Plant Pathol. J. 22(4) : 386-390 (2006)

The Plant Pathology Journal

©The Korean Society of Plant Pathology

Note

Agroinfiltration-based Potato Virus X Replicons to Dissect the Requirements of Viral Infection

Sang-Ho Park and Kook-Hyung Kim*

Department of Agricultural Biotechnology and Center for Plant Molecular Genetics and Breeding Research, Seoul National University, Seoul 151-921, Korea

(Received on November 11, 2006; Accepted on November 20, 2006)

Extensive research of the *Potato virus X* (PVX) has been performed in *in vitro* transcription system using the bacteriophage T7 promoter. We constructed an efficient T-DNA based binary vector, pSNU1, and modified vectors carrying PVX replicons. The suitability of the construct to transiently express PVX RNA using *Agrobacterium tumefaciens* was tested by analysis of infectivity in plants. The expressed PVX RNA was infectous and systemically spread in three plant species including *Nicotiana benthamiana*, *N. tabacum* cv. Xanthi-nc, and *Capsicum annuum* cv. Chilsungcho. The PVX full length construct, pSPVXp31, was caused severe mosaic symptoms on *N. benthamiana*, severe necrotic lesions on *C. annuum* while milder symptoms and delayed mosaic symptoms were appeared on the systemic leaves on *N. tabaccum*. RT-PCR analysis confirmed the presence of PVX RNAs on both inoculated and systemic leaves in all three plant species tested. Our results indicated that PVX replicons were efficiently expressed PVX RNA in at least three tested species. Further investigation will be needed to elucidate the mechanism of PVX replication, translation, movement and assembly/disassembly processes.

Keywords : agroinfiltration, PVX replicon, T-DNA based binary vector

---

Potato virus X (PVX), the type member of the genus *Potexvirus*, is a flexuous rod-shaped virus species containing a 6.4 kb plus-stranded RNA genome (Bercks, 1969; ICTV et al., 2000; Milne, 1988). PVX genomic RNA is capped at 5' end and polyadenylated at 3' end and contains five open reading frames (ORFs). The first ORF encodes the PVX RNA-dependent RNA polymerase (RdRp) required for RNA synthesis. Viral RdRp and host factors form replicase complex during virus infection and synthesizes minus-strand RNA, subsequent genomic (g) and subgenomic (sg) plus-strand RNAs (Buck, 1996; Lai, 1998; Longstaff et al., 1993; Plante et al., 2000). The ORF2, 3 and 4 are partially overlapped and encode the triple gene block (TGB) proteins that are required for virus cell-to-cell movement (Angell et al., 1996; Lough et al., 2000). The ORF5 encodes coat protein (CP) which is required for encapsidation and movement of the virus (Baulcombe et al., 1995; Chapman et al., 1992).

Replication of the PVX viral genome involves the synthesis of minus-strand RNA from a positive-strand and of the genomic and subgenomic plus-stand RNAs. To replicate viral genome successfully in host, the replication process requires various RNA-RNA, RNA-protein, and protein-protein interactions between viral and host factors (Kushner et al., 2003). To date, the molecular analysis of replication, host range and virulence of the PVX have been generally facilitated by infectious transcripts derived from viral cDNA clones. Recent studies have showed that insertion of foreign sequences into the T-DNA of *Agrobacterium tumefaciens* has successfully launched a number of plant DNA and RNA viruses (Annamalai and Rao, 2005; Gopinath et al., 2005; Grimsley et al., 1986; Leiser et al., 1992; Liu and Lomonossoff, 2002; Ratcliff et al., 2001; Rigden et al., 1996; Turpen et al., 1993). To identify the requirements of viral replication, an efficient infection system can be essentially established. We describe here *Agrobacterium*-mediated expression (hereafter referred to as agroinfiltration) system for PVX.

