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Allexivirus Transmitted by Eriophyid Mites in Garlic Plants

KANG, SANG GU[1,2], BONG JIN KOO[2], EUN TAG LEE[1,2], AND MOO UNG CHANG[2,3]*

[1]*Molecular Genetics Laboratory, Department of Biotechnology, School of Biotechnology, Yeungnam University, Gyeongsan 712-749, Korea*
[2]*Institute of Biotechnology, Yeungnam University, Gyeongsan 712-749, Korea*
[3]*Department of Biology, Yeungnam University, Gyeongsan 712-749, Korea*

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Abstract Viruses in garlic plants (*Allium sativum* L.) have accumulated and evolved over generations, resulting in serious consequences for the garlic trade around the world. These viral epidemics are also known to be caused by aphids and eriophyid mites (*Aceria tulipae*) carrying *Potyviruses*, *Carlaviruses*, and *Allexiviruses*. However, little is known about viral epidemics in garlic plants caused by eriophyid mites. Therefore, this study investigated the infection of garlic plants with *Allexiviruses* by eriophyid mites. When healthy garlic plants were cocultured with eriophyid mites, the leaves of the garlic plants developed yellow mosaic strips and became distorted. In extracts from the eriophyid mites, *Allexiviruses* were observed using immunosorbent electron microscopy (ISEM). From an immunoblot analysis, coat proteins against an *Allexivirus* garlic-virus antiserum were clearly identified in purified extracts from collected viral-infected garlic plants, eriophyid mites, and garlic plants infected by eriophyid mites. A new strain of GarV-B was isolated and named GarV-B Korea isolate 1 (GarV-B1). The ORF1 and ORF2 in GarV-B1 contained a typical viral helicase, RNA-directed RNA polymerase (RdRp), and triple gene block protein (TGBp) for viral movement between cells. The newly identified GarV-B1 was phylogenetically grouped with GarV-C and GarV-X in the *Allexivirus* genus. All the results in this study demonstrated that eriophyid mites are a transmitter insect species for *Allexiviruses*.

Keywords: Garlic virus (Gar-V), garlic plant (*Allium sativum* L.), *Aceria tulipae*, *Allexivirus*, triple gene block protein (TGBp)

As garlic plants (*Allium sativum* L.) are vegetatively propagated, viruses in garlic plants have accumulated and evolved over generations [7, 15, 28], with widespread detrimental results on the garlic industry around the world [9, 11, 32].

*Corresponding author
Phone: 82-53-810-2374; Fax: 82-53-816-8498;
E-mail: muchang@ynu.ac.kr

The three major garlic viruses are the garlic mosaic virus (GMV) from the *Potyvirus* genus, garlic latent virus (GarLV) from the *Carlavirus* genus, and garlic virus (GarV) from the *Allexivirus* genus, all of which have been abundantly detected in garlic plants [3, 4, 17, 20, 22, 27, 29, 31].

The *Allexivirus* GarV is a flexuous rod-shaped virus containing a single-stranded positive-sense polyadenylated RNA genome [5, 17, 23, 28]. In addition to this RNA genome, several other *Allexiviruses*, including GarV-A [28], GarV-B [29], GarV-C [28], GarV-D [29], GarV-X [5, 27], and GarV-E [4], have also been reported, consisting of six ORFs [28]. ORF 1 encodes an approximately 190 kDa replicase, containing methyl transferase, helicase, and RNA-dependent RNA polymerase motifs for viral replication; ORF2 and 3 encode approximately 27 kDa and 11 kDa proteins, respectively, where each is in a different reading frame of a putative triple gene block protein (TGBp) involved in cell-to-cell movement; ORF 4 encodes 40 kDa serine-rich proteins with no homology to any other reported viral proteins; ORF 5 encodes a 27 kDa protein of a viral coat protein; and ORF 6 encodes a 15 kDa protein of a nucleic-acid binding protein (NABP) [17, 27, 28].

Aphids are major vectors for *Potyviruses* and *Carlaviruses* [13, 32, 33]. However, a previous report suggested that eriophyid mites (*Aceria tulipae*) also represent a major vector [31], yet little is known about the epidemics and family distribution of the *Allexivirus* genus in garlic plants. Accordingly, this paper explores the role of the eriophyid mite as a possible significant vector of *Allexiviruses*, plus a new strain, GarV-B1, was obtained from garlic plants co-infected with the mites.

Materials and Methods

Viral Diseases in Garlic Plants

Garlic plants showing viral disease symptoms were randomly collected from 41 major garlic-farming sites in Korea. The garlic

plant symptoms included mosaic patterns, stripes, yellow color, and stunted growth. The garlic leaves and bulbs were harvested and used for viral isolation and serological tests.

