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Molecular characterization of *Hop mosaic virus*: its serological and molecular relationships to *Hop latent virus**

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Summary. The 3′-terminal sequence of hop mosaic virus (HpMV) genomic RNA was determined. A cDNA of approximately 1.8 kbp was amplified from the HpMV genome by 3′ RACE using a degenerate primer, which was designed to anneal to the overlapping region of open reading frames (ORFs) 2 and 3 of eight carlavirus genomes. The sequence contained three ORFs, encoding proteins of 7-, 34-, and 11-kDa, which corresponded to ORFs 4, 5, and 6 of the carlavirus genome, respectively. The amino acid sequence of ORF 5, encoding the coat protein (CP) of HpMV, shows the highest identity (67%) to that of *Hop latent virus* (HpLV). The HpMV CP N-terminal sequence differs from that of HpLV, but the central and C-terminal sequences of the CP of both viruses are similar. The sequence similarity possibly causes the cross-reaction of heterologous antibodies of HpMV and HpLV. Phylogenetic analyses based on the CP amino acid and 3′ non-coding region sequences indicate close relationships among HpMV, HpLV, and *Potato virus M*. We report here the first molecular characterization of HpMV genomic RNA.

Introduction

Hop mosaic virus (HpMV) is one of three carlaviruses that infect hop plants (*Humulus lupulus*), along with *Hop latent virus* (HpLV) and *American hop latent virus* (AHLV). The three carlaviruses are distinguished by their host ranges

*The nucleotide sequence reported in this paper has been submitted to the DDBJ/EMBL/GenBank nucleotide sequence databases under accession number AB051109.

and their symptomatology in infected plants. HpLV occurs symptomlessly in most hop cultivars, and it has no known reliable diagnostic host [6]. Likewise, AHLV infects most hop cultivars symptomlessly, except that it induces a faint ring-and-line pattern in an American seedling clone. AHLV can be assayed by necrotic lesions it induces in the inoculated leaves of *Datura stramonium* and by systemic infection in *Chenopodium quinoa* [7]. In many tolerant hop cultivars, HpMV also fails to induce conspicuous symptoms. However, sensitive Golding-type cultivars infected with HpMV show severe symptoms, such as chlorotic vein-banding, leaf distortion and stunting, and poor yield; infected plants usually die prematurely [5].

HpMV is found in Europe, Australia, North America, and China [5, 32], and most tolerant cultivars, accounting for over 90% of hop plants grown in England, were reported to be infected with HpMV [1]. In Japan, HpMV was detected in cultivars that had been introduced for breeding [18, 23]. HpMV infects *Nicotiana clevelandii* systemically without causing symptoms, and no hosts of the virus with local lesion symptoms have been found [1, 5]. However, Kanno et al. [18] reported that an HpMV isolate detected in hop cv. Sunshine showing a mosaic symptom pattern, infected only the inoculated leaves of *N. clevelandii*, without causing systemic infection. Assuming that the infectivity of HpMV in *N. clevelandii* depends on plant growth conditions or the biological properties of the HpMV isolate, HpMV is difficult to distinguish from HpLV by observation of host reactions, with the exception of those in the Golding-type hop cultivars.

Although the three carlaviruses can also be distinguished serologically, cross-reaction of both HpMV and HpLV to a heterologous antiserum has been reported [1, 3, 18]. Recently, we reported the complete sequence of the HpLV genome [13]; however, the HpMV genomic RNA has not been characterized. To compare HpLV and HpMV at the molecular level, we determined the HpMV genomic RNA 3′-terminal sequence from cDNA cloned by 3′ RACE (rapid amplification of cDNA ends) using a degenerate primer for the carlavirus genomes. The serological and molecular relationships of HpMV and other carlaviruses are discussed.

Materials and methods

Virus purification

HpMV was partially purified from an asymptomatic hop cv. Bullion, which has been maintained for breeding in the hop garden of Sapporo Breweries Ltd., Hokkaido, Japan. The hop plant was infected with HpMV, but free from HpLV, according to an enzyme-linked immunosorbent assay (ELISA) [10]. Young buds and shoots (100 g) were homogenized in 4 volumes of 0.5 M potassium phosphate buffer, pH 7.2, containing 1% (w/v) Na_2SO_3 and filtered through cheesecloth. The sap was clarified by adding Triton X-100 to 2% (v/v) and stirring for 1 h at 6 °C, followed by centrifugation at 6,000 × **g** for 15 min. The supernatant was further clarified by adding 1/3 volume carbon tetrachloride, with stirring for 3 min on ice, followed by centrifugation at 6, 000 × **g** for 15 min. HpMV was precipitated from the aqueous phase with 5% (w/v) polyethylene glycol 6,000 and 0.6% (w/v) NaCl with stirring at 6 °C for 1 h. The sediment was collected by centrifugation at 6,000 × **g** for 40 min. The pellet was resuspended in 0.05 M potassium phosphate buffer containing 1% (v/v) Triton X-100. After centrifugation at 18, 000 × **g** for 10 min, the supernatant was layered on a 20%

(w/v) sucrose cushion in 0.05 M potassium phosphate buffer, then centrifuged at 100,000 × **g** for 90 min. The virus in the pellet was further purified by a second cycle of differential centrifugation. Electron microscopy was used to monitor the presence of viral particles. Since several attempts at further purification by sucrose density gradient centrifugation were unsuccessful, the partially purified virus, after differential centrifugation, was stored at −80 °C and used for experiments in this study.

