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RT-PCR Detection of Citrus Tristeza Virus from Early Satsuma Mandarin and Yuzu in Cheju Island

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Citrus tristeza virus (CTV) was identified from CTV-infected early satsuma mandarin (*Citrus unshiu*) and yuzu (*C. junos*) by RT-PCR. The total RNAs were isolated from citrus bark and leaf tissues infected with CTV and reverse transcription was followed with primers designed for amplifying CTV coat protein gene. DNA fragments of 738 bp were amplified by RT-PCR and these products were cloned for sequence analysis. Based on the sequence analysis, this PCR product has 97% sequence homology to CTV (T-385) CP gene isolated from USA. RT-PCR assay for CTV detection was more sensitivity than ELISA assay which was done with anti-CTV CP antibody. This is the frist report about CTV identification in Cheju island, Korea.

Keywords : citrus tristeza virus, coat protein, ELISA, RT-PCR.

Citrus tristeza virus (CTV) is the most economically important virus pathogen of citrus and one of the major disease problems affecting citrus production world wide (Mehta et al., 1997; Lopez et al., 1998; Whiteside et al., 1993). It is responsible for several different disease syndromes in citrus plants, including 'stem pitting', 'weak tree vigor' and 'quick decline', in which phloem necrosis frequently results in death of tree (Mary et al., 1991; Mark et al., 1998; Mc clean et al., 1955).

CTV was first reported in Italy in 1955 in 'Meyer' lemon and the virus was identified to one number of phloem-limited closterovirus, consisting of 11 nm × 2,000 nm flexible particles and has a positive sense genomic RNA of around 19 Kb nucleotide, organized in 12 open reading frames potentially encoding at least 17 protein products (Areddia et al., 1992; Bar-Joseph et al., 1976; Mehta et al, 1997).

CTV is able to be infectious to most species, cultivars, and intergenic hybrids of citrus and some citrus relatives. Symptom expression in citrus host is in highly variable and affected by environment, host species, and the severity of the isolate (Price, 1966). In general, mandarins are especially tolerant to CTV infection, but CTV is infectious to sweet orange, sour orange, grape fruit, pummelos, and some citrus hybrids (Whiteside et al., 1993; Garnsey et al., 1997). CTV is transmitted by aphids (*Toxoptera citricida, Aphis gossypii* and *A. spiralcola*) in a noncirculative semi-persistent manner (Hermoso de Mendoza et al., 1984; Mehta et al., 1997).

Enzyme-linked immunosorbent assay (ELISA) is very useful tool in identification of virus on plants, but some problems are encountered with that assay. Those are the problem of the interpretation of the ambiguous results when ELISA values are not at least 2.5 times higher than the value of the healthy control plant and the results are sometimes only reliably during certain season, where virus titer is high (Mathews et al., 1997). Therefore, sensitive molecular techniques such as reverse transcriptase polymerase chain reaction (RT-PCR) may be able to resolve these problems and differentiate the virus strain.

In this study, we present detection of CTV using RT-PCR assay, a comparison of ELISA and RT-PCR assay for diagnosis of samples harvested in citrus orchards and we also compared the coat protein (CP) gene between CTV strains.

Leaves and barks of young twigs were collected from yuzu (*Citrus junos*) and early satsuma mandarin (*C. unshiu*) grown at the citrus orchards (Table 1). The positive control was used with CTV-infected yuzu, which appeared the typical stem-pitting symptoms and was determined to be CTV positive by ELISA. Virus-free stocks of *C. junos* from micrografted shoot-tips were used for the negative control and this was determined to be CTV-free by ELISA. ELISA was carried out using a double-antibody sandwitch ELISA method in a microplate with alkaline phosphate conjugate of monoclonal antibodies supplied by Dr. H. G. Su, National Taiwan University. The absorbance was measured at 405 nm with a ELISA reader (Bio Rad 550) after 30-60 min of substrate development at room temperature. When the ELISA values were at least 2 times higher than the value of the negative controls, the sample was identified as a CTV-positive.

Total RNA was extracted by using of RNAgents Total

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RNA Isolation System kit from 0.1 g of yuzu bark and leaf tissues. RNA concentration was determined with a spectrophotometer. Total RNA was used as a template in a reverse transcriptase-mediated PCR. RT-PCR was conducted following protocols provided by the manufacturer. A set of primers (CTCpo1 5'-TACCGTCCCCAAACCAACTA-3' and CTCpo2 5'-CATGGCAGGTTATACAGTAC-3') was used to detect CTV based on the nucleotide sequences of 27 K coat protein gene of CTV. Downstream primer, CTCpo1 was complementary to nucleotide 16079-16091 and upstream primer CTCpo2 corresponded to nucleotides 15318-15337 of the CTV determined (Mary et al., 1991). After PCR amplification, the product was electrophoresed in a 1.0% agarose gel in TAE buffer and visualized by ethidium bromide staining.

