A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Identification of Target Proteins Involved in Cell Death Signaling Induced by a *Phytophthora infestans* Core Effector in *Nicotiana benthamiana*

감자 역병균 effector의 세포 사멸 반응에 관여하는 식물 단백질 연구

FEBRUARY, 2017

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SUBMITTED TO THE FACULTY OF THE GRADUCATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

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ABSTRACT

In nature, plants have developed a multi-layered defense system, since they are exposed to various environmental challenges such as pathogen attacks. A programmed cell death is induced to the formation of necrotic lesions that prevents further spread of the pathogens from the infection site. Recent studies showed that diverse pathogen effectors, pathogen-specific secreted molecules, interact with plant proteins to induce the cell death. In this study, I examine of plant target proteins of a *Phytophthora infestans* core effector 82 (CE82) which induces cell death in *Nicotiana benthamiana* and pepper to investigate how the pathogen effector triggers cell death in plant cells. The candidates of target protein interacting with CE82 were identified by co-immunoprecipitation (co-IP) and LC-

MS/MS. Among the target protein candidates, four candidates are related to the cellular vesicle trafficking. In order to verify the function of four target protein candidates in CE82-induced cell death responses, virus-induced gene silencing (VIGS) was performed with the candidates. Although the gene expressions of the candidate genes were suppressed in *N. benthamiana*, there were no significant changes in cell death. Furthermore, I performed agro-infiltrated transient overexpression of the target genes for the gain-of-function study. Interestingly, CE 82-induced cell death was accelerated when CE82 was coexpressed with MON2 which is one of the target proteins related to vesicle trafficking. The cellular localization study revealed the localization of CE82 in vesicle-like or Golgi-like membrane. However, localization of CE82 was changed when it was coexpressed with MON2. I performed interaction studies using yeast-two-hybrid (Y2H) and bimolecular fluorescence complementation (BiFC) between CE82 and MON2. Although direct interaction was not evident in Y2H, BiFC result indicated that CE82 interacts with MON2 *in planta*. Study between effectors of *P. infestans* and target proteins provides important information contributing to plant immunity.

Keywords: *P. infestans*, Cell death, Effector, Monensin sensitivity 2, Target protein

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LIST OF ABBREVIATIONS

BiFC	Bimolecular fluorescence complementation
CE	Core effector
co-IP	co-immunonoprecipitation
ETI	Effector-triggered immunity
PTI	PAMP-triggered immunity
PRRs	Pattern recognition receptors
RxLR	Arg-x-Leu-Arg
MON2	Monensin sensitivity 2
VIGS	Virus-induced gene silencing
Y2H	Yeast two hybrid

INTRODUCTION

Plant defense system has been established to resist potential attacks by a broad range of pathogens in nature. The plant defense system can be divided into two: constitutive immune system and induced immune system. The constitutive immune system includes preformed structural barriers such as cell walls, waxy epidermal cuticles and secondary metabolites to prevent or attenuate invasion by invaders (Pieterse et al. 2009). In addition, plants have the ability to detect invading pathogens and to respond with induced immune responses. Pattern recognition receptors (PRRs) in the plasma membrane recognize pathogen microbial elicitors known as pathogen associated molecular patterns (PAMPs). This recognition is referred to as PAMP-triggered immunity (PTI). To suppress defense responses activated by PTI and to promote parasitic colonization, pathogens secrete a variety of pathogen-specific secreted molecules called effectors into plant cells. Certain effectors are recognized by resistance genes which mainly encode nucleotide-binding leucine-rich repeat proteins (NB-LRR, also called NLR), and this recognition induces effector-triggered immunity (ETI) (Pieterse et al. 2009; Jones and Dangl 2006). ETI is considered more durable and stronger resistance response than PTI. ETI is often accompanied by hypersensitive response (HR), a form of programmed cell death. The HR cell death is characterized by deliberate cell suicide at the infection site and leads to the

formation of necrotic lesion that can prevent further spread of the pathogen from the infection site (Tao et al. 2003; Hardham and Cahill 2010; Jones and Dangl 2006).

The oomycete pathogens are known as that they secrete a large number of effectors to promote successful infection by forming biotrophic structures called haustoria (Whisson et al. 2007). These effectors, are generally defined as secreted molecules that manipulate host cell structure and function thereby facilitating infection and/or triggering defense responses (Kamoun 2006). Two classes of cytoplasmic effectors, RxLR and Crinkler (CRN), are known in oomycetes. RxLR effectors carry a conserved amino-terminus motif, Arg-x-Leu-Arg (RxLR), required for their translocation inside of plant cytoplasm followed by a functional domain (referred as effector domain) (Haas et al. 2009; Cooke et al. 2012). Similar to the RxLR effectors, the CRN effectors contain a signal motif, Phe-Leu-Ala-Lys (FLAK), required for translocation into plant cytoplasm.. The functions and mechanisms of the CRN effectors in pathogenesis are mostly unknown. Several CRN effectors have been shown to target the host nucleus, rarely leads to cell death in plants, but may inhibit cell death triggered by elicitors (Shen et al. 2013). The RxLR effectors are well characterized and are involved in the suppression of plant basal resistance and cell death induced by corresponding R proteins (Bos et al. 2006). The analysis of *P. infestans* T30-4 genome revealed that there are 550 RxLR effector genes in its genome. Among the conserved

effectors, 57 effectors were categorized as core effectors. They are highly induced at 2 or 3 days post infiltration (dpi) *P. infestans* inoculation on potato and are commonly present in the most aggressive isolates including T30-4, 13_A2, NL07434 and US22.

Recent studies demonstrated that effectors can interact and modify plant proteins to manipulate plant defense response, such as proteins functioning in protein degradation (Bozkurt et al. 2011), transcriptional regulation of effector proteins (McLellan et al. 2013), ubiquitination (Bos et al. 2007) and vesicle trafficking (Nomura 2006; Du et al. 2015). For example, *Pseudomonas syringae* HopM1 destabilizes AtMIN7 which is an adenosine diphosphate ribosylation factor (ARF) guanine nucleotide exchange factor (GEF) having roles in intracellular vesicle trafficking (Nomura 2006; Nomura et al. 2011). This destabilization enhances pathogen growth by inhibiting AtMIN7 activity to suppress accumulation of callose at pathogen infection site. In case of *P. infestans* RxLR effector, AVR1, was found to interact with the potato Sec5 which is a component of exocyst complex involved in vesicle trafficking and is required for secretion of pathogenesis-related protein 1 (PR1) protein to the apoplast. AVR1 inhibits Sec5 to suppress accumulation of callose and PR1 at the pathogen infection site and enhances pathogen proliferation (Du et al. 2015).

In this study, I investigated plant target proteins involved in cell death signaling

induced by a *P. infestans* core effector in *N. benthamiana*. A CE82 inducing cell death in *N. benthamiana* and pepper was selected. I identified 16 target protein candidates interacting with CE82 through co-IP and LC/MS-MS. 4 out of 16 candidates are related to vesicle trafficking. Among them, MON2 has a positive effect on CE82-induced cell death.

LITERATUREL REVIEW

Plant immunity

In nature, plants have developed multiple defense strategies to inhibit a broad range of pathogen infections. Plant defense systems against pathogens can be divided into two classes: the preformed immune system and the induced immune system (Chisholm et al. 2006; Dangl and Jones 2001). First, structural barriers and antimicrobial secondary metabolites prevent or attenuate invasion by potential invaders (Pieterse et al. 2009). On the other hand, the induced immune system is activated by pathogen infection. During infection, pathogens send microbial elicitors, known as pathogen-associated molecular patterns (PAMPs) such as the bacterial flagellin or the fungal chitin, into the apoplast of plant cells (Jones and Dangl 2006). Pattern recognition receptors (PRRs) in the plasma membrane recognize PAMPs and lead to PAMP-triggered immunity (PTI). In PTI, influx of Ca^{2+} is increased, mitogen activated protein kinases (MAPKs) cascades are activated, transcription of pathogen-responsive genes is induced and reactive oxygen species are accumulated at the cell wall. These responses all contribute to prevention of pathogen growth (Pieterse et al., 2009, Vera Gohre 2008, Nürnberger et al. 2004). To suppress plant defense responses activated by PTI as

well as to promote parasitic colonization, pathogens secrete virulence molecules called effectors into cytoplasm of plant cells (Mitch et al. 2011). In response to secretion, plants have evolved diverse R genes to recognize certain/specific effectors directly or indirectly (Pieterse et al. 2009). This recognition induces effector-triggered immunity (ETI) (Jones and Dangl 2006). Generally, although PTI and ETI share common signaling components/pathways, ETI forms stronger and more prolong resistance response than PTI and is often accompanied by HR cell death which is the formation of necrotic lesion to prevents further spread of the pathogen from the infection site (Tao et al. 2003; Hardham and Cahill 2010; Jones and Dangl 2006).

Phytophthora infestans

One of oomycete pathogens, *P. infestans* has an extensive host range in the Solanaceae family such as tomato, eggplant, tobacco and potato. This pathogen is known as a causal agent of late blight and caused the Irish potato famine in the 1840s. Since then, *P. infestans* has been one of the most economically important disease threatening the worldwide potato production (Birch et al. 2012). Estimated cost of the chemical control and potential yield losses associated with *P. infestans* exceeds €6.7Million in a year (Haverkort et al. 2009). Moreover, protection by spraying fungicides during the growing season results in

emergence of aggressive new pathogen strains. Recently, with newly developed approaches in biology and genomics, studies to understand the mechanism of late blight pathogenesis have been focused on interaction between plant proteins and pathogen proteins.

