Synthesis of a Functional Single-Chain Antibody Against Citrus Tristeza Closterovirus in Bacteria

K. L. Manjunath, M. Hooker, H. R. Pappu, S. S. Pappu, C. A. Powell, M. Bar-Joseph, C. L. Niblett, and R. F. Lee

ABSTRACT. A synthetic gene that encodes the antigen-binding regions of the monoclonal antibody (MAb) 17G11, which is specific for citrus tristeza closterovirus (CTV), was constructed and expressed in *Escherichia coli*. MAb 17G11 reacts with a broad spectrum of CTV isolates and recognizes a surface epitope which is destroyed when treated with sodium dodecyl sulfate. The polymerase chain reaction products from the cDNAs of the variable regions of heavy and light chains of the immunoglobulin leader sequence were linked by a synthetic peptide. This construct was cloned downstream of the pelB leader sequence in pET 22b vector and expressed in *E. coli*. The expressed protein, purified by affinity chromatography, was found to have antigen binding properties similar to 17G11. The single chain antibody gene construct will be used for transgenic expression in plants to study its role in control of CTV.

Key words. Bacterial expression, coat protein, sequence, plantibodies, monoclonal antibodies.

Citrus tristeza closterovirus (CTV) is one of the most destructive diseases of citrus worldwide. Various control measures for control and prevention of CTV include quarantine, use of disease-free budwood, mild strain cross protection, tolerant scion-rootstock combinations, and biological control of vectors (11). The virus is still spreading into new areas, and continues to cause severe losses throughout the world and warrants development of better control strategies. During the last 10 years, various molecular biological strategies have been developed for the control of virus diseases (16). Pathogen-derived resistance involves expression of viral gene sequences in plants and has been found to be very effective (16). Expression of antibodies against viral genes in plants provides an alternative approach for virus disease control that avoids introduction of pathogen genes into transformed plants (14).

Immunoglobulins are key molecules in animal immune systems. Specific interaction of these molecules with the antigen is one of the first of several complex processes in the immune system. Antibody genes have been cloned and expressed in heterologous systems like bacteria, yeasts and plants (1, 17). Even though plants lack an immune system, production of a specific antigen-binding antibody may interfere with the virus replication (14) and prevent disease. Single-chain antibody (SCAB) proteins are novel recombinant proteins consisting of the variable regions of heavy (VH) and light chain (VL) molecules of immunoglobulins joined by a linker peptide (1). Compared to immunoglobulins, the SCAB proteins are much smaller in size and do not require elaborate assembly in the cell. These proteins were found to retain the same binding specificities of the parent MAbs whose VH and VL sequences were used (1, 17). Both complete (3, 5) and engineered antibodies (9) have been expressed in plants. Attenuation of viral symptoms by expression of both full-length and engineered single-chain antibodies in plants has been reported (14, 15). Here, we report the bacterial expression of a CTV-specific SCAB gene with the objective of studying its binding properties and comparing it with the parent MAb. This is a necessary step for its future utilization in plant

expression and assessment of its activity against CTV.

MATERIALS AND METHODS

Selection of monoclonal antibody. A panel of 11 MAbs (Table 1) was screened by using a double antibody sandwich indirect (DASI) ELISA against 60 CTV isolates. These isolates were from nine countries and represent a full range of biological diversity among the CTV groups. Isolates inducing decline, seedling yellows, grapefruit stem pitting, sweet orange stem pitting and mild isolates were included (4, 11). MAbs 1-7 were developed using purified virus preparations of CTV isolates T36 and B185 as antigens (6); MAbs 8-10 were developed against B227 antigen (Manjunath et al., unpublished data); and MAb F10 was kindly provided by Lochy Batista (Instituto de Investigaciones de Citricos, La Habana, Cuba). Microtiter plates were coated with protein-A-sepharose purified immunoglobulin-G preparation of a polyclonal antibody, 1051 at 1 µg/ml. Antigen extractions were used at 1:20 dilution. Ascites of MAb F10 was used at 1:10,000 dilution. Hybridoma supernatants of all other MAbs were used at 1:3 dilution.

