농학석사학위논문

Functional and structural analyses of *Nicotiana benthamiana* NbPCIP1 interacting with *Potato virus X* (PVX) coat protein and its homolog, NbPCIP2, during PVX infection cycle

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감염 중에 미치는 영향 비교 연구

2015 년 2 월

서울대학교 대학원

농생명공학부 식물미생물학 전공

강동우

A THESIS FOR DEGREE OF MASTER SCIENCE

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February 2015

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2015년 2월

서울대학교 대학원

농생명공학부 식물미생물학전공

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강동우의 석사학위논문을 인준함

2015년 2월

위 원 장 _____ (인)

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UNDER THE DIRECTION OF
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DEPARTMENT OF AGRICUTURAL BIOTECHNOLOGY

FEBRURY 2015

APPROVED AS A QUALIFIED THESIS OF DONGWOO KANG
FOR THE DEGREE OF MASTER OF SCIENCE
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ABSTRACT

Functional and structural analyses of *Nicotiana benthamiana* NbPCIP1 interacting with *Potato virus X* (PVX) coat protein and its homolog, NbPCIP2, during PVX infection cycle

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Plant RNA viruses are one of small pathogens encoding few viral proteins. Their replication usually takes place within cytoplasm using many host proteins as well as virus-encoded proteins. A previous study, a *Nicotiana benthamiana* cDNA library was screened to identify host proteins interacting with *Potato virus X* (PVX) coat protein (CP) using yeast-two hybrid (Y2H) assay. It demonstrated that one of identified host proteins, named as *N. benthamiana* PVX CP-Interacting Protein 1 (NbPCIP1), interacts with PVX

CP by Y2H assay, pull-down assay, and BiFC approaches. Interestingly, it revealed that at least one gene which is homologous to *NbPCIP1* is present in *N. benthamian* genome. This gene was referred as *NbPCIP2*. In previous research, it shows that NbPCIP1 interacts with PVX CP, whereas NbPCIP2 does not interact with PVX CP by Y2H assay. Here, to find crucial amino acid (aa) residue(s) of NbPCIP1 for the NbPCIP1 and PVX CP interaction, several deletion and substitution mutants for NbPCIP1 and NbPCIP2 were generated and subjected for Y2H assay. The tetrapeptide (HYGS) sequence is present in NbPCIP1, but not in NbPCIP2. Y2H assay result showed that HYGS is not essential factor for interaction between NbPCIP1 and PVX CP. To further identify crucial aa residue(s), additional single aa substitution mutants were constructed. Data revealed a single aa residue at position 72 aa might be crucial for the interaction between NbPCIP1 and PVX CP. Predicted three-dimensional (3D) structures of NbPCIP1 and NbPCIP2 showed significant differences between NbPCIP1 and NbPCIP2. Overexpression of NbPCIP1 and NbPCIP2 tagged with green fluorescent protein (GFP), respectively, showed that the two proteins localized at different sites in *N. benthamiana* plant cells. NbPCIP1-GFP was mostly localized at granular-like structure in the endoplasmic reticulum (ER), whereas the NbPCIP2-GFP was localized at plastid-like structure. Taken together, these results suggest that two homologous proteins, NbPCIP1 and NbPCIP2, might have different functions in response to PVX infection. In further study, the functional role of 72 aa position of NbPCIP1 required for interacting with PVX CP will be elucidated and the possible role of NbPCIP2 associated with viral replication and movement will be characterized.

Key words: *Potato virus X*, coat protein, *Nicotiana benthamiana*, host protein, interaction, NbPCIP1, NbPCIP2, yeast-two hybrid assay, green fluorescent protein

Student number: 2013-21159

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INTRODUCTION

Plant RNA viruses are one of small pathogens and encode few viral proteins. Their replication usually takes place within cytoplasm using host proteins as well as virus-encoded proteins. During virus infection, viral components interact with host factors for their infection and replication (Ahlquist et al., 2003). After viral RNA infects a cell, the viral replication complex composed of viral proteins and some host factors is formed and this complex is required for the life cycle of a plus-sense single-stranded RNA ((+) ssRNA) plant virus (Roosinck, 2005).

Viral coat protein (CP) plays important roles in symptom development, viral RNA replication, cell-to-cell and long distance movement, cross protection in many RNA plant viruses (Callaway et al., 2001; Carrington et al., 1996; Lazarowitz and Beachy, 1999; Lu et al., 1998). During their initial viral infection, CP out of other viral proteins was exposed to the surrounding environment. Therefore, CP might be the first protein interacting with host factors (Callaway et al., 2001).

Several host proteins interacting with diverse viral CPs have been reported. For example, the *Nicotiana tabacum* IP-L protein interacts with *Tomato mosaic virus* (ToMV) CP and facilitates long-distance movement of ToMV in *N. tabacum* (Li et al., 2005). PNbMTS1 interacts with *Bamboo mosaic virus* (BaMV) CP and over-expression of PNbMTS1 decreased the accumulation of viral CP in *N. benthamiana* (Cheng et al., 2009). NbDnaJ plays a role in *Potato Virus X* (PVX) replication and movement by interacting with PVX CP (Cho et al., 2012).

PVX, a member of the genus *Potexvirus*, is a flexuous rod-shaped virus consisted of a 6.4 kb (+) ssRNA genome (Bercks, 1970). PVX genome is capped and polyadenlyated. PVX contains an 84 nucleotide (nt) at the 5` non-translated region (NTR), five open reading frames (ORFs), and a 72 nt at 3` NTR (Bercks, 1970; Huisman et al., 1988; Skryabin et al., 1988). The 5` NTR includes AC-rich sequences and several repeat ACCA motifs which are important for PVX RNA replication (Park et al., 2008). N-terminal region of PVX CP containing its helical structure plays a major role for the interaction with NbPCIP1 (Park et al., 2013)

Several previous studies reported *N. benthamiana* proteins interacting with PVX CP. To identify host factors interacting with PVX CP during PVX infection of plants, an *N. benthamiana* cDNA library was constructed and subjected for the screen using yeast-two hybrid (Y2H) assay. Of identified host proteins interacting with PVX CP, we further characterized a host protein named as *N. benthamiana* PVX CP-Interacting Protein 1, NbPCIP1. The subcellular localization study confirmed that NbPCIP1 localizes at ER or an ER-associated granular-like structure of epidermal cells. However, NbPCIP1 co-localizes with PVX CP in nucleus and formed the inclusion body-like complexes in areas surrounding the nucleus in *N. benthamiana* cells. Functional study demonstrated that NbPCIP1 enhances viral replication and movement in *N. benthamiana* during PVX infection. The *N. benthamiana* genome contains an additional gene homologous to NbPCIP1. The gene was subsequently named as NbPCIP2. PVX CP interacts with NbPCIP1 while NbPCIP2 cannot interact with PVX CP by Y2H assay (Park et al., 2009).

Although we have shown that NbPCIP1 positively affect viral and systemic movement.

Furthermore, the crucial amino acid (aa) residues of NbPCIP1 required for the interaction between NbPCIP1 and PVX CP were identified based on Y2H assays, quantitative β-galactosidase assays, bimolecular fluorescent complementation (BiFC) assay using several deletion and substitution mutants of NbPCIP1 and NbPCIP2. Here, our results suggest that NbPCIP1 and NbPCIP2 might have different functions associated with PVX infection cycle. Moreover, the aa position 72 of NbPCIP1 gene might be crucial for the interaction between NbPCIP1 and PVX CP.

MATERIAL AND METHODS

1. Construction of NbPCIP1 deletion and substitution mutants

To find crucial aa residues of NbPCIP1 required for the interaction between NbPCIP1 and PVX CP, several NbPCIP1 deletion and substitution mutants were constructed based on NbPCIP1 and NbPCIP2 genes. Each mutant sequence was amplified by PCR using pNbPCIP1-AD as a template (Table 1)(Park et al., 2009). PCR products were cloned into the pACT2 vector (Clontech Laboratories, lnc., USA) by digestion with *Sma*I restriction enzyme for Y2H assays.

For the construction of substitution mutants, mutants were prepared by site-directed mutagenesis. Site-directed mutagenesis was performed by PCR using NbPCIP(+2)-*EcoR*I-F and substituted primers (Table 1). PCR products for substituted full-length NbPCIP1 mutants (PCIP1-H12P, C22S, S25G, P64H, H72Y, S90P, G104A, Q128H, and L146F) were amplified by mega primer (Fig 1) and NbPCIP-*EcoR*I-R (Table 1). pNbPCIP1-AD was used as a template. Each PCR product was cloned into pACT-2 vector using *Sma*I restriction enzyme for Y2H assays.