When *P. infestans* infects plant, hundreds of apoplastic and cytoplasmic effectors are delivered into plant cells to suppress the action of host defense thereby enabling parasitic colonization (Kamoun et al. 1997). The best studied cytoplasmic effectors are the RxLR class. They contain a conserved amino terminal motif, RxLR, required for their translocation into inside of plant cells (Haas et al. 2009; Cooke et al. 2012). A functional domain called the effector domain follows RxLR sequence. In the genome of *P. infestans*, approximately 550 RxLR effector genes were predicted (Haas et al. 2009). A major function of the RxLR effectors is to suppress the signal transduction pathways that mediate plant defense responses (Chisholm et al. 2006; Jones and Dangl 2006; Robatzek 2008). Recently, several studies reported that RXLR effectors interact with plant proteins to modulate plant defense response (Chisholm et al. 2006). For example, the RXLR effector Pi04089 interacts with a putative potato K-homology (KH) RNA-binding protein, StKRBP1, and uses it as a useful factor to enhance colonization of *P. infestans* (Wang et al. 2015). Another RXLR effector, Pi04314, interacts with isoforms of plant protein phosphatase type 1c and promotes *P. infestans* proliferation by suppressing induction of jasmonic acid (JA)- and SA-

responsive genes (Boevink et al. 2016).

Vesicle trafficking

Vesicle trafficking occurs between Golgi apparatus and plasma membrane in various different ways during plant development (Surpin and Raikhel 2004), cell growth (Hepler et al. 2001), cell polarity (Dhonukshe et al. 2008) and defense signaling (Robatzek 2007). It has become evident that plant intracellular vesicle trafficking is engaged in the plant defense responses against potential pathogen infections (Robatzek 2007). The well-characterized PRR, *Arabidopsis* FLAGELLIN-SENSING 2, is an LRR receptor kinase that recognizes bacterial flagellin in the plasma membrane (Chinchilla et al. 2006). This recognition is mediated by ubiquitination of endocytic trafficking and it activates PTI responses (Frey and Robatzek 2009). In the case of *Arabidopsis* penetration 1 (PEN1), it confers pre-invasive resistance to powdery mildew fungi at the cell surface by exocytosis defense response (Kwon et al. 2008b). During powdery mildew infection, PEN1 accumulates at the penetration sites within papillae using vesicle cycling to suppress pathogen pre-invasion (Collins et al., 2003).

In addition, pathogens appear to have evolved to utilize plant vesicle trafficking for the suppression of plant defense responses or the benefit of pathogen growth

(Kwon et al. 2008a). For example, *Pseudomonas syringae* HopM1 targets *Arabidopsis* AtMIN7 and destabilizes it in a proteasome dependent manner (Nomura 2006; Nomura et al. 2011). AtMIN7 is a major regulator of in cellular vesicle formation and trafficking. Destabilization of AtMIN7 by HopM1 suppresses SA-regulated callose deposition in PTI as well as during ETI in the cell wall and leads to pathogen proliferation. In case of *P. infestans* RxLR effector, AVR1, was found to interact with the potato Sec5 which is a component of exocyst complex involved in vesicle trafficking and is required for secretion of PR1 protein to the apoplast. The *N. benthamiana* which was silenced of *Sec5* genes showed increased lesion size by *P. infestans* inoculation and secretion of PR1 protein was decreased (Du et al. 2015). This report showed Sec5 plays a role in defense-associated vesicle trafficking.

MATERIALS AND METHODS

Plant materials and growth conditions

Seeds of *Nicotiana benthamiana* were sown in 200-plug form tray filled with horticultural bed soil (BioGreen, Seoul, Korea) and were grown at 22-24℃ with relative humidity of 50-60 % in a controlled walk-in chamber under a 16h light/8h darkness photoperiod. After about 2 weeks, the first true leaf fully expanded and seedlings were transplanted into plastic pots (104 mm diameter and 79 mm deep, one seeding per pot).

Plasmid constructions

Agrobacterium Agl1 carrying *P. infestans* core effectors were provided from Dr. Sophien Kamoun (The Sainsbury Laboratory, Norwich, UK). CE82 was cloned into a binary *Potato Virus X* (PVX)-based vector pICH31160. I also cloned 3xFLAG (DYKDDDK) at N-terminus of CE82. Target protein candidates were amplified with cDNA of *N. benthamiana*. CE82 and target protein candidates were cloned into donor vectors (pdonr221) and destination vectors (pK7FWG2, pK7WG2, pDEST32, pDEST22, pDEST-SCYCE, pDEST-VYNE) using

GATEWAY cloning system (Invitrogen, Seoul, Korea). For *in planta* expression, all plasmids were transferred to *Agrobacterium* GV3101.

Transient overexpression by agro-infiltration

Agrobacterium GV3101 containing the various constructs were cultured overnight in YEP medium with appropriate antibiotics at 28℃. The culture was centrifuged, resuspended in infiltration buffer (10 mM MgCl2, 10 mM MES (pH 5.6), and 150 μM acetosyringone) to a desired $O.D_{600}$ and kept at room temperature for 2 hours before infiltration. Recombinant *Agrobacterium* was infiltrated into expanded leaves of four-week-old *N. benthamiana* using a needleless syringe. For cell death screening, the infiltrated plants were placed in walk-in chamber to show cell death response during 3~6 days after infiltration. Transient co-expression of CE82 with *MON2* was performed as follows. *Agrobacterium* GV3101 carrying the respective constructs were mixed in a 1:1 ratio to a final OD_{600} of 0.4 and infiltrated in *N. benthamiana* leaves.

Immunoblot and co-immunoprecipitation

For protein extraction, leaves of four-week-old *N. benthamiana* were infiltrated with *Agrobacterium* GV3101 containing plasmid of interest to a final $O.D_{600}$ of

0.5 and leaves were harvested 2~3 (dpi) and grinded in liquid nitrogen. Total proteins were extracted using extraction buffer (GTEN buffer (10% glycerol, 25 mM Tris pH 7.5, 1 mM EDTA, 150 mM NaCl with 2% (w/v) PVPP and add 10 mM DTT, 1x protease inhibitor cocktail). The total extracted proteins were boiled for 10 min at 95℃ with 5x SDS-PAGE loading buffer (LPS solution, Daejeon, Korea) and separated on SDS-PAGE gel with Tris glycine SDS buffer (LPS solution, Daejeon, Korea) at 100 V for 90 min in 4℃ room. After SDS-PAGE, the proteins were transferred to a 0.45μm PVDF (Polyvinylidene Difluoride) membrane (Merck Millipore, Darmstadt, Germany) using Trans-Blot Turbo Blotting System (Bio-rad, California, USA). When the transfer run was completed, western blotting can be progressed.

For western blotting, the membrane was blocked in T-PBS solution containing 6% skimmed milk for 1h. For detection of FLAG fused proteins, I use FLAG antibody (Sigma-Aldrich, Darmstadt, Germany) and GFP antibody (Abcam, Cambridge, UK) as primary antibody at 1:20,000 dilution, and anti-mouse antibody conjugated to horseradish peroxidase (Thermo fisher scientific, Waltham, USA) was used as a secondary antibody at 1:25,000 dilutions, for 1h. After antibody incubation, the membrane was rinsed and washed with T-PBS 2 times or 3 times for 10 min and developed for 5 min on ECL (GE Healthcare, Little Chalfont, UK) followed by capture using Fusion imaging system (Vilber Lourmat,

MArne-la-Vallée, France). The membrane was stained with Ponceau S (LPS solution, Daejeon, Korea) to estimate protein loading.

For co-immunoprecipitation (Co-IP), the protocol described in Saunders et al was modified (Saunders et al. 2012). 10 mg total protein extract was mixed with α-FLAG affinity gel (BioLegend, California, USA). Fill it up to 4 ml with IP buffer and incubated at 4℃ for 2 hours on a rotating platform. Protein complexes attached to the beads were washed three times with IP buffer and collected, and then boiled for 10 min with SDS-PAGE loading buffer (LPS solution, Daejeon, Korea). For LC-MS/MS, the precipitate should be confirmed by Immunoblot.

Virus-induced gene silencing (VIGS)

The fragments of gene for silencing were amplified with cDNA of *N. benthamiana* and cloned into TRV2 vector using ligation-independent cloning. Constructs TRV:ΔGFP was used as a positive control and TRV:PDS (phytoene desaturase gene) was used as a negative control. Transformed *Agrobacterium grown,* harvested and resuspended in infiltration buffer as described above. After incubation at room temperature for 2–4 h, *Agrobacterium* carrying the TRV1 and TRV2 constructs was mixed in a 1:1 ratio, and infiltrated into the leaves of *N. benthamiana* at the four-leaf stage. Two

or three weeks later, the fourth and fifty leaves above the infiltrated leaf of *N. benthamiana* were used for agroinfiltration. Silenced leaves were sampled for RNA extraction and RT-PCR analysis.

Reverse transcription-PCR (RT-PCR)

Total RNA was extracted from *N. benthamiana* leaves using TRIzol reagent (Invitrogen, California, USA). First-strand cDNA was synthesized using 4 μg total RNA with oligo (dT) and SuperScript II reverse transcriptase (Invitrogen, California, USA). To determine the target gene expression, PCR was performed using target gene specific primers avoiding the VIGS regions. The following cycling conditions were used : 1 cycle of 95℃ for 3 m; 26 cycles of 95℃ for 15 s, 58℃ for 20 s and 72℃ for 30 s. The actin gene was used as a control for comparing transcript levels.

