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Characterization and Partial Nucleotide Sequence of *Potato Virus X* Isolated from Potato in Korea

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***Potato virus X* (PVX-KO) showing mild mosaic and stunting symptoms on potato (*Solanum tuberosum*) in Kangwon area has been isolated and characterized. EM observation of the purified virus particles showed flexuous rod shape of about 520 nm in length. The coat protein (CP) of the virus had a molecular weight of 31 kDa in SDS-PAGE analysis, and the viral RNA was approximately 6.4 kb in size in denatured agarose gel electrophoresis. In gel-immunodiffusion tests, it reacted strongly with an antiserum to common PVX from BIOREBA AG (USA). A rabbit antiserum was produced using purified virus and used for routine PVX detection by ELISA. Cultivated potatoes in Kangwon and other areas were frequently infected with PVX-KO. Both *Datura stramonium* and *Nicotiana tabaccum* cultivars developed necrotic local lesions 5 days after inoculation, and systemic mosaic symptoms with vein clearing 2 weeks after inoculation. All the features agree with the description of other PVX strains. To confirm and determine PVX strains, reverse transcription-polymerase chain reaction experiment was conducted using specific primers for viral CP. Amplified DNA fragments were cloned and sequenced. Results showed nucleotide sequence homologies of about 88 to 99% to other PVX strains. Based on CP amino acid sequence deduced from nucleotide sequences and host range studies PVX-KO is considered a member of the type X subgroup of PVX.**

Keywords : PVX strain KO, ELISA, RT-PCR, CP gene, sequence homology.

Potato virus X (PVX), the type member of the potexvirus group, is an important virus infecting many economically important crops including potatoes (*Solanum tuberosum* L.), tobaccos, and peppers. PVX can be found on potato plants in most potato-cultivated areas and frequently occurs together with other potato virus (Smith and Markham, 1945; Dowley, 1973) causing a considerable yield reduction. PVX is easily mechanically transmitted in nature and various strains showing differences in host range and pathogenicity has been described (Jones and Fribourg, 1979; Querci et al., 1995; Salazar and Harrison, 1978). Symptoms caused by PVX are variable and depend mainly on the strain. Most common isolates induce very mild mosaic or latent infection. Other strains cause severe mosaic, crinkling, or even rugosity of leaves (Manzer et al., 1978).

PVX is a flexuous rod-shaped virus containing a 6.4 kb plus-stranded RNA genome (Bercks, 1970; Hiebert and Dougherty, 1988). The PVX is capped at the 5' end and polyadenylated at the 3' end of the genome. PVX encodes five open reading frames (ORFs), as indicated in Fig. 1 (Bercks, 1970; Hiebert and Dougherty, 1988; Huisman et al., 1988). The viral replicase protein (165 kDa) is encoded by ORF1 and is the only viral protein absolutely required for PVX RNA synthesis. ORFs 2-4 encode the triple gene block (TB) that has been shown to be necessary for viral cell-to-cell transport (Angell et al., 1996; Beck et al., 1991). The product of ORF5, coat protein (CP), is involved in both virus movement and encapsidation (Chapman et al., 1992; Oparka et al., 1996).

PVX has the potential to cause serious problems in some potato cultivars and has similar potential threat in Korea. In the past two decades, PVX of potato has occurred in successive potato crops in Korea and caused increasing crop yield losses and massive PVX outbreaks on potato have

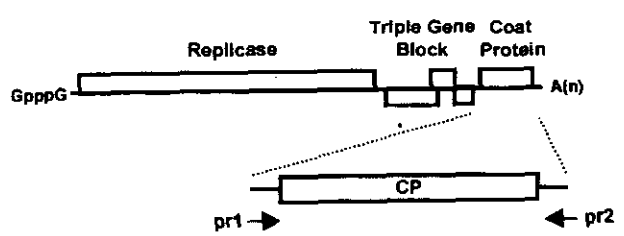

Fig. 1. Genome organization of *Potato virus X* (PVX). The five open reading frames (ORFs) encoded by the PVX genome is denoted by open boxes. Two arrows drown below the coat protein (CP) ORF are labeled as pr1 and pr2, respectively, and represent relative locations of primers used for RT-PCR assay for amplification of PVX specific DNAs containing CP gene.

