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Sequence Analysis of Monoclonal Antibodies Specific for CMV Coat Protein Subgroup II and Measuring their Affinity Constants with Competitive ELISA

Haggag S. Zein[1,3*] • Jaime A. Teixeira da Silva[2] • Kazutaka Miyatake[3]

[1] Department of Genetics, Faculty of Agriculture, Cairo University, 12 Gamma Street, Giza, 12613, Egypt
[2] Faculty of Agriculture and Graduate School of Agriculture, Kagawa University, Miki-cho, Ikenobe 2393, Kagawa-ken, 761-0795, Japan
[3] Department of Applied Biological Chemistry, Graduate School of Agriculture and Biological Sciences, Osaka Prefecture University 1-1, Gakuen-Cho, Sakai, Osaka, 599-8531, Japan

Corresponding author: * **haggagsalah@gmail.com** or **drzein@cu.edu.eg**

ABSTRACT

Availability of diagnostic methods for plant viruses provides greater flexibility, increased sensitivity, and specificity for rapid diagnosis of virus diseases in disease surveys and epidemiological studies. A total of four hybridomas were generated from BALB/c mice immunized with the m2 strain of *Cucumber mosaic virus* coat protein subgroup II (CMV-CPII). A modified reverse-transcriptase PCR protocol was used to amplify and sequence the light (V_L) and heavy chains (V_H) of the V-genes in order to produce monoclonal antibodies (mAbs) with high specific affinity for CMV-CPII. The V_H and V_L of mAbs produced were characterized and sequenced and compared with the GenBank database. Database analysis of the sequences that encoded the V-genes showed that the light chains of the four hybridomas expressed the family Vκ1A gene, *bb1.1*, while four of the heavy chains genes expressed four genes of the family V_H1/J558, gene V_H130. There was frequent addition of random nucleotides of the N region and expected variation in the lengths of CDR3 regions, which form the center of the antigen binding site. Somatic mutation, junctional diversity and alternative light chains collectively impart specificity to these serologically distinct epitopes. Apparent dissociation constants (K_d) of mAbs were determined by indirect competitive ELISA yielding K_d = 2.7-5.2×10^{-7} M. The serological differentiation of CMV isolates is of importance in breeding for disease resistance and in studying disease epidemiology, so specific mAbs were developed, as one of the goals, to provide tools for serotyping isolates of the virus.

Keywords: affinity, antibody genes, antigen binding site, *Cucumber mosaic virus*, enzyme-linked immunosorbent assay, hybridoma
Abbreviations: ELISA, enzyme-linked immunosorbent assay; **FR**, framework region; **HRP**, horseradish peroxidase; **mAb**, monoclonal antibody; **pAb**, polyclonal antibody; **PBS**, phosphate-buffered saline; **scFv**, single chain Fv; **V_H**, variable heavy chain; **V_L**, variable light chain

INTRODUCTION

The detection of plant pathogens has increased considerably over the last two decades. In addition to time benefits, there is a great advantage in terms of specificity when using serological techniques with specific monoclonal or recombinant antibodies. Among the major developments that have taken place over the past 25 years, it is obvious that the enzyme linked immunosorbent assay (ELISA) is the most significant advance. As an example, the use of ELISA for the detection of *Citrus tristeza virus* has allowed more than three million tests to be performed in Spain from 1997 to 2003 (Fagoaga *et al*. 2005). One of the most widespread viruses in horticultural crops, including some monocotyledons and a great number of dicotyledons, is *Cucumber mosaic virus* (CMV). It is the type species of the *Cucumovirus* genus in the *Bromoviridae* family (Palukaitis *et al*. 1992; van Regenmortel *et al*. 2000). CMV is a tripartite, positive-sense plant RNA virus. CMV occurs naturally worldwide; RNA1 encodes the 1a protein, which, together with the RNA2-encoded 2a protein, forms the viral component of the replicase complex (Hayes *et al*. 1990). RNA2 also encodes a second protein, 2b. The 2b coding region overlaps with the coding region for the C-terminal portion of the 2a protein but is in a different reading frame register. The CMV 2b protein functions in host-specific long-distance movement (Ding *et al*. 1994, 1995) and as a virulence determinant by suppressing post-transcriptional gene silencing. RNA3 encodes two proteins. The 3a protein is a cell-to-cell movement protein (MP), and the 3b protein is the capsid protein (CP), which is also involved in cell-to-cell movement and aphid-mediated CMV transmission from plant to plant (Perry *et al*. 1994, 1998). CMV attacks several economically important plants world-wide and this requires quick and sensitive methods for the reliable identification of mild strains from virulent strains. CMV-CPII strains occur in low concentrations, which may not be easily detectable by ELISA (Yu *et al*. 2005; Zein *et al*. 2007). Availability of these diagnostic methods provides greater flexibility, increased sensitivity, and specificity for rapid diagnosis of virus diseases in disease surveys, epidemiological studies, plant quarantine and seed certification, and breeding programs. Accurate diagnosis depends on the affinity and specificity of the antibody preparation used since high affinity antibodies are essential for the detection of very small amounts of pathogen. Monoclonal antibodies (mAbs) are the most highly selective yet versatile of all biochemical isolation tools (Zimmermann *et al*. 1998; Schillberg *et al*. 2001; Zein *et al*. 2009b). Diagnostic techniques for viruses fall into two broad categories: biological properties related to the interaction of the virus with its host and/or vector (e.g., symptomatology and transmission tests) and intrinsic properties of the virus itself (coat protein (CP) and nucleic acid). Detection methods based on CP include precipitation/agglutination tests; ELISA has been well established in our previous report (Zein *et al*. 2007, 2009b). Viral

