

**The study of salicylic acid-mediated defense
responses by a tobacco calmodulin-like protein**

–タバコカルモジュリン様タンパク質により誘導される
サリチル酸を介した防御応答に関する研究–

Hokkaido University Graduate School of Agriculture
Division of Agrobiolology Doctor Course

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September 2017

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HOKKAIDO
UNIVERSITY

Thesis submitted to Graduate School of Agriculture, Hokkaido University

In partial fulfillment of the requirements for the degree of Doctor of Philosophy

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September 2017

CONTENTS

	Page
GENERAL INTRODUCTION	1
MATERIALS AND METHODS	10
1. CHAPTER I : rgs-CaM induces SA signaling for antiviral defense response in tobacco	21
1-1. Introduction	22
1-2. Results	27
1-3. Discussion	52
2. CHAPTER II: Virus infection depending on autophagy gene, beclin1	56
2-1. Introduction	57
2-2. Results	59
2-3. Discussion	76
GENERAL DISCUSSION	78
SUMMARY	83
REFERENCES	86
ACKNOWLEDGMENT	103

LIST OF FIGURES

Figure	Title	Page
1	Resistance and response to CMV-Y infection in tobacco plants and schematic model of antiviral defense in tobacco.	26
1-1	Transgenic tobacco plants overexpressing rgs-CaM elicits immune responses in tobacco.	34
1-2	The phenotypic characteristics and defense responses shown in transgenic lines 16 and 23.	35
1-3	Necrotic symptom or cell death and SA signaling were induced by transient expression of rgs-CaM.	37
1-4	Susceptibility of rgs-CaM–knockdown tobacco plants to PVX, and SA signaling in response to PVX infection.	38
1-5	Implication of rgs-CaM involvement in SA signaling in response to infection by CMV.	40
1-6	Infectivity of CMV Δ 2b in SAR-induced tobacco	41
1-7	<i>PR1a</i> expression was dependent on wounding treatment by microperforation	42
1-8	Induction of SA signaling in viral RNA silencing suppressor (RSS)-expressing tobacco plants with Ca ²⁺ influx.	43
1-9	<i>PR1a</i> induction depends on rgs-CaM.	45
1-10	Induction of SA signaling has a phase change aged tobacco plants.	47
1-11	Induction of SA signaling was changed in different nutritive conditioned tobacco plants.	48

1-12	Infectivity of PVX in SAR-induced tobacco plants	49
1-13	The change of symptoms in SAR-induced and rgs-CaM-silenced plants inoculated with PVX	50
1-14	Enhanced resistance against CMV-Y depends on rgs-CaM in aged tobacco plants	51
2-1	Making of transgenic tobacco plants, in which the <i>beclin1</i> gene is suppressed by RNA silencing	63
2-2	Transcriptional levels of <i>Beclin1</i> were investigated by quantitative real-time RT-PCR in wild type and in <i>beclin1</i> knockdown tobacco lines on T1.	64
2-3	The severity of necrotic symptoms induced by CMV-N2b in WT and IR- <i>beclin1</i>	65
2-4	Symptoms, SA signaling and virus accumulation in WT (<i>xanthi-nc</i>) and <i>beclin1</i> knock down tobaccos inoculated with PVX	66
2-5	Virus accumulation and rgs-CaM expression in leaves of WT and <i>beclin1</i> knock down tobacco lines that were inoculated with PVX/2b.	67
2-6	Images of BBWV2-expressing GFP infection on inoculated leaves of aged plants	68
2-7	BBWV2-GFP infection in WT and IR- <i>beclin1</i> lines	69
2-8	BBWV2 spreading was affected by autophagy deficiency in aging leaves.	70
2-9	Images of BBWV2 infection in young leaves	71

2-10	The characteristic of BBWV2 infection was exposed to inadequate nutrition condition in WT and IR-beclin1 <i>N.benthamiana</i> .	72
2-11	Systemic infection in nutritionally deficient state of benthamiana.	73
2-12	Effect of salicylic acid and jasmonic acid treatment on necrotic symptoms developed on leaves of <i>N.benthamiana</i> plants inoculated with BBWV2.	74
2-13	Systemic symptoms affected by SA and JA treatments	75
3-1	Model of SA signaling in response to CMV infection in tobacco plants.	80
3-2	Schematic models of detection and counteraction of viral RSSs by rgs-CaM.	81

LIST OF TABLES

Table	Title	Page
1	Primers used in chapter 1 experiments	19
2	Primers used in chapter 2 experiments	20
3	Several CaM and CMLs known for involving in plant immunity.	25

ABBREVIATIONS

PRR	Pattern recognition receptor
PAMPs	Pathogen-associated molecular patterns
PTI	PAMP-triggered immunity
ETI	Effector-triggered immunity
HR	Hypersensitive response
PCD	Programmed cell death
SA	Salicylic acid
ROS	Reactive oxygen species
RSS	RNA silencing suppressor
SAR	Systemic acquired resistance
CaM	Calmodulin
CML	Calmodulin-like
VIGS	Virus-induced gene silencing
CMV	Cucumber mosaic virus
PVX	Potato virus X
PI 3-kinase	Phosphoinositide 3-kinase

GERNERAL INTRODUCTION

The earth incubates numerous species and they form complex network called ecosystem. All organisms have overcome biological battle on an evolutionary scale through a long history. To live on earth, they have to cooperate or compete with others as occasion needs so there are 6 relationships between them such as mutualism, commensalism, neutralism, competition, and exploitation. Exploitative interaction is a form that one species increase its fitness accompanying with expense and/or sacrifice of another species. Prey and predator is a simple example of exploitation. Infection is another instance; pathogens attack and loot host's materials and resources to propagate themselves (Berg, 2009; Pal and Gardener, 2006).

Since organisms are exposed to pathogens constitutively, they have evolved protective mechanisms individually called immunity. Immunity is well-studied in vertebrates and they develop innate-adaptive immune mechanisms and specialized cells for the immune systems. Plants also develop their own immune mechanisms. Although plants do not have mobile and active defender cells like T and B cells and adaptive immune system, they depend on innate immune systems equipped in each cells (Jones and Dangl, 2006). Innate immunity of Plants are composed of recognition of pathogens and downstream steps that trigger defense pathways (Nimchuk et al., 2003).

Plants have two types of recognition mechanisms against various external pathogen attacks. One is the recognition via receptor-like kinase and receptor-like proteins, which function as a pattern recognition receptor (PRR) such as toll-like interleukin receptor (TLR) of animals to detect pathogens. The other is the recognition via the R genes that encode the conserved nucleotide binding (NB) and leucine rich repeat (LRR) proteins

(Mandadi and Scholthof, 2013). These immune systems were proposed to be evolutionally developed through arms race between plants and pathogens, and coordinately act against pathogens in a zigzag model (Jones and Dangl, 2006); microbial or pathogen associated molecular patterns (MAMPs or PAMPs) are recognized by PRR, leading to activation of pattern-triggered immunity (PTI). The molecular components of PAMPs are well conserved among most microbes, but the compounds do not exist in host plants. Plants activate diverse signaling events through innate immune responses after recognition of PAMPs in plants. Above all, well-known examples of PAMP-PRR are bacterial flagellin/flagellin sensing 2 (FLS2) and fungal chitin (Chisholm et al., 2006; Nicaise et al., 2009). When plants are attacked by host-adapted pathogens that cause disease, these pathogens secrete effector molecules, which facilitate their infection by suppressing PTI, into plant cells. Then, plants have developed the R genes, which encode the NB-LRR proteins to specifically recognize the effector molecules and counter the pathogens with effectors. Plant disease resistance caused by interactions between those NB-LRR proteins and effectors is referred to an effector-triggered immunity (ETI).

Plant responses against pathogen

There are diverse responses of plants against pathogens. Homeostatic changes including increase of membrane permeability and production of defense-related materials are common in pathogen resistance. Defense mechanisms are associated with transcriptional activation of lytic enzymes, anti-microbial proteins, and secondary metabolite for resistance (Nürnberg et al., 2004). Ion channels on plasma membrane are also stimulated by pathogen recognition. For example, potassium influx channel is

closed by infection circumstance but efflux channel is activated simultaneously. Thus, netflux of potassium ion shows outward movement from cytosol. Furthermore, an internal calcium level, which is related to activation of calcium influx channel, increases but ER sequestered calcium may be also influence this calcium elevation phenomenon (Nürnberg and Scheel, 2001).

Ca^{2+} is known as a secondary messenger molecule regardless of animal and plant kingdom. It mediates enormous external cues and regulates numerous signal cascades. As mentioned above, transient Ca^{2+} elevation with biphasic pattern is observed after pathogen perception. Cytosolic Ca^{2+} elevation leads to several molecular responses such as activation of Ca^{2+} dependent protein kinases and mitogen-activated protein kinases and generation of nitric oxide (NO) and reactive oxygens (ROS). These signal cascades turn on down-stream defense gene transcription, and thus act as mobile signals to neighboring cells, and also can work as oxidative weapons (Ma and Berkowitz, 2007; Nürnberg and Scheel, 2001).

In addition, Ca^{2+} is recognized and interacted by calmodulin (CaM), calmodulin-like protein (CML), and calcineurin B-like proteins (CBL) and their interacting protein kinases (CIPK) (DeFalco et al., 2010). It was discovered that at least, inductions of several gene expression among CaM-related genes involve increasing Ca^{2+} concentration in cytoplasm by external signals (Polisensky et al., 1996). CaM has also known to be related to the signaling pathway in response to light, and cold (Lu et al., 1996; Polisensky et al., 1996; Sinclair et al., 1996; Xing et al., 1997). In plant immune system, CaMs bind to diverse transcription factors including pathogen-induced ones. Genetic studies have shown that CaM genes-silenced or mutated plants increase bacterial susceptibility but CaM-overexpressed plants accelerate resistance (Cheval et

al., 2013; Ma et al., 2009).

Pathogen resistance of plants as well as animals is often accompanied with programmed cell death (PCD). This typical cell death triggered by ETI is called hypersensitive reaction (HR). HR is induced at the region of pathogen invasion including bacteria, fungi, and viruses. HR shows a necrotic phenotype such as cytoplasmic shrinkage, chromatin condensation, mitochondrial swelling, vacuolization and chloroplast disruption (Coll et al., 2011). HR includes metabolic changes of several defense pathways for example SA, jasmonic acid (JA), and ROS etc. (Carr and Palukaitis, 2010; Culver and Padmanabhan, 2007; Pallas and Garcia, 2011). Moreover HR is entangled with Ca^{2+} level in cells (Mur et al., 2008).

Phytohormones in plant defense

Phytohormones are well-known signal compounds for regulators of plant growth and survival. Most of phytohormones are modulators of plant immune system and SA and JA with its derivatives are major hormones for defense (Pieterse et al., 2012). SA influences seed germination, seedling establishment, cell growth, senescence, expression of genes related to them, and reactions to biotic stresses etc (Klessig and Malamy, 1994; Morris et al., 2000; Metwally et al., 2003; Clarke et al., 2004; Rajjou et al., 2006). Additionally, SA is famous as a defense hormone. SA accumulates by infection in whole plant body, and SA treatment externally also confers resistance to viral, bacterial, and fungal infection on plants. The importance of SA in plant defense has been proven by the studies of SA contents among plant ecotypes and species and genetic mutants of SA biosynthesis (Pieterse et al., 2009; Vlot et al., 2009). Furthermore, SA was reported to directly effect on expression of pathogenesis-related (PR) genes, HR

against pathogen, and systemic acquired resistance (SAR) (Malamy et al., 1990; Ryals et al., 1996; Mur et al., 2008; Coll et al., 2011). Particularly, SA is considered to be an important signal to mediate ETI and PTI against diverse pathogens.

JA is also related to physiological processes such like germination, root growth, tuber formation, tendril coiling, fruit ripening, leaf senescence, stomatal opening, and even flowering and the development of flower meristem (Bari and Jones, 2009; Yuan and Zhang, 2015), but its main function is also known as a defense regulator. The defensive function of JA was revealed by its concentration, which is increased by infection and injury. Exogenous treatment of JA elevates transcription levels of defense-related genes. Genetic studies for jasmonate biosynthesis and signaling with their transgenic lines and mutants have been also performed to prove the defensive function of JA (Ballaré, 2011). There are antagonistic interaction between SA and JA; SA is involved in defense against biotrophic and hemi-biotrophic pathogens and JA is related to defense against necrotrophic pathogens and herbivorous insects, which are mutually antagonistic (Bari and Jones, 2009; Pieterse et al., 2009).

Although plants have not any mobile cells like animals, there are transportable immune signals which pass vascular tissues as a duct. These mobile signals moved from the site of infection to uninfected tissues, and then trigger resistant against subsequent pathogen invasion. This phenomenon is called SAR. SAR is typically induced by ETI and is recruited against various biotrophic pathogens (Pieterse et al., 2012; Spoel and Dong, 2012). SA is not only associated with local disease resistance but it also has been studied particularly in SAR-induced plants. Although SA itself is not mobile signal for SAR (which is proved by leaf detachment experiment and chimeric grafting experiment), SA accumulation and signal are required in SAR (Spoel and Dong, 2012;

Vlot et al., 2009).

In *Arabidopsis*, the primed state of SAR is partly attributed to the action of non-expressor of PR gene (NPR) proteins NPR1, NPR3, and NPR4, which functions downstream of SA to mediate regulations of defense genes (Attaran and He, 2012; Cao et al., 1997; Fu et al., 2012; Wu et al., 2012). In addition, epigenetic modifications in SAR-induced plants have been suggested to be involved in the primed state (Conrath et al., 2015). The effect of transgenerational SAR (Luna et al., 2012) supports the involvement of epigenetic modifications because such modifications can be inherited in plants (Hauser et al., 2011). Thus, the role of NPR1 in transgenerational SAR (Luna et al., 2012) indicates that SA is also involved in the epigenetic modifications. Although systemic SA biosynthesis (i.e., including parts distant from the site of infection) is required for induction of SAR (Delaney et al., 1994), SA derivatives and other chemical molecules have been recently identified as the systemic signaling molecules (Gao et al., 2015). In contrast to whether the mechanisms of SAR is induced and maintained by resistance responses even in the next generation, especially viruses, at secondary infection sites in SAR-induced plants remain unclear.

RNA silencing in plant defense

One major defense mechanism against diverse viruses is RNA silencing, which is induced by viral double-stranded RNA (dsRNA) and act as antiviral defense system (Liu et al., 2010, Mishina and Zeier, 2007). The RNA silencing mechanism is conserved among eukaryotes. Once infected virus generates dsRNA, it is diced into small interfering RNAs (siRNA) by an endonuclease, which is named Dicer. Then, the siRNAs are integrated in RNA-induced silencing complex (RISC) to degrade cognate

RNAs (Pumplin and Voinnet, 2013). In addition to posttranscriptional gene silencing (PTGS) by siRNAs, there are other silencing mechanisms; that is, one is induced by endogenous small RNA, micro RNA (miRNA) and target diverse endogenous genes, and another is transcriptional gene silencing (TGS) that directs methylation of DNA and histone, resulting in silencing genes at transcriptional level. These silencing processes are partially overlapping but distinguished in plants (Revers and Nicaise 2014).

Majority of plant viruses are RNA viruses, of which genomic RNAs form secondary structure and thus dsRNA partly. Moreover, they produce dsRNA as an intermediate of their RNA genome replication. These dsRNAs are considered to be targeted by RNA silencing (Revers and Nicaise 2014). Many of plant viruses have a RSS such as 2b and HC-Pro to interfere with RNA silencing or evolve diverse mechanisms to avoid silencing (Diaz-Pendon and Ding, 2008). Some RSSs are to repressed maturation of RNA-induced silencing complex (RISC) or degrade specific RNA sequences (Ding and Voinnet, 2007). For example, 2b of cucumber mosaic virus (CMV) bound to small RNAs (siRNAs and miRNAs) and suppressing PTGS pathway (Goto et al., 2007).

Viruses

CMV belongs to the genus *Cucumovirus* in the family *Bromoviridae* and is single-stranded RNA virus. The virus genome has three RNAs (RNA 1, RNA 2, and RNA 3) and RNA4 from the minus-strand copy of RNA3. It is responsible for agricultural economical loss in many crops worldwide. CMV has been known as a model for research concerning viral life cycle including viral replication and -movement, interaction with host response, and evolution for more than decades (Jacquemond, 2012).

Potato virus X (PVX) is a member of the genus *Potexvirus* (Koenig and Leseman, 1989). PVX is also a single-stranded RNA virus, of which genome size is about 5.9 kb. This virus has been used as a vector to express a gene in plants by many researches (Chapman et al., 1992).

Autophagy in plant defense

Autophagy is one of degradation process. The term 'Autophagy' is comes from the Greek word meaning 'eating of self'. The molecular mechanism of autophagy has been investigated about its function, since early of the 1950s (Shibutani et al., 2015). The difference things of autophagy compared to other degradation pathway are that it decomposes huge compound such like intracellular organelles and protein aggregates. Autophagy machinery is well-conserved within organisms and the mutations of autophagy genes in various species share vulnerable phenotypes to infection (Levine et al., 2011; Shibutani et al., 2015).

The process of autophagy, double membrane formed in cytoplasm more expands gradually when cells are in condition of starvation and pathogen infection. Then this cell absorbs waste protein in cytoplasm. Preferentially, autophagosome containing mitochondria and several organelles with lysosome is fused edge of the cells. And then, intracellular organelles are degraded by autolysosome. That is, autophagy mechanism is a bulk degradation system. This degradation reaction occurred for self-eating as large-scale in cytoplasm. In a sense, the process could be dangerous to living organism but it is valuable mechanism to maintain cellular homeostasis self-control by removal damaged intracellular organelles. Three different types of Autophagy have been defined: macro-autophagy, micro-autophagy, and chaperone-mediated autophagy (CMA). These

pathways leading to degradation through mechanistically separated route (Yang and Klionsky, 2010). Microautophagy is involved in directly engulfment of cytoplasm promoted by arm like structure at lysosomal membrane in yeast. CMA translocates unfolded proteins directly across membrane of the lysosome. On the other hand, macroautophagy sequesters intact organelles to isolated membrane via phagophore. Then this phagophore become double membrane called to autophagosome (Chiramel et al., 2013).

Autophagy is known to be associated with several molecular processes such as cancer, neurodegeneration, and aging in animals. Similarly, it is linked to endurance of biotic and abiotic stress and senescence. Nutrient deprivation is a major cause of autophagy induction; autophagy helps material recycling and redistribution which are also related to senescence. Furthermore, autophagy is also important in vascular formation, shoot elimination, leaf shape formation, and pollen germination and pollen tube growth (Liu and Bassham, 2012). In terms of defense, autophagy is necessary for immune system both in animals and in plants. The components in the autophagy machinery are interacted with pathogen recognition receptors and their cascades. Moreover, in autophagy-deficit mutant, host cells are more susceptible to pathogen invasion, and replication rate of pathogen is dramatically enhanced (Hayward et al., 2009).

MATERIALS AND METHODS

1. Plant materials and growth conditions

Mature seeds of *Nicotiana tabacum* cv. Xanthi and *N. tabacum* cv. Bright Yellow (BY) were grown as wild type plants. Transgenic tobacco plants (*N. tabacum* cv. Xanthi), in which *rgs-CaM* was either overexpressed or knocked down, were made previously (Nakahara et al., 2012). Transgenic tobacco plants (*N. tabacum* cv. BY4) expressing viral RSSs were also made previously (Nakahara et al., 2012). Transgenic tobacco plants expressing CMV CP and NahG were made similarly to those expressing viral RSSs (Nakahara et al., 2012). T2 or later generations of transgenic tobacco plants, all of which were shown to be kanamycin resistant, were grown under a 16-hr light/8-hr dark photoperiod at 25°C in a growth chamber or environmental control system for virus inoculation and other experiments.

