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Identification of a Potexvirus in Korean Garlic Plants

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Abstract : To understand the molecular structure of Korean garlic viruses, cDNA cloning of virus genomic RNA was attempted. Virus particles were isolated from virus-infected garlic leaves and a cDNA library was constructed from garlic virus RNA. One of these clones, S81, selected by random sequencing has been identified as a member of potexvirus group other than potyvirus and carlavirus. The clone is 873 bp long contains most of the coat protein (CP) coding region and 3'-noncoding region including poly(A) tail. A putative polyadenylation signal sequence (AAUAAA) and the hexanucleotide motif (ACUUAA), a replicational *cis*-acting element conserved in the 3'-noncoding region of potexvirus RNAs are noticed. The clone S81 shows about 30-40% identity in both nucleotide and amino acid sequences with CPs of potexviruses. The genome size of the virus was analyzed to be 7.46 knt by Northern blot analysis, which was longer than those of other potexviruses. The open reading frame encoding CP was expressed as a fusion protein (S81CP) in *Escherichia coli* and the recombinant protein was purified by immobilized metal binding affinity chromatography. Polyclonal antibody was raised against S81CP in rabbit to examine the occurrence of garlic potexvirus in Korean garlic plants by immunoblot analysis. Two virus protein bands of Mr 27,000 and 29,000 from garlic leaf extract of various cultivars reacted with the antibody. It was shown that Mr 27,000 band might not be a degradation product of Mr 29,000 band, suggesting that two types of potexvirus different in size of coat protein could exist in Korean garlic plants(Received January 5, 1995; accepted February 7, 1995).

Introduction

Most of the garlic plants cultivated throughout the world are infected with viruses which give rise to yellow streak on leaves and reduction of yield.[1)] The wide occurrence of the viral disease in garlic is due to the facts that there are no practical control for the disease and the production of garlic has solely depended on vegetative propagation. Although there has been some attempts to produce the virus-free seed garlic by tissue culture techniques, viruses were often not successfully eliminated.[2)] In most cases, moreover, virus-free garlic would be infected again with viruses when planted in field. It is necessary to understand the molecular structure of the virus to control those viruses infecting garlic plants.

Virus-like particles most commonly found in garlic are filamentous and range in length from 600 to 750 nm. Those are thought to belong to carlavirus[3)] and potyvirus group[4)] and therefore named to be garlic latent virus (GLV) and garlic mosaic virus (GMV), respectively.[1)] The partial cDNA clones for garlic viruses have been reported. Garlic virus (GV) 1 is a member of carlavirus group and GV2, a potyvirus.[5)] Other rod-shaped flexuous viruses similar to the carla- and potyviruses were also partially purified from garlic leaves showing typical viral disease symptoms.[6)] Those new group of viruses are refered to as GV-A, GV-B, GV-C and GV-D, whose genomic structures are different from carla- and potyvirus. The length of the viral particles is about 700 nm as determined by electronmicroscopy. It is, however, not clear yet how many different viruses are infecting garlic plants in field.

In this paper we characterized a cDNA clone for a different group of garlic virus from infected leaves. It belongs to potexvirus by comparison of nucleoteide and deduced amino acid sequences of the cDNA clone with those of coat proteins (CPs) of other potexviruses. Potexviruses including potato virus X (PVX) cause usually mosaic or ringspot symptoms in a wide range of monocotyledonous and dicotyledonous plants.[7)] Using antibody raised against recombinant CP from the cDNA clone S81, occurrence of potexviruses from Korean garlic plants is studied.

