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Sequence Analysis of the Coat Protein Gene of Citrus Tristeza Virus Isolated from Cheju Island

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Citrus tristeza virus (CTV) is the causal agent of one of the most important diseases of citrus. Recently, CTV has been detected in Cheju Island by ELISA. The coat protein (CP) gene of CTV isolated from Cheju Island was cloned by RT-PCR and the nucleotide was analyzed in this study. Citrus leaves were collected from trees showing decline symptoms from various region of Cheju Island in the summer of 1998 and 1999. The CP gene open reading frame is composed of 670 nucleotides and encodes a polypeptide of 223 amino acids. Sequence analysis the CP gene revealed that two CTV strains present in Cheju Island. Viruses collected from Sogwipo area and Cheju City area in 1999 showed 91-93% nucleotide sequence homology with CTV T36 strain. Viruses collected from Cheju City area in 1999 and Sogwipo City in 1998 showed 94-98% nucleotide sequence homology with CTV SY568 strain. A efficient viral RNA extraction methods was developed by modifying procedure for animal virus RNA purification methods and PCR product was detected from one tenth of RNA purified from as small as 45 mg fresh or frozen tissue.

Keywords : CTV-T36, CTV-SY568, Mild Strain, RT-PCR.

Virus and virus like diseases have a major impact on citrus production worldwide and often become the dominant yield-limiting factor in grove management. Among those viruses, citrus tristeza virus (CTV) is the major virus pathogen of citrus species.

CTV is a filamentous plant virus with flexible virions composed of one molecule of single-stranded RNA of positive polarity and two coat proteins (CPs) of 25, 27 kDa, coating 95 and 5% of the particle length, respectively (Febres et al., 1996). CTV is the largest known plant virus with the genome of ca. 20 kb (Bar-Joseph, 1985). At least 17 protein products are expressed from polyprotein processing, frameshift and 3'-coterminal subgenomic mRNAs (Hilf et al., 1995; Mawassi et al., 1995a). Those proteins include two papain-like protease (P-PRO), a methyltransferase (MT), a helycase (HEL) and RNA polymerase, homologue of HSP70 of heat shock protein and several structural gene and two CPs and putative RNA binding protein (RBP) (Karaseve et al., 1994; Pappu et al., 1994).

Symptoms caused by CTV are various but depend on the host species and cultivars (Moreno and Guerri, 1997). These symptoms include i) decline and death of plants grafted on sour orange, ii) stunting and yellowing of sour orange, grapefruit or lemon seedlings and iii) stem pitting of different citrus cultivars on their own roots or grafted on any rootstock. Complete nucleotide sequences of a decline isolate (CTV-T36) from Florida (Karaseve et al., 1994; Pappu et al., 1994), seedling yellows isolate (CTV-VT) from Israel (Mawassi et al., 1996), severe stem pitting isolate (CTV-SY568) from California (Yang et al., 1999) and a Spanish mild isolate (CTV-T385) (Vives et al., 1999) have been determined. Although complete relationships between the symptom phenotypes and genomic sequences are not clear, there are sequence differences among these isolates.

As other plant viruses, CTV could be detected by enzyme linked immunosorbent assay (ELISA) and reverse transcription-polymerase chain reaction (RT-PCR). However, low titer of CTV only in phloem area and nonspecific reaction are the obstacles in the serological diagnostic method (Rocha-Pena and Lee, 1991). Likewise, difficulty in RNA extraction from woody plant is the barrier in CTV detection with RT-PCR (Zhang et al., 1998).

Recently, CTV has been detected from Cheju Island by ELISA (Kim et al., 1999), but not confirmed by other methods. In this report, we have detected CTV from citrus plant in Cheju Island with RT-PCR and the PCR products were sequenced to characterize their molecular relatedness with known CTV isolates. We also introduce a simple and efficient method for CTV RNA extraction from diseased plant samples.

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Materials and Methods

Sample preparation. Citrus leaves were collected from citrus farms in Cheju City and Sogwipo City of Cheju Island in the summer of 1998 to 1999 years. Samples were collected from trees showing decline and yellow symptoms. Leaf discs of 6 mm diameter were made with a small paper punch and processed immediately or stored in a -80°C freezer.

