A Novel Member of the Genus *Nepovirus* Isolated from *Cucumis melo* in Japan

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ABSTRACT

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An unusual virus was isolated from a Japanese *Cucumis melo* cv. Prince melon plant showing mild mottling of the leaves. The virus had a broad experimental host range including at least 19 plant species in five families, with most infected plants showing no symptoms on inoculated and uninoculated systemically infected leaves. The virus particles were spherical, approximately 28 nm in diameter, and the coat protein (CP)

had an apparent molecular mass of about 55 kDa. The virus possessed a bi-partite genome with two RNA species, of approximately 8,000 and 4,000 nucleotides. Both genome components for the new virus were sequenced. Amino acid sequence identities in CP between the new virus and previously characterized nepoviruses were found to be low (less than 27%); however, in phylogenetic reconstructions the closest relationship was revealed between the new virus and subgroup A nepoviruses. These results suggest that the new virus represents a novel member of the genus *Nepovirus*. A new name, Melon mild mottle virus, has been proposed for this new virus.

Cucurbits are important vegetable crops in Japan being cultivated throughout the country. Melon (*Cucumis melo* L.), widely cultivated in tropical and temperate regions of the world, is especially popular. Several cultivars of melon are bred in Japan, and are classified into four major types: green flesh netted melon, red flesh netted melon, smooth skin melon, and oriental pickling melon (19). Oriental pickling melons such as *C. melo* cv. Prince, bred by crossing *C. melo* cv. *makuwa* smooth skin and others, are widely cultivated and distributed throughout Japan and are popular because of their lower prices (10). To support a lower cost cultivation system for such melons, Japan usually imports cheap melon seedlings.

Several pathogenic agents of melons, including viral diseases, have previously been reported worldwide. These include *Cucumber mosaic virus* (CMV), *Cucumber green mottle mosaic virus* (CGMMV), *Melon necrotic spot virus* (MNSV), *Squash mosaic virus* (SMV), *Tobacco ringspot virus* (TRSV), *Tomato ringspot virus* (ToRSV), and *Watermelon mosaic virus* (WMV) (25). These viruses induce severe mosaic or necrotic symptoms on infected melon leaves and fruits, and cause major economic losses. There are no reports of cucurbit viruses that do not cause serious symptoms in a natural infection. In Japan, ToRSV is the only nepovirus reported to infect *Cucumis* plants (25). ToRSV infection induces chlorotic ringspots and spots on upper non-inoculated melon leaves, leading to the development of typical mosaic symptoms. However, these symptoms do not appear on newly emerging leaves.

During a field survey in 2008, a virus-like disease was observed on the oriental pickling melon, *C. melo* cv. Prince, in the Tottori Prefecture, Japan. Infected plants showed a mild mottling of the true leaves, which was only observed during the early growth period. Therefore, this infection did not lead to economic damage or losses in fruit production. The phenomenon suggested a

pathogenic infection, but it was distinct from previously reported viral infections of melon. To identify the cause of the infection, we first isolated the pathogen and determined its host range, molecular properties and complete genome sequence. In addition, phylogenetic analyses of genomic sequences were conducted. These analyses indicated that the virus is a novel member of the genus *Nepovirus*.

MATERIALS AND METHODS

Virus isolation and host range test. Virus isolation was carried out from a single local lesion using *Chenopodium quinoa*. *Nicotiana benthamiana* was used for virus propagation. Host range analysis was examined by mechanical inoculation with sap prepared from infected *N. benthamiana* leaves using Carborundum as an abrasive. The plants tested and symptoms observed are listed in Table 1. Inoculated plants were kept in a greenhouse at 23°C under conditions of 16 h day/8 h night. The symptoms were observed for 3 weeks, and virus infection of inoculated plants was confirmed by back-inoculation to *C. quinoa* and reverse transcription-polymerase chain reaction (RT-PCR).

