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Generation of Infectious Transcripts from Korean Strain and Mild Mottle Strain of Potato Virus X

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Full-length cDNAs of two different strains of *Potato virus X* (PVX-Kr and PVX-Mo) have been directly amplified by long template reverse transcription polymerase chain reaction (RT-PCR) using the 5'-end primer containing a SP6 or T7 RNA promoter sequence and the virus-specific 3'-end primer, and then constructed in plasmid vectors. Capped *in vitro* transcripts from cloned full-length cDNAs as well as those RT-PCR amplicons proved to be infectious systemically on tobacco plants. Symptom expression on tobacco plants from PVX-Mo transcripts was faster and severer than that from PVX-Kr. In replication stability test of transcripts derived from PVX clones, progeny viruses showed stable replication according to sequencing through passages. This highly infectious transcript system from the full-length cDNA clones for PVX can be useful for recombinant molecules for functional analysis of viral proteins in plant-virus interaction study as well as for expression of foreign protein in planta.

***Keywords*:** Potato virus X, *in vitro* transcription, infectious cDNA clone, plant virus, mild mottle strain, Korean strain

Potato virus X (PVX) having many strains or isolates reported throughout the world is a type species of the genus *Potexvirus* which is well described and characterized biologically and molecularly. Especially, PVX has been used widely as a vector for foreign gene expression (Chapman *et al*., 1992; Baulcombe *et al.*, 1995), and also has been used for RNA silencing mechanism (Hamilton and Baulcombe, 1999; Hamilton *et al*., 2002). As many reports indicated, PVX has been used as a virus-induced gene silencing vector to down regulate specific gene (Baulcombe *et al*., 1995; Verchot-Lubicz *et al*., 2007). Genomic RNA from PVX was characterized as containing five open reading frames coding for proteins of 165 kDa (viral replicase), 25 kDa [triple gene block (TGB)-1], 12 kDa (TGB-2), 8 kDa (TGB-3), and 25 kDa (coat protein) from the 5' to 3' end. Especially, among TGB proteins, 25 kDa protein (p25) is known to be responsible for silencing suppressor (Vionnet *et al*., 2000). We analyzed coat protein (CP) sequences of Korean strain (Kr) and mild mottle strain (Mo) of PVX to compare between them before synthesizing infectious transcripts from cDNA of these strains. In addition we analyzed those transcripts from PVX cDNAs whether similar or different in their symptomatic reaction and infection mechanism compared to wild type.

For functional study of the virus genome in plant-pathogen interactions and for developing a viral vector such as PVX vector from UK3 strain (Kavanagh *et al*., 1992), we have constructed full-length cDNA clones from which infectious transcripts of the two kinds of strains could be synthesized.

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Materials and Methods

Virus source and RNA extraction

PVX Korean strain (PVX-Kr) and mild mottle strain (PVX-Mo) were propagated in *Nicotiana tabacum* cv. Xanthi-nc and used as sources of viruses. Kr strain was originally obtained from potato showing typical PVX symptom in Kangwon province, Korea (Jung *et al*., 2000), and Mo strain was from American Type Culture Collection (ATCC) (PV-197, USA). Those were purified by polyethylene glycol precipitation and differential centrifugation methods (Jung *et al*., 2000). Viral genomic RNA was extracted from purified virus particles by SDS, proteinase-K/phenol extraction followed by ethanol precipitation (Ryu and Park, 1995).

