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Expression of *Citrus tristeza virus* coat protein gene in *Escherichia coli*

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Abstract: *Citrus tristeza virus* (CTV) is among the most destructive pathogens of citrus and causes substantial economic losses in citrus-growing industry worldwide. Considering recent distribution of this pathogen and its capability of transmission by existing aphid vectors in Iran, detection of this virus is enforceable for controlling the damage caused by this pathogen in Iran, as one of the major citrus producing countries. Toward this aim, developing a reliable and sensitive detection method such as enzyme- linked immunosorbant assay (ELISA) would be the first step to detect CTV in large scale screenings of field samples. As the serological method requires great amounts of specific antibody, the consequent preparation of a large scale antigen source for immunization process is necessary. In this study the coat protein gene of CTV (CP25) was amplified by polymerase chain reaction from a cloned CP25 gene in pTZ57R/T and subcloned in pET26b expression vector and named pET-CP25. Two *Escherichia coli* strains of BL21 and Rosetta Gami (DE3) were transformed by pET-CP25. Expression of recombinant protein was induced by IPTG. The authenticity of recombinant protein was confirmed by western immunoblot analysis using a polyclonal antiserum against CTV particles. The results indicated that CTV coat protein gene was expressed in *E.coli.* This recombinant protein could be used as a source of antigen for immunization process.

Keywords: recombinant protein; western blot analysis; ELISA

Introduction

Tristeza is the most destructive and important disease of citrus worldwide that is caused by *Citrus tristeza virus* (CTV) (Bar-Joseph *et al.*, 1989). This virus is transmitted by aphids in a semi-persistent manner and among its vectors, the brown aphid of citrus (*Toxoptera citricida* Kirkaldy) the most efficient in transmission of the virus, has not been found in Iran. Occurrence of Tristeza in Iran was first reported from Mahdasht orchards in Sari, Mazandaran province, in Satsuma mandarin (*Citrus reticulata*) CTV infected seedlings imported from Japan (Ebrahim-nesbeat and Nienhaus, 1978). Considering the nonentity of the brown aphid in Iran, this disease was considered to be restricted to Mahdasht gardens for about three decades. Studies in recent years have demonstrated the existence of natural transmission of some CTV isolates by cotton aphid (*Aphis gossypii* Golver) in north of Iran (Rahimian *et al.*, 2000). The presence of CTV in south of Iran and its widespread distribution was reported in 1996 (Shafiee and Izadpanah, 1996; Izadpanah *et al.*, 2002). A more recent study has shown

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60% infection in citrus samples from Kerman province, southeast of Iran (Ahmadi *et al*., 2007). Adopting an appropriate strategy to prevent or decrease the damage caused by CTV will in the first place depend on identifying the infected plants that is usually achieved by enzyme linked immunosorbent assay (ELISA) as a reliable technique (Rocha-Pena and Lee, 1991). Large scale detection of CTV by ELISA requires great amounts of specific antibody and continuous antigen for immunization process. As CTV is restricted to phloem tissue, purification of the virus particles is too difficult and even in the best methods contamination with plant cell materials has been unavoidable (Lee *et al.*, 1987). Contaminations with plant-derived immunogens result in the occurrence of cross reactions with healthy crude extracts. One way to overcome this difficulty would be to induce the expression of viral coat protein gene as a recombinant protein in an appropriate host with no or fewer difficulties in purification and lower procedural cost (Nikolaeva *et al*., 1995). Up to now, several polyclonal and monoclonal antibodies have been produced against some plant viruses, recombinant structural (Shams-Bakhsh and Symons, 2004; Cerovska *et al.,* 2010) or non structural (Osman and Buck 1991) viral proteins are applied for production of diagnostic antibodies. In this investigation the coat protein gene of CTV was expressed in bacterial host to prepare a source for continuous antigen supply.

Materials and Methods

The CP25 gene source and plasmids

The 25kDa coat protein of CTV is the major portion of the virus proteins and covers 95% of the length of the virus particle. The related gene that had been cloned in pTZ57R/T was obtained from Dr. Ali Reza Afsharifar, Shiraz University and named pTZ-CP25.

pET26b was used as an expression vector. It is a member of pET expression vectors whose transcription of cloned gene is performed by T7 RNA polymerase under T7 promoter that is very specific (Studier and Moffat, 1986; Giordano *et al.*, 1989).

