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벼줄무늬잎마름병바이러스 단백질들의 항체 생성과 상호작용 연구

Generation of antibodies and characterization of interactions for *Rice stripe virus* proteins

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Generation of antibodies and characterization of interactions for *Rice stripe virus* proteins

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ABSTRACT

Rice stripe virus (RSV) is one of the most destructive viruses of rice, and greatly reduces rice production in China, Japan, and Korea, where mostly *japonica* cultivars of rice are grown. RSV is transmitted by the small brown plant-hopper (SBPH) in a persistent and circulative-propagative manner. RSV belongs to the genus *Tenuivirus* but has not yet been assigned to a virus family. The RSV genome is composed of four single-strand RNAs (RNA1 to RNA4) and encodes seven proteins. In the present study, we investigated the protein–protein interaction of RSV proteins. First, in order to detect RSV proteins in RSV infected rice, antibodies for RSV were generated on the basis of recombinant proteins and synthetic polypeptides as antigens. Among seven proteins in RSV, genes encoding NCP and NS3 proteins were cloned into the expression vector carrying His-tag after RT-PCR amplification. We obtained purified His-tagged recombinant proteins which were produced in *Escherichia coli*. Alternately, we also

applied synthetic polypeptides of RSV proteins which are suitable to raise antiserum for antibody production. A total of six polyclonal antibodies consisted of two recombinant proteins and four synthetic polypeptides were raised in rabbits. Of two recombinant proteins, only anti-NCP displayed stable hybridization signals in western blot analysis. In case of synthetic polypeptides, antibodies for NP and NCP were very effective to detect RSV in both RSV infected rice and weed plants. However, antibodies for NS3 and NSvc4 showed weak specific bands as well as strong non-specific background due to the difference of viral proteins produced in the infected leaves. In summary, the antibodies generated for RSV in this study will be useful for various assays such as RSV diagnostic detection, immunoprecipitation, protein purification, and western blot analysis. Second, we investigated interactions between six of the RSV proteins by yeast-two hybrid (Y2H) assay *in vitro* and by bimolecular fluorescence complementation (BiFC) *in planta*. Y2H identified self-interaction of the nucleocapsid protein (NP) and NS3, while BiFC revealed self-interaction of NP, NS3, and NCP. To identify regions(s) and/or crucial amino acid (aa) residues required for NP self-interaction, we generated various truncated and aa substitution mutants. Y2H assay showed that the N-terminal region of NP (aa 1–56) is necessary for NP self-interaction. Further analysis with

substitution mutants demonstrated that additional aa residues located at 42–47 affected their interaction with full-length NP. These results indicate that the N-terminal region (aa 1–36 and 42–47) is required for NP self-interaction. BiFC and co-localization studies showed that the region required for NP self-interaction is also required for NP localization at the nucleus. Overall, our results indicate that the N-terminal region (aa 1–47) of the NP is important for NP self-interaction and that six aa residues (42–47) are essential for both NP self-interaction and nuclear localization.

Keywords: *Rice stripe virus*, rice, antibody, nonstructural disease-specific protein, protein–protein interactions, nucleocapsid protein, yeast two-hybrid system, nuclear localization.

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GENERNAL INTRODUCTION

I. *Rice stripe virus* (RSV)

Rice (*Oryza sativa*) is one of the most important crops in the world and is a main food in Asian countries including China, India, Indonesia, Japan, Korea, and Philippines (Wang et al., 2005). RSV is the member of genus *Tenuivirus* which is not assigned to any virus family yet. RSV mainly infects rice plants, but also other plant species including maize, wheat, oat, foxtail millet, and several weeds (Falk and Tsai, 1998; Lian et al., 2011). RSV can infect *Arabidopsis* which is widely used as a model plant (Sun et al., 2011). Over the past several decades RSV has been frequently reported in three countries including China, Japan, and Korea. In Korea, RSV was previously reported only from southern areas, but has been recently observed in large ranges of altitudes, suggesting that the virus is rapidly spreading (Lee et al., 2008). In China, RSV has recently reemerged in Zhejiang province, eastern China, and the spread of RSV has been associated with climate change (Wang et al., 2008); this study demonstrated that RSV frequently infected the earliest sown seedlings and that young seedlings tend to be more susceptible than older plants (Wang et al., 2008). Since 2000, RSV has

appeared in the Yangtze river region and generally caused a 30%–40% of yield losses in eastern China (Wu *et al*., 2009). The occurrence of RSV in western Japan in 2008 was caused by viruliferous planthoppers that migrated from China (Otuka et al., 2010). RSV used to be reported only in China, Japan, Korea, and Taiwan where *japonica* cultivars, which are susceptible to RSV, are usually grown (Abo and Sy, 1997), but RSV was also recently reported in Southeast Asia for the first time (Ren et al., 2013).

RSV is transmitted by the small brown plant-hopper (SBPH) (*Laodelphax striatellus* Fallén) in a persistent and circulative-propagative manner (Falk and Tsai, 1998). However, the ability to acquire RSV is highly variable among SBPH isolates (Kisimoto, 1967). Specifically, RSV could be acquired from frozen infected leaves and then transmitted by the SBPH in the laboratory condition (Sun et al., 2011). Furthermore, RSV can also infect *Nicotiana benthamiana* by sap infiltration (Yao et al., 2012).

II. Identification and detection of RSV in rice plants and SBPH vectors

Disease symptoms in rice plants infected by RSV include chlorosis, weakness, necrosis, and stunted growth (Satoh et al., 2010). Several methods have been developed to detect RSV in infected rice plants or SBPH

vectors, and these include enzyme-linked immunosorbent assay (ELISA), western blot analysis, reverse transcription-polymerase chain reaction (RT-PCR), and RT loop-mediated isothermal amplification (LAMP). ELISA, which has been widely used for detection of RSV in high-throughput experiments, required the development of several antibodies specific to RSV proteins (Lian et al., 2011; Liang et al., 2005b; Suzuki et al., 1992). Such antibodies can be generated by recombinant proteins or synthetic polypeptides as antigens (Lian et al., 2011). The developed antibodies have also been used to localize specific RSV protein in rice plants and SBPH *in vivo* (Liang et al., 2005b; Suzuki et al., 1992). Western blot analysis with six antibodies detected all six RSV proteins in RSV-infected plants (Liang et al., 2005b). A limitation of both ELISA and western blot analysis is the time and cost required for antibody production. Recently, RT-PCR has being widely used to detect RSV (Cai et al., 2003; Zhang et al., 2008). Although RT-PCR is very specific and sensitive, it requires expensive equipment and long amplification time. Immune- and virion capture RT-PCR have been developed, and these have the advantage of not requiring the extraction of RNA from samples (Lian et al., 2011; Liang et al., 2005; Suzuki et al., 1992). Recently, a LAMP approach was developed to detect nine viruses including RSV in rice (Higgins et al., 2010). LAMP, which does not require

thermo-cycling, is specific, fast, accurate, and simple (Notomi et al., 2000).

III. Genome organization of RSV

The RSV genome is composed of four single-stranded RNAs that encode seven proteins (Fig. 1). The morphological characteristics of RSV RNAs were examined after the separation of RNAs using sucrose density gradient centrifugation (Ishikawa et al., 1989). Each of four components contained circular filaments and the average length of RNAs 1−4 were 2,110 nm, 840 nm, 610 nm, and 510 nm, respectively (Ishikawa et al., 1989). RNA1 is the largest RNA segment and is a negative sense RNA. A single ORF in the viral-complementary (vc) sense of RNA1 (vc RNA1) encodes a 337 kDa protein referred to as the RNA-dependent RNA polymerase (RdRp) (Barbier et al., 1992; Toriyama et al., 1994). Based on RdRp sequence, RSV is regarded as a member of the genus *Tenuivirus* (Toriyama et al., 1994).

RNA2 encodes NS2 (silencing suppressor; 22.8kDa) from the viral-sense RNA2 (vRNA2) and NSvc2 (glycoprotein; 94 kDa) from the vcRNA2 (Takahashi et al., 1993; Zhu et al., 1992; Zhu et al., 1991). The NS3 (nonstructural protein; 23.9 kDa) and NP (nucleocapsid protein; 35 kDa) are produced from vRNA3 and vcRNA3, respectively (Hayano et al.,

1990; Kakutani et al., 1991). And a nonstructural disease-specific protein (NCP; 20.5 kDa) and MP (movement protein; 32 kDa) are encoded by vRNA4 and vcRNA4, respectively (Hayano et al., 1990; Kakutani et al., 1990). Interestingly, 5'- and 3' terminal sequences of RSV were complementary to each other which might form panhandles (Takahashi et al., 1990).

A previous study examined nucleotide sequences of the 5' termini of RNA3 and RNA4 found that the mRNAs from both viral and viral complementary sequences possessed from 10 to 23 non-viral sequences which might be originated from host cellular mRNAs and thought to be for efficient translation of viral mRNA (Shimizu et al., 1996). Coinfection with RSV and *Cucumber mosaic virus* (CMV) in *Nicotiana benthamiana* exhibited that CMV served as cap donors for RSV transcription initiation (Yao et al., 2012). The 5' end of the cleaved CMV RNAs was complementary to the 3' end of the RSV genome sequences. Repetitive priming and realignment converted short CMV cap donors into longer sizes, which could be useful for long-distance elongation of RSV (Yao et al., 2012). This "cap-snatching mechanism" has been well characterized in hantavirus, influenza virus, and other viruses (Cheng and Mir, 2012; Dias et al., 2009).

IV. Genetic diversity of RSV isolated from different geographical regions

Complete genome sequences of various RSV isolates are currently available. A total of 13 RSV isolates from Korea were used for complete genome sequencing (Jonson et al., 2009a; Jonson et al., 2009b); significant differences could be detected in the intergenic regions of each RSV RNA, and comparative phylogenetic analysis with other isolates from China and Japan suggested that RSV isolates in Korea may have originated from distinctive ancestors by either reassortment or recombination events. To elucidate genetic diversity and population structure of RSV isolates in China, nucleotide sequences were determined for a large number of RSV isolates during 1997-2004 (Wei et al., 2009). RSV isolates in China could be divided into three subtypes. The population from eastern China was composed only of subtype I/IB isolates, while the population from Yunnan province (southwest China) contained mostly subtype II isolates. The RSV isolates from two different districts in China displayed low genetic diversity indicating substantial gene flow (Wei et al., 2009). The full genome sequence of RSV isolate ‘Zhejiang’ revealed that RdRp contains a domain

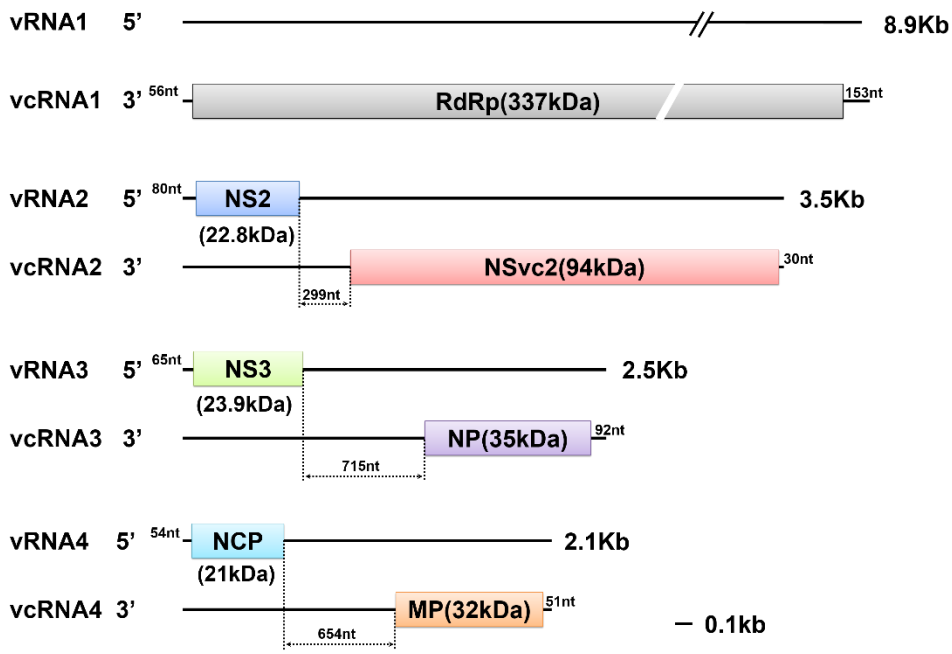

Fig. 1. Genome organization of RSV. RSV is consisted of four RNAs including RNA1, RNA2, RNA3, and RNA4. Viral RNA and viral complementary RNA in each RSV RNA are abbreviated as vRNA and vcRNA with corresponding RNA number, respectively. The black line indicates the length of nucleotides for each RNA and the colored rectangular represents corresponding ORF with a predicted size of protein. The sizes of 5’, 3’, and intergenic regions are also indicated as base pairs. The name of each protein is as follows: RdRp in RNA1, NS2 and NSvc2 (glycoprotein) in RNA2, NS3 (nonstructural protein) and NP (nucleocapsid protein), NCP (nonstructural disease-specific protein) and MP (movement protein) in RNA4.

referred as ovarian tumour (OTU), and indicated that RdRp might produce two different proteins; a novel inverted repeat motif was detected within the intergenic regions of ambisense RNAs (Zhang et al., 2007). Both Zhang et al. (2007a) and a previous study (Wei et al., 2003) that analyzed the intergenic region of RNA4 for 22 isolates in China suggested the possible role of the intergenic region in transcription termination. Using available RdRp amino acid sequences, we constructed a phylogenetic tree in which RSV isolates were divided into two groups (Fig. 2). The first group includes several RSV isolates from Korea, China, and Japan while the second group possesses seven RSV isolates only from China. These results suggested the RSV isolates in the first group might be diverged from the same RSV ancestor.

