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Isolation and Characterization of Pepper Genes Interacting with CMV-P1 Helicase Domain

CMV-P1 Helicase domain과 상호작용하는

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ABSTRACT

Cucumber mosaic virus (CMV), which has the broadest host range among plant viruses, is a very destructive pathogen in pepper production. *Capsicum annuum* 'Bukang' contains a single dominant resistance gene, *Cmr1* (*Cucumber mosaic resistance 1*) to CMV. *C. annuum* 'Bukang' is resistant to most of CMV strains, but susceptible to CMV-P1. CMV-P1 is a new strain overcoming *Cmr1*recently identified in South Korea. Previous study reported that CMV-P1 RNA1 helicase domain is responsible for overcoming *Cmr1*. To identify plant host factors involved in CMV-P1 replication and movement, a yeast two-hybrid system derived from *C. annuum* 'Bukang' cDNA library was used. A total of 156 potential

clones interacting with the CMV-P1 RNA1 helicase domain were isolated from about 100,000 clones in the first screening. Beta-galactosidase filter lift assay, PCR screening, and sequencing analysis narrowed the candidates to 10 host genes. These genes are known to be involved in virus infection, replication, or virus movement. To elucidate functions of these genes, the host genes were silenced in *Nicotiana benthamiana*, which were then inoculated with CMV-P1 expressing the green fluorescent protein (GFP). Gene silencing was confirmed by semi-quantitative RT-PCR. Plants silenced for 7 genes showed normal development comparable to *N. benthamiana* wild type, whereas plants silenced for the other three genes showed developmental defects including stunting and severe distortion. Virus accumulation in silenced plants was assessed by monitoring GFP fluorescence and enzyme-linked immunosorbent assay (ELISA). Among the 7 candidates, silencing the *cysteine synthase* gene showed rapid and high accumulation of CMV while silencing two host genes, *formate dehydrogenase* and *calreticulin-3 precursor*, showed reduced virus accumulation. In the case of the *cysteine synthase*-silenced plants, infection foci were observed in both inoculated and upper leaves, and GFP signals were detected earlier than TRV::00. *Formate dehydrogenase*-silenced plants showed local infection in inoculated leaves, and there were no GFP signal in upper leaves. In the case of *calreticulin-3 precursor*, no GFP signals were observed in both the inoculated and upper leaves. ELISA results confirmed the confocal observation results. These results demonstrate that *formate dehydrogenase* and *calreticulin-3 precursor* are required for CMV-P1 infection

Keywords: *Capsicum annuum*, *Cucumber mosaic virus*, host factor, virus resistance, *formate dehydrogenase*, *calreticulin-3 precursor*

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LIST OF ABBREVIATIONS

AD	Activation domain
BAC	Bacterial artificial chromosome
BBWV2	*Broad bean wilt virus 2*
BD	Binding domain
BLAST	Basic local alignment search tool
BMV	*Bromo mosaic virus*
cDNA	Complimentary deoxyribonucleic acid
CMV	*Cucumber mosaic virus*
CMV-Kor	CMV-Korean
Co-IP	Co-Immunoprecipitation
CP	Coat protein
CPMV	*Cowpea mosaic virus*
Cmr1	*Cucumber mosaic resistance 1*
dpi	Days post inoculation
eIF4E	Eukaryotic translation initiation factors 4E
eIF4G	Eukaryotic translation initiation factors 4G
ELISA	Enzyme-linked immunosorbent assay
ER	Extreme resistance
GFP	Green fluorescent protein

His	Histidine
HR	Hypersensitive response
INSV	*Impatiens necrotic spot virus*
Leu	Leucine
LIC	Ligation-independent cloning
MCS	Multiple cloning site
MP	Movement protein
*Nb*PDS	*Nicotiana benthamiana phytoene desaturase*
OD	Optical density
ORMV	*Oilseed rape mosaic virus*
PCR	Polymerase chain reaction
PMMoV	*Pepper mild mottle virus*
PepMoV	*Pepper mottle virus*
PD	Plasmodesmata
PVX	*Potato virus X*
PVY	*Potato virus Y*
RdRp	RNA-dependent RNA polymerase
SE	Sieve elements
Semi qRT-PCR	Semi quantitative reverse transcription -PCR
SYNV	*Sonchus yellow net virus*
TEV	*Tobacco etch virus*

TMGMV	*Tobacco mild green mosaic virus*
TMV	*Tobacco mosaic virus*
TRV	*Tobacco rattle virus*
Try	Tryptophan
TSWV	*Tomato spotted wilt virus*
TVCV	*Turnip vein clearing virus*
TYMV	*Tobacco yellow mottle virus*
VIGS	Virus-induced gene silencing

INTRODUCTION

Since plant viruses contain small number of genes in their genome, viruses are dependent on hosts, and viral proteins divert host factors for virus infection through interactions between viral and host proteins. These interactions are required for replication, cell-to-cell movement, and systemic movement. The outcome of interactions determines host specificity and tissue specificity strains. Accordingly, host factors constitute essential parts in most steps of virus infection (Ahlquist et al., 2003; Stapleford et al., 2010). Without the interactions, viruses are unable to infect plants, hence, virus-host interactions can be utilized for developing virus resistant crops.

Plant virologists have tried to identify host factors and to study host factor mutations (Kang et al., 2005, Whitham et al., 2004). For example, *NtTLP1*, which directly interacts with *Cucumber mosaic virus* (CMV) 1a protein, plays an important role in CMV replication and/or movement (Kim et al., 2005). *Tcoi1* directly interacts with CMV 1a methyltransferase (MT) domain, and overexpression of *Tcoi1* enhances the CMV infection while silencing of *Tcoi1* decreases virus infection (Kim et al., 2008). *Tsip1* strongly interacts with CMV 1a protein and CMV 2a protein that has the GDD motif typical for RNA-dependent RNA polymerase (RdRp), and forms virus replicase complex. This replicase complex controls CMV multiplication in tobacco plants (Huh et al., 2011). Additionally, knockout of the *ssi-2* gene, which encodes a *plastid-localized stearoyl-ACP desaturase*, enhances resistance to CMV in *Arabidopsis*,

and suppress viral multiplication and systemic movement (Sekine KT et al., 2004).

CMV has a very wide host range, and is one of the most destructive viruses in pepper production. CMV belongs to the *cucumovirus* group in the *Bromoviridae* family. CMV encodes five proteins from its tripartite genome (Edwardson et al., 1991; Palukaitis et al., 1992). CMV RNA 1 encodes CMV 1a protein which is composed of the MT domain and the helicase domain (Kim et al., 2005). CMV RNA 2 encodes CMV 2a protein including RdRp and CMV 2b protein. CMV RNA3 encodes movement protein (MP) and coat protein (CP).

To protect crops from the virus, various resistance sources against CMV have been identified by plant breeders. One of them is *Capsicum annuum* 'Bukang', which contains a single dominant resistance gene, *Cmr1* (*Cucumber mosaic resistance 1*). *C. annuum* 'Bukang' is resistant to most of CMV strains (CMV-Kor and CMV-Fny) (Kang et al., 2010).Recently, a new strain CMV-P1, which breaks *Cmr1*, was identified in South Korea. CMV-P1 systemically *C. annuum* cv. 'Bukang', and affects plant growth, vigor, quality, and yield (Lee et al., 2006).

To clarify which RNA genome is involved in breaking *Cmr1*, chimeric CMV viruses were constructed by combining CMV-Fny and CMV-P1 cDNA clones. The 3' end of CMV-P1 RNA1 helicase domain was implicated to play a key role in viral replication and systemic infection (Kang et al., 2012). In the helicase domain, it was shown that there were four amino acids differences between CMV-Fny and CMV-P1 (Kang et al., 2012) which determines virulence of CMV-P1.

In this study, I tried to identify host genes that interact with CMV-P1 helicase domain using a yeast two-hybrid system. To validate requirement of selected host genes in CMV-P1 infection, selected genes were silenced via VIGS and silenced plants were challenged with CMV-P1 harboring the green fluorescent protein (GFP). Later, virus accumulation in silenced plants was assessed by monitoring GFP fluorescence and enzyme-linked immunosorbent assay (ELISA). Through these steps it was revealed that *formate dehydrogenase* and *calreticulin-3 precursor* are essential host genes required for CMV-P1 infection.

LITERATURE REVIEW

1. Importance of pepper and pepper virus in Korea

Peppers (*Capsicum* sp.) are native to Southern Peru and Bolivia in South America (Pyou et al., 1980). Pepper is one of the most widely cultivated crops, and they grow in various temperature and climatic conditions in over 60 countries (Maretelli et al., 1983; Pyou et al., 1980). A pepper fruit includes high content of vitamin A, vitamin C, flavor, and capsaicin. Peppers have been used as an ingredient for food and medicine and become a key component in regional dishes. In South Korea, peppers are one of the most important vegetable crops. Cultivation area of peppers was 50,521 ha, and pepper production amounted to 350,436 ton in 2009 (www.krei.re.kr). Annual pepper production cost is more than one trillion Won in these days.

Pepper is easily infected with fungi, bacteria, and virus. Among them, plant viruses are difficult to control and affect pepper production by reducing yields and deteriorating quality. 66 viruses were identified to infect peppers worldwide. Six viruses including CMV, *Pepper mottle virus* (PepMoV), *Pepper mild mottle virus* (PMMoV), *Broad bean wilt virus 2* (BBWV2), *Tobacco mild green mosaic virus* (TMGMV), and *Tomato spotted wilt virus* (TSWV) were identified in infected peppers in South Korea. In 1980, the incidence ratio of *Tobamovirus* was over 90%, and that of CMV was 10% (Kim et al., 1990). To inhibit increase of *Tobamoviruses* which are spread by infected soil and seeds, cultivation methods were changed. As a result, infection of *Tobamoviruses*

(PMMoV and *Tobacco mosaic virus*) was decreased in 2002. However, infection of CMV and BBWV2, which are transmitted by aphids, was increased. In 2006, the incidence ratio of CMV, PepMoV, PMMoV, and BBWV2 was 29.4%, 13.6%, 14.3%, and 25.6%, respectively. Recently, it is easy to observe mixed infections more than ever before. The incidence ratio of CMV+PepMoV was 62.6% in 2002. In case of CMV+BBWV2 mixed infection, it was also increased to 83.2% in 2006. The triplex and quadruplex infections relating to CMV also have been identified (Cho et al., 2007).

Chemical methods and cultivation methods have been used for a long time, but, nevertheless, prevention of virus infection is still a difficult part of agriculture. Some viruses acquired resistance to chemical methods, and as a result, agricultural pesticides have become destructive. To overcome virus infection, virus research is essential, and breeding based on the research is also necessary for virus control.

2. *Cucumber mosaic virus*

CMV has a very wide host range and has been observed worldwide. It is one of the most destructive viruses which damage pepper production. CMV belongs to the *cucumovirus* group in the *Bromoviridae* family. And the CMV genome is composed of tripartite positive-sense RNAs (Palukaitis et al., 2003). The genome contains five proteins encoded by capped messenger-sense RNAs: RNA 1, RNA 2 and RNA 3 (Edwardson et al., 1991; Palukaitis et al., 1992). CMV RNA 1 encodes the CMV 1a protein which is composed of the MT domain in its N-terminal part and the helicase domain in the C-terminal part (Kim et al., 2005). And

methylation of the CMV 1a protein is concerned in regulation of systemic movement (Kim et al., 2008). CMV RNA 2 encodes the CMV 2a protein which has the GDD motif typical for RdRp, and CMV RNA 2 also encodes the CMV 2b protein which is expressed from an open reading frame (ORF) overlapping the 3'-terminal part of ORF 2a. The CMV 2b protein is related to host-specific virus accumulation and long distance movement of CMV (Ding et al., 1995; Lucy et al., 2000; Zhang et al., 2006). CMV RNA 3 encodes two proteins, MP and CP. And these proteins are significantly related to cell-to-cell movement of CMV (Li et al., 2001).

