Arch Virol (1998) 143: 1093–1107

Archives of Virology

Differentiation of *Allium* carlaviruses isolated from different parts of the world based on the viral coat protein sequence

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Accepted January 22, 1998

Summary. Common primers which amplify the 3′ terminal genomic RNAs of *Allium* carlaviruses were designed based on the nucleotide sequence of shallot latent virus (SLV), garlic latent virus (GLV) and garlic common latent virus (GCLV). A total of fifteen cDNAs encoding the coat protein (CP) of the carlaviruses, including the biologically identified isolates SLV, GLV and GCLV as well as viruses from infected *Allium* plants cultivated in different parts of the world, were amplified by RT-PCR with the common primers. The cDNAs were then cloned and sequenced. The predicted viral CP amino acid sequence as well as the nucleotide sequence revealed that SLV and GLV, previously considered as separate viruses on the basis of their biological and physical properties, belong to the same species of the genus *Carlavirus.* Both viruses are clearly differentiated from GCLV. In addition, every SLV and GLV isolate from the *Allium* plants in Taiwan showed characteristic and common variations in their CP sequences, suggesting the possible presence of geographical variants. However, no apparent sequence variations of SLV and GLV related to their host plant species, including *A. sativum, A. wakegi, A. chinense, A. fistulosum, A. cepa* and *A. ampeloprasum,* were observed. These findings suggested that the sequence variations observed in the respective virus isolates do not correlate with the specificity of their infectivities for *Allium* species.

Introduction

Allium species are commonly infected with a complex of two or more viruses, these often being members of the *Potyvirus* and *Carlavirus* genera [3, 7, 17, 23, 26–31]. Recently, novel and unassigned viruses with a unique genome organization have been characterized from garlic [22, 33] and shallot [10]. The complexity

of virus infection, together with their strictly restricted host ranges and lack of sensitive methods for detecting individual viruses have led to difficulties in differentiating and characterizing these *Allium* viruses. Recent advances in molecular biology, however, have provided new tools for differentiation and identification of viruses. Such molecular properties as the viral genome sequence and CP amino acid sequence are shown to be useful taxonomic criteria for distinguishing viruses from strains and for determining the relationships between genera, species, and subspecies of distinct viruses [2, 6, 9, 20, 21, 32]. The sequences of several viruses infecting *Allium* species, including leek yellow stripe virus (LYSV) [13, 23], onion yellow dwarf virus (OYDV) [11, 25], wakegi yellow dwarf virus (WYDV) [24, 25], garlic latent virus (GLV) [13, 23], garlic virus-A to -D (Gar V-A to -D) [22, 33] and shallot virus X (ShVX) [10] have been published. In previous papers [24, 25], we showed that at least 3 distinct potyviruses, which are distinguished by the difference in their CP and 3′ noncoding sequences, infect *Allium* plants around the world. These viruses were identified as LYSV, OYDV and WYDV. Following the success with potyviruses, we intended to characterize *Allium* carlaviruses with the same strategy.

Shallot latent virus (SLV) [4, 5] and garlic common latent virus (GCLV) are well characterized carlaviruses infecting *Allium* species [28]. Another carlavirus, namely garlic latent virus (GLV), has been isolated from garlic in Japan [12]. SLV and GLV are serologically closely related but differ in host reactions and physical properties such as thermoinactivation point (TIP) [4, 5, 12, 28]. Based on the serology, host range and symptomatology, Van Dijk [28] concluded that GLV was a garlic strain of SLV (SLV-G). We have determined the 3′ terminal nucleotide sequences, encoding the viral CP, of GLVs from garlic [23] and *A. wakegi* (DDBJ/EMBL/GenBank data base accession No. D73379). Additionally, two GLV sequences from garlic were deposited (accession Nos. D28591, Z68502). A partial cDNA sequence of GCLV was also deposited (accession No. X81138) by J. Schubert. However, detailed relationships between *Allium* carlaviruses based on the nucleotide sequence of genomic RNA or the amino acid sequence of viral CP have not been analyzed.

Based on the available nucleotide sequences, we designed several primers for amplifying the 3′ terminal regions of the genomes of *Allium* carlaviruses. To clarify the relations among SLV, GLV and GCLV at the nucleotide sequence level, we cloned the viral cDNAs of the purified isolates from garlic, Chinese leek, Welsh onion and rakkyo in Taiwan and/or Japan after amplification of the cDNAs by RT-PCR procedure. Furthermore, we cloned viral cDNAs from infected *Allium* plants from different parts of the world, including China, Indonesia, the Netherlands and Germany. We here describe the molecular differentiation of the *Allium* carlaviruses SLV, GLV and GCLV, based on the nucleotide sequences of the 3′ terminal regions including viral CP genes. We also present evidence suggesting that sequence variation probably depends on geographic location, but not on the host *Allium* species.

Materials and methods

Plant materials

Commercially available garlic bulbs produced in the Netherlands, Germany and China were purchased in the market. Indonesian garlic (*Allium sativum*), leek (*A. ampeloprasum* var. *porrum*), Welsh onion (*A. fistulosum*) and shallot (*A. cepa* var. *ascalonicum*) were collected in Indonesia. Japanese garlic cultivar Isshu-wase and *A. wakegi* plants were provided by Y. Ikeda (Hiroshima Prefectural Agriculture Research Center).

Virus isolates

GCLV isolate GF1 (GCLV-GF1) was originally obtained from garlic in Taiwan and purified after three local-lesion transfers on *Chenopodium quinoa*. SLVs from garlic (SLV-Gtwn), Chinese leek (SLV-CLtwn) and Welsh onion (SLV-WOtwn) in Taiwan were single local-lesion isolates from *C. quinoa*. GLV isolates from Chinese leek (GLV-CLjpn), rakkyo (*A. chinense*) (GLV-Rjpn) and *A. wakegi* (GLV-AwM77) in Japan were purified after several local-lesion transers on *Vicia faba*. The respective virus isolates were maintained in *C. quinoa* for GCLV-GF1, SLV-CLtwn and SLV-WOtwn, *A. fistulosum* for SLV-Gtwn and *V. faba* for GLV-CLjpn, -Rjpn and -AwM77.