Three PCR products form yuzu (Y-1, Y-6 and Y-20) and one PCR product from early satsuma mandarin (ESM-6) were isolated from a 1.5% agarose gel and cloned into pGEM-T easy vector (Promaga). These clones were sequenced with 17-mer oligonucleotides using the Prism sequencing kit (Perkin Elmer). The reaction products were resolved on an ABI PRISM 310 genetic analyzer (Perkin Elmer). Sequences were analyzed by Genebank search. These sequences of 4 CTV CP gene showed 97% homology to CTV-US strain 385. Therefore this sequence analysis confirmed that the virus isolated from yuzu and early satuma mandarin were one of CTV strains.

Twenty two yuzu trees and 14 early satsuma mandarin trees cultivated in four different areas of Cheju island were investigated for CTV screening (Table 1). The typical CTV symptoms among yuzu trees were shown on Y-1, Y-6 and Y-20 which produced severe stem-pittings and stunted stems (Fig. 1). But 7 out of 20 Yuzu trees were ambiguous to be infected or not. And 7 yuzu trees looked healthy just like the virus free stock as a negetive control. However RT-PCR and ELISA analysis indicated that 18 trees of 20 tested were infected by CTV (Table 1). By the PCR analysis, CTV CP specific gene was amplified (Fig. 2) and it was confirmed by sequence analysis. The sequence analysis data indicated that CTV-Cheju strain shared 97% sequence homology with CTV-US strain T385 (Fig. 3). The responses of early satuma mandarin trees to CTV were somewhat different from yuzu trees. Fourteen early satuma mandarin trees looked healthy but there were also infected by CTV (Table 1). Eight out of 14 early satuma mandarins were identified to contain CTV in leaves by PCR analysis (Table 1). The infectivity of early satuma mandarins was much reduced in comparison of yuzu trees. This result implicates that early satuma mandarin seems to be more tolerant to CTV and yuzu is more sensitive to CTV. This conclusion may need more infection assay to be clear with more yuzu and early satuma mandarin trees harvested from different places, different tissues and different seasons.

Table 1. List of isolates of citrus tristeza virus collected from citrus trees in Cheju, 1999 and the comparison of CTV detection sensitivities

Isolate	Source	Collection place	Symptoms[x]	Virus detecton	
				RT-PCR	ELISA
Y-1	*C junos*	Namwon	+++	+	+
Y-2	*C junos*	Namwon	–	–	–
Y-3	*C. junos*	Namwon	–	+	+
Y-4	*C. junos*	Namwon	–	+	+
Y-5	*C junos*	Namwon	–	–	+
Y-6	*C. junos*	Harye	+++	+	+
Y-7	*C. junos*	Harye	±	+	+
Y-8	*C junos*	Harye	±	+	–
Y-9	*C. junos*	Harye	–	+	+
Y-10	*C. junos*	Harye	±	+	+
Y-11	*C. junos*	Harye	+	+	+
Y-12	*C. junos*	Harye	±	+	+
Y-13	*C. junos*	Harye	++	+	+
Y-14	*C. junos*	Harye	–	+	+
Y-15	*C. junos*	Harye	+	+	+
Y-16	*C. junos*	Harye	–	+	+
Y-17	*C junos*	Harye	±	+	+
Y-18	*C. junos*	Harye	±	+	+
Y-19	*C. junos*	Pyosun	±	+	+
Y-20	*C. junos*	Pyosun	+++	+	+
Y-21[y]	*C. junos*	Harye	–	–	–
Y-22[z]	*C. junos*	Harye	–	–	–
ESM-1	*C unshiu*	Harye	–	–	–
ESM-2	*C. unshiu*	Harye	–	–	–
ESM-3	*C. unshiu*	Harye	–	–	–
ESM-4	*C. unshiu*	Harye	–	–	–
ESM-5	*C unshiu*	Harye	–	+	+
ESM-6	*C unshiu*	Harye	–	+	+
ESM-7	*C. unshiu*	Seowgipo	–	+	–
ESM-8	*C. unshiu*	Seowgipo	–	–	–
ESM-9	*C unshiu*	Seowgipo	–	+	+
ESM-10	*C. unshiu*	Seowgipo	–	+	+
ESM-11	*C unshiu*	Seowgipo	–	+	–
ESM-12	*C unshiu*	Seowgipo	–	+	–
ESM-13	*C. unshiu*	Seowgipo	–	–	–
ESM-14	*C. unshiu*	Seowgipo	–	+	+

