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STUDY OF THE INITIAL STEPS OF POTATO VIRUS X ASSEMBLY

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Abstract

Potato virus X (*Potexvirus*) is an important pathogen of number of economically significant agricultural plants of the *Solanaceae* family. Potato virus X (PVX) virions are flexible filamentous particles 515 nm in length and 13.5 nm in diameter with a helical structure. PVX genome consists of a single-stranded RNA (6435 nucleotides) which is capped and polyadenylated. The study of various stages of the infection process of plant viruses during infection, including the assembly of viral particles is of great practical interest. Identification of these mechanisms can be the basis for developing new approaches in virus-free crop production. Herein, the initial stages of potato virus X (PVX) virion assembly were examined on the example of virus-like particles (VLPs) produced by incubation of PVX RNA with PVX coat protein (CP) in vitro. The formation of identical set of VLP with discrete size under different incubation conditions (ionic strength, pH) was shown. However, the amount of VLP of a different size changes depending on incubation conditions. Most efficient VLP similar to the native virion size assembly has been achieved in a buffer of low ionic strength at pH 8.5. PVX RNA fragments from 800 to 5700 nt in length which were protected by coat protein within VLPs were isolated. Their individual analysis confirmed that all of them represent the 5′-terminal fragments of the genomic PVX RNA of different lengths. Thus, it was revealed that RNAs within VLP are genomic RNA 5′-terminal fragments of different lengths. PVX virions assembly initiation at the RNA 5′-end which is cooperatively extending in the 5′- to 3′-end direction was confirmed. Nucleotide sequence analysis of RNA fragments isolated from VLP of different sizes showed that sites capable to form RNA hairpins were discovered near the RNA 3′-end. They could act as «stop-signals» that prevent CP and RNA interaction and continuous cooperative assembling PVX VLP and virions. Probably, CP could «melt» RNA hairpins more or less efficiently and overcome «stop-signals» during the viral particles formation, depending on the conditions of incubation with the RNA in vitro.

Keywords: plant viruses, potato virus X, virus-like particles, RNA, coat protein, virion assembly.

The study of various stages of the infection process of plant viruses including the assembly of viral particles is of great practical interest. Identification of these mechanisms can be the basis for developing new approaches in virus-free crop production. Potato virus X (PVX), a typical *Potexvirus* (*Alphaflexiviridae*), is a common pathogen of many plant species of *Solanaceae* family, including number of economically significant agricultural plants, particularly potato plants. It should be noted that potato, together with rice, wheat and corn, are the main food stuffs in the world.

PVX virions are flexible filamentous particles 515 nm in length and 13.5 nm in diameter with a helical structure. PVX genome consists of a single-stranded RNA (6435 nucleotides) which is capped and polyadenylated. About 1300 subunits of viral coat protein (CP) are polymerized to form polar helix with a pitch of 3.6 nm, each coil consist of 8-9 CP subunits, with viral RNA being encapsulated into the CP helix (1).

PVX is the first filamentous plant virus reconstructed in vitro from RNA and CP (2). No differences between native virions and the reconstructed parti-

cles (i.e. virus-like particles, or VLP, and viral ribonucleoproteins, or vRNP) have been found. Furthermore, it was shown that the CP of PVX is unable to polymerize without RNA (3), and in vitro can form vRNP not only with RNA of PVX but also with heterologous nucleic acids (2). In further study of structure and characteristics of these vRNP or VLP, their homology to native virions has been identified (4, 5).

K.H. Kim and C. Hemenway (6) suggested that 5′-end of PVX RNA can be involved into coordination of viral part assembly. The region responsible for initiation of PVX assembling is not completely characterized, though in different publications there are evidences for its location at the 5′-end of RNA molecule (7-9). Recently published data suggest a key role of a cap-structure at the 5′-end of RNA in assembling vRNP of PVX through changing conformation of the 5′-end of RNA and, as a result, the CP recognition at initiation of the particle assembly (10).

Herein, the initial stages of potato virus X (PVX) virion assembly were examined on the example of virus-like particles (VLPs) produced by incubation of PVX RNA with PVX coat protein (CP) in vitro.

