

Differentiation Among Garlic Viruses in Mixed Infections Based on RT-PCR Procedures and Direct Tissue Blotting Immunoassays

Tadamitsu Tsuneyoshi and Shin-ichiro Sumi

Institute for Biotechnology Research, Wakunaga Pharmaceutical Co., Ltd., 1624 Shimokotachi, Koda-Cho, Takata-Gun, Hiroshima 739-11, Japan.

We thank S. Kasuga (OTC Institute) for making the electron microscopic observations. We also thank K. Yamashita (Aomori Green Bio Center) and Y. Ikeda (Hiroshima Prefectural Agriculture Research Center) who provided the virus isolates.

Accepted for publication 6 November 1995.

ABSTRACT

Tsuneyoshi, T., and Sumi, S. 1996. Differentiation among garlic viruses in mixed infections based on RT-PCR procedures and direct tissue blotting immunoassays. *Phytopathology* 86:253-259.

A total of six viruses belonging to at least three distinct genera, *Potyvirus*, *Carlavirus*, and a novel unclassified genus, were identified from infected garlic plants in Japan based on partial cDNA cloning and sequencing of genomes. We developed procedures that combined reverse-transcription polymerase chain reaction (RT-PCR) with restriction analysis for identification of each of the six viruses. The respective viral coat protein genes were expressed as fusion proteins in *Escherichia coli*, and the products were used as immunogens for producing antibodies that

reacted against viral particles. The antisera obtained specifically recognized each of three types of rod-shaped virus particles according to immunoelectron microscopy and enzyme-linked immunosorbent assay. Furthermore, a convenient serological method based on direct tissue blotting immunoassay (DTBIA) was developed. Survey of virus incidence using both DTBIA and RT-PCR presented direct evidence for mixed infections of garlic plants with several viruses. In addition, DTBIA is suitable for large-scale and routine diagnosis of garlic viruses.

Additional keyword: garlic latent virus, garlic mite-borne mosaic virus, garlic mosaic virus, leek yellow stripe virus.

Viral diseases of garlic (*Allium sativum* L.) are widespread throughout the world, and losses in crop yields and deterioration of quality due to virus infection are serious problems because garlic plants are propagated vegetatively (12,15,28,29). A number of reports have described the causal viruses of garlic diseases. Identification of the viruses that infect garlic, however, is complicated, and the pertinent literature is confusing (6,24,25,26,30).

In Japan, a potyvirus, designated garlic mosaic virus (GMV), and a carlavirus, designated garlic latent virus (GLV), have been detected in garlic plants (1,2,10). Although a close serological relationship between GLV and shallot latent virus (SLV) is suggested (21,26), a precise assignment has not yet been done. Therefore, we use GLV throughout this manuscript. GMV is suggested as the causative agent of a mosaic disease of garlic, whereas GLV can infect garlic systemically but causes no apparent disease symptoms (10,21). It has been reported that double infection with GMV and GLV results in more severe symptoms than those induced by infection with GMV alone (21). However, GMV and GLV have not been biochemically or physically analyzed, and there is no information about their interrelationship. Recently, Yamashita (32) isolated a mite-borne virus from a diseased garlic plant and provisionally designated it as garlic mite-borne mosaic virus (GMbMV). Based on the differences in both host range and serology, Van Dijk (24,25,26) differentiated two potyviruses, leek yellow stripe virus (LYSV) and onion yellow dwarf virus (OYDV); two carlaviruses, SLV and garlic common latent virus; and mite-borne rymovirus(es) among rod-shaped garlic viruses. In any case, most unselected commercial garlic cultivars are infected with a complex of two or more viruses. The relatively restricted host ranges and

the lack of sensitive methods for detecting the individual viruses have led to confusion in differentiating among garlic viruses.

Recent advances in molecular biology have provided new tools for classification and identification of viruses. Such molecular characteristics as viral genome sequence and organization have been useful for distinguishing viruses from strains and for determining the relationships between genera of distinct viruses (4,5,23,31). Indeed, we previously detected four independent viral cDNA clones (23), designated GV-A, -B, -C, and -D and indicated that they represent a novel and unclassified group of plant viruses and that they may be similar to shallot virus X (ShVX), which was recently reported by Kanyuka et al. (9). These garlic viruses recently were described as GarV-A, -B, -C, and -D under "unassigned viruses" in the "6th Report of the International Committee on Taxonomy of Viruses" (16). Nagakubo et al. (17) recently reported cDNA cloning and nucleotide sequencing of the 3' regions of a garlic potyvirus and of a carlavirus using a cloning strategy similar to ours (23). In addition, isolation and characterization of viruses based on their cDNAs make it possible to prepare virus-specific antisera raised against the bacterially expressed viral coat protein (CP) gene, primers, or probes to help in identifying unknown viruses that are difficult to isolate by traditional methods.

Here we report cDNA cloning of RNAs derived from a potyvirus (cDNA clone GV-7) and a carlavirus (cDNA clone GV-H) infecting garlic plants and describe the development of diagnostic methods for each of six viruses. Finally, we present evidence for mixed infections of garlic plants with several viruses.

MATERIALS AND METHODS

Plant materials. The garlic cultivars Fukuchi-howaito, Hokkaido-zairai, and Ishu-wase; an unknown Chinese cultivar; and clones of fertilized wild species (7) were used. Fukuchi-howaito plants with virus disease symptoms were collected from the In-

Corresponding author: S. Sumi; E-mail address: ldv03233@niftyserve.or.jp

Publication no. P-1996-0126-01R

© 1996 The American Phytopathological Society

stitute for Biotechnology Research, Wakunaga Pharmaceutical Co., experimental farm in Hokkaido, Japan, and the leaves were used for virion preparation. Hokkaido-zairai and clones of the fertilized wild species also were from the experimental farm. Garlic bulbs of Ishu-wase and the unknown Chinese cultivar were purchased in the market and grown in a greenhouse.

Virus isolates. Isolates of GLV and GMbMV (provisional designation) (32) were provided by Y. Ikeda (Hiroshima Prefectural Agriculture Research Center) and K. Yamashita (Aomori Green Bio Center), respectively. GLV was a single local-lesion isolate from *Vicia faba*. GMbMV was maintained through several transmissions by a mite (*Eriophyes tulipae*).

Purification of virions and cDNA cloning. Virions were partially purified from garlic leaves collected in the field that showed mosaic or yellow streak symptoms according to the procedures of Mohamed and Young (15). cDNA synthesis and cloning were done as described previously (23).