Nucleic acid extraction and cDNA cloning

Total RNA was extracted from approximately 20 mg of dried or 200 mg of fresh leaves of *C. quinoa, A. fistulosum*, and *V. faba* or from infected *Allium* plants, using ISOGEN (Nippon Gene, Toyama, Japan). cDNA synthesis and cloning were done as described previously [24].

Reverse transcription and the polymerase chain reaction

Reverse transcription (RT) was done with approximately 1 μg of the RNA and 0.2 μg of *Not*I-d$(T)_{18}$, 5′-AACTGGAAGAATTCGCGGCCGCAGGAAT$_{18}$-3′, as the primer in a reaction volume of 15 μl using a First-strand cDNA Synthesis Kit (Pharmacia Biotech, Uppsala, Sweden). For the PCR, 1 μl of the RT mix was added to a 100 μl polymerase reaction mixture containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each dNTP, 2.5 U *AmpliTaq* DNA polymerase (PERKIN ELMER, Foster City, CA) and 100 ng each of the upstream and downstream primers. The 5′ primers for amplification of the CP genes of SLV and GLV (designated CAR-V1 and -V2) were 5′-AAACCTTTTGG-TTCACTTTAGG-3′ and 5′-AGGTGCATTGTTATCATTACTGG-3′ and that for GCLV (designated CAR-V3) was 5′-GCTAGACATTGGTAGCCTTAGG-3′. The 3′ primer (designated PC-R4) 5′-ACCGATTCAACTGGAAGAATTCGCGG-3′ corresponds to a part of the *Not*I-d$(T)_{18}$ primer sequence and was universally used for amplifying the full 3′ terminal regions. In amplification of only viral CP gene sequences of SLVs or GLVs, the 3′ common primer CAR-CP3, 5′-GTATGCAACTTAAATATAGCACGC-3′, was used. The thirty reaction cycles used had periods of 30 sec for annealing at 55 °C, 60 sec for synthesis at 72 °C, and 30 sec for melting at 94 °C.

Cloning and sequencing of amplified fragments

Amplified fragments were purified by 1% agarose gel electrophoresis and cloned directly into the linearized plasmid vector, pT7Blue T (Novagen, Madison, WI). The chimeric plasmid was used to transform *E. coli* JM109. To increase the sequence accuracy we randomly selected 3 independent clones from each transformant and determined their nucleotide sequences using a *Bca*BEST Dideoxy Sequencing Kit (Takara Shuzo Co., Ltd., Otsu, Japan)

or a Thermo Sequenase fluorescent labeled primer cycle sequencing kit (Amersham International, Buckinghamshire, UK) according to the manufacturer's instructions. The nucleotide sequences determined in this paper were deposited in DDBJ/EMBL/GenBank database and their accession numbers are listed in Table 1, along with those of the previously reported sequences.

Results

Design of primers for amplification of the 3′ terminal regions of the Allium *carlaviruses*

A search for cDNA sequence data of the *Allium* carlaviruses in DDBJ/EMBL/GenBank DNA database found four GLV sequences (accession Nos.: D11161, D28591, D73379, Z68502) and one GCLV (accession No.: X81138) sequence, which cover entire viral CP genes. The nucleotide sequences of 7 kDa protein genes upstream of the CP genes were aligned and relatively conserved sequences at local areas were selected (data not shown). Three oligonucleotide sequences, respectively designated CAR-V1, -V2 and -V3, were designed as possible upstream primers. The common downstream primer PC-R4 corresponds to a part of the *Not*I-d$(T)_{18}$ primer for cDNA synthesis. These primer sets were tested by RT-PCR with total RNA extracted from the local-lesion host plants infected with the respective *Allium* carlavirus isolates. Results of the analyses of the amplified DNA fragments in 1% agarose gels are shown in Fig. 1. The primers CAR-V1 and CAR-V2 were effective for amplifying the 3′ terminal genomic RNAs of SLV and GLV isolates to produce DNA fragments of expected size (approximately 1.4 kb), but not for GCLV (Fig. 1). In contrast, the primer CAR-V3 was effective for

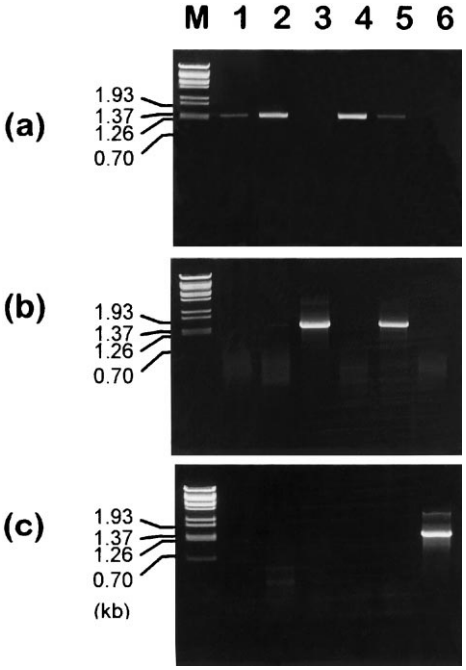

Fig. 1. DNA fragment amplification using the upstream primers CAR-V1 (**a**), CAR-V2 (**b**) and CAR-V3 (**c**) designed as possible common primers to SLV, GLV and GCLV. The primer PC-R4 was commonly used as the downstream primer. The polymerase chain reaction was done with 1 μl of an RT mix obtained by reverse transcription of the total RNA extracted from dried leaves of *C. quinoa*, *A. fistulosum* and *V. faba* maintaining the purified virus isolates. A 10 μl portion of the 100 μl PCR mixture was analyzed in a 1% agarose gel. *1–6*, respectively, show PCR products from total RNA extracted from the local legion host plants infected with GLV-Rjpn, GLV-CLjpn, SLV-CLtwn, SLV-WOtwn, SLV-Gtwn and GCLV-GF1. M is λDNA digested by *Bst*PI as the size marker

