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Detection and subgrouping of Cucumber mosaic virus isolates by TAS-ELISA and immunocapture RT-PCR☆

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Abstract

Eight mouse hybridoma cell lines secreting monoclonal antibodies (MAbs) against Cucumber mosaic virus (CMV) were produced. Analysis of the specificities of the MAbs against CMV isolates by triple antibody sandwich (TAS)-ELISA demonstrated that four MAbs were specific for subgroup I (S-I) isolates and two for subgroup II (S-II) isolates, whereas another two MAbs could detect both S-I and S-II isolates. TAS-ELISA and immunocapture RT-PCR (IC-RT-PCR) methods were then established for reliable and efficient detection and subgrouping of CMV isolates using the produced MAbs. When 197 field samples collected from six provinces in China were tested by TAS-ELISA, 130 samples were found to be infected by CMV. Among them, 121 samples were infected by S-I isolates (93.1%) and another nine samples by S-II isolates (6.9%). In IC-RT-PCR using the MAbs and specific primers in the region of the *coat protein* (*CP*) gene, samples shown to contain S-I isolates by TAS-ELISA gave one specific band about 500 nucleotides in length, whereas samples containing S-II isolates produced a single band with the length of approximately 600 nucleotides. The validity and reliability of the results of TAS-ELISA and IC-RT-PCR was confirmed by sequencing and phylogenetic analysis of nearly full-length *CP* genes of the isolates.

Keywords: Cucumber mosaic virus; Monoclonal antibody; Subgroup; TAS-ELISA; IC-RT-PCR

1. Introduction

Cucumber mosaic virus (CMV), a positive-sense RNA plant virus with a tripartite genome, is the type member of the genus C*ucumovirus*. CMV has a world-wide distribution and exists as a variety of isolates that differ in host range and pathogenicity (Palukaitis et al., 1992). CMV causes great losses in vegetables, ornamentals and fruits, and is destructive due to its rapid spread by more than 60 aphid species in the field.

Many CMV isolates have been described and they can be divided into two main subgroups, subgroup I (S-I) and subgroup II (S-II), on the basis of serological relationships, peptide mapping of the coat protein (CP), nucleic acid hybridization and nucleotide sequence identity (Palukaitis et al., 1992). More recently, a further division of subgroup I into IA and IB has been proposed based on the nucleotide sequences of the 5′ non-translated region (NTRs) and *CP* gene of RNA3 (Roossinck, 2002; Roossinck et al., 1999). Due to its economic importance, several serological methods have been developed for detection and differentiation of CMV isolates. Because the two subgroups are closely related serologically according to tests with polyclonal antibodies (PAbs), monoclonal antibodies (MAbs) raised against S-I and S-II isolates have been produced and used for differentiating the two subgroups (Porta et al., 1989; Hsu et al., 2000). Molecular tools such as RT-PCR and nucleotide sequencing are also available (Rizos et al., 1992; Roossinck et al., 1999; Choi et al., 1999), but they are expensive for epidemiological studies.

In China, CMV isolates have been obtained from over 120 species in 38 families of host plants. Several MAbs

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against CMV have been produced, but their use for practical detection was not reported (Yu et al., 1986; Cai et al., 1989). Furthermore, few data are available about the subgrouping of CMV isolates that occur in China. In attempts to understand better the epidemiology of CMV in China, MAbs against CMV isolates in both subgroups were produced and two reliable methods for efficient identification and subgrouping of CMV isolates were developed in the present study.

2. Materials and methods

2.1. Virus sources

CMV S-I isolates RB and P1 (CMV-RB and CMV-P1) were characterized and maintained by our lab (Zhou et al., 1999), isolate Fny (CMV-Fny) was kindly provided by Prof. Jialin Yu, China Agriculture University, China. CMV S-I isolate G1 (CMV-G1) and CMV S-II isolate G2 (CMV-G2) were provided by Dr. Joerg Schubert, Institute for Resistance Research and Pathogen Diagnostics, Germany. These isolates were propagated in *Nicotiana glutinosa* and purified as described previously (Zhou et al., 1995). The CMV S-II positive control LPC44800 was purchased from Agdia Inc. (Elkhart, IN, USA). A total of 197 samples were collected from field crops such as tobacco, tomato, pepper, squash, radish and banana showing mosaic symptoms in Yunnan, Jiangxi, Guizhou, Guangdong, Guangxi, and Zhejiang provinces of China from 2000 to 2003.

