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Molecular Cloning of the 3'-Terminal Region of Garlic Potyviruses and Immunological Detection of Their Coat Proteins

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cDNAs complementary to the 3'-terminal regions of two potyvirus genomes were cloned and sequenced. The clone G7 contains one open reading frame (ORF) of 1,338 nucleotides and a 3' untranslated region (3'-UTR) of 403 nucleotides at the 3'-end excluding the 3'-end poly(A) tail. The putative viral coat protein (CP) shows 55%-92% amino acid sequence homology to those of *Allium* potyviruses. The genome size of the virus was analyzed to be about 9.0 kb by Northern blot analysis. Five cDNA clones were screened out using GPV2 oligonucleotide as a probe. One of these clones, DEA72, which has a longest cDNA insert, contains one ORF of 1,459 nucleotides and a 3'-UTR of 590 nucleotides at the 3'-end excluding the 3'-end poly(A) tail. The putative viral CP shows 57%-88% amino acid sequence homologies to those of *Allium* potyviruses. The genome size of the virus was analyzed to be about 9.6 kb by Northern blot analysis. The results of immunoblot and Northern blot analyses suggest that almost all of the tested garlic plants showing mosaic or streak symptoms are infected with DEA72-potyvirus in variable degrees but rarely infected with G7-potyvirus. Immunoelectron microscopy using anti-DEA72 CP antibody shows that this potyvirus is about 750 nm long and flexuous rod shaped.

Keywords : coat protein, garlic, immunoblot, immunodecoration, potyvirus.

Garlic (*Allium sativum* L.) is an important vegetable crop and has long been cultivated extensively in Korea. More recently it has gained importance as a source of certain pharmaceuticals. This additional use has also contributed to the increasing demand of garlic. Most of the garlic plants cultivated throughout the world are thought to be infected with viruses which give rise to mosaic symptom on leaves and thus reduction of yield (Ahlawat, 1974; Grimsley et al., 1987; Lee et al., 1979; Walkey, 1990). The wide occurrence of virus disease in garlic is due to the fact that garlic plants are propagated vegetatively and that there is no effective method of controlling the disease. Although there has been some attempts to produce the virus-free seed garlic by tissue culture techniques, viruses were often not successfully eliminated even from tissue cultured garlic (Chang et al., 1980; Havranek, 1973). In most cases, moreover, even virus-free garlic plants would become infected with viruses again when planted in the field (Kehr and Schaeffer, 1976; Lee, 1981).

In attempting to control viral diseases, the first essential step is to establish the identity of the viruses responsible for the diseases in garlic plants and to determine their molecular characteristics. Two types of flexuous, rod-shaped viruses belonging to the potyvirus and carlavirus groups, respectively, have been reported from garlic plants in Japan, based on their morphological and cytopathological properties (Lee et al., 1979). A potyvirus, termed garlic mosaic virus (GMV), was thought to be a causative agent of the garlic virus disease, whereas a carlavirus, termed garlic latent virus (GLV), infects garlic plants systemically, but gives no apparent disease symptoms. It has been reported that double infection by GMV and GLV results in more severe symptoms than those arising from a single infection with GMV (Sako, 1989), which indicates a possible synergistic relationship between these viruses in the development of virus disease. However, GMV and GLV have not been further characterized at the molecular level. Symptom due to the infection of GMV was mosaic in garlic plants and local necrotic lesions in *Gomphrena globosa*, whereas latent infection was detected in the inoculated leaves of *Chenopodium amaranticolor, C. quinoa* and *Tetragonia expansa*. Particles of GMV were flexuous rods of about 750 nm long. Pinwheel-type cytoplasmic inculsions were found in the infected garlic plant cell (Chang et al., 1988; Lee et al., 1979).

GMV, garlic yellow stripe virus (GYSV), and onion yellow dwarf virus (OYDV) are known as potyviruses infecting in *Allium* plants (Noda and Iniuye, 1989). Recently, the partial nucleotide sequences of these viruses were reported.

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Nagakubo et al. (1994) determined the partial nucleotide sequences of cDNA clone of virus infecting garlic plants in Japan, garlic virus 2 (GV2). Partial cDNA clones for leek yellow stripe potyvirus (LYSV) have been isolated and their nucleotide sequences determined (Schubert, 1995; Tsuneyoshi and Sumi, 1996; Tsuneyoshi et al., 1998). Partial cDNA clones for OYDV have also been isolated and their nucleotide sequences determined (Kobayashi, 1996; Tsuneyoshi and Sumi, 1996; van der Vlugt, 1997a).

In the review of potyvirus identification and classification, Shukla and Ward (1989) echoed the opinion of many virologists that potyvirus taxonomy is in an unsatisfactory state. Both the amino acid sequence of the coat protein (CP) gene (Shukla and Ward, 1988, 1989) and the nucleotide sequence of the 3'-untranslated region (3'-UTR) (Frenkel et al., 1989) have recently been proposed as tools for potyvirus classification. The identification and classification of garlic potyviruses, LSYVs and OYDVs are also not clear. Recently, Van Dijk (1993) differentiated four potyviruses: leek yellow strip virus, onion yellow dwarf virus, shallot yellow strip virus (SYSV) and Welsh onion yellow strip virus (WoYSV), as well as six strains of these viruses, based on differences in the host range and in the serology of aphid-borne potyviruses detected in nearly 5700 *Allium* plants from around the world.

In this study, we have isolated two cDNA clones for garlic potyviruses, G7 and DEA72, and determined the nucleotide sequences. G7-potyvirus and some OYDVs are strains of the same virus and DEA72-potyvirus, GV2 and LYSV are strains of another same virus. Northern and immunoblot analyses showed that G7-potyvirus rarely infected garlic plants, however almost all of the tested garlic plants were infected with garlic potyvirus from which the cDNA clone DEA72 was drived.

Materials and Methods

Plant materials. Garlic (*A. sativum* L.) plants used in this study were collected from various regions of America, China, Japan and Korea. Leaves of garlic plants were harvested and kept at -70°C for further use.

Virus purification and viral RNA preparation. Garlic viruses were isolated from virus-infected garlic leaves by sucrose gradient sedimentation as described previously (La, 1973; Langenberg, 1973). The virus suspension was treated with 250 mg/ml proteinase K, 1% sodium dodecyl sulfate and 25 mM EDTA for 15 min at 37°C, after which the RNA was extracted twice with phenol-chloroform, once with chloroform, and then precipitated with 2.5 volumes of cold ethanol in the presence of 0.2 M sodium chloride. The RNA precipitate was washed with 70% ethanol, dissolved in sterile water and stored frozen at -70°C.

cDNA synthesis, molecular cloning and nucleotide sequencing. The double-stranded cDNAs of the garlic viruses were synthesized with SuperScript II reverse transcriptase according to the GIBCO BRL manual using oligo(dT)$_{12-18}$ as a primer. Blunt-ended cDNA was ligated into a *Sma*I-digested pUC18 plasmid and transformed into *Escherichia coli* MC1061. A nested series of exonuclease III deletions were generated from the original cDNA clones by use of the Erase-a-Base system (Promega). Dideoxynucleotide sequencing of both strands of dsDNA templates was carried out using [α-^{32}P]dATP and modified T7 DNA polymerase (Sequenase, U.S. Biochemicals). Northern blot analysis was carried out with garlic virus RNAs in an 0.8% formaldehyde agarose gel (Sambrook et al., 1989).