Subcloning of CP25 gene in pET26b

Considering the sequences of pTZ-CP25 and multiple cloning site of the expression vector pET26b, two oligonucleotide primers were designed as CTV-CP-F(5′-ATGAATTCGATGGACGACGAAACAAAG-3′) and CTV-CP-R (5′-TTTGTCGACACGTGTGTTGAATTTCCC-3') with recognition sites for restriction enzymes *Eco*RI and *Sal*I (underlined) in F and R primers, respectively. Plasmid pTZ-CP25 was extracted from bacteria using alkaline lysis (Sambrook and Russel, 2001). Polymerase chain reaction (PCR) was performed in 25 µl solutions containing *Taq* DNA polymerase 5 U/µl (SinnaGen, Iran), dNTPs 200µM, primers each 0.1µM, plasmid pTZ-CP 25 100 ng, PCR buffer (75mM Tris-HCl pH 8.8, 20 mM $(NH_4)_2SO_4$, Tween 20 0.01% v/v and 2 mM $MgCl_2$ with primary denaturation at 94 °C, 25 cycles of (denaturation at 94 °C for 25 s; annealing at 58 °C for 15s; extension at 72 °C for 45s) and a final extension of 5 minutes at 72 °C.

PCR product was separated in a gel electrophoresis of 1% agarose, stained with ethidium bromide 0.5 µg/ml and extracted by a gel extraction kit (Qiagen, Germany) and 300 ng of purified PCR product was digested with one unit each of *Eco*RI and *Sal*I each in total volume of 30 µl buffer (Fermentas, Lithuania) at 37 °C for 2 hours. Enzymes were inactivated at 65 °C in 20 minutes, about 1µg of expression vector pET26b was digested in the same conditions as described for PCR product and electrophoresed in 1% agarose gel then was purified by the gel extraction kit (Qiagen, Germany). Ligation was preformed with molar ratio of 3 to 1, insert to vector with T4 DNA ligase (Fermentas, Lithuania) in a total volume 20 µl, at 22 °C for 2 hours. Competent cells of *E. coli* DH5α strain were prepared with transformation and storage

solution (TSS) buffer and subsequent transformation was performed by heat shock method (Sambrook and Russel, 2001). After overnight incubation at 37 °C, the transformed colonies appeared on in Luria-Bertaini (LB) medium (1% tryptone, 0.5% yeast extract, 1% NaCl; pH 7, and 1.7% agar to solidify) containing 25 µg/ml kanamycine and screened by colony PCR for the presence of CP25 gene. Plasmids were extracted from colonies containing the CP25 gene and were digested with *Bam*HI and *Sal*I in separate and consecutive reactions to make certain about the gene direction.

Expression of recombinant CP25 gene

Expression plasmids carrying the CP25 gene were transferred to TSS prepared competent cells of BL21 strain and colonies appeared on LB-Agar containing 30µg/ml kanamycine after overnight incubation at 37 °C. A single colony was used for inoculation of 5ml liquid LB containing antibiotic, and after overnight growth of bacteria, 50 µl of the overnight culture was used to inoculate 5ml of fresh LB medium. When growth density reached 0.6 absorbance at 600 nm, IPTG was added to a final concentration of 1mM and sampling was performed at one hour intervals after induction. One milliliter sample was taken from growing bacteria before or after induction, centrifuged at 13000 rpm immediately and the pellet was suspended in loading buffer (50mM Tris-HCl pH 6.8, 2% SDS, 100 mM, 10% glyserol, 0.1% bromophenol-blue, 5% β-mercaptoethanol freshly used) and incubated at 96 °C for 5 min and stored at 4 °C until loading to a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE and western blotting:

Total protein was separated by SDS-PAGE with 13% polyacrylamide and Tris-glycine-SDS buffer (Laemmli, 1970). Protein staining was preformed with Coomassie Brilliant Blue R-250 in 10% acetic acid and 40% methanol and destaining was performed in a same solution without Coomassie Brilliant Blue.

The gel containing expressed bands of protein was placed in blotting sandwich (2 pieces of sponge, 2 pieces of whatman paper and Nitrocellulose paper) and soaked in transfer buffer (glycine 14.4 g/L, Tris 3 g/L, methanol 20% v/v) for 5 minutes. The sandwich was placed between special plastic pads, positioned between two electrodes of Electro blotter (Bio-Rad, USA) and blotting was carried out. Blocking of the nitrocellulose paper was performed with blocking buffer 5% skim milk in washing buffer (Tris 1.2 g/L, NaCl 9.05 g/L pH 7.5)) for two hours. Blocked paper was washed three times each for 10 minutes then was reacted with primary antibody 1/2000 (2.5µl polyclonal antibody specific for CTV coat protein obtained from DSMZ company, Germany) for 2 hours in 200 rpm at room temperature. After another washing step, secondary antibody conjugated with alkaline phosphatase (promega) 1/5000 dilution reacted in 2 hours at room temperature and washed again and blotted bands appeared by adding 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitro blue tetrazolium (NBT) (SigmaFast tablets, Sigma) substrate.