3. Resistance to plant virus in nature

To infect host, virus processes multiple steps which include entrance into plant cells, uncoating of nucleic acid, translation of viral proteins, replication of viral nucleic acid, assembly of progeny virions, cell-to-cell movement, systemic movement, and plant-to-plant movement (Carrington et al., 1996). To initiate infection, virus particles enter through wounds caused by agricultural machine or insects. It has been suggested that release of viral genome from capsid to plant cytoplasm is not host-specific. In case of TMV and *Tobacco yellow mottle virus* (TYMV), they processed uncoating both in host and non-host plants (Kiho et al., 1972; Matthews et al., 1985). Even though uncoating is performed, successful virus infection in host requires resistance to barrier in host, and host proteins, which were used for virus survival, are also essential. In order to prevent infection, plants have various resistance mechanisms targeted to virus replication, cell-to-cell

movement and systemic movement (Kang et al., 2005).

3.1 Resistance by replication blocking

Resistance at the single cell level is characterized in inoculated cells, and virus replication does not occur or is not detected in cellular resistance plants. This kind of resistance has been termed extreme resistance (ER), cellular resistance, or immunity (Fraser RSS, 1986; Fraser RSS, 1990). *Cowpea mosaic virus* (CPMV) is a classic example of the resistance, and the virus was proposed that it is prevented by protease inhibitor for replication disturbance (Ponz F et al., 1988). For successful virus infection in entire part of a plant, virus requires host factors which are used for plant life cycles such as replication and plant component movement.

In *Arabidopsi*s, knockout of the *ssi-2* gene, which encodes a *plastid-localized stearoyl-ACP desaturase*, enhances resistance to CMV. Viral multiplication and systemic movement are decreased in the *ssi-2* mutant plant (Sekine et al., 2004). Here as elsewhere the *lsp1* gene, which encodes eukaryotic translation factor eIF(iso)4E, is identified in *Arabidopsi*s mutants, and this mutant plant does not support replication of *Tobacco etch virus* (TEV) and *Turnip mosaic virus* (TuMV) (Lellis et al., 2002). eIF(iso)4E is also identified from pepper, lettuce, pea, and tomato that implicate host translation factors in resistance to *potyviruses* and CMV (Kang et al., 2005; Nicaise et al., 2003; Parella et al., 2002; Pellio et al., 2005; Ruffel et al., 2002). In another case, knock out of the *tom1* gene, which encodes a transmembrane protein localized in the tonoplast, does not support TMV accumulation in single cells. And the *tom2A* gene, which also encodes a

transmembrane protein that interacts with *tom1*, also does not support TMV accumulation in single cells (Ishikawa et al., 1991; Ishikawa et al., 1993; Ohshima et al., 1998).

The type of mechanism that involves an active resistant response to virus infection occurs rapidly to limit virus replication before cell-to-cell movement. It causes resistance at the single cell level, and this phenomenon shows no symptoms or extremely limited necrosis (Kang et al., 2005). The potato *Ry* gene has been studied for ER. After PVY infection, plants including the *Ry* gene did not show any virus symptom. Virus accumulation was also prevented in plants (Solomon-Blackburn et al., 2001). *Rx* in potato (Bendahmane et al., 1999), *Sw5* in tomato (Brommonschenkel et al., 2000), and *Rsv* in soybean (Hajimorad et al., 2005) showed similar mechanism with that of the *Ry* gene.

3.2 Resistance by cell-to-cell movement blocking

Viruses cannot infect plants without cell-to-cell movement, even though virus replication is successful in inoculated cell. Virus movement from the initially infected cells to neighboring cells is necessary, and this movement also affects systemic infection. Plants show responses to virus infection for survival, and one of them is cell-to-cell movement blocking. Resistance responses of cell-to-cell movement also relate with host factors which interact with viral components (Carrington et al., 1996). Interaction between cellular components and virus CP or MP mediates transport via plasmodesmata (PD) into adjacent cells by modifying pre-existing pathways in the plant for macromolecular movement (Shoko et al.,

2010). Resistance at the level of cell-to-cell movement is caused by failure of the interactions or by disruption of active host defense responses that rapidly limit virus spread. A number of genes from a plant have been reported that the genes inhibit the cell-to-cell movement of plant viruses.

In *Arabidopsis*, the *cum1-1* and *cum2-1* mutant plants inhibit CMV movement. These genes encode eukaryotic translation initiation factors 4E (eIF4E) and eukaryotic translation initiation factors 4G (eIF4G), respectively. In the mutant plants, accumulation of CMV 3a protein, which is necessary for cell-to-cell movement, is strongly reduced (Yoshii et al., 1998; Yoshii et al., 2004). Member of a different viral family have been studied using tobacco, pepper, and pea, and the results were similar. In pepper, the *pvr1* gene, which encodes eIF4E, affects cell-to-cell movement of PVY (Ruffel et al., 2002). And another gene *sbm1*, which encodes eIF4E in pea, also affects cell-to-cell movement of PVY (Gao et al., 2004).

The case of active host defense responses that rapidly limit virus spread shows hypersensitive response (HR) for cell-to-cell movement prevention of plant viruses. After plant system recognizes viral components, the system inducts host defense responses such as oxidative H_2O_2 bursts and callose biosynthesis. By induction of host defense system, viral movement is limited to a small number of cells. These kinds of genes include the N gene in tobacco (Otsuki et al., 1972) and the *Tm-2* and *Tm-22* genes in tomato (Motoyoshi et al., 1975).

3.3 Resistance by systemic movement blocking

For systemic movement, plant viruses should pass from mesophyll to leaf

veins and vascular tissue. Viruses enter bundle sheath cells, phloem parenchyma, and phloem sieve elements (SE), which are originally used in plant component movement (Carrington JC et al., 1996). One of the important factors for systemic movement is PD, which is known as membrane-lined tunnels through the cell wall. Virus has big molecules such as movement protein, and for this reason, they interact and modify the PD for both cell-to-cell and long distance movement. The most significant barrier for long distance movement is the SE-companion cell complex (Ding et al., 1998; Wintermantel et al., 1997). Systemic movement is more difficult to study than cell-to-cell movement because of wide part observation and many kinds of plant components, and as results, the mechanisms of viral interaction with phloem are not largely known. Few host factors have been identified for potential R gene, which may be essential for this process (Kang et al., 2005).

TMV MP interacts with *pectin methylesterase*, which inhibits TMV movement, and the TMV virion may be blocked against exit from phloem (Chen et al., 2003). In potato, the recessive *ra* allele completely blocks *Potato virus A* (PVA) against vascular transport in inoculated plants (Hamalainen et al., 2000). In pepper, the plants which include resistance allele *pvr3* show PepMoV-FL accumulated in inoculated leaves. The virus moves into the stem but internal phloem is not observed for systemic movement to young tissues (Guerini et al., 1999; Murphy et al., 1995). In *Arabidopsis*, the *RTM1*, *RTM*2 and *RTM3* restrict long-distance movement of TEV. All these genes have been identified that they are expressed in SEs, but the mechanism of TEV movement inhibition is not understood (Mahajan et al., 1998; Whitham et al., 2000).

MATERIALS AND METHODS

Yeast two-hybrid screening

To construct a bait vector, primers were designed based on the CMV-P1 RNA1 helicase domain sequence. A CMV-P1 RNA1 helicase domain fragment was prepared by PCR from a CMV-P1 cDNA clone provided by Professor Kook-Hyung Kim. The CMV-P1 RNA1 helicase domain fragment was cloned into a vector pBD-GAL4 Cam (Agilent Technologies, Santa Clara, CA, USA). The bait vector (pBD-GAL4 Cam vector) was transformed into *Saccharomyces cerevisiae* strain YRG-2, and was selected on media lacking tryptophan (SD-Trp) for 4 days at 30℃. The *C. annuum* 'Bukang' cDNA library which was cloned into a prey vector pAD-GAL4-2.1 (Agilent Technologies, Santa Clara, CA, USA) was provided by Professor Doil Choi. *C. annuum* 'Bukang' cDNA library screening was conducted as described in the manufacturer's instruction (Agilent Technologies, Santa Clara, CA, USA). The prey vector (pAD-GAL4-2.1 vector) was transformed into YRG-2 containing the bait vector (pBD-GAL4 Cam vector), and was incubated in the selection media lacking tryptophan and leucine (SD-Trp, Leu) for 5 days at 30℃. After co-transformation, each colony was picked and streaked in the selection media lacking tryptophan, leucine and histidine (SD-Trp, Leu, His) for 5 days at 30℃. Empty vector pAD-GAL4-2.1 was used as a negative control. pLAM5'-1/pAS2-1 and pTD1-1/pACT2 (Clontech, Mountain View, CA, USA) were used as a

negative control, and pVA3-1/pAS2-1 and pTD1-1/pACT2 were used as a positive control.

β-galactosidase filter lift assay

To identify interaction between candidate cDNAs and the CMV-P1 RNA1 helicase domain, candidate clones were incubated in the liquid selection media lacking tryptophan, leucine and histidine (SD-Trp, Leu, His) for 3 days at 30℃ in a shaking incubator. After 3 days, each cell culture was adjusted to OD_{600} of 0.2 and incubated for 4 days at 30℃ until the colonies were 0.4 - 0.7 mm in diameter. A 3MM paper (Whatman, Maidstone, Kent, UK) was contacted with all of the clones. The 3MM paper was dipped in liquid nitrogen for 15 seconds and thawed for 1 minute. This step was repeated three times and the 3MM paper soaked in the Z buffer (pH 7.0) with X-gal. It was then incubated for 8 hours at 25℃ in the dark.

Cloning and sequencing

For PCR screening, primers were designed based on the pAD-GAL4-2.1 vector multiple cloning site (MCS) sequence: 5’-CCCCACCAAA CCCAAAAAAAGAG-3’ and 5’- GTTTTTCAGTATCTACGATTCATAGA TCT-3’ (Table 1). Using these primers, the DNA fragments were amplified from

the yeast clones containing candidate cDNAs by colony PCR. The PCR products were eluted for cloning. The T-Blunt PCR cloning system (Solgent, Daejeon, South Korea) was used for TA cloning. The ligated DNA fragments were transformed into *E. coli* strain DH10B and were incubated in LB media containing antibiotics (50 mg/L kanamycin) for selection. To confirm the cloning of PCR fragments DNA seuqence, colony PCR was performed using AD vector specific primers. The clones containing cDNAs fragments were incubated in liquid LB media containing antibiotics (50 mg/L kanamycin) for 1 day at 37℃ in a shaking incubator. Plasmids were isolated from cultured cells using AccuPrep® Plasmid Mini Extraction Kit (Bioneer, Daejeon, South Korea) and sequences were determined (NICEM, Seoul National University, Seoul, South Korea).

Sequence analysis of candidate genes

To predict the functions of DNA and candidate genes, nucleotide sequences were blasted against the Genbank database at the National Center for Biotechnology Information (NCBI, Bethesda, MA, USA). Copy numbers and full length sequences of the candidate genes were obtained from *Capsicum annuum* genome Database (Molecular Genomics Laboratory at Seoul national university, Seoul, South Korea).