To develop efficient PVX replicons, PVX full length cDNA construct was cloned into the T-DNA region of a modified T-DNA vector, pSNU1, derived from pCAMBIA0390 as described previously (Liu et al., 2002; Shi et al., 1997). pSNU1 binary vector was designed to initiate the transcription by the duplicated CaMV 35S promoter and a self-cleaving ribozyme sequence (Turpen et al., 1993) was included at the 3' end, which generated authentic 3' end of a inserted sequence by cis-preferential cleavage after transcription. In previous study, TMV-based expression vectors containing ribozyme sequence has been shown to increase the infectivity 3-fold (Turpen et al., 1993). Plasmid pSNU1 contains a multiple cloning region of twelve restriction enzyme sites between the 35S promoter and the ribozyme sequence as shown in Fig. 1. PVX

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

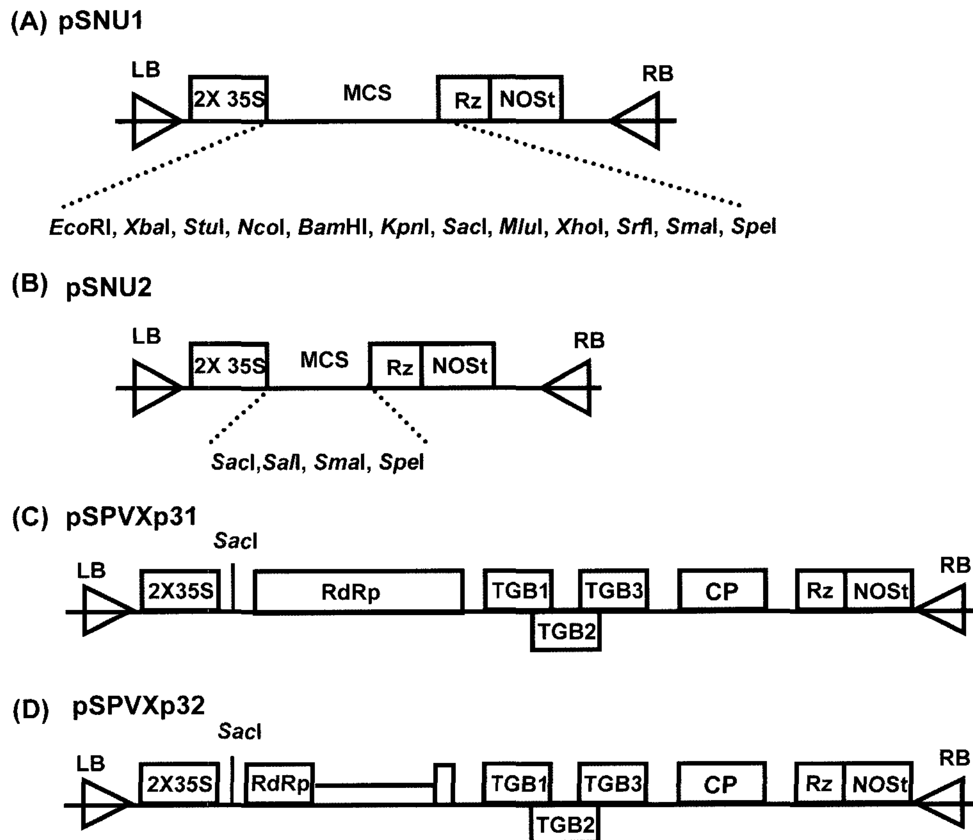

Fig. 1. Schematic representation of PVX cDNA construct for agroinfiltration. PVX cDNA clone in between the duplicated *Cauliflower mosaic virus* (CaMV) 35S promoter (2X35S) and the nopaline synthase terminator (NOSt) in a T-DNA vectors (pSNU1, pSNU2, pSPVXp31, and pSPVXp32). The boxes labeled LB and RB represents the left border and right border of the T-DNA sequence, respectively. Rz, self-cleaving ribozyme. MCS, multiple cloning sites.

