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Full Length Research Paper

Molecular characterization of *Citrus tristeza* virus isolates from Pakistan based on CPG/*Hin*f I restriction fragment length polymorphism (RFLP) groups analysis

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From six different districts of Punjab, Pakistan, 85 isolates of *Citrus tristeza* virus (CTV) were collected and characterized based on coat protein gene (CPG) analysis. All isolates were collected from field trees showing various CTV symptoms such as decline in most citrus varieties, inverse pitting on some sour orange rootstocks below bud union, mild-to-moderate stem-pitting on the trunk of some sweet orange. The CTV CP gene of all isolates was amplified by reverse transcriptase polymerase chain reaction (RT–PCR) using CP gene-specific primers yielding 672 bp. The maximum disease incidence was found in sweet orange followed by mandarin and grapefruit. These isolates were then subjected to CPG/*Hin*f I restriction fragment length polymorphism (RFLP) analysis. Mixed infection of CTV isolates was found very common in the field tress in Pakistan. The most dominant CPG/*Hin*f I RFLP groups III, I and VI are the basic causal epidemic in Pakistan. Moreover, based on symptoms in the field trees, CPG/*Hin*f I RFLP groups III, I and VI are considered to be the obvious causes of decline and stem-pitting in Pakistan.

Key words: *Citrus tristeza* virus, CPG/*Hin*f I restriction fragment length polymorphism (RFLP) groups, decline, stem-pitting.

INTRODUCTION

Citrus tristeza virus (CTV), in the family Closteroviridae is the casual agent of a destructive disease of citrus and has destroyed millions of trees throughout the world and continues to cause some of the more economically important diseases of citrus (Bar-Joseph et al., 1979). CTV infects all species, cultivars and hybrids of citrus, regardless of rootstock, inducing mild or masking conditions to severe symptoms. It seriously infects sweet orange or other varieties grafted on sour orange rootstock. It also induces disease in grapefruit, lemon and lime.

The virus is transmitted by several aphid species in a non-circulative, semi-persistent manner, among which the brown citrus aphid (*Toxoptera citricidus* Kirk.) is the most efficient vector in transmission and the disease is also dispersed to new citrus areas mostly by infected propagating material (Roistacher and Bar-Joseph, 1987). CTV is known to have great genetic and biological diversity among strains from mild isolates to severe isolates (Bar-Joseph and Lee, 1989; Garnsey et al., 2005; Yokomi et al., 2010). The biological characteristics of several CTV strains have been described. CTV is divided into seven restriction fragment length polymorphism (RFLP) groups by RFLP analysis of coat protein gene

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Abbreviations: CTV, *Citrus tristeza* virus; **CPG,** coat protein gene.

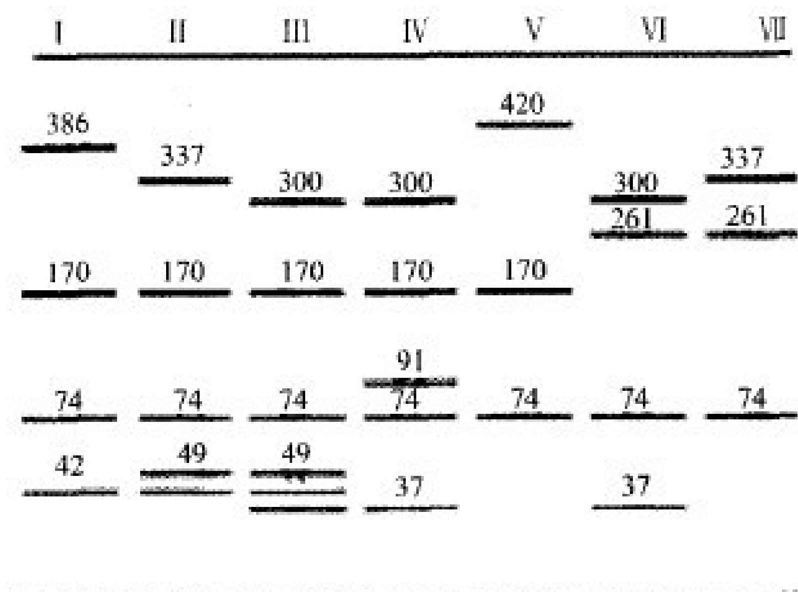

Figure 1. The diagram of seven CPG/*Hinf* I RFLP groups of CTV from Gillings (1993). The numbers on the top line represent the CPG/*Hinf* I RFLP groups; the number on the top of the each band represents the base pair size of that brand. Groups I, II, III, VI and VII were documented as severe strains, while groups IV and V as mild strains.