Viral Infection by Mites

To produce viral-free garlic plants, garlic bulbs were prepared by heat treatment at 37°C for 14 days. The heat-treated garlic bulbs were then germinated and grown with or without eriophyid mites in isolated pots.

Transmission Electron Microscopy (TEM) and Immunosorbent Electron Microscopy (ISEM)

The viral particles were observed under a transmission electron microscope (Hitachi H-600) using the direct negative (DN) staining method [10] and trap decoration method. The DN method was performed using cell extracts from ground leaf fragments with 2% (w/v) phosphotungstic acid (PTA), pH 7.0. The immunosorbent electron microscopy (ISEM) was then conducted as described previously [6, 19]. Briefly, antisera of the garlic mite-borne virus (Aomori Bio Greencenter, Japan) were diluted (1:1,000) and placed on a grid. The partially purified viruses were then extracted in a 0.1 M potassium phosphate buffer, added to the grid with the diluted antisera, incubated for 15 min, and washed with the phosphate buffer (pH 7.0). Finally, the viruses were stained with 2% uranyl acetate and observed under a transmission electron microscope.

Purification of Viral Particles and Isolation of Viral RNA

The viruses were isolated from the virus-infected garlic bulbs using CsCl-sucrose gradient sedimentation, as described by Yamashita *et al.* [34]. The viral RNA was then extracted from the purified viruses and treated with 2% bentonite, 50 mg/ml proteinase K, 2% sodium dodecyl sulfate, and 25 mM EDTA for 30 min at 37°C, followed by phenol/chloroform extraction and ethanol precipitation. The viral RNA was estimated by separation using 0.8% agarose gel electrophoresis under denaturing conditions with 2.2 M formaldehyde.

Cloning Viral cDNA and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

An RT-PCR was performed using primers designed for partial sequences of replicase and the TGB1 protein based on garlic virus sequences. The viral cDNA synthesis was carried out using an oligo d(T) primer with AMV reverse transcriptase (Gibco BRL, Rockville, U.S.A.) at 42°C for 45 min [26]. Any cDNA encoding a viral replicase and TGBp from garlic viruses was amplified by a PCR using a combination of degenerate primers: 5'-RGXCTTGTTAA-CCTCATGGRX-3' and 5'-XYGTTTGAGTGCTCAGTT-GYX-3' (X=A, T, C, G; Y=T, C; R=A, G). The PCR was 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 primer, and 2.5 units of *Taq* DNA polymerase in a 50 μl reaction mixture. The thermal cycle was 2 min at 95°C, followed by annealing at 55°C for 1.5 min and polymerization at 72°C for 1 min. A total of 30 cycles was completed, 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 the appropriate restriction enzymes, ligated into a T-Easy cloning vector (Promega, Madison, WI, U.S.A.), and transformed into XL1-Blue cells. The plasmids that contained cDNA inserts were selected for nucleotide sequencing, and the sequencing reactions performed with an ABI Prism 3100 DNA sequencer using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Forster City, CA, U.S.A.). The analyses of the nucleotide and the deduced amino acid sequences were performed using the BLAST program at NCBI [2], CLUSTAL W, and GCG (Genetics Computer Group, Madison, WI, U.S.A.) programs. The phylogenetic analyses were performed using the PHYLIP program. The sequences determined in this study have since been included in the DDBJ/GenBank/EMBL databases under the access number EF596816.

Protein Extraction and SDS-Polyacrylamide Gel Electrophoresis

Five-hundred μg of garlic leaves was harvested and ground in 3 ml of an extraction buffer [5 mM sodium phosphate buffer (pH 7.0), 1% (v/v) diethylcarbonate, 2% sodium dodecyl sulfate, 5% 2-mercaptoethanol]. The crude homogenates were centrifuged for 20 min at 4°C at 9,000 ×*g*, and then the supernatants were transferred to a new tube, leaving the cell debris behind, centrifuged again at 20,000 ×*g* for 20 min, and stored at −80°C until use. The protein contents in the final supernatants were determined using a Bradford assay (Bio-Rad, Hercules, CA, U.S.A.). For the SDS-PAGE analysis, 50 μg of the protein extracts was boiled for 3 min with 2-mercaptoethanol and loaded onto each lane of 12% acrylamide. Identical sets of SDS-PAGE were performed, and then one gel was stained for 1 h with Bio-Safe Coomassie blue (Biorad, Hercules, CA, U.S.A.) and destained with a destaining solution to visualize the bands, whereas the second gel was used directly for an immunoblotting analysis.

