Studies on the Coat Protein Genes of Four Isolates of Citrus Tristeza Closterovirus from India: Cloning, Sequencing and Expression*

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ABSTRACT. Four isolates of citrus tristeza closterovirus (CTV) from India causing stem pitting and vein flecking symptoms in lime, lemon, citron, sweet orange and grapefruit were used in this study. Two isolates caused severe stem pitting in mandarins, rough lemon and Cleopatra mandarin. The CTV coat protein gene (CPG) was selectively amplified by polymerase chain reaction from infected tissue extracts, cloned into the pUC 118 phagemid vector and sequenced. There was more than 90% similarity at the nucleotide level and above 95% at the deduced amino acid level among the sequences of these isolates. When compared to T36, a severe isolate from Florida, the same amino acid changes were found consistently at seven positions in all four Indian isolates. The coding region of the CPG of isolate B227 was expressed in *E. coli* BL21 (DE3) pLysS using the pETH3a vector. The identity of the *E. coli* expressed coat protein (ECP) was confirmed by Western blot analysis using polyclonal antibodies and MCA-13, a monoclonal antibody. Polyclonal antibodies raised against partially purified ECP expressed at 29 C reacted well with ECP and with CTV-infected tissue. The ECP can be produced in large quantities and can serve as a good source of antigen for production of antibodies and for use as a positive control in serological reactions.

Citrus tristeza virus (CTV) causes one of the most destructive disease syndromes of citrus. Quick decline on sour orange rootstock and stem pitting are two major components of the disease. Many new destructive isolates continue to be reported (12). The virus is spread by many aphid spp., *Toxoptera citricida* (Kirk.) being the most efficient (1). The virus is phloem-limited and the particles are approximately 2000-nm long flexuous rods consisting of a single type of coat protein with M_r of 26,000 (5,8,14) and a genome consisting of a single, positive strand RNA of about 20,000 nucleotides (1).

The CPGs of several biologically distinct CTV isolates have been sequenced (8,14). Information on amino acid and nucleotide sequences of different CTV isolates will be useful in developing strain specific antibodies and nucleic acid probes and in understanding the different host reactions caused by various isolates of CTV. Four severe isolates of CTV from India used in this study caused vein flecking and stem pitting on many tolerant varieties such as mandarin, rough lemon and cleopatra mandarin (6).

Rapid detection techniques are important in prevention of the disease by the use of disease free budwood and in the eradication of the disease. Enzyme linked immunosorbent assay (ELISA) is being used widely for detection of the virus in large citrus areas worldwide (3). Production of polyclonal antibodies to various isolates of CTV is often limited by various factors including production of sufficient quantities of infected tissue, international quarantine, low yields of virus, contamination by host proteins etc. To circumvent these obstacles, the CPG was cloned in an expression vector and the protein was expressed in *E. coli* cells. Antibodies were raised against the purified protein and tested for their efficacy in detecting the virus in the infected tissue.

MATERIALS AND METHODS

Virus isolates. The biological and serological characteristics of four severe isolates of CTV from India selected for the study are given in

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Table 1. B165 and B220 were aphid-transmitted isolates, while B194 and B227 were isolated from the field trees of 2-yr-old Coorg mandarin on rough lemon rootstock with stem pits on both the rootstock and the scion portions (Fig.1). These isolates were maintained in the Exotic Citrus Pathogen Collection under quarantine at the USDA facility in Beltsville, MD. The isolates were maintained by graft transmission in Madame Vinous sweet orange or Mexican lime seedlings under greenhouse conditions.

Extraction of nucleic acid and CPG amplification. Approximately 1 cm^2 leaf tissue was quick frozen in liquid nitrogen and ground to powder in a microfuge tube and extracted in 300µl of 0.1 M Tris-HCl, pH 8.0, 2 mM EDTA and 2% sodium dodecyl sulfate. Three-hundred µl of phenol-chloroform was added to the reaction tube, vortexed and heated at 70 C for 5 min followed by centrifugation for 5 min. The supernatant was passed through a 1 ml Sephadex G-50 column and the eluate was stored in liquid nitrogen. A one-

TABLE 1
BIOLOGICAL AND SEROLOGICAL CHARACTERISTICS OF FOUR INDIAN CTV ISOLATES

	B165	B194	B220	B227
Lime reaction	severe	mild	severe	severe
Quick decline	+[z]	ND	+	+
Stem pitting on lime & grapefruit	+	+	+	+
Stem pitting on Coorg mandarin, Rough lemon & Cleopatra mandarin	–	+	–	+
Vein flecking in Coorg mandarin	+	–	–	–
Reaction with MCA-13	+	+	+	+

[z] + = present; – = absent; ND = not determined.

