A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Molecular understanding of the cell death induced by *Phytophthora infestans* effectors in *Nicotiana benthamiana*

*Nicotiana benthamiana*에서 감자역병균 Effector 단백질에 의해 유도되는 세포사멸 기작

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ABSTRACT

Both pathogen and plant use cell death for their survival. Pathogen secretes virulence proteins called effector into host cell to disturb plant immune system and some effectors induce cell death in host. Plant nucleotide-binding leucine rich repeat (NLR) protein recognizes an avirulence effector and often induces programmed cell death to stop the pathogen growth. An array of effectors induce cell death in host but whether cell death induced by effectors is a plant immune response or a toxic effect caused by pathogen is still elusive in many cases.

Here, I characterized 25 effectors of *Phytophthora infestans* induce cell death in host *Nicotiana benthamiana*. Among them, 10 effectors induced

conspicuous cell death and were selected for further studies. To figure out whether the cell death induced by the effector is a plant immune response, NLR dependency of the cell death was investigated. To test that, two-step approach was designed using virus-induced gene silencing. In first step, the NLR signaling components (immune chaperone complex, helper NLRs and basal immunity related genes) were silenced and effector-induced cell death was observed. As results, all the effector-induced cell death was independent of three classes of NLR signaling components. In second step, genome-wide NLR gene silencing vectors were constructed by cloning 5 to 6 artificially synthesized NLR sequences in a TRV2 vector. Subsequently, NLRs were silenced using single vector covering 5 to 6 NLRs in a *N. benthamiana* plant and the change of effector-induced cell death was observed in the plant. As results, the cell death induced by 4 effectors was delayed compared to the control. These results may indicate that 4 *P. infestans* effectors require *N. benthamiana* NLRs to induce cell death. Further study could identify the corresponding NLR that recognizes the cell death-inducing cognite effector.

Keywords: *Phytophthora infestans*, *Nicotiana benthamiana*, RXLR effector, NLR, hypersensitive response, effector-triggered immunity

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LIST OF ABBREVIATIONS

PRR	Pattern recognition receptors
PAMP	Pathogen-associated molecular patterns
PTI	PAMP-triggered immunity
R	Resistance
ETI	Effector-triggered immunity
NLR	Nucleotide-binding leucine rich repeat
HR	Hypersensitive response
CNL	Coiled-coil NLR
TNL	Toll-interleukin 1 receptor type NLR
VIGS	Virus-induced gene silencing
GFP	Green fluorescent protein
qRT-PCR	Quantitative reverse transcription polymerase chain reaction

INTRODUCTION

Plants have evolved two-layered immune systems against pathogen infection. In first layer, pattern recognition receptors (PRRs) recognize pathogen-associated molecular patterns (PAMPs) which are conserved in the pathogens (Zipfel, 2014). PRRs activate defense signaling pathway and induce PAMP-triggered immunity (PTI). In turn, pathogens have adapted to particular host genotypes and have evolved virulence factors called effectors. Effectors are pathogen proteins secreted into the host cell. For suppressing PTI and host defenses (Jones and Dangl, 2006; Dodds and Rathjen, 2010). In second layer, plant have evolved resistance (R) proteins that recognize avirulence effectors and induce a robust immune response, effector-triggered immunity (ETI). ETI amplifies PTI-induced defense responses and plant resistance (Cui *et al*., 2015).

Many of R proteins are known as nucleotide-binding leucine rich repeat (NLR) proteins. NLRs function as intracellular immune receptors and are well conserved in plant species (Collier *et al*., 2011; Jacob *et al*., 2013). Recognizing the effectors directly or indirectly, NLRs activate downstream immunity signaling pathways (Qi and Innes, 2013).

Activated NLRs initiate immune signals which converge on mainly three classes of signaling components. First, helper NLRs are convergence points of immune signals from sensor NLRs which detect pathogen effectors. (Adachi *et al*., 2019). The helper NLR proteins in the NLR required for cell death (NRC) family function downstream of sensor NLRs including *R1*, *Rpi-blb2* and *Pto* (Wu *et al*., 2015;Wu *et al*., 2017). Helper NLR, N Requirement Gene 1 (NRG1) and Activated Disease Resistance 1 (ADR1) are downstream of specific type of NLRs. NRG1 and ADR1 are essential for many Toll-interleukin 1 receptor type NLRs (TNLs)-mediated resistance (Qi *et al*., 2018; Saile *et al*., 2020). Second, chaperone, which is a protein assisting the proper folding of macromolecules, are involved in NLR downstream signaling pathways (Beissinger and Buchner, 1998). Suppressor of the G2 allele of *SKP1* (SGT1), Required for MLA12 Resistance 1 (RAR1) and Heat Shock Protein 90 (HSP90) make up a cochaperone complex and they are required for the function of some NLRs (Takahashi *et al*., 2003; Shirasu, 2009). Third, plant basal immunity components such as Non-race-specific Disease Resistance 1 (NDR1) and Enhanced Disease Susceptibility 1 (EDS1) are convergence points of specific types of NLRs. NDR1 and EDS1 are required for coiled-coil NLRs (CNLs)- and TNLs-mediated resistance, respectively (Wiermer *et al*., 2005; Day et al.,

2006; Knepper et al., 2011). NLR downstream signaling pathways induce robust defense and often result in hypersensitive response (HR), a rapid programmed cell death at the infection site. HR restricts the spread of pathogens and contributes to the plant resistance (Jones and Dangl, 2006).

Phytophthora infestans is an oomycete pathogen and causes severe diseases on crops, especially causing late blight in potato and tomato (Fry, 2008). Genome sequencing of *P. infestans* revealed that it has over 500 RXLR effectors (Cooke et al., 2012). RXLR effectors have a conserved N-terminal RXLR (Arg-Xaa-Leu-Arg) motif for translocation signal into host cell and have highly diverse C-terminal effector domains (Whisson *et al*., 2007). A number of RXLR effectors of *P. infestans* induce or suppress cell death in plants (Bos *et al*., 2006; Lee *et al*., 2014). Some of them are known to have cognate R genes and function in plant immunity (Oh *et al*., 2014). However, it is still remaining question how the other hundreds of RXLR effectors modulate plant immunity and cell death.

In a previous study, multiple RXLR core effectors of *P. infestans* induced cell death in their host, *Nicotiana benthamiana* (SE Seo, unpublished). Core effectors are commonly present in four *P. infestans* isolates (T30-4, NL07434, P17777, 06_3928A) and also have conserved

RXLR motif (Cooke *et al*., 2012). Core effectors are highly expressed at biotrophic phase (2 or 3 dpi) after *P. infestans* infects on potato so these effectors are expected to play important roles in *P. infestans* infection (Cooke *et al*., 2012). The role of these effectors on colonization of *P. infestans* and subcellular localization in *N. benthamiana* were reported in prior research (Lee *et al*., 2018; Wang *et al*., 2019). However it is elusive whether cell death induced by effectors is a plant immune response or a toxic effect of pathogen.

Here, I demonstrated that 25 core effectors induced cell death in *N. benthamiana*. Among the effectors, 10 core effectors which induced conspicuous cell death. The cell death induced by 10 core effectors was independent of NLR signaling components (immune chaperone complex, helper NLRs and basal immunity related genes). Next, using genome-wide NLR gene silencing vectors, NLRs were silenced in a *N. benthamiana* and effector-induced cell death was observed. As results, cell death induced by CE65, CE70, CE77 and CE78 was delayed. These results may indicated that *N. benthamiana* NLRs are required to the cell death induced by 4 *P. infestans* effectors.

MATERIALS AND METHODS

Plant materials and growth conditions

Nicotiana benthamiana were used for in planta expression and virus-induced gene silencing. *N. benthamiana* seeds were germinated on the soil composed of vermiculite, cocopeat and peat moss (Farmhannong, Seoul, Korea). *N. benthamiana* plants were grown in a growth chamber at temperatures between 23°C and 24°C and the humidity about 60% under 16 h light / 8 h dark photoperiod. Two-week-old *N. benthamiana* seedlings were transplanted into and grown in separated pots (104 mm diameter X 79 mm height).