Immunoblotting Analysis

Nitrocellulose membranes were prewetted in 100% methanol, washed in distilled water for 5 min, and equilibrated in a transfer buffer for 10 min before the immunoblotting. The electrotransfer was performed in 25 mM Tris, 250 mM glycine, 0.1% SDS, and 20% methanol (pH 8.3) at 18 V, 350 mA for 12 h. The membrane was then 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 low speed. Thereafter, the membranes were rinsed briefly using two changes of a TTBS wash buffer. A rabbit antiserum against a garlic virus was diluted in TTBS (1:5,000) as the primary antibody and the membrane incubated for 1 h at room temperature with slow rotation. For the immunoblotting control, the rabbit antiserum against leek yellow-strip potyvirus-G (LYSV-G) (Aomori Bio Greencenter, Japan) was diluted in TTBS (1:5,000) and applied to the same garlic virus immunoblot. The membranes were initially rinsed with two changes of the wash buffer, and then washed in the wash buffer for 15 min at room temperature. For immunological detection, an anti-rabbit IgG horseradish peroxidase (HRP)-labeled secondary antibody (Amersham Biosciences) was diluted (1:2,000) in PBS, and the membranes incubated in the secondary antibody for 1 h at room temperature with slow rotation. The membranes were initially rinsed with two changes of the wash buffer, and then washed in the wash buffer for 5 min at room temperature, where the latter was repeated three times with fresh changes of the wash buffer. The reactions were detected using the ECL Western blotting detection reagent, as described in the ECL protocol (Amersham Biosciences), followed by exposure on X-ray film and development.

RESULTS

Symptoms of Viral-infected Garlic Plants

Viral infections were investigated in garlic plants from 41 different garlic-growing fields across Korea. For most of the sites, the viral diseases found in the garlic plants had already been identified. Whereas the leaves of the healthy garlic plants were thick, straight, and green under greenhouse conditions (Fig. 1A), the typical symptoms of the viral-infected garlic plants included retarded growth, plus curled yellow-strip mosaic patterns and distorted symptoms on the leaves (Fig. 1B). Some of the viral-infected garlic plants showed severely retarded leaf development (Fig. 1E) compared with the leaves of the healthy garlic plants (Fig. 1C). Furthermore, among the severely virus-infected garlic plants, eriophyid mites were exclusively found on the leaves (Figs. 1D and 1G), indicating that mites may be one of the main insect-vector transmitters of garlic viruses. Therefore, it was examined whether virus infection would occur in unaffected garlic plants cocultured with garlic mites. A virus transmission analysis revealed that the garlic plants cocultured with eriophyid mites developed curled, yellow-strip mosaic, and distorted symptoms on the leaves (Fig. 1F), whereas the leaves of the healthy garlic plants were straight, thick, and well developed (Fig. 1C), thereby confirming that eriophyid mites are one of the major insect vectors for *Allexiviruses*.

Isolation of Garlic Viruses from Garlic Plants Inoculated with Eriophyid Mites

The virus-infected garlic leaves revealed the development of large inclusion bodies, which became fully aggregated in the cytoplasm of the epidemic cells (Figs. 2A and 2B). The viruses from the virus-infected garlic plants were purified and further investigated using an antiserum against the garlic virus (GarV) from the *Allexivirus* genus. Rod-shaped and highly flexuous particles of about 600–800 nm in length were observed using immunosorbent electron

Fig. 1. Healthy and viral-diseased garlic plants and eriophyid mites.
A. Virus-free garlic plants in greenhouse; **B.** Field-grown garlic plants showing viral disease; **C.** Virus-free garlic plant leaf; **D.** Eriophyid mites: adults (m) and eggs (e); **E.** Comparison between viral-diseased (1) and healthy (2) garlic plants; **F.** Garlic plants infected with garlic mites; **G.** Eriophyid mites [17]. Note: the symptoms of the garlic plants with naturally infected garlic viruses were retarded growth and curled yellow-strip mosaic leaves.

