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Molecular Cloning and Sequencing of Heavy and Light Chain cDNAs from *Papaya ringspot* and *Cymbidium mosaic viruses*-Specific Monoclonal Antibodies

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ABSTRACT

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Two cDNA libraries were constructed from mRNAs isolated from hybridoma cell lines, 9G2D3 and 11A12F5, producing monoclonal antibodies specific to the coat proteins of *Papaya ringspot virus* (PRSV) and *Cymbidium mosaic virus* (CymMV), respectively. 9G2D3 hybridoma cell was determined to be a secretor of the IgG1 heavy chain and kappa light chain class. A full-length heavy and a light chain cDNAs (1571 and 966 bp), PRSV-H 10-9 and PRSV-L 3-8, were selected by hybridization with specific oligonucleotide probes, respectively. The sequence of PRSV-H 10-9 cDNA clone (accesion number: AY571285) contained a 5' untranslated region (34 bp, 1-34), an open reading frame (1380 bp, 35-1414) including a 19 amino acid leader peptide sequence (57 bp, 35-91), a variable region (348 bp, 92-439) and a constant region (975 bp, 440-1414). The sequence of the 3' end of this clone contained the untranslated region (100 bp, 1415-1514) with a prototype sequence of AATAAA (1482-1487) at position 27 nucleotides before the poly A region, and a poly A region (1515-1571). The sequence of PRSV-L 3-8 cDNA clone (accesion number: AY571286) contained a 5' untranslated region (3 bp, 1-3), an open reading frame (717 bp, 4-720) including a 20 amino acid leader peptide sequence (60 bp, 4-63), a variable region (333 bp, 64-396) and a constant region (324 bp, 397-720). The sequence of the 3' end of this clone contained the untranslated region (208 bp, 721-928) with a prototype sequence of AATAAA (906-911) at position 17 nucleotides before the poly A region, and a poly A region (929-966). 11A12F5 hybridoma cell was determined to be a secretor of the IgG3 heavy chain and kappa light chain class. A full-length heavy and a light chain cDNAs (1545 and 948 bp), CymMV-H 10-1 and CymMV-L 23, were selected by hybridization with specific oligonucleotide probes, respectively. The sequence of CymMV-H 10-1 cDNA clone (accesion number: AY571287) contained a 5' untranslated region (37 bp, 1-37), an open reading frame (1386 bp, 38-1423) including a 19 amino acid leader peptide sequence (57 bp, 38-94), a variable region (339 bp, 95-433) and a constant region (990 bp, 434-1423). The sequence of the 3' end of this clone contained the untranslated region (102 bp, 1424-1525) with a prototype sequence of AATAAA (1492-1497) at a position 28 nucleotides before the poly A region, and a poly A region (1526-1545). The sequence of CymMV 23 cDNA clone (accesion number: AY571284) contained a 5' untranslated region (2 bp, 1-2), an open reading frame (720 bp, 3-722) including a 20 amino acid leader peptide sequence (60 bp, 3-62), a variable region (336 bp, 63-398) and a constant region (324 bp, 399-722). The sequence of the 3' end of this clone contained the untranslated region (208 bp, 723-930) with a prototype sequence of AATAAA (908-913) at position 17 nucleotides before the poly A region, and a poly A region (931-948). The results in this study bring us closer to the use of transgenic plants expressing a functional single-chain variable fragement antibody specific to the viral coat proteins for controlling PRSV and CymMV.

Key words : molecular cloning, *Papaya ringspot virus*, *Cymbidium mosaic virus*, hybridoma, antibody gene

INTRODUCTION

Papaya ringspot virus (PRSV) is one of the most destructive pathogens infecting papaya in many areas of Hawaii, Florida, the Caribbean countries, South America, Africa, Australia, and the Far East. PRSV is a member of the genus *Potyvirus*, with flexuous, filamentous particles about 780 x 12 nm. It is transmitted mechanically and by many species of aphids in a nonpersistent manner. The virus is serologically indistinguishable from PRSV watermelon strain [18, 23, 26]. *Cymbidium mosaic virus* (CymMV) is a major limiting factor for orchid plants. Infected plants are unhealthy and produce lower quality flowers. CymMV is a member of the genus *Potexvirus*, with filamentous particles about 490 x 13 nm. It is transmitted mechanically [2, 10, 12, 27]. PRSV and CymMV can be effectively controlled by genetic engineering using transgenic plants with coat protein genes of these viruses [14, 25]. Antibody production in transgenic plants was first described by Hiatt *et al.* [7, 8]. Complementary cDNAs derived from a murine hybridoma's messenger RNA were used to transform tobacco leaf segments. The mature plants were crossed to yield progeny in which a functional antibody was expressed. This work opened the possibility for the control of plant virus diseases through the use of transgenic plants expressing specific antibodies to the pathogen [21, 22]. Tavladoraki *et al.* had shown that transgenic plants expressing a functional single-chain variable fragement antibody were specifically protected from virus attack [21]. In light of the recent works, we are going to investigate the possibility of controlling PRSV and CymMV through the use of transgenic plants expressing antibodies to the viral coat proteins. In this study, we describe the molecular cloning and sequencing of selected clones that code for the heavy and light chains immunoglobulin of monoclonal antibodies to the coat proteins of PRSV and CymMV.

MATERIALS AND METHODS

Hybridoma cell line

BALB/c mice were immunized initially by intraperitoneal injection with 100 μg of purified PRSV in 0.05 M potassium phosphate buffer, pH 7.2, mixed with Freund's Complete Adjuvant (1:1, v/v). Second immunization was done 3 wk later with the same antigen preparation except with Freund's Incomplete Adjuvant. Four weeks after the initial immunization, sera were collected by tail-bleeding from each mouse and sera titers were determined by indirect ELISA [15]. The mouse with the highest serum titer was given an intraperitoneal injection with 100 μg of purified antigen in 0.2 ml of 0.05 M potassium phosphate buffer, pH 7.2, two months after the initial immunization. Three days later, the spleen was harvested and gently ground through a sieve into fresh Dulbecco's Modified Eagle's Medium (DMEM) (Gibco). Cells were fused with mouse myeloma non-secreting cell line NS 1 (American Type Culture Collection). Fusion and cell maintenance protocols were adapted from that of Kohler and Milstein [12], except for the actual fusion procedure involving a 60-s exposure to 50% polyethylene glycol (MW 1450) and 0.05% dimethyl sulfoxide (DMSO). Cells were plated out in DMEM containing hypoxanthine, aminopterin, and thymidine for selection of hybridomas. Presence of virus-specific antibodies in culture medium was determined by indirect plate-trapped antigen (PTA)-ELISA [15]. Antibody-secreting cell lines were cloned using the limiting dilution method.