Serological tests

The serological relationships between HpMV and HpLV were examined by double-antibody sandwich (DAS) ELISA and immunoblot analysis. Rabbit antisera against HpMV and HpLV [1, 2], designated anti-HpMV-EM and anti-HpLV-EM, respectively, were kindly provided by Dr. A. N. Adams (Horticulture Research International, East Malling, U.K.). A rabbit antiserum against HpMV [18], designated anti-HpMV-IU, was kindly provided by Dr. N. Yoshikawa (Faculty of Agriculture, Iwate University, Japan). Rabbit antiserum against HpLV, designated anti-HpLV-HU, was prepared in a previous study [13]. The immunoglobulin (Ig) G purified from the anti-HpMV-IU and anti-HpLV-HU sera was coated at 5 μg/ml onto microplate wells (Nunc, Immunoplate II). HpLV purified from hop plants in a previous study [13] or the partially purified HpMV was used as an antigen in 2-fold serial dilutions, then reacted with each alkaline phosphatase-conjugated IgG in 1:600 dilutions. The purified HpLV was diluted from 10 μg/ml (2^0-dilution) to 19.5 ng/ml (2^9-dilution). Equivalent amounts of the partially purified HpMV was estimated according to the intensity of the coat protein (CP) stained with Coomassie brilliant blue (CBB) R-250 after sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The 12.5% SDS-PAGE was performed using Mini Protean™ II Dual Slab Cell (Bio-Rad) according to the manufacturer's instructions. In addition to HpMV and HpLV, purified *Potato virus S* (PVS), from the genus *Carlavirus*, and the hop isolate of *Apple mosaic virus* (ApMV), from the genus *Ilarvirus* [22], were electrophoresed as controls of an unrelated carlavirus and a virus from hop plants, respectively. For immunoblots, proteins were transferred from the gel to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore) using Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad) according to the manufacturer's instructions. The proteins on the membrane were reacted with anti-HpLV-HU, anti-HpMV-IU, anti-HpLV-EM, or anti-HpMV-EM primary antibodies at 3 μg/ml. After washing, the remaining primary antibodies were reacted with goat anti-rabbit IgG conjugated with alkaline phosphatase (Zymed) at a 1:800 dilution. Reactive protein bands were visualized using the substrate 5-bromo-4-chloro-3-indolyl phosphate in combination with nitroblue tetrazolium chloride.

3′ RACE and sequencing

HpMV genomic RNA was extracted from the partially purified viral particles as described [13]. The cDNA was synthesized by oligo (dT) priming and amplified by 3′ RACE method using a degenerate primer. To design degenerate primers for carlavirus genomes, available carlavirus sequences were aligned at both the nucleotide and amino acid levels using the program CLUSTAL W ver. 1.5 [26]. Two degenerate primers of 23 and 25 nucleotide lengths were used as sense primers in 3′ RACE. The primers oligo(dT)-AP2 [5′-CGATGGTACCTGCAGG CGCGCC$(T)_{18}$-3′] and 3NTR-AP2 [5′-CGATGGTACCTGCAGGCGCGCC-3′] were used for reverse transcription and as an antisense primer in 3′ RACE, respectively. After a 10-min denaturation at 65 °C, the viral RNA was annealed with the oligo(dT)-AP2 primer on ice for 2 min. First strand cDNA was transcribed using M-MLV reverse transcriptase (GIBCO BRL) at 42 °C for 1 h. Next, the cDNA was amplified using a degenerate primer and the 3NTR-AP2 primer with *Ex Taq* DNA polymerase (Takara Shuzo) or *Taq* DNA polymerase (Boehringer)

in a thermal cycler (Astec, Model PC-700). Program conditions were as follows: 94 °C, 1 min; 30 cycles of (98 °C, 20 sec; 68 °C, 5 min); 72 °C, 10 min.

The 3′ RACE products were phosphorylated with T4 DNA kinase (Toyobo), ligated into a dephosphorylated *Sma* I site of pUC119 or pUC118, and cloned in *Escherichia coli*, strain MV1184. Three independent clones were sequenced using an automated DNA sequencer (Li-Cor, Model 4000L) with a Thermo Sequenase™ fluorescent labeled primer cycle sequencing kit (Amersham). To determine the full sequence of the 3′ RACE clones, each HpMV cDNA was fragmented by restriction enzyme digestion and subcloned in pBlueScript II SK(-).

Sequence analysis

Sequences were analyzed using DNASIS software (Hitachi Software Engineering). For multiple sequence alignments, CLUSTAL X ver. 1.62b [27] was used with default parameters. Relationship dendrograms were calculated with 1,000 bootstrap replicates using the Neighbor-Joining option in CLUSTAL X and drawn using the program TreeView ver. 1.6.1 [21]. The DDBJ/EMBL/GenBank accession numbers of the carlavirus sequences discussed in this report are as follows: complete sequences of *Potato virus M* (PVM-R; X53062), *Blueberry scorch virus* (BBScV; L25658), *Garlic latent virus* (GarLV; Z68502), which is considered a garlic strain of *Shallot latent virus* (SLV) [29, 30], and *Hop latent virus* (HpLV; AB032469), partial sequences of *Carnation latent virus* (CLV; X55331, X55897 and AJ010697), *Chrysanthemum virus B* (CVB; S60150), *Garlic common latent virus* (GarCLV; AB004566), *Helenium virus S* (HVS; D01119 or D10454), *Lily symptomless virus* (LSV; X15343), *Poplar mosaic virus* (PopMV; D13364), *Potato virus S* (PVS-An; D00461, PVS-O; S45593), PVM-G (X57440), SLV (AB004456 and GCV-H; D11161), and whitefly-transmitted *Cowpea mild mottle virus* (CPMMV; AF024628) were used. *Potato virus X* (PVX; D00344) in the genus *Potexvirus* was used as the outgroup.