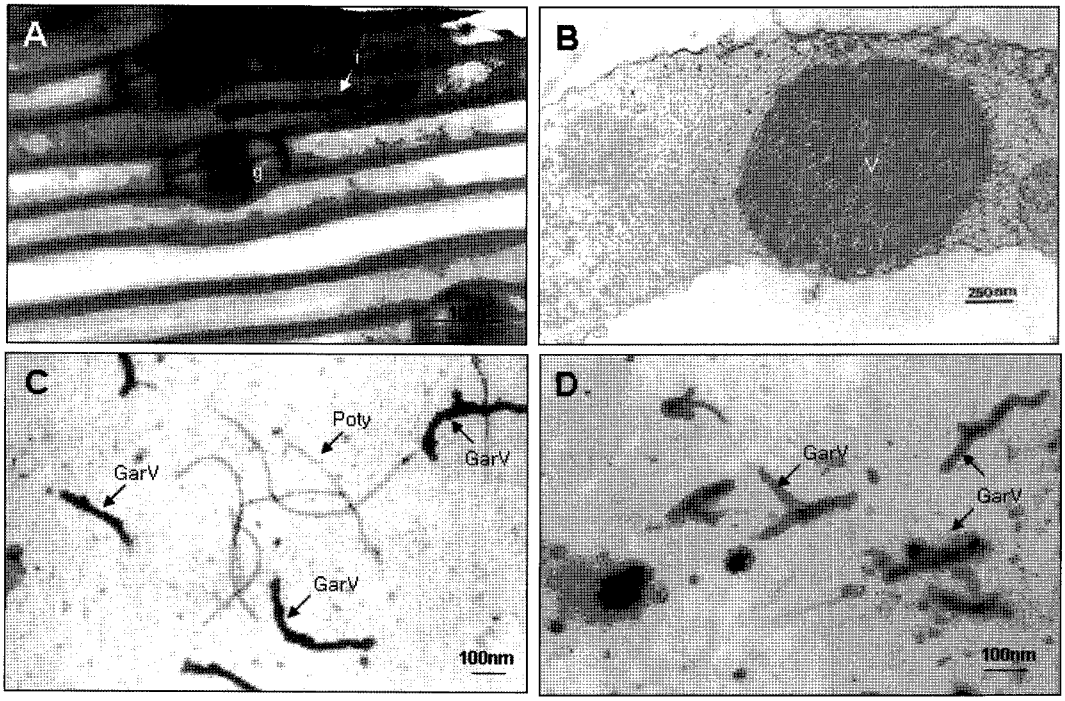

Fig. 2. Garlic viruses from garlic plants inoculated with mites.
A. Viral inclusion bodies accumulated in epidermal cells of leaf. Arrows with i and g indicate viral inclusion bodies and guard cells, respectively. **B.** Viral particles (v) aggregated in epidermal cells. **C.** Immunoelectron microscopy showing viral particles of garlic viruses (M) from garlic plants when using GarV antiserum. GarV indicates an *Allexivirus* and Poty indicates a *Potyvirus*, where the latter are not decorated with the GarV antiserum. **D.** Immunoelectron microscopy showing viral particles isolated from *A. tulipae*.

microscopy (ISEM) (Fig. 2C). However, under the ISEM, certain viruses were not decorated by the garlic-virus antiserum, suggesting that they were from the *Potyvirus* genus. The presence of the garlic virus from the *Allexivirus* genus was also investigated in the guts of *A. tulipae* using the same garlic-virus antiserum, and the results showed that the aggregated flexuous filamentous particles were decorated with the antiserum (Fig. 2D), suggesting that *A. tulipae* is a major transmitter of the garlic virus from the *Allexivirus* genus.

Immunoblot Analysis of Purified Viruses Extracted from Garlic Plants and Eriophyid Mites

Immunoblot analyses of the purified viruses extracted from the virus-infected plants and eriophyid mites were performed to confirm whether the mite is a true host of the *Allexivirus*. Fig. 3 shows that the protein extracts from the purified viruses and plants specifically cross-reacted with the antiserum for the *Allexivirus*. Extracts from the purified *Allexivirus* from the garlic plants cocultured with mites, adult eriophyid mites, and viral-diseased garlic plants collected from the farm fields were cross-reacted with the antiserum for the *Allexivirus*. In the virus-infected garlic plants, 28–30 kDa of coat proteins from GarV of the *Allexivirus* were clearly identified (Fig. 3). However, the extracts from the CMV virus, virus-free healthy garlic plants, and *Allium thunberaii* did not cross-react with the same antiserum. For the immunoblot control, a duplicated SDS-PAGE gel membrane with antiserum for LYSV-G was used, and no *Allexivirus* strain cross-reacted.

Virus Purification and Cloning Viral RNA Genome of GarV-B1 from Eriophyid Mite-infected Garlic Plants

The garlic virus from the virus-infected garlic leaves cocultured with eriophyid mites was purified using CsCl-sucrose gradient sedimentation. The viral RNA genome was then cloned using a reverse transcription PCR, and the nucleotide sequences identified as GarV-B strain Korea isolate 1 and named GarV-B1 (GenBank Accession No. EF596816). Fig. 4 shows a schematic diagram of the partial sequence of ORF1 and ORF2 from the GarV-B1 isolated in this study. The deduced amino acid sequences of the cloned viral cDNA contained the domains of a viral helicase, RNA-dependent RNA polymerase (RdRp) of ORF1, and triple gene block protein (TGBp) of ORF2 (Fig 4).

