Short Communication

CHARACTERIZATION OF A SUBGROUP II ISOLATE OF *CUCUMBER MOSAIC VIRUS* FROM BITTER GOURD IN CHINA

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SUMMARY

Diseased bitter gourd plants with severe leaf rolling symptoms were observed in Sichuan province (southwest China). Leaf dips prepared from symptomatic leaves contained particles *ca.* 30 nm in diameter. The virus recovered from all samples was identified as *Cucumber mosaic virus* (CMV) by Western blotting, RT-PCR and sequencing of amplicons. All obtained sequences were identical, and the virus isolate was designated SC-J1. The complete nucleotide sequence of the coat protein (CP) encoding gene (GenBank accession No. KT932936) of the isolate shared 99% identity with the corresponding regions of strain Hnt (CMV II). Phylogenetic analysis based on the nucleic acid sequence of the CP shows that SC-J1 belongs to CMV subgroup II. To our knowledge, this is the first report of CMV II infecting bitter gourd in China.

Keywords: bitter gourd, electron microscopy, Western blot, RT-PCR.

Bitter gourd (*Momordica charantia* L.), a vine grown for its bitter and edible fruit, is generally believed to be native to the Indian subcontinent, but was introduced into China in the 14th century. Bitter gourd has been widely planted in China, but is mainly concentrated in South China in the provinces of Guangdong, Guangxi, Hainan, and Sichuan. The total planting area of bitter gourd in Sichuan was about 9000 ha in 2013, and was mainly concentrated in the Chengdu, Shuangliu, and Pengshan regions. However, replication or spread of various kinds of pathogens contribute to great yield losses every year (Wang *et al.*, 2008; Jiang *et al.*, 2010).

Cucumber mosaic virus (CMV, genus *Cucumovirus*, family *Bromoviridae*), is one of the first plant viruses described (Jagger, 1916), and is transmitted by at least 75 species of aphids in the nonpersistent manner (Palukaitis *et al.*, 1992).

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Fig. 1. Symptoms of leaf curling caused by *Cucumber mosaic virus* subgroup II on bitter gourd. (a) healthy bitter gourd; (b) diseased bitter gourd.

Diseases associated with CMV have been reported for more than 1200 species distributed worldwide (Roossinck, 2001). The genome of CMV contains five open reading frames (ORFs) on three genomic RNAs. The 1a and 2a ORFs are the viral components of the replicase. The 2b ORF, which partially overlaps the 2a ORF, encodes a suppressor of posttranscriptional gene silencing (Brigneti *et al.*, 1998; Ding *et al.*, 1994). The 3a ORF codes for the movement protein and the 3b ORF codes for the virus coat protein (CP).

In the summer of 2014, a visual survey of plant viral symptoms on cucurbit crops was conducted in Sichuan (southwest China). Bitter gourd plants with severe leaf-rolling symptoms were found at several sites (Fig. 1). Fifteen leaf samples were collected, of which ten showed rolling symptoms, and five were asymptomatic. The leaves were cut out with a sterile scalpel, frozen immediately in liquid nitrogen in the field and stored at –80°C.

Total protein extraction was done from leaf samples. Protein concentrations were determined by the Bradford method (Bradford, 1976), and approximately 3 µg of protein from each sample was subjected to Western blotting tests using CMV (Xi *et al.*, 2007), *Tobacco mosaic virus* (TMV) and *Chilli veinal mottle virus* (ChiVMV) antibodies (Agdia,

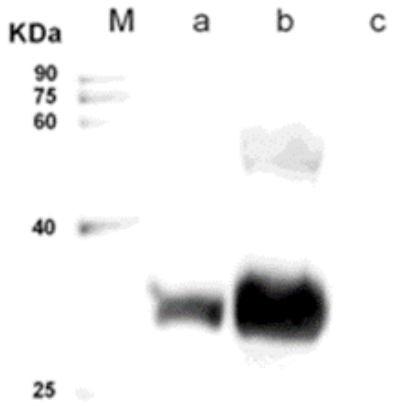

Fig. 2. Western blot analysis of virus preparations obtained from the collected plant samples, using anti-CMV sera. (a) diseased bitter gourd; (b) positive control; (c) healthy bitter gourd as negative control; (M) molecular weight-markers.