Construction of expression vectors for viral CP genes. The putative CP genes encoded on GV-7, -H, and -C cDNAs were amplified by polymerase chain reaction (PCR) using the viral cDNAs as templates. The primer sets for amplification of the CP genes of GV-7, -H, and -C were 5'-TGAAGCTTCTGCTAATGATGAATTAGA-3' and 5'-GTAGTCGACGGAAGCCATAACCGTTAC-3'; 5'-GCGACGCGTTGGCTAACGAAGAAGAAGAACTC-3' and 5'-GTAGTCGACTATAGCACGCAATAGTCTAC-3'; and 5'-TCAAGCTTCTAGTGGAGACAGCCTATC-3' and 5'-GGAGTCGACAAAGTAAATTTAA-3', respectively. The upstream primers for GV-7 and -C had the *Hind*III recognition sequence, and the primer for GV-H had the *Mlu*I site at the 5' end. These were intentionally added for ease in construction of the expression vectors. The *Sal*I site was similarly added to all downstream primers. Twenty-five cycles of PCR were performed using *Taq* DNA polymerase (GIBCO-BRL, Gaithersburg, MD) with denaturation at 94°C for 1 min, annealing at 45°C for 1 min, and extension at 72°C for 2 min. The amplified DNA fragments were purified by agarose gel electrophoresis and extracted by electroelution. The DNA fragments amplified from the cDNAs of GV-7, -H, and -C were ligated to the expression vector pTA1722 (19) or pTCGL1 (14) after double digestion with *Hind*III (or *Mlu*I) and *Sal*I. The chimeric expression plasmids that were obtained (designated pTACP-7, pTACP-C, and pTGCP-H) were used to transform *Escherichia coli* HB101. The transformants containing pTACP-7, pTACP-C, or pTGCP-H were destined to produce the fusion protein composed of the viral CP fused to the signal peptide of *E. coli* alkaline phosphatase (pTACP-7 and pTACP-C) or to the amino terminal 13 amino acids of human growth hormone (pTGCP-H).

Purification of the fusion proteins. *E. coli* HB101 containing pTACP-7, pTACP-C, or pTGCP-H was grown as described by Misoka et al. (14). The cells were collected by centrifugation and suspended in 50 mM Tris-HCl (pH 8.0), after which they were disrupted by sonication. The lysates were centrifuged at 12,000 × *g* for 10 min, washed three times with 50 mM Tris-HCl (pH 8.0), and resuspended in a glass homogenizer in buffer containing 8 M urea. After removing any undissolved materials by centrifugation (12,000 × *g* for 15 min), the supernatants were dialyzed, first against 10 mM Tris-HCl (pH 8.0) then against distilled water. The recombinant viral CPs were recovered mainly from the supernatants and were estimated roughly to be more than 70% pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Preparation of antisera. Approximately 1 mg of the partially purified fusion protein was mixed with an equal volume of complete Freund's adjuvant and injected subcutaneously twice into Japanese white rabbits at weekly intervals. The animals were re-immunized twice more at weekly intervals with about 1 mg of the fusion protein mixed with an equal volume of incomplete Freund's adjuvant. Serum was collected 2 weeks after the last immunization.

Immunoelectron microscopy. The trap decoration method (13) was used for immunoelectron microscopy observations of virus particles. Approximately 5- μ l amounts of each antiserum diluted 1/50 were placed on a grid. After standing for 5 min, about 10 μ l of the partially purified virion preparation from infected garlic plants was added to the grid, and the mixture was incubated for 15 min. After washing with phosphate buffer (pH 7), the virus particles trapped on the grid were treated with antisera as described above. The virions were stained with 2% uranyl acetate and were observed with a Hitachi H-300 (Hitachi Ltd., Ibaragi, Japan) electron microscope.

Enzyme-linked immunosorbent assay (ELISA). Serially diluted purified GLV or GMbMV particles in 100 mM sodium carbonate buffer (pH 9.2) were added to individual microliter wells (Costar Corp., Cambridge, MA), after which the plate was incubated at 4°C overnight to immobilize the viral particles. After blocking with phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) at room temperature for 2 h, antiserum diluted 1/1,000 with PBS containing 0.2% BSA was added to the wells, and the plate was incubated at 37°C for 1 h. Horseradish peroxidase-conjugated porcine anti-rabbit immunoglobulin (Ig) G (DAKO Japan Co., Ltd., Kyoto) was diluted 1/4,000 with PBS containing 0.2% BSA, after which 100 μ l of the conjugate was added to each well, and the plate was incubated at 37°C for 1 h. After three washes with PBS containing 0.05% Tween 20 (PBST), 100 μ l of 1,2-phenylenediamine dihydrochloride in 0.2 M sodium

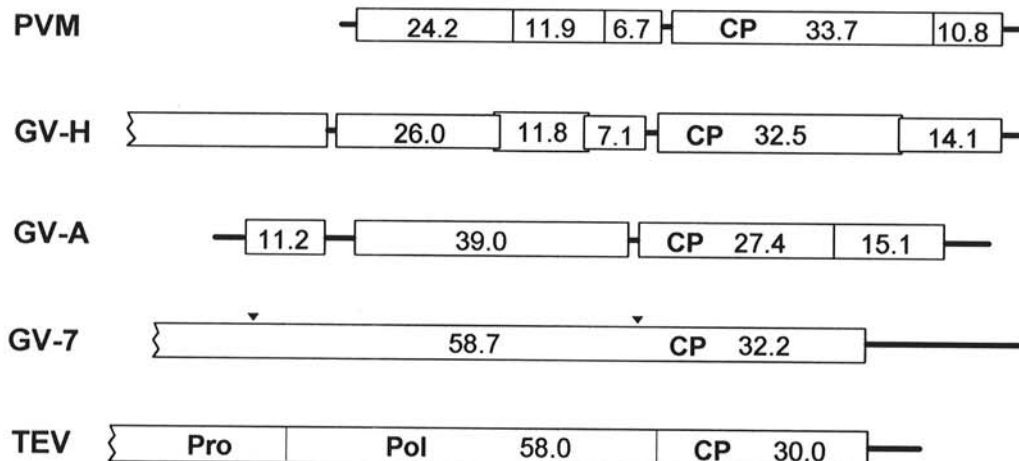


Fig. 1. Genomic organization of the 3' regions of the garlic viral cDNAs GV-H, -7 and -A, a representative of the novel-type viral cDNAs (GV-A to -D). The open reading frames are shown by boxes with molecular mass (kilodaltons) according to the putative translation products. The genomic organization of potato virus M (PVM) (20) and tobacco etch virus (TEV) (3) are shown as representatives of the genera *Carlavirus* and *Potyvirus*. Arrowheads indicate the predicted autoprocessing sites for the production of coat protein (CP) and RNA polymerase from a putative polyprotein encoded in GV-7 cDNA.

phosphate/0.1 M citric acid buffer (pH 5.0) containing 0.01% H₂O₂ was added as substrate, the plate was incubated at room temperature for 20 min, and absorbance at 490 nm was measured. Before the colorimetric measurement, 100 µl of 2 N H₂SO₄ was added to stop the reaction.