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occurred throughout Korea in the 1980s (Hahm et al., 1986, 1989). PVX is becoming one of the most important viruses on potato but little is known about the impact of PVX infection in field in Korea. Furthermore, there are only a few reports on PVX incidence (Choi, 1969; Hahm et al., 1986; Lee et al., 1977), and relatively little is known about the PVX strains on potato in Korea. Since each strain can cause strikingly different symptoms on its host plants including potato, it is very important to know which PVX strain is present in the field and to what extent. Stains of PVX have been determined by using different virus properties. For example, they have been classified into four groups according to their serological relationships; into three groups according to their thermal inactivation point; and into four groups according to the reaction of *S. tuberosum* cultivars carrying the hypersensitivity genes *Nx* and *Nb*, and their corresponding alleles (Cockerham, 1955; 1970).

In this paper, we describe the properties of PVX isolated from naturally infected potatoes in Korea. We isolated two different strains of PVX, PVX-KO1 and KO2, that showing differences of symptom development in some host plants. Virus specific polyclonal antibodies, reverse transcription-polymerase chain reaction (RT-PCR) were used to define subgroup of the isolated PVX strains. Our data indicate that PVX-KO1 and KO2 has nucleotide sequence homologies of about 88 to 99% to other PVX strains and is considered a member of the type X subgroup of PVX based on CP, amino acid sequence alignments and host range studies.

Materials and Methods

Virus. Leaf tissues were collected from an infected potato (*S. tuberosum* L) plant in the field located at Kangwon, Korea with symptoms of general crinkling, severe leaf mosaic and necrosis. The tissues were ground in 0.1M phosphate buffer, pH 7.0, and the extract was rubbed onto the leaves of young *Datura stramonium* plants. From systemically infected leaves, an isolate of the virus was obtained by four primary lesion passages in *D. stramonium* followed by propagation in the same host. The infected *D. stramonium* was then maintained in a greenhouse (22±3°C) and served as the inoculum sources.

Host Range. Sap was obtained from systemically infected *D. stramonium* tissues in the presence of 0.1 M phosphate buffer, pH 7.0 and rubbed onto Carborundum (200 mesh)-dusted leaves of 22 plant species and cultivars in 5 families (Table 1). Inoculation to each test plant was repeated two or three times using at least three plants for each inoculation. Symptomless leaves were tested for the presence of the virus by back inoculation to other plants.

Purification. Isolated virus was usually from freshly harvested infected *D. stramonium* tissue, 14 days after inoculation, using the method by Hu et al. (1995). Briefly, tissue was homogenized in extraction buffer (0.2 M sodium phosphate, pH 8.0, 0.2 M sodium sulfite, 0.05 M EDTA), filtered through cheesecloth, and centrifuged at 10,000 g for 10 min. The supernatant was treated with 8.5% butanol and clarified at 10,000 g for 10 min. Virus was precipitated with 8% polyethylene glycol, centrifuged at 10,000 g for 30 min, and resuspended in 0.02 M sodium phosphate buffer and 0.25% sodium sulfite. Further purification was by centrifugation through a 30% sucrose chusion at 150,000 g for 2 hr. After resuspension, the virus was subjected to fractionation on a 10-40% sucrose density gradient at 177,000 g for 3 hr. A single scattering zone was collected and dialyzed overnight against 0.02 M sodium phosphate buffer.

Electron microscopy. Diluted virus preparations were examined by electron microscope (Hitachi 30C) after negative staining with 2% potassium phosphotungstate, pH 6.0. Particle size was deter-

Table 1. Symptom developments on some plant species by inoculation with purified PVX-KO1 and KO2 strains

Plant species	Symptom[a]	
	Inoculated leaves	Systemic leaves
Amaranthaceae		
Gomphrena globosa	NL	–[b]
Chenopodiaceae		
Chenopodium amaranticolor	NS	–
C. quinoa	CS	–
C. murale	–	–
Cucurbitaceae		
Cucumis melo	–	–
C. sativus	–	–
Solanaseae		
Capsicum annum	SN	M
Datura stramonium	NL[c]	M, Mot, VC
Lycopersicon esculentum	–	M, Mot
Nicotiana bentamiana	N	N
N. debneyi	SN	M, N
N. glutinosa	–	M
N. rustica	NL	M, Mot
N. tabaccum cv. Samsun NN	NL[c]	M
N. tabaccum cv. Xanthi-nc	NL[c]	M
N. tabaccum cv. KY-57	NL[c]	M
Prtunia hybrida	–	–
Physalis floridana	N	M, Mot
Leguminosae	–	–
Phaseolus vulgaris	–	–
Pisum sativum	–	–
Vicia faba	–	–

[a] Symbols in the table indicate M, mosaic; Mot, mottle; N, necrosis; NL, necrotic local lesions; VC, vein clearing, –, no infection.

