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Mini-Review

Regulatory Viral and Cellular Elements Required for *Potato Virus X* Replication

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***Potato virus X* (PVX) is a flexuous rod-shaped virus containing a single plus-strand RNA. Viral RNA synthesis is precisely regulated by regulatory viral sequences and by viral and/or host proteins. RNA sequence element as well as a stable RNA stem-loop structure in the 5' end of the genome affect accumulation of genomic RNA and subgenomic RNA (sgRNA). The putative sgRNA promoter regions upstream of the PVX triple gene block (TB) and coat protein (CP) gene were critical for both TB and CP sgRNA accumulation. Mutations that disrupted complementarity between a region at the 5' end of the genomic RNA and the sequences located upstream of each sgRNA initiation site is important for PVX RNA accumulation. Compensatory mutations that restore complementarity restored sgRNA accumulation levels. However, the extent of reductions in RNA levels did not directly correlate with the degree of complementarity, suggesting that the sequences of these elements are also important. Gel-retardation assays showed that the 5' end of the positive-strand RNA formed an RNA-protein complex with cellular proteins, suggesting possible involvement of cellular proteins for PVX replication. Future studies on cellular protein binding to the PVX RNA and their role in virus replication will bring a fresh understanding of PVX RNA replication.**

Keywords : cellular protein, *cis*-acting regulatory elements, plant RNA virus, RNA replication, subgenomic RNA synthesis.

Potato virus X (PVX), the type member of the *Potexvirus* group, is a flexuous rod-shaped virus of 13 nm × 510-520 nm in size. Viral capsid is composed of 25 kDa protein monomers that are packed in a helical array. Each virion contains a single-stranded, plus-stranded RNA genome of 6.4 kb (Bercks, 1970; Huisman et al., 1988). Though potexviruses are distributed worldwide and infect a wide range of host plants, PVX has a rather narrow host range. The natural host range of PVX appears to be limited almost entirely to potato and tomato. It causes streak of tomato in combination with *Tomato mosaic virus* (Smith, 1972). Experimental hosts include many angiosperm species. Diagnostic hosts include *Datura stramonium*, *Gomphrena globosa*, and *Chenopodium amaranticolor* (see Table 1). Virus transmission occurs through mechanical contacts. There is no report on vector, flower, and seed transmission of PVX yet. PVX infection induces mosaic, necrosis, and no visible symptom depending on strains and host plant they infect. PVX has been recognized as a serious virus disease in potato cultivation in Korea since 1970 (Kim et al., 1983; Lee et al., 1977). When PVX is co-infected with another potato virus, *Potato virus Y* (PVY), it caused severe symptom development due to the interaction between PVX and PVY (Hahm et al., 1989).

The PVX genome, which is capped and polyadenylated, consists of an 84 nucleotide (nt) 5' nontranslated region (NTR), five open reading frames (ORFs), and a 72 nt 3' NTR (Fig. 1A) (Huisman et al., 1988; Skryabin et al., 1988). ORF1 encodes 165 kDa protein which has homology to viral replicase proteins of other viruses and is the only viral protein absolutely required for PVX RNA synthesis. ORFs 2 to 4 encode three polypeptides of 25, 12, and 8 kDa, respectively, involved in virus movement and referred to as the triple block (TB) genes (Beck et al., 1991). The product of 3' proximal ORF (ORF5), coat protein (CP), encapsidates viral RNA and is involved in the spread of PVX throughout a plant (Baulcombe et al., 1995; Chapman et al., 1992). During PVX infection, two major subgenomic RNAs (sgRNAs) are utilized for expression of the first TB gene (ORF2) and CP, respectively, whereas the other two TB genes (ORFs 3 and 4) are expressed from a less-abundant sgRNA (Morozov et al., 1991; Verchot et al., 1998).

