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Characterization of *Cucumber mosaic virus* Monoclonal Antibodies (mAbs) Specificity, Neutralization of Infectivity and Gene Sequence

Haggag Salah Zein[1,2*] • Mona Hashem Hussein[2] • Kazutaka Miyatake[1]

[1] Department of Applied Biological Chemistry, Graduate School of Agriculture and Biological Sciences, Osaka Prefecture University, 1-1, Gakuen-Cho, Sakai, Osaka, 599-8531, Japan

[2] Department of Genetics, College of Agriculture, Cairo University, 12 Gamaa Street, Giza, 12613, Egypt

Corresponding author: * **haggagsalah@gmail.com**, **hagzein8@yahoo.com**

ABSTRACT

Monoclonal antibodies (mAbs) were produced against the coat protein of subgroups I and II of two Japanese strains of *Cucumber mosaic virus*, namely pepo- and m2-CMV. We used direct ELISA to determine the apparent dissociation constants (Kd), which were 0.041-27.9 nM. The mAbs were screened for their ability to neutralize virus infectivity *in vitro*. Infectivity was almost completely inhibited when virus preparations were mixed *in vitro* with the homologous mAb prior to inoculation. Aggregation of CMV virions following incubation with mAbs was demonstrated using an immunobinding assay. The long-term goal of expressing these antibody genes is to make the recipient biovital and to exhibit virus resistance. V gene sequences of the mAbs specific subgroup I, V_L expressed from a germline family $V_\kappa 2$, gene bd2, while the V_H genes were derived from germline family V_HJ558, namely gene V_HJ558.45. On the other hand, the V gene sequence of subgroup II-specific mAbs, V_L, expressed from a different germline family $V_\kappa 1$A, gene bb1.1, while V_H genes derived from same germline family V_HJ558 resulted in a different gene, V130.3. The deduced amino acid sequences, i.e. V_H and V_L chains, established from our mAbs-specific subgroups I and II of Japanese strains shared a total nucleotide identity with previously reported antibodies specific to CMV. We concluded that high-affinity CMV-binding antibodies could arise without extensive somatic hypermutation in the variable-region genes because of the expression of appropriate HCDR3s. The present information permits us to analyze the relationship between antibody H and L chain genes and the antigenic domains on antigen.

Keywords: light and heavy chain gene, monoclonal antibodies, triple-antibody sandwich enzyme-linked immunosorbent assay

INTRODUCTION

Plant diseases caused by pathogens are of great economic importance. Damage to crop plants due to virus infections is difficult to assess and actual figures for global crop loss are not available. It is estimated that 20-40% of the world's food production is lost to pests and pathogens, even after a range of controls have been applied, and that without these controls losses would approach 80%. The cost of losses and controls are significant; in the UK in 1996 more than £30 million of wheat were lost to disease, even though £80 million was spent on wheat fungicides (Mathias 2003). *Cucumber mosaic virus* (CMV) attacks several economically important plants in Japan and 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) can be produced in potentially unlimited quantities, and the epitopes with which they react can be identified, thus making well-characterised preparations of these reagents the preferred choice for inclusion in standard assays. Therefore, successful farming is dependent on the ability to suppress the development of plant pathogens. Several methods have been employed to control pathogens, and these rely on vector management, crop rotation, production of pathogen-free plant stocks and seeds, as well as chemical controls, that bear highly undesirable environmental consequences. Traditional plant breeding has been successfully applied to develop resistance to a number of agronomically important crop pathogens. However, this strategy is time-consuming and does not guarantee that the selected resistance will be durable. To minimise losses caused by pathogen infection, the ultimate goal is the production of stable resistant varieties.

CMV is the type species of the genus *Cucumovirus* and is one of the most widespread plant viruses in the world. CMV has a very broad host range in wild and cultivated plants, with more than 1000 known hosts (Palukaitis *et al*. 1992; Roossinck 2002). 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. The strategy of using antibodies to increase pathogen resistance has been successfully demonstrated for human viruses (Marasco 1995) but the application in plant virology has been limited. 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.