[b] Inoculated and uninoculated systemic leaves not showing symptoms were tested for the presence of the virus by sap inoculation into *D. stramonium*.

[c] PVX-KO1 showed more severe early symptom developments in these plants. Total number of necrotic local lesions were more on PVX-KO1 inoculated plants than PVX-KO2 inoculated.

mined from a total of about 100 particles by examination in an electron microscope at × 20,000 and electron microscope stage micrometer was used to determine the particle length of the purified virus.

CP and RNA analyses. The purified virus was denatured and separated on 12% polyacrylamide gel along with Bio-Rad low molecular weight standard. Gels were visualized by staining with Coomassie blue (Sambrook et al., 1989). Viral RNAs were extracted from the purified virus using total RNA isolation system (Qiagen, Germany). Extracted RNA was denatured by incubation at 65°C for 10 min in formaldehyde-formamide-10× MOPS (morpholinepropanesulfonic acid) buffer (1.6:5:1, v/v/v) followed by electrophoresis in 1.2% agarose in 1 × MOPS buffer for 90 min at 60 V (Sambrook et al., 1989).

Antiserum production and serology. Purified virus preparations were injected weekly into New Zealand white rabbits at multiple sites on the back for 3 consecutive weeks as described (Hu et al., 1995). The first immunization consisted 1 ml of purified virus (1 mg) mixed with 1 ml of Freud's complete adjuvant. The two subsequent injections consisted of purified virus (0.5 mg) mixed with equal volume of Freud's incomplete adjuvant. The rabbits were bled 1 week after third injection and every week thereafter. Immunoglobulin G (IgG) was purified by protein A column chromatography (Goding, 1986). The purified IgG was conjugated to alkaline phosphatase type VII (Sigma, USA) for use in enzyme-linked immunosorbent assay (ELISA) (Clark and Adams, 1977; De Bokx et al., 1980). Heterologous antisera used to test the serological relatedness of isolate to other potato viruses were either produced in our laboratory or were generous gifts from National Institute of Agricultural Science and Technology & National Horticultural Research Institute, RDA, and were used in gel diffusion test.

Infectivity test. For the virulence test of PVX-KO on various potato cultivars, purified virus (3 mg/ml), diluted in 0.1 M phosphate buffer, pH 7.0, containing 0.01 M sodium sulfite, was mechanically inoculated onto virus free leaves of young potato cultivars (Table 2). All inoculated plants were kept in the greenhouse and visually monitored for symptom expression for up to 8 weeks post inoculation. Inoculation to each test plant was repeated three times using at least three plants for each cultivar including a virus free plant (control). Uninoculated systemic leaves were also tested by ELISA.

RT-PCR. First strand cDNA synthesis was carried out in a 20 μl reaction volume using 1st Strand cDNA Synthesis Kit for RT-PCR (Boehringer-Mannheim, Germany) according to the manufacture's instructions with 1 μg purified viral RNAs and 10 μg total RNAs from PVX-infected as well as healthy plant as a template using specific primer complementary to PVX RNA (pr2; 5'-GTAGGCGTCGGTTATGTA-3'). PCR reactions contained template cDNA (from cDNA synthesis reactions above), 20 pmol of amplification primers (pr1; 5'-ATTGATACTCGAAAGATG-3' and pr2; Fig. 1), 200 μM each dNTP, 1.5 mM $MgCl_2$, 50 mM KCl and 10 mM Tris-HCl, pH 8.3, in a 20 μl reaction volume. Template DNA denatured at 94°C for 3 min and then 2.5 units of *Taq* DNA polymerase was added. All PCR amplifications consisted of 35 cycles, each of 45 sec at 94°C for denaturation, 60 sec at 56°C for annealing, and 90 sec at 72°C for synthesis followed by 10 min extension at 72°C.

