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Expression of the RNA-dependent RNA Polymerase of *Citrus Tristeza Closterovirus* in *Eschericia coli* and Production of a Polyclonal Antibody

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ABSTRACT

The RNA-dependent RNA polymerase (RdRp) gene was first amplified by reverse transcription/polymerase chain reaction (RT/PCR) from dsRNA template isolated from citrus leaves infected with citrus tristeza closterovirus (CTV). Then the RdRp was cloned into expression vector and *Escherichia coli* was transformed with this vector containing the RdRp gene for expression. Analysis of total protein from *E. coli* by polyacrylamide gel electrophoresis showed that expression of a 60 kDa CTVRdRp protein was induced at 30 min and gradually increased for 3 hr after induction. Further analysis of the protein indicated that the RdRp was not soluble, and found only in the insoluble fractions of the *E. coli* cells. A polyclonal antiserum was produced by injecting a rabbit with the RdRp protein expressed in *E. coli* a collecting antiserum from it. Western blot analysis of the total protein from *E. coli* expressing the RdRp with this antiserum showed that the polyclonal antibody produced was specific to the RdRp of CTV.

Key words: CTV, RdRp, protein expression, antibody production

INTRODUCTION

The RNA-dependent RNA polymerase (RdRp) is universally conserved among different groups of RNA viruses; however, different viruses use different mechanism of expression for their RdRp. In some viruses, the RdRp is produced as a single peptide but, it is expressed as part of a polyprotein containing more than one viral protein in other viruses. The expression of the RdRp is controlled by proteolytic processing, translational readthrough or frameshifting in different viruses (Gallie, 1996). Understanding the expression of the RdRp and other viral proteins require molecular characterization of genes encoding them and analysis of their expressions *in vitro* and *in vivo*.

Low level of expression and accumulation of viral RdRp in plants makes very difficult to isolate functional RdRp from infected host cells. Although the RdRp of

some animal and plant viruses were isolated from partially purified extracts of their infected host cells (Graaff and Jaspar, 1994), most viral RdRps have been identified based on sequence analysis of conserved domains (O'Reilly and Kao, 1998). Among plant viruses, the partially purified extract of the RdRp of turnip yellow mosaic virus (TYMV) and brome mosaic virus (BMV) have been well-characterized (Singh and Dreher, 1997; Sun and Kao, 1997; Siegel et al. 1998). On the other hand, for most viruses the isolation of the partially purified active RdRp from the infected plants was unsuccessful due to host characteristics, low expression level, and/or poor solubility of the purified RdRp itself (Graaff and Jaspar. 1994).

Difficulties associated with the purification of the RdRp from the infected host cell can be overcome by expression of the RdRp in heterologouse systems such as *Escherichia coli.* Enzymatically active RdRps of poliovirus (Rothstein et al. 1988), hepatitis C virus (Behrens et al. 1996; Lohmann et al. 1997), tobacco vein mottling virus (Hong and Hunt, 1996) and bamboo mosaic virus (BaMV) (Li et al. 1998) were expressed in *E. coli,* and their function and catalytic activity were characterized. Some other viral RdRp were expressed in *E. coli* and the products were used for production of antibodies specific to viral RdRps. These antibodies were later used for detection, identification and functional dissection of RdRps of RNA viruses in the infected host tissue (Hayes et al. 1994; Scholthof et al. 1995).

Citrus tristeza virus (CTV) is a single-stranded positive-sense RNA virus belonging to *Closterovius* genus in the *Closterovridae* family. It has long thread-like, flexuous, filamentous particles about 2000 nm by 11 nm (Bar-Joseph and Lee, 1990). Virions of CTV consist of an about 20 kb single-stranded positive-sense RNA molecule encapsidated with major and minor capsid proteins (CPs) (Febres et al. 1996). The genome is organized into 12 open reading frames (ORF) potentially encoding 17 protein products and the 3' and 5' untranslated regions (UTR) (Pappu et al., 1994; Karasev et al. 1995). Sequence analysis of the CTV genome showed that a 56-kDa protein encoded by ORF1b which contained sequences similar to typical conserved motifs of RNA-dependent RNA polymerases (RdRp) of positive- stranded RNA viruses (Karasev et al., 1995).