V. Functions of RSV proteins

A recent study confirmed that PC2 tagged with enhanced GFP was cleaved into two different proteins, PC2-N and PC2-C (Zhao et al., 2012b). Both cleaved PC2 proteins are targeted to endoplasmic reticulum (ER) independently indicating that PC2 seems to contain three domains for ER targeting (Zhao et al., 2012b). Another study showed that PC2 protein,

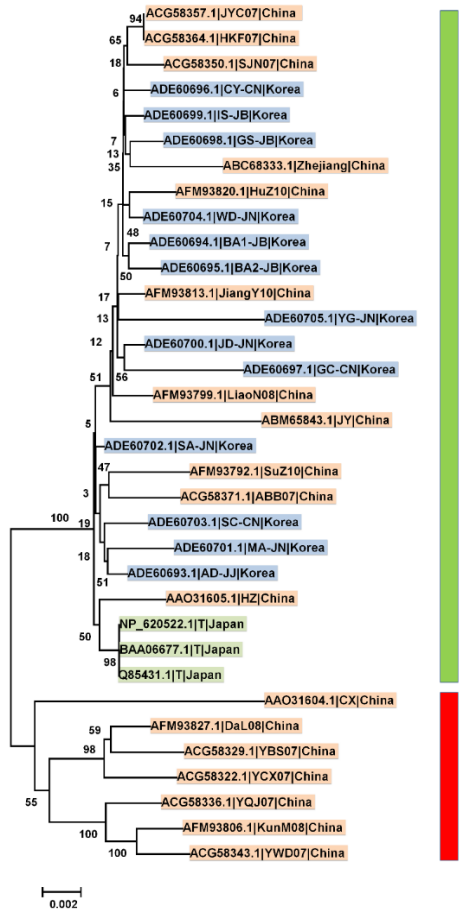

Fig. 2. The phylogenetic tree of 34 RSV isolates from China, Japan, and Korea A total of 34 RdRp amino sequences for 34 RSV isolates were retrieved. The phylogenetic tree was constructed based on the neighbor-joining method with 1000 bootstraps using MEGA5 program (www.megasoftware.net). Numbers at each node indicate bootstrap values. The scale bar represents 0.1 substitutions per amino acid site. Protein accession number, name, and country for each isolate were indicated. Red, green, and blue colors indicate isolates from China, Japan, and Korea, respectively. The phylogenetic tree displayed two groups of RSV isolates. The first group in green bar is composed of isolates from three countries while the second group in red bar contains isolates only from China.

which is similar to those membrane glycoproteins of the members belonging to the family *Bunyaviridae*, could be expressed on the surface of insect Sf9 cells (Zhao et al., 2012a).

The P3 protein could be expressed in the rice host as well as in SBPH vector and it forms inclusion bodies in both plant and insect cells (Takahashi et al., 2003). P3 protein was identified as a RNA silencing suppressor and can reduce the levels of small interfering RNAs (siRNAs) by binding to those small RNAs but not to double-stranded RNAs (Xiong et al., 2009). P3 tagged with green fluorescence protein (GFP) was targeted to nucleus and both N- and C-terminal regions of P3 are important for RNA silencing (Xiong et al., 2009). In contrast, a recent study with dsRNA-binding mutants and quantitative biochemical approaches revealed that dimeric P3 is a size independent dsRNA binding protein that can bind to long dsRNA with two or more copies (Shen et al., 2010). This study provided evidence that RNA silencing activity of P3 is is a size independent dsRNA-binding protein that can bind to long dsRNA with two or more copies (Shen et al., 2010). This study provided evidence that the RNA-silencing activity of P3 is correlated with its dsRNA-binding activity (Shen et al., 2010). A previous study that measured the nucleic acid binding activity of six RSV proteins suggested that three viral proteins (P3, PC3, and PC4) could bind to single-

and double-stranded RNA3 and RNA4 of RSV (Liang et al., 2005a).

PC4, encoded by RSV RNA4, supports the intercellular trafficking of a movement-deficient *Potato virus X* (PVX) in *Nicotiana benthamiana* leaves and suggest that PC4 is a viral-movement protein (Xiong et al., 2008). An *Agrobacterium*-mediated transient assay with enhanced green fluorescent fusion protein and immune cytochemistry suggested plasmodesmal localization (Xiong et al., 2008). In addition, a movement-deficient *Tobacco mosaic virus* (TMV) and mutagenesis were used to identify the regions of PC4 required for cell-to-cell movement, long-distance movement, and leaf necrosis response (Zhang et al., 2012). Moreover, PC4 was determined to target to plasmodesmata (PD) via ER to Golgi secretory pathway and by using actin-myosin VIII-1 motility system (Yuan et al., 2011).

VI. Identification of rice host factors interacting with RSV proteins

RSV uses host factors to complete its life cycles and it is therefore important to identify host factors that interact with RSV proteins or RNAs. However, only three rice host factors interacting with RSV proteins have been identified. Based on yeast two-hybrid (Y2H) systems, two rice chaperone proteins including a type I DnaJ and a small Hsp were identified as host

factors interacting with RSV MP and their specific interaction was confirmed by coimmunoprecipitation experiment (Lu et al., 2009). Y2H and biomolecular fluorescence complementation (BiFC) experiments demonstrated that another rice host protein, which is homologous to *Arabidopsis* suppressor of gene silencing (*AtSGS3*), can bind to RSV P2, (Du et al., 2011). Furthermore, the expression of RSV *P2* gene enhanced infectivity and pathogenicity of *Potato virus X* in *N. benthamiana* confirming the functional role of P2 as a silencing suppressor. A recent study identified five SBPH host proteins (a receptor for activated protein kinase C (RACK), glyceraldehyde-3-phosphate dehydrogenase (GAPDH3), and three ribosomal proteins) that interacted with RSV proteins (Li et al., 2011). a dot immunobinding assay indicated that all five proteins interacted with RSV particles; a Y2H experiment indicated, however, that RACK and GAPDH3 did not bind to RSV NP protein. RACK and GAPDH3 might be involved in RSV replication (Li et al., 2011).

VII. RSV proteins interaction studies in this work

In this study, protein–protein interaction study among RSV proteins was conducted to characterize the functions of RSV proteins. First, we

developed antibodies for RSV proteins using recombinant proteins and synthetic polypeptides used as antigens. A total of six polyclonal antibodies consisted of two recombinant proteins and four synthetic polypeptides were raised in rabbits. Of two recombinant proteins, only anti-NCP displayed stable hybridization signals in western blot analysis. In case of synthetic polypeptides, antibodies for NP and NCP were very effective to detect RSV in both RSV infected rice and weed plants. However, antibodies for NS3 and NSvc4 showed weak specific bands as well as strong non-specific background due to the difference of viral proteins produced in the infected leaves. Second, we investigated interactions between six of the RSV proteins by yeast-two hybrid (Y2H) assay *in vitro* and by bimolecular fluorescence complementation (BiFC) *in planta*. Y2H identified self-interaction of the nucleocapsid protein (NP) and NS3, while BiFC revealed self-interaction of NP, NS3, and NCP. Interaction study on NP self-interaction by NP deletion and substitution mutants indicate that the N-terminal region (aa 1–36 and 42–47) is required for NP self-interaction. BiFC and co-localization studies showed that the region required for NP self-interaction is also required for NP localization at the nucleus.

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CHAPTER I

Generation of antibodies to *Rice stripe virus* proteins based on recombinant proteins and synthetic polypeptides

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ABSTRACT

Rice stripe virus (RSV) is one of serious epidemic pathogens for rice species grown in mostly Asia. Therefore, it is necessary to produce a diagnostic detection kit applicable in fields for RSV detection. For that, antibodies for RSV were generated on the basis of recombinant proteins and synthetic polypeptides as antigens. Among seven proteins in RSV, genes encoding NCP and NS3 proteins were cloned into the expression vector carrying His-tag after RT-PCR amplification. We obtained purified His-tagged recombinant proteins which were produced in *E. coli*. Alternately, we also applied synthetic polypeptides of RSV proteins which are suitable to raise antiserum for antibody production. A total of six polyclonal antibodies consisted of two recombinant proteins and four synthetic polypeptides were raised in rabbits. Of two recombinant proteins, only anti-NCP displayed stable hybridization signals in western blot analysis. In case of synthetic polypeptides, antibodies for NP and NCP were very effective to detect RSV in both RSV infected rice and weed plants. However, antibodies for NS3 and NSvc4 showed weak specific bands as well as strong non-specific background due to the difference of viral proteins

produced in the infected leaves. In summary, the antibodies generated for RSV in this study will be useful for various assays such as RSV diagnostic detection, immunoprecipitation, protein purification, and western blot analysis.

INTRODUCTION

Rice (*Oryza sativa*) is one of most important crops in the world, especially, in Eastern Asia. Of known pathogens for rice, the rice stripe disease caused by *Rice stripe virus* (RSV) lead to dramatic hazardous impact on rice quantity and quality. RSV is a member of the genus *Tenuivirus* belonging to the family *Bunyaviridae*. RSV has known to be one of economically important virus with severe symptoms such as chlorotic stripes, chlorosis, and necrosis in leaves of many cultivated rice varieties (Hibino, 1996; Toriyama, 2000). Interestingly, most of Japanese paddy varieties are highly susceptible to RSV while Japanese upland varieties and Indica rice species varieties are resistant or tolerant to RSV (Maeda et al., 2006).

Since first report of RSV in 1965 in Korea, the RSV-mediated disease is gradually regarded as a severe disease (Toriyama, 2000). For example, during period 2007-2009, the RSV caused serious damage on rice production affecting 84% of total paddy field in many areas (Choi and Kim, unpublished). Since RSV was first reported in the early 1900s in Japan, this virus can be frequently occurred in temperate countries such as Korea, China, and Japan (Abo and Sy, 1998; Hibino, 1996). RSV can also infect not only rice but also plant species belonging to the family *Gramineae*

plants including maize, wheat, oat, foxtail millet, and several weeds (Toriyama, 2000). This virus is transmitted by mostly *Laodelphax striatellus* as well as three other plant hoppers.

The feature of RSV is a thin and filamentous shape without envelope. RSV is composed of four single stranded RNA segments named as RNA1, 2, 3, and 4 based on their segment size (Hamamatsu et al., 1993). For instance, RNA1 (~8.9 kb) is the largest RNA segment in RSV which encodes protein for RNA-dependent RNA polymerase (RdRp) in positive sense orientation. In contrast, each of RNA2, 3, and 4, encodes two proteins, respectively, in ambisense orientation. For example, RNA2 encodes NS2 (unknown function) and NSvc2 (glycoprotein). And NS3 and NP proteins are encoded by RNA3 whereas NCP (disease specific protein) and NSvc4 (movement protein) proteins are encoded by RNA4 (Xiong et al., 2008, 2009).

Recent phylogenetic studies have shown that RSV isolates in Korea showed mixtures of RSV populations from China and Japan suggesting invasion of RSV from other countries. Moreover, the RSV populations in China also displayed high divergence caused by frequent gene flow, however, it seems that there is no invasion of new RSV subtypes in China (Wei et al., 2009). In this study, we developed antibodies for RSV proteins using recombinant proteins and synthetic polypeptides used as antigens. To

test obtained antibodies for RSV detection, we performed western blot analysis using several RSV infected plants including rice and weeds.

MATERIALS AND METHODS

I. RNA isolation from RSV infected plants

RSV isolates were obtained and maintained as previously described (Jonson et al., 2009a, 2009b). Total RNA was prepared using Trizol as described previously (Jonson et al., 2009a, 2009b). The isolated total RNA was stored at -20 ℃ for further study.

II. Viral gene amplification and cloning to construct recombinant plasmid

We amplified two *NCP* (536 bp) and *NS3* (636 bp) genes of RSV by RT-PCR using gene specific primers designed upon previously obtained complete nucleotide sequences (Jonson et al., 2009a, 2009b). Each primer containing *Eco*RI and *Hin*dIII restriction sites is as follows: NCP_for 5'-AGA GAA TTC ATG CAA GAC GTA CAA AGG AC-3', NCP_rev 5'-TCT AAG CTT CTA TGT NTT GTG TAG AAG AG, NS3_for AGA GAA TTC ATG AAC GTG TTC ACA TCG TC, and NS3_rev TCT AAG CTT CTA CAG CAC AGT GGA GAG C-3'. The amplified PCR products

were purified and cloned into pGEM-T easy vector (Promega, Madison, USA) Cloned plasmids were transformed into *E. coli* DH10B. For the production of viral proteins fused with His-tag, DNA fragments for NCP and NS3 in pGEM-T easy vector were digested by *Eco*RI and *Hin*dIII enzymes (New England Biolabs, Ipswich, UK) and then were ligated into pET-28 and pET-30 expression vectors carrying His-tag at N-terminal region, respectively. His-tag fused proteins were expressed in *E. coli* BL21 and were purified according to manufacturer's instruction (Merck, Darmstadt, Germany).

