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Survey of Garlic Virus Disease and Phylogenetic Characterization of Garlic Viruses of the Genus *Allexivirus* Isolated in Korea

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A survey of virus infection in garlic plants cultivated in Korea was conducted for three years. Most virus-infected garlic plants (*Allium sativum*) showed typical symptoms on the leaves such as yellow mosaic, stripes, and distortion. Through immunosorbent electron microscopy and RT-PCR analysis, the complex mixtures of viruses including garlic viruses of the genus *Allexivirus*, garlic strain of *Leek yellow stripe virus* of the genus *Potyvirus*, and *Garlic latent virus* of the genus *Carlavirus* were identified in the virus-infected garlic plants. Among these viruses, *Allexivirus* was the most frequently detected in the regions surveyed. Using sets of differential primers for *Allexivirus* genomes, two members of the genus were amplified and sequenced from the purified viruses. The deduced amino acid sequences for the coat proteins and the nucleic acid binding proteins of two viruses showed high homologies to *Garlic virus A* (GarV-A) and *Garlic virus D* (GarV-D) of *Allexivirus*. This is the first report of GarV-A and GarV-D in Korea. This suggests that *Allexivirus* in garlic plants in Korea was mixed and varied. Phylogenetic analyses showed that the genus *Allexivirus* was diversified by the processes of accumulation and evolution of viruses in garlic plants due to the long period of repeated vegetative propagation.

Keywords : *Aceria tulipae*, *Allexivirus*, garlic, garlic virus, GMbMV.

Garlic (*Allium sativum* L.) plants are propagated vegetatively. Because of this, viruses accumulate and evolve in garlic plants (Coni et al., 1992; Lot et al., 1994; Summi et al., 1993). Viral diseases of garlic in *Allium* plants have became widespread, causing serious losses around the world (Delecolle and Lot 1981; Fujisawa, 1989; van Dijk, 1993a and b; Song et al., 1997).

Members in the genera *Potyvirus*, *Carlavirus*, and *Allexivirus* are major viruses in a complex mixture in garlic plants (Chen et al., 2001; Nagakubo et al., 1994; Sumi et al., 1993; Tsuneyoshi et al., 1998a and 1998b). All three genera, which include *Garlic mosaic virus* (GarMV) of *Potyvirus*, *Garlic latent virus* (GarLV) of *Carlavirus*, and *Garlic mite-borne mosaic virus* (GMbMV) and *Garlic virus X* (GarV-X) of *Allexivirus*, were detected in garlic plants in Korea (Chang et al., 1991 and 1992; Chung and Chang, 1979; Koo et al., 1998; Song et al., 1997). Koo et al. (1998) reported that the genus *Allexivirus* was widespread and caused mosaic or streak symptoms in cultivated garlic plants in Korea.

Allexivirus, which is known as GMbMV, is a flexuous rod-shaped virus containing a single-stranded positive sense RNA, and has recently been ratified as a genus (Pringle, 1999; Sumi et al., 1999). While *Potyviruses* and *Carlaviruses* are transmitted by aphids (van Dijk et al., 1991; van Dijk, 1994), *Allexiviruses* are transmitted by mites (*Aceria tulipae*). Several *Allexiviruses* including GarV-A (Sumi et al., 1993), GarV-B (Sumi et al., 1993), GarV-C (Sumi et al., 1999), GarV-D (Sumi et al., 1993), GarV-X (Song et al., 1997), and GarV-E (Chen et al., 2001) have been reported previously. Because garlic viruses accumulate in the bulbs by traditional vegetative propagation in the region several years after introduction, a wide diversity of viruses may evolve in regional cultivars. However, little is known about the distribution of the members of *Allexivirus*. Studies on regional distribution and molecular diversity of *Allexivirus* could be useful in the prevention of viral infection.

In this study, garlic-infecting viruses in garlic plants in Korea were found to be complex and mixed. These viruses were further characterized molecularly by using viral cDNAs cloning and sequence analysis of *Allexiviruses* from diseased garlic plants co-infected with their complex mixtures of viruses.