Table 1. cDNA clones of *Allium* carlaviruses and their accession numbers in DDBJ/EMBL/GenBank database

cDNA clone	Source (country)	Accession number	Reference
GCV-H	garlic cultivar Fukuchi-howaito (Japan)	D11161	22
GLV-AwM77	*Allium wakegi* (Japan)	D73379	this study
GLV-GV1	garlic (Japan)	D28591	13
GLV-CLjpn	Chinese leek (Japan)	AB004458	this study
GLV-Rjpn	rakkyo (Japan)	AB004565	this study
SLV-CLtwn	Chinese leek (Taiwan)	AB004456	this study
SLV-WOtwn	Welsh onion (Taiwan)	AB004544	this study
SLV-Gtwn	garlic (Taiwan)	AB004457	this study
GLV-Gnag	garlic cultivar Isshu-wase (Japan)	AB004567	this study
GLV-Gchi	garlic (China)	AB004684	this study
GLV-Guae	garlic (UAE)	AB004685	this study
GLV-Gind	garlic (Indonesia)	AB004686	this study
GLV-Sind	shallot (Indonesia)	AB004802	this study
GLV-Lind	leek (Indonesia)	AB004803	this study
GLV-GNS	garlic (Korea)	Z68502	Choi (unpubl.)
GCLV-GF1	garlic (Taiwan)	AB004566	this study
GCLV-Gams	garlic (the Netherlands)	AB004804	this study
GCLV-Ghav	garlic (Germany)	AB004805	this study
GCLV-CT1	garlic (Germany)	X81138	Schubert (unpubl.)

GCLV but not for SLV and GLV (Fig. 1). PCR performed with cDNAs of distinct *Allium* viruses, including LYSV, OYDV, WYDV and GarVs, as templates did not produce any amplified DNA fragments (data not shown).

Sequencing and comparison of the 3′ terminal sequence of Allium *carlavirus isolates*

The amplified DNA fragments from the *Allium* carlavirus isolates (GLVs from *A. wakegi*, Chinese leek and rakkyo in Japan; SLVs from garlic, Welsh onion and Chinese leek in Taiwan; GCLV from garlic in Taiwan) were cloned, then the nucleotide sequences of the respective cDNA clones were determined and deposited in DDBJ/EMBL/GenBank database (Table 1).

Two potential open reading frames (ORFs) which, respectively, encoded viral CP and nucleic acid binding protein (NABP), were detected in each cDNA sequence. The deduced amino acid sequences of CP and NABP shared high similarities of respectively 89.2 to 99.3% and 84.7 to 98.0% among the SLV and GLV isolates, but those of the GCLV isolate have limited similarities of approximately 45 and 20% with the SLV and GLV sequences, respectively. The length of the 3′ noncoding sequence of GCLV was remarkably different from SLV and GLV. The 3′ noncording region of GCLV was composed of only 13 nucleotides, in contrast with more than 90 nucleotides in SLV and GLV isolates.

Alignment of the respective CP amino acid sequences in addition to the GLV sequence from Japanese garlic, previously reported as cDNA clone GV-H [23], are shown in Fig. 2. Little difference was observed in overall CP sequences between the isolates which had been separated into SLV and GLV based on host range, symptoms and physical properties (data not shown). On the other hand, the sequence of GCLV differed significantly from those of SLV and GLV (Fig. 2 and Table 2). A characteristic deletion of 3 amino acids was found in the N-terminal sequence of the isolates from Taiwan, which was detected in every isolate from different Taiwanese *Allium* species, including garlic, Welsh onion and Chinese leek. In the GLV-AwM77 sequence form the *A. wakegi* isolate, the C to T transition in CGA codon for arginine at the second position to the C-terminus produced a termination codon TGA to delete the C-terminal 2 residues R and C. From the NABP amino acid sequence, SLV and GLV isolates were not distinguishable but were distinct from GCLV as shown in Table 2.

Comparison of sequences of carlaviruses from Allium *species in different parts of the world*

To clarify whether there were correlations between geographic location and the sequence variation, such as a 3-amino-acid deletion found in the CPs of the SLV and GLV isolates from Taiwan, we tested individual *Allium* plants, including garlic, leek and shallot plants, from different parts of the world by the RT-PCR procedure. In this experiment, the 3′ common primer designated CAR-CP3 was used to amplify only the CP gene regions of SLVs and GLVs. In RT-PCR using the 5′ CAR-V1 or -V2 combined with the 3′ primer CAR-CP3, approximately 1 kb DNA fragments were amplified from the total RNA of a Japanese garlic

Fig. 2. Multiple alignment of the predicted amino acid sequences of the viral CPs of SLV, GLV and GCLV encoded in the amplified DNA fragments by the RT-PCR. The available sequences of the previously reported *Allium* carlaviruses ([13, 23], DDBJ/EMBL/GenBank data base accession Nos. X81138 and Z68502) are introduced into the alignment. The amino acids identical to the GLV-CLjpn or GCLV-GF1 sequence are indicated by dots, and positions conserved in all the sequences are highlighted with asterisks. Characteristic variations found in the N-terminal sequence of the Taiwanian isolates, including a deletion of 3 amino acids and substitution from E to A at the second residue in the characteristic serial 4 acidic residues, are boxed. GLV-CLjpn, -Rjpn, -AwM77, -Gnag, -Gchi, -Guae, -Gind, -Sind and -Lind are the CP sequences of the respective viruses isolated from Japanese Chinese leek, rakkyo, *A. wakegi* and garlic cultivar Isshu-wase, from garlic clones of China, UAE and Indonesia, from Indonesian shallot and leek. SLV-CLtwn, -WOtwn and -Gtwn are the CP sequences of the Taiwanese SLV isolates from Chinese leek, Welsh onion and garlic, respectively. GCLV-GF1 is the CP sequence of GCLV isolated from garlic in Taiwan. GCLV-Gams and -Ghav are the CP sequences of the viruses isolated from garlic of Germany and the Netherlands, respectively. GCV-H [23], GLV-GV1 [13], GLV-GNS [DDBJ/EMBL/GenBank data base accession No. Z68502] and GCLV-CT1 (DDBJ/EMBL/GenBank data base accession No. X81138) are the CP sequences of previously reported *Allium* carlaviruses. Gaps are introduced for maximum alignment. Numbers to the right of the sequences show the positions from the N-terminus of the CP