RNA extraction and cDNA synthesis. Mouse hybridoma cells secreting 17G11 antibodies (6) were used for cDNA synthesis. Total RNA was extracted from the cells by using the ULTRASPEC™ RNA isolation system (Biotex Laboratories, Inc. Houston, TX). First strand cDNA was synthesized to the variable regions of heavy (VH) and light (VL) chains in separate reactions by using primers specific for the 3' end of the framework region 4 (FR4) of the variable domain (Table 2). Denaturation of RNA and primer annealing were done by mixing 10 µg of total RNA with 10 mM methyl mercuric hydroxide and an appropriate 3' end primer, and incubating at room temperature for 8 min followed by quick freezing in liquid nitrogen. The reaction tubes were thawed, and the reverse transcription reaction was carried out at 42°C for 45 min in a 25 µl reaction mix containing 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine, 10 mM dithiothreitol, 0.5 mM each of dATP, dCTP, dGTP and dTTP, RNasin (20 u) and AMV reverse transcriptase (10 u) (Promega).

Construction of the single chain Fv antibody (SCAB) gene. VH and VL regions were amplified by polymerase chain reaction (PCR) of the above cDNAs using degenerate consensus primers, a-d (Table 2). Ten µl of each of the above cDNA templates was used in 100 µl reaction mix containing 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM dNTPs, primers (100 *p*M each) and Taq polymerase (2.5 u). The reaction mixtures were incubated in an automatic thermocycler for 35 cycles at 94°C for 1 min, 57°C for 1 min and 72°C for 1 min followed by a final incubation at 72°C for 10 min. The PCR products obtained using VH and VL primers were then treated with the Klenow enzyme (Promega), phosphorylated with T4 polynucleotide kinase (US Biochemicals) and ligated to SmaI digested pUC 118 phagemid vector as described earlier (8). Transformation of *E. coli* (DH5), selection of recombinant colonies with gene sequences of VH (VH/118) and VL (VL/118) and plasmid purifications were conducted by using standard protocols (12). Double-stranded DNA templates from these colonies were then sequenced using the Sequenase version 2.0 sequencing kit (US Biochemicals) or by a DuPont Genesis 2000 DNA Analysis system at the DNA Sequencing Core Facility of the Interdisciplinary Center for Biotechnology Research (ICBR) at the University of Florida. Nucleic acid sequences were analyzed using computer programs of the University of

TABLE 1
REACTIONS OF MONOCLONAL ANTIBODIES TO 60 CTV ISOLATES[z] FROM DIFFERENT COUNTRIES CLASSIFIED IN TO 20 DIFFERENT ANTIGEN GROUPS[y]

Monoclonal antibody	Reaction pattern																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1. 18H9	-	-	-	+	+	+	+	+	+	+	-	+	+	-	-	+	-	+	+	+
2. 1D3	-	+	+	-	-	-	+	+	+	-	-	+	+	-	+	-	-	+	+	-
3. 17H9	-	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
4. 16G11	-	+	+	-	-	-	+	+	+	-	-	+	+	-	+	-	-	+	+	-
5. 17A4	-	-	-	+	+	+	+	+	+	-	-	-	-	+	+	-	+	+	+	+
6. 17G11	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7. 19C1	-	-	-	+	+	+	+	+	+	+	-	-	+	+	-	+	-	+	+	+
8. 12F2	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	-	+	+	-	+
9. 4B11	-	-	-	-	+	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-
10. 9F9	+	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+
11. F10	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
Type isolate	B16	B29	B144	B2	T28	T69	T36	B227	B129	T26	T38	B13	B220	B188	B151	B214	B190	B24	B7	B213
Nos. in the group	1	1	1	7	11	1	12	8	2	3	1	1	2	1	2	1	1	2	1	1

[z]Isolates from Florida are coded with T number and those from different citrus growing regions of the world with a B number and are maintained in the Exotic Citrus Pathogen Collection, United States Department of Agriculture, Beltsville, MD (4).
[y]+ denotes a positive reaction (at least twice the value of A_{405} over healthy controls) and - denotes a negative reaction in ELISA.