Table 2. Symptoms on various potato cultivars caused by purified PVX-KO1 and KO2 strain inoculations

Otato cultivars	Symptom	
	Inoculated leaves	Systemic leaves
Solanum tuberosum		
cv. Jopoong	NL[a]	M (0.162/0.167[b])
cv. Athantic	–	M (0.295/0.257)
cv. Superior	–	– (0.189/0.153)
cv. Irish Cobbler	–	M (0.245/0.383)
cv. Dajima	–	M (0.255/0.211)
cv. Gawon	NL	N (0.652/0.340)
Line Daekwan 1-67	NL	N, M (0.204/0.304)
Line Daekwan 1-68	NL	SM (0.729/0.162)
Line Daekwan 1-69	NL	N, SM (0.233/0.235)
Line Daekwan 1-70	NL	– (0.228/0.155)
Line Daekwan 2-1	–	SM (0.145/0.386)
Line Daekwan 2-2	–	M (0.357/0.323)
Line Daekwan 2-5	–	M (0.395/0.162)
Line Daekwan 2-6	–	SM (0.344/0.152)
Line Daekwan 2-7	–	SM (0.273/0.367)

[a]Symbols in the table indicate M, mosaic; SM, severe mosaic; N, necrosis; NL, necrotic local lesions; –, no symptom.
[b]ELISA absorbance values of noninoculated systemic leaves used to detect PVX replications.

Cloning and sequencing of PCR products. DNA fragments generated by PCR amplification were separated by electrophoresis in 1% agarose gels and purified according to the DNA extraction procedure using Agarose Gel DNA extraction kit (Boehringer-Mannheim, Germany) before further manipulation of the DNA. The RT-PCR generated cDNAs were then ligated into TOPO vector (Invitrogen, USA) and transformed into *Escherichia coli* DH5 10F' (Invitrogen, USA). Recombinants were analyzed by digestion with *Eco*RI. The plasmid that contained cDNA inserts of the correct sizes (750 bp for PVX-KO) was selected for nucleotide sequencing. All clones were sequenced in both orientations at least two times. The fragments sequenced using the ABI prismTM Terminator Cycle Sequencing Ready Reaction kit and ABI Prism 377 Genetic Analyzer (Perkin Elmer, USA) according to the manufacture's instructions. Sequence analysis was carried out using the Clustal W algorism of LaserGeneTM program (DNASTAR Inc.)

Results

Host range and symptomatology. The virus, when reinoculated onto several potato cultivars, produced symptoms identical to those observed on the original plant, namely systemic, severe mosaic, and crinkling. The results of

mechanical inoculations on selected indicator plants are summarized in Table 1. PVX-KO1 and KO2 were mechanically transmissible to 14 of the 22 test plants. Both PVX-KO strains caused necrotic spots on *Chenopodium amaranticolor* and *C. quinoa* 7 days post-inoculation. However on *D. stramonium* and *Nicotiana tabaccum* cultivars, PVX-KO1 produced more necrotic local lesions and caused little bit more severe symptoms on systemic leaves than PVX-KO2. Though they showed minor differences in early symptom development in some host plants, systemic mosaic and vein clearing was evident on new leaves of *N. tabaccum* cultivars and typical mosaic, severe vein clearing and mottling symptoms on *D. stramonium* 14 days post-inoculation. This type of symptom is similar to that of other PVX strains and other potexviruses (Bercks, 1970; Hahm et al., 1988; Jones and Fribourg, 1979).

Purification and properties. *D. stramonium* was chosen as the host for virus purification to minimize possible non-specific reactions against potato proteins following antiserum production. The average yield of the purified virus was 10-20 mg/100 g infected leaf tissue. Spectrophotometric analysis of preparations showed absorbance ratios of A260:A280nm of 1.1-1.3. Purified PVX-KO1 and KO2 particles as observed by the electron microscopy, were filamentous, approximately 11 nm in width and ranged in a clear modal length from 480 to 520 nm (Fig. 2A). Purified viral CP migrated as a single band with a molecular weight of 31 kDa in SDS-PAGE (Fig. 2B). The viral RNA was approximately 6.4 kb in size, as estimated by denaturing agarose gel electrophoresis (data not shown). Both experiments were repeated three times.

ELISA and Infectivity tests. The titer of the polyclonal antiserum produced was 1:50,000 in ELISA tests. PVX-KO tested positive in ELISA to the universal PVX common epitope antiserum but was not reacted with antisera to other potato viruses in this study (data now shown). All potato cultivar samples cultivated in Korea were tested for PVX-KO1 and KO2 infection and assayed by DAS-ELISA using PVX-KO1 polyclonal antibody. Both PVX strains produced severe mosaic and necrosis on cv. Gawon, line Daekwan 1-68, and line Daekwan 2 series samples, but not on cv. Superior (Table 2). PVX is easily detected on Potato cv. Superior in common field, but did not cause any symptom after mechanical inoculation. The results showed that there is clear differences in the infectivity of PVX-KO strains in various potato cultivars and that KO1 and KO2 behave similarly in tested potato cultivars despite their minor differences in other host plants as mentioned earlier.

