. 세포 사멸 반응을 유도하는 effector 후보군들에 의해 방어기작과 관련된 유전자들의 상향조절이 이루어질 뿐 아니라, 세포 사멸에 이르기 위해 과민성 세포 사멸의 하위 신호 전달 과정중에 관여하는 Supressor of the G2 allele of *SKP1* (SGT1)을 필요로 한다는 것을 통해 확인된 effector 후보군들이 *N. benthamiana*의 내생 NLR에 의해 인지되어 과민성 세포 사멸 반응을 일

으키는데 관여할 것이라는 가능성을 보여주고 있다. 이는 과민성 세포 사멸 반응의 분자생물학적 기작에 대한 이해를 높이는 데에 기여할 뿐 아니라, 고추역병균의 effector 레퍼토리를 확장하는 데에 이바지 할 것이다.

주요어: Effector-triggered immunity (ETI), Nucleotide-binding leucine rich repeat (NLR), 과민성 세포 사멸, RXLR effector, 고추역병균

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