Seeds of *beclin1* knockdown *benthamiana* were provided by Hukuda. I generated *beclin1* knockdown *N. tabacum* Xanthi-nc. Transgenic plants were selected with kanamycin.

2. Virus inoculation

Nicotiana benthamiana leaves infected with CMV-Y; CMV Δ 2b, which lacked 2b and was designated CMV-H1 in a previous study (Tsuda et al., 2013); and the PVX vectors were used as inocula for mechanical inoculation with carborundum and stored in a deep freezer at -80°C until needed.

The *rgs-CaM* open reading frame (ORF) and the ORF lacking its initial codon were cloned between the *Cla*I and *Sal*I sites of the PVX vector pP2C2S (Lecourieux et al.,

2006) to generate PVX-rgs-CaM and PVX-rgs-CaM(-atg) [VIGS(rgs-CaM)], respectively. After linearization of these plasmids by digestion with *SpeI*, infectious RNAs were transcribed from the linearized plasmids by T7 RNA polymerase with the 7-methylguanosine-5'-phosphate cap analog (Thermo Fisher Scientific Inc., Waltham, MA, USA) and used as inocula for mechanical inoculation. The *rgs-CaM* ORFs with/without the initiation codon were also cloned between the *XbaI* and *SacI* sites of pE2113 (Zhou et al., 2009).

The inoculum of CMV-N2b was provided by Prof. Masuta (2017). *Nicotiana benthamiana* plants infected with BBWV2-GFP were provided for inoculums by Atsumi (2013). The inoculums of BBWV2-GFP had applied to infect it using celite instead of carborundum for tender leaf tissue of *N. benthamiana* than *N. tabacum* with phosphate buffer pH.7.2.

3. RT-PCR, semi-quantitative RT-PCR, and real-time RT-PCR

(1) Extraction of RNA

Plant leaves about 0.05g - 0.1g were ground on mortar with liquid nitrogen for extraction of total RNA. After adding TRIzol reagent (Ambion, USA) to homogenize the samples, insoluble materials with TRIzol were precipitated by centrifugation at 14,000 rpm and discarded. Chloroform, one-fifth of TRIzol volume, was added into the supernatant and the supernatant was vortexed and centrifuged at 14000 rpm for 3 min. The aqueous layer was replaced into a new microcentrifuge tube and mixed with the equal volume of isopropanol and incubated on room temperature for 10 minutes. After precipitated by centrifugation at 14000 rpm, the pellet of nucleic acids was washed with 70% ethanol twice. After dried up, DNAs in nucleic acid samples were digested with

RNase-free DNase I (Roche Diagnostics, Basel, Switzerland) at 37°C for 20 min. To remove DNase and purify the RNA samples, 150µl of distilled water, 100µl of chloroform : isoamylalcohol (24:1) and 100µl of TE phenol were added into the treated samples and mixed and centrifuged at 14000 rpm for 4 min and then the supernatants were transferred into new tubes. They were mixed with one-tenth 3M sodium acetate and 2.5 times volume of 100% ethanol to a supernatant, respectively. After centrifugation at 14000 rpm for 10 min, the supernatant was removed completely. The RNA samples were washed with 70% ethanol and dried up. They were finally dissolved in distilled water. The RNA concentration was measured by a Nanodrop (spectrophotometer ND-1000).

First-strand cDNAs were synthesized from 1 µg of RNA extracts by a modified M-MLV reverse transcriptase, ReverTra Ace (Toyobo, Osaka, Japan). The mixture (20 µl) for cDNA synthesis containing 1 µg template RNA, 1 mM dNTP, and 100U ReverTra Ace was incubated in condition of 30°C for 10 min, 42°C for 30min, and 99°C for 5 min.

(2) RT-PCR

Accumulation of viral genomic RNAs and endogenous mRNAs was detected by PCR in a mixture (25 µl) containing cDNAs corresponding to 0.05 µg RNA, 0.4 µM of each of the specific primer pairs listed in Table 1, 0.2mM dNTP, and 0.625 U Ex Taq DNA polymerase (TaKaRa, Otsu, Japan). PCR mixtures for *PRI* were incubated for 2 min at 95°C, followed by 28 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 40 s, and PCR products were fractionated with 2% agarose gel electrophoresis.

(3) Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was done with temperatures cycles for rgs-CaM with primers listed in Table1 and Table2 (F-ATGGAAGAGGAGAGAAATAAGG, R-CTTGTCATCATAGCTTTGAACT), 24 cycles of 95°C for 30s, 59°C for 30s, and 72°C for 30s, and with those for 18S rRNA with primers set (F-CCGTAGTCCCTCTAAGAAGCTG, R-GGTCCAGACATAGTAAGGATTG), 15 cycles of 95°C for 30s, 58°C for 30s, and 72°C for 30s.

(4) Real-time PCR

Real-time PCR was performed by using the DNA Engine Opticon 2 system (Bio-Rad Laboratories, Hercules, CA, USA) according to the method in a previous study (Pruss et al., 2004). The reaction mixture (25 µl) contained 0.625 U of Ex Taq (TaKaRa), Ex Taq buffer, 0.2 mM dNTP, 0.2 µM (each) forward and reverse primers listed in Table 1, SYBR Green (30,000 × dilution) (Thermo Fisher Scientific), and cDNA corresponding to 12.5 ng of total RNA. Samples were incubated for 2 min at 95°C, followed by 39 cycles of 95°C for 10 s, 58°C for rgs-CaM or 59°C for PR1a for 20 s, and 72°C for 20 s. The internal control for real-time PCR was performed 18s rRNA.

4. Northern hybridization

(1) Preparation of RNA

Extracted RNA samples were adjusted of 5 µg/µl concentration and all procedures were on ice. To precipitate RNA, distilled water (40 µl), 100% ethanol (100 µl) and 3 M sodium acetate (4 µl) were added and mixed. After centrifugation of them at 14000 rpm, the supernatant was removed and dried up. Seven µl of RNA denature solution (20 × MOPS: formaldehyde : formamide=1:3.5:10, and 0.25% bromophenol blue) was added

into tubes with the pellets and mixed by pipetting carefully. The RNA was denatured in a heat block on 70°C for 5 min. The denatured-RNAs were fractionated in a 1.2% agarose gel (40 ml) containing 2 ml of 37% formaldehyde solution in 1 × MOPS buffer on conditions with 100V for 40–45 min.

(2) Membrane blotting

For blotting onto membrane, a tray was treated with 80% ethanol and washed with distilled water twice. Then, 20 × SSC buffer containing 3M NaCl and 0.3M sodium citrate was poured on area of the tray where a nylon membrane was put on. Chromatography paper or 3MM filter paper (Whatman) was cut into eight papers 1 cm over size of the gel, and soaked in 20 × SSC buffer. A nylon membrane (Hybond-N; GE Healthcare, Chicago, IL, USA) was also cut into one 0.5 cm over size of the gel and soaked in 20 × SSC buffer. Four pieces of filter paper, the overturned-gel, the membrane, and the four pieces of filter paper in order (pouring 20 × SSC buffer in all steps, continuously) were put on the tray with 20 × SSC. When the gel was put onto pieces of filter paper, air bubbles between them were removed carefully. After pieces of parafilm were shielded around papers, kitchen towels laid in piles on the paper. Lastly, a heavy block was put on the towels, and this blotting step took a time over 8hr. After blotting, all materials were discarded except the blotted membrane. Blotted RNA, which was stained with ethidium bromide, was checked by detecting and took a picture of the membrane under ultra violet light using a gel image system. I marked the location of first and second bands and combed on the membrane by a pencil. RNA on the membrane was fixed by UV crosslinker on 1200 t × 100 μJ/cm². The membrane was dehydrated in dry oven for 1–2 hours. The membrane which was dried up was kept at room temperature until doing following steps.

(3) Pre-hybridization

In advance to pre-hybridization, the membrane was absorbed in $5 \times$ SSC under shaking for 5 to 10 min (SSC buffer concentration is the same one of hybridization buffer in this step). After throw away of $5 \times$ SSC, the membrane was incubated with 10 ml of hybridization buffer (50% formamide, 7% SDS, 1% N-laurylsarcosine, 2% blocking reagent, 50 mM Na-Pi buffer, and $5 \times$ SSC) (Roche Diagnostics) under shaking at 55°C for 1 - 2 hours.

(4) Hybridization

Digoxigenin (DIG)-labelled cRNA probes were made from the target mRNA sequences, PVX genomic RNA sequence, and the conserved nucleotide sequence at the 3'-terminal regions of CMV genome segments using the primers listed in Table 1. A DIG-labelled cRNA probe for the target gene was denatured at 70°C on a heat block for 5 min, and then stood on ice. The membrane was set into a poly-pack and 3 ml of hybridization buffer and 1 μl of the DIG-labeled cRNA probe was added into the poly-pack. It was sealed after bubbles in it were removed, and incubated with shaking for overnight for hybridization (at 58°C for *PR1a* and at 60°C for the other targets).

(5) Membrane washing

The membrane was washed in $2 \times$ SSC with 0.1% SDS for 15 min twice and then washed again it in $0.2 \times$ SSC, 0.1% SDS for 15 minutes at 58°C . The membrane was next equivalent with MBS (100mM maleic acid and 150mM NaCl, pH 7.5) for 5 min and pre-incubated with blocking buffer for 30 min. The membrane was incubated with 1 μl anti-DIG-antibody in 10 ml of blocking buffer (10% blocking reagent in MBS buffer) for 60 min. The last washing was in 0.3% tween20 in MBS for 15 min two times and then it was pre-equivalent with AP buffer for 10 min.

(6) Detection

Mixture of 10 µl of CDP star in 3 ml of AP buffer was added into the membrane in a new poly-pack. Chemiluminescence signals were quantitatively detected by LAS-4000 mini PR Lumino-image analyzer (GE Healthcare) for adequate exposure time.

5. Western blot analysis

To prepare samples for western blotting, the sample tissues were homogenized with a mortar and then dissolved in 12-fold (volume/mass) urea-denaturing buffer containing 4.5 M urea, 1% (vol/vol) Triton X-100, 0.5% DTT, 0.0625 M Tris-HCl pH 6.8, 2% (wt/vol) SDS, 5% mercaptoethanol, 5% sucrose, and 0.002% bromophenol blue.

(1) SDS-PAGE and gel electrophoresis

The extracts were centrifuged to collect the supernatants. Equal amounts of samples were separated by 10% SDS polyacrylamide gel (SDS/PAGE). A separation gel and a stacking gel were made by using gel cast of 12% and 3% acrylamide respectively. A generated-SDS/PAGE was set into a gel running tank with tris-glycine (contained SDS) electrophoresis buffer. The prepared samples were loaded in the gel in the tank. The electrophoresis was done at 0.1A about 60 min.

(2) Electro-transfer and detection

Fractionated proteins depending on molecular weights in the SDS/PAGE were then transferred to Immobilon PVDF membranes (Millipore-Sigma) with Tris-glycine transfer buffer in electrophoretic transfer kit. The electrotransfer conditions were 15 V for 60 min, and then the gel was stained with coomassie brilliant blue G250 (CBB). Transferring proteins from the gel to a membrane were pretreated in a blocking buffer to interrupt nonspecific binding of antibodies to the membrane. The blots were incubated

with anti- PVX CP, CMV CP, 2b, and rgs-CaM rabbit polyclonal antibodies. Three μ l of primary antibody added into blocking buffer with the membrane and they were shaken for 30 min. After washed with blocking buffer for 5 min, the membrane was treated with a secondary antibody conjugated with an alkaline phosphatase for 1 hour. The membrane was washed in blocking buffer 3 times. Lastly, Proteins with the secondary antibodies were detected by treatment with CDP-Star solutions (Roche Diagnostics, Basel, Switzerland) for chemiluminescence detection. Chemiluminescent signals were quantitatively detected by a LAS-4000 mini PR Lumino-image analyzer (GE Healthcare).

6. BTH and Ca²⁺ ionophore treatment

A salicylic acid analog, benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH), was spread on tobacco leaves with cotton tufts that were dipped in 1mM BTH, 1.4% acetone as a solvent and 0.2% Tween-20.

In advance, calcium ionophore A23187 (Millipore Sigma, St. Louis, MO, USA) was dissolved in DMSO (5 mg/1 ml). Calcium ionophore A23187 solution (75 μ M), which diluted with phosphate-buffered saline (PBS; 137mM NaCl, 2.7mM KCl, 10mM Na₂HPO₄·12H₂O and 2mM KH₂PO₄ pH7.4) from the stock solution, was infiltrated into a leaf at a punched-hole using a syringe.

7. SA and JA treatment

1.1mM salicylic acid (SIGMA, USA) was sprayed directly on *N.benthamiana* leaves according to Lewsey and Carr (2009). In the case of JA treatment, the treatment was done according to the method (Yamada et al., 2012). Methyl Jasmonate (Wako, Japan)

dissolved in methanol was diluted at a final concentration of 100mM in water and then poured it in a tray under plants. After 24 h, the plants were replaced on new tray without Methyl Jasmonate. Both SA and JA treatments were performed three days before virus inoculation.

Table 1 Primers used in chapter 1 experiments

Gene name (accession number)	Primer sequences (5'→3') * ; used in making probe	Length
18S rRNA	F-CCGTAGTCCCTCTAAGAAGCTG R-GGTCCAGACATAGTAAGGATTG	185
pE2113_P35S	F-CCACTGACGTAAGGGATGACGC R-GTGTTCTCTCAAATGAAATGA	90
rgs-CaM (AF329729)	F-TGATAGGAGCATTGGAATGTATG R-ACTCATCAAAGTTGAGAACTCCATC *F-ACTATTACTACTGATTATCTTTGCA *R-GGGATCCTAATACGACTCACTATAGGGGCAAATGCTCCTATCAATTCAC	168 324
	F-ATGGAAGAGGAGAGAAAATAAGG R-CTTGTCATCATAGCTTTGAACT	218
PR1a (X06361 Y00707)	F-GAAGTGCGATTTTCATGACGGCTG R-CGAACCGAGTTACGCCAAACCACC *F-ATGGGATTTGTTCTCTTTTCAAAATTGCC *R-AATTCTAATACGACTCACTATAGGGGAAGGTTCTTGATATCAAGCAG	151 599
PVX	F-AGGGTCAACTACCTCAACTACCAC (for CP) R-TCCTTCAAATAGCCTCAATCTTGC F-ACCAATCTTTTACAGACTCCACCAC (for RdRp) R-CTCTAGATCATTAGCCGCTTCAACC *F-ATGTCAGCACCAGCTAGCACAACAC *R-AATTCTAATACGACTCACTATAGGGACATTATGGTGGTAGCGTGAC	171 134 739
CMV	*F-GGCGGGAGCTGAGTTGGCAGTTCTGC *R-AATTCTAATACGACTCACTATAGGGGTCTCCTTTGGAGGCCCCACGA	290

* Primers used for making DIG-cRNA probes for northern blotting

F: Sense primer

R: Antisense primer

Table 2 Primers used in chapter 2 experiments

Gene name (accession number)	Primer sequences (5'→3') * ; used in making probe	length
beclin1	F-CAATGCATGGAGCTGGTAG	137
	R-CTGGCTTGCATCTGGTTG	
18S rRNA	F-CCGTAGTCCCTCTAAGAAGCTG	185
	R-GGTCCAGACATAGTAAGGATTG	
PR1a (X06361 Y00707)	*F-ATGGGATTTGTTCTCTTTTCACAATTGCC	599
	*R-AATTCTAATACGACTCACTATAGGGGAAGGTTCTTGATATCAAGCAG	

* Primers used for making DIG-cRNA probes for northern blotting

F: Sense primer

R: Antisense primer

CHAPTER I

rgs-CaM induces SA signaling for antiviral defense response in tobacco

Introduction

By yeast two hybrid screening, tobacco calmodulin (CaM)-like protein, rgs-CaM was identified as a host factor that interacts with HC-Pro of tobacco etch virus. Since HC-Pro have been shown to suppress a post transcriptional gene silencing in the virus-infected plants, its counter partners including rgs-CaM were postulated candidates that were involved in suppression of gene silencing. Anandalakshmi *et al.* proved that rgs-CaM was an endogenous RSS binding to HC-Pro (Anandalakshmi et al., 2000). CaM is a well-conserved protein in all eukaryotes and shows a high degree of amino acid level conservation. The structural feature of CaM has four EF-hand motifs for calcium binding. The pairs of EF motifs consist of globular shapes separated by a flexible stick-like helix, therefore, total structure of CaM is similar to dumbbell which is a trademark of CaM (Yang and Poovaiah, 2003).

rgs-CaM is slightly different from an ordinary CaM protein, which is composed of C-terminal domain with three EF-hand motifs binding to the calcium and N-terminal domain with an additional extension of around 50 amino acids (Anandalakshmi et al., 2000). In plants, the number of canonical CaM is unexpectedly small, for example, 6 CaM were identified in *Arabidopsis* (McCormack et al., 2005). Therefore, various CaM-like proteins expect to play an important role in plant biology. Moreover, CaM and CaM-like protein do not have any domain that has an enzymatic activity, indicating that repertoires of their interacting proteins mediate and fine-tune signal transduction pathway for various internal and/or external cues (Kolukisaoglu et al., 2004; McCormack et al., 2005; Yang and Poovaiah, 2003).

It has been known that plant immunity is well balanced system that Ca²⁺-mediated

signaling stimulates ROS regulating hypersensitive response (HR) and defense reaction (Guo et al., 2003; Lecourieux et al., 2006; Mackey et al., 2003). For example, *Arabidopsis* calmodulin AtSR1, as known CAMTA3, regulates salicylic acid level with connecting the Ca²⁺ signal and immune responses (Du et al., 2009). AtSR1 has been proved to be a negative regulator of plants immunity using loss-of-function of *Arabidopsis AtSR* (Du et al., 2009). In addition, Ca²⁺ signaling mediates not only responses to biotic stress, but also those to abiotic stress such as cold, salinity, and drought damages. This signal process induced by abiotic stimuli have been discussed with CML physiological functions (DeFalco et al., 2010). Several CaM and CMLs have been known to be involved in plant immunity. The example's table was from Cheval et al., 2013 (Table 3).

In 2012, Nakahara et al. presented that the model of antiviral defense mediated by rgs-CaM in tobacco (Figure 1). When a virus infects plants, RNA silencing is induced by recognizing viral double-stranded RNA as antiviral defense system. Viruses have a RNA silencing suppressor (RSS) such as 2b, HC-Pro to interfere with RNA silencing. rgs-CaM binds to RSSs through its affinity to dsRNA binding domains of viral RSSs. Then, rgs-CaM directs degradation of its binding RSSs via autophagy. As that report, rgs-CaM reinforces antiviral RNA silencing in cells infected with viruses possessing RSS (Nakahara et al., 2012). In addition, an *Arabidopsis* CML, CML39, which was the most identical to rgs-CaM among *Arabidopsis* CMLs, was induced by AL2, the RSS protein of African cassava mosaic virus and mungbean yellow mosaic virus, overexpression of CML39 increased susceptibility against the geminivirus infection (Chung et al., 2014).

In this way, I wonder how rgs-CaM works for antiviral defense systems in tobacco plants. Thus, using knockdown and overexpressed rgs-CaM tobacco, I tried to verify its

role in the defense process in plants.

Table 3 Several CaM and CMLs known for involving in plant immunity. This table was from Cheval et al., 2013.