RT-PCR and sequence analysis. PVX specific DNA fragment containing CP gene was successfully amplified by RT-PCR using PVX specific primers, pr1 and pr2. Purified PVX-KO RNA as well as total RNAs from infected plant produced a PCR fragment of the expected size, 750 bp (data not shown). Two independent clones were generated for PVX-KO1 and KO2. The CP gene sequence of PVX-KO1 and KO2 strains were determined and deposited at the GenBank (accession numbers AF260640 and AF260641, respectively). No differences in nucleotide sequences were found between independent clones representing the same strain. Not surprisingly PVX-KO1 and KO2 displayed strong nucleotide sequence and deduced CP gene amino acid sequence homologies, 99.7% and 99.6%, respectively (Fig. 3). Compared to the other PVX strains, CP amino acid sequence was distantly related to the group B1 and group B2 strains (87.7% to 90.3%) while sharing the highest sequence homology with the strains belong to group X (98.3% to 99.6%) (data not shown). The extent of CP sequence differences suggests that PVX-KO1 and KO2 are members of PVX strains belonging to type X. A relationship dendrogram derived from amino acid comparison is shown in Fig. 4. A degree of uncertainty remains regarding the inclusion of PVX-KO1 and KO2 in subgroup X as they

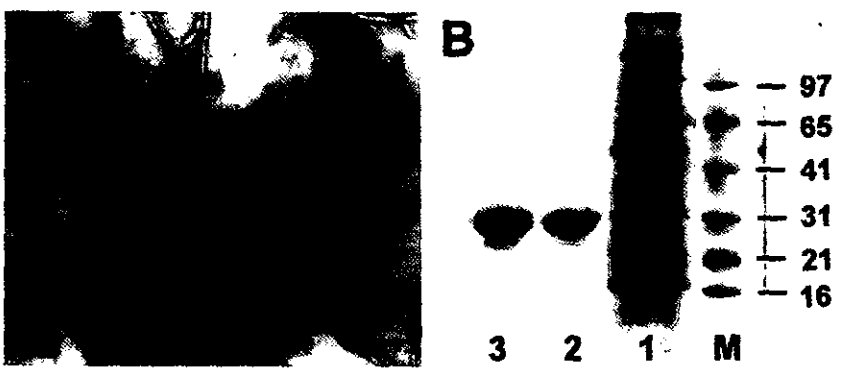

Fig. 2. Electron micrograph (EM) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of PVX strains KO1 and KO2. EM was taken after negatively staining purified PVX-KO particles with 2% phosphotunstate. Bar in (A) represents 200 nm. Total proteins and purified virus were separated on 12% SDS-PAGE (B). Gel was stained with Coomassie blue. Lane 1, Total proteins from PVX-KO infected potato; Lane 2, Purified PVX-KO1; Lane 3, Purified PVX-KO 2; Lane M, protein molecular weight markers.

