Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology)
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Monoclonal antibody-based serological methods for maize chlorotic mottle virus detection in China*

Jian-xiang WU[§], Qiang WANG[§], Huan LIU, Ya-juan QIAN, Yan XIE[†‡], Xue-ping ZHOU[†‡]

(*State Key Laboratory of Rice Biology, Institute of Biotechnology, Zhejiang University, Hangzhou 310058, China*)

[†]E-mail: xiey@zju.edu.cn; zzhou@zju.edu.cn

Received Oct. 10, 2012; Revision accepted Mar. 5, 2013; Crosschecked June 22, 2013

Abstract: Maize chlorotic mottle virus (MCMV) infects maize plants and causes significant losses in corn production worldwide. In this study, purified MCMV particles were used as the immunogen to produce monoclonal antibodies (MAbs) and polyclonal antibodies (PAbs). Four murine MAbs (4B8, 8C11, 6F4, and 9G1) against MCMV were obtained through the hybridoma technology. The triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA), dot-immunobinding assay (DIBA), and immunocapture reverse transcription-polymerase chain reaction (IC-RT-PCR) using the MAb 4B8 were then developed for sensitive, specific, and rapid detection of MCMV in fields. MCMV could be detected in infected leaf crude extracts at dilutions of 1:327 680, 1:64 000, and 1:3 276 800 (w/v, g/ml) by TAS-ELISA, DIBA, and IC-RT-PCR, respectively. One hundred and sixty-one maize field samples showing virus-like symptoms and sixty-nine symptomless maize field samples from ten different provinces of China were collected and screened for the presence of MCMV using the established serological methods. A phylogenetic tree was constructed based on the full length *CP* genes and Chinese MCMV isolates formed one branch with Thailand isolates. The detection results demonstrated that MCMV is one of most prevalent viruses infecting maize in the Yunnan and Sichuan provinces of China.

Key words: Maize chlorotic mottle virus (MCMV), Immunocapture reverse transcription-polymerase chain reaction (IC-RT-PCR), Triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA), Monoclonal antibody (MAb), Dot-immunobinding assay (DIBA)

doi:10.1631/jzus.B1200275 **Document code:** A **CLC number:** S432

1 Introduction

Maize chlorotic mottle virus (MCMV) is the only identified member of the genus *Machlomovirus* in the family Tombusviridae (King *et al.*, 2011) and it is most closely related to members of the genus *Carmovirus* (Nutter *et al.*, 1989). MCMV was first described in maize from Peru in 1974 (Castillo and Hebert, 1974) and thereafter was reported on maize plants in the United States and Mexico (Niblett and Clafin, 1978; Carrera-Martínez *et al.*, 1989). In China, it was reported first in Yunnan province in 2011 (Xie *et al.*, 2011). MCMV has an icosahedral particle with 30 nm in diameter, which is composed of a single 25 kDa capsid protein subunit encapsidating 4.4 kb single-stranded positive-sense genomic RNA (Nutter *et al.*, 1989; Lommel *et al.*, 1991b). Translation of the MCMV genome by a reticulocyte system results in polypeptides of 105, 52, 44, 41, 32, and 25 kDa. A sub-genomic RNA of 1090 nt was identified as the mRNA for the 25 kDa coat protein (CP) (Lommel *et al.*, 1991a).

The host range for MCMV is limited to members of the Gramineae family. Typical symptoms of MCMV include mild mosaic, severe stunting, leaf

‡ Corresponding authors
§ The two authors contributed equally to this work

* Project supported by the National Natural Science Foundation of China (No. 31272015), the Ministry of Education of China (No. 313052), and the Zhejiang Provincial Natural Science Foundation of China (No. Z3090039)

© Zhejiang University and Springer-Verlag Berlin Heidelberg 2013

necrosis, premature plant death, shortened male inflorescences with few spikes, and shortened, malformed, partially filled ears (Castillo and Hebert, 1974; Niblett and Clafin, 1978; Nault *et al.*, 1981; Uyemoto *et al.*, 1981). MCMV often induces corn lethal necrosis (CLN) resulting from synergistic interaction between this virus and maize dwarf mosaic virus (MDMV) (Niblett and Clafin, 1978; Goldberg and Brakke, 1987), wheat streak mosaic virus (WSMV) (Scheets, 1998), or sugarcane mosaic potyvirus (SCMV) (Uyemoto *et al.*, 1980), leading to serious yield losses in corn (Uyemoto, 1983; Scheets, 1998; Morales *et al.*, 1999; Xie *et al.*, 2011). Adults of six species of chrysomelid beetle were reported to transmit MCMV under experimental conditions (Nault *et al.*, 1978), and seed transmission was also reported for the virus (Jensen *et al.*, 1991; Zhang *et al.*, 2011). Recently, Dr. Bressan found thrips transmitting MCMV in Hawaii, USA (personal communication, Department of Plant and Environmental Protection Sciences, University of Hawaii, Honolulu, HI, USA).

