Plant Pathol. J. 24(3) : 289-295 (2008)

The Plant Pathology Journal

RNA-RNA Interactions between RNA Elements at the 5' end and at the Upstream of sgRNA of RNA Genome are Required for *Potato virus X* RNA Replication

Mi-Ri Park[1], Sang-Ho Park[1], Sang-Yun Cho[1], Cynthia L. Hemenway[2], Hong-Soo Choi[3], Seong Han Sohn[4] and Kook-Hyung Kim[1]*

[1]*Department of Agricultural Biotechnology, Seoul National Nniversity, Seoul 151-921, Korea*
[2]*Department of Molecular and Structural Biochemistry, Box 7622, North Carolina State University, Raleigh, North Carolina 27695-7622*
[3]*Department of Plant Pathology, National Institute of Agricultural Science and Technology, RDA, Suwon 441-707, Korea*
[4]*Cell and Genetics Division, National Institute of Agricultural Biotechnology, RDA, Suwon 441-707, Korea*

(Received on May 16, 2008; Accepted on July 8, 2008)

RNA–RNA interactions and the dynamic RNA conformations are important regulators in virus replication in several RNA virus systems and may also involved in the regulation of many important virus life cycle phases, including translation, replication, assembly, and switches in these important stages. The 5' non-translated region of *Potato virus X* (PVX) contains multiple *cis*-acting elements that facilitate various viral processes. It has previously been proposed that RNA-RNA interactions between various RNA elements present in PVX RNA genome are required for PVX RNA accumulation (Hu et al., 2007; Kim and Hemenway, 1999). This model was based on the potential base-pairing between conserved sequence elements at the upstream of subgenomic RNAs (sgRNAs) and at the 5' and 3' end of RNA genome. We now provide more evidence that RNA-RNA base-pairing between elements present at the 5' end and upstream of each sgRNA is required for efficient replication of genomic and subgenomic plus-strand RNA accumulation. Site-directed mutations introduced at the 5' end of plus-strand RNA replication defective mutant (Δ12) increasing base-pairing possibility with conserved sequence elements located upstream of each sgRNAs restored genomic and subgenomic plus-strand RNA accumulation and caused symptom development in inoculated *Nicotiana benthamiana* plants. Serial passage of a deletion mutant (Δ8) caused more severe symptoms and restored wild type sequences and thus retained possible RNA-RNA base-pairing. Altogether, these results indicate that the RNA element located at the 5' end of PVX genome involved in RNA-RNA interactions and play a key role in high-level accumulation of plus-strand RNA *in vivo*.

Keywords : plus-strand RNA accumulation, PVX, replication, RNA-RNA base-pairing

*Corresponding author.
Phone) +82-2-880-4677, FAX) +82-2-873-2317
E-mail) kookkim@snu.ac.kr

The genome of plus-strand RNA viruses participate in multiple processes during replication cycles: they serve as mRNA for making viral proteins including replicase, act as templates for genome replication, and facilitate packaging into virion. To accomplish each process successfully, RNA virus must have elaborate controlling mechanism. Although there are some advances in understanding life cycle of RNA viruses, but detailed mechanisms of RNA replication are still poorly understood. Recently, RNA-RNA interactions and the dynamic RNA conformations are known as important regulators in virus replication have been reported in several RNA virus systems such as *Alfalfa mosaic virus*, *Tomato bushy stunt virus*, *Turnip crinkle virus*, and *Dengue virus* (Alvarez et al., 2005; Olsthoorn et al., 1999; Ray et al., 2003; Zhang et al., 2006). It is possible that these RNA-RNA interactions may also involved in regulation of other phases in the virus life cycle including translation, assembly, and replication.

Potato virus X (PVX), the type member of the genus *Potexvirus*, is a flexuous rod-shaped virus containing a 6.4 kb plus-stranded RNA genome which is capped and polyadenylated at its 5' and 3' ends, respectively (Bercks, 1970; Huisman et al., 1988; Milne, 1988; Skryabin, 1988). The PVX genome contains five open reading frames (ORFs) encoding the RNA-dependent RNA polymerase protein (RdRp, 165 kDa), the triple gene block (TBs), and coat protein (CP) (Fig. 1). RdRp is the only viral protein absolutely required for PVX RNA synthesis (Rozanov et al., 1992). The TBs, products of ORFs 2-4, function in viral cell-to-cell transport (Angell et al., 1996; Beck et al., 1991), while the product of ORF5, CP, is involved in both virus movement and encapsidation (Chapman et al., 1992; Oparka et al., 1996).

