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Identification of the Capsid Protein-binding Region of the SL1(+) RNA Located at the 5' Region of the *Potato virus X* Genome

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Potato virus X (PVX) contains *cis*-acting elements including stem-loop 1 (SL1) RNA at the 5' region; SL1 is conserved among all potexviruses. The SL1 at the positive-sense RNA, SL1(+), is required for PVX RNA replication, cell-to-cell movement, and translation. Previous research demonstrated that SL1(+) RNA also serves as the origin of assembly for encapsidation of PVX RNA. To identify the essential sequences and/or regions for capsid protein (CP) subunit recognition within SL1(+) RNA, we used electrophoretic mobility shift assays (EMSA), UV cross-linking, and yeast three-hybrid analyses. The EMSA and UV cross-linking analyses with PVX CP subunits and RNA transcripts corresponding to the SL1(+) RNA showed that the SL1(+) RNA formed complexes with CP subunits. We also conducted EMSA and yeast three-hybrid analyses with RNAs containing various mutations of SL1(+) RNA elements. These analyses indicated that SL1(+) RNA is required for the interaction with PVX CP and that the RNA sequences located at the loop C and tetra loop of the SL1(+) are crucial for CP binding. These results indicate that, in addition to being important for RNA accumulation, the SL1(+) RNA from the 5' region of the PVX genome is also required for specific binding of PVX CP.

Keywords : assembly, *cis*-elements, EMSA, PVX, SL1(+) RNA-CP interaction

Potato virus X (PVX), the type species of the genus *Potexvirus* in the family *Alphaflexiviridae*, is a positive-sense single-stranded RNA containing both a 5' cap structure and a 3' poly-(A) tails (Huisman et al., 1988; Skryabin et al., 1988). The flexuous, rod-shaped virus contains a 6.4-kb RNA genome that encodes from 5' to 3': i) non-structural replicase component p165 (a 165-kDa protein) possessing the canonical RNA-dependent RNA poly-

merase and helicase motifs; ii) triple gene block proteins (TGBp1-3); and iii) a 25-kDa capsid protein (CP; Skryabin et al., 1988). TGB proteins, which are associated with viral cell-to-cell transport (Angell et al., 1996; Beck et al., 1991), and CP, which is involved in both virus movement and encapsidation (Chapman et al., 1992), are expressed from a subgenomic RNA (sgRNA) (Morozov et al., 1991; Verchot et al., 1998). Translation of sgRNA by the leaky ribosome scanning strategy results in synthesis of the ORF4 product, TGBp3 (Verchot et al., 1998).

The successful packaging of a viral genome is an essential step in the infection cycle of RNA viruses and is initiated via specific recognition of the viral genome by CP subunits. RNA packaging is essential for cell-to-cell movement and systemic spread for several plant RNA viruses (Dolja et al., 1994; Dolja et al., 1995; Vaewhongs and Lommel, 1995). Although the importance of these RNA-CP interactions in plant RNA virus movement and systemic spread has been demonstrated, the specific packaging signal or origin of assembly (OAS) has not been well characterized for many of these viruses. Previous *in vitro* binding studies with the CPs and cognate RNA-packaging signals of *Turnip crinkle virus* and *Brome mosaic virus* have shown that both specific and non-specific binding interactions occur (Duggal and Hall, 1993; Qu and Morris, 1997). In addition, the specific interaction between a 69-nucleotide (nt) *Tobacco mosaic virus* (TMV) RNA sequence including a stem-loop (SL) structure and the TMV CP leads to virion assembly (Butler, 1984). The specific RNA binding of CP to the OAS is essential to initiate and select cognate RNA and thus leads to the complete packaging of viral RNA into a mature virion.