Technique. PVX virions, the Russian strain, were isolated from *Datura stramonium* leaves (11), PVX coat protein was obtained by treatment of viral suspension with LiCl (12), and RNA was purified using phenolic method (13).

To obtain VLPs, PVX RNA and CP (1:700) were incubated for 10 min at room temperature in 0.01-0.001 M Tris-HCl buffer at pH 7.5, 8.0, or 8.5. The VLPs were treated with micrococcal nuclease (MN) («Fermentas», Lithuania), activated before use by 100 mM $CaCl_2$. After 10 min incubation at room temperature the reaction was stopped by adding 250 mM EGTA. RNA fragments were isolated from MN treated VLPs using tRNA as coprecipitator.

RT-PCR was carried out according to manufacturer's protocol («Promega», USA) with forward and reverse primers, complementary to PVX RNA sequences of 21-40 nt and 950-981 nt positions, respectively.

Nucleic acid study and analysis of RNA encapsulated in VLPs were conducted by gel retardation assay using 1 % agarose gel made with TAE (40 mM Tris acetate, 2 mM EDTA, pH 8.0; 0.025 μg ethidium bromide). After incubation, the RNA and CP mixtures were diluted in electrophoretic buffer containing 10 % glycerin and 0.2 % bromophenol blue, and then the samples were placed into the wells. Quick screen horizontal electrophoresis system QS-710 («IBI Scientific», USA) was used for the separation at constant voltage 70 V, with chemiluminiscent detection system ChemDOC XRS+ («Bio-Rad», USA; λ = 254 nm) applied for documentation.

For transmission electron microscopy of VLPs, the preparations were contrasted by 2 % uranyl acetate and examined in JEOL JEM-1011 (JEOL Ltd., Japan) at an accelerating voltage of 80 kV. The images were obtained with a digital camera (ES500W, Erlangshen, Gatan, USA) using DigitalMicrograph (Gatan, USA). To calculate VLPs lengths and diameters, micrographs were analyzed by scientific image manipulation software ImageJ (National Institutes of Health, USA).

The nucleotide sequences were analyzed using data and tools of The mfold Web Server (14).

Results. To investigate initial steps of assembly of PVX virions, the PVX RNA was incubated with PVX CP at the rate of 1:700 in different conditions. In a native PVX particle, the RNA to protein rate of 1:1300 is reported (1). So, the amount of CP used in our experiment was not enough for full RNA encapsulation, resulting in the VLPs in which the RNA molecule remains partly uncoated by protein. Single tailed particles (STP) with free 3′-end of RNA and rod-like

heads due to helical encapsulation of the 5′-end fragment of the viral RNA with CP, were reported earlier (9). STP probably is a transport form together with PVX virions (9). It should be noted that even if the molar rate of RNA to CP was the same as in the native virus, in most of the reconstructed preparations the VLPs were not full-sized (3).

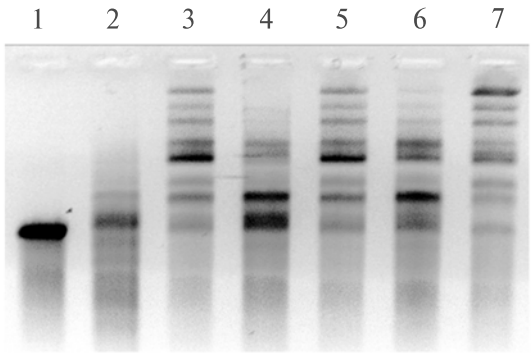

Fig. 1. Electrophoresis of virus-like particles reconstructed in vitro by reassembly from potato virus X (PVX) **RNA and coat protein** (CP) **at different pH and ionic strength:** 1 — control (RNA of PVX); 2, 4, 6 — 0.01 M Tris-HCl, pH 7.5, 8.0 and 8.5, respectively; 3, 5, 7 — 0.001 M Tris-HCl, pH 7.5, 8.0 and 8.5, respectively. RNA to CP molar rate is 1:700; 1 % agarose, staining with ethidium bromide.