Results

Serological relationship between HpMV and HpLV

Figure 1 shows the reactivity of HpMV and HpLV particles with either anti-HpMV-IU or anti-HpLV-HU antibody in DAS-ELISA. HpMV reacted with the anti-HpMV-IU antibody, but not with the anti-HpLV-HU antibody. By contrast, the HpLV reacted strongly with the anti-HpLV-HU antibody and weakly with the anti-HpMV-IU antibody. Figure 2 shows the reactivities of denatured HpMV and HpLV CPs with antibodies in immunoblot analyses. A single protein band is visualized in SDS-PAGE from the partially purified HpMV, and the HpMV CP migrated slightly more quickly than the HpLV CP (Fig. 2a). The HpMV CP reacted well with both anti-HpMV-EM and anti-HpMV-IU antibodies (Figs. 2b and 2c), but cross-reacted weakly with the anti-HpLV-EM and strongly with the anti-HpLV-HU antibody (Figs. 2d and 2e). The HpLV CP reacted well with the anti-HpLV-EM and anti-HpLV-HU antibodies (Figs. 2d and 2e), and cross-reacted strongly with the anti-HpMV-EM and anti-HpMV-IU antibodies (Figs. 2b and 2c).

cDNA amplification and 3′ sequence of the HpMV genome

A 25 nucleotide-long degenerate primer, designated CARORF3P, was designed to anneal to the overlapping region between open reading frames (ORFs) 2 and 3

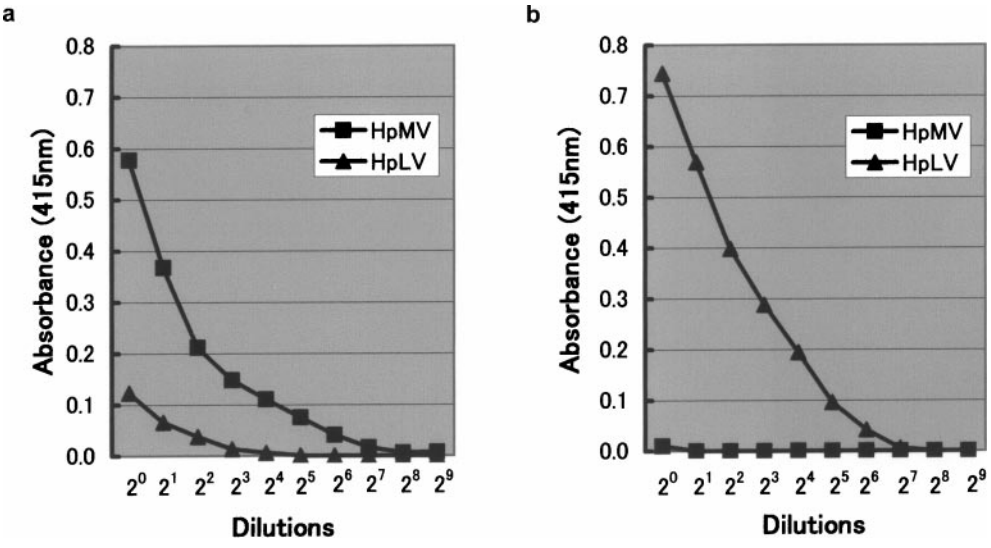

Fig. 1. Serological relationship between HpMV and HpLV in DAS-ELISA using anti-HpMV-IU (**a**) and anti-HpLV-HU (**b**) antibodies. Purified HpLV particles were used in 2-fold serial dilutions [10 μg/ml (2^0), 5 μg/ml (2^1), 2.5 μg/ml (2^2), 1.25 μg/ml (2^3), 625 ng/ml (2^4), 312.5 ng/ml (2^5), 156.3 ng/ml (2^6), 78.1 ng/ml (2^7), 39.1 ng/ml (2^8), 19.5 ng/ml (2^9)]. Estimations of equivalent amounts of partially purified HpMV particles were made according to the CBB-staining intensity of the CP after SDS-PAGE

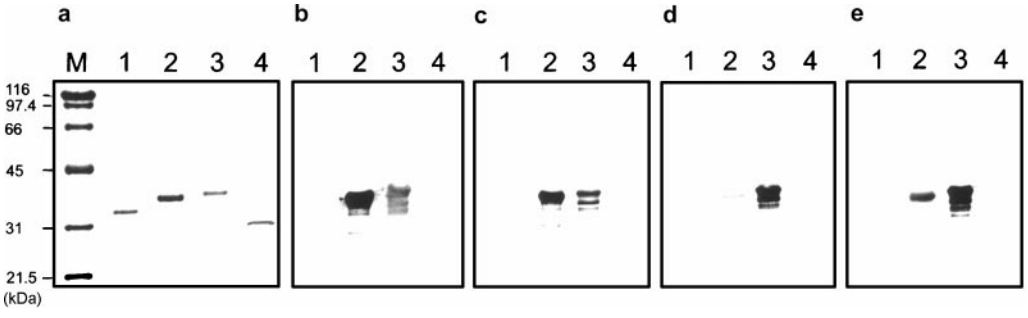

Fig. 2. Serological relationship between HpMV and HpLV CPs determined by immunoblotting. The CPs from viral particles of PVS (*1*), HpMV (*2*), HpLV (*3*), and ApMV (*4*) were subject to 12.5% SDS-PAGE, then stained with CBB R-250 (**a**) or transferred to PVDF membranes followed by reaction with anti-HpMV-EM (**b**), anti-HpMV-IU (**c**), anti-HpLV-EM (**d**), or anti-HpLV-HU (**e**) antibody. *M* Molecular weight marker (Bio-Rad)