The compact nature of PVX genome forces it to utilize the same sequence elements for multiple functions. The identified RNA elements located at the 5' PVX non-translated region (NTR) are AC-rich sequences and several

repeat ACCA motifs (Kim and Hemenway, 1996; Park et al., 2008) followed by at least one stable stem-loop, SL1 (Miller et al., 1998) which are all important for PVX RNA replication. A stable SL1 structure has been found to be critical for PVX RNA accumulation in tobacco protoplasts and in plants (Miller et al., 1999; Miller et al., 1998). It has been demonstrated that the CP-binding element is also located within this SL1 structure at the 5' region and that the binding of CP subunits to the SL1 structure was sufficient for the formation of virus like particles (Kwon et al., 2005). In addition, a SL1 structure acts as a *cis*-acting element essential for cell-to-cell movement of RNA (Lough et al., 2006), selectively encapsidates single-tailed RNA-CP particles, and modulates the nature of the infectious virus transport form through the binding with TGBp1 (Karpova et al., 2006).

It has also reported that conserved octanucleotide sequence elements located upstream of the two major PVX sgRNAs complementary to the 5' terminus (or 3' terminus of minus-strand RNA) are necessary for genomic and sgRNA plus-strand RNA accumulation (Kim and Hemenway, 1997; Kim and Hemenway, 1999). Reduced level of sgRNAs was observed in protoplasts inoculated with mutants containing modifications in the 5' NTR elements, despite no reduction in minus-strand RNA accumulation suggesting that initiation of PVX sgRNA synthesis is not solely dependent upon the presence of minus-strand RNA and the appropriate local signals. Hu et al. (2007) have shown that these conserved octanucleotide elements are also required for optimal transcription of minus-strand RNA both *in vitro* and *in vivo*.

Recently, we have reported the importance of the repeated ACCA motifs in the 5' PVX NTR for viral replication (Park et al., 2008). We analyzed the functional significance of these repeated ACCA-motifs by introducing deletions and site-directed mutations within the repeated ACCA sequences and by inoculating transcripts containing mutations onto NT1 protoplasts and *Nicotiana benthamiana* plants. However, the molecular nature of RNA-RNA associations and the details of how these repeated ACCA elements and how RNA-RNA interactions or conformational switches participate in PVX replication still remain to be elucidated. In this study, we further characterized the functional significance of these possible RNA interactions between different RNA elements present in PVX genome by introducing site-directed mutations. To further access the functional significance of possible RNA-RNA interactions, we inoculated transcripts containing deletions from the 5' end of the PVX genome (Δ8) onto *N. benthamiana* tobacco plants. Analysis of progeny RNAs recovered through several passages indicated that selection for stable RNA-RNA interactions between various *cis*-elements suggesting the stable RNA-RNA interactions was critical for PVX replication.

Materials and Methods

Materials. All restriction enzymes, modifying enzymes, polymerases, and m7GpppG cap analogue were purchased from New England BioLabs (MA, USA). RNasin and RQ1 RNase-free DNase and sequenase were obtained from Promega (WI, USA) and U.S. Biochemicals (MA, USA), respectively. Deoxy-, dideoxy- and ribonucleotides were purchased from Boehringer Ingelheim GmbH (Ingelheim, Germany) and oligonucleotides were synthesized by Sigma-Genosys (Sigma-Aldrich, MO, USA). Avian myeloblastosis virus reverse transcriptase and Trizol reagents were from Life Sciences (NJ, USA). Reagents for protoplast preparation were obtained from Yakult Honsha Co., Ltd (Tokyo, Japan) and Karlan Research Products (AZ, USA).

Site-directed mutagenesis and *in vitro* transcription. Mutations were introduced into the PVX cDNA clone, pSNU-PVX (Park and Kim, 2006) using the muta-Gene *in vitro* mutagenesis kit (Bio-Rad; Kunkel, 1985). Control plasmid, p32, was recloned onto pSNU1, and designated as pSNU-p32 (Park and Kim, 2006). All site-directed mutants were verified by sequencing, and a region containing the mutation was resected back into a wild type (wt) clone. *In vitro* transcription reactions were performed as described previously (Kim and Hemenway, 1996). The quality and relative concentrations of transcripts were checked by electrophoresis on a 1% agarose gel at 4°C, and visualized by ethidium bromide staining.