TABLE 2
PRIMERS USED FOR AMPLIFICATION OF VARIABLE REGIONS OF HEAVY AND LIGHT CHAINS OF MONOCLONAL ANTIBODY 17G11 AND CONSTRUCTION OF THE SINGLE CHAIN VARIABLE REGION ANTIBODY. RESTRICTION SITES, START AND STOP CODONS AND LINKER REGION SEQUENCES ARE SHOWN.

HEAVY CHAIN VARIABLE REGION	
a. 5' end primer	5'-(C/G)AGGT(C/G)(A/C)A(A/C)A(A/G)CTGCAG(C/G)AGTC(A/T)GG 3'
b. 3' end primer	5'-TGAGGAGACGGTGACCGTGGTCCCTTGGCCCC 3'
LIGHT CHAIN (KAPPA) VARIABLE REGION	
c. 5' end primer	5'-GACATCGAGCTCACCCAGTCTCCA 3'
d. 3' end primer	5'-CCGTTTCAGCTCGAGCTTGGTCCC 3'
SINGLE CHAIN ANTIBODY: HEAVY CHAIN	
	EcoRI start
e. 5' end primer	5'-AAGAATTCGAGCTCAGAAACCATGGAGGT(C/G)(A/C)A(A/G)CTGCAG(C/G)AG 3' SacI
f. 3' end primer	5'-TTCGGAGCCAGATCCTGAGGATTTACCCTCTGAGGAGACGGTGACCGTGGT 3' linker
LIGHT CHAIN	
g. 5' end primer	5'-TCAGGATCTGGCTCCGAATCCAAAGTCGACGACATCGAGCTCACCCAG 3' linker
	KpnI stop
h. 3' end primer	5'-AAGGTACCGGATCCTCACCGTTTCAGCTCGAGCTTGGTC 3' BamHI XhoI

Wisconsin Genetics Computer Group (UWGCG).

A second set of primers were designed for construction of the SCAB gene (Table 2, e-h) to include appropriate restriction sites at the ends, an initiation codon and a 14 amino acid long synthetic linker peptide, EGKSSGSGSESKVD (2) between VH and VL. The PCR strategy used for construction of the SCAB gene is shown in Fig. 1. The SCAB gene was assembled by overlap PCR of a mixture of re-amplified PCR products of VH and VL genes using the 5' primer of VH and the 3' primer of VL. The SCAB gene was then cloned into the EcoRI and BamHI sites of the pUC 118 vector and sequenced as before.

Expression and analysis of SCAB protein. The SCAB gene was subcloned from pUC 118 into the SacI and XhoI sites of the pET 22b vector (Novagen) leading to in-frame cloning with the pelB leader sequence encoding a 22 amino acid amino-terminal signal peptide for periplasmic transport of the expressed protein and a 6-histidine tag at the carboxy-terminus. The cloned gene was expressed in a T7 RNA polymerase-based expression system (13) using *E. coli*, strain BL 21 (DE3) pLysS, according to the manufacturer's protocol. Briefly, the cells were grown at 37°C until an A_{600nm} = 0.6 to 1.0 was reached. Expression of the cloned gene was induced by adding isopropyl N-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and continuing incubation for another 3 hr at 37°C. Bacterial expression was monitored by Western blotting using goat anti-mouse (Fab' specific) antibody conjugated to alkaline phosphatase (Sigma). The SCAB protein was purified from induced cells using Ni-NTA resin from Qiagen Inc. according to the manufacturer's instructions for purification of native cytoplasmic proteins. The pelleted cells from a 200 ml culture were resuspended in 20 ml of sonication buffer (50 mM sodium

