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Characterization and Partial Nucleotide Sequence Analysis of *Alfalfa Mosaic Alfamoviruses* Isolated from Potato and Azuki Bean in Korea

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Alfalfa mosaic alfamoviruses (AlMV) were isolated from infected potato (*Solanum tuberosum*) and azuki bean (*Paseolus angularis*) in Korea. Two AlMV isolated from potatoes were named as strain KR (AlMV-KR1 and KR2) and AlMV isolated from azuki bean was named as strain Az (AlMV-Az). Each isolated AlMV strain was characterized by using their host ranges, symptom developments, serological relations and nucleotide sequence analysis of coat protein (CP) gene. Strains KR1, KR2, and Az were readily transmitted to 20 of 22 inoculated plant species including bean, cowpea, tomato, tobacco, and potato. AlMV-KR1 and KR2 produced the typical symptoms like chlorotic or necrotic spots in *Chenopodium quinoa* and *Solanum tuberosum* cv. Superior. AlMV-Az caused bright yellow mosaic symptom and leaf malformation in *Nicotiana glauca*, which were different from the common mosaic symptom caused by AlMV-KR1 and KR2. Electron microscope observation of purified virus showed bacilliform virions containing a single-stranded plus-strand RNAs of 3.6, 2.6, 2.0 and 0.9 kbp in length, respectively, similar in size and appearance to those of *Alfamovirus*. In SDS-PAGE, the coat protein of the two viruses formed a consistent band that estimated to be about 24 kDa. The CP genes of the AlMV strains, KR1, KR2, and Az have been amplified by RT-PCR using the specific primers designed to amplify CP gene from viral RNA-3, cloned and sequenced. Computer aided analysis of the amplified cDNA fragment sequence revealed the presence of a single open reading frame capable of encoding 221 amino acids. The nucleotide and peptide sequence of viral CP gene showed that strain KR1, KR2, and Az shared highest nucleotide sequence identities with AlMV strain 425-M at 97.7%, 98.2%, and 97.2%, respectively. CP gene sequences of two strains were almost identical compared with each other. Altogether, physical, serological, biological and molecular properties of the purified virus isolated from potato and azuki bean established its identity as an AlMV.

Keywords : AlMV strain, potato, azuki bean, virus characteristics, dsRNA, CP gene, sequence homology.

Ever since its first report (Weimer, 1931), *Alfalfa mosaic virus* (AlMV) has been found in most countries infecting many plants. The virus is easily transmitted by sap inoculation and by several aphid species in a non-persistent manner to some 430 plant species in 51 dicotyledonous families including solanaceous crops such as pepper (*Capsicum* spp.), potato (*Solanum* spp.), and tobacco (*Nicotiana* spp.), where it can cause extensive yield losses and economic damage (Jaspars and Bos, 1980; van Regenmortel and Pinck, 1981). AlMV is one of the most biologically variable plant viruses and numerous natural variants having different pathogenicity have been reported (Crill et al., 1970; Paliwal, 1982; Walter et al., 1985; Hiruki and Miczynski, 1987; Hajimorad & Francki, 1988). Strains that have been studied in most detail are the Leiden and Madison isolates of strain 425 (425L and 425M) (Hagedorn and Hanson, 1963), the Strasbourg isolate (S) (Walter et al., 1985), the 15/64 strain (Hull, 1970) and the VRU strain (Hull, 1970).

AlMV, the type member of the *Alfamovirus* group, is a virus with a multi-components single-stranded positive-strand RNA genome consisting of three RNA species and a fourth subgenomic RNA (sgRNA). AlMV RNA is encapsidated with a single species of coat protein of MW 24,252 (van Beynum et al., 1977) to form bacilliform particles of the same width but differing in length depending on the RNA capsidated (Hull et al., 1969; van Regenmortel & Pinck, 1981). The complete nucleotide sequence of the AlMV RNAs has been determined (Barker et al., 1983; Cornelissen et al., 1983a, b). RNA-1 and RNA-2 contain single open reading frame (ORF) encoding the viral replicase subunits (P1 and P2, respectively). RNA-3 contains two ORFs encoding the movement protein and the coat

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protein (CP) (Taschncr et al., 1991). CP is expressed from a sgRNA.

Potato yellowing virus showing similar characteristics with AlMV in host range, particle size and shape has recently been reported (Fuentes and Jayasinghe, 1993) and been found in many crops worldwide, but it was believed to be uncommon in potato field (Colin, 1998). Though AlMV is now considered as a minor disease agent in potato, it appears to be common in native potato (*S. tuberosum*) cultivars (Salazar, 1996). AlMV causes calico or mild mottling in potato leaves and tubers, and vein necrosis in some cultivars (Oswald, 1950). In 1999, bright yellow mosaic symptoms known as calico (Smith, 1972) were observed in several potato cultivars in experimental fields of National Alpine Agricultural Experiment Station. In Korea, Lee (1981) first described the infection of AlMV from potato using host range, electron microscope (EM) observation and serological assay. Since the isolation and characterization of AlMV virus particles were not fully described, here we report the characteristics of AlMV isolated from a field-grown potato and azuki bean in Kangwon area. Although there are many available nucleotide sequences for AlMV strains, it would be interesting to know if AlMV nucleotide sequences show any sequence and/or structural differences compared to other reported strains. To obtain information on nucleotide as well as deduced CP gene sequences and to see whether they have differences, we determined the nucleotide sequence of the CP gene from cloned cDNA and showed similarity with other AlMV strains. Based on symptom developments, physical and molecular characteristics of the purified virion, and the partial nucleotide sequence of the AlMV RNA-3, here we report three strains of AlMV, KR1, KR2, and Az, isolated from native potato and azuki bean in field. We also present that biological, serological and molecular properties of each AlMV strains that can be used to identify different pathological characteristics.