2. Yeast two-hybrid (Y2H) assay

Y2H assay was conducted using Matchmaker Two-hybrid system 2 based on the yeast two-hybrid system protocols handbook (Clontech Laboratories, lnc., USA). In brief, *S. cerevisiae* strain AH109 was co-transformed with the pACT2 and pAS2-1.

Table 1. List of primers used for PCR amplification to generate several NbPCIP1 mutants.

Primers	Sequences (5′-3′)
NbPCIP(+2)-EcoRI-F	GGAATTCTGGTTCTCCAAACTC
NbPCIP-EcoRI-R	GGAATTCTTCCTCCARATCCTTAAGAG
NbPCIP2-BR	TTCCTCCAGATCCTTAAGA
NbPCIP1-H12P-R	GGATTGGGCTTGTTCAGC
NbPCIP1-C22S-R	GCTGCTATCGGAATAGTAGTTTTGTTCG
NbPCIP1-S25G-R	GGCTGCCATCGCAATAGTAG
NbPCIP1-P64H-R	GACCGCCATAGTGTCCGCC
NbPCIP1-H72Y-F	GTCCATAGTGATGGTGGCTA
NbPCIP1-S90P-F	CATGACCCCACCAACTTTTC
NbPCIP1-G104A-F	CATAGTGATGCTGGCTATG
NbPCIP1-Q128H-F	GACCAATTATCATGGCCATGG
NbPCIP1-L146F-R	GGAATTCTTATTCCTCCAGATCCTTAAGTGCCCAGTT GAACTTCTGGCTCTG
NbPCIP1-del(HYGS)-F	GCGGACATGAACATCATG
NbPCIP1-A4A-F	GCGGGCAGCAGCAGCATCA
NbPCIP1-A(MLSA)-F	CATATGTTGTCAGCCATGGGC
NbPCIP2-A(HYGS)-F	CGGCGGACACTATGGTAGTCATG
NbPCIP2-A4A-F	CGGCGGGCAGCAGCAGCATCATG
NbPCIP2-del(MLSA)-F	CCCATATGGGCATGGG

F, forward primer; R, reverse primer; Restriction sites used for cloning are underlined.

fusion derivatives by lithium acetate-mediated yeast transformation method and then selected on synthetic dropout (SD) medium lacking leucine, tryptophan, histidine, and adenine (−LWHA), and leucine and tryptophan (−LW) agar plates. The interactions of SV40 large T antigen(84-708, pTD1-1) and murine p53(72-390, pVA3-1) or human lamin C (66-230, pLAM5′-1) served as positive and negative controls, respectively. The results of Y2H assays were confirmed by quantitative β-galactosidase assays based on the protocol described in the Y2H system handbook.

3. Prediction of three-dimensional structure of the host proteins

The three-dimensional (3D) structures of the NbPCIP1 and NbPCIP2 were predicted and visualized by the I-TASSER server (http://zhanglab.ccmb.med.umich.edu/I-TASSER) and Vector Alignment Search Tool (VAST) (http://www.ncbi.nlm.nih.gov/Structure/VAST/vastsearch.html).

4. Subcellular localization of NbPCIP2 by transient assay

To prepare the sGFP fusion NbPCIP1 (pNbPCIP1-sGFP) and NbPCIP2 (pNbPCIP2-sGFP) constructs for the transient expression on *N. benthamiana* leaves, the NbPCIP1 and NbPCIP2 were amplified by PCR from pNbPCIP1-AD and pNbPCIP2-AD, respectively, using specific primers (Table 2). Amplified blunt-end NbPCIP1 product was cloned into the pPZP212 using *Stu*I site generating pNbPCIP1-sGFP and pNbPCIP2-sGFP. Pt-rb CD3-1000 was used as plastid marker (Nelson et al., 2007).

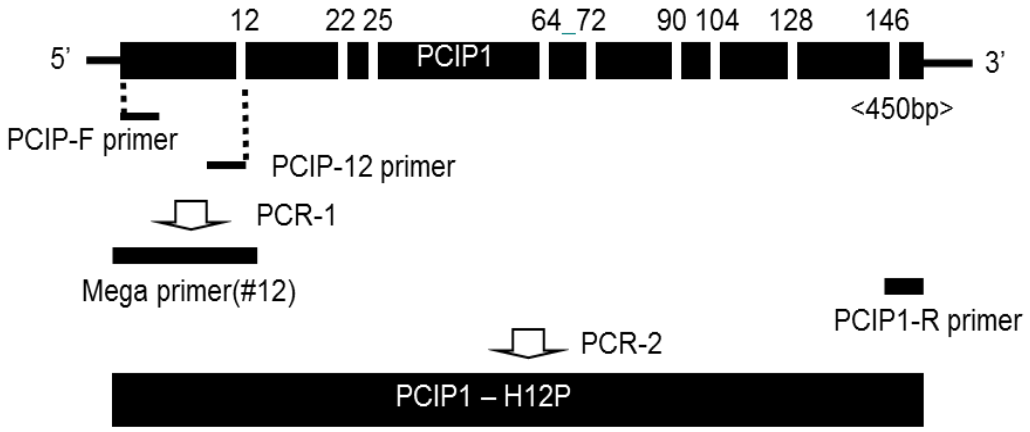


Fig. 1. NbPCIP1 mutants (PCIP1-H12P, C22S, S25G, P64H, H72Y, S90P, G104A, Q128H, L146F) were amplified by mega primer and NbPCIP-*EcoR*I-R. Primer binding sites are depicted.

Table 1. Primers used for the construction of pNbPCIP1-sGFP and pNbPCIP2-sGFP

Primers	Sequences (5′-3′)
NbPCIP-BF	GTTCTCCAAACTCAACTTG
NbPCIP1-BR	TTCCTCCAAATCCTTAAGA
NbPCIP2-BR	TTCCTCCAGATCCTTAAGA

F, forward primer; R, reverse primer

The generated constructs were then transformed into *Agrobacterium tumefaciens* strain GV2260 and agro-infiltrated together with the pTBSV p19 on the *N. benthamiana* leaves. At 3 days after agro-infiltration, infiltrated leaves were observed by fluorescence microscope (Carl Zeiss, Inc., Germany).

5. Bimolecular fluorescent complementation assay

For the construction of binary plasmids, NbPCIP1, NbPCIP2, NbPCIP1 mutants and PVX CP were amplified by PCR from pNbPCIP1-AD, pNbPCIP2-AD, pNbPCIP1-P64H-AD, pNbPCIP1-H72Y-AD, pNbPCIP1-S90P-AD, pNbPCIP1-G104A-AD, pNbPCIP1-del(HYGS)-AD, pNbPCIP1-A(MLSA)-AD, pPVX CP-BD using specific primers $His x_6$ NbPCIP-BF and NbPCIP1-BR for His_6 tagged-NbPCIP1, and $His x_6$ NbPCIP-BF and NbPCIP2-BR for His_6 tagged-NbPCIP2, and His_6 PVX CP-BF and PVX CP-BR for His_6 tagged-PVX CP (Table 3) and fused as blunt fragments to either the N-terminal portion (1−154 amino acid) of YFPR (NYFP) using *Stu*I site in the binary pPZP212 vector fused to *Tobacco etch virus* (TEV) translational leader (TL) sequence (pNbPCIP1-NYFP and pNbPCIP2-NYFP) or to the C-terminal portion (155−238) of YFP (CYFP) in the modified pPZP212 vector (pPVX CP-CYFP) (Morell et al., 2008). The generated constructs were transformed into *A. tumefaciens* strain GV2260 and grown on YEP medium containing selective antibiotics for 1 day. The transformed *A. tumefaciens* cells were harvested by centrifugation and suspended in MMA medium (10 mM MES salt (pH 5.6), 10 mM $MgCl_2$ and 200 μM acetosyringone) to an optical density at 600 nm of 0.7.

Table 3. List of primers used for the BiFC assay

Primers	Sequences (5′-3′)
His_{x6} NbPCIP-BF	CATCATCATCATCATCATAGGCCTGTTCTCCAAACTCAACTTG
NbPCIP1-BR	TTCCTCCAAATCCTTAAGA
NbPCIP2-BR	TTCCTCCAGATCCTTAAGA
His_6 PVX CP-BF	CATCATCATCATCATCATAGGCCTTCTTACGCAATTACTTCTCC
PVX CP-BR	TGGTGGTGGGAGA

F, forward primer; R, reverse primer

The cells were incubated at 30℃ for 2−4 h, and agro-infiltration was performed in pair-wise combinations together with the p19 silencing suppressor of *Tomato bushy stunt virus* (pTBSV-p19) into *N. benthamiana* leaves using a syringe without a needle. The pTBSV-p19 was used as a gene silencing suppresser. Three days after agro-infiltration, agro-infiltrated leaves were observed by super-resolution confocal microscope and checked Bimolecular fluorescent complementation (BiFC) assay pixel sum value by using LAS AF Lite (Leica Microsystems).

