Molecular analysis of monoclonal antibodies specific to *Cucumber mosaic virus* coat protein: restricted light chains and alternative heavy chain partners

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Abstract: A total of 10 hybridomas were generated from five fusions of BALB/c mice immunized with *Cucumber mosaic virus* coat protein (CMV-CP) subgroup I. A modified reverse-transcriptase PCR protocol was used to amplify and sequence the V-genes' light and heavy chains. Database analysis of the sequences that encoded the V-genes showed that the light chains of the 10 hybridomas expressed the family Vκ2 gene, bd2, while four of the heavy chains genes expressed four genes of the V_H1/J558 family, three of V_H5/V_H7183, and three of V_H8/V_H3609. There was frequent addition 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 direct ELISA yielding K_d = 3.0-38.5 nM. We conclude that high-affinity CMV-binding antibodies can arise without extensive somatic hypermutation in the variable-region genes because of the expression of appropriate HCDR3s. This information permits analysis, not previously possible, of the relationship between antibody H and L chain genes and the antigenic domains on antigen. Knowledge of the specific immunoglobulin genes for common epitope may lead to insights on pathogen-host co-evolution and helps blocking the virus infections in plants

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1. Introduction

Cucumber mosaic virus (CMV) is the type species in the genus *Cucumovirus*, family *Bromoviridae*. CMV has the broadest host range of any known virus, infecting more than 1,000 species of plants, including monocots and dicots, herbaceous plants, shrubs, and trees (Palukaitis et al. 1992). The antigenic structures of many plant viruses have been investigated through the identification of epitopes recognized by monoclonal and polyclonal antisera, which has been accomplished largely through the use of synthetic peptides, although early work was done with coat protein (CP)-derived peptide fragments and some information has emerged from sequence and mutational analysis. Serologically, the two subgroups are closely related, as shown by the cross reactions of polyclonal antibodies. Some monoclonal antibodies produced against the CPs of subgroups I and II can differentiate the two, indicating the presence of unique epitopes for each (Zein et al. 2007). Antibody genes from hybridomas, as well as diverse repertoires of antibody genes from immunised and non-immunised donors have been displayed in this way (Marks and Marks, 1996). Accurate diagnosis depends on the affinity and specificity of the antibody preparation used, and high affinity antibodies are essential for the detection of very small amounts of pathogen. Monoclonal and recombinant antibodies 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. Also, assay specificity can be designed to detect one or more antigens by incorporating several different antibodies (Zein and Miyatake, 2007). Previously, the molecular structure of CMV was determined at 23 Å resolution by cryoelectron microscopy and image reconstruction. In examining which capsid protein domains are clearly exposed on the virus, Liu et al. (2002) noted that the amino acid sequence of the βH-βI loop forms a conspicuous, negatively charged

electrostatic field on the surfaces of virions (Liu et al. 2002) and has fundamental aspects that are conserved among cucumoviruses: its structure plays a role in aphid vector transmission (Perry et al. 1998), A prominent feature on the surface of the *Cucumber mosaic virus* (CMV) capsid is a negatively charged loop structure, the βH-βI loop. Six of eight amino acids in this protein loop are highly conserved among strains of CMV and other members of the cucumovirus family. Liu et al. (2002) individually changed five of these amino acids to alanine or lysine (positively charged), creating nine mutants. The mutants formed virons and accumulated to wild-type levels, but eight of the nine mutants were defective in aphid vector transmission. Since the disruption of charged amino acid residues in the βH-βI loop reduces or eliminates vector transmissibility without grossly affecting infectivity or viron formation, they proposed that this sequence or structure has been conserved to facilitate aphid vector transmission. The long-term goal of generating CMV-resistant transgenic plants using antibody genes depends on the molecular basis of antibody structure and diversity. Knowledge of the molecular structure of epitopes provides the possibility to predict *in silico* the cross-reactivity of antibodies.

The epitopes can directly be used to screen the databases for identical or similar sequences, either by performing a general search or a specific search among family members. Antibody-based resistance is a novel strategy for generating transgenic plants resistant to pathogens (Schillberg et al. 2000). 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. Recent advances in gene isolation and an understanding of 'antibody-based resistance', an approach in which expressed antibodies bind to essential proteins, were used to interfere with pathogenesis. The introduction of antibodies is an attractive alternative and effective resistance against a number of plant viruses has been demonstrated (Zimmermann et al., 1998) as well resistance against other phytopathogens including fungi (Peschen et al., 2004).

The aim of this work was to examine the molecular structure, in particular, the nucleotide sequences of the heavy and light chain genes which are specific to CMV-coat protein (CP) specific for subgroup I, and their respective affinity. We also attempt to understand the antibody structure and the immunoglobulin genes that encode the biding sites of antibodies against CMV-CP, the high affinity heavy V_H and light V_L chains variable regions (Fab) of these antibodies for long-term CMV resistance of important transgenic crop plants.