Materials and Methods

Virus sources and propagation. Leaves from a field-grown AlMV-KR infected potato (*S. tuberosum* cv. Dejima) with calico yellowing symptom and light stunting (Fig. 1A & 1B) and AlMV-Az obtained from the naturally infected azuki bean plants (Fig. 1C), were collected from various areas in Kangwon province. These infected leaves were mechanically transmitted by macerating in 0.01M phosphate buffer (pH 7.0) to expanded leaves of *Chenopodium amaranticolor*, and single local lesions were taken from this leaves. Each isolate from the single local lesions was then propagated in *Nicotiana glutinosa*. AlMV-PV92, representatives of common strain, as supplied from ATCC and used as a control virus. Purified virus strains were served as an inoculum for host range tests and subsequent analysis.

Purification and host range study. Virus was purified from leaf tissues systemically infected *N. tabacum* cv. Xanthi-nc and *N. glutinosa*, 7-14 days post-inoculation according to Hajimorad and Francki (1991b). Viruses were purified by differential and sucrose cushion centrifugation and were fixed by the addition of glutaraldehyde to 0.25% (v/v) when required. Briefly, systemically infected leaves were homogenized in 2 volumes (w/v) of extraction buffer (0.2 M Na_2HPO_4; 0.3% 2-mercaptoethanol; 0.01 M EDTA, pH 7.6), and clarified by 10% cold mercaptoethanol; 0.01 M EDTA, pH 7.6), and clarified by 10% cold chloroform and butanol (1 : 1=v/v). After polyethylene-glycol (PEG, MW 6000, 8% w/v) precipitation, the sediments were resuspended in a buffer containing 0.02 M Na_2HPO_4, pH 7.6 and 1 mM EDTA followed by centrifugation through a 30% sucrose cushion for 2 h at 30000 rpm at 4°C in a RP50T rotor (Hitachi-SCP55H, Japan). The viruses were further purified by sucrose density gradient centrifugation at 28000 rpm for 2 h in a swinging-bucket 28.1 rotor (Takanami, 1981). The resulting virus solution was centrifuged at 30000 rpm for 1.5 h at 4°C in a RP50T rotor. The virus pellet was then resuspended in 0.5 mL TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) and stored at -70°C. Virus concentration was estimated using an assumed extinction coefficient of 4.9 (van Vloten-Doting and Jaspars, 1973).

Host range study was performed using the inoculated leaf

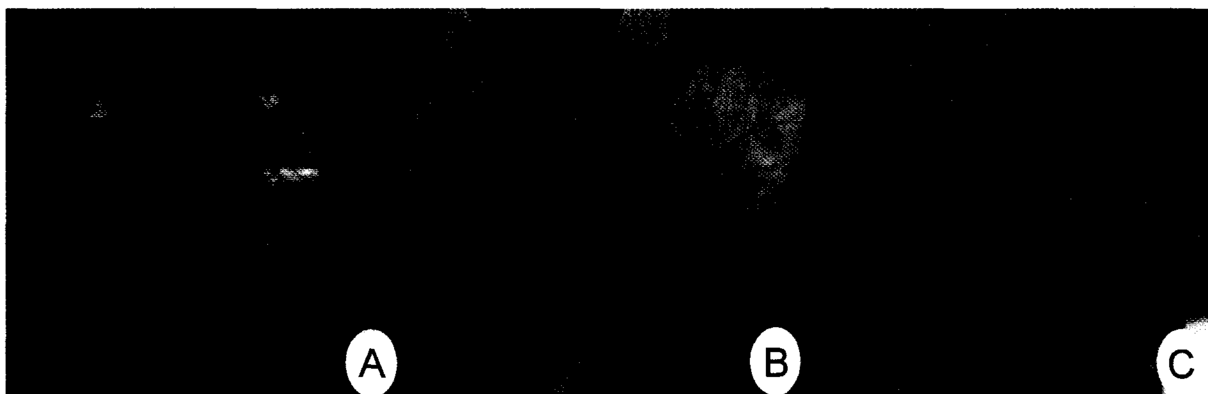

Fig. 1. Potato and azuki bean plants naturally infected with the AMV-KR1 (A), KR2 (B) and Az (C). Symptoms were calico yellow symptom on potato leaves and an interveinal chlorosis on azuki bean leaves, respectively.

extracts of *N. glauca*. Each leaf extract ground in extraction buffer (0.01 M Na_2HPO_4, pH 7.2; 0.01 M sodium sulfite; 1 mM EDTA) was inoculated onto *Capsicum annum, Chenopodium amaranticolor, C. quinoa, Datura stramonium, Gomphrena globosa, N. benthamiana, N. glauca, N. rustica, N. tabacum* cv. Xanthi-nc, *Phaseolus angularis, Vigna sinensis* and *Solanum tuberosum*. Each inoculation was repeated at least times in three test plants. All tested plants were kept in a greenhouse and monitored for symptom expression.

Electron microscopy. A small drop of 2% potassium phosphotungstate (PTA), pH 6.0, was placed on a carbon-coated Fomvar grid and a piece of infected leaf tissue of Xanthi-nc tobacco and/or *N. glutinosa* about 2 mm in width was dipped in the stain several times. Excess stain was removed by touching the edge of the grid with a filter paper. The purified virus preparations fixed with a drop of a 2% glutaraldehyde was placed on carbon-coated Formvar grids, stained 2% PTA.

