Proc. Int. Symp. on Biological Control
of Plant Diseases, pp. 86-102

Molecular Biological Studies on Korean Garlic Viruses

Jin Nam Choi, Jong Tae Song, Chan Seok Shin, Yong-Joon La[1], Jong Seob Lee[2], and Yang Do Choi

Department of Agricultural Chemistry and [1]Department of Agricultural Biology, Seoul National University, Suwon 441-744, Korea
[2]Department of Molecular Biology, Seoul National University, Seoul 151-742, Korea

ABSTRACT : To understand the molecular structure and pathogenesis mechanism of Korean garlic viruses, we have isolated cDNA clones for garlic viruses. The partial nucleotide sequences of 24 cDNA clones were determined and that of six clones containing poly (A) tail were compared with those of other plant viruses. One of those clones, V9 has 81.8% similarity in nucleotide sequence and 93.0% in deduced amino acid sequence, respectively, to the coat protein gene for garlic mosaic virus (GMV). Northern blot analysis with the clone V9 demonstrated that the genome of GMV is 7.8 kb long and has poly (A) tail. The anti-coat protein antibody for GMV recognizes 35 kDa polypeptide which could be the coat protein of GMV from infected garlic leaf extract or virus preparation. Clone G7 has about 62% of deduced amino acid sequence identity with the members of potyvirus group. Northern blot analysis with the clone G7 demonstrated that the genome of the potyvirus in garlic is 9.0 kb long and has poly (A) tail. The third clone, S81, shows 42% amino acid identity to the potexvirus. The other clones are under the characterization. To test the possibility of producing garlic virus resistant plant, we have designed a hairpin type ribozyme to cleave V9 RNA at the middle of the coat protein gene. From the cleavage reactions *in vitro* with two different sizes of RNA substrates, V9SUB (144 nucleotides) and V9 RNA (1,361 nucleotides), the ribozyme can cleave V9 sequence effectively at the predicted site. To study the activity of the ribozyme *in vivo*, plant transformation is in progress. Further possibilities to produce garlic virus resistant plant will be discussed.

Garlic (*Allium sativum L.*) is an important vegetable crop for the Korean people and has long been cultivated extensively in Korea. Currently garlic mosaic disease is considered to be the most important disease of garlic in Korea. Two sap-transmissible filamentous viruses from garlic plants were described and named as garlic latent virus (GLV) and garlic mosaic virus (GMV) based on the microscopic observation and the symptoms produced in infected garlic plants (41). Symptoms due to the infection of GMV was mosaic in garlic plants. Garlic plants inoculated with GLV produced visible symptoms at early stage of infection but less prominent at later stage (36, 38, 41). Particles of GMV were flexuous rods about 750 nm long and pinwheel-type cytoplasmic inclusions found in the infected garlic plant cells, as is most of the potyvirus (41). Particles of GLV were also flexuous rods, 650~700 nm long, and existed as either randomly or small aggregates in the cytoplasm of the infected plant cells as is most of the carlavirus. However, mixed infection of GLV and GMV were

found in most of the garlic plants showing mosaic symptoms collected from various parts of Korea and Japan (41). Present evidences indicate that most, if not all, of unselected commercial garlic cultivars contain a complex of two or more viruses (6, 8, 62). But the identity of the individual viruses contained in each complex is variable, particularly in materials from diverse geographical locations. Garlic mosaic virus (GMV), garlic yellow streak virus, leek yellow stripe virus and onion yellow dwarf virus are known as potyviruses occurring in vegetable Allium plants (49). Therefore, the identification of viruses infecting garlic is complex and the related literatures are not clear in this aspect (62).

To understand the molecular structure and identities of garlic viruses, molecular cloning of cDNAs for viral genome was attempted. Virus particles were isolated from field-grown garlic leaves and a cDNA library was constructed. Molecular evidences are described for the presence of multiple species of viruses in field-grown garlic.

To test the possibility of producing virus resistant plant in this study, we intended to try a newly designed hairpin ribozyme to cleave coding sequence of GMV coat protein at specific site. Further possibilities to produce virus resistant garlic by gene manipulation will be discussed.

Materials and Methods

Garlic samples. Garlic (*Allium sativum L.*) samples used in this experiment were collected from Danyang area and grown at the experimental farm of Seoul National University.

Virus preparation. Garlic virus particles were isolated from the virus-infected garlic leaves by sucrose gradient centrifugation as described by Langenberg (40) and La (37).

Construction of garlic virus cDNA library. cDNA library was constructed into pUC18 plasmid vector and gt11 phage vector by the method of Gubler and Hoffman (20) with minor modification. Garlic virus was treated with 1% SDS, 20 mM EDTA, and 0.25 mg/ml Proteinase K, incubated for 10 min at 60℃, extracted with phenol/chloroform/isoamylalcohol (25 : 24 : 1), and then precipitated with ethanol. Five μg of garlic virus RNA isolated from virus-infected garlic leaves was used as a template. Oligo (dT) was used as primer.

Nucleotide sequencing and recombinant DNA techniques. Nucleotide sequencing was carried out in plasmid pUC18 (61) by the dideoxynucleotide chain termination method of Sanger *et al.* (52). Universal M13 primers for reverse and forward reaction were used and the reaction products were analyzed by 6 M urea-polyacrylamide gel electrophoresis. Northern blot was carried out by the procedure of Kroczek and Siebert (35). Purification and manipulation of DNA were carried out according to the standard protocol of Sambrook *et al.* (51).

Design of oligodeoxynucleotides for ribozyme reaction. Since natural substrates in plant viral satellite RNAs for hairpin catalytic RNAs contain GUC or GUA sequence, one such site was selected from GMV RNA. We analyzed seconday structure of V9 RNA by 'PC-FOLD' program. The target region containing GUC (bases 278 to 410: $\triangle G$ value of -14.5 Kcal/mol) seems to the most open to access by a ribozyme compared with other regions contai-

ning GUC. Synthetic double stranded oligodeoxynucleotide determining target site was ligated with hairpin ribozyme encoding DNA. Hairpin ribozyme domain sequence was taken from (−)strand of TRSV (26).

Preparation of ribozyme and substrate RNA. Synthesized ribozyme DNA or V9 fragment was subcloned under the control of T7 polymerase in pBluescript SK transcription vector. The substrate and ribozyme RNA were prepared by *in vitro* transcription of the linearized plasmid with T7 RNA polymerase (Promega, technical manual, 1992). Then RNA products were analyzed by electrophoresis in 5% polyacrylamide/7M urea gel and by autoradiography.

Ribozyme cleavage reaction. The mixture for cleavage reactions contained 40 mM Tris-HCl (pH 8.0), 4 mM $MgCl_2$, 2 mM spermidine, 0.3 pmole each of ^{32}P-labeled substrate RNA and ribozyme RNA in a total volume of 10 μl. Mixtures were incubated for 1 hr at 50℃. After reaction, product was denatured by heating in loading mixture, fractionated on 5% polyacrylamide/7 M urea gels and autoradiographed.