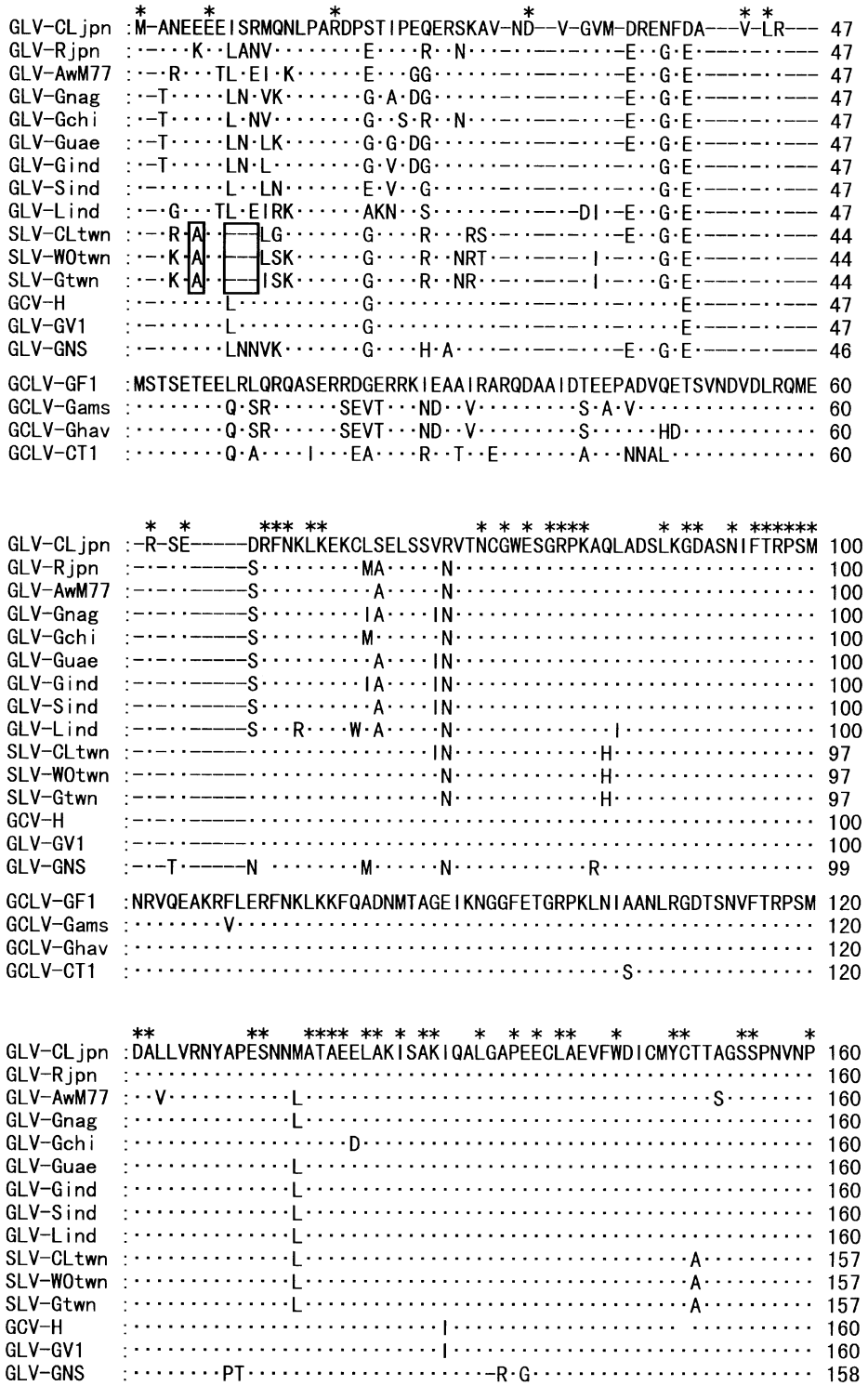

Fig. 2 (*continued*)

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GLV-CLjpn      :KGTISVGGKVVTRDMVVAVIKEYSTLRQVCRCYAPVVWNYMLLNEQPPANWDAKGFTENT 220
GLV-Rjpn       :·····························V·····V····························· 220
GLV-AwM77      :·····························V·····V····························· 220
GLV-Gnag       :·····························V·····V····························· 220
GLV-Gchi       :·····························V·····V····························· 220
GLV-Guae       :·····························V·····V····························· 220
GLV-Gind       :·····························V·····V····························· 220
GLV-Sind       :·····························V·····V····························· 220
GLV-Lind       :·····························V·····V····························· 220
SLV-CLtwn      :·····························T····F····························· 217
SLV-WOtwn      :·····························T····F····························· 217
SLV-Gtwn       :·····························T····F····························· 217
GCV-H          :·····························V·····V·····························S····· 220
GLV-GV1        :·····························V·····V·····························S····· 220
GLV-GNS        :·····························V·····V····························· 218

GCLV-GF1       :KGTFEYPGGAITRDAVYAVIKEVTTLRAFCRAFAPVVWNEMLIAKRPPTGWQTKGYTAST 240
GCLV-Gams      :·····························V·····V·····A····················· 240
GCLV-Ghav      :·····························V·····V·····A···A····T·· 240
GCLV-CT1       :·····························V·····V·····A···A·····A 240


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GLV-CLjpn      :KYAAFDTFDAVTNKAAIQPLEGLIRAPTDEERIAFATHKKLALAKNAQNSRYSNTSAEVT 280
GLV-Rjpn       :·····························V·····V·····N·····A····· 280
GLV-AwM77      :·····························V·····V·····T·····A···R·I· 280
GLV-Gnag       :·····························V·····V·····L·A····· 280
GLV-Gchi       :·····························V·····V·····A····· 280
GLV-Guae       :·····························V·····V·····A····· 280
GLV-Gind       :·····························V·····V·····A····· 280
GLV-Sind       :·····························V·····V·····A····· 280
GLV-Lind       :·····························V·····V·····T·····A····· 280
SLV-CLtwn      :·····························V·····V·····I····A····· 277
SLV-WOtwn      :·····························V·····V·····N····A····· 277
SLV-Gtwn       :·····························V·····V·····N····· 277
GCV-H          :·····························V·····V····S····· 280
GLV-GV1        :·····························V·····V····R····· 280
GLV-GNS        :·····························V·····V·-TR·····T·····A··A····· 277

GCLV-GF1       :KYAAFDTFDYVLNSACVQPLEGIIRVPTDEETIAHMTNKRIAIDRNRRNGRFSSTNSLVT 300
GCLV-Gams      :·F····························V·····V····· 300
GCLV-Ghav      :·····························V·····V····· 300
GCLV-CT1       :·····························V·····V····· 300