of eight carlavirus genomes, as shown in Fig. 3a. The primer sequence contains a portion of the sequence encoding M-P-L-T/S-P/A-P-P-$(X)_2$-T/S that is conserved at the N-terminus of carlavirus ORF 3 proteins [13]. 3′ RACE using CARORF3P and 3NTR-AP2 primers amplified the expected 1.8-kbp product for two carlaviruses: PVS and HpMV (Fig. 3b). The product amplified from the HpMV genome was cloned and three independent clones were used for sequencing.

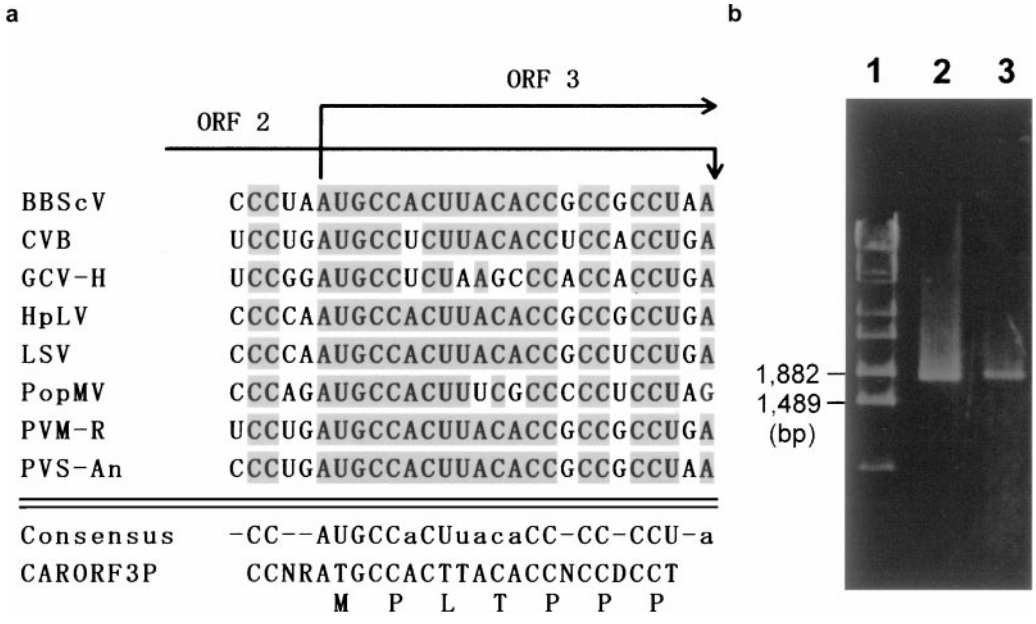

Fig. 3. cDNA amplification by 3′ RACE using a degenerate primer. **a** Design of the degenerate primer CARORF3P. The genomic sequences of eight carlaviruses were aligned using the program CLUSTAL W. Residues common to at least six genomes are shaded in gray and are indicated with uppercase (sequence common to all eight genomes) or lowercase (for six or seven genomes) letters as "Consensus". Dashes indicate non-conserved nucleotides; a mix of nucleotides [N (A, C, G and T), R (A and G), and D (A, G and T)] is arranged in the corresponding position in the CARORF3P primer. Seven amino acids encoded in ORF 3 are shown below. **b** Agarose gel (1%) analysis of 3′ RACE products from PVS (*2*) and HpMV (*3*). DNA size markers are *Sty* I-digested λ-DNA fragments (*1*)

Three subclones were generated from each 3′ RACE clone and sequenced as shown in Fig. 4. The determined sequence was 1,841 nucleotide long, excluding the poly(A) tail at the 3′ end. Four open ORFs corresponding to carlavirus ORFs 3, 4, 5, and 6 were found on the positive strand of the genome, although ORF 3 was incomplete because the 5′ sequence was replaced with the CARORF3P primer. ORF 3 probably terminates with a UAA codon at nucleotides 304–306 and encodes at least 101 amino acids (most likely 108 amino acids). The sequence H-X-L-P-[H/F]-G-G-X-Y-[R/K/Q]-D-G-T-K-X-[I/V]-X-Y-$[X]_2$-P conserved in the central region of carlavirus ORF 3 proteins [13] was found in the HpMV ORF 3 protein as H-Y-L-P-H-G-G-F-Y-R-D-G-T-K-V-I-R-Y-F-G-P. ORF 4 (nucleotides 297–503) in the HpMV genome shares a 10-nucleotide overlap with ORF 3 and potentially encodes a polypeptide of 68 amino acids with a calculated M_r of 7 k. The sequence C-X-U-X-U-X-G-$[X]_2$-&-X-&-$[X]_2$-C, where U is a bulky aliphatic residue (I, L, V, or M) and & is a bulky hydrophobic residue (that is, U plus aromatic residues F, Y, or W), conserved in the central region of carlavirus ORF 4 proteins [13], was found in the HpMV ORF 4 protein at amino acids 29–43 as C-I-V-V-L-T-G-E-S-V-R-F-Q-G-C. The amino acid sequences of