2. Material and Methods

2-1 Plant material and virus purification

Tobacco plants (*Nicotiana tabacum* cv. 'Xanthi-nc') and *Nicotiana benthamiana* plants at the five-leaf stage were used for inoculation. The pepo strain of CMV (subgroup IA) was originally obtained from *Cucurbita pepo* in Japan; CMV propagated in tobacco was purified as described by Nitta *et al.* (1988). Plants were inoculated mechanically with purified CMV strain pepo, diluted to a final concentration of 50 μg ml^{-1} in 100 mM phosphate buffer, pH 7.0. Inoculated plants were grown in a growth chamber (NK systems) at 24°C with a 14 h light/10 h dark cycle.

2-2 Immunization

Immunized eight-weeks old BALB/c mice (Nippon SLC Co., Japan) were injected subcutaneously with 100 μg of purified CMV strain pepo in 0.1 ml phosphate-buffered saline (PBS; 0.01 M phosphate and 0.015 M sodium chloride, pH 7.5), which was mixed with an equal volume of adjuvant containing TDM plus MPL (Sigma). 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 three 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 (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 amino protein, 16 μM thymidine, 6 mM Hepes, and 200 μM 2-mercaptoethanol). Clones that successfully secreted antibodies specific to CMV were examined by both ELISA and western blotting. Furthermore, these positive hybridoma cells were subcloned by a limiting dilution method in the presence of thymocytes of BALB/c mice as feeder cells according to standard protocols (Harlow and Lane, 1988).

2-3 Production of monoclonal antibodies (mAbs)

mAbs were produced following the intraperitoneal injection of 10^7 hybridoma cells into intraperitoneal 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.

2-4 Preparation of IgG

The immunoglobulin (IgG) fraction was separated from ascitic fluid by using 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.

2-5 Determination of the real binding Affinity mAbs to CMV-CP

The binding affinity of each mAb was determined by virus inhibition, with each mAb used at an appropriate concentration that gave 50% maximal binding (Fujii et al., 1998). Diluted optimum concentrations were prepared: mAbs-(4, 5, 8, and 52) used 100 ng/ml; mAb-(6 and 7) used 200 ng/ml; mAb-8 used 400 ng/ml. These dilutions were preincubated for 2 h at room temperature (RT) with an equal volume of buffer containing a range of CMV-CP concentrations (1, 3, 30, 90 μg/ml). Competitive ELISA was performed on 96-well microtiter plates coated with CMV-CP at a constant concentration (1 μg/ml in carbonate buffer (100 μl/well) at 4°C overnight. Wells were aspirated and remaining free sites on the microtiter plates were then saturated using 1% Block Ace (Nippon SLC Co., Japan) in TBS (200 μl/well), and incubated for 2 h at RT. Using polypropylene tubes, the CMV-CP was concentrated to(1, 3, 30, 90 μg/ml) into optimum concentrations of antibody solution, and the amount of free mAb in the antibody inhibitor mixture was placed for 30 min at RT using CMV-precoated plates. The average mAb affinity was calculated according to the method of Bobrovnik (2003).

1-6 RNA isolation and cDNA synthesis

Total RNAs prepared from about 10^7 hybridoma cells were cultured in minimum essential medium containing 15% (v/v) fetal bovine serum, 2% (v/v) glutamine (200 mM), and 1% (v/v) gentamicin (10 mg/ml) in a 5% CO_2 humidified incubator (BIO-RAD) using ISOGEN RNA extraction buffer (Nippon Gene Co., Tokyo, Japan). Chloroform was added, followed by vigorous agitation, and incubated at RT for 2-5 min, centrifuged, and the upper aqueous phase was retrieved then 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 $OD_{260/280}$. The mRNAs were isolated on Oligotex-dT30 (Super) columns (Takara, Kyoto, Japan), as specified by the manufacturer's instructions. The primers used in PCR amplification were based on data by Huse et al. (1989): for V_H, 5′-AGGTCCAACTG**CTCGAG**TCAGG-3′ and 5′-AGGCTT**ACTAGT**ACAATCCCTGGGCACAAT-3′, where the underlined portion of the 5′ primers incorporates an *Xho*I site and that of the 3′ primer an *Spe*I restriction site. Primers for the Vκ genes were 5′-CCAGATGT**GAGCTC**GTGATGACCCAGACT-CCA-3′ and 5′-GCGCCG**TCTAGA**ATTAACACT-CTTCCTGTTGAA-3′ where the underlined portion of the 5′ primer incorporates a *Sac*I restriction site and that of the 3′ primer an *Xba*I restriction site for amplification of the Fd and κLc regions, respectively. First-strand cDNA was synthesized from mRNA template with the Moloney murine leukemia virus M-MLV Reverse Transcriptase kit (Takara, Kyoto, Japan) using oligo-dT20 primers (Pharmacia Biotech). Variable heavy and light chains were amplified from first-strand cDNA using Ex-*Taq* DNA polymerase with 30 cycles of PCR (1 cycle = 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. PCR products of expected size 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 pGEM-T Easy Vector (1:1, 3:1, 10:1) respectively, according to manufacture protocol (Promega, Biotech) and ligated with Ligation High Kit (Takara, Kyoto, Japan), for the purpose of transforming into competent *E. coli* DH5α cells.