Results and Discussion

Heterogeneity of viruses infecting field-grown garlic. To study molecular structure of garlic viruses (GV), virus particles were isolated from virus-infected garlic leaves by Langenberg method (40) with minor modification. It was purified by 10~40% sucrose density gradient sedimentation and peak fraction was analyzed by electron microscopy and 12.5% SDS-polyacrylamide gel electrophoresis. Flexuous rod-shaped viruses of 600~800 nm was predominant as reported previously. At least five protein bands were resolved by SDS-PAGE and the molecular weights of the proteins were 27, 30, 34, 38, and 43 kDa (Fig. 1). These proteins could be regarded as structural proteins of garlic viruses. Therefore, the virus preparation can be considered to contain at least five different kinds of viruses, if each virus is made up of a single kind of coat protein. It also suggests that field grown garlic plants are indeed infected with multiple species of viruses. These results are consistent with Northern blot analysis using cDNA clones for GV as is shown later on. Purification of individual virus by serial infection in indicator plants didn't work even though partially successful.

To isolate the genomic nucleic acid from GV, the peak fraction was treated with SDS, EDTA, and Proteinase K and analyzed by 1.2% formaldehyde agarose gel electrophoresis (Fig. 2, lanes 1 and 2). The major band of about 8.2 kb was the same as the genome size predicted from the size of rod-shaped GV particles of about 700 nm. When the nucleic acid was treated with RNase A (50 μg/50 μl for 30 min at 37℃), the major band disappeared suggesting that GV is a RNA virus (Fig. 2, lane 3). It is not possible, however, to resolve individual viral genome at this range.

Multiple species of cDNA clones for GV are isolated. To isolate the cDNA clone for GV, garlic virus cDNA library was constructed. We used oligo (dT) as a primer because carlavirus and potyvirus of 600~800 nm long usually have poly (A) tail at the 3′ end of genome (30). We have isolated 24 cDNA clones for garlic viruses and determined their

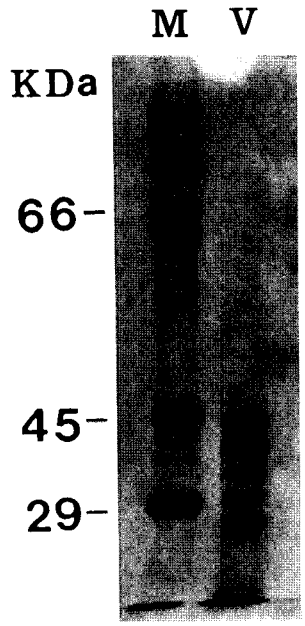

Fig. 1. SDS-PAGE analysis of garlic virus particles prepared from field-grown garlic leaves. Protein samples were analyzed by 12.5% SDS-PAGE and stained with Coomassie Blue. lane M: size marker, lane V: garlic virus particles from the leaves of garlic with mosaic symptom.

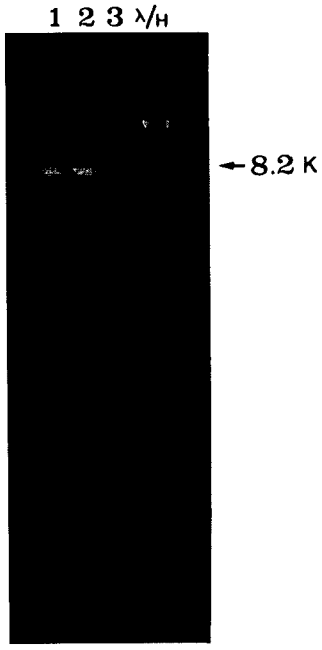

Fig. 2. Size analysis of garlic virus RNA. RNA was analyzed by 1.2% formaldehyde agarose gel electrophoresis and stained with ethidium bromide. Lanes 1 and 2 are from two different virus preparation. Virus genome of lane 1 was treated with RNase (50 μg/50 μl) for 30 min at 37℃ and analyzed in lane 3. λ/*Hind*III size marker is shown.

nucleotide sequences. Six different clones of them contained poly(A) tracts of 15~57 nucleotides at the 3′ end suggesting that they are cDNA clones from the 3′ end of viruses. The insert size of these clones was 2.0 kb(G7), 1.4 kb(V9), 1.5 kb(S83), 1.0 kb(S81), 1.0 kb(S106), and 0.55 kb(S64). As a result of Northern blot analysis, 6 clones strongly bind to the total GV RNA from field-grown garlic leaves and poly(A) RNA purified from total GV RNA by oligo (dT) cellulose chromatography (data not shown). Therefore, we confirmed that six clones are cDNA clones for garlic virus. The full nucleotide sequences of V9, G7, S83, S64, S81 and S106 were determined. The determined sequences were compared with those of other plant viruses in databank. First, these clones have no sequence similarity each other. This result further confirms that there are at least six different kinds of viruses in garlic as expected from previous SDS-PAGE analysis. The nucleotide sequencing analysis of cDNA clones are summarized in Table 1.

The clone V9 encodes a coat protein for GMV, a carlavirus. The full nucleotide sequence of clone V9 was determined (Fig. 3). It shows 81.8% similarity in 1,333 bp region in nucleotide sequence and 93.0% identity in 298 amino acids region in deduced amino acid sequence to the coat protein for GMV. The clone V9 consists of two open reading frames. The first ORF which corresponds to positions 55~945 seems to encode a GMV coat protein of 32

Table 1. The summary of nucleotide sequence analysis of 6 clones containing poly (A) tail

Clone	Size(bp)	Identity to the coat protein gene of			Group	Ref.
		Virus	Nucleotide sequence	Amino acid sequence		
G7	1,841	potato virus Y	54.0% in 1,675 bp	61.9% in 247 aa	Potyvirus	1
		tobacco etch virus	59.1% in 1,512 bp	62.4% in 255 aa		2
		soybean mosaic virus	55.9% in 1,159 bp	61.4% in 233 aa		3
S83	1,562	potato virus S	49.5% in 1,477 bp	39.4% in 282 aa	Carlavirus	4
		garlic mosaic virus	51.2% in 1,483 bp	49.8% in 241 aa		5
V9	1,346	garlic mosaic virus	81.8% in 1,333 bp	93.0% in 298 aa	Carlavirus	5
S81	867	potato virus X	48.2% in 767 bp	41.9% in 160 aa	Potexvirus	6
S64	498	garlic virus A	66.4% in 459 bp	55.0% in 111 aa	Unclassified	7
		garlic virus B	81.5% in 449 bp	79.1% in 110 aa		
		garlic virus C	71.8% in 451 bp	68.5% in 111 aa		
		garlic virus D	63.9% 452 bp	57.7% in 111 aa		
S106	1,000	not identified	?	?	Unclassified	