Analysis of genomic RNA and CP. Genomic RNAs were extracted from purified viruses by SDS-phenol-chloroform treatment followed by ethanol precipitation (Peden and Symons, 1973). After concentration by ethanol precipitation, the RNAs were resuspended in sterile TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.3) and kept at -70°C until required. RNAs were denatured by incubation at 70°C for 5 min in formaldehyde-formamide-10x MOPS (Sambrook et al., 1989). The analysis of RNAs under denatured gel conditions was done in submerged 1.5% agarose gels buffered in 1x MOPS buffer containing 0.5 mg/mL ethidium bromide.

For CP analysis, 2 μg of purified virus in 5 μl was placed in an equal volume of 2x Lammli buffer and boiled for 5 min. The denatured virus proteins were then electrophoresed through 12.5% polyacrylamide gels containing SDS (Lammli, 1970). Protein bands were stained with Coomassie blue R-250 (0.3% in 50% methanol/10% acetic acid) for several hours, followed by destaining in 30% methanol/10% acetic acid.

DsRNA analysis. Double stranded RNAs (dsRNAs) were purified from 7 g of leaf tissues of *N. glauca* infected with the viruses by the procedure of Morris and Dodds (1979). dsRNAs were analysed by electrophoresis through 6% polyacrylamide gel containing 1x TAE (40 mM Tris, 40 mM acetic acid, 2 mM EDTA, pH 7.8). The gel was stained with ethidium bromide and visualized under UV illumination.

Antiserum production and serology. All antisera were prepared in rabbits using glutaraldehyde-fixed AlMV-KR and AlMV-Az. Each virus isolate (1 mg) was emulsified in 0.5 mL of complete Freund's Adjuvant (CFA; Sigma, USA) and injected intramuscularly into rabbits on day one. One mg of AlMV isolates emulsified in 0.5 mL of incomplete FA (ICFA) was administered subcutaneously on day 14 and 28. Bleeding was begun at week three. Immunoglobulins were precipitated with ammonium sulfate, purified by protein An affinity column chromatography, and conjugated to alkaline phosphatase (Type VII-NA, Sigma Co., USA).

Double immunodiffusion tests in agar gel were used to determine titers of the antisera, and to establish the relationship of the isolates with antiserum to an AlMV strain (ATCC PV92). Diffusion gel contained 1% (w/v) agarose in 0.01 M phosphate buffer saline (PBS), pH 7.0. Leaf extracts in PBS from *N. glutinosa* inoculated with the AlMV isolates were used as antigens. Precipitin lines were observed after incubation for 12 hr at room temperature.

Enzymelinked imminosorbent assay (ELISA) methods employing the direct double-antibody sandwich method described by Clark and Adams (1977), were used. In ELISA tests, the crude antigen and antiserum were diluted 1 : 20 and 1 : 1000 in coating buffer, respectively. The enzyme conjugated antibodies were diluted 1 : 1000.

RT-PCR. PCR primers for AlMV were designed based on the sequence of the AlMV strain 425 Madison RNA-3 assembled from data in GenBank accession number K02703 (Barker et al., 1983). Down stream primer (AlMV-3pr:5'-GCATCCCTAGGGGCATTCATGCA-3') is complementary of nucleotides 2014 to 2037 of RNA-3, while the upstream primer (AlMV-5pr: 5'-ATCATTGATCGGTAATGGGCCGTT-3') is located between nucleotides 1136 and 1159, giving an expected amplification product of 901 bp. The primers are corresponding to intergenic and 3' non-coding regions covering full-length of coat protein (CP) gene on RNA-3.

RT-PCR reaction was performed as described previously (Koper-Zwarthoff et al., 1980; Neeleman et al., 1991; Vossen et al., 1995). The RNAs extracted from infected plant tissues were denatured at 70°C for 5 min and amplified with 0.5 units of Avian myeloblastosis virus reverse transcriptase (Promega), 2.5 units of *Taq* DNA polymerase (Promega), 0.2 mM dNTP and 0.3 μM of each primer per 100 μL reaction. RT-PCR was carried out with one cycle of RT reaction at 42°C for 45 min and 35 cycles of PCR amplification using the step program (94°C, 30 sec; 40°C, 1 min; 72°C, 1 min) followed by a 7 min final extension at 72°C. Ten mL of the amplified DNA fragments were separated by electrophoresis on a 1.2% agarose gel in TAE buffer and stained with ethidium bromide.

Cloning and sequencing. Cloning of PCR products and nucleotide sequencing were performed as described previously (Jung et al., 2000; Sambrook et al., 1989). PCR products of CP gene (4 μL), was ligated into the T-tailed vector pGEM-T (Promega) overnight at 16°C and/or TOPO vector (Invitrogen), and transformed into *E. coli* JM109 (Hanahan, 1985) and/or TOPO 10F cell (Invitrogen) according to the manufacture's instructions. Putative transformants were screened by digesting recombinant plasmids with *Sph*I, *Not*I and *Eco*RI, and electrophoresing in 1.2% agarose gels. The plasmid that contained cDNA inserts of the correct sizes (901 bp for AlMV-KR and Az) was selected for nucleotide sequencing (Sanger et al., 1980). All clones were sequenced using ABI prism™ Terminator Cycle Sequencing Ready Reaction Kit and ABI Prism 377 Genetic Analyzer (Perkin Elmer) according to the manufactures instructions. The nucleotide sequences of CP genes of the AlMV isolates were analyzed using the Clustal W algorism of LaserGene™ Program (DNASTAR Inc., USA) and compared with published data using programs of the DNA sequence analysis computer packages for PC (DNASTAR Inc., USA). The published AlMV sequences used in this study had the following GenBank accession numbers: L00162 (strain 425L) (Koper-Zwarthoff et al., 1980); K02703 (strain 425M) (Barker et al., 1983);

X00819 (strain S) (Ravelonandro et al., 1984); AF015716 (strain VRU) (Thole et al., 1998); AF015717 (strain15/64) (Thole et al., 1998).