P. infestans spore infection

P. infestans isolate 43072 provided by the RDA Gene Bank of Korea was used. It was grown on rye sucrose agar media as previously described at 17℃ in the dark for 10 days (Kamoun et al., 1998). To release the zoospores from sporangia, the plates were flooded with autoclaved distilled cold water, gently rubbed with a sterile cell scraper, and incubated at 4℃ for 1h. The zoospores were counted

under a hemocytometer, and the concentration was adjusted to 5×10^4 zoospores/ml. The detached leaves of *N. benthamiana* were placed in the rectangle plate overlaid with a wet paper towel to maintain high humidity. The 10 µl droplets of zoospores were applied onto the detached leaves and incubated at 17℃. Images are taken at 5 dpi.

Fluorescence microscopy

Fluorescence microscopy observation was performed using a microscope (ZEISS Axiocam 506 color). The emission wavelength was 450~490 nm for GFP and 546 nm for RFP. Images were processed with ZEN 2 lite software. After agroinfiltration of interest genes in *N. benthamiana* leaves, it is pared the abaxial and is observed by microscope.

Yeast two hybrid (Y2H)

Y2H was performed using the ProQuest™ Two-Hybrid system (Invitrogen Co., California, USA). Yeast strain MaV203 contains single copies of each of three reporter genes (*HIS3*, *URA3* and *lacZ*). Yeast was cotransformed with both pDEST32 and pDEST22 plasmids carrying CE82 as a bait and MON2 as a prey, respectively. Transformants were grown for 72 hours on synthetic complete (SC)

dropout media lacking amino acids leucine and tryptophan. Large colonies were picked up and resuspended in 30 μl of sterile water and 3 μl droplets were applied on selection media SCⅢ-Leu-Trp-His+25 mM 3-Amino-1,2,4-trizole(3AT) and SCⅢ-Leu-Trp-Ura media.

To test β-galactosidase activity, yeast transformants were transferred from SCⅡ plates to 3M papers, supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, 100 μg/ml) buffered with Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$). 3M papers were incubated for 4 hours at 28℃.

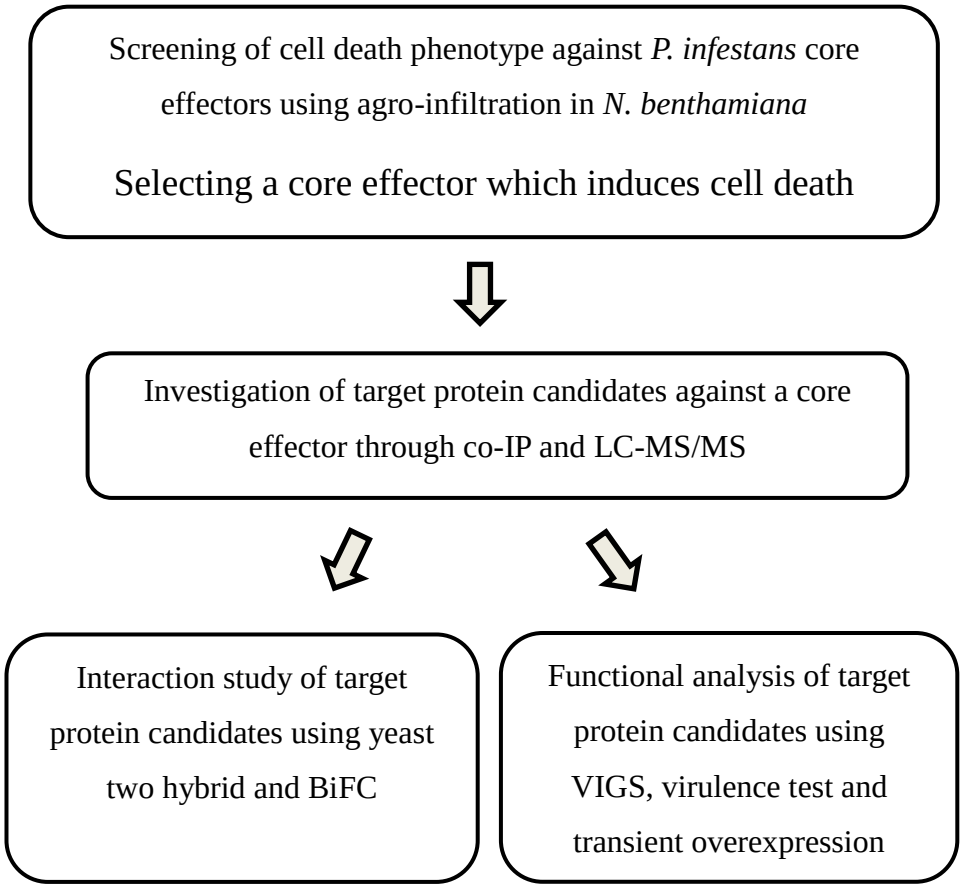

Figure 1. Experimental design in this study

RESULTS

CE82 induces cell death and is localized in vesicle-like structures in *N. benthamiana*

To investigate the biological function of *P. infestans CE82* in *N. benthamiana*, the construct of *CE82* was transiently over-expressed in *N. benthamiana* using agro-infiltration. Since several studies showed that the effector domains at the C-terminal region are sufficient to have the activity of effectors, I cloned the effector domain at the C-terminal region containing 87 amino acids of *CE82* and introduced into a *Potato Virus X* (PVX)-based vector pICH31160. BAX and mCherry were used as a positive and a negative control, respectively. After 3 dpi, *CE82* was induced localized cell death in *N. benthamiana* (Figure 2).

To investigate the localization of CE82, I examined cell imaging of CE82 using fluorescence microscope. The *CE82* tagged with GFP at the N-terminus was generated (referred as *GFP:CE82*). The *GFP:CE82* was agroinfiltrated and fluorescence was observed from 3dpi. The results represented that *GFP:CE82* were expressed in vesicle or Golgi like structures, suggesting that *GFP:CE82* likely function in vesicle trafficking (Figure 3).

In order to identify target proteins interacting with CE82, I generated *CE82* with three epitope tags of FLAG (DYKDDDK) at the N-termius (referred as *3xFLAG:CE82*). I verified whether the construct of *3xFLAG:CE82* is workable by testing the cell death phenotype in *N. benthamiana* using agro-infiltration. Although the cell death induced by *3xFLAG:CE82* was delayed than *CE82* without the tag, *3xFLAG:CE82* still induced cell death at 5dpi (Figure 2). Thus, this construct was used to for the further study.

To examine whether *CE82* affects proliferation of *P. infestans*, I inoculated *P. infestans* NL07434 zoospores on *N. benthamiana* leaves transiently overexpressing *CE82*. First, *CE82* was agroinfiltrated in the right half of *N. benthamiana* leaves, whereas empty vector in the left half. At 5days post inoculation, lesion of *P. infestans* were showed no significant difference. As a result, we identified *CE82* did not affect growth of *P. infestans*.

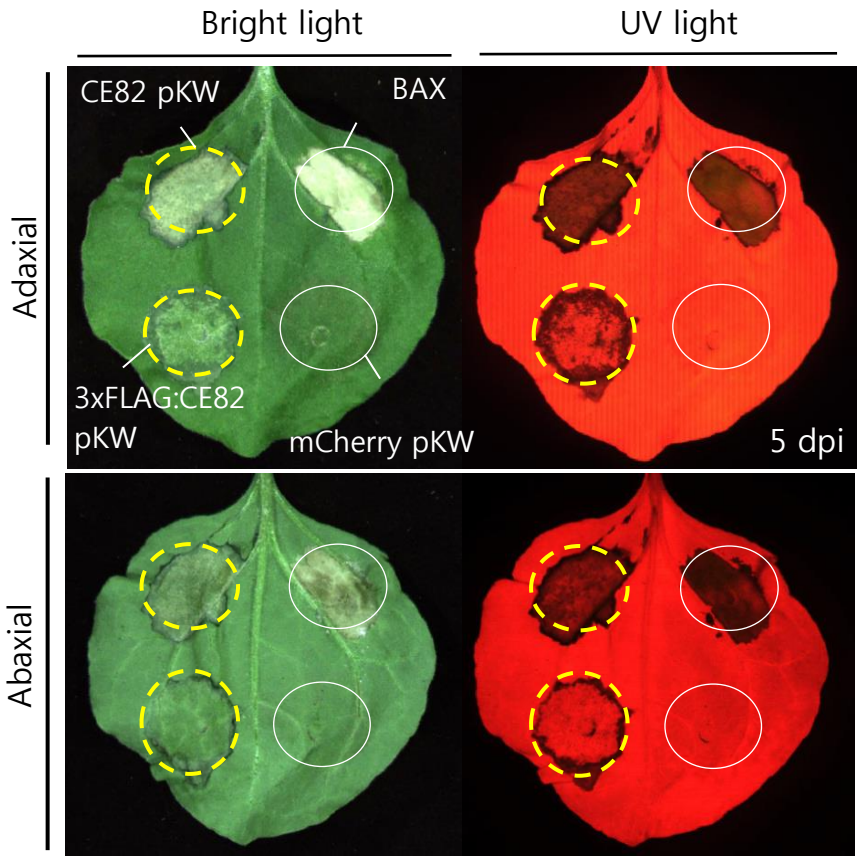

Figure 2. *CE82* of *P. infestans* induced cell death in *N. benthamiana*.

