Cloning and expression of coat protein gene of the most prevailing citrus tristeza virus isolate in Northern Iran

Accepted 6th September, 2015

ABSTRACT

Citrus tristeza is one of the most destructive diseases of citrus and its causing agent CTV was introduced from Japan to northern Iran in the late 1960s. After about three decades of restriction to originally infected Satsuma trees, natural spread of CTV has being undertaken and melon aphid (Aphis gossypii Glov.) was confirmed as a potent vector in these areas. Regarding the specificity of aphid-virus interaction, in this research we aimed to characterize the most dominating strain of CTV in northern Iran and accordingly express their CP25 gene as a source of antigen for antibody production. Ninety CTV isolates were recovered from originally infected satsuma trees and some other neighboring or distant citrus growing orchards and their coat protein gene was amplified using RT-PCR method. SSCP profile analysis of the amplified CP25 genes showed that all isolates are classified into 3 groups each with unique profile. However, CP25 gene nucleotide sequence of 3 representative isolates of each SSCP group showed high sequence identity (up to 98%) and close relationship to NUAgA severe seedling yellows strain of Japan. Biological properties of these representative isolates studied through graft inoculation onto known indicator plants confirmed that they produce seedling yellows-type symptoms. CP25 gene of a representative isolate was ligated to pET28a(+) expression vector and transformed into Bl21 competent cells to express recombinant coat protein. Protein expression was induced using 1 mM IPTG for 6 h and recombinant protein purified through Ni-NTA affinity chromatography. SDS-PAGE analysis confirmed the presence of a recombinant 25 KD protein.

Key words: Citrus tristeza virus, seedling yellows, recombinant coat protein, pET28.

INTRODUCTION

Citrus tristeza virus (CTV), a member of the genus Closterovirus, is the causal agent of one of the most destructive diseases of citrus worldwide (Bar-Joseph et al., 1989). CTV contains a single-stranded, positive-sense genomic RNA of 19.3 kb that potentially encode at least 17 protein products (Bar-Joseph et al., 1989; Karasev, 2000) and two capsid proteins of 25 and 27 kDa, coating for 97 and 3% of the virion, respectively (Febres et al., 1996; Karasev et al., 1995; Mawassi et al, 1996; Satyanarayana et al, 2004; Vives et al., 1999). CTV is naturally transmitted in a semi persistent manner by several aphid species mainly Toxoptera citricida and Aphis gossypii with different efficiencies (Brunt et al., 1990; Roistacher and Bar-Joseph, 1987; Rocha-Pen˜a et al., 1995). Aphid transmission significantly alter the composition of the CTV population leading to establishment and amplification of certain strains through genetic bottleneck (Albiach-Martí et al., 2000; d’Urso et al., 2000; Aylon et al., 2006; Brlansky et al.,
2003; Nolasco et al., 2008; Roy and Brlansky, 2009). Iran is among the major citrus-producing countries in the world. Mazandaran province in northern Iran shares 45% of total under cultivation areas and 47% of production rate of citrus (Alipour et al., 2013). CTV was massively introduced into Iran via importation of over 55,000 infected Satsuma mandarins (Citrusunshiu Marc.) on trifoliate orange [Poncirus trifoliata (L.) Raf.] rootstock from Japan to the north of Iran (Mahdasht orchards, Mazandaran province) in the late-1960s (Yamada and Tanaka, 1969; Ebrahim-Nesbeat and Nienhaus, 1978; Rahimian, 1994). After about three decades, restriction of the virus to the originally imported satsuma mandarins, A. gossypii has been gradually prevalent and started transmitting of CTV to other neighboring orchards (Ebrahimi et al., 1988; Rahimian et al., 2000; Rahimian and Zarei, 2002). Adopting an appropriate strategy to prevent or decrease of the damage caused by CTV are mainly dependent on identifying the infected plants which is usually achieved through antigen-antibody interaction in enzyme linked immunosorbent assay (ELISA) test (Rocha-Pena and Lee, 1991). Traditional methods for producing antibody using purified virus preparations as immunogens is very difficult because CTV is restricted to phloem tissue and even in the purified virus preparations as immunogens is very difficult because CTV is restricted to phloem tissue and even in the best methods contamination with plant cell materials which cause cross reactions has been inevitable (Gonsalves et al., 1978; Bar-Joseph and Malkinson, 1980; Permar et al., 1990; Lee et al., 1987). Recombinant protein technology has provided an opportunity to express coat protein in an appropriate host with no or fewer difficulties in purification (Nikolaeva et al., 1995). Several reports indicated that the commercial antibodies react inefficiently to CTV strains of northern Iran (Izadpanah et al., 2002). In this study, we characterize the dominating CTV strain in northern Iran and accordingly cloned and expressed its coat protein gene to prepare a source of antigen for antibody production.

