Pak. J. Biotechnol. Vol. 4 (1-2) 1-6 (2007) ISSN. 1812-1837
IBGE, University of Sindh, Jamshoro, Pakistan

PRODUCTION OF POLYCLONAL ANTIBODIES SPECIFIC TO CITRUS TRISTEZA VIRUS (CTV) USING THE 6X HIS-TAGGED FUSION COAT PROTEIN

Salama, M.I.[1]; El-Domyati, F.M. [2]; Salem, R.E.[1]; Nour El-Din, Hanan[1] and Sadik, A.S.[1,3]

[1]Agricultural Genetic Engineering Research Institute, ARC, 12619, Giza, Egypt, [2] Dept. Genetics, Faculty of Agric., Ain Shams University, Shobra El-Kheima, Egypt, [3]Dept. Agric. Microbiol., Faculty of Agric., Ain Shams University, Shobra El-Kheima, Egypt E-mail: atef_sadik@yahoo.com

ABSTRACT:

Citrus tristeza virus (CTV) is one of the most destructive and economically important diseases of commercial citrus worldwide. In this study, the coat protein (*cp*) gene of CTV was subcloned into PQE-30 vector at *Bam* HI and *Hind-III* restriction sites. The constructed plasmid that called pRMAF2 was transformed into *Escherichia coli* M15 strain for its expression and production of fusion protein for CTV-*cp* gene. The suitable condition for inductions of CTV-*cp* was in the presence of 1 mM IPTG and overnight incubation time. The bacterial culture was harvested and the expressed fusion protein(s) was purified by QIA express kit. Polyclonal antibodies (PAbs) were raised by injection of the purified fusion protein(s) into two white mice followed by western blotting analysis using the expressed fusion protein(s) and the CTV-infected citrus extracts as controls. It can be concluded that the immuno-enzymatic detection based on the produced antibodies against CTV-*cp*-ORF fusion proteins were safe, speed and sensitive for the detection of the virus.

INTRODUCTION

Citrus is one of the most widely grown and economically important fruit crops in the world for generation of income, foreign trade and nutrition. The world citrus production (about 100 million metric tons) has been increasing steadily to accommodate growing demands of domestic and international markets (Çevik, 2001).

CTV (Citrus triteza virus) is a member of the *Closteroviridae*, it is the causal agent of the most economically important viral disease of citrus worldwide (Bar-Joseph *et al*., 1989). The virus causes one of the most destructive diseases of citrus (Bar-Joseph *et al*., 1989). CTV isolates differ in genome sequences (Vives *et al*., 1999) and biological properties, such as symptoms induced on different hosts and aphid transmissibility. Some isolates caused decline of citrus varieties grafted on sour orange rootstock (*C.aurantium* L.), while other causes are stem pitting, lack of vigor in stem as well roots and reduce yield (Bar-Joseph *et al*., 1989) while few isolates induce very mild symptoms or symptomless, even in the most sensitive citrus species.

CTV virion is flexuous particle, about 2000 X 11 nm in size and contains a single positive-stranded genomic RNA of 19200–19300 nucleotides with 12 ORFs that encode up to 19 polypeptides (Karasev *et al*., 1995; Mawassi *et al*., 1996). The ORFs 25 and 27 code the capsid proteins of 25 and 27 KDa, respectively (Sekiya *et al*., 1991). The 25 KDa *CP* encapsidates 95%

of the particle length whiles the 27 KDa protein encapsi-dates one end of the particle forming a 'rattlesnake' structure (Febres *et al*., 1996).

Therefore, this study was aimed to produce of expressed fusion protein against *cp* gene and use it as antigen in producing of specific polyclonal antibody (PAb) for virus detection in immuno-serological reactions.