6. PVX RNA accumulation level in *N. benthamiana* protoplasts

Fully expanded leaves were collected from four- to five- week-old *N. benthamiana* seedlings and were surface-sterilized for 5 min with 0.8% Clorox (sodium hypochlorite), followed by rising with distilled water (three times for 5 min each). The leaves were sliced into 1-mm strips and were incubated in an enzyme mixture (0.5 M mannitol, 22mM KNO_3, 0.5 mM morpholineethanesulfonic acid [MES], 1 μM $CuSO_4$, 0.75 mM KH_2PO_4, 0.5% cellulase R10, and 1% macerozyme R10 (Yakult Honsha, Tokyo), pH 5.7 overnight at 25℃ in the dark. Protoplasts were released and passed through a 70-μM cell strainer before rinsing with wash buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid], 150 mM NaCl, 0.5 M mannitol, 10 mM $CaCl_2$, pH 5.7). Prepared protoplasts (2×10^5) were counted for transfection with 10 μg of the relevant plasmid (slowly added into a mixture of 100 μl of polyethylene glycol [PEG] solution [40% PEG, 10 mM $CaCl_2$] and 4.5 ml of wash buffer). The protoplast solution was mixed

well and kept on ice for 30 min before centrifugation at 40 × g for 7 min. The supernatant was discarded and the protoplast washed again with 5 ml of wash buffer to remove residual PEG. Protoplasts were incubated in petri dishes with 3 ml of protoplast culture medium (Murashige-Skoog medium containing 0.5 M mannitol and 10mM $CaCl_2$) at 25℃ in the dark.

Total RNA was extracted from the protoplasts using TRIzol (Invitrogen, Carlsbad, Cam U.S.A.). The RNA was treated with 2 U of RNase-free DNase (Promega) at 37℃ for 1 h and was recovered by ethanol precipitation. Total RNA extracted from protoplasts isolated from mock-inoculated and PVX-infected protoplasts, respectively, were carried out as described above with the addition of an RNase pre-incubation step on the isolated protoplasts (1 h on ice) prior to extraction (Schoelz and Zaitlin 1989). This additional step allowed for the degradation of any potentially contaminating RNA present on the outer surface of the protoplast envelope.

Semi-Quantitative RT-PCR assays were performed using specific primers His_6 PVX CP-BF and PVX CP-BR (Table 3). The dsDNA was amplified by five cycles and 15 cycles of PCR using Ex-Taq polymerase (TAKARA BIO, Japan). PCR conditions were at 94℃ for 2 min, each cycle for PCR was consisted of 20 seconds at 94℃, 30 seconds at 55℃, 50 seconds at 72℃. Amplified PCR products were checked by electrophoresis on a 1% agarose gel containing ethidium bromide and were visualized under UV light.

RESULTS

1. Generation of deletion and substitution mutants to identify crucial motifs of NbPCIP1 required for the interaction with PVX CP

To determine which amino acid(aa) residues of NbPCIP1 are required for the interaction between PVX CP and NbPCIP1, several deletion and substitution mutants for NbPCIP1 and NbPCIP2, respectively, were generated. The interaction between generated mutants and PVX CP was examined by Y2H assays. Comparative sequence analysis revealed that tetrapeptide (HYGS) sequence was only present in NbPCIP1, but not in NbPCIP2; however, HYGS was not required for the interaction with PVX CP (Fig 3).

To further identify crucial aa residues, several single aa substitution mutants were additionally constructed by using site-directed mutagenesis and Y2H assays were performed (Fig 4). Y2H assays showed that a single aa residue located at aa position 72 might be crucial aa for the interaction between NbPCIP1 and PVX CP.

Fig. 2. Alignment of amino acid sequences for the generated NbPCIP1 and NbPCIP2 mutants to find crucial domains for the interaction with PVX CP. The amino acids are represented by single letter amino acid code. The alignment was conducted using CLUSTALW program (DNAStar, USA).

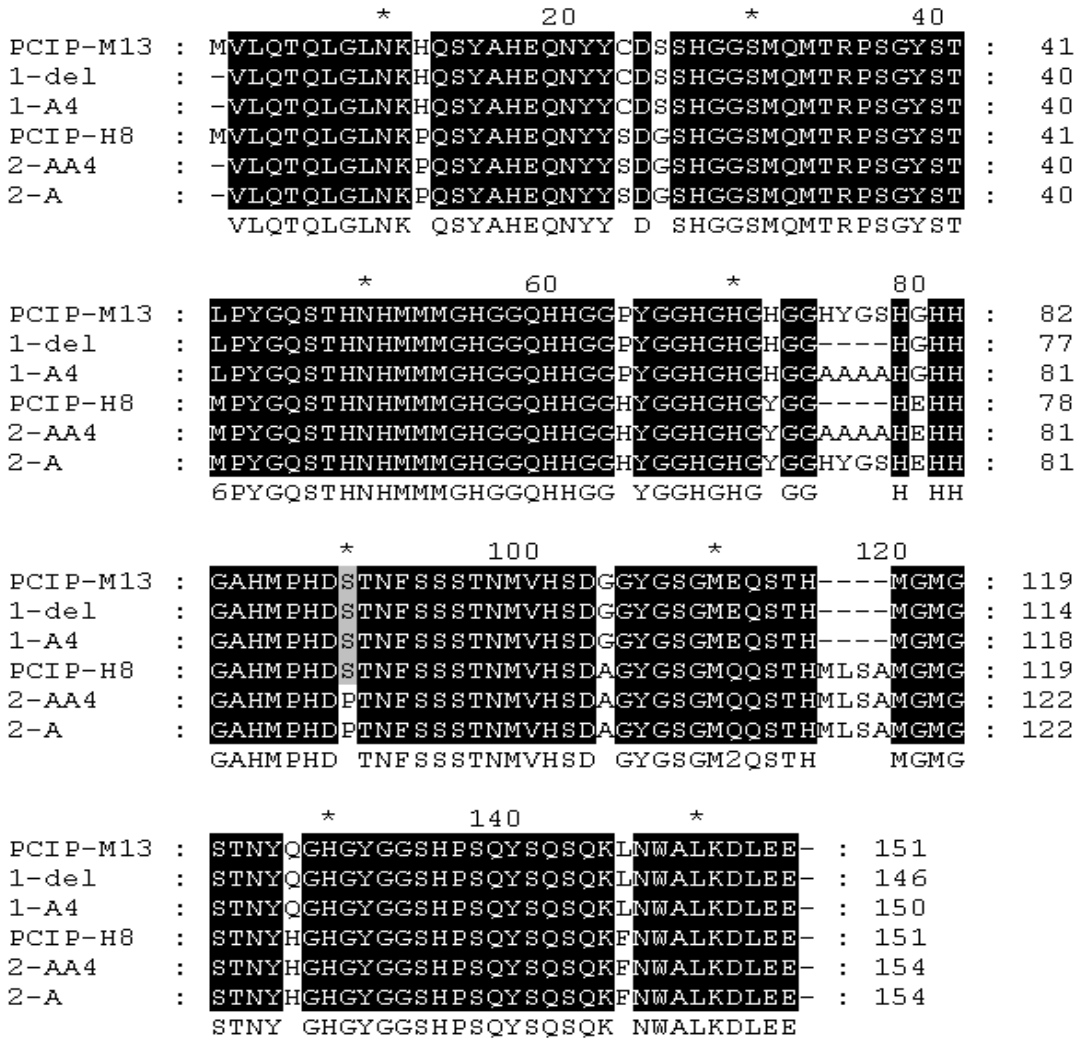


Fig. 3. The generated several mutants for NbPCIP1 and NbPCIP2 which were used for Y2H assays. The insertion, deletion, and substitution in each construct was depicted with corresponding nucleotide position. Interaction of each mutant with PVX CP was also indicated.