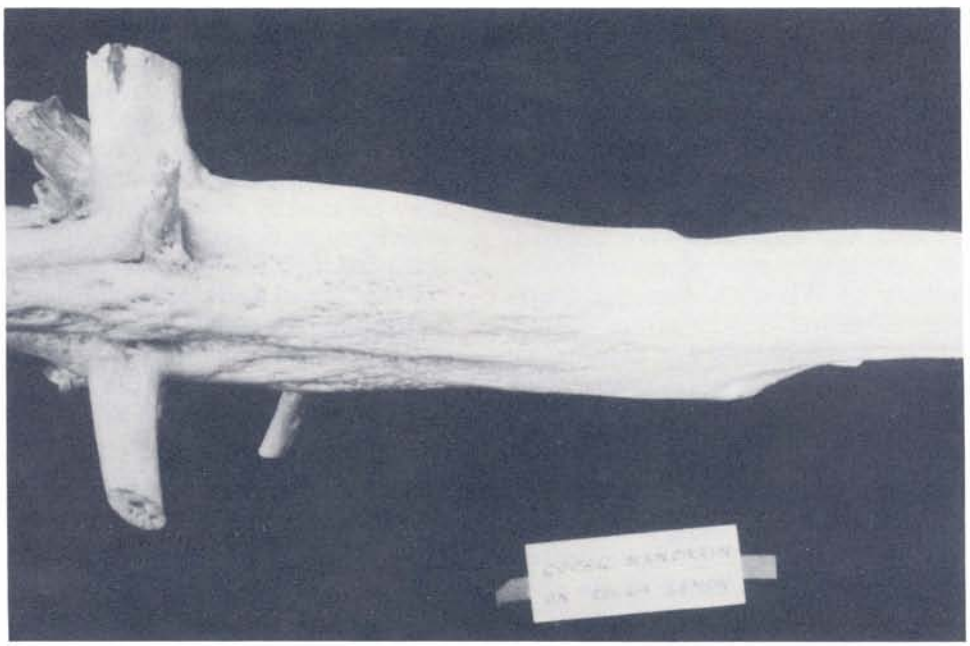

Fig. 1. The effect of CTV isolate B227 infection on a 2-yr-old Coorg mandarin tree on rough lemon stock. The bark was peeled to show the pitting near the bud union region.

tube reaction for reverse transcription of RNA of the CTV CPG to cDNA and its subsequent amplification was conducted as described previously (8, 9).

Cloning of CPG and DNA sequencing. The gene amplification products were purified by electrophoresis in 0.8% low melting point agarose (BRL Life Technologies Inc.), extracted with phenol-chloroform and precipitated in 70% ethanol. The cDNA was treated with Klenow enzyme (Promega) and phosphorylated with T4 polynucleotide kinase (US Biochemicals) and ligated to Sma-I digested pUC 118 phagemid vector (8). Competent cells of *E. coli*, strain DH5α were transformed (13). Selected recombinant colonies were tested for the presence of CTV CPG insert in the sense orientation with respect to the β-galactosidase gene by the presence of a 0.7 kb insert in EcoR1 digested plasmid preparations by agarose gel electrophoresis and the presence of coat protein in the *E. coli* protein extracts as detected by Western blotting using CTV-specific monoclonal antibody, MCA-13 (8).