Ref. : 1. Robaglia *et al.*, *J. Gen. Virol.* 70 : 935-947(1989).
2. Allison *et al.*, *Virology* 147 : 309-316(1985).
3. Eggenberget *et al.*, *J. Gen. Virol.* 70 : 1853-1880(1989).
4. Mackenzie *et al.*, *J. Gen. Virol.* 70 : 1053-1063(1989).
5. Sumi S. EMBL AC : D11161.
6. Kavanagh *et al.*, *Virology* 189 : 609-617(1992).
7. Sumi *et al.*, *J. Gen. Virol.* 74 : 1879-1885(1993).

```
1     AGCAAGCAAAACCTTTTGGTTCACTTTAGGTATACAGCGCTCTAAATTGATATTATGGCTAACGAAGAA   69
1                                                          M  A  N  E  E        5

70    GAAGAACTCAATAACGTTCAGAATTTGCCGACTCGCGACCCTGGGACTATCCCAGAGCATGAGCAGACG   138
6      E  E  L  N  N  V  Q  N  L  P  T  R  D  P  G  T  I  P  E  H  E  Q  T    28

139   AAAGCAGTGAATGATGTCGGCGTTATGGAGCGTGAGGGTTTCGAAGCCGTGCTACGAAGGACGGAAAAC   207
29     K  A  V  N  D  V  G  V  M  E  R  E  G  F  E  A  V  L  R  R  T  E  N    51

208   AGATTCAACAAACTTAAGGAAAAGTGCATGTCCGAATTATCCAGCGTGAATGTCACGAATTGCGGGTGG   276
52     R  F  N  K  L  K  E  K  C  M  S  E  L  S  S  V  N  V  T  N  C  G  W    74

277   GAGTCTGGACGCCCAAAAGCACAACTAGCTGACAGTCTCAAAGGTGACGCTTCTAATATTTTCACCCGC   345
75     E  S  G  R  P  K  A  Q  L  A  D  S  L  K  G  D  A  S  N  I  F  T  R    97

346   CCTTCTATGGATGCCCTCCTTGTTCGGAATTACGCACCTGAAAGTAACAACATGGCCACTGCTGAGGAA   414
98     P  S  M  D  A  L  L  V  R  N  Y  A  P  E  S  N  N  M  A  T  A  E  E   120

415   TTGGCAAAAATCTCCGCTAAGGTGCAGGCTCTTGGTGCCCCTGAAGAATGTTTAGCTGAAGTGTTCTGG   483
121    L  A  K  I  S  A  K  V  Q  A  L  G  A  P  E  E  C  L  A  E  V  F  W   143

484   GATATATGCATGTATTGCACCACTGCTGGAAGTTCTCCAAATGTAAATCCTAAAGGAACTATCTCTGTT   552
144    D  I  C  M  Y  C  T  T  A  G  S  S  P  N  V  N  P  K  G  T  I  S  V   166

553   GGTGGCAAGGTCGTTACTAGAGACATGGTTGTAGCTGTCATCAAAGAGTACTCCACATTACGCCAGGTC   621
167    G  G  K  V  V  T  R  D  M  V  V  A  V  I  K  E  Y  S  T  L  R  Q  V   189

622   TGCCGCTGCTACGCGCCTGTGGTCTGGAACTACATGTTATTGAATGAGCAACCACCAGCCAATTGGGAC   690
190    C  R  C  Y  A  P  V  V  W  N  Y  M  L  L  N  E  Q  P  P  A  N  W  D   212

691   GCAAAAGGTTTTACTGAGAACACTAAATATGCTGCCTTTGACACTTTTGATGCTGTCACGAATAAAGCT   759
213    A  K  G  F  T  E  N  T  K  Y  A  A  F  D  T  F  D  A  V  T  N  K  A   235

760   GCAATTCAACCTCTTGAGGGCCTGATTAGGGCCCCCACTGATGCAGAACGAATTGCCTTTGCAACGCAT   828
236    A  I  Q  P  L  E  G  L  I  R  A  P  T  D  A  E  R  I  A  F  A  T  H   258

829   AAAAAGTTGGCTCTAACCAAAAATTCTCAGAATGCTCGTTACGCCAATACTTCTGCTGATGTTACTGGA   897
259    K  K  L  A  L  T  K  N  S  Q  N  A  R  Y  A  N  T  S  A  D  V  T  G   281

898   GGTTTCTTCGGCTGTTTTCCAAAAAATAATTTCAGAGAGAATCGATGTTGATCAAACAGAGAACTTACC   966
282    G  F  F  G  C  F  P  K  N  N  F  R  E  N  R  C  *                     298
                                                    M  L  I  K  Q  R  T  Y

967   GTAGGCTACTGCGTGTTATATTTAAATTACATACTAATAAGAATTGTGTGGATCTGATAAATATAATCG   1035
       R  R  L  L  R  V  I  F  K  L  H  T  N  K  N  C  V  V  L  I  N  I  I

1036  TGAGTAAAATAGTGTGTGAAAGTGCGGGTGCTTCGAATGCTCGAGCTCGTAGGGCTAAGAGCATAGGCA   1104
       V  S  K  I  V  C  E  S  A  G  A  S  N  A  R  A  R  R  A  K  S  I  G

1105  GGTGCCCTCGCTGTTTTCGGTGTTCACCTGGTTTTTATTTCACTAAAAACTGTGACACGAAAAATTGTT   1173
       R  C  P  R  C  F  R  C  S  P  G  F  Y  F  T  K  N  C  D  T  K  N  C

1174  CCGCAGGTATTAACTATAATGCAAAAGTCAAGGATTTTATAGTTGATGGTGTAACAATGTGAAACCCTA   1242
       S  A  G  I  N  Y  N  A  K  V  K  D  F  I  V  D  G  V  T  M  *

1243  CTACAAACCTTGGCTAGTTGCCCATAAAACCTAAGTAATGCATAAGTGGGAACGTATAAAATAATTTGT   1311
1312  TTTTAAAATATTTTCGCAAAAAAAAAAAAAAAAAA                                    1346
```

Fig. 3. Nucleotide sequence and deduced amino acid sequence of the clone, V9, for garlic mosaic virus. Coat protein gene corresponds to the first open reading frame from the nucleotide 55-945. The second open reading frame encodes the 10 kDa protein.

kDa. In case of carlavirus and potyvirus, the average molecular weight of coat protein is known to be 31~34 kDa (29, 33). The second ORF which corresponds to positions 942~1232 encodes the 10 kDa protein. The 10 kDa protein, like the analogous proteins of other carlaviruses, contains a highly conserved region which comprises a basic arginine-rich domain and a putative zinc finger motif (32). Analogous 'finger' structures are found in other plant virus-specific proteins (53) and they have been shown to bind nucleic acids *in vitro* in the case of potato virus M (PVM) and chrysanthemum virus B (CVB) (19).

To figure out the RNA transcript corresponding to the isolated cDNA clone, V9, Northern blot analysis was carried out with the clone V9 as a molecular probe (data not shown). GV was isolated from field-grown garlic leaves showing yellow streak. Polyadenylated GV RNA was purified from total virus RNA by oligo (dT) cellulose chromatography. Forty four percent of total garlic virus RNA was retained by oligo (dT) cellulose chromatography. GV RNA and poly(A) GV RNA preparation show strong signal at the position of 7.8 kb and 2.0 kb. This result demonstrated that clone V9 is a cDNA clone for GMV and GMV genome has a poly(A) tail as most of other single stranded RNA virus of plants (30). Poly(A) tail sequence of 18 nucleotides at the 3′ end of the clone V9 supports this fact. The strong signal at the position of 7.8 kb corresponds to GMV genome, but about 2.0 kb long transcript seems to be a subgenomic RNA encoding the viral coat protein. To identify the V9 as a coat protein gene of GMV, cDNA fragment (nucleotides 227-1346) was constructed into pRSET-A expression vector and expressed in *E. coli*. pRSET vector allows high level expression of foreign gene in *E. coli* by the T7 promoter. Recombinant construct pRAT12 was transformed into *E. coli* BL21 and T7 RNA polymerase was induced by IPTG. The expressed GMV coat protein was purified by Ni^{2+}·NTA affinity chromatography because of an N-terminal metal binding domain on the fusion peptide provided by pRSET vector. The expressed protein was analyzed by 12.5% SDS-PAGE (data not shown). The IPTG-induced *E. coli* transformed with the pRAT12 shows the predicted 30 kDa polypeptide of recombinant protein compared to cell lyzate of control *E. coli* without coat protein gene. Purified coat protein was observed at the same size of 30 kDa in a single band.