RNA extraction RT-PCR for cDNA synthesis

Total RNA was extracted from leaves of *C. annuum* 'Bukang' using GeneAll^{R} Hybrid-R^{TM} (GeneAll Biotechnology, Seoul, South Korea) according to the manufacturer's protocol. First-strand cDNA was synthesized from 4 μg of total RNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA) and oligo-(d)T primers (Bioneer, Daejeon, South Korea)according to the manufacturer's protocol.

Vector construction for VIGS

The candidate genes were amplified from the *C. annuum* 'Bukang' cDNA using gene-specific primers. The gene-specific primers were designed based on *C. annuum* genome database (Molecular Genomics Laboratory at Seoul national university, Seoul, South Korea) (Table 1). A modified ligation-independent cloning system was used for cloning of the inserts into the TRV VIGS vector (Dong et al., 2007). All PCR products were purified by DNA Clean & Concentrator^{TM}-100 (Zymo Research, Irvine, CA , USA), and the TRV2-LIC vector was digested with *Pst1*. Fifteen fmol of the PCR products were mixed with 5 mM dATP, and treated with T4 DNA polymerase (Novagen, Darmstadt, Germany). The treated TRV2-LIC vector 22.5 fmol was mixed with 5mM dTTP, and treated with T4 DNA polymerase. After inactivating the T4 DNA polymerase, the inserts and the TRV2-LIC vector were mixed in a 5:1

ratio and incubated. The 3 ul of the mixture was transformed into *E. coli* DH10Band transformed colonies were selected by colony PCR using LIC primers (Table 1). Plasmids were extracted from identified colonies and sequenced.

Table 1. List of primers used in this study

Name	Sequence (5' → 3')[1]	Remark
ADMCS_F	CCCCACCAAACCCAAAAAAAGAG	PCR screening
ADMCS_R	GTTTTTCAGTATCTACGATTCATAGATCT	PCR screening
6_LIC_F	CGACGACAAGACCCTTCAACAATCCCTTCACTTC	TRV-LIC
6_LIC_R	GAGGAGAAGAGCCCTACTGCAACATGGCTCC	TRV-LIC
17_LIC_F	CGACGACAAGACCCTCTGGGACTACCATTGATG	TRV-LIC
17_LIC_R	GAGGAGAAGAGCCCTAGCGGACATGTTTCCA	TRV-LIC
20_LIC_F	CGACGACAAGACCCTTATCCGATCAAAAAGGATC	TRV-LIC
20_LIC_R	GAGGAGAAGAGCCCTTCATCCCAGTGATCAGG	TRV-LIC
66_LIC_F	CGACGACAAGACCCTGTCATTTGAAGATTCAATCTT	TRV-LIC
66_LIC_R	GAGGAGAAGAGCCCTTTGCAGCTAAAACCTCC	TRV-LIC
76_LIC_F	CGACGACAAGACCCTCGGCTGAGAGGTGGTA	TRV-LIC
76_LIC_R	GAGGAGAAGAGCCCTTCTGAACTCTCAACCTCCA	TRV-LIC
94_LIC_F	CGACGACAAGACCCTCTTTCCCTAACCTTTTTCA	TRV-LIC
94_LIC_R	GAGGAGAAGAGCCCTAGACCCTGAGTGTTCTGG	TRV-LIC
123_LIC_F	CGACGACAAGACCCTGGAGGACGAGAACGAGAA	TRV-LIC

123_LIC_R	GAGGAGAAGAGCCCTTCTCCACAAAGGACGAATC	TRV-LIC
132_LIC_F	CGACGACAAGACCCTCTTGTTCGTGAAATTGCT	TRV-LIC
132_LIC_R	GAGGAGAAGAGCCCTTCCACAACCATTGATACCT	TRV-LIC
144_LIC_F	CGACGACAAGACCCTAGGAAGTGGTTATTTTGAT	TRV-LIC
144_LIC_R	GAGGAGAAGAGCCCTACAAAGATTTCAAGTTCTTC	TRV-LIC
4178_LIC_F	CGACGACAAGACCCTAAAGAGAGGAACATTCGTT	TRV-LIC
4178_LIC_R	GAGGAGAAGAGCCCTGTCATTTCCTCCCTTGTAT	TRV-LIC
LIC_insert_F	TGTTACTCAAGGAAGCACGATGAGCT	Sequencing
LIC_insert_R	CAGGCACGGATCTACTTAAAGAACGTAG	Sequencing
Nb_actin_F	CCAGGTATTGCTGATAGAATGAG	Actin
Nb_actin_R	CTGAGGGAAGCCAAGATAGAG	Actin

[1]Adaptor sequences for LIC cloning are underlined.

Virus-induced gene silencing

Agrobactera containing TRV1 and 10 TRV2::candidate gene (or TRV2::PDS) were incubated in liquid LB media containing antibiotics (50 mg/L kanamycin and 50 mg/L rifampicin) for 20 hours at 30℃. The *Agrobacterium* cells were harvested and resuspended in infiltration media (10 mM $MgCl_2$, 10mM MES, 200 mM acetosyringone). *Agrobacterium* cells containing TRV2::PDS orTRV2::Candidate gene were adjusted to 0.4 OD_{600} and incubated at room temperature with shaking for 4 hours. *Agrobacterium* cells containing TRV1 was adjusted to 0.3 OD_{600} and incubated as described previously. TRV1 and TRV2::PDS (or 10 TRV2::Candidate gene) were mixed in a 1:1 ratio and infiltrated into *N. benthamiana* at the four-leaf stage by a 1 ml syringe needle. At 12 days after agro-infiltration, the silenced plants were used for further experiments

Semi quantitative RT-PCR analysis

RNA was extracted from leaves of silenced *N. benthamiana* plants and first-strand cDNA was synthesized to analyze the expression levels of candidate genes in silenced plants, gene-specific primers were designed using outside sequences of the genic region used for silencing (Table 2). The gene-specific primers were designed based *on N. benthamiana* homologs of pepper candidate

genes. Actin transcript was used as an endogenous control (Table 1) (Yeom et al., 2012).

Table 2. List of primers for gene silencing

Name	sequence(5’ → 3’)[1]	Application
NB_6_F	AAGGACAGGATAGGGTTCAGTATG	qRT-PCR
NB_6_R	CAAATTGTTGAAGGATATAGGCATC	qRT-PCR
NB_17_F	AAAGCAAACGAATATGCTGAAAT	qRT-PCR
NB_17_R	CCAGTGACCTCTGCTACTGTCAA	qRT-PCR
NB_20_F	TTCTTTGAAGAAAGATTTGATGATG	qRT-PCR
NB_20_R	ATAACATGAAGTTTCTTCGTCTGTGT A	qRT-PCR
NB_66_F	CCCAGACTCCAGGGGATG	qRT-PCR
NB_66_R	GGTTTCTCAGCAAATTGGATGAT	qRT-PCR
Nb_132F	CATAAAGTTCCTCTTCTCCTTCTGTAG T	qRT-PCR
Nb_132R	ACGAAGAGCAACGGTTCCA	qRT-PCR
Nb_144_F	GAGGACGTCGTACAGGCATG	qRT-PCR
Nb_144_R	GCAACACAGTAACGGATCTCTTC	qRT-PCR
Nb_4178_F	CATGAGATCTACGAGTCCGAGAG	qRT-PCR
Nb_4178_R	AAACAAAACGTACTACATTCATATTAT AATTTT	qRT-PCR

Inoculum preparation and virus inoculation

Agrobacterium tumefaciens strain GV2260 containing CMV-P1-GFP constructs were incubated in 3 ml LB media containing antibiotics (50 mg/L kanamycin and 50 mg/L rifampicin) at 30℃. After harvest, each cell culture was adjusted to 0.4 OD_{600}, and incubated for 4 hours. Then cell cultures were mixed in a 1:1:1 ratio and infiltrated into *N. benthamiana*. CMV-P1-GFP inoculum was prepared from infected *N. benthamiana* leaves after 14 days of inoculation. One gram of leaves was ground in 10 ml of 0.1 M potassium phosphate buffer (pH 7.5). Plants were inoculated by rub-inoculation with light carborundum dusting. Plants were kept in a growth chamber at 25℃ until symptom observation. Virus accumulation was tested at 5 and 10 days post inoculation (dpi) using DAS-ELISA according to the manufacturer's instructions (Agdia, Santa Fe Springs, CA, USA). Each sample was measured at 405 nm absorbance value.

Observation of virus infection using GFP

GFP fluorescence were observed in inoculated and upper leaves of virus inoculated plants at 5 and 10 dpi using a confocal laser-scanning microscope (Carl Zeiss-LSM510, Seoul National University, Seoul, South Korea). TRV::00 plants were used as a positive control.

RESULTS

Isolation of candidate genes interacting with CMV helicase domain

C. annuum 'Bukang' is resistant to CMV-P0 strains (CMV-Kor and CMV-Fny), but susceptible to CMV-P1. Kang et al (2012) showed that the 3' region of CMV-P1 RNA1 helicase domain is responsible for overcoming *Cmr1* and plays a role in viral replication and systemic infection (Kang et al., 2012). To identify host genes interacting with the CMV-P1 RNA1 helicase domain, a yeast two-hybrid library was screened using CMV-P1 helicase domain as a bait. A total of 100,080 yeast transformants were screened on the selection media lacking tryptophan, leucine and histidine (SD-Trp, Leu, His) and 156 candidate clones were selected. When 156 candidate clones were subjected to a β-galactosidase filter lift assay to confirm true interaction, only 82 clones showed positive response whereas other 74 clones failed to show the interaction with the CMV-P1 helicase domain. The resulted 82 clones interacting with the CMV-P1 helicase domain were used for isolation of genes (Table 3).

Sequence analysis of candidate genes

To obtain sequences of 82 candidate clones colony inserts were obtained by PCR using the primers designed based on the MCS sequence of the pAD-GAL4-2.1 vector. Among the 82 clones tested, inserts were successfully obtained

from 80 clones and amplified PCR products were used for sequence analysis (Table 3). The sequence analysis revealed that 78 candidate clone contained various kinds of genes. The inset size was varied from the smallest 401 bp to the largest 1498 bp. Through the blast search, host genes were classified by their functions, (Table 4). According to the results of NCBI DB and previous research, ten genes were identified that these genes are related to pathogenesis. Thirty five clones contained nucleotide sequences with unknown function. Three clones contained vector or *E.coli* sequence. Ten clones containing genes related to pathogensis were selected for further characterization These genes are *acireductone dioxygenase*, *ADP-glucose pyrophosphorylase*, *ADP-ribosylation factor 1*, *ADP-ribosylation factor*, *calreticulin-3 precursor*, *cysteine synthase*, *formate dehydrogenase*, *histone-H3*, *phosphomannomutase*, and *polyubiquitin 6PU11* (Table 6).

To obtain full length sequences and check copy number of the genes in the pepper genome, the sequences were blasted against pepper genome database. All candidate genes were turned out to be a single copy gene in pepper. Small sequence differences were detected between sequences obtained in this study and DB sequences because database sequences were generated from *C. annuum* 'CM334' whereas yeast two-hybrid library were made from RNA of *C. annuum* 'Bukang'.