**MATERIALS AND METHODS**

**Virus isolates**

Ninety CTV isolates were collected from Mahdasht orchards (Sari, Mazandaran) as the main region of infection and some other neighboring or distant orchards. Samples were recovered from originally infected symptomless satsuma mandarin trees or from symptomatic trees assumed to be naturally infected through aphid transmission or grafting processes.

**RNA extraction, cDNA synthesis and PCR amplification**

Total RNA was isolated from infected leaf midribs and barks using Total RNA Extraction Kit (Jena Bioscience, GmbH, Jena, Germany). Complementary DNA (cDNA) was synthesized on 5 µl isolated RNA template and 10PM of the reverse primer (CP25-R: GAACGCAACAGATCAACGTG) in a final volume of 20 µl using AccuPower RocketScript RT PreMix (Bioneer, Daejeon, Korea) according to the manufacturer’s instructions. The reaction condition included primer annealing at 54°C for 1 min, cDNA synthesis at 42°C for 1 h and heat inactivation at 95°C for 5 min. cDNA (0.5 µl) was added to PCR cocktail containing 1X PCR buffer (500 mM KCl; 200 mM Tris–HCl pH 8.4), 1.5 mM MgCl2, 200 µM dNTP, 0.3 µM of each forward (CP25-F: GTTTTGAATTATGGACGACGA) and reverse primers and 2.5 units of taq DNA polymerase. Amplification included an initial denaturation at 94°C for 4 min, 30 cycles of 1 min denaturation at 94°C, 1 min annealing at 54°C and 1 min elongation at 72°C and a final elongation step of 20 min at 72°C.

**SSCP analysis**

Collected isolates were totally classified through SSCP analysis of amplified CP25 gene. One micro liter (1 µl) of the amplified CP25 gene was added to 9 µl of denaturing solution (95% formamide, 20 mM EDTA (pH 8.0), 0.05% bromophenol blue and 0.05% xylene-cyanol), boiled for 10 min and immediately chilled on ice. Electrophoresis was performed in a 12% non-denaturing acrylamide gel at 350 volts for 3 h and visualized after silver staining (Beidler et al., 1982; Rubio et al., 1996).

**Biological characterization**

Biological features of three representative isolates each showing unique SSCP profile were determined by grafting budwood onto the indicator plants. The citrus species and cultivars used as indicator plants were grown from seeds, except for sweet oranges (cv. Thomson navel), which were 6- to 12-monthold buddings of nucellar origin. Plants were grown in a heat sterilized composite mix of bark shavings and sandy loam soil (1:1 v/v) in 4 L pots. Plants were kept in a greenhouse at 20–30°C and fertilized regularly. Three or four plants were graft-inoculated with each inoculum using three pieces of bark strip from each donor tree. One seedling of each test plant was left uninoculated as control. Two to 3 weeks after graft-inoculation, the plants were cut back to stimulate a flush of new growth and examined weekly for typical symptoms of CTV for about 24 months. The plants were periodically sprayed with aphicides and acaricides.

**Cloning, sequencing and sequence analysis**

The amplified CP25 gene of representative isolates was ligated into the pTZ57R/T vector using InsT/AcloneTM PCR
product cloning kit (Fermentas, Vilnius, Lithuania) according to manufacturer's instructions. The pTZ57R-CP25 cassette was transformed into *Escherichia coli* DH5α competent cells. Recombinant clones were screened and plasmids DNA were extracted using high pure plasmid extraction kit (Roche, Mannheim, Germany), ethanol participated and prepared for sequencing. For sequence determination, at least three clones of each isolate were sequenced at MWG Company (Ebersberg, Germany) using standard M13 primer pairs in both directions. The acquired sequences were aligned and compared with those available in databases using BLAST program (Altschul et al., 1990) (Table 1). Sequence alignments were done using ClustalW program and phylogenetic tree constructed by maximum likelihood method (Thompson et al., 1994). Percent identity was calculated using MegAlign software ver. 5.00 in DNASTAR package (DNASTAR Inc., Madison, WI, USA; Burland, 2000).