Double stranded DNA templates from the selected recombinant colonies were sequenced using the Sequenase version 2.0 sequencing kit (US Biochemicals) and "universal" forward and reverse primers. Synthetic internal primers prepared at the DNA Synthesis Core Facility, University of Florida, were used to obtain sequence farther from the "universal" primers. Nucleic acid sequences were analyzed by using the computer programs of University of Wisconsin Genetics Computer Group (UWGCG) (4) and Seqaid II, version 3.60 (11). The description and amino acid sequences of the other CTV isolates used in the dendrogram have been published (8).

Expression of the CPG in *E. coli*. The coding region of the CTV CPG was expressed in *E. coli* using the T7 RNA polymerase based-pETH vector system (7) which is a modification of the pET vector system (16). The CPG insert of CTV isolate B227 in pUC118 was subcloned into the SacI-EcoRI sites in the pETH3a polylinker resulting in the placement of the insert in frame with the initiation codon of the T7 coat protein gene. With this fusion, the CTV coat protein was extended by 25 amino acids from the vector at the N-terminus. The plasmid was used to transform *E. coli*, DH5α. After ascertaining the proper size and sequence at the initiation region, the plasmid was used to transform *E. coli*, BL21 (DE3) pLysS cells. The transformed cells were grown in 50 ml LB medium at three different temperatures (22, 29 and 37 C) until OD_{600} reached 0.6 to 1.0. Expression was induced by adding isopropylthio-β-galactoside (IPTG) to a final concentration of 0.4 mM and incubation was continued at the respective temperatures for 2.5 hr. Cells transformed with the vector alone (without the CPG) and induced at respective temperatures served as controls. Aliquots (50 µl) were taken at 30 min intervals up to 2.5 hr for estimating the protein expression. The aliquots were centrifuged and the cells were resuspended in 50 µl of SDS gel extraction buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol and 10% 2-mercaptoethanol). About 10 µl/well was used for running the protein minigels and Western blots using the CTV specific monoclonal antibody, MCA-13 (15, 18).

A modified technique described by Pognonec et al. (10) was used for partial purification of the soluble portion of coat protein. The induced cultures were transferred to ice for 5 min and the cells were pelleted by centrifugation. Cells were resuspended in 50 ml ice cold Tris NaCl (20 mM Tris-HCl, pH 7.4; 200 mM NaCl) and again pelleted by centrifugation. The pellets were stored at -70 C overnight. The cells were resuspended in 50 ml ice cold lysis buffer (20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 10% glycerol, 1 mM EDTA, 0.1% NP 40), sonicated, quickly frozen in liquid nitrogen and thawed at 37 C twice and centrifuged at 10,000 g for 10 min. Saturated ammonium sulfate was added dropwise to the supernatant with constant mixing to a final concentration of 33%. After 15 min on ice, the sample was centrifuged at 10,000 g for 10 min at 4 C. The pellet was resuspended in

5 ml lysis buffer with 100 mM NaCl and dialyzed against phosphate buffered saline (20 mM potassium phosphate buffer, pH 7.4; 100 mM NaCl) for 18 hr and stored at -20 C in 1 ml aliquots.

Production and assay of antibodies. Polyclonal antiserum (CREC-35) against the above fusion protein was raised in a New Zealand white rabbit at the Cocalico Biological Inc., Reamstown, PA. Initial injection was made with 1.5 mg of protein in 1 ml of PBS. Four booster injections were made at weekly intervals. Equal volume of Freund's incomplete adjuvant was used in all the injections. Three production bleeds (each of about 25 ml) were made at weekly intervals starting one week after the last injection. The titer of the antiserum was tested by double antibody sandwich ELISA (3). The coating antibody used was MCA-13. Tissue extracts at 1:10 dilution from healthy and CTV T36 infected plants and the *E. coli* - expressed coat protein ECP of isolate B227 were used as antigens. Antiserum CREC-35 was used as the detecting antibody, tested at different dilutions and probed with goat anti-rabbit antibody conjugated to alkaline phosphatase (Sigma). Protein A Sepharose-purified IgG of antiserum 1053 (OD_{280} 2.110), a polyclonal antibody raised against purified CTV preparation was used as a positive control.