A hybridoma cell line, 11A12F5, producing monoclonal antibody specific to the coat protein of CymMV were provided from Floral and Nursery Plants Research Unit, Beltsville Agricultural Research Center, Department of Agriculture, U.S.A.

Immunoassays

Hybridoma cell lines, 9G2D3 and 11A12F5, producing monoclonal antibody specific to the coat protein of PRSV and CymMV, respectively, were plated out in DMEM culture medium containing hypoxanthine, aminopterin, and thymidine for immunoassay. Two types of ELISA, PTA-ELISA and double antibody sandwich (DAS) ELISA, were used. In PTA-ELISA, antigens were allowed to bind directly to Immulon II plates (Dynatech Laboratories, Inc.) in 0.005 M coating buffer, pH 9.6 and incubated at 4 ℃ [15]. Plates were rinsed three times with phosphate-buffered saline (PBS) containing 0.05% Tween-20. After washing three times, plates were incubated with 1% bovine serum albumin (BSA) in PBS-Tween for 1 hr at room temperature, rinsed three times with PBS-Tween, and 100 μl/well of undiluted cell culture supernatants were added and incubated for additional 2.5 hr at 37 ℃. After another washing (three times with PBS-Tween), plates were incubated with a 1:1000 dilution (in PBS-Tween) of alkaline phosphatase-labeled goat anti-mouse IgG or IgM (Sigma Chemical Co.) at 100 μl/well. Plates were washed three times in PBS-Tween before addition of substrate. Reactions were read by an MR 580 Microplate Autoreader (Dynatech Instruments Inc.) at 405 nm.

In DAS-ELISA, plates were coated with purified immunoglobulin of rabbit antisera to PRSV or CymMV (1 μg/ml) for 2 hr at 37 ℃. Infected plant tissues were ground in 0.05 M potassium phosphate buffer, pH 7.2, and incubated overnight at 4 ℃. The remaining steps were the same as those in the PTA-ELISA after antigen incubation. The source of immunoglobulin mRNA of a hybridoma cell line, 9G2D3 and 11A12F5, were prepared and characterized in PTA- and DAS-ELISA tests for the specific antibody-antigen binding.

Isotype determination

Antibody class and subclass were determined by PTA-ELISA using reagents and the protocol supplied in a SBA Clonotyping Kit (Fisher Scientific Co.)

Isolation of total RNA and mRNA

RNAs were isolated from the 9G2D3 and 11A12F5 hybridomas as described by Chomczynski and Sacchi [4]. The denaturing solution (solution D) was 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0; 0.5% sarcosyl and 0.1 M 2-mercaptoethanol. The acid guanidinium-phenol-chloroform (AGPC) method [4] was used to isolate RNA from cultured cells. Cells (10^7) were collected from the medium and washed once with phosphate-buffered saline (PBS). The cells were then homogenized with 1 ml of solution D in a glass-Teflon homogenizer and subsequently transferred to a 4 ml polypropylene tube. Sequentially, 0.1 ml of 2 M sodium acetate, pH 4.0, 1 ml of water saturated phenol, and 0.2 ml of chloroform-isoamyl alcohol mixture (49:1) were added to the homogenate with thorough mixing by inversion after the addition of each reagent. The final suspension was shaken vigorously for 10 sec and cooled on ice for 15 min. Samples were then centrifuged at 10,000 g for 20 min at 4 ℃. After centrifugation, RNA was present in the aqueous phase whereas DNA and protein were present in the interphase and phenol phase. The aqueous phase was transferred to a fresh tube, mixed with 1 ml of isopropanol, and then placed at -20 ℃ for at least 1 hr to precipitate RNA. Sedimentation at 10,000 g for 20 min was again performed, and the resulting RNA pellet was dissolved in 0.3 ml of solution D, transferred into a 1.5 ml Eppendorf tube, and precipitated with 1 volume of isopropanol at -20 ℃ for 1 hr. After centrifugation in an Eppendorf centrifuge for 30 min at 4 ℃, the RNA pellet was washed with 70% ethanol, vacuum-dried, and dissolved in 20-50 μl diethyl pyrocarbonate (DEPC) treated water. Yield was calculated assuming that 25 $O.D._{260}$/ml = 1 mg RNA/ml. AGPC-isolated RNA was then subjected to oligo(dT) chromatography[1], and fractionated by electrophoresis through a gloxal-DMSO-containing 1.5% denaturing agarose gel as described by Sambrook *et al.*[19].

Construction of cDNA clones

$Poly(A)^+$ mRNA preparations were used as templates for the synthesis of cDNA library according to the protocol for ZAP-cDNA synthesis and cloning (Stratagene, La Jolla, CA, USA). An *Xho*I linker-poly(dT) primer was used to prime first-strand synthesis with MMLV reverse transcriptase, and RNase H and DNA polymerase I were used for second-strand synthesis. The double-stranded cDNA molecules were ligated with *Eco*RI adaptor at both ends. Following digestion with *Xho*I, cDNA molecules were ligated into the lambda ZAP-II vector (Stratagene) and packaged by capsid protein (Gold Packaging Extract, Stratagene).

Screening heavy and light chain cDNAs

Heavy and light chain cDNAs were screened by plaque hybridization (Stratagene). *E. coli* host strain XL1-Blue MRF' were preabsorbed with phage in 200 μl of 10 mM $MgSO_4$ at 37 ℃ for 15 min, and mixed with 3 ml of NZY top agar 〔0.5% NaCl, 0.2% $MgSO_4.7H_2O$, 0.5% yeast extract, 10% NZ amine (casein hydrolysate), 0.7% agar, pH 7.5〕, and then plated onto NZY plates. The plates were incubated 37℃ for 6-8 hr to form plaques and then chilled the plates for 2 hr at 4 ℃. The plaques were transferred onto nylon membranes (Colony/Plaque Screen, Du Pont Co.) for 2-3 min. Phage DNAs on the membranes were denatured in 0.5 N NaOH solution and neutralized in the buffer of 1.0 M Tris-HCl, pH 7.5. The DNAs were fixed to the membranes by UV cross linkage for 90 sec. The membranes were placed in prehybridization buffer for 1 hr and hybridized in hybridization buffer containing the Digoxigenin (DIG)-end-labeled single-stranded oligonucleotide probe (5'-ACAGTTGGTGCAGCATCAGC-3') corresponding to the part of the constant region of kappa light chain [5, 17], 5'-TACCAGGAGAGTGGGAGAGG-3' corresponding to the part of the constant region of IgG 1 [9,24] and 5'-TCTCATTTACCAGGGGAGCG-3', corresponding to the part of the constant and 3' untranslated region of IgG 3 [17] heavy chains, respectively. The oligonucleotides were end-labeled with DIG by T4 DNA polynucleotide kinase. The prehybridization, hybridization (58 ℃), and washing steps were done according to the protocol supplied by the vendor (Du Pont Co., USA).