Full-length cDNA amplification and cloning

Synthesis of first-strand cDNA of Kr was primed by oligonucleotide PKS3 which is complimentary to the 3'-terminal nucleotides of RNA of PVX-Kr (Fig. 1) based on nucleotide information obtained from GenBank (NCBI). This reverse primer contained four thymine at the end of complemented viral RNA sequences as four adenines (Fig. 1). The cDNA was used as templates for full-length cDNA amplification with forward primer PKS5 containing SP6 promoter site and reverse primer PKS3 by PCR using *Taq* DNA polymerase (Roche) (Choi *et al*., 2007). Double-stranded DNA molecules were cloned into *Sac*I/*Sph*I site of pUC19 vector. For PVX-Mo full-length cloning, we carried out PCR with PMT5 forward primer containing T7 promoter site and PKS3 reverse primer, and then full-length PCR product was inserted into pUC19 at the site of *Sal*I/*Sph*I (Fig. 1) Recombinant plasmids were propagated in *Escherichia coli* (strain JM109, Promega). Obtained PVX clones as well as

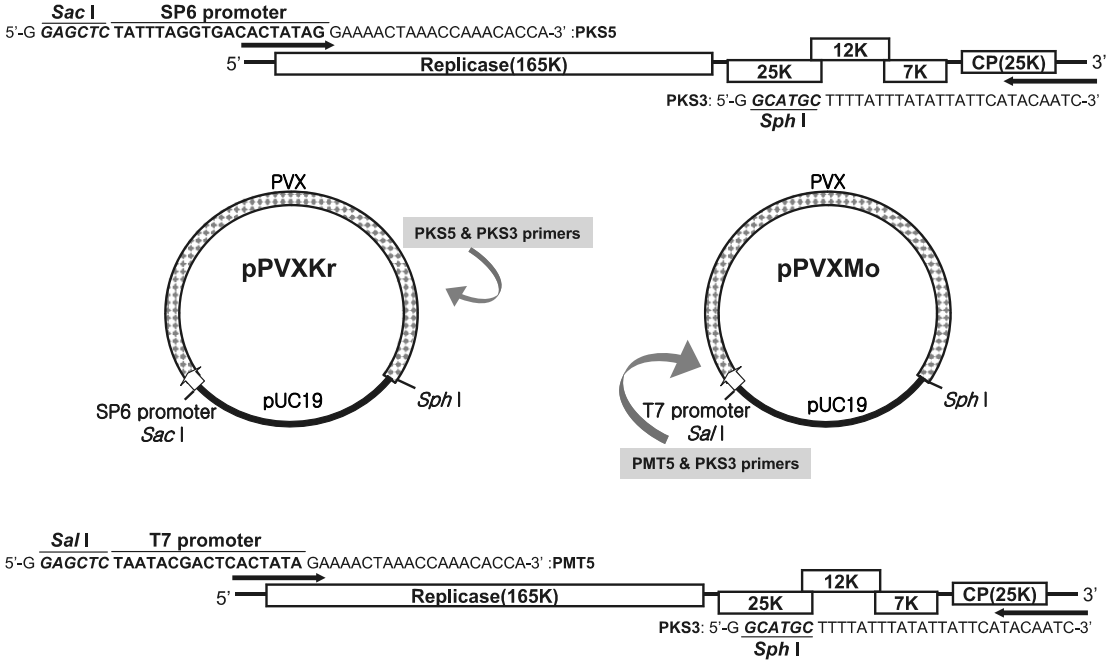

Fig. 1. Construction of full length cDNA clones (pPVXKr and pPVXMo) of *Potato virus X* (PVX-Kr, -Mo). SP6 RNA polymerase was used for construction of PVX-Kr clone and two specific endonuclease (*Sac*I and *Sph*I) recognition sites were used for positional cloning with pUC19 vector. T7 RNA polymerase was used for construction of PVX-Mo clone and two specific endonucleases (*Sal*I and *Sph*I) recognition sites were used for positional cloning with pUC19 vector.

RT-PCR amplicons were confirmed by their digestion site using various endonucleases.

Comparison of coat protein sequences between Kr and Mo strains

Sequences of CP and 3' nontranslatable region (NTR) were determined to compare Kr strain with Mo strain. Phylogenetic analysis was performed with other reported sequences from potato host in Korea to know the genetic relationship among them.