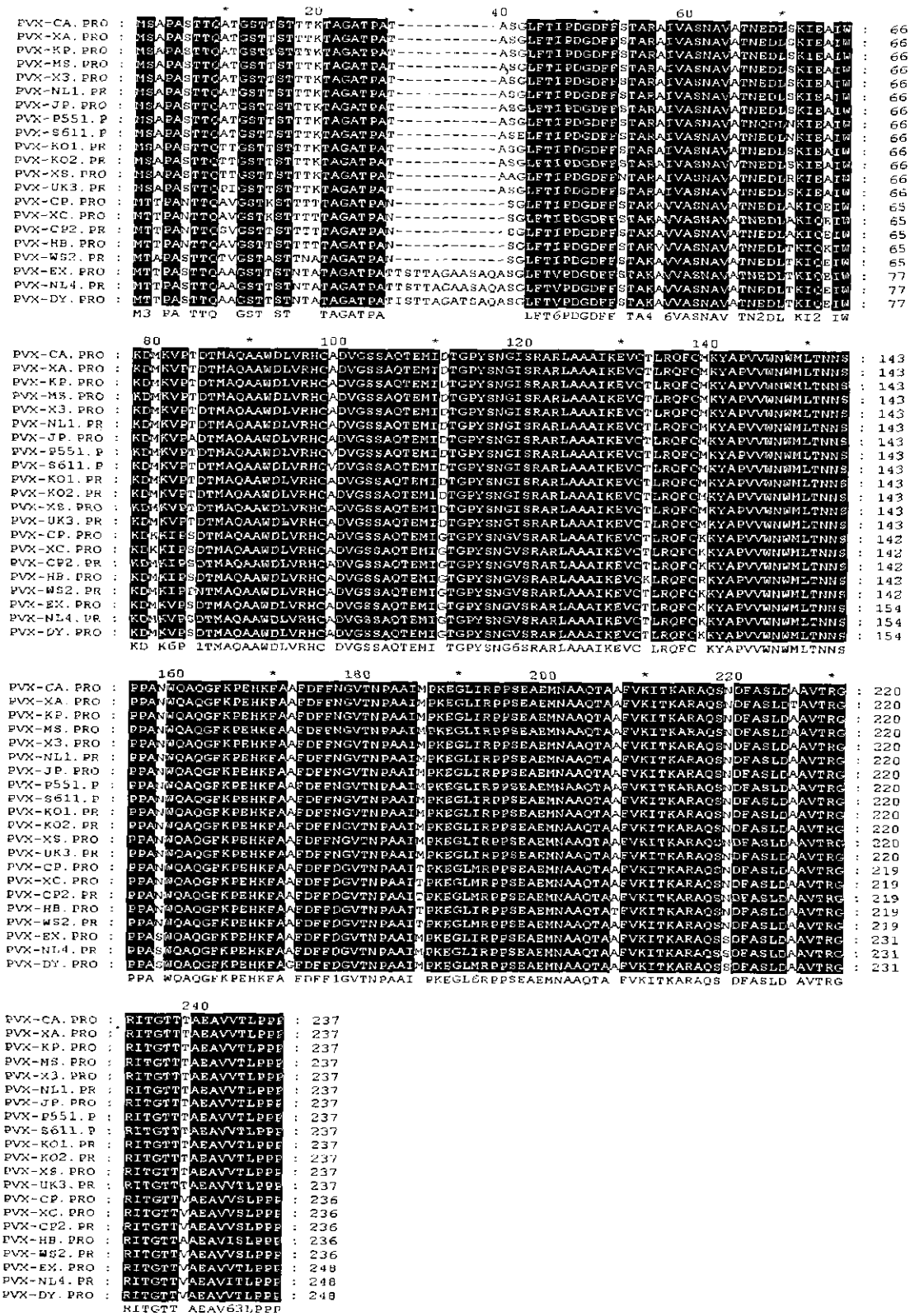

Fig. 3. Alignment of CP sequences between PVX-KO1 and KO2 and other PVX strains. Alignment was generated using the program Clustal W (DNASTAR). Conserved sequences are reverse shaded and indicated at the bottom of each column. References for CP sequences of the PVX strains are as follows: HB, UK3 (Kavanagh et al., 1992), CP, CP2 (Goulden et al., 1993), DX, NL1, NL4, DY, XS, EX, WS2, XA (Santa Cruz and Baulcombe, 1993, 1995), and P551, S6111, JP (GenBank Accession Numbers Z29333, Z29335, and D87692, respectively). CP sequences of strains CA, KP, MS, X3 were identical to strain NL1 and strain XC was identical to strain CP.

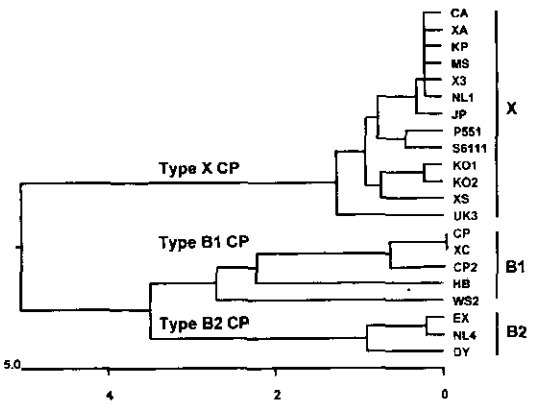

Fig. 4. Relationships between PVX CPs. The degree of relatedness was indicated by horizontal distance. The dendogram was generated by using Clustal W method (DNASTAR). CP subgroups are indicated.

were subgrouped based on sequence homology. To be sure, they need to be inoculated onto potato cultivars carrying different resistance genes.