Materials and Methods

Survey of virus infection in garlic plants cultivated in Korea. Garlic plants showing viral disease symptoms were randomly collected from 41 sites in major garlic farming sites in Korea from 1997 to 2000 (Fig. 1). Symptoms were identified as mosaic,

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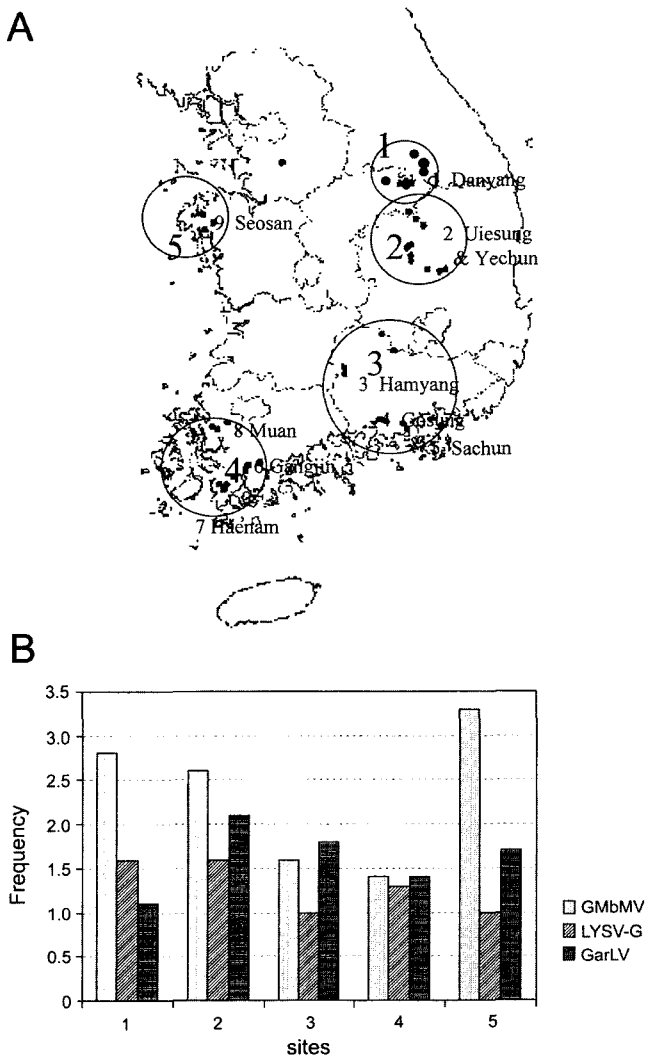

Fig. 1. Survey of virus infection in garlic plants. (**A**) Regional distribution of virus-infected garlic plants. The circled area indicates the five cultivation sites in South Korea. The forty-one fields in a region of a surveyed site are marked with dots. Samples were randomly collected by viral symptoms in each field. (**B**) Relative observed frequencies of the virus infection of GMbMV, LYSV, and GarLV. Antisera against each of the three specific types of viruses were used to detect by direct tissue blotting immunoassay. Frequencies were scored by strengths of activity, which were detected with alkaline phosphatase-labled goat anti-rabbit immunoglobulin antibodies in rabbit antisera raised against each virus. Scores were divided into four labels and marked in mean numbers of each site.

stripe, yellowing, and stunting. Garlic leaves and bulbs were used for virus isolation and serological tests.

Electron microscopy (EM). Viral particles were observed with a transmission electron microscope (Hitachi H-600, Hitachi Ltd., Ibaragi, Japan) by direct negative staining method (DN) and trap decoration method. DN method was performed using cell extracts by grinding the small pieces of leaves with 2% (w/v) phosphotungstic acid (PTA), pH 7.0. These were examined by immunosorbent electron microscopy (ISEM) as previously described by Miline and Luisoni (1977). Antisera against *Garlic latent virus* (GarLV), *Garlic mite-borne mosaic virus* (GMbMV) of *Allexivirus*, and *Leek yellow strip virus* (LYSV-G) were diluted to 1/1,000 (w/v) and used to perform ISEM. Approximately 5 μl of each diluted antiserum was placed on a grid. Partially purified viruses extracted in 0.1 M potassium phosphate buffer were added to the grid with the diluted antisera, and incubated for 15 minutes and washed with phosphate buffer (pH 7.0). The viruses were stained with 2% uranyl acetate and observed with a transmission electron microscope.

Direct tissue blotting immunoassay (DTBIA). Detection of viruses from tissues of virus-infected garlic plants by DTBIA was carried out as previously described by Tsuneyoshi and Sumi (1996).