III. Expression and purification of His-tag fusion proteins

Each cloned pET-28 or pET-30 plasmid was transformed into *E. coli* BL21 (DE3) by electroporation using a BTX Electro Square Porator^{TM} ECM830 (BTX Instrument Division, MA, USA). An overnight culture of transformed *E. coli* was diluted 1:100 in fresh LB (Luria-Bertani) medium and incubated at 30℃ until the OD_{600} reached 0.6. Expression of His-tagged RSV NCP and NS3 were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 hours at 30℃. The cells were immediately chilled on ice, collected by centrifugation at 5,000 rpm for 5 min using Gyro1524M

(Gyrozen, Daejeon, Korea), and pellet was frozen at -70℃, thawed, resuspended in extraction buffer (20 mM sodium phosphate, 500 mM NaCl, 10 mM Imidazole pH 7.4), and disrupted by sonication on ice. Cell lysate was centrifuged at 13,000 rpm for 20 min using Gyro1524M and the recombinant protein was purified from the soluble fraction by affinity chromatography using a Ni/NTA column (QIAGEN, Hilden, Germany). Purified proteins were first analyzed by SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel electrophoresis).

IV. Polypeptide design for antibody production

To select proper polypeptides for RSV protein antibody production, several peptides were designed by AbFrontier company (Young In Frontier, Seoul, Korea). For antibody production, optimal length of peptides is 15-25 amino acids (aa). Longer peptides more than 25 aa can be used with high cost and short peptides less than 10 aa may present limited number of epitope (http://www.anaspec.com/html/antibody_notes.html). As a result, several polypeptides up to six were designed (Table 2). Among five or six peptides for each target protein, two peptides which can most likely accessible to the full length protein were selected based on a prediction program and

expertise. Antibody was produced by co-immunizing with two selected peptides.

V. Antibody production

For the production of polyclonal antibodies, two different antigen types were used such as recombinant proteins for NCP and NS3 and synthetic peptides for NS3, NP, NCP, and NSvc4 proteins. Polyclonal antibodies were raised in rabbits according to instruction of AbFrontier company (Young In Frontier, Seoul, Korea). The detailed protocol for antigen preparation can be found in website of AbFrontier (https://www.abfrontier.com).

VI. Protein extraction from plant tissues

Leaves of RSV infected plants (rice and weeds) from field were harvested and were kept in -80℃. About 0.2 g of frozen plant materials were homogenized using a mortar and pestle. One milliliter of grinding buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA) was applied to the samples and was ground continually. To extract proteins, we added 0.5 mL of 2X Laemili's loading buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004%

bromphenol blue, 0.125 M Tris-HCl, 0.2 M dithiothreitol). Homogenized samples were boiled at 100℃ for 5 min. And denatured samples were centrifuged at 13,000 rpm for 3 min using Gyro1524M. After centrifugation, the supernatant were transferred into a 1.5 mL tube and deposited at -20℃.

VII. Western blot analysis

In advance to load protein samples in SDS-PAGE gel, proteins were boiled at 100℃ for 5 min and were resuspended in 30 μL of Laemmli loading buffer (Laemmli, 1970), electrophoresed on 12% SDS-polyacrylamide gel, and blotted onto PVDF transfer membranes (GE Healthcare, NJ, USA) (Towbin et al., 1992). The blots were probed with antiserum prepared against each His-tagged RSV protein or synthetic polypeptide. The products were visualized using a NBT-BCIP solution (Sigma-Aldrich, Munich, Germany).

RESULTS AND DISCUSSION

I. Generation of recombinant proteins for NS3 and NP proteins in *E. coli*

In order to generate antibodies to detect RSV infection, we first selected four functionally important proteins with small protein size, such as NS3 and NP proteins from RNA 3 segment, and NCP and NSvc4 from RNA 4 segment (Table 1). The protein size of four proteins ranged from 21.5 to 35.1 kDa. For the generation of antibodies, we used two different approaches. The first is to use recombinant proteins which are generated by cDNA cloning and protein expression in *E. coli* followed by protein purification. The other is to use synthetic polypeptides of interested protein.

For the production of recombinant proteins, we amplified genes encoding NCP and NS3 proteins by RT-PCR and purified PCR products were cloned into pET-28 and pET-30 expression vector, respectively. Two viral proteins fused with His-tag were expressed in *E. coli* BL21. To select proper bacterial cells expressing target proteins, we performed SDS-PAGE analysis separating cell lysates which were expressed in individual bacterial cell carrying the expression vector. From two independent bacterial cells, we

could detect an additional band about 24-28 kDa with presence of IPTG (Isopropyl β-D-1-thiogalactopyranoside) (Fig. 1A). In case of NCP-His-tag, proteins were expressed in four different bacterial cells with or without IPTG. Of them, we found an additional protein band from #5 cell with IPTG (Fig. 1B). The size of protein band was highly similar that of expected size (about 22 kDa). The obtained lysates were used for recombinant protein purification. For example, start material containing lysates and flow-through material from NS3-His-tag showed a large number of protein bands (Fig. 2). After washing, we obtained most strong protein signal from second eluate (E2) in NS3-His-tag (Fig. 2). In final, we could purify recombinant proteins for NS3 and NCP.

II. Assessment of generated antibodies to detect RSV in RSV infected rice and weeds.

For the recombinant protein-antibody, we used NCP and NS3 fused with His-tag. However, we obtained consistent positive results from only anti-NCP antibody (data not shown). To test anti-NCP antibody to detect RSV, we collected plant materials including rice and weeds which were infected by RSV. RT-PCR was performed to confirm whether the collected

Table 1. The list of RSV proteins used for antibody production

NS3 and NP from RNA3 genome as well as NCP and NSvc4 from RNA4 genome were used to produce antibodies.

	NS3	NP	NCP	NSvc4
Function	Gene silencing suppressor	Nucleocapsid protein	Pathogenesis related protein	Movement protein
MW	23.9 kDa	35.1 kDa	21.5 kDa	32.4 kDa
Length	636 bp	969 bp	536 bp	861 bp
Protein antiserum	Produced with negative result (-)	Not developed	Produced with positive result (+)	Not developed
Peptide antiserum	Produced with negative result (-)	Produced with positive result (+)	Produced with positive result (+)	Produced with negative result (-)

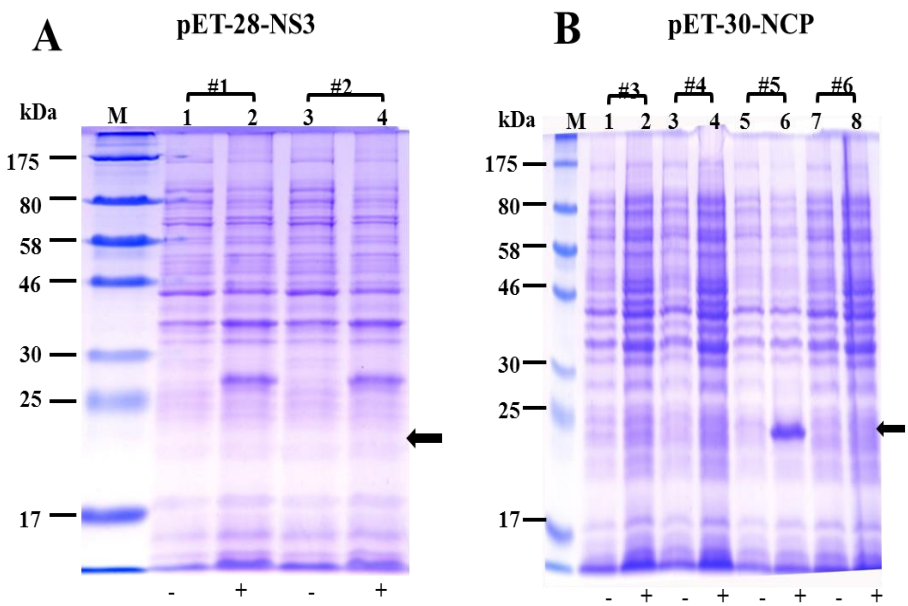

Fig. 1. SDS-PAGE analysis of the cell lysate derived from induced host bacteria *E. coli* BL21 carrying the recombinant expression vector. (A) Lysate of *E. coli* BL21 carrying pET-28-NS3 without IPTG (-) for lane 1 and 3, and with IPTG (+) for lane 2 and 4. The #1 and #2 indicate two independent cells carrying the pET-28-NS3 construct. (B) Lysate of *E. coli* BL21 carrying pET-30-NCP without IPTG (-) for lane 1, 3, 5, and 7, and with IPTG (+) for lane 2, 4, 6, and 8. The #3, #4, #5, and #6 indicate four independent cells carrying the pET-30-NCP construct. M: protein marker, Black arrows indicate expected bands of recombinant proteins. NS3-His-tag showed a large number of protein bands (Fig. 2). After washing, we obtained most strong protein signal from second eluate (E2) in NS3-His-tag (Fig. 2). In final, we could purify recombinant proteins for NS3 and NCP.

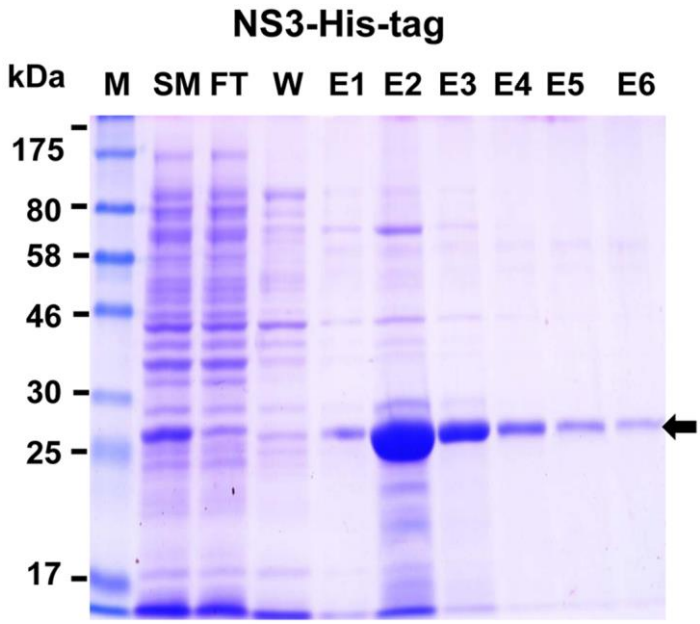

Fig. 2. SDS-PAGE analysis of purified His-tagged recombinant proteins using His GraviTrap™ columns. SDS-PAGE showed purified recombinant proteins for NS3-His-tag after expression in *E. coli* BL21. M: protein marker, SM: start material, FT: flow-through, W: washed material, E1-E6: eluate. Black arrow indicates an expected band of recombinant protein.

samples have been infected with RSV (Fig. 3A and 3B). We used RSV infected samples from two different regions for western blot analysis whether newly developed RSV antibodies can be applied for RSV detection regardless of their genotypes. RT-PCR results confirmed RSV infections except samples 1 and 5, all rice samples were infected by RSV (Fig. 3A). Western blot analysis identified bands with 21.5 kDa in rice sample 6 and 8 (Fig. 3C). Unfortunately, we could not detect any bands from samples 1-5 whereas sample 6 and 8 showed very strong bands. Although we loaded equal amount of proteins, we observed the differences in RSV infection severity from semi-quantitative RT-PCR (Fig. 3A and 3B) and thus the amount of viral proteins loaded in each lane might not be identical resulting in unsuccessful identification of virus infection. In addition, disease severity and/or infection time caused by RSV isolates of each collected sample might contribute to the production of individual RSV protein. Because we collected RSV infected plant leaves based on the symptoms but did not know the exact infection time and the relative amount of target RSV proteins. Although it is well known that rice is the major host for RSV, it can also infect other plants such as weeds. To test whether generated anti-NCP antibody can be also useful to detect RSV in RSV infected weeds, we collected three different kinds of weeds like green bristle grass (*Setaria*

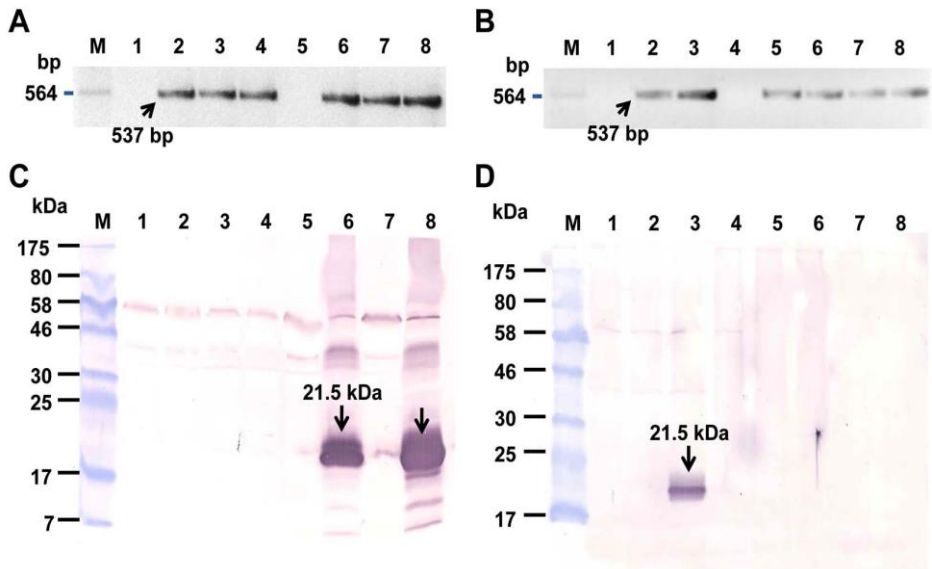

Fig. 3. RT-PCR and Western blot analysis to detect *Rice strip virus* (RSV) using recombinant protein-antibodies. (A & B) RT-PCR detection of RSV RNA. Accumulation of RSV RNA was determined by RT-PCR as described in the Materials and Methods. Except samples 1 and 5 in panel A and 1and 4 in panel B, all samples were positive to RSV by RT-PCR detection. (C & D) Western blot analysis. Proteins extracted from RSV infected rice and weeds were separated on SDS-PAGE gel. After blotting on the PVDF membrane, two protein-antibodies were applied for western blot analysis. Detection RSV in RSV infected rice (C) and weeds (D) by western blot analyses using anti-NCP antibody derived from the His-tag recombinant protein. (C) The rice samples were collected from Kimje (lanes 1-4) and Seocheon (lanes 5-8). (D) The weeds samples were collected from Anheung (green bristle grass; *Setaria viridis*; lanes 1-3 and andcrabgrass; *Digitaria sanguinalis*; lanes 4-6) and Seosan (barnyardgrass; *Echinochloa crus-galli*; lanes 7-8) and used for western blot analysis. Anti-NCP antibody was diluted to 1:20000.