The electorphoregrams (Fig. 1) show that the VLPs reassembly resulted in less intensity of the RNA band (see Fig. 1, track 1), while the slower moving products appeared due to particles with different level of encapsidation (see Fig. 1, tracks 2-7). The bands of the highest molecular weight corresponded to the VLPs with high extent of RNA encapsidation with CP. In 0.01 M Tris-HCl buffer, as pH increased from 7.5 to 8.5, the efficacy of reassembly notably rose and the number of products with higher molecular weight increased (see Fig. 1, tracks 2, 4, 6), while in 0.001 M Tris-HCl, there were no notable changes (see Fig. 1, tracks 3, 5, 7), except the maximum intensity of the band with the highest molecular weight (see Fig. 1, track 7). Thus, these conditions contribute to effective RNA encapsulation and formation of VLPs in which the PVX genome RNA is mostly coated by CP. It is also important that the intensity of the bands formed by VLPs of different sizes varied depending on the conditions of reassembling, while their positions in 1 % agarose remained unchanged. So, the stop signals seem to occur in the course of reassembly of CP on RNA from 5′- to 3′-end, and their overcoming is possible due to change of ionic strength and pH of incubation medium.

Electron microscopy of the VLPs which were obtained in the favorable conditions (0.001 M Tris-HCl, pH 8.5) confirmed that in the preparations there are particles of different size (Fig. 2, B) with the diameter and structure as those in PVX virions (see Fig. 2, A). Nevertheless, the VLPs were shorter than PVX virions, so, the RNA molecules were not completely packed into CP with «tails» remained free.

In the histogram, there are 8 discrete peaks corresponding to particle sizes of 60, 90, 160, 220, 300, 360, 420 and 460 nm (see Fig. 2, C). These data are consistent with results of gel retardation assay (see Fig. 1, track 7) according to which the obtained VLPs are a discrete set of particles heterogeneous in size. Considering that the native PVX virion of 515 nm in length contains the PVX genome RNA molecule of 6435 nt, the VLPs of specified sizes can presumably contain 800, 1100, 2000, 2700, 3700, 4500, 5200 and 5700 nt fragments of PVX genome RNA, respectively.

To characterize the RNA regions packed into VLPs, the particles were treated with MN in conditions when the RNA bound to CP in the particle is protected from the nuclease. Under electrophoresis of the samples obtained after the MN treatment, the fragments from 800 to 5700 nt were found (Fig. 3, A, track 1). To elucidate whether they are 5′-end regions, each individual fragment

(see Fig. 3, A, tracks 2-9) was isolated and used in RT-PCR.