Direct tissue blotting immunoassay (DTBIA). Detection of viruses by DTBIA was carried out according to Lin et al. (11) with some modifications. Sections were cut from leaf tissues with a razor blade for each garlic sample. Tissue blots were obtained by pressing the cut surface onto 0.45-µm pore, mixed ester-supported nitrocellulose membrane (Micron Separation, Westboro, MA). The membrane was immersed in 4% Block Ace solution (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) containing 1% of BSA and Triton X-100 for 60 min at room temperature. After washing twice with PBST, the nitrocellulose membrane was incubated with the antisera to bacterially expressed viral CPs diluted 1/4,000 with 0.4% Block Ace solution at room temperature for 1 h. After three successive washings in PBST solution, the membrane was incubated with alkaline phosphatase-labeled goat anti-rabbit IgG (Zymed Laboratories, South San Francisco) diluted 1/2,000 with 0.4% Block Ace solution for 1 h at room temperature. After washing three times with PBST, the membrane was incubated in Western Blue (Promega, Madison, WI) as a substrate solution for alkaline phosphatase for 5 to 10 min at room temperature. A positive result was indicated by the development of purple on the tissue blot.

Extraction of total RNA from garlic leaves. Thirty milligrams of garlic leaves was extracted in a microhomogenizer in 300 µl of 0.5 M borate buffer (pH 8.1) containing 0.1% thioglycolic acid. After centrifugation, total RNA was extracted from 178 µl of the supernatant with hot phenol/SDS (8) and precipitated with ethanol. Approximately 20 to 50 µg of RNA was obtained.

Oligonucleotide primers. Oligonucleotide primers were synthesized in an Applied Biosystems (Foster City, CA) DNA synthesizer. Various sets of primers for the different viral cDNAs were designed with the aid of the PCRPLAN program and the micro-computer software PC/GENE (IntelliGenetics, Mountain View, CA). The primer sequences are N-RT1/N-RT2 (common primer set to the novel-type viruses): 5'-CCTGCTAAGCTATATGCTGA-3' and 5'-GTAAGTTTAGCGATATCAAC-3'; C-RT2/C-RT3 (for the garlic carlavirus, based on GV-H sequence): 5'-AGAGCTAAGAGTATTGGTAG-3' and 5'-GGGTTTCACATTGTTACACC-3'; and P-RT3/

P-RT4 (for the garlic potyvirus, based on GV-7 sequence): 5'-AAGAGTCAACACTTGGTTTG-3' and 5'-GGTCTCAATCCTAGCTAGTC-3'.

Reverse transcription (RT) and PCR. RT was performed with approximately 1 to 2 µg of garlic total RNA in a 15-µl reaction volume using a First-Strand cDNA synthesis kit (Pharmacia, Uppsala, Sweden). For PCR, 1 µl of the RT mix was added to a 100-µl polymerase reaction mixture containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 200 µM each dNTP, 2.5 units of *Taq* polymerase (Gibco-BRL), and 100 ng of each of the upstream and downstream primers. Thirty reaction cycles were used, with periods of annealing at 50°C for 30 s, synthesis at 72°C for 45 s, and melting at 94°C for 45 s. After PCR, 10-µl portions of the reaction mixtures were analyzed on 1.5 or 4.0% agarose gels.

RESULTS

Sequencing of the 3' region of the viral cDNAs. To identify garlic viruses that are different from those identified in a previous study (corresponding to cDNAs GV-A, to -D [23]), we rescreened the cDNA libraries prepared from RNA extracted from partially purified virions from infected plants and selected the cDNA clones that failed to hybridize to the previously reported cDNAs. We obtained eight cDNA clones and studied them by means of cross-hybridization and restriction digestion. Finally, two independent cDNA clones, designated GV-7 and -H, were obtained. The nucleotide sequences of the 3' terminal regions of the respective 3,305 and 3,459 bases (excluding the poly(A) tail) of GV-7 and -H were determined. The data were deposited in the DDBJ (DNA Data Bank of Japan) database under the accession numbers D11118 and D11161, respectively.

The genomic organization and putative amino acid sequence of each possible open reading frame of the sequenced regions of these clones were distinct from those of previously identified novel-type viral cDNAs (Fig. 1) but similar to that of potyviruses and carlaviruses, respectively. In addition, the nucleotide sequences of these cDNAs were very close to those of the garlic carlavirus GV-1 and potyvirus GV-2 recently reported by Nagakubo et al. (17). The 1,377-bp nucleotide sequence of the GV-2 coding sequence, encoding viral CP and a truncated RNA-dependent RNA