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nucleic acid-based techniques like dot-blot hybridization assays and polymerase chain reaction are more sensitive than other methods. Availability of these diagnostic methods provides greater flexibility, increased sensitivity and specificity for rapid diagnosis of virus diseases in disease surveys (Zein *et al*. 2009b). Antibody-based resistance is a novel strategy for generating transgenic plants resistant to pathogens (Schillberg *et al*. 2001). Ectopic expression of recombinant antibodies (rAbs) has great potential to prevent viral infection (Boonrod *et al*. 2004; Zein *et al*. 2007). Advances in gene isolation and an understanding of 'antibody-based resistance', an approach in which expressed antibodies bind to essential proteins, can be used to interfere with pathogenesis (Stoger *et al*. 2004). Sequencing of genes of antibody variable domains provides fundamental information on antibody-producing cells and offers an extraordinarily reliable approach for the characterization of mAbs. Each heavy and light chain contains three hypervariable regions called complementarity-determining regions (CDR) that make up the antibody-binding site (Kramer and Hock 2003). V gene sequencing of the mAbs-specific CMV-CP has been poorly analysed. *In vitro* experiments detailed the ability of mAbs against CMV for neutralizing virus infectivity and block virus infection (Zein *et al*. 2007) while our more recent reports detail the antigenic properties and DNA hydrolyzing activity of these mAbs (Zein *et al*. 2009a, 2009b, 2010a, 2010b, unpublished). In this report we describe mAb production of specific CMV-CP-II and their specificity and binding affinity. This will be suitable for carrying out molecular epidemiology studies and diagnosis of CMV-infected plants. In addition, we attempt to understand the antibody structure and the immunoglobulin genes that encode the binding sites of antibodies against CMV-CPII. Knowledge of the specific immunoglobulin genes used to target common epitopes may lead to insights on pathogen-host co-evolution and block virus infection in plants.

MATERIALS AND METHODS

Virus purification and production of monoclonal antibodies

CMV was propagated in tobacco plant as described by Nitta *et al*. (1988). The plants used in our study were 8-10 week old tobacco (*Nicotiana tabacum* L.) cv. 'Xanthi-NC' with six fully developed leaves. Plants were grown in a greenhouse under normal conditions (20-25°C, 160 mE m^{-1} s^{-1} for 8 h d^{-1} supplemental light). Plants were inoculated mechanically with purified m2-CMV strain, diluted to a final concentration of 50 μg ml^{-1} in 100 mM phosphate buffer, pH 7.0 and CMV was purified from 30–100 g of systemically infected leaf tissue by differential centrifugation using a modified method by Natilla *et al*. (2006). Briefly, infected leaf tissues were ground in freshly-made cold 0.5 M Na-citrate, pH 6.5 buffer containing 0.1% thioglycollic acid and 2 ml chloroform/g of tissue. Extracts were centrifuged for 20 min at 6,000×g and the supernatant was stirred for 15 min with 10% PEG (8000) at 4°C. The solution was then allowed to sit for 1 h in an ice bath to precipitate the virus, followed by another centrifugation for 20 min at 6,000×g. The resultant pellet was resuspended in cold 0.05 M Na-citrate buffer (0.5 ml/g tissue used) containing 2% Triton×100 and adjusted to pH 7.0. The solution was centrifuged for 5 min at 10,000×g, the pellets were discarded, and the supernatant was centrifuged for 2.5 h at 39,000 rpm using an SW-41 rotor. The resulting pellet was resuspended in sterile water overnight at 4°C and centrifuged for 5 min at 10,000×g. The supernatant contained purified CMV. Quantification of protein concentrations in purified samples was performed by the Bradford assay (Sigma, St. Louis, MO). Aliquots were electrophoresed on pre-cast 10–20% Trisglycine gels (Invitrogen). Virus concentration was estimated by measuring the absorbance with a spectrophotometer at 260 nm and an extinction coefficient of 5.0 was used. The BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan). Immunized 8-weeks-old female BALB/c mice were injected subcutaneously with 100 μg in a 0.1 ml volume of phosphate-buffered saline (PBS; 0.01 M phosphate and 0.015 M sodium chloride, pH 7.5) of purified m2-CMV which was mixed with an equal volume of adjuvant purchased from RIBI, Immunochem Research Inc., and containing monophosphoryl lipid A MPL (25 μg), trehalose dicorynomycolate (TDM; 25 μg) and RIBI (Immunochem Research Inc.). 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 in 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 10^5 cells/well with HAT (Hypoxanthine Aminopterin Thymidine) medium (100 μM hypoxanthine, 0.4 μM aminoprotein, 16 μM thymidine, 6 mM Hepes, and 200 μM β-mercaptoethanol). Clones which successfully secreted antibodies specific to CMV were examined by both ELISA and Western blotting. In addition they were 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). After 8 days, the growing hybridoma cells in ELISA-positive wells were cloned by limiting the dilution as follows: the cells of each well were counted in a Burker chamber to reach a concentration of one cell per 200 μl of HAT medium in the well. After 3-4 days, ELISA-screened clones on the plates were coated with CMV-m2, and the cells from the positive wells were used to repeat the cloning procedure twice. Monoclonality of the cells in the wells was evaluated by colony morphology, and the cells were cryopreserved using Cellbanker (DIA-IATRON, Tokyo, Japan) and stored in liquid nitrogen. Since 1999, the above hybridoma clones have been used many times for the production of antibodies using repeated cultures (Zein *et al*. 2007). The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the Osaka University School of Medicine.