Purification of viral particles and isolation of viral RNA. GMbMV was isolated from virus-infected garlic bulbs by CsCl-sucrose gradient sedimentation as described previously (Yamashita et al., 1996). Viral RNA was extracted from the purified viruses treated with 2% bentonite, 50 mg/ml proteinase K, 2% sodium dodecyl sulfate, and 25 mM EDTA for 30 minutes at 37°C, followed by phenol/chloroform extraction and then ethanol precipitation. Viral RNA was estimated by separating on a 0.8% agarose gel electrophoresis under denaturing conditions with 2.2 M formaldehyde.

Reverse transcription polymerase chain reaction (RT-PCR). RT-PCR was performed with four differentially designed primers and one universal primer based on the sequences of the garlic viruses (Table 1). Viral cDNA synthesis was carried out using an oligo d(T) primer with AMV reverse transcriptase (Gibco BRL, Rockville, USA) at 42°C for 45 minutes. The cDNAs encoding the coat proteins and the 3' ends of garlic viruses were amplified by PCR using a combination of universal primer and differential primer (Table 1). PCR was performed in the mixture solution containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 10mM dNTP, 50 mM of each primer, and 2.5 units of *Taq* DNA polymerase in 50 μl reaction. Thermal cycle was set for 2 minutes at 95°C, followed by annealing at 55°C for 1.5 minutes, and polymerization at 72°C for 1 minute. A total of 30 cycles was followed by a 10-minute extension at 72°C. The amplified DNA fragments were examined by electrophoresis on 1% agarose gel.

Cloning and sequence analysis. The PCR products were digested with appropriate restriction enzymes, ligated into pBluescript SKII (Stratagene, LaJolla, CA, USA), and transformed into *Escherichia coli* strain XL1-Blue cells. The plasmid that contained cDNA inserts was selected for nucleotide sequencing. Sequencing reactions using ABI prism™ 377 DNA sequencer with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Forster City, CA, USA) were performed. Analysis of nucleotide and deduced amino acid sequences were performed using the programs BLAST at NCBI (Altschul et al., 1997), CLUSTAL and GCG softwares (Genetics Computer Group, Medison, WI, USA). Phylogenetic analyses were performed using programs in PHYLIP v. 3.5., and trees were constructed by a distance method NEIGHBOR. The sequences determined in this study were deposited at the GenBank with

Table 1. PCR primers for determination of coat protein genes and ORF6 of *Allexiviruses*

Primer	Sequence (5' to 3')	Restriction enzyme
Uni-1	CAG AAT TCA ATA CCC ATG GAC ATC A	*Eco*RI
Diff-A	CCA AGC TTT TTT TTT TTT TTT TTG TCA[a]	*Hin*dIII
Diff-C	CCA AGC TTT TTT TTT TTT TTT TTG TCC	*Hin*dIII
Diff-G	CCA AGC TTT TTT TTT TTT TTT TTG TCG	*Hin*dIII
Diff-T	CCA AGC TTT TTT TTT TTT TTT TTG TCT	*Hin*dIII

[a]Bold character: differential selection nucleotide of each primer.

accession number AF478197 (GarV-A) for clone pdifT and AF519572 (GarV-D) for clone pdifA.

Results

Electron microscopy of virus particles. The morphological properties of the virus particles purified from garlic plants were examined by electron microscopy. Rod-shaped and slightly or highly flexuous particles about 600-800 nm in length were observed (Fig. 2B, panel 1). Each particle was morphologically indistinguishable. It was difficult to determine whether the virus preparations employed here contained a single or several species of viruses, including GarLV of *Carlavirus,* LYSV of *Potyvirus*, and GMbMV of *Allexivirus*.

Immunosorbent electron microscopy (ISEM). To characterize each virus by viral particle morphology, ISEM was performed using three different antisera of GarLV, LYSV, and GMbMV. The results showed that: flexuous filamentous particles were decorated with antiserum against LYSV (Fig. 2B, panel 2), highly flexuous filamentous particles with antiserum against GMbMV (Fig. 2B, panel 3), and rigid filamentous particles with antiserum aganst GarLV (Fig. 2B, panel 4).