Protoplasts and plant inoculations. Protoplasts from a rapidly dividing *N. tabacum* suspension cell line, NT1, were prepared and inoculated as described previously (Kim and Hemenway, 1997). To determine if the protoplast data would be reflected in plant infection studies, especially on severity of symptoms, *N. benthamiana* plants were initially inoculated (passage 0; P0) with mutants, wt, replication defective control (p32) or with buffer. Extracts from these plants were passaged twice (P1 and P2), and symptom development was recorded for all passages. Each inoculated plants were maintained at 25°C in 12 h light and at 22°C in 12 h dark, and were observed for symptom development for 14 days post inoculation (dpi). Severity of symptoms was qualitatively determined and designated by the number of plus signs, with symptoms in wt plants indicated by three plus signs. To determine whether mutated sequences were maintained in progeny viruses, we amplified the dsDNA-containing mutated region by RT-PCR and sequenced 12 independent clones from leaf

samples. Protoplast and plant experiments with wt and mutants were performed separately at least three times.

RNA and protein analyses. Total RNA was isolated from inoculated protoplasts and *N. benthamiana* plants using Trizol reagent (Life Technologies, CA, USA) at 48 h post inoculation (hpi) and 14 dpi, respectively, as described previously (Mun et al., 2008). S1 nuclease protection assays were used for detection of minus- and plus-strand PVX RNAs, with single-stranded DNA probes (Kim and Hemenway, 1996; Kim and Hemenway, 1997). Accumulation of TB and CP sgRNAs was measured by primer extension with pr1 and pr2, respectively, according to methods described previously (Kim and Hemenway, 1996; Kim and Hemenway, 1997). Products from S1 nuclease digestion and from extension reactions were separated on 6% sequencing gels and visualized by autoradiography. Relative molar amounts of PVX RNAs in each sample were determined by using Image Quant software on a Molecular Dynamics Phosphoimager. Total proteins extracted from protoplasts and plants were resuspended in 30 μl of Laemmli loading buffer (Laemmli, 1970), electrophoresed on 12% SDS-polyacrylamide gels, blotted onto nitrocellulose membrane, probed with antiserum prepared against purified PVX, and were visualized using a Biotin/StreptAvidin kit (Amersham, Uppsala, Sweden).

Statistical analysis. SAS (version 9.1; SAS Institute Inc., USA) statistical analysis program was used to analyze data. Analysis of variance (ANOVA) was employed and means were compared using the Fisher's LSD (Least significant difference) test.

Results

PVX RNA replication was affected by mutations changing RNA-RNA interactions on NT1 protoplasts. The role of the RNA-RNA base-pairing between various *cis*-elements present at the PVX genome in PVX RNA synthesis were further defined by site-directed mutations onto plus-strand RNA synthesis defective mutant (Δ12) to restore possible RNA-RNA base-pairing with the other *cis*-elements present upstream of each sgRNA (Fig. 1B). Mutant Δ12-BP restored wt level of possible base-pairing between sequence elements located at the 5' end and upstream of each sgRNA while mutants Δ12-mut1 and Δ12-mut2 partially restored the possible RNA-RNA base-pairing (Fig. 1C). The effects of each mutation on PVX replication were assayed by inoculating mutants onto NT1 protoplasts and *N. benthamiana* plants. Accumulation of genomic plus- and minus-strand RNAs was analyzed by S1 nuclease protection assays using probes P1 and P2, respectively (Fig. 1A). Primer extension analyses for quan-