Lambda plaques selected by hybridization were transferred to SM buffer (0.1 M NaCl, 0.01 M Tris, 0.01 M EDTA, 0.0001% gelatin, pH 7.5). The ExAssist helper phage with *E. coli* XL1-Blue SOLR strain was used to allow efficient excision of pBluescript phagemid from the Uni-ZAP II vector (Stratagene). Clones hybridizing to the heavy and light chains constant-region probes were isolated, plasmid DNAs were purified using the boiling method as described by Sambrook *et al.* [19], digested with *Eco*RI and *Xho*I, and electrophoresed in a 1% agarose gel to determine the size of the cloned cDNA inserts. After electrophoresis, the DNA was transferred to nylon hybridization transfer membranes using 10 X SSC as the transfer buffer. The membrane was probed with the single-stranded oligonucleotide used in colony hybridization. Southern hybridization conditions were the same as those used in colony hybridization.

Sequence analysis of heavy and light chain cDNAs.

cDNA clones, PRSV-H 10-9, CymMV-H 10-1 and PRSV-L 3-8, CymMV-L 23 encoding the heavy and light chain constituents of the monoclonal antibodies to PRSV and CymMV, respectively, were selected for sequence analysis. Nucleotide sequences were obtained using the dideoxynucleotide chain termination sequencing method [20]. This was done from a super-coiled double-stranded template using universal and reverse primers for primer-directed double-stranded sequencing according to the procedure supplied in a ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, USA) and

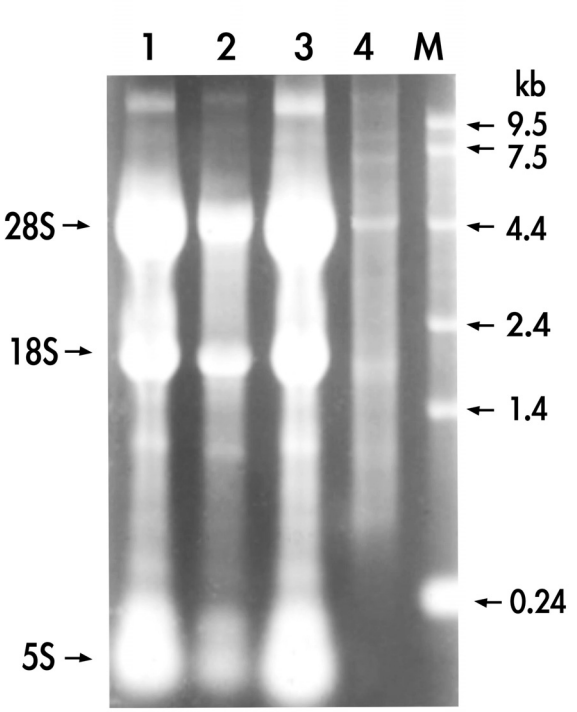

Fig. 1. Electrophoresis of total RNA and poly(A)$^+$ RNA from 9G2D3 and 11A12F5 hybridoma cells. Electrophoresis of total RNA isolated by the acid guanidinium thiocyanate-phenol-chloroform method before (lane 1: 9G2D3 and lane 3: 11A12F5) and after (lane 2: 9G2D3 and lane 4: 11A12F5) being chromatographed on an oligo (dT) column. Lane M contains an RNA marker (0.24-9.5 kb, BRL). RNA preparations were electrophoresed in a 1.5% gloxal-DMSO-agarose minigel. The gel was stained with ethidium bromide. 28S, 18S and 5S are ribosomal RNAs.

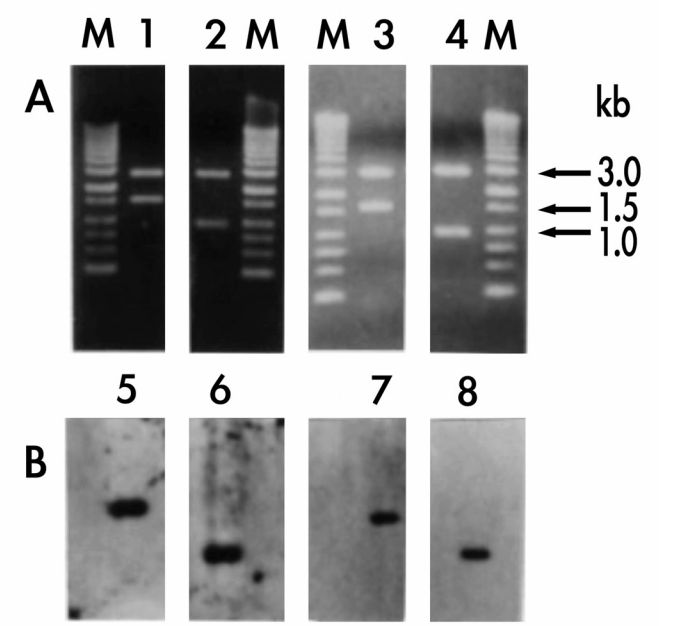

Fig. 2. Electrophoretic and southern blot analysis of heavy and light chains of cDNA clones. (A), electrophoretic analysis of heavy and light chain cDNAs (lane 1: PRSV-H 10-9 and 2: PRSV-L 3-8; lane 3: CymMV-H 10-1 and 4: CymMV-L 23). pBluescript plasmid DNAs with heavy and light chain cDNA inserts (lanes 1-4) were cleaved with EcoRI and XhoI, and run on an 1% agarose gel. The upper bands and lower bands in lanes 1-4 represent the 3.0-kb linearized pBluescript vector DNAs and the 1.6-kb heavy chain cDNA inserts in lane 1 and 3, and the 0.9-kb light chain cDNA inserts in lane 2 and 4, respectively. Lane M represents 1 kb DNA marker (Stratagene, USA). (B), southern blot analysis of the same DNA preparations as in (A) with DIG-labelled single-stranded oligonucleotide probes for the constant regions of heavy (lane 5: PRSV-H 10-9 and 7: CymMV-H 10-1) and light (lane 6: PRSV-L 3-8 and 8: CymMV-L 23) chains. The bands in lanes 5-8 correspond to the lower bands (heavy and light chain cDNA inserts) in lanes 1-4 in (A), repectively.

