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Detection of Allexiviruses in the Garlic Plants in Korea

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The genomes of different allexiviruses were isolated and cloned from virus-infected garlic plants (*Allium sativum*), which were collected from farm fields in the southern provinces in Korea. The partial nucleotide sequences of the genomes from different allexiviruses were clearly identified in the virus-infected garlic plants. The cloned partial genomes of viruses in garlic plants showed a greater than 90% homology to previously identified allexiviruses and classified into species of GarV-A, -B, -C, -D, -E, and -X, demonstrating that species of allexivirus found in the other countries in the world are also widely distributed in the garlic plants in Korea.

Keywords **:** *Allexivirus*, garlic plant, garlic virus, plant virus

Viruses in garlic (*Allium*) plants are widespread, causing economic loss around the world (Delecolle and Lot 1981; Fujisawa, 1989; Van Dijk, 1994). Because garlic plants (*Allium sativum*) propagated vegetatively, the viruses of garlic plants accumulated and evolved over generations, resulting in the evolution of a diversity of garlic viruses (Coni et al., 1992; Sumi et al., 1993; Lot et al., 1994; Koo et al., 2002).

Three viruses of genus *Potyvirus*, *Carlavirus*, and *Allexivirus* represent the majority of the world's garlic viruses. Amomg three genera, garlic mosaic virus (GMV) of the *Potyvirus*, garlic latent virus (GarLV) of the *Carlavirus* and garlic virus (GarV) of the *Allexivirus* were abundantly detected in garlic plants (Chang et al., 1992; Sumi et al., 1993; Nagakubo et al., 1994; Tsuneyoshi et al., 1998; Chen et al., 2001; Koo et al., 1998 and 2002). It has been known that *Potyviruses* and *Carlavirues* are transmitted by aphids (Van Dijk, 1994; Van Dijk et al., 1991). However, allexiviruses are also known to be transmitted by mites (*Aceria tulipae*) (Kang et al., 2007).

Allexivirus is a flexuous rod-shaped virus containing a single-stranded positive sense polyadenylated RNA genome consisting of six ORF's (Chen et al., 2004; Koo et al., 2002; Pringle, 1999; Sumi et al., 1999). To date, six variants of *Allexivirus* family have been reported in garlic plants and are made up of GarV-A (Koo et al., 2002; Sumi et al., 1999), GarV-B (Kang et al., 2007; Sumi et al., 1993), GarV-C (Sumi et al., 1999), GarV-D (Koo et al., 2002; Sumi et al., 1993), GarV-E (Chen et al., 2001) and GarV-X (Chen et al., 2004; Song et al., 1997). The genome sequences of GarV viruses are exclusive in six open reading frames (ORF) for functional proteins (Chen 2004; Song et al., 1997; Sumi et al., 1999). Conversely, six different allexiviruses demonstrated slightly different homologies in their corresponding ORF sequences. Therefore, the comparison of different amino acids of each ORF in viral proteins of allexiviruses may represent the complexity of the virus infection in garlic plants. Previous homology comparison for the ORF's of allexiviruses reported that two viruses including GarV-A and -D were mixed in a garlic plant (Koo et al., 2002). However, how many allexiviruses co-infected in garlic plants have not yet been revealed. Therefore, our goal for this study was to further investigate the distribution of allexiviruses using amplified cDNA fragments of the viral RNA genomes using reverse transcribed polymerase chain reactions (RT-PCR) from the garlic plants showing viral symptoms which were collected in farm fields in the southern provinces of Korea.

Materials and Methods

Virus disease in garlic plants. Garlic plants showing viral disease symptoms were randomly collected from 41 major garlic farming sites in Korea. Symptoms of garlic plants were examined as mosaic, stripe, yellow or stunt. The garlic leaves and bulbs were harvested and used for virus isolation and cloning the virus genome.

Transmission electron microscopy (TEM). For TEM analysis, leaf segments (1×5 mm^2) were cut from the leaves of virus-infected garlic plants. The samples were fixed with