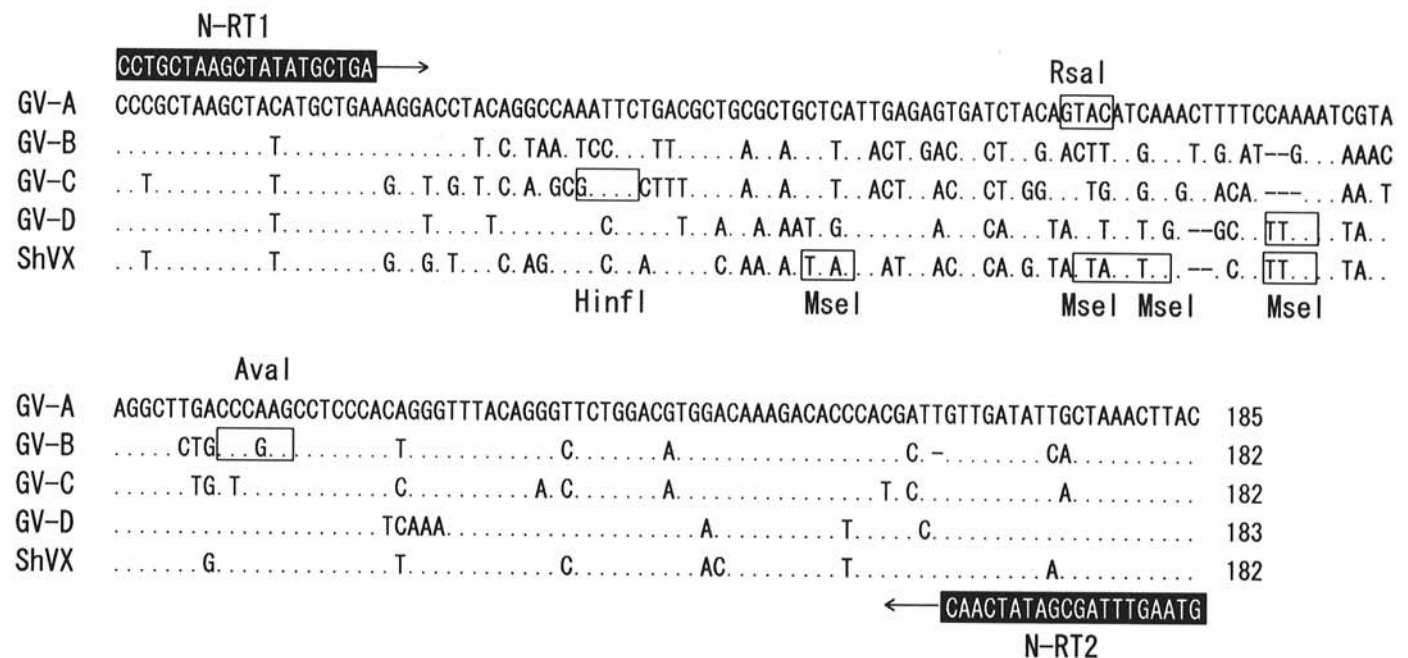


Fig. 2. Nucleotide sequences of the amplified regions of the garlic novel-type viral cDNAs (GV-A to -D) and shallot virus X detected by reverse-transcription polymerase chain reaction. The only nucleotides that differ from the GV-A sequence are indicated, and gaps are introduced for maximum alignment. The sequences of the upstream primer N-RT1 and the downstream primer N-RT2 are highlighted by shaded boxes. The restriction sites used to identify each virus are boxed.

polymerase, showed 98.8% identity with the corresponding 3' proximal GV-7 sequence. The 1,217-bp nucleotide sequence of the GV-1 coding sequence, encoding a zinc-finger protein and viral CP, showed 99.0% identity with the corresponding 3' proximal GV-H sequence. These results indicate that our cDNA clone GV-7 and clone GV-2 described by Nagakubo et al. (17) are probably derived from isolates of the same potyvirus and do not represent independent viruses or strains. Likewise, cDNA clones GV-H and -1 are representative of isolates of the same carlavirus.

RT-PCR for virus identification. So far, we have characterized six independent cDNA clones that possibly represent three distinct genera of garlic RNA viruses. We used RT-PCR to differentiate among these garlic viruses identified by their respective cDNA clones. In the case of the garlic carlavirus represented by cDNA clone GV-H and the potyvirus represented by cDNA clone GV-7, we selected several conserved sequences to design primers specific to a potyvirus and a carlavirus. In the case of RNA of the novel-type viruses represented by the cDNA clones GV-A, to -D, a highly conserved region in the 3' noncoding sequence was selected as the target of amplification. This region also is conserved in a mite-borne ShVX (9). As shown in Figure 2, the unique recognition sites of the restriction enzymes *RsaI*, *AvaI*, *HinfI*, and

MseI make it possible to identify individual viruses by restriction analysis of the amplified DNA fragments. All the primer sets were designed to amplify DNA fragments of approximately 180 bp (Fig. 3).

Several primer sets were tested using the viral cDNAs as templates for amplification by PCR, and the sets judged best with regard to specificity and PCR product yield were selected. We designated the specific sets of primers N-RT1/N-RT2 for the novel-type viral cDNAs, C-RT2/C-RT3 for GV-H, and P-RT3/P-RT4 for GV-7. Figure 3 shows results of the analyses of the amplified DNA fragments in 1.5% agarose gels after PCR with the selected sets of primers. Results of RT-PCR (Fig. 3B) done with the viral RNAs extracted from the GLV isolate or from partially purified virions from infected garlic plants suggest that our RT-PCR system effectively detects the individual viruses of the three types of garlic viruses.

Immunoelectron microscopy and ELISA with antisera against bacterially expressed viral CPs. To confirm the existence of the viruses indicated by the cDNAs and to characterize each virus as to viral particle morphology, we did the decoration test with antisera raised against the bacterially expressed viral CPs (Fig. 4). The rod-shaped virus particles decorated with antisera to the bacterially expressed CP derived from the novel-virus cDNA GV-C were flexuous and clearly distinguishable from the others. Viruses decorated with antisera to the bacterially expressed CPs from the garlic potyvirus cDNA GV-7 and the carlavirus cDNA GV-H were less flexuous and difficult to distinguish from each other. These findings point to the existence of at least three serologically distinct rod-shaped viruses (corresponding to cDNA clones GV-A, to -D; -7; and -H) that infect garlic plants in Japan. We have tentatively designated these viruses garlic potyvirus (GPV-7) and garlic carlavirus (GCV-H) in addition to garlic viruses A to D (GarV-A to -D [16]).

ELISA results with purified viral particles of GLV and GMbMV (Fig. 5) showed that the antisera raised against bacterially expressed CPs from GV-H and -C cDNA recognized GLV and GMbMV exclusively, respectively, and that the antiserum against bacterially expressed CP from GV-7 cDNA showed no cross-reactivity with these viral particles. These findings indicated that GCV-H and GarV-C are serologically closely related to GLV and GMbMV, respectively.