To figure out the specificity of anti-V9 antibody to garlic virus in garlic leaf extract and garlic virus preparation, immunoblot analysis was carried out (Fig. 4). The anti-V9 antibody recognizes four bands of 35, 32, 26 and 24 kDa from garlic virus preparation (Fig. 4, lane 1), but one band of 35 kDa from infected garlic leaf extract (Fig. 4, lane 2). It also recognizes V9 antigen (Fig. 4, lane 3), but don't recognizes 10 kDa as expectedly (Fig. 4, lane 4). Multiple bands were detected in garlic virus preparation than garlic leaf extract. This was perhaps a result of the lengthy purification procedure which would allow extensive degradation of the coat protein by proteolytic enzymes. To confirm proteolytic cleavage, infected garlic leaf extract was incubated at 37℃ at time intervals and each fraction was analyzed by immunoblotting. Considering that the intensity of 35 kDa band decreases, but that of lower bands increases, 32, 26, and 24 kDa bands are proteolytic cleavage products of GMV coat protein (data not shown).

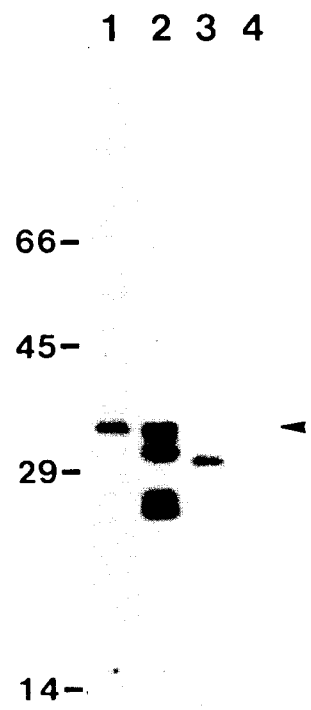

Fig. 4. Immunoblot analysis of GMV coat protein. Protein samples were separated on 12.5% SDS-PAGE, transferred onto nitrocellulose paper and probed with anti-coat protein antibody for GMV. Arrowhead indicates the nascent coat protein of GMV. lane 1: garlic leaf extract, lane 2: garlic virus preparations from cultivar Danyang, lane 3: affinity-purified coat protein, lane 4: affinity-purified 10 kDa protein.

The clone G7 encodes a coat protein for a potyvirus in garlic. To understand the genome structure of garlic potyvirus, the nucleotide sequence of the clone G7 was determined (Fig. 5). It has 50~60% similarity in nucleotide sequence and 60% identity in deduced amino acid sequence with the members of potyvirus group, potato virus Y (PVY), tobacco etch virus (TEV), plum pox virus (PPV) and soybean mosaic virus (SMV). We conclude, therefore, that the clone G7 is a cDNA clone for potyvirus in garlic. The clone G7 consists of two open reading frames. The first ORF which corresponds to nucleotide positions 1-570 seems to be C-terminus of potyvirus polymerase. The second ORF corresponding to nucleotide positions 571~1,338 encodes a coat protein of 256 amino acids. The molecular weight predicted from this coat protein gene is about 30 kDa. In case of carlavirus and potyvirus, the average molecular weight of coat protein is 31~34 kDa (29, 33). Arrowhead indicates the putative cleavage site for coat protein by viral encoded protease. In general, the potyvirus genome is translated as a larger polyprotein precursor. The precursor protein molecule is cleaved at susceptible Gln-Ser, Gln-Gly, Gln-Ala residues by virus-coded proteinases (13). The proteinase from TEV appears to recognize the consensus sequence motif Glu-X-X-Tyr-X-Gln-(Ser, Gly) found at five positions in the C-terminal portion of the polyprotein (7). The analogous putative cleavage site in tobacco vein mottling virus (TVMV) also contain

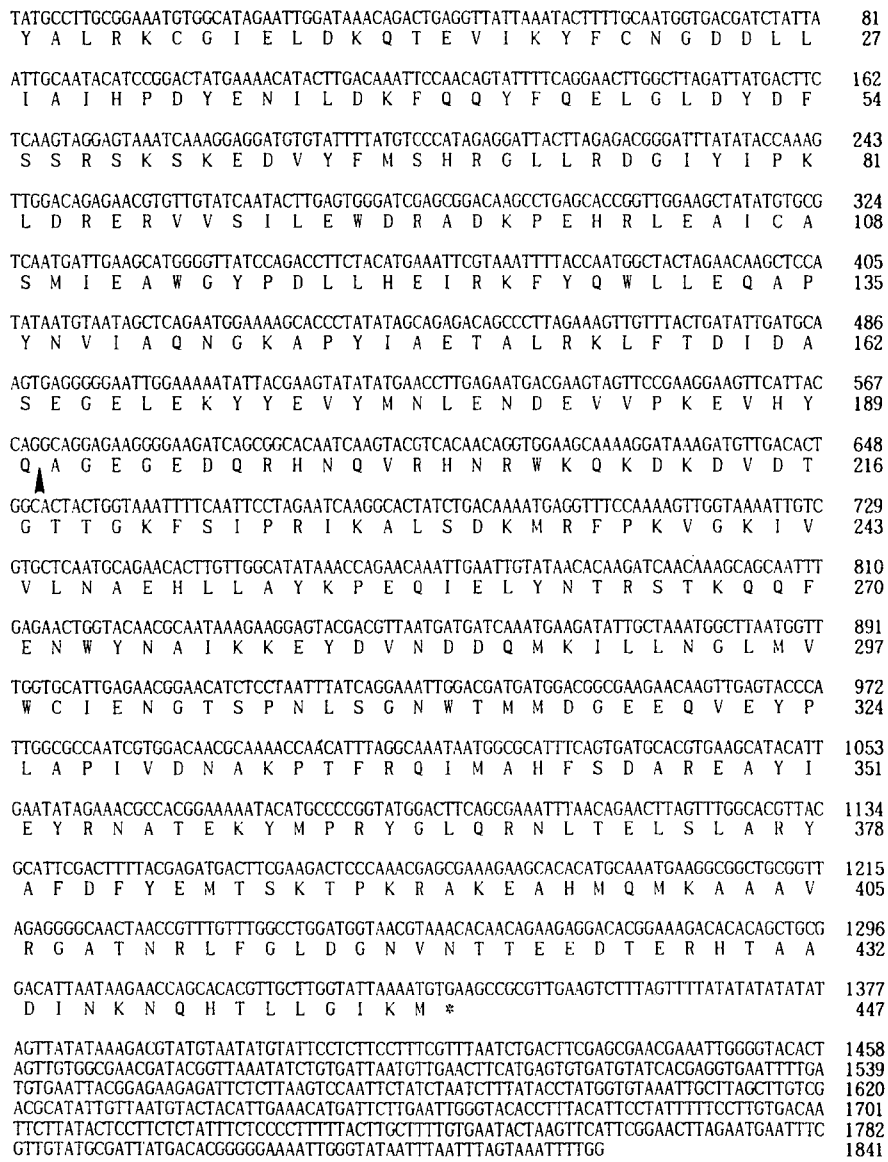

Fig. 5. Nucleotide sequence and deduced amino acid sequence of the clone G7. Partial polymerase gene corresponds to the first open reading frame from the nucleotide 1~570. The second open reading frame encodes coat protein from the nucleotide 571~1,338. Arrowhead indicates the putative cleavage site by virus encoded protease.