Direct tissue blotting immunoassay (DTBIA). DTBIA was employed as a convenient and practical detection technique for garlic viruses (Koo et al., 1998; Lin et al., 1990; Tsuneyoshi and Sumi, 1996). Virus infection was investigated using DTBIA for garlic plants collected from the 41 sites of the five garlic cultivation regions with three different antisera of GarLV, LYSV, and GMbMV. All three antisera reacted with leaf-tissue blots of infected garlic plants. However, no signal appeared on the blots from virus-free garlic samples produced by shoot-tip culture with three different antisera (data not shown). The strength of the cross-reaction of antisera against each virus was measured and scored (Fig. 1B). These results were consistent with those obtained by ISEM. This study concluded that DTBIA was applicable as a convenient and practical detection technique of garlic viruses in Korea.

Survey of virus infection in garlic plants. The incidence of virus infection in garlic plants collected from 41 sites in five garlic cultivation regions was surveyed using the DN method, ISEM, and DTBIA. Shoot-tip cultured virus-free garlic plants, which showed no symptoms in the first year of growth, were used as control to determine the rate of infection (Fig. 2). Figure 1 shows that all three viruses were detected in each garlic plant from the sites surveyed. Among the garlic viruses, GMbMV was the most common, while the other viruses were less abundant in the multiple virus-infected garlic plants (Fig. 1B).

Virus purification, RT-PCR, and sequencing of cDNAs of genomic RNAs of *Allexivirus*. Garlic-infecting *Allexivirus* was purified from the virus-infected garlic leaves and bulbs by CsCl-sucrose gradient sedimentation as described previously (Yamashita et al., 1996; Koo et al., 1998). Viral particles of *Allexivirus* were co-sedimented with 40% CsCl (data not shown). After precipitation of the viruses, differential RT-PCR analysis was performed to separate each garlic virus from a virus band of the *Allexivirus* (Fig. 3). PCRs were performed using a combination of differential primer and universal primer (Table 1). Figure 3 shows that approximately 1.5 kb fragments were amplified from all three sets of differential PCRs with Diff-A, Diff-C, and Diff-T primer coupled with a universal primer, Uni-1 (Table 1). Among them, two PCR products were cloned, named pdifA and pdif-T, and sequenced for further characterization. Nucleotide sequences of the clones pdifA and pdifT were 1,544 bp and 1,573 bp, respectively. The deduced amino acid sequences of these cDNAs contained each viral coat protein (CP) and ORF 6, which coded a nucleic acid binding protein (NABP) of the genus *Allexivirus*.

Characterization of garlic-infecting viruses of *Allexivirus* isolated in Korea. The deduced amino acid sequence of the clone pdifA showed more than 98% homology with the CP and 87% homology with ORF 6 of the 15 kDa NABP of GarV-D (Sumi, 1991). Therefore, this indicates pdifA was originated from GarV-D and virus was designated as GarV-D (K) to distinguish from other members of GarV-D. The clone pdifT showed 100% identity with CP and 99% identity with ORF 6 of GarV-A (Sumi et al., 1999). These results suggest that the clone pdifT was derived from GarV-A and named as GarV-A (K). Homology of the CPs of