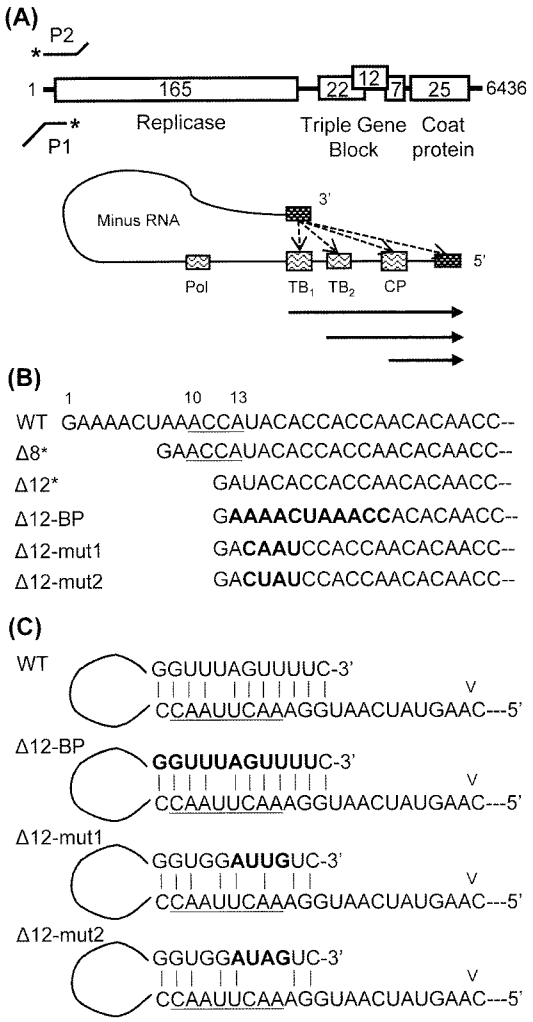

Fig. 1. Schematic representation of the PVX genome and model for long-distance interactions required for PVX replication (A). The five ORFs are denoted by open boxes and are labeled according to the sizes of the predicted polypeptides. The relative positions of the plus-strand RNA probe (P1) and minus-strand RNA probe (P2) for S1 nuclease protection assays are indicated; asterisks denote ^{32}P-labeled positions. Possible long-distance interactions are depicted in dotted arrows. Positions and sequences of *cis*-acting elements defined here and in previous reports (Hu et al., 2007; Kim and Hemenway, 1997; Kim and Hemenway, 1999; Park et al., 2008). The products of plus-strand sgRNA synthesis, including TB sgRNA (TB1), CP sgRNA (CP), and the putative sgRNA initiating near ORF3 (TB2) are also shown. (B) Schematic representation of deletion and site-directed mutations made at the 5' NTR of the genomic RNA. Two previously described mutants were also included in the analysis (asterisks; Kim et al., 2002; Park et al., 2008). The first ACCA of repeat ACCA elements is underlined. Mutated sequences introduced onto Δ12 mutant are indicated in bold character. (C) Predicted base pairs between the 5' end and the conserved octanucleotide element of PVX RNA. Predicted base pairing between the 3' terminus and internal conserved element located upstream of CP sgRNA (underlined) of mutants. Possible base pairing is illustrated using minus-strand RNA. Mutated sequences introduced onto Δ12 mutant are indicated in bold character.

tification of plus-strand sgRNAs were conducted.

As shown in Fig. 2, accumulation of genomic minus-strand RNA level was not significantly affected by any mutations introduced at the 5' end of PVX genome. We have previously reported that deletion of 12 nt (Δ12) almost abolished the accumulation of genomic plus-strand RNA (0.8%), while deletion of 8 nt (Δ8) did not (92.0%) (Table 1; Park et al., 2008). Interestingly, partial restoration of the possible long distance RNA-RNA base-pairing restored genomic plus-strand RNA accumulation (38.3% and 35.9% for Δ12-mut1 and Δ12-mut2, respectively). Complete restoration of the RNA-RNA base-pairing resulted in significantly increased level of genomic plus-strand RNA accumulation in inoculated NT1 protoplasts (Table 1). The accumulation of sgRNAs, as measured by primer extension analyses, also increased with increasing accumulation of genomic plus-strand RNA (Fig. 2; Table 1). Increased level of sgRNA accumulation subsequently affected the expression of CP as shown in the western blot (Fig. 3A).