The recent development of DNA recombination technologies, the ability to generate infectious viral RNA transcripts, and the development of numerous techniques that introduce various mutation on viral cDNAs have led us to elucidate the role(s) of viral gene products, their functional significance in symptom development and in host specificity. These findings also open new possibilities to study virus-host interactions in infected cells. In this review, the

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Table 1. A list of *Potato virus X* (PVX) diagnostic hosts and symptoms caused by PVX[a]

Host	Symptoms[b]
Nicotiana tabacum	L, S
N. clevelandii	S
N. glutinosa	S
N. debneyi	S
Physalis floridana	L, S
Lycopersicon esculentum	S
Datura stramonium	S
D. metal	S
Gomphrena globosa	L
Chenopodium amaranticolor	L
C. quinoa	L
Phaseolus vulgaris	–
Potato clone A6	L

[a] Data were obtained from URL http://biology.anu.edu.au/Groups/MES/vide/ by Brunt et al. (1996).
[b] –: absence of symptoms. Only local (L) and systemic (S) symptoms with diagnostic value are indicated for each virus.

process of PVX replication in infected cells, viral *cis*-acting elements and the host cellular factors required for PVX replication process will be discussed.

Cis-Acting Regulatory Elements in PVX Replication

Once PVX gets into a cell through damage on cell wall, it starts disassembly process to release viral RNA. Factors or elements required for this process are not studied yet for PVX. Many steps of PVX replication process and/or life cycle in the cell are still remained to be determined. Basically the PVX genomic RNA serves as an mRNA for the translation of 5' proximal ORF, replicase. Upon the synthesis of viral replicase, it uses input viral RNA as a template for the synthesis of genomic minus-strand RNAs. Newly synthesized RNAs are then used as templates for genomic and sgRNA synthesis. Gene products, ORF2 to 5, are synthesized from at least three different sgRNAs. By using at least two different regulation pathways for RNA transcription, PVX can control viral RNA synthesis and produce each gene product alternatively or simultaneously both in translation and in transcription level.

It is likely that specific sequence and/or structural elements in the 5' and 3' ends and in the upstream of each sgRNA transcription initiation sites and potentially elsewhere in the PVX genome contain *cis*-acting regulatory signals required for PVX replication. Smirnyagina et al. (1991) defined 5' NTR as the AC-rich α sequence containing nt 1-41 and the β sequence containing nt 42-4 that modulate efficient translation *in vitro* and *in vivo* (Pooggin and Skryabin, 1992; Smirnyagina et al., 1991; Tomashevskaya et al., 1993; Zelenina et al., 1992). It has been shown that multiple sequence and structural elements in the 5' NTR of the PVX RNA affect both gRNA and sgRNA accumulation (Kim and Hemenway, 1996; Miller et al., 1998). Deletions of more than 12 nucleotides from the 5' end, internal deletions or an insertion in the 5' NTR resulted in substantially decreased levels of PVX plus-strand RNA production when transcripts containing each mutation were inoculated onto suspension of cell protoplasts isolated from *Nicotiana tabacum* NT1 (Kim and Hemenway, 1996). Recently a prokaryotic-like ribosome recognition sequence was found in front of CP gene (Hefferon et al., 2000). This sequence element enables expression of reporter gene in *E. coli*. They also showed that the expression of CP from chloroplast and proposed that PVX may have employed this mode of expression prior to the development of sgRNAs (Hefferon et al., 2000).

Thermodynamic predictions and the solution structure analyses indicated that two thermodynamically favored stem-loop structures (SL1, nt 32-106 and SL2, nt 143-183; Fig. 1B) can form in the 5' region of PVX RNA (Miller et al., 1998). The importance of the SL1 structure was addressed by introducing site-directed mutations and inoculating mutated transcripts onto NT1 protoplasts. Mutational analysis of the stem regions of SL1 indicated that base-pairing was more important than sequence, which was consistent with the co-variation analysis. Alterations that increased length and stability of either stems were deleterious to plus-strand RNA accumulation. The formation of internal loop C between SC and SD, as well as specific nucleotides within this loop, were also required. Mutations that reduced genomic plus-strand RNA accumulation similarly affected CP accumulation, indicating that sg plus-strand RNA was also affected. These data indicate that both the sequence and structure of SL1 are required for one or more aspects of PVX plus-strand RNA accumulation.