RNA secondary structure predictions. Models for the secondary structure for the 3' NTR 179 nucleotides of AlMV-KR1 and KR2 RNA-3 were obtained with the Mfold program (Mathews et al., 1999; Zuker, 1989; Zuker et al., 1999) on Micheal Zukers internet location (http://mfold.wustl.edu/~folder/rna/form 1.cgi).

Results

Natural symptomatology of AlMV infection in potato and azuki bean. Natural infection of AlMV was confirmed by EM observation, serological tests, and RT-PCR indexing in the experimental fields for potato and azuki bean at National Alpine Agricultural Experiment Station and Kangwon Agriculture Technology Institute in 1998 and 1999 growing season, respectively. Symptoms in potatoes mainly induced bright yellow mosaic symptom known as calico (Fig. 1A & 1B) while causing severe mosaic leading to leaf desiccation to yellow mosaic or interveinal chlorosis in azuki bean (Fig. 1C).

Host range studies. Characteristic development symptoms of AlMV observed on potato, azuki bean and on selected indicator plants are summerized in Table 1. All strains of AlMV caused a few different symptoms on test plants (see Table 1). AlMV-KR1 was induced only local symptoms on *C. amaranticolor* and *G. globosa*. On the other hand, AlMV-KR2, Az, and PV92 were infected systemically.

Table 1. Reactions of indicator plants to mechanical inoculation of the AlMV strains

Host Plants[a]	Symptoms[b]			
	-KR1	-KR2	-Az	-PV92
Capsicum annum	MM/SM[c]	M/YM	M/YM	M/YM
Chenopodium amaranticolor	L/–	L/M	L/M	L/M
C. quinoa	L/–	–/SM	–/MM	–/MM
Datura stramonium	–/SM	–/M	–/M	–/M
Gomphrena globosa	L/–	L/YM	–/YM	L/YM
Nicotiana benthamiana	–/MM	–/M	–/M	–/M
N. glauca	–/MM	–/MM	–/YM	–/MM
N. rustica	L/SM	–/M	–/M	–/MM
N. tabacum cv. Xanthi-nc	L/SM	–/M	–/M	–/M
Phaseolus angularis	M/YM	M/YM	M/YM	M/YM
Vigna sinensis	L/MM	L/MM	L/MM	L/MM
Solanum tuberosum	–/YM, NS, N	–/M, NS	–/YM	–/MM

[a] Test plants were mechanically inoculated with AlMV strains.
[b] L, local lesions; M, mosaic; MM, mild mosaic; N, necrosis; NS, necrotic spots; SM, severe mosaic; YM, yellow mosaic; –, no infection.
[c] symptoms on inoculated leaves/upper of test plants

Necrotic spots of inoculated *S. tuberosum* cv. Superior leaves became calico type yellowing, necrosis and finally died. In general, more severe symptoms were induced by KR strains than that of Az in following plants; *C. quinoa*, *N. tabacum* cv. Xanthi-nc, *N. rustica*, *D. stramonium*, and *S. tuberosum* (See Table 1). Through symptoms were greatly influenced by environmental conditions, clear differences in symptoms between isolates of AlMV were obtained. The most characteristic symptom produced by AlMV-KR was severe yellow mosaic accompanying chlorotic and/or necrotic spots in *C. quinoa* and *S. tuberosum* cv. Superior. In contrast, AlMV-Az caused bright yellow mosaic and leaf malformation in *N. glauca* that are differed with common mosaic symptoms induced by the other two. Symptoms as well as systemic responses were also characteristic for some other *Alfamovirus* (Black and Price, 1940; Oswald, 1969; Valkonen et al., 1992).

Virus characteristics. The purified virus had an absorption spectrum typical of viral nucleoprotein. The $A_{260/280}$ value for the purified virus was 1.8. Absorbance at 260 nm (1 mg/mL) was 5.2 (Weimer, 1934; Jaspars and Boss, 1980). The yield of the virus was approximately 4-5 mg per 100 g of fresh leaves and the ELISA activity was A_{405}=1.29 after incubation of 1 μg of the virus substrate for 1h. Purified preparations of the both AlMV isolates were infectious and appeared to be relatively free from contamination as evidenced by EM observation (Fig. 2A). EM observation of AlMV-KR infected tobacco Xanthi-nc leaves revealed bacilliform particles in the sap. Most of particles were 30 to 60 nm long and approximately 23 nm wide. Purified virus preparations contain 4 types of the particles, B, M, Tb and Ta, containing seperately encapsidated ssRNAs 1 to 4, respectively (Hull, 1969; Kudela and Gallo, 1995). The diameters of 100 virions were measured; 20 were 30 nm, 42

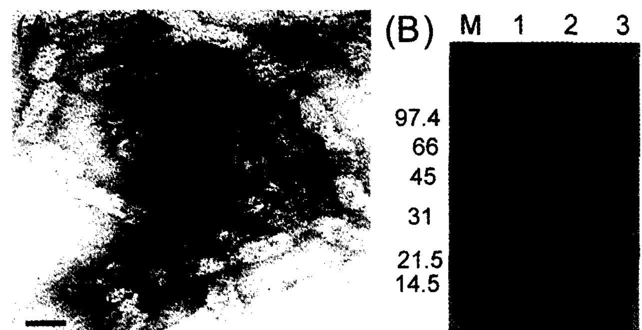

Fig. 2. Electron micrograph of purified particles of AlMV isolates (A) and SDS-PAGE of the coat proteins of AlMV isolates (B). The virus particles negatively stained with 2% PTA. Bar indicates approximately 30 nm. Electrophoresis was performed in 12.5% polyacrylamide gels using the Laemmli system. Lane M: BioRad low molecular weight protein size standards; lane 1: AlMV-KR; lane 2: AlMV-Az; lane 3: AlMV-PV92.