Production of antibodies

The frozen hybridomas were retrieved from liquid nitrogen, rapidly thawed in D-MEM medium (Sigma) containing 15% FCS (10^6/ml of medium). Two female mice (BALB/c, 10 weeks old) were treated with 0.5 ml of pristane (Sigma) 10 days prior to inoculation. The mice were injected intraperitoneally with 10^6 hybridoma cells. The hybridomas grew as ascitic tumors in the peritoneal space, and ascitic fluids containing antibodies were collected 10~15 days after the inoculation. The collected ascitic fluids were pooled, and the fluids or corresponding IgG fractions were stored at 4°C until further use.

Purification of mAbs

Either culture supernatants (200 ml) or ascitic fluid (5-10 ml) were precipitated with 50% saturated ammonium sulfate, dialyzed twice for 4 h against 500 vol of 20 mM Tris HCl (pH 8.0) at 4°C. Samples were diluted with the same amount of binding buffer (1.5 M glycine/3.0 M NaCl, pH 8.9), the crude mAbs solution was applied to a protein A-agarose affinity chromatography column (1 ml) HiTrap™ Protein A (Amersham Biosciences), washed with 10 vol of binding buffer, followed with 10 vol of binding buffer containing 1% Triton X-100, and washed with 10 vol of binding buffer. The antibodies were eluted (1-ml fraction) with elution buffer (0.1 M glycine, pH 2.6), and the eluted antibodies was neutralized with collection buffer (1.0 M Tris, pH 9.0) according to the manufacturer's procedure (Amersham Biosciences).

Indirect ELISA procedure

CMV (100 μl; 1 μg/ml) in coating buffer was added to each well of microtiter plates. The plates were incubated overnight in the dark at 4°C. After the buffer was removed, the wells were washed three times with 200 μl of PBST. Standard (100 μl) in the assay buffer and 50 μl of ascitic fluid (or IgGs) diluted in PBS-0.05% Tween (PBST) was added into the wells. After 1 h of incubation at 37°C, the unbound compounds were removed by washing (five times) with assay buffer. Goat anti-mouse IgG (100 μl; H+L specific) conjugated with horseradish peroxidase HRP (Amersham

Pharmacia Biotech, Sweden) in plates were incubated at 37°C for 1 h. After the solution was removed, each well was washed four times with the washing solution (PBS, 0.05% Tween). The absorbance of the developed color was measured at 450 nm. Briefly Western blotting was performed as follows: m2-CMV were loaded onto 12% SDS-PAGE (Bio-Rad), and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech). The culture supernatant was incubated with membrane strips for 2 h at room temperature (RT), and goat anti-mouse IgG conjugated with HRP were used as described above except that enhanced chemiluminescence (Amersham Pharmacia Biotech). The staining signal was detected using the enhanced chemiluminescence detection system according to manufacturer's instructions followed by exposure to Kodak X-ray film (Eastman Kodak, Rochester, NY).

SDS-polyacrylamide gel electrophoresis of proteins

Purified virus CP was analyzed by SDS-PAGE using 10% polyacrylamide gels. CMVCP-II mixed with 5 × protein loading buffer (80 mM Tris HCl, 50% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, 10% (w/v) SDS, pH 6.8 to a final concentration of 1× and heat-denatured by incubation for 5 min in a boiling water bath. SDS-PAGE was performed using pre-cast 10–20% Trisglycine gels (Invitrogen) in an electrophoresis apparatus (Bio-Rad) at 250 V with running buffer (Tris 125 mM, pH 8.3), glycine 960 mM, (w/v) 0.5% SDS until the bromophenol blue band run out of the gel. The gels were stained for 20 min with 0.2% (w/v) Coomassie Brilliant Blue R-250 in 20% methanol and 0.5% (v/v) acetic acid. Destaining was performed with 30% ethanol and 10% acetic acid to a background dye level suitably for photography.