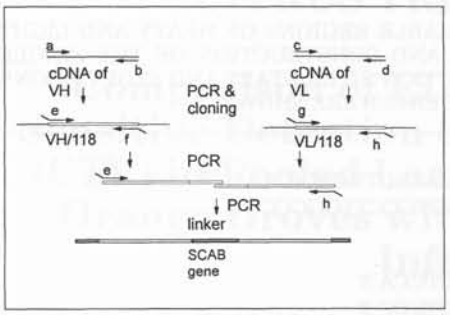

Fig. 1. Strategy for the synthesis of the SCAB gene by PCR: VH and VL regions from cDNA were amplified using primers a-d (Table 1) and cloned in to SmaI site of the pUC 118 vector. They were reamplified using a second set of primers (e-h, Table 1). A mixture of the reamplified VH and VL were used for PCR-construction of the SCAB gene with the primers e and h. The shaded areas of the SCAB gene represent restriction sites at the ends and the linker peptide region in the middle.

phosphate, pH 8.0; 300 mM NaCl) and stored frozen overnight. The lysate was either treated with DNase I (20 u/ml), or sonicated for 10-15 brief pulses at a high output setting. Ten ml of the soluble protein fraction was loaded on an equilibrated 3 ml Ni-NTA column and washed with about 25 volumes of wash buffer (50 mM sodium phosphate, pH 6.0; 300 mM NaCl; 10% glycerol). The column-bound protein was eluted with a 30 ml gradient of 0 to 0.5 M imidazole in wash buffer. The eluate was collected in 1 ml aliquots and analyzed by spectrophotometry and Western blotting. Fractions containing SCAB protein were pooled and dialyzed against phosphate buffered saline (50 mM phosphate, pH 7.4; 100 mM NaCl).

To study the extracellular transport of the expressed protein, the periplasmic fraction was extracted from 3 ml of induced culture by using the osmotic shock protocol provided by the manufacturer (Novagen). DASI ELISA was used to compare the binding specificities of the periplasmic extract containing SCAB with its parent MAb, 17G11. Microtiter plates were coated with