2-7 DNA sequence of variable region of the heavy and light chain genes

Direct sequencing of the treated DNA fragments was made using M13 primer and ABI PRISM BigDye Primer Cycle Sequencing Kit reagent following the manufacturer's instructions (Applied Biosystems) and run on an ABI Prism 310 Genetic

Analyzer (Applied Biosystems) using ABI Prism Sequencing Analysis 3.7 software for data analysis. The PCR product was analyzed and sequenced using M13 primer sequencing of the V regions. Cyclic sequencing of these DNAs was performed in both directions using a commercial kit (Thermo Sequence kit, Amersham Pharmacia Biotech), M13 forward (5′-CACGACG-TTGTAAAAACGAC-3′) and reverse (5′-GGATAACAATTTCAC-ACAGG-3′) primers set (Pharmacia, Biotech). 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. CDR position and numbering adopted Kabat numbering (Martin, 1996) and CDR definition was adopted from Andrew's web site (www.bioinf.org.uk/abs/).

3. Results

3-1 Production and characterisation of CMV-CP specific mAbs

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 that secret a single monoclonal antibody with specific binding, and 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, and one of the goals in their development being to provide tools for serotyping viral isolates. mAbs with serotype specificity have been previously described (Hsu et al. 2000). The antibodies bind a broad variety of antigens with high affinity and specificity and the structural information about the molecular interactions between them helps us to understand the effect of mutations on the affinity and specificity of the antibodies.

In this study, 10 mouse hybridoma cell lines secreting monoclonal antibodies specific to CMV-CP subgroup I were established, and the immunoglobulin classes and subclasses for each were determined (Table 1). The antibodies were produced in ascites fluids in mice, and the optimum dilution of the antibody solutions for use in ELISA ranged from 0.05 to 0.4 µg/ml (Table 1). In testing the recognition specificity of the mAbs, the binding affinity and specificity of each mAb were examined with ELISA and western blotting (data not shown).

3-2 Measurement of the Affinity constant of mAbs to CMV-CP by ELISA

The affinity constant of MAbs to CMV-CP was measured by ELISA binding specificity by demonstrating that soluble CMV-CP inhibited mAbs interactions with immobilized CMV. The mAb was 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 binding constant was calculated from the experimental curve with equation [1/Ai vs. (A0-Ai)* Li] plot linear regression analysis according to Bobrovnik (2003) (Figure 1). Two mAbs, -5 and -8, showed the highest binding activity, 3.0 and 5.0 $\times 10^{-9}$, respectively while the binding activity of mAbs-(4, 52, 521, and 6) against CMV-CP was 1.36, 1.43, 2.7, and 3.85 $\times 10^{-8}$, respectively (Table 1). This method was valuable for measuring true dissociation and was found to be simple, reproducible, and accurate. It is therefore possible to assess the contribution of different germlines in the assay by affinity maturation even when they have

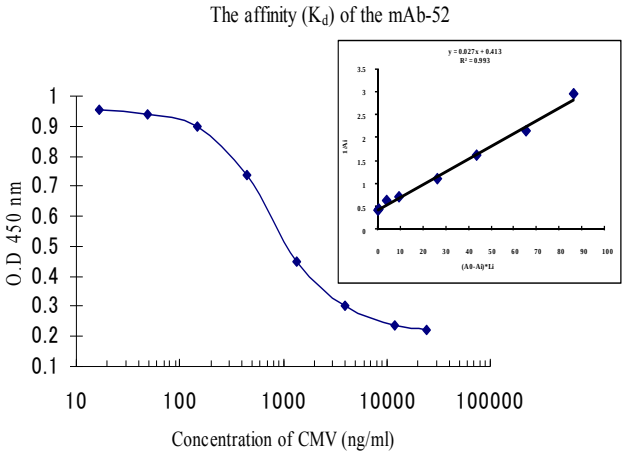

Figure 1. Competitive-ELISA for measuring the binding affinity of CMV-specific mAb-52. Different concentrations of CMV were incubated with a constant concentration of MAb-52 (0.45 µg/ml). The binding constant was calculated from the experimental curve with equation [1/Ai vs. (A0-Ai)* Li] plot linear regression analysis according to Bobrovnik (2003).

A. V_H-8/V_H 3609.CB17

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          ←--------------HFW1-------------><-HCDR1->←----HFW2-----><------HCDR2-----><---------HFW3--
                                      31! 35!ab             50!             !65
      CB17  QVTLKESGPGILQPSQTLSLTCSFSGFSLS TSNMGIG WIRQPSGKGLEWLA HIWWNDDKYYNPSLKS RLTISKDTSNNQV
       VH6  ............................. ..G..V. ............. ......S.....A... ........Y....
       VH7  ............................. ..G..V. ............. ..L...S.....A... ........Y....
     VH122  ............................. ..D..V. .....P.......L ..L...S.....A... ........Y....
             --------------------><---------HCDR3--------><---HFW4--->
            82!  abc      90!        100!abcdefghijk    !102
      CB17  FLKITTVDTADTATYXXXX N         D      J
       VH6  ....AS.........YCAR MGVI YYYGSSYVGY- FDV WGQGTTLTVSS
       VH7  ....AN..............  ....  ...........  ...  ...........
     VH122  ....AN..............  ....  ...........  ...  ...........