Preparation of antibody and immunoblot analysis. Two oligonucleotides containing an initiation or termination codon of CP, were synthesized. Polymerase chain reaction (PCR) was carried out with these oligonucleotides as primers and the clone DEA72 as a template (Sambrook et al., 1989). The PCR product was digested with restriction enzymes *Bam*HI and *Kpn*I and ligated into the pRSET-A expression vector, which was digested with the same enzymes. The recombinant construct plasmid was transformed into *E. coli* BL21, and the synthesis of T7 RNA polymerase was induced by 0.75 mM of IPTG. The expressed recombinant CP of DEA72-potyvirus was purified by Ni^{2+}-NTA affinity chromatography. The expressed protein was analyzed by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Sambrook et al., 1989).

Antibody against the CP of garlic potyvirus was prepared with recombinant CP expressed in *E. coli*. The purified recombinant CP was mixed with an equal volume of Freund's adjuvant and injected into a rabbit (New Zealand White) 3 times at two-week intervals as the procedure of Garvey et al. (1980). Serum was processed by the standard method after bleeding.

Immunoblot analysis was carried out according to the procedure of Burnette (1981). Garlic leaf extract prepared in SDS-PAGE sample buffer was analyzed by 15% SDS-PAGE and electrotransferred onto nitrocellulose paper. The blot was incubated first with anti-CP antibody (1 : 200 dilution) and then with goat anti-rabbit second antibody labeled with peroxidase (Sigma). ECL detection reagents 1 and 2 (Pierce Co.) were used as substrates for peroxidase.

Immunoelectron microscopy. Garlic viruses purified from leaves by sucrose gradient centrifugation were adsorbed onto a grid coated with formvar and carbon, and were charge-treated for 5 min. To reduce non-specific binding, the grid was treated with phosphate-buffered saline (PBS) solution containing 0.5% BSA for 30 min. The grid was incubated with 10 µl of anti-poty CP antibody (1 . 10 dilution) for 1 hr, washed with PBS and drained. It was negatively stained with 2% phosphotungstic acid and then observed using a transmission electron microscope (Hitachi-800).

Results and Discussion

Cloning and sequencing of viral cDNA clones for garlic potyviruses, G7 and DEA72. To identify garlic potyviruses, a cDNA library was constructed using viral RNA isolated from garlic plants (cultivar Danyang) which showed yellow streak or mosaic symptoms. Recombinant colonies

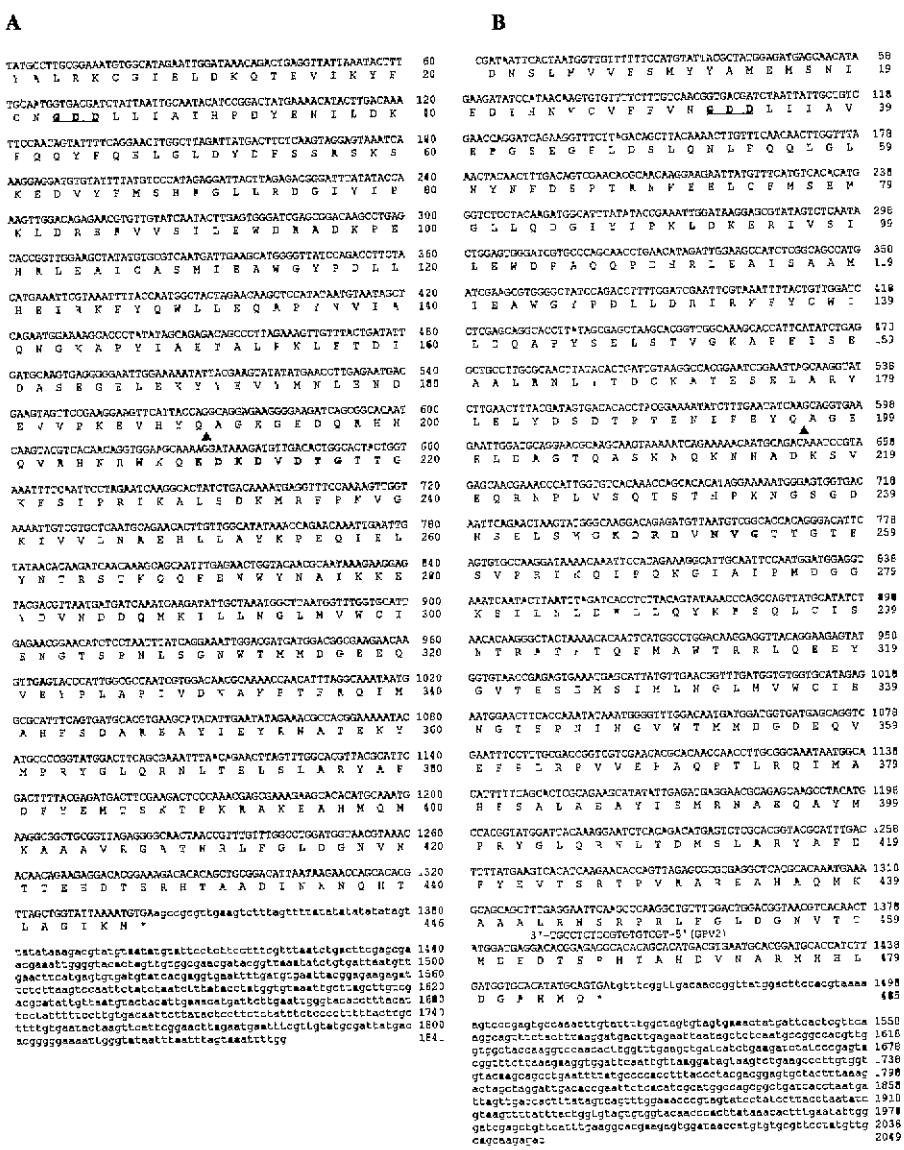


Fig. 1. Nucleotide and deduced amino acid sequences of the clone G7 (**A**) and DEA72 (**B**). The GDD motif is indicated by underline. Predict NIb/CP polyprotein cleavage site is indicated by arrow (▲). The nucleotide sequences of the cDNAs are shown as the equivalent of the viral positive-strand RNA. Coding sequences for polyproteins are shown in uppercase letters and the deduced amino acid sequences are presented under the nucleotide sequence. 3'-UTR is shown in lowercase letters. Termination codon is shown by asterisk (*) The conserved amino acid sequence motifs are shown in bold.