Materials and Methods

Virus preparation

Garlic virus particles were isolated from garlic leaves showing yellow streak of virus symptom by the proce-

Key words : garlic, garlic virus, coat protein, carlavirus, garlic latent virus (GLV), immunoblot.
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dure described previously,[8] which was adopted from Langenberg[9] and La.[10]

Construction of garlic virus cDNA library

A cDNA library was constructed into pUC18 plasmid vector by the method of Gubler and Hoffman[11] with minor modification as described in Choi *et al.*[8]

Nucleotide sequencing and recombinant DNA techniques

Nucleotide sequencing was carried out in plasmid pUC 18[12] by the dideoxynucleotide chain-termination method of Sanger *et al.*[13] Universal M13 primers for reverse and forward reactions were used and the reaction products were analyzed by 6 M urea-polyacrylamide gel electrophoresis. Northern blot hybridization was carried out by the procedure of Kroczek and Siebert.[14] Purification and manipulation of DNA were carried out according to the standard protocols of Sambrook *et al.*[15]

Overexpression and purification of recombinant CP

Overexpression and purification of recombinant CP were carried out according to the protocol provided by Qiagen.[16]

Preparation of antibody

Antibody was prepared by the procedure of Sambrook *et al.*[15] Purified recombinant CP for the clone S81 was mixed with equal volume of Freund's adjuvant and injected into rabbit (Newzealand White) 3 times at an interval of 2 weeks. Serum was processed by the standard method after bleeding.[17]

Immunoblot analysis

Protein samples were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).[8] Blotting of proteins from SDS-PAGE gels onto nitrocellulose paper was carried out by electrotransfer at 0.15 A for 4 hr in 50 mM Tris-glycine, pH 9.1, containing 20% methanol. The nitrocellulose blot was treated essentially according to Burnette[18] except that 5% nonfat dry milk was used instead of BSA as a blocking reagents.[19] The nitrocellulose blot was incubated first with anti-S81CP antibody (1 : 200 dilution) and then with goat anti-rabbit second antibody labeled with peroxidase. ECL detection reagents 1 and 2 (Amersham Co.) were used as substrates for peroxidase.

Results and Discussion

Field-grown garlic plants are infected with multiple species of viruses

Virus particles were isolated from garlic leaves showing yellow streak of virus symptom according to the method described by Langenberg[9], with minor modification.[8] It was purified by 10~40% sucrose density gradient sedimentation and analyzed by 15% SDS-PAGE. Although there were some variations by garlic cultivars, at least ten different protein bands were resolved by SDS-PAGE and the molecular weights of the proteins ranged from 22,000 to 37,000 (Fig. 1). The major bands are observed at the position of Mr 37,000, 33,000, 31,000, 29,000, 27,000 and 26,000. These proteins could be regarded as structural CPs of garlic viruses. The minor bands could be degradation products resulting from lengthy virus preparation procedure or from viruses existing in lower concentration in garlic plants.[16] It is known that the N- and C-termini of CP of potyvirus are degraded during purification and storage by enzymes of plant origin which cosediment with the virus particles.[20] Storage of a purified preparation of tobacco etch virus (TEV) at 4℃ resulted in the conversion of CP from Mr 32,000 to 26,000.[21] Proteolysis degradation of carlavirus CP is also reported.[16,22] Therefore, field-grown garlic plants could be considered to contain multiple species of virus even though exact number of virus species are not clear.