Table 2. List of RSV peptides identified by bioinformatic program for antibody production.

Protein	No.	Peptide sequences	Position[a]	Length	Antibody[b]
NP	1	TLADLQKAINDISKD-C	8-22	16	
	2	KDKGGDTLAKDMTM-C	56-70	15	
	3	FVRDVTKKVKVAAGS-C	81-95	16	
	4	C-KTMPEGKKKERGLTK	199-214	17	
	5	C-KKKEDGRKKVLDEF	237-251	16	O
	6	C-LVKEFFEDEAEGADD	308-322	16	O
NS3	1	MQDVQRTIEVSVGP-C	1-14	15	
	2	C-PDLKDPERVTEDTKK	38-52	16	O
	3	GGEDDDNALIDIG	93-105	13	
	4	KNFLRHPNRMSKDQIK-C	121-136	17	
	5	C-AESSDTEEYEKVWKK	147-162	17	O
NCP	1	MNVFTSSVGSVEFD-C	1-14	15	
	2	C-YIYDIHSSRHPSIDEHQ	41-58	18	
	3	C-SHDKNLPEEYRLPT	84-97	15	O
	4	C-NRKTLYRSPSKKRHK	162-177	17	O
	5	C-KYVDSSALEPSPGSSP	193-208	17	
NSvc4	1	C-LSEESQKRVDNKNRK	18-33	17	O
	2	KQDYSVKAHRKATFN-C	77-91	16	
	3	KSYVNSSDQVEVEVR-C	136-150	16	
	4	C-EFEPLEDKAINHLSS	218-232	16	
	5	C-KQPKKIPITKKSKSEVS	267-283	18	O

[a]Position indicates the first and last amino acid covering the sequence in each viral protein.

[b]Peptides used for antibody production.

viridis), crabgrass (*Digitaria sanguinalis*), and barnyardgrass (*Echinochloa crus-galli*). Of three RSV infected weeds, like green bristle grass (*Setaria viridis*), crabgrass (*Digitaria sanguinalis*), and barnyardgrass (*Echinochloa crus-galli*). Of three RSV infected weeds, only green bristle grass showed a clear band about 21.5 kDa (Fig. 3B). This result suggests that the generated anti-NCP can be implicated in detection of RSV at least in rice and green bristle grass and also suggest that there is a sensitivity difference between RT-PCR and antibody based detection methods as previously reported.

In addition to antibodies generated from recombinant proteins, we also tested antiserums derived from synthetic peptides. A total of four antiserums for NCP, NP, NS3, and NSvc4 were used for western analysis (Table 1). To optimize titer of each antiserum, we first tested individual antibody with various titers. Finally, optimal titer was obtained such as 1: 50,000 titer for NCP, 1: 10,000 titer for NP, 1: 1,000 titer for NS3, and 1: 1,000 titer for NSvc4. To compare efficiency of each antibody, we isolated proteins from RSV infected rice and two kinds of weeds like green bristle grass and crabgrass. Extracted proteins were separated on SDS-PAGE and visualized by staining with Commassie Brilliant Blue. Although same amount of proteins were loaded, the band pattern were totally different from each other. For example, proteins in lane 1-4 were extracted from rice, however, lane 1

and 3 showed similar banding pattern with strong bands about 55 kDa whereas land 2 and 4 displayed two strong bands about 35 kDa and 25 kDa imposing that these two additional bands might also be derived from RSV infection (Fig. 4A). Lanes 5-8 for weed proteins showed relatively weaker bands as compared to rice proteins (Fig. 4A). Despite of difference in band pattern, all proteins in lane 1-8 have also common bands like about 28 kDa and 55 kDa (Fig. 4A).

Using antiserum for NCP and NP, we detected NCP and NP protein from three samples including two rice and one green bristle grass (Fig. 4B and 4C). Based on the intensity of band, rice samples possessed more replicated RSV than that of green bristle grass. Antiserums for NP and NS3 revealed RSV only from rice samples (Fig. 4D). Especially, the detected band size (about 28 kDa) for anti-NS3 derived from synthetic peptides was slightly higher as compared to the expected band size (about 23.9 kDa) (Fig. 4D). This result is highly correlated with the result from purified His-tagged NS3 protein (Fig. 2). The increased size of recombinant protein or antibody for NS3 might be caused by protein aggregation. Anti-NSvc4 antibody detected only RSV protein in one rice sample (Fig. 4E). As shown in RT-PCR results, the amount of RSV RNAs seems to be highly correlated with that of RSV proteins (Fig. 4F). Although we used a same SDS-PAGE

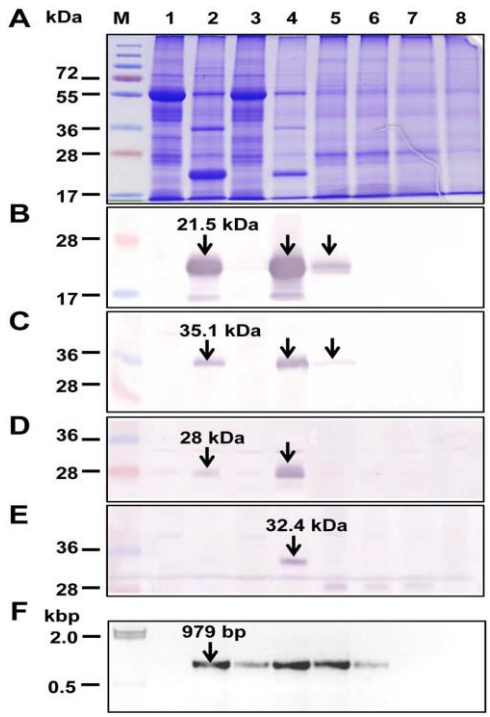

Fig. 4. Western blot analysis to detect RSV using peptide-antibodies. Proteins extracted from RSV infected rice and weeds were separated on SDS-PAGE gel. To visualize separated proteins, SDS-PAGE gel was stained with Commasie Brilliant Blue (A). The blot on PVDF membrane was probed with peptide-antibody for NCP (B), NP (C), NS3 (D), and NSvc4 (E) proteins, respectively. Individual RSV viral protein was detected after reaction with freshly prepared NBT-BCIP substrate. M: Molecular marker. Lane 1-4: Rice samples collected from Seocheon region. Lane 5-7: Green bristle grass (*Setaria viridis*) samples collected from Anheung region. Lane 8: Crabgrass (*Digitaria Sanguinalis*) samples collected from Anheung region. Antibody dilution rates for NCP, NP, NS3, and NSvc4 proteins were 1: 50,000 titer, 1: 10,000 titer, 1: 1,000 titer, and 1: 1,000 titer, respectively. (F) RT-PCR detection of RSV RNA. Accumulation of RSV RNA was determined by RT-PCR as described in the Materials and Methods. Except samples loaded in lanes 1, 7 and 8, all samples were positive to RSV by RT-PCR detection.

for western blot analysis, the obtained intensity for each protein band was varied. This might be caused by different titer of antibody. Of four antibodies produced from synthetic peptides, anti-NCP seems to be superiors with reliable protein detection ability including rice and green bristle grass.

In this study, we generated antibodies for RSV proteins using recombinant proteins and synthetic peptides as antigens. So far, several groups have developed monoclonal or polyclonal antibodies against RSV (Omura et al., 1986; Liang et al., 2005). And most of them were produced using recombinant proteins which require several different procedures such as cloning and purification. As compared to recombinant protein based approach, synthetic peptides have many advantages. For instance, as we have shown in this study, it does not need cloning and purification steps. Most important step is the selection of proper peptides which represent the target viral protein. Moreover, antigens using synthetic peptides produced more effective antibodies with high rate of success and specificity. However, synthetic peptides have also weak points such as lower affinity and longer immunization time as compared to recombinant proteins. Regardless of such disadvantages, our study showed that antibodies produced from synthetic peptides were superior to those generated from recombinant proteins.

Antibodies for viral proteins can be practically used in a wide range of application. First of all, they can be used to detect RSV in both RSV infected plants including rice and weeds and viruliferous insects like brown planthoppers by western blot and enzyme-linked immunosorbent assay (ELISA). Furthermore, the generated antibodies can be applied to make agricultural plant virus diagnostic kits. Moreover, localization of RSV in rice plants can be also observed by immunofluorescent or immunogold microscopy using the viral antibodies. It is also possible to find host factors that interact with viral proteins by immunoprecipitation.

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CHAPTER II

Interaction study of *Rice stripe virus* proteins reveals a region of the nucleocapsid protein (NP) required for NP self-interaction and nuclear localization

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ABSTRACT

Rice stripe virus (RSV), which belongs to the genus *Tenuivirus*, is an emergent virus problem. The RSV genome is composed of four single-strand RNAs (RNA1 to RNA4) and encodes seven proteins. We investigated interactions between six of the RSV proteins by yeast-two hybrid (Y2H) assay *in vitro* and by bimolecular fluorescence complementation (BiFC) *in planta*. Y2H identified self-interaction of the nucleocapsid protein (NP) and NS3, while BiFC revealed self-interaction of NP, NS3, and NCP. To identify regions(s) and/or crucial amino acid (aa) residues required for NP self-interaction, we generated various truncated and aa substitution mutants. Y2H assay showed that the N-terminal region of NP (aa 1–56) is necessary for NP self-interaction. Further analysis with substitution mutants demonstrated that additional aa residues located at 42–47 affected their interaction with full-length NP. These results indicate that the N-terminal region (aa 1–36 and 42–47) is required for NP self-interaction. BiFC and co-localization studies showed that the region required for NP self-interaction is also required for NP localization at the nucleus. Overall, our results indicate that the N-terminal region (aa 1–47) of the NP is important for NP self-interaction and that six aa residues (42–47) are essential for both NP self-interaction and nuclear localization.

INTRODUCTION

As an emergent pathogen, *Rice stripe virus* (RSV) is reducing rice production in Far East Asian countries including China, Japan, and Korea (Cho et al., 2013; Hibino, 1996; Wei et al., 2009a). RSV was also recently reported in Southeast Asia for the first time (Ren et al., 2013). RSV is usually transmitted by the small brown plant-hopper (SBPH) (*Laodelphax striatellus* Fallén) in a persistent and circulative-propagative manner (Falk and Tsai, 1998). The natural hosts of RSV include rice, maize, wheat, oat, and foxtail millet (Falk and Tsai, 1998; Lian et al., 2011). RSV can also infect *Arabidopsis* and *Nicotiana benthamiana* under laboratory conditions (Sun et al., 2011; Yao et al., 2012).

RSV belongs to the genus *Tenuivirus* but has not yet been assigned to a virus family. The RSV genome consists of four single-stranded RNAs designated RNA1 to RNA4. RNA1 is the largest RNA segment, and viral-complementary sense RNA1 (vcRNA1) encodes a 337-kDa protein known as the RNA-dependent RNA polymerase (RdRp) (Toriyama et al., 1994). The viral-sense RNA2 (vRNA2) and the vcRNA2 in RNA2 encode P2 (unknown function, NS2; 22.8 kDa) and PC2 (glycoprotein, NSvc2; 94 kDa), respectively (Takahashi et al., 1993). Interestingly, NSvc2 can be

cleaved into two proteins, NSvc2-N and NSvc2-C (Zhao et al., 2012b). The P3 (a suppressor of gene silencing, NS3; 23.9 kDa) and nucleocapsid protein (NP; 35 kDa) are encoded by vRNA3 and vcRNA3, respectively (Hayano et al., 1990b; Kakutani et al., 1991). The vRNA4 and vcRNA4 in RNA4 encode a nonstructural disease-specific protein (NCP; 20.5 kDa) and PC4 (movement protein, MP; 32 kDa), respectively (Hayano et al., 1990b; Kakutani et al., 1990).