ABI PRISM 377 DNA Sequencer. Computer-aided nucleotide and amino acid sequence analyses to search for open reading frames (ORFs) and hydrophobicity were done with the software DNASIS for windows (DNASIS version 2.0, Hitachi).

RESULTS

Immunoassays

The source of immunoglobulin mRNAs were from hybridoma cell lines, 9G2D3 and 11A12F5, expressing gamma 1 and gamma 3 heavy chain as well as kappa light chain, respectively. The monoclonal antibodies of 9G2D3 and 11A12F5 reacted to PRSV and CymMV in PTA- and DAS-ELISA tests, respectively, with O.D. 405 nm values over 2.0 30 min after addition of substrate.

Isolation of total RNA and mRNA

The A260/280 ratio of the isolated RNA preparations were 1.8 $\sim$ 2.0. The yields of RNA were 200 μg/10^7 cells for 9G2D3 and 210 μg/10^7 cells for 11A12F5. Agarose-gel resolution patterns of total RNA isolated from hybridoma cells and of total poly(A)$^+$ RNA fractionated with an oligo (dT)-cellulose column showed that most rRNAs were removed and poly(A)$^+$ RNAs were visualized (Fig. 1).

Isolation of heavy and light chain cDNAs

Two cDNA libraries were prepared from RNAs of the hybridoma cell lines, 9G2D3 and 11A12F5, and screened with single-stranded DIG-end-labeled oligonucleotide probes

Table 1. Lengths in nucleotide (nt) and amino acid (aa) of genomic regions of antibody genes of *Papaya ringspot virus* (PRSV) and *Cymbidium mosaic virus* (CymMV)

Fragments	Antibody gene clones	Genomic regions					
		Full-length	5' NTR	Leader signal peptide sequence	Variable region	Constant region	3' NTR
nt	PRSV-H 10-9	1517	34	57	348	975	100
	CymMV-H 10-1	1545	37	57	339	990	102
	PRSV-L 3-8	966	3	60	333	324	208
	CymMV-L 23	948	2	60	336	324	208
aa	PRSV-H 10-9			19	115	324	
	CymMV-H 10-1			19	112	329	
	PRSV-L 3-8			20	110	107	
	CymMV-L 23			20	111	107	

Table 2. Amino acid sequences of leader signal peptide of heavy and light chains of cDNA clones of *Papaya ringspot virus* (PRSV) and *Cymbidium mosaic virus* (CymMV)[1]

Antibody genes	No. of amino acid	Amino acid sequences
PRSV-H 10-9	19	MGWSWIFLFLLSGTAGVLS[2]
CymMV-H 10-1	19	MGWSWIFLFLLSGTAGVLS
PRSV-L 3-8	20	METDTILLWVLLLWVPGSTG
CymMV-L 23	20	MMSPAQFLFLLVLWIRETNG

[1.] Von Heijne method for signal sequence recognition [16] (PSORT II server: http://psort.nibb.ac.jp:8800).
[2.] Letters with under line represent hydrophobic amino acids.

corresponding to the constant region of gamma 1 heavy chain, gamma 3 heavy chain and kappa light chain. Four clones, PRSV-H 10-9、PRSV-L 3-8 and CymMV-H 10-1、CymMV-L 23, that hybridized with the heavy and light chain probes were obtained, respectively. Digestion of cDNAs isolated from these four clones with *Eco*RI and *Xho*I yielded two bands for plasmid and insert DNA (Fig. 2, A), respectively. The cDNA insert of PRV-H 10-9 and CymMV-H 10-1, PRV-L 3-8 and CymMV-L 23 clones were determined to be approximately 1.6 kb and 0.9 kb in length, the expected sizes of full-length heavy and chain cDNAs, respectively. Southern analysis confirmed that all cDNA inserts from the four clones reacted with the heavy and light chain probes, respectively (Fig. 2, B).

Sequence analysis of heavy and light chain cDNAs

Heavy and light chain cDNAs, PRSV-H 10-9、PRSV-L 3-8 and CymMV-H 10-1、CymMV-L 23, were subjected to sequence analysis. The nucleotide sequence of PRSV-H 10-9 cDNA (1571 bp) is shown in Fig. 3. A 5' end untranslated region occurs at 1-34 position of PRV-H 10-9 cDNA followed by ATG triplet at 35-37 position and begins an open reading frame (1380 bp, 35-1414) which is translated into a polypeptide of 459 amino acids. Analysis of the 1380 bp sequence of the PRSV-H 10-9 cDNA revealed a leader signal peptide sequence (57 bp, 35-91), a variable region (348 bp, 92-439), and a sequence of 975 bp (440-1414) (Table 1) identical to the constant region of published sequences of gamma 1 heavy chain [9, 24]. The sequence of 19 amino acids translated from signal peptide sequence shows high hydrophobicity (Table 2). The 1571 bp sequence also included a 3' end untranslated region (1415-1514), a poly A region at the 3' end and the hexamer AATAAA at a position 27 nucleotides before the 5' end to the poly A region (Fig. 3). The nucleotide sequences of PRSV-L 3-8 cDNA (966 bp) is shown in Fig. 4. A 5' end untranslated region occurs at 1-3 position of PRSV-L 3-8 followed by ATG triplet at 4-6 position and begins an open reading frame (717 bp, 4-720) which is translated into a polypeptide of 238 amino acid. Analysis of the 717 bp sequence of the PRV-L 3-8 cDNA revealed a leader signal peptide sequence (60 bp, 4-63), a variable region (333 bp, 64-396), and a sequence of 324 bp (397-720) (Table 1) identical to the constant region of published sequences of kappa light chain [5, 17]. The sequence of 20 amino acids translated from signal peptide sequence shows high hydrophobicity (Table 2). The 966 bp sequence also included a 3' end untranslated region (721-928), a poly A region at the 3' end and the hexamer AATAAA at a position 17 nucleotides before the 5' end to the poly A region (Fig. 4).