cDNA synthesis, PCR amplification of immunoglobulin variable regions

The total RNAs were prepared from about 10^7 hybridoma cells using ISOGEN (Nippon Gene Co., Tokyo, Japan). Chloroform was added, followed by vigorous agitation, and incubation at RT for 2-5 min, centrifugation at 800 × rpm, and the upper aqueous phase was procured and incubated with isopropanol at RT for 10 min to precipitate the RNA. The RNA pellet was washed with 75% ethanol, air-dried, and dissolved in 0.1% diethylpyrocarbonate water (Sigma). RNA concentration and purity were gauged using absorbance at $OD_{260/280}$. The mRNAs were isolated with Oligotex-dT30 (Super) columns (Takara, Kyoto, Japan) according to manufacturer's instructions. The primers used in the PCR amplification were based on previously published data (Huse *et al*. 1989): for V_H these were 5′-AGGTCCAACTGCTCGAGTCAGG-3′ (forward primer) and 5′-AGGC TTACTAGTACAATCCCTGG GCACAAT-3′ (reverse primer), where the underlined portion of the 5′ primers incorporates an *Xho*I site and that of the 3′ primer an *Spe*I restriction site. The primers for the kappa light chain (Vκ) genes were 5′-CCAGATGTGAGCTCGTGAT-GACCCAGACTC CA-3′ (forward primer) and 5′-GCGCCGTCTAGAATTAACACT-CATTCCTGTTGAA-3′ (reverse primer) where the underlined portion of the 5′ primers incorporate a *Sac*I restriction site and that of the Reverse primers an *Xba*I restriction site for amplification of the Fd and κLc regions, respectively. First-strand cDNA was synthesized from mRNA template with a Moloney murine leukemia virus M-MLV Reverse Transcriptase kit (Takara, Kyoto, Japan) using oligo-dT20 primers (Pharmacia Biotech). The variable regions of V_H and Vκ were amplified from first-strand cDNA using Ex-*Taq* DNA polymerase with 30 cycles of PCR (1 cycle of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C) in 50 µl of the following reaction mixture: 78 mM Tris-HCl (pH 8.8), 17 mM $(NH_4)_2SO_4$, 10 mM β-mercaptoethanol, 2 mM $MgCl_2$, 0.05% W-1 detergent (Takara, Kyoto, Japan), 0.2 mg of BSA/ml, 200 mM each of dATP, dCTP, dGTP, and dTTP, 1 mM of each primer, 10 ng of cDNA, and 2.5 U of Ex-*Taq* DNA polymerase (Takara, Kyoto, Japan). The PCR products were analyzed on a 2% low-melting-point agarose-Tris acetate-EDTA (TAE) gel and visualized with ethidium bromide (final concentration = 0.5 ug/ml). PCR products of an expected size of about 650 bp were excised from the gel and purified with a QIAGEN gel extraction kit as specified by the manufacturer. The amplified fragments were cloned into separated vectors pGEM-T Easy Vector (Promega Biotech), while PCR products were ligated into the plasmid pGEM-T Easy with (3:1~10:1) respectively of a ligation kit (Takara, Kyoto, Japan), for the purpose of transfer into competent cells *E. coli* DH5α.

Sequencing of V regions

The target DNA fragments cloned into pGEM-T Easy were propagated and purified from *E. coli* DH5α by alkaline lysis and sequenced directly with Sequenase (ABI PRISM 310 genetic Analyzer). Cyclic sequencing of these DNAs was performed in both directions using a commercial kit (Thermo Sequence kit, Amersham Pharmacia Biotech) and the M13 forward (5′-CAC GACGTTGTAAAACGAC-3′) and reverse (5′-GGATAACAA-TTTCACACAGG-3′) primers set (Pharmacia Biotech) using an ABI PRISM BigDye Primer Cycle Sequencing Kit.

Determination of mAb-binding affinity

Four mAbs (M2-1, M2-2, M2-3, and M2-4) were raised from two different fusions specific to m2-CMV with no cross reactivity with the other subgroup I. Apparent dissociation constants (Kd) of mAbs were determined by direct ELISA by virus inhibition, and when each mAbs was used at an appropriate concentration it gave 50% maximal binding. The optimum concentration of mAbs-(M21 and M2-2) was 2 µg/ml, while for mAb-M2-4 it was 10 µg/ml (**Table 1**). The mAbs were preincubated for 2 h at RT with an equal volume of buffer with increasing concentration of m2-CMV-CP (30~300 µg/ml). Competitive ELISA was performed in 96-well microtiter plates coated with a constant concentration (1 µg/ml) in carbonate buffer (100 µl/well) of CMV-CPII at 4°C overnight. Aspirate wells and remaining free sites on the microtiter plates were then saturated using 1% Block Ace in TBS (200 µl/well) and incubated for 2 hr at RT. Using polypropylene tubes to dilute antigen into antibody solution, the amount of free mAb in the antibody inhibitor mixture was then measured in an anti-CMV indirect ELISA using CMV-precoated plates which sat for 30 min at RT. The value of Kd was determined by measuring the slope of the linear relationship between li/(A0-Ai) and 1/Ai according to a previously described method (Bobrovnik 2003).

The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Osaka University School of Medicine.

Table 1 *Cucumber mosaic virus* specific monoclonal antibodies and their real affinity.

Fusion	Clones	Immunogen	Optimum µg/ml	Affinity [a] (Kd) M
1	M2-1	m2-CMV	2.0	4.1×10^{-7}
	M2-2		2.0	2.7×10^{-7}
2	M2-3	m2-CMV	nd	nd
	M2-4		10.0	5.2×10^{-7}

[a] The real affinity of the mAbs was determined with competitive enzyme linked immunosorbent assay. The value of Kd can determined by measuring the slope of the linear relationship between [li/(A0_Ai) and 1/Ai] according to a previously described method (Bobrovnik 2003).