Previous studies have indicated that the 5' and 3'-terminal nt of the PVX genome can form conserved SL structures (Miller et al., 1998; Pillai-Nair et al., 2003). The SL structure formed by the 5'-terminal nt containing the AC-rich single-stranded region and the SL1 secondary structure is more stable than that formed by the 3'-terminal nt. A SL1 secondary structure at the 5' end of positive-sense RNA, SL1(+) (Fig. 1A), is important for plus-strand RNA

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Fig. 1. Schematic representation of SL1(+) RNAs in the 5' end of the PVX genome. (A) Structure of wild-type SL1(+). Individual characters of SL1(+) are labeled as four stem regions (Stems A-D), three internal loops (Loops A-C), and the terminal loop (Tetra loop). (B) Predicted RNA structures of selected SL1(+) mutants. Mutations of SL1(+) were depicted in previous report (Miller et al., 1998). The mutated region of each RNA is shown in a grey box.

accumulation (Kim and Hemenway, 1996). Multiple sequence and structural elements including SL1(+) affect various aspects of the replication process (Kim and Hemenway, 1996; Kim and Hemenway, 1997; Miller et al., 1998). Recent studies have indicated that the top region, i.e., stem C (SC), loop C (LC), stem D (SD), and tetra loop (TL), of SL1(+) forms complexes with cellular proteins and also serves as an OAS for PVX assembly (Kim et al., 2002; Kwon and Kim, 2006; Kwon et al., 2005). The SL1(+) at the 5' end of PVX sequences is also required for cell-to-cell movement (Lough et al., 2006). Although the SL1(+) of PVX is likely to play crucial roles in virus assembly, sequences and/or regions on the SL1(+) required for CP recognition and binding have not been clearly identified.

In this study, we used the electrophoretic mobility shift assay (EMSA), UV cross-linking, and yeast three-hybrid analyses to identify sequences and/or regions of the SL1(+) RNA that are required for CP binding. To identify these

Fig. 2. UV cross-linking assay and electrophoretic mobility gel shift assay (EMSA) of SL1(+) and CP subunit complexes. (A) UV cross-linking assay of 32 P-labeled SL1(+) and *E. coli*-purified CP subunit. The arrowhead (in lane SL1(+)-CP) indicates PVX CP recombinant protein (about 30 kDa). (B) EMSA. Purified CP (0, 1, 2, 3, 4, 5, 6, and 7 μ g; indicated by numbers at the top) were mixed with 32 P-labeled SL1(+) and separated on a 5% non-denaturing polyacrylamide gel. In lane R, only labeled RNA was loaded.

crucial sequences, we tested the binding efficiency of wild-type (wt) and mutants of SL1(+) RNA to CP subunits expressed in *Escherichia coli*. We found that three major regions of the SL1(+) including SC, LC, and TL are crucial for binding with CP subunits.

To construct His-tagged PVX CP for EMSA, we cloned full-length fragments of PVX CP into the pET16b vector (Novagen). A 32 P-labeled SL1(+) was synthesized by *in vitro* transcription as described previously (Kwon et al., 2006). After incubation of this RNA with the CP, the RNA was cross-linked to the protein by UV irradiation. Unbound RNA was removed by digestion with RNaseA. Analysis of the protein by SDS-PAGE and autoradiography revealed a band in the position expected for the size of PVX CP (about 30 kDa; Fig. 2A). These results indicate that the *E. coli*-expressed PVX CP subunits bind to 32 P-labeled SL1(+).

Radiolabeled 32 P-SL1(+) transcript (20,000 cpm) was incubated with purified CP (0, 1, 2, 3, 4, 5, 6, and 7 μ g) on ice for 10 min with 10 μ l of binding buffer containing 5 mM HEPES, pH 7.9, 2 mM $MgCl_2$, 25 mM KCl, 5 mM DTT, 3.8% glycerol, 1 unit of RNase inhibitor, and 1 μ l of protease cocktail solution (Sigma-Aldrich). The reaction mixtures were analyzed on 5% nondenaturing polyacrylamide gels in $0.5 \times$ TBE buffer at 120 V for 30 min. The gel was exposed to an imaging plate and visualized with BAS-2500 (Fujifilm). Several bands of retarded RNAs were detected from 2–7 μ g of CP protein (Fig. 2B). Based on these results, we used 4 μ g of CP subunits for EMSA analysis with mutant SL1(+) RNAs.