Table 3. Summary of screening of host genes interacting with the CMV-P1 helicase domain

Total number Of clones	Number of selected clones			
	Y2H screening	β-galactosidase filter lift assay	PCR screening	Sequence analysis
100,080	156	82	80	78

Table 4. List of host genes identified by a yeast two-hybrid analysis

Clone name	Insert size	Putative function	Species of functional study	Number of clones[1]	Relation with pathogenesis
11-2-21	1488	*Ethylene-responsive element binding protein*	*Capsicum annuum*	1	
11-2-4178	586	*Phosphomannomutase*	*Solanum lycopersicum*	1	Viral pathogen
11-3-17	401	*Formate dehydrogenase*	*Solanum lycopersicum*	2	Viral pathogen
12-1-20	1176	*Calreticulin-3 precursor*	*Zea mays*	1	Viral pathogen
12-1-32	671	*Antimicrobial peptide snakin*	*Capsicum annuum*	1	
12-2-44	813	*Photosystem I subunit XI*	*Nicotiana attenuata*	1	
12-2-59	694	*Histone H2B*	*Capsicum annuum*	1	
12-3-76	1105	*Hexameric polyubiquitin 6PU11*	*Capsicum annuum*	1	Viral pathogen
12-4-77	913	*24K germin like protein*	*Nicotiana tabacum*	1	
12-5-4	963	*Chloroplast ferredoxin-$NADP^+$ oxidoreductase*	*Capsicum annuum*	1	
12-5-6	1011	*Cysteine synthase*	*Capsicum annuum*	1	Viral pathogen
12-5-12	982	*Methylketone synthase Ib*	*Solanum lycopersicum*	1	
12-6-37	734	*40S ribosomal protein S12*	*Vitis vinifera*	1	
12-6-45	805	*Photosystem I reaction centre PSI-D subunit precursor*	*Solanum tuberosum*	1	

12-6-49	611	*Thionin-like protein*	*Nicotiana tabacum*	1	
12-6-53	1387	*Ribosomal protein L3*	*Solanum lycopersicum*	1	
12-7-57	1158	*Membrane channel protein*	*Medicago sativa*	1	
12-7-64	759	*Translation factor SUI1*	*Brachypodium distachyon*	1	
12-7-68	902	*Secretory peroxidase*	*Nicotiana tabacum*	1	
12-8-66	801	*ADP-glucose pyrophosphorylase*	*Lycopersicon esculentum*	1	Viral pathogen
12-8-83	1616	*Polyketide cyclase/dehydrase and lipid transport protein*	*Arabidopsis thaliana*	1	
12-8-94	770	*ADP-ribosylation factor 1*	*Nicotiana benthamiana*	1	Fungal pathogen
12-8-98	849	*Enolase*	*Solanum lycopersicum*	2	
12-9-42	819	*Membrane steroid-binding protein*	*Medicago truncatula*	1	
12-9-102	788	*Ribulose 1,5-bisphosphate carboxylase*	*Capsicum annuum*	1	
12-9-123	957	*ADP-ribosylation factor*	*Capsicum annuum*	1	Fungal pathogen
12-10-106	796	*Peptide transporter PTR3-A*	*Medicago truncatula*	1	
12-10-107	725	*Ribosomal protein L12*	*Nicotiana tabacum*	1	
12-10-108	747	*Glycine-rich RNA-binding protein*	*Nicotiana sylvestris*	1	
12-11-114	648	*Glycoprotein-like protein*	*Solanum tuberosum*	1	
12-11-116	931	*Photosystem II 23 kda protein,*	*Solanum lycopersicum*	1	
12-12-119	689	*Ribosomal protein L25*	*Solanum tuberosum*	1	

12-12-132	673	*Histone H3*	*Capsicum annuum*	1	Virus infection
12-13-18	911	*18S ribosomal protein*	*Lycium barbarum*	1	
12-13-52	823	*TCP transcription factor 14*	*Solanum lycopersicum*	1	
12-13-144	733	*Acireductone dioxygenase*	*Solanum tuberosum*	1	Fungal pathogen
12-17-124	637	*40S ribosomal protein S23*	*Ricinus communis*	1	
12-17-125	1498	*NADP-dependent Isocitrate dehydrogenase*	*Nicotiana tabacum*	1	
		Unknown function		35	
		False sequence		3	

[1]A number of repetition that included same sequence

Table 5. List of candidate genes interacting with the CMV-P1 helicase domain.

Candidate clones	NCBI ID	Function	Copy number	Reference
11-2-4178	NM_001246872.1	*Phosphomannomutase*	1	Hung et al., 2002; Cotter et al., 2008; Deepak et al., 2008
11-3-17	NM_001247928.1	*Formate dehydrogenase*	1	Zuo et al., 2005; Senthil et al., 2005; Valcu et al., 2009; David et al., 2010
12-1-20	EU961843.1	*Calreticulin-3 precursor*	1	Whitham et al., 2003; Chen et al., 2005
12-3-76	AY496112.1	*Hexameric polyubiquitin 6PU11*	1	Reichel et al., 2000
12-5-6	AB029512.2	*Cysteine synthase*	1	Yang et al., 2007; Polesani et al., 2008
12-8-66	U85496.1	*ADP-glucose pyrophosphorylase*	1	Sindelar et al., 1982; Schaad et al., 2000
12-8-94	DQ531849.1	*ADP-ribosylation factor 1*	1	Lee et al., 2003
12-9-123	AF108891.1	*ADP-ribosylation factor*	1	Lee et al., 2003
12-12-132	AY491505.1	*Histone-H3*	1	Tesar et al., 1990; Kong et al., 2002; Senthil et al., 2005; Zhou et al., 2011
12-13-144	AK323870.1	*Acireductone dioxygenase*	1	Mukhtar et al., 2008

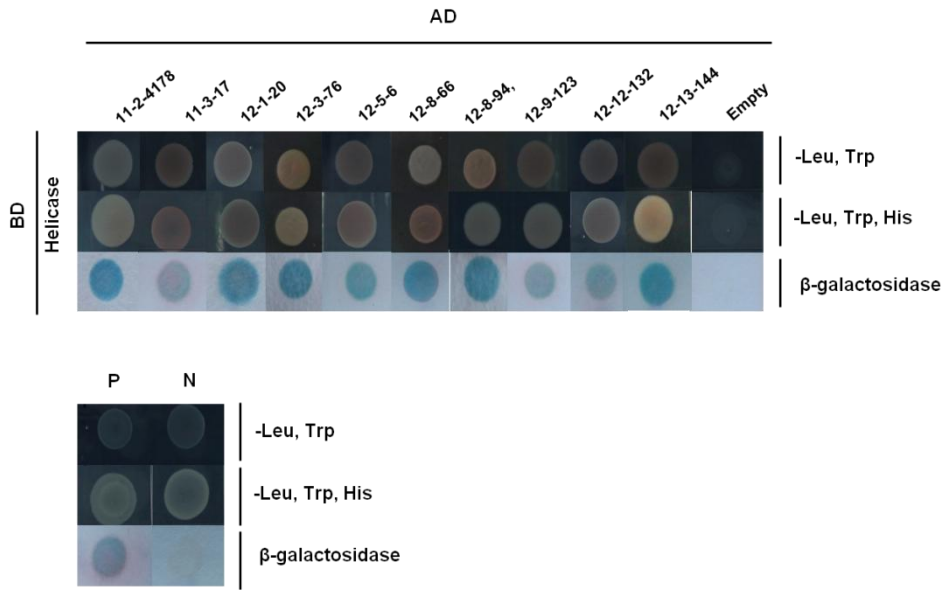


Figure 1. Interactions of candidate clones and the CMV-P1 RNA1 helicase domain using β-galactosidase filter lift assay.

The blue color development resulted from β-galactosidase activity started within 8 hours. AD and BD represent activation domain and BD binding domain, respectively. 11-2-4178, 11-3-17, 12-1-20, 12-3-76, 12-5-6, 12-8-66, 12-8-94, 12-9-123, 12-12-132 and 12-13-144 are clone numbers. Empty is a bait vector alone and P and N are positive and clones provided by Clontech (Mountain View, CA, USA).

Silencing selected genes and observation of plant development

In the previous studies, host proteins interacting with viral proteins played roles at different steps of virus life such as virus replication, cell-to-cell movement, and systemic movement (Huang et al., 2012; Vijayapalani et al., 2012). Therefore, it was expected that the candidate genes identified in this study may also play a certain role in virus infection. If selected genes are required for virus infection, knock-down of the expression of the selected genes will result in suppression of virus infection. It is also expected that silencing of the genes could result in developmental defects if this gene is essential for plant survival. To test the requirement of selected genes for CMV infection and plant development, the selected genes were silenced by a TRV-based VIGS system. Ten selected genes were cloned into the TRV2-LIC vector. Each gene was silenced in *N. benthamiana* and the effects of silencing the genes in plant growth were observed. *Phytoene desaturase* (PDS)-silenced plants were used as a silencing control. As expected, the silencing of PDS resulted in photo-bleaching at 10 to 12 dpi (data not shown). To check the level of silenced gene expression, semi qRT-PCR using gene-specific primers (Table 2) was performed. Infiltrated TRV::00 plant was used for comparison with candidate gene-silenced plants. *N. benthamiana* actin was used for actin quantification. qRT-PCR analysis showed that expression levels of 6 candidate genes (*cysteine synthase*, *formate dehydrogenase*, *calreticulin-3 precursor*, *ADP-glucose pyrophosphorylase*, *acireductone dioxygenase*, *phosphomannomutase*) were significantly reduced, whereas expression level of

histone-H3 was not reduced. In addition, when co-silencing of selected genes was tested, silencing of one gene did not affect other gene expression (Figure 3).

Silenced plants showed various developmental phenotypes depending on the silenced gene, which include curved leaves, stunting, and arrested growth. To observe development phenotype, silenced plants were compared with wild type *N. benthamiana*. Among 10 genes, silencing of three genes including *ADP-ribosylation factor 1*, *ADP-ribosylation factor*, and *polyubiquitin 6PU11* showed severe growth inhibition at 6 dpi, and leaf color was changed green to yellow at 10 dpi. Eventually, the silenced plants died at 12 dpi. TRV::00 plant showed typical TRV symptoms such as feeble curved leaves, and the plants grew slower than wild type. The rest of silenced plants including *acireductone dioxygenase, ADP-glucose pyrophosphorylase, calreticulin-3 precursor, cysteine synthase, formate dehydrogenase, histone-H3,* and *phosphomannomutase* showed similar phenotype with wild type (Figure 2).

The results demonstrate that *ADP-ribosylation factor 1*, *ADP-ribosylation factor*, and *polyubiquitin 6PU11* are essential factors for plant growth in *N. benthamiana.* The 7 candidate genes do not affect plant growth, and therefore they can be useful in studying virus infection of *N. benthamiana.*.

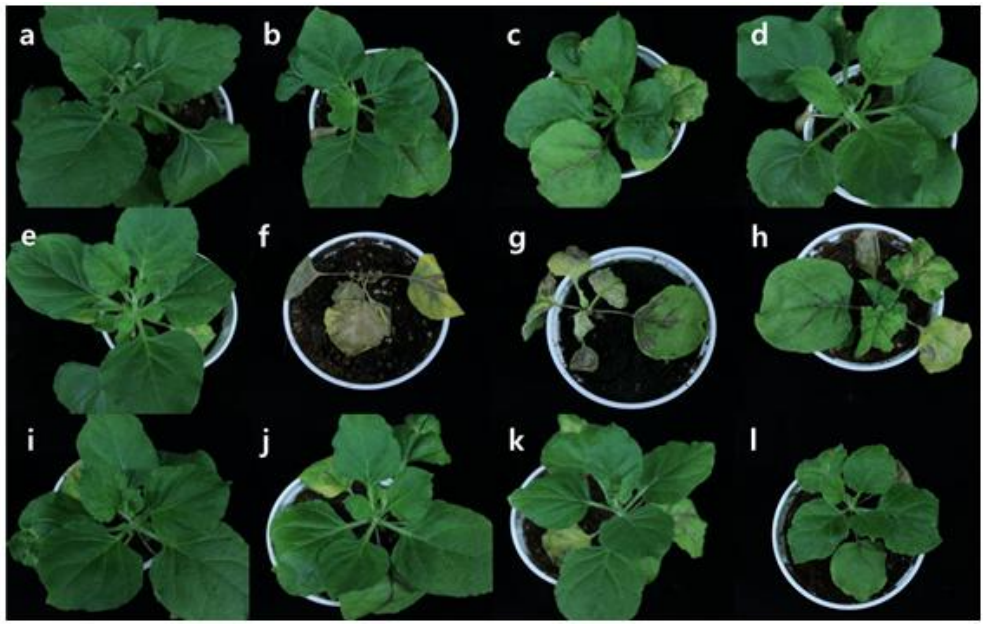


Figure 2. Developmental phenotypes of *N. benthamiana* plants silenced with genes encoding putative host factors.