Sequence analysis

Sequence analysis was performed using Genetix-Win 5.1 software (Software Development Co., Ltd., Japan). Sequences were initially aligned and checked for stop codons with reference to the IMGT standard for codon numbering (Lefranc and Lefranc 2001). Three genes had stop codons and/or frame-shift deletions in the sequence and were therefore assumed to be pseudogenes. These are not reported in this paper. Since there is the possibility of PCR crossover error, especially with the slower proof reading polymerase used in this study, the alignments were studied carefully to see if any of the sequences could have arisen by PCR crossover. If there was any doubt as to the validity of a sequence after this scrutiny, the sequence was excluded from the results. The percentage similarity between the V_H and V_L sequences with an open reading frame (ORF) and the closest matched mice sequences was determined ("blasted") against the publicly accessible "Ig-Blast" database of

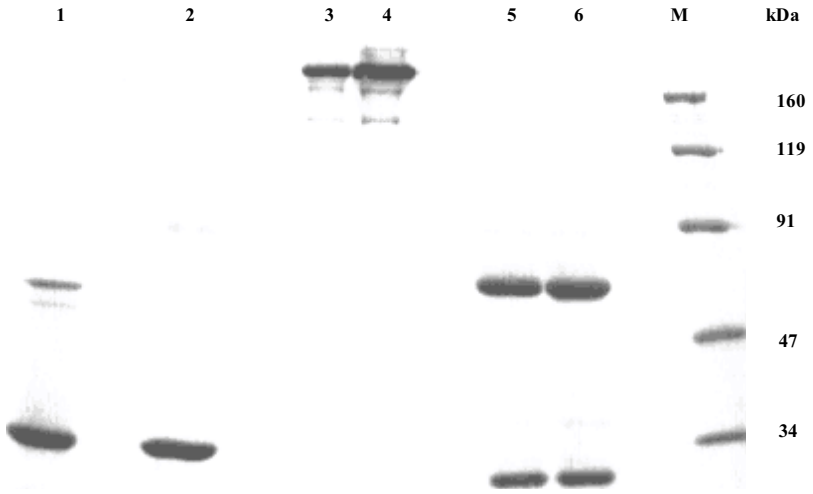

Fig. 1 Purity of the pepo and m2-CMV strains (lanes 1 and 2), mAbs -M1 and M2 under nonreducing condition (lanes 3 and 4) reducing condition (β-mercaptoethanol) (lanes 5 and 6) revealed by 10% SDS-PAGE. M indicates protein molecular weight marker. Numbers on the right side indicate the molecular weight in kDa.

mouse Ig sequences at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/igblast) to determine the closest germline gene of origin, and to identify potential mutations. The CDR position and numbering scheme adopted matched the Kabat numbering (Martin, 1996) and a CDR definition was adopted from Andrew's web site (www.bioinf.org.uk/abs/).

RESULTS

Specific antibody production of CMV-CPII

Immunization of nonautoimmune BALB/c mice with native CMV-CP stimulated antibodies was intriguing. Hybridoma technology allows the production of hybrid cell lines from B cells which secret a single mAb with specific binding, and which can potentially produce unlimited quantities. The serological differentiation of CMV isolates is important in plant breeding for disease resistance and to study disease epidemiology. Specific mAbs have been developed, one of the goals in their development being to provide tools for serotyping isolates of the virus. The present work has focused on CMV- CPII specific mAbs which were produced against the m2-CMV strain. However, the main original finding is the production of CMV subgroup-specific antibodies using a purified m2-CMV CP as the immunogen (**Table 1**). Panels of four hybridoma-specific CMV-CPII were produced by immunization of BALB/c mice, with purified virus preparations. An isotyping test revealed that the mAbs were IgG1. The purity of both virus CP- and IgG-specific CMV were subjected to SDS-PAGE, which revealed proteins with a molecular mass of approximately 26 kDa for the m2-CMV CP, 27 kDa for the mAb-light chains and 55 kDa for the mAb-heavy chains in denaturation conditions, and 180 kDa for IgG in non-denaturation conditions (**Fig. 1**). The binding reactivity of the mAbs against CMV-CPII, based on the use of mAbs for capture and a rabbit polyclonal antibody (pAb) to CMV for detection was developed in infected tobacco plants (**Fig. 2A**). The affinity constant of mAbs to CMV-CPII was measured by competitive ELISA, binding specificity by demonstrating that soluble CMV-CP inhibited mAbs interactions with immobilized CMV-CPII. Consequently, these mAbs will facilitate the development of more specific and standardized diagnostic techniques for CMV-CPII. The reactivity of the mAbs against CMV-CP adsorbed onto solid phase ELISA plates was more sensitive than the direct antigen capture antibody. Given that virus particles may be distorted after adsorption onto a solid phase, this suggests that these mAbs recognize linear epitopes rather than conformational epitopes. On the other hand, polyclonal antisera are unable to differentiate between CMV strains subgroups (Hsu *et al*. 2000; Zein and Miyatake 2007).