Virion purification. The virus isolate was propagated in and purified from *N. benthamiana*. Inoculated leaves were homogenized in 0.1 M phosphate buffer (pH 7.0) containing 10 mM sodium sulfite as an antioxidant. The extract was filtered through double-gauze and a 30% volume of chloroform was added to the extract and mixed well. The mixture was centrifuged at 4,500 × g for 15 min at 4°C and the supernatant centrifuged at 140,000 × g for 120 min at 4°C. The precipitate was resuspended in 0.05 M phosphate buffer (pH 7.0) and further clarified by 10 to 40% (wt/wt) linear sucrose density gradient centrifugation at 140,000 × g for 180 min at 4°C. Fractionated virus particles were resuspended in 0.05 M phosphate buffer (pH 7.0).

Electron microscopy. Purified virus particles were negatively stained with 2% phosphotungstic acid solution and observed under a transmission electron microscope (JEOL, Tokyo, Japan). Average diameter of virus particles was calculated from 20 particles.

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Analysis of viral coat protein and RNAs. Virus particle proteins were analyzed by subjecting purified virus to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and staining of the separated bands with Coomassie Brilliant Blue. Amino acid sequence of the N-terminus of coat protein (CP) was determined using the procise 494 HT protein sequencing system by Takara Bio Inc. (Shiga, Japan). Viral RNAs were extracted from purified virus using the RNeasy plant RNA isolation kit (QIAGEN, Hilden, Germany). Viral RNAs were loaded on 0.1 M MOPS agarose gels containing formamide, which were stained with ethidium bromide. RNA from *Tobacco mosaic virus* OM isolate was used as a size marker.

RT-PCR. Because the melon virus was suspected to be related to nepoviruses of subgroup A or B based on the analysis of viral morphology, CP, and RNAs, the nucleotide sequences of RNA1 and RNA2 were aligned and degenerate primers (Table 2) were designed from each conserved domain. For RNA1, A1F2/A1R2, A1F3/A1R3, A1F1/oligo dT, A1F17/A1R5, and A1F9/A1R4 primer sets were used for amplification. A1R8 primer was used for 5' rapid amplification of cDNA ends (RACE) analysis. For RNA2, A2F1/oligo dT and A2F2/A2R1 primer sets were used for amplification. A2R6 primer was used for 5' RACE analysis. Nucleotide sequences of the amplicons were determined using additional virus-specific primers (Table 2). Primers used for

amplification and sequencing are listed in Table 2. First-strand cDNA synthesis was performed using ReverTra Ace reverse transcriptase (TOYOBO, Tokyo, Japan). After RT for 60 min at 42°C, PCR was carried out with KOD plus polymerase (TOYOBO) and the following conditions: 96°C for 2 min followed by 30 cycles of 30 s at 96°C, 30 s at 45 or 50°C, and 3 min at 68°C, with a 10-min extension at 68°C. PCR products were separated by 1% agarose gel electrophoresis. Expected cDNA fragments were extracted from the gels using the QIAquick Gel Extraction Kit (QIAGEN). To detect the virus in host range tests, we designed the viral-specific primer A1F7 (5'-GACTTCCACATTATGCGGCT-3'). A1F7 and oligo dT primers were used for RT-PCR to detect this new melon virus. This sequence is located near the 3' end of RNA1 (nucleotide [nt] 6974 to 6993).

Sequencing. Nucleotide sequences of extracted cDNA fragments were determined by a primer walking method using BigDye Terminator v1.1 Cycle Sequencing Ready Reaction Kit and Applied Biosystems Genetic Analyzer DNA model 3100 (Applied Biosystems, Foster City, CA). Assembly of sequence fragments was performed with BIOEDIT version 5.0.9 (7) or ATGC version 4.0 (Genetyx Corporation) programs. 5'-terminal sequences of the virus genome were determined using 5'-RACE system for rapid amplification of cDNA ends version 2.0 (Invitrogen, Carlsbad, CA), according to the manufacturer's instruc-