The wide repertoire of antibody specificities and affinities that can be generated by natural and artificial immune systems provides a rich pool of potential reagents for analytical use. For each antibody, the affinity, kinetic, and antigen recognition properties, expressed most often in terms of the assay sensitivity and cross-reactivity, determine its utility. Although some novel approaches and methods of producing antibodies, such as recombination, have emerged in recent years, monoclonal antibody technology producing antibodies derived from a single cell line (hybridoma) so far has been a strong approach to obtain antibodies with desired properties (Köhler and Milstein 1975). Sequencing of genes of antibody variable domains provides fundamental infor-

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mation on the antibody-producing cells and offers an extraordinarily reliable approach to the characterization of monoclonal antibodies. The domains are usually constant among antibodies of the same class, except for the N-terminal end of the Fab regions, where the amino acid sequence is different in each antibody. Within the variable domain, there is a framework region, actually constant, and hypervariable loops. Each heavy and light chain contains three hypervariable areas called complementarity-determining regions (CDR) that make up the antibody-binding site. V gene sequencing of the monoclonal antibodies-specific CMV coat protein has been poorly analysed. Ziegler *et al*. (1995) isolated different clones from a human synthetic antibody library, which are specific to CMV-CP, their V_H genes belonging to the human germline family V_HI. Chae *et al*. (2001) isolated one scFv specific to both CMV subgroup I and II, with a phage display library which were contracted from mice that had been immunized with CMV-CP, its V_H gene sequence belonging to germline family V_HJ558, gene V130.3, while the V_L gene belonged to germline family V_κ4/5, gene ap4. Chua *et al*. (2003) synthesized a scFv antibody targeting CMV-CP, V_H which belonged to germline family V_HJ558, gene V_HF102, while V_L belonged to germline family V_κ4/5, gene at4. Recently, van Wyngaardt *et al*. (2004) isolated several clones with high reactivity against CMV-CP from a large semi-synthetic scFv phage display library based on chicken immunoglobulin genes.

This report describes the monoclonal antibody production of specific CMV-CP with their specificity and binding affinity. In addition, we attempt to understand the antibody structure and the immunoglobulin genes that encode the binding sites of antibodies against CMV-CP. The *in vitro* experiments to assess the ability of monoclonal antibodies to react against CMV neutralized virus infectivity and blocked virus infection. 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

Japanese strains pepo- and m2-CMV were propagated in tobacco plants *Nicotiana tabacum* L. 'Xanthi-nc' in a greenhouse and experiments 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 10^5 cells/well with HAT medium (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 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 (Hypoxanthine Aminopterin Thymidine medium) HAT medium in the well. After 3-4 days, ELISA-screened clones on the plates were coated with CMV-CP, and the cells from the positive wells were used to repeat the cloning procedure twice more. 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*. 2006).

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 ascites 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.

Preparation of IgG

The immunoglobulin fraction was separated from ascitic fluid by using a protein A affinity purification kit (Bio-Rad, Hercules, CA). Ascitic fluid was diluted with binding buffer 1:2 (v/v), centrifuged, and filtered through a PF syringe filter, 0.2 μm Acrodisc. Nine milliliters of the diluted ascitic fluid was applied to a column filled with 3 ml of sorbent and then allowed to flow through the chromatographic column with immobilized protein A. The column was washed with 10 bed volumes of washing buffer, eluted with 3-4 bed volumes of eluting buffer, and neutralized with Tris-HCl buffer. After dialysis against PBS buffer, the IgGs were incorporated into indirect ELISA to establish assay parameters of the respective antibodies.

Indirect ELISA procedure

One hundred microliters of CMV (1 μg/ml) in the coating buffer was added to each well of microtiter plates. The plates were incubated overnight at 4°C. After the buffer was removed, the wells were washed three times with 200 μl of PBST. One hundred microliters of standard 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. One hundred microliters of goat anti-mouse IgG (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. The absorbance of the developed color was measured at 450 nm. Western blotting, briefly, pepo-CMV, m2-CMV, were loaded onto 12% SDS-PAGE (Bio-Rad), and transferred to nitrocellulose membrane (Amersham Pharmacia Biotech). The culture supernatant was incubated with membrane strips for 2 h at RT, and goat anti-mouse IgG conjugated with HRP were used as described above except that enhanced chemoilluminescence (Amersham Pharmacia Biotech) was used to detect signals followed by exposure on X-ray film.

Virus neutralizing tests

The neutralizing assay was carried out according to Gera *et al*. (2000) with some modifications. In brief, highly purified mAb-8 specific CMV (final concentration 50 μg ml^{-1}) were mixed with freshly purified CMV (final concentration 5 μg ml^{-1}) and incubated for 4 hours at 4°C. Fifteen healthy tobacco plants at the four-leaf stage predusted with carborondum were mechanically inoculated with potassium phosphate buffer pH 7.2. The negative control was five plants inoculated with pepo-CMV.