were randomly chosen and their nucleotide sequences were determined. We have isolated 24 cDNA clones for garlic viruses and determined their nucleotide sequences. One of these clones, G7, contains a cDNA insert of 1,841 bp except for poly(A) tail. Sequence analysis reveals that clone G7 contains a single large open reading frame (ORF) of 1,341 nucleotides in reading frame 1 of the virus-sense cDNA and terminating with TGA codon followed by a 3'-UTR of 500 nucleotides at the 3'-end excluding the 3'-end poly(A) tail (Fig. 1A). The putative viral protein shows high nucleotide sequence homology to potyvirus group. When colony hybridization was carried out using the clone G7 as a molecular probe, however, no other colonies hybridized with the probe. Furthermore, immunoblot analysis revealed that almost all of the tested garlic plants showed no signal if any (data not shown). These results suggest that the clone G7 may be a partial cDNA clone of garlic potyvirus; however, the extent of infection by this potyvirus of garlic plants may be extremely rare.

To isolate more abundant garlic potyviruses through comparison of nucleotide sequences for CP from leek yellow strip virus (LYSV; Tsuneyoshi and Sumi, 1996), GV2

(Nagakubo et al., 1994), plum pox virus (Teycheney et al., 1989), potato virus Y (PVY; Robalia et al., 1989), and soybean mosaic virus (SMV; Jayaram et al., 1992), highly conserved regions were identified and an oligonucleotide GPV2 (3'-TGCCTCTCCGTGTGTCGT-5') was designed (Fig. 1B). Five cDNA clones were screened out using GPV2 oligonucleotide as a probe. One of the clone DEA72 contains a longest cDNA insert of about 2,500 bp and poly(A) tail. The nucleotide sequence of clone DEA72 was determined (Fig. 1B). Sequence analysis reveals that clone DEA72 contains one large ORF of 1,459 nucleotides of the virus-sense cDNA and terminating with TGA codon followed by a 3'-UTR of 590 nucleotides at the 3'-end excluding the 3'-end poly(A) tail.

The length of 3'-UTRs of reported potyviruses ranges 169 to 475 nucleotides (Hammond and Hammond, 1989; Gough et al., 1987). Clones G7 and DEA72 have longer 3'-UTR regions of 500 and 590 nucleotides, respectively. The 3'-UTR of DEA72 is unusually longer than that of other potyviruses. Neither of the potential eukaryotic polyadenylation signals AAUAAA (Nevins, 1983) or UAUGU (Zaret and Sherman, 1982), which have been reported for several other potyviruses, could be found in this region of both virus. This fact supports the hypothesis of Hammond and Hammond (1989) that 'signals' are fortuitous and not functional.

Frenkel et al. (1989) have proposed that the nucleotide sequence similarities between the 3'-UTRs of potyviral genomes are useful for taxonomic purposes. They determined that percentage sequence similarities between accepted virus strains ranged from 83% to 99%, whereas similarities between distinct viruses ranged between 39% to 53%. The nucleotide sequence similarity in 3'-UTR between G7 and DEA72 is only 45%. It suggests that G7 and DEA72-potyviruses are distinct potyvirus from each other.

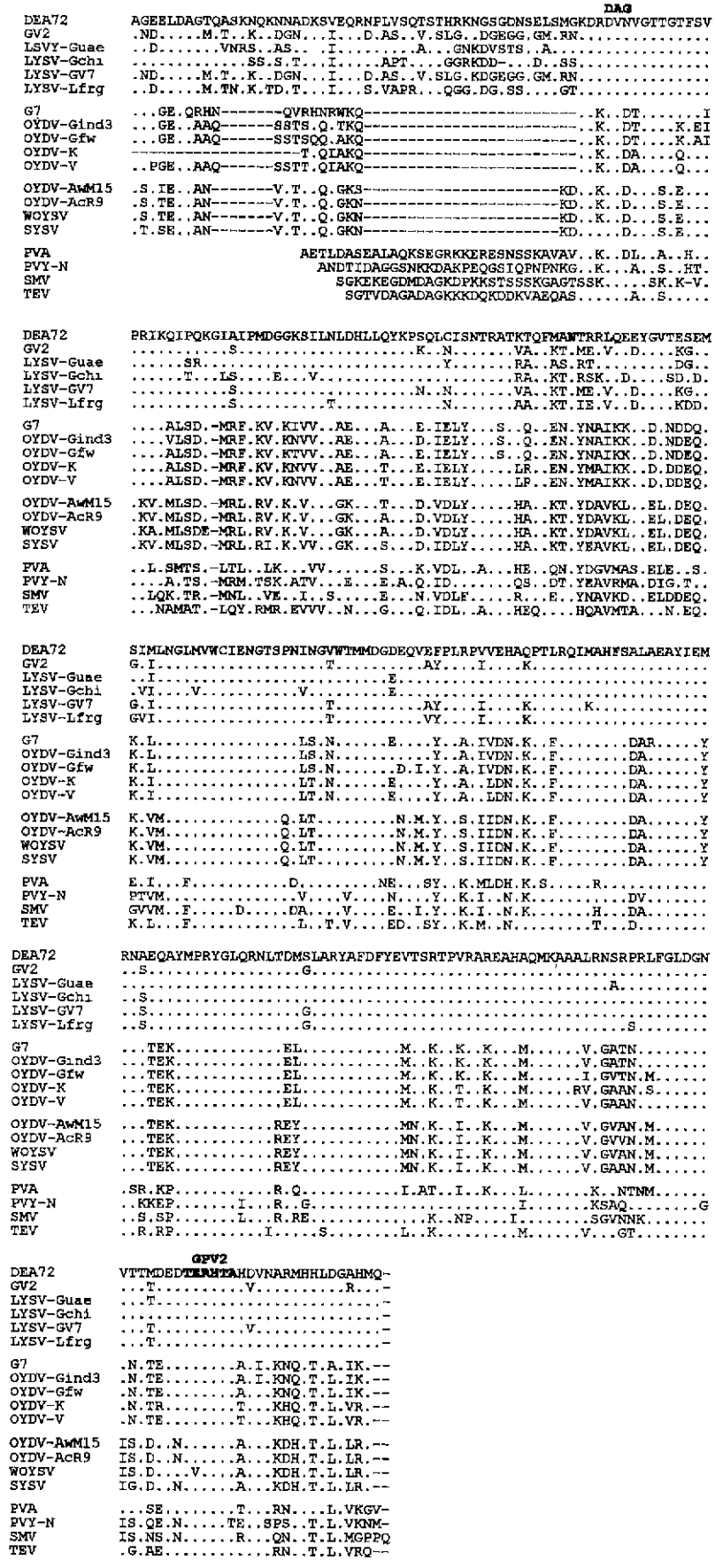
GMV, GYSV and OYDV are known as potyviruses infecting in *Allium* plants (Noda and Iniuye, 1989). There are partial nucleotide sequences of *Allium* potyviruses in EMBL data bank (Table 1). Clone G7 showed about 80% nucleotide sequence similarity in the 3'-UTR with OYDV-K and OYDV-V. The clone DEA72 showed about 88% nucleotide sequence similarity with GV2, LYSV-GV7 and LYSV-Lfrg. Therefore, G7-potyvirus, OYDV-K and OYDV-V are strains of the same virus and DEA72-potyvirus, GV2 and LYSVs are strains of another same virus by Frenkel's criteria (1989).