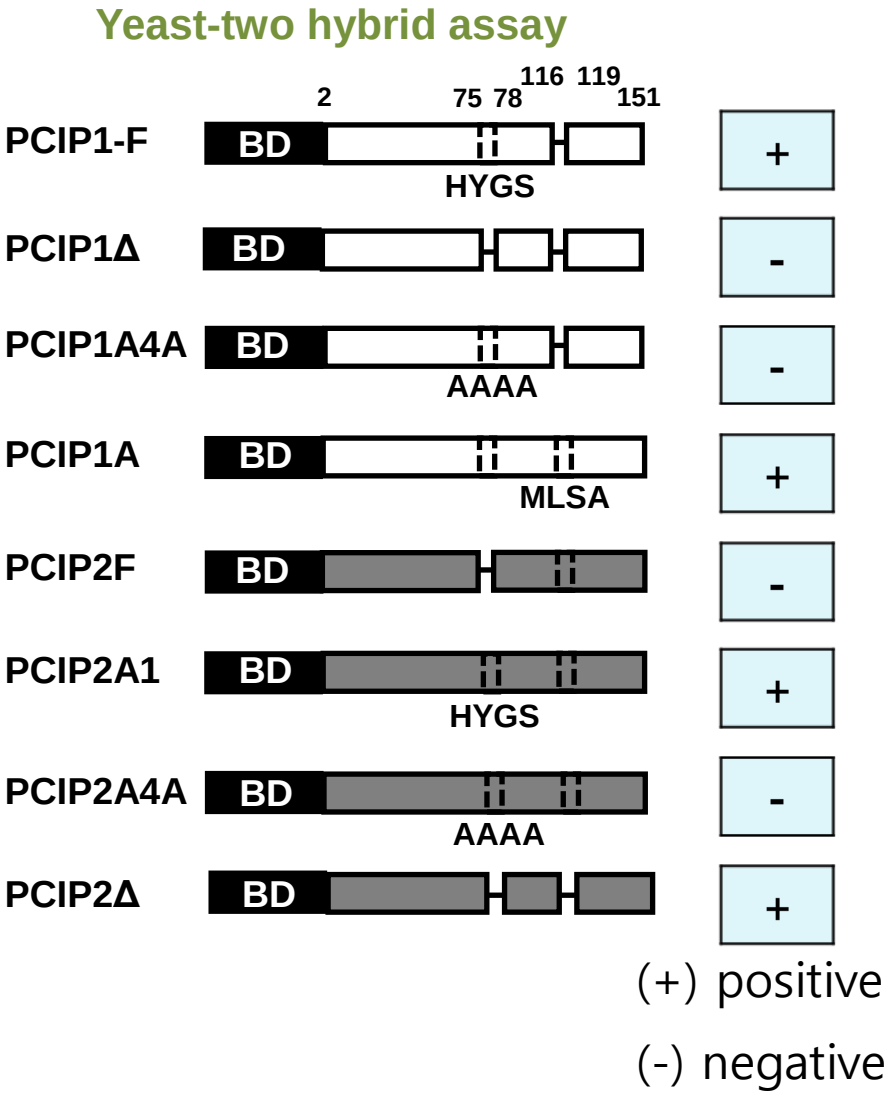


Fig. 4. Alignment of amino acid(aa) sequences of NbPCIP1 and NbPCIP2 mutants which were generated using a single position mutation. The aa sequences are represented by single letter aa codes. Alignment was conducted using CLUSTALW program (DNAStar, USA).

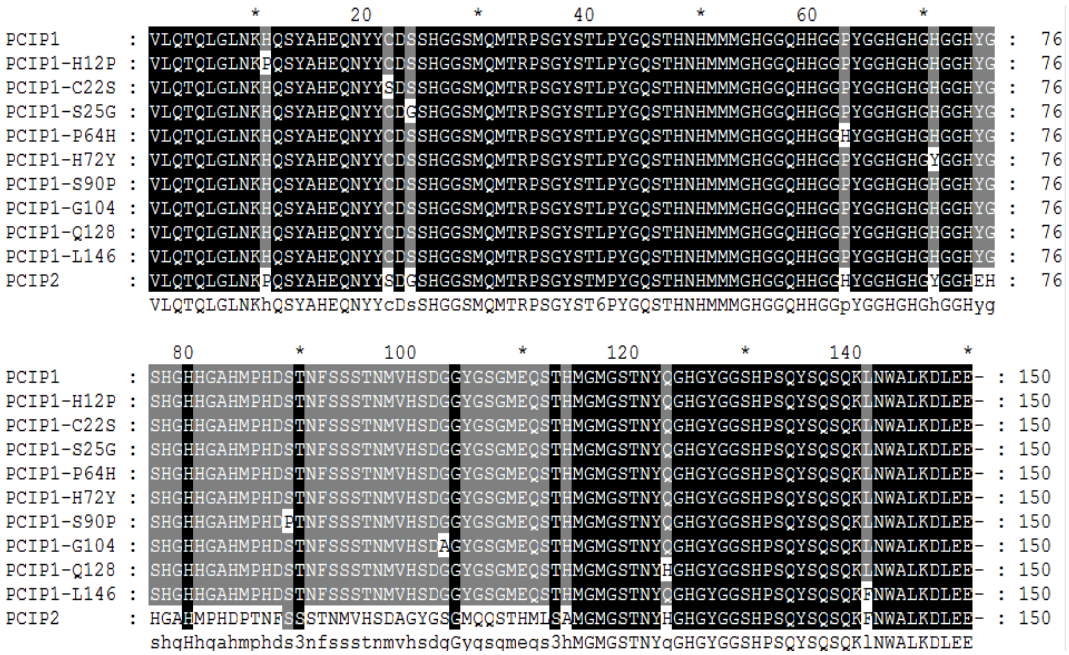


Fig. 5. Protein interaction results between the generated mutants and PVX CP by Y2H assays.

	-LW	-LWHA		-LW	-LWHA
pVA 3-1(+)	+	+	PCIP1-P64H	+	+
pLAM 5-1(-)	+	−	PCIP1-H72Y	+	−
PCIP1(WT)	+	+	PCIP1-S90P	+	+
PCIP2(WT)	+	−	PCIP1-G104A	+	+
PCIP1-H12P	+	+	PCIP1-Q128H	+	+
PCIP1-C22S	+	+	PCIP1-L146F	+	+
PCIP1-S25G	+	+			

(+) positive

(-) negative

2. Prediction of three-dimensional protein structures for NbPCIP1 and NbPCIP2

Three-dimensional (3D) protein structures for NbPCIP1, NbPCIP2, and NbPCIP1 mutants were generated by the I-TASSER server (http://zhanglab.ccmb.med.umich.edu/I-TASSER) and VAST (Vector Alignment Search Tool) (http://www.ncbi.nlm.nih.gov/Structure/VAST/vastsearch.html). Two different protein structures were obtained (Fig. 6A and 6B). As shown in the previous study, the NbPCIP1 exhibits strong nucleotide similarity (89.5%) with NbPCIP2 (Park et al., 2009). Comparative analysis found different regions of 3D protein structures (Fig. 6C and 6D) between NbPCIP1 and NbPCIP2. The predicted 3D protein structure for NbPCIP1 mutants were analyzed as compared to those of NbPCIP1 and NbPCIP2 structures (Fig. 7).

Fig. 6. Predicted 3D protein structures for NbPCIP1, NbPCIP2, and NbPCIP1 mutants using I-Tasser and VAST programs.