CE82, 3xFLAG:CE82, BCL2 associated X (BAX) as a positive control and mCherry as a negative control were infiltrated to a final $O.D_{600}$ 0.5 in 4-week-old *N. benthamiana*. The photographs were taken at 5 dpi under bright and UV light

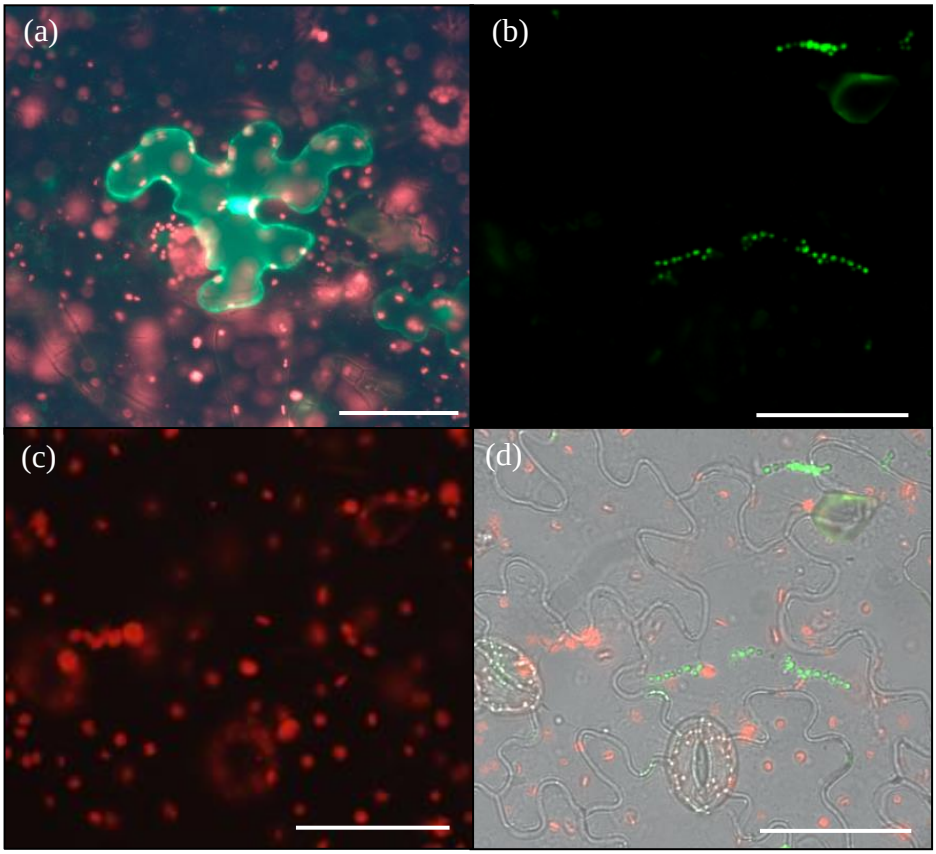

Figure 3. CE82 is localized in vesicle-like structures

GFP:CE82 was agroinfiltrated in *N. benthamiana*. At 3dpi, leaves were pared the abaxial and they were observed by green/red (a) green (b) and red light filter (c) using fluorescence microscopy. The images of green, red and bright light were merged (d). Scale bar=50µm

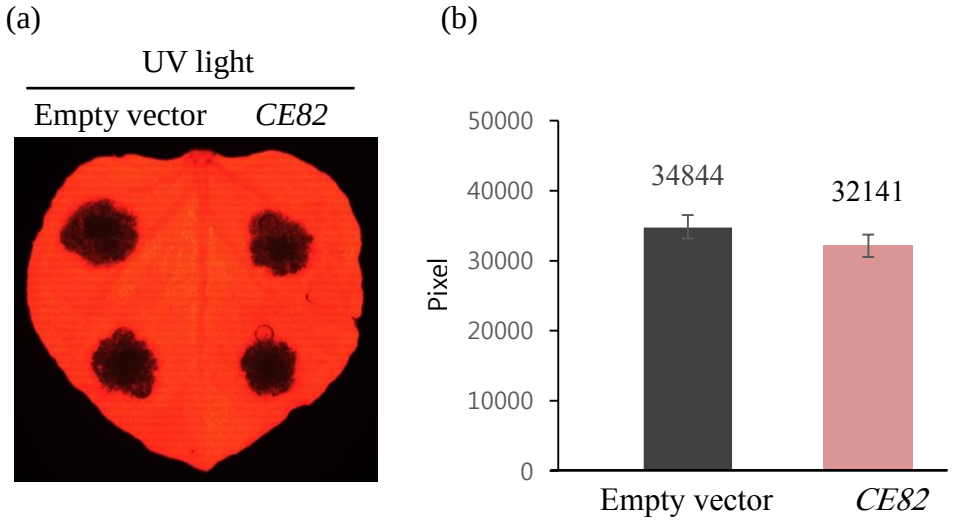

Figure 4. Overexpression of *CE82* did not affect pathogenicity of *P. infestans* in *N. benthamiana*

(a) The representative image shows *P. infestans* growth on the agroinfiltrated *N. benthamiana*. *CE82* was agroinfiltrated in the right half of *N. benthamiana* leaves, whereas empty vector in the left half followed by inoculation with *P. infestans* NL07434. At 5dpi, photograph was taken under UV light. (b) A graph represents average lesion size of *P. infestans* growth using Image J. Lesion size was calculated by pixel number. A one-way ANOVA test was performed to assess significant differences in lesion size (p-value= 0.2482).

Identification of target protein candidates with CE82 in *N. benthamiana*

In order to investigate target proteins interacting with CE82, co-IP using 3xFLAG:CE82 was performed. First, the protein expression of 3xFLAG:CE82 was confirmed by western blot using α-FLAG antibody. *N. benthamiana leaves were infiltrated with Agrobacterium* carrying *3xFLAG:CE82* and harvested at 2 and 3 dpi. Total protein extract of uninfiltrated *N. benthamiana* was used as a negative control. Westernblot results showed that protein 3xFLAG:CE82 is expressed both 2 and 3 dpi, and especially at 3dpi (Figure 5a). I decided to use 3 days time point for co-IP.

To find target protein candidates of CE82, pull-down and LC-MS/MS were performed. I proceeded α-FLAG IP in the same conditions of westernblot and IP using α-FLAG affinity gels was conducted with *N. benthamiana* leaves agroinfiltrated of *3xFLAG:CE82*. To validate its precipitation, α-FLAG immune blot was performed using α-FLAG-IPed precipitates. Total protein extract was used as input. Three times biological replicates were repeated. Candidates of target proteins were identified through LC-MS/MS. To exclude false protein selection, I eliminated redundant proteins which were identified through co-IP and LC-MS/MS with uninfiltrated *N. benthamiana*. As a result, 16 target protein candidates were hit more than two times in three biological replicates. Among 16

target protein candidates, 4 candidates (*MON2*, *GNOM1*, *GNOM-like* and *GNOM2*) commonly encode an guanine nucleotide exchange factor (GEF) which regulates intercellular vesicle trafficking at a variety of organisms such as yeast, mammalian and plants (Donaldson and Jackson 2000). With consideration of the localization of CE82, 4 candidates may have roles in CE82-induced cell death signaling. Thus, I proceeded to functional study of target protein candidates.

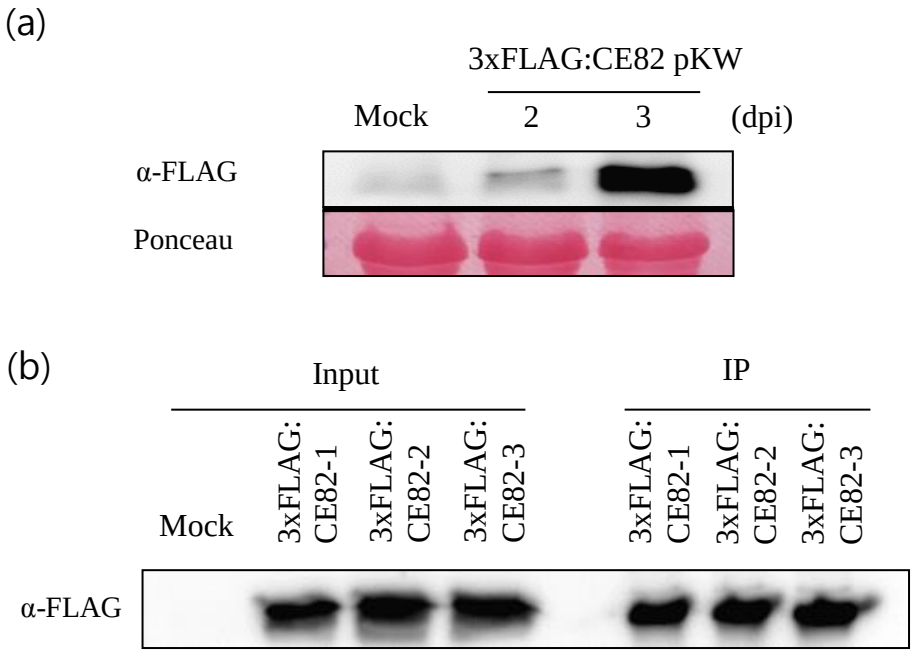

Figure 5. Immunodetection and co-IP of 3xFLAG:CE82

(a) Protein expression of 3xFLAG:CE82 was detected by western blot in *N. benthamiana* at 2~3 dpi. Total protein extract in each lane was loaded 40 μg/line and was detected using α-FLAG antibody. (b) Total protein extracts of uninfiltrated leaves were loaded as an input in 1 lane. Total protein extracts of three biological replicates for α-FLAG co-IP were loaded as inputs in 2, 3, 4 lanes. α-FLAG IPed samples were loaded in 6, 7, 8 lanes.