In contrast, all modified transcripts containing site-directed or deletion mutations at the 5' region were functional for genomic minus-strand RNA production, indicating that sequences or elements in the 5' NTR are not essential for the synthesis of PVX minus-strand RNA. Levels of sgRNAs were also reduced when genomic plus-strand RNA production is decreased in inoculated protoplasts, despite no reduction in minus-strand RNA accumulation. These results suggested that initiation of PVX sgRNA synthesis is not solely dependent upon the presence of minus-strand RNA and the appropriate local signals. Given that both the sequence element located at the 5' end and the SL1 are required for PVX plus-strand RNA synthesis, it appears that multiple elements throughout the PVX 5' region function locally and/or distantly to regulate RNA replication. Further progeny viral RNA analysis of the

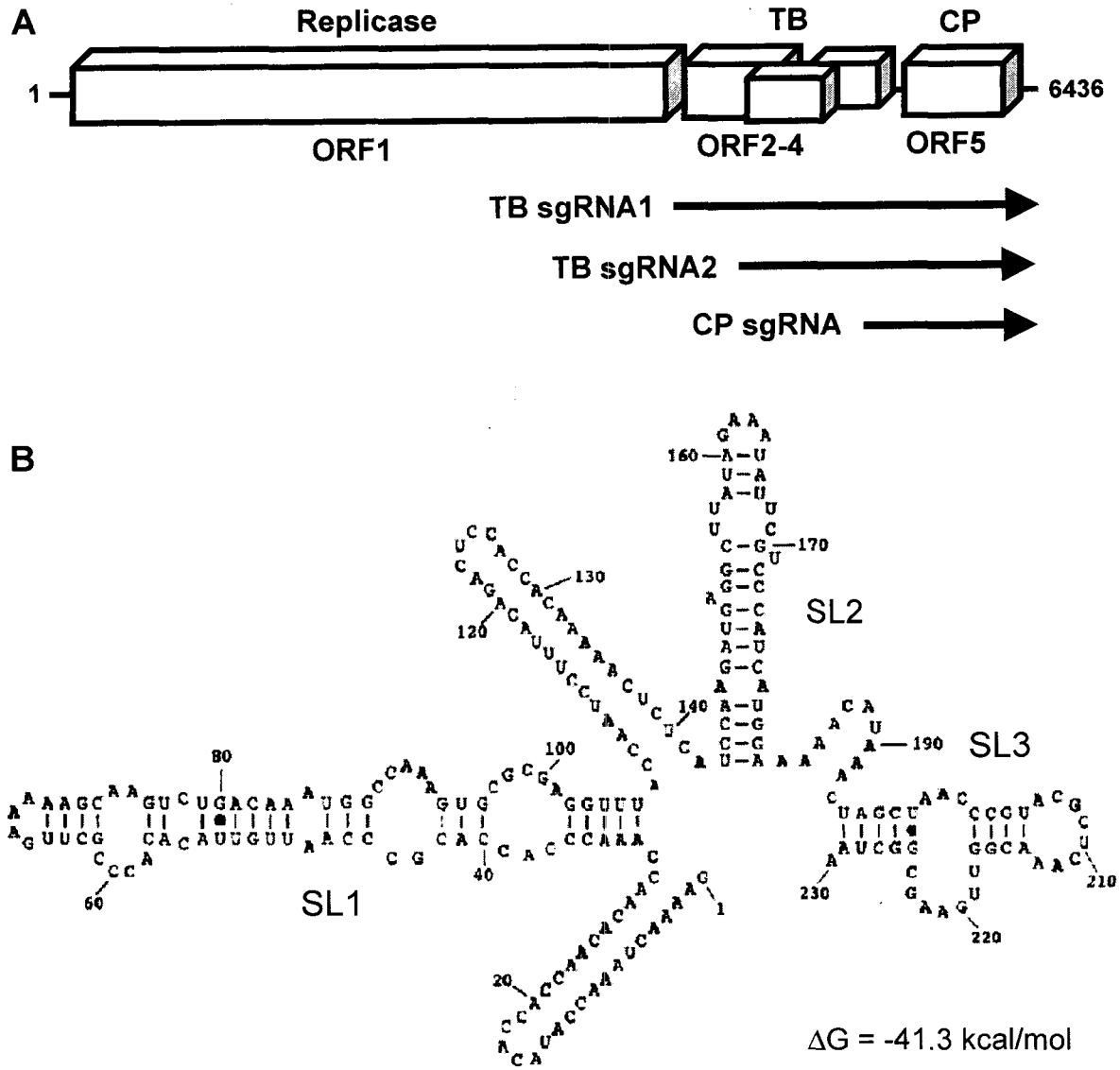

Fig. 1. *Potato virus X* genome organization. Five open reading frames (ORFs) are illustrated as open boxes and the three subgenomic RNAs (sgRNAs) are shown as arrows below the genome. RNA secondary structure in panel B was adapted from Miller et al. (1998).

stem-loop mutants through several passages also indicated the selection of the original stem-loop structure (Miller et al., 1999).