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2.5% (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.0) and incubated at 4°C for 30 min. Sample fixation was repeated by five time changes with the fixative. The samples were then transferred to 4°C for 12 h. Following fixation, the samples were washed with 0.1 M sodium phosphate buffer (pH 7.0), and incubated for 20 min at 4°C. After washing, samples were post-fixed for 6 h in a 1% (v/v) osmium tetroxide (Sigma-Aldrich, USA) in sodium phosphate buffer (pH 7.0). After washing three times in buffer, the samples were dehydrated through a graded series of ethanol concentrations. After incubating for 15 min in propylene oxide, the samples were further incubated in epoxy resin solution [0.46 M EMBED-818, 0.28 M nadic methyl anhydride, 0.25 M dodecenyl succinic anhydride, 17.2 mM 2,4,6-Tri(Dimethylaminoethyl) phenol in propylene oxide at 1:1 (v/v) volume] for 4 h at room temperature and once by changing the solution at 2:1 v/v for 12 h at room temperature. Next, the samples were embedded in fresh epoxy resin solution and were polymerized overnight at 70°C. Ultrathin sections were cut using an MT-X ultramicrotome (RMC, AZ, USA). The sections were viewed in an H-7600 transmission electron microscope (Hitachi Ltd., Japan) operating at 80.0 kV, which was located at the microscopic analysis center of Yeungnam University, Korea.

Immunosorbent electron microscopy (ISEM). Viral particles were observed under a transmission electron microscope (Hitachi H-7600) by the direct negative staining method (DN) (Doi et al., 1969) as well as the trap decoration method. The DN method was performed using cell extracts by grinding the small pieces of leaves with 2% (W/V) phosphotungstic acid (PTA) at a pH of 7.0. The ISEM was examined as described in Miline and Luisoni (1977). Briefly, antiserums of garlic virus (Aomori Bio Greencenter, City, Japan) were diluted (1:1,000) and used to perform ISEM. Diluted antiserum (1:1,000) was placed on a grid. The partially purified viruses were extracted in 0.1 M of potassium phosphate buffer, added to the grid with the diluted antiserums, incubated for 15 min and washed with phosphate buffer (pH 7.0). The viruses were stained with 2% uranyl acetate and viewed with a transmission electron microscope.

Purification of viral particles and isolation of viral RNA. The virus was isolated from virus-infected garlic bulbs by CsCl-sucrose gradient sedimentation as described by Yamashita et al. (1996). The viral RNA was extracted from the purified viruses, which were treated with 2% bentonite, 50 mg/mL proteinase K, 2% sodium dodecyl sulfate (SDS) and 25 mM of EDTA for 30 min at 37°C, followed by phenol/chloroform extraction and ethanol precipitation (Kwak et al., 2007). The viral RNA was estimated by separation on a 0.8% agarose gel electrophoresis under denaturing conditions with 2.2 M of formaldehyde. Furthermore, the total RNA was isolated by phenol:CIA extraction methods according to Koo et al. (2002).

Cloning viral cDNA and reverse transcription polymerase chain reaction (RT-PCR). RT-PCR was performed as described previously (Chung et al., 2007). The viral cDNA synthesis was carried out using an oligo d(T) primer with an AMV reverse transcriptase at 42°C for 45 min and cloned into pBluscript SK(+) phagemid plasmids (Startagene, USA) according to the manufacturer's instruction. For the RT-PCR, most reactions were performed in a solution containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM dNTP, 50 mM of each equivalent primer, and 2.5 units of Taq DNA polymerase in each reaction. A thermal cycle was set for 2 min at 95°C, followed by annealing at 55°C for 1.5 min and polymerization at 72°C for 1 min. Total of 30 cycles were completed and followed by a 10-min extension at 72°C. The amplified DNA fragments were examined by electrophoresis on a 1% agarose gel.

Nucleotide sequence analysis. The PCR products were digested with appropriate restriction enzymes, ligated into T-Easy cloning vector (Promega, USA) and transformed into XL1-Blue cells. The plasmid containing the cDNA inserts was selected for nucleotide sequencing and the sequencing reactions were performed using an ABI prism™ 3100 DNA sequencer using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, USA). The analysis of the nucleotide and the deduced amino acid sequences were performed with the BLAST at NCBI data base (Altschul et al., 1997), CLUSTAL W and DNAsis programs.

Protein extraction and SDS polyacrylamide gel electrophoresis. Five hundred micrograms of garlic leaves were harvested and ground in 3 mL of extraction buffer [5 mM sodium phosphate buffer (pH 7.0), 1% (v/v) diethylcabonate, 2% SDS, 5% 2-mercaptoethnaol]. Moreover, the crude homogenates were centrifuged at 9,000 *g* for 20 min at 4°C. Next, the supernatants were transferred to a new tube, leaving the cell debris behind, and centrifuged again at 20,000 *g* for 20 min and stored at −80°C until further use. The protein contents in the final supernatants were determined by the Bradford assay (Bio-Rad, USA). Moreover, the SDS-PAGE gel electrophoresis analysis involved boiling 50 μg of the protein extracts for 3 min, with 2-mercaptoethnol and loaded onto each lane of 12%

acrylamide. In addition, identical sets of SDS-PAGE gel electrophoreses were performed with one gel stained for 1 h in Bio-Safe Coomassie Blue (Biorad, USA) and de-stained to visualize the bands using a de-staining solution, while the second gel was used directly for the immunoblotting analysis.