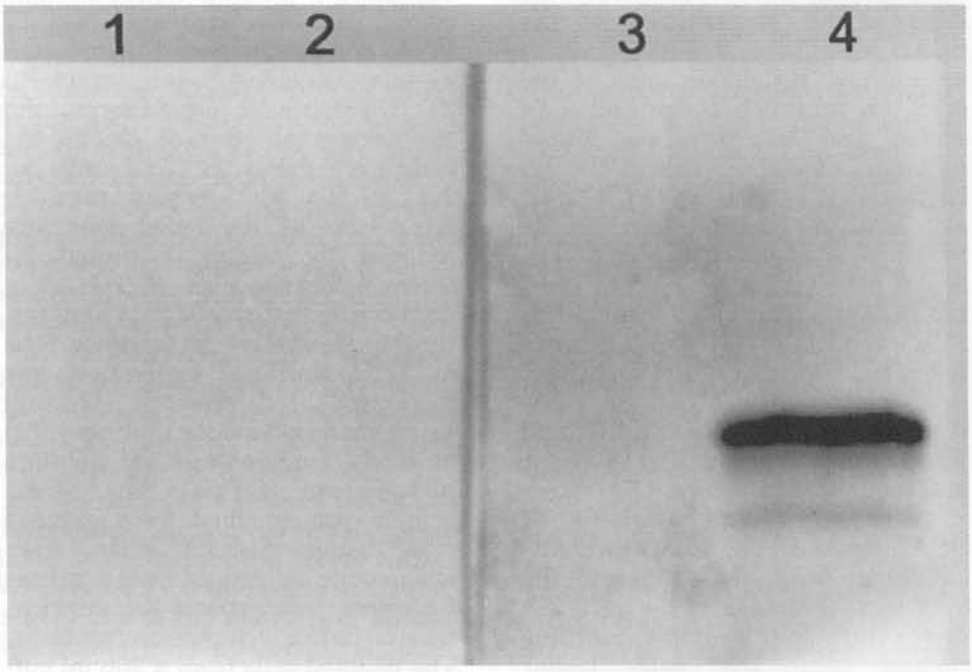

Fig. 2. Western blot analysis of healthy (lanes 1 and 3) and CTV (isolate T36) infected citrus (lanes 2 and 4) by two MAbs, 17G11 (left panel) and MCA-13 (right panel).

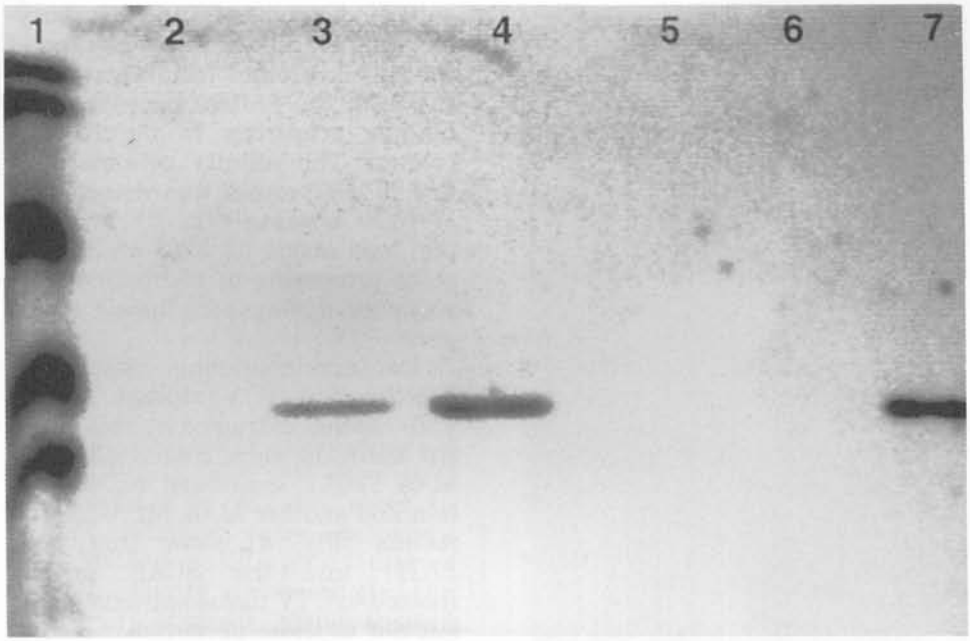

Fig. 3. Western blot analysis of the SCAB protein during different steps of affinity purification using goat anti-mouse antibody (Fab' specific) conjugated to alkaline phosphatase (Sigma). Lane 1 shows Protein molecular weight markers (from top to bottom, in kDa, 112, 84, 53.2, 34.9, 28.7). Total bacterial protein from non-transformed cells (lane 2), transformed cells before (lane 3) and after induction with IPTG (lane 4) are shown. The cell extract was passed through an affinity column as described in materials and methods and the SCAB protein was not detected in either unbound fraction (lane 5) or the wash solution (lane 6). Column bound proteins were eluted by using a gradient of imidazole and the fractions with an A_{280nm} over 0.5 were pooled and tested (lane 7).

CREC-35, a polyclonal antibody against CTV at 1 µg/ml, followed by tissue extracts from healthy and CTV-infected (isolate T36) citrus plants. Different dilutions (Fig. 5) of SCAB protein (50 µl stock from a 3 ml culture) and the MAb 17G11 (ascites) were tested. Goat anti-mouse antibody (Fab' specific) conjugated to alkaline phosphatase (Sigma) was used for detection.