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          1                    *          *          *
B165      MDDETKKLKNKNKETKEGDDVVAAESSFGPLNLHIDPTLIAMNDVRQLGT
B194      MDDETKKLKNKNKETKEGDDVVAAESSFGSMNLHIDPTLIAMNDVRQLGT
B220      MDDETKKLKNKNKEKKEGDDVVAAESSFGSMNLHIDPTLIAMNDVRQLGT
B227      MDDETKKLKNKNKETKEGDDVVAAESSFGSMNLHIDPTLIAMNDVRQLGT
T36       MDDETKKLKNKNKETKEGDDVVAAESSFSSVNLHIDPTLITMNDVRQLST

          51         *          *          *
B165      QQNAALNRDLFLTLKGKYPNLSDKDKDFHIAMMLYRLAVKSSSLQSDDDT
B194      QQNAALNRDLFLTLKGKYPNLPDKDKDFHIAMMLYRLAVKSSSLQSDDDT
B220      QQNAALNRDLFLTLKGKYPNLPDKDKDFHIASMLYRLAVKSSSLQSDDDT
B227      QQNAALNRDLFLTLKGKYPNLPDKDKDFHIAMMLYRLAVKSSSLQSDDDT
T36       QQNAALNRDLFLTLKGKHPNLPDKDKDFRIAMMLYRLAVKSSSLQSDDDA

          101
B165      TGITYTREGVEVDLSDKLWTDVVFNSKGIGNRTNALRVWGRSNDALYLAF
B194      TGITYTREGVEVDLSDKLWTDVVFNSKGIGNRTNALRVWGRTNDALYLAF
B220      TGITYTREGVEVDLSDKLWTDVVFNSKGIGNRTNALRVWGRTNDALYLAF
B227      TGITYTREGVEVDLSDKLWTDVVFNSKGIGNRTNALRVWGRTNDALYLAF
T36       TGITYTREGVEVDLSDKLWTDVVFNSKGIGNRTNALRVWGRTNDALYLAF

          151
B165      CRQNRRNLSYGGRPLDAGIPAGYHYLCADFLTGAGLTDLECAVYIQAKEQL
B194      CRQNRRNLSYGGRPLDAGIPAGYHYLCADFLTGAGLTDLECAVYIQAKEQL
B220      CRQNRRNLSYGGRPLDAGIPAGYHYLCADFLTGAGLTDLECAVYIQAKEQL
B227      CRQNRRNLSYGGRPLDAGIPAGYHYLCADFLTGAGLTDLECAVYIQAKEQL
T36       CRQNRRNLSYGGRPLDAGIPAGYHYLCADFLTGAGLTDLECAVYIQAKEQL

          201    *          223
B165      LKKRGADEVVVTNVRQLGKFNTR
B194      LKKRGADEVVVTNVRQLGKFNTR
B220      LKKRGADEVVVTNVRQLGKFNTR
B227      LKKRGADEVVVTNVRQLGKFNTR
T36       LKKRGADDVVVTNVRQLGKFNTR
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Fig. 2 Alignment of deduced amino acid sequences of coat protein genes of four CTV isolates from India and T36, a severe isolate from Florida.

RESULTS AND DISCUSSION

The coat protein genes of the four isolates used in this study were 669 nucleotides in length and the deduced amino

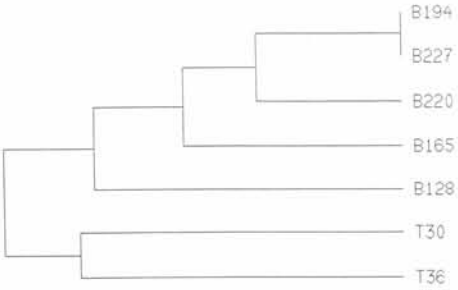

Fig. 3. A dendrogram showing the clustering relationships among the deduced amino acid sequences of the coat proteins of CTV isolates. All isolates with B numbers cause stem pitting while T30 and T36 do not cause stem pitting. The dendrogram was generated by UWGCG's Pileup and Figure programs.