Analysis of coding regions. Alignment of amino acid sequences of the putative proteins from G7 and DEA72 with polyproteins of known potyviruses reveals that they have high similarity with corresponding portions of the polyprotein of other potyviruses, starting at the C terminal of the nuclear inclusion protein (NIb), beginning 5' to the

Table 1. Homology (%) of the amino acid sequences of the coat protein of *Allium* potyviruses

Allium potyvirus[a]	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)
(1) DEA72	-			**(80~99)**											
(2) GV2	82	-								**(54~65)**				**(54~62)**	
(3) LSVY-Guae	88	82	-												
(4) LYSV-Gchi	85	80	85	-											
(5) LYSV-GV7	82	99	82	81	-										
(6) LYSV-Lfrg	82	88	82	82	88	-									
(7) G7	64	56	65	62	55	63	-			**(85~98)**					
(8) OYDV-Gind3	62	62	56	55	55	54	92	-						**(71~78)**	
(9) OYDV-Gfw	61	61	55	55	54	54	90	95	-						
(10) OYDV-K	63	62	65	62	62	61	89	87	86	-					
(11) OYDV-V	63	61	65	63	61	60	85	89	88	98	-				
(12) OYDV-AwM15	59	60	55	54	54	54	75	76	76	77	76	-		**(89~99)**	
(13) OYDV-AcR9	59	62	55	54	54	54	72	76	76	76	75	99	-		
(14) WOYSV	57	58	58	59	57	57	71	75	75	77	74	92	92	-	
(15) SYSV	61	58	60	60	58	62	71	76	74	78	75	90	89	89	-

[a] (1) and (7) in this study. (2) garlic virus 2 (GV2) from EMBL accession number D28590 (Nagakubo et al., 1994). (3) leek yellow stripe potyvirus (LYSV-Guae) from EMBL accession number AB005611 (Tsuneyoshi, et al., 1998), (4) leek yellow stripe potyvirus (LYSV-Gchi) from EMBL accession number AB005610 (Tsuneyoshi, et al., 1998). (5) leek yellow stripe potyvirus (LYSV-GV7) from EMBL accession number D11118 (Tsuneyoshi and Sumi, 1996). (6) leek yellow stripe potyvirus (LYSV-Lfrg) from EMBL accession number X89711 (Schubert, 1995), (8) onion yellow dwarf virus (OYDV-Gind3) from EMBL accession number AB000841 (Tsuneyoshi, et al , 1998), (9) onion yellow dwarf virus (OYDV-Gfw) from EMBL accession number AB000838 (Tsuneyoshi, et al., 1998), (10) onion yellow dwarf virus (OYDV-K) from EMBL accession number X95874 (Kobayashi, 1996), (11) onion yellow dwarf virus (OYDV-V) from EMBL accession number Y11826 (van der Vlugt, 1997a), (12) onion yellow dwarf virus (OYDV-AwM15) from EMBL accession number D73378 (Sumi, 1997), (13) onion yellow dwarf virus (OYDV-AcR9) from EMBL accession number AB000474 (Tsuneyoshi, et al., 1997), (14) welsh onion yellow stripe virus (WOYSV) from EMBL accession number Y11748 (van der Vlugt, 1997b), (15) shallot yellow stripe virus (SYSV) from EMBL accession number Y11747 (van der Vlugt, 1996).