The binding properties of affinity-purified SCAB protein (1mg/ml) was tested at 1:1,000 dilution and compared to MAbs 17G11 and MCA-13 (both at 1:50,000 dilution) in dot immunobinding studies (10) using antigen extracts at 1:20 dilution of healthy and CTV (isolate T36) infected citrus tissue in TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and Laemmli buffer (7).

RESULTS

A panel of 11 MAbs was screened against 60 different CTV isolates and 20 reaction patterns were recognized. Isolates reacting similarly to different MAbs were grouped together. Representative virus isolates in each group and total number of isolates in each group are shown in Table 1. Amongst the 11 MAbs tested, MAb 17G11 reacted well to most of the isolates except B144 to which it gave a very weak reaction. However, in Western blot analyses, MAb 17G11 did not react with CTV-infected tissue extract (Fig. 2) suggesting that 17G11 recognizes a neotope which is lost upon SDS treatment.

PCR of cDNA of VH and VL genes gave expected size products of about

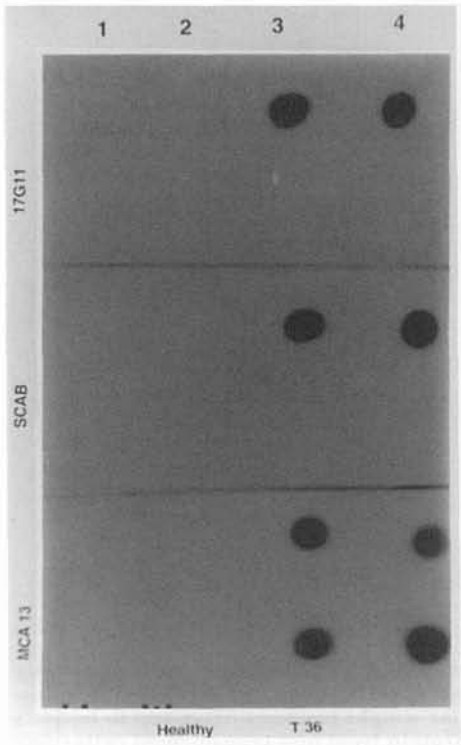

Fig. 4. Dot immunobinding assay of healthy (1 and 2) and CTV isolate T36 infected citrus tissue (3 and 4) by using bacterial expressed scab protein and two MAbs, 17G11 and MCA-13, on nitrocellulose membranes. Tissue extractions in TE buffer were spotted on the top portion of each membrane while extracts made in Laemmli buffer were spotted at the bottom.

350 bp. Their sequences were determined by double-stranded DNA sequencing using VH/118 and VL/118 plasmids. By a two-step PCR of VH and VL genes, the SCAB gene was constructed as shown in Fig. 1. The SCAB gene was cloned into pUC 118 vector and sequenced for confirmation (data not shown). The close relationship of these sequences to other published IgG variable region sequences was established by using "Blast" computer program of UWGCG (data not shown). The SCAB gene was cloned in pET 22b vector at the SacI and XhoI sites resulting in an in-frame cloning with an N-terminal 38 amino acid fusion peptide including a 22 amino acid pelB leader sequence and a carboxy-terminal 6-His tag. The 6-His tag of the fusion protein facilitates purification of the protein because of its binding properties to the Ni-NTA column. The affinity column purified SCAB protein was detected by Western blotting (Fig. 3). The protein was about 32 kDa which suggests processing of the pelB leader sequence during periplasmic transport.

Dot-immunobinding assays of healthy and CTV-infected (isolate T36) tissues, extracted by two different methods, were conducted using MAb 17G11, expressed SCAB protein and another MAb, MCA-13. The results (Fig. 4) show that both 17G11 and the SCAB protein reacted to CTV tissue extracts in TE, but not to those prepared in Laemmli buffer while MCA-13 detected CTV prepared in both the buffers. The SCAB protein did not show significant binding to healthy and TMV-infected tobacco, bovine serum albumin and ovalbumin (data not shown). In ELISA tests, the SCAB protein, from the periplasmic fraction, bound to CTV infected samples at significantly higher levels as compared to healthy tissue (Fig. 5), even though at much lower levels compared to MAb 17G11.