MATERIAL AND METHODS

1. **Subcloning of CTV-ORF-*cp* gene and bacterial transformation:** The pRMAF1 (pGEM-T easy vector + *cp*-ORF) was separately digested with *Bam H1* and *Hind III* restriction endonuclease enzy-mes and the released fragment was then subcloned into digested PQE-30 vector (QIAexpress® Type IV kit), with the same previous enzymes. Recombinant plasmid (pRMAF2-PQE-30 + *cp*-ORF) was transformed into *E. coli* strain M15 ($pREP_4$) for expression and production of fusion protein as (Nour El-Din *et al.,* (2005).
2. **Purification of the 6X His-tagged *cp*-fusion protein:** After confirmation and expression processes, purification process was carried out in a large scale using nickel-nitrilotriacetic acid (Ni-NTA) batch chromatography under denaturing conditions as recommended by Rajamohan *et al*. (1999) and finally analyzed by SDS-PAGE (Sambrook *et al*., 1989).
3. **Determination of the specificity of purified expressed protein:** Serial dilutions from the purified expressed protein were prepared and used for DAS-ELISA (Clark and Adams, 1977) using PAbs that purchased from Valanzano, Italy.
4. **Production of antiserum specific to the 6X His-tagged CTV-*cp* fusion protein:** Antiserum was produced by injecting two white mice with 100 µg\injection purified 6X His-tagged fusion protein(s) in which the first injection emulsified with an equal volume of complete freund's adjuvant and the second injection emulsified with an incomplete adjuvent for four subsequent intramuscular injections at weekly intervals. The blood was collected then incubated at 37°C for 1 h and centrifuged at 4000 rpm at room temperature (RT). The antiserum was collected and stored at 4°C until use.
5. **Western blotting analysis:** Western blotting analysis (Towbin *et al.* 1979) was carried out to determine the specificity of the produced immunoglobulin G (IgGs) to the *cp*-fusion protein(s) in the presence of healthy and CTV-infected citrus plants as well as the purified fusion proteins (as non-infectious positive control). After electrophoresing at 12% SDS-PAGE, the polypeptides were blotted onto a membrane (Immobilon ® PVDF membrane, Millipore cooperation, Bed ford, MA 01730) using a trans-blot apparatus (Bio-Rad).

RESULT

Expression and purification of fusion protein: The expression process was induced by adding IPTG in a final concentration of 1 mM and the level of protein expression was analyzed at different incubation times (1,2 h and ON). Results showed that the protein(s) was determined by using 12% SDS-PAGE (Figure 1), and after ON incubation, the fusion protein was produced at high concentration. Results in Figure-2 showed matography from the harvested bacterial cultures

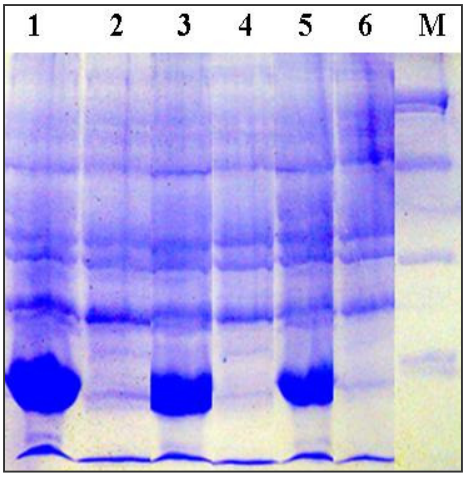

Figure-1: SDS-PAGE gel (12%) stained with Commassie Brilliant Blue shows time course study of expression of the CTV-Eg-*cp* gene in *E. coli* M15 after induction by IPTG. Lanes 1, 3 and 5, represents induction time after 1, 2 h and overnight inductions. Lanes 2, 4 and 6 represent M15 strain of *E. coli* carrying the insert free PQE-30 vector. M represents standard protein marker (Bio-lab) with Mr, 119,99,52,37,28,19, 6 KDa.

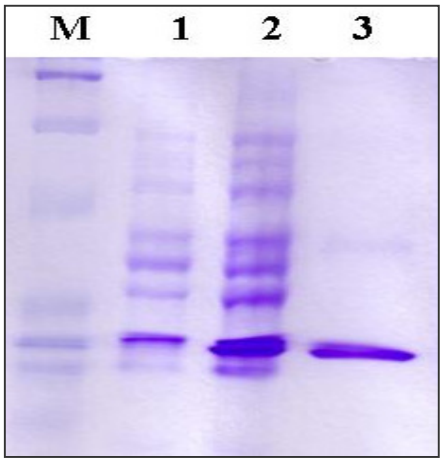

Figure-2: SDS-PAGE gel (12%) stained with Commassie Brilliant Blue shows purification of fusion CTV-Eg-*cp* protein. Lane 1 represents M15 carries the free insert PQE-30 vector. Lane 2 represents M15 carries the PQE-30+CTV-Eg-*cp* gene. Lane 3 represents the purified CTV-Eg- *cp* fusion protein and M represents standard protein marker (Biolab) Mr, 119, 99, 52, 37, 28, 19, 6 KDa .

Determination of the specificity of purified expressed protein by using DAS-ELISA: To reach such aim, a number of serial dilutions from the expressed fusion protein were prepared. These dilutions were subjected to DAS-ELISA detection of CTV as mentioned before. Results in Table-1 showed that positive ELISA values ranged between 1.077 and 2.429 compared to the negative (0.433). This result confirmed that the produced fusion protein is belonging to CTV-*cp* protein.