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, and the upper aqueous phase was procured and incubated with isopropanol at RT for 10 min to precipitate the RNA. The RNA pellet 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 instruction. 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′ (5′ primer) and 5′-AGGC TTACTAGTACAATCCCTG-GGCACAAT-3′ (3′ 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 Vκ genes were 5′-CCAGATGTGAGCTCGTGATGACCCAG ACTCCA-3′ (5′ primer) and 5′-GCGCCGTC-TAGAATTAACAC TCATTCCTGT-TGAA-3′ (3′ primer) where the underlined portion of the 5′ primers incorporate a *Sac*I restriction site and that of the 3′ primers an *Xba*I restriction site for amplification of the Fd and kappa Lc regions, respectively. First-strand cDNA was synthesized from mRNA template with Moloney murine leukemia virus M-MLV Reverse Transcriptase kit (Takara, Kyoto, Japan) using oligo-dT20 primers (Pharmacia Biotech). The variable regions of heavy chain (V_H) and kappa light chain (Vκ) were amplified from first-strand cDNA using Ex-Taq DNA polymerase with 30 cycles of PCR (1 cycle of 1 min at 94ºC, 1 min at 55ºC, and 2 min at 72ºC) 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 dATP, dCTP, dGTP, and dTTP, 1 mM each primer, 10 ng of cDNA , and 2.5 U of EXTaq 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. PCR products of the expected size 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 (Promega) 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′-CACGACGT TGTAAAACGAC-3′) and reverse (5′-GGATAACAATTTCACA CAGG-3′) primers set (Pharmacia Biotech) using a ABI PRISM BigDye Primer Cycle Sequencing Kit.

Fd or Lc sequences were "blasted" against the publicly accessible "Ig-Blast" database of 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/).

Determination of mAb-binding affinity

Apparent dissociation constants (K_d) 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. Diluted optimum concentration (**Table 1**) involved mAb-8 at 0.4 µg/ml, and m2-1 at 10 µg/ml which were preincubated for 2 h at room temperature with an equal volume of buffer with increasing concentration of CMV-CP (1~10 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-CP 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), incubated for 2 hr at room temperature. Using polypropylene tubes dilute antigen into antibody solution, the amount of free mAb in the antibody inhibitor mixture were then measured in an anti-CMV indirect ELISA using CMV-precoated plates which sat for 30 min at room temperature. The average of the mAbs affinity was calculated 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.

RESULTS AND DISCUSSION

Hybridoma technology allows the production of hybrid cell lines from B cells which secret a single, monoclonal antibody 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 monoclonal antibodies were developed, as one of the goals, to provide tools for serotyping isolates of the virus. Monoclonal antibodies with serotype specificity have been described by Hsu *et al*. (2000). The long-term goal was to express the antibody genes for exhibiting virus resistance through the mechanism of "antibody-based resistance". In this concept, the antibodies bind a broad variety of antigens with high affinity and specificity and the structural information about the molecular interactions between the antibodies and antigens helps us to understand the effect of mutations on the affinity and specificity of the antibody. Nine mouse hybridoma cell lines secreting monoclonal antibodies specific to CMV-CP subgoup I and II were established, and the immunoglobulin classes and subclasses for each were determined (**Table 1**). The antibodies were produced in ascitic fluid in mice, and the optimum dilution of the antibody solutions for use in ELISA ranged from 0.05 to 10 µg/ml (**Table 1**). In testing the recognition specificity of the monoclonal antibodies, ELISA was used as a reference in all as-

Table 1 *Cucumber mosaic virus* immunoglobulin class and subclasses of monoclonal antibodies, and the cross reactivity to CMV subgroups.

Fusion	Clones	Immunogen	Subclass	Optimum	Subgroup specificity [a]	
				µg/ml	I	II
1	4	pepo	IgG1	0.20	++	-
	5			0.05	+++	-
	6			0.15	++	-
2	8	pepo	IgG1	0.45	++	-
3	10	pepo	IgM	0.80	+	-
4	M2-1			2.0	-	++
	M2-2	m2	IgG1	2.0	-	++
5	M2-3	m2 + pepo	IgG1	5.0	-	+
	M2-4		IgG1	10.0	++	++

[a] The specific reactivity of the mAbs specific binding activity against CMV-CP. The prescreening of the mAbs with enzyme linked immunosorbent assay were scored as (+ or -) for measurements corresponding to absorbance (450 nm) values < 0.5 (-), 0.5-1 (+), 1-1.5 (++), 1.5 -2 (+++)