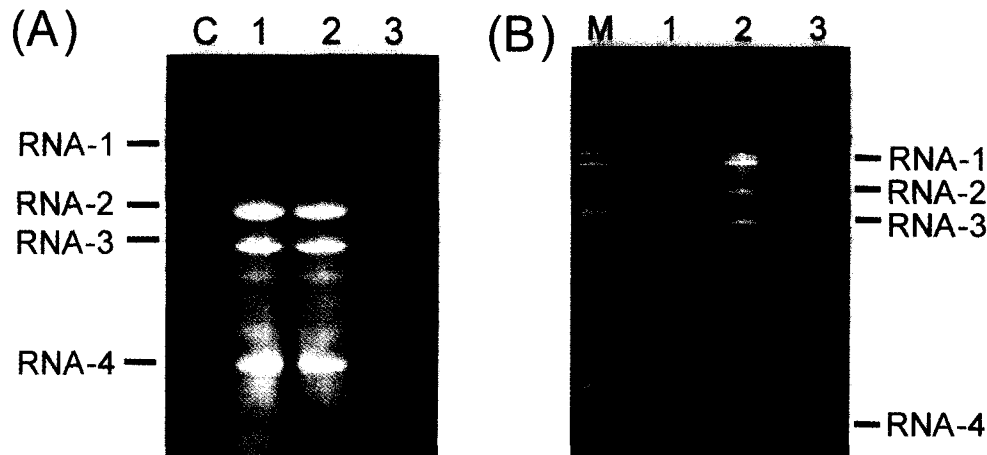

Fig. 3. Agarose gel electrophoresis of RNA species from purified virions of the AlMV isolates (A) and double stranded RNA (dsRNA) profiles of the AMV isolates extracted from infected *Nicotiana glauca* plants (B). Electrophoresis was performed in 1.2% agarose gels (A) and 6% polyacrylamide gels (B). Lane C; genomic RNA of cucumber mosaic virus (CMV), lane M: dsRNA of CMV as a size marker; lane 1: AlMV-KR; lane 2: AlMV-Az; lane 3: AlMV-PV92.

were 30-40 nm, and 38 were 60 nm. The purified virions of AlMV were found to encapsidate four species of RNAs of approximate size of 3.6, 2.6, 2.0 and 0.9 kb, respectively and co-migrated with RNAs 1-4 of AlMV-PV92 common strain obtained from ATCC in agarose gel (Fig. 3A). The thermal inactivation point (TIP) of AlMV isolates is observed to be 60°C and the dilution end point (DEP) was 10^{-4}. The longevity *in vitro* was a week at 20°C.

DsRNA analysis. The sizes and numbers of the dsRNA species produced during infection of the isolates of AlMV in *N. glutinosa* compared to that of Cucumber mosaic virus strain Mf (CMV-Mf) used as a size marker are shown in Fig. 3B. All isolates revealed four dsRNA species with estimated molecular sizes of 3.6, 2.6, 2.0 and 0.9 kbp and the mobility of the species of dsRNA was similar for all isolates.

Serology and field sample test. Crude sap extracted from *N. glutinosa* infected with AlMV isolates was tested by double immunodiffusion test in agar gel against to AlMV-PV92 antiserum from ATCC. All antigens of the isolates reacted positively with the antiserum, giving a single precipitin line closely fused. Preliminary serological studies demonstrated that antisera prepared against purified preparations of both AlMV-KR and Az formed infected plants. The antisera to each virus had titers of 1/128 to 1/64 in Ouchterlony gel diffusion tests with sonicated virus and 1/2048 to 1/1024 in microprecipitin serology tests (data not shown). ELISA tests demonstrated that the antisera of AlMV isolates reacted with purified viruses and gave a positive reaction to AlMV infected tissue from potato, pepper, tomato, cowpea and a number of tobacco species and did not produce non-specific reaction in various serological tests (Bancroft et al., 1960).

RT-PCR and sequence analysis. The nucleotide sequence of the AlMV RNA was not described previously in Korea. To determine whether the AlMV-KR and Az belong to the common type AlMV strain or whether it is a new strain of AlMV, we utilized RT-PCR assay. RT-PCR performed with AlMV-5pr and AlMV-3pr primers resulted in a product of 901 bp containing CP gene and 3' NTR of RNA-3 as expected (data not shown). No amplification products were seen when total RNAs from healthy plants were used.

The nucleotide sequence containing CP gene and 3 NTR of AlMV-KR were obtained and submitted to GenBank under the accession number AF294432 and AF294433, respectively. No differences in nucleotide sequences were found between independent clones representing the same strain. Of the amplified 901 bp cDNA, computer-aided analysis of the 666 bp sequence revealed the presence of CP open reading frame capable of encoding 221 amino acids. Compared to the other AlMV strains, CP amino acid sequences of KR1, KR2, and Az were distantly related to the strains 15-64, VRU, and NZ2 (91.9% to 95.0%) while sharing the highest sequence homology with the strain 425-M (97.7% to 98.2%) (Fig. 4, Table 2). The alignments of sequences demonstrate that isolated strains are similar to strains of 425M, N, and S suggesting that closer relationships exist between these viruses than them of the other strains. Comparison of the CP amino acid sequences deduced from the nucleotide sequences of the three isolates and eight reference strains of AlMV revealed that among several amino acid variations one non-conservative alterations, Gln at position 133, are found exclusively in among isolate CPs (Fig. 4). Several additional non-conservative amino acid changes are also observed including Ile at position 182 in strains KR1 and KR2, and His, D, and Y at posi-