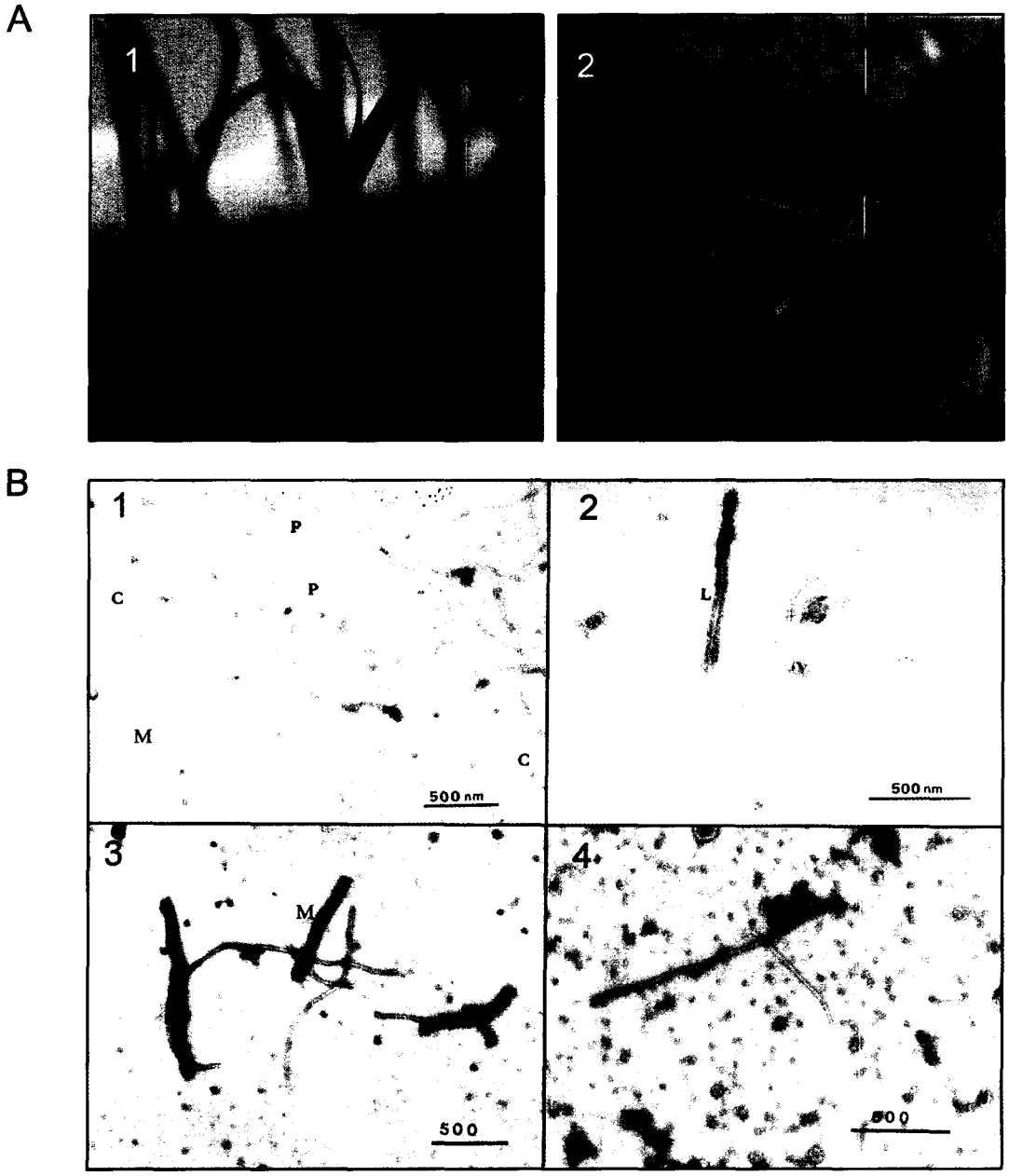

Fig. 2. Virus-free and virus-infected garlic plants. (**A**) Tissue cultured virus-free garlic plants (panel 1). Mosaic and streak symptoms in *Allium sativum* naturally infected with garlic viruses by mites (panel 2). (**B**) Garlic viruses. An electron microscope image of garlic viruses those were isolated from garlic leaves showing typical mosaic symptoms. Three different virus particles were observed (C, *Carlavirus*; M, GMbMV *Allexivirus*; P, *Potyvirus*) (panel 1). Immunosorbent electron microscopy of garlic viruses treated with antisera against LYSV-G of *Potyvirus* (panel 2), GMbMV of *Allexivirus* (panel 3), and GarLV of *Carlavirus* (panel 4). Bars represent 500 nm.

GarV-D (K) and GarV-A (K) was 73%. The C-terminus of the CPs was remarkably conserved in all the members of *Allexiviruses* (Fig. 4). The identities of CPs of GarV-D (K) with other GarVs were shared between 98% and 64% with GarVs. In addition, the identities of NABP (ORF 6) were shared from 87% to 53% with those of GarVs (Fig. 5).

The 27 kDa CP also shared the conserved amino acid sequences of GarV *Allexiviruses* (Fig. 5). The identities of NABPs of GarV-D (K) and -A (K) with other GarV viruses were from 99% to 54%. The homology of the NABPs of GarV-D (K) and GarV-A (K) showed 70% identity. As shown in Figure 4, the CP contains a conserved motif found in that of *Carlavirus*.