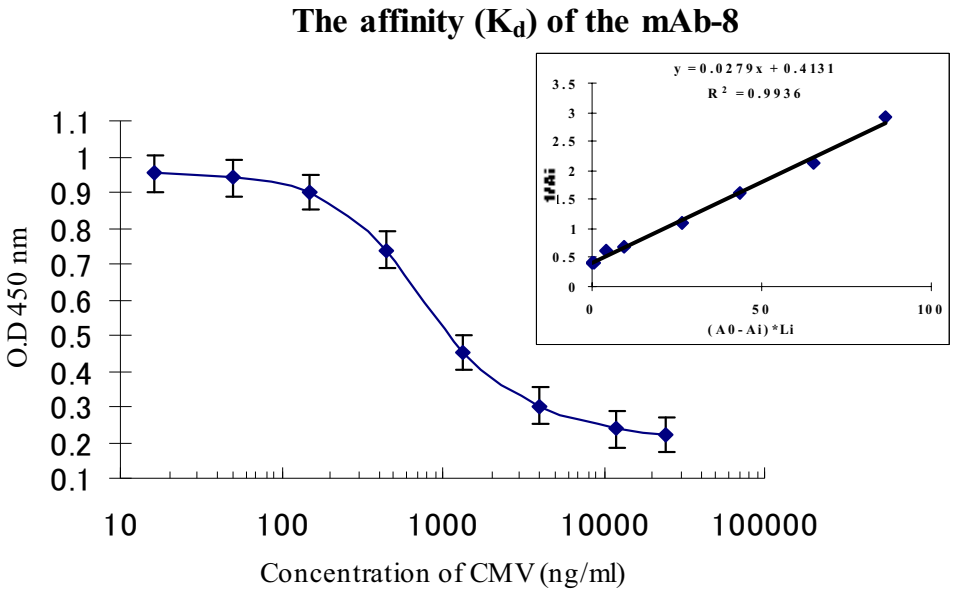

Fig. 1 Competitive ELISA of the binding affinity of mAb-8 against pepo-CMV. The inhibition ability of the different concentration of CMV to constant concentration of mAb-8 (0.45 µg/ml). The binding constant was calculated from the experimental curve by non-linear regression analysis.

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

Heavy Chain							Light Chain					CMV
Clone	Isotype	V_H	Germline	Homology (%)	D gene	J_H	Vκ	Germline	Homology (%)	Jκ	Kd (M)	
8*	IgG1	J558	J558.45	94	DSP2.11	2	Vκ2	bd2	99	2	27.910^{-9}	Sub-I
M2-1*	IgG1	J558	V130.3	97	DSP2.11	2	Vκ1A	bb1	99	4	4.1×10^{-7}	Sub-II
4H10	IgG1	J558	V130.3	93	unknown	2	Vκ2	bd2	99	2	nd	Sub-I
7B3	IgG1	J558	V130.3	94	DSP2.13	2	Vκ2	bd2	96	1	nd	Sub-II
ScFv	IgM	J558	V130.3	99	DSP2.13	3	Vκ4/5	ap4	99	4	nd	Sub-I +II

*Present work

says. The binding affinity and specificity of each mAb was examined with ELISA.

The Affinity constant of two different specificity mAbs to CMV-CP was measured by competitive ELISA. Binding specificity was confirmed by the soluble CMV-CP which inhibited the mAb interactions with immobilized CMV as mentioned above in the Materials and Methods. The monoclonal antibody was incubated in solution with the antigen until an equilibrium was reached; then the free antibodies, which remain unsaturated at equilibrium, were captured by binding to antigen on the microtiter plate and were measured by classical indirect ELISA. The binding constant was calculated from the experimental curve by nonlinear regression analysis according to Bobrovnik (2003). Interestingly, mAb-8 showed a very high specific binding affinity against pepo-CMV strain: 27.9×10^{-9} (**Fig. 1**). However, mAb-M2-1 showed specific reactivity against the m2-CMV strain at 4.1×10^{-7}, ~1000 times lower than mAb-8 (**Table 2**). Therefore, we strongly believe that the high affinity antibodies play a biovital role in virus inhibition in plants.