                ** **   *
GLV-CLjpn      :GGFFGCFPKHNFRENRC     297
GLV-Rjpn       :·········N·····       297
GLV-AwM77      :·····V··N·····--      295
GLV-Gnag       :·········N·····       297
GLV-Gchi       :·········N·····       297
GLV-Guae       :·········N·····       297
GLV-Gind       :·····L··N·····        297
GLV-Sind       :·········N·····       297
GLV-Lind       :·····V··N···QD··      297
SLV-CLtwn      :·····I·········       294
SLV-WOtwn      :·····V·········       294
SLV-Gtwn       :·····V·········       294
GCV-H          :···············       297
GLV-GV1        :···············       297
GLV-GNS        :·········N·····       294

GCLV-GF1       :GGMFGKDIKTNFNGSNNAD   319
GCLV-Gams      :·····V·····T·····     319
GCLV-Ghav      :·····V·····T·····     319
GCLV-CT1       :·····V·····T·····     319
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(Fig. 2)

Table 2. Percent homology of the predicted amino acid sequences of CP and NABP between *Allium* carlaviruses

		NABP (nucleic acid binding protein)																	
		1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.	15.	16.	17.	18.	19.
CP	1.GCV-H		83.7	95.9	95.9	88.8	89.8	89.8	88.8	83.7	-	-	-	-	-	-	18.8	20.1	19.5	21.1
	2.GLV-AwM77	90.2		80.6	84.7	85.7	88.8	86.7	86.7	80.6	-	-	-	-	-	-	15.6	17.5	16.3	19.1
	3.GLV-GV1	99.3	89.6		92.9	85.7	86.7	86.7	85.7	81.6	-	-	-	-	-	-	18.2	19.5	18.8	20.4
	4.GLV-CLjpn	97.6	89.2	97.3		89.8	90.8	91.8	90.8	84.7	-	-	-	-	-	-	14.9	16.2	17.4	19.1
	5.GLV-Rjpn	93.3	92.2	93.3	92.3		91.8	92.9	92.9	88.8	-	-	-	-	-	-	17.5	18.2	15.3	20.4
	6.SLV-CLtwn	92.9	89.8	92.0	91.5	93.0		96.9	94.9	90.8	-	-	-	-	-	-	17.5	18.8	16.3	21.8
	7.SLV-WOtwn	92.9	89.8	92.0	91.5	92.5	96.6		98.0	90.8	-	-	-	-	-	-	18.2	18.2	16.3	21.4
	8.SLV-Gtwn	92.9	90.1	92.0	92.2	92.5	96.3	99.3		90.8	-	-	-	-	-	-	16.9	17.5	16.3	19.7
	9.GLV-GNS	91.2	88.1	90.2	89.8	93.2	89.5	88.4	88.4		-	-	-	-	-	-	18.2	18.8	15.3	22.5
	10.GLV-Gnag	92.6	91.5	92.6	91.3	94.3	92.9	91.2	91.2	92.2		-	-	-	-	-	-	-	-	-
	11.GLV-Gchi	93.9	91.2	94.0	93.3	96.6	92.9	92.9	92.9	93.2	94.6		-	-	-	-	-	-	-	-
	12.GLV-Guae	93.3	92.2	93.3	91.9	94.3	93.5	91.8	91.8	92.2	98.0	94.6		-	-	-	-	-	-	-
	13.GLV-Gind	93.6	92.2	93.6	93.0	94.3	93.2	92.2	92.2	91.5	97.6	94.6	98.0		-	-	-	-	-	-
	14.GLV-Sind	94.6	93.2	94.6	93.6	95.3	93.9	93.2	93.2	91.8	96.3	94.6	97.3	97.6		-	-	-	-	-
	15.GLV-Lind	88.6	91.9	88.6	87.5	90.2	88.8	89.8	90.1	87.4	89.9	89.6	91.3	90.2	91.6		-	-	-	-
	16.GCLV-GF1	45.8	45.1	44.0	47.8	45.5	46.6	47.6	45.9	40.5	47.8	45.8	47.1	47.5	42.4	46.5		90.3	90.9	76.1
	17.GCLV-Gams	45.5	45.1	44.0	45.8	44.8	47.6	46.6	46.6	44.2	42.4	45.1	41.4	45.1	42.1	46.8	94.7		96.1	76.2
	18.GCLV-Ghav	45.5	45.1	44.0	47.8	45.1	46.6	45.9	46.3	44.6	45.8	45.5	41.8	45.1	45.1	46.8	94.4	97.2		78.2
	19.GCLV-CT1	46.5	44.4	44.0	47.1	42.1	48.0	46.6	45.2	44.6	42.1	45.1	41.4	40.7	42.1	45.1	93.7	92.5	92.8	

GCV-H is a GLV isolate from Japanese garlic cultivar Fukuchi-howaito [23]. GLV-AwM77 is the isolate from *A. wakegi*. GLV-GV1 is a previously reported isolate from Japanese garlic [13]. GLV-CLjpn and -Rjpn are the viruses from Chinese leek and rakkyo plant in Japan. SLV-CLtwn, -WOtwn and -Gtwn are the purified viruses isolated from Taiwanese Chinese leek, Welsh onion and garlic, respectively. GLV-GNS is a Korean isolate from garlic reported by Choi (accession No. Z68502). GLV-Gnag, -Gchi, -Guae, -Gind, -Sind and -Lind are the respective viruses from garlic of Japanese cultivar Isshu-wase, China, the UAE and Indonesia, and from Indonesian shallot and leek. GCLV-GF1 is the purified GCLV isolated from garlic in Taiwan. GCLV-Gams and -Ghav, respectively, are the viruses isolated from garlic in the Netherlands and Germany. GCLV-CT1 is a isolate from German garlic reported by Schubert (accession No. X81138)