The interactions among viral proteins are important aspects of the virus life cycle in that they affect the formation of virus replication complexes, the assembly of virions, virus movement, and virus transmission by vectors (Guo et al., 2001). It follows that an understanding of interactions among viral proteins is necessary to understand the virus infection cycle and virus–host interactions. Protein–protein interactions have been reported for many viruses. For instance, protein–protein interaction maps for potyviruses such as potato virus A, pea seed-borne mosaic virus (PSbMV), tobacco vein mottling virus, and soybean mosaic virus (SMV) have been constructed using yeast two-hybrid (Y2H) and bimolecular fluorescence complementation (BiFC) assays (Guo et al., 2001; Hong et al., 1995; Kang et al., 2004). In addition, a previous study identified 52 of interactions between the mature proteins of plum pox virus (PPV) by BiFC approaches

and revealed the self-interactions of CI, 6K2, VPg, NIa-Pro, NIb, and CP (Zilian and Maiss, 2011). These studies with potyviruses identified some unique protein–protein interactions, such as an interaction between helper component-proteinase (HC-Pro) and viral protein (VPg), and CP self-interactions (Guo et al., 2001; Kang et al., 2004; Yambao et al., 2003). In the case of animal and human viruses, genome-wide interaction maps for vaccinia virus and hepatitis C virus have been reported (Flajolet et al., 2000; McCraith et al., 2000). The functional roles of viral protein–protein interactions have also been studied. For instance, self-interaction of the cucumber mosaic virus 2b protein plays a vital role in the suppression of RNA silencing and the induction of viral symptoms (Xu et al., 2013).

Recently, several protein–protein interactions have been also identified in negative-strand plant RNA viruses. For example, a recent study showed N and NSm protein self-interactions and an NSm–N interaction in impatiens necrotic spot virus by BiFC (Dietzgen et al., 2012). In addition, BiFC analyses confirmed that nucleoprotein (N)–phosphoprotein (P) and matrix protein (M)–M interactions were common in the members of the family Rhabdoviridae such as lettuce necrotic yellows virus, sonchus yellow net virus, and potato yellow dwarf virus (Martin et al., 2012). Furthermore, interaction between N and P proteins of orchid fleck virus (OFV) in nucleus

was identified by BiFC and is required for viroplasm formation of OFV (Kondo et al., 2013).

In the case of RSV, a previous study that used co-immunoprecipitation detected NP self-interaction but did not detect interaction among NP, NCP, and MP (Lu et al., 2008). Interactions among other RSV proteins have not been reported, and important regions of the viral proteins required for interaction have not been determined. In the present study, we used Y2H *in vitro* and BiFC *in planta* to construct a protein–protein interaction map for six RSV proteins. We also used various truncated and substitution mutants to investigate the domain of RSV NP that is required for self-interaction and nuclear localization.

MATERIALS AND METHODS

I. Preparation of plasmids

Each RSV gene was amplified by using previously constructed plasmids including RNA2 (GenBank: GQ229106.1), RNA3 (GenBank: FJ602681.1), and RNA4 (GenBank: FJ602694.1) (Jonson et al., 2009a; Jonson et al., 2009b). PCR was performed using *i-Pfu* DNA polymerase (European Biotech Network, Brussels, Belgium) or *Pfu Ultra* II fusion HS DNA polymerase (Agilent Technologies, California, USA) enzyme mix. The amplified PCR product was cloned into the pENTR/D-TOPO vector following the manufacturer's instructions (Invitrogen, Carlsbad, USA). The primers used to amplify RSV genes are listed in Table S1. NP, MP, NCP, NS3, and NS2 were cloned and expressed as whole and NSvc2 was expressed in two parts as it is cleaved (Zhao et al., 2012b). NSvc2-N (from amino acids (aa) 1–381) and NSvc2-C (from aa 382–834) were cloned separately. As a result, a total of seven entry clones were constructed. To generate expression clones, we performed *in vitro* recombination reactions which is catalyzed by Gateway® LR Clonase® II enzyme mix. Detailed information for Gateway system can be found in manufacturer's manual

(Invitrogen).

II. Generation of deletion and substitution mutants of NP

The coding regions of C-terminal truncated mutants ΔNP(1–36), ΔNP(1–47), ΔNP(1–56), ΔNP(1–76), ΔNP(1–96), and ΔNP(1–162) were amplified from full-length NP using PCR to introduce a stop codon after the indicated amino acids in reverse primers. The coding region of N terminal truncated mutant, ΔNP(163–322), was amplified from full-length NP using PCR to introduce a start codon before amino acid 163 in the forward primer. The amplified PCR product was cloned into the pENTR/D-TOPO vector following the manufacturer's instructions (Invitrogen). To construct an alpha helices deletion mutant, ΔNP(1–8+28–322), a pair of primers that jointed the indicated regions were designed. First, PCR was conducted using M13 forward primer and reverse connection primer to amplify the coding region of N-terminal, and forward connection primer and M13 reverse primers were used to amplify the coding region of C-terminal segment using the full-length NP entry clone plasmid as template. Second, joint PCR was conducted as previously described (Yu et al., 2004) to connect the segments coding N- and C-terminal. Therefore, a DNA segment was generated that

contained attL domains and a ΔNP(1–8+28–322) segment; this DNA segment was used for an LR reaction in order to clone ΔNP(1–8+28–322) into destination vectors.

To construct substitution mutants NP(^{37}FAGQI^{41}-5A) and NP(^{42}EYAGYD^{47}-6A), long primers which join the indicated regions were designed in which amino acids 37 to 41 (FAGQI) and 42 to 47 (EYAGYD) were replaced with five or six alanines. Similarly, separate PCRs were performed to clone the coding regions of N- and C-terminus using full-length NP as template, and the two segments were then connected by joint PCR. The amplified PCR product was cloned into the pENTR/D-TOPO vector following the manufacturer's instructions (Invitrogen). The primers used to generate NP deletion and substitution mutants are listed in Table S1.

III. Y2H assay

For protein interaction study, we used the ProQuest™ Two-Hybrid System (Invitrogen), a genetic method that detects interactions between proteins *in vivo* in the yeast S*accharomyces cerevisiae* (Fields and Song, 1989; Vidal et al., 1996). Each entry clone was cloned into the destination vectors

pDEST22 and pDEST32 using the Gateway LR Clonase II enzyme mix and then were co-transformed into the yeast strain MaV203 according to the manufacturer's protocol (Invitrogen). Four plasmids provided by the ProQuest™ Two-Hybrid System were used as controls. These plasmids included Krev1 (also termed Rap1A; a member of the Ras family of GTP-binding proteins) with RalGDS (the Ral guanine nucleotide dissociation stimulator protein (Herrmann et al., 1996; Serebriiskii et al., 1999). The RalGDS mutants RalGDS-m1 and RalGDS-m2 suppress the interaction with Krev1 and were generated using the SureFrame™ Allele Library Construction Kit.

IV. BiFC assay

To facilitate BiFC analysis by agroinfiltration, we modified the previous designed vectors pSAT4-DEST-nEYFP-C1 and pSAT5-DEST-cEYFP-C1 (Tzfira et al., 2005). The two vectors were digested with *I-Sce*I and *I-Ceu*I (NEB, Ipswich, USA), respectively, and then the two expression cassettes were ligated into the pPZP vector, which was obtained from pPZP-RCS2-*bar* after digestion with *I-Sce*I and *I-Ceu*I (NEB), respectively. As a result, pPZP-DEST-nEYFP-C1 and pPZP-DEST-cEYFP-C1 vectors

were constructed for BiFC destination vectors. Each individual RSV gene in the entry vector was cloned into BiFC destination vectors producing N-terminal or C-terminal yellow fluorescence protein (YFP) fusions. The vectors were transformed into *Agrobacterium tumefaciens* strain GV2260 by heat shock and used for agroinfiltration. Vector-transformed agrobacterium cultures were agroinfiltrated into the leaves of 4-week-old *N. benthamiana* plants, which were grown in a growth chamber at 25℃ under a 16/8 light/dark photoperiod. The expression of YFP was observed by fluorescent microscopy (Carl Zeiss, New York, USA).

V. Confocal microscopy

To confirm subcellular localization of each RSV viral protein, we used a plasma membrane marker (mCherry-AtPIP2A) (Cutler et al., 2000; Nelson et al., 2007) and a nuclear marker (mCherry-VirD2NLS) (Jaedicke et al., 2011). After co-inoculation, we observed GFP and RFP expression using a laser scanning confocal microscope equipped with lasers spanning the spectral range of 405-633 nm (Carl Zeiss).

RESULTS

I. Interactions between six RSV viral proteins as indicated by Y2H and BiFC

We performed Y2H to establish the interaction map for the RSV viral proteins NP, MP, NCP, NS3, NS2, and NSvc2. In the Y2H assay, only NP and NS3 showed self-interaction (Table 1, Fig. 1A). The self-interaction was much stronger for NP than for NS3. No other interactions among RSV viral proteins were detected by Y2H, regardless of which RSV proteins were fused downstream of the GAL4 DNA-binding domain (GAL4 DBD) or the GAL4 activation domain (GAL4 AD) (Table 1).

Next, we conducted BiFC assays to detect protein–protein interactions *in planta*. We used modified gateway compatible destination vectors for BiFC (Citovsky et al., 2006). BiFC confirmed self-interaction for both NP and NS3 (Fig. 1B). The BiFC assay also revealed self-interaction of NCP, a nonstructural RSV protein (Table 1), and interactions between NP, NS3, and NCP (Fig. 1B).

II. Subcellular localization of interacting proteins by BiFC

In addition to revealing the interaction between two proteins, BiFC assay can determine the subcellular localization of the interacting proteins. Thus, self-interacting protein complexes for NP, NS3, and NCP were localized at both the nucleus and plasma membrane (PM), but interacting protein complexes involving two different proteins were localized at the PM (Fig. 1B). To confirm the BiFC results, we tagged GFP to the N-terminal region of NP, NS3, and NCP and performed a co-localization study with a PM marker and a nuclear marker. As expected, the three proteins were co-localized with PM and nuclear markers, indicating their subcellular localization at the PM and nucleus (Fig. 2).

III. Characterization of region(s) required for NP self-interaction using truncated mutants

To identify the region required for RSV NP self-interaction, we generated several truncated mutants. Based on domains predicted by the SMART program (http://smart.embl-heidelberg.de), RSV NP was divided into two truncated parts, ΔNP(1–162) and ΔNP(163–322), which contain aa 1–162

Table 1. Summary of interactions between six RSV proteins as determined by Y2H and BiFC.

	NS2	NSvc2-N	NSvc2-C	NS3	NP	NCP	MP
NS2	-	-	-	-	-	-	-
NSvc2-N	-	-	-	-	-	-	-
NSvc2-C	-	-	-	-	-	-	-
NS3	-	-	-	++	-+	-+	-
NP	-	-	-	-+	++	-+	-
NCP	-	-	-	-+	-+	-+	-
MP	-	-	-	-	-	-	-

"-", no interaction; "++", interaction was detected by both Y2H and BiFC; "-+", interaction was detected by BiFC but not by Y2H.

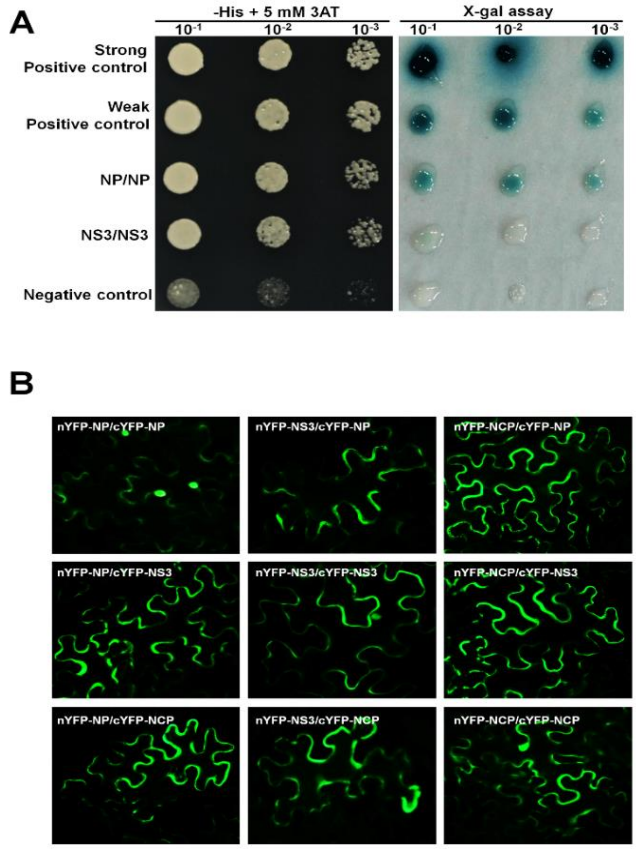

Fig. 1. Interaction study of RSV NP, NS3, and NCP by Y2H and BiFC. (A) Self-interaction of RSV NP and NS3 using Y2H. Colonies from -LT medium (SC medium without leucine or tryptophan) were verified on -His + 5 mM 3AT medium (SC medium without leucine, tryptophan, or histidine but with 5 mM 3-Amino-1,2,4-triazole) and in 5-bromo-5-chloro-3-indolyl-β-D-galactoside (X-gal) assay with dilutions of yeast cells at cell concentration of 10^{-1}, 10^{-2}, 10^{-3}. Three controls representing strong interaction, weak interaction, and no interaction were generated by co-transforming control plasmids provided by ProQuest™ Two-Hybrid System (Invitrogen). (B) Interaction study of NP, NS3, and NCP using BiFC. NP, NS3, and NCP were tagged with nYFP or cYFP at the N terminal. The three RSV proteins showed strong self-interaction and also interacted with each other.

and 163–322, respectively. NP truncated mutants and the full-length NP gene were fused downstream of GAL4 DBD and downstream of GAL4 AD, respectively. Self-interaction of truncated mutants as well as the interaction between truncated mutants and full-length NP were tested. ΔNP(1–162) and full-length NP interacted in that they expressed all reporter genes (*lac*Z, *HIS3*, and *URA3*), while ΔNP(163–322) did not interact with full-length NP (Fig. 3A). β-galactosidase (β-gal) activity was almost 50-times greater for the interaction between ΔNP(1–162) and full-length NP than for the NP self-interaction (Fig. 3A). Self-interactionof truncated mutants or interaction between two truncated mutants was not detected (Fig. 5). This result confirmed that the N-terminal region including aa 1–162 of RSV NP is important for NP self-interaction.