Virus neutralization tests

As expected, the subgroup specific antibodies all neutralized infectivity in homologous tests of mechanical inoculation. Subgroup-I specific mAb-8 neutralized virus infectivity as did other mAbs (data not shown). The inhibition by mAb-8 was significantly very high and consequently, virus infection was completely inhibited. Fifteen tobacco plants inoculated with virus pretreated with mAb-8 were clearly symptomless (**Fig. 2**). Additionally, the CMV coat protein disappeared in plants and could not be detected either by TAS-ELISA (**Fig. 3**) or Western blotting (**Fig. 4**). The mechanism of neutralization has not been fully explored. Binding of antibodies might alter the surface configuration of virions leading to interference with virus uncoating and release nucleic acids from protein coats within plant cells (Saunal *et al*. 1993). Loss of infectivity has been measured by a local lesion assay (Gera *et al*. 2000) proposed due to aggregation of virions by antibodies, reducing the number

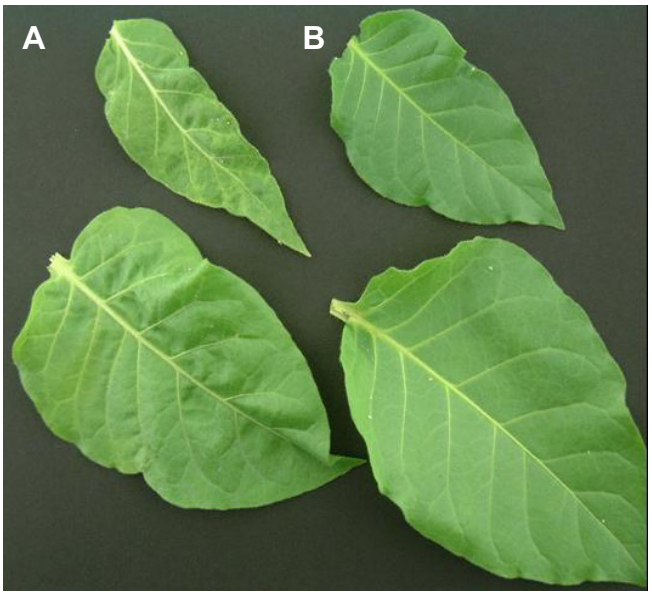

Fig. 2 CMV typical symptoms caused by the virus separated from tobacco plants. Mottle and mosaic in either inoculated or non-inoculated (upper leaves) with CMV, 2 weeks d.p.i (**A**). Plant leaves inoculated with CMV pretreated with mAb-8, either inoculated or non-inoculated leaves showed symptomless (**B**).

of infectious units. To test this hypothesis the effect of antibody treatment on virion configuration and aggregation was analyzed. The virus particles were examined with high sensitivity ELISA as well by Western blotting, and, following antibody treatment, disappeared almost completely with an increase in mAb concentration. These results indicate that besides the aggregation of virions, virus inactivation could also be attributed to covering surface epitopes essential for the host pathogen interaction. The binding of mAbs might inhibit the virus entering the plant cell or interfere with vi-

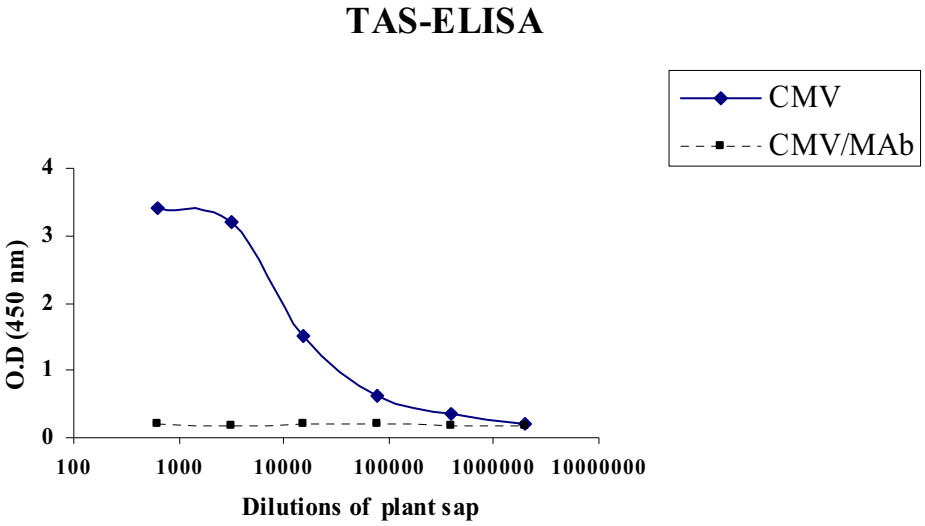

Fig. 3 Triple antibody sandwich enzyme liked immunosorbent assay. Detection of the CMV particles with mAb-8 by TAS-ELISA of inoculated tobacco leaves with CMV or CMV pretreated with mAb-8 at 7-d.p.i. plates were coated with polyclonal rabbit antibodies 1/5000, serial dilution of either CMV or CMV/mAb-8 inoculated leaves extracted plant sap; mAb-8 (0.45 µg/ml) was used for detection the CMV particles trapped with polyclonal antibodies. Goat anti-mouse-HRP conjugated 1/5000, Absorbance was measured at $OD_{450\ nm}$.

