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BRIEF REPORT

Detection of pepper mild mottle virus in pepper sauce in China

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Abstract Pepper mild mottle virus (PMMoV) was detected by RT-PCR in all 42 pepper sauce samples from the 10 main manufacturing provinces in China at concentrations ranging from 3.8 to 8.8 (Log_{10} copies/mL). Their coat protein nucleotide sequences had 97.4 to 100 % identity to each other and 92.4 to 100 % to other published isolates. The samples remained infectious to *N. benthamiana*, indicating that commercial trade in sauce could contribute to the natural spread of PMMoV.

Keywords Pepper mild mottle virus · Pepper sauce · Tobacco mosaic virus · Tomato mosaic virus · Tobacco mild green mosaic virus · Cucumber mosaic virus

Pepper mild mottle virus (PMMoV) belongs to the genus *Tobamovirus* and has a positive-strand RNA genome encoding four proteins: a 126-kDa viral replicase, a 183-kDa readthrough protein, a movement protein (MP) and a coat protein (CP) [1, 2]. The virus infects various solanaceous plants worldwide, and there have been several recent reports that it is universally present in human feces and can be used as an indicator of human fecal pollution of water [5–8]. Food products containing pepper or spices are thought to be common sources for viral ingestion. In one study PMMoV was detected in 12 out of 21 pepper- or spice-containing food products, and the virus in these food products had remained infectious to plants [4]. Pepper sauce, processed from fresh pepper and containing a high concentration of salt (NaCl), is a very common and favorite seasoning in China, but it has not previously been examined for the presence of PMMoV. We now report the results of tests on 42 pepper sauce samples from the 10 main manufacturing provinces in China.

The pepper sauce samples were made locally between November 2013 and September 2014 in Jiangxi, Hunan, Guizhou, Xinjiang, Yunnan, Hubei, Sichuan, Guangxi, Jiangsu and Shandong provinces. We first examined the samples for viral particles by negative staining electron microscopy as described previously [4]. Nearly all samples contained rod-shaped particles 300-400 nm long, characteristic of tobamoviruses (Fig. 1A). Next, reverse-transcription PCR was used to detect PMMoV. For analysis, total RNAs from a 2-mL sample were extracted and purified with EASYspin (Aidlab) according to the manufacturer's instructions. Total RNAs were then reverse transcribed to cDNAs in a reaction mixture containing 10 pmol of reverse primer M4T (5′-GTTTTCCCAGTCACGAC$(T)_{15}$-3′) [3], 40 U of RNase inhibitor (Takara), 15 U of reverse transcriptase (Takara) and 4 μL 10 mol dNTP (Takara). The first-strand cDNA was then used as template for PCR with 10 pmol of forward primer (PMMoV-CP f: 5′-ATGGCTTACACAGTTTCCAGT-3′) and reverse primer (PMMoV-CP r: 5′-CTAAGGAGTTGTAGCCCAGGTG-3′) and 2.5 U of Hot ExTaq DNA polymerase (Takara) under the following conditions: pre-denaturation at 94 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C