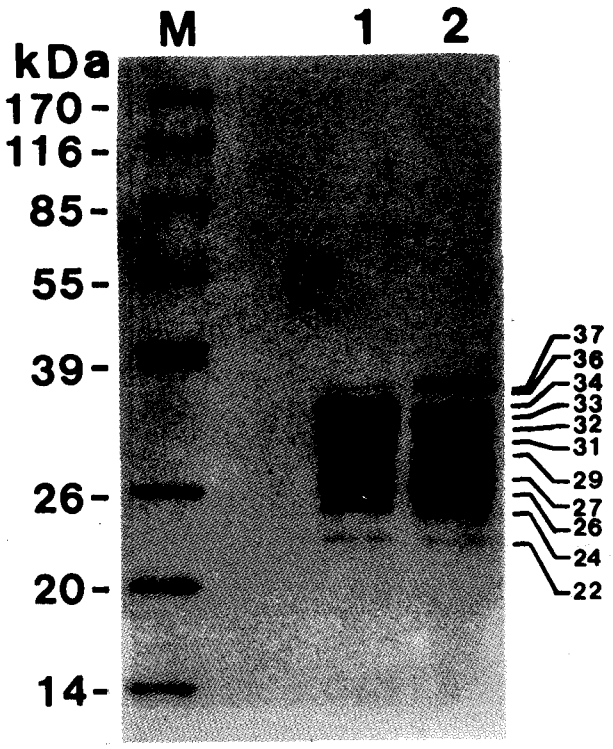

Fig. 1. SDS-PAGE analysis of garlic virus particles prepared from field-grown garlic leaves. Protein extracts were analyzed by 15% SDS-PAGE and stained with Coomassie Blue. Lane M, size marker; lane 1, garlic virus preparation from the cultivar Danyang; lane 2, garlic virus preparation from the cultivar Seosan

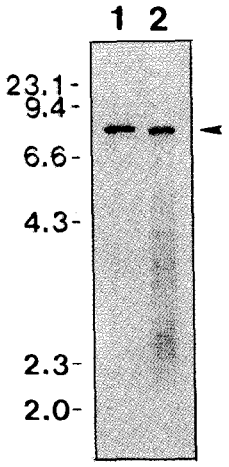

Fig. 2. Northern blot analysis of garlic virus RNA and garlic virus poly(A)$^+$ RNA. RNA was separated by 0.8% formaldehyde agarose gel electrophoresis and transferred onto nylon membrane. The filter was probed with the random primer-extended clone S81. Lane M, λ/HindIII size marker; Lane 1, garlic virus RNA from field-grown garlic leaves; Lane 2, poly(A)$^+$ RNA selected from garlic virus RNA of lane 1 by oligo(dT)-cellulose chromatography

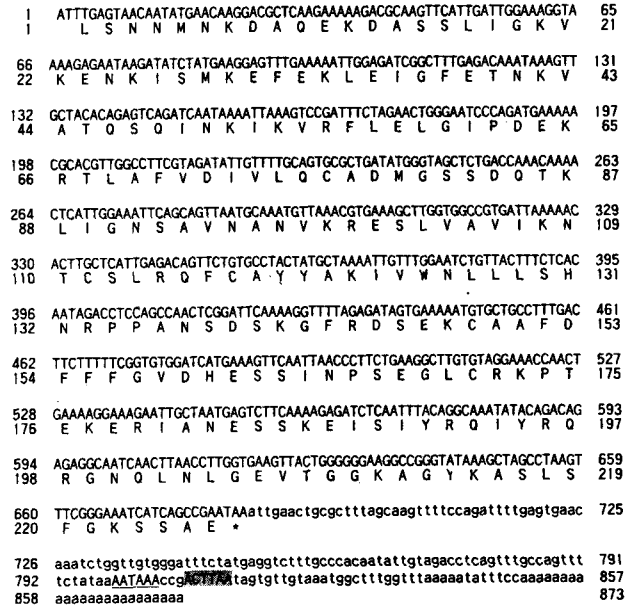

Fig. 3. Nucleotide sequence and deduced amino acid sequence of the cDNA clone S81. Partial coat protein gene corresponds to the open reading frame from the nucleotide 1-683. The deduced amino acid sequence is given in single-letter code and the termination codon is indicated by an asterisk (*). The putative polyadenylation signal AAUAAA is underlined and the hexanucleotide sequence ACTTAA, a *cis*-acting element for viral RNA replication, is in shade.