Based on sequence analysis it was previously suggested that ORF1b of CTV encoding the RdRp overlaps with ORF1a encoding two papain-like proteases and two replication associated proteins, a methyltransferase and a helicase. It was also proposed that the CTV RdRp is expressed by a +1 translational frameshifting at the carboxy terminus of the polyprotein encoded by ORF1a\1b (Karasev et al. 1995). We recently demonstrated that the putative RdRp of CTV is expressed by a +1 translational frameshift at the carboxy terminus of the polyprotein (Cevik et al., 2001). However, the expression of the RdRp of CTV has not been detected in infected plants and the fate of the RdRp is still unknown due to unavailability of antibodies specific to this protein. In this study, the RdRp gene of CTV isolate T36 was cloned and expressed in *E. coli* and a polyclonal antibody specific to the expressed RdRp protein was produced.

MATERIALS and METHODS

Amplification of the CTV RdRp Gene

Double-stranded RNA (dsRNA) was isolated from T36-infected bark tissue obtained from the Collection of Exotic Citrus Pathogens, in Beltsville, MD, USA using previously reported CF11 cellulose-based protocol (Valverde et al., 1990). The cDNA specific to the RdRp region of CTV isolate T36 was synthesized from the dsRNA template using Superscript II reverse transcriptase (GIBCO/BRL) with primer CN257 (5' CTACTCGAGATCTATCAATCGATCAGCCGGTT 3') according to the manufacturer's instructions. The RdRp gene was amplified from this cDNA by polymerase chain reaction (PCR) using Taq DNA polymerase and primers CN256 (5' TGTAAGCTT**ATG**AGACAC CGCCCCTCCT 3') and CN257. These primers were designed based on published sequence of isolate T36 (Karasev et al., 1995) and since the RdRp gene does not have an initiation codon an ATG codon (bold) was incorporated into the plus-sense primer CN256. In addition, specific recognition sequences for the restriction enzymes *Hind*III and *Bgl*II (underlined sequences) were incorporated into primers CN256 and CN257, respectively, to facilitate the cloning into the expression vector. The aamplification was conducted in 50 µl reaction containing 50 mM KCl, 10mM Tris-HCl pH 9.0, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 0.1 mM of each deoxyribonucleotide triphosphate (dNTP), 100 pmol of each primer, and 2-10 µl of cDNA template. The mixture was incubated at 94 °C for 2.5 min for initial denaturation, and 94 °C for 1 min, 45 °C for 1 min, and 72 °C for 2 min. for 40 cycles followed by one cycle at 72 °C for 10 min.

Cloning and Expression of the CTV RdRp Gene

The amplified RdRp gene was first cloned into the pGEM-T cloning vector (Promega) using the T-A cloning method with T4 DNA ligase. Then the *Hind*III/*Bgl*II fragment of the pGEM-T clone containing the RdRp gene was cut with *Hind*III and *Bgl*II restriction enzymes and it was sub-cloned into the pETh-3c expression vector (Invitrogen) cut with the same enzymes to generate pETh3c-CTVRdRp clone for expression in *E.coli*. The presence and the integrity of the CTV RdRp ORF in the pETh3c vector were confirmed by sequencing.

Competent cells of *E. coli* strain BL21 was transformed with the pETh3c-CTVRdRp by incubation at 42 °C for 90 second chilled on ice. Transformed cells were grown in liquid 2X YT medium (1.6% bacto-trytone, 1% bacto-yeast extract, 1.5% bacto-agar and 0.5 NaCl) for 1 hr at 37 °C and plated on 2X YT medium containing 75 mg/l ampicilin and incubated at 37 °C for 16 hr to obtained individual colonies. A single colony was selected and inoculated into 5 ml 2XYT media with 75 mg/l ampicilin and incubated at 37 °C overnight. An aliquot was taken from the overnight culture and inoculated into fresh 2X YT culture media with 75 mg/l ampicilin. The expression of the RdRp protein was induced by addition of 0.4 M isopropyl thio-β-D-galactoside (IPTG) 3h after incubation at 37 °C. The expression of the RdRp protein was monitored for 3 hrs by taking samples from the bacterial cultures induced by IPTG. The total bacterial proteins from *E. coli* with the pETh3c and pETh3c-CTVRdRp

plasmids were analyzed on a 10% SDS-polyacrylamide gel by electrophoresis (SDS-PAGE) to separate CTVRdRP from bacterial proteins. The gel was stained with Coomassie brilliant blue G-250 and destained with acetic acid solution (Sambrook et al. 1989) to visualize the expressed RdRp protein.