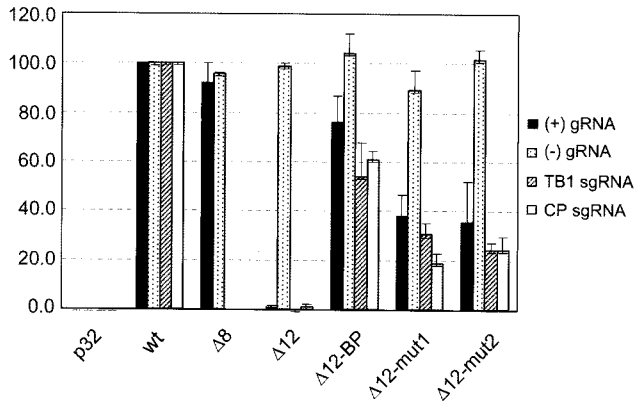

Fig. 2. Effects of mutations introduced into PVX genomic RNA on viral replication in *N. tabacum* NT1 protoplasts. Effect of mutation on PVX RNA accumulation was analyzed using S1 nuclease protection and primer extension analyses. Accumulation of PVX RNA inoculated with negative control transcripts (p32), positive control (wt), and mutants in protoplasts were measured using a Molecular Dynamics Photoimager. Each value is the mean compared to that of the wild type (wt) as a percentage of the wt and the standard error compiled from at least three independent experiments.

Possible RNA-RNA base-pairings between various *cis*-elements were restored during serial passage of D8 mutant in plants. Plants inoculated with wt generally developed symptoms at 5-7 dpi. When mutants containing the Δ12-mut1 and Δ12-mut2 mutations were inoculated onto *N. benthamiana* plants, no symptom development was

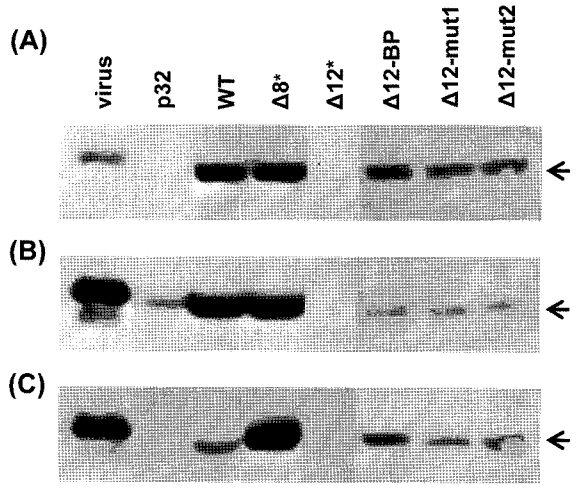

Fig. 3. Accumulation of CP subunits inoculated with negative control transcripts (p32), positive control (wt), and mutants in protoplasts (A) and in *N. benthamiana* plants (B and C). Total proteins extracted from protoplasts, inoculated (B) and upper systemic (C) leaves were electrophoresed on 12% SDS-polyacrylamide gels, blotted onto nitrocellulose membrane, probed with antiserum prepared against purified PVX, and were visualized using a Biotin/StreptAvidin kit (Amersham Phamacia Biotech, Uppsala, Sweden). The first lane in F contains the detected band obtained using Western blotting with antiserum prepared against purified PVX.

Table 1. Accumulation of genomic and subgenomic RNAs PVX on *N. tabacum* NT1 protoplasts inoculated with PVX mutants

Mutants	(+) RNA[a]	(−) RNA[a]	TB_1 sgRNA[a]	CP sgRNA[a]
wt	100.0 a	100.0 a	100.0 a	100.0 a
p32	0.0 d	0.0 b	0.0 d	0.0 d
Δ8	92.0[b] ab	96.0[b] a	na[c]	na
Δ12	0.8 d	98.8[b] a	0.0 d	0.9 d
Δ12-BP	76.5 b	103.9 a	54.0 b	61.0 b
Δ12-mut1	38.3 c	89.3 a	30.5 c	18.7 c
Δ12-mut2	35.9 c	101.7 a	24.3 c	24.3 c

[a] Data were analyzed by ANOVA and means were compared using the Fisher's LSD test using SAS program (version 9.1). Means with the same letter are not significantly different at $P \leq 0.05$. Each value is the mean compared to that of the wild type (wt) as a percentage of the wt compiled from at least three independent experiments.

[b] Accumulation of genomic plus-and minus-strand RNAs was described previously (Park et al., 2008).

