Production of Polyclonal Antisera to the Coat Protein of Citrus Tristeza Virus Expressed in *Escherichia coli*: Application for Immunodiagnosis

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This work was partially funded through the USDA-ARS cooperative agreements 58-5310-1-205 (University of California, Riverside) and 58-43YK-0-0008 (IFAS, University of Florida), and the California Citrus Advisory Board.

We thank J. Diaz and N. Berger for excellent technical assistance in some enzyme-linked immunosorbent assay experiments.

Accepted for publication 1 March 1995.

ABSTRACT

Nikolaeva, O. V., Karasev, A. V., Gumpf, D. J., Lee, R. F., and Garnsey, S. M. 1995. Production of polyclonal antisera to the coat protein of citrus tristeza virus expressed in *Escherichia coli*: Application for immunodiagnosis. Phytopathology 85:691-694.

Using specific primers based on the sequence of the Florida isolate T36 of citrus tristeza virus (CTV), the coat protein (CP) gene was amplified by RT-PCR (reverse transcription–polymerase chain reaction) from the severe California isolate SY568 of CTV. The RT-PCR product was cloned, sequenced, and subcloned into an expression vector pMAL-c2. The CTV CP was expressed as a fusion product containing a fragment of the *Escherichia coli* maltose-binding protein (MBP). This MBP-CP fusion protein reacted with CTV-specific antisera in immunoblotting and enzyme-linked immunosorbent assay (ELISA). After cell disruption, the MBP-CP fusion protein was purified to near homogeneity by amylose resin affinity column chromatography giving a yield of 1 mg of fusion protein per 10 ml of *E. coli* culture. Antisera obtained from rabbits after injection with MBP-CP protein were specific to CTV, with a titer of about 10^5 in an indirect ELISA, and were suitable in ELISA for trapping. These polyclonal antisera reacted with a wide range of CTV isolates from different geographic sources, and of different biological properties.

Citrus tristeza virus (CTV) is the most destructive pathogen of citrus and causes substantial economic losses in the citrus-growing industry worldwide (3,8). Strategies aimed at minimizing the destructive effect of the tristeza disease include regulatory methods that are heavily dependent on appropriate diagnostic procedures. For example, in California, extensive indexing is required for certification of budwood and identification of infected trees for eradication. Since 1963, several million trees have been checked for the presence of CTV. Indexing under this program is currently performed by the enzyme-linked immunosorbent assay (ELISA), a convenient and reliable method for the detection of CTV in the field samples (13). However, the enormous scale of the indexing program requires large amounts of specific antisera and a consistent supply of antigen for immunization purposes.

CTV has long, flexuous, filamentous particles containing a single species of 25-kDa coat protein (6,17). The host range of CTV is very narrow, and, like other closteroviruses, in *Citrus* spp. the virus is associated with the phloem (2). Because of this association, virus purification is difficult and the yields of purified CTV virions are usually low (1,7). Even the best methods may produce CTV preparations that are still contaminated with host components. As a result, polyclonal antisera are often unsatisfactory for the accurate diagnosis of CTV. Different approaches have been used to overcome these drawbacks, including the use of CTV coat protein (CP), fractionated on sodium dodecyl sulfate (SDS) polyacrylamide gels as immunogen (9), cross-absorption of the antisera with healthy plant extracts (14), and generation of monoclonal antibodies (MAbs) (12,19).

Recently, the CP gene of the Florida T36 isolate of CTV was cloned and sequenced (17). This provided the opportunity to utilize molecular biology methods as an alternative approach to obtaining the CTV CP antigen. In this paper we report preparation of a fusion protein containing the CP from the California severe SY568 CTV isolate in *Escherichia coli* cells, and use of the purified fusion protein as antigen to successfully raise wide-spectrum, polyclonal, CTV-specific antisera.

MATERIALS AND METHODS

Virus source and purification. SY568, a severe California CTV isolate, was maintained in Mexican lime (*Citrus aurantiifolia* (L.)) under greenhouse conditions. The virus was isolated from young bark tissue and purified by the method used for purification of beet yellows virus (15), except 0.1 M Tris-HCl buffer, pH 7.9, containing 0.2% 2-mercapthoethanol, 0.1% Triton X-100, and 1 mM phenyl-methyl-sulfonyl-fluoride was used throughout the purification procedure. Viral RNA was extracted by the phenol-chloroform-SDS method and ethanol precipitated (16).