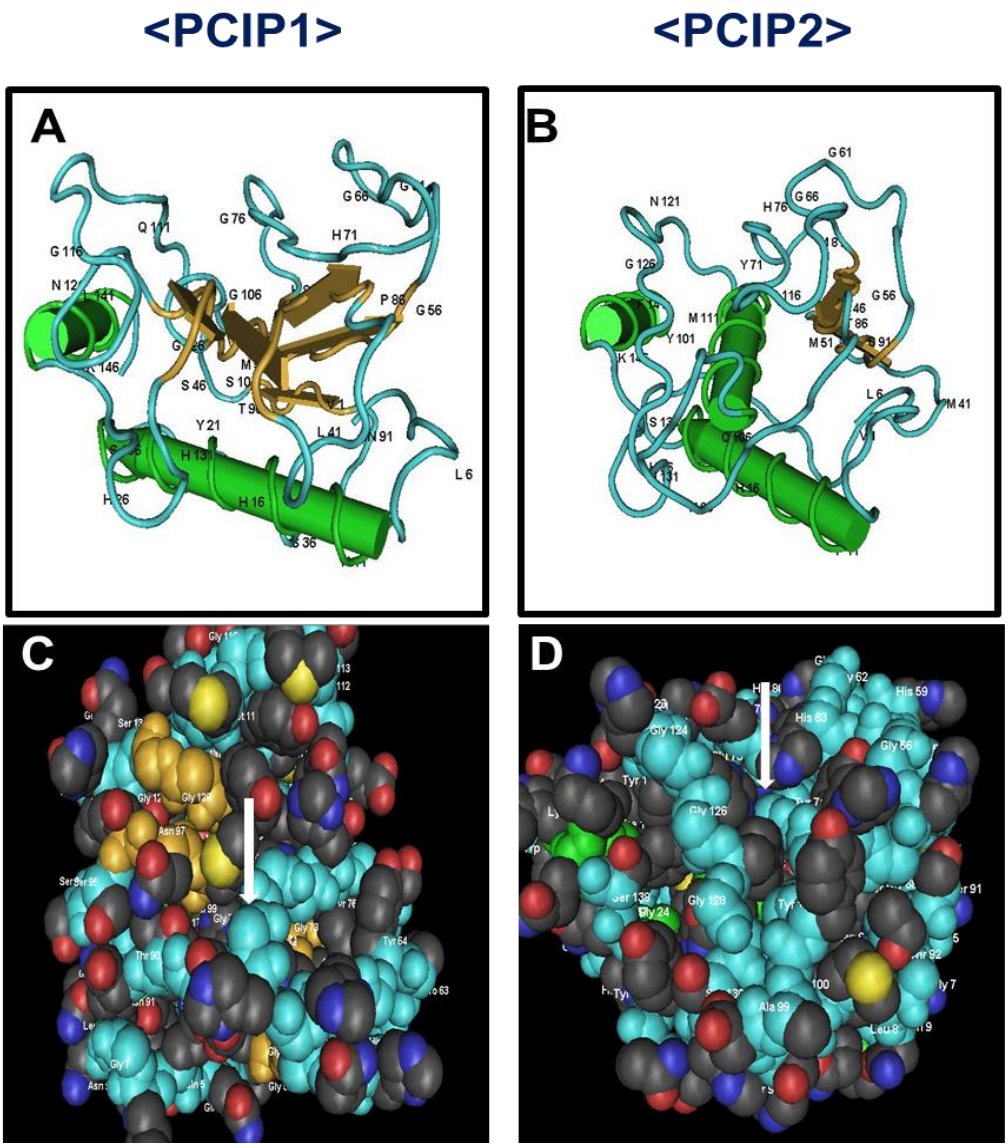


Fig.7. The possible effects of 3D protein structures of generated mutants on protein interaction with PVX CP. The Y2H assay results and 3D structure results were summarized.

Mutants	Yeast-two hybrid	Structure Similarity	72aa
PCIP1(WT)	+	PCIP1	External
PCIP2(WT)	−	PCIP2	Internal
PCIP1-H12P	+	Ex	External
PCIP1-C22S	+	PCIP2	External
PCIP1-S25G	+	PCIP1	Internal
PCIP1-P64H	+	PCIP1	External
PCIP1-H72Y	−	PCIP2	internal
PCIP1-S90P	+	Ex	External
PCIP1-G104A	+	PCIP1	Internal
PCIP1-Q128H	+	PCIP1	Internal
PCIP1-L146F	+	Ex	Internal

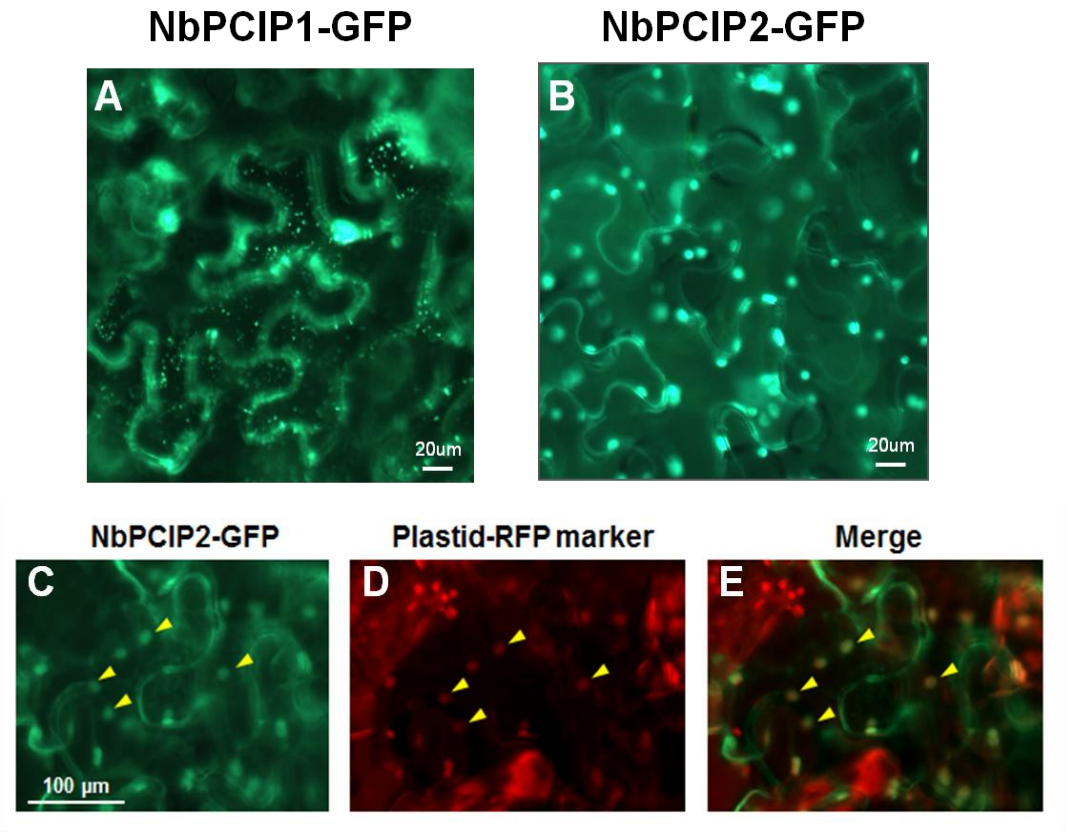
Ex : except for PCIP1 and PCIP2

3. Subcellular localization of NbPCIP2-GFP in *N. benthamiana*

To examine subcellular localization of NbPCIP2 in plant cells, the green fluorescent protein (GFP) was tagged to NbPCIP2 (pNbPCIP2-GFP). Agro-infiltration was performed to express pNbPCIP2-GFP transiently on the leaves of *N. benthamiana*. The pTBSV-p19 and GFP fusion NbPCIP1 (pNbPCIP1-GFP) were used as a silencing suppressor and a positive control, respectively. GFP fluorescence signals were observed using epifluorescence microscope at 3 days after agro-infiltration. The NbPCIP1-GFP was expressed showing granular-like structures throughout the cytoplasm in *N. benthamiana* leaves (Fig. 8B).

To determine whether the localization of NbPCIP2 structures is associated with plastid, NbPCIP2-GFP was transiently co-expressed with Pt-rb CD3-1000 plastid RFP marker (Nelson et al., 2007). Fluorescence images demonstrated co-expression of plastid and NbPCIP2-GFP within plastids (Fig.8E).

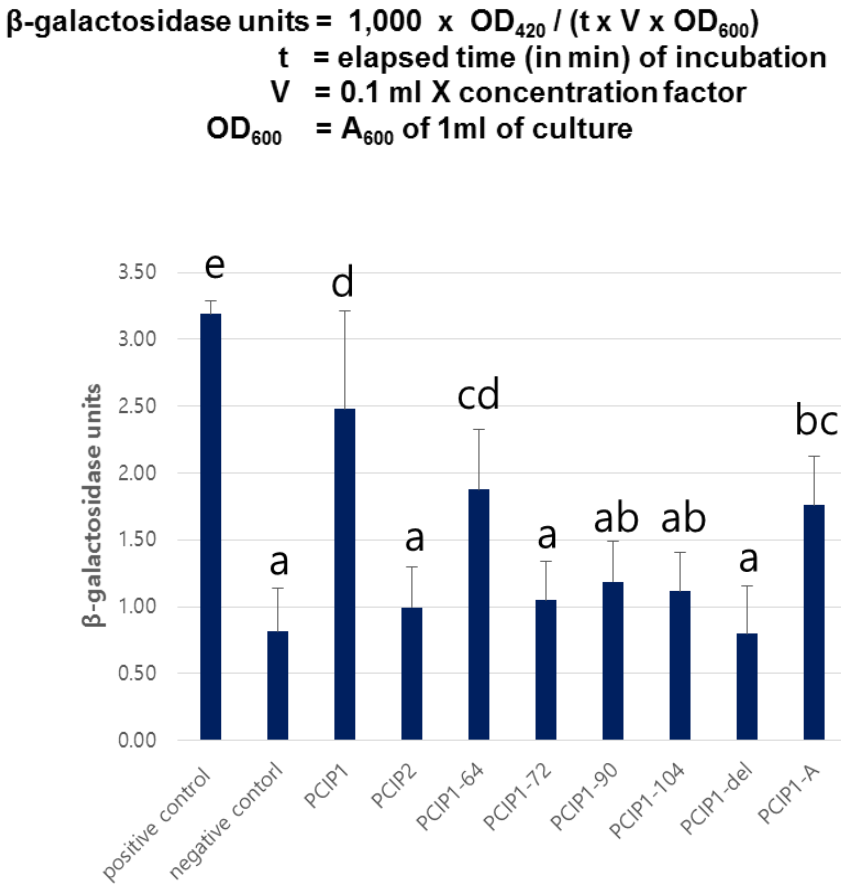
Fig.8. Subcellular localization of NbPCIP1 and NbPCIP2. To identify subcellular localization of NbPCIP1 and NbPCIP2, these GFP fusion proteins were transiently expressed on the leaves of *N. benthamiana*. Plastid-RFP marker was co-infiltrated.