(CPG) digested with the restriction enzyme *Hinf* I CPG/*Hinf* I). CTV isolates of groups I, II, III, VI and VII showed severe symptoms in indicator plants, while those with CPG/*Hinf* I groups IV and V gave mild symptom (Gillings et al., 1993). Hence, the CPG/*Hinf* I RFLP analysis offers rapid differentiation of CTV strains. The diagram of CPG/*Hinf* I RFLP groups of CTV isolates is shown in Figure 1.

CTV virions are flexuous, about 2000 × 11 nm in size and encapsidate a single-stranded, positive sense genomic RNA ca. 20 kb in size, comprising 12 open reading frames (ORFs) potentially encoding at least 17 protein products and two untranslated regions (UTRs) of about 107 and 273 nt at the 5′ and 3′ termini, respectively (Karasev et al., 1995; Mawassi et al., 1996; Yang et al., 1999; Vives et al., 1999). CTV has two genes encoding a major coat protein (CP) of 25 kDa and a minor coat protein (CPm) of 27 kDa, encoded by ORF 7 and 8 and encapsidate about 97 and 3% of the virion length, respectively (Febres et al., 1996; Satyanarayana et al., 2004).

Pakistan is generally considered to be among the top 10 countries of the world in citrus production and its quality and the leading producer in Kinnow mandarin with production centralized in the province of Punjab, which produces more than 96% of the total citrus crop of the country. It covers an area of 193,211 ha and has an annual production of 2,459,500 tonnes (Anon, 2008). The presence of CTV in the citrus species grown in Pakistan was confirmed through ELISA and electron microscopy (Catara et al., 1988; Anwar and Mirza, 1992; Iftikhar et al., 2009). Brown citrus aphid (*T. citricidus* Kirk.), is fortunately not reported from Pakistan. However, there are important aphid species such as *Toxoptera aurantii, Myzus persicae* and *Aphis gossypii*, which may be considered as putative vectors as no disease transmission has been made so far. This study reports molecular characterization of CTV isolates and CPG/*Hinf* 1 group analysis to evaluate the population structure and genetic diversity of CTV isolates from Pakistan.

MATERIALS AND METHODS

Plant samples

A survey was conducted in the citrus growing areas of Punjab, Pakistan from May 2008 to September 2008, and 230 citrus samples were collected from the orchards of 10 years old or more from 20 localities of five districts: Sargodha, Bhalwal, Faisalabad, Toba Tek Singh and Sahiwal. Most of the samples collected were Mosambi, Succari and Red blood sweet oranges, Kinnow and Feutrell's early mandarin and grapefruits. Leaves, twigs and shoots were collected randomly from three different directions of each sample tree in orchards.

Direct tissue blot immuno assay (DTBIA)

All of the 230 citrus samples collected were tested by DTBIA (Garnsey et al., 1993) and the positive samples were selected for

Table 1. Sequence of oligonucleotide primers used in this study.

Primer name	Sequence (5´ to 3´)	Fragment (bp)	Target gene	Reference
CP1 (+)	ATGGACGACGAAACAAAG	672	*p25*	Gillings et al. (1993)
CP3 (-)	TCAACGTGTGTTGAATTT		*p25*	

Figure 2. Direct tissue blots immunoassay for CTV isolates from Pakistan on a nitrocellulose membrane. 1: Positive control; 2: negative control; rest of the boxes: blotted freshly cut surface of stem samples on a nitrocellulose membrane.

further analysis and for the detection of mild and severe CTV isolates from Pakistan. The stems of new flush or the petioles of the leaves were cut transversely with razor blade and three to eight cross sections of shoots or leaves from each sample were pressed on nitrocellulose membrane (NCM) in 1 cm^2. The further analysis was done by following Garnsey's method (Garnsey et al., 1993).

Nucleic acid extraction and RT-PCR

Nucleic acid extraction method (Zhou et al., 2001) was used to extract total nucleic acid from CTV infected and healthy leaves and stems of citrus plants. The CP gene was reverse-transcribed (RT) and amplified by polymerase chain reaction (PCR). Sequence of oligonucleotide primers used in this study is shown in (Table 1).

TNA extract was heat denatured at 95°C for 5 min and then was reverse-transcribed by incubation at 42°C for 20 to 40 min in a (10 µl) reaction mixture. Thermal cycling conditions used were as follows: 94°C for 4 min, 52°C for 15 s, 72°C for 40 s; 94°C for 30 s, 52°C for 15 s, 72°C for 40 s, 3 cycles; 94°C for 30 s, 50°C for 15 s, 72°C for 40 s, 3 cycles; 94°C for 30 s, 48°C for 15 s, 72°C for 40 s, 3 cycles; 94°C for 30 s, 46°C for 15 s, 72°C for 40 s, 3 cycles; 94°C for 30 s, 44°C for 15 s, 72°C for 40 s, 3 cycles; 94°C for 30 s, 42°C for 15 s, 72°C for 40 s, 3 cycles, 94°C for 30 s, 40°C for 15 s, 72°C for 40 s, 3 cycles. Finally, 72°C for 10 min, 10°C pause and end reaction. RT-PCR products were analyzed on 1.2% agarose gels in TBE buffer (Tris-borate 90 mM, pH 8.0, 2 mM EDTA), stained with ethidium bromide and photographed by using Bio-Rad Gel documentation system or UVI tec gel analysis system (Cambridge) under ultraviolet light.