full length cDNA construct, p31, containing *Sac*I site at +1 of pMON8453 (Hemenway et al., 1990), was used as the parent plasmid for the construction of pSPVXp31. The plasmid p31 contains extra 6-nucleotide (nt) between T7 promoter and PVX genome. pSNU2, a variant of pSNU1, was constructed by amplifying pSNU1 with a primer prSNU2F (5'-**CGAGCTC***GTCGAC*CCCGGGACTAGT GTCTGTACTTATATCAGTACACTGACGA GTCCCTA-3'; boldface, italics, underlined and rectangle sequence represent *Sac*I, *Sal*I, *Sma*I, and *Spe*I sites, respectively) and a primer prSNU2R (5'-CGAGCTCTTGGGCGTGGTGG-GTTTGGTTTTCCCTCTCCAAATGAAATGAACTTCC TTATATAGAGGAAGGG-3'; *Sac*I site is underlined) The PCR product was digested with *Sac*I and ligated by itself. The resulting plasmid, pSNU2, contains multi cloning sites (MCS) consisting of four restriction sites, *Sac*I, *Sal*I, *Sma*I, and *Spe*I. Plasmid p31 was digested with *Sac*I and *Spe*I and ligated into similarly treated pSNU2 vector to yield pSPVXp31. The construct, p32, was generated by digestion pMON8453 with *Bsi*WI and *Afl*II and the modification implied deletion of most of the replicase gene. Plasmid p32 was digested with *Mfe*I and *Xho*I and ligated into similarly treated pSPVXp31 to yield pSPVXp32. All of the constructs were mobilized into *A. tumefaciens* strain GV2260 by electroporation.

To verify whether PVX mRNA derived from PVX replicons transiently expressed *in vivo* and efficiently infect plants, *Nicotiana benthamiana*, *Nicotinana tabacum* cv. Xanthi-nc, and *Capsicum annuum* cv. Chilsungcho leaves were agroinfiltrated with Agrobacterium culture transformed with pSNU1, pSNU2, pSPVXp31, and pSPVXp32. The agroinfiltration procedure was performed as described previously (Llave et al., 2000). Individual *Agrobacterium* colonies containing desired transformants were grown for 20 h in 2-ml cultures in LB medium with the appropriate antibiotics (50 μg/ml of kanamycin and 50 μg/ml of rifampicin) at 30°C. Ten microliter of the culture was used to inoculate a 10-ml culture LB broth containing the above antibiotics, which was grown for 16-20 h at 30°C. The *Agrobacterium* cultures containing desired transformants were spun down at 3000 rpm for 10 min and resuspended in infiltration medium (10 mM $MgCl_2$/10 mM MES, pH 5.7;

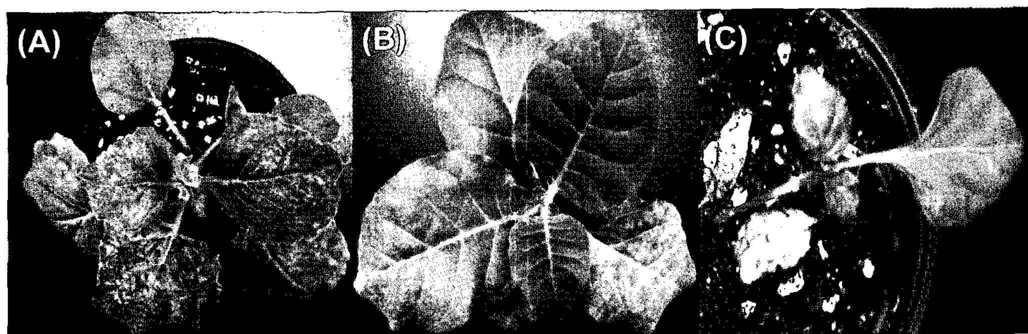

Fig. 2. Symptoms of *Nicotiana benthamiana* (A), *N. tabacum* cv. Xanthi nc (B) and *Capsicum annuum* cv. Chilsungcho (C) plants infected by agroinfiltration with pSPVXp31. Plants were photographed 14 days after agroinfiltration. Severe mosaic symptoms on *N. benthamiana*, mild symptoms in upper leaves on *N. tabacum* cv. Xanthi nc and severe necrotic symptoms on *Capsicum annuum* cv. Chilsungcho were observed.