The cDNA clone S81 was isolated by random sequencing

To isolate cDNA clones for garlic virus, a cDNA library was constructed from the virus preparation. The cDNA clones for garlic viruses containing poly(A) tails were isolated by random sequencing and one of these is the clone S81. Plant viruses of 600~800 nm long usually have poly(A) tail at the 3' end of their genomes.[23] Northern blot analysis revealed that the clone strongly binds to the band of 7,460 nucleotides (nt) when garlic virus RNA was prepared from field-grown garlic leaves and poly(A)$^+$ RNA selected from garlic virus RNA by oligo(dT)-cellulose chromatography (Fig. 2). Therefore, it is concluded that the clone S81 is a partial cDNA clone for garlic virus and its genome size is 7,460 nt. The genome size is a little longer than those of the other potexvirus: papaya mosaic virus (PMV), 6.7 knt,[24] PVX, 6.5 knt,[25] clover yellow mosaic virus (CYMV), 7.0 knt.[26] It was smaller than carlavirus and potyvirus which are 8.5 knt long[27] and 10 knt long[28], respectively. As the size of RNA hybridize with the clone S81 was slightly smaller than that of the major viral RNA genome analyzed by formaldehyde agarose gel, it may not be very abundant in Korean garlic plants (data not shown).

The clone S81 encodes CP of garlic potexvirus

The nucleotide sequence of the clone S81 was determined (Fig. 3). There is a partial open reading frame from the nucleotide 1 to 683 nt, the 3'-noncoding sequence and poly(A) tail. The sequence of the clone S81 was compared with those of other plant viruses in the EMBL Database. It shows 48.2% over 767 bp region in nucleotide sequence and 41.9% identity over 160 amino acids in the open reading frame to CP gene of PVX, a potexvirus. The potexvirus forms a large group of flexuous filamentous plant viruses with the length 470~580 nm[7] and positive-sense ssRNA genomes of about Mr 2.1×10^6. The capsid protein consists of identical subunits with a Mr varing from 18,000 to 27,000. The cDNA sequence of the genomic RNA of some members of the potexvirus group has determined : white clover mosaic virus (WCIMV)[29], PVX strain X3[23], narcissus mosaic virus (NMV)[30], PMV[24] and CYMV[26]. The amino acid sequences alignment of S81 and other potexviruses are shown in Fig. 4. N- and C-terminal region of CP are diverged whereas the internal region shows relatively higher positional identity. A stretch of highly conserved amino acid residues, KFAAFDFFDGV, is also noticed.[33] The homology between nucleotide sequences of CPs from various potexviruses shows about 40~50% and the amino acid sequence identity between CPs of the potexviruses is about 30~40% as shown in Table 1. The clone S81 shows the highest homology with PVX among the potexviruses. Compared with the coat protein region of PVX, the clone S81 seems to lack of 3 amino acids in N-terminal region. Considering the missing 3 amino acids, the Mr of coat protein for garlic potexvirus