[x] Degree of symptoms severity +++; Severe, ++: Moderate, +; Slight, ±: Ambiguous, –; No stem-pitting, y; Stunting symptom.
[y, z] virus-free stocks as a negative control

The virus detection sensitivity by RT-PCR and ELISA were tested with yuzu and early satuma mandarin trees. Basically RT-PCT approach was more sensitive to ELISA assay in this study (Table 1). But these two assays showed strong correlation of CTV detection on trees. In this study,

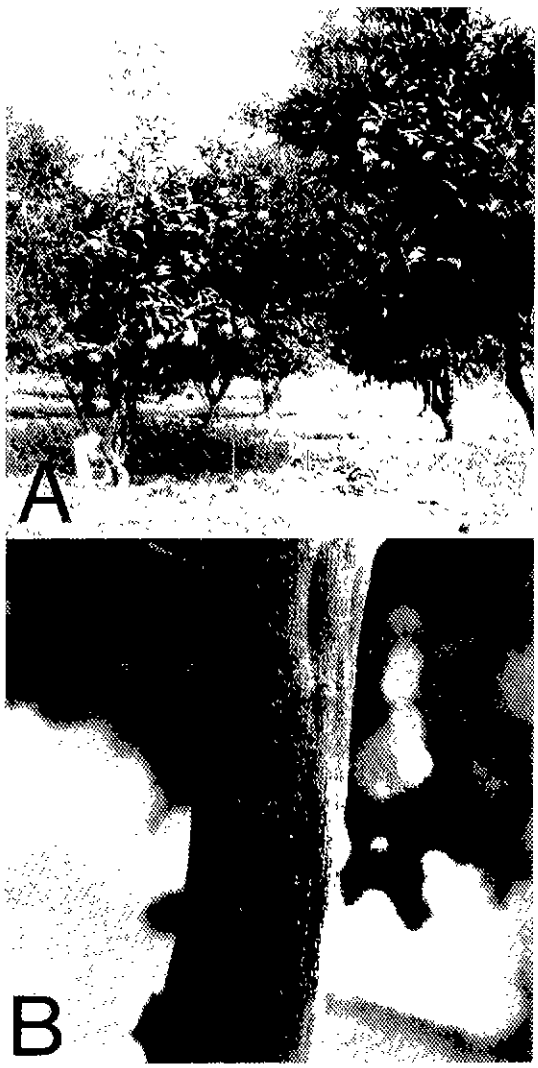

Fig. 1. Typical disease symptoms caused by CTV on yuzu. (A) CTV-infected plant is stunted on left side and virus-free plant is on the right. (B) Magnification of stem-pitting symptom shown on CTV-infected plant.

we applied the general recommendation of the Centrol California Tristeza Eradication Agency (CCTEA) for scoring the infectivity on ELISA assay. The threshold used for scoring a positive is at least 2.5 times higher ELISA value than the value of negative controls recommended by CCTEA and we also applied to 2.0-2.5 times values for it.

Even though two virus detection approaches were good enough to tell the infectivity against CTV on yuzu and early satuma mandarin trees, RT-PCR was little bit more sensitive to ELIZA assay (Table 1). In this point, Mathews et al. (1997) also reported that CTV was not reliablely detected by ELISA because CTV titer is affected by environmental factors such as temperatures and humidity as well as the biological factor such as specific tissue types tested. By contrast, RT-PCR methods gave more positive results for

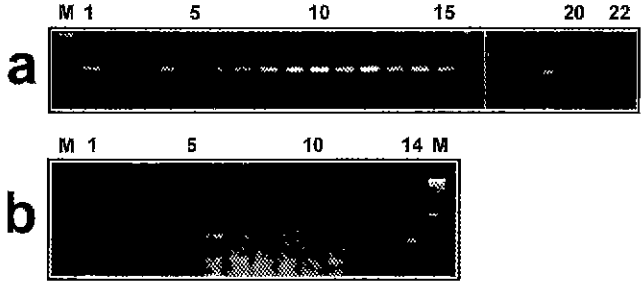

Fig. 2. CTV detection by RT-PCR. (A) *Citrus junos*: lane M, 1 kb DNA ladder; lane 1-20, yuzu tissues and lane 21 and 22, healthy and virus-free stock, respectively. (B) *C. unshiu*: lane M, 1 kb DNA ladder; lane 1-13, early satsuma mandarin tissues and lane 14, positive control