200 μM acetosyringone) to 0.8 OD at 600 nm and incubated at 30°C for a minimum of 3 h. These cultures were infiltrated into abaxial surface of the *N. benthamiana*, *N. tabaccum* cv. Xanthi nc, and *C. annuum* cv. Chilsungcho leaves (3 to 4 weeks old) by gently pressing using a 1-ml syringe without a needle. Infiltrated plants were grown at 25°C with a 16 h light cycle. After two weeks, infiltration of the clone pSPVXp31 was caused severe mosaic symptoms on *N. benthamiana*, severe necrotic lesion on *C. annuum* cv. Chilsungcho, while milder symptoms and delayed mosaic symptoms were showed on upper leaves of *N. tabaccum* cv. Xanthi (Fig. 2). No symptom was observed on clones pSNU1, pSNU2, and pSPVXp32 infiltrated plants (data not shown).

To determine whether RNA transcripts generated from DNA constructs induced these symptoms, total RNA was extracted from the infected plants and conducted RT-PCR analysis at five days post infiltration (dpi). The PVX viral RNA was detected by amplifying CP region with primers prPVX-CPF (5'-ATGTCAGCACCAGCTAGCACAACA-CAGG-3') and prPVX-CPR (5'-TTATGGTGGT GGGA-GAGTGACAACAGCCTCAG-3'). Amplified DNAs corresponding to the PVX CP region were separated on the 1% agarose gel and visualized with ethidium bromide-staining. As shown in Fig. 3, distinct DNA band was amplified by RT-PCR using total RNAs extracted from upper (systemic) leaves of the clone pSPVXp31 infiltrated plants. In contrast, no DNA was amplified from mock, pSNU1, pSNU2, and pSPVXp32 treated plants (data not shown). The construct pSPVXp32 contained large deletion of replicase gene but contains all known *cis*-acting elements required for PVX replication. Therefore, the construct pSPVXp32 might be used in replication defective control and in *trans*-acting studies of replicase and as vectors for foreign gene expression. An additional restriction enzyme site (*Sac*I) was introduced between duplicated CaMV promoter and PVX genome, which had no effects on PVX

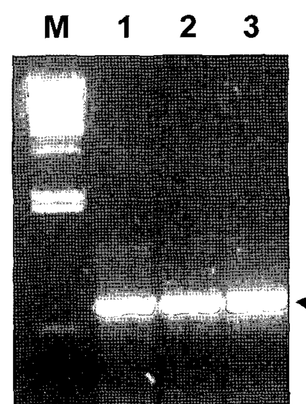

Fig. 3. RT-PCR analysis of RNA from upper leaves of agroinfected plants with pSPVX31 at five days post infiltration (dpi). Lanes 1-3 indicate RT-PCR amplified DNAs using total RNAs from pSPVX31 infected *N. benthamiana*, *N. tabacum* cv. Xanthi nc, and *Capsicum annuum* cv. Chilsungcho, respectively. M denotes lamda molecular mass size markers digested with *Hind*III.

infection. However, an unfavorable effect of six extra nt at the 5' end in transiently expressed PVX mRNA is not excluded. It remains to be determined how the 6 extra nt at the 5' end of pSPVXp31 affects the relative efficiency of replication compared to that of parental PVX replicon.