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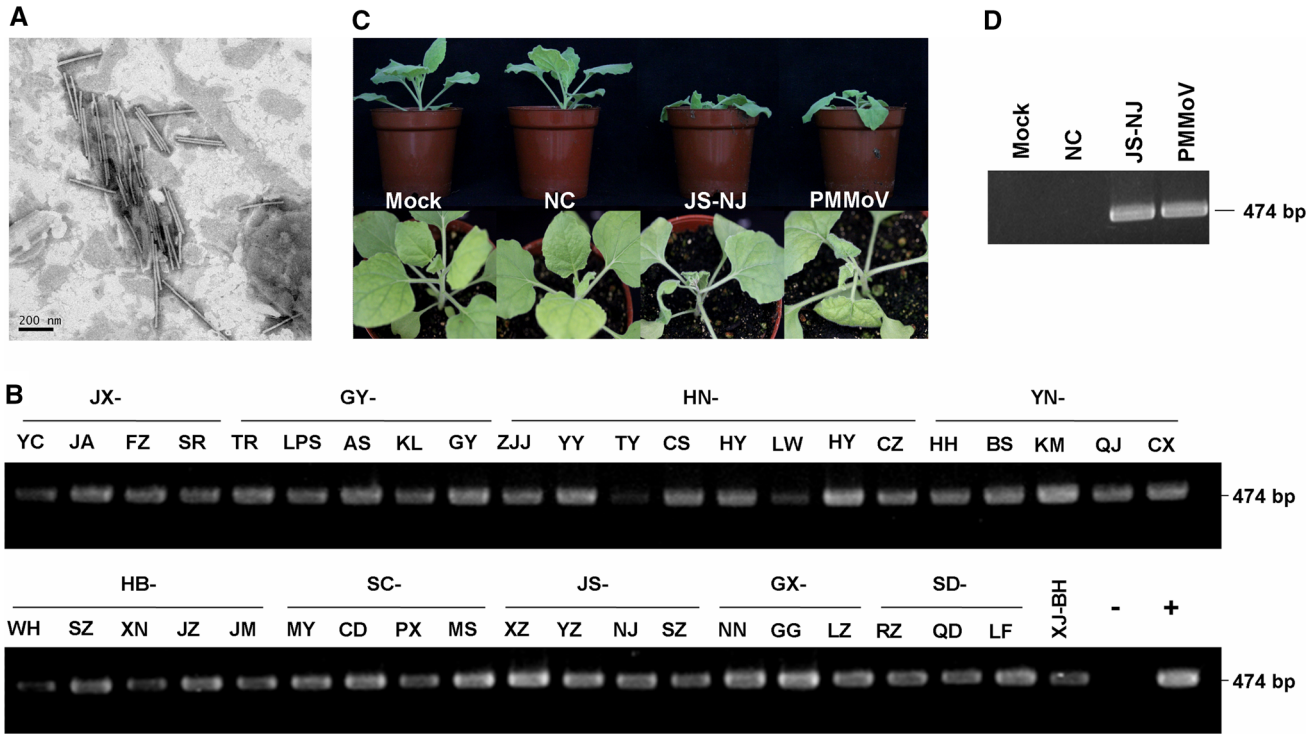

Fig. 1 Detection of PMMoV in pepper sauce samples and the infectivity of PMMoV in the JS-NJ sample to *N. benthamiana* plants. A, rod-shaped particles observed by electron microscopy. B, the predicted 474-bp fragment amplified from all 42 samples. C, at 7 dpi, wilt and stem necrosis on plants inoculated with JS-NJ and the positive control, but not on mock-inoculated and negative control (NC) plants. The upper panel shows a side view of the plants, while the lower panel shows an enlarged top view. D, confirmation of systemic infection by PMMoV on the treated and positive control plants by RT-PCR

for 30 s. The products were isolated on a 1.5 % agarose gel.

The predicted 474-bp fragment of the PMMoV genome was amplified from all 42 samples (Fig. 1B). RT-PCR products were purified using a QIAquick Gel Extraction Kit Kit (QIAGEN) and ligated into pGEM-T Easy Vector (Promega) for sequencing. Sequencing results showed that the sequences of these fragments (accession numbers: KP877405-KP877446) had >98 % identity to PMMoV sequences (accession numbers: AY859497.1 and AB000709.2), demonstrating that PMMoV was present in all samples. Using specific primers, the concentration of PMMoV in the pepper sauce samples was determined by real-time PCR according to a previously published method [4]. Concentrations of PMMoV in pepper sauces ranged from 3.83 to 8.75 (Log_{10} copies/mL) (Table 1). These data are similar to those from US sauce and vegetable samples (nearly 10^7 PMMoV RNA copies/mL) [4]. The relatively high concentration of PMMoV in the sample from GZ-TR manufactured on Nov. 22, 2013, shows that the virus had been stable for at least 13 months (Table 1).

Each sauce sample used here was made with pepper harvested locally. To examine viral variability, the full-length CP sequence of PMMoV was determined from each sample (a minimum of five replicate colonies per sample) and used for phylogenetic analysis. These nucleotide sequences had 97.4 to 100 % identity to each other and 92.4 to 100.0 % identity to other PMMoV sequences in GenBank (data not shown).

To determine whether PMMoV in these samples remained infectious, we collected 2 mL of pepper sauce from the JS-NJ sample and mixed it with 1 mL of PBS. After centrifugation at 13,000*g* for 20 min at 4 °C, the supernatant was collected and used to inoculate healthy *Nicotiana benthamiana* leaves. PMMoV-free pepper sauce was used as a negative control. At 7 days after inoculation, wilting and stem necrosis occurred on the inoculated plant and on the positive control inoculated with PMMoV purified directly from an infected plant, whereas the two negative controls showed no obvious symptoms (Fig. 1C). RT-PCR analysis of the samples confirmed that the plants inoculated with the JS-NJ sample and the positive control were systemically infected with PMMoV, while the mock-inoculated plant and the plant inoculated with a virus-free pepper sauce tested negative. This demonstrated that the PMMoV in the sauce had remained infectious to