At present, several methods have been developed for detection of MCMV (Uyemoto, 1983; Morales *et al.*, 1999; Stenger *et al.*, 2007; Stenger and French, 2008; Zhang *et al.*, 2011). Among these detection methods, serological methods are more frequently used to detect large numbers of samples in field surveys. However, the detection results of serological methods rely on the quality of antibodies. In this study, three monoclonal antibody (MAb)-based serological methods, triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA), immunocapture reverse transcription-polymerase chain reaction (IC-RT-PCR), and dot-immunobinding assay (DIBA) were developed for sensitive and specific detection of MCMV.

2 Materials and methods

2.1 Viruses, kit, and field maize samples

The MCMV Yunnan isolate was previously collected (Xie *et al.*, 2011). SCMV, southern rice black-streaked dwarf virus (SRBSDV), and rice black-streaked dwarf virus (RBSDV) were characterized and maintained in the authors' laboratory and used to determine the specificity of antibodies and establish the detection methods. MCMV was maintained and propagated on maize plants by sap mechanical inoculation in an insect-proof greenhouse. The viruses in the infected maize leaves were purified as described (Xie *et al.*, 2011). Double antibody sandwich ELISA (DAS-ELISA) kits for MDMV, WSMV, and SCMV detection were obtained from Agdia (Elkhart, IN, USA).

From 2010 to 2012, 161 field maize leaf samples showing virus-like symptoms and 69 symptomless field samples were collected from the Yunnan, Sichuan, Guizhou, Guangxi, Shandong, Heilongjiang, Liaoning, Zhejiang, Henan, and Hebei provinces of China. The collected samples were stored in a refrigerator.

2.2 Preparation of polyclonal antibodies (PAbs) and monoclonal antibodies (MAbs) against MCMV

Purified MCMV virions were used as the immunogen and PAbs against MCMV were prepared in New Zealand rabbits according to the previously described method (Wu *et al.*, 2009). The immunized rabbit was bled a week after the 5th immunization, and the antisera were used as the capture antibodies in TAS-ELISA for MCMV detection.

Four BALB/c mice were immunized with purified MCMV virions and hybridomas secreting MAbs to MCMV and MAbs were prepared according to the previously described method (Wu *et al.*, 2011). Titer determination, isotyping, specificity analyses of MAbs, and the purification of IgG were carried out according to the previously described method (Shang *et al.*, 2011).

2.3 Western blot

Western blot was conducted according to the method of Wu *et al.* (2007). MCMV-infected and healthy maize tissues were analyzed using the MAbs as the probe in the Western blot.

2.4 ELISA

MCMV in purified preparations or in crude extracts of infected plant tissues was detected according to the procedures of antigen-coated plate-ELISA (ACP-ELISA) (Jiang and Zhou, 2002) and TAS-ELISA (Shang *et al.*, 2011). Negative and positive control wells were added with crude extracts from healthy and MCMV-infected maize tissues,

respectively. Samples were considered to be positive when the absorbance value was at least three times greater than that of the negative controls. The detection results of ACP-ELISA and TAS-ELISA were further examined by RT-PCR described by Xie *et al.* (2011).

2.5 DIBA

DIBA was carried out according to the method of Shang *et al*. (2011) with a slight modification. Briefly, maize samples were ground to a fine powder with a mortar and pestle in liquid nitrogen and homogenized further in 0.01 mol/L phosphate buffered saline (PBS; 1 g leaf tissue in 10 ml PBS). The tissue homogenates were centrifuged at 8000×*g* for 5 min. The clarified crude extracts were spotted onto nitrocellulose membranes (2 μl/spot) and air-dried at room temperature. The dried membrane was blocked with 5% skimmed milk (0.05 g/ml) for 30–60 min, and then incubated in suitably diluted MAb for 1 h. After washing four times with 0.01 mol/L PBS containing 0.05% Tween-20 (PBST), the membrane was incubated in a suitable dilution of goat anti-mouse IgG conjugated with alkaline phosphatase (Sigma-Aldrich, St. Louis, MO, USA) for 1 h. Finally, the membrane was washed five times with PBST, and then incubated to be color-developed in alkaline phosphatase substrate buffer containing nitro-blue tetrazolium chloride/5-bromo-4-chloro-3′-indolyphosphate *p*-toluidine salt (NBT/BCIP; Promega, Madison, WI, USA).

2.6 IC-RT-PCR

Based on the completely conserved part of three MCMV genomic sequences (JQ982468, JQ982469, JQ982470) of Chinese isolates in GenBank, a forward primer (5′-ACAGGACACCGTTGCCGTTTAT-3′, corresponding to 3301–3322 nt) and a reverse primer (5′-CGATTTAGGCTCCCAGACACTT-3′, corresponding to 4187–4166 nt) were designed for amplifying the full-length *CP* gene of the MCMV by IC-RT-PCR (Wu *et al.*, 2011).