To better define the protein region required for NP self-interaction, we generated four truncated mutants based on the domain prediction and secondary structure prediction by I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER): ΔNP(1–36), ΔNP(1–56), ΔNP(1–76), and ΔNP(1–96). Y2H assays revealed that ΔNP(1–56), ΔNP(1–76), and ΔNP(1–96) but not ΔNP(1–36) could interact with full-length NP (Fig. 3A). Interestingly, the interactions were stronger between truncated mutants and full-length NP than in the NP self-interaction. Among the three

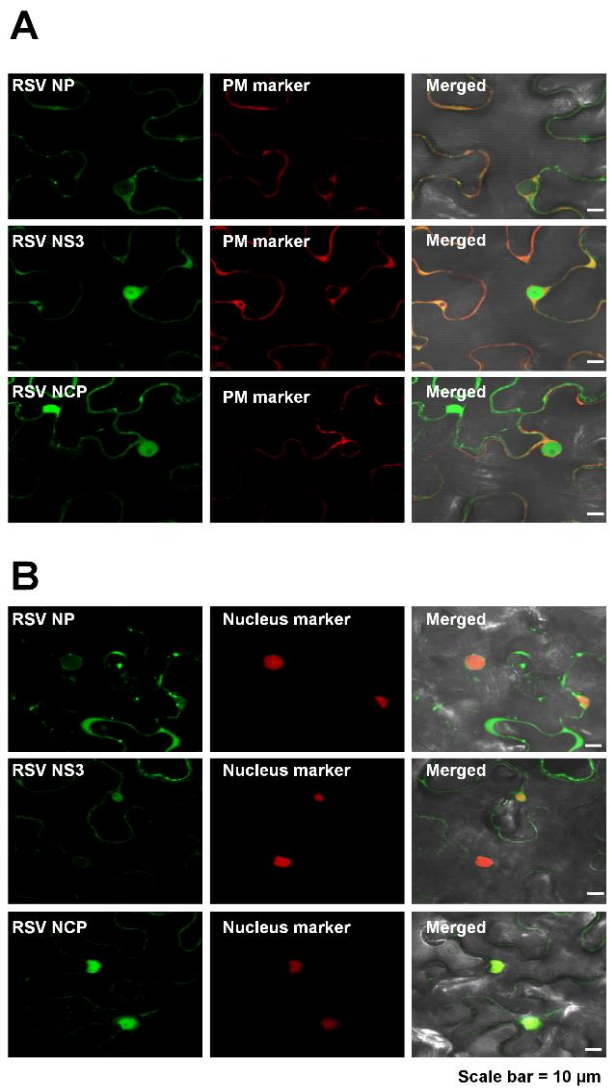

Fig. 2. Subcellular localization of NP, NS3, and NCP. (A) Co-localization study of NP, NS3, and NCP with a plasma membrane. (B) Co-localization study of NP, NS3, and NCP with a nuclear marker VirD2NLS. Each RSV protein was tagged with GFP at the N terminal, and the PM and nuclear markers were tagged with mCherry. At 36 h after co-infiltration of *Nicotiana benthamiana* leaves, green and red fluorescence were observed using confocal microscopy.

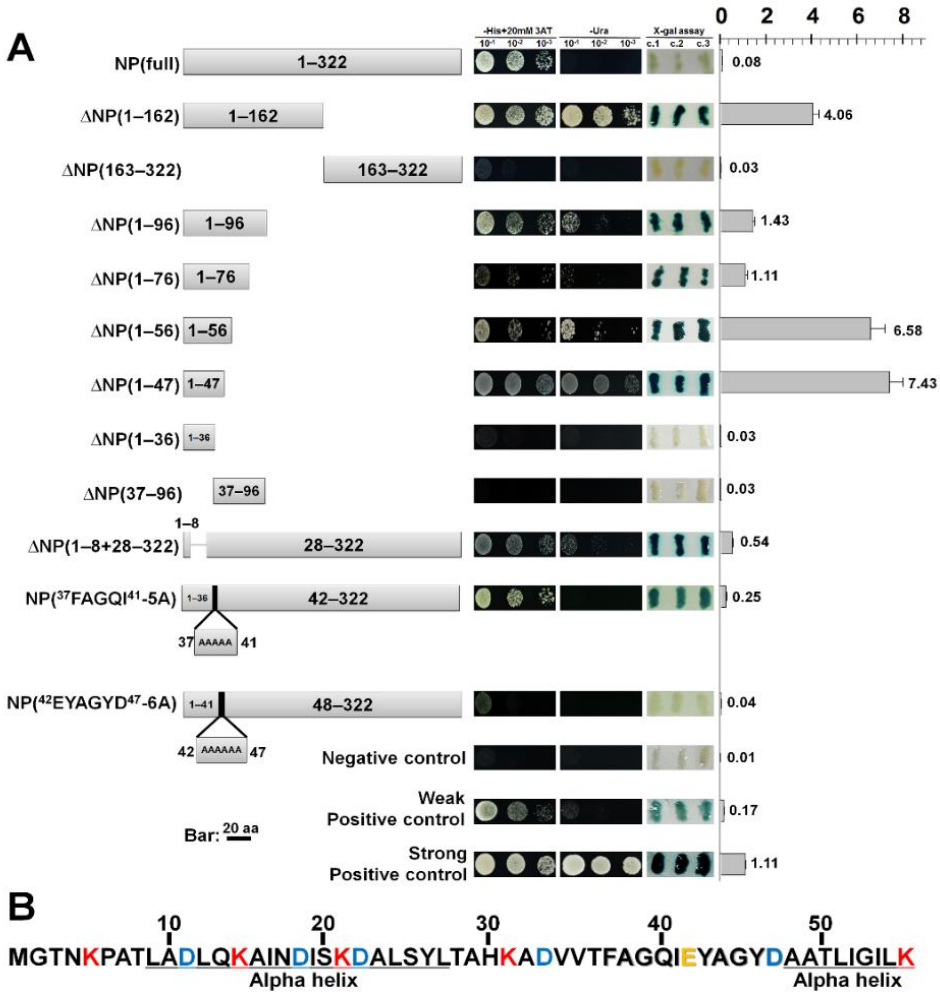

Fig. 3. Identification of the RSV NP region that is important for NP self-interaction. (A) Detailed information for all truncated mutants and their interaction with full-length NP by Y2H. Colonies from -LT medium (SC medium without leucine or tryptophan) were verified on -His + 20 mM 3AT medium (SC medium without leucine, tryptophan, or histidine but with 5 mM 3-Amino-1,2,4-triazole), -Ura (SC medium without leucine, tryptophan, or uracil) with dilutions of yeast cells at cell concentration of 10^{-1}, 10^{-2}, 10^{-3}, and three different colonies were used for X-gal assay. The interaction strength was calculated by quantitative assays for β-galactosidase activity in liquid medium. three controls (representing a strong interaction, a weak interaction, and no interaction)

were generated by co-transforming control plasmids provided by ProQuest™ Two-Hybrid System (Invitrogen). (B) Amino acid sequences of RSV NP (from 1 to 56). Two alpha helices, located at aa 9–27 and 48–56, were predicted by I-TASSER ONLINE. The colored amino acids indicate charged amino acids.

truncated mutants, ΔNP(1–56) showed the strongest interaction with full-length NP (Fig. 3A). Next, we generated the deletion mutant ΔNP(37–96) to test whether aa 37–96 of NP is important for the interaction. Surprisingly, ΔNP(37–96) did not interact with full-length NP by Y2H (Fig. 3A). Therefore, it seems that a region containing aa 1–56 at the N terminal region could be important for NP self-interaction.

We studied the interactions by Y2H among all truncated mutants and confirmed the interaction by BiFC. However, no significant interaction was found among the NP truncated mutants (Fig. 5). Therefore, the full-length of NP is necessary as an interaction partner.

IV. Mapping of the NP self-interaction domain

To further define an important region for NP self-interaction, we predicted the 3D structure of ΔNP(1–56) based on aa sequences using the I-TASSER program. Two alpha helices, located at aa 9–27 and aa 48–56, were predicted (Fig. 3B). The alpha helix is one of the most abundant secondary structural motifs and is involved in a wide range of protein interactions (Azzarito et al., 2013). To determine whether these two alpha helices are involved in NP self-interaction, we generated two deletion mutants, ΔNP(1–

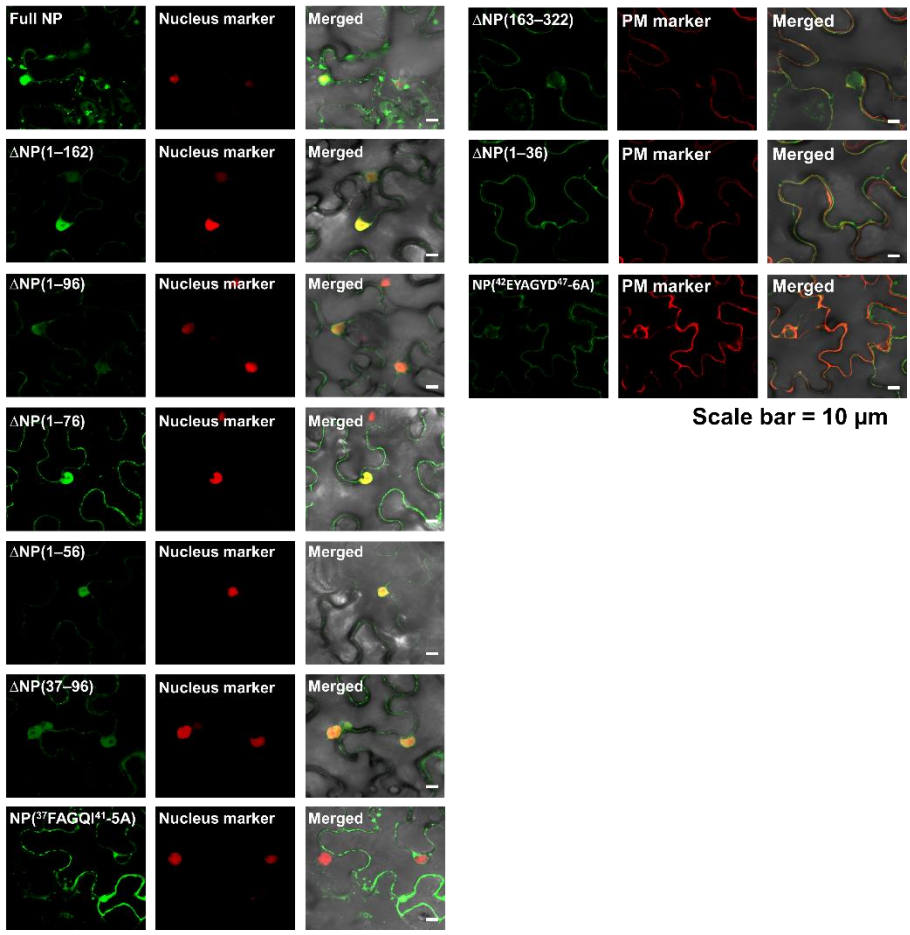

Fig. 4. Co-localization study of NP truncated mutants with a nuclear maker. Truncated mutants tagged with GFP were co-infiltrated with a nuclear or PM marker. The mutants ΔNP(1–162), ΔNP(1–96), ΔNP(1–76), ΔNP(1–56), ΔNP(37–96), and NP(^{37}FAGQI^{41}-5A) were co-localized with a nuclear marker, while ΔNP(163–322), ΔNP(1–36), and NP(^{42}EYAGYD^{47}-6A) were co-localized with a PM marker.