Viral RNA extraction. Viral RNA was directly extracted from the infected tissue by using the viral RNA purification kit (Boehringer Mannheim) designed for animal viruses by modifying the procedures provided by the manufacture. A microcentrifuge tube containing seven leaf discs, weighing approximately 100 mg was immersed in liquid nitrogen and the sample was ground with a pestle. Powered tissue was first extracted with 250 μl of 1× STE buffer. Supernatant of 200 μl was collected after 5 min centrifugation with a microcentrifuge at the maximum speed. To the supernatant, 200 μl of lysis/binding buffer (poly A carrier, 4.5 M guanidin hydrochloride, 50 mM Tris-HCl (pH 6.6), 30% Triton X-100 and glass fiber) of kit was added. After 10 min incubation at room temperature, the mixture was loaded onto a spin column. The column was centrifuged for 15 sec and then washed twice with 450 μl of washing buffer provided in the kit. The viral RNA was eluted with 100 μl elution buffer in the kit

Primers. Primers for RT-PCR were designed from published sequence of CTV-T36 (Karasev et al., 1995). Primer CTCPN (5'-TAACATCATATGATCCCATGA-3') and primer CTVC (5'-AGCTGCCTGACATTAGTAAC-3') which locate at 16077-16097, 16785-16804 from the 5' end of CTV-T36, respectively were used for the amplification of the N-terminal two third of the CP gene. Primer CTVN (5'-CGAGTCTTCTTTCGGTTCC-3') and primer CTCPC (5'-TCTTCATTAGGATCCTCTAAC-3') which locate at 16226-16244, 16908-16928 from the 5' end of CTV-T36, respectively were used for the amplification of the C-terminal two third of the CP gene. Primers CTVN and CTVC were used for amplification of the middle region of the coat protein gene for CTV detection and strain identification. The expected size of PCR products with CTCPN-CTVC, CTVN-CTVC and CTVN-CTCPC primer pairs are 714 bp, 560 bp and 766 bp, respectively (Fig. 1).

RT-PCR. RT-PCR was performed by access RT-PCR kit (Promega). Reaction mixture of 50 μl was made with 10 μl of purified viral RNA. RT-PCR reaction condition was as follows: RT at 48°C for 45 min, pre-denaturation at 95°C for 2 min, 40 cycles of 30 sec denaturation at 94°C, 30 sec annealing at 55°C, 30 sec extension at 68°C, followed by a 7 min post-extension at 72°C.

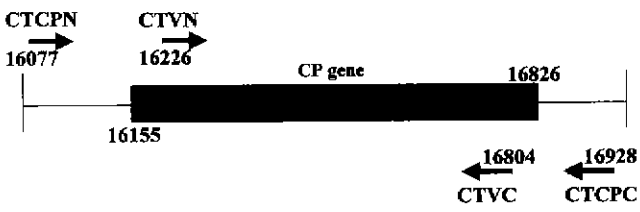

Fig. 1. Target site for CTV detection by RT-PCR. The coat protein gene are showed as thick bar and the primers used in RT-PCR are designated with PCR products as arrows. Primer CTCPN and primer CTVC which locate at 16077-16097, 16785-16804 from the 5' end of CTV-T36. Primer CTVN and primer CTCPC which locate at 16226-16244, 16908-16928 from the 5' end of CTV-T36.

Cloning and Sequencing. PCR products were separated on an agarose gel, eluted from the gel and cloned into the pGEM-T Easy vector system (Promega). Sequencing was performed by PCR with T7 and SP6 primers and the products were analyzed with the ABI PRISMTM 310 analyzer (Perkin Elmer). All clones were sequenced completely in both directions. Nucleic acid sequences were compared with the National Center for Biotechnology Information (NCBI) database using the BLAST program. Multiple sequence alignments were made using the Clastal W (Thomson et al., 1994).

Detection Limit by RT-PCR. In order to determine the minimum sample weight for viral detection, RNA was extracted from one disc (15 mg), two discs (30 mg), three discs (45 mg), five discs (75 mg), seven discs (100 mg) by using virus RNA extraction kit as described above. RNA extracted from 100 mg of tissue by using virus RNA purification kit was diluted serially into 1/2, 1/5, 1/10, 1/100, 1/1000, 1/10000 and 10 μl of the diluted RNAs were used for RT-PCR to determine the dilution end point.