2.7 Sequencing and sequence analysis

PCR products were cloned in a T-vector and then sequenced. All nucleotide sequences were aligned and their nucleotide identities were analyzed with the Clustal W method of the MegAlign procedure supplement within the DNASTAR package (version 7.0, DNAStar Inc., Madison, WI, USA). A dendrogram of the full-length *CP* gene of the MCMV was established with the neighbour-joining method in Mega software version 5.05.

3 Results

3.1 Virus purification and production of MAbs and PAbs against MCMV

To produce MAbs and PAbs of MCMV, purified MCMV particles were obtained by differential centrifugation. Isometric virions about 30 nm in diameter were observed by a transmission electron microscopy in purified preparation (data not shown).

Among four mice immunized with purified MCMV particles, two mice showed high titer of antibodies to MCMV (above 1:500000), and these two mice were used to prepare hybridomas. In two cell fusion experiments, each well of the culture plate contained at least one hybridoma clone. Cell culture supernatants were screened for the presence of antibodies against MCMV by an indirect-ELISA, and 123 culture plate wells were positive. Hybridomas secreting strong positive antibodies were cloned by the limiting dilution method and four hybridoma cell lines (4B8, 8C11, 6F4, and 9G1) secreting anti-MCMV antibodies were obtained. Hybridoma cells were injected intraperitoneally into mineral oil-primed BALB/c mice to produce the ascites and MAbs were purified from the ascitic fluid using a protein-G affinity column. Isotypes and subclasses of 4B8, 8C11, and 6F4 belonged to IgG1, κ light chain, while 9G1 belonged to IgG2b, κ light chain. The titer of MAbs in ascitic fluid ranged from 10^{-6} to 10^{-7} by an indirect-ELISA (Table 1). The IgG yield in ascites fluids ranged from 3.11 to 10.33 mg/ml (Table 1).

Table 1 Properties of MAbs to MCMV

MAb	Isotypes	Ascites titer	IgG yield in ascites (mg/ml)
4B8	IgG1, κ chain	10^{-7*}	3.11
8C11	IgG1, κ chain	10^{-6}	4.69
6F4	IgG1, κ chain	10^{-7}	4.08
9G1	IgG2b, κ chain	10^{-7}	10.33

* The MAb titer was the last dilution that yielded an absorption value three times more than that of the negative control

The ACP-ELISA was used to analyze the specificity of MAb. The analytic results demonstrated that all MAbs reacted strongly with the crude extract of the MCMV-infected maize tissues including all Yunnan and Sichuan isolates. However, all the four MAbs did not react with healthy maize tissues and the other three maize viruses (SRBSDV, RBSDV, and SCMV), which are known to occur in maize plants in China (Fang *et al.*, 2001; Jiang and Zhou, 2002; Zhou *et al.*, 2008; Yin *et al.*, 2011) (Fig. 1). These results indicate that all MAbs were very specific for MCMV. The specificity of the MAb was further analyzed by Western blot, and the results demonstrated that all four MAbs reacted strongly with the MCMV CP, while no positive signals were observed with the healthy plant tissue used as the negative control (Fig. 2).

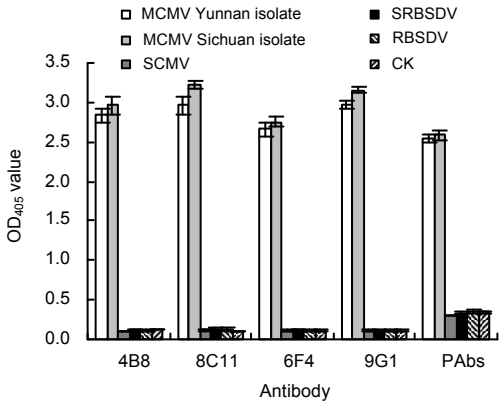

Fig. 1 Specificity analyses of MAbs and PAbs by ACP-ELISA

Samples were added at a 1:30 dilution (w/v) and probed with 1:5000 diluted MAbs or PAbs followed by alkaline phosphatase-labeled goat anti-mouse or anti-rabbit antibodies. The absorbance value was the mean value (±standard deviation (SD)) obtained from three independent assays at 30 min after adding the substrate at room temperature

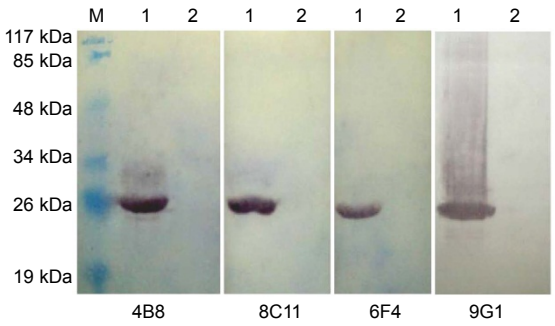

Fig. 2 Western blot analysis of MCMV coat protein with MAbs

Lane M: protein molecular weight marker; Lanes 1 and 2: MCMV-infected and healthy maize plants, respectively

ACP-ELISA was used to analyze sensitivities of MAbs, and the results revealed that all the four MAbs (4B8, 8C11, 6F4, and 9G1) could detect minimum viruses in infected tissue extracts diluted at 1:81920 (w/v, g/ml), indicating that all four MAbs were sensitive for MCMV detection (Fig. 3).