4. Quantitative β-galactosidase assay in liquid cultures (ONPG assay)

The *o*-nitrophenyl-β-D-galactose (ONPG) assay was used for the quantitative assay of β-galactosidase activity according to the manufacturer's instructions (CLONTECH). β-galactosidase units were measured. One unit of β-galactosidase is defined as the amount which hydrolyzes one μmol of ONPG to *o*-nitrophenyl and D-galactose per min per cell (Miler, 1972; Miler, 1992)(Fig 9). ONPG assay showed similar result of Y2H (Fig. 9). As a result, aa 72 position might be an important residue for the interaction between NbPCIP1 and PVX CP.

Fig. 9. Quantitative β-galactosidase assays to confirm Y2H assays results.



5. Protein interaction between NbPCIP1 mutants and PVX CP by BiFC assays

We identified the interaction of NbPCIP1 mutants with PVX CP by the Y2H assays. To further check the interaction of NbPCIP1 mutants with PVC CP, bimolecular fluorescent complementation (BiFC) assays were performed by *Agrobacterium*-infiltration on the leaves of *N. benthamiana*. Yellow fluorescent protein (YFP) fluorescence signals were observed by super-resolution confocal microscope (Leica Microsystems). BiFC assay has been used as one of the most promising techniques to check protein binding *in vivo* and is a very useful method for detecting protein-protein interactions (Morell et al., 2008). To conduct BiFC assay in *N. bethamiana* plants, NbPCIP1 mutants were fused to N-terminal YFP fragment (NYFP) resulting in pNbPCIP1-NYFP, pNbPCIP2-NYFP, pPVX CP –CYFP (Park et al., 2009). Co-expression of NYFP-fused NbPCIP1 mutants (pNbPCIP1-64-NYFP, pNbPCIP1-72-NYFP, pNbPCIP1-90-NYFP, pNbPCIP1-104-NYFP, pNbPCIP1-del(HYGS)-NYFP, pNbPCIP1-A(MLSA)-NYFP and CYFP-fused PVX CP (pPVX CP –CYFP) induced YFP fluorescence signals in agro-infiltrated *N. benthamiana* epidermal cells (Fig. 10). YFP fluorescence did not show in NYFP + CYFP, NbPCIP1-NYFP + CYFP, NbPCIP1-NYFP + CYFP, NbPCIP2-NYFP + CYFP, NbPCIP1-64-NYFP + CYFP, NbPCIP1-72-NYFP + CYFP, NbPCIP1-90-NYFP + CYFP, NbPCIP1-104-NYFP + CYFP, NbPCIP1-del (HYGS)-NYFP + CYFP, NbPCIP1-del (HYGS)-NYFP + CYFP-expressed *N. benthamiana* epidermal cells as negative controls (Fig. 12).

Fig. 10. Measurement of BiFC assays

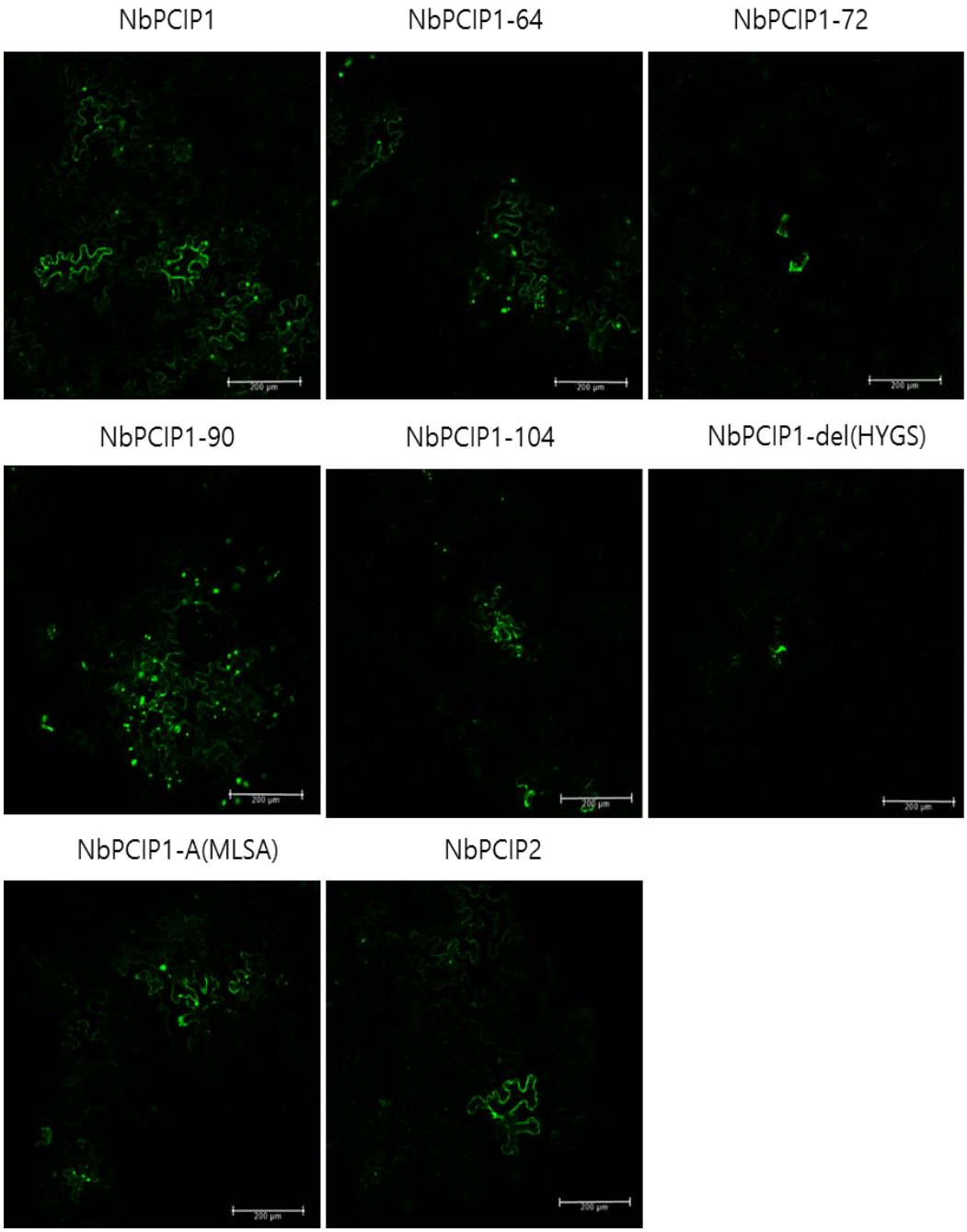
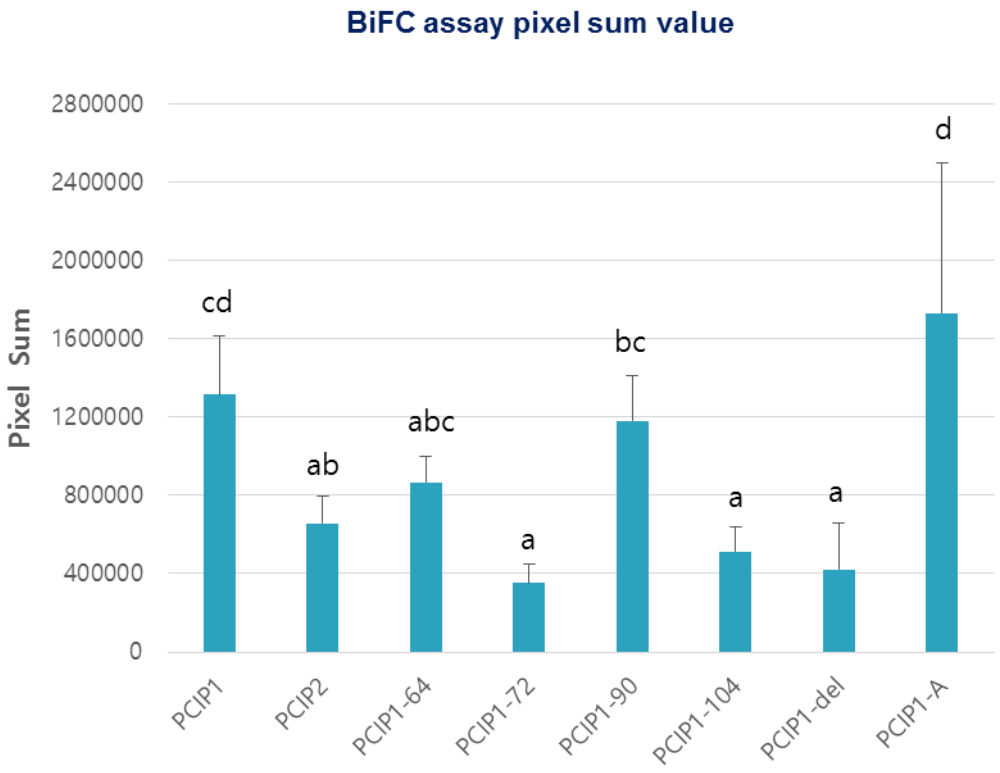
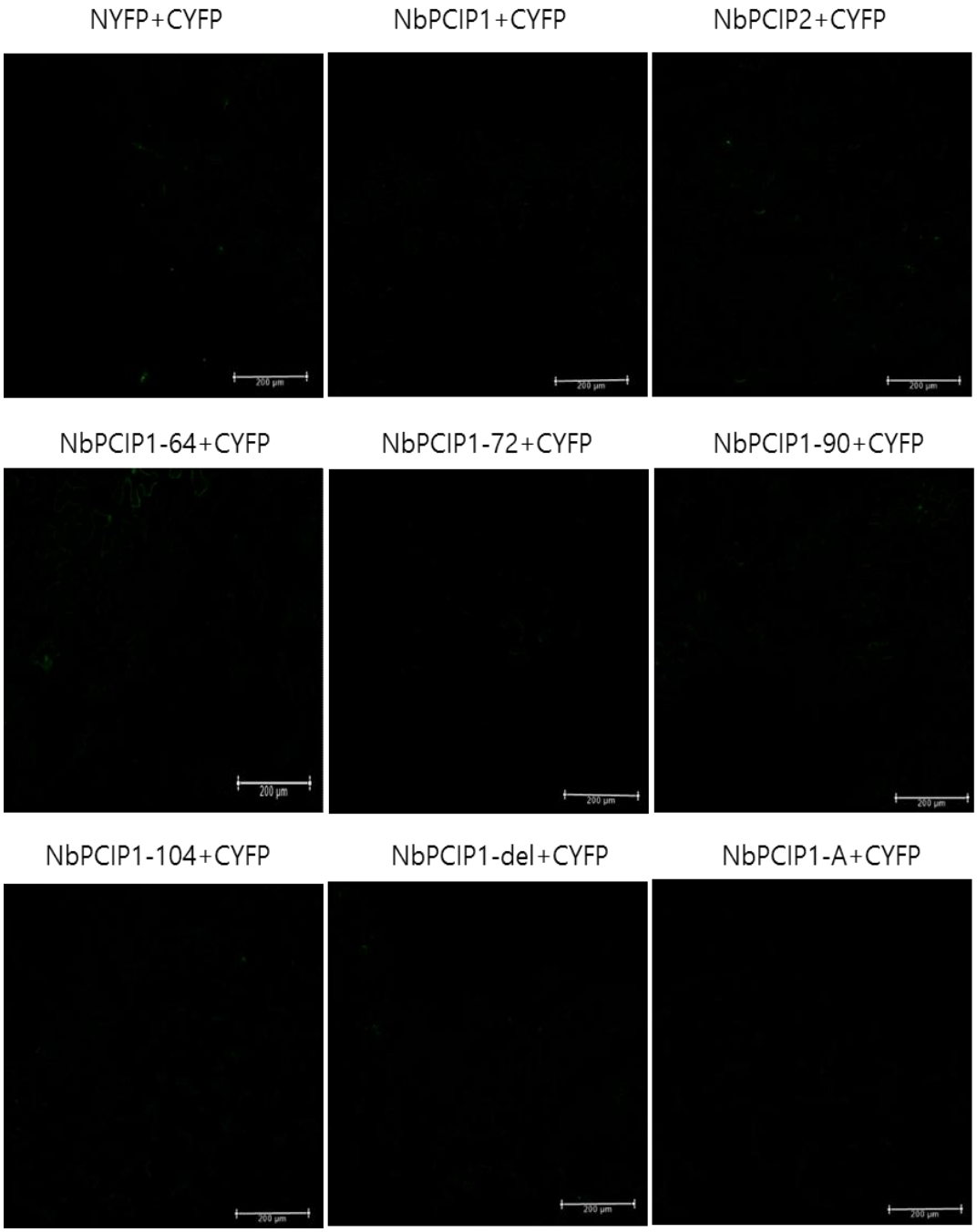