Table-1: DAS-ELISA for determination the specificity of the purified expressed protein

Dilutions	ELISA detection	
	ELISA value	Result
1:10	1.077	+
1:20	0.763	-
1:100	0.504	-
1:1000	0.435	-
Positive control	2.429	+
Negative control	0.433	-

Positive control: Original concentration of purified expressed protein. **+** =Positive. **-** = Negative.

Western blotting analysis: The specificity of the resultant antiserum was evaluated by western blot analysis using protein extracts of healthy citrus, protein CTV-infected and protein extracts from transformed (PQE-30+CTV-Eg-*cp* gene) untransformed bacterial cells Figure-3**.** The antiserum was reacted with the protein of about 28 kDa presented in the induced culture of *E. coli* harboring plasmid pRMAF2, purified protein. Moreover, the antiserum was also reacted with the protein of about 28 kDa presented in CTV-infected extract. No reaction was observed in extract from healthy citrus.

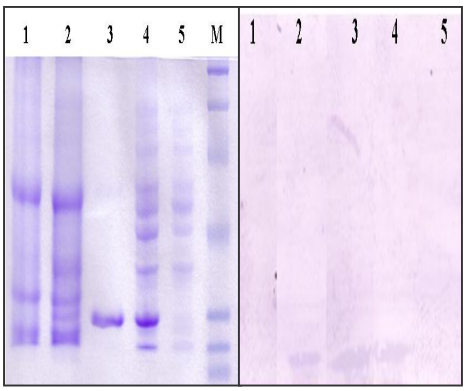

Figure-3: Western immunoblot analysis of proteins that probed with PAbs raised against CTV-Egy-*cp*-ORF fusion protein. M represents standard protein marker (Biolab) with Mr, 119, 99, 52, 37, 28, 19, 6 KDa. Lane 1 represents protein extracts of healthy citrus sample. Lane 2 protein extracts of CTV-infected sample. Lane 3 represents the purified CTV-Eg- *cp* fusion protein. Lane 4 represents M15 carries the PQE-30+CTV-Eg-*cp* gene. Lane 5 represents protein extracts of untransformed bacterial cells.

DISCUSSION

Citrus tristeza virus (CTV) is the most destructive pathogen of citrus and causes substantial economic losses in the citrus-growing industry of the world (Bar-Joseph *et al.*, 1989 and Lee and Rocha-pena, 1992). Strategies aimed at mini-mizing the destructive effect of the tristeza disease include regulatory methods that are heavily dependent on appropriate diagnostic procedures. For example, indexing is required for certi-fication of budwood and identification of infected trees for eradication. Indexing is performed by the ELISA, a convenient and reliable methods for the detection of CTV in the field sample (Rocha-pena and Lee (1991). However, the enormous scale of the indexing program requires large amounts of specific antisera and a consistent supply of antigen for immu-nization purposes. CTV is very narrow and like other Closteroviruses, in citrus spp. The virus is associated with the phloem (Bar-joseph et. al., 1989). Accordingly, virus purification is difficult and the yields of purified CTV virions are usually low (Bar-joseph *et al*., 1985 and Lee *et al*., 1987). The best methods to produce CTV are still contaminated with host components. As a result, polyclonal antisera are often unsatisfactory for the accurate diagnosis of CTV.

In this study, we utilized molecular biology methods as an alternative approach to obtain the CTV-CP antigen as a fusion protein in *E. coli* cells and used the purified fusion protein as an antigen to successfully raise PAbs, CTV-specific antiserum. The specificity of the produced antiserum was evaluated by western blot analysis and the antiserum was reacted strongly with the protein of about 28 KDa presented in CTV-infected extract, the purified fusion protein(s) and the induced culture of *E. coli* harboring plasmid pRMAF2. No reaction was observed in extract from healthy citrus.

Wanitchakorn *et al.* (1997) evaluated the specificity of the antiserum by western immunoblots using crude healthy and

BBTV-infected plant extracts, as well as the fusion protein. The antiserum had a high affinity (titer more than 10^4) for the 20 KDa BBTV-CP. The recombinant virus *cp* expressed in bacterial cells have great potential as an alternative source of antigens for raising specific antibodies to plant viruses and can be produced in large quantities and manipulated as needed for specific uses (Nikollaeva *et al*., 1995). De Sa *et al.* (2000) reported that the lyophilized expressed coat protein would be partially useful as a positive non-infectious control in serological tests for virus detection. Nour El-Din *et al*. (2005) produced recombinant 6X His-tagged BBTV-cp-ORF fusion protein, which used as an antigen in the production of specific polyclonal antibodies in high titer and free from plant contaminants.