Agroinfiltration has been widely used in plant biology for identification of disease resistance genes (Bendahmane et al., 2000) as well as induction and suppression of post transcriptional gene silencing (Johansen and Carrington, 2001; Voinnet et al., 2003). Recently, Annamalai and Rao (2005) have reported that an Agrobacterium-mediated T-DNA gene delivery system can be used to study the *cis*- and *trans*-acting requirements for *Brome mosaic virus* (BMV) RNA replication in plants and significant differences can exist for BMV RNA replication in different hosts. These results show that different virus systems are used to analyze the requirements for viral infection.

In this study, we showed that the PVX agroinfiltration system allowed PVX replicon efficiently to infect into local and systemic leaves and to replicate PVX RNA in large quantities in three different plant species including *N. benthamiana*, *N. tabaccum* cv. Xanthi-nc, and *C. annuum* cv. Chilsungcho. The biologically active T-DNA construct harboring PVX full length cDNA, pSPVXp31, can enable us to study the requirements for viral infection. Further studies of PVX replicons derived from pSPVXp31 and pSPVXp32 will be needed to characterize viral and host factors required for replication and other steps during PVX infection.

Acknowledgements

This research was supported in part by grants from the Center for Plant Molecular Genetics and Breeding Research funded by the Ministry of Science and Technology of the Republic of Korea; Research Project from the Rural Development Administration (RDA); and the BioGreen21 Program, RDA. SHP was supported by graduate fellowship from the Ministry of Education through the Brain Korea 21 Project.

References

Angell, S. M., Davies, C. and Baulcombe, D. C. 1996. Cell-to-cell movement of potato virus X is associated with a change in the size-exclusion limit of plasmodesmata in trichome cells of Nicotiana clevelandii. *Virology* 216:197-201.

Annamalai, P. and Rao, A. L. 2005. Replication-independent expression of genome components and capsid protein of brome mosaic virus in planta: a functional role for viral replicase in RNA packaging. *Virology* 338:96-111.

Baulcombe, D. C., Chapman, S. and Santa Cruz, S. 1995. Jellyfish green fluorescent protein as a reporter for virus infections. *Plant J.* 7:1045-1053.

Bendahmane, A., Querci, M., Kanyuka, K. and Baulcombe, D. C. 2000. Agrobacterium transient expression system as a tool for the isolation of disease resistance genes: application to the Rx2 locus in potato. *Plant J.* 21:73-81.

Bercks, R. 1969. The current status of knowledge of potato virus X group. *Zentralbl Bakteriol Parasitenkd Infektionskr Hyg.* 123:204-8.

Buck, K. W. 1996. Comparison of the replication of positive-stranded RNA viruses of plants and animals. *Adv. Virus Res.* 47:159-251.

Chapman, S., Hills, G., Watts, J. and Baulcombe, D. 1992. Mutational analysis of the coat protein gene of potato virus X: effects on virion morphology and viral pathogenicity. *Virology* 191:223-230.

Gopinath, K., Dragnea, B. and Kao, C. 2005. Interaction between Brome mosaic virus proteins and RNAs: effects on RNA replication, protein expression, and RNA stability. *J. Virol.* 79:14222-14234.

Grimsley, N., Hohn, B., Hohn, T. and Walden, R. 1986. "Agroinfection", an alternative route for viral infection of plants by using the Ti plasmid. *Proc. Natl. Acad. Sci. USA* 83:3282-3286.

Hemenway, C., Weiss, J., O'Connell, K. and Tumer, N. E. 1990. Characterization of infectious transcripts from a potato virus X cDNA clone. *Virology* 175:365-371.

International Committee on Taxonomy of Viruses., Van Regenmortel, M. H. V. and International Union of Microbiological Societies. Virology Division. 2000. *Virus taxonomy: classification and nomenclature of viruses: seventh report of the International committee on taxonomy of viruses.* San Diego: Academic Press.

Johansen, L. K. and Carrington, J. C. 2001. Silencing on the spot. Induction and suppression of RNA silencing in the Agrobacterium-mediated transient expression system. *Plant Physiol.* 126:930-938.