DTBIA for detection of viruses. DTBIA (11) was employed as a convenient and practical detection technique of garlic viruses. Using antisera against bacterially expressed cloned CP genes, antigens of GPV-7, GCV-H, and GarV-A to -D were readily detected in infected leaf-tissue blots on nitrocellulose membranes, as shown in Figure 6. On the other hand, no signal appeared on the blots from virus-free garlic samples produced by shoot-tip cul-

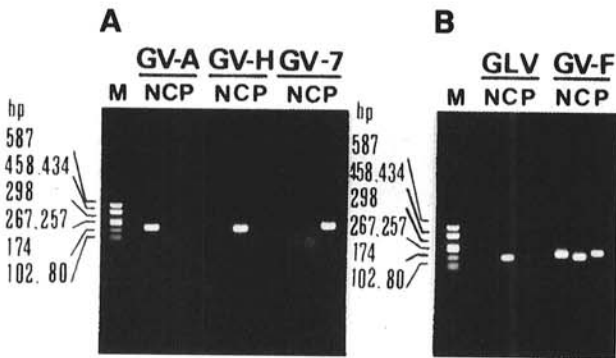


Fig. 3. Amplification of DNA fragments using specific primers to each of three types of garlic viruses: garlic novel-type virus (GarV), garlic potyvirus (GPV)-7, and garlic carlavirus (GCV)-H. **A**, Reverse-transcription polymerase chain reaction (RT-PCR) for 1 ng of each type of the cloned viral cDNAs. GV-A cDNA was the representative GarV-type virus used. **B**, RT-PCR for viral RNAs extracted from garlic latent virus (GLV) isolates and partially purified virions (GV-F) from infected garlic plants. An aliquot (10 μ l) of the 100- μ l PCR mixture was analyzed in a 1.5% agarose gel. N, C, and P above each lane indicate the set of primers for the GarV-type virus, the carlavirus, and the potyvirus cDNA sequences, respectively. Lane M shows the size marker *HaeIII* digests of pUC19.

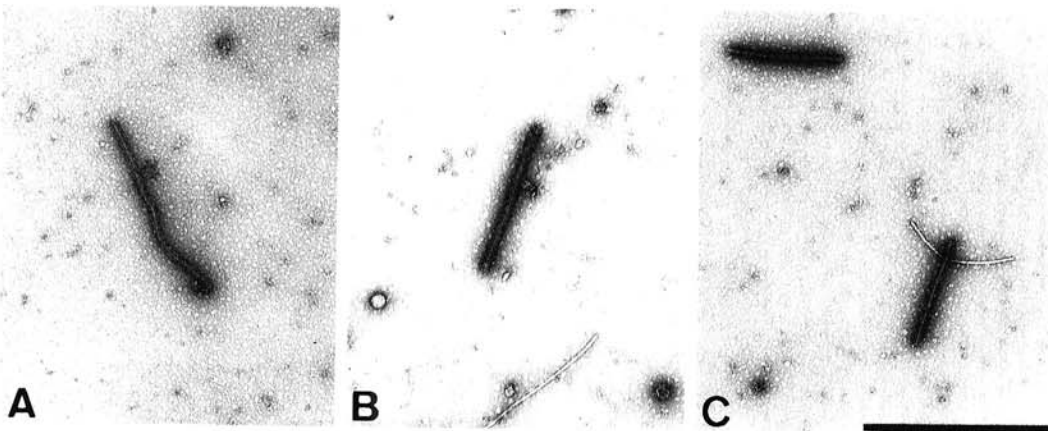


Fig. 4. Immunoelectron microscopy of garlic viruses treated with antisera raised against bacterially expressed viral coat proteins (CPs). **A**, **B**, and **C**, represent antisera used in trap decoration that were raised against the bacterially expressed CPs of the novel-type garlic virus (GarV)-C, the garlic potyvirus (GPV)-7, and the garlic carlavirus (GCV)-H, respectively. Undecorated particles with antisera were virions nonspecifically trapped on the grid. Bar = 1 μ m.

ture with any antisera (Fig. 6). These results agreed with those obtained by RT-PCR (data not shown). The sensitivity of this method for detection was estimated to be comparable to that of ELISA by comparing the result of DTBIA with that obtained in ELISA of isolated GLV and GMbMV particles (data not shown). From these results, we concluded DTBIA was applicable as a practical technique for detecting viruses infecting garlic plants.

Survey of virus infection in garlic. We surveyed the incidence of virus infection in several garlic cultivars using DTBIA and RT-PCR with total RNA extracted from small pieces of leaf tissue. Two to three clones of the Japanese cultivars Fukuchi-howaito, Ishu-wase, and Hokkaido-zairai; a novel fertilized wild species (7); and a commercially available Chinese cultivar were analyzed. Results are shown in Table 1. In the RT-PCR of total RNAs from almost all the garlic plants tested (with one exception, W8-1), two or more sets of the primers used for detecting GPV-7, GCV-H, and GarV-A to -D were able to prime the amplification of a specific DNA fragment with the expected size, indicating there was a mixed infection by a GarV-type virus, carlavirus, or potyvirus. In contrast, no RT-PCR product was found in virus-free garlic (Table 1, F-3).

Next, we made restriction analyses of some of the amplified DNA fragments using the primer set N-RT1/N-RT2 to identify the

individual GarV-type viruses. All the amplified fragments analyzed were partially or completely (Fig. 7, lane R of W8-1) digested by *RsaI*, and two DNA fragments of approximately 80 and 110 bp appeared on agarose gel (Fig. 7, lanes R). The sizes of these fragments agreed with predicted values (76 and 109 bp) estimated from the sequence in the corresponding region of GV-A cDNA, indicating infection with GarV-A. *AvaI* digestion also produced two DNA fragments with the expected sizes from the sequence of GV-B cDNA, but the amounts of these fragments varied with the RT-PCR product (Fig. 7, lanes A). This indicates infection with GarV-B. *HinI* digestion showed infection of the fertilized garlic wild species with GarV-C (Fig. 7, lane H of N1-1, N4-1, and N6-1). In addition, the relatively weak intensity of the bands, very weak especially in N1-1 and N4-1, on the agarose gel compared with those produced by the other enzymes suggests the concentration of the GarV-C compared to other GarV-type viruses was low in the affected garlic plants. *MseI* digestion of the amplified fragments of N1-1, N4-1, and N6-1 produced an apparently single band of approximately 100 bp (Fig. 7, lanes Ms). From the GV-D cDNA sequence, two fragments of nearly the same size (89 and 94 bp) should be produced by *MseI*, which could not be separated on the 4% agarose gel. Therefore, we concluded that these garlic plants were infected with GarV-D.