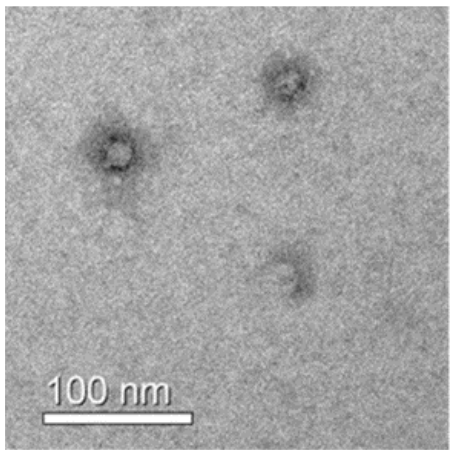

Fig. 3. Electron micrograph of viral particles from the sap of diseased bitter gourd leaves. Bar = 100 nm.

Elkhart, IN; Yunnan Academy of Agricultural Science, respectively). Bitter gourd plants displaying severe leaf-rolling symptoms contained a large amount of CMV-CP, but neither TMV nor ChiVMV (Fig. 2). Scanning electron microscopy of dips prepared from leaf samples revealed the virus particles to be about 30 nm in diameter (Fig. 3).

Identification of the virus was confirmed by molecular methods. Total RNA was extracted from 50 mg fresh diseased leaf samples using a Plant Total RNA Isolation Kit (FOREGENE Biotechnology Co., Ltd, China). The first-strand cDNA was synthesized using MMLV-reverse transcriptase (Promega, USA), following the protocol provided by the supplier, and CMV-R (5'-CTGGATGGACAACCCGTTC-3') as the initial primer. Reverse transcription polymerase chain reaction (RT-PCR) was performed and the complete CP gene and partial 3'-UTR of CMV was amplified with a pair of degenerate primers CMV-F (5'-ATGGACAAATCTGRATCWMCC-3') and CMV-R (Xi *et al*., 2006). PCR was performed in a total volume of 50 µl containing 3 µl cDNA solution, 5 µl 10 × PCR buffer, 4 µl dNTPs mixture (2.5 mM), 3 µl $MgCl_2$ (25 mM),

Fig. 4. A maximum-likelihood method for phylogenetic tree of SC-J1 and CMV isolates base on the nucleotide sequence of CP. ER-PSV (U15730) as an outgroup.

0.5 µl each primers (10 µM) and 0.5 µl of 5 U/µl Taq DNA polymerase (Takara, Japan). The PCR protocol was: 94°C for 3 min, followed by 35 cycles of 94°C for 45 s, 54°C for 45 s, 72°C for 70 s, and a final extension step at 72°C for 10 min. Electrophoresis in a 1% agarose gel showed only a single band (788 bp). No visible band was detected from non-infected control plants.

PCR products were purified with a Gel Extraction Kit (Omega Biotek, USA) after electrophoresis in a 1.5% agarose gel, cloned into the pMD-19T vector (Takara, Japan), transformed into competent cells of *Escherichia coli* strain DH5α according to the manufacturer's instructions, and sequenced (Shanghai, China). The sequences of all amplicons obtained from 10 samples were aligned using the DNAMAN 6 software. All clones of the amplicon were identical, and a single contiguous sequence for this isolate (SC-J1) was submitted to the GenBank (accession No. KT932936). Sequence similarity searches were performed using the BLAST program from the NCBI database (http://www.ncbi.nlm.nih.gov/). Nucleotide BLAST analysis revealed that SC-J1 had the highest nucleotide sequence identity (99%) to the CP gene of CMV isolate Hnt (KC407999).

Phylogenetic relationships were determined by the MEGA software package version 6.06 (Edgar *et al*., 2004; Tamura *et al*., 2013). Trees were constructed using 1000 bootstrap replicates. The virus strains and GenBank accession numbers used in this study are listed in Table 1.