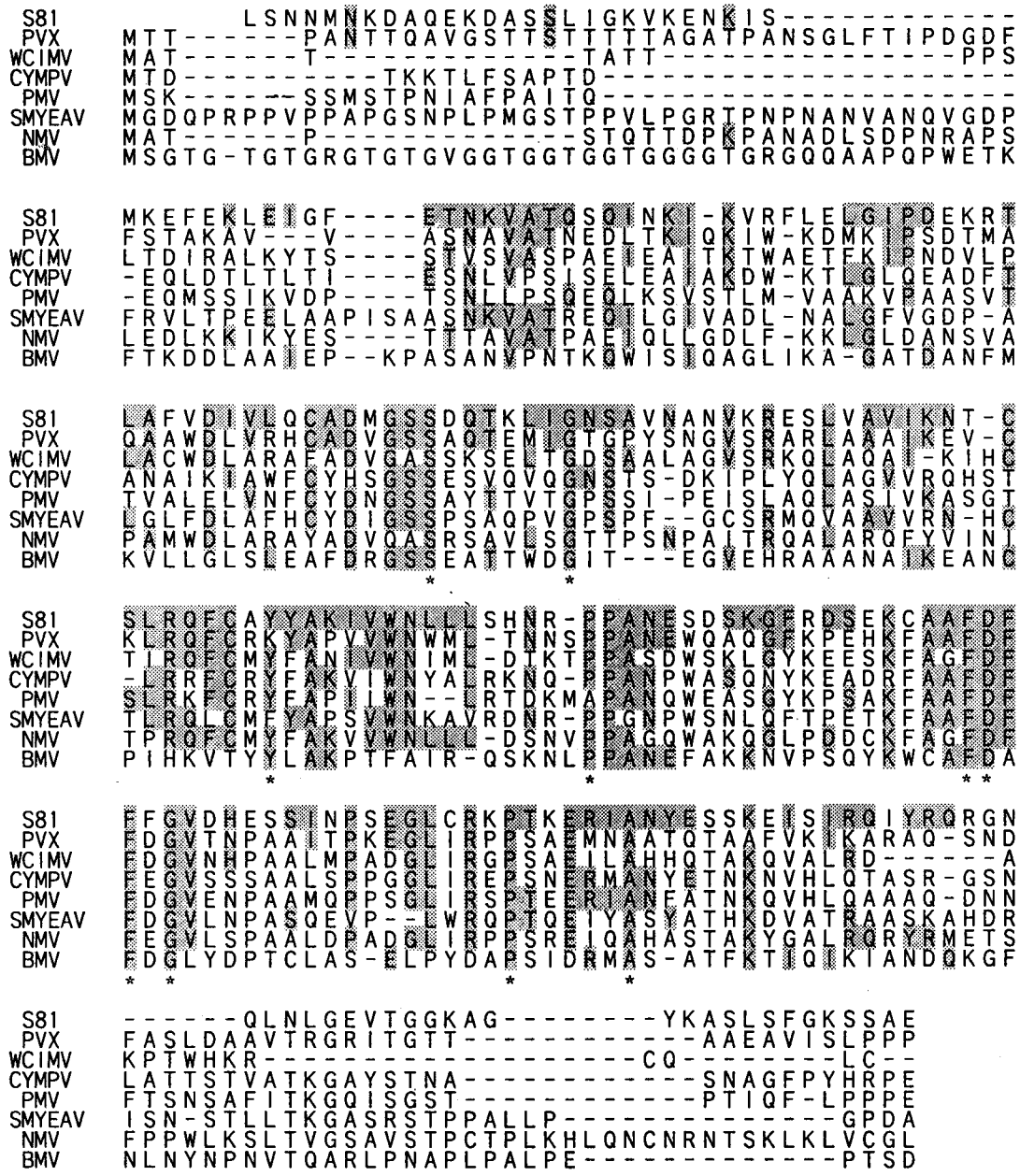

Fig. 4. A comparison of amino acid sequences of coat proteins from eight distinct potexviruses: PVX, potato virus X[23]; WCIMV, white clover mosaic virus[29]; CYMPV, clover yellow mosaic potexvirus[26]; PMV, papaya mosaic potexvirus[24]; SMYEAV, strawberry mild yellow edge-associated virus[31]; NMV, narcissus mosaic virus[30]; BMV, bamboo mosaic potexvirus[32]. Gaps were introduced for best fitting using the CLUSTSAL V alignment program. The positional identies among the potexviruses are shown in shade. The astrisk (*) indicates highly conserved amino acids among the eight coat proteins.

is expected to be 26,000. The Mr of potexvirus coat protein usually ranges from 18,000 to 27,000.[34]

The 3'-noncoding region contains a putative polyadenylation signal AAUAAA located at 51 nucleotides upstream from poly(A) tail (Fig. 3). The position of the polyadenylation signal from the poly(A) tail in the potexviruses are more variable[33] than it is in eukaryotic messenger RNAs in which the AAUAAA is usually 20 nt upstream of poly(A) tail.[35] In WCIMV RNA, it is located at 13 nt upstream of poly(A) whereas in PMV, it is at 124 nt upstream of poly(A). It is even absent from PVX. Thus, the role of this putative polyadenylation signal seems not clear.[36]

In potexviruses a hexanucleotide motif, 5'-ACUUAA, is conserved in the 3'-noncoding region. It is shown in shade in the 3'-noncoding region of the clone S81 (Fig. 3). This sequence was proposed to be involved in the production of negative-strand genomic and positive-strand subgenomic RNA by serving as a *cis*-acting element involved in replicase recognition of the template.[37]

Table 1. Homologies between nucleotide and amino acid sequences of coat proteins from various potexviruses.