To prepare SL1(+) RNA constructs for the initial binding assay, we amplified each DNA fragment from previously

Fig. 3. EMSA of WT and mutants of SL1(+)-CP complexes. (A) Radiolabeled 32 P-RNA transcripts were each mixed with 4 μ g of purified CP (lane CP). In lanes Free, no CP was added. Arrowheads indicate position of RNA-CP complexes. (B) Binding ratio of retarded signals/total signals. Quantification of binding ratio was measured by the Multi Gauge program. Error bars represent the standard deviation. Means with different letters are significantly different ($P \leq 0.05$) according to the Duncan's multiple range tests.

constructed full-length PVX clones (Miller et al., 1998) using PCR with specific primers T7-PVX SL1-F 5'-TAATACGACTCACTATAGACCAAACCCACCACGC C-3' and PVX SL1-R 5'-TAAACCTCGCGCACTTTGGC-3'. Thirteen mutants (Fig. 1B) were incubated with purified CP to evaluate the importance of base-pairing and/or sequence in the binding of SL1(+) to CP. The band intensities were analyzed with Fuji Multi Gauge v2.2 (Fujifilm) and Excel (Microsoft) software. Interestingly, mutants SC12 (increase stem length), LC3 (CCC-to-CAC), LC4 (CCC-to-AAA), and TL2 (G-to-A) showed significantly decreased binding efficiency (Fig. 3B). These data suggest that the spacing of SC and the sequences of LC and TL are important for CP binding. In addition, TL6 and TL8 (substitution of sequences) also showed decreased binding, which suggested that the GNRA tetra loop sequence is important for specific CP recognition.

To analyze SL1 RNAs-CP complexes *in vivo*, we performed yeast three-hybrid assays. All constructs used were based on plasmids of the RNA-Protein Hybrid Hunter System (Invitrogen). Yeast three-hybrid analysis was performed in *Saccharomyces cerevisiae* strain L40-ura3. The SL1 RNAs were cloned into the expression vector pRH5', and full-length CP was introduced to the pYESTrp3 vector. Co-transformation and screening SL1 RNA-CP interactions

were conducted according to the manufacturer's instructions. To confirm interactions between SL1 RNAs and CP, we assessed the expression of *lacZ* based on the appearance of blue colonies in the presence of X-gal (Fig. 4A). In the filter assay, SC11 (UGU-to-ACA), SD1 (insertion of two base-pairs at SD), LC2 (A-to-C), and TL5 (A-to-G) led to the constitutive expression of *lacZ*, suggesting that RNA sequences at the certain position of SC, LC, TL as well as stem length of SD did not affect specific recognition of CP. In contrast, *lacZ* expression was weak in SC7 (deletion), SC12 (insertion of two base-pairs at SC), SD2 (unstable stem), TL2, TL6, and TL8 (sequence substitutions). The strength of the interaction between SL1(+) RNAs and CP was subsequently quantified by β -galactosidase activity using a liquid-culture assay with o-nitrophenyl β -D-galactopyranoside (ONPG) as a substrate (Yeast Protocol Handbook, Clontech). Measurements were done with cells prepared from three independent assays. Chromatic reactions produced by β -galactosidase activity from co-transformed yeast cells corresponded exactly to the results of the SD-agar-medium filter assay (Fig. 4B). Taken together, the *in vitro* and *in vivo* assays revealed that the stem length of SC and the sequences at LC (CCC) and TL (GNAA) are important for CP recognition of the SL1(+) RNA.

Previous studies indicated that sequences and/or region

Fig. 4. Yeast three-hybrid assay. (A) β -galactosidase filter assay. *lacZ* reporter activation was assayed by β -galactosidase filter assay. (+) and (-) indicate positive and negative controls for the yeast assay. WT represents interaction between the WT SL1(+) RNA and CP subunits. (B) Liquid culture assay. Substrate ONPG (o-nitrophenyl- β -d-galactopyranoside) was used for an assay in which β -galactosidase was the reporter enzyme. Error bars represent the standard deviation. Means with different letters are significantly different ($P \leq 0.05$) according to the Duncan's multiple range tests.

for CP recognition are located on the SL1(+) RNA of PVX (Kwon et al., 2005). In this work, we investigated this region in detail, using mutants containing site-directed sequence changes, deletions, and additions. By conducting EMSA, UV cross-linking, and yeast three-hybrid analyses, we identified those sequences and/or regions of the PVX SL1(+) RNA that are required for CP binding. The SL structures at the 5' end of the positive-strand RNA of the genomes contain at least three well-defined stem-loop structures (SL1, SL2, and SL3 in the 3' to 5' direction) and are well conserved in most reported potexviruses (Chen et al., 2010; Miller et al., 1998; Pillai-Nair et al., 2003). In this regard, it is worth noting that SL1(+) structures are identical in all sequenced PVX strains.