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16038 acgccatcccatgagcactgctttaagggtcgttaattgacgactcttgatatttactaa
16098 gtttgaatta tggacgacgagacaaagaaattgaagaacaaaaacaaggaaacgaaagaa
   1          M  D  D  E  T  K  K  L  K  N  K  N  K  E  T  K  E
16158 ggcgacgatgttgttgcagcggagtcttctttcggttccttaaacttgcacatcgatccg
  18  G  D  D  V  V  A  A  E  S  S  F  G  S  L  N  L  H  I  D  P
16218 actctgatagcgatgaacgatgtgcgtcagttaggtacccaacagaacgccgctttgaac
  38  T  L  I  A  M  N  D  V  R  Q  L  G  T  Q  Q  N  A  A  L  N
16278 agagatttattccttactttgaaagggaagtatcctagtttgtctgataaagataaggac
  58  R  D  L  F  L  T  L  K  G  K  Y  P  S  L  S  D  K  D  K  D
16338 tttcacatagctatgatgttgtatcgtttagcggttaagagttcatcattgcaaagtgac
  78  F  H  I  A  M  M  L  Y  R  L  A  V  K  S  S  S  L  Q  S  D
16398 gacgacaccacgggcataacgtacactcgggagggcgtcgaagtggatttgtctgacaaa
  98  D  D  T  T  G  I  T  Y  T  R  E  G  V  E  V  D  L  S  D  K
16458 ctttggactgacgtcgtgtttaattctaagggtatcggtaaccgtactaacgccctlcga
 118  L  W  T  D  V  V  F  N  S  K  G  I  G  N  R  T  N  A  L  R
16518 gtctggggtagaagtagcgatgccctttatttagcgttttgtagacagaatcgcaatttg
 138  V  W  G  R  S  S  D  A  L  Y  L  A  F  C  R  Q  N  R  N  L
16578 agttatggcggacgtccgctagatgcagggattcggccgggtatcataacctgtgtgca
 158  S  Y  G  G  R  P  L  D  A  G  I  P  A  G  Y  H  N  L  C  A
16638 gatttcttgaccggagctggcttggctgatttagaatgtgctgtgtacatacaagctaaa
 178  D  F  L  T  G  A  G  L  A  D  L  E  C  A  V  Y  I  Q  A  K
16698 gaacaattgttgaagaagcgaggggctgatgaagtcgtagttaccaatgtcaggcagctt
 198  E  Q  L  L  K  K  R  G  A  D  E  V  V  V  T  N  V  R  Q  L
16758 gggaaatttaacacacgttgatctattgcgttgcgattctcgatttctatcgggatggtg
 218  G  K  F  N  T  R  *
16818 gagtttcatcgtgaacgtgggtgatataatcgttcggttlgcgttacatgttcctagtga
16878 aga
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Fig. 2. Nucleotide and deduced amino acid sequence of the coat protein gene of CTV Cheju isolate. This is a combined sequence of PCR products of CTCPN/CTVC primers and CTVN/CTCPC primers using CTV isolated from Kwangryung (Cheju City) in 1999. The start and stop codons for the coat protein are designated in bold.

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession No. AF249279.

Results and Discussion

Sequence of the CP gene of CTV isolated from Cheju Island. There are two CP of 25, 27 kDa, coating 95 and 5% of the particle length CTV, respectively (Febres et al., 1996). By combining the sequence of PCR product from CTCPN-CTVC and CTVN-CTCPC, a complete nucleotide sequence of the 25 kDa CP gene of a Cheju isolate of CTV was constructed. As shown in Fig. 2, the CP gene open reading frame is composed of 670 nucleotides encoding a polypeptide of 223 amino acids. The CP gene of Cheju isolate exhibited 98% and 93% homology in nucleotide sequence and 91% and 87% homology in amino acid sequence to CTV-SY568 (accession number : AF001623) and CTV-T36 (accession number : U16304) strain, respectively. This high sequence homology to other CTV isolates has been observed in other report, in which there was a greater than 80% sequence homology at both nucleotide and amino acid level (Pappu et al., 1993). In the sequence comparison of biologically diverse isolates of CTV in Israel, Mawassi et al. (1993) have found at least two CTV coat subspecies displaying extensive differences in their CP genes. In addition, they found two amino acids differences between the CP gene of *Aphis gossypii* transmissible and non-transmissible isolates. Although the genome of ca. 20 kb and 17 proteins encoded by the genome are the obstacles, further analyses of CTV genome could find the relationships between the CP sequence and the biological characteristics of each CTV isolate.