TABLE 1. Host range and symptomatology of the virus

Plant			Symptoms ^a	
Family	Species	Cultivar	Inoculated leaves	Upper leaves
Amaranthaceae	<i>Gomphrena globosa</i>		NS	+
Chenopodiaceae	<i>Chenopodium amaranticolor</i>		NS	NS
	<i>Chenopodium quinoa</i>		NS	Ma, M
	<i>Spinacia oleracea</i>	Atlas	+	+
	<i>Spinacia oleracea</i>	Titan	+	+
	<i>Spinacia oleracea</i>	Mahoroba	+	+
Cucurbitaceae	<i>Benincasa hispida</i>	Daimaru	+	—
	<i>Cucumis melo</i>	Arnest shunzhu	+	+
		Arlus knight shunzhu	+	+
		Arlus seinu	+	+
		Benechia harutachi	+	+
		Einea	+	+
		Miyabi	+	+
		Mone	+	+
		Prince	+	Mo*
	<i>Cucumis melo</i> var. <i>conomon</i>	Sanuki shirouri	—	—
	<i>Cucumis melo</i> var. <i>makuwa</i>	Kintaro	+	Mo*
	<i>Cucumis sativa</i>	Akimidori	—	—
	<i>Cucurbita pepo</i>	Ebisu	—	—
		Bochan	—	—
		Kabuki	—	—
		New Kodama	—	—
		Shimabeni	—	—
		Ibuki	—	—
		Not known	—	—
		Don-K	—	—
		Sakigake	—	—
		Zhukanme daimaru	—	—
Fabaceae	<i>Phaseolus vulgaris</i>	Kentucky wonder	—	—
	<i>Vicia fava</i>	Ryosei issun	—	—
	<i>Vigna unguiculata</i>	Akatane sanshaku	CS	CS*
Solanaceae	<i>Datura stramonium</i>		+	—
	<i>Nicotiana benthamiana</i>		CS	Mo*
	<i>N. clevelandii</i>		+	+
	<i>N. glutinosa</i>		+	+
	<i>N. tabacum</i>	Samsun	+	+
	<i>N. tabacum</i>	Xanthi-nc	+	+
	<i>Petunia hybrida</i>		+	+
	<i>Solanum lycopersicum</i>	Rutgers	+	+
	<i>Solanum lycopersicum</i>	Momotaro	+	+

^a Symptoms on inoculated and upper leaves. At least three plants were inoculated. CS, chlorotic spot; M, mosaic; Ma, malformation; Mo, mottling; NS, necrotic spot; +, latent infection; —, no infection; and *, symptom recovery of newly emerged leaves on inoculated plants.

tions. The complete nucleotide sequences of the virus RNA1 and RNA2 were deposited in GenBank (accession numbers AB518485 and AB518486, respectively).

Identities and phylogenetic analyses. Percentage of identities between the deduced amino acid sequence of the new melon virus and that of other *Nepovirus* species were calculated using GENETYX version 10 (Genetyx Corporation). Multiple sequence alignment was performed using the CLUSTALX program (9). Gaps were removed by BIOEDIT version 5.0.9 (7). The genetic relationships of the viruses were investigated by the maximum-likelihood (ML) algorithm of TREEPUZZLE version 5.0 (22,23). In this analysis, 1,000 puzzling steps were calculated using the Dayhoff matrix (1). Inferred gaps in the multiple alignments were treated as missing data. The calculated trees were displayed using TREEVIEW (18).

RESULTS AND DISCUSSION

A virus-like infection occurred suddenly on *C. melo* cv. Prince at Tottori Prefecture, Japan in 2008 (Fig. 1A). Although the plant showed mild mottling on young systemic leaves during the early growth stage just after transplantation into the fields (Fig. 1B), these symptoms disappeared quickly, resulting in no economic damage or loss of melon production. Based on these symptoms, it was suspected that a virus was the causal agent. Therefore, sap from original melon leaves was used to inoculate 2-week-old *C. quinoa* plants. As necrotic lesions appeared on inoculated *C. quinoa* leaves, single lesion isolation was subsequently carried out a total of three times. Pathogens established as isolates were

then propagated in *N. benthamiana* for use in subsequent experiments. To fulfill Koch's postulates, the isolated pathogen was inoculated to *C. melo* cv. Prince cotyledons. Two weeks after inoculation, three to four true leaves showed slight mottling that was identical to the symptoms in the original infected plant. Following back inoculation, the isolated virus was later identified by RT-PCR and thus confirmed as a pathogen of this disease. In addition, *C. quinoa* and *C. melo* cv. Prince were inoculated with a purified preparation of this new melon virus which also induced the original symptoms. Thus, Koch's postulates were fulfilled.