[c] na, not assayed.

observed in P0 plants (Table 2). Surprisingly, mild symptoms began to appear during P1 and P2 for mutants Δ12-mut1 or Δ12-mut2 and Δ12, respectively. P0 plants inoculated with Δ12-BP exhibited an attenuated phenotype, with less severe symptoms than plants inoculated with wt. Symptoms on Δ8 P0 plants were similar to plants inoculated with wt. In general, increased symptom development was evident on each inoculated plants after passages (Table 2). Similar levels of PVX RNA replication on inoculated and on upper systemic leaves were observed as determined by western blot analyses (Figs. 3B and 3C).

Twelve independent clones containing mutated region from leaf samples for Δ8 mutant were obtained by RT-PCR. All progeny clone recovered from wt infected plants resulted in wt sequences (data not shown). Surprisingly, insertion of deleted nt residues began to appear during P0 (Table 3). While two groups of reversion population was observed, only one major group was evident in P1. In P2, symptoms on infected plants were similar to wt, and 100% of recovered clones (12 of 12) had wt sequence. Selection during passage of Δ8 mutant resulted in the recovery of wt sequence. These data suggest that viral RNA continues to evolve to obtain an optimum template for replication, especially to maintain possible RNA-RNA base-pairing for the nt sequence at the 5' end.

Table 2. Symptom development from *N. benthamiana* leaves inoculated with mutated transcripts

Mutants	Symptoms[a]		
	P0	P1	P2
Mock	–	–	–
p32	–	–	–
WT	+++	+++	+++
Δ8	++	+++	+++
Δ12	–	–	–/+
Δ12-BP	+	++	+++
Δ12-mut1	–	+	++
Δ12-mut1	–	+	++

[a]Symptom development was observed for 14 days post-inoculation (dpi). The experiment was conducted more than three times using at least three plants for each inoculation. For each passage (P0, P1, and P2), relative levels of symptom development at 14 dpi are indicated by (–) or (+), with wt symptoms designated as (+++).

Discussion

In this study, we provided more evidences of the RNA-RNA base-pairing between the sequences in the 5' end and upstream of PVX plus-strand sgRNA. Site-directed mutagenesis of the PVX 5' end sequence restoring base-pairing ability resulted in increased RNA replication both in protoplasts and in plants. Significantly, the deleted sequences at the 5' end of PVX genome are recovered to wt sequences that maintain suitable base-pairing between various *cis*-elements suggesting the functional importance of RNA-RNA long distance interactions.

Deletion of the nucleotide sequence at the 5' end of the PVX genome, containing the first ACCA element, affects PVX plus-strand RNA synthesis as contrast with minus-strand genomic RNA accumulation in NT1 protoplasts (Kim and Hemenway, 1996). It has been reported that the functional significance of the repeated ACCA sequence elements in the 5' NTR region by analyzing the effects of deletion and site-directed mutations on PVX replication in *N. benthamiana* plants and NT1 protoplasts (Kim et al., 2002; Park et al., 2008). Single-, double-, and four-nucleotide substitution mutations introduced in the first (nt 10-13) element in the 5' NTR of the PVX RNA significantly affected viral replication, while mutations introduced in the second (nt 17-20) and third (nt 20-23) elements did not (Park et al., 2008). These results revealed the importance of the first ACCA located at nt positions 10 and 13.

We have also previously reported that mutations in the 5' NTR that reduced complementarity resulted in lower genomic RNA (gRNA) and sgRNA levels indicating that long distance RNA-RNA interactions are required for PVX plus-strand RNA accumulation (Kim and Hemenway, 1999).

Table 3. Analysis of progeny viral RNA isolated from inoculated *N. benthamiana* leaves with mutated transcripts

Mutants	Sequence[a]	# of clones[b] recovered
Wild-type	$_{1}$GAAAACUAAACCAUACACCACCAACACAACC-----	
Δ8	^{8}GAACCAUACACCACCAACACAACC-----	
P0	$_{1}$GAAA**CG**UAAACCAUACACCACCAACACAACC-----	(9/12)
	$_{1}$GAAAA**G**UAAACCAUACACCACCAACACAACC-----	(3/12)
P1	$_{1}$GAAAA**G**UAAACCAUACACCACCAACACAACC-----	(12/12)
P2	$_{1}$GAAAACUAAACCAUACACCACCAACACAACC-----	(12/12)

[a]Sequences of recovered clones corresponding to the repeat ACCA sequence are underlined. Mutated sequences are indicated in bold characters.
[b]The dsDNA-containing mutated region were amplified by RT-PCR and sequenced 12 independent clones for each mutant using total RNAs extracted at 14 dpi. Numbers represent # of clones for sequence analysis samples/twelve clones.