The 15kDa of NABPs of the two clones contained a zinc-finger domain. The core-like sequence structure of the zinc-finger domain (CX_2CX_7HXC) was found (Fig. 5). The

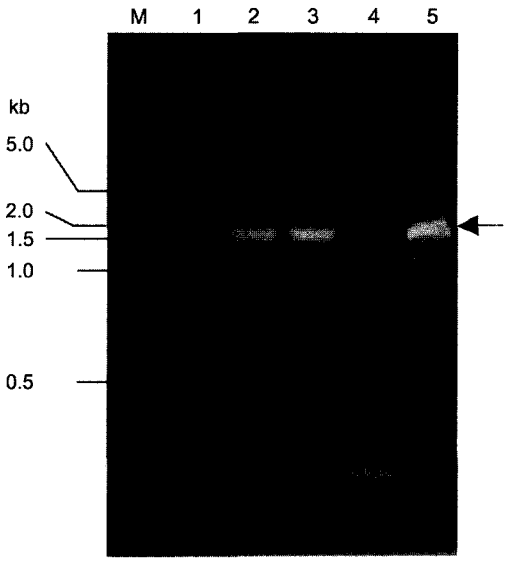

Fig. 3. Reverse-transcription polymerase chain reaction (RT-PCR) for garlic viruses using specific primers for each GarV. The cDNA fragments approximately 1.5 kb long were amplified using a universal primer and differential primers for each 3' sequence of *Allexiviruses*. Lanes indicate each universal primer and differential primer used for oligo d(T) (lane 1), Diff-A (lane 2), Diff-C (lane 3), Diff-G (lane 4), and Diff-T(lane 5). Lane M; 1 kb DNA marker.

basic amino acid cluster with arginine residues was conserved before the zinc-finger domain. These domains in NABPs were shared in plant virus (Sehnke et al., 1989).

Phylogenetic implications in the genus *Allexivirus*. In order to understand the phylogenetic relationships of the Korean isolated garlic viruses in the newly ratified genus *Allexivirus*, amino acids were aligned using the CLUSTAL W program. Results of phylogenetic analysis of amino acid comparison of CPs and NABPs produced a cluster dendrogram (Fig. 6). The alignments of the dendrogram showed that GarV-D (K) was grouped with GarV-D, whereas, clone GarV-A (K) was grouped with GarV-A. Therefore, it was concluded that viruses pdifA and pdifT were the first identified GarV-D and GarV-A species in Korea in the genus *Allexivirus*, respectively.

Discussion

The incidence of viral infection in traditional cultivation sites in Korea was surveyed, and members of the recently ratified genus *Allexivirus* were characterized. Based on the DTBIA and ISEM analyses, three different garlic viruses, GarV, LYSV-G, and GarLV, were detected in most surveyed sites (Fig. 1B). Among these identified viruses, *Allexivirus* was the most abundant, indicating that members of this virus were widespread in Korea. In symptomology and immunological analyses, it was observed that mixed-