Gene name	Plant species	Experimental evidence	Biological effect
<i>CaM1</i>	<i>Capsicum annuum</i>	Transient expression	Local resistance to <i>Xanthomonas campestris</i> pv. <i>vesicatoria</i>
<i>CaM13</i>	<i>Nicotiana tabacum</i>	Virus-induced gene silencing	Enhanced susceptibility to tobacco mosaic virus, <i>Ralstonia solanacearum</i> , <i>Rhizoctonia solani</i>
<i>Rgs-CaM</i>	<i>Nicotiana tabacum</i>	Knockdown/ overexpression	Impaired resistance to cucumber mosaic virus
<i>APR134</i>	<i>Solanum lycopersicum</i>	Virus-induced gene silencing	Loss of HR to avirulent <i>Pst</i> (<i>avrPto</i>)
<i>CML43</i>	<i>Arabidopsis thaliana</i>	Overexpression	Accelerated HR to avirulent <i>Pst</i> (<i>avrRpt2</i>)
<i>CML24</i>	<i>Arabidopsis thaliana</i>	Point mutation	Impaired HR to avirulent <i>Pst</i> (<i>avrRpt2</i>)
<i>CML42</i>	<i>Arabidopsis thaliana</i>	Insertional mutation	Enhanced resistance to <i>Spodoptera littoralis</i>
<i>CML9</i>	<i>Arabidopsis thaliana</i>	Insertional mutation/ overexpression	Impaired susceptibility to virulent <i>Pst</i>

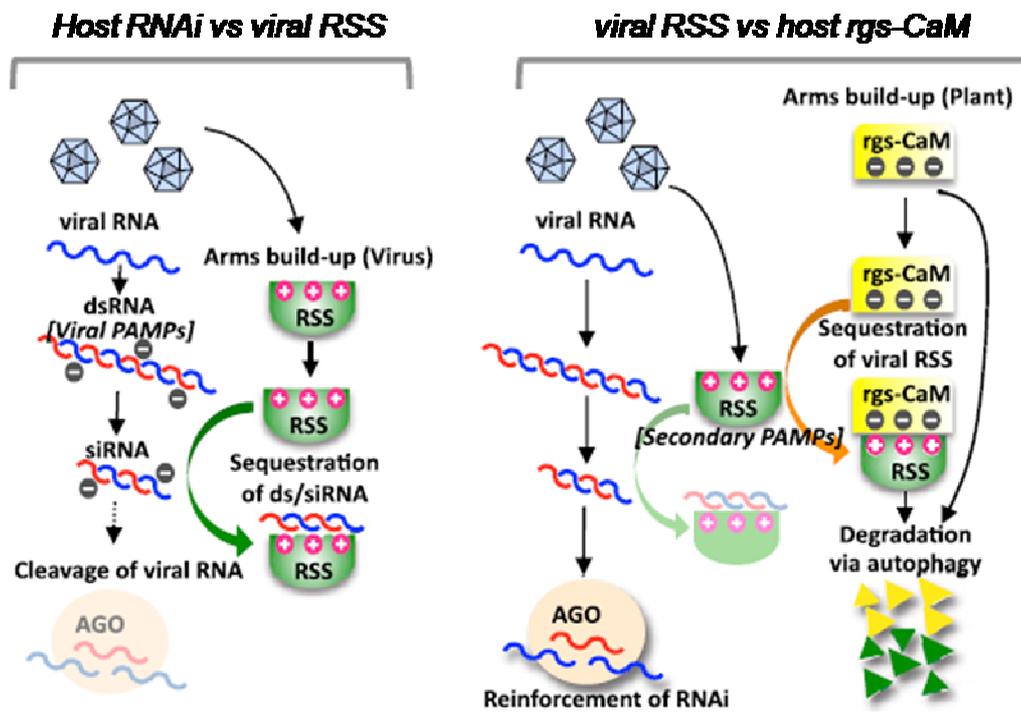


Figure 1 Resistance and response to CMV-Y infection in tobacco plants and schematic model of antiviral defense in tobacco. This model is from Nakahara et al. (2012).

Results

1. Overexpressed and ectopically expressed rgs-CaM induces defense reactions and SA signaling

I became aware of the association between rgs-CaM and other defense reactions, in addition to RNA silencing, by observing transgenic tobacco plants overexpressed the *rgs-CaM* gene. The construct was made based on the binary vector (Mitsuhara et al., 1996) to make overexpressing rgs-CaM tobacco (Figure 1-1A). Among transgenic tobacco plants that constitutively overexpressed the *rgs-CaM* gene under the control of the cauliflower mosaic virus 35S promoter, I selected two lines which showed dwarfing, deformation, and necrotic parts on their leaves (Fig 1-1B). I checked the existence of the construct in transgenic lines with PCR using two primer sets (pE2113_P35S; F-CCACTGACGTAAGGGATGACGC, R-GTGTTCTCTCCAAATGAAATGA, P35S-*rgs-CaM*; R-ACTCATCAAAGTTGAGAACTCCATC) and then confirmed that all tested transgenic plants had the construct though the siblings showed heterogeneous phenotypes (Figure 1-1A).

The phenotypic characteristics of each transgenic lines 16 and 23 are unusual including necrotic cell death and yellowing. The phenotypes of transgenic lines were similar to those of lesion mimic mutants that involve hypersensitive response-like programmed cell death, which are accompanied by induction of defense signaling components, including SA (Lorrain et al., 2003, Tang et al., 1999). I compared two overexpressed plants showing the phenotypes to wild type plants, cv. Xanthi, grown under the same conditions (Figure 1-2A). The transgenic plants were arranged in order of the severity of necrotic phenotype. In addition to cell death, mRNA of the gene for

pathogenesis-related protein 1a (*PR1a*), an indicator of activation of SA signaling (Ohshima et al., 1990), was induced in the leaves (Figure 1-2B). Accumulation of the rgs-CaM protein level in transgenic plants increased compared with wild type plants especially those at the position of 25 kDa. *PR1a* was induced in transgenic leaves and those levels showed positive correlation with phenotypic severity and accumulation of the rgs-CaM protein levels (Figure 1-2B). However, the increasing pattern was not exactly coincided with that of *PR1a*. These observation and results indicate the possibility that the overexpressed rgs-CaM can induce cell death and SA signaling. This possibility was confirmed using the PVX vector expressing rgs-CaM. Infection with this vector caused necrotic spots and induced *PR1a*, whereas infection with the empty PVX vector did not (Figure1-3). Taken together, these data suggest that overexpressed and ectopically expressed rgs-CaM induces cell death and SA signaling.

2. rgs-CaM induces SA signaling via perception of both Ca^{2+} and viral RSS

The abovementioned results led me to suspect that endogenous rgs-CaM is also capable of inducing immuneresponses and SA signaling. I assume that rgs-CaM is involved in SA signaling when it is induced. rgs-CaM is strongly induced by wounding stress on leaves of tobacco plants (Tadamura et al., 2012). However, wounding stress has been reported to induce jasmonic acid signaling rather than SA signaling (Leon et al., 2001). On one hand, rgs-CaM was also shown to be induced by infection with various viruses (Anandalakshmi et al., 2000; Li et al., 2014; Nakahara et al., 2012) and thus rgs-CaM may be involved in SA signaling in response to virus infection. I tested this possibility with PVX because rgs-CaM was induced by PVX infection (Nakahara et al., 2012) and facilitated multiplication of PVX via its endogenous RSS activity in

infected *Nicotiana benthamiana* plants (Anandalskshmi et al., 2000).

Compared with the GFP signals in leaves of wild-type tobacco plants inoculated with PVX that expressed GFP, as consist with the previous study (Anandalakshmi et al., 2000), reduced GFP signals were observed in the T1 generation of three out of five kanamycin-resistant lines of transgenic tobacco plants (Figure 1-4A) in which rgs-CaM was suppressed by RNA silencing (Nakahara et al., 2012). I used one of the three lines (IR-rgs-CaM1) as a source of rgs-CaM–knockdown tobacco plants for further experiments because the other lines were infertile. When rgs-CaM–knockdown tobacco plants, in which rgs-CaM was suppressed by an inverted repeat (IR) transgene (Nakahara et al., 2012), were inoculated with PVX without a GFP tag, the levels of cDNA from PVX RNAs (CP), including both genomic and subgenomic ones, and PVX genomic RNA (RdRp) accumulated slightly more in inoculated leaves of the rgs-CaM–knockdown plants, but a statistically significant difference was detected only for RdRp cDNA, corresponding to PVX genomic RNA (Figure 1-4B). In non-inoculated upper leaves, PVX RNAs appeared to accumulate more in the rgs-CaM–knockdown plants than in wild-type plants, but the difference was not statistically significant. I then examined whether SA signaling was induced in these plants by examining the mRNA level of *PR1a*. The *PR1a* mRNA level increased slightly but significantly in non-inoculated upper leaves of wild-type tobacco plants (Figure 1-4B). Similar results were obtained in the rgs-CaM–knockdown plants but the differences with the wild-type plants were not significant. The results suggest that, even if rgs-CaM is involved in defense and induction of SA signaling against PVX infection, its contribution is minimal.

When cucumber mosaic virus (CMV) was inoculated into wild-type and rgs-CaM–

knockdown tobacco plants, *PR1a* was strongly induced in inoculated leaves of wild-type plants but not in those of *rgs*-CaM-knock down plants (Figure 1-5), suggested the possibility that endogenous *rgs*-CaM is also involved in induction of SA signaling. This pattern was dependent on the viral RSS, the 2b protein of CMV because CMV without 2b did not show any differences of infection efficiency between WT plants and *rgs*-CaM knock-downed transgenic lines (Figure 1-6). Although, the same experimental methods were not same, it is better to use both northern to compare the results. This almost unchanged pattern of them was same both inoculated leaves and upper leaves implying that 2b protein was a target of the *rgs*-CaM dependent immunity.

Since mechanical inoculation caused wounding and thus Ca^{2+} influx on the epidermal cells of inoculated leaves, I suspected the relationship between SA signaling induced in leaves that were inoculated with CMV and wounding (Ca^{2+} inflow). If wounding and Ca^{2+} inflow are required for induction of SA signaling, wounding or Ca^{2+} inflow could induces SA signaling in tobacco leaves in the presence of CMV 2b without CMV infection. To test this hypothesis, I tested wounding effect on transgenic tobacco plants expressing viral RSS, 2b or clover yellow vein virus HC-Pro. As expected, the *PR1a* mRNA level increased in these leaves of transgenic lines 24h after wounding (Figure 1-7). Calcium ionophore (A23187) causes Ca^{2+} inflow into cytoplasm by increasing penetrability of Ca^{2+} through cell membrane. It was often used to induce Ca^{2+} inflow in living cells (Lee et al., 2009, Heo et al., 1999). Similarly, induction of Ca^{2+} inflow with ionophore A23187 also induced *PR1a* expression in transgenic lines (Figure 1-8). These results suggest that tobacco plants induced SA signaling in response to CMV infection via perception of Ca^{2+} and 2b. Since *rgs*-CaM binds to both Ca^{2+} and 2b, I wondered whether it is involved in the perception of them to induce SA signaling.

If rgs-CaM perceives 2b and Ca²⁺ influx for induction of SA signaling, expression of *PR1a* would be changed in transgenic lines, in which the rgs-CaM expression is suppressed. To confirm the involvement of rgs-CaM, the expression of rgs-CaM was suppressed by virus-induced gene silencing (VIGS) of rgs-CaM using the PVX vector. *PR1a* was induced in transgenic tobacco plants constitutively expressing viral RSSs, 2b of CMV and HC-Pro of CIYVV when the PVX empty vector was inoculated into these plants with/without Ca²⁺ influx (Figure 1-9A). Additionally, SA signal was induced by Ca²⁺ influx in PVX-infected WT plants, suggesting that the presence of two out of three: RSSs, Ca²⁺ influx and PVX infection, is necessary and sufficient for SA signaling. VIGS of rgs-CaM compromised the inductions of SA signaling by combinations of either RSS and PVX infection or Ca²⁺ influx and PVX infection, indicating SA signaling by these combinations depends on rgs-CaM (Figure 1-9A).

In tobacco plants 5 weeks after sowing, expression level of *PR1a* increased in rgs-CaM-knockdown tobacco plants by A23187 treatment but not in wild-type tobacco plants (Figure 1-10B, left panel). This result suggested that rgs-CaM have negative role in SA signals in response to Ca²⁺ influx because Ca²⁺ influx provoked SA signals. This result was not coincident with data presented above for the rgs-CaM knockdown transgenic line; Induction of SA signaling by CMV infection was compromised in rgs-CaM-knockdown plants. Since sampling age was only different point between each experiment, I performed a same experiment with older tobacco plants 10 weeks after sowing. Interestingly, reverse phenotype was observed and thus this result suggested that rgs-CaM is required for SA induction which was turned on by Ca²⁺ influx (Figure 1-10B, right panel). These results suggested that the function of rgs-CaM in SA induction was changed according to age. Moreover, I prepared wild type and

knockdown rgs-CaM tobacco planting on big and small cup at the same period after seedling. As a result, rgs-CaM-knockdown tobacco induced *PR1a* in tobacco plants in a big cup 24 h after treatment with ionophore A23187 (Figure 1-11, left). On the contrary, in small cups *PR1a* was induced in wild-type tobacco plants with Ca^{2+} influx (Figure 1-11, right). These data suggested that rgs-CaM could work negatively or positively for SA signaling in response to Ca^{2+} influx and aging and growth conditions of plants oppositely change the rgs-CaM function in SA signaling. In aged plants or plants grown in small cup (small nutrition), SA signaling is induced only by Ca^{2+} influx without viral infection.

In NahG transgenic lines, PVX infection with Ca^{2+} could not induce *PR1a* expression, meaning that this pathway was entirely dependent on SA (Figure 1-9B) because SA is digested by the NahG protein. I then inoculated CMV- Δ 2b, which lacks the RSS 2b gene. It did not induce *PR1a*. But, *PR1a* was induced in infected leaves treated with the Ca^{2+} influx. These results confirmed that the SA signal induction does not necessarily require viral RSS under artificial Ca^{2+} influx by ionophore A23187 and imply another unknown infection cue to induce *PR1a*.

3. rgs-CaM was not involved in enhanced resistance against PVX and CMV Δ 2b infection

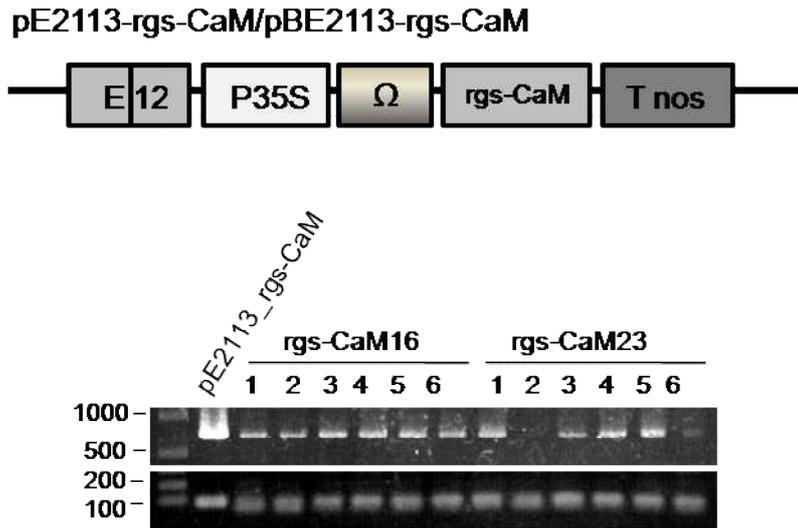
rgs-CaM has previously been shown to be involved in enhanced resistance against CMV-Y in systemic acquired resistance (SAR)-induced plants (Tadamura, 2014). When I artificially induced SA signal with BTH treatment, putative downstream cascade of the rgs-CaM immunity, the pattern of PVX infection was similar in tobacco plants with/without rgs-CaM (Figure 1-12). Then, this result was confirmed using the PVX

vector inducing VIGS against rgs-CaM. In wild-type tobacco plants inoculated with the empty PVX vector, the PVX CP accumulation reduced by BTH treatment, indicating enhanced resistance against PVX in SAR-induced tobacco plants. Similar results were obtained in plants inoculated with the PVX-VIGS (rgs-CaM) though PVX CP accumulated to the lesser extent in leaves, in which SAR was not induced compared with those inoculated with the PVX empty vector (Figure 1-13). When CMV Δ 2b was inoculated onto wild-type and rgs-CaM-knockdown tobacco plants, CMV infection was strongly inhibited in both wild-type and rgs-CaM-knockdown tobacco plants (Figure 1-6), suggesting that rgs-CaM hardly contribute to enhanced resistance against PVX and CMV Δ 2b probably because PVX and CMV Δ 2b did not have an RSS that was a target of rgs-CaM such as CMV 2b.

4. rgs-CaM is necessary for resistance against CMV in aged tobacco plants

In addition to the induction of SA signaling, I found that rgs-CaM is involved in SA-mediated antiviral defense. When CMV was inoculated into relatively old tobacco plants as shown Figure 1-14 (7 weeks after seeding), the rgs-CaM-knockdown plants developed systemic yellowing of leaves earlier than did the inoculated wild-type plants. Accumulation of CMV CP was only detected in inoculated IR-rgs-CaM tobacco at 16dpi (Figure 1-14B).

A



B

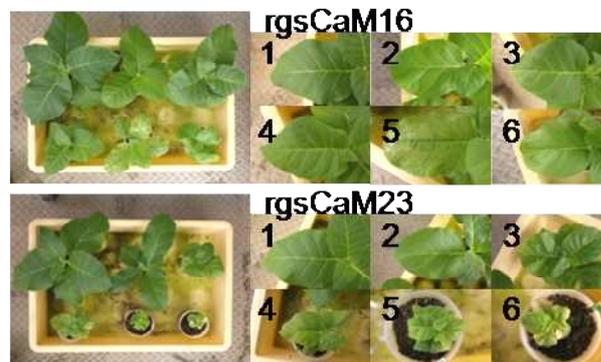


Figure 1-1 Transgenic tobacco plants overexpressing rgs-CaM elicits immune responses in tobacco. (A) This diagram of the constructs made based on Mitsuhashi et al., the expression vectors (1996). These individual plants of transgenic lines were confirmed to have the rgs-CaM transgene by detecting the 35S and rgs-CaM nucleotide sequences by PCR. (B) Within each of these two transgenic lines, severity of the lesion mimic phenotype was variable. Individual plants from each line are shown in order from mild (1) to severe (6) phenotypes. Ω; 5'-untranslated sequence of TMV.