Wild-type (a), phenotype of *cysteine synthase* (b), *formate dehydrogenase* (c), *calreticulin-3 precursor* (d), *ADP-glucose pyrophosphorylase* (e), *polyubiquitin 6PU11* (f), *ADP-ribosylation factor 1* (g), *ADP-ribosylation factor* (h), *histone-H3* (i), *acireductone dioxygenase* (j), *phosphomannomutase* (k)-silenced *N. benthamiana*, TRV::00 plant (l) at 12 dpi.

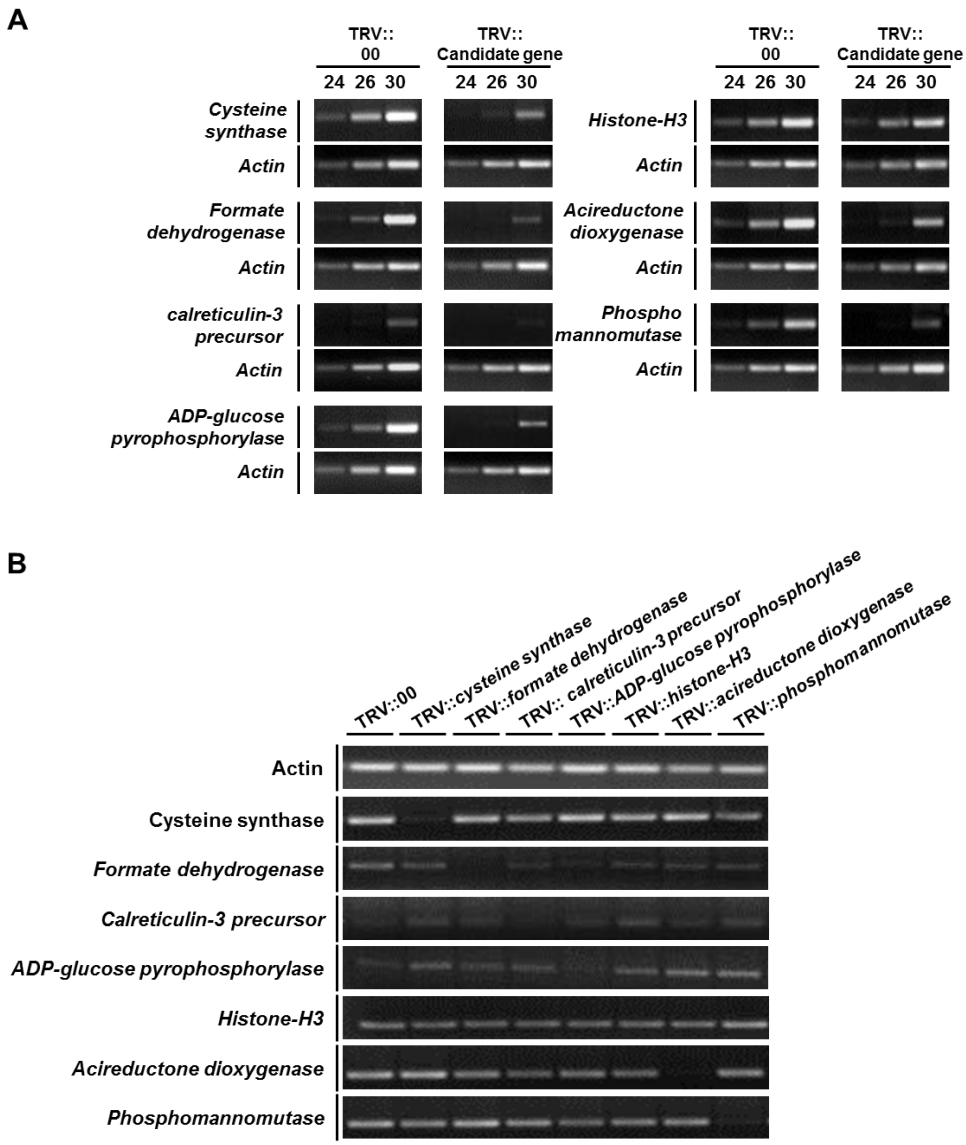


Figure 3. Semi-quantitative real-time PCR analysis of host factor gene expression of in silenced plants.

(A) The mRNA expression level of each candidate gene was evaluated by semi qRT-PCR analysis at 12 dpi. To normalize the expression level, *N. benthamiana* actin transcript was used as control. The words which are located left side of images is candidate gene-specific primers. Each candidate gene cDNA was amplified at 24, 26, and 30 cycles by PCR. **(B)** Identification of co-silencing at 26 cycles by PCR.

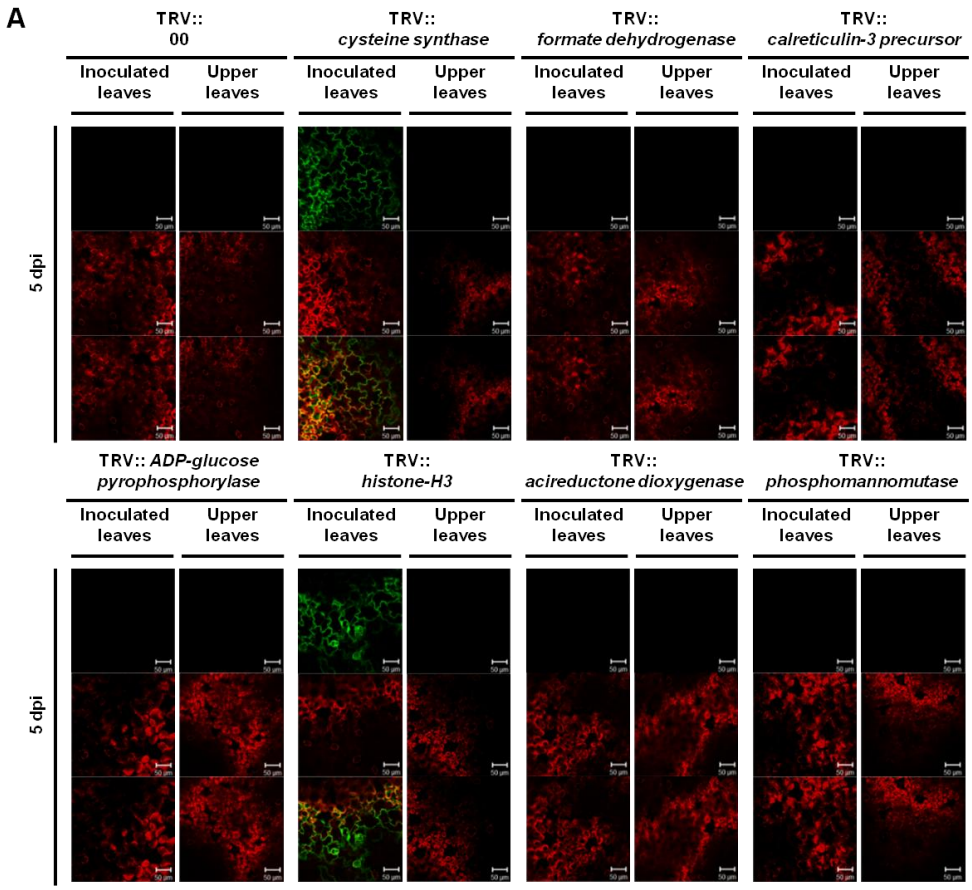
Functions of candidate genes in CMV infection

To investigate whether selected genes play a key role in CMV-P1 infection, gene-silenced plants were challenged with CMV-P1. After infection, virus spreads in plants were observed under a confocal laser-scanning microscope. GFP fluorescence in inoculated and systemic leaves of silenced plants were monitored at 5 dpi and 10 for systemic movement. TRV::00 plant was used as a positive control. At 5 dpi, there was no CMV-mediated GFP signal in the inoculated leaves of TRV::00 plant whereas *cysteine synthase* and *histone-H3*-silenced plants showed GFP signals in the inoculated leaves. Plants silenced with other genes did not show any GFP signal. TRV::00 plants showed GFP signals in the whole inoculated leaves at 10 dpi as well as upper leaves. In the case of *cysteine synthase* silenced plants, CMV—mediated GFP signal was detected in the whole upper leaves. *Formate dehydrogenase* silenced plants showed local infection in inoculated leaves and no CMV-P1-GFP signal in upper leaves. In the case of *calreticulin-3 precursor* silenced plants, there was no CMV-P1-GFP signal in both inoculated and upper leaves. *ADP-glucose pyrophosphorylase* silenced plants showed CMV-P1-GFP signals in only inoculated leaves whereas *cysteine synthase*, *histone-H3, acireductone dioxygenase* and *phosphomannomutase*-silenced plants showed CMV-P1-GFP signals both in inoculated and upper leaves (Figure 4).

To confirm the virus infection in the candidate gene-silenced plants, CMV-P1 CP was detected by ELISA at 5 and 10 dpi. Two leaf discs of the inoculated and the upper leaves of the inoculated plants were used. Inoculated wild type plants were used as a positive control. Mock inoculated wild type plants were used as a negative control. As was observed in confocal microscopy analysis,

accumulation of CMV-P1-GFP was increased significantly in the inoculated and upper leaves of *cysteine synthase*-silenced plants. In contrast, *formate dehydrogenase*-silenced plants showed small amount of CMV-P1 CP compared to the wild type. *Calreticulin-3 precursor*–silenced plants showed very low amount of CMV-P1 CP in the inoculated and upper leaves (Figure 5). In the leaves of *formate dehydrogenase*-silenced and *calreticulin-3 precursor*–silenced plants, CMV-P1 coat protein accumulation were greatly reduced and the same results were obtained in the upper leaves. According to the results, these 2 genes support the replication of CMV-P1.

Taken together, these results strongly suggest that *formate dehydrogenase* and *Calreticulin-3 precursor* plays a positive role in CMV-P1 replication.