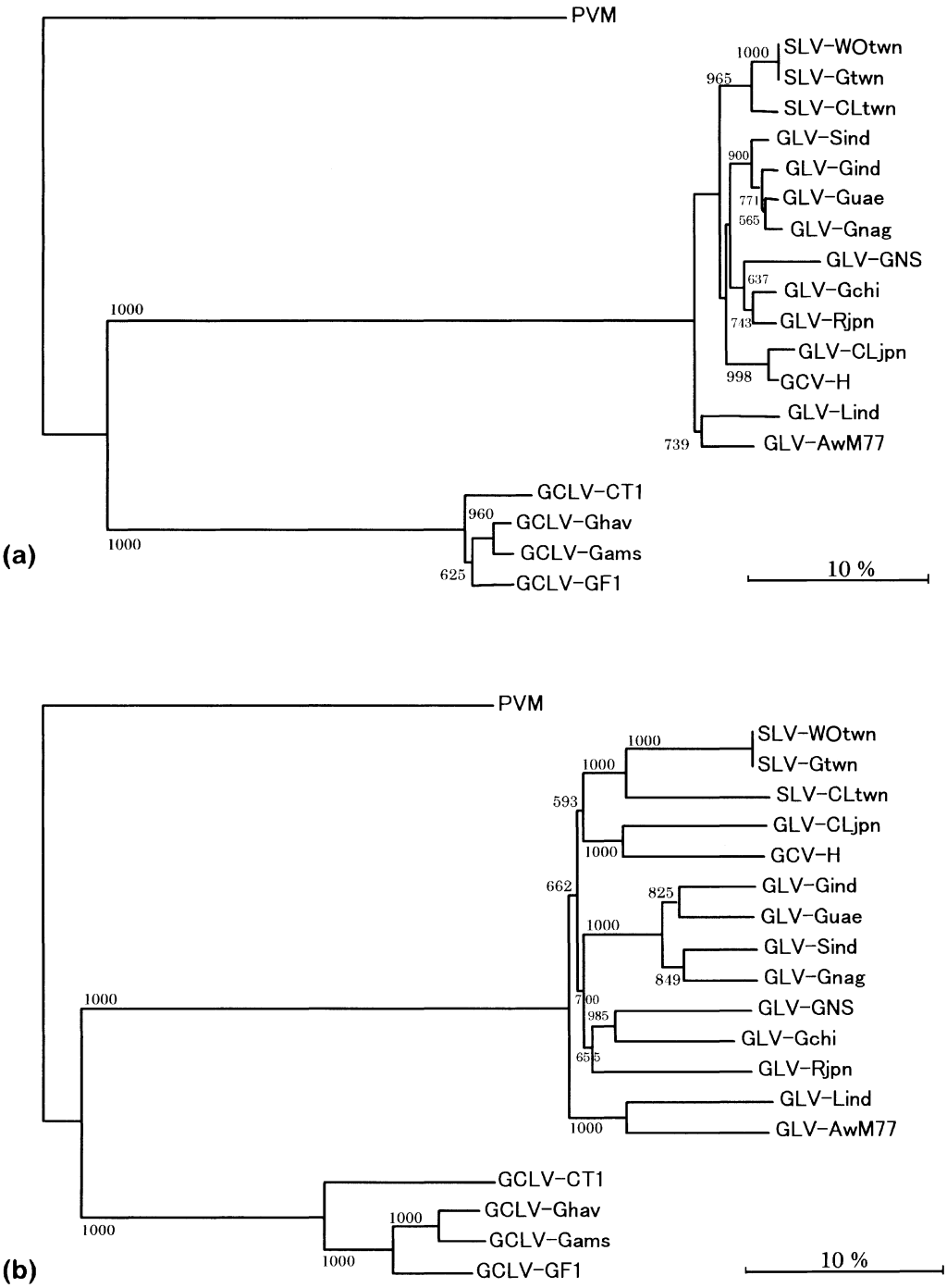

Fig. 3. Cluster dendrograms showing the relationships of *Allium* carlaviruses as deduced from comparison of the predicted amino acid sequences (**a**) and the nucleotide sequences (**b**) of the viral CP. Phylogenetic analyses were done with CLUSTAL W Vers. 1.6, then relationship dendrograms were calculated with bootstrapping (1 000 replicates) using the neighbor-joining option in CLUSTAL W. Potato virus M (PVM) [16] was used as a carlavirus out-group. All horizontal distances are proportional to sequence differences indicated by inset scale, but vertical distances are arbitrary. Numbers adjacent to nodes are bootstrap scores (out of 1 000 replicates). Abbreviations are the same as in Fig. 2

cultivar Isshu-wase and garlic plants in China, the United Arab Emirates (UAE) and Indonesia as well as from Indonesian leek and shallot (data not shown). The primer set of CAR-V3 and PC-R4 specific for GCLV amplified a DNA fragment of the expected approximately 1.4 kb in the RT-PCR done with the total RNA of garlic from the Netherlands and Germany (data not shown). After cloning the respective DNA fragments in pT7Blue vector, their nucleotide sequences were determined and deposited in the DDBJ/EMBL/GenBank database (Table 1). It should be noted that the cDNA clones of *Allium* carlaviruses obtained through RT-PCR with the common primers CAR-V1 or -V2 and CAR-CP3 likely comprised those of GLV, SLV and the related virus(es), but were provisionally designated as GLV clones in this experiment.

The deduced amino acid sequences of the respective viral CPs encoded in the 1 kb DNA fragments produced by the RT-PCR done with CAR-V1/CAR-CP3 or CAR-V2/CAR-CP3 shared high similarities of 89.6 to 98.0% each other (Table 2). In addition, they showed high degree of similarity of 87.5 to 96.6% with the viruses which were purified and identified as SLV or GLV (SLV-CLtwn, -WOtwn, -Gtwn, GLV-CL jpn, -Rjpn and -AwM77) (Table 2). It should be noted that the similarity of the viral CP sequence from Indonesian leek with the other sequences was a little lower, being 87.5 to 91.9%, in comparison with the similarities among the sequences from garlic in Japan, China, UAE and Indonesia as well as Indonesian shallot, 91.2 to 98.0%.

On the other hand, the amino acid sequences of the putative CP encoded in the amplified 1.4 kb DNA fragment in the RT-PCR using the primer set CAR-V3/PC-R4 specific for the GCLV sequence showed very limited similarities of lower than 48% to the SLV or GLV sequences. The CP sequences of GCLV isolates from the Netherlands, Germany and Taiwan shared high homologies of more than 94.7% each other and with the sequence deposited in DDBJ/EMBL/GenBank database as GCLV from garlic by J. Schubert. The putative NABP amino acid sequences showed high similarities of 76 to 96% among the same GCLV isolates but differed remarkably from those of the SLV and GLV isolates (less than 23%).