Transcription and inoculation of systemic host plants

RT-PCR amplicons and full-length cDNA clones were directly used as template DNA for *in vitro* transcription. The full length clones of Kr and Mo were linearized with *Sph*I, and digested DNAs were purified by phenol/chloroform extraction. *In vitro* transcription was done in the presence of cap analogue using SP6 RNA polymerase for PVX-Kr or T7 RNA polymerase for PVX-Mo. Transcripts were from 50 ng template of each strain in a final volume of 50 µl. Poly adenine (A) tail was synthesized to 3'-end of each transcript from Kr and Mo clone as well as PCR products by *E. coli* poly A polymerase (Roche). They were inoculated onto *Nicotiana tabacum* cv. Samsun or cv. Xanthi-nc and *N. benthamiana* plants with same transcript concentration of each strain. Plants at four leaf stage were inoculated with 20 µl *in vitro* transcripts. Tobacco plants were grown in growth chamber at 25°C/15°C (daytime/nighttime). Virus detection was performed by western blot analysis and RT-PCR to confirm their infectivity.

RT-PCR and immunoblotting for infectivity of *in vitro* transcripts of PVX-Kr and PVX-Mo

Virus infection was assessed by RT-PCR and Western blotting at 10 days post inoculation (dpi). Samplings for detection were done in both inoculated and systemic leaves of tobacco plants showing symptom. Total nucleic acids were obtained from five leaf disks (1 cm diameter) and purified from the supernatant by phenol/chloroform extraction and ethanol precipitation. PCR amplification was performed in a reaction tube with total RNA and CP specific primers (PVXCP5; 5'-GTTTCCAGTGATAATTGAAAG-3' and PVXCP3; 5'-GTCGGTTATGTGGACGTAG-3') to generate 750 bp product containing *Bst*XI restriction site which could be used for confirmation (Lee *et al*., 2007).

Total proteins for western blotting were obtained from the inter-phase in the phenol/chloroform extracted solution. Western blotting was performed with prepared total proteins from inoculated or systemic leaves inoculated with transcripts of PVX-Kr or -Mo. Hybridizations of nitrocellulose (NC) membrane binding coat proteins with anti-serum specific for CP were carried out to detect CP accumulation level and to compare with RT-PCR data. NC membrane was probed with antibody (1:2,500 dilutions) against PVX CP, and then membrane was incubated with an alkaline phosphatase (AP)-conjugated secondary antibody (1:7,500 dilution; Promega, USA). To visualize antibody-specific proteins, membrane was reacted with AP-substrate solution (Western Blue Stabilized Substrate Solution, Promega, USA).

Table 1. Sequence similarity and distance of PVX strains based on their coat protein nucleotide or amino acid sequences

		Mo		KO1		KO2		Kr	
		n.t.[a]	a.a[b]	n.t.	a.a	n.t.	a.a	n.t.	a.a
Mo	(ATCC)			100[c]	100	99.7	99.6	95.9	99.6
KO1	(AF260640)	0.0	0.0			99.7	99.6	95.9	99.6
KO2	(AF260641)	0.3	0.4	0.3	0.4			95.7	99.2
Kr	(AF373782)	4.2	0.4	4.2	0.4	4.5	0.8		

[a] nucleotide level
[b] amino acid level
[c] Numbers in upper right corner refer to percentage of similarity and numbers in lower left corner refer to distance.

Results

Comparison of CP sequences between PVX-Kr and PVX-Mo

Two kinds of PVX strains were used to analyze CP nucleotide sequences. Those sequences were determined using PVXCP5 primer in 5'→3' direction. Determined CP of PVX-Kr and -Mo were 714 nucleotides like strains of ROTH1 (AF111193) or X3 (D00344), and 3'NTR sequences of both strains consisted of 73 bp like almost reported strains. To compare with PVX KO1 (AF260640) and KO2 (AF260641) strains (Jung *et al*., 2000), Kr strain was analyzed by CLUSTAL W alignment method of MegAlign (Lasergene ver. 6 DNASTAR) including Mo strain. Homology percentage showed 95.9 and 99.6 between Kr and KO1, at CP nucleotide level and amino acid level, respectively. It also showed 95.7 and 99.2 between Kr and KO2, at CP nucleotide level and amino acid level, respectively (Table 1). Sequences of Mo in CP was identical with KO1 (Table 1), not with KO2 as 99.7% at nucleotide level and 99.6% at amino acid level. Between Kr and Mo in coat protein sequences, there was 95.9% nucleotide homology and 99.6% amino acid homology. There was no different nucleotide in 3'NTR (73 bp) from both PVX-Kr and -Mo.