Replicase of GarV-B1 and Phylogenetic Analysis

The deduced amino acid sequences of the partial ORF1 of the cloned viral RNA in this study showed a 97% homology in 474 amino acid sequences with the GarV-B replicase (GenBank ID 2826153), including the viral helicase and RdRp [28] (Fig. 5). A further amino acid

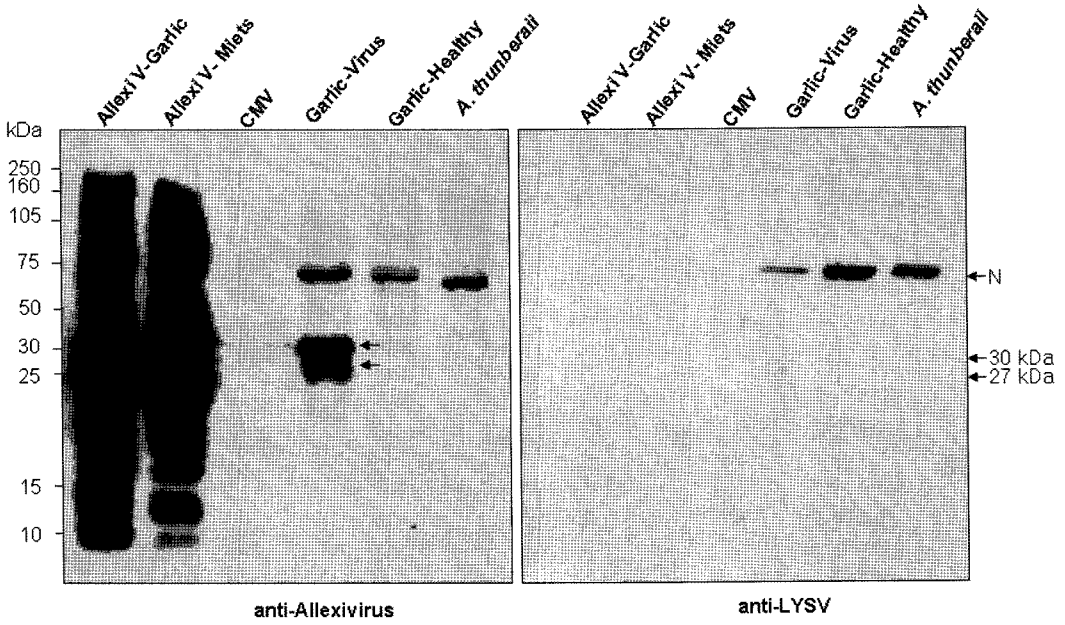

Fig. 3. Immunoblotting analysis of purified viruses extracted from virus-infected plants.
The protein extracts from the purified viruses and plants were assayed using an *Allexivirus* antiserum (left panel) and LYSV antiserum (right panel). Allexi V-Garlic, extract from the purified *Allexivirus* from the garlic plants cocultured with mites; Allexi V-mites, mites; CMV, CMV virus; Garlic-Virus, viral-diseased garlic plants collected from farm fields; Garlic-Healthy, virus-free healthy garlic plants; and *A. thunberaii, Allium thunberaii*. 30kDa and 27 kDa coat proteins are indicated by arrows. N indicates a nonspecific cross-reacted protein from plants.

comparison revealed a more than 76% homology with the *Allexivirus* genus family, including GarV-C (NP_569132), GarV-X (CAC83699), GarV-E (NP_659010), GarV-A (NP_569126), and ShV-X (NP_620648) (Fig. 6A). Among the domains contained in ORF1 from GarV-B1, the conserved motifs revealed a typical viral helicase and RdRp from the *Allexivirus* and *Potexvirus* genera. Thus, using the deduced amino acid sequences of the helicase and RdRp, the phylogenetic relationships of GarV-B1 produced a cluster dendrogram in the genus *Allexivirus* (Fig. 6B). The alignments of the dendrogram showed that GarV-B1 was grouped with GarV-C and GarV-X, whereas GarV-E, GarV-A, and ShV-X were grouped separately.