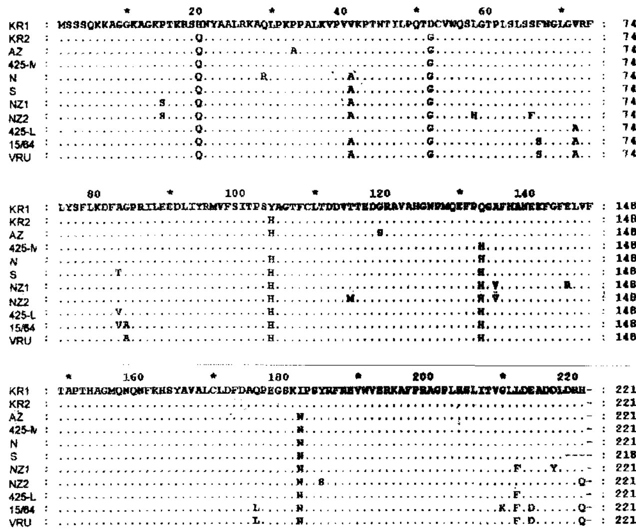

Fig. 4. Aligment of the AlMV CP gene sequences among AlMV-KR1, KR2, Az, 425-M (GenBank accession no. K02703; Barker et al., 1983), 425-L (GenBank accession no. L00162; Brederode et al., 1980; Koper-Zwarthoff et al., 1979), NZ1 (GenBank accession no. U12509), NZ2 (GenBank accession no. U12510), VRU and 15/64 (GenBank accession nos. AF015716 and AF 015717, respectively; Thole et al., 1998), N (GenBank accession no. M59241, Neeleman et al., 1991), and S (GenBank accession no. X00819; Ravelonandro et al., 1984).

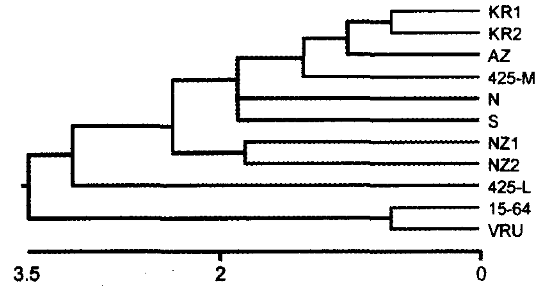

Fig. 5. Relationships of CP gene sequences between isolated AlMV strains and the other AlMV strains. The degree of relatedness was indicated by horizontal distance. The dendrogram was constructed from aligned CP gene sequences using Clustal W method (DNASTAR).

Table 2. Homology of the CP gene amino acid sequence among AlMV isolates and corresponding regions of other AlMV strains

Strains	CP gene sequence		
	KR1	KR2	Az
KR1		98.2	98.6
KR2	98.2		98.3
Az	98.2	98.6	
425-M	97.7	98.2	98.2
N	94.6	97.7	97.7
S	96.4	97.7	97.7
NZ1	94.6	95.9	95.9
NZ2	93.7	95.0	95.0
425-L	95.5	96.8	96.8
15-64	91.9	93.2	93.2
VRU	92.3	93.7	93.7

tion 20, 52, and 104, respectively, in strain KR1. Phylogenetic analysis of both nucleotide and predicted amino acid sequences indicated that the virus was a closely related to generic *Alfamovirus*. A relationship dendrogram derived from CP amino acid sequence comparison is shown in Fig. 5. When we align 3 NTR sequence of AlMV-KR, similar results were obtained as we align CP gene (data not shown). The 3 NTR sequence showed 99.4% to 100% sequence homology with the strain 425-M while showing 94.3% to 97.8% similarities with the strains 15-64, VRU, and NZ2 (data not shown). In general, sequences in the 3' NTR were more conserved among reported strains.

Discussion

AlMV is distributed world-wide and causes diseases of many economically important crops including the families *Solanaceae* and *Leguminosae* (Crill et al., 1970; Smith, 1972; Hiruki and Miczynski, 1987), but AlMV was considered as economically less important potato virus (Salazar, 1996; Colin, 1998). Three virus strains isolated from potatoes and azuki bean are closely related with an AlMV strain PV92 that used as control virus. A close relationship between each isolates was established based on biological assay, particle morphology, dsRNA analysis, serological reaction and molecular works including RT-PCR and sequence analysis for compositional properties of genomic RNA and coat protein.

In the present work, the virus characteristics are studied using purified virus preparations as well as infected plant materials and compared the strain identity using sequence similarities of the CP gene and 3' NTR with other reported AlMV strains (Houser-Scott et al., 1994). Each isolated strains was shown to be AlMV by the following evidence: i) its bacilliform particles of three different sizes, ii) symptom developments on many test plants, iii) serological reactions with common AlMV antiserum, and iv) the nucleotide sequences of CP gene and 3' NTR. Altogether these results indicated that native field potatoes and azuki bean are infected with AlMV in Kangwon areas in Korea. Although the last evidence was derived from only a partial nucleotide sequences, it has previously been shown that comparison of CP gene sequence give almost as good a picture of relationship with the genus *Alfamovirus* as do comparisons of longer sequences (Pietersen et al., 1985; Neeleman et al., 1991).