a consensus sequence motif, but the motif, Val-(Arg,Lys)-X-Gln-(Ser,Gly), is different from that in TEV (12). The finding of different sequence motifs for the two potyvirus proteinases is surprising, particularly when analysis of the 3′-terminal sequence for SMV-V (21), PPV-AT, and PPV-NAT (45, 46) show they all conform to the Val-X-X-Gln sequence but without a basic residue at the -3 position. Closer inspection of the putative cleavage sites in TEV reveals that four of the six (at residues 262, 772, 1849, and 2279) also conform to the Val-

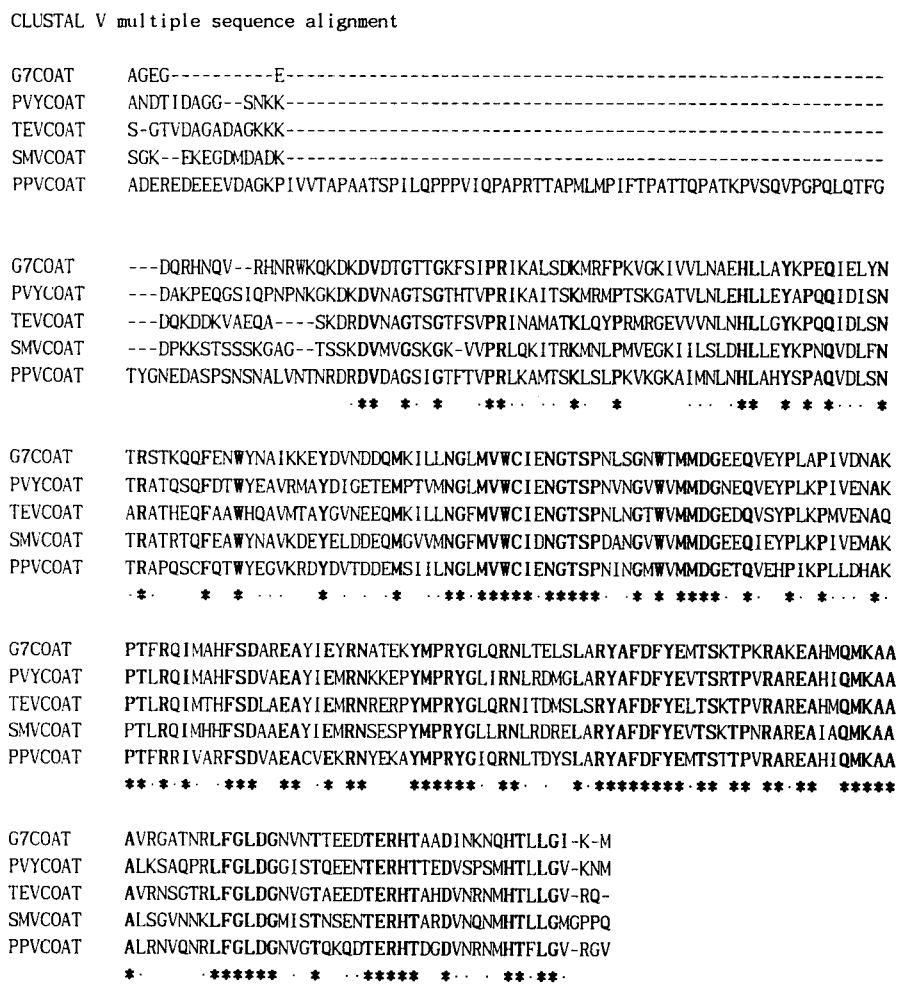

Fig. 6. A comparison of amino acid sequences of coat proteins from potyviruses. The asterisk (*) and dot (·) indicate identical and equivalent amino acid among the five proteins, respectively.

X-X-Gln-(Gly, Ser, Ala) motif. Clone G7 seems to be cleaved at Val-X-X-Gln-Ala site which may be the initiation site of coat protein indicated by arrowhead in Fig. 5.

A comparison of amino acid sequences of coat proteins from five potyviruses is shown in Fig. 6. The asterisk (*) and dot (·) indicate the identical amino acid and the equivalent amino acid among five proteins, respectively. High similarity in the majority of coat protein sequence but low similarity in N-terminus of coat protein is observed. The coat proteins from the potyviruses vary considerably in size because of differences in length of their N-termini. These variations in length at the N-terminal region may reflect different locations of the specific cleavage sites in this highly variable region of the potyvirus polyprotein (3, 12). In contrast the C-terminal ends vary in length by only one or two residues. Fig. 6 shows that the N-terminal ends vary considerably in sequence but the striking homology commences around residue 23 and extends through the C-terminal region.

To figure out the RNA transcript corresponding to the isolated cDNA clone G7, Northern blot analysis was carried out with the clone G7 as a molecular probe (data not shown). GV RNA was isolated from field-grown garlic leaves showing yellow streak and poly(A) RNA was purified from total garlic virus RNA by oligo (dT) cellulose chromatography. GV RNA and poly(A)$^+$ GV RNA preparation show strong signal at the position of 9.0 kb. This result demonstrated that the clone G7 is a cDNA clone for potyvirus and potyvirus genome has a poly(A) tail as other single stranded RNA virus of plants (30). There is a weak signal about 12.8 kb long. This could be probably the genomic RNA covalently linked to VPg at the 5′ terminus. It is reported that VPg is about 6 kDa in size for TEV (23) but larger (24 kDa) for TVMV (54). But a role for the VPg polypeptide has not been established. In addition, a cDNA clone, S81, for a potexvirus was also isolated.

Degradation of viral genome by ribozyme. To test the possibility of producing garlic virus resistant plant by transgene expression, we designed a hairpin ribozyme to cleave V9 RNA. Several self-cleaving RNA catalysts have been adapted for cleavage of a target RNA in trans (trans-activity). The group I self-splicing structure of the Tetrahymena ribosomal RNA intron, the hairpin self-cleaving structure of the minus srand of the satellite RNA of tobacco ringspot virus, and the hammerhead self-cleaving structure from the positive strand of the satellite RNA of tobacco ringspot virus have all been disigned for trans-activity (14, 26). Hairpin ribozyme is known to be more effective than hammerhead type (31). These ribozyme is to cleave 5′-phosphoester bond of non-base paired GUC flanked by basepaired region between ribozyme and substrate RNA. Since interactions between ribozymes and substrates are RNA-RNA interactions, ribozymes with altered sequence specificities can be generated. The substrate could be any RNA molecule provided it contained a GUC or similar trinucleotide at the target site, and the ribozyme could be made as a ~40-nucleotide RNA with specific base pairing to the substrate (25). This leads to the expectation that engineered ribozymes may serve as useful RNA cleavage tools to destroy specific mRNA *in vitro* and *in vivo* (9, 10, 55). Transgenic plant can be produced by using ribozyme in agriculture. Endogenous RNA transcripts encoded by the plant cell or by the plant pathogens are possible targets for ribozymes (14). To prevent virus replication early in infection, cleavage of the genomic RNA in the region encoding the RNA-dependent RNA polymerase should stop translation of this polymerase and inhibit production of progeny viral RNA. The ribozyme targeted against TMV RNA inhibited replication of TMV *in vivo* (15).