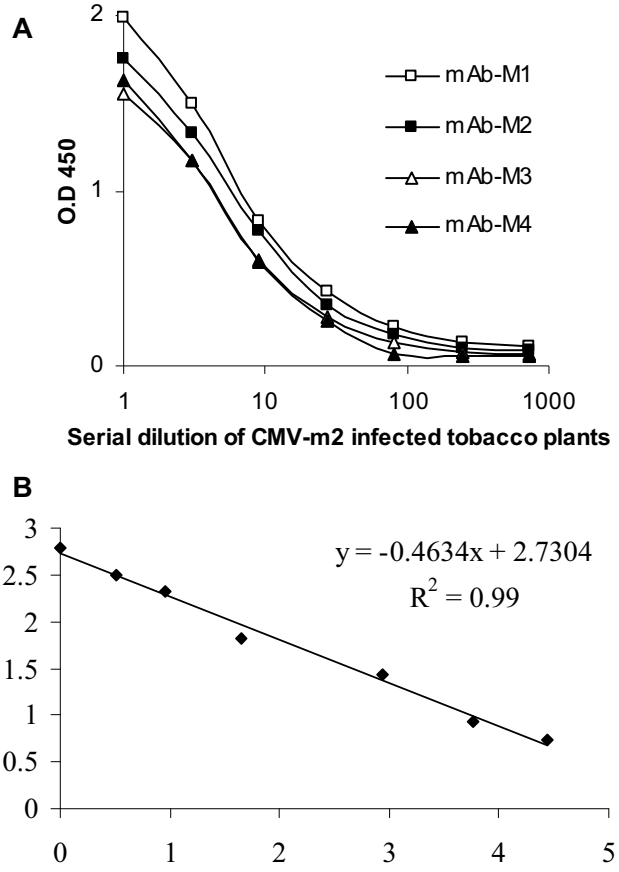

Fig. 2 The relative binding activity of CMV-CPII-specific mAbs in infected tobacco plants. (**A**) Competitive-ELISA for measuring the binding affinity of CMV-CPII specific mAbs, different concentrations of CMV were incubated with a constant concentration of each mAb (2-10 µg/ml). (**B**) The binding constant was calculated from an experimental curve with equation [li/(A0-Ai) and 1/Ai] plot linear regression analysis according to Bobrovnik (2003).

Measurement of the mAbs constant affinity with competitive ELISA

The real affinity and specificity of the mAbs were established with competitive ELISA (**Table 1**). The mAbs were produced in ascitic fluid in mice, and the optimum dilution

Table 2 Summary of variable region gene V, (D), and J genes of the mAbs specific to *Cucumber mosaic virus* coat protein.

Heavy chain						Light chain						
Accession Number	**Isotype**	**V_H Family**	**Germline gene**	**Homology with germline (%)**	**D gene**	**J_H**	**Accession Number**	**Vκ Family**	**Germline gene**	**Homology with germline (%)**	**Jκ**	**CMV Subgroup**
EF672207	IgG1	J558	V130.3	97	DSP2.11	2	EF672221	Vκ1A	bb1	99	4	II
EF672208	IgG1	J558	V130.3	97	DSP2.11	2	EF672222	Vκ1A	bb1	100	4	II
EF672209	IgG1	J558	V130.3	95	DSP2.11	2	EF672223	Vκ1A	bb1	99	4	II
EF672210	IgG1	J558	V130.3	94	DSP2.11	2	EF672224	Vκ1A	bb1	100	4	II

[a] Closest matches from GenBank Databases. Germ line assignments were based on published DNA sequences.

of the mAb solutions for use in competitive ELISA ranged from 2 to 10 μg/ml. The affinity constant of mAbs to CMV-CPII was measured by competitive ELISA, the binding specificity demonstrating that a soluble CMV-CP inhibited mAbs interactions with immobilized CMV-CPII. The mAbs were incubated in solution with the antigen until equilibrium was reached. Then, the antibody, which remains free at equilibrium, was captured by binding to antigen on the microtiter plate and measured by classical indirect ELISA. The Kd value can be determined by measuring the slope of the linear relationship between li/(A0-Ai) and 1/Ai (**Fig. 2B**) according to Bobrovnik (2003). The binding affinity of mAb-M2-2 (2.7×10^{-7}) was about two times higher than that of the other mAbs (M2-1 and M2-4 at 4.1×10^{-7}, 5.2×10^{-7}, respectively) (**Table 1**).

Molecular cloning, sequences and characterisation of mAbs VH and VL genes

Four independent V_H and V_L were cloned and sequenced, the nucleotide sequences of the light and heavy chain V regions and the translated amino acid sequences of the antibodies were deposited in GenBank database. The identities of the V genes were determined by homology to known V genes using the BLAST protocol (Altschul *et al*. 1997). Four antibodies revealed the germline family Vκ1A, gene bb1.1. GenBank accession nos. (EF672221, EF672222, EF672223 and EF672224; **Table 2**). The light chain and heavy chain sequences of the antibodies belong to the κ and γ1 isotypes, respectively. The nucleotide and deduced amino acid sequences of the expressed V_H genes are shown in **Fig. 3**. The V_H genes belonged to V_H1/V_HJ558 GenBank accession nos. EF672207, EF672208, EF672209 and EF672210. The V_L gene specific to m2-CMV strain served as the possible sequence germline family Vκ1A, gene bb1.11 with 99~100% identity (**Table 2**) (Liang *et al*. 2003). However, the V_H genes belonged to germline family V_HJ558, gene V130.3, with 97, 97, 95, and 94% identity, respectively (Chang and Mohan 2005). The D segments belonged to DSP2.11 joining with J_H2 throughout early lymphocyte development (**Table 1**). Abnormal sequences which result in a frame shift or a stop codon were not observed, indicating that these genes were functional (Kabat *et al*. 1991). V_H genes of V_H1/V_HJ558 served as possible sequence of the mAbs-specific CMV-CPII (**Fig. 3**); the amino acid sequences shows a sequence that is essentially identical to V_H V130.3 except for some changes: two silent mutations and four substitutions lead to the replacement of the Aspartic acid at position 32 with Tyrosine ($Asp32^HTyr$), as well as $Asp56^HAsn$ and $Ser61^HPro$, $Ile69^HMet$. In contrast, the V_H-M2-(3 and 4) substitution led to the following replacements: $Met34^HLue$, $Gln82^HHis$, $Ala93^HAsn$ and $Arg94^HTyr$. As the frequency of the PCR error used in this study was 1/5000-10,000 nucleotides, the intraclonal sequence heterogeneity observed here is highly likely not to be derived from PCR errors.