DAG
DEA72 AGEELDAGTQASKNQKNNADKSVEQRNPLVSQTSTHRKNGSGDNSELSMGKDRDVNVGTTGTFSV
GV2 .ND.....M.T..K..DGN...I...D.AS..V.SLG..DGEGG.GM.RN.....
LSVY-Guae ..D.....VNRS..AS.. .I.....A...GNKDVSTS..A.....
LYSV-Gchi .....SS.S.T...I.....APT....GGRKDD-...D..SS.....
LYSV-GV7 .ND.....M.T..K..DGN...I...D.AS..V.SLG.KDGEGG.GM.RN.....
LYSV-Lfrg ..D.....M.TN.K.TD.T...I...S.VAPR...QGG.DG.SS....GT.....
G7 ...GE.QRHN-------QVRHNRWKQ----------..K..DT.....I
OYDV-Gind3 ...GE..AAQ------SSTS.Q.TKQ----------..K..DT....K.EI
OYDV-Gfw ...GE..AAQ------SSTSQQ.AKQ----------..K..DT....K.AI
OYDV-K ----------T.QIAKQ----------..K..DA....Q...
OYDV-V ..PGE..AAQ------SSTT.QIAKQ----------..K..DA....Q...
OYDV-AwM15 .S.IE..AN-------V.T..Q.GKS----------KD..K..D...S.E...
OYDV-AcR9 .S.TE..AN-------V.T..Q.GKN----------KD..K..D...S.E...
WOYSV .S.TE..AN--------V.T..Q.GKN----------KD..K..D...S.E...
SYSV .T.SE..AN--------V.T..Q.GKN----------KD.....D...S.E...
PVA AETLDASEALAQKSEGRKKERESNSSKAVAV..K..DL..A..H..
PVY-N ANDTIDAGGSNKKDAKPEQGSIQPNPNKG..K...A..S..HT.
SMV SGKEKEGDMDAGKDPKKSTSSSKGAGTSSK.....SK.K-V.
TEV SGTVDAGADAGKKKDQKDDKVAEQAS......A..S.....
DEA72 PRIKQIPQKGIAIPMDGGKSILNLDHLLQYKPSQLCISNTRATKTQPMANTRRLQEEYGVTESEM
GV2 .....S.....K..N.....VA..KT.ME.V..D....KG..
LYSV-Guae .....SR.....Y.....RA..AS.RT.....DG..
LYSV-Gchi .....T..LS....E...V.....RA..KT.RSK..D....SD.D.
LYSV-GV7 .....S.....N..N.....VA..KT.ME.V..D....KG..
LYSV-Lfrg .....S.....T.....N.....AA..KT.IE.V..D....KDD.
G7 ....ALSD.-MRF.KV.KIVV..AE...A...E.IELY...S..Q..EN.YNAIKK..D.NDDQ.
OYDV-Gind3 ....VLSD.-MRF.KV.KNVV..AE...A...D.IELY...S..Q..EN.YNAIKK..D.NDEQ.
OYDV-Gfw ....ALSD.-MRF.KV.KTVV..AE...A...E.IELY...S..Q..EN.YNAIKK..D.NDEQ.
OYDV-K ....ALSD.-MRF.KV.KNVV..AE...T...E.IELY.....LR..EN.YMAIKK..D.DDEQ.
OYDV-V ....ALSD.-MRF.KV.KNVV..AE...T...E.IELY.....LP..EN.YMAIKK..D.DDEQ.
OYDV-AwM15 .KV.MLSD.-MRL.RV.K.V...GK...T...D.VDLY.....HA..KT.YDAVKL..EL.DEQ.
OYDV-AcR9 .KV.MLSD.-MRL.RV.K.V...GK...A...D.VDLY.....HA..KT.YDAVKL..EL.DEQ.
WOYSV .KA.MLSDE-MRL.RV.K.V...GK...A...D.VDLY.....HA..KT.YDAVKL..EL.DEQ.
SYSV .KV.MLSD.-MRL.RI.K.VV..GK...S...D.IDLY.....HA..KT.YEAVKL..EL.DEQ.
PVA ..L.SMTS.-LTL..LK...VV.....S...K.VDL..A...HE..QN.YDGVMAS.ELE..S.
PVY-N ....A.TS.-MRM.TSK.ATV...E...E.A.Q.ID.....QS..DT.YEAVRMA.DIG.T..
SMV ..LQK.TR.-MNL..VE..I..S.....E...N.VDLF.....R...E..YNAVKD..ELDDEQ.
TEV ...NAMAT.-LQY.RMR.EVVV..N...G...Q.IDL..A...HEQ....HQAVMTA...N.EQ.
DEA72 SIMLNGLMVWCIENGTSPNINGVWTMMDGDEQVEFPLRPVVEHAQPTLRQIMAHFSALAEAYIEM
GV2 G.I.....T.....AY....I....K.....
LYSV-Guae ..I.....E.....
LYSV-Gchi .VI....V.....V.....E.....
LYSV-GV7 G.I.....T.....AY....I....K.....K.....
LYSV-Lfrg GVI.....T.....VY....I....K.....
G7 K.L.....LS.N.....E....Y..A.IVDN.K..F.....DAR.....Y
OYDV-Gind3 K.L.....LS.N.....Y..A.IVDN.K..F.....DA.....Y
OYDV-Gfw K.L.....LS.N.....D.I.Y..A.IVDN.K..F.....DA.....Y
OYDV-K K.I.....LT.N.....E....Y..A..LDN.K..F.....DA.....Y
OYDV-V K.I.....LT.N.....E....Y..A..LDN.K..F.....DA.....Y
OYDV-AwM15 K.VM.....Q.LT.....N.M.Y..S.IIDN.K..F.....DA.....Y
OYDV-AcR9 K.VM.....Q.LT.....N.M.Y..S.IIDN.K..F.....DA.....Y
WOYSV K.VM.....Q.LT.....N.M.Y..S.IIDN.K..F.....DA.....Y
SYSV K.VM.....Q.LT.....N.M.Y..S.IIDN.K..F.....DA.....Y
PVA E.I...F.....D.....NE...SY..K.MLDH.K.S.....R.....
PVY-N PTVM.....V....V....N....Y..K.I..N.K.....DV.....
SMV GVVM..F.....D.....DA....V....E..I.Y..K.I..N.K.....H...DA.....
TEV K.L...F.....L..T.V....ED..SY..K.M..N.....T...D.....
DEA72 RNAEQAYMPRYGLQRNLTDMSLARYAFDFYEVTSRTPVRAREAHAQMKAAALRNSRPRLFGLDGN
GV2 ..S.....G.....
LYSV-Guae .....A.....
LYSV-Gchi ..S.....
LYSV-GV7 ..S.....G.....
LYSV-Lfrg ..S.....G.....S.....
G7 ...TEK.....EL.....M..K..K..K...M.....V.GATN.....
OYDV-Gind3 ...TEK.....EL.....M..K..K..K...M.....V.GATN.....
OYDV-Gfw ...TEK.....EL.....M..K..K..K...M.....I.GVTN.M.....
OYDV-K ...TEK.....EL.....M..K..T..K...M.....RV.GAAN.S.....
OYDV-V ...TEK.....EL.....M..K..T..K...M.....V.GAAN.....
OYDV-AwM15 ...TEK.....REY.....MN.K..I..K...M.....V.GVAN.M.....
OYDV-AcR9 ...TEK.....REY.....MN.K..I..K...M.....V.GVVN.M.....
WOYSV ...TEK.....REY.....MN.K..I..K...M.....V.GVAN.M.....
SYSV ...TEK.....REY.....MN.K..I..K...M.....V.GAAN.M.....
PVA .SR.KP.....R.Q.....I.AT..I..K...L.....K..NTNM.....
PVY-N ..KKEP.....I...R..G.....I.....KSAQ.....G
SMV ..S.SP.....L...R.RE.....K..NP....I.....SGVNNK.....
TEV ..R.RP.....I.....S.....L..K.....M.....V...GT.....
GFV2
DEA72 VTTMDEDTERHTAHDVNARMHHLDGAHMQ-
GV2 ...T.....V.....R...-
LYSV-Guae ...T.....-
LYSV-Gchi .....-
LYSV-GV7 ...T.....V.....-
LYSV-Lfrg ...T.....-
G7 .N.TE.....A.I.KNQ.T.A.IK.--
OYDV-Gind3 .N.TE.....A.I.KNQ.T.L.IK.--
OYDV-Gfw .N.TE.....A...KNQ.T.L.IK.--
OYDV-K .N.TR.....T...KHQ.T.L.VR.--
OYDV-V .N.TE.....T...KHQ.T.L.VR.--
OYDV-AwM15 IS.D..N.....A...KDH.T.L.LR.--
OYDV-AcR9 IS.D..N.....A...KDH.T.L.LR.--
WOYSV IS.D....V....A...KDH.T.L.LR.--
SYSV IG.D..N.....A...KDH.T.L.LR.--
PVA ...SE.....T...RN....L.VKGV-
PVY-N IS.QE.N.....TE..SPS..T.L.VKNM-
SMV IS.NS.N.....R...QN..T.L.MGPPQ
TEV .G.AE.....RN..T.L.VRQ--