Clone construction and restriction pattern between two strains

Full length cDNAs of PVX-Kr and Mo were amplified by long-template RT-PCR techniques and cloned into plasmid vector. Full length RT-PCR products derived from both strains and their recombinant cDNA clones were validated by RFLP analysis (Fig. 2). Endonucleases used in this study were recommended from other reported PVX sequences (Orman *et al*., 1990; Kavanagh *et al*., 1992; Querci *et al*., 1993; Malcuit *et al*., 2000; Kagiwada *et al*., 2002). Twelve endonucleases were used for obtaining PVX-Kr pattern (Fig. 2A and B), and thirteen endonucleases were used for PVX-Mo pattern for creating (Fig. 2C and D). Based on these restriction patterns, we could confirm that there were typical restriction sites in each strain and each clone had identical restriction sites in their sequences with PCR products.

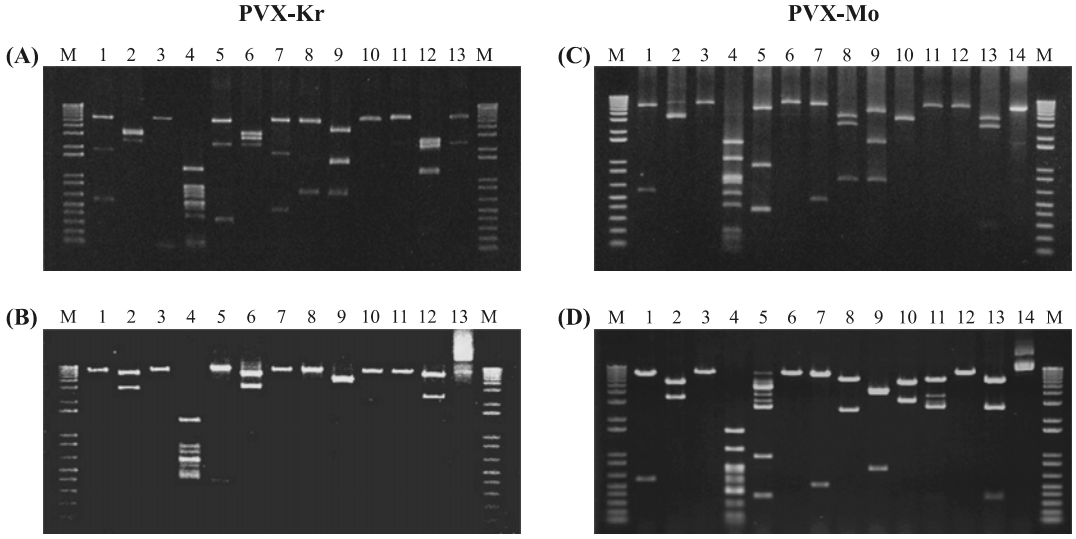

Fig. 2. RFLP analysis of full length PCR amplicons and clones for PVX-Kr and PVX-Mo. For confirming PCR product of PVX-Kr (A) and pPVXKr clone (B), twelve endonucleases (*Bam*HI, *Eco*RI, *Eco*RV, *Hae*III, *Hinc*II, *Hin*dIII, *Kpn*I, *Nco*I, *Pst*I, *Sac*I, *Sph*I, and *Xba*I; lane 1~12 from left) were treated. For confirming PVX-Mo PCR product (C), and pPVXMo clone (D), thirteen endonucleases (*Bam*HI, *Eco*RI, *Eco*RV, *Hae*III, *Hinc*II, *Hin*dIII, *Kpn*I, *Nco*I, *Pst*I, *Sac*I, *Sal*I, *Sph*I, and *Xba*I; lane 1~13 from left) were used. In lane 13 (A and B) and lane 14 (C and D), intact PCR products and clones were loaded.