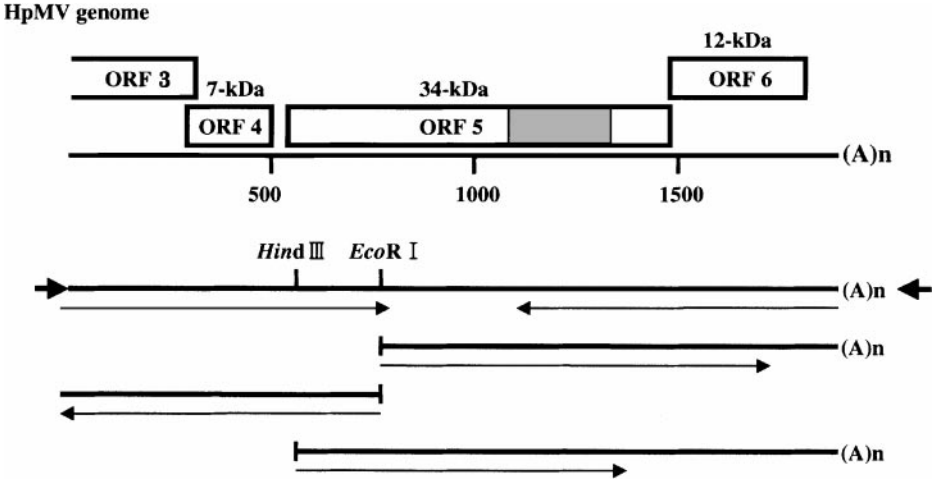

Fig. 4. Partial genomic organization of HpMV and sequence strategy for the 3′-terminal region of the HpMV genome. The 3′ RACE clone amplified using the CARORF3P and 3NTR-AP2 primers (large arrows) was digested with *Hin*d III or *Eco*R I for subcloning. Small arrows indicate the region and direction of sequencing. The gray region in ORF 5 indicates the core region [16]

ORFs 3 and 4 show hydrophobic properties, similar to their counterparts in other carlaviruses [16].

The carlavirus ORF 5 is expressed from subgenomic RNA and the sequence [C/U] UUAGGU is located in a putative subgenomic RNA promoter [20]. This motif is also present at nucleotides 488–494 in the intergenic region between ORFs 4 and 5 of the HpMV genome. ORF 5 (nucleotides 520–1,443) potentially encodes a polypeptide of 307 amino acids with a calculated M_r of 34 k, which correlates well with the 34 kDa size of the CP as estimated by SDS-PAGE [1]. The sequence [R/K]-[F/Y/W]-A-[G/A/P]-F-D-X-F-$[X]_2$-[L/V]-$[X]_3$-$[G/A/S/T]_2$, conserved in the CP of potexviruses and carlaviruses [14], except HpLV, where the underlined F is replaced with L, is found in the HpMV ORF 5 protein (CP) at amino acids 231–246 as R-F-A-A-F-D-C-F-D-Y-V-E-N-A-A-A. The sequence T-G-G-X-X-G conserved in the C-terminal regions of carlavirus CPs [13] is also present in the HpMV ORF 5 at amino acids 290–295. ORF 6 (nucleotides 1,443–1,751) shares one nucleotide with ORF 5 and potentially encodes a polypeptide of 102 amino acids with a calculated M_r of 12 k. Four cysteine residues involved in forming a zinc-binding finger and an upstream basic sequence [12] are also found in the HpMV ORF 6 protein sequence. The HpMV 3′ non-coding region (3′ NCR) consists of 90 nucleotides, excluding the poly(A) tail. A possible polyadenylation signal (AAUAAA) is found at nucleotides 1,811–1,816 of the HpMV genome as in PVM, HVS, and HpLV; this signal is absent in other carlaviruses. The hexanucleotide motif (UAUUUU) found within the 3′ NCR of carlaviruses [13] was identified at nucleotides 1831–1836 of the HpMV genome.

Table 1. Identities (%) in the sequence of the genomic regions of HpLV and other carlaviruses

	ORF 4	ORF 5 (CP)		ORF 6	3′ NCR
		total	core		
BBScV	40	46	67	36	69
CLV	38	42	52	37	47
CPMMV	33	53	67	40	51
CVB	37	46	59	47	60
GarCLV	ND	39	53	25	17
GarLV	34	41	50	38	65
HpLV	44	67	82	51	78
HVS	ND	50	63	44	61
LSV	37	48	64	36	50
PopMV	31	38	45	40	ND
PVM-R	48	64	85	47	74
PVM-G	ND	64	83	49	75
PVS-An	38	47	67	38	68
PVS-O	ND	47	67	42	66
SLV	ND	41	50	37	67

Numbers indicate the maximum identity in the overall sequence based on amino acids for the ORFs or nucleotides for the 3′ NCR. The CP core regions are divided based on Hillman and Lawrence [16]. *ND* No data