B. VH-5/VH7183.68-5N
          ←--------------HFW1------><-HCDR1->←----HFW2-----><------HCDR2-------><---------HFW3-------
                                      31!   !35             50! a!             !65
      68-5N      GLVQPGGSLKLSCAASGFTFS SYGMS WVRQTPDKRLELVA TINSNGGSTYYPDSVKG RFTISRDNAKNTLYLQMSS
       VH-8  ESGG..................... ..... ............... ................. ...................
              -------------><---HCDR--->←---HFW4--->
            82! abc 90!    95!               !102
      68-5N  LKSEDTAMYYCAR N   D      J
       VH-8  .............  EE YY GKAWFVY WGQGTLVTVSA

C.VH-5/VH7183.14
            ←--------------HFW1-------------><-HCDR1->←----HFW2-----><------HCDR2-----><---------HFW3----
                                      31!    !35            50! a!              !65
   VH7183.14  EVKLVESGGGLVKPGGSLKLSCAASGFAFS SYDMS WVRQTPEKRLEWVA TISSGGSYTYYPDSVKG RFTISRDNARNTLY
       VH5  ............................. ..... ............... ......GS......... .........K....
       VH52 ............................. ..... ............... Y.....GS.....T... .........K....

            ------------------->←-----HCDR----><---HFW4---->
            82! abc       90!     95!              !102
   VH7183.14  LQMSSLRSEDTALYYCAR N   D      J
       VH5  ......K.....M..... EE YY GKAWFVY WGQGTLVTVSA
       VH52 ......K.....M..... .G .. NGYEGD. .....S....S

D. VH-1/VHJ558.104B
            ←--------------HFW1-------------><-HCDR1->←----HFW2-----><------HCDR2--->←---------HFW3----
                                      31!   !35              50! a!              !65
     VH104B  QVQLQQPGSVLVRPGTSVKLSCKASGYTFT SYWMH WAKQRPGQGLEWIG EIHPNCGNINYNEKFKG KATLTVDTSSSTAY
       VH-11  ............................. ..... .V............ .....S........... ..............
            ------------------><-HCDR--><---HFW4--->
            82! abc        90!    95!         !102
     VH104B  VDLSSLTSEDSAVYYCAR N   D  J
       VH-11  ................I GG YY FDY WGQGTTLTVAS

E.VH-1/VHJ558.45
            ←--------------HFW1-------------><-HCDR1->←----HFW2-----><------HCDR2--->←---------HFW3----
                                      31!   !35              50! a!              !65
     J558.45  QVQLQQSGAELAKPGASVKMSCKASGYTFT SYWMH WVKQRPGQGLEWIG YINPSTGYTEYNQKFKD KATLTADKSSSTAY
       VH-521 .....E..P..................... K.... .............. .....S...D......G .....V........
              ------------------->←-HCDR-->←---HFW4--->
             82! abc        90!    95!         !102
     J558.45  MQLSSLTSEDSAVYYCAR N   D   J
       VH-521 .E.H...........TN   PYY RYDY WGQGTTLTVSS

F. VH-1/VHJ558.51
            ←--------------HFW1-------------><-HCDR1->←----HFW2-----><------HCDR2--->←---------HFW3--
                                      31!   !35              50! a!            !65
     J558.51  QVQLQQSGAELVRPGASVTLSCKASGYTFT DYEMH WVKQTPVHGLEWIG AIDPETGGTAYNQK FKGKATLTADKSSSTAY
       VH4  ..................K...R.LI.... ...V. .............. ..L.GN.N.....................
       VH9  ..................K...R.LI.... ...V. .............. ..L.GN.N.....I .................
              ------------------><--HCDR--->←---HFW4--->
            82! abc        90!    95!       !102
     J558.51  MELRSLTSEDSAVYYCTL N   D   J
       VH4  ...S............N   PYY RFDY WGQGTTLTVSS
       VH9  ...S............N   ... .Y.. ...........
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Figure 2. Multalignments of the amino acid sequences of V_H genes with germline gene in GenBank database. (A) Alignment of amino acid of V_H genes of the mAbs-(6, 7, and 122) with the most closely related germline family V_H-8/V_H 3609, gene CB17. (B) Alignment of amino acid of V_H genes of mAb-8 with the most closely related germline family V_H-5/V_H7183, gene 68-5N. (C) Alignment of amino acid of V_H genes of the mAbs-(5 and 52) with the most closely related germline family V_H-5/V_H7183, gene 14. (D) Alignment of amino acid of V_H genes of mAb-11 with the most closely related germline family V_H-1/V_HJ558, gene 104B. (E) Alignment of amino acid of V_H genes of mAb-521 with the most closely related germline family V_H-1/V_HJ558, gene 45. (F) Alignment of amino acid of V_H genes of mAbs-(4 and 9) with the most closely related germline family V_H-1/V_HJ558, gene 51. A dot in the individual sequences denotes amino acids that are the same as the consensus. A dash in the individual sequences denotes a deletion. The framework and complementarity determining regions (CDR) are indicated above the appropriate sequence segments. The numbering of amino acid residues is according to Kabat (1991).