Fig. 11. Measurement of BiFC assay pixel value.



In NbPCIP1-72-NYFP, NbPCIP1-104-NYFP, NbPCIP1-del(HYGS)-NYFP and PVX CP-CYFP, YFP fluorescence signals which reveal the interaction with PVX CP showed significantly weaker than in NbPCIP2-NYFP and PVX CP-CYFP and did not spread to adjacent cells in agro-infiltrated *N. benthamiana* epidermal cells (Fig 10). To measure the interaction of NbPCIP1 mutants with PVX CP accurately, pixel sum value was measured by using LAS AF Lite (Leica Microsystems). As expected, lower fluorescence pixel sum value was observed from NbPCIP-72-NYFP, NbPCIP1-104-NYFP, NbPCIP1-del(HYGS)-NYFP and PVX CP-CTFP (Fig. 11). These results showed additional evidence supporting amino acid position 72 might be crucial for the interaction between NbPCIP1 and PVX CP.

Fig. 12. Results of BiFC to confirm Y2H assay

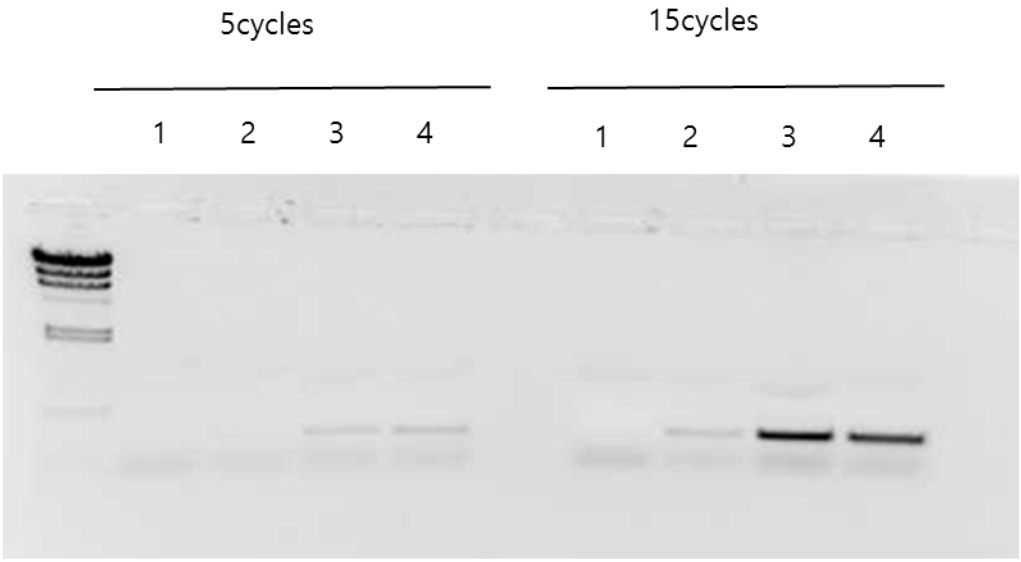


6. Semi-quantitative RT-PCR to detect PVX RNA accumulation level in *N. benthamiana* protoplasts overexpressing NbPCIP1 and NbPCIP2, respectively, after PVX infection

To examine the functional role of NbPCIP1 aa position 72 associated with PVX RNA replication in *N. benthamiana* protoplasts, PVX RNA accumulation was examined in protoplast overexpressing NbPCIP1 and NbPCIP2, respectively, after PVX infection. pNbPCIP1-GFP, pNbPCIP2-GFP, and pTBSV-p19 constructs were prepared for transient over-expression assay. Agro-infiltration was performed using mock (pPZP212-GFP + pTBSV-p19) and over-expression (pNbPCIP1-GFP + pTBSV-p19 and pNbPCIP2-GFP + pTBSV-p19) constructs on the leaves of *N. benthamiana* leaves. Protoplasts at 3 day post agro-infiltration (dpi) were harvested. PVX infectious clone, pSPVX-GFP, was used for PVX-challenge inoculation on protoplasts at 1 day post challenge inoculation (dpci). Total RNA was extracted from the protoplasts using TRIzol (Invitrogen, Carlsbad, Cam U.S.A.) and used for semi-Quantitative RT-PCR assays.

RT-PCR results show significant differences in the expression levels of PVX CP accumulation in samples extracted from mock-inoculated and PVX-infected plants (Fig 13). These results support both NbPCIP1 and NbPCIP2 positively affect PVX RNA replication; however, the level of PVX RNA replication was different between NbPCIP1 and NbPCIP2 overexpressing plants.