Host range studies and serological reaction with AlMV antisera showed that virus was one of the *Alfamovirus*, previously reported on several crops (Weimer, 1931, 1934; Hull, 1969; Roger, 1969; Paliwal, 1982; Valkonen et al., 1992). The symptoms on some test plants including *D. stramonium, G. globosa, N. rustica*, and *N. tabacum* inoculated with AlMV-KR showed some differences as compared to that of previously described AlMV isolated from soybean in Korea (Lee, 1985; Kudela and Gallo, 1995). Lee (1985) reported that *D. stramonium* infected with AlMV from soybean developed severe mosaic symptom systemically while no symptom was apparent in *N. rustica*. In contrast, AlMV-KR induced systemic mosaic symptoms on *D. stramonium* and *N. rustica*. Similarly, AlMV from Soybean induced necrotic spots only on inoculated *N. tabacum* leaves (Zaumeyer, 1953, 1963; Paliwal, 1982; Knorr et al., 1983) and systemic symptoms on *Chenopodiacea* plants while AlMV-KR1 caused systemic symptoms on *N. tabacum* and chlorotic and/or necrotic spots on *Chenopodiacea* plants. However, AlMV-KRs and Az could be clearly differentiated by host reactions in some indicator plants such as *N. glauca, C. quinoa* and *S. tuberosum*. From these results, although these three isolates have almost identical serological and biochemical properties, they have distinctive biological characteristics.

AlMV appears to be common in native field grown potato (Oswald, 1950; Salazar, 1996; Colin, 1998) and was detected in many native potato fields in Korea. Although Lee (1981) mentioned the detection of AlMV from potato, this is the first report to fully describe the isolation and characterization of virus particles associated with potato and azuki bean viral diseases (Jaspars and Boss, 1980). In this study, leaves with calico yellowing symptoms and light stunting were collected from different potato growing areas in Kangwon province and tested by ELISA (data not shown). AlMV-KR causes calico or mild mottling in potato leaves and tubers in cv. Dejima and appeared to be common in potato field where Dejima potato is grown. However, AlMV was found rarely in other potato cultivars including cultivars Atlantic, Irish Cobbler, Jopoong, Gawon in many areas of Korea and was not easily detected serologically. Due to their characteristic symptoms on Dejima potatoes, symptom developments often used as diagnostic tools for AlMV detection (Knorr et al., 1983; Salazar, 1996). We collected 10 potato samples showing calico and/or mild mottling symptoms but only 5 samples were infected with AlMV. Therefore it is worth mentioning that there are abnormalities similar to those caused by AlMV but caused by other agents or factors (Oswald, 1950; Valkoene et al., 1992). In infectivity test, AlMV-KR was inoculated onto virus free young potato cultivars and assayed by ELISA using AlMV polyclonal antibody. Both AlMV strains produced systemically severe yellow mosaic symptoms and necrosis on cv. Athlantic, Jopoong, Gawon and Superior, and induced symptomless on other cultivars. This result was not agreed to one of field tests as above.

Glutaraldehyde solution seems to be essential to stabilize the virus particles of AlMV-KR1, KR2, and Az. This is in agreement with the previous report showing that glutaraldehyde-fixation stabilizes the structural integrity of AlMV particles as well as increases their immunogenicity and antigenic reactivity (Hajimorad and Francki, 1991a). Keeping the virions structurally stable is extremely important because the low yield of particles often necessitates the bulking of virus preparations that may involve combining purified materials over several weeks to months (Pietersen et al., 1985; Valkonen et al., 1992). In general TIP of AlMV is reported to be between 50 and 65°C, DEP is between 10^{-3} and 5×10^{-3}, and inactivation by aging in sap is between 4 hr and 4 days with a half-life of 15 min (Hull, 1970; Jaspars and Bos, 1980). Loss of infectivity started at a relatively low temperature and extended over a range of 20°C or more (Pietersen et al., 1985). The actual inactivation point is depends on the concentration of the virus. AlMV infected potato and azuki bean partially purified preparations had numerous protein bands when electrophoresed in SDS-PAGE (figure not shown), but purified virus particles had a single protein band with strained with Coomassie Brilliant Blue R-250 (Fig. 2B) (Sehnke and Johnson, 1994). We did not detect serological differences between the particles of AlMV isolates and AlMV-PV92 strain by direct ELISA and Ouchtelony gel diffusion tests (data not shown). Our purified preparations contained high numbers of virus particles, so titer of our antiserum to AlMV-KR was high. Furthermore, the antiserum produced could be used for reliable ELISA tests to detect AlMV infection. This is the first report on the production of an antiserum to AlMV and its use for serodiagnosis of AlMV on potato plants in Korea.

AlMV strains is numerous and diverse (Hagedorn and Hanson, 1963; Bancroft et al., 1969; Hull, 1970; Hajimorad and Francki, 1991b). They infect many wild and cultivated plant species, often causing devastative diseases. Previously, it has been difficult to classify AlMV strains, but now they can be differentiated more accurately based upon molecular properties (Cornellisen et al., 1983a and b; Houser-Scott et al., 1994; Kudela and Gallo, 1995). To allow further identification of the viruses it is necessary to obtain viral genomic RNA sequence data. PCR primers for AlMV were designed complementary to two highly conserved sequences from the intergenic region and 3' non-coding region of AlMV-RNA 3 covering entire CP gene. Both isolates supported amplification of the expected fragments, which was also found in other AlMV strains. The amplified DNA fragments were subjected to sequence anal-

ysis. In this study, our results show that the level of amino acid sequence identity in the CP gene and the nucleotide sequence identity in the 3 NTR between isolated AlMV strains and other AlMV subgroup members were 91.9 to 100%, respectively. Although AlMV-KR1 and KR2 showed some differences in the symptom development in test plants, their pathogenicity was not different in potato cultivars. AlMV-KR1 produced more severe symptoms than other isolates and showed some differences (see Table 1). When looked at deduced amino acid sequence of CP gene, H20, D52 and Y104 in AlMV-KR1 were substituted with Q, G, and H in AlMV-KR2 (see Fig. 4). Whether these three amino acid substitutions between KR1 and KR2 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). Further detail molecular characteristics such as pseudo-recombinants or full-length genomic RNA sequences will be required in order to define different pathological characteristics between the AlMV isolates.