The results obtained by RT-PCR with the specific set of primers to each novel-type viral cDNA coincided with those of restriction analysis of PCR products (data not shown). These findings reveal mixed infection of garlic plants by GarV-type viruses. Furthermore, it is notable that in N4-1, two additional bands (indicated by arrowheads) of about 80 and 110 bp are present. This may sig-

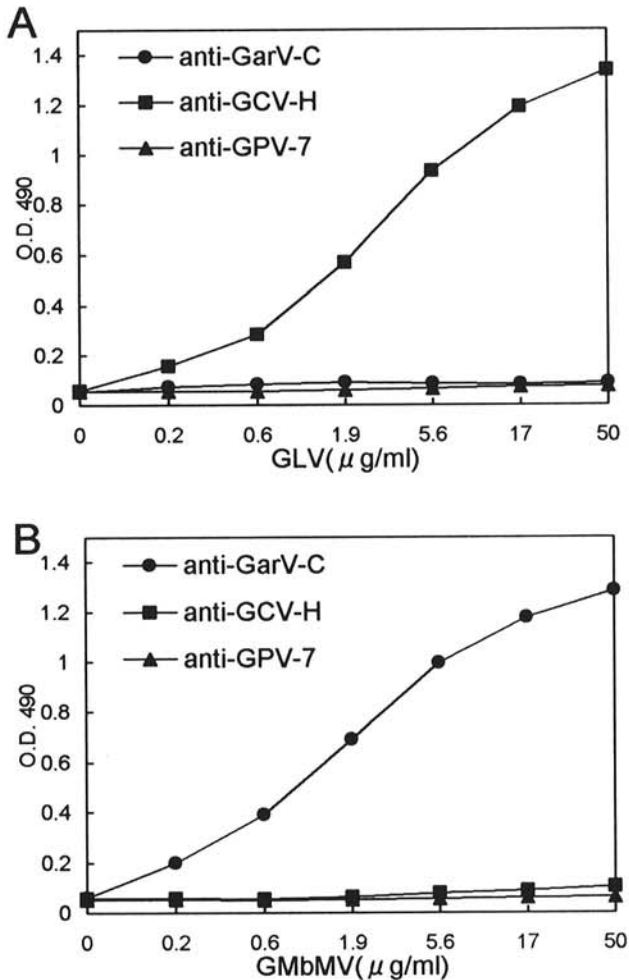


Fig. 5. Characterization of antisera raised against bacterially expressed garlic viral coat proteins by enzyme-linked immunosorbent assay: garlic novel-type virus (GarV)-C, garlic carlavirus (GCV)-H, and garlic potyvirus (GPV)-7. Serially diluted (0.2 to 50 µg/ml) **A**, garlic latent virus (GLV) and **B**, garlic miteborne mosaic virus (GMbMV) isolates were coated on microtiter wells, and each antiserum, diluted 1/1,000, was added and reacted at 37°C for 1 h. Antisera binding to virus particles were colorimetrically detected by absorbance at 490 nm produced by horseradish peroxidase-conjugated porcine anti-rabbit immunoglobulin G.

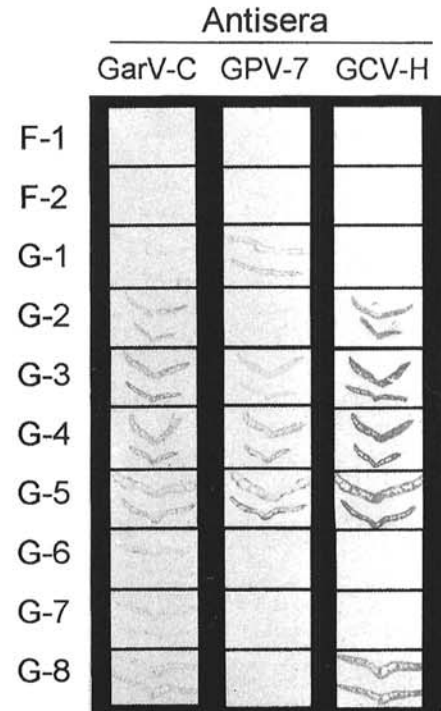


Fig. 6. Immunochemical detection of three types of garlic viruses: novel-type garlic virus (GarV)-C, garlic potyvirus (GPV)-7, and garlic carlavirus (GCV)-H, by direct tissue blotting immunoassay. Three sets of duplicate blots on nitrocellulose membrane were prepared for each garlic sample by briefly pressing the cut surface of a leaf onto the membrane. For every blotting, a newly cut surface was made by cutting just above the location of the previous cut surface with a razor blade. The blots were reacted with each rabbit antiserum raised against bacterially expressed viral coat proteins of GarV-C, GPV-7, and GCV-H, and the resultant immunocomplex was detected with alkaline phosphatase-labeled goat anti-rabbit immunoglobulin antibodies. F-1 and -2 are from virus-free garlic produced by shoot-tip culture as a negative control. G-1 to -8 are from cultivated garlic plants collected from our company's experimental farm.

nify the presence of yet another virus that is different from GarV-A to -D or ShVX.

Serological detection by DTBIA gave the same results as those obtained in RT-PCR. In addition, antisera against bacterially expressed GarV-C CP detected virus antigens in garlic plants that were infected with GarV-A, -B, or -D but not with GarV-C, including W3-1 and W8-1 in Figure 7 (data not shown). These results suggest that the antiserum to GarV-C CP can recognize all the novel-type viruses detected in garlic plants.

DISCUSSION

We detected three types of viral cDNAs, represented by GV-7, -H, and GV-A to -D in Japanese garlic plants (this study; [23]). Recently, nucleotide sequences of several viral cDNAs from *Allium* plants, including garlic, have been published (9,17) and deposited in a DNA database. A homology search of six viral cDNAs cloned by us with the GenBank database showed that the 3' proximal coding sequence between GV-7 and -2 (17), GV-H and -1 (17), GV-C and GMbMV (deposited by Yamashita et al. 1995, accession number D49443), and GV-D and JGVFRA (deposited by Ryabov et al. 1995, accession number L38892) have a very high sequence similarity, and more than 96% of the nucleotides in the respective region of 1.1 to 1.3 kb was identical between each pair. Shukla

TABLE 1. Reverse-transcription polymerase chain reaction (RT-PCR) detection of three types of viruses infecting garlic

Garlic ^a	PCR product		
	Novel-type virus	Carlavirus	Potyvirus
F-3	-	-	-
W-3	+	-	+
W-4	+	-	+
W-8	+	-	-
N-1	+	+	-
N-4	+	+	+
N-6	+	+	+
N-7	+	+	-
IW-1	-	+	+
IW-2	-	+	+
IW-3	-	+	+
P-1	-	+	+
P-3	+	+	+
P-4	-	+	+
C-1	+	+	+
C-2	+	+	+

^a F-3: virus-free plant; N-1, N-4, N-6, and N-7: clones of fertilized wild species; P-1, P-3, and P-4: clones of the Japanese cultivar Hokkaido-zairai; W-3, W-4, and W-8: clones of the Japanese cultivar Fukuchi-howaito; IW-1, IW-2, and IW-3: clones of the Japanese cultivar Ishu-wase; and C-1 and C-2: clones of the Chinese cultivar.