The recombinant RdRp protein produced in *E. coli* and separated from other bacterial proteins by SDS-PAGE and stained with 0.3 M $CuCl_2$ to visualize the bands. The protein band was excised from the gel, and the gel slice destained in 0.25 M EDTA/Tris-HCl, pH 9.0. The protein in the gel slice (about 5-7.5 mg) was used for production of polyclonal antibodies in a rabbit by injecting about 1.0-1.5 mg of expressed protein into the rabbit weekly for four weeks (Cocalico Biologicals Inc.). Antiserum was collected after the forth injection and tested for specificity to the expressed protein by Western blot analysis. To increase the antiserum titer, one more injection with about 2.5 mg protein was performed after the fourth collection of antiserum.

Western Blot Analysis

Total protein was extracted from *E. coli* strain BL21 with or without the CTV RdRp gene. The extracts were separated on 10% SDS-PAGE and transferred to nitrocellulose membranes using a semi-dry blotter (Bio-Rad). The membranes were then probed with different dilutions of antibody raised against the recombinant CTVRdRp followed by alkaline phosphatase (AP) conjugated anti-rabbit secondary antibody. The blots were developed by colorimetric detection system for the AP using the BCIP and NBT substrates (Sigma).

RESULTS and DISCUSSION

Expression of the RdRp of CTV in *E. coli*

Expression of CTV RdRp was induced in E. *coli* strain BL21 transformed with the pETh3c-CTVRdRp plasmid by addition of isopropyl thio-D-galactoside (IPTG). The CTV RdRp was expressed as a fusion protein with an estimated molecular weight of about 60 kDa, which contains about 3 kDa from the vector and 57 kDa from the CTV RdRp ORF. Analysis of bacterial proteins by SDS-PAGE showed that expression of the 60 kDa CTVRdRp was induced 30 min after addition of IPTG and it was accumulated for 3 hrs in the bacterial cells containing the pETh3c-CTV RdRp plasmid (Figure 1 lane 4-7). The protein was not expressed in non-induced cells with the same plasmid or induced cells containing the pETh3c vector alone (Figure 1A lanes 3 and 2, respectively). Since the expression of the protein was specifically induced and its size corresponded to the expected size, it was concluded that this protein was the CTV RdRp.

Analysis of the proteins from bacterial cells indicated that the recombinant RdRp was not soluble, and was found only in the insoluble fractions of the *E. coli* cells (Figure 2 lanes 6 and 7). To produce soluble CTV RdRp, expression was induced at lower temperatures including 30 and 35 °C. Although the expression of the RdRp was

induced at both temperature, the expression level did not reach the level of expression at 37 °C, and the protein could not be solubilized and always remained in the insoluble fractions of the cell (Figure 4B lanes 3-4 and 8-9) In addition, several different solvents were tested to solubilize the recombinant CTVRdRp, but failed to solubilize it. Aggregation of the CTVRdRp in *E. coli* prevented the purification of the RdRp protein for functional analysis. This type of problem was observed with the expression the of RdRp of other plant and animal viruses in E.coli. Therefore, it was expected that the CTV RdRp may expressed in E. coli as an insoluble protein. In some cases, this problem was resolved with deletion of some hydrophobic region involved in aggregation of the RdRp (Behrens et al. 1996; Lohmann et al. 1997).

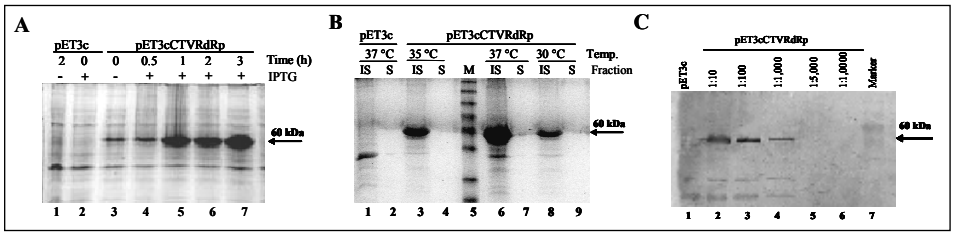

Figure 1. The expression of the RdRp of CTV in *E. coli* for production of a polyclonal antibody (A) SDS-polyacrylamide gel electrophoresis (SDS-PAGE) stained with Coomassie brilliant blue G-250 showing the induction of the expression of the CTV RdRp by IPTG in *E. coli* strain BL21 (B) Accumulation of the expressed RdRp of CTV in soluble (S) and insoluble (IS) fractions of *E. coli* BL21grown at different temperatures at 3 hours after induction. (C) Western blot analysis showing detection of the RdRp of CTV expresses in *E. coli* by the polyclonal antibody raised against to the recombinant the RdRp of CTV expresses in *E. coli*.