To estimate the host range of the virus, 26 plant species from five families were used, as shown in Table 1. The virus mainly infected plants of the family *Cucurbitaceae*, and the infection was symptomless in most of them. Only *C. melo* cv. Prince and *C. melo* var. *makuwa* cv. Kintaro showed mottle symptom on systemic leaves, but these symptoms did not appear on newly emerged leaves after several weeks. *C. quinoa* and *C. amaranticolor* were susceptible to the virus, showing clear necrotic spots on inoculated and upper noninoculated leaves. Viral infection was established in *Vigna unguiculata* but not *Phaseolus vulgaris* or *V. fava*. Infection of most *Solanaceae* plants was also symptomless on inoculated and upper noninoculated leaves (Table 1). Mottling symptoms were observed on leaves of some infected melons.

To determine the morphology of the virus particles, viral fractions were subjected to electron microscopy. Numerous spherical virus-like particles were observed with an average diameter of approximately 28 nm (Fig. 2A). Both empty and intact particles were observed in purified fractions, while these were not seen in extracts derived from healthy melon plants. To analyze the

TABLE 2. Primers used for amplification and sequencing of genome of the new melon virus in this study

Genome	Primer name	Sequence (5' to 3')	Nucleotide position	Polarity
RNA1	Oligo dT	TTTTTTTTTTTTTTTTT	PolyA tail	Antisense
	A1F2	TACCKSCWCTTGTGCRGGA	427–446	Sense
	A1R2	ATAAAATTGAYRCCATTYWAAG	650–671	Antisense
	A1F3	AAGMRAGCWATAGGTGAAC	2095–2114	Sense
	A1R3	TTCCAGAYTGTGAAGCACCAAA	2485–2507	Antisense
	A1F1	CGTGTACGGAGATGACAATCT	5676–5696	Sense
	A1F4	CTTGTCATGTCAGAGCGAA	5917–5936	Sense
	A1F5	TTATATTGGAGCTTGACAGC	6474–6494	Sense
	A1F6	CAAGTTTGGCGCTACTGCTAA	6818–6839	Sense
	A1F7	GACTTCCACATTATGCGGCT	6974–6993	Sense
	A1F8	GAGTTACTAACCCGATGAGC	7419–7438	Sense
	A1R4	TATCAAGAGGCGCTAGAACCT	5876–5896	Antisense
	A1F9	GATTAGTGGTGTTCCTCGACG	2340–2360	Sense
	A1F10	AATTTGCCAAGGGATTTCGAT	2557–2576	Sense
	A1F11	CTCACCTATTGTCGTTACCAC	2760–2780	Sense
	A1F12	GTTGCAGACAATGGAAGATCC	3629–3648	Sense
	A1F13	GTAAGTTTCAGAGCGCATTCG	4196–4215	Sense
	A1F14	TTGCCAGTGGATCAGACACC	4573–4592	Sense
	A1F15	GTTGCTATCAATGGTGCGGA	4900–4919	Sense
	A1F16	TCACGTAGTGGTTGGACAT	5304–5324	Sense
	A1F17	CATCTGGGTGTAAGGCAATG	488–507	Sense
	A1R5	GAGAGTATCGGCAATATTGCC	2140–2160	Antisense
	A1R6	ATTACAGACAAGACGTGAGAT	1577–1597	Antisense
	A1R7	CTTACATGCCATGGTCTCAGA	961–981	Antisense
	A1R8	CATTGCCTTACACCCAGATG	488–507	Antisense
RNA2	Oligo dT	TTTTTTTTTTTTTTTTT	PolyA tail	Antisense
	A2F1	GGTTYRNTTTYATGG	3440–3457	Sense
	A2F2	GAAYTNCCDTGGAGTACCC	611–630	Sense
	A2R1	GCTGCTCATCGGGTTAGTAA	3549–3568	Antisense
	A2F3	CAACACTCATTGGGAGTTAC	3533–3552	Sense
	A2R2	ACTCAGTCAAAGCCTTGCTGGTA	3340–3362	Antisense
	A2R3	ATCCCGTGGTGAATTCTATGG	3213–3233	Antisense
	A2R4	GGATCCTTACTACGTAACGG	2837–2856	Antisense
	A2R5	GCTGCTGCAAGAACTGAGTA	2440–2459	Antisense
	A2F4	AATCGCAATGTGGCCCTGAC	917–936	Sense
	A2F5	AAGTCATTGGGTGTTGGCAC	1267–1287	Sense
	A2F6	CACGATTCAGCCTATAGAGA	1651–1670	Sense
	A2F7	AACGGGGTCTGTATCTAGG	1848–1867	Sense
	A2R6	ACGACTCGGCCTGCTCAGA	827–845	Antisense
	A2R7	TCTCCTCACGAGGAAGATCG	325–344	Antisense