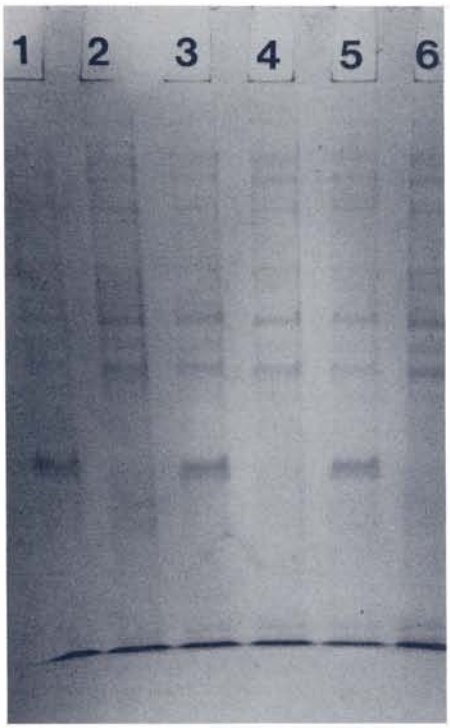

Fig. 4. Polyacrylamide gel electrophoresis of protein extracts of *E. coli*, grown at 37 C (lanes 1 & 2), 29 C (lanes 3 & 4) and 22 C (lanes 5 & 6). Bacteria transformed with the vector containing the CTV CPG insert are shown in lanes 1, 3 and 5 and those containing vector alone are shown in lanes 2, 4, and 6. The gel was stained with Coomassie Brilliant blue.

acid sequences were 223 residues long in agreement with the previously characterized CTV CPGs (8, 14). There was more than 90% direct sequence similarity at the nucleotide level and over 95% similarity at the amino acid level among the Indian isolates. Both B194 and B227 cause stem pitting in mandarins (6) and their deduced CP amino acid sequences were identical. When compared to T36, a severe isolate from Florida, 7 to 11 amino acid residues were different in the Indian isolates. Seven amino acid residues at positions 29, 41, 49, 68, 79, 100 and 208 were found to be common in the Indian isolates, but different in T36 (Fig. 2). The amino acid sequences of the Indian isolates, T36, T30 (mild isolate from Florida) and B128 (severe stem pitting isolate from Columbia) were used to construct a cluster dendrogram (Fig. 3). The Indian isolates and B128 all of which cause stem pitting, formed one cluster. T30 and T36 do not cause stem pitting and formed a different cluster.

Upon SDS-PAGE and Coomassie Brilliant blue staining, the *E. coli* expressed protein samples showed that the coat protein was expressed at the three temperatures tested (Fig. 4). The identity of the coat protein was established by Western blot using MCA-13 (Fig. 5). The faster migrating band in lane 2 is a proteolytic product of coat protein in the host plant (14). A time course study of the synthesis of CTV-CP showed that the level of protein expression increased rapidly up to 2 hr. (Fig. 6). High levels of expression often leads to formation of insoluble proteins in the bacterial expression system (16). Initially, we conducted protein expression at 37 C, but only a very small portion