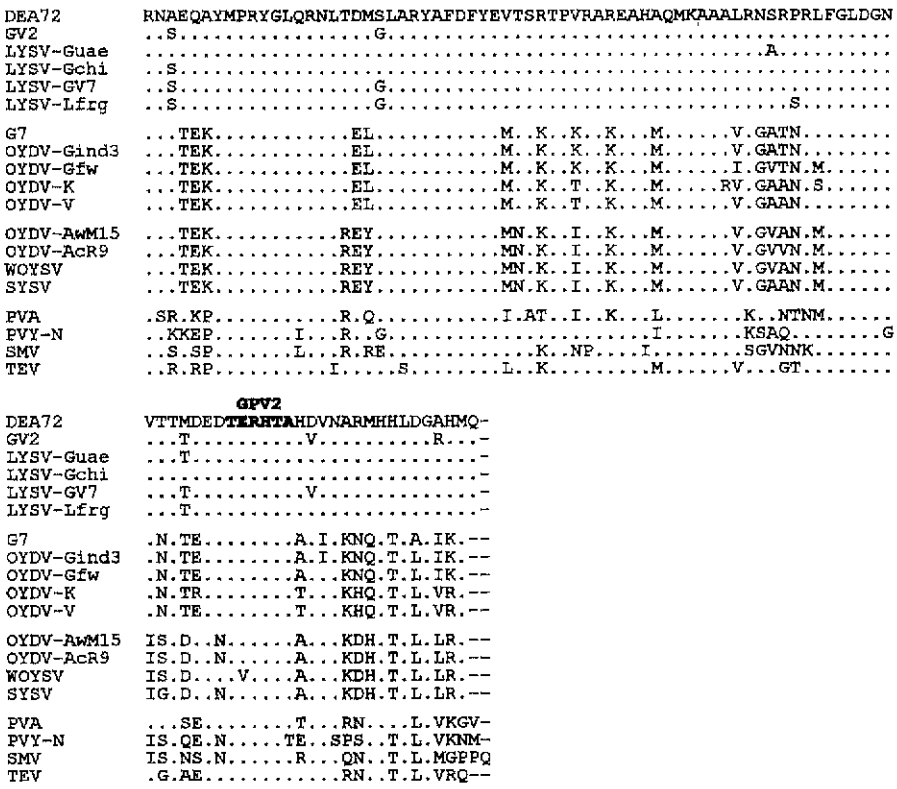


Fig. 2. Comparison of coat protein (CP) deduced amino acid sequences among G7, DEA72, nine other *Allium* potyviruses, and four other potyviruses. The dot (.) indicates identical amino acid among CPs. Gaps (---) were introduced to optimize alignments using the CLUSTAL W alignment program. The amino acid sequence of CP for garlic virus 2 (GV2) from EMBL accession number D28590 (Nagakubo et al., 1994), leek yellow stripe potyvirus (LYSV-Guae) from EMBL accession number AB005611 (Tsuneyoshi, et al., 1998), leek yellow stripe potyvirus (LYSV-Gchi) from EMBL accession number AB005610 (Tsuneyoshi, et al., 1998), leek yellow stripe potyvirus (LYSV-GV7) from EMBL accession number D11118 (Tsuneyoshi and Sumi, 1996), leek yellow stripe potyvirus (LYSV-Lfrg) from EMBL accession number X89711 (Schubert, 1995), onion yellow dwarf virus (OYDV-Gind3) from EMBL accession number AB000841 (Tsuneyoshi, et al., 1998), onion yellow dwarf virus (OYDV-Gfw) from EMBL accession number AB000838 (Tsuneyoshi, et al., 1998), onion yellow dwarf virus (OYDV-K) from EMBL accession number X95874 (Kobayashi, 1996), onion yellow dwarf virus (OYDV-V) from EMBL accession number Y11826 (van der Vlugt, 1997a), onion yellow dwarf virus (OYDV-AwM15) from EMBL accession number D73378 (Sumi, 1997), onion yellow dwarf virus (OYDV-AcR9) from EMBL accession number AB000474 (Tsuneyoshi, et al., 1997), welsh onion yellow stripe virus (WOYSV) from EMBL accession number Y11748 (van der Vlugt, 1997b), shallot yellow stripe virus (SYSV) from EMBL accession number Y11747 (van der Vlugt, 1996), potato virus A (PVA) from Puurand et al. (1994), potato virus Y (PVY-N) from Robalia et al. (1989), soybean mosaic virus (SMV) from Jayaram et al. (1992), and tobacco etch virus (TEV) from Allison et al. (1986).

GDD motif, and containing full-length coat protein (Fig. 1). The location of the genes of G7 and DEA72 CP are determined by comparison of their deduced partial polyprotein amino acid sequences with those of several other potyviruses. Using the proposed consensus polyprotein cleavage sites, V(R or K)FQ/(G or S) for tobacco vein mottling virus (TVMV; Domier et al., 1986), E--Y-Q/(G or S) for tobacco etch virus (TEV; Carrington and Dougherty, 1987) and V-(H or E)Q/(G or S or Q) for PVY-N (Robaglia et al., 1989), as indicators, CPs are predicted to be cleaved from the putative polyprotein between the Q/A dipeptide sequence at amino acid position as shown in Fig. 1A and B. Another potential cleavage site in this region of DEA72 was the Q/A dipeptide at amino acid position 142/143; however cleavage at this point would result in an N-terminal extension not shared by other potyviral CPs. The molecular mass of 32.5 kDa was calculated for the 289 amino acid putative DEA72 CP, which is in reasonable agreement with the molecular mass of 34.0 kDa determined experimentally by immunoblotting analysis. The putative CP sequences of G7 and DEA72 were aligned with and compared to those of several other potyviruses (Fig. 2) and the C-terminal sequence showed far greater similarity than the N-terminal sequence, as is true for all known potyviruses (Shukla and Ward, 1988, 1989).

Shukla and Ward (1988, 1989) reported that distinct members of the potyvirus group exhibited sequence homologies ranged from 38% to 71% (average 54%) with major

differences in the length and sequence of their N termini and high sequence homology in the C-terminal half of CPs. However, strains of individual viruses exhibited sequence homologies of 90% to 99% (average 95%) and had very similar N-terminal sequence. As shown in Table 1, the amino acid sequence similarity of CPs between G7- and DEA72-potyvirus is 64%. Furthermore, the lengths of their N termini of CPs are different (Fig. 2). These data also suggest that they are distinct potyvirus by this criterion. Our data clearly suggests clearly G7- and DEA72-potyvirus to be a distinct potyvirus from potato virus A (PVA; Puurand et al., 1994), PVY (N strain; Robalia et al., 1989), SMV (Jayaram et al., 1992) and TEV (Allison et al., 1986).