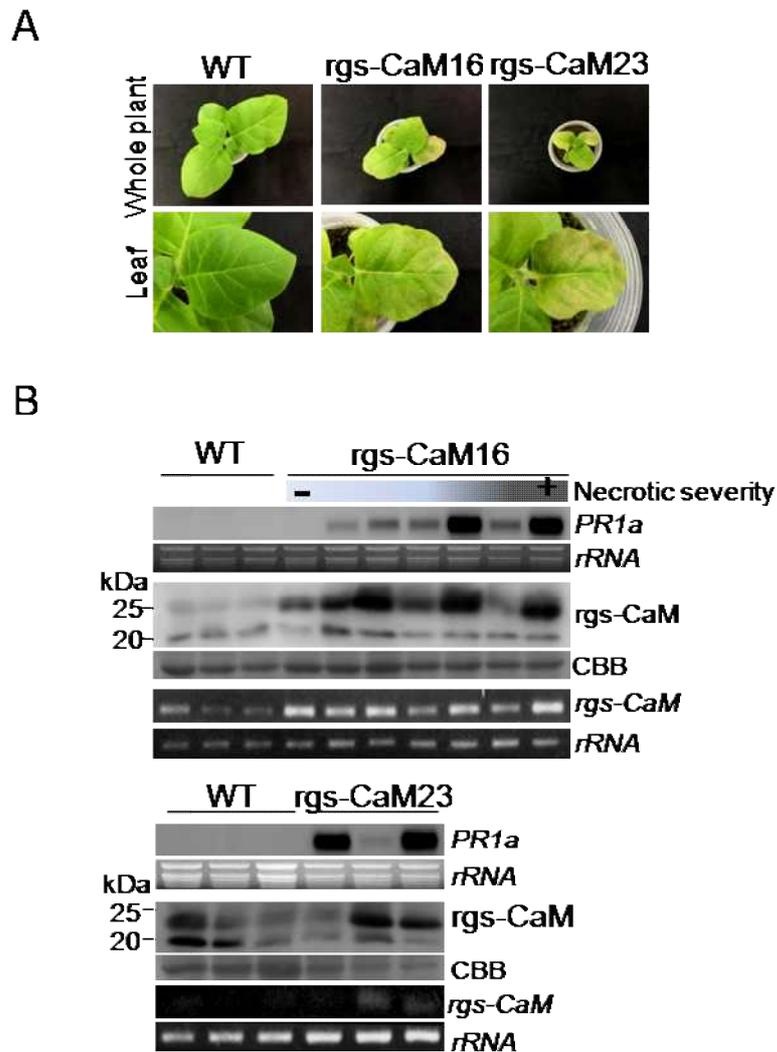
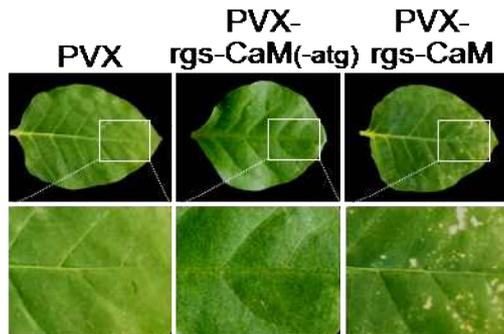


Figure 1-2 The phenotypic characteristics and defense responses shown in transgenic lines 16 and 23. (A) Transgenic tobacco plants overexpressing rgs-CaM showed phenotypic characteristics suggesting activation of immune responses, such as necrosis and dwarfing, at 7 weeks after seeding of transgenic lines 16 (rgs-CaM16) and 23 (rgs-CaM23). (B) Expression of *PR1a*, an indicator of salicylic acid signaling, was investigated by northern blotting. Samples from seven plants of transgenic line 16 were ordered from left to right in increasing severity of the phenotype. The *PR1a* mRNA level was investigated by northern blotting. Overexpression of rgs-CaM in these plants

was confirmed by western blotting for its protein and semi-quantitative RT-PCR for its mRNA. Wild-type (WT) tobacco was used as a control. Transgenic line 23 (the bottom panel), which overexpressed rgs-CaM and showed a similar phenotype to line 16, was also shown to induce *PR1a* expression by northern blotting; as in the case of line 16, its expression level varied within the line. Coomassie brilliant blue–stained (CBB) and ethidium bromide–stained (*rRNA*) gels are shown as loading controls.

A



B

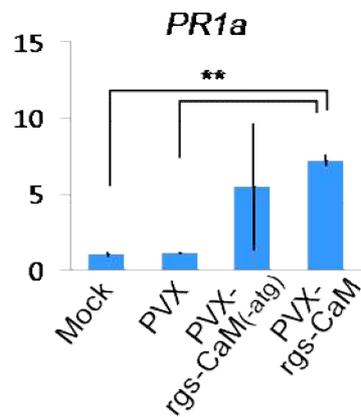


Figure 1-3 Necrotic symptom or cell death and SA signaling were induced by transient expression of rgs-CaM. (A) PVX vector expressing rgs-CaM (PVX-rgs-CaM), a PVX vector expressing the subgenomic RNA containing the rgs-CaM open reading frame without its initiation codon [PVX-rgs-CaM(-atg)], and an empty vector (PVX) were inoculated into wild-type tobacco (cv. Xanthi) plants. Inoculated leaves at 7 days post-inoculation are shown. (B) Their *PR1a* expression was investigated by real-time PCR. The mRNA levels relative to that of mock-inoculated plants are shown in the bar graph (n = 4). Error bars indicate SE. Student's t test was applied to the data and ** indicates P value of <0.01.

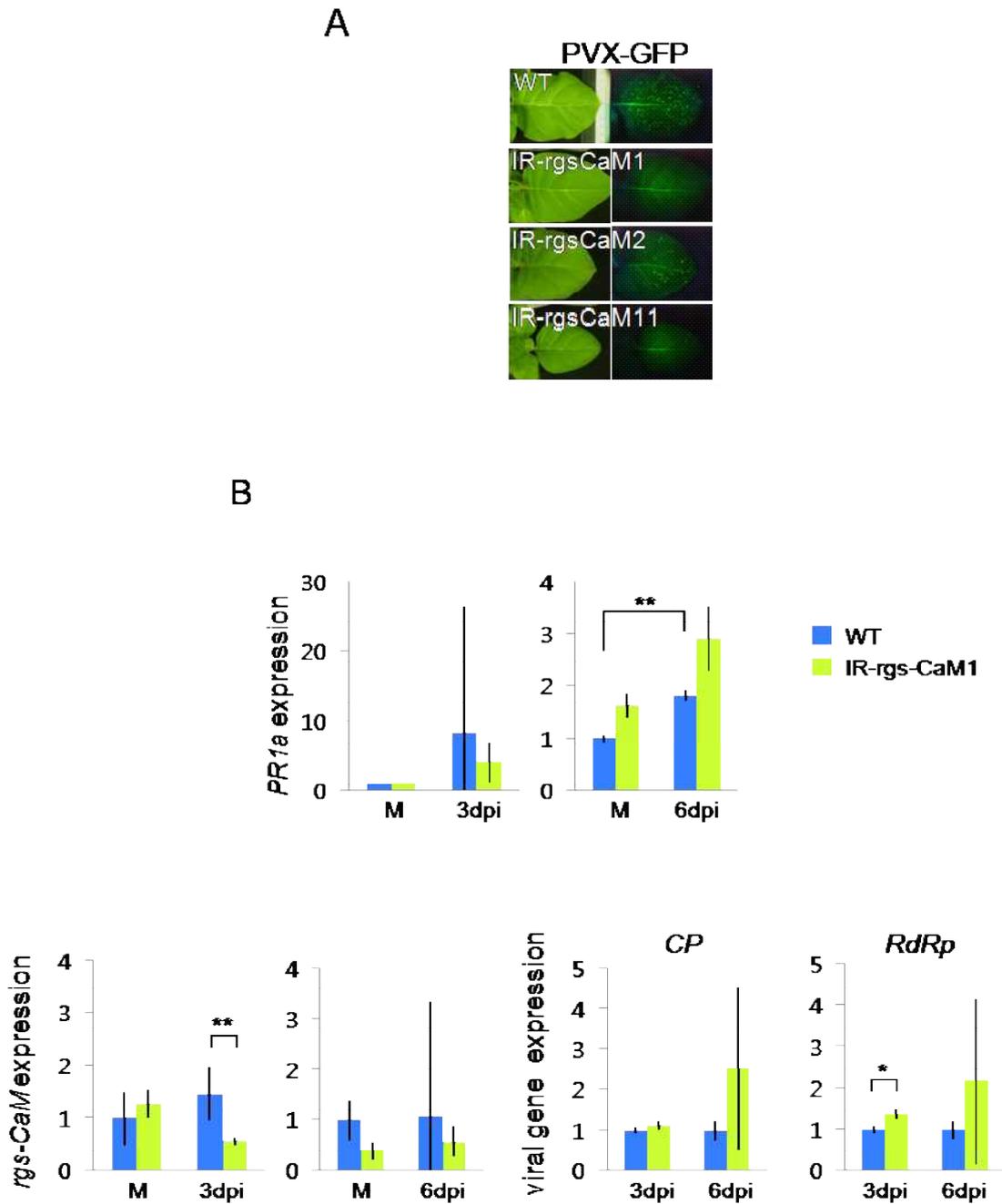


Figure 1-4 Susceptibility of rgs-CaM–knockdown tobacco plants to PVX, and SA signaling in response to PVX infection. (A) T1 or T2 progeny of rgs-CaM–knockdown tobacco plants (IR-rgs-CaM) were inoculated with PVX expressing GFP (PVX-GFP). The GFP signals on inoculated leaves are shown as 4dpi. (B) The levels of

PR1a and rgs-CaM mRNA were investigated by real-time PCR (n = 5). mRNA levels relative to those of mock-inoculated plants are shown. The same type of inoculation as in (A) was done with more individual plants (n = 8). Accumulation of PVX genomic RNA was measured by real-time PCR using a pair of primers for amplification of a partial cDNA sequence of viral RNA-dependent RNA polymerase (RdRp). Similarly, accumulation of PVX RNAs including both genomic and subgenomic RNAs was measured with a pair of primers for amplification of a partial cDNA of viral coat protein (CP). M, mock treatment. Bars indicate SE. Student's t test was applied to the data and * indicates P values of <0.05.

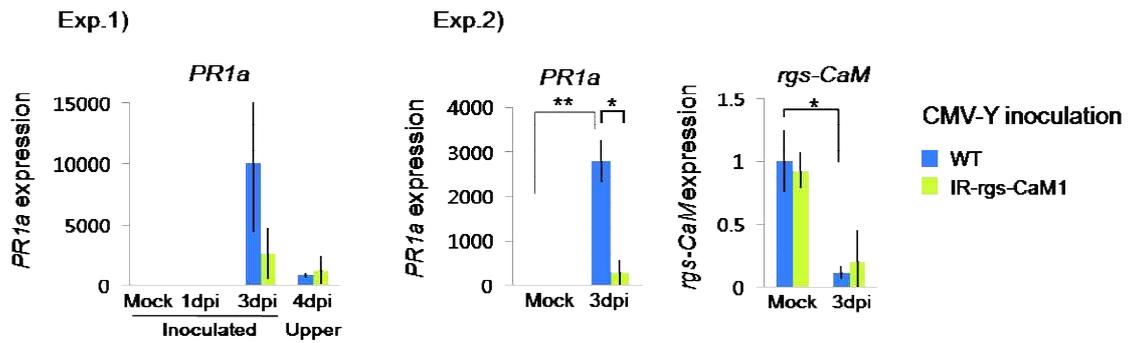


Figure 1-5 Implication of *rgs-CaM* involvement in SA signaling in response to infection by CMV. CMV-Y was inoculated into wild-type (WT) and *rgs-CaM*-knockdown (IR-*rgs-CaM*) tobacco plants. The *PR1a* and *rgs-CaM* mRNAs were investigated (n = 3) as done in (exp.1) and the same type of inoculation (exp.2) was done with more individual plants (n = 9) and investigated the *PR1a* mRNA level. Error bars indicate SE. Student's t test was applied to the data and * and ** indicate P value of <0.05 and <0.01, respectively. These results of exp.1 were of Tadamura's thesis of master (2014).

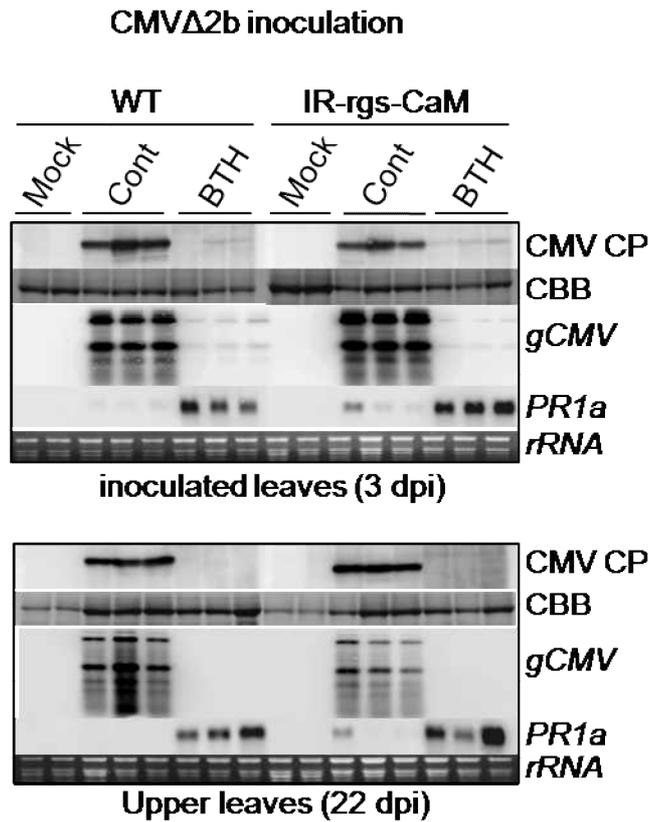
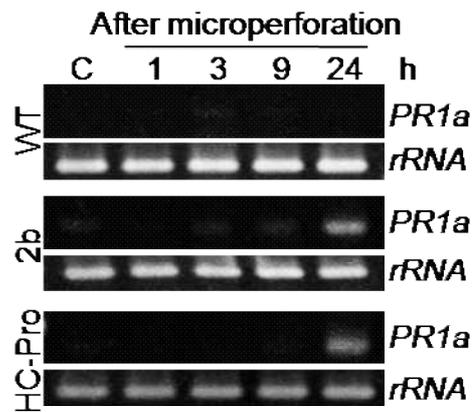


Figure 1-6 Infectivity of CMV Δ 2b in SAR-induced tobacco Five days after SAR induction by treatment with benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH), WT and IR-rgs-CaM tobacco plants were inoculated with CMV Δ 2b. Control plants (Cont) were treated with a solution containing 1.4% (vol/vol) acetone and 0.2% Tween-20 (the solution used to dissolve BTH). CMV CP was detected by western blotting. CMV genomic and subgenomic RNAs (*gCMV* and *sgCMV*, respectively) and the *PR1a* mRNA were detected by northern blotting. Coomassie brilliant blue-stained (CBB) and ethidium bromide-stained gels are shown as loading controls.

A



B

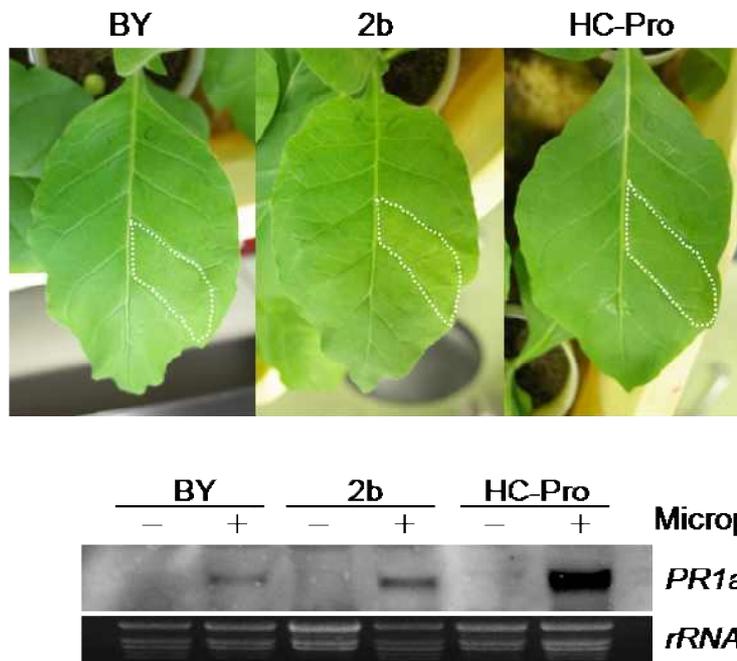


Figure 1-7 *PR1a* expression was dependent on wounding treatment by microperforation (A) Expression of *PR1a* was investigated by RT-PCR at different time points after microperforation of tobacco leaves. (B) More than hundred needles stamp induced microperforation for wounding treatment in WT and tobaccos expressing viral protein 2b and HC-Pro. Northern blot analysis was performed to detect *PR1a* expression using extraction from region of microperforation treated after 24 hours.

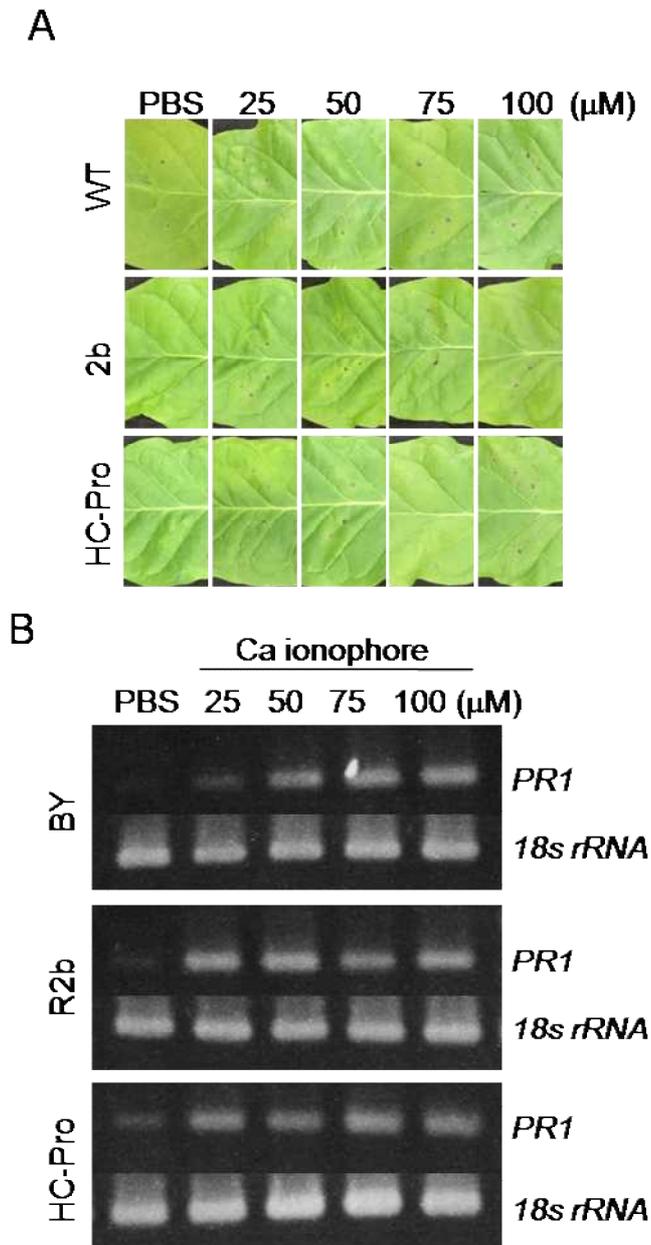


Figure 1-8 Induction of SA signaling in viral RNA silencing suppressor (RSS)-expressing tobacco plants with Ca^{2+} influx. (A) Tobacco leaves were infiltrated with A23187. A23187 was dissolved in PBS at the indicated concentrations and used to infiltrate wild-type (WT) and transgenic tobacco expressing RNA silencing suppressors, CMV 2b and CIYVV HC-Pro. Photographs were taken 24 h after infiltration with

A23187. (B) *PR1a* expression was detected in RNA extracts from region of leaves (A) which were infiltrated with A23187 by RT-PCR.