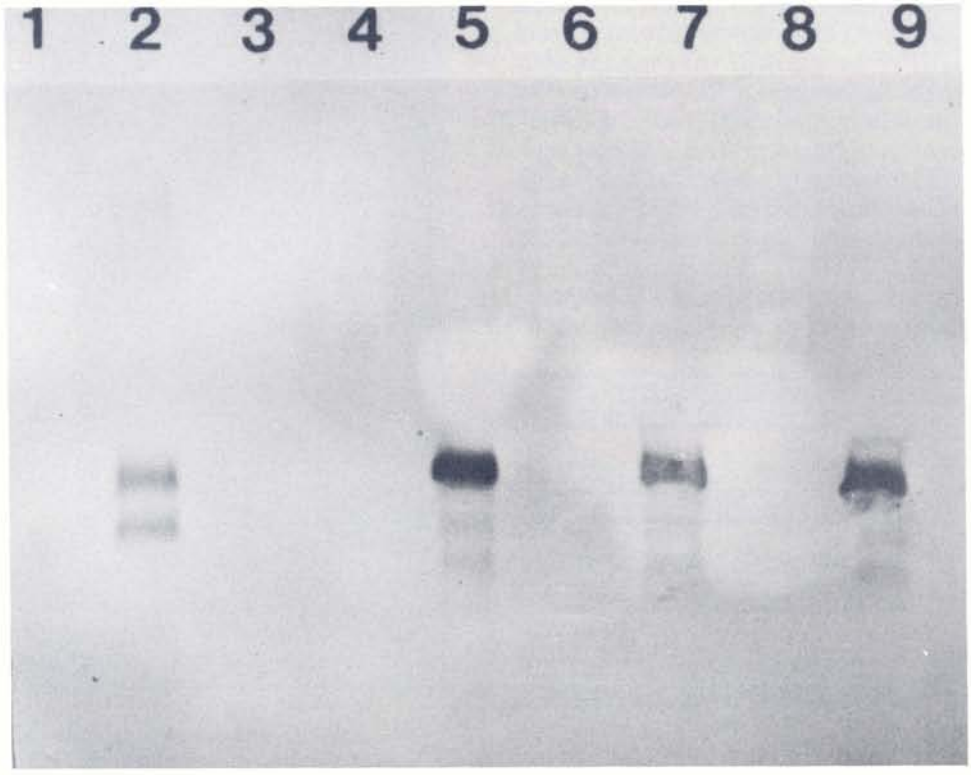

Fig. 5. Western blot analysis of the CTV coat protein expressed at 22 C (lane 5), 29 C (lane 7) and 37 C (lane 9). Extracts from the cells transformed with the vector alone (without the CPG gene) and expressed at the respective temperatures are shown in lanes 4, 6 and 8. Extracts of uninfected citrus tissue (lane 1), T36-infected citrus tissue (lane 2), and non-transformed *E. coli* cells (lane 3) were used as additional controls.

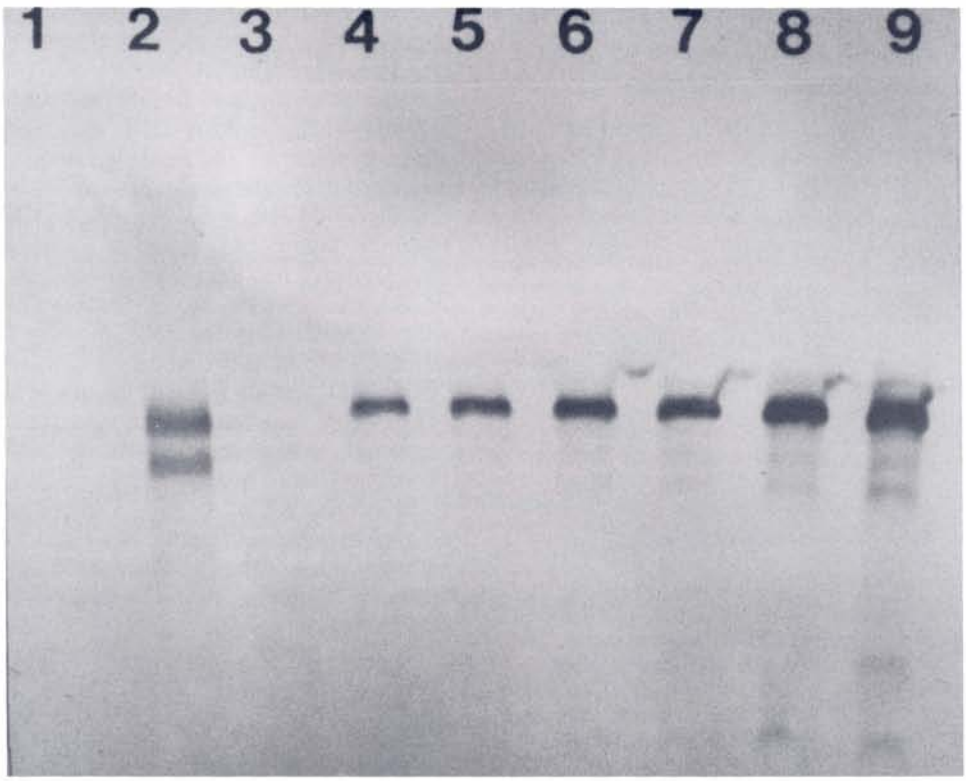

Fig. 6. Time course study of the expression of CTV-CP by Western blot. Lanes 4 to 9 show the levels of CTV coat protein expressed by transformed bacterial cells at 0, 0.5, 1.0, 1.5, 2.0 and 2.5 hr respectively after induction with IPTG. Extracts from uninfected citrus tissue (lane 1), T36-infected citrus tissue (lane 2) and *E. coli* transformed with vector alone (lane 3) were used as controls.