A
TRV:: 00
TRV:: cysteine synthase
TRV:: formate dehydrogenase
TRV:: calreticulin-3 precursor
Inoculated leaves
Upper leaves
5 dpi
TRV:: ADP-glucose pyrophosphorylase
TRV:: histone-H3
TRV:: acireductone dioxygenase
TRV:: phosphomannomutase

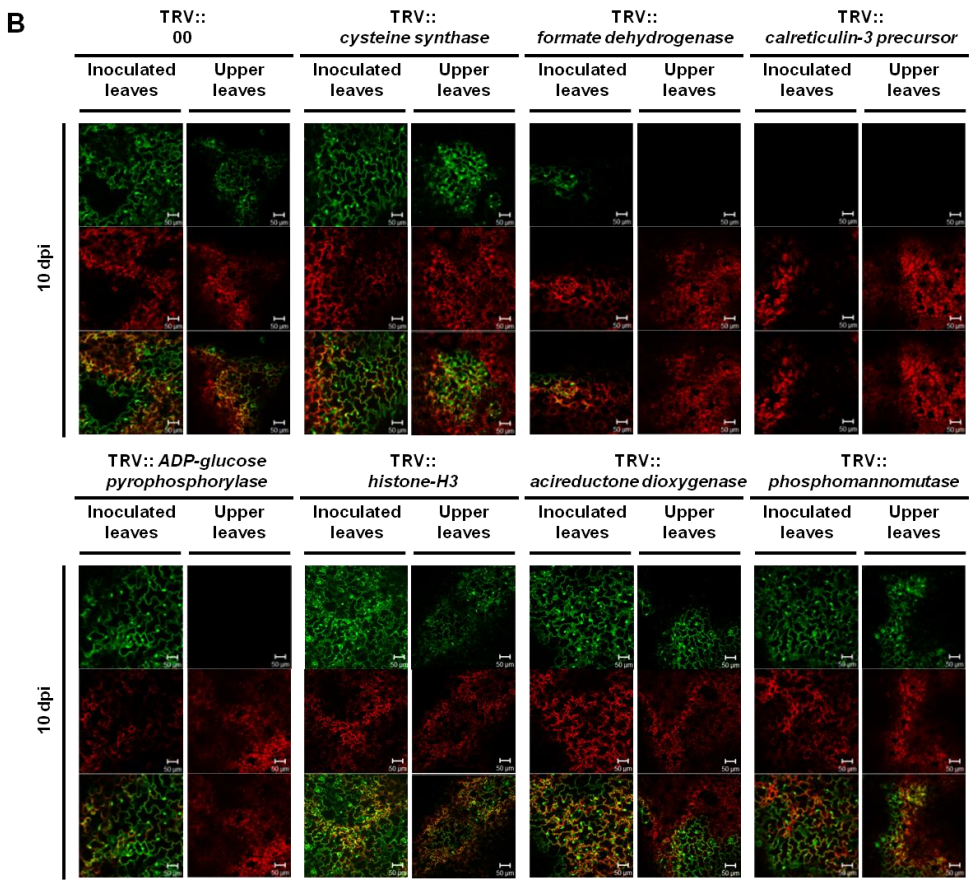


Figure 4. Monitoring CMV replication and movement by GFP fluorescence in inoculated and upper leaves of silenced plants inoculated with CMV-P1-GFP.

(A) GFP fluorescence was observed at 5 dpi in candidate gene-silenced plants, and TRV::00 plant was used as a positive control. **(B)** GFP fluorescence was observed at 10 dpi in candidate gene-silenced plants. Images on the left panel are optical sections of the inoculated leaves, and on the right panel are optical sections in the upper leaves. Images top panel to bottom are GFP, auto fluorescence, and merged image, respectively. The green fluorescence signal indicates CMV-P1 expressing GFP, and red fluorescence signal indicates chloroplast. Scale bars = 50 μm.

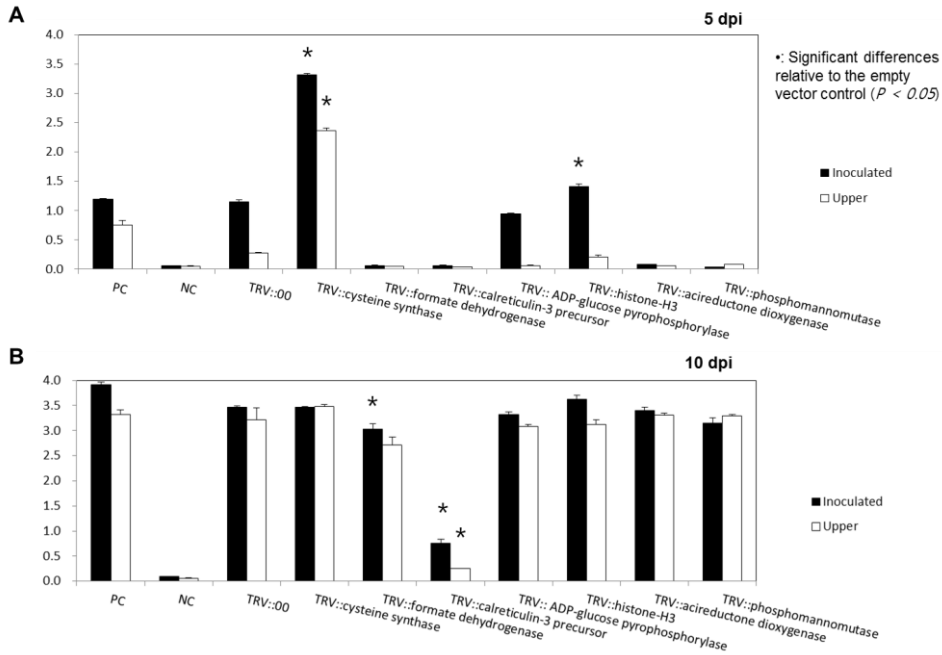


Figure 5. CMV accumulation in plants silenced with candidate host factors.

Virus accumulation was detected by ELISA. **(A)** Two leaf discs of the inoculated and the upper leaves of the inoculated plants were sampled at 5 dpi. **(B)** Two leaf discs of the inoculated and upper leaves of the inoculated plants were sampled at 10 dpi. PC (Positive control), NC (negative control), * indicates significant differences relative to the empty vector control ($P < 0.05$).

DISCUSSION

In the present study, the results showed that CMV-P1 helicase domain interacted with novel host factors which are essential for CMV infection. A systemic approach using a yeast two-hybrid system and virus induced gene silencing revealed that CMV-P1 helicase interacted with various kinds of genes, and *formate dehydrogenase* and *calreticulin-3 precursor* are most critical among the host factors .

Viruses have limited genome and require host factors to support their infection cycle, and viral components interaction with host factors (Ahlquist et al., 2003). By disrupting these interactions, genetic resistance can be engineered. Especially cellular-level resistance is effective, and can be induced by silencing targeted host gene. Several host factors which interact with CMV have been reported in different host plants and viruses(Kang et al., 2005, Whitham et al., 2004). In this study, to understand the virus infection mechanism and to develop virus resistant crops, host factors interacting with CMV-P1were identified.

In the previous study, one of host genes in tobacco, *NtTLP1*, which directly interacts with *Cucumber mosaic virus* (CMV) 1a protein, were identified and showed an important role in CMV replication and movement (Kim et al., 2005). *Tcoi1* directly interacts with CMV 1a methyltransferase (MT) domain in tobacco, and overexpression of *Tcoi1* enhanced the CMV infection, while silencing of *Tcoi1* decreased virus infection (Kim et al., 2008). This study focused on CMV 1a, specifically methyltransferase (MT) domain. In this study, helicase domain were

used to identify interacting host genes. CMV helicase domain includes distinguishing motifs; NTP-binding region (motifs Ⅰand Ⅲ), ATPase activity, RNA binding activity (motif Ⅵ). These motifs affect CMV 1a function, and control RNA2 and 3 replication (Kadaré et al., 1997 Roossinck et al., 1997). Kang et al (2012) showed that mutations several amino acids in the helicase domain enabled CMV-FNY to replicate and regulate systemic movement in the resistant cultivar (Kang et al., 2012). Therefore, identification of host factors interacting with helicase domain will elucidate the CMV infection mechanism. Y2H hybrid screening revealed various host genes interacting with CMV-P1 helicase domain. Among the host two host genes *formate dehydrogenase* and *calreticulin* were characterized further.

formate dehydrogenase is one of the most abundant soluble proteins in mitochondria, and regulates the concentration of one-carbon metabolites in plant. *Formate dehydrogenase* also regulates stress-signaling pathway, and has also been reported to be a product of CO_2 reduction under certain conditions of photosynthesis (Hermana et al., 2002). Although this protein has not been studied much in terms of biotic stresses, there are several reports showing this gene is required for pathogenesis. *Formate dehydrogenase* was up-regulated by *Sonchus yellow net virus* (SYNV) and *Impatiens necrotic spot virus* (INSV) in *N. benthamiana* (Senthil et al., 2005). After *Verticillium dahliae* infection, 131 EST are overexpressed, one of which was identified as *formate dehydrogenase* (Zuo et al., 2005). And *formate dehydrogenase* of *Phaseolus vulgaris* was up-regulated by fungus *Collectotrichum lindemuthianum* (David et al., 2010). This gene was also upregulated in *Fagus sylvatica* seedlings by infection with the root pathogen

Phytophthora citricola (Valcu et al., 2009). These results show that *formate dehydrogenase* is important factor for pathogenesis in plants. In this study, the results indicated interaction between *formate dehydrogenase* and CMV helicase domain is necessary, and further study is required to reveal .

calreticulin is located in the ER membrane and nucleus, suggesting that it may play a role in transcription regulation. After inoculation of *Turnip vein clearing virus* (TVCV), *Oilseed rape mosaic virus* (ORMV), *Potato virus X* (PVX), CMV strain Y, or TuMV in *Arabidopsis*, *calreticulin* expression was up-regulated (Whitham et al., 2003). Also, *calreticulin* was identified to interact with TMV MP in *N. tabacum*, and overexpressed *calreticulin* interferes with MP function (Chen et al., 2005). These results show that various viruses utilize *calreticulin* and this gene may be a common factor in virus infection.

Host genes essential for plant survival cannot be a target for engineering resistance to pathogens. Plants silenced with two host genes showed normal growth as the wild type *N. benthamiana* (Figure 2). These results suggested that *formate dehydrogenase* and *calreticulin-3 precursor* mutation or knockouts may provide a new strategy for breeding CMV resistances in crop plants. To develop resistant plants, plants carrying mutations in these host factors can be obtained by EMS mutant screening or germplasm screening.

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REFERENCES

Ahlquist P, Noueiry AO, Lee WM, Kushner DB and Dye BT. 2003. Host factors in positive-strand RNA virus genome replication. J. Virol. 77:8181-8186.

Bendahmane A, Kanyuka K and Baulcombe DC. 1999. The *Rx* gene from potato controls separate virus resistance and cell death responses. Plant Cell 11:781–792.

Brommonschenkel SH, Frary A and Tanksley SD. 2000. The broad-spectrum tospovirus resistance gene *Sw-5* of tomato is a homolog of the root-knot nematode resistance gene *Mi*. Mol. Plant. Microbe. In. 13:1130–1138.

Carrington JC, Kasschau KD, Mahajan SK, and Schaad MC. 1996. Cell-to-cell and long-distance transport of viruses in plants. Plant Cell 8:1669–1681.

Chen MH and Citovsky V. 2003. Systemic movement of a *tobamovirus* requires host cell pectin methylesterase. Plant J. 35:386–392.

Chen M, Tian G, Gafni Y and Citovsky V. 2005. Effects of *calreticulin* on viral cell-to-cell movement. Plant Physiol. 138:1866–1876.

Cho JD, Kim JS, Lee SH, Choi GS and Chung BN. 2007. Viruses and symptoms on peppers, and their infection types in Korea. Res. Plant Disease 13:75-81.

Choi SK, Yoon JY, Canto T, and Palukaitis P. 2011. Replication of *cucumber mosaic virus* RNA1 in cis requires functional helicase-like motifs of the 1a protein. Virus Res. 158: 271–276

Cotter JD, Storfer A, Page RB, Beachy CK and Voss SR. 2008. Transcriptional response of Mexican axolotls to *Ambystoma tigrinum virus* (ATV) infection. BMC Genomics. 9:493-511.