The phenotypes of CE82-induced cell death showed no significant difference under low-level gene expression of target genes.

To investigate how these target protein candidates were involved in CE82-induced cell death signaling, a loss-of-function study was performed using tobacco rattle virus (TRV) based virus-induced gene silencing (VIGS) (Liu et al. 2002). I designed VIGS constructs targeting *MON2* alone (*TRV2::MON2*), *GNOM1* and *GNOM2* together (*TRV2::GNOM1, 2*). *GNOM-like* was excluded in VIGS experiment for that it could not be amplified from *N. benthamiana* cDNA. Since mitogen-activated protein kinase (MAPK) cascade is known to play a key role in cell death signal transduction in response to pathogen infection, VIGS constructs targeting *N. benthamiana MAPK WIPK, SIPK* and *N. benthamiana MEK2* were generated (Ichimura et al. 2002). Constructs TRV2::ΔGFP (green fluorescent protein) was used as a negative control and TRV2::PDS (phytoene desaturase gene) was used as a positive control for VIGS, respectively. After 2~3 weeks, *CE82* was transiently overexpressed to compare with agroinfiltration of VIGS construct. BAX and INF1 were used as positive controls and mCherry as a negative control in cell death, respectively. To verify suppression of target gene expression, semi-quantitative RT-PCR was performed. The primers were designed at the target gene outside the region targeted for silencing. Actin was used as a

loading control (Figure 6). As a result, there were no significant differences in CE82-induced cell death phenotype with suppression of the target genes or MAPK cascade gene-silenced plants compared with TRV2::ΔGFP control plants (Figure 6). These results suggested that target proteins do not effect on CE82-induced cell death under low-level gene expression of target genes and MAPK cascade genes.

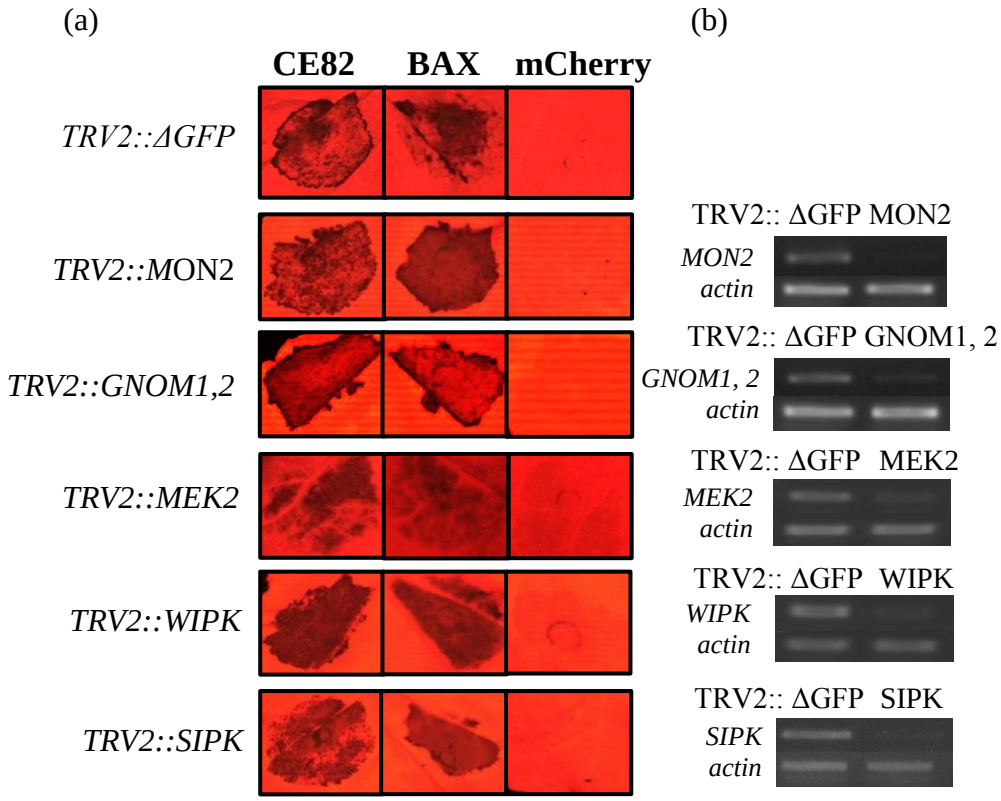

Figure 6. The phenotypes of CE82-induced cell death showed no significant difference in *N. benthamiana* silenced for target genes expression

(a) The images show sites of agro-infiltration of each constructs. TRV2:ΔGFP was suppressed expression of GFP partial gene and used as a negative control of VIGS. CE82, BAX as a positive control and mCherry as a negative control of infiltration were infiltrated to a final $O.D_{600}$ 0.5.. The photographs were taken at 3 dpi under UV light. (b) Suppression of target genes were confirmed using RT-PCR. Actin was used as a loading control.

MON2 accelerates CE82-induced cell death and translocates localization of CE82

To perform gain-of-function study, *MON2* was first cloned based on *N. benthamiana* sequence (https://solgenomics.net/). *MON2* was introduced into a pK7FG2 vector for transient expression. To investigate whether *MON2* has effect on CE82-induced cell death phenotype, *CE82* was transient co-expressed with *MON2* and compared with only *CE82* infiltrated. BAX as a positive control and empty vector pK7F as a negative control were used. When *MON2* was expressed alone, it didn't induce cell death response. I compared the cell death phenotype of *CE82* with *MON2* or empty vector. The region of co-infiltration of *CE82* and *MON2* showed cell death from 3dpi, earlier than *CE82* with the empty vector (Figure 7). At 4dpi, CE82-induced cell death was observed in both co-infiltration site, indicating that MON2 accelerates CE82-induced cell death responses.

Since *MON2* is known as a key regulator in intracellular vesicle trafficking and localized in trans-Golgi network, I tested whether *MON2* affects localization of *CE82* using fluorescent cell imaging. I co-infiltrated *GFP:CE82* with *MON2* in *N. benthamiana* and observed GFP expressions at 3dpi. As a result, I observed GFP:CE82 was localized in the plasma membrane and cytoplasm when co-expressed with *MON2* (Figure 8). With the previous results of the localization and cell death phenotype, it suggest that translocalization of CE82 with MON2 may

affect the acceleration of cell death.

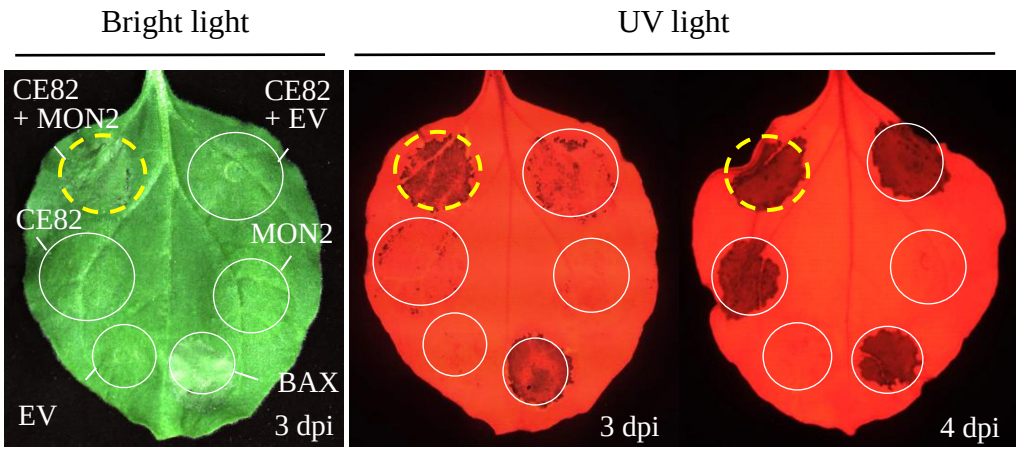

Figure 7. CE82-induced cell death is accelerated when co-expressed with *MON2* in *N. benthamiana*

CE82, MON2, BAX as a positive control and empty vector (EV) pK7F as a negative control were infiltrated to a final $O.D_{600}$ 0.4 in 4-week-old *N. benthamiana*. The photographs were taken at 3 ~ 4dpi under bright and UV light.

