Short Communication

Detection and Diagnosis of Cucumber Mosaic Virus in Infected Plants Using Monoclonal Antibodies by Enzyme-linked Immunosorbent Assays

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ABSTRACT

A comparison of methods to detect and diagnosis Cucumber mosaic virus by using enzyme-linked immunosorbent assays (ELISA) and an Immunocapture Reverse Transcriptase Polymerase Chain Reaction (IC-RT-PCR) was made to determine the detection limit of each method. We produced monoclonal antibodies for two Japanese strains Pepo, M2-Cucumber mosaic virus (CMV) as antigens. Tests to detect the pure virus protein with ELISA and IC-RT-PCR were performed and IC-RT-PCR was found to be more sensitive, reaching 10 pg/ml for the pure virus protein, while ELISA method levels were 0.7 ng/ml. Furthermore, we concluded that the present method is very useful for the diagnosis of infected plants.

Key words: Cucumber mosaic virus, Monoclonal antibodies, Immunoassay, Immuno-capture-RT-PCR, Early detection of virus infection

1. Introduction

Cucumber mosaic virus (CMV), one of the oldest plant diseases, exists worldwide and has the largest host range of all plant viruses. It infects more than 1,000 species of plants, causing many diseases in vegetables, fruit and ornamental crops, and causing severe economic losses (Roossinck, 2002). Due to its economic importance, the serological differentiation of CMV isolates is importance in breeding disease resistant plants and studying disease epidemiology. Viruses cause many important plant diseases and are responsible for huge losses in crop production and quality in all parts of the world.

Damage to crop plants due to virus infections is difficult to assess and actual figures for global crop loss are not available. Plant diseases losses are estimated to be $60 billion annually (Hsu, 2002). Losses due to virus are considered to be second to those caused by fungi. The ability of the virus to spread so efficiently and rapidly makes it necessary to develop a vital, quick and reliable method for diagnosing virus infections in order to increase plant production, particularly in developing
countries. It could be that only the lack of resources for the diagnosis of hard to identify viruses, and also in developed countries in closed systems such as green-houses and plant factories. Unlike for fungal and bacterial disease no direct method for the control of viral diseases is yet available. The early detection of plant viruses constitutes, therefore, one of the main ways of controlling these diseases, and so sensitive detection systems are essential.

Serological data, peptide mapping of the coat protein, and nucleic acid hybridization have divided the CMV strains into two subgroups, designated I and II (Hsu et al., 2000). Several strategies, including expression of pathogen-derived sequences or anti-pathogenic agents, have been developed to engineer improved pathogen resistance in transgenic plants. Antibody-based resistance is a novel strategy for generating transgenic plant resistance to pathogens (Schillberg et al., 2001). The aim of the present work is production and characterization of a high affinity and specificity of monoclonal antibodies against CMV coat proteins, which is the first step on the way to antibody based diagnosis of infected plants and resistance. The second objective, an attempt to evaluate immunocapture reverse transcriptase polymerase chain reactions, compared with double-antibody sandwich enzyme-linked immunosorbent assay for local CMV isolates in infected tobacco plants, is a better strategy for the prevention of the spread of viruses depending on general early detection and removal of infected plants.

2. Materials and Methods

Japanese strains and isolates of CMV were propagated in tobacco plants (Nicotiana tabacum, cv Xanthi) in a greenhouse. Virus purifications were performed in a long linear sucrose gradient as described previously (Osaki et al., 1973).

Virus concentration was estimated by measuring the absorbance with a spectrophotometer at 260 nm and an extinction coefficient of 5.0 was used.

Immunized eight-weeks old BALB/c mice were injected subcutaneously with 100 µg in a volume 0.1 ml phosphate-buffered saline (PBS; 0.01 M phosphate and 0.015 M sodium chloride, pH 7.5) of purified CMV of either strain Pepo or M2, which were mixed with an equal volume of adjuvant containing TDM plus MPL. After three injections were administered at two-week intervals, three days after the fourth injection, the mice were given a peritoneal injection of 200 µg of virus in 0.2 ml PBS. The mice were sacrificed 3 days later and their spleens were harvested. Fusion experiments were carried out in which lymphocytes from the spleens of the immunized mice were mixed at a 5:1 ratio with non-secreting P3X63-Ag8-U1 myeloma cells in Polyethylene glycol 6000 at 50% (w/v). The cells were distributed to 96 well plates at a concentration of 10⁶ cells/well with medium HAT (100 µM hypoxanthine, 0.4 µM aminoprotein, 16 µM thymidine, 6 mM Hepes, and 200 µM 2-mercaptoethanol). Clones which successfully secreted antibodies specific to CMV were examined with both ELISA and western blotting, and additionally, subcloned by limiting the dilution method in the presence of thymocytes of BALB/c mice as feeder cells according to standard protocols (Harlow and Lane, 1988).