molecular mass of the viral CP, purified viral particles were subjected to SDS-PAGE analysis. A major protein band migrated at approximately 55-kDa (Fig. 2B). To investigate the cleavage site between movement protein (MP) and CP, amino acid sequence of the N-terminus of CP was analyzed and found to be Ala-Asp-Cys-Pro-Ile. Nucleic acids extracted from the purified virus were completely digested by RNase A (WAKO, Osaka, Japan), but not DNase I (QIAGEN) treatment, indicating that they were represented by a single-stranded RNA genome. Nucleic acids were loaded on a MOPS agarose gel containing formamide, and two species (RNA1 and RNA2) were observed, approximately 8,000 and 4,000 nucleotides in length, respectively (Fig. 2C).

The particle morphology, number and size of the genomic RNA molecules, and the size of CP suggested that it resembled species from the genus *Nepovirus*, family *Secoviridae* (21). Particles of bipartite members of the family *Secoviridae* sediment

as three components in sucrose gradient, components M and B contain a single molecule of RNA1 and RNA2, respectively, and component T contains empty particles (3). The virus particles in the present study formed three adjacent bands in a sucrose density-gradient centrifugation, indicating that the virus is composed of the same components T, M, and B. Comoviruses and fabaviruses have two coat polypeptides (about 40 and 20 kDa), whereas nepoviruses have a single coat polypeptide species (about 55 kDa) (3,8,17). Thus, the particle properties of the new melon virus resembled those of *Nepovirus* species.

To address this possibility, the complete nucleotide sequences of RNA1 and RNA2 were determined. Schematic genome organization revealed is shown in Figure 3. RNA1 is 7,727 nts long, excluding poly (A) tail. RNA1 encodes a single open reading frame (ORF) that starts at the 5' AUG (nt 112 to 114) and terminates at UAG (nt 7054 to 7056). The polypeptide encoded

Fig. 1. Typical symptoms on *Cucumis melo* cv. Prince observed in field on A, leaves and B, tip of infected plant.

Fig. 2. A, Electron micrograph of purified viral particles. Scale bar, 50 nm. B, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of coat protein. Lanes 1 and 2 indicate molecular marker and coat protein of melon virus, respectively. C, Electrophoresis of genomic RNA of isolate. Lanes 1 and 2 indicate genomic RNA species of *Tobacco mosaic virus* OM and melon virus, respectively.