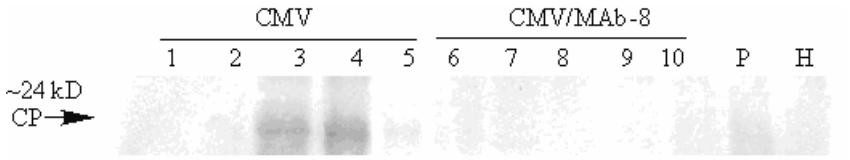

Fig. 4 Western blot analysis of CMV coat protein in inoculated and systemically infected tobacco leaves during one week after inoculation of CMV and CMV/mAb plants. Sample lanes 1-4 represent extracts from control plants inoculated leaves, lanes (1-4) were 1, 2, 5, and 7 d.p.i., respectively while lane 5, was systemic leaves (15 d.p.i) the position of the CMV coat protein (CP) is indicated by an arrow for the ~24 kDa monomer. Lanes 6-10: collected at the same time from plants inoculated with CMV pretreated with mAb-8. Lane P, positive infected plants with CMV, lane H, healthy plant.

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          <-------LFW1-------><-----LCDR1----><----LFW2---><-LCDR2-><----------LFW3-----------><-LCDR3--><---LFW4-->
                              24!  abcde     !34          50!    !56                        89!     !97 Jk
AY556403  DVVMTQTPLTLSVTIGQPASTSC KSSQTLLRSDGKTYLN WLLQRPGQSPKRLIYL VSKLDS GVPARFTGSGSGTDFTLKISRVEAEDLGLYYC WQGTHFPR TFGGGTKLEIK
  8       ....................I.. ....S..D........ ................ ...... ...D.....................V... .......W ...........
AY556401  ---------------------- --------------------------..... ..... ...D.....................V... .......Y ...........
    M2    .........S.P.SL.DQ..I.. R...S.VH.N.N...H .Y..K......L...K ..NRF. ...D..S.....................V.F. S.S..V.F ...S.......
AF193442  .IEL..S.AIM.ASP.EKVTIT. SA.SSV------S.MH .FQ.K..T...LW..S T.N.A. ......S......SYS.T...M....VAT... Q.RSSY.F ...S.......
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Fig. 5 The amino acid sequences of the light chains. Comparison analysis and alignments of the consensus amino acid sequences of V_L regions of the mAbs specific CMV-CP belonging to the germline family Vκ2, gene bd2: (AY556403, pepo, and AY556401), Vκ1A, gene bb1.1: (m2-CMV), and Vκ4/5, gene ap4: (AF193442) from V_L regions database. A dot in the individual sequences denotes amino acids that are the same as the consensus dash donates missed aa for alignments. The framework and complementarily determining regions (CDR) are indicated above the appropriate sequence segments in the figure. The amino acid residue numbering is according to Kabat numbering.

rus disassembly (Saunal *et al*. 1993; Gera *et al*. 2000). Neutralization of infectivity by immune serum is a test that allows differentiation of viruses or virus strains. The technique has been widely used with animal viruses (Morgan 1945). Although not extensively utilized in studies of plant viruses, neutralization has become one of the most important applications in medical virology (Meyer *et al*. 1994). It provides a means not only for identification of a virus but also protective immunity in vaccination. Only a limited number of reports regarding plant virus neutralization are described in the literature (Gold and Duffus 1967; Rochow and Ball 1967; Hsu *et al*. 1984; Aebig *et al*. 1987). This is mainly due to the lack of sensitive and precise assays for measurement of virus infectivity and nonspecific interference with infectivity by proteins present in sera (Rappaport and Siegel 1955; Rappaport *et al*. 1957). mAbs prepared against TMV were found to reduce TMV infectivity strongly when they were incubated with the virus prior to inoculation (Dietzgen 1986). Similar results were obtained with mAbs prepared against CMV (Gu *et al*. 1987; Gera *et al*. 2000).