Potexvirus strain	*Ref.*	Percentage identity, nt (a.a.)*						
		S81	PVX	WCIMV	CYMV	PMV	SMYEAV	NMV
PVX	23	48.8 (41.9)						
WCIMV	29	49.5 (36.3)	52.7 (47.8)					
CYMV	26	49.4 (32.4)	49.4 (31.7)	50.2 (36.6)				
PMV	24	46.8 (32.1)	52.7 (40.8)	49.5 (35.4)	53.2 (42.4)			
SMYEAV	31	49.4 (36.0)	51.2 (40.0)	52.3 (35.8)	51.2 (37.8)	51.4 (34.1)		
NMV	30	49.4 (34.5)	50.7 (34.4)	54.8 (47.8)	50.4 (37.1)	48.9 (34.0)	50.3 (30.7)	
BMV	32	42.5 (25.4)	51.3 (24.0)	45.6 (29.5)	46.2 (23.2)	43.8 (25.7)	43.9 (24.4)	45.9 (23.6)

*nt denotes nucleotide sequence and a.a. denotes amino acid sequence identities

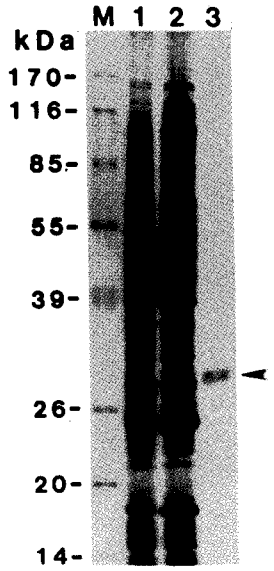

Fig. 5. SDS-PAGE analysis of garlic potexvirus coat protein, S81 CP expressed in *E.coli* BL21. It was purified by Ni^{2+}·NTA affinity chromatography. Lane M, size marker; Lane 1, cell lysate of control *E.coli* without coat protein gene; Lane 2, IPTG -induced *E.coli* transformed with the pS81CP; Lane 3, IMAC-purified S81CP

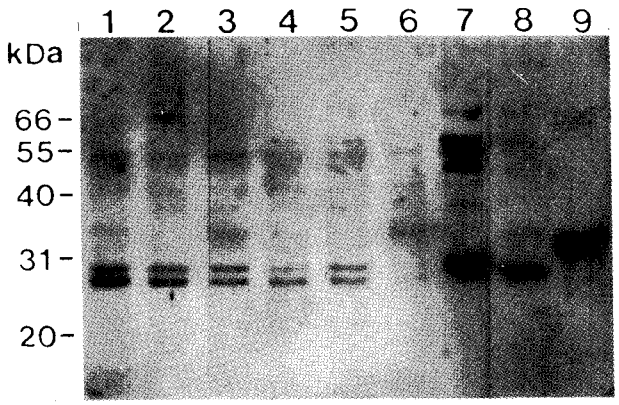

Fig. 6. Immunoblot analysis of garlic potexvirus coat proteins from garlic leaf extract of various cultivars. Protein samples were separated on 15% SDS-PAGE, transfered onto nitrocellulose paper and probed with anti-S81 protein antibody for Korean garlic potexvirus. Each sample is garlic leaf extract of cultivars Euiseong 1 (lane 1), Euiseong 2 (lane 2), Euiseong 3 (lane 3), Daegang (lane 4), Danseong (lane 5), Eosangcheon (lane 6), Wonju (lane 7), Japanese elephant garlic (lane 8) and affinity-purified coat protein used as an antigen (land 9).