As a conclusion, we are in full agreement with reported by Nour El-Din *et al.* (2005), who mentioned that such antibodies could overcome the difficulties of yield of purified virus, in addition to its role in identification of virus-infected plants as early as possible followed by removing the diseased plants and replanting with virus-free citrus plants.

REFERENCES

Bar-Joseph, M., D.J. Gumpf; J.A. Dodds, A. Rosner and I. Ginzberg, A simple purification method for citrus tristeza virus and estimation of its genome size. Phytopathology 75: 195-198 (1985).

Bar-Joseph, M., R. Marcus and R.F. Lee. The control continuous challenge of citrus tristeza virus control. Annu. Rev. Phytopathology 27:291-316 (1989).

Çevik, B., Characterization of the RNA-dependent RNA polymerase gene of citrus tristeza closterovirus. Ph.D Dissertation. University of Florida, Gainesville, FL. pp. 133 (2001).

Clark,M.F. and A.N.Adams, Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. Journal of General Virology 34:475-483 (1977).

De Sa, P.B., E. Hiebert and D.E. Purcifull Molecular characterization and coat protein serology of watermelon leaf mottle virus (Potyvirus). Archive of Virology 145: 641-650 (2000).

Febres, V.J., L. Ashoulin, M.Mawassi, A. Frank; M. Bar-joseph, K.L. Manjunath, R.F. Lee and C.L. Niblett, The p27 protein is present at one end of citrus tristeza virus particles. Phytopathology 86: 1331-1335 (1996).

Karasev, A.V., V.P. Boyko, S. Gowda, O.V. Nikolaeva, M.E. Hilf, E.V. Koonin, C.L. Niblett, K. Cline, D. J. Bumpf and R.F. Lee, Complete sequence of the citrus tristeza virus RNA genome. Virology 208: 511-520 (1995)

Lee, R.F, S.M. Garnsey, R.H. Bransky and A.C. Goheen, A purification procedure for enhancement of citrus tristeza virus yields and its application to other phloem-limited viruses. Phytopathology 77:1221-1226 (1987).

Lee, R. F. and M. A. Rocha-Pena. Citrus tristeza virus in: Kumar, J., Chaube, H. S., Singh,U.S. and Mukhopadhyay. A.N (eds). Plant Disease of Inter-national Importance. Disease of fruit crops. Prentice. Pp 226-249 (1992).

Mawassi,M., E.Mietkiewska, R.Gofman, G. Yang and M. Bar-Joseph, Unusual sequence relationships between two isolates of citrus tristeza virus. J. Gen. Virol. 77: 2359-2364 (1996).

Nikollaeva, O.V., A.V. Karasev, D.J. Gumpf, R.F. Lee and S.M. Garnsey, Production of polyclonal antisera to the

coat protein of citrus tristeza virus expressed in E.coli: application for immunodiagnosis. Phytopathology 85: 691-694 (1995).

Nour El-Din, H., M.I. Salama, A.B. Barakat, A.M. Saleim and A.S. Sadik, Nucleotide sequence of BBTV-cp gene and using its fusion protein for producing specific polyclonal antib-odies. Arab Journal of Biotechnology 8(2): 355-368 (2005).

Rajamohan, F., C.R. Engestrom., T.J. Denton, L.A. Engen, I. Kourinove and F.M. Uckun, High-level expression and purification of biological active recombinant pokweed antiviral protein. Protein Expression and Purification 16: 359-368 (1999).

Rocha-Pena,M.A. and R.F.Lee, Serological technique for detection of citrus tristeza virus. J. Virol. Methods 34: 311-331 (1991).

Sambrook, J., E.F. Fritsch and T. Maniatis. Molecular Cloning: A Laboratory Manual Cold Spring Harbor Laboratory, Cold Spring Harbor New York (1989)

Sekiya, M.E., D. Lawrence, M. Mc Caffery and K. Cline, Molecular cloning and nucleotide sequencing of the coat protein gene of citrus tristeza virus. J. Gen. Virol 72: 1013-1020 (1991).

Towbin, H., T. Staehelin and J. Gordon, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Nat. Acad. Sci. 76: 4350-4354 (1979).

Vives, M.C., L. Rubio, C. Lopez, J. Navas-Castillo, M.R. Albiach-Marti, W.O. Dawson, J. Guerri, R. Flores and P. Moreno, The complete genome sequence of the major component of a mild citrus tristeza virus isolate. J. Gen. Virol. 80: 811-816 (1999).

Wanitchakorn, R., R. M. Harding and J. L. Dale, Banana bunchy top virus DNA-3 encodes the viral coat protein. Arch. Virol. 142: 1673-1680 (1997).