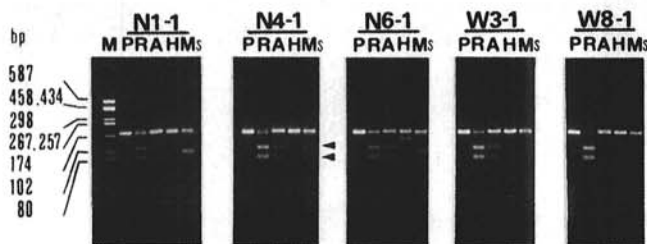


Fig. 7. Identification of individual garlic novel-type virus by restriction analysis of amplified DNA fragments. Restriction analyses were made for representative polymerase chain reaction (PCR) products of the fertilized wild garlic species (N1-1, N4-1, and N6-1) and the Japanese cultivar Fukuchi-howaito (W3-1 and W8-1). A 10- μ l portion of each reaction mixture was digested with lane R, *Rsa*I; lane A, *Ava*I; lane H, *Hinf*I; and lane Ms, *Mse*I, then layered on 4% agarose gels. Lanes P and M show the PCR product and *Hae*III digest of pUC19, the size marker, respectively. Arrowheads indicate the additional bands produced by *Mse*I, suggestive of a different type(s) of novel virus(es).

and Ward (22) showed that distinct members of the potyvirus group exhibit sequence identities of 38 to 71%, and strains of individual viruses have identities of 90 to 99%, judging from computer analyses of CP sequence data from 20 strains of nine distinct potyviruses.

ELISA for GLV and GMbMV virions with antisera raised against bacterially expressed viral CPs (Fig. 5) indicated a close serological relationship between GCV-H and GLV and GarV-C and GMbMV, respectively. In addition, Yamashita et al. (33) recently reported that the nucleotide sequence of the 3' proximal 816 bp of the cloned cDNA from a rod-shaped garlic virus, which was identified as LYSV based on biological and immunochemical characterization including host specificity, immunoelectron microscopy, and transmission vector, showed 90 or 97% identity with that of GV-7 or -2 cDNA, respectively, and concluded they all were derived from LYSV. Taking all of these findings into consideration, it is suggested that cDNAs GV-7 and -2 are derived from the same potyvirus, LYSV, and GV-H and -1 are derived from carlavirus GLV, respectively; cDNAs GV-C and GMbMV, as well as GV-D and cDNA of a mite-borne filamentous virus isolated from garlic plants in England by E. Ryabov, E. Generozov, A. Krutov, H. Vetten, and S. Zavriev (*unpublished*), respectively, are from the same virus infecting garlic plants.

We have developed methods for detecting viruses infecting garlic plants based on RT-PCR and DTBIA by preparing specific primers and antisera raised against bacterially expressed viral CPs. RT-PCR can identify the six viruses isolated as cDNA clones but is unsuitable for large-scale indexing or for studies of plant virus epidemiology because it is laborious and time-consuming. On the other hand, DTBIA is convenient, simple, and as sensitive as ELISA. DTBIA, however, does not distinguish among the novel-type viruses GarV-A to -D. The results from surveying virus incidence by DTBIA in approximately 100 garlic samples randomly collected in fields were consistent with those obtained by RT-PCR (data not shown). In addition, antisera prepared with bacterially expressed viral CP as an antigen developed little background color on nitrocellulose membrane without the need for pretreatments, such as cross-absorption with healthy plant extract and fractionation of viral CP on SDS-PAGE (18). All these results indicate the usefulness of molecular biology methods obtaining viral CP antigens.

DTBIA and RT-PCR combined with restriction analysis showed that some garlic plants were infected concomitantly with all six of the identified viruses: potyvirus GPV-7, carlavirus GCV-H, and four GarV-type viruses—GarV-A, -B, -C, and -D (Table 1; Fig. 7). ShVX, closely related to GarV-type viruses in molecular biology parameters, such as genomic organization, nucleotide sequence, and CP sequence, is isolated from shallot plants resistant to the Mongolian isolate of OYDV (27). This fact, along with the observation that a mixed infection of garlic by carlavirus GLV and potyvirus GMV enhances apparent disease symptoms (21), may suggest some interaction among the viruses in development of disease symptoms. We are now investigating a possible correlation between mixed infections with various combinations of the six garlic viruses and development and severity of symptoms.

In addition, GarV-type viruses have been detected in garlic plants cultivated in China, the United States, and Argentina, as well as in the United Kingdom (from the sequence data deposited by Ryabov et al. 1995, in GenBank). This is in contrast to the fact that an *Allium*-infecting potyvirus, OYDV, is widespread in garlic in Europe and the Philippines but rarely has been detected in Japanese garlic (17).

Our strategy for cloning viral cDNAs differs from previous strategies in that we cloned viral cDNA directly from diseased garlic plants, which probably were infected with a complex of viruses, without isolating the individual viruses in the appropriate host plants. The isolation and characterization of viruses as their cDNAs makes it possible to prepare virus-specific antisera, primers, or probes to aid in identifying unknown viruses that are difficult to

isolate by traditional methods. Furthermore, there is also a high probability of detecting variants difficult to distinguish by serology. In fact, we detected variants of both GV-H and -7 cDNAs whose nucleotide sequences at the 3' terminal region differed by as much as 14.4%.