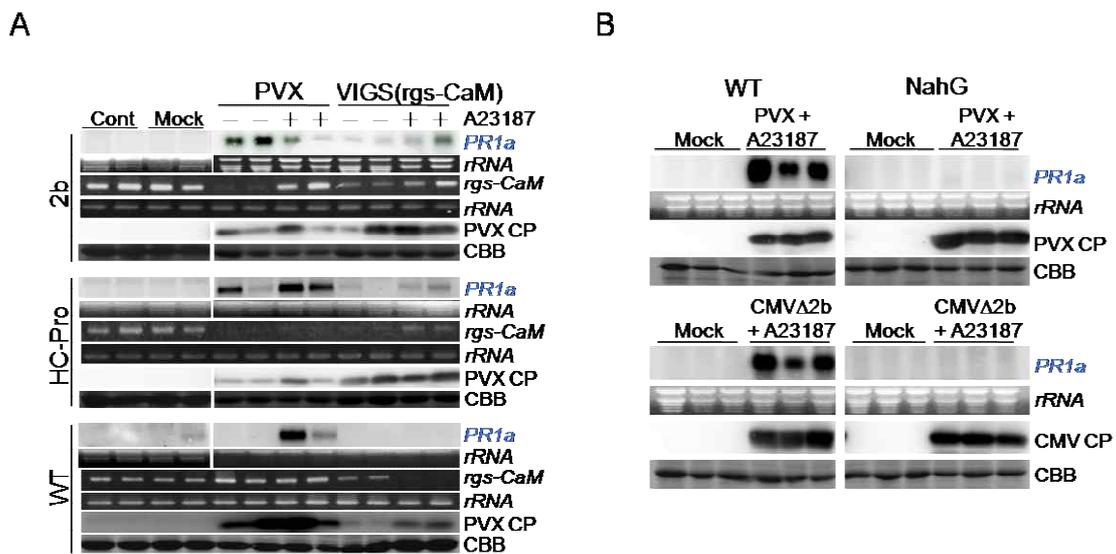


Figure 1-9 PR1a induction depends on rgs-CaM. (A) Wild-type (WT) and transgenic tobacco expressing RNA silencing suppressors, CMV 2b and CIYVV HC-Pro, were inoculated with a PVX empty vector (PVX) and a PVX vector expressing the rgs-CaM ORF sequence lacking the initiation codon as a means of inducing VIGS of rgs-CaM [VIGS(rgs-CaM)]. These inoculated leaves were infiltrated with A23187 (+) or buffer alone (-), 3 days after inoculation with PVX. The levels of *PR1a* mRNA, PVX CP, and rgs-CaM mRNA were investigated by northern blotting, western blotting, and semi-quantitative RT-PCR, respectively, 24 h after infiltration with A23187. Samples were also prepared from plants that were inoculated with buffer but not infiltrated (Mock) and those that were neither inoculated nor infiltrated (Cont). (B) WT and the transgenic tobacco plants expressing salicylate hydroxylase (NahG), which antagonizes salicylic acid signaling, were inoculated with PVX and CMVΔ2b and infiltrated with A23187 at 3 days after inoculation. The levels of *PR1a* mRNA and viral CPs were investigated by

northern and western blotting, respectively, 24 h after infiltration with A23187. Samples were also prepared from buffer-inoculated plants without infiltration (Mock). Coomassie brilliant blue–stained (CBB) and ethidium bromide–stained (rRNA) gels are shown as loading controls.

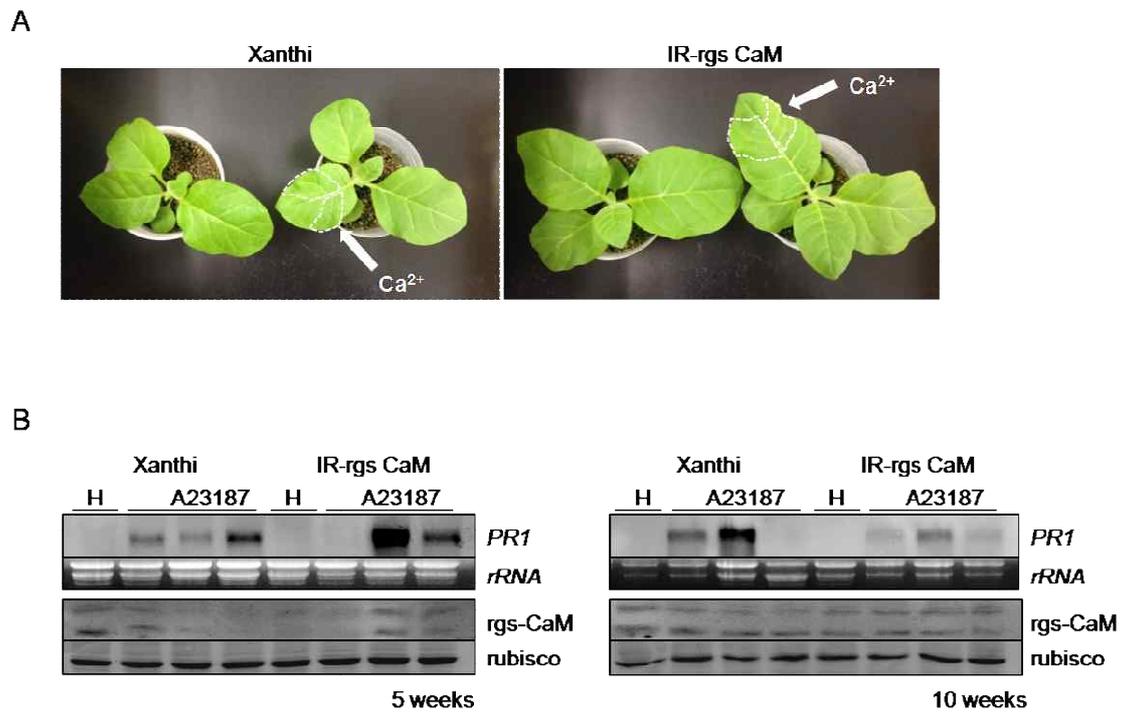


Figure 1-10 Induction of SA signaling has a phase change aged tobacco plants (A) Tobacco leaves were infiltrated with 75 μ M A23187 under the young aged (5-weeks) condition. (B) After 24 hours of A23187 infiltration, total RNA and protein were extracted from the leaves that were under the condition young (5-week) or aging (10-week) plants for northern and western blot assays, respectively.

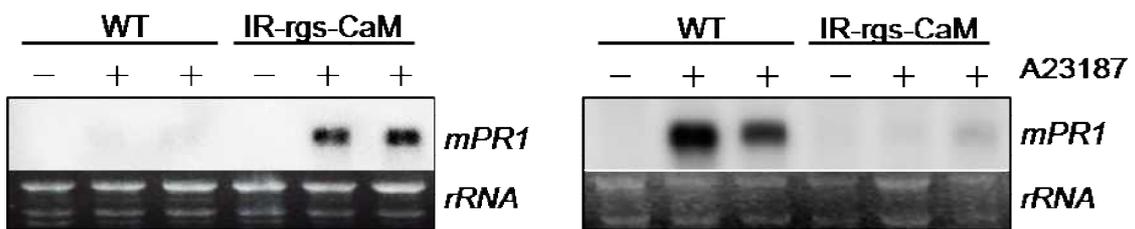


Figure 1-11 Induction of SA signaling was changed in different nutritive conditioned tobacco plants. Tobacco plants were planted and separated into big (left panel) and small cups (right panel) adjusted nutritive condition. Then, *PR1a* induction was analyzed by northern blot.

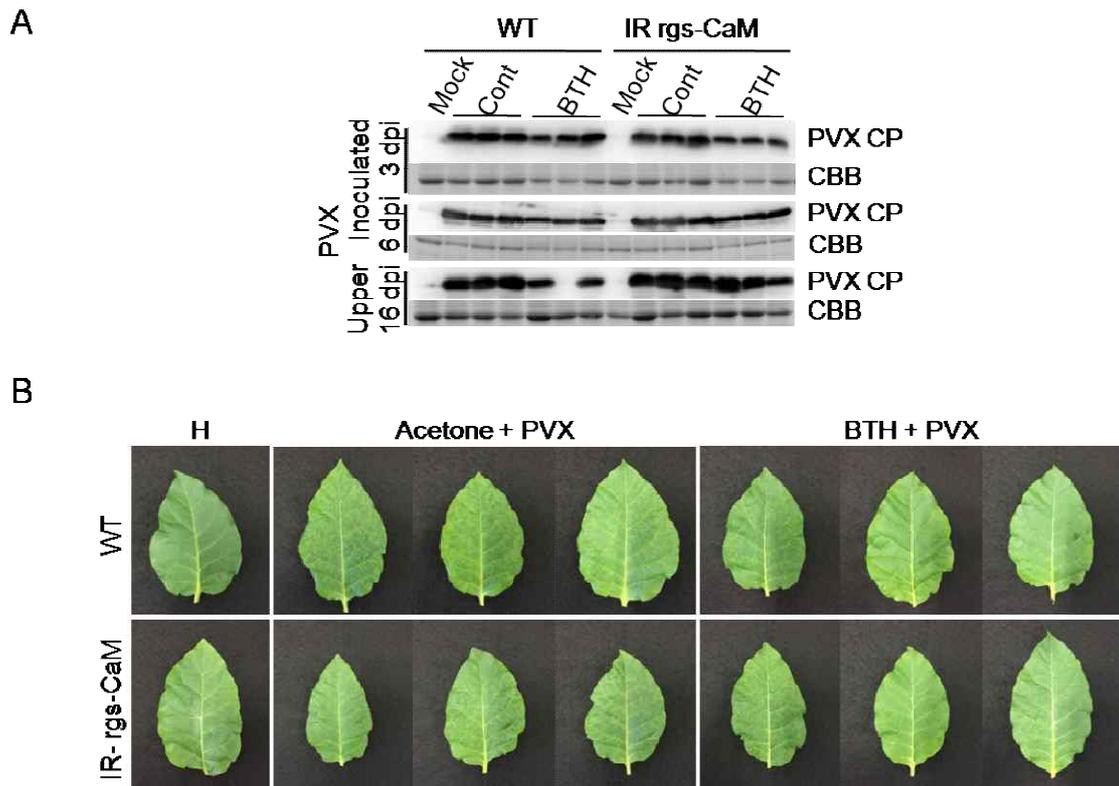


Figure 1-12 Infectivity of PVX in SAR-induced tobacco plants (A) PVX CP accumulation in plants inoculated with PVX at 5 days after BTH treatment. Accumulation of PVX CP was detected in inoculated and non-inoculated upper leaves by western blotting. CBB-stained gels are shown as loading controls. Control samples were prepared from buffer-inoculated plants (Mock). (B) Symptoms on upper leaves were photographed at sixteen days after inoculation.

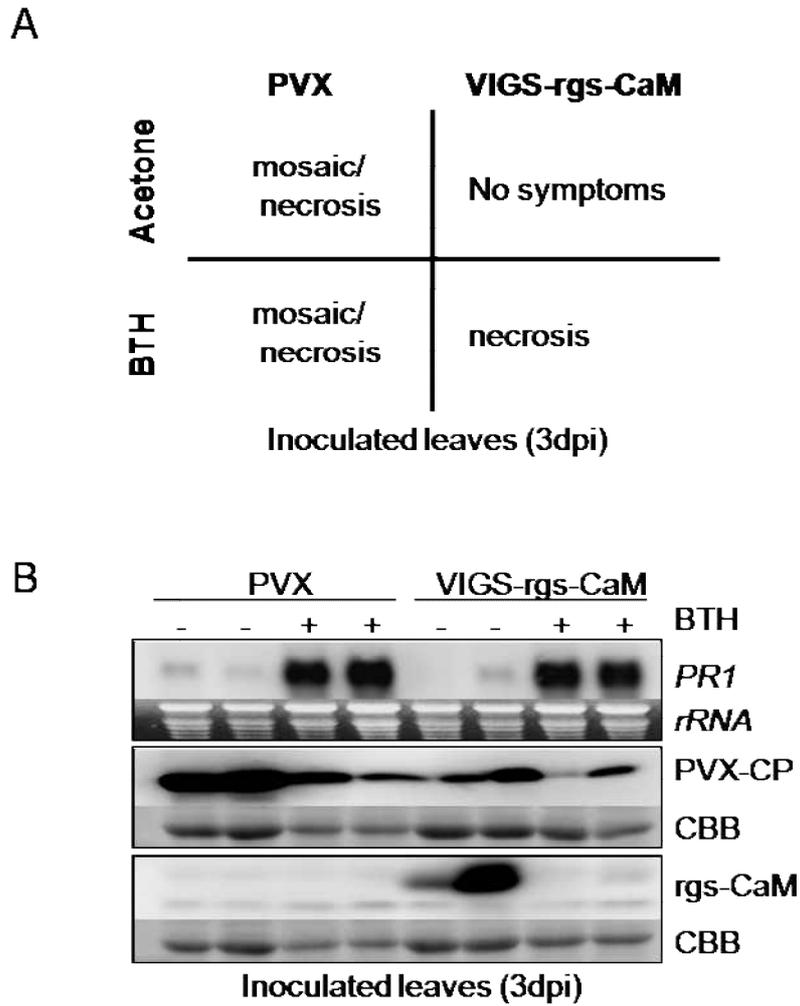


Figure 1-13 The change of symptoms in SAR-induced and rgs-CaM-silenced plants inoculated with PVX (A) The symptoms on leaves inoculated with PVX. These plants were pretreated with BTH to induce SAR or buffer (Acetone). (B) PVX CP and rgs-CaM accumulation in plants inoculated with PVX 5 days after BTH treatment were detected by western blotting. CBB-stained gels are shown as loading controls. PR1a expression was analyzed by RNA hybridization. Both of these blotting techniques were performed with inoculated and systemic leaves at 3dpi.

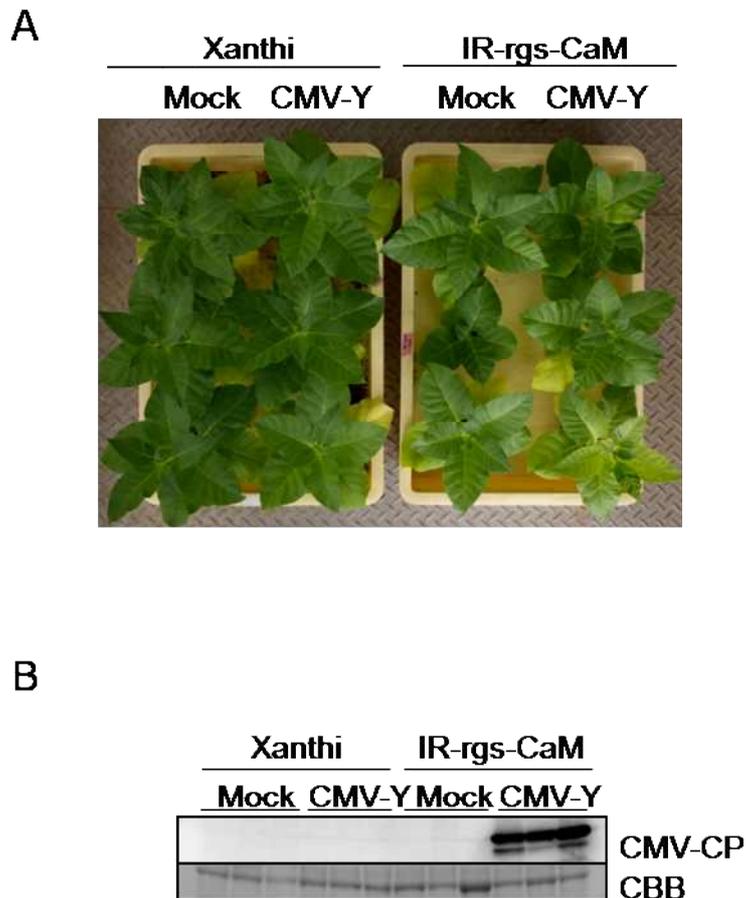


Figure 1-14 Enhanced resistance against CMV-Y depends on rgs-CaM in aged tobacco plants (A) Comparison of symptoms (yellowing) on non-inoculated upper leaves of tobacco plants inoculated with CMV-Y. CMV-Y was inoculated into wild-type (WT) and rgs-CaM–knockdown (IR-rgs-CaM) tobacco plants 7 weeks after sowing. The photograph was taken at 16 days post inoculation (dpi) with CMV-Y. All of the rgs-CaM–knockdown tobacco plants that were inoculated with CMV-Y developed systemic symptoms on their leaves, but wild-type tobacco plants did not express symptoms. (B) The difference in susceptibility between wild-type and rgs-CaM–knockdown plants was confirmed by detecting CMV CP in non-inoculated upper leaves of these plants by western blotting.

Discussion

The Ca^{2+} and calmodulin pathway is known to trigger SA signaling (Du et al., 2009). SA signaling is one of the major antiviral defense mechanisms, which is involved in systemic signaling after recognition of pathogens, resulting in long-lasting immunity in the whole plant body (An and Mou, 2011). Therefore, artificial activation of SA-mediated defense mechanisms may be useful to confer antiviral resistance on diverse crops because available resources of the plants that are resistant to viruses are limited. However, constitutive activation of plant immune systems results in inhibition of plant growth according to its trade-off relationship with growth (Huot et al., 2014). Consistent with this trade-off, *rgs-CaM*-overexpressed plants showed growth retardation and ectopic necrosis (Figure 1-1). There were positive relationships among phenotypic severity, *rgs-CaM* overexpression, and *PR1a* gene expression, which support the trade-off and suggest the link of the signaling derived from *rgs-CaM* with SA (Figure 1-1, 1-3).

rgs-CaM has been known as a binding partner to exogenous proteins, diverse viral RSSs (including potyvirus HC-Pro, CMV, and TAV 2b and human immune deficiency virus TAT), presumably via affinity to their positively charged dsRNA binding sites (Anandalakshmi et al., 2000; Nakahara et al., 2012), even though there is no conserved amino acid sequence motif among these dsRNA binding domains. Infection efficiencies of CMV-N2b increased in *rgs-CaM* knock-downed condition but CMV Δ 2b did not, which imply the contribution of the interaction of *rgs-CaM* with viral RSSs to antiviral defense. That is, *rgs-CaM* appears to effectively inhibit CMV infection through its interaction with CMV 2b. On the other hand, as the case of PVX that does not have an

RSS that interacts with rgs-CaM, the rgs-CaM-knockdown did not affect PVX infection (Figure 1-12). These results suggest that CaMs and/or CMLs are hub proteins for binding to various substrate proteins by their relatively disordered binding sites (Patil and Nakamura, 2006). Homology modeling of rgs-CaM (Makiyama et al., 2016; Nakahara et al., 2012) implies that rgs-CaM has a negatively charged disordered binding site for substrates. This site probably allows rgs-CaM to bind to diverse viral RSSs via affinity to positively charged dsRNA binding domains.

rgs-CaM perceives not only viral RSS but also Ca^{2+} cues that induce salicylic acid signaling. Inducing Ca^{2+} influx provokes SA signaling but this signal is attenuated in rgs-CaM knock-downed plants. Thus, *PR1a* expression triggered by Ca^{2+} requires functional rgs-CaM (Figure 1-8). A recent structural and thermodynamic study revealed that rgs-CaM actually binds to Ca^{2+} at three EF-hand motifs (Makiyama et al., 2016). Moreover, Makiyama et al. (2016) suggested that Ca^{2+} binding at the two EF-hands that show higher affinity to Ca^{2+} alters the conformation of rgs-CaM so that the negatively charged binding sites are more exposed, supporting the rgs-CaM dual perception of viral RSS and Ca^{2+} . Thus, rgs-CaM activated by Ca^{2+} recognizes RSS well and rgs-CaM bound RSS can be more sensitive to calcium vice versa (Figure 1-9). Because plant cells are surrounded by a cell wall, virus invasion seems to require mechanical wounding or feeding by vector organisms, which would cause Ca^{2+} influx in the virus-invaded cells. In general, defense responses against various abiotic and biotic stress responses involve Ca^{2+} fluxes (Lecourieux et al., 2006), and virus infection is known to lead to an increase of the cytoplasmic Ca^{2+} concentration (Zhou et al., 2009). Therefore, the dual perception of a viral component and Ca^{2+} seems suitable for reinforcing recognition of viral infection and induction of immune responses. Moreover, as rgs-CaM can be

activated by either Ca^{2+} influx and substrate protein binding, this mechanism may be able to supplement disease perception as a bypass. For example, the rgs-CaM pathway can be turned on by pathogenic attacks accompanying with Ca^{2+} influx even if the pathogens do not have any factors that interact with rgs-CaM.

The rgs-CaM-mediated immune responses do not appear to prevent primary virus infection; rather, they participate in SAR induction and thus prevent subsequent infection by viruses possessing RSSs that interact with rgs-CaM. First, the induction of rgs-CaM-mediated SA signaling after wounding of transgenic plants expressing viral RSSs takes 24 hr (Figure 1-7), which is slower than that seen with ETI (HR) (Tsuda et al., 2013). Second, efficiency of CMV-Y infection was not changed in inoculated leaves (Tadamura, 2014) but CMV-Y spread to upper leaves earlier in rgs-CaM knock-downed lines (Figure 1-14). Therefore, the rgs-CaM-induced immune responses do not necessarily need to be induced immediately but is required for preventing further infection and inducing SAR.