Discussion

Earliest efforts in identifying viruses were based on its biological and physico-chemical properties such as disease symptoms, transmission characteristics, serology, and EM observation. However, in some host different viruses could cause very similar symptoms while similar strains of one virus could cause very different symptoms (Matthews, 1991). The EM proved to be a valuable tool for routine diagnosis of viruses but viruses within same group or species can be morphologically very related. These difficulties led to the requirement of additional identification tests. Recently, RT-PCR and subsequent sequencing of amplified nucleotide using virus or strain specific primers have been used for detection and strain identification of many viruses (Hadidi et al., 1995; Kim, 1999).

PVX strains have been classified into subgroups based on symptom development in their host plants especially by their ability to overcome resistance genes. Cockerham (1955) divided PVX strains into 4 groups by reaction conferred by two hypersensitive genes, *Nx* and *Nb*, in potato cultivars. Group 1 strains induce a necrotic response on potato cultivars carrying either *Nx* or *Nb*. Group 2 and 3 strains induce hypersensitive response (HR) only on *Nb* and *Nx* potatoes, respectively. Group 4 strains overcome both of these resistance genes. Another resistance gene, *Rx* gene, from *S. andigena* and *S. acaule* was found and confer extreme resistance to all PVX strains with the exception of group 4 strain, coded as PVX-HB (Moreira et al., 1980). A strain showing similar characteristics was also reported (PVX-MS; Tozzini et al., 1994). However grouping based on HR reactions to resistance genes does require precise phenotype transition from an avirulent to a virulent type which is not usually clear and in many times different strains in different groups are not serologically distinguishable in many cases. To overcome this difficulty, Santa Cruz and Baulcombe (1995) inoculated PVX strains to potato cultivars carrying different resistance genes. They suggested all of the strains avirulent on the *Nx* cultivars as type X CP whereas strains overcoming the *Nx* resistance as type B. Data obtained from this study indicate that PVX KO1 and KO2 have type X CP (Fig. 3 & 4). Both PVX-KO1 and KO2 have 98.3 to 99.6% CP amino acid sequence similarity while they has 87.3 to 90.3% similarity to type B CP.

ELISA has been used for routine virus diagnostics in seed potato production. ELISA was also used in this study to see any correlation between symptom development and ELISA value. In general, PVX-KO1 inoculated potatoes showed higher absorbance value in ELISA than that of PVX-KO2 with the exception of several potatoes. However, symptom development and ELISA value between PVX-KO1 and PVX-KO2 on potato lines did not correlate well in some potato cultivars (Table 2). Potato cultivars Superior and Daekwan 1-70 showed relatively high ELISA values though they showed no symptoms on leaves. Hahm et al. (1988, 1989) reported that potato cv. Superior had a tendency to show symptomless disease development, so called mature-plant resistance, during a growing season though it was infected with PVX in field.

Korean stains of PVX showed a high degree of similarity in the symptom development and pathogenicity in potato cultivars with only a few differences limited to a small number of plants. Symptom development on *D. stramonium* and *N. tabaccum* plants inoculated with PVX-KO1 and KO2 showed some differences. PVX-KO1 inoculated plants showed more severe symptoms in early disease development than that of PVX-KO2. When looked at deduced amino acid sequence of CP gene, A54 in PVX-KO1 was substituted with V in KO2 strain. Chapman et al. (1992) showed that PVX CP production and encapsidation is required for efficient accumulation and virus spread. Goulden and Baulcombe (1993) also suggested that a feature of PVX CP, following the interaction with host cell, activates an induced response in the plant cell. Whether this single amino acid substitution between PVX-KO1 and KO2 is responsible for the symptom differences displayed by these two strains remained to be determined.

The importance of the viral CP in symptom development has been shown for several virus-plant combinations (Neeleman et al., 1991). Kavanagh et al. (1992) also showed that the PVX CP gene plays an important role in viral pathogenicity. However, it is worth noting that some other regions of PVX genome as well as CP gene could be

responsible for the biological differences between PVX-KO1 and PVX-KO2. Knowledge of the genetic basis of this differences in the interaction of virus strains and host cells could provide clues to understand the underlying mechanism. Studies are underway to further define the effect of single amino acid substitution in the CP gene of PVX RNA on symptom development of KO1 and KO2 strain.

Acknowledgements

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