The nucleotide sequences of CymMV-H 10-1 cDNA (1545 bp) is shown in Fig. 5. A 5' end untranslated region occurs at 1-37 position of CymMV-H 10-1 cDNA followed by ATG triplet at 38-40 position and begins an open reading frame (1386 bp, 38-1423) which is translated into a polypeptide of 461 amino acids. Analysis of the 1386 bp sequence of the CymMV-H 10-1 cDNA revealed a leader signal peptide sequence (57 bp, 38-94), a variable region (339 bp, 95-433), and a sequence of 990 bp (434-1423) (Table 1) identical to the constant region of published sequences of gamma 3 heavy chain [17]. The sequence of 20 amino acids

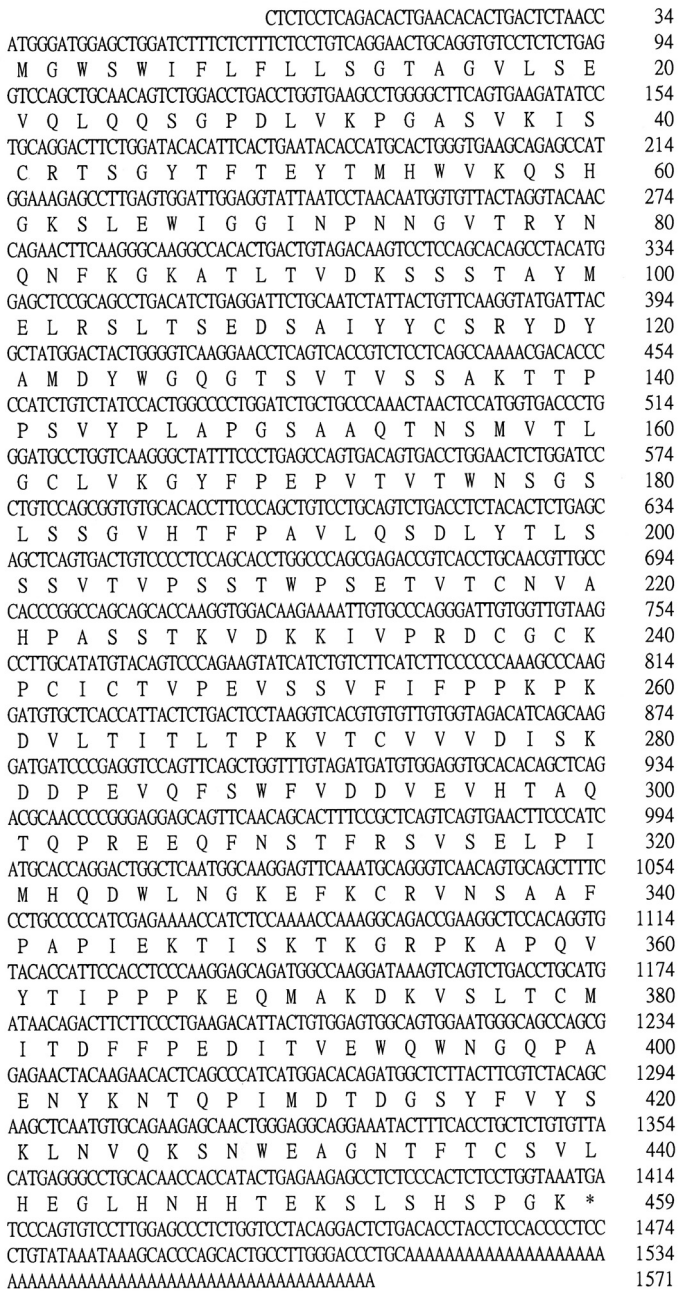

Fig. 3. Nucleotide and deduced amino acid sequences of PRSV-H 10-9 heavy chain (accesion number: AY571285). A 5' end untranslated region occurs at 1-34 position followed by ATG triplet at 35-37 position and begins an open reading frame (1380 bp, 35-1414) which is translated into a polypeptide of 459 amino acids. The 1380 bp sequence of the PRSV-H 10-9 cDNA contains a leader signal peptide sequence (57 bp, 35-91), a variable region (348 bp, 92-439), and a constant region sequence of 975 bp. The 1571 bp sequence also included a 3' end untranslated region (1415-1514), a poly A region at the 3' end and the hexamer AATAAA at a position 27 nucleotides before the 5' end to the poly A region.

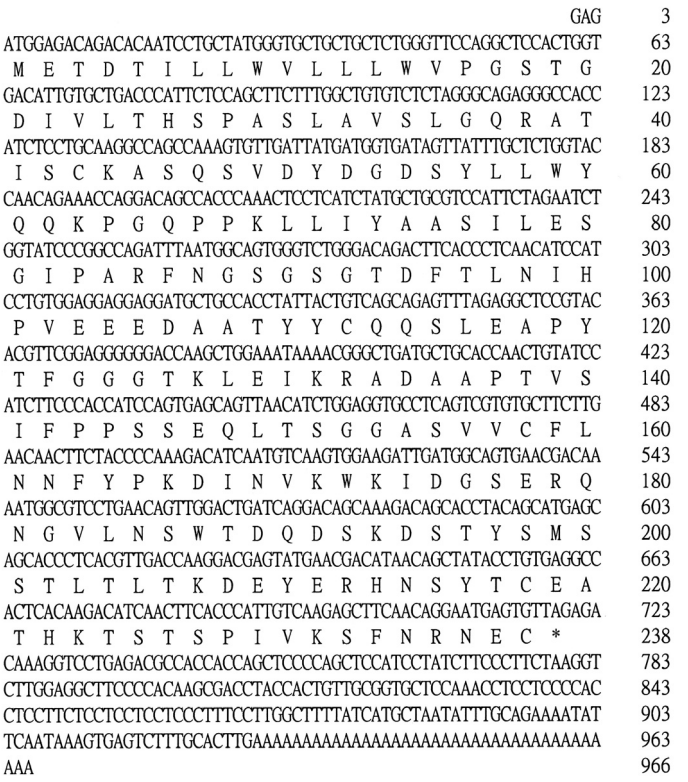

Fig. 4. Nucleotide and deduced amino acid sequences of PRSV-L 3-8 light chain (accesion number: AY571286). A 5' end untranslated region occurs at 1-3 position followed by ATG triplet at 4-6 position and begins an open reading frame (717 bp, 4-720) which is translated into a polypeptide of 238 amino acids. The 717 bp sequence contains a leader signal peptide sequence (60 bp, 4-63), a variable region (333 bp, 64-396), and a constant region sequence of 324 bp (397-720). The 966 bp sequence also included a 3' end untranslated region (721-928), a poly A region at the 3' end and the hexamer AATAAA at a position 17 nucleotides before the 5' end to the poly A region.