Cloning and sequencing of the CTV CP gene. CTV RNA (approximately 2 µg) was denatured with 20 mM methylmercury hydroxide, and the first cDNA strand was synthesized for 2 h at 37°C in a final volume of 50 µl containing 500 units of murine Moloney leukaemia virus reverse transcriptase (United States Biochemical Corp. [USB], Cleveland, Ohio), 0.5 mM dNTPs, 50 µg of random hexamers, and 50 units of ribonuclease inhibitor. First-strand buffer provided by the manufacturer (USB) was used. The reaction was stopped by addition of 2 µl of 0.5 M EDTA, and

the first-strand cDNA was extracted with phenol/chloroform and precipitated with ethanol.

Two pairs of primers synthesized based on the known sequence of the T36 CTV CP gene (17) were used for amplification of the SY568 CP gene. The first pair, primers I (cagctgcAGTCTATGTTAGCTAGACGTCA) and II (gacggtaCCTTCTAAACGATCGACCACAGT), was used to amplify and clone the CP gene including additional upstream and downstream regions. The virus-sense primer I corresponds to nucleotide (nt) positions 15,982 to 16,003 of the complete CTV sequence, while complementary-sense primer II corresponds to nt 17,002 to 17,024 (4). Virus-specific nucleotides are typed in upper-case letters and the *Pst*I and *Kpn*I sites introduced to facilitate cloning of the polymerase chain reaction (PCR) product are underlined. The second pair, primers III (ccgaattcGACGACGAAACAAAGAAATTG) and IV (gttggatCCGGGAATCGGAACGCAACAGATCAA), was used to amplify the CP coding region, and the resultant PCR product was cloned into pMAL-c2 expression vector in-frame with the fragment of maltose-binding protein (MBP). The virus-sense primer III corresponds to nt positions 16,158 to 16,178 of the complete CTV sequence, while the complementary-sense primer IV corresponds to nt 16,823 to 16,848 (4). Virus-specific nucleotides are typed in upper-case letters and the *Eco*RI and *Bam*HI sites introduced to facilitate this cloning are underlined. PCR was performed using approximately 1/20th of the first-strand cDNAs precipitated from the 50-µl reaction volume. The standard 100-µl PCR reaction contained 1 unit of *Taq*-polymerase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), 0.2 mM dNTPs, and 140 µM of each primer. The buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 mg per ml gelatine) supplied by the manufacturer (Boehringer Mannheim) was used throughout. The amplification profile included 2 min at 94 C (1 cycle); 30 s at 94 C, 30 s at 54 C, 1 min at 72 C (30 cycles); and 10 min at 72 C (1 cycle). The synthesized PCR products were analyzed in 1% agarose gels in Tris-acetate-EDTA buffer.

The PCR products to be cloned were extracted with phenol/chloroform, precipitated with ethanol, and digested with the respective pair of restriction endonucleases. After digestion, the DNA fragments were fractionated in low melting point agarose gels, extracted by standard procedures (16), ligated into the vector digested with the same pair of restriction enzymes, and transformed into XL1-Blue competent cells (Stratagene, La Jolla, Calif.) prepared according to the method of Hanahan (16). Two plasmid vectors, pBluescript SK (Stratagene), and pMAL-c2 (New England Biolabs, Beverly, Mass.) were used for sequencing and expression of fusion protein, respectively. All constructs were verified by sequencing; the Sequenase Version 2.0 kit (USB) and T7, T3 and CTV-CP primers were used for sequencing. All primers were synthesized by the Biotechnology Instrumentation Facilities of the University of California at Riverside.

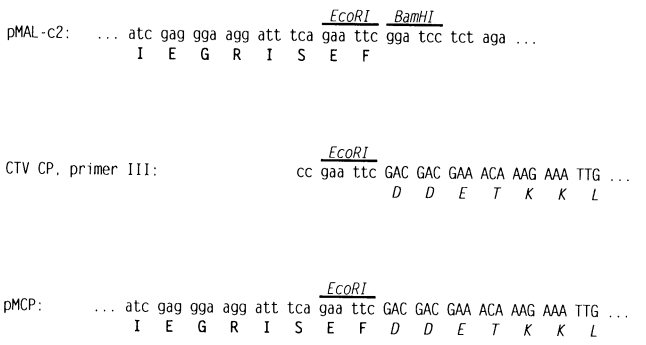

Fig. 1. Schematic representation of the fusion site between the maltose-binding protein (MBP)-encoding portion of the pMAL-c2 plasmid and the coat protein (CP) gene of CTV. CTV CP gene flanked by *Eco*RI and *Bam*HI sites was cloned into the pMAL-c2 vector digested with *Eco*RI and *Bam*HI. This resulted in the in-frame fusion of the MBP and CP open reading frames and generation of the MBP-CP expressing plasmid pMCP.