Immunoblotting analysis. The nitrocellulose membranes were pre-wetted in 100% methanol, washed in distilled water for 5 min and equilibrated in a transfer buffer for 10 min before immunoblotting. The electro-transfer was performed in 25 mM Tris, 250 mM glycine, 0.1% SDS, 20% methanol (pH 8.3) at 18 V and 350 mA for 12 h. The membrane was blocked with 5% nonfat dry milk in TTBS (25 mM Tris-HCl, 150 mM NaCl, and 0.2% Tween-20) for 1 h at room temperature with constant rotation, using an orbital shaker at a low speed. The membranes were briefly rinsed with two changes of the TTBS wash buffer. Next, rabbit antiserum against the garlic virus was diluted in TTBS (1:5000) as a primary antibody. Furthermore, the membrane was incubated for 1 h at room temperature with slow rotation. The membranes were briefly rinsed with two changes of wash buffer and subsequently washed once again for 15 min at room temperature. Anti-rabbit IgG Horseradish Peroxidase (HRP) labeled secondary antibody (Amersham Biosciences, USA) was diluted (1:2000) in PBS for immunological detection. The membrane was incubated in the secondary antibody for 1 h at room temperature with slow rotation. The membrane was rinsed with two changes of the wash buffer and then washed again in wash buffer for 5 min. This was repeated three times with fresh changes of wash buffer at room temperature at each time. The reaction was detected using the ECL Western blotting detection reagent, as described in the ECL protocol (Amersham Biosciences, USA), followed by exposure on X-ray film and development.

Results and Discussion

Virus diseases were widespread in galic plants in farms. We surveyed the viral infection of garlic plants from garlic-growing fields in the Gyeongbook area, Korea. In most investigated sites, the viral diseases on the garlic plants have been identified (Fig. 1). Figure 1 depicted the virus-infected garlic plants, which have retarded growth, curled yellow-strip mosaic and distorted symptoms on the leaves. Some of the virus-infected garlic plants showed severely retarded and curled leaf development (Fig. 1E). Previously,