Fig. 3

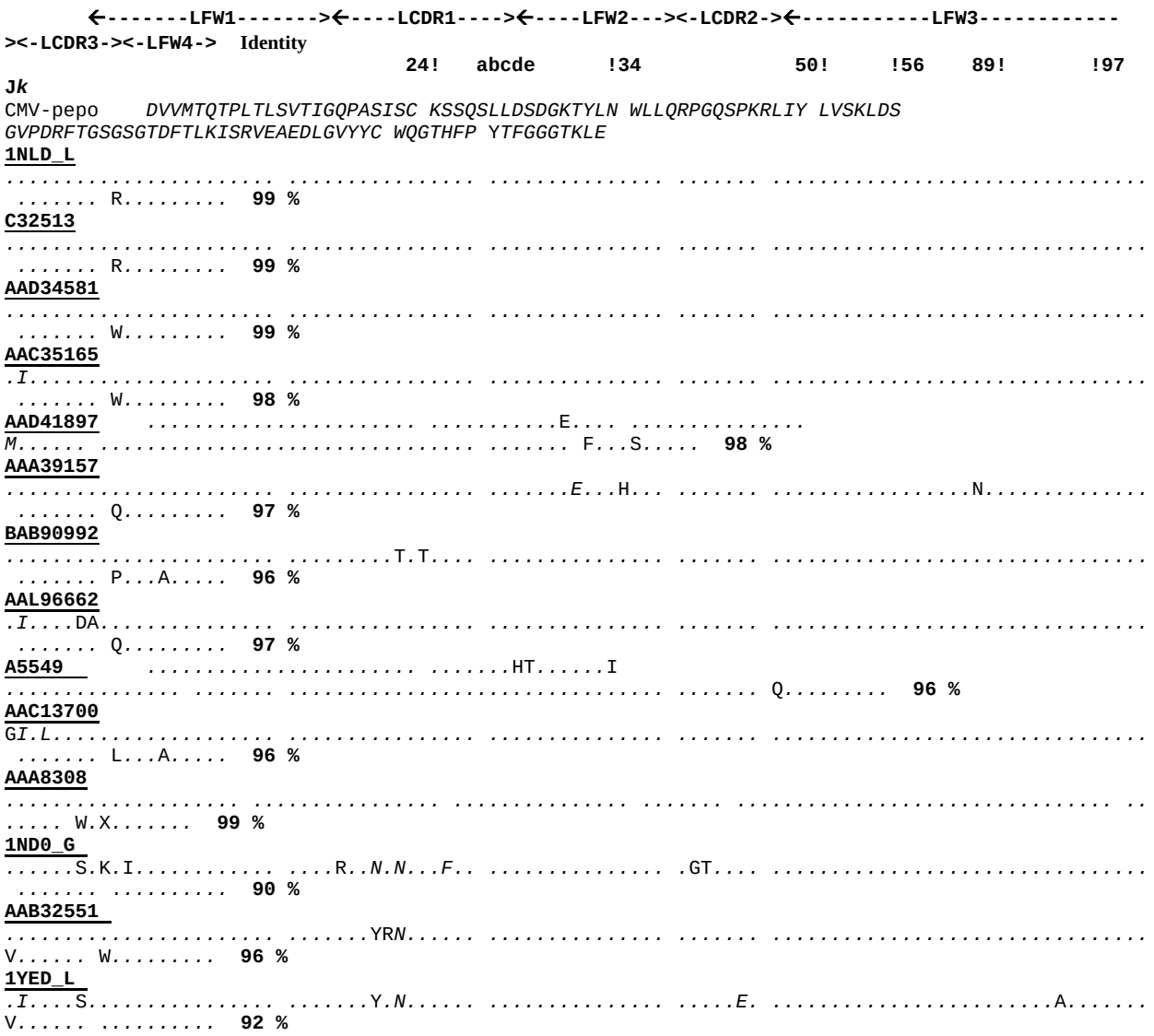

Figure 3. Multialignments of the amino acid sequence of light chain gene of CMV-specific mAbs with GenBank database VL accession numbers. Dots represent residues identical to the corresponding germline. The numbering of amino acid residues is according to Kabat (1991).

Table 1. The real affinity of CMV-CP specific mAbs with indirect competitive ELISA

Fusion	Clones	Immunogen	Optimum μg/ml	Affinity [a] (Kd) M
1	4	Pepo	0.20	1.36×10^{-8}
	9		nd	nd
	11		nd	nd
	521		0.10	1.43×10^{-8}
2	5	Pepo	0.05	3.0×10^{-9}

	8		0.10	5.0×10^{-9}
	52		0.45	2.7×10^{-8}
3	6	Pepo	0.2	3.85×10^{-8}
	7		nd	nd
	122		nd	nd

[a] The specific reactivity of the mAbs to CMV-CP. The affinity of the MAbs was determined by indirect enzyme linked immunosorbent assay as described in the materials and methods.