Agrobacterium-mediated transient overexpression and cell death assay

For transient expression in plant, *Agrobacterium tumerfaciens* strain GV3101 containing the respective plasmid were grown overnight in LB medium with appropriate antibiotics (50 μg / ml of rifampicin, 50 μg / ml of kanamycin, 50 μg / ml of gentamicin) at 28°C. *Agrobacterium* were centrifugated at 3,000 rpm for 10 min and resuspended in

infiltration buffer (10 mM MES pH 5.6, 10 mM $MgCl_2$, 200 μM acetosyringone) and incubated for 2 h at room temperature before infiltration. *Agrobacterium* solution were adjusted to the required OD_{600} before infiltration into *N. benthamiana* leaves. For co-infiltration, each *Agrobacterium* suspension was mixed with equal volume. The suspension was pressure-infiltrated into 4- to 5-week-old *N. benthamiana* using needleless syringe. Inoculated leaves were harvested at 3-4 days post infiltration (dpi) and cell death phenotype was observed.

Visualization of cell death and quantification for cell death intensity

Cell death images of *N. benthamiana* leaves were taken by FOBI device (Neoscience, Seoul, Korea) under white light or red filter. For quantification for cell death intensity, the maximum quantum yield of photosystem II (Fv/Fm) was measured in a closed Fluorcam 7.0 system (Photon Systems Instruments, Drasov, Czech Republic).

Construction of single or double NLR silencing fragment

Annotated sequences of 307 *N. benthamiana* NLR candidates were obtained from Solanaceae-RenSeq database (https://github.com/staskb/Solanaceae-RenSeq/tree/master/Annotation/CDS) and used for references. Constructs of 48 multiple NLR silencing vectors were kindly provided by Dr. Keehoon Sohn (Pohang University of Science and Technology, Korea). An NLR silencing fragment was designed to cover a nucleotide-binding adaptor shared by APAF-1, certain *R* gene products and CED-4 (NB-ARC) domain (Pfam signature PF00931) of NLR. Each NLR silencing fragment was 150 bp long and designed to target the partial region of NB-ARC domain of single or double NLR candidates with 100% identity.

Construction of multiple NLR silencing fragment and multiple NLR silencing vector

Multiple NLR silencing fragments were designed to consist of randomly selected 5 or 6 NLR silencing fragments. Multiple NLR silencing fragments were artificially synthesized into a single pTwist cloning vector (LNCbio, Seoul, Korea). Multiple NLR silencing

fragments were amplified and digested from pTwist vector using EcoR1 and BamHI. pTRV2 vector was linearized using EcoRI and BamHI and each amplicon of multiple NLR silencing fragments was inserted with pTRV2 vector using T4 DNA ligase (NEB, Ipswich, UK).

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

For VIGS, the binary TRV vectors pTRV1 and pTRV2 were prepared. pTRV2::*GFP*, pTRV2::*NbSGT1*, pTRV2::*RAR1*, pTRV2::*NRC2/3/4*, pTRV2::*NRG1/ADR1*, pTRV2::*NDR1* and pTRV2::*EDS1* were transformed into *A. tumefaciens* strain GV3101 by heat shock method (An., 1987). Multiple NLR silencing vectors were transformed into *A. tumefaciens* strain AGL1 by heat shock method (An., 1987). Each strain was grown overnight in LB medium with appropriate antibiotics (50 μg / ml of carbenicillin, 50 μg / ml of kanamycin in AGL1, 50 μg / ml of gentamicin, 50 μg / ml of rifampicin, 50 μg / ml of kanamycin in GV3101). *Agrobacterium* were centrifuged (3,000 rpm, 10 min, 20 °C) and resuspended in infiltration buffer (10 mM MES pH 5.6, 10 mM $MgCl_2$, 200 μM acetosyringone). *Agrobacterium* containing the pTRV1 vector and pTRV2 with target genes were mixed at a 1:1 ratio and adjusted to final OD_{600} = 0.25 before infiltration. Two leaves of 2-

week-old *N. benthamiana* were inoculated. Two upper leaves were used for cell death assay at 2 or 3 week after VIGS.

Quantitative reverse transcription PCR (qRT-PCR)

Six leaf disks were harvested from *N. benthamiana* using a cork borer and total RNA was extracted using TRIZOL reagent according to the manufacturer's instructions (MRC, OH, USA). First strand cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen, CA, USA) and 2 μg total RNA for qRT-PCR. qRT-PCR was performed at 95℃ for 5 min followed by denaturation for 40 cycles at 95℃ for 15 sec and 55℃ for 1 min with gene-specific primers (Table 1).

Phylogenetic analysis of NLR genes

The NB-ARC domain (SUPERFAMILY signature SSF52540) of target NLRs of NLR-com55 and NLR-com62 were extracted using Geneious Prime (v2021.1.1) (http://www.geneious.com). The NB-ARC domain of reference plant NLR proteins (RefPlantNLRs) were obtained from previous study (Kourelis *et al*., 2020). Extracted NB-ARC domain sequences were aligned using ClusterW (Higgins *et al*., 1994). Less than 90% coverage in all positions were removed using partial deletion

treatment. MEGA X software (Kumar *et al*., 2018) was used for phylogenetic analysis using the Maximum Likelihood method based on the Jones-Taylor-Thornton (JTT) model (Jones *et al*., 1992). Bootstrap values were calculated from 1000 bootstrap replicates.

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

Primer name	Sequence (5'-3')
SGT1_qRT_F	TTTGTGGAATCTAATGGGAC
SGT1_qRT_R	GTGACGTTTGTTTTGTTTG
RAR1_qRT_F	TCTGTAATGTCTGAAGTTTTGACAG
RAR1_qRT_R	TCTTGTGTTTTGCTGTGAGATTG
NRC2_qRT_F	GCTAAGCTTCACAAGGACAAAG
NRC2_qRT_R	CAGATTGATCTTCATCTTGAAGG
NRC3_qRT_F	GACGATAAAAATAAATTTGCTCAGTGG
NRC3_qRT_R	CATCAGCAACCCCTTTTGCTC
NRC4_qRT_F	GAACAGTGGAAAGTGTTGGTGG
NRC4_qRT_R	GGTCATGAATTCCTTTGACCTC
NRG1_qRT_F	GTTCGTGCTGATTTCCAGAAAG
NRG1_qRT_R	CATTCCCCTTGATGTTGATATATAG
ADR1_qRT_F	GGAAAAGTTCTCAACTCTGGTC
ADR1_qRT_R	CTCTGTTCCATATTAAACCTAACG
EDS1_qRT_F	CAAAGGTGTTCGTTACGATG
EDS1_qRT_R	CCTGAACCTGCAATTGTAAAAC
NDR1_qRT_F	TTTTACAGCTCCATATTACGCCAG
NDR1_qRT_R	ACAAGCAAAGCAGTTCCTAGG
EF1-α_qRT_F	GTATGCCTGGGTGCTTGAC
EF1-α_qRT_R	ACAGGGACAGTTCCAATACCA

RESULTS

Multiple RXLR core effectors of *P. infestans* induce cell death in *N. benthamiana*

In a previous study, 25 out of 57 *P. infestans* RXLR core effectors were identified to induce cell death in *N. benthamiana* using *Agrobacterium*-mediated transient overexpression. We validated the cell death induced by the 25 core effectors to select effectors which induce stable and conspicuous cell death for further studies. mCherry which does not induce cell death was used as a negative control. INF1 which is a *P. infestans* protein and induce HR cell death was used as negative and positive control. All 25 core effectors induced cell death at 4-day after infiltration (dpi) (Figure 1A). These results were in accordance with previous study. Of note, the intensity of cell death detected by the naked eyes or under UV light was different between effectors. Core effector such as CE68 and CE69 induced weak cell death which was observed only in the abaxial side, not in adaxial side of the leaves. On the other hand, core effector such as CE82 and CE86 induced strong cell death observed in both abaxial and adaxial sides of the infiltrated area. To quantify the intensity of cell death induced by core effectors, the

quantum yield of photosystem II (Fv/Fm) was measured (Figure 2B). The Fv/Fm ratios of cell death-induced by 19 core effectors, except for CE26, CE29, CE38, CE56, CE68 and CE69, were significantly reduced compared to the negative control, mCherry. Among these 19 core effectors, 10 core effectors induced stable and conspicuous cell death at 3- or 4-day post infiltration (dpi). The cell death was similar with INF1-induced HR cell death. (Figure 2A). Moreover, the Fv/Fm ratios of cell death induced by 10 core effectors were similar with or less than those of INF1 (Figure 2B). Next, we investigated the gene expression patterns of 10 core effectors during *P. infestans* infection of *Solanum tuberosum*. The transcriptome data of core effectors was provided by Sophien Dr. Kamoun (The Sainsbury Laboratory). All 10 core effectors were expressed highly at 2 dpi after *P. infestans* infection (Figure 2C). Taken together, these results indicated that 10 core effectors induce stable and conspicuous cell death in *N. benthamiana*. Therefore, we selected these effectors for further studies.