Phylogenetic analyses based on both the predicted amino acid sequence and the nucleotide sequence of CP indicated that the SLV and GLV isolates were not obviously separated and distributed in the same domain, but the GCLVs comprised a distinct domain from the SLV/GLV (Fig. 3).

Discussion

We cloned the cDNAs of the 3′ terminal region encoding viral CP gene of *Allium* carlaviruses which were biologically isolated and then identified as SLV, GLV and GCLV, respectively, in addition to those of viruses from infected *Allium* plants cultivated in different parts of the world, after amplification of the cDNAs by RT-PCR with the common primers. The predicted CP sequences of the SLV isolates shared 89.8 to 93.0% similarity with those of the GLV isolates and they were not clearly discriminated on the basis of their CP sequences. These results coincided with the previous observation that SLV and GLV are serologically closely related.

In addition, the similarities of the CP sequences of the viruses from the infected *Allium* plants ranged from 89.6 to 98.0%. Previous reports on SLV and GLV noted that they differed in host specificity, host reactions and physical properties; TIP, for instance, of SLV was 80 °C but that of GLV 55 to 60 °C [4, 12]. It is intriguing that in spite of the remarkable differences in biological and physical properties, we did not find any significant or characteristic alterations in the predicted CP sequences between SLV and GLV. These findings may suggest that possible variations in viral non-structural components other than CP caused the biological and physical differences reported previously. Indeed, in tobacco mosaic virus, it was reported that alterations in RNA replicase affected the viral infectivity [8, 14].

The CP sequences of the SLVs and GLVs from *Allium* plants showed high overall similarities, but the N-terminal sequence was frequently altered. Only 8 of the N-terminal 30 residues were common, although more than 90% of the residues in the following sequence were identical among all the sequences. As the N-terminal region of the CP seemed to carry the major virion-specific epitopes as demonstrated in the potyviruses [1, 19], the respective isolates could be serologically differentiated by specific antisera, or in analyses using monoclonal antibodies. However, the N-terminal sequence variations occurred randomly and no characteristic sequence was detected even in the isolates from the same *Allium* species such as garlic. These findings suggested that the sequence variations found in the viral CPs were independent of the host species, and that, the respective viruses may not show restricted host specificity to each *Allium* plant. Taking all of these results into consideration, we concluded that SLV and GLV should be recognized as strains of the same virus species, rather than as distinct species, as suggested by Van Dijk [28].

Viral CP was composed of 297 amino acid residues in the SLV and GLV isolates except for those from Japanese *A. wakegi*, Taiwanese *Allium* plants and Korean garlic. In the sequence of *A. wakegi*, the transition of cytosine to uridine occurred in the codon, CGA, directing the second amino acid residue R to the C-terminus of the CP. This point mutation produced a termination codon UGA, resulting in deletion of the C-terminal 2 amino acids RC. Interestingly, all the SLV isolate from *Allium* plants in Taiwan, which were independently isolated from garlic, Chinese leek and Welsh onion, have a deletion of 3 amino acids at the positions 11 to 13. The second residue in the characteristic group of four charged residues, typically EEEE, at positions 4 to 7 was also substituted by a neutral residue A in these Taiwanese isolates. These sequence variations were observed only in the isolates from Taiwan and not in those from *Allium* plants cultivated in neighboring Asian countries, including China, Japan and Indonesia. Furthermore, the similarities of the CP sequence of the respective Taiwanese isolates were 96.3 to 99.3%, irrespective of host plant species from which they were isolated. These findings suggested that there are geographical variations restricted to Taiwan and both their development and transmission pathway are of interest.

On the other hand, GCLV was clearly distinguished from SLV/GLV based on the similarities of CP and NABP. The overall similarity of the CP sequence

between GCLV and SLV/GLV was limited to only 40.5 to 48.0%. Besides, the N-terminal 50 amino acids differed remarkably, in that only 4 residues were commonly conserved. These sequence data supported the previous serological differentiation of GCLV from SLV/GLV.

Van Dijk [28] described another *Allium* carlavirus, namely Sint-Jan's onion latent virus (SjoLV), which was isolated from Utrechtse Sint-Jan's onion maintained in the Netherlands and detected in ever-ready onion, pearl onion, rakkyo, shallot, and Welsh onion, together with SLV. SjoLV reacted equally well with the antisera to SLV and GCLV in electron microscope decoration tests and Van Dijk described that "Garlic latent virus" isolated from hosts other than garlic in Japan should be regarded as SLV, SjoLV, or a mixture of these viruses [28]. We have not detected viruses which are clearly differentiated from SLV/GLV and GCLV based on their CP sequence in the survey of Japanese *Allium* plants, including Welsh onion, rakkyo and *A. wakegi.* A search for any possible epitope sequences common in the respective SLV and GCLV CPs, which were predicted according to the methods of Sette et al. [18] and Rothbard and Taylor [15], was unsuccessful (data not shown). These results suggest that SjoLV may be a strain of SLV or GCLV, but sequence data of viruses identified as SjoLV on the basis of host range and symptomatology are required to confirm this suggestion.

In conclusion, cDNA cloning and sequencing of the 3′ terminal region of *Allium* carlaviruses revealed that at least 2 distinct species belonging to *Carlavirus* are present in *Allium* plants, one being represented by SLV and the other by GCLV. It was also confirmed that the virus isolate previous described as GLV is a strain of SLV.