3-3 Utilization of the V gene segments of the H and L chain genes

The V_H and Vκ regions of 10 CMV-specific mAbs generated from three different fusions of BALB/c mice were sequenced. These sequences were almost homologous with corresponding germline genes published in the GenBank database, outlined in Table 2, which summarizes the V_H, D, and J_H fragments of variable heavy chain genes and Vκ, and Jκ of variable light chain genes. The nucleotide and deduced amino acid sequences of the expressed light chain germline gene assignments confidently to very restricted germline family Vκ2, gene bd2 (ten mAbs), GenBank accession nos. (**EF672211**, **EF672212**, **EF672213**, **EF672214**, **EF672215**, **EF672216, EF672217, EF672218**, **EF672219**, and **EF672220)** (Table 2). The identity of the V genes used was determined by searching the GenBank database for homologies to known V genes using the BLAST protocol (Altschul et al., 1997). On the other hand, the nucleotide and deduced amino acid sequences of the expressed V_H genes of the 10 anti-CMV antibodies are shown in Figs. 2, 3, and 4. The V_H genes belong to the following GenBank accession nos.: V_H1/V_HJ558 (four antibodies) (**EF672206**, **EF672197**, **EF672202**, **EF672203**); V_H5/V_H7183 (three antibodies) (**EF672198**, **EF672205**, **EF672201**); V_H8/V_H3609 (three antibodies) **(EF672199**, **EF672200**, **EF672204**) (Table 2). In addition, the V_H genes of the IgG antibodies were more somatically mutated. D segment usage also appears to be restricted with 7 mAbs of V_H using the DSP2 segment, while three mAbs were used for another segment, DFL16 (Figs 2, 3, and 4). On the other hand, it does not appear to be an obvious restriction in J_H segment usage. Interestingly, most antibodies could group into three sets based on their use of the same or highly similar V_H and V_L genes. Gene rearrangement entails the joining of heavy-chain V, D and J germline genes followed by the joining of light-chain V and J genes. The heavy chains belonged to three different families classified into three subgroups. The first included four mAbs (4, 9, 11, and 521) and belongs to the V_HJ558 germline family with different genes; the homology of the amino acid sequences were: $V_HJ558.51$ (89%), $V_HJ558.51$ (93%), $V_HJ558.45$ (94%), and V_H104B (99%) (Table 2; Cohen and Givol, 1983; Haines et al., 2001). The second subgroup included three mAbs-(5, 8, and 52) whose V_H gene segments were from the V_H7183 germline family (Gubbins et al., 2004). The mAbs-(5 and 52) V_H genes were derived from the same germline gene $V_H7183.14$ with 97 and 95% amino acid homology, respectively (Table 2; Chukwuocha et al., 1994). The third subgroup included mAbs-(6, 7, and 122) V_H genes which were derived from the same V_H3609 germline family, CB17H.10 gene (Gubbins et al., 2004) with 96, 96, and 95% homology, respectively (Table 2).

3-4 Somatic mutation and affinity maturation

Based on the sequence analyses of V genes in specific acquired immune responses to foreign antigens, somatic hypermutations were found to occur mainly in complementarity-determining regions (CDR) of V genes during the process of affinity maturation. The combined processes of immunoglobulin gene rearrangement and somatic hypermutation allowed for the creation of an extremely diverse antibody repertoire. V_H-521 showed 16 mutations, five of which were silent, while 11 others led to the mutation of amino acid no. 6 glutamine in germline to glutamic acid ($Gln6^HGlu$); $Ala9^HPro$; $Ser31^HLys$; $Thr54^HSer$; $Glu58^HAsp$; $Asp65^HGly$; $Ala71^HVal$; $Gln80^HGlu$; $Ser82^HHis$; $Ala94^HThr$; and $Arg95^HAsn$ (Fig. 2F). V_H-(4 and 9) showed 18 mutants, 7 silent and 11 amino acid replacements: $Thr19^HLys$; $Lys23^HArg$; $Ser25^HLue$; $Gly26^HIle$; $Met34^HVal$; $Asp52^HLue$; $Glu53^HGly$; $The54^HAsn$; and $Gly56^HAsn$; $Arg82A^HSer$; and $Lue94^HAsn$. The only difference between two antibodies is a one-point mutation in the V_H gene in CDRH2 $Lys65^HIle$ and another in the DSP2 segment of $Phe99^HTyr$ (Fig. 2H). In contrast, V_H-11 reveled only two substitutions, the first in CDRH2 with $Cys54^HSer$ and the second in FW3 with $Arg94^HIle$ (Fig. 2D). V_H-5 revealed 7 mutants: 2 were silent and 5 were substitutions: $Ser55^HGly$; $Tyr56^HSer$; $Arg75^HLys$; $Arg83^HLys$; $Lue89^HMet$. V_H-52 revealed 10 mutations: 3 were silent and 7 were substitutions, 5 being typical as Fd-5 with two more substitutions; $Thr50^HTyr$ and $Ser62^HThr$ (Fig. 2C). V_H-6 has 10