Another significant finding of this study is uncovering reverse response of defense depending on internal status like aging and nutrient. In younger plants, rgs-CaM acted as a repressor of SA response triggered by Ca^{2+} influx. However, rgs-CaM was required for induction of SA signaling in older plants under the same treatment. Similarly, in rich environment, rgs-CaM was a repressor of SA response but it was the sine qua non for PR1a expression in poor environment. These phenomena may reflect some trade-off between plant defense and cell maintenance. For example, healthy plant is lesser need of SA-dependent mechanism for defense or exhibits fast turn-over of SA. Further studies will be necessary to elusive these opposite reaction by rgs-CaM. Since there are

various repertoires of CaMs and CMLs and have been reported to be Ca^{2+} sensors that play important roles in various development processes and abiotic and biotic stress responses in plants (Bender and Snedden, 2013; Cheval et al., 2013), it will be worth examining whether there are CaMs and CMLs that oppositely function each other.

Furthermore, trade-off between immunity and growth in plants has driven the evolution of immune receptors for recognition of pathogen invasion that effectively induce defense mechanisms only when needed. This study revealed that a novel class of protein, calmodulin-like protein rgs-CaM, functions as an immune receptor for CMV infection and induces salicylic acid signaling, which is characteristic of immune responses against biotrophic pathogens, including viruses (Palukaitis and Carr, 2008), and is required for SAR induction (Delaney et al., 1994; Fu and Dong, 2013).

SA signaling is one of the major antiviral defense signaling. rgs-CaM knock down tobacco plants were more susceptible to CMV than wild-type tobacco plants. rgs-CaM seems to effectively inhibit CMV infection through 2b recognition and degradation by rgs-CaM, leading to reinforcement of antiviral RNA silencing and other SA-mediated antiviral responses.

CHAPTER II

Virus infection depending on autophagy gene, beclin1

Introduction

It has become common that autophagy is not only induced through nourishment depletion but also other factors such as pathogen infection, aging in the cell. Moreover, recent researches indicated that autophagy is induced by ROS and regulates hypersensitive response programmed cell death (HR-PCD) while plants defend against pathogens (Liu et al., 2005; Patel et al., 2008; Scherz-Shouval et al., 2010). It has been argued whether autophagy acts as a pro-survival or a pro-death process. Autophagy plays a part as an immune response regulator to degrade death signals or interfere with replication of pathogen during HR-PCD (Baehrecke, 2005; Hayward et al., 2009). Nakahara et al. (2012) reported autophagy degrades rgs-CaM and its direct binding to RSSs in virus infected plants. On the other hand, polerovirus RSS, P0, suppress RNA silencing through directing degradation of Argonaute 1 via autophagy (Derrien et al., 2012).

Beclin1 is a coiled-coil protein that is a homolog of human beclin1, yeast ATG6/VPS30, and Arabidopsis Atbeclin1 in *Nicotiana benthamiana* (Liu et al., 2005). This protein is a component of PI 3-kinase complex, which is a key regulator of autophagosome formation (Gozuacik and Kimchi, 2004). Human beclin1 can compensate for yeast atg6 mutant, indicating that the function of them may be well-conserved beyond taxonomical kingdom (Kametaka et al., 1998). Atbeclin1 and Nbbeclin1 were also autophagy-related genes and they have important roles from autophagy to HR-PCD in pathogen-defense mechanism. As expected, compromised beclin1 in *Arabidopsis* and *Nicotiana* provoked pathogen susceptibility against to *Pseudomonas* and TMV (Liu et al., 2005; Patel and Dinesh-Kumar, 2008). Since cell

death caused by HR-PCD keeps fresh tissues from spreading infection, malfunction of autophagy process should perturb plant defense mechanism.

Our laboratory had already showed that viral effector proteins, RNA silencing suppressor for antiviral RNA silencing were removed by rgs-CaM through autophagy process (Nakahara et al., 2012).

Therefore, from previous researches, it seemed like that autophagy might be a convergent point of pathogen perception and defense. To deal with infection, plant recruits variable defense mechanisms and autophagy could be involved in them. For example, autophagy was negative controller to SA-dependent cell death (Yoshimoto et al., 2009). If autophagy is main controller in plant defense system, I had a question whether autophagy, especially about beclin1 and beclin1-dependent processes, could control the infection processes of viruses.

Results

To test the roles of beclin1 in *Nicotiana tabacum*, I generated Ntbeclin1 knock-downed line individually (Figure 2-1). Among them, line #8, #20 showed highly suppressive expression patterns of beclin1 and line #3, #18 represented moderate expression patterns of it (Figure 2-2). Three lines (#2, #16, and #20) among them well grown in selective media. For infection, I used CMV-N2b strain containing with 2b protein rather than CMV-Y because CMV-N caused clear and extensive necrosis symptoms (Murota et al., 2017). I inoculated CMV-N2b strain containing with 2b protein to 5 weeks WT and beclin1 knock-downed tobacco plants. After 1 week later, I found that the symptom of CMV could be observed in un-inoculated leaves, which means that CMV were transferred to entire plants via vascular and/or apoplastic pathways. However, in beclin1 knock-downed lines, there are fewer plants appearing symptoms on upper leaves than WT (Figure 2-3). This result indicated that CMV propagation and/or movement were compromised in autophagy-deficient plants.

If beclin1 was required for efficient CMV infection, I wondered whether beclin1 and beclin1-dependent autophagy were generally required for other viral infection. I inoculated PVX to WT *N. tabacum* and beclin1 knock-downed 6 weeks after sowing. Then, I detected virus accumulation through western blot for detection of PVX coat proteins and *PR1a* expression, which is an indicator of elevation of SA signal (Figure 2-4). I found that PVXs CP accumulated in beclin1 knock-downed lines slightly increase but there were not significant changes for virus transmission and propagation in un-inoculated leaves. Therefore, deficient of beclin1 did not affect to PVX contrary to CMV.

As mentioned above, CMV 2b protein is a well-known suppressor of gene silencing. It targets AGO, small RNA producing machinery and reduces the accumulation of small RNAs of viral genes produced by plant siRNA process (Diaz-Pendon et al., 2007; González et al., 2010). Moreover, heterologous fused virus with CMV 2b accelerated infection ability, representing its virulent function (Chiba et al., 2006). Since PVX infection did not affected by abundancy of beclin1 contrary to CMV, I hypothesized that the differences were caused by interaction between CMV 2b and CMV 2b triggered immune response. When I integrated CMV 2b gene in PVX, accumulation of virus CP was not significantly changed in transgenic lines than wild type (Figure 2-5). This result suggested that CMV 2b was not related to beclin1-dependent CMV infection.

Since the inoculation tests with CMV and PVX did not show similar results, I chose alternative virus BBWV2-GFP. When I inoculated BBWV2-GFP into WT *N. tabacum* and beclin1 knock-downed plants, I observed more intensive GFP signal spots at transgenic lines than wild type (Figure 2-6). This result was coincident with CMV-N2b infection data, which suggested that beclin1 deficient was compromised about BBWV2 invading. However, when I checked virus accumulation through western blot of coat protein, they did not match to GFP signal intensities (Figure 2-7); there were not any significant differences in the CP accumulation in beclin1 deficient plants compared to WT.

Because BBWV2 infection was known to be restricted in inoculated leaves in *N. tabacum* (Atsumi et al., 2013) and I could not interpret the inconsistency between the GFP signal with BBWV2 and BBWV2 CP accumulation, I tried the same experiments in *N. benthamiana*. When I inoculated BBWV2 into 7 weeks WT *N. benthamiana* and beclin1 knock-downed plants, I observed more intensive GFP signals in transgenic lines

compared to WT (Figure 2-8). Moreover, when I checked GFP signals in un-inoculated leaves after 1 week inoculation, I found that beclin1-deficient lines showed spreads of GFP signals but WT did not. This result might support infection-prone phenotypes observed in *N. tabacum* transgenic lines but I do not have an idea behind a reason of the phenomena why accumulation of coat protein were not correlated with the GFP signals.

If beclin1-deficiency promoted infection spread rates of CMV and BBWV2, I wondered what autophagy-dependent protein degradation has a role to that. Autophagy process is activated not only by plant defense but also by aging (senescence) for reusing of materials and nutrients in old tissues (Avila-Ospina et al., 2014; Liu and Bassham, 2012; Thompson and Vierstra, 2005). I checked infection-prone phenotypes in younger knock-downed lines which were expected internal low activation of autophagy. Interestingly, when I introduced BBWV2 to 5 weeks plants, there were no differences for BBWV2 spreads between WT and transgenic plants (Figure 2-9). This data suggests that autophagy disturbs viral infection by aging of plants.

Starvation can trigger autophagy process that is required for material redistribution of entire body (Bassham et al., 2006; Liu and Bassham, 2012; Thompson and Vierstra, 2005). I prepared WT and transgenic plants with poor nutrient condition, then inoculated BBWV2. After 6 day infection, I compared infection efficiency of them (Figure 2-10). Unlike aging data, when I observed GFP signal, BBWV2 did not spread well to un-inoculated leaves of transgenic line under starvation situation (Figure 2-11). Consistent with this, wild *N. benthamiana* showed severe leaf necrotic symptoms than transgenic lines.

SA, JA, and ET are famous plant hormones for pathogen defense and programmed cell death (Hayward et al., 2009; Lam, 2004). Because autophagy was related to

programmed cell death that were known to involve these phytohormone pathways, I tested interactions of beclin1 deficiency with SA or JA. Before BBWV2 inoculation, I treated wild-type and beclin1-knockdown plants with SA and JA to turn on those signals. Due to pre-treating SA, cell death was occurred in beclin1 knock-downed plant inoculated with BBWV2 but JA treatment did not cause them (Figure 2-12 A). With or without BBWV2 inoculations was not a distinct point in this experiment. I detected BBWV2 CP by western blotting, SA treatment decreased viral protein accumulation but there was no different between WT and transgenic plants (Figure 2-12 B). Interestingly, systematic symptom such as paled green leaves was observed by SA treatment in transgenic lines. However, the same symptom was observed in wild-type plants treated with JA but not in beclin1-knockdown plants (Figure 2-13). The antagonistic relationships between SA and JA were well studied in plant defense field. This opposite pattern might be another example of it.

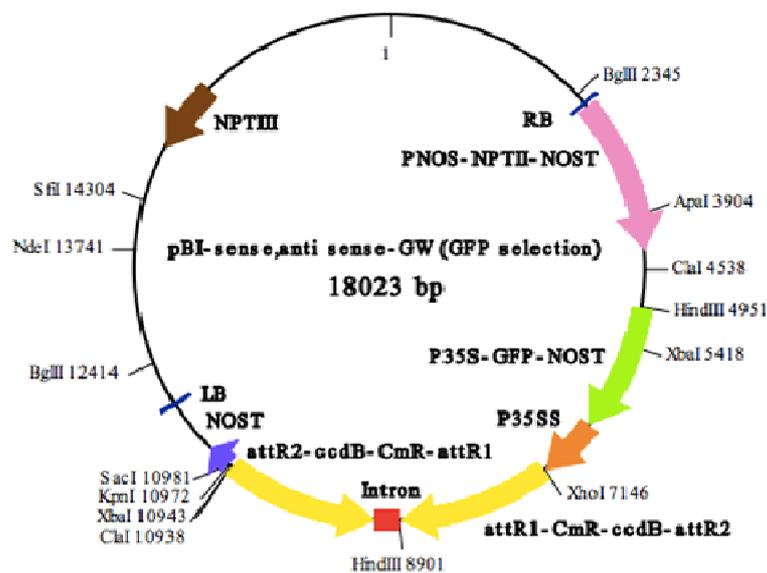


Figure 2-1 Making of transgenic tobacco plants, in which the *beclin1* gene is suppressed by RNA silencing. This map is of a binary vector that was used for generating beclin1 knock downed plants (yellow arrows were indicated as inserted gene about 600bp). Dr. Sun Hee Choi constructed the structure for beclin1 silencing, which was modified from IN3-VEC6 pBi-sense, anti sense-GW.

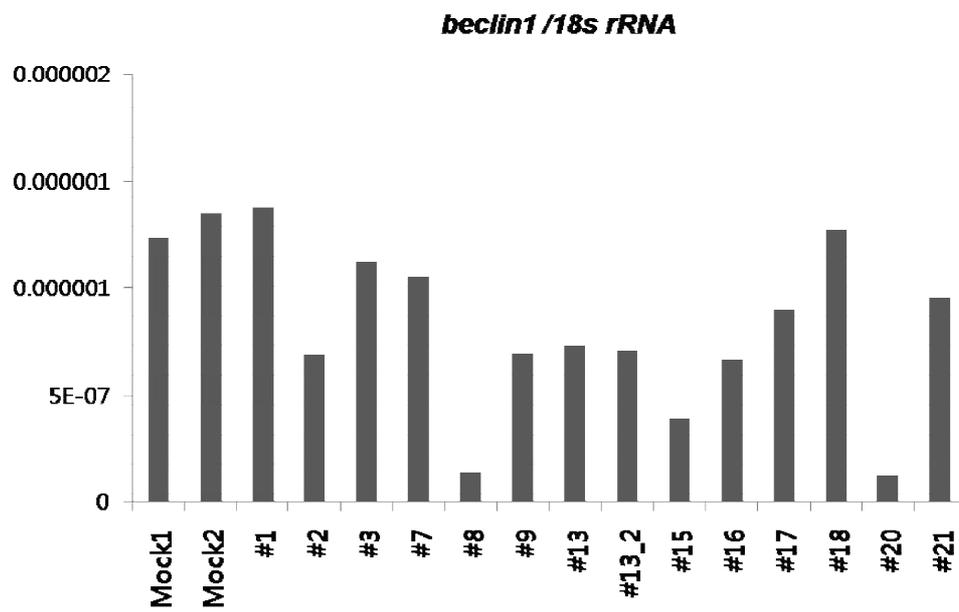
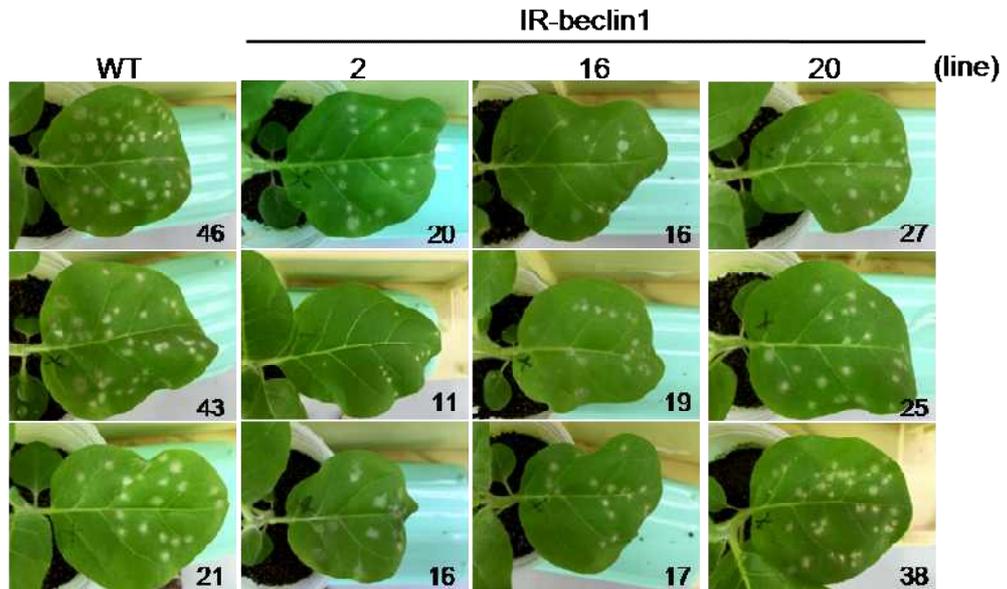


Figure 2-2 Transcriptional levels of *Beclin1* were investigated by quantitative real-time RT-PCR in wild type and in *beclin1* knockdown tobacco lines on T1.

A



B

	Plants number appearing necrotic spots on inoculated leaves	Plants number appearing necrotic spots on upper leaves
WT	12/18	12/18
2 (line)	18/19	8/19
IR-beclin1 16	16/16	8/17
20	11/18	5/18

Number of plants appearing necrotic spots on leaves / total inoculated plants

Figure 2-3 The severity of necrotic symptoms induced by CMV-N2b in WT and IR-beclin1 (A) Images for necrotic spots both of WT and IR-beclin1 on inoculated leaves at 4dpi. The number of bottom of pictures is counting the number of necrotic spots. (B) The table shows the number of plants appearing necrotic spots on inoculated leaves (4dpi) and upper leaves (18dpi). The number of plants was the sum of three times replications for each inoculation test.

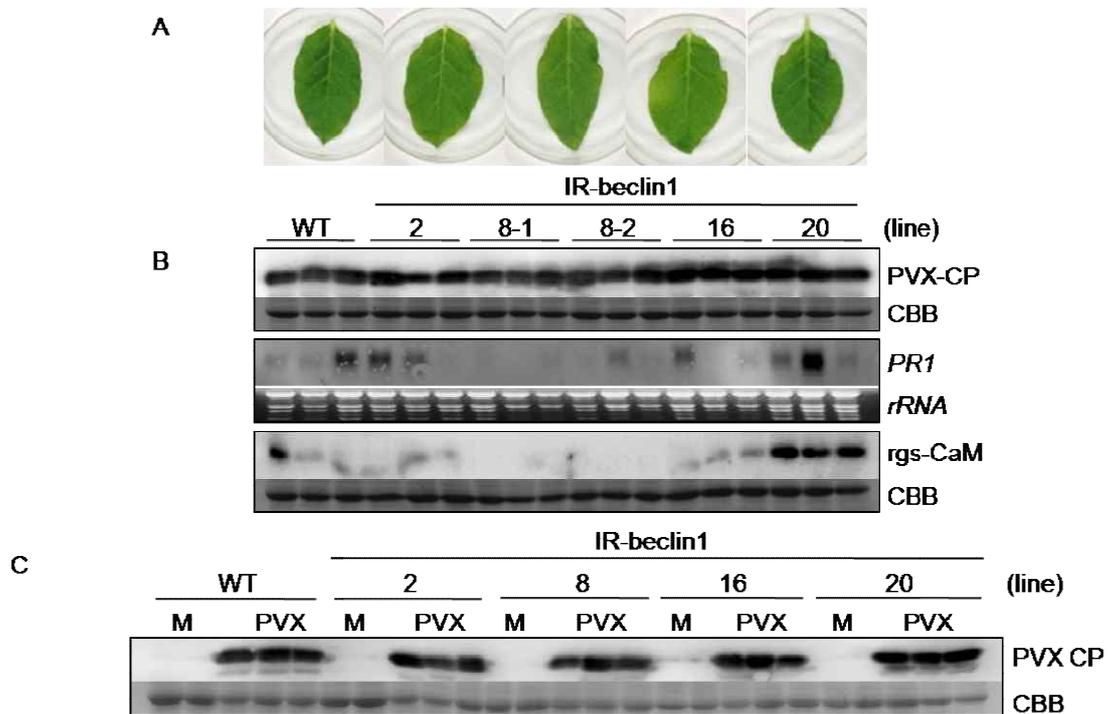


Figure 2-4 Symptoms, SA signaling and virus accumulation in WT (*xanthi-nc*) and *beclin1* knock down tobaccos inoculated with PVX. (A) The picture was shown mosaic symptoms on inoculated leaves at 4dpi. (B) Viral accumulation and rgs-CaM expression were detected by western blotting using anti-bodies of PVX-coat protein (CP) and rgs-CaM, respectively. Rubisco dyed with Coomassie brilliant blue was used as a loading control. Total RNA and protein samples were extracted from inoculated leaves at 4 days after inoculation. Northern blot was performed to detect PR1a of salicylic acid signal. Total RNA extracts were shown in lower panel. (C) Viral accumulation of PVX-CP was detected by western blotting for upper leaves. Total RNA and protein samples were extracted from inoculated leaves at 2 weeks after inoculation.