References

1. Allison RF, Dougherty WG, Parks TD, Willis L, Johnston RE, Kelly M, Armstrong FB (1985) Biochemical analysis of the capsid protein gene and capsid protein of tobacco etch virus: N-terminal amino acids are located on the virion's surface. Virology 147: 309–316
2. Atreya CD (1992) Application of genome sequence information in potyvirus taxonomy: an overview. Arch Virol [Suppl] 5: 17–23
3. Barg E, Lesemann D-E, Vettren HJ, Green SK (1994) Identification, partial characterization, and distribution of viruses infecting *Allium* crops in south and southeast Asia. Acta Horticult 358: 251–258
4. Bos L (1982) Shallot latent virus. CMI/AAB Descriptions of Plant Viruses, No. 250
5. Bos L (1983) Viruses and virus diseases of allium species. Acta Horticult 127: 11–29
6. Brunt AA (1992) The general properties of potyviruses. Arch Virol [Suppl] 5: 3–16
7. Conci V, Nome SF, Milne RG (1992) Filamentous viruses of garlic in Argentina. Plant Dis 76: 594–596
8. Ishikawa M, Meshi T, Motoyoshi F, Takamatsu N, Okada Y (1986) In vitro mutagenesis of the putative replicase genes of tobacco mosaic virus. Nucleic Acids Res 14: 8 291–8 304
9. Jordan R, Hammond J (1991) Comparison and differentiation of potyvirus isolates and identification of strain-, virus-, subgroup-specific and potyvirus group-common epitopes using monoclonal antibodies. J Gen Virol 72: 25–36

10. Kanyuka KV, Vishmichenko VK, Levay KE, Kondrikov DY, Ryabov EV, Zavriev SK (1992) Nucleotide sequence of shallot virus X RNA reveals a 5′-proximal cistron closely related to those of potexviruses and a unique arrangement of the 3′-proximal cistrons. J Gen Virol 73: 2 553–2 560
11. Kobayashi K, Rabinowicz P, Bravo-Almonacid F, Helguera M, Conci V, Lot H, Mentaberry A (1996) Coat protein gene sequences of garlic and onion isolates of the onion yellow dwarf potyvirus (OYDV). Arch Virol 141: 2 277–2 287
12. Lee YW, Yamazaki S, Osaki T, Inouye T (1979) Two elongated viruses in garlic, garlic latent virus and garlic mosaic virus. Ann Phytopathol Soc Jpn 45: 727–734 (in Japanese)
13. Nagakubo T, Kubo M, Oeda K (1994) Nucleotide sequences of the 3′ region of two major viruses from mosaic-diseased garlic: molecular evidence of mixed infection by a potyvirus and a carlavirus. Phytopathology 84: 640–645
14. Nishiguchi M, Kikuchi S, Kiho Y, Ohno T, Meshi T, Okada Y (1985) Molecular basis of plant viral virulence; the complete nucleotide sequence of an attenuated strain of tobacco mosaic virus. Nucleic Acids Res 13: 5 585–5 590
15. Rothbard JB, Taylor WR (1988) A sequence pattern common to T cell epitopes. EMBO J 7: 93–100
16. Rupasov VV, Morozov SYu, Kanyuka KV, Zavriev SK (1989) Partial nucleotide sequence of potato virus M RNA shows similarities to potexviruses in gene arrangement and the encoded amino acid sequences. J Gen Virol 70: 1 861–1 869
17. Sako I, Nakasone W, Okada K, Ohki S, Osaki T, Inouye T (1991) Yellow streak of rakkyo (*Allium chinense* G. Don), a newly recognized disease caused by garlic latent virus and onion yellow dwarf virus. Ann Phytopathol Soc Jpn 57: 65–69
18. Sette A, Buus S, Appella E, Smith JA, Chesnut R, Miles C, Colon SM, Gray HM (1989) Prediction of major histcompatibility complex binding region of protein antigens by sequence pattern analysis. Proc Natl Acad Sci USA 86: 3 296–3 300
19. Shukla DD, Strike PM, Tracy SL, Gough KH, Ward CW (1988) The N and C termini of the coat proteins of potyviruses are surface-located and the N terminus contains the major virus-specific epitopes. J Gen Virol 69: 1 497–1 508
20. Shukla DD, Ward CW (1989) Structure of potyvirus coat proteins and its application in the taxonomy of the potyvirus group. Adv Virus Res 36: 273–314
21. Shukla DD, Ward CW (1989) Identification and classification of potyviruses on the basis of coat protein sequence data and serology. Arch Virol 106: 171–200
22. Sumi S, Tsuneyoshi T, Furutani H (1993) Novel rod-shaped viruses isolated from garlic, *Allium sativum*, possessing a unique genome organization. J Gen Virol 74: 1 879–1 885
23. Tsuneyoshi T, Sumi S (1996) Differentiation among garlic viruses in mixed infections based on RT-PCR procedures and direct tissue blotting immunoassay. Phytopathology 86: 253–259
24. Tsuneyoshi T, Ikeda Y, Sumi S (1997) Nucleotide sequences of the 3′ terminal region of onion yellow dwarf virus isolates from *Allium* plants in Japan. Virus Genes 15: 73–77
25. Tsuneyoshi T, Matsumi T, Natsuaki TN, Sumi S (1998) Nucleotide sequence analysis of virus isolates indicates the presence of three potyvirus species in *Allium* plants. Arch Virol 143: 97–113
26. Van Dijk P (1991) Mite-borne virus isolates from cultivated *Allium* species and their classification into two new rymoviruses in the family *Potyviridae*. Neth J Plant Pathol 97: 381–399
27. Van Dijk P (1993) Survey and characterization of potyviruses and their strains of *Allium* species. Neth J Plant Pathol 99: 1–48

28. Van Dijk P (1993) Carlavirus isolates from cultivated *Allium* species represent three viruses. Neth J Plant Pathol 99: 233–257
29. Van Dijk P, Sutarya R (1992) Virus survey of garlic, shallot and Welsh onion in Java, Indonesia. Internal Communication of the DLO Centre for Plant Breeding and Reproduction Research, Wageningen, The Netherlands
30. Van Dijk P, Sutarya R (1992) Virus diseases of shallot, garlic and Welsh onion in Java, Indonesia, and prospects for their control. Onion Newsletter Tropics 4: 57–61
31. Walkey DGA (1990) Virus diseases. In: Rabinowitch HD, Brewster JL (eds) Onion and allied crops, vol 2. CRC Press, Boca Raton, pp 191–211
32. Ward CW, McKern NM, Frenkel MJ, Shukla DD (1992) Sequence data as the major criterion for potyvirus classification. In: Barnett OW (ed) Potyvirus taxonomy. Springer, Wien New York, pp 283–297 (Arch Virol [Suppl]5)
33. Yamashita K, Sakai J, Hanada K (1996) Characterization of a new virus from garlic (*Allium sativum* L.), garlic mite-borne mosaic virus. Ann Phytopathol Soc Jpn: 62 483–489

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Received October 14, 1997