Restriction fragment length polymorphism of CP gene

The PCR-amplified products of the CP gene of the different CTV isolates from Pakistan were analyzed and characterized by RFLP. And the products were subjected to digestion using *Hin*f I restriction enzyme at 37°C for 1 h. The products were electrophoresed in 3% ultra pure agarose and visualized with UV light after stained in ethidium bromide (Gillings et al., 1993).

RESULTS

DTBIA selection

The collected 230 samples from five districts of Punjab, Pakistan, were tested by DTBIA to determine the CTV infection. And 85 samples were found infected with CTV by DTBIA. At the end of the procedure, CTV infected samples changed the colour of the dots on the phloem on NCM into brown, which means infection by CTV, while healthy plants samples showed no particular change in the colour of the dots (Figure 2).

Table 2. Distribution and incidence of *C. tristeza* virus (CTV) in different districts of Punjab, Pakistan 0/0ª: no grapefruit and lime sample was collected from Sahiwal district and no lime sample from Toba Tek Singh district.

Host	Location	Noumber positive/number collected	CTV infection rate (%)
Sweet orange	Sargodha	22/42	52.38
	Faisalabad	3/6	50
	Bhalwal	9/17	53
	Toba Tek Singh	2/6	33.33
	Sahiwal	4/9	44.44
Mandarin	Sargodha	3/12	25
	Faisalabad	1/4	25
	Bhalwal	12/36	33.33
	Toba Tek Singh	3/8	37.5
	Sahiwal	5/18	28
Grapefruit	Sargodha	1/5	20
	Faisalabad	9/23	39
	Bhalwal	2/8	25
	Toba Tek Singh	0/4	0
	Sahiwal	0/0ª	0
Lime	Sargodha	1/6	17
	Faisalabad	5/18	28
	Bhalwal	3/12	25
	Toba Tek Singh	0/0ª	0
	Sahiwal	0/0ª	0

The results show that sweet orange was the most infected variety followed by mandarin, grapefruit and lime (Table 2).

Synthesis of CP gene by RT-PCR

All the 85 CTV isolates from Punjab, Pakistan were analyzed for CTV using RT-PCR. A 672 bp fragment, indicating the full length CTV coat protein gene was amplified from the positive samples after DTBIA, while absence of CTV and negative control did not produce any band at all (Figure 3).

RFLP profiles of the CP gene from different CTV isolates

DNA band digestion of these PCR products with the restriction enzyme *Hinf*I (Gillings et al., 1993) showed sequence diversity for these different isolates from Pakistan (Figure 4). The ratios of single and mixed infection were also compared between the sweet orange and mandarin samples and over all samples collected from field tress from Pakistan. The ratio of mixed and single infection was 35.6 and 64.2%, respectively, while the ratio of mixed infection in mandarin field tree was 57% and the ratio of single infection was 43%. The ratio of mixed and single infection in sweet orange was 35.7 and 64.28%, respectively (data not shown in table form). CPG/*Hinf* I RFLP group III was the main epidemic isolates in the CTV isolates from Pakistan, followed by group I with the rate 78.57 and 28.57%, respectively. CPG/*Hinf* I RFLP group VII was not found from Pakistan.

DISCUSION

The 230 samples assayed by DTBIA, from the field tress from Pakistan were analyzed in this study, of which 85 were found CTV positive. On the varietal basis, disease incidence was found from 17 to 52.38%, sweet orange was severely affected followed by mandarin (Table 1) and lime was the least affected variety in Punjab province of Pakistan. Our results supported Iftikhar's conclusion (Iftikhar et al., 2009) that sweet orange was the most affected variety in Punjab province. Overall, disease incidence was found about 37% which is alarming for the