Identification of two CTV strains in Cheju Island. In order to find any sequence variation among CTV present in Cheju Island, a PCR product of 560 bp encompassing the amino acids 18 to 216 of the CP gene were produced by RT-PCR with the primers CTVN and CTVC (Fig. 1), and sequenced after cloning into pGEM-T Easy vector. Among the CTV samples collected from more than 30 sites, PCR products of five isolates were cloned and sequenced. The location and year of collection are as follows; samples from Sangga, Kwangryung (Cheju City) and Namwon (Sogwipo City) in summer of 1999, two samples from Namwon (Sogwipo City) in 1998. In the multiple alignment of the sequence, the five isolates could be grouped into two known CTV strains, the CTV-T36 strain and CTV-SY568 strain (Table 1). In the pairwise alignment of all the isolates, the sequence homology among the isolates belong to T36 strain was 91-95%. The sequence homology among the isolates belong to SY568 was 94-98% and sequence homology between two groups was 91-96%. Although the grouping is based on the sequence of the CP gene, the presence of two CTV strains is of interest (Fig. 3). In the Unites States, the T36 strain was isolated from Florida (Rosner et al., 1986) and SY568 strain was isolated in California (Yang et al., 1999). In addition, a cluster dendrogram exhibiting greater similarities among groups of mild and severe Florida isolates that differed significantly from those of geographically distinct, exotic CTV isolates was obtained in the sequence comparison of CTV isolates originated from Florida and exotic isolates (Pappu et al., 1993). The

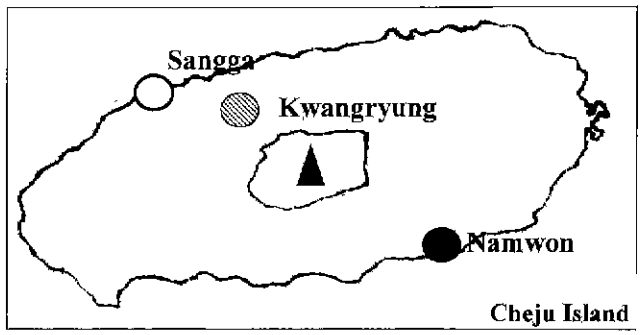

Fig. 3. Distribution of two CTV strains in Cheju Island. Open circle; T36 strain, hatched circle; SY568 strain, black circle; T36 and SY568.

Table 1. Sequence comparison of the coat protein genes of CTV isolates collected from Cheju Island. Years and collection sites are designated for each isolate. Strain names identified by multiple alignments are indicated in the parentheses. The asterisks represent nucleotides identical in all isolates. The sequence of CTV-T36 and SY-568 are from the GeneBank (Accession number U16304 and AF001623, respectively)

	99Namwon (T36)	99Sangga (T36 SY568)	STRAIN T36	STRAIN SY568	99kwang-Ryung (SY568)	98Namwon (SY568)	98Namwon (SY568)
99Namwon (T36)	–	95	91	91	93	91	91
99Sangga (T36 SY568)	95	–	93	93	96	92	92
STRAIN T36	91	93	–	93	92	92	92
STRAIN SY568	91	93	93	–	94	98	98
99kwang-Ryung (SY568)	93	96	92	94	–	94	94
98Namwon (SY568)	91	92	92	98	94	–	98
98Namwon (SY568)	91	92	92	98	94	98	–

sequence relatedness among strains of same area indicates appearance of new isolates from existing isolates by accumulation of mutation for long period of time. Another possible mechanism of new isolate origin is recombination between two different stains. Nucleotide sequence alignments of CTV-VT isolates showed that 3' half of the CTV-VT genome was considerably more similar to other sequenced isolates of CTV than the 5' half (Mawassi et al., 1996). One possible explanation for this difference could be recent recombination between two CTV isolates, which has been demonstrated both in CTV-VT and in other CTV isolates (Mawassi et al., 1995b). The origin of the two CTV strains in Cheju Island is not clear at this point. They could be distributed to citrus farms after originated from a particular citrus farm or they introduced from scions introduced from other area or other country. One positive aspect about the presence of CTV-SY568 strain is that this strain is very similar to CTV-T385, which is Spanish mild isolate (Vives et al., 1999). There is no complete control measure for CTV at this point but cross protection with a mild strain is one possible way of CTV control. Considering that there has not been much attention on citrus losses by CTV in Cheju Island for long time, it is possible that CTV mild strains present in Cheju Island. This possibility is also can be explained by the high incidence of CTV by ELISA (Kim et al., 1999). The high incidence of CTV without severe losses indicates that there are mild strains of CTV whose CP can be detected by antibody against severe CTV strain. Further molecular characterization of whole CTV genomes from more isolates in Cheju Island is necessary for the identification of mild strains and application in CTV control.