DISCUSSION

Antibody genes have been cloned and expressed in transgenic plants and shown to protect against viral attack (14, 15). Antibodies that recognize epitopes conserved in otherwise rapidly evolving RNA viruses are likely to be more effective in neutralization of the virus. A MAb of high binding affinity that reacts with a broad range of isolates representing most of the virus variability is an ideal candidate for studying *in vivo* attenuation of the disease. Eleven CTV-specific MAbs were tested against 60 isolates representing a broad spectrum of variability.

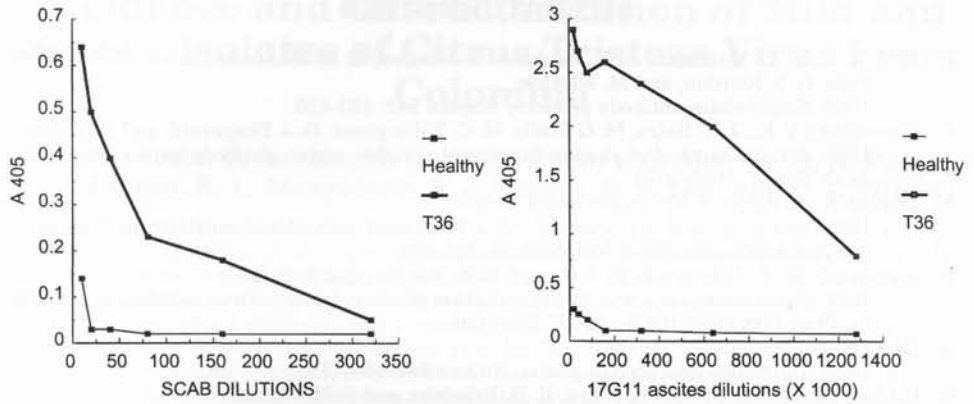

Fig. 5. Comparative titration of SCAB protein from periplasmic extract of *E. coli* (left panel) in comparison to the parent MAb 17G11 (right panel) by ELISA.

MAb 17G11 reacted strongly to all CTV isolates except B144 and was selected for construction of a SCAB gene. The inability to detect denatured antigen suggests that the epitope of MAb 17G11 is conformational.

As a first step in the investigation of the feasibility of using engineered antibodies for protection against CTV in citrus, a synthetic SCAB gene was constructed using the sequences of VH and VL regions of MAb 17G11, cloned and expressed in bacteria. Since the expressed protein showed binding affinities similar to its parent MAb 17G11 in dot-immunobinding assays, it indicates that the construct is functional in recognizing the antigen. The specificity of binding was demonstrated by its non-reactivity with other antigens. These studies were conducted with the objective of testing the functional properties of the expressed SCAB protein. Periplasmic fractions showed specific reaction to CTV in ELISA tests at levels much lower than the parent MAb 17G11. This may be either due to lower levels of SCAB in the periplasmic fraction or the reduced level of reaction of the SCAB protein with the conjugate. SCAB protein consists of only the variable domains of an immunoglobulin while the Fab' specific conjugate can react with two domains of heavy and light chains of a complete immunoglobulin. Antibodies specific to the SCAB protein are being produced for further evaluation. Studies are in progress on the development of transgenic plants using this synthetic gene to study the *in vivo* effect of SCAB protein on virus replication. In addition to having potential to develop virus resistant plants, the antibodies expressed in bacteria or plants may have great potential as future diagnostic reagents. Being much smaller in size than immunoglobulins, the single chain antibodies may also prove to be better reagents for *in situ* studies of virus infected plants.

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