translated from signal peptide sequence shows high hydrophobicity (Table 2). The 1545 bp sequence also included a 3' end untranslated region (102 bp, 1424-1525), a poly A region at the 3' end and the hexamer AATAAA at a position 28 nucleotides before the 5' end to the poly A region (Fig. 5). The nucleotide sequences of CymMV-L 23 cDNA (948 bp) is shown in Fig. 6. A 5' end untranslated region occurs at 1-2 position of CymMV-L 23 followed by ATG triplet at 3-5 position and begins an open reading frame (720 bp, 3-722) which is translated into a polypeptide of 239 amino acids. Analysis of the 720 bp sequence of the CymMV-L 23 cDNA revealed a leader signal peptide sequence (60 bp, 3-62), a variable region (336 bp, 63-398), and a sequence of 324 bp (399-722) (Table 1) identical to the constant region of published sequences of kappa light chain [5, 17]. The sequence of 20 amino acids translated from signal peptide sequence

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                                                GTCTTCTCCACAGACACTGAACACACTGACTCTAACC    37
ATGGGATGGAGCTGGATCTTTCTCTTTCTCCTGTCAGGAACTGCAGGTGTCCTCTCTGAG    97
 M  G  W  S  W  I  F  L  F  L  L  S  G  T  A  G  V  L  S  E    20
GTCCAGCTGCAACAGTCTGGACCTGACCTGGTGATGCCTGGGGCTTCAGTGAGGTTATCC   157
 V  Q  L  Q  Q  S  G  P  D  L  V  M  P  G  A  S  V  R  L  S    40
TGCAAGGCTTCTGGTTACTCGTTCACTGACTACTACATACACTGGGTGAGGCAAAGCCAT   217
 C  K  A  S  G  Y  S  F  T  D  Y  Y  I  H  W  V  R  Q  S  H    60
TTAAAGAGCCTTGAGTGGATTGGACGTCTTAATCCTTACAATGGTGCTCCTGACTACAAC   277
 L  K  S  L  E  W  I  G  R  L  N  P  Y  N  G  A  P  D  Y  N    80
CAGAATTTCAGGGCCAAGGCCAGCTTGACTTTAGATAAGTCCTCCAGCACAGCCTACATG   337
 Q  N  F  R  A  K  A  S  L  T  L  D  K  S  S  S  T  A  Y  M   100
GAGCTCACAGTCACATCTGAGGACTCTGCAGTCTATTACTGTGCAGCCAGTTTAGACTAC   397
 E  L  T  V  T  S  E  D  S  A  V  Y  Y  C  A  A  S  L  D  Y   120
TGGGGCCAAGGCACCACTCTCACAGTCTCCTCAGCTACAACAACAGCCCCATCTGTCTAT   457
 W  G  Q  G  T  T  L  T  V  S  S  A  T  T  T  A  P  S  V  Y   140
CCCTTGGTCCCTGGCTGCAGTGACACATCTGGATCCTCGGTGACACTGGGATGCCTTGTC   517
 P  L  V  P  G  C  S  D  T  S  G  S  S  V  T  L  G  C  L  V   160
AAAGGCTACTTCCCTGAGCCGGTAACTGTAAAATGGAACTATGGAGCCCTGTCCAGCGGT   577
 K  G  Y  F  P  E  P  V  T  V  K  W  N  Y  G  A  L  S  S  G   180
GTGCGCACAGTCTCATCTGTCCTGCAGTCTGGGTTCTATTCCCTCAGCAGCTTGGTGACT   637
 V  R  T  V  S  S  V  L  Q  S  G  F  Y  S  L  S  S  L  V  T   200
GTACCCTCCAGCACCTGGCCCAGCCAGACTGTCATCTGCAACGTAGCCCACCCAGCCAGC   697
 V  P  S  S  T  W  P  S  Q  T  V  I  C  N  V  A  H  P  A  S   220
AAGACTGAGTTGATCAAGAGAATCGAGCCTAGAATACCCAAGCCCAGTACCCCCCCAGGT   757
 K  T  E  L  I  K  R  I  E  P  R  I  P  K  P  S  T  P  P  G   240
TCTTCATGCCCACCTGGTAACATCTTGGGTGGACCATCCGTCTTCATCTTCCCCCCAAAG   817
 S  S  C  P  P  G  N  I  L  G  G  P  S  V  F  I  F  P  P  K   260
CCCAAGGATGCACTCATGATCTCCCTAACCCCCAAGGTTACGTGTGTGGTGGTGGATGTG   877
 P  K  D  A  L  M  I  S  L  T  P  K  V  T  C  V  V  V  D  V   280
AGCGAGGATGACCCAGATGTCCATGTCAGCTGGTTTGTGGACAACAAAGAAGTACACACA   937
 S  E  D  D  P  D  V  H  V  S  W  F  V  D  N  K  E  V  H  T   300
GCCTGGACACAGCCCCGTGAAGCTCAGTACAACAGTACCTTCCGAGTGGTCAGTGCCCTC   997
 A  W  T  Q  P  R  E  A  Q  Y  N  S  T  F  R  V  V  S  A  L   320
CCCATCCAGCACCAGGACTGGATGAGGGGCAAGGAGTTCAAATGCAAGGTCAACAACAAA  1057
 P  I  Q  H  Q  D  W  M  R  G  K  E  F  K  C  K  V  N  N  K   340
GCCCTCCCAGCCCCCATCGAGAGAACCATCTCAAAACCCAAAGGAAGAGCCCAGACACCT  1117
 A  L  P  A  P  I  E  R  T  I  S  K  P  K  G  R  A  Q  T  P   360
CAAGTATACACCATACCCCCACCTCGTGAACAAATGTCCAAGAAGAAGGTTAGTCTGACC  1177
 Q  V  Y  T  I  P  P  P  R  E  Q  M  S  K  K  K  V  S  L  T   380
TGCCTGGTCACCAACTTCTTCTCTGAAGCCATCAGTGTGGAGTGGGAAAGGAACGGAGAA  1237
 C  L  V  T  N  F  F  S  E  A  I  S  V  E  W  E  R  N  G  E   400
CTGGAGCAGGATTACAAGAACACTCCACCCATCCTGGACTCAGATGGGACCTACTTCCTC  1297
 L  E  Q  D  Y  K  N  T  P  P  I  L  D  S  D  G  T  Y  F  L   420
TACAGCAAGCTCACTGTGGATACAGACAGTTGGTTGCAAGGAGAAATTTTTACCTGCTCC  1357
 Y  S  K  L  T  V  D  T  D  S  W  L  Q  G  E  I  F  T  C  S   440
GTGGTGCATGAGGCTCTCCATAACCACCACACACAGAAGAACCTGTCTCGCTCCCCTGGT  1417
 V  V  H  E  A  L  H  N  H  H  T  Q  K  N  L  S  R  S  P  G   460
AAATGAGAACAGCACCTAGCCATTCCTCGGGTCTTACAAGACACTGATACCAGCTCTAAC  1477
 K  *
TGGTGAACCCTATAAATAAAGCACCCAGAGATGGGACCTTGTGAGATTAAAAAAAAAAAA  1537
AAAAAAAA                                                       1545
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Fig. 5. Nucleotide and deduced amino acid sequences of CymMV-H 10-1 heavy chain (accesion number: AY571287). A 5' end untranslated region occurs at 1-37 position followed by ATG triplet at 38-40 position and begins an open reading frame (1386 bp, 38-1423) which is translated into a polypeptide of 461 amino acids. The 1386 bp sequence of the CymMV-H 10-1 cDNA contains a leader signal peptide sequence (57 bp, 38-94), a variable region (339 bp, 95-433), and a constant region sequence of 990 bp (434-1423). The 1545 bp sequence also included a 3' end untranslated region (102 bp, 1424-1525), a poly A region at the 3' end and the hexamer AATAAA at a position 28 nucleotides before the 5' end to the poly A region.