mutants, 3 silent and 7 substitutions: Asn33^{H}Gly; Ile35A^{H}Val; Asp56^{H}Ser; Ser62^{H}Ala; Ser74^{H}Tyr; Thr82A^{H}Ala; and Thr82B^{H}Ser. V_H-72 showed 11 mutants, 3 silent and 8 substitutions, similar to Fd-6 substitutions, except for Trp52^{H}Lue and Thr82B^{H}Asn. V_H-122 showed 13 mutants, 3 silent and 10 substitutions, similar to Fd-72, except for Asn33^{H}Asp and Ser41^{H}Pro; Ala49^{H}Lue. As the frequency of the PCR error used in this study was one in 5000-10000 nucleotides, the intraclonal sequence heterogeneity observed here might not be derived from PCR errors.

3-5 CDR3 length, D regions, and number of N insertions

The length of the H-CDR3 varied from 27 nucleotides in mAb-4 to 51 nucleotides in mAb-6 (Table 3). It has been suggested that the presence of Tyr and Trp residues in H-CDR3 confer flexibility upon the Ab molecule. Consequently, V_H-(6, 7, and 122) has five Tyr residues in this region, while the other V_H has three (Table 3). There are different D and J_H regions used in the CMV-specific V_H and the number of N insertions between these regions (Table 3). On the basis of N insertions at both the V-D and the D-J_H junctions, a third subgroup, V_H showed 10 nucleotides on the V_H-D side and three nucleotides on the other side, D-J_H. V_H-(5 and 8) showed 6 and 4 nucleotides on the V_H-D side, respectively, while only one nucleotide on the D-J_H side. V_H-52 showed 7 nucleotides on the V_H-D side and 5 nucleotides in the D-J_H side. V_H of the first subgroup showed only one sided V_H-D, with 7 or 5 nucleotide insertions.

Table 2 Summary of variable region gene V, (D), and J genes of CMV-CP specific mAbs.

Heavy Chain								Light Chain				
Accession Number	**Clone**	**Isotype**	**V_H**	**Germline gene**	**Homology germline (%)**	**D gene**	**J_H**	**Accession Number**	**V_K**	**Germline gene**	**Homology Germline (%)**	**J_K**
EF672206	521	IgG1	J558	J558.45	94	DSP2.11	2	EF672220	V_K2	bd2	99	2
EF672197	4	IgG1	J558	J558.51	94	DSP2.11	2	EF672211	V_K2	bd2	100	1
EF672202	9	IgG1	J558	J558.51	93	DSP2.11	2	EF672216	V_K2	bd2	99	2
EF672203	11	IgG1	J558	VH104B	99	DSP2.9	2	EF672217	V_K2	bd2	100	2
EF672198	5	IgG1	7183	7183.14	97	DSP2.7	3	EF672212	V_K2	bd2	100	2
EF672205	52	IgG1	7183	7183.14	95	DFL16.2	4	EF672219	V_K2	bd2	98	1
EF672201	8	IgG1	7183	68-5N	100	DSP2.7	3	EF672215	V_K2	bd2	100	2
EF672199	6	IgG1	3609	CB17H.10	96	DFL16.1	1	EF672213	V_K2	bd2	98	1
EF672200	7	IgG2b	3609	CB17H.10	96	DFL16.1	1	EF672214	V_K2	bd2	99	1
EF672204	122	IgG1	3609	CB17H.10	95	DFL16.1	1	EF672218	V_K2	bd2	100	2

[a] Closest matches from either the GenBank Database, the germline assignments were based on the published DNA sequences.

Table 3. Nucleotide sequence of the CDR3 region of the V_H CMV-specific mAbs.

mAbs	N	D segment	N	J_H	Length
V_H 6,7, 122	ATGGGGGTGA	TTTATTACTACGGTAGTAGCTAC	GTA		
GGGTACTTCGATGTCTGGGGCGCAGGGACCACGGTCACCGTCTCCTCA				J_H1	84
V_H 5	GAAGAA	TACTATGGTAA	A		
GCCTGGTTTGCTTACTGGGGCCAAGGGACTCTGGTCACTGTCTCTGCA				J_H3	66
V_H 8	AGAA	TACTATGGTAA	A		
GCCTGGTTTGTTTACTGGGGCCAAGGGACTCTGGTCACTGTCTCTGCA				J_H3	64
V_H 52	AGGGTTA	TTATAACGGCTACG	AGGGG		
GACTACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTC				J_H4	64
V_H 521	ACAAACC	CCTACTATAGGTAC			
GACTACTGGGGCCAAGGCACCACTCTCACAGTCTCCTCA				J_H2	60
V_H 4	AAACC	CCTACTATAGG			
TTCGACTACTGGGGCCAAGGCACCACTCTCACAGTCTCCTCA				J_H2	58
V_H 9	AAACC	CCTACTATAGG			
TACGACTACTGGGGCCAAGGCACCACTCTCACAGTCTCCTCA				J_H2	58

V_H 11		ATCGG	CGGTTA	
CTACTTTGACTACTGGGGCCAAGGCACCACTCTCACAGTCGCCTCA			J_H2	57

Contribution of N- "random nucleotides insertion between V_H and D, D and J_H"