Monoclonal antibody production has been done by intraperitoneal injection of 10⁷ hybridoma cells into cavities of BALB/c mice primed two-weeks previously with 0.5 ml pristine (2, 6, 10, 14-tetramethylpentadecane), and the antibodies were purified from the isolated ascetic fluid by affinity chromatography protein A-Sepharose 1 ml high tarp (Amersham Pharmacia Biotech), according to procedures given by the manufacturer. Biotinylation of MABs (Biotinylated-MAB), N-hydroxysuccinimide-biotin dissolved in dimethyl-formamide reacted with purified antibodies in a 1:1 (w/w biotin/antibodies) ratio according to the manufacturer’s instructions (PIERCE). Rabbit polyclonal antibodies (PAbs) were produced by immunizing with 500 µg of Pepo-CMV strain as immunogen emulsified with an equal volume of Freund’s incomplete adjuvant (FIA). Upon getting a satisfactory response, after about three to five injections, antisera were assayed by the ability to interact with the CMV. The antisera were collected 10 days after the last injection and the antibodies were purified like MABs as mentioned above.

Enzyme-linked immunosorbent assay (ELISA) procedures, antigen-coated plates (ACP), and double antibody sandwich (DAS) using standard buffers have been used previously (Clark and Adams, 1977). Briefly, ACP-ELISA plates were coated directly with purified CMV 1 µg/ml from each strain, and incubated 2 h at 37ºC. While DAS-ELISA were first coated with PAbs 1 µg/ml for trapping the virus coat proteins, the plates were washed five times between each step using a plate washer (BIO-RAD, Hercules, CA, USA, Mod. 1575), blocked with 1% Block Ace, and followed by biotinylated-MABs 100 ng/ml. Streptavidin-HRP conjugate (dilution rate: 1/10000) (PIERCE) was added to react with biotinylated-MABs. The substrate solution was o-phenylenediamine 0.4 mg/ml in a citric
phosphate solution. Finally, the plates were incubated at 37°C for 15 min. and absorbance was read at 450 nm using a Microplate Reader 550 (BIO-RAD).

Competitive ELISA plates were coated with a constant (1 µg/ml) concentration of Pepo-CMV then blocked as mentioned above. Serial dilution (0.01~100 µg/ml) of non-biotinylated MAb-(S, 6, and 8) as inhibitors were incubated for 2 h at 37°C then followed by 100 ng/ml biotinylated MAb-4. The aforementioned was detected with Streptavidin-HRP.

IC-RT-PCR analysis was done according to a cited paper (Jansen et al., 1990). First strand cDNA synthesis was carried out by adding 0.5 U of M-MLV reverse transcriptase and 2 U of RNase inhibitor (TOYOBO, Osaka, Japan) with primer P3R123Bam (5'-CCGGATCCCTGGTCTCCTTTTGGAGGCCC-3') which is complementary to 3’ proximal 20 nucleotides of CMV RNAs according to (Nitta et al., 1988), and incubation at 37°C for 90 min. was performed. Nested PCR reaction was done using an oligonucleotide specific to genomic RNA3 pepoCPF (5'-CCTCTAGACTTCTCCGCAGATTGCG-3') and p3CPR3/4 (5'-CCGAGCTCTCAAGCTTGAACTCCAGATGCAG-3'). The amplification product was completed up to 100 µl with distilled water. The PCR reaction mixture was prepared by adding 1.2 µl (2.5 mM dNTP's mix); 1.2 µl MgCl2 (25 mM); 1.2 µl each primer (10 mM) as mentioned above; 1 µl of the cDNA; 1.2 µl high buffer (10X); 0.2 µl Taq-DNA polymerase 5 U/µl; and nuclease free distilled water up to a final volume 12 µl. The cycling program was 5 min. at 94°C without adding Taq-polymerase and subsequently, 30 cycles after adding Taq-polymerase (92°C, 58°C, and 72°C for 30 sec., respectively), and then final incubation for 5 min. at 72°C. Nested PCR amplification products were viewed under UV light after electrophoresis in 1% agarose gel Tris-Acetate-EDTA buffer and stained with ethidium bromide. All other chemicals were purchased from (Nacalai Tesque, Inc. Kyoto, Japan).