Expression of the CTV CP in *E. coli* and purification of the fusion protein. Four hundred milliliters of Luria-Bertani broth, containing 0.2% glucose and 100 µg per ml ampicillin, was inoculated with 4 ml of fresh overnight culture of XL1-Blue cells bearing the recombinant plasmid pMCP (Fig. 1) and incubated at 37°C with vigorous shaking. When OD_{600} of the suspension reached approximately 0.5, isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to a final concentration 0.3 mM and incubation was continued at 37°C for 3 h. Cells were harvested by centrifugation at 4,000 *g* for 20 min, resuspended in 20 ml of lysis buffer (10 mM sodium phosphate, pH 7.0 buffer containing 30 mM NaCl, 0.25% Tween 20, 10 mM 2-mercapthoethanol, 10 mM EDTA, and 10 mM ethyleneglycol-*bis*-(β-amino-ethyl ether)N, N′-tetra-acetic acid (EGTA), and frozen at –20°C overnight. The frozen suspension was thawed, cells were disrupted by sonication, and NaCl was added to a final concentration 0.5 M. After centrifugation at 9,000 *g* for 30 min the resulting supernatant (crude extract) was saved for purification of the fusion protein. The pre-swelled amylose resin (New England Biolabs), 1.5 g per 50-ml column buffer (10 mM Na-phosphate buffer, pH 7.2, 0.5 M NaCl, 1 mM Na-azide, 10 mM 2-mercapthoethanol, 1mM EGTA), was packed into a 1.5 × 10 cm column. The column was washed with 3 volumes of the column buffer containing 0.25% Tween 20 and the total crude extract was applied. The column was washed with 3 volumes of column buffer + 0.25% Tween 20, followed by 5 volumes of column buffer without Tween 20, and the fusion protein was eluted with column buffer containing 10 mM maltose. Concentration of the eluted protein was estimated spectrophotometrically using the extinction coefficient $A_{280,1cm}$ = 2.0.

Electrophoresis of proteins and immunoblotting. Proteins were analysed by electrophoresis in 8 to 20% gradient polyacrylamide gels using the denaturing discontinuous Tris-glycine-SDS system (5). Samples were denatured prior to polyacrylamide gel electrophoresis (PAGE) by boiling for 5 min in the presence of 1% SDS and 7 mM 2-mercapthoethanol. Pharmacia LMW protein markers were used (Pharmacia-LKB, Piscataway, N.J.): phosphorylase B (94-kDa), bovine serum albumin (BSA, 67-kDa), ovalbumin (43-kDa), carbonic anhydrase (30-kDa), soybean trypsin inhibitor (20.1-kDa) and α-lactalbumin (14.4-kDa). After electrophoresis the polyacrylamide gels were stained with Coomassie Brilliant Blue R-250.

For further analysis by immunoblotting, PAGE-separated proteins were electrophoretically transfered onto nitrocellulose (BAS-85, Schleicher & Schuell, Keene, N.H.) according to the procedure of Towbin et al. (18). Immediately after the transfer, the nitrocellulose was soaked in Tris-buffered saline (TBS) buffer (100 mM Tris-HCl, pH 7.5, 0.9% NaCl) containing 3% BSA for 15 h at room temperature and reacted with CTV-specific chicken antisera prepared to SDS-denatured CP (1/2,000 dilution) (9) or MBP-specific rabbit antisera (1/10,000 dilution, New England Biolabs) in TBS buffer containing 0.1% Tween 20 and 3% BSA. After extensive washing in TBS buffer containing 0.1% Tween 20, the membranes were incubated with either anti-chicken (Sigma, St. Louis, Mo.) or anti-rabbit (Sigma) antibodies conjugated with alkaline phosphatase. CTV-specific and MBP-specific bands were visualized with NBT/BCIP as a substrate. A set of pre-stained marker proteins (Bio-Rad, Richmond, Calif.) consisting of phosphorylase B (142.9-kDa), BSA (97.2-kDa), ovalbumin (50-kDa), carbonic anhydrase (35.1-kDa), soybean trypsin inhibitor (29.7-kDa), and lysozyme (21.9-kDa) was used for immunoblot analyses.