The ribozyme, V9R, was designed to cleave V9 RNA of GMV at the specific site between nucleotides A310 and G311 as shown in Fig. 7. Short RNA fragment of nt 278-410, V9S, was taken as a substrate. Each construct under the control of T7 promoter was transcribed *in vitro* with T7 RNA polymerase and the labeled transcript, V9S, was incubated with synthetic ribozyme, V9R. The product was analyzed by 5% polyacrylamide gel electrophoresis. From the mobility of products, it was possible to show that a specific cleavage occurred at the predicted site. The cleavage reaction required Mg^{2+} and the optimal concentration was 4 mM. it was the most active at 50℃. The Na^+ concentration between 0 and 500 mM had

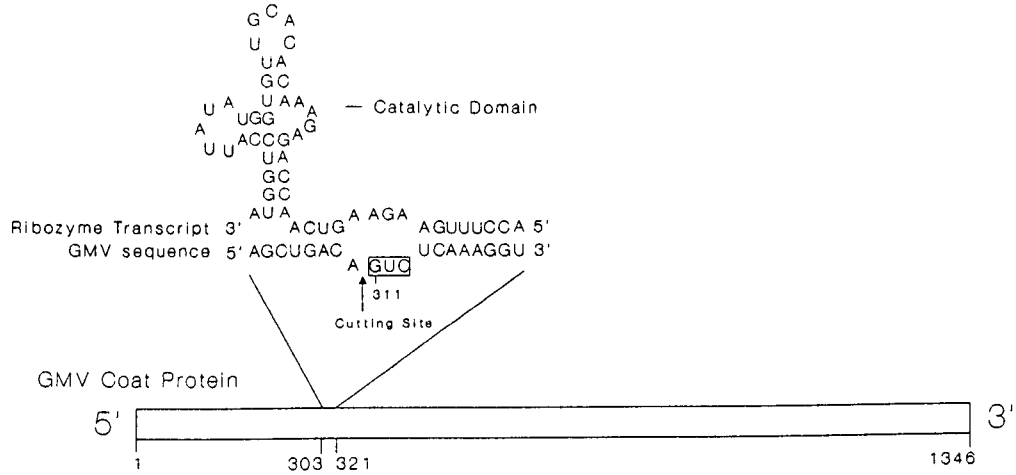

Fig. 7. Structure of hairpin ribozyme and base pairing with its target site within positive strand RNA of GMV. The ribozyme is targeted to the GUC (boxed) at position 311~313 of GMV RNA encoding the coat protein of the virus. The ribozyme catalytic domain and cleavage site are also indicated.

no significant effect on the cleavage. The reaction was optimal at pH 8.0. There was about 10 min of lag and the reaction rate was linear thereafter. Hydrolysis reaches maximum after 1.5 hr. Half of the substrate RNA was cleaved in 35 min and over 68% of substrate RNA was cleaved after 1.5 hr in optimal condition.

Ribozyme-mediated cleavage of natural substrates appears to be more complex than cleavage of a short transcript having limited secondary structure (10, 22, 25, 34, 63). This is evident when excess ribozyme is required to cleave large targets such as plant viral RNA (39). To show the ribozyme activity on V9 RNA rather than on a short fragment of substrate, it was synthesized from the clone V9 in pGEM-3 vector with SP6 RNA polymerase. The mixture for cleavage reactions contained 40 mM Tris-HCl (pH 8.0), 4 mM $MgCl_2$, 2 mM spermidine, 0.3 pmole each of ^{32}P-labeled substrate RNA and ribozyme RNA in a total volume of 10 μl. Mixtures were incubated for 1 hr at 50℃. The labeled transcripts, V9R (68 nucleotides) and V9 RNA (1,361 nucleotides), and the reaction products (P1: 1,054 nucleotides and P2: 307 nucleotides) were analyzed by 3% polyacrylamide gel electrophoresis (Fig. 8). From the mobilities of products, it was possible to show a specific cleavage occurred at the predicted site. These results demonstrated that the hairpin ribozyme could cleave effectively and specifically both V9 RNA (long substrate) and V9S (short substrate) *in vitro* and suggesting the possibility of utilizing the ribozyme to produce GMV-resistant plant.

Virus resistant transgenic plants. To control viral disease by molecular biological method, transgenic plants resistant to viral infection have also been developed other than using a ribozyme. Viral coat protein (CP) genes have been expressed in transgenic tobacco, tomato and potato plants, resulting in resistance to the virus from which the CP gene was isolated and to related viruses (1, 11, 27, 28, 43, 57~60). Recently, a field test of transgenic tomato plants that expressed the CP gene from tobacco mosaic virus (TMV) illustrated the practical appli-

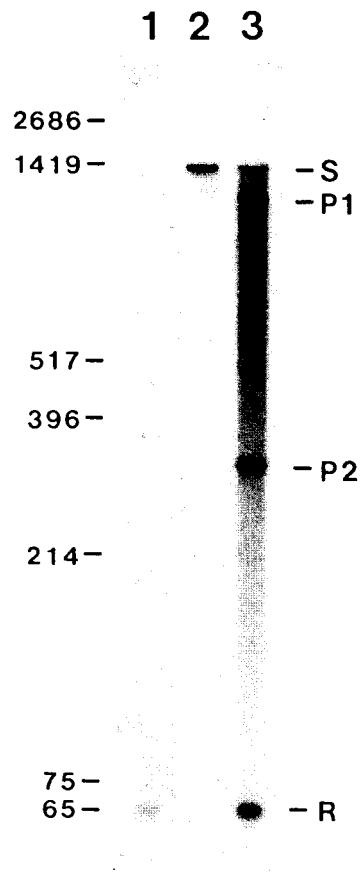

Fig. 8. Cleavage of GMV RNA sequence by the ribozyme. Reaction was carried out with V9 RNA as a substrate and V9R as a ribozyme. Mixture was analyzed in 3% polyacrylamide/7 M urea gel. Molecular size marker and the identities of RNA species are indicated. R denotes V9R of 68 nt, S for V9 RNA of 1,361 nt and P1 and P2 for reaction products of 1,054 nt and 307 nt, respectively. lane 1: Ribozyme, V9 R, lane 2 Substrate, V9 RNA, lane 3 : Cleavage reaction products.

cation of the resistance (48). Antisense RNA copies of viral CP genes have been expressed in transgenic tobacco plants and produced plants that were resistant to infection by low concentrations of virus (2, 11, 27).

Several recent reports have described virus resistance resulting from transgenic expression of the putative RNA polymerase of plant RNA viruses. The best characterized of these 'replicase-mediated resistance' effects is in tobacco transformed to express a modified form of the 54 kDa putative component of the tobacco mosaic virus (TMV) replicase complex in which there is extreme, but strain specific, resistance to TMV (18). Also, expression of amino-terminal portions or full-length viral replicase genes in transgenic plants confers resistance to potato virus X (PVX) infection. The first open reading frame (ORF1) of PVX encodes a putative replicase gene. Transgenic tobacco lines expressing ORF1 are resistant to PVX infection when inoculated with either PVX or PVX RNA (4). However, transgenic

expression of the putative replicase component of RNA viruses of plants does not always lead to resistance. In fact, with both alfalfa mosaic virus (56) and brome mosaic virus (47), transgenic expression of the replicase components had a positive rather than a negative effect on virus accumulation in that there was complementation of mutations in the inoculated viruses.