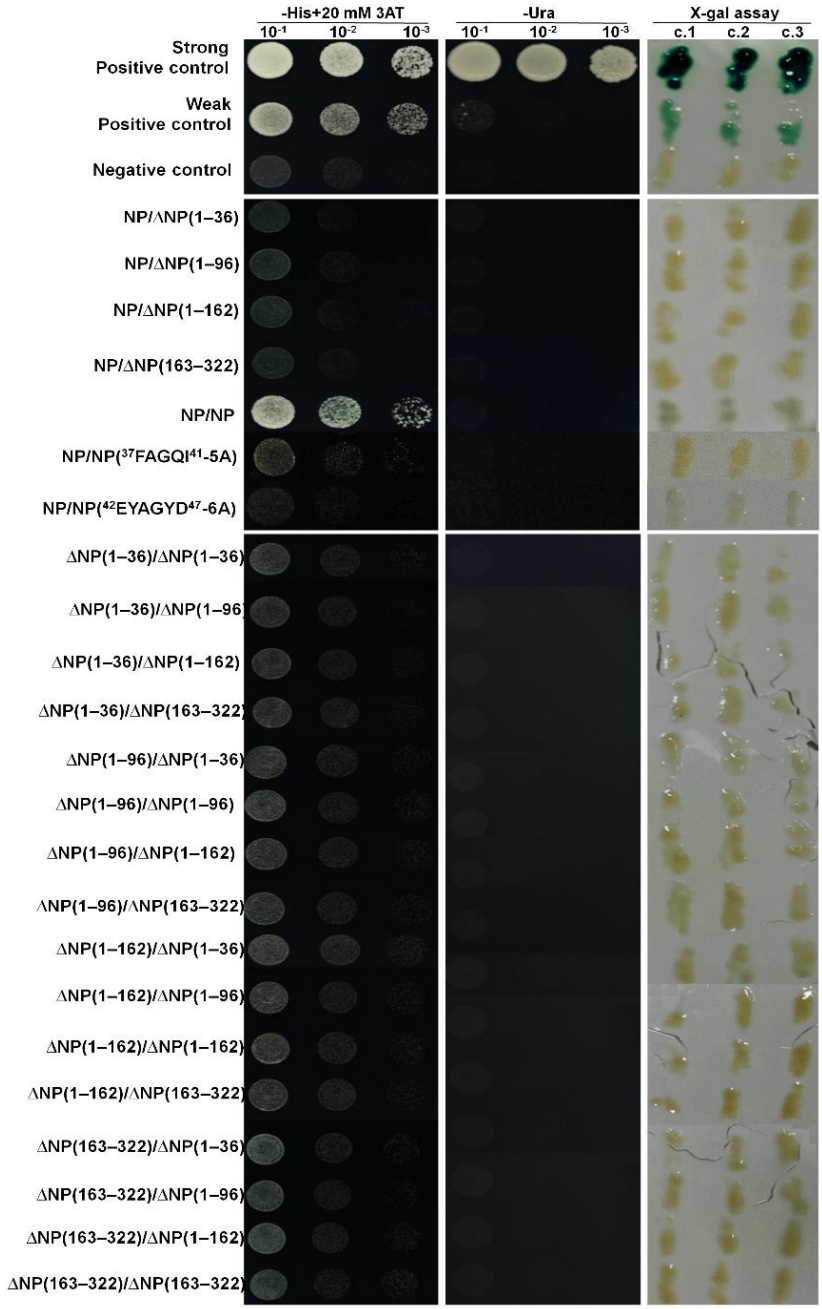

Fig. 5. Interaction study between NP truncated mutants and full-length NP by Y2H. Colonies from -LT medium (SC medium without leucine or tryptophan) were verified on -His + 20mM 3AT medium (SC medium without leucine, tryptophan, or histidine

but with 5 mM 3-Amino-1,2,4-triazole), -Ura (SC medium without leucine, tryptophan, or uracil) with dilutions of yeast cells at cell concentration of 10^{-1}, 10^{-2}, 10^{-3}, and three different colonies were used for X-gal assay. There was no interaction between NP truncated mutants and full-length NP. Three controls (representing a strong interaction, a weak interaction, and no interaction) were generated by co-transforming control plasmids provided by the ProQuest™ Two-Hybrid System (Invitrogen).

8+28–322) and ΔNP(1–47), in which one of the alpha helices was deleted. Surprisingly, these two mutants still interacted with full-length NP, and their interactions were stronger than the full-length NP self-interaction (Fig. 3A). In particular, quantitative β-gal activity assays showed that the interaction of ΔNP(1–47) with full-length NP was stronger than other interactions between the generated mutants and full-length NP. These results suggest that the two predicted alpha helices are not important for NP self-interaction.

Next, we focused on the 11 residues from aa 37–47 and generated the substitution mutants NP(^{37}FAGQI^{41}-5A) and NP(^{42}EYAGYD^{47}-6A) by substituting the residues with alanine at 37–41 aa and 42–47 aa, respectively. Interestingly, NP(^{37}FAGQI^{41}-5A) interacted with full-length NP, and the interaction was stronger than NP–NP self-interaction, whereas NP(^{42}EYAGYD^{47}-6A) did not interact with full-length NP by Y2H (Fig. 3A). These results demonstrated that the aa residues 42–47 are important for NP–NP self-interaction.

To confirm the Y2H results, we performed BiFC assays. All NP mutants were cloned into BiFC destination vectors separately tagged with nYFP or cYFP. The interactions between NP mutants and full-length NP were tested by agro-infiltration of *N. benthamiana* leaves. The BiFC results were consistent with Y2H results in that the truncated mutants ΔNP(1–56),

ΔNP(1–76), ΔNP(1–96), and ΔNP(1–162) as well as the substitution mutant NP(^{37}FAGQI^{41}-5A) interacted with full-length NP. However, ΔNP(163–322), ΔNP(1–36), ΔNP(37–96), and NP(^{42}EYAGYD^{47}-6A) did not interact with full-length NP (Fig. 6). To determine whether the swapping of GAL4 AD with GAL4 DBD affects the interaction, we fused all NP truncated mutants downstream of GAL4 AD and fused full-length NP downstream of GAL4 DBD. Unexpectedly, Y2H did not detect any interaction between NP and the NP mutants (Fig. 5).

We then fused the substitution mutants NP(^{37}FAGQI^{41}-5A) and NP(^{42}EYAGYD^{47}-6A) downstream of GAL4 AD and examined the interaction between all NP mutants and the two substitution mutants. Interestingly, the truncated mutants ΔNP(1–96), ΔNP(1–76), ΔNP(1–56), ΔNP(1–47), and NP(^{37}FAGQI^{41}-5A) interacted with NP(^{37}FAGQI^{41}-5A). On the other hand, full-length NP, ΔNP(1–161), and ΔNP(1–8+28–322) did not interact with NP(^{37}FAGQI^{41}-5A) although they can interact with full-length NP (Fig. 7A). These results demonstrated that substitution of the five amino acids (from 37 to 41) with alanine still resulted in disturbance of NP self-interaction. In contrast, no interaction was detected between any of the NP mutants and NP(^{42}EYAGYD^{47}-6A) (Fig. 7A). These results indicate that amino acids 42–47 are important for the interaction. At the same time,

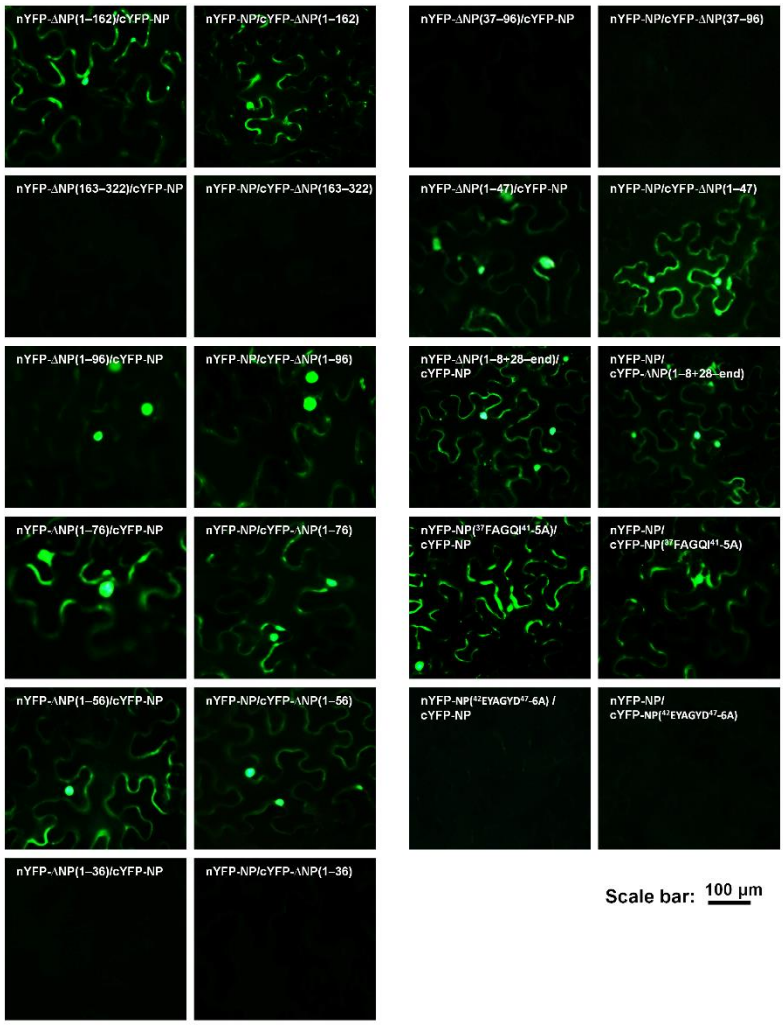

Fig. 6. Interaction study between RSV NP mutants and full-length NP by BiFC assay in *Nicotiana benthamiana*. NP truncated mutants and full-length NP were tagged with nYFP or cYFP.

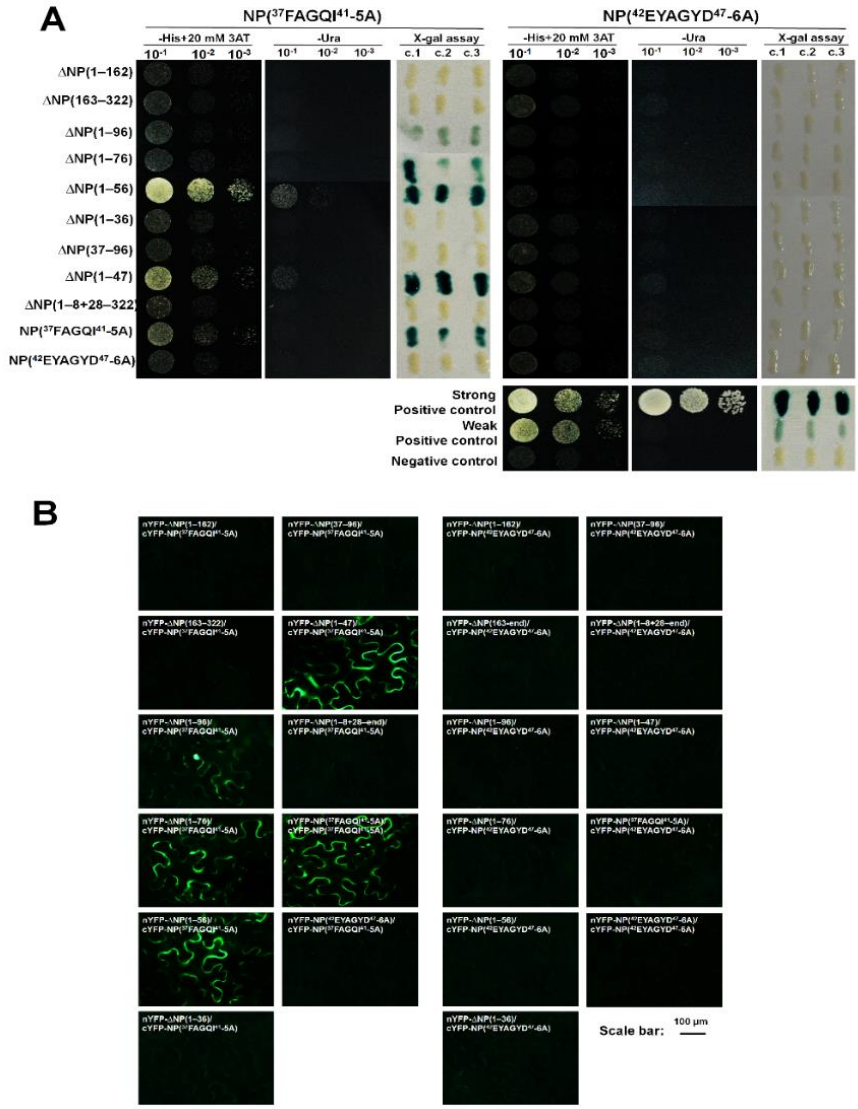

Fig. 7. Interaction study between NP truncated mutants and substitution mutants. (A) Interaction study between NP truncated mutants and substitution mutants NP(^{37}FAGQI^{41}-5A) and NP(^{42}EYAGYD^{47}-6A) by Y2H. Colonies from -LT medium (SC medium without leucine or tryptophan) were verified on -His + 20 mM 3AT medium (SC medium without leucine, tryptophan, or histidine but with 5 mM 3-Amino-1,2,4-triazole), -Ura (SC medium without leucine, tryptophan, or uracil) with dilutions of yeast cells at cell concentration of 10^{-1}, 10^{-2}, 10^{-3}, and three different

colonies were used for X-gal assay. Several mutants interacted with but no mutant interacted with NP(^{42}EYAGYD^{47}-6A). (B) Interaction study between NP truncated mutants and substitution mutants by BiFC assay in *N. benthamiana*. NP mutants and substitution mutants were targeted with nYFP and cYFP at N terminus. The interaction results obtained with BiFC were consistent with those obtained with Y2H.

the results indicated that the other amino acids at the N terminal also participate in the interaction because ΔNP(37–96) did not interact with full-length NP although the six amino acids (42–47) were included in the segment. BiFC assay results were consistent with Y2H results (Fig. 7B).