Molecular relationships of HpMV and other carlaviruses

Each genomic region of HpMV was compared with its counterpart in other carlaviruses. Their identities (%) are shown in Table 1. The amino acid sequence of the HpMV ORF 4 protein shows less than 50% identity with its counterparts. The HpMV CP shares a higher sequence identity with CPs of HpLV (67%), PVM-R (64%), and PVM-G (64%) than those of other carlaviruses. The phylogenetic analysis of carlaviruses based on the CP amino acid sequences indicates that HpMV is closely related to HpLV and PVM (Fig. 5a). Figure 5b shows the CP amino acid sequence comparison among HpMV, HpLV, and PVM. The major sequence differences between the three viruses are located at the N-terminal sequence, and the central and C-terminal sequences have high identity. Interestingly, the HpMV CP core region shares 85, 83, and 82% identities with the corresponding regions in PVM-R, PVM-G, and HpLV, respectively. The HpMV ORF 6 amino acid sequence is most similar to the HpLV ORF 6 (51%). The 3′ NCR of HpMV shares the highest sequence identity (78%) with the 3′ NCR of HpLV, 75% with that of PVM-G, and 74% with that of PVM-R. The phylogenetic analysis of carlaviruses based on the 3′ NCR sequences indicates that HpMV is most closely related to PVM rather than HpLV (Fig. 6a). The relationship between HpMV and PVM, based on 3′ NCR sequences, resembles that between strains of the same virus, as in PVS-An and PVS-O, and among GarLV, GCV-H, and SLV. Figure 6b

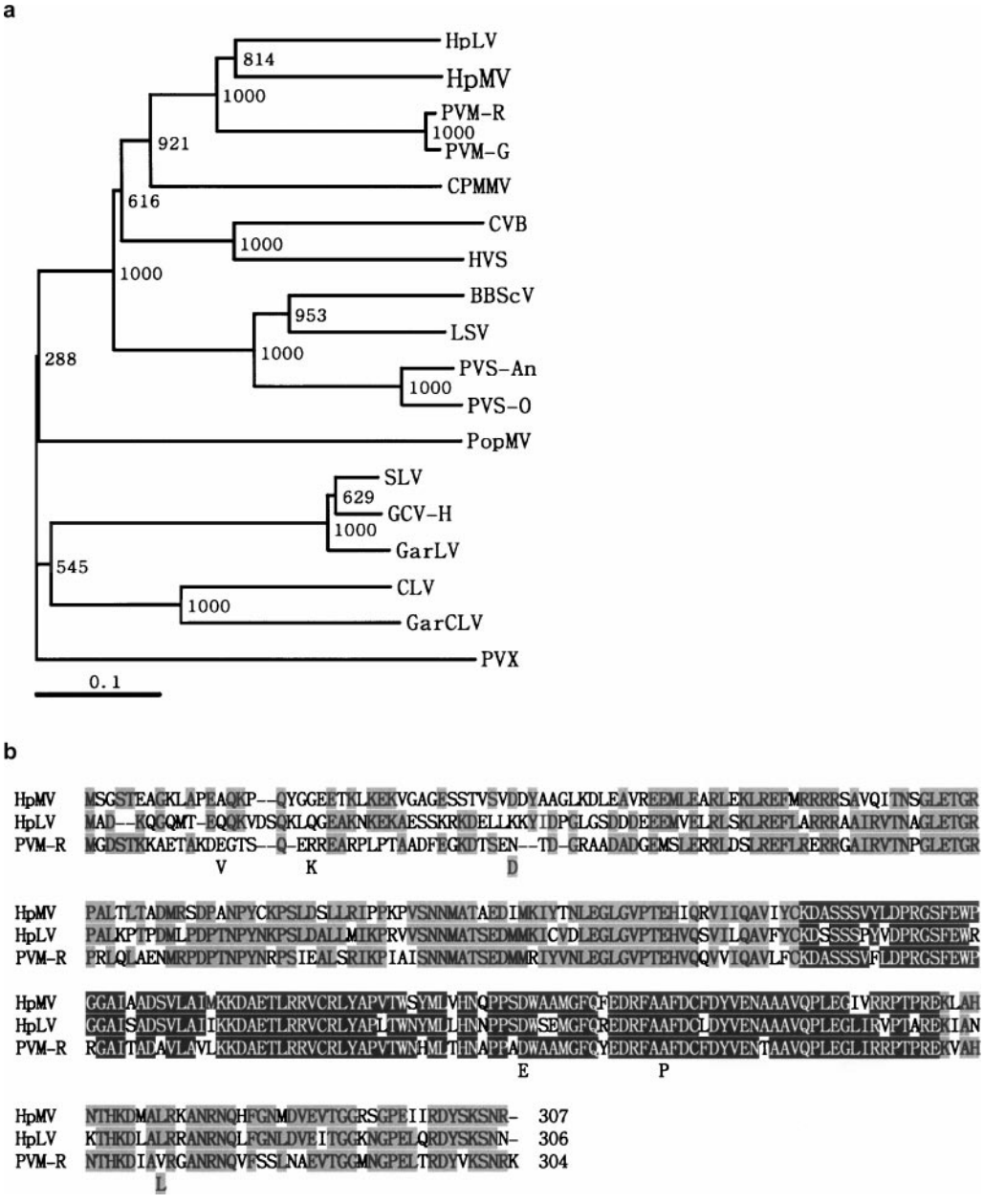

Fig. 5. Molecular relationships of HpMV and other carlaviruses based on CP amino acid sequences. **a** Phylogenetic analysis of carlaviruses based on CP amino acid sequences. Numbers adjacent to the nodes indicate bootstrap confidence values (for 1,000 replicates). PVX is used as an outgroup. **b** CP amino acid sequence comparison of HpMV, HpLV, and PVM. The six amino acids that differ between PVM-G and PVM-R are shown below PVM-R. Gaps (-) have been introduced for maximum alignment. Amino acids identical in at least two sequences are shaded in gray or are shown as white letters on a black background in the core sequence [16]