ORF2 of GarV-B1 and Phylogenetic Analysis

The ORF2 of GarV-B1 showed a significant amino acid sequence homology to the triple gene block protein 1 (TGBp1) of GarV-B (BAA61813) (Fig. 6A). Furthermore, the TGBp1 of GarV-B showed a more than 80% homology with GarV-X (NP_044572), GarV-C (NP_569133), ShV-X (NP_620649), GarV-D (BAA74945), GarV-E (NP_659011), and GarV-A (NP_569127) (Fig. 7A). The molecular mass of the putative TGBp1 protein of GarV-B1 was expected to be 27 kDa. Within the *Allexivirus* genus, the phylogenetic

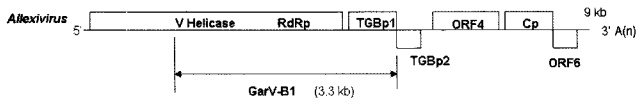

Fig. 4. Schematic diagram of the genome organization of *Allexivirus*. The nucleotide sequences of GarV-B1 cloned in this study are indicated with arrows. Helicase, viral helicase; RdRp, RNA-dependent RNA polymerase; TGBp, triple gene block proteins; ORF, open reading frame; Cp, coat protein.

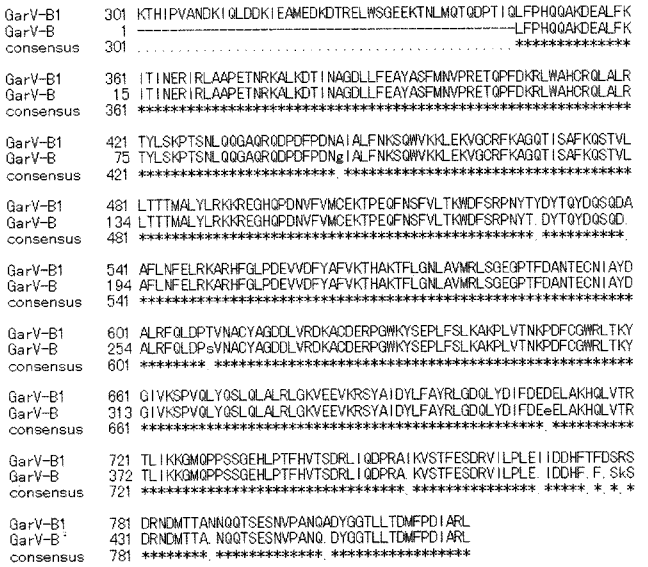

Fig. 5. Alignment of the amino acid sequences of replicases of GarV-B1 with GarV-B.

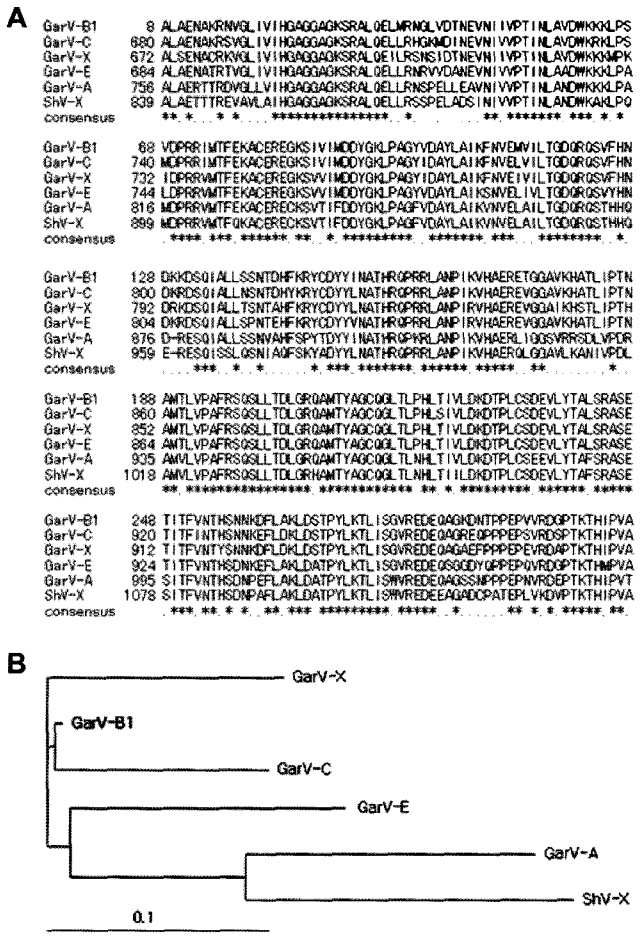

Fig. 6. Multiple alignments of amino acid sequences for replicases from *Allexivirus* and their phylogenetic relationships.
A. Identical amino acids are marked with an asterisk (*) and equivalent amino acids are marked with a dot (.). Gaps depicted as dashes (-) were introduced for maximum homology using the CLUSTAL W alignment program. **B**. Phylogenetic tree of viral replicases from *Allexivirus*. The trees were constructed using a cluster algorithm based on a neighbor analysis. The values at the forks indicate bootstrap scores out of 100 replicates. The scale bar shows the number of substitutions per base. The scale bar indicates a 0.1% substitution per amino acid site. The database accession numbers for the selected *Allexiviruses* are GarV-B1, EF596816; GarV-C, NP_569132; GarV-X, CAC83699; GarV-E, NP_659010; GarV-A, NP_569126; and ShV-X, NP_620648.