ELISA. All tests were performed using the indirect ELISA method. Wells of Immulon 2 microtiter plates (Dynatech, Chantilly, Va.) were coated with the goat anti-CTV IgG (2 µg per ml in 0.2 M sodium-carbonate buffer, pH 9.6). After a 2 to 3 h incuba-

tion at 37 C, the wells were washed three times with phosphate-buffered saline containing 0.1% Tween 20 (PBST) and loaded with the 200-µl sample of plant tissue extract prepared by grinding 1 g of fresh citrus bark in 10 ml of PBS buffer. The plates were incubated overnight at 4°C, washed extensively with PBST, and loaded with dilutions of the MBP-CP antiserum. After incubation for 2 h at 37°C, the plates were washed with PBST, incubated with goat anti-rabbit alkaline phosphatase conjugate (Sigma, #A-9919, 1:30,000 dilution) for 2 to 3 h at 37°C, and washed extensively with PBST. After loading with the substrate solution, 0.6 mg per ml p-nitrophenyl phosphate (Sigma), plates were incubated at room temperature for 30 min, and the ELISA reactions were recorded using an ELISA reader at $A_{405\ nm}$.

RESULTS AND DISCUSSION

The DNA fragment amplified with the use of primers I and II and the SY568 template cDNA was cloned into the pBluescript SK plasmid. The sequence determined differed from the respective sequence of the Florida T36 CTV isolate (17) in only 3 nucleotide substitutions (data not shown). Of these three, only one resulted in an amino acid change, Arg at amino acid position 79 (17) was substituted by His, in agreement with the corrected sequence of the CTV T36 coat protein (10,11).

The CTV-CP gene, starting with the second codon (Fig. 1), was amplified with the use of primers III and IV, and after digestion with *Eco*RI and *Bam*HI, cloned into the pMAL-c2 plasmid. This expression vector utilizes a 42-kDa fragment of bacterial MBP as a tag for fusion constructions. The resulting recombinant plasmid pMCP (Fig. 1) was verified to have correct junction sites by sequencing.

After induction with IPTG, XL1-Blue cells transformed with pMCP produced an additional 67-kDa protein that was absent from the control cells transformed with pMAL-c2 plasmid. The size of this additional protein was in good agreement with the expected molecular weight of the fusion protein, MBP + CP. This 67-kDa protein reacted with antisera specific to MBP (data not shown) as well as with CTV antisera (Fig. 2) in immunoblotting assays. Therefore, this 67-kDa protein will be referred to as MBP-CP fusion protein. Time-course experiments suggest that the optimal time interval between induction and harvesting *E. coli* XL1-Blue cells to be 3 h, while the optimal concentration of IPTG for induction was found to be 0.3 mM (data not shown). The induced MBP-CP fusion protein was purified from the cell extracts in a single affinity column chromatography step, using the amylose resin as a column matrix. This purification protocol was very simple and produced virtually homogeneous MBP-CP protein (Fig. 3). The yield of the purified fusion protein was estimated by A_{280} measurements at 40 mg per 400 ml of growing, transformed *E. coli* suspension.

The MBP-CP fusion protein was injected into rabbits at approximately 1 mg per ml directly in a column elution buffer (10 mM Na-phosphate buffer, pH 7.2, containing 0.5 M NaCl, 10 mM 2-mercapthoethanol, 1 mM EGTA, and 10 mM maltose). A series of subcutaneous and intramuscular injections were done at 1-week intervals, and sera were collected at 2-week intervals after the last injection. The titer against CTV, as determined by indirect ELISA, reached approximately 10^5 4 weeks after the last injection. The sera raised against the 67-kDa fusion protein gave exceptionally low background with healthy plant tissues when used as a second antibody in indirect ELISA and could be used for detecting CTV infections in field-grown trees, at dilutions of 1:4,000 to 1:10,000 (data not shown).

To illustrate the specificity of the antisera produced we performed an immunoblot analysis of total proteins from CTV-infected and healthy sweet orange plants (Fig. 4). The antiserum to MBP-CP fusion protein gave a strong specific reaction with the CTV CP band of approximately 25-kDa from CTV-infected plants. The reaction was equally strong for a severe, quick decline isolate T36, and for a mild, symptomless isolate T30 (both from Florida), suggesting a broad reaction range of this anti-MBP-CP serum toward different CTV isolates. This was further confirmed in ELISA; this antiserum demonstrated a broad reaction toward CTV isolates from California, Florida, Hawaii, Spain, Japan, Israel, the People's Republic of China, Brazil, the Philippines, and South Africa (data not shown).