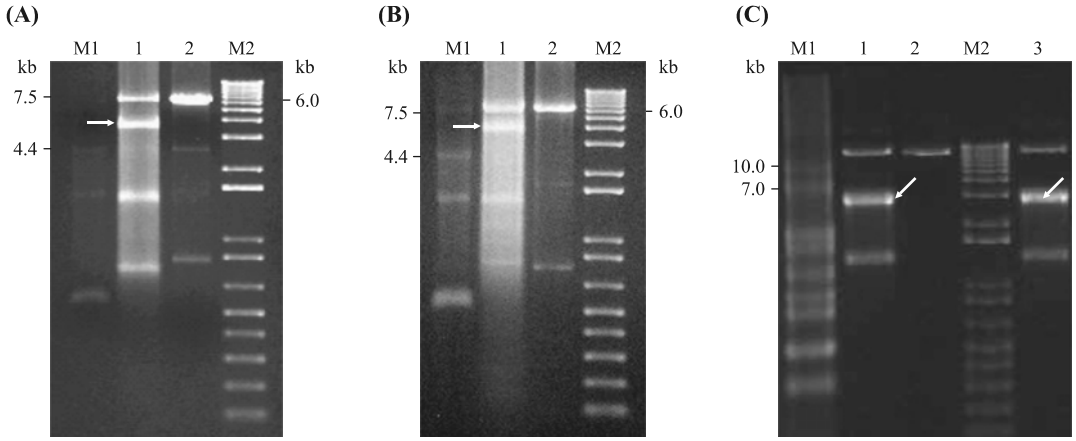

Fig. 3. *In vitro* transcripts from PCR amplicons and clones of PVX-Kr and PVX–Mo. Transcripts from PVX-Kr (A) and PVX-Mo (B) PCR products (M1, RNA siaze marker; 1, transcripts; 2, cDNA templates; M2, DNA size marker). Transcripts from clones (1, transcripts from pPVXKr; 2, linearized clone; 3, transcripts from pPVXMo). White arrows indicate *in vitro* transcripts in each agarose gel.