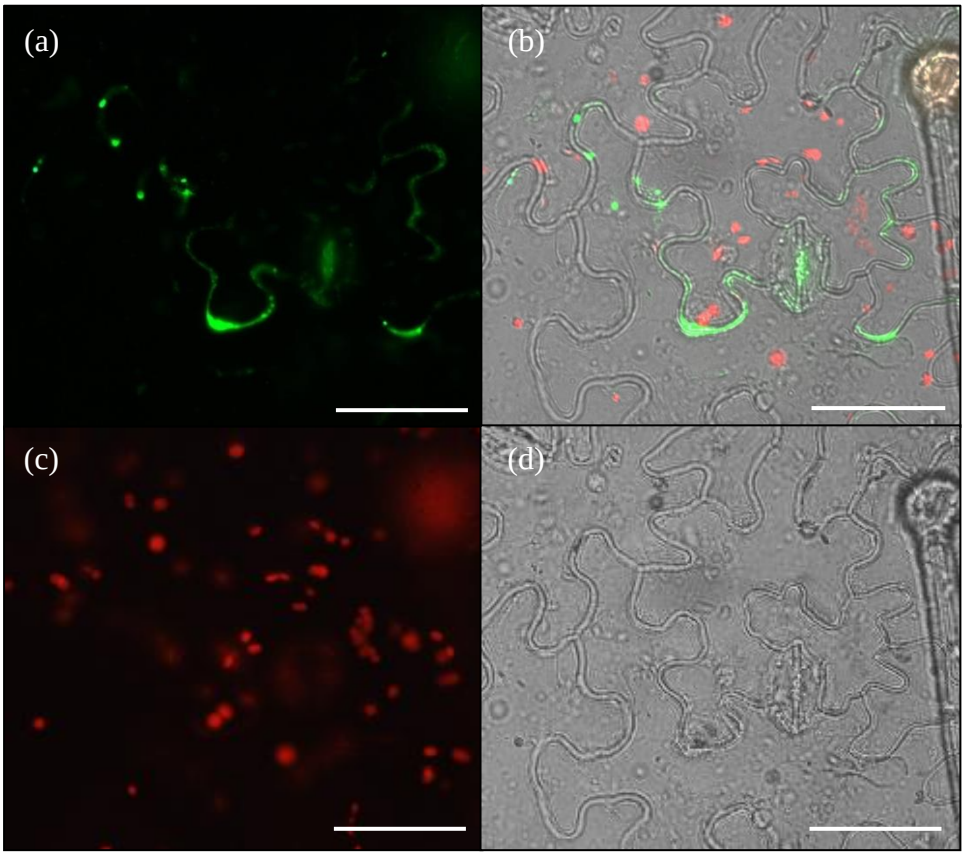

Figure 8. Translocalization of GFP:CE82 by MON2 in *N. benthamiana*

GFP:CE82 and MON2 were co-agroinfiltrated in *N. benthamiana*. At 3dpi, leaves were pared the abaxial and they were observed by green (a) red (c) bright light filter (d) using fluorescence microscopy. The images of green, red and bright light were merged (b). Scale bar=50μm

CE82 interacts with MON2 *in planta*

To investigate whether CE82 interacts with MON2, bimolecular fluorescence complementation (BiFC) experiment was performed. For BiFC, *CE82* was introduced into a pDEST-SCYCE vector containing SCFP3A (N-terminal region of GFP) at the C-terminus (referred as *CE82:SCFP3A*) and MON2 was cloned into a pDEST-VYNE vector which contains Venus (C-terminal region of GFP) at the C-terminus (referred as *MON2:Venus*). When *CE82:SCFP3A* and *MON2:Venus* were co-infiltrated in *N. benthamiana*, green fluorescence was observed at 3dpi, indicating that CE82 interacts with MON2 *in planta*.

To confirm direct interaction between CE82 and MON2, I performed yeast two hybrid assay using ProQuest™ two-hybrid system (Invitrogen, Cat.PQ10001-01). *CE82* and *MON2* were introduced into pDEST32 and pDEST22 for construction of the bait and prey plasmid, respectively. pEXP32-Krev1/pEXP22-RalGDSwt and pEXP32-Krev1/pEXP22-RalGDSm1 were used as positive controls showing strong and weak interaction, respectively. Empty vectors, pDEST32 and pDEST22, were used as a negative control. As a result, yeast co-expressing CE82 with MON2 did not grow on selective media lacking uracil or histidine and did not induce of β-galactosidase activity, whereas the strong positive control activates all reporter genes (Figure 10). These results suggest that CE82 does not directly interact with MON2.

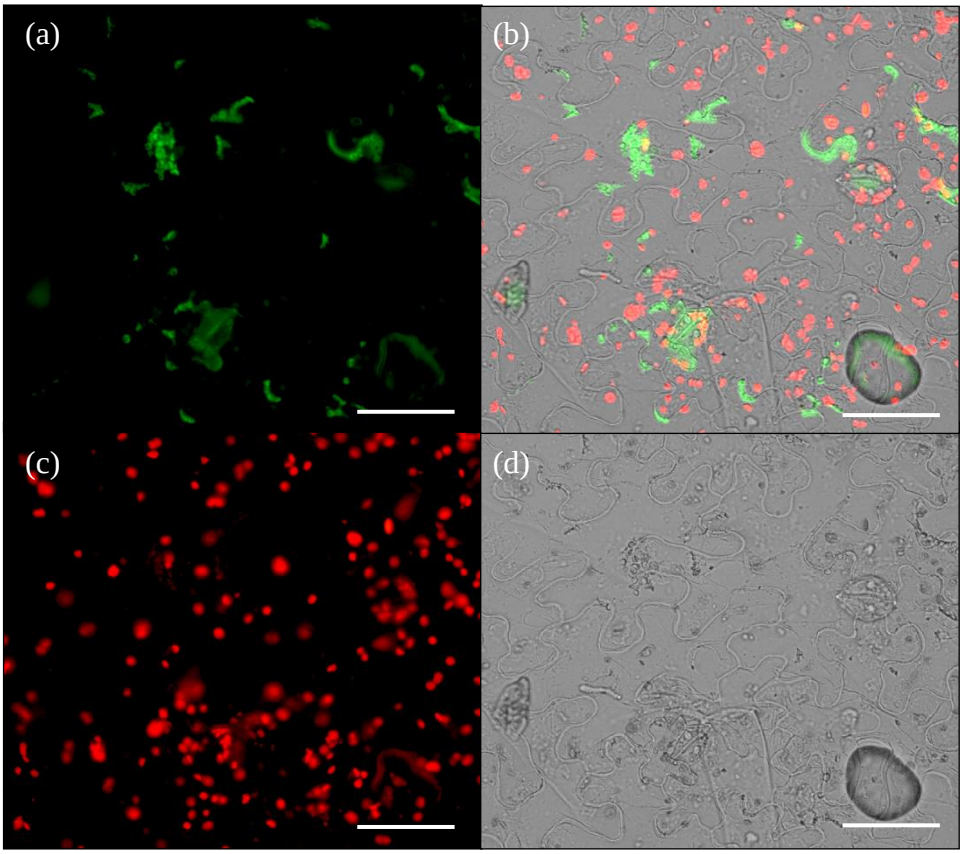

Figure 9. BiFC shows *in planta* interaction between CE82 with MON2

CE82:SCFP3A and MON2:Venus were co-agroinfiltrated in *N. benthamiana*. At 3dpi, leaves were pared the abaxial and they were observed by green (a) red (c), and bright light filter (d) using fluorescence microscopy. The images of green, red and bright light were merged (b). Scale bar=50µm

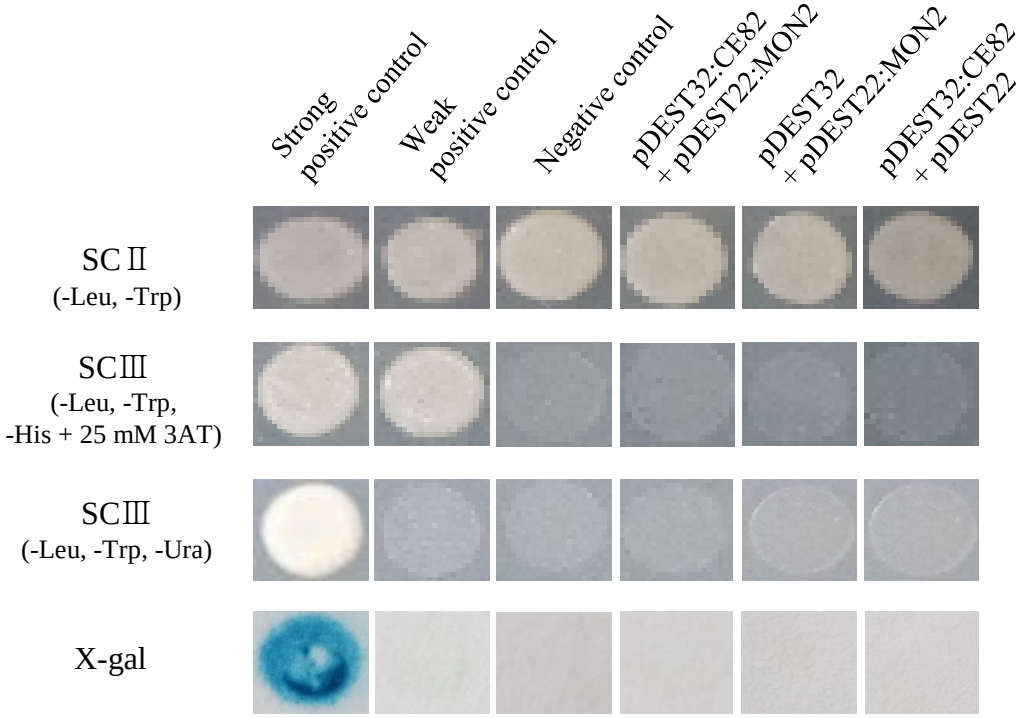

Figure 10. CE82 does not directly interact with MON2

Yeast cotransformed with indicated genes were grown on SC media lacking indicated amino acids. pEXP32-Krev1/pEXP22-RalGDS-wt and pEXP32-Krev1/pEXP22-RalGDS-m1 were used as strong and weak positive controls, respectively. pDEST32/pDEST22, pDEST32/pDEST22:MON2 and pDEST32:CE82/pDEST22 were used as negative controls. SCⅡ media was used to confirm the transformation of both plasmids and SCⅢ media lacking His or Ura were used to identify weak or strong interaction, respectively. X-gal assays validate yeast growth on SCⅢ media.