G7- and DEA72-potyvirus showed 55% to 88%, and 54 % to 82% amino acid sequence similarities of CPs to other *Allium* potyviruses, respectively (Table 1). The CP of G7-potyvirus, however, showed 85% to 92% amino acid sequence similarities with OYDV-Gind3, -Gfw, -K, and V. G7-potyvirus also showed 71% to 75 amino acid sequence similarity with WoYSV. SYSV, OYDV-AwM15 and OYDV-AcR9 in CP gene. However, it showed only 55% to 58% nucleotide sequence similarity with OYDV-AwM15, WoYSV and SYSV in the 3'-NC region. Therefore, G7, OYDV-Gind3, -Gfw, -K, -V are strains of the same virus, but they are distinct members of potyvirus group from OYDV-Awm15, OYDV-AcR9, WoYSV, and SYSV by Frenkel's criteria (1989) and Shukla and Ward's criteria (1988, 1989). DEA72-potyvirus showed about 81% to 88% amino acid sequence similarity with GV2, LYSV-Gue, -Gchi, GV7, and Lfrg (Table 1). The value was in middle range from distinct to strains (38% to 71% and 90% to 99%). However, DEA72-potyvirus showed about 88% nucleotide sequence similarity with GV2 and LYSV-GV7 in 3'-NC region. These data suggest that DEA72-potyvirus, GV2 and LYSVs are strains of the same virus by Frenkel's criteria (1989) and Shukla and Ward's criteria (1988, 1989). Recently, Tsuneyoshi et al. (1998) cloned cDNAs of potyviruses from *Allium* plants by RT-PCR method. The degree of their nucleotide sequence similarities clearly differentiated the respective viruses into three groups; namely OYDV garlic-type, OYDV wakegi-type, and LYSV group. Our results consist with their result. According to them, DEA72-potyvirus belong to LYSV group and G7-potyvirus to OYDV garlic-type group, respectively.

In potyviruses, it is demonstrated that critical amino acid residues, namely DAG or DAG-like motif, involved in virus transmission by aphids are located near the N-terminus of the CP and their mutation result in loss of insect transmissibility (Atreya et al., 1990; Atreya et al., 1991). Recently, the CP of several tobamoviruses has been shown to be a determinant of host range through its involvement in the long-distance spread of the virus as well as in virus particle assembly (Hilf and Dawson, 1993; Berzal-herranz et al., 1995). DEA72-potyviruses (LSYV group) contain longer N-termini of CP than those of G7-potyviruses (OYDV-garlic group) and OYDV wakegi-type group. Each group showed different amino acid sequences for DAG-like motif: DEA72-potyviruses (LYSV group), NVG; G7-potyviruses (OYDV-garlic group). D(T/A)G; OYDV-wakegi-type, DVG. These features also support that each group is different kinds of distinct potyvirus groups.

We conclude that potyviruses infecting *Allium* plants could be classified into at least three kinds of distinct potyvirus groups on the basis of nucleotide sequence similarities in the 3'-NC region (Frenkel et al., 1989) and amino acid sequence similarities in CPs (Shukla and Ward, 1988, 1989). (1) G7-potyvirus and OYDV-K and OYDV-V are strains of the same virus (OYDV garlic-type group, Tsuneyoshi et al., 1998). (2) DEA72-potyvirus, GV2 and LYSVs are strains of another same virus (LYSV group, Tsuneyoshi et al., 1998). (3) OYDV-S, WoYSV and SYSV are strains of the other same virus (OYDV wakegi-type group, Tsuneyoshi et al., 1998).

Northern blot and immunoblotting analysis. To figure out the length of genomic RNA, Northern blot analysis was carried out. The genome size of G7-potyvirus and DEA72-potyvirus were analyzed to be approximately 9.0 kb and 9.6 kb by Northern blot analysis, respectively. This result also supports that they are distinct potyvirus each other. North-

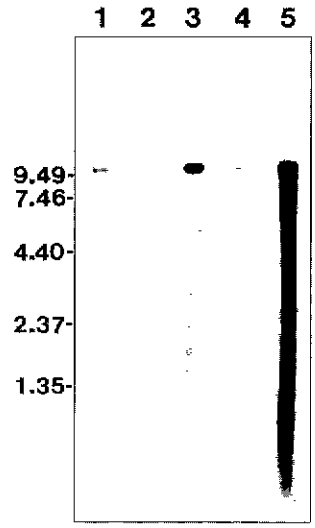


Fig. 3. Northern blot analysis of garlic virus RNAs from different garlic plants. Viral RNAs were separated by 0.8% formaldehyde agarose gel electrophoresis and transferred onto nylon membrane. The filter was probed with the random primer-extended clone DEA72. Each sample is garlic virus RNA prepared from garlic plants cultivated in Eosangcheon (lane 1), Daegang (lane 2), Wonju (lane 3), Euiseong 1 (lane 4), and Euiseong 2 (lane 5) The positions of markers are shown at the left side of the blot.

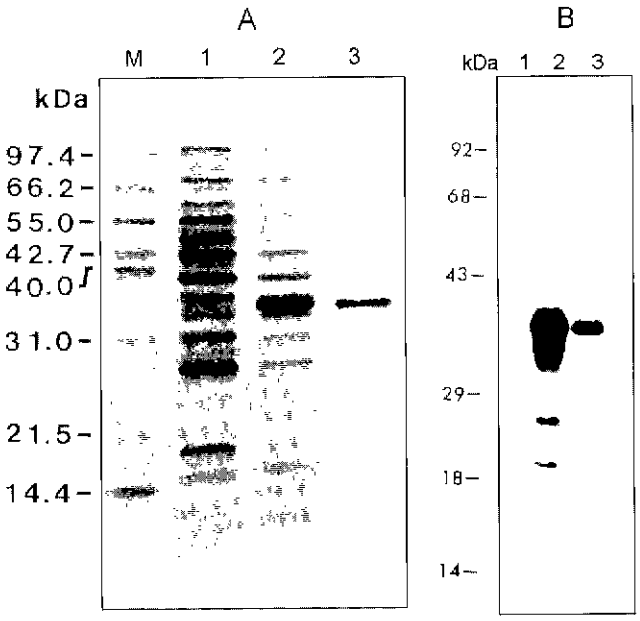


Fig. 4. SDS-PAGE and immunoblotting analysis of the recombinant DEA72-potyvirus CP. **A**; SDS-PAGE analysis of DEA72-potyvirus CP fusion protein expressed in *E. coli* BL21. Each lane contains total cell lysate of *E. coli* transformed with the vector only (lane 1), lysate of IPTG-induced *E. coli* transformed with the pDEA72-CP (lane 2), or the Ni^{2+}-NTA-purified recombinant CP (lane 3). **B**: Immunoblot analysis of the recombinant DEA72-potyvirus CP. Protein samples were analyzed by 12.5% SDS-PAGE, transferred onto nitrocellulose paper and probed with an anti-CP antibody for DEA72-potyvirus. Each lane contains total cell lyzate of *E. coli* transformed with vector only (lane 1), lyzate of IPTG-induced *E. coli* transformed with the pDEA72-CP (lane 2), or the Ni^{2+}-NTA-purified recombinant CP (lane 3).

ern blot analysis reveals that almost all of the tested garlic plants were infected with DEA72-potyvirus (Fig. 3), but rarely with G7-potyvirus (data not shown).