David P, Francs-Small CC, Se´vignac M, Thareau V, Macadre´ C, Langin T and Geffroy V. 2010. Three highly similar *formate dehydrogenase* genes located in the vicinity of the B4 resistance gene cluster are differentially expressed under biotic and abiotic stresses in *Phaseolus vulgaris*. Theor. Appl. Genet. 121:87–103.

Deepak SA, Shibato J, Ishii H, Ogawa Y, Yoshida Y, Iwahashi H, Masuo Y, Agrawal GK and Rakwal R. 2008. Proteomics approach for investigating the disease resistance using cucumber as model plant. Am. J. Biochem. Biotechnol. 4:231-238.

Dinesh-Kumar SP, Anandalakshmi R, Marathe R, Schiff M and Liu Y. 2003. Virus-induced gene silencing. Meth. Mol. Biol. 236:287-294.

Ding B, Li Q, Nguyen L,Palukaitis P and Lucas WJ. 1995. *Cucumber mosaic virus* 3a protein potentiates cell-to-cell trafficking of CMV RNA in tobacco plants. Virology. 207: 345–353.

Ding SW, Anderson BJ, Haase HR and Symons RH. 1994. New overlapping gene encoded by the *Cucumber mosaic virus* genome. Virology. 198:593–601

Ding XS, Carter SA, Deom CM and Nelson RS. 1998. *Tobamovirus* and *potyvirus* accumulation in minor veins of inoculated leaves from representatives of the *Solanaceae* and *Fabaceae*. Plant Physiol. 116:125–136.

Dong Y, Burch-Smith TM, Liu Y, Mamillapalli P and Dinesh-Kumar SP. 2007. A ligation-independent cloning *tobacco rattle virus* vector for high-

throughput virus-induced gene silencing identifies roles for *NbMADS4-1* and *-2* in floral development. Plant Physiol. 145:1161-1170.

Edwardson JR and Christie RG. 1991. Cucumoviruses. CRC handbook of viruses infecting legumes. CRC Press. 293–319.

Fraser RSS. 1986. Genes for resistance to plant viruses. Crit. Rev. Plant Sci. 3:257–294.

Fraser RSS. 1990. The genetics of resistance to plant viruses. Annu. Rev. Phytopathol. 28:179–200.

Gal-On A, Kaplan I, Roossinck MJ and Palukaitis P. 1994. The kinetics of infection of zucchini squash by *cucumber mosaic virus* indicate a function for RNA 1 in virus movement. Virology. 205:280-289.

Gao Z, Johansen E, Eyers S, Thomas CL, Noel Ellis TH and Maule AJ. 2004. The potyvirus recessive resistance gene, *sbm1*, identifies a novel role for translation initiation factor eIF4E in cell-to-cell trafficking. Plant J. 40:376–385.

Gibb KS, Hellman GM, and Pirone TP. 1989. Nature of resistance of a tobacco cultivar to *Tobacco vein mottling virus*. Mol. Plant. Microbe. In. 2:332–239.

Guerini MN, and Murphy JF. 1999. Resistance of *Capsicum annuum* ‘Avelar’ to pepper mottle *potyvirus* and alleviation of this resistance by co-infection with cucumber mosaic *cucumovirus* are associated with virus movement. Journal of General Virology. 80:2785–2793.

Hajimorad MR, Eggenberger AL and Hill JH. 2005. Loss and gain of elicitor function of *soybean mosaic virus G7* provoking *Rsv1*-mediated lethal

systemic hypersensitive response maps to P3. J. Virol. 79:1215–1222.

Hamalainen JH, Kekarainen T, Gebhardt C,Watanabe KN and Valkonen JP. 2000. Recessive and dominant genes interfere with the vascular transport of Potato virus A in diploid potatoes. Mol. Plant. Microbe. In. 13:402–412.

Hayes RJ and Buck KW. 1990. Complete replication of a eukaryotic virus RNA in vitro by a purified RNA-dependent RNA polymerase. Cell. 63:363–368.

Hermana PL, Ramberga H, Baack RD, Markwell J and Osterman JC. 2002. *Formate dehydrogenase* in *Arabidopsis thaliana*: overexpression and subcellular localization in leaves. Plant Sci. 163:1137–1145.

Huang YW, Hu CC, Liou MR, Chang BY and Tsai CH. 2012. *Hsp90* interacts specifically with viral RNA and differentially regulates replication initiation of *Bamboo mosaic virus* and associated satellite RNA. PLoS Pathog. 8:e1002726.

Huh SU, Kim MJ, Ham BK and Paek KH. 2011. A zinc finger protein *Tsip1* controls *Cucumber mosaic virus* infection by interacting with the replication complex on vacuolar membranes of the tobacco plant. New Phytol. 191:746–762

Hung CH, Wu HC, and Tseng YH. 2002. Mutation in the *Xanthomonas campestris xanA* gene required for synthesis of xanthan and lipopolysaccharide drastically reduces the efficiency of bacteriophage ØL7 adsorption. Biochem. Biophys. Res. Commun. 291:338–343.

Ishikawa M, Obata F, Kumagai T and Ohno T. 1991. Isolation of mutants of *Arabidopsis thaliana* in which accumulation of *tobacco mosaic virus* coat protein is reduced to low levels. Mol. Genet. Genomics. 230:33–38.

Ishikawa M, Naito S and Ohno T. 1993. Effects of the *tom1* mutation of *Arabidopsis thaliana* on the multiplication of *tobacco mosaic virus* RNA in protoplasts. J. Virol. 67:5328–5338.

Kang BC, Yeam I, Frantz JD, Murphy JF and Jahn MM. 2005. The *pvr1* locus in pepper encodes a translation initiation factor eIF4E that interacts with *Tobacco etch virus* VPg. Plant J. 42:392–405.

Kang BC, Yeam I and Jahn MM. 2005. Genetics of plant virus resistance. Annu. Rev. Phytopathol. 43:581-612.

Kang WH, Hoang NH, Yang HB, Kwon JK, Jo SH, Seo JK, Kim KH, Kim D and Kang BC. 2010. Molecular mapping and characterization of a single dominant gene controlling CMV resistance in peppers (*Capsicum annuum L.*). Theor. Appl. Genet. 120:1587–1596.

Kang WH, Seo JK, Chung BN, Kim KH and Kang BC. 2012. Helicase domain encoded by *Cucumber mosaic virus* RNA 1 determines systemic infection of *Cmr1* in pepper. PLoS One 7:e43136.

Kadaré G and Haenni AL. 1997. Virus-encoded RNA helicases. J Virol. 71:2583–2590.

Kiho Y, Machida H and Oshima N. 1972. Mechanism determining the host specificity of *tobacco mosaic virus*. Formation of polysomes containing infecting viral genome in various plants. Jpn. J. Microbiol. 16:451–459

Kim JS, Kim SK, Choi GS and Lee MW. 1990. Virus disease incidence and symptom appearance in red pepper. Korean J. Plant Pathol. 6:125-132.

Kim MJ, Ham BK, Kim HR, Kim IJ, Kim YJ, Ryu KH, Park YI and Paek KH. 2005. In vitro and in planta interaction evidence between *Nicotiana*

tabacum thaumatin-like protein 1 (*TLP1*) and *Cucumber mosaic virus* proteins. Plant Mol. Biol. 59:981–994.

Kim MJ, Ham BK and Paek KH. 2006. Novel protein *kinase* interacts with the *Cucumber mosaic virus* 1a methyltransferase domain. Biochem. Biophys. Res. Commun. 340:228-235

Kim MJ, Huh SU, Ham BK and Paek KH. 2008. A novel methyltransferase methylates *Cucumber mosaic virus* 1a protein and promotes systemic spread. J. Virol. 82:4823–4833

Kong L and Hanley-Bowdoin L. 2002. A *Geminivirus* replication protein interacts with a protein kinase and a motor protein that display different expression patterns during plant development and infection. Plant Cell 14:1817–1832.

Lee MY, Lee JH, Ahn HI, Yoon JY, Her NH, Choi JK, Choi GS, Kim DS, Harn CH and Ryu KH. 2006. Identification and sequence analysis of RNA3 of a resistance-breaking *Cucumber mosaic virus* isolate on *Capsicum annuum*. Plant Pathol. J. 22:265-270.

Lee WY, Hong JK, Kim CY, Chun HJ, Park HC, Kim JC, Yun D, Chung WS, Lee S, Lee SY, Cho MJ and Lim CO. 2003. Over-expressed rice *ADP-ribosylation factor 1* (*RARF1*) induces pathogenesis-related genes and pathogen resistance in tobacco plants. Physiol. Plant. 119:573–581.

Lellis AD, Kasschau KD, Whitham SA and Carrington JC. 2002. Loss-of susceptibility mutants of *Arabidopsis thaliana* reveal an essential role for eIF(iso)4E during *potyvirus* infection. Curr. Biol. 12:1046–1051.

Li Q, Ryu KH and Palukaitis P. 2001. *Cucumber mosaic virus*-plant interactions:

identification of 3a protein sequences affecting infectivity, cell-to-cell movement, and long-distance movement. Mol. Plant. Microbe. In. 14:378–385.

Lu R, Martin-Hernandez AM, Peart JR, Malcuit I and Baulcombe DC. 2003. Virus-induced gene silencing in plants. Methods. 30:296–303.

Lucy AP, Guo HS, Li WX and Ding SW. 2000. Suppression of posttranscriptional gene silencing by a plant viral protein localized in the nucleus. EMBO J. 19:1672–1680.

Mahajan SK, Chisholm ST, Whitham SA andCarrington JC. 1998. Identification and characterization of a locus (*RTM1*) that restricts long-distancemovement of *tobacco etch virus* in *Arabidopsis thaliana*. Plant J. 14:177–186.

Martelli GP and Quacquarelli A. 1983. The present status of tomato and pepper viruses. Acta Horticulturae. 127:39-64.

Matthews REF and Witz J. 1985. Uncoating of *Turnip yellow mosaic virus* in vivo. Virology. 144:318–327.

Motoyoshi F, and Oshima N. 1975. Infection of leaf mesophyll protoplasts from susceptible and resistance lines of tomato. J. Gen. Virol. 29:81–91.

Mukhtar MS, Deslandes L, Auriac MC, Marco Y and Somssich IE. 2008. The *Arabidopsis* transcription factor *WRKY27* influences wilt disease symptom development caused by *Ralstonia solanacearum*. Plant J. 56:935-947.

Murphy JF and Kyle MM. 1995. Alleviation of restricted systemic spread of pepper mottle *potyvirus* in *Capsicum annuum vs. Avelar* by coinfection with a Cucumovirus. Phytopathol. 85:561–566.

Nicaise V, German-Retana S, Sanjuan R, Dubrana MP and Mazier M. 2003. The eukaryotic translation initiation factor 4E controls lettuce susceptibility to the *potyvirus Lettuce mosaic virus*. Plant Physiol. 132:1272–1282.

Nitta N, Takanami Y, Kuwata S and kubo S. 1988. Inoculation with RNAs 1 and 2 of *Cucumber mosaic virus* induces viral RNA replicase activity in tobacco mesophyll protoplasts. J. Gen. Virol. 69:2695-2700.

Ohshima K, Taniyama T, Yamanaka T, Ishikawa M and Naito S. 1998. Isolation of a mutant of *Arabidopsis thaliana* carrying two simultaneous mutations affecting *tobacco mosaic virus* multiplication within a single cell. Virology 243:472–481.