Antibody was prepared against recombinant CP

To identify the potexvirus encoded by the clone S81, cDNA fragment was cloned into pRSET-B vector and expressed in *E. coli*. The pRSET vector allows high level expression of foreign gene in *E. coli* by the T7 promoter. The recombinant construct pS81CP was transformed into *E. coli* BL21 and T7 RNA polymerase was induced by IPTG. The expressed recombinant coat protein was purified by Ni^{2+} · NTA affinity chromatography utilizing the N-terminal metal binding domain on the fusion peptide provided by the pRSET vector. The expressed protein was analyzed by 12.5% SDS-PAGE. The IPTG-induced *E. coli* transformed with the pS81CP produced 30 kDa band as expected (Fig. 5). Induced protein was purified by IMAC and injected into rabbit to raise a polyclonal antibody.

Occurrence of potexvirus in Korean garlic plants was examined.

Immunoblot analysis was carried out with garlic leaf extract to examine the occurrence of potexvirus corresponding to the clone S81 in Korean garlic plants (Fig. 6). The anti-S81 antibody recognizes two bands of Mr 29,000 and 27,000 from infected garlic leaf extracts. Most of the samples show the doublets at Mr 29,000 and 27,000 but some of them show only one band, suggesting that those could be the virus CPs of closely related two different potexviruses. Cultivar Wonju shows only upper band, but Japanese elephant garlic shows only lower band. Virus concentration in cultivar Eosangcheon seems relatively low.

It is possible that the lower band could be a degradation product of upper band, resulting from the lengthy sample preparation procedure which would allow exten-

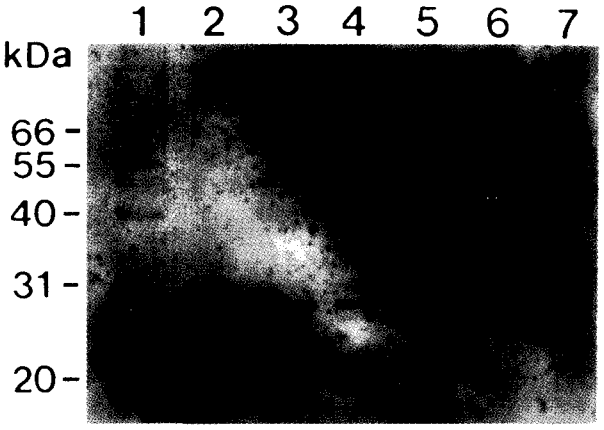

Fig. 7. Immunoblot analysis of garlic potexvirus coat proteins from garlic leaf extract of a cultivar according to time course. Garlic leaf extracts from cultivar Euiseong 1 was incubated at 37℃ for 0 min (lane 1), 30 min (lane 2), 1 hr (lane 3), 2 hrs (lane 4), 4 hrs (lane 5), 8 hrs (lane 6) or 16 hrs (lane 7) and protein samples were separated on 15% SDS-PAGE, transfered onto nitrocellulose paper and probed with anti-S81 antibody for Korean garlic potexvirus.

sive degradation of the coat protein by endogenous proteolytic enzymes. To test this possibility, garlic leaf extract was incubated at 37℃, and samples were taken at various time intervals and analyzed by immunoblotting (Fig. 7). After 2 hrs the lower band disappears and after 4 hrs the upper band also disappears, suggesting that they are degraded differently, hence precursor-product relationship was noticed. These results argue for the presence of 2 closely related potexviruses in Korean garlic samples. The molecular details of these variants in two viruses are not clear at this moment.

The bands of Mr 29,000 and 27,000 were also observed in SDS-PAGE of virus preparation (Fig. 1). The band of Mr 27,000 is noticed in Danyang and Seosan cultivars, but Mr 29,000 was major in cultivar Danyang and minor in cultivar Seosan. When immunoblot analysis was carried out with anti-S81 antibody to garlic virus preparation, several bands including Mr 27,000 was visualized but Mr 29,000 was not (data not shown). All of the bands detected were smaller than Mr 27,000. These are considered to be degradation products of Mr 29,000 and 27,000 resulting from lengthy virus preparation procedure and, therefore, we can not know exactly at this moment which band on the SDS-PAGE shown in Figure 1 corresponds to the potexvirus encoded by the clone S81.