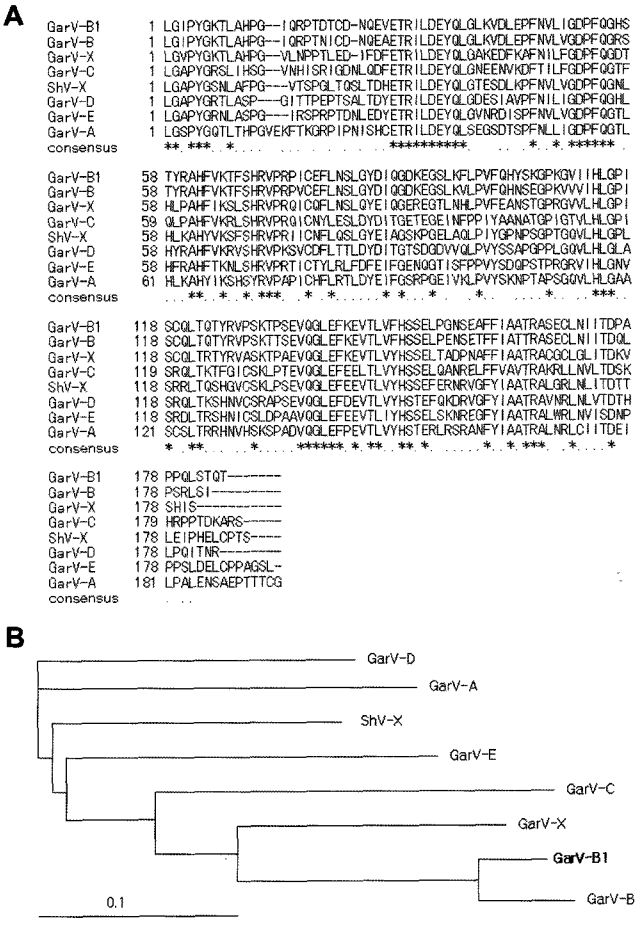

Fig. 7. Multiple alignments of amino acid sequences of triple gene block protein 1 (TGBp1) in the ORF2 from *Allexivirus* and their phylogenetic relationships.
A. Alignment of TGBp1 sequences. The amino acids marked with an asterisk (*) or dot (.) indicate an identical or equivalent amino acid, respectively. Gaps (-) were introduced for maximum homology using the CLUSTAL W alignment program. **B**. Phylogenetic tree of TGBp1 from *Allexivirus*. The scale bar indicates a 0.1% substitution per amino acid site. The database accession numbers for the selected *Allexiviruses* are GarV-B1, EF596816; GarV-B, BAA61813; GarV-X, NP_044572; GarV-C, NP_569133; ShV-X, NP_620649; GarV-D, BAA74945; GarV-E, NP_659011; and GarV-A, NP_569127.

relationships of GarV-B1 based on the amino acid sequence homologies of TGBp1 showed a close relationship with GarV-X and GarV-C (Fig. 7B).

DISCUSSION

The widespread occurrence of viral diseases in garlic plants across Korea has resulted in serious economic loss, as observed in other countries [9, 11, 33]. The ubiquitous viral diseases in garlic plants may be caused by the accumulation and evolution of viruses in garlic plants attributable to repeated vegetative propagation in garlic farming.

As shown in Fig. 1, the symptoms of the virus-infected garlic plants collected from farm fields included curled leaves and yellow-mosaic symptoms on the leaves, resulting in severely retarded growth caused by multiple viral infections, including the garlic mosaic virus (GMV) from the *Potyvirus* genus, garlic latent virus (GarLV) from the *Carlavirus* genus, and garlic virus (GarV) from the *Allexivirus* genus [4, 17, 27, 29]. Previous research has suggested that many viruses, including the onion mite-borne latent virus (OMbLV), shallot mite-borne latent virus (SMbLV), and garlic virus (GarV) are transmitted by

eriophyid mites [29, 30, 33]. This study also found that garlic plants cocultured with eriophyid mites produced curled and yellow-mosaic strips on the leaves, indicating that the mites are major insect vectors.