Kushner, D. B., Lindenbach, B. D., Grdzelishvili, V. Z., Noueiry, A. O., Paul, S. M. and Ahlquist, P. 2003. Systematic, genome-wide identification of host genes affecting replication of a positive-strand RNA virus. *Proc. Natl. Acad. Sci. USA* 100: 15764-15769.

Lai, M. M. 1998. Cellular factors in the transcription and replication of viral RNA genomes: a parallel to DNA-dependent RNA transcription. *Virology* 244:1-12.

Leiser, R. M., Ziegler-Graff, V., Reutenauer, A., Herrbach, E., Lemaire, O., Guilley, H., Richards, K. and Jonard, G. 1992. Agroinfection as an alternative to insects for infecting plants with beet western yellows luteovirus. *Proc. Natl. Acad. Sci. USA* 89:9136-9140.

Liu, L. and Lomonossoff, G. 2002. Agroinfection as a rapid method for propagating *Cowpea mosaic virus*-based constructs. *J. Virol. Methods* 105:343-348.

Liu, Y., Schiff, M., Marathe, R. and Dinesh-Kumar, S. P. 2002. Tobacco Rar1, EDS1 and NPR1/NIM1 like genes are required for N-mediated resistance to *Tobacco mosaic virus*. *Plant J.* 30:415-429.

Llave, C., Kasschau, K. D. and Carrington, J. C. 2000. Virus-encoded suppressor of posttranscriptional gene silencing targets a maintenance step in the silencing pathway. *Proc. Natl. Acad. Sci. USA* 97:13401-13406.

Longstaff, M., Brigneti, G., Boccard, F., Chapman, S. and Baulcombe, D. 1993. Extreme resistance to potato virus X infection in plants expressing a modified component of the putative viral replicase. *EMBO J.* 12:379-386.

Lough, T. J., Netzler, N. E., Emerson, S. J., Sutherland, P., Carr, F., Beck, D. L., Lucas, W. J. and Forster, R. L. 2000. Cell-to-cell movement of potexviruses: evidence for a ribonucleoprotein complex involving the coat protein and first triple gene block protein. *Mol. Plant Microbe Interact.* 13:962-974.

Milne, R. G. 1988. *The Filamentous plant viruses, The Plant viruses; v. 4.* New York: Plenum Press.

Plante, C. A., Kim, K. H., Pillai-Nair, N., Osman, T. A., Buck, K. W. and Hemenway, C. L. 2000. Soluble, template-dependent extracts from Nicotiana benthamiana plants infected with

potato virus X transcribe both plus- and minus-strand RNA templates. *Virology* 275:444-451.

Ratcliff, F., Martin-Hernandez, A. M. and Baulcombe, D. C. 2001. Technical Advance. Tobacco rattle virus as a vector for analysis of gene function by silencing. *Plant J.* 25:237-245.

Rigden, J. E., Dry, I. B., Krake, L. R. and Rezaian, M. A. 1996. Plant virus DNA replication processes in Agrobacterium: insight into the origins of geminiviruses? *Proc. Natl. Acad. Sci. USA* 93:10280-10284.

Shi, B. J., Ding, S. W. and Symons, R. H. 1997. Plasmid vector for cloning infectious cDNAs from plant RNA viruses: high infectivity of cDNA clones of tomato aspermy cucumovirus. *J. Gen. Virol.* 78:1181-1185.

Turpen, T. H., Turpen, A. M., Weinzettl, N., Kumagai, M. H. and Dawson, W. O. 1993. Transfection of whole plants from wounds inoculated with Agrobacterium tumefaciens containing cDNA of tobacco mosaic virus. *J. Virol. Methods* 42:227-239.

Voinnet, O., Rivas, S., Mestre, P. and Baulcombe, D. 2003. An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. *Plant J.* 33:949-956.