The obtained gene sequence CP25 was analyzed for its accordance to codon usage of *E. coli* K12 strain using the bioinformatic tool of http://gcua.schoedl.de website.

Results and discussion

In this study, the coat protein gene of CTV was amplified and isolated from a template of recombinant plasmid (pTZ-CP25) using specific primers (CTV-CP-F and CTV-CP-R), it produced an approximately 700 bp fragment and was subcloned in an expression vector of pET26b which was named pET-CP25. Restriction enzyme analysis of pET-CP25 using *Bam*HI and *Sal*I confirmed the orientation of the inserted gene (data not shown).

CTV-CP25 gene was expressed in two strains of BL21 and Rossetta Gami (DE3). The size of protein expressed in BL21 strain was smaller than that expected and was about 14KDa (Fig. 1). The sequence analysis of CTV-CP25 gene obtained from Shiraz University with ExPASy tool (Expert Protein Analysis System, www.expasy.org) showed many low-usage codons in the sequence. Two of the low usage codons CUU (136) for Leucine and CGA for Arginine (137) were consecutive and the polypeptide size from first amino acid to the 136th was about 14KDa. Therefore, it may be concluded that protein is truncated in this region as has also been reported in other studies (kane, 1995). Noticeable characteristic of Rossetta Gami strain is the production of tRNAs with rare codons. Western blot analysis using polyclonal antibody against purified CTV (DSMZ, Germany) developed two bands in lanes related to BL21 and one band for Rossetta Gami strain, which supports the hypothesis that two consecutive rare codons caused the truncation. No reaction was observed in non induced samples and transformed *E. coli* cells with pET26b plasmid which demonstrated its specificity for immunization (Fig 2). The expressed protein size in Rossetta Gami strain was less than expected which may be related to the nature of protein segregation in SDS-PAGE and nonentity of disulphide bands reduction (Sallantin *et al*., 1990) or other unknown reasons. However because the aim of this study was to prepare a suitable antigen source for polyclonal antibody production, higher expression of truncated recombinant CTV-CP25 in BL21 with higher growth rate is preferable to less concentration but longer protein produced in Rossetta Gami with lower growth rate, becasue another property of Rossetta Gami strain is chromosomal-based resistance to antibiotics of kanamycine, tetracycline and chloramphenicol. As the selectable marker of pET26b vector is kanamycine resistance, to prevent plasmid loss and exert appropriate selective force, higher concentration of kanamycine (300 µg/ml) was applied in culture medium of Rossetta Gami bacteria bearing the expression vector, at which concentration no growth of untransformed Rossetta Gami was observed. This application of higher antibiotic concentration resulted in lower growth rate. In 1995 the CP25 gene of SY568 isolate from California was expressed in *E. coli* using pMAL-c2 expression vector (Nikolaeva *et al*., 1995). In this vector the recombinant protein is conjugated with maltose binding protein (MBP) that is employed for purification. In this research pET26b expression vector was employed which contains *pel* B signal peptide sequence that assists in the solubility of expressed protein. Furthermore, the foreign gene is inserted into the pET26b bacterial expression vector downstream or upstream of a 6 × His-tag and expressed in *E. coli*, this helps to purify the expressed recombinant protein by Ni-NTA-agarose column.

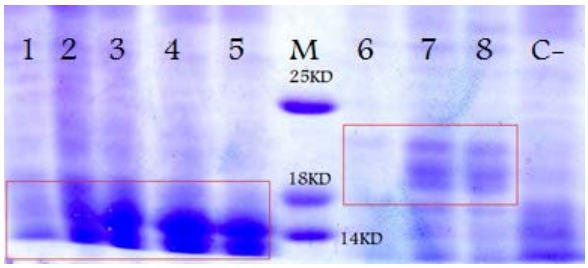

Figure 1 SDS-PAGE of recombinant CP25 expression 1-5: Expression of CP25 gene of recombinant plasmid pET-CP25 in BL21 strain: 1- Before induction 2: An hour after induction 3: two hours after induction 4: three hours after induction 5: four hours after induction. M: molecular weight marker SM0431 (Fermentas, Lithuania). 6 to 8: Expression of CP25 gene of pET-CP25 in Rossetta Gami Strain. 6: before induction, 7: three hours after induction, 8: five hours after induction. C: negative control: three hours after induction of BL21 strain carrying pET26b.