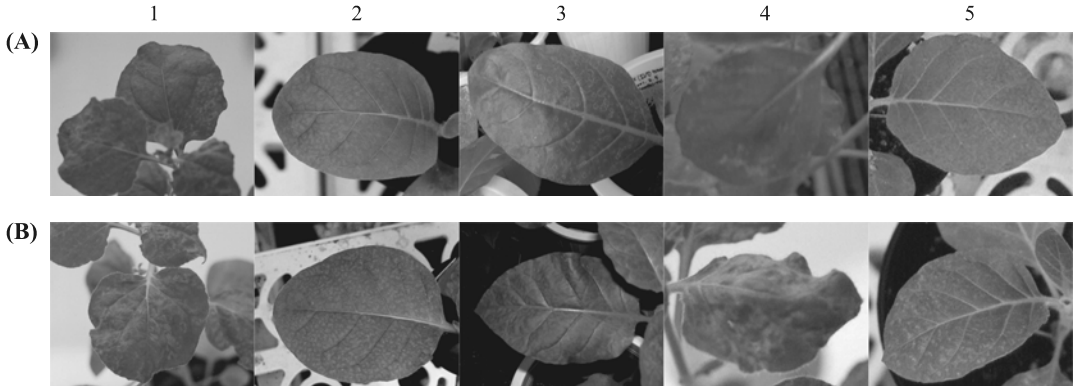

Fig. 4. Symptom expression in systemic upper leaves from various *Nicotiana* plants inoculated by *in vitro* transcripts of PVX-Kr and PVX-Mo or wild type PVX. (A) transcripts of PVX-Kr (1, *Nicotiana benthamiana*; 2, *N. tabacum* cv. Samsun; 3, *N. tabacum* cv. Xanthi-nc) or wild type PVX-Kr (4, *N. benthamiana*; 5, *N. tabacum* cv. Samsun). (B) transcripts of PVX-Mo (1, *N. benthamiana*; 2, *N. tabacum* cv. Samsun; 3, *N. tabacum* cv. Xanthi-nc) or wild type PVX-Mo (4, *N. benthamiana*; 5, *N. tabacum* cv. Samsun).

Production of *in vitro* transcripts from RT-PCR amplicons of PVX-Kr and -Mo and their infectivities

Full-length RNA transcripts of 6.4 kb could be transcribed *in vitro* from RT-PCR products of PVX-Kr and -Mo (Fig. 3). High amount over 10 μg of transcripts from 50 ng of DNA template was obtained by transcription reaction under SP6 RNA polymerase or T7. Typical mottle symptoms followed by mechanical inoculation with transcripts synthesized from PCR product of each strain were developed in the inoculated tobacco as well as *N. benthamiana* leaves (Fig. 4). Symptoms were observed in upper systemic leaves inoculated PVX-Kr and PVX-Mo independently at 8 dpi. Until 22 days after inoculation, symptoms were proliferated and showed systemic movement upwards. Compared with wild type virion of PVX-Kr or PVX-Mo, there is no distinct symptom differences in *Nicotiana* plants inoculated by each transcripts (Fig. 4). After observing symptom in transcripts-treated plants, virus detection PCR was carried out using

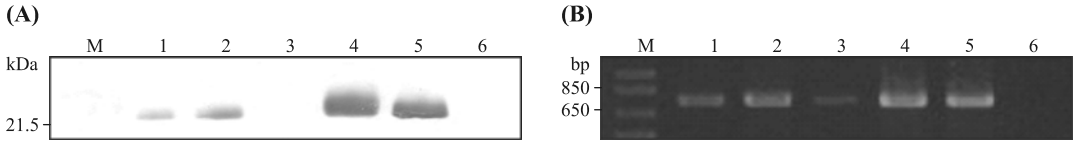

Fig. 5. Virus detection from *N. tabacum* inoculated by *in vitro* transcripts of PVX PCR amplicons. (A) Western blot analysis of PVX coat protein immunoprobed with PVX antibody. (B) detection of the coat protein gene of PVX by RT-PCR. M, DNA or protein size marker; 1, inoculated leaf by PVX-Kr transcripts; 2, systemic upper leaf inoculated by PVX-Kr transcripts; 3, inoculated leaf by PVX-Mo transcripts; 4, systemic upper leaf inoculated by PVX-Mo transcripts; 5, wild-type PVX; 6, negative control.

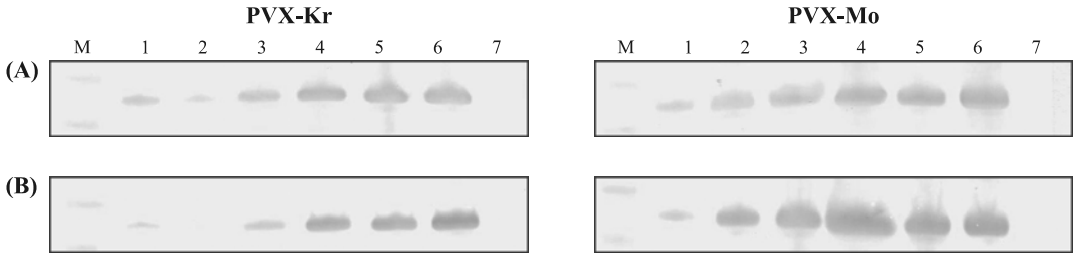

Fig. 6. Systemic movement check by *in vitro* transcripts from PCR amplicons of PVX-Kr and PVX-Mo strain at 15 dpi. (A) *N. tabacum* cv. Xanthi-nc (B) *N. tabacum* cv. Samsun. M, protein size marker; 1, total protein from inoculated leaf; 2, first upper leaf from inoculated leaf; 3, second upper leaf; 4, third upper leaf; 5, fourth upper leaf; 6, fifth upper leaf; 7, negative control.