of the protein was soluble. The insoluble protein was detected by Western blots but was not highly reactive in ELISA using a polyclonal antibody (1053) developed against purified CTV particles. Aggregation of proteins at abnormally high concentrations and improper folding of the expressed protein may result in loss of native epitopes for the polyclonal antibody. The yield of functional recombinant proteins in *E. coli* can be increased dramatically by growing and inducing the cells at suboptimal temperatures (2,17). Under these conditions of growth, folding of the polypeptide into a native conformation is facilitated. Growth and induction of cells at 22 and 29 C resulted in detectable levels of protein in the supernatant of the sonicated cell extracts. Since only a small portion of the protein was soluble, the volume of the bacterial culture was increased from 5 ml to 50 ml. Protein expressed at 22 C was detectable in ELISA at 1:5,000 dilution while at 29 C, the protein was detectable at 1:10,000 dilution. About 14 mg of soluble protein was obtained from a 50 ml culture induced at 29 C. This preparation was used for injecting the rabbit to raise polyclonal antibodies.

In ELISA, the antiserum CREC-35 detected both the ECP and T36-infected tissue when compared with the IgG preparations of polyclonal antibody, 1053 (Fig. 7). Results of ELISA tests suggest that the expression of CP at lower temperature probably helped to maintain the native foldings of the protein and that the antigenicity of the expressed coat protein was comparable to that of the native coat protein of the intact virus particle. Ammonium sulfate precipitation was preferred to chromatographic purification of the protein to avoid denaturation of the

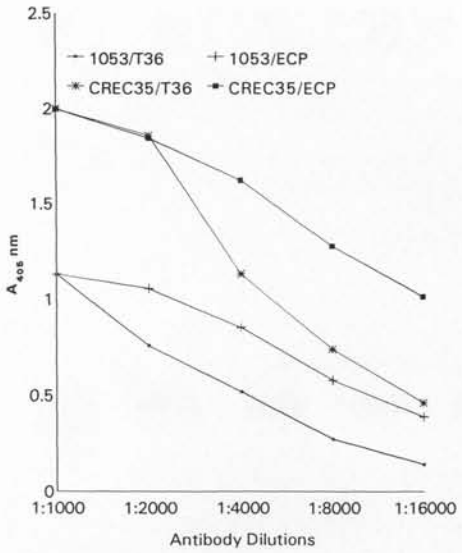

Fig. 7. ELISA reactions comparing the reactivities of polyclonal antiserum CREC-35 produced against CTV CP expressed in *E. coli* with the purified IgG preparation of polyclonal antiserum 1053. Antibodies at the indicated dilutions were reacted with the expressed coat protein (ECP) at 0.75 μg/ml or extracts of T36-infected tissue at 1:10 dilution (mg/ml). Substrate reactions were terminated after 30 min at room temperature. Antibody/antigen combinations tested are shown at the top.

protein by degradation due to SDS or in elution steps. Results of this study show that large quantities of CTV CP can be readily produced in *E. coli*. Since plasmids or the bacterial cells can be stored for longer periods, this source of CP provides an alternative approach to the laborious and expensive procedures for purification of CTV for use as an antigen to produce antibodies. Furthermore, ECP can also serve as a known and reliable positive standard in the serological reactions to detect CTV. This would eliminate the need to carry the current positive standards such as desiccated or lyophilized tissue extracts of CTV into politically sensitive locations.

Note added in proof: The sequence of the coat protein gene of isolate T36 of CTV (14) used for comparison in this study was resequenced and the deducted amino acid at position 79 was found to be histidine (8) instead of arginine as was reported originally (14). This reduced the number of amino acid differences between Indian isolates and T36 from seven to six.

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