As mentioned above, TB and CP gene products are synthesized from sgRNA produced during replication process in infected cell. Time course studies indicated that TB sgRNA accumulated few hours prior to CP sgRNA, but the relative levels of the TB sgRNA were approximately 2-fold lower than those for CP sgRNA at 12 hpi, and 10-fold lower at 42 hpi in tobacco protoplasts inoculated with infectious transcripts. Chapman et al. (1992) reported a similar accumulation pattern for these two PVX sgRNAs. This general pattern of expression has also been reported for several other plant viruses that synthesize multiple sgRNAs (Johnston and Rochon, 1995; Lehto et al., 1990). Alignment of several potexvirus RNA sequences has revealed nucleotide conservation upstream of the putative sgRNA initiation codons (Bancroft et al., 1991; Sit et al., 1989; Skryabin et al., 1988; White and Mackie, 1990; Zuidema et al., 1989). Additionally, White et al. (1992) observed that the conserved sequences are similar to the hexanucleotide element located near the 3' end of potexviral genomic RNA, and proposed that these elements may represent recognition sites for the replicase.

It has been shown that a conserved octanucleotide element upstream of the TB and CP genes is important for sgRNA accumulation, and that modifications in this region differentially affect levels of the corresponding RNAs (Kim

and Hemenway, 1997). Alteration in these elements drastically decreased the corresponding sgRNA levels, indicating that these sequences may function similarly for both genes. Many of the mutants displayed some common properties. Generally, modification to sequences upstream of one gene did not substantially alter accumulation of the other major sgRNA species, indicating that the elements function independently. If sgRNA synthesis is sequential from the 3' end of the minus-strand, these data suggested that CP sgRNA synthesis is not dependent upon prior synthesis of the TB sgRNA. For most mutants, genomic plus- and minus-strand RNA accumulation were not significantly altered. Although reduced CP levels may lead to reduced genomic plus-strand accumulation, the overall effect on genomic RNA levels may be determined by the extent to which various aspects of RNA synthesis or the coordination of this process are affected by a given mutation. A correlation between sgRNA synthesis and levels of viral RNA was reported for the *Mouse hepatitis virus* (Genus: *Coronavirus*), where inhibition of sgRNA synthesis from a defective-interfering (DI) RNA resulted in increased DI replication *in cis* (Jeong and Makino, 1992), apparently due to increased minus-strand accumulation (Lin et al., 1994).

Critical sequence elements located at the 5' end of PVX plus-strand RNA, in the region predicted to be unstructured, exhibit sequence complementarity to the conserved sequences located upstream of each sgRNA transcription initiation sites (Fig. 2). The role of this sequence complementarity was further addressed by introducing site-directed mutations to both regions. It has been reported that both the sequences of regulatory elements and the complementarity between terminal sequences and internal conserved elements are necessary for PVX plus-strand RNA synthesis (Kim and Hemenway, 1999). Mutations in the 5' NTR region that reduced complementarity resulted in lower genomic RNA and sgRNA levels, whereas mutations to the octanucleotide elements affected only the corresponding sgRNA levels. However, for both the NTR and octanucleotide mutants, the extent of reductions in RNA levels did not directly correlate with the degree of complementarity. These data indicate that long distance RNA-RNA interactions and sequence recognition by components of the replication complex are required for PVX plus-strand RNA accumulation. Similar modulation of sgRNA synthesis is also reported in other plus-strand plant RNA viruses (Sit et al., 1998; Zhang et al., 1999).