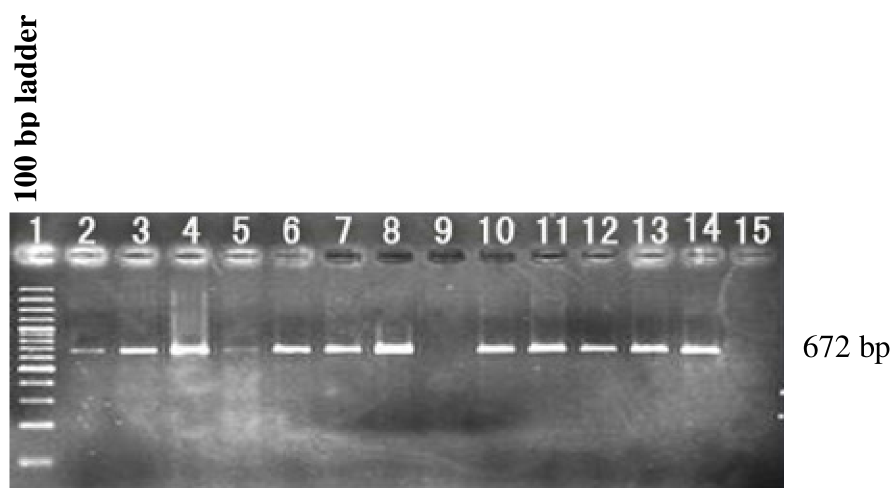

Figure 3. RT-PCR products of CP gene about 672 bp. Lane 1: DNA marker with 100 bp ladder; lanes: 2 to 13 samples from 8 citrus growing areas of Punjab; lane: 9 CTV-negative sample (by DTBIA); 14 positive control; 15 negative control.

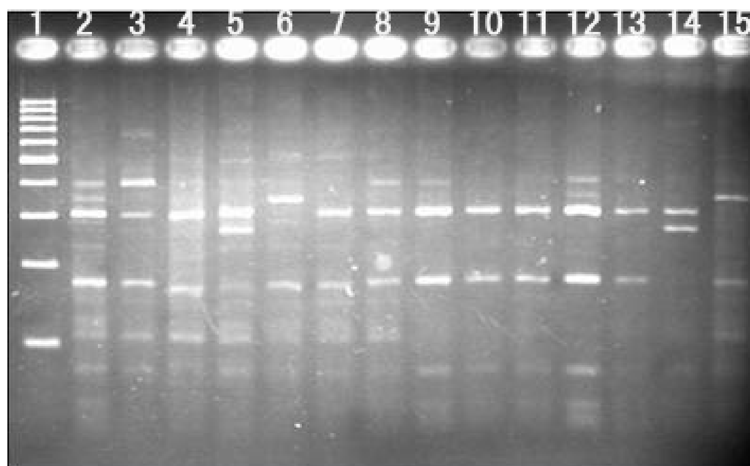

Figure 4. CPG/*Hinf* I RFLP group analysis in CTV infected samples collected from Pakistan. Lane 1, 100 bp DNA marker; lane 2, 12, I, II, III groups; lane 3, I, III, groups; lane 4, 7, 9,10,11,13, III group; lane 5, III, VI groups; lane 6, 15, II group; lane 14, VI group.

citrus industry of Pakistan.

In this study, the CPG/*Hinf* I restriction fragments revealed that the CTV population structure from Pakistan is a complex. Among all the CTV isolates analyzed, we found the mixed infections of members from I to VI RFLP groups, but the group VII was not found at all among isolates from Pakistan. Most of the mixed infection found was between the group I, III and group VI. Group III was the main dominant isolate and group I and VI were second and third accordingly. These results show the same tendency of mixed infection of group III and I on almost all citrus varieties analyzed from Pakistan. According to Gillings et al. (1993), group I, III and VI were documented as severe strains, we have found the mixed infection of these three groups. Therefore, basic conclusion could be made that CTV isolates from Pakistan have severe strains on sour orange rootstock. The same results of mixed infection were found by (Zhou et al., 2005, 2007; Xu et al., 2006; Bo et al., 2008) in China and that most interestingly, group III was the main epidemic and dominant group followed by group I and VI, respectively found on molecular and biological basis in China. Our results were consistent with these results of mixed infection and dominant isolates. Barzegar et al.

(2006) using RFLP profile, revealed that all the isolates in northern Iran were severe strains and produced symptoms such as stem-pitting on Mexican lime and stunting of new growth on sour orange and grapefruit seedlings. These symptoms have quite resemblance with the symptoms found in Pakistan on field trees of citrus. The results also confirmed that *Hin*fI/RFLP analysis could efficiently discriminate mild strains from severe strains (Gillings et al., 1993). To our knowledge, this is the first report on the molecular characterization of CTV from Pakistan and strain differentiation of those isolates by CPG/*Hin*f I RFLP groups' analysis.

In conclusion, the presence of severe strains and mixed infection of CTV in Pakistan where sour orange still remains the dominant rootstock, places the citrus industry of this entire region at high risk for an imminent, destructive outbreak of tristeza. Hence, this study will provide basis for conducting mild strain cross protection (MSCP) in the future in Pakistan and can also be helpful for neighboring countries.

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