4. Discussions

Antibody fragments may have advantages for treatment of infections due to their small size and lack of Fc portion. It has been known for some time that small Ab molecules such as Fv's could have the potential for therapeutic application. However non-covalently assembled V_H and V_L tend to dissociate in the range of 10^{-4}-10^{-7} M depending on the sequence of interacting complementarity-determining regions (King et al., 1995). Therefore techniques have been developed to covalently link both V regions and stabilize them for *in vivo* applications. Using data from known crystal structures of Antibody and computer modeling, a series of linkers were designed and evaluated as potential candidates to genetically connect the V_H and V_L regions. The resulting scFv molecules were evaluated for their functional activities and relative affinity (Wörn and Plückthun, 2001). Very little molecular characterization of CMV with monoclonal antibodies (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 the subgroup of both isolates I and II. V_H belongs to germline family V_HJ558 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. Briefly, the procedure followed included the isolation of the variable heavy and light chain domains of the murine monoclonal antibody from mRNA of hybridoma cells, followed by cloning, sequencing and characterization of the 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 10 anti-CMV mAbs generated from three different fusions of BALB/c mice were immunized with native CMV-CP, and the V_H, D, J_H, Vκ, and Jκ were determined (Table 2). Based on nucleotide sequence homology of the mAbs, V_H and V_L genes were classified into three subgroups. All the antibodies were found to derive from distinct B cells because they had utilized diverse V_H, D_H, and J_H gene combinations, and because the length of the CDR3 region ranged from 7 to 17 amino acid residues (Table 2). An abundance of V_H genes from the J558 family was observed (4/10) but each represented a separate member of the family (Table 2). CMV-CP is capable of inducing a variety of B cells that have distinct phenotypic and genotypic paratopes. Antibody-binding kinetics measured by surface plasmon resonance showed that antibodies from 'naïve' repertoires have comparable on-rates for antibodies from immune repertoires but faster off-rates (Winter et al., 1994). Frequently it is necessary to improve the affinity of antibodies isolated from a 'naïve' library by affinity maturation techniques that include chain shuffling, error-prone PCR (Fujii et. al., 1998), or parsimonious mutagenesis (mutagenesis in a few sites in which most residues remain parental) (Glaser et. al., 1992). Interestingly, the high affinity antibody specificity was encoded by germline genes such as mAb-8 (Figure 1). Furthermore, analysis of the affinity measurements and nucleotide sequences shows a strong correlation with the germline heavy chains, in which mAbs-(5, 52 and 521) were derived from V_H7183, showing high affinity to CMV-CP (Table 1). Although it is unlikely that all of these mutated residues are involved in CMV binding, increasing mutation in the heavy chain CDRs with increasing affinity of the antibodies is quite striking. It is difficult to determine the contribution of the CDR3 of the heavy chain, or of individual amino acids to affinity. Higher affinity scFv were produced by evolving the center of the antigen binding pocket by sequentially randomizing amino acid residues located within the heavy and light chain CDR3. Amino acid residues conferring higher affinity were located in loops within the CDRs and had solvent accessible side chains. In contrast, residues known to have a structural role were conserved. Isolation of higher affinity antibodies required a stringent selection process using limiting concentrations of soluble biotiylated antigen, use of a biosensor-based screening process

to identify higher affinity antibodies, and optimization of elution conditions (Schier et al., 1996).

One of the important aspects of V_L and V_H amino acid sequences is the study of the structural analysis of the antigen binding loops by molecular modeling and simulation of molecular dynamics (research currently in progress). Through these findings, it will be suggested that amino acids residues may play a crucial role in the antigen-antibody interaction.

Knowledge of specific immunoglobulin genes for a common epitope may lead to insight on pathogen-host co-evolution and help block a virus from infecting plants over the long-term, which is useful for antibody-based resistance. CMV-CP domains are clearly exposed on the virus; in addition, the amino acid sequence of the βH-βI loop forms a conspicuous, negatively charged electrostatic field on the surfaces of virions, which is a fundamental aspect that is conserved among *Cucumoviruses*, and this structure plays a role in aphid vector transmission (Liu et al., 2002). The βH-βI loop of the CP is a critical determinant of viral pathogenicity and has been shown to contain major immunodominant neutralization domains (He et al., 1998). 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 (Liu et al., 2002). 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 (Fellouse et al., 2004). 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 is suggested. We propose that high-affinity CMV-binding antibodies can arise without extensive somatic hypermutation in the variable-region genes because of the expression of appropriate HCDR3s. We consider that the negative charge on the acetate group in the CMV-CP was partially neutralized by a hydrogen bond with the phenolic hydroxyl group of tyrosine, which exists in HCDR3. We consider that the negative charge on the acetate group in the CMV-CP was partially neutralized by a hydrogen bond with the phenolic hydroxyl group of tyrosine that exists in HCDR3. Therefore, we speculate and expect that the HCDR3-peptide be used as tool for plant virus resistance depending on the peptide-neutralizing epitope.

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