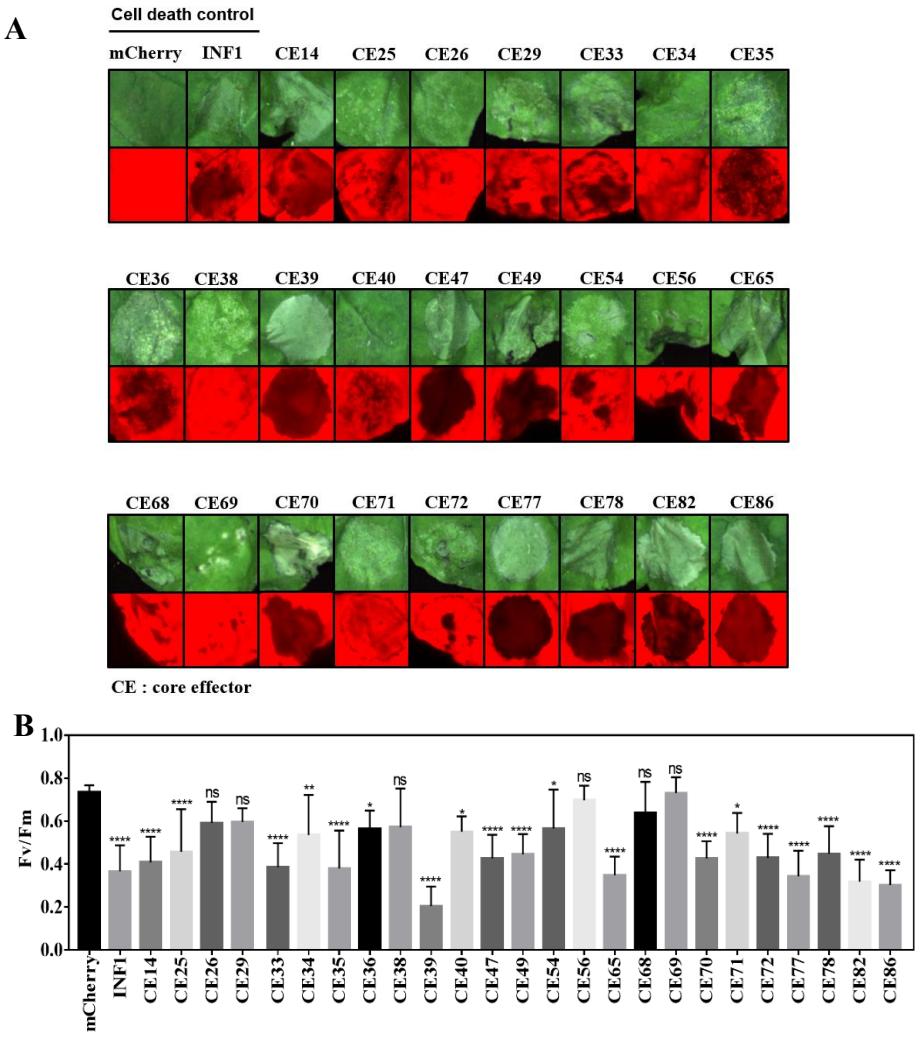

Figure 1. Multiple RXLR effectors of *P. infestans* induce cell death in *N. benthamiana*.

(A) 25 *P. infestans* RXLR core effectors induced cell death in *N. benthamiana*. The cell death phenotype was visualized under white and UV light at 4-day post infiltration (dpi). mCherry and INF1 were used as a negative and positive control, respectively. (B) The quantum yield of photosystem II (Fv/Fm) was measured for cell death intensity. The bars indicate the mean value of Fv/Fm and the error bars represent standard deviation for more than 12 replicates. The statistical significance was determined by one-way ANOVA test (****, $p<0.0001$; *, $p\leq0.05$; ns, not significant).

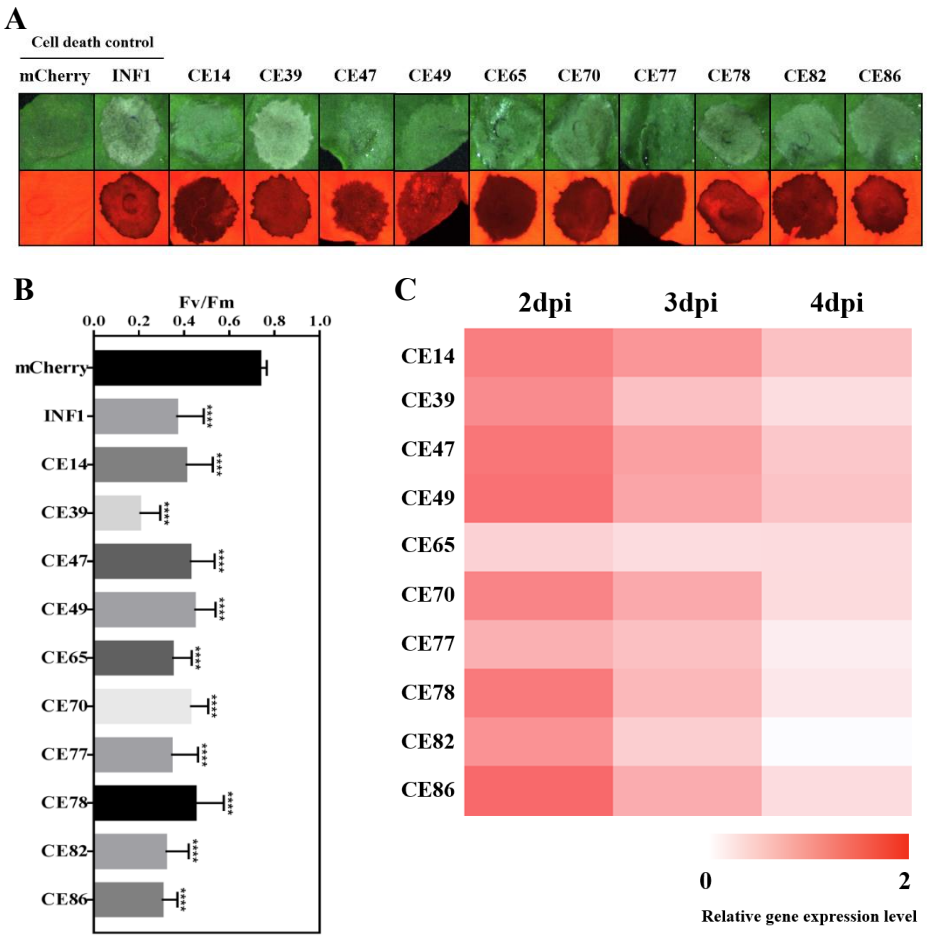

Figure 2. Ten effectors reproducibly induce HR-like cell death on *N. benthamiana*. (A) Cell death induced by 10 core effectors were similar with INF1-induced HR cell death. The cell death phenotype was visualized under white and UV light at 4 dpi. mCherry and INF1 were used as a negative and positive control, respectively. (B) The Fv/Fm ratios were measured for cell death intensity. The bars indicate the mean value of Fv/Fm and the error bars represent standard deviation for more than 12 replicates. The statistical significance was determined by one-way ANOVA test (****, $p<0.0001$). (C) Relative gene expression levels of 10 core effectors. The transcriptome data of core effectors were analyzed following *P. infestans* infection on potato.

Two-step approach for cell death screening assay

Previous studies reported that NLRs and NLR downstream signaling pathways are required for cell death induced by effector (Shirasu *et al*., 2009; Cui *et al*., 2016; Wu *et al*., 2017). To investigate whether the NLRs or NLR downstream signaling pathways are required for cell death induced by core effector, two-step approach for cell death screening was designed using VIGS. In the first step, we performed cell death screening to identify whether the NLR signaling pathways are required for cell death induced by core effector (Figure 3B). Three classes of NLR signaling components (immune chaperone complex, helper NLRs and basal immunity components) were used in this study because several *P. infestans* effectors are known to require signaling components of these classes to induce cell death (Shirasu *et al*., 2009; Cui *et al*., 2016; Wu *et al*., 2017). VIGS was performed to silence NLR signaling components in *N. benthamiana*. 10 core effectors were transiently overexpressed in NLR signaling components-silenced *N. benthamiana.* In first approach, we planned to select core effectors which induce NLR downstream signaling pathway-dependent cell death to use them in second approach because these core effectors might have potential to be recognized by NLRs which are upstream activators of

NLR downstream signaling pathways.