The nucleotide sequences of the CP genes of AlMV-KR2 and AlMV-Az show very few differences from either each other or those of the strains 425M, N, and S reported. The predicted amino acid sequences of the CPs of AlMV-KR and AlMV-Az were compared with those of other strains well known. There are only three amino acid differences between the CPs of AlMV-KR2 and AlMV-Az (Fig. 4). Computer analysis of the amino acid sequences for CPs indicated about 97 to 98% homology of sequences with strains of 425M, N, and S, and about 93% homology between strains of VRU and 15/64. Therefore, our primers from remarkably highly conserved regions of AlMV RNA-3 covering CP gene should detect all AlMV strains, and these results confirm that AlMV of both subgroups is detected by the assay as described by Neeleman et al. (1991). Interestingly, the AlMV-KR1, KR2, and Az isolates derived in this work both have changed in His to Gln at position 133 of the CP relative to the other reported AlMV strains. Furthermore, several amino acid substitutions between the AlMV-KR1, KR2, and Az are detected at non-conservative region. We do not know, however, whether these amino acid changes in CPs are related with their different phenotypes in several test plants. The major function of the CP is believed to be to protect the viral RNA within the infected cell and to permit transmission of the viral genome from plant to plant, either mechanically or by vectors such as insects, or nematodes (van Beynum et al., 1977; Vossen et al., 1995). On the other hand, for most plant viruses CP is required for systemic movement of the virus from leaf-to-leaf through the vascular system of the plant (Carrington et al., 1996). Previously, van der Kuyl et al. (1991) reported that differences in symptoms caused by AlMV strains YSMV and 425M on inoculated tobacco leaves are due to an amino acid substitution at position 29 of the CP. However, genetic determinants for the differences in systemic symptoms are more complex (Neeleman et al., 1991). Further detailed molecular characteristics such as pseudo-recombinants or full-length genomic RNA sequences will be required in order to define different pathological characteristics between the AlMV isolates.

Models for the secondary structure of the 3' NTR 179 nucleotides of AlMV-KRs RNA-3 were obtained with the Mfold program (Zuker, 1989; Mathews et al., 1999; Zuker

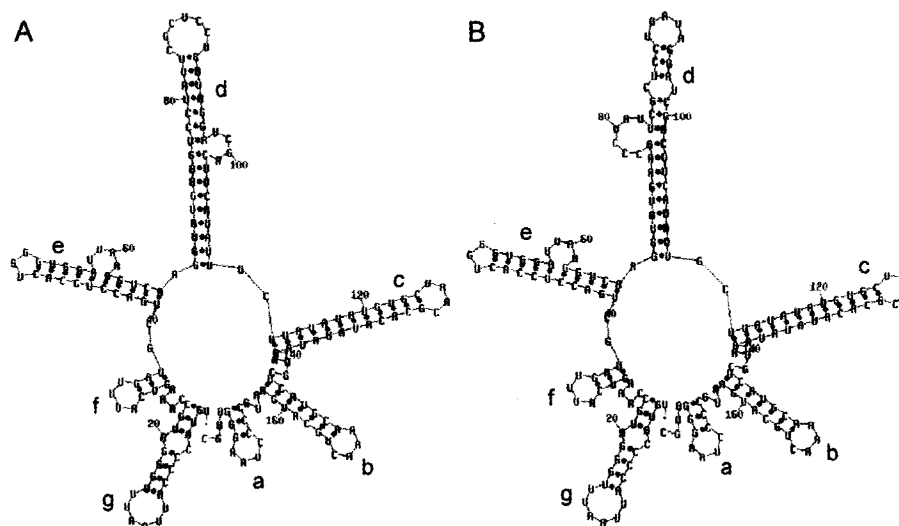

Fig. 6. RNA secondary structures predicted at the 3' NTR of RNA-3. Five stem-loop structures (a to g) were predicted using the 3'-terminal 179 nucleotides of AlMV KR1 (A) and KR2 (B) RNA-3 genome. Note that one nucleotide change at position 79 (U in KR1 and C in KR2) predicted strikingly different RNA secondary structure in stem-loop d.

et al., 1999) and are shown in Fig. 6. The 3-terminal sequence of 179 nucleotides that is 99.4% homologous between KR1 and KR2 can be folded into 7 stem-loop structures (labeled a-g in Fig. 6). Almost identical stem-loop structures are predicted (Koper-Zwarthoff and Bol, 1980; Houser-Scott et al., 1994) and supported by the data obtained from enzymatic structure probing (Quigley et al., 1984). Stem-loops a to e were flanked by single-stranded AUGC tetranucleotide sequences. The unique property of the *Alfamovirus* is the necessity of CP to initiate infection (Bol et al., 1971). Therefore, the genome of AlMV is not infectious unless RNA-1, -2, and -3 are supplemented CP subunits (Taschner et al., 1991). It has been shown that these AUGC tetranucleotide sequences and stem-loops are involved in CP binding that is required for genome activation (Houwing and Jaspars, 1982; Jaspars, 1985; Bol, 1999). It is interesting to mention that one nucleotide difference between KR1 and KR2 in the 3' NTR affected the RNA secondary structure in the stem-loop e (Fig. 6). Since stem-loop e is one of CP binding sites, these structural differences may affect virus replication and eventually symptom developments in host plants though it remained to be determined whether these different structures are actually formed in vivo (Houwing & Jaspars, 1982). Future experiments will determine if this sequence and RNA secondary structure difference in stem-loop e affect CP binding that facilitates RNA genome activation or responsible for symptom differences in tobacco Xanthi-nc plant.

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