Hybridoma technology allows the production of hybrid cell lines from B cells which secret a single mAb with one binding specificity, which can potentially produce unlimited quantities. The serological differentiation of CMV isolates is of importance in breeding for disease resistance and in studying disease epidemiology, so specific mAbs were developed, as one of the goals, to provide tools for serotyping isolates of the virus. mAbs-specific CMV-CP with serotype specificity has been described by Hsu *et al*. (2000). The long-term goal was to express the antibody genes to exhibit virus resistance through the mechanism of "antibody-based resistance" (Stoger *et al*. 2004). The present work indicates that the high specific affinity of mAbs to CMV-CPII (**Table 1**) plays a crucial role in the effectiveness of antibody-based viral resistance. Furthermore, it may be increased if antibodies are targeted against viral proteins crucial for replication, movement, and transmission. The CMV-CP has a multi-functional role in viral pathogenesis and involved in vector transmission. In this concept, the antibodies bind a broad variety of antigens with high affinity and specificity. Structural information about the molecular interactions between antibodies and antigens helps us to understand the effect of mutations on the affinity and specificity of the antibody (Dougan *et al*. 1998).

The V_L gene of the M2 strain belonged to Vκ1A, gene bb1.11 with ~100% identity (**Table 2**). Moreover, the V_H gene belonged to subgroup-1 V_HJ558, gene $V_HV130.3$ with 95, 95, 93, and 92% identity, respectively. The D segments in the M2 subgroup were DSP2.11 for clones M2-(1, 2, and 3) and DSP2.12 for clone M2-4. In all four clones the J_H were J_H2 antibodies produced and purified from their ascitic fluids by affinity chromatography showing a purity of more

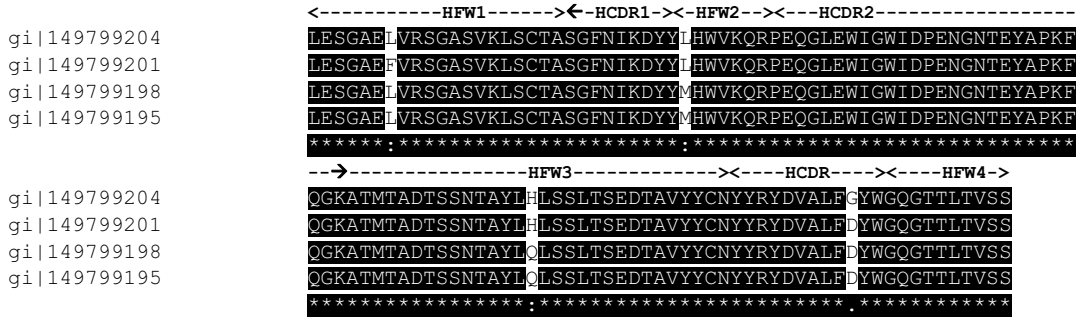

Fig. 3 Multialignments of the amino acid sequences of V_H genes with germline gene in the GenBank database. Amino acid sequence alignment of the heavy chain regions of the CMV-CPII-specific antibodies and germline precursors germline family V_HJ558, gene $V_H130.3$ were identified, through a homology search of the Kabat database. The amino acid residue numbering is according to Kabat numbering. Dots represent residues identical to the corresponding germline gene. CDR, complementarily-determining region; HFW, framework region.

than 95%, as shown in **Fig. 1**.

The expressed V region genes from all the hybridomas were cloned and sequenced from isolated mRNA using constant region gene-specific primers for the V_H and V_L chains. The expressed V_H and V_L genes of these hybridomas are listed in **Table 1**. Their affinity constants were determined to be 2.7-5.2 × 10^{-7} M, measured by competitive ELISA. The heavy and light chain variable regions of four of these antibodies were cloned and sequenced and a predominant anti-CMVCP response was observed.