3. Results and Discussion

Most strains of CMV had weak immunogens; nevertheless, in the present study of the M2-CMV strain, the amount of accumulated virus in an infected tobacco plant was so low it required about a month to yield 2 mg from 100 gm of infected leaves. Accordingly, it had very weak immunogens probably due to its instability, and yield low-tittered antisera (Palukaitis et al., 1992).

On the contrary, strain Pepo-CMV had very fast accumulation rate requiring only about 5 days to accumulate a high virus yield of 1 mg from 1 gm of leaves and had a powerful immunogen (data not shown). Nine stable hybridoma cell lines secreting MAbS specific to CMV coat protein were obtained from five fusion experiments, and the immunoglobulin classes and subclasses for each were determined, as shown in Table 1. The specific reactivity of the monoclonal antibodies depending on ACP-ELISA could be assigned to three major categories based on their specificities. Five MAbS (4, 5, 6, 8, and 10) reacted with all the tested strains of subgroup-I (S-I), but did not react with CMV subgroup-II strains (S-II). Three MAbS (M2-2, M2-3, and M2-4) reacted with tested strains of S-II, but did not react with S-I. One MAb (M2-1) reacted with all the strains and isolates. Accordingly, these monoclonal antibodies are subgroup specific with unique epitopes. Furthermore, rabbit antisera detected all the CMV strains and isolates. Neither MAbS nor PAbS reacted with Tomato aspermy virus or Peanut stunt virus (data not shown).

Epitopes mapping of CMV coat protein by competitive ELISA was performed for determining the antibody binding site specificity, which is illustrated in Figure 1. The identification of antibody binding sites or epitopes on protein antigens provides essential information for predictions of efficacy in the antibody development. Generally, our MAbS detected CMV in ELISA as

![Graph](image-url)
well as in western blotting (data not shown); consequently their epitope sites are linear epitope. The reactivity of biotinylated MAb-4 against CMV coat protein was pre-incubated with non-biotinylated MAbs (5, 6, and 8). Obviously, these antibodies could be classified into three categories. First, MAb-5 showed no competition with biotinylated MAb-4; therefore MAb-5 has a completely different epitope site, while MAb-6 shows slight competition, thus the epitope is predicted to overlap the MAb-4 epitope. Additionally, a high concentration of MAb-8 completely inhibited MAb-4 binding activity. For that reason, MAb-8 has the same combining site as specified epitopes. In contrast, in the antibodies’ specific group there is no cross reaction between each other (Table 1). Consequently, these MAbs show a different recognition-binding site on CMV coat proteins.

Detection of CMV by IC-RT-PCR is a simple and sensitive analysis of multiple samples without a preliminary purification of the virus particles or viral RNA. By undertaking IC-RT-PCR using primer pair pepoCFPF/p3CPR, we investigated the effects of temperature. The results indicated that the critical point in this experiment is the temperature for the virus particles lyse. We used a range of temperatures of 65°C to 90°C with different time exposures from 5 min. up to 30 min. 65°C for 25 min. gave the optimal detection and it was possible to specifically detect purified as well as infected tobacco plants. In both cases the products observed were of the correct predicted size of about 595 bp, as shown in Figure 2. The relative sensitivities of IC-RT-PCR (Figure 2A) were compared by using serial dilutions of purified Pepo-CMV (100 ng/ml), serially ten-fold diluted lanes 1 to 5 respectively; with a minimum detection limit of 0.01 ng/ml. No reaction occurred when the virus was substituted with buffer lane 6. On the other hand, for the detection of CMV infected plant sap, we investigated the crude extract RNA concentration on specific amplification by testing different dilutions of the standard crude extracts. The dilution rate of infected plants: 1/100 correctly predicted a size about of 595 bp, as shown in Figure 2B. In addition, importantly, twelve hours post inoculation was obviously enough to detect the virus by this method for lane 2. On the other hand, the accumulations of the virus gradually increased with time, in lanes 3 to 5 respectively. Remarkably, a high dilution rate 10^{-5} of infected plant sap was detected in lane 6; however, there was no

### Table 1

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The specific reactivity of the monoclonal and polyclonal antibodies for CMV coat protein strains and isolates.