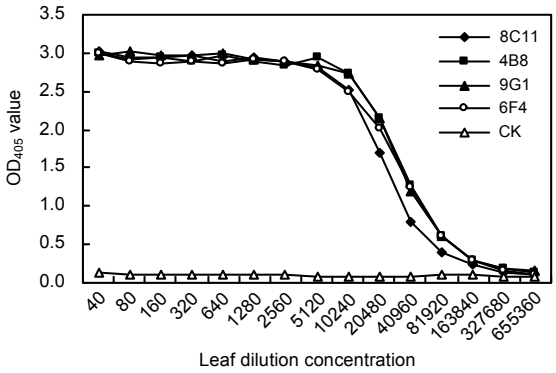

Fig. 3 Sensitivity analyses of MAbs by ACP-ELISA

MCMV-infected leaf crude extracts and healthy leaf crude extracts (CK) were two-fold diluted in PBS buffer from 1:40 to 1:655360 (w/v, g/ml)

3.2 TAS-ELISA for MCMV detection

In TAS-ELISA, rabbit PAbs against MCMV and the MAb (4B8) were used as the capture antibodies and the first antibody, respectively. The results of the three replicated tests revealed that the dilutions of PAbs at 1:5000, MAb 4B8 at 1:6000, and goat anti-mouse IgG conjugated with alkaline phosphatase at 1:8000 were optimal in TAS-ELISA. Purified MCMV and MCMV-infected maize tissues were used to analyze the sensitivity of TAS-ELISA. Dilution endpoint of infected tissue extracts by TAS-ELISA for MCMV detection was 1 in 327680 (w/v, g/ml) in 0.01 mol/L PBS (pH 7.4), and purified MCMV could be detected by TAS-ELISA at a minimum concentration of 25 pg/ml (Fig. 4), which revealed that the TAS-ELISA was sensitive enough for MCMV detection in maize plants. One hundred and sixty-one maize leaf samples showing virus-like symptoms and sixty-nine symptomless maize samples from ten provinces of China were collected. Field maize samples were screened for the presence of MCMV using the TAS-ELISA. The results demonstrated that 28 of the 161 samples with virus-like symptoms and none of 69 symptomless samples were positive (Table 2). All the positive samples detected by TAS-ELISA were also confirmed by the RT-PCR, but only one

symptomless sample was determined to be positive by RT-PCR. All the positive samples were collected from Yunnan and Sichuan provinces, indicating that MCMV was one of the most prevalent viruses infecting maize in these provinces (Table 2).

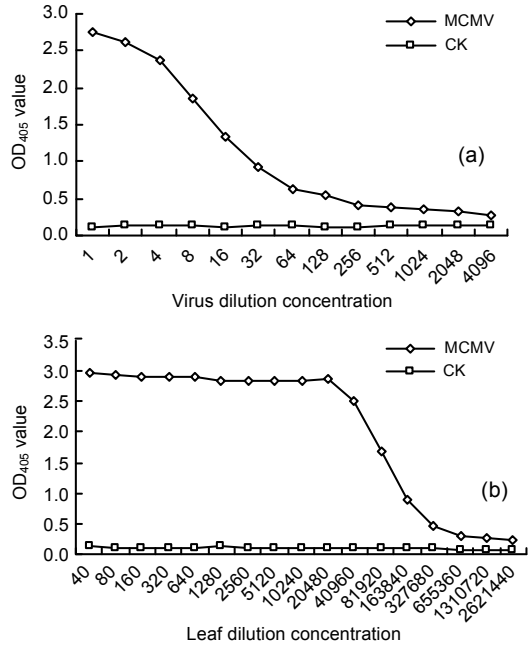

Fig. 4 Sensitivity analysis of the TAS-ELISA with purified MCMV (a) or MCMV-infected tissue extracts (b)
The purified virus preparation was two-fold diluted in PBS buffer from 1:1 to 1:4096 (w/v, g/ml). The original purified virus concentration was 256.0 ng/ml. MCMV-infected leaf extracts and healthy leaf extracts (CK) were two-fold diluted in PBS buffer from 1:40 to 1:2621440 (w/v, g/ml)

Table 2 Detection of MCMV in field maize samples by TAS-ELISA, DIBA, and IC-RT-PCR

Sample source	$n_{MCMV+}/n_{symptom}$	$n_{MCMV+}/n_{symptomless}$
Yunnan	18, 18, 18/36[a]	0, 0, 1/15
Sichuan	10, 10, 10/28	0, 0, 0/11
Guizhou	0, 0, 0/10	0, 0, 0/5
Guangxi	0, 0, 0/8	0, 0, 0/4
Heilongjiang	0, 0, 0/12	0, 0, 0/3
Shandong	0, 0, 0/15	0, 0, 0/7
Liaoning	0, 0, 0/8	0, 0, 0/4
Zhejiang	0, 0, 0/21	0, 0, 0/8
Henan	0, 0, 0/11	0, 0, 0/8
Hebei	0, 0, 0/12	0, 0, 0/4
Total	28, 28, 28/161	0, 0, 1/69