Otsuki Y, Takebe I, Honda Y and Matsui C. 1972. Ultrastructure of infection of tobacco mesophyll protoplasts by *tobacco mosaic virus*. Virology 49:188–194.

Palukaitis P and García-Arenal F. 2003. Cucumoviruses. Adv. Virus. Res. 62:241-323.

Palukaitis P, Roossinck MJ, Dietzgen RG and Francki RI. 1992. *Cucumber mosaic virus*. Adv. Virus. Res. 41:281–348.

Parella G, Ruffel S, Moretti A, Morel C, Palloix A and Caranta C. 2002. Recessive resistance genes against potyviruses are localized in colinear genomic regions of the tomato (*Lysopersicon* spp.) and pepper (*Capsicum* spp.) genomes. Theor. Appl. Genet. 105:855–861.

Pellio B, Streng S, Bauer E, Stein N and Perovic D. 2005. High-resolution mapping of the *Rym4/Rym5* locus conferring resistance to the *barley yellow mosaic virus* complex (BaMMV, BaYMV, BaYMV-2) in barley (*Hordeum vulgare* ssp. *vulgare* L.). Theor. Appl. Genet. 110:283–293.

Polesani M, Desario F, Alberto Ferrarini1, Zamboni A, Pezzotti M, Kortekamp A and Polverari A. 2008. cDNA-AFLP analysis of plant and pathogen genes expressed in grapevine infected with *Plasmopara viticola*. BMC Genomics 9:142-155.

Ponz F, Russel ML, Rowhani A and Bruening G. 1988. A cowpea line has distinct genes for resistance to *tobacco ringspot virus* and *cowpea mosaic virus*. Phytopathol. 78:1124–1128.

Pyou HG, Chol JI and Lee KH. 1980. Peppers In: Horeticultural cropes. Hwangmunsa, Korea 398P

Reichel C and Beachy RN. 2000. Degradation of *Tobacco Mosaic Virus* movement protein by the 26S proteasome. J. Virol. 74:3330–3337.

Restrepo-Hartwig MA and Ahlquist P. 1996. *Brome mosaic virus* helicase- and polymerase-like proteins colocalize on the endoplasmic reticulum at sites of viral RNA synthesis. J. Virol. 70:8908–8916.

Roossinck MJ, Kaplan I and Palukaitis P. 1997. Support of a *cucumber mosaic virus* satellite RNA maps to a single amino acid proximal to the helicase domain of the helper virus. J. Virol. 71:608-612.

Ruffel S, Dussault MH, Palloix A, Moury B and Bendahmane A. 2002. A natural recessive resistance gene against *potato virus Y* in pepper corresponds to the eukaryotic initiation factor 4E (eIF4E). Plant J. 32:1067–1075.

Schaad MC, Anderberg RJ and Carrington JC. 2000. Strain-specific interaction of the *Tobacco Etch Virus NIa* protein with the translation initiation factor eIF4E in the yeast two-hybrid system. Virology. 273:300-306.

Sekine KT, Nandi A, Ishihara T, Hase S and Ikegami M. 2004. Enhanced resistance to *Cucumber mosaic virus* in the *Arabidopsis thaliana ssi2* mutant is mediated via an SA-independent mechanism. Mol. Plant. Microbe. In. 17:623–632.

Senthil G, Liu H, Puram VG, Clark A, Stromberg A and Goodin MM. 2005. Specific and common changes in *Nicotiana benthamiana* gene expression in response to infection by enveloped viruses. J. Gen. Virol. 86:2615–2625.

Shoko U, Roman S, Danielle MN and Vitaly C. 2010. *ANK*, a host cytoplasmic receptor for the *Tobacco mosaic virus* cell-to-cell movement protein, facilitates intercellular transport through plasmodesmata. PLoS Pathog. 6: e1001201.

Šindelář L, Hanušová M, and Makovcová O. 1982. Regulation of ADPglucose Pyrophosphorylase in cucumber plants infected with the *Cucumber Mosaic Virus*. Biologia Plantarum. 24:142-151.

Solomon-Blackburn RM and Barker H. 2001. Breeding virus resistant potatoes (*Solanum tuberosum*): a review of traditional and molecular approaches. Heredity. 86:17–35.

Stapleford KA and Miller DJ. 2010. Role of cellular lipids in positive-sense RNA virus replication complex assembly and function. Viruses 2:1055-1068.

Tesar M and Marquardt O. 1990. Foot-and-mouth disease virus protease 3C inhibits cellular transcription and mediates cleavage of histone H3. Virology. 174:364–374.

Valcu C, Junqueira M, Shevchenko A and Schlink K. 2009. Comparative proteomic analysis of responses to pathogen infection and wounding in *Fagus sylvatica*. J. Proteome Res. 8:4077-4091.

Van Etten JL, Lane LC, and Dunigan DD. 2010. DNA Viruses: The really big ones (Giruses). Annu. Rev. Microbiol. 64:83–99.

Vijayapalani P, Maeshima M, Nagasaki-Takekuchi N and Miller WA. 2012. Interaction of the trans-frame Potyvirus protein *P3NPIPO* with host protein *PCaP1* facilitates Potyvirus movement. PLoS Patho. 8:e1002639

Whitham SA, Anderberg RJ, Chisholm ST and Carrington JC. 2000. *Arabidopsis RTM2* gene is necessary for specific restriction of *tobacco etch virus* and encodes an unusual small heat shock-like protein. Plant Cell 12:569–582.

Whitham SA, Quan S, Chang H, Cooper B, Estes B, Zhu T, Wang X and Hou Y. 2003. Diverse RNA viruses elicit the expression of common sets of genes in susceptible *Arabidopsis thaliana* plants. Plant J. 33:271–283.

Whitham SA and Wang Y. 2004. Roles for host factors in plant viral pathogenicity. Curr. Opin. Plant Biol. 7:365–371.

Wintermantel WM, Banerjee N, Oliver JC, Paolillo DJ and Zaitlin M. 1997. *Cucumber mosaic virus* is restricted from entering minor veins in transgenic tobacco exhibiting replicase-mediated resistance. Virology 231:248–257.

Yang C, Guo R, Jie F, Nettleton D, Peng J, Carr T, Yeakley JM, Fan J and Whitham

SA. 2007. Spatial analysis of *Arabidopsis thaliana* gene expression in response to *Turnip mosaic virus* infection. Mol. Plant. Microbe. In. 20:358–370.

Yeom SI, Seo E, Oh SK, Kim KW and C Doil. 2012. A common plant cell-wall protein HyPRP1 has dual roles as a positive regulator of cell death and a negative regulator of basal defense against pathogens. Plant J. 69:755–768.

Yoshii M, Nishikiori M, Tomita K, Yoshioka N, Kozuka R, Naito S and Ishikawa M. 2004. The *Arabidopsis cucumovirus* multiplication 1 and 2 loci encode translation initiation factors 4E and 4G. J. Virol. 78:6102–6111.

Yoshii M, Yoshioka N, Ishikawa M and Naito S. 1998. Isolation of an *Arabidopsis thaliana* mutant in which accumulation of *cucumber mosaic virus* coat protein is delayed. Plant J. 13:211–19.

Yoshii M, Yoshioka N, Ishikawa M and Naito S. 1998. Isolation of an *Arabidopsis thaliana* mutant in which the multiplication of both *cucumber mosaic virus* and *turnip crinkle virus* is affected. J. Virol. 72:8731–8737.

Zhang X, Yuan YR, Pei Y, Lin SS, Tuschl T, Patel DJ and Chua NH. 2006. *Cucumber mosaic virus*-encoded 2b suppressor inhibits Arabidopsis argonaute1 cleavage activity to counter plant defense. Genes Dev. 20: 3255–3268.

Zhou Y, Rojas MR, Park M, Seo Y, Lucas WJ and Gilbertson RL. 2011. *Histone H3* interacts and colocalizes with the nuclear shuttle protein and the movement protein of a *geminivirus*. J. Virol. 85:11821–11832.

Zuo K, Wang J, Wu W, Chai Y, Sun X and Tang K. 2005. Identification and characterization of differentially expressed ESTs of *Gossypium barbadense* Infected by *Verticillium dahlia* with suppression subtractive hybridization. Mol. Biol.. 39:191–199.

초 록

Cucumber mosaic virus(CMV)는 가장 넓은 기주 범위를 가지고 있는 식물 바이러스의 한 종류로 고추 생산에 있어 매우 중요한 병원체이다. *Capsicum annuum* '부강(Bukang)'은 CMV에 단일 우성 저항성 유전자인 *Cmr1(Cucumber mosaic resistance 1)*을 가지고 있다. '부강'은 대부분의 CMV-P0 계통에 저항성을 가지고 있으나, 최근 한국에서 발견된 새로운 변종인 CMV-P1에는 이병성인것으로 확인되었다. CMV-P1 RNA1 helicase domain이 *Cmr1*을 극복하는 것으로 이전 연구에서 보고된 바 있다. CMV-P1 복제와 이동에 관련되는 기주 단백질을 구명하기 위해 *C. annuum* '부강' cDNA library를 이용하여 yeast two-hybrid screening을 진행하였다. 총 100,000개 clone의 screening 결과, CMV-P1 RNA1 helicase domain과 상호작용이 있는 것으로 판단되는 156개의 clone을 분리하였다. β-galactosidase filter lift assay, PCR screening 및 sequencing 분석을 통해 상호작용의 가능성이 있는 10개의 유전자로 그 범위를 좁힐 수 있었다. 이 유전자들이 바이러스 감염, 복제 및 이동에 연관이 있다는 것은 이전 연구에서 이미 알려진 바 있다. 이 유전자들의 기능들을 확인하기 위해, 이 10개의 유전자들을 *Nicotiana benthamiana*에서 silencing 시킨 후 GFP(green fluorescent protein)를 발현하는 CMV-P1를 접종하였다. 유전자 silencing은 semi quantitative RT-PCR을 이용하여 확인하였다. 7개 유전자 경우에는 유전자

를 silencing 시킨 식물체들이 정상 *N. benthamiana*와 유사한 수준의 정상적인 성장을 보였고, 나머지 세 유전자의 경우에는 유전자가 silencing된 식물체들이 왜소화 및 심각한 뒤틀림을 포함하는 성장결함을 보였다. GFP 형광물질의 모니터링과 ELISA(enzyme-linked immunosorbent assay)를 통해 식물체에서의 바이러스 축적을 평가하였다. 7개의 후보 유전자 중에서 *cysteine synthase* 유전자의 silencing은 CMV의 이른 축적을 보였고, *formate dehydrogenase* 와 *calreticulin-3 precursor* 두 유전자의 silencing은 모두 감소된 바이러스 축적을 보였다. *cysteine synthase*가 silencing된 식물체의 경우 접종엽과 상엽 모두에서 감염 부위들이 관찰되었으며 TRV::00 보다 이른 시간에 GFP 신호들이 감지되었다. *Formate dehydrogenase*가 silencing된 식물체들은 접종엽에서 국부적 감염을 보였고 상엽에는 GFP 신호가 보이지 않았다. *calreticulin-3 precursor*의 경우에는 접종엽 상엽 모두에서 GFP 신호가 확인 되지 않았다. 이 현미경 관찰 결과는 ELISA 결과로 재확인되었다. 이상의 결과들은 CMV-P1 감염에 *formate dehydrogenase* 와 *calreticulin-3 precursor* 두 유전자가 매우 중요한 역할을 하고 있음을 보여준다.

주요 단어: *Capsicum annuum, Cucumber mosaic virus,* 기주 단백질, 바이러스 저항성, *formate dehydrogenase, calreticulin-3 precursor*