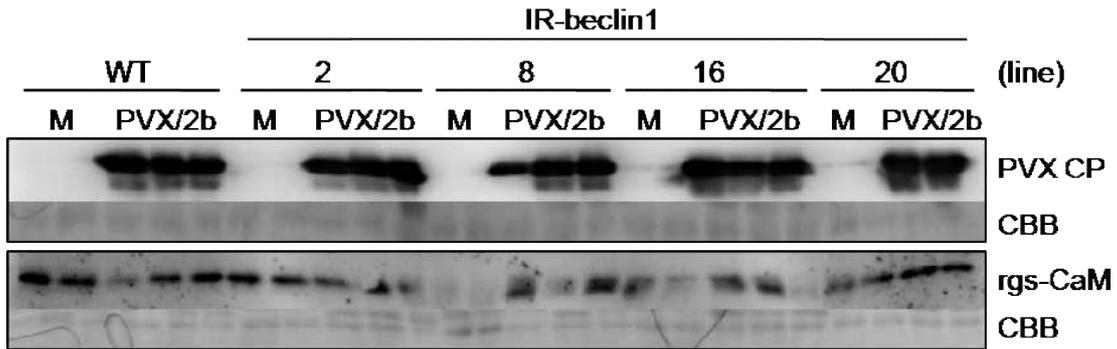


Figure 2-5 Virus accumulation and rgs-CaM expression in leaves of WT and beclin1 knock down tobacco lines that were inoculated with PVX/2b. PVX expressing CMV 2b (PVX/2b) was inoculated on WT and IR-beclin1 lines. 5 weeks old plants were inoculated and their leaves were harvested at 7 days after inoculation.

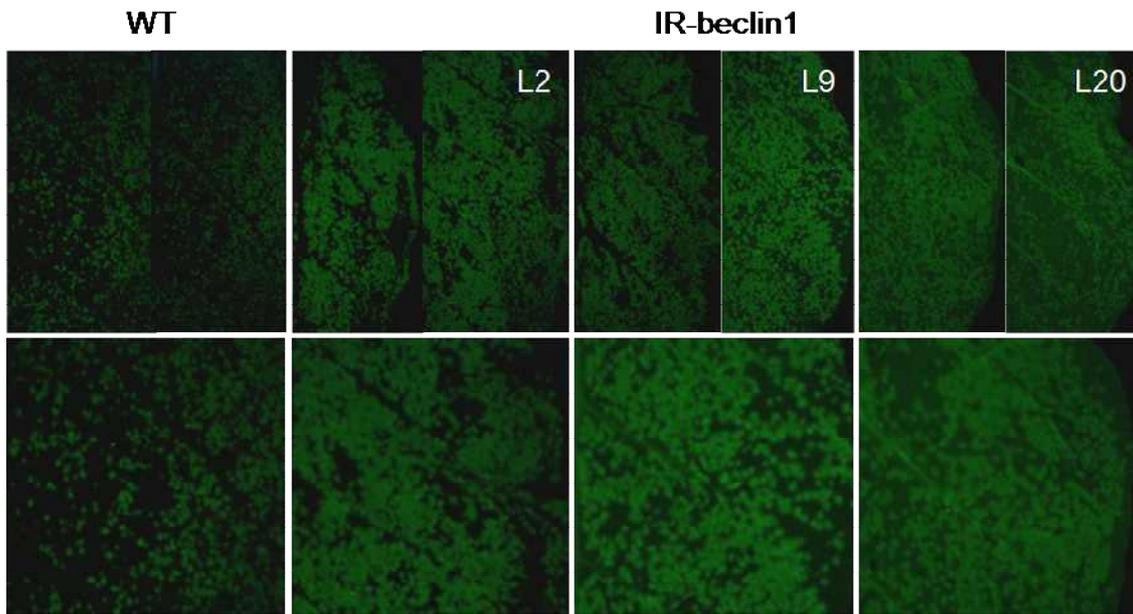


Figure 2-6 Images of BBWV2-expressing GFP infection on inoculated leaves of aged plants. The GFP signals indicate spreading viral infection observed by microscopic filter at 8 weeks old plants.

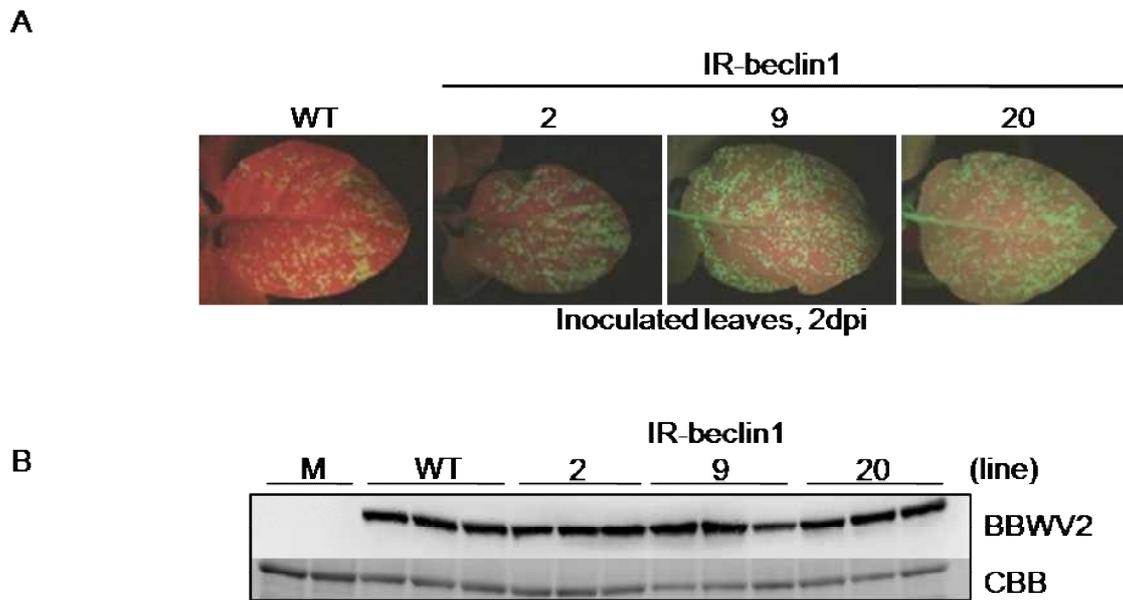


Figure 2-7 BBWV2-GFP infection in WT and IR-beclin1 lines. (A) BBWV2 infection and spread were detected by GFP signals. 6-week old tobaccos were used and harvested at 2 days after inoculation. (B) Western blot analysis of GFP co-expressed with BBWV2. Total protein was extracted at 3 day after inoculation.

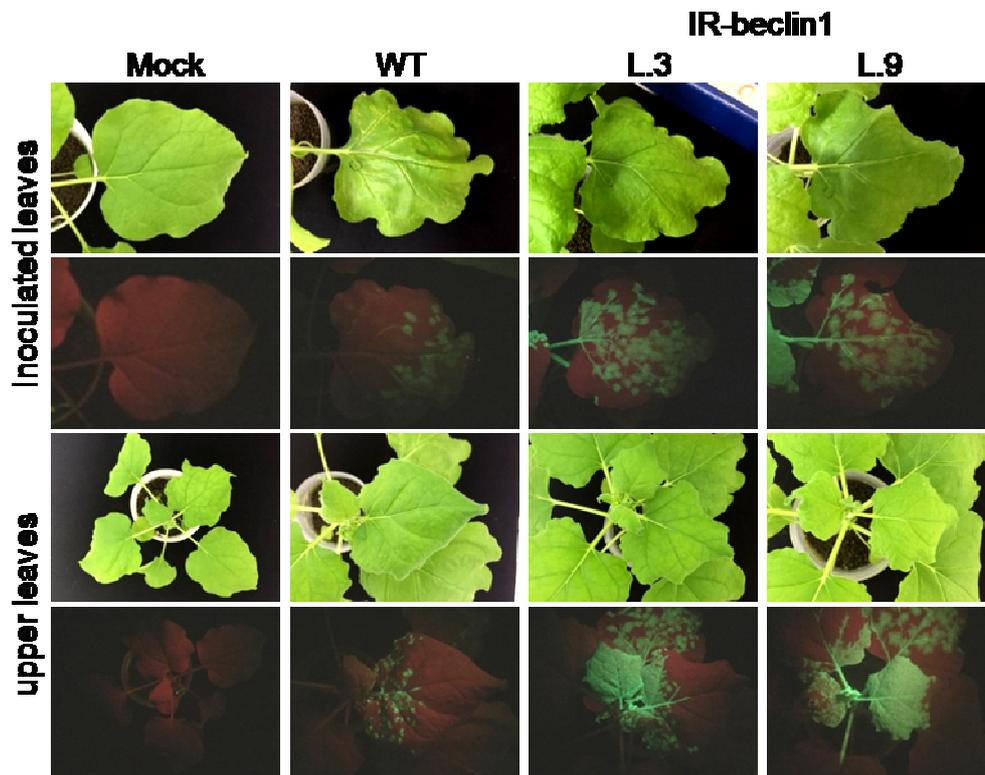


Figure 2-8 BBWV2 spreading was affected by autophagy deficiency in aging leaves.

The aging benthamiana infected with BBWV2-GFP showed fluorescent signal as degree of infection on inoculated and upper leaves at 7dpi.

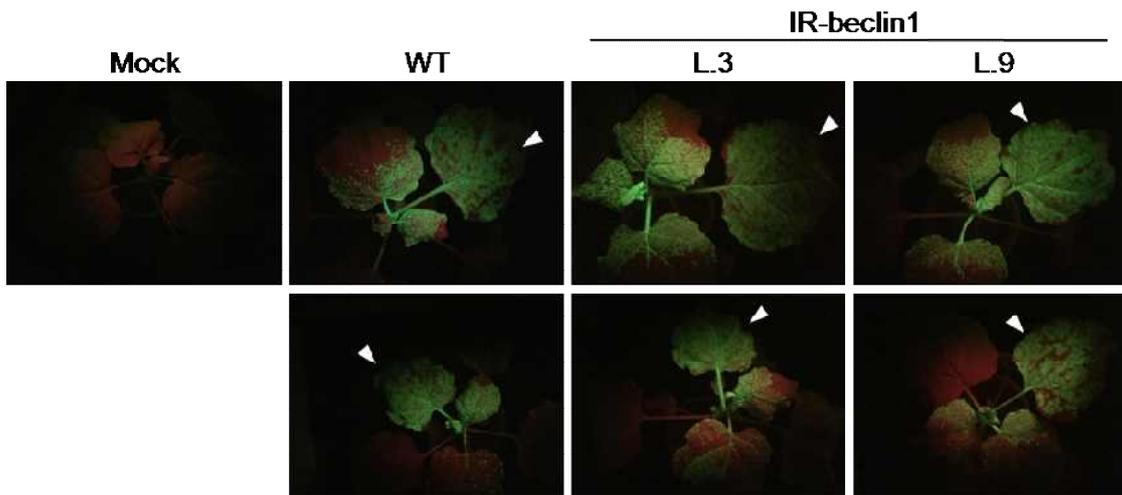


Figure 2-9 Images of BBWV2 infection in young leaves. The young plants infected with BBWV2-GFP showed fluorescent signal as degree of infection on inoculated and upper leaves at 4dpi. Arrow heads indicate inoculated leaves.

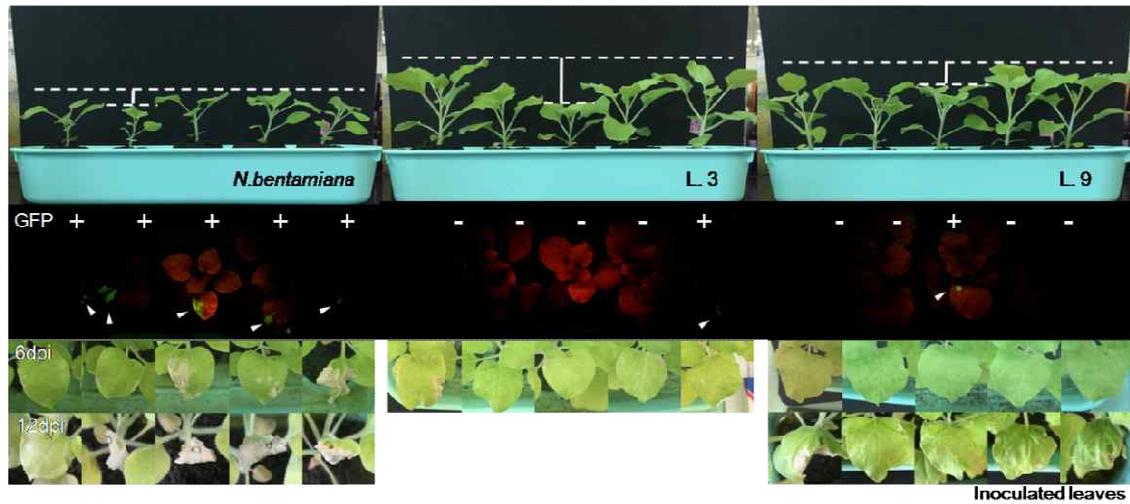


Figure 2-10 The characteristic of BBWV2 infection was exposed to inadequate nutrition condition in WT and IR-beclin1 *N.benthamiana*. White arrowheads indicate GFP signals.

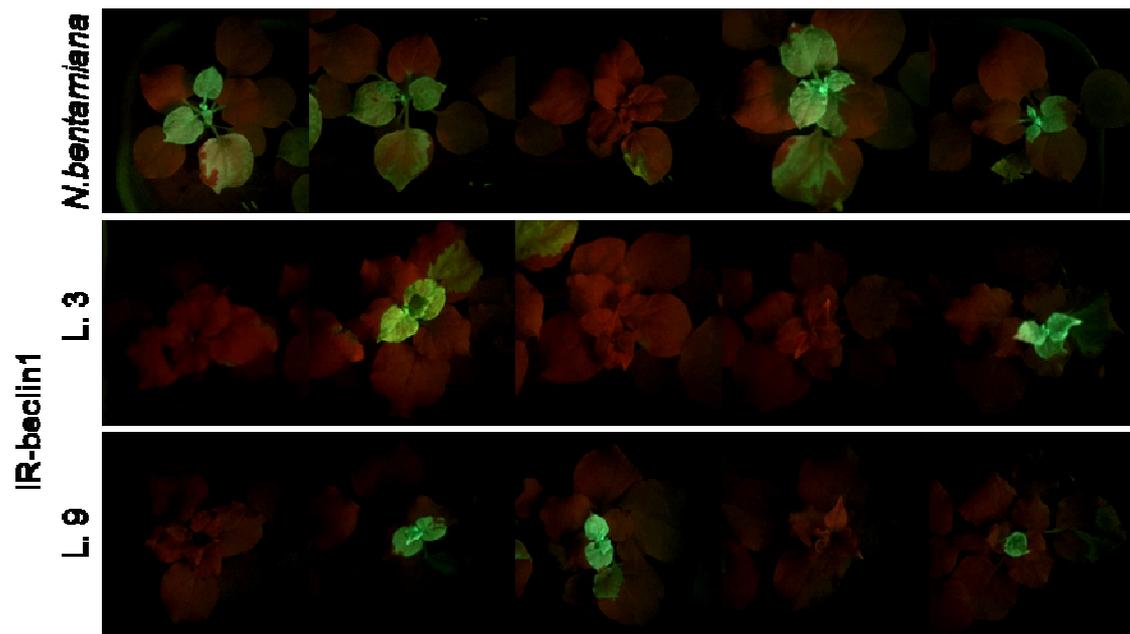


Figure 2-11 Systemic infection in nutritionally deficient benthamiana. BBWV2 virus was spread up to upper leaves in all of WT benthamiana but not in beclin1-silenced plants 24 days after inoculation.

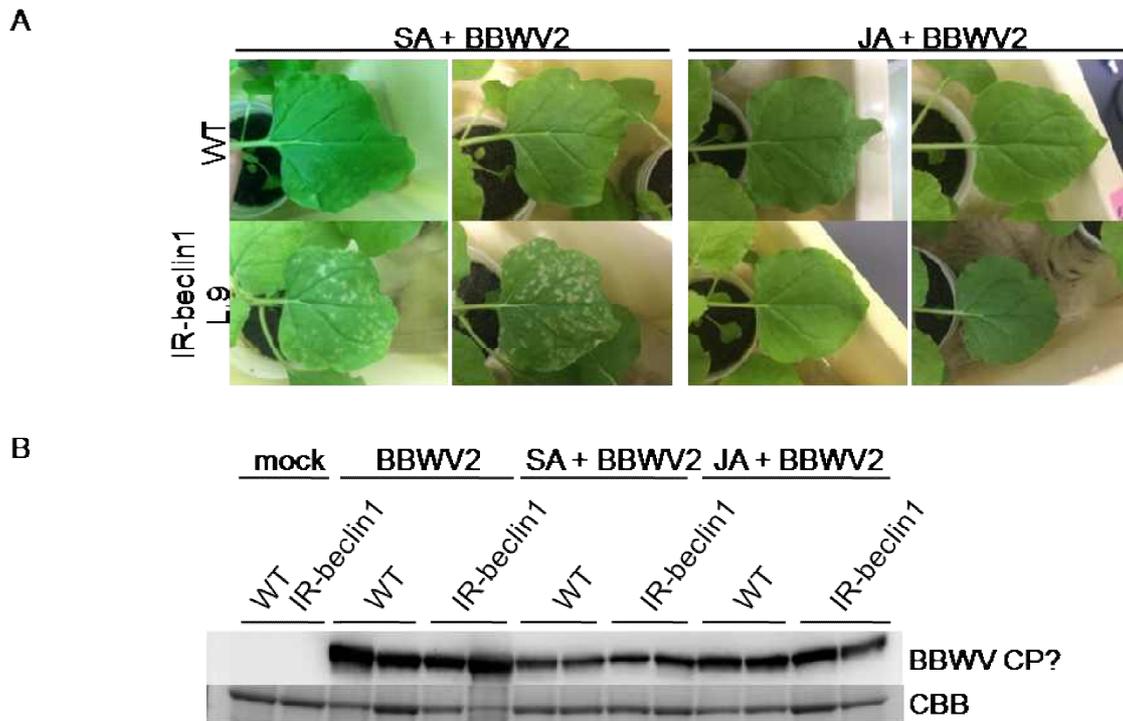


Figure 2-12 Effect of salicylic acid and jasmonic acid treatment on necrotic symptoms developed on leaves of *N. benthamiana* plants inoculated with BBWV2. (A) 5-week aged *N. benthamiana* was treated with 1.1 mM salicylic acid or 100 uM jasmonic acid 3 days later, inoculated with BBWV2-GFP. The inoculated leaves were observed at 3 dpi. (B) Western blot analysis was performed to verify viral accumulation in total protein extracts from inoculated leaves.