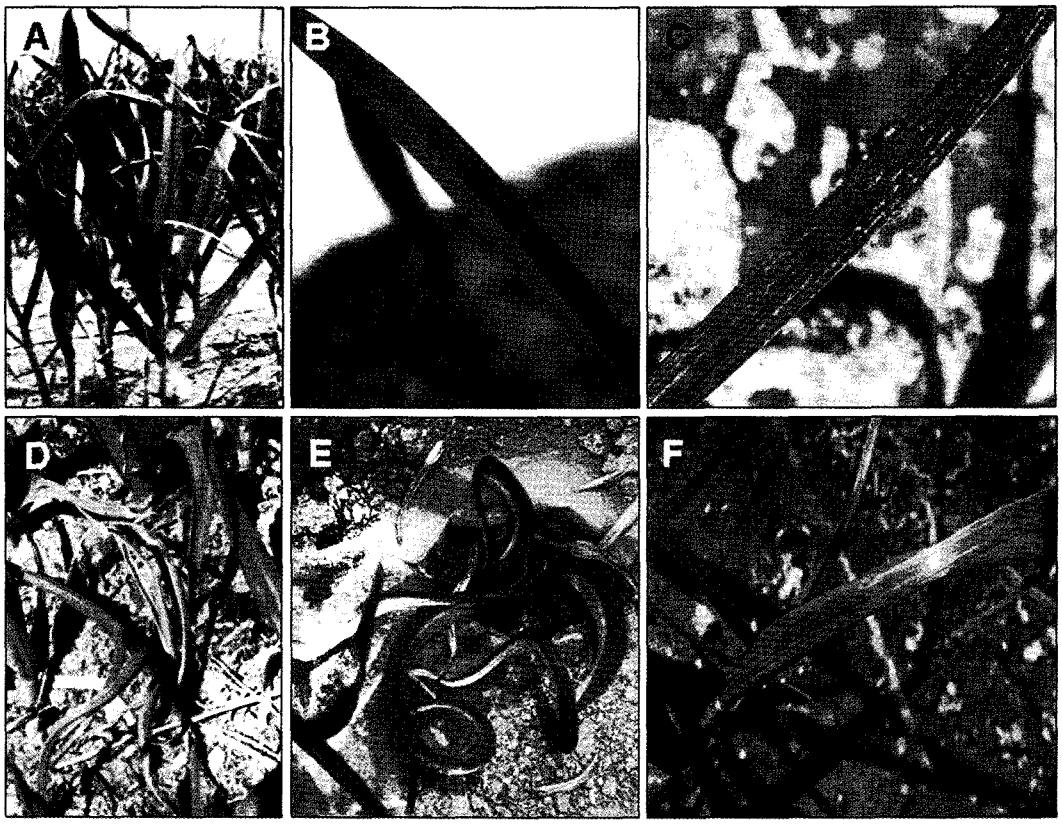

Fig. 1. Viral diseased garlic plants. (**A**) Healthy garlic plants grown in a greenhouse. (**B**) A leaf of a healthy garlic plant grown in a green house. (**C**) A leaf of a virus-infected garlic plant. (**D**, **E** and **F**) Farm fields grown garlic plants showing the viral disease. Note: The symptoms are showing retarded growth and curled yellow-strip mosaics on leaves.

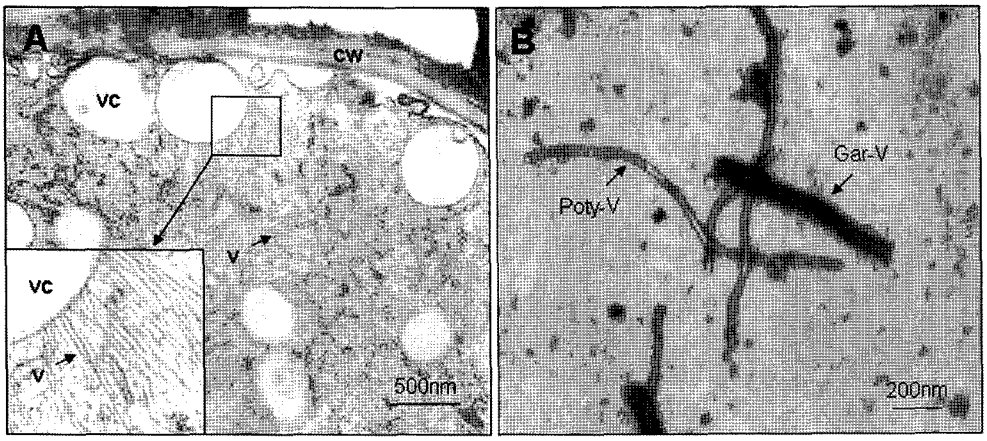

Fig. 2. Detection of garlic viruses in cells of a virus-infected garlic plant. (**A**) The virus accumulated in the epidermal cells of leaves. Arrow with *v* indicates a virus. (**B**) Immunoelectron microscopy showed of the viral particles of allexiviruses using antiserum against allexiviruses.. Poty-V is *Potyvirus* which is not decorated with antiserum against allexiviruses.

we found that garlic plants curled and produced yellow-mosaic strips on the leaves caused by Garlic viruses (GarV), which were propagated by eriophyid mites (*Aceria tulipae*) (Kang et al., 2007).

This retarded leaf development of garlic plants caused small bulbs, low yields and poor bulb quality, which resulted in large losses for the farm economy. As in previous investigations, which reported that the viral diseases of garlic plants were widespread around the world (Delecolle and Lot 1981; Fujisawa, 1989; Song et al., 1997; Sumi et al., 1993; Van Dijk, 1994; Van Dijk et al., 1991), virus disease in garlic plants was also widespread in Korea (Koo et al., 2002), resulting in loose economic acquirements in the farms of Korea.

Allexiviruses were found in the virus-infected leaves of garlic plants. We investigated the virus-infected leaves of garlic plants and found that a large number of viruses were amplified and fully aggregated in the cytoplasm of epidemic cells (Fig. 2A). We further purified the viruses from virus-infected garlic plants and performed further investigation using antiserum against *Allexivirus*. Based on the ISEM data, about 600-800 nm in length of the rod shaped *Allexivirus* particles were observed (Fig. 2B). However, under the ISEM, the other shaped of virueses, which are presumably potyviruses, were not decorated by the antiserum against allexiviruses. The results showed that different viruses were mixed within a garlic plant.