Alternatively, it is also possible that two bands of Mr 29,000 and 27,000 analyzed by immunoblotting with anti-S81CP antibody could result from cross-reactivity between garlic viruses. When garlic crude extract was treated with antibody against GLV or GMV, neither bands of Mr 29,000 and 27,000 were detected (data not shown). It is, therefore, presumed for the double bands to be specific coat proteins for potexviruses in garlic plants.

It appears that two types of garlic potexvirus is present in Korean garlic plants with different quantities in different cultivars. We have also cloned and sequenced another type of garlic viral cDNA homologous to S81. It argues for the presence of another potexvirus in Korean garlic.

Garlic potexvirus is less abundant than GLV in Korean garlic plants considering the results of immunoblot analysis. This is also consistent with that of Northern blot analysis. But it is not clear whether potexvirus is responsible for yellow symptom of garlic at this moment. A potexvirus in garlic plants has not been described by immunological method or electron microscopy in Korea and Japan. It is not known whether the virus affects garlic mosaic symptom or is involved in the synergistic effect with other garlic viruses. Further studies are necessary to characterize the the potexvirus in garlic.

Acknowledgement

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한국 마늘 Potexvirus의 cDNA 유전자 분리 및 분포에 관한 연구

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초록 : 한국 마늘 바이러스의 유전자 구조와 병 발생 메카니즘을 연구하기 위하여, 바이러스가 감염된 마늘 잎으로부터 바이러스 입자를 분리하고 바이러스로부터 RNA 를 추출하였다. 그 virus RNA 를 이용하여 마늘 바이러스 cDNA 유전자 은행을 만들어 일부 clone 의 염기서열을 결정하였다. 여기에서 얻은 cDNA clones 중에서 poly(A) tail 을 갖는 clone S81 를 분리하고 873 bp의 전체 염기서열을 결정하였다. Clone S81 의 염기서열을 다른 식물 바이러스와 비교한 결과 potexvirus 의 껍질단백질 부분의 염기서열과 30~40%의 유사성을 보여주었다. Clone S81 은 바이러스 RNA의 3' 말단 부위에 해당하고, 껍질단백질의 N-terminal 3개 아미노산이 빠진 open reading frame (ORF) 및 3'-noncoding region을 포함하고 있다. 3' 말단 부분에는 바이러스 복제과정에서 *cis*-acting element로 작용한다고 여겨지는 hexamer motif와 polyadenylation signal이 존재한다. 이 clone을 probe로 하여 Northern blot을 실시한 결과 genome의 크기는 7.5 knt라는 것을 알 수 있었고 clone S81은 potexvirus의 cDNA clone이라는 결론을 얻었다. 한국 마늘에서 이 바이러스의 분포 양상을 알아보기 위해 껍질단백질에 대한 항체를 만들었다. 먼저 발현벡터를 이용하여 대장균에서 대량으로 발현시키고 affinity chromatography로 껍질단백질을 정제하였다. 그 단백질을 토끼에 주사하여 껍질단백질에 대한 항체를 얻었다. 이 항체를 사용하여 다양한 지역에서 재배되는 마늘잎의 추출액에 대해 immunoblot을 실시하였다. 그 결과 분자량 29,000과 27,000 위치에서 signal을 보였다. 분자량 27,000 단백질이 29,000이 분해되어 생긴 산물인지 알아보기 위하여 그 추출액을 37℃에서 시간을 달리하여 incubation한 후 immunoblotting 하였다. 그 결과도 마찬가지로 같은 위치에서 signal을 보여줬다. 따라서 한국 마늘에는 재배되는 지역에 따라 다소 다르기는 하지만 대체로 두 종류의 potexvirus로 감염되어 있다고 추정된다.

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