$n_{MCMV+}/n_{symptom}$: number of MCMV-positive samples/number of samples with virus-like symptoms; $n_{MCMV+}/n_{symptomless}$: number of MCMV-positive samples/number of symptomless samples. [a] 18, 18, 18/36 represent 18, 18, 18 of 120 rice samples were positive by TAS-ELISA, DIBA, and IC-RT-PCR, respectively

3.3 DIBA for MCMV detection

Nitrocellulose membranes were used as the sample support, and a DIBA for detecting MCMV in infected maize tissues was carried out. The working concentrations of the MAb (4B8) and goat anti-mouse IgG conjugated with alkaline phosphatase in DIBA procedures were determined by phalanx tests. The results of three repeated phalanx tests demonstrated that the dilutions of MAb 4B8 at 1:5000 and goat anti-mouse IgG conjugated with alkaline phosphatase at 1:7000 were optimal for DIBA. The developed DIBA could detect minimum viruses in infected tissue crude extracts diluted at 1:64000 (w/v, g/ml) (Fig. 5a). Spots containing MCMV were brown, while no color or green spots of healthy tissue crude extracts diluted at 1:100 (w/v, g/ml) were visible (Fig. 5a). MCMV in the field maize samples was also detected by the developed DIBA under optimal conditions and the result is same as that of TAS-ELISA (Table 2; Fig. 5b).

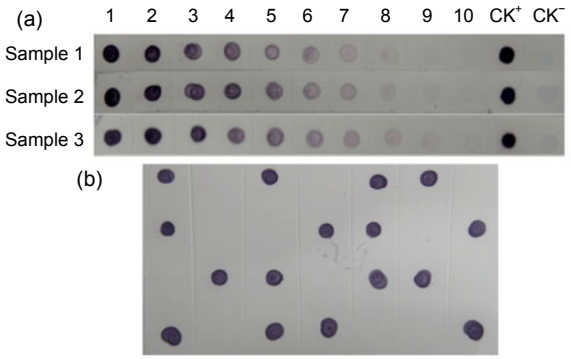

Fig. 5 Sensitivity analysis of DIBA detection for MCMV in infected plants (a) and detection result for field samples (b)
CK^- and CK^+: healthy and MCMV-infected leaf tissue extracts diluted at 1:100 (w/v, g/ml), respectively. Lanes 1–10: MCMV-infected leaf tissue extracts two-fold diluted from 1:1000 to 1:512000 (w/v, g/ml). Brown color spots indicate positive reaction and no color spots indicate negative reaction (Note: for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

3.4 IC-RT-PCR for MCMV detection and sequence analysis of *CP* gene

An IC-RT-PCR method was successfully developed to detect MCMV in maize plant samples. A single 880-bp band including the MCMV *CP* gene was amplified by IC-RT-PCR from each of the MCMV-infected maize samples, while no signals

were observed from the healthy, RBSDV-, SRBSDV-, or SCMV-infected maize plants (Fig. 6a).

The sensitivity analysis revealed that the minimum MCMV could be detected in infected tissue crude extracts diluted at 1:3276800 (w/v, g/ml) (Fig. 6b). Above 161 maize samples with virus-like symptoms and 69 symptomless maize samples were further screened for the presence of MCMV by IC-RT-PCR, and 28 samples that were tested to be positive with TAS-ELISA were found to be infected by MCMV, and furthermore, the one symptomless sample that was positive by RT-PCR also was found to be infected by MCMV (Table 2).

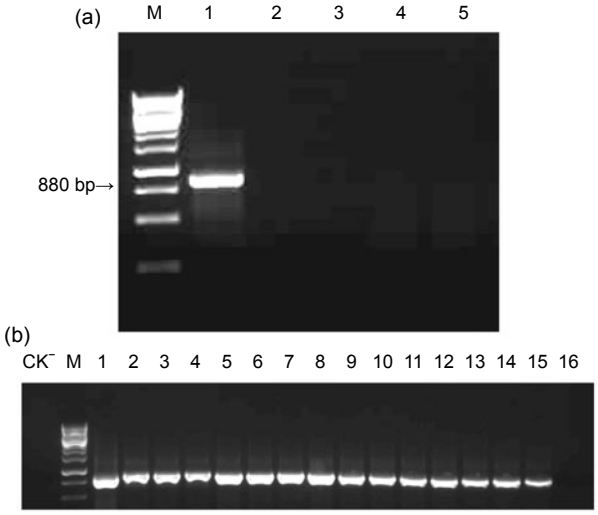

Fig. 6 Specificity (a) and sensitivity (b) analyses of IC-RT-PCR for MCMV detection