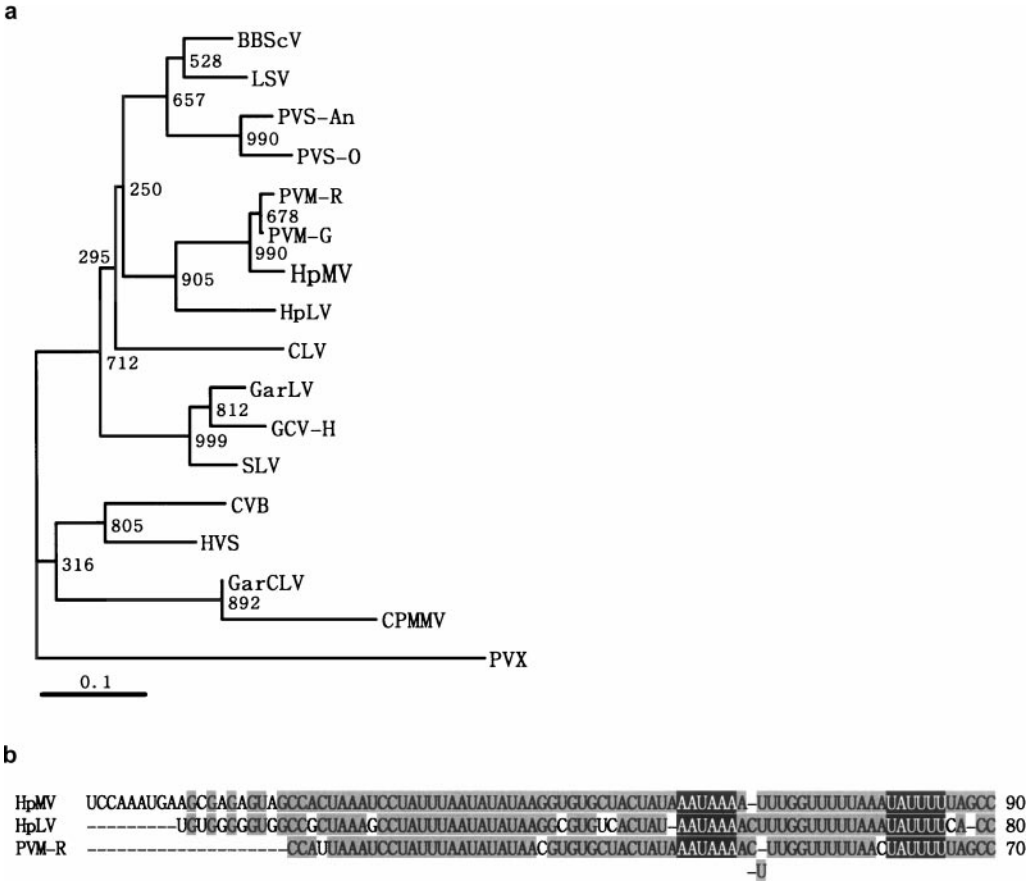

Fig. 6. Molecular relationships of HpMV and other carlaviruses based on the 3′ NCR. **a** Phylogenetic analysis of carlaviruses based on 3′ NCR nucleotide sequences. Numbers adjacent to the nodes indicate bootstrap confidence values (for 1,000 replicates). PVX is used as an outgroup. **b** Nucleotide sequence comparison of 3′ NCRs of HpMV, HpLV, and PVM. The single nucleotide differing between PVM-G and PVM-R is shown below PVM-R. Gaps (-) have been introduced for maximum alignment. Nucleotides identical in at least two sequences are shaded in gray. A possible poly(A) signal and a hexanucleotide motif conserved within the 3′ NCRs of carlaviruses are shown with white letters on a black background

compares the 3′ NCRs of HpMV, HpLV, and PVM. The carlavirus 3′ NCR sequences have diverse lengths, from 13 nucleotides in GarCLV to 118 nucleotides in CPMMV. The 3′ NCRs of HpMV, HpLV, and PVM consist of 90, 80, and 70 nucleotides, respectively. The HpMV 3′ NCR sequence resembles the PVM 3′ NCR sequence, plus a 20-nt 5′ extension. The 3′ 70 nucleotides of the HpMV 3′ NCR shares 94 and 95% identity with the 3′ NCR of PVM-R and PVM-G, respectively, and 86% with the 3′ 70 nucleotides of the HpLV 3′ NCR. The 3′ 70 nucleotides within the HpLV 3′ NCR share 82% identity with both PVM-R and PVM-G 3′ NCRs.

Discussion

HpMV isolates have been purified from systemically infected *N. clevelandii* plants in the U.K. and China [1, 32]. However, one HpMV isolate was reported to infect symptomlessly only the inoculated leaves of *N. clevelandii* and, consequently, was purified from systemically infected *N. occidentalis*, a newly-identified host plant [18]. Attempts to infect *N. clevelandii* and *N. occidentalis* with our HpMV isolate failed to result in systemic infection, possibly due to plant growth conditions. Therefore, we partially purified HpMV directly from hop plants and amplified the cDNA of the HpMV genome through to the 3′-terminal region by 3′ RACE, using a degenerate primer designed from the consensus of several carlavirus genome sequences. Badge et al. [4] reported a 3′ RACE using a carlavirus-specific primer, Carla-Uni, designed from ORF 6 and 3′ NCR of carlaviruses. The Carla-Uni primer was useful for cDNA amplification of many carlaviruses: PVS, PVM, LSV, AHLV, HpMV, and CPMMV. The Carla-Uni primer has only one nucleotide mismatch to the HpMV sequence presented here. It is unknown whether our CARORF3P primer will be useful for cDNA amplification of multiple carlavirus genomes. However, if the CARORF3P primer is able to amplify the cDNA of an unsequenced carlavirus genome, the resulting product would provide more sequence information than that using the Carla-Uni primer.