PVX CP specific primers at 10 dpi. Performed PCR produced 750 bp of designated amplicons, and we also confirmed virus infection by immunoblotting with anti-PVX serum (Fig. 5). Virus was detected in both inoculated leaves and systemic upper leaves of tobacco plants inoculated with transcripts from PVX-Kr or Mo full length PCR product (Fig. 5). We also chased virus in order to see initial transcripts can replicate stably and replicons can move through vascular system to upper parts of tobacco plants. Results from western blotting at 15 dpi showed that virus from PCR transcripts of both strains could move through vascular system to upper leaves in tobacco cv. Xanthi-nc as well as cv. Samsun (Fig. 6). According to this data, virus accumulation seemed to be detected more in systemic upper leaves of cultivar Samsun treated with PVX-Mo transcript (Fig. 6).

Production of *in vitro* transcripts from PVX-Kr and -Mo clones and their infectivities

PVX transcripts containing four adenines at 3' terminal of viral RNA from full-length cDNA clones which were linearized by *Sph*I were synthesized in the presence of cap analogue at first. And we treated *in vitro* polyadenylation to transcripts already made right before inoculation. Polyadenylated transcripts from each strain seemed to be highly infectious on its host plants. The symptoms from these transcripts were indistinguishable from those generated by virions and their viral RNA (data not shown). But their symptoms were delayed about 5 or 6 days compared with wild type produced symptom. The presence of PVX in inoculated and upper leaves of the plants were detected by RT-PCR producing 750 bp PCR fragment with CP specific primers (Fig. 7B). Restriction enzyme *Bst*XI was treated to confirm correctness of PCR fragment (Fig. 7C). Western blotting data showed binding specificity between samples and anti-PVX serum (Fig. 7A). In progeny stability test of transcripts from PVX clones by nucleotide sequencing, there was no difference between progeny virus and initial transcripts from PVX clones when the progeny was transferred to their host plants several times.

Discussion

Kr strain was differentiated from other three strains according to sequence analysis. We thought at first Kr strain might be the same as KO1 or KO2 strain which have been reported (Jung *et al*., 2000). But there was sequence difference in CP region among Kr, KO1, and KO2 (Table 1). Furthermore, nucleotide sequences of Mo strain was identical with KO1 strain and was very highly homologous (99.7%) with KO2. Although Mo stands for mild mottle as described in the ATCC, it is interesting that coat protein region of PVX-Mo is identical with another Korean isolate of PVX. However, we could find out that several isolates showed identical sequence homology at their amino acid level in CP region (Yu *et al*., 2008). According to previous report about KO1 and KO2 (Jung *et al*., 2000), these strains were categorized into type X CP which was avirulent on the *Nx* cultivars in potato based on their CP sequences (Santa Cruz and Baulcombe, 1995). Therefore, we could think Mo and Kr strains are included in type X CP with high homology above 95.7%. Although the RFLP pattern of Kr and Mo seemed to be different due to their sequence dissimilarity except for fragments by *Eco*RV, *Pst*I, *Sac*I, and *Sph*I (Fig. 2), sequences in CP showed high similarity between Kr and Mo strain (Table 1). Further extensive sequencing might be needed to find out characteristics between Mo and KO1