**Expression and purification**

The primer pairs CTVIF-3 (TGACATATGGAGACGAGACAAAGAATTTG) and CTVir-3 (CGCAAGCTTTCAACGTGGTTGAAATTTCC) harboring Ndel and HindIII restriction sites, respectively (underlined), were designed to amplify and subclone the CP25 gene into the Ndel and HindIII restriction sites of pET28a(+) expression vector which provides six His residues at the N-terminus of the expressed protein. pTZ57R-CP25 cassette (100 ng) extracted from E.coli DH5α was applied in a PCR reaction with the same conditions as above-except annealing temperature at 58°C to amplify CP25 gene. The amplified fragments and pET28a (+) were separately digested by Ndel and HindIII restriction enzymes (fermentase) and purified by the GF-1 Gel DNA Recovery (extraction) Kit (Vivantis, Malaysia). Ligation reaction was performed by T4 DNA ligase (Vivantis, Malaysia) at 16°C over night and transformed into the BL21 (DE3) pLysS competent cells by heat shock method (Sambrook and Russel, 2001). The transformed bacteria were selected through growth on LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl; pH 7, 1.7% agar) containing kanamycin (50 μg/ml) and checked by colony PCR to screen those receiving recombinant pET28a-CP25 cassettes. A single colony was used for inoculation of 5 ml liquid LB medium. Fifty micro liters (50 μl) of the overnight culture was added to 5 ml of fresh LB and incubate continued until the absorbance at 600 nm reached 0.6. Subsequently, IPTG (Isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 1 mM and sampling was performed at one, three and six hours after induction. Bacterial cells were then collected by centrifugation and resuspended in 100 μl phosphates lysis buffer (20 mM sodium phosphate, 500 mM NaCl, 0.2% Triton X-100, 1 mg/ml lysozyme, pH 7.8). The cells were lysed on ice by 5 cycles sonication (Ultrasonic processor, Hielerscher), 1 min each with 2 min intervals and centrifuged for 20 min at 6000 g. Recombinant CP25 was purified by Ni-NTA affinity chromatography (Qiagen, US). The column containing 5 ml Ni-NTA resin was equilibrated with phosphate buffer (20 mM sodium phosphate, 500 mM NaCl, pH 7.8). The column was loaded by supernatant and washed with 10 volumes of washing buffer (20 mM sodium phosphate, 500 mM NaCl, pH 6) and 10 volumes of washing buffer pH 5.5. Bound proteins were eluted by elution buffer (20 mM sodium phosphate, 500 mM NaCl, pH 4). Purity of the fractions was studied by SDS-PAGE and coomassie blue staining and recombinant protein concentration was determined spectrophotometrically.

**RESULTS**

**PCR amplification, SSCP analysis and sequence analysis**

RT-PCR amplification of the CP25 gene of different CTV isolates yielded a single DNA band of 672 bp in 1% agarose gel (Figure 1). Comparison of the CP25 gene SSCP profiles of different isolates showed that they are categorized into three groups each with unique profiles (Figure 2); Group I: included those isolates recovered from originally imported satsuma trees; group II: those collected from neighboring orchards verified to be infected through natural transmission and group III: the subisolates produced via multiple aphid transmission. Sequence database searches of acquired CP25 sequences of representative isolates of each

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Country</th>
<th>Accession number</th>
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<tbody>
<tr>
<td>T30</td>
<td>FL. U.S.A.</td>
<td>AF260651</td>
</tr>
<tr>
<td>T36</td>
<td>FL. U.S.A.</td>
<td>U16304</td>
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<td>T385</td>
<td>Spain</td>
<td>Y18420</td>
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<td>VT</td>
<td>Israel</td>
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<tr>
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<td>Egypt</td>
<td>AY340974</td>
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<td>Jordan</td>
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<td>AY550252</td>
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Figure 1. Electrophoresis of the amplified CP25 gene in 1% Agarose gel. 1. CP25 gene; 2. 100-10000 bp DNA size marker (Fermentas, Lithuania).

Figure 2. Discrete SSCP patterns of the amplified CP25 gene of representative CTV isolates in 12% non denaturing acrylamide gel. Group 1: Lane 1; group II: Lanes 2, 3, 4, 5; group III: Lane 6.

group by the online BLAST tool confirmed these products as the CP gene of 672 bp in length with ATG and TGA as the start and stop codons, respectively. Multiple sequence alignment showed 98.7–100% sequence identity among the CP25 gene nucleotide sequences of these isolates. Comparison with CP25 gene sequence of other CTV isolates showed that the Iranian isolates have the highest sequence identity to NUagA (97.6–98.5%) seedling yellows strains of Japan. Furthermore, in the phylogenetic tree constructed by Maximum likelihood method the Iranian isolates were
closely clustered with NUagA strain with the most distances to T385 mild strain (Figure 3).

**Biological features**

All Mexican lime, Alemow, and *Citrus hysterix* seedlings inoculated directly with buds from declining field isolates of group I and II isolates had new leaves with vein clearing and stunting of new growth. Some Mexican lime seedlings developed vein corking and dieback as well. Alemow seedlings also had occasional vein corking. Sour orange and grapefruit seedlings inoculated with all three representative isolates (Group I, II and III) showed severe yellowing and stunting of new growth. In some sour orange seedlings, the yellows reaction was followed by vein clearing and severe leaf stunting and cupping, and the growth of the subsequent flushes was severely arrested within 12 months of inoculation. Sweet orange budding on sour orange rootstocks grew poorly and was chlorotic, and developed decline symptoms by 9–12 months after inoculation.