(a) M: 1 kb DNA marker; Lanes 1–4: maize plants infected with MCMV, RBSDV, SRBSDV, and SCMV, respectively; Lane 5: a healthy maize as a negative control. (b) Lanes 2–16: MCMV-infected leaf tissue crude extracts at two-fold dilutions from 1:400 to 1:6553600 (w/v, g/ml); Lane 1: a positive control; CK^-: a negative control; M: 1 kb DNA marker. The IC-RT-PCR could detect minimum viruses in MCMV-infected leaf tissue crude extracts at 1:3276800 dilution (w/v, g/ml)

IC-RT-PCR amplified products were sequenced. Nucleotide sequences of PCR-amplified products were compared with that of the MCMV *CP* gene deposited in GenBank. Nucleotide sequence analyses of PCR-amplified products indicated that the PCR-amplified products had 96.3%–99.9% sequence identities with the *CP* of MCMV isolates in GenBank.

The *CP* sequences of MCMV isolates from Yunnan (GU138674, JQ943666, JQ943667, JQ943668, JQ943669, JQ943670, JQ943671, JQ943672, JQ943673, JQ943674) and Sichuan (JQ943675) were aligned with MCMV *CP* sequences deposited in the GenBank database. Chinese isolates have over 99.2% nucleotide sequence identities with each other, have more than 96.3% nucleotide sequence identities with the reported MCMV isolates X14736.2, EU358605, AM490791, AM490792, AM490793, and AY587605, and have the highest sequence identities (98.0%–99.6%) with the reported Thailand isolates AM490791, AM490792, AM490793, and AY587605.

A phylogenetic tree was constructed based on the full-length *CP* genes of the 12 Chinese MCMV isolates together with other eight MCMV isolates (Fig. 7). The Chinese isolates formed one branch with Thailand isolates, while isolates NE (EU358605) and KS (X14736.2) formed another branch.

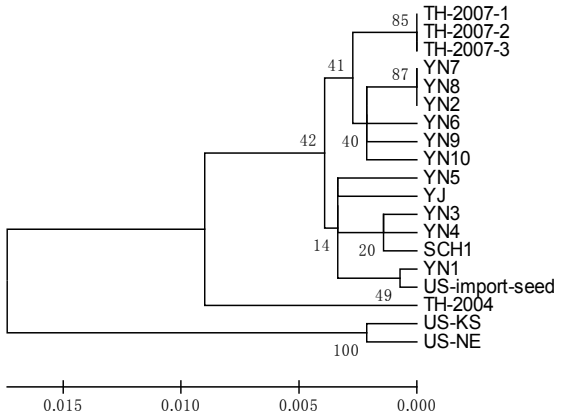

Fig. 7 Phylogeny of *CP* nucleotide sequences of Chinese MCMV isolates and all MCMV isolates available in GenBank

The phylogenic tree was constructed by using the neighbor-joining method and bootstrapped with 1000 replicates. Scale bar indicates nucleotide substitution per site. YN1–10 (GU138674, JQ943666, JQ943667, JQ943668, JQ943669, JQ943670, JQ943671, JQ943672, JQ943673, JQ943674) and YJ (JF422772): 11 Yunnan isolates, China; SCH1 (JQ943675): a Sichuan isolate, China; TH (AM490791, AM490792, AM490793, and AY587605): four Thailand isolates; KS (X14736.2): a Kansas isolate, USA; NE (EU358605): a Nebraska isolate, USA; US-import-seed (GU594293): an isolate from maize seeds imported from USA

4 Discussion

Since MCMV is an important pathogen in maize, accurate detection and identification of this virus are important for controlling this virus-induced disease.

The serological detection method is a suitable large-scale detection technique due to its high sensitivity, rapidity, and specificity. It has been widely used to detect and diagnose plant viruses (Jensen *et al.*, 1991; Yu *et al.*, 2005; Wu *et al.*, 2009; Shang *et al.*, 2011). Jensen *et al.* (1991)'s work, a DAS-ELISA for MCMV detection based on the prepared PAbs, was established and the virus was easily detectable in homogenates of infected leaves at a dilution of 1:4000 (w/v, g/ml). However, the MAb is used more often than PAbs, because of its high specificity, high sensitivity, and complete homogeneity. Furthermore, once a hybridoma cell line secreting MAb against the virus has been established, MAbs can be easily and continuously produced. It is well known that serological detection assays based on high-quality MAbs are often more sensitive and specific than assays based on PAbs. We prepared four highly specific and sensitive MAbs against MCMV and the three serological methods (DIBA, TAS-ELISA, and IC-RT-PCR) were then established for MCMV detection based on the produced MAbs. There is a commercially available PAb (Agdia, Elkhart, IN, USA) against MCMV to detect MCMV. We compared our MAbs with the commercially available PAbs and the results indicated that both specificity and sensitivity of MAbs are five times higher than those of the commercially available PAbs in ACP-ELISA (data not shown). The established TAS-ELISA, DIBA, and IC-RT-PCR methods could detect the minimum MCMV in infected leaf tissue crude extracts specifically with the dilutions of 1:327680, 1:64000, and 1:3276800 (w/v, g/ml), respectively. IC-RT-PCR is the most sensitive assay among the three methods. Moreover, information on the viral genome can be obtained from sequencing analyses of amplified products of IC-RT-PCR (Wu *et al.*, 2011). Despite this sensitivity, DIBA and TAS-ELISA are more suitable to detect field samples on a large scale. The results of our study indicate that MCMV has only occurred in Yunnan and Sichuan provinces, indicating that MCMV has not yet spread in other parts of China. Since MCMV can be introduced into corn production areas through seed transmission (Jensen *et al.*, 1991; Zhang *et al.*, 2011), quarantine measures are necessary to prevent the further spread of MCMV in China.