LITERATURE CITED

1. Abiko, K., Watanabe, Y., and Nishi, Y. 1980. Studies on garlic mosaic. I. Causal virus. Veg. Ornamental Crops Res. Stn. Ser. A. Bull. 7:139-147. In Japanese.
2. Abiko, K., Watanabe, Y., and Nishi, Y. 1980. Studies on garlic mosaic. II. Several factors affecting the detection of garlic mosaic virus following juice inoculation. Veg. Ornamental Crops Res. Stn. Ser. A. Bull. 7:149-154. In Japanese.
3. Allison, R. F., Johnston, R. E., and Dougherty, W. G. 1986. The nucleotide sequence of the coding region of tobacco etch virus genomic RNA: Evidence for the synthesis of a single polypeptide. Virology 154:9-20.
4. Atreya, C. D. 1992. Application of genome sequence information in potyvirus taxonomy: An overview. Arch. Virol. 20 (Suppl. 5):17-23.
5. Brunt, A. A. 1992. The general properties of potyviruses. Arch. Virol. 20 (Suppl. 5):3-16.
6. Conci, V., Nome, S. F., Milne, R. G. 1992. Filamentous viruses of garlic in Argentina. Plant Dis. 76:594-596.
7. Etoh, T. 1986. Fertility of garlic clones collected in Soviet Central Asia. J. Jpn. Soc. Hortic. Sci. 55:312-319.
8. Hari, V. 1981. The RNA of tobacco etch virus: Further characterization and detection of protein linked to RNA. Virology 112:391-399.
9. Kanyuka, K. V., Vishnichenko, V. K., Levay, K. E., Kondrikov, D. Y., Ryabov, E. V., and Zavriev, S. K. 1992. Nucleotide sequence of shallot virus X RNA reveals a 5'-proximal cistron closely related to those of potexviruses and a unique arrangement of the 3'-proximal cistrons. J. Gen. Virol. 73:2553-2560.
10. Lee, Y. W., Yamazaki, S., Osaki, T., and Inouye, T. 1979. Two elongated viruses in garlic, garlic latent virus and garlic mosaic virus. Ann. Phytopathol. Soc. Jpn. 45:727-734. In Japanese.
11. Lin, N. S., Hsu, Y. H., and Hsu, H. T. 1990. Immunological detection of plant viruses and a mycoplasma-like organism by direct tissue blotting on nitrocellulose membranes. Phytopathology 80:824-828.
12. Lot, H., Delecolle, B., Boccoardo, G., Marzachi, C., and Milne, G. 1994. Partial characterization of reovirus-like particles associated with garlic dwarf disease. Plant Pathol. 43:537-546.
13. Milne, R. G., and Luisoni, E. 1977. Rapid immune electron microscopy of virus preparations. Pages 265-281 in: Methods in Virology. Vol. 6. K. Maramorosch and H. Koprowski, eds. Academic Press, New York.
14. Misoka, F., Miyake, T., Miyoshi, K., Sugiyama, M., Sakamoto, S., and Fuwa, T. 1989. Overproduction of human insulin-like growth factor-II in *Escherichia coli*. Biotechnol. Lett. 11:839-844.
15. Mohamed, N. A., and Young, B. R. 1981. Garlic yellow streak virus, a potyvirus infecting garlic in New Zealand. Ann. App. Biol. 97:65-74.
16. Murphy, F. A., Fauquet, C. M., Bishop, D. H. L., Ghabrial, S. A., Martelli, G. P., Mayo, A., and Summers, M. D. 1995. Virus taxonomy. 6th Rep. Int. Commit. Taxonomy Viruses. Springer-Verlag, Vienna.
17. Nagakubo, T., Kubo, M., and Oeda, K. 1994. Nucleotide sequences of the 3' region of two major viruses from mosaic-diseased garlic: Molecular evidence of mixed infection by a potyvirus and a carlavirus. Phytopathology 84:640-645.
18. Nikolaeva, O. V., Karasev, A. V., Gumpf, D. J., Lee, R. F., and Garnsey S. M. 1995. Production of polyclonal antisera to the coat protein of citrus tristeza virus expressed in *Escherichia coli*: Application for immunodiagnosis. Phytopathology 85:691-694.
19. Oka, T., Sumi, S., Fuwa, T., Yoda, K., Yamazaki, M., Tamura, G., and Miyake, T. 1987. Efficacies of different secretion vectors for secretion of human epidermal growth factor by *Escherichia coli*. Agric. Biol. Chem. 51:1099-1104.
20. Rupasov, V. V., Morosov, S. Yu., Kanyuka, K., and Zavriev, S. K. 1989. Partial nucleotide sequence of potato virus M RNA shows similarities to potexviruses in gene arrangement and the encoded amino acid sequences. J. Gen. Virol. 70:1861-1869.
21. Sako, I. 1989. Occurrence of garlic latent virus in *Allium* species. Plant Prot. 43:389-392. In Japanese.
22. Shukla, D. D., and Ward, C. W. 1989. Structure of potyvirus coat proteins and its application in the taxonomy of the potyvirus group. Adv. Virus Res. 36:273-314.
23. Sumi, S., Tsuneyoshi, T., and Furutani, H. 1993. Novel rod-shaped viruses isolated from garlic (*Allium sativum*) possessing a unique genome organization. J. Gen. Virol. 74:1879-1885.
24. Van Dijk, P. 1991. Mite-borne virus isolates from cultivated *Allium* species and their classification into two new rymoviruses in the family Potyviridae. Neth. J. Plant Pathol. 97:381-399.
25. Van Dijk, P. 1993. Survey and characterization of potyviruses and their strains of *Allium* species. Neth. J. Plant Pathol. 99 (Suppl. 2):1-48.
26. Van Dijk, P. 1993. Carlavirus isolates from cultivated *Allium* species represent three viruses. Neth. J. Plant Pathol. 99:233-257.
27. Vishnichenko, V. K., Konareva, T. N., and Zabriev, S. K. 1993. A new filamentous virus in shallot. Plant Pathol. 42:121-126.
28. Walkey, D. G. A. 1990. Virus diseases. Pages 191-211 in: Onion and Allied Crops. Vol. 2. H. D. Rabinowitch and J. L. Brewster, eds. CRC Press, Boca Raton, FL.
29. Walkey, D. G. A., and Antill, D. N. 1989. Agronomic evaluation of virus-free and virus-infected garlic (*Allium sativum* L.). J. Hortic. Sci. 64:53-60.
30. Walkey, D. G. A., Webb, M. J. W., Bolland, C. J., and Miller, A. 1987. Production of virus-free garlic (*Allium sativum* L.) and shallot (*A. ascalonicum* L.) by meristem-tip culture. J. Hortic. Sci. 62:211-220.
31. Ward, C. W., and Shukla, D. D. 1991. Taxonomy of potyviruses: Current problems and some solutions. Intervirology 32:269-296.
32. Yamashita, K. 1993. Purification and serology of garlic mite-borne mosaic virus (GMbMV). (Abstr.) Ann. Phytopathol. Soc. Jpn. 59:57. In Japanese.
33. Yamashita, K., Sakai, J., and Hanada, K. 1995. Leek yellow stripe virus (LYSV) isolated from garlic and its relationship to garlic mosaic virus (GMV). (Abstr.) Ann. Phytopathol. Soc. Jpn. 61:273. In Japanese.