DISCUSSION

Characterisation of CMV-CP subgroup-specific mAbs

A reliable plant pathogen diagnostic method requires high sensitivity and specificity, especially when it is used to determine the sanitary status of plant materials to be vegetatively propagated. In addition, this is essential when indexing fruit trees as virus-free plants in a nursery from which many thousands of plants will originate by grafting (Helguera *et al*. 2001). Finally, it is essential in testing greenhouse plant material to be used as explant material for tissue culture or micropropagation. A variety of properties have been used to group CMV isolates into three subgroups: IA, IB, and II including antigenic specificity, symptomology, peptide mapping, and nucleic acid analyses (Quemada *et al*. 1989; Palukaitis and Zaitlin 1997; Roossinck *et al*. 1999). For the large-scale diagnosis of viral diseases and to elucidate the antigenic structure of plant viruses at the molecular level, mAbs that are specific to plant viruses have been found to be very useful (van Regenmortel 1984). In addition to the diagnosis, mAbs were applied in identifying the functional regions of the CMV RNA replicase (Hayes *et al*. 1994). However, it is known that some plant viruses, including CMV, are poor immunogens for preparation of antisera by conventional means (Palukaitis *et al*. 1992). The production of mAbs and pAbs are an efficient means of using long-term maintenance to produce antibodies. The mAbs from ascites can yield high amounts and titers of antibody comparable to a cell cultured supernatant. The CMV-CP has a large number of different epitopes on the virion's CP (Hsu *et al*. 2000) with newly discovered and different functions (Zein *et al*. 2009a, 2009b, 2010a, 2010b, unpublished). mAbs have been widely used for disease diagnosis and therapy. Bashir *et al*. (2006) reported that although ELISA with pAbs detected CMV, TAS-ELISA with CMV S-II-specific several mAbs did not produce any positive result and suggested that only CMV S-I genotypes caused infections in cucurbits. Failure of the S-II-specific mAbs was possibly due to two reasons. First, S-II strains occur in low concentrations, which may not be easily detectable by ELISA (Yu *et al*. 2005). Second, although mAbs are more reliable than pAbs, sometimes they can be problematic due to their high specificity. This is because they react with a small portion of a protein such as a virus capsid, usually comprising a very short chain of amino acid residues (epitopes). Nucleotide substitutions can result in different amino acids, which change epitope conformation, a crucial factor in antibody-specificity (Kroese and Bos 1999). The identification of antibody binding sites or epitopes on protein antigens provides essential information for predicting efficacy in antibody development (Liu *et al*. 2002). Most CMV strains are weak immunogens (Palukaitis *et al*. 1992). Nevertheless, in the present study of the m2-CMV strain, the amount of accumulated virus in infected tobacco plants was very low and it required about a month to yield 2 mg/100 g of infected leaves. The weakness of the immunogens were probably due to its instability, yielding low-titered antiserum; in contrast, pepo-CMV strain showed a very fast accumulation rate requiring only five days to accumulate a high yield of virions (1 g/100 g of infected tobacco leaves) and was thus revealed to be a powerful immunogen. Immunization of nonautoimmune BALB/c mice with native CMV-CP strain m2 took over three months with very low-titer antiserum.

Molecular sequence analysis of CMV specific V_H and V_L genes

The expressed V region genes from all the hybridomas were cloned and sequenced from isolated mRNA using constant region gene-specific primers for the V_H and V_L chains. The expressed V_H and V_L genes of these hybridomas are listed in **Table 2**. Their affinity constants were determined to be 2.7-5.2 × 10^{-7} M, measured by competitive ELISA. The heavy and light chain variable regions of four of these antibodies were cloned and sequenced and a predominant anti-CMVCP response was observed. Antigen-specific antibodies develop over the course of the immune response by sequence diversification of germ line genes encoding the V domains followed by selective antigen binding by B cell receptors with the greatest affinity, which stimulates clonal proliferation (Planque *et al*. 2003). Briefly, the procedure followed included the isolation of the variable heavy and light chain domains of the murine mAb from mRNA of hybridoma cells, followed by cloning, sequencing and characterization of Fab. V_H-gene usage was determined and compared to V_H-genes used by antibody fragments of a germline database. The V_H and Vκ regions of four anti-CMV-CPII mAbs generated from two different fusions of BALB/c mice were immunized with native CMV-CP strain m2, and V_H, D, J_H, Vκ, and Jκ were determined (**Table 2**). The light chains of these antibodies could be assigned to one major Vκ1A group with sequence identity between the different light chains of each class ranging from 65 to 100% at the amino acid level (Zein *et al*. 2009, 2010a, 2010b, unpublished). The identity of the V region genes used was determined by searching the GenBank database for homologies to known V region genes using the BLAST protocol while the V_H of this hybridoma derived from the V_H J558 germline family, V130 gene, rearranged with DSP2 and J_H2. These two hybridomas have 100% identity in the nucleotide sequence of the H and L chains, including the sequence at the V(D)J junction, and are assigned to clone A (**Table 2**).

GenBank database of the CMV-specific V_H and V_L

Very little molecular characterization of CMV with mAbs has been achieved so far. Three different clones of a human synthetic antibody library specific to CMV-CP have been isolated; their V_H belongs to the human V_H1 family (Ziegler *et al*. 1995). A scFv phage display library was constructed from mice immunized with CMV specific to both isolates I and II subgroup. V_H belongs to germline family V_H J558 and subfamily V130.3, while the V_L gene belongs to germline Vκ4/5, gene ap4. Synthesis of an scFv antibody targeting CMV-CP, V_H belongs to germline family V_HI/J558, gene V_HF102, while V_L belongs to germline Vκ4/5, gene at4 (Chae *et al*. 2001). Several clones with high reactivity against CMV-CP were isolated from a large semi-synthetic scFv phage display library based on chicken immunoglobulin genes (van Wyngaardt *et al*. 2004). Due to the activation of the immune system as a response to a foreign antigen, maturation of the antibody response takes place, resulting in the production of specific, high-affinity antibodies. Therefore, specific antibodies can be selected using a relatively small, random combinatorial V-gene library derived from an immunized donor (Clarkson *et al*. 1991).

Serology has been shown to be a very useful criterion for the identification and classification of members of several plant virus groups. Virus- or group-specific mAb probes to CMV can be produced, and antibody heavy and light chains can provide an alternative route to mAb coating the surface of the virus, physically covering the sites that bind to plant cell or aphids. Thus, we strongly speculate that production in plant cells of antibody genes with high specificity and binding affinity against CP could interrupt the virus assembly and mediate resistance in plants. The bin-

ding of IgG to the virus protein may prevent effective release of nucleic acid from the CP. Alternatively, it may block a virus from attaching to some site within the cell at an early stage of infection. A systemic study using these procedures may illuminate the mechanism of resistance. Therefore, by expressing this affinity scFv for CMV-CP in transgenic plants strongly supports the concept of antibody-based resistance.

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