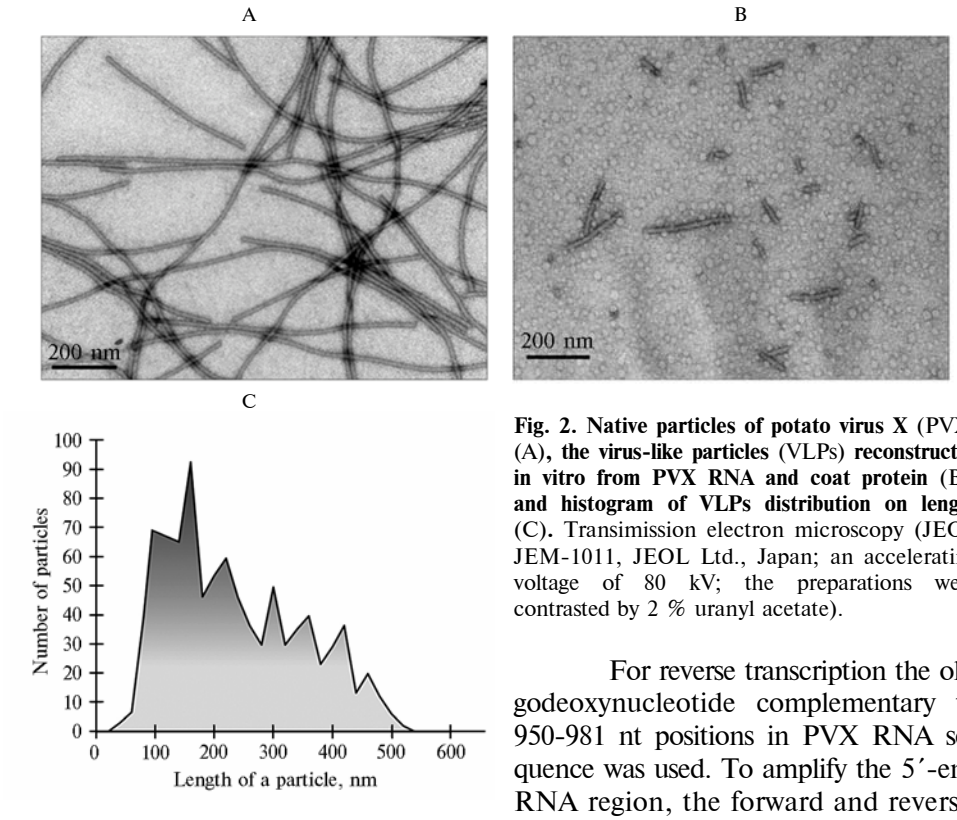

Fig. 2. Native particles of potato virus X (PVX) (A)**, the virus-like particles** (VLPs) **reconstructed in vitro from PVX RNA and coat protein** (B)**, and histogram of VLPs distribution on length** (C)**.** Transimission electron microscopy (JEOL JEM-1011, JEOL Ltd., Japan; an accelerating voltage of 80 kV; the preparations were contrasted by 2 % uranyl acetate).

For reverse transcription the oligodeoxynucleotide complementary to 950-981 nt positions in PVX RNA sequence was used. To amplify the 5′-end RNA region, the forward and reverse primers, complementary to the PVX RNA sequences at 21-40 nt and 950-981 nt positions, respectively, were used. The expected amplification product (see Fig. 3, B) was obtained for most fragments (see Fig. 3, tracks 2-8), except the fragment the length of which was less than 876 nt (see Fig. 3, A, track 9). So, it could not have the site for binding reverse primer complementary to RNA sequence at 950-981 nt positions. Thus, the RNAs within VLP are genomic RNA 5′-terminal fragments of different lengths, and PVX virions assembly initiation at the RNA 5′-end which is cooperatively extending in the 5′- to 3′-end direction is confirmed.

Using SELEX method, S.J. Kwon et al. (7) earlier found a number of RNA sequences having high affinity to PVX CP. By prediction of their secondary structure using computer modeling, a capability to form RNA hairpins was demonstrated. According to our suggestion, the similar hairpins could be distributed along the genome RNA of PVX affecting interaction between RNA and CP.

Indeed, the computer analysis of predicted secondary structure of the 3′-ends in individual RNA fragment from VLPs of different sizes confirmed a probability of hairpins formation.

The figure 4 illustrates the predicted hairpin structures for 3′-ends in individual fragments (see Fig. 3) of 1100, 2700, 5200 and 5700 nt in length calculated on the base of histogram of VLPs distribution according to their size (see Fig. 2, C). A capability to form similar RNA hairpins was also demonstrated for other individual fragments observed in our experiments (800, 2000, 3700 and 4500 nt, data not shown).

It can be assumed, that these hairpins act as stop signals preventing cooperative assembly of VLPs. In early experiments it was shown that coat protein

of potexviruses is capable of melting secondary structure in RNA (15).