Cellular Proteins Required for PVX Replication

In general, viral and host proteins modulate RNA virus replication process by stabilizing or destabilizing RNA-RNA, RNA-protein, and protein-protein interactions in many points during replication (Andino et al., 1999; Diez et al., 2000; Gamarnik and Andino, 1998; Ishikawa et al., 1997; Lai, 1998; Strauss and Strauss, 1999). Several cellular proteins have been characterized for many RNA viruses and their requirements for viral replication are being extensively studied in these days.

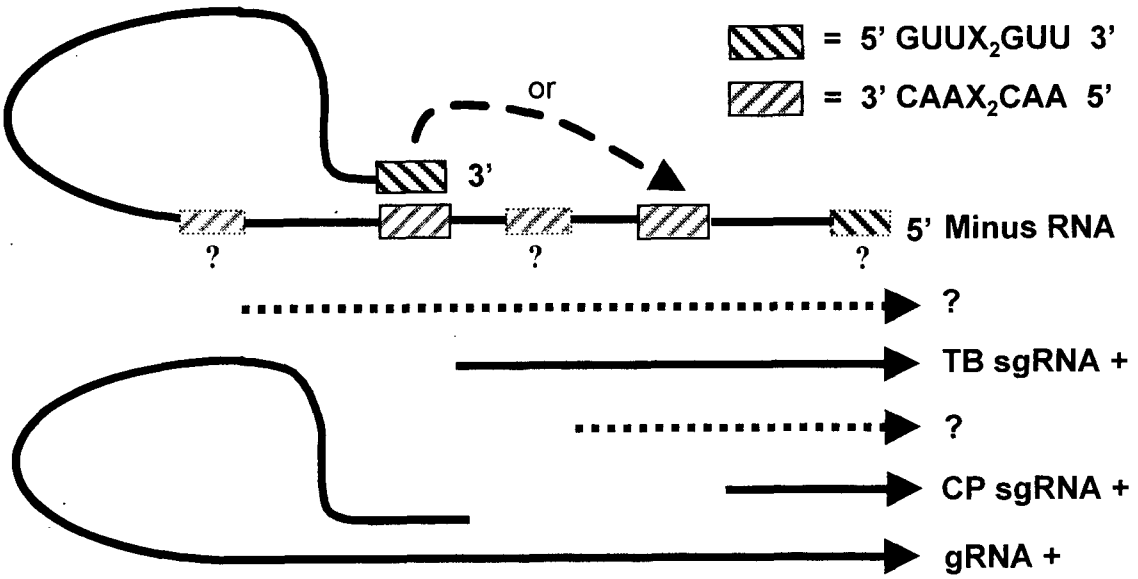

Fig. 2. Elements and interactions involved in *Potato virus X* RNA accumulation. The black hatched box corresponds to the element (5'-GUUAGUU-3') located at the 3' end of minus-strand RNA and the gray hatched box represent the TGB and CP conserved sequence (3'-CAAUUCAA-5'). Other putative boxes are marked with smaller gray-hatched dotted boxes with a ? mark below it. Adapted and modified from Kim and Hemenway (1999).

However, the protein composition of the transcription or replication machineries for PVX RNA synthesis has not been precisely characterized. Presumably, viral replicase, which is encoded by the ORF1 of PVX genome, is involved in both of these processes. In view of multiple functions required for viral RNA replication, it would not be surprising that the binding of cellular proteins with the PVX sequences may also involved in regulating PVX replication especially for sgRNA transcription. Binding of host proteins (28 and 32 kDa) to 3' region of PVX RNA and their possible role in RNA replication has been reported (Sriskanda et al., 1996). Furthermore, they also showed that the 8-nucleotide U-rich motif within the PVX 3' NTR is required for these cellular protein bindings by deletion mapping and site-directed mutations. Mutations that reduce cellular protein binding also affected PVX replication, suggesting that 28 and 32 kDa cellular protein bindings to the 3' NTR of PVX RNA play a role in the process.

A cellular protein that binds to the 5' end of a PVX RNA genome was found using gel retardation and UV cross-linking assays. Gel-retardation analysis of RNA-protein binding was carried out using NT-1 cell extracts and RNA probes representing 5 end of PVX positive-strand RNA. The cDNA constructs (p13) was used to generate RNA probe of 46 nucleotide, labeled as 5' 46(+), which contains single-stranded region (nt 1-31) and portion of stem-loop structures formed at nt 32-106 of plus-strand RNA after *Mun*I digestion followed by in vitro RNA transcription. The results showed that a distinct RNA-protein complex showing slower electrophoretic mobility was formed between 46 nt RNA probe and the NT-1 cell extracts (Fig. 3A).