The *CP* sequences of MCMV isolates from Yunnan and Sichuan of China and Thailand (AM490791, AM490792, AM490793, and AY587605) were very closely related, and the high degree of sequence identity (98.0%–99.6%) suggests that these isolates have evolved from a common ancestor. Many corn seeds planted in China originated from foreign countries such as the United States and Thailand. We think it is very likely that MCMV was introduced into China from other countries based on analysis of *CP* sequences and the lack of MCMV in China before 2009.

The synergistic interactions between MCMV and either MDMV, WSMV, or SCMV lead to corn lethal necrosis (CLN) (Niblett and Clafin, 1978; Uyemoto *et al.*, 1980; Goldberg and Brakke, 1987; Scheets, 1998). All the leaf samples used in this study showed lethal necrosis symptoms. Accordingly, the MCMV-positive samples were also tested for the presence of MDMV, WSMV and SCMV using commercially available ELISA kits (Agdia, Elkhart, IN, USA), and the detection results indicated that all MCMV-positive samples were mix-infections with SCMV (data not shown). Since SCMV is a very common pathogen in many areas in China (Jiang and Zhou, 2002), synergistic interactions between MCMV and SCMV may increase the severity of MCMV damage to corn production in China.

Compliance with ethics guidelines

Jian-xiang WU, Qiang WANG, Huan LIU, Ya-juan QIAN, Yan XIE, and Xue-ping ZHOU declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

References

Carrera-Martínez, H., Losoya-Saldaña, H., Mendoza-Zamora, C., Alvizo-Villasana, H., 1989. Inmunoabsorción enzimática (ELISA) en la identificación y distribución del virus moteado clorótico del maíz (VMCM) en el estado de México. *Rev. Mex. Fitopatol.*, **7**:20-25 (in Mexican Spanish).

Castillo, J., Hebert, T., 1974. Nueva enfermedad virosa afectando al maiz en el Peru. *Fitopatologia*, **9**(2):79-84 (in Spanish).

Fang, S., Yu, J., Feng, J., Han, C., Li, D., Liu, Y., 2001. Identification of rice black-streaked dwarf *Fijivirus* in maize with rough dwarf disease in China. *Arch. Virol.*, **146**(1):167-170. [doi:10.1007/s007050170200]

Goldberg, K.B., Brakke, M.K., 1987. Concentration of maize chlorotic mottle virus increased in mixed infections with maize dwarf mosaic virus, strain B. *Phytopathology*, **77**(2):162-167. [doi:10.1094/Phyto-77-162]

Jensen, S., Wysong, D., Ball, E., Higley, P., 1991. Seed transmission of maize chlorotic mottle virus. *Plant Dis*., **75**(5):497-498. [doi:10.1094/PD-75-0497]

Jiang, J., Zhou, X., 2002. Maize dwarf mosaic disease in different regions of China is caused by sugarcane mosaic virus. *Arch.Virol*., **147**(12):2437-2443.

King, A.M.Q., Lefkowitz, E., Adams, M.J., Carstens, E.B., 2011. Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses. Elsevier Academic Press, San Diego, CA, Vol 9, p.256-267.

Lommel, S., Kendall, T., Siu, N., Nutter, R., 1991a. Characterization of maize chlorotic mottle virus. *Phytopathology*, **81**(8):819-823. [doi:10.1094/Phyto-81-819]

Lommel, S., Kendall, T., Xiong, Z., Nutter, R., 1991b. Identification of the maize chlorotic mottle virus capsid protein cistron and characterization of its subgenomic messenger RNA. *Virology*, **181**(1):382-385. [doi:10.1016/0042-6822(91)90509-A]

Morales, F., Arroyave, J., Castillo, J., Leon, C., 1999. Cytopathology of maize chlorotic mottle virus in *Zea mays* L. *Maydica*, **44**(3):231-235.

Nault, L., Styer, W., Coffey, M., Gordon, D., Negi, L., Niblett, C., 1978. Transmission of maize chlorotic mottle virus by chrysomelid beetles. *Phytopathology*, **68**(7):1071-1074. [doi:10.1094/Phyto-68-1071]

Nault, L.R., Gordon, D.T., Loayza, J.C., 1981. Maize virus and mycoplasma diseases in Peru. *Trop. Pest Manage.*, **27**(3):363-369. [doi:10.1080/09670878109413806]

Niblett, C., Clafin, L., 1978. Corn lethal necrosis, a new virus disease of corn in Kansas. *Plant Dis. Rep*., **62**:15-19.