by this ORF has a molecular mass of 252,145-Da. The 5' and 3' untranslated regions are 111 and 671 nts long, respectively. BLAST analysis of the amino acid sequence showed that the virus was definitely related to nepovirus sequences. The ORF contains consensus motifs I to VIII conserved in the RNA dependent RNA polymerases of positive-strand RNA viruses (11) (Fig. 3), and motifs A to C of the NTP-binding helicase (HEL) of superfamily 3 of positive-strand RNA viruses (6,11). In addition, it encodes the catalytic triad of H, E/D, and C conserved in cysteine proteases (C-Pro) (2,5,15) (Fig. 3). A leucine, at amino acid position 1412 of the deduced polyprotein of RNA1, was found in the substrate binding pocket of protease as described for nepoviruses of subgroup A or B (15). The viral genome-linked protein (VPg) is thought to be located between the HEL and C-Pro domains, by analogy with comoviruses and nepoviruses, although we were unable to find the nepovirus and comovirus VPg consensus sequence reported by Mayo and Fritsch (16).

RNA2 is 3,854 nts long excluding its poly (A) tail. RNA2 encodes an ORF that starts at AUG (nt 122 to 124) and stops at UGA (nt 3,482 to 3,484). The polypeptide encoded by the ORF has molecular mass of 122,184-Da. The 5' and 3' untranslated regions are 121 and 370 nts long, respectively. BLAST analysis of the nucleotide and amino acid sequences of RNA2 showed them to be related to nepoviruses. The cleavage site between MP and CP was defined as M/A based on amino acid sequencing of the N-terminus of the CP (Fig. 3). Protease from subgroup C nepoviruses has a histidine as a conserved amino acid in the substrate binding pocket, whereas subgroup A and B nepoviruses and the new melon virus have a leucine in the substrate-binding pocket of their proteases (21 and this study). The proteases of subgroup A and B nepoviruses cleave a variety of cleavage sites including K/A, K/S, R/G, R/A, C/A, C/S, or G/E. Interestingly, the M/A cleavage site observed at the junction between MP and CP in the new melon virus is a new cleavage site. The deduced molecular mass of the CP was 58,360-Da, which is close to the apparent molecular mass determined by the SDS-PAGE analysis. The conserved nepovirus CP motif (13,14) was located in RNA2, including the FYGR motif in the C-terminus. In addition, the ORF also contained the LPL motif in the putative MP domain (12). Nepovirus subgroup C has a long 3' UTR (approximately 1.4 kb) (3,4,20) in comparison with those of subgroups A and B, which are less than 1 kb in length. The 3' UTR of the melon virus RNA1 is 671 nts long, while that of RNA2 is 370 nts long. The genome organization and a set of conserved motifs of the new melon virus confirmed that the virus is a member of the genus *Nepovirus*, subgroup A or B.

Viral species of the family *Secoviridae* are classified according to identities of CP and Pro-Pol, defined as the sequence comprised between the conserved protease CG motif and the conserved polymerase GDD motif sequences (3,21). We therefore compared amino acid sequences between the virus and other *Nepovirus* species (Table 3). Pro-Pol region amino acid sequence identities were remarkably low when compared with subgroups A to C with ranges of 31.3 to 52.2%, 42.7 to 45.4%, and 39.4 to 47.0%, respectively. The percentages of amino acid sequence identity in the CP gene were also remarkably low, with ranges of 25.3 to 26.1%, 21.8 to 26.9%, and 22.1 to 25.8% for subgroups A, B, and C, respectively (Table 3). Deduced amino acid sequences from the entire polyprotein from RNA1 and RNA2 were compared with those of other nepoviruses and again found to have low levels of identities: 15.2 to 35.7% for RNA1 and 14.3 to 23.1% for RNA2. Criteria demarcating species in the genus *Nepovirus* are less than 75% sequence identity in the deduced amino acid sequence of CP and less than 80% in the deduced amino acid sequence of the Pro-Pol domain (21). Our results

TABLE 3. Percentage of sequence identity between the deduced amino acid sequence of various regions of the genome of the new melon virus and those of other nepovirus species