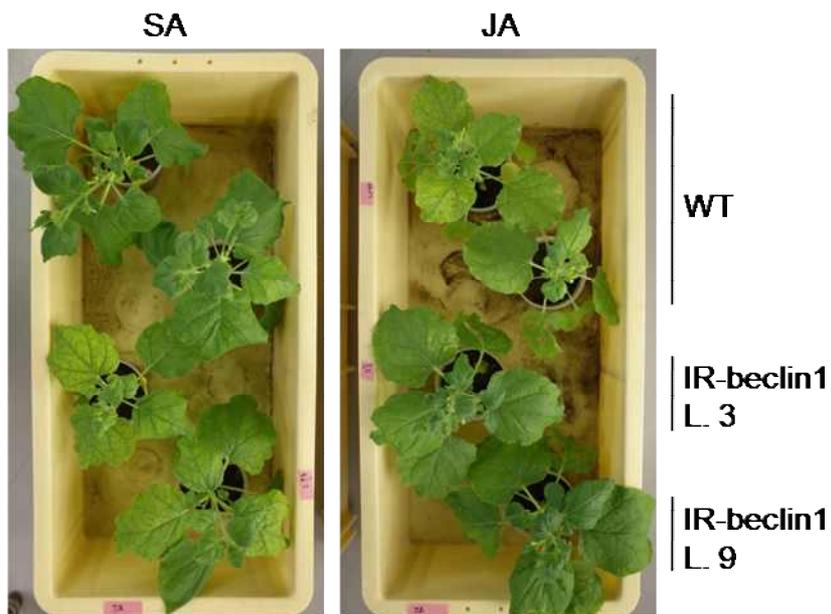


Figure 2-13 Systemic symptoms affected by SA and JA treatments BBWV2 was inoculated into WT and beclin1 knockdown *N.benthamiana*. The photograph was taken at 3 weeks post inoculation (3wpi). Plants growth defect and symptoms (chlorosis) such as chlorophyll destroyed in response to induced artificial defense signals were observed.

Discussions

Autophagy is one of processes for degradation of cellular components for detoxification or material recycling or clearing cellular contents (Bassham, 2007). It is also important in pathogen defense and the mechanism of it is convergent in plant kingdom (Hayward et al., 2009). Since ETI interaction by rgs-CaM involved autophagy to compromise virulent protein of CMV 2b, I generated beclin1 knock-downed tobacco plant for suppressing autophagy process. Autophagy deficient plants showed more susceptible to CMV and BBWV2 infection but not to PVX. Therefore, autophagy appears to prevent CMV and BBWV2 from invading into tobacco plants. Unlike data with CMV, infection of PVX-2b fusion virus was not changed by beclin1 deficiency. This result perhaps suggests the possibilities that other gene(s) of CMV in addition to the 2b protein is also interacted with autophagy. Further experiments with the PVX vector expressing other CMV proteins will be required for proving this suggestion.

Senescence and starvation, trigger of autophagy, were also related to virus infection. Old beclin1 deficient transgenic plants were vulnerable to virus infection but young transgenic plants were not. Similarly, with less nutrition, transgenic plants were susceptible to infection. These results suggest that autophagy function is required for active defense, especially in needy condition such like chlorophyll degrading senescence or material reusing starvation.

SA is a well-known phytohormone involved in various stresses. Among the roles of SA, it regulates biotic stresses and induces programmed cell death, etc (Hayat et al., 2010). Pre-activating SA signal could prevent plants from infection but there were little interaction between SA and beclin1. To verify this data, it would be required to

quantification of virus or time scales of infection efficiency. When I checked transcription levels of beclin1 with variable condition, SA deficiency caused decrease of beclin1 expression and the phenomenon of beclin1 decrease was recovered by wounding. It suggests that SA induced by stresses like wounding can activate beclin1 and beclin1 dependent autophagy. However, SA can suppress viral production via autophagy independent manner (Ryu et al., 2004).

So far, I tried to figure out interactions between autophagy and different viruses. There were not simple relationships but complicated patterns. Physiological and molecular data represented such discrete evidences, which may reflect complex response of plant defense depending on internal status and environmental cues.

GENERAL DISCUSSION

Although plant immune system was apparently considered to be less complex than vertebrate one, it is becoming clear that plants develop complex immune mechanisms to counter diverse pathogens. It has membrane-anchored and intracellular receptors to surveil pathogen invasion and to detect pathogenic molecules. When cells are under attack, they figure out what kind of invasion threat, which is a result of evolutionary arm race by natural selection. Cytosolic processes including homeostasis, metabolism, and trafficking are changed and rearranged for preparing and/or aiding defense response (Mukhtar et al., 2011; Robatzek, 2007). At the same time, according to relative internal signal cascades, cells recruit proper defense hormones and determine whether hypersensitive reaction is need against biotrophic invaders or cell death process should be blocked against necrotic pathogens (Hofius et al., 2007; Pieterse et al., 2009).

rgs-CaM is a plant specific calmodulin-related protein and interacts with viral RSSs. It suggests that rgs-CaM is not only ordinary calcium cation binding protein but also intracellular receptor against pathogenic elicitors. Therefore, rgs-CaM is a convergent point of both calcium signal and pathogen-derived molecules. Moreover, the influence of rgs-CaM is different depending on developmental times and nutrient support, which suggests that the modulation of rgs-CaM is flexible by internal environment such as aging and metabolism. Since it is obvious that the system has evolved by natural selection, it may reflect an active preparedness of plant to pathogen. For example, if necrotrophic pathogens prefer younger plants to invasion, plant need to reduce sensitivity for cell death signals. On the other hand, plants and/or individual cells may make a judgment whether they should expense resources to recover or abandon infected

region and suicide themselves. If there is some decision making process, it sounds interesting what the key molecule is and how the process is working, etc.

Autophagy is a final step or a trigger of programmed cell death, programmed cell death is an active process that expels infected cells to sequester and prison pathogens, which blocks pathogen propagation and prevents spread of disease. I showed that autophagy deficiency is susceptible for infection not only inoculation region but also far apart regions from introduction. Like rgs-CaM deficiency condition, the influence of silencing beclin1 is different depending on aging and nutrient status of plants. This may be a trivial result because autophagy is one of downstream steps under the rgs-CaM-mediated signal cascade. Therefore, natural interactions between pathogen varieties and endogenous plant stasis will be worth studying. I hope that my research will be one of fundamental approaches to demonstrate such interactions in natural context.

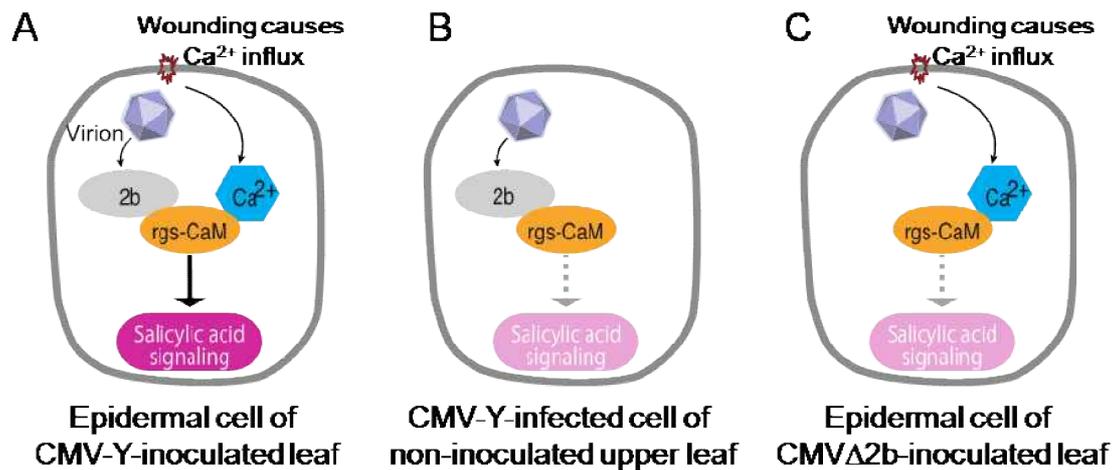
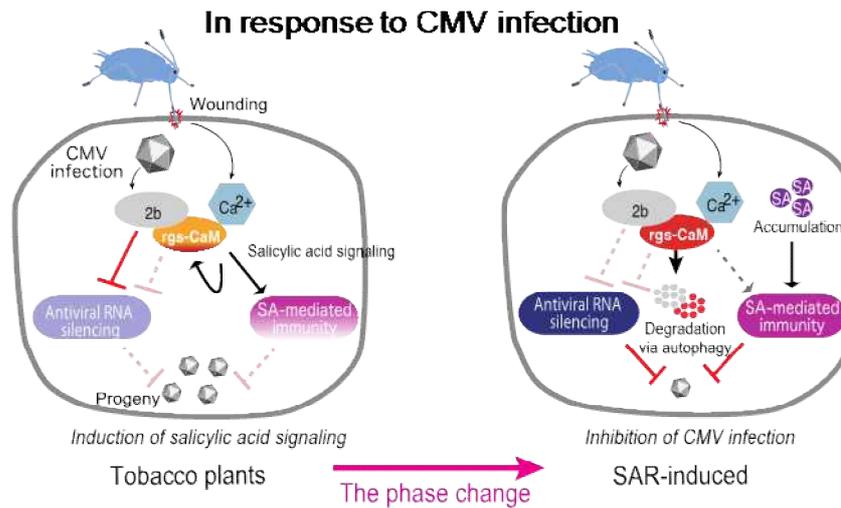


Figure 3-1 Model of SA signaling in response to CMV infection in tobacco plants.

In this model, rgs-CaM functions as an immune receptor that perceives viral RSS and Ca²⁺. Tobacco plants induce SA signaling when rgs-CaM perceives both 2b and Ca²⁺ as CMV infection cues in an inoculated leaf (A) but not when rgs-CaM perceives either 2b or Ca²⁺ alone, e.g., in a non-inoculated upper leaf (B) or in a leaf inoculated with CMV lacking 2b (CMVΔ2b) (C).

A



B

Salicylic acid signaling in response to PVX and CMV Δ 2b infection and artificial Ca^{2+} influx

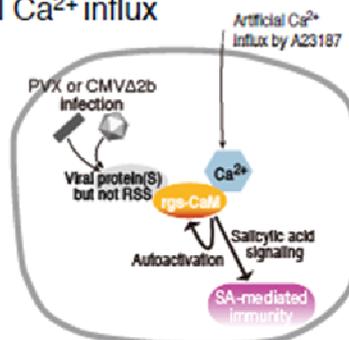


Figure 3-2 Schematic models of detection and counteraction of viral RSSs by rgs-CaM. (A, left) In normally growing tobacco plants, the rgs-CaM– mediated defense system does not inhibit CMV infection but induces SA signaling via perception of CMV 2b and Ca^{2+} as CMV infection cues, which perhaps leads to induction of SAR. (A, right) When the phase of rgs-CaM is changed by SAR induction, subsequent CMV infection is inhibited by rgs-CaM–mediated anti-RSS defense reactions. rgs-CaM directs

degradation of RSS (CMV 2b) via autophagy, resulting in reinforcement of antiviral RNA silencing in addition to SA-mediated antiviral immunity. (B) When plants are infected with PVX or CMV Δ 2b and Ca²⁺ influx is artificially induced with A23187, SA signaling is induced, probably via perception by rgs-CaM of Ca²⁺ and viral proteins other than RSS.

SUMMARY

Plant calmodulin (CaMs) and calmodulin-like protein (CMLs) are calcium ion (Ca^{2+}) sensors that play important roles in development and stress responses. An increase in the Ca^{2+} concentration in the cytoplasm is one of the earliest events following exposure to environmental stresses and Ca^{2+} is a crucial secondary messenger in the perception of these stresses. In plants, CaMs and CMLs constitute a relatively large family of Ca^{2+} sensor genes. One of the tobacco CMLs, *rgs-CaM*, uniquely binds to exogenous proteins, diverse viral RNA silencing suppressors (RSSs), presumably via affinity to their positively charged dsRNA-binding sites. *rgs-CaM* has previously been shown to be antiviral protein; that is, it directs degradation of viral RSSs via autophagy, resulting in reinforcement of antiviral RNA silencing.

In this study, *rgs-CaM* was shown to function as an immune receptor to recognize virus infection and induce defense reactions. Among transgenic tobacco plants that constitutively overexpressed the *rgs-CaM* gene under the control of the cauliflower mosaic virus 35S promoter, two lines showed dwarfing, deformation, and necrotic symptoms on their leaves. These phenotypes were similar to those of lesion mimic mutants that involve hypersensitive response-like programmed cell death, which are accompanied by induction of defense signaling components, including SA. In the transgenic plants showing these phenotypes, mRNA of the gene for pathogenesis-related protein 1a (*PR1a*), an indicator of activation of SA signaling, was induced in the leaves. Infection with the PVX vector expressing *rgs-CaM* caused necrotic spots and induced *PR1a*, whereas infection with the empty PVX vector did not. Taken together, these data suggest that overexpressed and ectopically expressed *rgs-CaM* induces cell death and

SA signaling.

When cucumber mosaic virus (CMV) was inoculated into wild-type and rgs-CaM-knock down tobacco plants, PR1a was strongly induced in inoculated leaves of wild-type plants but not in those of rgs-CaM-knock down plants, suggested the possibility that endogenous rgs-CaM is also involved in induction of SA signaling. *PR1a* was expressed in leaves of transgenic tobacco plants expressing viral RSS, 2b or clover yellow vein virus HC-Pro 24 h after wounding and induction of Ca²⁺ influx with ionophore A23187. This *PR1a* induction was attenuated by silencing of the rgs-CaM expression. These results suggest that tobacco plants induced SA signaling in response to CMV infection via perception of Ca²⁺ and 2b by rgs-CaM. SA signaling is one of the major antiviral defense signaling. rgs-CaM knock down tobacco plants were more susceptible to CMV than WT tobacco plants. rgs-CaM seems to effectively inhibit CMV infection through 2b recognition and degradation by rgs-CaM, leading to reinforcement of antiviral RNA silencing and other SA-mediated antiviral responses.

Since the rgs-CaM-mediated antiviral responses involve autophagy, using autophagy deficient tobacco plants, I examined whether autophagy was involved in defense responses against viruses, including salicylic acid. Beclin 1, the ortholog of yeast Atg6, is well-conserved in diverse organisms, including plants, and performs functions to assemble autophagy stimulator and suppressors regulate the formation of autophagosome (Kang et al., 2011). To prepare autophagy deficient tobacco plants, I generated transgenic plants, in which the *beclin1* gene was knocked-down. Then, I inoculated CMV, potato virus X (PVX) and broad bean wilt virus 2 (BBWV2) to these transgenic *N.tabacum* and *N.benthamiana* plants. As the results, autophagy defect

affected the symptom development with CMV infection and the systemic spread of BBWV2 expressing GFP but not PVX infection.

When pretreated with SA analogue before BBWV2 inoculation, autophagy deficient plants developed obvious necrotic symptoms but WT plants hardly did though there was no difference in viral accumulation compared with WT. Activation of SA signaling enhanced BBWV virulence in autophagy deficient plants, suggesting the association of autophagy with SA in BBWV2-infected plants.

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Acknowledgment

I am so happy to finally I completed this special English course in Graduated School of Agriculture, Hokkaido University. My long-life as a student has come to an end. It doesn't mean a real end but it marks a new beginning (or chapter) for my next journey. I believe that my five-year experience in Japan would give me strength in pursuing my career as a researcher and giving me a better understanding about life.

I would have not been possible to finish my study without the supports from people around me. I would like to express my deepest gratitude to Prof. Dr. Chikara Masuta and assistant professor, Dr. Tatsuji Hataya and Dr. Kenji Nakahara from Pathogen-Plant Interactions Laboratory for supporting my research. Special thanks go to Dr. Tsuyoshi Inukai, Assistant professor of Cell biology, for his kind advice. I am grateful to all of my lab members whom I had pleasure to work with, especially Suzuki, Hisa, Lee, Tadamura, Hukuda, Yamane, Nakano, Murakami, Takahashi, Abe, Nishi, Takahashi (kaho) and Uyeda. Thanks for your friendship, support, and great times for these past five years. I would like to specifically acknowledge Miran and Tsuchida Marumi. Thanks for being good counselors to me.

I would like to thank Ministry of Education, Culture, Sports, Science and Technology (MEXT) for support me financially and giving me an opportunity to continue my study. I would like to extend my sincere thanks to Dr. Hyun Hee Kim, professor of Life Science, Sahmyook University for leading/inspiring me to a research field. I am also grateful to Dr. Ki Hyun Ryu, professor of Seoul Women's University for giving me a chance to study abroad. I always appreciate to colleagues of Plant Genetic laboratory in Sahmyook University. Last but not least, my sincere gratitude goes to my friends. No

words can express my appreciation to my family and my lover for their respects and supports during my ups and downs. Without them, this experience would not have been possible. I really feel a sense of respect and appreciation beyond expression to my family and lover.

감사의 글

학위 과정을 마무리하는 시간이 오긴 오네요. 돌아보니 늘 미흡하고 부족한 나를 발견하는 경험이 대부분이었기 때문에 힘든 시간이 많았던 것 같습니다. 때로는 감사함에 가슴이 벅차 오르고 때로는 감당하기 무거운 짐으로 느껴져 눈물도 흘러가며 보내온 낯선 땅에서의 유학생살을 되돌아보며 한없는 그분의 큰 은혜에 다시 감사하게 됩니다. 다시는 돌아오지 않을 소중한 시간을 보내며 얻은 경험은 토대로 앞으로 저에게 주어질 새로운 세상에서 조금은 성숙해진 마음가짐으로 임하게 되기를 저 스스로 기대해봅니다.

일본 문부과학성의 실제적인 지원으로 5년간 경제적인 어려움 없이 학업을 지속할 수 있었습니다. 식물-병원 연구실의 増田 교수님, 畑谷 교수님, 犬飼 교수님, 그리고 저의 지도 교수님인 中原 교수님께 감사합니다. 여러 선생님들의 덕분에 석·박사 과정을 완주 할 수 있었습니다. 일본 생활에 잘 적응하도록 도와준 鈴木, 比佐, 착한 동기들-李, 忠村, 福田, 山根, 中野, 후배들-高橋, 阿部, 村上, 西, 上田, 高橋, 宮下를 포함한 모든 연구실원 멤버들 고맙습니다. 그리고 학생실에서 속마음을 나눌 수 있었던 미란씨, 土田 고맙습니다.

연구의 길에 들어서게 해주신 그리고 유학이라는 새로운 경험과 기회를 열어주신 삼육대 김현희 교수님 감사합니다. 지금은 모교의 교수님이 되신 자랑스러운 황윤정 선배님 고맙습니다. 삼육대학교 식물유전학 연구실 선후배 식구들 고맙습니다. 유학 오기 전 농진청에서 배우면서 일할 수 있게 도움주신 안병옥 연구관님 감사합니다. 그리고 친절하게 많은 것을 가르쳐주신 황현주 박사님, 오미진 박사님 감사합니다.

친절한 샤프로 교회 목사님을 비롯한 일본 교우님들, 교회친구이자 입학동기 Ikabongo, 그리고 Nana, Saringya, 일본에서 만난 동생 万純 여러분들이 저의 외로움을 달래주어서 잘 견딜 수 있었습니다. 고맙습니다. 동생 정현이도 진심으로 고맙다.

늘 따뜻한 마음을 담아 말씀해 주시고 기도로 응원해주시는 김신섭 목사님 감사합니다. 늘 기도로 큰 힘 주시는 엄기호 목사님, 사랑하는 친구 진경, 라현, 우리, 혜선, 혜미, 진영, 신혜, 희섭 다들 사랑하고 고마워. 조금만 더 버티라고 용기준 류연이도 늘 고맙다. 어떤 부탁도 마다 않고 들어주는 창원오빠 정말 고마워.

박사과정 1년에 갑자기 아빠를 잃게 되어 마지막 시간을 함께 못한 점이 마음에 깊은 상처로 남아있지만 아빠의 사랑 기억하며 앞으로도 힘을 내겠습니다. 지금의 나를 있게 한 엄마의 수고와 끊임없는 기도와 사랑에 온 마음을 다해 감사합니다. 그리고

존경하고 사랑합니다. 하나뿐인 우리 언니에게는 늘 표현이 부족한데 언제나 응원하고 사랑합니다. 투정하고 불평해도 늘 이해해주고 잘 참아준 남자친구 고맙고 미안하고 사랑합니다.