To determine whether there is a difference in RNA-protein complex formation between healthy and infected cell extracts, NT-1 protoplasts were inoculated with PVX RNA and used to prepare cell extracts. It was previously reported that the maximal level of positive-strand PVX RNAs accumulated in infected cell after 20-24 h post-inoculation (Kim and Hemenway, 1996). Subsequently, extracts from infected and uninfected cells were prepared at 24 h post-inoculation. No obvious difference was observed in the number of or the electrophoretic mobilities of the RNA-protein complexes that formed with extracts from uninfected (Fig. 3A) or PVX RNA-infected (data not shown) NT-1 cells.

PVX 5' 46(+) was capable of forming RNA-protein complexes with cellular protein and the formation of the complex is dose-dependent (Fig. 3B). Gel shift competitor analyses with a number of competitors were carried out to evaluate the specificity of host protein binding to PVX' 5 RNA probe. Unlabeled PVX 5' NTR RNA was used as a specific competitor and *E. coli* tRNA, poly(I)-poly(C), BSA, plasmid pBS(+) RNA, or 5' region of PVX DNA were used as non-specific competitors. When increasing amounts of the unlabeled PVX 5' RNA were incubated with the NT-1 cell extracts prior to the addition of the ^{32}P labeled PVX RNA, formation of RNA-protein complexes was proportionally decreased (data not shown). In contrast, no inhibition of the complex formations was detected when *E. coli*

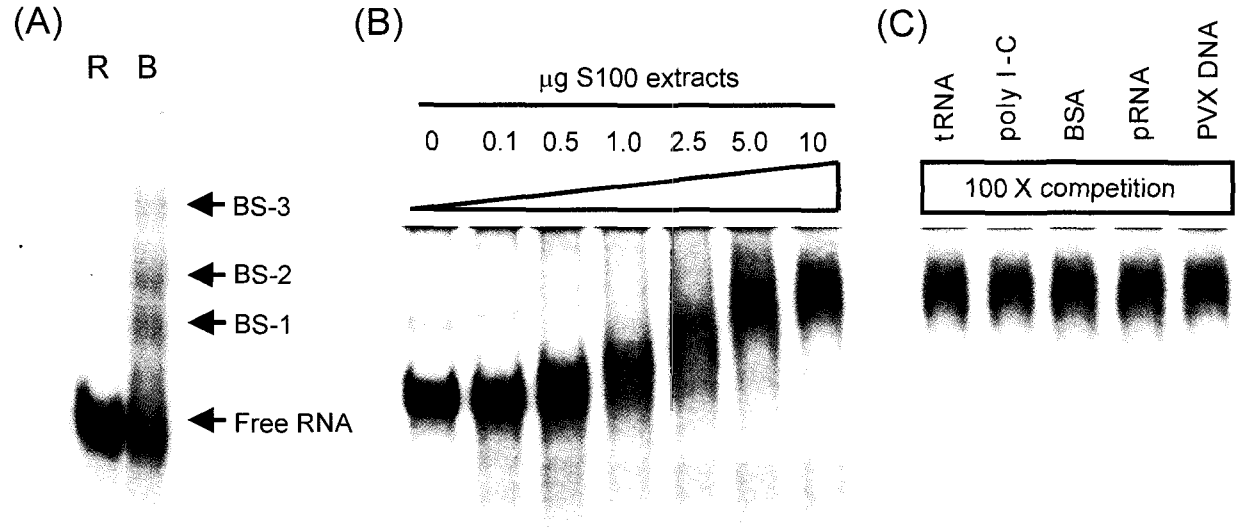

Fig. 3. Identification and specificity analysis of *Potato virus X* 5' 46(+) RNA-cellular protein complex performed by gel mobility shift assay. (**A**) PVX 5' 46(+) RNA was incubated with *Nicotiana benthamiana* NT1 S100 extracts. Lanes: R, free labeled RNA probe; B, probe with uninfected NT1 S100 extracts. Three retarded bands (BS-1, BS-2, and BS-3, respectively) were observed. (**B**) Gel shift assay with a fixed amount of the labeled PVX 5' 46(+) RNA (25 pg RNA in each lane) and the various amount of S100 extracts. The amount of cell extracts was indicated. (**C**) Non-specific competition assay with 100-fold molar excess of each competitor. Used competitors were indicated above the gel.