Cross-reactions of HpMV to the anti-HpLV-EM serum in tube precipitation tests, $F(ab')_2$ ELISA, and immunosorbent electron microscopy (ISEM) have been reported [1, 3, 18]. The HpMV particles did not react with the heterologous anti-HpLV-HU antibody in DAS-ELISA; however, weak cross-reaction of the HpMV CP with the anti-HpLV-EM antibody and strong cross-reaction with the anti-HpLV-HU antibody was observed (Figs. 1b, 2d, and 2e). The strong cross-reaction of the HpMV CP with the anti-HpLV-HU antibody was probably caused by a cryptotope, a continuous epitope(s) hidden within the CP in the intact virus particle that appears in the denatured CP. On the other hand, cross-reactivity of HpLV particles to the anti-HpMV antibody was observed in both DAS-ELISA and immunoblot analysis (Figs. 1a, 2b, and 2c). Cross-reaction of HpLV to the anti-HpMV-EM antibody occurred in $F(ab')_2$ ELISA [3], although HpLV did not react with antiserum against HpMV in tube precipitation tests [1], and few HpLV particles were trapped with the anti-HpMV-EM antiserum in ISEM [17]. We found that the purified HpLV produced multiple bands in the immunoblot analysis, indicating degradation of the HpLV CP (Figs. 2b–2e), perhaps due to long-term storage at $-80\,^{\circ}C$ and several freeze-thaw cycles. The N- and C-termini of carlavirus CPs appear to be located on the surface of virus particles and to be an immunodominant region, like other filamentous viruses in the genera *Potexvirus* and *Potyvirus* [9, 24]. Degradation of potyvirus particles during storage occurs from the surface-exposed N- and C-terminal regions of the CP [15, 24], and similar degradation appears to occur in carlaviruses. Amino acid sequence comparisons between HpMV and HpLV CPs illustrate the differences within the N-terminal sequences, but the central and C-terminal sequences share high

identity (Fig. 5b). Since cross-reactions occurred with both the intact and degraded CP in immunoblot analysis, the cross-reactivity of HpLV to the anti-HpMV antibody probably depends on the similarity of the central and C-terminal regions of HpMV and HpLV. We hypothesize that the epitope(s) located at the N-terminus is related to virus-specificity in intact viral particles, the epitope(s) located at the C-terminus is related to the weak cross-reactivity seen in intact virus particles, and the central region is related to the cross-reactivity of the denatured CP. The difference in cross-reactivity of HpMV to the anti-HpLV-EM and anti-HpLV-HU antibodies in immunoblotting is due to the recognition of the CP central region by the antibody and may reflect the quality of the viral preparation used as the immunogen.

In addition to cross-reacting with anti-HpLV antiserum, HpMV reacts in tube precipitation tests with antiserum against CLV, PVM, or CPMMV, but not PVS, CVB, or *Red clover vein mosaic virus* in the genus *Carlavirus* [1]. Sequence comparison of the CP does not explain the HpMV reaction with antiserum to CLV. Many carlaviruses react with CLV antiserum [19], and a number of inexplicable cross-reactions of carlaviruses to heterologous antisera have been reported [2, 8, 19]. Some of these may be related to the discontinuous epitope(s) in the conformation of the virus particles, although the cross-reactions in immunoblot analyses are still inexplicable [8]. However, the cross-reaction of HpMV with anti-PVM antiserum can be explained by the sequence similarity in the central and C-terminal regions of the CP (Fig. 5b). The results indicate that the serological relationships among HpMV, HpLV, and PVM could arise from the similarity in the central and C-terminal regions of the three viral CPs and could depend on the quality of the viral particles used as immunogens for antiserum production (i.e., intact vs. degraded).

We have reported that HpLV is closely related to PVM at the molecular level [13]. In addition, here we report that the HpMV is closely related to HpLV and PVM according to phylogenetic analyses based on the CP amino acid and 3′ NCR nucleotide sequences (Figs. 5 and 6). However, HpMV is a distinct species, based on the amino acid sequences of ORFs 4 and 6 (Table 1). The 3′ NCR of polyadenylated positive-sense RNA viruses plays an important role in RNA replication [11, 25, 28, 31]. The 3′ NCRs of the three carlaviruses containing a possible poly(A) signal have high sequence similarity, suggesting similar tertiary structures, such as a putative 3′-terminal pseudoknot structure required for the efficient replication of potexvirus RNA [28]. The similarity also suggests that the 3′-terminal sequences originated from a common sequence, such as a host gene or a common ancestral viral genome. Although no common host is known for the two hop carlaviruses and PVM, the three carlavirus possibly originated from a common ancestor. Host proteins have been reported to bind the 3′ NCR of potexvirus and probably play a role in the process of viral multiplication [25]. HpMV and HpLV most probably use similar 3′ NCR-binding hop proteins in some aspect of viral replication, and possibly evolved from a common ancestor.

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