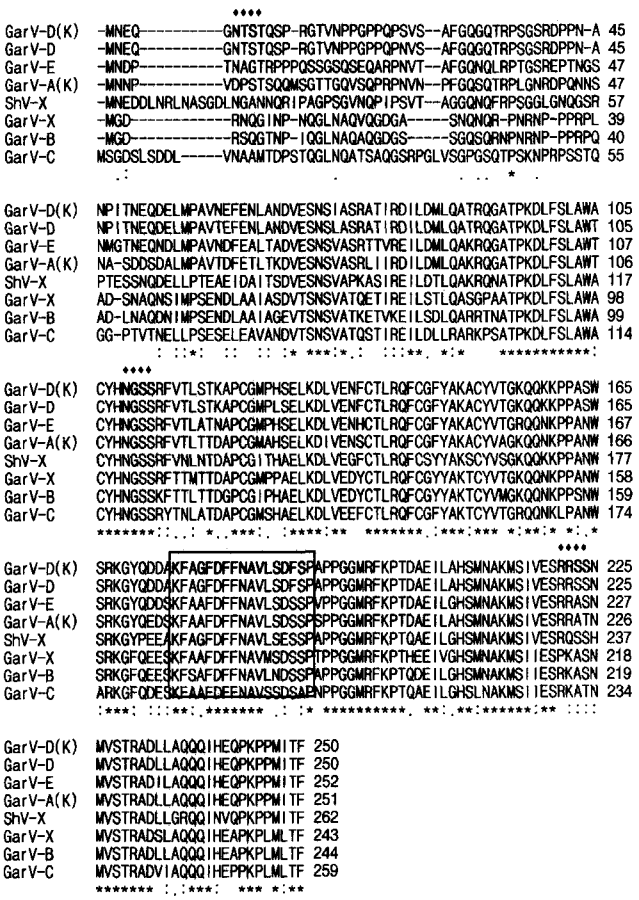

Fig. 4. Multiple alignments of the amino acid sequences of the coat proteins of *Allexivirus*. The identical amino acids are marked with an asterisk (*) and equivalent amino acid marked with dots (: and .). Gaps (–) were introduced for maximum homology using the CLUSTAL alignment program. Motifs were searched using the GCG program. Three glycosylation sites were marked with diamond (◆). A motif found in coat proteins of *Carlavirus* was boxed. Database accession numbers for the selected *Allexiviruses* are GarV-D (K) (AF519572), GarV-D (AB010303), GarV-E (AJ292230), GarV-A (NC_003375), GarV-A (K) (AF478197), ShV-X (L76292), GarV-X (AJ292229), GarV-B (AB010301), GMbMV (D49443), and GarV-C (NC_003376).

virus infected garlic plants showed the most severe symptoms. This proved that complex infection of the viruses caused malignant disease symptoms. Tissue cultured virus-free garlic plants were used as control to measure the strength of symptoms in this survey. These virus-free garlic plants did not show any symptoms in the first year after field growth. However, from the second year, virus symptoms were observed in 50% of the garlic plants (data not shown), indicating that aphid- and mite-mediated viral transmissions are unavoidable. Six different garlic viruses (GarV-A, -B, -C, -D, -E, and -X) have been identified as *Allexivirus* in East Asia (Chen et al., 2001; Sumi et al., 1999). However, only GarV-X was identified

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GarV-D(K)  MHPHDFNLLCCLHFSQPSLPKDLKEFIFSHCVDCCKLVRKINQNKPFQGIXKCAKRRRAK 60
GarV-D     MHPHDFNLLCCLHFSQPPLPKDLKEFIFSRCVDCCKLVRRTNQNKPFQGVSKCAKRRRAK 60
GarV-E     MHPHDFNLLCCLHFSPNHLPKDLQILIFSRVTTECKL-RRLSENKPFRGRSKFAMRRRAK 59
GarV-A(K)  MIPQDFNILCCLHFAKPFIPQDLKAHLFFTCVNECKLVRIARENKPFLGTSKCAQRRRAK 60
ShV-X      MHPHDLNLLCCLHFSKPSLPNDLKTLLFRACETSCKLNRRLLDNKPFQGTSKCAKRRRAK 60
GarV-X     MHSYDFNLLACLQFSKPTLPSDVRIIIYNLTFDSRKLGRKAQQNKPFTGTSKCAARRRAK 60
GarV-B     MHTYDFNFLACLQFAQPNLPSDVRISIYMLSSASRKIGRKSQQNKPFTGTSKCAARRRAK 60
GarV-C     MHPYNLNLLCCLHFSKPTLPLELRLYIYNLAAPNLLLGRKMLQNKPFQGTSKCAARRRAK 60

GarV-D(K)  RYNRCFDCGAFLVDGHRCKVFVSKAHSDVLAVIHEGPVKLYAERSYRPNSDAAQLIENDI 120
GarV-D     RYNRCFDCGAFLVDGHRCKVFVSKAHSDVLAVIHEGPAKLYAERSYRPNSDAAQLIENDI 120
GarV-E     RYNRCFDCGAVLIDDHQCKVLTSRAQSDVLTVIREGPAKLYAERTYRPNSDAARLIEDDI 119
GarV-A(K)  RYNRCFECGAYLLDNHKCRIFVSRAQSDVLAVIHEGPAKLHAERTYRPNSDAALLIESDL 120
ShV-X      RYNRCFDCGAYLYDDHVCKRFTSRSNSDCLSVIHQGPAKLYAEGAYRANSDAEQLIMNDM 120
GarV-X     RYGRCFDCGALLEANHVCKLFTTRSNSDCLSVIHEGPAKLYAERSFRKSSFAEQLIRNDL 120
GarV-B     RYNRCFDCGALLNTDHVCKLFTSRASTDCLHVIREGPAKLYAERTFRKSSFAEQLILDDL 120
GarV-C     RYNRCFDCGGYLLNNHVCKQFPTRASTSCFNVIHEGPAKLYAEGSFRRDSFAEQLILNDL 120