Virus detection limit by RT-PCR. Although PCR is a very effective method for virus detection with its extreme sensitivity and specificity, there are still a considerable numbers of problems in application. With plant viruses, the majority of problems have to do with initial nucleic acid purification. Especially nucleic acid preparation from woody plant such as citrus is problematic because of high content of phenolic compound and low virus titer in phloem area (Olmos et al., 1999). CTV has been recently reported from Cheju Island by ELISA and the incidence was 69.8% although there was a significant differences in the incidence depend on the cultivars. In this experiment RNA was extracted from plant showing decline and tested for the presence of CTV by RT-PCR. Amplified PCR products of expected size were detected from all the samples collected in 1998 and 1999 in Cheju City and Sogwipo City. This was expected because the samples had been collected from plant showing CTV symptoms. Zhang et al. (1998) developed a small-scale procedure for nucleic acids from woody plants based on the cellulose chromatography for dsRNA purification (Morris and Dodds, 1979). There are several

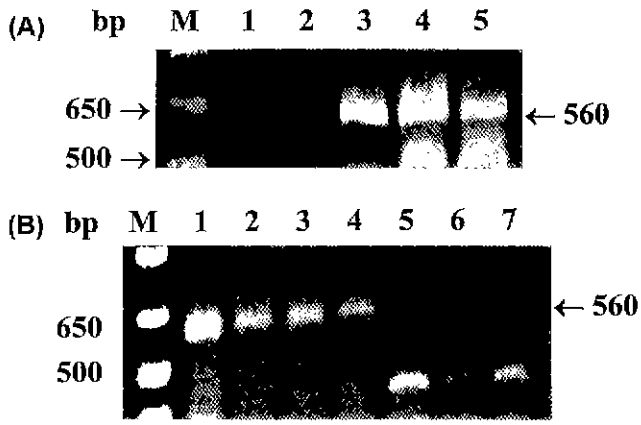

Fig. 4. A, Minimum tissue required for virus detection. CTV genomic RNA was prepared from different amount of leaf tissue in final volume of 100 µl PCR product from viral RNA without DNAse treatment elute, and 10 µl was used for RT-PCR. Lane M; DNA size marker, Lanes 1-5; RNA prepared from 15, 30, 45, 75 and 100 mg tissue, respectively. B, Dilution end point for RT-PCR. Viral RNA from 100 mg of leaf tissue was prepared in 100 µl and serially diluted before RT-PCR. For each reaction, 10 µl of diluted RNA was for RT-PCR in 50 µl reaction. Lane M, DNA size marker, Lanes 1-7; RNA diluted to 1/1, 1/2, 1/5, 1/10, 1/100, 1/1000 and 1/10000, respectively.

viral RNA extraction kit developed for animal virus detection. In our experiments, one of the viral RNA extraction kit was used for plant virus RNA extraction by modifying the RNA extraction procedures provided by the manufacturer. RT-PCR results revealed that purified viral RNA was better than dsRNA for CTV detection by RT-PCR (not shown). In order to determine the minimum amount of tissue sample or viral RNA required for virus detection by RT-PCR, viral RNA was prepared from different amount of sample leaf discs by using virus RNA extraction kit. As shown in Fig. 4-A, PCR product was detected from as few as three leaf discs, which is 45 mg in fresh weight. Considering that only 10 µl from total of 100 µl eluted RNA was used for each PCR reaction, this methods is very effective method for virus detection from small-scale samples. The sensitivity was also determined by serial dilution of purified viral RNA. As shown in Fig. 4-B, PCR product was detected until the purified RNA was diluted one tenth. The appearance of smaller PCR products from higher dilution could be nonspecific binding of primer because of high primer/template ratio. Thus, appropriate ratio between the RNA template and primers is necessary for the best result in virus detection by RT-PCR. Although the RNA extraction kit is little expensive for routine virus detection, this method can be used for virus detection from stock plant for scions. In order to evaluate possible usage of this method in other plant virus detection, viral RNA was extracted from lily infected with lily symptomless virus (LSV). PCR product

of expected size was detected from RT-PCR with LSV specific primer set (data not shown). Thus, this method seems to be suitable for viral RNA extraction from woody plants such as citrus and plant with high contents of sticky polysaccharides such as lily.

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