Coat proteins of allexiviruses were identified from virus infected garlic plants by immunoblot analysis. We performed an immunoblot analysis to identify allexiviruses extracted from virus-infected garlic plants and symptomless garlic plants. Fig. 3 shows that the protein extracted from the leaves showing the curled yellow-strip mosaic symptom of garlic plants were specifically cross-reacted to antiserum against coat proteins of allexiviruses. In the virus-infected garlic plants, 27 kDa and 30 kDa of coat proteins in GarV of the *Allexivirus* were clearly identified (Fig. 3). Further analysis demonstrated that most farm grown garlic plants showed yellow-strip mosaic on leaves which were cross-reacted with the antiserum. However, leaf extracts of symptomless garlic plants did not show strong cross-react signals in response to the same antiserum.

Nucleotide sequence analysis and identification of *Allexivirus* species. The viruses were purified from the virus-infected garlic leaves by CsCl-sucrose gradient sedimentation. Moreover, the viral RNA genome was

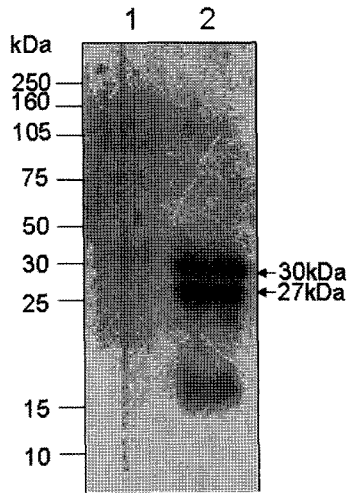

Fig. 3. Identification of allexiviruses by immunoblotting analysis of leaf extracts from virus infected garlic plants. The leaf protein extracts from the leaves of symptomless garlic plants (lane 1) and the leaves showing curled yellow-strip mosaic symptom of garlic plants (lane 2) were loaded. Anti-*Allexivirus* antibodies were reacted against 30 kDa and 27 kDa of coat proteins (arrows).

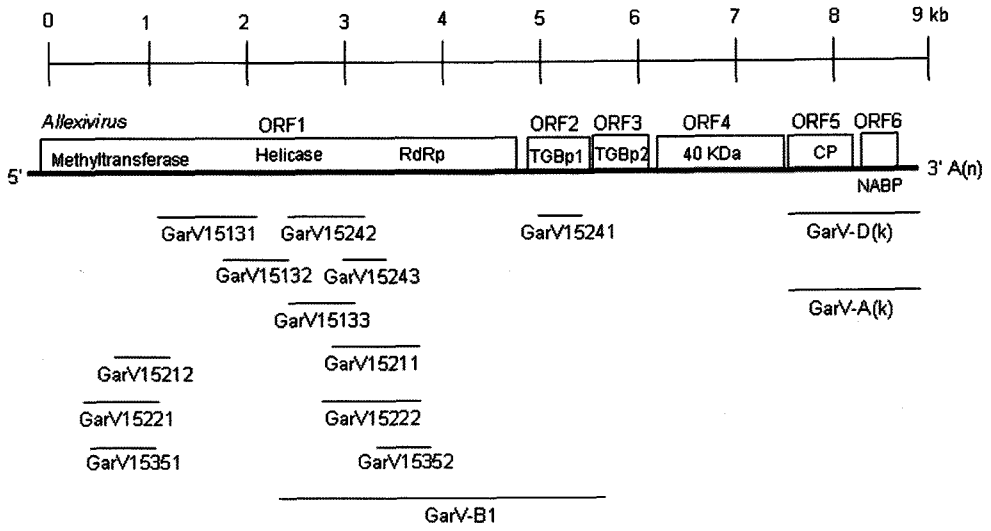

Fig. 4. Schematic diagram for the genome organization of *Allexivirus* and nucleotide sequences of viruses cloned from virus-infected garlic plants. The deduced amino acid sequences of individual clones were aligned with those of registered garlic viruses in data-bases and located under corresponding sequences of allexiviruses. Open reading frame (ORF); RNA-dependent RNA polymerase (RdRp); and triple gene block proteins (TGBp).

cloned with reverse transcription PCR and the nucleotide sequences were elucidated. Fig. 4 presents a schematic constitution of the genome of *Allexivirus* genus. Fifteen different cDNA sequences were aligned with the different parts of genome sequences of *Allexivirus* (Fig 4). The deduced amino acid sequences of these partial sequences were often aligned with helicase and RNA-dependent RNA polymerase (RdRp) in the ORF1 of *Allexivirus*. In particular, a clone of GarV-B1 coded for helicase, RdRp of ORF1 and a triple gene block protein (TGBp) from the ORF2 of GarV-B (EF596816) (Kang et al., 2007). Two sequences from GarV-D(k) (AF519572) and GarV-A(k) (AF478197) were aligned with coat protein and nucleic acid binding proteins from the *Allexivirus* genome (Koo et al., 2002).