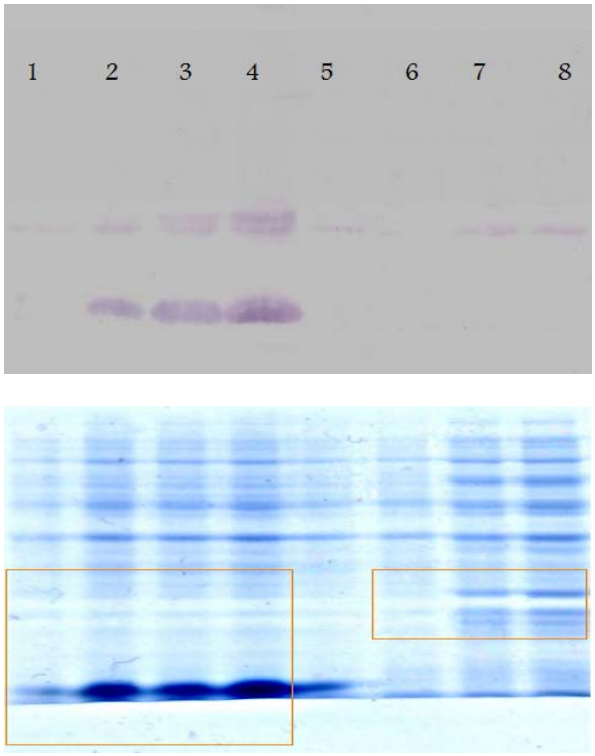

Figure 2 SDS-PAGE and western blotting. 1-4: expression of recombinant CP25 in Bl21 strain 1: before induction, 2: an hour after induction, 3: two hours after induction, 4: three hours after induction, 5: negative control three hours after induction in BL21 strain containing pET26b. 6 to 8: Expression of CP25 gene of pET-CP25 in Rossetta Gami Strain. 6: before induction, 7: three hours after induction, 8: five hours after induction.

Although it seems that the expressed protein was truncated the outcome suffices the final purpose of this investigation which is to prepare an available and cost effective antigen source for immunization process. In conclusion, the large scale production and avoidance of drawbacks of viral purification and contamination with plant proteins makes the recombinant protein technology an efficient strategy for antiserum preparation.

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بیان ژن پروتئین پوششی ویروس تریستزای مرکبات در *Escherichia coli*

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چکیده: ویروس تریستزای مرکبات (CTV) *Citrus tristeza virus* یکی از مهم‌ترین بیمارگرهای مخرب مرکبات می‌باشد و سالانه خسارت هنگفتی به صنعت تولید این محصول در دنیا وارد می‌کند. به‌دلیل گسترش و شیوع این بیمارگر و همچنین قابلیت انتقال آن توسط شته‌های ناقل موجود در ایران به‌عنوان یکی از کشورهای اصلی تولید مرکبات، ردیابی ویروس برای مدیریت کنترل خسارت آن اهمیت به‌سزایی یافته است. به‌منظور دست‌یابی به این هدف، معرفی یک روش ردیابی دقیق و حساس مانند Enzyme Linked Immunosorbant Assay (ELISA) اولین مرحله ردیابی ویروس تریستزای مرکبات برای تشخیص نمونه‌ها در سطوح وسیع است. از آنجایی‌که روش‌های سرولوژی نیازمند مقادیر فراوانی از آنتی بادی اختصاصی است، تأمین منبع آنتی‌ژن ویروسی برای استفاده در فرآیند تولید آنتی‌بادی پلی‌کلونال ضروری می‌باشد. در این پژوهش ژن رمزکننده پروتئین پوششی CTV، (CTV-CP25) جدا شده از ایران که در ناقل pTZ57R/T همسانه‌سازی شده بود با استفاده از واکنش زنجیره‌ای پلیمراز تکثیر و در ناقل بیانی pET26b همسانه‌سازی و pET-CP25 نامیده شد. دو سویه BL21 و Rosetta Gami (DE3) باکتری *Escherichia coli*، با pET-CP25 تراریخته شد. بیان پروتئین پوششی نوترکیب با IPTG القاء گردید. پس از تأیید بیان پروتئین نوترکیب، به‌منظور بررسی ماهیت آن، لکه‌گذاری وسترن با استفاده از آنتی‌بادی پلی‌کلونال اختصاصی علیه پیکره ویروس تریستزای مرکبات انجام گردید. نتایج این پژوهش نشان داد که ژن رمزکننده پروتئین پوششی CTV در سلول باکتری بیان شد. این پروتئین نوترکیب می‌تواند برای تولید انبوه آنتی ژن و نهایتاً آنتی‌بادی علیه آن استفاده شود.

واژگان کلیدی: پروتئین نوترکیب، لکه گذاری وسترن، الیزا