The relative reactivity of the MAbs to CMV coat protein. ELISA plates were coated with each strain 1 μg/ml, followed by either 1 μg/ml of MAbs or PAbs antibodies which were detected with goat anti-mouse,-rabbit HRP conjugate (dilution rate: 1/5000), respectively. Absorbance at (O.D 450 nm) were scored as < 0.1 (-), > 0.1 to 1 (+), >1 to 1.7 (++) , and >1.7 to 2.7 (+++). The optical density for the buffer ranged from 0.009 to 0.070 and healthy tobacco plants ranged from 0.082 to 0.097.
detection in the healthy plants in lane 1.

The IC-RT-PCR approach was chosen rather than hybridization or RT-PCR tests because of high sensitivity due to an initial immunocapture enrichment step followed by PCR amplification. The high specificity was due to the combination of virus specific antibody captures and primer specificity, and virus detection directly in crude plant extracts rather than in partially or highly purified nucleic acid preparations. Efficacies of these treatments appear to vary substantially between different viruses, and therefore, the best condition for any particular viruses are difficult to predict and need to be assessed individually. Effective control of viral infection in plants depends on selecting and propagating virus-free plants and eradicating diseased plants.

A reliable plant pathogen diagnostic method requires high sensitivity and specificity, especially when it is used for determining the sanitary status of the plant material to be vegetatively propagated as well as the plants seeds, and as breeders and specialty propagators of vegetative plant material. In our system, sensitivity and specificity for the detection of the DAS-ELISA either purified or infected plants. However, while DAS-ELISA allowed reliable results, MAbs were compared for the detection of CMV both in a purified virus preparation detected with high sensitivity with a minimum detection level 0.7 ng/ml and 10^-5 dilution of infected plant sap. MAb-6, which has a higher relative affinity than the MAb-4, about hundred times for the purified state, and a thousand times for infected plants, as shown in Figure 3. We concluded that a panel of monoclonal antibodies produced against CMV coat protein was used to characterize some of their strains and isolates, and different formats of enzyme-linked immunosorbent assays were compared to establish the most suitable one for diagnosis of infected plants and for serotype differentiation. Since most MAbs retained their activity when used for coating microtiter plates, a dual MAb-type assay was found to be most suitable. The assay using only one type of monoclonal antibody did not lack sensitivity because presumably, unlimited epitopes were available.

Because of the ability of MAbs to recognize subtle conformational alterations in the viral antigen, the ability of certain monoclonal antibodies to block the co-translational disassembly of virions during the infection process was found to be linked to the precise location of their complementary epitopes and not to their binding affinity. Such blocking of the antibodies that act by satisfactorily preventing the interaction between virions and ribosomes may, when expressed in plants,
be useful for controlling virus infection.

From the above results, we concluded that the present monoclonal antibodies are useful for diagnosis and disease resistance in plants especially in a recently developed highly efficient plant growth machine, a so called salad machine (Takai et al., 2004) in a closed system, especially in closed plant growth systems such as green-houses and plant factories. No direct chemical control means are available to combat virus infections in plants. Control of viral disease is achieved primarily by sanitary practices that involve reducing the source of inoculums from infected plants, preventing spreading within the crop. Virus disease testing programs are now common in many parts of the world where the economic importance of growing virus-free plants is recognized, and testing provides assurance of virus-free production materials. Early detection of virus in a field or green-house and removal of infected plants minimizes spread of the virus, helps to protect healthy plants.

4. Conclusion

Nine monoclonal antibodies were obtained from five fusion experiments performed after immunizing mice with two Japanese strains Pepo-, M2-CMV as antigens. These MAbs showed a diverse reactivity for the different subgroup of the isolates. Of these MAbs, five MAbs specifically reacted with CMV subgroup-I, three MAbs were capable of identifying the subgroup-II. One of the monoclonal antibodies was found to be non-specific for CMV-coat proteins. We compared them to detect the limit of the pure virus protein with three different enzyme-linked immunosorbent assays. IC-RT-PCR was the most sensitive and detected as 10 pg/ml for the pure virus protein, while other methods’ levels were 10 ng/ml. From the results, we concluded that the present method is very useful for diagnosis of infected plants.

References


Acronym : Antigen-coating plate (ACP), Cucumber mosaic virus (CMV), Double-Antibody Sandwich Enzyme-Linked Immunosorbent Assay (DAS-ELISA), Immunocapture Reverse Transcriptase Polymerase Chain Reaction (IC-RT-PCR), Monoclonal Antibodies (MAbs), Polyclonal Antibodies (PAbs)