Table 1. Subgroups, accession numbers, country of origin and host plant species of CMV isolates.

Isolate	Subgroup	Geographical origin	Host	Accession
ToCMV5-2	IA	Japan	*Nicotiana tabacum*	AB448692.1
PoCMV7-5	IA	Syria	*Solanum tuberosum*	AB448694.1
PoCMV9-11C	IA	Syria	*Solanum lycopersicum*	AB448696.1
Jalgon	IA	India	*Musa* sp. (cultivar 'Grandnaine')	EU531469.1
KZN_BM4	IA	South Africa	*Cucurbita pepo* L.	KJ789895.1
Gera-EG	IA	Egypt	*Pelargonium* sp. (geranium)	JQ013954.1
21Tahaa	IB	French Polynesia	*Momordica charantia*	FN554692.1
Gerbera Zing.	IB	India	*Gerbera jamesonii* cv. 'Zingaro'	JX913531.1
akil-p-1	IB	India	*Pisum sativum* (pea)	EU140547.1
BQ6	IB	China	*Eleocharis dulcis*	KF268463.1
HB1	IB	China	*Capsicum annuum*	FJ788413.1
PF	II	Japan	*Solanum lycopersicum*	AB368501.1
Mb	II	China	*Musa basjoo*	GU002300.1
Cynthia 2-12	II	United States	*Hosta* sp.	JX898520.1
D-1	II	Poland	*Dahlia cultorum* Thorsr.	DQ018292.1
Hnt	II	China	*Nicotiana tabacum*	KC407999.1
Tsh	II	China	*Solanum lycopersicum*	EF202597.1
Del	II	Poland	*Delphinium* sp.	EU191025.1
C2	II	Iran	*Spinacia oleracea* L.	KC763473.1
Cn2	II	Poland	*Cucurbita pepo* cv. *Atena Polka F1*	HM480051.1
232Mrg	II	Japan	*Cnidium officinale*	AB086848.1
Ld	II	Iran	*Lepidium draba*	EF050074.1
CMVHF4	II	China	*Nandina domestica*	KP735939.1
PaFM	II	Korea	*Capsicum annuum* var. *grossum*	AB109908.1

Previous work (Roossinck *et al*., 1999) with the sequence of the CP ORF, and rearrangements in the 5' non-translated region (NTR) of RNA 3, divided CMV isolates into three subgroups, IA, IB and II, that displayed as much as 25% divergence at the nucleotide level. Phylogenetic analyses of the nucleotide sequence of SC-J1 characterized in this study and 29 isolates available in GenBank indicated three lineages (Table 1, Fig. 4). The sequence of SC-J1 clustered into the CMV subgroup II clade.

In this study, we identified the virus (CMV II) collected from diseased bitter gourd plants in Chengdu, China. The diseased bitter gourd plants were scattered throughout the fields and observed to be infested with green peach aphid (*Myzus persicae*, Sulzer). However, multiple infections by plant viruses are common, and complex interactions of the viruses in mixed infections result in unpredictable disease phenotypes in infected plants (Hammond *et al*., 1999). Although we failed to detect TMV and ChiVMV, there might be multiple infections with another virus naturally involved in development of these especially severe disease symptoms. Recently, we detected a PRSV type P (*Papaya ringspot virus* type P, genus *Potyvirus*, family *Potyviridae*) isolate derived from the original diseased plants (unpublished), which may be involved in the expression of these unusually severe symptoms. Most recently, CMV II has been identified in several kinds of vegetables, showing severe symptoms and yield loss (data not shown). According to previous research results, CMV I, rather than CMV II, has been widely reported in China. To our knowledge, CMV subgroup II was reported only from lily, tobacco, tomato, banana, passion fruit, *Tagetes erecta*, heavenly bamboo and Chinese mallow (*Malva verticillata*) in China (Palukaitis *et al*., 1992; Peng *et al*., 2015); this is the first report of CMV II infecting cucurbits in China. The current situation of CMV II outbreak in Sichuan is therefore troubling and better management of seed, vector and greenhouse sanitation is necessary to control the disease.

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