The expression of virus satellite RNAs in transgenic tobacco plants affects virus protection or ameliorates disease symptoms caused by the helper virus (17, 24). Expression of human interferon in transgenic tobacco also gives resistant to TMV.

Ribosome-inactivating proteins (RIP) inhibit protein synthesis by specific RNA N-glycosidase modification of 28S rRNA (16). Expression of pokeweed antiviral protein (PAP) in transgenic plants offers the possibility of developing resistant to a broad spectrum of plnt viruses by expression of a single gene (42). The successful use of RIPs in crop protection will depend on the extent to each their cytotoxicity is detrimental to the host cells, although introduction of the barley RIP cDNA under control of a wound-inducible promoter that is also strongly active in pollen and floral organs did not cause infertility in the primary transformants, and tobacco plants expressing the cDNA under the control of the CaMV 35S promoter apparently grow norally (44).

In light of these reports, new virus resistant crops should be available for commercial use shortly. To develop such a transgenic plant by gene manipulation, however, understanding of structure and infection machanism of target virus is required.

References

1. Abel, P. P., Nelson, R. S., De, B., Hoffmann, N., Rogers, S. G., Fraley, R. T. and Beachy, R. N. 1986. Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. *Science* 232 : 738-743.
2. Abel, P. P., Stark D. M., Sanders, P. and Beachy, R. N. 1989. Protection against tobacco mosaic virus in transgenic plants that express TMV antisense RNA. Proc. Natl. Acad. Sci. USA (in press).
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 polyprotein. *Virology* 154 : 9-20.
4. Braun, C. J. and Hemenway, C. L. 1992. Expression of Amino-Terminal Portions or Full-Length Viral Replicase Genes in Transgenic Plants Confers Resistance to Potato Virus X Infection. *The Plant Cell* 4 : 735-744.
5. Burnette, W. N. 1981. "Western blotting" : Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Analytical Biochemistry* 112 : 195-203.
6. Cadilahc, B., Quiot, J. B., Marrow, J. and Leroux, J. P. 1976. Mise en evidence au microscope electronique de deux virus differents infectant l'ail (*Allium sativum*) et l' echalotte (*Allium cepa* var. ascalonicum). *Ann. Phytopath.* 8 : 65-72.
7. Carrington, J. C. and Dougherty, W. G. 1987. Processing of tobacco etch virus 49 kD protease requires autoproteolysis. *Virology* 160 : 355-362.

8. Chang, M. U., Park, W. W., Chung, J. D., Lim, K. B. and La, Y. J. 1988. Distribution of garlic latent virus and garlic mosaic virus in infected garlic tissues. *J. Kor. Soc. Hort. Sci.* 29 : 253-265.
9. Cotten, M. and Birnstiel, M. L. 1989. Ribozyme mediated destruction of RNA *in vivo*. *EMBO J.* 8 : 3861-3866.
10. Cotten, M., Schaffner, G. and Birnstiel, M. L. 1989. Ribozyme, Antisense RNA and Antisense DNA Inhibition of U7 Small Nuclear Ribonucleoprotein-Mediated Histone Pre-mRNA Processing *In Vitro*. *Mol. Cell. Biol.* 9 : 4479-4487.
11. Cuozzo M., O'Connell, K. M., Kaniewski, W., Fang, R. X., Chua, N. H., and Tumer, N. E. 1988. Viral protection in transgenic plants expressing the cucumber mosaic virus coat protein or its antisense RNA. *Bio/Technology* 6 : 549-557.
12. Domier, L. L., Franklin, K. M., Shahabuddin, M., Hellman, G. M., Overmeyer, J. H., Hiremath, S. T., Siaw, M. E. E., Lomonossoff, G. P., Shaw, J. G. and Rhoades, E. 1986. The nucleotide sequenc of tobacc vein mottling virus. *Nucleic Acids Res.* 14 : 5417-5430.
13. Dougherty, W. G. and Carrington, J. C. 1988. Expression and function of potyviral gene products. *Annu. Rev. Phytopathol.* 26 : 123-143.
14. Edington, B. V. and Nelson, R. S. 1992. In : *Gene Regulation : Biology of Antisence RNA and DNA.* by Erickson, R. P. and Izant, J. G. pp.209-221. Raven Press, Ltd., New York.
15. Edington, B. V., Dixon, R. A. and Nelson, R. S. 1993. In : *Transgenic Plants. : Ribozymes : Descriptions and Uses.* by Andrew Hiatt. pp.301-323. Marcel Dekker, Inc. New York.
16. Endo, Y., Tsurugi, K. and Ebert, R. F. 1988. The mechanism of action of barley toxin : a type 1 ribosome-inactivating protein with RNA N-glycosidase activity. *Biochim. Biophys. Acta* 154 : 1194-1197.
17. Gerlach, W. L., Buzayan, J. M., Scheider, I. R. and Breuning, G. 1986. Satellite tobacco ringspot virus RNA : Biological activity of DNA clones and their *in vitro* transcripts. *Virology* 151 : 172-185.
18. Golemboski, D. B., Lomonossoff, G. P. and Zaitlin, M. 1990. Plants transformed with a tobacco mosaic virus nonstructural gene sequence are resistant to the virus. *Proc. Natl. Acad. Sci. USA* 87 : 6311-6315.
19. Gramstat, A., Courtpozanis, A. and Rohde, W. 1990. The 12 kDa protein of potato virus M displays properties of a nucleic acid-binding regulatory protein. *FEBS Letters* 276 : 34-38.
20. Gubler, U. and Hoffman, B. J. 1983. A simple and very efficient method for generating cDNA libraries. *Gene* 25 : 263-269.
21. Gunyuzlu, P. L., Tolin, S. A. and Johnson, J. L. 1987. The nucleotide sequence of the 3-terminus of soybean mosaic virus. *Phytopathology* 77 : 1766-1766.
22. Hampel, A., Tritx, R., Hicks, M. and Cruz, P. 1990. Hairpin catalytic RNA model : Evidence for helices and sequence equirement for substrate RNA. *Nucleic Acids Res.* 299-304.
23. Hari, V. 1981. The RNA of tobacco etch virus : Further characterisation and detection of protein linked to the RNA. *Virology* 112 : 391-399.
24. Harrison, B. D., Mayo, M. A. and Baulcombe, D. C. 1987. Virus resistance in transgenic plants that express cucumber mosaic virus satellite RNA. *Nature* 334 : 799-802.
25. Haseloff J. and Gerlach, W. L. 1988. Simple RNA enzymes with new and highly specific endoribonuclease activities. *Nature* 334 : 585-591.
26. Hawpel, A. and Tritz, R. 1989. RNA Catalytic Properties of the Minimum (−)sTRSV Sequence. *Biochemistry* 28 : 4929-4933.
27. Hemenway C., Fang, R. X., Kaniewski, W., K., Chua, N. H. and Tumer, N. E. 1988. Analysis of the mechanism of protection in transgenic plants expressing the potato virus X coat protein or its antisense RNA. *EMBO J.* 7 : 1273-1280.