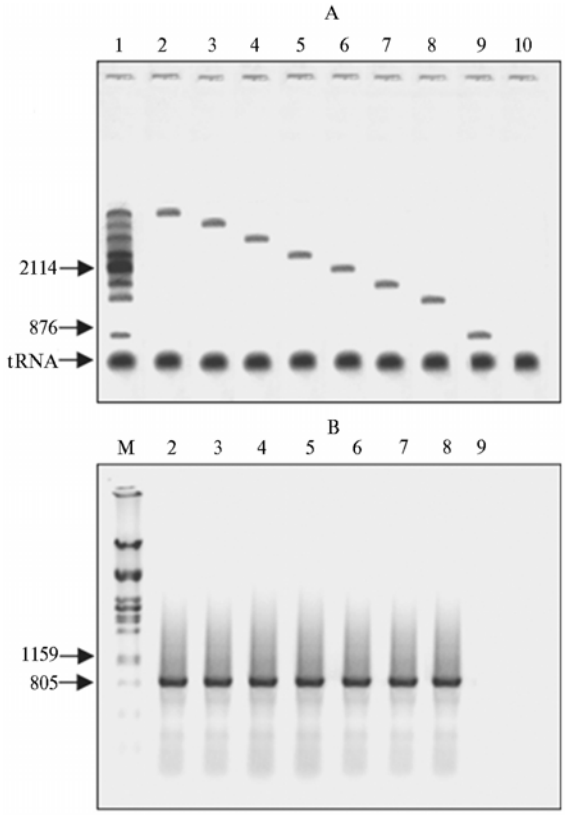

Fig. 3. Analysis of potato virus X (PVX) **RNA fragments from in vitro reconstructed virus-like particles** (VLPs) **of different sizes treated with micrococcal nuclease** (MN)**:** A — electrophoretic pattern, B — RT-PCR analysis of individual fragments; 1 — RNA fragments isolated from VLPs (A), 2-9 — individual RNA fragments, 10 — tRNA as coprecipitator (A); M — DNA molecular weight marker (B). The arrows indicate the markers of RNA length, nt (A), and DNA length, bp (B); 1 % agarose, staining with ethidium bromide. In RT-PCR the forward and reverse primers, complementary to the PVX RNA sequences at 21-40 nt and 950-981 nt positions, respectively, were used.

To overcome stop signals, the coat protein should be detained at such a region, melt the hairpin, and only after that the process of assembling virion could be continued.

Thus we have shown that incubation of potato virus X (PVX) RNA and coat protein (CP) under different conditions (ionic strength, pH) formed one and the same pattern of virus-like particles (VLPs) of specific size. Nevertheless, the amount of individual VLP

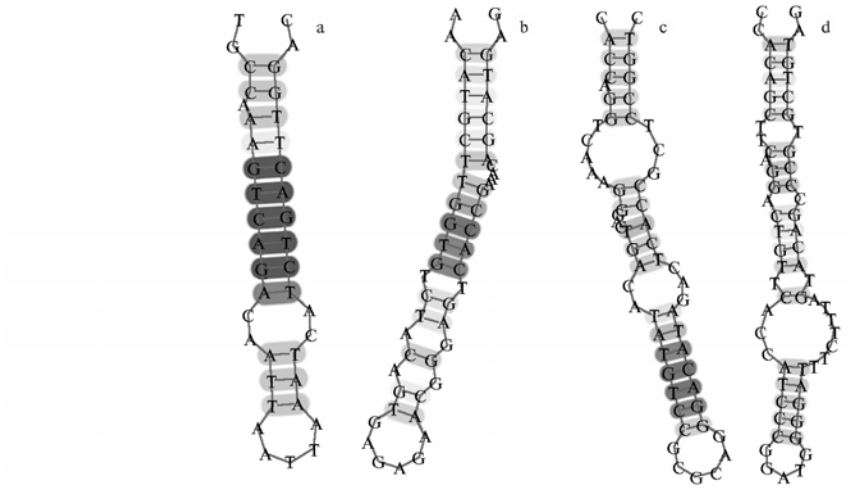

Fig. 4. Schemes of a predicted secondary structure in the 3′-ends of RNA fragments isolated from virus-like particles (VLPs) **of different sizes reconstructed in vitro from potato virus X** (PVX) **RNA and coat protein** (CP)**:** a — 1078-1117 nt, b — 2705-2757 nt, c — 5125-5183 nt, d — 5730-5792 nt. Grayscale colors reflect the strength of interaction between nucleotides. The images were obtained with the use of The mfold Web Server (http://mfold.rit.albany.edu/).

depends on the conditions during incubation. Most efficient VLP similar to the native virion size assembly has been achieved in a buffer of low ionic strength at

pH 8.5. From VLPs the RNA fragments protected by coat protein helix were isolated. All of them were shown to be the 5′-proximal fragments of PVX genome RNA and have different length. More evidences are obtained that the PVX assembly is initiated at the 5′-end of the RNA molecule, being cooperatively extended to its 3′-end. Due to analysis of nucleotide sequences of 3′-regions of RNA fragments isolated from the reconstructed VLPs, the sites were discovered where the RNA-hairpins could be probably formed. These RNA-hairpins may play a role of stop signals and prevent cooperative assembling VLPs. The changes of incubation conditions probably affect the CP capability of melting and overcoming these hairpins.

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