DISCUSSION

Plants utilize cell death for a multitude of biological processes including growth, development and immune responses (Greenberg 1997; Michayluk 1975; Van Breusegem and Dat 2006). A specialized form of cell death called HR is often occurred as a consequence of direct or indirect interactions between pathogen effectors and the corresponding R proteins during ETI (Jones and Dangl 2006; Dangl et al. 2013). Many RXLR effectors trigger ETI in plant possessing cognate R proteins. However, they also contribute to virulence through the suppression of PTI and ETI. The *P. infestans* RxLR effectors display extensive sequence diversity and have expended in repetitive DNA-rich regions, suggesting that they have rapidly evolved and may carry distinct repertoires of function.

CE82-induced cell could result from an effector that contributes to phytotoxicity or an HR due to recognition of R proteins. However, CE82 did not have an enhancing effect on virulence of *P. infestans* in *N. benthamiana.* Since CE82 is highly induced at biotrophic phage of *P. infestans*, biological functions of CE82 may be correlated with an infection cycle. Especially, the CE82 may modulate the plant immune system in biotrophic phase and triggers cell death signaling. It suggests that CE82 is not an enhancing factor for pathogen virulence but a modulator of plant defense responses through interacting with plant proteins.

In case of *Crinkler* (CRN) effectors in *Phytophthora capsici*, three out of the eleven CRN effectors induce cell death and but only one enhances *P. capsici* virulence in *N. benthamiana* (Stam, Howden et al. 2013). Since CRN effectors expanded in hemi-biotrophic and necrotrophic pathogens, it suggests that CRN effectors may have specific contributions to the necrotrophic phase (Stam, Jupe, et al. 2013). CE82-induced cell death may not be a direct virulence function, it represents an important phenotype, suited to study interaction between CE82 and target proteins functions.

The CE82-induced cell death phenotype showed no significant difference in *N. benthamiana* silenced for target genes. When *MON2* was deleted in yeast and mammalian cell, it accelerated the distribution of vesicles in endocytic trafficking from endosome to Golgi, therefore suggesting that MON2 could normally suppress endosome-to-Golgi trafficking (Mahajan et al. 2013). I suggest that if MON2 play a role as a MON2 of yeast and mammalian, it does not affect that accelerated trafficking from endosome to Golgi in CE82-induced cell death signaling.

In case of *GNOM1* and *GNOM2*, they have conserved Sec7 domain which plays a key role in activation of ARF GTPase (Donaldson and Jackson 2000). There are 17 genes having Sec7 domain in *N. benthamiana*. Although we silenced *GNOM1* and *GNOM2* together in *N. benthamiana*, they can be functionally

redundant with other 15 genes which show similarity up to 50%. Therefore, it was difficult to distinguish cell death phenotype in *N. benthamiana* silenced for *GNOM1* and *GNOM2* expression.

The CE82 was translocalized in plasma membrane, cytoplasm and vesicle-like structures when coexpressed with *MON2*. With consideration of that MON2 accelerates CE82-induced cell death, the translocalization of CE82 by MON2 is may critical role in CE82-induced cell death. Recent research may support this suggestion. They reported that a potato resistance protein R3a translocalizes from the cytoplasm to vesicle when co-expressed with *P. infestans* effector form $AVR3a^{KI}$ but not with $AVR3a^{EM}$. The recognition of $AVR3a^{KI}$ by R3a induce dell death in *N. benthamiana* but not with $AVR3a^{KI}$. From these results, they concluded that effector recognition and cell death signaling by *R3a* require its translocalization to endosomal compartments in the vesicle trafficking pathway (Engelhardt et al. 2012).

REFERENCES

Birch, P. R. J., Bryan, G., Fenton, B., Gilroy, E. M., Hein, I., Jones, J. T., Prashar, A., Taylor, M. A., Torrance, L., and Toth, I. K. (2012). Crops that feed the world 8 : Potato : are the trends of increased global production sustainable? Food Security 4: 447

Boevink, P. C., Wang, X., Mclellan, H., He, Q., Naqvi, S., Armstrong, M. R., Zhang, W., Hein, I., Gilroy, E. M., Tian, Z., and Birch, P. R. J. (2016). PP1c isoforms that promote late blight disease. Nature Communications 7:1–14

Bos, J. I. B., Armstrong, M. R., Gilroy, E. M., Boevink, P. C., Hein, I., Taylor, R. M., Zhendong, T., Engelhardt, S., Vetukuri, R. R., Harrower, B., Dixelius, C., Bryan, G., Sadanandom, A., Whisson, S. C., Kamoun, S., and Birch, P. R. J. (2007). Phytophthora infestans effector AVR3a is essential for virulence and manipulates plant immunity by stabilizing host E3 ligase CMPG1. Proceedings of the National Academy of Sciences, USA 107:2–7

Bos, J. I. B., Kanneganti, T. D., Young, C., Cakir, C., Huitema, E., Win, J., Armstrong, M. R., Birch, P. R. J., and Kamoun, S. (2006). The C-terminal half of Phytophthora infestans RXLR effector AVR3a is sufficient to trigger R3a-mediated hypersensitivity and suppress INF1-induced cell death in Nicotiana benthamiana. Plant Journal 48:165–176

Bozkurt, T. O., Schornack, S., Win, J., Shindo, T., Ilyas, M., Oliva, R., Cano, L. M., Jones, a. M. E., Huitema, E., van der Hoorn, R. a. L., and Kamoun, S. (2011). Phytophthora infestans effector AVRblb2 prevents secretion of a plant immune protease at the haustorial interface. Proceedings of the National Academy of Sciences, USA 108:20832–20837

Van Breusegem, F., and Dat, J. F. (2006). Reactive oxygen species in plant cell death. Plant Physiology 141:384–390

Chinchilla, D., Bauer, Z., Regenass, M., Boller, T., and Felix, G. (2006). The Arabidopsis receptor kinase FLS2 binds flg22 and determines the specificity of flagellin perception. Plant Cell 18:465–476

Chisholm, S. T., Coaker, G., Day, B., and Staskawicz, B. J. (2006). Review Host-Microbe Interactions : Shaping the Evolution of the Plant Immune Response. Cell 124:803–814

Cooke, D. E. L., Cano, L. M., Raffaele, S., Bain, R. A., Cooke, L. R., Etherington, G. J., Deahl, K. L., Farrer, R. A., Gilroy, E. M., Goss, E. M., Hein, I., Maclean, D., Mcnicol, J. W., Randall, E., Oliva, R. F., Pel, M. A., Shaw, D. S., Squires, J. N., Taylor, M. C., Vleeshouwers, V. G. A. A., Birch, P. R. J., Lees, A. K., and Kamoun, S. (2012). Genome analyses of an aggressive and invasive lineage of the Irish potato famine pathogen. PLOS Pathogens 8.10: e1002940.

Dangl, J. L., Horvath, D. M., and Staskawicz, B. J. (2013). Pivoting the plant immune system from dissection to deployment. Science 563:7931–7935

Dangl, J. L., and Jones, J. D. G. (2001). Plant pathogens and integrated defence responses to infection. Nature 411.6839:826-833.

Dhonukshe, P., Tanaka, H., Goh, T., Ebine, K., Mähönen, A. P., Prasad, K., Blilou, I., Geldner, N., Xu, J., Uemura, T., Chory, J., Ueda, T., Nakano, A., Scheres, B., and Friml, J. (2008). Generation of cell polarity in plants links endocytosis, auxin distribution and cell fate decisions. Nature 456:962–966

Donaldson, J. G., and Jackson, C. L. (2000). Regulators and effectors of the ARF GTPases. Current Opinion in Cell Biology 12.4:475–482

Du, Y., Mpina, M. H., Birch, P. R. J., Bouwmeester, K., and Govers, F. (2015). *Phytophthora infestans* RXLR effector AVR1 interacts with exocyst component Sec5 to manipulate plant immunity. Plant Physiology 169:1975–1990

Engelhardt, S., Boevink, P. C., Armstrong, M. R., Ramos, M. B., Hein, I., and Birch, P. R. J. (2012). Relocalization of late blight resistance protein R3a to endosomal

Compartments is associated with effector recognition and required for the immune response. Plant Cell 24:5142–5158

Frey, N. F. d, and Robatzek, S. (2009). Trafficking vesicles: pro or contra pathogens? Current Opinion in Plant Biology 12:437–443

Greenberg, J. T. (1997). Programmed cell death in plant-pathogen interactions. Annual Review of Plant Biology 48.1: 525-545

Haas, B. J., Kamoun, S., Zody, M. C., Jiang, R. H. Y., Handsaker, R. E., Cano, L. M., Grabherr, M., Kodira, C. D., Raffaele, S., Torto-alalibo, T., Bozkurt, T. O., Ah-fong, A. M. V, Alvarado, L., Anderson, V. L., Armstrong, M. R., Avrova, A., Baxter, L., Beynon, J., Boevink, P. C., Bollmann, S. R., Bos, J. I. B., Bulone, V., Cai, G., Cakir, C., Carrington, J. C., Chawner, M., Conti, L., Costanzo, S., Ewan, R., Fahlgren, N., Horn, K., Horner, N. R., Hu, C., Huitema, E., Jeong, D., Jones, A. M. E., Jones, J. D. G., Jones, R. W., Karlsson, E. K., Kunjeti, S. G., Lamour, K., Liu, Z., Ma, L., Maclean, D., Chibucos, M. C., Mcdonald, H., Mcwalters, J., Meijer, H. J. G., Morgan, W., Morris, P. F., Munro, C. A., Pritchard, L., Ramsahoye, B., Ren, Q., Restrepo, S., Roy, S., Sadanandom, A., Savidor, A., Schornack, S., Schwartz, D. C., Schumann, U. D., Schwessinger, B., Seyer, L., Sharpe, T., Silvar, C., Song, J., Studholme, D. J., Sykes, S., Thines, M., Vondervoort, P. J. I. Van De, Phuntumart, V., Wawra, S., Weide, R., Win, J., Young, C., Zhou, S., Fry, W., Meyers, B. C., West, P. Van, Ristaino, J., Govers, F., Birch, P. R. J., Whisson, S. C., Judelson, H. S., and Nusbaum, C. (2009). Genome sequence and analysis of the Irish potato famine pathogen Phytophthora infestans. Nature 461:393–398

Hardham, A. R., and Cahill, and D. M. (2010). The role of oomycete effectors in plant – pathogen interactions. Functional Plant Biology 37:919–925

Haverkort, A. J., Struik, P. C., Visser, R. G. F., and Jacobsen, E. (2009). Applied biotechnology to combat late blight in potato caused by *Phytophthora infestans*. Potato Research 52.3:249–264

Hepler, P. K., Vidali, L., and Cheung, A. Y. (2001). Polarized cell growth in higher plants. Annual Review of Cell and Developmental Biology 17.1:159-187.