GarV-D(K)  LYIKALKL--- 128
GarV-D     LYIRALKL--- 128
GarV-E     LYIKTLKL--- 127
GarV-A(K)  QYIKLFQNRKA 131
ShV-X      LLIKSLKL--- 128
GarV-X     QLMKLYK----- 127
GarV-B     ELMKLYE----- 127
GarV-C     ELMKLHNN--- 128
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Fig. 5. Multiple alignments of the amino acid sequences of ORF-VI of cDNA of *Allexivirus*. The amino acids marked asterisk (*) and the dots (: and .) indicate identical and equivalent amino acid, respectively. Gaps (–) were introduced for maximum homology using the CLUSTAL alignment program. Motifs were searched using GCG program. A phosphotidyl site is in bold and lined. A zinc-finger motif, CX_2CX_7HXC, was boxed. Three glycosylation sites are marked with diamond (◆). A motif found in coat proteins of *Carlavirus* was boxed.

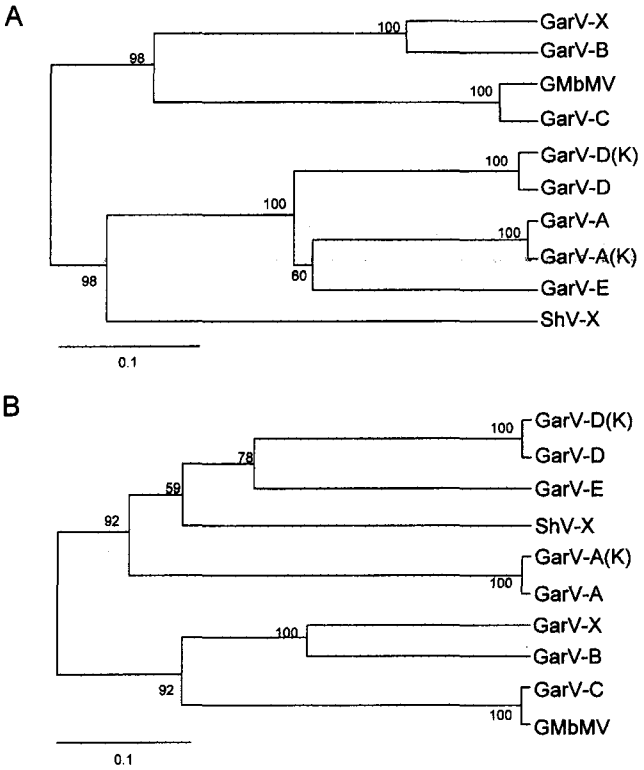

Fig. 6. Phylogenetic trees of the amino acid sequences of coat proteins (**A**) and ORF6 NABP (**B**) of members of the genus *Allexivirus* obtained. Phylogenetic analysis were performed with CLUSTAL W. Trees were constructed by cluster algorithm using neighbor analysis. The values at the forks indicate bootstrap scores out of 100 replicates. The scale bar shows the number of substitutions per base. Selected garlic viruses for analysis were the same accession numbers used in alignments in figures 3 and 4. Clone pdifA was renamed GarV-D1 (AF519572) and clone pdifT was renamed GarV-A (AF478197).

(Song et al., 1997) in Korea. In this report, two more members of GarVs, GarV-A, and -D, were found in Korea. This suggests that all members of the genus *Allexivirus* may exist in mixed infection in garlic plants in the country. It was also found that GarV-A and -D contained specific motifs found in the coat proteins of the genus *Carlavirus*. The 15 kDa NABP contained a zinc-finger domain. These domains in the NABP are shared in plant viruses including *Carlaviruses* and *Tobraviruses* (Haylor et al., 1990; Rupasov et al., 1989; Sehnke et al., 1989). This suggests that the genus *Allexivirus* may be closely related with *Carlavirus*. Interestingly, all three viruses found in Korea were grouped distinctly in the cluster dendrogam. Phylogenic trees based on alignments of the coat protein and NABP showed that the isolated GarV-D and GarV-A were grouped with GarV-E and relatively related with ShV-X (Kanyuka et al., 1992), whereas, GarV-X was grouped with GarV-B and -C (Fig. 5).

Through traditional vegetative propagation using seed bulbs, GarVs of *Allexivirus* have been transmitted through generations resulting to a substantial diversity. It is predicted that the common ancestor of *Allexivirus* might have been introduced to the closely related host garlic strains, which were introduced in China, Korea, and Japan. In conclusion, it was found that the two new members of the genus *Allexivirus* were the most abundant in the complex mixtures of viruses infecting garlic plants in Korea, indicating that members of the genus *Allexivirus* were widespread in the country.

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