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Y-6     1     atggcaggttatacagtacttcctaataccgatgacaaagaaatggatccggtgagtgcc 60
              ||||||||||||||  ||||||||||||||||||||||||||||||||||||||||||||
T-385   15294 atggcaggttatacgacacttcctaataccgatgacaaagaaatggatccggtgagtgcc 15353
Y-6   . 61    gctgtacccggtaagtatccggatgtcattgaaaaatttgtggccaataggtccgtagac 120
              ||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
T-385   15354 gctgtacccggtaagtatccggatgtcattgaaaaatttgtggccaacaggtccgtagac 15413
Y-6     121   gcgttaatagaaggcgttataagtaagttggataccaattcaatatacgaagattccact 180
              ||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
T-385   15414 gcgttaatagaaggcgtcataagtaagttggataccaattcaatatacgaagattccact 15473
Y-6     181   gaaaaatttactggtgaacacttgaaatacgttatggttactatggatactttcttattg 240
              ||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
T-385   15474 gaaaaatttactggtgaacacttgaaatacgttatggttactatggatgctttcttatta 15533
Y-6   . 241   gaaaactacaagacgaaaacggaagatctgttggttcacttagctatgatccaaaagagg 300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
T-385. 15534  gaaaactacaagacgaaaacggaagatctgttggttcacttagctatgatccaaaagagg 15593
Y-6     301   ttgtgcactatatccacgagtactaaaaccaagttccgcgataaaggttgtattagttac 360
              ||||||||| |||||||| ||||||||||||||||||||||||||||||||||||||||||
T-385: 15594  ttgtgcactacatccacgagcactaaaaccaagttccgcgataaaggttgtattagttac 15653
Y-6     361   gtacaagggggttcgcgatacacgttaatggataaagtagtttttccttcattatatcg 420
              || ||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
T-385. 15654  gtgcaagggggttcgcgatacaagttaatggataaagtagtttttccttcattatatcg 15713
Y-6   . 421   aaatttaccgatagggagactccgaacgctctacgtaagtatgcttgcactttcgaggag 480
              |||||||| | ||||||||||||||||||||||||||||||||||||||||||||||||
T-385   15714 aaatttacccacagggagactccgaacgctctacgtaagtatgcttgcactttcgaggag 15773
Y-6     481   ttacatttgtgtatggctaggttgagacccgacttatacgaaaataaaaggacgaccaaa 540
              ||||| |||||||||||||||||||||||||||||||||||||||||||| || |||
T-385   15774 ttacacttgtgtatggctaggttgagacccgacttatacgaaaataaaaggactactaaa 15833
Y-6     541   gccgggactccacacttaaagggctacttatcagccgactttctttcgggttctctccca 600
              |||||||||||||| |||||||||||| ||||||||||||||||||||||||||||||||
T-385. 15834  gccgggactccacatttaaagggctatttatcagccgactttctttcgggttctctccca 15893
Y-6   . 601   gggtactccgaacatgaacgaggcatcattcttcgagcgtctgagtctatgttagctaga 660
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
T-385   15894 gggtactccgaacatgaacgaggcatcattcttcgagcgtctgagtctatgttagctaga 15953
Y-6   : 661   cgtcaaggttacgaggaggcaaccgagcttcttaa-ctacgtgatttgggtaagtacttg 719
              |||||||||||||||||||||||||||||||| |||||||||||||||||||||||||
T-385: 15954  cgtcaaggttacgaggaggcaaccgagcttcttaacctacgtgatttgggtaagtacttg 16013
Y-6   : 720   tagttggtttggggacggtaa 740
              |||||||||||||||||||||
T-385: 16014  tagttggtttggggacggtaa 16034
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Fig. 3. Comparison of the nucleotide sequence of the CTV-CP gene. The nucleotide are numbered starting with the first nucleotide of the insert. T-385 represents the 27 K coat protein gene of CTV-US strain 385 and Y-6 was the coat protein PCR product amplified in this study

CTV detection in samples collected for this study.

Here, we showed that yuzu and early satuma mandarin

were infected by CTV in Cheju island. We confirmed the CTV infection on trees by RT-PCR, ELISA and sequence analysis but we also found that there might be several CTV strains in Cheju island (data not shown). We amplified CTV CP gene from yuzu and early satuma mandarin trees tested in this study by RT-PCR and the PCR fragments were treated with different restriction enzymes. The band patterns were classified into two or three groups among the tested trees. Interestingly, this band patterns were correlated to symptom severity so that this might be possible to identify mild and severe strain of CTV. This experiment will give us the clue of virus protection way such as a cross protection. Now we are cloning the PCR fragments of different groups for further analysis.

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