In second approach, cell death screening was performed to identify whether the NLRs are required for cell death induced by core effectors (Figure 3C). 10 core effectors were transiently overexpressed in NLRs-silenced *N. benthamiana.* To reduce labor-intensive and time-consuming process of silencing NLRs, we constructed multiple NLR silencing vectors for genome-wide NLR silencing in a second approach described in a later chapter (Figure 7).

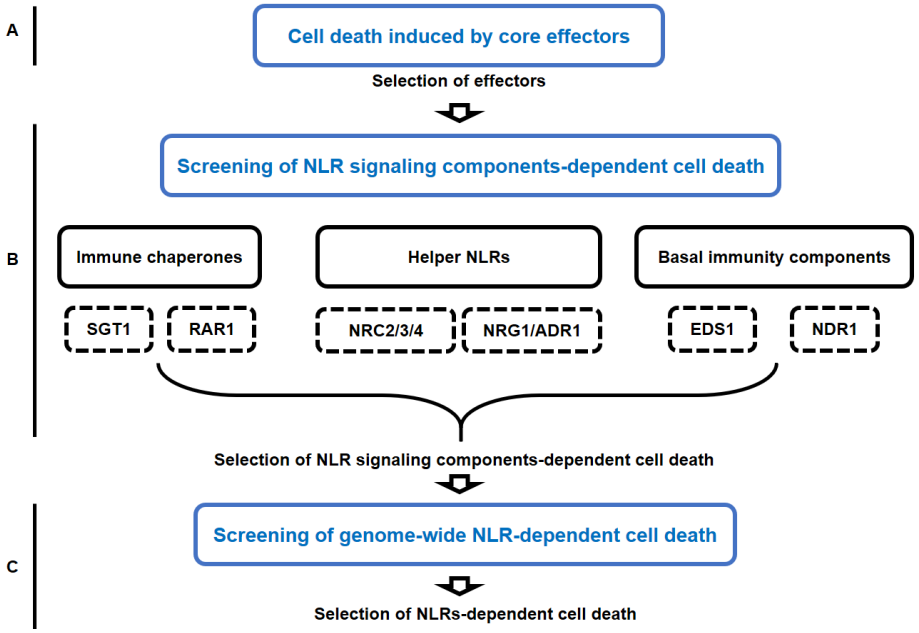

Figure 3.Scheme for identification of *N. benthamiana* NLR that recognize cell death-inducing *P. infestans* effector

(A) 10 core effectors inducing HR-like cell death were selected for further studies. (B), (C) Two-step approach for cell death screening was designed. (B) In first approach, VIGS was performed to investigate NLR signaling components (immune chaperones, helper NLRs, basal immunity components)-dependent cell death. (C) In second approach, VIGS was performed to investigate genome-wide NLRs-dependent cell death. To increase the practicability of genome-wide NLR silencing, multiple NLR silencing vectors was constructed. This will be addressed in detail in Figure 7.

Cell death induced by core effector is independent of immune chaperone complex

In first approach, we first investigated whether core effector induced-cell death requires the major immune chaperone complex. Various pairs of NLR and recognized effector require SGT1 to induce cell death and RAR1 is required for the NLR Rpm1 and the effector AvrRpm1 inducing cell death (Wu *et al*., 2017; Tornero *et al*., 2002). The components of the immune chaperone complex, *SGT1* and *RAR1*, were therefore selected for the gene silencing.

VIGS was performed to silence *NbSGT1* and *NbRAR1*. GFP was used for VIGS control. Necrosis-inducing protein (*NIP*) and the NLR-effector pair Rpbiblb2-Avrblb2 were used as a negative and positive cell death control to confirm silencing of *NbSGT1*. 10 core effectors were transiently overexpressed in silenced *N. benthamiana*. 4-day after infiltration, all 10 core effectors induced cell death at the infiltration site in similar manner in *NbSGT1*-, *NbRAR1*- or *GFP*- silenced *N. benthamiana* (Figure 4A). The silencing efficiency of *NbSGT1* and *NbRAR1* was measured by qRT- PCR (Figure 4B). These results suggest that cell death induced by 10 core effectors is independent of immune chaperone complex.

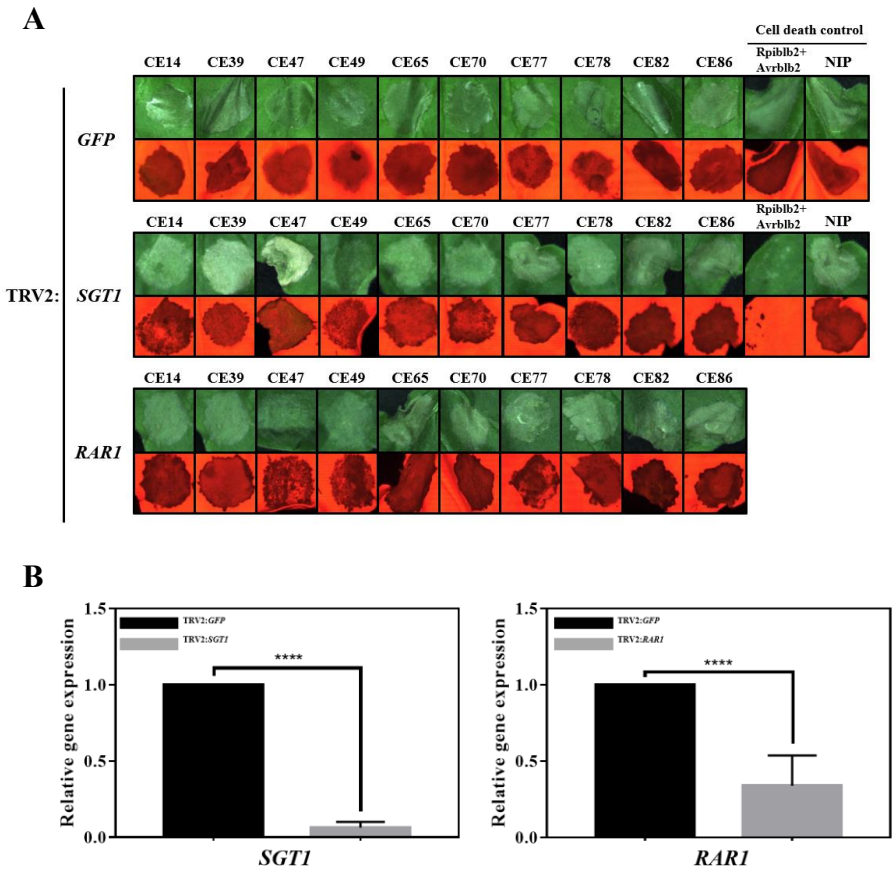

Figure 4. Cell death induced by effectors is independent of the immune chaperone complex.

(A) Cell death phenotypes induced by 10 core effectors in *GFP*- or immune chaperone complex-silenced *N. benthamiana*. Necrosis-inducing protein (*NIP*) and the NLR-effector pair *Rpbiblb2-Avrblb2* were respectively used as a negative and positive control to confirm silencing of *SGT1*. (B) The silencing efficiency was measured by qRT-PCR after VIGS. The mean values for transcript levels were normalized to those of *EF1*-α. The transcript level of each gene in *GFP*-silenced *N. benthamiana* was set to 1. The error bars represent standard deviation of three biological replicates. The statistical significance was determined by t-test. (****, $p<0.0001$).

Cell death induced by core effector is independent of helper NLRs

Next, we investigated whether core effector induced-cell death requires helper NLRs. NRC family (NRC2, NRC3, NRC4), a helper NLR family required for *R1* and *Rpi-blb2*-mediated cell death, and helper NLRs NRG1 and ADR1, essential for TNLs- mediated HR, were selected for the gene silencing (Wu *et al*., 2017; Qi *et al*., 2018; Saile *et al*., 2020).