**Expression and purification**

The recombinant pET28a-CP25 cassette was transformed into *E. coli* BL21 strain and expression of the desired protein was induced upon IPTG application. Expressed CP25 protein was purified by Ni-NTA affinity chromatography and resolved via SDS-PAGE method. Results showed that a protein with the expected molecular mass of about 25 kDa was appeared in acrylamide gel. Results of protein expression in different condition indicated that the best conditions for recombinant protein expression achieved when 1 mM of IPTG was applied on a bacterial concentration of 6.0 at 600nm for six hours (Figure 4).

**DISCUSSION**

Citrus tristeza disease was established in Iran when infected Satsuma mandarins (*Citrus unshiu* Marc.) on trifoliate orange (*Poncirus trifoliata* (L.) Raf.) rootstock were imported from Japan to the Mahdasht orchards (Sari, Mazandaran Province, northern Iran) in the late 1960s (Bove, 1995; Rahimian, 1994). After about three decades, restriction to originally satsuma trees, *A. gossypii* has gradually evolved to transmit virus and CTV is now present in most citrus growing areas of northern Iran where declining trees are increasing rapidly (Ebrahimi et al., 1988; Rahimian et al., 2000; Rahimian and Zarei, 2002). In most field trees, CTV occurs as a mix of different virus strains or subisolates that are known to differ in their aphid transmissibility (Brlansky et al., 2003; Broadbent et al., 1996; Raccah et al., 1980). Many papers refer to the occurrence of strain segregation as a result of transmission by aphid (Brlansky et al., 2003; Broadbent et al., 1996; Kano and Koizumi 1991; Marais et al., 2000; Tsai et al., 2000; van Vuuren et al., 2000; Nolasco et al., 2008; Roy and Brlansky, 2009). So, specific CTV strain is probably transmitting and dominating in northern Iran. In this paper, we characterized the most prevailing CTV strain in northern Iran and accordingly express their CP25 gene as a source for antiserum production. Ninety isolates included in this study were recovered from originally infected satsuma trees and neighboring orchards verified to be infected through natural transmission. Comparison of the SSCP profiles of their amplified CP25 gene showed that they classified into three groups. However, CP25 gene nucleotide sequence analysis of representative isolates showed high
level of sequence identity. In the phylogenetic constructed by clustal W method CTV isolates were closely related to severe seedling yellows NUagA strain of Japan with more than 97% identity and had the least similarity to the mild T385 strain. Biological properties of three representative isolates studied on known indicator plants showed that all isolates mostly create severe yellowing and stunting symptoms. Based on biological and molecular properties, these isolates considered as seedling yellows strain. So, melon aphid are spreading severe seedling yellows strain in northern Iran and it would be a serious threat for citrus industry as sour orange is still the main citrus rootstock used in these areas. Roy and Bransky (2009) characterized the most frequently transmitted genotypes from a naturally mixed infected CTV isolate FS627 and its AT subisolates. Their results showed that T30 is the most frequently transmitted genotype when it is mixed with either T36 or VT genotypes. They suggested that, under certain conditions, brown citrus aphid transmit T30-like CTV population more frequently than T36 (Roy and Bransky, 2009). Nolasco et al. (2008) investigated the involvement of T. citricida in strain segregation and genetic bottleneck events by comparing the nucleotide diversity of CTV coat protein (CP) gene variants present in field-grown trees with that of variants retrieved from single apterus aphids. Results showed a reduction of more than one order of magnitude in most cases (Nolasco et al., 2008). Izadpanah et al. (2002) compared the capability of commercial (BioReba) versus locally produced antiserum in a direct double-antibody sandwich ELISA test to detect CTV in citrus-growing regions of Fars and Bushehr provinces in southern Iran. Most southern isolates reacted strongly with the locally-produced antiserum whereas weak reactions were obtained with BioReba antibody (Izadpanah et al., 2002). Regarding the incidence of seedling yellows strain of CTV in northern Iran and its aphid transmission ability, we expressed recombinant CP25 protein of a representative seedling yellows isolate. CP25 gene was ligated into the pET28a expression vector and transformed into the BL21 strains of E. coli. Different conditions tested to produce high amounts of recombinant protein. Best results were acquired when 1 mM of IPTG was applied on a bacterial concentration of 6.0 at 600 nm for six hours. Recombinant CP25 protein was purified by Ni-NTA affinity chromatography. SDS-PAGE analysis confirmed the expression of full length CP25 (25 KD) in this situation. In previous study in Iran, Amirisadeghan et al. (2013) cloned CTV-CP25 gene in pET26b and expressed in two strains of BL21 and Rosetta Gami (DE3). However, the size of expressed protein was smaller than expected (Amirisadeghan et al., 2013). The full length protein produced in this study can be used as a good source of antigen in preparation of CP25 related antibodies.

REFERENCES


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