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                                                          AA     2
ATGATGAGTCCTGCCCAGTTCCTGTTTCTGTTAGTGCTCTGGATTCGGGAAACCAACGGT    62
 M  M  S  P  A  Q  F  L  F  L  L  V  L  W  I  R  E  T  N  G    20
GATGTTGTGATGACCCAGACTCCACTCACTTTGTCGGTTACCATTGGACAACCAGCCTCC   122
 D  V  V  M  T  Q  T  P  L  T  L  S  V  T  I  G  Q  P  A  S    40
ATCTCTTGCAAGTCAAGTCAGAGCCTCTTAAATAGTGATGGAAAGACATATTTGAGTTGG   182
 I  S  C  K  S  S  Q  S  L  L  N  S  D  G  K  T  Y  L  S  W    60
TTGTTACAGAGGCCAGGCCAGTCTCCAAAGCGCCTAATCTATCTGGTGTCTAAACTGGAC   242
 L  L  Q  R  P  G  Q  S  P  K  R  L  I  Y  L  V  S  K  L  D    80
TCTGGAGTCCCTGACAGGTTCACTGGCAGTGGATCAGGGACAGATTTCACACTGAAAATC   302
 S  G  V  P  D  R  F  T  G  S  G  S  G  T  D  F  T  L  K  I   100
AGCAGAGTGGAGGCTGAGGATTTGGGAATTTATTATTGCTGGCAAGGTACACATTTTCCT   362
 S  R  V  E  A  E  D  L  G  I  Y  Y  C  W  Q  G  T  H  F  P   120
CGGACGTTCGGTGGAGGCACCAAGCTGGAAATCAAACGGGCTGATGCTGCACCAACTGTA   422
 R  T  F  G  G  G  T  K  L  E  I  K  R  A  D  A  A  P  T  V   140
TCCATCTTCCCACCATCCAGTGAGCAGTTAACATCTGGAGGTGCCTCAGTCGTGTGCTTC   482
 S  I  F  P  P  S  S  E  Q  L  T  S  G  G  A  S  V  V  C  F   160
TTGAACAACTTCTACCCCAAAGACATCAATGTCAAGTGGAAGATTGATGGCAGTGAACGA   542
 L  N  N  F  Y  P  K  D  I  N  V  K  W  K  I  D  G  S  E  R   180
CAAAATGGCGTCCTGAACAGTTGGACTGATCAGGACAGCAAAGACAGCACCTACAGCATG   602
 Q  N  G  V  L  N  S  W  T  D  Q  D  S  K  D  S  T  Y  S  M   200
AGCAGCACCCTCACGTTGACCAAGGACGAGTATGAACGACATAACAGCTATACCTGTGAG   662
 S  S  T  L  T  L  T  K  D  E  Y  E  R  H  N  S  Y  T  C  E   220
GCCACTCACAAGACATCAACTTCACCCATTGTCAAGAGCTTCAACAGGAATGAGTGTTAG   722
 A  T  H  K  T  S  T  S  P  I  V  K  S  F  N  R  N  E  C  *   239
AGACAAAGGTCCTGAGACGCCACCACCAGCTCCCCAGCTCCATCCTATCTTCCCTTCTAA   782
GGTCTTGGAGGCTTCCCCACAAGCGACCTACCACTGTTGCGGTGCTCCAAACCTCCTCCC   842
CACCTCCTTCTCCTCCTCCTCCCTTTCCTTGGCTTTTATCATGCTAATATTTGCAGAAAA   902
TATTCAATAAAGTGAGTCTTTGCACTTGAAAAAAAAAAAAAAAAA                 948
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Fig. 6. Nucleotide and deduced amino acid sequences of CymMV-L 23 light chain (accesion number: AY571284). A 5' end untranslated region occurs at 1-2 position of CymMV-L 23 followed by ATG triplet at 3-5 position and begins an open reading frame (720 bp, 3-722) which is translated into a polypeptide of 239 amino acids. The 720 bp sequence contains a leader signal peptide sequence (60 bp, 3-62), a variable region (336 bp, 63-398), and a constant region sequence of 324 bp (399-722). The 948 bp sequence also included a 3' end untranslated region (208 bp, 723-930), a poly A region at the 3' end and the hexamer AATAAA at a position 17 nucleotides before the 5' end to the poly A region.

shows high hydrophobicity (Table 2). The 948 bp sequence also included a 3' end untranslated region (208 bp, 723-930), a poly A region at the 3' end and the hexamer AATAAA at a position 17 nucleotides before the 5' end to the poly A region (Fig. 6).

DISCUSSION

By using the AGPC extraction method [4] along with oligo (dT) cellulose chromatography[1], total RNA and poly(A)$^+$ RNA from hybridoma cells could be visualized in a electrophoresis gel (Fig. 1). The presence of 28S and 18S rRNA bands in a ratio of ~2:1 indicates that intact RNAs were isolated [6]. If the ratio was less than 1:1, further extraction cycles would have to be performed to remove the residual RNase activity. Results from spectrophotometry also indicate that RNA preparation using this method contained

less contaminating proteins, as seen by the $A_{260/280}$ ratio. The AGPC extraction method also provides high yield of RNA (200 and 210 μg/10^7 cells) and can be completed within 6 hr in our laboratory.