The morphological properties of the garlic virus from the *Allexivirus* genus isolated in this study were rod-shaped and flexuous particles of about 600–800 nm in length (Fig. 2). In extracts from the viral-diseased garlic plants and eriophyid mites, antiserum to the garlic-mite-borne mosaic virus cross-reacted with the flexuous filamentous particles in an ISEM analysis (Figs. 2C and 2D). As shown in Fig. 2, members of the *Allexivirus* genus and *Potyvirus* genus were both found in the virus-infected garlic plants, supporting the idea that a complex mixture of viruses from the *Potyvirus*, *Carlavirus*, and *Allexivirus* genera are the major virus groups infecting garlic plants [4, 17, 20, 27, 29, 31]. However, in the extracts from the eriophyid mites, large aggregates of viruses were highly decorated with the *Allexivirus* garlic-virus antiserum (Fig. 2D), indicating that eriophyid mites are a host of garlic viruses from this genus. Furthermore, coat proteins against the garlic-virus antiserum were clearly identified in the purified extracts from the virus-infected garlic plants collected from the farms, eriophyd mites, and garlic plants infected with the mites (Fig. 3).

The molecular mass of the coat protein from the *Allexivirus* garlic virus was approximately 27–28 kDa, as derived from the deduced amino acid sequences [17, 27, 29). However, after a further immunoblotting analysis, 27 kDa and 30 kDa coat proteins from garlic viruses were found in the leaf extracts from the garlic plants cocultured with mites (Fig. 3). The 30 kDa protein was also considered to be a coat protein from the garlic viruses, and the molecular mass of the coat proteins was calculated based on the deduced amino acid sequences, which slightly increased the protein mass, as frequently observed on an SDS-PAGE gel, because of its high hydrophilic properties. Similar to the present observation, a size discrepancy for coat proteins was previously reported in an immunoblotting analysis of ShV-X, GMbMV, and the tobacco streak virus [15, 25, 34].

The partial genome of a new GarV-B1 virus was isolated and sequenced from the virus-infected garlic plants with eriophyid mites. In the ORF1 of GarV-B1, the conserved motifs showed a typical viral helicase and RdRp from the *Allexivirus* and *Potexvirus* genera. The RNA helicases in a single-stranded RNA (ssRNA) virus are thought to be involved in duplex unwinding during viral RNA replication [1, 12, 24]. RdRp (E.C. 2.7.7.48) is an essential protein in most ssRNA viruses with no DNA stage, and catalyzes the synthesis of a complementary RNA strand to virus RNA as a template [21]. This has also been found in positive-strand ssRNA viruses, such as *Potexvirus*, *Bromovirus*, and *Tobamovirus* [8, 14]. The genome of the GarV-B1 newly identified in this study contained the movement protein TGBp, which interacts with the plasmodesmata of plant cells and functions to allow the movement of viral particles from cell to cell [8]. This feature of TGBp in GarV-B1 is shared among all the garlic viruses (GarV) in the *Allexivirus* genus. These viral movement proteins are known to exist as a polypeptide in *Capilovirus*, *Tymovirus*, and *Vitivirus* or triple gene block (TGB) in *Allexivirus*, *Carlavirus*, and *Potexvirus*.

Using the deduced amino acid sequences of the helicase and RdRp, the phylogenetic relationships of GarV-B1 produced a cluster within the genus *Allexivirus*. Moreover, the alignments of the dendrogram showed that GarV-B1 was grouped with GarV-C and GarV-X, whereas GarV-E, GarV-A, and ShV-X were associated in a separate group. This grouping was further supported by a phylogenetic analysis using TGBp1 proteins, as the TGBp1 protein of GarV-B1 was also grouped with GarV-X and GarV-C (Fig. 7B).

Previous studies have shown that three main garlic viruses are the widespread cause of garlic plant infections across Korea, including GarV from the *Allexivirus* genus, LYSV-G from the *Potyvirus* genus, and GLV from the *Carlavirus* genus, all of which seem to be present in a complex mixture, even in single garlic plants showing viral infection [17]. To date, six different garlic viruses (GarV-A, -B, -C, -D, -E, and -X) have been identified from the *Allexivirus* genus [4, 28], and GarV-A, -B, -D, and -X have all been found in Korea [17, 27]. However, these viruses are not only amplified by long-term vegetative propagation, but also by transmission through aphids and plant eriophyid mites, resulting in serious economic loss [9, 11, 16, 17, 27, 33]. Thus, it is highly recommended that garlic farmers take measures to control aphids and mites.

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