VIGS was performed to silence *NbNRC2/3/4* and *NbNRG1/ADR1*. The N gene-p50 pair and the NLR-effector pair R8-Avr8 were used as negative and positive cell death controls to confirm silencing of *NbNRC2/3/4* and *NbNRG1/ADR1*. 10 core effectors were transiently overexpressed in silenced *N. benthamiana*. 4-day after infiltration, 10 core effectors induced cell death at the infiltration site in similar manner in *NbNRC2/3/4*-, *NbNRG1/ADR1*- and *GFP*- silenced *N. benthamiana* (Figure 5A). The silencing efficiency of *NbNRC2/3/4* and *NbNRG1/ADR1* was measured by qRT-PCR (Figure 5B). Taken together, these results suggest that cell death induced by 10 core effectors is independent of helper NLRs.

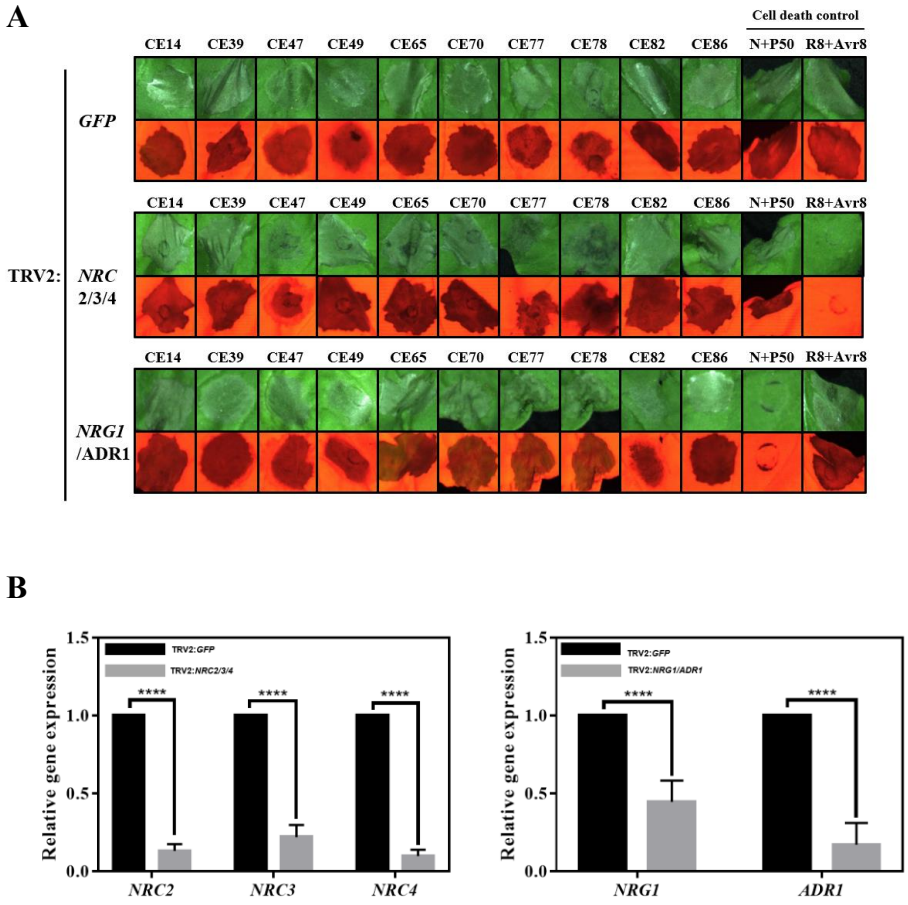

Figure 5. Cell death induced by effectors is independent of the helper NLRs.

(A) Cell death phenotypes induced by 10 core effectors in *GFP*- or helper NLRs-silenced *N. benthamiana*. The *N* gene-*p50* pair and the NLR-effector pair R8-Avr8 were used as cell death controls. (B) The silencing efficiency was measured by qRT-PCR after VIGS. The mean values for transcript levels were normalized to those of *EF1*-α. The transcript level of each gene in *GFP*-silenced *N. benthamiana* was set to 1. The error bars represent standard deviation of three biological replicates. The statistical significance was determined by t-test. (****, $p<0.0001$).

Cell death induced by core effector is independent of basal immunity components

Next, we investigated whether cell death induced by core effectors requires basal immunity components. EDS1 which is necessary for TNLs-mediated cell death and NDR1 which is recruited by multiple CNLs were selected for the gene silencing (Cui *et al*., 2015).

VIGS was performed to silence *NbEDS1* and *NbNDR1*. The *N* gene-*p50* pair and the NLR-effector pair R8-Avr8 were used as a positive and negative control to confirm silencing of *NbEDS1*. 10 core effectors were transiently overexpressed in silenced *N. benthamiana*. 4-day after infiltration, 10 core effectors induced cell death at the infiltration site in similar manner in *NbEDS1*-, *NbNDR1*- and *GFP*- silenced *N. benthamiana* (Figure 6A). The silencing efficiency of *NbEDS1* and *NbNDR1* was measured by qRT-PCR (Figure 6B). Taken together, these results suggest that cell death induced by 10 core effectors is independent of basal immunity components.

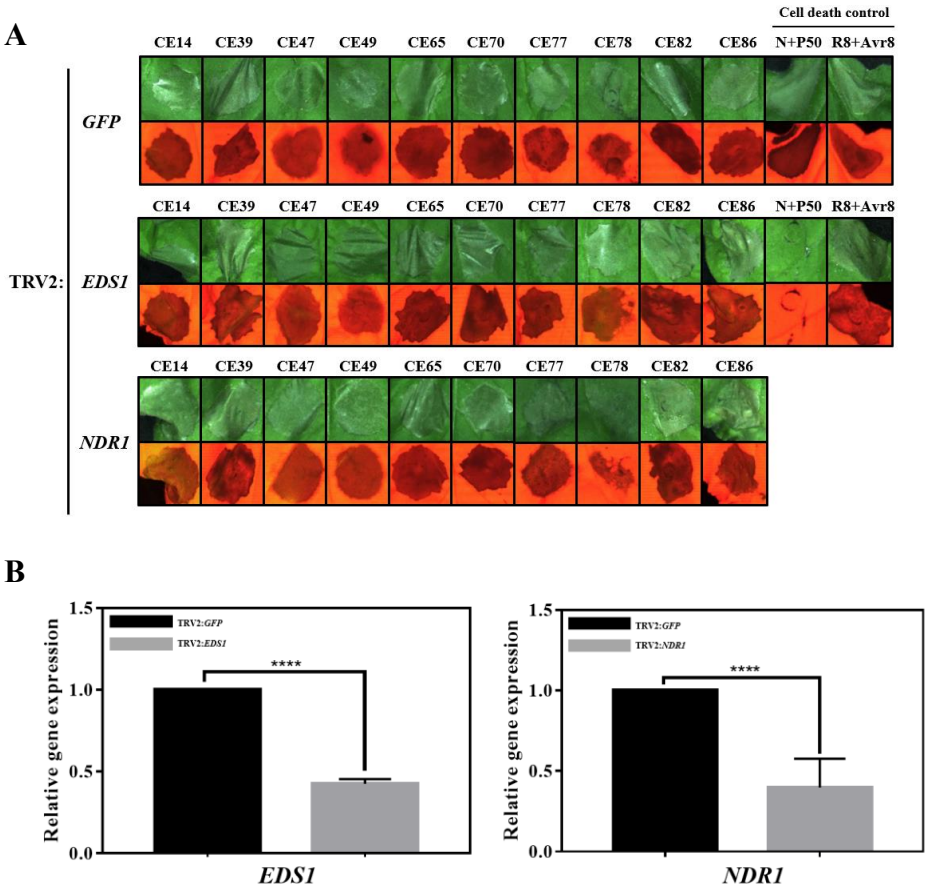

Figure 6. Cell death induced by effectors is independent of the basal immunity components.

(A) Cell death phenotype induced by 10 core effectors in *GFP*- or basal immunity components-silenced *N. benthamiana*. The *N* gene-*p50* pair and the NLR-effector pair R8-Avr8 were used as cell death controls. (B) The silencing efficiency was measured by qRT-PCR after VIGS. The mean values for transcript levels were normalized to those of *EF1*-α. The transcript level of each gene in *GFP*-silenced *N. benthamiana* was set to 1. The error bars represent standard deviation of three biological replicates. The statistical significance was determined by t-test. (****, $p<0.0001$).