Utilization of the V gene segments of the CMV-specific H and L chain

Data obtained from DNA analysis were employed for manipulating the affinity and the fine specificity of the monoclonal antibody towards CMV-CP. In our laboratory, we produced monoclonal antibodies against the CMV-CP with unique diversity of binding and kinetic characteristics. Some of these antibodies have found a wide application in the development of immunoanalytical techniques including ELISA, Dot-immunobinding Assay, and Immunocapture RT-PCR (Zein *et al*. 2006). The unique differences in immunoassay characteristics prompted us to examine nucleotide sequences of variable regions of heavy (V_H) and light (V_L) chains to gain information on the identity of the antibodies and the character of their binding sites. For this purpose, we employed a set of primers that binds to the conserved framework region flanking the CDRs. The V_H and V_L regions were prepared from the respective mRNAs by RT-PCR and cloned in order to establish the nucleotide sequences. Overlapping the results of the DNA analysis with the respective immunochemical data obtained for corresponding antibodies was a primary aim of this study. Additionally, amino acid sequences of selected antibodies were

deduced from DNA analysis (**Fig. 5**). We determined the molecular sequence of the expressed and rearranged immunoglobulin V-genes by sequencing. The expressed V region genes from hybridoma were cloned and sequenced from isolated mRNA using constant region gene-specific primers for the H and L chains. 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 (Altschul *et al*. 1997). The V_H and V_κ regions of mAbs anti-CMV specifically generated from two different fusions of BALB/c mice were sequenced and showed sequences almost homologous with corresponding germline genes published in the GenBank Database, which are outlined the V_H, D, J_H, V_κ, and J_κ segments (**Table 2**).

DNA analysis and assessment of new antibody characteristics

The antibodies produced by mAbs- (8 and M2-1) were chosen first for the nucleotide sequence investigation because differences in specificity in their assay and kinetic parameters postulated that interesting changes would appear in the variable regions of their heavy and light chains. Thus, mAb-8 was supposed to be one of the most sensitive toward pepo-CMV in the long term and therefore, it was most often used for the development of various immunoanalytical formats.

The results of the deduced amino acid sequences analysis are presented in **Table 2**. Unpredictably, variable regions of the V_H and V_L chains, established for the mAbs- 8 and M2 clones, shared a total nucleotide identity with previously deduced antibody-specific antibodies although their binging activities were different (**Table 2**). CMV-CP antigenic variation elicits recurrent V_H genes with alternative light chain usage in B cells of the murine host. There is recurrent use of germline antibody gene assemblages to encode antibody-binding domains to coat protein. Comparisons of the molecular sequences of M2 specific antibodies germline family V_HJ558, gene $V_HV130.3$ (**Fig. 6**), indicate that the V_H-domains share a high degree of sequence identity with a previous report (Hsu *et al*. 2000) and have been isolated from numerous hybridoma cells, which are CMV-specific mAbs. Furthermore, the V genes from some of these mAbs have been determined i.e., mAb-10F10, Genebank accession no. (AY556406) cross reacts with CMV (both subgroups), mAb-4H10, Genebank accession no. (AY556402) was sub-I specific, and 7B3, Genebank accession no. AY556404 was sub-II specific (Hsu *et al*. unpublished). On the other hand, Chae *et al*. (2001) isolated the V genes of the mAb-IgM which cross-reacted with both CMV subgroups I+II, Genebank accession no. AF193442. We did a comparative analysis between our mAbs light chain with two mAbs- (4H10, 7B3). Interestingly, V_L has a high identity at the nucleotide level with the used restricted germline gene Vκ2/bd2 (**Table 2**). However, the V_H gene-specific CMV showed recurrent use, where, mAbs M2, 4H10, 7B3, and scFv were also driven from the same gemline family V_HJ558, gene V130.3, while HCDR3 was distinct and caused the antibody specificity (**Fig. 6**). In general, V_HJ558 is the largest V_H family in the mouse and has the most distinct members in the germline configuration. The V_HJ558 germline family is known to be predominantly expressed in murine B cells although this varies between mouse strains (Haines *et al*. 2001). Recently murine J558 alleles have also been used to encode mAbs to distinct protein epitopes on the SARS-CoV (Gubbins *et al*. 2004).