The deduced amino acid sequences of the cloned viral genome fragments in this study revealed similarities ranging from 66 to 90% with the viruses classified in the *Allexivirus* genus (Table 1). Further amino acid comparison showed that 15 different clones belonging to species of garlic viruses in the *Allexivirus* genus, including the GarV-A (NP-569126), GarV-B (BBA61813), GarV-C (NP_569132),

Table 1. Identification of the garlic virus proteins cloned from virus infected garlic plants collected in Korea.

Clone Name	Numbers of amino acid sequences	Definition (ORF)	Homologous garlic virus	Homologous protein accession numbers	Identities (%)	Positives (%)	References
GarV15131	305	Replicase(ORF1)	GarV-A	NP_569126	294/305 (96%)	296/305 (97%)	Sumi et al., 1999
GarV15132	168	Replicase(ORF1)	GarV-X	NP_044571	82/168 (48%)	111/168 (66%)	Song et al., 1998
GarV15133	192	Helicase1(ORF1)	GarV-C	NP_569132	174/192 (90%)	189/192 (98%)	Chen et al., 2001
GarV15211	133	Replicase(ORF1)	GarV-C	NP_569132	96/109 (88%)	104/109 (95%)	Chen et al., 2001
GarV15212	55	Replicase(ORF1)	GarV-X	NP_044571	47/55 (85%)	52/55 (94%)	Song et al., 1998
GarV15351	154	Replicase(ORF1)	GarV-X	NP_044571	102/109 (93%)	107/109 (98%)	Song et al., 1998
GarV15352	51	Replicase(ORF1)	GarV-A	NP_569126	37/39 (94%)	37/39 (94%)	Sumi et al., 1999
GarV15221	55	RdRp (ORF1)	GarV-X	CAC83699	48/55 (87%)	52/55 (94%)	Chen et al., 2001
GarV15222	159	Replicase(ORF1)	GarV-C	NP_569132	138/159 (86%)	146/159 (91%)	Chen et al., 2001
GarV15241	68	TGBp1 (ORF2)	GarV-B	BAA61813	63/68 (92%)	65/68 (95%)	Sumi et al., 1993
GarV15242	105	Replicase(ORF1)	GarV-C	NP_569132	97/105 (92%)	103/105 (98%)	Sumi et al., 1999
GarV15243	95	Replicase(ORF1)	GarV-E	NP_659010	43/45 (95%)	43/45 (95%)	Chen et al., 2001
GarV-B1	783	Replicase(ORF1)	GarV-C	NP_569132	701/779 (89%)	743/779 (95%)	Sumi et al., 1999
GarV-D(K)	250	Coatprotein(ORF5)	GarV-D	BAA61825	245/250 (98%)	247/250 (98%)	Koo et al., 2002
GarV-A(K)	250	Coatprotein(ORF5)	GarV-A	NP_569130	250/250 (100%)	250/250 (100%)	Koo et al., 2002

*Gaps were 0%.

GarV-D (BAA61825), GarV-E (NP_659010), and GarV-X (CAC83699) (Table 1). To date, six different genomes of allexiviruses (GarV-A, -B, -C. -D, -E, and -X) have been registered in the public nucleotide databases (Chen et al., 2001; Kang et al., 2007; Koo et al., 2002 and 1998; Song et al., 1997; Sumi et al., 1993, 1999). Based on this study, the genome distribution of the allexiviruses demonstrated that identified six species of the genus *Allexivirus* also widespread in garlic plants in Korea. Because allexiviruses are highly homologous in genomic nucleotide sequences observed in this study, various garlic viruses may have evolved genetically in garlic plants. This statement is consistent to previous suggestions, which state that the vegetative propagation of garlic plants over several generations may result in the evolution of the garlic virus (Coni et al., 1992; Koo et al., 2002; Lot et al., 1994; Sumi et al., 1993). Both international garlic trades and traditional long term-vegetative propagations using seed bulbs in farms might be one of major causes of virus accumulation in garlic plants. To overcome the economic losses by the allexiviruses, it is highly recommended that a virus treatment in seed bulbs as well as controlling insects by insecticides.

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