tRNA, poly(I)-poly(C), BSA, plasmid pBS(+) RNA, or 5' region of PVX DNA were used as a competitor (Fig. 3C). The results showed that none of the non-homologous competitor was able to compete for cellular proteins with the PVX 5' NTR RNA prove even at 250-fold molar excess, whereas the homologous competitor significantly reduced the formation of RNA-protein complexes at 50-fold molar excess. These results indicate that the interaction between cellular protein and PVX 5' NTR RNA is specific.

Functional significance of the cellular protein binding was assayed by inoculation of transcripts containing mutations onto *N. tabacum* protoplasts and the effects of each mutation on virus replication were compared to that of cellular protein binding to the RNA probes containing same mutations. The data suggest that binding of a cellular protein to the 5' end of PVX genome is important for both genomic and sg plus-strand RNA accumulation (data not shown) and that the cellular protein is involved in the process of PVX RNA replication.

Recently, soluble and template-dependent membrane-bound replication complexes were isolated from PVX infected *N. benthamiana* plants (Plante et al., 2000). Upon the addition of full-length plus- or minus- strand PVX transcripts, the corresponding-size products were detected while no synthesis was observed when *Red clover necrotic mosaic Dianthovirus* RNA-2 templates were added. This result indicates that the extracted membrane-bound replication complexes have template specificity for PVX transcripts. These membrane-bound and soluble template-dependent systems will facilitate analyses of cellular or viral components required for PVX replication.

Concluding remarks

Understanding each step or point in virus replication is an important frontier for the research of viral diseases. Although general features and some of *cis*- and *trans*-acting elements required for PVX replication are described in this paper, detailed molecular and biochemical studies on each replication steps are still remained to be determined. Upon identifying and characterizing viral and cellular factors, virus replication and symptom development on different host plants as well as control measures for PVX will be illuminated.

The regulation of viral RNA replication or transcription may involve several cellular proteins that specifically recognize either 5' or 3' NTR, and other sequences in the genome. For example, different cellular proteins that specifically bind to the sequences in the 5' or 3' NTR were observed (Blackwell and Brinton, 1995; Furuya and Lai, 1993; Ito and Lai, 1997; Lai, 1997; Shi et al., 1996; Shi et al., 2000; Zhang and Lai, 1995). Recently, one of the cellular proteins that binds to negative strand leader and IG sequences of MHV RNA have been characterized as a heterogeneous nuclear ribonucleoprotein (hnRNP) A1, involved in alternative splicing of cellular RNAs (Li et al., 1997). Using the ability of *Brome mosaic virus* (BMV) to replicate in yeast (Janda and Ahlquist, 1993), Diez et al. (2000) showed that the efficient BMV RNA replication requires Lsm1p, a yeast protein related to core RNA splicing factors, in an early template selection step by using Lsm1p mutant. They suggested that Lsm1p facilitates the effective interaction of some factors with nonpolyadenylated RNAs and facilitates the transition of mRNA from translation to a new fate.

Development of RNA template-dependent RNA polymerase system will enable us to analyze the mechanisms of RNA synthesis and to determine the extent to which regulatory elements required *in vitro* are similar to sequences/structures that are shown to be important for PVX RNA accumulation *in vivo*. An advantage of the *in vitro* system is that it will enable us to obtain information specific to RNA synthesis rather than other aspects of the replication cycle, such as translation and encapsidation, which are frequently difficult to distinguish *in vivo*. A general understanding of the PVX replication complex will provide new information on the cellular and/or viral factors involved in replication.

Future research on RNA-RNA long-distance interaction and potential cellular factors involved in this process and on the cellular protein recognition by other regulatory sequence and/or structure elements will bring fresh understanding of the PVX replication processes. We should also learn other aspects in PVX life cycles including whether the synthesis of sgRNA occurs on minus- or plus-strand RNAs or how virus switch from the translation to the RNA replication steps.

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