Multiple NLR silencing vector construction for genome-wide NLR silencing

In first approach, cell death induced by 10 core effectors was independent of NLR signaling components. But we performed second approach because there is a possibility that unknown NLR signaling pathways may be involved in cell death. We used all 10 core effectors in second approach.

Over 300 NLR candidates have been annotated in *N. benthamiana* (Seong *et al*., 2017) so it is labor-intensive and time-consuming process to silence all NLRs one-by-one in each *N. benthamiana*. In a previous study, multiple NLR silencing vectors were constructed and provided by Dr.Sohn (POSTECH). A multiple NLR silencing vector consists of 5 or 6 NLR silencing fragments, each of which could silence single or double NLRs. Therefore, multiple NLRs could be silenced using a multiple NLR silencing vector. These multiple NLR silencing vectors were constructed to target NLRs annotated based on *N. benthamiana* genome Niben.genome.v0.4.4. But we selected 307 NLR candidates which were recently annotated based on *N. benthamiana* genome Niben.genome.v1.0.1 for this study (Seong *et al*., 2017). By BLAST search, we found that 182 recently annotated NLRs were

matched with previously constructed NLR silencing fragments over 98% identity and over 95% query coverage. Therefore, we designed additional multiple NLR silencing vectors for silencing the unmatched 125 NLRs.

A NLR silencing fragment is a 150 bp long DNA which have 100% identity with the partial region of the NLR domain, nucleotide-binding adaptor shared by APAF-1, certain *R* gene products and CED-4 (NB-ARC) domain (Figure 7A). Each NLR silencing fragment was 150 bp long and designed to silence single NLR candidate or double NLR candidates which have conserved NB-ARC domain (Pfam signature PF00931). A total of 121 single and 3 double NLR silencing fragments were designed. Among them 2 double NLR silencing fragments respectively covered 2 NLR candidates, which were already targeted by previously constructed NLR silencing fragments. Therefore, a total of 124 NLR silencing fragments were designed to silence 127 NLR candidates. Next, we designed multiple NLR silencing fragments which consist of randomly selected 5 or 6 NLR silencing fragments (Figure 7B). A total of 21 multiple NLR silencing fragments were designed and artificially synthesized into pTwist cloning vectors. In turn, the multiple NLR silencing fragments were cloned into pTRV2 silencing vectors (Figure 7C). As a result, a total of 21 multiple NLR silencing plasmids

which could silence 127 NLR candidates were constructed. Taken together, 69 multiple NLR silencing vectors (newly constructed 21 multiple NLR silencing vectors and previously constructed 48 multiple NLR silencing vectors) were used for further studies.

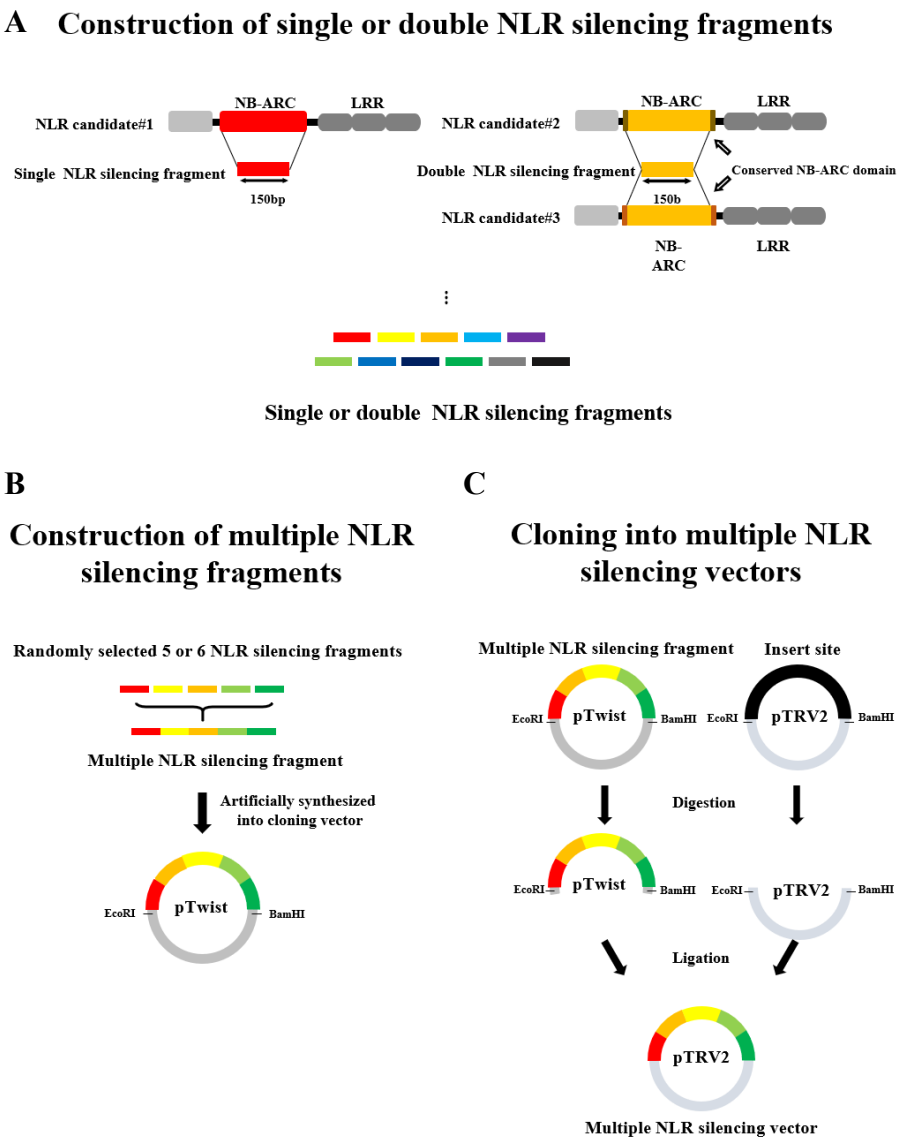

Figure 7. Pipeline for the genome-wide multiple NLR silencing vector construction. (A) Single or double NLR silencing fragments were constructed to have 100% identity with the partial region of the NB-ARC domain of NLRs. Each NLR silencing fragment was 150 bp long. (B) A multiple NLR silencing fragment was constructed to consist of randomly selected 5 or 6 NLR silencing fragments. Multiple NLR silencing fragments were artificially synthesized into pTwist cloning vectors. (C) Cloning of multiple NLR

silencing fragments in TRV2 silencing vectors. Multiple NLR silencing fragments in pTwist vectors and pTRV2 silencing vectors were digested with the restriction enzyme BamHI and EcoRI. Digested multiple NLR silencing fragments were cloned into pTRV2 vectors with T4 ligase.

Cell death induced by core effectors is delayed using multiple NLR silencing vectors

To investigate whether cell death induced by core effectors requires NLRs, VIGS was performed to silence NLR candidates using 56 of 69 multiple NLR silencing vectors. 10 core effectors were transiently overexpressed in silenced *N. benthamiana*.

After 4-day after infiltration, it was observed that CE65-, CE70-, CE77-, CE78-induced cell death was delayed using the cognate multiple NLR silencing vectors (Multi-vector#8 or Multi-vector#15) compared with using the control TRV2:*GFP* (Figure 8). In detail, CE65, CE77 and CE 70 induced cell death only at the edge of infiltration region. CE77 and CE78 induced cell death overall at the infiltration region, but the intensity of cell death was weaker than that of the control. These results suggest that cell death induced by 4 core effector requires NLR.

Next, we investigated evolutionary diversity about target NLRs of Multi-vector#8 and Multi-vector#15. The reference NLR dataset, which consists of experimentally validated 415 NLR sequences and were explored evolutionary relationships previously, were used for reference dataset of phylogenetic analysis (Kourelis *et al*., 2020). The NB-ARC domain (SUPERFAMILY signature SSF52540) of reference NLR

dataset and the target NLRs of Multi-vector#8 and Multi-vector#15 were extracted, except for 3 target NLRs in Multi-vector#8 which have no NB-ARC domain (SUPERFAMILY signature SSF52540). We performed the phylogenetic analysis using the extracted NB-ARC domains. The phylogenetic tree was classified into well-defined NLR clades, CNL, TNL, RPW8-type CNL (RNL), and a monophyletic G10 group of CNL (G10-CNL). It was confirmed that 6 target NLRs were grouped in CNL subclade, 3 target NLRs were grouped in TNL subclade and 1 target NLR was grouped in G10 subclade (Figure 9). Overall, these results imply that target NLRs of Multi-vector#8 and Multi-vector#8 are involved in cell death induced by core effectors. In addition, these results present the possibility that 4 core effectors require NLRs independent of downstream signaling pathway related with immune chaperone complex, helper NLRs and basal immunity components.