Fig. 13. Semi-quantitative RT-PCR to measure PVX replication in the protoplasts overexpressing NbPCIP1 and NbPCIP2.



1. P19
2. P19 + PVX
3. P19 + PVX + PCIP1
4. P19 + PVX + PCIP2

DISCUSSION

Viruses encode a small number of proteins. Therefore, viruses rely on their life cycle on the mechanism of infected hosts by utilizing host genes and proteins. In case of plant viruses, viruses are infected in the host by insects or mechanical wounding. After infection, the viral coat protein is removed and viral RNA is unveiled. The viral RNA is replicated by interaction between viral proteins and host proteins. Subsequently, the viral RNA is accumulated and viral movement protein (MP) and coat protein (CP) are synthesized. Using MP and CP, new viral particle is formed and moves to neighboring cells via plasmodesmata (PD). Therefore, it is necessary for plant viruses to utilize host proteins for their life cycles.

Of known plant viruses, the *Potato virus X* (PVX) belonging to the genus *Potexvirus* in the family *Flexiviridae* is regarded as an important viral pathogen resulting in serious damage in the production of the family Solanaceae including potato, tomato, tobacco, and pepper. Of known viral proteins, CP is the major protein which binds to various host proteins. Several previous studies reported host proteins with viral CP. A majority of host proteins were derived from *Nicotiana tabaccum* and *N. benthamiana*, which are susceptible for many plant viruses and used as model plants for plant molecular virology.

To identify host proteins interacting with viral proteins, Y2H assay of known several approaches is widely used. In the previous study, *N. benthamiana* cDNA library compatible for Y2H assay has been established and used for Y2H assay to find host

proteins interacting with PVX CP. As a result, several host proteins were identified. Of them, NbPCIP1 interacting with PVX CP has been characterized.

In this study, a homolog of NbPCIP1 referred as NbPCIP2 has been identified and characterized. Sequence analysis found that two proteins have KDLE motif which is known as endoplasmic reticulum (ER) retention motif. Transient expression of NbPCIP1 tagged with GFP confirmed localization of NbPCIP1 in ER. In addition, Y2H assays showed that NbPCIP1 interacts with PVX CP; however, NbPCIP2 did not interact with PVX CP. It has been known that NbPCIP1 is a positive regulator for PVX movement and replication. To find amino acid(aa) region which determines the interaction with PVX CP, aa sequences between NbPCIP1 and NbPCIP2 were compared. NbPCIP1 contains HYGS aa residue while NbPCIP2 does not contain it. Transient expression of NbPCIP2 tagged with GFP revealed its subcellular localization within plastid.

In this study, a crucial aa of NbPCIP1 required for interaction with PVX CP was identified using several deletion and substitution mutants. Y2H assay found that the aa 72 is an important aa for interaction with PVX CP between two proteins. BiFC assays confirmed the results of Y2H assays and measured protein interactions based of pixel sum value. Moreover, the quantitative beta-galacotosidase assay provides the aa position 72 of NbPCIP1 is an important for the interaction with PVX CP. In addition, the predicted 3D protein structure identified the position of aa 70 is different from each other. Although NbPCIP1-72-NYFP, NbPCIP1-104-NYFP, NbPCIP1-del(HYGS)-NYFP and PVX CP-CYFP, YFP fluorescence signals which reveal the interaction with PVX CP showed significantly weaker than in the interaction of NbPCIP2-NYFP with PVX CP-CYFP and

did not spread to adjacent cells in agro-infiltrated *N. benthamiana* epidermal cells (Fig. 10). Amino acid position 72 of NbPCIP1 might relate with viral cell-teo-cell movement. In previous study, PVX more quickly moved to upper leaves in NbPCIP1 over-expressed *N. benthamiana* plants than in control plants. It revealed that NbPCIP1 affects viral movement in *N. benthamiana* plants. In this study, NbPCIP1-H72Y mutant and NbPCIP1-del(HYGS) mutant did not interact with PVX CP in yeast and did not spread to adjacent cells.

To reveal whether the aa 72 position is important for viral replication, semi-quantitative RT-PCR was performed using total RNA extracted from protoplast overexpressing NbPCIP1 and NbPCIP2 after PVX infection. Both genes positively regulate PVX replication.

In this study, it demonstrated that two homologous genes, NbPCIP1 and NbPCIP2, have been evolved differently in response to viral infection. For example, NbPCIP1 containing the aa 72 localizes in ER and interacts PVX CP; however, NbPCIP2 without the aa 72 localizes in plastid and does not interact with PVX CP. Functional study also demonstrated that the direct interaction with PVX CP results in strong positive regulation on PVX infection. Taken together, a single amino substitution which might be evolved differently from a single gene contribute the subcellular localization and functions of two homologous genes in response to viral infection. Further study will be possibility that the interaction between aa position 72 of NbPCIP1 and PVX CP is involved in virus cell-to-cell movement.

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Potato virus X 외피단백질과 상호작용을 하는 *N. benthamiana* NbPCIP1과 상동체인 NbPCIP2가 PVX 감염 중에 미치는 영향 비교 연구

강동우

초록

식물 RNA 바이러스는 적은 단백질을 코딩 하는 매우 작은 병원체이다. 이들의 복제는 일반적으로 바이러스에 코딩 된 단백질뿐만 아니라 기주 단백질을 이용하여 세포질 내에서 일어난다. 이전 연구에서 PVX Coat Protein (CP) 와 상호작용을 하는 찾기 위해서 *N. benthamiana*의 cDNA library를 제작하였고, yeast-two hybrid system을 이용하여 PVX CP와 상호작용을 하는 기주 단백질 (NbPCIP1)을 선별하였다. Yeast-two hybrid assay, protein-protein binding assay, BiFC assay를 이용하여 NbPCIP1이 PVX CP와 상호작용함을 확인하였다. 염기서열을 분석하여 NbPCIP1과 유사한 NbPCIP2를 선별하였는데, Yeast-two hybrid assay를 통해 NbPCIP1는 PVX CP와 상호작용을 하였지만 NbPCIP2는 상호작용을 하지 않았다 (Park et al., 2009). NbPCIP1과 PVX CP와 상호작용을 하는데 있어 필요한 아미노산 부분을 확인하기 위하여 NbPCIP1과 NbPCIP2의 염기서열 제거 및

서열 치환을 한 돌연변이 유전자들을 만들었고 yeast-two hybrid assay를 통하여 상호작용을 하는지 확인하였다. 그 결과 NbPCIP1에는 있고 NbPCIP2에는 없는 tetrapeptide인 HYGS 아미노산 부분이 PVX CP와 상호작용을 하는데 있어 필요한 아미노산 부분이 아님을 확인하였다. NbPCIP1의 염기서열을 바탕으로 아미노산 한 개를 치환하는 돌연변이들을 만들었고 yeast-two hybrid assay를 통해 PVX CP와 상호작용 여부를 확인하였다. 상호작용을 확인해본 결과 한 개의 아미노산을 치환하였을 때 72번 위치의 아미노산이 NbPCIP1과 PVX CP가 상호작용을 하는데 있어 중요한 부분이 될 수 있는 가능성을 확인하였다. NbPCIP1과 NbPCIP2의 3차원 구조를 예측해본 결과 NbPCIP1과 NbPCIP2 사이에 큰 차이가 있음을 보여주었다. 게다가 green fluorescent protein (NbPCIP1-sGFP, NbPCIP2-sGFP)를 붙여 *N. benthamiana*에서 발현을 시켜 형광현미경으로 관찰한 결과, 작은 입자 형태 세포 내에 산발적으로 분포하였고 소포체에 위치하는 NbPCIP1과 달리 NbPCIP2는 보다 더 큰 입자 형태로 분포되어 있었고 엽록체와 같은 구조에 위치하는 것을 확인하였다. 이러한 결과들이 NbPCIP1과 NbPCIP2가 비록 염기서열이 서로 비슷하지만 PVX 감염 중에 각자 다른 기능을 갖고 있다고 본다. 추가적인 연구를 통해 NbPCIP1의 72번 아미노산 위치가 PVX CP와 상호작용을 하는데 중요한 위치인지 확인할 수 있고, NbPCIP1과 비교하여 NbPCIP2가 바이러스 복제와 이동에 차이를 나타내는지 확인할 수 있다.

주요어 : PVX Coat protein (CP), *Nicotiana benthamiana* PVX CP-Interacting Protein 1, *Nicotiana benthamiana* PVX CP-Interacting Protein 2,**염기서열 치환 돌연변이**, yeast-two hybrid assay, green fluorescent protein (GFP)

학 번 : 2013-21159