2.2. Preparation of hybridomas secreting MAbs

Hybridomas secreting MAbs against CMV were produced by fusion of spleen cells from immunized BALB/c female mice and the mouse myeloma cell line SP2/0 as described (Jiang et al., 2003). The procedures for cell fusion, screening, cloning and subcloning by limiting dilution were carried out as described previously (Hsu et al., 1990).

2.3. Production of MAbs in ascitic fluids

Ascitic fluids containing MAbs were produced by injecting about 10^7 hybridoma cells into the peritoneal cavity of healthy BALB/c mice, which had been injected with pristine 1 week earlier. After 2 weeks, ascitic fluids were collected and their titres tested by ELISA. Immunoglobulins (IgGs) of MAbs were purified by ammonium sulphate precipitation followed by dialysis against three changes of 0.01 M PBS (pH 7.4) and the concentrations were determined spectrophotometrically. The subclass of each MAbs was determined by an agar diffusion test using the mouse monoclonal antibody isotyping reagents as described by the manufacturer (Sigma, St. Louis, Mo, USA).

2.4. ELISA

Triple antibody sandwich (TAS)-ELISA (Zhou et al., 1997) and antigen-coated plate (ACP)-ELISA (Jiang et al., 2003) were used for detection and characterization of MAbs against CMV with an alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Sigma). Healthy tobacco plants and buffer alone were used as controls. The positive threshold was fixed at twice the average of the optical density obtained with the healthy controls. All tests were duplicated.

2.5. Immunocapture RT-PCR (IC-RT-PCR)

Three CMV specific primers were designed based on the cDNA sequences of the CMV *CP* gene deposited in the GenBank database. The forward primers CMV I-F (5′-CGACTTAATAAGACGTTAGCAGC-3′, corresponding to nucleotides 121–143 of *CP* gene of CMV S-I isolates) and CMV II-F (5′-TCCCAATGCTAGTAGAACCTCC-3′, corresponding to nucleotides 18–39 of *CP* gene of CMV S-II isolates) located at the upstream end of *CP* gene were specific for CMV S-I and CMV S-II isolates, respectively. The reverse primer CMV-R (5′-TGCTCRAYGTCRACATGAAG-3′, complementary to nucleotides 601–620 of *CP* gene of CMV S-I and S-II isolates) was degenerate and based on the conserved sequence of CMV *CP* genes in both subgroups. The immunocapture was carried out as described (Jiang and Zhou, 2002).

2.6. Sequence determination and analysis

PCR products were recovered, cloned and sequenced as described previously (Jiang and Zhou, 2002). All sequences were aligned and their identities determined by the Clustal V method of the MegAlign procedure supplement within the DNASTAR package (version 7.0, DNAStar Inc., Madison, WI, USA). A dendrogram was constructed using the neighbour-joining method in DNAMAN software (Version 5.0, Lynnon Biosoft, Quebec, Canada). The database accession numbers of other CMV *CP* gene sequences used for comparison are: CMV-P1 (AJ006988), CMV-Fny (D10538), CMV-SD (AB008777), CMV-RB (AJ006990), CMV-Ix (U20219), CMV-Oahu (U31220), CMV-WL (D00463), CMV-Q (M21464) and CMV-YNb (AJ242585). The Peanut stunt virus (PSV) *CP* gene (U 15730) was used as an outgroup for comparison.

3. Results

3.1. Characterization of MAbs against CMV

A total of eight hybridoma cell lines secreting MAbs against CMV were obtained. MAbs 3C9, 1D9, 1B6, and 1H5 were produced by immunization with CMV-RB, while MAbs 3C12, 4B2, 4C12 and 3F5 were obtained after immunization