28. Hoekma, A., Huisman, M. J., Molendijk, L, van den Elzen, P. J. M. and Cornelissen, B. J. C. 1989. The genetic engineering of two commercial potato cultivars for resistance to potato virus X. *Bio/Technology* 7 : 273-278.
29. Hollings, M. and Brunt, A. A. 1981. Potyvirus group. *CMI/AAB Descriptions of Plant Viruses.* No. 245.
30. Huisman, M. J., Linthorst, H. J. M., Bol, J. F. and Cornelissen, B. J. C. 1988. The complete nucleotide sequence of potato virus X and its homologies at the amino acid level with various plus-stranded RNA viruses. *J. Gen. Virol.* 69 : 1789-1798.
31. Kicuchi, Y. and Sasaki, N. 1991. Site-specific cleavage of natural mRNA sequences by newly designed hairpin catalytic RNAs. *Nucleic Acids Res.* 19 : 6751-6755.
32. Klug, A. and Rhodes, D. 1987. 'Zinc fingers' : a novel protein motif for nucleic acid recognition. *Trends in Biochemical Sciences* 12 : 464-469.
33. Koenig, R. 1982. Carlavirus group. *CMI/AAB Descriptions of Plant Viruses.* No. 259.
34. Koizumi, M., Iwai, S. and Ohtsuka, E. 1988. Cleavage of specific sites of RNA by designed ribozymes. *FEBS.* 239 : 285-288.
35. Kroczek, R. A. and Siebert, E. 1990. Optimization of Northern analysis by vacuum-blotting, RNA transfer, visualization and ultraviolet fixation. *Analytical Biochemistry* 184 : 90-95.
36. La, Y. J. 1972. Local lesion hosts for a garlic virus. *Korean J. Bot.* 15 : 42-42.
37. La, Y. J. 1973. Studies on garlic mosaic virus. *Korean J. Pl. Prot.* 12 : 93-107.
38. La, Y. J. and Choi, I. R. 1987. Studies on the transmission of garlic viruses I. Search for a systemic assay host. *Res. Rept. RDA (Agri. Institutional cooperation)* : 129-132.
39. Lamb, J. W. and Hay, R. T. 1990. Ribozymes that cleve potato leafroll virus RNA within coat protein and polymerase genes. *J. Gen. Virol.* 71 : 2257-2264.
40. Langenberg, W. G. 1973. Serology, physical properties and purification of unaggregated infectious maize dwarf mosaic virus. *Phytopathology* 63 : 149-154.
41. Lee, Y. W., Yamazaki, S., Osaki, T. and Inouye, T. 1979. Two elongated viruses in garlic, garlic latent virus and garlic mosaic virus. *Ann. Phytopath. Soc. Japan* 45 : 727-734.
42. Lodge, J. K., Kaniewski, W. K. and Tumer, N. E. 1993. Broad-spectrum virus resistance in transgenic plants expressing pokeweed antiviral protein. *Proc. Natl. Acad. Sci. USA* 90 : 7089-7093.
43. Loesch-Fries, L. S., Merlo, D., Zinnen, T., Burhop, L., Hill, K., Krahn, K., Jarvis, N., Nelson, S. and Halk, E. 1987. Expression of alfalfa mosaic virus RNA4 in transgenic plants confers virus resistance. *EMBO J.* 6 : 1845-1851.
44. Logemann, J., Jach, G., Tommerup, H., Mundy, J. and Schell, J. 1992. Expression of a barley ribosome-inactivating protein leads to increased fungal protection in transgenic tobacco plants. *Bio/Technology* 10 : 305-308.
45. Maiss, E., Breyl, E., Brisske, A. and Casper, R. 1988. Molecular cloning of cDNA complimentary to the RNA genome of plum pox virus (PPV). *J. Phytopathol.* 122 : 222-231.
46. Maiss, E., Timpe, U., Brisske, A., Jelkmen, U., Casper, R., Himmler, G., Mattanovich, D. and Katinger, H. W. D. 1989. The complete nucleotide sequence of plum pox virus (PPV). *J. Gen. Virol.,* in press.
47. Mori, M., Mise, K., Okuno, T. and Furusawa, I. 1992. Expression of brome mosaic virus encoded replicase gene in transgenic tobacco plant. *J. Gen. Virol.* 73 : 169-172.
48. Nelson, R. S., McCormick, S. M., Delannay, X., Dube, P., Layton, J., Anderson, E. J., Kaniewska, M., Proksch, R. K., Horsch, R. B., Rogers, S. G., Fraley, R. T. and Beachy, R. N. 1988. Virus tolerance, plant growth and field performance of transgenic tomato plants expressing coat protein from tobacco mosaic virus. *Bio/Technology* 6 : 403-409.

49. Noda, C. and Inouye, N. 1989. Leek yellow stripe virus isolated from an ornamental Allium plant in Japan. *Ann. Phytopath. Soc. Japan* 55 : 208-215.
50. Perriman, R., Delves, A. and Gerlach, W. L. 1992. *Proc. Nat. Acad. Sci. USA,* 86 : 9139-9143.
51. Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. Molecular cloning : a laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press.
52. Sanger, F., Nicklen, S. and Coulson, A. R. 1977. DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA,* 74 : 5463-5467.
53. Sehnke, P. C., Mason, A. M., Hood, S. J., Lister, R. M. and Johnson, J. E. 1989. A 'zinc finger'-type binding domain in tobacco streak virus coat protein. *Virology* 168 : 48-56.
54. Siaw, M. F. E., Shahabuddin, M., Ballard, S., Shaw, J. and Rhoads, R. E. 1985. Identification of a protein covalently linked to the 5' terminus of tobacco vein mottling virus RNA. *Virology* 142 : 134-143.
55. Steinecke, P., Herget, T. and Schreier, P. H. 1992. Expression of a chimetric ribozyme gene results in endonucleolytic cleavage of target mRNA and a concomitant reduction of gene expression *in vivo. EMBO J.* 11 : 1525-1530.
56. Taschner, P. E. M., van der Kuyl, A., Neeleman, L. and Bol, J. F. 1991. Replication of an incomplete alfalfa mosaic virus genome in plants transformed with viral replicase genes. *Virology* 181 : 445-450.
57. Tumer, N. E., O'Connell, K. M., Nelson, R. S., Sanders, P. R. and Beachy, R. N. 1987. Expression of alfalfa mosaic virus coat protein gene confers cross-protection in transgenic tobacco and tomato plants. *EMBO J.* 6 : 1181-1188.
58. van Dun, C. M. P. and Bol, J. F. 1988a. Transgenic tobacco plants accumulating tobacco rattle virus coat protein resist infection with tobacco rattle virus and pea early browning virus. *Virology* 167 : 649-652.
59. van Dun, C. M. P., Bol, J. F. and van Vloten-Doting, L. 1987. Expression of alfalfa mosaic virus and tobacco rattle virus coat protein genes in transgenic tobacco plants. *Virology* 159 : 299-305.
60. van Dun, C. M. P., Overduin, B., van Vloten-Doting, L. and Bol, J. F. 1988b. Transgenic tobacco expressing tobacco streak virus or mutaed alfalfa mosaic virus coat protein does not cross-protect against alfalfa mosaic virus infection. *Virology* 164 : 383-389.
61. Vieira, J. and Messing, J. 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and seqencing with synthetic universal primers. *Gene* 19 : 259-268.
62. Walkey, B. 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. Horticultural Science* 62 : 211-220.
63. Zang, A. J., Been, M. D. and Cech, T. R. 1986. The tetrahymena ribozyme acts like an RNA restriction endonuclease. *Nature* 324 : 429-433.
64. Zuker, M. 1989. In : *Characterizatin of RNAs. : Computer Prediction of RNA Structure. Meth. Enzymology* 180 : 262-288.