The cloning strategy presented here was designed to produce long cDNA. Poly $(A)^+$ mRNA fractions substantially enriched for heavy and light chain mRNAs were used to screen for cDNA clones. All of the heavy and light chain cDNA clones isolated had the expected full-length cDNA inserts, and the frequency recovered was 1%. Sambrook *et al.* reported that the mRNA species which code for immunoglobulin and ovalbumin can comprise as much as 50-90% of the total poly$(A)^+$ cytoplasmic RNA isolated from specific types of differentiated cells [19]. The sequences of the PRSV-H 10-9、PRSV-L 3-8 and CymMV-H 10-1、CymMV-L 23 clones each contains a single open reading frame from the ATG which agrees with those of the heavy and light chains [5, 9, 17, 24]. The first 19 and 20 amino acid sequences translated from the heavy and light chains of nucleotides are highly hydrophobic, indicating those are leader peptide sequences which are necessary for secretion of the protein [3, 13]. The sequences of constant region of PRSV-H 10-9、PRSV-L 3-8 and CymMV-H 10-1、CymMV-L 23 clones are the same as those of gamma 1 and gamma 3 heavy chain and kappa light chain previously reported [5, 9, 17, 24]. The prototype sequence AATAAA has been found approximately 20 nucleotides from the poly$(A)^+$ attachment site at the 3' end in all eukaryotic nontranslated regions sequenced to date [5, 17]. As expected, it occurs in the PRSV-H 10-9 and CymMV-H 10-1 sequences 27 and 28 nucleotides, respectively, and PRSV-L 3-8 and CymMV-L 23 sequences 17 nucleotides from the poly A region.

Using these methods for mRNA isolation and cDNA cloning and sequencing, full length cDNA clones, PRSV-H 10-9、PRSV-L 3-8 and CymMV-H 10-1、CymMV-L 23, coding for the heavy and light chain of the monoclonal antibodies to PRSV and CymMV were successfully obtained. Recently, expression of antibody to viral protein in plant could provide the engineered resistance to viral infection [21]. Therefore, the progress made in this study brings us closer to the use of antibody mediated protection for controlling PRSV and CymMV.

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摘 要

王惠亮[1,3]、李慶孝[1]、徐惠迪[2]. 2004. 木瓜輪點與喜姆比蘭嵌紋病毒單元抗體基因選殖與核苷酸序列譯讀. 植病會刊 13：7-16. ([1]高雄市 國立高雄師範大學生物科學研究所；[2]美國馬里蘭州 農業部 Beltsville 農業研究中心；[3]聯絡作者：電子郵件 hlwang@nknucc.nknu.edu.tw; 傳眞: +886-7-7169030)

木瓜輪點病毒 (*Papaya ringspot virus*, PRSV) 和喜姆比蘭嵌紋病毒 (*Cymbidium mosaic virus*, CymMV) 鞘蛋白單元抗體，分別爲 9G2D3 具 IgG1 重鏈和 kappa 輕鏈和 11A12F5 具 IgG3 重鏈和 kappa 輕鏈之單元抗體。由產生單元抗體之融合瘤細胞萃取獲得抗體基因之訊息核酸 (mRNA)，並建立兩病毒抗體基因之互補去氧核醣核酸 (cDNAs)。分別選殖對抗體基因重鏈和 kappa 輕鏈固定區 (constant region) 核苷酸序列具專一性之寡核苷酸探針，成功選殖到木瓜輪點病毒全長度之鞘蛋白單元抗體重鏈 (PRSV-H 10-9, 1571 bp; accesion number :AY571285) 和輕鏈 (PRSV-L 3-8, 966 bp; accesion number :AY571286)，喜姆比蘭嵌紋病毒全長度之鞘蛋白單元抗體重鏈 (CymMV-H 10-1, 1545 bp; accesion number :AY571287) 和輕鏈 (CymMV-L 23, 948 bp; accesion number :AY571284)。PRSV-H 10-9 全長度核苷酸序列包含一個 34 bp (1-34) 之 5' 端非轉譯區，一個 1380 bp (35-1414) 之開放轉譯架構 (open reading frame) 轉譯成 459 個胺基酸序列，其中包含 57 bp (35-91) 轉譯成 19 個前導胺基酸序列 (leader peptide sequence)，348 bp (92-439) 變異區 (variable region) 序列和 975 bp (440-1414) 固定區序列，一個 100 bp (1415-1514) 3' 端非轉譯區和一個 poly A 尾區序列 (1515-1571)。PRSV-L 3-8 全長度核苷酸序列包含一個 3 bp (1-3) 之 5' 端非轉譯區，一個 717 bp (4-720) 之開放轉譯架構轉譯成 238 個胺基酸序列，其中包含 60 bp (4-63) 轉譯成 20 個前導胺基酸序列，333 bp (64-396) 變異區序列和 324 bp (397-720) 固定區序列，一個 208 bp (721-928) 3' 端非轉譯區和一個 poly A 尾區序列 (929-966)。CymMV-H 10-1全長度核苷酸序列包含一個 37 bp (1-37) 之5' 端非轉譯區，一個 1386 bp (38-1423) 之開放轉譯架構轉譯成 461 個胺基酸序列，其中包含 57 bp (38-94) 轉譯成 19 個前導胺基酸序列，339 bp (95-433) 變異區序列和 990 bp (434-1423) 固定區序列，一個 102 bp (1424-1525) 3' 端非轉譯區和一個 poly A 尾區序列 (1526-1545)。CymMV-L 23 全長度核苷酸序列包含一個 2 bp (1-2)之5' 端非轉譯區，一個 720 bp (3-722) 之開放轉譯架構轉譯成 239 個胺基酸序列，其中包含 60 bp (3-62) 轉譯成 20 個前導胺基酸序列，336 bp (63-398) 變異區前導序列和 324 bp (399-722) 固定區序列，一個 208 bp (723-930) 3' 端非轉譯區和一個 poly A 尾區序列 (931-948)。本試驗結果可提供進一步合成重鏈和輕鏈變異區單鏈抗體分子，進行轉殖植物之育成，達到防治木瓜輪點病毒和喜姆比蘭嵌紋病毒之目的。

關鍵詞: 分子選殖、木瓜輪點病毒、喜姆比蘭嵌紋病毒、融合瘤、抗體基因