Ichimura, K., Shinozaki, K., Tena, G., Sheen, J., Henry, Y., Champion, A., Kreis, M., Zhang, S., Hirt, H., Wilson, C., Heberle-Bors, E., Ellis, B. E., Morris, P. C., Innes, R. W., Ecker, J. R., Scheel, D., Klessig, D. F., Machida, Y., Mundy, J., Ohashi, Y., and Walker, J. C. (2002). Mitogen-activated protein kinase cascades in plants: a new nomenclature. Trends in Plant Science 7:301–308

Jones, J. D. G., and Dangl, J. L. (2006). The plant immune system. Nature 444:323–329

Kamoun, S. (2006). A catalogue of the effector secretome of plant pathogenic oomycetes. Annual Review Phytopathology 44:41–60

Kamoun, S., West, P. Van, Jong, A. J. De, Groot, K. E. De, Vleeshouwers, V. G. A. A., and Govers, F. (1997). A gene encoding a protein elicitor of *Phytophthora infestans* is down-regulated during infection of potato. Molecular Plant-Microbe Interactions 10:13–20

Kwon, C., Bednarek, P., and Schulze-Lefert, P. (2008a). Secretory pathways in plant immune response. Plant Physiology 147:1575–1583

Kwon, C., Neu, C., Pajonk, S., Yun, H. S., Lipka, U., Humphry, M., Bau, S., Straus, M., Kwaaitaal, M., Rampelt, H., El Kasmi, F., Jürgens, G., Parker, J., Panstruga, R., Lipka, V., and Schulze-Lefert, P. (2008b). Co-option of a default secretory pathway for plant immune responses. Nature 451:835–40

Liu, Y., Liu, Y., and Schiff, M. (2002). Virus-induced gene silencing in tomato. The Plant Journal 31.6:777-786.

Mahajan, D., Boh, B. K., Zhou, Y., Chen, L., Cornvik, T. C., Hong, W., and Lu, L. (2013). Mammalian Mon2/Ysl2 regulates endosome-to-Golgi trafficking but possesses no guanine nucleotide exchange activity toward Arl1 GTPase. Scientific Reports 3:3362

McLellan, H., Boevink, P. C., Armstrong, M. R., Pritchard, L., Gomez, S., Morales, J., Whisson, S. C., Beynon, J. L., and Birch, P. R. J. (2013). An RxLR effector from *Phytophthora infestans* prevents re-localisation of two plant NAC transcription factors from the endoplasmic reticulum to the nucleus. PLOS Pathogens 9.10:e1003670.9

Michayluk, M. R. (1975). Nutritional requirements for growth of vicia hajastana cells and protoplasts at a very low population density in liquid media. Planta 126.2:105–110

Mitch, J., Lin, Z. D., and Coaker, G. (2011). Plant NB-LRR signaling : upstreams and downstreams. Current Opinion in Plant Biology 14:365–371

Nomura, K. (2006). A bacterial virulence protein suppresses host innate immunity to cause plant disease. Science 313:220–223

Nomura, K., Mecey, C., Lee, Y.-N., Imboden, L. A., Chang, J. H., and He, S. Y. (2011). Effector-triggered immunity blocks pathogen degradation of an immunity-associated vesicle traffic regulator in Arabidopsis. Proceedings of the National Academy of Sciences, USA 108:10774–10779

Nürnberger, T., Brunner, F., Kemmerling, B., Piater, L., Nurnberger, T., Brunner, F., Kemmerling, B., and Piater, L. (2004). Innate immunity in plants and animals: strinking similarities and obvious differences. Immunological Reviews 198:249–266

Pieterse, C. M. J., Leon-reyes, A., Ent, S. Van Der, and Wees, S. C. M. Van. (2009). Networking by small-molecule hormones in plant immunity. Nature Chemical Biology 5.5:308–316

Robatzek, S. (2007). Vesicle trafficking in plant immune responses. Cellular Microbiology 9:1–8

Robatzek, V. G. and S. (2008). Breaking the barriers : microbial effector molecules subvert plant immunity. Annual Review of Phytopathology 46:189-215

Saunders, D. G. O., Breen, S., Win, J., Schornack, S., Hein, I., Bozkurt, T. O., Champouret, N., Vleeshouwers, V. G. a. a., Birch, P. R. J., Gilroy, E. M., and Kamoun, S. (2012). Host protein BSL1 associates with phytophthora infestans RXLR effector AVR2 and the solanum demissumimmune receptor R2 to mediate disease resistance. Plant Cell 24:3420–3434

Shen, D., Liu, T., Ye, W., Liu, L., Liu, P., Wu, Y., Wang, Y., and Dou, D. (2013). Gene duplication and fragment recombination drive functional diversification of a superfamily of cytoplasmic effectors in *Phytophthora sojae*. PLOS One 8.7: e70036

Stam, R., Howden, A. J. M., Delgado-Cerezo, M., M M Amaro, T. M., Motion, G. B., Pham, J., and Huitema, E. (2013a). Characterization of cell death inducing *Phytophthora capsici* CRN effectors suggests diverse activities in the host nucleus. Frontiers in Plant Science 4:387

Stam, R., Jupe, J., Howden, A. J. M., Morris, J. A., Boevink, P. C., Hedley, P. E., and Huitema, E. (2013b). Identification and characterisation CRN effectors in *Phytophthora capsici* shows modularity and functional diversity. PLOS One 8:1–13

Surpin, M., and Raikhel, N. (2004). Traffic jams affect plant development and signal transduction. Nature Reviews Molecular Cell Biology 5:100–109

Tao, Y., Xie, Z., Chen, W., Glazebrook, J., Chang, H., Han, B., Zhu, T., Zou, G., and Katagiri, F. (2003). Quantitative nature of *Arabidopsis* responses during compatible and incompatible interactions with the bacterial pathogen *Pseudomonas syringae*. The Plant Cell 15:317–330

Wang, X., Boevink, P., Mclellan, H., Armstrong, M., Bukharova, T., Qin, Z., and Birch, P. R. J. (2015). A host KH RNA-binding protein is a susceptibility factor targeted by an RXLR effector to promote late blight disease. Molecular Plant 8:1385–1395

Whisson, S. C., Boevink, P. C., Moleleki, L., Avrova, A. O., Morales, J. G., Gilroy, E. M., Armstrong, M. R., Grouffaud, S., West, P. Van, Chapman, S., Hein, I.,

Toth, I. K., Pritchard, L., and Birch, P. R. J. (2007). A translocation signal for delivery of oomycete effector proteins into host plant cells. Nature 450.7166:115-118

초록

식물은 병원균의 공격에 대응하는 다양한 방어 기작을 가지고 있다. 그 중 식물은 병원균의 강력한 병원성을 가진 effector에 대해 면역반응의 결과로 세포 사멸를 일으키기도 한다. 세포 사멸은 병원균이 공격한 세포를 괴사시켜 병원균의 증식을 막는 것을 말한다.

본 연구는 담배에서 감자역병균의 core effector 82 (CE82)가 일으키는 세포 괴사 반응에 관여하는 식물 단백질을 확인하기 위해 진행되었다. CE82는 비기주인 고추와 기주인 담배 잎에서 세포 사멸을 일으키는 effector로, 담배에서 소포 관련 기구에 발현하는 것을 확인하였다. 하지만 CE82는 감자역병균의 증식에 직접적으로 영향을 주지 않았다. Immunoprecipitation 과 LC-MS/MS를 통해 CE82와 상호작용 할 것이라 예상되는 식물 타겟 단백질 후보군 16개를 확인하였다. 이 중 4개가 (MON2, GNOM1, GNOM-like, GNOM2) 소포 운반 관련 단백질로 알려져있다. 그 중 MON2, GNOM1, GNOM2의 발현을 낮추었을 때, CE82에 의한 세포괴사 속도나 반응에 차이가 없는 것을 확인하였다. MON2를 CE82와 같이 과발현 시켰을 때, 세포사멸이 빨라지고 CE82의 발현 위치가 소포 관련 기구뿐만 아니라 세포막과 세포질에서도 확인되었다. MON2와 CE82가 간접적으로 상호작용하는 것을 bimolecular fluorescence complementation을 통해 확인하였다. 이를 통해 CE82에 의한 세포 사멸이 소포 운반 기능과 관련이 있을 것이라 예상할 수 있고, 또한 이 작용에 MON2가 관여하고 있을 것이라 추측할 수 있다.