Virus	RNA1 ^a	RNA2	Pro-Pol	CP
Subgroup A				
ArMV	35.3	15.7	49.9	25.3
GDeV	— ^b	15.9	—	25.9
GFLV	35.7	16.8	52.2	26.1
RpRSV	25.2	19.6	31.3	N.A. ^c
TRSV	32.1	26.1	45.4	27.4
Subgroup B				
BRSV	26.0	20.5	42.7	23.0
CNSV	26.3	20.1	45.4	21.8
GCMV	22.7	23.1	43.7	26.9
OLRSV	—	16.4	—	25.4
TBRV	27.0	20.9	44.6	23.7
Subgroup C				
BRV	27.9	23.1	47.0	25.8
BLMoV	—	23.0	—	23.7
CLRV	—	21.5	—	23.5
PRMV	15.2	—	39.4	—
ToRSV	23.8	14.3	43.3	22.1
AYRSV	—	19.1	—	22.2

^a RNA1 and RNA2 indicate the RNA1- and RNA2-encoded polyprotein, respectively. Pro-Pol is the region located at protease and polymerase gene (21).
^b Amino acid sequence of the isolates not shown.
^c Unavailable amino acid sequences as cleavage site is unknown.

Fig. 3. Schematic genome organization of virus. Protein cleavage site between movement protein and coat protein was shown by sequencing of amino acid of N-terminus of coat protein. Gray zones indicate conserved motifs observed in positive-strand RNA viruses. Numbers indicate position of amino acid sequence calculated from N-terminus of viral open reading frame.

suggest that the new virus is a distinct species in the genus *Nepovirus*. Phylogenetic analyses of Pro-Pol and CP regions were conducted using the ML method (Fig. 4). The virus was grouped with subgroup A nepoviruses in both phylogenies, but distinct from other members reported previously.

Some nepoviruses are transmitted by nematodes of the genera *Longidorus*, *Paralongidorus*, and *Xiphinema* (24). Although we attempted to detect these nematodes in soil collected from melon fields where virus-infected melon plants were found, we were unable to do so. It is also known that nepoviruses are widely spread by pollen and seed transmission (3). Therefore, viral transmission by these modes should be investigated for the virus in the present study. We conclusively demonstrated that the virus could

not infect *Lagenaria siceraria* var. *hispida* used as rootstock of *C. melo* cv. Prince in the field, perhaps suggesting that it is spread by seed transmission rather than nematode vectors in the field.

In conclusion, biological and molecular characterization of the virus suggests that it is a new species in the genus *Nepovirus* (subgroup A). We propose the name Melon mild mottle virus (MMMoV) for the new virus.

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Fig. 4. Maximum likelihood phylogenetic tree based on amino acid sequence of polyprotein of **A**, RNA1 and **B**, RNA2, **C**, Pro-Pol region, and **D**, coat protein of nepovirus species. Horizontal branch length indicates 0.1 replacements per site. Node numbers indicate percentage of supporting puzzling steps. Subgroups of isolates referred from ICTV eighth report (3). GenBank accession numbers, RNA1 and RNA2, and abbreviation used for each virus are as follows: *Arabidopsis mosaic virus* (ArMV, AY303786, AY017339), *Grapevine deformation virus* (GDeV, not shown, AY291208), *Grapevine fan leaf virus* (GFLV, NC_003615, NC_003623), *Raspberry ringspot virus* (RpRSV, AY303787, AY303788), *Tobacco ringspot virus* (TRSV, U50869, NC_005096), *Beet ringspot virus* (BRSV, NC_003693, NC_003694), *Cycas necrotic stunt virus* (CNSV, NC_003791, NC_003792), *Grapevine chrome mosaic virus* (GCMV, NC_003622, NC_003621), *Olive latent ringspot virus* (OLRSV, not shown, AJ277435), *Tomato black ring virus* (TBRV, NC_004439, NC_004440), *Black current reversion virus* (BRV, NC_003509, NC_003502), *Blueberry leaf mottle virus* (BLMoV, U20622, U20621), *Cherry leaf roll virus* (CLRV, not shown, AAB27443), *Peach rosette mosaic virus* (PRMV, AF016626, not shown), *Tomato ringspot virus* (ToRSV, NC_003840, NC_003839), and *Artichoke yellow ringspot virus* (AYRSV, not shown, ABM65096).

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