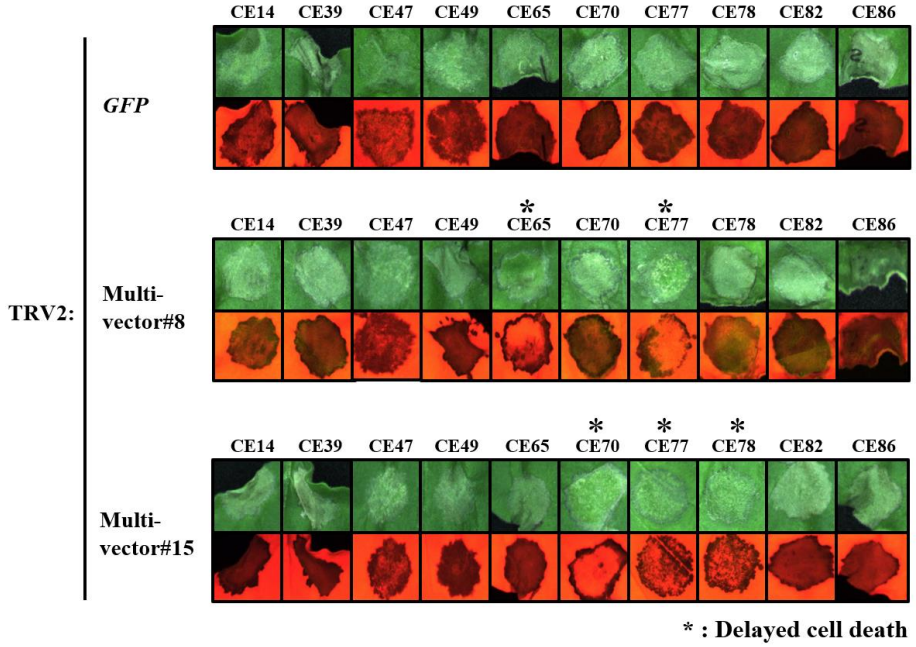

Figure 8. Cell death induced by CE65, CE70, CE77 and CE78 was delayed using multiple NLR silencing vectors.

Phenotypes of cell death induced by core effectors in *N. benthamiana* silenced by Multi-vector#8 or Multi-vector#15. 10 core effectors were transiently overexpressed into silenced *N. benthamiana* 2 or 3 weeks after VIGS. The asterisk (*) means delayed cell death in *N. benthamiana* silenced by cognate multiple NLR silencing vectors compared with *GFP*-silenced *N. benthamiana*.

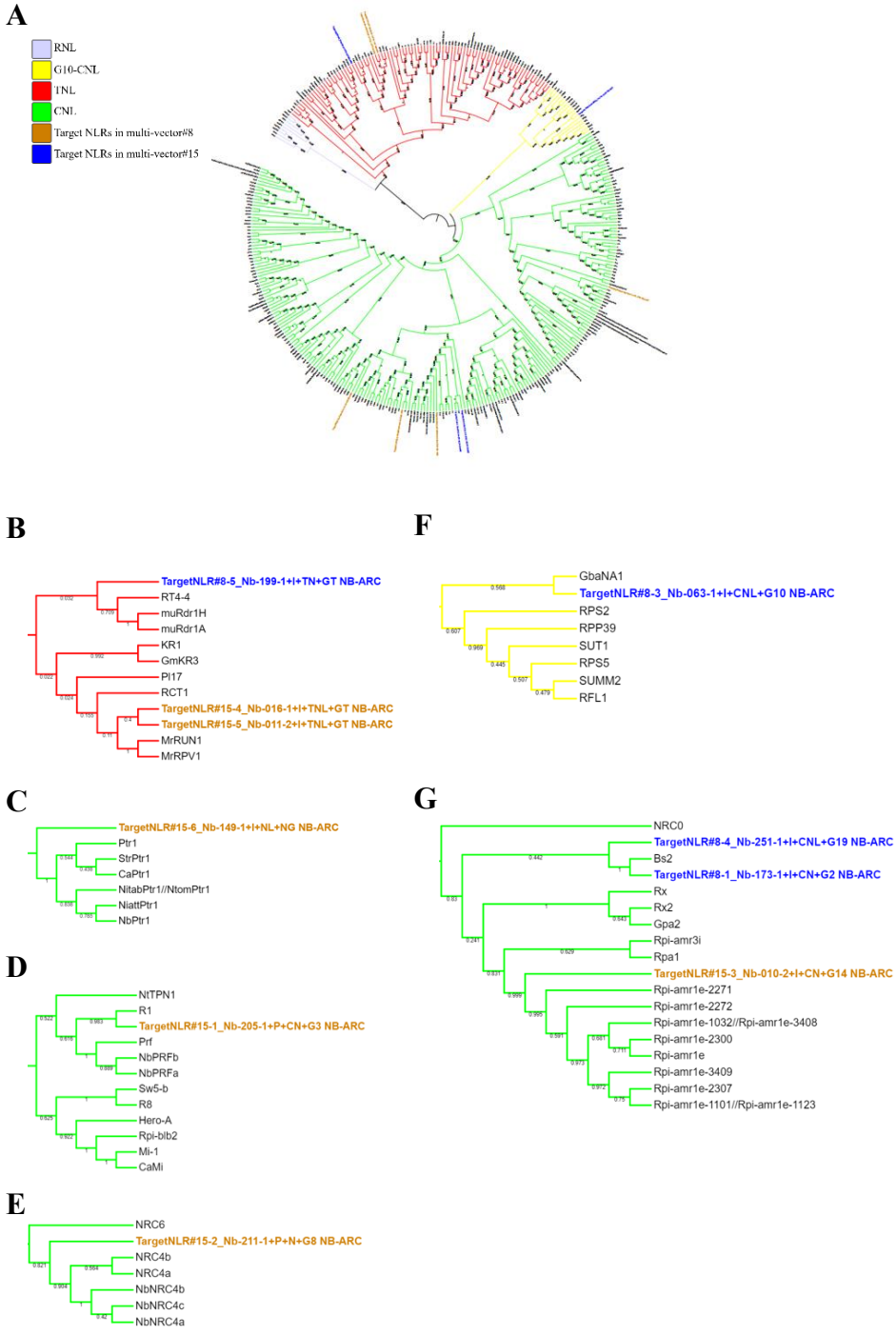
A
RNL
G10-CNL
TNL
CNL
Target NLRs in multi-vector#8
Target NLRs in multi-vector#15
B
TargetNLR#8-5_Nb-199-1+I+TN+GT NB-ARC
RT4-4
muRdr1H
muRdr1A
KR1
GmKR3
PI17
RCT1
TargetNLR#15-4_Nb-016-1+I+TNL+GT NB-ARC
TargetNLR#15-5_Nb-011-2+I+TNL+GT NB-ARC
MrRUN1
MrRPV1
C
TargetNLR#15-6_Nb-149-1+I+NL+NG NB-ARC
Ptr1
StrPtr1
CaPtr1
NitabPtr1//NtomPtr1
NiattPtr1
NbPtr1
D
NtTPN1
R1
TargetNLR#15-1_Nb-205-1+P+CN+G3 NB-ARC
Prf
NbPRFb
NbPRFa
Sw5-b
R8
Hero-A
Rpi-blb2
Mi-1
CaMi
E
NRC6
TargetNLR#15-2_Nb-211-1+P+N+G8 NB-ARC
NRC4b
NRC4a
NbNRC4b
NbNRC4c
NbNRC4a
F
GbaNA1
TargetNLR#8-3_Nb-063-1+I+CNL+G10 NB-ARC
RPS2
RPP39
SUT1
RPS5
SUMM2
RFL1
G
NRC0
TargetNLR#8-4_Nb-251-1+I+CNL+G19 NB-ARC
Bs2
TargetNLR#8-1_Nb-173-1+I+CN+G2 NB-ARC
Rx
Rx2
Gpa2
Rpi-amr3i
Rpa1
TargetNLR#15-3_Nb-010-2+I+CN+G14 NB-ARC
Rpi-amr1e-2271
Rpi-amr1e-2272
Rpi-amr1e-1032//Rpi-amr1e-3408
Rpi-amr1e-2300
Rpi-amr1e
Rpi-amr1e-3409
Rpi-amr1e-2307
Rpi-amr1e-1101//Rpi-amr1e-1123

Figure 9. Phylogenetic diversity of NLRs in the silencing vectors NLR-com55 and NLR-com62.