In addition, long-distance interactions between 5' and 3' terminal sequences are required for all PVX RNA accumulation (Hu et al., 2007). In this regard, it is worth noting that the restoration of possible RNA-RNA base-pairing from the 5' end of Δ12 (Δ12-BP) restored accumulation of PVX RNAs (Fig. 1C). Addition of nt to the 5' end of Δ8 in inoculated plants that maintain RNA-RNA interactions also support the importance of base-pairing. When mutations affect RNA-RNA long-distance interactions, such that the normal RNA-RNA base-pairing between the various *cis*-elements is restored, sgRNA and gRNA syntheses are increased. These results indicate the importance of multiple RNA-RNA long-distance interactions and correlate with results previously observed for plus-strand RNA accumulation *in vivo*. Although we have not looked for the second site reversions that may also affect the fitness of progeny RNA, our data indicate that the preferred RNA sequence and RNA-RNA interactions between various *cis*-elements are selected for during PVX replication, as suggested previously (Miller et al., 1999).

It is a common notion that various RNA *cis*-elements at the 5' and 3' ends of viral RNA genome modulate translation, transcription, replication, and assembly. This view is now changing as accumulating evidences indicate that many RNA *cis*-elements are function in more than one important step of virus life cycle. It has been reported that the sequences located at the 5' end of PVX genome are required for translation, plus-strand RNA accumulation, assembly, and cell-to-cell movement (Kim and Hemenway, 1996; Kim and Hemenway, 1999; Kim et al., 2002; Kwon et al., 2005) and are in agreement with functional overlaps of RNA elements observed in many other RNA virus systems (Iwakawa et al., 2007; Shen and Miller, 2007).

In this paper, we observed long distance RNA-RNA interactions in the absence of viral and/or cellular proteins that are likely to modulate these RNA interactions. Our previous data suggested that the sequences located at the 5' end of the RNA, especially sequences at nt 10-13 ACCA, contains a binding site for at least one cellular protein, p54 (Kim et al., 2002). Mutations that decreased the affinity of the template RNA for the cellular factor decreased PVX plus-strand RNA accumulation in protoplasts suggesting that the p54 may function in PVX RNA replication by binding to the 5' terminus of the viral genomic RNA (Kim et al., 2002). It is also possible that the other cellular factors and/or viral proteins might also involve in modulating long distance RNA-RNA interactions. In fact, it has been reported that several other host factors also bind to the 5' 46 nt of the PVX RNA (Kim et al., 2002). Multiple RNA-RNA, RNA-protein, and protein-protein interactions might be involved in accomplishing efficient virus replication. The characterization of these other host factors that bind to the 5' AC-rich sequences and SL1 RNA structure and determination of their possible roles will shed light on our understanding in modulating the virus replication cycle.

Acknowledgements

We thank M. Gilda Jonson for critical reading of the manuscript. This research was supported in part by grants from the Korea Science and Engineering Foundation (KOSEF) grant (No. R01-2008-000-10087-0) funded by the Korea government (MEST); the Agriculture Specific Research Project funded by the Rural Development Administration; the BioGreen21 Program (No. 20080401034028), Rural Development Administration to KHK. SHP and SYC were supported by graduate fellowships from the Ministry of Education through the Brain Korea 21 Project.

References

Alvarez, D. E., Lodeiro, M. F., Luduena, S. J., Pietrasanta, L. I. and Gamarnik, A. V. 2005. Long-range RNA-RNA interactions circularize the dengue virus genome. *J. Virol.* 79:6631-6643.

Hu, B., Pillai-Nair, N. and Hemenway, C. 2007. Long-distance RNA-RNA interactions between terminal elements and the same subset of internal elements on the *potato virus* X genome mediate minus- and plus-strand RNA synthesis. *RNA* 13:267-280.