The polyclonal antisera prepared using the methods described had a titer of approximately 10^5 in an indirect ELISA against CTV antigen. The titer was calculated on the assumption that this is the highest antiserum dilution at which the signal in ELISA is at least 3-fold higher than the signal for the respective healthy

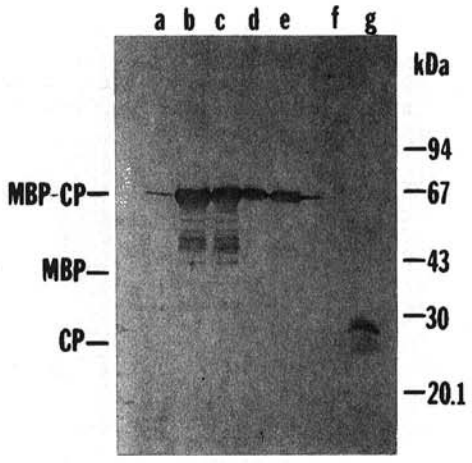

Fig. 2. Immunoblot analysis of proteins synthesized in bacterial cells harboring the pMCP plasmid after analysis in 8 to 20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Detection with the chicken anti-CTV serum diluted 1:2,000: (a) bacterial extract, no IPTG induction; (b) bacterial extract, IPTG induction; (c) bacterial extract, IPTG induction, supernatant after sonication and low-speed clarification; (d) same as in (c), pellet; (e) supernatant from (c) purified by a single cycle of affinity chromatography; (f) bacterial extract containing control maltose-binding protein; (g) partially purified CTV, SY568 isolate.

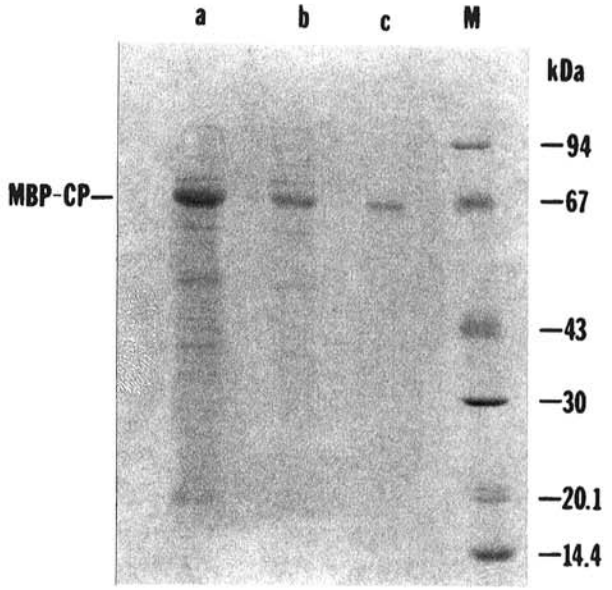

Fig. 3. Electrophoretic analysis in a 8 to 20% polyacrylamide gel electrophoresis of (a) extract from *Escherichia coli* transformed with pMCP after isopropyl-beta-D-thiogalactopyranoside induction; (b) same as in (a) after sonication and low-speed clarification; (c) same as in (b) after a single step of affinity collumn chromatography; (M) marker proteins. After electrophoresis the gel was stained with Coomassie Brilliant Blue G-250.

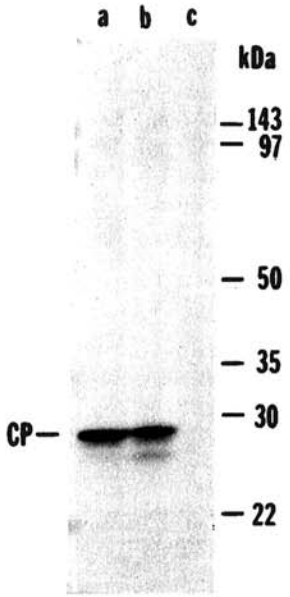

Fig. 4. Immunoblotting of total proteins from the sweet orange bark tissue after electrophoretic analysis in a 8 to 20% polyacrylamide gel electrophoresis. The immobilized proteins were treated with anti–maltose-binding protein–coat protein antiserum (dilution 1:3,000): (a) plant infected with CTV isolate T30; (b) plant infected with CTV isolate T36; (c) healthy plant. Positions of marker proteins are indicated.

extract. However, in immunoblots these antisera demonstrated substantially higher titers (exceeding 10^6), which suggested preferential detection of denatured CTV coat protein. Preliminary tests were made with MBP-CP antisera as a source of trapping antibodies in double antibody sandwich ELISA in combination with selected CTV-specific MAbs (data not shown). Further development of fusion protein antibodies optimized for trapping CTV is underway.

The antiserum to MBP-CP fusion protein was used during the past 2 years in California for indexing, certification, and surveys of CTV infections in the Central Valley with highly satisfactory results. We believe that the recombinant virus coat proteins expressed in bacterial cells have great potential as an alternative source of antigens for raising specific antibodies to plant viruses. They can be produced in large quantities and can be manipulated or modified as needed for specific uses.

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