To raise antibodies against CPs of G7- and DEA72-potyvirus, coding region of CP gene was recombined into pRSET expression vector. The pRSET vector allows high level expression of a foreign gene in *E. coli* by the T7 promoter. Antibodies against CPs of G7- and DEA72-potyviruses were prepared with the recombinant CPs (Fig. 4A). To figure out the specificity of anti-DEA72 CP polyclonal antibody, immunoblot analysis was carried out (Fig. 4B). The anti-DEA72 CP antibody recognized the recombinant CP of DEA72-potyvirus antigen (Fig. 4B, lane 2 and 3). This result demonstrated the specificity of the antibody.

To investigate the extent of infection by garlic potyvirus of garlic plants, individual garlic plants were subjected to immunoblot analysis. Immunoblot analysis with anti-G7 CP antibody showed that only one of the tested garlic plants showed signal (data not shown). Immunoblot analysis with anti-DEA72 CP antibody showed that almost all of the tested garlic plants showed a signal at 34 kDa. The typical

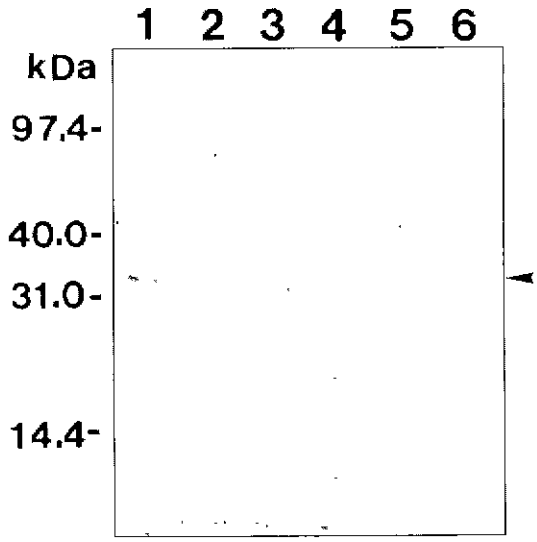


Fig. 5. Immunoblotting analysis of DEA72-potyvirus CP from various garlic plants. Equal amounts of leaf extracts from individual garlic plants collected from various regions were separated by 15% SDS-PAGE, transferred onto nitrocellulose paper and probed with anti-DEA72 CP antibody. Garlic leaf extracts of Wonju (lane 1), Dongyang (lane 2), Danyang (lane 3), Euiseong (lane 4), Sunsan (lane 5), Danbook (lane 6), Eosangcheon (lane 7), Oakpochun (lane 8) and Saljukdae (lane 9). Position of CP is shown by arrowhead.

results are shown in Fig. 5. The intensity of these signals, however, was variable, as observed in the Northern blot analysis. These data suggest that almost all of the tested garlic plants infected with DEA72-potyvirus, but rarely with G7-potyvirus. At this time, however, it is not clear whether the varying intensity of the signals reflects the relative amount of DEA72-potyvirus infecting the garlic plants, or not. The other possibility is that there are more variations depending on the strains of the DEA72-potyvirus (LSYV group) and the amino sequence variation in CPs of this group could make the varying intensity.

The size of DEA72-potyvirus particles was 750 nm long. To identify the DEA72-potyvirus particles from infected garlic plants, immunoelectron microscopy was carried out using an anti-DEA72 CP polyclonal antibody (Fig. 6). There were viruses of various lengths in the microscopic field. The virus particles of flexuous rod-shape were decorated no more than 5% and the length of the decorated virus particles was estimated to be 750 ± 15 nm long. This result shows that DEA72-potyviurs is one of the viruses infecting garlic plants and its length is about 750 nm long. It has been reported that carlaviruses are 610-700 nm long, flexuous rod shaped, while potyviruses are 720-900 nm long (Hollings and Brunt, 1981; Koenig, 1982). Therefore, DEA72-potyvirus was pretty much the same size as the other potyviruses as observed by electron microscopy.

A potyvirus, named GMV, was thought to be a causative

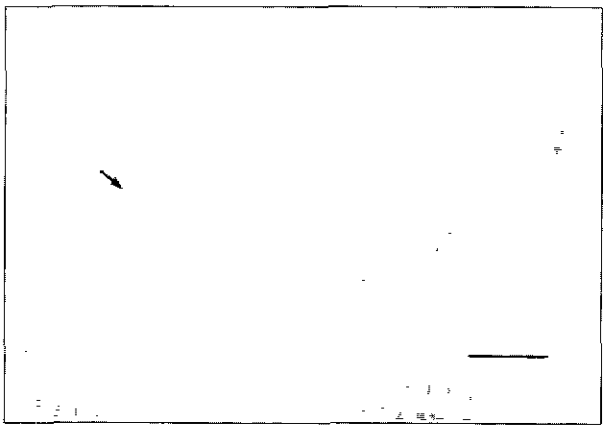


Fig. 6. Detection of DEA72-potyvirus particles by immunoelectron microscopy. Virions in a virus preparation from a garlic plant from Wonju were subjected to a primary decoration using anti-DEA72 CP polyclonal antibody. A decorated virus is indicated by arrow. The bar represents 500 nm.

agent of the garlic virus disease. GMV particles were flexuous rods of about 750 nm long and pinwheel-type cytoplasmic inculsions were found in the infected garlic plant cell (Chang et al., 1988; Lee et al., 1979). However, GMV has not been further characterized at the molecular level, so it is not clear at molecular level what is GMV in garlic potyviruses. DEA72-potyvirus infected garlic plants, which showed streak or mosaic symptoms and these virus particles were flexuous, rod-shaped, and about 750 nm long as determined by immunoelectron microscopy. These features of DEA72-potyvirus (LYSV group) are the same to those of GMV. From these data, we suggest that DEA72-potyvirus and GMV could be the same virus.

DEA72-potyvirus (LYSV group) was isolated from Korean garlic plants showing mosaic or streak symptoms. Northern blot and immunoblotting analysis with anti-DEA72 CP antibody showed that almost all of the tested Korean garlic plants were infected with this virus. These results suggest that Korean garlic plants are mainly infected with DEA72-potyvirus (LYSV group), but rarely infected with G7-potyviruses (OYDV garlic-type group). Even though there is no clear method for identification of garlic viruses, garlic plants are usually mixed-infected with various viruses including carlaviruses, GarV-type viruses, potexviruses and potyviruses (Sumi, et al., 1993, Choi et al., 1995, Song et al., 1995, Tsuneyoshi and Sumi 1996, Song et al., 1997, Song et al., 1998, Tsuneyoshi et al., 1998). So, molecular identification studies including genome characterization should be carried out.

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