The assembly of the mature virion involves numerous and complex CP-CP and CP-viral RNA interactions. Previous studies involving *in vitro* assembly have generally used CP isolated from purified virus (Choi and Rao, 2003; Wengler et al., 1984). The identification of an RNA element essential for assembly initiation has been studied for many viruses (Choi and Rao, 2003; Hacker et al., 1992; Lok and Abouhaidar, 1986; Qu and Morris, 1997). Here, we developed an EMSA system using *E. coli*-expressed PVX CP protein and determined the binding efficiencies

with RNA transcripts containing site-directed mutations within the SL1(+) located at the 5' region of PVX RNA. These findings suggest that several SL1(+) RNA mutations may disturb a selective binding to CP subunits and thus affect formation of virions. Because the binding of the CP subunits to the SL1(+) RNA (OAS) and the initiation of virion formation were closely co-related, it is tempting to speculate that SL1(+) RNA is essential in part because of the specificity and strength of its binding to the CP subunit. It is possible, however, that virus assembly depends on other viral or cellular proteins that recognize and bind to the SL1(+) RNA located at the 5' end of PVX genome. Future experiments should characterize the CP interaction domain(s) with SL1(+) RNA and determine whether other viral and cellular proteins are involved in these specific interactions.

All mutations incorporated in the SL1(+) RNA except for those substituting sequences at the loops affected the predicted secondary structure of the RNA (Fig. 1B). Generating site-directed mutants within the SL1(+) RNA identified the important regions and/or sequences for CP subunit recognition. We have clearly shown that the SC, LC, and TL are important for recognition of the PVX CP. This result is consistent with the findings of Kwon et al. (2005), who

showed that chimeric RNAs with only the SL1(+) region formed virus-like particles, indicating specific recognition between SL1(+) RNA and CP subunits. This region probably plays a role in recognizing CP subunits and thus in initiating virion assembly. A previous report indicated that alterations that increase the length and stability of either SC or SD were deleterious to plus-strand RNA accumulation (Miller et al., 1998). Mutations that change the formation of the internal LC between SC and SD as well as specific nucleotides within TL also affected virus replication. In general, mutations that reduced genomic plus-strand RNA accumulation also had fewer RNA-CP interactions, suggesting the possible involvement of CP binding on positive-strand RNA replication. Recent research indicates that SL1 RNA may have an additional role(s) in the PVX infection cycle by specifically interacting with host proteins (Cho et al., 2012b, 2012c). Several host proteins play roles in PVX replication and movement by interacting with SL1 RNAs and PVX CP (Cho et al., 2012a). These data indicate that multiple features of SL1 are critical for PVX infection cycles.

In summary, we have determined and characterized the region of the SL1(+) RNA that is required for specific CP recognition in PVX. Characterization of this region is important because previous research has demonstrated that the SL1(+) RNAs located at the 5' end of positive-strand RNAs are highly conserved among potexviruses and are essential for many important steps in the virus infection cycle including translation, replication, cell-to-cell movement, and assembly. We hypothesize that 1) SL1(+) RNA-CP interactions might contribute to the proper initiation of PVX assembly; 2) the upper region of SL1 (nt 43–96) that contains SC, LC, and TL might be key for the *cis*-acting element that is required for many viral infection steps; and 3) extensive interactions with viral proteins as well as with host proteins in this region would confer stability of viral RNA. Given that the SL1(+) RNA is essential for PVX infection and is highly conserved among potexviruses, we also suspect that this region could have a pivotal function in the infection cycle of all potexviruses.

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