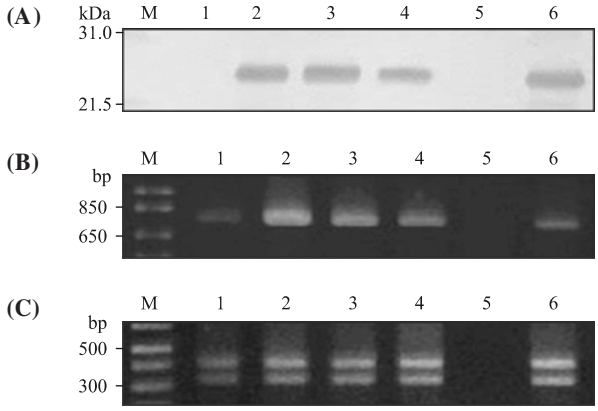

Fig. 7. The infectivity test of pPVXKr and pPVXMo *in vitro* transcripts by western blotting, RT-PCR, and confirmation of PCR with endonuclease (*Bst*XI) digestion. (A) western blot analysis of PVX coat protein immunoprobed with PVX antibody. (B) detection of the coat protein gene of PVX by RT-PCR. (C) restricted PCR fragment by *Bst*XI digestion. M, DNA or protein size marker; 1, inoculated leaf by pPVXKr transcripts; 2, systemic upper leaf inoculated by pPVXKr transcripts; 3, inoculated leaf by pPVXMo transcripts; 4, systemic upper leaf inoculated by pPVXMo transcripts; 5, healthy leaf; 6, systemic upper leaf inoculated by wild-type PVX viral RNA.

strain and host range test of Mo strain seemed to be necessarily recommended also.

Replication stability and infectivity of transcripts from Kr and Mo strain were investigated. Infectious transcripts were produced from full length PCR products of PVX-Kr and PVX-Mo independently. Both transcripts from PCR products caused symptom expression on tobacco plants and *N. benthamiana* plant, and their progeny viruses were detected from systemic leaves stably. Progeny viruses derived from infectious transcripts were efficiently transmitted by sap inoculation on their host plants, and its physical and biochemical properties were the same as wild-type virus. Thus, the resulting transcripts proved to be infectious because virus was detected by RT-PCR or western blotting detection (Fig. 5). Detection of infection in systemic leaves in different tobacco plant inoculated by sap of tobacco plants which were initially inoculated with transcripts, was also indicating virus multiplication in plant was stable through passages. Also virus infection at 10 dpi was detected in inoculated, 1st, 2nd, 3rd, 4th, and 5th systemic leaves above the inoculated leaf (Fig. 6). These transcripts from both strains showed stable replication of virus even in the uppermost leaf, and virus replication in cv. Samsun appeared a bit more than in cv. Xanthi-nc. Transcripts without polyadenylation from full length clones of Kr and Mo strain could not induce any symptom and diagnostic band was not detected in inoculated or systemic leaves (data not shown). To overcome this problem, we treated polyadenylation to transcripts made from both clones right after confirmation of transcript production on agarose gel. These polyadenylated transcripts in their 3'-end of RNA were infectious in tobacco plant, and they could induce typical symptom. Our data seem to be accordant with the previous report by Guilford *et al*. (1991), which 3'-terminal sequences of PVX influenced the infectivity of the viral RNA from white clover mosaic virus. There are supporting evidences with our result about correlation between numbers of adenine and infectivity. According to Chapman *et al*. (1992), PVX containing eight adenines at 3' terminal of RNA showed relatively very low infectivity but containing eighty adenines showed 50% of infectivity compared with viral RNA. Twenty-four adenine-tailed transcripts showed only 5% of infection as Kavanagh and his colleagues reported (1992). Therefore, poly adenine tailing seemed to be essential for clone's infectivity. But cDNA with long 3' adenine tail was thought to be unstable during cloning process in common *Escherichia coli* strain in this study, so we decided to add poly adenine after transcription to avoid cloning problem. We think that this highly infectious transcript system from full-length cDNA for PVX-Kr and -Mo would be useful for recombinant molecules for expression of foreign gene *in planta* as well as for reverse genetics for plant-PVX interaction study.

Acknowledgements

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