Production and Analysis of Polyclonal Antibody

A polyclonal antibody specific to the CTV RdRp were raised by injecting a rabbit with a total of 7.5-10 mg the recombinant RdRp. After four weekly injections of 1.0-1.5 mg protein, antiserum was collected and tested for specificity by western blot analysis. When different amounts of the recombinant protein was analyzed using different dilution of the antiserum, it reacted strongly with the 60 kDa protein expressed in bacterial cells containing the pETh3c-CTV RdRp plasmid, but not with proteins in cells carrying the pETh3c plasmid alone (Figure 3 lanes 1-4). A weak reaction also occurred with two other smaller proteins in bacterial cells containing the pETh3c plasmid with or without the CTVRdRp gene (Figure 3 lanes 1-4). These results indicated that the antibody was specific to the recombinant CTVRdRp protein. Different dilutions of the expressed CTVRdRp tested by western blot analysis to determine the sensitivity of the antibody showed that a 1:10,000 dilution of the antibody could detect up to a 1:1000 dilution (about 10-100 ng) of the expressed protein. However, best results were obtained using 1:1000 dilution of the antibody (Figure 3; lanes1-4).

Although a number of monoclonal and polyclonal antibodies specific to virion and the structural protein (minor and major coat protein) of CTV are available for detection and identification of CTV (Bar-Joseph et. al., 1979; Vela et al., 1986; Permar

et al 1990 Febres et al., 1994), no antibody specific to non-structural proteins has been produced to date. This study provides the first antibody specific to one of the replication-associated proteins of CTV. This antibody is important reagent for studying *in vitro* and *in vivo* expression of the RdRp to answer important question about the structure and functions of the RdRp in replication. The antibody can be used to determine if the RdRp of CTV is expressed by a +1 translational frameshift and if the RdRp is expressed and stayed as a part of the poliprotein or cleaved and function as an individual protein.

ÖZET

CİTRUS TRİSTEZA VİRÜSÜ'NÜN RNA-BAĞIMLI RNA POLİMERAZININ *ESCHERİCHİA COLİ*'DA EKSPRESYONU VE POLİKLONAL ANTİKOR ÜRETİLMESİ

Citrus tristeza vürüsünün (CTV) RNA-bağımlı RNA polimeraz (RdRp) geni CTV'üyle bulaşık turunçgilden elde edilen dsRNA kullanılarak ters transkripsiyon/polimeraz zincir reaksiyonuyla (RT/PCR) çoğaltılmıştır. Daha sonra bu gen bakteriyel ekspresyon vektörüne klonlanarak *Escherichia coli* bakterisine aktarılmıştır. Bu *E. coli* bakterisinden elde edilen toplam proteinlerin poliakrilamid jel elektroforez yöntemiyle analiz edilmesi sonucunda uyarılmadan 30 dakika sonra 60 kDa büyüklüğündeki CTV RdRp proteininin bakteride üretilmeye başlandığı ve üretiminden 3 saat sonrasına kadar dereceli olarak arttığı gösterilmiştir. Daha sonraki analizler RdRp proteininin çözünür olmadığını ve bu proteinin *E. coli* bakteri hücresinin çözülmeyen kısmında bulunduğunu ortaya koymuştur. *E. coli*'da üretilen bu RdRp proteini bir tavşana enjekte edildikten bir süre sonra bu tavşandan antiserum toplanarak poliklonal antikor üretimi sağlanmıştır. RdRp genini taşıyan *E. coli* bakterisinden elde edilen toplam proteinin üretilen bu antibodinin kullanımıyla western blot yöntemiyle analiz edilerek üretilen antikorun CTV'nün RdRp proteinine spesifik olduğu belirlenmiştir.

Anahtar Kelimeler: CTV, RdRp. Protein ekspresyonu, antikor üretimi

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