V. Dependency of NP self-interaction and nuclear localization on the six aa residues

Because BiFC assay indicated that the interaction complexes involving NP were localized at the nucleus, we hypothesized that the domain required for NP self-interaction might be also important for subcellular localization of NP at the nucleus. To determine the subcellular location of NP mutants, we tagged the truncated mutants ΔNP(1–162), ΔNP(163–322), ΔNP(1–96), and ΔNP(1–36) as well as the substitution mutants NP(^{37}FAGQI^{41}-5A) and NP(^{42}EYAGYD^{47}-6A) with GFP at the N terminal region.Co-localization study with a nuclear marker (mCherry-VirD2NLS) indicated that ΔNP(1–162), ΔNP(1–96), ΔNP(1–76), ΔNP(1–56), and NP(^{37}FAGQI^{41}-5A), which can interact with full-length NP, localized at the nucleus while ΔNP(163–322), ΔNP(1–36), and NP(^{42}EYAGYD^{47}-6A), which do not interact with full-length NP, did not localize at the nucleus (Fig. 4). Even though it did

not interact with full-length NP, the truncated mutant ΔNP(37–96) localized at the nucleus, apparently because it contains the six amino acids, i.e., aa 42–47 (Fig. 4). Taken together, those results demonstrated that aa 42–47 play an important role in the subcellular location of NP at the nucleus.

DISCUSSION

In this study, we used Y2H and BiFC analyses to investigate the interactions among six RSV viral proteins. While a previous study with RSV detected only NP self-interaction by co-immunoprecipitation (Lu et al., 2008), we identified strong self-interaction for the RSV proteins NP, NS3, and NCP. Previous reports have documented CP self-interaction in many viruses. For example, CP self-interaction is common for potyviruses and is involved in the virus infection cycle (Guo et al., 2001; Kang et al., 2006; Kang et al., 2004; Zilian and Maiss, 2011). Although RSV NP functions in pathogenicity (Yuan et al., 2013), how NP self-interaction contributes to the RSV life cycle is unknown. In another example, the tomato bushy stunt virus (TBSV) protein P19 specifically interacts with itself and the host protein Hin19 in the suppression of defense-related gene silencing (Park et al., 2004). Self-interaction has also been reported for NS3, which, like P3, is an RSV suppressor of gene silencing (Shen et al., 2010b). The nonstructural protein p5 of rice grassy stunt virus exhibits self-interaction and forms oligomeric complexes *in vitro* and *in vivo* (Chomchan et al., 2003). We also identified self-interaction of RSV NCP in the current study. It seems that self-interaction of nonstructural proteins is common in the genus *Tenuivirus*.

Previously, Takahashi et al. (Takahashi et al., 2003a) demonstrated that NCPs aggregated in virus-infected cells forming massive inclusion bodies in the cytoplasm. This report somehow is different from our result which showed nuclear localization of NCP. It is possible that subcellular localization of NCP could be affected by RSV infection since the previously performed NCP localization study was conducted in RSV infected condition whereas our study was performed in non-infected condition. Based on our results, we are currently conducting experiments to identify host proteins that interact with RSV NP, NS3, and NCP in order to reveal the possible functions of such interactions in viral pathogenicity and gene silencing during the RSV life cycle.

Interestingly, the BiFC assay but not the Y2H assay revealed that NP, NS3, and NCP can interact each other. That the BiFC assay often detects more interactions than Y2H might have several explanations. First, protein–protein interactions may be easier to detect *in planta* with the BiFC assay than *in vitro* with the Y2H assay because conditions *in planta* are more natural (Zilian and Maiss, 2011). Second, the interaction between two proteins might require a third component such as a host protein, which would be present in the BiFC assay but not in the Y2H assay.

In addition, we found that the interactions between NP mutants and

full-length NP depended on the directionality of the fusion protein. Such results were also reported for other plant viruses. For example, interactions between PSbMV P3 and NIa were only detected when these proteins were fused with GAL4 DBD and AD, respectively (Guo et al., 2001). Similarly, the interaction was stronger between GAL4 DBD:NIb and GAL4 AD:NIa than between GAL4 DBD:NIa and GAL4AD:Nib. The reason for such disparity in our study as well as the previous study might be that the direction of fusion may affect protein folding or exposure of binding sites, which in turn may affect the probability of interaction.

The Y2H results with various truncated and substitution mutants indicated that the N-terminal region (aa 1–47) of RSV NP is necessary for self-interaction. Moreover, the six amino acids (aa 42–47) were determined to be the most important residues for NP self-interaction. The BiFC and co-localization study confirmed that the six residues are also important for subcellular localization of NP in nucleus. Therefore, we infer that a specific region of a protein can be important for both protein interaction and subcellular localization of the interacting proteins. Although alpha helices at the N-terminal region of NP are not absolutely required for the interaction, it is apparent, however, that the N-terminal domain enhances the reactivity of the absolutely required residues 42-EYAGYD-47. In addition, it is possible

that presence of either (or both) of the alpha helices sufficiently alters the exposure for binding.

It was very interesting results that truncated or substituted mutants of NP displayed much stronger interaction than the full-length NP self-interaction. Although we do not have experimental evidence for explaining this phenomenon, there had been similar results reported for the protein–protein interaction studies. For instance, 87 aa at N-terminal of UL36 protein of pseudorabies virus which is an important domain for interaction, had stronger interaction than that full length UL36 protein with UL37 protein (Klupp et al., 2002; Vittone et al., 2005). Moreover, replacement of the two tyrosine residues at the C-terminal regions of human insulin receptor resulted in a 2-fold gain of function for binding of the receptor to, hGrb10, the Src homology-2 domain-containing protein (Dong et al., 1997). Overall, there might be two possible explanations for that. First, exposure of the important interaction domain or protein folding was changed by deletion or substitution of amino acids. Second, there might be a specific protein region in NP which inhibits or disrupts protein interaction and this protein region might be missing or structurally changed in truncated or substituted mutants.

In plant viruses, CP is a multifunctional protein that is involved in

nearly every stage of the viral infection cycle (Ivanov and Mäkinen, 2012). In plant viruses with nuclear replication (like pararetroviruses and geminiviruses), CPs are imported into the nucleus and are involved in viral replication (Guerra-Peraza et al., 2005). While the N-terminal region of RSV NP is required for NP self-interaction, the C-terminal region is an important domain for CP self-interaction and thought to be involved in virus assembly for potyviruses like SMV (Kang et al., 2006). Although the general structure of the CP of potyviruses, flexiviruses, and rod-shaped viruses like tobacco mosaic virus (TMV) has been suggested to follow a similar folding pattern, we are not aware of any structural analysis of the tenuivirus CP to suggest a similar structure. In this regard, it is worth noting that the C-terminal R-domain of CPs for some isometric viruses including tombusviruses is internal to the virion rather exposed to the exterior as in the potyviruses, flexiviruses, and TMV. Therefore, it is possible that structure of the tenuivirus NP might follow similar folding pattern to those of tombusvirus and thus the C-terminal region of NP reside internal to the virion. For example, the CP of cucumber necrosis virus, a member of the genus *Tombusvirus* like TBSV, is consisted with three major domains including the R domain which interacts with the RNA genome and forms internal scaffold (Katpally et al., 2007). Furthermore, a nuclear localization

signal in the N-terminal region of cauliflower mosaic virus (CaMV) CP is important for viral infectivity (Leclerc et al., 1999). Similarly, a recent report demonstrated that RSV NP contributes to pathogenicity and induces mosaic symptoms on *N. benthamiana* leaves (Yuan et al., 2013). Although geminiviruses and caulimoviruses are not closely related with tenuiviruses like RSV, we supposed that there might be common functional roles between RSV NP and other CPs in virus life cycle. It has to be acknowledged, however, that our data presented here are from Y2H and BiFC assays which lacks the links to biology of RSV life cycle in infected cells and thus might not represent actual interactions between viral proteins.

Although no study has determined where RSV replicates within host cells, our results indicate that three self-interacting RSV proteins–NP, NS3, and NCP–are localized in the nucleus. It is not surprising that NS3 should localize to the nucleus, as several other viral silencing suppressors such as CMV 2b and CaMV P6 have been reported to localize to the nucleus (González et al., 2010; Haas et al., 2008). Altogether, this suggests that the RSV life cycle might be connected with the host nucleus. However, it is not known whether NP self-interaction and nuclear localization are important for RSV replication and assembly. Future research should determine whether the domain for NP self-interaction and nuclear localization is

involved in RSV symptom development. Future research should also identify host factors interacting with NP.

In conclusion, we used several truncated and substitution mutants to determine that amino acid residues 42–47 of the RSV NP subunit are required for RSV NP self-interaction. These six aa residues of the NP are also required for the formation of a functional protein complex and for localization at the nucleus. The information in this study will also be useful for the investigation of virus–host protein interactions.

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벼줄무늬잎마름병바이러스 단백질들의 항체 생성과 상호작용 연구

리안센

초록

벼줄무늬잎마름병바이러스 (RSV) 는 벼에게 있어서 가장 치명적인 바이러스 중 하나이며, 벼의 대부분인 자포니카 품종을 재배하고 있는 중국, 일본과 한국의 쌀 생산량을 현저하게 감소시킨다. RSV 는 지속적이고 순환적 전파 방식으로 작은 애멸구 (SBPH) 를 통해 전염된다. RSV 는 현재 *Tenuivirus* 종에 포함되지만, 아직 바이러스의 속에 해당 되지 않는다. RSV 의 게놈은 네 개의 단일 가닥 RNA (RNA1–RNA4) 로 구성되어 있으며, 일곱개의 단백질을 암호화 한다. 본 연구에서는 RSV 단백질의 단백질-단백질간의 상호작용을 중점적으로 다루었다. 먼저, RSV 에 감염된 벼로부터 RSV 단백질을 검출하기 위해서, RSV 에 대한 항체는 재조합

단백질과 합성된 폴리팹타이드를 이용하여 생성되었다. 7 개 RSV 단백질 가운데, NCP 와 NS3 단백질을 암호화하는 유전자는 RT-PCR 를 통해 증폭 후 재조합 단백질 발현벡터에 클로닝하였고 *Escherichia coli* 를 이용한 과발현 방법을 이용, 최종적으로 정제된 재조합 단백질을 얻을 수 있었다. 항체 생성을 위한 다른 방법으로는 RSV 단백질 염기서열을 이용한 폴리펩타이드를 합성하고, 이를 이용하여 항체를 생성하였다. 최종적으로 두 개의 재조합단백질과 네 개의 합성 폴리팹타이드를 포함하는 여섯 개의 다클론성항체들을 확보하였다. Western blot analysis 을 통하여 NCP 를 항원으로 사용하여 제작한 항체가 안정적인 단백질 검출 결과를 보였고, 합성 폴리팹타이드의 경우, NP 와 NCP 합성 펩타이드를 이용한 항체를 이용할 때 RSV 에 감염된 벼와 담배로부터 RSV 를 검출하는데 성공하였다. 그러나 NS3 와 NSvc4 항체의 경우, 다른 RSV 단백질과의 비특이적인 상호작용으로 인하여 약하거나 비 특이적 결합반응을 보여주었다. 요약해보면, 본 연구를 통하여 제작된 RSV 항체를 이용하여 이병식물에서 RSV 를 진단, Immunoprecipitation, 단백질 정제와 Western blot analysis 등 다방면에서 유용할 것으로 사료된다. 둘째, 본

연구에서는 yeast-two hybrid (Y2H) 분석과 Bimolecular Fluorescence Complementation (BiFC) 을 통하여 RSV 의 여섯 개의 단백질 간의 상호작용 관계를 조사하였다. Nucleocapsid protein (NP) 와 NS3 단백질과의 상호작용을 연구하기 위하여 Y2H 연구를 수행하였고 BiFC 연구를 통하여 NP, NS3, NCP 사이의 상호작용에 관한 결론을 도출하였다. NP 단백질 상호작용에 관련한 중요한 아미노산 (AA) 의 잔기들과 위치를 알아내기 위하여 도메인 및 아미노산 치환 돌연변이를 제작하여 연구에 사용하였다. 또한 Y2H 분석을 통해 NP 상호 작용에 있어서 NP 의 N--아미노말단 영역 (AA 1-56) 필요하다는 실험적인 결과를 얻었다. 추가적인 아미노산 치환 돌연변이를 이용하여 42 번째 부터 47 번째 있는 아미노산이 전반적인 NP 상호작용에 영향을 준다는 것을 보여줬다. 이러한 결과들은 NP 단백질의 N-아미노 말단 영역 (AA 36 과 AA 42-47) 이 NP 단백질의 상호 작용을 위해 필요하다는 것을 보여준다. BiFC 와 Co-localization 연구에서도 NP 단백질이 상호작용을 보이는 위치가 식물 세포 핵 주변이고 NP 단백질의 핵 이동에 N-아미노말단 영역이 필요하다는 결론을 보여줬다.

결과적으로, 본 연구를 통해 NP 의 N-아미노 말단 영역 (AA 1-47) 은 NP 상호 작용의 중요한 요소이고, 여섯 개의 아미노산 잔기들 (42-47) 은 NP 상호 작용뿐만 아니라 핵 국소화에 필수불가결하다는 사실이 도출되었다.

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