(A) Phylogenetic tree was constructed based on the NB-ARC domain (SUPERFAMILY signature SSF52540). The Maximum Likelihood method based on the Jones-Taylor-Thornton (JTT) model (Jones *et al*., 1992) and bootstrap analysis with 1,000 replicates were performed. The blue and brown labeled genes are target NLRs of Multi-vector#8 or Multi-vector#15, respectively. CNL : coiled-coil NLR; TNL : Toll-interleukin 1 recetopr type NLRs; RNL : RPW8-type CNL; G10-CNL : a monophyletic G10 group of CNL. (B) ~ (G) The phylogenetic subgroup of target NLRs.

DISCUSSION

Pathogen and plant use cell death for their survival. Multiple RXLR core effectors of *P. infestans* induce cell death in host *N. benthamiana* and *P. infestans* still can cause disease. Although the impacts on pathogenicity of these core effectors have been investigated (Lee *et al*., 2018; Wang *et al*., 2019), whether cell death induced by core effector is plant defense response or toxic reaction by pathogen is an unanswered question.

In this study, we performed transient overexpression assay to investigate the cell death induced by core effectors. It was confirmed that 25 core effectors induce cell death in *N. benthamiana* (Figure 1). This result is consistent with a previous study (Seo, unpublished). Also we found that 10 core effectors induced HR-like strong and conspicuous cell death (Figure 2). In general, HR cell death is induced when the NLRs recognize the effectors and the NLR signaling pathways are activated (Jones and Dangl, 2006; Cui *et al*., 2015). This implies that NLRs and NLR signaling pathways are might be required for cell death induced by core effectors. 10 core effectors were selected for further studies.

A two-step approach for cell death screening was designed to

investigate whether the cell death induced by core effectors requires NLR signaling pathways (first step, Figure 3B) and NLRs (second step, Figure 3C). In first approach, NLR signaling components (immune chaperone complex, helper NLRs, basal immunity components) were silenced in *N. benthamiana* for cell death screening assay. SGT1 and RAR1 which are involved in cell death induced by several *P. infestans* effectors were selected for immune chaperone complex. NRC family (NRC2, NRC3, NRC4) and NRG1 and ADR1 were selected for the helper NLRs. For basal immunity components, EDS1 and NDR1 were selected for silencing. None of cell death induced by 10 core effectors was compromised in NLR signaling components-silenced *N. benthamiana* (Figure 4, Figure 5 and Figure 6). This result implies that cell death induced by core effectors may require unique signaling pathways because many *P. infestans* effectors which have known NLR partners require NLR signaling pathways used in this study to induce HR (Tornero *et al*., 2002; Cui *et al*., 2015; Wu *et al*., 2017). But there is also effector-NLR pair such as PITG_15039-*RpiEdn2* which induces cell death but has not yet been reported to have related NLR signaling pathway (Chatziavgerinos, Wageningen University, unpublished). We performed second approach with 10 core effectors because there is a possibility that cell death induced by core effectors may require unknown

NLR signaling pathways.

In second approach, multiple NLR silencing vectors which could efficiently silence genome-wide NLRs were constructed and used for cell death screening (Figure 7). Multiple NLR silencing vector was designed to consist of 5 or 6 NLR silencing fragments. Each NLR silencing fragment have 100% identity with partial region of NB-ARC domain (Pfam signature PF00931) of NLR. Randomly selected 5 or 6 NLR silencing fragments were arranged in order and constructed as a multiple NLR silencing fragment. Multiple NLR silencing fragments were cloned into TRV2 silencing vectors and used for cell death screening assay.

Cell death induced by 4 core effectors was delayed using 2 multiple NLR silencing vectors compared with using the control vector TRV2:*GFP*. CE65-, CE77- induced cell death and CE70-, CE77-, CE78-induced cell death was delayed using Multi-vector#8 and Multi-vector#15, respectively. This result imply that 4 core effectors require target NLRs of Multi-vector#8 and Multi-vector#15 to induce cell death. Interestingly, cell death induced by 4 core effectors was delayed but not fully compromised. This result may be attributed to the limitation of VIGS system in which the target genes are not completely knockout. In addition, when using multiple NLR silencing vector the silencing

efficiency may be lower than using silencing vector which silence single genes. To confirm which NLR is involved in delayed cell death, each target NLR of Multi-vector#8 and Multi-vector#15 is required to be silenced separately in *N. benthamiana*.

Overall, it can be speculated that cell death induced by 4 *P. infestans* core effectors may require NLRs but may be independent of NLR signaling components used in this study. It is remaining question how *P. infestans* cause disease in *N. benthamiana* despite multiple core effectors induce cell death. There might be possible answer to this questions. First, cell death induced by core effectors may be required for pathogenicity. Recently studies reported that cell death-inducing RXLR effectors are essential for virulence or enhances pathogen growth in host plant (Lee *et al*., 2018; Li *et al*;, 2019). Also it was reported that pathogen even tricks the NLR-mediated immunity using its effector. A necrotrophic pathogen *Cochliobolus victoriae* secretes an effector victorin which activates the NLR protein LOV1 in *Arabidopsis* (Lorang *et al*., 2012). Activation of LOV1 induces HR-like response which results in disease susceptibility. *P. infestans* may use their cell death-inducing effectors through the similar strategy of *C. victoriae*. Second, *P. infestans* may modulate the function of effectors. A hundreds of effectors are secreted into host cell and it would be important for pathogen to

finely tune the function of effectors. For example, *P. infestans* effector AVR3a induces cell death in presence of a NLR protein R3a, but it could also suppress INF1-induced cell death in *N. benthamiana* (Bos *et al*., 2006). By modulating effectors and cell death properly, pathogen may promote pathogenicity. But it is difficult work to understand the mode of action of whole effectors. The identification of corresponding NLRs required for cell death induced by core effector could provide insight to understanding of how the core effectors induce cell death and their function in pathogenicity.

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ABSTRACT IN KOREAN

병원균과 식물은 생존을 위해 세포사멸을 이용한다. 병원균은 숙주 세포 내로 병원성단백질인 effector를 분비하여 식물 면역체계를 방해하는데 일부 effector는 숙주에서 세포사멸을 일으킨다. 식물의 nucleotide-binding leucine rich repeat (NLR) 단백질은 effector를 인지하여 병원균의 증식을 막기 위해 종종 programmed cell death를 일으키기도 한다. 다양한 effector가 숙주에서 세포사멸을 일으키지만 effector에 의한 세포사멸이 식물 면역반응에 의한 것인지 혹은 병원균에 의한 독성반응인지 알려진 경우는 거의 없다.

본 연구에서 감자역병균의 25개 effector가 숙주인 *N. benthamiana*에서 세포사멸을 일으키는 것이 확인되었다. 그 중 10개의 effector가 강력하고 안정적인 세포사멸을 일으켜서 이후 실험에 사용되었다. Effector에 의한 세포사멸이 식물 면역반응에 의해 유도되는 것인 것 확인하기 위해, 이러한 세포사멸이 NLR을 필요로 할 것이라고 가정됐다. 가설을 검증하기 위해, 바이러스를 이용한 유전자 침묵법을 이용하여 effector에 의한 세포사멸에 필요한 NLR 신호전달물질과 NLR을 찾는 두 단계로 이루어진 접근방법이 고안됐다. 첫 번째 단계에서는 10개 effector가 NLR 신호전달물질인 면역샤페론단백질 복합체, helper NLRs, 면역관련 기본요소들과 무관하게 세포사멸을 유도하는 것을 밝혔다. 두 번째 단계를 진행하기 위해, 효율적으로 NLR 유전자를 침묵시킬 수 있는 다중 NLR

침묵 벡터를 제작했다. 다중 NLR 침묵 벡터를 사용했을 때 4개의 effector에 의한 세포사멸이, GFP 유전자가 침묵된 대조군에 비해 늦게 유도됨이 확인되었다. 이러한 결과들을 종합했을 때 NLR들이 effector에 의한 세포사멸에 관여한다는 것을 시사한다.