Table 1 Detection of viruses in 42 pepper sauce samples

No.	Sample		Virus					Manufacturing date	Concentration of PMMoV[a]
			PMMoV	TMV	TMGMV	ToMV	CMV		
1	Jiangxi	JX-YC	+		+			Aug. 11, 2014	5.14 ± 0.0427
2		JX-JA	+		+			May. 6, 2014	6.95 ± 0.0046
3		JX-FZ	+		+		+	Aug. 10, 2014	6.05 ± 0.0165
4		JX-SR	+		+			Aug. 12, 2014	7.49 ± 0.0710
5	Guizhou	GZ-TR	+	+	+		+	Nov. 22, 2013	7.39 ± 0.0135
6		GZ-LPS	+			+		Aug. 11, 2014	6.83 ± 0.1705
7		GZ-AS	+		+			Jul. 16, 2014	7.80 ± 0.0303
8		GZ-KL	+		+			Jul. 27, 2014	7.25 ± 0.0120
9		GZ-GY	+					Aug. 28, 2014	5.36 ± 0.0645
10	Hunan	HN-ZJJ	+					Jul. 13, 2014	6.78 ± 0.1003
11		HN-YY	+		+			May. 6, 2014	5.49 ± 0.1737
12		HN-CS	+		+			Sep. 2, 2014	6.73 ± 0.0919
13		HN-HY	+	+	+		+	Jul. 12, 2014	5.69 ± 0.0361
14		HN-TY	+					Aug. 11, 2014	4.23 ± 0.1646
15		HN-XT	+					Aug. 10, 2014	3.83 ± 0.7193
16		HN-LW	+				+	Jul. 21, 2014	4.12 ± 0.0974
17		HN-CZ	+	+	+			Aug. 12, 2014	5.64 ± 0.0410
18	Yunnan	YN-HH	+	+	+	+	+	Aug. 10, 2014	8.71 ± 0.1090
19		YN-BS	+	+		+	+	Aug. 10, 2014	5.45 ± 0.0504
20		YN-KM	+					Aug. 27, 2014	6.51 ± 0.0401
21		YN-CX	+				+	May. 15, 2014	4.69 ± 0.1554
22		YN-QJ	+	+	+			Sep. 1, 2014	6.12 ± 0.0414
23	Hubei	HB-WH	+					Aug. 11, 2014	7.93 ± 0.0472
24		HB-SZ	+					Aug. 11, 2014	7.73 ± 0.0657
25		HB-XN	+					Aug. 12, 2014	5.95 ± 0.1251
26		HB-JZ	+	+	+	+	+	Apr. 18, 2014	7.89 ± 0.3019
27		HB-JM	+					Feb. 15, 2014	5.08 ± 0.0462
28	Sichuan	SC-MY	+	+	+			Aug. 14, 2014	8.36 ± 0.0261
29		SC-CD	+		+		+	May. 19, 2014	6.44 ± 0.0392
30		SC-PX	+		+			Sep. 2, 2014	7.66 ± 0.0694
31		SC-MS	+					Aug. 20, 2014	4.95 ± 0.0639
32	Jiangsu	JS-XZ	+		+			Jul. 26, 2014	7.86 ± 0.0549
33		JS-YZ	+	+	+	+	+	Aug. 5, 2014	8.75 ± 0.0652
34		JS-NJ	+					Aug. 13, 2014	7.02 ± 0.0076
35		JS-SZ	+		+			Aug. 11, 2014	5.35 ± 0.1439
36	Guangxi	GX-NN	+		+		+	Aug. 1, 2014	5.42 ± 0.0481
37		GX-GG	+		+	+	+	Aug. 15, 2014	4.22 ± 0.2293
38		GX-LZ	+		+			Sep. 2, 2014	6.43 ± 0.3833
39	Shandong	SD-RZ	+		+	+		Aug. 2, 2014	6.47 ± 0.0215
40		SD-QD	+		+	+		Aug. 5, 2014	6.53 ± 0.0195
41		SD-LF	+	+	+		+	Aug. 13, 2014	5.20 ± 0.3833
42	Xinjiang	XJ-BH	+					Aug. 11, 2014	4.65 ± 0.0905
Detection rate			100 %	23.81 %	61.90 %	19.05 %	30.95 %		

[a] The concentration of PMMoV as Log_{10} copies/mL (mean ± SD)

plants (Fig. 1D). These results indicate that commercial trade in sauce could contribute to the natural spread of PMMoV.

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