Restricted immunoglobulin gene usage has been observed in immunoglobulins reactive to other TI-antigens such as *H. influenza* (Senn *et al*. 2003), *Streptococcus pneumoniae* (Shaw *et al*. 1995), and *C. neoformans* (Pirofski *et al*. 1995) in mice, and to *H. influenza* in humans (Adderson *et al*. 1991). Similar recurrent antibody responses have been observed in monoclonal antibodies to antigens on other infectious pathogens, including VSV (Kalinke *et al*. 1996) and Influenza A (Kavaler *et al*. 1990). These findings collectively reinforce earlier findings of restricted antibody responses in mice to simple synthetic hapten antigens such as phosphatidyl choline (Seidl *et al*. 1997), phenyl oxalozone (Delassus *et al*. 1995). In contrast, antibody responses to other antigens can be encoded by extremely diverse antibody genes (Akolkar *et al*. 1987) although there may be less biological significance to responses against the types of haptenic-antigens used by those investigators. Recurrent antibody responses are not limited to single strains of mice as anti-oxazolone hapten antibody responses utilize the same V-genes in 10 different strains of mice independent of MHC background (Kaartinen *et al*. 1991). V_H gene usage to PS antigens has been most extensively studied in murine monoclonal antibodies produced in mice. Antibody responses to *C. neoformans* are encoded by a highly restricted use of V_H gene families although in contrast the murine response to different serogroups results in use of different V_H gene families (Pirofski *et al*. 1995).

The central role of HCDR3 in establishing antigen specificity is the consequence of the vast potential for diversity in the way that its sequence and length (structure) are generated. Diversity in HCDR3 derives from the inclusion of a D_H gene segment, great flexibility in the site of gene segment rearrangement, and the random addition of non-templated (N regions) and templated (P junctions) nucleotides

Fig. 6 Comparison analysis and alignments of the consensus amino acid sequences of V_H regions of the mAbs specific CMV-CP belonging to the V_HJ558 V-gene family, VH130.3 germ-line V-gene. MAb-M2 and three different mAbs from data base. A dot in the individual sequences denotes amino acids that are the same as the consensus. Dash denotes non-amino acid which for alignment. The framework and complementarily determining regions (CDR) are indicated above the appropriate sequence segments in the figure. The amino acid residue numbering Kabat numbering.

at the rearrangement junctions this diversity makes HCDR3 the focus of the initial somatic diversification of the antibody repertoire Kirkham and Schroeder (1994) (**Fig. 6**). Tyrosine side chains were capable of mediating most of the contacts necessary for high-affinity antigen recognition, and thus, it seems likely that the overabundance of tyrosine in natural antigen-binding sites is a consequence of the side chain being particularly well suited for making productive contacts with antigen. Interestingly the genes encoding the heavy chain variable region of these antibodies displayed evidence of only minimal somatic hypermutation. The crucial role of heavy-chain CDR3 in high–affinity CMV recognition was suggested to be dominant. We suggest that high-affinity CMV-binding antibodies can arise without extensive somatic hypermutation in the variable-region genes because of the expression of appropriate HCDR3s. Furthermore, we decided that the negative charge on the acetate group in the CMV coat protein was partially neutralized by a hydrogen bond with the phenolic hydroxyl group of tyrosine that exists in HCDR3 (**Table 2**).

The βH-βI loop of the coat protein is a critical determinant of viral pathogenicity and has been shown to contain major immunodominant neutralization domains. The decapeptide sequence DDKLEKDE (aa198–205) probably contains essential contact residues, in which K (lysine) and E (glutamic acid) are both hydrophilic and negatively charged and might be important to constitute the epitope. Tyrosine side chains that exist in the antigen combining site might be capable of mediating most of the contacts necessary for high-affinity antigen recognition, and, thus, it seems likely that the overabundance of tyrosine in natural antigen-binding sites is a consequence of the side chain being particularly well suited for making productive contacts with antigen. Interestingly the genes encoding the heavy chain variable region of these antibodies displayed evidence of only minimal somatic hypermutation. The light chain distribution of anti-CMV Ab, which is characterized by the predominance of kappa light chains, has shown that V_{κ}II light chains are the most commonly expressed light chains among clonally purified serum anti-CMV antibodies from responses to immunization. Furthermore, a number of these antibodies show identical amino acid homology with the translated amino acid sequence of the germline V_{κ}IA, gene bb1.1

Thus, we strongly speculate that production in plant cells of antibody genes with high specificity and binding affinity against coat protein could interrupt the virus assembly and mediate resistance in plants. The binding of IgG to the virus protein may prevent effective release of nucleic acid from the protein coat. 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. Antibody-based resistance from the results allowed us to conclude that CMV-CP reacted with high affinity and specificity.

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