Nutter, R.C., Scheets, K., Panganiban, L., Lommel, S., 1989. The complete nucleotide sequence of the maize chlorotic mottle virus genome. *Nucleic Acids Res*., **17**(8):3163-3177. [doi:10.1093/nar/17.8.3163]

Scheets, K., 1998. Maize chlorotic mottle machlomovirus and wheat streak mosaic rymovirus concentrations increase in the synergistic disease corn lethal necrosis. *Virology*, **242**(1):28-38. [doi:10.1006/viro.1997.8989]

Shang, H., Xie, Y., Zhou, X., Qian, Y., Wu, J., 2011. Monoclonal antibody-based serological methods for detection of cucumber green mottle mosaic virus. *Virol. J*., **8**(1):228-236. [doi:10.1186/1743-422X-8-228]

Stenger, D.C., French, R., 2008. Complete nucleotide sequence of a maize chlorotic mottle virus isolate from Nebraska. *Arch. Virol*., **153**(5):995-997. [doi:10.1007/s00705-008-0069-y]

Stenger, D.C., Young, B.A., Qu, F., Morris, T.J., French, R., 2007. Wheat streak mosaic virus lacking helper component-proteinase is competent to produce disease synergism in double infections with maize chlorotic mottle virus. *Phytopathology*, **97**(10):1213-1221. [doi:10.1094/PHYTO-97-10-1213]

Uyemoto, J., 1983. Biology and control of maize chlorotic mottle virus. *Plant Dis*., **67**(1):7-10. [doi:10.1094/PD-67-7]

Uyemoto, J., Bockelman, D., Claflin, L., 1980. Severe outbreak of corn lethal necrosis disease in Kansas. *Plant Dis*., **64**(1):99-100. [doi:10.1094/PD-64-99]

Uyemoto, J., Claflin, L., Wilson, D., Raney, R., 1981. Maize chlorotic mottle and maize dwarf mosaic viruses: effect of single and double inoculations on symptomatology and yield. *Plant Dis*., **65**(1):39-40. [doi:10.1094/PD-65-39]

Wu, J., Yu, L., Li, L., Hu, J., Zhou, J., Zhou, X., 2007. Oral immunization with transgenic rice seeds expressing vp2 protein of infectious bursal disease virus induces protective immune responses in chickens. *Plant Biotechnol. J*., **5**(5):570-578. [doi:10.1111/j.1467-7652.2007.00270.x]

Wu, J., Yu, C., Yang, C., Zhou, X., 2009. Monoclonal antibodies against the recombinant nucleocapsid protein of tomato spotted wilt virus and its application in virus detection. *J. Phytopathol*., **157**(6):344-349. [doi:10.1111/j.1439-0434.2008.01498.x]

Wu, J., Meng, C., Shang, H., Rong, S., Zhang, C., Hong, J., Zhou, X., 2011. Monoclonal antibody-based triple antibody sandwich-enzyme-linked immunosorbent assay and immunocapture reverse transcription-polymerase chain reaction for odontoglossum ringspot virus detection. *J. Virol. Methods*, **171**(1):40-45. [doi:10.1016/j.jviromet.2010.09.027]

Xie, L., Zhang, J., Wang, Q., Meng, C., Hong, J., Zhou, X., 2011. Characterization of maize chlorotic mottle virus associated with maize lethal necrosis disease in China. *J. Phytopathol*., **159**(3):191-193. [doi:10.1111/j.1439-0434.2010.01745.x]

Yin, X., Xu, F., Zheng, F., Li, X., Liu, B., Zhang, C., 2011. Molecular characterization of segments s7 to s10 of a southern rice black-streaked dwarf virus isolate from maize in northern China. *Virol. Sin*., **26**(1):47-53. [doi:10.1007/s12250-011-3170-9]

Yu, C., Wu, J., Zhou, X., 2005. Detection and subgrouping of cucumber mosaic virus isolates by TAS-ELISA and immunocapture RT-PCR. *J. Virol. Methods*, **123**(2):155-161. [doi:10.1016/j.jviromet.2004.09.014]

Zhang, Y., Zhao, W., Li, M., Chen, H., Zhu, S., Fan, Z., 2011. Real-time Taqman RT-PCR for detection of maize chlorotic mottle virus in maize seeds. *J. Virol. Methods*, **171**(1):292-294. [doi:10.1016/j.jviromet.2010.11.002]

Zhou, G.H., Wen, J.J., Cai, D.J., Li, P., Xu, D.L., Zhang, S.G., 2008. Southern rice black-streaked dwarf virus: a new proposed *Fijivirus* species in the family reoviridae. *Chin. Sci. Bull*., **53**(23):3677-3685. [doi:10.1007/s11434-008-0467-2]