Iwakawa, H. O., Kaido, M., Mise, K. and Okuno, T. 2007. *cis*-Acting core RNA elements required for negative-strand RNA synthesis and cap-independent translation are separated in the 3'-untranslated region of Red clover necrotic mosaic virus RNA1. *Virology* 369:168-181.

Karpova, O. V., Arkhipenko, M. V., Zaiakina, O. V., Nikitin, N. A., Kiseleva, O. I., Kozlovskii, S. V., Rodionova, N. P. and Atabekov, I. G. 2006. [Translational regulation of *potato virus* X RNA-coat protein complexes: the key role of a coat protein N-terminal peptide]. *Mol. Biol. (Mosk)* 40:703-710.

Kim, K.-H. and Hemenway, C. L. 1996. The 5' nontranslated region of *potato virus* X RNA affects both genomic and subgenomic RNA synthesis. *J. Virol.* 70:5533-5540.

Kim, K.-H. and Hemenway, C. L. 1997. Mutations that alter a conserved element upstream of the *potato virus* X triple block and coat protein genes affect subgenomic RNA accumulation. *Virology* 232:187-197.

Kim, K.-H. and Hemenway, C. L. 1999. Long-distance RNA-RNA interactions and conserved sequence elements affect *potato virus* X plus-strand RNA accumulation. *RNA* 5:636-645.

Kim, K.-H., Kwon, S.-J. and Hemenway, C. L. 2002. Cellular protein binds to sequences near the 5' terminus of *potato virus* X RNA that are important for virus replication. *Virology* 301:305-312.

Kunkel, T. A. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA*

82:488-492.

Kwon, S.-J., Park, M.-R., Kim, K.-W., Plante, C. A., Hemenway, C. L. and Kim, K.-H. 2005. *cis*-Acting sequences required for coat protein binding and *in vitro* assembly of *Potato virus* X. *Virology* 334:83-97.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.

Lough, T. J., Lee, R. H., Emerson, S. J., Forster, R. L. and Lucas, W. J. 2006. Functional analysis of the 5' untranslated region of potexvirus RNA reveals a role in viral replication and cell-to-cell movement. *Virology* 351:455-465.

Miller, E. D., Kim, K.-H. and Hemenway, C. L. 1999. Restoration of a stem-loop structure required for *potato virus* X RNA accumulation indicates selection for a mismatch and a GNRA tetraloop. *Virology* 260:342-353.

Miller, E. D., Plante, C. A., Kim, K.-H., Brown, J. W. and Hemenway, C. L. 1998. Stem-loop structure in the 5' region of *Potato virus* X genome required for plus-strand RNA accumulation. *J. Mol. Biol.* 284:591-608.

Mun, H.-Y., Park, M.-R., Lee, H.-B. and Kim, K.-H. 2008. Outbreak of *Cucumber mosaic virus* and *Tomato spotted wilt virus* on bell pepper grown in Jeonnam province in Korea. *Plant Pathol. J.* 24:93-96.

Olsthoorn, R. C., Mertens, S., Brederode, F. T. and Bol, J. F. 1999. A conformational switch at the 3' end of a plant virus RNA regulates viral replication. *EMBO J.* 18:4856-4864.

Park, M.-R., Kwon, S.-J., Choi, H.-S., Hemenway, C. L. and Kim, K.-H. 2008. Mutations that alter a repeated ACCA element located at the 5' end of the *Potato virus* X genome affect RNA accumulation. *Virology* 378:133-141.

Park, S.-H. and Kim, K.-H. 2006. Agroinfiltration-based *Potato virus* X replications to dissect the requirements of viral infection. *Plant Pathol. J.* 22:386-390.

Ray, D., Wu, B. and White, K. A. 2003. A second functional RNA domain in the 5' UTR of the Tomato bushy stunt virus genome: intra- and interdomain interactions mediate viral RNA replication. *RNA* 9:1232-1245.

Shen, R. and Miller, W. A. 2007. Structures required for poly(A) tail-independent translation overlap with, but are distinct from, cap-independent translation and RNA replication signals at the 3' end of *Tobacco necrosis virus* RNA. *Virology* 358:448-458.

Zhang, J., Zhang, G., Guo, R., Shapiro, B. A. and Simon, A. E. 2006. A pseudoknot in a preactive form of a viral RNA is part of a structural switch activating minus-strand synthesis. *J. Virol.* 80:9181-9191.